Universidad Michoacana de San Nicolás de Hidalgo

Instituto de Investigaciones Químico Biológicas

"Estudio de la participación de las *N*-acil-L-homoserina lactonas y ciclodipéptidos en la regulación del desarrollo vegetal por bacterias del género *Pseudomonas*"

Presenta:

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DR. JOSÉ LÓPEZ BUCIO
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PRESENTE

Por este conducto nos permitimos comunicarle que después de haber revisado el manuscrito final de la Tesis Titulada: "Estudio de la participación de las *N*-acil-*L*-homoserina lactonas y ciclodipeptidos en la regulación del desarrollo vegetal por bacterias del Género *Pseudomonas*"presentado por el M.C RANDY ORTIZ CASTRO, consideramos que reúne los requisitos suficientes para ser publicado y defendido en Examen de Grado de Doctor en Ciencias.

Sin otro particular por el momento, reiteramos a usted un cordial saludo.

ATENTAMENTE
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Este trabajo fue realizado en el Laboratorio de Biología del Desarrollo Vegetal del Instituto de Investigaciones Químico Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo, bajo la dirección del D. en C. José López Bucio y con la co-asesoría del D. en C. Jesús Campos García.

Este trabajo está dedicado a:

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A mis padres Alfonso y Petra por su cariño, amor y apoyo incondicional siempre. Gracias por brindarme sus ánimos y enseñanzas. Siempre los llevo en mi corazón.

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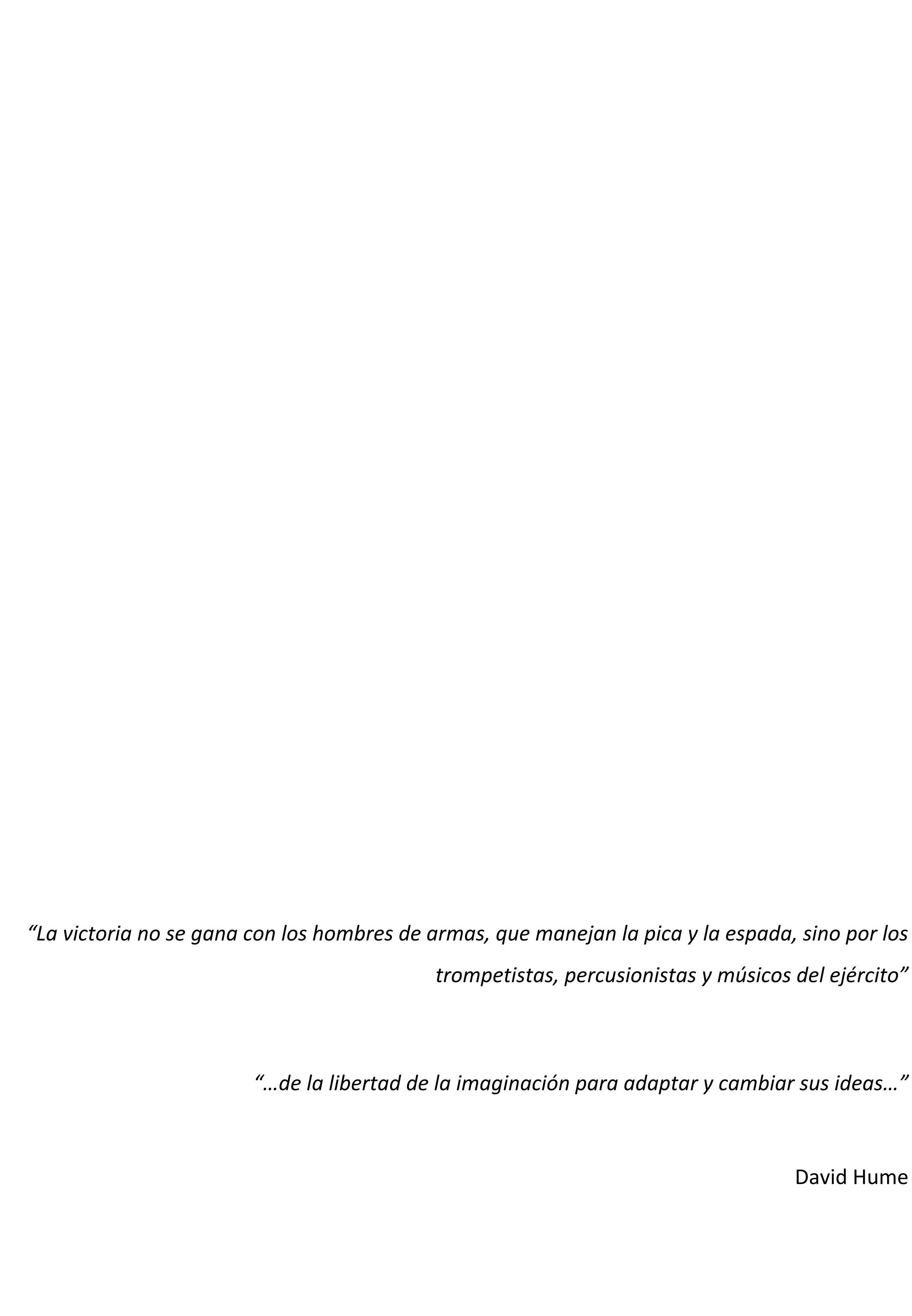

“La victoria no se gana con los hombres de armas, que manejan la pica y la espada, sino por los trompetistas, percusionistas y músicos del ejército”

“…de la libertad de la imaginación para adaptar y cambiar sus ideas…”

David Hume

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1. RESUMEN

Los microorganismos y sus huéspedes eucariontes se comunican a través de múltiples y variadas señales químicas. Ambos grupos de organismos compiten constantemente por los recursos del ambiente, de tal forma que durante las interacciones que se establecen entre plantas y bacterias, el reconocimiento existente puede conducir a eventos de patogénesis o simbiosis. Las auxinas, incluyendo al ácido indol-3-acético (AIA), son hormonas que participan en el crecimiento y desarrollo en las plantas. La producción de estos compuestos no es exclusiva de los tejidos vegetales, ya que muchas especies bacterianas se caracterizan por la producción de tales substancias, sin embargo, hasta la fecha se desconoce como las auxinas, o moléculas relacionadas, afectan la fisiología bacteriana.

Las bacterias Gram-negativas regulan la proliferación celular y la expresión genética través de la producción de *N*-acil-L-homoserina lactonas (AHLs) en un proceso denominado "quorum-sensing" (QS, por sus siglas en inglés). Existe una gama muy amplia de AHLs con variaciones en la longitud del ácido graso. También se han descrito compuestos de estructura química diferente que mimetizan la acción de las AHLs, entre las que se encuentran diversos ciclodipeptidos (CDP's) y sus derivados las dicetopiperazinas (DCPs). Estudios previos demostraron que las plantas pueden percibir a las AHLs modulando la arquitectura radicular y las respuestas de defensa. No obstante, esta información es sustentada en estudios farmacológicos, se carecen de evidencias genéticas o moleculares que contribuyan a elucidar cómo los compuestos moduladores del QS afectan las interacciones entre bacterias Gram negativas y plantas. Para obtener información al respecto, en este trabajo se estableció un sistema *in vitro* para caracterizar la interacción de *Pseudomonas aeruginosa*-*Arabidopsis thaliana* y determinar su impacto en el crecimiento vegetal, así como incursionar en los mecanismos moleculares involucrados. Nuestros resultados indican que en *P. aeruginosa*, el sistema de QS LasI controla la producción de tres CDP's, ciclo(L-Pro-L-Val), ciclo(L-Pro-L-Phe) y ciclo(L-Pro-L-Tyr), los cuales participan en la estimulación del crecimiento vegetal. El análisis de los efectos de los tres compuestos indicó una actividad auxínica débil, que fue suficiente para modificar la arquitectura del sistema radicular (ASR) y la expresión de genes de respuesta a

auxinas. Estos efectos no ocurren en las mutantes de *Arabidopsis tir1, tir1afb2afb3, arf7, arf19* y *arf7arf19* relacionadas con la vía de señalización de auxinas.

La similitud estructural del ácido indol-3-acético (AIA) con los CDP's y un análisis bioinformático sugirió que estos cuatro compuestos se pueden unir en el mismo sitio al receptor de auxinas TIR1. Esta primera serie de resultados proporciona evidencia de que la producción de los CDP's está regulada negativamente por el sistema de QS LasI, siendo sobreproducidos en la mutante *LasI* de *P. aeruginosa.* Respecto al mecanismo involucrado se encontró que los CDP's actúan a través de la señalización de auxinas promoviendo el crecimiento y desarrollo vegetal.

Diferentes especies del género *Pseudomonas* se han catalogado como rizobacterias promotoras del crecimiento vegetal (PGPR) por su habilidad de producir fitohormonas o inducir la inmunidad. Entre ellas se encuentran diferentes aislados de *Pseudomonas putida* y *Pseudomonas fluorescens*. En nuestro estudio, la inoculación de *Arabidopsis* con *P. putida* y *P. fluorescens* estimuló la formación de raíces laterales y pelos radiculares e incrementó la producción de biomasa. Nuevamente se evidenció la inducción de genes de respuesta a auxinas a través de una vía que involucra a los receptores de la familia de TIR1. Estudios adicionales con la mutante *root hair defective 6* (*rhd6*) de *Arabidopsis*, carente de pelos radiculares confirmó que *P. putida* y *P. fluorescens* producen compuestos con actividad auxínica. Las alteraciones tan marcadas en la arquitectura radicular en plantas silvestres mediante el incremento del grado de ramificación de la raíz y la formación extensa de pelos radiculares, nos motivó a examinar si las mutantes producían los mismos, u otros, CDP's encontrados en *P. aeruginosa*. Se encontró que las dos rizobacterias producen ciclo(L-Pro-L-Val), ciclo(L-Pro-L-Phe) y ciclo(L-Pro-L-Tyr) en grandes cantidades, capaces de inducir la expresión de genes de respuesta a auxinas en la planta. Estos resultados indican que los CDP's en *P. putida* y *P. fluorescens* participan en eventos de señalización celular en el sistema radicular, modificando las relaciones planta-bacteria.

P. aeruginosa es la más estudiada por su importancia como patógeno de humanos y plantas. Uno de los principales mecanismos de virulencia de *P. aeruginosa* es la producción de piocianina (PCN). En nuestro estudio evaluamos el efecto de la piocianina sobre el crecimiento

y desarrollo de plántulas de *Arabidopsis*. Plantas de Arabidopsis inoculadas con *P. aeruginosa* PAO1 mostraron síntomas de enfermedad. En contraste, cuando fueron inoculadas con mutantes *lasI-* y la doble mutante *rhlI-/lasI-* de *P. aeruginosa* afectadas en la producción de piocianina mostraron una disminución en la virulencia con un incremento en la fitoestimulación. Los tratamientos con piocianina mostraron una modulación en la arquitectura del sistema radicular inhibiendo el crecimiento de la raíz primaria y promoviendo la formación de raíces laterales y pelos radiculares sin afectar la viabilidad del meristemo o muerte celular. Estos efectos se correlacionan con una alterada proporción de peróxido de hidrógeno y anión superóxido en la punta de la raíz y con una inhibición de la división y elongación celular. El análisis de mutantes mostró que la modulación de la piocianina sobre el crecimiento de la raíz fue independiente de auxina, citocinina y ácido abscísico, pero requiere de la señalización de etileno tal como las mutantes relacionadas a etileno *etr1-1, ein2-1* y *ein3-1* de Arabidopsis fueron menos sensibles a la inhibición de la raíz inducida por piocianina y la distribución de especies reactivas de oxigeno (ROS). Nuestros resultados sugieren que la piocianina es un factor importante para la modulación de la interacción entre la producción de ROS y la arquitectura del sistema radicular por una señalización dependiente de etileno.

En conjunto, nuestros resultados muestran la sofisticada red de señalización que se establece entre las bacterias y las plantas, y el impacto de tales interacciones en la arquitectura radicular de *Arabidopsis*, la cual se refleja en la productividad vegetal. Los resultados obtenidos en este trabajo dan las bases científicas para desarrollos tecnológicos con potencial de aplicación en el campo de los bioinoculantes para su aplicación en campo, como se describe en las revisiones científicas que acompañan el anexo del presente trabajo, así como en la solicitud de patente generada.

2. INTRODUCCIÓN

Las plantas son organismos sésiles y altamente sensibles que compiten constantemente por recursos ambientales mediante el desarrollo de tres partes bien definidas: i) la raíz, órgano de la planta que crece por debajo del suelo, provee anclaje, participa en la captura de agua y nutrientes y es el sitio donde se llevan a cabo interacciones con microorganismos del suelo; ii) el tallo, el cual da soporte a las hojas, flores y frutos; y iii) el follaje, sitio donde se lleva a cabo la fotosíntesis y se forman las estructuras reproductivas. Esta organización determina la arquitectura de la planta y ha sido un criterio para la clasificación de estos organismos. Es de resaltar que la denominada "revolución verde", que ha incrementado la producción de cereales a nivel global estuvo basada en la modificación de la arquitectura foliar para la selección de nuevas variedades con ventajas agronómicas (López-Bucio *et al.,* 2005; Ross *et al.,* 2005; Peng *et al.,* 1999; Reinhardt y Kuhlemeler, 2002; Wang y Li, 2008).

El crecimiento y desarrollo en plantas involucran la integración de varias señales endógenas y ambientales, que junto con el programa genético intrínseco, determinan la forma y función de los diferentes órganos incluyendo la raíz. Las plantas responden activamente a factores bióticos y abióticos, de igual manera, tienen mecanismos para la comunicación entre diferentes órganos y tejidos mediados por diferentes sustancias químicas incluidas las fitohormonas. Una hormona regula una gama amplia de procesos celulares y morfogenéticos, en tanto que múltiples hormonas pueden afectar simultáneamente un mismo proceso (Gray, 2004; Suárez-López, 2005). Las fitohormonas poseen diferentes identidades químicas e incluyen compuestos volátiles como el etileno y el ácido jasmónico (AJ), o pueden ser compuestos orgánicos tales como el ácido indol-3-acético (AIA ó auxina), citocininas, el ácido giberélico (AG), el ácido abscísico (AAB), brasinosteroides (BRs), ácido salicílico (AS) y lípidos (Wayers y Peterson, 2001; Figura 1). En general, estos compuestos regulan cada aspecto del ciclo de vida de la planta modificando la división, expansión, diferenciación y muerte celular, modulando así procesos como la germinación, floración, fructificación y senescencia (Bishopp *et al.,* 2006; Santner *et al.,* 2009).

La arquitectura del sistema radicular (ASR) presenta una gran plasticidad en su morfología y fisiología en respuesta a factores abióticos incluyendo la disponibilidad de nutrientes minerales como fosfato, nitrato y sulfato, así como al estrés biótico, basado en

interacciones planta-planta o planta-microorganismo, que conlleva a modificaciones en la formación de raíces laterales, pelos radiculares y raíces adventicias (Nibau *et al.,* 2008; López-Bucio *et al.,* 2002; López-Bucio *et al.,* 2003; Chen *et al.,* 2007; Ortiz-Castro *et al.,* 2008). Las auxinas juegan un papel muy importante en la regulación de la ASR, ya que la aplicación de auxinas naturales y sintéticas incrementa el desarrollo de raíces laterales y pelos radiculares (Reed *et al.,* 1998; Casimiro *et al.,* 2001). Recientemente, se ha descrito el papel de nuevas moléculas señal tales como las *N*-acil-etanolamidas, alcamidas, *N*-acil-homoserina lactonas (AHLs) y dicetopiperazinas (DCPs) como reguladores de procesos de desarrollo de plantas y mediadores de interacciones planta-bacteria (Blancaflor *et al.,* 2003; Chapman, 2004; López-Bucio *et al.,* 2006; López-Bucio *et al.,* 2007; Morquecho-Contreras y López-Bucio, 2007; Morquecho-Contreras *et al.,* 2010; Méndez-Bravo *et al.,* 2010; Ortiz-Castro *et al.,* 2011).

3. ANTECEDENTES

3.1. Estructura y función de la raíz de *Arabidopsis thaliana*

Arabidopsis thaliana es una planta perteneciente a la familia Brasicaceae que, a diferencia de otras plantas utilizadas en investigación, reúne numerosas ventajas que incluyen: un tamaño pequeño de alrededor de 30 cm, un ciclo de vida corto de 6-8 semanas, una alta fecundidad, ya que una planta puede producir hasta 10,000 semillas y la posibilidad de crecerla *in vitro,* además de tener su genoma totalmente secuenciado (Bennetzen, 2001). Adicionalmente, el uso extensivo de *Arabidopsis* durante las dos últimas décadas ha permitido la creación de poblaciones de semilla mutagenizada, así como una amplia colección de mutantes y líneas transgénicas. Con la secuencia completa del genoma disponible, ahora es posible adquirir por catálogo mutantes insercionales afectadas en genes específicos para analizar la participación de diferentes rutas de señalización en los programas de crecimiento y desarrollo (Dinneny y Benfey, 2009).

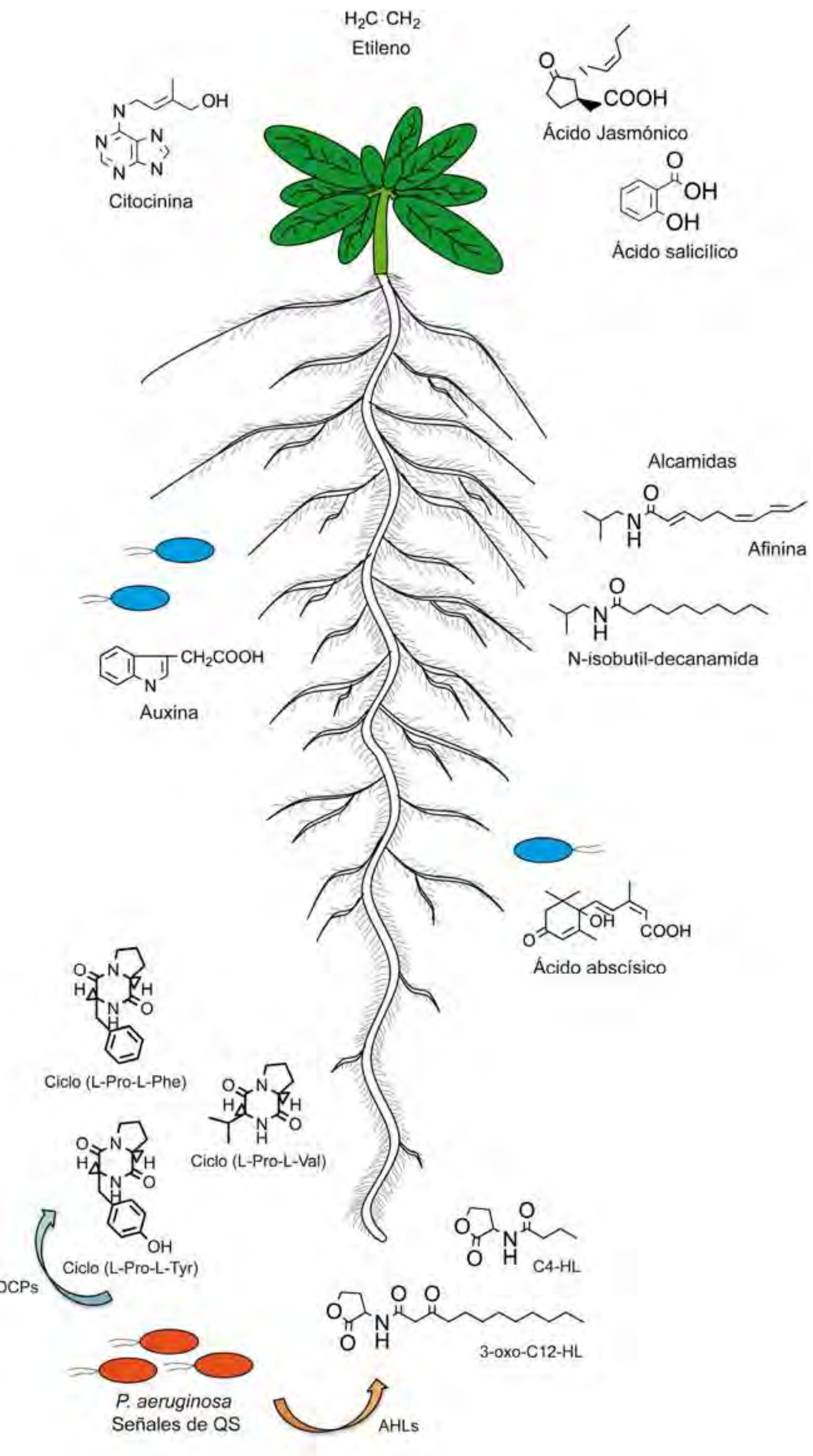

Figura 1. Pequeñas moléculas señal que regulan la arquitectura de las plantas. Se muestran las seis hormonas clásicas: auxina, citocininas, etileno, ácido jasmónico, ácido salicílico y ácido abscícico. También se ilustran nuevos compuestos con actividad biológica como las alcamidas, *N*-acil-homoserina lactonas (AHLs) y dicetopiperazinas (DCPs). Una sola hormona puede afectar múltiples procesos de desarrollo y varias señales pueden impactar en el mismo proceso morfogenético. Todos estos reguladores orquestan la arquitectura de la planta.

En la raíz de *Arabidopsis*, un número pequeño de células madre generan todos los tejidos a través de división celular localizada, seguida de eventos de expansión y diferenciación celular. Debido a que el crecimiento de la raíz es indeterminado, estos procesos son continuos, presentándose todos los estados de desarrollo al mismo tiempo, aunque en diferente región de la raíz. Las células madre, también llamadas células iniciales, se localizan en el ápice de la raíz (Dolan *et al*., 1993). En contacto con las células iniciales se encuentra un número pequeño de células que son mitóticamente inactivas, denominadas centro quiescente (QC; Figura 2). Los linajes celulares se pueden reconocer por la formación de columnas o filas de células organizadas a lo largo del eje longitudinal de la raíz (Schiefelbein *et al.,* 1997). Otro aspecto importante para estudios citológicos es el patrón radial de la raíz, el cual está conformado por anillos o capas concéntricas de tejidos que forman la epidermis, córtex, endodermis, periciclo y los tejidos vasculares, con un número constante de ocho células por anillo para las capas de la corteza y la endodermis (Figura 2; Dolan *et al.,* 1993; Celenza *et al.,* 1995).

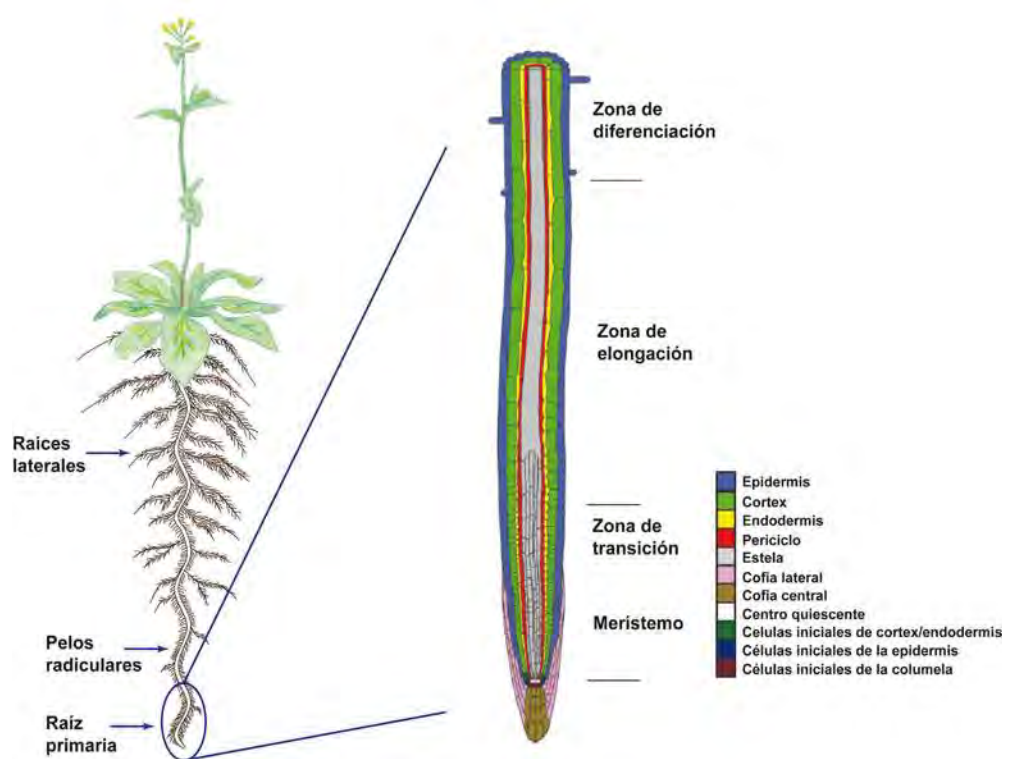

Figura 2. Estructura de la raíz de *Arabidopsis thaliana*. Se esquematiza la zona de crecimiento de la raíz primaria y las diferentes capas de tejidos en un corte longitudinal (Modificado de Ubeda-Tomas *et al*., 2012).

La expansión celular y la diferenciación son factores determinantes en la formación de tejidos y órganos (Dolan y Davies, 2004). Las células de la raíz se forman en el meristemo, en

esta zona la tasa de crecimiento es nula o muy baja, pero a medida que las células salen del meristemo aumentan su longitud a través de toda la zona de alargamiento. Al final de la zona de alargamiento, la tasa de crecimiento disminuye y las células se diferencian hasta alcanzar su forma y función final.

3.2. Raíces laterales

Después de que el patrón de crecimiento de la raíz primaria ha sido establecido y las capas celulares individuales han comenzado a diferenciarse, el aumento en biomasa está basado en eventos de ramificación de la raíz primaria mediante la formación de raíces laterales (RLs). Las RLs se forman en la zona de diferenciación a partir de células del periciclo posicionadas adyacentes a los polos del xilema (Figura 2; Dolan *et al.,* 1993; Petricka *et al.,* 2012).

Las células del periciclo, una vez estimuladas, se diferencian y proliferan formando un primordio de raíz lateral (PRL). La aplicación exógena de auxinas induce la iniciación de PRL y estimula el desarrollo de RL. Los patrones de desarrollo que dan origen a PRL son diferentes de los que ocurren en la formación de la raíz primaria (Casimiro *et al*., 2001). Se ha sugerido que la formación de PRL puede ocurrir a través de dos procesos: i) La estimulación de la diferenciación y proliferación en la capa del periciclo que forma el PRL; ii) La formación del meristemo de la raíz lateral (Celenza *et al*., 1995; Cheng *et al*., 1995; Laskowski *et al*., 1995). La primera división periclinal del periciclo representa el criterio utilizado para definir el principio de formación de raíces laterales. Se han definido siete etapas de desarrollo que preceden la emergencia de raíces laterales (Malamy y Benfey, 1997). Sin embargo, en *Arabidopsis*, la primera división periclinal ocurre dentro de grupos de células iniciales pequeñas, indicando que en las células fundadoras deben ocurrir también una serie de divisiones transversales (Malamy y Benfey, 1997).

El primer evento morfológico relacionado con la iniciación de RL ocurre en dos células fundadoras del periciclo dentro de la misma fila celular, adyacente a uno de los polos del xilema. Ambas células fundadoras se dividen simultáneamente formándose cuatro células pequeñas. Las células hijas continúan dividiéndose simétricamente y asimétricamente, desde el centro hacia arriba y hacia abajo, creando grupos con un máximo de diez células cortas que son

similares en longitud. Con frecuencia se observa plasticidad en el orden preciso de estas divisiones (Figura 3).

Siguiendo un periodo de expansión radial, las células hijas se dividen periclinalmente, originando un primordio compuesto de capas internas y externas definido como etapa II (Malamy y Benfey, 1997). La figura 3 resume las divisiones subsecuentes dentro del primordio de la raíz lateral, denominadas etapas III-VII, que conducen por último a la emergencia, etapa VIII. El primordio de la raíz lateral se expande notablemente a medida que emerge desde la raíz parental, etapa VIII. En el estado III, la forma de domo del primordio de raíz lateral (PRL) es evidente debido a las divisiones periclinales de la capa exterior y a la ausencia de estas divisiones en las células más periféricas. En el estado V, las células de todas las capas se someten a divisiones anticlinales para generar el PRL que comienza a empujar a través de la capa del córtex de la raíz primaria. En etapas subsecuentes, el PRL está a punto de emerger del interior de la raíz primaria y se activa el programa de división que conducirá al crecimiento indeterminado característico de este tipo de órganos (Casimiro *et al*., 2003; Petricka *et al.,* 2012).

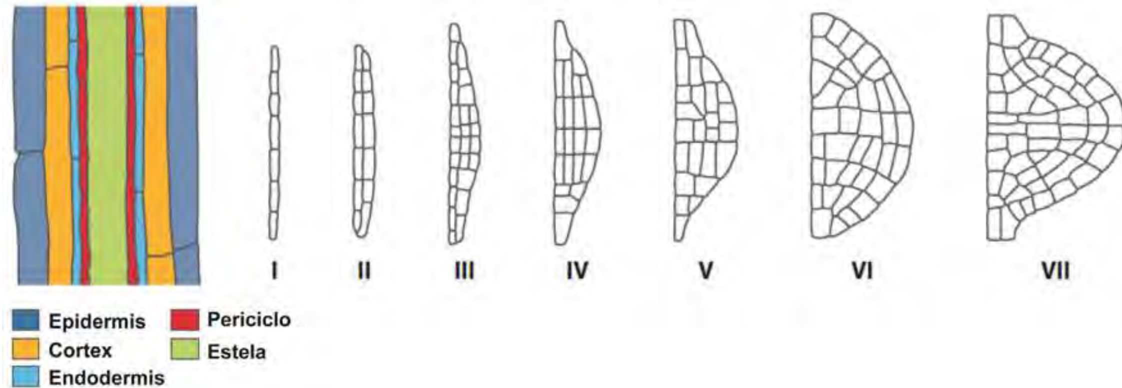

Figura 3. Desarrollo de la raíz lateral. Esquema de la zona de diferenciación mostrando un corte longitudinal de la raíz primaria (izquierda). Las raíces laterales se forman en el periciclo (rojo). También se muestran los estados de desarrollo de la raíz lateral con cada una de las etapas que lo conforman (Etapas I-VII, Modificado de Petricka *et al*., 2012)

3.3. Pelos radiculares

Los pelos radiculares son células epidérmicas diferenciadas que contribuyen con cerca del 77% del área superficial total de la raíz, representando el principal punto de contacto entre la planta y la rizósfera. Cada pelo radicular es una extensión de forma tubular con crecimiento

apical desde la base de una célula epidérmica especializada llamada tricoblasto (Dolan *et al*., 1994; Foreman y Dolan, 2001). Los pelos radiculares son los responsables directos de la captación de agua y nutrientes mediante la expresión de transportadores de alta afinidad y el incremento en la superficie de absorción de la raíz primaria y de las raíces laterales.

Mediante técnicas de microscopia con video de lapsos de tiempo, cryo-SEM y microscopía de luz se han identificado tres fases estructurales del desarrollo del pelo radicular. El primer estado es la aparición de un bulbo en la parte basal del tricoblasto. Durante el segundo estado, se forma una estructura cilíndrica a partir del bulbo. El tercer estado, el cual comienza cuando el pelo radicular mide entre 20 a 40 μm de longitud, involucra un incremento sostenido en la tasa de crecimiento (Dolan *et al*., 1994).

El análisis genético de las fases de formación de los pelos radiculares ha permitido identificar diversas proteínas involucradas en el desarrollo de estas estructuras. Se han aislado mutantes de *A. thaliana* incapaces de formar pelos radiculares que definen genes involucrados en los estados tempranos de formación y crecimiento del pelo tales como *RHL1, RHL2* y *RHL3*. Las tres mutantes tienen fenotipos pleiotrópicos y las plantas son extremadamente enanas, lo que sugiere un efecto generalizado en la expansión celular. *RHL1* codifica una pequeña proteína hidrofílica que contiene una señal de localización nuclear (Scheneider *et al*., 1997). En el análisis del crecimiento de los pelos radiculares, se han identificado cuatro grupos principales de genes. Los genes del primer grupo son requeridos para el inicio en la formación del pelo radicular, estos incluyen *RHD6*, *SHV3, CEN2, RHD3, SCN1* y *TIP1. RHD6* está involucrada en el ensamblaje de componentes celulares en el sitio de la iniciación del pelo radicular (Parker *et al*., 2000). El segundo grupo de genes son requeridos para la formación del abultamiento. Solamente dos genes se encuentran en esta clase, *TIP1* y *RHD1.* Se ha encontrado que estos genes actúan en paralelo, ya que la doble mutante *rhd1/tip1* no muestra el bulbo característico del tricoblasto. El tercer grupo de genes son requeridos en la transición de la formación del bulbo a un crecimiento apical. Están implicados 11 genes: *RHD2, SHV1, SHV2, SHV3, TIP1, BST1, RHD3, CEN1, CEN2, CEN3* y *SCN1.* Los genes del cuarto grupo son requeridos para la elongación del pelo radicular mediante un crecimiento apical. Este grupo de genes incluye *TIP1, SCN1, COW1, RHD3, CEN1, CEN2, CEN3* y *BST1* (Foreman y Dolan, 2001).

Otros factores que afectan la formación de los pelos radiculares son el etileno y las auxinas. Las mutantes en las rutas de auxinas o etileno, *axr2, aux1* y *etr1,* muestran pelos

radiculares más cortos (Pitts *et al*., 1998, Grierson y Schiefelbein, 2002). Sin embargo, se demostró que el bloqueo de la ruta endógena de etileno no es eficiente para alterar la formación de pelos radiculares (Cho y Cosgrove, 2002), lo que suscribe el efecto de esta fitohormona en el proceso de elongación celular que determina el tamaño de estas estructuras.

3.4. Los reguladores del crecimiento vegetal

Las plantas compiten constantemente por los recursos del ambiente como la luz, el agua y disponibilidad de nutrientes. Diversas fitohormonas actúan como integradores esenciales de los programas de desarrollo y las señales del ambiente. Un reto fundamental en la biología ha sido entender los mecanismos moleculares que subyacen la acción hormonal. En otras palabras, cómo las señales químicas son percibidas y transducidas al interior de la célula para activar la expresión génica. Este proceso puede ser dividido dentro de tres principales pasos: percepción de la señal, transducción de la señal a través de una cascada de eventos bioquímicos que permiten la inducción del paso final, la respuesta a nivel de expresión de los genes (Benavente y Alonso, 2006). La señalización química en la planta y entre plantas es compleja, se han identificado más de 20 grupos de moléculas con función reguladora. Una gran cantidad de sustancias diferentes conocidas como metabolitos secundarios se producen en la raíz, que pueden también afectar su crecimiento y desarrollo. Esta diversidad es necesaria considerando la gran abundancia de microbios, insectos y plantas competidoras.

Los reguladores del crecimiento incluyendo a las auxinas, las citocininas, las giberelinas, el ácido abscísico, los brasinosteroides, el etileno, el ácido salicílico y el ácido jasmónico, se producen en la planta a través de varias rutas metabólicas, que pueden alterar el crecimiento y desarrollo vegetal en pequeñas concentraciones (Figura 1). En la actualidad, se acepta que los reguladores del crecimiento forman un grupo de moléculas encargadas de la integración de la información del ambiente, cuyas vías de señalización interactúan con frecuencia para regular la morfogénesis vegetal. Los reguladores del crecimiento mas estudiados son las auxinas, las citocininas, las giberelinas, el ácido abscísico y el etileno (Gray, 2004), aunque el número de compuestos con función señalizadora ha venido incrementándose. Dentro de estos nuevos compuestos se pueden incluir el ácido jasmónico, los brasinoesteroides, el ácido salicílico, las *N*-acil etanolaminas, el glutamato y el óxido nítrico, entre otros (López-Bucio *et al*., 2006;

Morquecho-Contreras y López-Bucio, 2007; Santner *et al*., 2009). Dichas sustancias pueden transportarse entre los diferentes tejidos a través del sistema vascular, como en el caso de las auxinas, o difundirse libremente a través de las membranas como el etileno (Gray, 2004). Prácticamente todos los aspectos del desarrollo vegetal en cierta medida están bajo el control de los reguladores del crecimiento. Un solo regulador puede afectar una gran variedad de aspectos celulares y procesos del desarrollo, en tanto que varios reguladores pueden influir en un mismo proceso (Gray, 2004).

3.4.1. Auxinas

Las auxinas, cuyo nombre deriva de la palabra griega "*auxein*" que significa crecer, forman parte de uno de los principales grupos de reguladores del crecimiento. Las auxinas fueron identificadas por estimular el crecimiento diferencial en respuesta a luz en hipocotilos de maíz y avena. El ácido índol-3-acético (AIA) es la principal auxina en plantas (Bonner y Bandurski, 1952), que es un derivado del triptófano que regula diferentes procesos como la división, expansión, diferenciación celular, formación de raíces laterales, floración y respuestas trópicas (Woodward y Bartel, 2005; Bishopp *et al.,* 2006). En general, los efectos de las auxinas dependen de la concentración, ya que en concentraciones bajas promueven el crecimiento induciendo elongación de hipocotilos, de tallos y de raíces, en tanto que en concentraciones elevadas los efectos son opuestos inhibiendo la elongación celular (Woodward y Bartel, 2005; Benjamins y Scheres, 2008). Existen varias auxinas naturales o sintéticas como el AIA, el ácido indol-3-butírico (AIB), el ácido 2-fenilacético (APA), el ácido 4-cloroindol-3-acético (4-Cl-AIA), el ácido 1-naftalen acético (ANA), el ácido 2,4-diclorofenoxiacético (2,4-D), la dicamba y el picloram (Figura 4). La aplicación de AIA o auxinas sintéticas causa cambios profundos en el crecimiento y desarrollo de las plantas (Kende y Zeevaart, 1997; Bonner y Bandurski, 1952).

Las auxinas son de gran importancia para el desarrollo de la raíz, incluyendo la formación y emergencia de raíces laterales, la organización del meristemo y la respuesta gravitrópica. La biosíntesis de auxinas ocurre tanto en la parte aérea de la planta como en la raíz, la auxina requerida para el desarrollo de la raíz puede sintetizarse tanto en el follaje como localmente. Las partes aéreas de las plantas principalmente las hojas jóvenes son un sitio de gran importancia en la biosíntesis de estos compuestos (Ljung *et al*., 2001).

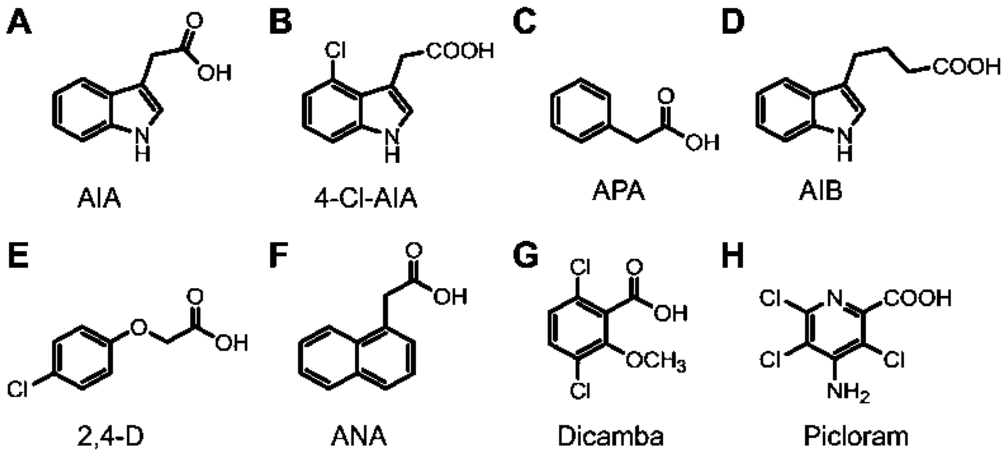

Figura 4. Estructura química de auxinas naturales y sintéticas. (A-B) Auxinas naturales: ácido indol-3-acético (AIA), ácido 4-cloroindol-3-acético (4-Cl-AIA), ácido 2-fenilacético (APA), ácido indol-3-butírico (AIB). (E-H). Auxinas sintéticas: ácido 2,4-diclorofenoxiacético (2,4-D), ácido naftalen acético (ANA), ácido 2-metoxi-3,6-diclorobenzoíco (dicamba), ácido 4-amino-3,5,6-tricloropicolinico (picloram).

En *A. thaliana* la concentración más alta de AIA se localiza en las regiones meristemáticas y en aquellas con crecimiento activo como las hojas jóvenes, cotiledones y en los primordios de las raíces, siendo dichos tejidos los de mayor capacidad biosintética de este compuesto (Ljung *et al.,* 2001). Estudios genéticos y bioquímicos indican que el triptófano (Trp) es el principal precursor para la síntesis de AIA en plantas (Woodward y Bartel, 2005; Zhao, 2010). Alternativamente, se ha propuesto una ruta independiente del triptófano para la síntesis de AIA, pero sus bases genéticas aún no han sido esclarecidas (Zhao, 2010; Strader y Bartel, 2008). Se han propuesto cuatro rutas para la biosíntesis de AIA a partir del Trp en la planta: 1) La ruta YUCCA (YUC); 2) la ruta del ácido indol-3-pirúvico (AIP); 3) La ruta del indol-3-acetamida (IAM); y 4) La ruta del indol-3-acetaldoxima (IAOx; Zhao, 2010; Sugawara *et al.,* 2009). La ruta IAM implica la conversión de IAM a AIA y es llevada a cabo por una amidasa (AMI1) en *Arabidopsis* (Pollmann *et al.,* 2003). La ruta YUC es esencial para la embriogénesis, desarrollo floral, crecimiento de las plántulas y patrón vascular (Tobeña-Santamaría *et al.,* 2002; Cheng *et al.,* 2006; Cheng *et al.,* 2007; Yamamoto *et al.,* 2007). La familia YUC codifica proteínas tipo flavin monooxigenasa que catalizan la conversión de triptamina (TAM) a *N*-hidroxi-TAM (HTAM; Zhao *et al.,* 2001; Kim *et al.,* 2007). Se han propuesto a los intermediarios IAOx y al indol-3-acetonitrilo (IAN) como posibles productos en la conversión de HTAM a AIA, sin embargo, aún no se ha esclarecido totalmente este proceso (Zhao *et al.,* 2001). Estudios recientes han mostrado la participación de una TRIPTOFANO AMINOTRANSFERASA de *Arabidopsis* (TAA1) en

la conversión del Trp a IPA (Tao *et al.,* 2008; Stepanova *et al.,* 2008; Yamada *et al.,* 2009). Se había sugerido que las rutas del IPA y YUC actúan de manera independiente en la síntesis del AIA. Sin embargo, un estudio reciente en *A. thaliana* a través de evidencias genética, enzimática y metabólica indica que las familias TAA y YUC funcionan en la misma ruta biosintética, concluyendo que YUC está implicada directamente en la conversión de IPA a IAA (Mashiguchi *et al.,* 2011; Won *et al.,* 2011).

Las auxinas derivadas del follaje se transportan a través de proteínas membranales hacia la raíz. Esto permite la formación de un gradiente de auxinas con una mayor acumulación en la parte proximal al tallo. Esta acumulación diferencial de auxinas permite una redistribución posterior hacia la zona de elongación celular y diferenciación, donde es esencial para distintos procesos morfogenéticos (Rashotte *et al*., 2000; Casimiro *et al*., 2001). Se han descrito dos vías principales de distribución de auxinas: la primera de transporte rápido, desde los tejidos jóvenes del follaje hacia los sitios de demanda en la raíz. Este tipo de distribución de auxina se realiza a través del floema (Marchant *et al.,* 2002). El otro tipo es de transporte lento, importante en la distribución de auxina a distancias más cortas. En este último, el transporte ocurre célula a célula y requiere de proteínas de expulsión, una de las características de este tipo de transporte es que su direccionalidad está estrictamente controlada dentro de un tejido mediante la localización diferencial de transportadores membranales (Rubery y Scheldrake, 1974; Raven, 1975)

En el transporte polar de auxinas (PAT; Figura 5) participan proteínas transportadoras de influjo como AUX1 y de expulsión de la familia PIN, MDR y PGP que en conjunto regulan la distribución controlada del AIA para generar gradientes de concentración en tejidos específicos. Dichos gradientes son importantes debido a que el AIA lleva a cabo su efecto a través de la formación de gradientes de concentración, con máximos bien definidos en las regiones meristemáticas (Benková *et al*., 2003; Rashotte *et al*., 2000, Vieten *et al*., 2007; Vanneste *et al.,* 2009).

El AIA al ser un ácido débil puede encontrarse en forma protonada o no protonada dependiendo del pH. El pH apoplástico es ~5.5 favoreciendo que las moléculas del AIA se encuentren en su forma disociada (AIA^-) en un ~83% y en su forma protonada (AIAH) en un ~17% (Zazímolova *et al*., 2010). Esta última forma se puede difundir libremente hacia el interior de la célula (difusión pasiva) o a través del transportador de entrada *AUX1*. Sin embargo, una

vez dentro de la célula el cambio de pH del medio favorece la forma disociada del AIA^- casi en su totalidad, por lo que las moléculas en dicha forma, requieren de un mecanismo de transporte activo a través de múltiples proteínas de expulsión como las familias PIN y MDR/PGP's (Swarup *et al*., 2004; Mravec *et al*., 2008; Benjamins y Scheres, 2008; Zazímolova *et al*., 2010).

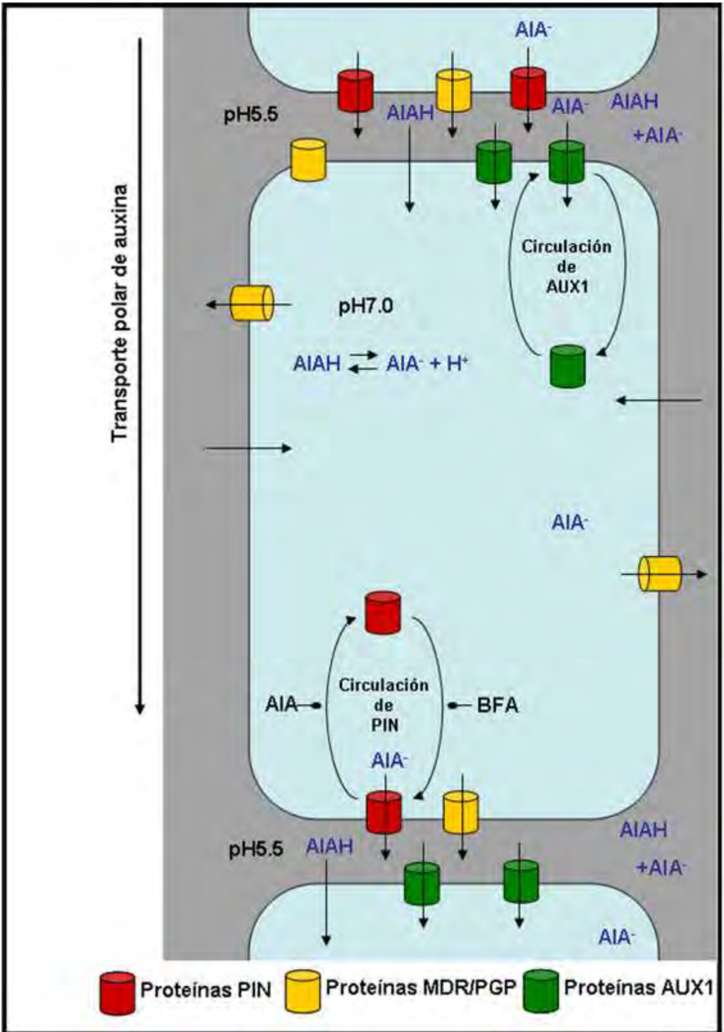

Figura 5. Transporte polar de auxinas. De acuerdo con la hipótesis quimiosmótica, un gradiente de pH a través de la membrana plasmática permite la acumulación de AIA en la célula. Un alto pH en el interior causa la disociación del AIAH, haciéndola incapaz de pasar de manera pasiva a través de la membrana. Los acarreadores de expulsión de auxinas (PINs, algunos MDR/PGPs) son necesarios para transportar auxinas hacia el exterior de la célula. Además de la difusión simple de moléculas de AIA, los acarreadores de entrada (AUX1) transportan los aniones de auxina (AIA^-) hacia el interior de la célula. La localización subcelular polar de las proteínas PINs es importante para la dirección del transporte de auxinas (Modificado de Vieten *et al*., 2007).

Los genes PIN codifican proteínas con 10 dominios transmembranales que tienen similitud con otras proteínas que participan en el transporte a través de la membrana plasmática. En consecuencia, las proteínas PIN muestran una localización asimétrica en la

membrana plasmática consistente con el papel de controlar la polaridad del movimiento de auxinas (Benjamins y Scheres, 2008). Un segundo componente proteico del sistema de salida, es el sitio de unión de los inhibidores de transporte de auxinas tales como el ácido N-naftiltalámico (NPA) y el ácido triyodobenzóico (TIBA), el cual lleva a cabo una función regulatoria (Swarup *et al*., 2004; Mravec *et al*., 2008).

El movimiento acropétalo de AIA de la parte aérea hacia la raíz se ha implicado en el control del crecimiento de las raíces laterales, mientras que el movimiento basipétalo de AIA del extremo radicular es requerido para la respuesta gravitrópica, pero también se ha sugerido que afecta la división celular durante la formación de las raíces laterales (Swarup *et al*., 2004; Mravec *et al*., 2008).

Durante el establecimiento de la arquitectura de la raíz, señales del interior y exterior de la planta son transmitidas a las células del periciclo donde ocurre la formación de las raíces laterales (Bhalerao *et al*., 2002). Estudios genéticos han mostrado que mutantes deficientes en la respuesta a auxinas *axr2*, muestran un crecimiento de raíz y follaje agravitrópico, un hipocotilo corto y un crecimiento de la raíz primaria resistente a auxinas (Wilson *et al*., 1990; Nagpal *et al*., 2000) o un desarrollo de raíces laterales alterado (Casimiro *et al*., 2003). Se ha determinado que la escasez de auxinas mantiene las células del periciclo en la fase G1 y la posterior adición promueve la transición de la fase de G1-S, activándose así la formación de raíces laterales (Himanen *et al*., 2002). En *A. thaliana* las respuestas inducidas por auxinas comprenden una red de procesos compleja y altamente regulada que incluye la síntesis de auxinas, la disponibilidad en su forma activa, la regulación de su transporte, su percepción y señalización, además de la interacciones con otras señales tanto endógenas como del ambiente (Kieffer *et al.,* 2010).

Análisis genéticos y bioquímicos han permitido identificar varios genes involucrados en la percepción de auxinas (Leyser, 2006). Mutaciones en uno de estos genes, *TIR1*, causa defectos en varias respuestas mediadas por auxina tales como inhibición de la elongación de la raíz e inducción en la formación de raíces laterales (Ruegger *et al.,* 1998). La proteína TIR1 es un componente del sistema ubiquitina-proteosoma (UPS) que media la degradación de proteínas (Gray *et al.,* 1999). TIR1 fue una de las primeras proteínas de la familia F-Box (FBP) identificadas en plantas, la cual interacciona con el complejo SCF-ubiquitin ligasa (E3) para la subsecuente degradación por el proteosoma 26S de los represores transcripcionales Aux/IAA en respuesta a

auxina (Cardozo y Pagano, 2004; Gray *et al.,* 2001). Las auxinas regulan la transcripción a través de la acción de dos grandes familias de factores transcripcionales denominados proteínas auxina/ácido indol-3-acético (Aux/IAA) y los factores de respuesta a auxinas (ARFs). Los ARFs se unen directamente al DNA, activando o inhibiendo la transcripción dependiendo del tipo de ARF (Guilfoyle y Hagen, 2007). Las proteínas Aux/IAA se unen a los ARFs y reprimen la transcripción regulada por auxinas mediante el reclutamiento de un co-represor denominado TOPLESS (TPL; Szemenyei *et al.,* 2008; Figura 6).

El sistema ubiquitina proteosoma juega un papel muy importante en la regulación de diferentes actividades en eucariotes. En mamíferos, la degradación de proteínas mediada por proteosoma regula el ciclo celular, la reparación del DNA, la apoptosis, las respuesta inmunes y el metabolismo (Viestra, 2009). La ubiquitina y proteínas relacionadas son conjugadas a otras proteínas a través de la acción secuencial de tres enzimas llamadas enzima de activación de ubiquitina (E1), enzima de conjugación de ubiquitina (E2) y ubiquitina ligasa (E3) que confiere especificidad al sustrato, que dependerán de su estado de fosforilación, defosforilación, hidroxilación y glicosilación para realizar sus funciones (Pickart, 2001). Finalmente, la proteína blanco que es ubiquitinada es degradada por el proteosoma 26S (Hershko y Ciechanover,1998; Callis y Vierstra, 2000). Se han identificado cuatro tipos de ligasas E3 en plantas, entre ellas se encuentra el complejo proteico SCF (Skp1-culina-F-caja/RINGH2) (Callis y Vierstra, 2000, Patton *et al*., 1998). El nombre del complejo proviene de las primeras tres subunidades identificadas en levadura, Skp1 (ASK1), Cdc53 (o culina) y una proteína de la caja F. Se identificó una cuarta subunidad, con un motivo RING-H2 típico de las proteínas Rbx1/Hrt1/ROC1 como un componente esencial del complejo (Tyers y Willems, 1999). En *Arabidopsis* existen 18 genes relacionados a *Skp1*, 5 culinas y alrededor de 300 proteínas de caja F, siendo estas últimas las que funcionan en el reconocimiento del sustrato y representan los más diversos y específicos componentes del complejo. El alto número de genes que codifican para proteínas que contienen las cajas F, muestra la importancia potencial del complejo SCF y la función que cumple en la degradación de proteínas en varios procesos celulares (Dharmasiri y Estelle, 2002).

Estudios detallados han demostrado que para la respuesta a auxinas se requiere la participación del complejo SCF^{TIR1} (Gray *et al*., 1999). *TIR1* codifica para una proteína de 594 aminoácidos que contiene un dominio de caja F y seis repetidos ricos en leucina (Ruegger *et al*., 1998). El motivo de caja F está localizado en el extremo N-terminal y se requiere para el

ensamblaje de la proteína de caja F con el centro del complejo SCF mediante su unión a las proteínas Skp1 (Patton *et al*., 1998).

Se ha demostrado que varias proteínas AUX/IAA, como AXR2/IAA7, AXR3/IAA17 y SHY2/IAA3 interactúan con el complejo SCF^{TIR1} cuando estos se añaden a extractos de plantas y que dicha interacción es estimulada por las auxinas (Gray *et al*., 2001). Una vez formado el complejo AUX/IAA-SCF^{TIR1}, dependiente de auxinas, ocurre la ubiquitinación y degradación de dichas proteínas represoras. En concentraciones bajas de auxinas, las proteínas Aux/IAA son degradadas a nivel basal, cuando la concentración de auxinas se incrementa, una interacción eficiente entre proteínas AUX/IAA y TIR1 promueve la degradación de los represores AUX/IAA dando como resultado un incremento en la transcripción regulada por auxinas (Guilfoyle, 2007).

Las genes *Aux/IAA* fueron identificados como genes regulados por auxinas en chícharo y soya, encontrando que sus niveles de transcritos se incrementaban rápidamente (en menos de 60 minutos) en tratamientos con auxina, por lo cual fueron referidos como "genes de respuesta temprana". Además de los genes *Aux/IAA* se encuentran familias de los genes *SMALL AUXIN UP RNAs (SAUR)* y *GH3* (Abel y Theologis, 1996; Hagen y Guilfoyle, 2002). El genoma de *Arabidopsis* contiene 29 miembros de la familia de genes Aux/IAA (Abel y Theologis, 1996; Remington *et al.,* 2004; Overvoorde *et al.,* 2005), aunque muchos genes Aux/IAA son inducidos por auxinas, algunos como IAA28, muestran poco o nula respuesta a auxina (Rogg *et al.,* 2001). IAA7/AXR2, IAA12/BDL, y IAA17/IAA17 interaccionan directamente con $SCF^{TIR1/AFB1-3}$ de una manera dependiente de auxina (Kepiski y Leyser 2004; Dharmasiri *et al.,* 2005a; Dharmasiri *et al.,* 2005b; Kepiski y Leyser, 2005). En presencia de auxina, SCFTIR1 incrementa su afinidad por Aux/IAA, acelerando la degradación de los represores (Ulmasov *et al.,* 1997; Worley *et al.,* 2000; Gray *et al.,* 2001; Tiwari *et al.,* 2001; Zenser *et al.,* 2001; Tiwari *et al.,* 2004).

Las proteínas nucleares Aux/IAA contienen cuatro dominios conservados (Mockaitis y Estelle, 2008). El dominio I es un dominio represor que contiene el motivo EAR (*Ethylene Response Factor* [ERF] (LxLxL), responsable del reclutamiento del co-represor transcripcional TOPLESS (TPL; Long *et al.,* 2006). El dominio II contribuye a la rápida degradación de Aux/IAA (Mockaitis y Estelle, 2008). Los dominios III y IV muestran una alta homología con los dos dominios de las proteínas ARF, también nombrados como III y IV y median la homo y heterodimerización entre los Aux/IAA y ARFs (Kim *et al.,* 1997; Ulmasov *et al.,* 1997). Las 23 proteínas ARF en *Arabidopsis* actúan como factores de transcripción que al unirse al ADN

activan o reprimen genes de respuesta a auxinas por la unión a AuxREs (elementos de respuesta a auxina) en los promotores de estos genes (Okushima *et al.,* 2005). Los fenotipos observados en mutantes con ganancia de función en proteínas Aux/IAA, muestran una represión constitutiva de las proteínas ARF. La inhibición de formación de raíces laterales en las mutantes *slr/iaa14* es similar a los defectos observados en la mutante *arf7 arf19* (Hamann *et al.,* 2002; Weijers *et al.,* 2005; Muto *et al.,* 2007). Recientemente, en un estudio utilizando el sistema de doble híbrido en levadura y ensayos de unión a auxina utilizando proteínas purificadas, se demostró que diferentes combinaciones de proteínas TIR1 y Aux/IAA forman complejos con un rango amplio de afinidad a auxina, la cual era determinada por la proteína Aux/IAA. Además se demostró que el co-receptor AFB5-Aux/IAA se une al herbicida picloram (Calderon-Villalobos *et al.,* 2012).

Respecto al receptor TIR1, se obtuvo un avance espectacular de conocimiento en su función cuando se estableció un sistema libre de células para estudiar la señalización por auxinas (Dharmarsiri *et al*., 2003). Se observó que la adición de las auxinas a un extracto crudo de plantas promovió la interacción entre las proteínas AUX/IAA y el complejo SCFTIR1, indicando la presencia de un receptor soluble. Posteriormente, dos reportes revelaron la simplicidad de la ruta de auxinas, cuando demostraron con la adición de auxinas marcadas radiactivamente a los extractos crudos de plantas, que la hormona co-purificaba con el complejo SCFTIR1-AUX/IAA (Kepinski y Leyser, 2005; Dharmarsiri *et al*., 2005a). Además, encontraron que la actividad de unión fue saturable, específica para auxinas activas y posee la afinidad esperada de un receptor de auxinas. Sin embargo, esta evidencia solo sugería, que el receptor formaba parte del complejo SCFTIR1, pero el descubrimiento de que TIR1 al ser expresado en células animales también interactúa con los AUX/IAA en una manera dependiente de auxinas, proporcionó la evidencia adicional de que TIR1 es uno de los receptores de auxinas (Figura 6; Dharmasiri *et al*., 2005).

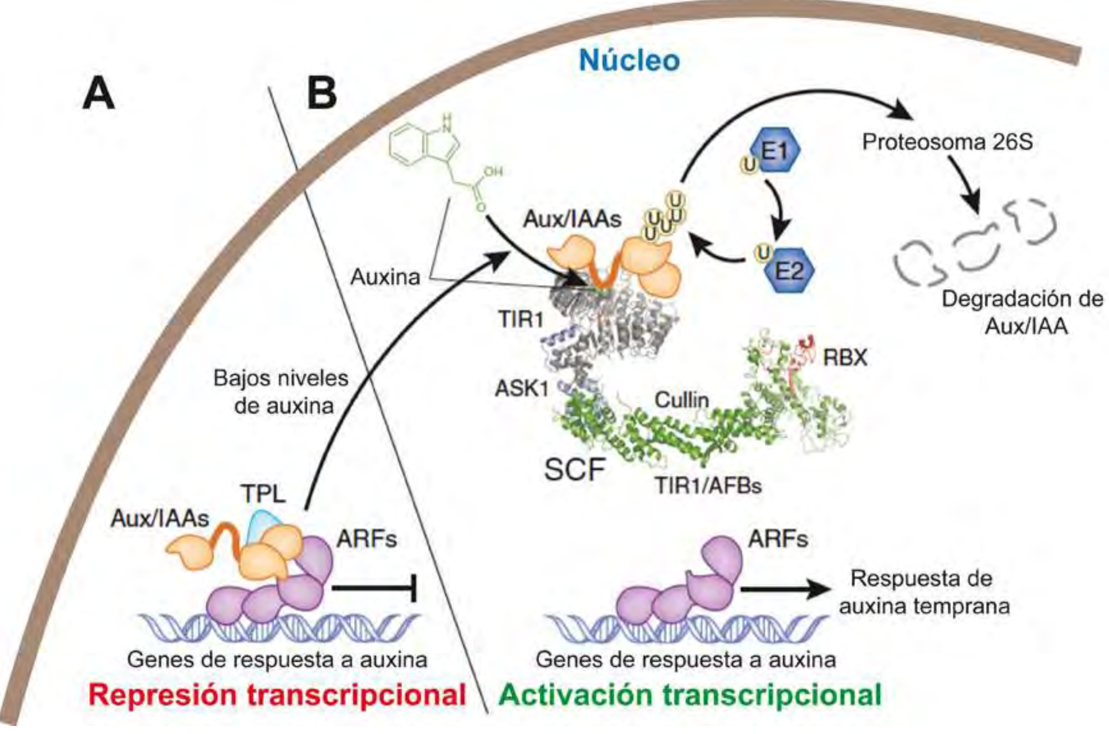

Figura 6. Modelo de la señalización por auxinas en *Arabidopsis*. TIR1 es una proteína F-box que se une directamente a auxinas y activa la degradación de las proteínas Aux/IAA. (A) En bajos niveles de auxina, la transcripción dependiente de los ARF es reprimida por las proteínas Aux/IAA y el co-represor TPL. (B) Altos niveles de auxina resultan en la formación del complejo TIR1-Aux/IAA permitiendo la ubiquitinación de Aux/IAA y su subsecuente degradación, permitiendo a las proteínas ARF la activación transcripcional de genes regulados por auxina en la planta (Modificado de Santner *et al.,* 2009).

Por otra parte, Tan *et al*., (2007) establecieron el primer modelo estructural del receptor TIR1-auxinas, donde la estructura cristalográfica de TIR1 muestra que el dominio de elementos repetidos ricos en leucina del receptor TIR1 forma una especie de bolsa, la cual contenía como cofactor inositol hexakisfosfato mismo que reconoce a las auxinas y al polipéptido AUX/IAA (Guilfoyle, 2007). Este cofactor se encuentra localizado hacia el fondo de la superficie en forma de bolsa de TIR1 y es el sitio en donde se unen varios análogos de auxinas (IAA, NAA y 2,4-D). Por otra parte, acoplado en la parte superior de la auxina, el sustrato (AUX/IAA) ocupa el resto de la bolsa de TIR1 y termina completando el sitio de unión de la hormona. Las auxinas aumentan las interacciones de TIR1 con su sustrato actuando como un "pegamento molecular", coordinando el ensamblaje del complejo con TIR1 y el dominio II de los AUX/IAA en contacto con la hormona. Estudios recientes, han mostrado que el óxido nítrico (ON) facilita la interacción de TIR1-Aux/IAA modulando la señalización de auxina a través de la S-nitrosilación del receptor de auxina TIR1, facilitando la degradación de Aux/IAA y promoviendo la expresión de genes regulados por auxina (Terrile *et al.,* 2012). En otro estudio, Yu *et al.* (2013), mostró que

mutaciones en dos residuos (D170E y M473L) del receptor TIR1 de auxina incrementan la afinidad entre TIR1 y Aux/IAA, facilitando la actividad del complejo SCF^{TIR1}, dando lugar a una rápida degradación de los represores Aux/IAA e incrementando la transcripción de los genes de respuesta a auxina en la planta.

3.4.2 Etileno

Entre las hormonas de las plantas, el etileno se distingue por ser un hidrocarburo (C_2H_4) de naturaleza gaseosa. Esta molécula juega papeles importantes en el crecimiento y desarrollo de la planta por influir en un amplio rango de procesos a través de todo el ciclo de vida, desde la germinación, floración, maduración de frutos y senescencia (Bleecker y kende, 2000). Uno de los efectos más dramáticos del etileno en la morfogénesis de la planta es la clásica "triple respuesta" mostrada cuando las plantas son crecidas en obscuridad y expuestas a etileno. La triple respuesta en *Arabidopsis* se caracteriza por una curvatura exagerada del gancho apical, ensanchamiento radial e inhibición del hipocotílo y crecimiento de la raíz (Figura 7; Guzman y Ecker, 1990).

La síntesis del etileno comienza con el aminoácido metionina, que además de fungir como bloque constructor en la síntesis de proteínas, es precursor químico de otros compuestos biológicos. El etileno tiene su origen en los carbonos 3 y 4 de dicho aminoácido. En un primer paso y por medio de la acción de la enzima S-adenosil-metionina (SAM) sintetasa, la metionina es convertida en S-AdoMet (S-adenosil-metionina) a expensas de ATP. Este compuesto entra en el llamado ciclo de Yang donde una fracción del mismo es convertida en ACC (ácido1-carboxílico-1-aminociclopropano) por la enzima ACC sintetasa (ACS) y la otra nuevamente en metionina, asegurando una segunda ronda disponible para la producción de etileno sin la necesidad de aumentar la demanda celular del aminoácido. En la parte final del proceso, el ACC es oxidado por la enzima ACC oxidasa (ACO) para producir etileno, CO_2 y cianuro. El paso clave en la regulación espacio-temporal de la biosíntesis del etileno es aquel que involucra la acción de la ACS (Kende, 1989, Schaller y Kieber, 2002). Se han utilizado ampliamente inhibidores en el proceso de maduración, como el aminoetoxi-vinilglicina (AVG) que bloquea la conversión de AdoMet a ACC, y los iones de cobalto (Co^{+2}), que bloquean la conversión de ACC a etileno por la

ACC oxidasa en el último paso de la síntesis del etileno (Yu y Yang, 1979; Abeles *et al.,* 1992; Saltveit, 2005; Lau y Yang, 1976).

Figura 7. Fenotipo de la triple respuesta en *Arabidopsis*. Plantas de *A. thaliana* de tres días de edad crecidas en condiciones de obscuridad sin tratamiento hormonal (planta de la izquierda) o suplementada con 10 μM del precursor de etileno ACC (Modificado de Benavente y Alonso, 2006).

El etileno es reconocido por el receptor ETR1, que codifica para una histidina cinasa muy similar a las histidina cinasas de dos componentes presentes en bacterias (Chang *et al.,* 1993). Diversos estudios indican que el etileno interactúa con una familia de receptores de cinco miembros: *ETR1*, *ethylene response2 (etr2), ethylene insensitive4 (ein4), ethylene resistant1 (ers1) y ethylene resistant2 (ers2)* (Chang *et al.,* 1993; Hua *et al.,* 1998; Sakai *et al.,* 1998). La unión del etileno a ETR1 está mediada por un cofactor de cobre que está localizado en los dominios transmembranales amino terminal de los receptores. Particularmente, los residuos Cis65 e His69 juegan un papel fundamental en la interacción hormona-metal-proteína. La unión del etileno causa un cambio conformacional en el sitio de unión que es transmitido al dominio carboxilo terminal, iniciando la cascada de señalización. Los receptores de etileno residen en la membrana del retículo endoplásmico y están asociados a la proteína cinasa CTR1, en la ausencia de etileno, los receptores promueven la actividad de la cinasa CTR1, los cuales reprimen las respuestas de etileno, cuando el etileno está presente, los receptores, y por tanto CTR1, están inactivos (Gao *et al.,* 2003; Huang *et al.,* 2003; Kieber *et al.,* 1993).

CTR1 consiste de un único dominio regulatorio amino terminal y un dominio cinasa serina/treonina en el carboxilo terminal similar al de cinasas activadas por mitógeno (MAPKKK), lo que ha postulado un posible inicio de la señalización a través de la cascada MAPK que

culminaría con la activación de EIN2 (Novikova *et al.,* 2000; Ouaked *et al.,* 2003; Zhao y Guo, 2011; Hahn y Harter, 2009; Ecker, 2004), sin embargo, la existencia de una cascada MAPK en la señalización de etileno ha sido controversial. Recientemente, se ha mostrado que CTR1 fosforila directamente a EIN2 reprimiendo la función de EIN2 y que sustituciones en los residuos Ser645 y Ser924 por Ala, juegan un papel importante en la fosforilación de EIN2 (Ju *et al.,* 2012; Qiao *et al.,* 2012).

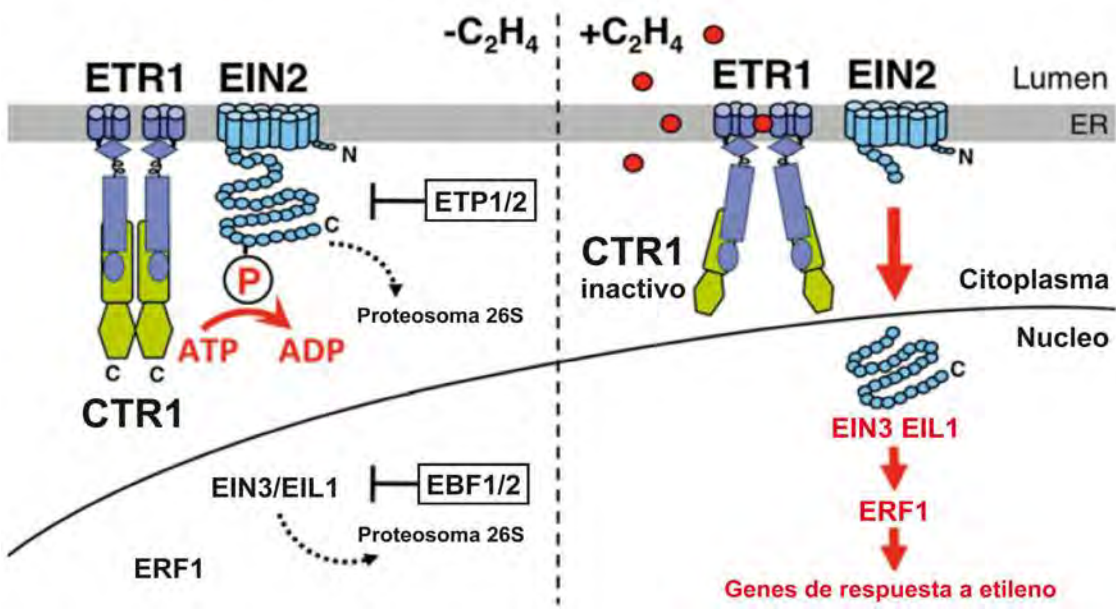

Figura 8. Modelo de la señalización de etileno. En la ausencia de etileno (izquierda), los receptores de etileno (ej. ETR1) en la membrana del retículo endoplásmico activan la proteína cinasa CTR1 que fosforila el dominio carboxilo terminal de EIN2, evitando su localización nuclear. Sin etileno, EIN2 es etiquetado por las proteínas F-box ETP1/2 para su degradación por el proteosoma 26S. Los factores de transcripción EIN3/EIL1 de igual manera son etiquetados por las proteínas EBF1/EBF2 para su degradación. En la presencia de etileno (derecha), los receptores son inactivados y por tanto la cinasa CTR1 es desactivada. La ausencia de la fosforilación en el dominio carboxilo terminal de EIN2 es liberado y dirigido al núcleo donde puede activar río abajo la cascada transcripcional modulando los genes de respuesta a etileno en la planta (Modificado de Ju *et al.,* 2012).

EIN2 es un regulador positivo de las respuestas de etileno que actúa cascada abajo de CTR1. EIN2 es una proteína integral que consiste de un dominio amino terminal de 12 hélices transmembranales, cuya secuencia es muy similar a los transportadores de metales tipo Nramp, seguido de un dominio carboxilo terminal citosólico (Alonso *et al.,* 1999). Este último dominio, por la acción de una proteasa aún desconocida, es removido de la membrana del retículo cuando EIN2 se encuentra desfosforilada. En la ausencia de etileno, los niveles de proteína decrecen por un mecanismo de ubiquitinación, donde el complejo $SCF^{ETP1/2}$ marca a EIN2 con ubiquitina para su degradación por el proteosoma 26S (Qiao *et al.,* 2009), corriente abajo de

EIN2 se localizan varios factores transcripcionales (ej. EIN3 y ERF1) que modulan las respuestas transcripcionales a etileno (An *et al.,* 2010; Solano *et al.,* 1998).

ETHYLENE INSENSITIVE3 (EIN3), es una proteína nuclear requerida para la señalización de etileno regulada por EIN2 (Chao *et al.,* 1997). EIN3 pertenece una familia de cinco proteínas EIN3-Like (EIL) con características de factores transcripcionales, lo cual sugiere que EIN3 y al menos uno de los EILs puede actuar como un regulador transcripcional de las respuestas a etileno (Chao *et al.,* 1997). Investigaciones en tabaco han permitido identificar una familia de proteínas denominadas ETHYLENE-RESPONSE-ELEMENT-BINDING-PROTEINS (EREBPs) las cuáles se unen a los elementos *cis* de la caja GCC en los promotores de genes regulados por etileno, los cuales median la respuesta al ataque por patógenos (Ohme-Takagi y Shinshi, 1995). EIN3, EIL1 y EIL2 se unen a una región palindrómica, conocida como sitio de unión a EIN3 (EBS), en los promotores de miembros de la familia *ETHYLENE RESPONSE FACTOR1 (ERF1).* ERF1, es un factor transcripcional que actúa cascada abajo de EIN3 y EIL1 y es responsable de la modulación de genes de respuesta a etileno (Solano *et al.,* 1998). Al igual que EIN2, los niveles de EIN3 y EIL1 están regulados por un complejo $SCF^{EBF1/2}$ y su posterior degradación por el proteosoma 26S. En presencia de etileno, los niveles de proteínas de EIN3 BINDING FACTOR1 (EBF1), que forman parte del complejo SCF disminuyen, incrementando los niveles de EIN3 y EIL1 (Figura 8; An *et al.,* 2010).

3.5. Nuevos reguladores del crecimiento vegetal

3.5.1. *N*-acil etanolamidas

Las *N*-acil-etanolamidas (NAEs) se producen por la hidrólisis por una fosfolipasa D (PLD) de un fosfolípido precursor, la *N*-acilfosfatidiletanolamida (NAPE), que constituye entre el 1-5% de los fosfolípidos de la membrana plasmática de plantas y animales. En plantas de algodón, los contenidos de NAPE varían entre 1.9 y 3.2% del total de fosfolípidos dependiendo del tejido y estado de desarrollo. Sin embargo, los niveles de NAPE cambian en respuesta al estrés. Se ha reportado que durante la respuesta a elicitores de patógenos tales como la xilanasa, los cultivos de células de tabaco en suspensión disminuyen en 5 veces los niveles de NAPE-(C_{14}) con un

correspondiente incremento de alrededor de seis veces en el contenido de NAE-(C_{14}), lo cual sugiere una correspondiente co-regulación de los niveles de NAPE y NAE (Kilaru *et al*., 2007).

Las NAEs de angiospermas son etanolamidas de ácidos grasos cuya longitud de la cadena acilo varía de 12 a 18 carbonos, que puede ser saturada o insaturada. Generalmente las NAEs más abundantes encontradas en plantas y vertebrados son similares, incluyendo NAE 16:0, 18:1, 18:2 y 18:3. Las NAEs se acumulan sobre todo en semillas, más que en tejido vegetativo. Inicialmente las NAEs fueron reportadas en semillas secas, encontrándose cantidades de 0.75 μg/g en tomate, 1.1 μg/g en maíz, 0.75 μg/g en algodón y en *Arabidopsis thaliana* de 2 μg/g de peso fresco, siendo las NAEs 16:0 y 18:2 las más abundantes (Kilaru *et al*., 2007; Chapman, 2004).

La anandamida es una de las NAEs de mayor importancia en vertebrados debido a su actividad como un neurotransmisor y neuromodulador y por regular algunos comportamientos como la alimentación, el miedo y la ansiedad. En la actualidad se ha establecido que la anandamida y otras NAEs poliinsaturadas se unen a los receptores canabinoides CB1 y CB2, en tanto que las NAEs saturadas o monoinsaturadas no son capaces de hacerlo (Alger, 2004). En vertebrados, las NAEs participan en la ruta de señalización de los endocanabinoides, esta ruta es inhibida por la activación de una enzima, la amido hidrolasa de ácidos grasos (*fatty acid amide hydrolase*, FAAH), la cual hidroliza a las NAEs para producir su correspondiente ácido graso libre y etanolamina. Estudios recientes con ratones deficientes en FAAH revelan altos niveles de NAE 20:4, 16:0 y 18:1 en sus tejidos comparados a ratones silvestres, lo cual sugiere un papel importantes de la FAAH en el metabolismo de las NAEs. Esta ruta también se ha estudiado en plantas, en donde se ha encontrado que líneas de *Arabidopsis* con expresión alterada de FAAH muestran cambios en los niveles endógenos de NAEs. Los niveles de NAEs en plantas mutantes en FAAH fueron 30% más altos, mientras que en plantas sobreexpresantes de FAAH fueron 50% menores comparados con los encontrados en las plantas silvestres.

En diversos trabajos se ha reportado que las NAEs ejercen un efecto en el crecimiento y desarrollo de las plantas. Blancaflor *et al*., (2003) evaluaron los efectos de altas concentraciones de *N*-lauroiletanolamida (NAE 12:0) sobre el desarrollo temprano de la raíz de *Arabidopsis*, encontrando que se inhibe la elongación de la raíz primaria, en tanto se incrementa la longitud de las raíces laterales y se muestra un efecto inhibitorio en la formación de pelos radiculares a altas concentraciones de C12:0. Estos efectos morfogenéticos estuvieron asociados con

alteraciones en la división celular, la organización endomembranal y el trafico de vesículas, sugiriendo que la NAE 12:0 podría jugar un papel esencial en procesos celulares (Figura 9).

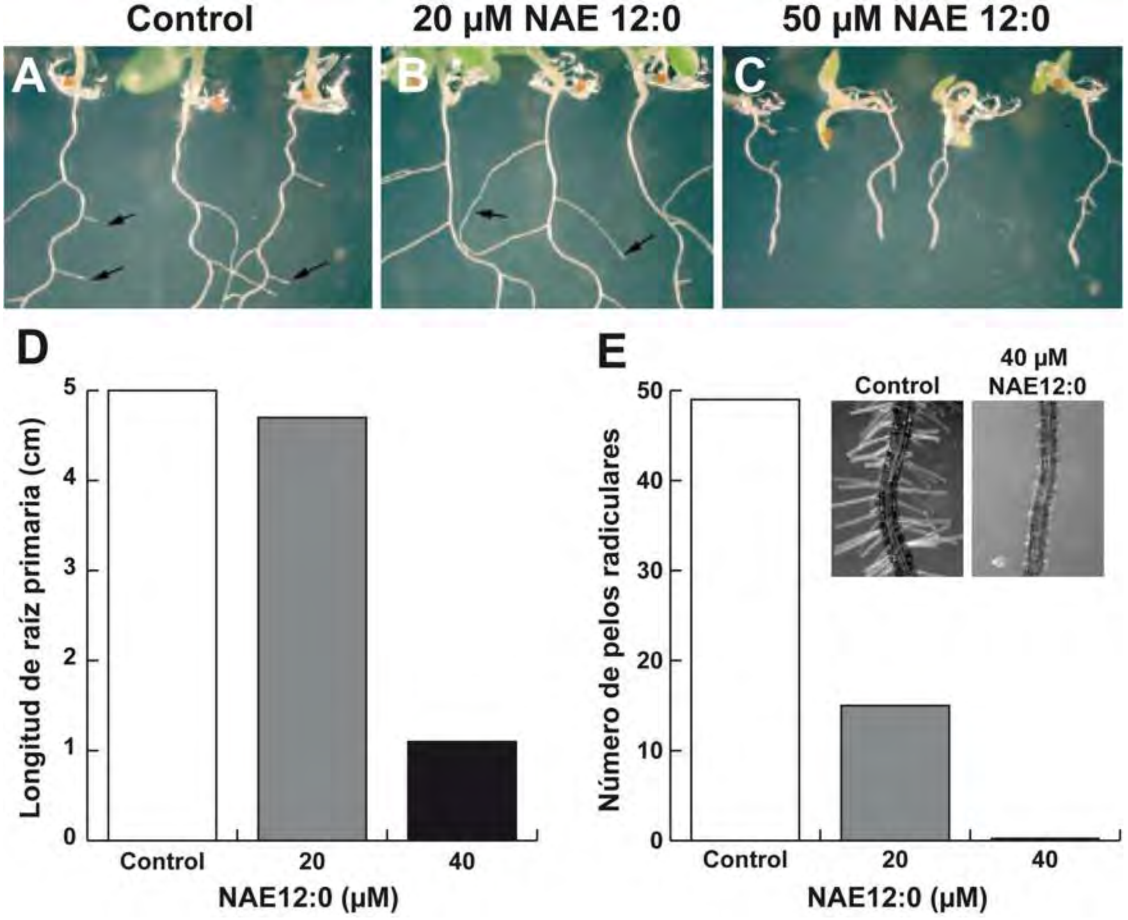

Figura 9. Efectos de la NAE12:0 sobre la arquitectura radicular de plántulas de *Arabidopsis thaliana*. (A-B) Imágenes representativas del crecimiento radicular de plantas de 9 días crecidas en medio control (A), o suplementadas con 20 μM (B) y 50 μM (C) de NAE12:0. Se puede observar como se incrementa la longitud de las RLs en dosis bajas (20 μM; indicadas por las flechas) comparadas con el control. (D) Crecimiento de la raíz primaria, (E) Número de pelos radiculares. Imágenes representativas del desarrollo de los pelos radiculares, se puede apreciar la inhibición del crecimiento de los pelos radiculares de las plantas crecidas en medios suplementados con NAE12:0 (Modificado de Blancaflor *et al*., 2003).

Si bien se ha encontrado que las NAEs se acumulan en grandes cantidades en las semillas, sus niveles disminuyen drásticamente cuando estas germinan. Teaster *et al.* (2007) evaluaron la participación de la vía de señalización del ABA y si ésta era requerida en las respuestas mediadas por las NAEs, encontrando que la aplicación combinada de concentraciones micromolares de ambas, NAE y ABA, producen una disminución dramática en el crecimiento de la raíz, distinto al observado cuando los compuestos se aplican por separado. Esta inhibición en el crecimiento fue más notable en las mutantes de la vía de señalización del

ABA (*abi1, abi2, abi3* y *abi5*), lo cual sugiere que la acción de las NAEs requiere de una vía de señalización intacta del ABA.

3.5.2. Alcamidas

Las alcamidas son metabolitos secundarios que comprenden más de 200 compuestos relacionados estructuralmente y de amplia distribución en plantas. Estas amidas han sido encontradas en más de 10 familias de angiospermas: Aristolochiaceae, Asteraceae, Brasicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae y Solanaceae (Ramírez-Chávez *et al*., 2004). La estructura general de las alcamidas se origina de la condensación de una cadena acilo insaturada y una amida. Aunque existen alcamidas de diferente longitud en su cadena acilo, en general contienen un doble enlace 2*E* conjugado al grupo carbonilo sustituido con un grupo *N*-isobutilo (Ríos-Chávez *et al*., 2003).

Entre las plantas que acumulan alcamidas en sus tejidos están *Echinacea purpurea*, *Echinacea angustifolia* y *Heliopsis longipes,* comúnmente los extractos de estas plantas son utilizados en el tratamiento del resfriado común así como en el tratamiento de infecciones cutáneas y de las vías respiratorias (Ramírez-Chávez *et al*., 2004; Raduner *et al*., 2006).

La afinina (*N*-isobutil-2*E*,6*Z*,8*E*-decatrienamida) es la principal alcamida producida por la raíz de *Heliopsis longipes* (Figura 10A), representando hasta el 1% del peso seco total. Molina-Torres *et al*. (2004) evaluaron el efecto fungistático y bacteriostático de la afinina y dos alcamidas obtenidas por reducción catalítica de la afinina: *N*-isobutil-2*E*-decanamida y *N*-isobutil-decanamida, encontrando que solamente la afinina presenta una actividad fungitóxica, mientras que la *N*-isobutil-2E-decanamida mostró una alta actividad bacteriostática contra *Escherichia coli* y *Erwinia caratovora*.

Las alcamidas modulan el crecimiento vegetal como lo reportan Kanbe *et al*. (1993) quienes encontraron que la amidenina, una alcamida aislada del actinomiceto *Amycolatopsis* sp., estimula la producción de biomasa en plantas de arroz (*Oryza sativa*). Ramírez-Chávez *et al*. (2004) evaluaron el efecto de la afinina y dos alcamidas derivadas de la afinina: la *N*-isobutil-2*E*-decanamida y la *N*-isobutil decanamida, sobre el desarrollo de la raíz de *Arabidopsis*. La afinina en concentración de 1.2×10^{-4} M inhibió el crecimiento de la raíz primaria en un 70% comparada al control, mientras que las otras dos alcamidas derivadas de la afinina resultaron ser más

activas en la estimulación del crecimiento de los pelos radiculares. El efecto de las alcamida fue independiente de la vía de señalización de auxinas, como lo reveló el desarrollo normal de mutantes resistentes a auxinas en respuesta a tratamientos con alcamidas y por la nula capacidad de estos compuestos para afectar la expresión de los genes marcadores de auxinas *DR5:uidA* y *BA3:uidA* (Figura 10).

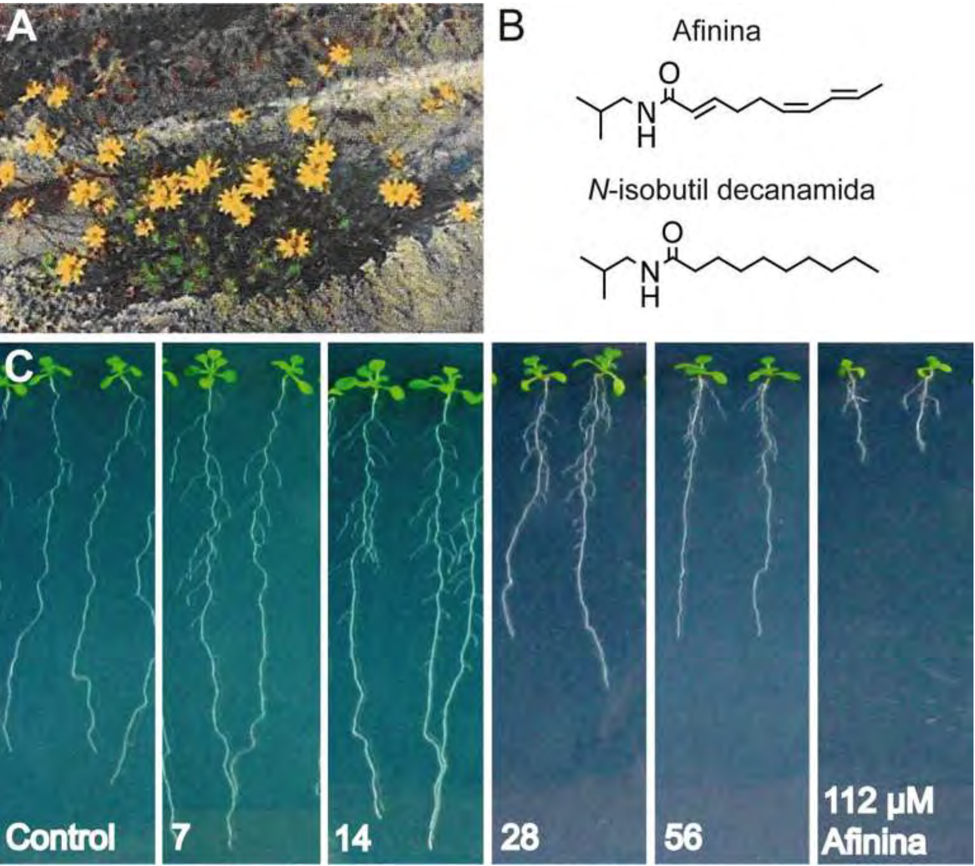

Figura 10. Efecto de las alcamidas en el sistema radicular de *Arabidopsis thaliana*. (A) Fotografía de una planta de *Heliopsis longipes*, en la raíz de esta planta se puede encontrar hasta el 1% de peso fresco a la afinina. Estructura química de dos alcamidas, afinina y *N*-isobutil decanamida. (C) Efecto de la afinina sobre la arquitectura de la raíz de *A. thaliana* (Modificado de Ramírez-Chávez *et al*., 2004)

Un estudio reciente en nuestro grupo de trabajo, indica que la longitud de la cadena acilo y el grupo amida juegan un papel crucial en la actividad biológica de las alcamidas, encontrando que la *N*-isobutil decanamida es el compuesto de mayor actividad regulando el crecimiento de la raíz primaria y la formación de raíces laterales (López-Bucio *et al*., 2007). Este compuesto indujo la formación de estructuras similares a callos en raíces primarias, láminas foliares ectópicas a lo largo de los peciolos de las rosetas y desarrollo de callos sobre la lámina foliar en lugar de los tricomas. Esta formación de órganos ectópicos por la *N*-isobutil

decanamida correlaciona con una expresión aumentada del marcador de división celular *CyCB1:uidA* y el incremento en la expresión del marcador inducible por citocininas *ARR5:uidA* tanto en las raíces como en el follaje. Además, se encontró que la actividad de la *N*-isobutil decanamida interactúa con la ruta de señalización de citocininas, ya que la triple mutante de los receptores de citocininas *CRE1/AHK2/AHK3* de *A. thaliana* mostró la ausencia de órganos ectópicos y estructuras callosas en las laminas de las hojas (López-Bucio *et al*., 2007).

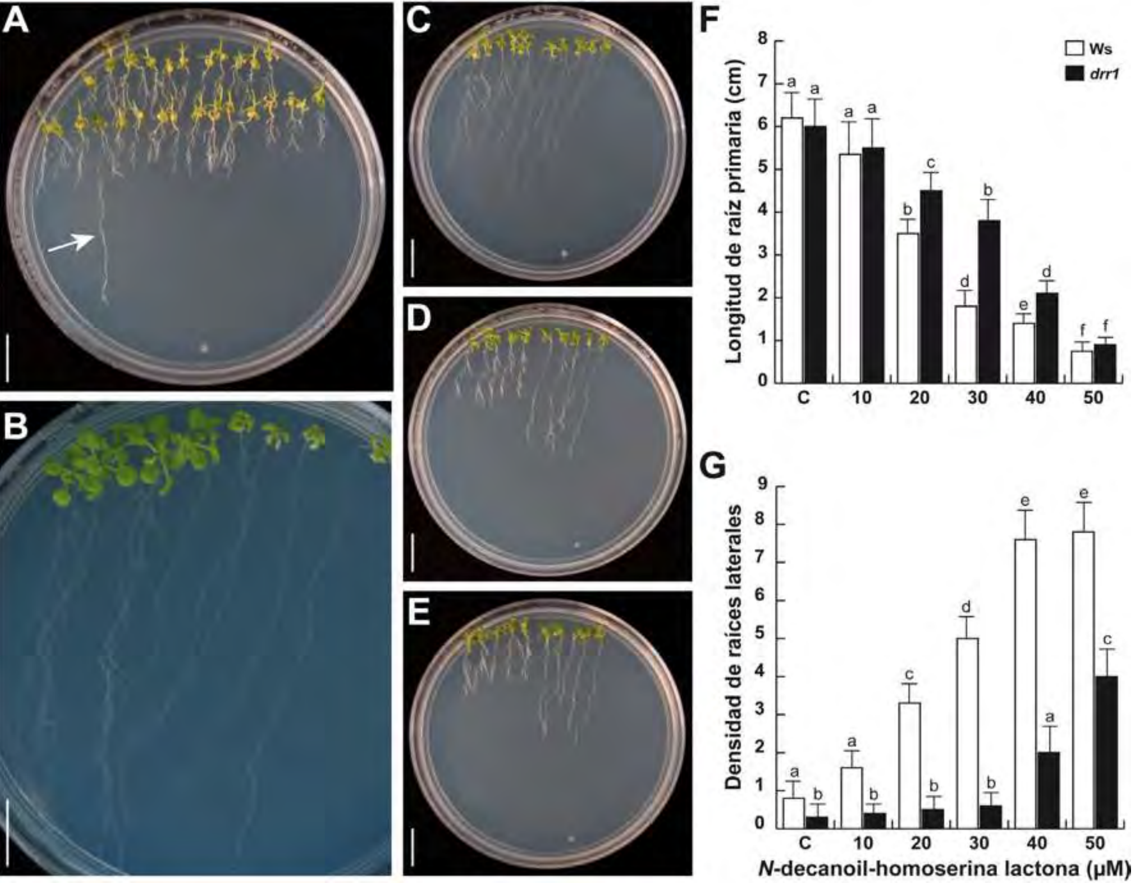

Figura 11. Escrutinio genético y caracterización fenotípica de la mutante *drr1*. (A) Imagen representativa del aislamiento de la mutante *drr1* (indicada por la flecha) resistente a la inhibición del crecimiento de la raíz primaria por decanamida. (B-C) Fotografías representativas del crecimiento lado a lado de plantas silvestres Ws y la mutante *drr1* crecidas en medios MS 0.2x (B), o suplementados con diferentes concentraciones de decanamida 20 µM (C), 25 µM (D) y 30 µM (E). (F-G) Efecto de la *N*-decanoil-homoserina lactona en el crecimiento de la raíz primaria (F) y densidad de raíces laterales (G) en la mutante *drr1* (Modificado de Morquecho-Contreras *et al.,* 2010).

Un análisis global de cambios en la expresión de genes en *A. thaliana*, en respuesta a *N*-isobutil decanamida, reveló la participación de redes transcripcionales de genes de defensa, en particular, genes que codifican enzimas para la biosíntesis de ácido jasmónico (AJ) y el receptor de AJ COI1 se indujeron en respuesta a alcamidas. Además, se demostró la participación del

óxido nítrico (NO) y peróxido de hidrógeno (H_2O_2) como mediadores de la respuestas a alcamida induciendo cambios en el desarrollo y confiriendo resistencia contra el patógeno *Botrytis cinerea* (Méndez-Bravo *et al.,* 2010; Méndez-Bravo *et al.,* 2011).

Para esclarecer el mecanismo de señalización de las alcamidas en el desarrollo, Morquecho *et al.* (2010), aislaron y caracterizaron una mutante resistente a la aplicación exógena de la *N*-isobutil decanamida que se caracteriza por un mayor crecimiento de la raíz primaría e inhibición en la formación de raíces laterales, la cual fue nombrada como *drr1* (*decanamide resistant root 1*; Figura 11).

El análisis de primordios de raíces laterales reveló que DRR1 es un elemento requerido en la activación de células del periciclo para la formación de primordios de raíces laterales en respuesta a *N*-isobutil decanamida. Interesantemente, las mutantes *drr1* crecidas en medios MS 0.2x suplementados con diferentes concentraciones de *N*-decanoil homoserina lactona (AHLs), molécula señal en bacterias Gram-negativas con una alta similitud estructural con las NAEs y alcamidas (Figura 12), mostraron resistencia a la inhibición del crecimiento de la raíz primaria. Estos resultados en su conjunto indican que las plantas poseen mecanismos para percibir NAEs, alcamidas y AHLs, las cuales modulan el desarrollo radicular (Ortiz-Castro *et al.,* 2008; Morquecho-Contreras *et al.,* 2010).

Bacteria

N-decanoil-homoserina lactona

Planta

N-etanol decanamida

N-isobutil decanamida

Figura 12. Similitud estructural de la *N*-decanoil homoserina lactona (C10-HL) y compuestos relacionados de plantas, *N*-etanol decanamida (NAE 10:0) y *N*-isobutil decanamida.

3.6 El quorum-sensing y la comunicación celular en las bacterias

Las bacterias utilizan moléculas pequeñas como señales químicas para la comunicación celular, esta comunicación necesita la producción, liberación y detección de factores hormonales, conocidos como autoinductores (Schauder y Bassler, 2001). Esto permite a las bacterias coordinar la expresión de genes dependientes de la densidad poblacional, en un proceso comúnmente denominado como "quorum-sensing" (QS) (Fuqua *et al*., 1994, Taga y Bassler, 2003; Reading y Sperandio, 2006). Cuando el número de células de una colonia bacteriana se incrementa, aumenta la concentración del autoinductor. Una vez que se alcanza una concentración umbral, se produce la unión del autoinductor al receptor dentro de las células bacterianas, activando las cascadas de transducción de señales que resulta en cambios en la expresión de genes en toda la población bacteriana. Entonces, mediante la producción de autoinductores, las bacterias actúan cooperativamente (Atkinson y Williams, 2009).

El QS juega un papel crítico en interacciones de bacterias patogénicas y simbióticas con el huésped. En relaciones simbióticas, los fenotipos modulados por QS incluyen bioluminescencia (e.j. *Vibrio fischeri*) o la formación de nódulos en la raíz para la fijación de nitrógeno (e.j. *Rhizobium leguminosarum*) (Praneenavat *et al.,* 2009; González y Keshavan, 2006; Williams, 2007; Sanchez-Contreras *et al.,* 2007; Williams *et al.,* 2007). Varios patógenos de importancia clínica usan el sistema de QS para regular procesos asociados con la virulencia; esto favorece la perspectiva de sobrevivencia porque se coordina la producción de factores de virulencia cuando la población bacteriana alcanza una alta densidad poblacional, incrementando la probabilidad de que las defensas del huésped sean abatidas con éxito (Geske *et al.,* 2007; Raina *et al.,* 2009). En *Pseudomonas aeruginosa*, el sistema de QS está involucrado en la formación de biopelículas y tolerancia a agentes antimicrobianos y al sistema inmune del huésped. Esta bacteria es una de los patógenos con más prevalencia en infecciones hospitalarias potencialmente mortales y es una de las principales causas de la mortalidad en pacientes con fibrosis quística (Rasmusen y Givskov, 2006; Popat *et al.,* 2008; Williams *et al.,* 2007).

Existen diferentes formas de comunicación entre bacterias, que se pueden agrupar de dos maneras: una es la comunicación vía péptidos y la otra es la comunicación vía acil homoserina lactonas, que son utilizadas por bacterias Gram positivas y Gram negativas,

respectivamente (Vandeville *et al.,* 2005; De Keersmaecker *et al.,* 2006; Xavier y Bassler, 2003; Duan *et al.,* 2003).

3.6.1. Regulación del quorum-sensing por *N*-acil-homoserina lactonas en bacterias Gram negativas

Diversas especies de bacterias Gram negativas usan *N*-acil homoserina lactonas (AHLs) para regular su comportamiento (Bosgelmez-Tinaz, 2003). Estos compuestos contienen un anillo homoserina lactona (HL) unido a una cadena de ácido graso mediante un enlace amida. La longitud de la cadena de ácido graso puede variar entre 4 a 18 carbonos, usualmente por incrementos de 2 carbonos. Estos pueden ser saturados o insaturados y con o sin sustituyentes en el carbono 3 (C-3) (Waters y Bassler, 2005; Camili y Bassler, 2006, Fuqua y Greenberg, 2002).

Uno de los primeros sistemas de QS descritos es el de *Vibrio fischeri* una bacteria marina bioluminiscente que se encuentra asociada con organismos marinos incluyendo peces y calamares (Nealson y Hastings, 1979). El sistema de quorum-sensing en *V. fischeri* está regulado por dos proteínas, LuxI y LuxR, que controlan la expresión del operón de la luciferasa (*luxICDABE*) requeridas para la producción de luz (Figura 13).

LuxI es la sintasa que produce el autoinductor 3-oxo-hexanoil-homoserina lactona (3-oxo-C6-HL), y LuxR es el receptor citoplásmico, cuyo complejo LuxR+3-oxo-C6-HL actúa como un activador transcripcional que se une al DNA, este complejo activa la transcripción del operón que codifica para la luciferasa, a su vez el complejo LuxR+3-oxo-C6-HL induce la expresión de *luxI* que se encuentra codificado en el operón de la luciferasa (Waters y Bassler, 2005). El circuito de QS descrito para *Vibrio fischeri* representa el paradigma básico de la señalización mediada por AHLs. En efecto, varias proteínas involucradas en la producción de AHLs han sido caracterizadas como AHL sintasas tipo LuxI, con una longitud de 190-230 aminoácidos y poseen 30-35% de identidad, manteniendo un residuo conservado de treonina en la posición 143 (Manefiel y Turner, 2002; Ng y Bassler, 2009). Estas señales químicas son producidas por enzimas específicas y detectadas por proteínas reguladoras (Parsek *et al*., 1999; Pearson *et al*., 1999). Además, se ha encontrado que la actividad específica de los diferentes compuestos puede estar determinada por el anillo lactona, el grupo amida y la longitud de la cadena de ácido graso (Tabla I) (Zhu *et al.,* 1998; Vannini *et al.,* 2002; Raffa *et al.,* 2004).

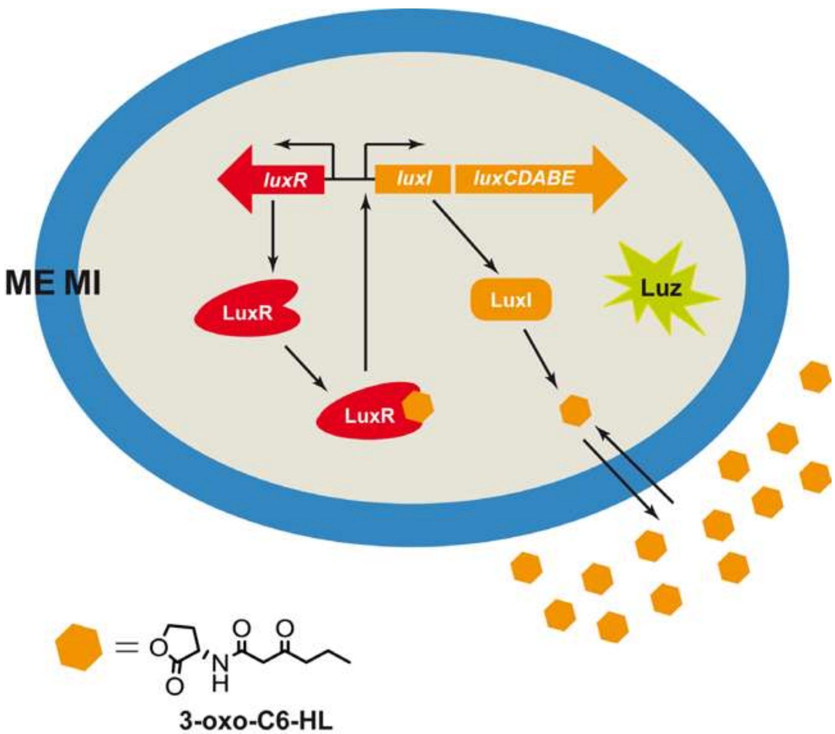

Figura 13. Mecanismo de quorum-sensing en *Vibrio fischeri*. El autoinductor 3-oxo-hexanoil homoserina lactona (3-oxo-C6-HL) es producido por la sintasa LuxI y secretado hacia el exterior de la célula. Un receptor tipo LuxR reconoce 3-oxo-C6-HL y se une a la región del promotor tipo Lux que regula la expresión de genes. ME, membrana externa; MI, membrana interna (Modificado de Waters y Bassler, 2005).

Los procesos de señalización por QS que ocurren en algunas bacterias Gram-negativas son con frecuencia más complejos que el descrito para *V. fischeri*. Varias especies bacterianas utilizan dos o más señales AHL, o inclusive otros tipos de moléculas para regular procesos celulares (Ng y Bassler, 2009; Geske *et al.,* 2008).

La síntesis de las AHLs por las sintasas del tipo LuxI generalmente proceden vía un mecanismo de reacción ordenado secuencialmente utilizando a la S-adenosilmetionina (SAM) como el donador amino para la formación del anillo homoserina lactona y una proteína transportadora del acilo (ACP) como el precursor de la cadena (Figura 14).

Tabla I. Ejemplos de sistemas de quorum-sensing tipo LuxR/LuxI de bacterias Gram negativas

Bacteria	Regulador	Señal	Función regulada
Vibrio fischeri	LuxR/LuxI	3-oxo-C6-HL	Bioluminiscencia.
Pseudomonas aeruginosa	LasR/LasI RhlR/RhlI	3-oxo-C12-HL C4-HL	Virulencia y desarrollo de biopelículas. Virulencia, producción de ramnolípidos y piocianina.
Agrobacterium tumefaciens	TraR/TraI	3-oxo-C8-HL	Número de copias del plasmido Ti y conjugación bacteriana.
Erwinia caratovora	CarR/CarI	3-oxo-C6-HL	Producción de antibióticos carbapenem y exoenzimas.
Burkholderia cenocepacia	CepR/CepI	C8-HL	Factores de virulencia, proteasas, lipasas.
Pseudomonas putida	PpuR/PpuI	3-oxo-C12-HL	Biopelículas.

Pseudomonas aeruginosa es una especie bacteriana en la que mejor se conoce el mecanismo de QS. Esta bacteria utiliza al menos tres sistemas, dos de los cuáles están mediados por AHLs, el sistema LasR-LasI y el sistema RhlR-RhlI regulados por 3-oxo-dodecanoil-HL (3-oxo-C12-HL) y butanoil-HL (C4-HL), respectivamente. Estos sistemas de señalización están integrados con un tercer sistema que emplea una molécula químicamente distinta a las AHL, la 2-heptil-3-hidroxi-4(1H)-quinolona, denominada como señal dependiente de quinolona de *Pseudomonas* o PQS (Figura 15; Williams *et al.,* 2007).

En *P. aeruginosa*, el activador transcripcional LasR funciona en conjunto con la *N*-3-oxo-dodecanoil-homoserina lactona (3-oxo-C12-HL), sintetizada por la sintasa LasI, el complejo LasR-3-oxo-C12-HL regula la expresión de genes de virulencia *lasB, lasA, aprA* y *toxA,* además de *lasI* por si misma. Un gen adicional *rsaL*, está bajo el control regulatorio de LasR-3-oxo-C12-HL, el

producto regula negativamente el QS de *P. aeruginosa* por la inhibición de la expresión de LasI (De Kievit *et al.,* 2000).

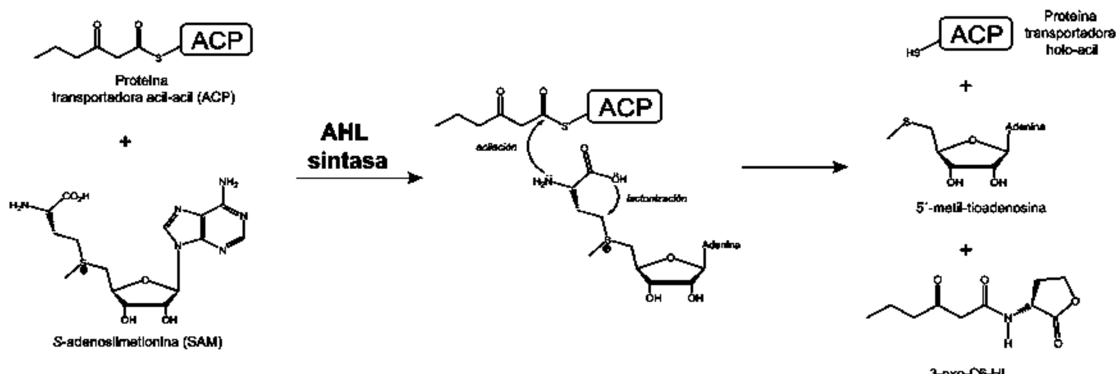

Figura 14. Esquema representativo que ilustra de manera general la ruta biosintética de las AHLs. ACP, proteína transportadora del acilo; SAM, S-adenosilmetionina y la acilo-ACP se unen a la sintasas de AHL (una sintasa tipo LuxI), donde se llevan a cabo reacciones de lactonización y acilación. La AHL (en este caso 3-oxo-C6-HL) es liberada junto con otros dos productos holo-ACP y 5'-metil-tioadenosina (Modificado de Galloway *et al.,* 2011).

El sistema rhl del QS consiste del activador transcripcional RhlR y la sintasa RhlI que dirige la síntesis de *N*-butanoil-homoserina lactona (C4-HL). El complejo RhlR-C4-HL regula la expresión de *rhlAB* requerido para producción de ramnolípidos, *lasB, aprA,* el factor de la fase estacionaria *RpoS* y la producción de metabolitos secundarios como piocianina y cianuro (De Kievit *et al*., 2000).

El quorum sensing en *P. aeruginosa* juega un papel importante en la patogenicidad de este organismo. El análisis de las mutantes *lasI*, *rhlI* y una doble mutante *lasI-rhlI*, han mostrado una reducción de su virulencia, particularmente en la doble mutante (De Kievit *et al.,* 2000, Pearson *et al.,* 2000). La idea de que LasR se encuentra en la parte superior del sistema regulatorio de QS en *P. aeruginosa* y que LasR regula la actividad de RhlR y PqsR, se basa en el efecto de mutaciones nulas en *lasR* que efectivamente inhabilitan el QS. Sin embargo, se han encontrado tanto en laboratorio como en aislados clínicos mutantes nulas LasR, con los sistemas RhlR y PqsR activos produciendo factores de virulencia, lo que nos habla de la complejidad del sistema. En un estudio reciente, Lee *et al.,* (2013) identificaron un gen que codifica para una sintasa de péptidos no ribosomales denominada *ambB,* que cuando es mutada afecta la activación de LasR, RhlR y PqsR en condiciones de deficiencia de fosfato. Bajo las condiciones de deficiencia de fosfato la bacteria no produce la señal que activa a RhlR y PqsR,

indicando que AmbB y otras tres proteínas codifican para el operón *ambBCDE* el cual está involucrado en la producción de la señal 2-(2-hidroxilfenil)-tiazol-4-carbaldehido (IQS). Se ha encontrado que la producción de la señal IQS esta dada por la proteína PhoB, la cual se activa en condiciones de deficiencia de fosfato en la ausencia de LasR.

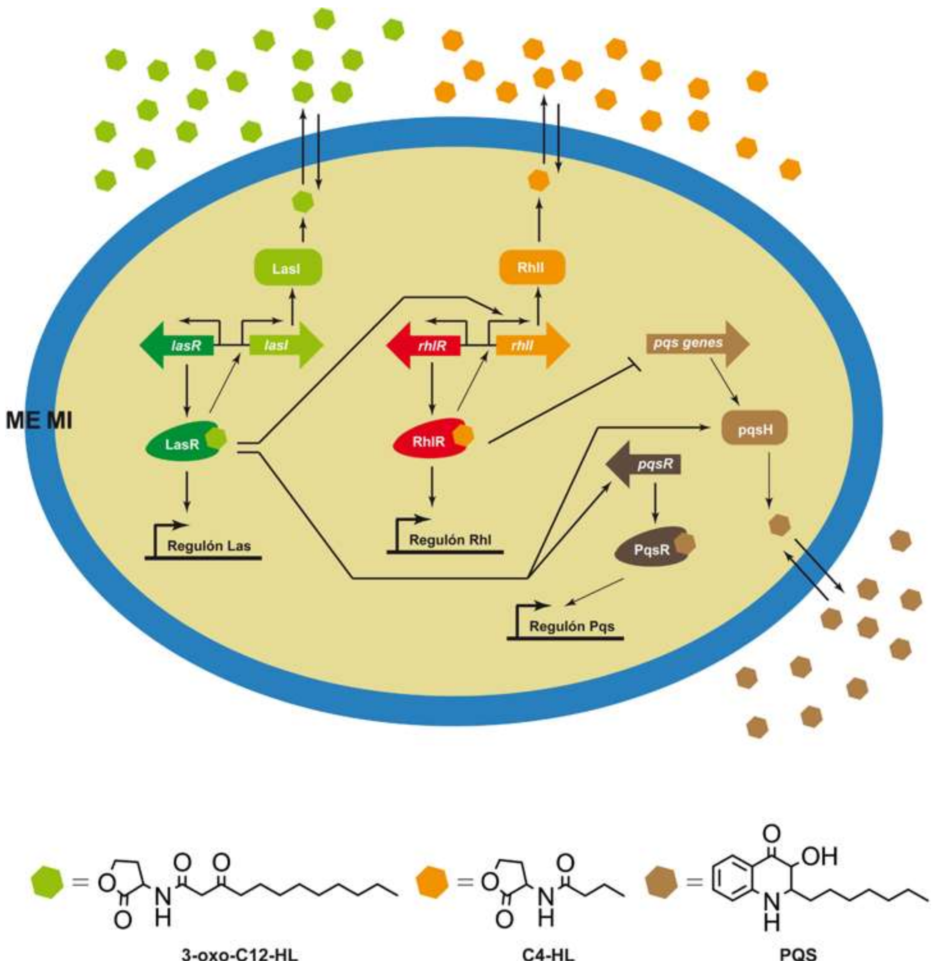

Figura 15. Sistema de QS Las, Rhl y Qsc en *Pseudomonas aeruginosa*. El sistema de QS de *P. aeruginosa* implica la participación de tres factores de transcripción, LasR, RhlR y PqsR. LasR se sitúa en la parte superior del circuito, entonces cuando la señal, 3-oxo-C12-HL, alcanza una concentración adecuada, LasR activa la trascripción de genes (regulón Las). El regulón Las incluye RhlR y PqsR, los cuales se activan en respuesta a sus señales blanco C4-HL y PQS, activando regulones Rhl y Pqs, respectivamente, que interaccionan a su vez con el regulón Las (Modificado de Williams *et al*., 2007; Lee *et al*., 2013).

3. 6. 2. AHLs como nuevos blancos en la regulación del sistema de quorum-sensing

Diversas bacterias patogénicas emplean el sistema de QS para el control de la expresión de genes y la producción de factores de virulencia. Se ha discutido acerca del papel de inhibidores de QS (QSI) en el control de factores de virulencia. La mayoría de los estudios en la

modulación química de la síntesis de AHLs están basados en el uso de varios análogos de la SAM, por ejemplo, S-adenosil-homocisteina (SAH), sinefungina y butiril SAM, son potentes inhibidores *in vitro* de la sintasa RhlI de *P. aeruginosa*. Se han examinado otros compuestos que bloquean la sintasa del autoinductor, entre ellos homologos y análogos de nucleótidos de purina, derivados de tiol y derivados de tiol alquilados, así como derivados de homoserina lactona (Figura 16; Galloway *et al.,* 2011).

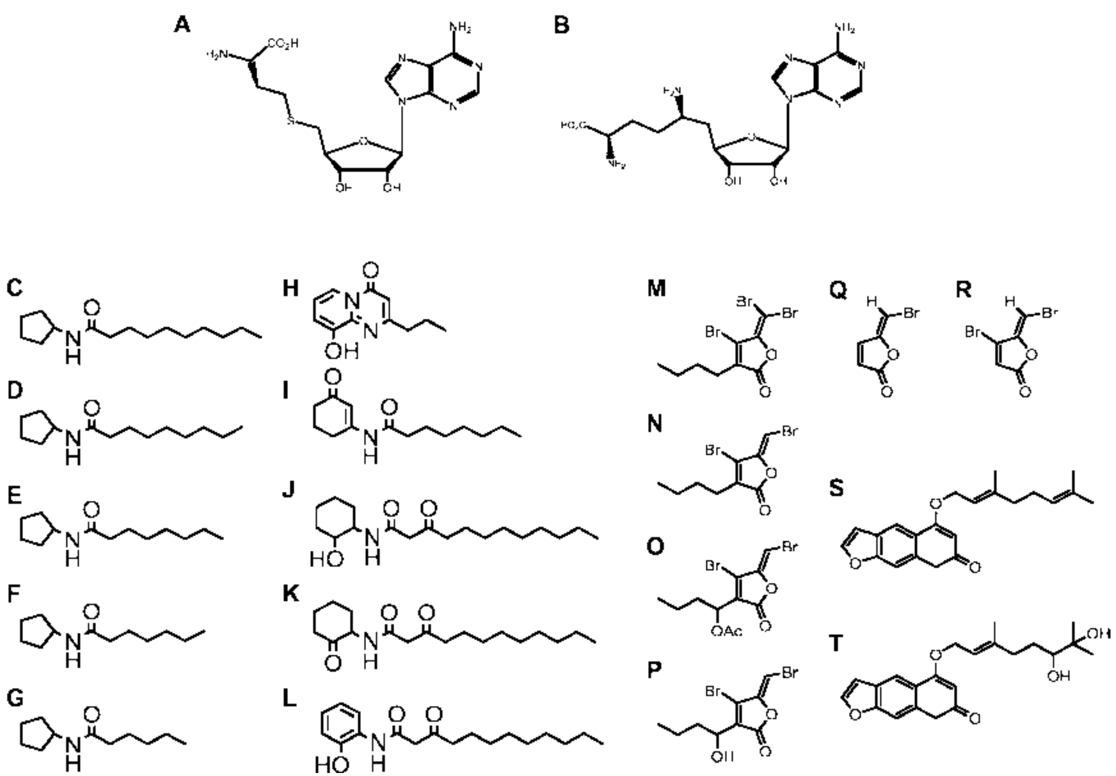

Figura 16. Inhibidores del quorum sensing. (A-B) Análogos en la inhibición de la síntesis de AHLs: (A) S-adenosilhomocisteína (SAH), (B) Sinefungina. (C-G) Diferentes *N*-acil-ciclopentilaminas con actividad biológica; C10-CPA (C), C9-CPA (D), C8-CPA (E), C7-CPA (F), C6-CPA (G). (H-I) Inhibidores de unión a AHL sintetasas y reguladores: J8-C8 (H), E9C-3-oxo-C6 (I), *N*-2-hidroxiciclohexil-3-oxododecanamida (J), N-2-oxociclohexil-3-oxododecanamida (K), N-2-hidroxifenil-3-oxododecanamida (L). (M-P) Furanonas naturales derivadas de *D. pulchra*. (Q-R) Furaronas sintéticas derivadas con actividad inhibitoria. (S-T) Compuestos aislados del jugo de toronja con actividad inhibitoria del QS, Bergamotina (S) y dihidroxibergamotina (T) (Modificado de Rasmussen *et al*., 2005, Ishida *et al*., 2007, Galloway *et al.,* 2011).

Algunos metabolitos secundarios de plantas pueden regular el QS en *P. aeruginosa* (Givskov *et al.,* 1996). *Delissea pulchra* produce furanonas halogenadas, que son antagonistas naturales del QS, afectando la motilidad en *Serratia liquefaciens* (Hentzer *et al.,* 2002; Rassmusen *et al.,* 2000; Figura 16). Las furanonas naturales de *D. pulchra* son incapaces de inhibir el sistema de QS de *P. aeruginosa*. Con base en lo anterior se han dirigido investigaciones

en la identificación de nuevos compuestos naturales y análogos sintéticos (Rassmussen y Givskov, 2006; Hentzer y Givskov, 2003). Girennavar *et al.,* (2008) aislaron dos furocoumarinas, la bergamotina y la hidroxibergamotina, del jugo de toronja. Estos compuestos estructuralmente parecidos a las furanonas de *D. pulchra* afectan funciones reguladas por el QS en diferentes especies bacterianas, incluyendo la formación de biopelículas en *E. coli* O157:H7, *Salmonella entérica* y *P. aeruginosa* (Girennavar *et al.,* 2008). Por otra parte, Hentzer *et al.* (2003), reportaron la síntesis de un análogo que carece de la cadena alquilo de la furanona de *D. pulcra* que es capaz de interferir con el sistema de QS mediado por AHL en *P. aeruginosa*, en un sistema de infección pulmonar en ratones, disminuyó la severidad del daño pulmonar y severidad de la infección.

En un estudio de Ishida *et al*., (2007), se encontró que la *N*-decanoil-ciclopentilamida (C10-CPA) es un fuerte inhibidor del QS en *P. aeruginosa* y las concentraciones requeridas para la inhibición máxima media de la expresión de *lasB-lacZ* y *rhlA-lacZ* fueron de 80 y 90 μM, respectivamente. Además, la C10-CPA inhibe la producción de factores de virulencia, incluyendo elastasa, piocianina y ramnolípidos, y formación de biopelículas sin afectar su crecimiento. También se afectó la inducción de *lasI-lacZ* por *N*-3-oxo-dodecanoil-homoserina lactona y *rhlA-lacZ* por *N*-butanoil-homoserina lactona en la mutante *lasI-rhlI* de *P. aeruginosa,* indicando que C10-CPA interfiere con los sistemas de quórum-sensing *lasI* y *rhlI* vía la interacción entre sus reguladores (LasR y RhlR) y el autoinductor. Las Cn-CPA de cadena corta, C6, C7 y C8-CPA inhibieron el sistema de QS Lux de *V. fischeri* (Wang *et al.,* 2008), por lo que las ciclopentilamidas representan una clase general de análogos de AHLs capaces de antagonizar los sistemas de QS LuxR, LasR, Rhl y Spn, este último sistema de *Serratia marcescens*, regulado por C9-CPA (Morohoshi *et al.,* 2007).

Burkholderia glumae BGR1 produce una toxoflavina, una fitotoxina que actúa como un factor de virulencia en la pudrición bacteriana del grano de arroz, en esta bacteria la síntesis de la toxina es controlada por un sistema de QS TofI/TofR mediado por la AHL, *N*-octanoil-HL (C8-HL). Chung *et al.* (2011), sintetizaron dos compuestos con actividad inhibitoria, encontrando que el compuesto J8-C8 inhibe la sintasa, uniéndose a TofI, ocupando el sitio de unión de cadena acilo del sustrato de la proteína transportadora de acilos. Por otra, parte se identificó un segundo inhibidor, E9C-3-oxo-C6, el cual compite con C8-HL para unirse a TofR (Figura 16).

Estudios recientes por Dong *et al.* (2000) mostraron que una enzima de *Bacillus* sp. 240 B1 codificada por el gen *aiiA* es capaz de degradar AHLs*.* La enzima cataliza la hidrólisis del enlace lactona de la AHL. Plantas transgénicas que expresan esta lactonasa, muestran resistencia a la infección por *E. carotovora* y retardan el desarrollo de los síntomas de la pudrición. Por otra parte, Lin *et al.,* (2003) clonó al gen *AiiD*, que codifica para una nueva y potente acilasa de AHLs en un aislado de *Ralstonia*. *AiiD* hidroliza la amida, liberando la homoserina lactona y el correspondiente ácido graso. La expresión de *AiiD* en *P. aeruginosa* PAO1 reprime el quórum-sensing en esta bacteria, disminuyendo la producción de elastasa y piocianina.

Las alcamidas y NAEs representan un interesante grupo de compuestos naturales, los cuales podrían interferir con el sistema de QS en la bacteria. La similitud estructural de NAEs y alcamidas está muy relacionada con las *N*-acil ciclopentamidas (*N*-acil-CPA), las cuales muestran una fuerte actividad para inhibir el QS, interesantemente, la *N*-decanoil-ciclopentilamida (C10-CPA) es la que muestra una mayor actividad inhibitoria de los factores de virulencia, incluyendo elastasa y pyocyanina e interfiere con los sistemas de QS *rhl* y *las* de *P. aeruginosa* (Ishida *et al.,* 2007).

3.6.3. Papel de las rizobacterias en la estimulación del crecimiento y desarrollo vegetal

Las raíces de las plantas están rodeadas por una porción de suelo, denominada rizósfera (Bais *et al.,* 2006; Walker *et al.,* 2003), a la que se liberan una amplia variedad de compuestos incluyendo azúcares, ácidos orgánicos y vitaminas, los cuales son utilizadas como nutrientes o señales por las bacterias (Badri *et al.,* 2009; Rudrappa *et al.,* 2008). Una gran variedad de bacterias benéficas proliferan en esta región (Hiltner, 1904; Smalla *et al.,* 2006; van Loon, 2007). Por otra parte, las bacterias liberan fitohormonas (ej. AIA, AAB, citocinina ó giberelinas) afectando procesos celulares en sus huéspedes eucariotas (Tsavkelova *et al.,* 2005; Tsavkelova *et al.,* 2006; Costacurta y Vanderleyden, 1995; van Loon, 2007).

El efecto positivo de rizobacterias promotoras del crecimiento vegetal (PGPRs) ha sido estudiado en cultivos anuales, tales como trigo, haba, lechuga, frijol, maíz y cebada (Kloepper *et al.,* 1990; Barazani y Friedman, 1999; Badri *et al.,* 2009). Las PGPRs ejercen su efecto a través de la fijación del nitrógeno atmosférico (ej. la simbiosis de *Rhizobium* con leguminosas) o la

producción de fitohormonas y otros compuestos que influyen en el desarrollo vegetal como en la producción de AIA por *Azospirillum brasilense* (Figura 17; van Loon, 2007; Zakharova *et al.,* 1999; Barazani y Friedman, 1999; Brencic y Winans, 2005; Gray y Smith, 2005).

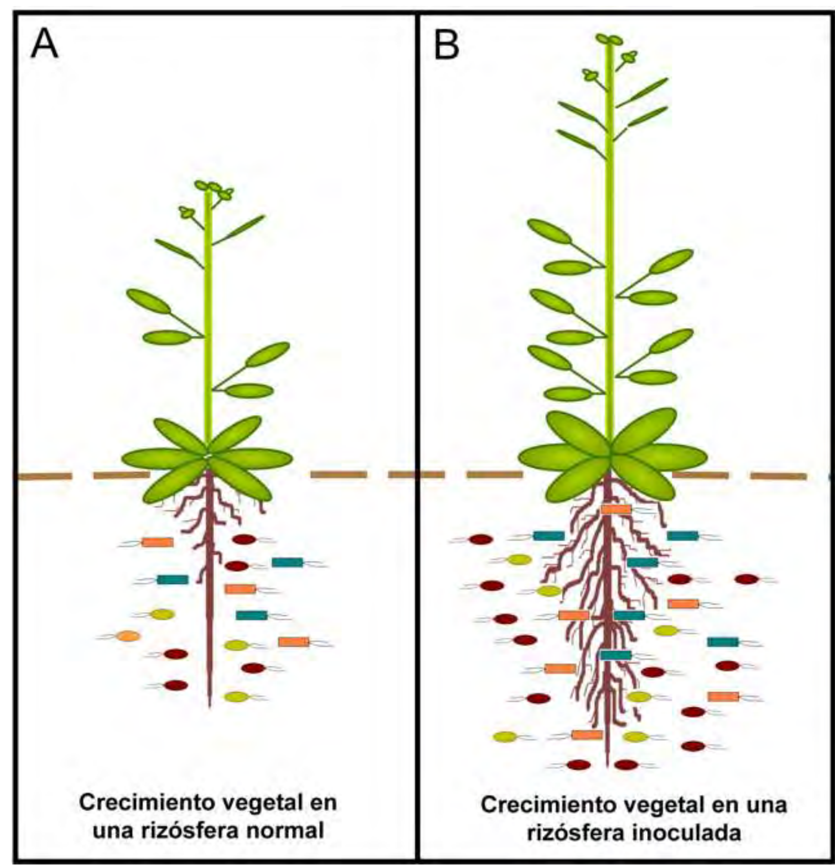

Figura 17. Las rizobacterias promotoras del crecimiento vegetal (PGPRs). Las bacterias de la rizosfera tienen la capacidad de producir fitohormonas (ej. auxinas, citocininas), las cuales modifican el sistema radicular para la toma de agua y nutrientes e incrementan la productiva vegetal y/o activan respuestas inmunes (Modificado de Ortiz-Castro *et al*., 2009).

Varias PGPRs pueden conferir inmunidad contra el ataque de patógenos foliares mediante la activación de defensas de las planta, reduciendo de este modo la susceptibilidad al daño (van Loon *et al.,* 2007).

La estimulación del desarrollo radicular es uno de los principales efectos de las PGPRs (Glick *et al.,* 1995; Patten and Glick, 2002). El rápido establecimiento de las raíces, ya sea por la proliferación de las raíces adventicias o pelos radiculares, resulta en un ventaja para las plantas incrementando el potencial exploratorio del sistema radicular necesario para la toma de agua y nutrientes. Diversas PGPR sintetizan fitohormonas incluyendo al AIA, AIB, o sus precursores, de esta manera regulan positivamente el crecimiento radicular (Spaepen *et al.,* 2007; Ortiz-Castro

et al., 2008; Dodd *et al.,* 2010; Martínez-Morales *et al.,* 2003; Spaepen *et al.,* 2007). Las auxinas son cuantitativamente las fitohormonas más abundantes secretadas por especies de *Azospirillum*, y en general se considera que la producción de auxinas es el principal factor responsable de la estimulación del desarrollo del sistema radicular y la promoción del crecimiento vegetal (Spaepen *et al.,* 2007).

3.6.4. Participación de las *N*-acil-L-homoserina lactonas en la interacción planta-bacteria

Las bacterias asociadas a plantas son capaces de comunicarse por medio de AHLs, esto es crucial para interacción de patógenos de plantas y simbiontes con el huésped, lo que ha planteado la posibilidad de que las plantas sean capaces de reconocer estos compuestos y responder ajustando su metabolismo (Elasri *et al.,* 2001; Cha *et al.*, 1998; Khmel *et al.,* 2002; D'Angelo-Piccard *et al.,* 2005; Pierson y Pierson, 2007). Las angiospermas producen compuestos que afectan las respuestas reguladas por el QS en la bacteria, ya que exudados de la raíz de plántulas de chícharo (*Pisum sativum*) o *Medicago truncatula* contienen sustancias que mimetizan AHLs y afectan diversos procesos celulares en bacterias asociadas a plantas (Teplitski *et al.,* 2000; Gao *et al.,* 2003).

Un primer reporte indicando que las plantas pueden percibir las AHLs fue publicado por Mathesius *et al.* (2003), quienes mostraron que *Medicago truncatula* responde a la aplicación de AHLs mediante cambios en la producción de proteínas, afectando respuestas de defensa y estrés, procesamiento de proteínas, producción de hormonas y elementos de citoesqueleto, así como también el metabolismo primario y secundario. Posteriormente, se encontró que la presencia de bacterias productoras de AHLs en la rizósfera de tomate induce respuestas de defensa dependientes de ácido salicílico y etileno, las cuales juegan un papel importante en la activación de resistencia sistémica en plantas y confiere resistencia al hongo patógeno *Alternaria alternata* (Schuhegger *et al.,* 2006). El hecho de que ciertas mutantes de *Rhizobium* alteradas en la producción o respuesta a AHLs fueran incapaces de nodular plantas de leguminosas respalda la hipótesis de que las AHLs pueden participar en la interacción simbiótica (Rosemeyer *et al.,* 1998; Daniels *et al.,* 2002; Zheng *et al.,* 2006).

Gao *et al.* (2003) encontraron en exudados de la raíz de *Medicago truncatula,* al menos 15 sustancias separables cromatográficamente capaces de estimular o inhibir respuestas en

bacterias que expresan genes reporteros de QS. Otro estudio muestra que los metabolitos secundarios de plantas como el ácido salicílico, que juega un papel importante en varios procesos fisiológicos, como la resistencia sistémica al ataque de patógenos, regula los factores de virulencia de *P. aeruginosa* PA14 la cual muestra una reducida formación de biopelícula en las raíces de *A. thaliana* (Prithiviraj *et al*., 2005).

En un estudio reciente en nuestro grupo de trabajo, se evaluó el efecto de AHLs con diferente longitud de la cadena del ácido graso desde 4 hasta 14 carbonos en la cadena saturada, encontrando que las AHLs modulan la arquitectura del sistema radicular inhibiendo el crecimiento de la raíz primaria y promoviendo la formación de raíces laterales y el desarrollo de pelos radiculares. La *N*-decanoil-HL (C10-HL) fue el compuesto con mayor actividad biológica induciendo la formación de raíces laterales y desarrollo de pelos radiculares (Figura 18), la cual está muy relacionada en su estructura química con las NAEs y alcamidas de plantas, esto abre la posibilidad de que las plantas puedan percibir a las AHLs, NAEs y alcamidas por un mecanismo genético común (Ortiz-Castro *et al.,* 2008; López-Bucio *et al.,* 2006).

Utilizando una estrategia transcriptómica en *A. thaliana*, von Rad *et al.* (2008) documentaron cambios en la expresión de genes en la planta en respuesta a *N*-hexanoil-HL (C6-HL), una señal de QS producida por *Serratia liquefaciens* MG1. En este trabajo se demostró que la C6-HL modula la expresión de genes involucrados en la biosíntesis de auxinas, mientras que los niveles de citocininas fueron reducidos indicando que las AHLs modifican la proporción de auxinas/citocininas. La C10-HL causa una diferenciación de células en la región del meristemo primario, la cual esta relacionada con la reducción en la expresión de marcadores de división celular *CycB1:GUS* y *pPRZ:GUS*. Esta respuesta ocurre a través de una vía independiente de la señalización de auxinas, ya que la C10-HL no incrementa la expresión de genes de respuesta a auxinas y mutantes relacionadas a auxinas, *aux1-7*, *axr2* y *doc1*, muestran una inhibición del crecimiento similar al observado en plantas silvestres. Las líneas mutantes y sobreexpresoras de un gen de la amido hidrolasa de ácidos grasos (AtFAAH) tienen alteradas las respuestas de crecimiento a C10-HL, esto sugiere que la planta posee la maquinaria genética para metabolizar AHLs (Ortiz-Castro *et al.,* 2008).

Jin *et al.* (2012) demostraron que la percepción de las AHLs por las plantas requiere de la participación de dos receptores, Cand2 y Cand7, acoplados a proteínas G (GPCRs), los cuales regulan el crecimiento de la raíz y las interacciones entre plantas y bacterias. Por otra parte, se

demostró que en raíces de *Arabidopsis* tratadas con *N*-3-oxo-hexanoil-HL (3-oxo-C6-HL) y *N*-3-oxo-octanoil-HL (3-oxo-C8-HL) ocurre un incremento en la elongación celular en la raíz. El análisis genético revela que las mutantes insercionales de T-ADN de *gcr1*, las cuales codifican para una GPCR GCR1 fueron insensibles a 3-oxo-C6-HL o 3-oxo-C8-HL en la estimulación del crecimiento de la raíz. Mutantes con pérdida de función de la subunidad Gα de GPA1 no muestran respuesta a la AHL en la elongación de la raíz, mientra que plantas con ganancia de función en la subunidad Gα muestran un efecto exagerado en la elongación de la raíz por las AHLs. Además, la expresión de GCR1 y GPA1 fueron sobrereguladas bajo tratamientos con AHLs, indicando que las proteínas GCR1 y GPA1 están probablemente involucradas en elongación de las raíces de *Arabidopsis* mediadas por las AHLs (Liu *et al.,* 2012).

En las plantas, los procesos de desarrollo están modulados por la participación de segundos mensajeros como Ca^{2+}, óxido nítrico (NO) y peróxido de hidrógeno (H_2O_2) que afectan múltiples procesos fisiológicos, incluyendo resistencia sistémica adquirida, respuesta hipersensible, senescencia foliar, muerte celular programada, cierre estomático, gravitropismo, estructura de la pared celular, desarrollo de la raíz, e interacciones polen-estigma (Tuteja y Mahajan, 2007; Knight *et al,* 1997; Grant y Loake, 200; Neill *et al.,* 2002; Hu *et al.,* 2004; Mitter *et al.,* 2004; Shuhegger *et al.,* 2006; Xu *et al.,* 2004; Mitter *et al.,* 2004; Xu *et al.,* 2008). En plántulas de *Arabidopsis* tratadas con 10 µM de C4-HL, ocurrió un incremento en la concentración del Ca^{2+} citosólico libre. Este estudio sugiere que C4-HL puede actuar como un elicitor o molécula señal en plantas a través del Ca^{2+} (Sung *et al.,* 2011). Bai *et al.,* (2012), demostraron que la 3-oxo-C10-HL induce la formación de raíces adventicias y la expresión de genes regulados por auxinas en explantes de *Vigna radiata* vía la señalización de cGMP, dependiente de H_2O_2 y ON (Bai *et al.,* 2012). La capacidad de las plantas para detectar AHLs producidas por las rizobacterias en la rizosfera y la similitud de estas señales de QS a las alcamidas y NAEs abre la posibilidad de que en las plantas hayan evolucionado proteínas receptoras y rutas de transducción de señales para comunicarse con las rizobacterias.

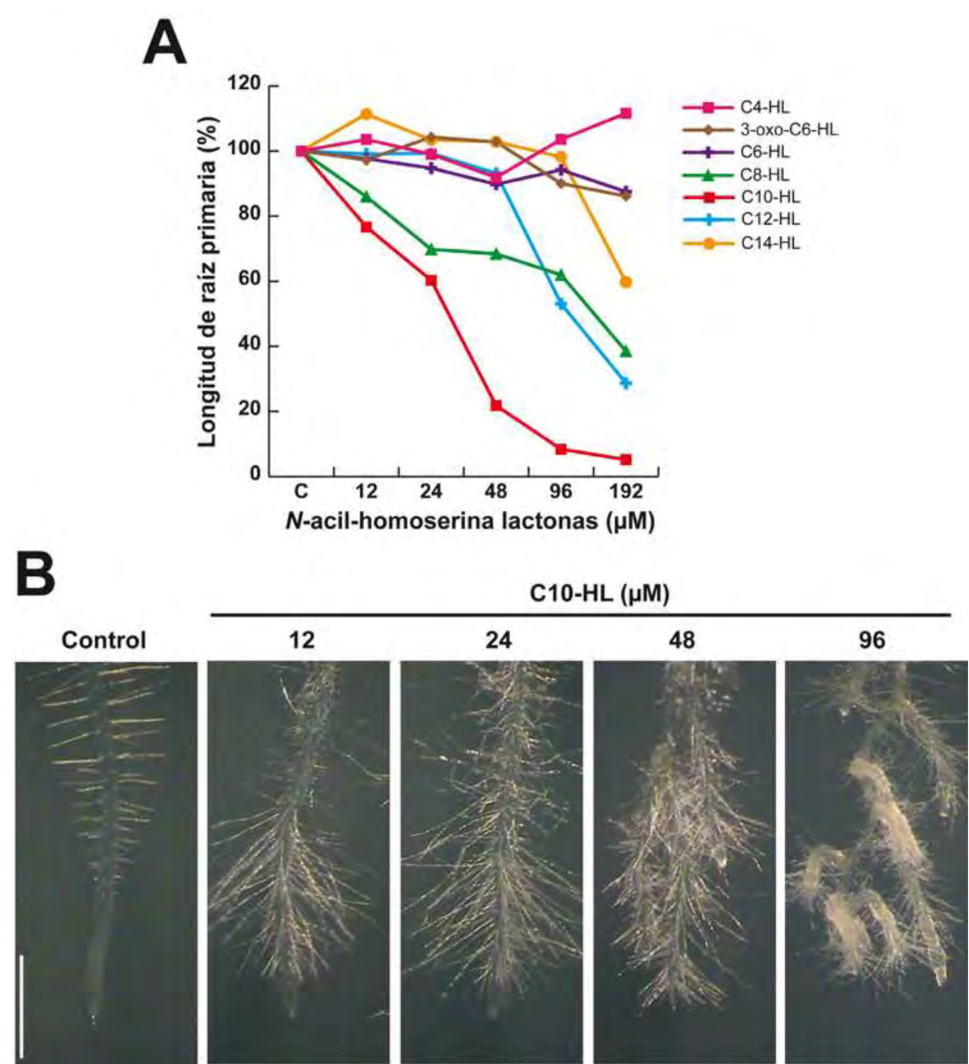

Figura 18. Efecto de las *N*-acil homoserina lactonas sobre la arquitectura del sistema radicular de *Arabidopsis*. Las plantas de *A. thaliana* fueron crecidas en medio MS 0.2x suplementados con diferentes concentraciones de AHLs, de diferente longitud desde 4 hasta 14 carbonos en su cadena de ácido graso. (A) Longitud de la raíz primaria. (B) Fotografías representativas de la raíz primaria de plantas de *Arabidopsis* de 9 días de edad crecidas en la presencia de la AHL indicada (Modificado de Ortiz-Castro *et al*., 2008).

3.6.5. Papel de los ciclodipéptidos en las interacciones planta-bacteria

Los ciclodipéptidos (CDPs) y sus derivados las dicetopiperazinas (DCPs) también conocidas como 2,5-dioxopiperazinas, 2,5-dicetopiperazinas o dipeptidos anhidros, forman parte de una clase novedosa de metabolitos secundarios sintetizados por una gran variedad de microorganismos (Gondry *et al.,* 2009), incluyendo especies bacterianas como *Vibrio* sp (Park *et al.,* 2006), *Pseudomonas putida* (Degrasi *et al.,* 2002), *Lactobacillus* sp. (Lee *et al.,* 2011; Ström

et al., 2002), *Bacillus subtilis* (Lu *et al.,* 2009), *Burkholderia cepacia* (Wang *et al.,* 2010) y en la anémona marina *Nemastostelle vectensis* (Seguin *et al.,* 2011) (Figura 19).

Las ciclodipeptido sintetasas (CDPSs) constituyen una familia de enzimas formadoras de enlaces peptídicos que usan aminoacil-tARNs (aa-tARNs) como sustrato para formar varios ciclodipeptidos. La familia de CDPS incluye al menos nueve miembros identificados en varias especies (Sauguet *et al.,* 2011). La CDPS AlbC de *Streptomyces noursei* utiliza principalmente phenilalanina-tARN^{Phe} (Phe-tARN^{Phe}) y leucil-tARN^{Leu} (Leu-tARN^{Leu}) como sustratos para sintetizar ciclo(L-Phe-L-Leu) (Sauguet *et al.,* 2001) y la anemona marina *Nemastostelle vectensis*, sintetiza varios CDs que preferencialmente contienen triptófano (Seguin *et al.,* 2011). Algunos DCPs, tales como ciclo(His-Gly), ciclo(His-Ala), y ciclo(L-His-L-Phe) muestran actividad antitumoral reduciendo la viabilidad de las células HeLa, WHCO3 y MCF-7 de células de carcinoma de cuello uterino, esófago y mamaria (Kanoh *et al.,* 1999; Lucietto *et al.,* 2006). Otros compuestos incluyendo ciclo(L-Phe-L-Pro) y ciclo(L-Ile-L-Pro) que fueron aislados de cepas de *Propionibacterium* muestran actividad contra *Aspergillus fumigatus* y *Rhodotorula mucilaginosa* (Lind *et al.,* 2007), mientras que el CD ciclo(L-Arg-D-Pro) inhibe el crecimiento del patógeno humano *Candida albicans* (Houston *et al.,* 2002).

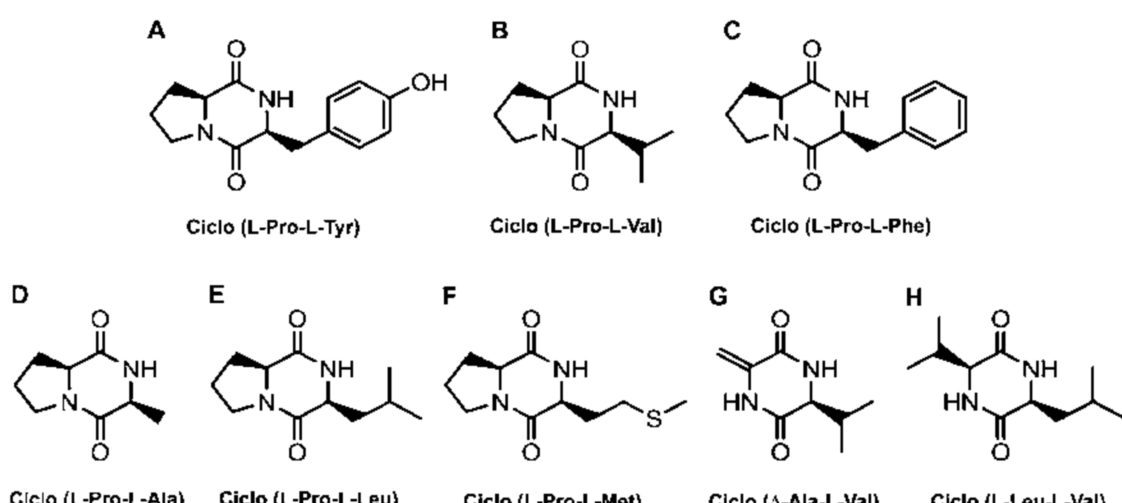

Figura 19. Estructura química de algunos ciclodipeptidos con actividad biológica.

En la búsqueda de moléculas de QS de *Vibrio vulnificus* que pudieran estimular cepas reporteras dependientes de AHLs, Park *et al.,* (2006), identificaron el ciclodipeptido ciclo(Phe-Pro). El ciclo(Phe-Pro) es liberado al medio de cultivo de una manera dependiente de la

densidad poblacional, con un máximo de concentración cuando la bacteria se encuentra en la fase estacionaria. La adición del ciclo(Pro-Phe) ya sea purificado o sintetizado, altera la expresión de los factores de virulencia en varias especies de *Vibrio* sp., representando una posible molécula regulatoria que contribuye en la patogénesis de esta bacteria (Park *et al.,* 2006; Klose, 2006). Se ha propuesto que los DCPs pueden actuar como bloqueadores del QS, por ejemplo el ciclo(L-Pro-L-Phe) inhibe la luminescencia en *V. fischeri* (Campbell *et al.,* 2009). Estudios de competencia muestran que ciclo(L-Pro-L-Tyr) y ciclo(L-Phe-L-Pro) antagonizan al 3-oxo-C6-HL en la inducción de la bioluminiscencia, sugiriendo que esos DCPs pueden competir por el mismo sitio de unión de las AHLs (Holden *et al.,* 1999). Por otro parte, el ciclo(L-Leu-L-Pro) señal producida por *Achromobacter xylosoxidans* inhibe la producción de la aflatoxina de *Aspergillus parasiticus*, reprimiendo la transcripción de genes relacionados con la biosíntesis de la aflatoxina (Yan *et al.,* 2004).

La producción de los DCPs no solamente se ha reportado en especias patogénicas, también en bacterias tipificadas como benéficas de plantas (PGPRs) y animales. Degrassi *et al.,* (2002), encontraron que la bacteria promotora del crecimiento vegetal *Pseudomonas putida* WCS358 produce y secreta ciclo(L-Phe-L-Pro), ciclo(L-Tyr-L-Pro), ciclo(L-Leu-L-Pro) y ciclo(L-Leu-L-Val) con un posible papel en la comunicación célula a célula y promoción del crecimiento vegetal. Por otra parte, en un reporte reciente se demostró que la bacteria benéfica de humanos *Lactobacillus reuteri* produce los ciclodipeptidos ciclo(L-Tyr-L-Pro) y ciclo(L-Phe-L-Pro), los cuales regulan negativamente la expresión de la toxina-1 de *Staphylococcus aureus*, un patógeno humano y la toxina de cólera y producción de pilus regulados por la toxina en *Vibrio cholerae*, disminuyendo su virulencia (Bina y Bina, 2010; Li *et al.,* 2011). Estos hallazgos vinculan directamente a los CDPs con las propiedades de virulencia o estimulación del crecimiento de las bacterias en su interacción con organismos de diferentes reinos.

4. JUSTIFICACIÓN

La producción de AHLs por *Pseudomonas aeruginosa* regula la arquitectura de la raíz de *Arabidopsis thaliana* y es un factor clave en la estimulación del crecimiento vegetal. Esto evidencia la existencia de mecanismos de señalización que regulan las respuestas del crecimiento y desarrollo de las plantas a las alcamidas y AHLs y determinan las relaciones de patogenicidad y simbiosis con bacterias del género *Pseudomonas.* Sin embargo, no se conocen los mecanismos implicados en esta interacción y se desconocen los efectos de las alcamidas de las plantas sobre procesos celulares bacterianos. Investigar estos procesos sin duda contribuirá al mejor entendimiento de las relaciones planta-bacteria con un amplio potencial biotecnológico en el campo de la agricultura.

5. HIPÓTESIS

Las bacterias Gram negativas estimulan el crecimiento vegetal a través de la producción de *N*-acil-homoserina lactonas (AHLs) o moléculas cuya producción está regulada por el mecanismo de quórum-sensing.

6. OBJETIVOS

6.1. OBJETIVO GENERAL

Evaluar el papel *in vivo* de la producción de compuestos bioactivos en bacterias del género *Pseudomonas* sobre el crecimiento y desarrollo vegetal de *Arabidopsis thaliana*.

6.2. OBJETIVOS PARTICULARES

1. Identificar los compuestos producidos por especies de *Pseudomonas* que modifican el crecimiento y desarrollo de *A. thaliana*.
2. Elucidar el mecanismo de acción de los compuestos bacterianos identificados en las especies de *Pseudomonas* a nivel celular, fisiológico y molecular en *A. thaliana*.

7. RESULTADOS.

Los principales resultados generados durante la realización del presente proyecto de tesis se presentan en los siguientes capítulos:

7.1. CAPÍTULO I

Ortiz-Castro R., Díaz-Pérez C., Martínez-Trujillo M., del Río R., Campos-García J., López-Bucio J. (2011). Trans-kingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proc. Natl. Acad. Sci.* USA. 108(17):7253-7258.

7.2. CAPÍTULO II

Ortiz-Castro R., López-Bucio J. (2013). Small molecules involved in transkingdom communication between plants and rhizobacteria. *Molecular Microbial Ecology of the Rhizosphere*. Frans J. De Bruijn (Ed). Vol. I, Wiley-Blackwell, New Jersey. pp. 295-307.

7.3. CAPÍTULO III

Ortiz-Castro R., Campos-García J., López-Bucio J. (2013). Rapid identification of plant-growth-promoting rhizobacteria using an agar plate cocultivation system with *Arabidopsis*. *Molecular Microbial Ecology of the Rhizosphere*. Frans J. De Bruijn (Ed). Vol. I, Wiley-Blackwell, New Jersey. pp. 345-353.

7.4. CAPITULO IV

En este capítulo se muestran los resultados sobre el efecto de *Pseudomonas putida* y *Pseudomonas fluorescens* en la estimulación del crecimiento vegetal. Este trabajo se encuentra en revisión en la revista *Plant and Soil.*

7.5. CAPITULO V

En este capítulo se muestran los resultados obtenidos sobre el efecto de la piocianina, un factor de virulencia producido por *Pseudomonas aeruginosa*, en el desarrollo post-embrionario de *Arabidopsis thaliana*. Este trabajo se encuentra en revisión en la revista *Molecular Plant-Microbe Interactions*.

CAPÍTULO I.

Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants

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Edited* by Luis Herrera Estrella, Center for Research and Advanced Studies, Irapuato, Mexico, and approved March 17, 2011 (received for review May 13, 2010)

Microorganisms and their hosts communicate with each other through an array of signals. The plant hormone auxin (indole-3-acetic acid; IAA) is central in many aspects of plant development. Cyclodipeptides and their derivative diketopiperazines (DKPs) constitute a large class of small molecules synthesized by microorganisms with diverse and noteworthy activities. Here, we present genetic, chemical, and plant-growth data showing that in *Pseudomonas aeruginosa*, the LasI quorum-sensing (QS) system controls the production of three DKPs—namely, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr)—that are involved in plant growth promotion by this bacterium. Analysis of all three bacterial DKPs in *Arabidopsis thaliana* seedlings provided detailed information indicative of an auxin-like activity, based on their efficacy at modulating root architecture, activation of auxin-regulated gene expression, and response of auxin-signaling mutants *tir1*, *tir1 afb2 afb3*, *arf7*, *arf19*, and *arf7arf19*. The observation that QS-regulated bacterial production of DKPs modulates auxin signaling and plant growth promotion establishes an important function for DKPs mediating prokaryote/eukaryote transkingdom signaling.

root development | lateral roots | root hairs | phytostimulation | plant–bacteria interactions

The communication between bacteria and their hosts through interkingdom signaling is a recent field of research. This field evolved from the initial observation that bacteria can communicate through hormone-like signals—a process known as quorum sensing (QS) (1). The field expanded with the realization that these bacterial signals can modulate mammalian (2) and plant (3) cell-signal transduction, and that host hormones can cross-signal with QS molecules to modulate bacterial gene expression (4, 5). A predominant type of small-molecule autoinducer, *N*-acyl-L-homoserine lactone (AHL), is used by Gram-negative bacteria (6, 7). AHLs are synthesized from S-adenosyl methionine (SAM) and particular fatty acid carrier proteins by AHL synthases (1). AHLs all share the core homoserine lactone moiety, but distinct fatty acid side chains are incorporated into the signal molecules by their respective AHL enzymes. Small-to-medium–chained AHLs cross membranes freely and bind in the cytoplasm to transcription factors, which upon ligand binding, regulate the transcription of QS-controlled genes (1, 4).

Both pathogenic and symbiotic plant-associated bacteria require QS to successfully interact with their hosts (8, 9). However, plants have evolved multiple mechanisms to interpret these QS signals. Small concentrations of AHLs caused substantial changes in gene expression in *Medicago truncatula* and *Arabidopsis thaliana*, affecting primary metabolism, plant-hormone responses, and root system architecture (3, 10, 11). Bacteria that inhabit the rhizosphere may also influence plant growth by producing phytohormones, such as auxins (12). Application of indole-3-acetic acid (IAA) or IAA-related metabolites stimulates lateral root (LR) and root hair formation, which may increase water and nutrient acquisition, leading to increased biomass production (12). Consistent with this, several *Arabidopsis* mutants with defective auxin transport, perception, or signaling, including *aux1*, *axr2*, *tir1*, and *tir3*/*doc1*/*big* have been identified that show reduced root hair and LR formation or decreased plant size (13).

Plant/bacteria communication can be achieved by means of different metabolites, some of which can mimic the activity of endogenous phytohormones. Cyclodipeptides and their derivative diketopiperazines (DKPs) constitute a class of small molecules synthesized by a wide range of microorganisms that exhibit diverse and useful biological activities. For example, cyclo(L-Phe-L-Pro) and cyclo(L-Phe-*trans*-4-OH-L-Pro) act as antifungal compounds (14), and epipolythiodioxopiperazines show antitumor, antibacterial, antiviral, and immunosuppressive properties (15, 16). These compounds are synthesized by a family of tRNA-dependent peptide bond-forming enzymes termed cyclodipeptide synthases (17). Although DKPs are noteworthy bioactive molecules, there is limited information concerning the regulation of DKP biosynthesis in bacteria and its role in plant signaling.

Results

QS-Modulated Plant Growth Promotion by *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* has been used as a bacterial model to understand QS regulated by AHLs. *P. aeruginosa* has two mainly AHL-dependent QS systems—the *las* and *rhl* systems. In the *las* system, the LasI AHL synthase directs the synthesis of 3-oxo-C12-AHL, which interacts with the transcription factor LasR to target gene promoters. In the *rhl* system, the RhlI synthase directs the synthesis of C4-AHL, which interacts with the cognate regulator RhlR and controls transcription of target genes (6, 7, 18). We tested the in vivo effect of *P. aeruginosa* on plant growth by cocultivating 4-d-old *Arabidopsis thaliana* seedlings grown on agar plates containing 0.2× Murashige and Skoog (MS) medium with $\sim 2.8 \times 10^8$ cfu of *P. aeruginosa* PAO1 WT and the *P. aeruginosa* AHL synthase-deficient mutants *lasI*, *rhlI*, and *rhlI*/*lasI* double mutant by streaking the bacteria on the surface of the medium at a 5-cm distance from the primary root tip (Fig. 1*A*). After 8 d of growth in the presence of *P. aeruginosa* WT, a significant increase in shoot and root biomass production was observed (Fig. 1 *B* and *C*), which correlated with altered *Arabidopsis* root system architecture (RSA; Fig. 1 *D* and *E*). With comparable growth for all bacterial WT and mutant strains on the plant-bacteria interacting medium, the *lasI* single and *rhlI*/*lasI* double mutant exhibited lower primary root growth inhibition but greater formation of lateral roots and root hairs compared with the WT strain or the *rhlI* single *P. aeruginosa* mutant (Fig. 1 *D* and *E* and Fig. S1). Interestingly, altered RSA correlated with significantly increased shoot and root biomass

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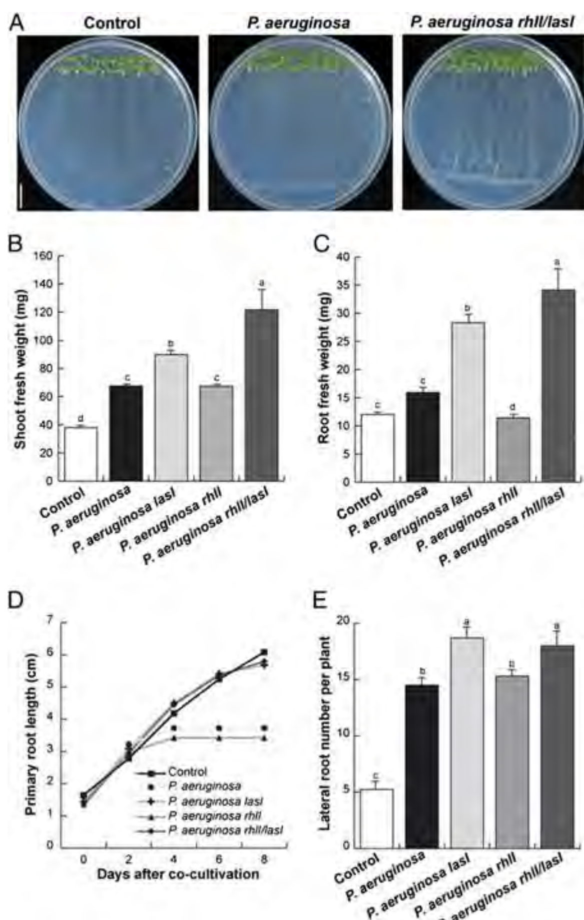

Fig. 1. Effect of cocultivation with *P. aeruginosa* WT and QS mutant strains on root development and plant growth promotion. Four-day-old *A. thaliana* seedlings were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or LasI/RhlI at a distance of 5 cm from the primary root tip, and grown for an additional 8-d period. (*A*) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings cocultivated with WT *P. aeruginosa* and *P. aeruginosa rhlI/lasI* double mutant. (Scale bar = 1 cm.) (*B*) Effect of bacterial cocultivation on shoot biomass production or (*C*) root biomass production. Data from *B* and *C* show the means ± SD from three groups of 30 seedlings. (*D*) Effect of bacterial cocultivation on *Arabidopsis* primary root growth. Day 0 indicates the length reached by the primary root at the moment of bacterial application. Mean ± SD values were plotted at the indicated days in the kinetic experiment (n = 30). (*E*) Effect of bacterial cocultivation on lateral root formation. Date points represent mean ± SD (n = 30). These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P < 0.05$.

production in plants cocultivated with *P. aeruginosa lasI* single mutants and with a nearly threefold-enhanced growth promotion by the *rhlI*/*lasI* double mutant (Fig. 1 *B* and *C*). This plant growth-promoting (PGP) effect could also be observed in plants cocultivated with bacteria at very close (1 cm) distance from the root tip, in which *P. aeruginosa lasI* and *rhlI*/*lasI* strains could directly contact the root system, and increased by fivefold shoot and root fresh weight (Fig. S2). These findings suggest that AHL signals produced by the AHL synthases LasI and RhlI modulate the production of compounds directly involved in biomass production, and cell division and differentiation processes in the root.

P. aeruginosa Produces DKPs Capable of Stimulating LR Development in _Arabidopsis_. Diverse bacterial species possess the ability to produce the auxin phytohormone IAA (12). To search for IAA or IAA-related substances, EtOAc extracts of WT *P. aeruginosa* cell-free culture supernatants were assayed for their ability to stimulate LR initiation by counting lateral root primordia (LRP; Fig. 2 *A* and *B*). Three active fractions were identified (P6, P7, and P8) with peak retention times of 7, 7.5, and 12 min, respectively. The supply of purified fractions strongly increased stage A LRP production (Fig. 2*B*). The corresponding active peaks dramatically accumulated in *P. aeruginosa lasI* and *rhlI*/*lasI* mutant extracts (Fig. 2*C*). The molecular identity of purified peaks 6, 7, and 8 was resolved by GC/mass spectrometry and further confirmed by ^{1}H NMR and ^{13}C NMR spectra analysis as diketopiperazines (DKPs) cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe), respectively (Fig. 2 *D*–*F* and Fig. S3). ^{13}C NMR (100 MHz) spectra analysis and δ-values data of each purified compound show that the carbon number is in agreement with DKP molecular structures obtained for MS and ^{1}H NMR analysis (Fig. S3). Our finding that in the *P. aeruginosa lasI* and *rhlI*/*lasI* mutants all three DKPs increase in concentration suggests that DKP biosynthesis is regulated by the LasI/LasR QS system.

Chemical Complementation of _P. aeruginosa lasI_ and _rhlI/lasI_ Mutants. Application of commercially available C4-AHL and 3-oxo-C12-AHL compounds to the growth medium of WT and *P. aeruginosa*

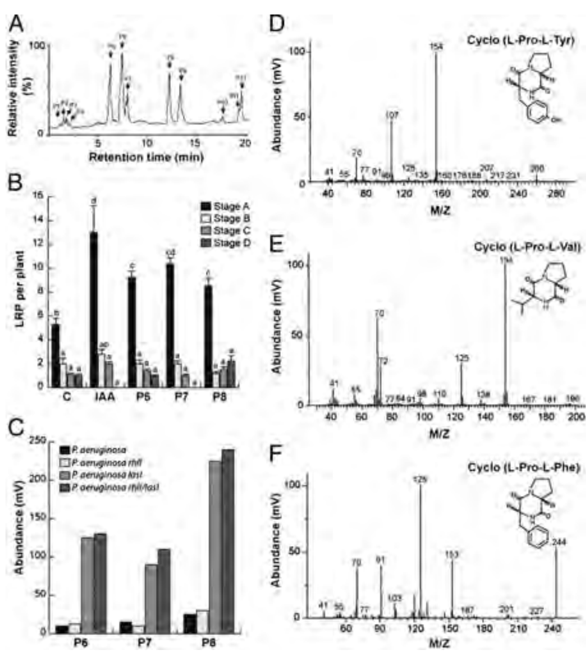

Fig. 2. Identification and characterization of DKPs produced by *P. aeruginosa*. (*A*) Representative HPLC semipreparative chromatogram from culture supernatants of WT *P. aeruginosa*. Arrows indicate the collected peaks that were tested for activity on lateral root development in *A. thaliana*. (*B*) *Arabidopsis* Col-0 seedlings were germinated and grown for 6 d on the surface of agar plates containing 0.2× MS medium and transferred into 24-well cell culture plates (10 seedlings per well) and grown in 0.2× MS liquid medium containing 3 μM IAA or 30 μM of each DKP for 12 h, then cleared and LRP recorded according to Zhang et al. (39) for 10 independent roots. Data points represent mean ± SD. (*C*) Relative abundance of P6, P7, and P8 in ethyl acetate extracts from 1-L cultures of WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or LasI/RhlI subjected to GC/mass spectrometry. Data points represent the mean relative abundance (×10^7 mV). (*D*–*F*) Mass spectra of P6, P7, and P8 fractions purified by HPLC and analyzed under GC/mass spectrometry. (*D*) P6, cyclo(L-Pro-L-Tyr; *m/z* = 260), (*E*) P7, cyclo(L-Pro-L-Val; *m/z* = 196), and (*F*) P8, cyclo(L-Pro-L-Phe; *m/z* = 244).

mutant strains cocultivated with *A. thaliana* seedlings showed that only 3-oxo-C12-AHL normalized primary root growth inhibition and root hair development by *lasI* or *rhlI*/*lasI* strains, as observed in seedlings cocultivated with WT *P. aeruginosa* or *rhlI* single mutant (Fig. S4). Chemical complementation of single *lasI* or double *rhlI*/*lasI* mutants by 3-oxo-C12-AHL revealed that regulation of plant growth and development by *P. aeruginosa* is likely controlled by the LasI QS system.

Bacterial DKPs Modulate Auxin Responses in *Arabidopsis*. Lateral root growth and root hair formation are tightly regulated by auxin (13). The peculiar heterocyclic system of DKPs can be found in IAA and other compounds with auxin activity (19). The finding that cocultivation of *Arabidopsis* seedlings with *lasI* and *rhlI*/*lasI* mutants also leads to plants with enhanced lateral root and root hair formation prompted us to evaluate whether DKPs could act as auxin signal mimics. To determine if the enhanced production of DKPs by *P. aeruginosa lasI* mutants could affect auxin signaling in plants, *Arabidopsis* transgenic seedlings expressing the auxin-inducible *DR5:uidA* marker (20) were cocultivated with WT *P. aeruginosa* or *lasI* mutant. In aseptically grown seedlings, *DR5:uidA* is expressed primarily in the root tip region (Fig. 3*A*). *DR5:uidA* seedlings supplied with 3 μM IAA showed a strong GUS activity throughout the primary root (Fig. 3*B*), indicating activated auxin responses. The pattern of GUS expression in *DR5:uidA* seedlings cocultivated with WT *P. aeruginosa* remained similar to that observed in axenically grown plants (Fig. 3*C*). In contrast, in plants cocultivated with *P. aeruginosa lasI* mutant, there was a very clear increase in expression of this marker in the entire primary root (Fig. 3), indicating that the LasI QS system regulates the biosynthesis of compound(s) with auxin activity. Next, we tested the activity of all three DKPs on *DR5:uidA* expression in root tips by transferring 6-d-old seedlings grown on 0.2× MS solidified medium to 0.2× MS liquid medium supplied with IAA or DKPs, respectively. Fig. 3 *E* and *F* shows histochemical staining for transgenic *DR5:uidA* seedlings that were treated with IAA or the different DKPs. A dose-dependent GUS expression in plants treated with cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Val) was clearly observed, whereas cyclo (L-Pro-L-Phe) showed less activity (Fig. 3*F*). In a similar assay, a second auxin response marker—namely, *BA3:uidA* (21)—was activated by IAA and all three DKPs (Fig. S5). These results show that bacterial DKPs can activate auxin-inducible gene expression in *Arabidopsis* seedlings.

The biological activity of IAA and cyclo(L-Pro-L-Tyr) was also tested in relation to primary root growth. IAA inhibited primary root growth in nanomolar concentrations, whereas much greater concentrations of cyclo(L-Pro-L-Tyr) were required for growth-repressing effects (Fig. S5), indicating weak auxin activity for this DKP.

DKPs Enhance Aux/IAA Protein Degradation and Require a Canonical Auxin Signaling Pathway for Activity. Auxin is perceived by direct binding to the transport inhibitor response 1 (TIR1) protein, a member of a small family of F-box proteins (22, 23). This interaction accelerates the Skp1, Cdc53/Cullin1, F-box protein ubiquitin ligase-catalyzed degradation of Aux/IAA repressor proteins, allowing derepression of auxin-regulated genes by auxin response transcription factors (ARFs) (24). We next compared the effect of IAA and DKPs on auxin-mediated degradation of Aux/IAA proteins using the *Arabidopsis HS::AXR3NT-GUS* line (24). Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37 °C for 2 h and further treated with 3 μM IAA, or 30 μM of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) for 60 min. Treatment with DKPs showed enhanced degradation of the fusion protein in a similar way to IAA, but greater

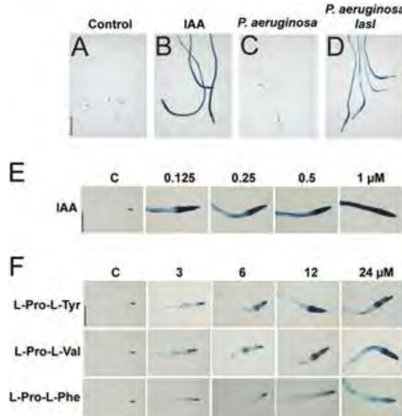

Fig. 3. Effect of bacterial DKPs on auxin responses in *A. thaliana*. (*A–F*) Twelve hours of β-glucuronidase (GUS) staining of *DR5:uidA* primary roots supplied with the solvent (*A*), with 3 μM IAA (*B*), cocultivated with WT *P. aeruginosa* for 8 d (*C*) or with *P. aeruginosa lasI* mutant for 8 d (*D*). (*E*) Effect of IAA or (*F*) purified DKPs on *DR5:uidA* gene expression in transgenic seedlings grown on 0.2× MS agar medium for 6 d and then transferred into 24-well cell culture plates (10 seedlings per well) containing 2 mL 0.2× MS liquid medium supplied with the indicated concentrations of compounds and incubated for 10 h. Seedlings were stained for GUS activity and cleared for microscopy analysis. Photographs show representative individuals from at least 30 stained plants. (Scale bars = 500 μm.)

concentrations of the compounds were required to achieve the same effect on *HS::AXR3NT-GUS* degradation (Fig. S5). Our data indicate that DKPs likely act in the auxin-mediated signaling pathway, possibly by direct binding to an auxin receptor, which rapidly destabilizes the AXR3 protein.

We performed a computational molecular docking analysis of DKP affinity to the *Arabidopsis* TIR1 receptor using the published crystallized TIR1 structure with the Aux/IAA7 peptide. This analysis revealed only one conformation cluster for all three DKPs with the same orientation into TIR1, which mimics the binding of IAA or 2,4-D (Fig. S6), suggesting that DKPs can fit in the TIR1 binding pocket.

To determine whether the TIR1 family of auxin receptors and ARFs are involved in *Arabidopsis* responses to DKPs, we analyzed LR formation in response to cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) in WT Col-0 *Arabidopsis* seedlings and in *tir1-1* and *tir1afb2afb3*, single and triple mutants, respectively, and in *arf7-1*, *arf19-1*, and *arf7arf19* mutants. In solvent-treated WT seedlings, cyclo(L-Pro-L-Val) and cyclo(L-Pro-L-Phe) increased total LR number per seedling (Fig. 4). *tir1-1* mutants had a roughly 25% reduction in LR number, observed in WT seedlings in solvent media. Interestingly, the increase in LR formation observed in WT seedlings when treated with DKPs was clearly reduced in *tir1-1* mutants (Fig. 4). When a triple *tir1afb2afb3* mutant was analyzed, it was found that LR formation was not stimulated by DKP treatment (Fig. 4). The single *arf7-1* mutant displayed a significant reduction in LR number compared with WT seedlings in solvent media; in addition, the stimulation of LR formation by DKPs was reduced in both *arf7-1* and *arf19-1* single mutants. The *arf7arf19* double mutant was completely insensitive to DKPs in terms of increased lateral root formation (Fig. 4). A kinetic experiment monitoring primary root growth and lateral root formation revealed that WT *P. aeruginosa* similarly inhibited primary root growth in WT and all five auxin-related mutant lines tested, whereas *arf7arf19* and *tir1afb2afb3* lines were resistant to lateral root induction both by *P. aeruginosa* WT or *lasI* mutants (Fig. S7).

Discussion

LasI QS-Controlled DKP Production Enhances Plant Growth Promotion Capability of *P. aeruginosa* In this work, we explored genetically whether AHL QS is involved in growth and development of *Arabidopsis* evaluating the effects of cocultivation with *P. aeruginosa lasI*, *rhlI* and *rhlI*/*lasI* single and double mutants on plant biomass production and root architectural changes. We found that both shoot and root biomass production increased in *Arabidopsis* seedlings cocultivated with WT *P. aeruginosa*. Interestingly, growth promotion capability was further potentiated in *lasI* and *rhlI*/*lasI* mutants (Fig. 1 and Fig. S2), which correlated with decreased primary root growth inhibitory effect with the mutants compared with WT *P. aeruginosa* (Fig. 1). This primary root growth normalizing effect is apparently independent of the second bacterial AHL QS system RhlI, which produces C4-AHL and can be reverted by the inclusion of 3-oxo-C12-AHL in the agar medium (Fig. S4). It was noticeable that *lasI* and *rhlI* cocultivation strongly promoted LR formation and root hair development in *Arabidopsis* WT seedlings in a way that suggests that the effects of the bacteria are mediated by auxin (Fig. 1 and Figs. S1 and S2).

Our data are apparently contradictory to previous reports showing that *P. aeruginosa* is pathogenic to *Arabidopsis* (25–27). However, in their initial screen, Rahme et al. (25) evaluated a collection of 75 *P. aeruginosa* strains (30 human, 20 soil, and 25 plant isolates) for their ability to cause disease on leaves of four different *A. thaliana* ecotypes. Most strains elicited no symptoms, and only two strains, UCBPP-PA14, a human isolate, and UCBPP-PA29, a plant isolate, caused severe soft-rot symptoms in leaves of some, but not all, of the ecotypes tested (25). It is also important to note that *P. aeruginosa* pathogenicity tests have focused mainly on leaves, infiltrating thousands of bacteria into plant tissues, which are able to secrete a variety of potent degradative enzymes and virulence factors (25–27). In contrast, accumulating information shows the potential of *P. aeruginosa* as a phytostimulant (28). It is tempting to speculate that the issue of whether *P. aeruginosa* is a pathogen or a plant growth promoting bacterium or both would depend upon the specific assays and even with the initial concentrations of the inoculums.

Our detailed study is unique in that it characterizes the responses of *Arabidopsis* roots to *P. aeruginosa*. Our data shows that cocultivating *P. aeruginosa* in the vicinity of the primary root did not cause cell death or cell damage (Fig. S8), as revealed by *CycB1:uidA* (29) and *AtPRZ:uidA* (30) marker gene expression in the primary root meristem and expression of the cell nuclei marker *AtHistH2B:YFP* (31) by confocal laser scanning microscopy in seedlings stained with propidium iodide (PI). This finding suggests that root architecture remodeling under these conditions is unlikely due to a toxic effect, but instead by induction of cell differentiation processes at the root meristem region. Secretion of 3-oxo-C12-AHL by WT *P. aeruginosa* likely contributes to primary root inhibition by decreasing proliferative cell activity in the meristem, as application of the purified compound arrested cell division in the root meristem (Fig. S9). In consonance with these results, no symptoms of chlorosis or necrosis were detected in leaves of plants cocultivated with WT *P. aeruginosa* or QS-related mutants (Fig. S2).

In our experiments, we were unable to detect IAA from bacterial extracts, but instead found that three DKPs are produced by WT *P. aeruginosa* and are negatively regulated by the LasI AHL QS-controlled pathway. Each compound was purified to homogeneity by semipreparative HPLC, and its structure confirmed by MS and NMR spectroscopy as cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe; Fig. 2 and Fig. S3). Certain DKPs from *P. aeruginosa* and other Gram-negative bacteria, including cyclo(L-Ala-L-Val), cyclo(L-Pro-L-Tyr), and cyclo(L-Pro-L-Phe),

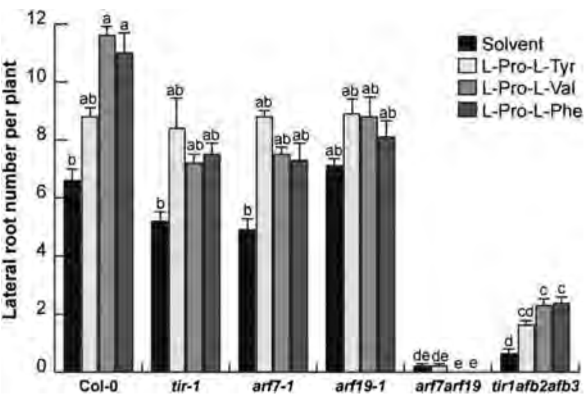

Fig. 4. *A. thaliana* WT and *tir1-1*, *arf7-1*, *arf19-1*, *arf7arf19*, and *tir1afb2afb3* mutant lines were germinated and grown for 6 d on 0.2× MS agar medium and transferred into 24-well cell culture plates (10 seedlings per well) and grown in 2 mL 0.2× MS liquid medium supplemented with 30 μM of each DKP for two additional days. Data points show the mean lateral root number per plant ± SD. Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated twice with similar results.

have been described as QS factors affecting bioluminescence and swarming motility (32). Whether these molecules modulate AHL-mediated QS in the producer organism affecting pathogenic or symbiotic relationships with plants, or in other organisms occupying a similar ecological niche, remains to be established.

Bacterial DKPs Show Auxin-Like Activity in *Arabidopsis*. The structure/activity relationship of auxin has been extensively investigated. Among more than 200 auxinic compounds identified, only two common features can be recognized as critical for auxin activity: a planar aromatic ring structure and a carboxyl group-containing side chain (19). The ring structure and its attached atoms on known auxinic compounds can vary significantly, suggesting a large degree of promiscuity. However, the two common features alone do not necessarily give rise to an auxin-like molecule. The DKPs identified in this work possess a heterocyclic system also found in IAA and other compounds with auxin activity. The effects of IAA, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr), and cyclo(L-Pro-L-Phe) on auxin-regulated gene expression suggests that all three DKPs show a weak auxin activity. Cocultivation of transgenic *Arabidopsis* seedlings expressing the auxin-inducible reporter constructs *DR5:uidA* or *BA3:uidA* with *P. aeruginosa lasI* mutant, or treatment with DKPs, clearly activated GUS expression in the root system. However, greater concentrations than IAA were required for DKPs to activate auxin-inducible gene expression (Fig. 3 and Fig. S5). Four additional lines of evidence indicate that DKPs may act as auxin signal mimics: (*i*) the effect of cyclo(L-Pro-L-Tyr) inhibiting primary root growth (Fig. S5); (*ii*) the effects of cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr), and cyclo(L-Pro-L-Phe) on Aux/IAA stability using the *Arabidopsis HS::AXR3NT-GUS* line (Fig. S5); (*iii*) the promotion of LR formation by DKP application to *Arabidopsis* WT seedlings; and (*iv*) the finding that DKPs no longer stimulate lateral root formation in auxin receptor mutants *tir1afb2afb3* and in *arf7arf19* (Fig. 4 and Fig. S7). Molecular docking analysis further predicted that DKPs might interact with the TIR1 auxin receptor (Fig. S6). These data suggest that the planar structure of DKPs is likely responsible of their activity as auxin signal mimics. These compounds might bind to the promiscuous auxin binding pocket of TIR1 with different affinities, as shown for synthetic auxins with varied structure such as naphthalene acetic acid (1-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichoropicolinic acid (picloram) (19, 33). The interaction between the F-box–containing protein (SCF) and the Aux/IAA was previously demonstrated in a pull-down assay in which TIR1-myc was recovered from plant extracts using recombinant Aux/IAA proteins in the absence and presence of auxin (34). These experiments showed that the interaction between TIR1 and the Aux/IAA proteins is dramatically enhanced by auxin. Whether DKPs elicit auxin responses by direct binding to TIR1 or AFB auxin receptors remains to be determined.

Role of DKPs in Plant/Bacteria Interactions. Plant hormones control plant growth by affecting the spatial and temporal expression of genes involved in cell division, elongation, and differentiation. The potential of single bacterial strains to interfere with plant hormone levels remains one of the major challenges toward better understanding, predicting, and possibly controlling plant hormone responses in complex plant-associated bacterial communities. We propose a model to explain the effects of *P. aeruginosa* in *Arabidopsis* (Fig. S10). WT *P. aeruginosa* alters RSA by at least two mechanisms: one that likely involves 3-oxo-C12-AHL and is independent of auxin signaling leads to primary root growth inhibition, and one where it promotes root branching interacting with auxin as it requires normal functioning of auxin receptors *TIR1*, *AFB2*, and *AFB3* and transcriptional regulators *ARF7* and *ARF19* acting downstream (Fig. S7). In our model, the primary root growth inhibition is no longer observed in plants cocultivated with *lasI* or *rhlI*/*lasI*, but instead an increase in root hair and lateral root formation occurs that correlates with plant growth promotion and production of DKPs. Root branching induced by *lasI* or *rhlI*/*lasI* mutants indeed required normal auxin signaling and could be a particular response to all three DKPs secreted by the mutants (Figs. S7 and S10). Our results showing the involvement of DKPs in RSA modulation add to the plethora of potential functions of these intriguing molecules. Based on their auxin-like activity, DKPs can be regarded as broad-spectrum molecules used to modulate the activity of both prokaryotic and eukaryotic cells, and thus represent a novel class of signals enabling interkingdom communication. An interesting study by Degrassi et al. (35) showed that plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four DKPs, which as shown in the present work may be involved in plant growth promotion. Manipulating AHL-dependent QS signaling and DKP biosynthesis may be a promising strategy for development of bacterial inoculants to enhance crop yields by means of auxin signaling and root architecture modulation.

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis thaliana* (Col-0); the transgenic lines *DR5::uidA* (20), *BA3::uidA* (21), *HS::AXR3NT-GUS* (22), *CycB1:uidA* (29), *AtPRZ:uidA* (30), and *AtHistH2B:YFP* (31); and the mutant lines

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tir1-1, *tir1afb2afb3* (36), *arf7-1*, *arf19-1*, and *arf7arf19* (37) were used for all experiments. Seeds were surface sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2× Murashige and Skoog medium (Murashige and Skoog basal salts mixture, M5524; Sigma). The suggested formulation is 4.3 $g \cdot L^{-1}$ of salts for 1× medium; we used 0.9 $g \cdot L^{-1}$, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light, 8 h of darkness, a light intensity of 100 $\mu mol \cdot m^2 \cdot s^{-1}$, and a temperature of 22 °C.

In Vitro Plant/Bacteria Cocultivation Assay. Bacterial strains used in this work were *P. aeruginosa* PAO1 (WT), *P. aeruginosa lasI*, *rhlI*, and *rhlI/lasI* single and double mutants, respectively (38). The bacterial strains were evaluated in vitro for their plant growth-promotion ability, using the *Arabidopsis* Col-0 ecotype. Bacterial densities of 2.5×10^8 cfu were cocultivated by streaking on agar plates containing 0.2× MS medium. Six-day-old germinated *Arabidopsis* seedlings (20 seedlings per plate) were grown to one side of the plate, opposite to the bacterial streak site at a 5-cm distance from the root tip. The seedlings were grown for a further 8-d period by placing the plates in the growth chamber in a completely randomized design. All experiments were replicated at least three times.

Hormone Treatments. For all experiments, 0.2× MS medium was supplemented with IAA or DKPs. Ethanol-dissolved compounds were added to cooled (50 °C) molten medium and poured into plates. Control plates were supplied with the greatest concentration of ethanol used in the AHL treatments. IAA was purchased from Sigma. DKPs were directly purified from WT *P. aeruginosa* and *lasI* mutant cultures.

Aux/IAA Protein Degradation Assay. Six-day-old *HS::AXR3NT-GUS Arabidopsis* transgenic seedlings were incubated on liquid 0.2× MS medium for 2 h at 37 °C, followed by transfer of the seedlings into liquid 0.2× MS medium supplied with the different DKP or IAA compounds for 60 min at 22 °C. The seedlings were washed with fresh 0.2× MS medium and for 12–14 h histochemically stained for GUS activity.

Analysis of Growth, Statistics, and Histochemical and Microscopy Analysis. The detailed analysis of growth, purification, and chemical characterization of cyclodipeptides, statistics, histochemical, and microscopy analysis are described in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Analysis of Growth and Statistical Analysis. Growth of primary roots was registered using a rule. Lateral root number was determined by counting the lateral roots present in the primary root from the tip to the root/stem transition. Fresh weight of plants was determined with an analytical balance (Ohaus Corp.) with a 0.0001-g precision value. For all experiments, the overall data were statistically analyzed in the SPSS 10 software (SPSS). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in WT and mutant seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Histochemical Analysis. Transgenic plants that express the *uidA* reporter gene were stained in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer [NaH_2PO_4 and Na_2HPO_4, 0.1 M (pH 7)] with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37 °C (1). Plants were cleared and fixed as previously described (2). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.

Microscopy. The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6; Leica Microsystems). Total lateral roots were counted at 30× magnification. Primary root meristems were analyzed in semipermanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus; Carl Zeiss) at 100× or 400× magnifications. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss).

Propidium Iodide Staining and YFP Detection. For fluorescent staining with propidium iodide (PI), plants were transferred from the growth medium to 10 mg·mL^{-1} of PI solution for 3 min. Seedlings were rinsed in water and mounted in 50% glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568-nm excitation line and emission window of 585–610 nm, and YFP emission with 505- to 550-nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1000), after which the two images were merged to produce the final image.

Solvent Extraction, Purification, and Chemical Characterization of Cyclodipeptides from *P. aeruginosa*. A 2.5 × 10^8 cfu inoculum of WT *P. aeruginosa* and *lasI* mutant strains was placed in 1.5 L Luria Bertani (LB) medium and incubated in a growth cabinet 24 h at 37 °C with shaking for bacterial growth. Cell-free supernatants were prepared by centrifugation (10,000 × *g*, 25 °C for 10 min) in an Eppendorf centrifuge 5810R. The resulting supernatant was extracted twice with ethyl acetate supplied with acetic acid (0.1 mL/L). The extracts were evaporated to dryness (Rotavapor R-210; Buchi) at 60 °C. The residue was redissolved in methanol:acetonitrile (1:1), concentrated, and redissolved to 1 mL in HPLC-grade acetonitrile.

For purification of extracts, 1 mL of extract of ethyl acetate was applied to HPLC system using a C_{18} semipreparative column (Econosil C_{18} 10 U, 250 mm × 22 mm; Alltech). Fractions were eluted with water:acetonitrile, starting with an equilibration step 0:100; followed by a gradient linear up 60:40, with a flow 5 mL·min^{-1} by 40 min; a gradient linear up 90:10, with a flow 5 mL·min^{-1} by 10 min; a gradient linear up 0:100 with a flow 5 mL·min^{-1}; and ending with an equilibration of 15 min with a flow 5 mL·min^{-1}. The deionized water and HPLC-grade acetonitrile were filtrated and degasified. The fractions were collected and concentrated by Freezone 6 lyophilizer (Labconco). The purified fractions were subsequently used for biological activity evaluation and chemical determination.

The purified fractions were analyzed in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector (model 5973) and a 30 m × 0.2 mm × 0.25 mm 5% phenyl methyl siloxane capillary column. Operating conditions used helium as carrier gas, 1 mL/min, with a detector temperature of 300 °C and an injector temperature of 250 °C. The volume of injected sample was 1 µL. The column was held for 3 min at 80 °C and programmed at 6 °C/min to a final temperature of 230 °C for 5 min. A SCAN analysis was used to verify the presence of cycle dipeptides. ^{1}H NMR spectrum of the fractions (1 mg) dissolved in $CDCl_3$ with biology activity was analyzed (Varian Mercury Plus 400 MHz NMR magnet), and the signal assignment was correlated with ^{13}C NMR (Varian Mercury Plus 100 MHz NMR magnet) spectra. The date were analyzed and compared with described compounds and atoms in molecules designed (Fig. S5).

Molecular Docking Analysis of Interactions Between DKPs and TIR1. AutoDock Vina and AutoDock 4.2 software for rigid and flexible (C405, S438, and R489) molecular docking calculations were used. The structure of TIR1 with Aux/IAA7 peptide (PDB ID code 2P1Q) from *A. thaliana* was used as a target. Small molecules used as ligands were cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), cyclo (L-Pro-L-Phe), and Probe P8 (4). Modeling was carried out with VI EWDD molecular editor (Rome Center for Molecular Design Laboratory). The TIR1 docking site was determined from the TIR1 crystallized structure with IAA, 2,4-D (3), and α-alkyl-IAA (4); a water molecule from the TIR1 auxin-binding site was conserved. A grid box was set to cover the whole docking site (12, 14, 14 grid with a grid spacing of 1.0 Å). One hundred docking runs were performed using the Lamarckian genetic algorithm when AutoDock 4.2 was used. A file comprising all possible conformers was analyzed by AutoDock Tools (5) and figures was generated with PyMOL version 0.99 software (6). The DKP docked conformers were tested according to (*i*) DKP conformers with similar orientation to IAA in the TIR1 model; (*ii*) DKP orientation overlapping with the IAA and 2,4-D binding site in TIRl; (*iii*) DKP orientation selected as present in conformer clusters; (*iv*) DKP conformers with the minimum binding energy to TIR1; and (*v*) DKPs and IAA ability to produce ionic interaction with TIR1 residues involved in molecular binding to polar residue Arg403.

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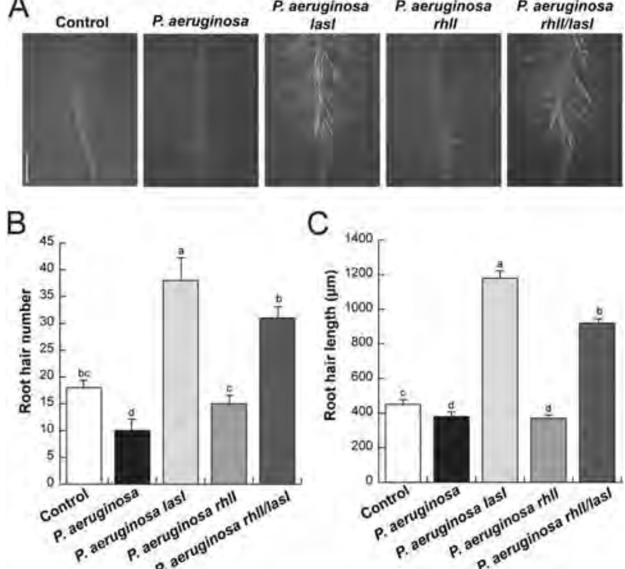

Fig. S1. Effect of cocultivation with *P. aeruginosa* WT and QS mutant strains on root hair development. Four-day-old *A. thaliana* seedlings were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at a distance of 5 cm from the primary root tip and grown for an additional 8 d. (*A*) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings cocultivated with WT *P. aeruginosa* and *P. aeruginosa* QS mutants. (Scale bars = 500 μm.) (*B*) Effect of bacterial cocultivation on root hair number or (*C*) root hair length. Data from *B* and *C* show the means ± SD from 30 seedlings. These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P < 0.05$.

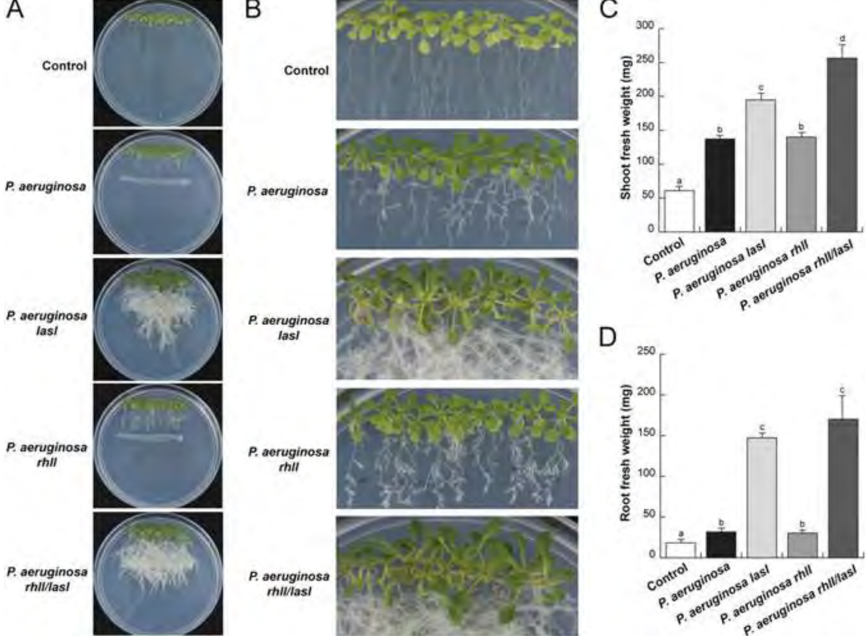

Fig. S2. Effect of cocultivation with *P. aeruginosa* WT and QS mutant strains on plant growth. Four-day-old *A. thaliana* seedlings were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at a distance of 1 cm from the primary root tip and grown for 12 d. (*A*) representative photographs were taken for plates from each treatment. This experiment includes five different plates per treatment and was repeated three times with similar results. Notice the strong biomass accumulation effect of *P. aeruginosa lasI* and *rhlI/lasI* mutants and the robust induction of lateral roots when the *Arabidopsis* root system establishes physical contact with the bacterial inoculum. (*B*) Close-up photographs from axenically grown plants or plants cocultivated with each bacterial strain. (*C*) Effect of bacterial cocultivation on shoot fresh weight or (*D*) root fresh weight. Data from *C* and *D* show the means ± SD from three groups of 30 seedlings. These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P < 0.05$.

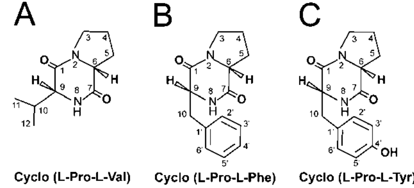

Fig. S3. MS, ^{1}H NMR, and ^{13}C NMR data of DKPs. GC/mass spectrometry, ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz, $CDCl_3$), spectra δ values data of atom number of (*A*) cyclo(L-Pro-L-Val) [retention time (Rt) = 7.5 min]: mass spectrum shows a molecular ion with mass of 196 (*m/z*), corresponding to molecular formula $C_{10}H_{16}N_2O_2$ [*m/z* (%): 196 (2), 154 (100), 138 (6), 125 (35), 110 (7), 98 (7), 72 (30), 70 (65), 55 (9), 41 (12)]; ^{1}H NMR data: 0.91(3H, d, J = 6.9 Hz CH_3-Val); 1.06 (3H, d, J = 6.9 Hz CH_3-Val); 1.88–2.18 (3H, m, Pro 4-Ha,b, Pro 3-Ha); 2.39 (1H, m, Pro 3-Hb); 2.63 (1H, m, Val H-10); 3.50–3.75 (2H, m, Pro 5-Ha,b); 3.95 (1H, bs, Val H-9); 4.09 (1H, bt, J = 6.9 Hz, Pro H-6); 5.70 (1H, bs, NH); ^{13}C NMR data: 164.8 (C-1), 45.1(C-3), 22.4(C-4), 28.3(C-5), 60.3(C-6), 169.9(C-7), 56.8(C-9), 28.5(C-10), 16(C-11), 19.3 (C-12); (*B*) cyclo(L-Pro-L-Phe) (Rt = 12 min): mass spectrum shows a molecular ion with mass of 244 (*m/z*), corresponding to molecular formula $C_{14}H_{16}N_2O_2$ [*m/z* (%): 244 (58), 153 (48), 125 (100), 120 (20), 103 (6), 91 (42), 70 (40), 55 (5), 41 (6)]; ^{1}H NMR data: 1.85–2.10 (3H, m, Pro 4-Ha,b, Pro 3-Ha); 2.34 (1H, m, Pro 3-Hb); 2.78 (1H, dd, J = 10.6, 14.0 Hz, Phe 10-Ha); 3.55–3.73 (3H, m, Pro 5-Ha,b, Phe 10-Hb); 4.09 (1H, bt, J = 7.3 Hz, Pro H-6); 4.28 (1H, dd, J = 2.2, 10.6 Hz, Phe H-9); 5.62 (1H, bs, NH); 7.22–7.38 (5H, m, Phe); ^{13}C NMR data: 165(C-1), 45.5(C-3), 22.6(C-4), 28.3(C-5), 59.1(C-6), 169.4(C-7), 56.2(C-9), 36.8(C-10), 135.9(C-1′), 129.3(C-3′, C-5′), 129.1 (C-2′, C-6′), 127.6(C-4′); (*C*) cyclo(L-Pro-L-Tyr) (Rt = 7 min): mass spectrum shows a molecular ion with mass of 260 (*m/z*), corresponding to molecular formula $C_{14}H_{16}N_2O_3$ [*m/z* (%): 260 (8), 154 (100), 125 (3), 107 (50), 77 (5), 70 (18), 41 (2)]; ^{1}H NMR data: 1.85–2.10 (3H, m, Pro 4-Ha,b, Pro 3-Ha); 2.4 (1H, m, Pro 3-Hb); 2.74 (1H, dd, J = 10.6, 14.7 Hz, Tyr 10-Ha); 3.40–3.70 (3H, m, Pro 5-Ha,b, Tyr-10-Hb to Tyr 10-Hb); 4.09 (1H, bt, J = 7.7 Hz, Pro H-6); 4.22 (1H, dd, J = 2.2, 10.6 Hz, Tyr H-9); 5.72 (1H, bs, NH); 5.95 (1H, bs, OH); 6.82 (2H, d, J = 8.2 Hz, H-3′,5′ Tyr); 7.07(2H, d, J = 8.2 Hz, H-Tyr Phe); ^{13}C NMR data: 165(C-1), 45.4(C-3), 22.5(C-4), 28.3(C-5), 59.1(C-6), 169.6(C-7), 56.2(C-9), 35.9(C-10), 127.3(C-1′), 116.1(C-3′, C-5′), 130.3(C-2′, C-6′), 155.3(C-4′).

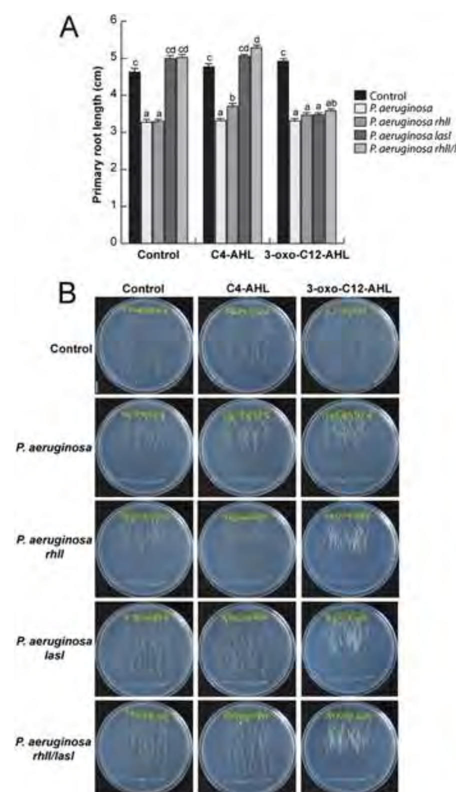

Fig. S4. Chemical complementation of *P. aeruginosa* QS mutants. *A. thaliana* seedlings were germinated and grown on 0.2× MS agar medium supplemented with or without 10 μM C4-AHL or 3-oxo-C12-AHL. Four-day-old seedlings were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at a distance of 5 cm from primary root tip and grown for an additional 8 d. Data points shown in *A* represent mean primary root length ± SD (n = 30). (*B*) Representative photographs of plants from the different treatments. (Scale bars = 1 cm.) These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P < 0.05$.

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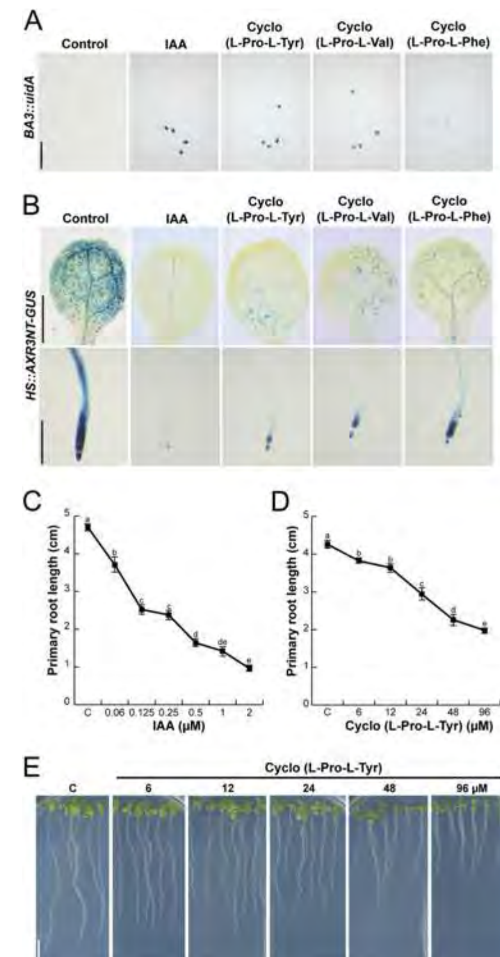

Fig. S5. Effects of IAA and bacterial DKPs on auxin-regulated gene expression and primary root growth. (*A*) *BA3:uidA* auxin-regulated gene expression. Six-day-old *BA3:uidA* seedlings were transferred for 12 h of β-glucuronidase staining to 24-well plates containing 2 mL of liquid 0.2× MS medium supplied with 3 μM IAA or 30 μM DKP. (*B*) Effect of DKPs on AXR3 degradation. Six-day-old *HS:AXR3NT-GUS* seedlings grown on 0.2× MS medium were heat shocked for 2 h a 37 °C and then transferred for 60 min to 0.2× MS liquid medium containing 3 μM IAA or 30 μM DKPs. Plants were stained for β-glucuronidase activity and cleared to show gene expression. Photographs show representative individuals from at least 10 stained plants. (Scale bars = 500 μm.) (*C–E*) Effects of IAA and cyclo(L-Pro-L-Tyr) on primary root growth. WT *A. thaliana* (Col-0) seedlings were germinated and grown for 8 d on 0.2× MS agar medium supplemented with the indicated concentrations of IAA (C) or cyclo(L-Pro-L-Tyr) (*D*) and primary root length recorded for 15 seedlings. (*E*) Representative photographs of *Arabidopsis* seedlings treated with cyclo(L-Pro-L-Tyr). Data points show the mean primary root length ± SD. Different letters indicate means that differ statistically at $P < 0.05$. (Scale bars = 1 cm.)

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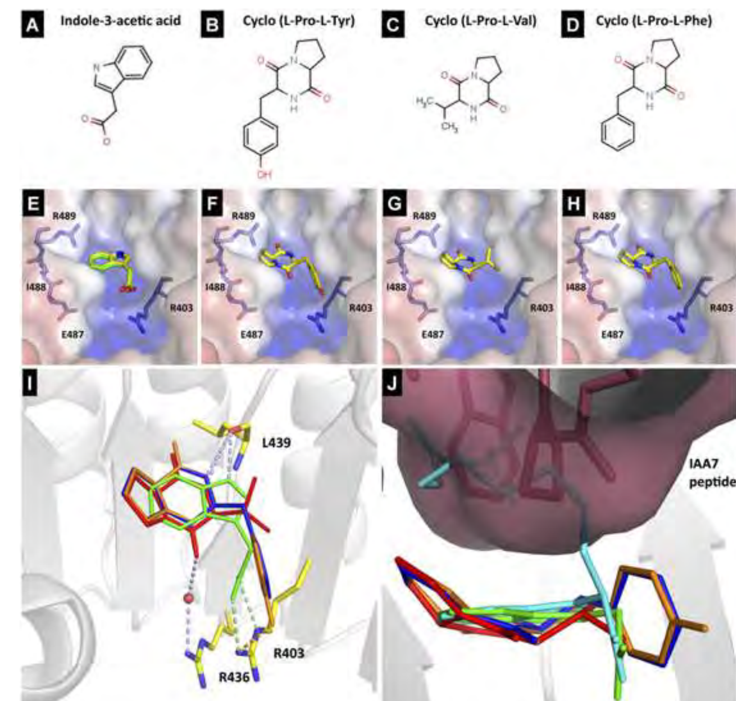


Fig. S6. Modeling of *P. aeruginosa* DKPs in the TIR1-IAA auxin receptor of *A. thaliana* using the software AutoDock Vina and Autodock 4.2 (2). Chemical structures of IAA and DKPs from *P. aeruginosa*: (*A*) IAA, (*B*) cyclo(L-Pro-L-Tyr), (*C*) cyclo(L-Pro-L-Val), (*D*) cyclo(L-Pro-L-Phe), and (*E–H*) structural position of IAA and DKPs, respectively, in the auxin-binding cavity of TIR1. Residues involved in pocket formation (E487, I488, and R489) and residue (R403) involved in the interaction with auxin carboxyl group are indicated in surface representation. (*I*) A close-up view of the IAA and DKPs superposition in the auxin-binding site of TIR1. IAA (green), cyclo(L-Pro-L-Tyr) (orange), cyclo(L-Pro-L-Val) (red), cyclo(L-Pro-L-Phe) (blue), and a water molecule (red sphere). Interacting residues of IAA and DKPs with TIR1 are indicated by dashed lines. (*J*) Modeling of IAA, DKPs, and Aux/IAA7 degron peptide superimposed in the crystal structure of the TIR1 auxin-binding site. IAA and DKPs are shown as in *I*, antiauxinic molecule α-butyl-indole-3-acetic acid (probe 8) (2) is shown as light blue. The DKP-TIR1 binding energy obtained was near the range of redocked auxins (IAA = −8.1 kcal/mol; 2,4-D = −7.2 kcal/mol; cyclo(L-Pro-L-Tyr) = −3.7 kcal/mol, cyclo(L-Pro-L-Val) = −5.3 kcal/mol, and cyclo(L-Pro-L-Phe) = −4.0 kcal/mol). Also, the dimension (5.5 Å) of *P. aeruginosa* DKPs identified was similar to IAA (4.66 Å), NAA (4.86 Å), and 2,4-D (5.27 Å).

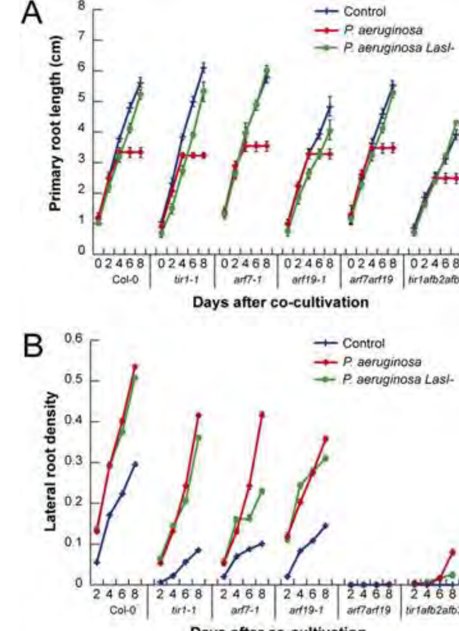


Fig. S7. Effect of cocultivation with *P. aeruginosa* WT and QS-related mutant strains in RSA development in *Arabidopsis* WT and auxin-related mutant lines. Four-day-old *A. thaliana* WT and *tir1-1*, *arf7-1*, *arf19-1*, *arf7arf19*, and *tir1afb2afb3* mutant lines were inoculated with WT *P. aeruginosa* or with mutants defective on the AHL synthases LasI at a distance of 5 cm from the primary root tip and grown for an additional 8 d. The effect of bacterial cocultivation on primary root length (*A*) or lateral root density (*B*) was monitored every 2 d. Day 0 indicates the length reached by the primary root at the moment of bacterial inoculation. Data points show the mean ± SD. The experiment was repeated twice with similar results.

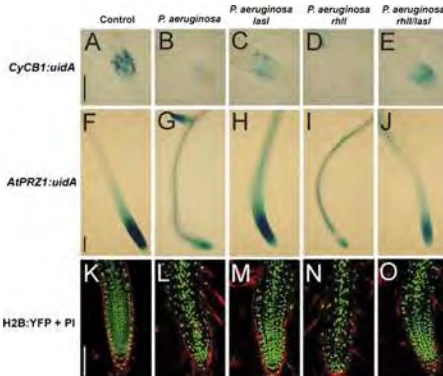


Fig. S8. Effect of cocultivation with *P. aeruginosa* WT and QS mutant strains on cell division and meristem cell viability. Four-day-old *A. thaliana* seedlings expressing the *CyCB1:uidA*, *AtPRZ1:uidA*, or *AtHistH2B:YFP* markers were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at a distance of 1 cm from primary root tip and grown for an additional 6 d. (*A*–*J*) Plants were stained for GUS activity and cleared to show gene expression. (*K*–*O*) Transgenic *Arabidopsis* seedlings expressing the *AtHistH2B:YFP* marker were stained with PI to determine cell structure and viability. Photographs show representative individuals from at least 20 stained plants. The experiment was replicated twice with similar results. (Scale bar = 100 μm.)

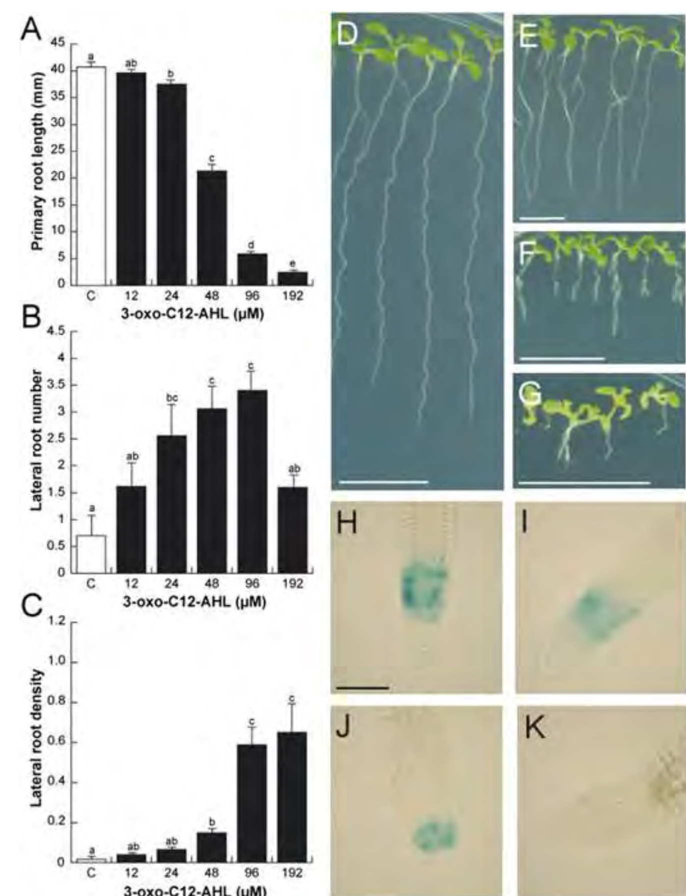
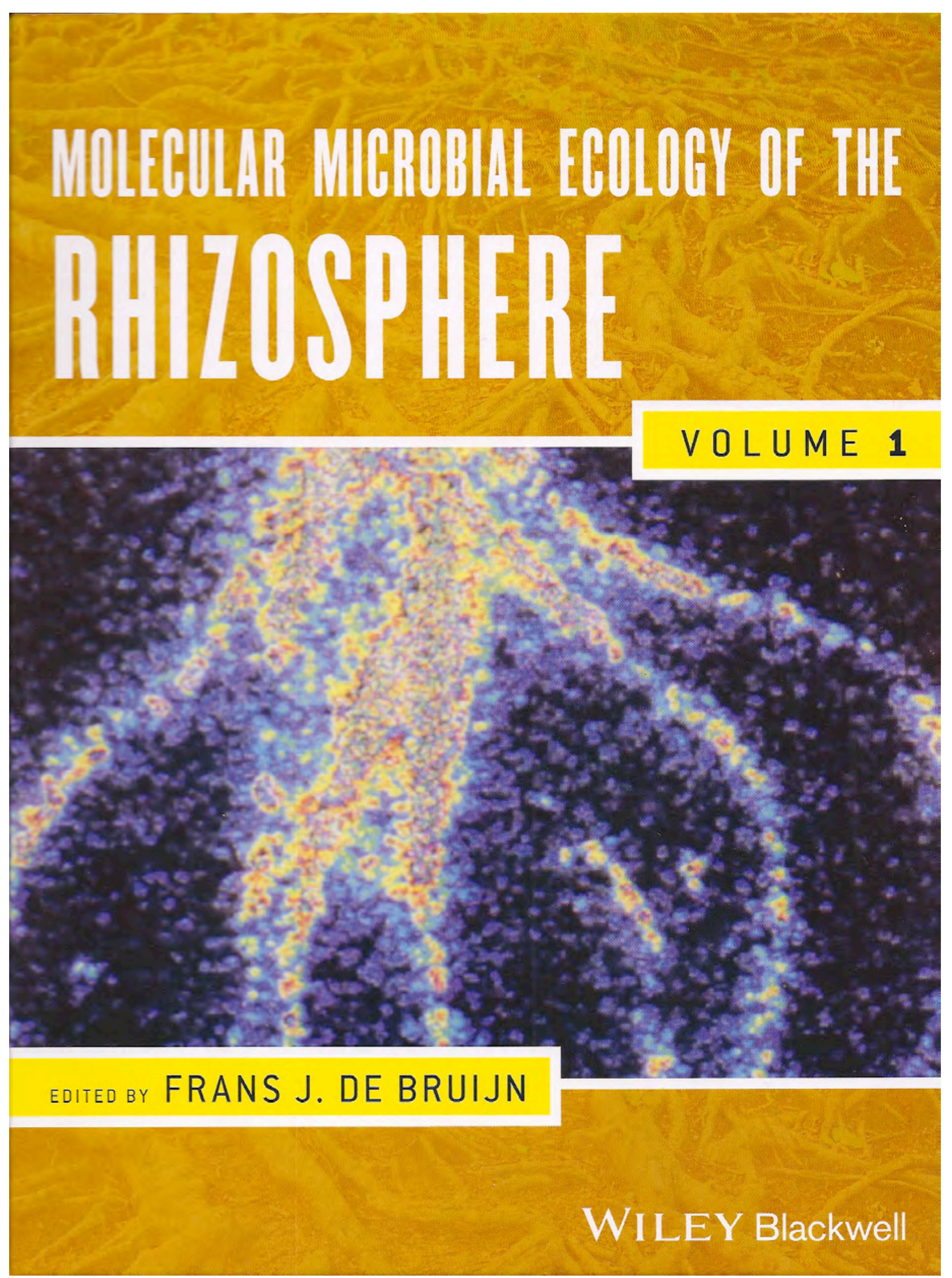


Fig. S9. Effects of 3-oxo-C12-AHL on root system architecture and cell division marker expression in *Arabidopsis*. *A. thaliana* (Col-0) seedlings were germinated and grown for 10 d on 0.2× MS agar medium supplemented with the indicated concentrations of 3-oxo-C12-AHL, and primary root length (*A*), lateral root number (*B*), and lateral root density (*C*) were recorded for 30 seedlings. Representative photographs of *Arabidopsis* seedlings treated with solvent (*D*) or 48 μM (*E*), 96 μM (*F*), and 192 μM (*G*) of 3-oxo-C12-AHL. Data points in *A*–*C* show the mean ± SD from 30 seedlings. Different letters indicate means that differ statistically at $P < 0.05$. (*H*–*K*) Transgenic *Arabidopsis* seedlings expressing the *CyCB1:uidA* marker were grown for 10 d in medium with the solvent—48, 96, or 192 μM of 3-oxo-C12-AHL, respectively. Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from at least 20 stained plants. The experiment was replicated twice with similar results. (Scale bars: *D*–*G*, 1 cm; *H*, 100 μm.)

Fig. S10. Root architectural responses of *Arabidopsis* to WT *P. aeruginosa* and QS-related mutants.

CAPITULO II.



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Chapter 28

Small Molecules Involved in Transkingdom Communication between Plants and Rhizobacteria

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28.1 INTRODUCTION

Intensive agriculture based on an overuse of fertilizers and water has been critical in the supply of food and grains for the increasing human population. Negative impacts of agricultural practices on soils and water have stimulated the commercialization of rhizobacterial inoculants to sustain crop growth and yield (Conway and Pretty, 1988; Loneragan, 1997; Berg, 2009).

The use of plant growth-promoting rhizobacteria (PGPR) that impact on plant hormone status may have positive effects on plant biomass production by modifying root architecture to capture existing soil resources, including nutrients such as phosphorus (P), nitrogen (N), and iron (Fe) and enhance water acquisition (Dodd et al., 2010; see Chapter 53). At least three well-defined parts can be recognized in the developing plant: (i) the root, the below-ground part of the plant, which provides anchorage and plays an essential role in interactions with PGPR; (ii) the stem, which supports the leaves, flowers, and fruits; and (iii) the shoot, with important functions in reproduction and photosynthesis. The three-dimensional organization of plant organs is known as *plant architecture* and has long been considered a major target for crop improvement. Notably, the green revolution, which greatly contributed to grain production in the past decades, was based on the modification of plant architecture for selection of crop varieties of agronomic relevance (Peng et al., 1999; Reinhardt and Kuhlemeler, 2002; López-Bucio et al., 2005; Ross et al., 2005; Wang and Li, 2008).

Growth and development of plants involve the integration of a myriad of endogenous and environmental signals which, together with the intrinsic genetic program, determine plant architecture. Plants have a sophisticated system to integrate information from the environment and to actively respond to biotic and abiotic factors; likewise, they have developed mechanisms for communication among plants and between plants, and their associate microorganisms through transkingdom signaling (see also Chapter 27).

Virtually every aspect of development of the plant, from embryogenesis to senescence, is subject to regulation mediated by different chemical substances known as *phytohormones* or *plant growth regulators*. A single hormone may target a wide range of cellular and morphogenetic processes, while simultaneously, multiple hormones may influence the same developmental process (Gray, 2004; Suárez-López, 2005).

Plants produce diverse phytohormones with different chemical identity including volatiles, such as ethylene and jasmonic acid (JA), small organic compounds, such as indole-3-acetic acid (IAA or auxin), cytokinins, gibberellic acid (GA), abscisic acid (ABA), brassinosteroids (BRs), and lipids (Weyers and Paterson, 2001). In general, these compounds regulate every aspect of plant life and all major developmental transitions including germination,

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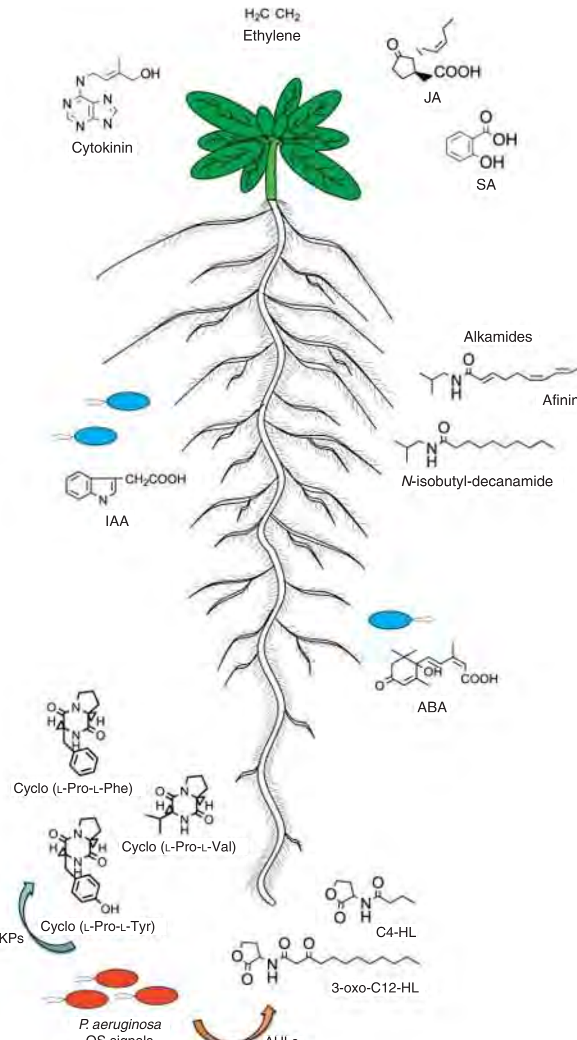

Figure 28.1 Small molecule signals that regulate plant architecture. The six classic phytohormones, auxin (IAA), cytokinin, ethylene, jasmonic acid, salicylic acid, and ABA are shown. Novel plant signals such as alkamides and bacterially-produced *N*-acyl-L-homoserine lactones and diketopiperazines are also illustrated. A single hormone can affect multiple developmental processes, while multiple signals may have impact on the same morphogenetic process. All together, these regulators orchestrate plant architecture.

vegetative development, flowering, fruit production, and senescence (Bishopp et al., 2006; Santner et al., 2009) (Fig. 28.1).

Root system architecture (RSA) displays considerable plasticity in its morphology and physiology in response to abiotic (i.e., nutrient availability, heavy metal stress) or biotic signaling (i.e., plant-to-plant or plant–microbe interactions) modifying lateral root, root hair, and adventitious root formation (López-Bucio et al., 2002; López-Bucio et al., 2003; Chen et al., 2007; Nibau et al., 2008; Ortiz-Castro et al., 2008a). Classic phytohormones, such as auxins and cytokinins, play important roles in the regulation of RSA and also affect defense responses, indicating multilevel interactions in the physiology

of plants (Reed et al., 1998; Casimiro et al., 2001). Moreover, research conducted in the past 10 years has identified the *N*-acylethanolamines (NAEs), alkamides, and *N*-acyl-L-homoserine lactones (AHLs; see Chapters 70–77) as regulators of plant developmental processes and as mediators of bacterial–plant interactions (Blancaflor et al., 2003; Chapman, 2004; López-Bucio et al., 2006, 2007a; Morquecho-Contreras and López-Bucio, 2007; Campos-Cuevas et al., 2008; Morquecho-Contreras et al., 2010; Méndez-Bravo et al., 2010). The aim of this chapter is to summarize recent findings about the signals involved in the interaction of plants with PGPR, particularly auxin and cytokinins, because these hormones play essential roles in growth and developmental processes. We also present and discuss some recent information on plant perception of bacterial quorum-sensing (QS) signals, which may be relevant toward the identification of beneficial agricultural traits modulated by PGPR.

28.2 PLANT–BACTERIA INTERACTIONS IN THE RHIZOSPHERE

Since the advent of the "green revolution," crop productivity has been highly dependent on the use of chemical fertilizers such as N and P. This comes at a heavy price. Besides, the leaching of fertilizers into aquatic systems accounts for increasing plant and algal blooms, now a global problem. The rising costs of N and P fertilizers driven by the rising costs of fossil fuels and the need for a long-term agricultural sustainability are making natural alternatives to chemical fertilizers even more attractive.

Plant roots are surrounded by a portion of soil, which is known as the *rhizosphere* (Walker et al., 2003; Bais et al., 2006). Plants produce a wide range of compounds including sugars, organic acids, and vitamins, which are used as nutrients or signals by bacteria (Badri et al., 2009; see Chapter 22). Plant-beneficial bacteria commonly proliferate in close proximity to the root system, establishing an intimate relationship with plants with a profound effect on plant immunity and health. At least in part, these effects can be explained because bacteria release phytohormones (i.e., IAA, ABA, SA, JA, cytokinins, or gibberellins) or may impact on the homeostasis of several hormone pathways by affecting biosynthesis, degradation, and/or signaling through receptors and/or transcription factors (Costacurta and Vanderleyden, 1995; Tsavkelova et al., 2006; van Loon, 2007; Dodd et al., 2010). The growth-promoting potential of PGPR has been studied in annual crops, such as wheat, soybean, lettuce, beans, maize, and barley (Barazani and Friedman, 1999; Badri et al., 2009; see Chapter 53). Some PGPR may fix atmospheric nitrogen as in the *Rhizobium*–legume symbiosis (see Chapters 44, 45), or may confer immunity against foliar pathogens by activating plant defenses, thereby improving plant growth under different environments (van Loon et al., 2007).

Promotion of root growth is one of the major markers of PGPR (Glick et al., 1995; Patten and Glick, 2002). Rapid establishment of roots, either by proliferation of lateral or adventitious roots or root hairs, is advantageous for plants as it increases the exploratory potential of the root system. Many PGPR synthesize plant hormones, and in this way they may positively affect root growth (Spaepen et al., 2007; Ortiz-Castro et al., 2008b; Dodd et al., 2010). The production of phytohormones and other compounds that influence plant development by PGPR is well documented (van Loon, 2007; Barazani and Friedman, 1999; Gray, 2004). The switch between indeterminate and determinate growth in roots and in shoots is frequently regulated by endogenous or environmental signals that impact on cell division and/or differentiation programs.

28.2.1 Auxin–Cytokinin Ratio in Plant Developmental Processes

The control of plant growth by auxin and cytokinins is a well-known example of hormone interactions that modulate developmental transitions, particularly in apical dominance and in root and shoot morphogenesis. The balance between auxin and cytokinin is a key regulator of *in vitro* organogenesis. Exposing callus cultures to a high auxin-to-cytokinin ratio results in root formation, whereas a low ratio of these hormones promotes shoot development (Howell et al., 2003). Apical dominance is one of the classical developmental events believed to be controlled by the ratio of auxin to cytokinin. This is supported by phenotypic observations in *Arabidopsis* mutants impaired in different aspects of auxin and/or cytokinin signaling. Several mutants overproducing auxin have been described for *Arabidopsis thaliana*. The *sur1/alf1/rty/hsl3* (Boerjan et al., 1995), *sur2* (Barlier et al., 2000) and its stronger allele *rnt* (Bak et al., 2001), and *yucca* (Zhao et al., 2001) mutants display similar developmental alterations correlated with increased auxin levels. These include an increased apical dominance, root formation, cell elongation, and the formation of epinastic cotyledons and leaves. In contrast, the cytokinin-overproducing *bus1* and the allelic *sps* mutant exhibit the formation of bushy shoots, retarded onset of vascularization, and upward curling leaves (Reintanz et al., 2001; Tantikanjana et al., 2001). Moreover, many experiments have demonstrated the existence of synergistic, antagonistic, and additive interactions between these two plant hormones, suggesting complex signal interactions (Nordstrom et al., 2004). Cytokinin and auxin have antagonistic roles in root development: auxin promotes the formation of lateral

and adventitious roots (Dubrovsky et al., 2008), whereas cytokinins inhibit root formation interfering with the auxin effect (Laplaze et al., 2007).

In many aspects of plant development, it is reasonable to believe that mechanisms of importance for the homeostatic control of the auxin–cytokinin ratio should be relatively rapid. The site of synthesis is a critical question for understanding the cross talk of the two hormones and how they interact. Although, both, CK and IAA can be produced in roots and shoots (Ljung et al., 2001; Nordstrom et al., 2004), the production of these major hormonal signals does not occur randomly but is regulated by the location of the synthesizing cells in the plant body and their developmental stage, and is influenced by environmental conditions and by microorganisms. Young shoot organs are the major sites of IAA production (Ljung et al., 2001; Bhalerao et al., 2002), while root tips are major sites of CK synthesis (Aloni et al., 2006). From the sites of hormone production, the signals move in specific structural pathways and by different mechanisms to regulate plant development.

28.2.2 Auxin in Plant Responses to PGPR

Diverse bacterial species produce auxins as part of their metabolism, including IAA, indole-3-butyric acid (IBA), or their precursors (Martínez-Morales et al., 2003; Spaepen et al., 2007; see Chapters 27, 29). Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum* species, and it is generally agreed that auxin production is the major factor responsible for the stimulation of root system development and growth promotion by this bacterium (Spaepen et al., 2007). Auxin synthesis has been demonstrated in *Azospirillum brasilense* (Dobbelaere et al., 1999), in symbiotic N-fixing cyanobacteria (Sergeeva et al., 2002), in the actinomycete *Frankia* (Wheeler et al., 1984), and in *Rhizobia* (Mathesius, 2008). The exudation of various compounds from plants has been shown to stimulate IAA biosynthesis in bacteria, which likely use tryptophan exuded by roots as a precursor of IAA (see Chapter 29). Flavonoids, which are produced from legume roots to stimulate IAA synthesis, have also been reported to induce IAA synthesis in *Rhizobium* sp. (Theunis et al., 2004; see Chapter 51). There is evidence that auxin synthesis by bacteria alters root architecture in non-nodulating plants. For example, auxin synthesis by *Erwinia herbicola* pathovar *gypsophilae* stimulates the formation of tumors in its plant host *Gypsophila paniculata* L. (Clark et al., 1993), while bacterially produced auxin may also explain the stimulation of root elongation in canola by *Pseudomonas putida* (Xie et al., 1996).

28.2.3 Role of Cytokinins in Growth Promotion by PGPR

Cytokinins were discovered in the search for compounds that enhanced division of plant cells in culture. Cytokinins are *N*6-substituted adenine derivatives that contain an isoprenoid derivative side chain. These hormones influence numerous aspects of plant development and physiology, including seed germination, de-etiolation chloroplast differentiation, apical dominance, flower and fruit development, leaf senescence, and plant–pathogen interactions (Ferreira and Kieber, 2005). Plants continuously use cytokinins to maintain the pools of totipotent stem cells in their shoot and root meristems (Howell et al., 2003; Leibfried et al., 2005).

The positive effect of cytokinins on growth at the whole plant level has been demonstrated by the identification of genes involved in cytokinin perception and signaling. Three sensor histidine kinases, *CRE1/AHK4/WOL*, *AHK2*, and *AHK3*, have been shown to act as cytokinin receptors (Kakimoto, 2003). These receptors activate the expression of several response regulators in a cytokinin-dependent manner (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Further downstream, cytokinin signaling stimulates the G1/S transition of the cell cycle, which has been proposed to be mediated by the transcriptional induction of the *CYCD3* gene that encodes a D-type cyclin (Riou-Khamlichi et al., 1999). The cytokinin receptors play redundant functions in transducing the signal to downstream factors. When grown on soil, none of the single cytokinin receptor mutants of *Arabidopsis* (*cre1-12*, *ahk2-2*, *ahk3-3*) exhibited significant defective phenotype. However, the *ahk2-2 ahk3-3* double mutants had smaller leaves and shorter stems than did the wild-type plants. All single and double mutants produced apparently normal flowers that yielded viable seeds. Interestingly, the *cre1-12 ahk2-2 ahk3-3* triple mutants showed a dwarf phenotype with reduced root and shoot growth and smaller meristems. These mutants also produced inflorescences with nonfunctional flowers, which failed to produce seeds (Higuchi et al., 2004). These data suggest that cytokinin receptors are important for plant viability and normal growth. Cytokinins can be produced by microorganisms. Their production by PGPR has been well documented and correlated with increased growth of plants (Nieto and Frankenberger, 1990; García de Salamone et al., 2001; Arkhipova et al., 2005). Until recently, little was known on the genetic basis and signal transduction components that mediate the beneficial effects of cytokinin-producing PGPR. However, a recent report has provided important information on the role played by cytokinin receptors in plant growth promotion by *Bacillus megaterium* rhizobacteria. *B. megaterium* UMCV1 strain was initially isolated from the rhizosphere

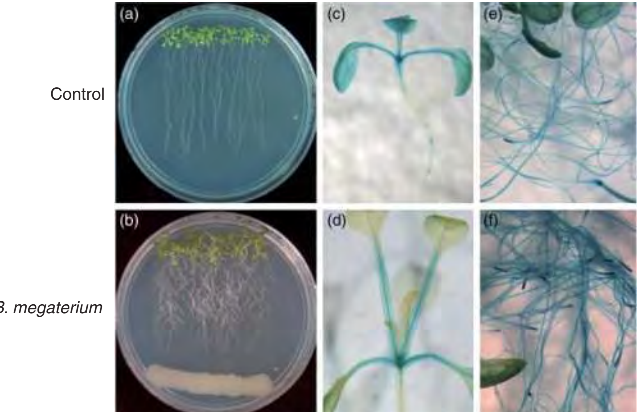

Figure 28.2 *Bacillus megaterium* promotes *Arabidopsis* growth and development through cytokinin signaling. (a) *Arabidopsis* WT (Col-0) seedlings were grown on the surface of agar plates with 0.2× MS medium or cocultivated with *B. megaterium* at a distance of 5 cm from the root tip. (b). *Arabidopsis* transgenic seedlings expressing the cytokinin-inducible *ARR5:uidA* reporter were germinated and grown for 5 days on MS 0.2×. (c and e) or cocultivated with *B. megaterium*. (d and f) at a distance of 5 cm from the primary root tip. Notice the increased growth promotion and enhanced expression of the cytokinin reporter in roots of plants cocultivated with *B. megaterium*.

of bean (*Phaseolus vulgaris* L.) plants. Cocultivation with this bacterium promoted biomass production of *A. thaliana* and bean plants *in vitro* and in soil (López-Bucio et al., 2007b). This effect was related to altered RSA in inoculated plants with an inhibition in primary root growth followed by an increase in lateral root formation and root hair length. The effects of bacterial inoculation on plant growth and development were found to be independent of auxin- and ethylene-signaling as revealed by normal responses of auxin-resistant mutants *aux1-7*, *axr4-1*, and *eir1*, and ethylene-response mutants *etr1* and *ein2*, and the failure to activate the expression of auxin-reporter markers.

The involvement of cytokinin signaling in mediating plant growth promotion by *B. megaterium* in plants was further investigated using *A. thaliana* mutants lacking one, two, or three of the cytokinin receptors, and *RPN12*, a gene involved in cytokinin signaling acting downstream of the receptors. It was found that growth promotion was reduced in *AHK2-2* single- and double-mutant combinations and in *RPN12*. Furthermore, growth promotion and lateral root induction was completely abolished in the *cre1-12 ahk2-2 ahk3-3* triple mutant, indicating the importance of cytokinin perception in the plant's response to *B. megaterium* (Ortiz-Castro et al., 2008b) (Fig. 28.2).

Later, it was found that cytokinin signaling is important for plant perception of alkamides, which comprise a novel class of plant signals related to bacterial QS hormones. The alkamides have been reported to affect, both, shoot and root system architecture in plants (López-Bucio et al., 2007a).

The study of the effect of rhizobacterial determinants on plant growth promotion and defense responses by PGPR revealed that root-associated bacterial volatile organic compounds (VOCs) are responsible, at least in part, for the induction of, both, plant host resistance and growth promotion (Ryu et al., 2003; Ryu et al., 2004; see Chapter 63). Bacterial VOCs-mediated plant growth promotion was absent in the cytokinin receptor loss-of-function *cre1* mutant in *Arabidopsis*, supporting a critical role of the plant growth hormone cytokinin in plant growth promotion. Moreover, the interplay between cytokinin and SA has been suggested as a molecular mechanism of plant immunity. Cytokinin signaling through *AHK2* and *AHK3* receptors activates SA signaling during interaction with *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000), a hemibiotrophic bacterial pathogen (Choi et al., 2010). Application of *trans*-zeatin or the overproduction of endogenous cytokinins enhanced the plant immune response, which is compromised in *ahk2 ahk3* knockout mutants. Cytokinins also activated type-B *Arabidopsis response regulator* (ARR) transcription factor ARR2, which binds to the promoter of the SA marker genes *Pathogenesis related 1* and *2* (*PR1* and *2*). Cytokinins have therefore emerged as strong candidates in mediating the cross talk between plant growth promotion and ISR triggered by bacterial VOCs.

28.3 QUORUM-SENSING SIGNALS ON PLANT GROWTH AND DEVELOPMENT

Many bacterial species use small molecule signaling to communicate with each other and to coordinate their growth activities. This cell-to-cell communication mechanism is known as *quorum sensing* (QS) and relies on the production, detection, and response to

diffusible signals in a cell density-dependent manner (Fuqua et al., 1994; Fuqua and Greenberg, 2002; see Section 9). QS processes are important to many bacterial species in the regulation of a variety of functions such as symbiosis, virulence, antibiotic production, biofilm formation, exopolysaccharide synthesis, toxin production, extracellular enzyme production, motility, and plasmid transfer (Schauder and Bassler, 2001; Marketon et al., 2003; Quiñones et al., 2005).

Diverse Gram-negative bacteria produce and use AHLs to regulate QS (Fuqua and Greenberg, 2002). These compounds contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain. The acyl groups of naturally occurring AHLs vary from 4 to 18 carbons in length; they can be saturated or unsaturated and with or without a C-3 substituent (Waters and Bassler, 2005). These chemical signals are produced by specific enzymes and they are detected by specific receptors (Pearson et al., 1994; Vannini et al., 2002). The specific activity of the different compounds can be determined by the lactone ring, the amide group, and the fatty acid chain length (Vannini et al., 2002; Fuqua and Greenberg, 2002).

Several reports indicate that AHL production is common among plant-associated *Pseudomonas* but less frequent in free-living soil isolates (Elasri et al., 2001; Khmel et al., 2002; D'Angelo-Picard et al., 2005), thus implicating an important role of AHL QS in plant–bacteria interactions. A recent study showed that AHLs modulate RSA, inhibiting primary root growth and inducing lateral root and root hair development (Ortiz-Castro et al., 2008a). Interestingly, the AHLs share structural chemical similarity with NAEs and alkamides from plants, and this opens the possibility that plants can sense AHLs, NAEs, and alkamides by a common genetic mechanism (López-Bucio et al., 2006).

Bacteria have evolved molecular mechanisms to perceive particular AHLs. The first AHL-type QS signal was described in *Vibrio fischeri* (Eberhard et al., 1981), in which the enzyme LuxI produces the 3-oxo-C6-HL signal that interacts with its receptor LuxR and consequently induces the transcriptional expression of the *lux* genes encoding proteins involved in bioluminescence (Engebrecht et al., 1983; Swartzman et al., 1990; see Chapter 73). Another well-known QS system is that of *Pseudomonas aeruginosa*, an opportunistic pathogen of animals and plants. In *P. aeruginosa*, between 5% and 20% of its genes and proteins are directly or indirectly subjected to QS regulation (Bauer et al., 2005). *P. aeruginosa* has two AHL QS sensor proteins, LasR and RhlR, that are regulated by 3-oxo-C12-HL and C4-HL, respectively.

Recent information has shown that bacteria can communicate with plants via AHLs, and this is crucial for the interaction of PGPR as well as plant pathogens with plant hosts (Cha et al., 1998; Elasri et al., 2001; Khmel et al., 2002; González and Marketon, 2003; D'Angelo-Picard et al., 2005; Pierson and Pierson, 2007; see Chapters 71, 73). Certain *Rhizobium* mutants that fail to produce or sense AHLs were unable to induce nodule formation in legume plants, suggesting that AHLs might participate in symbiotic interactions (Rosemeyer et al., 1998; Daniels et al., 2002; Zheng et al., 2006). Higher plants produce compounds that affect QS-regulated responses in bacteria, which are present in root exudates of pea (*Pisum sativum*) and *Medicago truncatula* (Teplitski et al., 2000; Gao et al., 2003). This indicates that secretion of compounds by plant roots, which act as AHL signal mimics, may affect AHL-regulated behaviors in bacteria.

The first report that plants can sense AHLs used *M. truncatula*. It was found that AHLs modulated defense and stress responses, protein processing, responses to plant hormones and cytoskeletal elements, as well as primary and secondary metabolism (Mathesius et al., 2003). The presence of AHL-producing bacteria in the rhizosphere of tomato induced the salicylic acid- and ethylene-dependent defense response, which plays an important role in the activation of systemic resistance in plants and conferred protection against the fungal pathogen *Alternaria alternata* (Schuhegger et al., 2006).

By using a transcriptomic strategy in *A. thaliana*, von Rad et al. (2008) documented the changes in gene expression in the plant in response to *N*-hexanoyl-DL-homoserine lactone (C6-HL), a QS signal produced by the soil bacterium *Serratia liquefaciens* MG1. This AHL modulated the expression of genes involved in auxin biosynthesis and response, whereas the levels of cytokinins were reduced indicating that AHLs may increase the auxin-to-cytokinin ratio. Interestingly, unlike most other bacterial signals, C6-HL influenced only a few defense-related transcripts and did not induce plant systemic resistance against *Pseudomonas syringae*. Evidence was provided that *Arabidopsis* takes up bacterial C6-HL and allows its systemic distribution throughout the plant (von Rad et al., 2008).

The analysis of RSA in *A. thaliana* seedlings treated with increasing concentrations of AHLs ranging from 4 to 14 carbons in length was performed by Ortiz-Castro and associates (2008a). Medium chained (C8-C12) AHLs modulated primary root growth, lateral root formation, and root hair development, and in particular, *N*-decanoyl-HL (C10-HL) was the most active compound inducing lateral root formation and root hair development (Fig. 28.3).

C10-HL caused the differentiation of cells at the primary root meristem region, which was related to a reduction in the expression of cell division markers *CycB1*:*GUS* and *pPRZ:GUS*. The response of primary roots to C10-HL was unlikely mediated by auxin signaling, because C10-HL did not increase auxin-responsive gene expression and the auxin-related mutants, *aux1-7*,

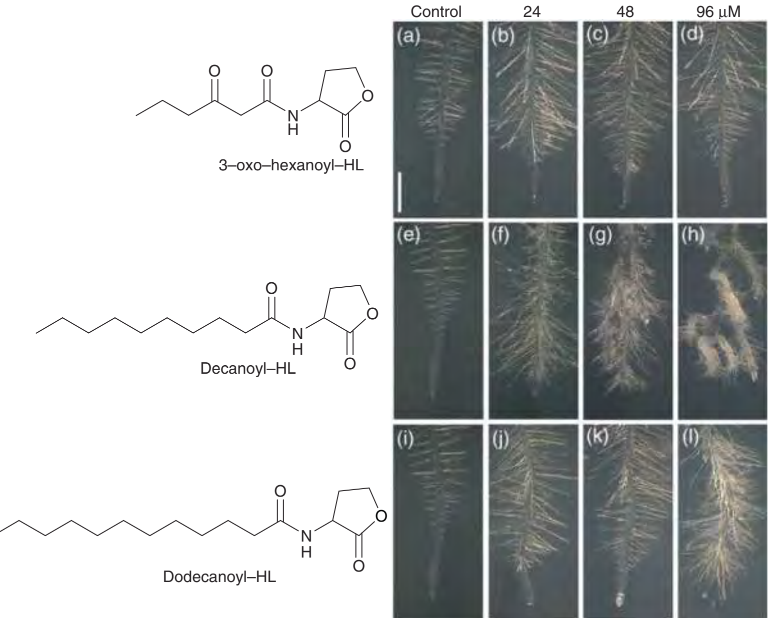

Figure 28.3 *N*-Acyl-L-homoserine lactones regulate *Arabidopsis* root system architecture. Representative photographs of primary roots of 9-day-old *Arabidopsis* seedlings grown in the presence of the indicated compounds.

axr2, and *doc1*, showed similar growth inhibition to C10-HL as observed in wild-type seedlings. It was also found that mutant and overexpressor lines for an *Arabidopsis* fatty acid amide hydrolase gene (AtFAAH) sustained contrasting growth response to C10-HL, thus suggesting that plants possess the enzymatic machinery to metabolize AHLs (Ortiz-Castro et al., 2008a).

Certain fatty acid amides from plants, including NAEs and alkamides, are strong candidates as AHL signal mimics. These compounds share chemical similarity to AHLs and are also capable of regulating root and shoot architecture (Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008; Morquecho-Contreras et al., 2010). Morquecho-Contreras and associates (2010) isolated an *Arabidopsis* recessive mutant termed *decanamide-resistant root1* (*drr1*) after screening a mutant population for primary root growth resistance under treatment with 30 μM of *N*-isobutyl-decanamide that represses growth in wild-typeseedlings. The *DRR1* locus was required at an early stage of pericycle cell activation to form lateral root primodia. As the *drr1* mutants were also resistant to inhibition of primary root growth in a medium containing C10-HL, this study strongly suggests that plants have evolved a genetic mechanism to perceive NAEs, alkamides, and AHLs, all of which modulate root development (Ortiz-Castro et al., 2008a; Morquecho-Contreras et al., 2010).

Alkamides and NAEs represent an interesting group of natural products, which may interfere with bacterial QS. For instance, the related *N*-acyl-cyclopentylamides (*N*-acyl-CPA) showed strong activity to inhibit QS, *N*-decanoyl-cyclodipentylamide (C10-CPA) being the strongest inhibitor of virulence factors, including elastase and pyocyanin. This compound interferes with the *las* and *rhl* QS systems in *P. aeruginosa* (Ishida et al., 2007).

Perception of AHLs by plants required the participation of Cand2 and Cand7, two candidate G-protein-coupled receptors (GPCRs), regulating root growth by the bacterial AHLs and modulating interactions between plants and microbes (Jin et al., 2012). In a separate study, it was reported that the treatment of *Arabidopsis* roots with *N*-3-oxo-hexanoyl-homoserine lactone (3OC6-HL) and *N*-3-oxo-octanoyl-homoserine lactone (3OC8-HL) resulted in significant root elongation. The genetic analysis revealed that the T-DNA insertional mutants of gcr1, encoding a GPCR GCR1,

were insensitive to 3OC6-HL or 3OC8-HL in assays of root growth. The loss-of-function mutants of the sole canonical Gα subunit GPA1 showed no response to AHL promotion of root elongation, while Gα gain-of-function plants overexpressing either the wild type or a constitutively active version of *Arabidopsis* Gα exhibited the exaggerated effect on root elongation caused by AHLs. Furthermore, the expressions of GCR1 and gpa1 were significantly upregulated after plants were contacted with both AHLs, indicating that GCR1 and GPA1 are likely involved in AHL-mediated elongation of *Arabidopsis* roots (Liu et al., 2012). Taken together, the available evidence suggests that AHLs regulate morphogenetic processes in the root in a dose- and structure-dependent manner, promoting growth in low concentrations and repressing growth at high levels. This provides insight into the mechanism of plant responses to bacterial QS signals (see Chapter 73).

In plants, the activity of phytohormones is modulated through the participation of intracellular second messengers including Ca^{2+}, nitric oxide (NO), and hydrogen peroxide (H_2O_2), which participate in multiple physiological processes, such as systemic acquired resistance, the hypersensitive response, leaf senescence, programmed cell death, stomatal closure, root gravitropism, cell wall formation, and root development (Grant and Loake, 2000; Neill et al., 2002; Mittler et al., 2004; Schuhegger et al., 2006; Tuteja and Mahajan, 2007; Xuan et al., 2008). A global analysis of gene expression changes in *A. thaliana*, in response to *N*-isobutyl-decanamide, revealed the participation of defense-responsive transcriptional networks, in particular, genes encoding enzymes for JA biosynthesis and the JA receptor COI1 in plant responses to alkamides. Moreover, the participation of NO and H_2O_2 as mediators of alkamides and AHLs inducing both developmental changes and conferring resistance to the pathogen *Botrytis cinerea* was recently evidenced (Méndez-Bravo et al., 2010; Méndez-Bravo et al., 2011). In consonance with these results, it was found that 3-oxo-C10-HL induces the formation of adventitious roots in explants of mung bean (*Vigna radiata*) seedlings via H_2O_2- and NO-dependent cGMP signaling (Bai et al., 2012).

Compelling evidence that 3-oxo-C14-HL conferred resistance in *Arabidopsis* against the biotrophic fungi *Golovinomyces orontii* and the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 was provided by Schikora et al. (2011). The AHL promoted the activation of mitogen-activated protein kinases AtMPK3, an AtMPK6 followed by a higher expression of the defense-related transcription factors WKRY22 and WKRY29 and *PATHOGENESIS-RELATED1* gene (Schikora et al., 2011). Changes in the cytosolic Ca^{2+} concentration in root cells were documented in *Arabidopsis* seedlings that were treated with 10 μM C4-HL. This was the first evidence suggesting that C4-HL may act as an elicitor from bacteria to plants through Ca^{2+} signaling, connecting bacterial QS signaling to developmental processes (Sung et al., 2011). The ability of plants to detect AHLs produced by rhizobacteria in the rhizosphere and the similarity of these QS signals to alkamides and NAEs open the possibility that plants have evolved particular receptor proteins and signal transduction pathways to communicate with rhizobacteria. This will represent an ongoing area for research in the plant–bacteria interaction mechanisms.

28.4 CYCLIC DIPEPTIDES MODULATE PLANT–BACTERIA INTERACTIONS

The structural simplicity of bacteria belies their extraordinary sophistication in manipulating their environment. Nowhere is their versatility more apparent than in their ability to communicate with higher organisms. In a screen for QS molecules from *Vibrio vulnificus* that could stimulate AHL-dependent QS reporter strains, Park and associates (2006) identified a cyclic dipeptide cyclo(Phe-Pro) rather than an AHL. Cyclo(Phe-Pro) was released into bacterial cell-free culture medium in a density-dependent manner, with maximum concentrations present as cells enter stationary phase. Addition of either purified or chemically synthesized cyclo(Phe-Pro) altered expression of the major virulence factors in several *Vibrio* spp., thus representing a potential QS molecule that contributes to the pathogenesis of these bacteria (Park et al., 2006; Klose, 2006). The widespread distribution of cyclodipeptides (CDs) and their derivate diketopiperazines (DKPs) indicates that these signals could be part of a function that is common to many bacterial species (Gondry et al., 2009). However, the DKPs have been reported not only in pathogenic species but also in bacteria typified as beneficial to plants such as *Pseudomonas putida* (Degrassi et al., 2002) or to animals such as *Lactobacillus* sp. (Ström et al., 2002; Li et al., 2011). These exciting findings add further complexity to transkingdom cell-to-cell signaling.

DKPs are synthesized by cyclodipeptide synthases (CDPSs), which constitute a family of peptide-bond forming enzymes that use aminoacyl-tRNAs (aa-tRNAs) as substrates to form various cyclodipeptides. The CDPS family includes at least eight identified members found in various bacterial species (Sauguet et al., 2011; Seguin et al., 2011). The CDPS AlbC from *Streptomyces noursei* uses mainly phenylalanyl-$tRNA^{Phe}$ (Phe-$tRNA^{Phe}$) and leucyl-$tRNA^{Leu}$ (Leu-$tRNA^{Leu}$) as substrates to

synthesize cyclo(L-Phe-L-Leu) (Sauguet et al., 2011). Certain DKPs such as cyclo(His-Gly), cyclo(His-Ala), and cyclo(L-His-L-Phe) show antitumor activity reducing the viability of HeLa, WHCO3 and MCF-7 cells from cervical, esophageal, and mammary carcinoma (Kanoh et al., 1999; Lucietto et al., 2006). Other compounds including cyclo(L-Phe-L-Pro) and cyclo(L-Ile-L-Pro) that were isolated from *Propionibacterium* strains showed activity against *Aspergillus fumigatus* and *Rhodotorula mucilaginosa* (Lind et al., 2007), whereas the cyclic dipeptide cyclo(L-Arg-D-Pro) was found to inhibit the growth of the human pathogen *Candida albicans* (Houston et al., 2002). Most interesting is the proposed role of DKPs as QS blockers because cyclo(L-Pro-L-Phe) was capable of inhibiting luminescence in *V. fischeri* (Campbell et al., 2009). Competition studies showed that cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) antagonize the 3-oxo-C6-HL-mediated induction of bioluminescense, suggesting that these DKPs may compete for the same binding site as AHLs (Holden et al., 1999). On the other hand, the cyclo(L-Leu-L-Pro) signal produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*, modulating the repression of transcription of the aflatoxin-related genes. This is the first report of a cyclodipeptide that affects aflatoxin production (Yan et al., 2004).

Even though DKPs seem to play many different functions in bacteria, recent information points to a very important role in ecological processes. Ortiz-Castro et al. (2011) explored one facet of *P. aeruginosa/Arabidopsis* interspecies relationships by showing that QS pathways in *P. aeruginosa* regulate the biosynthesis of bacterial DKPs that in turn mimic the activity of the plant growth hormone auxin. When grown nearby, *P. aeruginosa* could enhance the growth of *A. thaliana* seedlings by modulating RSA. This effect was enhanced when the bacterial strains contained mutations in components of the *LasI* QS system. Profiling bacterial extracts for fractions that could enhance lateral root growth in *Arabidopsis* led to the identification of three DKPs, namely cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr), which were more abundant in the QS mutant strains. DKPs induced the auxin reporters *DR5:GUS* and *BA3:GUS* in plant roots, and their growth-promoting activity was dependent on key components of the auxin signaling system. Taken together, these data outline a molecular mechanism by which QS modulates bacterial metabolism to facilitate communication with its plant host.

Although other Rhizobacteria such as *Bacillus* spp. or several environmental strains of *Pseudomonas* can induce plant growth by direct or indirect means, there is limited information about the early signaling events that take place during plant perception of bacteria. Plants are faced with the challenge of how to recognize and exclude pathogens that pose a genuine threat, while tolerating more benign organisms. Importantly, the DKPs identified in *P. aeruginosa* clearly show the importance of the *LasI* QS system in plant growth promotion by this bacterium and revealed that DKPs are likely involved in phytostimulation through modulating auxin responses.

The beneficial effects of *P. aeruginosa LasI* mutants to *Arabidopsis in vitro* seem to be contradictory with the notion of *P. aeruginosa* as a plant pathogen. It is tempting to speculate that overproduction of DKPs is beneficial to plants not only because they directly activate hormonal responses but also because they decreasing virulence factors in the bacterium. Evidence supporting this hypothesis comes from a recent report demonstrating that the human beneficial bacteria *Lactobacillus reuteri* produces the cyclic dipeptides cyclo(L-Tyr-L-Pro) and cyclo(L-Phe-L-Pro), which negatively regulate the expression of toxic shock syndrome toxin-1 of *Staphylococcus aureus*, a human pathogen, and of cholera toxin and toxin-regulated pilus production in *Vibrio cholerae*, thus decreasing their virulence (Bina and Bina, 2010; Li et al., 2011). All this information contributes to a better understanding of interspecies cell-to-cell communication between *Lactobacillus* and *Staphylococcus*, and provides a unique mechanism by which endogenous or PGPR strains may attenuate virulence factors in bacterial pathogens that associate with eukaryotic hosts.

28.5 CONCLUSION

Bacteria interact extensively with plants and develop into complex multicellular populations. The relevance of these interactions to plant health and disease is just beginning to be understood and appreciated. Accumulating information has shown the importance of classic phytohormones such as auxins and cytokinins in plant growth promotion by rhizobacteria, particularly in the regulation of RSA. The root system can sense and respond to bacterially produced AHLs and DKPs. Among the reported activities of DKPs, their auxin-like activity as well as the inhibitory effects on virulence factors in plant pathogenic bacteria deserve further attention. Moreover, many plant species are able to produce compounds by roots that structurally mimic AHLs, including alkamides and NAEs, perhaps to modulate the behaviors of their associate bacteria. Cross-kingdom communication between bacteria and eukaryotic organisms is still a young field. The coming years of research should help to establish the generalities and specific facets of the communication between plants and rhizobacteria by means of small molecule signaling, opening new strategies for agricultural management based on bioinoculants or their products.

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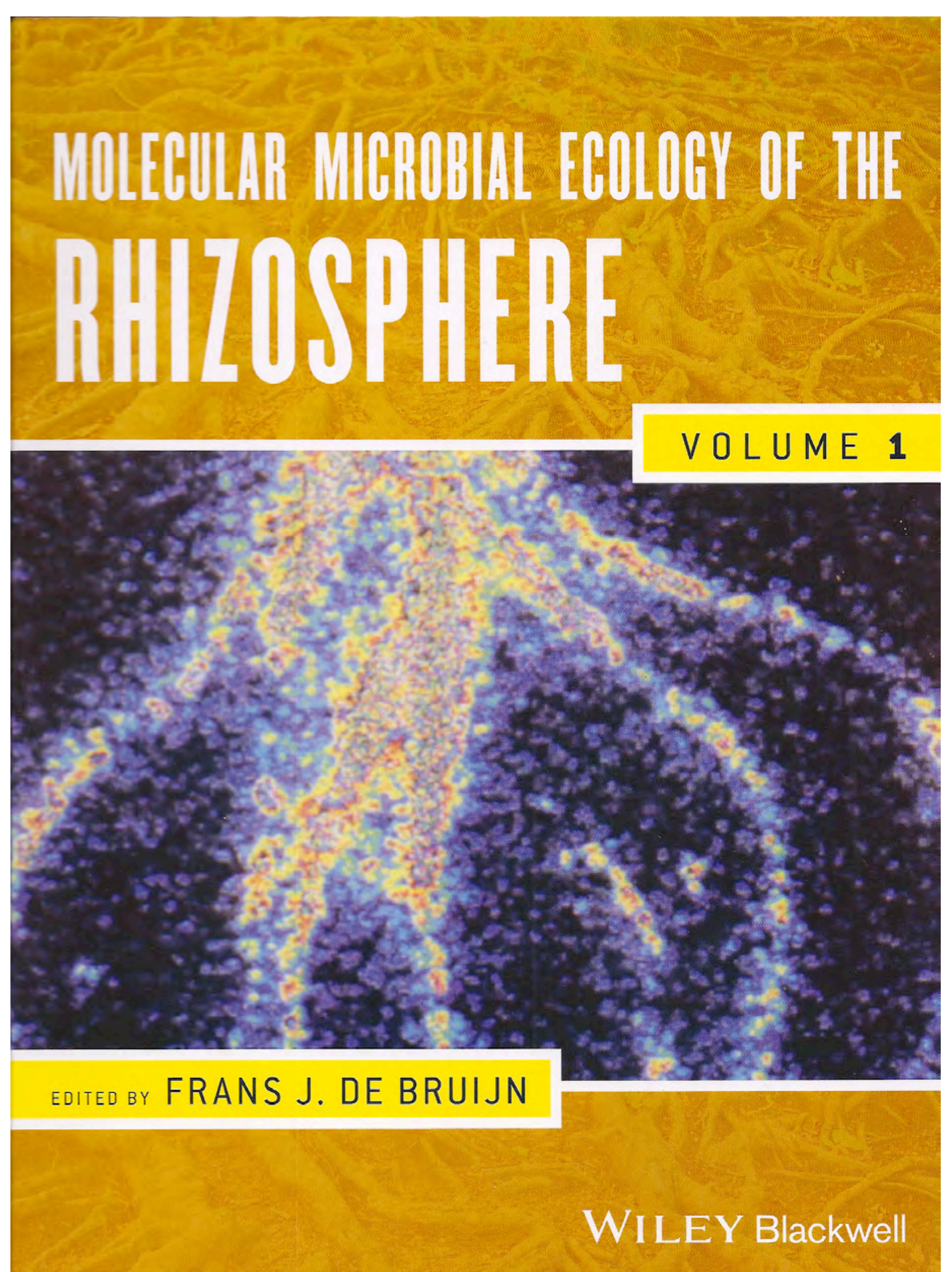
CAPITULO III.



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Chapter 32

Rapid Identification of Plant-Growth-Promoting Rhizobacteria Using an Agar Plate Cocultivation System with *Arabidopsis*

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32.1 INTRODUCTION

Over the last 50 years, the major challenge of providing sufficient food for the increasing human population has been facilitated by the application of high inputs of chemical fertilizers. Current production methods in agriculture using large amounts of nitrogen (N) and phosphorus (P) sources are not only costly but they also lead to several environmental and health problems (Conway and Pretty, 1988). In addition, in crops such as wheat and maize, intensive arable cultivation is no longer sustainable because it often leads to soil degradation (Loneragan, 1997).

Root-associated microorganisms play important roles for plant growth and health. Plant-growth-promoting rhizobacteria (PGPR) proliferate in the rhizosphere, the region of the root in contact with the soil, which can be modified by root exudates (Bloemberg and Lugtenberg, 2001; Lugtenberg et al., 2002; Persello-Cartieaux et al., 2003; see Chapter 53). These bacteria can confer immunity or resistance against a wide range of foliar diseases by activating plant defenses, thereby reducing the susceptibility to pathogen attack (Van Loon et al., 1998; Compant et al., 2005; see Chapter 54). Direct plant growth promotion by PGPR is also based on improved nutrient acquisition and production of signals involved in root morphogenesis, biomass accumulation, and gas exchange (López-Bucio et al., 2007; Ortíz-Castro et al., 2008a, 2008b, 2009; Dodd et al., 2010; see Chapters 27, 28).

The use of PGPR-based inoculants in agriculture as "biofertilizers" may reduce the need for chemical fertilizers. Currently, there is a strong growing market for microbial inoculants worldwide with an annual growth rate of approximately 10% (Vessey, 2003; Berg, 2009; Pérez-García et al., 2011). However, interaction of PGPR with host plants is an intricate and interdependent relationship, involving not only the two partners but also other biotic and abiotic factors. The impact of bacterial inoculants on plant productivity under field conditions has not always been consistent, with positive, neutral, variable, or deleterious effects. This can be attributed to several factors, including an inefficient colonization of roots by inoculated bacteria, the amount of bacteria inoculated, rhizosphere competence, the bioactivity of the strain toward plant roots, and the root response itself, among others (Berg, 2009; Pérez-García et al., 2011).

Promotion of root growth is one of the major markers of PGPR (Glick et al., 1995; Patten and Glick, 2002). The root system displays considerable plasticity in its morphology and physiology in response to variability within

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its environment. Rapid establishment of roots either by proliferation of lateral or adventitious roots or root hairs is advantageous for plants as it increases the exploratory potential of the root system. Many PGPR synthesize plant hormones, and in this way, they may positively affect root growth (Spaepen et al., 2007; Ortiz-Castro et al., 2009; Dodd et al., 2010; see Chapters 27, 28, 29).

Although economically important species such as maize, tomato, pea, and barley have been traditionally chosen as experimental material to assess the impact of PGPR, the small crucifer *Arabidopsis thaliana* possesses a number of attributes that make it particularly well suited as a model species (Persello-Cartieaux et al., 2001; Ryu et al., 2003, 2005; López-Bucio et al., 2007; Contesto et al., 2010; Ortiz-Castro et al., 2011). Several rhizobacteria are well known with regard to their mode of action in phytostimulation. These comprise members of the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Strenotrophomonas*, and *Arthrobacter* (Ryan et al., 2009; Gutiérrez-Luna et al., 2010; Velázquez-Becerra et al., 2011). The *Pseudomonas* genus is particularly interesting because of its ability to undergo transitions to become an important and dangerous plant pathogen (i.e., *P. syringae, Pseudomonas aeruginosa*). However, it is also studied for its ability to colonize the rhizosphere (i.e., *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. aureofaciens*, and *P. chloraphilis*), where it can act as plant-beneficial bacteria (Venturi, 2006).

Pseudomonas aeruginosa and other species both produce and use *N*-acyl- L-homoserine lactones (AHLs) for communication through a regulatory mechanism named quorum sensing (QS), which links perception of bacterial cell density to gene expression (Fuqua et al., 1994; see Section 9). QS modulates many physiological processes such as symbiosis, virulence, resistance to oxidative stress, antibiotic production, motility, and biofilm formation (Miller and Bassler, 2001). *P. aeruginosa* possesses at least two AHL QS systems, the *las* and *rhl* systems (Pesci et al., 1997); in the *las* system, LasI directs the synthesis of the AHL signal *N*-(3-oxo-dodecanoyl)- L-homoserine lactone (3-oxo-C12-AHL), which interacts with LasR and activates or represses target promoters (Passador et al., 1993; Pearson et al., 1994). In the *rhl* system, RhlI directs the synthesis of another AHL signal, *N*-(butanoyl)- L-homoserine lactone (C4-AHL), which interacts with the cognate regulator RhlR and modulates the transcription of target genes as well (Pearson et al., 1995). The Las and Rhl systems are intimately connected and regulate the production of multiple virulence factors and biofilm formation as well as PGPR traits (Smith and Iglewski, 2003; Ortiz-Castro et al., 2011).

In a previous report we showed the potential of *P. aeruginosa* in phytostimulation. Genetic, chemical, and plant growth data indicated that in this bacterium, the *LasI* QS system controls the production of three diketopiperazines (DKPs)—namely, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr), which possess a weak auxin activity (Ortiz-Castro et al., 2011).

In this chapter, we highlight the use of *A. thaliana* as a plant model to characterize PGPR. The effects of cocultivation with a collection of five *P. aeruginosa* strains, including *P. aeruginosa* WT PAO1, AHL synthase-deficient mutants *lasI*, *rhlI*, *rhlI/lasI*, and the *atuD* mutant affected in catabolic pathways independent of the *LasI* or *rhlI* QS systems, on root and shoot biomass production are shown. In addition, we compared the plant-growth-promotion potential of three rhizobacterial isolates including *Escherichia coli*, *P. fluorescens*, and *P. putida*. Out of all bioassays performed, we consistently found that the *P. aeruginosa* QS mutant *lasI*, as well as WT strains of *P. putida* and *P. fluorescens*, dramatically increased the growth of *Arabidopsis* seedlings *in vitro*. Thus, by testing rhizobacterial species that impact on root architecture, it may be possible to select PGPR for further assessment in crop production.

32.2 METHODS

32.2.1 Plant Material and Growth Conditions

A. thaliana (Col-0) seeds were surface sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2× Murashige and Skoog medium (Murashige and Skoog basal salts mixture, M5524; Sigma). The suggested formulation is 4.3 g/l of salts for 1× medium; we used 0.9 g/l, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 μmol·m^2/s, and temperature of 22 °C.

32.2.2 *In vitro* Plant/Bacteria Cocultivation Assay

Bacterial strains used in this work were the *P. aeruginosa* PAO1 WT strain, *P. aeruginosa* QS-related *lasI*, *rhlI*, and *rhlI/lasI* single and double mutants (Pesci et al., 1997; Ortiz-Castro et al., 2011), and the *P. aeruginosa atuD* mutant, which is defective in citronellyl-CoA dehydrogenase (Aguilar et al., 2006; Díaz-Pérez et al., 2007). Rhizosphere strains of *E. coli*, *P. fluorescens*, and *P. putida* were also included in the experiments. All bacterial strains

were evaluated *in vitro* for their plant-growth-promotion ability, using the *Arabidopsis* Col-0 ecotype. Bacterial cultures with a density of 2.5×10^8 CFU were streaked on agar plates containing 0.2× MS medium. Subsequently, 6-day-old germinated *Arabidopsis* seedlings (20 seedlings per plate) were grown on one side of the plate, opposite to the bacterial streak site at 5 or 1 cm distance from the root tip. Alternatively, 6-day-old seedlings grown on 0.2× MS medium were transferred to plates with a bacterial inoculum, by placing the primary root over the bacterial streak. The seedlings were grown for a further 8-day period in the growth chamber, in a completely randomized design. All experiments were replicated three times. The fresh weight of plants was determined with an analytical balance (Ohaus Corp.) with a 0.0001 g precision value. For all experiments, the overall data were statistically analyzed using the SPSS 10 software (SPSS). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in shoot and root biomass production. Representative photographs were taken to highlight the impact of the bacteria on the phenotype of the *Arabidopsis* root system.

32.3 RESULTS

32.3.1 Strategy for Identifying PGPR *In Vitro*

Plant-associated bacteria may cause beneficial, neutral, or pathogenic effects on plants upon inoculation. The growth-promoting activity of bacteria on plant morphogenesis mediated by diffusible compounds or volatile organic compounds (VOCs) was tested by cocultivation of *Arabidopsis* seedlings with a bacterial streak at a long (5 cm) or short (1 cm) distance (Fig. 32.1).

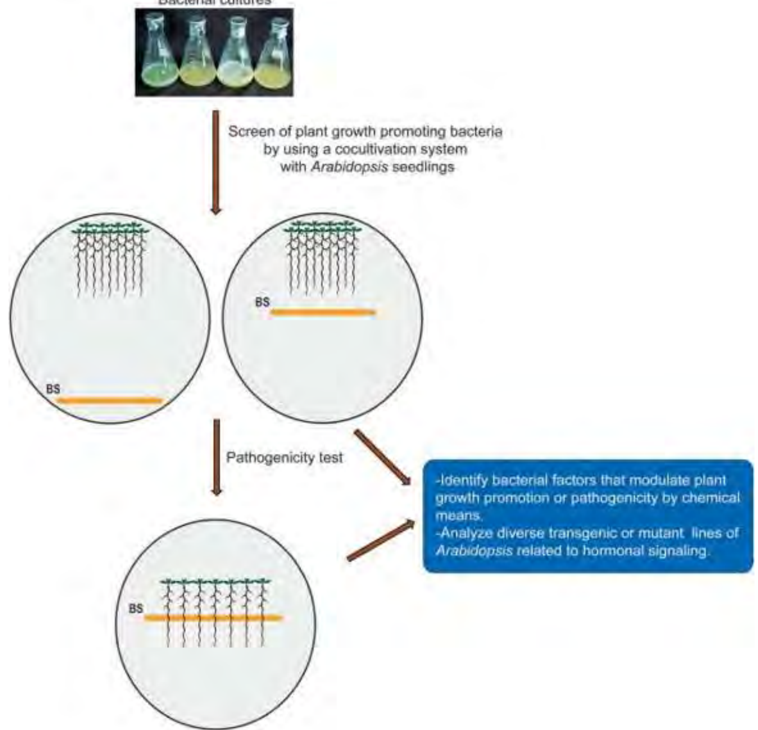

Figure 32.1 A novel procedure for rapid identification of plant growth-promoting rhizobacteria.This figure illustrates how the model plant *Arabidopsis* can be cocultivated with bacterial isolates to select those that are beneficial to plants or determine potential plant deleterious effects. BS = Bacterial streak.

To determine potential pathogenic effects, the plants were transplanted to establish direct contact of the root with the bacterial proliferation site. Such cocultivation strategy may help to identify bacteria that are likely to cause soft-rot symptoms or plant growth deleterious effects, as the bacterial cells may produce antibiotics or secrete a variety of potent degradative enzymes. The use of *Arabidopsis* for testing PGPR traits provides at least two important advantages over crop species: (i) the rapid identification of bacterial factors/signals that modulate plant growth or pathogenicity, which can be assessed by chemical means, and (ii) further dissection of the hormonal pathways that participate in plant responses by using genetic tools (i.e., mutant/overexpressor lines and inducible hormone markers) available for *Arabidopsis*.

32.3.2 Growth Promotion of *A. thaliana* Cocultivated at Distance with QS-Related Mutants of *P. aeruginosa*

P. aeruginosa has been known to infect a number of plants, including *A. thaliana*, and some virulence factors that cause disease have been investigated (Rahme et al., 1995; Plotnikova et al., 2000; Walker et al., 2004). However, a quick search in the literature yielded several reports showing the benefits of *P. aeruginosa* to plants grown under different conditions (Devliegher et al., 1995; Steindler et al., 2009). *P. aeruginosa* represents an interesting bacterial model to test the interactions with *A. thaliana* because this bacterium uses AHL signals for cell-to-cell communication and the *rhlI*, *lasI*, and *rhlI/lasI* QS-related mutants are available to test the contribution of QS in plant growth promotion. Moreover, AHL signals have been found to modulate root-system architecture in *Arabidopsis*, thus representing potential hormone-like signals involved in phytostimulation (Ortiz-Castro et al., 2008b; see Chapters 71, 73).

We tested the *in vivo* effect of *P. aeruginosa* on plant growth by cocultivating 6-day-old *Arabidopsis* seedlings, grown on agar plates containing 0.2× MS medium, with 2.8×10^8 cfu of *P. aeruginosa* PAO1 WT and the AHL synthase-deficient mutants *lasI*, *rhlI*, and *rhlI/lasI* double mutant by streaking the bacteria on the surface of the medium at a long (5 cm; Fig. 32.2a–e) or short (1 cm; Fig. 32.2f–j) distance from the primary root tip.

After 8 days of growth using this cocultivation system, a significant increase in shoot and root biomass production was observed in plants cocultivated with WT and all *P. aeruginosa* mutants (Fig. 32.2k and l). Interestingly, the *lasI* single and *rhlI/lasI* double mutant exhibited the

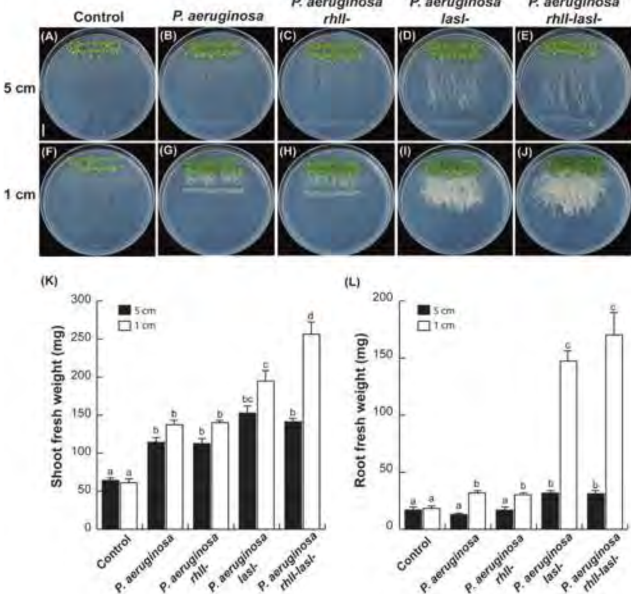

Figure 32.2 Effect of cocultivation with *Pseudomonas aeruginosa* WT and QS mutant strains on plant growth. Six-days-old *Arabidopsis thaliana* seedlings were cocultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at a distance of 5 cm (a–e) or 1 cm (f–j) from the primary root tip and grown for 8 days. Representative photographs were taken for plates from each treatment. The experiment included five different plates per treatment and was repeated three times with similar results. (k) Effect of bacterial cocultivation on shoot fresh weight or (l) root fresh weight. Data from (k) and (l) show the means ± SD from three groups of 30 seedlings. Different letters indicate means statistically different at *P* 0.05. Scale bar = 1 cm.

greatest stimulatory effect on shoot and root biomass production that correlated with a prolific induction of lateral roots and root hairs when cultivated in close proximity to the root system (Fig. 32.2i and j). These findings suggest that AHL signals produced by the AHL synthases LasI and RhlI modulate the production of compounds directly involved in biomass production, cell division, and differentiation processes in the root. AHL-deficient mutants were the most effective bacteria promoting plant growth because they produce high amounts of DKPs with an auxin-like activity, which directly increases lateral root and root hair formation (Ortiz-Castro et al., 2011); at the same time, the DKPs might decrease the expression of virulence factors as occurs in human pathogenic bacteria (Bina and Bina, 2010; Li et al., 2011).

32.3.3 Biomass Accumulation in *Arabidopsis* Seedlings Cocultivated in Direct Contact with Bacteria

Signal exchange between rhizobacteria and plant cells generally occurs across the barrier of the plant cell wall at the root epidermis. To determine the potential pathogenic effects of wild-type (WT) *Pseudomonas* when the roots were in direct contact with the root system, a transplanting method was devised in which *Arabidopsis* seedlings were germinated and grown for 6 days in Petri plates containing solidified 0.2× MS nutrient medium and then transferred to the same medium containing a streak of each of the following bacteria: *P. aeruginosa* PAO1 WT strain, *P. aeruginosa* QS-related *lasI*, *rhlI*, and *rhlI/lasI* single and double mutants, respectively, *P. aeruginosa atuD* mutant, and rhizosphere strains of *E. coli*, *P. fluorescens*, and *P. putida*. Direct contact of roots with the bacterial inoculum of *P. aeruginosa* PAO1 (Fig. 32.3c), the *rhlI* (Fig. 32.3d), and *atuD* (Fig. 32.3e) mutants caused a general delay in the growth of *Arabidopsis* compared with uninoculated seedlings or seedlings inoculated with *E. coli* (Fig. 32.3a and b). However, direct cocultivation of plant roots with the *lasI* and *rhlI/lasI* mutants (Fig. 32.3f and g), as well as *P. putida* (Fig. 32.3h) and *P. fluorescens* (Fig. 32.3i), had up to tenfold increase in root fresh weight and a threefold increase in shoot fresh weight when compared to axenically grown seedlings or plants cocultivated with *E. coli* (Fig. 32.3j and k).

Our results indicate that AHL-dependent QS leads to a reduction and/or delay in plant growth as shown in seedlings cocultivated with WT *P. aeruginosa* PAO1, which are in agreement with previous reports indicating that direct contact of plants with *P. aeruginosa* affects plant growth (Rahme et al., 1995; Walker et al., 2004). Again, *lasI* and *rhlI/lasI* mutants had the biggest positive effect, which is somewhat comparable with that of *P. putida* and *P. fluorescens*, which are well-known PGPR. This methodology can be used to select putative PGPR that strongly promote plant growth likely by modulating root architectural processes.

32.4 DISCUSSION

Plant agronomists have traditionally chosen economically important species such as maize, tomato, pea, and barley as their experimental material to assess the impact of PGPR. Unfortunately, these species are in several respects not ideal for genetic studies: the generation times are long, and relatively large amounts of space are required for cultivation. In contrast, the small crucifer *A. thaliana* possesses a number of attributes that make it particularly well suited as a model species. A substantial base of genomic information already exists and a diverse collection of mutants and transgenic lines are available for both forward and reverse genetics (Meyerowitz, 2001). Moreover, its suitability to be grown *in vitro* and the availability of methods for cocultivation with rhizobacteria have been exploited (Persello-Cartieaux et al., 2001; Ryu et al., 2003, 2005; López-Bucio et al., 2007; Contesto et al., 2010; Ortiz-Castro et al., 2011).

Recent developments in techniques for studying rhizobacteria–plant interactions under controlled conditions are important in the assessment of effectiveness and consistent performance of microbial strains to be used in crop production and protection. After isolation of rhizobacteria, different tests can be performed to select putative PGPR. Among the biochemical tests traditionally used to find PGPR traits, the most common ones include the production of plant-growth-regulating substances (i.e., auxins and cytokinins) (Spaepen et al., 2007; Ortiz-Castro et al., 2008b; Dodd et al., 2010), degradation of the ethylene precursor ACC by ACC deaminase (Glick, 2005), phosphate solubilization (Hameeda et al., 2008), siderophore production (Masalha et al., 2000; Rroco et al., 2003), iron reduction (Valencia-Cantero et al., 2007), and/or nitrogen fixation (Zhang et al., 1996; Dixon and Kahn, 2004). These different approaches have proved to be effective strategies to isolate PGPR. However, an important limitation is that some of the biochemical traits described earlier are inducible; that is, they are expressed only under certain growth conditions.

Roots play many essential adaptive functions in plants including anchorage to soil, acquisition of nutrients and water, and production of compounds with nutritional or regulatory properties for microorganisms. Secretion of carbon sources by roots dramatically modifies the local environment, supplying nutrients to rhizosphere bacteria, including sugars, organic acids, amino acids, phenolics, and vitamins, which are collectively termed as root exudates (Rudrappa et al., 2008; Badri and Vivanco,

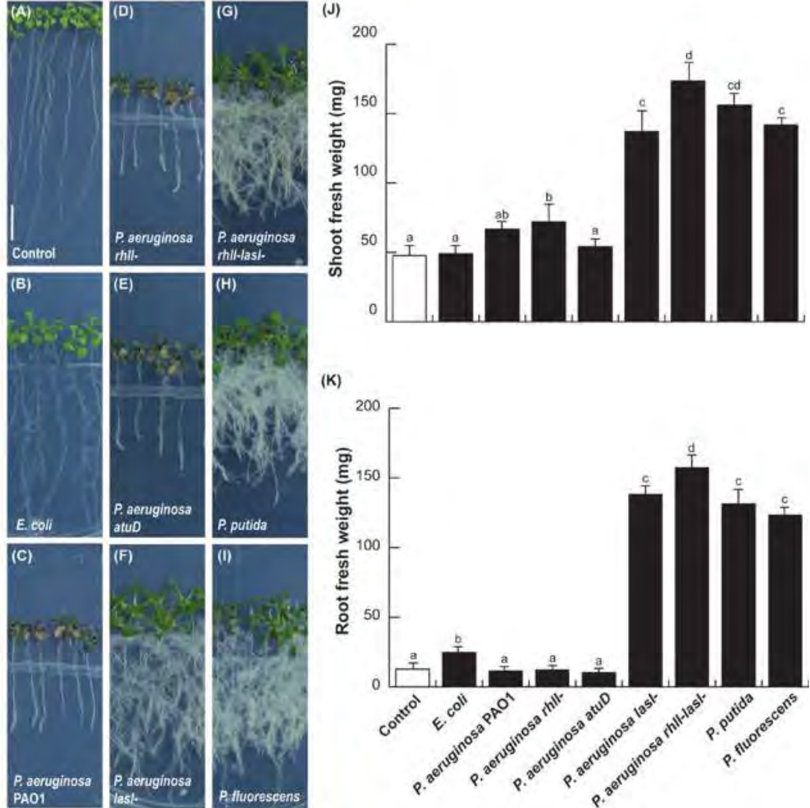

Figure 32.3 Growth promotion in plants grown in direct contact with a bacterial inoculum. Bacteria are allowed to colonize the root system. Six-days-old *A. thaliana* seedlings were transferred to 0.2× MS medium without bacteria (a) or placed over a streak of bacteria including *Escherichia coli* (b), WT *P. aeruginosa* PAO1 (c), *P. aeruginosa rhlI* (d), *atuD* (e) or *lasI* (f), *rhlI/lasI* (g) and plant growth-promoting rhizobacteria *Pseudomonas putida* (h) and *Pseudomonas fluorescens* (i) and grown for 8 days. Effect of bacterial cocultivation on shoot fresh weight (j) or root fresh weight (k). Data from (j) and (k) show the means ± SD from three groups of 30 seedlings. These analyses were repeated three times with similar results. Different letters indicate means statistically different at *P* 0.05. Scale bar = 1 cm.

2009; see Chapter 22). Thus, the rhizosphere of wild plant species appears to be the best source from which to isolate PGPR. However, the plant–bacteria interaction in the rhizosphere can be beneficial, neutral, variable, or deleterious depending on the communication established by the two partners and upon the biotic and abiotic factors prevalent during the interaction.

Currently, it is not easy to draw a clear distinction between pathogenic and plant-growth-promoting *Pseudomonas* species. They colonize the same ecological niches and possess similar mechanisms for plant colonization (Preston, 2004). The interactions of *P. aeruginosa* with plants are not completely understood. In their initial screening to identify *P. aeruginosa* strains pathogenic to plants, Rahme et al. (1995) evaluated a collection of 75 *P. aeruginosa* strains, of which 30 were human, 20 soil, and 25 plant isolates, for their ability to cause disease on leaves of four different *A. thaliana* ecotypes. Most strains elicited no symptoms, and only two strains, UCBPP-PA14, a human isolate, and UCBPP-PA29, a plant isolate caused severe soft-rot symptoms in the leaves of some, but not all, of the ecotypes tested (Rahme et al., 1995). This challenged the notion that all *P. aeruginosa* strains are pathogenic. In contrast, accumulating information shows the potential of *P. aeruginosa* as a phytostimulant bacterium (Devliegher et al., 1995; Steindler et al., 2009). Our data show the possibility that environmental *P. aeruginosa* isolates with alterations on the LasI QS system may have growth-promoting, instead of pathogenic, effects on plants. It is also important to

clarify that *P. aeruginosa* pathogenicity tests have focused mainly on leaves, infiltrating thousands of bacteria into plant tissues (Rahme et al., 1995; Plotnikova et al., 2000; Walker et al., 2004). Under such conditions, it is reasonable to argue that the inoculation strategy likely causes soft-rot symptoms in plants as bacterial cells secrete a variety of antibiotics and/or degradative enzymes.

Although several rhizobacteria from the *Pseudomonas* genus can induce plant growth by direct or indirect means, there is limited information about the early signaling events that take place during plant perception of bacteria. Plants are faced with the challenge of how to recognize and exclude pathogens that pose a genuine threat, while tolerating more benign organisms. Our research recently showed the importance of the LasI QS system of *P. aeruginosa* in plant growth promotion by this bacterium and identified a novel class of signals, the DKPs, which were involved in phytostimulation, possibly modulating auxin responses (Ortiz-Castro et al., 2011).

It is well known that bacteria that colonize plant surfaces produce and respond to a diverse mixture of AHL signals. Both bacteria and plants likely utilize such kind of communication for influencing the outcome of their relationship. The non-pathogenic bacteria *P. fluorescens* and *P. putida* produce AHLs and DKPs (Loh et al., 2002; Degrassi et al., 2002), whether this property is related to their strong stimulatory effect on plant growth remains to be determined. Higher plants produce compounds that affect QS-regulated responses in bacteria, which are present in root exudates of pea (*Pisum sativum*) and *Medicago truncatula* (Teplitski et al., 2000; Gao et al., 2003). Advances in this field have identified the *N*-acyl-ethanolamines and alkamides, which share high structural similarity to AHLs (Ortiz-Castro et al., 2008b). This indicates that secretion of compounds by plant roots, which act as AHL signal mimics, may affect AHL-regulated behaviors in bacteria.

Bacterial communication also depends on the production of DKPs. The beneficial human bacteria *Lactobacillus reuteri* RC-14 produces DKPs that are able to interfere with the *staphylococcal* QS system *agr*, a key regulator of virulence genes (Li et al., 2011). Ortiz-Castro et al. (2011) reported that the *lasI* single and *rhlI/lasI* double mutants, which exhibited the greatest stimulatory effect on shoot and root biomass production in *Arabidopsis*, produce vast amounts of cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr). This increased production of DKPs may play a dual function in the interaction of bacteria with plants, first, by inhibiting or decreasing the production of virulence factors in the bacteria, and second, by acting as auxin-like signals they can promote lateral root and root hair development in the plant. This explains why AHL production in *P. aeruginosa* seems to repress the PGPR effect.

The use of *Arabidopsis*–rhizobacteria cocultivation protocols discussed here might not only contribute to identify putative PGPR but may also provide a novel strategy by which beneficial and/or virulence factor production by bacterial isolates can be investigated.

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CAPITULO IV.

Plant and Soil

Pseudomonas putida and Pseudomonas fluorescens regulate Arabidopsis root architecture through an auxin mediated pathway and produce bioactive cyclodipeptides

--Manuscript Draft--

Manuscript Number:	
Full Title:	Pseudomonas putida and Pseudomonas fluorescens regulate Arabidopsis root architecture through an auxin mediated pathway and produce bioactive cyclodipeptides
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Abstract:	Aims: This work was conducted to examine the effects of Pseudomonas putida and Pseudomonas fluorescens, in biomass production, root development and activation of auxin signaling of Arabidopsis thaliana. Methods: Selected strains of P. putida and P. fluorescens were tested for activation of DR5:uidA auxin inducible marker, and to promote growth and modulate root system architecture in WT and tir1, tir1afb2afb3, arf7-1, arf19-1, arf7arf19 and rhd6 auxin-related mutants. Ciclodipeptides released by the bacteria were determined by gas chromatography-mass spectrometry. Results: P. putida and P. fluorescens stimulated lateral root and root hair formation and increased plant biomass, which correlated with induction of auxin response. Genetic analyses suggest that growth promotion involves auxin signaling as tir1, tir1afb2afb3, arf7-1, arf19-1 and arf7arf19 mutants show decreased lateral root response to inoculation and because P. putida and P. fluorescens normalize root hair development in the rhd6 mutant. It was found that the bacteria produce the cyclodipeptides cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Tyr), which were able to induce auxin-responsive gene expression in roots. Conclusions: Our results indicate that an important mechanism of phytostimulation by P. putida and P. fluorescens implies an improved root development through activating auxin signaling and that bioactive cyclodipeptides likely participates in plant growth promotion.
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Full title:

Pseudomonas putida and *Pseudomonas fluorescens* regulate *Arabidopsis* root architecture through an auxin mediated pathway and produce bioactive cyclodipeptides

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Keywords: Plant Growth Promoting Rhizobacteria, *Arabidopsis*, growth and development, ciclodipeptides, auxin

Abstract

Aims: This work was conducted to examine the effects of *Pseudomonas putida and Pseudomonas fluorescens*, in biomass production, root development and activation of auxin signaling of *Arabidopsis thaliana.*

Methods: Selected strains of *P. putida and P. fluorescens* were tested for activation of *DR5:uidA* auxin inducible marker, and to promote growth and modulate root system architecture in WT and *tir1, tir1afb2afb3, arf7-1, arf19-1, arf7arf19* and *rhd6* auxin-related mutants. Ciclodipeptides released by the bacteria were determined by gas chromatography-mass spectrometry.

Results: *P. putida* and *P. fluorescens* stimulated lateral root and root hair formation and increased plant biomass, which correlated with induction of auxin response. Genetic analyses suggest that growth promotion involves auxin signaling as *tir1, tir1afb2afb3, arf7-1, arf19-1 and arf7arf19* mutants show decreased lateral root response to inoculation and because *P. putida* and *P. fluorescens* normalize root hair development in the *rhd6* mutant. It was found that the bacteria produce the cyclodipeptides cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Tyr), which were able to induce auxin-responsive gene expression in roots.

Conclusions: Our results indicate that an important mechanism of phytostimulation by *P. putida* and *P. fluorescens* implies an improved root development through activating auxin signaling and that bioactive cyclodipeptides likely participates in plant growth promotion.

.

Keywords: Plant Growth Promoting Rhizobacteria; *Arabidopsis*; root development; auxin signaling; cyclodipeptides.

Abbreviations

ARFs: Auxin response transcription factors

CDPs: Cyclic dipeptides

DKPs: Diketopiperazines

IAA: Indole-3-acetic acid

LRD: Lateral root density

LRN: Lateral root number

PGPR: Plant Growth Promoting Rhizobacteria

QS: Quorum-sensing

Introduction

Plants and bacteria have coexisted for millions of years. As a result, beneficial or detrimental relationships have been established in which signaling molecules play an important role (Hughes and Sperandio 2008). Plants produce many compounds including sugars, organic acids and vitamins, which are used as nutrients or signals by bacteria (Badri et al. 2009; Rudrappa et al. 2008). On the other hand, bacteria release phytohormones, small molecules and volatile compounds, which may act directly or indirectly to activate plant immunity or to regulate plant growth and development (Ortiz-Castro et al. 2009).

The root system provides anchorage to plants and aids in nutrient and water uptake. Compounds released as exudates attract bacteria that may positively influence plant growth as described for plant-growth-promoting rhizobateria (PGPR). This beneficial effect has been reported in annual crops, such as wheat, soybean, lettuce, beans, maize and barley (Kloepper et al. 1990; Barazani and Friedman 1999). Certain rhizobacterial strains may fix atmosferic nitrogen as in the *Rhizobium*-legume symbiosis, or can produce phytohormones and other compounds that influence plant development such as indole-3-acetic acid (auxin, IAA) (Barazani and Friedman 1999; van Loon 2007; Zakharova et al. 1999; Brencic and Winans 2005; Gray and Smith 2005). Moreover, several PGPR may confer immunity against pathogens by activating plant defenses, thereby improving plant fitness in their natural environment (van Loon 2007).

The *Pseudomonas* genus comprises ubiquitous Gram-negative bacteria distributed in different environments and contains species dangerous to plants (i.e. *P. syringae, P. aeruginosa*). Other species have the ability to colonize the rhizosphere (i.e. *P. fluorescens, P. putida, P. aureofaciens* and *P. chloraphilis*), where they can act as plant-beneficial bacteria by antagonizing pathogens or through production of compounds that influence plant-disease resistance and growth (Venturi 2006). Gram-negative bacteria produce and use *N*-acyl-L-homoserine lactones (AHLs) for cell-to-cell communication through a regulatory mechanism known as quorum-sensing (QS), which links perception of bacterial cell density to gene expression (Fuqua et al. 1994). QS coordinates many physiological processes such as symbiosis, production of virulence factors, resistance to oxidative stress, antibiotic production, motility and biofilm formation (Miller and Bassler 2001). Several PGPR, including *P. fluorescens, P. Putida, Rhizobium etli, Burkholderia*

cepacia, and *Sinorhizobium meliloti* exhibit QS systems based on AHL production for rhizosphere communication. Accumulating information indicates that AHL production is common among plant associated-*Pseudomonas*, but less frequent in free-living soil isolates, thus implying an important role of AHLs in plant-bacteria interactions (Elasri et al. 2001; Khmel et al. 2002; D'Angelo-Picard et al. 2005). AHLs can be perceived by plants. In *A. thaliana*, C8-to-C12 AHLs affect primary root growth, lateral root formation and root hair development (Ortiz-Castro et al. 2008). In *Medicago truncatula* and *A. thaliana*, supply of AHLs resulted in differential gene expression that leads to alterations in both defense and development (Mathesius et al. 2003; Von Rad et al. 2008).

Very recently, a second group of QS-like signals, namely cyclic dipeptides (CDPs) or diketopiperazines (DKP's), which in bacteria are capable to interact with LuxR-type receptors, have been found to modify root development (Campbell et al. 2009; Ortiz-Castro et al. 2011). Thus, CDPs may act sometimes as AHL mimics for QS modulation in bacteria or affecting phytohormone responses in plants. DKP and CDP synthesis are catalyzed by a family of tRNA-dependent peptide bond-forming enzymes termed non-ribosomal peptide synthases. DCPs can be synthesized by a wide range of organisms including bacteria, fungi, and animals (Gondry et al. 2009; Furtado et al. 2005; Seguin et al. 2011). Some CDPs such as cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) act as antifungal compounds (Ström et al. 2002), while epipolytiodioxopiperazine, another DKP, shows antitumoral, antibacterial, antiviral, and inmunomodulation activities (Kanoh et al. 1999; Williams et al. 1998). *P. putida* WCS358, a plant growth-promoting bacterium produces four CDPs (Degrassi et al. 2002), but the role of these compounds in bacterial cell physiology or during plant-bacteria interactions remains unknown. Recently, Ortiz-Castro et al. (2011) described that mutants of *Pseudomonas aeruginosa* defective on the AHL synthase *LasI* promoted root growth and biomass production in Arabidopsis. The decreased virulence and enhanced phytostimulation was related with production of three CDPs, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-Tyr), which showed weak auxin activity. Thus, CDPs may represent a particular signature of certain PGPRs but their occurrence in plant beneficial bacteria and the mechanisms of action in plants remains to be elucidated.

In this work we characterized the effects of *P. putida* and *P. fluorescens* in plant growth and development in *A. thaliana*. Our data show that growth promotion and modulation of root system architecture are integral responses of seedlings to these rhizobacteria. We also evidenced the requirement of the auxin pathway for phytostimulation and report on the production of CDPs by *P. putida* and *P. fluorescens* that may play a role in plant morphogenesis.

Materials and methods

Plant material and growth conditions

Arabidopsis (Col-0), the transgenic lines *CyCB1:uidA* (Colón-Carmona et al. 1999), *AtPRZ1:uidA* (Sieberer et al. 2003), *DR5:uidA* (Ulmasov et al. 1997) and mutant lines *rhd6* (Masucci and Schiefelbein 1994), *tir1, tir1afb2afb3* (Dharmasiri et al. 2005), *arf7-1, arf19-1, arf7arf19* (Okushima et al. 2007) were used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2x Murashige and Skoog (MS) medium (Murashige and Skoog 1962). MS medium basal salts mixture (Sigma, St. Louis, MO, USA). The suggested formulation was prepared with 0.9 g L^{-1} of salts for 0.2x concentration of medium, lacking amino acids and vitamins and adding phytagar micropropagation grade at 10 g L^{-1} (Phytotechnology, Shawnee Mission, KS, USA), and sucrose 6 g L^{-1}. Plants were placed in a plant growth chamber (Percival Scientific AR-95L, USA) with a photoperiod of light by 16 h (light intensity of 100 µmol m^2s^{-1}), darkness 8 h, humidity 80 % and temperature of 22 ^oC.

In vitro root co-cultivation assay

The bacterial strains of *P. putida* and *P. fluorescens* (Aguilar et al. 2006) were evaluated *in vitro* for their ability to promote growth of plants, using the *A. thaliana* Col-0 ecotype. Bacterial densities of $2.5x10^8$ CFU were inoculated by streaking on agar plates containing 0.2x MS medium. Six-day-old germinated *Arabidopsis* seedlings (20-30 seedlings per plate) were grown opposite to the inoculation site. Bacteria inoculation was performed at 5 cm distance from the

root tip. Seedlings placed on the MS plates were grown for further 8-day period in growth chamber in randomized design under the conditions described above.

Hormone treatments

For liquid induction of auxin expression experiments, MS 0.2x media were supplemented with indole-3-acetic acid (IAA, Sigma Co. St. Louis, USA) or ethyl acetate extract from bacteria grown on Luria Bertani medium (LB) at 37 ^{o}C with shaking (Ortiz-Castro *et al.* 2011). Ethanol-dissolved compounds were added to cooled (40 ^{o}C) melted medium and poured into Petri plates.

Analysis of plant growth and statistics

Growth of primary roots was registered using a rule. Lateral root number (LRN) and lateral root density (LRD) was determined by counting the lateral roots present in the primary root from the tip to root/stem transition. LRD was determined by dividing the lateral root number by the primary root length and expressed as LRD cm^{-1}. The meristem length was measured as the distance between the quiescent center to the cell file where cells started to elongate. Fresh weight of plants was determined with an analytical balance with a 0.0001 g precision value (Ohaus Corporation, Pine Brook, NJ, USA). For all experiments, data were statistically analyzed in the SPSS 10 program (SPSS, Chicago, IL, USA). One-way and two-way ANOVA analyses with Tukey's post hoc test were used for testing differences in growth and root developmental responses in wild type (WT). Different letters are used to indicate means that differ significantly ($P < 0.05$).

Histochemical analysis

Transgenic plants that express the *uidA* reporter gene (Jefferson et al. 1987) were stained in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, for 12 h at 37 ^{o}C. Plants were cleared and fixed as previously described by Malamy and Benfey, (Malamy and Benfey 1997). The processed roots were included in glass slips and sealed with

commercial nail varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.

Microscopy

Arabidopsis thaliana root system was analyzed with a stereoscopic microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). Total lateral roots were counted at 30X magnification. Primary root meristems were analyzed in semi-permanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus, Carl Zeiss, Göttingen, Germany) at 100X or 400X magnifications. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony Electonics Inc., Oradell, NJ, USA) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss, Göttingen, Germany).

Solvent extraction, purification and chemical characterization of cyclo dipeptides from *P. putida and P. fluorescens*

For bacterial growth, 2.5×10^8 CFU inoculum of *P. putida* and *P. fluorescens* strains were placed in 1.5 L LB liquid medium and incubated by 24 h at 37 ^{o}C with 150 rpm shaking. Cell-free supernatants were prepared by centrifugation (10,000 x g at room temperature by 10 min; Termo IEC-CL5R Centrifuge, Termo Electron Co, Milford, MA, USA). The supernatant was extracted twice with ethyl acetate supplied with acetic acid (0.1 mL L^{-1}). Extracts were evaporated to dryness using a rotavapor (Rotavapor R-210; Büchi, Switzerland) at 60 ^{o}C with vaccum and residues were dissolved with 2 mL of methanol:acetonitrile (1:1).

For purification of CDPs, 100–500 µL of ethyl acetate extracts obtained from the *Pseudomonas* strains were separated using a HPLC system with a C_{18} semipreparative column (Econosil C_{18},10 U, 250 mm x 22 mm; Alltech). Fractions were eluted with water:acetonitrile, starting with 0:100; followed by a linear gradient 60:40, after following by 40 min; linear gradient up 90:10 by 10 min, and finishing with 0:100 by 10 min with stabilization time of 15 min. The desionized water and HPLC-grade acetonitrile were filtrated and degasified. Compound detection was performed at 210 nm using a UV detector (Perkin Elmer), collecting the fractions that correspond to

observed peaks in the chromatograms. Fractions were concentrated by lyophilization using a Freezone 6 lyophilizer (Labconco, Missouri, USA) and dissolved in ethanol. Purified fractions were used for biological activity evaluation and chemical analysis.

Purified fractions were analyzed in Agilent 6850 Series II gas chromatograph using 30 m x 0.2 mm x 0.25 mm, 5% phenyl methyl siloxane capillary column (HP-5 MS), equipped with an Agilent MS detector model 5973 (Agilent Co. USA). Operating conditions in GC-MS analysis were: helium as carrier gas at 1 mL min^{-1}, 300 ^{o}C detector temperature, and 250 ^{o}C injector temperature, injecting 1 µL of sample. Column was held for 3 min at 80 ^{o}C and temperature ramp was programmed at 6 ^{o}C min^{-1} to final temperature of 230 ^{o}C by 5 min. A SCAN analysis was used for to obtain the mass fragmentation of the compounds. ^{1}H-NMR and ^{13}C-NMR spectrometry analyses were carried out for to confirm the structure and nature of the purified compounds as described (Ortiz-Castro et al. 2011).

Results

Plant-growth promotion by *P. putida and P. fluorescens*

Pseudomonas species may act as PGPR, but little is known about the mechanisms of phytostimulation. To test the responses of *Arabidopsis* seedlings to environmental *Pseudomonas* species, we co-inoculated 5d-old seedlings grown on agar plates containing 0.2x Murashige and Skoog (MS) medium with 2.8 x 10^{8} CFU of *P. putida* or *P. fluorescens* by streaking the bacterial inoculum on the surface of the medium at 5 cm distance from the primary root tip. By using this plant-bacteria interaction system, it has been possible to monitor the effects of diffusible bacterial compounds on plant growth without physical direct interaction between the plant and bacterium (López-Bucio et al. 2007; Ortiz-Castro et al. 2008; Ortiz-Castro et al. 2013). After 8 days of growth in the presence of *P. putida* or *P. fluorescens* significant increases in shoot and root biomass production were observed (Fig. 1A-E). Interestingly, co-inoculation with each bacterium did not affect primary root growth in the first 6 days of interaction and only a slight reduction was observed after 8 d. In contrast, enhanced lateral root initiation, particularly in the number of primordia emerging from the primary root (stage D) were clearly documented

(Fig. 1G). In addition, both bacterial species increased the number and length of root hairs (Fig. 2A-E). This growth promotion effect could also be observed in plants co-inoculated in closer proximity to the root tip, in which *P. putida* and *P. fluorescens* colonies were in direct contact with the root system. In this later condition, there was an increase of two and five-fold in shoot and root fresh weight, respectively (Fig. 3A-E).

***P. putida* and *P. fluorescens* did not affect cell division on primary root meristems**

To investigate whether bacterial co-cultivation could affect cell division on primary root meristems, we analyzed Arabidopsis transgenic plants that express the *CycB1:uidA* marker, which is expressed the G2/M phase of the cell cycle and is commonly used as a marker of mitotic activity (Colón-Carmona et al. 1999). Additionally, the marker *pPRZ1:uidA* was used to determine the length of the root meristem domain (Sieberer et al. 2003). Bacterial co-cultivation did not clearly affect primary root growth after 8 days, but markedly increased lateral root and root hair proliferation (Fig. 4A-C). In axenically-grown seedlings, and in bacterially influenced roots, both *CycB1:uidA* and *pPRZ1:uidA* were expressed in the root meristem (Fig. 4D). No differences between control and inoculated seedlings were evident in *CycB1:uidA* expressing cells or in meristem length in *pPRZ1:uidA* seedlings (Fig. 4E-F). These results indicate that *P. putida* or *P. fluorescens* did not affect cell proliferation in the root meristem as reveled by the unaffected expression of meristem markers.

P. putida* and *P. fluorescens* require a canonical auxin signaling pathway for plant-growth promotion in *Arabidopsis

The effects of *P. putida* and *P. fluorescens* increasing lateral root formation, root hair development and plant growth are reminiscent of activated auxin responses. Several auxin-related mutants have been identified in screenings for auxin responses that show root architectural alterations. Auxin-related mutations have been found to alter root hair development as in the *rhd6* mutant, which is defective on root hair initiation and can be

normalized by auxin supplementation (Masucci and Schiefelbein 1994). On the other hand, the *DR5:uidA* Arabidopsis transgenic line has been used as an excellent marker of auxin responses at the molecular level (Ulmasov et al. 1997). Therefore, we used the *rhd6* mutant and *DR5:uidA* lines to determine whether auxin signaling is involved in the interaction of *Arabidopsis* with *P. putida* and *P. fluorescens*. We found that co-cultivation with these bacterial species clearly induced root hair development in WT (Col-0) seedlings (Fig. 5A-C). In uninoculated *rhd6* mutants grown in axenic conditions, the lack of root hairs was evident (Fig. 5D), while in inoculated seedlings the formation of long root hairs was clearly observed. These results show that *P. putida* and *P. fluorescens* normalize root hair development in *rhd6* seedlings (Fig. 5E-F). Concomitantly, an increase in *DR5:uidA* expression marker was shown in Arabidopsis seedlings co-cultivated with *P. putida* and *P. fluorescens* compared to uninoculated seedlings (Fig. 5G-I), indicating the activation of auxin responses in primary roots.

***P. putida* and *P. fluorescens* stimulate lateral root formation through the canonical auxin pathway**

Auxin is perceived by direct binding to the transport inhibitor response 1 (TIR1) protein, this interaction accelerates the degradation of Aux/IAA repressor proteins, allowing de-repression of auxin-regulated genes by auxin response transcriptors factors (ARFs). To determine whether the TIR1 family of auxin receptors and ARFs were involved in root architectural responses to *P. putida* and *P. fluorescens*, we analyzed the effect of co-cultivation with these bacteria on primary root growth, lateral root number and lateral root density in WT (Col-0), *tir1-1*, *arf7-1*, *arf19-1*, *arf7arf19* and *tir1afb2afb3* mutants. The results showed that *P. putida* or *P. fluorescens* did not affect primary root growth in WT and mutants lines of Arabidopsis seedlings, except in *arf19-1* and *arf7arf19* single and double mutants, respectively. In WT seedlings co-inoculated with *P. putida* or *P. fluorescens* increments in lateral root number (LRN) and lateral root density (LRD) were found (Fig. 6B, C). The single mutants *tir1*, *arf7-1* and *arf19-1* displayed a reduction in LRN and LRD compared with WT seedlings when grown axenically, but the bacteria clearly induced lateral root formation in these mutants. In contrast, the *arf7arf19* double mutant and the *tir1afb2afb3* triple mutant were completely insensitive to *P. putida* and *P. fluorescens*

strains in terms of LRN and LRD indicating an important function of these genes in lateral root induction by the bacteria (Fig. 6B-C).

P. putida* and *P. fluorescens* produces DKPs capable of modulating auxin responses in *Arabidopsis

Growth promotion and root architectural alterations observed in seedlings co-cultivated with *P. putida* and *P. fluorescens* are similar of the effects of *Arabidopsis* seedlings co-cultivated with the *P. aeruginosa* mutant *LasI*, which produces increasing amounts of cyclodipeptides (Ortiz-Castro et al. 2011). Therefore, we compared the production of these compounds in *P. aeruginosa*, *P. putida* and *P. fluorescens.* Ethylacetate (EtOAc) extracts of cell-free supernatants from the growth medium of all three bacteria mentioned above were analyzed by GC-MS to determine CDP production. The results showed that in *P. putida* and *P. fluorescens* cultures, the production of cyclo-L-Pro-L-Tyr (RT12 min), cyclo-L-Pro-L-Val (RT 25 min), and cyclo-L-Pro-L-Phe (RT 27 min) were much increased as compared to the amounts produced by *P. aeruginosa* (Fig. 7A-B).

EtOAc extracts of *P. putida* and *P. fluorescens* cell-free supernatants were subjected to semi-preparative purification and the fractions collected were assayed for their ability to induce auxin-gene expression by using *Arabidopsis* transgenic seedlings expressing the *DR5:uidA* marker. In seedlings grown on MS 0.2x medium, the *DR5:uidA* marker was expressed mainly in the root tip region (Fig. 8A). As expected, treatment with 3 μM IAA strongly increased GUS activity throughout the primary root (Fig. 8B), indicating an activation of auxin responses. The pattern of GUS expression in *DR5:uidA* seedlings supplied with EtOAc extracts of *P. putida* and *P. fluorescens* was also increased in the primary root (Fig. 8C-D), indicating that *P. putida* and *P. fluorescens* produce compounds with auxin activity. Purified fractions from *P. putida* and *P. fluorescens* (RT's 11.8, 17.6, and 27.8 min, respectively) showed auxin activity by inducing the *DR5:uidA* marker in the primary root tip of *Arabidopsis* seedlings (Fig. 8F-H and 8J-L). The results obtained indicate that the cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val) and cyclo(L-Pro-L-Phe) are likely responsible of the *DR5:uidA* induction in primary roots.

Discussion

The root surface and the rhizosphere are rich in nutrients, which act as signals for microbial attraction, including pathogens and free living species that compete for these nutrients (Raaijmakers et al. 2009). Once microorganisms are attracted, the next step to define the resulting interaction as neutral, pathogenic or beneficial to plants is the recognition process, which can occur through a variety of diffusible molecules or through physical interactions (Ryu et al. 2004; Göhre and Robatzek 2008; Nürnberger and Kemmerling 2009).

The *Pseudomonas* genus includes species that can be pathogenic (i.e. *P. aeruginosa*) as well as PGPR (Persello-Cartieux et al. 2003). Particularly, *P. putida* and *P. fluorescens* are well known for their growth promoting capacity, as reported for different cultivars (Preston 2004). In this work, both *P. putida* and *P. fluorescens* stimulated the growth of *Arabidopsis* seedlings *in vitro*, confirming the proposed PGPR role for these bacteria. *P. putida* or *P. fluorescens* co-cultivation increased the shoot and root fresh weight compared to axenically-grown *Arabidopsis* seedlings (Fig. 1A-E). This beneficial effect correlated with a strong capacity to activate lateral root formation (Fig. 1G) and root hair development (Fig. 2) and could be observed when the bacterial streak is placed at a distance (5 cm, Fig. 1A-C), or in direct contact with the root (Fig. 3 A-C). These results highlight the potential of using co-cultivation systems to characterize the impact of bacterial isolates to identify promising PGPR towards its potential use as biofertilizers. Since the root system exhibits an amazing diversity of architectures through changes in root hair, lateral and adventitious root formation and primary root growth, which plays an important role in anchor to the soil and in water and nutrient acquisition (López-Bucio et al. 2003; Nibau et al. 2008), alteration on key aspects of RSA configuration by these PGPR might be of practical value in agriculture.

Several rhizobacteria may affect root growth by inhibiting cell division in primary roots that often leads to short roots with increased lateral root branching potential (Ortiz-Castro et al. 2011; López-Bucio et al. 2007; Contesto et al. 2010). It was previously reported that AHLs can stimulate or inhibit root growth depending on the AHL structure and concentration in the medium (Ortiz-Castro et al. 2008; Von Rad et al. 2008). Particularly, the AHL-depending effect was correlated with an inhibition of primary root growth and increased lateral root and root hair

formation depending on AHL structure (Ortiz-Castro et al. 2011). In contrast, *P. putida* and *P. fluorescens* only inhibited primary root growth at later stages during the interaction and in a modest manner, indicating that their effects are likely independent of AHL production. However, the branching capacity of roots was highly increased by the bacteria suggesting that the LR forming capacity is independent of primary root growth inhibition. In agreement with this result, cell proliferation in PR meristems and expression of *CycB1:uidA* and *PRZ1:uidA* gene markers in primary roots remained unchanged (Fig. 4D-F). Therefore, *P. putida* and *P. fluorescens* regulate root architecture by activating pericycle cells to divide without affecting cell proliferation in root meristems.

Lateral roots originate after embryogenesis from the pericycle layer and emerge in the differentiation zone of the primary root (Malamy and Benfey 1997). The pericycle layer consists of quiescent cells that are competent to proliferate (Péret et al. 2009). Every pericycle cell has the ability to divide in response to elevated auxin levels (Boerjan et al. 1995; Dubrovsky et al. 2008). Auxin promotes lateral root initiation, as demonstrated by exogenous auxin application (Blakely and Evans 1979) and by the analysis of mutants affected in auxin biosynthesis (Barlier et al. 2000) or response (Fukaki et al. 2002). Organ formation in *Arabidopsis* involves dynamic gradients of the signaling molecule auxin with maxima at the primordium tip (Benková et al. 2003). These gradients are mediated by cellular efflux requiring asymmetrically localized PIN proteins (Benková et al. 2003). *P. putida* and *P. fluorescens* promoted LRP formation, principally at early stages of development (Fig. 1G) and induced root hair formation (Fig. 5A-C). An analysis of root hair development in the *rhd6* mutant, revealed that co-cultivation with *P. putida* or *P. fluorescens* normalize root-hair formation in this mutant (Fig. 5D-F). Since the root hair defect of *rhd6* can be reversed by treatment with auxin (Masucci and Schiefelbein 1994), these results show the key role of auxin signaling in the root responses to the bacteria.

The induction of lateral roots and root hairs by *Pseudomonas* species correlate with activation of the auxin inducible marker *DR5:uidA* in *Arabidopis* primary roots and in lateral root primordia observed as blue spots arising from the primary root (Fig. 5G-I). Benková et al. (2003) showed that auxin accumulates at the position of future LRP. This auxin accumulation correlated with the localization of *DR5:uidA* expression. Therefore, this might explain why seedlings co-cultivated with the bacteria forms LRs at early times as well as their great capacity to form LRs.

Several studies have reported the effect of plant hormones or bacterial metabolites on root system architecture. Lopez-Bucio et al. (2007) showed that increased growth of lateral roots in *A. thaliana* co-cultivated with *Bacillus megaterium* was independent of auxin. In contrast, a bacterial antibiotic 2,4-diacetylphloroglucinol (DAPG) produced by *P. fluorescens* inhibit primary root growth and stimulates lateral root production in tomato seedlings. Using auxin-resistant *diageotropica* tomato mutant seedlings and the auxin-inducible GH3 promoter fused to luciferase in tobacco plants, the authors showed that DAPG induced changes in root architecture were related to auxin signaling (Boezclton et al. 2008). Similarly, *Phylobacterium brassicacearum* strain STM196 induced the expression of *DR5:uidA* in *Arabidopsis* primary and lateral root tips (Contesto et al. 2010). This information highlights the complexity of hormonal crosstalk in plant-bacteria interactions and the multiple facets of plant development targeted by microbe signals.

IAA is the most widely recognized plant hormone involved in root architecture regulation. Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms, and signal perception through a family of IAA receptors (Woodward and Bartel 2005). The production of IAA, as well as auxin precursors or auxin signal mimics seems to be a common feature of microorganisms that associate with plants. The early phase of the interaction between *Arabidopsis* roots and microbes, prior to symbiosis establishment, is accompanied by the stimulation of lateral root development and may involve detection of volatile and diffusible signals, some of which could affect auxin homeostasis (Sukumar et al. 2013).

Diverse bacterial species produce auxins as part of their metabolism including indole-3-acetic acid, indole-3-butyric acid or their precursors (Martínez-Morales et al. 2003, Spaepen et al. 2007). Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum*, and it is generally agreed that auxin production is the major factor responsible for the stimulation of root system development and growth promotion by this bacterium (Kepinski and Leyser 2005). Auxin is perceived by direct binding to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) protein, a member of a small family of F-box proteins (Dharmasiri et al. 2005; Kepinski & Leyser 2005). This interaction accelerates the Skp1, Cdc53/Cullin1, F-box protein ubiquitin

ligase-catalyzed degradation of Aux/IAA repressor proteins, allowing de-repression of auxin-regulated genes by auxin response transcription factors (ARFs) (Gray 2004). To determine whether the TIR1 family auxin receptors and ARFs are involved in the plant growth-promoting observed for inoculation with *P. putida* and *P. fluorescens*, we analyzed the root system in WT (Col-0) *Arabidopsis* seedlings and in *tir1* and *tir1afb2afb3*, single and triple mutants respectively, and in *arf7-1, arf19-1*, and *arf7arf19* mutants. As expected, co-cultivation with *Pseudomonas* species did not inhibit primary root growth in WT seedlings and in *tir1, arf7-1*, *arf19-1, arf7arf19* and *tir1afb2afb3* mutants. However, the increase in lateral root number and density observed in WT seedlings co-cultivated with *P. putida* and *P. fluorescens* was completely abolished in *arf7arf19* and *tir1afb2afb3* (Fig. 6). Besides, the induction of root hair development in *tir1afb2afb3* was decreased compared with WT seedlings (Supplementary Fig. 1). All together, these results suggest that compounds with auxin-like activity produced by *P. putida* and *P. fluorescens* affect an auxin signaling pathway linked to development and growth promotion in *Arabidopsis.*

In our study, we were unable to detect IAA from *P. putida* and *P. fluorescens* bacterial extracts, but we found three DKPs, cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Phe) and cyclo(L-Pro-L-Val) (Fig. 7), the same cyclodipeptides previously reported in *Pseudomonas aeruginosa* (Ortiz-Castro et al. 2011). When analyzing the interaction of *Arabidopsis* seedlings with *P. aeruginosa* WT PAO1 and mutants affected in AHL sintases *lasI, rhlI* and *lasIrhlI*, it was found that inhibition of primary root growth correlated with production of C12AHL, as *P. aeruginosa LasI* mutant defective on C12 AHL synthase did not inhibit primary root growth. In this mutant, however, the prolific induction of lateral roots was related to the production of vast amounts of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Phe) and cyclo(L-Pro-L-Val) (Ortiz-Castro et al. 2011). In a separate study, *P. putida* WCS358 was found to produce four cyclodipeptides, cyclo(L-Phe-L-Pro), cyclo(L-Tyr-L-Pro), cyclo(L-Leu-L-Pro) and cyclo(L-Leu-L-Val) (Degrassi et al. 2002). Purification of all three CDPs cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Phe) from *P. putida* and *P. fluorescens* extracts and comparison to the effects of IAA, on auxin-regulated gene expression suggests that these CDPs have a weak auxin activity, different enough to that of IAA (Fig. 8). These results may explain the different effects of auxin and CDPs regulating primary root growth, and lateral root formation.

The CDPs are compounds with varied biological activities and recent information points to their key role in prokaryote-eukaryote communication (Ortiz-Castro et al. 2011). It has been proposed that certain CDPs can act as QS-inhibitors as cyclo(L-Pro-L-Phe) inhibited bioluminescence in *Vibrio fischeri* (Campbell et al. 2009). Cyclo(L-Leu-L-Pro) produced by *Achromobacter xylosoxidans* inhibed aflatoxin production of *Aspergillus parasiticus,* modulating the expression of genes related to aflatoxin biosynthesis (Yan et al. 2004). It is tempting to speculate that the beneficial effect of bacteria that produce and secrete CDPs is due to decreased virulence, as these compounds repress expression of genes related to pathogenicity, as well as their putative role modifying cellular process in plants because of their auxinic activity. It is expected that manipulating AHL-dependent QS signaling and CDP biosynthesis could be a promising strategy to obtain new bacterial strains with high plant growth-promotion capacity, establishing beneficial relationship with plants by means of cell-to-cell communication.

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Figure legends

Figure 1. Effect of co-inoculation with *P. putida* and *P. fluorescens* on growth promotion and root development in *A. thaliana*. Four-day-old *A. thaliana* seedlings were co-inoculated with *P. putida* or *P. fluorescens* at 5 cm distance of the primary root tip, and grown by an additional 8-d period. (a-c) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings co-inoculated with *P. putida* or *P. fluorescens,* respectively (Scale bar = 1 cm). (d) Effect of bacterial co-cultivation on shoot biomass production or (e) root biomass production. Data from D and E show the means ± SD from three groups of 30 seedlings. (f) Effect of bacterial co-inoculation on *Arabidopsis* primary root growth. Day 0 indicates the length reached by the primary root at the bacterial application time. Mean ± SD values were plotted at the indicated days (n = 30). (g) Effect of bacterial co-inoculation on lateral root primordia formation. Data points represent mean ± SD (n = 30). These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P<0.05$.

Figure 2. Effect of co-cultivation with *P. putida* and *P. fluorescens* on root hair development in *A. thaliana*. Four-day-old *A. thaliana* seedlings were co-cultivated with *P. putida* or *P. fluorescens* at 5 cm distance from the primary root tip, and grown by additional 8-d. (a-c) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings co-cultivated with *P. putida* or *P. fluorescens* (Scale bar = 100 µm). (d) Effect of bacterial co-cultivation on root hair number or (e) root hair length. Data from d and e show the means ± SD from 30 seedlings. These analyses were repeated three times with similar results. Different letter indicate means statistically different at $P<0.05$.

Figure 3. Effect of co-cultivation with *P. putida* and *P. fluorescens* by direct contact with the root system in *Arabidopsis* growth and development. Six-day-old *A. thaliana* seedlings were co-cultivated with *P. putida* or *P. fluorescens* by direct contact and grown for additional 8-d period. (a-c) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings co-inoculated with *P. putida* or *P. fluorescens* (Scale bar = 1 cm). (d) Effect of bacterial co-cultivation on shoot fresh weight and (e) root fresh weight. Data from d and e show the means

± SD from 30 seedlings. These analyses were repeated three times with similar results. Different letter indicate means statistically different at P<0.05.

Figure 4. Effect of *P. putida* or *P. fluorescens* on cell division and meristem viability in *A. thaliana*. Four-day-old *A. thaliana* seedlings expressing the *CyCB1:uidA*, or *PRZ1:uidA* markers were co-cultivated with *P. putida* or *P. fluorescens* at a distance of 5 cm from primary root tip and grown in direct contact with the bacterial colony. (a-c) Representative photographs of axenically grown *Arabidopsis* seedlings or seedlings co-cultivated with *P. putida* or *P. fluorescens*. (Scale bar = 1 cm). (d) Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from at least 20 stained plants. The experiment was replicated two times with similar results (Scale bar = 100 μM). (e) Number of *CyCB1:uidA* expressing cells. (f) Meristem length. Data from e and f show the means ± SD from 30 seedlings. These analyses were repeated three times with similar results. Different letter indicate means statistically different at P<0.05.

Figure 5. Effect of *P. putida* and *P. fluorescens* on root hair development and auxin-inducible gene expression. Four-day-old *A. thaliana* wild-type Col-0, *rhd6* Arabidopsis mutant and *DR5:uidA* marker seedlings were co-cultivated with *P. putida* or *P. fluorescens* at 5 cm distance of the from primary root tip and grown for additional 8-d. (a-c) Representative photographs of wild-type (Col-0) root hair formation on axenically grown *Arabidopsis* seedlings or seedlings cocultivated with *P. putida* or *P. fluorescens*. (d-f) Formation of root hairs in *rhd6* roots grown axenically or co-cultivated with *Pseudomonas* species. (g-i) Twelve hours of GUS staining of *DR5:uidA* primary roots of axenically grown Arabidopsis seedlings or seedlings co-cultivated with *P. putida* or *P. fluorescens* (Scale bar = 100 μm).

Figure 6. Effect of *P. putida* and *P. fluorescens* on root system architecture of *A. thaliana* wild-type (Col-0) seedlings and auxin-related mutants. *A. thaliana* WT and *tir1-1, arf7, arf19, arf7arf19* and *tir1afb2afb3* mutant seedlings were germinated and grown for 6 d on 0.2x-MS medium and co-cultivated with *P. putida* or *P. fluorescens* at a 5 cm distance from the primary root tip and grown for additional 8-d. Effect of bacterial co-inoculation on primary root length

(a), lateral root number (b) and lateral root density (c). Data show the means ± SD from 30 seedlings. These analyses were repeated three times with similar results. Different letters indicate means statistically different at $P<0.05$.

Figure 7. Identification and quantification of CDPs produced in cultures of *P. aeruginosa, P. putida* and *P. fluorescens*. (a) Representative GC-MS chromatograph from culture supernatants of *P. aeruginosa*, *P. putida* and *P. fluorescens*. The Arrows indicate the fractions corresponding to DKPs, cyclo (L-Pro-L-Val; m/z=196), cyclo(L-Pro-L-Tyr; m/z=260) and cyclo (L-Pro-L-Phe; m/z=244). (b) Relative abundante of DCPs, cyclo (L-Pro-L-Tyr), cyclo(L-Pro-L-Val) and cyclo(L-Pro-L-Phe) in ethyl-acetate extracts from 1-L cultures of *Pseudomonas* analyzed by GC-MS. Data points represent the mean relative abundance ($x10^7$ mV).

Figure 8. Effect of the bacterial CDPs on auxin responsive gene expression in *A. thaliana* seedlings. (a-d) Twelve hours of β-glucoronidase (GUS) staining of *DR5:uidA* primary roots supplied with the solvent (a), with 3 µM IAA (b), ethyl-acetate extracts of *P. putida* (c), or *P. fluorescens* (d). Effect of purified CDPs from *P. putida* (e-h) or *P. fluorescens* (i-l) on *DR5:uidA* gene expression in transgenic seedlings grown on 0.2x-MS agar medium for 6 d and then transferred into 24-well cell culture plates (10 seedlings per well) containing 2 mL 0.2x-MS liquid medium supplied with 30 µM CDPs cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Val) and cyclo (L-Pro-L-Phe) and incubated for 10 h. Seedlings were stained for GUS activity and cleared for microscopy analysis. Photographs show representative individuals from at least 30 stained plants. (Scale bars = 100 µm).

Figure S1. Effect of co-cultivation with *P. putida* and *P. fluorescens* strains on root hair development in *A. thaliana* Col-0 and auxin-related Arabidopsis mutants. Six-day-old *A. thaliana* seedlings were co-inoculated with *P. putida* or *P. fluorescens* at a distance of 5 cm from the primary root tip, and grown for additional 8-d period. Representative photographs of axenically grown Arabidopsis seedlings or seedlings co-inoculated with *P. putida* or *P. fluorescens*. (Scale bar = 100 µm). These analyses were repeated three times with similar results.

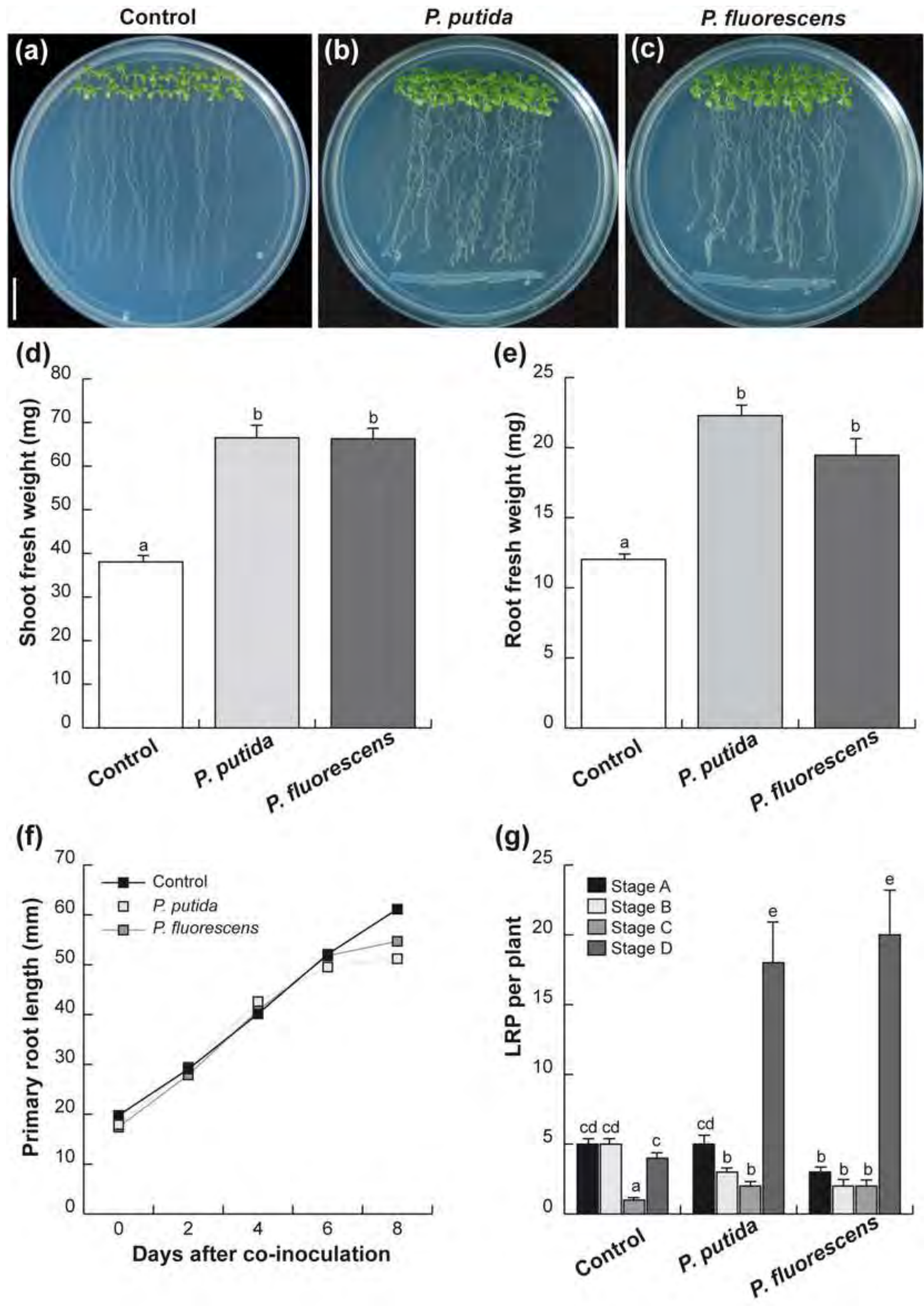
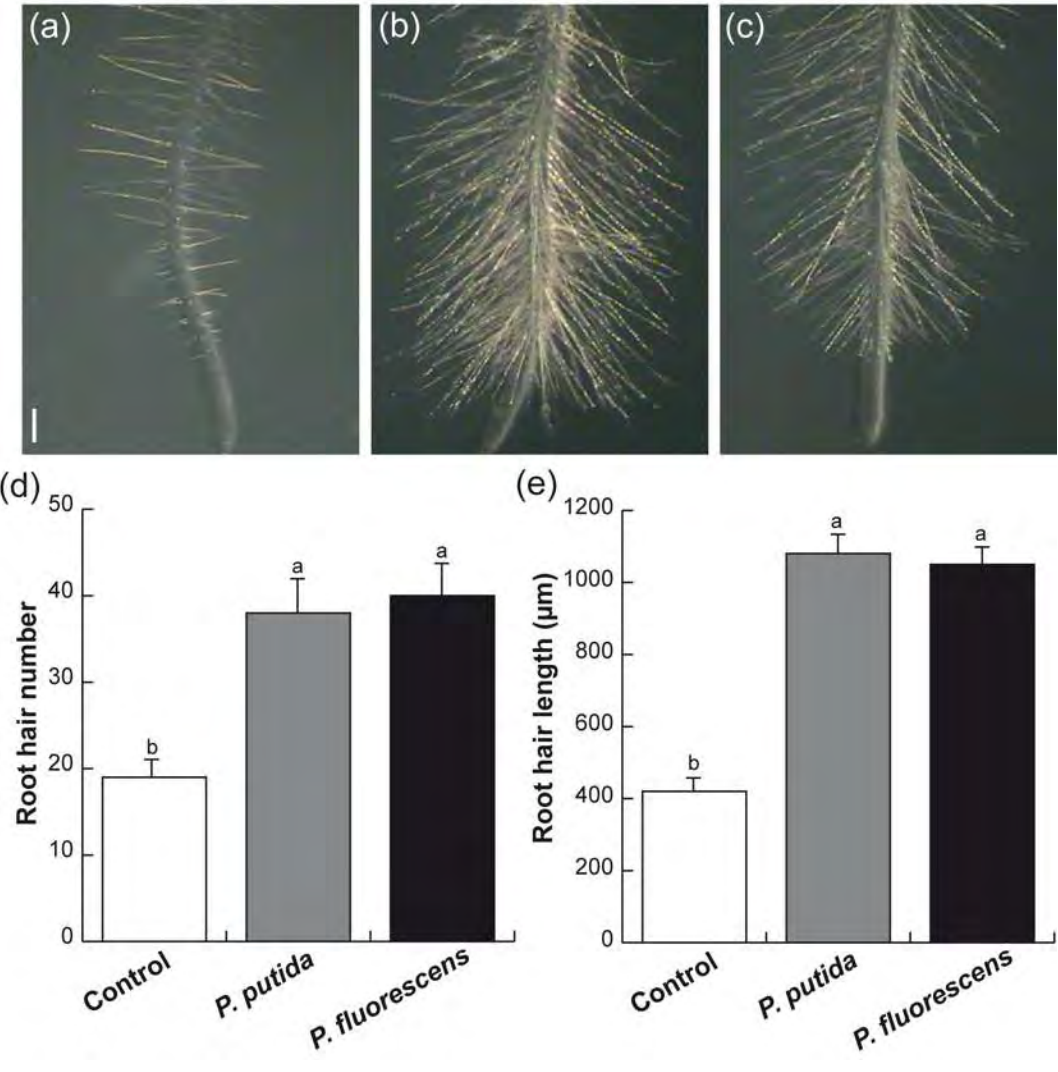


Figure 1



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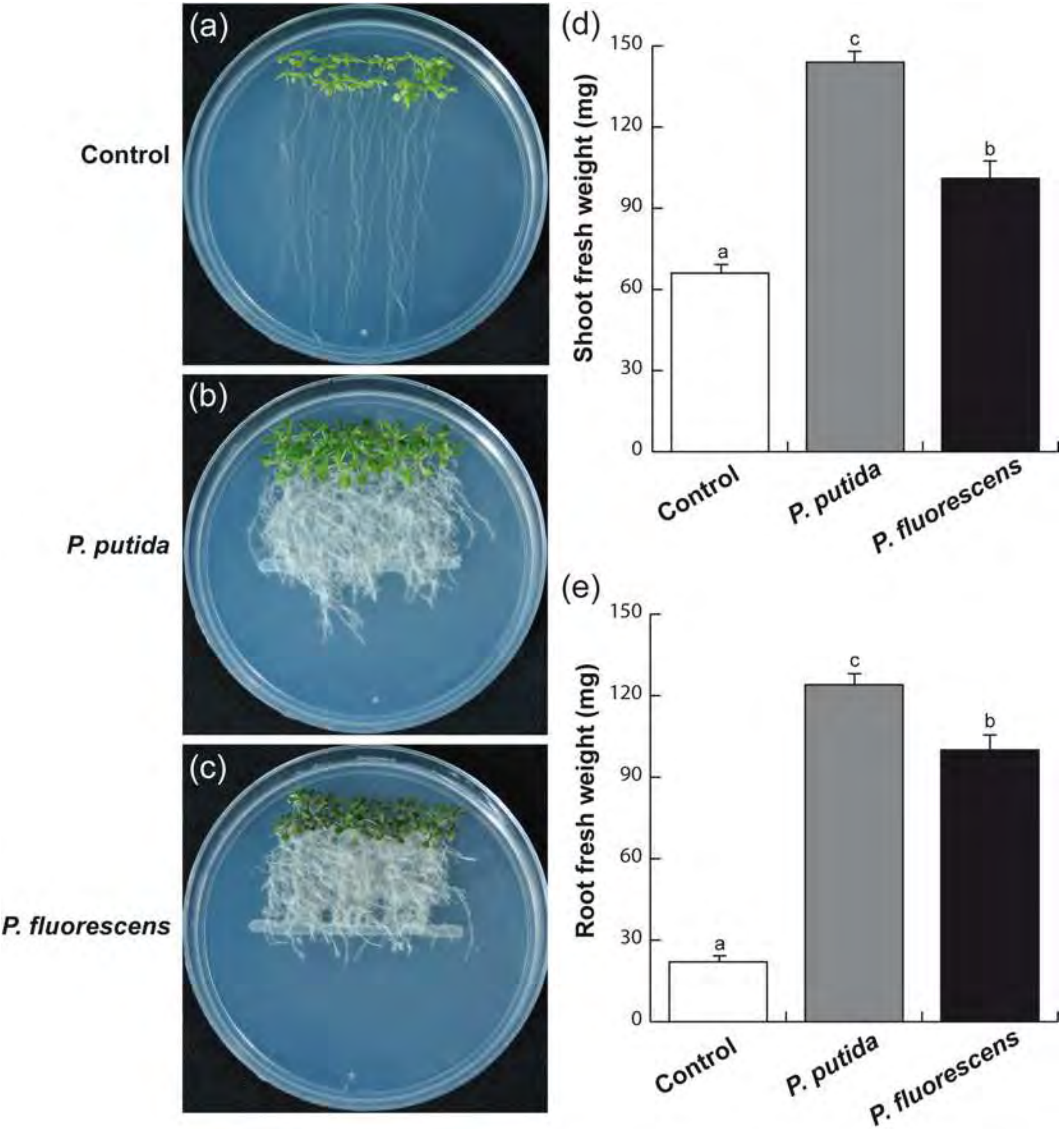

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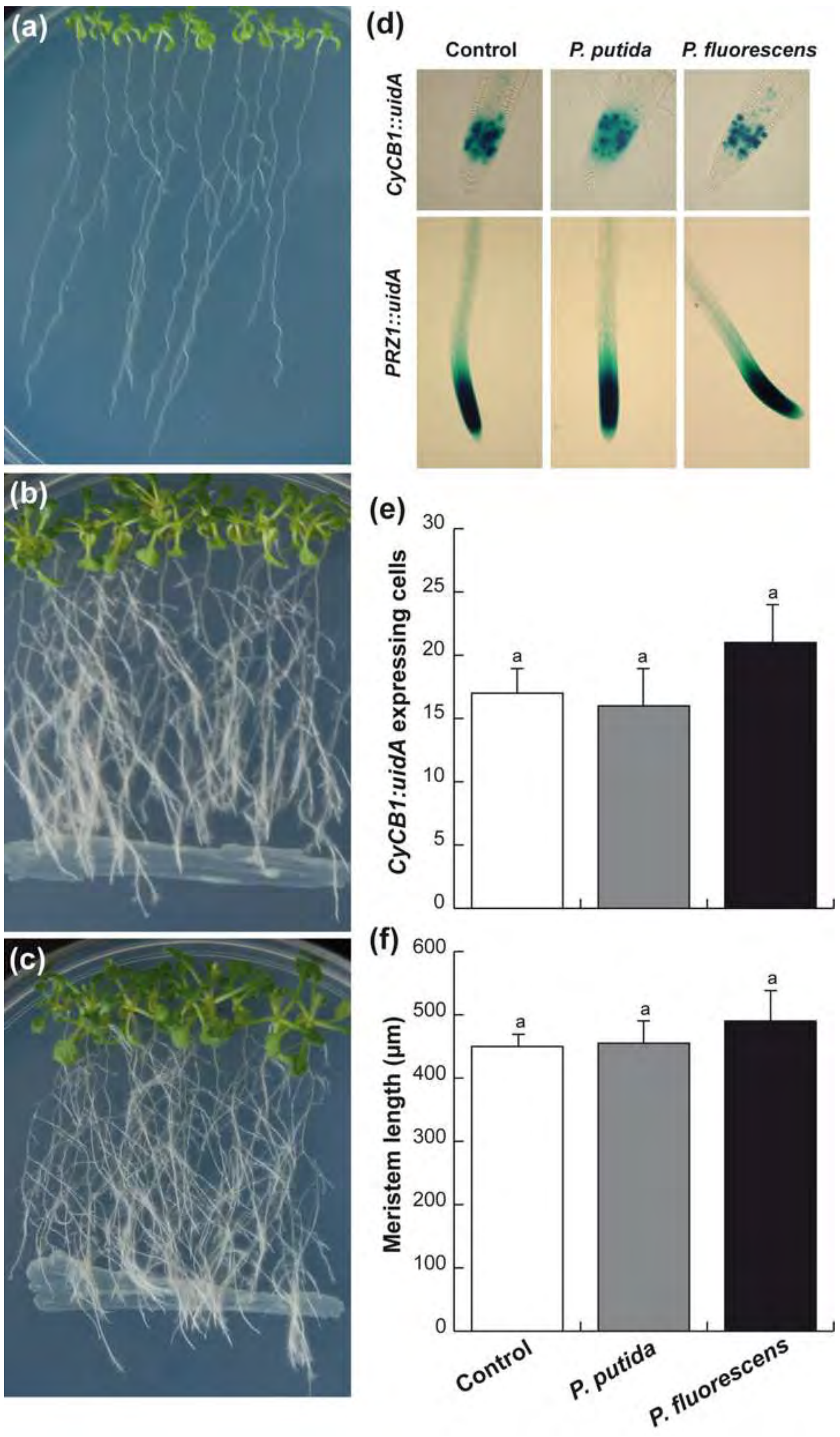

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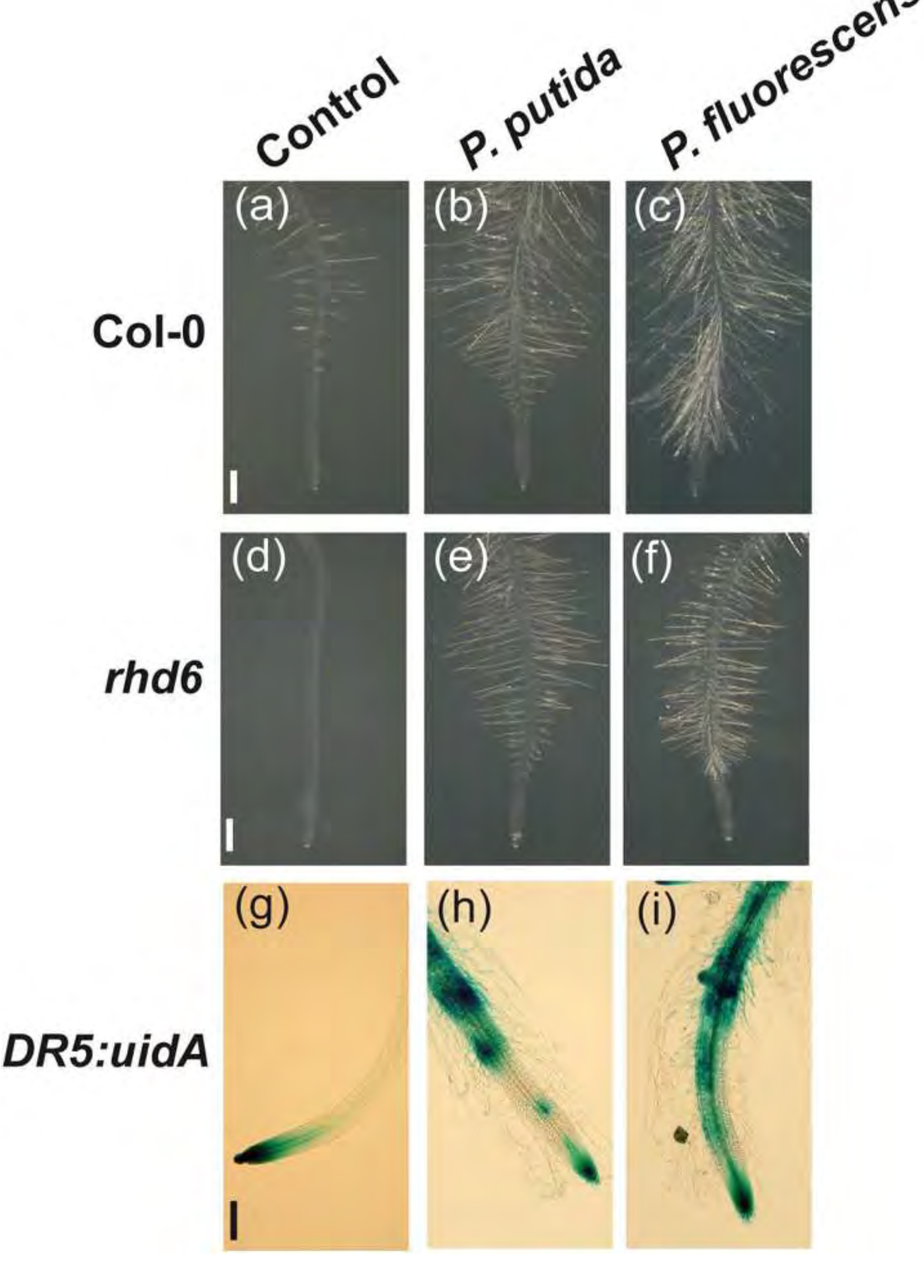

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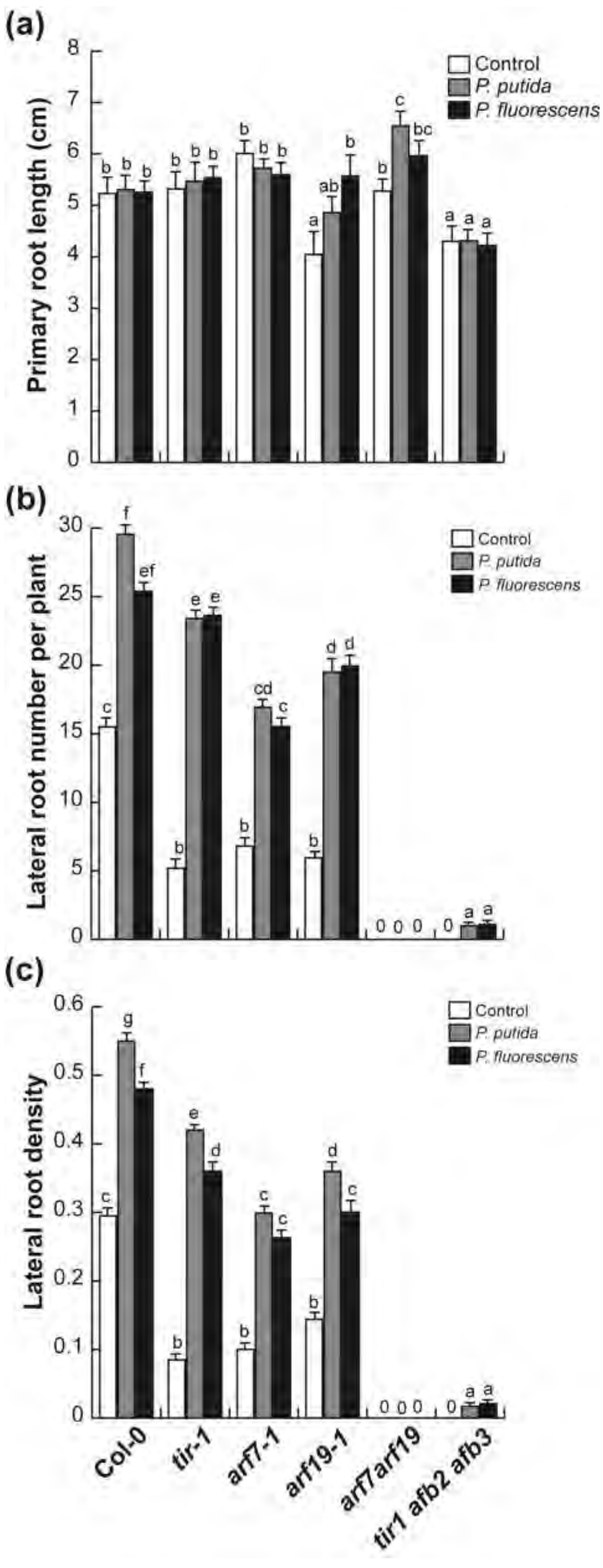

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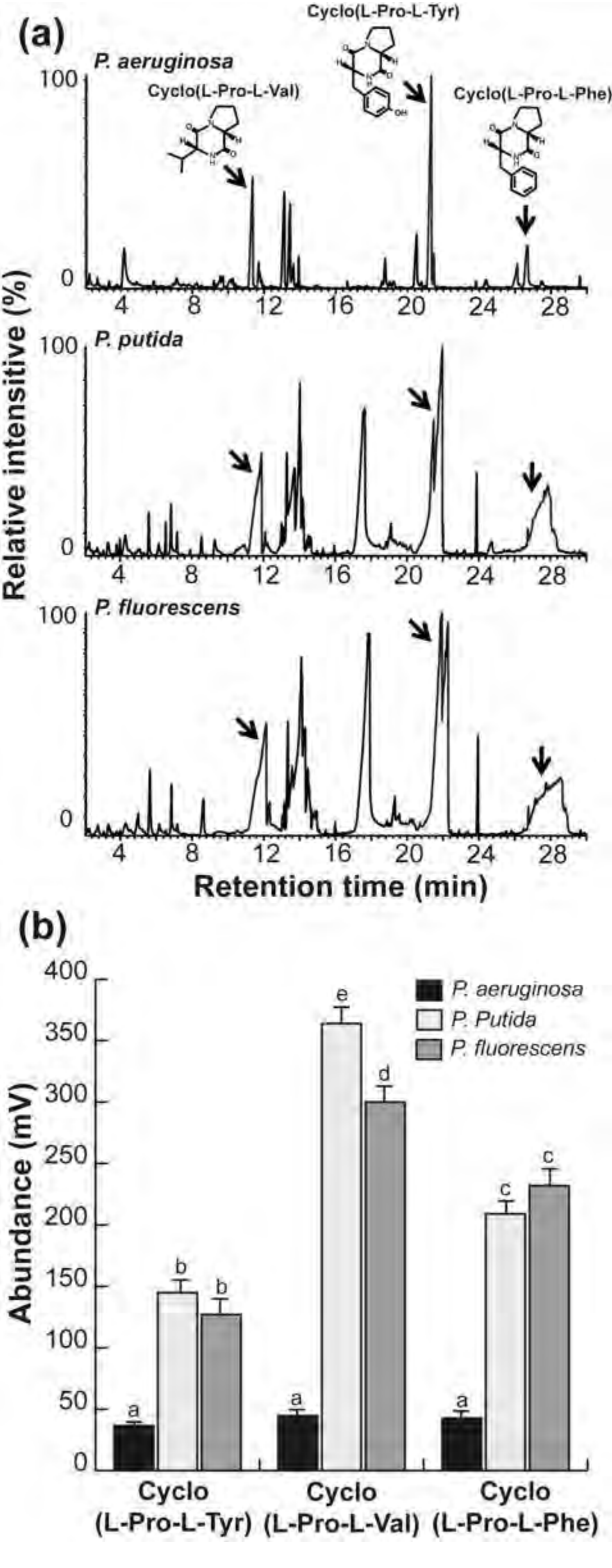

Figure 7

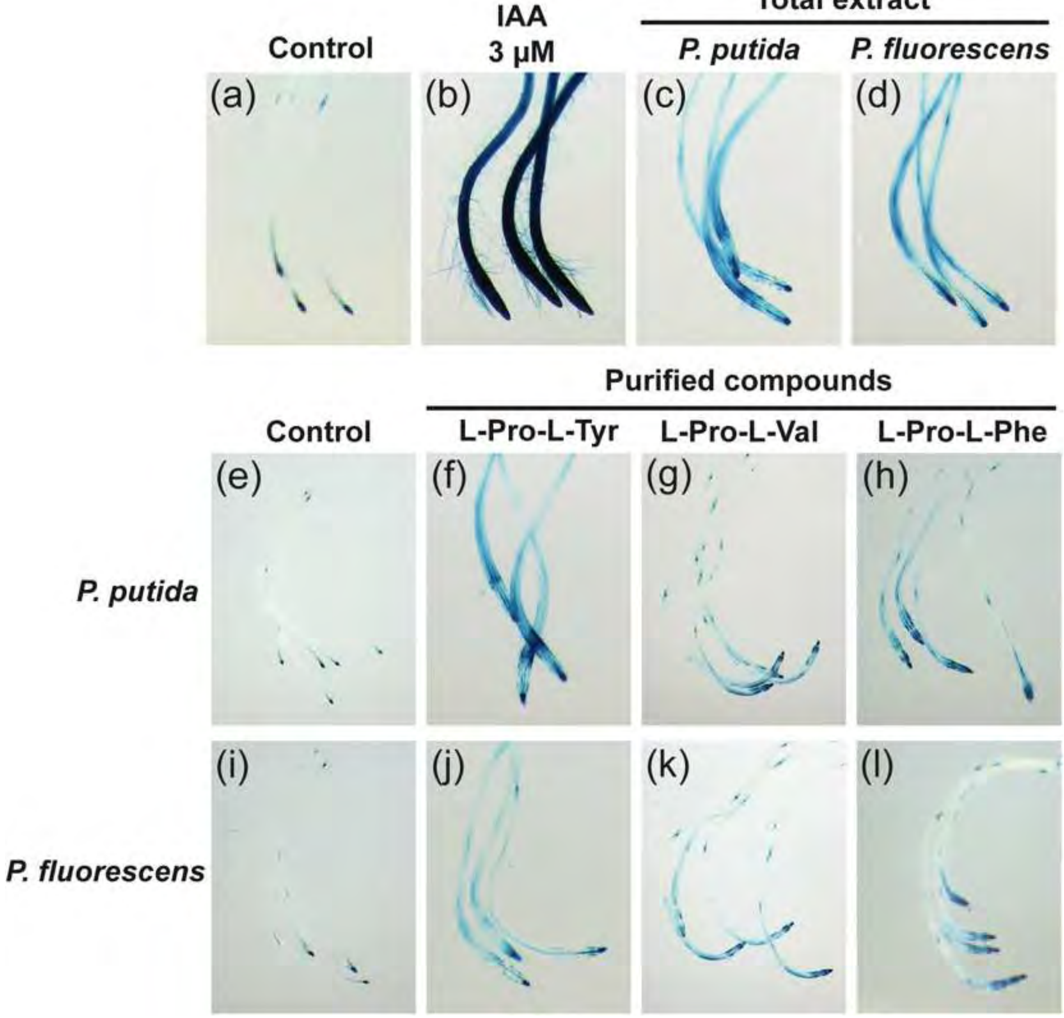

Figure 8

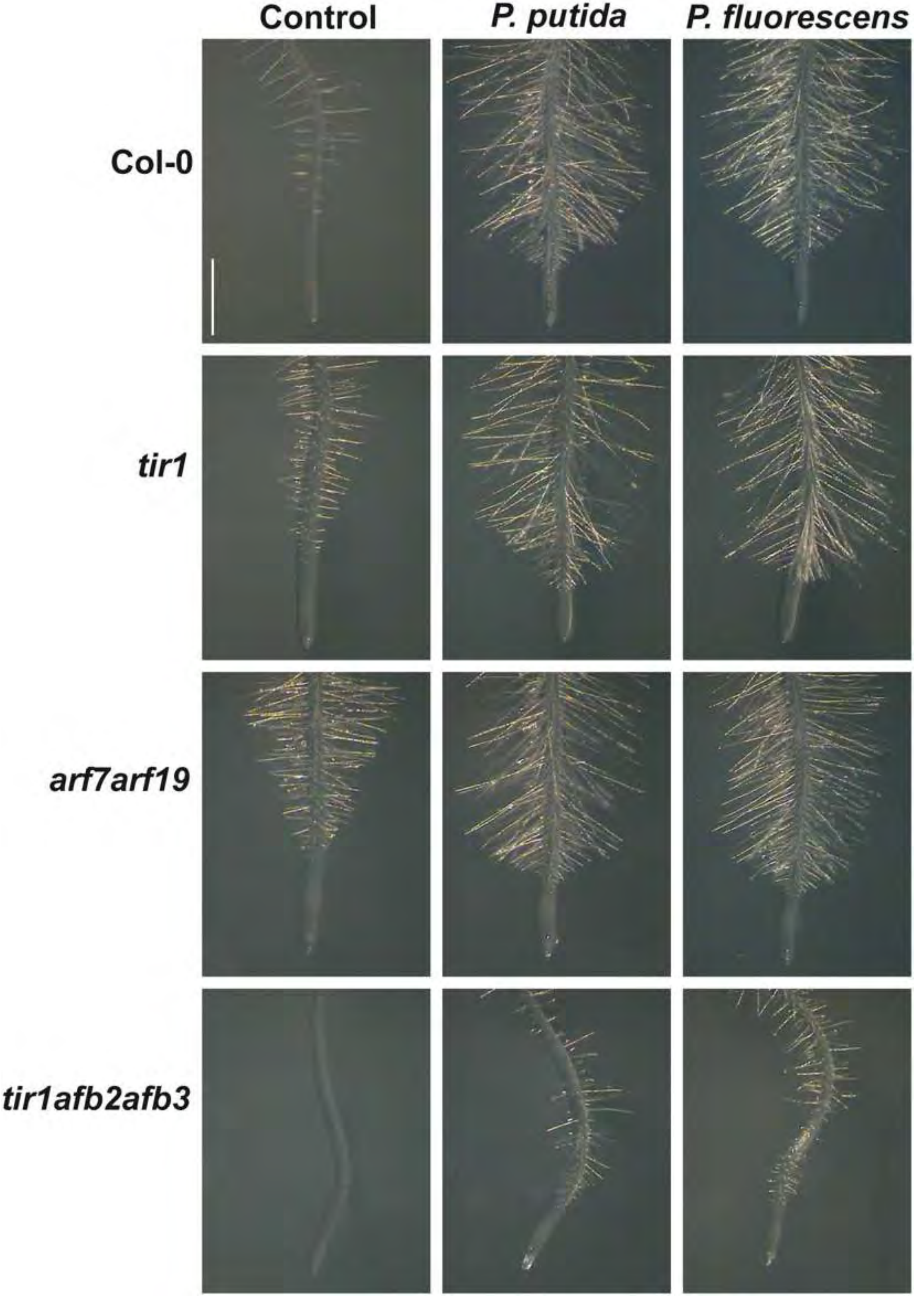

Figure S1

Molecular Plant-Microbe Interactions

Pyocyanin, a virulence factor produced by Pseudomonas aeruginosa, alters root development through reactive oxygen species and ethylene signaling in Arabidopsis

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Pyocyanin, a virulence factor produced by *Pseudomonas aeruginosa,* alters root development through reactive oxygen species and ethylene signaling in *Arabidopsis*

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ABSTRACT

Pyocyanin acts as a virulence factor in *Pseudomonas aeruginosa*, a plant and animal pathogen. In this study we evaluated the effect of pyocyanin on growth and development of *Arabidopsis* seedlings. Root inoculation with *P. aeruginosa* PAO1 strain caused disease symptoms in WT *Arabidopsis* seedlings. In contrast, single *lasI-* and double *rhlI-/lasI-* mutants of *P. aeruginosa* affected in pyocyanin production showed decreased virulence concomitant with an increased phytostimulation. Treatment with pyocyanin modulates root system architecture inhibiting primary root growth and promoting lateral root and root hair formation without affecting meristem viability or causing cell death. These effects correlated with altered proportions of hydrogen peroxide and superoxide in root tips and with an inhibition of cell division and elongation. Mutant analyses showed that pyocyanin modulation of root growth was likely independent of auxin, cytokinin and abscisic acid, but required ethylene signaling as the *Arabidopsis etr1-1*, *ein2-1* and *ein3-1* ethylene-related mutants were less sensitive to pyocyanin-induced root stoppage and reactive oxygen species (ROS) distribution. Our findings suggest that pyocyanin is an important factor modulating the interplay between ROS production and root system architecture by an ethylene dependent signaling.

Keywords: *Arabidopsis*, *Pseudomonas aeruginosa*, quorum-sensing, pyocyanin, ethylene-signaling, reactive oxygen species.

INTRODUCTION

The ecophysiology of plants cannot be understood without the microbial populations that proliferate outside and inside roots. Rhizobacterial species may impact root physiology through production of plant hormones such as auxin or cytokinins, by stimulating root growth or altering root system architecture. Moreover, many bacterial species provide protection against pathogens, tolerance to abiotic stress, resistance to insect or herbivore attack and even allelopathy may be due to root-associated microorganisms (Friesen et al. 2011). Bacteria communicate with plants through secreted signaling factors. These are small, diffusible molecules that are specifically released and then recognized by eukaryotic tissues. By producing different classes of signals, the bacteria can be recognized as pathogens or symbionts leading to very different host responses (Ortiz-Castro et al. 2011; Ortiz-Castro et al. 2013).

The *Pseudomonas* genus comprises ubiquitous Gram-negative bacteria distributed in different environments and contains pathogenic species for plants (i.e. *P. syringae, P. aeruginosa*). Other species have the ability to colonize the rhizosphere (i.e. *P. fluorescens, P. putida, P. aureofaciens* and *P. chloraphilis*), where they can act as plant-beneficial bacteria by antagonizing pathogens or through production of compounds that influence plant-disease resistance and growth (Venturi 2006). Gram-negative bacteria produce and use *N*-acyl-*L*-homoserine lactones (AHLs) for cell-to-cell communication through a regulatory mechanism known as quorum-sensing (QS), which links perception of bacterial cell density to gene expression (Fuqua et al. 1994). QS coordinates many physiological processes such as symbiosis, production of virulence factors, resistance to oxidative stress, antibiotic resistance, motility and biofilm formation (Miller and Bassler 2001). In *P. aeruginosa*, two main QS signals, namely *N*-(3-oxododecanoyl)-*L*-homoserine lactone (3-oxo-C12-AHL, C12-AHL) and *N*-butyryl-*L*-homoserine lactone (C4-AHL), are synthesized by the AHL synthases encoded by the *lasI* and *rhlI* genes, respectively. At high bacterial density, the transcription factor LasR binds to C12-AHL; whereas RhlR, another transcriptional regulator binds to C4-AHL to activate the transcription of virulence genes (de Kievit and Iglewski 2000; Rumbaugh et al. 2000; Fuqua and Greenberg 2002; Bosgelmez-Tinaz 2003).

Roots have developed the capacity to recognize bacterial QS signals and adjust growth and development in response to these metabolites (Mathesius et al. 2003; Ortiz-Castro et al. 2008; von Rad et al. 2008). Recently, genetic, chemical, and plant-growth data was presented showing that in *Pseudomonas aeruginosa*, the *lasI* QS system controls the production of three diketopiperazines (DKPs)—namely, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr)—that were involved in plant growth promotion by this bacterium. Analysis of all three bacterial DKPs in *Arabidopsis thaliana* seedlings provided detailed information indicative of an auxin-like activity, based on their efficacy at modulating root architecture, activation of auxin-regulated gene expression, and response of auxin-signaling related mutants. Moreover, the enhanced phytostimulation observed in *P. aeruginosa lasI-* mutant was indicative of reduced virulence of this particular strain (Ortiz-Castro et al. 2011).

P. aeruginosa is most studied for their importance as a human and plant pathogen. Surprisingly, many studies have revealed extensive conservation in its virulence mechanisms to infect evolutionary divergent hosts. One of these conserved virulence factors is pyocyanin (PCN). For example, PCN participates in the fast killing of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*, likely by producing reactive oxygen species (Mahajan-Miklos et al. 1999; Lau et al. 2003; Lau et al. 2004a; Lau et al. 2004b). PCN is synthesized from chorismate through a series of complex steps mediated by proteins encoded by two *phzABCDEF*G operons, and by the *phzH*, *phzM* and *phzS* genes, which modify precursors into the tricyclic compound (Mavrodi et al. 2001; Rada and Leto 2013). PCN synthesis is regulated by QS, as several reports indicate that mutations in the *lasI-lasR*, *rhlI-rhlR* QS systems result in the loss of PCN production (de Kievit and Iglewski 2000; Rumbaugh et al. 2000; Schaber et al. 2004; Siehnel et al. 2010). Moreover, PCN itself functions as a QS signal as indicated by: (i) cell density-dependent accumulation, (ii) it is a small diffusible molecule that is recognized by adjacent cells and (iii) triggers a specific transcriptional response (Dietrich et al. 2006), further complicating our understanding of its mechanisms of action.

Despite *in vitro* studies demonstrating that PCN interferes with multiple cellular functions in animals, its importance during bacteria-plant interactions is uncertain. This is partially caused by the difficulty in defining the contribution of PCN among the numerous virulence factors produced by *P. aeruginosa* during infection. Currently, the response of plant cells or whole

organs to *P. aeruginosa*-produced PCN is unknown and whether this compound causes cell damage or regulates fundamental cellular processes in plants remains to be clarified. To gain insight into how PCN might be functionally integrated into *P. aeruginosa* physiology during interaction with plants, the contribution of PCN to virulence was assessed by comparing the *Arabidopsis* growth responses to *P. aeruginosa* PAO1 and the QS-related mutants *rhlI-*, *lasI-* and *rhlI-/lasI-* in direct interaction of the bacteria with the root system. We also provide detailed pharmacological evidence of PCN bioactivity on *Arabidopsis* seedlings and analyzed the growth of primary roots in response to PCN in WT, auxin, cytokinin, ethylene, and abscisic acid-related *Arabidopsis* mutants. Our data conclusively indicate that PCN acts as a signaling molecule for root development likely affecting ROS production and ethylene signaling.

RESULTS

AHL-mediated quorum-sensing plays a role in disease development of *Arabidopsis* induced by *P. aeruginosa*

We first tested whether direct colonization of the *Arabidopsis* root with *P. aeruginosa* PAO1 and QS-related single *rhlI-*, *lasI-*, and double *rhlI-/lasI-* mutants could cause disease of seedlings. In several experiments and times of co-cultivation, *P. aeruginosa* PAO1 clearly induced root damage, and seedlings were unable to grow (Fig. 1B, G, and L). These effects were similar to those caused by the *P. aeruginosa rhlI-* mutant, defective on the AHL synthase that produces C4-AHL (Fig. 1C, H, and M). In contrast, co-cultivation with the *P. aeruginosa lasI-* single mutant defective on 3-oxo-C12-AHL synthesis or with the *rhlI-/lasI-* double mutant failed to cause any disease symptom and dramatically increased growth of seedlings at 3, 6 and 9 days of co-cultivation (Fig. 1D, I, and N; 1E, J and O, respectively). These data indicate that 3-oxo-C12-AHL mediated QS, controls the production of virulence factors underlying plant disease symptoms. Interestingly, an analysis of hydrogen peroxide in root tips of *Arabidopsis* seedlings co-cultivated 9 days with WT *P. aeruginosa* and AHL-related mutants, revealed a decrease in H_2O_2 in roots co-cultivated with WT and *rhlI* mutant, and an increased accumulation of H_2O_2 in both *lasI-* and *rhlI-/lasI-* mutants (Fig. 2A-E). This highly contrasting response indicates that diffusible factors

released by WT *P. aeruginosa* modulates the levels of H_2O_2 and perhaps other ROS likely involved in root system adjustment

AHL-mediated quorum-sensing regulates pyocyanin production in *P. aeruginosa*

Pseudomonas aeruginosa releases PCN as a main virulence factor (De Vleesschauwer et al. 2006). To determine whether QS-related single *rhlI-*, *lasI-*, and double *rhlI-/lasI-* mutants of *P. aeruginosa* could be defective on the production of PCN, we determined production of this metabolite in bacterial cell cultures by spectrophotometric analyses. It was found that PCN levels drastically decreased in single *rhlI-*, *lasI-*, and double *rhlI-/lasI-* mutants when compared to *P. aeruginosa* PAO1 (Fig. 3). These data show that AHL-modulated QS plays an important role in PCN production.

Pyocyanin alters *Arabidopsis thaliana* root system architecture

To determine if *Arabidopsis* plants could sense PCN and investigate how this compound affects plant morphogenesis, we evaluated *Arabidopsis* root developmental responses to pharmacological application of PCN. With this aim, *Arabidopsis* seedlings were germinated and grown on 0.2x MS-agar medium supplemented with PCN concentrations from 0.6 to 40 µM and primary root growth measured 10 d after germination (d.a.g.). PCN treatments showed a dose-dependent inhibitory effect of primary root growth, with 10 µM PCN causing a 70% reduction in primary root length (Fig. 4A; Fig. S1). In contrast, an induction of lateral root formation was evident from 0.6-to-2.5 µM PCN, while inhibitory effects were recorded at higher concentrations (Fig. 4B). A stimulatory effect of PCN in lateral root density was also observed with a three-fold increase from 2.5-to-20 µM concentration of this compound when compared to solvent-treated seedlings (Fig. 4C).

Pyocyanin alters root hair development

Root hairs are root epidermal cells that participate in nutrient and water uptake and increase the exploratory potential of the root system. To analyze whether PCN could alter root hair development, we performed experiments in which *Arabidopsis* WT (Col-0) seedlings were germinated and grown on the surface of agar plates containing different concentrations of PCN from 0.6 to 40 µM. Root hair parameters were analyzed at 7 d.a.g. on primary roots of solvent-

treated or PCN-treated seedlings. To investigate the effects of PCN on root hair density, we measured the trichoblast length and root hair length on seedlings subjected to different concentrations of this compound. Trichoblasts are the hair forming root epidermal cells that form cell files along the root surface. We found a dose-dependent decrease in trichoblast length in response to PCN treatment (Fig. 5A), which correlated with increased root hair number and root hair length (Fig. 5B and C). Root hair development was located closer to the primary root tip in plants grown in medium supplied with 40 µM PCN (Fig. S2), clearly indicating the progression of cell differentiation toward the root meristem region. These results suggest that pyocyanin can be perceived by roots and alter root system architecture and root hair development.

Pyocyanin alters cell division without affecting cell viability or integrity

Previous reports evidenced the toxicity of PCN in different organisms. However, the effects of PCN inhibiting primary root growth of *Arabidopsis* seedlings suggested that this compound could play an important role in cell division and/or elongation. To investigate the patters of cell division in response to PCN, we analyzed the expression of *CycB1:uidA*, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al. 1999) and *pPRZ1:uidA*, which marks only active meristems (Sieberer et al. 2003). The inhibition of primary root growth under 5 µM or higher PCN concentrations correlated with the reduction in the number of cells expressing *CycB1:uidA* in the primary root meristem and GUS expression of *pPRZ1:uidA* transgenic seedlings (Fig. 6A-H). We also analyzed the gene expression of the cell nuclei marker *AtHistH2B:YFP* by confocal laser scanning microscopy in seedlings stained with propidium iodide (PI) to determine whether PCN could cause cell death or damage of root tissues. Visualization of *AtHistH2B:YFP* in the nuclei of cells in 7 d.a.g. showed that PCN-treated roots were indeed viable. In these cells, PI was unable to penetrate, even at concentrations higher than 40 µM (Fig. 6I-L). Quantification of meristem length and number of cells expressing *CycB1:uidA* clearly documented the repressing effects of PCN on cell proliferation in primary roots (Fig. 6M and N). These results suggest that pyocyanin regulates cell division without affecting cell integrity or meristem viability.

Pyocyanin did not activate auxin inducible gene expression in *Arabidopsis* roots

Auxin is an important phytohormone involved in the modulation of several development processes in the root system. To test whether pyocyanin may or not function via auxin-regulated processes, we analyzed expression of *DR5:uidA* (Ulmasov et al. 1997) and *BA3:uidA* (Oono et al. 1998) auxin-inducible markers in transgenic *Arabidopsis* seedlings treated with PCN. Figure 7 shows histochemical staining of roots of transgenic *DR5:uidA* and *BA3:uidA* seedlings that were grown for 10 d on 0.2x MS medium supplemented with solvent, indole-3-acetic acid (IAA) or the indicated concentrations of PCN. *DR5:uidA* expression in solvent-treated seedlings is located at the edges of the cotyledons and mainly at the root tip region (Fig. 7A and G). *DR5:uidA* seedlings grown in a concentration of 3 µM IAA showed GUS activity throughout the shoot and primary root (Fig. 7B and H), whereas *BA3:uidA* seedlings supplied with the same IAA concentration expressed GUS specifically at the root elongation region (Fig. 7N and T). When *DR5:uidA* and *BA3:uidA* seedlings were grown on PCN supplied medium, the GUS expression remained similar in the shoot and primary root tip (Fig. 7C-F, I-L, O-R, and U-X). These results suggest that PCN did not induce auxin responsive gene expression in *Arabidopsis* seedlings.

Pyocyanin inhibits primary root growth of auxin, cytokinin and abscisic acid related *Arabidopsis* mutants

Several mutants with alterations in root development have been identified using screens for resistance to growth inhibitory amounts of phytohormones. Since PCN did not activate auxin-inducible markers, we decided to confirm whether PCN operates or not in a genetically defined auxin-pathway. With this aim *Arabidopsis* WT (Col-0) seedlings and auxin-related mutants *tir1afb2afb3*, *arf7arf19*, *axr1-3* and *aux1-7* were evaluated in primary root growth response assays to 10 µM PCN. As shown in Fig. S3, PCN treatment caused a 70% inhibition in primary root growth in WT plants compared to solvent-treated seedlings. When *tir1afb2afb3, arf7arf19, axr1-3* and *aux1-7* were grown in medium supplied with 10 µM PCN, the inhibition in primary root growth was similar to that observed in WT plants (Fig. S3). The results of both auxin-responsive gene expression and the root response of auxin-related mutants to PCN suggest that auxin is not involved in plant perception of PCN.

In addition, we evaluated the involvement of cytokinin, abscisic acid and ethylene signaling in response to PCN by evaluating the primary root growth of *Arabidopsis* double mutants defective

on cytokinin receptors (*cre1-12ahk2-2*; *cre1-12ahk3-3*), abscisic acid signaling (*abi1*; *abi3*) and ethylene signaling (*ein2-1* and *ein3-1*). The primary root growth of auxin, cytokinin and abscisic acid mutants was normally inhibited by PCN, indicating that these phytohormones are unlikely mediating the cellular effects of PCN. Interestingly, an analysis of ethylene response mutants (*ein2-1* and *ein3-1*) showed a small, yet statistically significant resistance of primary root growth to inhibition by PCN (Fig. S3), indicating that ethylene might be a signal that mediates the plant response to PCN.

A role of ethylene signaling in root response to pyocyanin

To further define the particular role of ethylene signaling in the *Arabidopsis* developmental responses to PCN, we investigated the sensitivity of primary root responses to several PCN concentrations of *Arabidopsis* WT seedlings and *etr1-1, ein2-1*, and *ein3-1* mutants. PCN was supplied to the growth medium in concentrations from 0.3 to 2.5 µM and the primary root growth of all four lines was measured. Interestingly, we found than *etr1-1* and *ein2-1* showed resistance to inhibition of primary root growth compared to WT seedlings, while *ein3-1* did not show resistance to PCN (Fig. 8A). To further determine the participation of ethylene signaling in the responses to PCN, we used $AgNO_3$, a well-known blocker of ethylene action. We found that when *Arabidopsis* seedlings were grown on medium supplemented with 5 µM PCN and 5 µM $AgNO_{3,}$ the inhibitor normalized in part both primary root growth and root hair development (Fig. S4 and S5). This restoration of primary root growth in plants grown on medium supplemented with PCN and $AgNO_3$ correlated with normalization of mitotic activity in the primary root meristem and reduced the accelerated root hair differentiation process caused by PCN (Fig. S5). These results suggest that ethylene signaling plays a role in root architectural responses to PCN.

Pyocyanin induces ROS production dependent of ethylene-signaling

An important toxicity mechanism by which PCN damages eukaryotic hosts is the production of ROS (Liu and Nizet 2009). To test if the effects of PCN on primary root growth were accompanied by an induction of ROS, we analyzed ROS accumulation in primary root tips by

confocal microscopy using fluorochromes to detect total ROS, and superoxide (O^{2-}). To test the role of ethylene signaling in ROS induction by PCN, we grew *Arabidopsis* seedlings on MS 0.2x medium supplemented with or without 5 µM PCN, $AgNO_3$ or PCN plus $AgNO_3$ and seven days after germination the seedlings were incubated with H2DCFC-DA or DHE to detect total ROS and O^{2-} in the primary root tip by confocal microscopy. As expected, we found that total ROS and O^{-2} increased in plants treated with PCN (Fig. 9C and H). This increase in ROS was similar to that induced by paraquat, a generator of ROS commonly used to evaluate ROS production in different systems (Fig. 9E and J). Interestingly, when plants are supplied with the ethylene perception blocker $AgNO_3$ the levels of ROS and O^{-2} were reduced in control seedlings (Fig. 9B and G) or in seedlings supplied with PCN (Fig. 9D and I). Quantification of fluorescence confirms that pyocyanin provokes a ROS accumulation which is dependent of ethylene signaling (Fig. 9K and L).

In another series of experiments, we analyzed the levels of H_2O_2 in ethylene-related mutants treated with different concentrations of PCN, whose levels clearly changed in *Arabidopsis* root tips (Fig. 10). We found that PCN reduces in a dose-dependent way the levels of H_2O_2 on primary root tips (Fig. 10A-F). However, in *etr1-1*, *ein2-1* and *ein3-1* seedlings the levels of H_2O_2 were sustained even at concentrations of 1.25 and 2.5 µM PCN that drastically affect root growth (Fig. 10G-X). This sustained production of H_2O_2 indicates that the PCN mechanism of signaling involves the ethylene pathway and that it is probably related with the resistance of primary root growth when the plants are grown on pyocyanin.

DISCUSSION

The root system is an essential plant organ that displays a high developmental plasticity in response to environmental stimuli. Rhizobacteria can influence root architecture, most prominently, by enhancing lateral root formation and root hair development. This can be done by producing phytohormones or bacterial quorum-sensing signals that are perceived at the root tip to adjust cell proliferation and growth (Ortiz-Castro et al. 2009).

Root growth depends on maintaining the proper balance between cell division and differentiation. In the primary root, cells originate from a stem cell center at the tip. Progeny of these stem cells rapidly divide in a transit-amplifying zone know as the meristem, after which they undergo massive increases in cell volume in the elongation zone. Once fully elongated, cells enter the maturation zone in which they differentiate into various cell types. Previous work has shown that co-cultivation of *Arabidopsis* seedlings with *P. aeruginosa* inhibits primary root growth, which is determined by the rate of cell division in the meristematic zone and the extent of cell expansion in the elongation zone. This leads to an acceleration of lateral root growth as a result of increased rates of cell division in the pericycle (Ortiz-Castro et al. 2011). Interestingly, co-cultivation of *Arabidopsis* with the QS-related mutants *rhlI-*, *lasI-* and *rhlI-/lasI-* caused a decreased virulence and a concomitant phytostimulation (Fig. 1), which can be likely explained either by a decreased production of virulence factors or by stimulation of root developmental processes as *P. aeruginosa lasI-* and *rhlI-/lasI-* mutants overproduce cyclodipeptides with auxin activity (Ortiz-Castro et al. 2011). Most likely, the beneficial effects of co-cultivation with *lasI-* and *rhlI-/lasI-* bacterial strains may be due to a combination of both processes.

One of the factors of virulence and survival of *P. aeruginosa* is the production of secondary metabolites (i.e. phenazines, which have antibiotic properties) including pyocyanin (PCN, 1-hydroxy-5-methylphenazine), a blue-green pigment with redox properties (Lau et al. 2004a; Liu and Nizet 2009). PCN synthesis is regulated by the *lasR* and *rhlR* QS systems (de Kievit and Iglewski 2000; Rumbaugh et al. 2000; Schaber et al. 2004; Siehnel et al. 2010) and in agreement with these previous results we found a decreased production of PCN in *lasI* and *rhlI/lasI P. aeruginosa* mutants (Fig. 3). PCN can easily penetrate biological membranes and directly accept electrons from reducing agents such as NADPH and reduced glutathione, then transfer the electrons to oxygen to generate ROS such as hydrogen peroxide and superoxide at the expenses of host antioxidant systems such as glutathione and catalase (O´Malley et al. 2004). Based on the finding that PCN is capable of killing fungi and is toxic to nematodes, we hypothesized that the eukaryotic cellular pathways that are affected by PCN could be evolutionary conserved and therefore, by using a plant model system it could be defined whether PCN acts causing toxicity to cells or regulating fundamental cellular processes such as division, elongation and/or differentiation. Moreover, diverse bacterial species proliferate in the rhizosphere and release

PCN and other phenazines with potential biocontrol activities (Fuqua and Greenberg 2002; Bosgelmez-Tinaz 2003). This would suggest that natural phenazines like phenazine-1-carboxylic acid (PCA) and PCN can accumulate in the plant rhizosphere in amounts sufficient not only for inter- and intraspecies signaling but also for the direct inhibition of competing organisms.

Despite numerous reports of the PCN-mediated cellular injuries, the response of plant cells or whole organs to *P. aeruginosa*-produced PCN is unknown. A lack of information might have lead to an under-estimation or miss-estimation of the mechanisms by which *P. aeruginosa* causes cell damage or phytostimulation. Knowledge about the activity of PCN in plants and the cellular pathways that are affected may be of practical value in agriculture and it was the objective of this research to clarify some aspects of PCN activity in *Arabidopsis* seedlings. We found that PCN can be directly perceived by roots to adjust growth and development. PCN was found to inhibit primary root growth and stimulate lateral root and root hair formation in a dose-dependent way (Figs. 4-5, S1-S2). In this regard, PCN activity is similar to the previously reported activities of other bacterial QS signals, namely *N*-acyl-*L*-homoserine lactones, which regulate root system architecture in a highly specific way, depending on the length of the acyl-side chain (Ortiz-Castro et al. 2008). These results indicate that bacteria may affect root development not only by producing AHLs but also PCN, and possibly other phenazines.

Accumulating evidence indicates that ROS play an essential role in the basic mechanism of cell growth and in the establishment of cell shape. This fundamental role in cell growth is likely to be widespread in plant parts, as shown in the polarized tip growth of root hairs. These structures are long thin extensions growing out perpendicularly from trichoblasts, one of the cell types of the root epidermis. The presence of root hairs greatly increases the surface area of the root available for the absorption of nutrients and water and for interaction with soil particles and bacteria. Since PCN decreases trichoblast cell length and increases root hair elongation (Fig. 5 and S2), it is tempting to speculate that plant perception of QS signals and PCN affect both the production and localization of ROS and then the growth mechanisms that determine the shape of trichoblast change. Our data that PCN affects primary roots, root hairs and lateral root development through production of ROS, is consistent with available genetic and pharmacological evidences. For instance, the ROOT HAIR DEFECTIVE2 (RHD2)/AtrbohC protein defective on a respiratory burst oxidase homolog (RBOH) enzyme, which catalyzes the reduction

of oxygen to generate superoxide anion, is required for root hair elongation. The roots of plants homozygous for loss-of-function *rhd2* mutations have decreased levels of ROS and are 20% shorter than the wild-type, indicating that cell expansion is defective in these plants (Foreman et al. 2003; Renew et al. 2005). On the other hand, by using inhibitors such as diphenylene iodonium (DPI), it has been suggested that NOX-derived ROS control cell expansion in maize (*Zea mays*) roots (Liszkay et al. 2004). Recently, it was found that silencing PvRbohB in transgenic *Phaseolus vulgaris* roots had a negative impact on lateral root density. In this work, the down-regulation of PvRbohB affected both the growth and ROS levels in young lateral roots. Interestingly, the PvRbohB promoter was induced during lateral root primordium initiation in the pericycle, and remained active throughout lateral root development. This study identifies RBOHs as potentially important players in lateral root development in *P. vulgaris.* The particular impact of such regulation of root hair and lateral root growth by bacterial molecules such as PCN in the interactions between plants and bacteria remain to be determined. The above described information indicates that ROS-mediated configuration of the root system is not an *Arabidopsis*-specific response and thus PCN might be active in crops.

Although root treatment with PCN did not induce visible cell death in transgenic *Arabidopsis* seedlings expressing the *AtHisH2B:YFP* marker stained with propidium iodide, a marked reduction in root meristem length and expression of *CycB1:uidA*, and *AtPRZ1:uidA* was observed (Fig. 6), indicating that PCN repress cell division. It could be proposed that the PCN-induced generation of ROS might lower proliferating cell activity, thus decreasing primary root growth. These results suggest that redox regulation plays an important role in maintaining root meristem activity. Moreover, this is supported by previous findings that differences in superoxide and hydrogen peroxide accumulation in the root tip significantly affect root growth and differentiation (Dunand et al. 2007; Tsukagoshi et al. 2010). Our data indicate that PCN modulates the balance between cell proliferation and differentiation by directly regulating the accumulation of ROS in the root tip.

Contradictory information exists regarding the role of plant hormones in regulation of ROS production. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the *Arabidopsis* primary root. This pathway seems to function independently

of auxin and cytokinin signaling (Tsukagoshi et al. 2010). In contrast, in *Lepidium sativum* ('cress'), five respiratory burst oxidase homologs (Lesarbohs) were sequenced and it was found that their expression patterns were similar to their *Arabidopsis* orthologues throughout the life cycle. Cress plants in which *LesarbohB* expression was knocked down showed a root phenotype associated with defective auxin-related genes (Müller et al. 2012). These transgenic plants further displayed altered expression of auxin marker genes including those encoding the auxin responsive proteins 14 and 5 (IAA14 and IAA5), and LBD16 (LATERAL ORGAN BOUNDARIES DOMAIN16), an auxin-responsive protein implicated in lateral root initiation. It was speculated that ROS produced by rbohs play a role in root development via auxin signaling. Our data are in agreement with those of Tsukagoshi et al. (2010) in that the effects of PCN on ROS induction are independent of auxin signaling considering the following evidences. First, PCN did not activate the expression of the auxin-inducible reporter markers *DR5:uidA* and *BA3:uidA* (Fig. 7), second, the auxin-related *tir1afb2afb3*, *arf7 arf19*, *axr1-3, aux1-7* mutants displayed similar primary root growth inhibition in response to PCN when compared to WT seedlings (Fig. S3). In addition, the primary root growth of cytokinin and abscisic acid- related mutants also were normally inhibited by PCN, indicating that the genes defective in these mutants are unlikely mediating the cellular effects of PCN in the primary root.

The ROS distribution at the primary root tip evidences that localized superoxide accumulation in the meristematic zone is necessary for proliferation, whereas H_2O_2 accumulates in the elongation zone when cells arrest division and begin differentiation (Tsukagoshi et al. 2010). Because PCN is a redox-active compound and has been demonstrated before to be capable of generating ROS in animal systems, we investigated whether PCN treatment activates the oxidative machinery of *Arabidopsis* roots. By means of a combination of fluorophores that specifically react with ROS and using confocal microscopy, we found that PCN supply to *Arabidopsis* seedlings grown *in vitro* leads to enhanced ROS, and superoxide levels in primary root tips. PCN treatment increased superoxide accumulation in the root elongation zone (Fig. 9), while co-cultivation with *P. aeruginosa* (Fig. 2) or PCN supply (Fig. 10) decreased H_2O_2 accumulation in the same region, which was coincident with the inhibitory effects of PCN on cell division and elongation. Thus, disrupting the spatial distribution of superoxide and/or hydrogen peroxide may compromise normal root growth. Staining for the presence of these ROS in the

root showed a clear correlation between growth rate and the relative distribution of different ROS species in the meristematic and elongation zones. Interestingly, differences in the localization of superoxide and hydrogen peroxide in seedlings treated with PCN (Fig. 9), or co-cultivated with *P. aeruginosa* WT and QS-related mutants *lasI*, *rhlI* and *rhlI/lasI* (Fig. 2) suggest that these ROS can function as intercellular signaling molecules.

The PCN-elicited accumulation of ROS was partially blocked when supplied together with the ethylene blocker $AgNO_3$ (Fig. 9) and the hydrogen peroxide decrease was lower in the ethylene related mutants *etr1-1*, *ein2-1* and *ein3-1* than in WT seedlings. These data correlate with the greater resistance of ethylene-related mutants to primary root growth inhibition caused by PCN (Fig. 8), further indicating that ethylene plays an important role in mediating the ROS response to PCN. To the best of our knowledge, the particular distribution of superoxide and hydrogen peroxide in primary root tips of ethylene-related mutants has not been previously investigated. However, while analyzing the flg22-triggered ROS production in *Arabidopsis* seedlings, Mersmann et al. (2010) identified ethylene signaling as a critical component of the oxidative burst in response to this bacterial elicitor as *etr1-1* and *ein2-1* mutants were strongly diminished in flg22-induced ROS accumulation. Ethylene has diverse functions in plant-microbe interactions (van Loon et al. 2006). It is important for defense against necrotrophic fungi (Chagué et al. 2006), but its contribution to bacterial resistance remains unclear. Our data demonstrated that among the PCN responses tested, the ethylene-insensitive mutants were resistant to the PCN effect decreasing hydrogen peroxide accumulation in the elongation zone of the primary root (Fig. 10). This suggests that ethylene plays a dual function in response to bacterially produced PCN, it may contribute to defense responses, possibly through regulation of ROS production, and at the same time in ROS modulated root system architecture. Our work underscores the importance of PCN as a signaling molecule in plant-bacteria interactions as a modulator of cellular programs that determine the configuration of the root system. Understanding the contribution of quorum-sensing in pathogenesis and symbiosis, particularly the role played by AHLs in the production of virulence factors and/or compounds with a role in auxin (i.e. Ortiz-Castro et al., 2011) or ethylene signaling (this work) should contribute to the development of new strategies for protecting plants against pathogens or increase plant productivity.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (Col-0), the transgenic lines *CycB1:uidA* (Colón-Carmona et al. 1999), *AtPRZ1:uidA* (Sieberer et al. 2003), *DR5:uidA* (Ulmasov et al. 1997), *BA3:uidA* (Oono et al. 1998), histone *AtHisH2B:YFP* (Boisnard-Lorig et al. 2001), and mutant lines *etr1-1* (Hua and Meyerowitz 1998), *ein2-1* (Guzmán and Ecker 1990), *ein3-1* (Chao et al. 1997), *tir1afb2afb3* (Dharmasiri et al. 2005), *arf7arf19* (Okushima et al. 2007), *aux1-7* (Pickett et al. 1990), *axr1-3* (Lincoln et al. 1990), *abi1* (Ma et al. 2009), *abi3* (Koornneef et al. 1984; Nambara et al. 1992), *cre1-12ahk2-2, cre1-12ahk3-3* (Higuchi et al. 2004; Mähönen et al. 2006) were used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2x MS medium (Murashige and Skoog 1962). MS medium (Murashige and Skoog basal salts mixture, Cat. M5524) was purchased from Sigma (St. Louis, MO, USA). The suggested formulation is 4.3 g L^{-1} of salts for a 1x concentration of medium; we used 0.9 g L^{-1}, which we consider and refer to as MS 0.2x. This medium lacks amino acids and vitamins. Pyocyanin was purchased from Sigma. The compound was dissolved in DMSO and used at the indicated concentrations. In control seedlings, we added the solvent in equal amounts as present in the greatest concentration of compound tested. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, USA). Plants were placed in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 100 µmol m^2s^{-1} and temperature of 22 ^oC.

In vitro plant-bacteria co-cultivation assays

Bacterial strains used in this work were *P. aeruginosa* PAO1 (WT), *P. aeruginosa rhlI-, lasI-* and *rhlI-/lasI-* single and double mutant respectively (Li et al. 2007). The bacterial strains were evaluated *in vitro* for their pathogenic or plant growth-promotion ability, using the *Arabidopsis* Col-0 ecotype. Bacterial densities of 2.5 x 10^8 cfu were inoculated by streaking on agar plates containing 0.2x MS medium. Six-day-old germinated *Arabidopsis* seedlings (10 seedlings per

plate) were transferred and located over the bacterial streak site and grown for a further 3, 6 and 9-d period. The plates were placed in the growth chamber (Percival Scientific AR-95L) in a completely randomized design. All experiments were replicated at least three times.

Analysis of plant growth and statistical analysis

Growth of primary roots was registered using a rule. Lateral root number (LRN) was determined by counting the lateral roots present in the primary root from the tip to root/stem transition. Lateral root density (LRD) was determined by dividing the lateral root number by the primary root length and expressed as LRD cm^{-1}. The length of the meristems was determined as the distance between the quiescent center to the cell file where cells started to elongate. For all experiments, data were statistically analyzed in the SPSS 10 program (SPSS, Chicago, IL, USA). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in wild-type (WT) and ethylene related mutants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Microscopy

Arabidopsis thaliana root system was analyzed with a stereoscopic microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). Total lateral roots were counted at 30X magnification. Primary root meristems were analyzed in semi-permanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus, Carl Zeiss, Göttingen, Germany) at 100X or 400X magnifications. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony Electonics Inc., Oradell, NJ, USA) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss).

Histochemical analysis

Transgenic plants that express the *uidA* reporter gene (Jefferson et al. 1987) were stained in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, for 12 h at 37 ^{o}C. Plants were cleared and fixed as previously described by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail

varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.

H_2O_2 production was detected by the endogenous peroxidase dependent staining procedure using 3,3-diaminobenzidine (DAB) uptake (Thordal-Christensen et al. 1997). Control or PCN-treated of *A. thaliana* WT and ethylene mutant seedlings were placed in a solution of 1 mg mL^{-1} DAB, pH 3.8, and incubated in dark for 2 h. Subsequently, they were immersed in boiling 96% (v/v) ethanol for 10 min and then stored in 96% (v/v) ethanol. For each treatment, at least 15 treated seedlings were analyzed. A representative plant was chosen for each treatment. H_2O_2 production was visualized as a reddish brown precipitated coloration and photographed using a stereoscopic microscope.

Propidium iodide staining and YFP detection

For confocal microscopy, solvent- or pyocyanin-treated transgenic *Arabidopsis* seedlings expressing the histone *AtHisH2B:YFP* construct (Boisnard-Lorig et al. 2001) were mounted on microscope slides into a solution of propidium iodide (PI). For fluorescent staining with PI, recently collected plants with intact root systems were transferred to a solution of 10 mg/ml PI for 3 min. Seedlings were rinsed in water and mounted in 50% glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568 nm excitation line and a emission window of 585–610 nm, and YFP emission with a 505–550 nm band pass emission filter (488 nm excitation line), after which the two images were merged to produce the final image. Primary root meristems were analyzed by imaging mounted samples with an inverted confocal microscope (Olympus FV1000).

Pyocyanin quantification

Pyocyanin was extracted from the supernatant fraction of *P. aeruginosa* grown in LB medium at 37 ^{o}C for 48 h. 1 mL of supernatant was mixed with 1 mL of chloroform and the lower organic layer was separated. To this layer, 1 mL 0.2 HCl was added and the pyocyanin-rich organic layer was separated to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant were determined according to Essar et al. 1990.

ROS and superoxide detection

General ROS and specific superoxide anion (O^{-2}) were monitored by incubating *Arabidopsis* seedlings with 10 µM of the fluorescent probes 2',7'-dichlorofluorescein diacetate (H2DCF-DA), and dihydroethidium (DHE) in 10 mM Tris-HCl (pH 7.4), respectively (Gomes et al. 2005). *Arabidopsis*-treated seedlings were incubated for 30 min in darkness, and washed three times for 5 min with fresh buffer. Fluorescence signals from at least 10 treated and control seedlings were detected using a confocal microscope (Olympus FV1000). Fluorescence signals were quantified by counting pixel number in the green channel by employing ImageJ software.

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FIGURE LEGENDS

Figure 1. Effect of co-cultivation with *P. aeruginosa* WT and QS mutant strains on plant growth. Six-day-old *A. thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at direct contact and grown for 3 (A-E), 6 (F-J) and 9 (K-O) days. Representative photographs were taken for plates from each treatment. This experiment was repeated three times with similar results. Notice the damage and inhibitory on root system architecture caused by the *P. aeruginosa* WT and *rhlI-* mutant and the strong induction on root system architecture and greening plants effect of the *P. aeruginosa lasI-* and *rhlI-/lasI-* mutants. Scale bar = 1cm.

Figure 2. Effect of co-cultivation of *P. aeruginosa* WT and QS mutant strains on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* seedlings. Four-day-old *A. thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases RhlI, LasI, or RhlI/LasI at a distance of 5 cm from the primary root tip and grown for 8 d. Representative photographs of primary root of control seedlings (A) or co-cultivated with WT *P. aeruginosa* (B), and *rhlI* (C), *lasI* (D) or *rhlI/lasI* (E) mutants. *Arabidopsis* seedlings were treated with a solution of 3,3’-diaminobenzidine (DAB). In the presence of H_2O_2, DAB polymerizes, forming a dark red-brown coloration in plant tissues. This experiment was repeated three times with similar results. Scale bar = 500 µm.

Figure 3. Pyocyanin production in *Pseudomonas aeruginosa* WT and QS mutant strains. (A) Pyocyanin production, cells were grown in LB medium at 37 ^{o}C for 48 h, the supernatant fractions were separated, and the amount of pyocyanin (µg mL^{-1}) in each fraction was determined by the chloroform:acid extraction procedure. Values represent the mean of three

independent experiments ± SD. (B) Representative photograph of pyocyanin production as observed by the green color of culture grown in liquid medium.

Figure 4. Effect of pyocyanin on *Arabidopsis* root system architecture. *Arabidopsis* WT (Col-0) seedlings were germinated and grown for 10 d under increasing pyocyanin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent the mean ± SD (*n*=30). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

Figure 5. Effects of pyocyanin on epidermal cell differentiation. (A) Trichoblasts length. (B) Root hair number. (C) Root hair length. *Arabidopsis thaliana* seedlings were grown for 5 d on 0.2x MS medium supplemented with the indicated concentrations of pyocyanin. Date points indicated mean ± SD (*n* = 20). The results show mean of 10 epidermal cells located in the root hair forming zone of the primary root. This experiment was repeated twice with similar results. Different letters indicate statistical differences at $P<0.05$.

Figure 6. Effect of pyocyanin on cell division and meristem viability. *A. thaliana* seedlings expressing the *CycB1:uidA*, *AtPRZ1:uidA* or *AtHistH2B:YFP* markers were grown for 5 d on 0.2x MS medium supplemented with the indicated concentrations of pyocyanin. (A-H) Plants were stained for β-glucoronidase activity and cleared to show gene expression. (I-L) Transgenic *Arabidopsis* seedlings expressing the *AtHisH2B:YFP* marker were stained with propidium iodide to determine cell structure and viability. Photographs show representative individuals from at least 20 stained plants. The experiment was replicated twice with similar results. Scale bar = 100 µm. (M) the *CycB1:uidA* expression domain in response to pyocyanin was measured and cells expressing this marker were counted (N). Data points represent the mean ± SD (*n* = 20). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P<0.05$.

Figure 7. Effect of pyocyanin on auxin-regulated gene expression. (A-L) *DR5:uidA* and (M-X) *BA3:uidA* gene expression in transgenic seedlings grown on MS 0.2x agar medium for 6 d and then transferred into 24-well cell culture plates (10 seedlings per well) containing 2 mL 0.2x MS

liquid medium supplied with the indicated concentrations of IAA or pyocyanin and incubated for 10 h. Seedlings were stained for GUS activity and cleared for microscopy analysis. Photographs show representative individuals from at least 30 stained plants (Scale bars = 500 µm).

Figure 8. Effect of pyocyanin on primary root growth of *Arabidopsis thaliana* WT (Col-0) and ethylene related mutants *etr1-1*, *ein2-1,* and *ein3-1*. (A) *Arabidopsis thaliana* WT and *etr1-1, ein2-1*, and *ein3-1* ethylene mutant seedlings were grown for 12 d on 0.2x MS medium supplemented with the indicated concentrations of pyocyanin. (B) Representative photographs of the growth of WT and *ein2-1* seedlings showing the resistance of the mutants to pyocyanin. Data points in (A) show the mean ± SD. Different letters indicate means that differ statistically at $P<0.05$. The experiment was repeated three times with similar results. Scale bar = 1cm.

Figure 9. Role of ethylene signaling in ROS-induced production by pyocyanin. (A-D) Representative photographs of ROS (A-E) and O^{-2} (F-J) determined in primary roots of *Arabidopsis* seedlings grown for 7 d on 0.2x MS agar medium supplemented with 5 µM of pyocyanin and $AgNO_3$ or with 0.1 µM of paraquat. Fluorescence signals from primary root tips (n = 10) for ROS (K) and O^{-2} (L) were quantified using the ImageJ program. The graph is expressed in arbitrary units. Values in K and L represent the mean ± SD (*n* = 30). Different letters are used to indicate means that differ statistically at $P<0.05$. The experiment was repeated three times with similar results. Photographs are representative individuals of at least 10 seedlings analyzed. Scale bar = 100 µm.

Figure 10. Effect of pyocyanin on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* WT (Col-0) and ethylene-related mutants *etr1*, *ein2* and *ein3*. Histochemical detection of H_2O_2 with DAB staining in (A-F). *A. thaliana* WT (Col-0) and (G-L) *etr1-1*, (M-R) *ein2-1*, and (S-X) *ein3-1*. *Arabidopsis* seedlings were grown for 7-d on 0.2x MS medium supplemented with the indicated concentrations of pyocyanin. Photographs show representative individuals from at least 30 stained plants. Scale bar = 500 µm.

Figure S1. Effect of pyocyanin on *Arabidopsis* root system architecture. *Arabidopsis* WT (Col-0) seedlings were germinated and grown for 12 d under increasing pyocyanin concentrations. Representative photographs are shown. The experiment was repeated three times with similar results. Scale bar = 1 cm.

Figure S2. Effect of pyocyanin on root hair development. Representative photographs of root hairs formed at the differentiation zone of the primary root in 9-day-old *Arabidopsis* seedlings grown in 0.2x MS medium supplemented with the indicated concentrations of pyocyanin are shown. The experiment was repeated three times with similar results. Scale bar = 500 µm.

Figure S3. Effect of pyocyanin on primary root growth of *Arabidopsis thaliana* WT (Col-0) and in mutants defective on several hormone pathways. WT (Col-0) and the auxin-related *tir1afb2afb3*, *arf7arf19*, *axr1-3, aux1-7* mutants; cytokinin-related *cre1-12ahk2-2* and *cre1-12ahk3-3*; abscisic acid-related *abi1* and *abi3*; and ethylene-related *ein2-1* and *ein3-1* mutants were included in the experiments. WT and mutant seedlings were grown for 12 d on 0.2x MS medium supplemented with the indicated concentrations of pyocyanin and primary root length was determined. Data show the mean ± SD. Different letters indicate means that differ statistically at $P<0.05$. The experiment was repeated two times with similar results.

Figure S4. Role of ethylene signaling in pyocyanin-induced primary root inhibition of *Arabidopsis* seedlings. *Arabidopsis* seedlings were grown for 12 d on MS 0.2x agar medium supplemented with 5 µM of pyocyanin and $AgNO_3$. Data points show the mean ± SD (n = 30). Different letters indicate means that differ statistically at $P<0.05$. The experiment was repeated three times with similar results.

Figure S5. Role of ethylene signaling in the effect of pyocyanin in *Arabidopsis* root system architecture and root hair development. *Arabidopsis* WT (Col-0) seedlings were germinated and grown for 12 d in 0.2x MS medium supplemented with the 5 µM of pyocyanin and $AgNO_3$. Representative photographs of *Arabidopsis* root system architecture (A-D), root hair development (E-H), and *pPRZ1:uidA* expression (I-L) under the different treatments are shown. The experiment was repeated three times with similar results. Scale bar in (A-D) = 1 cm, in (E-L) = 500 µm.

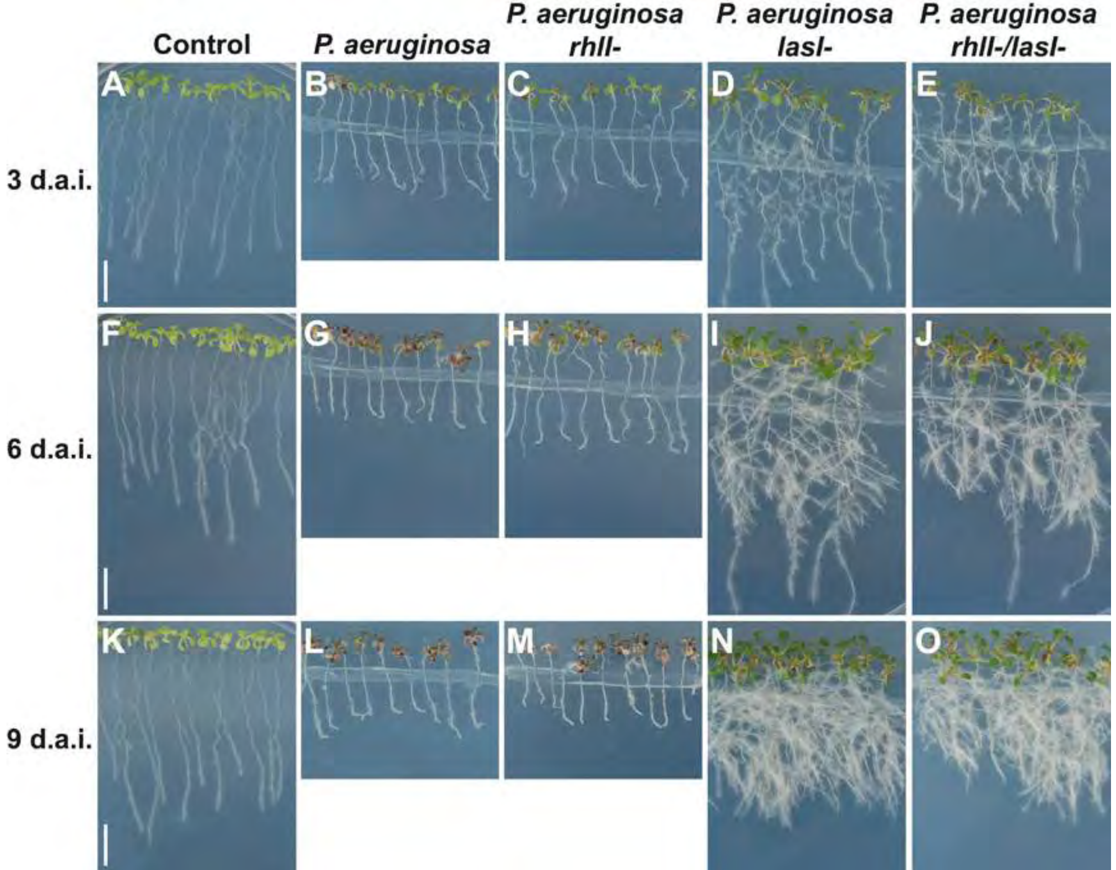
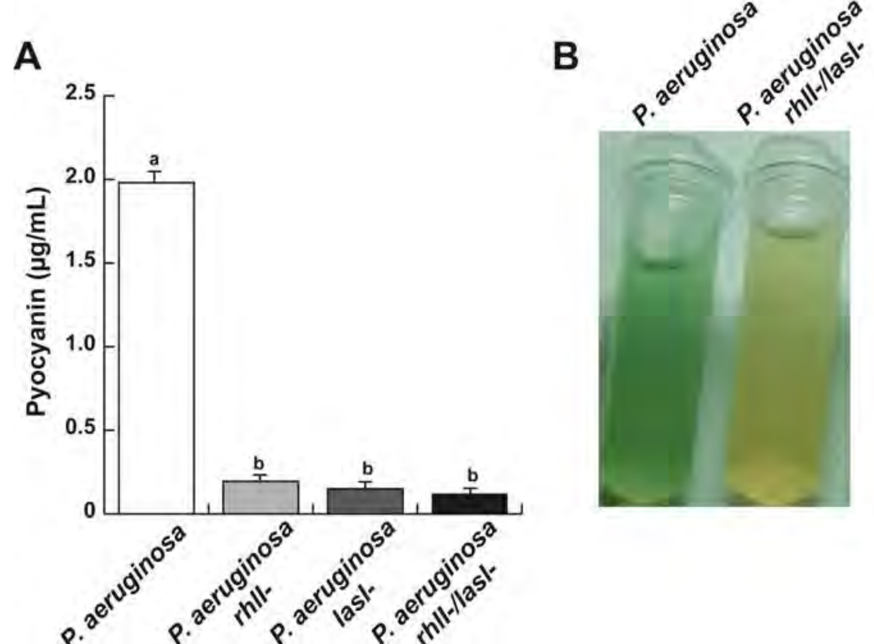


Figure 1.

Figure 2.



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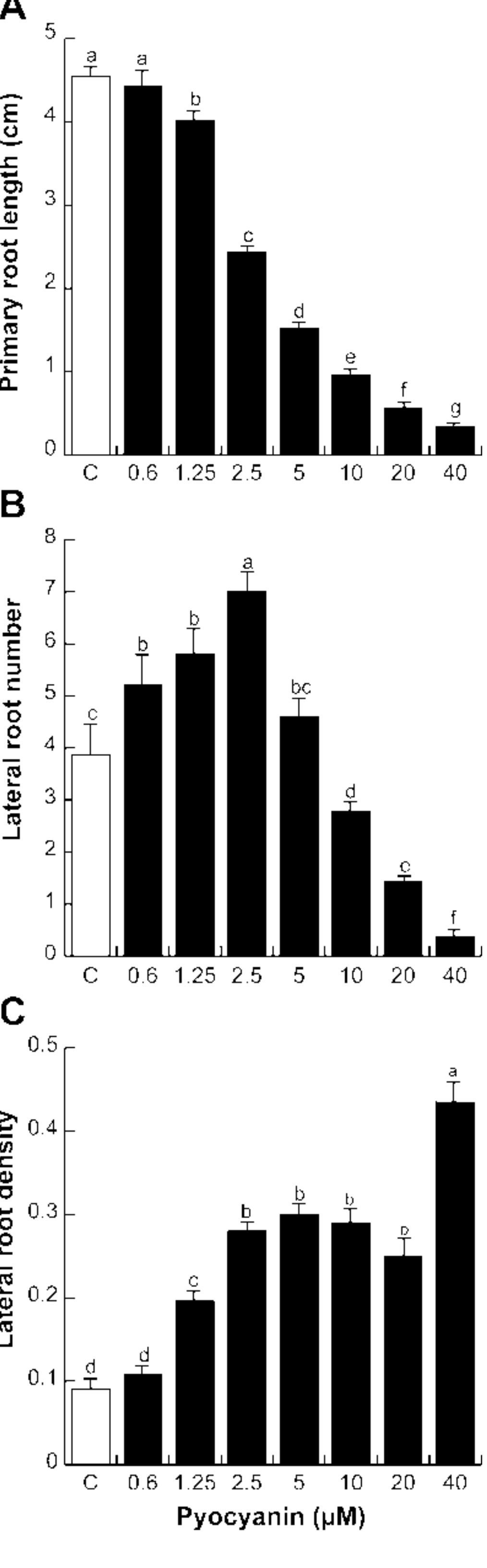

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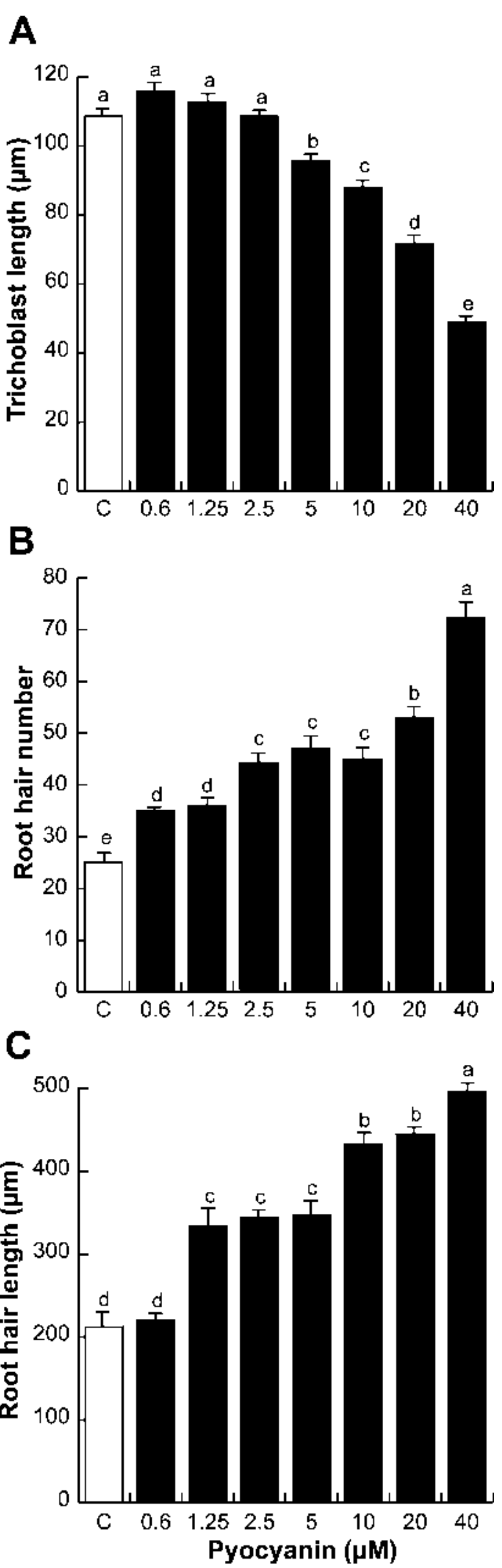

A
Trichoblast length (μm)
120
100
80
60
40
20
0
a
a
a
a
b
c
d
e
C
0.6
1.25
2.5
5
10
20
40
B
Root hair number
80
70
60
50
40
30
20
10
0
e
d
d
c
c
c
b
a
C
0.6
1.25
2.5
5
10
20
40
C
Root hair length (μm)
500
400
300
200
100
0
d
d
c
c
c
b
b
a
C
0.6
1.25
2.5
5
10
20
40
Pyocyanin (μM)

Figure 5.

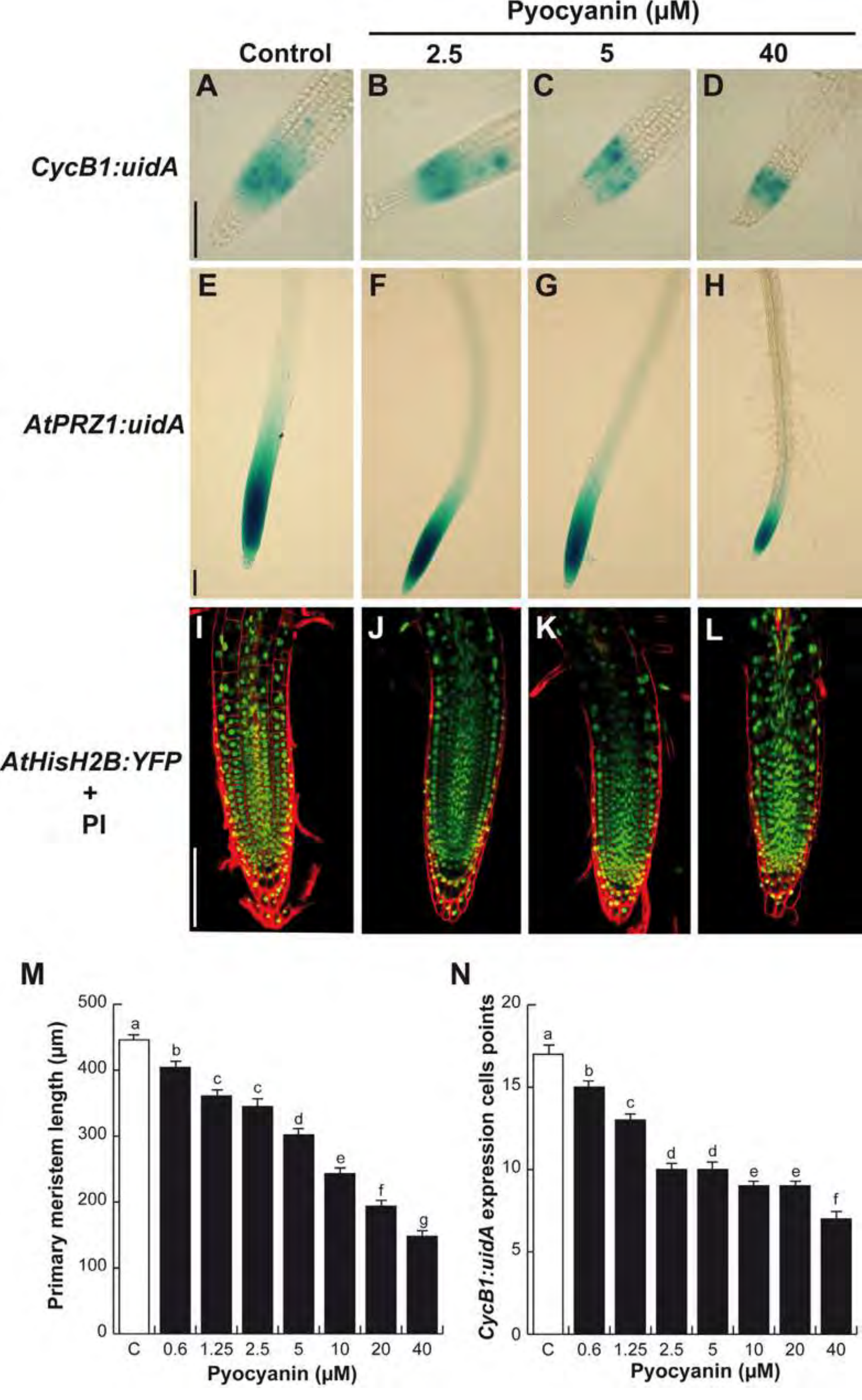
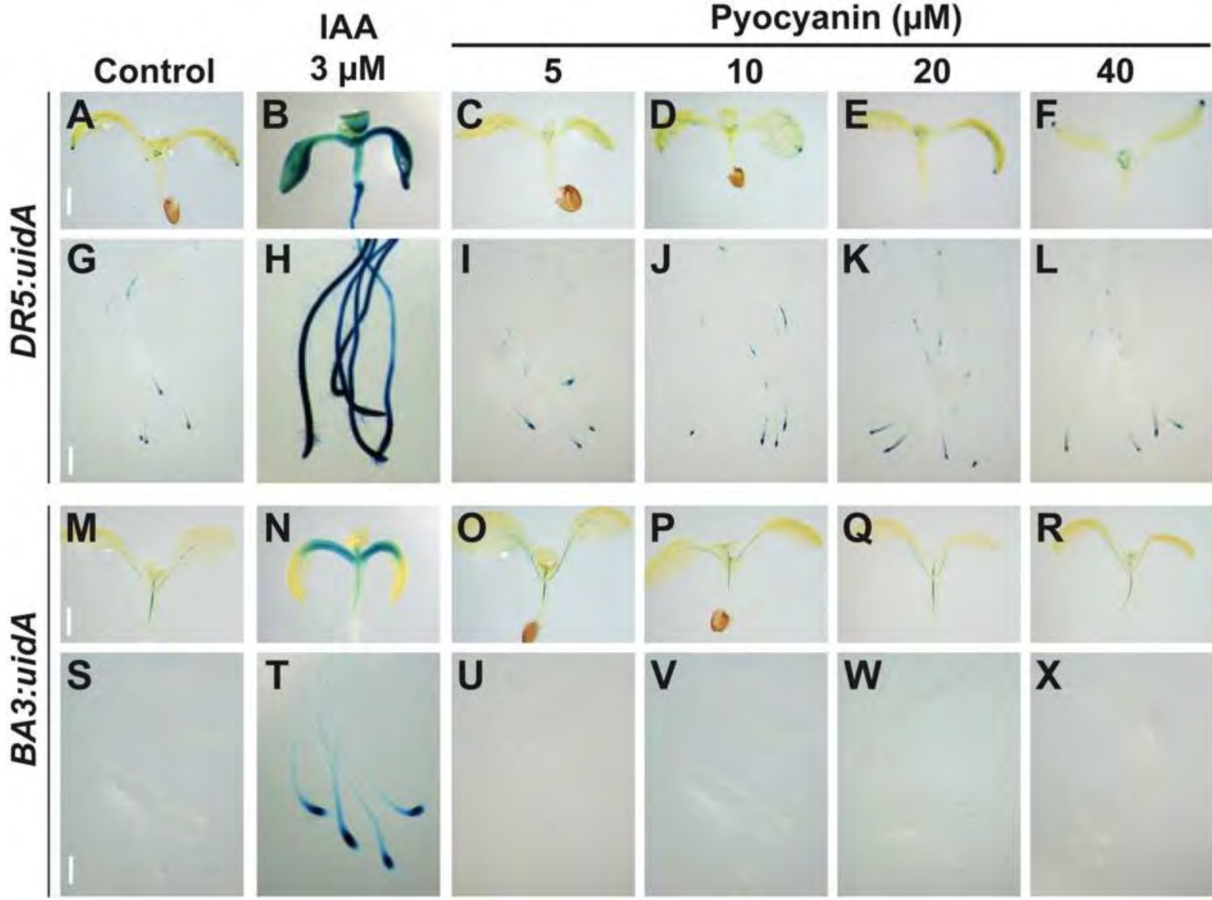


Figure 6.



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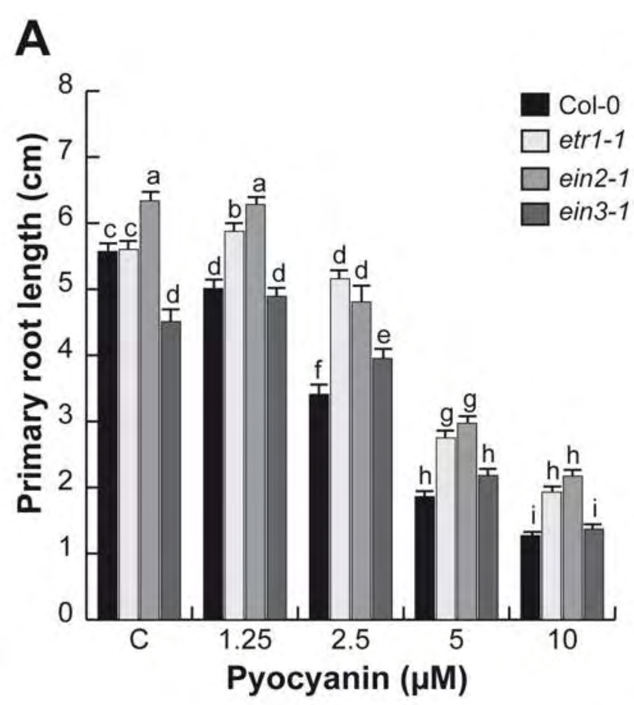

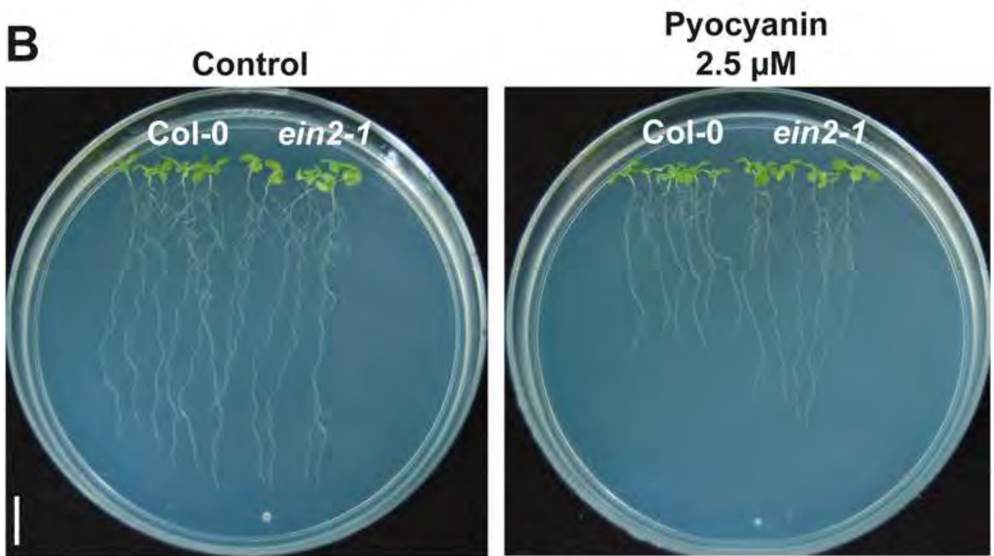

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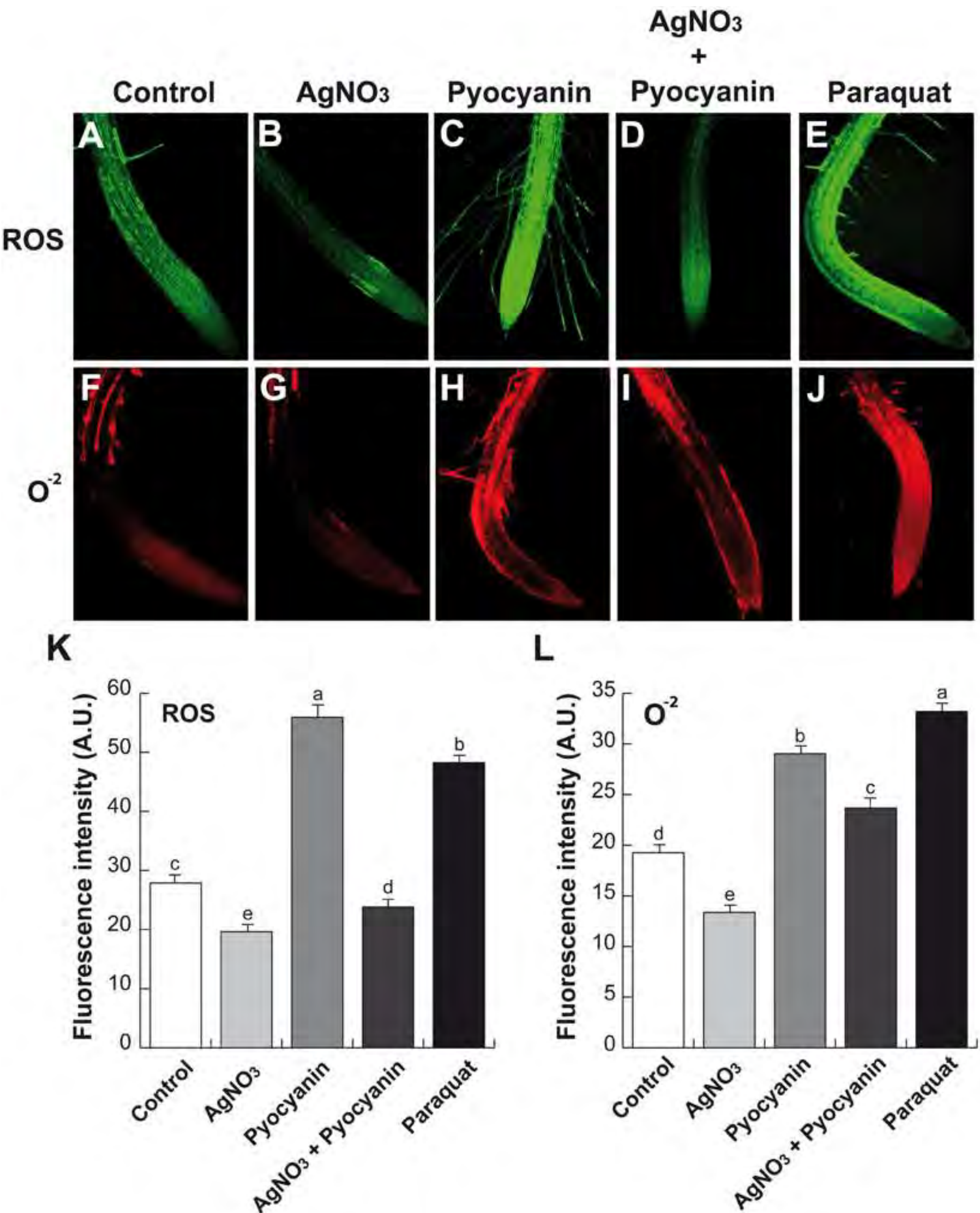

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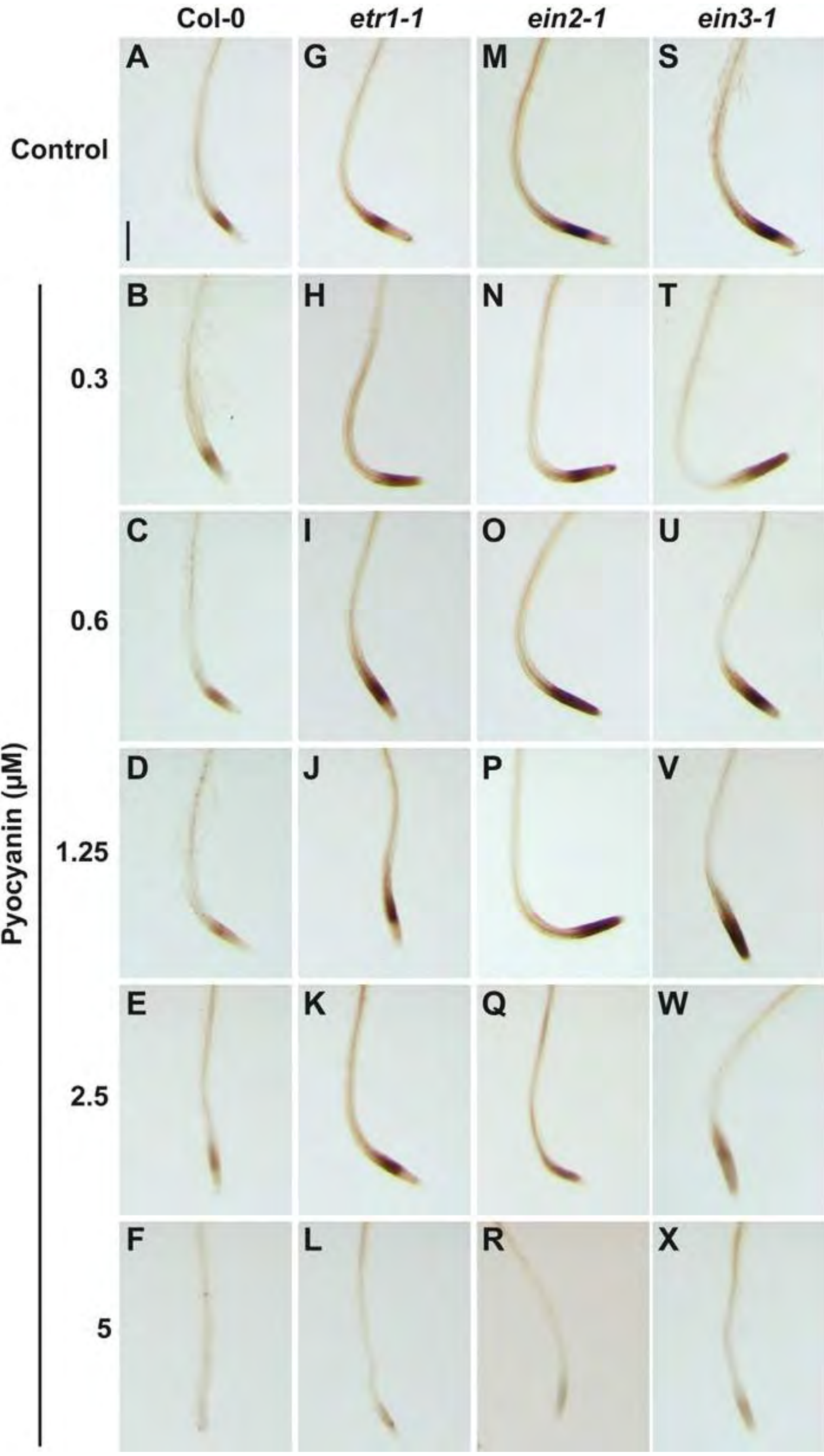

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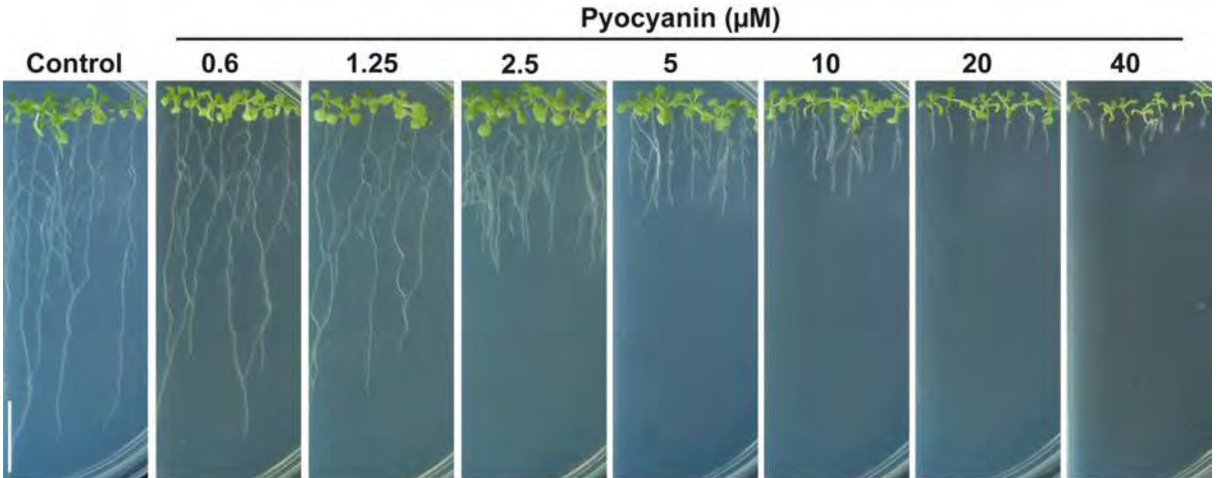
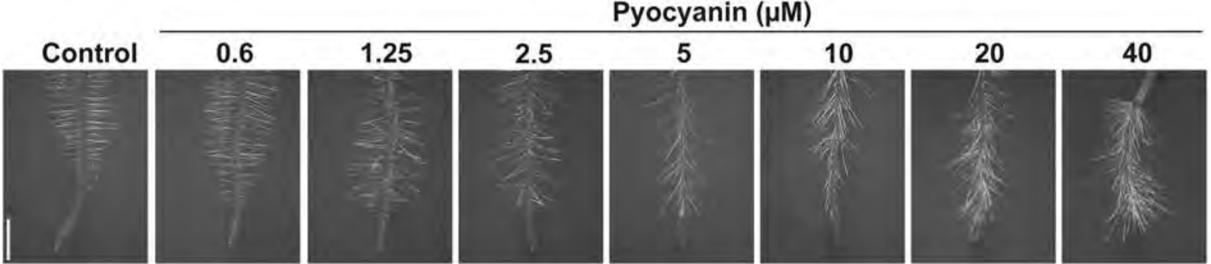


Figure S1.



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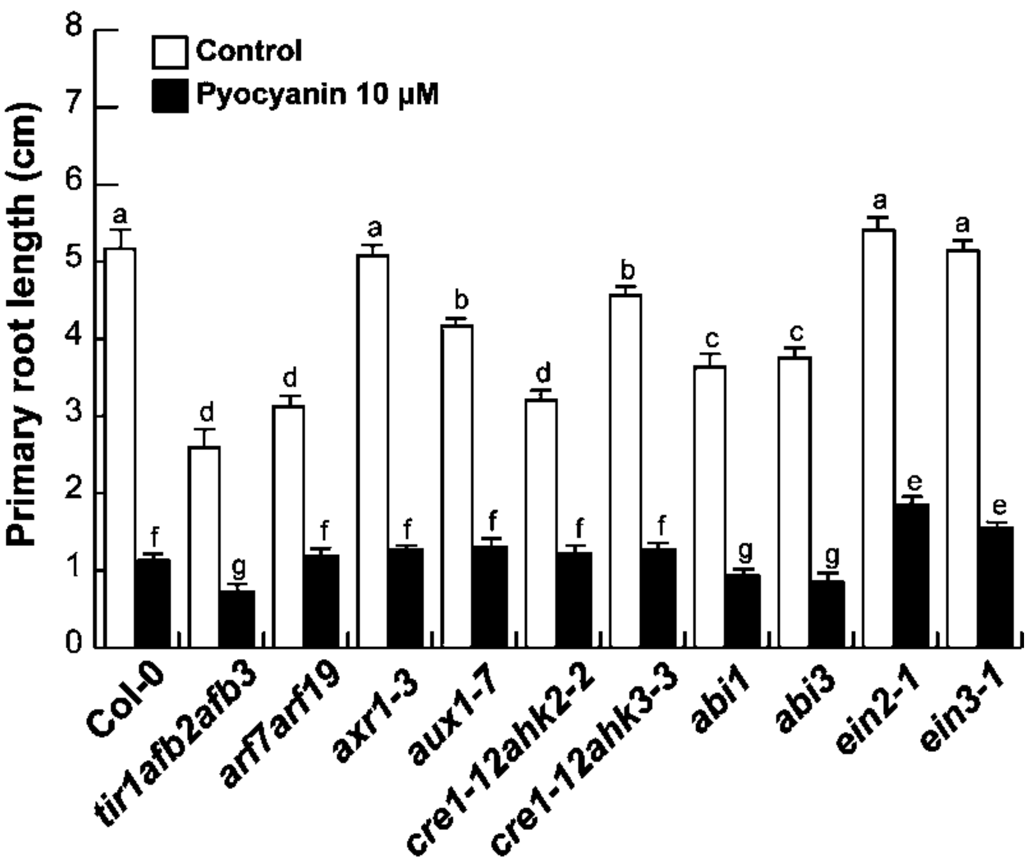

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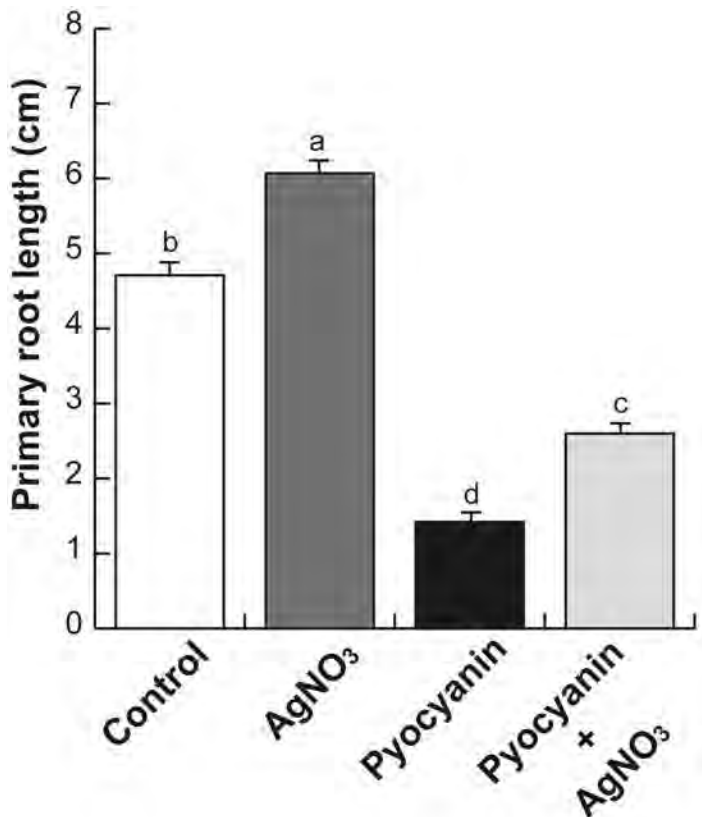

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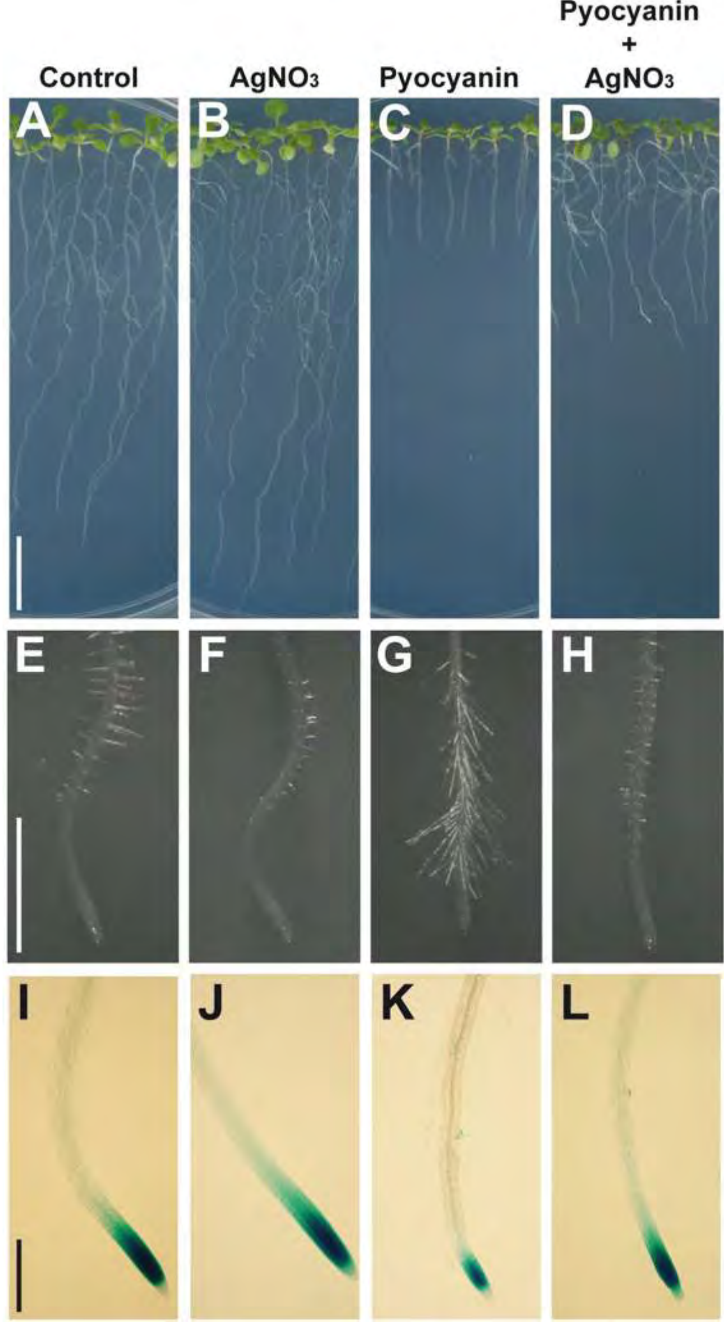

Figure S5.

8. DISCUSION Y CONCLUSIONES

Las plantas son sensibles a los cambios en los factores bióticos y abióticos. Tanto el sistema foliar como el radicular responden ajustando su arquitectura ante las situaciones cambiantes del ambiente, mediante procesos celulares regulados por diferentes fitohormonas. En el caso de la raíz, la interacción que se establecen con microorganismos del suelo como bacterias, protozoarios y hongos, entre otros organismos, determinan en gran medida el potencial de crecimiento, ya que algunas especies microbianas pueden ser patogénicas, o como se ha empezado a evidenciar, muchas de ellas son benéficas y promueven el crecimiento o activan la inmunidad.

Diversas interacciones planta-bacteria ocurren en la rizosfera, donde las condiciones de abundancia de fuentes de carbono a través de exudados radiculares atraen a las poblaciones bacterianas (Bais *et al.,* 2006). Tanto en la comunicación bacteria-bacteria como bacteria-planta, participan diferentes clases de compuestos. Las bacterias regulan la proliferación celular a través de la producción y detección de moléculas señal o "autoinductores" de una manera dependiente de la densidad poblacional, proceso comúnmente denominado *quorum-sensing* (QS) (Fuqua *et al.,* 1994). Particularmente, en Gram-negativas, las *N*-acil-homoserina lactonas (AHLs) actúan como señales que modulan la expresión genética y el comportamiento. Varios reportes han demostrado que la producción de AHLs es común en especies de *Pseudomonas* asociadas a plantas y mucho menos frecuente en especies de vida libre (Fuqua y Greenberg, 2002; Elasri *et al.,* 2001, Khmel *et al.,* 2002, D'Angelo-Picard *et al.,* 2005). En un primer estudio se demostró que las plantas perciben a las AHL y se inducen respuestas de defensa y estrés (Mathesius *et al.,* 2003). Por otra parte, en un trabajo de nuestro grupo, se encontró que las plantas responden a las AHLs mediante cambios en la arquitectura de la raíz. En ese reporte se encontró que las AHLs están estructuralmente relacionadas a alcamidas y NAEs, compuestos típicos de plantas con actividad sobre procesos de organogenesis (Figura 18; Ortiz-Castro *et al.,* 2008). Estos resultados sugieren que las plantas pueden detectar AHLs, NAEs y alcamidas por un mecanismo genético común.

Pseudomonas aeruginosa es una bacteria Gram negativa y un patógeno oportunista en humanos inmunocomprometidos o en pacientes con fibrosis quística, además de infectar otros organismos como insectos, nematodos y plantas (Walker *et al.,* 2004). En *P. aeruginosa* entre el 5 al 20% de los genes están sujetos a una regulación por el QS. Esta especie ha sido ampliamente utilizada para entender los mecanismos de regulación mediados por AHLs, en la que previamente se describieron los sistemas LasR-LasI y RhlR-RhlI, regulados por la 3-oxo-dodecanoil-HL (3-oxo-C12-HL) y butanoil-HL (C4-HL), respectivamente (Williams *et al.,* 2007).

Para entender el papel *in vivo* de la producción de AHLs en la interacción *A. thaliana - P. aeruginosa,* en este trabajo exploramos genéticamente la participación de las AHLs en el crecimiento y desarrollo de *Arabidopsis*, utilizando un sistema de co-inoculación *in vitro* (López-Bucio *et al.,* 2007; Ortiz-Castro *et al.,* 2013) con cepas silvestres de *P. aeruginosa* y las mutantes *lasI, rhlI* y *rhlIlasI* afectadas en las sintasas de AHL, sobre la producción de biomasa y cambios en la arquitectura radicular. Se encontró que la biomasa del follaje y la raíz se incrementa en plantas co-cultivadas con *P. aeruginosa*, esta estimulación del crecimiento fue potenciada en las mutantes *lasI* y *rhlI lasI*, la cual correlaciona con la disminución en la inhibición del crecimiento de la raíz primaria comparada con las plantas co-cultivadas con las cepas silvestres de *P. aeruginosa*. Este efecto en la inhibición de la raíz primaria es dependiente de la 3-oxo-C12-HL, lo cual se pudo observar al complementar la mutante *lasI* cuando se agrega la 3-oxo-C12-HL al medio de crecimiento, el efecto inhibitorio se restaura al igual cuando las plantas son co-cultivadas con *P. aeruginosa*. Fue muy relevante observar que la inoculación con *lasI* y *rhlIlasI* promueve la formación de abundantes raíces laterales y pelos radiculares, este efecto es muy similar a los efectos mediados por auxinas.

Reportes previos habían sugerido que *P. aeruginosa* es patógena de *Arabidopsis*. Rahme *et al.* (1995) evaluaron una colección de 75 cepas de *P. aeruginosa* al causar enfermedad sobre las hojas de cuatro ecotipos de *A. thaliana*, identificando sólo dos cepas UCBPP-PA14, un aislado de humano, y UCBPP-PA29, aislada de plantas, las cuáles causaron necrosis en las hojas en algunos de los ecotipos de *Arabidopsis* evaluados. Sin embargo, estos ensayos de patogenicidad se realizaron mediante infiltraciones de miles de bacterias dentro de los tejidos vegetales, con las consecuencias negativas al producir enzimas degradativas, factores de virulencia y compuestos que causan estrés oxidativo, como la piocianina (Rahme *et al.,* 1995). En contraste,

otros estudios habían demostrado el papel promotor del crecimiento vegetal de algunas cepas ambientales de *P. aeruginosa*, lo cual sugiere que *P. aeruginosa* puede actuar como un patógeno o promoviendo el crecimiento, dependiendo de diversos factores, y que existen variantes genéticas con efectos contrastantes sobre las plantas con las que interactúan.

Para determinar si la inhibición del crecimiento de la raíz primaria producida por *P. aeruginosa*, causa un daño en el tejido o afecta la proliferación o diferenciación celular, se utilizaron los marcadores *CycB1:uidA (*Colón-Carmona *et al.,* 1999*), AtPRZ:uidA* (Sieberer *et al.,* 2003) y *AtHistH2B:YFP* (Boisnard-Lorig *et al.,* 2001), los cuales mostraron que no existe un daño o muerte celular en la raíz primaria. Esto sugiere que los cambios en la arquitectura radicular se deben a un proceso de diferenciación celular inducido en la zona de crecimiento de la raíz. Posiblemente, la inhibición de la raíz primaria sea debida a la producción de la 3-oxo-C12-HL por *P. aeruginosa*, tal como se observó cuando las plantas fueron crecidas en medios suplementados con el compuesto purificado, ya que se encontró un efecto dosis dependiente en la inhibición del crecimiento de la raíz primaria e induciendo la formación de raíces laterales. El principal efecto del co-cultivo de *Arabidopsis* con las mutantes de *P. aeruginosa* afectadas en la producción de AHLs, particularmente en la mutante *LasI*, fue un incremento notable en la formación de pelos radiculares y de raíces laterales, muy cercanas al ápice de la raíz, sugiriendo un efecto auxínico sobre la planta.

Las bacterias tienen la capacidad de producir auxinas (Spaepen *et al.,* 2007), por lo que se determinó la producción de AIA por las cepas bacterianas. No se detectó la producción de este compuesto en el sobrenadante del medio de crecimiento de las diferentes especies y mutantes de *Pseudomonas* evaluadas. Por lo contrario, fue posible identificar tres ciclodipeptidos producidos por *P. aeruginosa*, cuya biosíntesis está regulada negativamente por el sistema de QS LasI. Las fracciones del extracto libre de células del medio de crecimiento fueron resueltas por HPLC y la estructura de los diferentes compuestos confirmada por MS y NMR, identificando en las tres fracciones con actividad biológica a los dipeptidos ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe). La actividad biológica de los compuestos es variable, lo que coincide con reportes previos en que se sugirió a los dipeptidos como inhibidores del QS en *Vibrio fischeri* afectando la producción de bioluminiscencia, pero en concentraciones mayores en las que actúa el inductor natural (Campbell *et al.,* 2009). Yan *et al.* (2004) mostraron

que la producción de ciclo(L-Leu-L-Pro) por *Achromobacter xylosoxidans* inhibe la producción de aflatoxina en *Aspergillus parasiticus*, modulando la expresión de genes relacionados a la producción del metabolito. Estudios de competencia muestran que ciclo(L-Pro-L-Tyr) y ciclo(L-Pro-L-Phe) antagonizan a la 3-oxo-C6-HL en la inducción de bioluminiscencia, sugiriendo que estos compuestos pueden competir por el mismo sitio de unión de las AHLs (Holden *et al.,* 1999). La producción de los dipeptidos no solamente ha sido reportada en especies patogénicas, Degrassi *et al.* (2002) indicaron que la bacteria promotora del crecimiento vegetal *Pseudomonas putida* WCS358 produce y secreta ciclo(L-Phe-L-Pro), ciclo(L-Tyr-L-Pro), ciclo(L-Leu-L-Pro) y ciclo(L-Leu-L-Val) con un posible papel en la comunicación célula a célula y su interacción con la planta.

El sistema heterocíclico de los dipeptidos es muy similar al encontrado en el ácido indol-3-acético (AIA) y otros compuestos con actividad auxínica. Nuestros resultados indican que los tres CDPs ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) tienen actividad sobre la expresión de genes regulados por AIA. Cuatro evidencias adicionales sugieren que los dipeptidos mimetizan la acción de las auxinas; 1) El efecto del ciclo(L-Pro-L-Tyr) inhibiendo el crecimiento de la raíz primaria. 2) Los efectos de los tres CDPs ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) en la estabilidad de los represores Aux/IAA, y de los factores de respuesta a auxina (ARFs) utilizando la línea reportera *HS::AXR3NT-GUS* (Gray *et al.,* 2001), en la que observamos una degradación del represor liberando al ARF y activando la expresión de genes regulados por auxina, 3) El efecto promotor en la formación de raíces laterales, 4) La respuesta alterada de la mutantes *tir1afb2afb3* (triple mutante afectada en los receptores de auxina) y *arf7arf19* (doble mutante afectada en los factores de respuesta a auxina). Además, el modelaje molecular de la unión de los CDPs al receptor TIR1, sugiere que la estructura plana de los tres CDPs es probablemente la responsable de la actividad, esta unión a TIR1 es muy similar al de otras auxinas sintéticas como el ácido naftalen acético (ANA), 2,4-D, y picloram. Estos resultados sugieren que los dipeptidos tienen una actividad auxínica débil y que requieren de una vía de señalización de auxinas para afectar los procesos del desarrollo anteriormente descritos.

El género *Pseudomonas* incluye especies que pueden ser patogénicas (e.g. *P. aeruginosa*) así como bacterias promotoras del crecimiento vegetal (PGPR) (Persello-Cartieux *et al.,* 2003). *P. putida* y *P. fluorescens* han sido ampliamente utilizadas por su capacidad en la estimulación del

crecimiento vegetal y por aumentar la productividad en diferentes cultivos (e.g. maíz, frijol, lechuga, trigo, cebada) (Persello-Cartieux *et al.,* 2003), sin embargo, el mecanismo por el cual estas PGPR modulan el desarrollo de la planta ha sido poco estudiado. En nuestro trabajo, evaluamos el papel promotor de *P. putida* y *P. fluorescens* en *Arabidopsis* utilizando un sistema de co-cultivo *in vitro*. La inoculación con *P. putida* y *P. fluorescens* incrementó la biomasa del follaje y raíz, comparada con las plantas crecidas en condiciones axénicas. Este efecto se correlaciona con un incremento en la formación de raíces laterales y desarrollo de pelos radiculares. A diferencia del efecto mostrado en la inhibición del crecimiento de la raíz primaria cuando las plantas de *A. thaliana* se co-cultivan con cepas silvestres de *P. aeruginosa* o crecen en medio suplementado con 3-oxo-C12-HL, durante el co-cultivo con *P. putida* o *P. fluorescens* solamente se observa una ligera inhibición en el crecimiento de la raíz primaria, este resultado se correlaciona con la expresión de *CycB1:uidA* y *PRZ1:uidA* en el meristemo de la raíz primaria, ya que su expresión es similar a las plantas control.

La inoculación de *P. putida* y *P. fluorescens* estimuló la formación de raíces laterales y pelos radiculares, cambios que evidencian una respuesta a auxinas alterada en la planta. Para determinar si el efecto de la inoculación está mediado por auxinas, analizamos el desarrollo de los pelos radiculares en la mutante *rhd6*. Esta mutante está afectada en la iniciación de los pelos radiculares y la carencia de estas células absorbentes puede ser normalizada por la adición exógena de auxinas o el precursor de etileno ACC al medio de crecimiento (Masucci y Schiefelbein, 1994). Se encontró que la inoculación con *P. putida* o *P. fluorescens* restaura la formación de pelos radiculares en esta mutante. Esta inducción en la formación de raíces laterales y pelos radiculares en plántulas de *Arabidopsis* inoculadas con *P. putida* o *P. fluorescens*, correlaciona con la activación del marcador de respuesta a auxinas *DR5:uidA* en la raíz primaria, lo que resalta el papel de la señalización de las auxinas en la respuesta a ambas bacterias. Varios estudios han mostrado el efecto de hormonas de plantas o metabolitos bacterianos sobre la arquitectura de la raíz. López-Bucio *et al.* (2007) encontró que el incremento de crecimiento de raíces laterales en plantas de *A. thaliana* inoculadas con *Bacillus megaterium* es independiente de auxinas. Por otra parte, el antibiótico bacteriano 2,4-diacetilfloroglucinol (DAPG) producido por *P. fluorescens* inhibe el crecimiento de la raíz primaria y estimula la formación de raíces laterales en plantas de tomate. Utilizando plántulas

de tabaco que expresan un gen reportero de la luciferasa bajo el promotor inducible por auxinas GH3, los autores encontraron que los cambios inducidos por DAPG en la arquitectura de la raíz son dependientes de la señalización de auxinas (Boezclton *et al.,* 2008). La producción de IAA, así como precursores de auxina o compuestos similares a las auxinas es una característica común de microorganismos asociados con plantas. La fase temprana de la interacción entre raíces de *Arabidopsis* y algunos microorganismos, previo al establecimiento de la simbiosis, es acompañado por la estimulación del desarrollo de raíces laterales y puede involucrar la detección de señales volátiles y difusibles, algunos de los cuales pueden afectar la homeostasis de auxinas (Sukumar *et al.,* 2013).

Diversas especies bacterianas han sido tipificadas como PGPR por la característica de producir auxinas como parte de su metabolismo incluyendo al ácido indol-3-acético (AIA), ácido indol-3-butírico (AIB) o sus precursores (Spaepen *et al.,* 2007). Las auxinas son cuantitativamente las fitohormonas más abundantes secretadas por *Azospirillum*, y se ha visto que la producción de auxinas es el factor responsable de la estimulación del sistema radicular y promoción del crecimiento por esta bacteria.

Las auxinas son percibidas por la proteína TRANSPORT INHIBITOR RESPONSE 1 (TIR1), un miembro de una pequeña familia de proteínas F-box (Dharmasiri *et al.,* 2005; Kepinski y Leyser, 2005). Esta interacción acelera la degradación catalizada por la ubiquitin ligasa de las proteínas represoras Aux/IAA, permitiendo la des-represión de genes regulados por auxinas por los factores transcripcionales (ARFs) (Gray, 2004). Para determinar si la familia de receptores TIR1 y ARFs están involucrados en la promoción del crecimiento vegetal observados por la inoculación con *P. putida* y *P. fluorescens*, se analizó el sistema radicular en plántulas silvestres de *Arabidopsis* y mutantes afectadas en los receptores de auxinas *tir1* y *tir1afb2afb3,* sencilla y triple, respectivamente, y en las mutantes *arf7-1*, *arf19-1*, y *arf7arf19*, afectadas en los ARFs. La inoculación con *P. putida* y *P. fluorescens* no afectó el crecimiento de la raíz primaria en plántulas silvestres ni en las mutantes *tir1, arf7-1, arf7-19, arf7arf19* y *tir1afb2afb3*. Sin embargo, el incremento en el número y densidad de raíces laterales observado en plantas silvestres co-cultivadas con ambas bacterias disminuyó en *arf7arf19* y *tir1afb2afb3.* Además, la inducción del desarrollo de los pelos radiculares disminuyó en la mutante *tir1afb2afb3* comparado con las plantas silvestres. En conjunto, estos resultados sugieren que *P. putida* y *P.*

fluorescens producen compuestos con actividad auxínica afectando una vía de señalización fundamental para el crecimiento y desarrollo de las plantas.

En nuestro estudio, no se logró detectar AIA en el medio de crecimiento de *P. putida* y *P. fluorescens*, pero en cambio, encontramos tres ciclodipeptidos, ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Phe) y ciclo(L-Pro-L-Val), mismos que previamente habíamos identificado en la mutante afectada en la sintasa LasI de *P. aeruginosa* (Ortiz-Castro *et al.,* 2011). Degrassi *et al.* (2002) mostró que *P. putida* WCS358 produce ciclo(L-Phe-L-Pro), ciclo(L-Tyr-L-Pro), ciclo(L-Leu-L-Pro) y ciclo(L-Leu-L-Val). Los compuestos purificados a partir del medio de crecimiento de las dos bacterias activan la expresión de genes regulados por auxinas, aunque en menor grado que el AIA, lo que sugiere que una actividad débil puede afectar los procesos de morfogénesis induciendo raíces laterales y pelos radiculares. Alternativamente, la combinación de los tres dipeptidos podría tener un efecto sinérgico en la respuesta auxínica global causando alteraciones sobre la arquitectura de la raíz como las que se observan en plantas co-cultivadas con *P. putida* y *P. fluorescens*.

Las fitohormonas controlan el crecimiento afectando la expresión espacial y temporal de genes involucrados en la división celular, elongación y diferenciación. El potencial de cepas bacterianas que alteran los niveles de los reguladores del crecimiento está aún inexplorado y podría representar una alternativa para formular bioinoculantes para la aplicación en cultivos. En su conjunto, los resultados derivados de este trabajo muestran el papel dual de las AHLs y los CDPs bacterianos en la modulación de la arquitectura de la raíz, indicando la existencia de una regulación altamente sofisticada para la comunicación entre organismos de diferentes reinos. Ambas clases de compuestos incluyen moléculas de amplio espectro con actividad tanto en procariotas como eucariotas. En particular, la vasta producción de CDPs en *P. putida* y *P. fluorescens* podría estar asociada con las propiedades de estimulación del crecimiento vegetal, lo cual abre la posibilidad de aplicaciones adicionales en la biotecnología agrícola, la modulación de la arquitectura radicular y su impacto en la captación de agua y nutrientes minerales.

9. REFERENCIAS

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10. ADDENDA.

En este apartado se presentan las publicaciones derivadas de la colaboración con el grupo de trabajo del laboratorio durante el desarrollo de la tesis.

I. Artículos publicados con arbitraje internacional

1. **Ortiz-Castro R.**, Contreras-Cornejo H.A., Macías-Rodríguez L., López-Bucio J. (2009). The role of microbial signals in plant growth and development. *Plant Signaling and Behavior* 4: 1-12.
2. Morquecho-Contreras A., Méndez-Bravo A., Pelagio-Flores R., Raya-González J., **Ortiz-Castro R.,** López-Bucio J. (2010). Characterization of *drr1*, an alkamide resistant mutant of Arabidopsis, reveals an important role for small lipid amides in lateral root development and plant senescence. *Plant Physiology* 152:1659-1673.
3. Pelagio-Flores R., **Ortiz-Castro R**., Méndez-Bravo A., Macías-Rodríguez L., López-Bucio J. (2011). Serotonin, a tryptophan-derived signal conserved in plants and animals, regulates root system architecture probably acting as a natural auxin inhibitor in *Arabidopsis thaliana*. *Plant Cell Physiology* 52:490-508.
4. Pelagio-Flores R., Muñoz-Parra E., **Ortiz-Castro R**., López-Bucio J. (2012). Melatonin regulates Arabidopsis root system architecture likely acting independently of auxin signaling. *Journal of Pineal Research* 53:279-288.
5. Pelagio-Flores R., **Ortiz-Castro R**., López-Bucio J. (2013). *dhm1*, an Arabidopsis mutant with increased sensitivity to alkamides shows tumorours shoot development and enhanced lateral root formation. *Plant Molecular Biology* 81:609-625.

II. Capítulos de libros publicados

1. **Ortiz-Castro R.,** Méndez-Bravo A., López-Bucio J. (2010). Amino compound containing lipids: A novel class of signal regulating plant development. *Plant developmental biology-biotechnological perspectives.* Eng-Chong P., Davey M. (Eds). Volume 2. Springer, Germany. pp. 209-226.

2. **Ortiz-Castro R.,** López-Bucio J. (2010). La arquitectura de las plantas. *Fronteras en la biología del desarrollo de las plantas.* Beltrán-Peña E., López-Bucio J. (Eds). Universidad Michoacana de San Nicolás de Hidalgo. pp. 1-14.
3. Contreras-Cornejo HA., **Ortiz-Castro R.,** López-Bucio J. (2013). Promotion of plant growth and the induction of systemic defence by Trichoderma: Physiology, genetics and gene expression. *Trichoderma – Biology and Applications*. Mukherjee PK., Horwitz BA., Singh US., Mukherjee M., Scmoll M. (Eds). CAB International. pp. 175-196.

III. Artículos publicados con arbitraje nacional

1. **Ortiz-Castro R.,** Valencia-Cantero E., López-Bucio J. (2009). Participación de las citocininas en la estimulación del crecimiento vegetal por *Bacillus megaterium*. *Biológicas* 11:64-72.
2. Martínez-Trujillo M., Santíz-Gómez M., **Ortiz-Castro R.,** Carreón-Abud Y. (2009). Efecto del cobre en el crecimiento y la arquitectura de la raíz de *Arabidopsis thaliana*. *Biológicas* 11:122-131.
3. **Ortiz-Castro R**., Campos-García J., López-Bucio J. (2012). Comunicación planta bacteria basada en ciclodipéptidos de origen microbiano con actividad auxínica. *Ciencia Nicolaita* 56:59-74.
4. Barrera-Ortiz S., **Ortiz-Castro R**., López-Bucio J. Beltran-Peña E. (2012). Bases moleculares de la señalización del ácido abscísico y etileno en plantas. *Ciencia Nicolaita* 56:20-34.

IV. Artículos de divulgación

1. **Ortiz-Castro R**., López-Bucio J. (2010). Alcamidas: alternativa en la producción agrícola. *Investigación y Desarrollo. Suplemento La Jornada*. Marzo de 2010. Número 267. Año XVIII.
2. **Ortíz-Castro R**., López-Bucio J (2010). Las alcamidas: un grupo nuevo de reguladores del crecimiento y sus aplicaciones en la biotecnología agrícola. *Claridades Agropecuarias*. 205:36-42.

V. Patentes

1. Ortiz-Castro Randy, López-Bucio José, Campos-García Jesús**.** En esta patente se describe la propiedad auxínica de los ciclodipeptidos producidos por la *Pseudomonas aeruginosa* y su potencial aplicación en el área de los bioinoculantes para su aplicación en el campo agrícola. Actualmente la patente se encuentra en la aprobación del examen de forma ante el Instituto Mexicano de la Propiedad Industrial (IMPI).

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Review

The role of microbial signals in plant growth and development

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Plant growth and development involves a tight coordination of the spatial and temporal organization of cell division, cell expansion and cell differentiation. Orchestration of these events requires the exchange of signaling molecules between the root and shoot, which can be affected by both biotic and abiotic factors. The interactions that occur between plants and their associated microorganisms have long been of interest, as knowledge of these processes could lead to the development of novel agricultural applications. Plants produce a wide range of organic compounds including sugars, organic acids and vitamins, which can be used as nutrients or signals by microbial populations. On the other hand, microorganisms release phytohormones, small molecules or volatile compounds, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis. In this review, we focus on recent developments in the identification of signals from free-living bacteria and fungi that interact with plants in a beneficial way. Evidence has accumulated indicating that classic plant signals such as auxins and cytokinins can be produced by microorganisms to efficiently colonize the root and modulate root system architecture. Other classes of signals, including *N*-acyl-L-homoserine lactones, which are used by bacteria for cell-to-cell communication, can be perceived by plants to modulate gene expression, metabolism and growth. Finally, we discuss the role played by volatile organic compounds released by certain plant growth-promoting rhizobacteria in plant immunity and developmental processes. The picture that emerges is one in which plants and microbes communicate themselves through transkingdom signaling systems involving classic and novel signals.

Introduction

Plants are sessile, multicellular organisms, which rely on developmental and metabolic changes for growth. At least three well defined parts can be recognized in the developing plant, (1) the root, the below-ground part of the plant, which provides anchorage and plays an important role in water and nutrient uptake from the soil, (2) the stem, which performs essential functions as a supporting structure for the leaves and as a conduit for water and nutrients moving from one part of the plant to another, and (3) the shoot, which produces leaves, flowers and fruits that enables efficient light capture and provides a means for reproduction and seed dispersal. The anatomical configuration of the root, stem and shoot systems to build a plant is known as plant architecture. Plant architecture is a crucial factor in the agronomic success of crops and is a vital consideration for plant breeders.[1,2]

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The general developmental pattern in plants depends on indeterminate growth and iterative organogenesis, characterized by continued cell division in the meristematic regions. The shoot apical meristem represents a source of undifferentiated cells that divide and contribute to the new leaf primordia during vegetative growth, and to inflorescence and floral meristems during the reproductive phase of the life cycle. At the other end of the plant, the root apical meristem provides new cells, which add to the pre-existing files extending back into the mature root.[3,4] Behind the root and shoot meristems, the newly added cells expand before fully differentiating, in the maturation zone, into the varied cell types that build the plant.[5]

The root system displays considerable plasticity in its morphology and physiology in response to variability within its environment. By contrast to the primary root that is formed during embryogenesis, lateral and adventitious roots are formed postembryonically. Lateral roots are formed typically from the pericycle, as a consequence of the activation of the cell cycle in specific groups of so-called 'founder cells', which undergo a series of periclinal and anticlinal divisions to generate a meristem de novo.[6] Activation of pericycle cells for lateral root initiation might take place in the basal meristem and correlated with elevated auxin sensitivity in this part of the root.[6,7] Once the developing lateral root primordium has formed its own meristem, this mew meristem produces cells that expand and push the new root tip through the ground and epidermal layers to the outside.[8-10]

Extensive communication occurs between plants and microorganisms during different stages of plant development in which signaling molecules from the two partners play an important role. Fungal and bacterial species are able to detect the plant host and initiate their colonization strategies in the rhizosphere by

producing canonical plant growth-regulating substances such as auxins or cytokinins. On the other hand, plants are able to recognize microbe-derived compounds and adjust their defense and growth responses according to the type of microorganism encountered. This molecular dialogue will determine the final outcome of the relationship, ranging from pathogenesis to symbiosis, usually through highly coordinated cellular proceses.[11] Bacterial and fungal phytopathogens are not restricted to infecting aerial or root tissues exclusively, as such, communication between the shoot and root can confer a survival advantage to the plant and potentially limit or prevent diseases. For instance, beneficial soil bacteria and fungi can confer immunity against a wide range of foliar diseases by activating plant defenses, thereby reducing a plant's susceptibility to pathogen attack.[12] For many years, this was considered the basis by which beneficial microorganisms could increase plant yield when inoculated in crops, however, it is increasingly appreciated that classic and novel microbial signals may also directly participate on plant morphogenesis.

Signaling during plant-pathogen interactions has been a central topic in phytopathology for many years, whereas more recent efforts are being made to discover the signals involved in plant communication with non pathogenic microbes, especially those that enhance plant productivity. Among the latter, nitrogen-fixing bacteria belonging to Rhizobium, as well as mycorrhizal fungi, which are able to establish endosymbiosis with plants, have already been extensively reviewed[13-17] and will therefore not be further discussed here. Our aim is rather to discuss recent findings about the signals involved in the interaction of plants with free-living, beneficial microbes including filamentous fungi of the genus Trichoderma and plant growth promoting rhizobacteria (PGPR), for which important recent discoveries have been made on their chemical communication, the biological processes they sustain and the benefits to plants involved in these interactions.

Plants: Contribution to Microbes

Diverse compounds released by different parts of the root system create a unique environment in the surrounding soil, which is known as the rhizosphere. These compounds are collectively termed as root exudates and belong to three main classes: (1) Low-molecular weigth, (2) High-molecular weigth and (3) Volatile organic compounds (VOCs). Low-molecular weigth compounds represent the main portion of exudates and consist of sugars, amino acids, organic acids, phenolics, vitamins and various secondary metabolites. High-molecular weigth compounds consist of mucilage and proteins, while carbon dioxide, certain secondary metabolites, alcohols and aldehydes constitute volatiles.[18,19] Different plant species contain many common constituents of each of these categories but may vary in their amounts and time of release by the root. Several factors such as temperature, light, age and soil type can affect the nature and timing of exudation.[19-21] In plants grown under low phosphate availability or in the presence of toxic concentration of aluminum, the exudation of organic acids such as oxalic acid, malic acid and citric acid is particularly increased.[22-24] These compounds may act as signals for microbial attraction such as malate (see below), or be used as carbon sources for microbial nutrition.

The physical, biochemical and ecologic characteristics of the rhizosphere are defined by the balance between different compounds released, timing of release and any unique substances that are produced constitutively or in an inducible manner. It is estimated that between 20 to 40% of all photosynthetically fixed carbon is eventually transferred to the rhizosphere. This high cost may be borne by the plant owing to the significant influence the rhizosphere exerts on plant health by affecting proceses such as nutrient and water uptake and establishment of beneficial interactions with soil microbial populations.[11,19]

The types of microorganisms within a rhizosphere include bacteria, fungi, actinomycetes and algae. Microbial populations react to the exudates released by plant roots, their numbers can vary by as much as 10–100-fold in the rhizosphere from those found in the soil.[25] Microorganisms and their products also affect the roots in a variety of positive, negative and neutral ways.[26,27] The rhizosphere is therefore a dynamic system in which interactions and communication between the root and microorganisms play an important role in continuing to maintain plant growth and productivity. Managing the rhizosphere may represent an important area for biotechnology improvement with the aim of boosting the intrinsic yield and biomass production with a minimum input of water, fertilizers and agrochemicals. This can be achieved by inoculating rhizospheres with selected beneficial microorganisms or by engineering plants to modify the nature and level of exudate compounds (Fig. 1).

The role of root exudates as signaling molecules has been recently addressed by Rudrappa and associates, who showed that root-secreted malic acid recruits the beneficial soil bacteria *Bacillus subtilis* to the root and this interaction plays a role in plant protection against the foliar pathogen *Pseudomonas syringae*.[28] Similarly, in tobacco and alfalfa plants genetically engineered to overproduce citric or malic acid, an increased colonization by mycorrhizal fungi and rhizobacteria have been reported, which highlights the role played by organic acids in plant-microbe interactions.[29,30] These results also suggest that manipulation of organic acid biosynthesis and exudation in transgenic plants may represent an attractive technology to modify the rhizosphere with potential novel applications in agriculture.

Elicitors are molecules involved in plant defense responses, many of them are directly derived from beneficial or pathogenic microbes.[31] Roots of elicited plants exude an array of compounds not detected in the exudates of non-elicited plants. Exogenous application of defence signalling molecules, such as salicylic acid, methyl jasmonate and nitric oxide induces the accumulation of a wide range of secondary metabolites including indole glucosinolates, phytoalexins and alkamides, which may play a role in communication with microbial populations.[32-34] The demonstration that roots selectively secrete organic compounds to effectively signal bacteria and fungi establishes a regulatory role of plant metabolites in recruitment of beneficial microbes. In addition, the effects of elicitors and other microbe-derived molecules on root exudation underscore the breadth and sophistication of plant microbe interactions.

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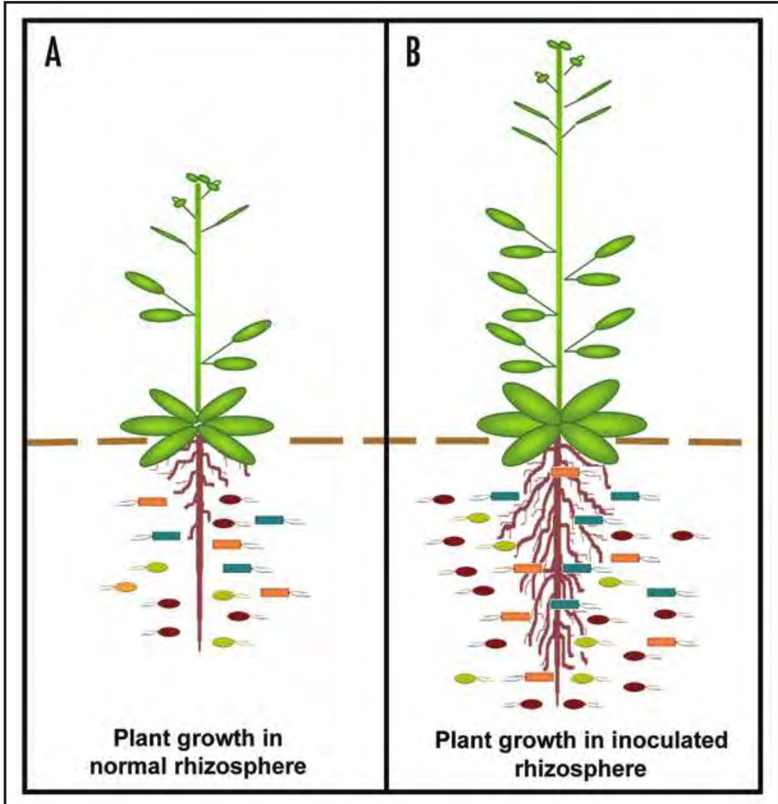

Figure 1. Rhizosphere modification improves plant productivity. Inoculation with plant beneficial microorganisms offers many advantages to crops, including enhanced rooting, activation of immunity and improved plant yield.

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Microbes: Contribution to Plants

The release of carbon compounds from plants into the rhizosphere increases microbial biomass and activity. Free-living microbes including filamentous fungi of the genus Trichoderma and a variety of plant growth-promoting rhizobacteria (PGPR) are able to suppress soil-borne plant pathogens and to stimulate plant growth by different direct or indirect mechanisms, such as production of phytohormones, mycoparasitism and competence with plant pathogens, decomposition and mineralization of organic mater and enhancing the bioavailability of mineral nutrients such as phosphorus and iron.[35] Furthermore, microorganisms may also contribute to plant immunity by producing elicitor molecules. In the course of their life, plants are exposed to many potential pathogens. To counter these attacks, they have evolved a large set of defence responses. These defences include pre-existing physical and chemical barriers, as well as inducible responses that are activated after pathogen perception.[36] This recognition step can be achieved by the means of molecules common to many classes of microbes known as microbe-associated molecular patterns (MAMPs).[31] MAMPs, also known as general elicitors in plants are involved in non-specific immunity and the associated resistance is effective against a broad range of pathogens.[37] MAMPs belong to different families including proteins, glycans and lipids. Certain beneficial bacteria produce rhamnolipids, which are novel MAMPs conferring resistance to pathogenic fungi in grapevine.[38]

Although not directly economically important, the model plant *Arabidopsis thaliana* offers a number of experimental advantages over crop species, including its small size, short life cycle, and the suitability to be grown under axenic conditions. The adoption of this model in plant-microbe interactions research has increased our knowledge about the molecular and physiological mechanisms by which microbial inoculation modulates growth and development (Fig. 2).

Next, we discuss more in depth the molecular mechanisms involved in plant growth promotion by two classes of beneficial microorganisms, namely Trichoderma fungi and plant growth promoting rhizobacteria as a means to show the importance of free-living microbes that proliferate in close proximity to plant roots.

Free-Living Beneficial Fungi

Fungi have the advantage over bacterial inoculants in that they are generally more effective at spreading through the soil and rhizosphere. The mechanisms involved in plant growth promotion by fungi are competition with fungal pathogens, antibiotic production and elicitation of defence responses.[39] In addition, many plant beneficial fungi are able to parasitize spores, sclerotia or hyphae of pathogenic fungi, resulting in biocontrol. Mycoparasitism is initiated by host sensing, which is generally followed by direct growth towards it, recognition, penetration and degradation. Production of a number of degradative enzymes, including chitinases, proteases and glucanases is involved in the biocontrol process.[39]

Trichoderma species belong to a class of free-living fungi beneficial to plants that are common in the rhizosphere. In addition to their mycoparasitic capabilities, many Trichoderma strains are able to colonize and grow in association with plant roots and significantly increase plant growth and development. Colonization by Trichoderma very rarely is detrimental to the plant or results in a pathogenic interaction.[39] In contrast, root colonization by Trichoderma frequently is associated with induction of both local and systemic resistanse, which depend on the production of a protein elicitor by the fungus designated *Sm1* (*small protein 1*). *Sm1* lacks toxic activity to plants and microbes. Instead, native, purified *Sm1*, triggers production of reactive oxygen species in rice and cotton seedlings, and induces the expression of defence-related genes both locally and systemically.[40] The beneficial effects of Trichoderma on plant growth and development may also dependent on more direct mechanisms as a recent report has shown that certain species including *T. virens* and *T. atroviride* can produce indole-3-acetic acid (IAA) and other auxin-related compounds. In Arabidopsis, normal auxin perception is a prerequisite for growth enhancement when inoculated with *T. virens*.[41]

Plant Growth-Promoting Rhizobacteria (PGPR)

PGPR are natural rhizosphere-inhabiting bacteria, which belong to diverse genera such as Pseudomonas and Bacillus species. These microorganisms have been isolated from a wide variety of wild and

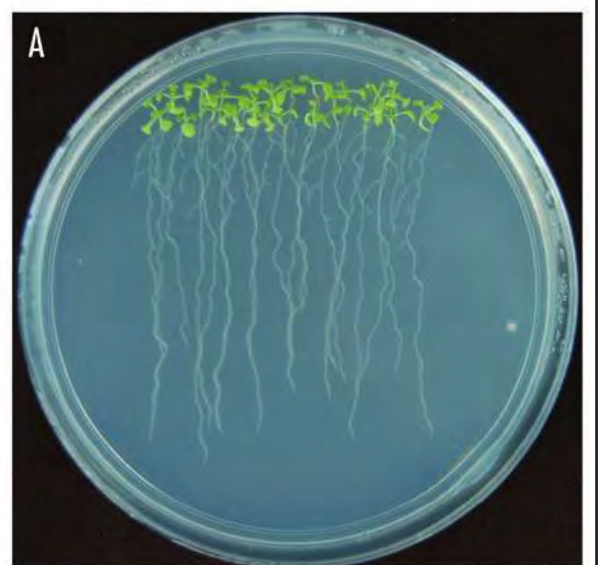

Figure 2. Use of *Arabidopsis thaliana* for research in plant-microbe interactions. Representative photographs showing uninoculated Arabidopsis seedlings grown in a 0.2x Murashige and Skoog medium (A), inoculated with *Trichoderma atroviride* (B), or with *Bacillus megaterium* (C). Note the elicitation of shoot growth by both the fungus and bacterium and the formation of branched root systems.

cultivated plant species such as Arabidopsis, barley, rice, canola and bean.[42] PGPR are used as inoculants for biofertilization, phytoestimulation and biocontrol. The general effect of PGPR is an increased growth and productivity of plants. Their contribution can be exerted through different mechanisms including root system architecture modulation and increased shoot growth by production of phytohormones such as auxins and cytokinins. Other indirect mechanisms include the effects of products such as antibiotics and hydrogen cyanide, which promote plant growth by inhibiting the growth of deleterious microorganisms in the rhizosphere. PGPR can induce defence programs such as systemic acquired resistance and induced systemic resistance, thus reducing phytotoxic microbial communities. They also can elicit induced systemic tolerance (IST) to abiotic stress.[43-45]

Pseudomonas comprises a genus of ubiquitous Gram-negative bacteria that can live in several environmental niches such as the rhizosphere. Although a few Pseudomonas are studied for their roles as plant pathogens (i.e., *Pseudomonas syringae*), there are many species such as *P. fluorescens*, *P. putida*, *P. aeaureofasciens* and *P. chloraphis*, which may act as plant beneficial bacteria by antagonizing plant pathogens and through the production of traits that directly influence plant disease resistance and growth.[46] *Pseudomonas fluorescens* strain FII3 was isolated from the roots hairs of sugar beet and found to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG). The growth of plant pathogenic fungi *Pythium ultimum*, *Phoma beta*, *Rhizopus stolonifera* and *Fusarium oxisporum* were found to be inhibited by strain F113 in vitro due to the production of DAPG.[47] This strain was also found to protect sugar beet seedlings from damping-off in soil infested by Pythium.[48] Pseudomonas strains, therefore, have considerable potential as biocontrol agents.

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Recent information indicates that different Pseudomonas may also regulate plant development by production of autoinducer signals, namely *N*-acyl-L-homoserine lactones.[49] Free-living bacteria belonging to the Azospirrillum and Bacillus genuses can also promote growth of plants by production of auxins or cytokinins.

Microbial Signals Involved in Plant Growth and Development

A wide range of microorganisms found in the rhizhosphere are able to produce substances that regulate plant growth and development. Bacterial and fungal production of phytohormones such as auxins and cytokinins can affect cell proliferation in the shoot leading to tumorous growth as in the case of *Agrobacterium tumefaciens* or *Ustilago maydis* infection, or modify root system architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake. Therefore, the balance between auxin-to-cytokinin and the site of hormone

accumulation in the plant may determine whether a microbial interaction may be beneficial or detrimental. In the last five years, additional signals form microbes have been found to play a role in plant morphogenetic processes, including the above mentioned *N*-acyl-L-homoserine lactones (AHLs) and volatile organic compounds (VOCs). AHLs belong to a class of bacterial quorum-sensing signals from Gram negative bacteria such as Pseudomonas. These compounds enable bacterial cells to regulate gene expression depending on population density. Very recently, it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots and modulate defence and cell growth responses. In a similar way, bacterial volatiles such as acetoin and 2,3-butanediol produced by certain PGPR can be used for plant-bacteria communication, and as a plant growth promotion triggers.

Auxins and Cytokinins

Auxins and cytokinins interact in the control of many important developmental processes in plants, particularly in apical dominance, and root and shoot development. The balance between auxin and cytokinin is a key regulator of in vitro organogenesis. Exposing callus cultures to a high auxin-to-cytokinin ratio results in root formation, whereas a low ratio of these hormones promotes shoot development. Many experiments have demonstrated the existence of synergistic, antagonistic or additive interactions between auxins and cytokinins, suggesting complex signal interactions involved in the modulation of root and shoot architecture.[50,51]

Although both auxins and cytokinins can be produced in roots and shoots, the production of these signals does not occur randomly but is regulated by the location of the producing tissues, the developmental stage of the plant and environmental gowth conditions such as light and temperature. Young shoot organs such as the first true leaves and developing primary and lateral roots are important sites of IAA production.[52-54] This can be visualized in transgenic Arabidopsis seedlings expressing the auxin-response marker *DR5:GUS*,[55] while the root cap is the major site of cytokinin synthesis.[56] From the tissues involved in hormone production, the signals move through specific transport systems and different mechanisms to regulate plant growth and development, IAA can be transported from the shoot to the root through the vascular tissue. In addition to long distance auxin transport, local transport of IAA along and across tissues is important for auxin localization in small groups of cells, for example in an emerging lateral root primordium or in the root cap during gravitropism. This can be achieved by the action of specific influx and efflux transporter systems. Auxin importers include members of the amino acid permease family *AUX1* (*auxin resistant 1*), *LAX* (*like-aux1*) and *PGP4*, this later belong to the *MDR/PGP* (*multidrug resistanse/P-glycoprotein*).[57-59] Auxin is exported by transporters of the *PIN* (*pin-formed*) and *PGP* families, including *PIN1* to *PIN7*, *PGP1* and *PGP19* in Arabidopsis.[60,61] Conversely, the root cap produces cytokinins, which appear to regulate primary root growth and gravitropism.[56] Free bioactive cytokinins can be visualized by the expression of *ARR5:GUS*, which consists of the citokinin-activated promoter of the response regulator *ARR5* fused to the β-glucoronidase reporter gene.[62] Transgenic Arabidopsis seedlings expressing this construct react to cytokinins in a concentration dependent manner.[62] The use of *ARR5:GUS* expressing seedlings shows that the cap of the primary root produces elevated concentrations of free bioactive cytokinins. Root synthesized cytokinins in Arabidopsis is distributed to the shoot by the transpiration stream.[63]

Auxins

The architecture of the root system is modified by the endogenous auxin level and by environmental stimuli such as the availability of water and mineral nutrients.[64-66] Therefore, the beneficial activities of rhizosphere microorganisms can be related to the production of auxins or to the solubilization of nutrients as well, which may affect the initiation of lateral roots, the growth of lateral roots or both developmental processes, giving rise to root systems with increased exploratory capacity.

Diverse bacterial species produce auxins as part of their metabolism including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or their precursors.[67,68] Evidences indicating that IAA is a positive regulator of plant growth comes from the analysis of mutants that overproduce it, such as *super root* and *yucca*, which have long hypocotyls and increased numbers of lateral roots and root hairs,[69,70] and the positive effect of IAA application on growth of excised stems and hypocotyls and of auxin analogs in intact Arabidopsis seedlings.[71] Plant growth-promoting activity of certain microorganisms has been related to the production of auxins. Loper and Schroth (1986) found that 12 of 14 PGPR isolates produced IAA in culture filtrates and there was a significant relationship between IAA production, decreased root elongation, and increased shoot-to-root ratios in sugar beet seedlings.[72] A positive correlation between auxin production and growth-promoting activity of diverse PGPR has been also reported in *Brassica juncea* and wheat.[73,74] Auxins are quantitatively the most abundant phytohormones secreted by Azospirillum species, and it is generally agreed that auxin production is the major factor responsible for the stimulation of root system development and growth promotion by this bacterium.[68]

Many fungal species also produce auxins. Recent findings about the role of fungal-produced IAA in different plant-fungus interacting systems open the possibility that fungi may use IAA and related compounds to interact with plants as part of its colonization strategy, eading tol plant growth stimulation and modification of basal plant defense mechanisms.[41,75] In maize (*Zea mays*) and *Arabidopsis thaliana*, Trichoderma inoculation affected root system architecture, which was related to increased yield of plants. Reported developmental effects include increased lateral root formation and root hair growth.[41,76,77]

The signaling mechanisms by which *Trichoderma virens* promote growth and development were further investigated in *Arabidopsis thaliana* by Contreras-Cornejo and associates (2009). It was found that mutations in genes involved in auxin transport or signaling, *AUX1*, *BIG*, *EIR1* and *AXR1*, reduced the growth-promoting and root developmental effects of Trichoderma inoculation.[41] When grown under axenic conditions *T. virens* produced IAA and the IAA-related substances indole-3-acetaldehyde (IAAld)

and indole-3-ethanol (IEt). Interestingly, application of all three compounds to Arabidopsis seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (mM range). These results indicate that the effects of inoculation with auxin-producing fungi in plants under natural conditions may depend on the type and concentration of auxins produced by the fungi.

Cytokinins

Cytokinins are purine derivatives that promote and maintain plant cell division in cultures and are also involved in various differentiation processes including shoot formation, primary root growth and callus formation. Plants continuously use cytokinins to maintain the pools of totipotent stem cells in their shoot and root meristems.[78,79] Endogenous cytokinin overproduction in transgenic plants causes pleiotropic phenotypic alterations including cytokinin-auxotrophic growth of calli in vitro.[78] Analysis of cytokinin-overproducing and cytokinin-deficient mutants has confirmed a stimulatory role for these compounds in the regulation of cell division activity in the shoot meristem and young leaves.[80-82] Recent data indicate regulatory interactions between cytokinins and alkamides, these later compounds belong to a novel class of plant signals reported to affect both shoot and root system architecture in plants.[83]

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The positive effect of cytokinins on growth at the whole plant level has been demonstrated by the identification of genes involved in cytokinin perception and signaling. Three sensor histidine kinases, *CRE1/AHK4/WOL*, *AHK2*, *AHK3* have been shown to act as cytokinin receptors.[84] These receptors activate the expression of several response regulators in a cytokinin-dependent manner.[85,86] Further downstream, cytokinin signaling stimulates the G_1/S transition of the cell cycle, which has been proposed to be mediated by the transcriptional induction of the *CYCD3* gene that encodes a D-type cyclin.[87] The cytokinin receptors play redundant functions in transducing the signal to downstream factors. When grown on soil, none of the single cytokinin receptor mutants of Arabidopsis (*cre1-12*, *ahk2-2*, *ahk3-3*) exhibited significant defective phenotype. However, the *ahk2-2 ahk3-3* double mutants had smaller leaves and shorter stems than did the wild-type plants. All single and double mutants produced apparently normal flowers that yielded viable seeds. Interestingly, the *cre1-12 ahk2-2 ahk3-3* triple mutants showed a dwarf phenotype with reduced root and shoot growth and smaller meristems. These mutants also produced inflorescences with nonfunctional flowers, which failed to produce seeds.[83,88] These data suggest that cytokinin receptors are important for plant viability and normal growth.

Cytokinins can be produced by microorganisms. Their production by PGPR has been well documented and correlated with increased growth of plants.[89-91] Until recently, little was known on the genetic basis and signal transduction components that mediate the beneficial effects of these PGPR. A recent report has provided important information on the role played by cytokinin receptors in plant growth promotion by *Bacillus megaterium* rhizobacteria. *B. megaterium* UMCV1 strain was initially isolated from the rhizosphere of bean (*Phaseolus vulgaris* L.) plants. Inoculation with this bacterium was found to promote biomass production of *Arabidopsis thaliana* and bean plants in vitro and *in soil*.[44] This effect was related to altered root system architecture in inoculated plants, with an inhibition in primary root growth followed by an increase in lateral root formation and root hair length (Fig. 2). The effects of bacterial inoculation on plant growth and development were found to be independent of auxin- and ethylene-signaling as revealed by normal responses of auxin resistant mutants *aux1-7*, *axr4-1* and *eir1* and ethylene-response mutants *etr1* and *ein2*, and the failure to activate the expression of auxin-reporter markers.[44]

The involvement of cytokinin signaling in mediating plant growth promotion by *Bacillus megaterium* in plants was further investigated using *A. thaliana* mutants lacking one, two or three of the cytokinin receptors and *RPN12*, a gene involved in cytokinin signaling acting downstream of the receptors. It was found that growth promotion was reduced in *AHK2-2* single and double mutant combinations and in *RPN12*. Furthermore, growth promotion and lateral root induction was completely abolished in the *cre1-12 ahk2-2 ahk3-3* triple mutant, indicating the importance of cytokinin perception in the plant's response to *B. megaterium*.[92]

It is expected that in the coming years novel molecular components involved in the interaction between plants and PGPR will be discovered with the continued use of Arabidopsis as a model system. It is also expected that the knowledge gained on the role of auxin and cytokinin in plant-microbe interactions will open new avenues to use microbial strains with a capability to produce these phytohormones for plant improvement under controlled and field conditions.

N-acyl-L-homoserine Lactones

Many bacteria regulate diverse cellular processes in concert with their population size—a process commonly referred to as quorum-sensing (QS).[93] Bacterial cell-to-cell communication utilizes small diffusible signals, which the bacteria both produce and perceive. The bacteria couple gene expression to population density by eliciting a response only when the signaling reaches a critical threshold. The population as a whole is thus able to modify its behavior as a single unit. In Gram-negative bacteria, the quorum-sensing signals most commonly used are *N*-acyl-L-homoserine lactones (AHLs). It is now apparent that AHLs are used for regulating diverse behaviors in rhizosphere inhabiting bacteria and that plants may produce their own metabolites, which may interfere with quorum-sensing signaling.

AHLs are composed of a homoserine lactone residue linked to an acyl-side chain. The specificity derives from the length of the acyl chain (4–18 carbon atoms), substitution at the C3 position and saturation level within the acyl chain.[94-96] AHLs can be broadly classified as long, medium or short-chained depending on whether their acyl moiety consists of more than eight, between eight-to-twelve or less than twelve carbon atoms, respectively.[49,97] These molecules are freely diffused through the bacterial membrane and distribute within the rhizosphere.[98-100]

Examples of AHL-regulated gene systems are diverse and include virulence, biolumiscense, sporulation, swarming,

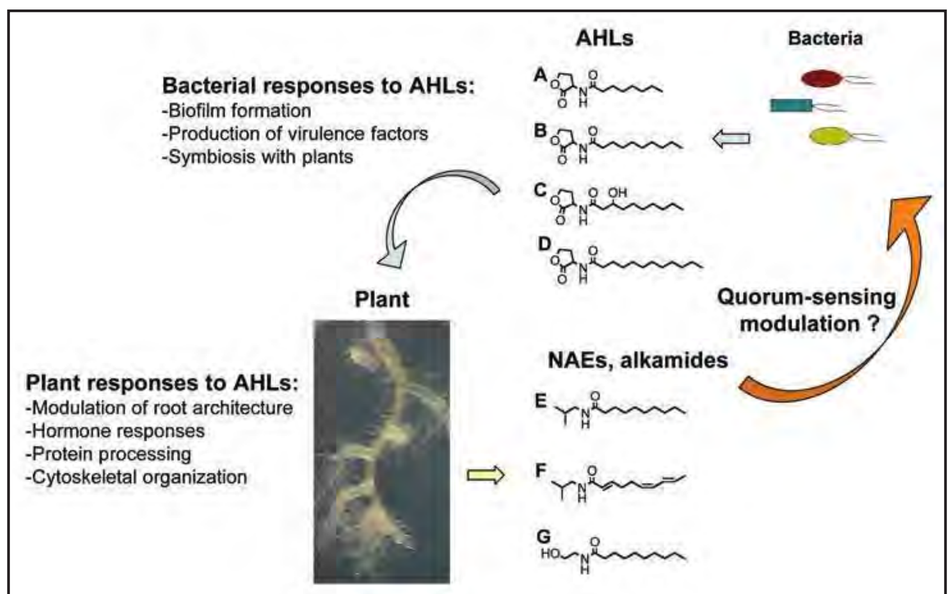

Figure 3. Communication beteen plants and bacteria mediated by AHLs and plant-produced signals. (A) *N*-octanoyl-homoserine lactone (C8-HL), (B) *N*-decanoyl-homoserine lactone (C10-HL), (C) *N*-3-oxo-decanoyl-homoserine lactone, (D) *N*-dodecanoyl-homoserine lactone (C12-HL), (E) *N*-isobutil decanamide, (F) *N*-isobutyl-*2E,6Z,8E*-decatrienamide (Affinin), (G) *N*-ethanol decanamide (NAE 10:0). The bacteria produce AHLs, which modulate root system architecture and the plant produces NAEs and alkamides, which may interfere with bacterial quorum-sensing.

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siderophore production, antibiotic biosynthesis and plasmid conjugal transfer.[101,102] AHLs orchestrate important processes of many beneficial rhizosphere colonizing bacteria. For example: deletion of the gene *pco1* responsible for the production of the AHLs 3-oxo-C6-HL and 3-oxo-C8-HL in *Pseudomonas fluorescens* 2P24 caused the mutant to be defective in biofilm formation, colonization of wheat rhizosphere and biocontrol ability against wheat take-all, while complementation of *pco1* restored the biocontrol activity to the wild-type level.[103] While bacteria make use of AHLs for signaling, until recently it was unknown if plants functionally respond to these same signals. The first work showing that plants are able to perceive AHLs was published by Mathesius and Coworkers (2003), they found that in *Medicago truncatula* plants grown axenically, application of nanomolar concentrations of two different AHL types, 3-oxo-C12-HL and 3-oxo-C16:1-HL caused significant changes in the accumulation of over 150 proteins.[104] These proteins were found to have functions in plant defence, stress response, energetic and metabolic activities, transcriptional regulation, protein processing, cytoskeletal activities and hormone responses.[104] These results were further confirmed by microarray expression analysis in *Arabidopsis thaliana*.[105]

The presence of AHL-producing bacteria in the rhizosphere of tomato induced the salicylic acid and ethylene-dependent defence responses, which play an important role in the activation of systemic resistance in plants and conferred protection against the fungal pathogen *Alternaria alternata*.[100] Furthermore, AHLs were found to be taken up by plants in a process dependent on the length of the acyl chain.[105,106] Application of a homoserine lactone, a breakdown product of AHL by means of soil bacteria, to bean roots leads to an increase in stomatal conductance and transpiration in shoots. This in turn is beneficial for both the plant and the bacteria through an increase in mineral nutrient uptake.[107]

Plants produce substances that mimic AHLs, at least 10 chromatographically separable active compounds can be detected in root exudates of *Medicago truncatula*.[108] These plant compounds can affect QS responses in bacteria indicating that plants produce AHL signal mimics.[108,109] Some of these signals may belong to the *N*-acylethanolamines (NAEs) and alkamide groups, which are structurally similar to AHLs. NAEs are naturally produced by plants and accumulate in desiccated seeds. Their synthesis can also be stimulated in leaves in response to elicitor treatment.[110] Alkamides are present in at least 20 plant families and preferentially accumulate in certain medicinal plants such as *Echinacea angustifolia* en *Heliopsis longipes*.[111,112] Exogenous application of NAEs and alkamides to Arabidopsis seedlings were found to alter root system architecture and shoot development in a dose-dependent way, indicating that these signals may play a role in plant morphogenetic processes.[83,113-115] Thus, the possibility is open the NAEs and alkamides can be used by plants to interfere with bacterial QS (Fig. 3).

In two recent reports, the root developmental responses of Arabidopsis seedlings to exogenous application of AHLs were presented.[49,105] It was found that low micromolar concentrations of C4-HL and C6-HL increased growth of roots, while C10-HL decreased growth of roots and rosettes.[105] In this way, Ortíz-Castro and coworkers (2008) performed a detailed analysis of root architectural responses to a variety of AHLs ranging form 4 to 14 carbons. The compounds affected primary root growth, lateral root formation and root hair development in Arabidopsis seedlings. From the different AHLs evaluated, C10-HL was found to be the most active compound in altering root architecture. Developmental changes altered by C10-HL were related to changes in cell division, cell elongation and cell differentiation and its mechanism of action was found to be independent of auxin signaling.[49] Interestingly, mutant and overexpresor lines of Arabidopsis with altered levels of the enzyme fatty acid amide hydrolase, which play a role in NAE metabolism in plants,[116] sustained differential growth and developmental responses to C10-HL, indicating that this enzyme may play a role in AHL degradation under natural conditions.

Several plant species have been genetically transformed to express enzymes involved in AHL synthesis or degradation. Tobacco and potato plants expressing the *yenI* gene coding for the enzyme AHL synthase from *Yersinia enterocolitica*, responsible for short-chain AHL production as well as tobacco and tomato plants engineered

to produce the AHL synthase *LasI* from *Pseudomonas aeruginosa*, responsible for the synthesis of medium-chain AHLs have been obtained.[97,117-119] In these plants, the AHLs produced diffused freely across the plastid and plasma membranes and were released to the rhizosphere,[97] where they have the potential to affect bacterial processes regulated by such molecules.

The information above discussed suggests that organisms from different kingdoms, namely plants and bacteria, have acquired mechanisms to sense and respond to each other's signaling molecules. We further propose that in the rhizosphere, AHLs, NAEs and alkamides are excellent candidates for mediating this interkingdom communication. By increasing our understanding on this communication, it could then be possible to develop novel strategies to increase sustained plant production.

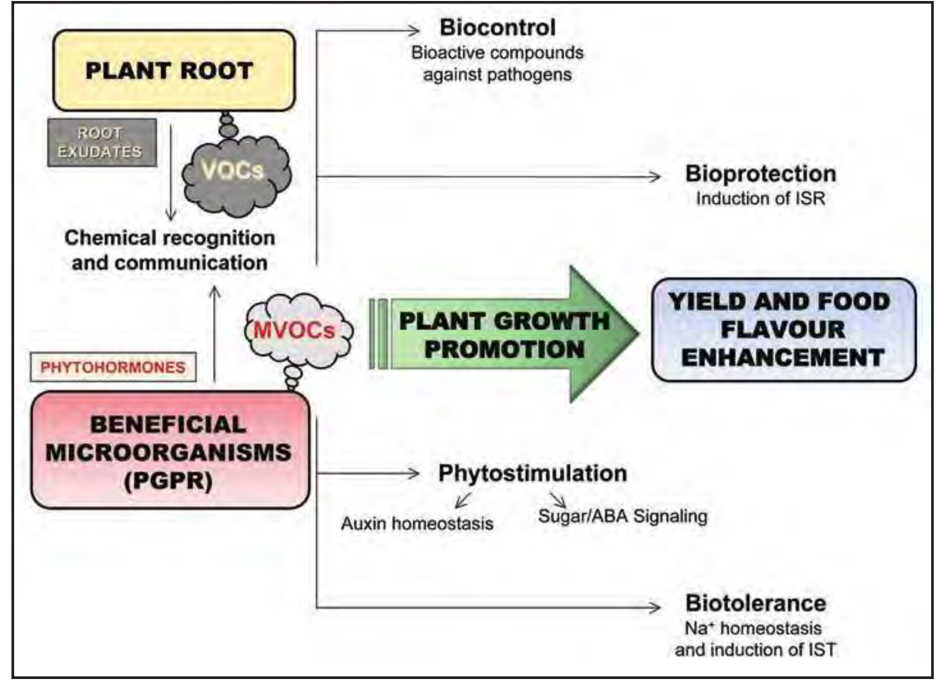

Figure 4. Mechanisms involved in volatile organic compound modulation of plant growth. Microorganisms produce VOCs, which can be sensed by plants to alter morphogenesis or activate defense and stress-related responses.

Volatile Organic Compounds

Volatile organic compounds (VOCs) are defined as compounds that have high enough vapor pressures under normal conditions to significantly vaporize and enter the atmosphere. This class of chemicals includes compounds of low molecular weight (<300 g/mol^{-1}), such as alcohols, aldehydes, ketones and hydrocarbons.[120,121]

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VOCs are efficient mediators for communication acting universally as attractants, repellents or warning signals in organisms from all kingdoms. Mammals can detect at least 10,000 different VOCs as odors from different sources, which act as matting signals, alert signals and sensory molecules. How the olfactory system can distinguish so many VOCs was a longstanding mystery until 1991, when Richard Axel and Linda Buck identified a gene family that encodes about 1,000 different types of olfactory receptors in the mouse, and a smaller number, about 350 in humans.[122] These findings provided important information on the mechanism of VOC perception, for which Axel and Buck were awarded the Nobel Prize in Physiology or Medicine in 2004.

It is increasingly appreciated that sensory experiences based on VOCs represent a beautifully orchestrated response to a wide range of stimuli not only in animals, but also in plants and microbes. The diversity, distribution and function of these compounds are starting to be revealed.[121] Choudhary and coworkers (2008) have shown that VOCs are actively produced and used as a sophisticated "language" by plants to pursue communication with other organisms.[123] In fact, due to the volatility properties of this kind of compounds it is tempting to speculate that roots emit volatiles to be sensed quickly and effectively by other organisms such as microbes in order to establish a communication. On this way, Steeghs and coworkers (2004) explored the Arabidopsis rhizosphere for VOC emission and their induction by biotic stresses.[124] They found alcohols, aldehydes, acids, ketones, esters and terpenes, which can be produced constitutively or induced specifically as a result of different positive or negative interactions with microorganisms.

Recent studies have been conducted in order to clarify the role of microbial VOCs during positive plant-microbe interactions. The ecological functions of bacterial volatiles are not understood in detail, but several functions such as communication, defence and plant growth-promotion have been suggested.[125] Available information indicates that the diversity of bacterial volatiles has a comparable complexity to that known for plants. Recent investigations using gas chromatography (GC) and mass spectrometry (MS) illustrate the splendid capacity of bacteria to produce a wealth of VOCs and the appearance of a characteristic volatile profile or compound is attributable to the specific metabolism or metabolic pathway(s) that are active in the bacteria, which can vary depending on the growth media and growth conditions; more information on this topic has been reported by Schulz and Dickschat, (2007), who made an excellent work in summarizing most known bacterial compounds reported to be released by bacteria, their occurrence and biosynthesis.[18]

In most of the mechanisms that PGPR use to interact with plants, VOC emission has a crucial participation (Fig. 4). The role of VOCs on antibiosis and the biocontrol of plant pathogens is the mechanism that has received most attention in the last decade, as the finding that certain volatiles having antifungal properties determine to a large extent the biocontrol performance of many rhizobacteria.[120,126-128] There are numerous reports showing that the production of volatiles produced by bacteria such as ammonia, butyrolactonas, HCN, phenazine-1-carboxylic acid, alcohols,

among others, may have activity in vivo in different fungal species.[126-128] The effects of these volatiles on fungi range from mycelium growth inhibition and promotion to the stimulation or reduction of sporulation. Therefore, volatiles can be used for communication between bacteria and their eukaryotic neighbours. Kai et al. (2009) discussed PGPR species that produce bioactive volatiles with activity in fungi.[125] It was shown that the volatiles from any one bacterial strain do not cause the same effect or the same degree of response in all fungi; rather the responses depend on the specific fungal-bacteria combination.[125]

The ability of PGPR VOCs to act as bioprotectants via induced systemic resistance has been demonstrated. ISR occurs when the plant's defence mechanism is stimulated and primed to resist infection by pathogens,[129] and recently it has been reported that PGPR volatiles may play a key role in this process.[129] For example, volatiles secreted by *Bacillus subtilis* GB03 and *B. amyloquefaciens* IN937a were able to activate an ISR pathway in Arabidopsis seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora*. The majority of bacteria that activate ISR appear to do so via a SA-independent pathway involving jasmonate and ethylene signals. VOCs from strain IN937a triggered ISR through an ethylene-independent signaling pathway, whereas VOCs from strain GB03 appear to operate through an ethylene-dependent, albeit independent of the salicylic acid or jasmonic acid signaling pathways. This finding provided new insight into the role of VOCs as initiators of defense responses in plants.

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The fact that PGPR VOCs emitted by *B. subtilis* GB03 can trigger different hormonal signaling networks in *A. thaliana*, involving cytokinins, brassinosteroids, auxin, salicylic acid and gibberellins,[130-133] opens new expectations on the role of volatiles during plant-microorganism relationship with regard to plant development. To investigate how PGPR VOCs trigger growth promotion in *A. thaliana*, Zhang et al. (2007) examined mRNA levels of Arabidopsis seedlings exposed to volatiles of *B. subtilis* GB03 using oligonucleotide microarrays. In screening over 26,000 protein-coded transcripts, a group of approximately 600 differentially expressed genes related to cell wall modifications, primary and secondary metabolism, stress responses and auxin homeostasis were identified.[132] These data for the first time implicate VOCs as modulators of auxin homeostasis and cell expansion and suggest that VOCs can directly impact pathways involved in plant morphogenesis. More recently, the same group demonstrated that *B. subtilis* GB03 VOCs augment photosynthesis capacity by increasing photosynthetic efficiency and chlorophyll content in Arabidopsis through the modulation of endogenous sugar/ABA signaling.[133] This strain produces different kinds of volatiles such as short-chained alcohols, aldehydes, acids, esters, ketones, hydrocarbons and sulphur-containing compounds.[134] The specific roles of these VOCs in plant signaling and the mechanism of perception by plants merit further research.

The term "induced-systemic tolerance" (IST) was recently proposed for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress.[43,45,135] The role of VOCs emitted from *B. subtilis* GB03 on IST to salt stress (100 mM NaCl) in Arabidopsis was evaluated by Zhang et al. (2008b). The authors observed that VOCs concurrently downregulated *HKT1* (*High-affinity K^+ transporter1*) expression in roots, but upregulated it in shoots, resulting in lower Na^+ accumulation throughout the plant.[135]

Studies on the effect that PGPR VOCs have on secondary metabolite production in plants such as accumulation of aroma compounds for flavor enhancement in agronomic crops have begun to emerge,[136] so the addition of highly active but cheap compounds to plants for increasing plant productivity and immunity, induction of IST, and enhance food flavor, might represent a novel and promising strategy in agriculture.

Concluding Remarks

Plants and microorganisms have coexisted for million of years. Plants maintain a complex interaction with their rhizospheric populations, which is crucial for nutrient assimilation, development and activation of defense mechanisms. These mutually beneficial associations are possible because plants and microorganisms can communicate with each other through various signaling mechanisms.

In this review, we have considered four major classes of signals that participate in the interactions that occur between plants and beneficial microorganisms, auxins, cytokinins, AHLs and VOCs. It can be generally appreciated that plants are able to sense and respond to rhizosphere-inhabiting bacterial and fungal populations and their products. Aside from the discovery of different types of chemical communication for interkingdom signaling, a challenge for the future is to begin to address the possibility that there is a significant specific communication. A further challenge is to determine the role played by phytohormones and other plant-derived metabolites such as NAEs, alkamides and VOCs in the physiology of microorganisms. In addition, the mechanisms of NAE, alkamide and AHL perception in plants remains as a mystery. In *Vibrio harveyi*, an AHL called autoinducer-1 (AI-1) acts as a species-specific quorum-sensing signal, which activates a two-component signaling system.[137,139] Plants possess two-component signaling systems underlying the regulation of growth and development in response to cytokinins and ethylene.[140] In this regard, recent research has revealed an important interaction between alkamides and cytokinin signaling as the level of cytokinin receptors.[83] Whether small lipid amides act as ligands of cytokinin receptors remains to be determined, however, the possibility is open that AHLs, NAEs and alkamides could regulate plant development by modulate two-component signaling systems.

Studies of transkingdom signaling between plants and bacteria based on small lipid signals (i.e., AHLs) are just beginning to reveal its diverse roles in healthy plants and the field of plant-microbe interactions will undoubtedly provide good examples of the molecular mechanisms involved in the interaction. Exploring further the interactions by means of global gene expression analyses and proteomic strategies along with the identification of plant mutants defective on signal perception/transduction should help increase our knowledge on the mechanisms used by plants to communicate with beneficial microbial populations.

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Characterization of *drr1*, an Alkamide-Resistant Mutant of Arabidopsis, Reveals an Important Role for Small Lipid Amides in Lateral Root Development and Plant Senescence[1[C][W][OA]]

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Alkamides belong to a class of small lipid signals of wide distribution in plants, which are structurally related to the bacterial quorum-sensing signals *N*-acyl-L-homoserine lactones. Arabidopsis (*Arabidopsis thaliana*) seedlings display a number of root developmental responses to alkamides, including primary root growth inhibition and greater formation of lateral roots. To gain insight into the regulatory mechanisms by which these compounds alter plant development, we performed a mutant screen for identifying Arabidopsis mutants that fail to inhibit primary root growth when grown under a high concentration of *N*-isobutyl decanamide. A recessive *N*-isobutyl decanamide-resistant mutant (*decanamide resistant root* [*drr1*]) was isolated because of its continued primary root growth and reduced lateral root formation in response to this alkamide. Detailed characterization of lateral root primordia development in the wild type and *drr1* mutants revealed that *DRR1* is required at an early stage of pericycle cell activation to form lateral root primordia in response to both *N*-isobutyl decanamide and *N*-decanoyl-L-homoserine lactone, a highly active bacterial quorum-sensing signal. Exogenously supplied auxin similarly inhibited primary root growth and promoted lateral root formation in wild-type and *drr1* seedlings, suggesting that alkamides and auxin act by different mechanisms to alter root system architecture. When grown both in vitro and in soil, *drr1* mutants showed dramatically increased longevity and reduced hormone- and age-dependent senescence, which were related to reduced lateral root formation when exposed to stimulatory concentrations of jasmonic acid. Taken together, our results provide genetic evidence indicating that alkamides and *N*-acyl-L-homoserine lactones can be perceived by plants to modulate root architecture and senescence-related processes possibly by interacting with jasmonic acid signaling.

Plant growth and development require the integration of a variety of environmental and endogenous signals, which together with the intrinsic genetic program determine plant form and longevity. Lipids have long been recognized as signals that have the capacity to trigger profound physiological responses. In animals, ceramides and sphingosines are lipids that have proapoptotic and antiproliferative actions (Wymann and Schneiter, 2008). In plants, ceramides, sphingosines, and phosphatidic acid are involved in mediating plant growth, development, and responses to environmental stimuli (Worrall et al., 2003; Wang, 2004).

In the past few years, additional small lipids have been found to acts as plant signals, including alkamides and *N*-acyl-ethanolamines (NAEs). Alkamides comprise at least 200 amides with varied acyl chain lengths and saturation grades (for review, see López-Bucio et al., 2006; Morquecho-Contreras and López-Bucio, 2007). These compounds have been found to alter root and shoot system architecture in Arabidopsis (*Arabidopsis thaliana*; Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008). NAEs represent compounds with aminoalcohol linked as an amide to the fatty acid. They are likely produced from the hydrolysis of *N*-acyl-phosphatidylethanolamines, a minor constituent of cell membranes, by phospholipase D (Chapman, 2004). NAEs have been found to accumulate in seeds of some higher plants, including cotton (*Gossypium hirsutum*), corn (*Zea mays*), Arabidopsis, soybean (*Glycine max*), tomato (*Solanum lycopersicum*), and pea (*Pisum sativum*), and their levels decline during germination (Wang et al., 2006).

Many gram-negative bacteria produce alkamide-related substances termed *N*-acyl-L-homoserine lactones (AHLs). These compounds participate in

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cell-to-cell signaling that is usually referred to as quorum sensing (Pearson et al., 1994). The AHL signals contain a conserved HL ring and an *N*-linked acyl side chain. The acyl-chain moiety of naturally occurring AHLs can differ in length and substitution at position C3, which is either unmodified or carries an oxo or hydroxyl group (Pearson et al., 1994; Parsek et al., 1999). These molecules are freely diffused through the bacterial membrane, which is to some extent dependent upon the length of the acyl side chain and the nature of any C3 substitutions and distribute within the rhizosphere (Pearson et al., 1999; Schuhegger et al., 2006; Scott et al., 2006). Evidence has accumulated indicating that plants are able to perceive AHLs. The application of AHLs to *Medicago truncatula* and Arabidopsis plants resulted in differential transcriptional changes in roots and shoots, affecting the expression of genes potentially involved in development (Mathesius et al., 2003; Von Rad et al., 2008). Ortíz-Castro et al. (2008) evaluated Arabidopsis growth responses to a variety of saturated AHLs ranging from four to 14 carbons in length, focusing on alterations in postembryonic root development. The compounds affected primary root growth, lateral root (LR) formation, and root hair development. While this information clearly indicates that plants are able to sense a variety of small lipid signals, including alkamides, NAEs, and AHLs, which modulate root architecture, the genetic mechanisms involved in signal perception to these compounds are unknown.

The Arabidopsis root system is an excellent model to dissect the genetic and developmental processes that determine plant architecture. It mainly consists of an embryonic primary root and postembryonic developed LRs (López-Bucio et al., 2005). LR formation is influenced by a wide range of environmental cues, such as nutrients and water availability in the soil (López-Bucio et al., 2003; Malamy, 2005; Nibau et al., 2008). The plasticity of LR formation is of critical importance, allowing plants to compete for resources and adapt to constantly changing growth conditions. LRs originate from pericycle founder cells located opposite to xylem poles, which undergo several rounds of anticlinal divisions to create a single-layered primordium composed of up to 10 small cells of equal length (termed stage I; Dolan et al., 1993; Malamy and Benfey, 1997; Dubrovsky et al., 2001). Further anticlinal and periclinal divisions create a dome-shaped primordium (spanning stages III–VII), which eventually emerges from the parental root (Malamy and Benfey, 1997; Casimiro et al., 2003; Péret et al., 2009).

The phytohormone auxin (indole-3-acetic acid [IAA]) plays an important role during each stage of LR formation (De Smet et al., 2006; Fukaki et al., 2007; Dubrovsky et al., 2008; Fukaki and Tasaka, 2009). Application of IAA or synthetic auxins such as 2, 4-dichlorophenoxyacetic acid or naphthaleneacetic acid (NAA) stimulates LR formation (Celenza et al., 1995; Woodward and Bartel, 2005), whereas polar auxin transport inhibitors such as *N*-(1-naphthyl)-phthalamic acid and 2,3,5-triiodobenzoic acid prevent LR formation (Casimiro et al., 2001; Himanen et al., 2002). Consistently, Arabidopsis mutants with increased auxin levels, such as *rooty* and its alleles *aberrant lateral root formation1* and *superroot1*, have increased numbers of LRs (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants with defective auxin transport, perception, or signaling, including *aux1*, *axr1*, and *tir3/doc1/big*, show reduced LR formation (Lincoln et al., 1990; Gil et al., 2001; Swarup et al., 2001). In contrast to auxin, less is known about the action of alkamides, AHLs, and other related small lipid signals on LR formation and whole plant development.

To identify the genetic components responsible for the root architectural responses to alkamides, we performed a visual screening for Arabidopsis mutants that under high *N*-isobutyl decanamide concentration do not manifest primary root growth reduction. We isolated an *N*-isobutyl decanamide-resistant mutant (*decanamide resistant root* [*drr1*]) defective in a single recessive trait. Detailed cellular and developmental studies of wild-type and *drr1* plants indicate that *drr1* mutants show resistance to primary root growth inhibition and LR growth promotion induced by both an alkamide (*N*-isobutyl decanamide) and a bacterial quorum-sensing signal (*N*-decanoyl-L-homoserine lactone [C10-AHL]). We further show that *DRR1* is a crucial component of the regulation of plant senescence, which likely links alkamide and jasmonic acid (JA) in modulating plant longevity and LR development.

RESULTS

Isolation of *drr1*, an Arabidopsis Mutant with Altered Primary Root Growth Response to *N*-Isobutyl Decanamide

From a group of similar chain length alkamides and NAEs, López-Bucio and coworkers (2007) identified *N*-isobutyl decanamide, a C10 saturated alkamide that is naturally produced in *Acmella radicans* (Ríos-Chávez et al., 2003) and *Cissampelos glaberrima* (Laurerio-Rosario et al., 1996), as the most active compound in inhibiting primary root growth and stimulating LR formation in Arabidopsis.

To investigate the genetic basis of plant responses to alkamides, we screened 25,000 lines from T-DNA insertion mutant collections (Krysan et al., 1999) by inspecting the root architecture of plants grown over the surface of 0.2× Murashige and Skoog (MS) agar plates supplied with 30 μM *N*-isobutyl decanamide. A mutant line was isolated that, in contrast to the wild type, was able to sustain primary root growth under this inhibitory concentration of the alkamide (Fig. 1A). The mutant was backcrossed to wild-type plants (Wassilewskija [Ws] ecotype) three times prior to detailed phenotypical analysis. In F2 progeny from these crosses, in plants grown in medium supplied

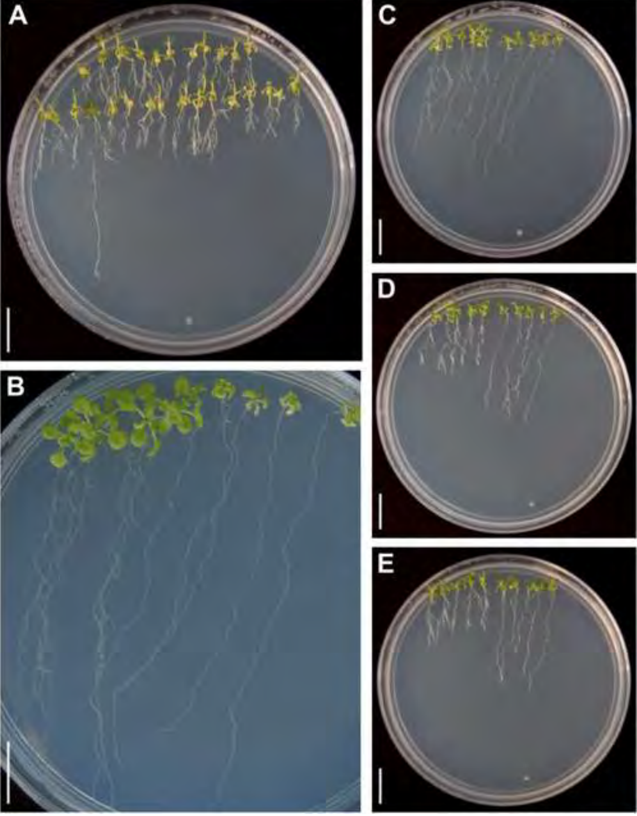


Figure 1. Genetic screen and phenotypic characterization of *drr1* mutants. A, Photograph of an agar plate supplied with 30 μM *N*-isobutyl decanamide showing a putative *drr1* mutant with long primary root. B, Five 14-d-old wild-type (Ws) and *drr1* seedlings grown side by side on the surface of agar plates containing 0.2× MS medium lacking *N*-isobutyl decanamide. C to E, Photographs of agar plates supplied with 20 μM (C), 25 μM (D), or 30 μM (E) *N*-isobutyl decanamide, showing five wild-type (left) and *drr1* (right) plants grown side by side. Photographs in B and C are representative individuals of four plates per treatment. Bars = 1 cm. [See online article for color version of this figure.]

with 30 μM *N*-isobutyl decanamide, the line segregated the mutant phenotype in a 1:3 ratio (Table I). These results indicate that the primary root growth resistance to the alkamide resulted from a recessive single-gene mutation. We named this locus *drr1*. To further study the developmental alterations induced by *N*-isobutyl decanamide in wild-type and *drr1* plants, we grew ecotype Ws and *drr1* plants side by side on vertically oriented agar plates with varied alkamide contents. Wild-type plants grown in 0.2× MS agar medium without *N*-isobutyl decanamide showed a typical root system, consisting of a long primary root with many LRs forming in a gradient from the root/shoot junction to the primary root tip (Fig. 1B). In the same medium, *drr1* mutants developed a long primary root lacking visible LRs (Fig. 1B), thus indicating that *DRR1* is important for normal LR development under normal growth conditions. In wild-type plants treated with 20, 25, or 30 μM *N*-isobutyl decanamide, there was a dose-dependent inhibitory effect of the alkamide on primary root growth, which correlates with an increase in LR formation. In these plants, multiple LRs developed, giving rise to a highly exploratory root system with different architecture from that observed in plants grown in medium without *N*-isobutyl decanamide (Fig. 1, C–E). In contrast, alkamide-treated *drr1* mutants showed longer primary roots and reduced LR formation when compared with wild-type plants in most concentrations of *N*-isobutyl decanamide tested (Fig. 1, C–E).

drr1 Mediates the Root Architecture Responses of Arabidopsis to *N*-Isobutyl Decanamide

To more clearly define the alterations in the root architectural response to *N*-isobutyl decanamide caused by mutation in *DRR1*, we performed temporal and single-point measurements of primary root length, LR number per plant, and LR density in wild-type and *drr1* mutants treated with varied concentrations of *N*-isobutyl decanamide. Primary root growth was similar in wild-type and *drr1* plants in concentrations of up to 15 μM *N*-isobutyl decanamide, while in concentrations of 20, 25, and 30 μM of this compound, *drr1* primary roots were significantly longer that wild-type plants (Fig. 2A). *N*-Isobutyl decanamide increased the number of emerged LRs in wild-type plants, while *drr1* plants were resistant to this effect (Fig. 2B). The density of emerged LRs dramatically increased in response to alkamide treatment in wild-type plants, but the mutants again showed reduced responses. The most contrasting responses between wild-type and *drr1* plants were observed in 25 μM *N*-isobutyl decanamide, in which wild-type plants showed a highly branched root system harboring second- and third-order LRs (Fig. 1, C–E), with a 6-fold increased density of LRs (Fig. 2C). In this alkamide concentration, *drr1* mutant plants produced less than 15% of the LRs observed in wild-type plants. Interestingly, although *drr1* mutants produced

Table I. *Segregation ratio of progeny resulting from crosses between drr1 mutant and wild-type seedlings*

Generation	Phenotype of Progeny		Ratio Obtained, Wild Type:*drr1*	Ratio Tested, Wild Type:*drr1*	χ^{2a}
	Many LRs (Wild Type)	Few LRs (*drr1*)			
F1	128	0			
F2	730	260	2.81:1	3:1	0.84

[a]With one degree of freedom and a critical value of 5%, the hypothesis is accepted if the χ^2 is smaller than 3.841.

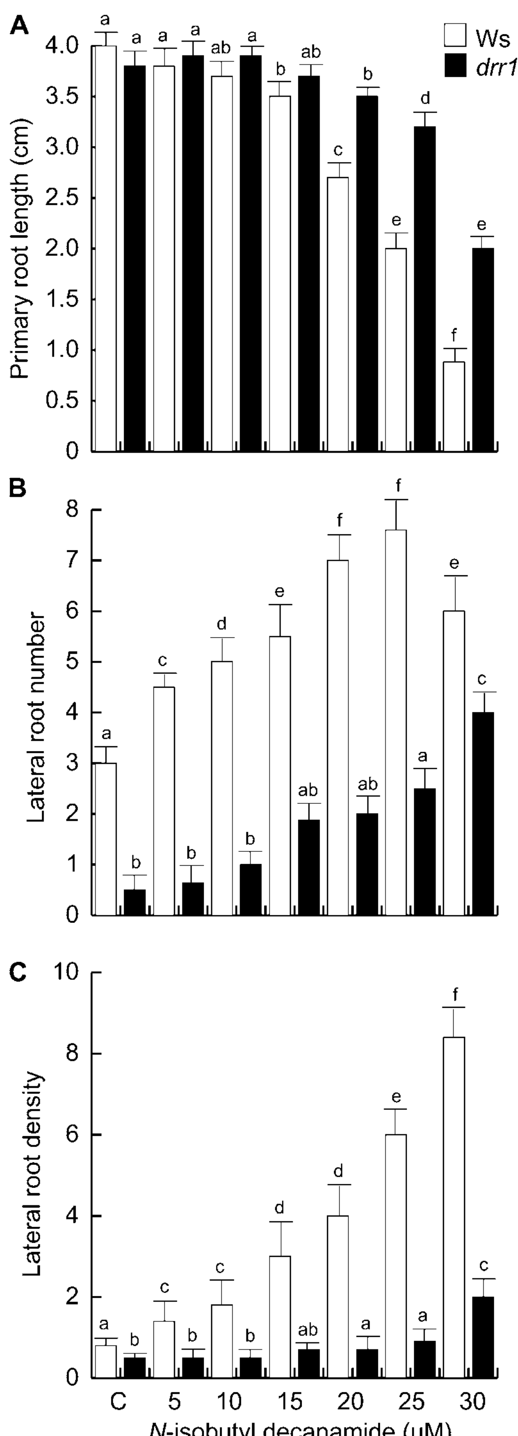


Figure 2. Effects of *N*-isobutyl decanamide on the root system architecture of wild-type (Ws) and *drr1* plants. A, Primary root length. B, Number of emerged LRs per plant. C, LR density expressed as the number of LRs per centimeter. Data were recorded at 12 d after germination. Values shown are means ± SD (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

consistently fewer LRs compared with the wild type in most *N*-isobutyl decanamide treatments, exposure to 30 μM *N*-isobutyl decanamide caused an 8-fold increase in LR number and a 2-fold increase in LR density (Fig. 2, B and C), indicating that the mutants are not completely insensitive to the alkamide.

drr1 Mutants Are Resistant to Inhibitory Effects of *N*-Isobutyl Decanamide on Cell Division in Primary Root Meristems

An important factor determining primary root growth reduction in wild-type seedlings grown in high *N*-isobutyl decanamide concentrations is the reduction in cell division in the root meristem (López-Bucio et al., 2007). To analyze the cell division responses of *drr1* mutants to alkamide treatment, we crossed *drr1* with a transgenic plant harboring the *CycB1*:*uidA* construct, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al., 1999). *CycB1*:*uidA* seedlings and *drr1* seedlings were grown in 0.2× MS agar medium supplied with the solvent or with 20, 25, and 30 μM *N*-isobutyl decanamide. In both wild-type *CycB1*:*uidA* and *drr1* mutant seedlings supplied with the solvent only, a patchy pattern of single cells expressing *CycB1*:*uidA* was observed in the primary root meristem (Fig. 3, A and B). In wild-type plants subjected to treatment with 30 μM *N*-isobutyl decanamide, GUS expression in the primary root tip decreased and root hairs were formed in close proximity to the root meristem (Fig. 3C). Interestingly, *CycB1*:*uidA* expression in the primary root apex of *drr1* seedlings treated with the alkamide was not as much inhibited as in the wild type, and their root meristems were anatomically similar to those of solvent only-treated seedlings (Fig. 3D). Root hair formation close to the root meristem was not observed in *drr1* seedlings treated with the alkamide (Fig. 3D). Next, we quantified the length of the primary root meristems in wild-type and *drr1* plants at 4 and 12 d after germination. At these developmental stages, increased concentrations of *N*-isobutyl decanamide decreased the length of the meristem in wild-type plants, while *drr1* mutants were resistant to this effect (Fig. 3E).

drr1 Is Defective in *N*-Isobutyl Decanamide-Induced LR Primordia Development

LR formation is a major determinant of root system architecture. Next, we investigated the effects of *N*-isobutyl decanamide on lateral root primordia (LRP) development and LR emergence in wild-type and *drr1* plants. LRP originating in the primary root and emerged LRs were counted at 6 d after germination in plants grown in 0.2× MS agar medium supplied with the solvent only or with 30 μM *N*-isobutyl decanamide. The developmental stage of each LRP was classified according to Malamy and Benfey (1997; see "Materials and Methods"). In solvent-treated wild-type plants,

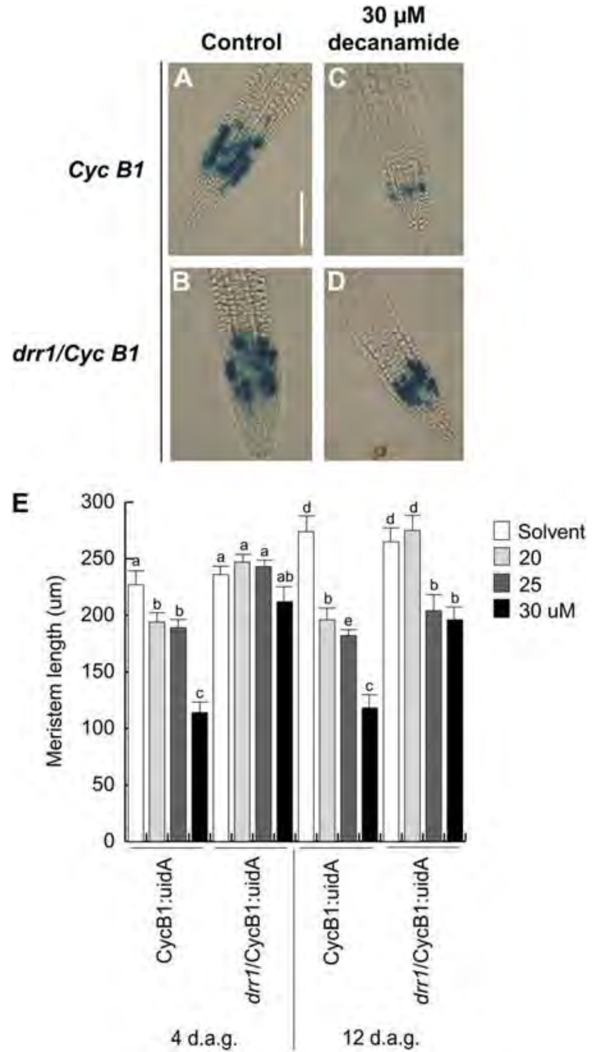


Figure 3. *CycB1*:*uidA* expression in transgenic wild-type and *drr1* seedlings. Twelve-hour GUS staining is shown for *CycB1*:*uidA* primary roots in wild-type and *drr1* Arabidopsis seedlings grown on agar-solidified 0.2× MS medium with or without *N*-isobutyl decanamide. A and B, Solvent-treated seedlings. C and D, Plants supplied with 30 μM *N*-isobutyl decanamide. E, Meristem length. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Bar = 100 μm. d.a.g., Days after germination. [See online article for color version of this figure.]

most LRP remained at an early developmental stage (stage I). Interestingly, *N*-isobutyl decanamide treatment increased both the number of LRP at stage I and the density of emerged LRs (Fig. 4). Solvent-treated *drr1* mutants showed similar LRP density to wild-type plants (Fig. 4A) but dramatically decreased density of emerged LRs (Fig. 4B), indicating that the mutant is not inherently defective in LRP initiation but rather shows a retardation in the maturation of LRP. When treated with *N*-isobutyl decanamide, *drr1* mutant seedlings did not show an increase in stage I LRP or in LRP emergence observed in wild-type plants (Fig. 4), indicating the *DRR1* locus is involved in alkamide responses in the pericycle and during LRP development. Although alkamide treatment significantly increased the density of emerged LRs in *drr1* mutants, *drr1* always showed lower LR density than wild-type plants in the different growth conditions (Fig. 4B). These results indicate that *N*-isobutyl decanamide modifies root system architecture both by inducing more pericycle cells to form stage I LRP and by accelerating the emergence of LRP from the primary root to form mature LRs. Mutations in *drr1* interfere with both of these processes.

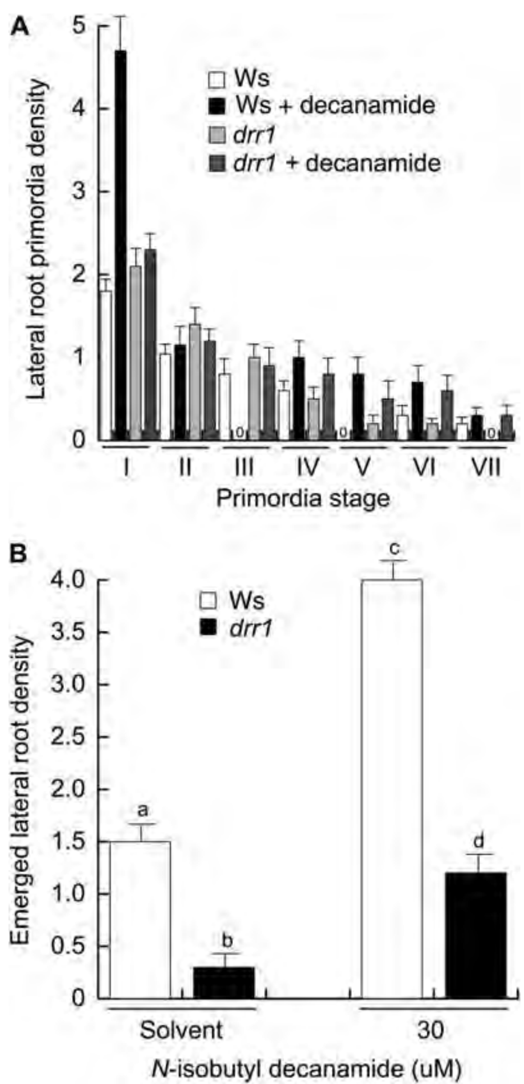


Figure 4. Effects of *N*-isobutyl decanamide on wild-type (Ws) and *drr1* LR development. A, LRP stage distribution in 6-d-old primary roots grown on medium supplied with the solvent only or with 30 μM *N*-isobutyl decanamide (indicated as decanamide). B, Emerged LR density in the same experiment. Wild-type and *drr1* seedlings were cleared, and the number and stage of LRP were recorded according to Malamy and Benfey (1997). Values shown are means ± SD (n = 15). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results.

drr1 Is Defective in Root Architectural Responses to C10-AHL, a Quorum-Sensing Signal from Bacteria

Previous studies documented that AHLs, a class of alkamide-related quorum-sensing signals from bacteria, modulate root system architecture in Arabidopsis (Ortíz-Castro et al., 2008; Von Rad et al., 2008). To determine if *DRR1* is involved in AHL responses, we tested the primary root growth and LR responses of *drr1* seedlings to C10-AHL over a range of concentrations of this compound as compared with wild-type plants. *drr1* had a level of resistance to primary root growth inhibition by C10-AHL over most concentrations tested (Fig. 5A). At 30 μM C10-AHL in wild-type plants, about 60% inhibition of growth occurred, whereas in the mutant, it was about 30%. As previously reported (Ortíz-Castro et al., 2008), C10-AHL stimulated LR formation (Fig. 5B). A dose-dependent effect increasing LR density was observed (Fig. 5C), confirming the positive role of AHLs in LR induction. In contrast, *drr1* mutants showed reduced LR formation when compared with wild-type seedlings over most concentrations of C10-AHL tested (Fig. 5, B and C).

drr1 Shows Normal Auxin Responses

Several auxin-related mutants have been characterized in screens for primary root growth resistance to inhibitory amounts of IAA, which display alterations in LR formation (Rogg et al., 2001; Swarup et al., 2001; Fukaki et al., 2002). To determine if *drr1* operates in a genetically defined auxin pathway, wild-type Arabidopsis seedlings (ecotype Columbia [Col-0] and Ws), *drr1* seedlings, and the auxin-related mutants *aux1-7* and *axr2* were evaluated in primary root growth response assays to IAA. First, to confirm the auxin resistance of auxin-related mutant lines, homozygous *aux1-7* and *axr2-1* seedlings were screened for resistance to IAA based on primary root growth. In these experiments, *aux1-7* and *axr2* were resistant to the inhibition of primary root elongation by IAA when compared with wild-type Col-0 seedlings (Fig. 6A). These mutants also failed to form abundant root hairs at the root tip region in response to increasing IAA concentration in the medium, a phenotype associated with increased auxin resistance (Fig. 6B). In contrast, the auxin response in *drr1* mutants was equally sensitive to IAA than the wild-type (Ws ecotype) both in primary root growth assays (Fig. 6A) and toward induction of root hair formation close to the root tip (Fig. 6B). Because *drr1* mutants showed normal root responses to IAA, we conclude that auxin signaling is unaffected in the mutant.

To better understand the role played by auxin in LR formation in wild-type and *drr1* plants, we tested the effects of NAA to activate LR formation in a transfer assay. In these experiments, wild-type and *drr1* plants were first germinated and grown for 7 d in 0.2× MS agar medium. At day 7 after germination, plants were

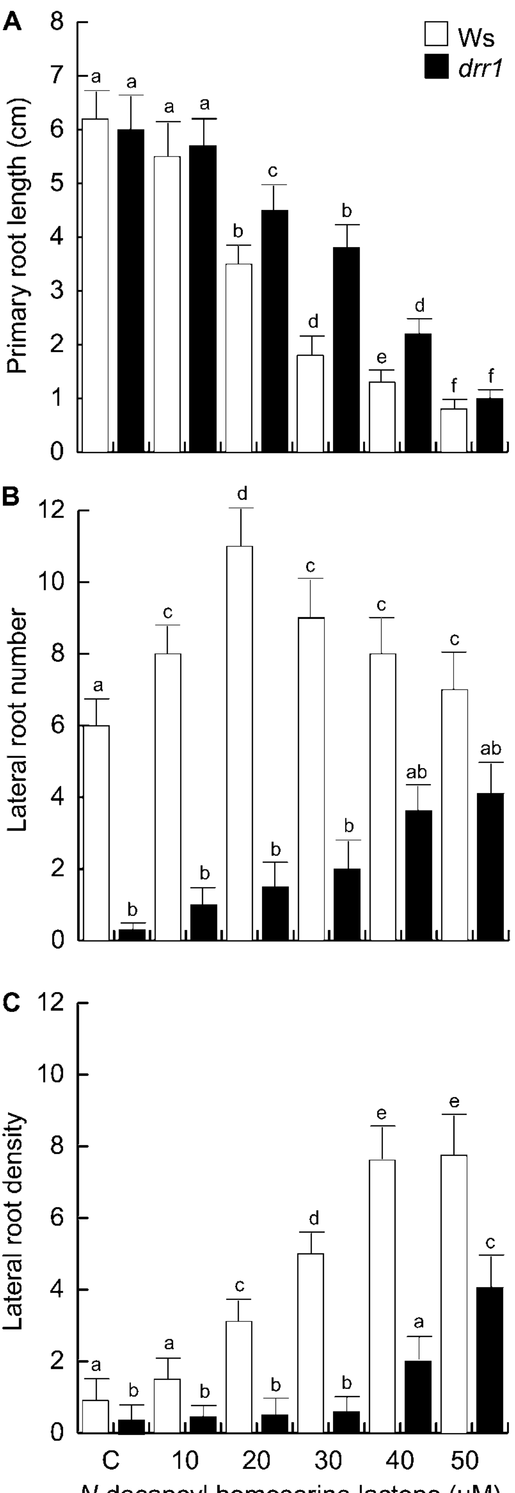


Figure 5. Effects of C10-AHL on the root system architecture of wild-type (Ws) and *drr1* plants. A, Primary root length. B, Number of emerged LRs per plant. C, LR density expressed as the number of LRs per centimeter. Data were recorded at 14 d after germination. Values shown are means ± SD (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

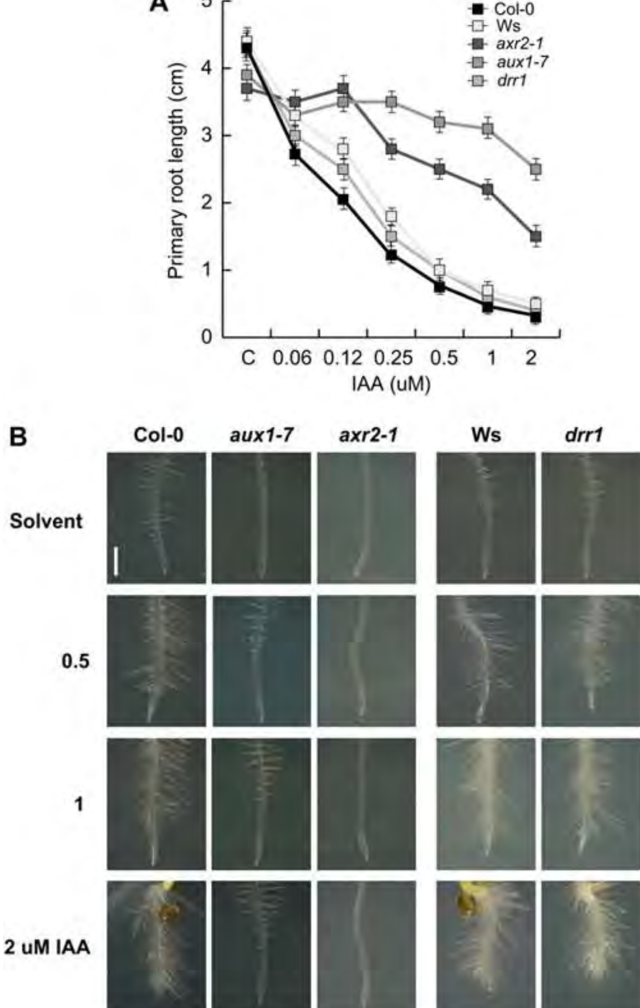


Figure 6. Auxin responses in wild-type and *drr1* seedling roots. A, Primary root growth in 12-d-old primary roots of wild-type (Col-0 and Ws), *axr2-1* and *aux1-7* auxin-resistant mutants, and *drr1* Arabidopsis mutants grown on medium supplied with the solvent only or with varied IAA concentrations. B, Morphology of root tips of wild-type and mutant lines exposed to IAA. Seedlings were photographed at 7 d after germination using a digital camera connected to a dissecting microscope. Values shown in A are means ± SD ($n = 30$). The experiment was repeated twice with similar results. [See online article for color version of this figure.]

transferred to 0.2× MS liquid medium supplied with the solvent or varied concentrations of NAA for an additional 4-d period. At this stage, the number and density of LRs were determined. As shown in Figure 7, NAA treatment caused a dose-response effect in LR formation (Fig. 7A), which was similar between wild-type and *drr1* plants. Both wild-type and *drr1* plants produced highly branched root systems with normal LR growth (Fig. 7, B–I). These results indicate that *drr1* seedlings are not inherently defective in pericycle cell activation to form LRs and are able to correctly sense and respond to auxins.

drr1 Mutants Show Extended Longevity

To study the role of *DRR1* in plant growth and development, we compared the phenotype of wild-type and homozygous *drr1* plants of the same age that were first germinated and grown for 10 d on 0.2× MS agar medium and then transferred to soil. Wild-type and *drr1* plants were grown side by side during their entire life cycle. The young and adult phenotypes of plants are shown in Figure 8. During the first 28 d after transfer, a general delay in the growth of *drr1* mutants was observed, as illustrated by their delay in stem formation (Figs. 8, A and B, and 9A) and significantly decreased rosette size during early stages of vegetative growth (Fig. 9B). At 28 d after transfer, the rosette leaves of wild-type plants had already turned yellow and stem growth ceased, but *drr1* leaves remained

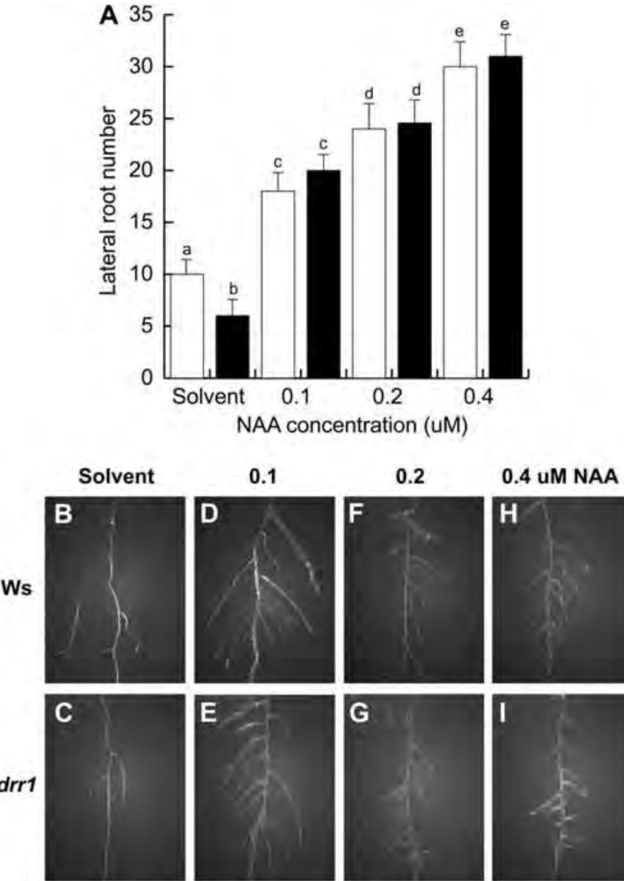


Figure 7. Auxin restoration of LR development in *drr1* plants. A, Total LR number per plant in 11-d-old wild-type (Ws) and *drr1* plants that were first grown for 7 d in 0.2× MS agar medium and then transferred to 0.2× MS liquid medium supplied with the solvent or with varied concentrations of NAA for an additional 4-d growth period. B to I, Representative photographs of wild-type and *drr1* LRs in plants exposed to NAA. Values shown in A are means ± SD ($n = 15$). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results. [See online article for color version of this figure.]

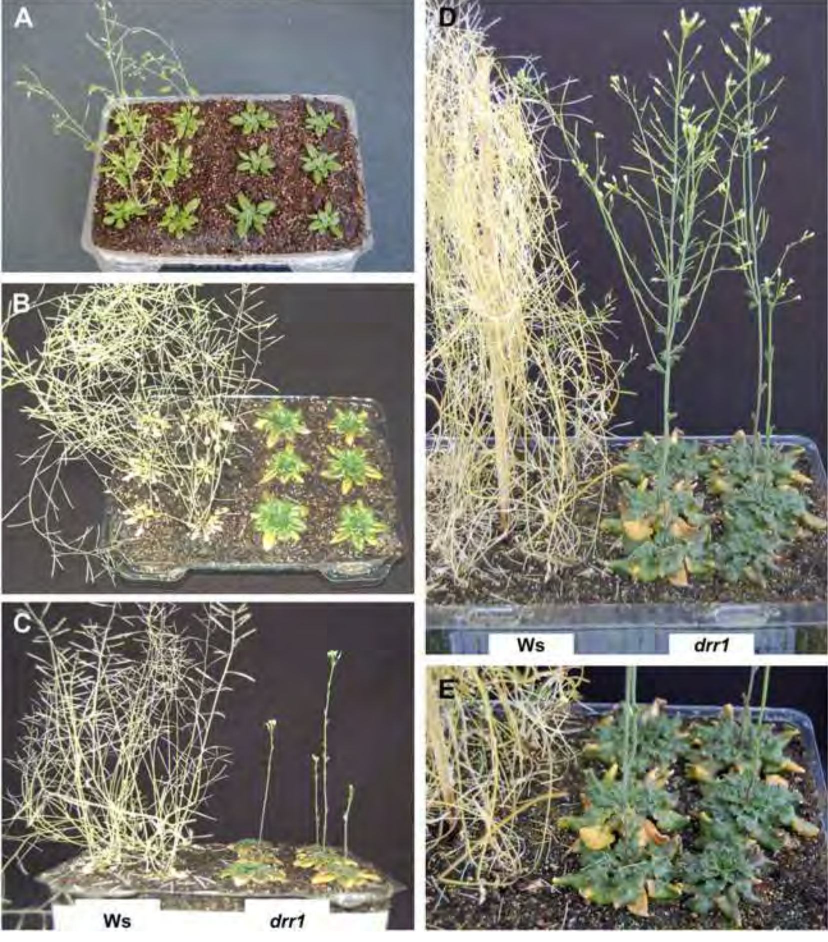


Figure 8. Phenotypes of wild-type and *drr1* plants grown in soil. A to D, Phenotypes of wild-type (Ws; left) and *drr1* (right) plants grown side by side at 14, 28, 56, or 84 d after transfer to soil. E, Closeup of rosette leaves at 84 d after transfer. Plants were grown with a 16-h-light/8-h-dark cycle at 22°C in a growth chamber. The retarded leaf senescence in *drr1* was related to the retarded emergence of floral stems and flowering time. [See online article for color version of this figure.]

green, and 7 d later, the stems just started to be formed (Figs. 8, A–C, and 9, A and B). At 35 d after transfer, wild-type leaves had turned completely yellow and showed signs of death with drying (Fig. 8C). In contrast, the *drr1* mutant leaves retained a significant amount of chlorophyll and maintained the integrity of the leaf shape (Fig. 8C). The extension of leaf longevity at a whole plant level dramatically increased in *drr1* mutants with time. Delayed flowering was accompanied by the generation of new leaves, increased rosette size, and greater stem length in *drr1* mutants when compared with wild-type plants (Figs. 8, D and E, and 9, A–C). *drr1* sustained chlorophyll production for a longer time period (Fig. 9D). In addition, the shoot architecture of *drr1* mutants was different from that observed in wild-type seedlings, producing only one primary stem with reduced branches, which suggests increased apical dominance in the mutants (Fig. 9, E and F). Aside from the delayed senescence and altered shoot architecture, *drr1* mutants produced fertile flowers that yielded fruits with fully viable seeds (Fig. 8D). We determined that the longevity in *drr1* mutants was extended by approximately 2-fold when compared with wild-type plants, which correlates with a 3- to 4-fold increase in the number of visible leaves and overall increased plant size (Fig. 9, A–C).

The *drr1* Mutant Shows Delayed Senescence Symptoms in JA- and Alkamide-Induced Senescence

Leaf senescence is modulated by JA (Schommer et al., 2008). Therefore, the possibility was open that the *drr1* mutant could be deficient in the JA-induced senescence program. We compared the effects of JA and *N*-isobutyl decanamide in wild-type and *drr1* plants in a senescence-induced assay for detached leaves (Fig. 10). In this assay, after 6 d of incubation in water, wild-type detached leaves gradually lost chlorophyll content (Fig. 10, A and C). A deficient senescence program for detached *drr1* plants incubated in water was evident (Fig. 10, B and D). In response to treatments with JA and *N*-isobutyl decanamide, wild-type leaves showed severe senescence symptoms that were reduced in *drr1* mutants (Fig. 10, E–I). Taking together the increased *drr1* longevity in soil and the delaying response to hormone-induced senescence, we conclude that *DRR1* plays an important role in the senescence process modulated by JA and *N*-isobutyl decanamide as well as in age-dependent senescence.

drr1 Is Altered in Jasmonate-Mediated LR Induction

Jasmonates are signals involved in root system architecture modulation (Wasternack, 2007). The in-

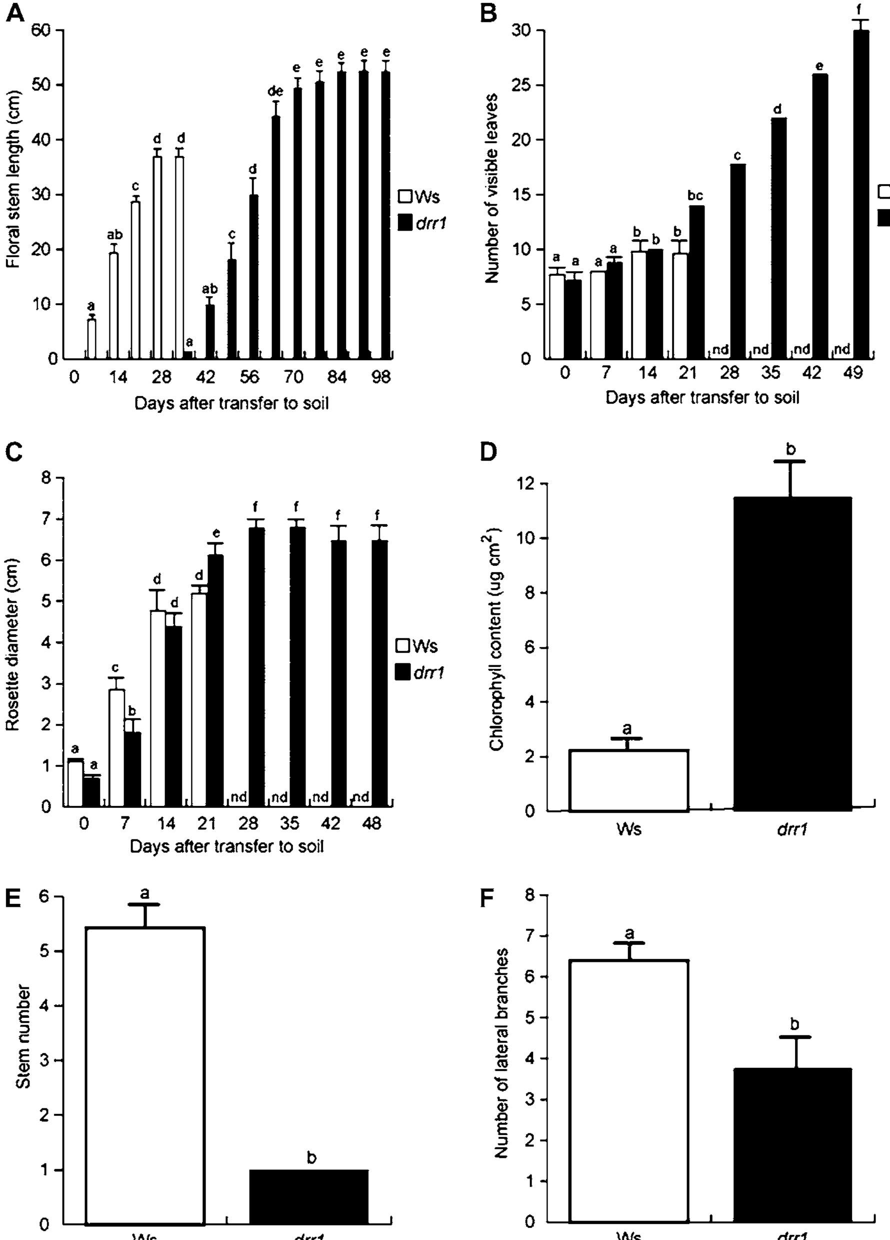
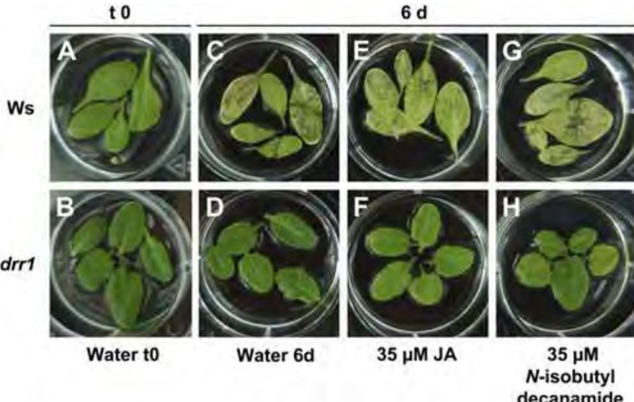


Figure 9. Age-dependent senescence symptoms and other developmental traits of wild-type (Ws) and *drr1* plants grown in soil. A, Age-dependent stem size. B, Rosette diameter. C, Number of visible rosette leaves. D, Chlorophyll content in rosette leaves at 28 d after transfer to soil. E, Stem number. F, Number of stem branches. Plants were grown with a 16-h-light/8-h-dark cycle at 22°C in a growth chamber, and developmental traits were monitored during their entire life cycle. Values shown are means ± SD ($n = 18$). Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results. nd, Not determined.

hibitory effect of methyl jasmonate (MeJA) on primary root growth has been well recognized and widely employed as a useful trait to identify jasmonate-related mutants in Arabidopsis. Recently, it has been reported that MeJA also promotes LR formation (Sun et al., 2009). The MeJA dose response of *drr1* in primary root growth and LR formation was compared with *jar1*, a MeJA-insensitive mutant, and wild-type seedlings of the Ws and Col-0 ecotypes; the Col-0 ecotype provided the genetic background for *jar1* and therefore was included as an additional control. When compared with Ws and Col-0 plants, the *jar1* mutant showed strong resistance to MeJA-induced primary root growth inhibition over most concentrations tested (Fig. 11A). The primary root growth inhibition in *drr1* was essentially the same as in Ws seedlings (Fig. 11A). Interestingly, MeJA, at concentrations of 30 to 45 μM, increased emerged LRs in wild-type seedlings and in *jar1* mutants by 70% to 150% (Fig. 11B; Supplemental Fig. S1). In the absence of MeJA, LR formation in *drr1* was significantly reduced compared with wild-type and *jar1* plants. However, *drr1* mutants failed to produce increased numbers of LRs when grown on medium containing a range of concentrations of MeJA (Fig. 11B; Supplemental Fig. S1). Our data reveal that *drr1* encodes a novel genetic locus modulating the effects of MeJA on LR formation.



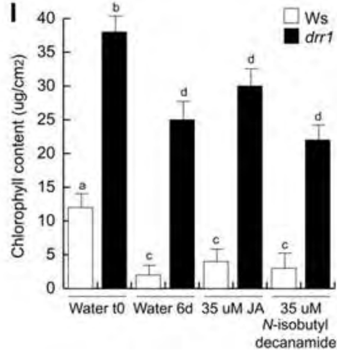


Figure 10. Hormone-dependent senescence symptoms in the *drr1* mutant. Detached leaves of wild-type (Ws) and *drr1* plants were incubated in 2-mL water solutions supplied with the indicated concentrations of compounds. The plates were included in a growth chamber (Percival ARR95L) under dark conditions, and representative photographs of leaves subjected to the different treatments were taken 6 d later (A–H) and chlorophyll determination was performed (I). The experiment included at least three independent samples of five leaves each and was replicated three times with similar results. [See online article for color version of this figure.]

DISCUSSION

drr1 Mutants Define a Locus Involved in Root Architectural Responses to Both Alkamides and AHLs

This report describes the identification and characterization of an Arabidopsis mutant that was defective in its root response to *N*-isobutyl decanamide but with additional characteristics, which suggest that alkamides play a role in plant longevity. Our characterization of root architectural responses in the wild type and *drr1* to *N*-isobutyl decanamide provided insights into the genetic mechanisms mediating the responses to alkamides. While *N*-isobutyl decanamide inhibited primary root growth and promoted LR formation in wild-type seedlings, resistance to the repressive effect of this alkamide on primary root growth and the failure to increase LR formation typified the *drr1* phenotype (Figs. 1 and 2).

Detailed cellular analysis of wild-type and *drr1* plants showed that the mutants sustained almost normal root meristematic activity when grown under inhibitory concentrations of *N*-isobutyl decanamide, as revealed by cell counts and *CycB1*:*uidA* expression in the primary root meristem (Fig. 3). Interestingly, the typical increase in LR primordia initiation and LR emergence observed in wild-type plants treated with the alkamide was reduced in *drr1* (Fig. 4). Several types of reported experimental evidence suggested that conditions that reduce primary root meristematic activity, including destruction of the primary root meristem by cell ablation and physical decapitation of the root tip, elicit an increase in LR number (Tsugeki and Fedoroff, 1999). Our findings that *drr1* mutants grown in medium lacking alkamides sustain normal primary root growth but reduced LR formation (Figs. 1 and 2) indicate that LR proliferation in response to *N*-isobutyl decanamide is not a direct consequence of

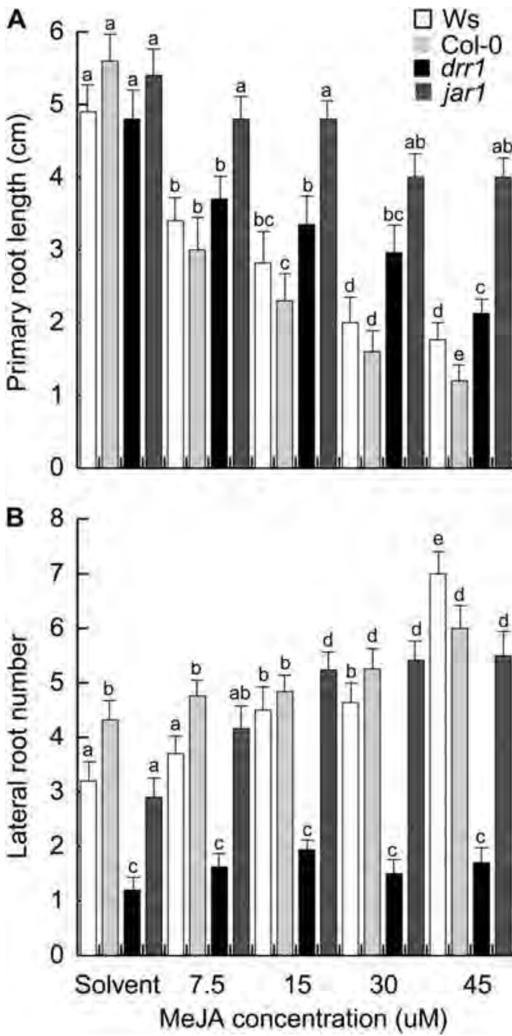


Figure 11. Effects of JA on primary root growth and LR development in wild-type (Ws and Col-0) and mutant (*drr1* and *jar1*) lines. A, Primary root length. B, Number of emerged LRs per plant. Data were recorded at 12 d after germination. Values shown are means ± SD (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

primary root growth inhibition but rather suggest a positive effect of the alkamide on pericycle cells to produce more LRP (Fig. 4).

Many bacterial species use small molecule signaling to communicate with each other and to coordinate their growth activities, a process commonly referred to as quorum sensing (Taga and Bassler, 2003; Reading and Sperandio, 2006). Diverse gram-negative bacteria produce AHLs, and these compounds contain a conserved HL ring and an *N*-linked acyl side chain. Our previous work indicated that saturated medium (C8–C14)-chained AHL compounds showed a dose-dependent effect on root architecture, inhibiting primary root growth and promoting LR formation (Ortíz-Castro et al., 2008). In this work, we used the *drr1* mutant to determine whether alkamides and AHLs could be perceived by similar genetic mechanisms. We show that C10-AHL inhibited primary root growth and promoted LR formation in Arabidopsis wild-type seedlings (Fig. 5). *drr1* mutants showed reduced sensitivity to both *N*-isobutyl decanamide and C10-AHL, indicating a potential genetic interaction in plant responses to alkamides and AHLs in roots. These results also suggest that plants have evolved the capacity to sense AHLs in order to activate developmental responses.

Several reports indicate that bacteria commonly associated with plants are capable to produce a variety of AHLs (Cha et al., 1998; Elasri et al., 2001; Khmel et al., 2002; D'Angelo-Picard et al., 2005). Several strains of *Pseudomonas* have been studied for their ability to colonize plant-related niches, such as the rhizosphere (i.e. *P. aeruginosa*, *P. fluorescens*, and *P. putida*), where they can act as plant growth-promoting rhizobacteria by antagonizing plant-deleterious microorganisms and through the production of traits that directly influence plant disease resistance and growth (Venturi, 2006). The rhizospheric *P. putida* plant beneficial strains WCS358 and IsoF produce 3-oxo-C_{12}-AHL, whereas in the rhizosphere-colonizing biocontrol *P. fluorescens* strain $F_1$13, the production of three AHL molecules, including C10-AHL, has been reported (Laue et al., 2000; Venturi, 2006). Interestingly, C10-AHL and C12-AHL seem to be also produced in the nitrogen-fixing bacterial symbiont *Sinorhizobium meliloti* (Marketon et al., 2002; Teplitski et al., 2003). The marked resistance of *drr1* to C10-AHL on root development opens new possibilities to identify novel genetic determinants involved in plant-bacteria interactions. Furthermore, the *drr1* mutant can be used as a tool to identify novel plant growth-promoting bacterial strains, which could modulate root system architecture through AHL production.

Two recent reports suggested that auxin signaling might be involved in plant responses to AHLs (Mathesius et al., 2003; Von Rad et al., 2008). Auxins are signaling molecules that regulate the asymmetric pericycle cell divisions and thereby influence the patterning of newly initiated LRP. Thus, the possibility was open that altered auxin responses could be responsible for reduced LR formation in *drr1* mutants. Our results, however, showed that *drr1* mutants are not resistant to IAA or NAA treatments in terms of primary root growth inhibition or LR formation (Figs. 6 and 7), indicating that *DRR1* might not be directly connected to the auxin response pathway to modulate plant growth and development. These results are in agreement with our previous research showing that both alkamides and AHLs modulate root system architecture, likely through auxin-independent signaling mechanisms (Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008; Ortíz-Castro et al., 2008).

DRR1 Plays a Role in Senescence-Related Processes

Senescence is a developmental process that limits the longevity of an organism. Genetic studies of longevity mutants have also suggested that some common mechanisms, such as alterations in energy metabolism and oxidative damage, might play a role in determining life span in animals as divergent as nematodes, *Drosophila*, and mammals (Lin et al., 1998; Parkes et al., 1998). Plants also undergo a distinctive senescence process at the organ and/or organism level. A number of studies have provided evidence suggesting that leaf senescence is an active process controlled by a genetic program (Woo et al., 2001, 2002, Schommer et al., 2008). However, our understanding of how senescence and longevity are controlled at the whole plant level remains quite limited. Our results suggest that LR development and age-dependent plant senescence are directly connected through *DRR1*. Obvious alterations were seen in *drr1* plants grown in soil under long days (16-h-light/8-h-dark conditions). In *drr1* plants, leaf senescence was delayed by about 4 to 5 weeks when compared with wild-type plants (Figs. 8 and 9). The extended longevity of leaves was related to an extended growth period as well as to slower onset and/or progression of senescence after the maturation stage. The reduced growth observed at early stages of development in *drr1* mutants opens the possibility that it may contribute to extended longevity. Consistent with this hypothesis, we observed that reduced LR formation was not caused by the failure of the pericycle to produce these structures but by the retarded development of LRP to emerge from the primary root (Fig. 4). In this way, the *drr1* mutation shows a senescence character that differs from the delayed leaf senescence phenotype observed in the *oresara* (*ore*) and *teosinte branched/cycloidea/PCF* (*tcp*) mutants described previously (Woo et al., 2001, 2002; Schommer et al., 2008). In *ore* mutants, the retarded senescence phenotype seems to be specifically observed in leaves. For instance, the leaf longevity in *ore9-1* was extended only by about 27%, without affecting other developmental traits such as flowering time and/or plant size (Woo et al., 2001). To our knowledge, no LR phenotypes have been reported for leaf senescence mutants such as the *ore* and *tcp* lines. Interestingly, the *drr1* mutants also show that

plants that bolt and senesce late produce more leaves and increase in size (Figs. 8 and 9), which could lead to potential agricultural applications. Together, our findings suggest that *DRR1* may function normally as a positive regulator of senescence in Arabidopsis, limiting longevity at the whole plant level. Because the *drr1* mutation affects a wide variety of age-dependent developmental and senescence responses (Fig. 10), *DRR1* may function upstream in the regulatory cascade of senescence pathways.

drr1 Mutants Reveal Cross Talk between Alkamides and Jasmonate in LR Formation

Cross-resistance of mutants to multiple hormones is well documented (Wilson et al., 1990; Hobbie and Estelle, 1994; Tiryaki and Staswick, 2002) and suggests that the action of hormones is coordinated by common intermediates or modulators. Several phytohormones are involved in leaf senescence, including ethylene, cytokinin, and JA (Schommer et al., 2008). High concentrations of *N*-isobutyl decanamide have been found to induce callus formation in leaves and in roots (López-Bucio et al., 2007). Although not explicitly tested here, preliminary information shows that *drr1* plants are also resistant to callus formation (data not shown). The proliferative growth activity elicited by *N*-isobutyl decanamide on callus formation in leaves and LR formation in roots was previously shown to be decreased or even absent in Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors *CRE1*, *AHK2*, and *AHK3* (López-Bucio et al., 2007). The triple cytokinin receptor mutant *cre1-12/ahk2-2/ahk3-3* was particularly insensitive to high alkamide concentrations in terms of developmental alterations, indicating that *N*-isobutyl decanamide requires, at least in part, a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes. However, the primary root growth response of the *drr1* mutants to kinetin, a highly active cytokinin in modulating root development, was similar to that observed in wild-type plants (Supplemental Fig. S2A), indicating that *drr1* is not resistant to root inhibition by cytokinin. However, we cannot exclude the possibility that cross talk between alkamide and cytokinin responses may account for the increased longevity and/or reduced senescence of *drr1* mutants. Abscisic acid and ethylene are two growth regulators also involved in senescence; *DRR1* mutation rendered the *drr1* seedlings more sensitive to the primary root growth inhibitory effect of low abscisic acid concentrations than wild-type seedlings (Supplemental Fig. S2B), whereas the ethylene precursor 1-aminocyclopropane-1-carboxylic acid similarly inhibited growth (Supplemental Fig. S2C).

The plant hormone JA plays a key role in the environmental stress responses and developmental processes of plants. A recent report has revealed an important role of JA in LR development. In such work (Sun et al., 2009), it was shown that exogenous MeJA promotes LR formation in Arabidopsis wild-type plants but not in *anthranilate synthase1* mutants, leading to the proposal that localized auxin biosynthesis in response to jasmonate could be important for fine-tuned modulation of LR formation. Our detailed morphological comparison among wild-type, *drr1*, and *jar1* plants indicated that, when grown on JA-free medium, LR development in *drr1* was significantly lower than in wild-type or *jar1* plants. Interestingly, while JA application led to increased LR numbers in wild-type and *jar1* plants, it failed to activate LR formation in *drr1* (Fig. 11; Supplemental Fig. S1). Comparison of the primary root response to JA also showed that *drr1* behaves essentially different from *jar1*, which was very insensitive to primary root growth inhibition by JA but responded similarly to wild-type plants in LR induction by this compound. Therefore, *drr1* shows alkamide resistance in terms of primary and LR growth, whereas it has resistance to jasmonate in LR formation only. Taking into account these results, it is tempting to speculate that further cross talk of alkamide signaling with phytohormones such as cytokinins or jasmonates might vary in different tissues or in a developmental context, possibly explaining why *drr1* mutants exhibit no defects in primary root growth inhibition assays to these phytohormones.

In summary, we have provided evidence that alkamide and AHL signaling are under genetic control in Arabidopsis and that normal responses to these signals are important for plant development. Elucidation of the genetic identity of the *DRR1* product is critical to understand the molecular mechanisms underlying the distinct effects of these and other small lipid signals on root architecture adjustment and their role in plant longevity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type plants (Col-0 and/or Ws ecotypes), the transgenic line *CyCB1:uidA* (Colón-Carmona et al., 1999), and the mutant lines *jar1* (Tiryaki and Staswick, 2002), *axr2-1* (Timpte et al., 1994), and *aux1-7* (Picket et al., 1990) were used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog, 1962). MS medium (MS basal salts mixture; catalog no. M5524) was purchased from Sigma. The suggested formulation is 4.3 g L^{-1} salts for a 1× concentration of medium; we used 0.9 g L^{-1}, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant grown chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 μmol m^{-2} s^{-1}, and temperature of 22°C.

Mutant Isolation Procedure

T-DNA lines (Ws; Krysan et al., 1999) were provided by the Ohio Arabidopsis Seed Stock Center. Seeds were surface sterilized and plated on 0.2× MS medium supplied with 30 μM *N*-isobutyl decanamide. A number of approximately 25,000 T-DNA lines were screened for reduced LR formation

by placing seeds on nutrient agar plates (20–25 seeds per plate). The seeds were distributed in two rows on the agar surface at a density of one seed per centimeter, stratified at 4°C for 48 h, and then incubated at 22°C. Fourteen days after germination, *N*-isobutyl decanamide-treated plants have a short primary root and a large number of LRs are formed. Putative mutants with long primary roots and a reduced number of LRs were selected, transferred to soil, and allowed to self-fertilize. Homozygous M3 seeds were rescreened for sustained primary root growth in medium supplied with 30 μM *N*-isobutyl decanamide, transferred to soil, and backcrossed three times to the wild type (Ws) to remove unlinked mutations.

Genetic Analysis of *drr1* Mutants

To determine the segregation pattern of the *drr1* phenotype, 990 F2 seedlings derived from the cross *drr1* × Ws were analyzed in MS 0.2× agar medium supplied with 30 μM *N*-isobutyl decanamide. A typical 3:1 recessive segregation was observed for the wild-type/*drr1* phenotype. Cosegregation of primary root growth resistance and increased longevity was further confirmed in *drr1* seedlings grown in soil.

Hormone Treatments

For all experiments, MS 0.2× nutrient medium was supplemented with *N*-isobutyl decanamide, C10-AHL, or the indicated phytohormones. Ethanol-dissolved compounds were added to cooled (50°C) molten medium and poured onto plates. Control plates were supplied with the greatest concentration of solvent used in the treatments. For hormone-induced senescence, leaves at 22 d after leaf emergence were detached and floated on sterilized water in the presence or absence of 35 μM JA or 35 μM *N*-isobutyl decanamide for 6 d. All treatments were performed at 22°C under dark conditions. Chemicals were purchased from Sigma Chemical.

Analysis of Growth and Statistical Analysis

Growth of primary roots was registered using a rule. LR number and LR density were determined by counting the LRs present in the primary root from the tip to the root/stem transition. LR density was determined by dividing the LR number by the primary root length and expressed as LR density per centimeter. The length of the meristem was determined as the distance between the quiescent center and the cell file where cells started to elongate. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with a Tukey's posthoc test were used for testing differences in growth and root developmental responses in wild-type and mutant lines. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Determination of Developmental Stages of LRP

LRP were quantified at day 4 after germination. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LR primordium was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follows. Stage I, LRP initiation; in the longitudinal plane, approximately eight to 10 "short" pericycle cells are formed. Stage II, the formed LR primordium is divided into two layers by a periclinal division. Stage III, the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV, LR primordium with four cell layers. Stage V, the LR primordium is midway through the parent cortex. Stage VI, the LR primordium has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII, the LR primordium appears to be just about to emerge from the parent root.

Chlorophyll Determination

We used leaves from wild-type (Ws) and *drr1* plants germinated and grown on 0.2× MS medium and then transferred to soil for 35 d. Wild-type leaves were yellowed as a result of age-dependent senescence; *drr1* leaves remained green at this stage. We used a hand-held chlorophyll meter (CCM-200; Opti-Sciences) to calculate a chlorophyll content index based on absorbance measurements at 660 and 940 nm on 15 independent leaves. Five separate measurements with the hand-held meter were made on each leaf. Chlorophyll content was finally determined as described previously (Richardson et al., 2002).

Histochemical Analysis of GUS Activity

Transgenic plants that express the *uidA* reporter gene (Jefferson et al., 1987) were stained in 0.1% 5-bromo-4-chlorium-3-indolyl-β-D-glucuronide in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37 °C. Plants were cleared and fixed as described previously by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.

Microscopy

The Arabidopsis root system was analyzed with a stereoscopic microscope (MZ6; Leica Microsystems). Total LRs were counted at 30× magnification. Primary root meristems were analyzed in semipermanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus; Carl Zeiss) at 100× or 400× magnification. Images were captured with a Cyber-shot DSC-S75 digital camera (Sony Electronics) adapted to the microscope and processed with the Axio Vision 4AC software (Carl Zeiss).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *drr1* shows defective LR formation in response to JA treatment.

Supplemental Figure S2. Effects of kinetin, abscisic acid, and ethylene on primary root growth in wild-type and *drr1* plants.

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Regular Paper

Serotonin, a Tryptophan-Derived Signal Conserved in Plants and Animals, Regulates Root System Architecture Probably Acting as a Natural Auxin Inhibitor in *Arabidopsis thaliana*

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Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter in mammals and is widely distributed in plants. This compound is synthesized from tryptophan and shares structural similarity with IAA. To date, little is known about the morphological, physiological and molecular responses of plants to serotonin. In this study, we characterized the effects of serotonin on growth and development in *Arabidopsis thaliana* seedlings. Gas chromatography–mass spectrometry (GC-MS) analysis showed that plants are able to take up serotonin from the growth medium, which coincided with greatly stimulated lateral root development at concentrations from 10 to 160 μM. In contrast, higher doses of serotonin repressed lateral root growth, primary root growth and root hair development, but stimulated adventitious root formation. To investigate the role of serotonin in modulating auxin responses, we performed experiments using transgenic Arabidopsis lines expressing the auxin-responsive marker constructs *DR5:uidA*, *BA3:uidA* and *HS::AXR3NT-GUS*, as well as a variety of Arabidopsis mutants defective at the *AUX1*, *AXR1*, *AXR2* and *AXR4* auxin-related loci. We found that serotonin strongly inhibited both *DR5:uidA* and *BA3:uidA* gene expression in primary and adventitious roots and in lateral root primordia. This compound also abolished the effects of IAA or naphthaleneacetic acid on auxin-regulated developmental and genetic responses, indicating an anti-auxin activity in the plant. Mutant analysis further showed that lateral root induction elicited by serotonin was independent of the *AUX1* and *AXR4* loci but required *AXR1* and *AXR2*. Our results show that serotonin regulates root development probably by acting as a natural auxin inhibitor.

Keywords: Arabidopsis • Auxin signaling • Root architecture • Serotonin.

Abbreviations: ARF, auxin response factor; ER, endoplasmic reticulum; GC-MS, gas chromatography–mass spectrometry; GUS, β-glucuronidase; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; NPA, 1-naphthylphthalamic acid; LR, lateral root; LRP, lateral root primordium; PAT, polar auxin transport; PCIB, *p*-chlorophenoxyisobutyric acid; RNAi, RNA interference; TDC, tryptophan decarboxylase; TIBA, triiodobenzoic acid; WT, wild-type; YFP, yellow fluorescent protein.

Introduction

Plants synthesize and use a variety of signals to adjust growth and development throughout their life cycle. Auxins, including IAA, comprise a group of tryptophan-derived signals, which are involved in most aspects of plant development (Woodward and Bartel 2005). Extensive studies over the past decade have investigated the factors involved in the regulation of plant morphogenesis by auxins. These compounds exert a strong biological activity at very low concentrations in both in vivo and in vitro systems and are essential for maintenance of physiological and morphogenetic processes including gravity and light responses, root hair development, and lateral root (LR), adventitious root and shoot system development (Woodward and Bartel 2005). Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms of auxin, and signal perception through a family of IAA receptors (Ljung et al. 2002, Leyser 2006, Mockaitis and Estelle 2008).

Although IAA is among the most highly characterized metabolites of tryptophan, relatively high levels of IAA-related compounds have been reported in plants such as the mammalian neurotransmitter serotonin (5-hydroxytryptamine). This compound is a ubiquitous signal, which plays multiple roles in neurotransmission, hormone and mitogenic functions as well as acting in immunomodulatory and anti-inflammatory processes in animal cells (Frazer and Hensler 1999). In plants, serotonin has been found in roots, leaves, fruits and seeds from

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at least 42 species (Grose 1982, Engstrom et al. 1992, Roshchina 2001). Serotonin has been implicated in important physiological and developmental functions including flowering, senescence, shoot formation and defense responses (Odjakova and Hadjiivanova 1997, Murch et al. 2001, Roshchina 2001, Ishihara et al. 2008, Kang et al. 2009a).

Serotonin biosynthesis occurs via two enzymatic steps. Tryptophan decarboxylase (TDC) catalyzes the conversion of tryptophan into tryptamine, followed by tryptamine 5-hydroxylase (T5H), which hydroxylates the C-5 position of tryptamine to form serotonin (Kang et al. 2007a, Kang et al. 2007b, Kang et al. 2008, Kang et al, 2009b). In leaves and seeds of rice (*Oryza sativa*) plants, TDC expression is very low or negligible. In leaves, the serotonin concentration was reported to be around $0.3\,\mu g\,g^{-1}$ FW. However, levels greatly increase in response to senescence signals such as nutrient deprivation or leaf detachment, and serotonin synthesis is closely coupled with transcriptional and enzymatic induction of tryptophan biosynthetic genes as well as TDC in senescing leaves (Kang et al. 2009a). Transgenic rice plants overexpressing TDC produced 25-fold higher serotonin levels than wild-type (WT) plants and showed delayed leaf senescence, whereas lines in which expression of TDC was suppressed through an RNA interference (RNAi) system produced less serotonin and senesced faster than the WT line (Kang et al. 2009a). Serotonin accumulation was also reported to occur in rice leaves infected by the fungal pathogen *Bipolaris oryzae* (Ishihara et al. 2008). Serotonin accumulation was preceded by a transient increase in tryptamine content and by marked activation of TDC. Serotonin treatment suppressed the growth of fungal hyphae, indicating that the activation of the tryptophan pathway is involved in the establishment of effective defenses against the pathogen through serotonin production in rice plants. Collectively, this information indicates that serotonin levels in plant tissues may increase by demand, under particular developmental transitions or when challenged by pathogens.

Although serotonin is naturally present in a wide variety of plants, little is known about the molecular mechanisms involved in plant developmental responses to this compound. The Arabidopsis root system is an excellent model to characterize the effects of compounds with novel and interesting activities in plants (López-Bucio et al. 2006, Contreras-Cornejo et al. 2009). Roots perform the essential activities of providing water, nutrients and physical support to the plant. The primary root originates in the embryo and produces many LRs during the lifetime of a plant, and each of these will produce more LRs. The quantity and placement of these structures determine the architecture of the root system, and this in turn plays a major role in determining whether a plant will survive in a particular climate or environment (Malamy and Benfey 1997b, Casimiro et al. 2003, López-Bucio et al. 2005). During the post-embryonic development of plants, new axes of growth emerge from shoot tissues through adventitious organogenesis. This is particularly important in crops such as maize, in which adventitious root formation provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or after injury. While LRs typically form from lateral root primordia (LRPs) initiated on the primary root pericycle, adventitious roots form naturally from stem tissue. LR and adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones such as auxin, and environmental factors such as light and nutrient deprivation (Casimiro et al. 2003, López-Bucio et al. 2003, Péret et al. 2009).

The control of post-embryonic root growth and LR formation is tightly regulated by auxin (IAA). IAA moves throughout the plant in the phloem or by a more controlled polar transport system [polar auxin transport (PAT)]. PAT is a process regulated by AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) uptake proteins, PIN-FORMED (PIN) efflux carriers and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Swarup et al. 2004; Mravec et al. 2008). There are several Arabidopsis mutants defective in the production of auxin transport proteins or in the correct location of these proteins that show auxin-related phenotypes, including *aux1-7* and *axr4-1*. The *aux1-7* mutant is defective at the *AUX1* locus encoding an auxin influx transporter (Swarup et al. 2004), while the *axr4-1* mutant is defective in an accessory protein of the endoplasmic reticulum (ER) that regulates localization of AUX1 proteins. Loss of AXR4 results in abnormal accumulation of AUX1 in the ER of epidermal cells, indicating that the *axr4* agravitropic phenotype is caused by defective AUX1 trafficking in the root epidermis (Dharmasiri et al. 2006).

Auxin is perceived by the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN 1-3 (TIR1/AFB1-3) receptor family. TIR1 is part of the ubiquitin–ligase complex $SCF^{TIR1/AFB}$ that catalyzes the ubiquitination and destruction of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (Gray et al. 2001, Dharmasiri et al. 2005, Kepinski et al. 2005). These proteins, under low auxin concentrations, form dimers with the auxin response transcription factors (ARFs), thereby blocking their activity. The Arabidopsis *auxin-resistant 1* (*axr1*) mutants were initially isolated in a screen for auxin non-responsive seedlings (Lincoln et al. 1990). Subsequent analysis demonstrated that AXR1 is a subunit in the related to ubiquitin (RUB)-activating enzyme, the first enzyme in the pathway that conjugates the ubiquitin-related protein RUB to members of the ubiquitin protein ligases (del Pozo et al. 2002). Once freed from the AUX/IAAs, ARFs regulate the expression of auxin-responsive genes (Lau et al. 2008, Kieffer et al. 2010). Gain-of-function mutations in *IAA3/SHY2*, *IAA7/AXR2*, *IAA12/BDL*, *IAA14/SLR*, *IAA18/CRANE*, *IAA19/MSG2* and *IAA28* genes lead to plants with altered root development (Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Uehara et al. 2008). The *slr* and *iaa28* gain-of-function mutants show a strong reduction in LR formation (Rogg et al. 2001, Fukaki et al. 2002). These observations indicate that the AUX/IAA proteins function as negative regulators of LR development, although a detailed direct

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comparison of the LR phenotypes of these mutants has not been performed.

Based on its structural similarity to IAA, we hypothesized that serotonin might act through a canonical auxin signaling pathway to modulate developmental responses by either activating or repressing auxin responses. We therefore characterized the effects of serotonin on root system architecture and auxin-mediated responses in *Arabidopsis thaliana*. Interestingly, our results show that the supply of serotonin has a dual effect on LR formation, stimulating it at low (10–160 μM) concentrations, but with repressing effects at higher (150–600 μM) concentrations. Although at high concentrations serotonin also induced adventitious root formation, it repressed typical auxin responses such as primary root growth and root hair formation. Moreover, an analysis of root architecture responses in the *aux1-7*, *axr1-3*, *axr2-1* and *axr4-1* Arabidopsis auxin-related mutants and auxin-inducible gene expression tests revealed that serotonin may act as a natural auxin inhibitor in plants.

Results

Serotonin affects *Arabidopsis thaliana* root system architecture

Serotonin and IAA are tryptophan-derived compounds, with similar chemical structures (**Fig. 1**). To evaluate the effects of serotonin on plant growth and development, we used *A. thaliana* as a model system. *Arabidopsis thaliana* (Col-0) seedlings were grown in Petri plates containing solid 0.2× Murashige and Skoog (MS) medium supplemented with the solvent or with increasing concentrations of serotonin from 10 to 160 μM. Twelve days after germination, the primary root length, LR number and LR density were determined for 30 seedlings. We found that serotonin strongly promoted LR development, without affecting primary root growth. This leads to plants with increased LR number and density (LR cm^{-1}) (**Fig. 2A–D**).

An important developmental trait widely used to monitor auxin responses is primary root growth (Woodward and Bartel 2005). To determine whether serotonin treatments of >160 μM could affect primary root growth and other root architectural parameters, Arabidopsis seedlings were supplied with 150–600 μM serotonin. It could be seen that both LR number and density increased at a serotonin concentration of 150 μM but decreased at greater concentrations of this compound (**Fig. 3B, C**). **Supplementary Fig. S1** illustrates the root architectural responses of Arabidopsis seedlings to high serotonin concentrations; it can be seen that this compound dramatically inhibits primary root growth while promoting root branching caused by proliferation of adventitious roots. Our results show that serotonin has a dual effect in modulating root system architecture, promoting LR development at low concentrations (10–160 μM) but inhibiting primary root growth and LR development at higher concentrations.

Fig. 1 Comparative chemical structures of serotonin (*N*-acetyl-5-hydroxytryptamine), IAA and their common precursor tryptophan.

Serotonin affects cell division and cell growth in Arabidopsis roots

The post-embryonic root developmental effects of high serotonin concentrations in Arabidopsis seedlings suggested that this compound could play an important role in cell division and/or cell elongation. To study the effects of this compound on cell division and elongation, we measured the length of both fully developed cortical cells from the differentiation region and the primary root meristem from 7 d old WT Arabidopsis (Col-0) seedlings. In addition, we analyzed the expression of *pPRZ1:uidA*, which marks only active meristems (Sieberer et al. 2003), and *CyCB1:uidA*, which is expressed only in cells in the G_2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al. 1999). Strong primary root growth inhibition under concentrations of serotonin ≥300 μM correlated with both decreased cell size of cortical cells and the loss of β-glucuronidase (GUS) expression in the primary root meristem of *pPRZ1:uidA* and *CyCB1:uidA* transgenic seedlings (**Fig. 4A–C**). In addition to these effects, meristem length significantly decreased from 300 μm in solvent-treated seedlings to 220 μm at a concentration of 600 μM serotonin. These results indicate that serotonin inhibits primary root growth by affecting both cell division and elongation.

Serotonin induces lateral root growth but not lateral root primordia initiation

To determine whether serotonin promotes LR development by stimulating LRP growth or inducing de novo formation of LRPs, or modulating both of these processes, we investigated the stages of LRP development affected by serotonin. LRPs were quantified 7 d after germination in plants treated with the solvent or with 150 μM serotonin, which increases LR number and density without affecting primary root growth (**Fig. 2**). Seedling roots were first cleared to enable LRPs at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997a). We found that the stage distribution of LRPs was affected by treatment with serotonin. In particular, LRP stage I, which describes LRPs at the earliest stage of

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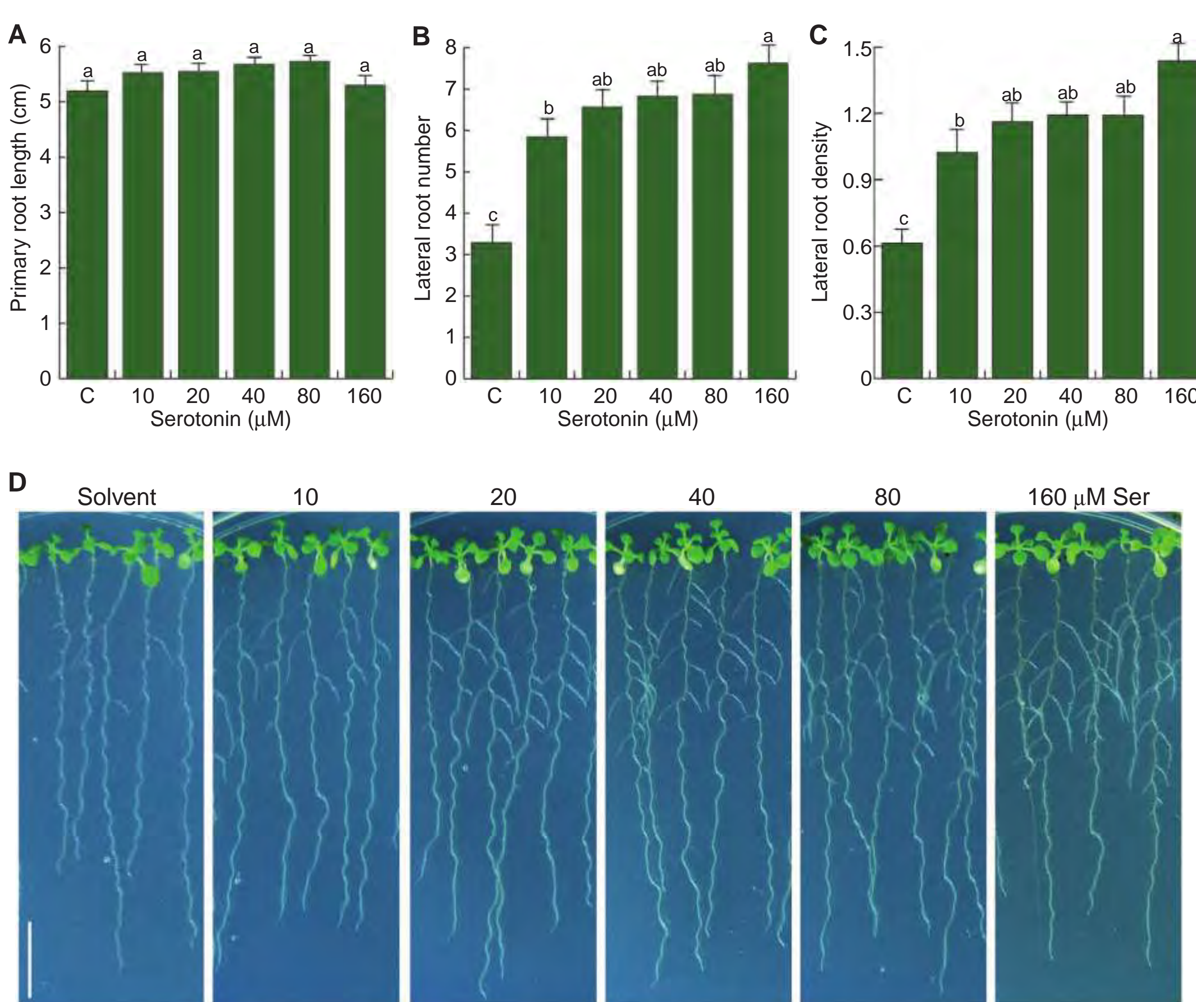

Fig. 2 Effects of low serotonin concentrations on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 12 d under increasing serotonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. (D) Representative photographs of Arabidopsis seedlings grown in the indicated serotonin treatments. The experiment was repeated twice with similar results. Scale bar = 1 cm.

development, was significantly decreased in serotonin-treated seedlings (Fig. 5A). In marked contrast, LRP stage VII, covering the most developed LRPs giving rise to emerged LRs, was induced 3-fold by serotonin (Fig. 5A). The total number of LRPs per seedling did not change in response to serotonin treatments (Fig. 5B). These data suggest that serotonin did not induce de novo LRP initiation and probably increases root branching in Arabidopsis by inducing the maturation of pre-formed LRPs from pericycle cells.

Serotonin promotes adventitious root development

To determine whether serotonin is involved in regulation of proliferative events in the shoot system, we assessed its regenerative properties by cultivating stem explants from etiolated Arabidopsis seedlings under increasing concentrations of serotonin and monitoring adventitious root formation as reported by Campos-Cuevas et al. (2008). Arabidopsis explants treated with 150–600 μM serotonin showed a roughly 2-fold increase in adventitious root number compared with solvent-treated explants (Fig. 6A). Fig. 6B and C shows representative photographs of the effects of serotonin on adventitious root formation. This result illustrates that serotonin is a compound with a strong effect on Arabidopsis adventitious root organogenesis.

Serotonin inhibits root hair development and expansin gene expression

The serotonin effects of inhibiting primary root growth and promoting adventitious root formation are reminiscent of those caused by treating plants with auxins (Woodward and Bartel 2005). Auxins have also been found to induce root hair development in several plant species (Parker et al. 2000). To determine whether serotonin could affect root hair development, we performed experiments in which Arabidopsis seedlings were germinated and grown in Petri plates containing 0.2× agar–MS medium supplemented with increased

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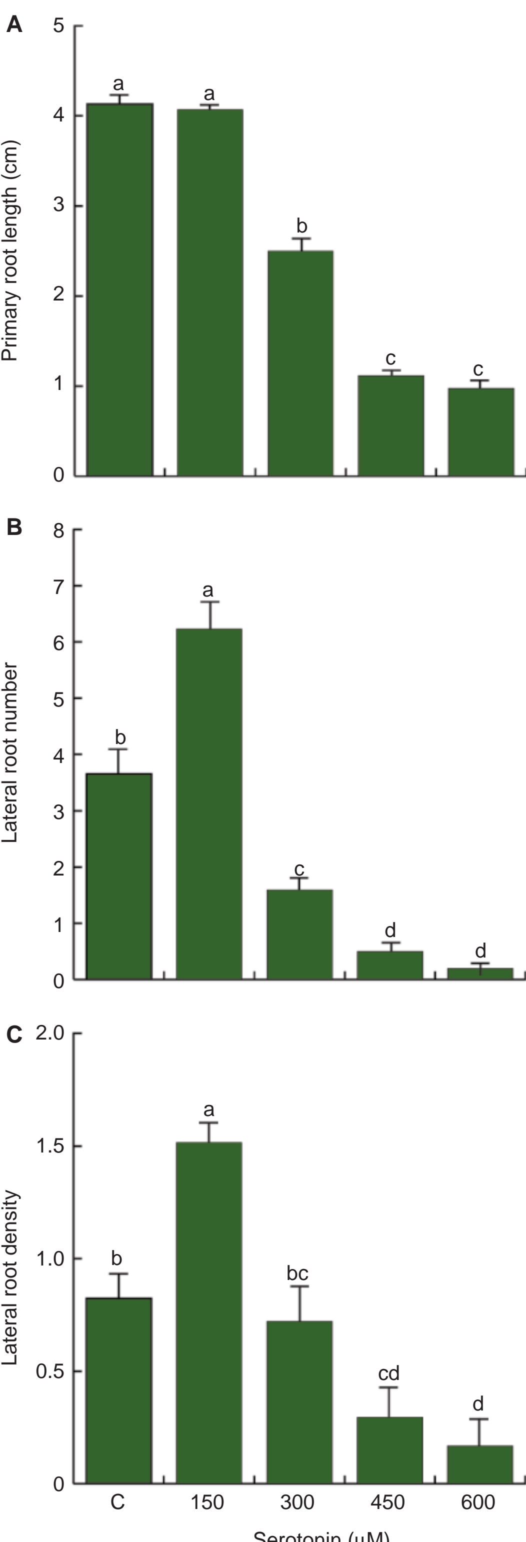

Fig. 3 Effects of high serotonin concentrations on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 10 d under increasing serotonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

concentrations of the compound, and 5 d after germination root hairs from the differentiation and maturation zones of the primary root were analyzed. In marked contrast to adventitious root development, serotonin treatments dramatically inhibited root hair development both in the differentiation zone and in the maturation zone (**Fig. 7A**). Next, we determined whether the compound altered root hair initiation, root hair elongation or both, by microscopically counting and measuring trichoblast cells present in the maturation zone of the primary root. It was found that 150 μM serotonin significantly inhibited root hair growth, while increased concentrations dramatically blocked hair growth (**Fig. 7B**). The root hair density analysis showed that serotonin also inhibited root hair formation in a dose-dependent way (**Fig. 7C**). To determine whether serotonin affects root hair formation at an early step in development, we used the *pAtEXP7:uidA* transgenic line, which expresses GUS in trichoblast cells and is a marker of root hair initiation (Cho and Cosgrove 2002). Serotonin produced a deficient cell differentiation program in root epidermal cells, evidenced by loss of GUS expression in trichoblast cells in serotonin-treated seedlings (**Fig. 7D**).

GC-MS analysis of serotonin levels in *A. thaliana* seedlings

To determine whether serotonin is naturally produced in *A. thaliana* and if the effects observed on root system architecture correlated with serotonin accumulation in plant tissues, we performed experiments to quantify serotonin from root and shoot tissues from solvent- or serotonin-treated WT (Col-0) seedlings by using gas chromatography–mass spectrometry (GC-MS) analysis. Small yet detectable amounts of serotonin were identified in root and shoot tissue of solvent-treated Arabidopsis seedlings; however, serotonin levels in plant tissues dramatically increased in seedlings treated with this compound (**Fig. 8A**). Serotonin is acetylated in the derivatization process by acetic anhydride, producing *N*-acetylserotonin (**Fig. 8B**). **Fig. 8C and D** shows mass spectra of the *N*-acetylserotonin standard and the extracted sample, respectively. Representative chromatograms of root and shoot samples from solvent- or serotonin-treated seedlings are shown in **Fig. 8E–H**. These findings provide the first evidence that serotonin is produced naturally in Arabidopsis, and that plants are able to take up serotonin from the growth medium.

Serotonin inhibits auxin-inducible gene expression

We next investigated whether serotonin acts in an auxin-related signaling pathway by analyzing the expression of the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers. Since low serotonin concentrations activate LR development by inducing LRP outgrowth (**Figs. 2, 5**), we first determined histochemical GUS expression during LRP development in 7 d transgenic *DR5:uidA* Arabidopsis seedlings, in response to the solvent or 150 μM serotonin. Interestingly, serotonin clearly

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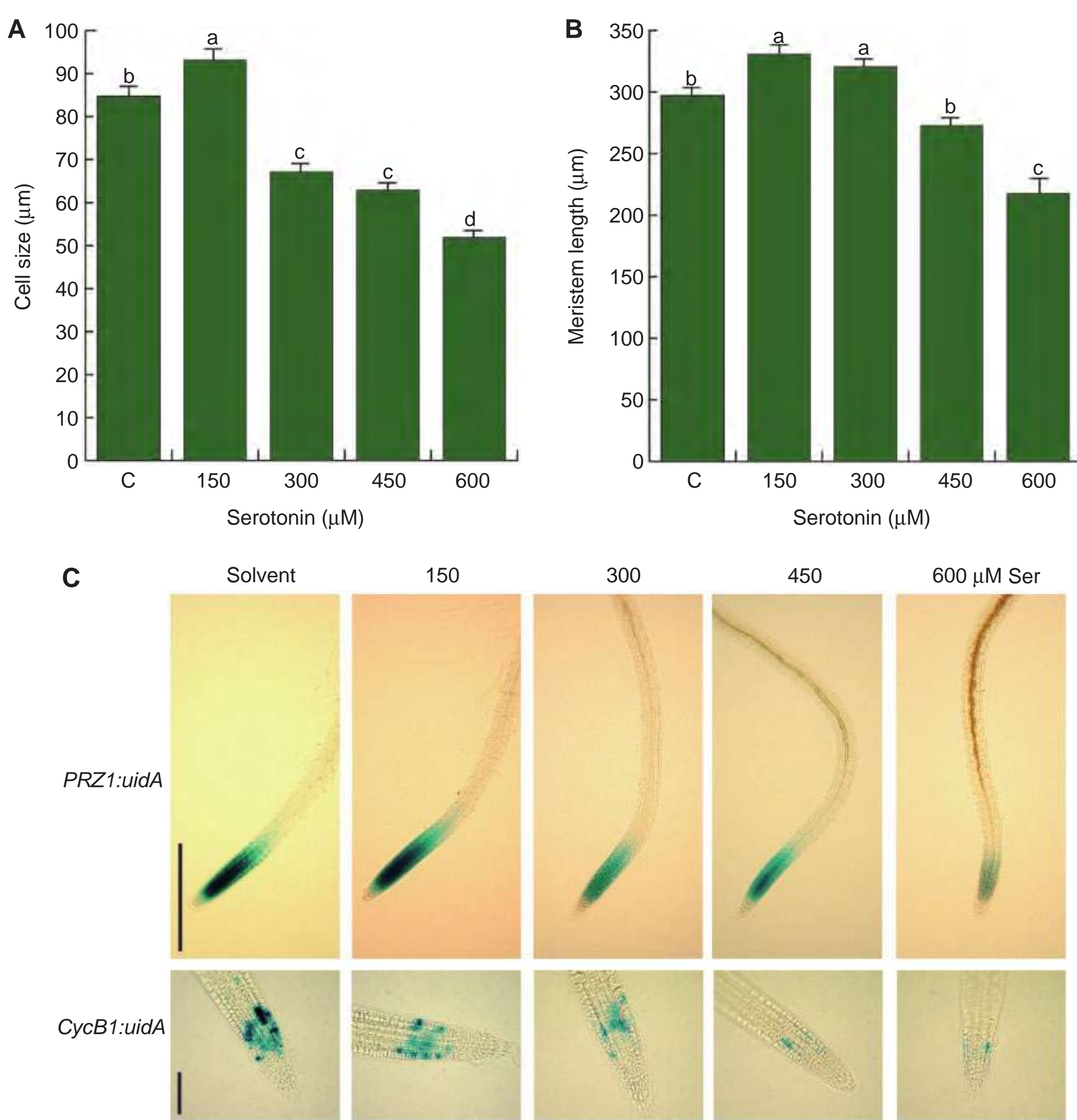

Fig. 4 Effect of serotonin on cell division and elongation. WT (Col-0), *pPRZ:uidA* and *CycB1:uidA A. thaliana* seedlings were grown for 7 d on 0.2× MS medium supplemented with the indicated concentrations of serotonin. (A) Mean epidermal cell length. (B) Meristem length. Data points represent the mean ± SD ($n = 30$). (C) Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from at least 15 stained plants. The experiment was replicated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Scale bars in *pPRZ:uidA* images = 500 µm, and in *CycB1:uidA* images = 50 µm.

inhibited *DR5:uidA* expression in LRPs from all tested developmental stages (**Fig. 9A**). We also tested the response of the markers to a high serotonin concentration by analyzing histochemical staining of transgenic *DR5:uidA* and *BA3:uidA* Arabidopsis seedlings that were grown for 7 d in 0.2× agar–MS medium and then transferred to liquid 0.2× MS medium supplemented with the solvent, 5 µM IAA or 450 µM serotonin, and incubated for 9 h at 22°C. As previously reported (Ulmasov et al. 1997), in solvent-treated *DR5:uidA* seedlings, GUS expression was absent from cotyledons and leaves and was expressed primarily in the root tip region (**Fig. 9B**). *DR5:uidA* seedlings grown under a concentration of 5 µM IAA showed strong GUS activity throughout the plant (**Fig. 9B**). The pattern of GUS expression in *DR5:uidA* seedlings treated with 450 µM serotonin further decreased when compared with solvent-treated plants (**Fig. 9B**), indicating the lack of auxin activity for this compound. Untreated *BA3:uidA* seedlings did not show detectable levels of GUS activity (**Fig. 9C**), whereas, when treated with 5 µM IAA, they showed GUS expression mainly in the petioles of the cotyledons (**Fig. 9C**) and in the root elongation zone (**Fig. 9C**). GUS expression in seedlings treated with serotonin was undetectable (**Fig. 9C**), indicating that this compound failed to activate *BA3:uidA* expression. We also analyzed *DR5:uidA* expression in developing adventitious roots from

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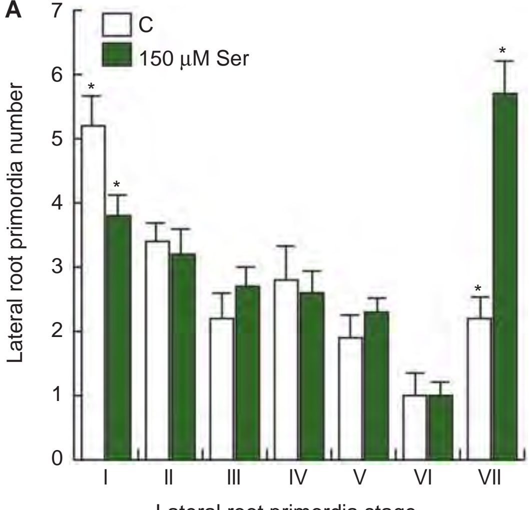

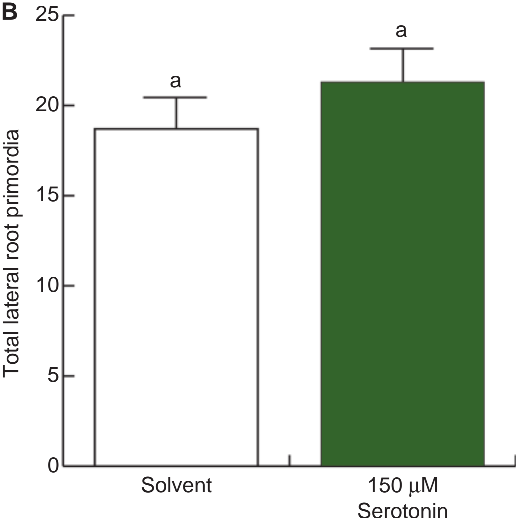

Fig. 5 Effects of serotonin on lateral root primordia development. Arabidopsis Col-0 seedlings were grown for 7 d on agar plates supplemented with the solvent or with 150 μM serotonin. Data are presented for LRP developmental stages (A) and total LRPs per seedling (B). LRP stages were recorded according to Malamy and Benfey (1997a). Values shown represent the mean of 15 seedlings ± SD. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Arabidopsis seedlings treated with 150–600 μM serotonin (**Supplementary Fig. S2**). A dose–response inhibitory effect on GUS expression was clearly observed, indicating that serotonin did not stimulate but rather repressed auxin-inducible gene expression.

To determine in more detail the possible mechanism of action of serotonin, we performed competence assays by using the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers. Transgenic Arabidopsis seedlings expressing each of these markers were grown for 7 d in 0.2× agar–MS medium and then transferred to liquid 0.2× MS medium supplemented with the solvent, 1 μM IAA or naphthaleneacetic acid (NAA), 450 μM serotonin or each auxin in combination with serotonin. When serotonin and IAA were supplied together, a marked reduction of auxin-induced *DR5:uidA* and *BA3:uidA* expression was evident (**Fig. 10A**). Serotonin also antagonized the effects of NAA on auxin-inducible gene expression when supplied at 450 μM (**Fig. 10B**) or under a lower concentration, namely 150 μM (**Supplementary Fig. S3**). These results suggest that serotonin may act as a competitive inhibitor of auxin-regulated gene expression in Arabidopsis.

Serotonin did not antagonize auxin-mediated Aux/IAA protein degradation

Auxin promotes the degradation of Aux/IAA repressor proteins via the ubiquitin–proteasome pathway and thereby induces primary auxin-responsive gene expression (Gray et al. 2001). To address the effect of serotonin on auxin-mediated degradation of Aux/IAA proteins, we examined the effect of IAA and serotonin on Aux/IAA stability using the Arabidopsis *HS::AXR3NT-GUS* line, in which a translational fusion between domains I and II of AXR3 and the GUS reporter protein is expressed under the control of a heat shock promoter (Gray et al. 2001). Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37°C for 2 h and further treated with 5 μM IAA, 450 μM serotonin or 5 μM IAA plus 450 μM serotonin for 10, 30 and 60 min. Treatment with IAA showed enhanced degradation of the fusion protein in cotyledons and in the primary root, but serotonin failed to induce degradation of the fusion protein even after 60 min of treatment (**Fig. 11A–L**). Moreover, this compound stabilized the expression of this marker in cotyledons (compare **Fig. 11A–C with I–K**), indicating auxin antagonist activity. When both compounds were supplied together, the effect of IAA predominates, giving rise to a GUS expression pattern similar to that observed in Arabidopsis seedlings treated with IAA alone (**Fig. 11M–P**). Our data indicate that serotonin acts independently of auxin or downstream of auxin receptors, which modulate the degradation of the AXR3 protein.

Effects of serotonin on auxin-induced lateral root formation

Because serotonin strongly inhibited the expression of the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers, it was possible that the repressing effects of this compound on root hair and LR development could be due to serotonin acting as an auxin inhibitor. We next evaluated the LR responses of WT (Col-0) seedlings to serotonin and NAA by growing Arabidopsis WT (Col-0) seedlings on Petri plates containing 0.2× agar–MS medium supplemented with different concentrations of serotonin, NAA or NAA plus serotonin. Seven days after germination, primary root length and LR number were quantified. In these experiments, serotonin concentrations of 150 μM did not affect primary root growth (**Fig. 12A**), but significantly increased LR number (**Fig. 12B**). NAA treatment or NAA plus

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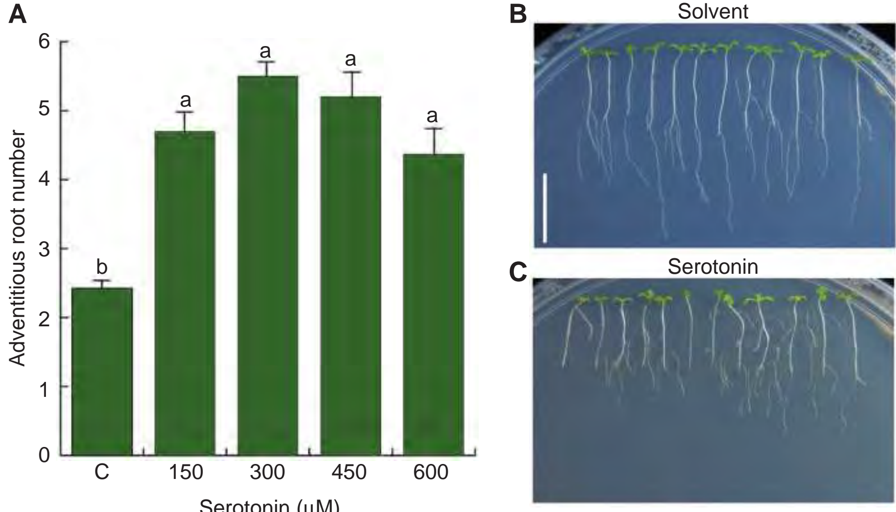

Fig. 6 Effects of serotonin on adventitious root development from Arabidopsis shoot explants. Arabidopsis seedlings were germinated and grown in darkness for 5 d on the surface of agar plates containing 0.2× MS medium and hypocotyl explants were obtained. Hypocotyl explants were transferred to 0.2× MS medium containing the indicated concentrations of the compound and cultivated for a further 9 d period to quantify adventitious root formation. (A) Adventitious root number in response to serotonin treatments. (B) Representative photograph of solvent-treated Arabidopsis (Col-0) explants. (C) Photograph of explants supplied with 450 μM serotonin. Values shown in (A) represent the mean ± SD ($n = 20$). Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. Scale bar = 1 cm.

150 μM serotonin showed a strong effect of induced root branching. Interestingly, serotonin specifically induced LR growth when supplied together with NAA, indicating that it alleviates the growth-repressing effects of NAA on LR elongation (**Fig. 12C, D**). Supply of 300 and 450 μM serotonin inhibited both primary root growth and LR formation. In contrast, concentrations of 0.1 and 0.2 μM NAA, which inhibit primary root growth, strongly stimulated LR formation (**Supplementary Fig. S4**). Although treatment of seedlings with both serotonin and NAA showed an additive effect on primary root growth inhibition, we found that serotonin had an antagonistic effect on LR response to NAA by decreasing LR formation (**Supplementary Fig. S4**). These results imply that although the effects of serotonin and NAA on primary root growth are similar, they act in an opposite fashion to regulate LR development.

Effect of serotonin on root architectural traits in auxin-related Arabidopsis mutants

To evaluate at the genetic level the role played by selected auxin-related loci in serotonin responses, we compared the primary root growth and adventitious root formation of WT (Col-0) seedlings and the *axr2-1*, *axr4-1*, *aux1-7* and *axr1-3* Arabidopsis mutants in response to 150 μM serotonin treatment. Serotonin significantly induced both LR number and density in WT and in *axr2-1*, *axr4-1* and *aux1-7* seedlings but not in *axr1-3* mutants (**Fig. 13A–C**). Surprisingly, the *axr2-1* mutants showed increased LR numbers when grown in medium without serotonin, indicating that this mutant is inherently potentiated in LR formation (**Fig. 13B, C**). We also tested the effects of a high serotonin concentration on adventitious root development in intact Arabidopsis seedlings. Supply of 450 μM serotonin caused an 80% inhibition in primary root growth in WT seedlings compared with solvent-treated control seedlings. All four auxin-resistant mutants *aux1-7*, *axr1-3*, *axr2-1* and *axr4-1* showed similarly inhibited primary root growth to WT plants (**Supplementary Fig. S5**). When grown in medium without serotonin, WT and mutant seedlings showed an absence of adventitious roots, whereas when treated with the compound the formation of 3–6 adventitious roots was observed. This effect was similar in WT seedlings and in *axr4-1* and *aux1-7* mutants (**Supplementary Fig.S5**). In contrast, *axr2-1* mutants showed exacerbated responses to the compound while *axr1-3* mutants showed decreased adventitious root numbers (**Supplementary Fig. S5**). **Supplementary Fig. S6** illustrates the root architectural responses of Arabidopsis seedlings to IAA treatments; the primary root growth resistance of the lines used in this study with an almost normal adventitious root induction except in *axr1-3* can be seen. Our results indicate that LR and adventitious root induction by serotonin are independent of the *axr2-1*, *axr4-1* and *aux1-7* loci but require an intact *axr1-3* locus.

Discussion

Serotonin is a highly conserved indolic compound occurring in evolutionarily distinct organisms from humans to plants. The results of research with serotonin have uncovered several facts:

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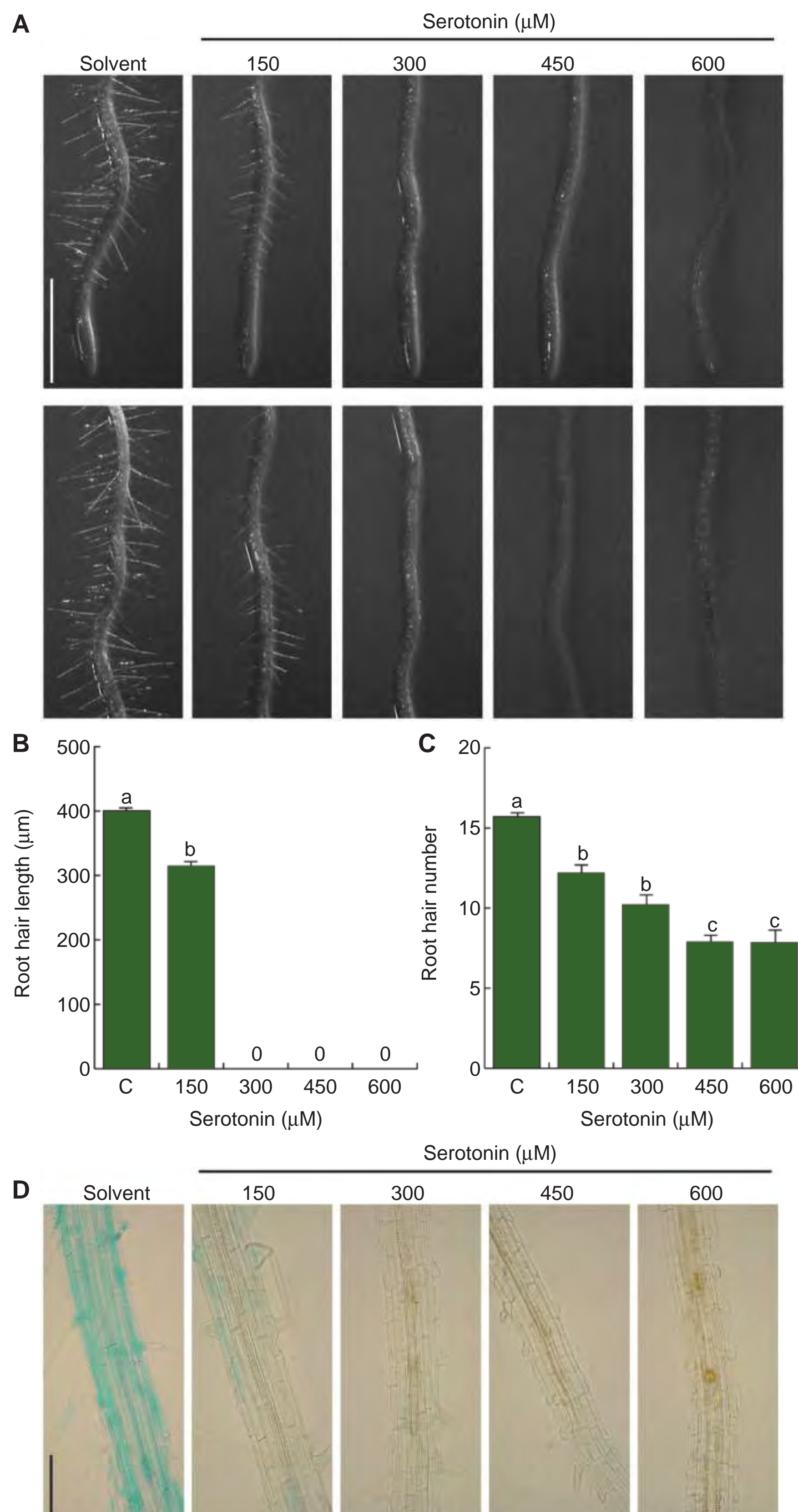

Fig. 7 Effects of serotonin on root hair development. *Arabidopsis thaliana* seedlings were grown for 5 d on 0.2× MS medium supplemented with the indicated concentrations of serotonin. (A) Representative photographs of root hairs formed at the differentiation and maturation regions of

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(i) it is present in a wide number of plant species; (ii) it is produced from tryptophan; and (iii) its concentration may vary in plant tissues or in response to environmental conditions, suggesting important developmental and adaptive functions (Ishihara et al. 2008, Kang et al. 2009a). However, little is known about its signaling role in the plant. In a previous study by Kang et al. (2009a), it was found that serotonin is greatly accumulated in rice (*O. sativa*) leaves undergoing senescence induced by either nutrient deprivation or detachment, and its synthesis is closely coupled with transcriptional and enzymatic induction of the tryptophan biosynthetic genes as well as TDC. Transgenic rice plants that overexpressed TDC accumulated higher levels of serotonin than the WT and showed delayed senescence of rice leaves. In contrast, transgenic rice plants, in which expression of TDC was suppressed through an RNAi system, produced less serotonin and senesced faster than the WT, suggesting that serotonin is involved in attenuating leaf senescence.

Since the role of serotonin in plant development is not well understood, in this work we tested the hypothesis that it could act as a plant growth regulator by probably acting as an auxin or interfering with auxin action. Pharmacological tools that have increased our understanding of auxin signaling are auxin response inhibitors, which belong to two main classes: those that alter auxin transport and those that perturb auxin signaling. Most studies have employed synthetic inhibitors, such as 1-naphthylphthalamic acid (NPA), *p*-chlorophenoxyisobutyric acid (PCIB) and triiodobenzoic acid (TIBA), or small molecule antagonists of the TIR1 receptor function produced by introducing different alkyl chains to the α-position of IAA (Fujita and Syono 1996, Casimiro et al. 2001, Oono et al. 2003, Hayashi et al. 2008). While synthetic auxin inhibitors have provided important information about the molecular mechanisms involved in auxin action, the in planta role of these unnatural compounds is questionable. More recently, naturally occurring tryptophan derivatives such as tryptophan conjugates of jasmonic acid and IAA have been found to interfere with a broad range of auxin-mediated processes (Staswick 2009). Our results show that serotonin may also act as an endogenous auxin inhibitor. We used the Arabidopsis root system to test the effects of exogenously supplied serotonin on several morphogenetic processes including primary root growth, LR formation, adventitious root formation and root hair development, most of which are auxin-regulated processes. We found that serotonin stimulated LR development at concentrations of 10–160 μM by inducing LRP maturation (**Figs. 2**, **5**). This effect correlated with decreased expression of the auxin response marker *DR5:uidA* in LRPs (**Fig. 9A**). These results suggest that under normal growth conditions, auxin synthesis/response in developing LRPs is supraoptimal for LR growth. Serotonin might thus increase LRP maturation by antagonizing auxin signaling in LRPs. However, it also repressed LR formation and root hair growth at higher concentrations but increased adventitious root formation from intact plants and from shoot explants (**Fig. 6**, **7**; **Supplementary Fig. S1**). The activity of serotonin in modulating root growth was lower than that observed for auxins. **Fig. 2** shows that this compound only modestly slowed root growth. Even at 300 μM, primary root growth inhibition was <50% of the control value. By comparison, under similar growth conditions and using the same growth medium, IAA or auxin-related signals produced 50% inhibition at concentrations of at least two orders of magnitude lower than this (Contreras-Cornejo et al. 2009). Intriguingly, another animal neurotransmitter, glutamate, exerted a 60% primary root growth inhibition in WT Arabidopsis (Col-0) seedlings at a 500 μM concentration (Walch-Liu et al. 2006). Similarly to glutamate, serotonin did not interfere with LR initiation but potentiated LR outgrowth. An important difference in the mode of action of serotonin compared with glutamate is the role played by auxin signaling in plant responses to these signals. Two loss-of-function mutants at the *AXR1* locus (*axr1-3* and *axr1-12*) were hypersensitive to glutamate in primary root growth inhibition, whereas *aux1-7* was resistant, indicating that auxin transport and signaling might be important for root responses to glutamate (Walch-Liu et al. 2006). In contrast, primary root growth in both *axr1-3* and *aux1-7* was inhibited similarly to that in WT seedlings when treated with 450 μM serotonin (**Supplementary Fig. S5**).

The effects of serotonin on inhibiting primary root growth and repressing LR formation are similar to those caused by auxin influx or efflux inhibitors such as TIBA and NPA (Fujita and Syono 1996, Casimiro et al. 2001). Serotonin effects also resemble those caused by application of yokonolide B, an inhibitor of auxin action isolated from *Streptomyces diastatochromogenes*, which stimulated LR formation at low concentrations, whereas at higher concentrations it promoted adventitious root development (Hayashi et al. 2003). Although serotonin was detected at low levels in roots and shoots of solvent-treated Arabidopsis seedlings (**Fig. 8**), it may still play a significant role since it increases in concentration under particular developmental transitions and in response to pathogen attack (Ishihara et al. 2008, Kang et al. 2009a). Auxin is very important for root architecture remodeling and it is highly regulated by a complex network of interacting mechanisms; therefore, serotonin as an endogenous auxin inhibitor might be expected to remain low in most tissues under conditions of normal growth.

Fig. 7 Continued
the primary root of 5-day-old Arabidopsis seedlings grown on the surface of agar plates supplemented with the indicated concentrations of serotonin. (B and C) Data points indicate the mean ± SD for root hair length (B) or root hair number (C) of 10 epidermal cells located in a fully differentiated zone of the primary root from 20 seedlings analyzed. (D) *AtExp7:uidA* expression in response to serotonin treatments. These experiments were repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Scale bars in (A) = 500 μm and in (D) = 100 μm.

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Fig. 8 Determination of serotonin from control or serotonin-treated Arabidopsis seedlings by GC-MS. Arabidopsis WT seedlings were germinated and grown for 22 d in 0.2× MS medium supplemented with the solvent or with increased concentrations of serotonin. (A) Serotonin quantification in root and shoot. (B) Acetylation reaction of serotonin with acetic anhydride. (C) The 70 eV electron impact full scan mass spectra from *m/z* 50 to 500 of the *N*-acetylserotonin standard. (D) *N*-Acetylserotonin in a plant extract. (E) Total ion chromatogram of *N*-acetylserotonin from solvent-treated shoots or roots (F), or from shoot and roots of plants treated with 450 µM serotonin, respectively (G–H).

Interestingly, supplementation of serotonin in the growth medium dramatically increased serotonin concentrations in both root and shoot tissues, indicating that Arabidopsis seedlings are able to take up serotonin from the medium and transport it within different plant tissues (**Fig. 8**).

To test whether serotonin may act on auxin transport or signaling, we examined the effect of serotonin on *DR5:uidA* and *BA3:uidA* gene expression induced by auxins with different transport properties. IAA is a substrate for auxin influx carriers, while NAA freely diffuses through membranes (Delbarre et al. 1996, Marchant et al. 1999). Serotonin similarly antagonized auxin-inducible gene expression revealed by both marker lines in response to both IAA and NAA (**Figs. 9**, **10**), suggesting that serotonin does not perturb auxin transport.

Auxin alters the stability of AUX/IAA repressors, and therefore serotonin may act by blocking AUX/IAA protein degradation, thus explaining the inhibitory effects of this compound on *DR5:uida* and *BA3:uidA* gene expression. To test this possibility, we analyzed the effects of serotonin on auxin-induced degradation of an AUX/IAA protein. The Arabidopsis *HS::AXR3NT-GUS* transgenic line strongly expresses an IAA17/AXR3 translational fusion protein under control of a heat shock

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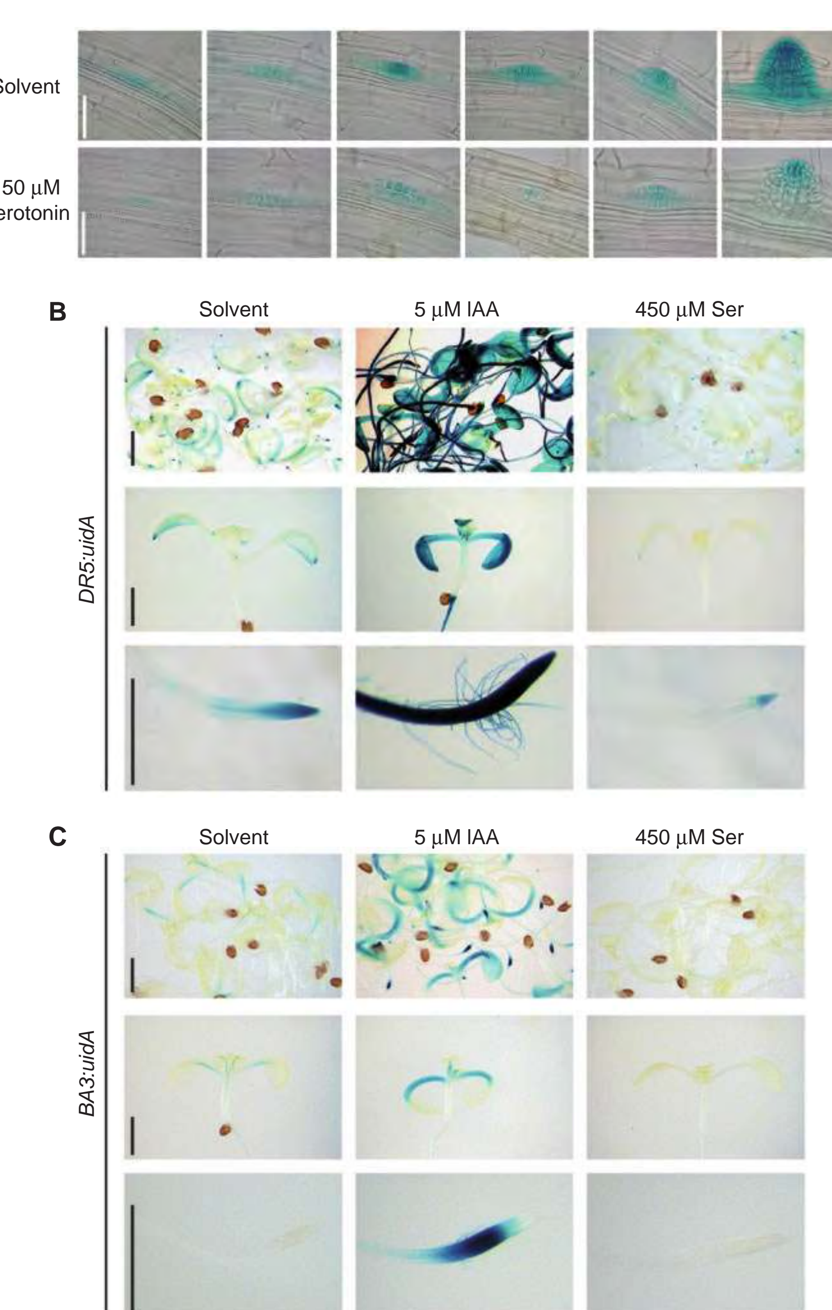

Fig. 9 Effect of serotonin on auxin-regulated gene expression. Twelve hour GUS staining of *DR5:uidA* or *BA3:uidA* Arabidopsis seedlings grown for 7 d on agar plates containing 0.2× MS medium or medium supplemented with IAA or serotonin. (A) *DR5:uidA* expression in lateral root primordia. Comparative effect of IAA and serotonin on *DR5:uidA* (B) and *BA3:uidA* expression (C) Notice the decrease in GUS expression in LRPs, shoots and roots in the treatments with serotonin. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Scale bars in (A) = 50 µm and in (B, C) = 500 µm.

promoter (Gray et al. 2001). The degradation rate of the AUX/IAA fusion protein is rapid and was enhanced by IAA treatment (**Fig. 11**). In contrast, serotonin failed to induce degradation of the fusion protein, indicative of the lack of an auxin activity. When IAA and serotonin are supplemented together, the degradation rate of the AUX/IAA fusion protein is rapid and resembles the effects of applying IAA alone (**Fig. 11**). These data suggest that serotonin may not compete for auxin binding to its receptors or that it acts downstream of auxin receptors, which modulate the degradation of the AXR3 protein.

Serotonin did not suppress the inhibition of primary root growth caused by IAA or NAA treatment (**Fig. 12**, **Supplementary Fig. S4**). However, it stimulates LR growth at low concentrations in the absence of exogenous auxin and

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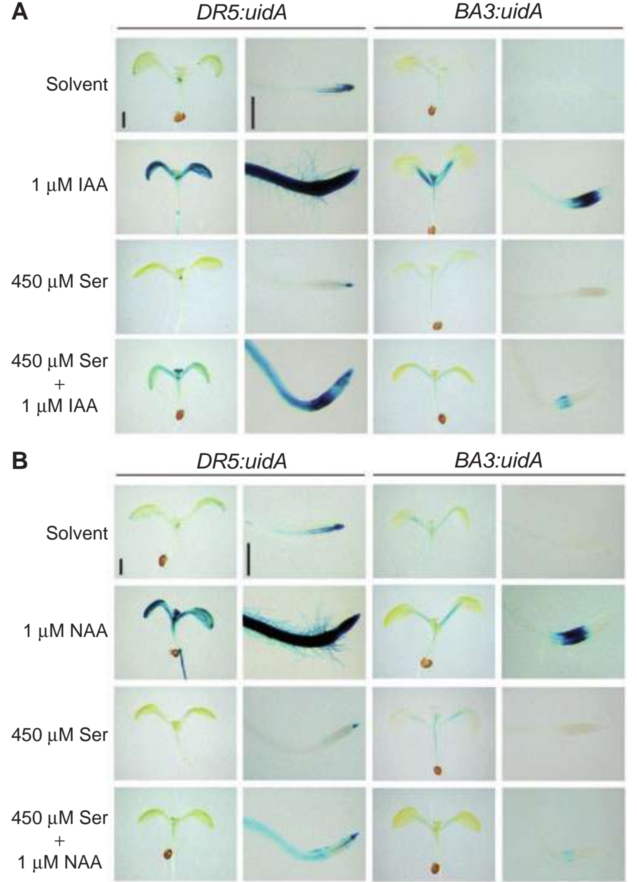

Fig. 10 Serotonin antagonizes the auxin-inducible expression modulated by IAA or NAA treatment. Twelve hour GUS staining of *DR5:uidA* and *BA3:uidA* Arabidopsis seedlings grown for 7 d on agar plates containing 0.2× agar–MS medium and then transferred to 0.2× MS liquid medium supplemented with auxins, with serotonin or both. Note the decrease in GUS expression in shoots and roots in both IAA (A) and NAA (B) treatments by serotonin. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Scale bars = 250 μm.

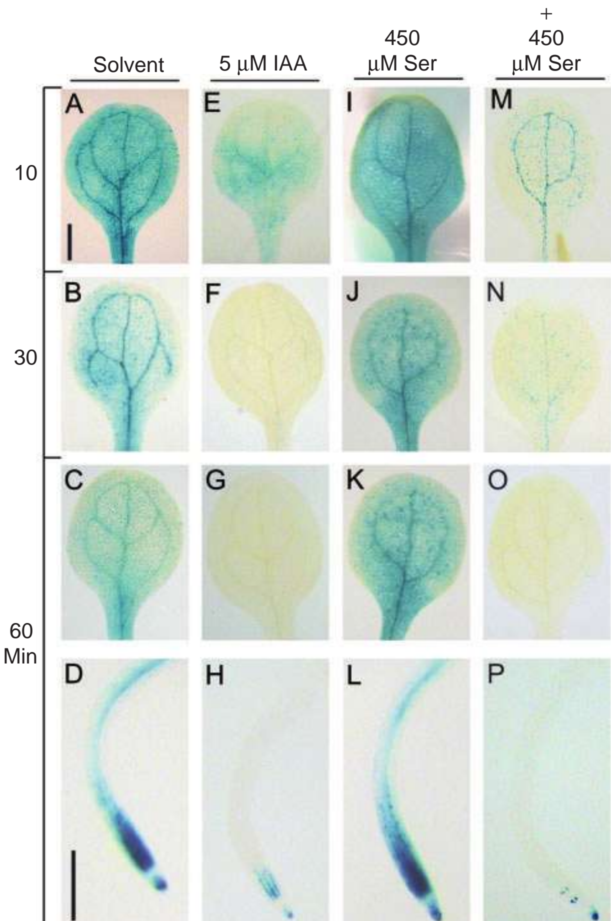

Fig. 11 Analysis of AUX/IAA stability with a *HS::AXR3NT-GUS* fusion. Transgenic seedlings expressing the *HS::AXR3NT-GUS* constructs were heat shocked at 37°C for 2 h. After heat induction, the seedlings were treated with IAA, serotonin or IAA plus serotonin for different times at the indicated concentrations, and stained overnight for GUS activity. Representative photographs of cotyledons from at least 20 stained seedlings are shown. Similar results were obtained in two independent experiments. Scale bars = 250 μm.

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exacerbates the effects of NAA by inducing growth of LRs (**Fig. 12C, D**). High serotonin concentrations (i.e. 450 μM) blocked the stimulation of lateral rooting in response to exogenous auxin (**Supplementary Fig. S4**). Auxin antagonists vary significantly in how they affect root architecture. For example, terfestatin A and a synthetic auxin inhibitor having an alkyl substitution at the α-position of IAA stimulated primary root growth, which was attributed to the inhibition of endogenous auxin (Yamazoe et al. 2005, Hayashi et al. 2008). In contrast, both PCIB and yokonolide B suppressed primary root growth, and this was dependent on TIR1 and AUX/IAA7, suggesting it was not a toxic effect (Hayashi et al. 2003, Oono et al. 2003). By using transgenic Arabidopsis seedlings expressing *AtHistH2B:YFP* (yellow fluorescent protein) and vital staining with propidium iodide, we determined that the primary root growth inhibitory effect of serotonin was not due to toxicity, as serotonin-treated seedlings did not show meristem cell damage (**Supplementary Fig. S7**). Instead, it could be due to serotonin modulating cell division and elongation (**Fig. 4**).

Several auxin-resistant Arabidopsis mutants such as *axr1* and *aux1-7*, which are defective in auxin signaling and transport, respectively, exhibit fewer LRs and reduced root hair formation. Since similar phenotypes were observed in WT Arabidopsis (Col-0) roots treated with serotonin, it is possible that high levels of serotonin in the plant impair the cellular auxin response and thereby inhibit the initiation of LRs and root hairs. To determine whether auxin-related mutants were also resistant to serotonin, we tested the effects of this compound on LR development, primary root growth inhibition and adventitious root formation in WT Arabidopsis seedlings and in *axr2-1*, *axr4-1*, *aux1-7* and *axr1-3* auxin-related mutants. We

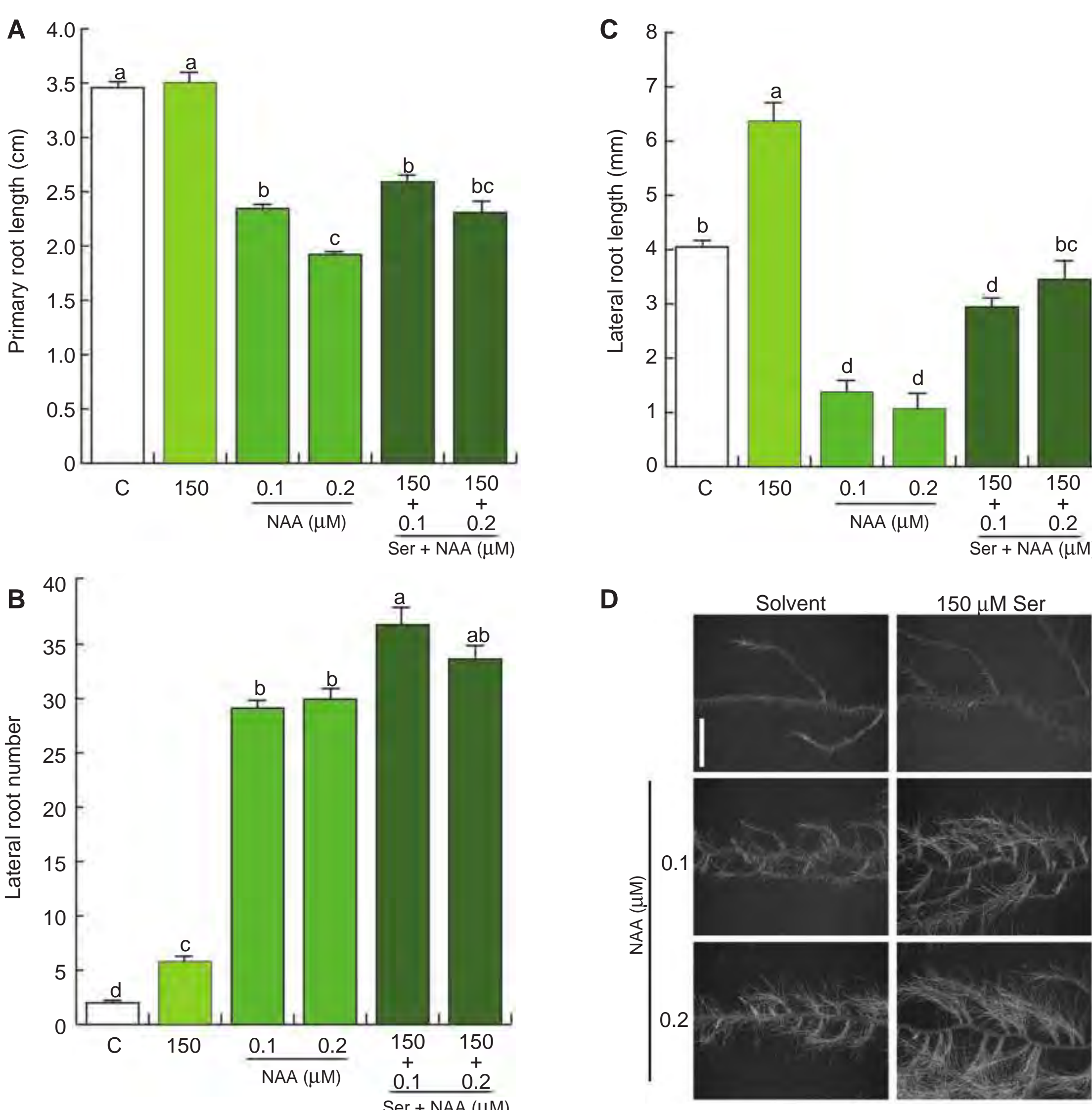

Fig. 12 Serotonin induces lateral growth in combination with auxin. Arabidopsis (Col-0) seedlings were germinated and grown for 7 d on agar solidified 0.2× MS medium supplemented with NAA, 150 μM serotonin or both compounds in combination. (A) Primary root length. (B) Lateral root number per plant. (C) Lateral root length. (D) Representative photographs of lateral roots grown in the indicated treatments. Data points indicate the mean ± SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiments were repeated twice with similar results. Scale bar = 1 mm.

found that 150 μM serotonin significantly increased LR number and density in WT, *axr2-1*, *axr4-1* and *aux1-7*, but not in *axr1-3* (**Fig. 13B, C**). Surprisingly, the dominant *axr2-1* mutant, with a gain of function in *IAA7/AXR2*, caused increased LR formation both under normal growth conditions and in response to serotonin. This result suggests that *IAA7/AXR2* plays a positive role in LR development, in agreement with previously published information (Nagpal et al. 2000). The lack of response of *axr1-3* to serotonin indicates that this compound requires an intact AXR1 protein to activate LR development. Treatment with 450 μM serotonin also showed exacerbated adventitious root production in *axr2-1* and decreased adventitious root response in *axr1-3* when compared with WT plants (**Supplementary Fig. S6**). Although the exact mechanism of action of serotonin is still unclear, several lines of evidence indicate that serotonin probably acts as a natural auxin inhibitor. (i) Serotonin is present in Arabidopsis tissues at low concentrations. (ii) Serotonin treatment stimulates LRP maturation by decreasing auxin responses during LRP development. (iii) Exogenous application of serotonin inhibited root developmental processes which are under auxin control, such as primary root growth, LR formation and root hair development. (iv) Serotonin blocked auxin-responsive *DR5:uidA* and *BA3:uidA* gene expression and auxin-regulated LR formation. (v) Mutant analyses indicate that serotonin inhibits primary root growth and promotes adventitious root formation

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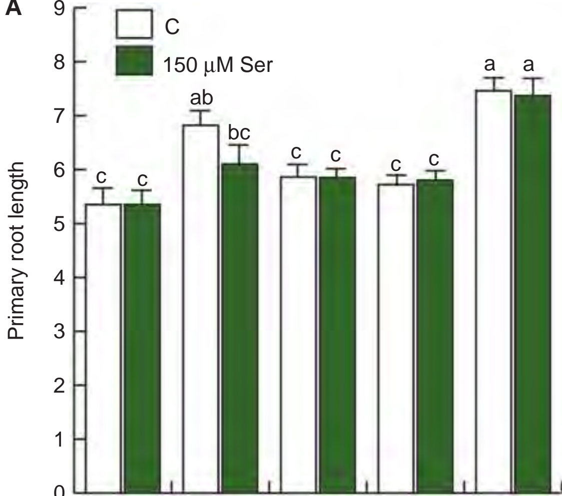

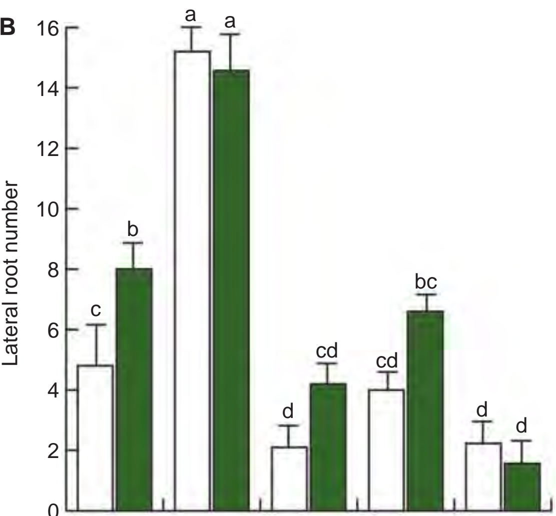

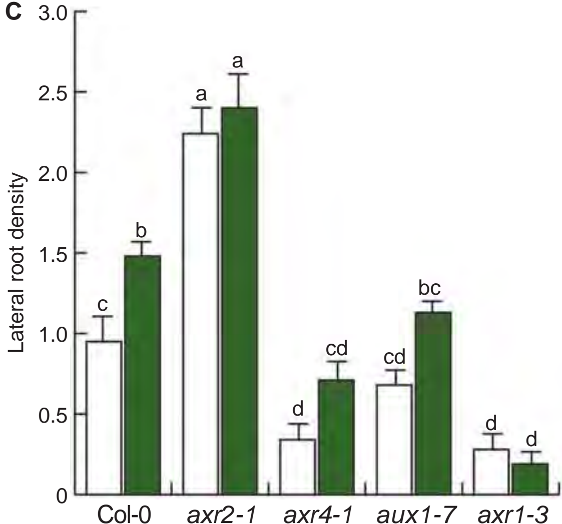

Fig. 13 Effects of serotonin on primary root growth and lateral root formation in WT Arabidopsis (Col-0) seedlings and auxin-related mutants. Arabidopsis WT and mutant seedlings were germinated and grown for 12 d in 0.2× MS medium supplemented with the solvent or 150 µM serotonin. (A) Primary root length. (B) Lateral root number per seedling. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. The experiment was repeated three times with similar results.

independently of at least three auxin-related loci, namely *axr2-1*, *axr4-1* and *aux1-7*.

Plant neurobiology has recently emerged as an integrated view of plant signaling. Plants process the information from a changing environment to develop and reproduce. Communication between cells and tissues is essential for plant fitness, which involves an integrated signaling system that includes long-distance electrical signals, vesicle-mediated transport of IAA and production of chemicals known to be neuronal in animals (Baluska et al. 2005, Brenner et al. 2006). Among the animal neurotransmitters, acetylcholine, catecholamines, histamine, serotonin, dopamine, melatonin and glutamate are the most common in the animal nervous system, playing roles in information processing and development. It is of interest that each of these compounds is present in plants. Similarly to IAA, serotonin and melatonin are tryptophan derivatives. Interestingly, IAA, which is transported from cell to cell has some characteristics reminiscent of neurotransmitters, such as a poorly understood vesicle-based process that involves IAA transporters and recycling between the plasma membrane and endosomes (Geldner et al. 2003, Schlicht et al. 2006). Our results showing that serotonin regulates root architecture in a similar way to another neurotransmitter, glutamate, and that these compound can also affect auxin-mediated responses in Arabidopsis are in agreement with the proposed role of IAA in the plant neurobiological perspective. The possible roles played by other neurotransmitter signals in plant processes may be further clarified by using the molecular tools available in *A. thaliana* and other model plants.

Materials and Methods

Plant material and growth conditions

Arabidopsis (*A. thaliana* Col-0), the transgenic Arabidopsis lines *HS::AXR3NT-GUS* (Gray et al. 2001), *DR5:uidA* (Ulmasov et al. 1997), *BA3:uidA* (Oono et al. 1998), *pPRZ1:uidA* (Sieberer et al. 2003) and *CyCB1:uidA* (Colón-Carmona et al. 1999), histone *H2B:YFP* (Biosnard-Lorig et al. 2001), and the mutant lines *axr1-3* (Lincoln et al. 1990), *aux1-7* (Picket et al. 1990), *axr2-1* (Timpte et al. 1994) and *axr4-1* (Hobbie and Estelle 1995) were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and with 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar

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(commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h light/8 h darkness, light intensity of 100 μmol m^2 s^{-1} and temperature of 22°C.

Analysis of growth

Growth of primary roots was registered using a ruler. LR numbers were determined by counting the LRs present in the primary root, from the tip to the root/stem transition. LR densities were determined by dividing the LR number by the primary root length and expressed as LRP cm^{-1}. Root hairs were measured in a 500 μm region from the primary root tip. The average length of root hairs was determined by measuring 100 hairs for each root, taking as a reference the root protoxylematic plane to locate the radical hair base in the epidermal cell.

Determination of developmental stages of LRP

LRPs were quantified 7 d after germination. Seedling roots were first cleared to enable LRPs at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as folllows, Stage I: LRP initiation. In the longitudinal plane, approximately 8–10 'short' pericycle cells are formed. Stage II: the LRP is divided into two layers by a periclinal division. Stage III: the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV: an LRP with four cell layers. Stage V: the LRP is midway through the parent cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appears to be just about to emerge from the parent root.

Histochemical analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were stained and incubated overnight at 37°C in a GUS reaction buffer (0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62°C. The solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40, 20 and 10% (v/v) for a 24 h period each, and fixed in 50% glycerol (v/v). The processed roots were placed on glass slides and sealed with commercial nail varnish. For each marker line and for each treatment, at least 20 transgenic plants were analyzed.

Aux/IAA protein degradation assay

Six-day-old *HS::AXR3NT-GUS* transgenic Arabidopsis seedlings were incubated on 0.2× liquid MS medium for 2 h at 37°C, followed by transfer of the seedlings into 0.2× liquid MS medium supplemented with IAA or serotonin or both for 10, 30 or 60 min at 22°C. The seedlings were washed with fresh 0.2× MS medium and, 12–14 h later, histochemically stained for GUS activity.

Serotonin determination by GC-MS

The extraction of serotonin from leaves and roots of *A. thaliana* Col-0 (0.1 g) was done with 3 ml of methanol with continuous agitation for 3 h. The extract was evaporated to complete dryness under a stream of nitrogen. Serotonin was acetylated with acetic anhydride (1.5 ml) and 1 ml of dichloromethane, and then sonicated for 15 min and heated at 75°C for 1.5 h. The *N*-acetylserotonin was evaporated under a stream of nitrogen and redissolved in 50 μl of dichloromethane. *N*-Acetylserotonin was analyzed in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973, and a 30 m × 0.2 mm × 0.25 mm, 5% phenyl methyl silicone capillary column (HP-5 MS). Operating conditions used helium as carrier gas, 1 ml min^{-1}; detector temperature of 300°C and injector temperature of 250°C. The volume of injected sample was 1 μl. The column was held for 3 min at 150°C and programmed at 6°C min^{-1} to a final temperature of 278°C for 5 min. *N*-Acetylserotonin was identified by comparison with a mass spectra library (NIST/EPA/NIH, 'Chem Station' Hewlett Packard). The identity of the *N*-acetylserotonin was further confirmed by the comparison of the retention time in the tissue extract with a sample of the pure serotonin standard (Sigma) derivatized following the same procedure mentioned above. A SIM analysis was used to verify the presence of this compound in the sample. The molecular ions were monitored after electron impact ionization (70 eV). They were *m/z* 218, *m/z* 159 and *m/z* 146. To estimate the amount of compound produced by the plant, we constructed an individual calibration curve for the derivatized standard using concentrations from 0.1 to 30 μg.

Microscopy

The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6, Leica Microsystems). Total LRs were counted at 30× magnification. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony Electronics Inc.) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss). For confocal microscopy, solvent- or serotonin-treated transgenic Arabidopsis seedlings expressing the histone *H2B:YFP* construct (Boisnard-Lorig et al. 2001) were mounted on microscope slides into a solution of propidium iodide (10 mg ml^{-1}). Primary root meristems were analyzed by imaging mounted samples with an inverted confocal microscope (Zeiss Axiovert 200 LSM). For propidium iodide detection, wavelengths employed were an excitation line of 568 nm with an emission window of 585–610 nm. YFP was excited with 488 nm line and emission detected at 505–550 nm.

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Data analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All LRs emerging from the primary root and observed under the 30× objective were taken into account for LR number data. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with Tukey's post-hoc test were used for testing differences in growth and root developmental responses in WT and mutant plants. In the figures, different letters are used to indicate means that differ significantly ($P = 0.05$).

Supplementary data

Supplementary data are available at PCP online.

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Molecular, Biological, Physiological and Clinical Aspects of Melatonin
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Melatonin regulates Arabidopsis root system architecture likely acting independently of auxin signaling

Abstract: Melatonin (*N*-acetyl-5-methoxytryptamine) is a tryptophan-derived signal with important physiological roles in mammals. Although the presence of melatonin in plants may be universal, its endogenous function in plant tissues is unknown. On the basis of its structural similarity to indole-3-acetic acid, recent studies mainly focusing on root growth in several plant species have suggested a potential auxin-like activity of melatonin. However, direct evidence about the mechanisms of action of this regulator is lacking. In this work, we used *Arabidopsis thaliana* seedlings as a model system to evaluate the effects of melatonin on plant growth and development. Melatonin modulated root system architecture by stimulating lateral and adventitious root formation but minimally affected primary root growth or root hair development. The auxin activity of melatonin in roots was investigated using the auxin-responsive marker constructs *DR5:uidA, BA3:uidA,* and *HS::AXR3NT-GUS*. Our results show that melatonin neither activates auxin-inducible gene expression nor induces the degradation of *HS::AXR3NT-GUS*, indicating that root developmental changes elicited by melatonin were independent of auxin signaling. Taken together, our results suggest that melatonin is beneficial to plants by increasing root branching and that root development processes elicited by this novel plant signal are likely independent of auxin responses.

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Key words: Arabidopsis, auxin signaling, melatonin, root development

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a well-known animal hormone, which modulates sleep, mood, sexual behavior, seasonal reproductive physiology, circadian rhythms and functions as an antioxidant [1–3]. Recent information indicates that melatonin is highly conserved across all life kingdoms and is present in at least twenty plant families including Alliaceae, Araceae, Asparagaceae, Bromeliaceae, Musaceae, Poaceae (Gramineae), Solanaceae, and Cruciferae [4, 5]. This indoleamine can be found in different organs including roots, stems, leaves, flowers, fruits, and seeds at concentrations usually ranging from picograms to nanograms per gram of tissue [6, 7]. However, since its identification in plants [4, 5], an increasing number of research reports have investigated the possible physiological roles and its mechanism of action, suggesting that melatonin is involved in photoperiod responses and regulation of plant development and may act as an antioxidant (reviewed in [8–10]).

Melatonin is structurally related to indole-3-acetic acid (IAA), the most abundant natural auxin in plants. IAA is involved in a wide variety of physiological process throughout the plant life cycle including tropic responses toward light, gravity, and touch stimuli as well as in root and shoot system establishment [11]. In vertebrates, the biosynthetic pathway and metabolism of melatonin have been well characterized. Beginning with the amino acid precursor tryptophan, four enzymes are sequentially involved in melatonin biosynthesis: they include tryptophan hydroxylase (TPH), which converts tryptophan to 5-hydroxytryptophan; aromatic amino acid decarboxylase (AAAD), which converts 5-hydroxytryptophan to serotonin; arylalkylamine *N*-acetyl-transferase (AANAT), which synthesizes *N*-acetylserotonin from serotonin; and *N*-acetylserotonin *O*-methyltransferase (ASMT), which forms melatonin. In addition, melatonin can be enzymatically or nonenzymatically transformed to several biologically active metabolites [12–15]. Currently, the available evidence suggests that plants have the molecular machinery for melatonin biosynthesis [13–18].

On the basis of the chemical similarity between melatonin and IAA and the effects of both compounds on plant morphogenesis, previous studies have suggested that melatonin could act as a growth promoting compound, probably increasing auxin levels or showing an auxin-like activity. In St. John's wort (*Hypericum perforatum* L.) explants, melatonin regulated root formation [19]. When etiolated hypocotyls from *Lupinus albus* are treated with a range of melatonin and IAA concentrations, both compounds elicited plant growth at micromolar concentrations but repressed the growth at higher concentrations [20]. It was also confirmed that melatonin acts as a growth promoter in coleoptiles of wheat, barley, canary grass, and oat. However, its activity was lower in comparison with IAA [21]. Melatonin also affected the regeneration of lateral

and adventitious roots and the expansion of cotyledons in etiolated seedlings of *L. albus,* and in *Brassica juncea* young seedlings, lower concentrations of melatonin have been found to stimulate the root growth and to raise the endogenous levels of IAA, but higher concentrations have inhibitory effects, which was observed by comparing the effect of varied concentrations of melatonin and IAA supplied to the growth media [22–24].

Recently, our research documented the activity of the melatonin precursor serotonin in the growth and development of *Arabidopsis thaliana* seedlings. Serotonin was identified as an important regulator of root development processes, probably by acting as a natural auxin inhibitor [25]. The information described above suggests that the role of melatonin in plant growth and developmental processes may be complex, with auxin-related or unrelated activity depending on the plant system and the process under study.

To more deeply investigate the role of melatonin in plants, in this work we evaluated the effects of exogenously supplied melatonin on root system architecture in *A. thaliana*. Detailed analysis of morphological parameters, including primary root growth, lateral and adventitious root formation, and root hair development, showed that melatonin can be perceived by plants and modulate a subset of root architectural responses such as lateral and adventitious root formation, but is less active in regulating primary root growth and root hair formation. Because most of these root developmental traits are under auxin control, we performed auxin-responsive gene expression analyses in transgenic *Arabidopsis* seedlings expressing the *DR5:uidA*, *BA3:uidA,* and *HS::AXR3NT-GUS* gene markers in response to melatonin treatments. Our results show that melatonin likely acts through auxin-independent signaling mechanisms.

Materials and methods

Plant material and growth conditions

Arabidopsis (*A. thaliana* Col-0) transgenic lines *HS::AXR3NT-GUS* [26], *DR5:uidA* [27], *BA3:uidA* [28] and *CycB1:uidA* [29] were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium [30]. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Phytagar (Micropropagation grade) was purchased from *Phyto*Technology Laboratories, Lenexa, KS, USA. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival Scientific AR95L, Perry, IA, USA) with a photoperiod of 16-hr light/8-hr darkness, light intensity of 100 μmol/m^2/s, and temperature of 22°C. For dark-grown plants, seeds were sown on the surface of agar plates and the plates covered by four layers of aluminum foil. Plants were included in the growth chamber for 5 day until development of long hypocotyls. Etiolated seedlings were selected on the basis of the continuous growth of the stem that ensures a suitable source of plant tissue. Etiolated hypocotyls were used to determine the effects of melatonin on adventitious root formation.

Analysis of growth

Growth of primary roots was registered using a rule. Lateral root numbers were determined by counting the lateral roots present in the primary root, from the tip to the root/stem transition using an AFX-II-A stereoscopic microscope (Nikon, Tokyo, Japan). Lateral root densities were determined by dividing the lateral root number by the primary root length. Root hairs were measured in a 500-μm region from the primary root tip. The average length of root hairs was determined upon measuring 100 hairs for each treatment, taking as a reference the root protoxylem plane to locate the radical hair base in the epidermal cell. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses with Tukey's post hoc test were used for testing the differences in growth and root developmental responses in wild-type and mutant plants. Different letters are used to indicate the means that differ significantly ($P < 0.05$).

Histochemical analysis

Transgenic plants that express the *uidA* reporter gene [31] were stained in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, for 12 hr at 37°C. Plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62°C. The solution was substituted for 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40%, 20% and 10% (v/v) for a 24-hr period each, and fixed in 50% glycerol (v/v). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and for each treatment, at least 20 transgenic plants were analyzed.

Aux/indole-3-acetic acid protein degradation assay

Six-day-old *HS::AXR3NT-GUS* Arabidopsis transgenic seedlings were incubated in 0.2× MS liquid medium and heat shocked for 2 hr at 37°C, followed by transfer of the seedlings into liquid 0.2× MS medium, supplied with IAA, or melatonin for 10, 30, or 60 min at 22°C. The seedlings were washed with fresh 0.2× MS liquid medium and stained 12 hr for histochemical analysis of β-glucuronidase (GUS) activity using GUS reaction buffer.

Microscopy

The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany). Total lateral roots were counted at 30× magnification. Images were captured with a Sony Cybershot DSC-S75 digital camera (Sony Electronics Inc.,

Oradell, NJ, USA) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss Inc., New York, NY, USA).

Results

Melatonin is widely distributed in plants but information about its physiological role in these organisms is scarce. To clarify the possible mechanisms of melatonin action in plants, we tested the effects of this compound on plant growth and development using *A. thaliana* as a model system. Arabidopsis (Col-0, Ws and Ler ecotypes) seedlings were germinated and grown on 0.2× MS-agar media supplied with solvent or 100- and 200-μM melatonin concentrations. Ten days after germination (dag), primary root length, lateral root number, and lateral root density were analyzed. We found that melatonin did not significantly inhibit the primary root growth even at concentrations of 200 μM (Fig. 1A), but this compound clearly increased both lateral root number and density in all three ecotypes tested (Fig. 1B,C). Fig. S1 shows the effects of melatonin on root system architecture in Col-0, Ws, and Ler ecotypes. As the above-described results indicate similar developmental effects in the Arabidopsis ecotypes analyzed, we continued our study focusing our experiments on the Col-0 ecotype.

To characterize in more detail the effects of melatonin in root system architecture, we evaluated the effects of increasing melatonin concentrations from 150 to 600 μM. The primary root growth was not affected even at 600-μM melatonin (Fig. 2A). However, 150–600-μM melatonin concentrations increased lateral root number by three-fold, compared to solvent-treated seedlings (Fig. 2B). Fig. 2C shows comparative photographs of Arabidopsis seedlings that were treated with the solvent only or with 600-μM melatonin. It can be clearly observed the formation of branched root systems in response to the compound caused by increased formation of lateral roots, without significantly affecting the primary root growth.

Auxin regulates primary root growth in Arabidopsis by modulating cell division. To investigate whether melatonin could affect cell division, we analyzed the expression of the *CycB1:uidA* marker, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity [29]. *CycB1:uidA*-expressing seedlings were grown on 0.2× agar medium supplied with the solvent or with increased concentrations of melatonin (150–600 μM). Melatonin did not significantly affect *CycB1:uidA* in primary root tips (Fig. 3). These results indicate that the cell division of primary roots in Arabidopsis is unaffected by melatonin treatments.

To determine whether melatonin promotes lateral root development by stimulating lateral root primordia (LRP) growth or inducing de novo formation of LRPs, or modulating both of these processes, we investigated the stages of LRP development affected by melatonin. LRPs were quantified 7 days after germination in plants treated with the solvent or with 100–200-μM melatonin. Seedling roots were first cleared to enable LRPs at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey [32]. We found that the stage distribution of LRPs was affected by treatment with melatonin. In particular, LRP stage I, which describes LRPs at the earliest stage of development, was significantly decreased in melatonin-treated seedlings, in contrast to that observed in stages IV–V (Fig. 4A). The total number of LRPs per seedling did not change in response to melatonin treatments (Fig. 4B). These data suggest that melatonin did not induce de novo LRP initiation and probably increases root branching in Arabidopsis by inducing the maturation of preformed LRPs from pericycle cells.

Previously, melatonin was suggested to be involved in the regeneration of adventitious root in a similar way to IAA. Next, we assessed the regenerative properties of melatonin in adventitious root formation by using shoot explants from Arabidopsis etiolated seedlings, which were treated with the solvent or with increasing concentrations of melatonin by using the experimental system described in detail by Campos-Cuevas et al. [33]. Hypocotyl explants were obtained and transferred to agar plates containing 0.2× MS medium supplied with the solvent or with increased concentrations of melatonin. Seven days after transfer, the organogenic properties of melatonin were evaluated by monitoring adventitious root formation. We found that increasing concentrations of melatonin showed

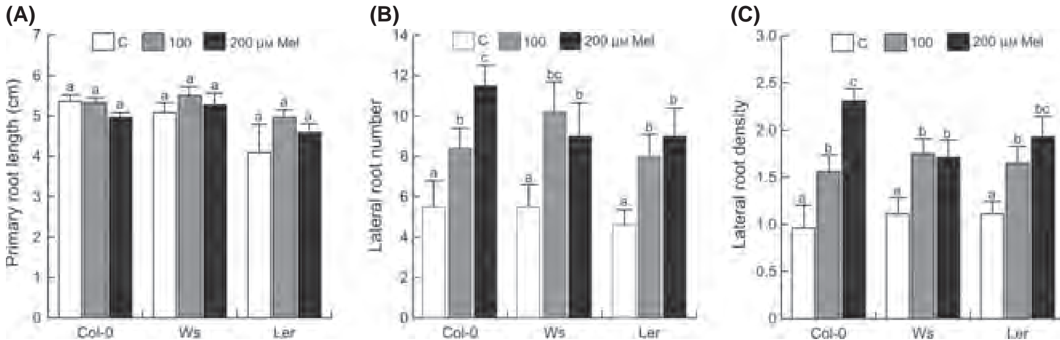

Fig. 1. Effects of melatonin on Arabidopsis root system architecture. Arabidopsis seedlings of three different ecotypes (Col-0, Ws and Ler) were germinated and grown for 10 day under increasing melatonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent means of 30 seedlings ± S.D. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

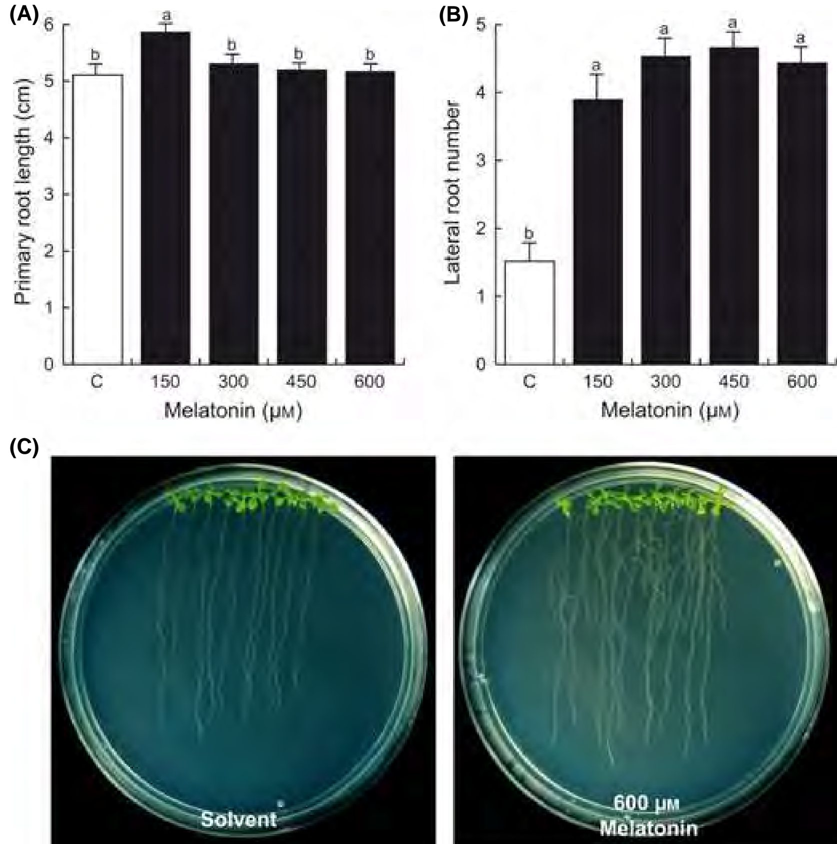

Fig. 2. Effects of melatonin on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 10 day under increasing melatonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Photographs show representative plates of WT (Col-0) seedlings grown in medium supplied with the solvent only or with 600-μM melatonin. Notice the promoting effects of the compound in lateral root formation. Values shown represent means of 30 seedlings ± S.D. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

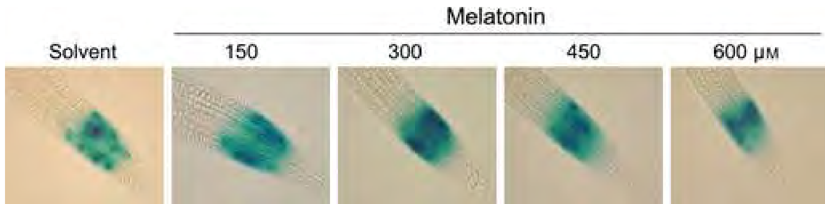

Fig. 3. Effect of melatonin on cell division. *CycB1:uidA* Arabidopsis seedlings were grown for 7 day on 0.2× MS medium supplemented with the indicated concentrations of melatonin. Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from at least 15 stained plants. The experiment was replicated twice with similar results.

a clear increase in adventitious root formation in Arabidopsis explants (Fig. 5A); this effect can be observed in representative photographs (Fig. 5B). Interestingly, explants treated with melatonin but at a further developmental stage (12 days) showed a strong increase in secondary adventitious root number (Fig. 6A,B).

Root hairs are epidermal cells involved in nutrient and water uptake. Root hair development is a process regulated by auxins in several plant species including Arabidopsis [34]. To determine whether melatonin could affect root hair development, we performed experiments in which Arabidopsis seedlings were germinated and grown under increasing concentrations of melatonin in Petri plates containing 0.2× MS-agar medium; 5-day after germination, root hairs were analyzed and counted from the differentiation and maturation zones of the primary root. To test whether melatonin could alter root hair initiation, root hair elongation or both, we analyzed trichoblast cells present in the maturation zone of the primary root. In contrast to melatonin effects on lateral and adventitious root, this analysis showed that melatonin did not affect root hair formation (Fig. 7A). However, a small yet significant effect of melatonin repressing root hair growth could be observed (Fig. 7B). Fig. 7C shows representative photographs of

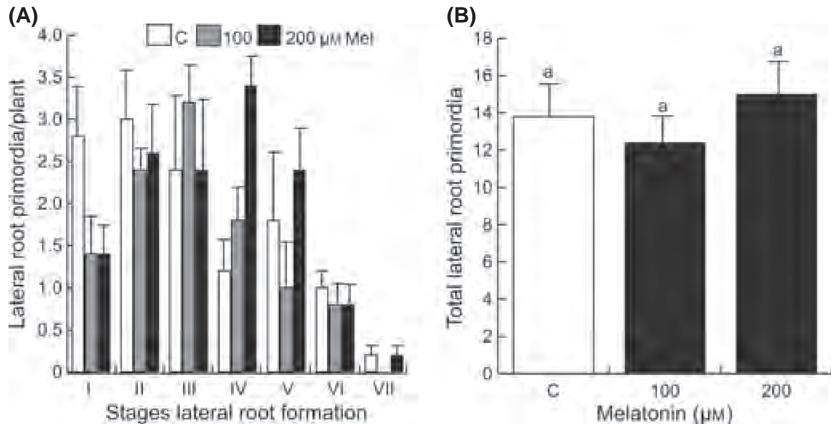

Fig. 4. Effects of melatonin on lateral root primordia development. Arabidopsis Col-0 seedlings were grown for 7 day on agar plates supplemented with the solvent or with 100- and 200-μM melatonin. Data are presented for LRP developmental stages (A) and total LRPs per seedling (B). LRP stages were recorded according to Malamy and Benfey [32]. Values shown represent the mean of 15 seedlings $\pm$ S.D. Different letters are used to indicate the means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

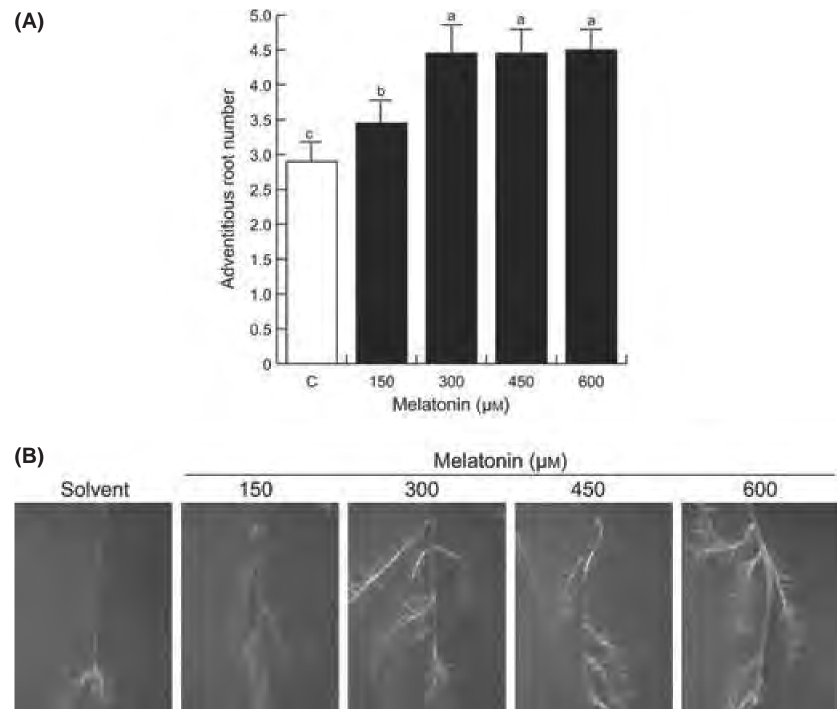

Fig. 5. Effects of melatonin on adventitious root development from Arabidopsis shoot explants. Arabidopsis seedlings were germinated and grown in darkness for 5 day on the surface of agar plates containing 0.2× MS medium. Hypocotyl explants were transferred to MS 0.2× medium containing the indicated concentrations of melatonin and cultivated for a further 7-day period to quantify adventitious root formation. (A) Adventitious root number in response to melatonin. (B) Representative photographs of Arabidopsis (Col-0) explants grown on the surface of agar plates containing 0.2× MS medium or in the same medium supplied with increasing melatonin concentrations. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results.

root hair development both in the differentiation zone and in the maturation zone grown under varied melatonin concentrations.

The above-described effects of melatonin in lateral and adventitious root development are in consonance with an auxin-like activity. However, the inhibitory effect of this compound on root hair growth indicates that this compound may possess a more complex mode of action on root morphogenesis. To determine whether melatonin could act in an auxin-related signaling pathway, we analyzed the expression of auxin-responsive gene markers *DR5:uidA* and *BA3:uidA* in transgenic Arabidopsis seedlings treated with IAA and melatonin. Fig. 8 shows histochemical staining for transgenic *DR5:uidA* and *BA3:uidA* seedlings that were grown 6 day in 0.2× MS-agar medium and then transferred to 0.2× MS liquid medium supplied with 5-μM IAA

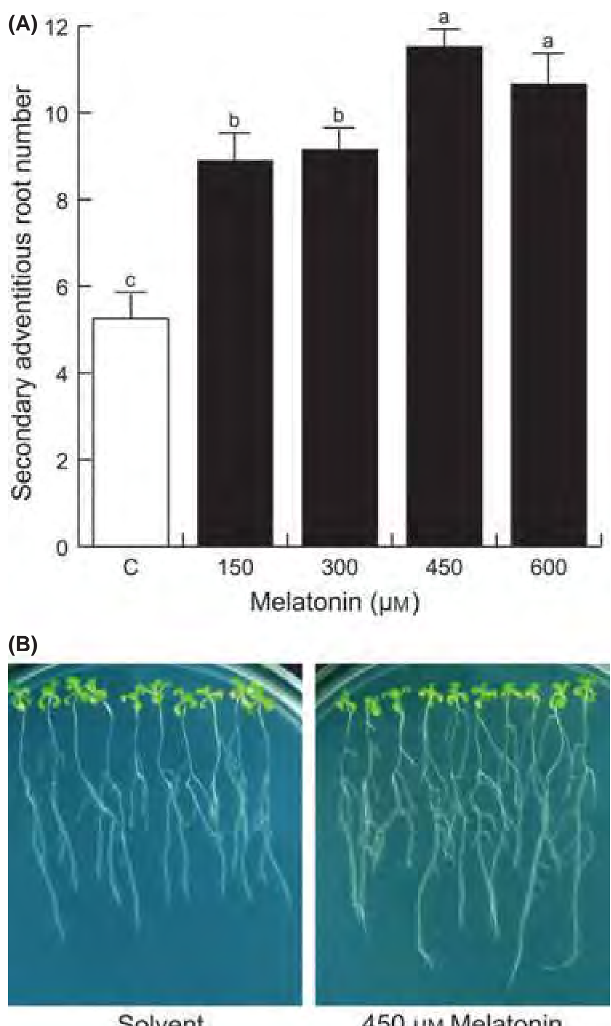

Fig. 6. Effects of melatonin on secondary adventitious root development. Arabidopsis seedlings were germinated and grown in darkness for 5 day on the surface of agar plates containing 0.2× MS medium. Hypocotyl explants were transferred to MS 0.2× medium containing the indicated concentrations of melatonin and cultivated for a further 12-day period to quantify secondary adventitious root formation. (A) Secondary adventitious root number. (B) Representative photographs of Arabidopsis (Col-0) explants grown on the surface of agar plates containing 0.2× MS medium or in the same medium supplied with melatonin. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results.

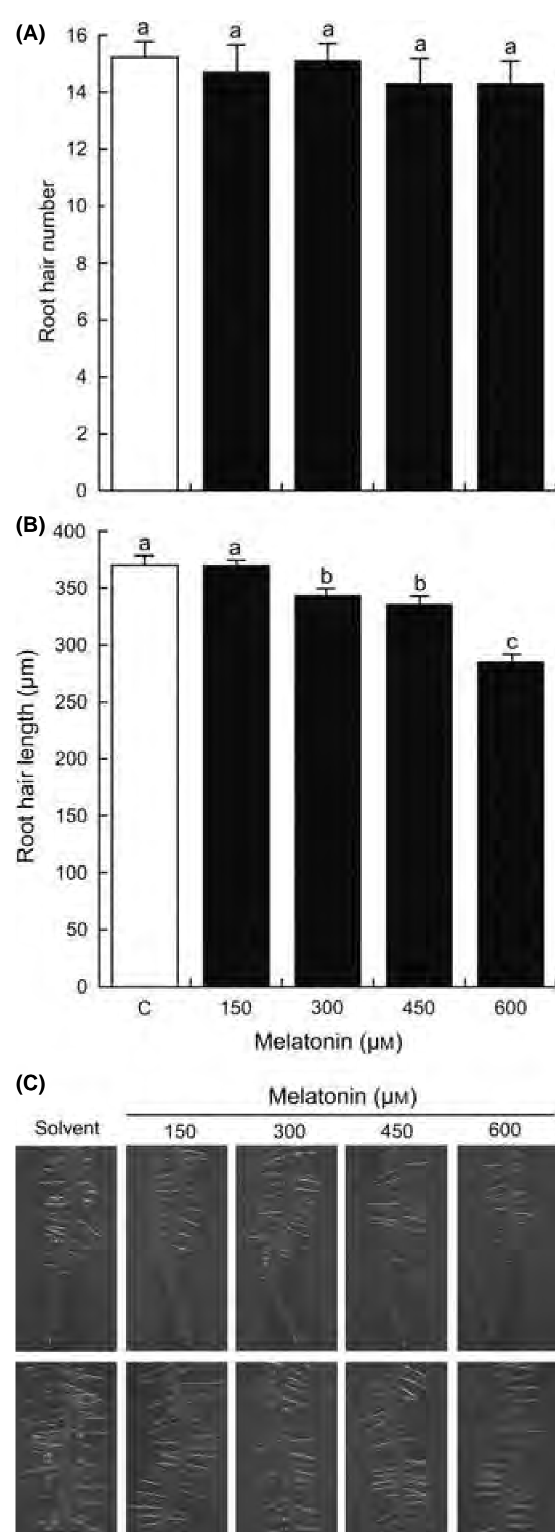

Fig. 7. Effects of melatonin on root hair development. *Arabidopsis thaliana* seedlings were grown for 5 day on MS 0.2× media supplemented with the indicated concentrations of melatonin. (A) Root hair number. (B) Root hair length. (C) Representative photographs of root hairs formed at the differentiation and maturation region of the primary root. Different letters indicated statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

or 450-μM melatonin and incubated for 9 hr. As previously reported [27], in solvent-treated control plants, *DR5:uidA* is expressed primarily in the root tip zone (Fig. 8A–C). *DR5:uidA* seedlings grown under a concentration of 5-μM IAA showed GUS activity throughout the plant (Fig. 8D–F). In contrast, *DR5:uidA* seedlings treated with 450-μM melatonin did not show an increase in GUS expression (Fig. 8G–I), indicating that melatonin act through an auxin-independent way. Expression of *DR5:uidA* marker in adventitious roots from etiolated seedlings treated with increasing concentrations of melatonin showed similar staining patterns to solvent-only-treated seedlings (Fig. S2). Untreated *BA3:uidA* plants did not show detectable levels

of GUS activity in the root and only low expression in petioles could be observed (Fig. 8J–L), whereas when treated with 5-μM IAA, they showed a clear GUS expression mainly in petioles of cotyledons (Fig. 8M,N) and in the root elongation zone (Fig. 8O). GUS expression in plants treated with 450-μM melatonin was undetectable (Fig. 8P–R), indicating that this compound failed to activate *BA3:uidA* expression even at high concentrations. These results suggest that melatonin did not posses an auxin-like activity inducing auxin-responsive gene expression.

Auxin signaling involves Aux/IAA proteins, which are auxin-responsive repressors, and degradation of these proteins by the ubiquitin–proteasome pathway activates auxin-responsive gene expression [26]. We next compared the effect of IAA and melatonin on auxin-mediated degradation of Aux/IAA proteins using the Arabidopsis *HS::AXR3NT-GUS* line, in which a translational fusion between domains I and II of AXR3 and the GUS reporter protein is expressed under the control of a heat shock promoter [26]. Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37°C for 2 hr and further treated with 5-μM IAA or 450-μM melatonin for 10, 30, and 60 min. In solvent-treated control seedlings, GUS expression was observed in cotyledons and roots (Fig. 9A–D). Treatment with IAA clearly showed enhanced degradation of the fusion protein throughout the plant (Fig. 9E–H), but melatonin failed to achieve the same effect on *HS::AXR3NT-GUS* degradation (Fig. 9I–L). Our data indicate that melatonin likely act in an auxin-independent signaling pathway.

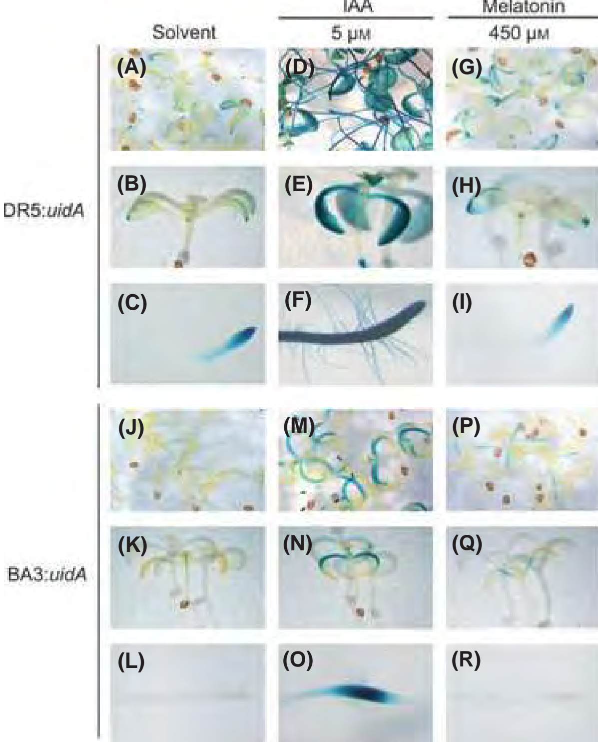

Fig. 8. Effect of melatonin on auxin-regulated gene expression. Twelve-hour GUS staining of *DR5:uidA* Arabidopsis seedlings treated with the solvent (A–C), in medium supplied with 5-μM indole-3-acetic acid (IAA)(D–F) or 450-μM melatonin (G–I) and incubated for 9 hr at 22°C. Twelve-hour GUS staining of *BA3:uidA* Arabidopsis seedlings treated with the solvent (J–L), in medium supplied with 5-μM IAA (M–O) or 450-μM melatonin (P–R). Notice the failure to activate GUS expression by the treatments with melatonin. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

Discussion

Melatonin is a ubiquitous compound, which has been found in many evolutionary distinct organisms including

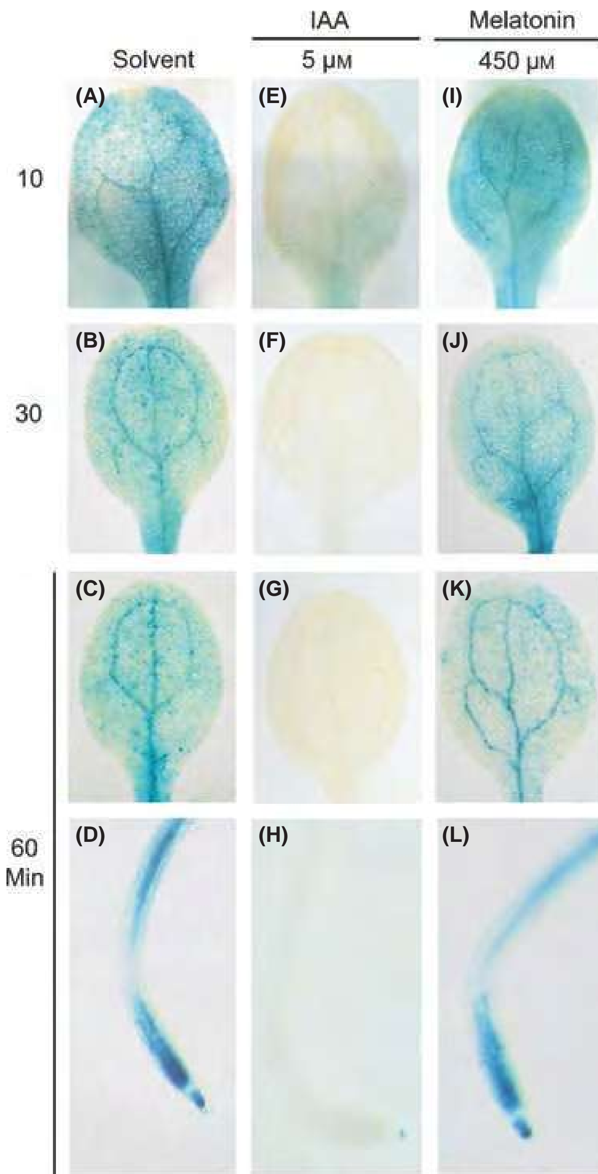

Fig. 9. Analysis of AUX/indole-3-acetic acid (IAA) stability with *HS::AXR3NT-GUS* fusions. Wild-type seedlings expressing the *HS::AXR3NT-GUS* constructs were heat shocked at 37°C for 2 hr. After heat induction, the seedlings were treated with IAA or melatonin for different time periods at the indicated concentrations and stained overnight for GUS activity. Representative photographs of cotyledons from at least 20 stained seedlings are shown. Similar results were obtained in two independent experiments.

bacteria, algae, invertebrates, mammals, and plants [4, 5, 9, 35–37]. In mammals, melatonin is mainly produced by the pineal gland and secreted into the blood stream. The signaling roles of melatonin in vertebrates include circadian rhythm and photoperiodism [38] as well as immunomodulatory and cytoprotective responses [39–41]. However, the roles and mechanisms of action of melatonin in plants are poorly characterized.

This study investigated the basis by which melatonin triggers root developmental changes in *A. thaliana*. Based mainly on its structural similarity to auxins and because both IAA and melatonin apparently regulate similar developmental processes, melatonin has been suggested to function as an auxin to promote root and vegetative growth in a number of plant species. Most experiments aimed at demonstrating an auxin-like activity of melatonin have been unsuccessful. A recent report by Chen et al. [23] showed that melatonin stimulates root growth in roots of etiolated seedlings of *B. juncea*. Our results nicely mesh with this previous report by showing that melatonin treatment of 150 μM promoted primary root growth in Arabidopsis seedlings grown in vitro under a photoperiod of 16-hr light/8-hr darkness (Fig. 2A). Interestingly, higher melatonin concentration of up to 600 μM did not significantly affect the primary root growth indicating the lack of an inhibitory effect of melatonin toward regulating primary root growth. In contrast, melatonin dramatically induced lateral root formation in a dose-dependent way (Fig. 2B), thus confirming the signaling role played for melatonin in growth and developmental processes.

To evaluate the organogenic properties of melatonin, we tested the effects of this compound in the formation of adventitious roots from hypocotyls of dark-grown *A. thaliana* seedlings. Our results extend the findings by Arnao and Hernández-Ruíz [22], which showed that etiolated hypocotyls from *Lupinus albus L.* produce increased numbers of adventitious roots in response to a range of concentrations of melatonin and IAA. Interestingly, our data suggest that the formation of lateral and adventitious roots by melatonin did not involve auxin signaling because *DR5:uidA* expression analysis in adventitious root tips and LRP did not increase in response to melatonin treatments (Fig. S2).

Root hairs are important cell structures involved in both water and nutrient acquisition. They are formed from specialized epidermal cells known as trichoblasts. Auxin treatments increase both root hair numbers and length of root hairs in Arabidopsis seedlings. Our analysis of root hair development shows that melatonin did not affect root hair number (Fig. 7A), but slightly decrease root hair length (Fig. 7B,C), indicating that the effects of melatonin on epidermal cell differentiation are different to those elicited by auxins.

Many growth and developmental responses in plants are mediated by phytohormones, such as auxin. IAA has been found to be the typical auxin in plants, mainly evaluated by cell elongation tests in hypocotyls, primary root growth, and lateral root responses [11]. Our comparative analysis of auxin activity for IAA and melatonin indicates that melatonin lacks an auxin-like activity. This hypothesis is supported by two lines of evidence: (i) the effect of the compound on *DR5:uidA* and *BA3:uidA* gene expression and (ii) the Aux/IAA stability analysis using the Arabidopsis *HS::AXR3NT-GUS* line. Treatment with IAA increased auxin-inducible gene expression revealed by the *DR5:uidA* and *BA3:uidA* gene markers, but melatonin did not stimulate the expression of these markers (Fig. 8). Furthermore, IAA showed enhanced degradation of the fusion protein *HS::AXR3NT-GUS*, but melatonin failed to induce the degradation of the fusion protein even after 60 min of treatment (Fig. 9). These data indicate that melatonin did not act in an auxin-mediated signaling pathway. Interestingly, exogenously supplied melatonin was found to stimulate lateral root formation at 100 μM or greater concentrations, which are much higher concentrations to that required for IAA or related auxin signals to affect the same developmental trait [11]. This indicates that although both IAA and melatonin regulate lateral root formation, their mechanisms of action may be rather different.

Recently, our work has uncovered an important role of serotonin, the precursor of melatonin on root system architecture. Concentrations of serotonin from 10- to 160-μM stimulated lateral root growth in *A. thaliana*. At higher concentrations, serotonin inhibited primary and lateral root growth, but promoted formation of adventitious roots. Interestingly, *Arabidopsis* lines expressing auxin-responsive marker constructs *DR5:uidA* and *BA3:uidA* indicated antiauxin effects of serotonin in LRP [25]. Our reported effects of melatonin on root development indicate that melatonin shows a different activity compared with serotonin in modulating morphogenetic processes. First, the induction of lateral roots by melatonin apparently is not related to a primary root growth inhibitory effect. Second, in contrast to serotonin, melatonin did not inhibit auxin-responsive gene expression during LRP development, indicating that it is not an antiauxin.

Plant neurobiology has recently emerged as an integrated view of cell signaling. Plants process the information from the environment to successfully develop and reproduce. Communication between cells and tissues is essential for plant adaptation, which involves an integrated signaling system that includes long-distance electrical signals, vesicle-mediated transport of IAA, and production of chemicals known to be neuronal in animals [42, 43].

Among the animal neurotransmitters, acetylcholine, catecholamines, histamine, serotonin, dopamine, melatonin, and glutamate are the most common in the animal nervous system, playing roles in information processing and development. It is of interest that each of these compounds is present in plants. Our analysis of the effects of serotonin [25] and melatonin in Arabidopsis have shown that neurotransmitter signals can be perceived by plants to modulate the morphogenetic processes. Serotonin posses both growth promoting and repressing effects on root developmental traits, while melatonin mostly have beneficial effects in *Arabidopsis* seedlings by promoting the branching of the root system, which could lead to a greater absorptive capacity for nutrient and water uptake from the soil. The utility of serotonin and melatonin in agricultural production is an important novel avenue in the research of

these indoleamines based on its important presumptive role in plant physiology.

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Author contributions

RP-F and JL-B designed the research; RP-F, RO-C, EM-P, and JL-B performed the research; JL-B contributed new reagents/analytic tools; RP-F, RO-C, EM-P, and JL-B analyzed the data; and RP-F and JL-B wrote the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of melatonin on Arabidopsis (Col-0, Ler and WS ecotypes) root system architecture.

Figure S2. Expression of auxin-response *DR5:uidA* gene in response to melatonin.

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dhm1, an Arabidopsis mutant with increased sensitivity to alkamides shows tumorous shoot development and enhanced lateral root formation

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Abstract The control of cell division by growth regulators is critical to proper shoot and root development. Alkamides belong to a class of small lipid amides involved in plant morphogenetic processes, from which *N*-isobutyl decanamide is one of the most active compounds identified. This work describes the isolation and characterization of an *N*-isobutyl decanamide-hypersensitive (*dhm1*) mutant of Arabidopsis (*Arabidopsis thaliana*). *dhm1* seedlings grown in vitro develop disorganized tumorous tissue in petioles, leaves and stems. *N*-isobutyl decanamide treatment exacerbates the *dhm1* phenotype resulting in widespread production of callus-like structures in the mutant. Together with these morphological alterations in shoot, *dhm1* seedlings sustained increased lateral root formation and greater sensitivity to alkamides in the inhibition of primary root growth. The mutants also show reduced etiolation when grown in darkness. When grown in soil, adult *dhm1* plants were characterized by reduced plant size, and decreased fertility. Genetic analysis indicated that the mutant phenotype segregates as a single recessive Mendelian trait. Developmental alterations in *dhm1* were related to an enhanced expression of the cell division marker *CycB1-uidA* both in the shoot and root system, which correlated with altered expression of auxin and cytokinin responsive gene markers. Pharmacological inhibition of auxin transport decreased LR formation in WT and *dhm1* seedlings in a similar manner, indicating that auxin transport is involved in the *dhm1* root phenotype. These data show an important role of alkamide signaling in cell proliferation and plant architecture remodeling likely acting through the *DHM1* protein.

Keywords Alkamides · Auxin · Cytokinins · Root development · Cell division

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Introduction

Plant growth and development are regulated by environmental stimuli and specific genetic programs. The characteristics of the different plant organs are determined by the balance between cell division, cell growth and differentiation. Cell proliferation takes place in meristems, in which cells divide at appropriate times to sustain plant growth (Sarkar et al. 2007; Stahl and Simon 2010). During shoot development, stem branches are initiated as primordia from apical and lateral meristems. Although most cells in organ primordia are meristematic and proliferate, they lose competence and withdraw from the cell cycle as organs develop (McSteen and Leyser 2007; Ongaro and Leyser 2008).

Root systems proliferate by lateral root (LR) formation, which involves the activation of pericycle founder cells located opposite to xylem poles. Founder cells undergo several rounds of anticlinal divisions to create a single layered primordium composed of up to ten small cells of equal length (termed stage 1) (Dolan et al. 1993; Malamy and Benfey 1997; Dubrovsky et al. 2008). Further anticlinal and periclinal divisions create a dome-shaped primordium (spanning stages III-VII), which eventually emerges from the parental root giving rise to a novel branch that

grows independently of the primary root (Malamy and Benfey 1997; Casimiro et al. 2003; Péret et al. 2009). Both in the shoot and in the root, the maintenance of meristem competence of cells is a key mechanism that mediates lateral organ growth by defining total cell production.

Cells located in meristems or within differentiated tissues, are instructed to perform formative divisions by a number of phytohormones including auxins, cytokinins, ethylene, lipooligosaccharides, steroids and peptides (Kyozuka 2007; Bleckmann and Simon 2009). Recent research has shown the importance of auxin and cytokinin for establishing organizing centres or influencing formative divisions, which shapes the plant body. In some cases, these phytohormones influence transition steps of the cell cycle. In particular, cytokinins drive the G1 to S transition by activation of cyclinD3 expression or dephosphorylation of CDK/cyclin complexes, while auxin promotes the G2-to-M transition through a mechanism involving cyclinB1 (Zhang et al. 1996; Rhiou-Khamlichi et al. 1999; Sabatini et al. 2000; Himanen et al. 2002; Fukaki and Tasaka 2009). Genetic and molecular analyses have revealed the main components of the cytokinin and auxin signal transduction pathways. Interestingly, manipulation of relevant genes in these pathways, including receptors and transcription factors, often leads to alterations in root or shoot structures with concomitant effects on plant growth (Howell et al. 2003; Kakimoto 2004; Woodward and Bartel 2005).

Hormonal crosstalk in plant morphogenesis has been well documented and suggests that the action of phytohormones is coordinated by some common intermediates or modulators such as second messengers, kinases, phosphatases and/or transcription factors (Wilson et al. 1990; Hobbie and Estelle 1994; Tiryaki and Staswick 2002). A recently described class of fatty acid amides, the alkamides have been found to interact with cytokinin and jasmonic acid signaling to modulate plant development. Alkamides comprise at least 200 compounds with varied acyl chain length and saturation grade and share structural similarity with bacterial quorum-sensing signals, thus representing potential inter-kingdom signals for plant-bacteria communication (López-Bucio et al. 2006; Morquecho-Contreras and López-Bucio 2007; Ortíz-Castro et al. 2008; Ortiz-Castro et al. 2011). Alkamides alter root and shoot system architecture and affect biomass production in Arabidopsis in a dose-dependent way (Ramírez-Chávez et al. 2004, Campos-Cuevas et al. 2008). High concentrations of *N*-isobutyl decanamide, an alkamide naturally present in plants, induced callus formation in leaves and increased LR formation indicating a strong morphogenetic bioactivity (López-Bucio et al. 2007). Interestingly, the proliferative growth activity elicited by *N*-isobutyl decanamide on callus formation in leaves and LR formation was decreased or lost in Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors *CRE1*, *AHK2*, and *AHK3* (López-Bucio et al. 2007). The triple cytokinin receptor mutant *cre1-12/ahk2-2/ahk3-3* was particularly insensitive to high alkamide concentrations in callus formation indicating that *N*-isobutyl decanamide requires, at least in part, a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes. Currently, the genetic mechanisms involved in plant responses to alkamides are poorly understood.

A recent screen for identifying Arabidopsis mutants that fail to inhibit primary root (PR) growth when grown under a high concentration of *N*-isobutyl decanamide identified a recessive mutant that was resistant to *N*-isobutyl decanamide termed *decanamide resistant root*-(*drr1*) (Morquecho-Contreras et al. 2010). Detailed characterization of root system architecture (RSA) and lateral root primordia (LRP) development in WT and *drr1* mutants revealed that *DRR1* is required at an early stage of pericycle cell activation to form LRP in response to *N*-isobutyl decanamide, which coincided with reduced LR formation in the mutants under normal growth conditions. Exogenously supplied auxin similarly inhibited primary root growth and restored normal LR formation in *drr1* seedlings, suggesting that alkamides and auxin act by different mechanisms to alter root development. It still remains to be determined whether the response to auxins in shoots of *drr1* mutants is altered.

To better understand the morphogenetic and hormonal modulation of growth by alkamides and their interactions with other classic plant signals, we identified Arabidopsis mutants that have increased sensitivity to *N*-isobutyl decanamide. A *decanamide hypersensitive mutant*-(*dhm1*) was isolated and genetically characterized. Detailed cellular and developmental studies of WT and *dhm1* plants indicate that *dhm1* mutants show both increased inhibition of PR growth and promotion of LRs in response to *N*-isobutyl decanamide treatments when compared to WT plants. The *dhm1* mutant phenotype is also characterized by a constitutive tumorous shoot development, which is more drastic in plants treated with the alkamide. In addition, *dhm1* seedlings showed significant alterations in both shoot and root development when grown in dark conditions. The analysis of hormonal markers *DR5:uidA*, *ARR5:uidA* and *TCS*::*GFP* as well as pharmacological evidence show that *DHM1* is a crucial component of regulation of cell division and proliferation, which likely links alkamide with auxin and cytokinins in modulating plant growth and development.

Results

Isolation of *dhm1,* an Arabidopsis mutant with altered shoot and root response to *N*-isobutyl decanamide

The alkamide *N*-isobutyl decanamide has been shown to induce formation of callus-like structures on leaves and ectopic blades along petioles of rosette leaves of WT

Arabidopsis seedlings (López-Bucio et al. 2007). To investigate in more detail the genetic basis of plant responses to alkamides, we performed a mutant screen to identify essential genes potentially involved in proper plant growth and developmental responses to *N*-isobutyl decanamide. Arabidopsis EMS-mutagenized seeds were germinated in Murashige and Skoog (MS) 0.2X medium supplied with 35 μM *N*-isobutyl decanamide and screened 10 days after germination (d.a.g.) for seedlings with exacerbated responses to the effects of this compound on shoot and root systems. Among 25,000 lines that were grown under these conditions, three mutants were isolated, which in contrast to WT seedlings, showed increased sensitivity to *N*-isobutyl decanamide evidenced by generalized callus-like structures in the shoot system (Fig. 1a–j). Only one mutant survived transfer to soil and yielded viable seed. The mutant was backcrossed to WT plants (Columbia 0 [Col-0] ecotype) three times prior to detailed phenotypical analysis. At this stage, genetic analyses were performed to test the nature of the mutation. Segregation analysis of the mutant phenotype in F1 and F2 populations showed that formation of tumors segregated as a single Mendelian recessive trait. Seedlings from a F1 population had a WT phenotype and the F2 progeny segregates in a ratio consistent with WT:tumorous phenotype of 3:1 (Table 1). These results indicate that the overproduction of callus in the mutant isolated, resulted from a recessive single gene mutation. We named this locus as *DHM1* from *decanamide hypersensitive mutant1*.

dhm1 mediates the root architecture responses of Arabidopsis to *N*-isobutyl decanamide

To more clearly define the developmental alterations in *dhm1* mutants in response to alkamide treatment, we grew WT and *dhm1* seedlings side by side in MS 0.2X agar plates supplied with increasing concentrations of *N*-isobutyl decanamide. Ten days after germination (d.a.g.) WT seedlings showed PRs of 3.6 cm length with a few LRs when grown in medium without *N*-isobutyl decanamide, while seedlings treated with increasing concentrations of this compound showed a dose-dependent inhibitory effect on PR growth but increased LR formation (Fig. 2a–c). Interestingly, *dhm1* mutants showed a longer PR than WT seedlings and enhanced LR formation in medium without the alkamide, while PR growth was more inhibited and LR formation exacerbated in *dhm1* seedlings treated with *N*-isobutyl decanamide (Fig. 2a–c). The greater sensitivity of *dhm1* to *N*-isobutyl decanamide treatments was evidenced by increasing differentiation processes typified by root hair (RH) and LR formation closer to PR meristems when compared to WT seedlings (Supplemental Figure S1). In addition, we analyzed the responses of WT and *dhm1* seedlings to *N*-isobutyl decanamide in shoot development. In WT seedlings, callus-like structures were observed only in treatments of 30 μM *N*-isobutyl decanamide (Supplemental Figure S2). We found that a higher formation of callus-like structures was already evident in shoots of *dhm1* mutants grown in medium without the alkamide, while these structures increased in a dose-dependent way in response to the compound (Supplemental Figure S2). These results indicate that *dhm1* mutant seedlings are oversensitive to *N*-isobutyl decanamide.

Root and shoot development of the *dhm1* mutant

To more closely investigate the alterations in plant architecture caused by mutation in the *DHM1* gene, we compared the growth of WT (Col-0) and *dhm1* seedlings grown in 0.2X MS medium. We performed growth kinetic assays to measure PR length, LR number per plant, LR density, and fresh weight both in roots and in shoots. These assays showed that *dhm1* PRs were significantly longer than those of WT seedlings. A clear difference in length between WT and *dhm1* PRs started to be evident at 4 d.a.g. and further increased 10 d.a.g. (Fig. 3a). We also found a greater increase in LR number and density in *dhm1* mutants with time (Fig. 3b, c), showing a more robust and branched root system when compared to WT seedlings (Fig. 3d, e). Next, we quantified biomass production both in roots and in shoots from WT and *dhm1* seedlings. Our results show that root biomass production in *dhm1* mutants was two-fold increased, while shoot biomass production was up to four-fold of that produced by WT seedlings (Fig. 3f). These results suggest that *DHM1* gene function normally as a negative regulator on lateral root formation as well as in cell division and differentiation control, both in shoot and root systems.

We next analyzed the phenotype of *dhm1* seedlings germinated and grown for 10 days on 0.2X MS agar medium and then transferred to soil. Growth and development were analyzed every 7 days after transfer to soil by quantifying the length of floral stems, rosette diameter and number of visible leaves. In contrast to the phenotype observed in vitro, *dhm1* seedlings were significantly smaller than WT plants, with length of the main stem of around 15 cm, while that of WT plants reached up to 25 cm (Fig. 4a, d). Moreover, rosette size and number of visible leaves were smaller in the mutants than in WT plants (Fig. 4b–f). These results are consistent with a general growth defect in *dhm1* seedlings in soil, suggesting that *DHM1* plays an important role in plant organogenesis.

dhm1 mutants show increased lateral root primordia initiation and adventitious root formation from hypocotyl explants

The phenotype observed in *dhm1* mutants on LR formation suggests an enhanced response on pericycle cells and/or on growth of lateral root primordia (LRP) to produce more

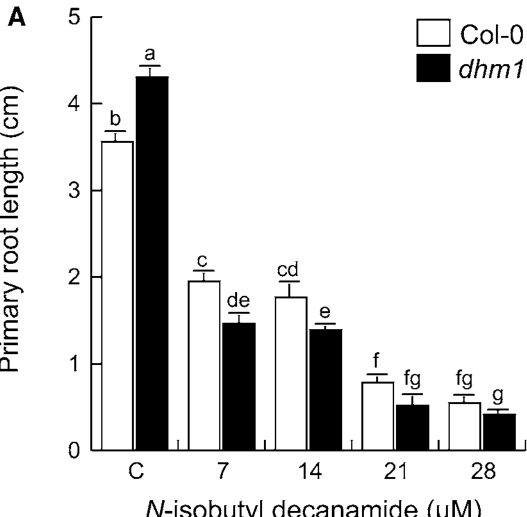
Fig. 1 Phenotypes of WT and *decanamide hypersensitive mutants* (*dhm*) of Arabidopsis. **a** Photograph of the shoot system of a WT (Col-0) seedling grown in 0.2X MS medium lacking *N*-isobutyl decanamide, or **b** a plant supplied with 35 μM of *N*-isobutyl decanamide. **c**–**e** putative alkamide-oversensitive mutants showing increased formation of callus-like structures in the shoot system. **f**–**j**, magnifications of photographs shown in **a**–**e**. *Bars* 500 μm

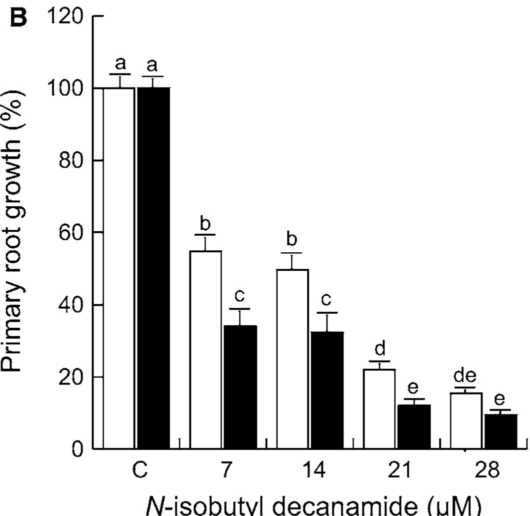
Table 1 Segregation ratio of progeny resulting from crosses between *dhm1* mutant and wild-type (WT) seedlings

Generation	Phenotype of progeny		Ratio obtained WT:*dhm1*	Ratio tested WT:Mutant	X^{2a}
	Normal leaves (WT)	Neoplasic leaves (*dhm1*)			
F_1	46	0			
F_2	175	54	3.24:1	3:1	2.46

[a] With one degree of freedom and critical value of 5 %, the hypothesis is accepted if the X^2 is smaller than 3.841

LRs. We analyzed the possible alterations on these processes in *dhm1* seedlings in LRP development and determined total LRP. WT and *dhm1* seedlings were grown side by side on the surface of agar plates containing 0.2X MS medium. Six days after germination seedling roots were first cleared to enable LRP at early stages of development





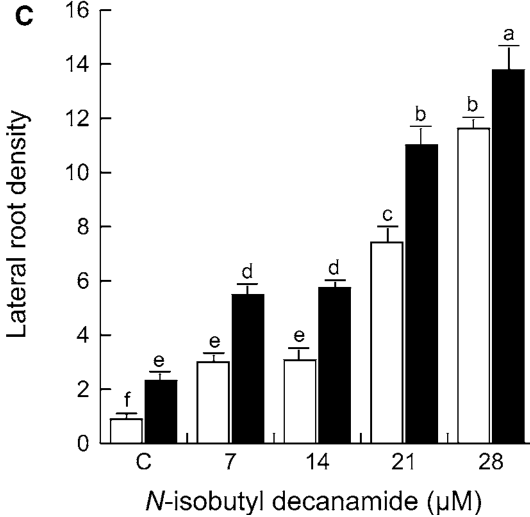


Fig. 2 Effects of *N*-isobutyl decanamide on root system architecture of WT (Col-0) and *dhm1* plants. **a** Primary root length. **b** Primary root growth (%). **c** Lateral root density, expressed as the number of lateral roots per centimeter. Data were recorded 10 days after germination. Values shown are mean ± SD (n = 20). *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results

to be visualized and counted and the developmental stage of each LRP was classified according to Malamy and Benfey (1997). We found increased density of LRP at stage I and II in *dhm1* seedlings compared with the WT (Supplemental Figure S3). The total number of LRP also increased significantly (Supplemental Figure S3), suggesting that *dhm1* PRs are more branched because they produce more LRP de novo from pericycle cells and because an increased maturation of pre-formed LRP.

In contrast to crops such as maize and rice, whose root systems are mainly composed by adventitious roots (Hochholdinger et al. 2004; Osmont et al. 2007), the formation of these lateral organs in Arabidopsis is scarse (Falasca et al. 2004). Nevertheless, auxins such as indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) have been found to increase adventitious root formation in this plant (Konishi and Sugiyama 2003; Sorín et al. 2005). To determine whether *dhm1* could be defective on adventitious root formation, we used hypocotyl explants as reported by Campos-Cuevas et al. (2008). WT and *dhm1* hypocotyl explants were obtained from 7 day-old etiolated seedlings and then grown 9 days side by side under light conditions in Petri plates containing agar solidified 0.2X MS medium. The number of adventitious root per explant was determined. Although *dhm1* explants were shorter than WT explants, a roughly twofold increase in adventitious root number in *dhm1* evidenced a strong organogenic capacity (Supplemental Figure S4). These data suggest that *DHM1* likely modulates the formation of lateral organs from hypocotyls.

dhm1 mutants show enhanced cell division in root and shoot meristems

The root and shoot system architectures in *dhm1* seedlings suggest an alteration in cell proliferation programs that precedes organ formation. We next analyzed the cell division responses of *dhm1* mutants by out-crossing a *dhm1* mutant with pollen from a transgenic *CycB1-uidA* seedling, because this is a good marker of mitotic activity since it is expressed only in cells in the G2/M phase of the cell cycle (Colón-Carmona et al. 1999). *CycB1-uidA* seedlings and homozygous *dhm1* seedlings harboring the *CycB1-uidA* construct were grown in 0.2X MS agar medium. Marker expression were analyzed in both root and shoot of *CycB1-uidA* and *dhm1* seedlings at 2, 4, 6 and 8 d.a.g. Cell division domains in *CycB1-uidA* seedlings were clearly visible in the shoot apical meristem (SAM) (Fig. 5a–d), and in the root apical meristem (RAM) (Fig. 5e–h).

Interestingly, *CycB1-uidA* expression in *dhm1* mutant seedlings was always higher than in *CycB1-uidA* seedlings (Fig. 5i–p). These results indicate that a longer PR and formation of callus-like structures in *dhm1* seedlings are related to an increase in cell division.

Development of *dhm1* mutants under dark conditions

The enhanced expression of *CycB1-uidA* cell division marker in *dhm1* seedlings explains, at least in part the tumorous phenotype of this mutant. However, exacerbate cell division may also lead to alterations in cell elongation and/or differentiation. To determine whether *dhm1* seedlings were defective on cell elongation and differentiation programs, we tested hypocotyl elongation and root system architecture in WT and *dhm1* seedlings grown side by side over the surface of MS 0.2X agar plates under dark conditions. Interestingly, while WT seedlings showed long hypocotyls with their apical hook well-defined (Supplemental Figure S5), *dhm1* hypocotyls were shorter and formed no apical hook (Supplemental Figure S5). Besides, WT seedlings showed a poorly developed root system

Fig. 3 Comparative root and shoot development of WT and *dhm1* seedlings grown in vitro. Arabidopsis plants were germinated and grown on agar plates containing 0.2X MS agar medium. Growth kinetic analyses were performed at the indicated times. **a** Primary root length. **b** LR number per plant. **c** LR density. **d** and **e** Photographs of representative WT (Col-0) and *dhm1* seedlings, respectively. **f** Fresh weight of root and shoot in WT and *dhm1* mutants grown in 0.2X MS medium. Values shown are mean ± SD (n = 60). *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results. *Scale bar* 1 cm

Fig. 4 Comparative development of WT and *dhm1* seedlings grown in soil. Arabidopsis plants were germinated and grown 10 days on agar plates containing 0.2X MS agar medium and then transferred to soil. Growth kinetic analyses were performed at the indicated times after transfer. **a** Floral stem length. **b** Rossette diameter. **c** Number of visible leaves. **d** Col-0 and *dhm1* seedlings 35 days after transfer to soil. **e** and **f** Close-up of rosette leaves 35 days after transfer. Values shown are means ± SD (n = 15). *Different letters* show means statistically different at the 0.05 level. This analysis was repeated twice with similar results

characterized by a short PR with small root hairs and absence of LRs (Supplemental Figure S5), which is consistent with their etiolation phenotype. In contrast *dhm1* seedlings showed a well-developed root system with longer PRs and root hairs than WT seedlings (Supplemental Figure S5). Moreover, *dhm1* roots were highly branched due to increments in LR and adventitious root formation (Table 2). The above-described characteristics of *dhm1* seedlings suggest an enhanced de-etiolation response consistent with an important role of *DHM1* in cell growth and differentiation.

dhm1 shows tissue specific modulation of auxin and cytokinin-responsive gene expression

Auxins control every aspect of plant development regulating cell division, elongation and/or differentiation processes. The *dhm1* root system, typified by greater LR and

Fig. 5 *CycB1-uidA* expression in transgenic WT and *dhm1* seedlings. *CycB1-uidA* and *dhm1/CycB1-uidA* were germinated and grown on agar solidified 0.2X MS medium. Twelve-hour GUS staining from 2, 4, 6 and 8 days old seedlings is shown for *CycB1-uidA* (**a**–**h**) and *dhm1/CycB1-uidA* (**i**–**p**). Seedlings were cleared to show representative individuals from at least 20 stained plants. The experiment was repeated three times with similar results. *Scale bar* 250 μm

Table 2 Enhanced de-etiolation of *dhm1* mutant seedlings

Developmental trait	(WT)	*dhm1*
Root length (cm)	1.6 ± 0.2	3.3 ± 0.7
Number of lateral roots	0.1 ± 0.3	13.0 ± 3.9
Hypocotyl length (cm)	1.9 ± 0.1	0.6 ± 0.1
Number of adventitious roots	0 ± 0	2.0 ± 0.2

RH forming capacity, is reminiscent of altered auxin responses. Therefore, the possibility was open that an enhanced auxin response could be related to the *dhm1* mutant phenotype. The involvement of auxin in mediating the observed effects on architecture of the root system in *dhm1* seedlings was tested using the auxin responsive marker gene *DR5:uidA*, which was transferred into *dhm1* by outcrosses. We first performed histochemical GUS expression to analyze LRP development in 6 day-old *DR5:uidA* and *dhm1/DR5:uidA* seedlings. GUS expression in LRP of *DR5:uidA* seedlings was always greater than in *dhm1/DR5:uidA* from all developmental stages investigated (Fig. 6a–p). These data suggest that unexpectedly, the *dhm1* mutant has a decreased auxin response in LRP. We also determined GUS expression in *DR5:uidA* and *dhm1/DR5:uidA* seedlings at 7 d.a.g. in the shoot system and in PRs. In contrast to the expression observed in LRP, an enhanced GUS expression in apical meristems and young leaves was evident in *dhm1/DR5:uidA* seedlings and interestingly, *dhm1/DR5:uidA* seedlings also showed an increased expression of the auxin-response marker in the PR tip and emerged LR tip (Fig. 7a–h).

To evaluate cytokinin response in *dhm1* seedlings, we out-crossed a *dhm1* mutant with pollen of a transgenic plant harboring the *ARR5:uidA* construct, which is induced by cytokinin (D'Agostino et al. 2000; Romanov et al. 2002). *ARR5:uidA* and *dhm1/ARR5:uidA* seedlings were

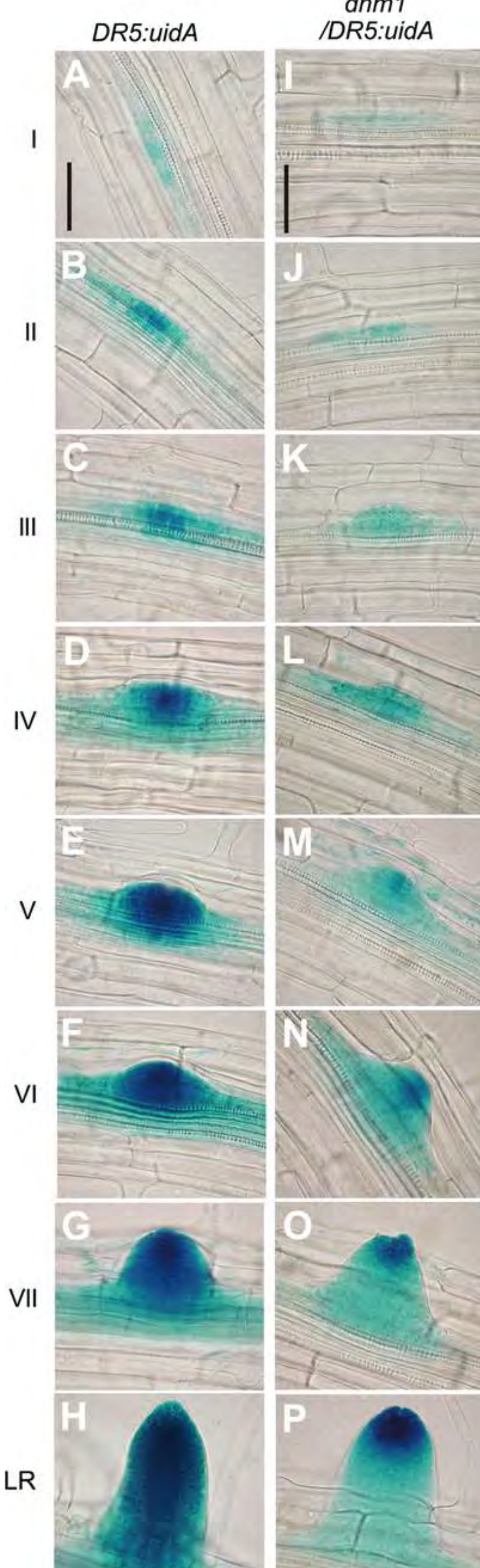


Fig. 6 *DR5:uidA* expression in lateral root primordia in WT and *dhm1* seedlings. Arabidopsis seedlings were grown 6 days on agar solidified 0.2X MS medium. Twelve-hour GUS staining is shown for *DR5:uidA* LRP in WT and *dhm1* seedlings. **a–h** *DR5:uidA* expression in all LRP stages analyzed in *DR5:uidA* seedlings. **i–p** *DR5:uidA* expression in *dhm1* LRP. Photographs are representative individuals of at least 20 stained seedlings analyzed. The experiment was repeated twice with similar results. *Scale bar* 50 μm

grown in 0.2X MS agar medium and 7 days after germination stained to reveal GUS activity. In WT seedlings, *ARR5:uidA* expression was detected mainly at the shoot meristem, in the root cap and in vasculature of the PR (Fig. 7i–l). *ARR5:uidA* expression in *dhm1* mutants was localized in the same regions that in *ARR5:uidA* seedlings, but interestingly, *dhm1* showed increased expression of this marker at most regions analyzed, including leaves, the vasculature, PR and in mature LRs (Fig. 7m–p). To uncover changes in cytokinin signaling in vivo, we followed *TCS*::*GFP* expression in *TCS*::*GFP* (Müller and Sheen 2008) and *dhm1/TCS*::*GFP* seedlings 7 days after germination by confocal microscopy. Under our growth conditions, from 10 representative seedlings analyzed, *TCS*::*GFP* expression domains were specifically located in the collumela regions in PR and LRs and in lower amounts in the shoot meristem. Interestingly, in *dhm1/TCS*::*GFP* seedlings, GFP expression was increased in LRs, in which the expression domain expanded towards the quiescent centre-root meristem region (Supplemental Figure S6).

NPA represses lateral root formation in *dhm1* seedlings

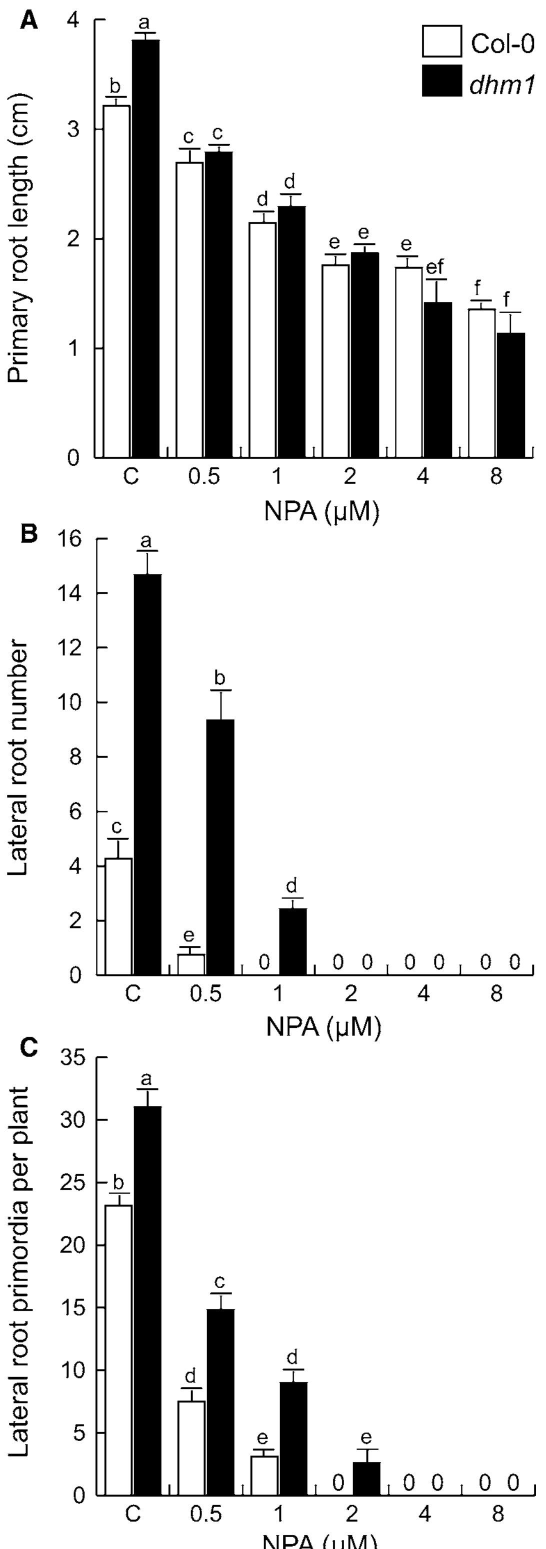
Expression analysis of the auxin-responsive gene marker *DR5:uidA* in *dhm1* LRP showed decreased auxin responsiveness, suggesting altered auxin transport and/or response. To examine the role of auxin transport in the increased formation of LRs in *dhm1* seedlings, WT and *dhm1* seedlings were grown side by side in agar plates containing MS 0.2X medium supplied with or without the auxin polar transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) and 8 d.a.g. RSA in both WT and *dhm1* seedlings was analyzed. PR length was decreased in a dose-dependent way in WT and *dhm1* seedlings by treatment with 0.5–8 μM NPA (Fig. 8a). These NPA concentrations decreased LR formation both in WT and *dhm1* seedlings, being WT seedlings more sensitive to concentrations of 0.5 and 1 μM NPA, as *dhm1* mutants were still able to form in average 10 and 3 LRs, respectively (Fig. 8b). Concentration of 2 μM NPA completely arrested LR formation in WT and *dhm1* seedlings (Fig. 8b). Because it is the initiation of LR meristems which determines the formation of

Fig. 7 Auxin and cytokinin-regulated gene expression in WT and *dhm1* seedlings. Twelve-hours of GUS staining of *DR5:uidA* (**a**–**d**) and *dhm1/DR5:uidA* (**e**–**h**) seedlings. GUS expression in *ARR5:uidA* (**i**–**l**) and *dhm1/ARR5:uidA* (**m**–**p**) seedlings after 6-h of GUS staining. Photographs are representative individuals of at least 20 plants stained. Plants were cleared to show representative individuals. The experiment was repeated twice with similar results. *Scale bars* 200 μm

LRs, we next tested the NPA effect on total LR primordia (LRP) per plant. It was found that NPA inhibited the formation of LRP in a dose dependent manner and that WT seedlings were more sensitive to NPA than *dhm1* seedlings from 0.5 to 2 μM NPA (Fig. 8c). The *DR5:uidA* expression patterns observed in WT and *dhm1* seedlings in response to 0.5 μM NPA during several stages of primordium development, showed that this auxin transport inhibitor decreases GUS expression at all stages analyzed in WT and *dhm1* mutants. This effect correlated with increased GUS expression in PR tips (Supplemental Figure S7), which was dose- dependent upon NPA treatment and is consistent with an accumulation of auxin in this zone. Greater concentrations of NPA further decreased the number of LRP expressing *DR5:uidA* and LRP number (Fig. 8c). These results suggest that the increase in LR formation in *dhm1* is dependent of auxin transport/signaling.

dhm1 shows normal auxin and cytokinin response on primary root development

The morphological and molecular responses related to auxin and cytokinins observed in *dhm1* seedlings indicate that the corresponding mutation might have altered the plant response to these regulators. To determine if *DHM1* is involved in auxin and/or cytokinin responses in PRs, we tested the PR growth response of *dhm1* seedlings to indole-3-acetic acid and kinetin, both of which are known to modulate PR growth. WT and *dhm1* seedlings were grown side by side on the surface of agar plates containing MS 0.2X medium supplied with a range of concentrations of these compounds and PR length determined. We found that the PR growth response of the *dhm1* mutants to indole-3-acetic acid and kinetin were similar to that observed in WT plants (Supplemental Figure S8). These assays indicate



◀ **Fig. 8** Effect of NPA on root system architecture in WT and *dhm1* seedlings. WT (Col-0) and *dhm1* seedlings were grown under increasing NPA concentrations on vertically oriented agar plates. 8 days after germination, the primary root length (**a**), lateral root number (**b**) and lateral root primordia number per plant (**c**) were determined. Values shown represent the mean ± SD (n = 30). *Different letters* indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results

that *dhm1* PRs are not resistant or hypersensitive to the exogenous supply of these compounds, but rather that an altered response to alkamides may affect the endogenous responsiveness to auxin and cytokinin in a tissue specific manner.

Discussion

dhm1 is an Arabidopsis mutant with increased sensitivity to alkamides

Alkamides and some related signals from plants and bacteria have been found to regulate diverse growth and developmental programs in plants (Ramírez-Chávez et al. 2004, López-Bucio et al. 2007; Campos-Cuevas et al. 2008; Coulon et al. 2012). In this report, we describe the identification and characterization of the Arabidopsis *decanamide hypersensitive mutant1* (*dhm1*) that shows increased sensitivity to *N*-isobutyl decanamide both in root and shoot systems, and present additional data that suggest an important role of *DHM1* gene in the regulation of cell division and differentiation, possibly through regulating auxin and cytokinin signaling. To our knowledge, this is the first report in plants of mutants selected for increased responses to fatty acid amides. Previous research from our group focused on alkamide signaling in several Arabidopsis accessions, including the analysis of mutants defective on auxin, jasmonic acid, and cytokinin signaling and the crosstalk with NAEs and AHLs (López-Bucio et al. 2007; Morquecho-Contreras et al. 2010). In this study, we selected for alkamide sensitivity with the goal of finding mutants with alterations in the targets of fatty acid amides that would be helpful to understand more in deep their role in basic cellular processes. It was our hypothesis that mutations that confer higher alkamide sensitivity might occur in genes that condition cell proliferation, as *N*-isobutyl decanamide treatment induces neoplasic growth in leaves, promotes lateral root formation in low concentrations and the formation of nodule-like structures instead of normal lateral roots at higher levels in Arabidopsis WT seedlings (López-Bucio et al. 2007).

The finding that several *dhm* mutants with neoplasic growth were identified (Fig. 1) indicates that the group of alkamide-sensitive mutants is genetically complex and that many more mutants will be needed to saturate the group. For instance, only one allele (*dhm1*) could be recovered because the rudimentary leaves of *dhm* seedlings compromised their survival in soil at early developmental stages. Following exposure to *N*-isobutyl decanamide, Arabidopsis *dhm1* seedlings show significant differences from the WT. *N*-isobutyl decanamide inhibited PR growth and stimulated

LR formation in WT seedlings in a dose-dependent manner, but *dhm1* showed exacerbated responses on these processes under treatments with the alkamide (Fig. 2; Supplemental Figure S1). *dhm1* seedlings were also oversensitive to *N*-isobutyl decanamide in the formation of callus-like structures in leaves (Supplemental Figure S2). More interesting, when grown in medium lacking alkamides in vitro, *dhm1* mutants sustained increased PR growth with prolific formation of LRs and LRP (Fig. 3; Supplemental Figure S3), thus indicating that *DHM1* plays an important role in RSA remodeling under normal growth conditions.

Given the apparent complexity of the *dhm1* phenotype evidenced in the shoot and the root system, it was surprising that only a few similar mutants were previously reported in the literature, these include mutants over-responsive for cytokinins or defective in cell wall biosynthesis (Frank et al. 2002; Bouton et al. 2002; Krupkova et al. 2007; Krupková and Schmülling 2009). It is possible that only alkamide-related mutants harboring weak alleles could be recovered as only plants defective in these and not stronger alleles are able to survive and produce viable seed. Among the few Arabidopsis mutants with formation of callus or tumors on leaves, alleles of *tumorous shoot development* (*TSD1* and *TSD2)* and *quasimodo1* (*QUA1*) have been characterized. Mutants defective in each of these loci show a disorganized cell proliferation program throughout the shoot system, commonly associated with alterations on cell wall composition and/or cell adhesion (Frank et al. 2002; Bouton et al. 2002; Krupkova et al. 2007; Krupková and Schmülling 2009). *TSD1*, *TSD2* and *QUA1* genes, encode enzymes involved in cellulose synthesis, cell adhesion and pectin synthesis, respectively (Bouton et al. 2002 Krupkova et al. 2007; Krupková and Schmülling 2009). Our detailed analyses of *dhm1* mutants show that they exhibit similar alterations on shoot development as *tsd2* mutants. However, the *dhm1* seedlings show marked differences in root system development when compared to the reported phenotype of *tsd2* seedlings, being *tsd2* characterized by decreased growth of the PR and reduced formation of LRs (Krupkova et al. 2007). In contrast, *dhm1* seedlings show enhanced PR growth and prolific formation of LRs (Fig. 3). It remains to be determined whether *tsd2* mutants have alterations in root hair or adventitious root development as shown for *dhm1* mutants (Supplemental Figure S4). These contrasting differences in root architecture indicate an opposite role between *DHM1* and *TSD2* on root development, suggesting that *DHM1* could be a novel locus involved in plant responses to alkamides that impact cell proliferation programs.

The *dhm1* phenotype is in contrast to that described for *decanamide resistant root1* (*drr1*) mutant, which was isolated because of its continued PR growth and reduced LR formation in response to *N*-isobutyl decanamide. Our previous characterization of LRP development in WT and *drr1* mutants revealed that *DRR1* is required at an early stage of pericycle cell activation to form LRP in response to *N*-isobutyl decanamide. When grown both in vitro and in soil, *drr1* mutants showed dramatically increased longevity and reduced hormone- and age-dependent senescence, which were related to reduced LR formation (Morquecho-Contreras et al. 2010). Our present results suggest that LR development and age-dependent plant senescence are also directly connected through *DHM1* as phenotype alterations were also seen in *dhm1* plants grown in soil under long days (16-h-light/8-h-dark conditions). In *dhm1* plants, leaf senescence occurred earlier than in WT seedlings, there was a decreased longevity, reduced production of leaves and decreased growth of stem and leaves (Fig. 4). In this way, the *dhm1* mutants show the opposite senescence and growth characteristics of *drr1* seedlings.

dhm1 mutants show enhanced expression of a cell division marker

Cell division normally ceases during leaf development (Donnelly et al. 1999; De Veylder et al. 2002) and is not observed in mature organs. Increased concentrations of *N*-isobutyl decanamide in the growth medium were found to induce the production of callus-like structures in roots and in leaves of WT Arabidopsis seedlings (López-Bucio et al. 2007). The appearance of callus on cotyledons, hypocotyls and leaves in *dhm1* seedlings are similar to the effects caused by alkamide treatment in WT Arabidopsis seedlings, suggesting that plant tissues have extended meristem activity that increases upon alkamide supply. To verify whether the ectopic organs originated from cells that expressed a meristem marker gene, we monitored expression of *CycB1* marker, which is specifically expressed during the G2-to-M phases of the cell cycle. Accordingly, *CycB1-uidA* fusion protein was expressed in the shoot and root apical meristems in WT seedlings (Fig. 5). Microscopical analyses of the shoot and root apex of young seedlings showed that the expression domain of *CycB1-uidA* increased in *dhm1* mutants. Moreover, in the epidermis of cotyledons, petioles and leaves of *dhm1* seedlings, clusters of cells expressing *CycB1-uidA* were observed (Fig. 5), GUS staining being present over a wider region than in non-treated plants, suggesting that cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. *dhm1* mutant seedlings also had a higher PR growth rate than WT seedlings (Fig. 3a), which coincided with an increased expression of the *CycB1-uidA* marker in the PR meristem (Fig. 5m–p). Taken together, these results suggest that alkamides alter several aspects of plant morphogenesis through the control

of meristematic activity involving the *DHM1* gene and that this gene acts to repress cell proliferation.

Skotomorphogenesis in *dhm1* mutants

In contrast to WT seedlings, *dhm1* mutants did not etiolate after two weeks under dark conditions. Microscope observations of dark-grown WT and *dhm1* seedlings showed that *dhm1* hypocotyls are plenty of callus-like structures. Later in development in the dark, *dhm1* seedlings produced longer primary roots with increased formation of LRs and RHs (Supplemental Figure S5). These results were similar to those observed in cytokinin-overproducing mutants *altered meristem program1* (*amp*) and *high organogenic capacity1* (*hoc1*), which have a high organogenic capacity for shoot regeneration but fail to etiolate normally (Chaudhury et al. 1993; Catterou et al. 2002). Catterou and associates (2002) indicated that the dark-grown characteristics of *hoc* mutants could be phenocopied by the application of exogenous cytokinins to WT dark-grown seedlings. Due to its dark-grown characteristics, *dhm1* appeared partially similar to photomorphogenic mutants *de-etiolated* (*det*) and *constitutive photomorphogenesis* (*cop*) (Deng and Quail 1992). Chory (1992) reported that cytokinins can replace normal light requirements in certain photomorphogenic responses, suggesting that cytokinins are able to act independently of light for promoting photomorphogenic response. Consequently, the skotomorphogenesis of dark-grown *dhm1* mutants might be induced by elevated cytokinin content or increased responsiveness to cytokinins.

Auxin is another hormone involved in photomorphogenesis. Auxin transport and/or signaling are required for light responses in hypocotyl growth in Arabidopsis. Steindler and coworkers (1999) reported that the light-regulated *ATHB-2* gene acts as a negative regulator of shade-induced hypocotyl elongation, and they found this response to be altered in the auxin response mutant *auxin resistant1* (*axr1*). A role for auxin transport in this response was supported by experiments in which treatments with NPA caused reduced hypocotyl elongation in response to low red light to far-red light (R:FR) ratios (Steindler et al. 1999). Additional data indicating a strong connection between light and auxin transport include changes in auxin transport rate and intensity induced by red light treatments in cucumber seedlings and differential growth mediated by lateral transport of auxin triggered by phototropic responses in pea and tobacco (Shinkle et al. 1998). Moreover, Gil et al. (2001) found that two mutants of Arabidopsis termed *dark overexpression of* CAB (*doc1*), which display altered expression of light-regulated genes, and *transport inhibitor response3* (*tir3*), known for its reduced auxin transport-have similar growth defects and define mutations in a single gene termed BIG. Expression-profiling experiments indicated that altered expression of multiple light-regulated genes in *doc1* mutants can be suppressed by elevated levels of auxin caused by overexpression of an auxin biosynthetic gene, suggesting that normal auxin distribution is required to maintain low-level expression of these genes under dark conditions. Double mutants of *tir3* with the auxin-related mutants *pin formed* (*pin1*), pinoid (*pid*), and *auxin resistant1* (*axr1*) display severe defects in auxin-dependent growth of the inflorescence. In contrast to what is known on the regulation of hypocotyl growth and photosynthesis, less is known about the development of the root system under contrasting light supply. The possibility is open that an altered auxin/cytokinin ratio in *dhm1* mutants is responsible of the increased development of the root system of the mutants under dark conditions.

Auxin and cytokinin responses in *dhm1* mutants

Morphologic alterations induced in Arabidopsis by *N*-isobutyl decanamide have been previously found to be independent of auxin and at least in part dependent of cytokinin signaling (López-Bucio et al. 2007). However, detailed physiological analysis of alterations induced by *DHM1* mutation in plants indicates that it might be the auxin-cytokinin ratio and not only cytokinin signaling alone, the critical factor underlying the phenotype of *dhm1* mutants. This hypothesis is supported because the LR promoting effect of *N*-isobutyl decanamide (Fig. 2) correlated with decreased expression of the auxin response marker *DR5:uidA* in LRP in *dhm1* mutants (Fig. 6). These results suggest that under normal growth conditions, auxin synthesis/response in developing LRPs is supraoptimal for LR growth and that overproduction of cytokinins or another antiauxin in LRP might have antagonistic effects in auxin-regulated gene expression in *dhm1* mutants. In support of this hypothesis, while documenting the activity of serotonin, a neurotransmitter from animals ubiquitous to plants in regulating Arabidopsis RSA, we found that 10-to-160 μM serotonin promoted LR formation. This effect correlated with a decreased expression of *DR5:uidA* in LRP (Pelagio-Flores et al. 2011). In addition, interactions have been defined where cytokinin signaling promotes the expression of auxin signaling inhibitors and regulates the complex network of auxin transport proteins to position zones highly responsive to auxin (Bishopp et al. 2011). Thus, LR formation seems to be coordinated by subtle spatial differences in the concentrations of auxin and cytokinins. Alkamides might thus promote LRP development by modulating such signaling crosstalk important for LR formation.

WT and *dhm1* seedlings were analyzed for differences in auxin and cytokinin signaling to detect an eventual

correlation between the mutant phenotype and changes in hormone responses. There was an increase in *DR5:uidA* expression in PR and LRs in *dhm1* mutants, whereas *ARR5:uidA* expression was thoroughly increased in the shoot system and in emerged LRs (Fig. 7). Another marker commonly used to monitor cytokinin signaling in vivo, namely *TCS*::*GFP*, expanded its expression domain in emerged LRs of *dhm1* seedlings (Supplemental Figure S6). In plants, the correlation between overexpression of genes encoding cell cycle regulators such as *CycB1* and its relationship with the hormonal status is poorly understood. The enhanced LR formation in *dhm1* mutants mimic an auxin phenotype, and the tumorous shoot phenotype seems to be more related to enhanced cytokinin responses. This is in agreement with our results showing stronger cytokinin-inducible *ARR5:uidA* expression in the shoot system. Two mutants that overproduce auxin have been previously reported in Arabidopsis. One mutant overproliferating LRs was independently isolated several times and called *sur1* (Boerjan et al. 1995), *rty* (King et al. 1995), *hls3* (Lehman et al. 1996), and *alf1* (Celenza et al. 1995). The *RTY/SUR1* gene encodes a protein similar to Tyr aminotransferases possibly implicated in auxin synthesis (Golparaj et al. 1996). The *dhm1* mutants are different to *sur1* mutants in several ways: (1) *sur1* mutants did not show formation of callus-like structures on leaves, (2) *sur1* mutants develop a stunted root system with dramatically increased formation of LRP but unable to sustain LR growth and (3) homozygous *sur1* mutants are unable to produce viable seeds. This information indicates that specific aspects of auxin signal transduction instead of general auxin biosynthesis are elicited in *dhm1* seedlings. Possibly, a loss-of-function mutation of a repressor that links auxin response to cell cycle progression may be responsible of the *dhm1* phenotype. Interestingly, treatments with NPA, an auxin transport inhibitor that block LR formation in WT seedlings were also effective in *dhm1* seedlings both decreasing emerged LRs and LRP (Fig. 8; Supplemental Figure S7), indicating that auxin transport is important for pericycle cell responses to *N*-isobutyl decanamide. In accordance, a recent study by Bai et al. (2012) showed that the alkamide-related signal from rhizobacteria 3-O-C10-HL stimulated adventitious root formation in mung bean explants by increasing polar auxin transport. It would be interesting to determine whether alkamides could affect the distribution of auxin transport proteins in plant tissues to induce organ formation.

From previous and present results, we provide a model to explain how alkamides, auxin and cytokinin signaling might interact at the level of the *DRR1* and *DHM1* loci to modulate plant morphogenesis (Fig. 9). Evidence is mostly based on the activity of *N*-isobutyl decanamide, although similar responses could be induced by other alkamides (i.e. affinin; López-Bucio et al. 2006) or alkamide-related signals such as *N*-acyl ethanolamines (NAEs; Blancaflor et al. 2003) and *N*-acyl-L-homoserine lactones (AHLs; Ortíz-Castro et al. 2008). In our model, *N*-isobutyl decanamide induces LRP emergence likely modulating repression of auxin signaling and activating nitric oxide accumulation (Campos-Cuevas et al. 2008; Méndez-Bravo et al. 2010). Cytokinin modulation of alkamide signaling is a two-step process involving LRP emergence from the pericycle by inhibiting auxin signaling and the further promotion of cell division in leaves to induce callus formation. Both LR induction and cell proliferation in shoots by *N*-isobutyl decanamide require normal *DRR1* and *DHM1* function, which play opposite functions in plant cell division. PR growth in *dhm1* seedlings was similarly affected by IAA and kinetin treatment than WT seedlings (Supplemental Figure S7), indicating that the mutants are not hypersensitive to auxin or cytokinin in PR growth.

Genetic evidence supports the hypothesis that NO acts downstream of auxin signaling to modulate cell cycle gene expression in pericycle cells either by acting as a second messenger modulating transcription of cell cycle genes (i.e. *CycB1*) or by direct binding to regulatory and/or structural

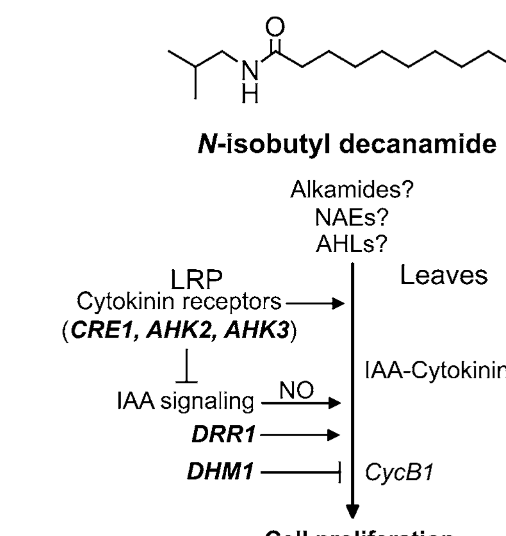


Fig. 9 Cell proliferation responses to *N*-isobutyl decanamide and their regulation. In this model, LR induction and cell proliferation by *N*-isobutyl decanamide require normal *DRR1* and *DHM1* function, with play opposite functions in plant cells. Previous evidence supports the hypothesis that NO acts downstream of auxin signaling to modulate cell cycle gene expression in pericycle cells either by acting as a second messenger modulating transcription of cell cycle genes (i.e. CycB1) or by direct binding to regulatory and/or structural proteins responsible for cell cycle transition, affecting their function by nitration or nitrosylation. The particular auxin-cytokinin interaction during alkamide responses is cell tissue specific and the amplitude of cell response to each plant growth regulator may determine the particular development outcome involved in determination of plant architecture

proteins responsible for cell cycle transition, affecting their function by nitration or nitrosylation (Méndez-Bravo et al. 2010). The particular auxin-cytokinin interaction during alkamide responses is cell tissue specific and the amplitude of cell response to each plant growth regulator may determine the particular development outcome involved in determination of shoot and root architecture.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana WT plants (Col-0 ecotype), *dhm1* mutant and the transgenic lines *CyCB1-uidA* (Colón-Carmona et al. 1999), *DR5:uidA* (Ulmasov et al. 1997), *ARR5:uidA* (D'Agostino et al. 2000) and *TCS*::*GFP* (Müller and Sheen 2008) were used for all experiments unless indicated otherwise. Seeds were surface sterilized with 95 % (v/v) ethanol for 5 min and 20 % (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2X MS medium (Murashige and Skoog 1962), pH 7, 0.6 % (w/v) Suc, and 1 % (w/v) phytagar. MS medium (MS basal salts mixture; catalog no. M5524) was purchased from Sigma. The suggested formulation is 4.3 g/l salts for a 1X concentration of medium; we used 0.9 g/l, which we consider and refer to as 0.2X MS. This medium lacks amino acids and vitamins. Phytagar (Micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant grown chamber (Percival Scientific AR-95L) with 16/8 h photoperiod at 100 μmol m^{-2} s^{-1} and temperature of 22 °C.

dhm1 mutant isolation and genetic analysis

Ethyl methanesulfonate (EMS)-mutagenized Col-0 seeds were purchased from Lehle Seeds (Round Rock Tx). Seeds were surface sterilized and germinated on MS 0.2X medium supplement with 35 μM *N*-isobutyl decanamide. Approximately 25,000 M2 seedlings were screened for short primary roots and callus formation in leaves. Fourteen days after germination, putative mutants with altered response to *N*-isobutyl decanamide were selected, transferred to soil and allowed to self-fertilize. Homozygous *dhm1* mutant plant was backcrossed with seedlings from the WT (Col-0) ecotype to remove unlinked mutations. To determine the segregation pattern of the *dhm1* phenotype, the F1 and F2 population derived from the cross between *dhm1* and Col-0 was analyzed. A typical 3:1 recessive segregation was observed for the WT/*dhm1* phenotype. The *CyCB1-uidA* (Colón-Carmona et al. 1999), *ARR5*:*uidA* (D'Agostino et al. 2000) and *DR5*:*uidA* (Ulmasov et al. 1997) genes were introduced into *dhm1* mutant by crossing them with seedlings from each transgenic line.

Analysis of growth and data analysis

The growth of PR was registered using a rule. LR number was determined counting all LRs emerged from the PR and observed under a 30X magnification with a stereoscopic microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). LR density was determined by dividing the LR number by the PR length and expressed as LR number per centimeter. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with a Tukey's posthoc test were used for testing differences in growth and root developmental responses in WT and *dhm1* mutants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Determination of developmental stages of LRP

Lateral root primordia were quantified at day 6 after germination. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LR primordium was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follows. Stage I, LRP initiation; in the longitudinal plane, approximately eight to 10 "short" pericycle cells are formed. Stage II, the formed LR primordium is divided into two layers by a periclinal division. Stage III, the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV, LR primordium with four cell layers. Stage V, the LR primordium is midway through the parent cortex. Stage VI, the LR primordium has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII, the LR primordium appears to be just about to emerge from the parent root.

Histochemical analysis of GUS activity

For histochemical analysis of GUS activity, transgenic Arabidopsis seedlings that express the *uidA* reporter gene (Jefferson et al. 1987) were stained in 0.1 % 5-bromo-4-chlorium-3-indolyl-β-D-glucuronide in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 100 mM, pH7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, for the indicated time at 37 °C. The stained seedlings were cleared with 0.24 N HCl in 20 % methanol (v/v) and incubated for 60 min at 62 °C. The solution was substituted by 7 % NaOH (w/v) in 60 % ethanol (v/v) for 20 min at room temperature. Plants were dehydrated with ethanol at 40, 20 and 10 % (v/v) for a 24 h period each. For each

marker line and for each treatment, at least 20 transgenic plants were analyzed using a stereoscopic microscope (Leica MZ6, Leica Microsystems).

Propidium iodide staining and *TCS:GFP* detection

For fluorescent staining with propidium iodide (PI), plants were transferred from the growth medium to 10 mg ml^{-1} of PI solution for 1 min. Seedlings were rinsed in water and mounted in 50 % glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568-nm excitation line and emission window of 585–610 nm, and *GFP* emission with 500- to 523-nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1000) after which the two images were merged to produce the final image.

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Chapter 11
Amino Compound-Containing Lipids: a Novel Class of Signals Regulating Plant Development

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11.1 Introduction

Plants produce compounds of different chemical identity that mediate a range of cellular functions, including volatiles such as ethylene and jasmonate, small organic hormones such as auxins, cytokinins, gibberellins and abscisic acid, brassinosteroids and lipids (Weyers and Paterson 2001). Lipids have long been recognized as signalling molecules that have the capacity of triggering profound physiological responses. In animals, ceramides and sphingosines are lipids that have pro-apoptotic and anti-proliferative actions (Wymann and Schneiter 2008). In plants, ceramides, sphingosines and phosphatidic acid are involved in mediating growth, development and responses to biotic and abiotic stimuli, and their production is regulated by a key set of lipid-modifying enzymes such as phospholipases, lipid kinases and/or phosphatases (Worrall et al. 2003; Wang 2004). Studies on the downstream targets and modes of action of signalling lipids are still in their early stages.

Alkamides and *N*-acyl ethanolamines (NAEs) comprise a group of bioactive acylamides with varied acyl chain length and saturation grade (Chapman 2004; López-Bucio et al. 2006; Morquecho-Contreras and López-Bucio 2007). In mammals, the metabolism of NAEs is part of the endocannabinoid signalling pathway wherein anandamide (NAE 20:4) acts as an endogenous agonist of G protein-coupled cannabinoid receptors that, in turn, regulate a wide array of physiological and behavioural processes, including modulation of neurotransmission in the central nervous system (Wilson and Nicoll 2002), synchronization of embryo development (Paria and Dey 2000) and vasodilation (Kunos et al. 2000). Recently, additional cellular targets of NAEs have been discovered, including ion channels

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(Movahed et al. 2005; Oz et al. 2005) and transcription factors (LoVerme et al. 2006).

Alkamides comprise more than 200 related compounds widely distributed in plants, ranging from lichens to angiosperms. An important role for these compounds in plant morphogenesis has been inferred from pharmacological application and mutant analysis in the model plant *Arabidopsis thaliana* (López-Bucio et al. 2006). In the past 5 years, accumulated evidence points to a role of NAEs and alkamides in diverse physiological processes, including seed germination and regulation of plant architecture. In this chapter, the recent information on NAEs and alkamides in plants is reviewed in the context of their occurrence, metabolism, interactions with other plant growth regulators and functions in plant development. Structurally related compounds from bacteria are the *N*-acyl homoserine lactones (AHLs). These compounds participate in cell-to-cell signalling between bacteria, usually referred to as quorum-sensing. It is proposed that small lipid signalling based on NAEs, alkamides and AHLs might be part of an ancestral inter-kingdom communication system between plants and their associated bacteria to regulate symbiotic and/or pathogenic behaviours.

11.2 Biosynthesis and Metabolism of Acylamides in Plants

Most organisms are known to contain in their inner and outer membranes amphipatic lipids based on one or two amino acids linked to a fatty acid through an amide bond. Three classes of compounds are beginning to be considered as plant signals based on their potent activities and strong morphogenic effects—NAEs, alkamides and AHLs (Table 11.1). NAEs represent compounds with aminoalcohol linked as an amide to the fatty acid. They are likely produced from the hydrolysis of *N*-acyl phosphatidylethanolamines (NAPEs), a minor constituent of cell membranes, by phospholipase D. NAPEs occur naturally in diverse biological systems. For example, NAPEs accumulate during cell injury or stress in animal tissues and dehydration in plant seeds (Hansen et al. 2002; Chapman 2004). The conformation of NAPEs in a lipid bilayer and their interaction with other lipids have been analyzed by spectroscopy studies (Lafrance et al. 1990; Swamy et al. 2000). For long-chain NAPEs, the *N*-acyl moiety is bent and buried in the hydrophobic phase of the phospholipid bilayer. By contrast, for short-chain NAPEs, the *N*-acyl chain remains at the level of the glycerol backbone, exposed to the aqueous milieu. NAPEs are the source of signalling NAEs, including anandamide (*N*-arachidonoyl ethanolamide; Table 11.1), which binds to cannabinoid receptors CB1 and CB2 and regulates important physiological processes in animals. The molecular determinants of the activity of the different types of phospholipases have been the subject of several structural studies (Caramelo et al. 2003; Okamoto et al. 2004). A novel NAPE-hydrolyzing phospholipase D (PLD) was cloned from mammals, which exhibited specificity towards NAPE substrates but not to other common membrane phospholipids, indicating that this is one of the enzymes responsible for converting NAPE to

Table 11.1 Characteristics of NAEs, alkamides and AHLs

Producing organism	Compound	Function
Animals	*N*-arachidonoyl ethanolamide (Anandamide)	Modulation of neurotransmission in central nervous system Synchronization of embryo development Brain development Cell proliferation Cardiovascular and immune regulation
Arabidopsis thaliana	*N*-acyl ethanolamine (NAE 12:0)	Regulation of seed germination Regulation of cell division and expansion Regulation of root architecture Control of cytoskeletal structure
Acmella radicans	*N*-isobutil decanamide	Regulation of cell division Regulation of root architecture Activation of cytokinin-signalling
Pseudomonas aeruginosa	*N*-3-oxo-dodecanoyl-HL	Virulence and biofilm formation
Pseudomonas fluorescens	*N*-decanoyl-HL	Population growth

NAE in vivo (Okamoto et al. 2004). Recent reports have shown that NAEs may be generated from NAPEs via phospholipase A (Simon and Cravatt 2006) or phospholipase C (Liu et al. 2006). In plants, PLD-β and PLD-γ catalyze the formation of NAEs from NAPEs in vitro (Pappan et al. 1998). PLDs from plants, animals and fungi share similarities in structure and catalytic mechanisms. However, the plant PLD family is much more complex than those of other organisms; for example, 12 PLD genes are present in *Arabidopsis*, whereas only two PLD genes are in mammals and one in yeast (*Saccharomyces cerevisiae*; Wang 2004). This complexity might account for the critical role of the PLD products phosphatidic acid and NAEs in plant signalling.

The enzymatic machinery for the degradation of NAEs is conserved between animals and plants. For example, an enzyme that rapidly hydrolyzes NAEs into ethanolamine and their corresponding fatty acids has been cloned from mammals. This enzyme, called fatty acid amide hydrolase (FAAH), belongs to a group of proteins containing a conserved amidase sequence (Shresta et al. 2003, 2006). The

amidase region of these proteins consists of about 125 amino acids. There is 18.5% identity between the *Arabidopsis* FAAH (*AtFAAH*) and rat FAAH when compared over the entire length of the proteins, whereas there is 37% identity within the amidase region. Functional homologues of *AtFAAH* were also identified in rice (*Oriza sativa*) and *Medicago truncatula*, supporting a common mechanism for the regulation of NAE hydrolysis in diverse plant species (Shresta et al. 2006). The possibility has been considered that a breakdown product of ceramide or other sphingolipids may result in metabolites similar to NAEs or alkamides (Ramírez-Chávez et al. 2004). Interestingly, a fatty acid amidase was identified recently as an alternate enzyme for NAE hydrolysis. This enzyme belongs to the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase (Tsuboi et al. 2005). The combined action of fatty acid hydrolases *AtFAAH* and ceramidases may account for the efficient metabolism of NAEs in different plant tissues.

11.3 Distribution of Acylamides

NAEs have been quantified in seeds of some higher plants, including cotton (*Medicago truncatula*), corn (*Zea mays*), *Arabidopsis*, soybean (*Glycine max*), tomato (*Lycopersicon esculentum*) and pea (*Pisum sativum*). The total NAE content varied among plant species from 500 to 1,600 ng/g fresh weight, with acyl chain length ranging from 12 to 20 carbon atoms (Chapman 2004). NAEs containing 16C and 18C were the most abundant compounds in dry seeds. Total NAE concentrations fall drastically following seed imbibition and during germination. In desiccated *Arabidopsis* seeds, total NAE content was of the order of 2,000 ng/g, and this declined to 500 ng/g within 96 h after sowing (Wang et al. 2006). The elevated concentrations of NAEs in seeds point to the possibility that these lipids may function in processes relevant to seed or seedling development.

Compared to seeds, the vegetative tissues of plants have lower NAE content and their NAE profile also differs, in that NAE 12:0 and NAE 14:0 appear to predominate (Chapman 2004). There is evidence that, in stem and leaves, medium-chain NAEs are synthesized on demand rather than being stored. For instance, the concentration of NAE 14:0 in tobacco (*Nicotiana tabacum*) leaves is approximately 5 ng/g on a fresh weight basis. Interestingly, a 10-min exposure to nanomolar concentrations of two protein elicitors, xylanase and cryptogein, caused a 10- and 50-fold increase in NAE 12:0 and NAE 14:0 respectively (Tripathy et al. 1999). Exogenous application of synthetic NAE 14:0 at a concentration of 1 μM induced a fourfold increase in phenylalanine ammonia lyase (PAL) expression in a manner similar to that elicited by xylanase and cryptogein in both cell suspensions and leaves of tobacco (Tripathy et al. 1999). These results suggest medium-chain NAEs may participate in the signal transduction events leading to plant defence responses.

Alkamides comprise over 200 related compounds that have been found in as many as ten plant families, namely the Aristolochiaceae, Asteraceae, Brassicaceae,

Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae and Solanaceae. Species containing high concentrations of alkamides are found in the Asteraceae, Piperaceae and Rutaceae (Christensen and Lam 1991; Laurerio-Rosario et al. 1996; Kashiwada et al. 1997). Certain alkamides, such as pellitorin, have been described as allelochemicals. Pellitorin is naturally present in *Stauranthus perforatus*, a rutaceous tree, and exerts a strong allelochemical effect on the growth of weeds (Anaya et al. 2005). At a concentration of 100 mg/ml supplied to the culture medium in vitro, pellitorin caused a 45 and 80% inhibition in root growth of *Amaranthus hypochondriacus* and *Echinocloa crusgalli* respectively. In a glasshouse experiment, the decomposition of leaves and roots of *S. perforatus* incorporated as green manures (2%) to the soil had a significant inhibitory effect on the growth of weeds. The allelopathic action of decomposition of plant tissues was comparable to that of DPCA (dimethyl tetrachloroterephthalate), a commercial herbicide (Anaya et al. 2005). Based on the results discussed above, it is tempting to speculate that pellitorin production by *S. perforatus* might represent a strategy for competition.

Alkamide-accumulating plants can occur in different plant families. Certain medicinal plants, such as *Echinacea angustifolia*, *Echinacea purpurea* and *Heliopsis longipes*, have been used in the past and present by different civilizations. These plants accumulate alkamides in plant tissues (Bauer and Reminger 1989; Molina-Torres et al. 1996). In *H. longipes*, a traditional herb endemic to central México, the alkamide affinin accumulates especially in roots, where it is present in as high as 1% (w/w) on a fresh weight basis (Molina-Torres et al. 1996). In *E. purpurea*, alkamides accumulate preferentially in flower heads and in roots. Their levels are low at the beginning of vegetative growth and increase at the flowering stage (Letchamo et al. 1999; Qu et al. 2005). For total alkamides, concentrations varied from 5 to 27.6 mg/g in roots and from 0.22 to 5.3 mg/g in vegetative tissues (Qu et al. 2005). The reason why certain plants accumulate alkamides is not clear. However, it could provide an advantage for competition, acting as allelochemicals as in the case of pellitorin, or might represent a mechanism to adjust plant growth and development, as their importance in cellular processes is increasingly being appreciated.

11.4 Role of NAEs and Alkamides in Plant Development

The plant kingdom is a vast storehouse of chemical substances manufactured and used by plants as defences against viruses, bacteria, fungi and insects. Physiological and ecological constraints play key roles in plant growth patterns. Plant activity at the cellular level can be classified in general terms as growth (cell division and enlargement) or differentiation (chemical and morphological changes leading to cell specialization). Some plants provision seeds with high concentrations of secondary metabolites, possibly to protect the seed and the rapid growing seedling before it has developed the capacity to synthesize significant quantities on its own

(Herms and Mattson 1992). Growth processes demand particularly high concentrations of limited plant resources. They are highly dependent on protein synthesis for the manufacture of photosynthetic, biosynthetic and regulatory enzymes, as well as for structural protein. The production of secondary metabolites competes directly with protein synthesis and, consequently, with growth. Thus, it is not surprising that plants exposed to pathogens or to sub-lethal abiotic stress conditions exhibit a broad range of morphogenic responses (Potters et al. 2007). Despite the diversity of phenotypes, a generic stress-induced growth response can be recognized that appears to be carefully orchestrated and comprises three components, namely inhibition of cell elongation, localized stimulation of cell division and alteration in cell differentiation (Herms and Mattson 1992; Potters et al. 2007). Although the stress-induced developmental responses seem to be part of a general acclimation strategy, whereby plant growth is redirected to diminish stress exposure, little is known about the molecular mechanism underlying this response. Altered phytohormone synthesis, transport and/or metabolism could be part of the physiological component of plant growth modulation. Five major hormones have been identified, namely auxins, abscicic acid (ABA), cytokinins, ethylene and gibberellin (GA). Other signals are currently being added to this list, including brassinosteroids and certain lipids such as sphingolipids and phosphatidic acid (Weyers and Paterson 2001; Wang 2004). In our studies, we have identified a new class of regulatory molecules in plants, the alkamides, which are structurally similar to NAEs. The alkamides were initially identified in terms of their similarity to ceramides, a group of key signals in yeast and animals (Ramírez-Chávez et al. 2004; López-Bucio et al. 2006). Until recently, NAEs and alkamides had been considered as a class of secondary metabolites that accumulated in particular plant species and tissues. Research using *Arabidopsis thaliana* suggested that they might have a pivotal role in integrating environmental signals into developmental transitions. Alkamides and NAEs appear to deliver a message regulating a particular plant function. The functions include germination and alteration of growth and differentiation in the course of development.

11.4.1 Seed Germination

Seed dormancy and germination are under the control of phytohormones and their signalling pathways. Genetic analysis of seed germination in *Arabidopsis* has revealed that GA and ABA are crucial regulators (Finch-Savage and Leubner-Metzger 2006). GA is regarded widely as a growth-promoting compound that positively regulates germination. By contrast, ABA has historically been considered to function as a germination inhibitor (Razem et al. 2006).

The notion that NAEs plays a role in seed germination is supported by their accumulation in desiccated seeds of a wide range of plant species but, during imbibition and germination, NAE concentrations decrease significantly and remain low during subsequent seedling growth (Venables et al. 2005). These observations

suggest that the rapid metabolism of NAEs is a prerequisite for germination. In fact, exogenous application of NAE 10:0, and the alkamides affinin and *N*-isobutyl decanamide regulated in a dose–response manner the germination of seeds from different plant species, with low concentrations (nm range) promoting germination and high concentrations (mM range) drastically inhibiting germination (Morquecho-Contreras and López-Bucio, unpublished data).

Important information about the in vivo role of NAEs in germination came from the manipulated expression of *AtFAAH*, an enzyme that hydrolyzes NAEs into ethanolamine and free fatty acids, in *Arabidopsis thaliana*. In this way, Wang et al. (2006) reported that *AtFAAH* expression and FAAH catalytic activity increased during seed germination and seedling growth, consistent with the timing of NAE depletion. Moreover, the authors identified T-DNA mutants of *A. thaliana* and generated transgenic plants overexpressing *AtFAAH*. It was found that seeds of *AtFAAH* mutants possessed elevated concentrations of endogenous NAEs, and seedling growth was hypersensitive to exogenously applied NAE 12:0. By contrast, seeds and seedlings of *AtFAAH*-overexpressing plants had lower endogenous NAE content and seedlings were less sensitive to exogenous NAE (Wang et al. 2006). Intriguingly, no phenotype on germination was reported for *AtFAAH* mutants or overexpressors, indicating that additional enzymes are likely involved in NAE metabolism with potential redundant functions.

The interaction between NAEs and abscisic acid in regulating seed germination was recently shown by Teaster and co-workers (2007), who reported that NAE and ABA concentrations were depleted during seed germination. Combined application of low concentrations of ABA and NAEs produced a more dramatic reduction in germination than either compound alone. Transcript profiling and gene expression studies in NAE-treated seedlings revealed elevated transcripts for a number of ABA-responsive genes and genes typically enriched in desiccated seeds (Teaster et al. 2007). These data suggest that NAEs act in concert with ABA to regulate seed germination. Whether alkamides interact with ABA or GA to affect germination remains to be determined.

11.4.2 Shoot Development

The shoot represents the aboveground part of higher plants. It is composed of the stem with its branches, and axillary meristems. The stem raises foliage and flowers for optimal light exposure and seed dispersal. The ultimate source of all aboveground organs is a small population of stem cells in the central zone of the shoot apical meristem (Laux and Mayer 1998). Leaf initiation at the shoot apical meristem involves a balance between cell proliferation and commitment to make primordia. *Arabidopsis* has a typical simple leaf, which consists of a petiole and a blade (Fig. 11.1a). The presence of a petiole is presumed to be important in the effective capture of light by ensuring that the leaf blades do not overlap. To produce this leaf shape, the cells on the proximal side of the leaf differentiate into petioles

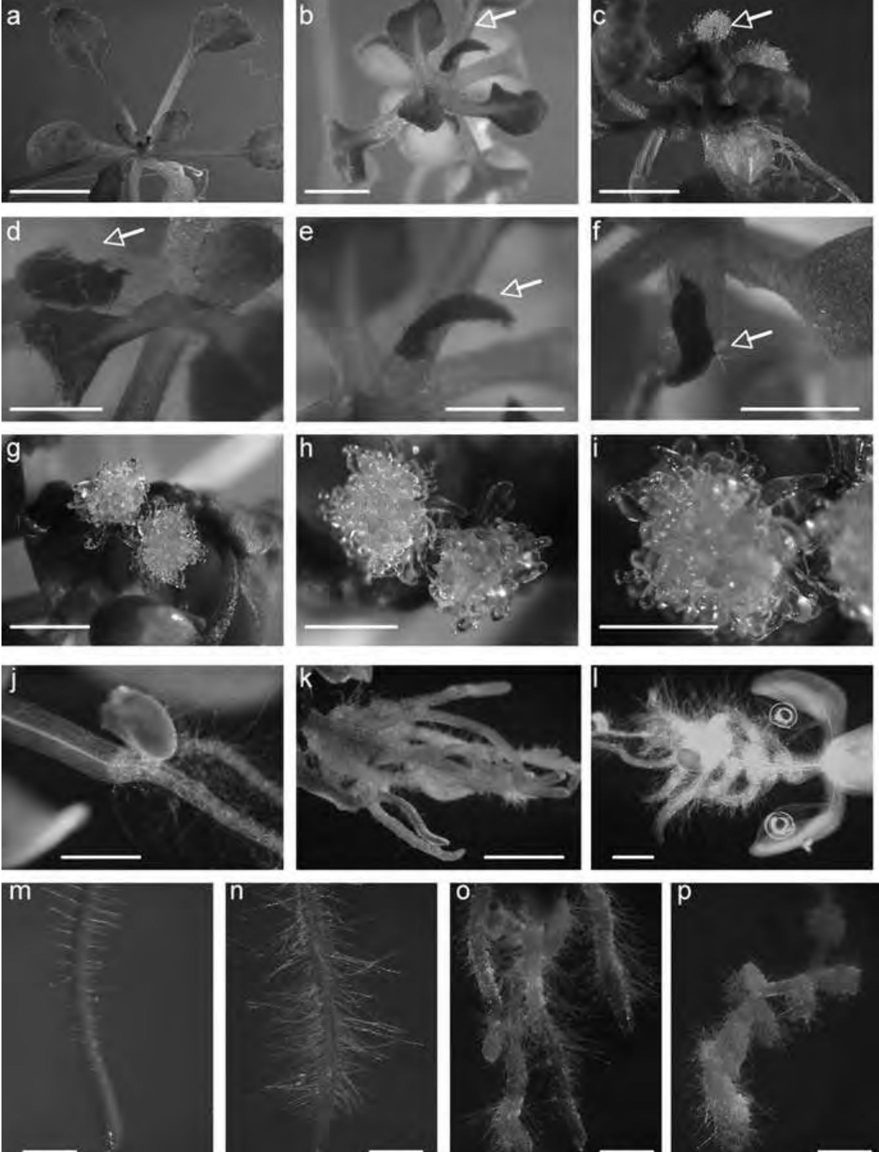

Fig. 11.1 Effects of *N*-isobutyl decanamide on *Arabidopsis* shoot and root development. (**a–c**) Shoot development of 21-day-old *Arabidopsis thaliana* grown on 0, 28 and 56 μM *N*-isobutyl decanamide respectively. Note the formation of blades on petioles and callus-like structures on leaf surfaces (*arrows*). (**d–f**) Close up of developing blades on petioles showing the presence of trichomes (*arrows*). (**g–i**) Close up of callus-like structures formed over the surface of leaves. (**j–l**) Adventitious root development of *Arabidopsis* seedlings grown for 18 days on 0 (**j**) or 56 μM *N*-isobutyl decanamide (**k**, **l**). Note the proliferation of adventitious roots on the stem region of *N*-isobutyl decanamide-treated plants. (**m–p**) Development of the root apical zone of 12-day-old

without producing blades or other organs (Ha et al. 2003). Two of the clearest effects of *N*-isobutyl decanamide on shoot development were the ectopic induction of outgrowths along the leaf petioles and the formation of callus-like structures on blades (Fig. 11.1b, c). The outgrowths formed on petioles resembled a leaf blade in that they showed the presence of trichomes, a class of differentiated epidermal cells commonly present in blades but not in petioles (Fig. 11.1d–f). In addition, *N*-isobutyl decanamide treatments were found to induce the production of callus-like structures on leaves (Fig. 11.1g–i). These structures sustained growth as plant development progressed and expressed genes indicative of proliferative activity such as cyclin B1, which is involved in cell cycle transitions (López-Bucio et al. 2007). These results suggest that, in plants treated with the alkamide, leaf cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. The fact that petiole cells do not undergo correct developmental specification and are diverted towards other developmental fates, such as blade formation, indicates that the alkamide is also capable of reprogramming petiole cells to initiate the de novo formation of organs in differentiated cells.

11.4.3 Root Development

Roots perform the essential activities of providing water, nutrients and physical support to the plant. The primary root originates in the embryo and produces many lateral roots during the lifetime of a plant, and each of these produces more lateral roots. The quantity and placement of these determine the architecture of the root system; in turn, this plays a major role in determining whether a plant will survive in a particular climate or environment (Malamy and Benfey 1997; Casimiro et al. 2003). During the post-embryonic development of plants, new axes of growth emerge from shoot tissues through adventitious organogenesis. This is particularly important in crops, such as maize, in which adventitious root formation provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or injury. While lateral roots typically form from the primary root pericycle, adventitious roots form naturally from stem tissue. Lateral and adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones such as auxin, and environmental factors such as light and wounding (Casimiro et al. 2003).

Different reports indicate that NAEs and alkamides may play an important role in regulating root architecture, with stimulating or repressing effects in biomass production depending on the compound, the concentration in the medium and

Fig. 11.1 (continued) *Arabidopsis* seedlings grown on 0, 14, 28 and 56 μM *N*-isobutyl decanamide respectively. The shorter distance between the root tip and the root hair zone is an indication of a faster differentiation process (compare **m** and **n**) and an increase in root hair density. Note the formation of lateral roots close to the root tip (**o**) and the conversion of the primary root tip and lateral roots into callus-like structures (**p**). Scale bars (**a**–**l**)=5 mm, (**m**–**p**)=500 μm

conditions of culture. Micromolar concentrations of NAE 12:0 and NAE 18:2 supplied to *A. thaliana* seedlings grown in agar plates inhibited primary root elongation and disrupted normal cell growth in a dose-dependent and selective manner (Blancaflor et al. 2003; Motes et al. 2005; Wang et al. 2006). Kanbe et al. (1993) showed that amidenin, a non-substituted alkamide isolated from the actinomycete *Amycolatopsis* sp., promoted the growth of rice plants at concentrations of 0.6 and 1.8×10^{-5} M and inhibited growth at concentration of 6×10^{-5} M. Similarly, the alkamides affinin and *N*-isobutyl decanamide showed a dose-dependent effect on biomass production in *A. thaliana*, which correlated with primary root growth inhibition and enhanced lateral root proliferation (Ramírez-Chávez et al. 2004).

As a first step in exploring the structure–activity relationships of NAEs and alkamides, López-Bucio and co-workers (2007) quantified the root growth response of *Arabidopsis* seedlings to natural and synthetic compounds. From a group of similar chain length NAEs and alkamides, they identified *N*-isobutyl decanamide, a C10 saturated alkamide that is naturally produced in *Acmella radicans* (Ríos-Chávez et al. 2003) and *Cissampelos glaberrima* (Laurerio-Rosario et al. 1996), as the most active compound in inhibiting primary root growth and stimulating lateral root formation. The plant regenerative properties of *N*-isobutyl decanamide were tested further in *Arabidopsis* explants that were treated with various concentrations of this compound. Cultivation of stem explants that harboured the shoot apical meristem on a nutrient-rich medium lacking alkamides resulted in the formation of plants with fully developed shoot and root systems. By contrast, explants obtained from stems or primary roots resulted in the development of adventitious roots in stem explants or of lateral roots in explants from primary roots (Campos-Cuevas et al. 2008). *N*-isobutyl decanamide treatments were found to induce adventitious root formation both in explants or intact *Arabidopsis* seedlings (Fig. 11.1j–l). This effect was accompanied by the differentiation of the primary root meristem, followed by root hair and lateral root formation close to the primary root tip (Fig. 11.1m–p). The stimulation of root branching by *N*-isobutyl decanamide was related to a general plant growth-promoting effect of this compound in plants regenerated from explants, indicating a potential use of alkamides in the propagation of plants and/or explants by tissue culture means (Campos-Cuevas et al. 2008).

11.5 Signals Interacting with NAEs and Alkamides

11.5.1 Auxins

The effects of NAEs and alkamides in root and shoot development suggest that phytohormones could be involved in the responses of plants to these compounds. Auxins are involved in altering primary root growth and in promoting lateral and adventitious root formation (Woodward and Bartel 2005). Several lines of evidence

indicate that the effects of alkamides on root system architecture are independent of auxin signalling. First, the examination of primary root growth of auxin-resistant mutants, *aux1-7*, *eir1* and *axr4-2*, in response to high affinin concentrations revealed a primary root growth inhibition similar to wild-type plants (Ramírez-Chávez et al. 2004). Second, affinin and *N*-isobutyl decanamide were able to induce high numbers of adventitious roots in shoot explants from auxin-resistant mutants (Campos-Cuevas et al. 2008), indicating a normal cell proliferating response. Third, affinin and *N*-isobutyl decanamide failed to activate the expression of the auxin-inducible gene markers *DR5:uidA* and *BA3:uidA* in primary roots and during adventitious root formation in explants (Ramírez-Chávez et al. 2004; Campos-Cuevas et al. 2008). This information suggests that alkamides regulate plant development by an auxin-independent signalling mechanism.

11.5.2 Cytokinins

Cytokinins are purine derivatives that promote and maintain plant cell division in cultures and are also involved in various differentiation processes, including shoot formation, primary root growth and callus formation (Howell et al. 2003). Three different cytokinin receptors have been described, which activate gene expression in a cytokinin-dependent manner. These receptors are sensor histidine kinases, encoded by the *cre1/ahk4/wol*, *ahk2* and *ahk3* genes in *Arabidopsis* (Kakimoto 2003).

The possibility that alkamides could regulate organ development in roots and shoots interacting with cytokinin signalling was investigated recently. This was achieved by evaluating the activation of cytokinin gene expression markers in response to *N*-isobutyl decanamide and testing the responses of single, double and triple mutant combinations for the three known cytokinin receptors to this alkamide (López-Bucio et al. 2007). Interestingly, the ectopic formation of blades on petioles and callus-like structures on leaves was related to an enhanced expression of *ARR5:uidA*, a cyokinin-inducible gene marker in shoots. The triple cytokinin receptor mutant *cre1-12/ahk2-2/ahk3-3* was insensitive to *N*-isobutyl decanamide treatment, showing absence of blades on petioles and callus-like structures in leaves under elevated concentrations of this alkamide. These results suggest that alkamides interact with cytokinin signalling to control cell division and differentiation processes during plant development. The molecular mechanisms underlying this interaction are currently under investigation.

11.5.3 Nitric Oxide

Nitric oxide (NO) is a critical signalling molecule in several vital processes in both mammals and plants. Because of its gaseous nature, it is a highly permeable molecule that freely diffuses thorough biological membranes. NO is synthesized

from the amino acid L-arginine. In plant systems, it can be produced principally by two routes: (1) from L-arginine by a nitric oxide synthase-like protein or (2) from nitrite via nitrate reductase and nitrite reductase enzyme action. Once synthesized and released, NO acts as mediator of developmental processes (Neill et al. 2003). Experimental data indicate the signalling roles for NO in processes such as xylem differentiation, programmed cell death, and lateral and adventitious root formation (Lamattina et al. 2003).

The relationship between NO and alkamides was investigated recently in *A. thaliana* shoot explants (Campos-Cuevas et al. 2008). *N*-isobutyl decanamide treatment was found to induce NO accumulation in different stages of plant development. NO was detected by confocal microscopic analysis at the sites of adventitious root formation, and its concentration increased with alkamide treatment. Whether NO mediates the adventitious root response and other morphogenetic responses of plants to alkamides remains to be investigated.

11.6 Cellular Alterations Underlying Plant Responses to NAEs and Alkamides: Cell Cycle Progression and Microtubule Stability

The molecular mechanisms underlying NAE and alkamide responses comprise parallel inhibition of cell elongation and localized stimulation of cell division. These processes are likely to function as focal points in their regulation of plant architecture (Blancaflor et al. 2003; Ramírez-Chávez et al. 2004).

11.6.1 Cell Cycle Progression

Cell division activity in plants is localized in small groups of cells, called meristems, which are already present in the embryo and are active during most of the cell cycle of the plant. The cell cycle that occurs in dividing cells consists of the alternating phases of DNA replication (S phase) and chromosome separation (mitosis, or M phase), interrupted by gaps known as G1 (interval between M and S phases) and G2 (interval between S and M phases). Important controls operate at the transition points as cells move from G1 into S phase, and from G2 into M phase (Beemster et al. 2003). Both the G1–S and G2–M phase transitions can be controlled in plant cells in response to phytohormones, such as auxins and cytokinins. An example of G2 control is found in the development of lateral root primordia, which are derived from pericycle cells arrested in G2. These cells move into M phase upon auxin stimulation and then continue to proliferate, producing a lateral root primordium that eventually emerges from the side of the primary root (Himanen et al. 2002).

Ramírez-Chávez et al. (2004) evaluated the effects of affinin on cell division by using the G2–M specific marker *CycB1:uidA*, which is expressed only in dividing

cells. In response to micromolar concentrations of affinin, a dose–response reduction in the number of cells expressing *CycB1:uidA* was observed in root meristems. Meristem cells of *Arabidopsis* roots grown in the presence of NAE 12:0 also displayed abnormal cell division patterns characterized by oblique wall formation and the appearance of numerous irregularly shaped cells that varied in size (Blancaflor et al. 2003). Interestingly, the opposite effect was observed in leaves exposed to *N*-isobutyl decanamide, in which the formation of blades on petioles and callus-like structures could be observed (López-Bucio et al. 2007). These results suggest that localized effects of alkamides are important for cell proliferating activity of these compounds.

11.6.2 Microtubule Stability

Cellular elongation in roots has been shown to be dependent on the cortical microtubule cytoskeleton. Drugs that either depolymerize or stabilize microtubules in roots cause a significant reduction in root growth rate that eventually leads to radial expansion (Baskin et al. 1994). In primary roots, cortical microtubules in cells of the elongation zone are typically arranged along to the longitudinal axis of the root and uniformly distributed (Fig. 11.2a). This orientation shifts to oblique or longitudinal arrays as the cells make their transition into the maturation zone. Continuous exposure to 50 μM NAE 12:0 (Blancaflor et al. 2003) or 36 μM *N*-isobutyl decanamide (Méndez-Bravo et al., unpublished data) caused radial swelling in roots of *Arabidopsis* seedlings (Fig. 11.2a, b). Instead of the typical cylindrical cell shape observed in the differentiation zone of untreated roots, cells of alkamide-treated roots were shorter and wider. Microtubules in cells with altered shape were oriented in random directions and appeared to be fragmented and disorganized (Fig. 11.2c, d). This change in microtubule orientation and size, and cell elongation creates a link between acylamides and root architecture.

11.7 AHLs: Inter-Kingdom Signals for Plant–Bacterial Interactions

Bacterial cells communicate with each other using chemical signals. Specifically, they release, detect, and respond to the accumulation of compounds that allow bacteria to coordinate their gene expression in responses to changes in the population density, a process commonly referred to as quorum-sensing (Waters and Bassler 2005). Many processes in bacteria are regulated by quorum-sensing, including symbiosis, virulence, antibiotic production and biofilm formation. Gram-negative bacteria use *N*-acyl-homoserine lactones (AHLs) to communicate and regulate their quorum-sensing; these compounds contain a conserved homoserine lactone (HL)

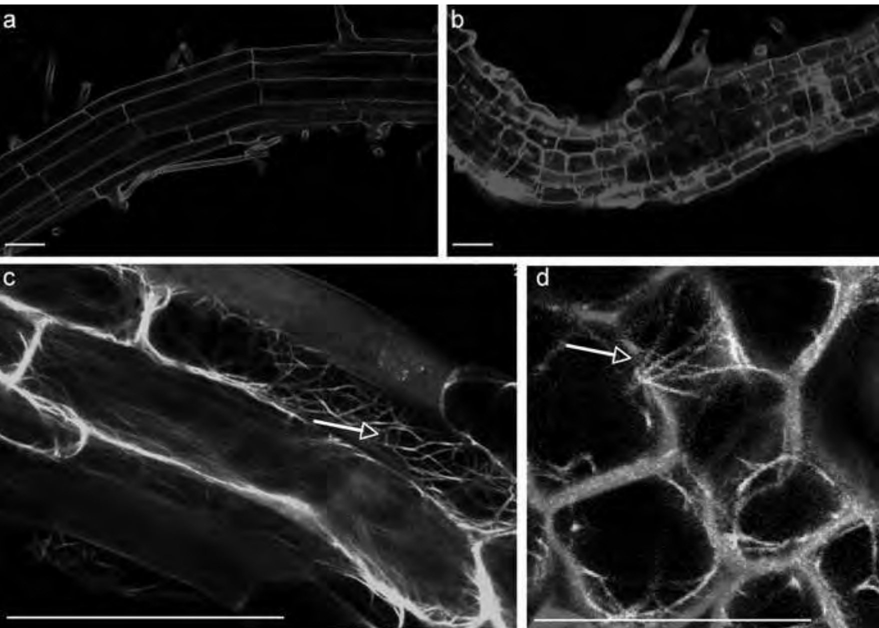

Fig. 11.2 Effects of *N*-isobutyl decanamide on cell elongation and microtubule stability. (**a**, **b**) Confocal microscope images of (respectively) 0 and 56 μM *N*-isobutyl decanamide-treated root epidermal cells of WT *Arabidopsis thaliana* seedlings. Note the shorter size of alkamide-treated cells. (**c**, **d**) Organization of epidermal microtubules in root cells of 12-day-old *Arabidopsis* transgenic seedlings expressing a fimbrin::GFP construct. **c** Microtubules in the epidermal cells of control roots and **d** roots treated with 56 μM *N*-isobutyl decanamide. Note the fragmented and randomly oriented microtubules in alkamide-treated cells. Scale bars = 50 μm

ring and an amide (*N*)-linked acyl side chain (Table 11.1). The acyl groups of naturally occurring AHLs range from 4 to 18 carbons in length; they can be saturated or unsaturated and with or without a C-3 substituent (Reading and Sperandio 2006). Recent evidence shows, however, that quorum-sensing signalling is not restricted to bacterial cell-to-cell communication, but also allows communication between plants and their prokaryote partners (Hughes and Sperandio 2008). The presence of AHL-producing bacteria in the rhizosphere of tomato induced the salicylic acid and ethylene-dependent defence response that plays an important role in the activation of systemic resistance in plants, and conferred resistance to the fungal pathogen *Alternaria alternata* (Schuhegger et al. 2006). In addition, certain *Rhizobium* mutants that fail to produce or sense AHLs were unable to nodulate legume plants, suggesting that AHLs may play a role in nodulation (Zheng et al. 2006). Our research aiming to clarify the role of AHLs in plant development revealed that these compounds exert strong and specific cellular responses similar to NAEs and alkamides, including arrested cell proliferation in the primary root meristem and enhanced lateral root formation (Ortíz-Castro et al. 2008).

11.8 Concluding Remarks

Most plant hormones are pleiotropic rather than specific, that is, each has more than one effect on the growth and development of plants. Auxin, for instance, stimulates the rate of cell elongation at low concentrations with inhibitory effects at high concentrations, it causes shoots to grow upwards and roots downwards, and it promotes the formation and growth of lateral roots and shoot branches. Auxin also causes the plant to produce a second hormone, ethylene, to elicit a plethora of additional responses. Ethylene, in turn, regulates auxin biosynthesis and alters auxin-responsive gene expression (Stephanova et al. 2007). The other well-known plant hormones gibberellins, abscisic acid and cytokinins have a similarly complex array of functions. Such interactions may also exist for NAEs and alkamides. These compounds are part of a vast array of amino compound-containing lipids ubiquitous in organisms from bacteria to mammals. Lipids are integral components of the cell membrane, the prime and essential limit of cells with its environment. Small signalling NAEs can be released from the membrane by PLD enzymes in response to biotic or abiotic stimuli (Bargmann and Munnik 2006). The presented information suggests that acylamides can be perceived by plants. NAEs and alkamides may actually function by activating other signalling pathways already important for plant development, such as cytokinins and abscisic acid, or by inducing accumulation of NO, a highly active messenger for cellular responses. This information suggests that these lipids are not merely structural components, but also important players involved in the orchestrated adjustment of anatomical characteristics. Such adjustment may account for the morphological adaptations to limit exposure to unfavourable environmental conditions.

NAEs and alkamides are believed to act, at least in part, by an endocannabinoid-like signalling mechanism similar to that described in animals (López-Bucio et al. 2006; Morquecho-Contreras and López-Bucio 2007). Recent findings add further complexity for plant responses to these lipids. In a search for alkamide-related compounds from bacteria that could modify root growth, we identified the *N*-acyl homoserine lactones, a class of bacterial quorum-sensing signals, as important regulators of plant morphogenesis (Ortíz-Castro et al. 2008). Thus, the possibility is open that amino compound-containing lipids could represent a novel class of signals for plant–bacterial communication. Elucidating how alkamides interact with classic and novel signals to regulate plant development remains a major challenge. The adoption of an integrated approach that combines genetics, molecular biology, cell biology and analytical chemistry should increase our knowledge on the signalling pathways involved in small lipid perception in plants.

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FRONTERAS EN LA BIOLOGÍA DEL DESARROLLO DE LAS PLANTAS

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Prefacio

El estudio del desarrollo de las plantas involucra una serie de conocimientos y herramientas de actualidad. Desde los experimentos clásicos que condujeron a la identificación de los primeros reguladores del crecimiento vegetal como las auxinas hasta las novedosas metodologías para el análisis de la expresión global de genes, de proteínas y metabolitos, dichas herramientas permiten comprender cómo las plantas, no obstante ser organismos sésiles, logran completar su ciclo de vida estando expuestas a innumerables factores bióticos y abióticos desfavorables para mantener la producción de biomasa que sostiene a las cadenas tróficas de los diferentes ecosistemas.

El desarrollo de las plantas implica rutas complejas de señalización, empezando por la percepción de señales extracelulares a través de receptores localizados en la membrana plasmática. Aunque este modelo canónico de respuesta química está conservado en las plantas como en otros eucariontes, diversos tipos de receptores incluyendo a los de auxinas, giberelinas y ácido jasmónico tienen una localización intracelular desde donde integran respuestas adaptativas transmitidas por cascadas de señalización, las cuáles pueden regular directamente la actividad enzimática y otras funciones celulares. Por otra parte, dichas cascadas también pueden modular la transcripción de genes y/o afectar la estabilidad de diversos tipos de proteínas, alterando el fenotipo. Si bien, el número de publicaciones sobre este tema en la literatura especializada es cada vez mayor, son pocos los materiales que presentan la complejidad de las rutas de señalización intracelular que regulan el crecimiento y desarrollo en las plantas en un lenguaje accesible y actualizado para estudiantes de nivel de Licenciatura o para estudiantes en etapas tempranas del Postgrado en Ciencias Biológicas.

La edición de este libro surge como uno de los objetivos del Curso de Biología del Desarrollo que se imparte a los alumnos del Programa de Maestría y Doctorado del Instituto de Investigaciones Químico Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo, como un ejercicio comprometido en la comprensión del tema y la difusión del conocimiento actual en este campo.

Elda Beltrán Peña
José López Bucio

Junio 2010

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La arquitectura de las plantas

Randy Ortíz-Castro y José López-Bucio

La arquitectura de las plantas determina la eficiencia de estos organismos para adaptarse al ambiente. El follaje participa en la fotosíntesis y la reproducción, en tanto que la raíz es importante para el anclaje al suelo y la captación de agua y nutrientes. El desarrollo de la raíz y del follaje está dirigido por mecanismos genéticos, pero la configuración final de la arquitectura de la planta está influenciada por factores ambientales tanto bióticos como abióticos.

Hasta la fecha se han esclarecido varios de los procesos que modulan la formación de órganos y tejidos incluyendo los tallos, las hojas, las flores y las raíces, en los que participan diferentes reguladores del crecimiento vegetal como las auxinas, las citocininas y el etileno.

También se ha encontrado que los nutrientes tales como el fósforo, el nitrógeno y el hierro pueden regular procesos celulares fundamentales. En este capítulo se discuten los procesos que modulan la arquitectura de las plantas y se analiza la importancia de este factor en la productividad vegetal y la adaptación a condiciones adversas.

1.1 Introducción

Las plantas son los productores primarios de los ecosistemas terrestres. La producción de follaje, semillas y frutos que es la base de las cadenas tróficas está ligada estrechamente a la arquitectura de estos organismos. En una planta podemos distinguir dos grandes sistemas de órganos, el follaje y la raíz. El follaje incluye las partes de la planta que crecen por encima del suelo, tales como el tallo, las ramas, las hojas, las flores y los frutos, cada una de ellas desempeña funciones específicas para el crecimiento, desarrollo y cumplimiento del ciclo de vida. Por otra parte, el sistema radicular está formado por la raíz primaria, las raíces laterales y adventicias y los pelos radiculares. Estas estructuras permiten el anclaje al suelo y facilitan la captación de agua y de nutrientes.

La arquitectura de la planta es un criterio fundamental para la clasificación sistemática y taxonómica, y aún en la actualidad representa el mejor medio para la identificación de nuevas especies vegetales. Notablemente, la llamada "revolución verde", conjunto de técnicas y sistema de manejo de las plantas que ha permitido un incremento sostenido en la productividad agrícola, está basado en la modificación de la arquitectura de la planta: la selección de variedades de trigo con tallos cortos y firmes dando lugar a plantas con una mayor productividad y resistencia al daño ocasionado por el viento y la lluvia (Peng *et al.*, 1999).

Todos los organismos poseen una estructura básica y una fisiología distintiva. En animales, los tejidos se forman a partir de procesos celulares determinados que ocurren en el embrión. Por el contrario, la actividad meristemática sostenida de las plantas posibilita programas de desarrollo indeterminados que permiten la formación de nuevos órganos como raíces, ramas y hojas durante todo el ciclo de vida (Niklas, 2000).

Las plantas vasculares poseen meristemos terminales, intercalados y en algunos casos meristemos laterales que les permiten una gran diversificación de la arquitectura básica. La producción de células en los meristemos, acompañada de eventos de elongación y diferenciación posibilita el recambio estacional de órganos, tales como la caída de las hojas durante el otoño y su reposición durante la primavera. También ayuda a recuperar tejidos que son dañados mecánicamente o que se pierden por el ataque de patógenos o por la exposición a diferentes tipos de estrés ambiental como la lluvia, el granizo o el viento. Entonces, las plantas pueden crecer continuamente mientras mantienen su actividad metabolica y fotosintética.

1.2 La evolución de la arquitectura de las plantas

Las primeras plantas vasculares conocidas, tales como *Cooksonia* y *Rhynia*, datan de los periodos Silúrico y Devónico, respectivamente. Estas fueron plantas pequeñas con tallos aéreos ramificados dicotómicamente, con esporangios terminales y estructuras tipo rizomas que se consideran ancentrales a las plantas vasculares de la actualidad.

Utilizando la estructura observada en los fósiles de *Rhynia,* Zimmermann (1953) propuso la "teoría del teloma" para explicar los saltos en la evolución del esporofito de la planta vascular. De acuerdo a esta teoría, *Rhynia* estaba compuesta de unidades morfológicas discretas. Las unidades terminales fueron llamadas telomas, y las unidades por debajo de las ramificaciones dicotómicas, análogas a los internodos de las plantas actuales, fueron nombradas mesomas. La teoría del teloma involucra cinco procesos elementales que, independientemente o en varias combinaciones, resultó en la evolución de las morfologías diversas de hojas, esporofitos, tallos y raíces presentes en las plantas vasculares (**Figura 1.1**). Estos procesos fueron: primero, dominancia apical, la cual resulta del desarrollo desigual de dos productos de un ápice; segundo, planación, el cual se produce por un sobrelapamiento de los telomas y mesomas en un solo plano; tercero, fusión, el cual ocurre en estructuras planares para producir la vena laminar de las hojas y entre telomas y mesomas para producir el tallo multivascularizado; cuarto, reducción, el cual produce diferencias en las longitudes de los telomas y mesomas; y quinto, recurvación, durante el cual los telomas fértiles se curvan hacia el plano de la ramificación durante la formación de estructuras reproductivas.

Por otra parte, se ha propuesto el concepto de "análisis arquitectónico" para tratar de entender cómo

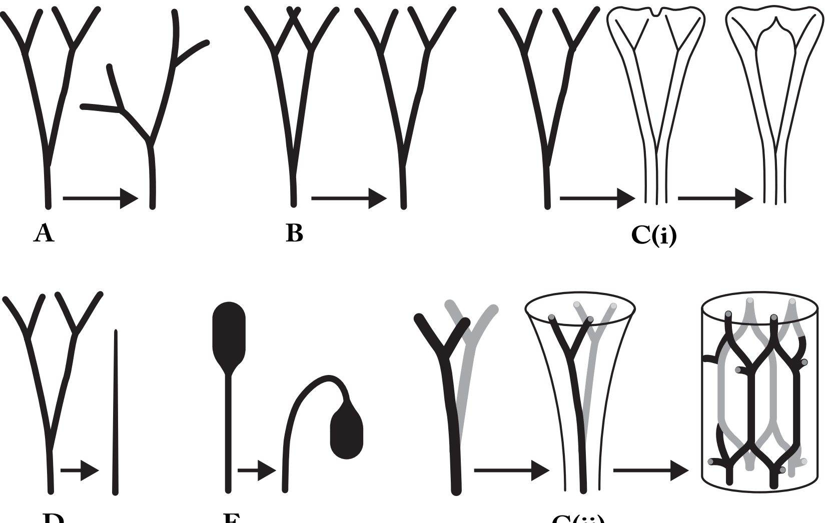

Figura 1.1. Los cinco procesos elementales que de acuerdo a la teoría del teloma, contribuyen a la diversificación morfológica en las primeras plantas vasculares. (A) dominancia apical, **(B)** planación, **(C)** **(i)** fusión en la formación de la hoja, **(ii)** fusión en la formación del tallo, **(D)** reducción, y **(E)** recurvación (Tomado de Sussex y Kerk, 2001).

es que ocurre la organización morfológica de las plantas vasculares. De acuerdo a este análisis, bajo condiciones óptimas de crecimiento, la arquitectura de la planta está modulado por las condiciones ambientales e interacciones con otros organismos los cuales afectan la expresión génica. Por lo tanto, la variación fenotípica y funcional surge del balance entre expresión genética, integración de estímulos y programas de crecimiento indeterminado o contínuo. Se han propuesto 23 modelos arquitectónicos que explican la morfología de árboles, matorrales, algunas lianas, plantas vasculares inferiores, e incluso plantas fósiles (**Figura 1.2**; Sussex y Kerk, 2001).

Los modelos arquitectónicos pueden ser explicados en términos del comportamiento de los meristemos y los fitómeros que ellos producen, dependiendo de una actividad celular proliferativa continua o intermitente; durante una transición del desarrollo o estacional (ej. transición vegetativa a reproductiva); si la ramificación es terminal o axilar; si las ramas crecen verticalmente u horizontalmente, o con alguna orientación intermedia, etc. (**Figura 1.3**). Los mecanismos celulares, fisiológicos y moleculares responsables de la funcionalidad del meristemo y de las yemas axilares están bajo estudio.

1.3 Arquitectura del follaje

1.3.1 Filotaxis

Durante el desarrollo vegetativo, las plantas forman continuamente nuevas hojas que son distribuidas con base en un patrón regular, con ángulos de divergencia bien definidos entre las hojas sucesivas. A este proceso se le conoce como filotaxis (Steeves y Sussex, 1989). Los patrones filotácticos más comunes son alternado, decusado y espiral (**Figura 1.4**). Las hojas se forman en la punta de las ramas gracias a la actividad del meristemo apical del follaje (SAM, *shoot apical meristem* por sus siglas en inglés). El meristemo integra la información proveniente del primordio de la hoja preexistente antes de que se produzca una hoja nueva, se ha propuesto que las hojas producen inhibidores que afectan la formación de las hojas nuevas (Steeves y Sussex, 1989). Dependiendo de su concentración y estabilidad, un inhibidor puede crear un campo que restringe la formación de hojas a posiciones con distancias mínimas definidas (**Figura 1.4**). La filotaxis es una característica multigénica, ya que hasta la fecha no ha sido posible

Figura 1.2. Modelos que describen la estructura del follaje en plantas vasculares (Tomado de Sussex y Kerk, 2001).

identificar mutantes con fenotipos filotácticos específicos. Sin embargo, se han descrito varias mutantes de *Arabidopsis thaliana* que exhiben defectos en la iniciación, separación, espaciamiento y arreglo de las hojas (Reinhardt y Kuhlemeier, 2001). Entre éstas, las mutantes *pin-formed1* (*pin1*), *pinoid* (*pid)* y *monópteros* (*mp*), están afectadas en genes que participan en el transporte o la respuesta a auxinas (Gälweiler *et al.*, 1998; Hardtke y Berleth, 1998; Christensen *et al.*, 2000; Benjamins *et al.*, 2001). La inhibición del transporte de auxinas, por una mutación en la proteína PIN1 o mediante el uso de inhibidores, disminuye la formación de hojas y ramas a partir del tallo, mientras que el crecimiento del tallo y la producción de células en el meristemo no se afecta (Okada *et al.*, 1991; Reinhardt *et al.*, 2000). Estos resultados indican que las auxinas desempeñan un papel fundamental en la filotaxis.

1.3.2 Ramificación y dominancia apical

Las plantas producen brotes laterales (ramas) a partir de los meristemos axilares que se encuentran en la parte axial de las hojas (**Figura 1.5**). El patrón de ramificación también está asociado a la filotaxis. En diversas especies, la actividad de los meristemos axilares está reprimida por los brotes apicales mediante el proceso conocido como "dominancia apical" (Davies, 1995). La dominancia apical inhibe la formación de ramas. Esta ha sido una de las principales características de selección durante la domesticación del maíz a partir de su ancestro el teosinte. En el teosinte se forman varios tallos dando lugar a una planta con arquitectura ramificada del follaje, por el contrario, el maíz desarrolla un tallo principal

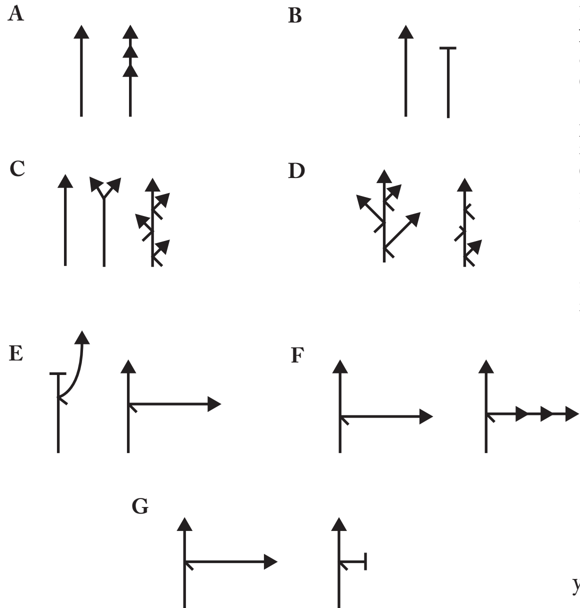

Figura 1.3. Patrones de la actividad meristemática que contribuye a la arquitectura del follaje. (A) El meristemo apical del follaje produce células continuamente (izquierda) o intermitentemente (derecha). **(B)** El meristemo apical del follaje produce células continuamente (izquierda) o inhibe su actividad en respuesta a un cambio del programa de desarrollo (derecha). **(C)** Formación de nuevos órganos: ramificación ausente (izquierda), terminal (centro) o axilar (derecha). **(D)** Crecimiento de todas las ramas axilares (izquierda) o las ramas axilares muestran potenciales de crecimiento diferente (derecha). **(E)** Las ramas axilares crecen verticalmente (izquierda) u horizontalmente (derecha). **(F)** Los meristemos axilares producen células continuamente (izquierda) o intermitentemente (derecha). **(G)** Los meristemos axilares producen células continuamente (izquierda) o su actividad se inhibe temporalmente (derecha) (Tomado de Sussex y Kerk, 2001).

a partir del cual se forman las hojas y las estructuras reproductivas. El incremento en la dominancia apical en el maíz está mediada principalmente por el gen *TEOSINTE BRANCHED1* (*TB1*) (Doebley *et al.*, 1997). En la mutante *tb1*, todos los meristemos axilares crecen, dando lugar a una planta altamente ramificada (**Figura 1.5**). Un efecto fenotípico diferente se ha descrito en la mutante de tomate *lateral suppressor* (*ls*), en la cual los meristemos axilares vegetativos están suprimidos, causando una reducción en la formación de ramas (Schumacher *et al.*, 1999). La existencia de mutantes afectadas específicamente en la ramificación y la dominancia apical demuestra que estas características se encuentran bajo un control genético.

1.3.3 Floración: crecimiento determinado e indeterminado

La floración puede afectar la arquitectura de la planta en diferentes maneras. En plantas como *Arabidopsis* y *Antirrhinum*, el meristemo del tallo principal es indeterminado, encontrándose activo durante todo el ciclo de vida de la planta produciendo primero hojas y después flores (**Figura 1.5**). Este tipo de crecimiento se conoce como monopodial (Schmitz y Theres, 1999). En contraste, el meristemo de las plantas de la familia *Solanaceae* (ej. jitomate) es determinado, éste termina su actividad produciendo una sola flor, y la producción de nuevos órganos ocurre mediante la formación de meristemos laterales. Este tipo de crecimiento es referido como crecimiento simpodial (Schmitz y Theres, 1999). Mutaciones en el gen *FLORICAULA* (*FLO*) de *Antirrhinum* y de su ortólogo *LEAFY* (*LFY)* de *Arabidopsis* transforman las flores en ramas axilares indeterminadas (**Figura 1.5**; Coen *et al.*, 1990; Weigel *et al.*, 1992). Esto indica que el desarrollo mediado por *FLO* y *LFY,* es suficiente para la transformación de meristemos axilares indeterminados a meristemos florales determinados y ha permitido proponer que las flores evolucionaron a partir de un meristemo axilar (Coen y Nugent, 1994).

1.3.4 Estructura de las hojas

Las hojas pueden ser simples, como en *Arabidopsis* y tabaco, o compuestas de varias subunidades, como en tomate y chícharo (Sinha, 1999). Una hoja común tiene tres ejes: el eje proximodistal que va desde la punta al peciolo, el eje dorsiventral que denota la parte superior o epidermis y la parte inferior o envés de la hoja, y el lateral de izquierda-derecha (Reinhardt y Kuchlemeier,

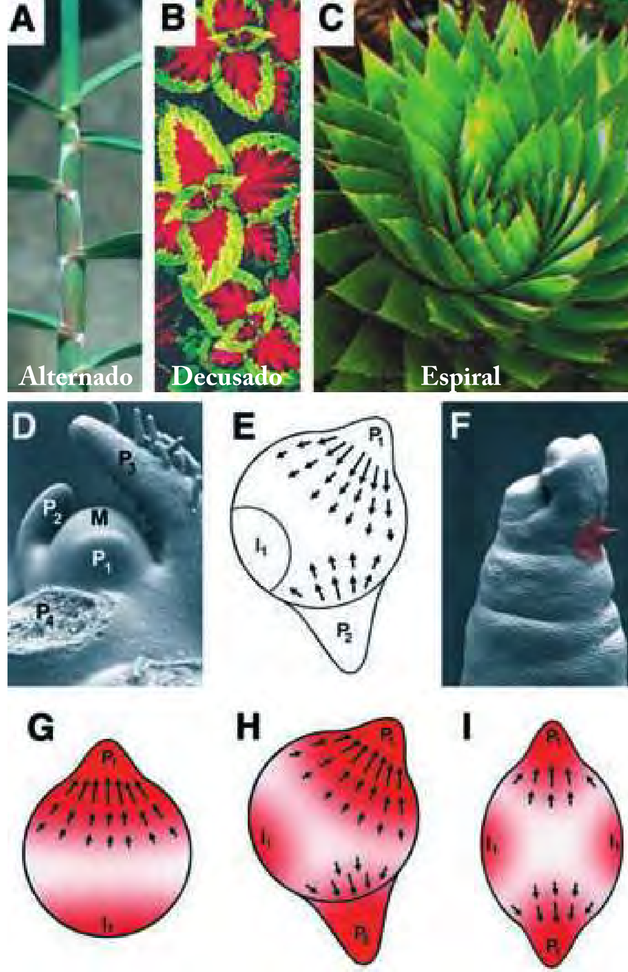

Figura 1.4. Regulación de la filotaxis. (A) Filotaxis alternada en *Trisetum distichophyllum*. Las hojas divergen 180º y alternan en dos filas opuestas. **(B)** Filotaxis decusada en *Solenostemon scutellarioides*. Se forma por pares de hojas opuestas, cada par de hojas sucesivas divergen en 90º. **(C)** Filotaxis espiral en *Aloe polyphylla*. Las hojas sucesivas inician con un ángulo divergente de 137º. **(D)** Ápice del tallo de una planta de jitomate con el primordio foliar más joven en la sucesión en espiral (P_1, P_2, P_3 y la base P_4) y el meristemo foliar apical (M). **(E)** Modelo de regulación filotáctica por un inhibidor (flechas) emanando de un primordio joven (P_1 y P_2). **(F)** La administración local de AIA (parche rojo) en la punta de una mutante de *Arabidopsis pinformed1* induce la formación de un primordio. **(G-I)** Modelo de transporte de auxina durante la filotaxis. Las auxinas se transportan en el meristemo desde un primordio de hoja hasta un nuevo sitio para inducir la proliferación celular. **(G)** Si solamente un primordio joven responde a la auxina, se establece la filotaxis alterna. **(H)** Si dos o mas primordios responden, se establece una filotaxis en espiral. **(I)** Si el tamaño del meristemo permite que coexistan dos máximos de auxinas, entonces se forman hojas opuestas, resultando en una filotoxis decusada (Tomado de Reinhardt y Kuhlemeier, 2002).

2002). Las variaciones en la forma de la hoja derivan, en parte, de las diferencias en la división de las células madre o iniciales (**Figura 1.6**). Solamente un grupo pequeño de células se activan a partir del flanco del meristemo del tallo en dicotiledóneas, tales como *Arabidopsis,* mientras en monocotiledóneas como el maíz, la hoja se forma de las células de la circunferencia del meristemo (Long y Barton, 2000; Poethig y Szymkowiak, 1995; Irish y Sussex, 1992). En el maíz, mutaciones en el factor de transcripción *NARROW SHEATH* resulta en la disminución de los márgenes de la hoja causado por una falta en la integración de las correspondientes células iniciales (Scanlon *et al.*, 2000; Scanlon y Freeling, 1997). Entonces, el fenotipo de la mutante *narrow sheath* sugiere que diferentes dominios a lo largo del eje medio-lateral participan en la formación de la hoja. Otras mutaciones en maíz que afectan la organización de las células iniciales incluyen *leafbladeless1* (*lbl1*) y *rough sheath2* (*rs2*) (Timmermans *et al.*, 1999; Timmermans *et al.*, 1998), las cuales afectan el desarrollo de la lámina foliar.

1.4 Arquitectura de la raíz

1.4.1 Sistemas radiculares

El sistema radicular de las plantas desempeña funciones adaptativas esenciales incluyendo la captación de agua y nutrientes, el anclaje al suelo y el establecimiento de interacciones bióticas y abióticas

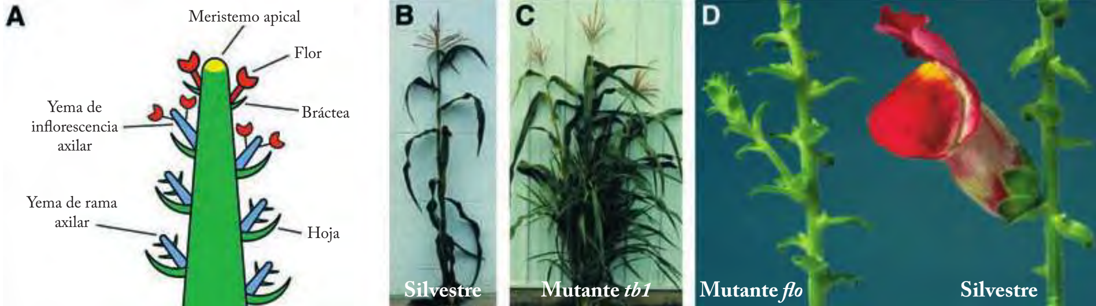

Figura 1.5. Regulación de la dominancia apical y la formación de ramas. (A) Estructura del tallo de una planta monopodial. El meristemo apical (amarillo) permanece activo durante el ciclo de vida de la planta. Dependiendo del estado de desarrollo, los brotes axilares (azul) forman las hojas o las flores. **(B-C)** Arquitectura de la planta de maíz y de la mutante *teosinte branched1* (*tb1*). **(D)** Formación de flores en plantas de *Antirrhinum* y en la mutante *flo* que produce ramas en lugar de flores (Tomado de Reinhardt y Kuhlemeier, 2002).

en la rizósfera. La primera raíz derivada de la planta se llama radícula, esta se forma después de la germinación de la semilla y conduce al desarrollo de una raíz primaria. Las raíces que se forman a partir de la raíz primaria se denominan raíces laterales o secundarias. Las raíces que se originan en otras partes de la planta, tales como el tallo o las hojas, se denominan raíces adventicias.

Existen tres procesos fundamentales que afectan la arquitectura del sistema radicular. Primero, la división celular en el meristemo de la raíz primaria otorga la capacidad de un crecimiento indeterminado por la incorporación de nuevas células a la raíz. Segundo, la formación de raíces laterales que incrementa la capacidad exploratoria del sistema radicular; y tercero, la formación de pelos radiculares que aumentan la superficie total de absorción de la raíz primaria y laterales (Dolan *et al.*, 1993). La alteración en alguno de estos procesos puede afectar la configuración del sistema radicular y modificar la capacidad de las plantas para crecer en suelos en los cuales la disponibilidad de nutrientes es limitada (López-Bucio *et al.*, 2003; Malamy y Benfey, 1997; Casimiro *et al.*, 2003; Dubrovsky *et al.*, 2006). Con el crecimiento continuo de la raíz primaria y sus patrones de ramificación, se producen los principales tipos de sistemas radiculares observados en las angiospermas, el sistema pivotante, el sistema fibroso y las raíces almacenadoras (López-Bucio *et al.*, 2005).

En el sistema pivotante, la raíz primaria alcanza una mayor profundidad que las raíces laterales. Las raíces laterales que se forman de la raíz pivotante se llaman raíces secundarias y pueden generar raíces terciarias e incluso cuaternarias mediante eventos sucesivos de ramificación. Las raíces fibrosas son típicas de plantas monocotiledóneas como el maíz, en ellas, la raíz primaria crece por un periodo limitado y en su lugar proliferan raíces adventicias que emergen del tallo, principalmente de los nodos. En la planta de maíz se han identificado mutantes afectadas en el desarrollo de la raíz. La mutante recesiva *rt1,* tiene una menor formación de raíces adventicias en tanto que en la mutante *des21*, las raíces seminales y los pelos radiculares se encuentran ausentes (Hochholdinger *et al.*, 2004; Gavazzi *et al.*, 1993).

Las plantas del desierto como los agaves y los cactus producen sistemas radiculares eficientes en la exploración de las capas superficiales del suelo para la captación de agua y nutrientes. El sistema radicular de las cactáceas muestra un crecimiento temporal o determinado, con un ciclo de vida corto de la raíz primaria, en el cual las células meristemáticas se dividen por pocos días después de la germinación (Dubrovsky, 1997). Durante esta inhibición del crecimiento de la raíz primaria, las células del periciclo se dividen para formar raíces laterales, incrementando con ello el área de superficie para la captación de agua. Otras raíces pueden especializarse en la acumulación de sustancias de reserva, incluyendo agua, minerales, carbohidratos y vitaminas. Estas raíces se denominan raíces acumuladoras.

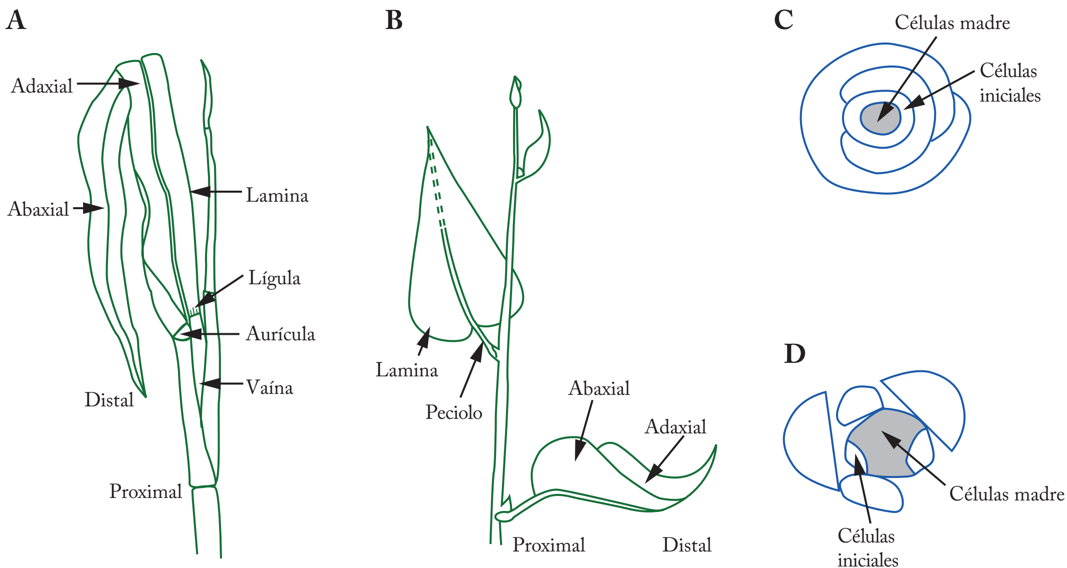

Figura 1.6. Desarrollo de la hoja en plantas monocotiledóneas y dicotiledóneas. (A) La planta monocotiledónea se muestra con una sola hoja. La vaina proximal está separada de la lámina distal por una aurícula y una lígula. **(B)** La planta dicotiledónea se muestra con varias hojas, indicándose el peciolo y la lámina foliar. Representación esquemática de secciones transversales de (**C**) un meristemo de maíz y **(D)** un meristemo de *Arabidopsis* mostrando las células iniciales dentro del meristemo (Tomado de Byrne *et al.*, 2001).

1.4.2 Regulación de la arquitectura radicular

1.4.2.1 Desarrollo embrionario de la raíz

En angiospermas, el meristemo de la raíz primaria se forma durante el desarrollo del embrión. El cigoto lleva a cabo divisiones asimétricas para formar una célula apical y una basal (Scheres *et al.*, 1994). La célula apical se divide mitóticamente hasta alcanzar un estado de ocho células, con dos filas de cuatro células cada una. La fila superior da lugar al meristemo del follaje y a los cotiledones, mientras que la inferior contribuye a la formación del hipocótilo y la raíz. La célula basal se divide para formar el suspensor. La célula superior del suspensor se conoce como hipófisis. El meristemo y la cofia de la raíz derivan directamente de la hipófisis (**Figura 1.7**).

1.4.2.2 Partes del sistema radicular

La raíz se diferencia del tallo por su estructura, por el modo en que se forma y por la ausencia de yemas y hojas. La raíz embrionaria se conoce como radícula. La raíz está formada de tres tipos principales de sistemas de tejidos: la epidermis, el cortex y el cilindro vascular. Algunas células de la epidermis se especializan en la absorción, tales como los pelos radiculares. Las células que forman los diferentes tejidos se producen a partir de cuatro células madre (células iniciales) localizadas en el ápice de la raíz (Dolan *et al.*, 1993). Debido a la restricción de ciertos eventos de desarrollo a regiones particulares, pueden ubicarse varias regiones con características bien conocidas: una región meristemática donde ocurre la división celular, una zona de elongación y una zona de diferenciación (**Figura 1.8**).

Un componente fundamental de la estructura radicular son las raíces laterales (RL), que tienen su origen de células del periciclo opuestas a los polos del xilema (Dolan *et al.*,1993). En los helechos, los primordios de raíces laterales se forman en la endodermis (Dubrovsky y Rost, 2003). Tanto en la raíz primaria como en las raíces laterales, ocurre la formación de pelos radiculares. Los pelos radiculares son células epidérmicas diferenciadas que contribuyen con cerca del 77% del área superficial total de la raíz, representando el principal punto de contacto entre la planta y la rizósfera.

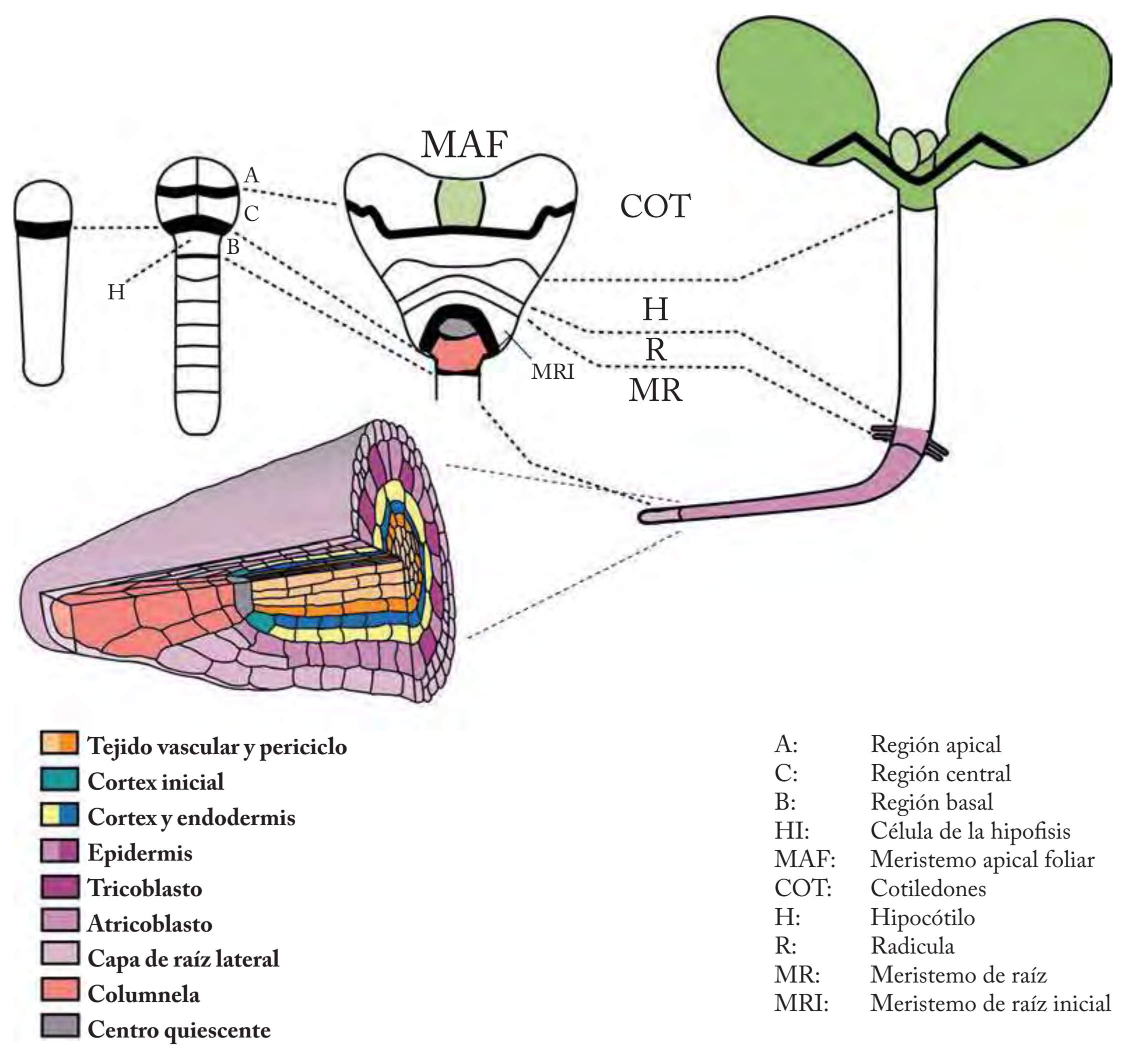

Figura 1.7. Orígen embrionario de la raíz de *Arabidopsis*. De izquierda a derecha: primera división cigótica; embrión en estado de ocho células; formación de la raíz y del follaje a partir del embrión (Modificado de Scheres *et al.*, 2002).

Cada pelo radicular es una extensión de forma tubular con crecimiento apical desde la base de una célula epidérmica especializada llamada tricoblasto (Dolan *et al.*, 1994; Foreman y Dolan, 2001). Los pelos radiculares son los responsables directos de la captación de agua y nutrientes.

El análisis genético de las diferentes fases de formación de los pelos radiculares ha permitido identificar diversas proteínas involucradas en el desarrollo de estas estructuras. Se han aislado mutantes de *Arabidopsis thaliana* incapaces de formar pelos radiculares que definen genes importantes para los estados tempranos del crecimiento del pelo tales como *rhl1*, *rhl2* y *rhl3*. Las tres mutantes tienen fenotipos pleiotrópicos y las plantas son extremadamente enanas (Scheneider *et al.*, 1997). RHL1 codifica una proteína hidrofílica que contiene una señal de localización nuclear. Otros factores que afectan la formación de

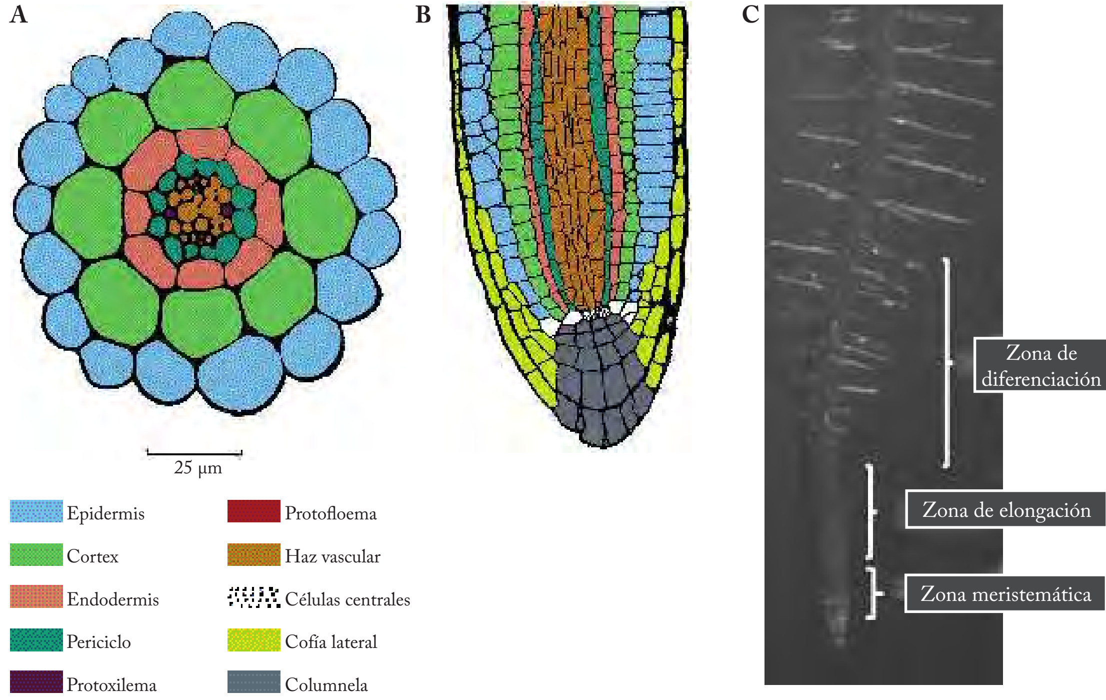

Figura 1.8. Estructura del sistema radicular. (A) Sección transversal de la raíz de *Arabidopsis*. **(B)** Sección longitudinal del ápice de la raíz. **(C)** Zonas de crecimiento y diferenciación en la raíz primaria de *Arabidopsis*.

los pelos radiculares son el etileno y las auxinas. Las mutantes en las rutas de auxinas o etileno, *axr2*, *aux1* y *etr1*, muestran pelos radiculares más cortos (Pitts *et al.*, 1998, Grierson y Schiefelbein, 2002). Sin embargo, se demostró que el bloqueo de la ruta endógena de etileno no es eficiente para inhibir totalmente la diferenciación de los tricoblastos, lo que suscribe los efectos de esta fitohormona al proceso de elongación celular que determina el tamaño de los pelos radiculares (Cho y Cosgrove, 2002).

1.4.2.3 Regulación por nutrientes

La respuesta de la raíz a la disponibilidad de nutrientes en el suelo es de fundamental importancia para la adaptación de las plantas al ambiente. Nutrientes minerales como el nitrato, el fosfato, el sulfato y el hierro actúan como señales que pueden ser percibidas en el meristemo. Estas señales dan origen a mecanismos moleculares que modifican la división celular y tienen un impacto profundo sobre la arquitectura de la raíz. El desarrollo de pelos radiculares, el crecimiento de la raíz primaria y la formación de raíces laterales, son particularmente sensibles a cambios internos y externos en la disponibilidad de nutrientes. Estas respuestas pueden ser mediadas por reguladores de crecimiento de la planta, tales como auxinas, citocininas y etileno, sugiriendo que los cambios en el estado nutricional están mediados por alteraciones en la síntesis de hormonas (López-Bucio *et al.*, 2003).

Las condiciones de baja disponibilidad de fosfato (P) y hierro (Fe) aumentan la longitud y densidad de los pelos radiculares, proporcionando una ventaja competitiva para las plantas. Sin embargo, aunque concentraciones de P y Fe tienen efectos similares sobre la densidad de los pelos radiculares, el análisis de mutantes de respuesta a auxinas sugieren que estas respuestas están mediadas por diferentes rutas de señalización (López-Bucio *et al.*, 2003).

1.5 Conclusiones

En este capítulo hemos presentado un esquema general de cómo las plantas se organizan en estructuras con funciones especializadas como el follaje y la raíz. Además de tener un amplio potencial biotecnológico, el estudio de los factores que afectan la arquitectura de las plantas ha conducido a un mejor entendimiento de cómo las plantas funcionan en su ambiente natural. Varios de los genes que se han identificado en plantas modelo tales como *Arabidopsis* tienen sus homólogos en plantas de interés agronómico como el maíz, no es por tanto de extrañar que la "revolución verde" estuviera sustentada en gran medida en el uso de variedades con arquitectura foliar y radicular distintiva. Algunas áreas de investigación promisorias estarán dirigidas a entender los mecanismos ecológicos, fisiológicos y moleculares que determinan la eficiencia de las plantas para adaptarse a ambientes extremosos, como las respuestas a contaminantes, a dióxido de carbono y al aumento de la temperatura. En los siguientes capítulos se abordan con mayor detalle algunos de estos aspectos. Sin duda alguna, solo a partir de este entendimiento se logrará un uso sostenible del recurso vegetal para la producción de alimentos ante el continuo reto del crecimiento poblacional

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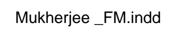

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Foreword

Trichoderma spp. have emerged as a group of fungi with immense impact on human welfare. The literature of these remarkable fungi expands daily, demanding that a timely and exclusive volume be conceived and brought to fruition as a benchmark publication on the biology and application of *Trichoderma*. Prasun Mukherjee and colleagues have presented such a prestigious collection of authoritative chapters on *Trichoderma*. The last such effort was made by Gary Harman and Christian Kubicek in 1998, and the face of *Trichoderma* research has changed considerably in the past 15 years, especially with the advancements in genetics and genomics. The current volume is an update on the advances in *Trichoderma* research, covering most of the aspects related to the biology, genetics, genomics and applications of *Trichoderma* in human welfare. The book starts with an introductory chapter by Mukherjee *et al*. (Chapter 1), which provides an overview of *Trichoderma* and its applications, and ends with an update on the negative impact of these fungi on human health (Hatvani *et al*., Chapter 17). All the chapters are written by authorities in the field with vast experience in their respective areas. The chapter on taxonomy (Atanasova *et al*., Chapter 2) covers the molecular phylogeny of 200 *Trichoderma* spp., which is the first treatise of its kind, and will certainly prove to be very useful in exploration and exploitation of *Trichoderma* spp. The chapter on *in vitro* sexual development (Schmoll, Chapter 4) is again a unique compilation of a recent development in the field and will provide guidance for applications of this novel breeding tool in strain improvement of these fungi. In place of a chapter on secondary metabolism in general, this book has two related chapters addressing this subject (Chapter 6 on volatile metabolites and Chapter 15 on metabolites from marine-derived *Trichoderma*), which demonstrates the novelty and importance of these compounds. The section on *Trichoderma*–plant interactions (Section II) will be of special interest to readers because the scope is broad and illustrates many of the recent developments in this rapidly unfolding and intensively interrogated field of *Trichoderma* research. All the chapters in this section represent an advanced treatise on this topic, providing rich and insightful text regarding the physiology, biochemistry and genetics of interactions of these beneficial fungi with plants (Chapters 8–12). The book also revisits some of the 'traditional' topics, but viewing them in new perspectives that reveal the applications of *Trichoderma* from plant health management (Chapter 14), to biofuels (Chapter 13) and cell factories (Chapter 16). Similarly, the much discussed topics on light response and asexual sporulation (Chapters 3 and 5) are enriched with new and absorbing information and details (especially related to the genetics and

genomics) that will help readers comprehend and understand these processes that have the potential to lead to more effective and economical formulation products. Overall, this book is a treat to all those involved in R&D activities dealing with *Trichoderma* and will prove to be an invaluable tool in furthering basic understanding as well as the commercial success of these economically important fungi.

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Preface

After decades of improvement of agriculture and chemicals production towards industrialized processes, environmental issues and sustainability needs initiated movement for a greener industry and agriculture. Several principles are needed to make agriculture sustainable. One of these is to use natural biological control, where possible. The idea to use members of the genus *Trichoderma* to control plant pathogens can be traced back to before the middle of the past century, but the past decade has seen a qualitative leap in the tools and approaches for understanding these beneficial fungi and their interaction with plants. In addition, however, the industrial production of chemicals and enzymes has started to shift towards biotechnological processes, in many cases applying filamentous fungi as work horses. Here also, *Trichoderma* is a major organism, especially with the research focus on second-generation biofuels.

The landmark achievement since the last monograph on *Trichoderma* is, without doubt, the publication of the genomes of three species. Other paradigm shifts are almost as important. One is the realization that induction of resistance in the plant rivals direct killing of pathogenic fungi as a mechanism for control of disease. Another is the ability to do genetic crosses in *Trichoderma* species that were thought to lack a sexual cycle under laboratory-defined conditions. Methods for engineering of strains have been optimized. Next-generation sequencing is becoming the best way to follow gene expression, as well as to identify the genes corresponding to classical mutant phenotypes.

The concept of this book grew, in part, from our participation in the genome projects. Sequencing shone a spotlight on the genus *Trichoderma*, the most important members of which from the biotechnological point of view are often hidden underground in the rhizosphere or within plants as endophtyes. We were further encouraged by our dialogue with an international community of researchers who focus in every aspect from molecular genetics to field applications. The chapters in this volume address basic biology, morphogenesis in response to light and other signals, genetics, interactions with plants and secondary metabolites, just to mention a few of the topics. The collection of diverse approaches should serve as a link between genomes and biology. Moreover, we trust that having this information, critically reviewed and within easy reach, will encourage the connections that start new research. Finally, we hope that the unfolding of the story of *Trichoderma* research will provide an enjoyable path through the myriad of biotechnological and genetic details.

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10 Promotion of Plant Growth and the Induction of Systemic Defence by *Trichoderma*: Physiology, Genetics and Gene Expression

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10.1 Introduction

Plants are essential to human life and to most organisms as well. They produce food, fuel, fibre, medicines and materials for humans and are integral to most ecosystems. Plant growth and development are greatly affected by environmental stresses such as drought, salinity, nutrient deficiency and adverse temperatures. Owing to climate changes these challenges are becoming even more intensified. Pathogens can also have a severe impact on plant health, decreasing agricultural production. For the past 50 years, the major challenge of providing sufficient food for the increasing human population has been facilitated by the application of high inputs of chemical fertilizers containing nitrogen (N), phosphorus (P) and potassium (K), which, together with advances in crop and agricultural techniques focusing on shoot biomass and seed yield, has resulted in increasing productivity (González *et al.*, 2009; Xing and Zhang, 2010). Current production methods based on high amounts of nutrients are not only costly but also lead to several environmental and health problems (Conway and Pretty, 1988). Additionally, in crops such as wheat and maize, intensive arable cultivation is no longer sustainable because it often leads to soil degradation (Loneragan, 1997). In this scenario, research with plants and microbes will be central in finding alternative methods to cope with the threat of food shortage.

There is a huge diversity of microorganisms that colonize plant roots and some of them play beneficial functions in biocontrol, protecting hosts from pests and diseases and promoting plant growth by releasing hormones or hormone-like signals (Ortiz-Castro *et al.*, 2009; Harman *et al.*, 2011; Berendsen *et al.*, 2012). A number of fungi are known to proliferate in the rhizosphere, the part of the soil that receives the influence of plant roots, or even penetrate plant tissues without causing disease. These include endo- and ecto-mycorrhizas, binucleate *Rhizoctonia* spp., *Piriformospora indica* and *Trichoderma* spp. (Waller *et al.*, 2005; Shoresh *et al.*, 2010; Harman *et al.*, 2011). Some of these organisms were initially appreciated because of their biocontrol properties antagonizing root pathogens

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and protecting plants from diseases, but recent studies have demonstrated that they may possess additional attributes for application in agriculture.

Small amounts of *Trichoderma* spp. supplied as bioinoculants may confer significant advantages on a wide variety of crops, including both monocotyledons and dicotyledons. These include systemic resistance to diseases through an induction of jasmonic acid and salicylic acid signalling (Segarra *et al.*, 2007; Contreras-Cornejo *et al.*, 2011; Salas-Marina *et al.*, 2011), improved adaptation to abiotic stress including drought, salt and temperature (Mastouri *et al.*, 2010), enhanced nutrient solubility (Yedidia *et al.*, 2001; Rudresh *et al.*, 2005; Azarmi *et al.*, 2011) and regulation of root system architecture (Björkman, 2004; Contreras-Cornejo *et al.*, 2009; Samolski *et al.*, 2012).

The relevance of the root system has often been overlooked in plant-breeding programmes aimed at increasing food supply. Nevertheless, the root system has indispensable functions in the plant such as the uptake of nutrients and water, anchorage in the soil and interaction with symbiotic microorganisms (López-Bucio *et al.*, 2003, 2005). Consequently, root system development is central for the plant to reach optimal growth and directly contributes to the levels of yield obtained in crops. The impact of the root on plant growth has become apparent not only in model plants such as *Arabidopsis thaliana*, *Medicago truncatula* and *Lotus japonicus* but also in important crops such as wheat (*Triticum aestivum*), rice (*Oriza sativa*) and maize (*Zea mays*) (Hochholdinger and Tuberosa, 2009; Coudert *et al.*, 2010). One way to minimize the negative impact of biotic and abiotic factors on yield is to manipulate root system architecture (RSA) towards a distribution of roots in the soil that optimizes water and nutrient uptake. It is now established that most of the genetic variation for RSA is driven by a suite of genetic and hormonal factors on the plant and is modulated by its interactions with microorganisms (Den Herder *et al.*, 2010; Berendsen *et al.*, 2012).

In the past 10 years, the role of genetic factors, plant hormones and nutrients on root biomass, root branching and root absorptive capacity has been studied in detail in various plant species (López-Bucio *et al.*, 2003, 2005; De Dorlodot *et al.*, 2007; Hochholdinger and Tuberosa, 2009; Coudert *et al.*, 2010). Many plant symbionts detect signals derived from roots for colonization, and use plant carbon sources such as organic acids, amino acids and sucrose for nutrition. Indeed, root colonization represents increased sink strength, thus providing an additional level of complexity in modulating plant growth through sugar distribution. Uptake, exchange and competition for sugar at plant–fungus membranes seem to be essential in determining the outcome of the *Trichoderma*–maize interactions (Vargas *et al.*, 2009) and we may think that both plants and fungi benefit from an increased root absorptive capacity, thus providing a new avenue to explore towards potential agricultural applications. The available data highlight the need for a better comprehension of cellular and molecular mechanisms involved in signalling exchange between fungi and their host plants.

10.2 The Rhizosphere and Plant Fitness

The capacity of plants to survive adverse conditions and reach reproductive maturity critically depends on their ability to continuously adapt to changes in the environment. Plants have therefore evolved an array of intricate regulatory mechanisms that involve the generation of signalling molecules mediating the activation of adaptive responses: in particular, the activation of pathogen-specific defence mechanisms upon infection, as well as the acquisition of architectural and physiological adjustments that permits survival, development and reproduction (Ortiz-Castro *et al.*, 2009).

Many complex interactions between plants and microorganisms occur at the rhizosphere, the soil zone in close contact with roots. The root system performs the essential activities of providing water, nutrients and physical support to the plant. The primary root originates in the embryo and produces many lateral roots during vegetative growth, and each of these will produce more lateral roots. The quantity and placement of these structures determine the architecture of the root

system, and this in turn plays a major role in determining whether a plant will survive in a particular climate or environment (Malamy and Benfey, 1997; Casimiro *et al.*, 2003; López-Bucio *et al.*, 2005). During the post-embryonic development of plants, new axes of growth emerge from shoot tissues through adventitious organogenesis. This is particularly important in crops such as maize, in which adventitious root formation provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or after injury (Hochholdinger and Tuberosa, 2009). Although lateral roots typically form from lateral root primordia initiated on the primary root pericycle, adventitious roots form naturally from stem tissue. Lateral and adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones such as auxin, and environmental factors such as light and nutrient deprivation (Casimiro *et al.*, 2003; López-Bucio *et al.*, 2003; Péret *et al.*, 2009).

A further adaptation to take in water and nutrients is performed by root hairs. These are long tubular-shaped outgrowths from root epidermal cells. In *Arabidopsis*, root hairs are approximately 10 μm in diameter and can grow to be 1 mm or more in length. A single rye (*Secale cereale* L.) plant may have 14 billion root hairs that provide 400 m^2 of surface area (Datta, 2011). Along with the vast increase in the root absorptive capacity and the root diameter conferred by root hairs, they are generally thought to aid plants to interact with microbes. This has been particularly demonstrated in the *Rhizobium*–legume symbiosis, in which a root hair forms a channel allowing penetration of the bacteria into the root tissues to form N-fixing nodules (Marx, 2004). Root hairs play an important role in the uptake of sparingly soluble nutrients that have low diffusion in the soil, such as phosphate. Because they have a small radius, root hairs explore a larger volume of soil per unit of surface area than thicker lateral roots. Root hairs also play a role in modulating the properties and composition of the rhizosphere because they exude high quantities of organic compounds, including organic acids, amino acids, sugars, proteins, mucilage, phenolics and secondary metabolites. In *Sorghum* spp. exudates seem to be produced solely by root hairs (Czarnota *et al.*, 2003) and exudation is positively correlated with root hair number and density (Yan *et al.*, 2004).

Root exudates perform diverse functions in the rhizosphere including mineral weathering, mobilization of nutrients, metal detoxification and growth inhibition of pathogenic bacteria, invertebrate herbivores or neighbouring plants (Badri and Vivanco, 2009). Some compounds such as organic acids can act as chemotactic signals to attract symbiotic fungi and bacteria (Rudrappa *et al.*, 2008), whereas sugar plays a fundamental role in interactions with mycorrhizal fungi and *Trichoderma* (Vargas *et al.*, 2009, 2011).

Microorganisms and plants emit signalling molecules for communication. Plants are able to recognize microbe-derived compounds and adjust their defence and growth responses according to the type of microorganism encountered. This molecular dialogue will determine the final outcome of the relationship, ranging from pathogenesis to symbiosis, usually through highly coordinated cellular processes (Ortiz-Castro *et al.*, 2009). Regarding their positive effects on plant growth, many rhizobacterial or fungal species that elicit plant biomass production or increase crop performance have been used as biofertilizers. Plant-growth-promoting rhizobacteria (PGPR) are natural rhizosphere-inhabiting bacteria that belong to diverse genera such as *Pseudomonas* and *Bacillus* species (Soleimani *et al.*, 2005). The general effect of PGPR is an increased growth and productivity of plants. Their contribution can be exerted through different mechanisms including modulation of root system architecture and increased biomass production through the release of phytohormones such as auxins or cytokinins (Lugtenberg *et al.*, 2002; López-Bucio *et al.*, 2007; Ortíz-Castro *et al.*, 2009). Besides, several fungi such as mycorrhizas, *Piriformospora indica* and *Trichoderma* spp. can interact with plants in many beneficial ways, some of which resemble those of PGPR.

Below, we present and discuss recent information on the mechanisms of growth promotion by the biocontrol agents of the *Trichoderma* genus.

10.3 Beneficial Effects of *Trichoderma* on Plants

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi and compete with deleterious plant microorganisms (Harman *et al.*, 2004a). Until recently, these traits were considered to be the basis for how *Trichoderma* exert beneficial effects on plant growth and development. It is clear, however, that certain strains also have substantial direct influence on plant development and crop productivity (Fig. 10.1) (Harman, 2006, 2011).

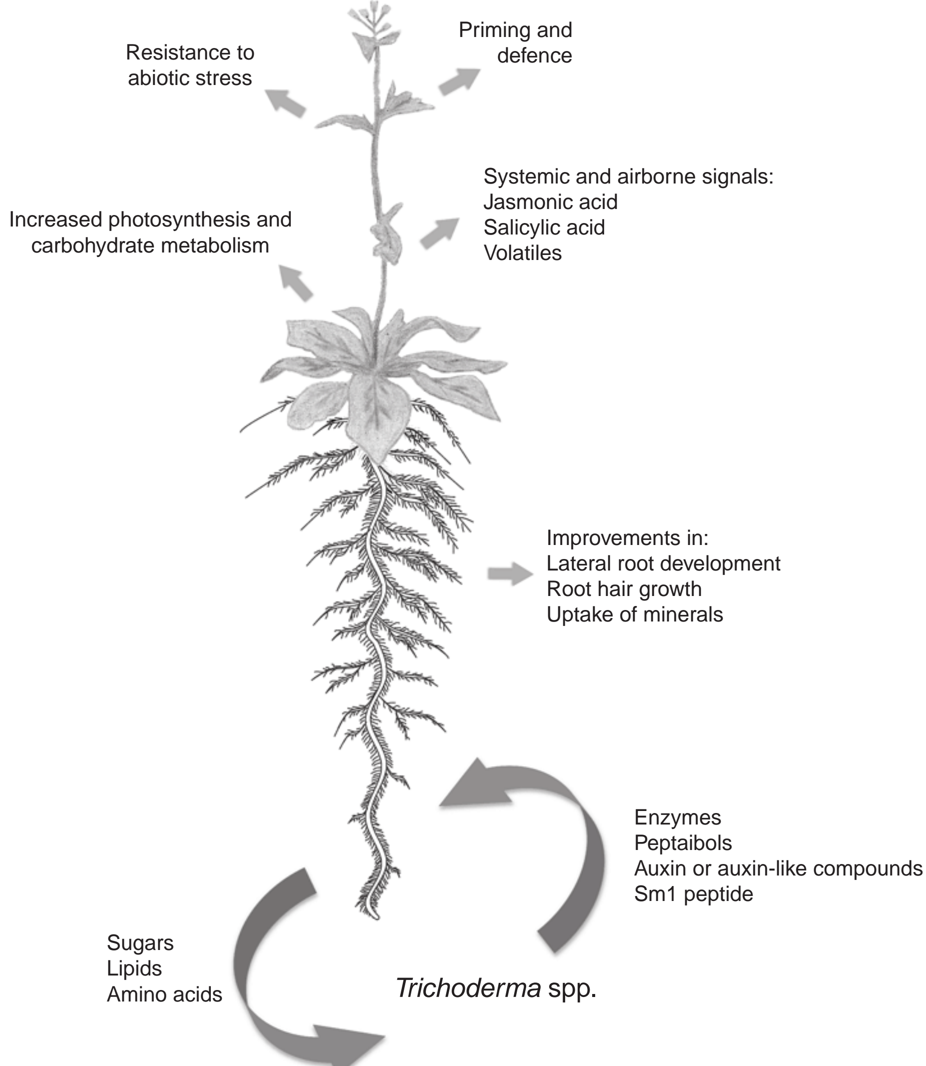


Fig. 10.1. Promotion of plant growth by *Trichoderma*. Root-derived nutrient sources such as sugars, lipids, organic acids and amino acids attract fungal symbionts, which colonize the root system and increase its absorptive capacity through root hair and lateral root production. Root colonization triggers the fungal and/or plant emission of diffusible signals such as jasmonic acid, salicylic acid and volatiles that increase photosynthesis, activate defence responses and confer protection against abiotic stress in distant parts of the plant.

10.3.1 Regulation of plant growth and development

Trichoderma enhancement of plant growth has been known for decades and can occur in axenic systems or in soil. Early reports of the effects of *Trichoderma* spp. on floricultural and horticultural crops such as cucumber, periwinkle and chrysanthemum indicated that these fungi impact on seed germination, flowering and vegetative growth (Chang *et al.*, 1986). It was reported that cucumber seedlings grown in soil amended with *Trichoderma harzianum* propagules sustain a 30% increase in seedling emergence 8 days after sowing. Three weeks later, these plants exhibited a 95% and 75% increase in root area and cumulative root length, respectively, and substantial increases in dry weight (80%), shoot length (45%) and leaf area (80%) were registered (Yedidia *et al.*, 2001). This report showed the correlation between increased root growth and shoot biomass production, which has been confirmed in maize plants (Bjorkman *et al.*, 1998; Harman *et al.*, 2004b; Vargas *et al.*, 2009).

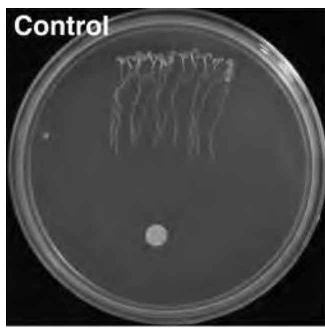
In a study comparing the effects of *T. harzianum* Rifai 1295-22 (also known as 'T22') and commercial formulations of ectomycorrhizal fungi in the establishment and growth of crack willow (*Salix fragilis*), Adams *et al.* (2007) found that after 5 weeks of growth in soil, tree saplings grown with *T. harzianum* T22 produced shoots and roots that were 40% longer than those of the controls and shoots that were 20% longer than those of saplings grown with ectomycorrhiza. Moreover, *T. harzianum* T22 saplings produced more than double the dry biomass of controls and more than 50% extra biomass than the ectomycorrhiza-treated saplings. These results highlight the potential of *Trichoderma* for establishment and early growth of trees in forest plantations. More recently, Tucci *et al.* (2011) showed that genetic variability among wild and cultivated tomato lines affected the outcome of the interaction with *Trichoderma atroviride* and *T. harzianum*. The beneficial response, which included enhanced growth and systemic resistance against the necrotrophic fungus *Botrytis cinerea*, was evident for some, but not all, of the tested lines. At least in one case (line M82), treatment with *Trichoderma* had no effect or was even detrimental for plant growth. In contrast, Azarmi *et al.* (2011) reported the beneficial effects of three *Trichoderma* isolates including *T. harzianum* isolate T969, *T. harzianum* isolate T447 and *Trichoderma* sp. isolate T in tomato seedling vigour. *Trichoderma* spore suspension was supplied either directly to seeds or to nursery soil with *Trichoderma*-fortified wheat. Seed germination rate was not affected by *Trichoderma* application, but shoot height, shoot diameter, shoot fresh and dry weight as well as root fresh and dry weight in tomato seedlings were increased when sown in *Trichoderma* sp. T and *T. harzianum* T969 fortified soil. Plants grown on soil amended with *Trichoderma* sp. T and *T. harzianum* T969 also had marked increases in leaf number, leaf area and chlorophyll content (Azarmi *et al.*, 2011).

The interaction between *T. harzianum* CECT 2413 strain and the tomato-root system was also studied during the early stages of root colonization by the fungus. When *T. harzianum* conidia were inoculated into the liquid medium of hydroponically grown tomato plants, profuse adhesion of hyphae to the roots as well as colonization of the root epidermis and cortex was observed. Confocal microscopy of a *T. harzianum* transformant that expressed the green fluorescent protein (GFP) revealed intercellular hyphal growth and the formation of plant-induced papilla-like hyphal tips. Analysis of the *T. harzianum*–tomato interaction in soil indicated that the contact between the fungus and roots persisted over a long period of time (Chacón *et al.*, 2007).

Arabidopsis thaliana has been established as an excellent model system to study the genetic and physiological mechanisms of *Trichoderma*–plant interactions and the influence of fungi on the basic elements of root architecture and adaptation to the environment. This has been possible because of the vast knowledge gained from plant developmental programmes, the availability of mutants and gene reporter lines, the small size of the plant and the ability to test the interaction under axenic conditions (Contreras-Cornejo *et al.*, 2009, 2011). *Trichoderma virens* and *T. atroviride* were found to promote *Arabidopsis* seedling growth with significant increases in root and shoot biomass production. Promotion of plant growth elicited by *Trichoderma* correlated

with prolific formation of lateral roots in wild-type plants of the Columbia-0 (Col-0), Wassilewskija (Ws) and Landsberg erecta (Ler) ecotypes (Fig. 10.2).

In order to detect any potential deleterious effect of *T. virens* or *T. atroviride* in *Arabidopsis*, a separate study was conducted to test temporal responses of plants to varied concentrations of conidia (Contreras-Cornejo *et al.*, 2011).



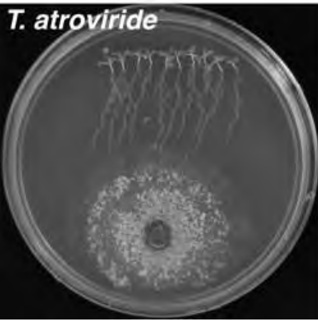


Fig. 10.2. *Trichoderma virens* and *Trichoderma atroviride* promote root branching in *Arabidopsis* seedlings. Photographs of *Arabidopsis* seedlings (ecotype Landsberg erecta, Ler) grown in a 0.2 × Murashige and Skoog medium and co-cultivated with *T. virens* or *T. atroviride*. Notice the great stimulatory effect of the fungi on lateral root formation.

Total plant biomass in control or inoculated seedlings was determined every two days after inoculation (dai). During the first two days, no significant differences were observed in biomass production between control plants and plants co-cultivated with *T. virens* or *T. atroviride*. However, from 4 to 8 dai, a 40% increase in total fresh weight was evident in *Trichoderma* co-cultivated plants. In this work, enhanced lateral root proliferation was a typical response of *Arabidopsis* roots colonized by the mycelia of *T. virens* or *T. atroviride*, and no deleterious symptoms such as chlorosis or necrosis could be observed in leaves. Interestingly, co-cultivation with *Trichoderma* increased anthocyanin production in leaves and the plants were more robust and greener, probably as a result of enhanced nutrient efficiency (Contreras-Cornejo *et al.*, 2011). Similar effects have been described in maize plants grown under field conditions (Harman *et al.*, 2011).

All the above-described information shows the potential of *Trichoderma* spp. stimulating the growth in a wide variety of plant families. Once the interaction with roots has been established, the growth-promoting effects to plants can last for the entire life cycle of the plant because the fungus continues to colonize the root system. The ability of *Trichoderma* to induce developmental changes in plants, resulting in improved root systems, may provide a competitive advantage through different mechanisms.

10.3.2 Contributions to plant nutrition

Plant growth and biomass production requires an adequate supply of nutrients, which act as structural components of cells or play roles in metabolism. Sixteen chemical elements are known to be important for plant growth and reproduction. They are divided into two main groups: macronutrients and micronutrients. Although both groups of elements are essential for plants to complete their life cycles, macronutrients such as N, P and K are required in greater amounts. In the soil–plant interface, both macronutrients and micronutrients undergo a complex dynamic equilibrium of

solubilization, uptake and transport that is greatly influenced by the soil pH and rhizospheric microorganisms. Certain nutrients such as N and P can directly acts as signals that alter post-embryonic root development, modifying primary and lateral root growth and root hair formation (López-Bucio *et al.*, 2003).

Roots interact with diverse populations of soil microorganisms, which have significant implication for growth and nutrition. Soil nutrients are transferred towards the root surface through the rhizosphere or, in the case of roots associated with mycorrizal fungi, through the fungal hyphae, prior to acquisition (Richardson *et al.*, 2009). Most plant species improve their mineral nutrition with the help of beneficial microorganisms such as fungi and bacteria, some of which are important in N fixation, P solubilization and micronutrient uptake (Tallapragada and Gudini, 2011). *Trichoderma* species may enhance nutrient uptake either by modifying RSA or through the exudation of substances that increase nutrient availability.

The potential of the biocontrol agent *T. harzianum* strain T-203 (later on identified as *Trichoderma asperellum* and recently as *Trichoderma asperelloides*) to induce a growth response in cucumber plants was correlated with improvements in the nutrition of plants. An increase of 90% and 30% in P and Fe concentration, respectively, was observed in *T. harzianum*-inoculated plants. An increased growth response was apparent as early as 5 days post-inoculation, resulting in an increase in root and shoot biomass production with a concomitant elevation in the concentration of Cu, P, Fe, Zn, Mn and Na in inoculated roots. In the shoots of these plants, the concentration of Zn, P and Mn increased by 25, 30 and 70%, respectively (Yedidia *et al.*, 2001). *T. harzianum* 1295-22 was reported to increase the solubility of P and several micronutrients such as Fe, Mn and Zn in a liquid sucrose–yeast extract medium *in vitro* (Altomare *et al.*, 1999). Rudresh *et al.* (2005) reported the ability of nine isolates of *Trichoderma* spp. to solubilize insoluble phosphate as compared with an efficient phosphate-solubilizing bacterium *Bacillus megaterium* subsp. *phospaticum* PB that was used as the reference strain. All nine *Trichoderma* isolates were found to solubilize tricalcium phosphate to various extents. *T. viride* (TV 97), *T. virens* (PDBCTVs 12), and *T. virens* (PDBCTVs 13) solubilized 70% of that solubilized by the reference bacterial strain. Pot culture and field evaluations further demonstrated that *T. harzianum* (PDBCTH 10), *T. viride* (TV 97), and *T. virens* (PDBCTVs 12) increased P uptake in chickpea (*Cicer arietinum* L.) plants supplied with rock phosphate as P source, which correlated with growth and yield parameters. *T. harzianum* retained its P solubilizing potential at varying concentrations of cadmium, indicating that *Trichoderma* may provide advantages to plants even in soils polluted with heavy metals (Rawat and Tewari, 2011). In another study, *T. harzianum* isolate T969, increased the concentrations of Ca^{2+}, Mg^{2+}, P and K compared with the control, with positive effects on shoot height, shoot diameter, and shoot fresh and dry weights in tomato seedlings (Azarmi *et al.*, 2011).

The use of high quantities of chemical fertilizers in agriculture causes pollution of soils and water bodies. Thus, a major goal of biotechnology is to develop novel strategies to optimize fertilizer use. With this aim, Molla *et al.* (2012) tested the ability of *Trichoderma* spp. to increase growth of tomato plants when supplied together with fertilizer. It was found that supplementation of fertilizer with *Trichoderma* enhanced plant production by 50% compared with a standard dose of NPK macronutrients, minimizing the use of fertilizer and their potential negative effects in the environment. A recent application in the field came from manipulation by genetic means of the *T. harzianum qid74* gene, which encodes a cysteine-rich cell-wall protein (Samolski *et al.*, 2012). Microscopic observations revealed more and longer root hairs in cucumber plants treated with the *qid74*-overexpressing strains and fewer and shorter hairs in roots treated with *qid74*-disrupted transformants, compared with those observed in plants inoculated with the wild-type strain. Modifications in root architecture induced by *qid74* increased the total absorptive surface, facilitating nutrient uptake and translocation of nutrients to the shoots, resulting in increased plant biomass through an efficient use of NPK and micronutrients. The nutrient uptake improvements in

plants conferred by *Trichoderma* spp. present the opportunity for more sustainable agricultural practices with a high yield, low cost and less polluting effects to fulfill the current demand for plant-derived products.

10.3.3 Induction of defence responses

It is generally believed that plants activate defence responses upon pathogen or insect attack. This means that plants save energy under enemy-free conditions and could invest photosynthetically fixed carbon in growth and reproduction. Interestingly, some types of soil can suppress the symptoms of plant diseases. Research has shown that the observed increased resistance in these plants is the result of the presence of rhizosphere microorganisms, including bacterial and fungal species, which exert their protective effect by directly inhibiting the growth of pathogens or by means of the activation of a part of the plant's immune system (Pieterse *et al.*, 2009). Plants possess various inducible defence mechanisms for protection against pathogens. An example of this is systemic acquired resistance (SAR), which is activated by a wide range of pathogens, especially those that cause tissue necrosis (Ryals *et al.*, 1996). Similarly, colonization of plant roots by certain non-pathogenic rhizobacteria can activate induced systemic resistance (ISR) in the host plant (Van Loon *et al.*, 1998; Conrath, 2011). Both pathogen-induced SAR and rhizobacteria-mediated ISR are effective against different types of pathogens, and are typically characterized by a restriction of pathogen growth and a suppression of disease development compared with primary infected, non-induced plants.

The signalling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR differ. Whereas SAR requires endogenous accumulation of salicylic acid (SA), the signalling pathway controlling ISR functions independently of SA and requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene (Pieterse *et al.*, 2009). Additionally, it has been established that accumulation of phytoalexins and other low molecular weight antimicrobial metabolites is integral to plant protection (Glawishnig, 2007). The chemical structures of phytoalexins vary among different plant families and include flavonoids, terpenoids and indoles. According to the classical vision, SA and JA play antagonistic relationships during defence responses. However, this traditional view of ISR seems to be more complex (Niu *et al.*, 2011), a notion that is confirmed by recent information on *Trichoderma*–plant interactions (Segarra *et al.*, 2007; Korolev *et al.*, 2008; Contreras-Cornejo *et al.*, 2011).

Several pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), microbe effectors or wound stimuli can initiate a stereotypical defence response, which involves the so-called priming of cells, both in tissues exposed to the stimuli and also in distant parts of the plant. Priming is defined as the physiological state that enables plants to respond to a stimulus in a very efficient way, in a more rapid and robust manner than non-primed plants. Priming has been found to be crucial in various types of systemic plant immunity, including SAR and ISR (Conrath, 2011). Like plant beneficial rhizobacteria, *Trichoderma* can induce priming for enhanced defence in plants (Alfano *et al.*, 2007; Mathys *et al.*, 2012). The mechanisms underlying this process are starting to be revealed (Segarra *et al.*, 2007; Contreras-Cornejo *et al.*, 2011; Mathys *et al.*, 2012).

The characterization of two kinds of ISR elicitors secreted by *T. virens* Gv29-8 has been described. Peptides with antimicrobial activity termed peptaibols have ISR effects and systemically induce defences in cucumber leaves (Viterbo *et al.*, 2007). The second ISR elicitor is the extracellular small protein Sm1, the gene expression of which was up-regulated in the presence of cotton plants (Djonovic *et al.*, 2006). Further *in vivo* studies, using reverse genetic analyses, demonstrated that expression of *SM1* is essential for triggering ISR in maize plants and providing protection against the foliar pathogen *Colletotrichum graminicola* (Djonovic *et al.*, 2007). In maize, the metabolic pathways that lead to the establishment of Sm1-mediated ISR involve the signalling networks associated with SA, green leafy volatiles and JA metabolism and

seem to be independent of pathogenesis-related proteins (Djonovic *et al.,* 2007).

Several recent reports have confirmed that the primed state of plants inoculated with *Trichoderma* is modulated by an intricate network of signalling pathways. Treatment with *Trichoderma hamatum* T382 primes *Arabidopsis* plants, resulting in an accelerated activation of the defence response against *B. cinerea* (Mathys *et al.,* 2012). Normalized microarray data were used to identify genes that were differentially expressed during priming, which were classified as involved in the plant-type hypersensitive response, as responsive to chitin and as defence-related genes responsive to SA and abscisic acid. Priming was also characterized by anthocyanin production and the stimulation of the transport of a variety of compounds in the plant such as phospholipids and ions (Mathys *et al.,* 2012).

The determination of plant growth regulators involved in the primed state induced by *Trichoderma* has confirmed the production of the phytohormones JA and SA in leaves. In the first hours of interaction between cucumber plant roots and *Trichoderma asperellum* strain T34, SA and JA levels and peroxidase activity increased in the cotyledons to different degrees, depending on the applied concentration of fungi (Segarra *et al.,* 2007). During co-cultivation of *Arabidopsis* roots with *T. virens* or *T. atroviride*, an induction of hydrogen peroxide, SA and JA was observed in leaves, which correlated with induction of pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA* (Contreras-Cornejo *et al.,* 2011). It was also found that both *T. virens* and *T. atroviride* increased accumulation of camalexin, a characteristic phytoalexin of *Arabidopsis*, in plants. All these combined responses seem to contribute to the *Trichoderma*-conferred resistance to *B. cinerea* because *Arabidopsis* mutants defective in genes from the respective pathways are compromised in the protection conferred by the biocontrol agents (Mathys *et al.,* 2012). Interestingly, co-cultivation of *Arabidopsis* seedlings with *T. virens* mutants defective in the 4-phosphopantetheinyl transferase 1 gene (*PPT1*) compromised the SA and camalexin responses, resulting in decreased protection against the pathogen (Velázquez-Robledo *et al.,* 2011). These data are in agreement with the gene expression data of Mathys and coworkers (2012), who observed a close similarity between *Trichoderma*-induced priming and SAR.

10.4 The Auxins from *Trichoderma*: Comparison with Other Plant Symbionts

Plants synthesize and use a variety of signals to adjust growth and development throughout their life cycle. Auxins, including indole-3-acetic acid (IAA), comprise a group of tryptophan (Trp)-derived signals that are involved in most aspects of plant development (Woodward and Bartel, 2005). Extensive studies over the past decade have investigated the factors involved in the regulation of plant morphogenesis by auxins. These compounds exert a strong biological activity at very low concentrations in both *in vivo* and *in vitro* systems and are essential for the maintenance of physiological and morphogenetic processes including gravity and light responses, root hair development, lateral root, adventitious root and shoot system development (Woodward and Bartel, 2005). Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms, and signal perception through a family of IAA receptors (Ljung *et al.,* 2002; Leyser, 2006; Mockaitis and Estelle, 2008).

Although the role of auxin signalling in symbiosis between plants and fungi still remains controversial, genetic evidences indicating that IAA is a positive regulator of plant growth comes from the analysis of *Arabidopsis* mutants that overproduce it, such as *super root* and *yucca*, which have long hypocotyls and increased numbers of lateral roots and root hairs (Boerjan *et al.,* 1995). Moreover, the positive effect of IAA application on growth of excised stems and hypocotyls and of auxin analogues in intact *Arabidopsis* seedlings has been described (Zhao *et al.,* 2001). The architecture of the root system is modified by the

endogenous auxin level and environmental stimuli that increases the auxin pool in the plant and/or affect auxin sensitivity such as temperature and the availability of water and mineral nutrients (Himanen *et al.*, 2002; López-Bucio *et al.*, 2003; Pérez-Torres *et al.*, 2008). A recent report has further shown that auxin-like signals produced from rhizosphere microorganisms could increase the exploratory capacity of the root system in *Arabidopsis* with a dramatic impact in plant biomass production (Ortiz-Castro *et al.*, 2011).

The potential of plant-associated microorganisms to produce free IAA, as already reported for *Trichoderma* spp., represents a means to influence the endogenous auxin pool of the host (Contreras-Cornejo *et al.*, 2009; Felten *et al.*, 2012; Hilbert *et al.*, 2012). Little is known, however, about the implication of this hormone in symbiosis. Auxin has often been suggested to play a role in the crosstalk between plant and fungal signalling during ectomycorrhizal establishment and in the colonization of barley roots by *Piriformospora indica* (Felten *et al.*, 2009, 2012; Hilbert *et al.*, 2012).

The early phase of the interaction between tree roots and ectomycorrhizal fungi, prior to symbiosis establishment, is accompanied by stimulation of lateral root development. For instance, plant inoculation with an IAA-overproducing strain of the ectomycorrhizal fungus *Hebeloma cylindrosporum* resulted in a faster and deeper colonization of the root compared with the wild-type strain and in a faster transcriptional response in the plant (Tranvan *et al.*, 2000). Another ectomycorrhizal fungus, *Laccaria bicolor*, increased lateral root development in poplar (*Populus tremula* × *Populus alba*) and *Arabidopsis*, which correlated with an increase in auxin accumulation at root apices. Blocking plant polar auxin transport with 1-naphthylphthalamic acid inhibited lateral root development and auxin accumulation. An oligoarray-based transcript profile of poplar roots exposed to molecules released by *L. bicolor* revealed the differential expression of 2945 genes, including several components of polar auxin transport (*PtaPIN* and *PtaAUX* genes), auxin conjugation (*PtaGH3* genes), and auxin signalling (*PtaIAA* genes). Transcripts of *PtaPIN9*, the homologue of *Arabidopsis AtPIN2*, and several *PtaIAAs* accumulated specifically during the early interaction phase (Felten *et al.*, 2009). These results reveal a critical role for auxin in root interactions with ectomycorrhiza.

Piriformospora indica, a newly described cultivable endophyte that colonizes roots, has been found to promote plant growth during its symbiotic relationship with a wide variety of plants (Waller *et al.*, 2005). *P. indica* can produce the phytohormones IAA and indole-3-lactate (ILA) through the intermediate indole-3-pyruvic acid (IPA). Time-course transcriptional analyses after exposure to tryptophan identified the tryptophan aminotransferase (*piTam1*) gene as a key player. *P. indica* strains in which the *piTam1* gene was silenced via an RNA interference (RNAi) approach were compromised in IAA and ILA production and displayed reduced colonization of barley (*Hordeum vulgare*) roots, but the elicitation of growth was not affected (Hilbert *et al.*, 2012).

Trichoderma species produce auxins as part of their metabolism including IAA and its precursors indole-3-ethanol, indole-3-acetaldehyde and indole-3-carboxaldehyde (Fig. 10.3; Contreras-Cornejo *et al.*, 2009, 2011).

The role of auxin signalling in *Trichoderma*–plant interactions was investigated in detail in *Arabidopsis thaliana* by Contreras-Cornejo and coworkers (2009). It was found that mutations in genes involved in auxin transport or signalling, *AUX1*, *BIG*, *EIR1* and *AXR1*, reduced the growth-promoting and root-developmental effects of *Trichoderma* inoculation. Colonization of plant roots by fungal hyphae activated the auxin-inducible reporter *DR5:uidA*, which correlated with an increased cell proliferation in primary and lateral root tips. Interestingly, the application of all three identified indolic compounds to *Arabidopsis* seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (μM range). Furthermore, *T. virens* also produced indole-3-carboxaldehyde (ICAld), a compound related to IAA metabolism probably involved in camalexin biosynthesis (Fig. 10.3;

CH_2COOH

Indole-3-acetic acid

CH_2CH_2OH

Indole-3-ethanol

CH_2CHO

Indole-3-acetaldehyde

CHO

Indole-3-carboxaldehyde

Fig. 10.3. Indolic compounds produced by *T. virens*. The chemical structures of all four compounds identified in *T. virens* cultures are shown. The levels of indoles increase when the culture medium is supplied with tryptophan (Trp), indicating that they probably derive from Trp metabolism.

Contreras-Cornejo *et al.,* 2011). The supply of ICAld to *Arabidopsis* seedlings inhibited primary root growth, induced adventitious root formation and increased camalexin levels (Contreras-Cornejo *et al.,* 2011).

At different stages of their life cycle, fungi release specific volatile organic compounds (VOCs) in order to interact with particular organisms (Splivallo *et al.,* 2011). *T. atroviride* produced at least 25 different VOCs including alcohols, ketones, alkanes, furanes, pyrones (mainly the bioactive 6-pentyl-alpha-pyrone), monoterpenes and sesquiterpenes (Stoppacher *et al.,* 2010). Vinale and co-workers (2008) reported an auxin-like effect in etiolated pea stems treated with harzianolide and 6-pentyl-α-pyrone, common metabolites produced by *Trichoderma* strains (Vinale *et al.,* 2008; Hermosa *et al.,* 2012). Certain VOCs from rhizobacteria could affect auxin biosynthesis and transport and have been proposed as candidate signals for plant growth promotion by PGPR (Zhang *et al.,* 2007). The possibility that VOCs from *Trichoderma* spp. could affect auxin homeostasis in plants remains to be determined.

The reported findings about the role of fungal-produced IAA in different plant–fungus interacting systems presents the possibility that fungi may use IAA and related compounds to communicate with plants as part of its colonization strategy, leading to plant growth stimulation and modification of basal plant defence mechanisms (Prusty *et al.,* 2004; Kazan and Manners, 2009). We speculate that the effects of inoculation with *Trichoderma* in plants under natural conditions may depend on the type and concentration of auxins produced by the fungi as well as the production of volatiles or auxin signal mimics. Perhaps auxin signalling may also play a role in colonization of roots by *Trichoderma* as already described for mycorrhizal fungi and *P. indica*. Confirmation of this hypothesis requires further experimentation.

10.5 Genes Regulated in the *Trichoderma*–Plant Interaction

With the advent of the genomics era, it has been possible to analyse the fungal and plant genes that are regulated in fungi during the interaction with plants as well as the plant genes responsive to root colonization by Trichoderma (Table 10.1).

In the following section, we will summarize the role played by some relevant genes in the plant–fungi interactions.

10.5.1 *Trichoderma* genes

To study the molecular mechanisms underlying the fungal ability to colonize the roots of tomato, the *T. harzianum* transcriptome was analysed during the early stages of the plant–fungus interaction. The expression of fungal genes related to redox reactions, lipid metabolism, detoxification and sugar or amino-acid transport increased in *T. harzianum* colonized roots (Chacón *et al.,* 2007). In another report, gene expression analysis of *T. harzianum* in the presence of tomato plants, chitin or glucose was performed through a high-density

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Table 10.1. Genes involved in *Trichoderma*–plant interactions.

Gene name	Interaction	Function	References
Fungal genes			
Endopolygalacturonase 1 (*Thpg1*)	*T. harzianum*–Tomato	Plant cell wall-degrading enzyme involved in root colonization and defence responses	Morán-Diez *et al.*, 2009
Invertase (*TvInv*)	*T. virens*–Maize.	Sugar metabolism	Vargas *et al.*, 2009
Cysteine-rich cell wall protein (*qid74*)	*T. harzianum*–Tomato	Adherence to hydrophobic surfaces and cell protection	Samolski *et al.*, 2012
1-Aminocyclopropane-1-carboxylate deaminase (*Tas-acdS*)	*T. asperellum T203*–Canola	Cleaves 1-aminocyclopropane-1-carboxylate to produce ethylene	Viterbo *et al.*, 2010
Small protein (*SM1/Epl1*)	*T. virens*–Cotton, maize *T. atroviride*–Maize	Induction of defence responses	Djonović *et al.*, 2006; Vargas *et al.*, 2008
4'-Phosphopantetheinyl transferase (*PPT1*)	*T. virens*–*Arabidopsis*	Activates enzymes involved in primary and secondary metabolism	Velázquez-Robledo *et al.*, 2011
Xylanase (*TvX/EIX*)	*T. viride*–Tobacco	β-1,4-endoxylanase, elicits plant defence responses and emission of ethylene	Sharon *et al.*, 1993
Swollenin (*TasSwo*)	*T. asperellum*–Cucumber	Involved in defence responses	Brotman *et al.*, 2008
Plant genes			
Transcription factor MYB77 (*MYB77*)	*T. asperelloides* T203–*Arabidopsis*	Modulates plant responses to auxin	Brotman *et al.*, 2012
Auxin responsive (*IAA29*)	*T. harzianum*–*Arabidopsis*	Member of the family of auxin repressors Aux/IAA	Morán-Diez *et al.*, 2012
Hookless 1 (*HLS1*)	*T. asperelloides* T203–*Arabidopsis*	Auxin signalling and cell growth	Brotman *et al.*, 2012
Root hair deficient (*RHD6*)	*T. virens*–*Arabidopsis.*	Cell differentiation	Contreras-Cornejo *et al.*, 2009
High indolic glucosinolate or transcription factor MYB51 (*HIG1*/*MYB51*)	*T. asperelloides* T203–*Arabidopsis*	Regulator of indolic glucosinolate biosynthesis	Brotman *et al.*, 2012
Transcription factor WRKY40 (*WRKY40*)	*T. asperelloides* T203–*Arabidopsis.*	Transcription factor induced by pathogens	Brotman *et al.*, 2012
Pathogenesis-related 1 (*PR-1*)	*T. virens*/*T. atroviride*–*Arabidopsis*	Defence responses (encodes a defensin)	Contreras-Cornejo *et al.*, 2011
Pathogenesis-related 2 (*PR-2*)	*T. asperellum* T203–Cucumber	β-1,3-glucanase.	Shoresh *et al.*, 2005
Chitinase (*EgCHI1/2/3*)	*T. harzianum*–Oil palm.	Hydrolizes glycosidic bonds in chitin	Naher *et al.*, 2012
Pathogenesis related 3 (*PR-3*)	*T. asperellum* T203–Cucumber.	Chitinase	Shoresh *et al.*, 2005

Lipoxygenase 1 (*LOX1*)	*T. asperellum* T203–Cucumber.	Biosynthesis of JA	Shoresh *et al.*, 2005
Lipoxygenase 2 (*LOX2*)	*T. virens*/*T. Atroviride*–*Arabidopsis*	Biosynthesis of JA	Contreras-Cornejo *et al.*, 2011
Phenylalanine ammonia lyase (*PAL*)	*T. asperellum* T-203–Cucumber *T. virens*–Maize	Biosynthesis of SA	Yedidia *et al.*, 2003; Shoresh *et al.*, 2005, 2008; Mukherjee *et al.*, 2012
Glutathione S-transferase (*GST*)	*T. harzianum* T22–Maize	Cell detoxification	Shoresh *et al.*, 2008
Hydroxyperoxide lyase (*HPL*)	*T. asperellum* T-203–Cucumber	Production of antimicrobial and wound-related C_6-volatiles	Yedidia *et al.*, 2003
Chitinase (*ChiB*).	*T. stromaticum*–Cacao	Chitinase	De Souza *et al.*, 2008
Endo-1,4-β-glucanase (*Glu-1*)	*T. stromaticum*–Cacao	Endo-1,4-β-glucanase	De Souza *et al.*, 2008
Mitogen activated protein kinase 3 (*MAPK3*)	*T. hamatum*–Cacao	Signal transduction cascades that regulate defence responses and abiotic stress resistance	Bae *et al.*, 2009
Trichoderma-induced MAPK (*TIPK*)	*T. asperellum*–Cucumber	Defence and wound responses	Shoresh *et al.*, 2006
Ethylene-overproducing 3 (*ETO3*)	*T. asperellum* T203–Cucumber	Ethylene biosynthesis	Brotman *et al.*, 2012
Ethylene receptor 1 (*ETR1*)	*T. asperellum* T203–Cucumber; *T. asperelloides* T203–*Arabidopsis*	Defence responses	Shoresh *et al.*, 2005; Brotman *et al.*, 2012
Constitutive triple response 1 (*CTR1*)	*T. asperellum* T203–Cucumber	Defence responses	Shoresh *et al.*, 2005
Calcineurin B-like proteins-interacting protein kinase (*OsCIPK14/15*)	*T. viride*–Rice	Recognition of microbe-associated molecular patterns.	Kurusu *et al.*, 2010
Stearoyl–acyl carrier protein desaturase (*SAD1/2*)	*T. harzianum*–Oil palm	Regulates cellular polyunsaturated fatty acid content	Alizadeh *et al.*, 2011
Lipid transferase protein 4 (*LTP4*)	*T. asperelloides*–*Arabidopsis*	Lipid transferase involved in resistance induced by *Trichoderma*	Brotman *et al.*, 2011
R gene (*HR4*)	*T. atroviride*–*Arabidopsis*	Recognition of specific microbe factors as signals of invasion	Saenz-Mata and Jimenez-Bremont, 2012

oligonucleotide microarray analysis. The results revealed 1617 probe sets showing differential expression in *T. harzianum* mycelium under at least one of the culture conditions tested as compared with one another. Hierarchical clustering and heat map representation showed that the expression patterns obtained in glucose medium clustered separately from the expression patterns observed in the presence of tomato plants and chitin. Interestingly, some up-regulated transcripts were predicted to encode proteins related to *Trichoderma*–plant interactions, such as Sm1/Elp1, proteases P6281 and PRA1, enchochitinase CHIT42, or QID74 protein. In this study, previously uncharacterized genes were also identified, including those responsible for the possible biosynthesis of nitric oxide, xenobiotic detoxification, mycelium development, and others related to the formation of infection structures (Samolski *et al.,* 2009).

By using *in vitro* and *in vivo* assays with *T. harzianum* CECT 2413 (T34), *T. virens* Gv29-8 (T87) and *T. hamatum* IMI 224801 (T7), Rubio *et al.* (2012) showed that these strains affected the growth and development of lateral roots in tomato plants in different ways, with beneficial effects reported for strains T7 and T34. After 20 h of incubation in the presence of tomato plants, using a high-density oligonucleotide microarray, the authors showed that carbohydrate metabolism was the most significantly over-represented process commonly observed in the three *Trichoderma* strains with an induction of the chitin degradation enzymes *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and chitinase. Strains T7 and T34, both of which stimulate plant development, were found to enhance hexokinase activity and the transcription of genes encoding a 40S ribosomal protein and a P23 tumour protein orthologue.

As mentioned earlier, sugars exuded by roots into the rhizosphere are crucial nutrient sources for the symbiotic association between *Trichoderma* and plants (Vargas *et al*., 2009). By using several bioinformatics tools, two genes likely to be involved in the uptake of sucrose by *T. virens*, an intracellular invertase (TvInv) and a plant-like sucrose transporter (TvSut), were recently identified. Genetic, biochemical and physiological studies were conducted to characterize the role of sucrose on invertase activity in the fungus and in the interactions with maize plants (Vargas *et al.,* 2009). The loss-of-function on *tvsut* caused a detrimental effect on fungal growth when sucrose was the sole source of carbon in the medium, and also affected the expression of genes involved in the symbiotic association (Vargas *et al.,* 2011). These results show that *T. virens* contains genes for sucrose uptake and metabolism, which play an important role during early stages of root colonization. These exciting results provide new insights into the mechanisms and roles of fungal genes in the *Trichoderma*–plant interaction; it might be of further interest to investigate the contribution of nitric oxide released by fungal hyphae to root growth because recent information suggests that it affects primary root growth and induces lateral root formation (Fernández-Marcos *et al.,* 2011; Méndez-Bravo *et al.,* 2011).

10.5.2 Plant genes

Genes and proteins regulated by *Trichoderma* have been discovered and characterized in *Arabidopsis thaliana,* tomato, maize, cacao, chilli pepper and oil palm plants. The first evidence for auxin signalling in mediating the observed developmental alterations by *T. virens* inoculation in plants was inferred from tests using the auxin-responsive marker constructs *DR5:uidA, BA3:uidA* and *HS::AXR3NT-GUS* and the analysis of *aux1-7, doc1, eir1* and *axr1* auxin-related mutants of *Arabidopsis*. The *aux1-7* mutant is defective at the AUX1 locus, which encodes an auxin influx facilitator participating in both acropetal and basipetal auxin transport at the root tip (Swarup *et al.,* 2001); *doc1* is a mutant allele of *BIG*, which encodes a protein important for the correct location of certain auxin transport proteins (Gil *et al.,* 2001), whereas *eir1* encodes the auxin transporter AtPIN2 (Luschnig *et al.,* 1998). Five days after plants were inoculated, *T. virens* increased (by 62%) shoot fresh weight in wild-type seedlings when compared with uninoculated seedlings. In contrast, all four mutant lines, *aux1-7, doc1, eir1* and *axr1-3*, showed decreased or null responses in growth

promotion by the fungus. Interestingly, it was found that *T. virens* induced up to a fourfold increase in lateral root number when compared with axenically grown plants, a reduction in lateral root formation when compared with inoculated wild-type plants was observed for *aux1-7* and *axr1-3* inoculated seedlings, and no lateral root induction was registered for uninoculated or inoculated *doc1* seedlings. These results support the hypothesis that both normal auxin transport and response are important for the effects of *T. virens* on plant growth and lateral root development (Contreras-Cornejo *et al.*, 2009).

Plants have large collections of so-called resistance proteins that recognize specific microbe factors as signals of invasion. One of these proteins is coded by the *Arabidopsis thaliana HR4* gene in the Col-0 ecotype that is homologous to *RPW8* genes present in the Ms-0 ecotype. In a recent study, Saenz-Mata and Jiménez-Bremont (2012) investigated the expression patterns of the *HR4* gene in *Arabidopsis* seedlings interacting with *T. atroviride*. It was observed that the induction of the *HR4* gene mainly occurred at 96 h post-inoculation, at a time when the fungus directly interacted with roots. To examine the effect of phytohormones involved in biotic stress signalling on the *HR4* gene, 15-day-old *Arabidopsis* (Col-0) seedlings were sprayed with the ethylene donor ethephon, SA and methyl jasmonate (MeJA) and harvested at 1, 3 and 24 h after spraying. Ethephon treatment induced the *HR4* gene at 1 and 3 h by about threefold, and this induction was maintained at 24 h. For SA and MeJA treatment, a strong initial induction (about tenfold at 1 h) of this gene was observed. The *HR4* gene was also differentially regulated in interactions with the beneficial bacterium *Pseudomonas fluorescens* and the pathogenic bacterium *Pseudomonas syringae*. Although the functional relevance of the *HR4* gene or its homologues in the *Trichoderma*–plant interaction still needs to be investigated by mutant and transgenic means, these results indicate that *HR4* and *RPW8* genes could play a role in the establishment of *Arabidopsis* interactions with beneficial microbes.

The molecular basis of the ISR in *A. thaliana* by *T. hamatum* T382 against the phytopathogen *B. cinerea* B05-10 was unravelled by microarray analysis both before and after pathogen inoculation (Mathys *et al.*, 2012). In general, the defence responses elicited by *Trichoderma* alone were similar to those activated after SAR, the systemic defence response that is triggered in plants upon pathogen infection leading to increased resistance toward additional infections. Root colonization with *T. hamatum* T382 primed the plant, resulting in an accelerated activation of the defence response genes against *B. cinerea*, which were dependent upon SA and JA signalling and the phenylpropanoid pathway. The involvement of different defence-related pathways identified in this transcriptomic study was validated using phenotypic analysis of *A. thaliana* disease signalling mutants related pathways including *npr1, sid2* and NahG for the SA pathway, *ein2* and *etr1* for the ethylene pathway and *myc2* for the JA pathway, or in defence-related mechanisms such as *tt, chs* and *f3h*, all carrying mutations in the phenylpropanoid pathway. The suppressive effect on *B. cinerea* disease, as observed earlier in wild-type *Arabidopsis* plants pre-treated with *T. hamatum* T382, was not detected in most of these mutants, indicating that the corresponding genes and pathways play an important role in this interaction. Indeed, mutants corresponding to key genes in SA- or JA-mediated signalling, or anthocyanin production did not display the *T. hamatum* T382-induced ISR against *B. cinerea*.

A major challenge of studying model plants, such as *Arabidopsis*, is transferring the knowledge and new tools to crop species; transcriptomic and proteomic approaches have proven to be effective toward this goal. The proteome and transcriptome of plants change in response to root colonization by *Trichoderma*, indicating that these fungi reprogram the expression of plant genes. Alfano and co-workers (2007) showed that root colonization by *T. hamatum* T382 protected plants against bacterial spot of tomato (*Xanthomonas euvesicatoria* 110c). To gain insight into the mechanism by which *T. hamatum* T382 induced resistance in tomato, microarrays were used to determine its effect on the expression pattern of 15925 genes in leaves just before inoculation with the pathogen. *T. hamatum* T382

modulated the expression of genes in leaves and 45 genes were found to be differentially expressed with functions associated with biotic or abiotic stress, as well as RNA, DNA and protein metabolism. Four extensin and extensin-like proteins were induced. This work showed that *T. hamatum* T382 actively induces systemic changes in plant leaves and disease resistance through systemic modulation of the expression of stress- and metabolism-associated genes.

Endophytic *Trichoderma* isolates collected in tropical environments have been evaluated for changes in gene expression in cacao (*Theobroma cacao)* and chilli pepper (*Capsicum annuum*). During the interaction between cacao seedlings and four endophytic *Trichoderma* isolates, *Trichoderma ovalisporum*-DIS 70a, *T. hamatum*-DIS 219b, *T. harzianum*-DIS 219f and *Trichoderma* sp.-DIS 172ai, seven cacao genes were induced during root colonization. These included putative genes for ornithine decarboxylase (P1), GST-like proteins (P4), zinc finger protein (P13), wound-induced protein (P26), EF-calcium-binding protein (P29), carbohydrate oxidase (P59) and an unknown protein (U4). Two plant expressed sequence tags (ESTs), extensin-like protein (P12) and major intrinsic protein (P31), were repressed owing to colonization. The plant gene expression profile was dependent on the *Trichoderma* isolate colonizing the cacao seedling (Bailey *et al.*, 2006). Six additional endophytic isolates were tested for induced resistance capabilities in pepper. The isolates induced defence reactions and conferred protection against *P. capsici. Trichoderma* endophytic colonization induced multiple lipid transferase protein (LTP)-like family members. The timing and intensity of induction varied between isolates. Expression of *CaLTP-N*, encoding a LTP-like protein in pepper, in *Nicotiana benthamiana* leaves reduced disease development in response to *P. nicotianae* inoculation, suggesting LTPs are functional components of resistance induced by *Trichoderma* species (Bae *et al.*, 2011). An additional LTP (*LTP4*) was regulated during the systemic defence response of *A. thaliana* plants to the leaf pathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) mediated by the beneficial fungus *T. asperelloides* T203. Among the defence-related genes affected by T203*, LTP4* was up-regulated, whereas the *WRKY40* transcription factor, known to contribute to *Arabidopsis* susceptibility to bacterial infection, showed reduced expression (Brotman *et al.*, 2011).

These data and other recent discoveries demonstrate that fatty acid metabolism pathways play significant roles in pathogen defence in addition to phytohormone-mediated defence pathways (Christensen and Kolomiets, 2011). A key regulator in the fatty acid biosynthetic pathway is stearoyl–acyl carrier protein desaturase (SAD). Plant SAD is known to regulate cellular polyunsaturated fatty acid content. This enzyme also catalyzes conversion of saturated stearic acid (18:0) to monounsaturated oleic acid (18:1) and plays essential roles in maintenance of biological membrane structure, and synthesis of storage lipids and signalling molecules. Alizadeh and coworkers (2011) investigated the effects of *T. harzianum* in *SAD1* and *SAD2* gene expression in the oil palm (*Elaeis guineensis*), which is one of the most profitable oil-bearing crops. In *T. harzianum* inoculated seedlings, the expression levels of *SAD1* and *SAD2* increased gradually and were stronger in roots than in leaves, which was consistent with the protection conferred by this fungus against the pathogen *Ganoderma boninense*.

Proteomic approaches using two-dimensional gel electrophoresis and mass spectrometry have provided additional information on the protein profiles modulated by *Trichoderma* in plants, particularly focusing on systemic changes. In the first hours of interaction between cucumber roots and *T. asperellum* strain T34, SA and JA levels and peroxidase activity increased in the cotyledons to different degrees depending on the applied concentration of the fungi. These effects correlated with changes in 28 proteins, 17 of which were up-regulated while 11 were down-regulated. Proteins involved in reactive oxygen species (ROS) scavenging, stress response, isoprenoid and ethylene biosynthesis, and in photosynthesis, photorespiration and carbohydrate metabolism were differentially regulated by *Trichoderma* (Segarra *et al.*, 2007). Another study was conducted to investigate changes in the proteome of maize leaves induced by a seed treatment, and subsequent root colonization by *T. harzianum* T22 (Shoresh *et al.*, 2010).

A large portion of the up-regulated proteins have putative functions in carbohydrate metabolism, photosynthesis, stress and defence responses. Other processes that were up-regulated were amino acid metabolism, cell wall metabolism and genetic information processing. Up-regulation of carbohydrate metabolism, stress response and plant defence correspond well with the enhanced growth response and induced resistance conferred by the *Trichoderma* inoculation.

10.6 Concluding Remarks

Trichoderma-based bioinoculants are increasingly used in agriculture, with several hundred formulations available as registered products worldwide. Several strategies have been applied to identify the genes and signals involved in the interactions of *Trichoderma* with plants. Proteome and genome analysis in crops as well as genetic analysis in the model plant *Arabidopsis* have greatly enhanced our knowledge on the signalling pathways by which these biocontrol agents promote plant growth and activate defence responses. Using these different approaches, a variety of novel genes and gene products have been identified, including enzymes that allow the fungus to metabolize plant-derived sugars, elicitors of induced resistance and plant proteins specifically induced by *Trichoderma*. The *Trichoderma*–plant interaction can be viewed as a mycorrhiza-like system in several respects: (i) it depends on carbon sources supplied by plants; (ii) it requires physical contact and possibly internal proliferation of the fungus in plant tissues; (iii) it involves the exchange of IAA and auxin-related signals; (iv) it improves plant nutrition increasing N, P, K and micronutrient content, thus boosting photosynthesis and carbon metabolism; and (v) it activates defence responses through JA-, SA- and phytoalexin-dependent mechanisms.

The crosstalk between hormones at both physiological and molecular levels is receiving increasing importance, bringing a new understanding of how they are able to act either antagonistically or synergistically in a tissue-specific fashion to influence plant growth and defence responses. The hormonal crosstalk in the plant induced by *Trichoderma* is dynamic and the expression of growth and defence-related genes of the auxin, JA/ethylene and/or SA pathways may overlap, depending on the *Trichoderma* strains and the amount of inoculum, the plant species, the developmental stage of the plant and the timing of the interaction. Therefore, there is a need for more studies aimed at testing the functional relevance of genes and proteins whose expression is modulated during the interaction both in the plant and the fungi, as well as characterizing the phenotypes of loss-of-function mutants and overexpressing lines during *Trichoderma*–plant interactions in the presence of pathogens and/or different types of abiotic stresses. The use of *Trichoderma* mutants impaired in the production of volatiles or secondary metabolites will be a powerful tool to establish the ecological roles of these signals.

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Participación de las citocininas en la estimulación del crecimiento vegetal por *Bacillus megaterium*

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PALABRAS CLAVE

Arabidopsis;

estimulación del crecimiento vegetal;

desarrollo de la raíz;

rizobacterias

Resumen

Las rizobacterias promotoras del crecimiento vegetal (PGPRs) influyen en el crecimiento y desarrollo de las plantas mediante, entre otros mecanismos, la producción de fitohormonas como las auxinas, giberelinas y citocininas. Se conoce poco sobre las rutas de señalización que median los efectos benéficos de las PGPRs en las plantas. Recientemente reportamos la identificación de una cepa de *Bacillus megaterium* que promueve el crecimiento de plántulas de *Arabidopsis thaliana* y *Phaseolus vulgaris*. En este trabajo se investigó el papel de la ruta de señalización de las citocininas en las respuestas de las plantas a la inoculación con *B. megaterium*, utilizando plantas silvestres de *A. thaliana* y mutantes carentes de uno, dos o los tres receptores probables de citocininas *CRE1*, *AHK2* y *AHK3*, y el gene *RPN12* que codifica para una subunidad del proteosoma, involucrado en la ruta de señalización de las citocininas. Se encontró que la promoción del crecimiento vegetal provocado por *B. megaterium* coincide con un efecto prolífico en la formación de las raíces laterales y que ambos efectos se reducen en las mutantes afectadas en el receptor de citocininas *AHK2-2*, en las combinaciones de dobles mutantes que incluyen este gen y en la mutante afectada en el gen RPN12. La triple mutante *CRE1-12/ AHK2-2/ AHK3* fue la más resistente a la inoculación en términos de promoción del crecimiento y modificación del desarrollo de la raíz. El uso de la línea reportera de citocininas *ARR5:GUS* confirma que *B. megaterium* activa la señalización por citocininas. Nuestros resultados indican que los receptores de citocininas tienen un papel fundamental en la estimulación del crecimiento ejercido por *B. megaterium* en *A. thaliana*.

Abstract

Accumulating evidence indicates that plant growth promoting rhizobacteria (PGPR) influence plant growth and development by the production of phytohormones such as auxins, gibberellins, and cytokinins. Little is known about the genetic basis and signal transduction components that mediate the beneficial effects of PGPRs in plants. We recently reported the identification of a *Bacillus megaterium* strain that stimulated growth of *Arabidopsis thaliana* and *Phaseolus vulgaris* seedlings. In this report, the role of cytokinin signaling in mediating plant responses to bacterial inoculation was investigated using *A. thaliana* mutants lacking one, two or three of the putative cytokinin receptors *CRE1*, *AHK2* and *AHK3*, and *RPN12* a gene codifying a subunit of the proteasome involved in cytokinin signaling. We show that plant growth promotion by *B. megaterium* is reduced in *AHK2* single and double mutant combinations and in *RPN12*. Furthermore, the triple cytokinin receptor *CRE1-12/AHK2-2/AHK3-3* knockout was the most resistant to inoculation in terms of growth promotion and root developmental responses. The use of the cytokinin reporter line *ARR5:GUS* confirms that *B. megaterium* activates cytokinin signaling. Our results indicate that cytokinin receptors play an important role in plant growth promotion by *B. megaterium*.

KEYWORDS

Arabidopsis;

plant growth stimulation;

root development;

rhizobacteria

Introducción

Existen diversas especies de rizobacterias que se asocian con las plantas y promueven el crecimiento vegetal. Estas se han denominado PGPRs por sus siglas en inglés *plant growth promoting rhizobacteria* y son organismos de vida libre, habitantes de la rizósfera con una influencia positiva en el crecimiento y desarrollo de las plantas (Bloemberg y Lugtenberg, 2001; Persello−Cartieaux et al., 2003).

Las PGPR pertenecen a diversos géneros tales como *Pseudomonas*, *Azospirillum*, y *Bacillus* y han sido aislados de una amplia gama de especies vegetales tales como la cebada, el arroz, la canola, el frijol y Arabidopsis (Alström 1991; Alexandre et al., 1996; Persello−Cartieaux et al., 2001). La contribución de las PGPRs al crecimiento de las plantas puede ser ejercido mediante mecanismos que incluyen la competencia con microorganismos deletéreos (Ongena et al., 1999), la activación de los sistemas de defensa de las plantas (Pieterse et al., 1996; Schuhegger et al., 2006) y la producción de substancias que regulan el crecimiento vegetal como las auxinas, las citocininas y diversos compuestos volátiles bacterianos (Selvadurai et al., 1991; Lebuhn et al., 1997; Arkhipova et al., 2005; Kai et al., 2009).

Las fitohormonas están involucradas en el control del crecimiento y en casi todos los procesos importantes para el desarrollo de las plantas. La secreción bacteriana de fitohormonas puede impactar la arquitectura de la raíz mediante la inducción de pelos radiculares y raíces laterales que participan en la absorción de agua y nutrimentos, contribuyendo así al crecimiento vegetal (Persello−Cartieaux et al., 2003).

Para dilucidar los mecanismos de señalización mediante los cuales las PGPRs promueven el crecimiento y modifican el desarrollo vegetal, se ha utilizado a *Arabidopsis thaliana* como planta modelo para identificar cepas bacterianas con capacidad de promover el crecimiento. De esta forma, en trabajos previos, identificamos una nueva cepa de *Bacillus megaterium* (UMCV1), que promueve el crecimiento de plantas de *Arabidopsis thaliana* y *Phaseolus vulgaris* tanto en condiciones *in vitro* como crecidas en el suelo (Valencia-Cantero et al., 2007; López-Bucio et al., 2007). La inoculación con *B. megaterium* afecta el sistema radicular de las plantas silvestres de *A. thaliana* en una forma que sugiere que el efecto está mediado por fitohormonas, incluyendo la inhibición en el crecimiento primario de la raíz, seguido por un incremento en el número de raíces laterales y la longitud de los pelos radiculares (López-Bucio et al., 2007). También se reportó que los efectos de la inoculación bacteriana en el crecimiento de las plántulas son independientes a las rutas de señalización de auxinas y etileno, como queda de manifiesto por un incremento en la producción de biomasa y la estimulación de las raíces laterales de las mutantes resistentes a auxinas *aux1-7*, *axr4-1* y *eir1-1* y las mutantes afectadas en la respuesta a etileno *etr1-1* y *ein2-1*. En este mismo trabajo, la inoculación de *B. megaterium* no incrementó la expresión del gen reportero inducible por auxinas *DR5:GUS*, indicando que esta bacteria no activa la vía de señalización de las auxinas (López-Bucio et al., 2007). Estas observaciones indican que las rutas de señalización involucradas en la estimulación del crecimiento en respuesta a la inoculación con *B. megaterium* están aún por ser identificadas en las plantas, siendo la ruta de señalización de las citocininas una clara candidata.

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Las citocininas son una clase de fitohormonas producidas tanto por plantas como por microorganismos (Aloni et al., 2006), su producción por bacterias asociadas a plantas ha sido bien documentada (Salamone et al., 2001; Nieto y Frankenberger, 1990). Por lo anterior, puede esperarse que la inoculación de plantas con bacterias capaces de producir citocininas conduzca a un incremento en la concentración de citocininas en los tejidos radiculares y que esto tenga un impacto en el crecimiento de la planta. A la fecha se han identificada tres receptores de citocininas, *CRE1*, *AHK2* y *AHK3* (Kakimoto, 2003; Higuchi et al., 2004; Nishimura et al., 2004; Mähönen et al., 2006). Las plantas con una sola mutación o mutantes doblemente afectadas en cualquiera de las combinaciones de los genes *CRE1*, *AHK2* y *AHK3* tienen un desarrollo normal de la raíz. Sin embargo la mutante triple *cre1-12/ahk2-2/ahk3-3* muestra un crecimiento menor de la raíz primaria y un arresto en el crecimiento de los

tallos indicando que la percepción de las citocininas es importante para el desarrollo normal de las plantas (Kakimoto, 2003; Higuchi et al., 2004).

Para determinar una posible participación de la vía de señalización de las citocininas en la estimulación del crecimiento vegetal por *B. megaterium*, en este trabajo se utilizó *Arabidopsis thaliana* como planta modelo y mutantes afectadas en cada uno de los tres receptores conocidos de citocininas *CRE1*, *AHK2* y *AHK3*, así como en el gen *RPN12*. Adicionalmente, se analizaron los efectos de la inoculación con esta bacteria sobre la expresión del marcador inducible por citocininas *ARR5:GUS* en plantas transgénicas de Arabidopsis que expresan este gen reportero. Con estas herramientas, en el presente estudio se presenta evidencia que indica que la ruta de señalización de las citocininas desempeña un papel importante en la estimulación del crecimiento y la regulación de la arquitectura de la raíz por *B. megaterium*.

Materiales y Métodos

Material biológico y condiciones de cultivo

Se emplearon plantas de *A. thaliana* ecotipos Col-0 y C24 (silvestres) y las plantas mutantes sencillas c*re1-12*, *ahk2-2tk*, *ahk3-3*; las mutantes dobles *cre1-12/ahk2-2tk*, *cre1-12/ahk3-3*, y *ahk2-2tk/ahk3-3*, y la triple mutante *cre1-12/ahk2-2/ahk3-3*. Estas mutantes han sido descritas previamente en Kakimoto (2003), Higuchi et al. (2004), Nishimura et al. (2004) y Mähönen et al., (2006).

La triple mutante *cre1-12/ahk2-2/ahk3-3* fue obtenida a partir de una población heterocigota de *cre1-12/cre-12, ahk2-2tk/ahk2-2tk* y *ahk3-3/AHK3*. En cajas de Petri con medio de Murashige y Skoog (Sigma) 0.2x solidificado con 8 g/L^{-1} de agar de micropropagación (medio MS). Las mutantes triples desarrollan una raíz primaria corta y pueden distinguirse fácilmente de las plantas heterocigotas *cre1-12/cre1-12, ahk2-2tk/ahk2-2tk* y *ahk3-3/AHK3*. Para seleccionar las triples mutantes, se seleccionó un conjunto de semillas producido por las plantas heterocigotas *cre1-12/cre1-12, ahk2-2tk/ahk2-2tk* y *ahk3-3/AHK3*, se desinfectaron superficialmente y se sembraron en cajas con medio MS. Después de 10 días las plántulas con la raíz primaria corta, fueron transferidas a cajas con medio MS fresco para realizar el experimento correspondiente.

También se empleó la mutante *rpn12a-1*, mutante con el fondo genético del ecotipo C-24 que es deficiente en una subunidad del proteosoma de Arabidopsis S26, que es importante para numerosas respuestas del crecimiento reguladas por citocininas (Smalle et al., 2002).

La bacteria empleada fue *B. megaterium* UMCV1 descrita en López-Bucio et al. (2007). *Bacillus megaterium* fue asilada en una búsqueda de bacterias promotoras del crecimiento de la rizósfera de plantas de maíz crecidas en suelo ligeramente ácido de una localidad de la Ciudad de Uruapan Michoacán, México (19° 23' 56.69'' N, 102° 02' 41.17'' O), en condiciones de invernadero y fue identificada como *B. megaterium* con base en un análisis de su gene ribosomal de 16S.

Las semillas de las plantas fueron esterilizadas superficialmente con etanol al 95% (vol/vol) por 5 min y blanqueador comercial al 20% (vol/vol) por 7 min. Después de 5 lavados con agua destilada esterilizada, las semillas fueron geminadas y crecidas en cajas de Petri con medio MS.

Las cajas con las semillas y/o plantas se colocaron verticalmente en un ángulo de 65° para propiciar que la raíz de las plantas creciera sobre la superficie del agar y permitir el crecimiento de los tallos. Las plantas fueron colocadas en una cámara de crecimiento Percival con fotoperiodo controlado de 16 h luz y 8 h obscuridad a una intensidad de 200 μmol m^2 s^{-1} y una temperatura de 24 °C.

Efecto de la inoculación de *B. megaterium* en el crecimiento y el desarrollo de la raíz de *A. thaliana*.

Para investigar si la inoculación de *B. megaterium* estimula a la ruta de señalización de citocininas, se evaluó el efecto de la inoculación bacteriana a 5 cm del meristemo de la raíz primaria de plantas silvestres y mutantes de 5 días de edad (5 días después de la germinación). En el caso de los experimentos con las triples mutantes la inoculación de *B. megaterium* se hizo tanto a 2 como a 5 cm del meristemo de las raíces

primarias y las plantas empleadas tuvieron 10 días al momento de ser inoculadas. En todos los casos se incluyeron controles consistentes de plantas no inoculadas.

Después de 6 días de crecimiento en presencia de *B. megaterium*, se cuantificó la biomasa de los tallos y las raíces de 4 grupos de 20 plantas por cada genotipo y cada distancia de inoculación. La significancia estadística se determinó mediante un análisis de varianza y una prueba de Tukey ($p < 0.05$).

Determinación de la actividad de la β-glucuronidasa (GUS)

Las plantas transgénicas que expresan el gene reportero ARR:*uidA* (De Agostino et al., 2000) se procesaron con X-Gluc 0.1% (5-bromo-4-cloro-3-indolil, β-D-glucurónido) en amortiguador de fosfatos (NaH_2PO_4 y Na_2HPO_4, 0.1M, pH 7) con 2 mM ferrocianuro de potasio y 2 mM ferricianuro de potasio, durante 12 horas a 37 °C. Las plantas se clarificaron y fijaron con 0.24 N HCl en metanol 20% (v/v) durante 60 min a 62 °C. La solución se sustituyó por NaOH 7% (v/v) en etanol 60% (v/v) y se incubó durante 20 min a temperatura ambiente. Las plantas se deshidrataron con

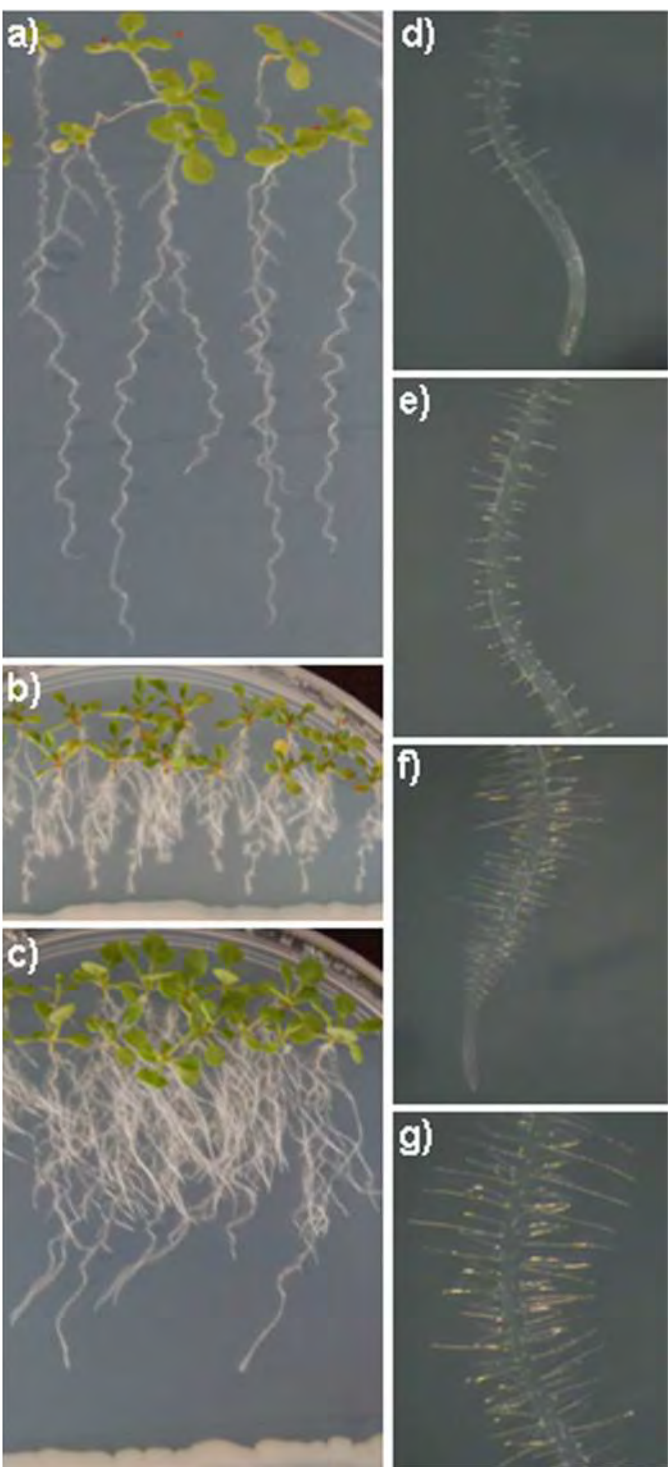

Figura 1. Efecto de *Bacillus megaterium* sobre el crecimiento y desarrollo de plantas silvestres de Arabidopsis (ecotipo Col-0). Se presentan fotografías representativas de a) plantas no inoculadas, b) plantas inoculadas a 2 cm de la punta de la raíz, c) plantas inoculadas a 5 cm. Los paneles d y e muestran el fenotipo de los pelos radiculares en plantas no inoculadas, en tanto que e y f presentan los efectos de la inoculación a 5 cm.

tratamientos de etanol a 40, 20 y 10% (v/v) por 24 horas en cada periodo. Finalmente se substituyó el etanol por glicerol 50% (v/v). Las raíces procesadas se incluyeron en portaobjetos para su análisis en un microscopio estereoscópico Leica L2.

Resultados

Bacillus megaterium promueve la formación de raíces laterales y pelos radiculares en plantas de *Arabidopsis thaliana*.

Las plantas silvestres de Arabidopsis thaliana (ecotipo Col-0) fueron germinadas y crecidas durante 5 días en medio de Murashige y Skoog (MS) al 0.2x. Este medio incluye todos los nutrientes necesarios para el crecimiento vegetal en concentraciones adecuadas (López-Bucio et al., 2007). En esta etapa, las plantas fueron inoculadas mediante un estriado con *B. megaterium* a 2 o 5 cm de distancia de las puntas de las raíces. Después de 6 días adicionales de crecimiento, las plantas no inoculadas formaron una raíz primaria con crecimiento indeterminado (pivotante o continuo) con raíces laterales en la región proximal a la base del tallo (Fig. 1a). Las plantas inoculadas a 2 o 5 cm se caracterizaron por la formación de sistemas radiculares altamente ramificados con una gran proliferación de raíces laterales (Fig. 1b-c). Para investigar con mayor detalle las alteraciones morfogenéticas inducidas por *B. megaterium*, también se evaluaron los efectos de la inoculación con esta bacteria en el desarrollo de los pelos radiculares, los cuáles son células epidérmicas especializadas en la captación de agua y de nutrientes. En plantas no inoculadas se observaron pelos radiculares en la zona de diferenciación próxima al meristemo de la raíz (Fig. 1d), alcanzando éstos su mayor tamaño en la zona media de la raíz (Fig. 1e). La inoculación bacteriana estimuló el crecimiento de los

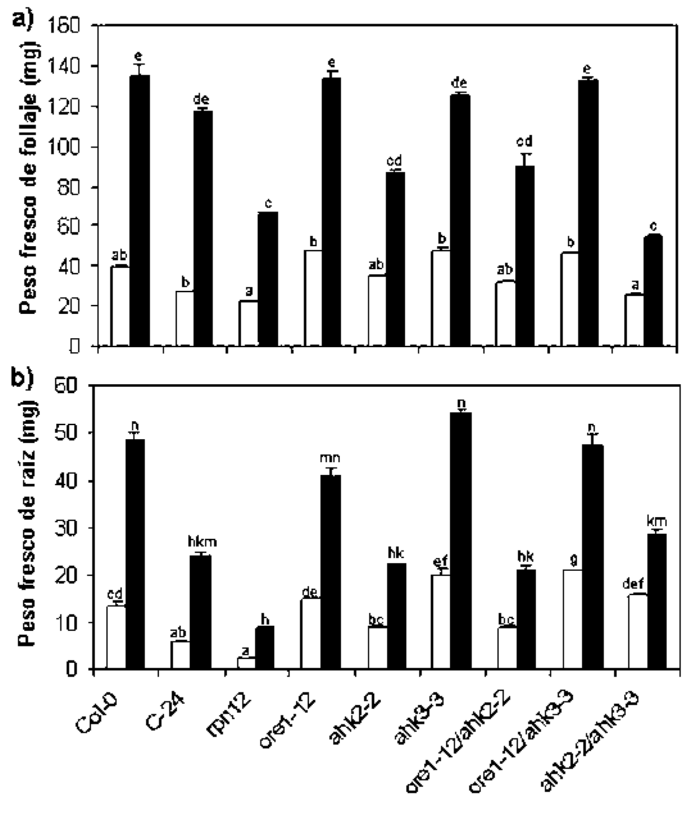

Figura 2. Efecto de *Bacillus megaterium* sobre la producción de biomasa de plantas silvestres de Arabidopsis (ecotipo Col-0) y de diferentes líneas mutantes afectadas en la ruta de señalización de citocininas. El material vegetal fue cosechado seis días después de la inoculación bacteriana a 5 cm de la punta de la raíz, cuantificándose por separado el peso fresco de la raíz y del follaje. Los pesos frescos de cada parte de la planta se cuantificaron en una balanza analítica. a) Peso fresco del follaje. b) Peso fresco de la raíz. Los valores representan las medias de cuatro grupos de 20 plántulas. Se usan diferentes letras para indicar medias que difieren significativamente ($p < 0.05$).

pelos radiculares tanto en la zona de diferenciación como en la región media de la raíz primaria (Fig. 1f-g), indicando un efecto positivo sobre la elongación celular.

Efecto de la inoculación con *Bacillus megaterium* en la producción de biomasa en plantas silvestres de *Arabidopsis thaliana* y en mutantes afectadas en la respuesta a citocininas.

Para investigar los efectos de *B. megaterium* en el crecimiento vegetal y el papel de las citocininas en este proceso, se comparó la producción de biomasa en plantas silvestres de los ecotipos Col-0 y C-24 con mutantes afectadas en la respuesta a citocininas reportadas previamente en la literatura. Estas mutantes incluyeron pérdidas de función para los receptores de citocininas CRE1, AHK2-2 y AHK3-3 y para el gen RPN12. Las plantas silvestres y las mutantes individuales o dobles fueron inoculadas a 5 cm de la punta de la raíz con *B. megaterium* y 7 días después se comparó el peso fresco del follaje y de la raíz con plantas no inoculadas. Como se aprecia en la Figura 2, la inoculación bacteriana causó un incremento de tres veces en el peso fresco de tallos y raíces en plantas silvestres de los ecotipos Col-0 y C24. En contraste los efectos en la promoción del crecimiento debido a la inoculación se redujeron en la mutante *rpn12a-1* (con el fondo genético C-24), en las mutantes en el receptor *AHK2-2* y en sus combinaciones en mutantes dobles (Fig. 2a-b).

Posteriormente se probaron los efectos de la inoculación de *B. megaterium* en el crecimiento y el desarrollo radicular de plantas de la línea triple mutante *cre1-12/ahk2-2/ahk3-3,* carente de los tres receptores de citocininas. En este experimento la inoculación bacteriana se realizó a 2 y a 5 cm de las puntas de las raíces ya que la mutante forma raíces muy cortas en condiciones normales de crecimiento, evaluando a los 6 días después de la inoculación de la bacteria el crecimiento general de las plantas y la arquitectura de la raíz. En los controles de plantas silvestres (Col-

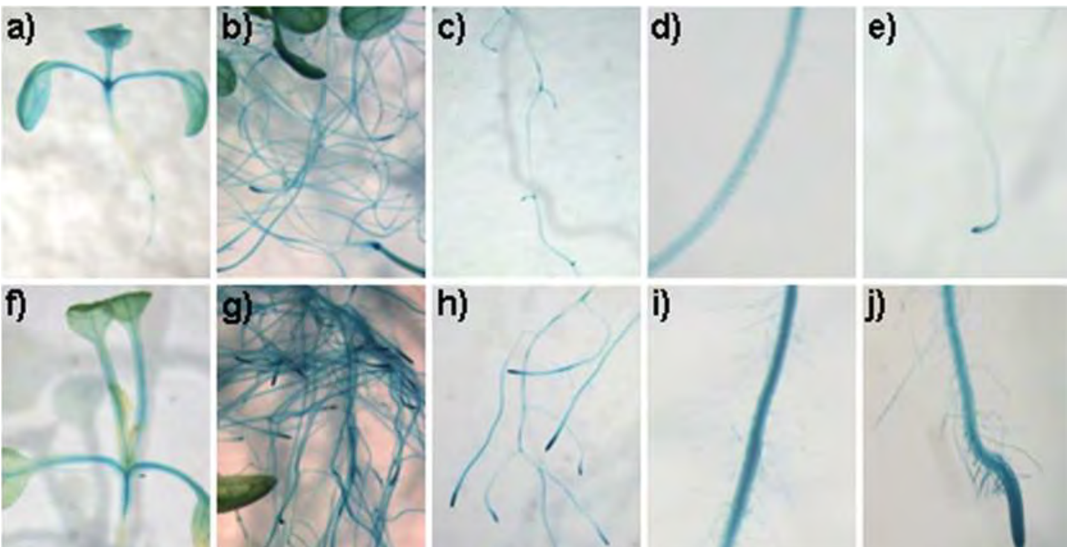

Figura 3. Efecto de *Bacillus megaterium* sobre la expresión del marcador de citocininas *ARR5:uidA*. Las plantas transgénicas de Arabidopsis que expresan *ARR5:uidA* se germinaron y crecieron durante 5 días en medio MS 0.2x e inoculadas posteriormente con *B. megaterium* a una distancia de 5 cm de las puntas de las raíces. El análisis de la expresión de *GUS* se realizó a los 6 días después de la inoculación. Se presentan fotografías representativas de la expresión de *GUS* en diferentes regiones de la planta. a-e) plantas no inoculadas, f-j) plantas inoculadas con *B. megaterium*. Notar el incremento de la expresión del marcador de citocininas en las raíces de las plantas inoculadas.

0), la inoculación bacteriana estimuló el crecimiento y el desarrollo. En particular, las plantas inoculadas desarrollaron su sistema radicular robusto con gran proliferación de raíces laterales (Fig. 3a-b). En triples mutantes *cre1-12/ahk2-2/ahk3*-3 no inoculadas, el crecimiento general de las plantas está afectado, dando lugar a plantas enanas con raíces pequeñas (Fig. 3c). En estas plantas la inoculación bacteriana a 2 o 5 cm falló en estimular el crecimiento y el desarrollo de la raíz (Fig. 3d-e).

Bacillus megaterium induce la expresión de un marcador de respuesta a citocininas

La respuesta alterada a *B. megaterium* observada en las mutantes de citocininas sugiere fuertemente que esta ruta de señalización podría participar mediando las respuestas del crecimiento y desarrollo de *Arabidopsis thaliana* durante la inoculación. Este resultado nos motivó a estudiar los efectos de la bacteria sobre la expresión de un marcador inducible por citocininas denominado *ARR5:GUS* en plantas transgénicas que expresan este marcador (De agostino et al., 2000). En las plantas no inoculadas, el marcador se expresa preferentemente en la zona meristemática del follaje y en las puntas de las raíces (Fig. 4a-e). La inoculación con *B. megaterium* incrementó claramente la expresión del marcador en las raíces, lo cual correlaciona con un crecimiento abundante de estas estructuras (Fig. 4f-j).

Discusión

Los efectos observados de la inoculación con *B. megaterium* en plantas silvestres de *Arabidopsis thaliana* sugieren que esta bacteria ejerce efectos benéficos sobre el crecimiento y desarrollo vegetal. El análisis de las respuestas de un grupo de mutantes afectadas en la señalización por citocininas muestra que los genes *AHK2* y *RPN12* juegan un papel preponderante

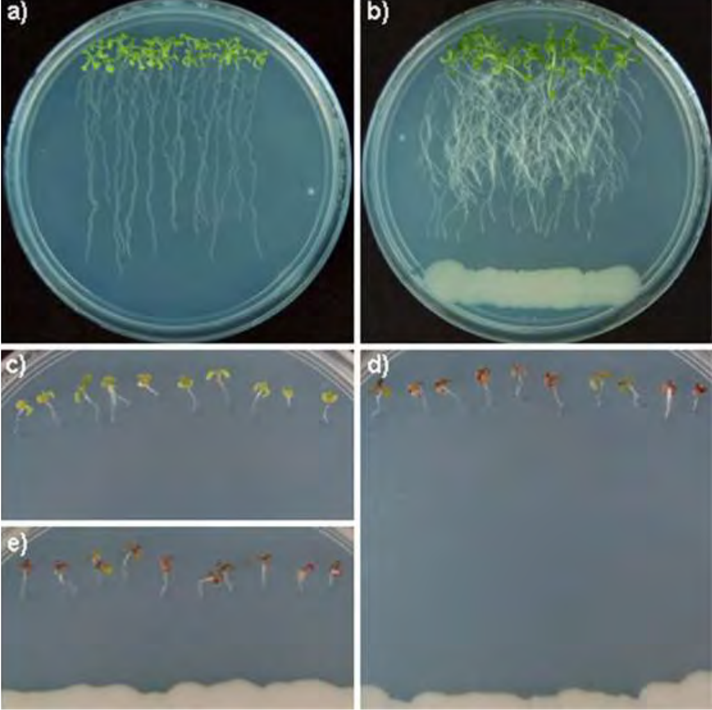

Figura 4. Efecto de *Bacillus megaterium* sobre el crecimiento y el desarrollo del ecotipo silvestre de Arabidopsis (Col-0) y su triple mutante en los receptores de citocininas *cre1-12/ahk2-2/ahk3-3*. (a) Plantas de Arabidopsis (Col-0) crecidas en la superficie de placas de agar suplementado con medio de Murashige y Skoog (MS). b) Plantas de Arabidopsis que fueron inoculadas con *B. megaterium* a una distancia de 5 cm de las puntas de las raíces a los 5 días después de la germinación y posteriormente crecidas durante un periodo de 6 días en presencia de la bacteria. c) Plantas de la triple mutante *cre1-12/ahk2-2/ahk3-3* no inoculadas, d) inoculadas a 5 cm o e) a 2 cm de la punta de la raíz primaria. Notar el efecto de la estimulación del crecimiento por inoculación bacteriana y la formación extensiva de raíces laterales en las plantas silvestres. Estos efectos estuvieron ausentes en la triple mutante de los receptores de citocininas. Las fotografías muestran unidades representativas de cuatro cajas por tratamiento.

en la estimulación del crecimiento y el desarrollo ejercida por *B. megaterium*, y que los tres receptores de citocininas (*CRE1*, *AHK2* y *AHK3*) son requeridos para que la planta efectúe una respuesta normal a la estimulación producida por la inoculación bacteriana.

No obstante que la red de señalización entre plantas y rizobacterias ha sido estudiada exhaustivamente durante los últimos 20 años, se han reportado hasta ahora muy pocos componentes moleculares involucrados en la interacción entre las bacterias y las plantas (Persello−Cartieaux et al., 2003). Nuestro trabajo aporta nuevos conocimientos mostrando que la estimulación del crecimiento vegetal por *B. megaterium* requiere de una vía de señalización de citocininas en *A. thaliana*. Esto es interesante, ya que reportes previos habían sugerido que diferentes PGPRs provenientes de la rizósfera pueden producir citocininas que ejercen un pronunciado efecto estimulador del crecimiento vegetal en cultivos de interés agrícola (Selvadurai et al., 1991; Arkhipova et al., 2005). Este efecto puede ser mediado por diferentes homólogos de receptores de citocininas.

El análisis de la expresión del marcador de respuesta a citocininas *ARR5:GUS* indica que *B. megaterium* activa mecanismos genéticos relacionados con la vía de señalización de las citocininas, esto podría ser explicado por una capacidad potencial de producción de citocininas por la bacteria. La difusión de los compuestos hacia la planta activaría directamente los receptores responsables de traducir la respuesta en patrones de crecimiento estimulando la producción de raíces laterales y pelos radiculares y en consecuencia aumentando la producción de biomasa foliar y radicular.

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Efecto del cobre en el crecimiento y la arquitectura de la raíz de *Arabidopsis thaliana* L.

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PALABRAS CLAVE

cobre;

raíz;

arquitectura

Resumen

Para estudiar los efectos del cobre en el crecimiento de *Arabidopsis thaliana*, se estableció un sistema *in vitro* utilizando placas de agar con medio MS 0.5X suplementadas con el metal. Las semillas que se utilizaron fueron de *A. thaliana* ecotipo Columbia y las líneas transformantes *DR5::uidA, CycB1;1::uidA* y *Exp7::uidA.* La inhibición total del crecimiento de la raíz primaria se presentó en las 3 líneas a una concentración de 90 µM y el crecimiento de raíces laterales se vio estimulado. La exposición de las raíces primarias a medios con cobre ocasionó una inhibición del crecimiento de manera proporcional al tiempo de exposición. En las condiciones experimentales utilizadas, la inhibición del crecimiento de la raíz primaria de Arabidopsis se correlacionó con la disminución de la actividad mitótica. Monitoreando la expresión del marcador *AtExp7:uidA* se encontró que la diferenciación celular ocurrió de manera más cercana al extremo de la raíz en condiciones de exposición al cobre. La expresión del marcador de respuesta a auxinas *DR5:uidA* mostró una posible participación de esta hormona en las respuestas de la raíz a este metal, sin embargo, se requieren estudios adicionales para tener conclusiones confiables.

Abstract

To study the effects of cupper on the growth of *Arabidopsis thaliana*, an *in vitro* system using agar plates with medium MS 0.5X supplemented with the metal was established. *A. thaliana* seeds ecotype Columbia were used and besides the transformed lines *DR5::uidA, CycB1;1::uidA* and *Exp7::uidA.* The total inhibition of the primary root growth in the three lines was at a concentration of 90 µM and the growth of the lateral roots was stimulated. The exposition of the primary roots to cupper media produced an inhibition of the growth proportionally to the time of exposition. In the experimental conditions used the inhibition of the root growth was correlated with the diminishing of the mitotic activity. Monitoring the expression of the *AtExp7:uidA* marker, the cellular differentiation occurred closer to the root tip when these were exposed to cupper. The expression of the *DR5:uidA* responding to auxines marker showed a possible participation of this hormone, however, additional studies are required to have trust conclusions.

KEYWORDS

cupper;

root;

architecture.

Introducción

No obstante la aparente simplicidad, las especies de plantas muestran una amplia variación en la forma arquitectónica del sistema de la raíz. Desde una perspectiva del desarrollo, la raíz de *Arabidopsis thaliana* presenta un ejemplo de simplicidad; un número pequeño de células madre generan todos los tipos celulares a través de divisiones estereotípicas seguidas de una diferenciación y expansión celular. La simetría radial de la raíz combinada con una carencia de movimiento de las células clonalmente relacionadas, son frecuentemente encontradas en todos los tipos de filas celulares. Estas células pueden ser localizadas hasta sus orígenes, identificándose cuatro células madre (o células iniciales) en el ápice de la raíz. La porción central de la capa de la raíz, la columnela, tiene su propio juego de células iniciales. El

cortex y endodermis, son generados por las divisiones iniciales endodermales del cortex. Finalmente, el tejido vascular y periciclo tiene sus propias células iniciales. Internamente y en contacto a todos los tipos de células iniciales se encuentra un número pequeño de células que son mitóticamente inactivas y son conocidas como centro quiescente (QC). (Dolan *et al.* 1993; Scheres *et al.* 1994).(Figura 1).

Se han identificado tres procesos principales que afectan la arquitectura de la raíz: a) La división celular de los meristemos formando nuevas células, b) La formación de raíces laterales aumentando la capacidad exploratoria y c) Formación de pelos radiculares incrementando el área de absorción. Se ha demostrado que entre los factores que afectan la arquitectura de la raíz de Arabidopsis se encuentra la baja disponibilidad de fósforo (López-Bucio *et al.* 2003, Sánchez-Calderón *et al.* 2005) y la presencia de metales como el cromo (Ortiz-Castro *et al.* 2007), el aluminio Elizarrarás (2005), el zinc y el plomo Vargas (2007).

El cobre es un micro nutriente que se encuentra presente en diversas enzimas o proteínas implicadas en los procesos de oxidación y reducción, por ejemplo, la citocromo oxidasa, una enzima respiratoria que se halla en las mitocondrias y la plastocianina, una proteína de los cloroplastos que cataliza la transferencia de electrones entre el citocromo b_6f y el fotosistema I (Raven *et al.* 1999). El receptor de etileno ETR1, una proteína transmembranal, requiere la unión de cobre para su funcionamiento (Rodríguez *et al.* 1999). Además, el cobre junto con el zinc es un cofactor de dos superóxido dismutasas (CDS1, CDS2) de las siete que se han encontrado en *Arabidopsis*; CDS1 es activa en el citosol mientras que CDS2 es activa en el estroma del cloroplasto (Bowler *et al.* 1994).

Las plantas rara vez tienen deficiencia de cobre, en parte porque lo requieren en cantidades muy pequeñas. En ausencia de cobre las hojas jóvenes con frecuenta adquieren un color verde oscuro y están arrugadas o deformes, y muchas veces exhiben manchones necróticos. (Salisbury y Ross 1991). En

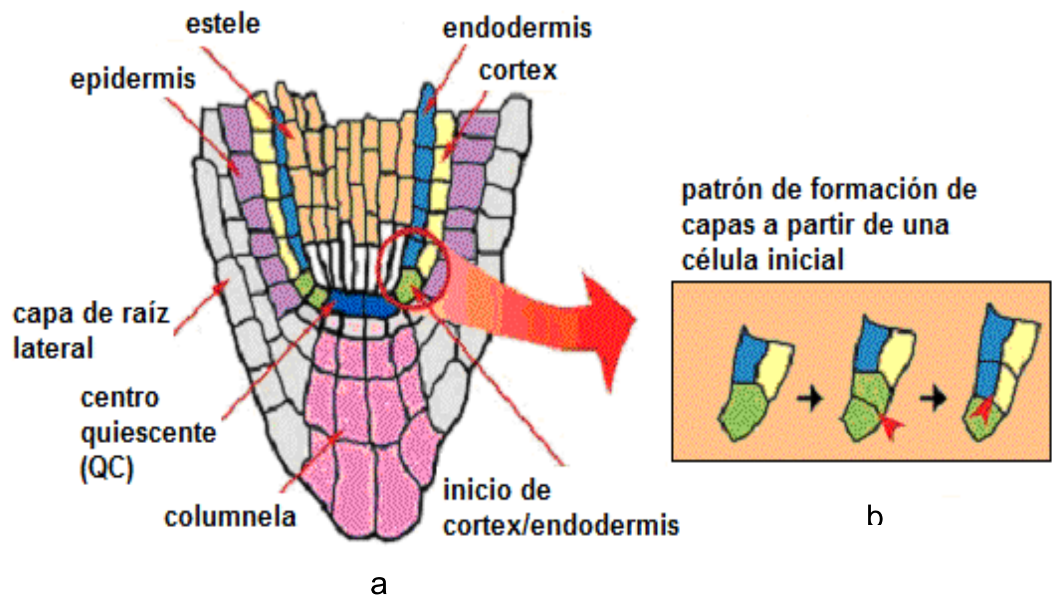

Figura 1. a) Sección longitudinal de la raíz de *A. thaliana,* la cual ha sido coloreada mostrando los diferentes tipos de células. b) Representación esquemática de los dos tipos de divisiones asimétricas que experimentan las células hijas del cortex-endodermis.

cantidades traza, varios de estos iones son requeridos para el metabolismo, crecimiento y desarrollo, pero los problemas se originan cuando las células son confrontadas con un exceso de estos iones vitales (Jonak *et al.* 2004). Para permitir la entrega suficiente del metal a las proteínas blanco, pero a la vez impedir un daño inducido por el ión metálico, los organismos han desarrollado sistemas compuestos de transportadores de membrana específicos y proteínas de unión solubles que juntos evitan la acumulación de iones metálicos libres en las células (Nelson 1999). La entrada de iones de cobre en las plantas depende de transportadores específicos, de los cuales se han identificado COPT1 y otros 4 homólogos en *A. thaliana* (Sancenon 2003).

En respuesta a niveles tóxicos de metales pesados las plantas sintetizan péptidos que unen estos metales, los cuales son ricos en cisteínas e incluyen las fitoquelatinas y las metalotioneínas; entre los metales que estos péptidos pueden quelar y secuestrar en la vacuola se encuentran el cobre y el cadmio, permitiendo de esta manera desintoxicar a las células. (Cobbett y Goldsbrough 2002). Además de las fitoquelatinas y metalotioneínas, el control interno de metales libres en las células depende de sistemas de transporte internos, los cuales juegan un papel importante en la homeostasis y tolerancia (Hall y Williams 2003). La entrega de cobre al lumen del tilacoide a la proteína plastocianina en el cloroplasto es necesaria para la fotosíntesis y se ha demostrado que en *A. thaliana* depende de proteínas transportadoras específicas, ubicadas una de ellas (PAA1) en la membrana interna del cloroplasto y otra (PAA2) en las membranas de los tilacoides (Abdel-Ghany *et al.* 2005). La entrega del cobre al estroma para el funcionamiento de la superóxido dismutasa CSD2 sólo requiere a la proteína transportadora PAA1. Otra proteína similar (RAN1) es requerida para la entrega del cobre a los receptores de etileno (Hirayama *et al.* 1999).

La presencia de niveles elevados de metales pesados dispara un rango amplio de respuestas celulares, incluyendo cambios en la expresión de genes. Jonak *et al.* (2004) reportan la activación de proteínas cinasas implicadas en la actividad mitótica, lo que sugiere que el exceso de estos metales induce diferentes mecanismos de señalización en raíces. No existe sin embargo una descripción detallada de los efectos del cobre en las raíces, lo cual se aborda en este trabajo utilizando la especie *A. thaliana.*

Materiales y métodos

Material biológico

Se utilizaron semillas de *A. thaliana* ecotipo Columbia. Del mismo ecotipo se utilizaron además las líneas transformantes *DR5::uidA, CycB1;1::uidA* y *AtExp7::uidA*, que contienen al gen reportero *uidA* (GUS) fusionado a los promotores de genes de auxinas, ciclinas y expansinas, respectivamente. (Ulmasov *et al.* 1997, Colón-Carmona 1999, Cho y Cosgrove 2002).

Condiciones de crecimiento de las plantas

La desinfección de semillas se llevó a cabo de acuerdo a lo reportado por Ortiz-Castro *et al.* (2007). Las semillas desinfectadas se sembraron en cajas de Petri con medio MS (Murashige y Skoog 1962) con 2% de sacarosa, con agar para plantas al 1%. Las condiciones de crecimiento fueron de acuerdo a la temperatura ambiental del laboratorio con el foto periodo normal del día. Las plantas de 6 días de edad se transfirieron a cajas de Petri con medio MS con diferentes concentraciones de cloruro de cobre.

Determinación de la concentración mínima inhibitoria del crecimiento de la raíz primaria

Se utilizaron plántulas de 6 días después de la germinación y se trasplantaron a los medios MS suplementados con cobre. Se marcó con un color la longitud inicial de la raíz primaria, cuidando que la raíz estuviera orientada en posición horizontal para distinguir los patrones de crecimiento. Las concentraciones iniciales que se utilizaron 0, 50, 65, 80 y 90 mM.

Cinética de inhibición del crecimiento de la raíz primaria

La concentración de cobre mínima inhibitoria

del crecimiento de la raíz primaria se utilizó para hacer una cinética de tiempo de exposición al metal. Plantas de 6 días crecidas en MS fueron colocadas por diferentes tiempos en los medios con cloruro de cobre y posteriormente fueron regresadas a medios MS sin el metal, para analizar el crecimiento de la raíz primaria.

Determinación de la actividad del gen reportero *uidA* (GUS)

Las plantas de 10 días de edad con actividad del gen reportero *uidA* (GUS), se tiñeron con X –Gluc (5- bromo- 4-cloro-3-indolil b-D-glucurónido) al 0.1% en buffer de fosfatos durante toda la noche a 37^0C de acuerdo a Jefferson *et al.* (1987). Las raíces fueron clarificadas, fijadas y montadas de acuerdo a lo reportado por Ortiz-Castro (2005).

Observación y mediciones de las raíces

Las muestras de raíces fueron observadas en un microscopio compuesto (Zeiss Axiostar Plus) y fotografiadas con cámara digital SONY DSC-S75. Las imágenes fueron analizadas y procesadas utilizando el software Zeiss AxioVision 4AC, con el cual se realizaron las mediciones de la zona de crecimiento, ancho de la raíz y las expresiones del gen reportero *uidA* en las líneas transformantes de Arabidopsis.

Resultados

Efecto del cobre en el sistema radicular de *A. thaliana*.

Para estudiar los efectos del cobre en el crecimiento de *A. thaliana* se estableció un sistema *in vitro* utilizando placas petri con medio MS 0.5X. En este sistema, plantas de *A. thaliana* fueron crecidas por seis días en medio MS 0.5X y posteriormente trasplantadas a medios MS 0.5X suplementados con cloruro de cobre y se analizó el crecimiento vegetal a los 72 horas después del trasplante (Figura 2).

Para analizar el efecto del cobre en el sistema radicular de *A. thaliana* se determinó la concentración mínima inhibitoria del crecimiento de la raíz primaria en tres líneas transformantes de Arabidopsis: *DR5::uidA*, *CycB1;1::uidA* y *AtEXP7::uidA*. Primeramente se probaron las concentraciones de 0, 50, 100, 150 y 200 µM, encontrándose que la concentración mínima inhibitoria fue de 100 µM (datos no mostrados). Posteriormente se utilizó el rango de concentraciones de 0, 50, 65, 80 y 90 µM. Para cada una de las líneas mencionadas se registró el crecimiento de la raíz primaria a las 24, 48 y 72 horas; los resultados para la línea *CycB1;1::uidA* se presentan en las figura 3, donde se observa que el crecimiento disminuyó con el aumento de la concentración de

Figura 2.- Sistema de crecimiento *in vitro* de *A. thaliana*. Plantas de transgénicas de *A. thaliana CyCB1::uidA* fueron crecidas en medios MS 0.5X por seis días y posteriormente fueron trasplantadas a medios MS 0.5X suplementados con cloruro de cobre. El crecimiento de las plantas fue registrado hasta las 72 hrs. Imágenes representativas de al menos dos repeticiones. La barra de escala representa 1 cm.

cobre observándose una inhibición total a 90 μM. El mismo comportamiento se observó para las líneas *DR5::uidA* y *AtEXP7::uidA* (datos no mostrados). No obstante que a 90 μM se inhibió el crecimiento de la raíz primaria, no hubo síntomas de toxicidad en otros aspectos, ya que el follaje continuó desarrollándose de la misma manera que las plantas no expuestas al cobre. A esta concentración se estimuló el desarrollo de raíces laterales modificando de esta manera la arquitectura de la raíz.

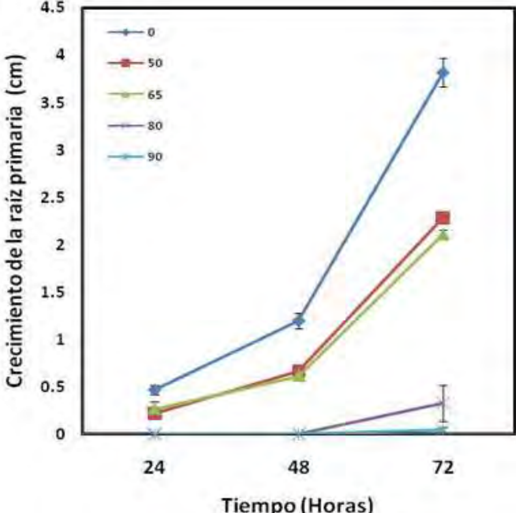

Figura 3. Inhibición del crecimiento de la raíz primaria por efecto del cobre en la línea *CycB1;1::uidA* de *A. thaliana*. Plantas de *CyCB1::uidA* fueron crecidas en medios MS 0.5X por seis días y posteriormente fueron trasplantadas a medios MS 0.5X suplementados con cloruro de cobre (0, 50, 65, 80 y 90 μM) por seis días. El crecimiento de la raíz primaria se registró a las 24, 48 y 72 hrs después del trasplante. Los datos mostrados representan el promedio ± intervalo de confianza.

Efecto del tiempo de exposición al cobre en el crecimiento de la raíz primaria

Una vez que se determinó que 90 μM de cobre fue la concentración mínima inhibitoria del crecimiento de la raíz primaria se realizó una cinética de tiempos para determinar cuanto tiempo de exposición al cobre (90 μM) es necesario para tener un efecto en el crecimiento de la raíz primaria de *A. thaliana*. Primeramente se hizo una cinética en tiempos de 0, 3, 6, 12, 24, 36, 48, 60 y 72 h de exposición al metal y se regresaron a medios MS para registrar el crecimiento de la raíz primaria; se determinó que a partir de las 3 horas de exposición al metal se inhibió por completo el crecimiento de la raíz primaria (datos no mostrados). Con base en lo anterior se utilizaron tiempos menores de exposición al metal. En una cinética con *CycB1;1::uidA* con tiempos de exposición al metal de 0, 0.5, 1 y 1.5 horas se registró el crecimiento en medio MS a 24, 48 y 72 horas (Figura 4). A 1.5 h de exposición al metal el crecimiento se reactivó hasta las 48 h en un 10% y a las 72 h en 30%. Cuando la exposición al cobre fue de 1 h el crecimiento se reactivó hasta las 72 h en un 15%. Se realizó también una cinética con la línea *DR5::uidA* con resultados similares a la línea *CycB1;1::uidA*.

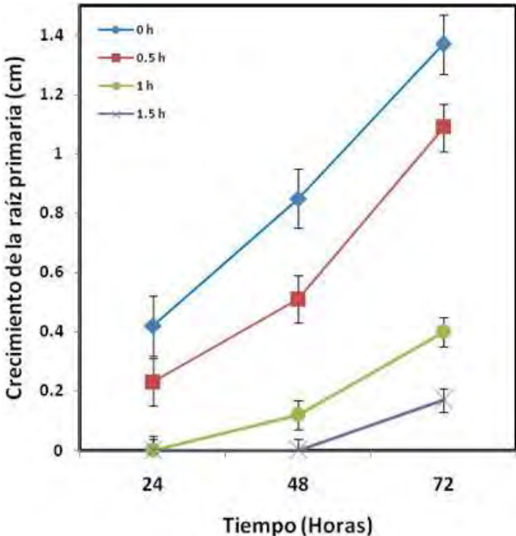

Figura 4. Cinética de inhibición del crecimiento de la raíz primaria por efecto del cobre en la línea *CycB1;1::uidA* de *Arabidopsis thaliana*. Se geminaron semillas de la línea transformante *CycB1;1::uidA* en medio MS sólido, se dejaron crecer durante 6 días a temperatura ambiente y se transfirieron a medios MS con 90 μM de cobre, en diferentes tiempos de exposición (0, 0.5, 1 y 1.5 h) para después regresarse a medios MS sin el metal. El crecimiento de la raíz primaria se registró a las 24, 48 y 72 horas. Los datos mostrados representan el promedio ± intervalo de confianza.

Efecto del cobre en la actividad mitótica de la raíz

En el crecimiento de las raíces existe una intensa actividad mitótica en los meristemos apicales, lo que da origen a los diferentes tipos celulares. Para determinar si la inhibición del crecimiento de la raíz primaria se debió a la pérdida de la actividad mitótica se utilizó la línea transformante *CycB1;1::uidA*. Después de 3 días de exposición a diferentes concentraciones de cloruro de cobre se determinó la actividad del gen reportero *uidA*. En la figura 5 se muestran resultados representativos. La actividad mitótica de la raíz primaria se mantuvo desde 0 hasta 65 µM de cobre, lo cual coincide con la presencia de crecimiento en la raíz primaria mientras que a 80 y 90 µM del metal se pierde esta actividad. En las raíces laterales la actividad mitótica es normal hasta 65 µM y disminuye en 80 y 90 µM, pero no se pierde por completo; esto se puede deber a que el crecimiento de las raíces laterales continúa aún en las concentraciones altas del metal, por razones no conocidas.

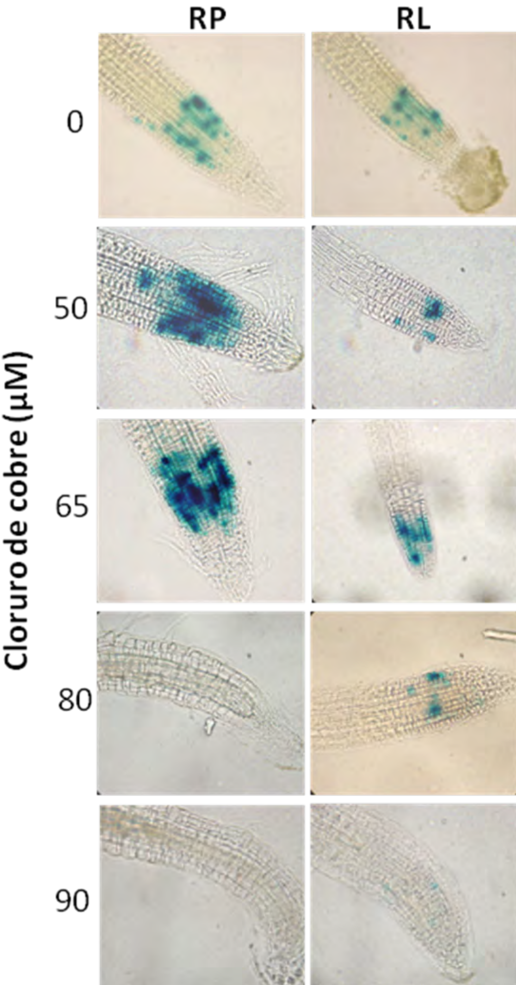

Figura 5. Efecto del cobre en la actividad mitótica de las raíces de *A. thaliana*. Se geminaron semillas de la línea transformante *CycB1;1::uidA* en medio MS sólido, se dejaron crecer durante 6 días a temperatura ambiente y se transfirieron a medios MS suplementados con diferentes concentraciones de cloruro de cobre (0, 50, 65, 80 y 90 µM). Las raíces fueron teñidas a las 72 horas de acuerdo a materiales y métodos, para detectar la actividad del gen reportero *uidA*. RP, raíces primarias; RL, raíces laterales.

Modificación del tamaño de la zona de crecimiento

Para determinar si la zona de crecimiento disminuyó debido a la exposición al cobre se utilizó la línea transformante *AtExp7:uidA*. Las plantas expuestas a diferentes concentraciones de cobre fueron teñidas para determinar la actividad del gen reportero *uidA* conducida por un promotor relacionado con la expansión celular. El análisis mostró un patrón de expresión cercano al ápice de la raíz en plantas tratadas con cobre (Figura 6). Con base en la expresión anterior las mediciones de la zona de crecimiento mostraron una reducción de 1000 µm a 200 µm en los tratamientos de 80 y 90 µM de cobre (Figura 7), mientras que las raíces laterales mantuvieron un tamaño de zona de crecimiento de alrededor de 350 µm.

Efecto del cobre en la distribución de respuesta a auxinas.

Las auxinas comprenden un grupo de sustancias naturales y sintéticas que participan en la regulación de múltiples aspectos del desarrollo de la raíz, incluyendo el crecimiento de la raíz primaria y la formación de raíces laterales y pelos radicales, por lo que se analizó su presencia en la raíz con la línea transformante *DR5::uidA*. Los tratamientos con cobre muestran que a 80 y 90 µM se pierde la respuesta a auxinas, mientras que en las raíces laterales no se alteran los patrones de distribución de éstas (Figura 8).

Discusión

Los resultados presentados en este trabajo revelaron diferentes efectos del cobre sobre el crecimiento y la morfogénesis de la raíz de *A. thaliana*. El crecimiento de la raíz primaria se inhibe gradualmente conforme se aumenta la concentración de este metal y en las condiciones experimentales utilizadas a 90 μM se inhibe por completo. Se ha reportado que el cobre sólo es limitante para la fotosíntesis en concentraciones menores de 0.1 μM, mientras que a concentraciones de 50 μM se presenta un efecto nocivo disminuyendo la cantidad de plastocianina y de clorofila, posiblemente por el daño ocasionado a las membranas de los tilacoides (Abdel-Ghany *et al.* 2005).

En este trabajo, no obstante la inhibición del crecimiento de la raíz primaria por efecto del cobre, las plantas continuaron su crecimiento debido a que desarrollaron abundantes raíces laterales, modificando la arquitectura de la raíz. Elizarrarás (2005) y Ortiz-Castro *et al.* (2007) demostraron que el aluminio y el dicromato, en concentraciones mínimas inhibitorias del crecimiento de la raíz primaria también estimulan el desarrollo de las raíces laterales, de manera similar a lo que ocurre con el cobre. Esta estrategia posiblemente le permite a la planta aumentar la superficie del sistema radical y sobrellevar la presencia de los metales mediante una mejor absorción de nutrientes.

La exposición al cobre en tiempos muy cortos determinó en cierto grado la inhibición del crecimiento de la raíz primaria (Figuras 2 y 3). Es posible que en una primera etapa el cobre se adsorba a la pared celular, ya que se ha reportado que los polisacáridos de ésta llevan cargas negativas, las cuales atraen a los iones positivos; en una segunda etapa estos iones pueden ser absorbidos y pasar a través de la membrana celular mediante transportadores específicos (Öpik y Rolfe 2005). Esto explica porque en tiempos cortos de exposición cobre (0.5 h) se encontró una inhibición del crecimiento (Figura 4). Uno de los transportadores implicados en la absorción a través de la raíz es COPT1, ya que estudios fisiológicos han demostrado que se expresa en este órgano, además de hacerlo en estomas y embriones en desarrollo (Sancenon *et al.* 2004).

En las condiciones experimentales utilizadas la inhibición del crecimiento de la raíz primaria de *Arabidopsis* se correlacionó con la disminución de la actividad mitótica (Figura 5). Este efecto fue acompañado por procesos de diferenciación que indujeron la expresión del marcador *AtExp7:uidA* en la región proximal al meristemo en plantas tratadas con altas concentraciones de cobre (Figura 6). Esto implica que las células se empiezan a expandir en una zona más cercana al ápice y pierden su capacidad

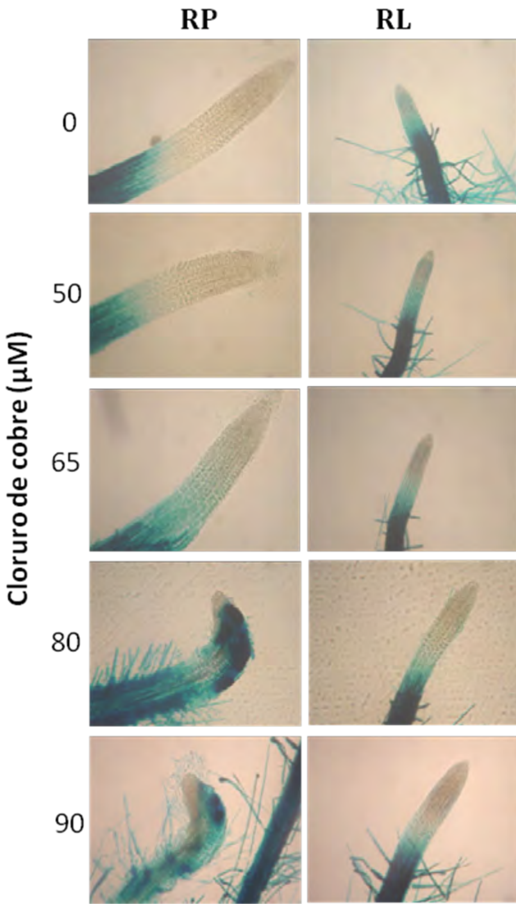

Figura 6. Efecto del cobre en la expansión de las raíces de *A. thaliana*. Se geminaron semillas de la línea transformante *AtEXP7::uidA* en medio MS sólido, se dejaron crecer durante 6 días a temperatura ambiente y se transfirieron a medios MS suplementados con diferentes concentraciones de cloruro de cobre (0, 50, 65, 80 y 90 μM). Las raíces fueron teñidas a las 72 horas de acuerdo a materiales y métodos, para detectar la actividad del gen reportero *uidA*. RP, raíces primarias; RL, raíces laterales.

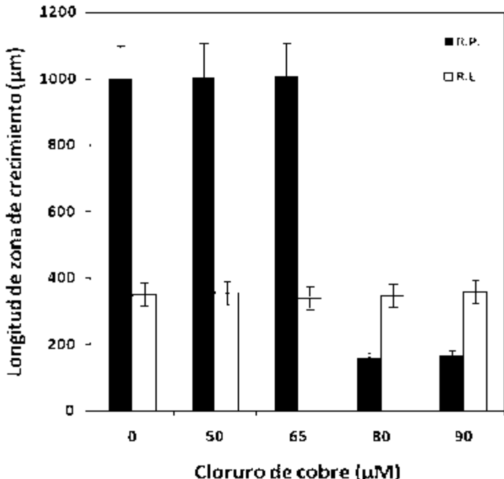

Figura 7. Efecto del cobre en el tamaño de la zona de crecimiento en las raíces de *A. thaliana*. Se geminaron semillas de la línea transformante *AtEXP7::uidA* en medio MS sólido, se dejaron crecer durante 6 días a temperatura ambiente y se transfirieron a medios MS suplementados con diferentes concentraciones de cloruro de cobre (0, 50, 65, 80 y 90 µM). Las raíces fueron teñidas a las 72 horas de acuerdo a materiales y métodos, para detectar la actividad del gen reportero *uidA*. RP, raíces primarias; RL, raíces laterales. Los datos mostrados representan el promedio ± intervalo de confianza.

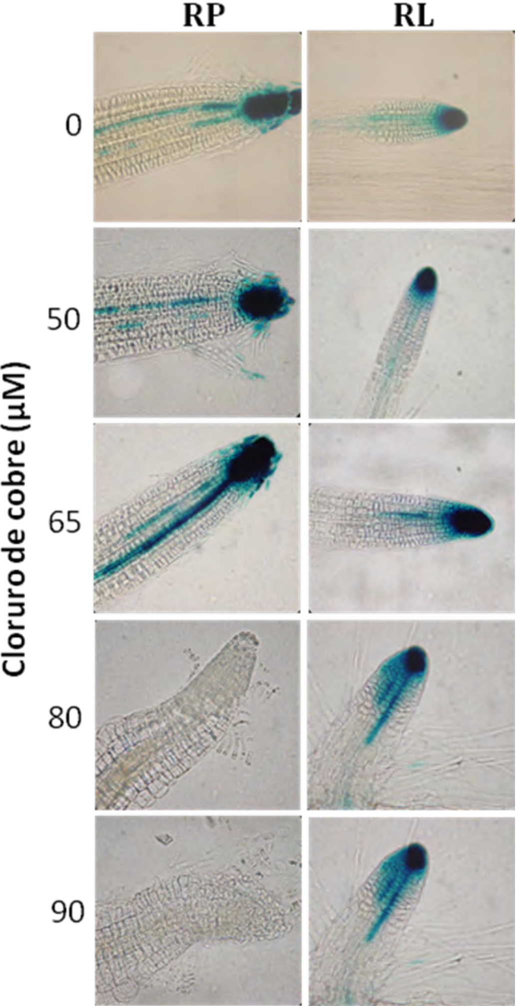

Figura 8. Efecto del cobre en la respuesta de distribución de auxinas de las raíces de *A. thaliana DR5::uidA*. Se geminaron semillas de la línea transformante *DR5::uidA* en medio MS sólido, se dejaron crecer durante 6 días a temperatura ambiente y se transfirieron a medios MS suplementados con diferentes concentraciones de cloruro de cobre (0, 50, 65, 80 y 90 µM). Las raíces fueron teñidas a las 72 horas de acuerdo a materiales y métodos, para detectar la actividad del gen reportero *uidA*. RP, raíces primarias; RL, raíces laterales.

mitótica. Alteraciones celulares similares han sido reportadas para plántulas de maíz sumergidas en una solución de medio de cultivo suplementado con 50 µM de aluminio (Doncheva *et al*. 2005) y para plantas sometidas a un estrés nutricional de fósforo, que también muestran una disminución en la actividad mitótica de la raíz primaria de *A. thaliana* (López-Bucio *et al.* 2003).

Se ha determinado que el desarrollo de pelos radicales, el crecimiento de la raíz primaria y la formación de raíces laterales, son particularmente sensibles a cambios internos y externos en la carencia de nutrimentos y pueden ser mediados por reguladores de crecimiento de la planta, como auxinas, citocininas y etileno (López-Bucio *et al.* 2003). En los diversos cambios de la arquitectura de la raíz generados por la exposición al cobre, los resultados del análisis de la expresión del marcador de respuesta a auxinas *DR5:uidA* sugieren una posible participación en la respuesta de la raíz a este metal, sin embargo, se requieren estudios adicionales para asegurar lo anterior.

En conjunto los resultados obtenidos permitieron concluir lo siguiente: a) El efecto del cobre en raíces

puede ser estudiado adecuadamente en un sistema *in vitro* utilizando *A. thaliana*. b) La concentración mínima inhibitoria del crecimiento de la raíz primaria por cloruro de cobre fue de 90 µM. c) El tiempo de exposición de las raíces de Arabidopsis al cobre inhibe el crecimiento de la raíz primaria de manera proporcional al tiempo de exposición. d) La inhibición del crecimiento de la raíz primaria se correlaciona con la pérdida de la actividad mitótica. e) La zona de crecimiento se hace más pequeña cuando la raíz se expone al metal, por la expansión de las células a una menor distancia del ápice. f) La participación de auxinas en la respuesta de la raíz al cobre requiere estudios adicionales.

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56 Ciencia Nicolaita

Comunicación planta-bacteria basada en ciclodipéptidos de origen microbiano con actividad auxínica

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Resumen

Los microorganismos y sus huéspedes eucariotas se comunican entre sí mediante diferentes señales químicas. Las auxinas, incluyendo el ácido-indol-3-acético (AIA), son hormonas importantes en muchos aspectos del desarrollo de las plantas. Los ciclodipéptidos y sus derivados las dicetopiperazinas (DCPs) constituyen una clase novedosa de moléculas pequeñas sintetizadas por microorganismos. En este trabajo presentamos evidencia de que en la bacteria *Pseudomonas aeruginosa* el sistema de percepción de quórum (quorum-sensing; QS) LasI controla la producción de tres DCPs, ciclo(L-Pro-L-Val), ciclo(L-Pro-L-Phe) y ciclo(L-Pro-L-Tyr), que participan en la estimulación del crecimiento vegetal. El análisis del efecto de los compuestos en plantas de *Arabidopsis thaliana*mostró cambios en la respuesta auxínica, afectando la arquitectura radicular y modificando la expresión de genes regulados por AIA en las plantas silvestres pero no en las mutantes afectadas en la señalización de auxinas *tir1, tir1afb2afb3, arf7, arf19* y *arf7arf19*. Los resultados obtenidos indican que la producción bacteriana de DCPs está regulada por el QS, que éstos compuestos modulan las vías de señalización de las auxinas y promueven el crecimiento vegetal,

permitiendo proponer una importante función de las DCPs en la comunicación planta-bacteria.

Palabras clave: auxinas, raíces laterales, pelos radiculares, fitoestimulación, dicetopiperazinas.

Abstract

Microorganisms and their eukaryotic hosts communicate through an array of chemical signals. Auxins, including indole-3-acetic acid (IAA) are hormones that participate in many developmental processes in plants. Cyclodipeptides and their derivate diketopiperazines (DCPs) belong to a novel class of small molecule signals, which are produced by bacteria. In this work, an *in vitro* system was established to study the interactions of bacteria with plants and their impact on plant growth. We present evidence that in *Pseudomonas aeruginosa*, the LasI quorum-sensing (QS) system controls the production of three DCPs, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Phe) y cyclo (L-Pro-L-Tyr), that promote biomass accumulation in *Arabidopsis thaliana*. Analysis of the effects of all three compounds in seedlings showed that they activate auxin responses, alter root architecture and auxin-regulated gene expression in WT seedlings but not in *tir1, tir1afb2afb3, arf7, arf19* y *arf7arf19* auxin-related mutants. Our observations that bacterial production of DCPs is negatively regulated by QS and that DCPs modulate auxin signaling to promote plant development establish an important function of DCPs in plant-bacteria communication.

Keywords: auxins, lateral roots, root hairs, phytostimulation, diketopiperazines.

Introducción

Un campo reciente y fundamental de la biología se relaciona con el estudio de los procesos que regulan la interacción entre las bacterias y los eucariontes. Este campo se ha visto influenciado con la observación inicial de que las bacterias pueden comunicarse a través de señales moleculares, en un proceso conocido como quorum-sensing (QS; Fuqua et al., 1996). Diferentes evidencias indican que estas señales o moléculas bacterianas pueden modular la transducción de señales en mamíferos (Telford et al., 1998) y plantas (Mathesius et al., 2003), y por otro lado, las hormonas del huésped pueden afectar la expresión de genes bacterianos (Sperandio et al., 2003; Gao et al., 2003). Un grupo muy importante de moléculas pequeñas descritas en procesos de QS bacteriano, denominadas autoinductores, entre las cuales se encuentran las *N*-acil-homoserinalactonas (AHLs), se sintetizan a partir de la S-adenosil metionina y de proteínas acarreadoras de ácidos grasos por las sintasas de AHLs (Pearson et al., 1994; Pearson et al., 1995; Fuqua et al., 1996). Las AHLs contienen un grupo homoserinalactona y una cadena de ácido graso de longitud variable que ayuda en la libre difusión a través de las membranas bacterianas, y ya en el citoplasma se unen a facto-

res transcripcionales para regular la transcripción de genes relacionados con el proceso de QS (Fuqua et al., 1996; Sperandio et al., 2003).

Las bacterias, tanto patógenas como simbiontes de las plantas, requieren del QS para comunicarse satisfactoriamente con sus huéspedes (Hussain et al., 2008; Rosemeyer et al., 1998). Las plantas por otra parte, han desarrollado múltiples mecanismos para percibir estas señales químicas. Por ejemplo, concentraciones micromolares de AHLs afectan sustancialmente la expresión de genes en *Medicago truncatula* y *Arabidopsis thaliana* impactando en el metabolismo primario, en las respuestas hormonales y en la arquitectura del sistema radicular (Mathesius et al., 2003; von Rad et al., 2008; Ortiz-Castro et al., 2008). Las bacterias que habitan la rizósfera pueden influir sobre el crecimiento vegetal mediante la producción de fitohormonas, tales como el ácido indol-3-acético (AIA), la principal auxina natural (Spaepen et al., 2007). La aplicación del AIA o metabolitos relacionados estimula la formación de raíces laterales y pelos radiculares, los cuales participan en la captación de agua y nutrientes y esto permite un incremento en la producción de biomasa (Spaepen et al., 2007). En consonancia con lo anterior, varias mutantes de *A. thaliana* afectadas en el transporte, percepción o señalización de auxinas, incluyendo *aux1*, *axr2*, *tir1* y *tir3/doc1/big*, manifiestan una reducción en la formación de raíces laterales y en el tamaño de la planta (Woodward et al., 2005).

La comunicación planta-bacteria puede ocurrir por medio de diferentes compuestos, algunos de los cuales mimetizan la actividad de fitohormonas endógenas. Los ciclodipéptidos y sus derivados las dicetopiperazinas (DCPs) constituyen una clase novedosa de moléculas pequeñas sintetizadas por microorganismos a las cuales se les ha encontrado diversas funciones biológicas. Por ejemplo, los compuestos ciclo (L-Phe-L-Pro) y ciclo(L-Phe-trans-4-OH-L-Pro) actúan como antifúngicos (Ström et al., 2002). Por otra parte, la epipolitiodioxopiperazina muestra propiedades antitumorales, antibacterianas, antivirales e inmunosupresoras (Kanoh et al., 1999; Williams et al., 1998). Estos compuestos se sintetizan por una familia de enzimas llamadas ciclodipéptidosintetasas (Gondry et al., 2009). Aunque las DCPs son moléculas bioactivas notables, existe poca información concerniente a su biosíntesis en bacterias y su papel en la comunicación con las plantas.

En este trabajo presentamos evidencia que en *P. aeruginosa*, el sistema de QS dependiente de la AHL-sintasa LasI, controla la producción de los DCPs ciclo(L-Pro-L-Val), ciclo(L-Pro-L-Phe) y ciclo(L-Pro-L-Tyr), los cuales están involucrados en la estimulación del crecimiento vegetal por esta bacteria, mediante la activación de una respuesta auxínica.

MATERIALES Y MÉTODOS

Material vegetal y condiciones de crecimiento

Se utilizaron plantas silvestres de *A. thaliana* (Col-0), las líneas transgénicas *DR5:uidA* (Ulmasov et al., 1997), *BA3:uidA* (Oono et al., 1998), *HS::AXR3NT-GUS* (Dhar-

masiri et al., 2005a), y las mutantes *tir1-1*, *tir1afb2afb3* (Dharmasiri et al., 2005b) y *arf7-1*, *arf19-1*, *arf7arf19* (Okushima et al., 2007). Las semillas se desinfectaron superficialmente con etanol al 95% (v/v) por 5 min e hipoclorito al 20% por 7 min. Después de cinco enjuagues con agua destilada esterilizada, las semillas fueron germinadas y crecidas en medio Murashige y Skoog (MS) 0.2x carente de aminoácidos y vitaminas (Murashige y Skoog basal salts mixture; Sigma). La formulación sugerida es de 4.3 g/L de sales para una concentración del medio de 1x. Se utilizó 0.9 g/L para referir a la solución MS 0.2x. El phytagar (grado micropropagación) fue adquirido de la casa comercial Phytotechnology. Las plantas fueron colocadas en una cámara de crecimiento (Percival Scientific AR-95L) con un fotoperiodo de 16 h de luz, 8 h de oscuridad, con una intensidad luminosa de 10 mol y una temperatura de 22 ^{o}C.

Ensayo *in vitro* de co-inoculación bacteriana de la raíz

Las cepas bacterianas utilizadas en este trabajo fueron *P. aeruginosa* PAO1 (cepa silvestre), *P. Aeruginosa lasI*$^{-}$, *rhlI*$^{-}$ y *rhlI*$^{-}$*/lasI*$^{-}$ mutantes sencillas y dobles, respectivamente (Li et al., 2007). Las cepas bacterianas fueron evaluadas *in vitro* en la estimulación del crecimiento vegetal, utilizando el ecotipo de *A. thaliana* Col-0. Se inocularon densidades bacterianas de 2.5x10^{8} UFC (unidades formadoras de colonias) mediante estriado sobre placas con medio MS 0.2x sólido. Plántulas de 6 días después de la germinación (8 plántulas por placa) fueron crecidas en el lado opuesto al sitio de inoculación bacteriana. La inoculación se realizó a una distancia de 5 cm de la punta de la raíz. Las plántulas fueron crecidas durante 8 días colocando las placas en una cámara de crecimiento bajo un diseño aleatorio. Todos los experimentos fueron repetidos al menos tres veces.

Tratamientos con hormonas vegetales y ciclodipéptidos

Para todos los experimentos, el medio MS fue suplementado con AIA (Sigma) o DCPs. Los compuestos fueron disueltos en etanol y se agregaron al medio a una temperatura de 50 ^{o}C y posteriormente vaciado en cajas de Petri. Las DCPs fueron purificadas del medio de cultivo Luria Bertani (LB) en el cual se creció la cepa mutante de *P. Aeruginosa lasI*$^{-}$.

Análisis del crecimiento y estadística

El crecimiento de las raíces primarias se registró utilizando una regla. El número de raíces laterales (RL) fue contado desde la punta de la raíz primaria hasta la transición de la raíz y el tallo. La densidad de raíces laterales (DRL) se determinó dividiendo el número de raíces laterales entre la longitud de la raíz primaria y expresada como DRL/cm. El peso fresco de las plantas se cuantificó pesando el material en una balanza analítica. Para todos los experimentos, los datos fueron analizados estadísticamente en el programa SPSS-10. Se realizaron análisis univariados y multivariados con una prueba de Tukey para estimar las diferencias de las respuestas.

Análisis histoquímico de la actividad de β-glucuronidasa (GUS)

Las plantas transgénicas que expresan el gen reportero *uidA* (Jefferson et al., 1987) fueron teñidas en 0.1% de X-Gluc (5-bromo-4-cloro-3-indolil, -D-glucurónido) en amortiguador de fosfatos (NaH_2PO_4 y Na_2HPO_4, 0.1 M, pH 7) suplementado con 2 mM de ferrocianuro de potasio, por 12 h a 37 °C. Las plantas se clarificaron y fijaron de acuerdo al protocolo de Malamy y Benfey (1997). Las raíces así procesadas fueron incluidas en un portaobjetos para su análisis microscópico. Se analizaron al menos 10 plantas transgénicas de cada línea marcadora y en cada tratamiento.

Análisis microscópico de plantas

El sistema radicular de *A. thaliana* se estudió con un microscopio estereoscópico (Leica MZ6). El número de raíces laterales totales se estimó bajo un aumento de 30X. Los meristemos de las raíces laterales se visualizaron en preparaciones semi-permanentes de raíces clarificadas usando un microscopio compuesto (AxiostarZeiss Plus) a un aumento de 100X o 400X. Las imágenes se capturaron con una cámara digital Sony Cyber-shot DSC-S75 adaptada al microscopio y procesadas con el programa Zeiss Axio Vision 4AC (Carl Zeiss).

Extracción, purificación y caracterización de los ciclo-dipéptidos de *P. aeruginosa*

Se inoculó 1.5 L de medio de cultivo Luria Bertani con un cultivo crecido de *P. aeruginosa* PAO1 y de la mutante *lasI* ($2.5x10^8$ UFC) en una cámara de crecimiento con agitación (125 rpm) por 24 h a 37°C. El cultivo bacteriano se centrifugó a 10,000x g por 10 min a 25°C en una centrífuga Eppendorf 5810R. El cultivo bacteriano libre de células se extrajo con dos volúmenes de acetato de etilo acidificado con ácido acético (0.1 mL/L). Los extractos se evaporaron utilizando un rotavapor a 60°C. El residuo fue concentrado con metanol:acetonitrilo (1:1) y disuelto en 1 mL de acetonitrilo grado HPLC.

Para la purificación de las DCPs, 1 mL del extracto obtenido con acetato de etilo fue separado en un sistema de HPLC utilizando una columna semi-preparativa C_{18} (Econosil C_{18}, Alltech). En el proceso de separación se utilizó el sistema de solventes agua:acetonitrilo, comenzando el programa de corrimiento con un paso de calibración 100:0, seguido por un gradiente linear 60:40, con un flujo de 8 mL min^{-1} por 25 min; un gradiente linear de 40:60, con un flujo de 8 mL min^{-1} por 3 min; un gradiente linear de 100:0 con un flujo de 8 mL min^{-1}por 3 min y terminando con una calibración de 12 minutos con un flujo de 8 mL min^{-1}. Los diferentes picos identificados se colectaron y las fracciones fueron concentradas por liofilización. Las fracciones colectadas y purificadas fueron posteriormente usadas para la evaluación de su actividad biológica y análisis estructural.

Las fracciones se analizaron mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS) en un equipo de GC Agilent 6850 Series II con un detector de MS Agilent modelo 5973, y una columna capilar HP-5 MS. Las condiciones de operación fueron:

helio como gas acarreador, 1 mL/min; una temperatura de detección a 300 °C y una temperatura de inyección de 250 °C. El volumen de la muestra inyectada fue de 1 µL. Se mantuvo la columna por 3 min a 80 °C y se programó una rampa a 6°C/min a una temperatura final de 230 °C sostenida durante 5 min. Se utilizó un análisis SCAN para verificar la presencia de los ciclodipéptidos. La espectrometría de resonancia magnética nuclear de hidrógeno (^{1}H-NMR) y de carbono (^{13}C-NMR) se realizó con el magneto de ^{1}H-NMR (Varian 400 MHz) y magneto de ^{13}C-NMR (Varian 100 MHz), respectivamente (Ortiz-Castro et al., 2011).Las muestras a analizar (1 mg) fueron disueltas en $CDCL_3$. Las respectivas estructuras fueron determinadas mediante un análisis estructural comparativo con bases de datos y compuestos comerciales de estructura conocida.

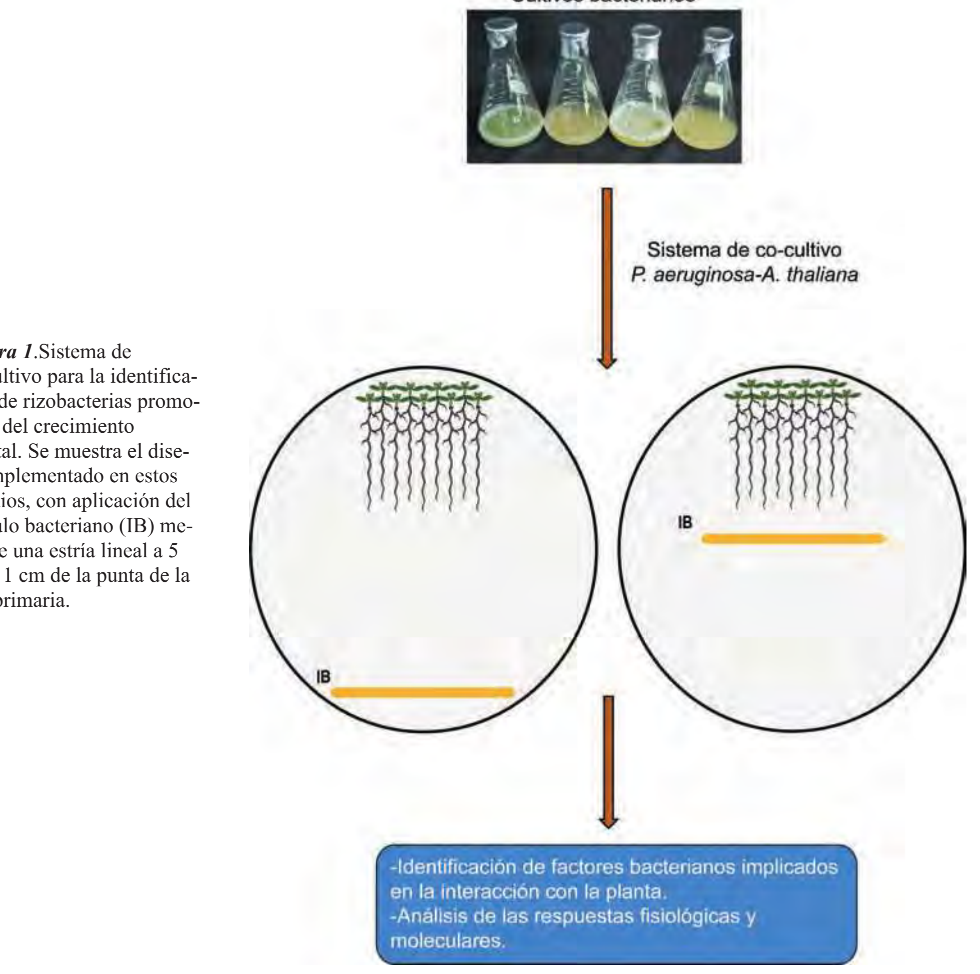


Figura 1.Sistema de co-cultivo para la identificación de rizobacterias promotoras del crecimiento vegetal. Se muestra el diseño implementado en estos estudios, con aplicación del inóculo bacteriano (IB) mediante una estría lineal a 5 cm o 1 cm de la punta de la raíz primaria.

RESULTADOS Y DISCUSIÓN

Estimulación del crecimiento vegetal por compuestos producidos por *Pseudomonas aeruginosa*

En *P. aeruginosa* se han descrito dos sistemas de QS dependientes de AHLs, los sistemas *las* y *rhl*. En el sistema *las*, la sintasa de AHLs LasI dirige la síntesis del compuesto 3-oxo-C12-AHL, el cual interacciona con el factor transcripcional LasR para unirse a los promotores de los genes blanco. En el sistema *rhl*, la sintasa RhlI dirige la síntesis de C4-AHL, la

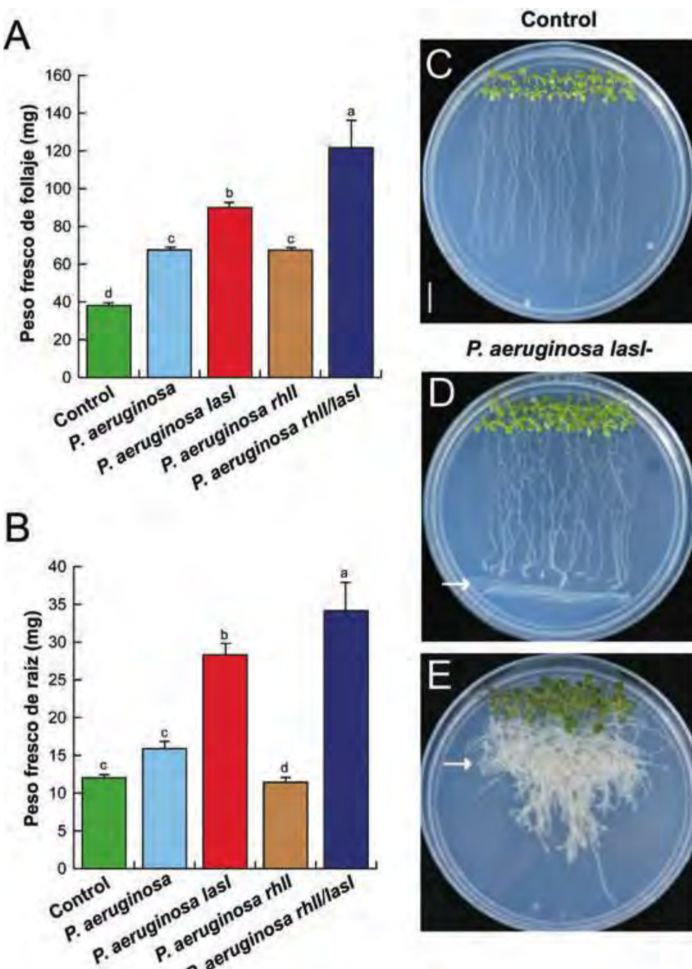


Figura 2. Efecto de *P. aeruginosa* PAO1 y cepas mutantes afectadas en la producción de AHLs sobre el desarrollo de la raíz y la estimulación del crecimiento vegetal. Plántulas de *A. thaliana* de 6 días de edad se inocularon con *P. aeruginosa* PAO1 o mutantes afectadas en las sintasas de AHLs *LasΓ*, *RhlΓ*, o *RhlΓ/LasΓ* a una distancia de 5 cm de la punta de la raíz primaria y crecidas por un periodo adicional de 8 días.(A) Efecto de la co-inoculación bacteriana sobre la producción de biomasa de follaje o (B) la biomasa de raíz. (C-E) Fotografías representativas del crecimiento en condiciones axénicas. (C) Co-inoculadas con la mutante de *P. Aeruginosa lasΓ* a una distancia de la raíz primaria de 5 cm (D) o 1 cm (E) (Escala de la barra = 1 cm). La flecha indica el sitio donde se realizó la inoculación bacteriana. Los datos representan la media D.E. (n = 30). Las diferentes letras indican diferencia estadística con una P<0.05.

cual interacciona con el regulador RhlR para controlar la transcripción de sus respectivos genes blanco (Pearson et al., 1994; Pearson et al., 1995; Pesci et al., 1997).

Inicialmente se evaluó el efecto de *P. aeruginosa* sobre el crecimiento vegetal utilizando un sistema de co-cultivo *in vitro* evaluando la co-inoculación bacteriana con plantas de *A. thaliana* mediante un estriado bacteriano a una distancia larga (5 cm) o corta (1 cm; Fig. 1). Para analizar el efecto de la bacteria en el crecimiento vegetal, plantas de *A. thaliana* de 4 días de edad crecidas en medio MS 0.2x se inocularon con 2.8×10^8 UFC de *P. aeruginosa* (PAO1) y mutantes de *P. aeruginosa* afectadas en las sintasas de AHLs (*lasI*$^-$*, rhlI*$^-$) y la doble mutante *rhlI*$^-$*/lasI*$^-$ mediante un estriado de cada bacteria (IB, inóculo bacteriano) sobre la su-

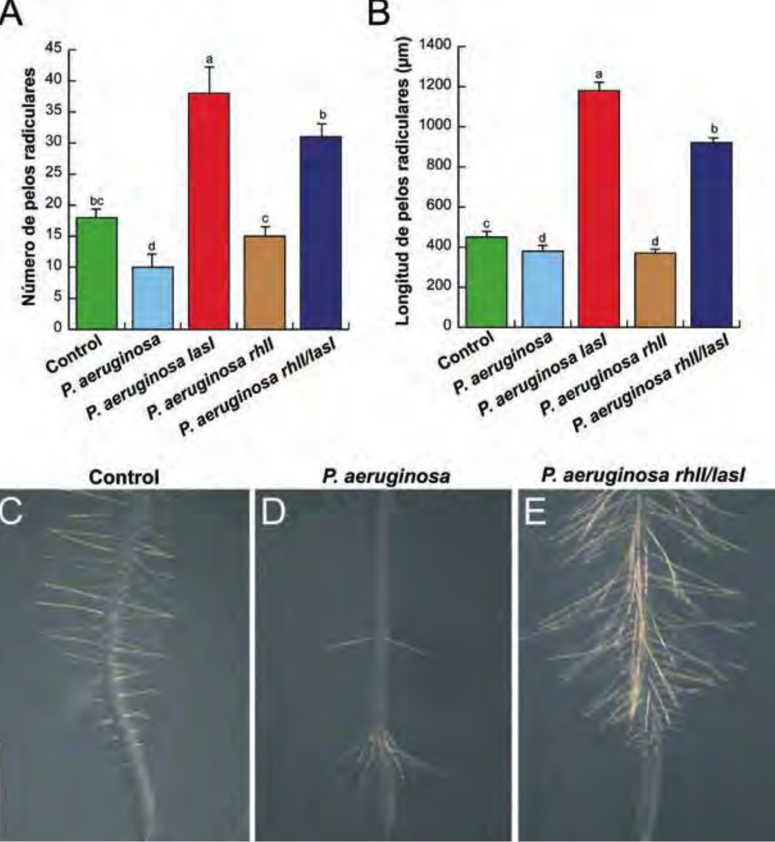


Figura 3. Efecto de *P. aeruginosa* PAO1 y mutantes afectadas en la producción de AHLs sobre el desarrollo de los pelos radiculares. Plántulas de *A. thaliana* de 4 días de edad se inocularon con *P. aeruginosa* PAO1 o mutantes afectadas en las sintasas de AHLs *LasI*$^-$, *RhlI*$^-$, o *RhlI*$^-$*/LasI*$^-$ a una distancia de 5 cm de la punta de la raíz primaria y se crecieron por un periodo adicional de 8 días. (A) Efecto de la co-inoculación bacteriana en el número de pelos radiculares ó (B) longitud de los pelos radiculares. Datos de A y B muestran la media D.E. de 30 plántulas. Diferentes letras indican diferencia estadística a $P<0.05$. (C-E) Fotografías representativas de plántulas de *A. thaliana*crecidas axénicamente(C), o inoculadas con *P. aeruginosa* PAO1 (D) o la doble mutante de *P. Aeruginosa rhlI*$^-$*/lasI*$^-$(E). (Barra de escala = 500 µm).

perficie del medio a una distancia de la punta de la raíz de 5 cm o 1 cm. Después de 8 días de crecimiento en presencia de la bacteria, se observó un incremento en la producción de biomasa en follaje y raíz (Fig. 2A y 2B), el cual se correlacionó con una arquitectura del sistema radicular alterada (Fig. 2C-E). Interesantemente, la mutante *lasI⁻* mostró el mayor efecto estimulador en la producción de biomasa de follaje y raíz aparentemente asociado con una inducción prolífica de raíces laterales (Fig. 2E) y pelos radiculares (Fig. 3A-E). Estos resultados sugieren que las AHLs producidas por la sintasa LasI modulan la producción de algún(os) compuesto(s) directamente involucrado(s) en la regulación del desarrollo vegetal, así como en procesos de división y diferenciación celular en la raíz de *A. thaliana*.

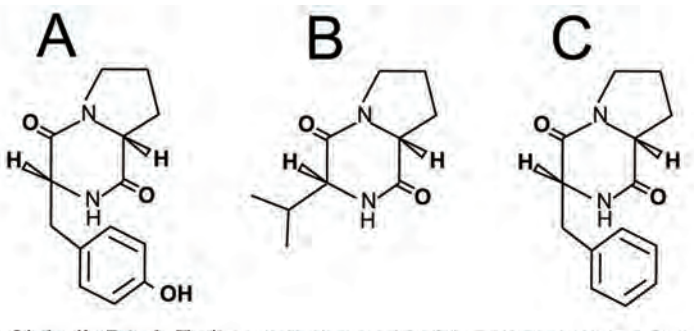
Las DCPs producidas por *P. aeruginosa* estimulan el desarrollo de raíces laterales en *A. thaliana*

Diversas especies bacterianas producen compuestos auxínicos como el AIA (Spaepen et al., 2007). Para determinar si esta(s) u otra(s) sustancia(s) podrían encontrarse en el sobrenadante de cultivos de *P. aeruginosa,* se realizó la extracción de los compuestos activos y se evaluó su efecto sobre la formación de raíces laterales mediante el conteo de primordios. Después de someter los extractos totales a separación por HPLC y colección de fracciones, se identificaron tres fracciones activas (P6, P7 y P8). La caracterización molecular de los extractos purificados P6, P7 y P8 se realizó mediante espectroscopía de gases acoplada a espectrometría de masas (GC/MS) y confirmada por análisis de los espectros de ^{1}H NMR y ^{13}C NMR. En estos extractos se evidenció la presencia mayoritaria (> 95%) de tres DCPs, ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) (Fig. 4A-C). Las DCPs encontradas se acumularon en los extractos de la mutante de *P. Aeruginosa lasI⁻* y en la doble mutante *rhlI⁻/lasI⁻* (Fig. 4D). Estos hallazgos muestran que en las mutantes de *P. Aeruginosa lasI⁻* y *rhlI⁻/lasI⁻*, se incrementan las concentraciones de las DCPs, lo cual sugiere que la biosíntesis de estos compuestos está regulada por el sistema de QS dependiente de LasI. Aunque un efecto biológico directo sobre *A.thaliana* es la modulación del desarrollo, la función que puedan estar jugando las DCPs en los mecanismos de comunicación molecular de *P. aeruginosa* con otras bacterias permanece sin dilucidar.

Las DCPs bacterianas modulan respuestas auxínicas en *A. thaliana*

El crecimiento de las raíces laterales y la formación de pelos radiculares están altamente regulados por auxinas (Woodward et al., 2005). El sistema heterocíclico peculiar de las DCPs está presente en el AIA y otros compuestos con actividad auxínica (Calderón-Villalobos et al., 2010). El hallazgo de que la inoculación de plantas de *A. thaliana* con las mutantes *lasI⁻ y rhlI⁻/lasI⁻* causa en las plantas un incremento en la formación de raíces laterales y pelos radiculares nos condujo a evaluar si las DCPs actúan mimetizando señales tipo auxina. Para esto, se utilizaron plantas transgénicas que expresan el marcador inducible por auxinas *DR5:uidA*(Ulmasov et al., 1997), mismas que fueron co-inoculadas con *P. aeruginosa* (PAO1) o la mutante *lasI⁻*. En las plantas crecidas en condiciones axénicas, el marcador

DR5:uidA se expresó principalmente en la punta de la raíz (Fig. 5). Por un lado, las plantas suplementadas con 3 µM de AIA mostraron una fuerte actividad de GUS a lo largo de toda la raíz primaria (Fig. 5B), indicando la inducción de la respuesta auxínica, mientras que el patrón de expresión de GUS en plantas co-inoculadas con *P. aeruginosa* (PAO1) fue similar al observado en condiciones axénicas (Fig. 5C). En contraste, en plantas co-inoculadas con la mutante de *P. Aeruginosa lasI⁻*, se observó un claro incremento en la expresión de este marcador en toda la raíz primaria (Fig. 5D), indicando que el sistema de QS LasI regula la biosíntesis de compuestos con actividad auxínica.



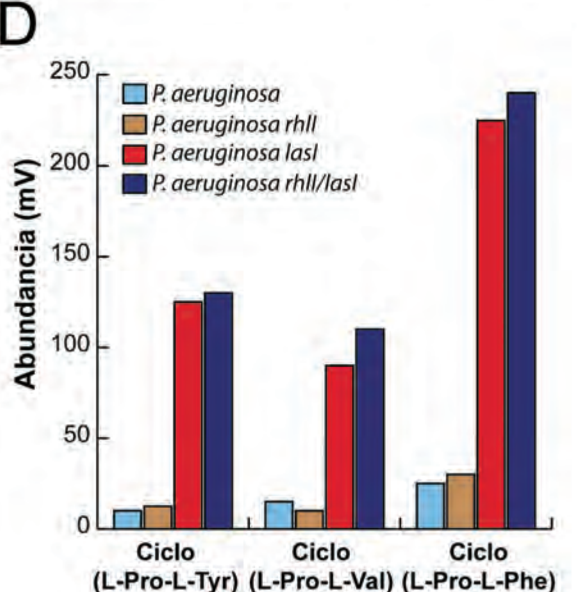


Figura 4. Identificación y caracterización de DCPs producidas por *P. aeruginosa*. *P. aeruginosa* PAO1 y mutantes afectadas en las sintasas de AHLs *LasI⁻*, *RhlI⁻*, o *RhlI⁻/LasI⁻* fueron crecidas en medio LB por 24 h y posteriormente el sobrenadante del cultivo se extrajo con acetato de etilo. Los extractos se fraccionaron y purificaron mediante HPLC. Se caracterizaron tres fracciones activas mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS), identificándose tres DCPs, **(A)** ciclo (L-Pro-L-Val; m/z = 196), **(B)** ciclo (L-Pro-L-Phe; m/z = 244), y **(C)** ciclo (L-Pro-L-Tyr; m/z = 260). **(D)** Abundancia relativa de las DCPs determinada por GC/MS. Los datos representan la media de la abundancia relativa (x10^7 mV).

Posteriormente, se evaluó la actividad de las tres DCPs sobre la expresión del marcador *DR5:uidA* en raíces mediante la transferencia de plantas de 6 días de edad a medios líquidos suplementados con AIA o DCPs, respectivamente. En la figura 5E se muestra la tinción histoquímica de plantas transgénicas *DR5:uidA* tratadas con AIA o las diferentes DCPs. Estos ensayos permitieron observar un aumento en la expresión de GUS en plantas tratadas con las DCPs, indicando la inducción de la respuesta auxínica, aunque en menor grado comparada con plantas tratadas con AIA (Fig. 5E).

Las auxinas actúan mediante su interacción con la proteína TIR1 (*transport inhibitor response1,* por sus siglas en inglés), un miembro de una familia pequeña de proteínas F-box

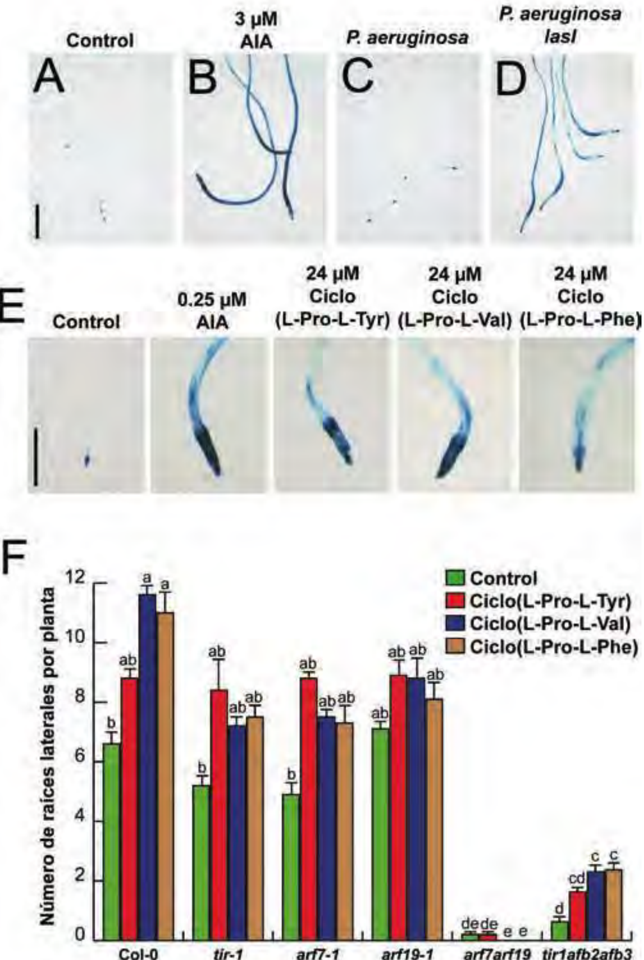


Figura 5. Efecto de las DCPs bacterianas en la respuesta auxínica en *A. thaliana*.(A-B) Expresión del marcador inducible por auxinas *DR5:uidA* en plantas transgénicas control **(A)**, con 3 μM AIA **(B)**, co-inoculadas con *P. aeruginosa* PAO1 **(C)** o con la mutante de *P. Aeruginosa lasΓ* **(D)**. **(E)** Efecto del AIA o DCPs purificadas sobre la expresión de *DR5:uidA*. Las fotografías muestran plantas representativas (Barra de escala = 500 μm). **(F)** Número de raíces laterales en plantas silvestres de *A. thaliana* y líneas mutantes *tir1-1*, *arf7-1,arf19-1*, *arf17arf19* y *tir-1afb2afb3*germinadas y crecidas en medio MS 0.2x solidificado por 6 días y posteriormente transferidas a medios líquidos MS 0.2x suplementados con 30 μM de cada DCP y tratadas por dos días. Los datos muestran la media D.E. Las diferentes letras indican diferencia estadística a $P< 0.05$.

(Dharmasiri et al., 2005b; Kepinski et al., 2005). En esta interacción se acelera la degradación catalizada por la ubiquitina ligasa de las proteínas represoras Aux/AIA, permitiendo la activación de genes regulados por factores de respuesta a auxinas (ARFs; *auxin response factors*) (Gray et al., 2001). Para determinar si los receptores de auxinas de la familia TIR1 y ARFs están involucrados en las respuestas de *A. thaliana* a las DCPs, se cuantificó la formación de raíces laterales en respuesta a ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) en plantas silvestres y en las mutantes *tir1-1* y *tir1afb2afb3*, *arf7-1, arf19-1* y *arf7arf19*. En plantas silvestres tratadas con el compuesto ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) se incrementó el número de raíces laterales por planta (Fig. 5F). La mutante *tir1-1* mostró una reducción de un 25% en el número de raíces laterales, con respecto al observado en plantas silvestres. Interesantemente, el incremento en la formación de raíces laterales observado en plantas silvestres tratadas con DCPs disminuyó en la mutante *tir1-1* (Fig. 5F). En la mutante *tir1afb2afb3*, la formación de raíces laterales no fue estimulada por el tratamiento con las DCPs (Fig. 5F). La mutante sencilla *arf7-1* muestra reducción en el número de raíces laterales comparado con las plantas silvestres; además, la estimulación de la formación de raíces laterales por las DCPs disminuyó en las mutantes *arf7-1* y *arf19-1* (Fig. 5F). Estos resultados sugieren que las DCPs muestran actividad auxínica y requieren para su efecto de una ruta de señalización intacta de auxinas.

Conclusiones

En este trabajo, se exploró genéticamente el papel del QS en *P. aeruginosa* en su interacción con la planta modelo *A. thaliana* mediante un sistema de co-cultivo *in vitro* (Fig. 1). Encontramos que la producción de biomasa en follaje y raíz se incrementó en plantas co-inoculadas con *P. aeruginosa*. Es importante resaltar la alta capacidad de estimulación de la mutante $lasI^{-}$ a una corta distancia del sistema radicular (Fig. 2). Estos resultados complementan reportes previos en los que se había mostrado que *P. aeruginosa* causa efectos dañinos en *A. thaliana* (Rahme et al., 1995; Plotnikova et al., 2000; Walker et al., 2004). Rahme et al., (2005) evaluó una colección de 75 cepas de *P. aeruginosa* potencialmente dañinas en hojas de cuatro ecotipos de *A. thaliana*, analizando su patogenicidad mediante infiltraciones de bacterias dentro del tejido vegetal. Estos autores encontraron que solamente dos cepas UCBPP-PA14, un aislado de humanos, y UCBPP-PA29, un aislado de plantas, causaron síntomas de ablandamiento de tejido, posiblemente por la secreción de enzimas digestivas y factores de virulencia, características que en *P. aeruginosa* se conocen a detalle y están regulados por los sistemas de QS. En contraste, información de otros grupos ha resaltado el potencial de algunas cepas ambientales de *P. aeruginosa* como un bio-inoculante (Preston, 2004), lo cual, con base en nuestros hallazgos, permite proponer que el comportamiento de *P. aeruginosa* como un patógeno o como una bacteria promotora del crecimiento vegetal dependerá del geno- y fenotipo de esta especie.

Por otro lado, en los experimentos realizados no se detectó AIA en los extractos bacterianos. Sin embargo, se encontraron tres DCPs producidas mayoritariamente en cultivos

de *P. aeruginosa*, cuya biosíntesis está regulada negativamente por el sistema de QS LasI. Cada compuesto se purificó y su estructura fue confirmada por GC-MS y NMR, llegando a la identificación de los compuestos ciclo(L-Pro-L-Tyr), ciclo(L-Pro-L-Val) y ciclo(L-Pro-L-Phe) (Ortíz-Castro et al., 2011). Los efectos del AIA y las DCPs sobre la expresión de genes regulados por auxinas en *A. thaliana* sugiere que las tres DCPs muestran una actividad auxínica débil comparada con el AIA (Fig. 5). Los hallazgos de que las DCPs no estimulan la formación de raíces laterales en la triple mutante de los receptores de auxinas *tir1afb2afb3* y la doble mutante en los factores de transcripción *arf7arf19* que median la señalización cascada abajo al receptor TIR1 de *A. thaliana* (Fig. 5), sugiere que estos compuestos requieren para su efecto de una ruta de señalización auxínica. Lo anterior establece una importante función de los DCPs de origen microbiano en la comunicación planta-bacteria. La manipulación de la señalización del QS dependiente de AHLs y la biosíntesis de DCPs puede ser una estrategia prometedora para el desarrollo de inoculantes bacterianos que incrementen la producción de cultivos por medio de una modulación de la señalización de auxinas y la regulación de la arquitectura radicular.

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Bases moleculares de la señalización del ácido abscísico y el etileno en plantas

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Resumen

Los reguladores del crecimiento son moléculas fundamentales en la modulación de diversos procesos del desarrollo de las plantas, incluyendo la germinación, la senescencia, la arquitectura de la raíz y del follaje y las respuestas a factores bióticos y abióticos. Evidencia reciente indica que el ácido abscísico (ABA) y el etileno interactúan antagónicamente en varios de estos procesos mediante la participación de proteínas involucradas tanto en la biosíntesis como en la señalización de cada regulador. En tal interacción, juegan un papel relevante algunas enzimas como la ACC sintasa y ACC oxidasa que participan en la biosíntesis de etileno, así como las proteínas ETR1 y EIN2 de su ruta de señalización. Por otra parte, las proteínas NCED y ABA2 son importantes en las respuestas celulares al ácido abscísico. Los mecanismos moleculares implicados en las interacciones de ambas hormonas se desconocen, no obstante, la caracterización a nivel genético o mediante técnicas de química analítica, farmacológicas y moleculares ha empezado a aportar información valiosa para entender su funcionalidad en los diferentes tejidos. En este trabajo se discuten los avances recientes en las vías de señalización del ABA y del etileno, con énfasis especial en la interacción entre ambas fitohormonas y su efecto sobre los procesos de organogénesis.

Palabras clave: Ácido abscísico, etileno, desarrollo vegetal, *Arabidopsis thaliana*.

Abstract

Plant growth regulating substances are fundamental in modulating diverse developmental processes in plants, including germination, senescence, root and shoot architecture and responses to biotic and abiotic factors. Recent evidence indicates that abscisic acid (ABA) and ethylene interact antagonistically in various of these processes through the participation of proteins involved in the biosynthesis and/or response to each regulator. In such interaction, the enzymes ACC synthase and ACC oxidase that participate in ethylene biosynthesis, as well as the ETR1 and EIN2 proteins belonging to the ethylene signaling pathway, play essential roles. On the other hand, the NCED and ABA2 proteins are important for cellular responses to ABA. Little is known about the mechanisms of ethylene-ABA interactions. However, genetic characterization of each pathway, analytic chemistry, pharmacology and molecular analysis, have started to provide information towards understanding their functional relevance in plant tissues. In this work, recent advances in ABA and ethylene signaling are discussed, with special emphasis on the interaction between both phytohormones and its effect on organogenesis processes.

Key words: Abscisic acid, ethylene, plant development, *Arabidopsis thaliana*.

Introducción

Las fitohormonas, también conocidas como reguladores del crecimiento vegetal, modulan el crecimiento y el desarrollo de las plantas. Entre ellas, las auxinas, citocininas, giberelinas, etileno, ácido abscísico, ácido jasmónico, ácido salicílico y brasinoesteroides han sido ampliamente estudiadas.

El ácido abscísico (ABA), además de regular la maduración y latencia de las semillas juega un papel crítico en la respuesta a sequía y salinidad, acumulándose en las células vegetales bajo estrés hídrico donde promueve el cierre estomático y regula la expresión de una gran cantidad de genes (Hirayama y Shinozaki 2007; Umezawa et al., 2010). Diversas investigaciones sobre el ABA llevaron a proponer un modelo complejo de señalización, donde se sugería como sus posibles receptores a proteínas enlazadas a las proteínas G (GCR2, GTG1/2) y no explicaba de qué forma estas últimas podrían regular las respuestas al ABA en plantas (Pandey et al., 2009). En años recientes, se ha podido establecer la ruta involucrada al descubrir un nuevo tipo de receptor de ABA, formado por las proteínas PYR/PYL/RCAR (Ma et al., 2009; Park et al., 2009), que se unen al complejo proteico fosfatasa-cinasa (PP2C-SnRK2). En presencia del ABA, la actividad de la fosfatasa se bloquea, quedando así las cinasas SnRK2 activas (Umezawa et al., 2009; Vlad et al., 2009).

Por otra parte, la participación del etileno como regulador del envejecimiento, la maduración de los frutos y la respuesta a patógenos es bien conocida. El etileno fue una de las fitohormonas descubiertas a finales del siglo XX, período en el que se documentó que el gas producido de emanaciones durante la extracción de la hulla causaba envejecimiento prema-

turo y abscisión de las hojas de las plantas (Abeles et al., 1992). Neljubov en 1901 identificó al etileno como el componente “activo” de dichas emanaciones y posteriormente se demostró que las plantas lo producen de manera natural, estableciéndose de esta manera que el etileno es un regulador endógeno del crecimiento y desarrollo vegetal (Schaller y Kieber, 2002). Las investigaciones con *Arabidopsis thaliana* han permitido identificar diversos componentes de la ruta de transducción de señales del etileno, incluyendo cuatro receptores membranales, intermediarios de la vía y dos familias de factores de transcripción.

Por su carácter antagónico en el desarrollo vegetal, donde el etileno promueve la senescencia y el ABA induce la germinación, se ha propuesto que estas dos fitohormonas interactúan en la modulación de procesos fisiológicos de crecimiento y desarrollo de las plantas. Este artículo resume los avances recientes sobre los componentes de las vías de señalización del ABA y del etileno, así como la relación antagónica entre ambos fitorreguladores y su efecto sobre la fisiología vegetal.

Ruta de señalización del ABA

Hasta hace unos pocos años no se tenía definido el mecanismo de respuesta al ABA en plantas. Park y colaboradores (2009), examinaron minuciosamente bibliotecas químicas y observaron que la “pirabactina” funciona como un agonista selectivo del ABA. Después de un escrutinio genético contra la pirabactina, se identificó al gen *PYRABACTIN RESISTANCE1* (*PYR1*). Otro grupo de investigadores, en un escrutinio de doble híbrido de levadura aislaron una proteína que interactúa con ABI1, a la cual denominaron *REGULATORY COMPONENT ABA RECEPTOR1* (*RCAR1*) (Ma et al., 2009). Posteriormente, en el genoma de *Arabidopsis* se identificó que *PYR* y *RCAR* corresponden a la misma familia de genes formada por 14 miembros, los cuales se conocen como *PYR1* y parecidos a *PYR1* (*PYL*) 1-13 o *RCAR1-RCAR14* (Umezawa et al., 2010). También se reportó que las proteínas PYR/PYL/RCAR podían unirse al ABA e interactuar con las fosfatasas de la familia 2C (PP2C) del grupo A, inhibiendo así la actividad de las PP2C. Los resultados antes mencionados permitieron sugerir que las PYR/PYL/RCARs funcionan como una subunidad reguladora negativa de las PP2C (Ma et al., 2009; Park et al., 2009). Debido a que las fosfatasas PP2C modulan negativamente la señalización de ABA, se propuso que la unión de PYR/PYL/RCAR al ABA activa su vía de señalización. La función de las distintas PYR/PYL/RCARs se confirmó por la insensibilidad al ABA de la mutante cuádruple *pyr1 pyl1 pyl2 pyl3* (Park et al., 2009) y, en forma contrastante, cuando se sobre-expresaron las proteínas RCAR1/PYL9, RCAR3/PYL8 o RCAR8/PYL5 en *Arabidopsis* se produjo hipersensibilidad al ABA y elevada tolerancia a la sequía (Ma et al., 2009; Santiago et al., 2009; Saavedra et al., 2010).

En complemento, en un escrutinio genético para aislar mutantes de *Arabidopsis* insensibles al ABA, se identificaron dos genes: *ABA-INSENSITIVE1* (*ABI1*) y *ABI2*, que codifican las fosfatasas PP2C (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodríguez et al., 1998). Se observó que las mutantes *abi1-1* y *abi2-1*eran insensibles al ABA

en varios tejidos y en diferentes etapas de desarrollo, sugiriendo con ello que las fosfatasas PP2C actúan como reguladores globales de la señalización del ABA. Recientemente, se determinó que la mutante de la fosfatasa PP2C, *abi1-1* es incapaz de interactuar con los receptores PYR/PYL/RCAR, lo que causa que dichos receptores no puedan inactivar a las fosfatasas que mantienen la vía apagada (Ma et al., 2009; Park et al., 2009).

La identificación y caracterización de las fosfatasas PP2C ha mostrado la importancia de los eventos de fosforilación en la señalización del ABA, donde varias cinasas han sido aisladas y tipificadas como componentes de esta vía (Hirayama y Shinozaki, 2007). Entre ellas, existen 10 cinasas SnRK2, designadas en *Arabidopsis* como SnRK2.1-SnRK2.10 (Hrabak et al., 2003) o SRK2A-SRK2J (Yoshida et al., 2002). Las cinasas SnRK2/SRK2 son desfosforiladas eficientemente por las fosfatasas tipo ABI1, inactivándolas (Umezawa et al.,

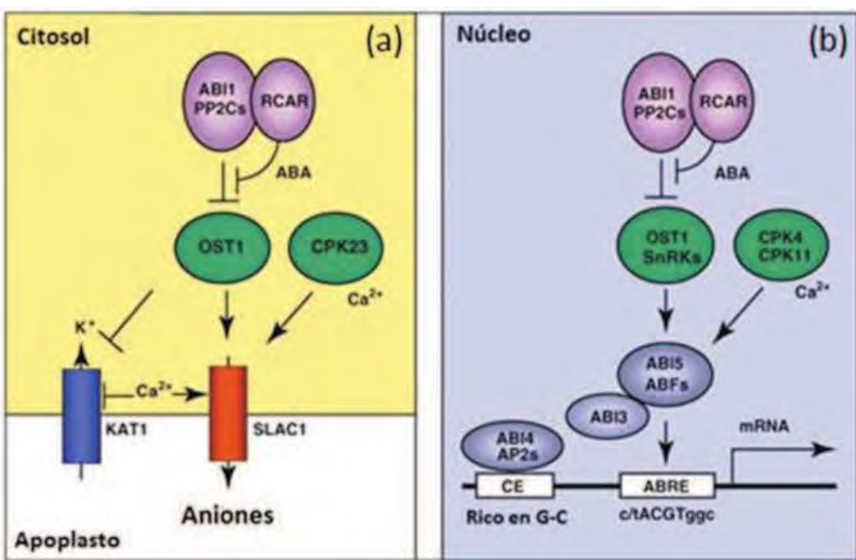

Figura 1. La señalización de ABA. El receptor de ABA está formado por el complejo heteromérico de una PP2C como ABI1 y una proteína de unión al ABA, RCAR (ambas en color rosa). El complejo receptor controla la señalización del ABA tanto en el citosol (a) como en el núcleo (b). La actividad fosfatasa de las PP2C inhibe la acción de la cinasa citosólica, OST1, y la nuclear, SnRKs (presentadas en verde) y posiblemente de las CPKs dependientes de Ca^{2+} como la CPK23. En presencia de ABA, la actividad fosfatasa del receptor es bloqueada. Como consecuencia, las cinasas son liberadas de la inhibición y quedan activas para regular a los blancos clave de la ruta de señalización de ABA. En las células guarda, las proteínas blanco son los canales iónicos SLAC1 y KAT1, los cuales son activados e inhibidos, respectivamente, por la acción de OST1. En el núcleo, los blancos son ABI5 y ABFs, factores de transcripción tipo cierre de leucina básico. Las ABFs fosforiladas y unidas como dímeros se enlazan a los elementos cis de respuesta a ABA (ABRE), promoviendo la expresión de los genes de respuesta a ABA y, en forma concertada con otros factores transcripcionales como ABI3, ABI4 y AP2s, inducen la expresión de genes de respuesta a ABA (los componentes son presentados en azul acero). ABI3 se une a ABI5 y aumenta su actividad, mientras que ABI4 y factores de transcripción tipo AP2 se unen al elemento CE rico en G-C para regular en forma óptima la expresión de genes dependientes de ABA (Modificado de Raghavendra et al., 2010).

2009; Vlad et al., 2009). En este tópico, Vlad y colaboradores (2009) identificaron a la serina 175 en la cinasa SRK2E/OST1 como el sitio blanco de las fosfatasas, lo cual proporcionó una evidencia fuerte de que dichas enzimas inactivan a las cinasas SnRK2. Finalmente, al reconstituir *in vitro* los componentes de la vía, se demostró que los receptores PYR/PYL/RCAR se unen e inactivan a las PP2C, impidiendo la desfosforilación de las cinasas SnRK2 de una forma dependiente del ABA (Umezawa et al., 2009).

Con relación a las cinasas SnRK2/SRK2, se ha reportado que pueden activar o inactivan canales iónicos de la membrana plasmática, o bien pueden afectar factores de transcripción en el núcleo. Por ejemplo, OST1/SRK2E actúa como regulador positivo del cierre de estomas (Mustilli et al., 2002), activando los canales aniónicos SLAC1 e inhibiendo a los canales catiónicos KAT1 (Fig. 1a) (Geiger et al., 2009; Lee et al., 2009). Ambos canales son regulados por la ruta de señalización del ABA y Ca^{2+} (Siegel et al., 2009). Se ha sugerido que la regulación dependiente de Ca^{2+} probablemente se debe a la estimulación de SLAC1 por la cinasa CPK23 (Fig. 1a) (Geiger et al., 2010).

Los reguladores transcripcionales clave en la expresión de genes dependientes de ABA son los factores de unión a elementos de respuesta a ABA -ABRE- (ABFs), del tipo cierre de leucina de región básica (bZIP) con ABI5 como un representante típico (Choi et al., 2005; Finkelstein et al., 2005). La fosforilación de este último por las SnRKs conduce a su activación, mientras que la sumoilación a través del marcaje de ABI5 con ubiquitina por una SUMO E3 ligasa para su degradación en el proteosoma, antagoniza la acción de ABI5 (Miura et al., 2009). ABI5 y otros ABFs son también fosforilados por las cinasas CPK4 y CPK11 dependientes de Ca^{2+} (Zhu et al., 2007). Otros factores de transcripción que contribuyen a la especificidad del ABA, como el ABI3 (reguladores tipo B3), se unen a ABI5 e incrementan su actividad. Además, ABI4 (factor transcripcional tipo AP2) y factores adicionales que incluyen a las MYC/MYB actúan también como reguladores positivos de respuesta al ABA (Yamaguchi-Shinozaki y Shinozaki, 2006). La heterodimerización de ABI3 con ABI5 y su interacción con la caja ABRE, además de la unión de ABI4 con los elementos ricos en G-C en los promotores de los genes de respuesta a ABA, permiten la transcripción inducible de estos genes (Fig. 1b) (Raghavendra et al., 2010).

Vía de señalización del etileno

Para dilucidar la vía de transducción de señales del etileno se requirió del aislamiento de mutantes afectadas de los intermediarios que la integran. Cabe mencionar que la obtención de dichas mutantes en *Arabidopsis* ha dependido casi exclusivamente del fenotipo en la respuesta triple que se presenta cuando las plántulas mutantes crecen en la oscuridad (Knight et al., 1910). Dicho fenotipo se caracteriza por la presencia de hipocotilos cortos y gruesos, la inhibición de la elongación de los hipocotilos y la formación de un gancho apical (Guzmán y Ecker, 1990) que contrasta con el fenotipo etiolado: crecimiento preferencial del hipocotilo en la oscuridad, observado en las plántulas silvestres (Wt) expuestas al aire (Fig. 2). Las mutaciones aisladas a través del fenotipo alterado en la respuesta al etileno pertene-

cen a dos grupos: i) insensibles y ii) de respuesta constitutiva al etileno. Un ejemplo de las primeras es la mutante *etr1-1*, que presenta ganancia de la función en un receptor (Bleecker et al., 1988; Chang et al., 1993) y *ein2*, con pérdida de la función en un elemento de la vía de señalización (Alonso et al., 1999). Mientras que las mutantes de respuesta constitutiva a etileno mostraron el fenotipo de hipersensibilidad a etileno, tanto cuando crecen en aire como en etileno; un ejemplo de ellas son las *eto3* (*ethylene overproducer3*) con una sobreproducción de etileno (Guzmán y Ecker, 1990). Alternativamente, también existen mutaciones en la ruta de transducción de señales como la *ctr1* que da como resultado la pérdida de la función del regulador negativo de la vía de señalización manteniendo constitutivamente activada la respuesta celular a etileno (Fig. 2) (Kieber et al., 1993).

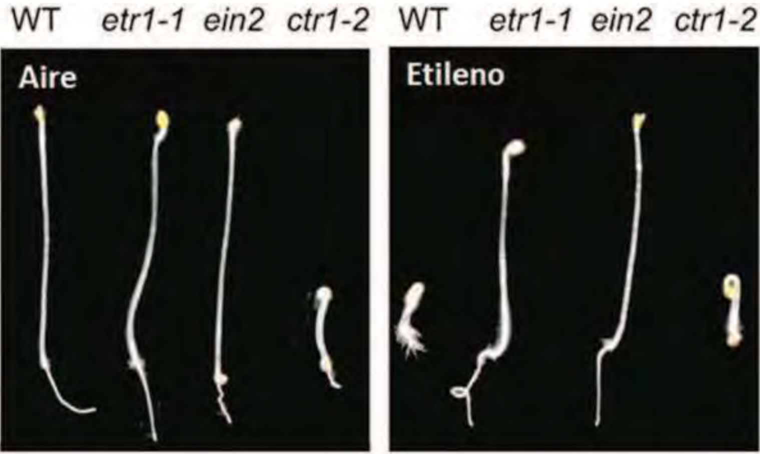

Figura 2. Mutantes de la ruta de transducción de señales del etileno. El efecto del etileno sobre la inducción de la respuesta triple en plántulas que crecen en la oscuridad para el tipo silvestre (Wt) y las mutantes insensibles a etileno *etr1-1* y *ein2* y de respuesta constitutiva al etileno *ctr1-2* (Modificado de Schaller y Kieber, 2002).

La cascada de señalización de etileno descrita hasta la fecha consta de cuatro componentes clave: el receptor ETR1, el regulador negativo de la ruta CTR1, el intermediario EIN2 y el factor de transcripción EIN3. Existen otras proteínas que pueden realizar la misma función debido a su alta similitud con los componentes de la ruta de etileno; tal es el caso de ETR2, ERS1, ERS2 y EIN4 que también funcionan como receptores, o EIL1, EIL2, EIL3 y ERFs, que lo hacen como factores de transcripción (Fig. 3).

ETR1 es una proteína de 738 aminoácidos del retículo endoplásmico que presenta tres dominios transmembranales, que comprenden el sitio de unión a etileno donde, al parecer, la unidad funcional de percepción de etileno es un dímero (Schaller et al., 1995). En la activación de dicho receptor se ha comprobado químicamente la actividad histidina cinasa, que implica la autofosforilación de la proteína en un residuo conservado de histidina, con la posterior transferencia de ese grupo fosfato a un residuo de ácido aspártico dentro de un do-

minio receptor (Gamble et al., 1998). Dicha actividad es típica de sistemas bacterianos de dos componentes, lo que sugiere que podría haberse heredado cuando se llevó a cabo la simbiosis que condujo al origen de los cloroplastos (Parkinson, 1993). La unión de alta afinidad del etileno está mediada por un cofactor de cobre coordinado por dos aminoácidos conservados (Cis65 e His69) (Schaller y Bleecker, 1995; Rodríguez et al., 1999). La unión del etileno al receptor ETR1 lo activa y le permite la interacción y activación de la proteína CTR1 (Clark et al., 1998).

El gen *CTR1* codifica una proteína de 821 aminoácidos, donde la mitad carboxilo terminal presenta un dominio similar al de la familia Raf de las cinasas serina/treonina. Estas últimas participan en la cascada de las proteínas cinasas activadas por mitogenos (MAPK) en animales, la cual consta de varias *MAPK* cinasa cinasa (MAPKKK) que fosforilan y activan a las MAPK cinasas (MAPKK), las cuales a su vez activan a las MAPK que transmitirán la señal fosforilando a sus blancos (Ichimura et al., 2000). Se ha sugerido que CTR1 podría funcionar como una MAPKKK en forma análoga a las proteínas Raf, e iniciar la señalización a través de la cascada MAPK en *Arabidopsis*, lo que culminaría con la inactivación de EIN2 (Novikova et al., 2000; Ouaked et al., 2003). Así, las mutaciones en *CTR1* que afectan su actividad provocan un fenotipo de respuesta constitutiva a etileno (Kieber et al., 1993). *EIN2* codifica una proteína integral de membrana de 1294 aminoácidos que contiene 12 dominios transmembranales y en la región amino terminal presenta una alta similitud con la familia de transportadores de cationes Nramp (Alonso et al., 1999). La región amino terminal es transmembranal. Las mutantes *ein2* exhiben el fenotipo insensible a

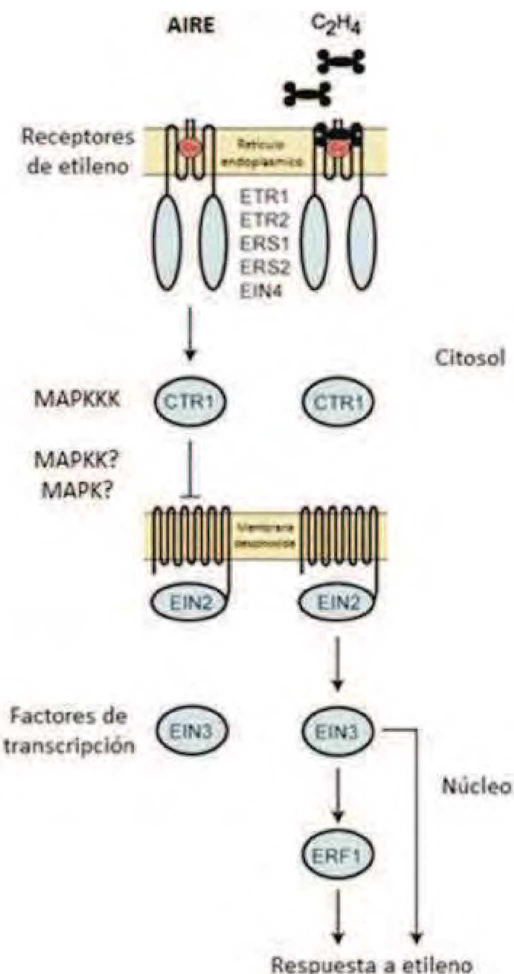

Figura 3. Modelo para la transducción de señales del etileno. En aire, los receptores del etileno mantienen a la cinasa CTR1 en estado inactivo, reprimiendo las respuestas a etileno, mientras que en etileno, la represión es liberada. La unión del etileno inactiva los receptores y por lo tanto a CTR1. Como resultado, EIN2 se activa encendiendo así la cascada que involucra a los factores de transcripción EIN3/EILs y ERFs,los cuales participan en la regulación de las respuestas al etileno. Los dominios solubles de las proteínas se muestran como círculos y las estructuras transmembranales predichas para los receptores de etileno y EIN2 se presentan como líneas que atraviesan la membrana (Modificado de Schaller y Kieber, 2002).

etileno más fuerte que el resto de las mutantes resistentes a etileno aisladas en *Arabidopsis*, lo que corrobora su papel crítico en la señalización. Los experimentos para demostrar la posible actividad de transportador de metales de EIN2 de plantas en sistemas heterólogos, mostraron que EIN2 de *Arabidopsis* no complementa cepas de levaduras deficientes en canales de metales (Thomine et al., 2000).

Por otra parte, corriente abajo de EIN2 se ha reportado una proteína blanco de 628 aminoácidos, conocida como EIN3,con características de factor de trascripción (Chao et al., 1997). EIN3 se encuentra en el núcleo y presenta dominios acídicos ricos en prolina y glutamina. Las mutaciones con pérdida de función para EIN3 presentan un fenotipo insensible a etileno, lo que sugiere que funciona como un regulador positivo de la transducción de señales del etileno. EIN3 es miembro de una familia que contiene al menos otras tres proteínas parecidas a EIN3 (EILs -EIN3-like) (Chao et al., 1997), las cuales se unen como homodímeros a un elemento promotor en el gen ERF1 promoviendo su transcripción (Solano et al., 1998). Los ERFs son también factores de transcripción que inducen la expresión de genes relacionados a la patogénesis, como la β-1-3-glucanasa, la quitinasa básica y la defensina (Ohme-Takagi y Shinshi, 1995; Solano et al., 1998). De esta manera, EIN3 y los EILs estimulan la transcripción de genes de respuesta a etileno y de otros factores de transcripción como los ERFs; estos últimos a su vez también promueven la transcripción de miembros de su misma familia, sugiriendo la existencia de una compleja red de regulación transcripcional en la ruta de señalización del etileno (Solano et al., 1998).

Relación antagónica entre el ABA y el etileno

La interacción entre ABA y etileno en las plantas se ha caracterizado a distintos niveles. Por ejemplo, existe un reporte que muestra que las mutantes deficientes de ABA, *flacca* y *notabillis* de tomate, contienen más del doble de etileno que las plantas silvestres (Sharp et al., 2000). Las mutantes *flacca* y *notabillis* presentan tamaño pequeño, biomasa reducida, tallo corto, hojas pequeñas y formación de raíces adventicias comparadas con plantas normales, las cuales son características típicas de una repuesta aumentada al etileno. También se observó que el tratamiento de dichas mutantes con tiosulfato de plata, un inhibidor de la acción del etileno, restaura parcialmente el crecimiento del follaje en las mutantes mencionadas. Esta información sugiere que el efecto causado por la deficiencia de ABA en el follaje puede ser atribuido a una sobreproducción de etileno (Sharp et al., 2000). Respecto al efecto de la alteración en la señalización del etileno, se ha reportado que las mutaciones con pérdida de la función en el gen *EIN2* muestran un incremento en la producción de ABA en *Arabidopsis* (Ghassemian et al., 2000; Wang et al. 2007; Cheng et al., 2009). Además, se ha observado que las mutaciones en *CTR1*, que inducen una respuesta constitutiva a etileno, y la *EIN2*, que provoca insensibilidad a etileno, aumentan o suprimen respectivamente el fenotipo de resistencia a la germinación de la mutante de *Arabidopsis abi1* cuando se suplementa con ABA de manera exógena (Beaudoin et al., 2000). Sin embargo, la mutación en *EIN2* no mostró un efecto supresor significativo en las mutantes *abi3* bajo las mismas condi-

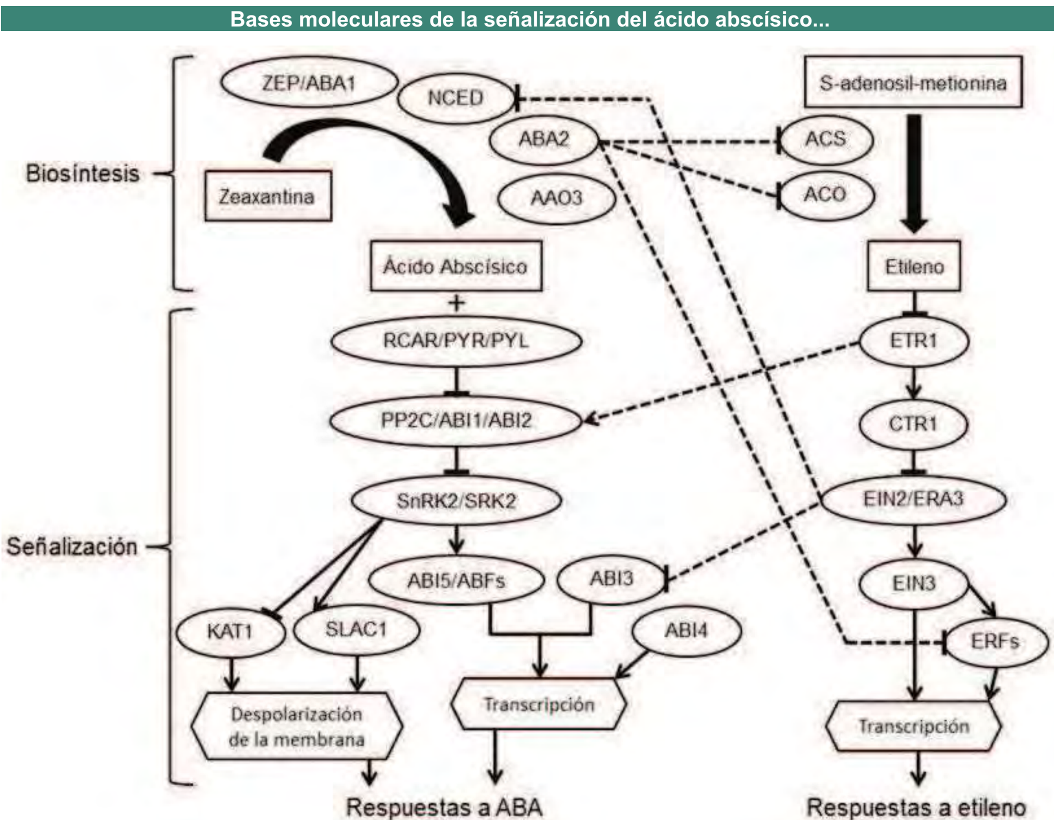

Figura 4. La interacción ABA-etileno implica a la vía de biosíntesis y señalización de ambas fitohormonas. Los componentes dentro de óvalos representan proteínas y dentro de rectángulos representan metabolitos, en tanto que los elementos delimitados por hexágonos muestran respuestas celulares. Las líneas continuas señalan procesos establecidos en tanto que las discontinuas indican los procesos para la interacción propuestos. La punta de flecha al final de las líneas indica la activación o promoción en tanto que la línea perpendicular señala la inactivación.

ciones (Beaudoin et al., 2000). Lo anterior indica que la cascada de señalización del etileno definida por ETR1, CTR1 y EIN2 inhibe la señalización de ABA probablemente cascada arriba del factor transcripcional ABI3 durante la germinación de la semilla. Otras evidencias de la interacción etileno y ABA provienen del análisis de las mutaciones que aumentan la producción de etileno en las mutantes *eto3* que presentan sensibilidad reducida a ABA durante la germinación (Subbiah y Reddy, 2010). Además, se ha observado que la aplicación del precursor de etileno ácido 1-amino-1-ciclopropano (ACC) reduce el efecto inhibitorio sobre la germinación en plantas silvestres de *Arabidopsis*; los análisis bioquímicos y moleculares identificaron a una mutante designada como *enhancer response* to *ABA3* (*era3*), la cual incrementó la sensibilidad a ABA en las semillas y acumuló ABA, sugiriendo que este gen es un regulador negativo de la biosíntesis de ABA (Ghassemian et al., 2000). Análisis genéti-

cos posteriores realizados por este mismo grupo, mostraron que *ERA3* representa un nuevo alelo en el locus *ETHYLENE INSENSITIVE2* (*EIN2*). Debido a que mutaciones que afectan la señalización de etileno modifican también los niveles de ABA y que mutaciones en la síntesis de ABA alteran la cantidad de etileno, un análisis de la expresión global de genes por microarreglos mostró que la mutación en *ABA2*, gen que codifica una enzima implicada en la biosíntesis del ABA, regula positivamente la expresión de los genes de biosíntesis del etileno ACO (ACC oxidasa) y el factor de transcripción de respuesta a etileno ERF053, mientras que el gen *9-CIS-EPOXICAROTENOID DIOXYGENASE 3* (*NCED3*) que codifica una enzima de la biosíntesis de ABA fue regulada negativamente en la mutante *ein2*. Además, también se observó que la mutación en el gen *ETR1* disminuye la expresión de los genes *ABSCISIC ACID INSENSITIVE1* (*ABI1*) y *CYP70742*, este último implicado en el catabolismo de ABA (Cheng et al., 2009). Los datos antes mencionados sugieren que el ABA y el etileno pueden controlar la biosíntesis, el catabolismo y la señalización hormonal, aumentando así sus efectos antagónicos en las plantas (Fig. 4).

Cabe mencionar que también se han reportado efectos distintos a una interacción netamente antagónica, por ejemplo, Zhang y col. (2009) observaron que en frutos de tomate el ABA induce la biosíntesis de etileno cuando se aplica de manera exógena a través del incremento en la expresión los genes que codifican para la ACC sintasa y la ACC oxidasa. Por otra parte, se ha observado que la aplicación exógena de auxinas incrementa la biosíntesis de etileno y la producción de ABA culminando con la inhibición del crecimiento en *Galiumaparine* (Hansen y Grossmann, 2000). Finalmente, existen datos donde la adición de la citocinina *N-6*-benzil-adenina (BA) revirtió la sensibilidad incrementada de resistencia a la germinación de las mutantes insensibles a etileno tratadas con ABA (Subbiah y Reddy, 2010). Estos últimos reportes sugieren la participación de otras hormonas en la regulación de procesos que implican la interacción ABA-etileno.

Conclusiones

La interacción del ABA-etileno modula el crecimiento y desarrollo de las plantas de una manera antagónica, donde cada una de estas fitohormonas puede regular la concentración de la otra. De esta forma, cuando una de las dos hormonas pierde o incrementa la capacidad de ejercer su efecto en la planta, el cambio fenotípico observado es el resultado de la alteración de la señal de ambas hormonas, ya que éstas se regulan entre sí. La mayoría de las evidencias de la interacción ABA-etileno presentadas en esta revisión muestran que la regulación principal se efectúa a través de la vía de biosíntesis de ambas hormonas, en la que el gen *EIN2* juega un papel fundamental. Además de la modulación transcripcional reportada, el hecho de que ambas rutas de señalización utilizan cascadas de fosforilación para transmitir la señal abre la posibilidad de una regulación post-transcripcional entre ellas. Como se mencionó anteriormente, existen reportes en los cuales la interacción entre el ABA y el etileno no es netamente antagónica; cabe destacar que dichos resultados provienen de análisis realizados en órganos específicos, lo cual no representaría un mecanismo de ac-

ción general sobre la planta. Además, la participación de otras hormonas, como las auxinas o citocininas, incrementa el número de variables ocasionando una interacción aún más compleja. La participación de las auxinas o las citocininas en la interacción ABA-etileno sugiere la existencia de redes de señalización que interactúan constantemente para lograr un crecimiento y desarrollo adecuado en los organismos vegetales.

En nuestro grupo de trabajo estamos caracterizando las respuestas que modifican la arquitectura radicular por efecto del suplemento exógeno de ABA y etileno a mutantes de *A. thaliana* sencillas y dobles en los componentes de las rutas de señalización del ABA y etileno para identificar a nivel genético los nodos de interacción entre ambas vías. Asimismo, la determinación de la actividad histoquímica en mutantes de la ruta de señalización del etileno llevando la línea transgénica con la secuencia que codifica la β-glucuronidasa fusionada a la región promotora del gen *ABI4* de la vía de señalización del ABA, permitirán la identificación de un posible mecanismo a través del cual las dos rutas de señalización interaccionan. También, la cuantificación del ABA en mutantes de la vía de señalización del etileno permitirá identificar los elementos de esta ruta que modifican la producción de ABA.

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Genes para enfrentar el estrés hídrico

INVESTIGADORES DEL IPN HAN IDENTIFICADO PLANTAS MEXICANAS QUE TOLERAN LA FALTA DE AGUA Y QUE PODRÍAN IMPULSAR EL CAMPO NACIONAL

Héctor de la Peña

En México los estados que concentran la mayor producción de granos se ubican al norte del territorio nacional, donde prevalece el clima árido y escasea el recurso hídrico, por lo que es necesario hacer uso de la tecnología de riego capaz de garantizar un óptimo aprovechamiento del agua.

A pesar de los buenos resultados obtenidos bajo esas condiciones, investigadores nacionales buscan otras alternativas a la escasez de agua en zonas agrícolas. En ese sentido la biotecnología está planteando nuevas opciones de mejoramiento al llamado estrés hídrico.

Un par de esas investigaciones se desarrolla en el Centro de Investigación en Biotecnología Aplicada del Instituto Politécnico Nacional (CIBA-IPN), en Tlaxcala, donde a partir de plantas de la región se busca obtener genes útiles para que en el futuro, cultivos comerciales puedan tolerar bajos niveles de riego.

Ambos trabajos están dirigidos por los doctores Analilia Arroyo Becerra y Miguel Angel Villalobos López, quienes se dedicaron a seleccionar durante cerca de un año aquellas especies de la región de Puebla y Tlaxcala que presentaran el fenómeno de "resurrección", que significa que en apariencia la planta está muerta debido a una pérdida total de agua, pero al rehidratarse recupera su vitalidad rápidamente.

Musgo *Plagiomnium cuspidatum*

"En la literatura científica se conoce y ha estudiado a profundidad una planta de resurrección africana (*Craterostigma plantagineum*) para obtener genes útiles frente al estrés hídrico. Pero nos preguntamos por qué seguir estudiando plantas de otros países si México tiene una gran biodiversidad, y es posible encontrar algunas con características de resurrección o tolerancia a condiciones extremas. Entonces, iniciamos la búsqueda y recolectamos alrededor de 20 especies diferentes", refirió Villalobos López.

Una vez identificadas las especies (con la ayuda de colaboradores de la Universidad Autónoma de Tlaxcala-UAT y de la Benemérita Universidad Autónoma de Puebla-BUAP), los investigadores realizaron otra selección más exhaustiva a nivel laboratorio, y a partir de ello decidieron estudiar sólo algunas especies, entre las que figuran los musgos *Plagiomnium cuspidatum* y *Ceratodon stenocarpus*, en el caso de la investigación del doctor Villalobos López, y *Selaginella sartorii* y zacate salado (*Distichlis spicata*), por parte de la doctora Arroyo Becerra.

En el caso del zacate salado, si bien no es una planta de resurrección, fue seleccionado debido a que en su ambiente natural resiste estrés hídrico, tolera inundaciones y también suelos con alta salinidad. "Creemos que su germoplasma (genes) tiene un valor científico enorme, por lo que decidimos integrarla a la investigación", comentó la especialista del CIBA-IPN.

En tanto, Villalobos López explicó que su selección de musgos fue en base a su rápida capacidad de recuperar sus funciones una vez que se les agrega agua, pues mientras reportes internacionales para otras plantas de resurrección indican que la mayoría de esas especies se recuperan en periodos de horas a días, las especies de musgos seleccionados lo consiguen en cuestión de minutos después de estar dos años totalmente deshidratados.

La siguiente etapa de la investigación consiste en obtener la información (caracterizar) de los genes que participan en la tolerancia a la falta de agua en las especies seleccionadas. Para secuenciar una parte de los genes clonados, los investigadores envían el material a colaboradores (doctor Miguel Angel Ramírez Romero) del Centro de Ciencias Genómicas y al Instituto de Biotecnología, ambos de la UNAM.

De acuerdo con los investigadores, la información hasta ahora recabada da indicios de que es posible emplear genes de las especies seleccionadas (musgos, selaginella y zacate) en la generación de plantas modificadas para conferir tolerancia a déficit hídrico. Empero, apuntaron que en ese tipo de investigación los grados de certeza son relativos, pues la introducción de un gen a una planta diferente no garantiza que funcione totalmente como lo hace en la especie de donde proviene.

"Hasta ahora no encontramos algún punto en contra con el que pudiéramos pensar que los genes no funcionarían en una planta de interés agronómico. Además, las mejoras no necesariamente van encaminadas a contender con escenarios extremos, sino a medianamente estresantes (poco riego), que son las que realmente se encuentran en una gran parte de los campos de cultivo", expresó la doctora Arroyo Becerra.

De tal forma, ambos investigadores politécnicos esperan que en un periodo no mayor a cuatro años tengan la capacidad de generar plantas de interés agronómico tolerantes a estrés hídrico, e iniciar el proceso de análisis experimental en invernaderos bajo condiciones controladas. ■

Alcamidas: alternativa en la productividad agrícola

Randy Ortiz Castro*
y José López Bucio**

Estas fitohormonas tienen la capacidad de estimular el crecimiento de las plantas sin afectar sus propiedades

La agricultura moderna tiene sus bases en la llamada "Revolución verde", que incorpora variedades mejoradas, suelos fértiles y un alto consumo de nutrimentos, pesticidas y otros insumos. Aunque esta tecnología ha posibilitado el suministro de granos durante las pasadas generaciones; el constante crecimiento poblacional, el deterioro cada vez más drástico de los suelos y la demanda de productos del campo libres de substancias potencialmente tóxicas para la salud humana precisan implementar nuevas estrategias con base en productos bioactivos generados de manera natural por las plantas y amigables con el ambiente.

Los reguladores del crecimiento o fitohormonas son compuestos de diferente naturaleza química que pueden alterar el desarrollo vegetal en concentraciones pequeñas. El concepto de hormona, tal como fue desarrollado para los animales no es fácil de extrapolar a las plantas. Por una parte, los vegetales no cuentan con un sistema de transporte tan eficiente como la circulación sanguínea, ni tampoco tienen el equivalente a un sistema nervioso central capaz de integrar o coordinar todas las actividades fisiológicas del organismo.

Sin embargo, las plantas tienen un sistema sofisticado para integrar información del ambiente y responder activamente a los estímulos bióticos como el frío o la salinidad, y abióticos como el ataque de patógenos; de igual manera, poseen mecanismos para la comunicación entre los diferentes órganos y tejidos. Actualmente se sabe que los reguladores del crecimiento forman un grupo de moléculas integradoras de información, cuyas vías de señalización interactúan con frecuencia a fin de regular la morfogénesis vegetal.

En fechas recientes se identificó un grupo novedoso de fitohormonas: las alcamidas, que abarcan unos 200 compuestos relacionados estructuralmente y son sustancias ampliamente distribuidas en la naturaleza; las encontramos tanto en los líquenes como en las plantas con flores, formando parte de las semillas, raíces y también del follaje, donde desempeñan funciones fisiológicas y adaptativas que van desde la germinación hasta la respuesta de las hojas ante ataques de plagas y patógenos.

Diferentes investigaciones sugieren que las alcamidas y algunas sustancias similares podrían utilizarse como promotores del crecimiento. Por ejemplo, investigadores de Japón encontraron que la amidenina (obtenida de un hongo) estimula el crecimiento de las plantas de arroz. Otros trabajos indican que las *N-acyl-homoserina lactonas* (moléculas de la misma familia de las alcamidas producidas por bacterias benéficas) controlan la germinación de las semillas y pueden modificar la forma y función de las raíces cuando se suministran a las plantas.

El conocimiento sobre las alcamidas y su participación en la fisiología de las plantas nos abre nuevos horizontes para su posible uso en la agricultura.

El primero como alternativa a la producción tradicional en el campo mediante la formulación de productos que estimulan el crecimiento de las plantas sin afectar al consumidor e inocuos con el ambiente. Otro es el aumento de la capacidad de los cultivos para captar agua y nutrientes minerales mediante aplicación de alcamidas que promueven el enraizamiento. Y un tercero como identificación de bacterias benéficas productoras de alcamidas presentes de manera natural en los suelos agrícolas.

La información aquí presentada permite vislumbrar que, en años venideros, las alcamidas serán incorporadas a los sistemas de producción agrícola para coadyuvar al aumento de la producción de alimentos tan necesario en este milenio que recién comienza. ■

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Las alcamidas: un grupo nuevo de reguladores del crecimiento y sus aplicaciones en la biotecnología agrícola ε↵

Palabras clave: *Arabidopsis thaliana*, alcamidas, estimulación del crecimiento, rizobacterias.

INTRODUCCIÓN

Las plantas producen diversas substancias que regulan su crecimiento y desarrollo. Estas substancias son denominadas hormonas vegetales o reguladores del crecimiento (RC) y ejercen sus funciones mediante programas de señalización intracelular específicos que conducen al encendido o apagado de genes. Por sus efectos biológicos y por su distribución amplia en el reino vegetal se consideran 6 clases principales de RC: las auxinas, las citocininas, las giberelinas, el etileno, el ácido abscísico y los brasinoesteroides. Todos estos compuestos son requeridos para la viabilidad celular, participan en procesos específicos de desarrollo y como integradores de señales ambientales.

La formación de órganos y tejidos ocurre por interacciones entre RC. Se ha descrito que las auxinas interactúan con las citocininas para regular el crecimiento de la raíz primaria, la formación de pelos radiculares y de las raíces laterales. De hecho, la combinación de diferentes concentraciones de auxinas y de citocininas en sistemas de cultivo de tejidos posibilita la regeneración de órganos y es la base para la propagación vegetal "*in vitro*".

Las plantas son los productores primarios de los ecosistemas en donde interactúan con una amplia variedad de factores bióticos y abióticos que modulan su crecimiento. El metabolismo vegetal produce una amplia variedad de substancias químicas, algunas que desempeñan funciones defensivas en respuesta al ataque de patógenos, otras que funcionan como atrayentes de especies microbianas benéficas. Nuestro grupo de trabajo ha enfocado sus esfuerzos hacia la identificación de compuestos que se producen de manera natural por las plantas que pueden actuar como estimuladores del crecimiento.

Recientemente, hemos documentado la existencia de un grupo nuevo de reguladores que incluye moléculas derivadas de lípidos como las *N-acil* etanolaminas (NAEs) y las alcamidas. Las alcamidas son compuestos conservados durante la evolución, se les encuentra en los animales regulando importantes rutas de señalización y recientemente en plantas donde regulan la ramificación de la raíz y las respuestas de defensa (Ramírez-Chávez *et al.*, 2004). En las bacterias también se han encontrado compuestos con estructura química similar a las alcamidas conocidas como N-*acil*-homoserina lactonas, las cuáles regulan la densidad poblacional. El estudio de los mecanismos de acción de esta gama de compuestos abre nuevas perspectivas para su aplicación en la agricultura.

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LA ARQUITECTURA DE LAS PLANTAS

Las plantas como organismos sésiles pasan su ciclo de vida ancladas a un sustrato. De manera general se pueden dividir en dos sistemas, la parte aérea o follaje y el sistema radicular. El follaje está constituido por el tallo, las ramas y las hojas, que se derivan de la actividad proliferativa del meristemo apical (Norberg *et al*., 2005). En las hojas ocurre la fotosíntesis, que es la principal fuente de carbohidratos para los tejidos consumidores de la planta incluyendo la raíz, las flores y los frutos. Las raíces tienen como función el anclaje al suelo y la captación de nutrientes y agua. El sistema radicular consta de tres partes principales diferenciadas: la raíz primaria, las raíces laterales y los pelos radiculares. El crecimiento de la raíz ocurre por la producción de nuevas células en el meristemo radicular, que se localiza en la parte más distal de la raíz protegido por la cofia y es la región donde se lleva a cabo la división celular. Después de dividirse, las células pasan a la zona de elongación donde aumentan de tamaño antes de diferenciarse. Las raíces laterales surgen a partir de las células del periciclo, que es un tejido adyacente al sistema vascular, para formar un nuevo meristemo. Su función es aumentar la superficie de absorción de la raíz y la exploración del suelo (Figura 1; Himanen *et al*., 2002; Oakenfull *et al*., 2002).

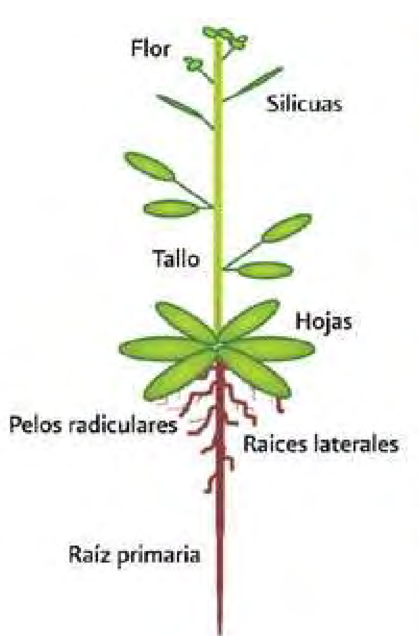

Figura 1. Plantas de Arabidopsis thaliana. A. thaliana es un buen modelo vegetal para el estudio de procesos fisiológicos y moleculares, tiene un ciclo de vida corto y la planta adulta es de tamaño pequeño, por lo que se le puede cultivar in Vitro utilizando cajas de Petri. La planta forma una roseta con varias hojas concéntricas, a partir de donde se produce el tallo que da lugar a las flores y los frutos (silicuas). La raíz primaria tiene un crecimiento continuo y permite la formación de raíces laterales que incrementan la superficie exploratoria y la captación de agua y nutrientes.

EL DESARROLLO VEGETAL

El desarrollo de las plantas difiere del de los animales en tres aspectos principales: 1) el desarrollo de los animales produce un embrión que antes del nacimiento ya posee la mayoría de los órganos para el funcionamiento del organismo, mientras que la embriogénesis de una planta se caracteriza por la producción de grupos de células totipotenciales (células madre) que darán lugar a los meristemos apical y distal del embrión. Estos meristemos se conservan durante el desarrollo postembrionario y permiten la formación de órganos y tejidos que constituyen a una planta adulta. De esta manera, el crecimiento post-embrionario en las plantas no sólo involucra el aumento de tamaño del organismo, como ocurre en los animales, sino también la producción de la mayoría de los órganos. 2) el crecimiento post-embrionario en las plantas es continuo e indeterminado, esto es que las células madres continuamente producen nuevos tejidos, en contraste con la restricción de crecimiento que tienen los animales en los que la organogénesis solo ocurre en la embriogénesis. 3) una diferencia igualmente importante entre los ciclos de vida de las plantas y los animales está relacionada con la naturaleza sésil de las plantas y la manera como ajustan su crecimiento y desarrollo en respuesta a los factores ambientales. Los factores como la luz, la temperatura y la disponibilidad de nutrientes pueden impactar la división, elongación y diferenciación celular y de esta forma regular el crecimiento, actuando como señales que operan mediante rutas de transducción específicas o bien a través de su interacción con reguladores del crecimiento (López-Bucio *et al*., 2003).

ARABIDOPSIS THALIANA

Arabidopsis thaliana es un excelente modelo para el estudio de los procesos fisiológicos y moleculares, ya que el ser una angiosperma de un tamaño pequeño (30 cm) y un ciclo de vida corto (6-8 semanas) tiene una alta fecundidad y se puede cultivar en un espacio reducido (Figura 1). La raíz de *Arabidopsis* tiene características que la hacen un buen

modelo para estudiar procesos de crecimiento y desarrollo. La simplicidad de su organización celular la hace manejable para manipulación experimental y para escrutinios de mutantes a gran escala cubriendo varios aspectos morfogenéticos (Scheres y Wolkenfelt, 1998). El crecimiento de la raíz primaria, la morfología de los pelos radiculares, la elongación de las células y la estructura del meristemo apical son marcadores biológicos fáciles de analizar para identificar nuevos mecanismos de señalización en plantas o nuevos RC (Scheres y Wolkenfelt, 1998).

REGULADORES DEL CRECIMIENTO

Los reguladores del crecimiento o fitohormonas son compuestos de diferente naturaleza química que pueden alterar el desarrollo vegetal en concentraciones pequeñas. El concepto de hormona tal como fue desarrollado para los animales no es fácil de extrapolar a las plantas. Por una parte, las plantas no cuentan con un sistema de transporte tan eficiente como la circulación sanguínea y no tienen el equivalente a un sistema nervioso central como el de los animales para la integración y la coordinación de todas las actividades fisiológicas del organismo.

Aún así, las plantas tienen un sistema sofisticado para integrar información del ambiente y responder activamente a los estímulos bióticos y abióticos; de igual manera, poseen mecanismos para la comunicación entre los diferentes órganos y tejidos. Actualmente se acepta que los reguladores del crecimiento forman un grupo de moléculas integradoras de información, cuyas vías de señalización interactúan con frecuencia para regular la morfogénesis vegetal. Los reguladores mas estudiados son las auxinas, las citocininas, las giberelinas, el ácido abscísico, el ácido jasmónico y el etileno (López-Bucio *et al.*, 2006).

Las hormonas vegetales son generalmente moléculas pequeñas, que ejercen sus efectos biológicos a concentraciones bajas y regulan aspectos celulares diversos. Estos compuestos se pueden transportar entre los diferentes tejidos a través del sistema vascular (xilema y floema) como en el caso de las auxinas o mediante los espacios intercelulares como el etileno (Gray, 2004).

Prácticamente todos los aspectos del crecimiento vegetal están bajo control de los RC, al menos en cierto grado. Un solo regulador puede afectar una amplia gama de aspectos celulares y procesos de desarrollo, mientras que por otro lado, un solo proceso puede ser influenciado, al mismo tiempo, por múltiples hormonas (Gray, 2004).

IDENTIFICACIÓN DE NUEVOS REGULADORES DEL CRECIMIENTO VEGETAL

En años recientes se han descubierto metabolitos con actividad fisiológica en las plantas, cuyo efecto en procesos celulares ya había sido descrito en sistemas animales, incluyendo el L-glutamato y el óxido nítrico. Del glutamato se conoce su capacidad de modificar el crecimiento de las raíces de Arabidopsis y el óxido nítrico afecta la arquitectura de la planta y la adaptación a estrés biótico y abiótico. Otros compuestos, incluyendo las poliaminas, el ácido salicílico y el péptido sistemina se han incorporado a la lista de potenciales hormonas vegetales (Pagnussat *et al.*, 2002; Walch-Liu *et al.*, 2006; Morquecho-Contreras y López-Bucio, 2007). De especial interés ha resultado la identificación de una familia de lípidos pequeños que incluye a las *N*-aciletanolamidas y las alcamidas debido a que están presentes en las bacterias, los animales y las plantas y se cree participan en procesos celulares importantes (Tabla 1; López-Bucio *et al.*, 2006; Morquecho-Contreras y López-Bucio, 2007).

TABLA 1. NUEVOS REGULADORES DEL CRECIMIENTO DE NATURALEZA LIPÍDICA. SE RESALTAN LAS ESTRUCTURAS DE LAS ALCAMIDAS, NAES Y AHLS, EL ORGANISMO QUE LAS PRODUCE Y SUS POSIBLES FUNCIONES FISIOLÓGICAS

Organismo	Compuesto	Función
Animales	Anandamida (NAE 20:4)	Transmisión del impulso eléctrico en el sistema nervioso central. Sincronización del desarrollo del embrión. Desarrollo del cerebro. Proliferación celular.
Arabidopsis thaliana	NAE 10:0	Germinación de la semilla. Proliferación celular. Respuestas de defensa. Regulación de la arquitectura radicular.
Heliopsis longipes	Afinina	Germinación de la semilla. Proliferación celular. Formación de raíces laterales y pelos radiculares.
	N-isobutil decanamida	Control de la estructura del citoesqueleto. Formación de raíces laterales.
Pseudomonas fluorescens	*N*-decanoil-homoserina lactona	Crecimiento poblacional. Formación de biopelículas.

N-ACILETANOLAMINAS

Las *N*-aciletanolaminas o NAEs fueron identificadas en la década de 1950 como constituyentes de la lecitina de soya y los cacahuates. La actividad biológica de estos compuestos en las plantas y su papel como agentes antiinflamatorios en animales ha sido el objeto de investigaciones recientes (Chapman, 2004).

Las NAEs son compuestos de identidad lipídica, constan de una cadena hidrocarbonada que puede tener hasta 20 átomos de carbono, con diferente grado de instauración, unida a un grupo amida y a una cadena lateral que porta un etanol. Una de las NAEs mas conocidas es la anandamida o N-araquidonil-etanolamina, (Tabla 1; NAE 20:4). En los animales, esta substancia ha cobrado importancia por tener actividad como un neurotransmisor y neuromodulador y por regular algunos comportamientos como la alimentación, el miedo y la ansiedad. Ahora está bien establecido que la anandamida y otras NAEs poli-insaturadas se unen a los receptores canabinoides CB1 y CB2, en tanto que las NAEs saturadas o monoinsaturadas no interactúan con estos receptores (Alger, 2004; Grotenhermer, 2006; Berdyshev *et al.*, 2001). En las plantas, las NAEs se acumulan en semillas de diferentes especies. En el maíz y el algodón el contenido total de NAEs varía entre 0.5 y 2.0 µg/g de peso fresco. En las semillas de *A. thaliana* se encuentran hasta en cantidades de 1.206 µg/g de peso fresco, siendo las más abundantes las NAEs 16:0 y 18:2 aunque las NAEs 14:0 y 12:0 se encuentran en cantidades detectables (Chapman, 2004).

Cabe mencionar que los niveles de NAEs identificados en las semillas disminuyen después de la germinación, lo cual indica que su catabolismo es un pre-requisito para la germinación y el crecimiento post-embrionario (Wang *et al.*, 2006). El tejido vegetativo de las plantas tiene niveles más bajos de NAEs en comparación con las semillas y difieren en que en estos tejidos predominan la NAE 12:0 y NAE 14:0, lo cual evidencia que en los tallos y hojas las NAEs de cadena mediana se sintetizan en respuesta a cierto tipo de estímulos. Esta acumulación en las hojas parece ser importante en la defensa de las plantas contra ataques por herbivoría o patógenos y en respuestas protectoras a estrés abiótico (Chapman, 2004; López-Bucio *et al.*, 2006; Morquecho-Contreras y López-Bucio, 2007).

ALCAMIDAS

Las alcamidas se consideraron inicialmente metabolitos secundarios. Este grupo incluye más de 200 compuestos con estructura química similar que están presentes en varias familias de plantas como las Aristolochiaceae, Asteraceae, Brassicaceae, Convolvula-

ceae, Euphorbiceae, Menispermaceae, Peperaceae, Poaceae, Rutaceae y Solanaceae. El interés por estos compuestos está en aumento por su gran variedad de actividades biológicas. Algunas especies vegetales que acumulan estos compuestos han sido utilizados como plantas medicinales por diferentes civilizaciones (Ramírez-Chávez *et al.*, 2004).

Entre las plantas que acumulan alcamidas en sus tejidos están *Echinacea purpurea, Echinacea angustifolia y Heliopsis longipes*, comúnmente, los extractos de estas plantas son utilizados para el tratamiento del resfriado común e infecciones de las vías respiratorias y se ha estudiado la posibilidad de que este efecto ocurra por la interacción de las alcamidas con el sistema inmune aunque esto todavía espera ser confirmado (Ramírez-Chávez *et al.*, 2004; Raduner *et al.*, 2006).

En las raíces de *Heliopsis longipes*, García-Chávez y col., en el 2004 encontraron un contenido total de alcamidas de 9.369 µg/g de peso seco de raíz repartido en 8 compuestos, de los cuales en cuanto a longitud de cadena prevalecían las decamidas (alcamidas de 10 átomos de carbono) seguidas por las undecamidas (11 átomos de carbono). La alcamida mayoritaria es la afinina, que se encuentra hasta en 1 por ciento de peso fresco y a ella se le atribuyen los efectos específicos observados a estas raíces, entre los que se pueden considerar su acción como anestésico local, el estímulo organoléptico, así como su actividad insecticida y bactericida, por lo que se considera que las alcamidas podrían constituir un elemento de defensa contra herbivoría y ataques de patógenos de las plantas que las contienen (García-Chávez et al., 2004; Ramírez-Chávez *et al.*, 2004).

Blancaflor y col. (2003) reportaron el efecto de diferentes NAEs sobre la arquitectura de la raíz de *Arabidopsis thaliana*. En particular, la lauriletanolamida (NAE12:0) en concentración de 20 µM estimuló la proliferación de raíces laterales. En una concentración de 50 µM se observó un 80% de inhibición del crecimiento de la raíz primaria que coincide con el crecimiento celular de la región meristemática. Los efectos morfogenéticos fueron atribuidos a alteraciones en la división celular, organización endomembranal y tráfico de vesículas causado por la NAE12:0 (Blancaflor *et al.*, 2003).

Existen dos reportes en la literatura que indican que las alcamidas también son capaces de modificar el crecimiento de las plantas y que tendrían un papel importante en el crecimiento y desarrollo. En el primer reporte se estudió el efecto de la amidenina, una alcamida aislada del actinomiceto *Amycolatopsis sp*, en el crecimiento de plantas de arroz (*Oriza sativa*). Se observó que la aplicación de este compuesto en concentraciones de 6 hasta 18 µM estimula en un 20 por ciento la producción de biomasa foliar y hasta en un 50 por ciento la biomasa radicular. En concentración de 60 µM la amidenina mostró un efecto represor del crecimiento tanto de la raíz como del follaje (Kanbe *et al.*, 1993).

El segundo reporte corresponde a investigaciones en que se analizaron los efectos de la afinina, la alcamida mayoritariamente presente en las raíces de *Heliopsis longipes*, sobre el crecimiento y desarrollo de

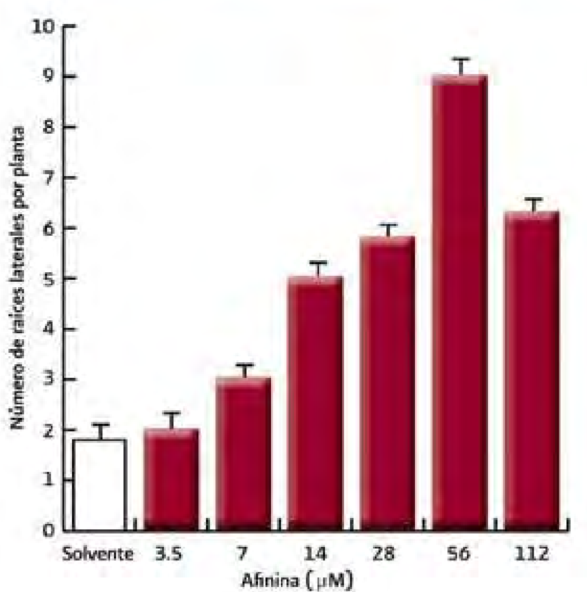

Figura 2. Estimulación del enraizamiento en plantas de *Arabidopsis thaliana* tratadas con la alcamida afinina. Las plantas fueron crecidas in Vitro y tratadas con diferentes concentraciones del compuesto. Nótese el efecto producido en la formación de raíces laterales, estructuras que promueven el anclaje de la planta al suelo y la captación de agua y nutrientes minerales.

la raíz de *Arabidopsis thaliana*. Se encontró que este compuesto altera el desarrollo vegetal de una manera dosis-dependiente. En bajas concentraciones (7 - 14 μM) se observa una estimulación del crecimiento de la raíz primaria y de la formación de raíces laterales, mientras que en concentraciones más altas (28 y 56 μM) se observa una inhibición del crecimiento de la raíz primaria y de las raíces laterales. Otro de los efectos encontrados por el tratamiento con afinina es la estimulación del crecimiento de los pelos radiculares (Figura 2; Ramírez-Chávez *et al.*, 2004). La información anteriormente presentada sugiere que las alcamidas y las NAEs forman parte de un grupo novedoso de reguladores del crecimiento con un amplio potencial biotecnológico.

LAS ALCAMIDAS EN LA INTERACCIÓN PLANTA-MICROORGANISMO

Las plantas interactúan con diversas especies de microorganismos en la rizósfera, porción de suelo que rodea a la raíz, en la cual los exudados radiculares modifican las características físicas y químicas del sustrato (Bais *et al.*, 2006). Se ha observado que los microorganismos son atraídos por los nutrientes exudados de las raíces de las plantas mediante un proceso de quimiotaxis. Estas interacciones pueden ser clasificadas como asociaciones positivas o negativas. Las interacciones positivas incluyen asociaciones simbióticas con hongos micorrízicos o la colonización de la raíz por bacterias promotoras del crecimiento vegetal como *Pseudomonas y Bacillus* (Figura 3; Bais *et al.*, 2006).

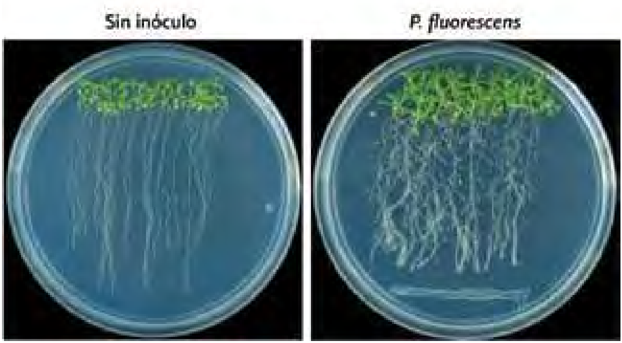

Figura 3. Estimulación del crecimiento de *Arabidopsis thaliana* por bacterias promotoras del crecimiento. Se presentan fotografías de plantas de *Arabidopsis thaliana* crecidas in Vitro. Nótese la estimulación del crecimiento foliar y el amplio desarrollo radicular promovido por la rizobacteria *Pseudomonas fluorescens*.

Estos microorganismos se encuentran asociados con plantas silvestres como *Arabidopsis thaliana* y cultivos como el trigo, el arroz y el frijol estimulando el crecimiento a través de diferentes mecanismos como la modulación de la arquitectura radical, la actividad fotosintética y la producción de fitohormonas como las auxinas, las giberelinas y las citocininas.

Las bacterias utilizan moléculas pequeñas como señales químicas para la comunicación celular, esta comunicación necesita la producción, liberación y detección de factores hormonales similares a las alcamidas conocidos como *N*-acil homoserina lactonas (AHLs) (Tabla 1; Schauder y Bassler, 2001). Nuestras investigaciones han demostrado que las plantas pueden responder a la producción de AHLs mediante cambios en la arquitectura radicular promoviéndose el crecimiento de la raíz primaria, la formación de raíces laterales y el desarrollo de pelos radiculares, contribuyendo a la capacidad total de la planta para la toma de agua y nutrientes (Figura 4; Ortíz-Castro *et al.*, 2008).

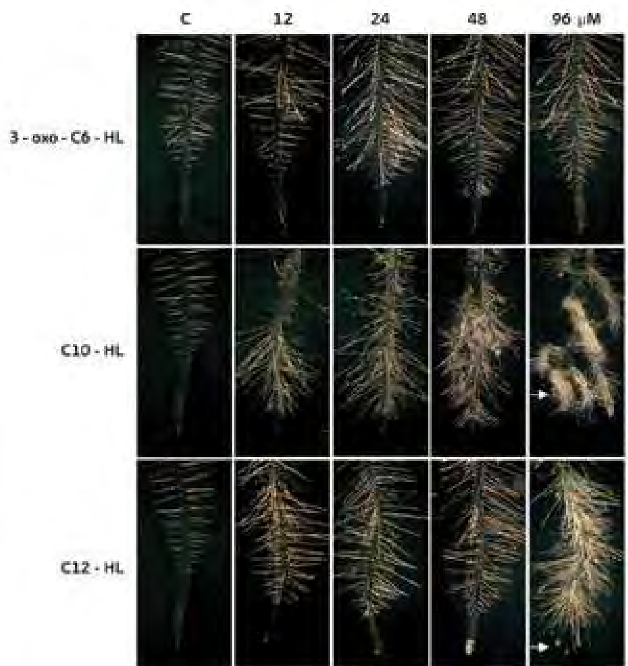

Figura 4. Las bacterias producen compuestos similares a alcamidas denominados N-acil-homoserina lactonas (AHLs) que promueven el desarrollo de los pelos radiculares. Se presentan fotografías representativas de pelos radiculares formados en la región del ápice de la raíz primaria de plantas de *Arabidopsis thaliana* tratadas con diferentes compuestos bacterianos. La flecha indica el ápice del meristemo de la raíz.

POTENCIAL BIOTECNOLÓGICO DE LAS ALCAMIDAS

La participación de las alcamidas en la morfogénesis vegetal y en las interacciones con microorganismos benéficos abre un panorama donde se vislumbran aplicaciones biotecnológicas importantes, por ejemplo, la posibilidad de identificar nuevas cepas de rizobacterias promotoras del crecimiento (Figu-

ra 3). Esto con base en la observación que muchas bacterias asociadas a plantas producen compuestos similares a las alcamidas para regular su densidad poblacional, la identificación del perfil de producción de estos compuestos nos podría llevar al desarrollo de nuevos biofertilizantes.

Adicionalmente, la aplicación en el campo de productos formulados con alcamidas a los cultivos permitiría la formación de sistemas radiculares más robustos, con un aumento en la producción de raíces laterales y un mejor desarrollo de pelos radiculares.

Esta manipulación dirigida de la arquitectura radicular se traduciría en una mejor captación de agua y nutrimentos, optimizando los recursos disponibles en el suelo, incrementando la producción y disminuyendo los costos de producción del cultivo.

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Expediente de Patente de Invención Normal **MX/a/2012/004547**

de **Asunto:** Resultado del Examen de Forma.

México, D.F., a 23 de abril de 2013

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Se tiene por satisfecho el examen de forma señalado por la Ley de la Propiedad Industrial y su Reglamento.

Cabe señalar que de conformidad con los artículos 52 de la Ley de la Propiedad Industrial; 39 y 40 del Reglamento de la Ley de la Propiedad Industrial vigentes, una vez concluido el examen de forma y, después del vencimiento del plazo de 18 meses, contado a partir de la fecha de presentación de la solicitud de patente, o en su caso, de prioridad reconocida, toda solicitud de patente será publicada en la Gaceta, conteniendo los datos bibliográficos comprendidos en la solicitud presentada, el resumen de la invención y, en su caso, el dibujo más ilustrativo de la misma o la fórmula química que mejor la caracterice.

No obstante, la Ley de la Propiedad Industrial y su Reglamento también prevén que, a petición del solicitante mediante escrito, la solicitud de patente podrá ser publicada de manera anticipada, siempre que la misma haya aprobado el examen de forma y el solicitante entere al Instituto el pago de la tarifa correspondiente (artículo 1d).

Así, una vez publicada la solicitud, de conformidad al artículo 52 BIS de la Ley de la Propiedad Industrial, cualquier persona podrá dentro de un plazo de seis meses posteriores a aquella, presentar ante el Instituto, información concerniente a los requisitos de patentabilidad y sus excepciones (artículos 16 y 19 de la Ley de la Propiedad Industrial), por lo que el Instituto dará inicio al examen de fondo hasta haber transcurrido los plazos especificados.

La suscrita firma el presente oficio con fundamento en los artículos 6° fracciones III y XI y 7° bis 2 de la Ley de la Propiedad Industrial (Diario Oficial de la Federación 27/06/1991, reformada el 02/08/1994, 25/10/1996, 26/12/1997, 17/05/1999, 26/01/2004, 16/06/2005, 25/01/2006, 06/05/2009, 06/01/2010, 18/06/2010, 28/06/2010, 27/01/2012 y 09/04/2012); artículos 1°, 2°, 3° fracción V, inciso a), sub inciso i), segundo guión, 4° , 5 ° y 12° fracciones I, II, III, IV y VI del Reglamento del Instituto Mexicano de la Propiedad Industrial (D.O.F. 14/12/1999, reformado el 01/07/2002, 15/07/2004, 28/07/2004 y 07/09/2007); artículos 1°, 2°, 3°, 4°, 5° fracción V inciso a) sub inciso i), segundo guión, 16 fracciones I, II, III, IV y VI y 30 del Estatuto Orgánico del Instituto Mexicano de la Propiedad Industrial (D.O.F. 27/12/1999, reformado el 10/10/2002, 29/07/2004, 04/08/2004 y 13/09/2007); artículos 1°, 3° y 5° inciso e) e i) y penúltimo párrafo del Acuerdo que delega facultades en los Directores Generales Adjuntos, Coordinador, Directores Divisionales, Titulares de las Oficinas Regionales, Subdirectores Divisionales, Coordinadores Departamentales y otros subalternos del Instituto Mexicano de la Propiedad Industrial. (D.O.F. 15/12/1999, reformado el 04/02/2000, 29/07/2004, 04/08/2004 y 13/09/2007).

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