UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO-BIOLÓGICAS
PROGRAMA INSTITUCIONAL DE DOCTORADO
EN CIENCIAS BIOLÓGICAS

"Bioactividad y mecanismos de acción de la melatonina y serotonina en la regulación de la arquitectura de la raíz de *Arabidopsis thaliana*"

Tesis que presenta:

Mtro. en Ciencias. Ramón Pelagio Flores

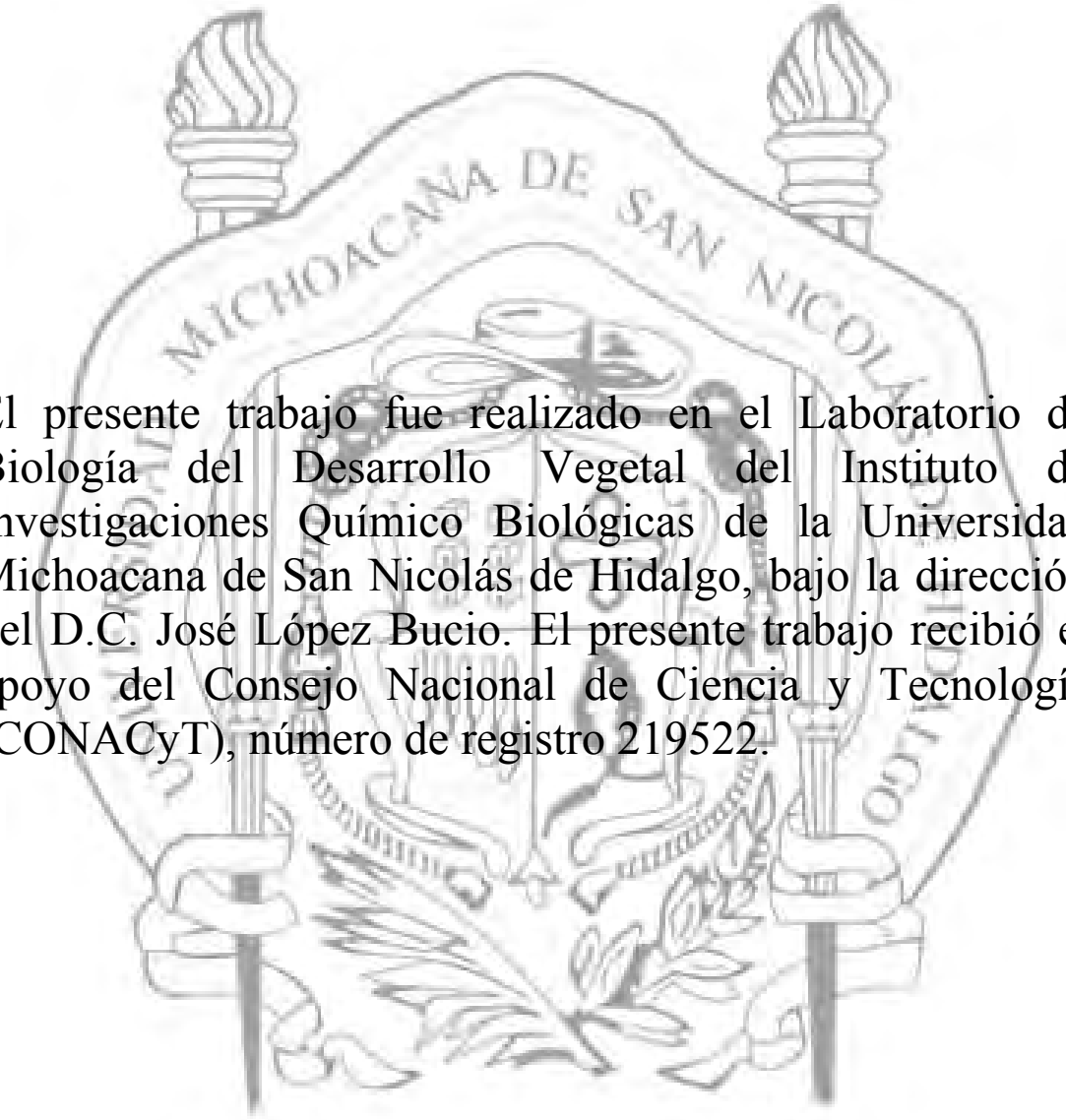
Para obtener el grado de:

Doctor en Ciencias en Biología Experimental

Asesor de Tesis

Dr. en Ciencias. José López Bucio

Morelia, Mich. Agosto de 2015



El presente 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.C. José López Bucio. El presente trabajo recibió el apoyo del Consejo Nacional de Ciencia y Tecnología (CONACyT), número de registro 219522.

DEDICATORIAS

Este trabajo está dedicado:

A mi esposa **Vicky** y mis hijos **América y Ramón**, por todo su amor, comprensión y por todos los momentos de felicidad que me brindan, este trabajo es de ustedes, los amo.

A mis padres, **Ramón Pelagio Urrutia** y **Gloria Flores Rivera** por todo su amor, apoyo incondicional, consejos, confianza y ejemplo de vida. Los amo y siempre estaré orgulloso de ustedes.

A mis hermanos **Chelo**, **Gélica**, **Manolo**, **Gonza**, **Carlos**, **Licha** y **Pepe**, por todo el apoyo, confianza y cariño incondicional que siempre me han brindado, gracias y a todos los quiero mucho. Y finalmente a la nueva generación que junto con mis hijos siempre llenan de alegría a de toda la familia, mis sobrinos el Güero, Chalo y Carlitos.

AGRADECIMIENTOS

Al **D.C. José López Bucio**

Por todo el apoyo y confianza que me brindo en la realización de este trabajo, por su paciencia, esmero y pasión con la que siempre impulsa e instruye a cada uno de sus estudiantes en su formación académica, sin escatimar en compartir sus conocimientos y por siempre enseñar con el ejemplo, ganándose la admiración y respeto de quienes lo conocemos, mi más sincero agradecimiento doc.

A mi comité

Los doctores en ciencias, **Eduardo Valencia Cantero, Elda Beltrán Peña, Lenin Sánchez Calderón** y **Lourdes Macías Rodríguez,** por acceder amablemente a ser parte de mi comité de evaluación, por su disposición, comentarios y sugerencias en cada una de las evaluaciones y por su apoyo en la revisión de este trabajo de tesis.

A mis compañeros y amigos

Alejandro, Amira, Aron, Arnoldo, Cesar, Cesar Peña, Chavacano, Chavita, Edith, Gus, Javier, León, Liz, Lupita, Marina, Poncho, Randy, Susana y Viridiana por su apoyo, trato y amistad les agradezco sinceramente.

A la UNSNH y al IIQB

Por ser mí casa durante muchos años y hacerme sentir orgulloso de ser Nicolaita.

Al **Consejo Nacional de Ciencia y Tecnología (CONACYT)**, por la beca otorgada, que permitió mi manutención durante la realización de este trabajo (Número de registro: 219522).

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1. RESUMEN

La serotonina y melatonina son dos indolaminas con amplia distribución en las plantas mejor conocidas por su funcionamiento como neurotransmisores en mamíferos. Debido a su similitud estructural con el ácido indol acético (AIA), algunos trabajos pioneros habían sugerido una posible actividad similar a las auxinas para ambos compuestos. En un reporte de nuestro grupo de trabajo en el que se caracterizaron los efectos de la serotonina sobre el crecimiento y desarrollo de plantas de *Arabidopsis thaliana* y su relación con la vía auxínica, se encontró que la serotonina regula diferentes procesos del desarrollo de la raíz actuando como un inhibidor de la respuesta a las auxinas. La presente investigación se realizó para determinar si la melatonina también tiene la capacidad de regular procesos del crecimiento y desarrollo de las plantas de manera similar a la serotonina o si puede actuar como una auxina, caracterizando sus efectos sobre la arquitectura del sistema radicular. A diferencia de lo observado con serotonina, la cual en altas concentraciones presenta efectos represores sobre el crecimiento de la raíz primaria, la formación de raíces laterales y en el desarrollo de pelos radiculares, la melatonina promovió la formación de raíces laterales y adventicias sin afectar de manera importante el crecimiento de la raíz primaria y solo mínimamente inhibió el desarrollo de los pelos radiculares. Para profundizar sobre el papel de la auxinas en mediar las respuestas del desarrollo inducidas por la melatonina, se usaron las líneas transgénicas *DR5:uidA*, *BA3:uidA* y *HS::AXR3NT–GUS* como marcadores de la respuesta auxínica, encontrándose que la melatonina no afecta la expresión de dichos marcadores y que sus efectos de regular el desarrollo de las plantas ocurre de manera específica e independiente de la respuesta auxínica.

Debido a que la distribución de especies reactivas de oxigeno (ROS) se ha asociado con alteraciones en el crecimiento de la raíz y puesto que la serotonina es un compuesto con alta capacidad antioxidante, en este trabajo también se determinó el papel de ROS sobre la regulación de los procesos del desarrollo inducidos en respuesta a la serotonina, evidenciando que el gen *RCD1* importante en la regulación de ROS, así como *JAR1* y *COI1,* dos genes esenciales en la vía de señalización del ácido jasmónico, y *ETR1*, *EIN2* y *EIN3*, los cuales median las respuestas al etileno, también juegan un papel muy importante en la regulación del crecimiento de la raíz en respuesta a la serotonina vía cambios en la distribución de ROS. Nuestros datos sugieren que la melatonina y la serotonina modulan diferencialmente el desarrollo de la raíz y que la serotonina altera la homeostasis de ROS a

través de un mecanismo en el que participa *RCD1* y las vías de señalización del AJ y el etileno.

Palabras clave: Arabidopsis, serotonina, melatonina, auxinas, ROS.

2. ABSTRACT

Serotonin and melatonin are two indolaminas widely distributed inplants, well-known due to their function as neurotransmitters in mammals. Since they share structural similarity with indole-3-acetic acid (IAA), early works suggested a possible auxin activity for both compounds. In a previous report from our research group, the serotonin effects on growth and development of *Arabidopsis* seedlings and its relationship with the auxin signaling pathway was characterized. It was found that serotonin regulates different root development processes acting as an inhibitor of auxin response. To determine if melatonin also could be able to regulate plants growth and development in a similar way to serotonin or if it can act as an auxin, we evaluated its effects on root system architecture. In contrast to serotonin, which represses primary root growth, lateral root formation and root hair development in high concentrations, melatonin had the opposite effect promoting lateral and adventitious root formation without affecting primary root growth, and minimally affecting root hair development. These data indicate that the two indolemines affect root morphogenesis in a different and contrasting manner. To investigate the role of auxins in mediating the development responses induced by melatonin, we used the transgenic lines *DR5:uidA*, *BA3:uidA* and *HS:AXR3NT-GUS* as auxin markers, we found that melatonin does not affect the expression of these markers and their effects on plants development are specific and independent of auxin responses.

Because the distribution reactive species of oxygen (ROS) has been associated with alterations in root growth and considering that serotonin is a compound with high antioxidant capacity, the role of ROS in the regulation of root development in response to serotonin was examined. It was found that RCD1 an important gene in ROS regulation as well as *JAR1* and *COI1*, two essential genes in the jasmonic acid signaling pathway and *ETR1*, *EIN2* and *EIN3*, which mediate the ethylene response, play an important role in root growth regulation in response to serotonin via changes in ROS distribution. Our data suggest that melatonin and serotonin differentially modulate the root development and that serotonin alters the homeostasis of ROS through a mechanism involving RCD1, jasmonic acid and ethylene signaling pathways.

3. INTRODUCCIÓN

El crecimiento y desarrollo de las plantas está dirigido por un programa genético intrínseco, el cual es influenciado por una gran variedad de factores endógenos y exógenos. Dentro de los factores endógenos se encuentran las fitohormonas o reguladores del crecimiento. Las auxinas constituyen uno de los principales grupos de reguladores debido a que controlan las respuestas a la luz y la gravedad, la formación de pelos radiculares, la formación de raíces laterales y adventicias, así como el desarrollo del sistema foliar (Woodward y Bartel 2005). El ácido indol-3-acético (AIA) es considerado como la principal auxina de las plantas, al que se han atribuido la mayoría de los efectos sobre la morfogénesis, por lo que la homeostasis de dicho regulador juega un papel muy importante en el mantenimiento de un óptimo crecimiento y desarrollo vegetal (Ljung et al. 2002; Leyser 2006; Mockaitis y Estelle 2008). Si bien el AIA es uno de los derivados del triptófano mejor caracterizados en plantas, existen otros derivados de este aminoácido que son sintetizados de manera natural por las plantas y que están relacionados estructuralmente a dicha auxina, mismos que podrían tener una función en la regulación del desarrollo de las plantas, como es el caso de la serotonina y la melatonina.

Las indolaminas serotonina y melatonina son moléculas bioactivas que en animales participan en la regulación de importantes procesos fisiológicos tales como la regulación de los ritmos circadianos, el humor, el sueño, la ansiedad, la temperatura corporal, el comportamiento sexual y la reproducción (Reiter 1993; Veenstra-VanderWeele et al. 2000; Galano et al. 2011). En plantas, ambos compuestos han sido identificados y cuantificados en un gran número de especies pertenecientes a diferentes familias encontrándose en un amplio rango de concentraciones que va desde pg/g hasta μg/g dependiendo de múltiples factores, incluidos la especie de planta, el tejido analizado las condiciones de crecimiento e incluso de los métodos de cuantificación (Reiter et al. 2007; Paredes et al. 2008; Ramakrishna 2011).

La serotonina ha sido identificada en al menos 42 especies de plantas pertenecientes a diferentes familias, en tejidos como las raíces, las hojas, las semillas y los frutos así como en derivados de plantas (vinos y alimentos procesados) (Grose 1982; Engstrom et al. 1992, Roshchina 2001). De igual manera, la melatonina se ha identificado en un gran número de especies, muchas de ellas utilizadas en la medicina tradicional, destacando además que ambos compuestos han sido encontrados en todas las plantas en las que se han buscado, razón por la cual, se han venido realizado diversas investigaciones para tratar de elucidar la

función que desempeñan en estos organismos. La serotonina se ha implicado en procesos del desarrollo como la floración, la senescencia, morfogénesis, respuestas de defensa y estrés, entre otros (Odjakova y Hadjiivanova 1997; Murch et al. 2001; Roshchina 2001; Ishihara et al. 2008; Kang et al. 2009a), mientras que la melatonina se ha asociado con la coordinación del fotoperiodo, el control de la fisiología reproductiva, la regulación de la apoptosis, la detoxificación de radicales libres y el crecimiento y desarrollo (Murch et al. 2001; Murch y Saxena 2002; Hernández-Ruiz et al. 2004; Paredes et al. 2008).

Dada la similitud estructural de la serotonina y la melatonina con AIA, inicialmente se creía que tanto la serotonina como la melatonina podrían ser compuestos con actividad auxínica y regular procesos del crecimiento y desarrollo de una manera similar al AIA (Murch et al. 2001; Hernández-Ruiz et al. 2004, 2005; Afreen et al. 2006; Arnao y Hernández-Ruiz 2006, 2007; Chen et al. 2009; Posmyk et al. 2009). Sin embargo, a diferencia de los sistemas animales, en los cuáles estas indolaminas han sido ampliamente estudiadas, la información existente respecto a su función, así como sus posibles mecanismos de acción es escasa.

En un trabajo previo, se estudiaron los efectos de la serotonina sobre el crecimiento y desarrollo de plantas de *Arabidopsis thaliana* y su relación con la vía auxinica, donde se encontró que la serotonina tiene la capacidad de modular la arquitectura del sistema radicular de manera dependiente de su concentración, probablemente actuando como un inhibidor de auxinas (Pelagio-Flores et al. 2011). Este estudio indicó que la serotonina tiene la capacidad de regular procesos del desarrollo de las plantas pero de manera independiente a la activación de la vía auxínica. Sin embargo, con el objetivo de esclarecer si la melatonina podría actuar de manera similar a la serotonina o si a diferencia de ésta, la melatonina contribuye a la respuesta auxínica, este trabajo se plantea con la finalidad de profundizar sobre los mecanismos de acción de la serotonina y melatonina. A diferencia de la serotonina, la melatonina promovió la formación de raíces laterales y adventicias sin afectar de manera importante el crecimiento de la raíz primaria y solo ligeramente el desarrollo de los pelos radiculares. También se investigó el papel de la señalización por auxinas en las alteraciones del desarrollo inducidas en respuesta a la melatonina en plantas de *Arabidopsis*, utilizando las líneas transgénicas *DR5:uidA*, *BA3:uidA* y *HS::AXR3NT–GUS* como marcadores de la respuesta auxínica. Se encontró que la melatonina no afecta la expresión de dichos marcadores de manera similar a como lo hacen las auxinas induciendo su expresión ni como lo hace la serotonina inhibiendo su expresión, lo que sugiere que la

melatonina tiene la capacidad de regular el desarrollo de las plantas de manera específica e independiente de la respuesta auxínica y diferencialmente a la serotonina.

Continuando con el estudio de los mecanismos que participan en la regulación de las respuestas de la planta a la serotonina, se investigó el papel de las especies reactivas de oxígeno (ROS) en la regulación de los procesos del desarrollo inducidos por la serotonina, debido a que la serotonina es un compuesto con alta capacidad antioxidante (Foyer y Noctor 2013, Wrzaczek et al. 2013, Kangasjärvi y Kangasjärvi 2014; Dunand et al. 2007, Tsukagoshi et al. 2010). Para determinar la participación de las ROS en mediar las respuestas a serotonina, se evaluó el efecto de la serotonina en un grupo de mutantes de *Arabidopsis thaliana* alteradas en vías de señalización que han sido relacionadas con ROS, como son la mutante *rcd1* alterada en las respuestas a ROS, las mutantes *coi1* y *jar1* de la vía de señalización del al ácido jasmónico, así como *etr1*, *ein2*, y *ein3* alteradas en la señalización del etileno, que han sido también asociadas directa o indirecta con la señalización de ROS (Ahlfors et al. 2004; Mittler et al. 2011). Interesantemente, todas las mutantes mencionadas mostraron una resistencia parcial a los efectos de la serotonina sobre la inhibición del crecimiento de la raíz primaria, comparado con las plantas silvestres (Col-0), efecto que correlaciona con una la acumulación de ROS en la punta de la raíz primaria de las plantas silvestres pero no en las mutantes. Estos resultados indican que la homeostasis de ROS juega un papel muy importante en la modulación del crecimiento de la raíz primaria en respuesta a serotonina, vía una interacción entre las vías de señalización de las ROS, el ácido jasmónico y el etileno.

4. ANTECEDENTES

4.1. Las plantas

Las plantas al igual que los animales, son organismos multicelulares en los cuales hay división de funciones entre las células que forman los diferentes tejidos y órganos, de tal manera que las funciones celulares están coordinadas de manera precisa para contribuir a la supervivencia del organismo. En general, las plantas se componen de dos sistemas, el sistema aéreo y el radicular. La parte aérea incluye órganos tales como las hojas, los brotes, las flores y los frutos y es el lugar donde se lleva a cabo la fotosíntesis y la reproducción, mientras que la raíz es el órgano responsable del anclaje de la planta al suelo y posee estructuras tales como los pelos radiculares y las raíces laterales que son responsables de la

captación de agua y nutrientes (Himanen et al. 2002). Dada su naturaleza sésil, las plantas tienen que responder eficientemente a los diferentes estímulos para sobrevivir, característica que las convierte en excelentes modelos de investigación para el estudio de procesos celulares, fisiológicos, morfogenéticos y adaptativos.

4.2. *Arabidopsis thaliana* como modelo de estudio

Arabidopsis thaliana es una dicotiledónea perteneciente a la familia de las Brasicáceas que a diferencia de otras plantas utilizadas en investigación, reúne numerosas ventajas, que incluyen un tamaño pequeño (alrededor de 30 cm), un ciclo de vida corto (6-8 semanas), una alta fecundidad (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 últimas décadas ha permitido la creación de bancos de semillas mutagenizadas, de colecciones 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). En particular, su raíz reúne una serie de características que la hacen un excelente modelo para caracterizar los efectos de compuestos con actividad biológica permitiendo estudiar una gran diversidad de procesos morfogenéticos que permiten entender las bases del desarrollo de las plantas (Scheres y Wolkenfelt 1998; López-Bucio et al. 2006).

4.2.1. Sistema radicular de *Arabidopsis*

El sistema radicular está formado por una raíz primaria, las raíces laterales y los pelos radiculares y en muchos casos también por raíces adventicias, la raíz tiene como principales funciones el anclaje al suelo para brindar soporte y la absorción de agua y nutrientes, además de ser la estructura que interactúa con el medio ambiente rizosférico. En *Arabidopsis,* la raíz está formada por diferentes tipos celulares agrupados de manera radial en capas o anillos concéntricos bien definidos, como son la epidermis, el córtex, la endodermis, el periciclo y el sistema vascular que incluye el xilema y el floema. Todas las cuales se originan a partir de un pequeño grupo de células localizadas en el ápice o meristemo de la raíz y que se conocen como células centrales, iniciales o fundadoras. A

medida que la raíz crece, se pueden distinguir tres zonas principales: la de división celular o meristemática compuesta por el meristemo apical (zona de mayor división celular) y el meristemo basal (disminución de la división celular pero mayor expansión celular), seguido de la zona de elongación (ya no ay división celular solo aumento de tamaño) y finalmente la de diferenciación (las células adquieren su forma y función final) (Fig. 1) (Dolan et al. 1993; Schiefelbein et al. 1997; Casimiro et al. 2001; Overvoorde et al. 2010).

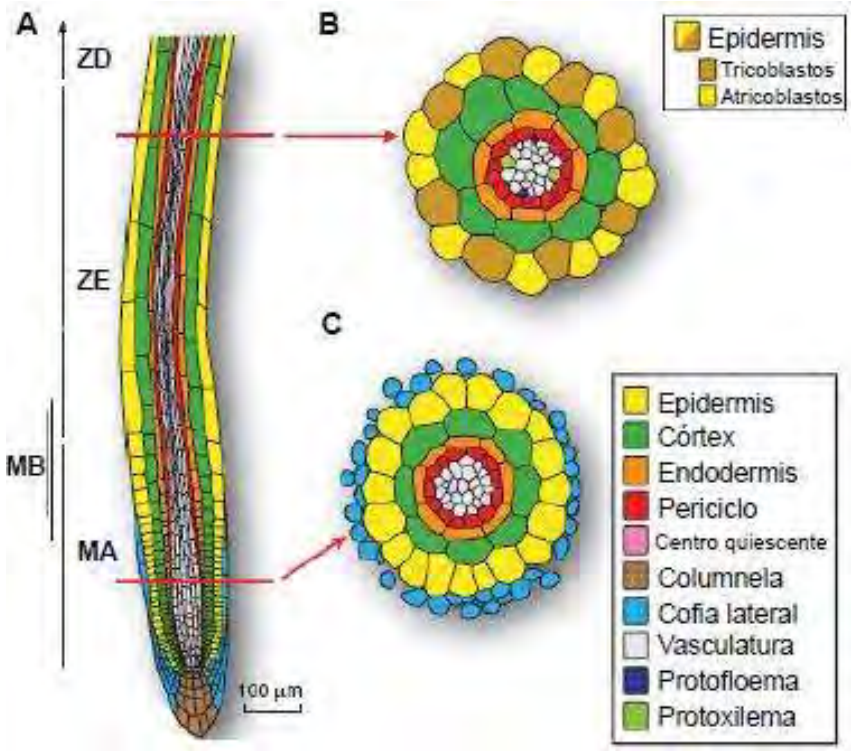

Figura 1. Organización celular de la raíz de *Arabidopsis thaliana*. Organización proximal-distal de la raíz de *Arabidopsis,* meristemo apical (MA), meristemo basal (MB), zona de elongación (ZE) y zona de diferenciación (ZD) (A). Organización radial de la zona indicando las capas celulares en la zona de elongación (B) y el meristemo (C) (Modificado de Overvoorde et al. 2010).

4.2.1.1. Pelos radiculares

En la epidermis de la raíz se desarrollan dos tipos de células, las que forman pelos y las que no los forman conocidas como tricoblastos y atricoblastos, respectivamente. Los pelos radiculares son proyecciones cilíndricas que emergen a partir de los tricoblastos (Dolan et al. 1994; Foreman y Dolan 2001). Los pelos radiculares tienen como función primordial la captación de agua y nutrientes, dichas células contribuyen de manera importante en los procesos antes mencionados, aumentando la superficie de absorción de la raíz. El mecanismo que especifica la identidad de dichas células está determinado por la

posición de las células epidérmicas con respecto a las células corticales, las células epidérmicas en contacto con 2 células corticales son las que se diferenciaran en pelos radiculares (células H), mientras que las células epidérmicas que se encuentren adyacentes a una sola célula del córtex (células N) no se diferenciaran en pelos radiculares, de tal manera que los pelos radiculares se forman invariablemente sobre las células epidérmicas que se encuentran encima de la pared celular entre dos células corticales (Dolan et al. 1994; Dolan y Costa, 2001). Una vez establecida la identidad de las células epidérmicas, aquellas que formarán pelos radiculares inician este proceso formando una protuberancia que formará el pelo radicular. Para el crecimiento de la protuberancia se requiere la expansión de la pared celular del tricoblasto, el rearreglo del citoesqueleto y la actividad de enzimas que modifican la pared celular tales como celulasas y expansinas, a través de un proceso en el cual se requieren auxinas, etileno y ROS. Mutantes alteradas en las respuestas a auxinas como *axr2* y *aux1*, a etileno como *etr1* y/o alteradas en la producción de ROS como *rhd2* por mencionar algunas, muestran alteraciones en el desarrollo de los pelos radiculares, como pueden ser defectos en la iniciación del pelo, en la formación de la protuberancia o en la elongación (Pitts et al., 1998; Grierson y Schiefelbein, 2002; Foreman et al. 2003). El desarrollo de los pelos radiculares, es un proceso ampliamente estudiado lo que han permitido la identificación de muchos de los genes implicados la formación y control en el desarrollo de dichas estructuras, por ejemplo los genes *CEN2*, *RHD3*, *RHD6*, *SHV3*, *SCN1* y *TIP1* de Arabidopsis se ha visto que participan en regular la iniciación de los pelos radiculares (Lee y Schiefelbein, 2002).

4.2.1.2. Las raíces laterales

Las raíces laterales son importantes en la captación de agua y nutrientes ya que un sistema radicular más ramificado abarca mayor superficie exploratoria en el suelo (López-Bucio et al. 2005). Las raíces laterales de *Arabidopsis* como en muchas dicotiledóneas, se forman a partir de las células del periciclo, opuestas a los polos del xilema en la capa de células más cercana al cilindro vascular de la raíz (Dolan et al. 1993; Dubrovsky y Rost, 2005). La formación de las raíces laterales comienza con una serie de divisiones periclinales y anticlinales a partir las células del periciclo pasando por diferentes etapas de desarrollo (I-VII) hasta la emergencia de una raíz lateral madura (Fig. 2) (Malamy y Benfey, 1997a; Casimiro et al. 2001, 2003). En general, la formación de las raíces laterales se puede dividir en 4 etapas principales: 1) la estimulación y des-diferenciación de células

del periciclo, 2) La división celular en las capas que forman el primordio, 3) la emergencia del primordio de la raíz primaria y 4) la activación del meristemo de la raíz lateral para continuar su crecimiento (Malamy y Benfey, 1997a).

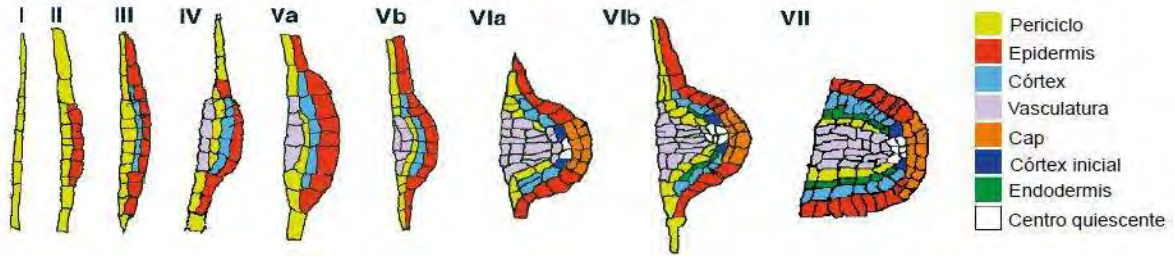

Figura 2. Etapas de desarrollo de las raíces laterales. Representación de las etapas del desarrollo de los primordios (I-VII), para la formación de raíces laterales (Modificado de Malamy y Benfey, 1997a).

4.2.1.3. Las raíces adventicias

Las raíces adventicias son esenciales para la supervivencia de un gran número de especies de plantas. En *Arabidopsis* a diferencia de las raíces laterales que surgen a partir de la raíz primaria, las raíces adventicias no se forman de los tejidos de la raíz sino de la parte aérea de la planta, principalmente a partir del hipocotilo en el follaje y son inducidas en respuesta a la aplicación de auxinas (Falasca y Altamura, 2003). En condiciones normales de crecimiento, la formación de este tipo de raíces en *Arabidopsis* es esporádica. Sin embargo, se han descrito mutantes que presentan una mayor formación de raíces adventicias, lo que sugiere que este proceso está regulado a nivel genético. Las raíces adventicias cumplen con funciones similares a las descritas para las raíces laterales y la raíz primaria e incluso, a pesar de producirse en la parte aérea. Este tipo de raíces presentan una organización y estructura similar a la de la raíz primaria y raíces laterales (Falasca y Altamura, 2003).

4.3. Reguladores del crecimiento

El crecimiento y desarrollo vegetal dependen de la integración de señales ambientales y endógenas, que junto con el programa genético de cada planta determinan su morfología (Gray 2004). Las fitohormonas y/o reguladores del crecimiento, son compuestos de diferente naturaleza química producidos de manera natural por las plantas. Estas moléculas funcionan como señales en la regulación del crecimiento y desarrollo a lo largo del ciclo de vida, integrando cada uno de los estímulos percibidos por la planta, para

ajustar su crecimiento a los diferentes ambientes a los que están expuestas. Los reguladores del crecimiento más representativos y estudiados, son las auxinas, las citocininas, las giberelinas, el ácido abscísico y el etileno, considerados como los reguladores clásicos de plantas (Gray 2004). El número de moléculas con función señalizadora ha venido incrementándose durante las últimas décadas y actualmente se han reportado a el ácido jasmónico, los brasinoesteroides, el ácido salicílico, las N-acil etanolaminas, el glutamato, el óxido nítrico y las especies reactivas de oxígeno, que regulan múltiples aspectos de la fisiología vegetal (Fig. 3) (López-Bucio et al. 2006; Pitzschke et al. 2006; Morquecho-Contreras y López-Bucio 2007; Santner et al. 2009).

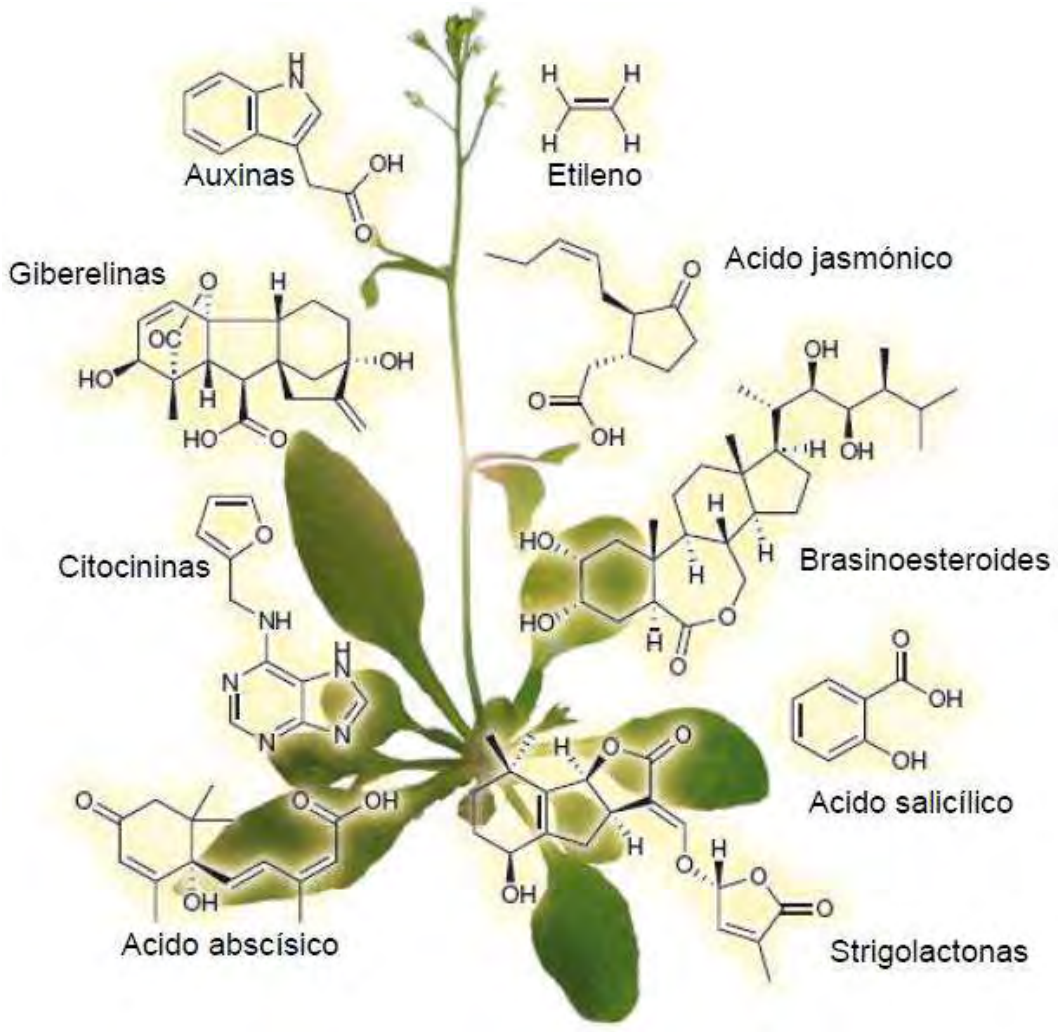

Figura 3. Reguladores del crecimiento y desarrollo de las plantas. Auxinas: regulan la división y expansión celular, procesos de diferenciación, el desarrollo de raíces laterales y la dominancia apical. Citocininas: promueven la división celular y están involucradas en germinación, senescencia y funcionamiento de los meristemos de la raíz y del follaje. Etileno: induce la maduración de fruto, la senescencia y respuestas de estrés biótico y abiótico. Giberelinas: inducen la germinación, elongación del tallo y la floración. Ácido abscísico: promueve la latencia de las semillas y participa en diferentes rutas de señalización por estrés. Ácido jasmónico: modula el desarrollo del polen y las respuestas de estrés y defensa. Brasinoesteroides: regulan la expansión celular y la fotomorfogénesis. Ácido salicílico: regula respuestas de defensa (Gray 2004; Santner et al. 2009).

4.3.1. Auxinas

Las auxinas, cuyo nombre deriva de la palabra griega ―auxein" que significa crecer, forman uno de los principales grupos de reguladores del crecimiento. En los tejidos de las plantas existe un gran número de metabolitos derivados del triptófano entre los que se encuentran las auxinas, fitoalexinas, alcaloides y compuestos indólicos (Radwanski y Last 1995). Sin embargo, el metabolito más estudiado en las plantas y su principal auxina es el ácido indol-3-acético (AIA) (Woodward y Bartel 2005). Esta auxina es de gran importancia para el desarrollo vegetal ya que participa en la regulación de un gran número de procesos durante todo el ciclo de vida: como la regulación de tropismos tanto a la luz como a la gravedad, la regulación de la arquitectura de la raíz y el follaje, la formación de órganos y el desarrollo vascular. El AIA también se encuentra involucrado en el control de la abscisión, incrementa la tasa de división en el cambium, ayuda al desarrollo del ovario y son responsables de la dominancia apical (Woodward y Bartel 2005).

En general los efectos de las auxinas dependen de la concentración, ya que en concentraciones bajas inducen la elongación de los hipocotilos, del follaje y de las raíces, en tanto que en concentraciones más elevadas los efectos son opuestos, ya que inhiben la elongación de la raíz y del tallo (Woodward y Bartel 2005; Benjamins y Scheres 2008). Adicionalmente, existen otras auxinas naturales como el ácido-4-cloroindol-3-acético, el ácido fenilacético, ácido indol-3-propiónico, el ácido indol butírico y las auxias sintéticas como el ácido naftalen-1-acético (ANA) y el ácido-2,4-diclorofenoxiacético (2,4-D) (Enders y Strader 2015).

En *Arabidopsis* las respuestas inducidas por auxinas comprenden una red de procesos regulada y compleja, que incluye la síntesis de auxinas, la disponibilidad en su forma activa, la regulación de su transporte a través de la planta, percepción y señalización, además de las interacciones con otras señales tanto endógenas como del ambiente (Fig. 4). Las proteínas involucradas en convertir dichas señales en gradientes de auxinas y traducirlas en respuestas del crecimiento ya han sido identificadas y caracterizadas (Kieffer et al. 2010; Wright y Nemhauser 2015).

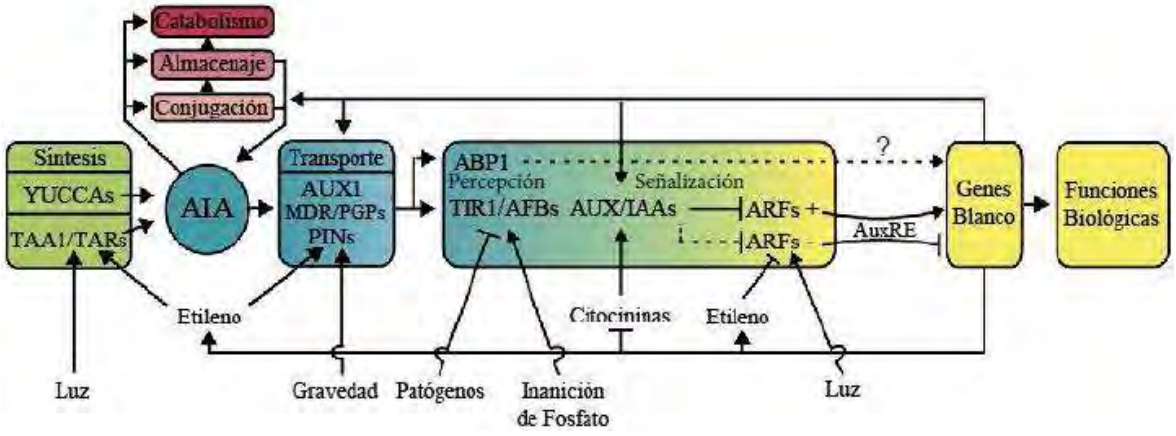

Figura 4. Representación esquemática de la red de procesos e interacciones en la regulación de las respuestas a auxinas. Aquí se incluye la síntesis de AIA (en verde los genes de la síntesis), la regulación de su disponibilidad en la forma activa (en rojo procesos de regulación), el transporte de la auxina (cuadro azul, transportadores), la percepción y señalización (rectángulo central receptores, y factores transcripcionales activadores y represores), además las diversas señales endógenas y ambientales que interactúan en la ruta ejemplificando la complejidad de las interacciones en la regulación de las respuestas a auxinas (Modificado de Kieffer et al. 2010).

4.3.1.1. Biosíntesis del ácido indol-3-acético

La síntesis de ácido indol-3-acético ocurre principalmente en los meristemos apical y radicular de la planta, así como en hojas jóvenes y aunque el AIA fue la primera hormona identificada, a la fecha la ruta biosintética a nivel genético sigue siendo poco clara. Se han propuesto dos principales rutas para la biosíntesis de AIA para contribuir al contenido de AIA en la planta: una independiente de triptófano y otra dependiente de este aminoácido (Ljung et al. 2001; Woodward y Bartel 2005; Delker et al. 2008). Para la vía independiente de triptófano se han sugerido al indol-3-glicerol e indol como probables precursores del AIA, aunque la información existente al respecto es limitada (Ouyang et al., 2000; Zhang et al., 2008). Para la biosíntesis de AIA a partir del triptófano se han propuesto 4 vías, la ruta del indol-3-acetamida (IAM), la del ácido indol-3-pirúvico (IPA), la ruta de la triptamina (TAM) y la del indol-3-acetaldoxima (IAOX), las cuales han sido parcialmente elucidadas. Adicionalmente el triptófano es usado para la síntesis de una diversidad de compuestos de plantas que contienen indol como los glucosinolatos, fitoalexinas y derivados de algunos derivados de triptamina como los indol alcaloides y la serotonina (Fig. 5) (Mano y Nemoto 2012).

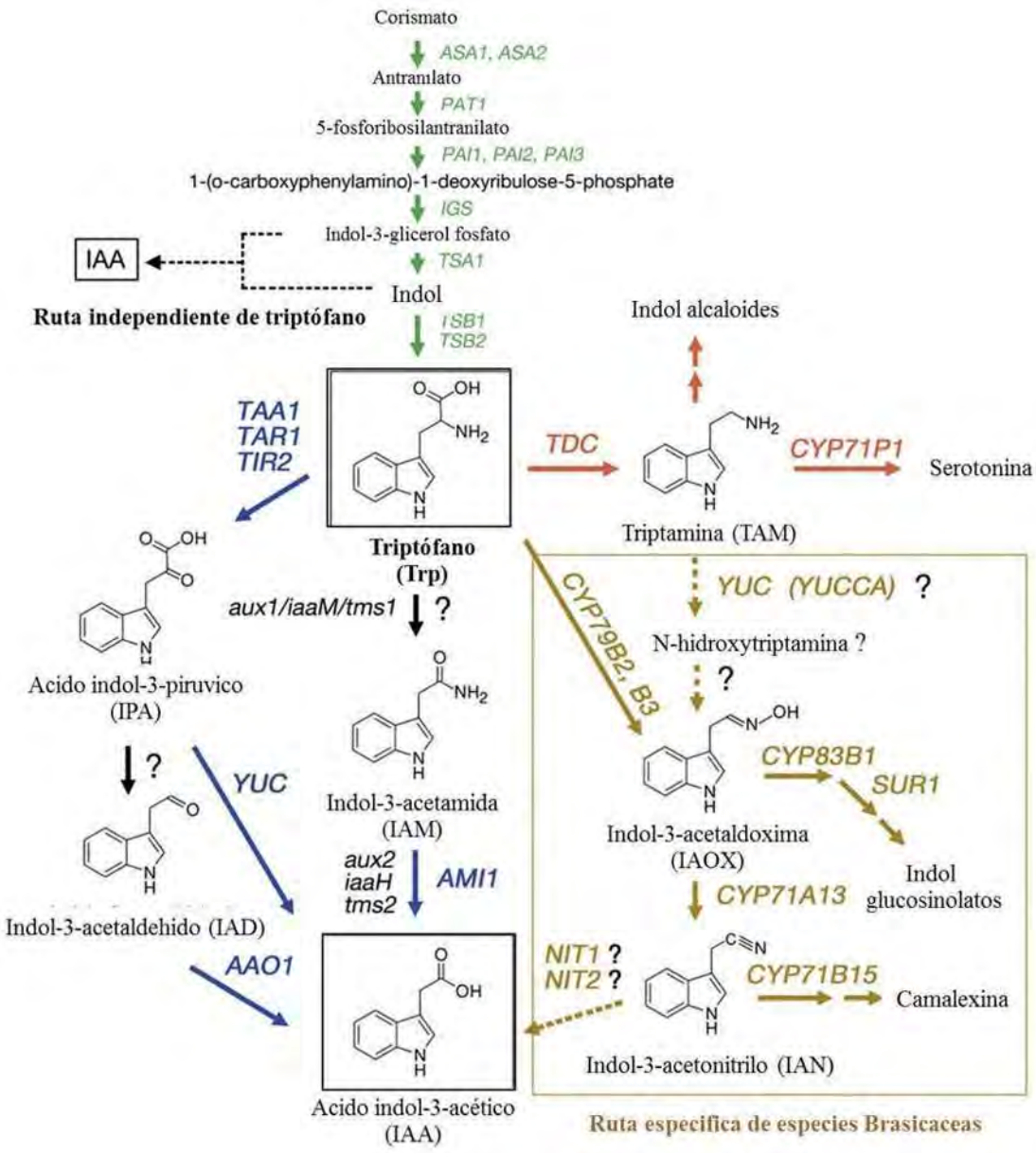

Figura 5. Rutas de la biosíntesis del ácido indol-3-acético (AIA). El triptófano es sintetizado en los cloroplastos a partir del corismato vía indol-3-glicerol (en verde). El AIA se puede sintetizar a través de rutas independientes (flecha negra punteada) y dependientes del triptófano, las enzimas cuyos genes están identificados en *Arabidopsis* se presentan en mayúsculas sin paréntesis y en minúsculas entre paréntesis las mutantes de *Arabidopsis* afectadas en diferentes pasos de la síntesis. El signo de interrogación indica que no se ha identificado los genes involucrado en dichas conversiones (modificado de Mano y Nemoto 2012).

4.3.1.2. Transporte de auxinas

Las auxinas son transportadas desde sus sitios de síntesis hasta tejidos específicos donde activan cascadas de señalización que causan las respuestas de desarrollo a través de diferentes mecanismos (Benjamins y Scheres 2008; Zazímalová et al. 2010). El transporte de auxinas permite la formación de gradientes de auxinas, importantes para la regulación de los diferentes procesos de desarrollo.

Si bien las auxinas son moléculas que pueden ser sintetizadas en un gran número de tejidos (Ljung et al. 2001), el flujo de auxinas a través de toda la planta hacia los diferentes sitios de acción, requiere de un complejo mecanismo de transporte el cual debe ser altamente regulado para un desarrollo normal de las plantas. El AIA se mueve a través de dos vías principales, por el floema conocido como transporte rápido no polar y célula a célula conocido como transporte polar de auxinas (TPA). El transporte polar de auxinas está regulado por proteínas transportadoras de eflujo de las familias PIN-FORMED (PIN), ATP-BINDING CASSETTE (ABC), MULTI-DRUG RESISTANCE (MDR) y P-GLYCOPROTEIN (PGP), que en conjunto regulan la distribución controlada de auxinas para generar gradientes o altas concentraciones en tejidos específicos. En la parte aérea, el transporte ocurre de manera unidireccional, de las células apicales a la base del tallo (transporte basipétalo), mientras que en la raíz se transporta de manera bidireccional aunque predominantemente la auxina se mueve desde la base de la raíz hacia el ápice (transporte acropétalo). El AIA es un ácido débil que dependiendo del pH se puede encontrar en forma protonado (AIAH) o no protonado (AIA^-), en el espacio intercelular el pH es de 5.5 lo favorece que el AIA se encuentre peferentemente en su forma protonada, la cual puede difundirse libremente al interior de la célula, aunque en su forma de anión puede ingresar a la célula a través de proteínas transportadoras de influjo como AUXIN RESISTANT 1 AUX1. Sin embargo, una vez dentro de la célula, debido al cambio de pH del medio (pH=7), se favorece la forma ionizada del AIA la cual requiere del transporte polar mediado por las proteínas transportadoras de eflujo antes mencionadas (PIN, ABC, MDR y PGP) (Swarup et al. 2004; Mravec et al. 2008; Benjamins y Scheres 2008).

4.3.1.3. Mecanismo de percepción y señalización de auxinas

La mayoría de las respuestas inducidas por las auxinas involucran la regulación de la expresión génica, a través de un mecanismo de transducción de señales que ha sido ampliamente estudiado y que actualmente es el más aceptado para explicar gran cantidad de los procesos del crecimiento y desarrollo regulados por auxinas. Dicho mecanismo de señalización depende de tres componentes principales: i) los receptores de auxinas de la familia TIR1 (TRANSPORT INHIBITOR RESPONSE1)/AFB's (AUXIN SIGNALING F-BOX) como parte del complejo Skp1/Cullin/F-box (SCF) E3, ii) la familia de factores transcripcionales de respuesta a auxinas ARFs (AUXIN RESPONSE FACTORS) y iii) los represores de las respuestas auxinas AUX/IAA (AUXIN/INDOLE-3-ACETIC ACID)

(Abel y Theologis 1996; Ulmasov et al. 1997a,b; Guilfoyle 2007). Los receptores de auxinas pertenecen a una familia de proteínas conocida como F-Box, TIR1 fue una de las primeras proteínas de este tipo identificadas en plantas y asociada a la percepción auxinas vía el sistema ubiquitina-proteosoma para la degradación de proteínas. En el proceso de señalización, la auxina se une al receptor en el complejo SCF ubiquitina-ligasa E3, lo que favorece también la interacción de las proteínas AUX/IAA con el complejo, donde son marcadas por ubiquitinación para ser degradadas por el proteosoma 26S, lo que permite la liberación de los ARFs, activando la transcripción de los genes regulados por auxinas. Normalmente bajo condiciones limitantes de auxinas las proteínas AUX/IAA junto con otras accesorias como TPL, se unen a los ARFs reprimiendo la transcripción, como se muestra en la figura 6 (Santner et al. 2009). Si bien este es uno de los modelos mejor conocidos y caracterizados para explicar la mayoria de los procesos en los que participan las auxinas, cabe señalar que algunas de las primeras respuestas inducidas por las auxinas, como la elongación celular, el flujo de iones en la membrana plasmatica y la inhibición de la endosiosis de un número de proteinas membranales, se ha visto que son reguladas a través de mecanismos independiente de la expresión génica mediada por los receptores F-Box (TIR1 y AFBs 1 al 5) (Schenck et al. 2010), procesos que habian sido mayormente sugeridos a ser regulados vía ABP1 (AUXIN BINDING PROTEIN1) (Sauer y Kleine-Vehn 2011). ABP1 es una proteina que durante muchos años ha sido objeto de investigación y se ha propuesto que media respuestas a auxinas actuando como un receptor de esta hormona (Jones 1994; Woo et al. 2002; Ljung 2013). Sin embargo, recientemente se han reportado evidencias que muestran que ABP1 no participa en mediar respuestas a auxinas, debido a que se observó que mutantes nulas en ABP1 no presentaron alteraciónes en el desarrollo asociadas a auxinas, ni repuestas diferenciales respecto a las plantas silvestres, en presencia de auxinas (Gao et al. 2015).

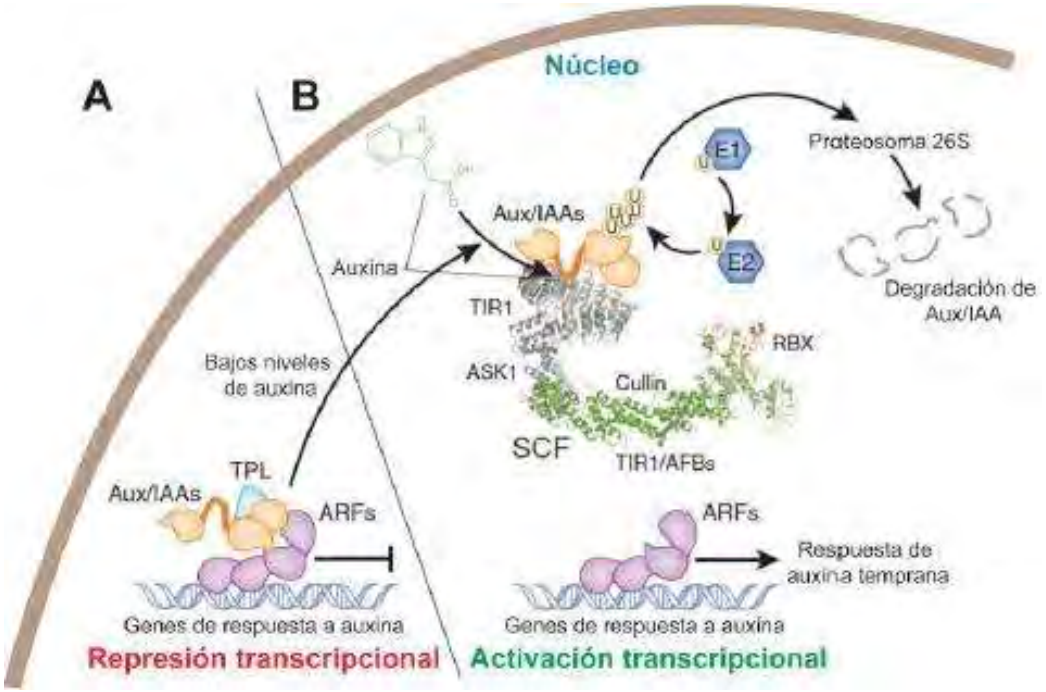

Figura 6. Mecanismo de señalización de las auxinas en *Arabidopsis.* (A) A bajos niveles o ausencia de auxinas las proteínas de la familia Aux/IAA junto con otras accesorias o co-represoras como TOPLESS (TPL), se unen a la familia de activadores transcripcionales conocidos como ARFs, esta unión reprime la transcripción de genes de respuesta a auxinas. (B) Cuando los niveles de auxina se incrementan, se favorece la ubiquitinación y posterior degradación de las proteínas represoras Aux/IAA vía el proteosoma 26S, permitiendo que los ARFs activen la transcripción de los genes de respuesta a auxinas (Modificado de Santner et al. 2009).

4.3.2. Ácido jasmónico

Los jasmonatos son compuestos de naturaleza lipídica, derivados de los ácidos grasos linoleico y linolénico, principalmente. Estos compuestos son reguladores del crecimiento que actúan como moléculas señalizadoras en las respuestas de las plantas para la regulación de numerosos procesos del crecimiento y desarrollo. El ácido jasmónico (AJ) regula aspectos de defensa y desarrollo. Los procesos en los que participa el AJ incluyen las repuestas de defensa inducidas tanto por heridas (mecánicas o bióticas), por organismos patógenos, en el desarrollo de las flores, en el crecimiento de la raíz, en la formación de las raíces laterales, pelos radiculares, la maduración de frutos, senescencia, y desarrollo del polen (Staswick et al. 1992; Berger 2002; Raya-González et al. 2012).

Al igual que las auxinas, el papel del AJ en las plantas y los mecanismos por los cuales modula cada uno de los procesos en los que participa, han sido ampliamente estudiados y actualmente se ha logrado la identificación y caracterización de la mayoría de los elementos que participan tanto en su biosíntesis, percepción y señalización.

4.3.2.1 Biosíntesis de ácido jasmónico

La biosíntesis de AJ comienza en los cloroplastos a partir del a ácido α-linolénico (18:3) liberado de galactolípidos de membranas celulares de los cloroplastos, por acción de la fosfolipasa A1 (PLA1). Posteriormente, a través de un proceso de oxidación en el que participan una familia de lipooxigenasas (LOX), una óxido sintasa (AOS) y una óxido ciclasa (AOC), forma el ácido oxofitodienoico (OPDA), el cual será transportado al peroxisoma en donde ocurre la síntesis de AJ. En el peroxisoma el OPDA es reducido por la reductasa de OPDA (OPR3) y la cadena de ácido carboxílico es cortada por la maquinaria de la β-oxidación de ácidos grasos hasta formar el AJ (Schaller y Stintzi 2009; Wasternack 2014). El AJ es liberado al citosol y conjugado con el amino ácido isoleucina, por la conjugasa JAR1 para dar como producto jasmonoil isoleucina, una forma más activa del AJ (Fonseca et al. 2009) que induce la expresión de los genes regulados por AJ (Fig. 7).

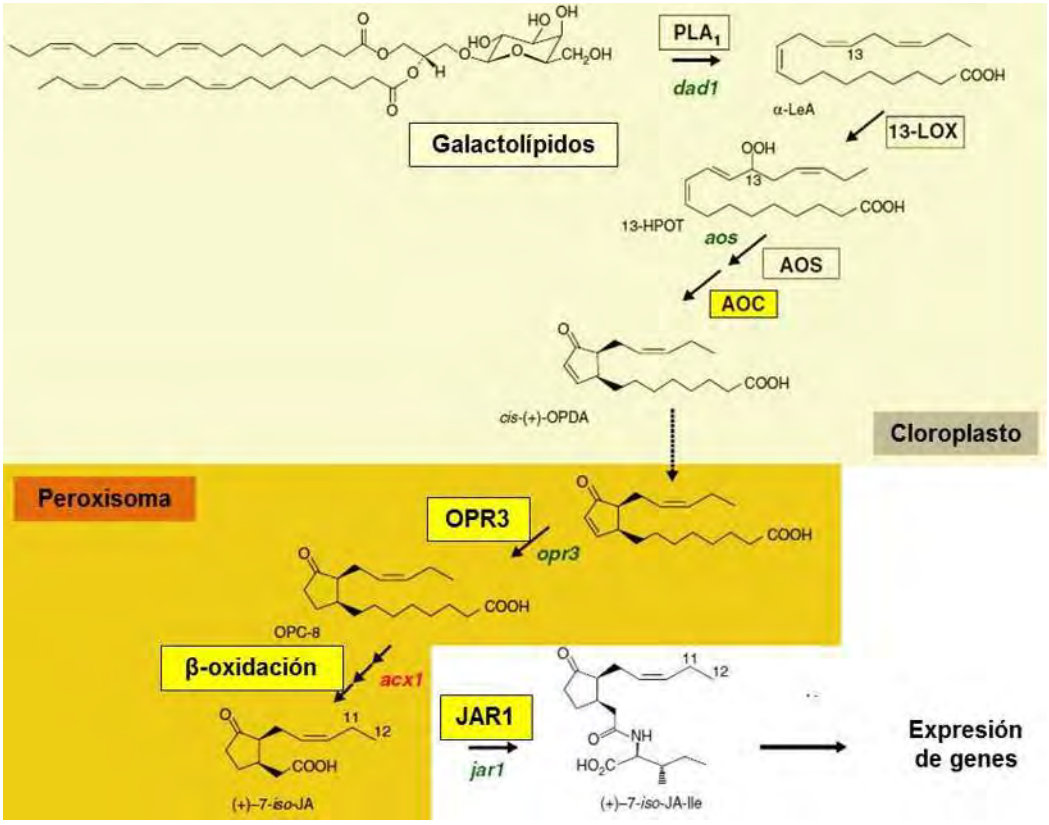

Figura 7. Biosíntesis de ácido jasmónico. La biosíntesis de AJ a partir de galactolipidos comienza en los cloroplastos hasta la formación de OPDA, el cual es transportado a los peroxisomas y convertido a AJ, el cual es liberado al citosol donde puede ser conjugado con el aminoácido isoleucina para dar como resultado la forma más activa del AJ. En mayúscula de indican las enzimas que participan en cada uno de los pasos de la síntesis de AJ y en cursiva las mutantes que se han caracterizado tanto en *Arabidopsis* (verde) como en jitomate (rojo) (Modificado de Wasternack 2014).

4.3.2.2. Mecanismo de percepción y señalización del ácido jasmónico

El mecanismo de señalización del AJ ha sido extensamente estudiado y se dispone de suficiente información respecto a los componentes que participan en cada paso, la mayoría de los cuales se han identificado gracias a la realización de escrutinios genéticos de mutantes con alteraciones en las respuestas al AJ (Turner et al. 2002). Interesantemente, la percepción de varias hormonas de las plantas (AJ, auxinas, giberelinas y etileno) es muy parecida ya que involucran la participación de un sistema de ubiquitinación y degradación de proteínas vía el proteosoma 26S (Kelley y Estelle 2012). En el caso del AJ, la percepción y señalización es particularmente similar a la de las auxinas (Perez y Goossens 2013). Al igual que las auxinas, la señalización del AJ también depende de tres componentes principales: i) un receptor nuclear del tipo F-box COI1 (CORONATINE INSENSITIVE1) como parte del complejo Skp1/Cullin/F-box (SCF), ii) los factores transcripcionales principalmente del tipo (MYC2) y iii) las proteínas represoras de la familia JAZ (JASMONATE ZIM-DOMAIN). La unión del AJ con el receptor COI1 en el complejo SCF ubiquitina-ligasa E3, conduce a la ubiquitinación y degradación de las proteínas JAZ las cuales reprimen la actividad de MYC2 y otros factores transcripcionales cuando los niveles de AJ son bajos, pero una vez degradadas las proteínas JAZ se activa la transcripción de los genes de respuesta al AJ (Fig. 8) (Wasternack 2007).

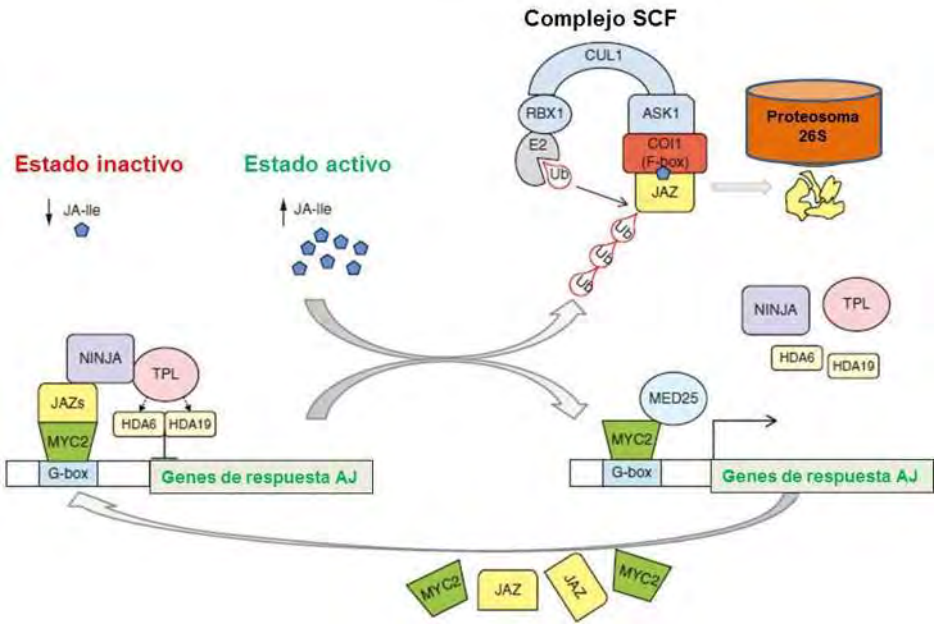

Figura 8. Percepción y señalización del ácido jasmónico. En bajos niveles o ausencia de AJ, las proteínas de la familia JAZ junto con otras proteínas accesorias como la proteína co-represora TPL y la proteína adaptadora NINJA, se unen a activadores trancripcionales como MYC2. MYC2 es uno de los factores transcripcionales que participa en la regulación de la mayoría de las respuestas inducidas por el AJ reprimiendo la transcripción de genes. Cuando los niveles de JA se incrementan, se favorece la

ubiquitinación y posterior degradación de las proteínas JAZ vía el proteosoma 26S, permitiendo así la activación transcripción de los genes (Modificado de Wasternack 2014).

4.3.3. Etileno

El etileno es un regulador de naturaleza gaseosa que participa en la regulación de importantes procesos del crecimiento y desarrollo, así como en diferentes respuestas e interacciones de las plantas con el medio ambiente. Usualmente, bajo condiciones normales de crecimiento las plantas producen pequeñas cantidades de etileno, las cuales se incrementan dependiente de factores como la edad, la senescencia, la maduración de frutos y en respuestas a diferentes estreses (Yang y Hoffman 1984; Kende 1993; Wang et al. 2002). Al igual que otros reguladores del crecimiento, el etileno también afecta la defensa, morfogénesis de raíz y follaje, germinación, senescencia, floración y maduración de frutos (Yoo et al., 2009). Dada su naturaleza gaseosa, a diferencia de la mayoría de los reguladores de crecimiento, el etileno no requiere de un sistema de transporte o de degradación, siendo la biosíntesis el único mecanismo para regular de los niveles de etileno presentes en la planta (Burstenbinder y Sauter 2012).

4.3.3.1. Biosíntesis del etileno

El etileno se sintetiza a partir del aminoácido metionina (Met), a través de una sencilla ruta biosintética, que comienza con la conversión de metionina en S-adenosil-L-metionina (SAM) por acción de la enzima SAM sintetasa, seguido de la conversión de SAM en ácido 1-aminociclopropano-1-carboxílico (ACC) por la ACC sintasa (ACS), que a su vez es oxidado por la ácido ACC oxidasa (ACO) para formar etileno (Fig. 9) (Adams y Yang 1977, 1979; Yang y Hoffman 1984; Kende 1993). En contraste con la simplicidad de la molécula y de su ruta biosintética, los mecanismos de regulación de la biosíntesis son complejos ya que implican la integración de un amplio número de señales endógenas y exógenas.

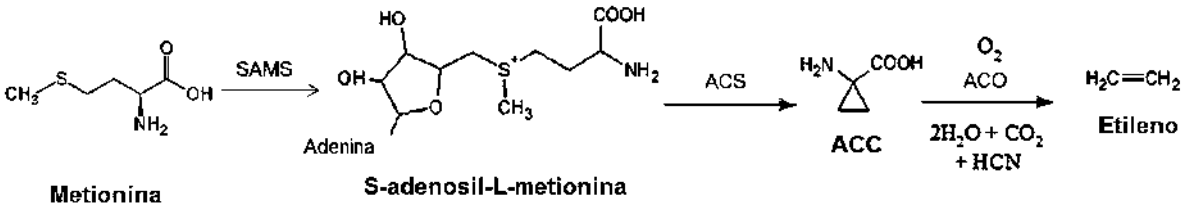

Figura 9. Biosíntesis de etileno en plantas. El etileno se sintetiza a partir S-adenosil-L-metionina (SAM) derivado de la metionina, seguido de la conversión al ácido 1-aminociclopropano-1-carboxílico (ACC) por

la enzima ACC sintasa (ACS) para finalmente ser oxidado a etileno por la enzima ACC oxidasa (ACO). (Van de Poel et al. 2015).

4.3.3.2. Mecanismo de señalización del etileno

Al igual que la biosíntesis, la vía de transducción de señales del etileno ha sido ampliamente estudiada lo que ha derivado en una vía para la señalización de etileno. Esta vía consiste en la percepción del etileno por una familia de receptores (*ETR1*, *ETR2*, *ERS1*, *ERS2*, y *EIN4*) principalmente de localización intracelular sobre la membrana del retículo endoplasmatico, los cuales están asociados con la proteína cinasa CTR1 regulando su actividad. En ausencia de etileno los receptores regulan positivamente a CTR1 reprimiendo las respuestas a etileno. Mientras que en presencia de etileno y su unión con el receptor inactiva CTR1, lo que resulta en la movilización de un fragmento de EIN2 hacia el núcleo a través de un mecanismo desconocido para activar a los factores transcripcionales encargados de mediar las respuestas inducidas por el etileno como EIN3 y ERF1 (Fig. 10) (Xie et al. 2006; Ju et al. 2012; Qiu et al. 2012).

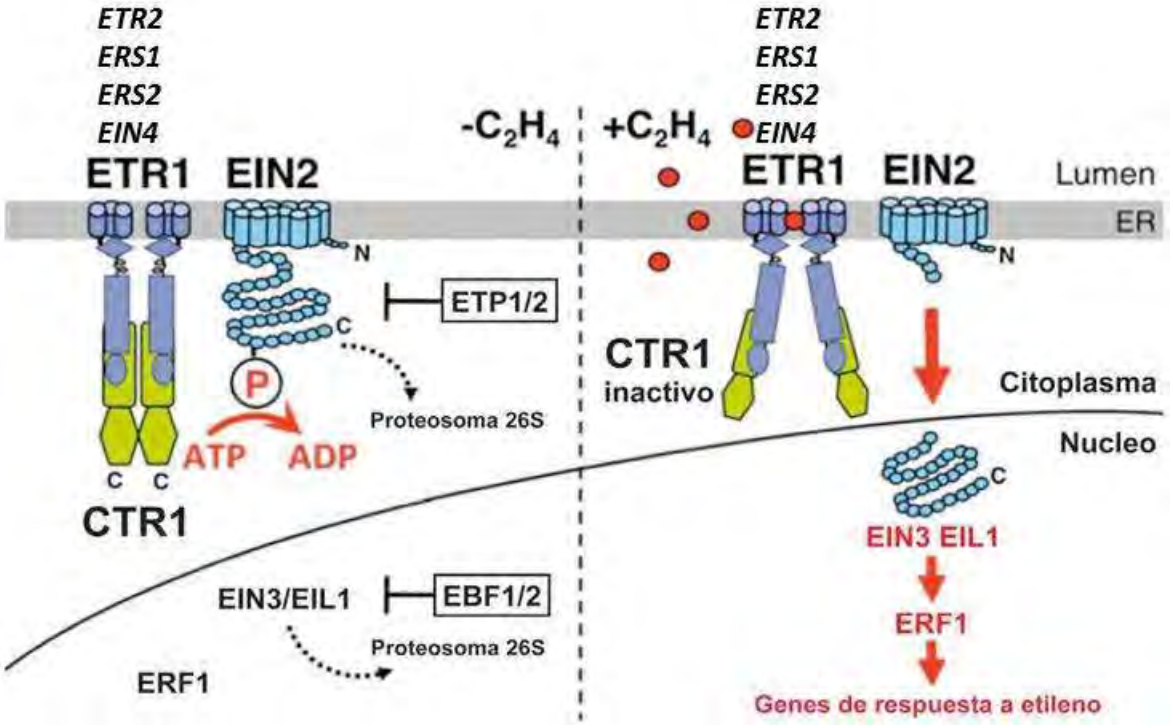

Figura 10. Vía de señalización del etileno. En ausencia de etileno los receptores de etileno mantienen activa a la proteína cinasa CTR1 que fosforila a la proteína EIN2 localizada en la membrana del RE en su dominio carboxilo terminal. Cuando este motivo esta fosforilado no puede ser cortado, lo que impide la movilización del fragmento proteico hacia el núcleo donde activa a los factores transcripcionales río abajo en la señalización. Además en ausencia de etileno tanto EIN2 como los factores transcripcionales son marcados

por las proteínas ETPs y EFBs para su degradación por el proteosoma 26S. Mientras que en pretendencia de etileno los receptores se inactivan, inactivando a CTR1 permitiendo la actividad de EIN2 e inducir las respuestas asociadas al etileno vía EIN3 y ERF1 (Modificado de Ju et al. 2012).

4.4. ROS como moléculas señal

Las especies reactivas de oxígeno o ROS por sus siglas en inglés (Reactive Oxygen Species), como su nombre lo indica son moléculas derivadas del oxígeno (O_2), las cuales son más reactivas que éste en su forma basal O_2, por ser menos estables y reaccionar fácilmente con la mayoría de compuestos de las células como las membranas plasmáticas, el ADN, las proteínas y los lípidos entre otros, mientras que el oxígeno en su forma basal es poco reactivo en condiciones normales. Entre las ROS se incluyen el ozono, el anión superóxido, el peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (OH^-) (Shapiguzov et al. 2012; Kangasjärvi y Kangasjärvi 2014). En plantas, las ROS son continuamente producidas como producto del metabolismo aeróbico, la fotosíntesis y la respiración, así como también en respuesta a diferentes estímulos internos y externos, siendo los cloroplastos y las mitocondrias los principales sitios de producción (Apel y Hirt 2004). Las ROS son mayormente conocidas como moléculas causantes de daño celular, sin embargo, sus efectos son más bien dependientes de los niveles en los que se encuentran presentes en los organismos. Altos niveles pueden causar estrés oxidativo dañando proteínas, DNA y lípidos lo que puede conducir a necrosis y finalmente la muerte, mientras que en bajos niveles actúan como moléculas señalizadoras en la regulación de múltiples procesos del crecimiento y desarrollo, defensa, respuestas a estrés y muerte celular programada en plantas (Pitzschke et al. 2006; Foyer y Noctor 2013, Wrzaczek et al. 2013, Kangasjärvi y Kangasjärvi 2014). En los últimos años, las ROS han surgido como importantes moléculas reguladoras de la división y diferenciación celular de plantas (Dunand et al. 2007, Tsukagoshi et al. 2010). Los mecanismos de regulación involucrados en mediar las respuestas de las plantas a ROS incluyen: 1) la participación de un mecanismo de señalización que involucra un sensor de ROS que inicia una cascada de señales que llevaran a la regulación de procesos biológicos específicos; 2) la regulación de la actividad de factores transcripcionales de manera directa afectando la expresión de genes y 3) actuando como segundos mensajeros para iniciar respuestas biológicas. El estado redox celular tiene un impacto importante en el desarrollo de los organismos y los mecanismos moleculares exactos a través del cual ROS lleva regulación durante el desarrollo celular recién comienzan a entenderse. Aunque varios sensores de ROS y cascadas de señalización

han sido identificados, estos representan sólo un primer avance sobre la señalización redox en la regulación del crecimiento y desarrollo de las plantas (Schmidt y Schippers 2015).

4.5. Indolaminas

Las indolaminas son una familia de aminas biogénicas que comparten una estructura molecular común. Como ejemplos más representativos de indolaminas se encuentra a la serotonina y la melatonina, las cuales son conocidas por su presencia en sistemas animales en los que funcionan en la regulación de procesos fisiológicos importantes por su actividad neurohormonal. Sin embargo, no son exclusivos de sistemas animales ya que se ha logrado su identificación en organismos pertenecientes a diferentes grupos taxonómicos, incluidas las plantas (Roschina 2001; Dubbels et al. 1995).

4.5.1. Serotonina en las plantas

La serotonina se encuentra ampliamente distribuida en las plantas en las cuales se ha encontrado en una gran variedad de especies pertenecientes a diferentes familias (Bowden et al. 1954; Roshchina 2001). Aparentemente, los frutos y las semillas son los principales tejidos en los que se acumula, aunque su contenido varía enormemente entre cada especie y tejido, en un rango de 0.007 μg/g en hojas frescas de lechuga hasta 2,000 μg/g en semillas de *Griffonia simplicifolia* (Fellows y Bell 1970). En plantas, la serotonina se ha asociado en procesos del crecimiento y desarrollo, por ejemplo, estimulación el desarrollo de raíces y la germinación de las semillas en plantas de cebada (Csaba y Pal 1982). Otras funciones en que se ha implicado a la serotonina es la exudación de savia por las raíces, la floración, la permeabilidad de iones, como un antioxidante y en morfogénesis (Odjakova y Hadjiivanova 1997; Murch et al. 2001; Roshchina 2001). Recientemente, la serotonina se ha implicado en la regulación de la senescencia y respuestas de defensa, estas últimas también asociadas con su capacidad antioxidante y mantenimiento de la integridad celular facilitando el reciclamiento eficiente de los nutrientes de las hojas senescentes a los tejidos en desarrollo y funcionando como barrera contra el ataque de organismos patógenos a través de la incorporación de dímeros de serotonina u otros derivados de serotonina en la pared celular fortaleciendo la integridad de la misma (Ishihara et al. 2008; Kang et al. 2009).

Con base en la similitud estructural de la serotonina con el AIA y considerando que ambos son derivados del triptófano, en un trabajo previo de nuestro grupo, se caracterizaron los efectos de la serotonina sobre el desarrollo más particularmente sobre la arquitectura del sistema radicular de plantas de *Arabidopsis thaliana* y su interacción con la señalización de las auxinas. Se encontró que la serotonina regula diferentes procesos del desarrollo de manera dependiente de la concentración ya que a bajas concentraciones (10 a 160 µM) estimula la maduración de raíces laterales sin afectar el crecimiento de la raíz primaria (Fig. 11) mientras que altas concentraciones (300 a 600 µM), reprimen la formación de raíces laterales el crecimiento de la raíz primaria y la formación de los pelos radiculares pero favorecen el desarrollo de raíces adventicias (Fig. 12).

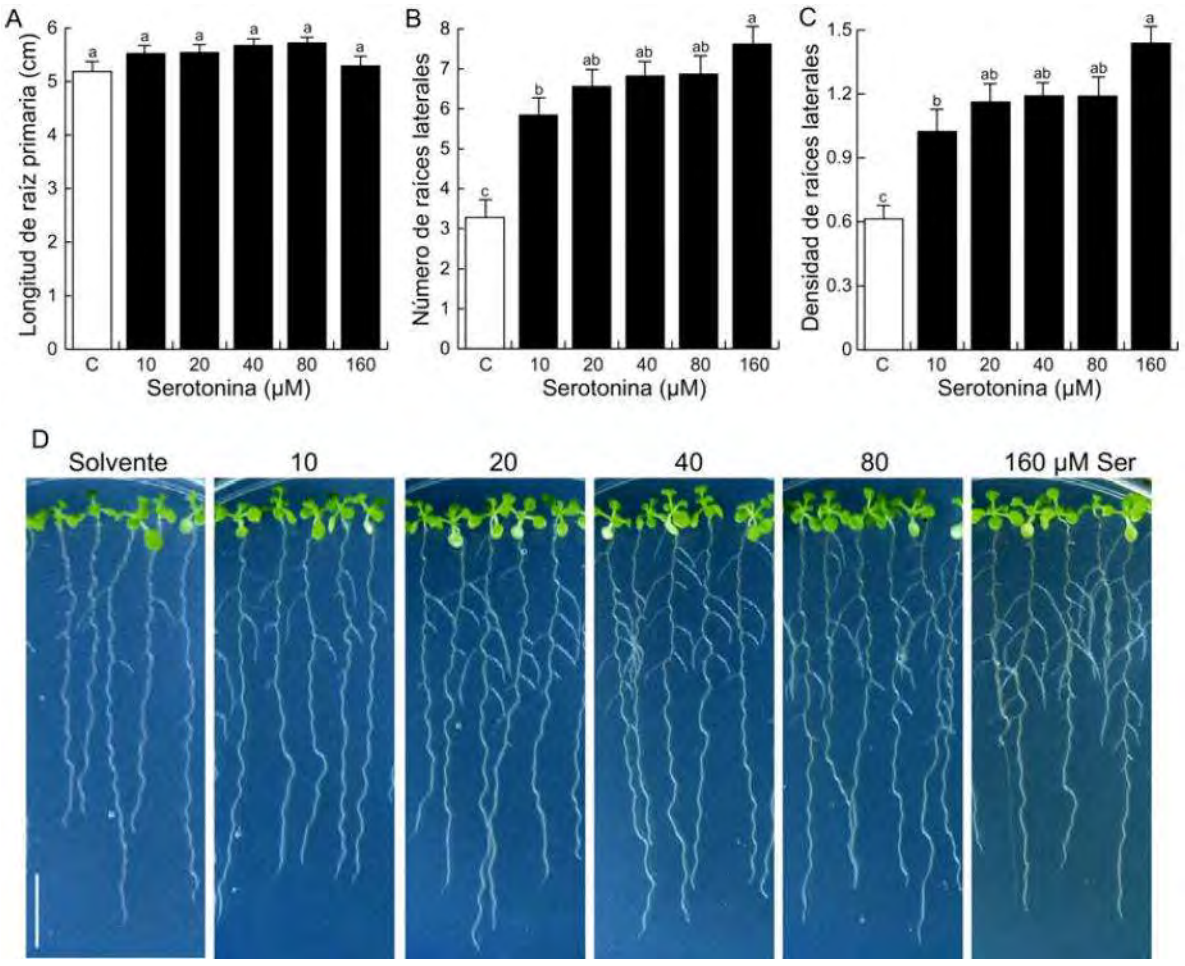

Figura 11. Efecto de la serotonina sobre la arquitectura del sistema radicular de *Arabidopsis thaliana*. (A) crecimiento de la raíz primaria, (B) número de raíces laterales, (C) densidad de raíces laterales, (D) fotografías representativas de los diferentes tratamientos. Se graficó la media ± desviación estándar (n=30). Las distintas letras indican una diferencia estadística con una $P < 0.05$. El experimento fue repetido tres veces con resultados similares (Pelagio-Flores et al. 2011).

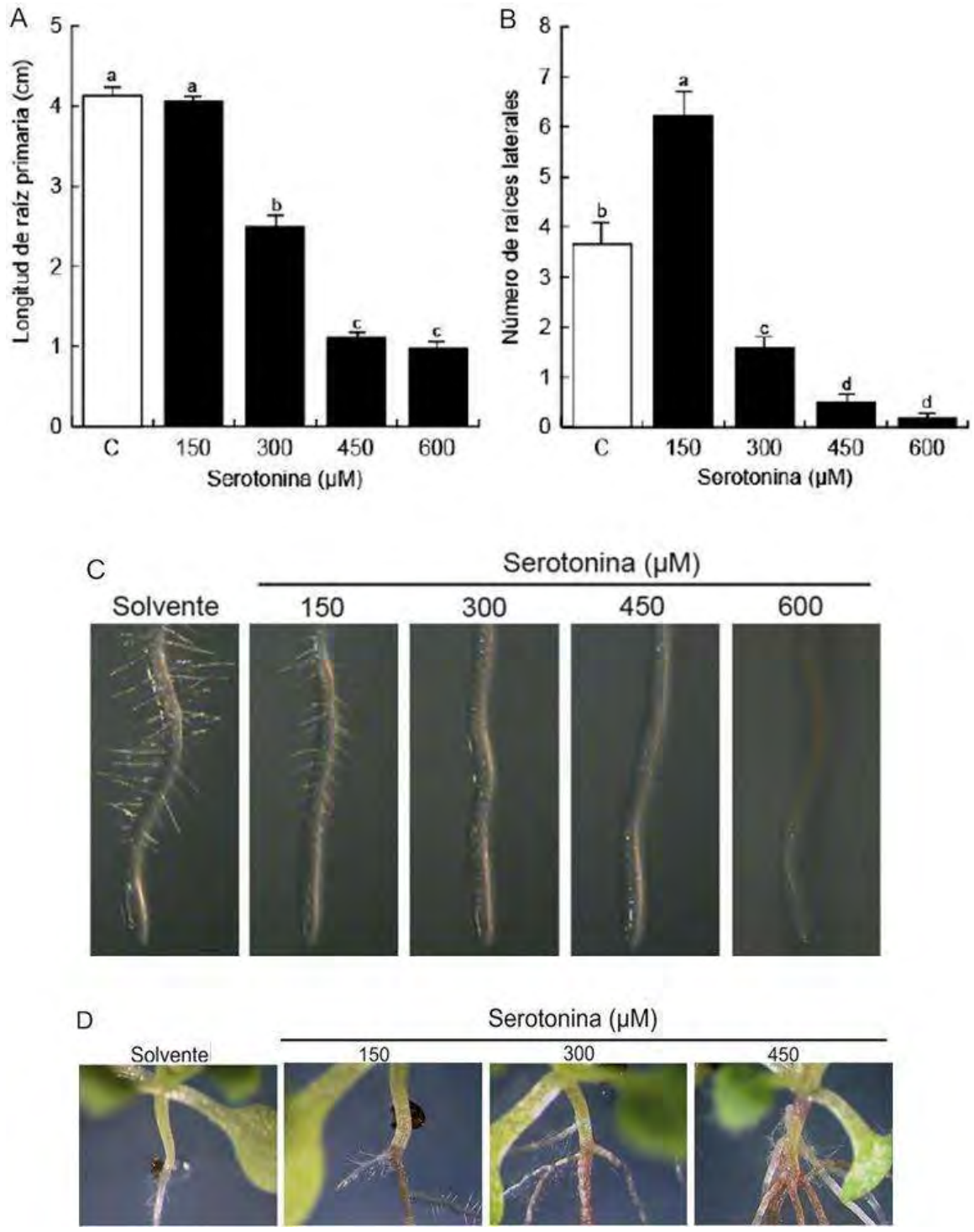

Figura 12. Efecto de altas concentraciones de serotonina sobre la arquitectura del sistema radicular de *Arabidopsis thaliana*. (A) Efecto de la serotonina sobre el crecimiento de la raíz primaria, (B) efecto en la formación de raíces laterales, (C) sobre la formación de pelos radiculares, (D) fotografías representativas del efecto de la serotonina en el desarrollo de raíces adventicias (Pelagio-Flores et al. 2011).

Dada la relación estructural de la serotonina con el AIA como ya hemos mencionado, se determinó el papel de las auxinas en mediar las respuestas a la serotonina analizando el efecto sobre la expresión de genes regulados por auxinas, utilizando las líneas transgénicas *DR5:uidA* y *BA3:uidA* como marcadoras de la respuesta auxínica, ya

que ambas líneas presentan una aumentada respuesta a auxinas y diferentes propiedades de expresión dependientes de la concentración de auxinas y del tejido (Ulmasov et al. 1997; Oono et al. 1998). Así como analizando su efecto sobre la arquitectura de la raíz de mutantes afectadas en genes que participan en mediar la respuesta a auxinas. El conjunto de resultados obtenidos en dicho trabajo mostraron que la serotonina regula la arquitectura la raíz de *Arabidopsis* regulando diferentes procesos del desarrollo posiblemente actuando como un inhibidor natural de auxinas en las plantas (Figura 13).

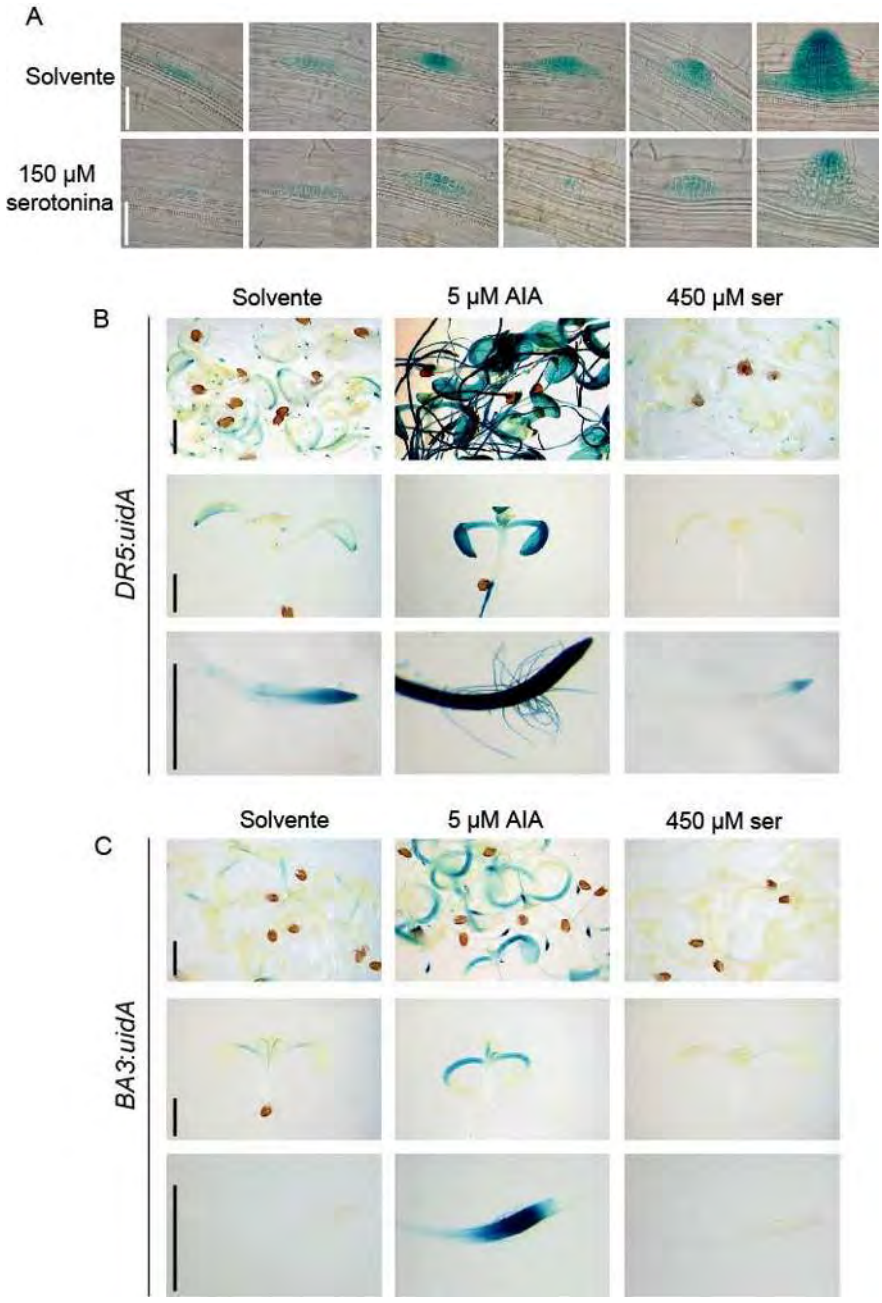

Figura 13. **Efecto de la serotonina sobre la expresión de genes regulados por auxinas**. (A) expresión de *DR5:uidA* en los primordios de RL, (B) efecto comparativo de la expresión con AIA y serotonina de *DR5:uidA*, (C) y de *BA3:uidA*. Nótese como en todos los casos las plantas tratadas con serotonina muestran una menor expresión del marcador indicando una actividad opuesta a la de las auxinas (Pelagio-Flores et al. 2011).

Sin embargo, a pesar de la creciente evidencia que muestra la participación de la serotonina en la regulación de diversos procesos del desarrollo de las plantas, más allá de la caracterización de la su ruta de biosíntesis en plantas de arroz, la información y el conocimiento respecto a los mecanismos genéticos y moleculares que participan en mediar las diferentes respuestas de la planta a esta indolamina, son muy limitados.

4.5.2. La melatonina en las plantas

Inicialmente, la melatonina era considerada o se creía exclusiva de mamíferos, sin embargo a partir de su identificación en el dinoflagelado marino *Gonyaulax poliedra* en 1991, esta creencia cambio e inspiró la búsqueda de esta molécula en otros organismos como por ejemplo en plantas, lográndose su identificación en estos organismos años más tarde (Dubbels et al. 1995; Kolar et al. 1995).

La melatonina se ha identificado en más de 100 especies de plantas pertenecientes a diferentes familias, tanto de monocotiledóneas como dicotiledóneas, estando presente en prácticamente todas las partes de las plantas incluidas raíces, hojas, tallos, flores, frutos y semillas, siendo las semillas y flores las partes en las que se ha cuantificado la mayor concentración de melatonina (Dubbels et al. 1995; Hernández-Ruíz et al. 2005). Aunque se ha visto que su contenido varía considerablemente dependiendo de la especie, de los tejidos analizados y del método de cuantificación (Kolár et al. 1995; Murch et al. 2000; Hernández-Ruíz et al. 2005).

Al igual que en animales, se ha propuesto que la melatonina podría desempeñar un papel importante en la regulación de diversos procesos. Algunos trabajos han asociado a la melatonina con defensa, estrés abiótico, senescencia y la regulación del crecimiento y desarrollo de las plantas. Por ejemplo se ha visto que la melatonina tiene una función protectora contra el estrés oxidativo causado por factores ambientales como la concentración de ozono, las altas y bajas temperaturas, la radiación UV y la contaminación (Van Tassel et al. 2001; Tan et al. 2000; 2002). Consistente con altos niveles de melatonina, el jacinto acuático es altamente tolerante a la contaminación ambiental (Tan et al. 2007a, b), lo que sugiere que esos altos niveles de melatonina probablemente ayudan a las plantas para protegerse contra el estrés ambiental causado por contaminantes del suelo y agua. Una de las propiedades o funciones más interesantes atribuídas a la melatonina es su participación en la regulación del desarrollo de las plantas, promoviendo el crecimiento

en un gran número de especies vegetales (Murch et al. 2001; Murch y Saxena 2002; Hernández-Ruíz et al. 2004, 2005). Al respecto uno de los trabajos pioneros en el cual se sugiere una función similar a las auxinas para la melatonina fue el realizado por Murch et al. (2001), en que se determinó que el aumento en la ramificación de la raíz de plantas de *Hypericum perforatum* L. en cultivos *in vitro*, coincide con un aumento en la concentración de AIA como de melatonina. En años posteriores un número creciente de publicaciones también sugiere que la melatonina participa como un regulador del crecimiento con actividad auxínica (Hernández-Ruiz et al. 2004, 2005; Afreen et al. 2006; Arnao y Hernández-Ruiz 2006, 2007; Chen et al. 2009; Posmyk y Janas 2009).

4.5.3 Biosíntesis de serotonina y melatonina

Las rutas biosintéticas de la serotonina y melatonina fueron primero caracterizadas en animales. En plantas, al igual que en animales la biosíntesis de estas indolaminas también se lleva a cabo a partir del triptófano aunque con algunas diferencias en las rutas de síntesis entre ambos organismos. En plantas se ha propuesto que la síntesis ocurre de la siguiente manera: primero el triptófano por acción de la triptofano descarboxilasa (TDC) es convertido a triptamina, la cual a su vez es convertida a serotonina por la enzima triptamina 5-hidroxilasa (T5H), seguido de la conversión de serotonina a N-acetilserotonina y posteriormente a melatonina por las enzimas serotonina N-acetiltransferasa (SNAT) y N-acetilserotonina O-metiltransferasa (ASMT), respectivamente (Fig. 13) (Tan et al. 2014). Actualmente, todos los genes involucrados en la biosisntesis de serotonina y melatonina en plantas (i.e. *TDC*, *T5H*, *SNAT* y *ASMT*) han sido clonados y caracterizados principalmente en plantas de arroz (Kang et al. 2007a,b; Kang et al. 2011; 2013). Siete genes *TDC* han sido encontrados en el genoma de plantas de arroz de los cuales solo 3 participan en la síntesis de serotonina y melatonina en plantas. Un gen *T5H* involucrado en la síntesis de serotonina pertenece a la familia de genes citocromo P450 identificado como *CYP71P1* (Fujiwara et al. 2010). Estudios recientes han mostrado que los genes *SNAT* de plantas no están relacionados con los *SNAT* de animales (AANAT) ya que no comparten homología ni localización celular. En contraste los genes *ASMT* de plantas y animales comparten homología y ambos tienen localización citoplásmica siendo altamente expresados en el sistema radicular (Byeon et al. 2014). Aunque es posible que en algunas plantas la síntesis de serotonina y melatonina ocurra igual que en animales, ya que se ha encontrado producción de 5-hidroxitriptofano como

intermediario o en la síntesis de estos compuestos (Murch et al. 2000; Park et al. 2012; Tan et al. 2012; 2013).

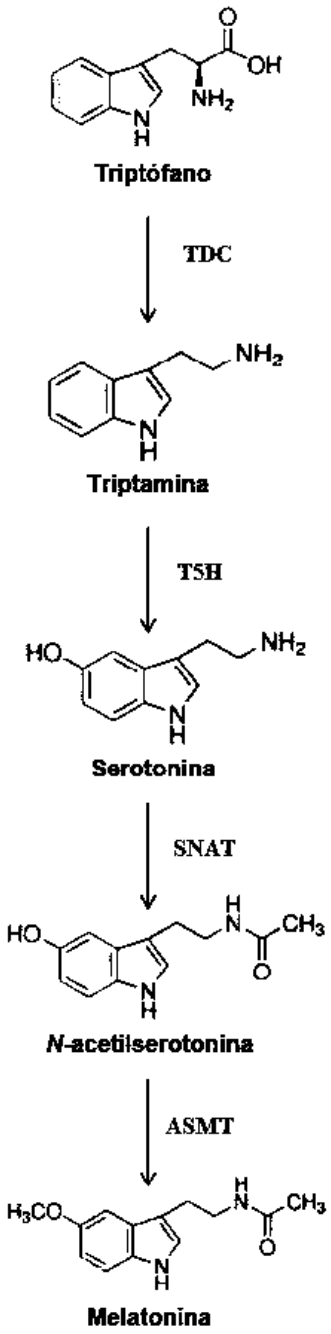

Figura 14. Biosíntesis de serotonina y melatonina en plantas. La síntesis ocurre partir del triptófano a través de una sencilla ruta donde la triptófano descarboxilasa (TDC) cataliza la conversión del triptófano a triptamina y la triptamina 5-hidroxilasa (T5H) cataliza la conversión de la triptamina en serotonina, seguido por la conversión de serotonina a N-acetilserotonina y finalmente esta última a melatonina por acción de las enzimas serotonina N-acetiltransferasa (SNAT) y N-acetilserotonina O-metiltransferasa (ASMT) respectivamente (Modificado de Kang et al. 2007a).

5. JUSTIFICACIÓN

Las indolaminas serotonina y melatonina son dos compuesto presentes de manera natural en las plantas, organismos en los cuales se ha sugerido su participación en la regulación de procesos del crecimiento y desarrollo a lo largo de su ciclo de vida. Sin embargo, la información y el conocimiento que se tiene respecto a su función en plantas, así como los mecanismos genéticos y moleculares involucrados en mediar las respuestas de ambos compuestos son muy limitados. La caracterización de los efectos de estos compuestos sobre el desarrollo de las plantas y el estudio de su interacción con otras vías de señalización mediante el uso mutantes y líneas transgénicas de *Arabidopsis*, podrían contribuir de manera importante al entendimiento del papel que desempeñan ambas indolaminas en el ciclo de vida de las plantas y de los mecanismos involucrados en mediar las respuestas de las plantas a estos compuestos.

6. HIPÓTESIS

La melatonina regula la arquitectura del sistema radicular de *Arabidopsis* de manera diferencial a la serotonina e independiente de la vía auxínica y que las especies reactivas de oxígeno juegan un papel importante en la regulación de las respuestas inducidas por la serotonina debido a su alta capacidad antioxidante.

7. OBJETIVOS

7.1. Objetivo general

Caracterizar el efecto de la melatonina sobre la arquitectura de la raíz de Arabidopsis y estudiar los posibles mecanismos de acción tanto de la melatonina como de la serotonina.

7.2. Objetivos particulares

1. Caracterizar el efecto de la melatonina sobre el la arquitectura de la raíz de *Arabidopsis thaliana* y su interacción con auxinas.
2. Determinar la participación de las especies reactivas de oxígeno en mediar las respuestas inducidas por la serotonina sobre la arquitectura de la raíz de *Arabidopsis*.
3. Estudiar las posibles interacciones de la serotonina con las vías de señalización de otros reguladores del crecimiento.

8. RESULTADOS

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

8.1. CAPÍTULO I

Melatonin regulates *Arabidopsis* root system architecture likely acting independently of auxin signaling. (Publicado en la revista Journal of Pineal Research con factor de impacto de 9.6) (En revisión pata el libro titulado el por la editorial

8.2. CAPÍTULO II

Serotonin modulates *Arabidopsis* root growth via changes in reactive oxygen species and jasmonic acid-ethylene signaling. (En revisión, en la revista Physiologia Plantarum con factor de impacto de 3.1)

8.3. CAPÍTULO III

Serotonin and melatonin in plant growth and development (En revisión para el libro titulado: ~~–~~Neurotransmitters Serotonin and Melatonin in plants: Their functional role in plants and implications in human nutrition" Taylor & Francis, India.)

J. Pineal Res. 2012; 53:279–288
Doi:10.1111/j.1600-079X.2012.00996.x

Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

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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Received December 28, 2011;
Accepted March 12, 2012.

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

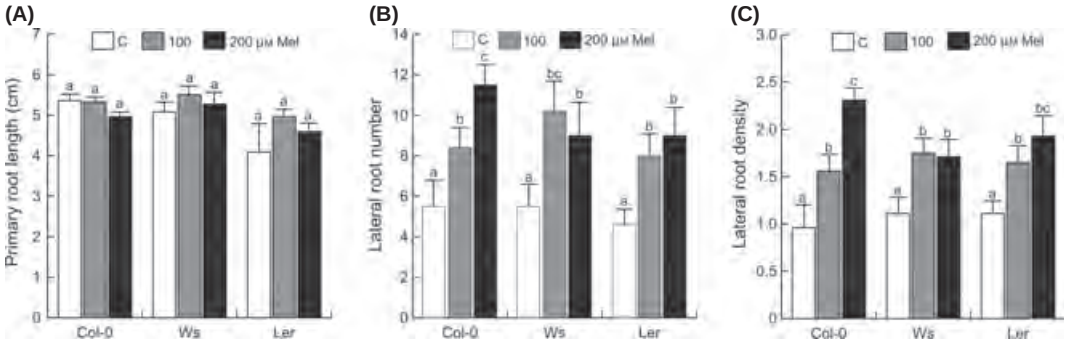


Fig. 1. Effects of melatonin on Arabidopsis root system architecture. Arabidopsis seedlings of three different ecotypes (Col-0, Ws and Ler) were germinated and grown for 10 day under increasing melatonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values 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.

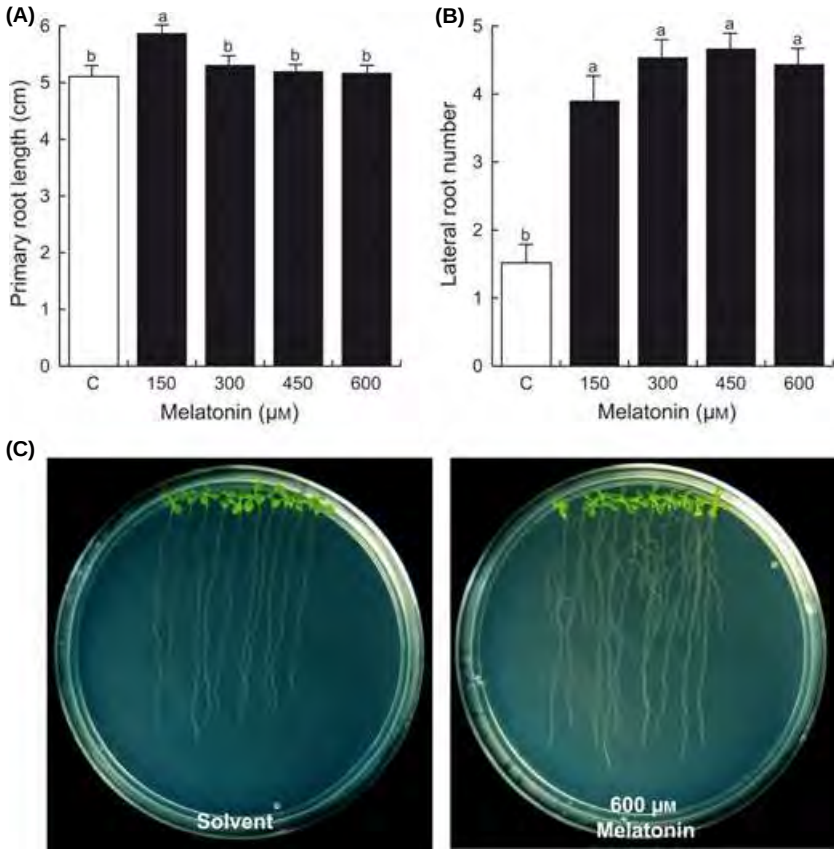


Fig. 2. Effects of melatonin on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 10 day under increasing melatonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Photographs show representative plates of WT (Col-0) seedlings grown 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.

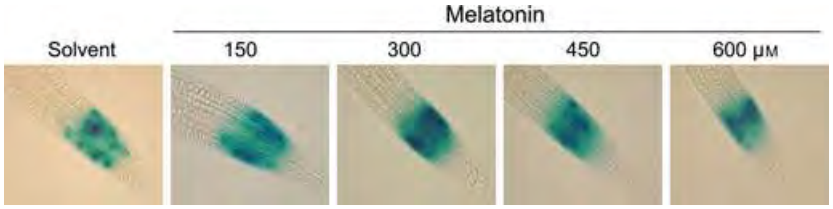


Fig. 3. Effect of melatonin on cell division. *CycB1:uidA* Arabidopsis seedlings were grown for 7 day on 0.2× MS medium supplemented with the indicated concentrations of melatonin. Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from 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

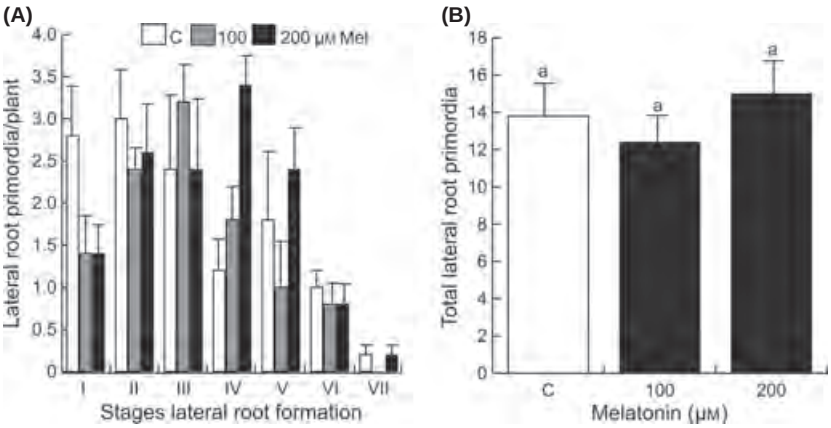
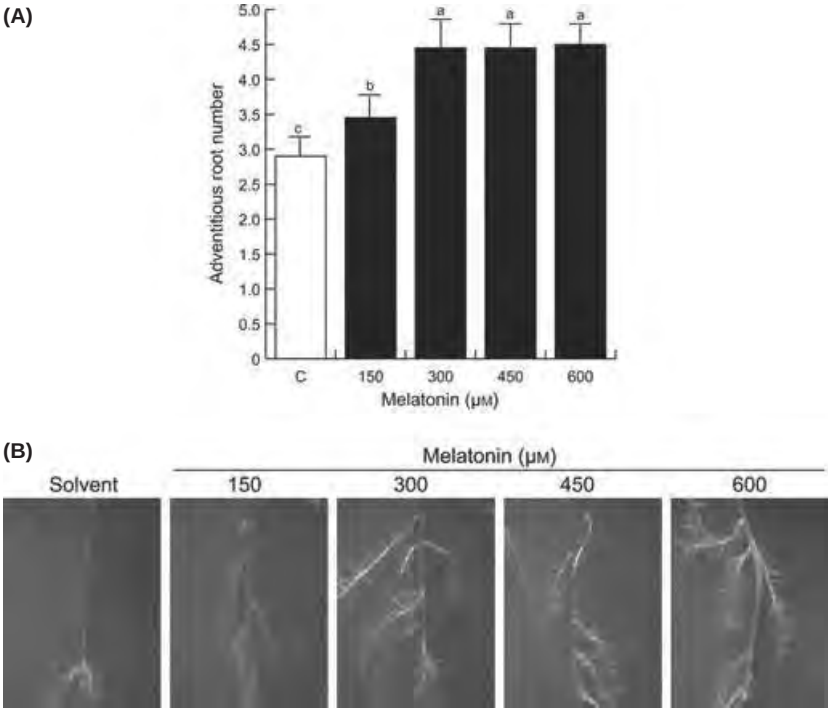


Fig. 4. Effects of melatonin on lateral root primordia development. Arabidopsis Col-0 seedlings were grown for 7 day on agar plates supplemented with the solvent or with 100- and 200-μM melatonin. Data are presented for LRP developmental stages (A) and total LRPs per seedling (B). LRP stages were recorded 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.



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

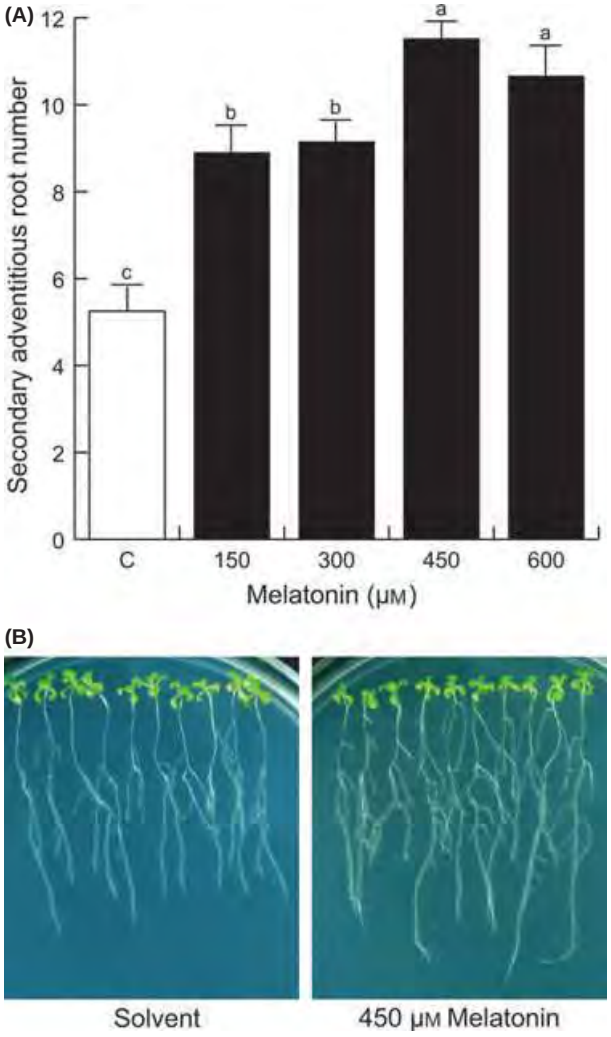


Fig. 6. Effects of melatonin on secondary adventitious root development. Arabidopsis seedlings were germinated and grown in darkness for 5 day on the surface of agar plates containing 0.2× MS medium. Hypocotyl explants were transferred to MS 0.2× medium containing the indicated concentrations of melatonin 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.

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

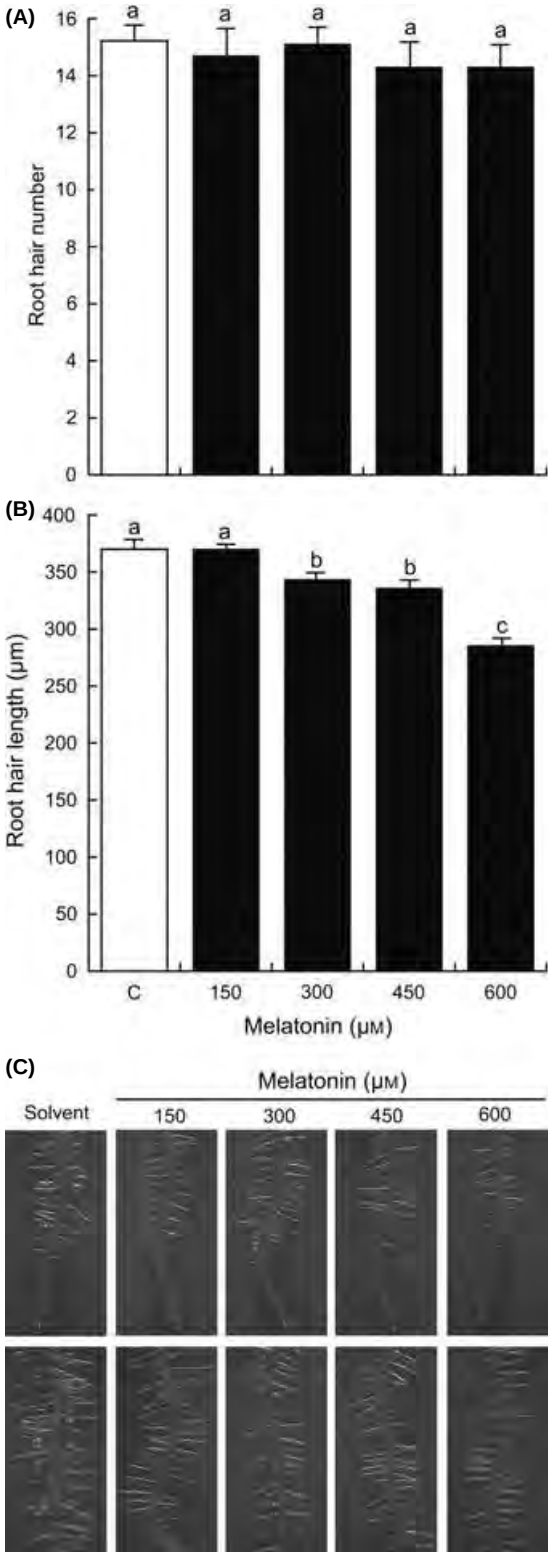


Fig. 7. Effects of melatonin on root hair development. *Arabidopsis thaliana* seedlings were grown for 5 day on MS 0.2× media supplemented with the indicated concentrations of melatonin. (A) Root hair number. (B) Root hair length. (C) Representative photographs of root hairs formed at the differentiation 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.

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.

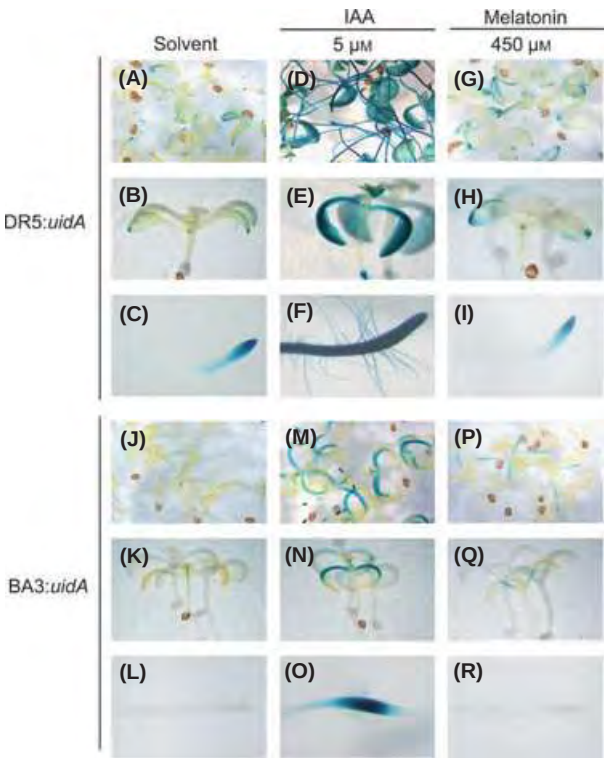


Fig. 8. Effect of melatonin on auxin-regulated gene expression. Twelve-hour GUS staining of *DR5:uidA* Arabidopsis seedlings treated with the solvent (A–C), in medium supplied with 5-μM indole-3-acetic acid (IAA)(D–F) or 450-μM melatonin (G–I) and incubated for 9 hr at 22°C. Twelve-hour GUS staining 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

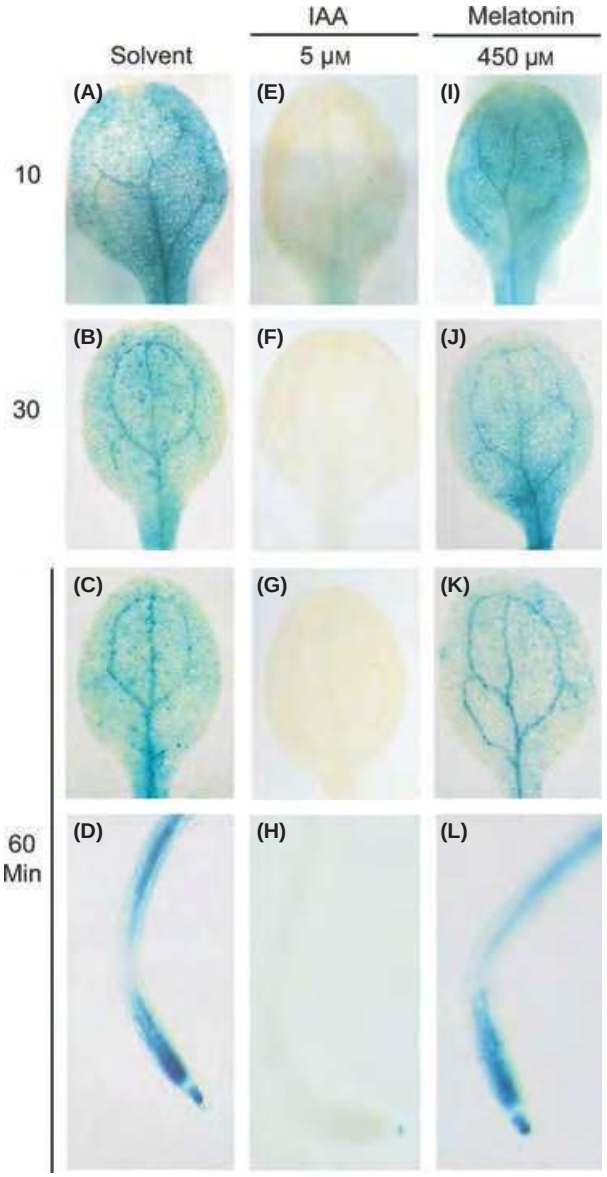


Fig. 9. Analysis of AUX/indole-3-acetic acid (IAA) stability with *HS::AXR3NT-GUS* fusions. Wild-type seedlings expressing the *HS::AXR3NT-GUS* constructs were heat shocked at 37°C for 2 hr. After heat induction, the seedlings were treated with IAA or melatonin for different time periods at the indicated 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.

Acknowledgements

We gratefully acknowledge Hyung Taeg Cho, Tom Guilfoyle, Athanasios Theologis, Mark Estelle, and Bonnie Bartel for kindly providing us with seeds of Arabidopsis transgenic lines. This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT, México, grant no. 80916), Consejo de la Investigación Científica (UMSNH, México, grant no. CIC 2.26), and Marcos Moshinsky Fellowship to JLB.

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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CAPITULO II

Physiologia Plantarum
An International Journal for Plant Biology

Serotonin modulates Arabidopsis root growth via changes in reactive oxygen species and jasmonic acid-ethylene signaling.

Journal:	*Physiologia Plantarum*
Manuscript ID:	Draft
Manuscript Type:	Regular manuscript - Development, growth and differentiation
Date Submitted by the Author:	n/a
Complete List of Authors:	Pelagio-Flores, Ramón; Universidad Michoacana de San Nicolas de Hidalgo, Instituto de Investigaciones Quimico Biologicas Ruiz-Herrera, León; Universidad Michoacana de San Nicolas de Hidalgo, Instituto de Investigaciones Quimico Biologicas López-Bucio, José; Universidad Michoacana de San Nicolás de Hidalgo, Instituto de Investigaciones Químico Biológicas
Key Words:	Arabidopsis, Serotonin, reactive oxygen species, ethylene, Jasmonic acid

Serotonin modulates *Arabidopsis* root growth via changes in reactive oxygen species and jasmonic acid-ethylene signaling.

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Abbreviations: ASMT, N-acetylserotonin O-methyltransferase; Et, ethylene; DAB, 3,3′-diaminobenzidine; DHE, dihydroethidium; H2DCF-DA, 2′,7′-dichlorofluorescein diacetate; JA, jasmonic acid; MeJA, methyl jasmonate; SNAT, serotonin N-acetyltransferase; ROS, reactive oxygen species; TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxilase.

Abstract

Serotonin (5-hydroxytryptamine) is a bioactive indoleamine with neurotransmitter function in vertebrates and represents an emerging signaling molecule in plants, playing key roles in development and defense. In this report, the role of reactive oxygen species (ROS) and jasmonic acid-ethylene signaling in root developmental alterations induced by serotonin was investigated. An *Arabidopsis thaliana* mutant defective at the RADICAL-INDUCED CELL DEATH1 (RCD1) locus was resistant to paraquat-induced ROS accumulation in primary roots and showed decreased inhibition or root growth in response to serotonin. A suite of jasmonic acid (JA) and ethylene (Et) related mutants including *coi1*, *jar1, etr1*, *ein2* and *ein3* showed tolerance to serotonin in the inhibition of primary root growth and ROS redistribution within the root tip when compared to wild-type seedlings. Competence assays between serotonin and $AgNO_3$, a well-known blocker of ethylene action showed that primary root growth in medium supplemented with serotonin was normalized by $AgNO_3$, whereas roots of *eto3,* an ethylene overproducer mutant were oversensitive to serotonin. Our results provide compelling evidence that serotonin affects ROS distribution in roots, involving RCD1 and components of the JA-Et signaling pathways.

Introduction

Serotonin (5-hydroxytryptamine) is a neurotransmitter that controls fundamental physiological processes in animals such as mood, sleep and anxiety (Veenstra-VanderWeele et al. 2000). It is the precursor in the synthesis of melatonin, another well-known molecule with regulatory functions (Paredes et al. 2009, Pelagio-Flores et al. 2012, Arnao 2014). Since the first identification of serotonin in the legume *Mucuna pruriens* (Bowden et al. 1954), an increasing number of reports have evidenced its wide distribution in the plant kingdom, occurring in a wide range of concentrations in wild and edible plants (Roschina 2001, Ramakrisna et al. 2011). Similarly to indole-3-acetic acid (auxin, IAA), serotonin and melatonin biosynthesis occurs from L-tryptophan through a reaction that first converts L-tryptophan to tryptamine by tryptophan decarboxylase (TDC) and then the tryptamine is converted to serotonin by the tryptamine 5-hydroxilase (T5H), followed by serotonin conversion to N-acetylserotonin and then into melatonin by the enzymes serotonin N-acetyltransferase (SNAT) and N-acetylserotonin O-methyltransferase (ASMT), respectively (Kang et al. 2007a, 2007b, Kang et al. 2011, 2013).

An increasing number of reports have evidenced the critical role of serotonin in modulating plant growth and developmental processes including germination, flowering, senescence, shoot branching, root architecture, adaptation to environmental stress, and protection against pathogens (Csaba and Pal 1982, Odjakova and Hadjiivanova 1997, Murch et al. 2001, Roshchina 2001, Ishihara et al. 2008a, 2008b, Kang et al. 2009, Pelagio-Flores et al. 2011). Serotonin modulates root morphogenesis via auxin dependent or independent mechanisms (Csaba and Pal 1982, Murch et al. 2001). In *Arabidopsis*, serotonin inhibited primary root growth and root hair formation while stimulating lateral and adventitious root formation probably acting as a natural auxin inhibitor (Pelagio-Flores et al. 2011). Rice plants that overexpress two putative tryptophan decarboxylase genes, *TDC-1* and *TDC-3* genes, had increased serotonin levels, stunted growth and low fertility, indicating that serotonin overproduction may be deleterious to plants (Kanjanaphachoat et al. 2012). In contrast, via its antioxidant properties and reinforcement of cell walls, serotonin confers plant protection to pathogens and herbivores and delays senescence (Ishihara et al. 2008a, 2008b, Kang et al. 2009).

Reactive oxygen species (ROS), which include the hydroxyl radical (HO^{-}), superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), are continuously produced as a result of the normal aerobic metabolism of plants, the photosynthesis process and in response to different exogenous and endogenous cues. ROS balance in cells is tightly regulated by a metabolic and signaling network that involve the production of antioxidant compounds, antioxidant enzymes and ROS-producing enzymes (Gechev et al. 2006, Mittler et al. 2011, Sharma et al. 2012, Wrzaczek et al. 2013). Initially, ROS were consider toxic products of cells that when accumulating could induce cell damage or death, but currently, ROS also are considered important players in the regulation of different plant growth and development processes such as defense, tolerance to stress, cell division and differentiation among others, by acting as signaling molecules (Foyer and Noctor 2013, Wrzaczek et al. 2013, Kangasjärvi and Kangasjärvi 2014). In plants, ROS signaling occurs through complex mechanisms and hormone response crosstalk via salicylic acid, jasmonic acid and ethylene genetic components (Mittler et al. 2011).

The gene *RADICAL-INDUCED CELL DEATH1* (*RCD1*), which encodes a protein belonging to the (ADP-ribosyl)transferase domain-containing subfamily of WWE protein-protein interaction domain protein family plays an important role in ROS homeostasis (Ahlfors et al. 2004). *Arabidopsis* mutants defective on *RCD1* show high sensitivity to extracellular ROS produced in response to ozone (Overmyer et al. 2000), but intriguingly, these mutants manifest tolerance to the intracellular ROS generated by the herbicide methyl viologen (paraquat), ultraviolet (UV)-B irradiation or freezing stress (Ahlfors et al. 2004, Fujibe et al. 2004, Overmyer et al. 2005). In addition, *RCD1* is involved in a subset of responses to ethylene and methyl jasmonate (MeJA), since *Arabidopsis rcd1* mutant seedlings exhibit decreased expression of MeJA and ethylene regulated genes (Ahlfors et al. 2004).

Jasmonic acid (JA) is a canonical component in the plant defense responses against insects and pathogens (Li et al. 2005, Browse and Howe 2008, Méndez-Bravo et al. 2011), also critical for adaptation to stressing growth conditions and root architecture remodeling (Staswick et al. 1992, Sun et al. 2009, Raya-González et al. 2012). Several *Arabidopsis* mutants deficient in JA signaling have been isolated and characterized including the *coronatine insensitive1 (coi1)* and *jasmonic acid resistant1 (jar1)* (Berger 2002, Wasternack 2007), which represent useful tools to study plant responses linking JA signaling, ROS accumulation and cellular responses. Ethylene is another important

plant hormone involved in defense as well as in growth and developmental processes such as root hair formation, root growth, germination and fruit ripening (Tanimoto et al. 1995, Johnson and Ecker 1998, Bleecker and Kende 2000). Many genes encoding components of the ethylene signaling pathway have been identified, including ethylene receptors such as *ETR1* (Bleecker et al. 1988) or components acting downstream such as *EIN2* (Guzman and Ecker 1990, Alonso et al. 1999), and *EIN3* (Chao et al. 1997). JA and Et signaling are known to interact and to share some common elements for the regulation of different plant responses (Sun et al. 2006, Zhu et al. 2006, Adams and Turner 2010). Currently, their precise connection with ROS regulated processes is under active investigation.

Because impaired ROS distribution has been related to an altered root growth (Dunand et al. 2007, Tsukagoshi et al. 2010) and serotonin possesses antioxidant properties, this work explores the possibility that the effects of serotonin on root growth could be due to a ROS imbalance affecting fundamental cellular processes. Our results show that *RCD1* is an important element in controlling the ROS balance involved in primary root growth adjustment by serotonin. Evidence is presented that *JAR1* and *COI1,* two essential genes in jasmonic acid signaling as well as *ETR1*, *EIN2* and *EIN3*, which mediate ethylene responses, play a key role in serotonin modulation of root growth via ROS distribution. We further show that primary root growth inhibition by serotonin in WT seedlings correlate with altered ROS distribution at the meristem and elongation zones, whereas in all JA-and Et-related mutants ROS levels and distribution in response to serotonin were minimally affected. These observations suggest that serotonin modulates root growth via affecting ROS homeostasis, *RCD1* and JA-Et signaling pathways.

Materials and Methods

Plant material and growth conditions

Arabidopsis ecotype Columbia (Col-0), the *rcd1* insertion line (SALK_116432), mutant lines *etr1-1* (Hua and Meyerowitz 1998), *ein2-1* (Guzmán and Ecker 1990), *ein3-1* (Chao et al. 1997), *eto3-1* (Kieber et al. 1993), *coi1-1* (Feys et al. 1994) and *jar1-1* (Staswick et al. 1992) were used for all experiments. Seed 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, seed were germinated and grown on agar plates containing

0.2x MS medium (Murashige and Skoog 1962). MS medium (Murashige and Skoog basal salts mixture; catalog M5524) was purchased from Sigma-Aldrich (St. Louis, MO). The suggested formulation is salts at 4.3 g liter^{-1} for a 1x concentration of medium; we used 0.9 g liter^{-1}, which we consider and refer to as 0.2x MS. This medium lacks amino acids and vitamins. Serotonin was purchased from Sigma-Aldrich (H9523). The compound was dissolved in dimethyl sulfoxide and used at the indicated concentrations. In control seedlings, we added the solvent in amounts equal to those present in the greatest concentration of compound tested. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, US.A.). 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 m2 s^{-1}, and temperature of 22°C.

Plant growth analysis and statistics

Growth of primary roots was registered using a rule. The length of 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). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in the WT and mutant seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$).

H_2O_2 detection

H_2O_2 production was detected by endogenous peroxidase-dependent staining procedure using 3,3′-diaminobenzidine (DAB) uptake (Thordal-Christensen et al. 1997). Control or serotonin-treated *A. thaliana* WT and mutant seedlings were placed in a solution of DAB at 1 mg ml–1, pH 3.8, and incubated in dark for 2 h. Subsequently, they were immersed in boiling 96% (vol/vol) ethanol for 10 min and then stored in 96% (vol/vol) 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.

ROS and O^{2-} detection

General ROS were visualized incubating *Arabidopsis* seedlings with 10 μM of 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) a cell-permeable non-fluorescent probe that is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation. O^{2-} anion was monitored by incubating the seedlings with dihydroethidium (DHE), in 10 mM Tris-HCl (pH 7.4) (Gomes et al. 2005). *Arabidopsis* 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 numbers in the green channel by employing ImageJ software.

Results

Arabidopsis mutants defective on RADICAL-INDUCED CELL DEATH1 exhibits tolerance to serotonin

Serotonin plays a role in different development processes via its ROS scavenging properties (Ramakrishna et al. 2011). To investigate the role of ROS in root developmental responses to serotonin, we compared primary root growth of WT (Col-0) seedlings and *rcd1* mutants, which show an enhanced sensitivity to extracellular ROS generated by O_3 exposure (Overmyer et al. 2000), but show resistance to the intracellular ROS generated in response to paraquat (Ahlfors et al. 2004, Fujibe et al. 2004).

We first tested the response of roots of WT and *rcd1* seedlings to paraquat. In agreement with previous reports, WT seedlings showed a dose-dependent primary root growth inhibition in medium supplied with increasing paraquat concentrations, whereas *rcd1* seedlings were able to sustain primary root growth at most concentrations of paraquat tested (Fig. S1). Using confocal imaging, ROS levels in WT and *rcd1* primary root tips were determined. Paraquat clearly increased ROS in WT but not in *rcd1* roots (Fig. S1), suggesting that increased ROS accumulation at the meristem/elongation regions likely determines primary root growth.

We next compared primary root growth of WT and *rcd1* seedlings that were grown side by side over the surface of agar plates supplied with MS 0.2x medium with or without 300 µM of serotonin. Interestingly, *rcd1* seedlings showed tolerance to the primary root growth inhibition caused by serotonin (Fig. 1). These results suggest that the primary root growth inhibition in response to serotonin is mediated, at least in part, via the action of the RCD1 gene controlling ROS homeostasis and/or distribution.

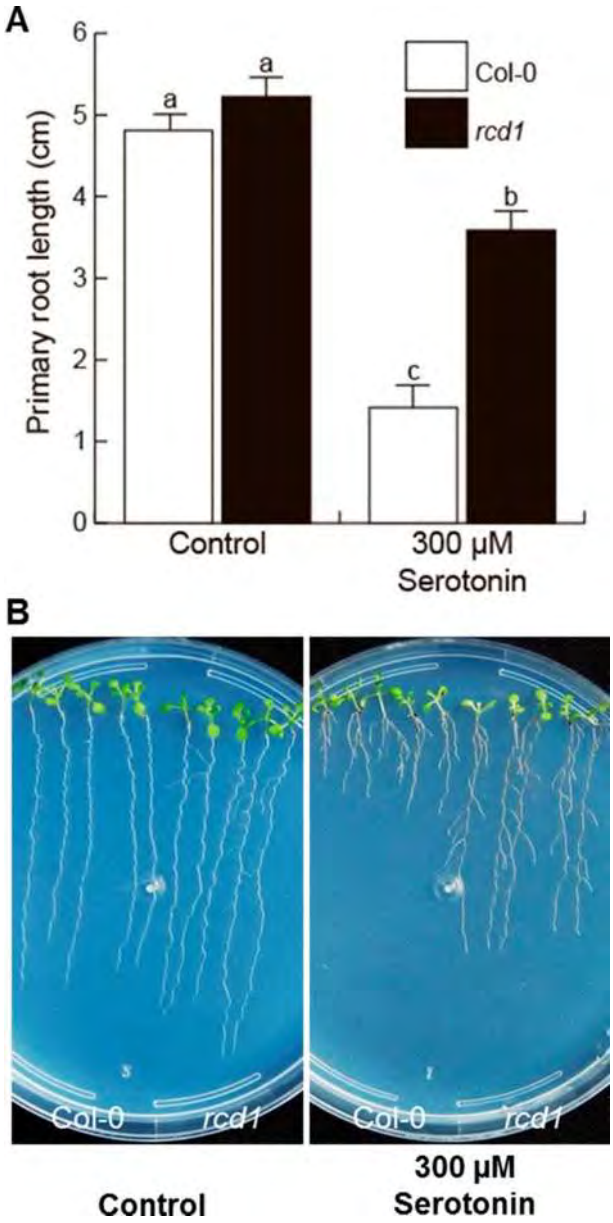

Fig. 1. Effects of serotonin on primary root growth of WT and *rcd1* seedlings. Wild-type and *rcd1* seedlings were grown side by side on 0.2x Murashige-Skoog (MS) medium with or without 300 µM of serotonin and analyzed 10 days after germination (d.a.g). (A) Primary root length, (B) representative photographs of WT and *rcd1* seedlings in response to serotonin. Values shown represent the mean ±SD (n=15).

Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

Involvement of jasmonic acid in primary root response to serotonin

The *RCD1* protein modulates jasmonic acid and ethylene responses, because *rcd1* mutants exhibit reduced sensitivity to these hormones (Ahlfors et al. 2004, Alonso et al. 1999, Overmyer et al. 2000, Cao et al. 2006). To determine if jasmonic acid signaling is involved in the plant responses to serotonin, we compared the primary root growth of WT, *jar1* and *coi1* seedlings, these later are defective in the synthesis, and perception of jasmonic acid, respectively (Staswick and Tiryaki 2004, Yan et al. 2009). An experiment was performed, in which 4 day-old WT and homozygous *coi1-1* seedlings were transferred to 0.2x MS agar-solidified medium supplemented with the solvent only or with 300 μM serotonin and the root length was measured 6 days after transfer. Serotonin inhibited primary root growth in WT plants compared to solvent-treated seedlings, whereas *coi1-1* mutants were clearly resistant to serotonin effects, which correlated with the mutants having longer root meristems and greater cells at the cell differentiation region (Fig. 2). In another set of experiments, the growth of WT and *jar1* seedlings was compared after 9 days of growth side by side on agar plates. Similarly to *coi1*, *jar1* seedlings were less affected by serotonin treatments on root traits including root length, root meristem size and cell length (Fig. 3). These results indicate that *COI1* and *JAR1* are important genetic elements mediating root response to serotonin.

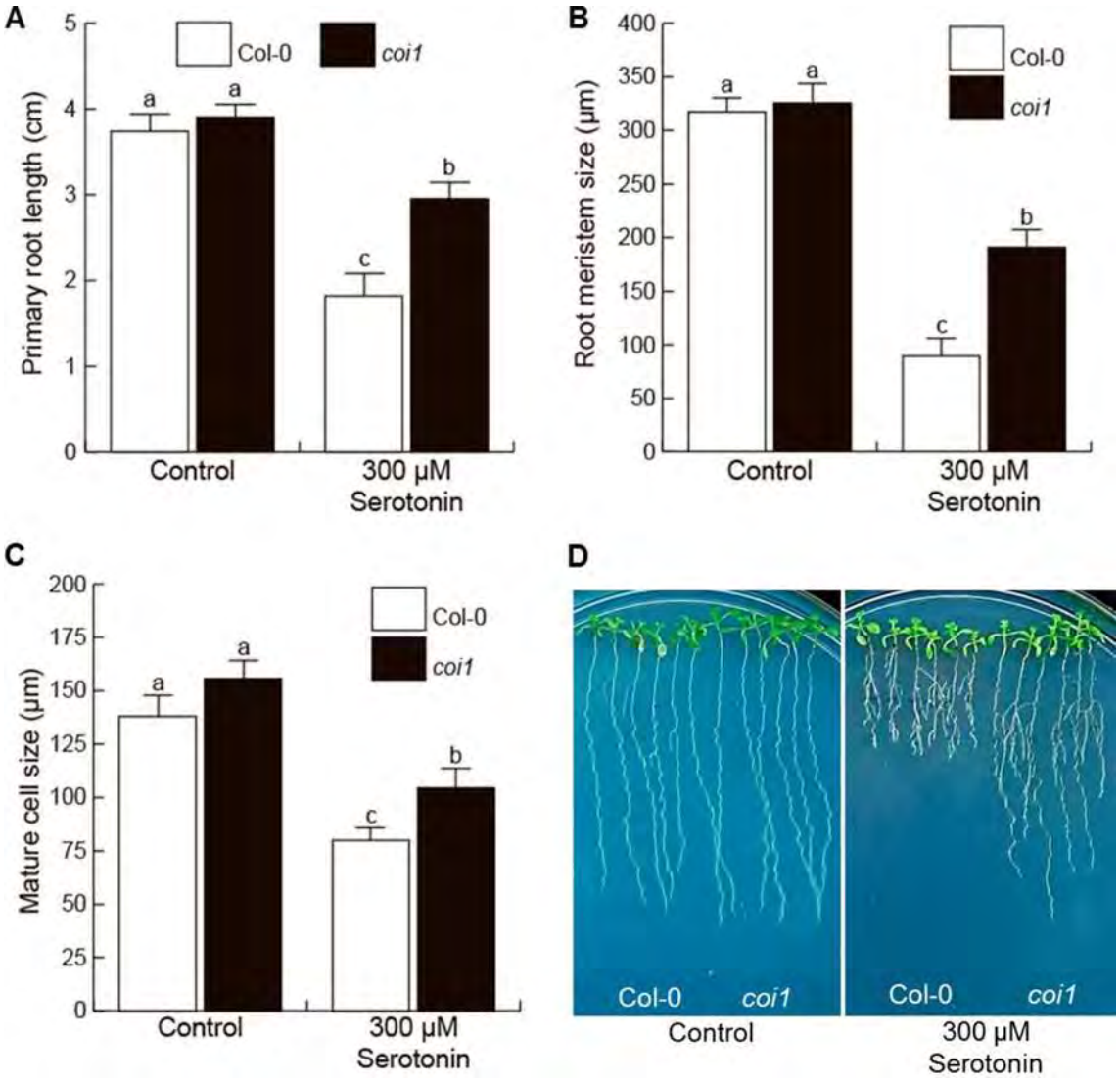

Fig. 2. Effects of serotonin on primary root growth of WT and *coi1* seedlings. WT seedlings were germinated and grown for 4d on 0.2x MS medium and homozygous *coi1* seedlings were selected from a *coi1/COI* segregating population in medium supplemented with 4 µM JA, next WT and *coi1* were transferred to fresh 0.2x MS media supplied with the solvent (control) or with 300 µM serotonin and analyzed 6 days later. (A) Primary root length, (B) meristem size, (C) cortical cell length. (D) Representative photographs of WT and *rcd1* seedlings. Values shown represent the mean ±SD (n=15). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

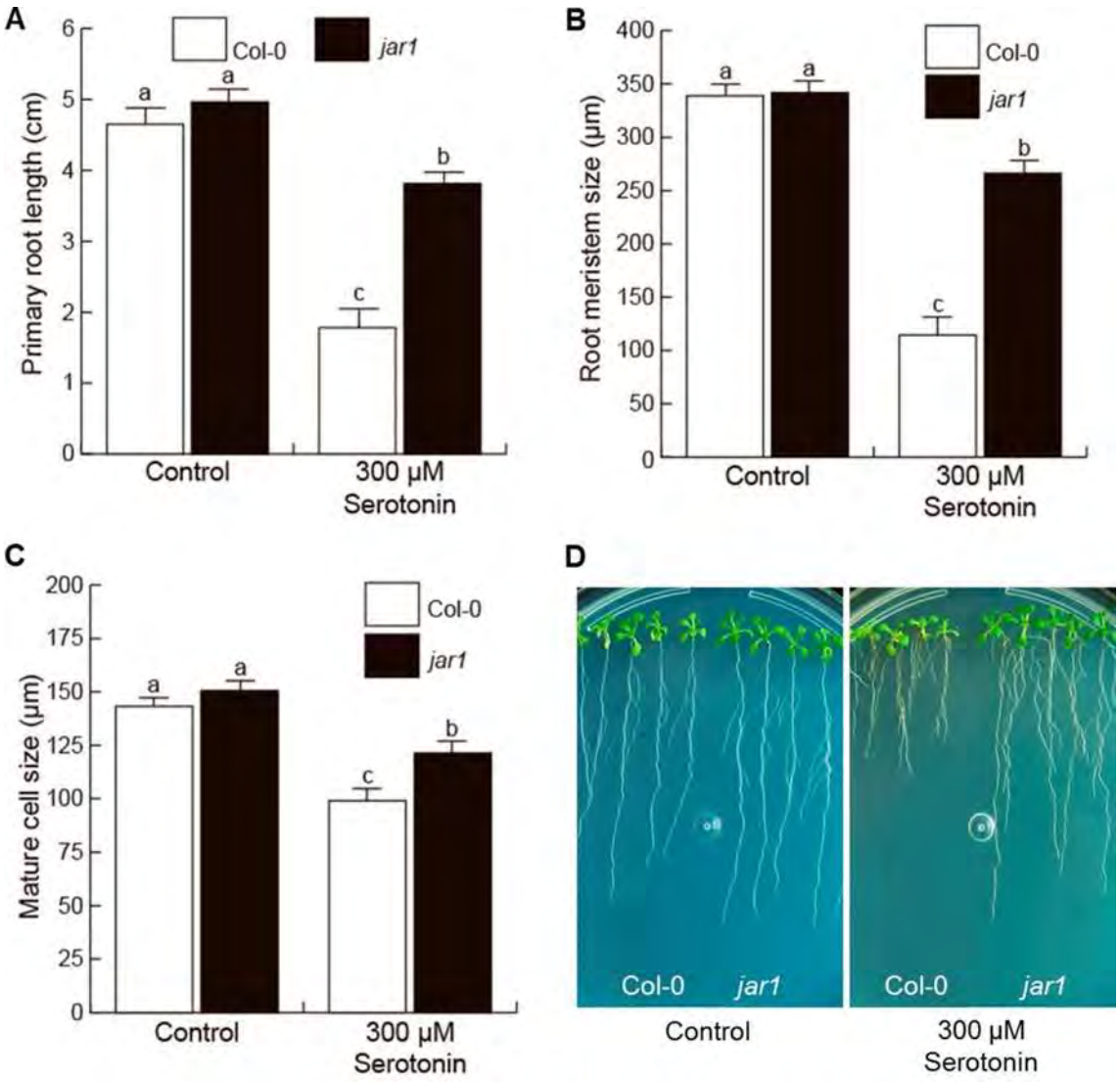

Fig. 3. Effects of serotonin on primary root growth of WT and *jar1* seedlings. Wild-type and *jar1* seedlings were grown side by side on 0.2x MS medium supplemented with the solvent (control) or with 300 µM of serotonin and analyzed 10 days after germination (d.a.g). (A) Primary root length, (B) meristem size, (C) cortical cell length. (D) Representative photographs of WT and *jar1* in response to serotonin. Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

ROS-JA interaction in serotonin response

The establishment and maintenance of cell division, elongation and differentiation programs in the root is linked to complex mechanisms of interaction between ROS and several hormonal signaling pathways (De Tullio et al. 2010, Tsukagoshi et al. 2010). To clarify whether the serotonin-induced primary root growth inhibition was related to an alteration in the levels and/or distribution of ROS in primary root tips and if this could be related to the observed serotonin-tolerance in *coi1* and *jar1* mutants, we analyzed ROS levels and distribution in response to serotonin in roots of WT, *coi1* and *jar1* mutants by using the probe H2DCF-DA and confocal imaging. WT seedlings grown under normal conditions had relatively low ROS levels, while in response to serotonin an enhanced accumulation of ROS could be detected in the meristematic zone of the primary root (Fig. 4A, B). Intriguingly, *coi1* and *jar1 Arabidopsis* mutants showed higher ROS levels under normal growth conditions and no further increases were evident in serotonin treatments (Fig. 4A, B). Quantification of the relative fluorescence in the root apical zone confirmed an increase of ROS in WT in response to serotonin (Fig. 4C, D), which was absent in *coi1* and *jar1* mutants. These results show that *coi1* and *jar1* are tolerant to serotonin-induced ROS accumulation, a property that correlates with the lower sensitivity to serotonin in the inhibition of primary root growth, suggesting that ROS redistribution within the root tip in response to serotonin are likely responsible of primary root growth inhibition.

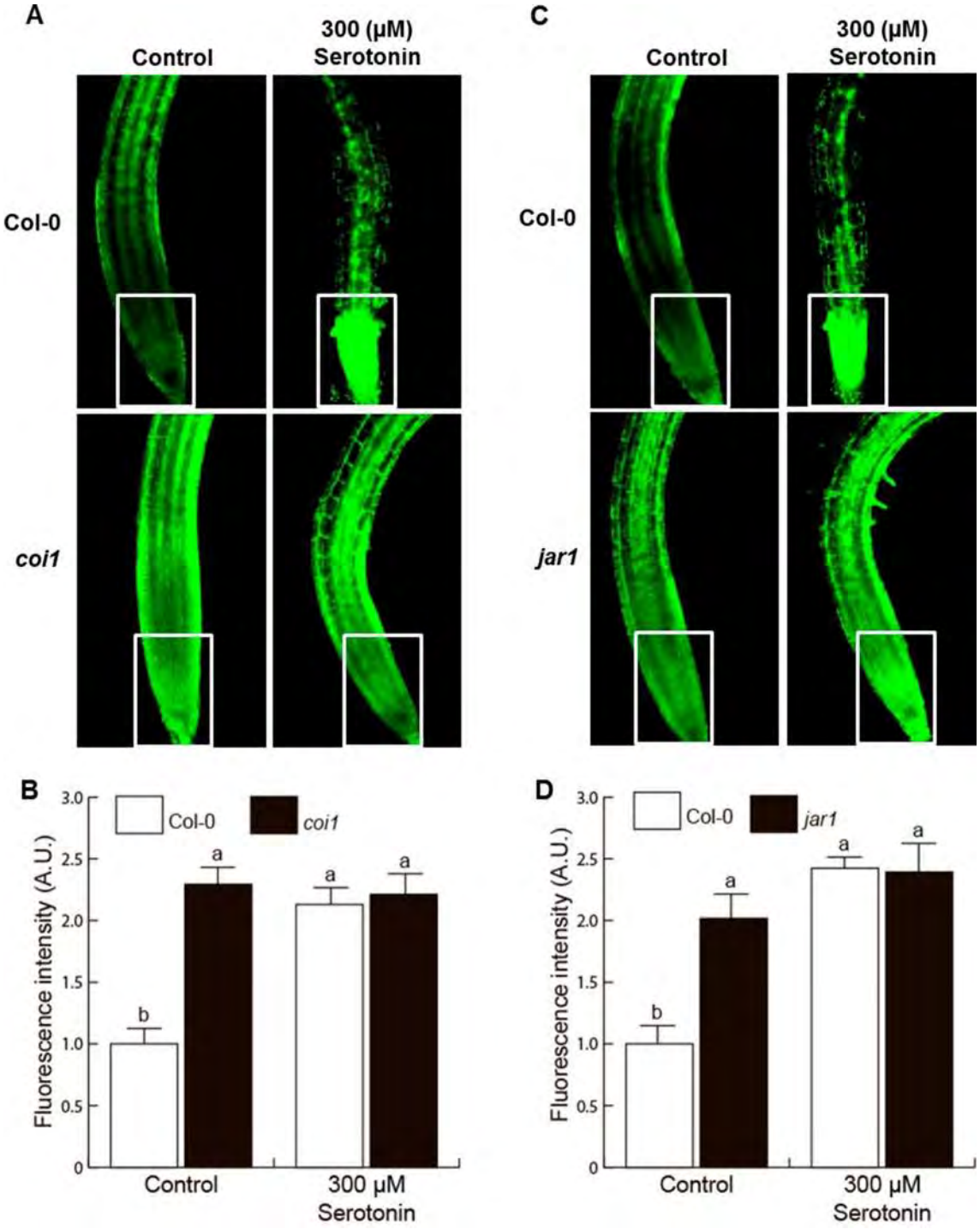

Fig. 4. Effect of serotonin on ROS levels in WT and the jasmonic acid related mutants *coi1* and *jar1*. *Arabidopsis* seedlings of the different lines were grown for 7 days on 0.2x MS medium supplemented with the solvent or with 300 µM of serotonin. (A and C) Representative photographs of the detection of endogenous ROS levels in primary roots of *coi1* and *jar1* compared to WT. (B and D) Fluorescence from primary root tips was quantified using the ImageJ program. The graph is expressed in arbitrary units (A.U.). Values shown represent the mean ±SD (n=10). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

Involvement of ethylene in root response to serotonin

The role of ethylene signaling and root responses to serotonin was evaluated by comparing primary root growth of WT and mutants defective in ethylene signaling *etr1*, *ein2* and *ein3* in medium with or without serotonin supply. Interestingly, while the primary root length of wild-type seedlings was strongly inhibited by 300 µM serotonin treatment, all three ethylene related mutants showed resistance to inhibition of primary root growth, sustaining longer primary roots than WT seedlings (Fig. 5A, B), suggesting that ethylene may be another signal in mediating the serotonin response. Silver ions have been found to block ethylene action via their ability to bind to the ethylene receptors and in this way affect downstream ethylene signaling (Bayer 1976, Rodriguez et al. 1999). To further explore the participation of ethylene in the effects exerted by serotonin, we performed competence assays between serotonin and $AgNO_3$. In these assays, WT seedlings were grown on MS 0.2x medium, supplemented with serotonin, $AgNO_3$, and serotonin plus $AgNO_3$. Interestingly, primary root growth in medium with serotonin was normalized by $AgNO_3$ (Fig. 6A, B). In addition, we used an ethylene overproducing mutant (*eto3*) known to produce 100-fold more ethylene than WT plants (Kieber et al. 1993). To test a possible susceptibility of *eto3* to serotonin, WT and *eto3* seedlings were grown on media supplemented with 0, 100, 200 and 300 µM serotonin and root growth was compared. The roots of *eto3* were shorter than the WT in medium lacking serotonin but interestingly, *eto3* mutant seedlings were oversensitive to serotonin in primary root growth inhibition already evident at 100 µM serotonin, which did not affect the primary root growth of WT seedlings but strongly decreased primary root growth in *eto3* seedlings (Fig. S2). These results suggest that ethylene plays an important role in modulating the primary root growth in response to serotonin.

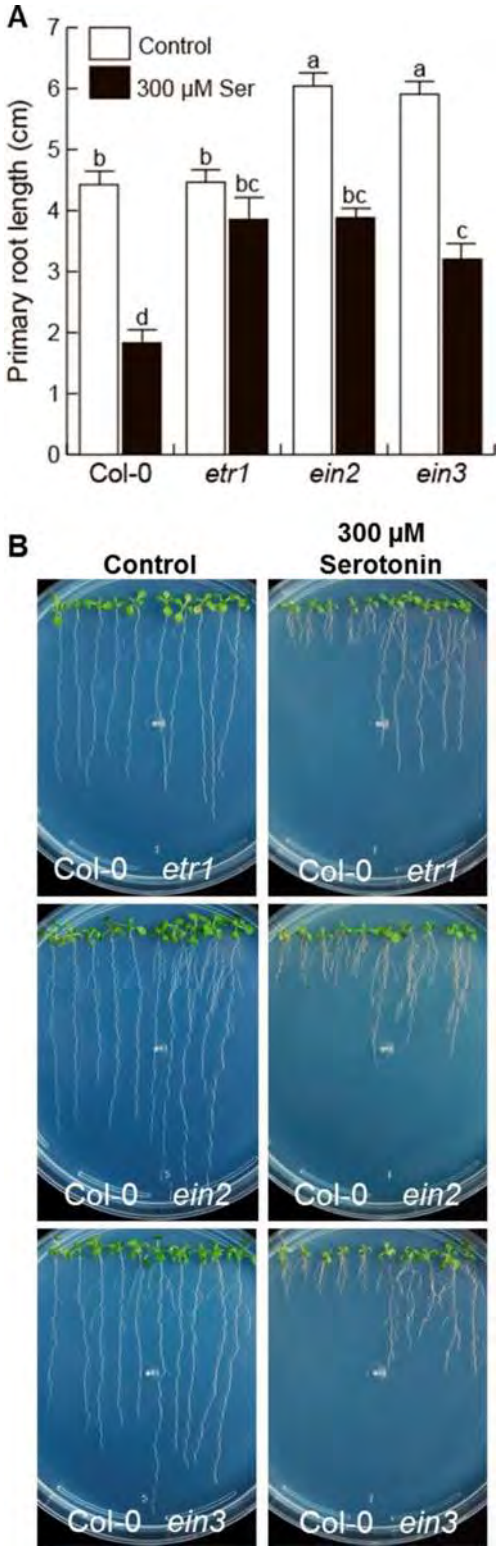

Fig. 5. Effects of serotonin on primary root growth of WT, *etr1, ein2 and ein3* seedlings. Wild-type and ethylene-related mutants were grown side by side on 0.2x MS medium supplemented with the solvent (control) or with 300 µM of serotonin and analyzed 10 d.a.g. (A) Primary root length, (B) Representative photographs of WT and ethylene mutants in media with or without serotonin. Values shown represent the mean ±SD (n=15). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

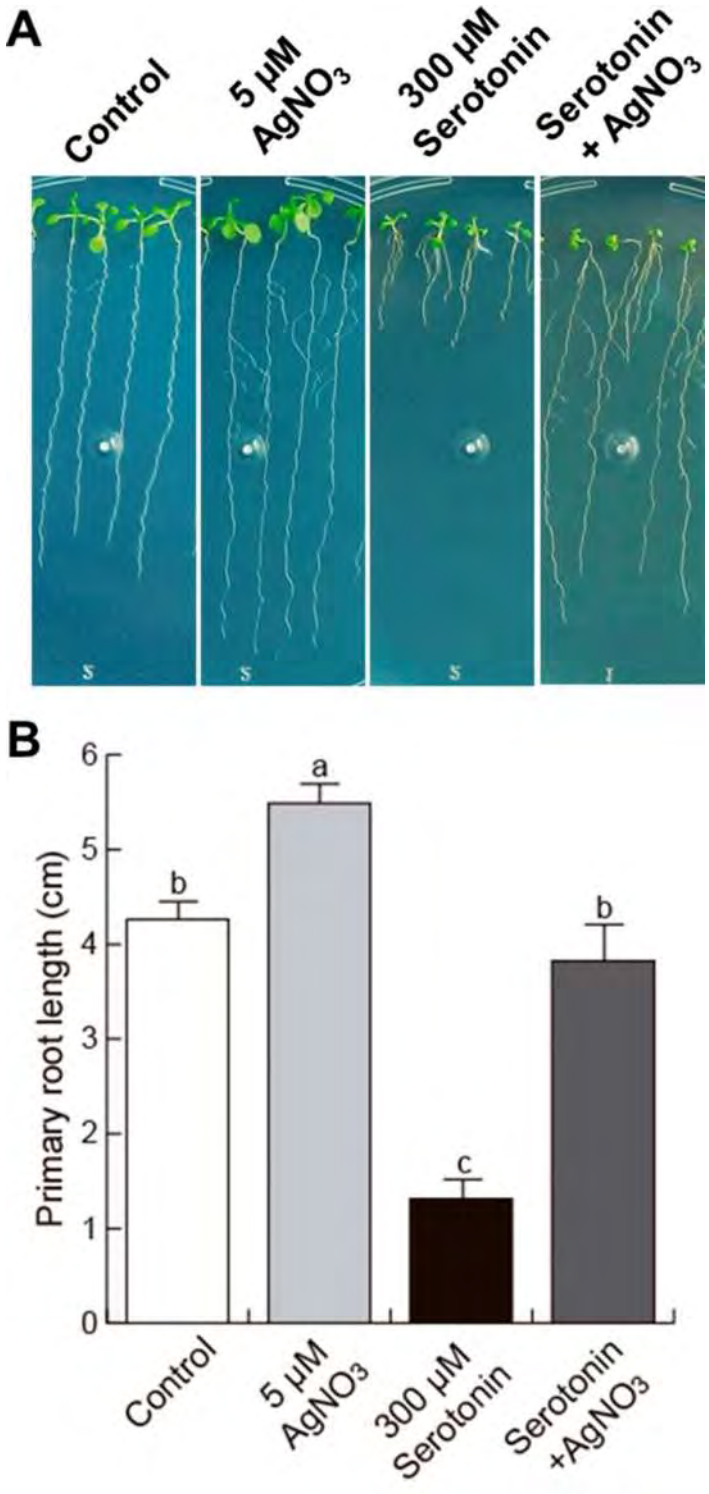

Fig. 6. Effect of $AgNO_3$ on *Arabidopsis* primary root growth inhibition induced by serotonin. Wild-type (Col-0) seedlings were grown for 10 days on 0.2x MS medium supplemented with 0, 5 µM of $AgNO_3$, 300 µM of serotonin and 300 µM of serotonin plus 5 µM of $AgNO_3$. (A) Representative photographs of *Arabidopsis* roots grown under the different treatments are shown. Notice that the $AgNO_3$ restored root growth. (B) Quantitative data of primary root growth. Values shown represent 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.

ROS-ethylene interaction in serotonin response

To test if the resistance to serotonin in the ethylene mutants is related to an impaired response to ROS as in the JA mutants, we analyzed ROS levels in WT, *etr1*, *ein2* and *ein3* seedling roots when grown under standard conditions or in response to 300 μM serotonin. 7 days after germination, seedlings were incubated with H2DCF-DA to visualize ROS in primary root tips by confocal microscopy. Similar to *jar1* and *coi1*, ethylene mutants showed higher ROS levels than WT seedlings in standard growth medium or in response to serotonin (Fig. 7A, B). Because accumulation of specific ROS changes differentially in response to diverse stimuli that affect root growth (Ortiz-Castro et al. 2014), we then analyzed the accumulation of O^{2-} and H_2O_2 in WT and ethylene-related mutants by using dihydroethidium (DHE) and 3,3′-diaminobenzidine (DAB), respectively. Interestingly, *etr1*, *ein2* and *ein3* mutants showed higher levels of O^{2-} and H_2O_2 when grown in standard growth conditions compared to WT seedlings, and did not show altered ROS species distribution in response to serotonin (Fig. 7C, D). We also analyzed the levels and distribution of ROS in *eto3* mutants. Intriguingly, similar to mutants resistant to serotonin, *eto3* seedlings grown under standard conditions had higher ROS levels, which further increase in the root tip in response to serotonin (Fig. S3). To further understand the relation between serotonin and ROS distribution in root growth, we tested the effect of paraquat in WT, *jar1, etr1*, *ein2* and *ein3* primary root growth and ROS distribution. The results show that all four mutants tested had a slight, yet statistically significant tolerance to paraquat (Fig. S4), and manifest a clear reduced accumulation of ROS at the very root tip in response to paraquat compared with WT seedlings (Fig. S4). These results support the idea that ROS accumulation at the meristematic and or elongation zones may be involved primary root growth inhibition by serotonin.

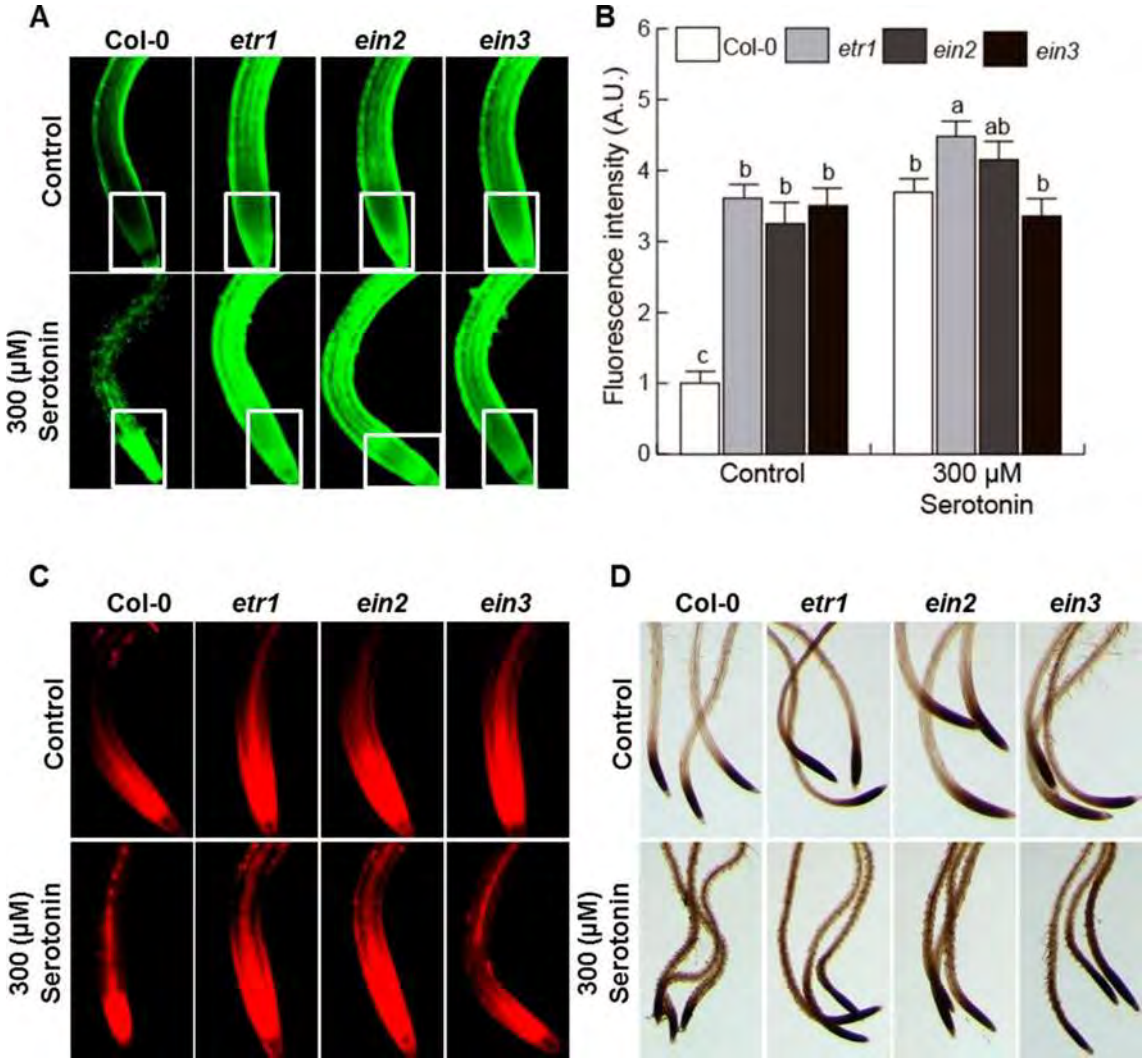

Fig. 7. Effect of serotonin on ROS levels in WT and ethylene-related mutants *etr1, ein2* and *ein3*. *Arabidopsis* seedlings of the different WT and mutant lines were grown for 7 days on 0.2x MS medium supplemented with the solvent or with 300 µM of serotonin. (A) Representative photographs of the detection of endogenous ROS levels in primary roots of ethylene mutants *etr1, ein2 and ein3* compared to WT. (B) Fluorescence from primary root tips was quantified using the ImageJ program. (C) Representative photographs of the detection of O^{2-} and (D) H_2O_2. The graph is expressed in arbitrary units. Values shown represent the mean ±SD (n=10). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

Discussion

Serotonin is a natural bioactive compound widely distributed in plants. In contrast to the signaling roles attributed to serotonin in animals, the genes and proteins mediating the serotonin responses in plants remain unknown. Serotonin regulates senescence and plant

defense responses likely scavenging ROS (Ishihara et al. 2008, Kang et al. 2009). Previously, we showed that serotonin modulates *Arabidopsis* root growth affecting both cell division and elongation, which was attributed to an anti-auxinic activity (Pelagio-Flores et al. 2011). Besides serotonin, many other signals including plant hormones auxin, jasmonic acid and ethylene inhibit primary root growth, which might be related to an impaired ROS accumulation and/or distribution within the primary root tip (Dunand et al. 2007, Tsukagoshi et al. 2010). Since serotonin is a compound with high antioxidant activity, we hypothesized that ROS homeostasis could be affected by serotonin. Therefore, the aim of this work was investigate the role of ROS in regulating primary root growth and the genetic mechanisms underlying serotonin perception. This was done by comparing primary root growth in WT and *rcd1 Arabidopsis* seedlings, these later are defective on the RADICAL-INDUCED CELL DEATH1 gene that controls growth and development via ROS signaling (Overmyer et al. 2000, Ahlfors et al. 2004), as well as in mutants impaired in jasmonic acid and ethylene responses, which have been related to RCD1 function (Overmyer et al. 2000, Ahlfors et al. 2004). The *RADICAL-INDUCED CELL DEATH1* protein plays a role in plant signaling likely via protein-protein interactions. It contains both a putative poly(ADP-ribose) polymerase catalytic domain and a WWE protein-protein interaction domain. Poly(ADP-ribose) polymerases mediate the attachment of ADP-ribose units from donor NAD^+ molecules to target proteins and have been implicated in a number of processes, including DNA repair, apoptosis, transcription, and chromatin remodeling. Consistent with previous reports, we observed a clear resistance of *rcd1* mutant seedlings to the application of paraquat in terms of primary root growth and ROS redistribution. Under standard growth conditions *rcd1* seedlings have been reported to accumulate ROS in leaves (Overmyer et al. 2000) and our current data show higher ROS levels in *rcd1* primary roots than in WT seedlings. Interestingly, *rcd1* primary roots were resistant to serotonin, suggesting that *RCD1* is required for serotonin responses in *Arabidopsis*. Since *rcd1* mutants have reduced responses to jasmonic acid and ethylene (Ahlfors et al. 2004), we tested the responses of WT and *jar1*, *coi1*, *etr1*, *ein2* and *ein3 Arabidopsis* mutants impaired in jasmonic and ethylene signaling. In WT seedlings ROS levels decreased at the primary root elongation region but strongly increased in the root meristem when exposed to serotonin treatment. Similarly to *rcd1*, all mutants tested were partially resistant to the primary root inhibition caused by serotonin and interestingly, all jasmonic acid and ethylene-related mutants sustained higher ROS levels under standard

growth conditions or in medium supplied with serotonin. The high and widely distributed ROS in primary roots of the corresponding mutants suggest that *RCD1*, *COI1*, *JAR1*, *ETR1*, *EIN2* and *EIN3* loci act as negative regulators of ROS accumulation and/or sensing. The ethylene role in ROS regulation seems to be more complex, since the high ROS levels observed in roots of *eto3* mutants, which overproduce ethylene suggest that ethylene acts as a ROS inducer, in agreement with a previous report in which inhibition of the ethylene signaling leads to a low ROS accumulation (Overmyer et al. 2000). Therefore, it is possible that at least in part the normalized primary root growth by $AgNO_3$ in seedling treated with serotonin is due to a decrease in ROS levels result of a suppressed ethylene action. Taken together our results suggest that the serotonin-induced root growth inhibition in WT seedlings is likely due to a ROS imbalance at the primary root tip likely affecting cell division, elongation or both of these processes. In this regard, reduced primary root growth in plants that overaccumulate ROS in root tips in response to different stimuli have been recently reported, such as reduced primary root growth in NO-deficient mutants such as in the triple mutant *nia1 nia2 noa1*, which correlate with an enhanced ROS accumulation and suppression of auxin activity (Sanz et al. 2014). ROS production in primary roots in response to boron deficiency was associated with a rapid inhibition of root cell elongation mediated by a signaling pathway triggered by an impaired cell wall integrity caused by the boron deficiency in a process that also involve the participation of ethylene and auxin (Camacho-Cristóbal et al. 2015). Indeed low phosphate availability, which is known to affect root system architecture inducing a determinate developmental program in primary roots of *Arabidopsis thaliana* (Sánchez-Calderon et al. 2005) induces ROS accumulation in root tips followed by callose deposition in root meristems and blocks cell-to-cell communication for maintenance of the stem cell niche (Müller et al. 2015). All this evidence supports the proposal that local distribution of ROS leads to alterations in cell wall integrity, thus affecting root growth via ethylene-auxin crosstalk (Tsang et al. 2011). It is also possible that serotonin could regulate primary root growth affecting callose deposition, since previous reports have documented that defense root response to pathogenic infections and phenotypic alterations associated to serotonin accumulation induced a brown pigmentation attributed to lignification (Ishihara et al. 2008, 2011, Kanjanaphachoat et al. 2012). If the brown pigment observed in roots of *Arabidopsis* seedling grown in presence of serotonin are due to callose deposition or to any serotonin derivative, which in turn modifies cell wall integrity affecting the cell-to-

cell communication leading to root growth inhibition remains to be investigated. In addition, decreased auxin responses in plants treated with serotonin (Pelagio-Flores et al. 2011) may be explained because ROS accumulation suppresses auxin activity in *Arabidopsis* primary root meristems (Sanz et al. 2014). In conclusion, we propose that serotonin-induced root growth inhibition is due to a ROS imbalance at the root tip, a zone occupied by stem cell populations, the meristem and cell elongation regions, in a process mediated by *RCD1* and ethylene-jasmonic acid crosstalk. Whether particular ROS and cell populations within primary root tips are particularly affected by serotonin is currently under investigation.

Author contributions

JLB, RPF. Conceived and designed the experiments; RPF, LFRH. Performed experiments; JLB RPF, LFRH. Analyzed the data; JLB. Contributed reagents/materials/analysis tools; RPF JLB. Wrote the paper.

Acknowledgments

We are thankful to Dr. Plinio Guzman for kindly providing us with *Arabidopsis* mutant seeds. This work was supported by Grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT, México, grant no.177775) and the Consejo de la Investigación Científica (UMSNH, México, Grant No. CIC 2.26). Ramón Pelagio Flores is indebted to CONACYT for a doctoral fellowship.

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SUPPORTING INFORMATION

Fig. S1. Paraquat resistance of *rcd1 Arabidopsis* mutant.

Fig. S2. Hypersensitivity of *eto3 Arabidopsis* mutant to serotonin.

Fig. S3. Effect of serotonin on ROS levels in *eto3*.

Fig. S3. Effects of paraquat on *etr1*, *ein2*, *ein3* and *jar1*.

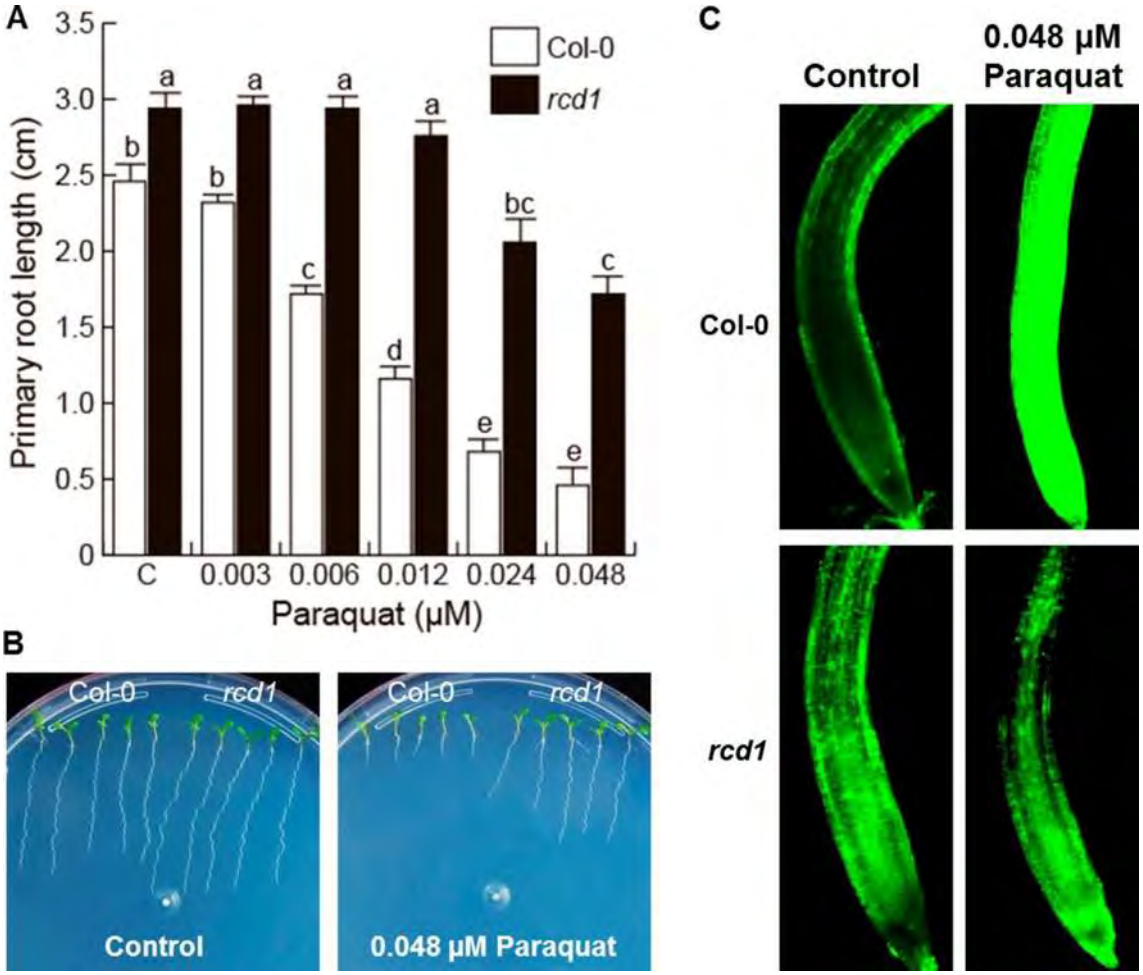

Fig. S1. Effects of paraquat on primary root growth and ROS accumulation in WT and *rcd1* mutants. Wild-type and *rcd1* seedlings were grown for 7 days on 0.2x Murashige-Skoog (MS) medium supplemented with increased concentrations of paraquat. (A) Primary root length, (B) representative photographs of WT and *rcd1* in response to paraquat (C) Confocal images of ROS accumulation in primary roots. Data points show the mean ± standard deviation (n = 30). Representative photographs of the detection of ROS with H2DCF-DA are shown. Values shown represent the mean ±SD (n=15). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

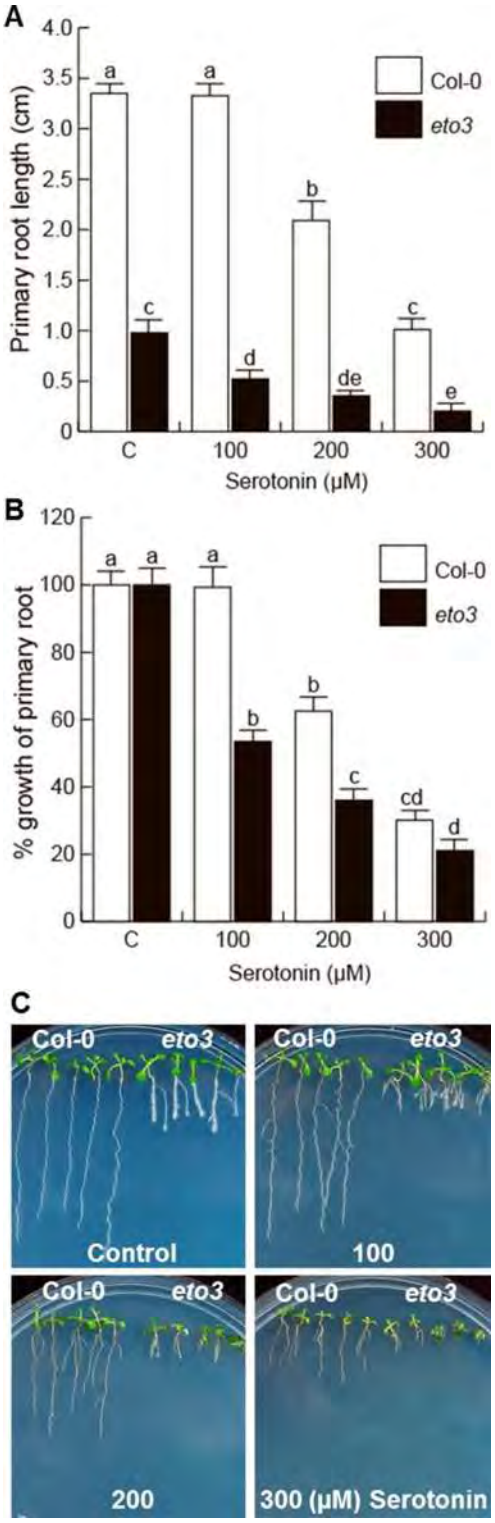

Fig. S2. Effects of serotonin on primary root growth of WT and *eto3* seedlings. Wild-type and *eto3* seedlings were grown side by side on 0.2x MS medium supplemented with the solvent (control) or with 300 µM of serotonin and analyzed 7 days after germination. (A) Primary root length, (B) % growth of primary roots, (C) representative photographs of WT and *eto3* mutants grown in medium supplied or not with serotonin. Values shown represent the mean ±SD (n=15). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

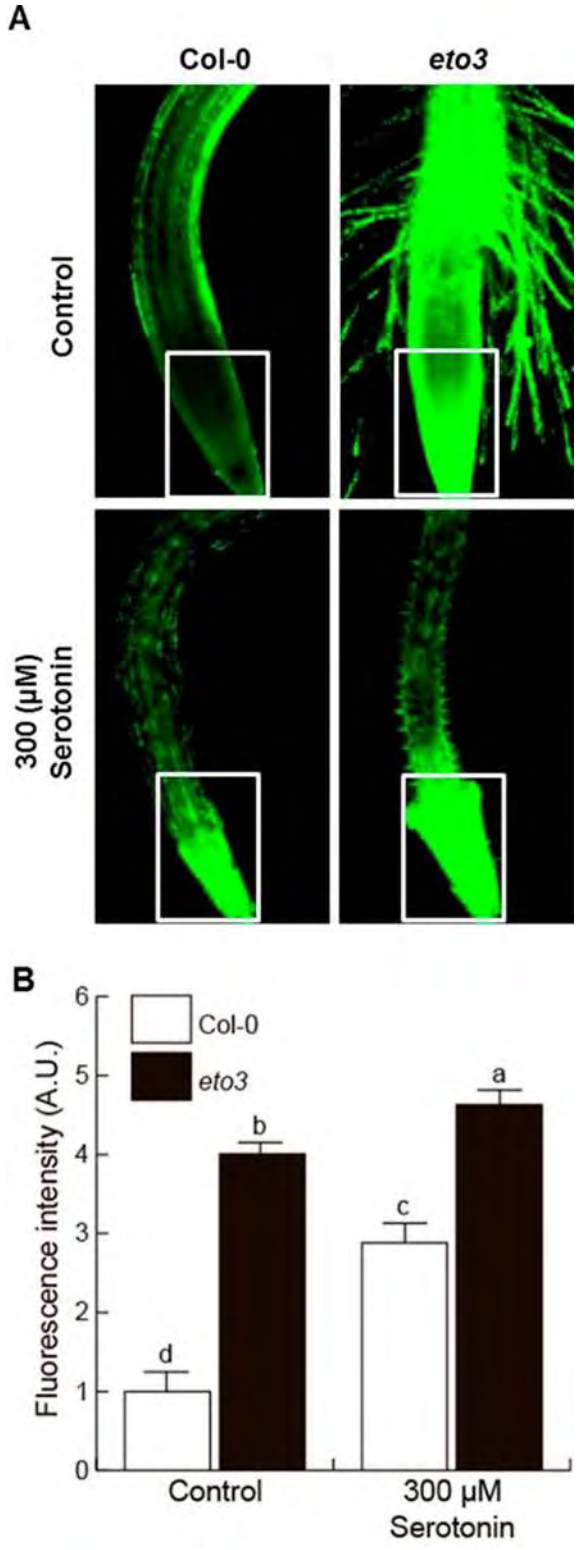

Fig. S3. Effect of serotonin on ROS levels in *eto3*. *Arabidopsis* seedlings of Col-0 and *eto3* were grown for 7 days on 0.2x MS medium supplemented with the solvent or with 300 µM of serotonin and analyzed by confocal to ROS detection. (A) ROS accumulation in primary roots (B) ROS quantification. The graph is expressed in arbitrary units. Values shown represent the mean ±SD (n=10). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

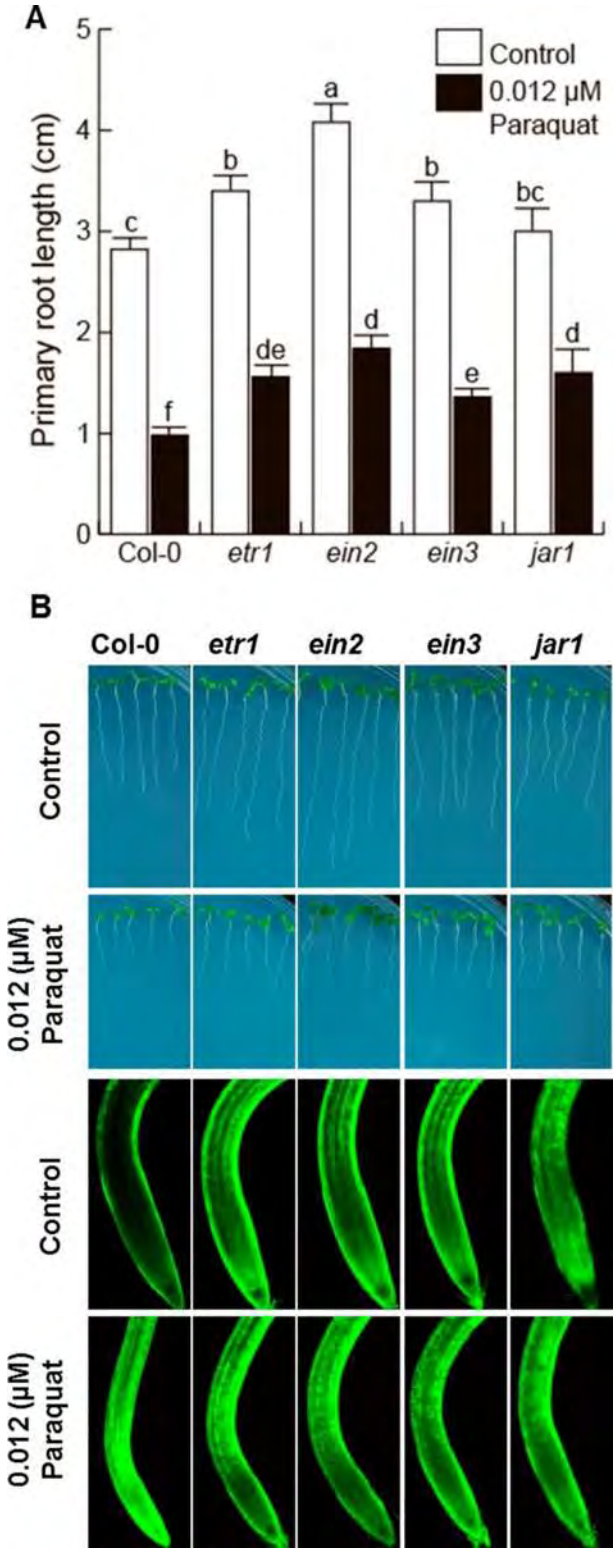

Fig. S4. Effects of paraquat on primary root growth of WT, *etr1, ein2, ein3* and *jar1*. Wild-type and mutant lines were grown on 0.2x MS medium supplemented with the solvent (control) or with 0.012 µM of paraquat and analyzed 7 days after germination (A) Primary root length, (B) representative photographs of the growth of WT and mutants and ROS accumulation in response to paraquat treatments. Values shown represent the mean ±SD (n=15). Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

CAPITULO III

Book title: Serotonin and melatonin: their functional role in plants and implications in human health.

Editors: Gokare A. Ravishankar, Akula Ramakrishna.

Publisher: Tailor & Francis Group.

Edition: USA.

Book chapter:

Serotonin and melatonin in plant growth and development

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Chapter outline:

Introduction

Serotonin (5-hydroxytryptamine) and melatonin (*N*-acetyl-5-methoxytryptamine) are two bioactive indoleamines derived from tryptophan, which modulate important physiological processes in mammals such as circadian rhythms, mood, sleep, anxiety, body temperature, sexual behavior and reproduction (Veenstra-VanderWeele et al. 2000; Reiter 1993; Galano et al. 2011). These compounds are present in organisms belonging to evolutionary distant taxonomic groups including Bacteria, Cyanobacteria, Dinoflagellata, Euglenoidea, Rhodophyta, Phaeophyta, and Plantae (Odjakova and Hadjiivanova 1997; Roshchina 2001; Murch et al. 2001; Kang et al. 2009a; Paredes et al. 2009; Ramakrishna et al. 2011). Melatonin and serotonin content varies considerably among plant species, and their different biological activities impact growth, development, defense, and adaptation to abiotic stress. Serotonin and melatonin are structurally related to indole-3-acetic acid (IAA), the main and most abundant auxin present naturally in plants. IAA is the most widely investigated plant growth regulator, involved in virtually every aspect of growth and development throughout the plant life cycle including tropic responses toward light, gravity, and touch stimuli as well as in root and shoot system establishment (Woodward and Bartel 2005). IAA is a tryptophan derived compound for which transporters, receptors and transcription factors mediate its role in plant morphogenesis. Since the molecular structure of IAA is closely related to serotonin and melatonin, the possibility that these compounds could act in similar ways in plant signaling was proposed (Murch et al. 2001; Murch and Saxena, 2002). Although research on serotonin and melatonin in plants is still in its infancy, in the last 10 years important advances have been made in this field that provide the basis not only for understanding the mechanisms of action of these compounds, but also regarding their possible applications as phytostimulants.

Serotonin and melatonin distribution in plants

Serotonin was identified in plants for the first time by Bowden et al. (1954), in the legume *Mudica Pruriens* and almost forty years later Van Tassel et al. (1993) described the presence of melatonin in the ivy morning glory (*Pharbitis nil*) plant and in tomato (*Solanum lycopersicum*) (Dubbels et al. 1995; Van Tassel et al. 1995; Kolar et al. 1995). Since then, serotonin and melatonin have been identified in an increasing number of species from different plant families, including edible and medicinal plants used in traditional medicine to treat a variety of chronobiological disorders or degenerative

diseases (Feldman and Lee 1985; Roshchina 2001; Murch et al. 1997; Badria 2002; Chen et al. 2003). Accordingly, some research has been conducted to identify plants with high levels of beneficial metabolites, including serotonin and melatonin, which may have applications on human health or agriculture. Up to 400 μg/g serotonin have been found in walnuts (*Juglans regia*), while melatonin content varies from 6 pg/g in the shoots of *Ipomoea nil* to 230 μg/g from *Pistachio* kernels, although lower levels can be found in different edible plant parts (Paredes et al. 2009; Ramakrishna et al. 2011; Arnao 2014; Hardeland 2014). In spite of the apparent ubiquity of these molecules in the plant kingdom, the precise roles and/or the importance of serotonin and melatonin for plant functional processes still needs to be explored.

Biosynthesis of serotonin and melatonin

The serotonin and melatonin biosynthetic pathways were first characterized in humans. Serotonin is synthesized from tryptophan in a short biosynthetic route that involves only two enzymatic steps where tryptophan is converted into 5-hydroxytriptophan and then into serotonin by the enzymes tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase (AADC), respectively (Veenstra-VenderWeele et al. 2000). Melatonin synthesis continues from serotonin by two following reactions catalyzed by the arylalkylamine N-acetyltransferase (AANAT) and the N-acetylserotonin methyltransferase (ASMT) to form N-acetylserotonin and melatonin, respectively (Yu and Reiter, 1993; Arnao and Hernández-Ruiz, 2006). In plants, serotonin and melatonin biosynthesis also occurs from L-tryptophan through a reaction that first converts L-tryptophan to tryptamine by the tryptophan decarboxylase (TDC) and then the tryptamine is converted to serotonin by the tryptamine 5-hydroxilase (T5H), followed by serotonin conversion to N-acetylserotonin and then into melatonin by the enzymes serotonin N-acetyltransferase (SNAT) and N-acetylserotonin O-methyltransferase (ASMT), respectively (Fig. 1) (Tan et al. 2014), interestingly, auxin biosynthesis also has been proposed to occur from a tryptamine pathway (Quittenden et al. 2009). Currently, most genes involved in serotonin and melatonin biosynthesis from tryptophan (TDC, T5H, SNAT and ASMT) have been cloned and characterized mostly from rice plants (Kang et al. 2007a,b; Kang et al. 2011; 2013). Seven TDC genes are present in the genome of rice plants and three of them have been demonstrated to play a role in serotonin and melatonin synthesis. A T5H gene involved in serotonin synthesis belongs to the cytochrome P450 gene family and has been

identified as cytochrome P450 monooxygenase subform CYP71P1 (Fujiwara et al. 2010). Plant SNAT seems to be unrelated to the SNAT from animals since they did not share homology or cellular localization, recent studies showed that plant SNAT is located in chloroplasts, where it acts as a rate-limiting enzyme in melatonin biosynthesis. In contrast, plant and animal ASMT share homology and have a cytoplasmic localization, plant ASMT is highly expressed in the root system suggesting particular organ specific roles (Byeon et al. 2014). The evidence already available thus suggests that serotonin and melatonin biosynthesis in plants and animals occurs in a different manner, as chloroplasts are plant specific organelles. However, some reports have shown some commonalities, since both plant and animals produce 5-hydroxytryptophan as intermediate compound in serotonin and melatonin biosynthesis (Murch et al. 2000; Park et al. 2012; Tan et al. 2012; 2013).

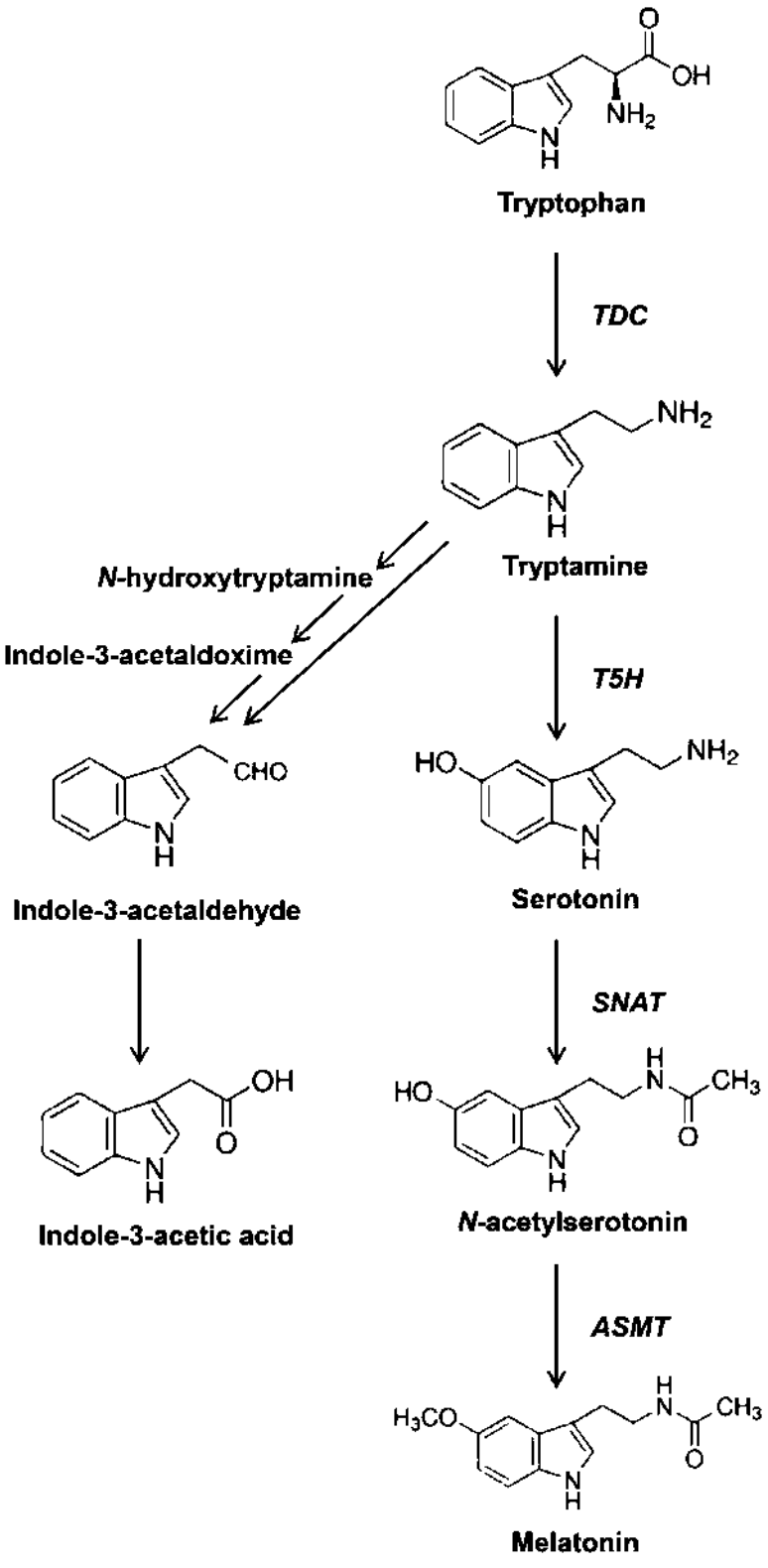

Figure 1. Proposed pathway for serotonin and melatonin biosynthesis in rice plants. The specific reactions catalyzed by tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin N-acetyltransferase (SNAT), and *N*-acetylserotonin-O-methyltransferase (ASMT) are shown.

Serotonin and melatonin as plant growth promoting compounds

Due to the structural similarity with auxin, initial attempts to explain the biological activities of serotonin and melatonin, particularly in root and shoot developmental processes were attributed to possible auxin-like activity (Csaba and Pal, 1982; Murch and Saxena 2002; Arnao and Hernández-Ruiz 2006; Ramakrishna et al. 2009; 2011). The function of serotonin and melatonin on plant growth regulation has been investigated in several studies. Csaba and Pal (1982) found that serotonin treatment increases root length in barley, showing a higher activity than the synthetic auxin naphthylacetic acid (NAA) and IAA. Years later, it was reported that both serotonin and melatonin have an important role on St. John's wort (*Hypericum performatum L.*) morphogenesis, since increased root and shoot formation were correlated with high levels of melatonin and serotonin, respectively, suggesting that a balance in the serotonin-melatonin ratio may affect plant morphogenesis (Murch et al. 2001). Melatonin promotes vegetative growth in dark-grown *Lupinus albus* L. hypocotyls, increases cotyledon expansion and stimulates adventitious and lateral root formation in this plant species in a similar manner to IAA, which led the authors to suggest an auxinic activity for melatonin on plant growth (Hernández-Ruiz et al. 2004; Arnao and Hernández Ruiz, 2007; Hernández-Ruiz and Arnao, 2008). Similarly, melatonin had a growth promoting effect in coleoptiles of wheat, barley, canary grass and oat, although in this case the melatonin activity was lower than that of IAA. In contrast, both melatonin and IAA showed an inhibitory effect in root growth in these monocotyledonous plants (Hernández-Ruiz et al. 2005). In agreement with the above described results, melatonin applied to etiolated seedlings of wild leaf mustard (*Brassica juncea*) stimulated root growth (Chen et al. 2009). Despite these seminal reports, until recently, clear evidence was lacking demonstrating the auxin activities of serotonin and melatonin at the molecular level. It was Pelagio-Flores and associates (2011; 2012) who characterized the effects of both indoleamines on the architecture of the root system in *Arabidopsis thaliana* seedlings, as well as their relationship with the auxin signaling pathway by analyzing auxin-inducible gene expression in transgenic seedling expressing the *DR5:uidA* and *BA3:uidA* gene markers. Both serotonin and melatonin modulated root system architecture in a highly specific and dose-response manner, affecting different processes of growth and development (Fig. 2A). Serotonin was found to modulate primary root growth, lateral and adventitious root formation and root hair development (Pelagio-Flores et al. 2011). Melatonin regulated root system architecture by stimulating lateral and

adventitious root formation but minimally affected primary root growth or root hair development (Pelagio-Flores et al. 2012). Intriguingly, the melatonin response was independent of the auxin signaling pathway, because melatonin treatments that promote lateral root growth did not affect auxin-responsive gene expression. In contrast, serotonin inhibited the expression of both *DR5:uidA* marker (Fig. 2B), indicating that it likely acts as an endogenous auxin inhibitor. Other recent reports have evidenced the rhizogenic capacity of melatonin, a pair of these reports were published by Sarropoulou et al. (2012a; 2012b), who observed an auxinic response to melatonin in explants of the sweet cherries, cherry rootstock PHL-C (*Prunus avium* × *Prunus cerasus*), cherry rootstocks CAB-6P (*Prunus cerasus* L.), Gisela 6 (*P. cerasus* × *P. canescens*), and MxM 60 (*P. avium* × *P. mahaleb*) on adventitious root formation and root growth. Moreover, the use of transgenic rice lines that overexpress serotonin N-acetyltransferase from sheep revealed that enhanced melatonin levels in these transgenic lines correlated with increasing adventitious root lengths and improved growth (Park and Back, 2012). In cucumber (*Cucumis sativus*) melatonin concentration of 300 μM promoted lateral root growth (Zhang et al. 2013; 2014). The beneficial effects of melatonin to plants via promotion of root branching were recently confirmed in *Punica granatum* cv. (Sarrou et al. 2014). An analysis of most reports about the effects of serotonin and melatonin on root growth, lateral and/or adventitious roots thus indicates that these indolamines may affect developmental processes either acting as auxin inhibitors or more likely in an auxin-independent pathway (Pelagio-Flores et al. 2011, 2012; Koyama et al. 2013). Thus, it is tempting to speculate that specific signal transduction pathways may exist, which control the cellular and developmental responses to internal levels of serotonin and melatonin. In agreement with this speculation, analysis of genes differentially expressed in response to melatonin in rice (Byeon et al. 2013), cucumber (Zhang et al. 2014) and *Arabidopsis* (Weeda et al. 2014) have shown that auxin-related genes are minimally represented or are mostly down-regulated after melatonin treatment, thus providing support to an auxin-unrelated or antagonist signaling pathway mediating melatonin response.

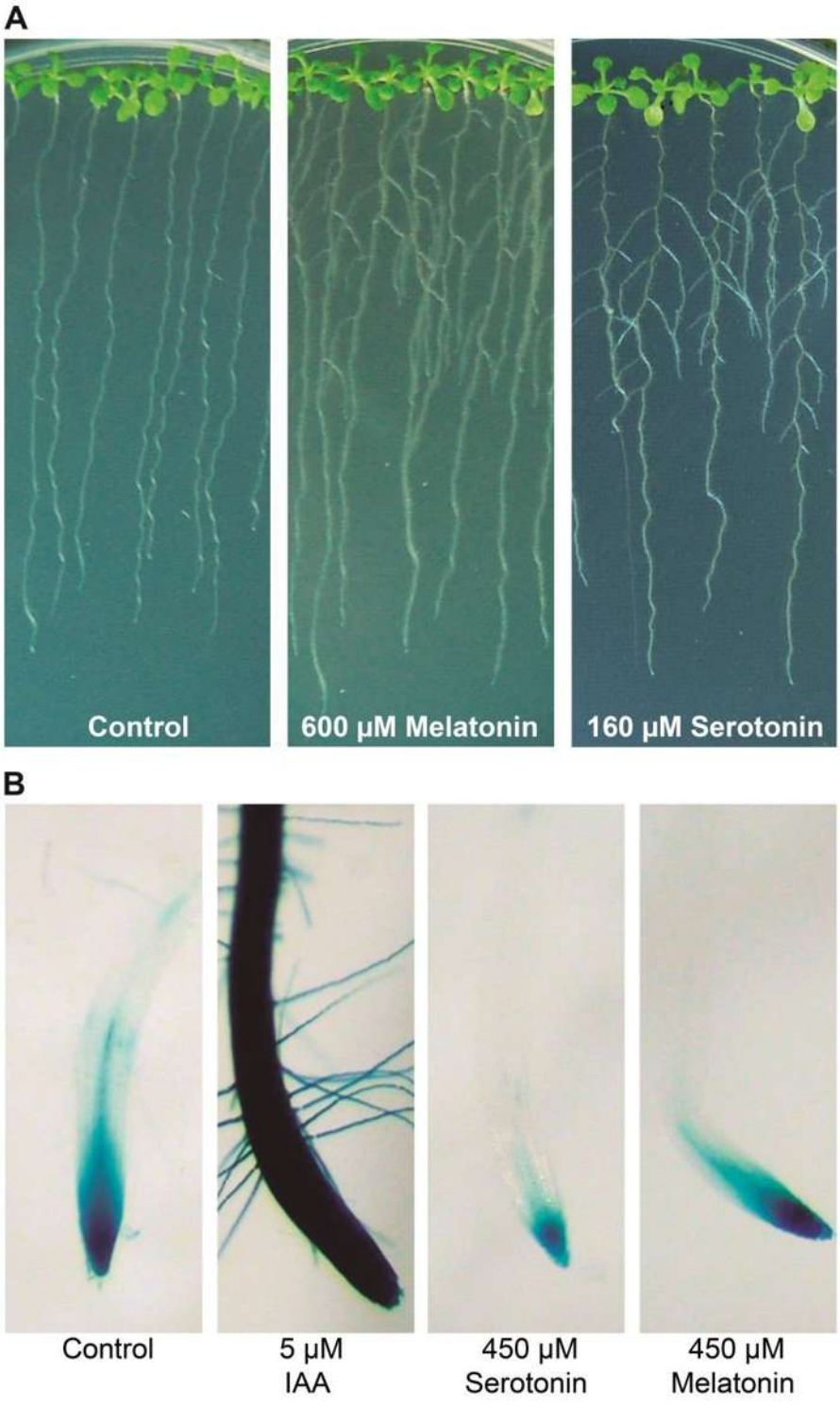

Figure 2. Effects of melatonin and serotonin on *Arabidopsis* root system architecture and auxin-regulated gene expression. (A) Melatonin and serotonin effects on primary and lateral root formation. (B) Comparative effect of IAA, melatonin and serotonin on the auxin-responsive marker *DR5:uidA*. Notice that serotonin or melatonin did not activate auxin-inducible gene expression, in contrast serotonin decreases expression of the markers in both shoots and roots. Representative photographs of Arabidopsis seedlings treated with the indicated compounds are shown.

Abiotic stress tolerance

Oxidative stress is an important inducer of damage and cellular death caused by an increase in the level of reactive oxygen species (ROS) and other free radicals. ROS play an important role in regulating plant growth and development, defense, stomatal closure and cell signaling (Wrzaczek et al. 2013; Kangasjärvi and Kangasjärvi, 2014). Plants as sessile organisms are exposed to diverse biotic and abiotic stresses that in most cases induce ROS accumulation, which together with the ROS generated as result of metabolism and the high photosynthetic activity of plants could lead to an oxidative stress that need to be controlled to avoid damage of plant tissues (Foyer and Noctor, 2013; Considine and Foyer, 2013).

Serotonin and melatonin possess a high antioxidant capacity that overpasses that of the antioxidants ascorbic acid and tocopherol (Poeggeler et al. 2002; Kang et al, 2009a). In this regard, many beneficial effects of melatonin in response to the majority of abiotic stresses have been evidenced. Increased melatonin levels are found in tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), and in alpine and Mediterranean plants naturally exposed to high UV levels when compared to the same species exposed to lower UV radiation (Dubbels et al. 1995; Tettamanti et al. 2000). Melatonin levels correlated with tolerance to ozone, a free radicals generator, suggesting an antioxidant role for melatonin (Hardeland and Pandi-Perumal, 2005). Similar data were obtained in *Glycyrrhyza uralensis*, in which plants grown in high-intensity UV-B radiation showed an enhanced melatonin accumulation in roots when compared with plants grown in low-intensity radiation, suggesting that the high-intensity UV-B radiation increases the melatonin levels as a natural response to protect the plants of oxidative stress (Afreen et al. 2006). In addition, the detrimental effects induced by abiotic stresses, such as drought, cold, heat, salinity, alkalinity, chemical pollutants, and herbicide treatments on plant growth can be alleviated by melatonin (Kang et al. 2010; Li et al. 2012; Park et al. 2013; Bajwa et al. 2014; Arnao and Hernández-Ruiz, 2014; Zhang et al. 2015; Liu et al. 2015), via ROS scavenging, and regulating the expression and activity of antioxidant enzymes.

The molecular mechanisms by which melatonin enhances stress tolerance have been poorly studied. However, recent findings indicate that melatonin decreases the drought stress in two *Malus* species by regulating expression of genes involved in ABA metabolism and decreasing the ABA content, it also acts as a scavenger of H_2O_2, and improves the stomatal function maintaining open stomata (Li et al. 2015). Multiple genes involved in diverse plant processes such as nitrogen and carbohydrate metabolism, the

tricarboxylic acid cycle, transport, hormone metabolism, metal homeostasis, redox reactions, and secondary metabolism are up-regulated in bermudagrass by melatonin [*Cynodon dactylon* (L). *Pers*.] (Shi et al. 2015). All the above mentioned evidences indicate that environmental stress can increase the level of endogenous melatonin in plants. Concomitantly, overexpression of the melatonin biosynthetic genes in crops could represent a promising avenue towards adapting plants to changing and/or challenging environments.

Regulation of senescence

Serotonin and melatonin modulate plant senescence via their antioxidant properties. Senescence is a development process inherent to all plants. The phytohormones abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) promote senescence, while cytokinins delay it. Serotonin and melatonin also delay the senescence process in different plants and under diverse growth conditions. Melatonin showed a clear protective effect delaying the senescence process in detached barley (*Hordeum vulgare* L.) leaves, evidenced by the greater chlorophyll content in leaves treated with melatonin, where it could act through an specific control of genes associated to senescence (SAGs) or via its role as antioxidant, preventing oxidative stress (Arnao and Hernández-Ruiz, 2009). Similarly, serotonin delayed senescence induced by nutrient deprivation or leaf detachment in rice. Transgenic rice plants with high serotonin levels showed delayed senescence, while plants with suppressed serotonin production senesced faster than the wild type and these effects were associated with the high antioxidant capacity of serotonin suggesting that it may protect the cellular integrity via facilitating efficient nutrient recycling from senescing leaves to sink tissues (Kang et al. 2009a).

Melatonin regulates senescence of apple leaves by modulating the levels and activity of antioxidative enzymes and suppression of senescence genes (Wang et al. 2012). Similar outcomes were observed in apple leaf senescence under drought conditions (Wang et al. 2013a) and in leaf senescence of *Malus hupehensis* (Wang et al. 2013b). In conclusion, the above-described effects of melatonin on senescence regulation has been closely related with their properties as free radical scavenger, preventing the ROS generation or by affecting directly or indirectly the antioxidant enzymes and/or senescence genes (Tan et al. 2002, 2012; Kang et al. 2009a; Galano et al. 2011, 2013). Although the molecular mechanisms of serotonin and melatonin action on senescence regulation remain unclear, a

recent transcriptome analysis from *Arabidopsis* in response to melatonin indicated that the transcription factors *AUXIN RESISTANT 3 (AXR3)/INDOLE-3-ACETIC ACID INDUCIBLE 17 (IAA17)* and *SHORT HYPOCOTYL 2 (SHY2)/IAA3*) related to auxin signaling, were down-regulated by melatonin (Weeda et al. 2014). Shi et al. (2014) found that melatonin levels in *Arabidopsis* rosette leaves were induced in an age-dependent manner and that leaf senescence is delayed by exogenous melatonin, which correlated with down-regulated expression of *AXR3/IAA17*. Besides, AtIAA17-overexpressing plants and AtIAA17 knockout mutants showed contrasting effects with early and delayed leaf senescence respectively, indicating that *AXR3/IAA17* plays an essential role in senescence regulation mediated by melatonin probably affecting the expression of senescence-related *SEN4* and *SAG12* genes (Shi et al. 2014).

Activation of defense responses

The plant defense includes physical and chemical barriers. The protective effects of serotonin and melatonin to biotic stress have also been documented both in plant defense against pathogens and herbivores. Serotonin may be part of defense responses as it accumulates in trichomes of nuttle plant (*Cnidoscolus texanus*), as part of protective chemicals (Lookadoo and Pollard, 1991). Some hydroxycinnamic acid amides have been implicated in the inducible defense of plants and are considered as phytoalexins, in this case, the hydroxycinnamic acid amides of serotonin such as *N*-*p*-coumaroylserotonin and *N*-feruloylserotonin, which are present in low amounts on healthy bamboo plants and are accumulated in diseased plants in response to pathogenic fungi play a role in defense via their antifungal activity (Tanaka et al. 2003).

Serotonin plays an important role in defense against the pathogen fungus *Bipolaris oryzae*, since serotonin and its hydroxycinnamic acid amides are accumulated and incorporated into the cell wall at damaged areas in infected rice leaves, possibly serotonin polymers form a physical barrier, which prevents the spread of the pathogen at the site of infection (Ishihara et al. 2008a). In another report, Ishihara et al. (2008b) evaluated the plant response against herbivore attack using the rice striped stem borer (*Chilo suppressalis*). The authors found increased levels of serotonin and *p*-coumaroylserotonin in the larvae-fed leaves, similarly to other reports of plants grown in natural conditions or upon pathogen infection (Ly et al. 2008; Kang et al. 2009b). Thus, the defense properties of serotonin are explained via reinforcement of cell walls, its strong antioxidant activity and antifungal

activity. Like serotonin, melatonin can modulate defense responses in apple plants (*Malus prunifolia* (Willd.) Borkh. cv. Donghongguo) against the damage caused by the fungus *Diplocarpon mali*. In addition, pretreatment of plants with melatonin improved the resistance to *Marssonina* apple blotch, by reducing the number of lesions, inhibiting pathogen expansion, maintaining a high potential efficiency of photosystem II, improving the total chlorophyll content, modulating the oxidation-reduction system and enhancing the activities of plant defense-related enzymes such as chitinase and β-1,3-glucanasa (Yin et al. 2013). Melatonin treatments limit the propagation of *Pseudomonas syringae* DC3000 in *Arabidopsis* leaves inhibiting bacterial growth by ten-fold compared to untreated leaves. These effects were related to induction of pathogenesis-related (PR) genes, as well as several genes induced in defense responses mediated by salicylic acid and ethylene. Further analysis of *Arabidopsis* mutants suggested that the mechanisms by which melatonin modulate the plant defense responses are mediated by the salicylic acid and ethylene signaling pathways (Lee et al. 2014).

Serotonin and melatonin in plant productivity

Yields of the major cereal crops such as rice, wheat, and maize have increased steadily over the past years. However, to meet cereal production demand in the next decade, increase of yields must continue. Crop yield might be influenced by several factors including root system architecture, environmental conditions and the senescence process, all of which are regulated by serotonin and melatonin (Janas and Posmyk 2013). Thus, it is not surprising that these indoleamines could have a positive effect on crop yield. Moreover, crop yields are limited by a combination of biotic stresses, abiotic stresses, and nutritional factors. Various analyses have suggested that drought, heat, cold or salinity—are the major factors that prevent crops from realizing their full yield. Recent interest in renewable fuels has led to a substantial increase in ethanol production from plant materials. Although the initial emphasis has been on using starch from corn, and to a lesser extent from other food grains, this is unlikely to be sustainable in the long term as maize is of primary importance for human nutrition; alternatives such as cellulose sources from crops with high biomass production such as sugarcane are likely to be a more important substrate for ethanol production. The challenge here is to increase total vegetative biomass rather than seed yield. It is expected that by increasing total leaf area and leaf number, while

decreasing senescence, the photosynthetic capacity of plants as well their overall yield should increase.

The potential of serotonin and melatonin to increase biomass production of plants has been found to depend on the plant species, growth conditions and time of application. Contrasting effects on yield have been reported for serotonin. An study conducted by Kanjanaphachoat and associates (2012), showed that in rice plants, overexpression of two putative tryptophan decarboxylase genes, TDC-1 and TDC-3 genes, involved in serotonin synthesis increases serotonin levels, causing a stunted growth, low fertility and dark-brown color. Enzymatic assays of both *TDC-1* and *TDC-3* recombinant proteins showed tryptophan decarboxylase activities that converted tryptophan to tryptamine, which could be converted to serotonin by a constitutively expressed tryptamine 5' hydroxylase (T5H). A mass spectrometry assay demonstrated that the dark brown leaves in the TDC-overexpressing lines were caused by the accumulation of serotonin but not tryptamine. These results represent the first evidence that over-expression of TDC results in deleterious effects to plants. In another report, the roots and shoot of rice seedlings grown in the presence of tryptamine exhibited a dose-dependent increase in serotonin in parallel with enhanced T5H enzyme activity. However, no detrimental growth was evidenced and the seedlings retained a normal phenotype (Kang et al. 2007a). In vitro experiments using *Arabidopsis* seedlings germinated and grown in media supplied with increasing concentrations of serotonin, revealed that serotonin indeed repress growth and causes dark-brown color in roots and shoots (Pelagio-Flores et al. 2011). More recent studies in which *Arabidopsis* seedlings were pretreated for 10 days with serotonin and then transferred to soil, indicated a delayed life cycle, and enhanced leaf biomass production and seed yield (Fig. 3A and B). Interestingly, serotonin pretreatment delayed the transition from vegetative growth to flowering in plants transferred to soil, thereby extending the period of vegetative growth of plants, which leads to greater biomass, larger leaves, thicker stems and more fruits and seeds (Fig. 3C and D). These results are promising towards the use of serotonin, which may be now incorporated into different agricultural biostimulants to increase leaf biomass and photosynthesis in crops for more extensive evaluation under field conditions.

Melatonin supply to cucumber and corn seeds had a beneficial effect on the growth of seedlings and crop production of plants, especially when subjected to cold (Posmyk et al. 2009) and water-stress (Zhang et al. 2013). In experiments conducted under field conditions, plants from seeds of corn (*Zea mays* L), mung bean (*Vigna radiata* L.) and

cucumber (*Cucumis sativus* L.), pretreated with melatonin had higher yield than untreated plants (Janas and Posmyk 2013). Treatment of seeds with melatonin also increased leaf size, plant height, seed production and fatty acid content in soybean. Melatonin also improved soybean tolerance to salt and drought stresses through up-regulation of genes related to secondary metabolic processes, cell division, photosynthesis, carbohydrate metabolism, fatty acid biosynthesis, and ascorbate metabolism, which are normally repressed by salt stress. These results show the potential of melatonin for improvement of growth and seed production in different crops (Wei et al. 2014).

Selection and breeding for high biomass-yielding varieties is a traditional strategy to increase total plant biomass. Alternatively, some effort is now going into the use of transgenic approaches to increase total plant biomass, in this regard, the genes for serotonin and melatonin biosynthesis are promising targets for potential biotechnological applications.

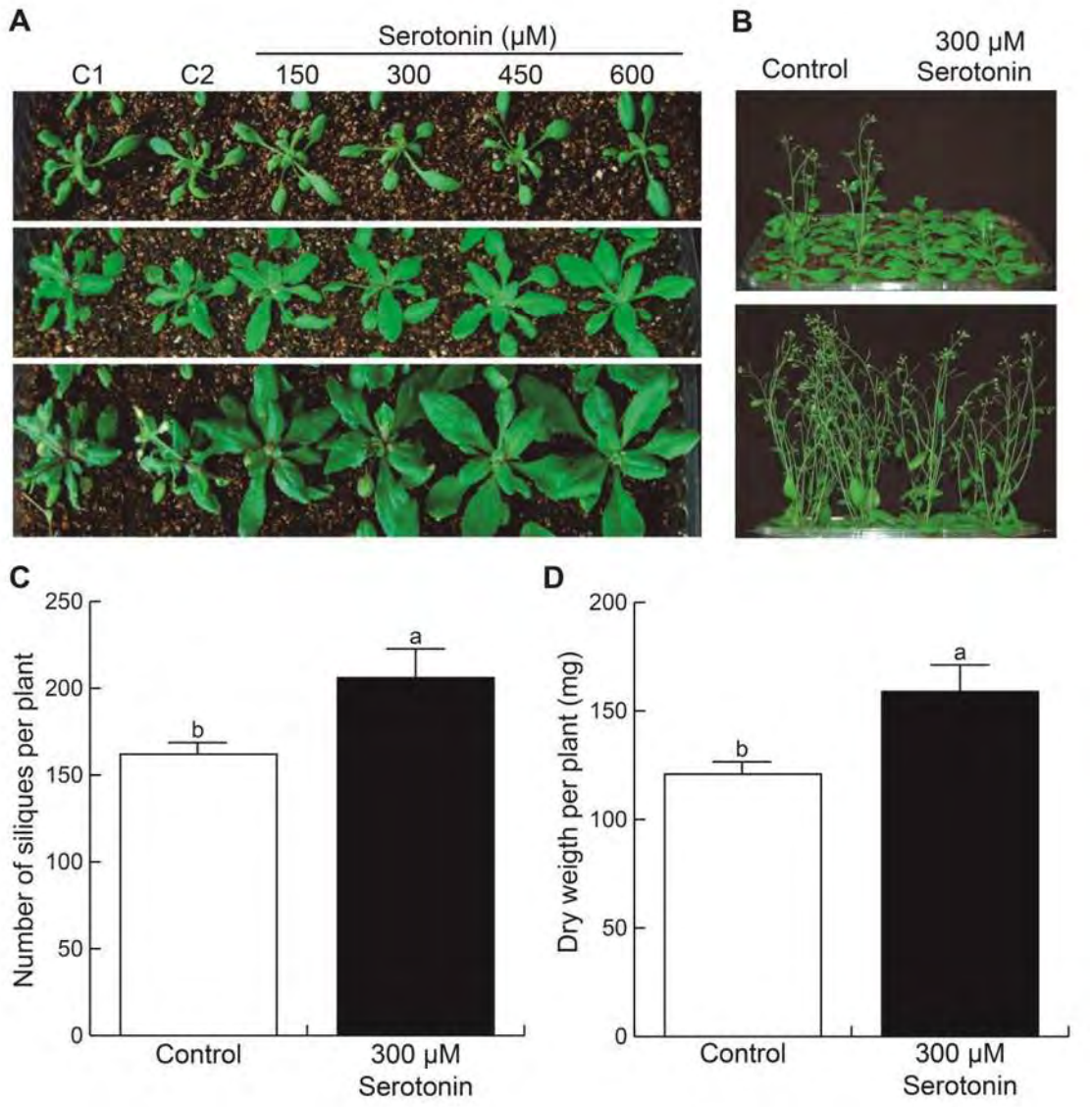

Figure 3. Effect of serotonin on *Arabidopsis* growth. *Arabidopsis* seedlings were grown 10 days in vitro at indicated serotonin concentrations and then transferred to soil to complete their life cycle. Note the positive impact of serotonin in rosette leaves (A, B), seed and biomass production (C and D, respectively). Plants were grown with a photoperiod of 16-h-light/8-h-darkness at 22°C in a growth chamber.

Concluding remarks

Serotonin and melatonin are emerging signals for the regulation of plant growth and development an play an important role in plant adaptation to stressing environmental conditions. Melatonin acts as a circadian regulator, cytoprotector and growth promoting substance. It also acts in rhizogenesis, cellular expansion and stress-protection. Serotonin modulates root branching affecting both de novo formation of organs and cell

differentiation of root epidermal cells. Currently, the main aspects of serotonin and melatonin research are (i) To elucidate the signal transduction pathways and mechanisms of action of these indoleamines, (ii) To explore the activity of these compounds in crops and vegetables, and (iii) To evaluate the impact of their application as biostimulators in agriculture. Regarding the first aspect, the use of *Arabidopsis* reporter and mutant lines indicates that serotonin and melatonin did not increase auxin response, instead, serotonin actually inhibits auxin-regulated gene expression. Still remains to be investigated the possible crosstalk of serotonin and melatonin response with ethylene, cytokinin, JA or SA signaling, particularly considering their proposed roles in defense and senescence. Application of melatonin improves root development and adaptation to soil stress such as drought, salinity and pollutants. This may be explained because roots are the critical sites of water and nutrient uptake by plants. Melatonin thus may enhance the rate of germination, and modulate developmental transitions during the plant life cycle with positive impact on plant productivity. This may be due acting as a retardant of stress-induced leaf senescence. From this information, it is tempting to speculate that increasing melatonin levels in crops or obtaining melatonin-overproducing plants may be a promising strategy to improve plant productivity under adverse environmental conditions that affect harvest index, or to better adapt plants to climate changes. Another proposal concerns the use of plants rich in melatonin as a tool in phytoremediation of heavy metal contaminated soils, a strategy that certainly deserves further attention. An interesting point to underline is that, although serotonin and melatonin appear to modulate the same physiological processes such as root development, they seem to act through different mechanisms. Indeed, future studies should be conducted to determine the factors that increase serotonin and melatonin biosynthesis and the relationship with auxin biosynthesis, particularly considering that all three compounds are produced from tryptophan as precursor.

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9. DISCUSION Y CONCLUSIONES

La serotonina y melatonina son dos indolaminas derivadas del aminoácido triptófano, presentes de manera natural en las plantas, en las que, al igual que en los animales, desempeñan un papel importante en la regulación de procesos fisiológicos y del desarrollo. Algunos de los procesos en los cuales se ha asociado la participación de estos compuestos incluyen la regulación de la senescencia, la organogénesis de la raíz, respuestas a estrés y defensa entre otros (Hernández-Ruiz et al. 2005; Arnao y Hernández-Ruiz 2006; Chen et al. 2009; Kang et al. 2010; Pelagio-Flores et al. 2011; Ramakrishna et al. 2011; Li et al. 2012; Sarropoulou et al. 2012a; 2012b; Park et al. 2013; Bajwa et al. 2014; Arnao y Hernández-Ruiz, 2014).

A partir del triptófano, se sintetiza el ácido indol acético, considerado como la principal auxina de plantas (Woodward y Bartel 2005). Lo anterior permitió plantear la hipótesis de que las indolaminas podrían tener actividad auxínica y regular el crecimiento y desarrollo de manera similar al AIA (Csaba y Pal. 1982; Murch et al. 2001; Murch y Saxena 2002; Hernández-Ruiz et al. 2004). Sin embargo, a diferencia de las auxinas y otros reguladores del crecimiento, los cuales han sido ampliamente estudiados y de los que se conoce en gran detalle su función y mecanismos de acción, la serotonina y la melatonina son dos compuestos de reciente interés en el campo de la investigación científica de plantas, por lo que la información existente respecto al papel que desempeñan en plantas, así como los mecanismos involucrados en mediar las respuestas celulares, genéticas y moleculares a dichos compuestos es limitada.

En un primer aporte de nuestro grupo de investigación para dilucidar los mecanismos de señalización de las indolaminas, se caracterizó el efecto de la serotonina sobre la regulación de la arquitectura del sistema radicular de *Arabidopsis thaliana* y su relación con la vía de señalización de las auxinas, en el cual se encontró que la serotonina tiene la capacidad de regular diferentes procesos del desarrollo de una manera independiente a la auxinas, actuando más bien a través de un mecanismo que involucra la inhibición de la respuesta auxínica (Pelagio-Flores et al. 2011). Considerando que las serotonina es intermediario en la síntesis de melatonina, como una continuación de dichas investigaciones, resultó interesante determinar si la melatonina también podía regular el desarrollo de las plantas lo hace la serotonina o las auxinas. A diferencia de la serotonina la cual inhibe el crecimiento de la raíz primaria, el desarrollo de los pelos radiculares y la formación de raíces laterales en altas concentraciones (Pelagio-Flores et al. 2011), la

melatonina no afectó significativamente el crecimiento de la raíz primaria y la formación de pelos radiculares fue solo mínimamente inhibida, además de que incrementa la formación de raíces laterales, en concentraciones en las que la serotonina inhibe el desarrollo de dicho órganos, lo que indica una alta capacidad organogénica. Para determinar el papel de las auxinas en este programa, utilizamos las líneas de *Arabidopsis DR5:uidA*, *BA3:uidA* y *HS::AXR3NT–GUS* como marcadores de la respuesta auxínica (Ulmasov et al. 1997; Oono et al. 1998; Ouellet et al. 2001). Encontrando qué la melatonina no activa la expresión *DR5:uidA* o *BA3:uidA*, lo que sugiere que la melatonina carece de actividad auxínica, lo cual fue apoyado por un experimento en el que se evaluó el efecto de la melatonina sobre la degradación de la proteína AXR3, perteneciente a la familia de represores de auxinas (Aux/IAA) que se sabe se degradan en respuesta a auxinas. A diferencia de la serotonina que inhibe la expresión de los marcadores *DR5:uidA* o de *BA3:uidA,* la melatonina no reprimió su expresión, consistente con el efecto diferencial que se observa entre ambas moléculas. En conjunto estos resultados muestran que la melatonina tiene la capacidad de regular procesos del desarrollo de las plantas de manera diferencial a la serotonina e independiente de la activación de la vía de señalización de auxinas. Nuestros resultados han sido confirmados por trabajos más recientes en los que se muestra que la melatonina regula el desarrollo de las plantas de manera independiente a las auxinas (Koyama et al. 2013), así como por el análisis de la expresión global de genes en respuesta a melatonina en plantas de arroz (Byeon et al. 2013), pepino (Zhang et al. 2014) y Arabidopsis (Weeda et al. 2014) que muestran que la melatonina no induce la expresión de genes regulados por auxinas o incluso estos genes son reprimidos después del tratamiento con melatonina. Otra evidencia que apoya que la serotonina y la melatonina carecen de actividad auxínica como se pensaba inicialmente, es la baja actividad de ambos compuestos comparada con la de las auxinas, las cuales actúan en concentraciones muy bajas en la modulación del crecimiento de la raíz, mientras que la serotonina y la melatonina como vimos en este trabajo actúan en concentraciones muy por muy por encima (al menos 100 veces más) de las requeridas de auxinas.

Nuestros resultados muestran que tanto la serotonina como la melatonina tienen la capacidad de regular la arquitectura de la raíz de *Arabidopsis thaliana*, impactando en el desarrollo de raíces laterales y adventicias, el desarrollo de pelos radiculares o el crecimiento de la raíz primaria de manera diferencial e independiente a la ruta de señalización de las auxinas, propiedades que bajo determinadas condiciones podrían

favorecer la supervivencia de las plantas o aumentar su capacidad para la captación de agua y nutrientes del suelo, lo que resultaría de gran utilidad en la agricultura para aumentar la productividad mediante el uso de bioestimulantes. En este sentido, un primer acercamiento sobre el impacto de la serotonina sobre el crecimiento de plantas de *Arabidopsis* crecidas en suelo, mostró que la serotonina impacta positivamente el crecimiento general de las plantas y aumenta la producción de frutos y el número de semillas por fruto, comparado con las plantas que no fueron tratadas (Capitulo III). Lo anterior posiblemente asociado al efecto de la serotonina en retardar la senescencia, ya que se ha reportado que las plantas con senescencia acelerada producen menos biomasa que aquellas con senescencia retardada, propiedad atribuida a altos niveles endógenos de serotonina (Gregersen et al. 2013). Consistente con lo anterior, las plantas tratadas con serotonina tardan más en desarrollar el tallo floral, producen mayor número de hojas y tardan en envejecer (Kang et al. 2009). Estos resultados sugieren que la serotonina puede ser es un compuesto con potencial para el uso en la agricultura, aunque se requiere de mayor evidencia experimental para profundizar en las nuevas posibles aplicaciones.

Por otra parte, tanto la serotonina como melatonina han sido consideradas como moléculas protectoras contra plagas, organismos patógenos y el agobio ambiental como la radiación, temperaturas extremas, agentes químicos y estrés hídrico entre otros. Dicho efecto protector se ha atribuido principalmente a la capacidad antioxidante que tienen ambos compuestos, ya que tales condiciones de estrés generalmente se asocian con la inducción especies reactivas de oxígeno (ROS) y la serotonina y la melatonina pueden actuar como atrapadores de radicales libres o regular los niveles y la actividad de enzimas antioxidantes (Ishihara et al. 2008a, 2008b; Kang et al. 2009b; Arnao y Hernández-Ruiz, 2009; Ramakrisna et al., 2011; Wang et al. 2012; Zhang et al. 2015). La capacidad antioxidante de ambas indolaminas, podría jugar un papel importante en mediar las respuestas de la planta a estos compuestos, debido a que se ha visto que las ROS no solo son moléculas que causan daño a los organismos, sino que más bien dependiendo de su distribución y concentración, pueden ser toxicas o actuar como moléculas señalizadoras. En plantas, las ROS actuando como moléculas de señalización participan en la regulación de importantes procesos fisiológicos, incluyendo el desarrollo del sistema radicular, la formación de raíces laterales y pelos radiculares, a través de una compleja interacción con otros reguladores del crecimiento (Cárdenas y Quinto 2008; Mittler et al. 2011; Manzano et al. 2014).

En este trabajo se investigó la participación de las ROS en mediar las respuestas a la serotonina, para lo cual se utilizó la mutante *rcd1* la cual es resistente a los efectos del Paraquat (generador de ROS), así como mutantes alteradas en las respuestas al ácido jasmónico (*coi1* y *jar1)* y al etileno *(etr1*, *ein2* y *ein3)*, debido a que se ha visto que la mutante *rcd1* muestra una reducida respuesta a tanto al ácido jasmónico como al etileno (Overmyer et al. 2000, Ahlfors et al. 2004). En este grupo de mutantes se evaluó el efecto de la serotonina sobre el crecimiento de la raíz primaria y se determinaron los niveles y la distribución de ROS comparado con las plantas silvestres. Interesantemente, todas las mutantes evaluadas mostraron resistencia a los efectos de la serotonina sobre la inhibición del crecimiento de la raíz primaria, comparado con las plantas silvestres, en las cuales la serotonina inhibe fuertemente el crecimiento de la raíz, mientras que las mutantes mantienen un mayor crecimiento evidenciado por una raíz primaria más larga. Para determinar la participación de las ROS en los efectos observados en respuesta a la serotonina, se determinaron los niveles de ROS tanto en condiciones control como en respuesta a la serotonina en todas las mutantes y se compararon con las plantas silvestres. Se encontró que todas las mutantes resistentes a la serotonina presentan un mayor contenido de ROS en la raíz primaria comparado con las plantas silvestres cuando son crecidas en condiciones control pero cuando son crecidas en presencia de serotonina, la inhibición del crecimiento de la raíz primaria en las plantas silvestres coincidió con un aumento en los niveles de ROS acumulándose principalmente en la punta de la raíz y disminuyendo en la zona de diferenciación, mientras que las mutantes resistentes mantienen una distribución y niveles similares a los observados en ausencia de serotonina, lo que sugiere que la inhibición de la raíz primaria se debe a la acumulación de ROS en la punta de la raíz. Aunque la resistencia observada en las mutantes *coi1*, *jar1*, *etr1*, *ein2* y *ein3* también sugiere que otras de las señales que podrían estar involucradas en mediar las respuestas a la serotonina pueden ser el AJ y el etileno. Experimentos adicionales para estudiar la posible participación del etileno en mediar las respuestas inducidas por serotonina, mostraron que el $AgNO_3$ (bloqueador de la acción del etileno) normaliza el crecimiento de la raíz de plantas tratadas con serotonina y que *eto3* (mutante sobreproductora de etileno), presenta una mayor sensibilidad tanto en la inhibición del crecimiento de la raíz primaria, evidenciando un papel fundamental del etileno en estos procesos. Los efectos de la serotonina no coinciden totalmente con las respuestas inducidas por etileno, por ejemplo, la formación de pelos radiculares es un proceso positivamente regulado por etileno mientras que la serotonina reprime la formación de éstos.

Considerando estos resultados nosotros proponemos que la inhibición del crecimiento de la raíz primaria en respuesta a la serotonina se debe a un cambio en la homeostasis de las ROS en la raíz primaria, a través de una compleja interacción ROS-JA-etileno en el que participan los genes *RCD1*, *COI1*, *JAR1*, *ETR1*, *EIN2* y *EIN3*.

La inhibición del crecimiento de la raíz primaria por acumulación de ROS en la punta de la raíz, es consistente con trabajos recientes en los que se muestra que la inhibición del crecimiento de la raíz primaria coincide con un aumento de ROS en la punta de la raíz, como en el caso de la triple mutante *nia1 nia2 noa1* deficiente en la producción de óxido nítrico, la cual presenta un fenotipo de raíz corta asociado a una mayor acumulación ROS, las cuales suprimen la respuesta a auxinas (Sanz et al. 2014). De manera similar, alteraciones en el crecimiento de la raíz primaria, en respuesta a la deficiencia de boro o fósforo, se han asociado con un aumento de ROS en la punta de la raíz, las cuales causan alteraciones en la integridad de las paredes celulares o inducen la deposición de callosa en el meristemo de la raíz, en ambos casos afectando la comunicación célula-célula para el mantenimiento del meristemo de la raíz, a través de un mecanismo que involucra la participación de auxinas y etileno (Camacho-Cristóbal et al. 2015; Müller et al. 2015). Los datos anteriores coinciden con un reporte previo que propone que las alteraciones en el crecimiento de la raíz, están asociadas a un cambio en la integridad de las paredes celulares causado por la distribución de ROS, vía la interacción auxinas-etileno (Tsang et al. 2011). Lo anterior sugiere la posibilidad de que la serotonina modifique la distribución de ROS en la raíz induciendo su acumulación en la punta de la raíz, lo que podría explicar el efecto represor de la serotonina sobre la expresión de los genes de respuesta a auxinas (Pelagio-Flores et al. 2011).

La serotonina y melatonina son dos compuestos naturales de plantas, con aparente función como moléculas señalizadoras evidenciada por la variedad de procesos en los que participan, razón por la cual en los últimos años han generado mayor interés en el campo de la investigación científica de plantas. Las diferentes publicaciones derivadas de nuestros resultados muestran que la melatonina tiene la capacidad de regular la arquitectura de la raíz de *Arabidopsis* de una manera diferencial a la serotonina e independiente de la señalización por auxinas, lo que contribuye al conocimiento respecto al papel de estos compuestos en las plantas. En este trabajo se muestran además, las primeras mutantes con respuesta alterada a serotonina (*rcd1*, *coi1*, *jar1*, *etr1*, *ein2* y *ein3*) y con ello los primeros elementos genéticos que participan en mediar sus respuestas en plantas.

En resumen, la serotonina y la melatonina al igual que el AIA son compuestos derivados del triptófano por lo que están relacionados estructuralmente. Nuestros resultados muestran que la serotonina y la melatonina tienen la capacidad de regular diferentes procesos del crecimiento y desarrollo sobre el sistema radicular de las plantas de manera diferencial y que actúan a través de mecanismos independientes. Por un lado las auxinas como es bien sabido regulan la arquitectura de la raíz principalmente por activar la vía de señalización de las auxínica. En contraste con auxinas, los mecanismos de acción de serotonina y melatonina recién comienzan a estudiarse, aquí nosotros sugerimos que la serotonina regula la arquitectura de la raíz por modular la homeostasis de ROS que a su vez suprime la respuesta auxínica, lo anterior a través un mecanismo que involucra la participación de los genes *RCD1*, *COI1*, *JAR1*, *ETR1*, *EIN2* y *EIN3*. La melatonina regula la arquitectura de la raíz a través de una vía independiente a auxinas y serotonina de la cual aún no se ha identificado ningún elemento genético (Fig. 15). Por lo que se requiere de más investigaciones para poder definir una vía de señalización especifica que integre todos los elementos que participan en mediar las respuestas de las plantas a ambos compuestos.

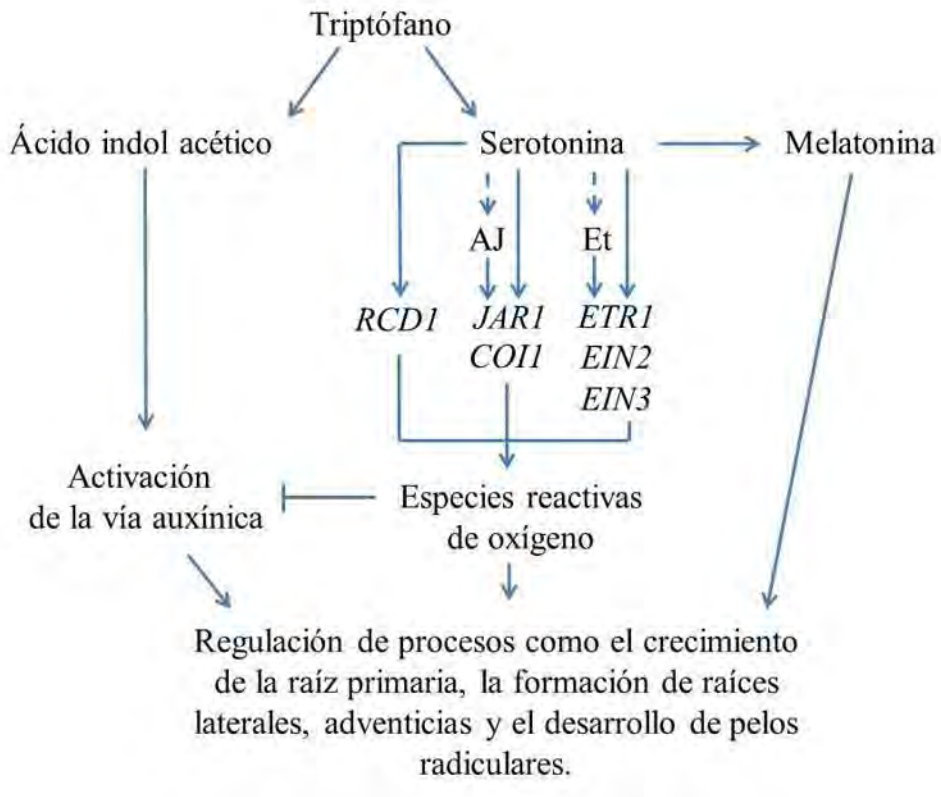

Figura 15. Esquema representativo de la regulación de la arquitectura del sistema radicular por el ácido indol acético, la serotonina y la melatonina. Los tres compuestos regulan la arquitectura de la raíz de manera independiente, la axina a través de la vía auxínica, la serotonina por modular la homeostasis de especies reactivas de oxígeno y la melatonina a través de un mecanismo aun desconocido.

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11. ADDENDA

En este apartado se presentan las publicaciones derivadas de otros proyectos de investigación realizados en colaboración con el grupo de trabajo durante mis estudios de doctorado.

I. Artículos publicados con arbitraje internacional

1. Raya-González J., **Pelagio-Flores R.,** López-Bucio J. (2012). The jasmonate receptor *COI1* plays a role in jasmonate-induced lateral root formation and lateral root positioning in *Arabidopsis thaliana*. *Journal of Plant Physiology* 169:1348-1358. (Factor de impacto: 2.5)
2. **Pelagio-Flores R,** Ortiz-Castro R, López-Bucio J (2013). *dhm1*, an *Arabidopsis* mutant with increased sensitivity to alkamides shows tumorous shoot development and enhanced lateral root formation. *Plant Molecular Biology* 81:609-625. (Factor de impacto: 4.2)
3. Ortiz-Castro R, **Pelagio-Flores R,** Méndez-Bravo A, Ruiz-Herrera LF, Campos-García J, López-Bucio J (2014). Pyocyanin, a virulence factor produced by *Pseudomonas aeruginosa*, alters root development through reactive oxygen species and ethylene signaling in Arabidopsis. *Molecular Plant-Microbe Interactions* 4:364-378. (Factor de impacto: 4.0)

II. Revisiones en revistas internacionales

1. José López-Bucio J, **Pelagio-Flores R**, Herrera-Estrella A *Trichoderma* as biostimulant: Exploiting the multilevel properties of a plant beneficial fungus. (aceptado en la revista *Scientia Horticulturae* con factor de impacto de 1.3)

III. Capítulos de libro

1. Muñoz-Parra E., **Pelagio-Flores R.,** López-Bucio J., Beltrán-Peña E. (2013) Factores de competencia en plantas. Beltrán-Peña E., López-Bucio J. (Eds). Universidad Michoacana de San Nicolás de Hidalgo. pp. 14-25.

Journal of Plant Physiology 169 (2012) 1348–1358

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

The jasmonate receptor COI1 plays a role in jasmonate-induced lateral root formation and lateral root positioning in *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 13 January 2012
Received in revised form 12 April 2012
Accepted 9 May 2012

Keywords:
Arabidopsis thaliana
Auxin signaling
Jasmonic acid
Plant development
Root system architecture

ABSTRACT

Jasmonic acid (JA) regulates a broad range of plant defense and developmental responses. COI1 has been recently found to act as JA receptor. In this report, we show that low micromolar concentrations of JA inhibited primary root (PR) growth and promoted lateral root (LR) formation in *Arabidopsis* wild-type (WT) seedlings. It was observed that the *coi1-1* mutant was less sensitive to JA on pericycle cell activation to induce lateral root primordia (LRP) formation and presented alterations in lateral root positioning and lateral root emergence on bends. To investigate JA-auxin interactions important for remodeling of root system (RS) architecture, we tested the expression of auxin-inducible markers *DR5:uidA* and *BA3:uidA* in WT and *coi1-1* seedlings in response to indole-3-acetic acid (IAA) and JA and analyzed the RS architecture of a suite of auxin-related mutants under JA treatments. We found that JA did not affect *DR5:uidA* and *BA3:uidA* expression in WT and *coi1-1* seedlings. Our data also showed that PR growth inhibition in response to JA was likely independent of auxin signaling and that the induction of LRP required *ARF7*, *ARF19*, *SLR*, *TIR1*, *AFB2*, *AFB3* and *AXR1* loci. We conclude that JA regulation of postembryonic root development involves both auxin-dependent and independent mechanisms.

Introduction

The capacity of plants to survive adverse environmental conditions depends on a remarkable repertoire of growth and developmental changes to shape their basic body plan and optimize their metabolism to given biotic and abiotic demands. The root system (RS) exhibits an amazing diversity of architectures through changes in root hair, lateral root (LR) and adventitious root formation, which play an important role in anchor to the soil and in water and nutrient acquisition (López-Bucio et al., 2003; Nibau et al., 2008).

LR formation, which occurs throughout the life cycle of the plant, is a major determinant of root system architecture that increases branching and the root exploratory capacity. LRs initiate from pericycle founder cells that undergo coordinated cell division programs giving rise to LR primordia (LRP) (Malamy and Benfey, 1997; Dubrovsky et al., 2008). The newly formed LRP will continue to grow, eventually emerging through the adjacent endodermis, cortex, and epidermal layers of the primary root (PR). Finally, a new apical meristem is established that controls the production of cells required for growth of LRs (Swarup et al., 2008; Fukaki and Tasaka, 2009).

Little is known about the mechanisms that control LR development. However, accumulating evidence indicates that LR initiation, the establishment of the meristem and LR emergence are regulated independently (Fukaki and Tasaka, 2009). The plant hormone auxin (indole-3-acetic acid [IAA]) is an important long- and short-distance signal that controls multiple developmental processes in the RS through changes in cell division, elongation and/or differentiation (Chapman and Estelle, 2009; Vanneste and Friml, 2009; Kieffer et al., 2010). 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 stimulates LR formation (Celenza et al., 1995; Woodward and Bartel, 2005), whereas polar auxin transport inhibitors such as N-(1-naphthyl)-phthalamic acid (NPA) 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 number of LRs (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants defective on auxin transport, perception, or signaling, including *aux1*, *axr1*, *tir3*, *slr* and *arf7*/*arf19*, show reduced LR

Abbreviations: BR, brassinosteroids; CK, cytokinin; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; LR, lateral root; LRP, lateral root primordia; NAA, naphthaleneacetic acid; NPA, N-(1-naphthyl)-phthalamic acid; PR, primary root; RS, root system; RSA, root system architecture; TIBA, 2,3,5-triiodobenzoic acid; WT, wild-type.

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http://dx.doi.org/10.1016/j.jplph.2012.05.002

formation (Lincoln et al., 1990; Gil et al., 2001; Swarup et al., 2001; Fukaki et al., 2002). Interestingly, recent information suggests a link between root waving and LR initiation, which depends on gravitropism/thigmotropism. Reorientation of PR growth in response to gravity (gravitropism) or touch stimuli (thigmotropism) depends on auxin fluxes, which are connected with LR formation (De Smet et al., 2007; Laskowski et al., 2008; Lucas et al., 2008). When roots bend, the concentration of IAA increases along the outside of the bend. In addition, a complex auxin flux pattern is generated that further enhances IAA levels through localized reflux loops. The auxin importer-AUX1-and efflux transporters-PIN2,3,7-are known to be regulated by auxin. AUX1 overexpression enhances the auxin maxima that specify the LR founder cells at the bend, while down-regulation of PIN proteins modulates the spacing of LRs along the PR axis (Laskowski et al., 2008).

Plant hormones operate in a complex framework of interacting responses rather than through isolated linear pathways. This hormonal crosstalk network can be modulated by a multitude of signals from developmental or environmental origins. Whereas auxin is a key hormone for LR development, other hormones are also involved in LR formation acting as positive or negative regulators. For example, cytokinin negatively regulates LR initiation (Li et al., 2006; Laplaze et al., 2007; Kuderová et al., 2008). In contrast, brassinosteroids promote LR formation acting synergistically with auxin (Bao et al., 2004).

The phytohormone jasmonic acid (JA) is a crucial component of the plant defense signaling system. JA and its metabolites, collectively called jasmonates, are lipid-derived signals produced during defense responses against insects and pathogens (Stintzi et al., 2001; Kessler et al., 2004; Li et al., 2005; Browse and Howe, 2008) but also under exposition to ozone, UV light, wounding, and other abiotic stresses (Wasternack, 2007). Reduction in root growth and carbon allocation patterns in several plant species upon mechanical wounding or by herbivory was ascribed to JA. In *Arabidopsis*, treatment with JA inhibits PR growth, which was likely due to the arrest of mitosis (Staswick et al., 1992; Feys et al., 1994; Yan et al., 2007; Zhang and Turner, 2008). JA also promotes LR formation by directly inducing the auxin biosynthesis gene *anthranilate synthase1* (ASA1) and/or by modulating endocytosis and plasma membrane accumulation of the PIN2 protein (Sun et al., 2009, 2011). This opens the possibility that jasmonates can impact LR formation on two levels. First, during LR initiation, and secondly, affecting the emergence of LRs from the PR. It is also tempting to speculate that an increase in JA levels is perhaps induced by mechanical stimulation or gravity-induced root waving, which has been reported to promote LR formation (De Smet et al., 2007; Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008), or in response to localized auxin maxima, as auxin has been recognized to induce JA biosynthesis (Tiryaki and Staswick, 2002; Hoffman et al., 2011). Currently, the exact cellular/tissue responses to jasmonates during the remodeling of RSA are not well understood.

Several *Arabidopsis* mutants that are deficient in jasmonate biosynthesis or signaling have been isolated and characterized including the *coronatine insensitive1* (*coi1*), *jasmonic acid resistant1* (*jar1*) and *auxin resistant1* (*axr1*) (Berger, 2002; Wasternack, 2007; Browse, 2009). Among these, the *coi1-1* mutant, which was isolated by its insensitivity to JA and coronatine in PR growth inhibition, has been shown to be defective in JA responses in most plant organs (Feys et al., 1994; Devoto et al., 2005), consistent with its role as a jasmonate receptor (Yan et al., 2009). The resistance of *axr1* to both JA and auxin in root growth indicates that these regulators interact in modulating developmental processes (Tiryaki and Staswick, 2002). Additional commonalities exist between perception mechanisms of jasmonates and auxin. They both use as receptor an SCF-type E3 ubiquitin ligase with a specific F-box protein for each hormone, COI1 for jasmonate and TIR1 or the closely related proteins AFB1, AFB2 and AFB3 for auxin (Santner et al., 2009). Although one of the most dramatic effects of applied JA on plants is the inhibition of PR growth, the signaling mechanisms by which jasmonates regulate other aspects of RSA, such as LR formation and patterning merit further research.

In the present work, we investigated the effects of JA on RSA of *Arabidopsis* seedlings. We provide evidence that JA inhibits PR growth and regulates LR formation in a dose-dependent manner. The LR responses correlated with an induction of LRP formation. To investigate the role of JA in modulating the environmental regulation of LR growth, we performed experiments to compare the LR patterning and induction of LR development on bends between *Arabidopsis* wild-type (WT) and *coi1-1* seedlings. We also tested the responses of WT and *coi1-1* lines expressing the auxin-responsive marker constructs *DR5:uidA* and *BA3:uidA* and analyzed the root architectural responses to JA in a variety of *Arabidopsis* mutants defective in auxin transport and signaling. Our results show that the *COI1* locus is involved in jasmonate-induced LR formation, LR positioning and LR emergence on root bends in *Arabidopsis* seedlings.

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana* Col-0), the transgenic *Arabidopsis* lines *CycB1:uidA* (Colón-Carmona et al., 1999), *DR5:uidA* (Ulmasov et al., 1997), *BA3:uidA* (Oono et al., 1998), *pLOX2:uidA* (Jensen et al., 2002) and mutant lines, *coi1-1* (Feys et al., 1994), *axr1-3* (Lincoln et al., 1990), *aux1-7* (Picket et al., 1990), *axr2-1* (Timpte et al., 1994), *axr4-1* (Hobbie and Estelle, 1995), *tir1/afb2/afb3* (Parry et al., 2009), *arf7-1/arf19-1* (Wilmoth et al., 2005), *slr* (Fukaki et al., 2002) and *eir1-3* (Luschnig et al., 1998), 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. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar (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 of light/8 h darkness, light intensity of 300 μmol/m^{-2}/s^{-1}, and temperature of 22 °C.

For transfer experiments, wild-type (WT) (Col-0) seeds were first sterilized and germinated on 0.2× MS medium as described above. For *coi1-1* mutant selection, 500 seeds from a *coi1-1/COI1* segregating population were screened for sustained PR growth in agar solidified MS 0.2× medium supplemented with 4 μM jasmonic acid (JA) by placing seeds on 100 cm^2 nutrient agar plates (20 seeds per plate). The seeds were distributed in two rows on the agar surface at a density of 1 seed/cm, stratified at 4 °C for 48 h, and then incubated at 22 °C. Putative JA resistant mutants with long PRs were selected and transferred to plates with the different treatments.

Chemicals

JA and indole-3-acetic acid (IAA) were purchased from Sigma. IAA was dissolved in dimethyl sulfoxide (DMSO), whereas JA was dissolved in ethanol. In control seedlings, we added the solvents in equal amounts as present in the greatest concentration of each compound tested.

Analysis of growth

Arabidopsis RS and PR meristem integrity were analyzed with a stereoscopic microscope (Leica, MZ6). All LRs emerged from the PR and observed with the 3× objective were taken into account for LR number data. Images were captured with a Samsung SCC 131-A digital color camera adapted to the microscope. PR length was determined for each root using a ruler. LR number was determined by counting the LRs per seedling, and LR density was determined by dividing the LR number value by the PR length values for each analyzed seedling. For transfer assays, the PR length and LR number and density were determined from the tip to the marked site of PR when the transfer was made. For all experiments with WT and mutant lines, the overall data were statistically analyzed using the SPSS 10 program. Univariate and multivariate analyses with Tukey's post hoc test were used for testing differences in growth and root development responses. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Determination of developmental stages of lateral root primordia (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 follows, 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.

Results

JA regulates Arabidopsis RS architecture

Previous reports have shown that JA regulates PR growth (Staswick et al., 1992) and LR formation (Sun et al., 2009). However a detailed connection between these developmental responses is still lacking. To determine whether PR growth inhibition was an important factor inducing de novo LR formation, we evaluated the effects of different JA concentrations in *Arabidopsis* seedlings (Col-0) germinated and cultivated 12 days on agar-solidified Petri plates supplied with 0.2× Murashige and Skoog (MS) medium. We found that low JA concentrations from 0.25-to-1 μM JA that modestly inhibited PR growth (10-to-20%) strongly increased (two-to-three-fold) the number of emerged LRs per seedling (Fig. 1A and B). Concentrations of 4 μM JA or higher inhibited 80% PR growth compared to solvent-treated seedlings but LR formation decreased when compared with 0.25 μM JA (Fig. 1A and B). The LR density (LR number per cm PR) increased in a dose-dependent way by JA (Fig. 1C), giving rise to a shift in RSA from a long PR with a low number of LRs to a short and more branched RS in JA-treated seedlings (Fig. 1E).

Interestingly, the length of LRs increased by 40% at 1 and 2 μM JA but decreased at 4 μM or greater JA concentrations (Fig. 1D and E).

PR growth depends on two basic developmental processes: cell division in the root apical meristem and elongation of cells that leave the root meristem (Blilou et al., 2002). To determine if JA could inhibit PR growth affecting any of these processes, we tested the responses of *Arabidopsis* roots to this compound by using the mitotic reporter *CycB1:uidA* line, which monitors cell cycle progression in the root meristem (Doerner et al., 1996). *Arabidopsis* transgenic seedlings expressing *CycB1:uidA* were grown in 0.2× MS medium supplied with the solvent (control) or with 1 and 4 μM JA. In solvent-treated seedlings, a patchy pattern of expression was observed in the PR meristem (Fig. S1). In plants subjected to treatments with 1 and 4 μM JA, GUS expression in the PR tip decreased compared with control plants (Fig. S1). Next, we quantified the length of the PR meristem in the same seedlings. At these similar developmental stages, 4 μM JA decreased 50% the length of the meristem, compared with control plants (Fig. S1). To determine the effects of JA on cell elongation, we measured fully developed cortical cells of the PR. In seedlings treated with 4 μM JA, cortical cell length was 45% reduced when compared with control plants (Fig. S1). These results show that JA may affect PR growth by inhibiting both cell division and elongation.

coi1-1 seedlings are defective in LRP development in response to JA

To understand the role played by the jasmonate receptor COI1 during LR formation, we investigated the effects of JA on LRP development in WT and *coi1-1* seedlings. An experiment was performed, in which 4 day-old WT and homozygous *coi1-1* seedlings were transferred to 0.2× MS agar-solidified medium supplemented with the solvent only or with 2 μM JA. Firstly, we analyzed LRP formation in both lines previous to transfer. It was found that *coi1-1* mutant seedlings developed a lower number of stage I LRP than WT seedlings (Fig. 2A), indicating that COI1 is important for pericycle cell activation. Two days after transfer to 2 μM JA, WT seedlings showed an increase in LRP stages I–V. In contrast, JA was unable to activate the same LRP stages in *coi1-1* mutants (Fig. 2B). In response to JA treatment, the LR density increased with time in WT seedlings but not in *coi1-1* mutants (Fig. 2C). These results indicate that COI1 is an important signaling component involved in LRP formation under normal growth conditions and in response to JA.

coi1-1 mutants are defective in LR positioning

LRs are spaced along the PR axis in a regular left-right alternating pattern that correlates with gravity-induced waving (De Smet et al., 2007; Laskowski et al., 2008; Lucas et al., 2008). To determine whether the mutation in *COI1* alters the LR formation pattern, we analyzed the left–right alternating pattern in WT (Col-0) and *coi1-1* seedlings. To investigate the correlation between PR growth and LR formation, the seedlings were transferred 4 days after germination from 0.2× MS medium to the same medium solidified with 1.5% agar and grown with an inclination angle of 45°. The PRs of *coi1-1* seedlings grew faster than those of WT seedlings. This effect was evident 4-to-6 d after transfer (Fig. 3A).

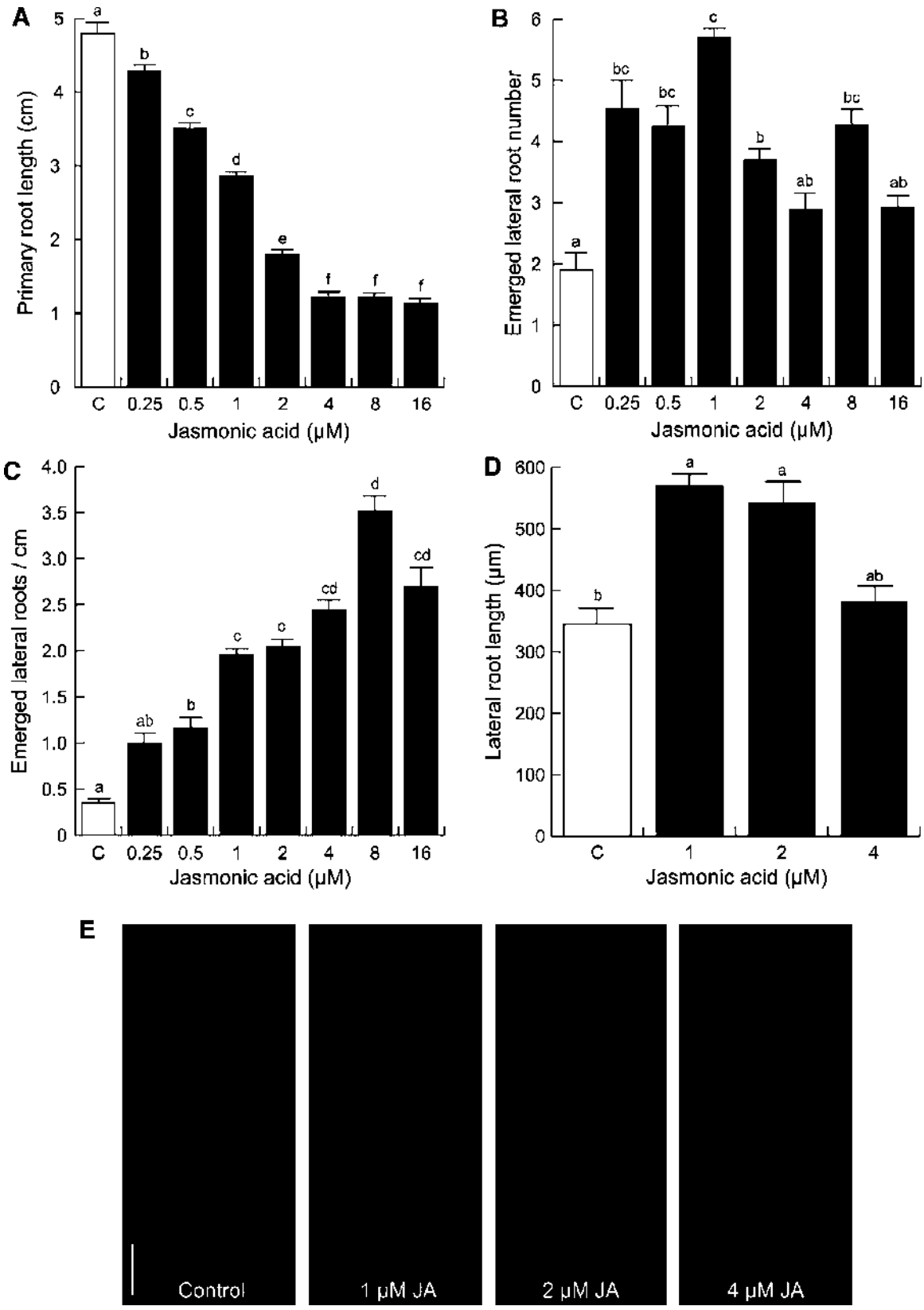

Fig. 1. Effect of JA on *Arabidopsis* RSA. *Arabidopsis* (Col-0) seedlings were germinated and grown for 12 d under increased JA concentrations. (A) PR length. (B) Number of emerged LRs. (C) LR density (number of emerged LRs per cm). (D) Length of emerged LRs. (E) Representative photographs of *Arabidopsis* seedlings grown in the indicated JA treatments. Values shown in (A–D) represent the means of 30 seedlings ± SD. Different letters represent means statistically different ($P < 0.05$). The experiment was repeated three times with similar results. Scale bar = 1 cm.

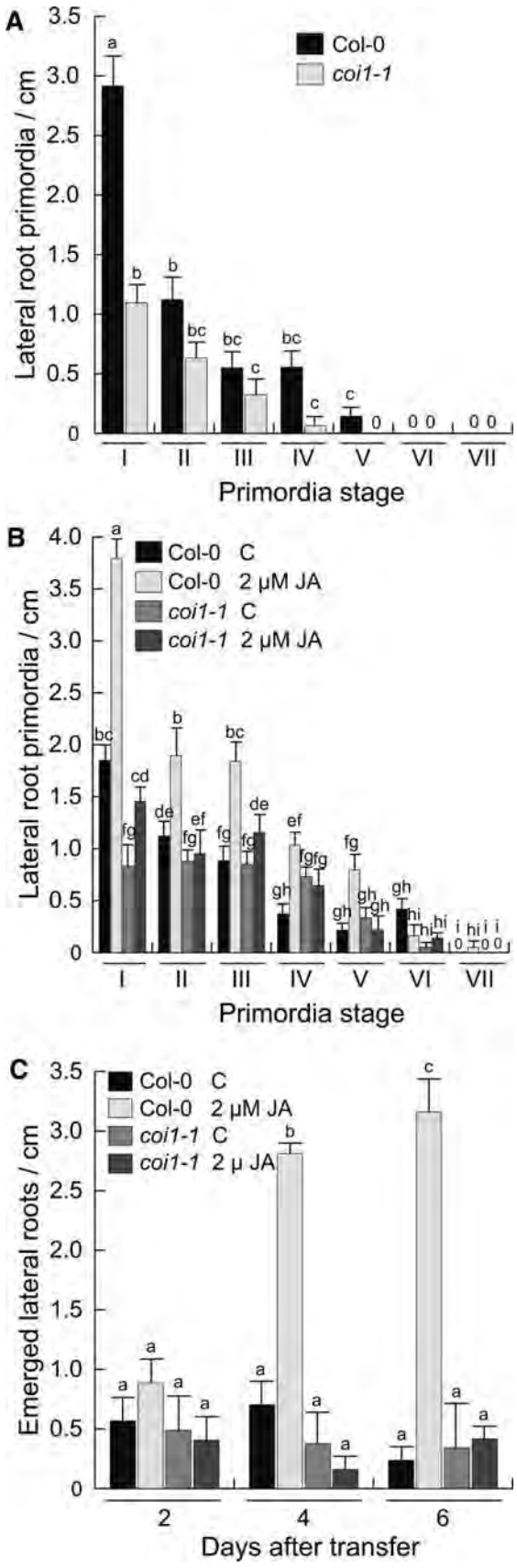

Fig. 2. Effect of JA on LR development in WT and *coi1-1* seedlings. WT seedlings were germinated and grown for 4d on 0.2× MS medium and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI* segregating population in medium supplemented with 4 µM JA. (A) LRP density in four-day-old WT and *coi1-1* seedlings prior to transfer. (B) LRP density in WT and *coi1-1* seedlings two days after transfer to fresh 0.2× MS media supplied with the solvent (control) or with 2 µM JA. (C) Kinetics of emerged LR density in WT and *coi1-1* seedlings 2, 4 and 6 days after transfer to 0.2× MS media supplemented with the solvent (control) or with 2 µM JA. Values shown represent the means of 20 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results.

Interestingly, in *Arabidopsis* WT seedlings 6 days after transfer, most LRs were formed on top of PR bends, with the wavy growth resulting in a left–right alternation pattern of equal distribution in both sides of the PR as previously reported by De Smet et al. (2007) (Fig. 3B and C). Moreover, *coi1-1* seedlings showed an alteration in the pattern of LR formation, in which LRs predominantly appeared to one side of the PR (63.9% right/36.1% left) (Fig. 3B and C). This uneven positioning of LRs resulted in a clear deviation from the right–left alternation pattern observed in WT seedlings.

To determine whether the uneven distribution of LRs in *coi1-1* mutants could be due to the presence of LRP arrested in development between emerged LRs, we performed a LRP analysis in *DR5:uidA* and in *coi1-1*/*DR5:uidA* seedlings. Interestingly, WT *DR5:uidA* seedlings form a few LRP between emerged LRs, whereas in the *coi1-1*/*DR5:uidA* line most LRPs remained dormant at this stage, and failed to emerge from the PR (Fig. S2). This explains why later in development *coi1* mutants develop high number of emerged LRs but with uneven distribution along the PR axis.

coi1-1 mutants are defective in development of LRs on bends

Both waving and the gravitropic response in root are mediated by differential growth. This causes a reorientation of the root tip toward the gravity vector and results in root bending. It has been demonstrated that in *Arabidopsis* LR initiation can be induced by either gravitropic curvature or by the transient bending of the PR by hand (Ditengou et al., 2008). We next tested whether changing the direction of root growth by rotating plants through an angle of 135° affects LR formation in WT and *coi1-1* seedlings by determining the emerged LRs after gravistimulation for 96 h. We observed the initiation of a LR at the convex side of the gravity-induced curve in WT seedlings as previously reported by Ditengou et al. (2008) (Fig. 4A). However, *coi1-1* seedlings that were grown side by side in the same plate with the WT failed to form LRs after gravistimulation (Fig. 4A). We also tested LR initiation by transient manual bending. When 10-day-old WT roots were bent with fine forceps through 135° and left to grow for 120 h after root bending, LR emergence was observed in 40% of plants ($n = 50$) (Fig. 4B). Under our growth conditions, *coi1-1* roots were also defective in this response with only 20% of roots forming LRs on the curves (Fig. 4B). We performed a LRP analysis in *DR5:uidA* and in *coi1-1*/*DR5:uidA* seedlings at the convex side of the gravity-induced curve. It was found that most LRP that were formed in WT *DR5:uidA* seedlings in response to gravity were active and emerged from the PR. However, in *coi1-1*/*DR5:uidA* seedlings many LRPs remained arrested in development inside the PR (Fig. 4C).

coi1-1 mutants show normal auxin-responsive gene expression

Auxins are a class of phytohormones that regulate PR growth and promote LR formation. To test whether JA could alter auxin-regulated gene expression and in this way affect RSA, we conducted analyses of the expression of the β-glucuronidase (GUS) reporter gene in *Arabidopsis* lines harboring the *DR5:uidA* and *BA3:uidA* gene constructs. WT and *coi1-1* seedlings harboring the marker constructs were grown for 7 d in agar solidified 0.2× MS supplemented with the solvent (control), 1 µM IAA or 4 µM JA, and incubated

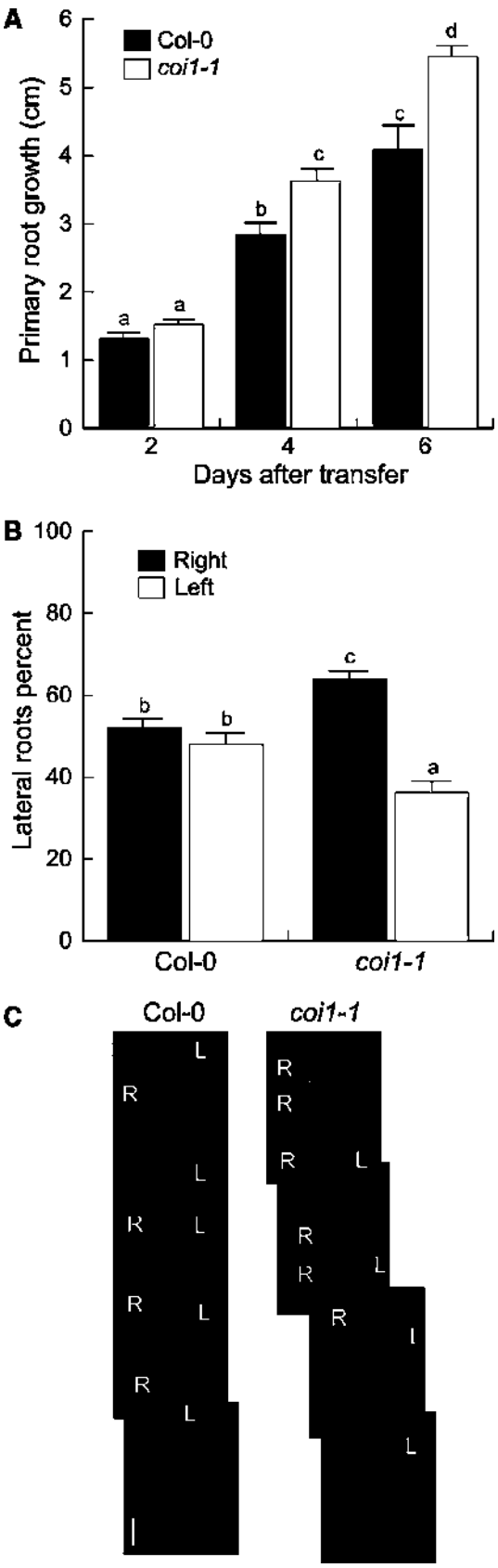

Fig. 3. Effects of JA on LR positioning in WT and *coi1-1* seedlings. WT seedlings were germinated and grown for 4 d on 0.2× MS medium and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI* segregating population in medium supplemented with 4 μM JA. Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates and PR growth measured (A). (B) LR positioning showing the right (R) – left (L) alternating LR formation. (C) Photographs of representative WT and *coi1-1* seedlings illustrating LR formation six days after transfer. Values shown represent the means of 20 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results. Scale bar = 500 μm.

for 12 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. 5A and D). *DR5:uidA* seedlings treated with a concentration of 1 μM IAA showed strong GUS activity throughout the plant (Fig. 5B and E), whereas seedlings treated with 4 μM JA showed a GUS activity similar to solvent-treated plants (Fig. 5C and F). *coi1-1*/*DR5:uidA* seedlings showed a similar GUS expression pattern both in shoots and in roots (Fig. 5G–L). Untreated *BA3:uidA* seedlings did not show detectable levels of GUS activity (Fig. 5M and P), similarly to results reported by Oono et al. (1998), whereas when treated with 1 μM IAA, they showed GUS expression mainly in the petioles of the cotyledons and in the root elongation zone (Fig. 5N and Q). GUS expression in *BA3:uidA* seedlings treated with JA was undetectable (Fig. 5O and R). This pattern of expression remained unchanged in *coi1-1*/*BA3:uidA* seedlings (Fig. 5S–X). As a further control, we tested GUS expression in WT and *coi1-1 Arabidopsis* lines harboring the *pLOX2:uidA* gene construct, which has been shown to be activated by JA (Jensen et al., 2002). As expected, JA clearly induced *pLOX2:uidA* expression in shoots in WT but not in *coi1-1* seedlings (Fig. S3). These results indicate that JA did not affect the general auxin-response in WT or in *coi1* mutants.

Effect of JA on RS architecture in auxin-related Arabidopsis mutants

To evaluate at the genetic level the role played by selected auxin-related loci in JA responses, we compared the PR growth and LR formation of WT (Col-0) seedlings and auxin-related mutants in response to JA treatment. Two types of auxin-related mutants were used: (i) mutants defective in auxin signaling including *tir1*/*afb2*/*afb3*, *arf7-1*/*arf19-1*, *slr*, *axr2-1* and *axr1-3*, and (ii) mutants defective in auxin transport including *aux1-7*, *eir1* and *axr4-1*. Treatments with 4 μM JA caused 70% inhibition in PR growth in WT seedlings compared to solvent-treated seedlings (Fig. 6A). In media lacking JA, all mutant lines tested showed longer PRs compared to WT plants. When WT and mutant seedlings were grown under 1 or 4 μM JA treatments, a similar inhibition in root growth was observed depending on the JA treatment, with exception of *axr1-3*, which was less inhibited (Fig. 6A and Fig. S4). In solvent-treated medium, the triple mutant *tir1*/*afb2*/*afb3*, double mutant *arf7-1*/*arf19-1* and the *slr* mutant showed PRs lacking LRs. Interestingly, all these mutant lines showed no LR induction in JA treatments, indicating an important role for auxin in pericycle cell activation in response to JA (Fig. 6B). A reduction in LR formation in response to JA was also evident in the auxin and JA resistant mutant *axr1-3* (Fig. S3). In contrast, the *aux1-7*, *eir1* and *axr4-1* showed strong induction of LR formation in response to JA (Fig. 6B). Given the fact that JA inhibits both cell division and elongation, the possibility was open that some LRs could be initiated but failed to develop. To test this possibility, we performed experiments to analyze LRP formation in WT, *tir1*/*afb2*/*afb3* and *arf7-1*/*arf19-1* seedlings in response to 1 μM JA. We found that *tir1*/*afb2*/*afb3* and *arf7-1*/*arf19-1* mutants did not increase LRP formation after JA treatment (Fig. 6C and D). Together, these data suggest that JA interacts with or act downstream of the canonical auxin-signaling pathway to promote LR initiation.

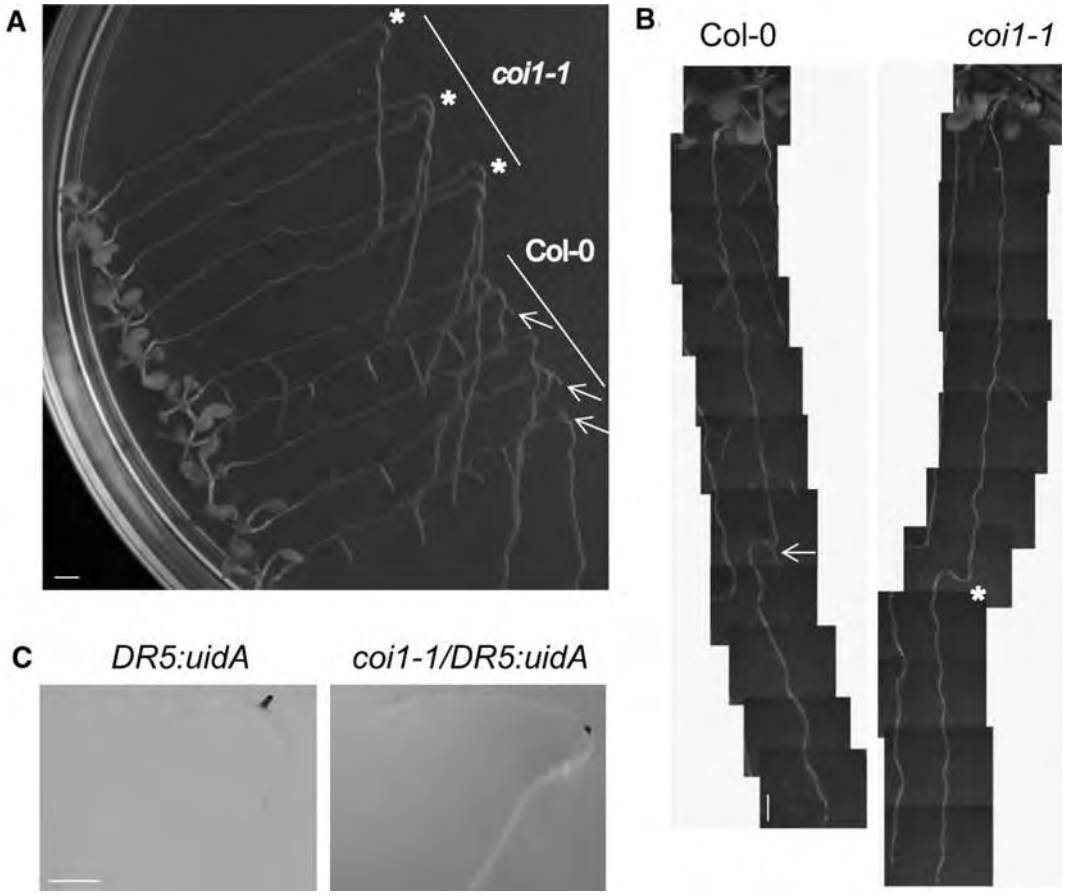

Fig. 4. LR formation after a change of gravity vector and root bending in WT and *coi1-1* mutants seedlings. (A) LR development after gravistimulation for 96 h; Ten-day-old WT and *coi1-1* seedlings grown on agar plates were rotated through 135°. (B) LRs emerged in six-day old WT and *coi1-1* seedlings with PRs bent and left to grow for 120 h after root bending. Arrows indicates fully developed LRs, asterisk (*) marks the absence of emerged LRs. (C) LRP development after gravistimulation in *DR5:uidA* and *coi1-1*/*DR5:uidA* seedlings. The assays were done as described by Ditengou et al. (2008). Similar results were obtained in two independent experiments ($n = 6$ plates). Scale bar = 500 μm.

Discussion

JA induces changes in RS architecture in Arabidopsis

The RS shares with the shoot the basic body plants and the pathways that are essential for organogenesis and growth (Veit, 2004). The site of LR initiation seems to depend on correct auxin transport to perycicle cells in the PR (Dubrovsky et al., 2000; López-Bucio et al., 2005), whereas the final architecture of the roots is coordinated by hormonally regulated processes that affect cell division, elongation and differentiation (Casson and Lindsey, 2003). Although IAA is considered the major plant growth-regulating substance underlying RSA adjustment, the discovery of novel signals such as jasmonates affecting PR growth and LR formation has been a recent goal in plant biology. In a previous report, we determined an important role of JA in LR development in our analysis of the *decanamide root resistant1* (*drr1*) *Arabidopsis* mutant (Morquecho-Contreras et al., 2010). The *drr1* mutant was isolated in a screen for identifying *Arabidopsis* mutants that fail to inhibit PR growth when grown under a high concentration of *N*-isobutyl decanamide, a plant alkamide very active in modulating RSA. Detailed 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. 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 when exposed to stimulatory concentrations of JA. These results provided genetic evidence indicating that alkamides can be perceived by plants to modulate RSA and senescence-related processes possibly by interacting with JA signaling and that JA is an important signal for LR development (Sun et al., 2009; Morquecho-Contreras et al., 2010).

The question of whether JA affect plant growth by influencing cell elongation or cell division has been a matter of debate. Evidence from cell culture studies and wounding of *Arabidopsis* plants suggests that plant growth inhibition mediated by JA occurs through a block in mitotic cell division, while wounding does not seem to affect leaf cell size (Swiatek et al., 2004; Zhang and Turner, 2008). In contrast, it has been shown that the requirement of jasmonate for pollen viability is not at the level of meiosis but at later stages (Devoto et al., 2002). Our results show that the reduction of PR elongation observed in JA-treated seedlings is a complex process that involves a decrease in both cell elongation and cell division (Fig. S1). JA likely affected cell division in the meristem as a consequence of reduced mitotic activity as observed using the *CycB1:uidA* reporter gene. A reduction in cell number in plants with high levels of endogenous jasmonates has been previously reported, and this effect was associated with altered expression of *CycB1;1* in the shoot apical meristem (Zhang and Turner, 2008).

COI1 regulates JA effects on RSA and is an important element in LR development

The F-box protein COI1 acts as a JA receptor, which directly binds to JA-isoleucine (JA–Ile) (Xie et al., 1998; Yan et al., 2009). The *coi1-1* null mutants are male-sterile, display insensitivity to JA

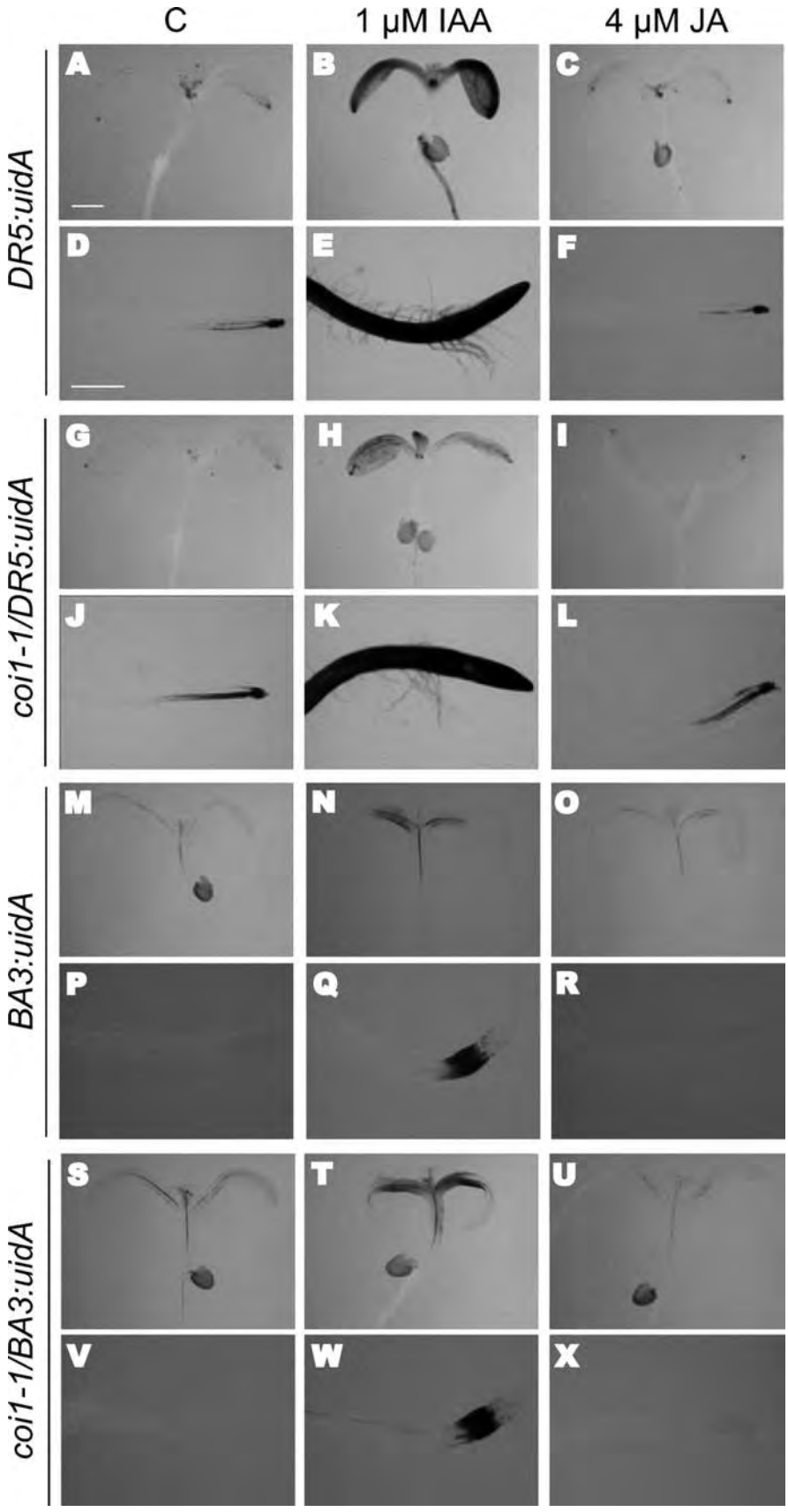

Fig. 5. Effect of JA and IAA on auxin-inducible gene expression in WT and *coi1-1* seedlings. (A-L) 12-h GUS staining *DR5:uidA* and *coi1-1*|*DR5:uidA Arabidopsis* seedlings that were grown for 7 d on agar plates containing 0.2× MS medium supplemented with the solvent (control) or 1 μM IAA or 4 μM JA. (M–X), 12-h GUS staining *BA3:uidA* and *coi1-1*|*BA3:uidA* seedlings that were grown for 7 d on agar plates containing 0.2× MS medium supplemented with the solvent (control) or 1 μM IAA or 4 μM JA. Photographs are representative individuals of at least 15 stained seedlings. The experiment was repeated three times with similar results. Scale bar = 200 μm.

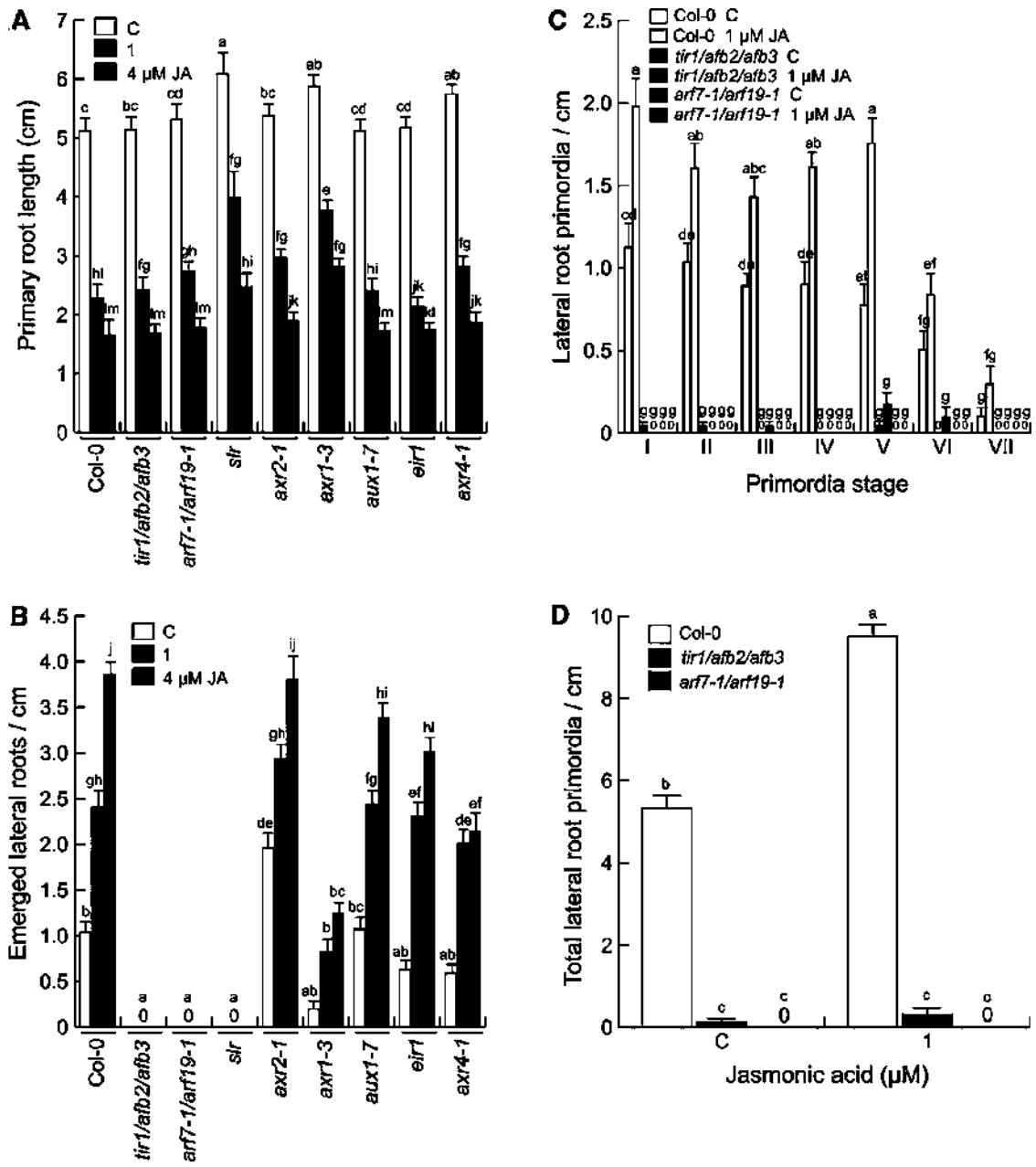

Fig. 6. Effects of JA on PR growth and LR development in WT seedlings and auxin-related mutants. *Arabidopsis* WT and *tir1/afb2/afb3*; *arf7-1/arf19-1*, *slr*, *axr2-1*, *axr1-3*, *aux1-7*, *eir1*, and *axr4-1* triple, double or single mutant seedlings, respectively, were germinated and grown for 12 d in 0.2× MS medium supplemented with the solvent (control), 1 μM or 4 μM JA. (A) PR length. (B) LR density. (C) LRP stage density. (D) Total LRP density. Values shown represent the means of 20 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results.

in PR growth assays and show susceptibility to insect attack and pathogen infection (Feys et al., 1994; Xie et al., 1998; Reymond et al., 2000). Although the resistance of *coi1-1* to jasmonates on PR growth is well characterized, the role played by this receptor during the induction of LRs by JA remains elusive. Special attention was devoted to the induction of LRs by JA, which apparently occurs without requiring PR growth arrest (Fig. 1). Interestingly, the formation of LRs was induced at stages I–V, whereas a minor alteration was observed at later stages of development (Fig. 2), indicating that JA exerted its effects mostly on the initiation of LRP.

Our results showed that *coi1-1* seedlings are very resistant to LR formation in response to JA (Fig. 2), which suggests that COI1 is required for the JA-induced signal transduction events in pericycle cells to form LRP. Although a reduced capacity of *coi1-1* mutants to form LRs when compared to WT seedlings was evidenced under normal growth conditions or in response to JA treatments, it should be noted that the capacity of this mutant to develop LRs under normal growth conditions is similar to that observed for WT seedlings (Fig. 3). Intriguingly, the total number of emerged LRs, which is spaced along the main axis in a regular left–right altering pattern changed in *coi1-1* mutants when compared to WT seedlings (Fig. 3). This suggests that *COI1* might be an element required for the emergence of LRs. Through the characterization of the *anthranilate synthase1* (*asa1*) mutant, which is defective in JA-induced LR formation, Sun et al. (2009) showed that in addition to promoting auxin biosynthesis through transcriptional activation of the *ASA1* gene, JA negatively regulates auxin transport through the reduction of PIN1 and PIN2 protein levels in the plasma membrane. The coordinated regulation of auxin transport/response by JA may account for the initiation of LRs in response to JA.

COI1 is involved in LR growth on bends

The phytohormone auxin is a key regulator of PR growth and LR development. It has been demonstrated that basipetal and acropetal auxin transport are required during the initiation and emergence phases of LR development acting as an instructive signal (Casimiro et al., 2001; Bhalerao et al., 2002; Swarup et al., 2008; Dubrovsky et al., 2008).

Recent information has shown that bending causes the initiation of LRs (Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008). The earliest observable event during this process was a change in PIN1 localization in differentiating xylem cells. The signaling events between the bending stimulus and PIN1 relocalization are currently unknown. However, based on the bending response of an *arf7*/*arf19* double mutant that normally forms no LRs but do so upon bending when the root tip is removed, Ditengou et al. (2008) have suggested that the bending stimulus is auxin-independent or acts downstream of *arf7*/*arf19* to specify LR identity. Our results that the *coi1-1* is defective on induction of LRs on bends are consistent with an important role of JA in RSA responses to root bending (Fig. 4). In this particular response, analysis of LRP formation on bends shows that the *coi1-1* mutants develop LRP, which are however, unable to emerge from the PR. Two additional lines of evidence indicate that JA is an important signal for LR formation: (i) the dose-dependent increase in LR numbers by JA treatments, and (ii) the failure of JA to induce auxin-inducible gene expression. Interestingly, JA was unable to activate the auxin-response markers *DR5:uidA* and *BA3:uidA* in the shoot system or in PR tips (Fig. 5). Moreover, when these markers were movilized into the *coi1-1* background, no further changes in expression were documented. These data indicate that JA is not a general inducer of auxin responses in the plant and provide support to the conclusion by Sun et al. (2009) that JA likely regulates LR development by specifically affecting auxin responses at earlier stages of LRP formation. In this context, JA and auxin may act in concert to modulate developmental processes. To address this question, we evaluated the impact on auxin response when JA and IAA are combined by using the *DR5:uidA* line. JA neither reduces nor activates auxin-inducible gene expression when supplied together with IAA (Fig. S5). From these results we hypothesize that the JA-auxin crosstalk may create a fine regulatory network whose net outputs largely depend on the action of specific phytohormone combinations rather than on the independent activities of separate hormones.

JA require a canonical auxin signaling pathway for inducing LR development

Auxin is perceived by direct binding to the TRANSPORT INHIBITOR RESPONSE1 (TIR1) protein, a member of a small family of F-box proteins (Dharmasiri et al., 2005; Kepinski and 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 of auxin receptors and ARFs are involved in *Arabidopsis* responses to JA, we analyzed PR growth and LR formation in response to JA in WT (Col-0) *Arabidopsis* seedlings, in *tir1*/*afb2*/*afb3* triple mutants in *arf7-1*/*arf19-1* double mutants, and in *slr*, *axr2-1*, *axr1-3*, *aux1-7*, *eir1* and *axr4-1* single mutants, which are well known for their resistance to auxin in PR growth. Seedlings from all mutant lines showed similar inhibition in PR growth by JA treatment, with exception of *slr* and *axr1-3*, which showed significant resistance when compared to WT seedlings (Fig. 6A and Fig. S4). Interestingly, the increase in both LR and LRP formation observed in WT seedlings when treated with JA was clearly reduced in *tir1*/*afb2*/*afb3*, *arf7-1*/*arf19-1* and *slr* mutants (Fig. 6A–D and Fig. S4).These results clearly show the dependence of an intact auxin signaling pathway for JA-induced LR formation.

In summary, we have provided evidence that RSA changes induced by JA in *Arabidopsis* including enhanced LR formation, LR positioning and induction of LR emergence on bends operate through the *COI1* locus. We also documented the role of auxin signaling in PR and LR responses to JA, which indicate that JA modulates postembryonic root development through auxin-dependent and independent effects. Whether environmental signals such as water and nutrient availability or biotic factors may affect RSA through increased JA biosynthesis and/or signaling is currently under investigation.

Acknowledgments

We thank Drs. Athanasios Theologis, Tom Guilfoyle, Mark A. Estelle, Peter Doerner, Bonnie Bartel, Masao Tasaka and Arturo Guevara-García for kindly providing us with seeds of transgenic and mutant lines. This work was supported by the Consejo Nacional de Ciencia y Tecnología (grant no. 80916), the Consejo de la Investigación Científica (CIC 2.26), and the Marcos Moshinsky Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.05.002.

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Plant Mol Biol (2013) 81:609–625
DOI 10.1007/s11103-013-0023-6

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

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Received: 20 July 2012 / Accepted: 3 February 2013 / Published online: 15 February 2013

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

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0023-6) contains supplementary material, which is available to authorized users.

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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

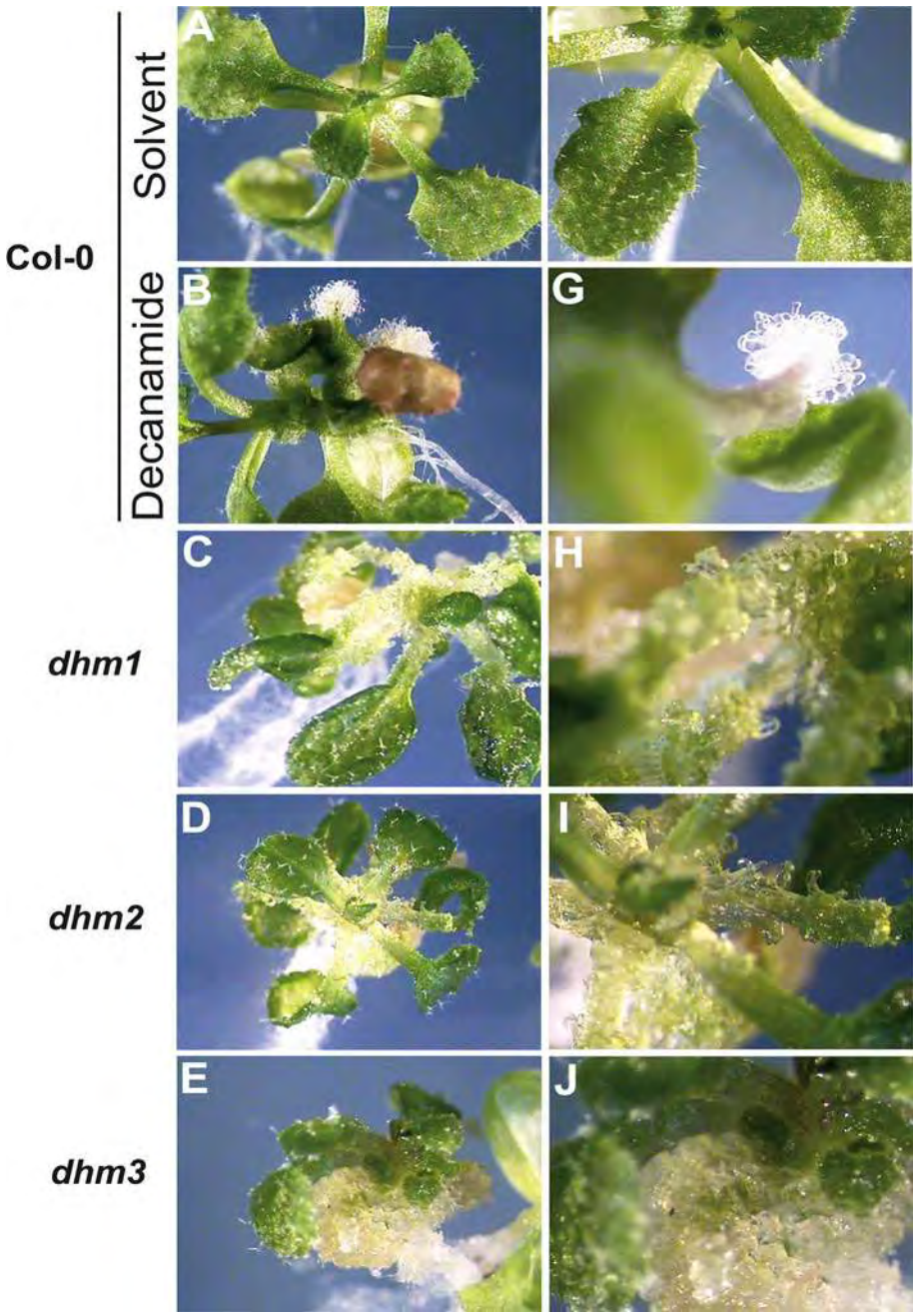


Fig. 1 Phenotypes of WT and *decanamide hypersensitive mutants* (*dhm*) of Arabidopsis. **a** Photograph of the shoot system of a WT (Col-0) seedling grown in 0.2X MS medium lacking *N*-isobutyl decanamide, or **b** a plant supplied with 35 μM of *N*-isobutyl decanamide. **c–e** putative alkamide-oversensitive 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

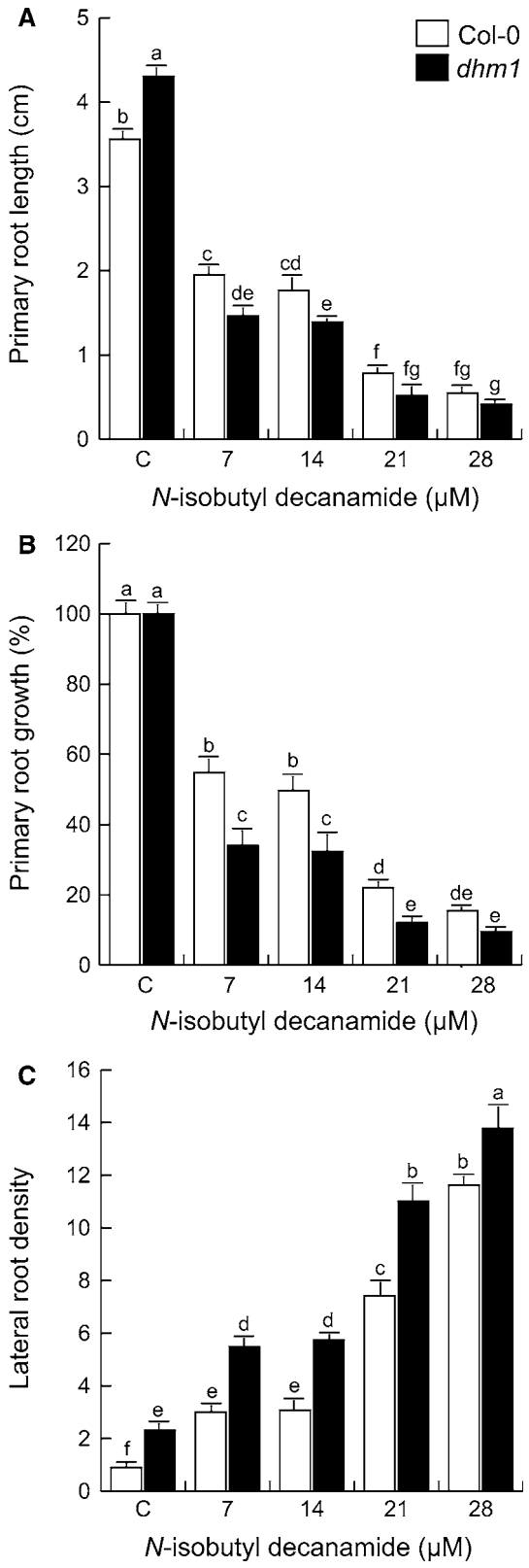


Fig. 2 Effects of *N*-isobutyl decanamide on root system architecture of WT (Col-0) and *dhm1* plants. **a** Primary root length. **b** Primary root growth (%). **c** Lateral root density, expressed as the number of lateral roots per centimeter. Data were recorded 10 days after germination. Values shown 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

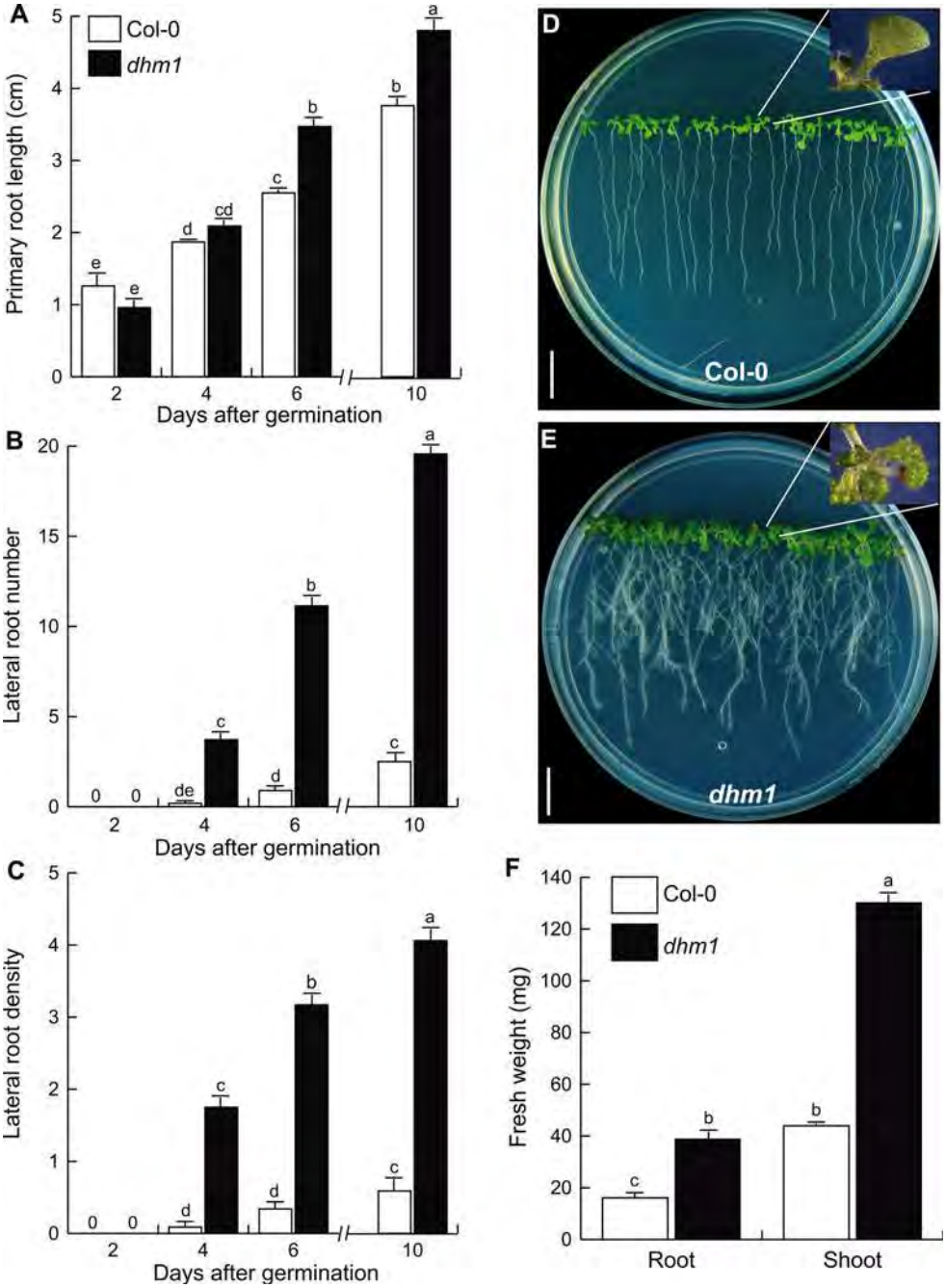


Fig. 3 Comparative root and shoot development of WT and *dhm1* seedlings grown in vitro. Arabidopsis plants were germinated and grown on agar plates containing 0.2X MS agar medium. Growth kinetic analyses were performed at the indicated times. **a** Primary root length. **b** LR number per plant. **c** 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

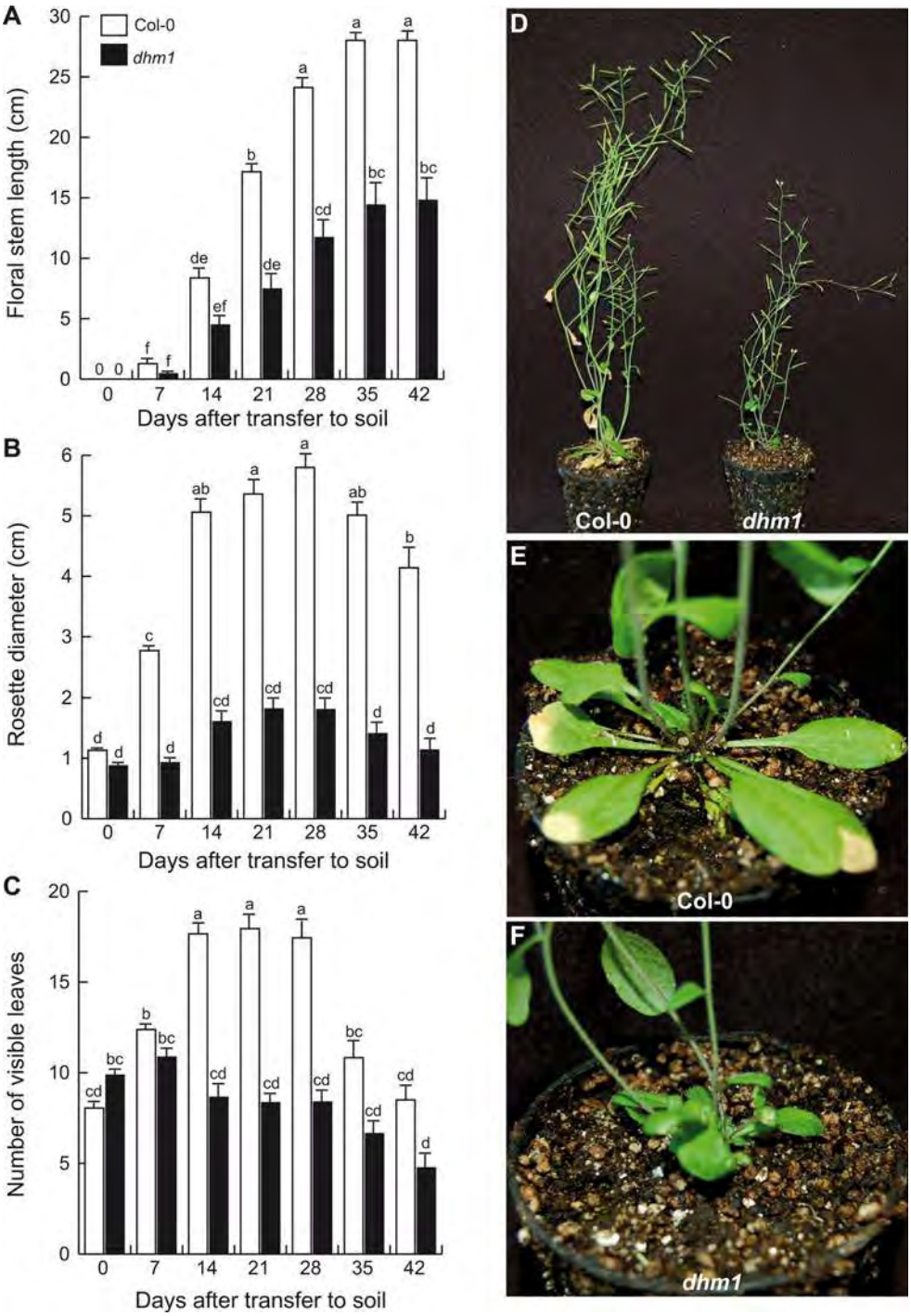


Fig. 4 Comparative development of WT and *dhm1* seedlings grown in soil. Arabidopsis plants were germinated and grown 10 days on agar plates containing 0.2X MS agar medium and then transferred to soil. Growth kinetic analyses were performed at the indicated times after transfer. **a** Floral stem length. **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

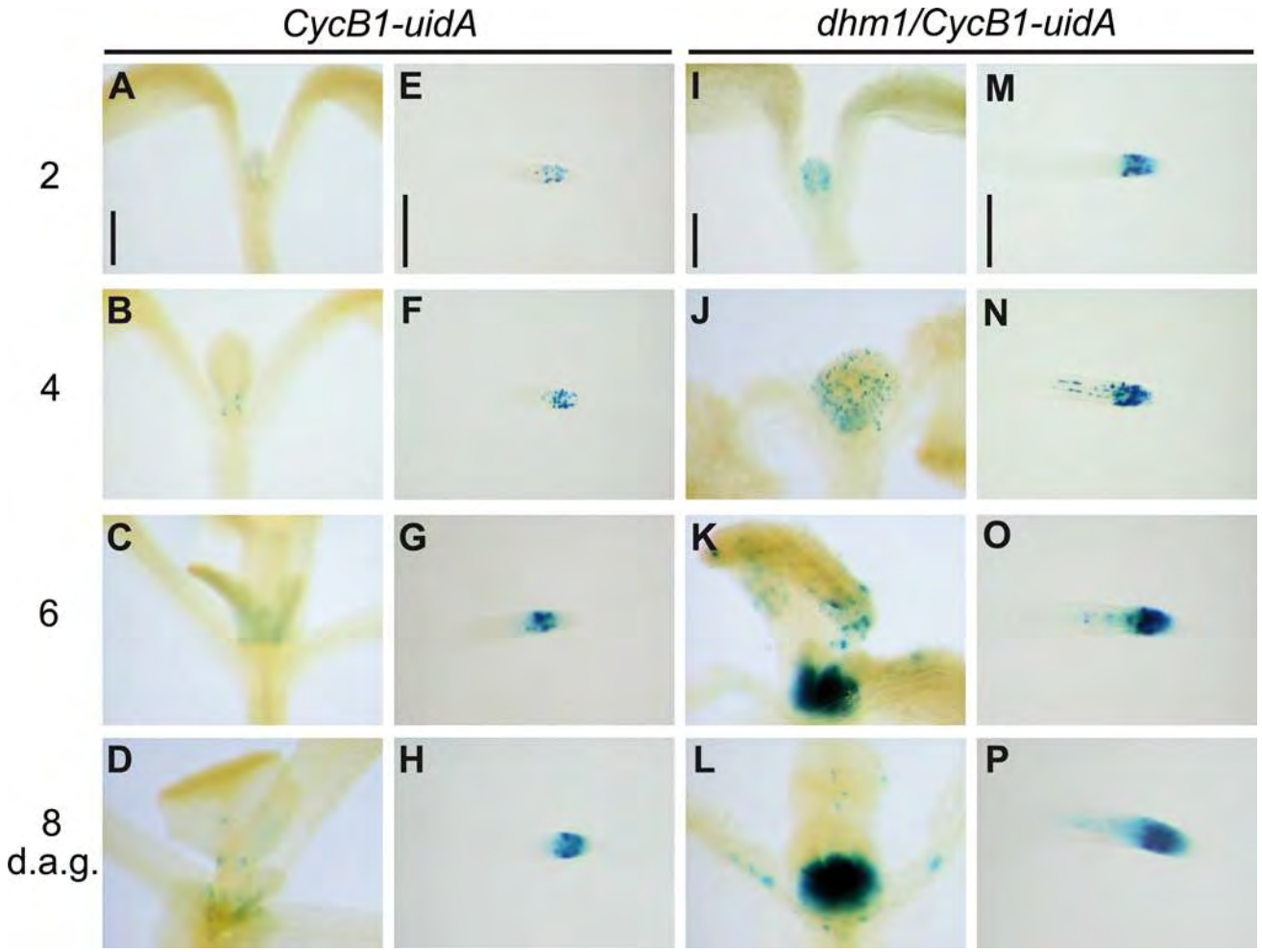


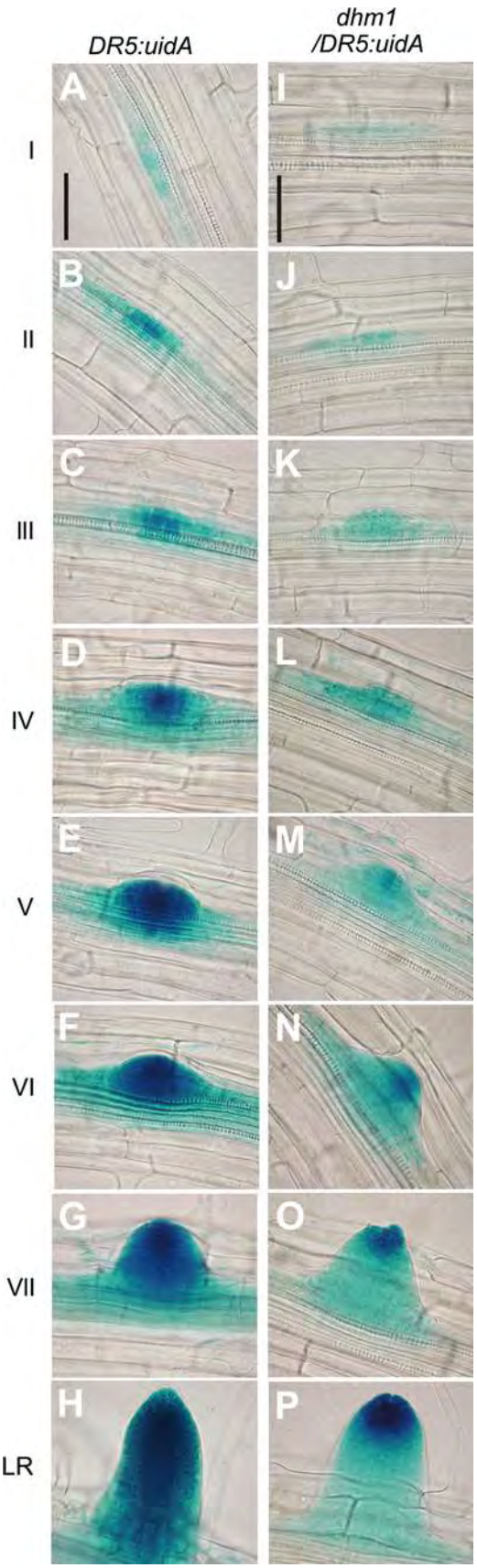
Fig. 5 *CycB1-uidA* expression in transgenic WT and *dhm1* seedlings. *CycB1-uidA* and *dhm1/CycB1-uidA* were germinated and grown on agar solidified 0.2X MS medium. Twelve-hour GUS staining from 2, 4, 6 and 8 days old seedlings is shown for *CycB1-uidA* (**a**–**h**) and *dhm1/CycB1-uidA* (**i**–**p**). 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

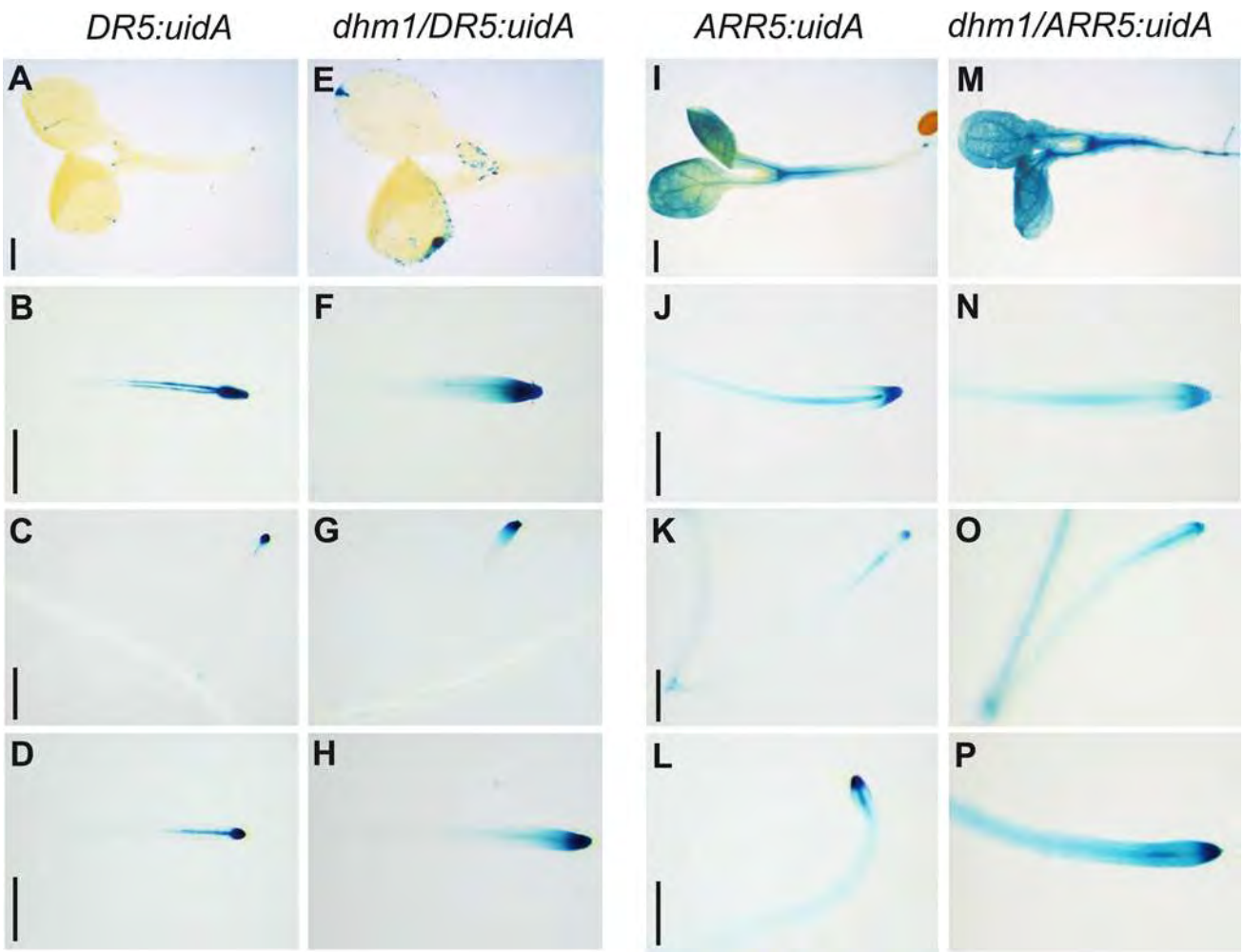
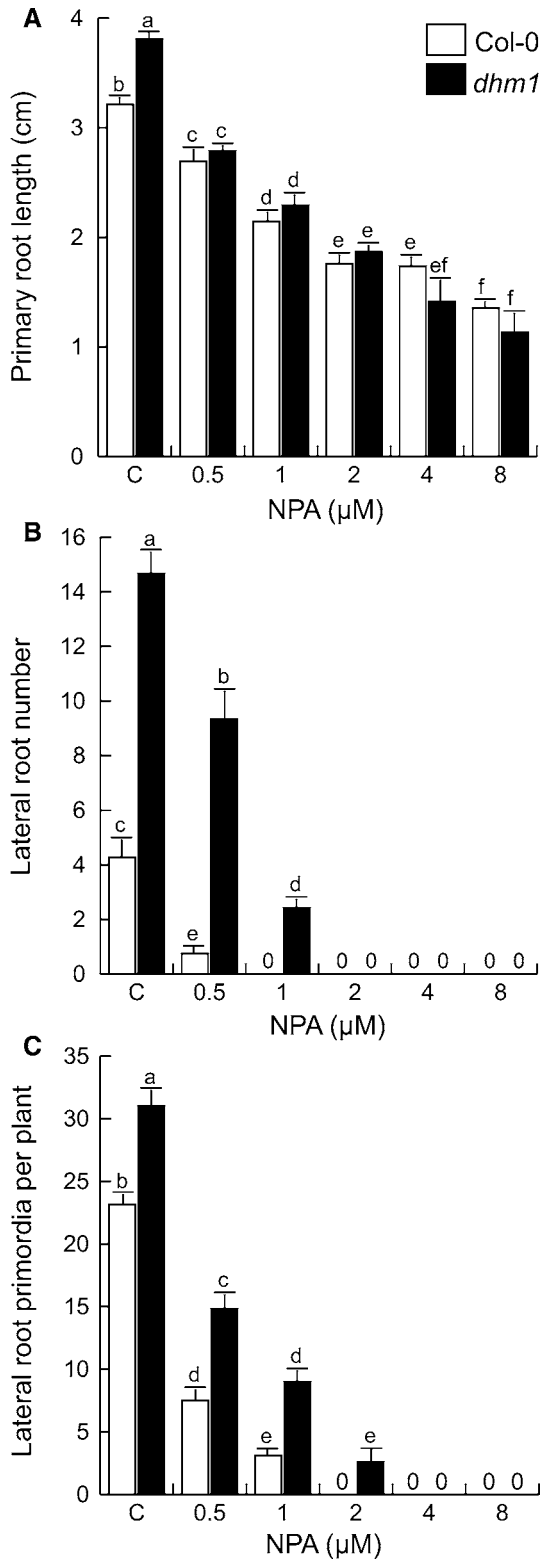


Fig. 7 Auxin and cytokinin-regulated gene expression in WT and *dhm1* seedlings. Twelve-hours of GUS staining of *DR5:uidA* (**a**–**d**) and *dhm1/DR5:uidA* (**e**–**h**) seedlings. GUS expression in *ARR5:uidA* (**i**–**l**) and *dhm1/ARR5:uidA* (**m**–**p**) seedlings after 6-h of GUS staining. Photographs 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



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 overresponsive 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

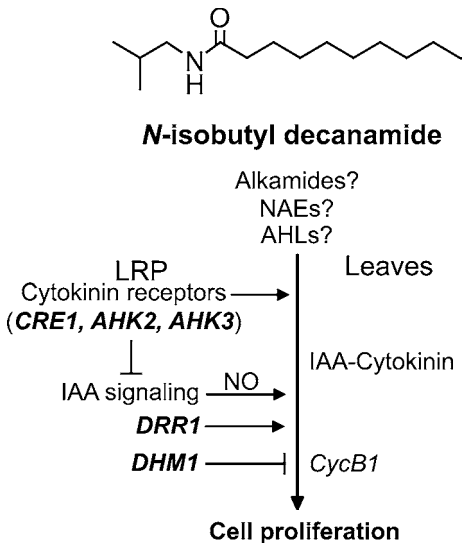
Morphologic alterations induced in Arabidopsis by *N*-isobutyl decanamide have been previously found to be independent of auxin and at least in part dependent of cytokinin signaling (López-Bucio et al. 2007). However, detailed physiological analysis of alterations induced by *DHM1* mutation in plants 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



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.

Acknowledgments We are thankful to Drs. Peter Doerner, Joseph J. Kieber, Thomas Guilfoyle and Jen Sheen for kindly providing us with seeds of transgenic and mutant lines. We appreciate the kind support by Dr. León Francisco Ruíz Herrera with confocal microscopy analysis. This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT, México, Grants no. 60999 and 177775), the Consejo de la Investigación Científica (UMSNH, México, Grant no. CIC 2.26), and Marcos Moshinsky Fellowship to JLB.

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MPMI Vol. 27, No. 4, 2014, pp. 364–378. http://dx.doi.org/10.1094/MPMI-08-13-0219-R.

e-Xtra*

Pyocyanin, a Virulence Factor Produced by *Pseudomonas aeruginosa,* Alters Root Development Through Reactive Oxygen Species and Ethylene Signaling in *Arabidopsis*

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Submitted 8 August 2013. Accepted 31 October 2013.

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 inhibited primary root growth in wild-type (WT) *Arabidopsis* seedlings. In contrast, single *lasI*– and double *rhlI–/lasI*– mutants of *P. aeruginosa* defective in pyocyanin production showed decreased root growth inhibition 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 because 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.

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 by altering root system architecture. Moreover, many bacterial species provide protection against pathogens, tolerance to abiotic stress, and resistance to insect or herbivore attack, and even allelopathy may be due to root-associated microorganisms (Friesen et al. 2011; Ortiz-Castro et al. 2009). 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 and López-Bucio 2013; Ortiz-Castro et al. 2011).

The *Pseudomonas* genus comprises ubiquitous gram-negative bacteria distributed in different environments and contains pathogenic species for plants (i.e., *Pseudomonas syringae* and *P. aeruginosa*). Other species have the ability to colonize the rhizosphere (i.e., *P. aeruginosa, 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). The ability of *P. fluorescens* CHA0 and *P. aeruginosa* 7NSK2 to induce resistance in grapevine against *Botrytis cinerea* was recently demonstrated. Both strains also triggered an oxidative burst and phytoalexin accumulation in grape cells and primed leaves for accelerated phytoalexin production upon challenge with *B. cinerea* (Verhagen et al. 2010). Redox-active pyocyanin (PCN) secreted by *P. aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice (De Vleeschauwer et al. 2006). These results suggest that *Pseudomonas*-derived metabolites can induce reactive oxygen species (ROS), which act as a double-edged sword in the interaction of rice with the hemibiotroph *M. grisea* and the necrotroph *R. solani.*

Gram-negative bacteria produce and use *N*-acyl-*L*-homoserine lactones (AHL) 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, *N*-(3-oxododecanoyl)-*L*-homoserine lactone (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 (Bosgelmez-Tinaz 2003; de Kievit and Iglewski 2000; Fuqua and Greenberg 2002; Rumbaugh et al. 2000).

Roots have developed the capacity to recognize bacterial QS signals and adjust growth and development in response to

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*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are published online and that Figures 1, 2, 3, 6, 7, 8, 9, and 10 appear in color online.

these metabolites (Mathesius et al. 2003; Ortiz-Castro et al. 2008; von Rad et al. 2008). Recently, genetic, chemical, and plant-growth data were presented showing that, in *P. aeruginosa*, the *lasI* QS system controls the production of three diketopiperazines (DKP)—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 DKP 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 (Ortiz-Castro et al. 2011).

P. aeruginosa is most studied for its 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 PCN. For example, PCN participates in the fast killing of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus*, likely by producing ROS (Lau et al. 2003, 2004a and b; Mahajan-Miklos et al. 1999). 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* and *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 the fact that i) it demonstrates cell density-dependent accumulation, ii) it is a small diffusible molecule that is recognized by adjacent cells, and iii) it 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 growth and development was assessed by comparing the *Arabidopsis* primary and lateral root 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 wild-type (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 QS plays a role in growth and development of *Arabidopsis* modulated by *P. aeruginosa.*

We first tested whether direct colonization of the *Arabidopsis* root with *P. aeruginosa* PAO1 and QS-related single *rhlI–* and *lasI–*, and double *rhlI–/lasI–* mutants could affect growth of seedlings. In several experiments and times of co-cultivation, *P. aeruginosa* PAO1 caused primary root growth inhibition, and the shoot system was 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 inhibition of growth and dramatically increased root and shoot biomass production of seedlings at 3, 6, and 9 days of co-cultivation (Fig. 1D, I, and N; and E, J and O, respectively). These data indicate that C12-AHL–mediated QS controls the production of factors that repress primary root growth. Interestingly, an analysis of hydrogen peroxide (H_2O_2) in root tips of *Arabidopsis* seedlings co-cultivated for 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* mutants and an increased accumulation of H_2O_2 in both *lasI–* and *rhlI–/lasI–* mutants (Fig. 2A to 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 QS regulates PCN production in *P. aeruginosa.*

P. aeruginosa releases PCN as a main virulence factor (De Vleesschauwer et al. 2006). To determine whether QS-related single *rhlI–* and *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–* and *lasI–* and double *rhlI–/lasI–* mutants when compared with *P. aeruginosa* PAO1 (Fig. 3). These data show that AHL-modulated QS plays an important role in PCN production.

PCN alters *A. thaliana* root system architecture.

To determine whether *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 0.2× Murashige-Skoog (MS) agar medium supplemented with PCN concentrations from 0.6 to 40 μM and primary root growth was measured 10 days 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; Supplementary 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 (LRD) was also observed with a threefold increase from 2.5 to 20 μM concentration of this compound when compared with solvent-treated seedlings (Fig. 4C).

PCN 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 (Supplementary Fig. S2), clearly indicating the progression of cell differentiation toward the root meristem region. These results suggest that PCN can be perceived by roots and alter root system architecture and root hair development.

PCN alters cell division without affecting cell viability or integrity.

Previous reports demonstrated 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 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 PCN concentrations of 5 μM or higher correlated with the reduction in the number of cells expressing *CycB1:uidA* in the primary root meristem and β-glucuronidase (GUS) expression of *pPRZ1:uidA* transgenic seedlings (Fig. 6A to H). We also analyzed the gene expression of the cell nuclei marker *AtHistH2B:YFP* (*yellow fluorescent protein*) 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 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 to 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 PCN regulates cell division without affecting cell integrity or meristem viability.

PCN 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

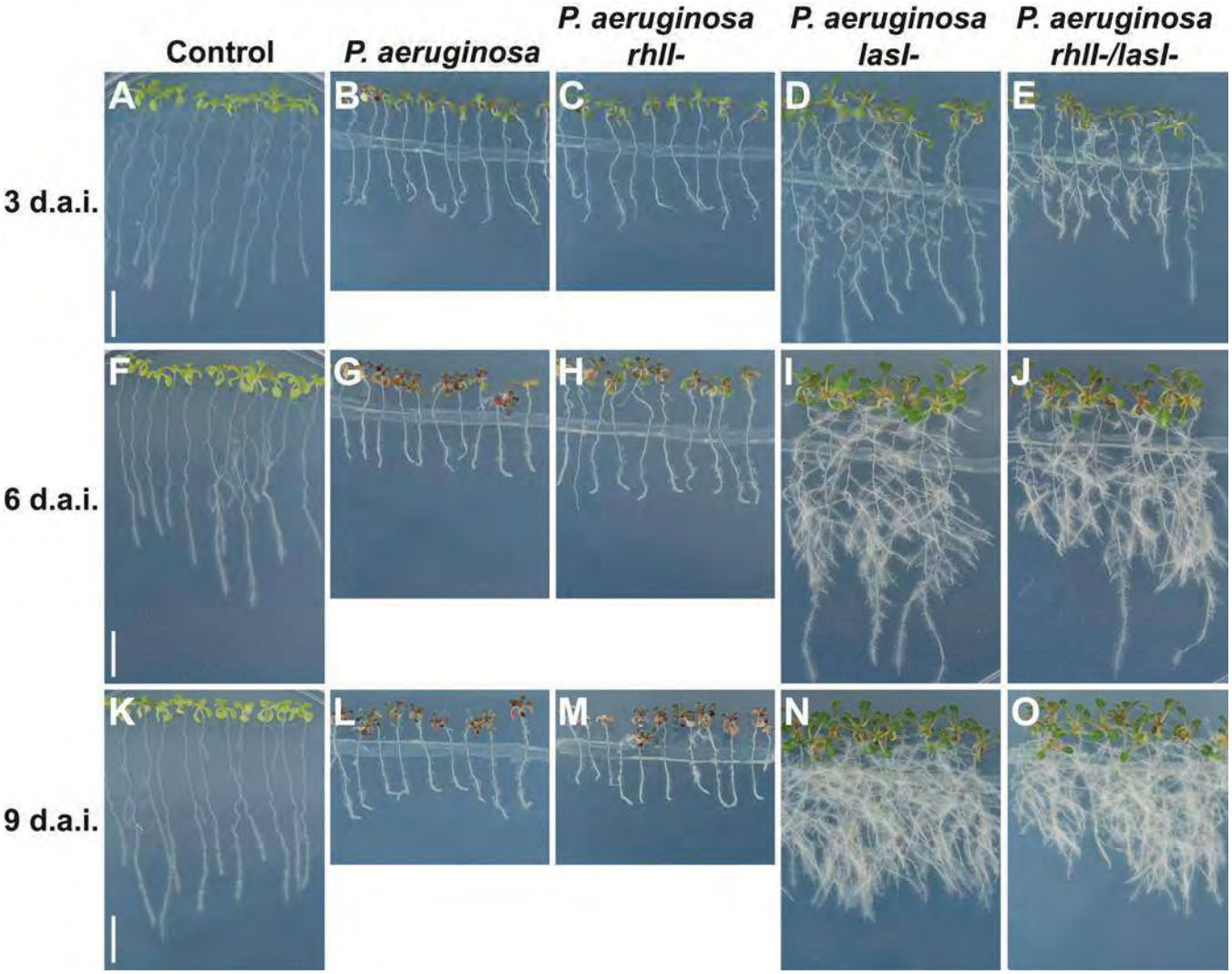

Fig. 1. Effect of co-cultivation with *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains on plant growth. Six-day-old *Arabidopsis thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases LasI, RhlI, or RhlI/LasI at direct contact and grown for **A** to **E,** 3; **F** to **J,** 6; and **K** to **O,** 9 days. Representative photographs were taken for plates from each treatment. This experiment was repeated three times with similar results. Notice the damage and inhibitory effect 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 = 1 cm.

test whether PCN 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. Histochemical staining of roots of transgenic *DR5:uidA* and *BA3:uidA* seedlings that were grown for 10 days on 0.2× MS medium supplemented with solvent, indole-3-acetic acid (IAA), or the indicated concentrations of PCN is shown in Figure 7. *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 to F, I to L, O to R, and U to X). These results suggest that PCN did not induce auxin-responsive gene expression in *Arabidopsis* seedlings.

PCN 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. Because PCN did not activate

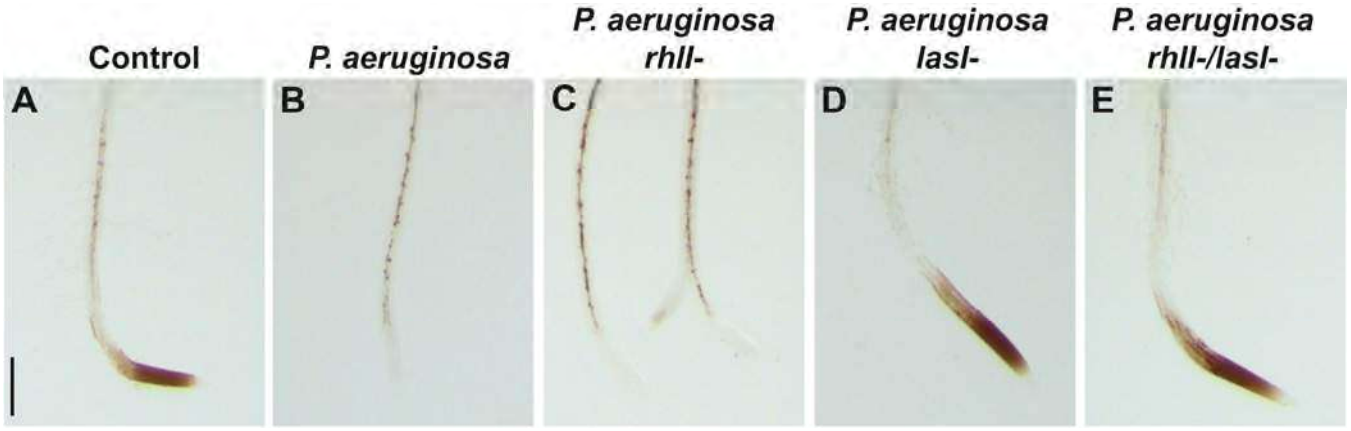

Fig. 2. Effect of co-cultivation of *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* seedlings. Four-day-old *A. thaliana* seedlings were co-cultivated with WT *P. aeruginosa* or mutants defective on the AHL synthases RhlI, LasI, or RhlI/LasI at a distance of 5 cm from the primary root tip and grown for 8 days. Representative photographs of primary root of **A,** control seedlings or co-cultivated with **B,** WT *P. aeruginosa* and **C,** *rhlI*; **D,** *lasI*; or **E,** *rhlI/lasI* 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.

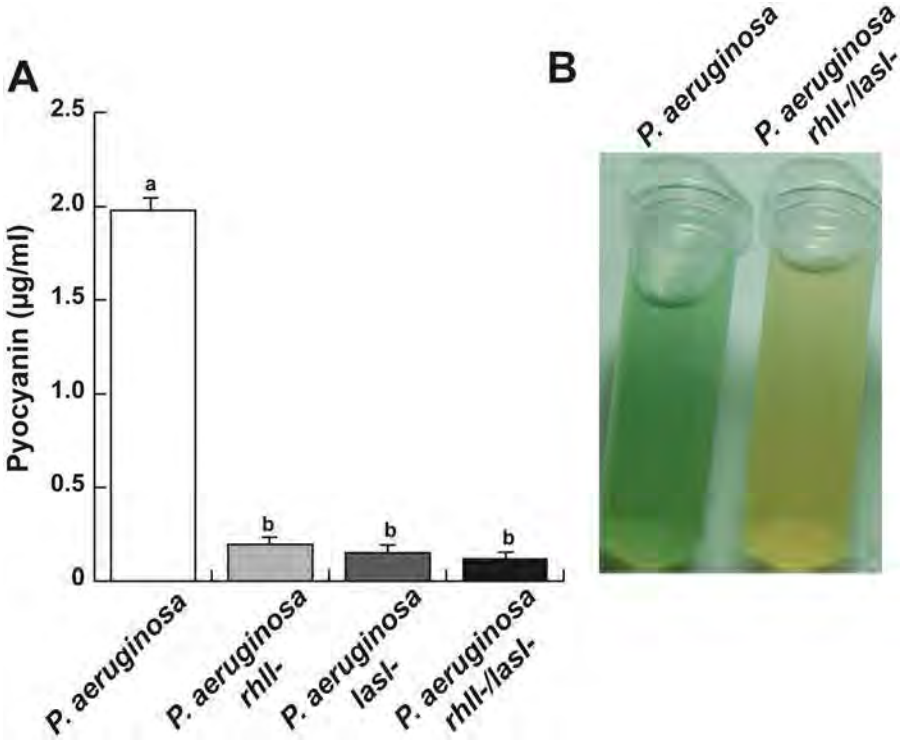

Fig. 3. Pyocyanin production in *Pseudomonas aeruginosa* wild-type (WT) and quorum-sensing (QS) mutant strains. **A,** Pyocyanin production: cells were grown in Luria-Bertani medium at 37°C for 48 h, the supernatant fractions were separated, and the amount of pyocyanin (μg ml^{-1}) in each fraction was determined by the chloroform:acid extraction procedure. Values represent the mean of three independent experiments ± standard deviation. **B,** Representative photograph of pyocyanin production as observed by the green color of culture grown in liquid medium.

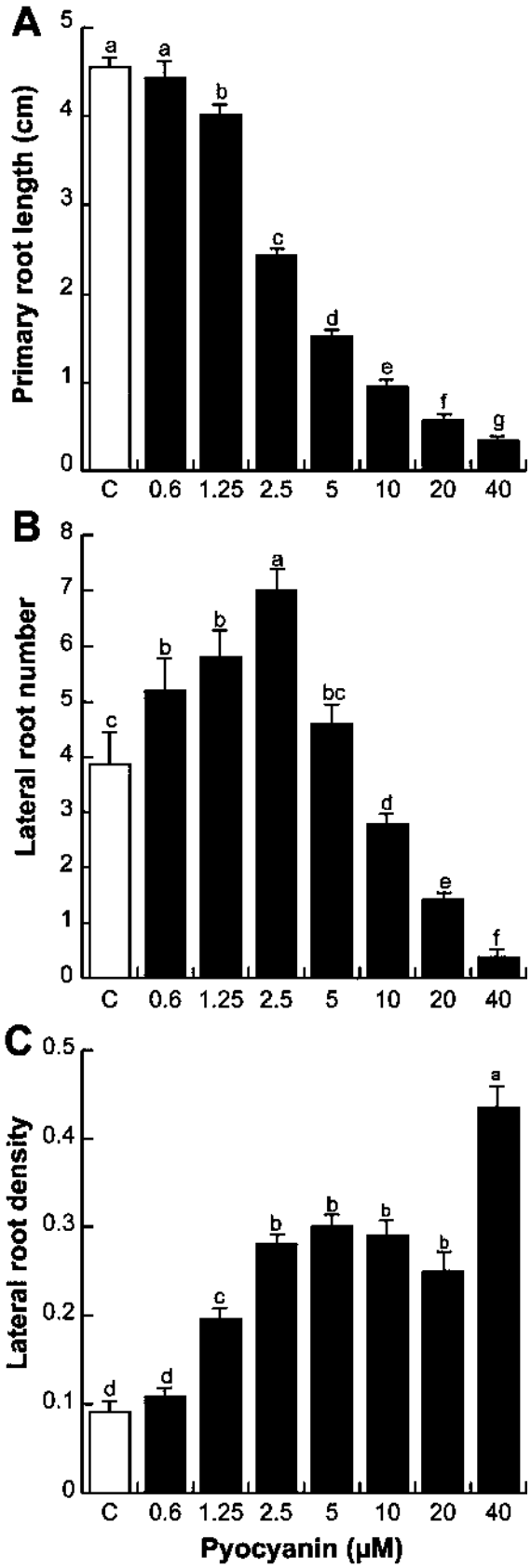

Fig. 4. Effect of pyocyanin on *Arabidopsis* root system architecture. *Arabidopsis* wild-type (WT) (Col-0) seedlings were germinated and grown for 10 days under increasing pyocyanin concentrations. **A,** Primary root length; **B,** lateral root number; **C,** lateral root density. Values shown represent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

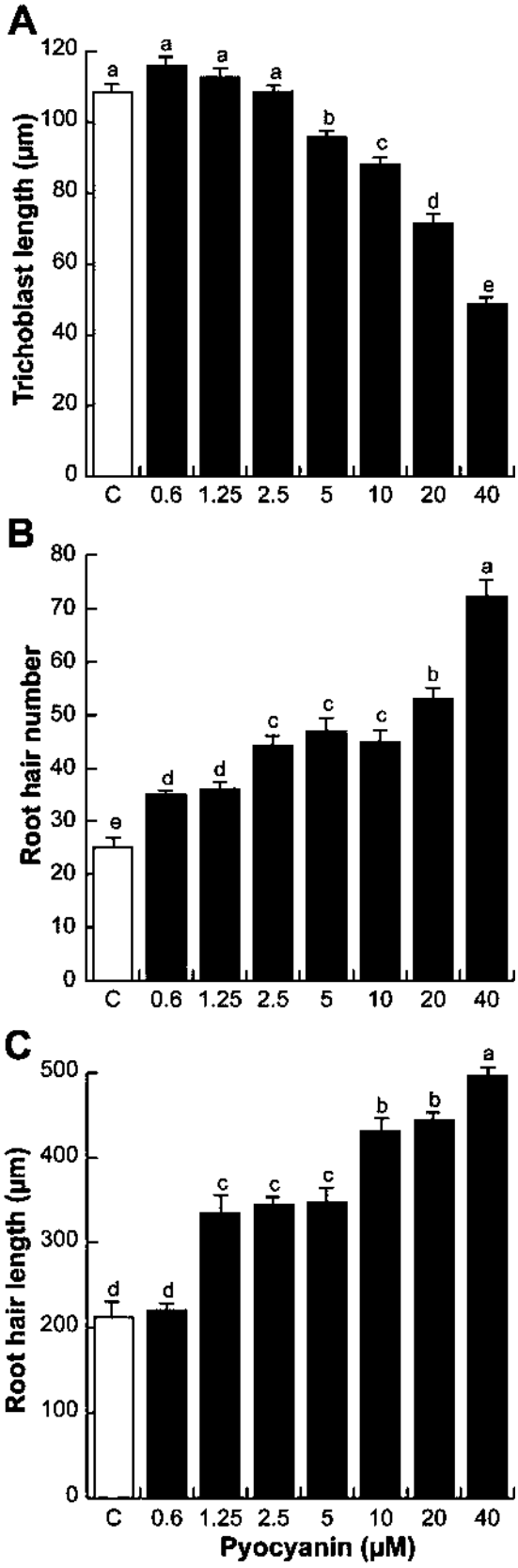

Fig. 5. Effects of pyocyanin on epidermal cell differentiation. **A,** Trichoblasts length; **B,** root hair number; **C,** root hair length. *Arabidopsis thaliana* seedlings were grown for 5 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. Data points indicated mean ± standard deviation (n = 20). 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 $P < 0.05$.

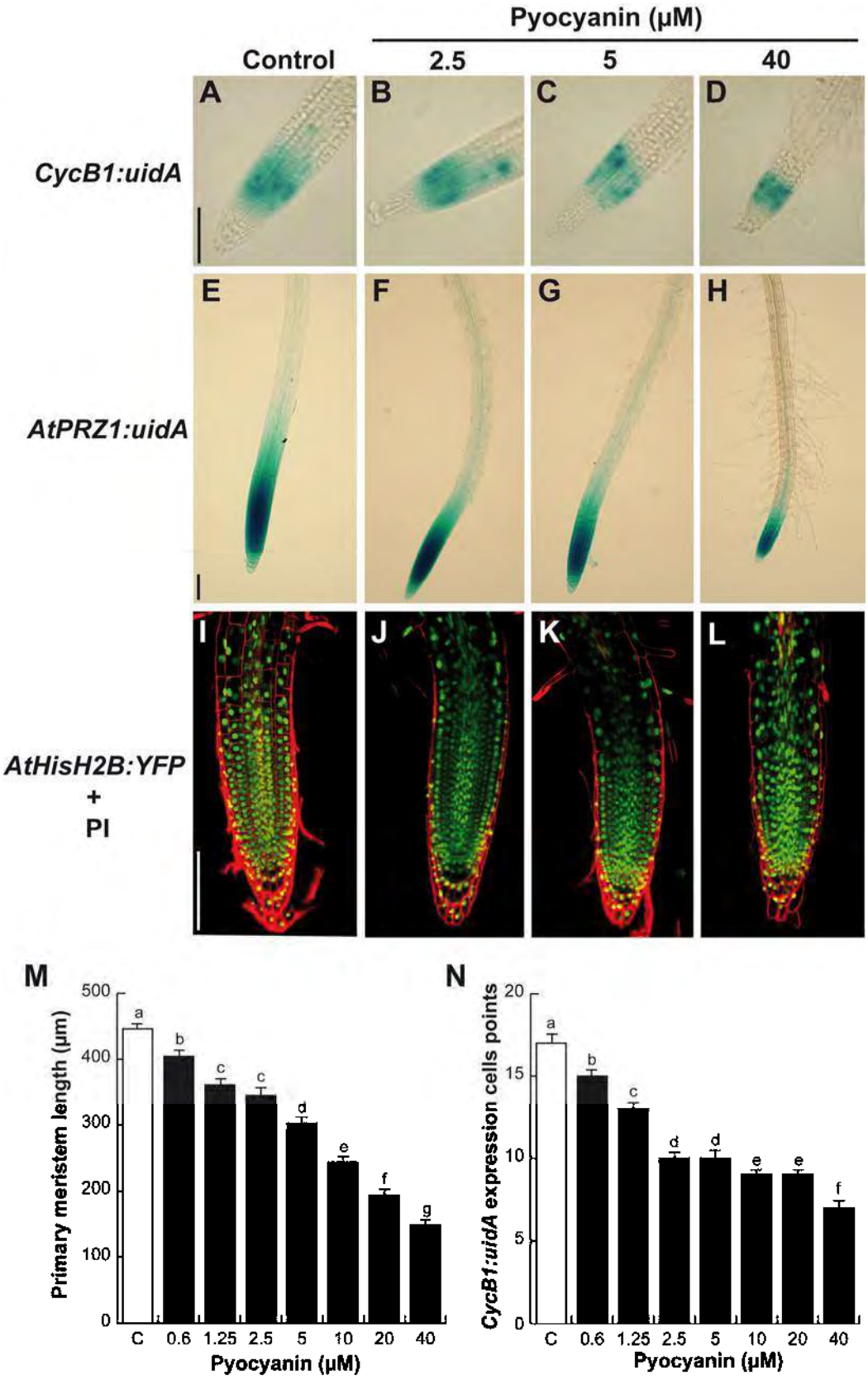

Fig. 6. Effect of pyocyanin on cell division and meristem viability. *Arabidopsis thaliana* seedlings expressing the *CycB1:uidA*, *AtPRZ1:uidA*, or *AtHistH2B:YFP* markers were grown for 5 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. **A** to **H,** Plants were stained for β-glucoronidase activity and cleared to show gene expression. **I** to **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 **N,** cells expressing this marker were counted. Data points represent the mean ± standard deviation (n = 20). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P < 0.05$.

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. PCN treatment caused a 70% inhibition in primary root growth in WT plants compared with solvent-treated seedlings (Supplementary Fig. S3). 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. 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* and *cre1-12ahk3-3*), abscisic acid signaling (*abi1* and *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, indicating that ethylene might be a signal that mediates the plant response to PCN.

A role of ethylene signaling in root response to PCN.

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 10 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 with WT seedlings, while *ein3-1* did not show clear 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 reduced the effect of PCN in both primary root growth and root hair development (Fig. 8B and C to J). This restoration of primary root growth in plants grown on medium supplemented with PCN and $AgNO_3$ correlated with normalization of root hair differentiation process caused by PCN (Fig. 8G to J). These results suggest that ethylene signaling plays a role in root architectural responses to PCN.

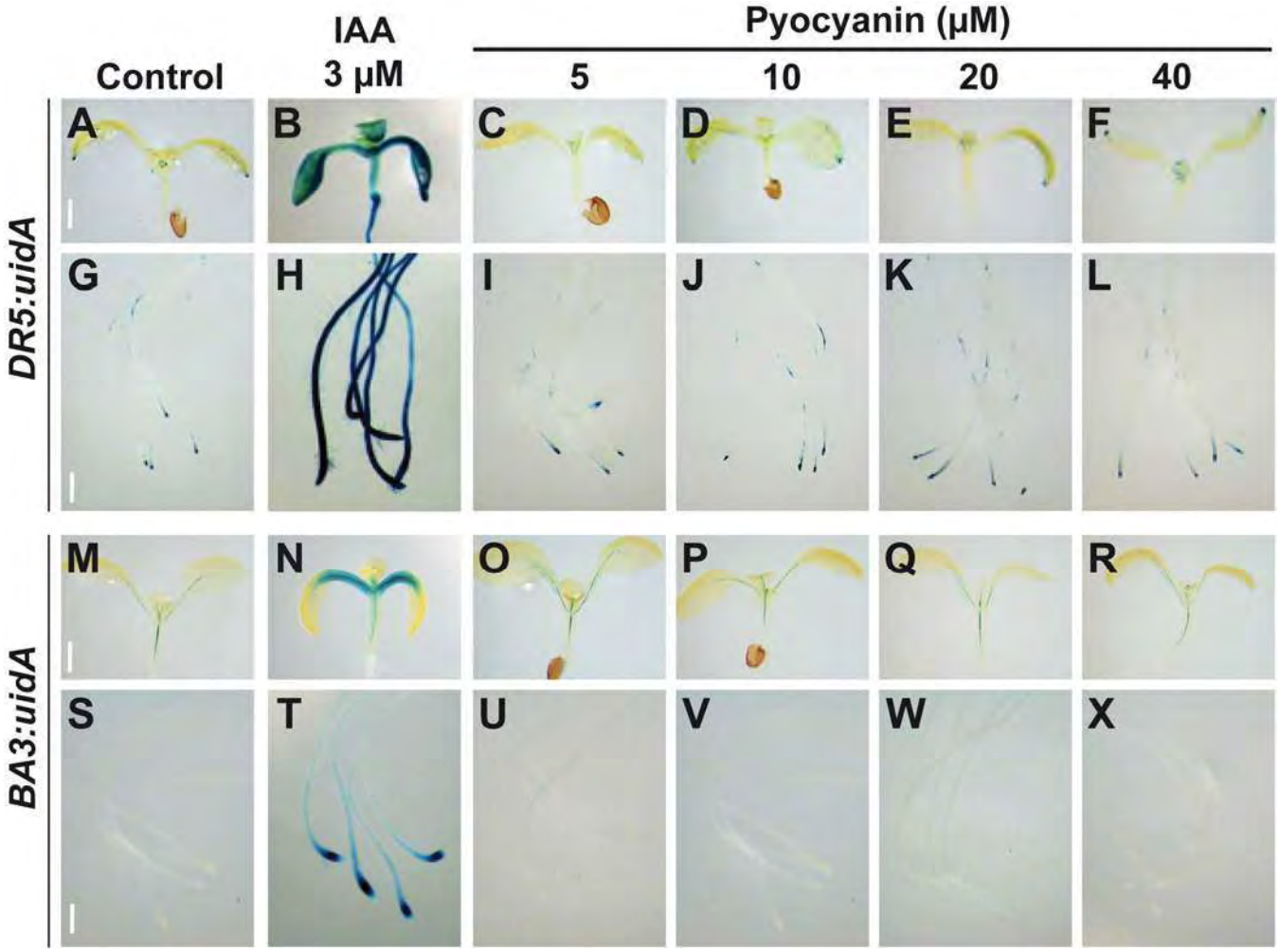

Fig. 7. Effect of pyocyanin on auxin-regulated gene expression. **A** to **L,** *DR5:uidA* and **M** to **X,** *BA3:uidA* gene expression in transgenic seedlings grown on 0.2× Murashige-Skoog (MS) agar medium for 6 days and then transferred into 24-well cell culture plates (10 seedlings per well) containing 2 ml of 0.2× MS liquid medium supplied with the indicated concentrations of indole-3-acetic acid (IAA) or pyocyanin and incubated for 10 h. Seedlings were stained for β-glucuronidase activity and cleared for microscopy analysis. Photographs show representative individuals from at least 30 stained plants (scale bars = 500 μm).

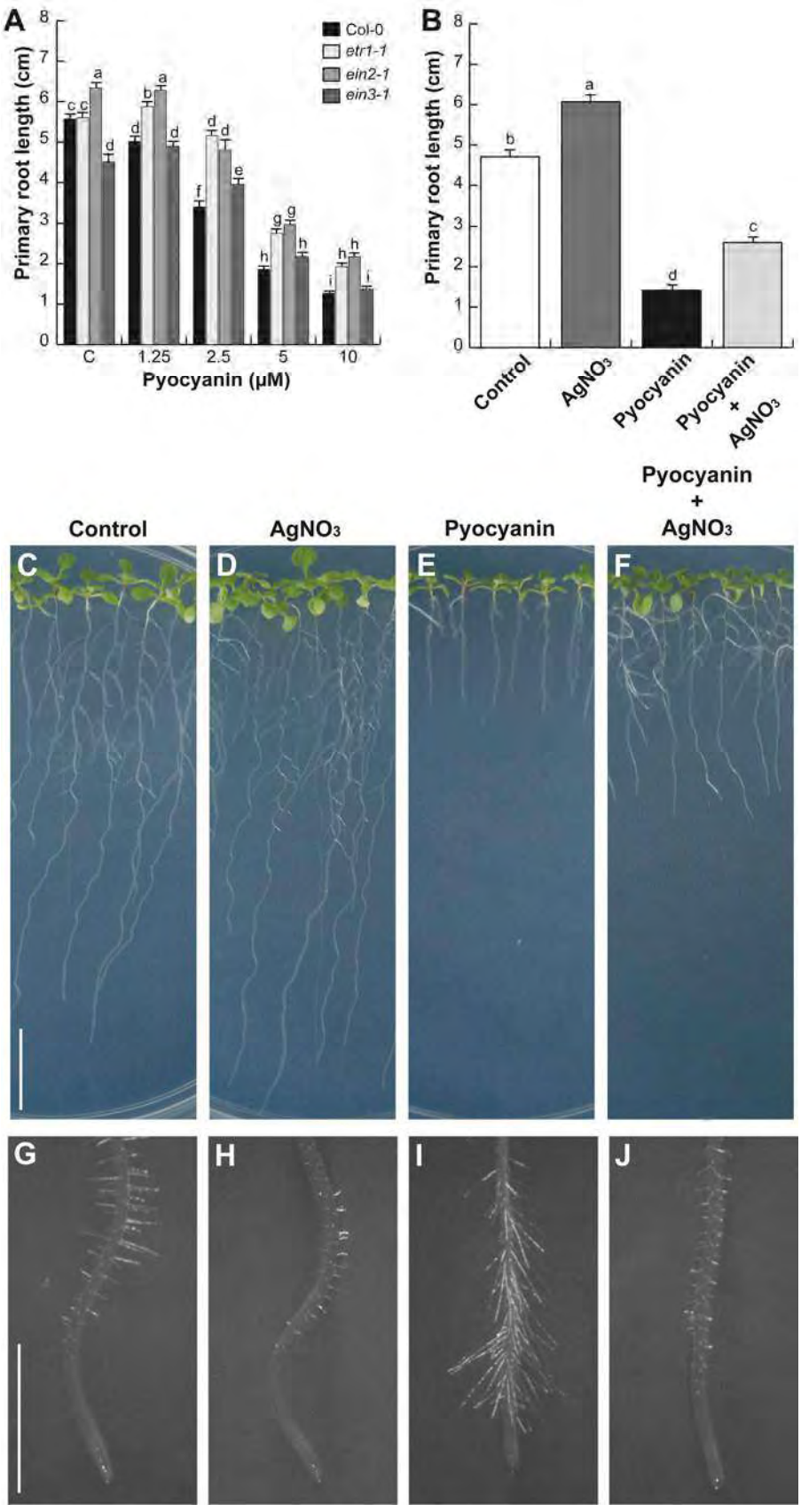

Fig. 8. Role of ethylene signaling in pyocyanin-induced primary root inhibition of *Arabidopsis* seedlings. **A,** *Arabidopsis thaliana* wild-type (WT) and *etr1-1, ein2-1*, and *ein3-1* ethylene mutant seedlings were grown for 12 days on 0.2× Murashige-Skoog (MS) medium supplemented with the indicated concentrations of pyocyanin. **B,** *Arabidopsis* WT (Col-0) seedlings were grown for 12 days on 0.2× MS agar medium supplemented with 5 μM pyocyanin and $AgNO_3$. Data points show the mean ± standard deviation (n = 30). Representative photographs of **C** to **F,** *Arabidopsis* root system architecture and **G** to **J,** root hair development under the different treatments are shown. Different letters indicate means that differ statistically at $P < 0.05$. The experiment was repeated three times with similar results.

PCN 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 whether 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 0.2× MS medium supplemented with or without 5 μM PCN, $AgNO_3$, or PCN plus $AgNO_3$ and, 7 d.a.g., the seedlings were incubated with 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) or dihydroethidium (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 PCN provokes an 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 the levels of H_2O_2 on primary root tips in a dose-dependent way (Fig. 10A to 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 to 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 to the resistance of primary root growth when the plants are grown on PCN.

DISCUSSION

Plant roots are colonized by an immense number of microbes, referred to as the root microbiome. Selected strains of beneficial soilborne bacteria can protect against abiotic stress and prime the plant immune system against a broad range of pathogens. *Pseudomonas* spp. rhizobacteria represent one of the most abundant genera of the root microbiome. 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 QS signals that are perceived at the root tip to adjust cell proliferation and growth.

Our 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 inhibition of root growth and a concomitant phytostimulation (Fig. 1), which can be likely explained by either a decreased production of virulence factors or 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.

The beneficial effect of *Pseudomonas* spp. to plants by means of regulating root architecture was recently confirmed by Zamioudis and associates (2013). By employing a germ-free experimental system, the authors showed the ability of selected *Pseudomonas* strains to promote plant growth and drive developmental plasticity in the roots of *Arabidopsis* by inhibiting primary root elongation and promoting lateral root and root hair formation. By studying cell-type-specific developmental markers and employing genetic and pharmacological approaches, it was demonstrated the crucial role of auxin signaling and transport in rhizobacteria-stimulated changes in the root system architecture of *Arabidopsis*. The authors further show that *Pseudomonas* spp.-elicited alterations in root morphology and that rhizobacteria-mediated systemic immunity are mediated by distinct signaling pathways.

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 known 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.

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 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). Although all three QS-related *P. aeruginosa* mutants tested have a significant defect in PCN production, the *rhlI* mutant inhibited growth and affected root architecture similarly to the WT Pao1 strain. This result indicates that loss of PCN production is not the only factor by which *P. aeruginosa* inhibits root growth or decreases H_2O_2 level in the root tip. A second factor which affects root growth and is present in *rhlI* mutants is C12-AHL. Our previous work demonstrated that C12-AHL but not C4- or C6-AHL is very active in inhibiting primary root growth (Ortiz-Castro et al. 2008), thus explaining why *rhlI* mutants still repress primary root growth.

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 H_2O_2 and O^{2-} at the expenses of host antioxidant systems such as glutathione and catalase (O'Malley et al. 2004). Several reports have documented that PCN is an important virulence factor of *P. aeruginosa*. Its induction through quorum signaling correlated with the biofilm growth stage of the bacterium. Although PCN has a wide range of toxic effects in animal cells, the proposed basis of its toxicity is production of O^{2-} anions and downstream ROS by oxidizing NADPH (O'Malley et al. 2004; Lau et al. 2004a; Liu and Nizet 2009). To the best of our knowledge, there is a lack of information about the levels of PCN released by *P. aeruginosa* when colonizing plant roots. However, some reports, mainly from animal systems, have shown that PCN accumulates in low micromolar levels in *P. aeruginosa* host cells. Wilson and associates (1998) showed that PCN levels varied between 78.5 and 128.5 μM in the sputum of patients with cystic fibrosis (CF). Hunter and associates (2012) analyzed the level of PCN of 47 CF patients, identifying concentrations up to 48 μM in patients with severe CF. In another study by Price-Whelan and associates (2007), the PCN concentration reached 100 μM in

the stationary phase. Dietrich and associates (2006) showed that PCN activates genes related to redox homeostasis, iron acquisition, and virulence by using DNA microarrays and quantitative reverse-transcriptase polymerase chain reaction. In this work, the authors reported that *P. aeruginosa* PAO1 and PA14 produce 10 and 55 μM PCN, respectively, during the stationary phase, and demonstrated that PCN can act as signaling molecule at these concentrations.

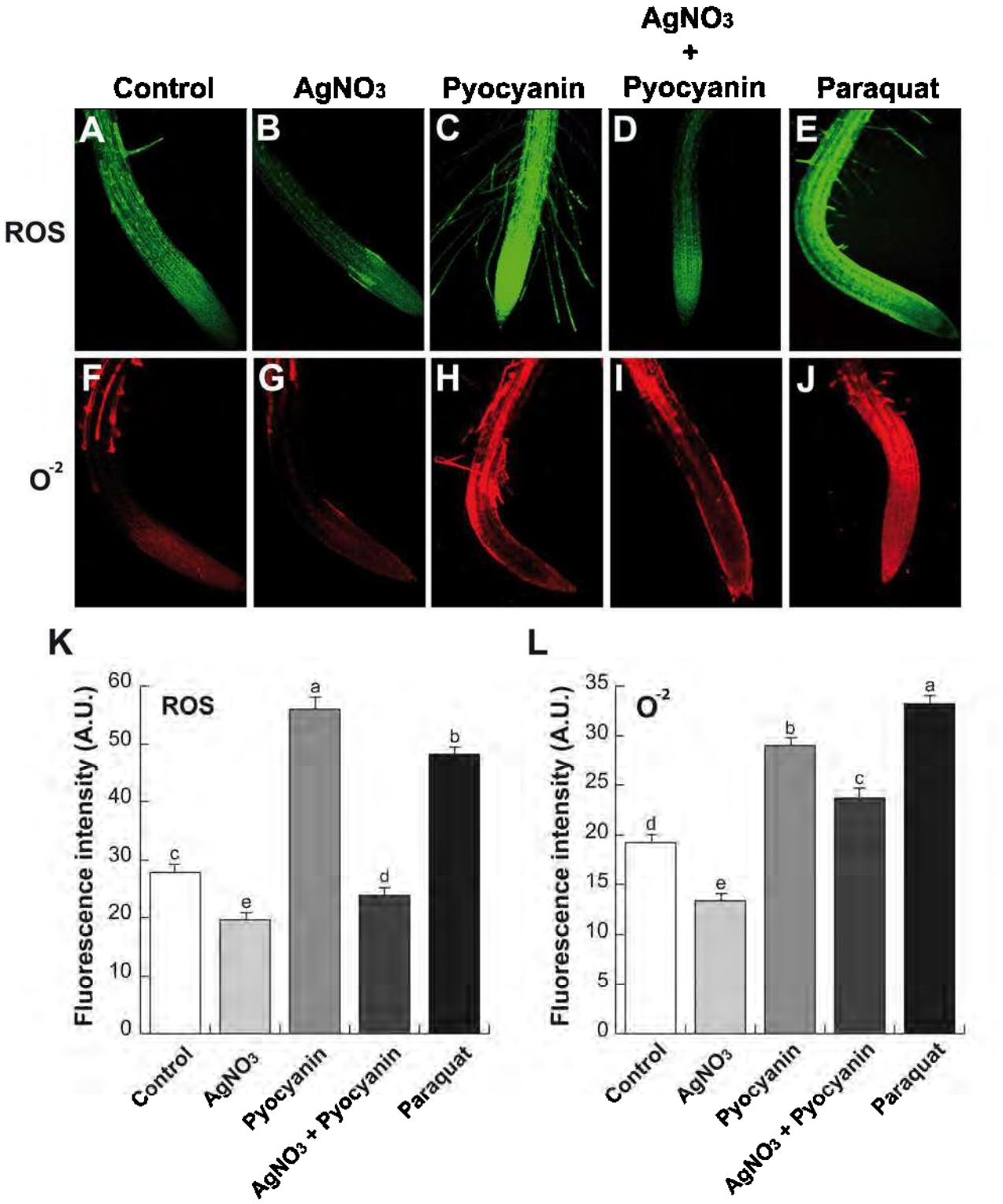

Fig. 9. Role of ethylene signaling in reactive oxygen species (ROS)-induced production by pyocyanin. Representative photographs of the detection of endogenous **A** to **E,** ROS and **F** to **J,** O^{2-} with 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) and dihydroethidium (DHE), respectively, which were determined in primary roots of *Arabidopsis* seedlings grown for 7 days on 0.2× Murashige-Skoog agar medium supplemented with 5 μM pyocyanin and $AgNO_3$ or with 0.1 μM paraquat. H2DCF-DA and DHE fluorescence signals from primary root tips (n = 10) for **K,** ROS and **L,** O^{2-} were quantified using the ImageJ program. The graph is expressed in arbitrary units. Values in K and L represent the mean ± standard deviation (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.

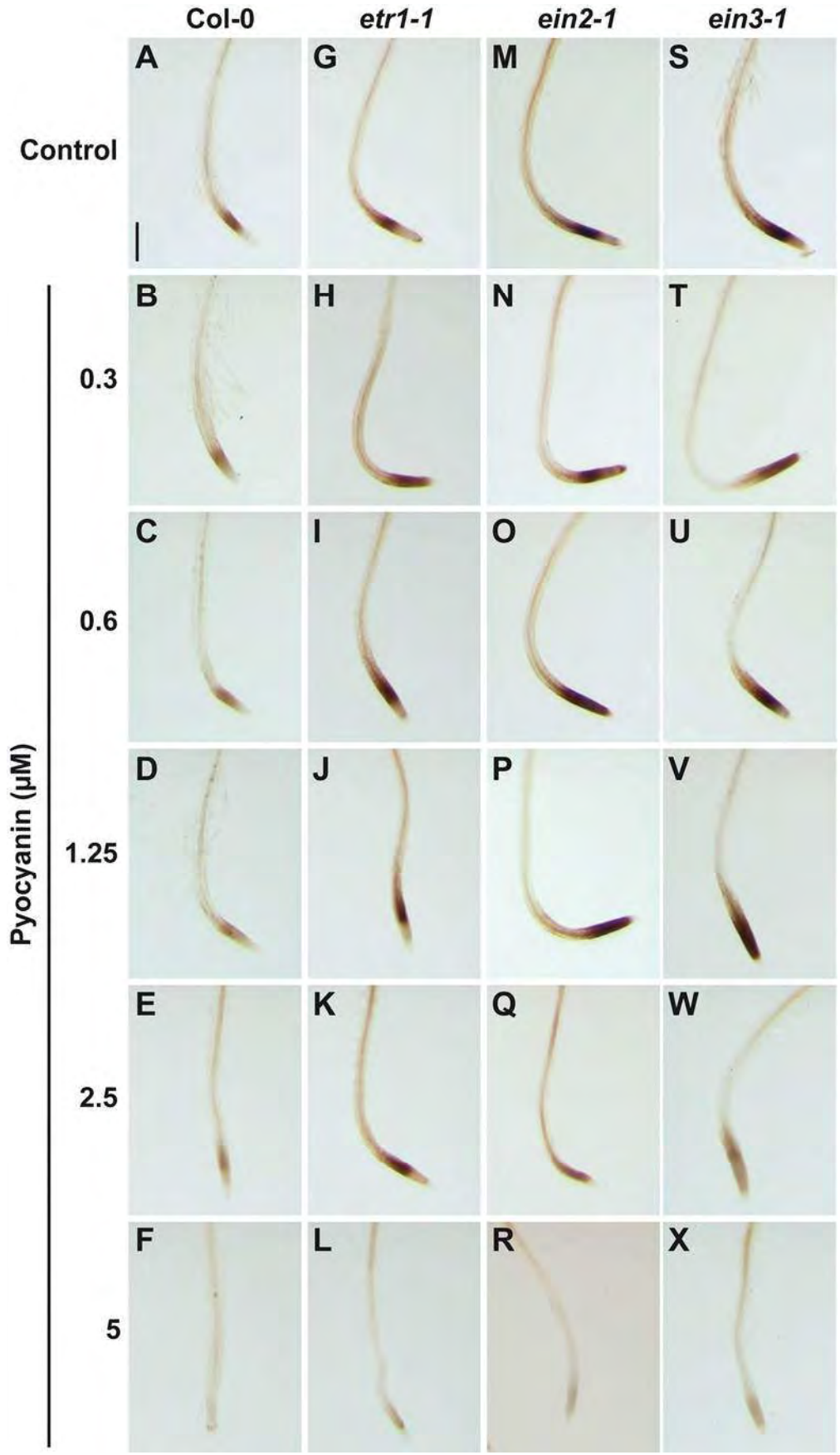

Fig. 10. Effect of pyocyanin on H_2O_2 accumulation in the primary root meristem of *Arabidopsis thaliana* wild-type (WT) (Col-0) and ethylene-related mutants *etr1*, *ein2,* and *ein3*. Histochemical detection of H_2O_2 with 3,3′-diaminobenzidine staining in **A** to **F,** *Arabidopsis thaliana* WT (Col-0) and **G** to **L,** *etr1-1*; **M** to **R,** *ein2-1*; and **S** to **X,** *ein3-1*. *Arabidopsis* seedlings were grown for 7 days on 0.2× Murashige-Skoog medium supplemented with the indicated concentrations of pyocyanin. Photographs show representative individuals from at least 30 stained plants. Scale bar = 500 µm.

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 evolutionarily conserved and, therefore, by using a plant model system, it would be possible to define whether PCN causes toxicity to cells or regulates fundamental cellular processes such as division, elongation, or differentiation at concentrations naturally present when colonizing host cells. Moreover, diverse bacterial species proliferate in the rhizosphere and release PCN and other phenazines with potential biocontrol activities (Bosgelmez-Tinaz 2003; Fuqua and Greenberg 2002). This would suggest that natural phenazines such as phenazine-1-carboxylic acid 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 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 led to an underestimation 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 and no toxicity symptoms were evident, indicating that the activity of PCN in plants might be rather different from that reported for animal cells. PCN was found to inhibit primary root growth and stimulate lateral root and root hair formation in a dose-dependent way in low micromolar concentrations (Figs. 4 and 5). In this regard, PCN activity is similar to the previously reported activities of other bacterial QS signals (namely, C12-AHL), 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 by producing not only AHL but also PCN and, possibly, other phenazines with signaling roles in plant cells.

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. Because PCN decreases trichoblast cell length and increases root hair elongation (Fig. 5), 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 indicating that PCN affects primary roots, root hairs, and lateral root development through production of ROS are 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 the O^{2-} 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 WT, 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, 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 LRD. In this work, the downregulation 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 remains 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 PI, 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 O^{2-} and H_2O_2 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* orthologs 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 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 and associates (2010) in that the effects of PCN on ROS induction are independent of auxin signaling, considering the following evidence. 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*, and *aux1-7* mutants displayed similar primary root growth inhibition in response to PCN when compared with WT seedlings. 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 shows that localized O^{2-} 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 O^{2-} levels in primary root tips. PCN treatment increased O^{2-} accumulation in the root elongation zone (Fig. 9), while co-cultivation with *Pseudomonas 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 O^{2-} or H_2O_2 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 O^{2-} and H_2O_2 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 and not only as toxicity factors, as reported in animal cells.

The PCN-elicited accumulation of ROS was partially blocked when supplied together with the ethylene blocker $AgNO_3$ (Fig. 9), and the H_2O_2 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 *etr1* and *ein2-1* 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 O^{2-} and H_2O_2 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 and associates (2010) identified ethylene signaling as a critical component of the oxidative burst in response to this bacterial elicitor because *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 H_2O_2 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 QS in pathogenesis and symbiosis, particularly the role played by AHL in the production of virulence factors or compounds with a role in auxin (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.

A. 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), and 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), and *cre1-12ahk2-2* and *cre1-12ahk3-3* (Higuchi et al. 2004; Mähönen et al. 2006) were used for all experiments. Seed 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, seed were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog 1962). MS medium (Murashige and Skoog basal salts mixture; catalog M5524) was purchased from Sigma-Aldrich (St. Louis). The suggested formulation is salts at 4.3 g $liter^{-1}$ for a 1× concentration of medium; we used 0.9 g $liter^{-1}$, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. PCN was purchased from Sigma-Aldrich. The compound was dissolved in dimethyl sulfoxide and used at the indicated concentrations. In control seedlings, we added the solvent in amounts equal to those present in the greatest concentration of compound tested. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, US.A.). 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^2 s^{-1}, and temperature of 22°C.

In vitro plant-bacteria co-cultivation assays.

Bacterial strains used in this work were *P. aeruginosa* PAO1 (WT) and *P. aeruginosa* single mutants *rhlI*– and *lasI*– and double mutant *rhlI–/lasI*– (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 2.5×10^8 CFU were inoculated by streaking on agar plates containing 0.2× 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-day 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. LRD was determined by dividing the LRN by the primary root length and was expressed as LRD cm^{-1}. The length of the meristems was determined as the distance between the quiescent centers to the cell file where cells started to elongate. For all experiments, data were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in the WT and ethylene-related mutants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

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. Primary root meristems were analyzed in semipermanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus; Carl Zeiss, Göttingen, Germany) at ×100 or ×400 magnifications. Images were captured with a Sony Cybershot DSC-S75 digital camera (Sony Electonics Inc., Oradell, NJ, U.S.A.) 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% 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 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 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 DAB at 1 mg ml^{-1}, pH 3.8, and incubated in dark for 2 h. Subsequently, they were immersed in boiling 96% (vol/vol) ethanol for 10 min and then stored in 96% (vol/vol) 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.

PI staining and YFP detection.

For confocal microscopy, solvent- or PCN-treated transgenic *Arabidopsis* seedlings expressing the histone *AtHisH2B:YFP* construct (Boisnard-Lorig et al. 2001) were mounted on microscope slides into a solution of PI. For fluorescent staining with PI, recently collected plants with intact root systems were transferred to a solution of PI at 10 mg/ml 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 585to 610 nm, and YFP emission with a 505- to 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).

PCN quantification.

PCN was extracted from the supernantant fraction of *P. aeruginosa* grown in Luria-Bertani medium at 37°C for 48 h. Supernatant (1 ml) was mixed with 1 ml of chloroform and the lower organic layer was separated. To this layer, 1 ml of 0.2 HCl was added and the PCN 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 PCN produced per milliliter of culture supernantant, were determined according to Essar and associates (1990).

ROS and O^{2-} detection

General ROS and specific O^{2-} anion were monitored by incubating *Arabidopsis* seedlings with 10 μM fluorescent probes H2DCF-DA and DHE, respectively, in 10 mM Tris-HCl (pH 7.4) (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 numbers in the green channel by employing ImageJ software.

ACKNOWLEDGMENTS

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT, México, grant numbers 60999 and 177775), the Consejo de la Investigación Científica (UMSNH, México, grant number CIC 2.26), and the Marcos Moshinsky Fellowship to J. López-Bucio. We thank P. Doerner, C. Luschnig, T. Guilfoyle, and P. Guzman for kindly providing us with seed of transgenic and mutant lines and A. Méndez Bravo for his kind support with confocal microscopy analysis.

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***Trichoderma* as biostimulant: Exploiting the multilevel properties of a plant beneficial fungus.**

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Abstract

Plant biostimulants are formulated with diverse microorganisms and/or substances that are applied to crops with the aim of enhancing growth, development and adaptation to abiotic stress. *Trichoderma*-based products have been particularly successful because of their capacity to control phytopathogenic fungi. Some *Trichoderma* strains have a predominant biostimulant action that makes them unique for their extended use in horticulture. They are safe for humans, livestock and crop plants and in their natural environment colonize plant roots without apparent adverse reactions. Both solid and liquid formulations containing conidia can be used to produce suitable quantities of active and viable inocula during product formulation and field use. The mechanisms of phytostimulation by *Trichoderma* involves multilevel communication with root and shoot systems, as it releases into the rhizosphere auxins, small peptides, volatiles and other active metabolites, which promote root branching and nutrient uptake capacity, thereby boosting plant growth and yield. Recent proteomic and genetic data suggest that *Trichoderma* activates the mitogen activated protein kinase 6, transcription factors and DNA processing proteins, which represent promising targets towards formulation of more efficient products.

Keywords: *Biostimulant, Trichoderma, Volatiles, Auxins, Formulation, Plant signaling.*

Index

1. Introduction

Currently, there are almost 7 billion people in the world for which plants should provide grains, fruits, seeds and energy resources. Moreover, it has been estimated that in the next 50 years total population will increase by at least 1.5 billion people, making it necessary to duplicate crop production in the same time to ensure food provision (IFA, 2012). The so called "green revolution" that employed rice, corn and wheat varieties almost doubled yields from 1.9 tons per hectare in 1950-64 to 3.5 tons in 1985-98. This has resulted in an increase in grain production from 824 million tons in 1960 to almost 2,400 million tons in 2011. However, this was possible only thanks to intensive application of nitrate (N) and phosphate (P) based fertilizers and depletion of soil and water resources (Den Herder et al., 2010). In the coming years, a global P crisis is expected to occur, due to exhaustion of most P reserves. In 2008, the phosphate rock price temporarily spiked at US $500 per ton, more than five times the average price in 2007 (Gilbert, 2009). To farmers, increasing P use efficiency through better agricultural practices is an urgent need to save money while maintaining yields.

Other major challenges of agriculture are globalization and climate change, which are predicted to modify the scale and frequency of emerging plant diseases, and threatening global food security. New highly virulent fungal strains are emerging, which adapt to warmer temperatures and overcome many of the main plant defense genes (Hubbard et al., 2015). Several below-ground characteristics of plants such as root architecture, P uptake and N fixation are promising features to strengthen the innate immune system of plants. Indeed, a more sustainable agriculture requires yield increases and product quality, while reducing the negative impact of agrochemicals on the environment, all of which might be fostered by biostimulants (Berg, 2009; Xiang et al., 2012; Calvo et al., 2014; Owen et al., 2015).

Biostimulant microbes have been important since the beginning of agriculture (i.e. *Rhizobium* in legumes) and current expectations include their commercialization as a complement to crop nutrition. The beneficial effects of microbes to plants depend upon sophisticated nutritional and chemical signaling as well as soil and climate factors. Plant roots release sugars, organic acids, amino acids and phenolics, which affect the composition of rhizosphere communities, leading to beneficial relationships (Ortiz-Castro et al., 2009). Symbiosis takes place between

crops and soil microoganisms, including plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF), which are considered natural biostimulants. *Trichoderma* spp. belong to a class of PGPF successfully used on a commercial scale for biological control of phytopathogens such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Armillaria mellea* and *Chondrostereum purpureum* (Benítez et al., 2004; El-Komy et al., 2015). There is a great variability on antagonistic capacity and biostimulant action of *Trichoderma* resulting in strains that have a predominant biostimulant action and others that have a predominant antagonistic activity. Therefore, some *Trichoderma* strains are more suitable for biological control as biopesticides and other for stimulating crop growth and nutrient uptake acting as biostimulants (Benítez et al., 2004; Shoresh et al., 2010; Contreras-Cornejo et al., 2013, 2014a; Zhao et al., 2014).

Trichoderma spp. help plants better resist environmental stresses such as salt and drought via reinforcing plant growth and reprograming gene expression in roots and shoots (Figure 1). The fungal mycelium secretes different compounds that increase the branching capacity of the root system, thus improving nutrient and water acquisition. This review focuses on recent advances in our understanding of the phytostimulant properties of *Trichoderma*, the underlying signaling mechanisms and their potential applications.

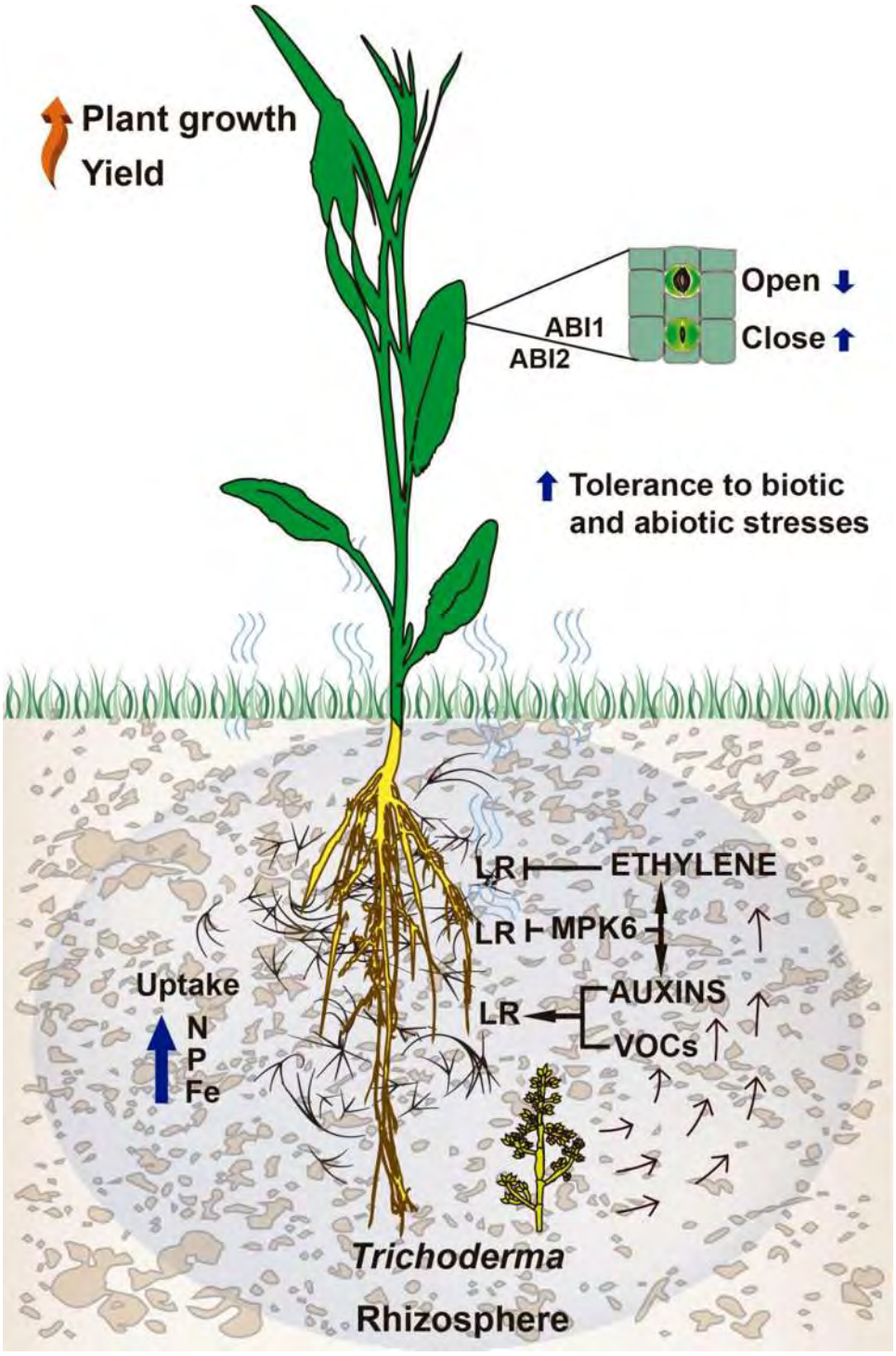

Figure 1. Biostimulant properties of *Trichoderma* depend on fungal-root communication via volatiles, ethylene and auxins. *Trichoderma* metabolites, including volatile blends, and the classic phytohormones indole-3-acetic acid, (auxin, IAA) and ethylene, regulate several aspects of root development including lateral root (LR) formation. *T. atroviride* induces the activation of plant mitogen activated protein 6 (MPK6) that translates fungal signals into root cellular responses. Root sensing of *Trichoderma* and its metabolites may increase nutrient uptake and stress tolerance while boosting plant health and yield.

2. Growth and habitat of *Trichoderma*

Trichoderma (teleomorph *Hypocrea*, Ascomycota, Dikarya) is a well-studied fungal genus that currently consists of more than 200 molecularly defined species (Atanasova et al., 2013). *Trichoderma* species are in general considered cosmopolitan and prevalent components of different ecosystems in a wide range of climatic zones (Kubicek et al., 2008). However, some species are ubiquitous while others are limited to specific geographical areas (Harman et al., 2004). Members of this genus are frequently found parasitizing other fungi, on dead wood and bark, in soil and rhizosphere, in marine sponges (Paz et al., 2010; Gal-Hemed et al., 2011), associated to woody and herbaceous plants (Jaklitsch, 2009; Chaverri et al., 2011; López-Quintero et al., 2013) and as endophytes (Zhang et al., 2007; Hanada et al., 2008; Mulaw et al., 2010), which exemplifies their ability to occupy various ecological niches. *Trichoderma* species grow and ramify as typical fungal hyphae, 5 to 10 μm in diameter (Figure 2A). Most laboratory strains produce asexual spores such as conidia and chlamydospores (Figure 2B), and in their natural environment some form ascospores in perithecia. Spores have either dispersal or resting functions and are also used as inocula. *Trichoderma* conidiophores appear as paired branches that assume a pyramidal aspect, ending in one or a few phialides. Phialides may be held in whorls or may be penicillate, and can be densely clustered on a wide main axis or solitary. Conidia are pigmented ranging from deep green to nearly grey and are less than 5 □m long and wide (Samuels, 1996). Although chlamydospores were discovered long time ago, our understanding of the genetic basis for their formation and proliferation is scarce. However, chlamydospores are abundantly produced in submerged culture and are the active propagules in some *Trichoderma*-based commercial formulations.

The versatile life style of *Trichoderma* is based on three major nutritional modes: saprotrophy, mycotrophy, and dependence upon plant-derived sugars. Most *Trichoderma* species can live as parasites of other fungi. In fact, *Trichoderma* fruiting bodies have been found on basidiomycetes, and several species of the genus can grow on a wide variety of fungi as necrotrophic hyperparasites (Druzhinina et al., 2011). However, parasitic interactions of a few species have been observed with other organisms and occasionally in immunocompromised humans (Kredics et al., 2003; Casas-Flores and Herrera-Estrella, 2007; Druzhinina et al.,

2008). Some species, such as *Trichoderma reesei, Trichoderma subeffusum, Trichoderma luteffusum, Trichoderma polysporum, Trichoderma phellinicola, Trichoderma sulphurea* and *Trichoderma longibranchiatum* are known for their capacity to produce cellulases, and to depend upon degrading dead or decayed organic matter and wood, suggesting that these enzymes are integral to *Trichoderma* nutrition (Jaklitsch, 2011; Xie et al., 2014). An analysis of the *Trichoderma* genomes sequenced up to date, indicates that the capacity to degrade cellulose is most likely present in the whole genus, although probably with varying efficiency. Based on these observations, it has been hypothesized that the *Trichoderma* ancestral species first parasitized on wood-rotting fungi and later explored wood as an alternative ecological niche, switching to living on pre-degraded wood rather than on a fungal host (Rossmann et al., 1999; Druzhinina et al., 2011).

Another niche occupied by *Trichoderma* species is the rhizosphere, which attracts them due to the presence of root-derived sugar and exudates. These strains are called „rhizosphere competent strains" and they are particularly suitable for use as inoculants due to their long persistence on the roots and beneficial properties (Fig. 2C, D; Vargas et al., 2009; Druzhinina et al., 2011). A mutualistic relationship with plants, in which the fungus penetrates the external tissues of the root system results in several beneficial effects to the plant, while *Trichoderma* benefits from a nutrient rich environment. Numerous *Trichoderma* species have been collected from different crop fields and plant hosts in diverse climatic zones of all continents (Jaklitsch, 2009; Chaverri et al., 2011; López-Quintero et al., 2013).

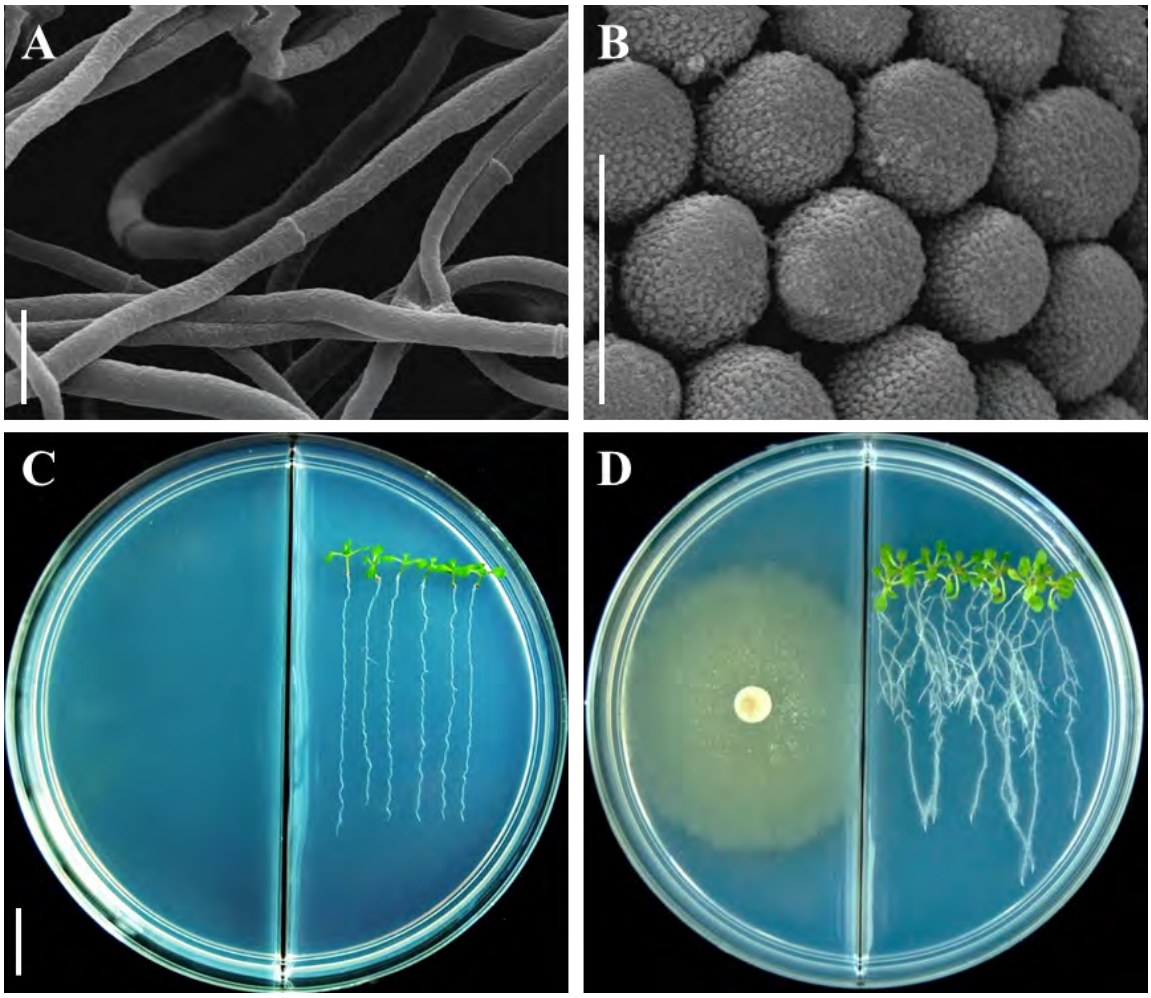

Figure 2. *Trichoderma virens* propagules and its interaction with *Arabidopsis*. Scanning electron microscopy images of hyphae and conidia of *T. virens*. A) Hyphae (scale bar 5 □m). B) Conidia (scale bar 1 □m). C) 12-day-old *Arabidopsis* (Col-0) seedlings grown on the surface of a divided agar plate containing 0.2X MS medium. D) 12-day-old *Arabidopsis* exposed to volatiles of *T. virens*. Seedlings were treated with sterilized deionized water at day 7 after germination (left plate), or inoculated with *T. virens* Tv29.8 at the opposite side of the plate (right plate) and photographed 5-days later. Scale bar 1 cm.

3. Uses of *Trichoderma* in horticulture

3.1. *Trichoderma* inocula

The ability of *Trichoderma* to sense, invade, and destroy other fungi has been the major trait behind their commercial success as biopesticides (Verma et al., 2007). Currently, more than 60% of all registered biopesticides contain a single *Trichoderma* isolate, or mixtures of *Trichoderma* species, in principle for greater bioactivity (Figure 3). The *Trichoderma* species

more frequently used in biocontrol and best studied regarding their mechanisms of action are *T. asperellum*, *T. atroviride*, *T. harzianum*, *T. virens*, and *T. viride*, most of which also exhibit high biostimulant action on horticultural crops. For instance, the mixture of three *Trichoderma* species (*T. harzianum*, *T. viride* and *T. virens*) increased plant height, total biomass and improved shoot and root P and N uptake in chickpea in glasshouse and field trials (Rudresh et al., 2005a). Mixtures like the one described in this example may provide a more consistent level of growth promotion and a broader spectrum of activity, due to the variations in the mechanism of action of the different species used, resulting in better performance and commercial success. Nevertheless, the use of different species in a formulation must be carefully examined, since at least *in vitro* different species of *Trichoderma* may antagonize each other (Gómez et al., 1997).

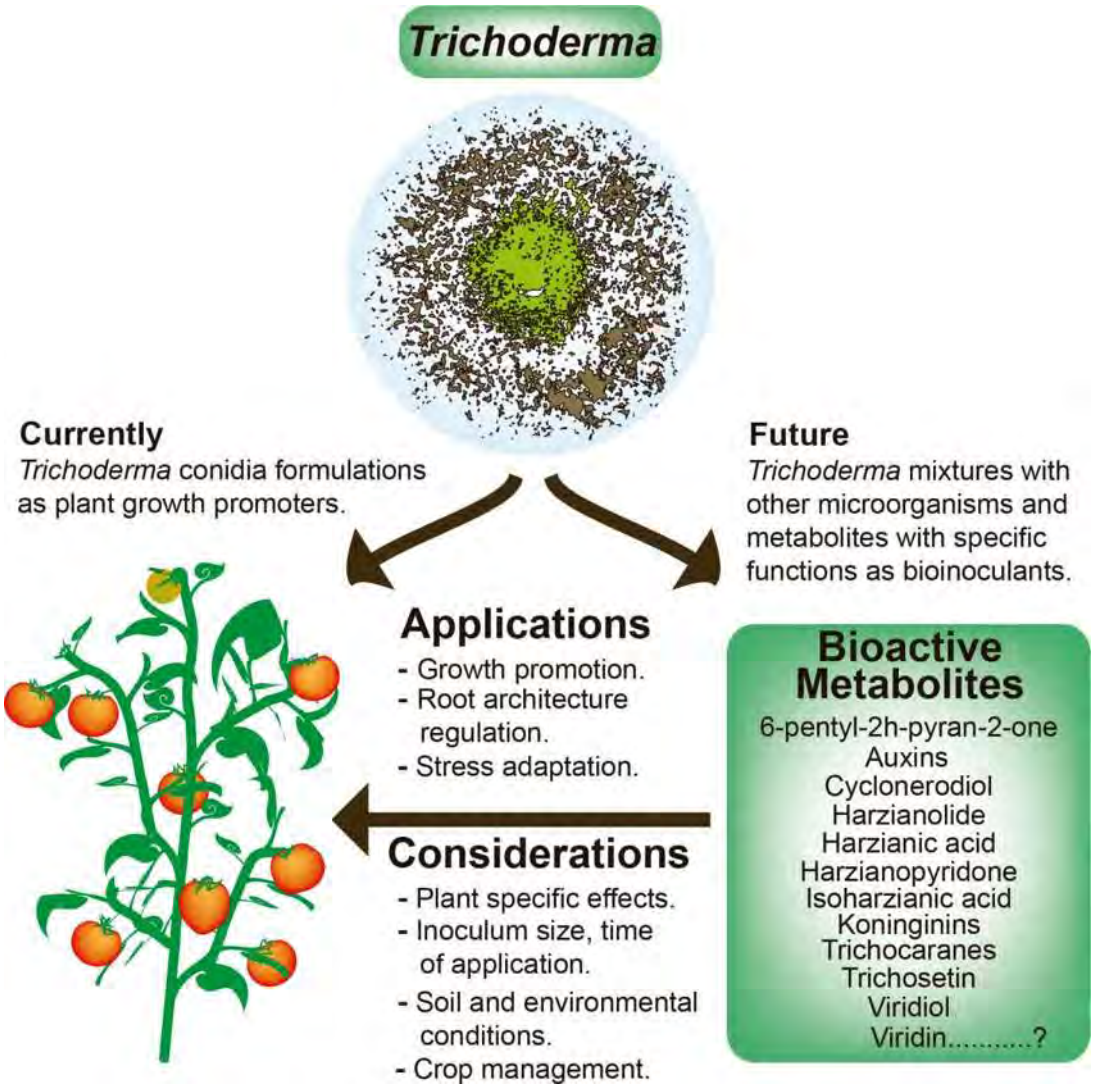

Figure 3. *Trichoderma* bioinoculants contain conidia for their extended agricultural use. Novel formulations are expected to improve the biostimulant potential as novel fungal metabolites with specific functions and plant targets are discovered. Volatiles such as 6-pentyl-2h-pyran-2-one and auxins increase root branching, secondary metabolites and small peptides can improve innate immunity of plants.

When grown at the rhizosphere or on the root surface, *Trichoderma* is expected to face frequent interactions with other plant microorganisms such as arbuscular mycorrhizal (AM) fungi. Indeed, such interactions have been investigated in the past, with contrasting results. In some cases, inoculation with both fungi either resulted in positive synergistic effects on plants or in the inhibition of plant growth (Rousseau et al., 1996; Chandanie et al., 2009; Colla et al., 2015a,b). Nevertheless, De Jaeger et al. (2011) showed that *Trichoderma harzianum* was able to parasitize the mycelium of an AM fungus, *Glomus sp*., thus affecting its viability. When ^{33}P was measured in the plant and in the fungal mycelium in the presence/absence of *T. harzianum*, an increased uptake of ^{33}P by the AM fungus in the presence of *T. harzianum* was evidenced, possibly related to a stress reaction caused by mycoparasitism. Regarding potential negative effects of *Trichoderma* on AM fungi, Rousseau et al. (1996) showed that *T. harzianum* proliferated at the spore surface and penetrated the thick host wall of *G. intraradices* through local hydrolysis of the wall polymers. At an advanced stage of the colonization process, the hyphae of *G. intraradices* were perforated in many places. Using *in vivo* imaging of green fluorescent protein-tagged lines, Lace and co-workers (2015) investigated the cellular interactions occurring between *Trichoderma atroviride*, *Medicago truncatula* and two *Gigaspora* species under *in vitro* culture conditions. *T. atroviride* parasitized *Gigaspora gigantea* and *Gigaspora margarita*. Despite these data suggest antagonistic *Trichoderma*-AM fungi interactions, recent research indicates that at least some *Trichoderma* combinations with other microbes can improve plant growth (Colla et al., 2015a). The highest shoot, root dry weight, chlorophyll content in lettuce, tomato and zucchini was observed in a *Glomus–Trichoderma* combination, followed by a single inoculation of *Glomus* or *Trichoderma*, whereas the lowest growth promoting values were recorded in the uninoculated plants. Mixtures of *Trichoderma* and rhizospheric bacteria have been evaluated for their growth promoting activity. A combination of *T. atroviride* and *Bacillus subtilis* greatly promoted growth of beans in terms of plant dry biomass (43% over the control)

compared with 2% for *Trichoderma* alone and 34% for *B. subtilis* alone (Yobo et al., 2011). Similarly, Jisha and Alagawadi (1996) showed that a mixture of *Bacillus polymyxa* and *T. harzianum* enhanced growth of sorghum more than either organism separately. The mechanism of growth promotion by mixtures was attributed to an increased nutrient uptake (Yobo et al., 2009), siderophore production and production of plant growth promoting substances (Yobo et al., 2011). In another set of experiments, a combination of *Rhizobium*, *Bacillus* and *Trichoderma* was applied to chickpea, resulting in greater effects of the mix than any of the individual organisms with increases in germination, nutrient uptake, height, nodulation and total biomass of plants (Rudresh et al., 2005b).

3.2. Mass propagation and inoculum formulation

The development of bio-inoculants depends on three crucial components: (1) effectiveness of the strain, (2) feasibility of the production of high levels of quality propagules, (3) and efficient delivery systems that provide a competitive advantage (Harman, 1991). Once an effective bio-inoculant has been identified, the method of mass production, formulation and application should be taken into consideration to stabilize the product during storage and to facilitate its delivery to the plant. Both solid and liquid formulations are used to produce suitable quantities of active and viable inocula of *Trichoderma* mainly as spores (conidia, Figure 2B), which are more tolerant to adverse environmental conditions during product formulation and field use, in contrast to their mycelial and chlamydospore forms (Amsellem et al., 1999; Howell, 2003; Jin et al., 1991; Papavizas, 1985). Nevertheless, the presence of a mycelial mass is also a key component for the production of relevant metabolites (Benhamou and Chet, 1993). Other ideal properties of biomass of a bio-inoculant summarized by Harman and coworkers (1991) include the following: (1) cost-effective production, (2) preservation against microbial contamination in dry powder form and low water availability, and (3) long shelf life. Engineers prefer submerged fermentation systems over alternative production systems, since they allow better control of nutrients, pH, temperature and other environmental factors thus reducing contamination, while improving biomass production and quality (Whipps, 1997). Consequently, liquid formulation is the preferred approach for biomass production in Europe and North America (Churchill, 1982). Inexpensive growth media such as molasses and yeast extracts are used for liquid formulations (Papavizas et al., 1984). In

general terms liquid formulations have limited shelf life and require storage and transportation at low temperatures to sustain viability of the microorganism, resulting in higher costs, unless subjected to a drying process. An alternative for liquid formulations that is gaining popularity among growers is the use of very simple tanks *in situ* to apply it immediately after production. However, this production system is very basic and does not easily permit adequate quality control of the inoculum.

An alternative system for production of inoculum is solid fermentation. In this case, agricultural waste materials such as rice and wheat straw, sugarcane bagasse, ground corncobs, sawdust, rice and wheat bran are used as either food base or substrate alone or in combination to grow *Trichoderma*. When using solid fermentation to produce the inoculum, the food base in the formulation should favor the bioinoculant (Papavizas et al., 1984) or even better, a food base that can only be utilized by desired microorganisms is strongly recommended (Nelson and Powelson, 1988). This method of formulation requires minimal cost and small scale production but it is bulky and requires large space for production, inoculation and storage including drying and milling. Furthermore, from an engineering point of view, control of pH and nutrient availability in solid fermentation systems is more complex, impacting quality control of the product. In both solid and liquid fermentation systems, it is required to dry the product to achieve higher stability and longer shelf life (Jin et al., 1992). Drying is essential in order to prevent spoilage by microbial contamination but results in losses of viability during the process, particularly when carried out at elevated temperatures (Jin and Custis, 2011). A dry product has the advantage of convenient storage and transport. Spray drying is perhaps the method of choice among the different drying techniques for large scale production due to its low cost (Morgan et al., 2006).

Microencapsulation of conidia with sugars, such as sucrose, trehalose, molasses or glycerol increases the survival of *Trichoderma* (Jin and Custis, 2011). Microencapsulation is defined as a process in which very small particles or droplets are surrounded by a protective coat, or embedded in a homogeneous or heterogeneous matrix, to produce small capsules (Gharsallaoui et al., 2007). This process results in prolonged shelf life and controls microbial release, thus enhancing its application efficiency (John et al., 2011; Rathore et al., 2013). Microencapsulation provides living cells with a physical barrier that protects them from

adverse environmental conditions (O'Riordan et al., 2001; Ross et al., 2005). For instance, the use of a maltodextrin-gum biopolymer matrix for microencapsulation of *T. harzianum* resulted in high conidia survival after spray-drying, 11-fold higher than non-encapsulated conidia (Muñoz-Celaya et al., 2012).

Some additives including protectants and carriers have been used to increase fungal survival under adverse environmental conditions, including fine clay, peat, vermiculite, alginate, wheat, bran, talc, diatomaceous earth, corn and sugarcane bagasse and pasteurized soil (Martínez-Medina et al., 2009; Doni et al., 2014). A study used bentonite–vermiculite as a solid substrate for both *T. harzianum* proliferation and as a carrier based on the consideration that both are harmless to the environment, low cost, and easily available. It investigated the survival and effectiveness of *T. harzianum* bentonite–vermiculite formulation and its growth promotion on melon plants under nursery conditions compared with the incorporation of this agent as conidia suspension (Martínez-Medina et al., 2009). The effectiveness of the application of *T. harzianum* to plants was related directly to its formulation, since the level of *T. harzianum* colony-forming units lasted after 8 weeks, whereas the supply of liquid suspension reduced it by two orders of magnitude. Plants treated with the bentonite–vermiculite formulation showed a higher shoot weight than untreated plants. The effectiveness of other carriers of *Trichoderma* sp. SL2, including ground corn or sugarcane bagasse on rice seedling growth was also tested (Doni et al., 2014). The use of potato dextrose agar as carrier was not practical for field application due to its short shelf life and high cost. When a *Trichoderma* sp. SL2 suspension mixed with ground corn was used as a treatment supplied to soil, it significantly enhanced rice root length, fresh weight and total biomass as compared to the mixture with sugarcane bagasse or the control, indicating its potential application as carrier for *Trichoderma* spp. inoculants. In another study, greater dry weight of radish shoots was evidenced when *Trichoderma* was applied to compost as a peat–bran inoculum in comparison to its supply as suspension of conidia (Baker et al., 1984). Similar observations were made by Kleifeld and Chet (1992) who found that a peat–bran preparation of *T. harzianum* T-203 was more effective in promoting plant growth in pepper and other plants than conidial suspensions or seed coatings. Importantly, in the peat–bran inoculated plants *Trichoderma* proliferated on roots for over 25 days, suggesting that the peat–bran could be used as a food reservoir for *Trichoderma*. Nevertheless, Bell et al. (2000) reported a contrasting effect on the presence

nutrients in a pellet formulation of *T. harzianum*, which inhibited growth of cucumber seedlings, whereas a seed coat formulation stimulated growth, leading the authors to suggest that the food source included in the pellets could have supported growth of inhibitory microbes in the soil.

Timing of delivery and application is crucial since it can be delivered already at planting and should limit growth of competitive microorganisms and provide conductive growth for the biocontrol agent. Regarding the use of *Trichoderma* as biostimulant, it is important to apply the inoculum at an early stage of the plant growth cycle to maximize its benefits in terms of root development and nutrient uptake. Increases in plant growth following *Trichoderma* treatment depend on the specific crop or plant genotype. For example, in a series of repeated trials, six strains of *Trichoderma* consistently promoted growth of lettuce (Ousley et al., 1994). However, when Baker (1988) tested the same isolate on pea and radish, he found only small increases in growth. In a more recent study, Tucci et al. (2011) showed that genetic variability among wild and cultivated tomato varieties affects the outcome of the interaction with two strains of *T. atroviride* and *T. harzianum*. The expected beneficial response, which included enhanced growth, was obtained for some, but not all tested varieties. Furthermore, at least in one case, treatment with *Trichoderma* had no effect or resulted even detrimental on crop growth. A simple explanation for the lack of beneficial effects is that the isolates differ in their capacity to colonize certain plant roots. However, it can not be discarded that the promotion or inhibition effects exhibited by the same strain in different interactions may be due to differential production of secondary metabolites, some of which may be detrimental to certain plant genotypes, and that may be the result of the *Trichoderma*-plant metabolic crosstalk.

Successful results do not only rely on the use of an effective bioinoculant but also on the method of delivery or application on the seed, root and soil. In this sense, either seed treatment or coating, are highly recommended and effective methods of application of *Trichoderma* for agricultural purposes (Mathre et al., 1999). It is preferable to apply in advance *Trichoderma* into the soil to build up a *Trichoderma* population, which may provide synergic effects with other agronomical practices such as organic fertilization. In contrast, soil fumigation or fungicide application may negatively affect native or incorporated *Trichoderma* populations.

4. The effects of *Trichoderma* on crop physiology

4.1. Plant growth

Trichoderma improves plant growth and development in axenic systems, in greenhouses, or in field (Table 1). Several reports have shown the beneficial effects of *Trichoderma* spp. on horticultural crops such as cucumber, periwinkle, chrysanthemum and lettuce on seed germination, vegetative growth and flowering (Chang et al., 1986; Hermosa et al., 2012; Studholme et al., 2013). Cucumber seedlings grown in soil amended with *T. harzianum* propagules sustained a 30% increase in seedling emergence 8 days after sowing. Three weeks later, these plants exhibited a 75% and 95% increase in root length and total root area, respectively, and substantial increases in dry weight, shoot length and leaf area (Yedidia et al., 2001). This early report showed correlation between increased root growth and shoot biomass production, which was further observed in crops such as maize, bean and tomato (Björkman et al., 1998; Björkman, 2004; Harman, 2011; Vargas et al., 2009, Azarmi et al., 2011; Pereira et al., 2014). *Trichoderma* isolates including *T. harzianum* isolate T-969, *T. harzianum* isolate T-447 and *Trichoderma* sp. isolate T were supplied either directly to tomato seeds or to nursery soil. Seed germination rate was not affected by *Trichoderma* application, whereas shoot height, shoot diameter, shoot fresh and dry weights were increased by *Trichoderma* sp. T and *T. harzianum* T-969. Plants grown on soil amended with *Trichoderma* sp. T and *T. harzianum* T-969 had also a marked increase 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. Inoculation of *T. harzianum* conidia into the liquid medium of hydroponically grown tomato plants caused profuse adhesion of hyphae to the roots as well as colonization of the root epidermis and cortex. Confocal microscopy of a *T. harzianum* strain 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). *T. parareesei* and *T. reesei,* which produce cellulases and xylanases of industrial interest also increased lateral root development and acclimatized tomato plants to grow under salt stress conditions (Rubio et al., 2014).

Propagation *in vitro* of cuttings is important for many horticultural crops. Increased vigor of rooted cuttings results in optimized propagation with reduced fungicide inputs, improved quality and faster stock turnout. Adams et al. (2007) compared the effects of *T. harzianum* Rifai 1295-22, also known as „T-22" and commercial formulations of ectomycorrhizal fungi in the establishment and growth of crack willow (*Salix fragilis* L.). Tree saplings grown with *T. harzianum* T-22 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* T-22 saplings produced more than double the dry biomass of controls and more than 50% extra biomass than the ectomycorrhiza treated saplings. In another work, the potential of 62 *Trichoderma* isolates to enhance root development on cuttings was evaluated in a screening bioassay using the ornamental plant *Impatiens walleriana* (Clouston et al., 2010). Six individual *Trichoderma* isolates and a commercial mixture of isolates were identified, which improved root length, root dry weight, shoot dry weight, and root/shoot ratio when compared to untreated cuttings. Sofo et al. (2010) tested the effects of *Trichoderma* T-22 on shoot growth and rooting of GiSeLa6 (*Prunus cerasus* x *P. canescens*) and of GF677 (*P. amygdalus* x *P. persica*), cultured *in vitro*. The results showed that early inoculation of the fungus at the stage of shoot transfer to root-inducing medium damaged both GiSeLa6 and GF677 plants, whereas, following later inoculation (7 d after shoot transfer to root-inducing medium), the plants survived and showed significant increases in shoot growth and root development. In particular, root lengths in GiSeLa6 and GF677 plants increased by 180% and 136%, respectively, compared to non-inoculated controls. These morphological characteristics could increase the quality and viability of nursery planting material and provide advantages during the plant acclimatization phase. Thus, selected strains of *Trichoderma* may be used to develop plant growth promotion products that hasten the rooting process and improve cutting establishment for nursery grown species.

Table I. Biostimulant effects of *Trichoderma* applications on agronomical and physiological aspects of horticultural crops, and *Arabidopsis*.

Crop	*Trichoderma* species/strain	Application mode	Experimental conditions	Effects	Reference
Arabidopsis	*Trichoderma virens* and *Trichoderma atroviride.*	Seedling were grown in Petri dishes and inoculated with $1x10^6$ conidia.	In vitro	Increased lateral root formation and biomass production.	Contreras-Cornejo et al., 2009.
Arabidopsis	*Trichoderma hamatum*	*Trichoderma* bran inoculum added to soil	Microcosms	Promoted root and shoot	Studholme et al., 2013

	strain GD12.	before sowing.		growth.	
Arabidopsis	*Trichoderma asperelloides* T203.	Root system was inoculated with a solution containing $1x10^5$ germinated spores[a]/ml or *Trichoderma* added to the soil at a concentration of 10^6 spores[a]/g.	Hydroponics	Improved seed germination.	Brotman et al., 2013
Arabidopsis	*Trichoderma virens* Tv29.8 and *Trichoderma atroviride* IMI 206040.	Seedling were grown in Petri dishes and inoculated with $1x10^6$ conidia.	In vitro	Improved plant growth under saline conditions, induced lateral root and root hair formation.	Contreras-Cornejo et al., 2014b
Arabidopsis	*Trichoderma virens* and *Trichoderma virens Δppt1*.	Seedling were grown in divided Petri dishes and exposed to fungal volatiles emitted from a colony developed from 1×10^6 conidia.	In vitro	Increased lateral root formation and growth.	Contreras-Cornejo et al., 2014c
Bean	*Trichoderma harzianum* (ALL 42).	Seeds were immersed in a spore suspension containing $2.4x10^8$ conidia/ml.	In vitro	Increased overall plant size and the number of lateral roots	Pereira et al., 2014
Cherries	*Trichoderma harzianum* T-22.	Plants were inoculated with approximately 50,000 conidia.	In vitro	Increased shoot growth and root development.	Sofo et al., 2010.
Chickpea	*Trichoderma harzianum*, *Trichoderma viride* and *Trichoderma virens.*	The growth medium or seeds were inoculated with a talc-based formulation of ground mycelium.	In vitro, glasshouse and field conditions	Increased shoot branching, biomass, grain yield and P and N uptake.	Rudresh et al., 2005
Chickpea	*Trichoderma* sp.	Agar plates were inoculated with a fungal mycelial disc of 5 mm diameter.	In vitro	Increased solubilization of inorganic phosphate.	Rawat and Tewari, 2011.
Chrysanthemum	*Trichoderma harzianum*.	$5x10^6$ conidia per g of soil or sprayed on roots at a concentration of $1x10^8$ conidia/ml.	Greenhouse	Promoted seed germination, vegetative growth and flowering.	Chang et al., 1986.
Cucumber	*Trichoderma harzianum*.	$5x10^6$ conidia per g of soil or sprayed on roots at a concentration of $1x10^8$ conidia/ml.	Greenhouse	Promoted seed germination, vegetative growth and flowering.	Chang et al., 1986.
Cucumber	*Trichoderma harzianum* T-203.	Mycelial inoculum added directly to the nutrient solution and applied to the soil.	Hydroponics and greenhouse	Increased root length, total root area, shoot length and leaf area.	Yedidia et al., 2001.
Cucumber	*Trichoderma harzianum.*	Plants grown in Petri dishes were inoculated	In vitro, hydroponics	Increased the length of root	Samolski et al., 2012.

		with $1x10^6$ spores. Seeds were coated with a spore suspension containing $2x10^8$ spores[a]/ml.	greenhouse	hairs and lateral roots.	
Cucumber	*Trichoderma asperelloides* T203.	Root system was inoculated with a solution containing $1x10^5$ germinated spores[a]/ml or *Trichoderma* added to the soil at a concentration of 10^6 spores[a]/g.	Hydroponics	Improved seed germination.	Brotman et al., 2013
Lettuce	*Trichoderma hamatum* strain GD12.	*Trichoderma* bran inoculum added to soil before sowing.	Microcosms	Promoted root and shoot growth.	Studholme et al., 2013
Lettuce	*Trichoderma atroviride* MUCL 45632.	The substrate was supplied with prepared tablets containing $4.5x10^5$ conidia.	In vitro, greenhouse, and field conditions	Enhanced shoot and root dry weight, and chlorophyll content.	Colla et al., 2015a
Maize	*Trichoderma harzianum* 1295-22.	Seeds were treated with a suspension of $5x10^6$ spores[a].	Greenhouse	Increased root and shoot growth.	Björkman et al., 1998.
Maize	*Trichoderma harzianum* T-22.	Seeds were treated with a commercial formulation, containing $1x10^9$ CFU (primarily conidia).	Greenhouse	Induced root and shoot growth, increased plant biomass.	Harman et al., 2004
Melon	*Trichoderma harzianum.*	Solid bentonite–vermiculite formulation containing $1x10^9$ conidia/g.	Nursery conditions	Increased plant growth and shoot biomass.	Martínez-Medina et al., 2009.
Melon	*Trichoderma atroviride* MUCL 45632.	The substrate was supplied with prepared tablets containing $4.5x10^5$ conidia.	In vitro, greenhouse, and field conditions	Enhanced shoot and root dry weight, and chlorophyll content.	Colla et al., 2015a
Pepper	*Trichoderma harzianum*.	$5x10^6$ conidia per g of soil or sprayed on roots at a concentration of $1x10^8$ conidia/ml.	Greenhouse	Promoted seed germination, vegetative growth and flowering.	Chang et al., 1986.
Pepper	*Trichoderma atroviride* MUCL 45632.	The substrate was supplied with prepared tablets containing $4.5x10^5$ conidia.	In vitro, greenhouse, and field conditions	Enhanced shoot and root dry weight, and chlorophyll content.	Colla et al., 2015a
Periwinkle	*Trichoderma harzianum*.	$5x10^6$ conidia per g of soil or sprayed on roots at a concentration of $1x10^8$ conidia/ml.	Greenhouse	Promoted seed germination, vegetative growth and flowering.	Chang et al., 1986.
Rice	*Trichoderma harzianum.*	Roots were treated with a mixed *Trichoderma* formulation in talcum powder and carboxy	Glasshouse	Promoted root growth.	Shukla et al., 2012

		methyl cellulose 10g/l.			
Tomato	*Trichoderma harzianum Rifai strain T-22.*	Seeds were treated with a conidial suspension of $2x10^7$ CFU per g of seed.	In vitro	Promoted seed germination and growth.	Mastouri et al., 2010.
Tomato	*Trichoderma harzianum* and *Trichoderma* sp.	Seeds were immersed in 1 ml of suspension containing 10^6-10^7 spores[a] per ml or the spores were added to the nursery soil.	Greenhouse	Promoted shoot and root growth, and increased leaf number, leaf area and chlorophyll content.	Azarmi et al., 2011.
Tomato	*Trichoderma harzianum.*	Plants grown in Petri dishes were inoculated with $1x10^6$ spores. Seeds were coated with a spore suspension containing $2x10^8$ spores[a]/ml.	In vitro, hydroponics greenhouse	Increased the length of root hairs and lateral roots.	Samolski et al., 2012.
Tomato	*Trichoderma parareesei.*	Plants were grown in Petri dishes and inoculated with $1x10^6$ conidia.	In vitro	Increased lateral root development and promoted growth.	Rubio et al., 2014
Tomato	*Trichoderma atroviride* MUCL 45632.	The substrate was supplied with prepared tablets containing $4.5x10^5$ conidia.	In vitro, greenhouse, and field conditions	Enhanced shoot and root dry weight, and chlorophyll content.	Colla et al., 2015a
Zucchini	*Trichoderma atroviride* MUCL 45632.	The substrate was supplied with prepared tablets containing $4.5x10^5$ conidia.	In vitro, greenhouse, and field conditions	Enhanced shoot and root dry weight, and chlorophyll content.	Colla et al., 2015a

[a]Authors do not specify the type of spores used (presumably conidia).

4.2. Plant nutrition

Intensive agriculture demands an adequate supply of nutrients, which act as structural components of cells or play roles in photosynthesis and metabolism. At the rhizosphere, both macronutrients and micronutrients undergo a complex dynamic equilibrium of solubilization, uptake and transport that is greatly influenced by root associated microorganisms. In particular, N and P can directly act as signals that alter post-embryonic root development, modifying primary and lateral root growth and root hair formation and may be involved in the success of plant-microbe associations (Giehl and von Wirén, 2014; Ruiz-Herrera et al., 2015).

Accumulating information has shown the efficacy of *Trichoderma* spp. as biofertilizers, since their application to soil, seeds or plant surfaces increases the solubility of nutrients as well as the nutrient uptake capacity of the root and/or their distribution within plant parts. These beneficial properties are explained via modulation of root architecture or through the exudation of substances that increase nutrient availability such as siderophores and organic acids (Samolski et al., 2012; Zhao et al., 2014). The importance of root architectural alterations induced by *Trichoderma* in plant nutrition was evident following the early work by Björkman et al. (1998). This report showed the correlation between increased root growth and shoot biomass production in maize, which was investigated in detail later in *Arabidopsis* and cucumber plants (Contreras-Cornejo et al., 2009; Samolski et al., 2012). *T. virens* and *T. atroviride* promoted *Arabidopsis* seedling growth increasing root and shoot biomass production via prolific formation of lateral roots (Contreras-Cornejo et al., 2009; Figure 2C and D). Inoculation of cucumber roots with a *T. harzianum* strain that overexpresses the qid74 gene, which encodes a cysteine-rich cell-wall protein, increases root hair initiation and growth, whereas fewer and shorter root hairs developed in roots inoculated with a strain defective on qid74. Modifications in root architecture in cucumber plants induced by WT or qid74 overexpressing strains increased plant biomass through an efficient use of N, P, K and micronutrients (Samolski et al., 2012).

Data showing the increase of nutrient content in horticultural and crop species as a result of *Trichoderma* inoculation confirm the biostimulation properties of these fungi. *T. harzianum* 1295-22 increased the availability of P and several micronutrients such as Fe, Mn and Zn in liquid sucrose–yeast extract medium *in vitro* (Altomare et al., 1999). *T. harzianum* strain T-203 (later on identified as *Trichoderma asperellum* and recently as *Trichoderma asperelloides*) increased 90% and 30% the P and Fe concentration in roots, respectively. 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 (Yedidia et al., 2001).

Phosphate solubilization is a major beneficial trait of *Trichoderma* regarding the depletion of phosphate mines and the concomitant increase in P fertilizer prices. Rudresh et al. (2005a) reported the ability of nine isolates of *Trichoderma* spp. to solubilize calcium phosphate as

compared with an efficient phosphate-solubilizing bacterium *Bacillus megaterium* subsp. *phosphaticum* PB that was used as the reference strain. *T. viride* (TV 97), *T. virens* (PDBCTVs 12) and *T. virens* (PDBCTVs 13) released 70% more P than 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 T-969, increased the concentrations of Ca, Mg, 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). An interesting possibility, still to be investigated, is that *Trichoderma* or its metabolites could up-regulate the expression of nutrient transporters in plants roots, thus improving nutrient uptake efficiency.

Iron (Fe) deficiency is an agriculturally important problem to cereals in alkaline soils due to the insolubility of Fe forms. To investigate the potential of *Trichoderma* to solubilize Fe in the rhizosphere, a high siderophore-producing strain of *T. asperellum* (T-6) was isolated from cucumber roots. Applying strain T-6 to sterilized soil could increase soil levels of Fe^{2+} and siderophores, as well as increase Fe^{2+} and Fe^{3+}-chelate reductase activity in cucumber roots (Zhao et al., 2014). Extracellular Fe^{3+} reducing activity and organic acid release were evidenced upon analysis of the culture filtrate of T-6. Moreover, Colla et al. (2015a) demonstrated that *T. atroviride* MUCL45632 was able to produce two types of siderophores: hydroxamate and catechol compounds. These results indicate that *Trichoderma* influences Fe nutrition of crops. Exudation of organic acids and release of Fe^{3+} reducing enzymes may contribute in the solubilization and reduction of insoluble Fe^{3+} to the more soluble Fe^{2+} boosting the root Fe uptake capacity.

The improvements of *Trichoderma*-based biostimulants may help farmers reduce the use of chemical fertilizers and 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 N, P, and K macronutrients, minimizing the use of fertilizer and their potential negative effects in the environment.

4.3. Abiotic stress tolerance

Heat, drought, cold, and salinity are the major abiotic stresses that induce severe cellular damage in crop plants. Fungal species are commonly found in association with plant communities resisting naturally to different types of stress (Marasco et al., 2012). *Trichoderma* colonized plants results in increased endogenous level of auxins, ethylene and gibberellins, plant enzymes, antioxidants and compatible solutes and compounds like phytoalexins and phenols that provide tolerance to environmental stress. Since ethylene is considered a plant growth regulating compound that inhibits root development and plant growth (Lewis et al., 2011), its production by fungi or in plants in response to fungal root colonization may be important to fine-tune induced biotic or abiotic stress responses.

4.3.1. Salt tolerance

The beneficial effects of *Trichoderma* species on alleviating the adverse effects of salt stress have been recently documented in *Arabidopsis* and crop plants (Mastouri et al., 2010, 2012; Rawat et al., 2013, Contreras-Cornejo et al., 2014b; Hashem et al., 2014). Mastouri et al. (2010) reported that *T. harzianum* T-22 treated seeds germinated faster and more uniformly than untreated seeds whether the stress applied was osmotic, salt, or suboptimal temperatures. Treatments with the antioxidant glutathione, or application of T-22, resulted in a similar protective effect. These findings support the notion that *T. harzianum* strain T-22 increases seedling vigor during osmotic stress by inducing physiological responses of plants against oxidative damage. Application of *T. hamatum* to *Ochradenus baccatus* plants grown under standard conditions stimulated plant growth. Salt treatments that increase salt levels in plant tissues decreased growth, pigment content, membrane stability, water content, and total lipid content (Hashem et al., 2014). These effects were explained because salt stress causes lipid peroxidation and thus adaptive responses mediate plant survival via biosynthesis of phenols, diacylglycerol, sterol esters, nonesterified fatty acids, and activation of antioxidant and ROS

detoxifying enzymes. *T. hamatum* alleviated the antagonistic effect of salinity on plant growth and metabolic processes.

Arabidopsis and cucumber plants treated with *Trichoderma asperelloides* prior to salt stress imposition showed significantly improved seed germination (Brotman et al., 2013). An evaluation of *Arabidopsis* roots inoculated with T-203 by microarray analysis coupled with qPCR studies revealed wide gene transcript reprogramming, involving genes related to osmo-protection and response to oxidative stress. The monodehydroascorbate reductase (MDAR), and superoxide dismutase (SOD) genes were up-regulated in *Arabidopsis* and cucumber, respectively, while an increase in the pool of ascorbic acid in plant hosts contributed to the osmoprotectant properties of *Trichoderma*.

Auxin signaling is a major target of salinity stress in plants (Zörb et al., 2013). Auxin production by *T. virens* (Tv29.8) and *T. atroviride* (IMI 206040) promoted plant growth in both normal and saline conditions, which was related to altered root architecture and biochemical changes. *Arabidopsis* seedlings grown under saline conditions inoculated with *Trichoderma* spp. showed increased levels of abscisic acid, L-proline, and ascorbic acid, and enhanced elimination of Na^+ through root exudates (Contreras-Cornejo et al., 2014b). These data show the critical role of auxin signaling and root architecture to salt tolerance in *Arabidopsis* and suggest that these fungi may enhance the plant IAA level as well as the antioxidant and osmoprotective status of plants under salt stress.

4.3.2. Drought tolerance

Trichoderma spp. may enhance drought tolerance to plants via improved root development (Contreras-Cornejo et al., 2009; Shukla et al., 2012), activating antioxidant protection against damage by dehydration (Mastouri et al., 2012; Brotman et al., 2013), and/or delaying drought induced changes in stomatal opening, photosynthesis and chlorophyll leaf content. The tolerance to water deficit of tomato seedlings was attributed to activation of antioxidant responses and higher activity of ascorbate and glutathione-recycling enzymes (Mastouri et al., 2012). Bae et al. (2009) reported that the total amino acid (histidine, arginine, proline, c-aminobutyric acid, valine, and leucine) content increased in response to drought in untreated cacao plants, but not in *T. hamatum* DIS 219b-colonized seedlings, suggesting that amino acid

accumulation can be a response to drought. Thus, *Trichoderma* diminished the negative effects of stress on plant metabolism. Shukla et al. (2012) showed that the primary direct effect of *Trichoderma* colonization in rice was promotion of root growth, regardless of water status, which caused delay in the drought responses of rice plants. Five *Trichoderma* isolates that were resistant to drought were identified, which delayed drought induced changes like stomatal conductance, net photosynthesis and leaf greenness. Drought conditions varying from 3 to 9 days of withholding water led to an increase in the concentration of many stress induced metabolites in rice leaves, while *Trichoderma* colonization caused a decrease in proline, MDA and H_2O_2 contents, and increased phenolic compound concentration (Shukla et al., 2012). Contreras-Cornejo et al. (2015a) investigated the potential of *T. virens* and *T. atroviride* in modulating stomatal aperture and plant transpiration. *Arabidopsis* wild type (WT) seedlings and ABA-insensitive mutants *abi1-1* and *abi2-1* were co-cultivated with these fungi under well watered conditions and stomatal aperture and water loss determined in leaves. *Arabidopsis* WT seedlings inoculated with *Trichoderma* showed both decreased stomatal aperture and reduced water loss when compared to uninoculated seedlings. This effect was absent in *abi1-1* and *abi2-1* mutants. *T. virens* and *T. atroviride* induced the abscisic acid inducible marker *abi4:uidA* and produced ABA, which might be involved together with auxins, in stress tolerance mechanisms. All together, these reports show a novel facet of *Trichoderma* in boosting plant health under drought stress. Moreover, fungal metabolites can be useful toward regulating stomatic aperture and water use efficiency of plants.

4.4. Molecular mechanisms of *Trichoderma* phytostimulation

Arabidopsis thaliana has been established as an excellent model system to study the genetic and physiological mechanisms by which *Trichoderma* promotes plant growth. This has been possible because of the vast knowledge gained from physiological processes and genetic pathways on this small weed, and the ability to test the *Trichoderma*-plant interaction under axenic conditions (Contreras-Cornejo et al., 2009, 2011). With the advent of genomics revolution, other crops such as maize (Shoresh and Harman, 2008; Vargas et al., 2009, 2011, Harman, 2011), tomato (Rubio et al., 2012) and bean (Pereira et al., 2014) have been used to advance the existing knowledge about the genes and proteins mediating the phytostimulation process.

Contreras-Cornejo and coworkers (2009) investigated in detail the role of auxin signaling in *Trichoderma*–plant interactions in *Arabidopsis thaliana*. The authors found that mutations in genes involved in auxin transport or signaling, *AUX1, BIG, EIR1* and *AXR1*, reduced the beneficial effects of *Trichoderma* on biomass production and root branching. Colonization of roots induced the auxin response, which correlated with increased development of lateral roots. The application of 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 (mM range). For example, the application of indole-3-carboxaldehyde to *Arabidopsis* seedlings inhibited primary root growth while inducing adventitious root formation (Contreras-Cornejo et al*.,* 2011). The connection between fungal produced auxin and root development elicited by *Trichoderma* was found to depend on mitogen activated protein kinase signaling (Contreras-Cornejo et al., 2015b). Inoculation of *Arabidopsis* with *T. atroviride* regulates root growth and root hair formation modulating MPK6 activity, likely depending of ethylene (ET) and auxin signaling. It was found that ET, and indole-3-acetic acid (IAA) produced by the fungus induce MPK6 activity. Further analysis of auxin-related gene expression in WT seedlings and ethylene-related mutants showed that the effect of ET on root growth and differentiation apparently is mediated by auxin signaling, thus indicating that an auxin-ethylene crosstalk involving MPK6 fine-tunes seedling growth and development in response to a beneficial soil fungus. In consequence, MPK6, and its MAP kinase associated cascade, whose components remain to be identified, seems to be a regulation node to maintain and/or amplify the hormonal effects underlying plant development.

Root colonization with *T. harzianum* (T-22) induced changes in the proteome of shoots of maize seedlings. Using a proteomics strategy, proteins that are involved in carbohydrate metabolism, photosynthesis or development were identified likely mediating the maize response to *Trichoderma* inoculation. 16 up-regulated proteins were related to DNA metabolism and genetic information processing including transcription factors and nuclear proteins such as RNA polymerase I, II, and III, RNA-binding and nuclear proteins, a BTB/POZ domain-containing protein, splicing factor SC35, FCP1-like phosphatase, and a DNA repair-recombination protein, RAD50 (Shoresh and Harman, 2008). This suggests that the differences observed in plants biostimulated with *Trichoderma* require the involvement of

regulatory proteins. Down regulated proteins included a β-glucosidase protein, which putatively hydrolyzes cytokinin-conjugates and releases free cytokinins during plant growth and development and a small peptide (DVL protein) found to affect *Arabidopsis* development (Shoresh and Harman, 2008).

T. harzianum (ALL-42), isolated from Brazilian Cerrado soil promoted common bean growth, overall size, foliar area, root area, and the number of lateral roots in comparison to plants grown without the fungus. A proteomic analysis revealed changes in protein profiles with potential regulatory functions including a histone acetyl-transferase complex component, and NAC1 domain protein. The transcription factors classified as NAC family members have a crucial role in a wide range of plant developmental processes, such as lateral root development. Changes in protein metabolism were also observed in the root proteomics maps, up-regulated for common bean plants challenged with phytopathogenic fungi and *T. harzianum*. An increase in fructokinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ferritin, which play a crucial role in carbohydrate metabolism, as well as iron nutrition, explains some of the biofertilizer properties of this fungus. Ferritin was highly expressed in the proteomic maps of *Trichoderma*-treated plants, suggesting an additional role in increasing the ability of plants to mobilize and transport iron (Pereira et al., 2014).

5. Bioactive compounds from *Trichoderma*

5.1. Auxins

Auxin signaling plays a role in nearly every aspect of plant development from embryogenesis to reproduction. Signals from the developmental program, physiological status, and encounters with root microorganisms all converge on the auxin pathway (Ortiz-Castro et al., 2011; Kazan, 2013; Simon et al., 2013). The potential of plant-associated microorganisms to produce free IAA, auxin precursors or auxin signal mimics influence the endogenous auxin response of the host and are important modulators of root system architecture (Contreras-Cornejo et al., 2009; Felten et al., 2009; Hilbert et al., 2012).

T. virens and *T. atroviride* produce a variety of molecules with auxin activity as part of their metabolism including IAA, indole-3-ethanol, indole-3-acetaldehyde and indole-3-

carboxaldehyde (Contreras-Cornejo et al., 2009, 2011). Following the hypothesis that direct plant growth-promoting activity of *T. harzianum* strain T-22 could depend of changes in the levels of different classes of plant growth regulators, Sofo et al. (2011) analyzed phytohormone levels in cherry rootstocks (*Prunus cerasus* x *P. canescens*) inoculated with this fungal strain. Root and shoot growth increased by 76 and 61%, respectively in plants treated with T-22, which correlated with 49 and 40% higher levels of IAA in both leaves and roots. In tomato plants, the phytostimulant properties of *Trichoderma* spp. also depended on auxin accumulation (Chowdappa et al., 2013; Martínez-Medina et al., 2014). *T. harzianum* OTPB3 strain was isolated from rhizosphere of field grown beans (*Phaseolus vulgaris* L.) and significantly increased root and shoot growth, leaf area and seedling vigor index in tomato. The level of IAA increased 45% in roots of tomato seedlings inoculated with OTPB3 when compared to uninoculated controls (Chowdappa et al., 2013). In another work, four *Trichoderma* isolates were collected from agricultural soils and identified as *T. harzianum* (two isolates), *Trichoderma ghanense*, and *Trichoderma hamatum*. Their plant growth-promoting activity on melon plants was examined *in vivo*, and compared to that of T-22. Several growth-related phytohormones were analyzed in the shoots of plants whose roots were colonized by the different *Trichoderma* isolates. An increase in auxin and a decrease in cytokinins and abscisic acid content were particular signatures of the isolates that promoted plant growth (Martínez-Medina et al., 2014). Although these later reports did not explain whether increased IAA content observed in tomato and melon originated from production by the fungi or the plant, the natural isolates might be able to produce auxin as reported for *T. virens*, alternatively, fungal colonization may up-regulate plant genes for IAA biosynthesis or down-regulate the genes involved in the catabolism of this hormone.

5.2. Secondary metabolites

Secondary metabolites comprise an heterogeneous group of natural compounds produced by plants and fungi, which aid the producing organism to survive and reinforce basic functions, such as competition or symbiosis (Demain and Fang, 2000; Bérdy, 2005). Accumulating evidence has revealed the interesting bioactivities of secondary metabolites in plant growth and development (Ramírez-Chávez et al., 2004; Vinale et al., 2008). In ecosystems, plants communicate with neighbors through volatile organic compounds (VOCs), which influence

plant growth but less is known about the physiological effects of fungal VOCs on plants (Ortiz-Castro et al., 2009).

The production of secondary metabolites in *Trichoderma* spp. is strain-dependent and includes volatile and non-volatile substances, such as 6-n-pentyl-6H-pyran-2-one (6PP), gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Vinale et al. 2008; Reino et al., 2008). *T. atroviride* produces at least 25 different VOCs including alcohols, ketones, alkanes, furanes, pyrones, mono terpenes and sesquiterpenes (Stoppacher et al., 2010). The VOC profiles vary depending on environmental/biological growth conditions, since comparison to the control cultures revealed an inhibiting effect of cultivation of *T. atroviride* in the presence of the mycotoxin fusaric acid (FA) on the biosynthesis of 6PP, α-phellandrene, β-phellandrene, p-menth-2-en-7-ol and bergamotene, whereas the production of 1-octen-3-ol, 3-octanol and 3-octanone was increased in the presence of FA. Two recent reports tested the effects of *Trichoderma* VOCs on *Arabidopsis* growth and development. Hung et al. (2013) exposed plants to VOCs released by mature colonies of *T. viride* cultured on Petri plates by placing them into a growth chamber with shared atmosphere without direct physical contact. Plants grown in the presence of *T. viride* volatiles produced 45% more total biomass, had increased size, flowered earlier, and developed more lateral roots when compared to controls. Similarly, *T. virens* improved plant growth and root branching via emission of VOCs (Figure 2D). GC–MS analysis of *T. viride* VOCs revealed 51 compounds of which isobutyl alcohol, isopentyl alcohol, and 3-methylbutanal were most abundant. Contreras-Cornejo et al. (2014c) examined the effects of VOCs from *T. virens* and those produced by a mutant strain defective on the 4-phosphopantetheinyl transferase 1 (TvPPT1), in plant growth promotion using divided Petri plates (Figure 2C and D). *T. virens* produced a series of hydrocarbon terpenes, including the sesquiterpenes β-caryophyllene, (−)-β-elemene, germacrene D, τ-cadinene, δ-cadinene, α- amorphene, and τ-selinene and the monoterpenes β-myrcene, trans-β-ocimene, and cis-β-ocimene, which were absent in *Δppt1* mutant. WT and *Δppt1* fungal emissions did not affect primary root growth, but the formation of lateral roots and growth of these structures was clearly induced, indicating that such VOCs promote root branching. Other major secondary metabolites produced by *T. harzianum* and *T. atroviride* biocontrol strains have been investigated for their effect on plant growth promotion. An auxin-like activity was observed on etiolated pea stems treated with 6PP, which also promoted the growth of tomato

and canola seedlings. Tomato plants sprayed with 6PP had increased total biomass and root biomass that correlated with a highly branched root system (Vinale et al., 2008).

T. harzianum produces harzianic acid (HA) and isoharzianic acid (iso-HA), a stereoisomer of HA, which promote plant growth. In a recent work, iso-HA improved the germination, stem and root lengths and leaf area in tomato (Vinale et al., 2014). HPLC-DAD experiments showed that the production of HA and iso-HA was affected by the presence of plant tissue in the liquid medium. In particular, tomato tissue elicited the production of HA but negatively modulated the biosynthesis of its analogue iso-HA, suggesting that different forms of the same *Trichoderma* secondary metabolite have specific roles in the molecular mechanism regulating the *Trichoderma*- plant interaction (Vinale et al., 2014). This report indicates that HA and the new secondary metabolite iso-HA are active in plants and can be useful for agricultural applications. The identification of molecules with plant growth promoting properties can support the development of new biostimulants based on fungal compounds as active ingredients, which represent an attractive alternative to products containing only living microorganisms.

Trichoderma elicitors can be used to stimulate the production of secondary metabolites in plants (Ming et al., 2013). *Trichoderma atroviride* D16 is an endophytic fungus isolated from the root of *Salvia miltiorrhiza*, which produces various diterpene quinones termed tanshinones, which have been widely used for the treatment of cardiovascular diseases and menstrual disorders, as well as for the prevention of inflammation. The extract of mycelium (EM) and the polysaccharide fraction (PSF) isolated from *T. atroviride* D16 promoted hairy root growth and stimulated the biosynthesis of tanshinones in hairy roots. Although the active constituent responsible for these biological activities of the extracts is still unknown, these results suggest that metabolites from *T. atroviride* could elicit chemical responses in the host plant likely by influencing the expression of genes involved in the secondary metabolite biosynthesis pathway.

6. Conclusions

The use of plant beneficial fungi in agriculture has greatly increased during the past decade mostly due to their multi-level properties, and their expected success as biofertilizers (Harman,

2011; Ansari et al., 2013). Beneficial fungi with broad-host range undergo long-term interactions with a large variety of plants, thereby playing a significant role in natural and managed ecosystems and in the adaptation of crops to global climate changes (Ansari et al., 2013; Ellouze et al., 2014). *Trichoderma* spp. are promising fungi towards the development of sustainable agriculture. As such, there is an ongoing necessity to accelerate their integration in agriculture production systems, the development of successful inoculation systems and modes of delivery. The constant increase in food demand throughout the world, the rise of biofuels production and the imminent depletion of phosphate stock provisioning strengthen the necessity to seriously support research on *Trichoderma* and translate their beneficial biostimulant potential to field-grown crops.

Nutrients are taken up at the root epidermis by root hairs, a process mediated by specific transport proteins. It is noteworthy that root tips are able of sensing the local and internal concentrations of nutrients to affect root hair growth and root system architecture (Ruiz-Herrera et al. 2015). Consequently, it would be expected that the root capacity to acquire macro and micronutrients boost in the presence of microbes via increased nutrient solubilization and/or transport. The recent identification of transporter proteins such as the NRT1.1 nitrate transporter, which plays a key role in mediating the adaptations of roots to fluctuating levels of NO_3^- availability, but also facilitates uptake of auxin (Krouk et al., 2010) opens an interesting novel avenue for research, as the transporters may not only promote localized root growth facilitating NO_3^- influx into cells, but also modulating auxin accumulation in roots. Intriguingly, silencing of another nutrient transporter, the high-affinity ammonium transporter PiAMT1 from barley, whose transcripts accumulate during N starvation, resulted in enhanced colonization of this host by the beneficial root endophyte *Piriformospora indica* (Lahrmann et al., 2013). Determining whether *Trichoderma* metabolites including auxins and VOCs may target any nutrient transporters such as NRT1.1 or AMT1 may provide the desired combined advantages towards improving both root development and nutrient acquisition.

Biostimulated plants resulting from root colonization by *Trichoderma* spp. better resist environmental stresses such as drought and salt, and have an improved capacity to survive certain bacterial and fungal pathogen attacks. Intracellular signaling processes that translate

fungal perception into genetic, metabolic and developmental responses in roots are the focus of current plant-microbe interactions research. Important signaling pathways involved in recognition of microbial elicitors, such as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), as well as auxin and other growth regulating substances, include mitogen-activated protein kinase (MAPK) cascades (Xu and Zhang, 2015). The recent findings that *Arabidopsis thaliana* MPK6 play a critical role in *Trichoderma* plant responses (Contreras-Cornejo et al., 2015b) open new interesting research questions, as this MAP kinase protein has been found to control plant defense responses, root development and P acquisition (Meng et al., 2013; López-Bucio et al., 2014; Lei et al., 2014). A very interesting possibility is that *Trichoderma* could enhance P uptake via MPK6 signaling, but it still remains to be determined whether this signaling module operates in crops and the signaling cascade acting upstream of MPK6 awaits further elucidation.

Acknowledgments

Research conducted by the groups of José López-Bucio and Alfredo Herrera-Estrella related to the subjects of this manuscript is currently supported in part by a grant from SEP-CONACYT (236825). We gratefully acknowledge Jesús Salvador López-Bucio for critical review of this manuscript.

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CAPÍTULO 2
Factores de competencia en plantas

Factores de competencia en plantas

Edith Muñoz-Parra, Ramón Pelagio-Flores, José López-Bucio y Elda Beltrán-Peña

En las comunidades vegetales, las plantas se encuentran expuestas a diversas condiciones ambientales a las cuales deben responder eficientemente para asegurar su supervivencia, ajustando su crecimiento a través de la síntesis y utilización de una gran variedad de compuestos. El crecimiento y desarrollo vegetal dependen de la integración de diferentes señales endógenas como son los reguladores del crecimiento vegetal o fitohormonas y señales ambientales como la disponibilidad de nutrientes y agua, la temperatura, y organismos vecinos. Cada uno de estos estímulos activa una serie de respuestas a nivel genético, bioquímico y fisiológico que impactan el fenotipo y la arquitectura de la raíz y el follaje. En este capítulo se describen los principales estímulos bióticos y abióticos, así como las correlaciones que se establecen entre el estímulo ambiental y algunos reguladores del crecimiento para dar lugar a la integración fenotípica y adaptativa mediante cambios en el desarrollo.

2.1. Introducción

Las plantas son organismos sésiles que se encuentran expuestos a diversas condiciones ambientales que en muchos de los casos son desfavorables para su desarrollo, por lo cual deben responder eficientemente para asegurar su supervivencia, ajustando su crecimiento a través de la síntesis y utilización de una gran variedad de compuestos. En general, el crecimiento y desarrollo vegetal dependen de la integración de diferentes señales endógenas y ambientales. Cada una de estas señales puede activar una serie de respuestas que le permitan adaptarse e ir modulando diversos procesos del desarrollo que conlleven a una integración final de estas señales dando lugar a características fenotípicas específicas. La integración de tales respuestas en programas morfogenéticos depende de varios reguladores del crecimiento llamados colectivamente hormonas vegetales. Entre los reguladores más estudiados se encuentran ocho clases principales: las auxinas, las giberelinas, el ácido abcísico, las citocininas, el etileno, los brasinoesteroides, los jasmonatos y las estrigolactonas (Depuydt y Hardtke, 2011), todos ellos han sido descritos con funciones en la regulación del crecimiento de maneras distintas y algunas veces de manera específica dependiente de las condiciones a las que se encuentra sometida la planta (Santner *et al.,* 2009; Santner y Estelle, 2009; Wolters y Jurgens, 2009). Estos reguladores actúan en concentraciones bajas para regular el crecimiento y desarrollo (Gray, 2004).

En este capítulo abordaremos de forma general el conocimiento que se tiene de las respuestas vegetales bajo diversos estímulos tanto bióticos como abióticos, y en las correlaciones que se establecen entre el estímulo ambiental, los reguladores del crecimiento y el fenotipo.

2.2. Las plantas, hábitat e interacciones biológicas

Los organismos vegetales se encuentran bajo estímulos tanto bióticos como abióticos. Estos estímulos incluyen a las interacciones biológicas, entre las que se pueden mencionar las siguientes:

1. **Neutralismo**. Cuando dos especies interactúan pero ninguna se afecta.
2. **Simbiosis**. Relación obligada entre dos especies, que puede o no beneficiar a ambas; existen varios tipos:
   - Mutualismo. Relación en que dos especies se benefician, puede ser temporal o permanente.
   - Amensalismo. Asociación perjudicial para una de las especies u organismos y neutral para la otra.
   - Comensalismo. Asociación benéfica para una especie y neutral para la otra, también conocida como inquilinismo.
   - Parasitismo. Interacción en la cual una especie se beneficia y otra es perjudicada.
3. **Competencia**. Interacción entre dos organismos o especies, en que ambos comparten uno o varios factores medioambientales limitantes para su crecimiento.
4. **Depredación**. Interacción en la que un organismo se alimenta de otro.

De las interacciones mencionadas, ha resultado interesante el estudio de la competencia, ya que es un factor que influye de manera importante en la estructura de las comunidades y no siempre es un fenómeno simple y directo sino que puede verse influenciado de múltiples maneras (Novoplansky, 2011).

2.3.

La competencia puede definirse como la interacción entre organismos o especies, en la cual la aptitud o adecuación de uno, se ve modificado por la presencia de otro. Esto ocurre debido a que existe una limitación en la cantidad de un recurso usado por ambos organismos o especies, el cual puede ser luz, agua, alimento, territorio, pareja y/o compuestos específicos necesarios para la sobrevivencia. La competencia se puede clasificar en dos tipos principales:

1. Competencia interespecífica. Entre individuos de diferentes especies.
2. Competencia intraespecífica. Entre individuos de la misma especie.

Mientras la densidad (número de individuos por unidad de área) de una población sea mayor, también se incrementa la probabilidad de relaciones intraespecíficas y se puede acentuar ésta aún más, si el recurso por el que se compite es limitado; lo que puede llevar a un proceso selectivo en el que sobreviven los organismos mejor adaptados.

2.3.1. Competencia en plantas

Las plantas son los productores primarios de los ecosistemas, razón por la cual, estudiar las interacciones con otros organismos resulta muy importante. Los vegetales tienen la capacidad de modificar tanto su arquitectura, -lo que ha sido llamado plasticidad fenotípica-, como sus procesos del desarrollo en respuesta a señales bióticas o abióticas del medio en que se encuentren; esta característica se ha seleccionado posiblemente para una mejor adaptación al medio y hacer más eficiente la competencia por recursos esenciales.

2.4. Morfología vegetal

La germinación de las semillas da lugar a la formación de un organismo completo, en este proceso intervienen básicamente dos hormonas, el ácido abcísico promoviendo la dormancia y el ácido giberélico de manera antagónica promoviendo la germinación (Koornneef y Karssen, 1994). Los dos sistemas principales que conforman la arquitectura de una planta son el aéreo, formado por tallo, hojas, flores y frutos; y el radicular que generalmente consiste de una o varias raíces primarias, de cuyo interior emergen raíces laterales, y de cuya superficie proliferan pelos radiculares o absorbentes (**Fig. 2.1**). Particularmente, la planta *Arabidopsis thaliana* se ha utilizado como el modelo ideal para estudiar procesos fisiológicos (Scheres y Wolkenfelt, 1998). Avances importantes en el entendimiento del crecimiento y desarrollo se han logrado gracias a los estudios con esta angiosperma.

En el sistema aéreo lo primero en emerger durante la germinación es el hipocotilo y el o los cotiledones, posteriormente se da lugar a la formación de las hojas verdaderas. Este sistema se encuentra adaptado para la realización de la fotosíntesis, que le proporciona a la planta la energía necesaria, gracias a una serie de reacciones a partir del CO_2 que capta del ambiente, el agua que es absorbida en las raíces y la energía luminosa que incide sobre ella, estos serán convertidos en O_2, ATP, glucosa y otros carbohidratos que el organismo utilizará en sus

El sistema radicular se encuentra adaptado para la captación de agua y nutrientes del suelo o del ambiente en que esté la planta, así como también para permitir su fijación al sustrato y proporcionar sostén a la parte aérea (Ortiz-Castro, 2005). Las raíces secretan una

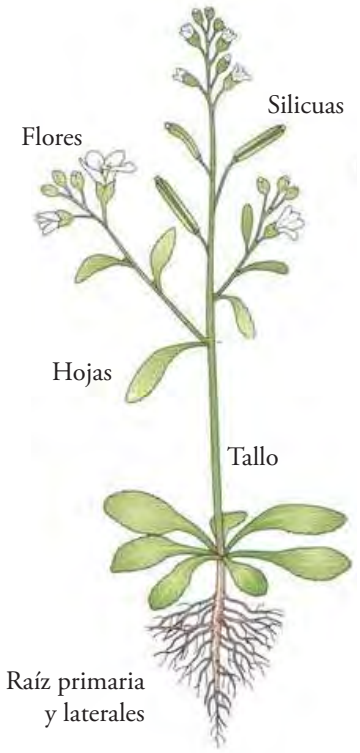

Figura 2.1. Esquema de la morfología de *Arabidopsis thaliana*. Se muestra el sistema aéreo formado por tallo, hojas, flores y frutos; y el radicular que consta de una raíz primaria, raíces laterales y pelos radiculares (Modificado de Taiz y Zeiger, 2002).

gran cantidad de compuestos al ambiente que le rodea conocido como rizosfera, incluyendo ácidos orgánicos, azúcares y mucílago (Narasimhan *et al.*, 2003). La función de estas substancias es lubricar la región de la cofia que se localiza en la punta de la raíz, protegerla de la abrasión por las partículas del suelo, así como permitir la comunicación con los microorganismos del suelo.

La raíz se diferencia del tallo por su estructura, el modo en que se forma y la falta de estructuras fotosintéticas. La primera raíz derivada de la planta se llama radícula, que se forma después de la germinación de la semilla y se diferencia en la raíz primaria o raíz principal. Las raíces desarrolladas a partir de la primaria se denominan raíces laterales o secundarias. Las raíces que crecen a partir de otra parte de la planta, como el tallo, se denominan adventicias (Scheres *et al.*, 2002).

Las raíces son órganos heterotróficos, puesto que su nutrición depende de la fotosíntesis efectuada por las hojas, sin embargo, la fotosíntesis depende de la captación de agua y minerales que son absorbidos por la raíz. Debido a que las plantas son sésiles, su estrategia para una mejor adaptación es la plasticidad en los programas de desarrollo que modifican su arquitectura. Mediante el crecimiento de una raíz más larga o la formación de nuevas raíces laterales o adventicias, la planta puede explorar una mayor área superficial en el suelo para la absorción de agua y nutrientes (Ortiz-Castro, 2005). De igual forma, en el sistema aéreo se modifica el tamaño del hipocotilo, tallo, peciolos y hojas en búsqueda de la luz necesaria mediante respuestas celulares específicas (Franklin *et al.*, 2003).

2.5. Principales factores de competencia

Dentro de las comunidades biológicas, todos los individuos presentan ciertos requerimientos básicos para su crecimiento y desarrollo. En los organismos vegetales los requerimientos principales son: el agua, la luz, el dióxido de carbono, nutrientes minerales, además de algunas señales abióticas importantes como la gravedad, la composición y estructura del suelo, y la percepción de algunos elementos volátiles del ambiente como el etileno que pueden influenciar el desarrollo (**Fig. 2.2**) (Schaller y Kieber, 2002).

Cuando alguno de estos requerimientos indispensables, es percibido como limitante o en escasez y dependiendo de la duración e intensidad de cada señal, se inducen en los organismos respuestas que les permiten adaptarse y/o competir por ellos, para asegurar un mejor aprovechamiento y así lograr la supervivencia.

2.6. Percepción de luz

Las plantas son extremadamente sensibles a la luz, constantemente monitorean su intensidad, longitud de onda y duración para modular diversos procesos del desarrollo. Las respuestas a la luz son de gran importancia adaptativa en los ecosistemas, especialmente bajo condiciones de vegetación densa

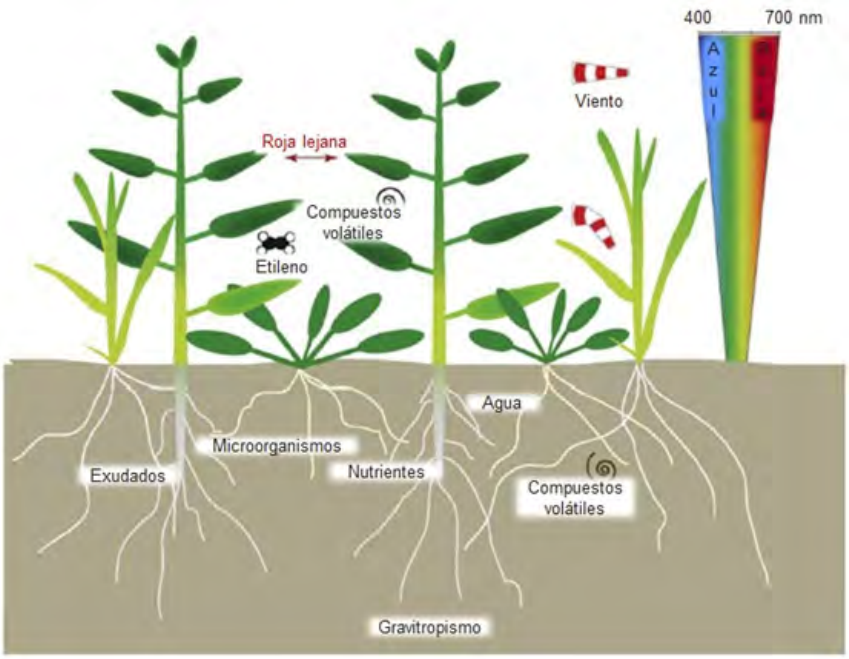

Figura 2.2. Factores de competencia. La percepción de luz de diversas longitudes de onda, la emisión de compuestos volátiles como el etileno y la exposición reducida al viento sirven como sensores ambientales. La interacción con plantas vecinas, patógenos o simbiontes, respuestas mecánicas, cambios en la disponibilidad de nutrientes, agua o exudados radiculares, así como las características bioquímicas del suelo son factores de competencia intra o interespecíficas (Modificado de Kegge y Pierik, 2010).

(Vanderbussche *et al.*, 2005).

Las respuestas fotomorfogénicas son especialmente evidentes en la etapa de plántula; dependiendo si la germinación se lleva a cabo en luz u oscuridad, las plántulas eligen dos distintas rutas del desarrollo (**Fig. 2.3**). En *Arabidopsis*, por ejemplo, si la germinación se lleva a cabo en oscuridad, los cotiledones permanecen cerrados y el hipocotilo se elonga en forma pronunciada, permitiendo a la plántula utilizar sus reservas de energía para emerger rápidamente en busca de fuentes de luz; en cambio en condiciones de luz directa, la plántula presenta el desarrollo de un hipocotilo más corto y los cotiledones se separan para extenderse y permitir el desarrollo de las primeras hojas verdaderas más rápidamente (Steindler *et al.*, 1999).

Los reguladores del crecimiento vegetal, tienen una participación directa sobre la regulación de procesos del desarrollo de las plantas, de entre los compuestos descritos, las giberelinas han sido involucradas en la promoción de la germinación. Actualmente se sabe que cambios en las intensidades de luz provocan una variación en las cantidades de giberelinas en las plantas; Elliott (1975) analizó el crecimiento de las hojas de *Pisum sativum* en respuesta a luz blanca, roja y oscuridad, mostrando que la longitud de las hojas varía de acuerdo al tratamiento, presentando un mayor crecimiento en luz blanca y casi nulo en oscuridad; el autor argumenta que estas respuestas están controladas por fitocromos y protoclorofila.

En plantas superiores se han identificado tres familias principales de fotoreceptores: fitocromos (PHY) absorbentes de luz roja/roja lejana (R/FR por sus siglas en inglés red/far-red), criptocromos absorbentes de luz azul (B) y luz UV y fototropinas (Quail, 2002). Las variaciones en la proporción de luz R/FR permiten percibir a las plantas vecinas (Franklin *et al.*, 2003).

En *Arabidopsis* se han aislado y secuenciado cinco genes codificantes de fitocromos, *PHYA-PHYE* (Mathews y Sharrock, 1997). Franklin *et al.* (2003) mediante análisis de mutantes *PHY* describen que la elongación de las hojas y la aceleración en la floración en respuesta a una baja relación R/FR está mediada por PHYB, D y E de una manera funcionalmente redundante, mientras que PHYA inhibe la elongación de las hojas independientemente de la luz R/FR.

También el tiempo de floración es una característica importante que se modifica por

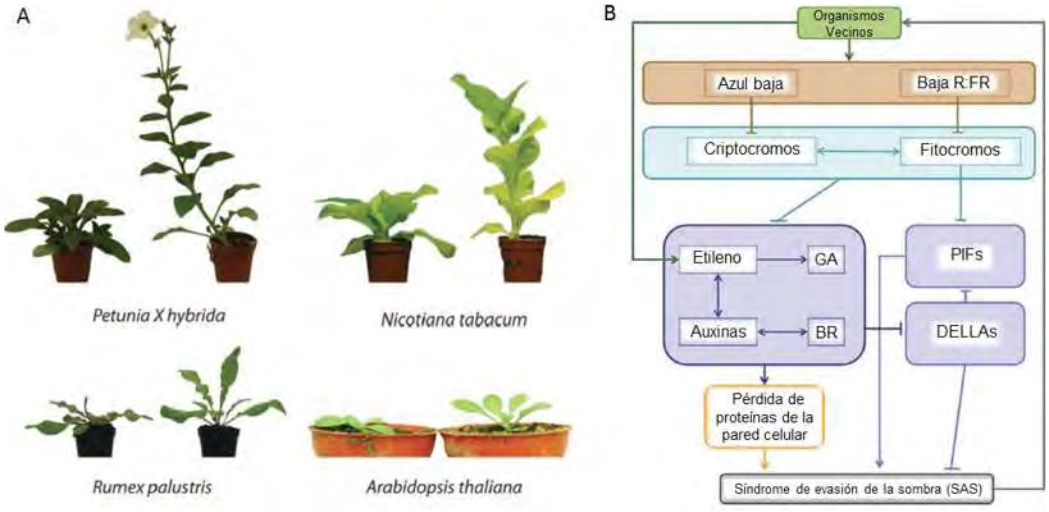

Figura 2.3. Regulación de las respuestas de evasión de sombra. (A) Fenotipo de evasión de la sombra en *Petunia X hibrida*, *Nicotiana tabacum CV* Samsun NN, *Rumex palustris* y *Arabidopsis thaliana* ecotipo Col-0. Para cada especie las plantas de la izquierda representan el control y las de la derecha son plantas crecidas en condición de escasa luz roja. Nótese que la disminución en luz roja conduce a la formación de hojas más verticales, peciolos alargados y floración temprana. **(B)** Esquema representativo de algunos eventos en la transducción de señales en el proceso de evasión de la sombra y la detección de organismos vecinos en alta densidad (Modificado de Keuskamp *et al.,* 2010).

la competencia. La temperatura al igual que la duración del fotoperiodo son estímulos que influyen de manera significativa sobre la floración. La ruta reguladora descrita para este tipo de respuestas, involucra a las proteínas PIF (PHYTOCHROME INTERACTING FACTORS), las cuales son una familia de factores transcripcionales que se unen al DNA para regular la transcripción de genes que forman parte de la transducción de señales de los fitocromos (Duek y Fankhauser, 2005; Keuskamp *et al.*, 2010). Recientemente, ha sido descrito que las PIFs interactúan con las proteínas DELLA (Feng *et al.*, 2008; de Lucas *et al.*, 2008), que son represoras del crecimiento; la regulación de estas proteínas parece ser una respuesta fotomorfogénica clave que está involucrada también en las respuestas a la percepción de vecinos o de evasión de la sombra. La estabilidad de las DELLAs está controlada por las giberelinas conectando así la señalización de los fitocromos con la acción hormonal. Sin embargo, la estabilidad de las DELLA se ve afectada también por otras hormonas como las auxinas y el etileno (Pierik *et al.*, 2009), lo que sugiere que las DELLA tienen un papel integrador de varias rutas hormonales.

2.7. Agua

Las especies vegetales requieren utilizar uno o más recursos que muchas veces se encuentran limitados en diversas partes del suelo, dependiendo de diversos factores como las distintas temporadas del año, lo que puede modificar la intensidad de competencia entre organismos y facilitar o no su coexistencia (Silvertown, 2004).

El agua es uno de estos recursos y tiene un impacto profundo e inmediato sobre cómo el sistema radicular se distribuye por el suelo. Las respuestas de las plantas a los gradientes de agua, han sido estudiadas por más de 100 años, pero los mecanismos que subyacen a estas respuestas permanecen desconocidos. Las respuestas más rápidas de las plantas al estrés osmótico involucran el transporte de iones y el incremento inmediato en los niveles de Ca^{2+} citoplásmico (Takahashi *et al.*, 1997; Cessna *et al.*, 2007).

Un grupo de osmosensores potenciales, son los receptores tipo histidina cinasa AHK1, AHK2-4, que a su vez son receptores de citocininas. En *Arabidopsis* se han encontrado mutantes para AHK1-4 y todas presentan sensibilidad a estrés osmótico y al ácido abscísico (ABA) y diferencias en la inducción de la expresión de genes de respuesta a ABA (Tran *et al.*, 2007; Wohlbach *et al.*, 2008), pero su papel en las

Eapen *et al.* (2003) identificaron mutantes de *Arabidopsis* como la *nhr1* (por sus siglas en inglés *no hydrotropic response*) con defectos en el desarrollo, mostrando un crecimiento postembrionario detenido cuando la mutación es homóciga, mientras que en la heteróciga se pierde completamente el hidrotropismo a la vez que aumenta la elongación celular, el gravitropismo y la curvatura de la raíz, junto con una disminución en la sensibilidad al ABA.

Debido a que realizar análisis en respuesta a gradientes de agua, resulta dificil, aún queda una gran cantidad de investigación por realizar en este campo para poder elucidar los mecanismos fisiológicos que se encuentran involucrados.

2.8. Nutrientes

En el grupo de los factores de competencia que pueden modificar los programas de desarrollo de las plantas, se encuentra la competencia por nutrientes. Siendo los nutrientes principales la fuente de carbono, el nitrógeno y el fósforo. Desde hace décadas ha sido objeto de investigación el conocer cuáles son los requerimientos para que cada especie vegetal pueda cultivarse con facilidad, mejorando así la producción de biomasa, principalmente en especies de importancia económica. En el campo de la investigación, también ha sido importante conocer los requerimientos de los organismos vegetales para facilitar su estudio. Murashige y Skoog (1962) establecieron un medio de crecimiento que hasta la fecha es de los más utilizados, debido a que contiene los nutrientes esenciales para el crecimiento de la mayoría de las plantas.

Deak y Malamy (2005), realizaron modificaciones en las concentraciones de sacarosa en el medio de cultivo para *Arabidopsis*, mostrando que un aumento en la concentración de sacarosa inhibe la formación y longitud de raíces laterales. MacGregor *et al.* (2008) mostraron que el contacto directo de los tejidos aéreos de las plantas en un medio con sacarosa es suficiente para promover la emergencia de raíces laterales a partir de los primordios de la raíz primaria.

Como mencionamos antes, uno de los nutrientes importantes para el desarrollo de las plantas es el fósforo, éste es uno de los principales limitantes de la productividad, debido a su baja movilidad en el suelo.

López-Bucio *et al.* (2002) analizaron la respuesta en la modulación de la arquitectura radicular de *Arabidopsis* en tratamientos con bajas cantidades de fósforo, encontrando que estas condiciones limitantes (concentraciones menores a 50 μM) conducen a cambios importantes como son la disminución en longitud de la raíz primaria, y aumento en número y densidad de pelos radiculares; lo cual correlaciona con un incremento en la sensibilidad a auxinas, los autores sugieren que esta sensibilidad podría indicar un papel fundamental en las modificaciones de la arquitectura de la raíz bajo la disponibilidad de fósforo (**Fig. 2.4**).

Otro nutriente importante para los organismos vegetales es el nitrógeno, un elemento primario, ya que se encuentra en los aminoácidos y por lo tanto en las proteínas, así como en nucleótidos, vitaminas, ácidos nucleicos, clorofila y fitohormonas como auxinas y citocininas.

Las formas iónicas mediante las cuales una raíz puede absorber el nitrógeno son el nitrato (NO_3^-) y el amonio (NH_4^+). La deficiencia de nitrógeno en plantas disminuye el crecimiento, el tamaño de las hojas se presenta reducido al igual que el de los frutos y no se puede sintetizar clorofila, de esta forma aparece la clorosis (hojas de color amarillo), que empieza en las hojas de mayor edad o en la parte inferior de la planta, las cuales pueden llegar a caerse y si la carencia es severa puede aparecer clorosis en las hojas más jóvenes.

Deak y Malamy (2005) reportaron en análisis realizados con *Arabidopsis*, que al reducir las concentraciones de nitrato en el medio de cultivo, se reprime la formación de raíces laterales, sugiriendo que las sales de nitrógeno son un componente regulador del desarrollo. Forde y Walch-Liu (2009) suplementaron el medio de cultivo con nitrato y glutamato, mostrando que estas sales presentan un efecto diferencial en la arquitectura de la raíz, estimulando el crecimiento de las raíces laterales en la

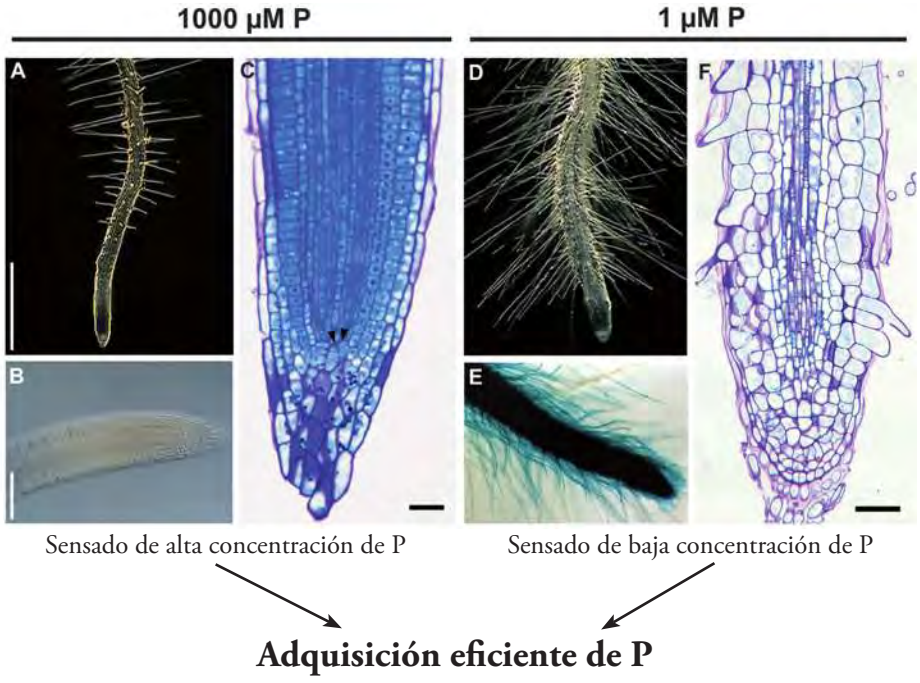

Figura 2.4. Efecto de la disponibilidad de fósforo (P) en la arquitectura del sistema radicular de *Arabidopsis*. (A-C) Imágenes de la zona de crecimiento de la raíz primaria de una plántula de 12 días crecida en condiciones óptimas de P. **(D-F)** Fotografías de las mismas regiones en una planta crecida bajo limitación de P (Modificado de López-Bucio *et al.,* 2002).

región que hace contacto con estas sustancias, pero el contacto con ácido glutámico induce una inhibición del crecimiento de la raíz primaria, que no se observa al contacto con nitrato (**Fig. 2.5**).

Así como los trabajos anteriores analizan la respuesta de las plantas a diversas cantidades de nutrientes específicos, de igual forma hay reportes en la literatura para otros macro y micronutrientes. Es importante destacar que la abundancia o carencia de nutrientes es también dependiente de la cantidad de individuos que haga uso de ellos, por lo que las respuestas a las diversas condiciones pueden también ser consideradas como respuestas de competencia, pero aún no se han descrito los procesos del desarrollo involucrados.

2.9. Compuestos volátiles

Adicionalmente a la competencia por nutrientes e intensidad de luz, se ha comprobado que existe una correlación entre la emisión de compuestos volátiles como etileno y jasmonatos, en función de la ubicación de plantas vecinas y disponibilidad de agua o luz. Se ha descrito que de forma similar a las partes aéreas de la planta, el sistema radicular también emite compuestos volátiles de forma regulada (Hiltpold y Turlings, 2008) y esto puede darse en respuesta a diversos estímulos ambientales, entre ellos el ataque de herbívoros, el cual ha sido estudiado en plantas de maíz y de algodón (Rasmann y Turlings, 2008).

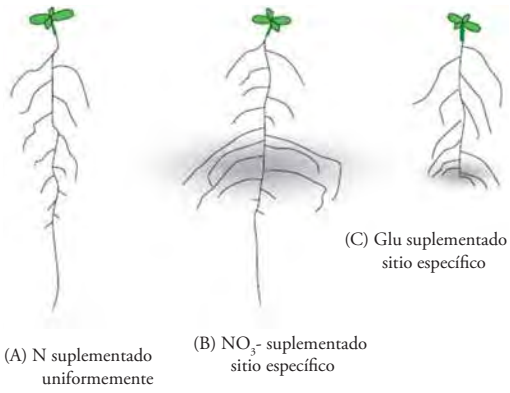

Figura 2.5. Respuestas contrastantes en la arquitectura de la raíz de *Arabidopsis* al localizar fuentes de nitrógeno de diferentes formas. **(A)** Suplemento uniforme de nitrato. **(B)** Adición de nitrato localizada y **(C)** Suplemento localizado de ácido glutámico (Modificado de Forde y Walch-Liu, 2009).

En experimentos *in vitro* con tabaco (*Nicotiana attenuata*) se ha demostrado que el etileno emitido por las plantas presenta una actividad alelopática, reduciendo el tamaño de las raíces de las plantas vecinas (Inderjit y Baldwin, 2009).

Desde hace décadas se ha comenzado la investigación en esta área pero aún falta utilizar enfoques multidisciplinarios combinando la ecología, bioquímica y fisiología molecular para revelar cómo es que la emisión de compuestos volátiles contribuyen a los procesos fundamentales del desarrollo vegetal (Sultan, 2010; Kegge y Pierik, 2010).

2.10. Microoganismos

Sabemos que en las comunidades, las interacciones entre organismos son esenciales para el desarrollo de los mismos. En los ecosistemas, las plantas interactúan con muchos individuos ya sea de la misma o diferente cabo mediante un conjunto de señales. En el caso de las interacciones entre microrganismos y el sistema radicular, se han realizado estudios analizando la respuesta de las plantas frente a hongos y bacterias.

Contreras-Cornejo *et al.* (2009) analizaron especies del hongo *Trichoderma* que es común en la rizosfera, y describen que *T. virens* produce compuestos relacionados a auxinas como el ácido indol-3-acético, el indol-3-acetaldehído e indol-3-etanol. Al colocar el hongo en un sistema de interacción con la planta *Arabidopsis thaliana,* se observó un aumento en la producción de biomasa, así como en la formación de raíces laterales. Lo anterior muestra que la producción de compuestos auxínicos por organismos vecinos tiene un papel importante en la modulación de la morfología vegetal.

Ortiz-Castro *et al.* (2011) analizaron las respuestas de *Arabidopsis* al interaccionar con cepas bacterianas de *Pseudomonas aeruginosa*, sus resultados indicaron que el sistema de quorum-sensing de *P. aeruginosa* controla la producción de ciclodipéptidos, los cuales mostraron tener un papel importante en la promoción del crecimiento de la planta (**Fig. 2.6**).

En conjunto se puede decir que la producción de compuestos con actividad alelopática no está restringida al reino vegetal, sino que existe una señalización entre reinos procariota y eucariota (Ortiz-Castro *et al.*, 2011) y que la modulación del desarrollo vegetal y su morfología son el resultado de una compleja red de señales percibidas y respuestas inducidas.

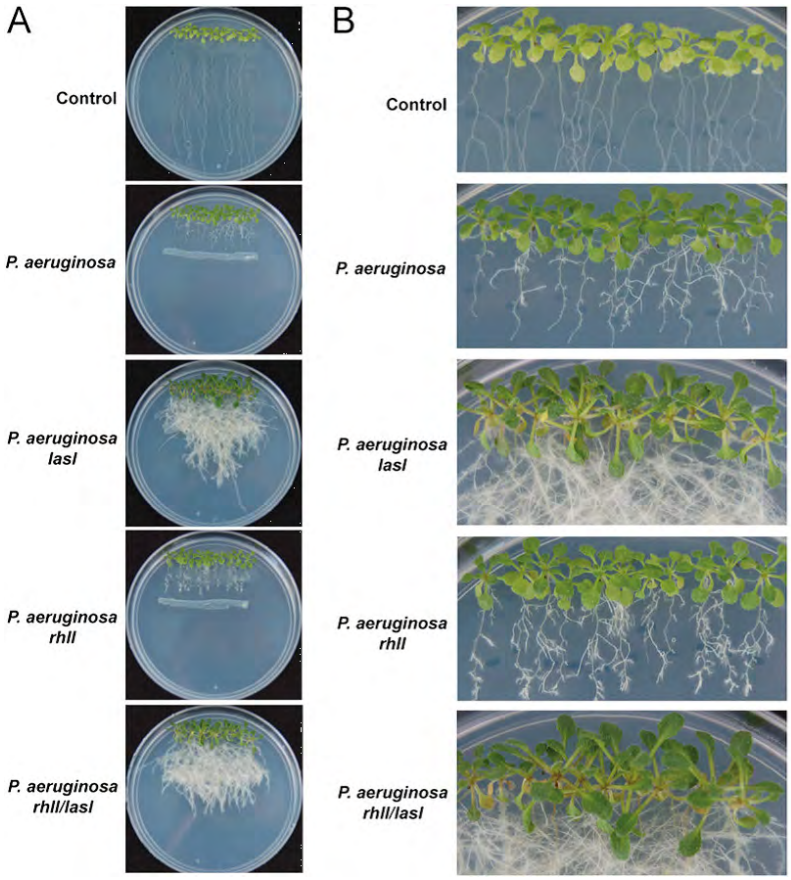

Figura 2.6. Efecto del co-cultivo de *P. aeruginosa* silvestre y cepas mutantes sobre el crecimiento vegetal. Las mutantes presentan defecto en las sintasas de acil homoserina lactonas *LasI*, *RhlI* o *RhlI/LasI*. **(A)** Fotografías representativas. **(B)** Acercamiento de A (Modificado de Ortiz-Castro *et al.,* 2011).

2.11. Exudados radiculares

La plasticidad fenotípica es una característica esencial de las plantas para responder a los estímulos ambientales, que usualmente son dinámicos. Adicionalmente las plantas usan una amplia variedad de señales externas y mecanismos de percepción internos, que son integrados posteriormente a nivel de transducción de señales, llevando a una respuesta fenotípica integral.

En plantas, las respuestas que se presentan pueden ser de tres tipos principales: i) tolerancia, ii) evasión o iii) confrontación, que en las raíces es también llamada alelopatía (Keuskamp *et al.,* 2010; Novoplansky, 2011).

2.12. Alelopatía

Las plantas responden de forma diferente al percibir a otras especies que a la misma especie, indicando lo anterior que el comportamiento de la planta es especializado. Las interacciones raíz-raíz no siempre están dadas por la competencia por algún recurso. Algunas de estas interacciones involucran señales químicas como la producción de compuestos alelopáticos y liberación de sustancias tóxicas para suprimir el desarrollo de las raíces vecinas (Falik *et al.,* 2003). Se ha sugerido que las raíces son capaces de identificarse a sí mismas y a otras plantas, aunque sus vecinas sean de la misma especie o incluso de la misma planta (Falik *et al.*, 2003).

Los agentes alelopáticos descritos a la fecha son principalmente metabolitos secundarios y los compuestos que se conocen han sido aislados de las plantas y su rizosfera. La naturaleza química de los agentes alelopáticos es muy variada. A medida que progresan las investigaciones en el tema se incorporan nuevos grupos a las cuales no se les atribuía esta actividad biológica. Normalmente se les ordena en los siguientes grupos: compuestos alifáticos, lactonas no saturadas, lípidos y ácidos grasos, terpenoides, glucósidos cianogénicos, compuestos aromáticos y alcaloides.

Narasimhan *et al.* (2003) mediante análisis de metabolomica de la rizosfera, identificaron una serie de compuestos exudados por la raíz en *Arabidopsis thaliana*, y describieron sustancias de cada uno de los grupos mencionados anteriormente.

Se ha descrito también que en la raíz hay zonas principales de secreción de compuestos, como son la de elongación en la región cercana a las células meristemáticas, así como también la punta de la raíz. Se ha propuesto que los principales mecanismos por los cuales se secretan compuestos, incluyen difusión, activación de canales iónicos y tráfico vesicular, así como emisión de compuestos volátiles (Badri y Vivanco, 2009; Kegge y Pierik, 2010).

2.13. Contacto mecánico

Las raíces de las plantas presentan un crecimiento direccional en respuesta a la estimulación por contacto (tigmotropismo), se han reportado tanto respuestas positivas como negativas. Adicionalmente, la estimulación mecánica permite a la raíz alterar sus programas de desarrollo y llevar a la formación de raíces laterales. Un programa endógeno relacionado al proceso antes mencionado, es la acumulación de auxinas en el meristemo que parece determinar los sitios de la iniciación subsecuente de las raíces laterales a partir de las células del pericicloo (Malamy y Benfey, 1997).

La identidad molecular de los mecanoreceptores que permiten a la raíz percibir los estímulos mecánicos, aún se desconocen. Mediante análisis electrofisiológicos se han identificado conductores de iones que se activan en distintas células de la plantas y se han reconocido cambios en el Ca^{2+} citosolico, que es una característica ubicua de la transducción de señales, sugiriendo que la permeabilidad de Ca^{2+} puede responder directamente al estrés mecánico que se presenta en la raíz (Monshausen *et al.*, 2008).

El genoma de *Arabidopsis* posee diez homólogos de los genes *MscS* (por sus siglas en inglés: *mechanically sensitive channels of small conductance*) de bacterias. En *E. coli* estos canales responden a la tensión membranal abriéndose y liberando solutos al exterior de la célula, por lo cual actúan como una válvula que previene la ruptura osmótica en estrés hiposmótico (Martinac *et al.*, 2008). La proteínas MSL 2 y 3 parecen tener un papel en la división de plastidios (Haswell y Meyerowitz, 2006), mientras que las MSL 4, 5, 6, 9 y 10 se han encontrado en la raíz de *Arabidopsis*. Mediante el análisis de mutantes se ha sugerido que las proteínas MSL 9 y 10 son los componentes principales de los canales de Cl^- mecanosensibles en la raíz (**Fig. 2.7**) (Haswell *et al.*, 2008).

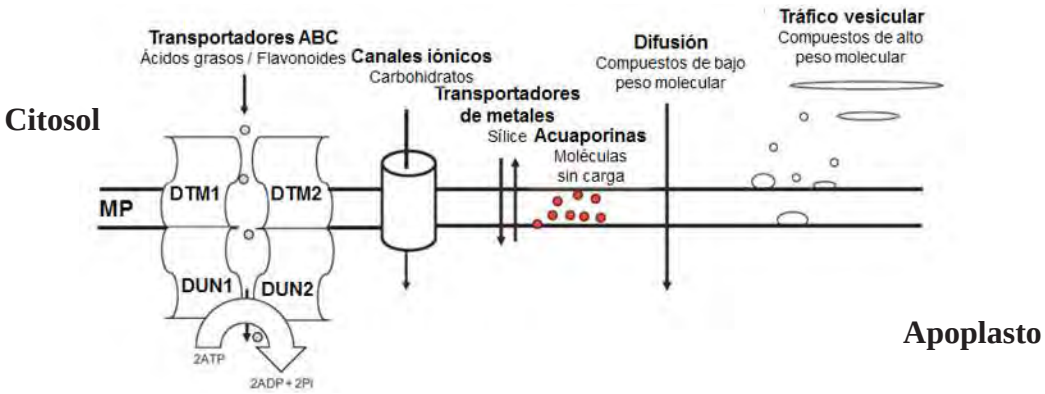

Figura 2.7. Mecanismos de exudación de compuestos a través de la membrana celular vegetal. MP, membrana plasmática; DTM, dominio transmembranal; DUN, dominio de unión a nucleótidos (Modificado de Badri y Vivanco, 2009).

2.14. Conclusiones

Las diversas condiciones ambientales a las que se encuentran expuestas las plantas en sus hábitats, se traducen en estímulos que activan diversas respuestas del crecimiento y desarrollo, lo que les permite adaptarse. Ahora sabemos que en este proceso participan factores endógenos como las giberelinas y las auxinas, que activan rutas de transducción de señales y afectan la arquitectura de la raíz y del follaje, así como el tiempo de floración. Actualmente se cuenta con herramientas tanto genéticas, como bioquímicas y de fisiología molecular que podrían ayudar a esclarecer los mecanismos de comunicación planta-planta y planta-microorganismo. Diversas aplicaciones agrícolas se podrían derivar de esta información, entre ellas conferir a los cultivos una protección más efectiva al ataque de patógenos o incrementar la producción de cereales mediante un aprovechamiento óptimo de los recursos minerales, agua y espacio.

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