UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO-BIOLÓGICAS UMSNH

INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS

"Mecanismos de bioestimulación de la 6-pentil-2H-piran-2-ona de *Trichoderma atroviride* y su papel en la regulación de la arquitectura radicular de *Arabidopsis thaliana*"

Tesis que presenta

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EL PRESENTE TRABAJO FUE REALIZADO EN LOS LABORATORIOS DE BIOQUÍMICA ECOLÓGICA Y BIOLOGÍA DEL DESARROLLO VEGETAL DEL INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS DE LA UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO, BAJO LA DIRECCIÓN DE LA D.C. LOURDES IVETH MACÍAS RODRÍGUEZ Y EL D.C. JOSÉ LÓPEZ BUCIO.

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A mis amados hijos Natalia y Luca.

“Para estar mañana en el recuerdo de tus hijos, debes estar presente en sus vidas hoy”

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RESUMEN

Las plantas son organismos que integran diversos estímulos ambientales para controlar su crecimiento y desarrollo, e interactúan con una amplia variedad de especies bacterianas y fúngicas mediante la emisión de moléculas bioactivas. Los hongos del género *Trichoderma* habitan comúnmente en la rizósfera y contribuyen mejorando la salud y el crecimiento vegetal. Estudios recientes sugieren que los compuestos orgánicos volátiles (COVs), en particular la 6-pentil-2H-piran-2-ona (6-PP) emitida por *T. atroviride*, regula programas de crecimiento y defensa en varias especies de plantas.

Este trabajo se planteó para esclarecer los mecanismos moleculares por los cuales la 6-PP estimula el crecimiento vegetal, así como dilucidar sus efectos sobre el desarrollo de la raíz en *Arabidopsis thaliana*. Mediante técnicas de microscopía confocal, se observó que la 6-PP afecta de manera diferencial la abundancia y distribución de los transportadores de auxinas PIN1, PIN2, PIN3 y PIN7 en la raíz primaria y en los primordios de las raíces laterales. La comparación del crecimiento de plantas silvestres y mutantes de *A. thaliana* afectadas en las vías de señalización de auxinas y etileno indica que la 6-PP promueve la formación de raíces laterales a través de los receptores de auxinas TIR1, AFB2 y AFB3 y los factores transcripcionales ARF7 y ARF19. En contraste, la inhibición del crecimiento de la raíz primaria en respuesta a tratamientos con 6-PP ocurre por un mecanismo independiente a la vía auxínica, en el que participa el gen *EIN2*. Estos resultados evidencian que la percepción de la 6-PP en la raíz involucra diversos componentes moleculares, ocurre de una manera tejido-específica y modifica aspectos celulares fundamentales para orquestar la morfogénesis vegetal y proporcionaron las primeras evidencias que una substancia volátil emitida por un hongo, activa cascadas de señalización molecular en plantas.

PALABRAS CLAVE: *Trichoderma*, volátiles, 6-pentil-2H-piran-2-ona (6-PP), auxinas, etileno.

ABSTRACT

Plants integrate diverse environmental stimuli to control their growth and development. They interact with bacterial and fungal species via releasing bioactive molecules. Fungi of the genus *Trichoderma* commonly inhabit the rhizosphere and contribute to improving plant health and survival. Recent studies suggest that volatile organic compounds (VOCs), in particular 6-pentyl-2H-pyran-2-one (6-PP) emitted by *T. atroviride*, act as signals regulating growth and defense programs in several plant species.

The aim of this work was to clarify the molecular mechanism by which 6-PP promotes plant growth and elucidate its effects on *Arabidopsis thaliana* root development. Using microscopy and confocal imaging techniques, it was found that 6-PP differentially affects the abundance and distribution of auxin transporters PIN1, PIN2, PIN3 and PIN7 in primary roots and in lateral root primordia. Comparison of growth of WT, auxin- and ethylene-related mutants indicated that 6-PP promotes lateral root formation through TIR1, AFB2 and AFB3 auxin receptors and ARF7 and ARF19 transcriptional factors. In contrast, 6-PP inhibited primary root growth in a process independent of auxin signaling and mediated by the *EIN2* gene. These results indicated that root perception of 6-PP involve components of auxin transport and the ethylene-response regulator EIN2 in a tissue specific manner and orchestrates fundamental processes during root system architecture configuration. Our work demonstrates for the first time that a fungal volatile can be recognized by plants to reprogram growth and patterning.

KEY WORDS: *Trichoderma*, volátiles, 6-pentil-2H-piran-2-ona (6-PP), auxin, ethylene.

1. INTRODUCCIÓN

Las plantas responden a un gran número de señales, tanto internas como procedentes del medio ambiente, que son fundamentales para su supervivencia (Chaiwanon *et al*., 2016). El sistema radicular desempeña funciones adaptativas esenciales incluyendo la captación de agua y nutrientes, el anclaje al suelo y el establecimiento de interacciones bióticas y abióticas. La rizósfera es una zona densamente poblada en la que las raíces interactúan con otras raíces de plantas vecinas y con los microorganismos del suelo, incluyendo bacterias y hongos (Bais *et al*., 2004). En esta zona, los metabolitos que son exudados contribuyen con el reconocimiento entre organismos de diferentes grupos taxonómicos, estableciéndose relaciones positivas, negativas o neutrales. Dichas interacciones pueden influir en el crecimiento y desarrollo, cambian la dinámica de los nutrientes y modifican la susceptibilidad al estrés biótico y abiótico (Morgan *et al*., 2005).

Los hongos del género *Trichoderma* se han utilizado como excelentes agentes de biocontrol debido a la producción de antibióticos, su capacidad de parasitar otros hongos y competir con otros microorganismos, además de que inducen el crecimiento y respuestas de defensa en sus hospederos vegetales (Contreras-Cornejo *et al*., 2013). Una característica de*Trichoderma* es su alta tasa de producción de metabolitos secundarios, los cuales le ayudan a sobrevivir y competir en su nicho ecológico, usualmente asociado con las raíces. Con base en la naturaleza química de los metabolitos de *Trichoderma,* se han reportado una gran variedad de productos naturales de origen volátil y no volátil, entre los que se encuentran peptaiboles, alcoholes, hidrocarburos, cetonas, pironas y terpenos, (Reino *et al*., 2008). Los compuestos orgánicos volátiles de *Trichoderma* funcionan como indicadores de la especie y del estado de crecimiento e incluyen moléculas que median la interacción con otros microorganismos y plantas (Zeilinger y Schuhmacher, 2013).

Uno de los primeros compuestos volátiles identificados en*Trichoderma* es la lactona insaturada 6-pentil-2H-piran-2-ona, la cual se produce abundantemente por *T. harzianum, T. atroviride, T. asperellum*, *T. viride, T. koningii, T. citrinoviride* y

T. hamatum y a la que se han atribuido funciones como antibiótico, inductor de respuestas de defensa y promotor del crecimiento vegetal, observándose que induce una mayor acumulación de biomasa en el follaje de plantas de jitomate, que correlaciona con la alta capacidad de ramificación de la raíz (Vinale *et al*., 2008; Kottb *et al*., 2015). Con base en la posible importancia de la 6-PP en las interacciones planta-hongo, resulta de interés conocer los componentes moleculares que regulan la percepción de la 6-PP en los diferentes tejidos vegetales *in vivo* e *in vitro*, así como dilucidar sus efectos en la configuración del sistema radicular.

2. ANTECEDENTES

2.1. La arquitectura radicular de *Arabidopsis thaliana.*

El sistema radicular de las plantas desempeña funciones indispensables, entre las cuales están el anclaje al suelo, la captación de agua y nutrientes y el establecimiento de interacciones bióticas y abióticas. La primera raíz derivada del embrión se llama radícula, esta se forma después de la germinación de la semilla y conduce al desarrollo de una raíz primaria. A partir de esta, se forman otras raíces denominadas raíces laterales o secundarias. Las raíces que se originan en otras partes de la planta, tales como el tallo o las hojas, se denominan raíces adventicias.

La arquitectura del sistema radicular puede ser modificada por tres procesos fundamentales: 1) la división celular en el meristemo de la raíz primaria, lo cual posibilita un crecimiento indeterminado por la incorporación de nuevas células a la raíz, 2) la formación de raíces laterales, por medio de las cuales se incrementa la capacidad exploratoria del sistema radicular y 3) la formación de pelos radiculares que aumenta la superficie de absorción de agua y nutrientes de la raíz primaria y las raíces laterales (Dolan *et al*., 1993).

La raíz de *Arabidopsis thaliana* representa un modelo sencillo y conveniente para estudiar procesos del desarrollo, así como sus respuestas adaptativas e interacciones con el medio ambiente (Jones y Ljung, 2011). Destaca por que en ella, un número pequeño de células genera todos los tejidos a través de divisiones mitóticas seguidas de procesos de expansión y diferenciación celular altamente sincronizados. Debido a que el crecimiento de la raíz primaria es indeterminado, estos procesos se llevan a cabo continuamente. El crecimiento de la raíz primaria ocurre gracias a la producción de células en el meristemo apical de la raíz (RAM*, root apical meristem,* por sus siglas en inglés). El RAM se establece durante la embriogénesis y el desarrollo post-embrionario de la raíz ocurre mediante la actividad de este reservorio de células progenitoras (Sozzani e Iyer-Pascuzzi, 2014).

En el RAM se producen células en dos direcciones, una capa de tejido llamado cofia, que abarca el extremo distal de las raíces y su función es de protección conforme ésta crece a través del suelo. Simultáneamente, la cofia percibe y procesa diversos estímulos ambientales regulando la dirección del crecimiento en función de la gravedad (gravitropismo), luz (fototropismo), obstáculos (tigmotropismo), temperatura (termotropismo), humedad (hidrotropismo), nutrientes y otras sustancias químicas (alelopatía o quimiotropismo) presentes en el medio circundante (Ishikawa y Evans 1990; Okada y Shimura, 1990).

El sistema radicular es una estructura compuesta por diferentes tejidos celulares, incluyendo la epidermis, la corteza, la endodermis, el periciclo y los haces vasculares (Fig. 1).

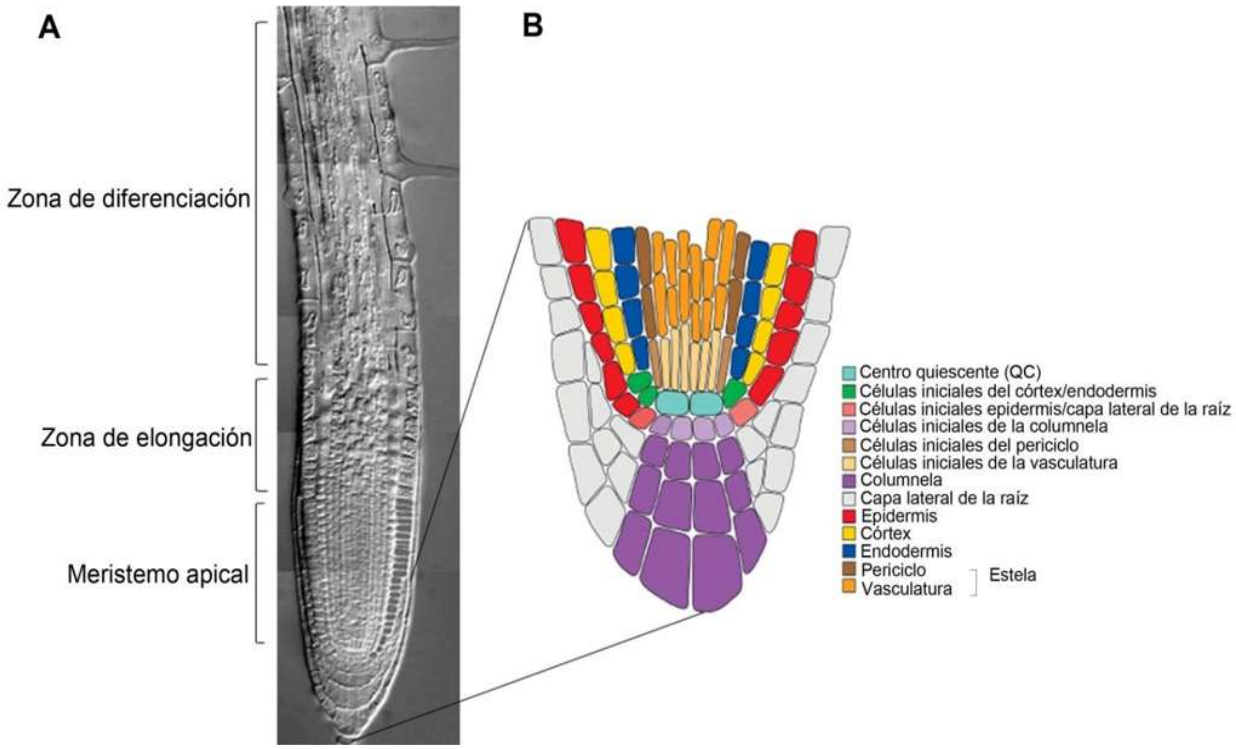

Figura 1. Estructura celular de la raíz de *Arabidopsis thaliana*. A) Acercamiento de la punta de la raíz primaria en la que se muestran la zona meristemática, de elongación y de diferenciación. B) Esquema de la zona meristemática de la raíz primaria donde se observan en colores los distintos tipos celulares (Modificado de Grieneisen *et al*., 2007; Stahl y Simon, 2005).

Las células que forman los diferentes tejidos se producen a partir de cuatro células madre (células iniciales) localizadas en el ápice de la raíz (Dolan *et al.,* 1993). Internamente y en contacto con las células iniciales, se encuentra un número pequeño de células llamado centro quiescente (QC, *quiescent center*), que presenta poca actividad mitótica, pero su función principal es la de mantener la organización de las células adyacentes. Conforme va creciendo la raíz, la zona de división celular (zona meristemática) dará paso a una fase de expansión celular y una vez que las células incrementan su tamaño, se diferencian en su forma y función finales. En la zona más distal de la raíz, este proceso se evidencia por la aparición de pelos radiculares, células epidérmicas especializadas en captar agua y nutrientes, en la región adyacente a la zona de elongación. Adicionalmente, mediante eventos de división celular en el periciclo, se originan las raíces laterales, órganos que incrementan la superficie total de exploración del suelo y contribuyen con un mejor anclaje.

2.1.1. Las raíces laterales.

Un componente fundamental de la arquitectura radicular son las raíces laterales (RL). Estas se originan a partir de la raíz primaria y su desarrollo está controlado por distintos factores que incluyen la disponibilidad de nutrientes, agua y substancias alelopáticas (López-Bucio *et al*., 2003). Las raíces laterales se originan a partir de células del periciclo (Fig. 2A) (Dolan *et al*., 1993). De acuerdo a su desarrollo, Malamy y Benfey (1997) las clasifican en las siguientes etapas: Etapa I: iniciación de un primordio de raíz lateral en un plano longitudinal con aproximadamente 8 divisiones a partir de una célula del periciclo. Etapa II: el primordio consta de dos capas celulares causadas por una división periclinal de la primera capa. Etapa III: se lleva a cabo otra división periclinal para dar lugar a la formación de una tercera capa de células. Etapa IV: el primordio consta de cuatro capas de células. Etapa V: el primordio alcanza el córtex de la raíz. Etapa VI: el primordio forma un domo que alcanza la epidermis. Etapa VII: ocurre la emergencia de la raíz, formándose una raíz lateral (Fig. 2B y C).

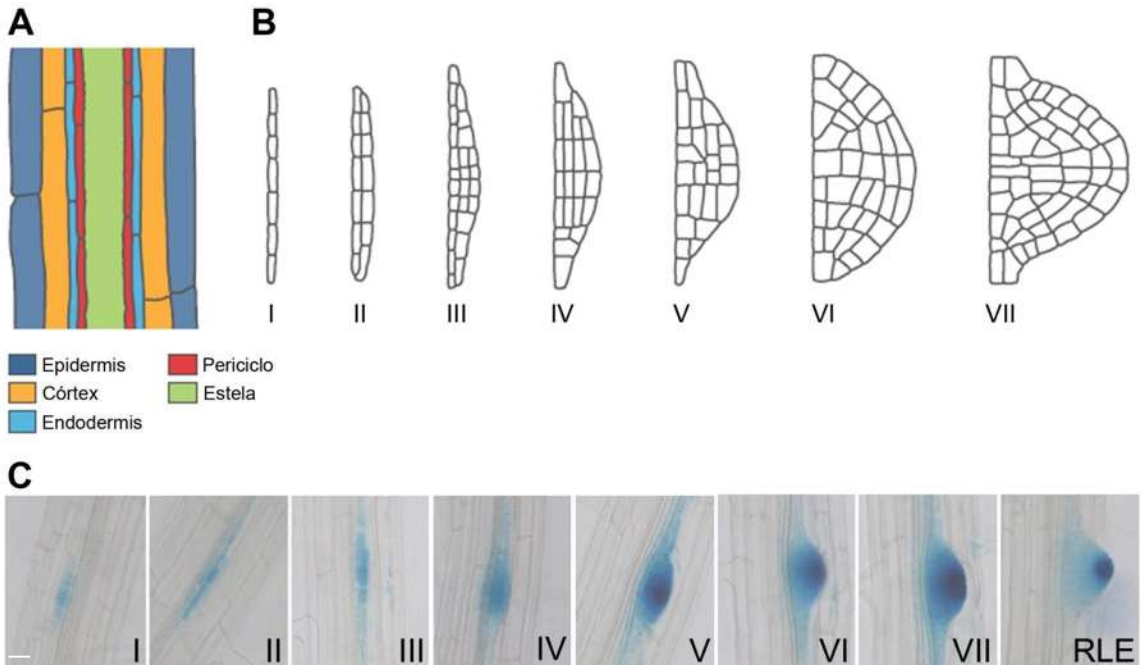

Figura 2. Formación y desarrollo de las raíces laterales en *Arabidopsis thaliana*. A) Esquema de la zona de diferenciación donde se observan las células del pericliclo que darán origen a las raíces laterales. B) Estadios de los primordios de raíces laterales (en números romanos). C) Fotografías de los primordios de raíces laterales donde se muestra el máximo de auxinas, indicado por el color azul en la línea transgénica *DR5:GUS.* RLE: raíz lateral emergida. Barras de escala: 20 µm (Modificado de Petricka *et al.,* 2009).

2.2. Los reguladores del crecimiento.

El crecimiento y desarrollo vegetal involucra la integración de diversas señales ambientales y fisiológicas, las cuales junto con el programa genético intrínseco determinan la forma y funcionamiento de la planta (Gray, 2004). Varias moléculas orgánicas pequeñas denominadas fitohormonas o reguladores del crecimiento son responsables de cada aspecto del desarrollo, desde la embriogénesis hasta la senescencia. Dicho control es llevado a cabo por la modulación de los programas celulares de división, expansión, diferenciación y muerte celular.

Los primeros reguladores del crecimiento descritos fueron las auxinas, el ácido abscísico, las citocininas, las giberelinas y el etileno. Recientemente, diversos compuestos han sido también reconocidos por su actividad biológica,

entre los que se incluyen a los brasinoesteroides, jasmonatos, ácido salicílico, óxido nítrico y algunos lípidos (Fig. 3) (Durbak *et al.,* 2012). Los reguladores del crecimiento funcionan como moléculas integradoras de información, cuyas vías de señalización interactúan con frecuencia para controlar la morfogénesis y su adaptación al ambiente (López-Bucio *et al*., 2006).

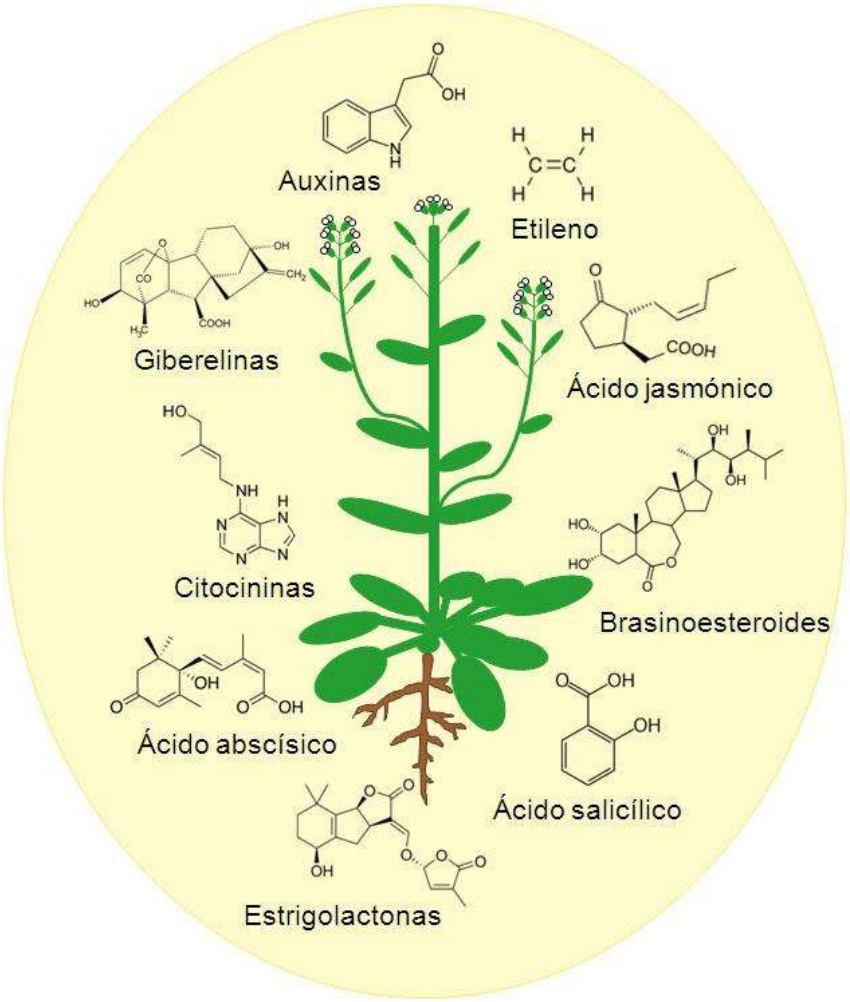

Figura 3. Reguladores del crecimiento vegetal. Auxinas: regulan los tropismos, la arquitectura de la raíz y follaje, el desarrollo vascular y el crecimiento en cultivo de tejidos. **Citocininas**: controlan la división celular, la diferenciación vascular, el desarrollo y ramificación del follaje, de la raíz e inflorescencias, el balance de nutrientes, senescencia y la tolerancia al estrés. **Etileno**: induce la maduración de frutos, la senescencia y respuestas de estrés biótico y abiótico. **Giberelinas**: controlan la germinación de las semillas, la elongación de tallos, la expansión de las hojas, el desarrollo de tricomas, flores y frutos. **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 y Estelle, 2009).

Las fitohormonas integran diversos estímulos ambientales con el programa genético de la planta y comparten diversas características: I) se encuentran en bajas concentraciones en el interior de los tejidos, II) su biosíntesis, transporte y percepción se incrementa en respuesta a factores ambientales, III) comparten módulos de señalización, por lo que un regulador puede afectar múltiples respuestas en la planta y/o diferentes compuestos pueden incidir sobre un mismo aspecto del desarrollo (Gray, 2004). Los efectos fisiológicos en la morfogénesis vegetal con frecuencia dependen de la interacción de varias rutas hormonales sobre los tejidos en los cuales inciden.

2.3. Las auxinas.

Las auxinas son los reguladores del crecimiento vegetal más estudiados, ya que participan en cada aspecto del crecimiento y desarrollo de la planta (Fig. 4), modulando procesos diversos como las respuestas a la luz y gravedad, la arquitectura general de la raíz y follaje, la organogénesis, el desarrollo vascular y el crecimiento de tejidos en cultivos *in vitro* (Teale *et al*., 2006). Se caracterizan por presentar en su molécula un grupo indol (compuesto por un anillo aromático unido a un anillo pirrólico) y un grupo carboxilo que le otorga una carga negativa a la molécula. La auxina más abundante es el ácido indol-3-acético (AIA), el cual está implicado en los tres procesos principales del desarrollo vegetal: división, elongación y diferenciación celular.

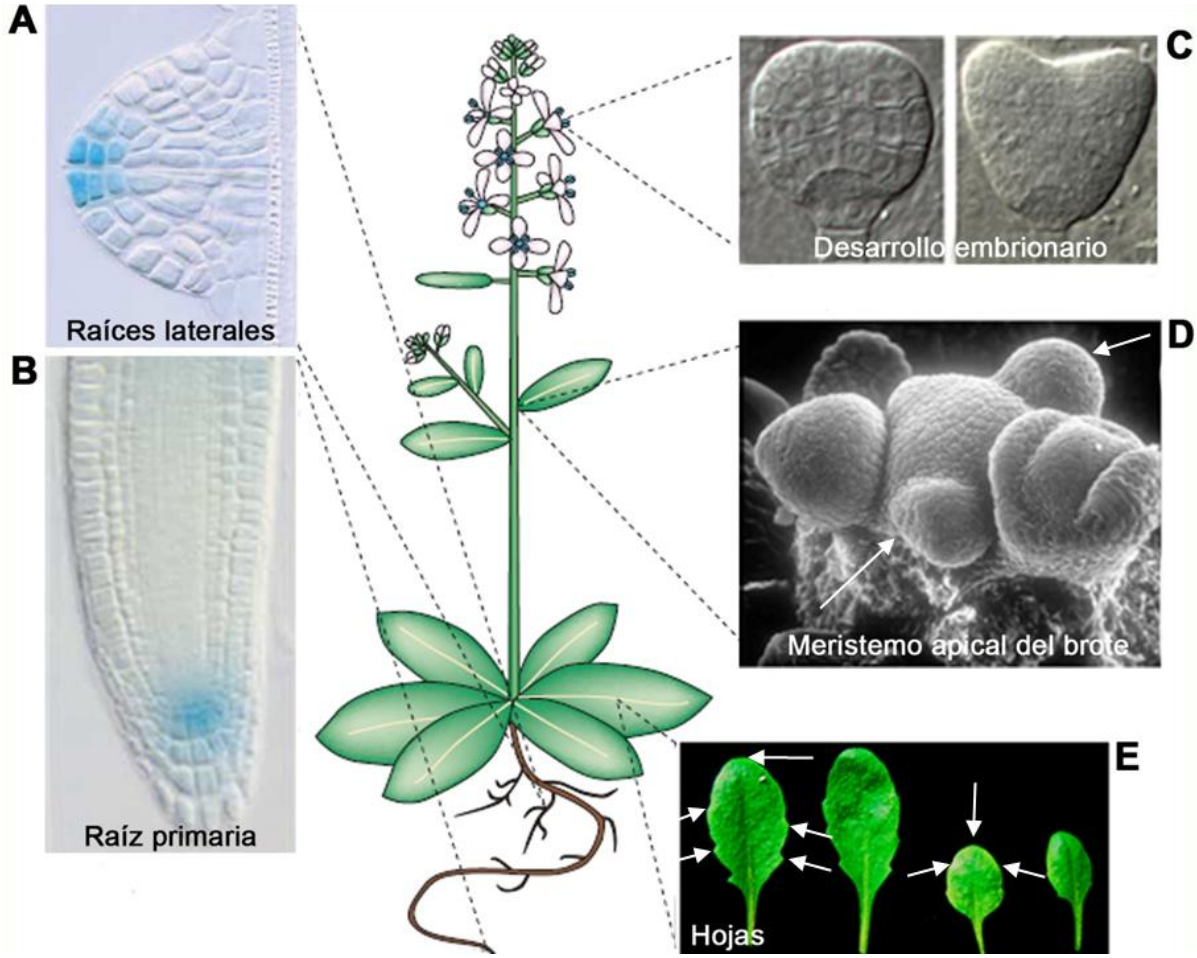

Figura 4. Procesos del desarrollo regulados por auxinas. A) Raíz lateral donde se observa el máximo de auxinas en la línea *DR5:GUS*. Las auxinas son trasportadas hasta su sitio de acción para la formación de raíces laterales. B) Punta de la raíz primaria donde se aprecia la expresión del marcador *DR5:GUS*. La regulación del flujo de auxinas determina las respuestas a la gravedad. C) Fotografía de un embrión en estado globular. Las auxinas se movilizan por medio de los transportadores de influjo (AUX1) y eflujo (PIN). D) Microfotografía de un meristemo apical del brote. Las auxinas son transportadas hacia los sitios de formación de hojas nuevas, indicados con flechas y E) fotografía de hojas de *Arabidopsis thaliana*, donde se señalan los sitios de producción de auxinas. (Modificado de Teale *et al.,* 2006; A y B tomadas de Benková *et al*., 2003, C, Tsugeki *et al*., 2009, D, Smyth *et al*., 1990, y E, Moustaka *et al*., 2015).

2.3.1. Biosíntesis de auxinas

Las auxinas se sintetizan localmente en los cotiledones, las hojas jóvenes y en el meristemo de la raíz. Las rutas de biosíntesis del AIA propuestas incluyen una dependiente del triptófano y otra independiente de este aminoácido. La vía dependiente involucra varios intermediarios como el indol-3-acetamida (IAM),

ácido indol-3-pirúvico (IPA), indol-3-acetaldoxima (IAOX), triptamina (TAM) y la vía donde participa la TRIPTÓFANO AMINOTRANSFERASA DE *ARABIDOPSIS* (TAA)/YUCCA (YUC), cada una culmina con la producción de ácido indol-3-acético. El precursor del AIA, L-Triptófano (L-Trp) se sintetiza a partir del corismato (Tzin y Galili, 2010). El L-Trp actúa como precursor para la síntesis de IAM por medio de la monooxigenasa-2-triptófano codificada por un gen homólogo al gen bacteriano *IAA1*, el cual convierte el L-Trp en AIA (Sugawara *et al*., 2009). En la ruta del IPA, la aminotransferasa de L-Trp (TAA1) convierte el L-Trp en IPA (Stepanova *et al*., 2008) y a partir de este, las enzimas YUCCA (YUC) sintetizan el AIA (Mashiguchi *et al*., 2011; Stepanova *et al*., 2011; Won *et al*., 2011). La triptófano descarboxilasa (TDC) es una enzima citosólica que convierte el L-Trp en TAM, otro precursor de AIA (Quittenden *et al*., 2009; Novák *et al*., 2012). En la ruta del IAOX, dos enzimas homólogas del citocromo P450, CYP79B2 y CYP79B3 convierten el L-Trp en el intermediario de la síntesis de AIA, IAOX (Mano y Nemoto, 2012).

Con base en el análisis de mutantes de biosíntesis de L-Trp, tales como *trp2-1* y *trp3-1*, se ha evidenciado una ruta biosintética independiente de L-Trp (Zhao *et al*., 2002). En dichas mutantes, los niveles de AIA libre se incrementan, sugiriendo el papel de estos genes como reguladores negativos de las enzimas biosintéticas (Ouyang *et al*., 2000). La vía independiente de triptófano no ha sido completamente elucidada, aunque se propone como precursor al indol-3-glicerol fosfato (IGP) (Finet y Jaillais, 2012). Recientemente, se sugirió que una enzima sintasa de indol de localización citosólica (INS) es clave en la vía independiente de triptófano, debido a que su pérdida de función resulta en defectos en la embriogénesis de la planta (Wang *et al*., 2015).

2.3.2. Transporte de auxinas.

La actividad de las auxinas depende de su concentración celular y tisular, por lo que una vez sintetizadas, se requiere de un mecanismo de transporte para la formación de gradientes en sitios específicos de las plantas. Desde las zonas de

síntesis, las auxinas son redistribuidas hacia otros tejidos y órganos donde se requieren para la división y elongación celular, formación de raíces laterales, dominancia apical, desarrollo de hojas y flores y para los tropismos (Davies, 2004). El transporte de auxinas ocurre de dos maneras: 1) el primer sistema es pasivo y ocurre a través del floema, donde el AIA producido en la parte aérea (hojas jóvenes y flores) puede viajar distancias relativamente largas. 2) El transporte polar de auxinas que ocurre a corta distancia, o célula-célula (TPA), mueve a las auxinas de la parte aérea hacia el ápice de la raíz (Petrásek y Friml, 2009). La direccionalidad del flujo auxínico está regulado por la naturaleza química de la molécula, el AIA es un ácido débil (pk_a= 4.75) y en el apoplasto de los tejidos donde se deposita, que es relativamente ácido (pH=5), se encuentra tanto en forma protonada (IAAH) como disociada ($AIA^- + H^+$). El IAAH se puede difundir a través de las membranas celulares bajo gradientes de concentración, así que, en el citosol con un pH cercano al 7.0, se disocia casi en su totalidad (Rubery y Sheldrake, 1974). La forma aniónica o disociada (AIA^-), no puede atravesar la membrana celular, de manera que se requieren transportadores de eflujo para el mantenimiento del gradiente local y para alcanzar una concentración óptima de auxinas principalmente en los tejidos en desarrollo (Krupinski y Jönsson, 2010). De acuerdo a este modelo, existen diversos transportadores de influjo y eflujo con características específicas.

En el transporte polar de auxinas, participan proteínas transportadoras de influjo como AUXIN RESISTANT1 (AUX1) y de salida, que incluye a varios integrantes de las familias PIN-FORMED (PIN) y POLYGLYCOPROTEIN (PGP) que en conjunto regulan la distribución controlada del AIA para generar gradientes de concentración (Geisler *et al*., 2005). Dichos gradientes son importantes debido a que el AIA lleva a cabo sus efectos a través de la formación de máximos de concentración que son percibidos por receptores de expresión constitutiva y/o tejido específica (Benková *et al*., 2003). La ubicación de las pozas de auxinas está determinada por la posición asimétrica de las proteínas transportadoras dentro de los tejidos y órganos. Los acarreadores de influjo AUX1/LIKE AUX1 (AUX1/LAX) se identificaron mediante el estudio de una mutante agravitrópica afectada en el

gen AUX1 en *Arabidopsis,* que codifica para una proteína transmembranal tipo permeasa, implicada en la toma de auxinas (Taiz y Zeiger, 2002).

Los procesos en los que se requiere transporte de auxinas a larga distancia comprenden la formación de los tejidos vasculares, así como la formación de pelos radiculares y raíces laterales (Overvoorde *et al*., 2010; Fàbregas *et al*., 2015;). Por otra parte, el eflujo de auxinas está mediado por los transportadores PIN que contienen en su secuencia de aminoácidos 10 a 12 regiones transmembranales. En *Arabidopsis* se han reportado ocho proteínas PIN, los PIN1, 2, 3, 4 y 7 son importantes en la organogénesis y en el gravitropismo de la raíz. La mayoría de las proteínas PIN presentan una distribución asimétrica (polar) en las membranas de algunos tejidos, mientras que en otros muestran actividad redundante y su localización es indistinta respecto a los planos apical-basal y lateral de las células, dando origen a un flujo auxínico con un patrón multidireccional (Fig. 5) (Křeček *et al*., 2009). PIN1 se localiza en el sistema vascular (Gälweiler *et al*., 1998; Friml *et al*., 2002a; Scarpella *et al*., 2006); PIN2 se expresa en la punta de la raíz de forma basal en las células del córtex y de forma apical en células de la epidermis y columnela (Müller *et al*., 1998); PIN3 se localiza lateralmente en células de la endodermis o periciclo y de forma simétrica en las células de la columnela (Friml *et al*., 2002b); PIN4 se encuentra en las células centrales del meristemo con polaridad hacia las células del centro quiescente (Friml *et al*., 2002a). PIN7 presenta un patrón de expresión similar a PIN3 (Blilou *et al*., 2005). A diferencia de los PIN de la membrana plasmática, PIN5, 6 y 8 se localizan en el retículo endoplásmico, donde modulan la homeostasis de auxinas mediante su conjugación con aminoácidos, limitando así su bioactividad.

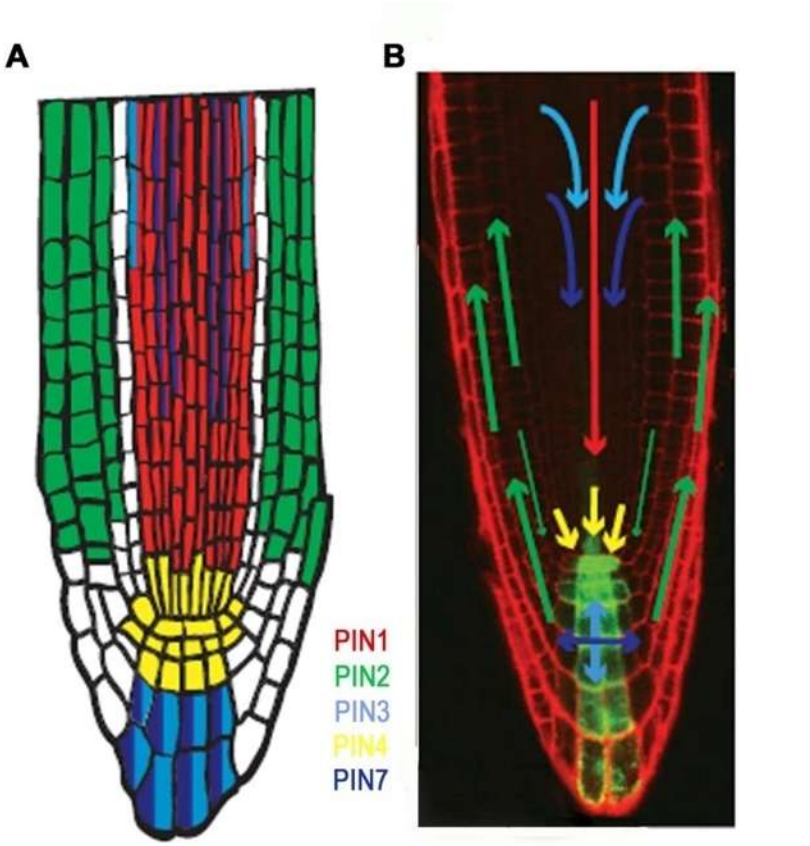

Figura 5. Transporte polar de auxinas. A) Esquema de la zona meristemática de la raíz primaria de *Arabidopsis* donde se muestra la distribución de los transportadores de eflujo de auxinas, indicando en colores los tejidos en los cuales se expresan las proteínas PIN. PIN1: células de la estela, PIN2: células de la epidermis y de la capa lateral de la raíz, PIN3: células de la columnela y lateralmente en células del periciclo, PIN4: en el centro quiescente y células que lo rodean (iniciales) y PIN7: células de la vasculatura y de la columnela. B) Fotografía de la raíz primaria donde se muestra el máximo de auxinas mediante la expresión del marcador *DR5::GFP*, indicando con flechas la dirección del transporte polar de auxinas (Modificada de Cederholm *et al*., 2012).

El mecanismo para el establecimiento y mantenimiento de la polaridad celular es extremadamente complejo y se relaciona con la estructura de la pared celular, al citoesqueleto, la señalización por fosfoinosítidos e implican al calcio como segundo mensajero, así como a eventos de transporte en la membrana plasmática y tráfico intracelular (Dhonukshe *et al*., 2007a; Kleine-Vehn *et al*., 2011; Mravec *et al*., 2011). Este último proceso es un factor determinante en el modelo de distribución de los transportadores de auxinas, que propone que las proteínas PIN son secretadas de manera no polar y su subsecuente endocitosis y reciclaje

establecen su localización en la célula (Dhonukshe *et al*., 2007b). Dicho reciclaje, se establece mediante la participación de la proteína endosomal GNOM (Geldner *et al*., 2003; Kleine-Vehn *et al*., 2006) y es promovida por la fosforilación de los transportadores PIN por varias cinasas, incluyendo PINOID (PID) (Kleine-Vehn *et al*., 2006), y por la subunidad reguladora de la proteína fosfatasa 2A (PP2A) (Michniewicz *et al*., 2007).

Otra familia de transportadores de eflujo, además de los PIN son los ABCB, que tienen dominios de unión al ATP en su secuencia y se localizan en la membrana de manera no polar. La subfamilia de P-glicoproteínas (PGP) de resistencia a drogas (ABCB/MDR/PGP) incluye los miembros ABCB1, 4, 19, 15 y 21 relacionados con el eflujo de auxinas, los cuales participan en la formación de pelos radiculares, respuestas en el crecimiento, formación de raíces adventicias y el fototropismo (Retzer *et al*., 2014). Los PGP pertenecen a una subfamilia de los transportadores ABCB que se descubrieron inicialmente en líneas cancerígenas de mamíferos, donde su sobreexpresión resultaba en una resistencia hacia el tratamiento a diversos fármacos (Cho *et al*., 2007). Al principio se sugirió que dichos transportadores funcionaban como bombas de eflujo en la detoxificación celular. En las plantas, se les atribuye un papel importante en el transporte de auxinas tanto en monocotiledóneas como en dicotiledóneas. Por ejemplo, la mutante *atpgp1* presenta reducción en el desarrollo de la raíz y gravitropismo alterado, relacionados con un menor transporte de auxinas (Geisler *et al.*, 2005). Además de la modulación del tráfico vesicular y endocitosis, los niveles de las proteínas PIN pueden ser afectados por su degradación en la vacuola. En el caso de PIN2, su concentración celular depende de la actividad del proteosoma que involucra al complejo SCF^{TIR1}/AFB (Baster *et al*., 2013). La traducción y distribución de las proteínas PIN está regulada por la acumulación de AIA en el citosol, por la interacción con vías de señalización de otros reguladores del crecimiento, así como por diversos factores ambientales y durante la interacción con microorganismos.

2.3.3. Señalización de auxinas.

La percepción de las auxinas inicia cuando la concentración intracelular de AIA se incrementa por eventos de biosíntesis o transporte, lo que les permite unirse a las proteínas que funcionan como receptores y éstas con los co-receptores, que actúan como represores transcripcionales (Tan *et al*., 2007). En *Arabidopsis*, se han reportado seis receptores para las auxinas localizados en el núcleo: TRANSPORT INHIBITOR RESPONSE 1 (TIR1) y las AUXIN SIGNALLING F-BOX PROTEIN 1, 2, 3, 4 y 5 (AFB1/2/3/4/5), proteínas con dominios F-box, que conforman el complejo SCF^{TIR1}/AFB (Dharmasiri *et al*., 2005; Parry *et al*., 2009). Los represores transcripcionales están codificados por una familia de genes llamada AUXIN/INDOLE-3-ACETIC ACID (Aux/IAAs) (Gray *et al*., 2001).

Las proteínas Aux/IAA reprimen la actividad de los factores de transcripción que pertenecen a la familia de los AUXIN RESPONSE FACTORS (ARFs) (Ulmasov *et al*., 1997). Existen 23 ARFs en *Arabidopsis*, algunos de ellos presentan un dominio rico en glutamina y se clasifican como activadores transcripcionales, mientras que otros albergan dominios ricos en serina y funcionan como represores en la ruta de señalización las auxinas (Guilfoyle y Hagen, 2007). En ausencia de la señal hormonal, los represores Aux/IAA reprimen la transcripción con la asistencia de un co-represor transcripcional denominado TOPLESS (TPL) (Long *et al*., 2006). Los represores Aux/IAAs pueden reclutar directamente al co-represor TPL a través de un motivo EAR. Los represores Aux/IAA no se unen a las auxinas, sino que interactúan con el receptor TIR1 a través de un dominio específico. Cuando los niveles de auxinas aumentan en la célula, estas moléculas se unen al complejo receptor, estabilizando de esta manera la unión del complejo SCF^{TIR1}/AFB con los represores Aux/IAAs, lo que permite la poli-ubiquitinación de los represores y su posterior degradación por el proteosoma, liberando así a las factores de transcripción que regulan la expresión de los genes de respuesta a auxinas (Fig. 6) (Dos Santos-Maraschin *et al*., 2009).

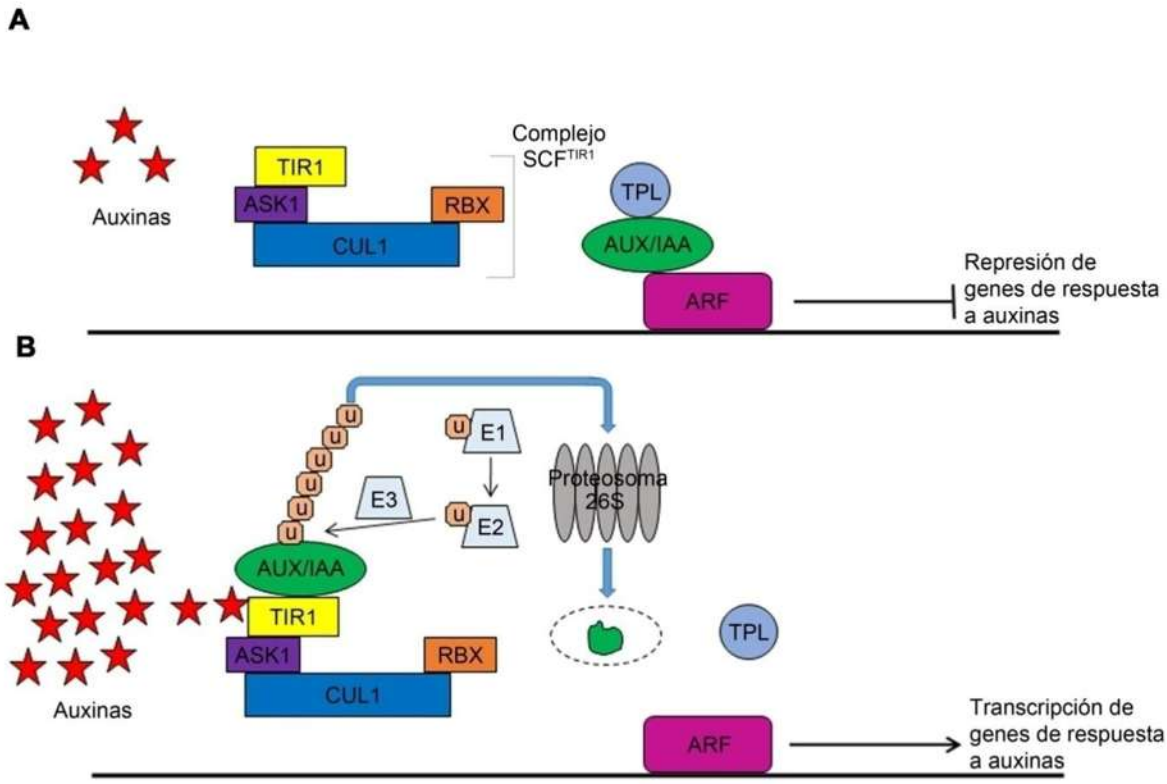

Figura 6. Señalización regulada por auxinas. A) En niveles bajos de auxinas, el co-represor TPL, reprime la transcripción de genes de respuesta auxinas regulando la unión de las proteínas AUX/IAA a los ARF. B) Cuando se incrementan los niveles de auxinas, éstas se unen al complejo SCF^{TIR1}, aumentando la afinidad por los AUX/IAA y promoviendo su degradación a través de la vía ubiquitina-proteosoma, conduciendo a la activación del ARF que posteriormente se une al promotor de genes de respuesta a auxinas (Modificado de Saini *et al*., 2013).

Las auxinas son fundamentales para el crecimiento vegetal, ya que actúan sinergística o antagónicamente con otros fitorreguladores en distintas etapas del ciclo de vida. Además, tienen un profundo efecto en el establecimiento de interacciones con microorganismos patógenos y benéficos (Robert-Seilaniantz *et al.*, 2011; Sukumar *et al*., 2013). Por ejemplo, mutantes de *Arabidopsis* afectadas en transporte y señalización de auxinas (*pin2/eir1* y *tir3*) mostraron resistencia a la colonización y a los síntomas de enfermedad producidos por el fitopatógeno *Fusarium oxysporum* (Kidd *et al*., 2011). En raíces de jitomate se demostró que es necesaria la señalización de auxinas en etapas tempranas de la colonización por el hongo simbionte *Glomus intraradices*, ya que la mutante resistente a auxinas

diageotropica (dgt), disminuye su afinidad para formar micorrizas (Hanlon y Coenen, 2011).

Algunos microorganismos pueden activar la señalización de auxinas por la producción de compuestos auxínicos o moléculas que mimetizan la acción de éstos fitorreguladores. Dos especies del hongo *Trichoderma virens* y *Trichoderma atroviride* incrementaron la biomasa y promovieron el crecimiento de raíces laterales en *Arabidopsis thaliana* gracias a la producción de AIA, indol-3-acetaldehído (IAAld) e indol-3-etanol (IEt) (Contreras-Cornejo *et al.,* 2009). Otro estudio señala que la bacteria *Pseudomonas aeruginosa* produce moléculas de tamaño pequeño denominados ciclodipéptidos y sus derivados dicetopiperazinas (DCPs), los cuales regulan el sistema de quorum-sensing (QS) de las bacterias y tienen un efecto promotor en plantas de *A. thaliana* modulando la expresión de genes y señalización de auxinas (Ortiz-Castro *et al.,* 2011). Estos hallazgos sugieren un papel primordial de la biosíntesis, transporte y señalización de auxinas para el crecimiento y desarrollo de la raíz en respuesta a las interacciones con los microorganismos que habitan en el suelo.

2.4. La rizósfera.

Las plantas interactúan con microorganismos que viven en el suelo, dichas interacciones son esenciales para el ensamble de la comunidad y para el funcionamiento armónico de los ecosistemas (Shi *et al*., 2016a). La zona que circunda las raíces de las plantas y que está habitada por una población única de microorganismos influenciada por los compuestos químicos producidos por el hospedero vegetal, se denomina rizósfera (Mendes *et al*., 2013). La rizósfera se compone de tres zonas, las cuales están definidas con base en la influencia y proximidad de la raíz (Fig. 7). 1) La endorrizósfera incluye porciones de la corteza y endodermis, en la cual los microorganismos ocupan el “espacio libre” entre las células (espacio apoplástico). 2) El rizoplano es la zona media directamente adyacente a la raíz que incluye la epidermis y la capa de mucílago secretada, y 3) la ectorrizósfera que se extiende a partir del rizoplano e influye en las

características del substrato. Debido a la complejidad inherente y la diversidad de los sistemas radiculares de las plantas, la rizósfera no es una región de tamaño o forma definible, sino que consiste en un gradiente de propiedades químicas, biológicas y físicas que cambian tanto radialmente como longitudinalmente a lo largo de la raíz (McNear, 2013).

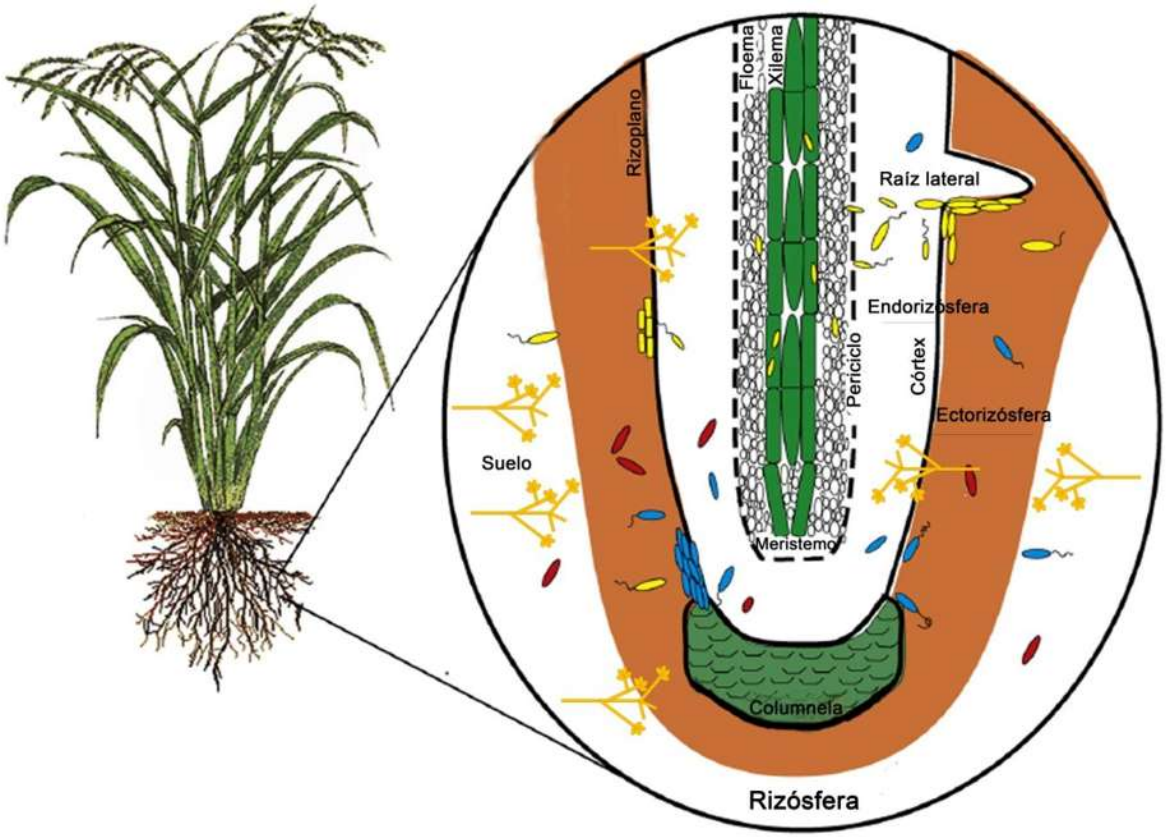

Figura 7. La rizósfera. Esquema de las distintas zonas de la rizósfera donde se muestran algunos tejidos vegetales y la interacción de los microorganismos que habitan en torno a ellos. La endorizósfera incluye el córtex y la endodermis, el rizoplano es la zona inmediata de la raíz, abarca la epidermis y el mucílago y la ectorrizósfera que incluye del rizoplano al suelo (Modificado de Hardoim *et al*., 2008).

La rizósfera es considerada como uno de los ecosistemas terrestres más complejos, en ella se encuentra una gran variedad de organismos que incluye bacterias, arqueas, protozoos, hongos, oomicetos, algas, nematodos y artrópodos. La mayoría de sus habitantes forman parte de una compleja red de alimentación

que inicia a partir de los nutrientes producidos y liberados por las raíces de las plantas, los cuales son clave para la regulación de la diversidad microbiana (Mendes *et al*., 2013). Las plantas modulan el microbioma de la rizósfera estimulando selectivamente la proliferación de microorganismos con características benéficas para el crecimiento y salud de las mismas (Cook *et al*., 1995).

2.5. El Género *Trichoderma.*

Trichodema es un hongo anaerobio facultativo que pertenece a la subdivisión Ascomycetes y se caracteriza por no presentar un estado sexual determinado (Harman *et al*., 2004). Se encuentra de manera natural en casi todos los tipos de suelo, especialmente en aquellos que contienen materia orgánica o desechos vegetales en descomposición, así como en residuos de cultivos, lo que lo caracteriza como saprófito (Wilson, 1997). Las especies de *Trichoderma* poseen gran diversidad genética y producen cientos de compuestos químicos de interés comercial y ecológico (Kubicek *et al*., 2011).

En su medio natural, estos hongos colonizan las raíces de las plantas estableciéndose en el apoplasto de las células de la epidermis y el córtex (Brotman *et al*., 2008). La colonización resulta en la estimulación del crecimiento de la raíz y en su protección contra patógenos. Además, algunas especies convierten en su forma disponible a macro y micronutrientes que no están disponibles en el suelo (Resende *et al*., 2014). También compiten con otros microorganismos por nutrientes y espacio (Elad, 1996; Harman, 2000). *Trichoderma spp*. puede parasitar a otros hongos, fenómeno conocido como micoparasitismo, controla nemátodos y otros fitopatógenos que atacan a los cultivos, ya que es capaz de producir diversos metabolitos secundarios y enzimas extracelulares tales como celulasas, quitinasas, glucanasas y proteasas (Harman *et al*., 2012). Todas las propiedades benéficas que poseen las especies de *Trichoderma* las convierten en uno de los principales agentes para el control

biológico con amplio potencial para el desarrollo de bioestimulantes y su aplicación en campo.

2.5.1. Metabolismo secundario de *Trichoderma.*

Para sobrevivir y competir en su nicho ecológico, *Trichoderma* produce metabolitos secundarios los cuales son específicos de cada especie y su concentración varía en relación a la biosíntesis y las tasas de transformación o degradación por otros microorganismos (Vinale *et al*., 2012). El término "metabolito secundario" se refiere a un grupo heterogéneo de compuestos químicamente diferentes (en general con un peso molecular menor a 3000 daltones), los cuales se relacionan con funciones de supervivencia del organismo productor, como la competencia y simbiosis (Demain y Fang, 2000). En la naturaleza, los metabolitos secundarios funcionan a muy bajas concentraciones (microgramos por litro) y pueden actuar como mediadores de la comunicación bioquímica entre los habitantes de la rizósfera, permitiendo así, el establecimiento de relaciones con otros organismos, incluyendo hongos, insectos, plantas y animales (Contreras-Cornejo, 2016).

De acuerdo con estudios químicos y analíticos, los hongos del género *Trichoderma* son excelentes productores de metabolitos secundarios entre los que destacan peptaiboles, terpenos y pironas volátiles y no volátiles, sideróforos y compuestos que contienen nitrógeno. Se han identificado alrededor de 373 diferentes moléculas, muchas de las cuales aún se desconoce su función (Reino *et al*., 2008; Crutcher *et al*., 2013).

2.5.2. Efectos de los metabolitos de *Trichoderma* sobre las plantas.

Regulación del crecimiento vegetal.

Trichoderma produce compuestos químicos que pueden causar cambios en el metabolismo de la planta que colonizan. Algunas cepas incrementan la

producción de biomasa, al respecto, Contreras-Cornejo *et al.* (2009) reportaron que plántulas de *A. thaliana* inoculadas con *T. virens* y *T. atroviride* acumularon mayor cantidad de biomasa foliar que las no inoculadas y este efecto se correlacionó con un incremento en el número de raíces laterales (Fig. 8). En este trabajo, se determinó que *T. virens* produce ácido indol-3-acético (AIA), indol-3-acetaldehído (IAAld), indol-3-etanol (IEt) e indol-3-carboxaldehído (ICAld) que actúan como precursores del AIA. Resultados similares se evidenciaron en plantas de frijol (*Phaseolus vulgaris*) inoculadas con distintas cepas de *Trichoderma*, donde el 60% de las cepas produjeron AIA y otros análogos de auxinas (Hoyos-Carvajal *et al*., 2009). La gran cantidad de compuestos indólicos que produce *Trichoderma spp.* influyen sobre el crecimiento y desarrollo de la planta hospedera.

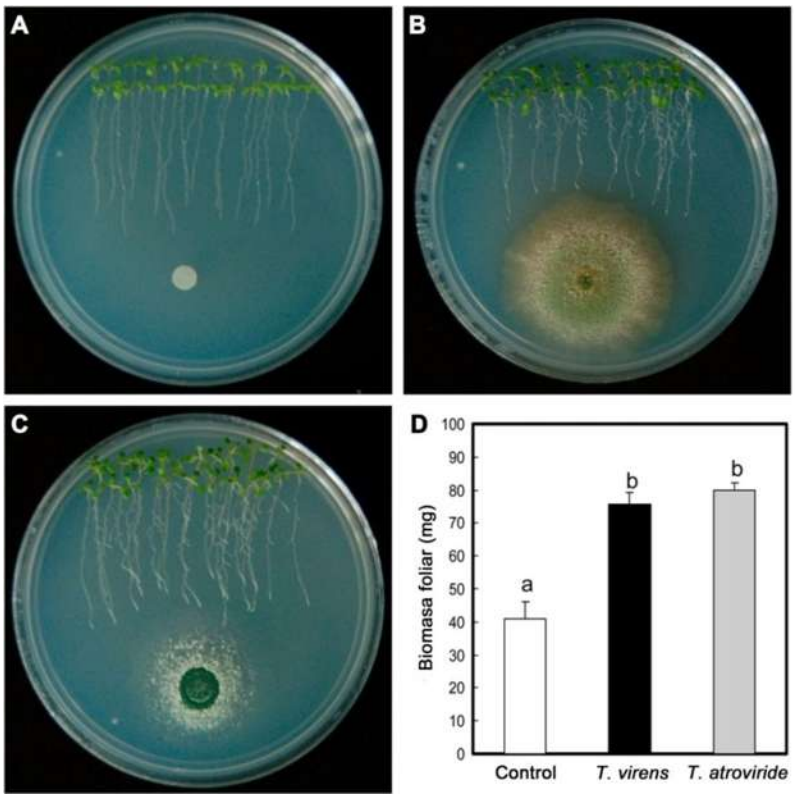

Figura 8. Efectos de *Trichoderma* sobre el crecimiento de *A. thaliana*. A) Plantas del ecotipo silvestre (Col-0) de *A. thaliana* crecidas en condiciones control. B) Efecto de la inoculación de *T. virens* y C) *T. atroviride*, donde se observa que ambas especies inducen el desarrollo de raíces laterales comparado con el tratamiento control. D) Efecto de *Trichoderma* sobre la acumulación de biomasa foliar (Adaptado de Contreras-Cornejo *et al.,* 2009).

Otro regulador del crecimiento vegetal producido por *Trichoderma* es el etileno (C_2H_4), un hidrocarburo gaseoso simple que modula procesos en las plantas como la germinación de las semillas, crecimiento, abscisión de hojas y pétalos, senescencia de órganos, respuestas de estrés y contra patógenos (Schaller y Kieber, 2002). *Trichoderma atroviride* produce etileno y AIA, los cuales inducen la actividad de la proteína cinasa activada por mitógenos (MPK6) que regula el crecimiento de la raíz primaria y la formación de pelos radiculares en *Arabidopsis thaliana*. El análisis de la expresión de genes de respuesta a auxinas en plántulas silvestres y mutantes de la vía del etileno mostraron que su efecto sobre el crecimiento y diferenciación de la raíz está mediado por la señalización de auxinas, indicando un nodo de señalización en MPK6 que ajusta el crecimiento y desarrollo de *Arabidopsis* en respuesta a *Trichoderma* (Contreras-Cornejo *et al.,* 2015a).

Además de la producción de algunos reguladores del crecimiento vegetal, se han determinado numerosos metabolitos que dependen del tipo de cepa, tal es el caso de las koninginines A-C, E, G, producidas por *T. koningii* , así como la lactona volátil 6-pentil-α-pirona (6-PP) producida por *T. harzianum, T. viride, T. atroviride y T. koningii*, la cual promueve el crecimiento de plantas de trigo, tomate, canola y *A. thaliana* (Cutler *et al*., 1986, Parker *et al*., 1995a, b, 1997, Stoppacher *et al*., 2010; Yang *et al*., 2012). Los compuestos producidos por *T. harzianum* como el ácido harziánico y derivados como el harzianólido inducen el crecimiento y actúan como elicitores de la resistencia sistémica en plantas de tomate (Cai *et al*., 2013; Vinale *et al*., 2013). Otros metabolitos como el viridiol y los trichocaranenos producidos por *T. virens*, se han reportado como inhibidores del crecimiento en trigo (Howell y Stipanovic; 1994; Macías *et al*., 2000). Algunos metabolitos secundarios de *Trichoderma* que regulan crecimiento y desarrollo en plantas presentan actividad promotora a bajas concentraciones e inhiben el crecimiento a altas dosis. Sin embargo, se requieren más estudios para elucidar los mecanismos de acción de diferentes metabolitos sobre procesos morfogenéticos específicos.

Inducción de respuestas de defensa

Las propiedades benéficas de *Trichoderma spp.* no están limitadas a su uso en el biocontrol (Schuster y Schmoll, 2010). Diversas clases de metabolitos secundarios pueden actuar como elicitores o inductores de resistencia durante la interacción *Trichoderma*-planta (Vinale *et al*., 2012). Estas moléculas incluyen: i) proteínas con actividad enzimática, como las xilanasas (Lotan y Fluhr, 1990), ii) productos de genes de avirulencia, los cuales activan reacciones de defensa (Harman *et al.,* 2004) y iii) compuestos de bajo peso molecular o derivados de paredes celulares vegetales por la acción de las enzimas de *Trichoderma.*

Algunos ejemplos de elicitores en plantas, son la 6-pentil-α-pirona, la (-)-harzianopiridona y el harzianólido, los cuales fueron purificados de *T. harzianum* (cepas *T22, T39 y A6*), *T. atroviride* (cepa *P1*) y posteriormente fueron aplicados a plantas de jitomate y canola inoculadas con los patógenos *Botrytis cinerea* y *Leptosphaeria maculans*. Los resultados de estos trabajos mostraron una reducción en los síntomas de enfermedad e indujeron la expresión del gen de respuesta a defensa *PR-1* (*pathogenesis-related protein 1*) (Vinale *et al*., 2008). Se ha descrito como elicitor a la proteína de la familia cerato-platanina Epl-1, ya que se observó que la mutante de *T*. *harzianum* Δ*epl-1*, mostró afectada su capacidad de confrontación contra otros hongos resultando en una reducida actividad micoparasítica. Además, se encontró que la mutante Δ*epl-1*, induce en frijol la expresión del gen de respuesta a ácido jasmónico *LOX1* y del gen *GLU1*, que codifica una β-1,3-glucanasa, con actividad en la degradación de la pared celular de hongos, lo que indica un papel importante en la inducción de la inmunidad vegetal (Gomes *et al*., 2015).

Una clase novedosa de elicitores son los peptaiboles, oligopéptidos ricos en aminoácidos sintetizados por péptido sintasas no ribosomales (NRPSs) (Szekeres *et al*., 2005). De acuerdo a su tamaño se han dividido en tres grupos: peptaiboles de cadena larga (18-20 residuos de aminoácidos), peptaiboles de cadena corta (11-16 residuos) y los lipopeptaiboles (6-10 residuos) (Daniel y Filho, 2007). La cepa de biocontrol *Trichoderma longibrachiatum* produce una gran variedad de

peptaiboles de cadena larga denominados Trichokoninas (TKs), los cuales inducen resistencia a la infección por la bacteria Gram (-) *Pectobacterium carotovorum subsp. carotovorum* en la col china, regulando la expresión del gen de defensa *PR-1*, así como la expresión de enzimas como la catalasa y la peroxidasa (Li *et al*., 2014). Recientemente, se encontró que la TK VI disminuye la longitud de la raíz primaria, inhibiendo división y elongación celular y ocasiona un desarreglo en las células del nicho celular. Además, incrementa el contenido de auxinas y modifica los gradientes de estas en la punta de la raíz modulando la síntesis local y el transporte polar (Shi *et al*., 2016b).

Los volátiles de *Trichoderma* también juegan un papel clave para la activación de la inmunidad. En un estudio realizado con plantas de *A. thaliana* infectadas con el patógeno necrótrófico *Botritis cinerea*, las cuales fueron expuestas a los compuestos orgánicos volátiles de *T. virens*, se observó una disminución importante en el daño ocasionado por este hongo (Contreras-Cornejo *et al*., 2014a). Otro estudio señala que en plantas de jitomate infectadas con el áfido *Macrosiphum euphorbiae* y el parasitoide *Aphidius ervi*, la inoculación con *Trichoderma harzianum T-22*, indujo la expresión de genes relacionados con la síntesis de volátiles y de genes de la vía de señalización del ácido salicílico, lo que sugiere que *T. harzianum T-22* induce defensa sistémica adquirida y atrae a los parasitoides de los áfidos (Coppola *et al*., 2017). Estas evidencias muestran que los metabolitos secundarios de *Trichoderma* están directamente involucrados con la activación de genes de defensa en las plantas y en aspectos de comunicación con microorganismos benéficos.

Solubilización de nutrientes.

Las plantas responden convenientemente a la disponibilidad de nutrientes en el suelo, lo cual es fundamental para su adaptación al ambiente. Existen dos grupos de elementos importantes para el crecimiento y reproducción de las plantas: macronutrientes y micronutrientes. Los macronutrientes como el N (nitrógeno), P (fósforo) y K (potasio) son requeridos en grandes cantidades. En el

suelo existe un equilibrio dinámico entre la solubilización, toma y transporte de los nutrientes, lo cual se encuentra bajo la influencia del pH y los microorganismos que habitan la rizósfera. Algunos nutrientes como el N y P, pueden actuar como señales que modifican el desarrollo de la raíz, modificando la longitud de la raíz primaria y el crecimiento y formación de raíces laterales y pelos radiculares (López-Bucio *et al*., 2003).

Un mecanismo por el cual *Trichoderma* induce crecimiento y productividad vegetal es la solubilización de nutrientes que se encuentran de forma no disponible en el suelo (Vinale *et al*., 2012). *Trichoderma harzianum 1295-22* incrementa la solubilidad de macronutrientes como el P y micronutrientes como Fe (Fierro), Mn (Manganeso) y Zn (zinc) cuando se inocula en medio líquido con extracto de levadura y sacarosa (Altomare *et al*., 1999). Hoyos-Carvajal y col. (2009) reportaron que 101 aislados de *Trichoderma spp*. provenientes de Colombia solubilizan fosfato, determinando que el 20% de estas cepas fueron capaces de producir formas solubles de fosfato a partir de roca fosfórica. *T. viridae* y *T. harzianum* pueden solubilizar P, aún en ambientes de agobio por metales pesados como el cadmio y bajo condiciones variables de pH (Rawat y Tewari, 2011). Se ha demostrado que las plantas de jitomate crecidas en condiciones deficientes de P, Fe, Cu, o Zn mejoran su crecimiento cuando se inocula *T. harzianum SQR-T037* promoviendo el desarrollo de raíces y aumentando la absorción de nutrientes mediante la exudación de ácidos orgánicos, por la reducción y quelación de Fe y Cu (Li *et al*., 2015a).

El hierro es un nutriente esencial debido a que se utiliza en distintas rutas metabólicas. Como metal de transición, sus propiedades redox le permiten dos estados de oxidación, ferroso (Fe^{2+}) y férrico (Fe^{3+}) para la donación y aceptación de electrones, respectivamente. Por lo tanto, el suministro adecuado de hierro es necesario para la supervivencia. Algunos microorganismos en condiciones limitantes de hierro utilizan un sistema de absorción de alta afinidad basado en la liberación de moléculas quelantes llamadas sideróforos. Diversas especies de *Trichoderma* producen sideróforos como el coprógeno B, fusarina C y ferricrocina en condiciones de deficiencia de hierro. Los sideróforos producidos por este hongo

pueden ser absorbidos y proporcionar hierro a las plantas en distintas condiciones ambientales (Anke *et al*., 1991).

Adaptación al estrés abiótico.

Las plantas están expuestas continuamente a distintos tipos de estrés como el calor, la sequía, el frío y la salinidad. Algunas cepas de *Trichoderma* pueden aminorar el efecto ocasionado por el estrés abiótico. En plantas de jitomate bajo estrés hídrico, la inoculación con *T. harzianum T-22* incrementó la expresión de genes que codifican para enzimas antioxidantes como la superóxido dismutasa, (SOD), catalasa (CAT) y la ascorbato peroxidasa en la raíz y follaje, lo que sugiere que *T. harzianum T-22* contribuye en la detoxificación de especies reactivas de oxígeno y las plantas resisten de mejor forma la deficiencia de agua u otros tipos de estrés (Mastouri *et al*., 2012). Contreras-Cornejo y col. (2015b), determinaron el papel de *Trichoderma* en la modulación de la apertura de los estomas y la transpiración en plantas de *Arabidopsis*. En este trabajo se comparó el crecimiento de plantas silvestres y mutantes insensibles al ácido abscísico (ABA) *abi1-1* y *abi2-1* inoculadas con *T. virens* y *T. atroviride* en condiciones normales de hidratación, a las cuales se les determinó la apertura estomática y la pérdida de agua en las hojas. Las plantas silvestres inoculadas con *Trichoderma* mostraron disminución de la apertura de los estomas y menor pérdida de agua, mientras que en las mutantes no se observó ningún efecto. Además, *T. virens* y *T. atroviride* indujeron la expresión del marcador inducible por ABA *abi4:GUS* y por medio de técnicas analíticas se determinó que producen este fitoregulador.

La salinidad afecta el crecimiento de las plantas y modifica la arquitectura de la raíz. Estos efectos están relacionados con la disminución del diámetro de la roseta y niveles bajos de clorofila en el follaje. Al inocular *A. thaliana* con *T. virens* y *T. atroviride* se mejoró la tolerancia al estrés salino probablemente por mecanismos mediados por la acumulación de ácido abscísico, el antioxidante ácido ascórbico y la L-prolina e incrementando la eliminación de Na^+ en los exudados radiculares (Contreras-Cornejo *et al*., 2014b), indicando que los

metabolitos de *Trichoderma* como las auxinas y el ABA tienen un papel clave para la bioestimulación del crecimiento y confieren tolerancia al estrés (Fig. 9).

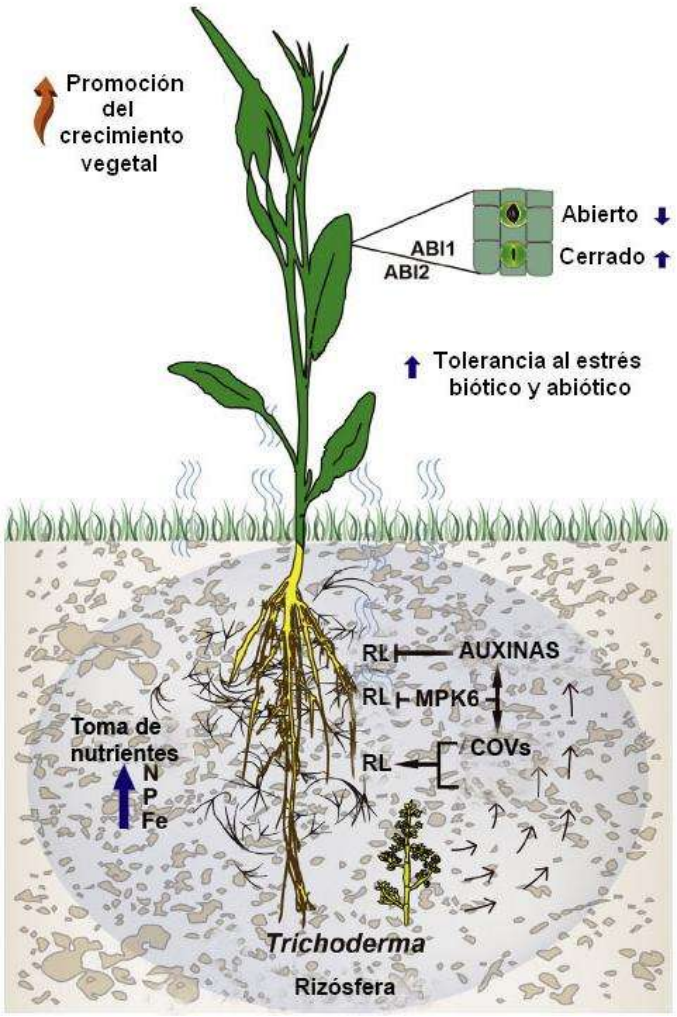

Figura 9. El papel multifuncional de*Trichoderma* en interacción con las plantas. *Trichoderma* produce compuestos volátiles y reguladores del crecimiento vegetal como las auxinas y etileno, los cuales regulan procesos del desarrollo como la ramificación de la raíz a través de la activación de la cinasa dependiente de mitógenos MPK6. La raíz, en respuesta al hongo modifica su exploración del suelo, la toma de nutrientes y la tolerancia al estrés, mejorando la productividad (Modificado de López-Bucio *et al*., 2015).

2.6. Compuestos orgánicos volátiles de *Trichoderma.*

Los hongos producen una amplia cantidad de productos naturales que reflejan su diversidad e incluye una gran variedad de estructuras químicas. Entre

los metabolitos secundarios producidos por los hongos se encuentran los compuestos orgánicos volátiles (COVs), que son un grupo de moléculas orgánicas, que tienen un peso <300 g/mol, lo cual hace que se evaporen fácilmente a temperatura ambiente y que difundan a través del aire y del suelo. Existe una gran variedad química de COVs que incluye alcoholes, aldehídos, componentes aromáticos, ésteres, furanos, cetonas, terpenos y compuestos que contienen azufre y nitrógeno (Macías-Rodríguez *et al*., 2015). De manera similar a otros productos naturales, la biosíntesis de COVs fúngicos está regulada por las condiciones de crecimiento tales como la disponibilidad de nutrientes, pH, temperatura y el fotoperiodo. Las proporciones relativas de los compuestos pueden cambiar con la edad del cultivo y en la interacción con otros organismos (Wheatley, 2002).

Trichoderma spp. produce una amplia gama de productos naturales de origen volátil. Entre los COVs reportados de*T. atroviride* se encuentran alcoholes, cetonas, ésteres y compuestos de ocho carbonos y en el caso de *T. virens*, se han identificado un gran número de sesquiterpenos (Stoppacher *et al*., 2010; Contreras-Cornejo *et al*., 2014a). Por medio de cromatografía de gases acoplada a espectrometría de masas (CG-MS) en *T. harzianum* cultivado en agar papa dextrosa, se identificaron 278 compuestos volátiles que incluyen hidrocarburos saturados, ciclohexanos, ciclopentanos, ácidos grasos, alcoholes, ésteres, compuestos que contienen azufre y la 6-pentil-α-pirona, la cual fue el metabolito más abundante (Siddiquee *et al*., 2012).

Los COVs de *Trichoderma* tienen funciones en el desarrollo del hongo, también se incluyen antimicrobianos y compuestos que median la comunicación con otros organismos (Effmert *et al*., 2012). Por ejemplo, los compuestos que proporcionan el olor característico a hongos como el 1-octen-3-ol, la 3-octanona y el 3-octanol se identificaron como inductores de la conidiación en *T.atroviride*, *T. harzianun* y *T. longibrachiatum*, con efectos dependientes de la concentración. (Nemcovic *et al*., 2008). Otras substancias como la 6-PP y diversos sesquiterpenos presentan actividad antifúngica y como elicitores de defensa en plantas (Vinale *et al*., 2008; Minerdi *et al*., 2011; Velázquez-Robledo *et al.*, 2011).

Interesantemente, se ha reportado que los sesquiterpenos también son producidos por plantas y pueden ser reconocidos por los microorganismos que habitan el suelo para establecer la colonización en las raíces (Bais *et al*., 2006).

En plantas de *Arabidopsis* expuestas a los volátiles de *T. viride*, se promueve la producción de biomasa foliar y radicular, que correlaciona con un mayor contenido de clorofila en la hojas (Hung *et al*., 2013). Específicamente, la 6-PP producida por algunas especies de *Trichoderma* es bioactivo en jitomate, canola, chícharo, trigo y *A. thaliana* (Parker *et al*., 1997; Vinale *et al*., 2008; Kottb *et al*., 2015), considerándose como un compuesto de interés tanto por su actividad antimicrobiana e influenciando los procesos de crecimiento y desarrollo en plantas.

2.6.1.6-pentil-2H-piran-2-ona (6-PP).

Uno de los primeros COVs identificados de *Trichoderma* fue la 6-PP. Este compuesto es el responsable del “olor a coco” asociado a ciertas especies de este hongo. Una vez descubierto, fue sintetizado como producto natural y actualmente es usado en la industria alimentaria como agente saborizante (Collins y Halim, 1972).

La 6-PP es una lactona insaturada, la única evidencia sobre su posible ruta biosintética indica que es sintetizada a partir del ácido linoleico, el cual es oxidado para formar el intermediario 13-hidroperóxido, el cual es metabolizado por β-oxidación e isomerización para formar el 5-hidroxi-2,4-ácido decenoico. La esterificación interna del intermediario hidroxi-ácido conduce a la formación de la 6-PP, cuando no se promueve la actividad de la 2,4-dienol-coenzima A reductasa (dependiente de NADPH) (Fig. 10) (Serrano-Carreón *et al*., 1993).

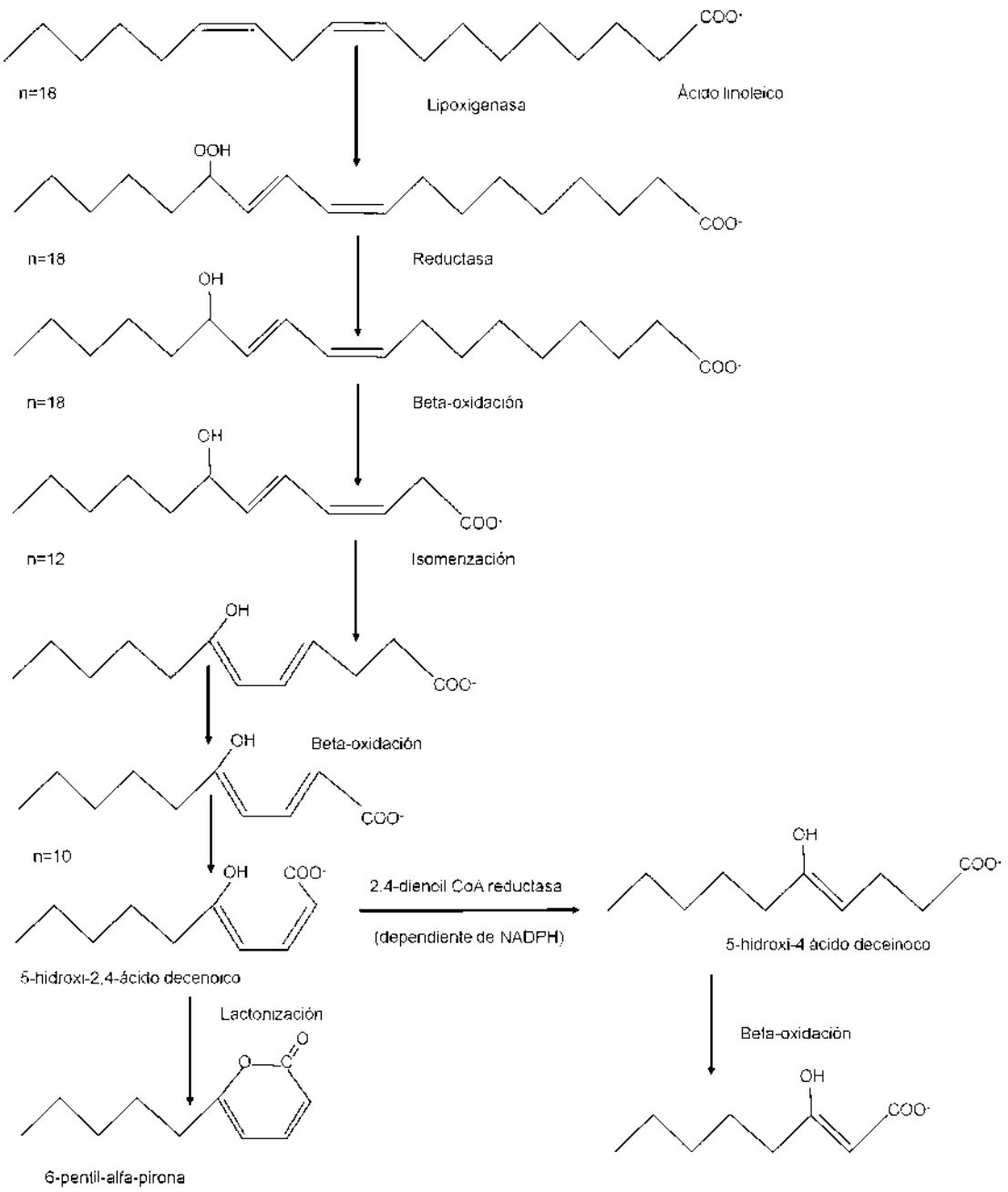

Figura 10. Biosíntesis de la 6-PP. El ácido linoleico es oxidado, dando origen al intermediario 13-hidroperóxido (13-HPOD), el cual por medio de β-oxidación es metabolizado e isomerizado para formar el 5-hidroxi-2,4-ácido decenoico. Finalmente, la lactonización ocurre por un ciclo adicional de β-oxidación (Modificado de Serrano-Carreón *et al*., 1993).

Desde hace más de 40 años se ha investigado el papel multifacético de la 6-PP, diversos estudios demuestran su capacidad de inhibir el crecimiento de

fitopatógenos como *Rhizoctonia solani*, *Botrytis cinerea* y varias especies de *Fusarium* (Dennis y Webster, 1971). En otro estudio, se determinó la concentración local de la 6-PP en la interacción *Trichoderma*-patógenos, donde la respuesta metabólica de *T. harzianum* varía de acuerdo al patógeno, resaltando un incremento en la producción de 6-PP del 300 al 700% en presencia de *B. cinerea* (Cooney y Lauren, 1998). La 6-PP inhibe la biosíntesis de micotoxinas como el deoxinivalenol (DON) de *Fusarium graminearum* y el ácido fusárico en *Fusarium moniliforme* (Cooney *et al*., 2001; El-Hasan *et al*., 2008).

Parker y col. (1997) estudiaron la bioactividad de la 6-PP y varios análogos, observando que estos compuestos inhiben el crecimiento de los coleóptilos de trigo y la germinación de semillas de lechuga. En otro estudio trataron con 6-PP plantas de jitomate y canola infectadas con *B. cinerea* y observaron una reducción de los síntomas de enfermedad. Las plantas de jitomate asperjadas con 6-PP incrementaron su biomasa total y radicular, que correlaciona con una mayor ramificación en la raíz, sugiriendo que la 6-PP tiene una actividad auxínica (Vinale *et al*., 2008). En *Arabidopsis thaliana* expuesta a este volátil, se observó una disminución del crecimiento de la raíz primaria, en tanto se indujo la expresión del gen PR-1 (proteína relacionada a patógenos inducida por ácido salicílico), el factor de transcripción involucrado en la formación de tricomas (GL3) y el gen activado por etileno VSP2 indicando que la 6-PP regula la expresión de varios genes de defensa en *Arabidopsis* (Kottb *et al*., 2015). Aún se desconocen los mecanismos genéticos y moleculares por el cual las plantas censan este volátil.

3. JUSTIFICACIÓN

La 6-PP es un compuesto volátil producido por *T.atroviride* que estimula el crecimiento vegetal, pero se desconocen los mecanismos de señalización involucrados en dicho efecto. La señalización por auxinas es considerada una vía hormonal canónica en el desarrollo de la raíz, por lo que resulta de interés conocer los elementos genéticos y moleculares que modulan las respuestas a la 6-PP y los elementos de respuesta a auxinas que podrían participar en la percepción molecular de éste volátil.

4. HIPÓTESIS

La 6-pentil-2H-piran-2-ona de *T.atroviride* regula el crecimiento y el desarrollo de *Arabidopsis* mediante mecanismos dependientes de la ruta de señalización de auxinas.

5. OBJETIVOS

5.1. Objetivo general

Determinar el efecto de la 6-pentil-2H-piran-2-ona en el crecimiento y desarrollo de *Arabidopsis thaliana* y su relación con las vías de señalización de las auxinas.

5.2. Objetivos específicos

1. Analizar los patrones de producción de 6-PP durante el crecimiento del hongo y en su interacción con *Arabidopsis.*
2. Caracterizar el efecto de la 6-PP sobre el crecimiento y desarrollo de *Arabidopsis.*
3. Estudiar la participación de elementos genéticos en las vías de señalización de auxinas en la modulación del crecimiento y desarrollo de la raíz en respuesta a 6-PP.

6. RESULTADOS

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6.1. CAPÍTULO I.

The volatile 6-pentyl-2*H*-pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and *ETHYLENE INSENSITIVE 2* functioning

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Summary

• Plants interact with root microbes via chemical signaling, which modulates competence or symbiosis. Although several volatile organic compounds (VOCs) from fungi may affect plant growth and development, the signal transduction pathways mediating VOC sensing are not fully understood.

• 6-pentyl-2*H*-pyran-2-one (6-PP) is a major VOC biosynthesized by *Trichoderma* spp. which is probably involved in plant–fungus cross-kingdom signaling. Using microscopy and confocal imaging, the effects of 6-PP on root morphogenesis were found to be correlated with *DR5:GFP*, *DR5:VENUS*, *H2B::GFP*, *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* gene expression. A genetic screen for primary root growth resistance to 6-PP in wild-type seedlings and auxin- and ethylene-related mutants allowed identification of genes controlling root architectural responses to this metabolite.

• *Trichoderma atroviride* produced 6-PP, which promoted plant growth and regulated root architecture, inhibiting primary root growth and inducing lateral root formation. 6-PP modulated expression of *PIN* auxin-transport proteins in a specific and dose-dependent manner in primary roots. *TIR1*, *AFB2* and *AFB3* auxin receptors and *ARF7* and *ARF19* transcription factors influenced the lateral root response to 6-PP, whereas *EIN2* modulated 6-PP sensing in primary roots.

• These results indicate that root responses to 6-PP involve components of auxin transport and signaling and the ethylene-response modulator *EIN2*.

Introduction

Providing healthy food sources, grains, fuels and fiber to an ever-increasing global population is one of the greatest challenges of this century. New techniques and products are needed for sustainable crop productivity without damaging soil and water resources. The *Trichoderma* genus includes species that naturally associate with plant roots and are considered highly versatile beneficial fungi (Harman *et al.*, 2004; Harman, 2011; Mukherjee *et al.*, 2013). Among their various attributes, *Trichoderma* spp. benefit agricultural activities, acting as biofungicides and in bioremediation of soils contaminated with metals or chemical wastes, and eliciting plant development and defense (Chang *et al.*, 1986; Björkman *et al.*, 1998; Björkman, 2004; Vargas *et al.*, 2009; Velázquez-Robledo *et al.*, 2011; Samolski *et al.*, 2012; Pereira *et al.*, 2014; Zhao *et al.*, 2014). These fungi produce plant growth-promoting compounds, which have the capacity to increase photosynthesis and biomass production and to elicit developmental programs via regulation of gene expression (Chacón *et al.*, 2007; Shoresh & Harman, 2008; Vargas *et al.*, 2009, 2011; Harman, 2011; Studholme *et al.*, 2013; Martínez-Medina *et al.*, 2014; Pereira *et al.*, 2014; Rubio *et al.*, 2014).

Trichoderma virens and *Trichoderma atroviride* produce the auxins indole-3-acetic acid (IAA), indole-3-ethanol (IET), indole-3-acetaldehyde (IALD) and indole-3-carboxaldehyde (ICALD). These compounds stimulate cell division, elongation and/or differentiation processes, ultimately increasing the growth and yield of the plant host (Contreras-Cornejo *et al.*, 2009, 2011). The role of auxins from *Trichoderma* in plant morphogenesis was investigated in detail in *Arabidopsis thaliana* by Contreras-Cornejo *et al.* (2009). Fungal colonization of *A. thaliana* roots induced the expression of the auxin-inducible gene marker *DR5:uidA* and increased development of lateral roots and root hairs. It was found that mutations in genes involved in auxin transport or signaling, including *AUX1*, *BIG*, *EIR1* and *AXR1*, reduced the beneficial effects of *Trichoderma* on biomass production and root branching. Interestingly, supplementation of *A. thaliana* seedlings with all identified *Trichoderma* auxins showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (mM range). In particular, application of ICALD

inhibited primary root growth, induced adventitious root formation and increased the camalexin concentration in leaves, thus suggesting a possible connection of auxin signaling with defense and development (Contreras-Cornejo *et al.*, 2011). Recent research has further highlighted the critical role of auxin production by *Trichoderma* in phystostimulation not only under standard growth conditions but also under stress imposed by abiotic factors (Mastouri *et al.*, 2010, 2012; Rawat *et al.*, 2013, Contreras-Cornejo *et al.*, 2014a; Hashem *et al.*, 2014).

The relationship between fungal produced auxins and root developmental programs elicited by *Trichoderma* was found to depend on mitogen activated protein kinase (MAPK) signaling (Contreras-Cornejo *et al.*, 2015). Co-cultivation of *A. thaliana* roots with *T. atroviride* modulated lateral root growth and root hair formation and increased MPK6 activity, these effects probably being dependent on ethylene (ET) and auxin signaling. It was also found that ET, IAA and IALD produced by the fungus induced MPK6 activity, while auxin-inducible *DR5:uidA* gene expression was concomitantly enhanced in *A. thaliana* mutants defective in the CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) protein, a negative regulator of the ethylene response pathway, which is thought to function as a MAPK kinase kinase. Detailed analysis of root hair and lateral root responses to *T. atroviride* in *A. thaliana* wild-type (WT) seedlings and ethylene-related mutants *etr1*, *ein2* and *ein3* showed that the effect of ET on root morphogenesis was apparently mediated by auxin–ethylene crosstalk involving MPK6, which fine-tunes seedling growth and development in response to *Trichoderma* (Contreras-Cornejo *et al.*, 2015). As a consequence, MPK6, and its MAP kinase associated cascade, probably involving CTR1 and other components still to be identified, seems to be a regulation node to maintain and/or amplify the hormonal effects underlying plant development and/or defense.

The production of bioactive metabolites in *Trichoderma* spp. is strain-dependent and, along with auxins, these metabolites include volatile and nonvolatile substances such as sesquiterpenes, 6-pentyl-2*H*-pyran-2-one (6-PP), gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Reino *et al.*, 2008; Vinale *et al.*, 2008). Exposure of *A. thaliana* seedlings to volatile organic compound (VOC) blends emitted by *Trichoderma* increased root branching and biomass production and accelerated flowering (Hung *et al.*, 2013; Contreras-Cornejo *et al.*, 2014b). Harzianolide and 6-PP promoted growth of pea (*Pisum sativum*) stems and tomato (*Lycopersicum esculentum*) and canola (*Brassica napus*) seedlings. Tomato plants sprayed with 6-PP had increased biomass and a highly branched root system, which may account for improved water and nutrient acquisition (Vinale *et al.*, 2008). These findings suggest that some *Trichoderma* metabolites may be interpreted by plants as transkingdom signals to modulate plant morphogenesis, but, currently, little is known about the cellular, genetic and molecular mechanisms by which plants sense these fungal metabolites.

Because 6-PP is involved in many developmental processes of fungal growth and has emerged as a plant bioactive metabolite (Vinale *et al.*, 2008), it is important to uncover molecular components specific to root architecture remodeling and their relationship with plant genetic programs. Here, we show that *Trichoderma atroviride* produces 6-PP, whose concentrations increase in co-cultivation with *A. thaliana* seedling. Supplying *A. thaliana* seedlings with 6-PP enhanced shoot and root biomass production in a dose-dependent manner and improved root branching and root hair growth. 6-PP did not induce an auxin or ethylene response in primary root tips or aerial plant parts, but increased auxin responsiveness in lateral root primordia and differentially modulated expression of auxin transporters PIN1, PIN2, PIN3 and PIN7. A genetic screen for 6-PP resistance established that this compound required auxin receptors TIR1, AFB2 and AFB3 and downstream transcription factors ARF7 and ARF19 to stimulate lateral root development. Intriguingly, strong primary root growth resistance to 6-PP was conferred by a loss-of-function mutant of the ethylene response regulator EIN2, which indicates that root response to 6-PP did not occur constitutively in all tissues but rather showed clear preference for specific root tissues and signaling components. The plant response to 6-PP further uncovered the contribution of a specific component in the ethylene pathway in root architectural remodeling and highlights the complex network of signaling molecules involved in the fungal–plant interaction.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L., Heynh.) Columbia (Col-0) ecotype, the transgenic *A. thaliana* lines *DR5:GFP* (Ottenschläger *et al.*, 2003), *DR5:VENUS* (Brunoud *et al.*, 2012); *H2B::RFP* (Boisnard-Lorig *et al.*, 2001); *CycB1:uidA* (Colón-Carmona *et al.*, 1999); *PIN1::PIN1::GFP* (Benkova *et al.*, 2003), *PIN2::PIN2::GFP* (Blilou *et al.*, 2005), *PIN3::PIN3::GFP* (Žádníková *et al.*, 2010) and *PIN7::PIN7::GFP* (Blilou *et al.*, 2005) and the mutant lines *axr1-3* (Lincoln *et al.*, 1990), *aux1-7* (Picket *et al.*, 1990), *tir1/afb2/afb3* (Parry *et al.*, 2009), *arf7-1/arf19-1* (Wilmoth *et al.*, 2005), *eir1* (Roman *et al.*, 1995), *etr1* (Hua & Meyerowitz, 1998), *ein2* (Guzmán & Ecker, 1990), and *ein3* (Chao *et al.*, 1997) 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 × Murashige and Skoog (MS) medium (Murashige and Skoog basal salts mixture). The MS medium was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL (Grand Island, NY, USA). Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L; Percival Scientific, Perry, IA, USA), with a photoperiod of 16 h : 8 h, light : dark, a light intensity of 300 μmol m^2 s^{-1}, and a temperature of 22°C.

Fungal growth and plant inoculation experiments

Trichoderma atroviride Karsten (formerly *Trichoderma harzianum*) IMI 206040 was used. An inoculum of 1×10^6

spores was placed at 5 cm from *A. thaliana* primary roots germinated and grown for 4 d on agar plates containing 0.2 × MS medium. The plates, which included 10 *A. thaliana* seedlings each, were arranged in a completely randomized design in a Percival AR95L growth chamber. After 3 and 5 d of co-cultivation, determinations of 6-PP accumulation and plant growth were performed.

Effect of 6-PP on plant growth and development

6-PP (purchased from Sigma) was dissolved in ethanol. To investigate whether 6-PP could have an effect on *A. thaliana* growth, the compound was supplied at different doses (0, 50, 75, 100, 125, 150, 175 and 200 μM) to the plant growth medium. In control conditions ('C' in most figure panels), we added an ethanol volume equal to that present in the highest compound concentration. Petri plates containing 30 plants under different treatments were placed in a Percival AR95L growth chamber for 10 d to estimate biomass production.

Arabidopsis thaliana root system and primary root (PR) meristem integrity were analyzed with a stereoscopic microscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany). All lateral roots (LRs) that emerged from the PR were counted at ×30 magnification. Images were taken with a Samsung SCC 131-A digital color camera adapted to the microscope and processed with the Zeiss AXIO VISION 4AC software (Carl Zeiss). PR length was measured for each root using a ruler. LR density was determined by dividing the LR number by the PR length for each seedling analyzed.

Propidium iodide staining and GFP, VENUS and RFP detection

For confocal microscopy, solvent- or 6-PP-treated transgenic *A. thaliana* seedlings were transferred from the growth medium to 10 mg ml^{-1} propidium iodide solution for 1 min. Seedlings were rinsed in water and mounted in 50% (v/v) glycerol on microscope slides. Each sample was analyzed separately for propidium iodide (with a 568-nm wavelength argon laser for excitation, and an emission window of 585–610 nm) and GFP, VENUS or RFP fluorescence (488 nm excitation/505–550 nm emission, 514 nm excitation/527 nm emission, and 532 nm excitation/588 nm emission, respectively), using a confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan), after which the two micrographs were merged to produce a final image. Fifteen independent seedlings were analyzed per line, and treatment representative images were selected for figure construction.

Determination of developmental stages of lateral root primordia

Lateral root primordia (LRPs) were quantified 6 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 & 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 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.

Analysis of VOCs and 6-PP determinations

The VOCs released by *T. atroviride* were analyzed in Petri dishes containing 0.2 × MS medium with a solid-phase microextraction (SPME) technique and GC-MS. The compounds were collected for 1 h with a blue SPME fiber (PDMS/DVB; Supelco Inc., Bellafonte, PA, USA) and desorbed at 180°C for 30 s in the injector port of a gas chromatograph (Agilent 7890B; Agilent, Foster City, CA, USA), equipped with an MS detector (5977A; Agilent) and Mass Hunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA) for data acquisition and processing. A free fatty acid-phase capillary column (HP-FFAP) (30 m × 0.25 mm ID; film thickness 0.25 μm) was used. In the operating conditions, helium was used as the carrier gas (1 ml min^{-1}) and the detector temperature was 250°C. The column was held for 1 min at 60°C, and then programmed to rise at a rate of 3°C min^{-1} to a final temperature of 180°C, which was maintained for 1 min. Three independent determinations were made. The mass fragments were analyzed using electron impact ionization at 70 eV and a scan rate of 1.9 scan s^{-1}. Fragments were read from 40 to 450 Da, and data were evaluated using total ion count (TIC). The chromatograms of the eluted compounds were deconvoluted and their mass spectra matched with those of the NIST 11 mass spectral database.

The identification of 6-PP was performed by comparing retention time (Rt) and the mass spectra from an authentic standard with those obtained in the sample. To estimate the amount of 6-PP produced by *T. atroviride* from 3 and 5 d of growth and during the interaction *T. atroviride–A. thaliana*, we constructed an external calibration curve using a 6-PP standard following a similar method to that established by Polizzi *et al.* (2011). A diluted solution of 6-PP in ethanol was prepared. Petri dishes were filled with 0.2 × MS medium; upon cooling of the agar, a piece of foil (1 cm^2) was placed on the top with different concentrations (10 μM to 10 mM) of 6-PP. The Petri plates were immediately closed and sealed with parafilm and analyzed under the same conditions as used for the fungal samples. A good linearity of the calibration curve ($r^2 = 0.999$) was found.

Data analyses

For all experiments with WT and mutant lines, the overall data were statistically analyzed using SPSS 10 Software (IBM Corp., Endicott, NY, USA). Univariate and multivariate analyses with Tukey's post hoc test were used to assess the significance of

differences in growth and root development responses. Different letters are used to indicate means that differ significantly ($P < 0.05$). GFP fluorescence in primary root tips was quantified by determining the green pixels present in an area comprised of the first 20 cells upward from the quiescent center, using the ImageJ software (http://rsbweb.nih.gov/ij/), in 15 micrographs per line and treatment. We then obtained an arbitrary unit value (AU = green pixels μm^{-2}) for each individual, and means were obtained from whole data sets. AU means for control conditions were given a value of 1, and those for 6-PP treatments were adjusted relative to these, and are thus referred to in figures as relative fluorescence. *DR5:GFP* fluorescence in LR formation zones was quantified similarly, except that the area measured comprised the whole micrographs and statistics for these were omitted because of technical difficulties in obtaining images on the same focal plane.

Results

6-PP is the most abundant compound within the VOC profile of *T. atroviride*

Previous reports have shown the VOC profile from *T. atroviride* grown in potato dextrose agar (PDA), malt extract agar (MEA), or biomalt medium (BM) (Keszler *et al.*, 2000; Stoppacher *et al.*, 2010; Siddiquee *et al.*, 2012; Jeleń *et al.*, 2014; Lee *et al.*, 2015). All this research identified the compound 6-PP within the corresponding VOC profile. To assess the possible roles of 6-PP during the interaction of *T. atroviride* with plants, in this study we analyzed the VOCs emitted from *T. atroviride* in fungal colonies grown for 5 d in Petri plates supplied with 0.2 × MS agar solidified medium. This medium was chosen because it is commonly used for *A. thaliana* growth and the effects of 6-PP on plants could then be evaluated. Table 1 shows that 6-PP is the major compound within the VOC profile (57.94%) from *T. atroviride*. This compound is an alkyl lactone, with an unsaturated six-membered ring containing one oxygen atom and a ketone functional group. The isomer found in *T. atroviride* according to GC-MS analysis is denoted 2-pyrone, with an alkyl group at the 6-position (Fig. 1a). The identification of 6-PP was made by comparison of the Rt (37.21 min) and mass spectra of a standard (Fig. 1b) with those obtained from *T. atroviride* colonies (Fig. 1c).

Table 1 Volatile organic compounds produced by *Trichoderma atroviride* after 5 d of growth in 0.2 × MS medium, analyzed by solid-phase microextraction (SPME)-GC-MS

Compound	Normalized amount of volatile compound (%)
1,3-Octadiene	1.24 ± 0.17
2-Heptanone	7.17 ± 0.83
3-Octanone	11.4 ± 1.17
2-Nonanone	1.11 ± 0.08
3-Octanol	1.08 ± 0.05
1-Octen-3-ol	6.82 ± 2.15
α-Bergamotene	5.51 ± 0.11
2-Undecanone	1.72 ± 0.13
3-Methyl-1-octene	0.88 ± 0.04
β-Sesquiphellandrene	1.49 ± 0.15
Unknown (a 204 mw sesquiterpene)	0.86 ± 0.08
Unknown (a 204 mw sesquiterpene)	1.71 ± 0.18
Unknown (a 204 mw sesquiterpene)	1.07 ± 0.09
6-Pentyl-2*H*-pyran-2-one (6-PP)	57.94 ± 2.70

Compounds were tentatively identified on the basis of NIST 11 MS Spectral library searches. Mean values ± SE of the sum of three independent determinations are given.

To determine whether plant interaction could affect 6-PP production by the fungus, we next estimated 6-PP amounts in the plates containing single *T. atroviride* colonies and at 3 and 5 d of direct interaction with *A. thaliana* seedlings. It was observed that 6-PP emission increased with time (Fig. 1d). Interestingly, at 5 d of interaction with plants, when fungi had physical contact with the root system, the emission of the compound increased by 40% compared with the level recorded for single colonies (Fig. 1d). At this stage, an induction of root branching by *T. atroviride* was evident (Fig. 1e), indicating the possible participation of 6-PP in the lateral root formation process.

6-PP increases biomass production, root branching and root hair development in *A. thaliana* seedlings

To investigate the plant growth-regulating activity of 6-PP, we tested the effects of increasing, low micromolar doses of this compound in *A. thaliana* (Col-0) seedlings, germinated and grown on Petri plates containing agar-solidified 0.2 × MS medium. The seedlings were treated with ethanol (control treatment) or with 50–200 μM 6-PP dissolved in ethanol. After 10 d of growth in medium supplied with 50–175 μM 6-PP, a roughly two-fold increase in shoot, root and total plant biomass was observed (Fig. 2a–c). By contrast, the greatest concentration (200 μM) of the compound did not increase biomass accumulation (Fig. 2a–c). Representative photographs of plates illustrating the effects of 6-PP are shown in Fig. 2(d–g) and photographs of individual plants are provided in Supporting Information Fig. S1. It is noteworthy that 6-PP treatments increased both lateral root number and density in a dose-dependent manner, while an inhibition of primary root growth was observed from 125 μM onwards (Fig. 3a–c). To determine if the toxic effects of high 6-PP are responsible for primary root growth inhibition, we analyzed the expression of the vital marker *H2B:RFP*, which is specifically expressed in the nuclei of living cells (Boisnard-Lorig *et al.*, 2001), by confocal microcopy. Our data indicate that 6-PP did not affect cell integrity in primary roots, as cells of roots supplied with 200 μM 6-PP did not show cell damage or an absence or nuclei (Fig. S2a–d). To investigate the pattern of cell division in response to 6-PP, we analyzed the expression of *CyCB1:uidA*, which is expressed only in cells in the G2/M transition of the cell cycle in the primary root meristem (Colón-Carmona *et al.*, 1999). Strong primary root growth inhibition under 150 μM or greater concentrations of 6-PP correlated with a reduction of GUS expression in the primary root meristem of *CycB1:uidA*-expressing seedlings

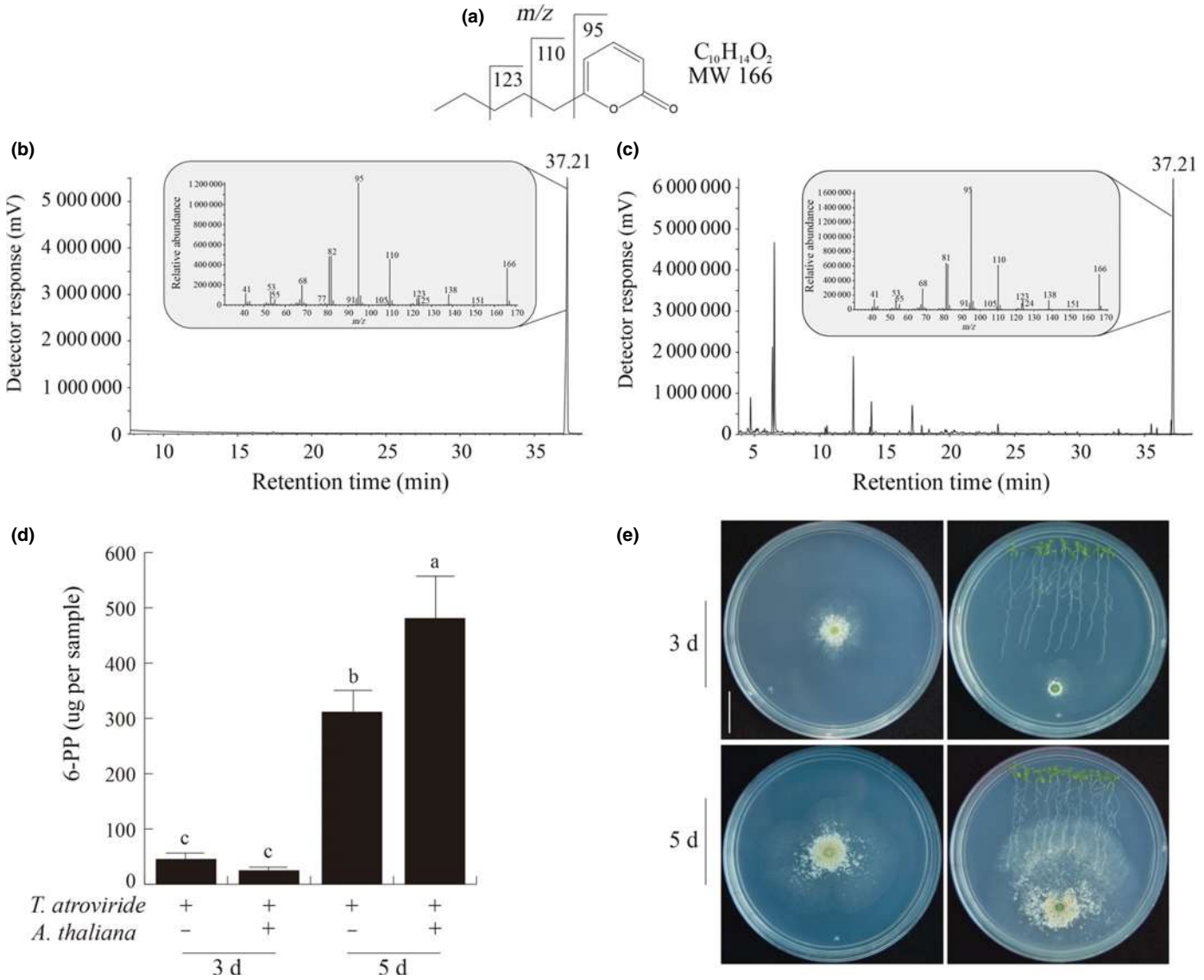

Fig. 1 Molecular characterization and production of 6-pentyl-2*H*-pyran-2-one (6-PP) by *Trichoderma atroviride* IMI 206040. (a) Chemical structure of 6-PP showing the major fragment ions (*m/z*) of electron ionization mass spectra. (b) Total ion chromatogram and mass spectra from commercial standard (6-PP; Rt = 37.21 min). (c) Total ion chromatogram of volatile organic compounds (VOCs) from the fungus, indicating the presence of 6-PP at a retention time (Rt) of 37.21 min. 6-PP was identified by comparison of mass spectra in the NIST 2011 library and those of a commercial standard. (d) Estimation of 6-PP content in *T. atroviride* and the *Arabidopsis thaliana*–*T. atroviride* interaction system. (e) Representative photographs of the fungal colonies at 3 and 5 d of growth and during the interaction with plants. Bar, 1 cm.

(Fig. S3a–j). The 6-PP effects on primary root growth were accompanied by increased root hair formation and elongation, but decreased mature trichoblast cell length (Fig. S4a–k), suggesting that high 6-PP concentrations inhibit root growth, affecting cell division and elongation programs.

We next determined the stages of LRP development affected by 6-PP by quantifying the number of stage I–VII LRPs originating from primary roots 6 d after germination (dag) in seedlings treated with the solvent (control) or 75 or 150 μM 6-PP; this last treatment strongly increased LR density (Fig. 3c). We found that the stage distribution of LRPs was clearly modulated by treatment with 6-PP. In particular, LRP stages I–VI, which represent young LRPs, were significantly decreased in 6-PP-treated seedlings (Fig. 4a). By contrast, the number of emerged LRs was increased two- or three-fold by 150 μM 6-PP in seedlings at 4 and 6 dag, respectively (Fig. 4b). The total number of LRPs per seedling decreased in response to 6-PP treatments (Fig. 4c), whereas the LRP density decreased (75 μM 6-PP) or did not significantly differ (150 μM 6-PP) among treatments (Fig. 4d). These data indicate that 6-PP probably increases LR branching by inducing the emergence of preformed LRPs from pericycle cells and accelerating the growth of LRs.

6-PP regulates primary and lateral root development through auxin signaling

LR development is tightly correlated with auxin signaling (Fukaki *et al.*, 2007). To understand the role played by 6-PP in

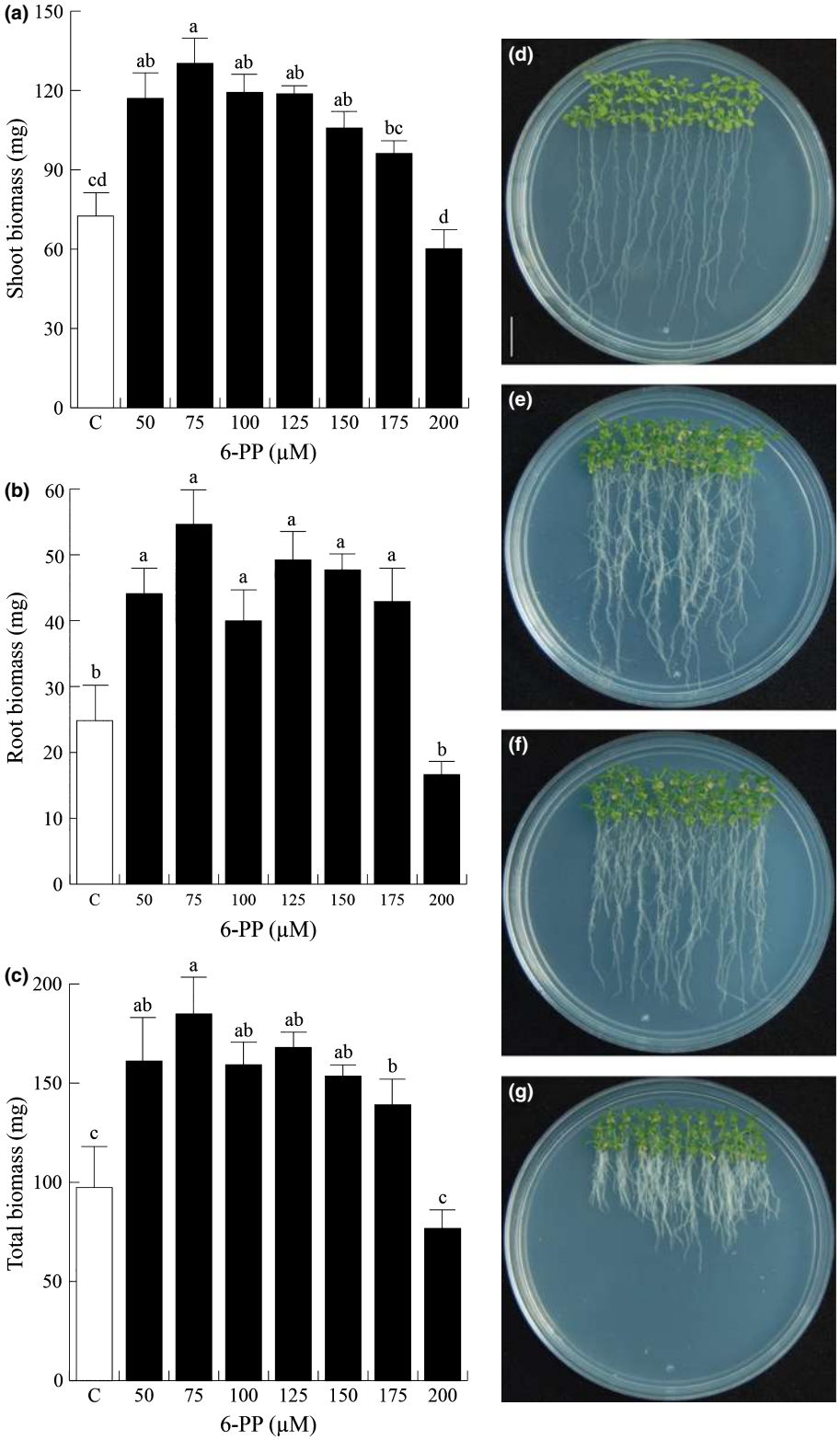

Fig. 2 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on plant biomass production. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 12 d under increasing 6-PP concentrations. (a) Shoot biomass. (b) Root biomass. (c) Total biomass. (d) Representative photographs of seedlings grown in (d) 0.2 × MS medium supplied with the solvent or (e) 75, (f) 125, and (g) 175 μM 6-PP supplemented media. Photographs show representative plates; each treatment included three plates. Data from (a–c) show the mean ± SD for three groups of 30 seedlings that were recovered from the medium, excised at the root–shoot junction, and weighed using an analytical scale. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results. Bar, 1 cm.

root system architecture remodeling and its possible relationship with auxin signaling, we analyzed the expression of the auxin responsive marker *DR5:GFP* in primary root tips, emerging LRs and LRPs in transgenic *A. thaliana* seedlings expressing this marker and exposed to 75 and 150 μM 6-PP. *DR5:GFP* expression did not increase in primary root tips or in emerging LRs at 75 μM or higher 6-PP concentrations (Fig. 5a–f). However, an analysis of *DR5:GFP* expression in stage II and V LRPs showed an enhanced auxin-inducible expression in the vasculature of primary roots and in developing primordia (Fig. 5g–l).

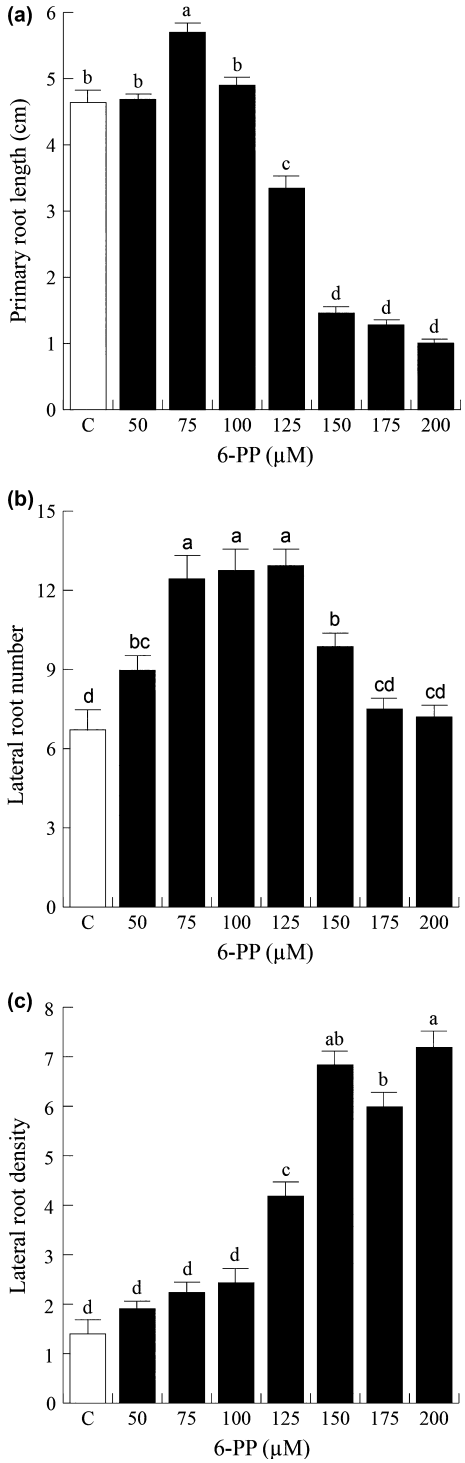

Fig. 3 6-pentyl-2*H*-pyran-2-one (6PP) regulates *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a) Primary root length. (b) Number of emerged lateral roots. (c) Lateral root density (number of emerged lateral roots cm^{-1}). Values represent the means of 30 seedlings ± SD. Different letters represent means that are statistically different ($P < 0.05$). The experiment was repeated three times with similar results.

To further analyze possible changes in auxin accumulation and/or responsiveness in primary root tips caused by 6-PP, a detailed analysis was conducted in *A. thaliana* seedlings expressing *DR5:VENUS* treated with a concentration of auxin (IAA), or an auxin transport inhibitor (*N*-(1-naphthyl)phthalamic acid (NPA)), which represses root growth, or with increasing 6-PP concentrations. As expected, both IAA and NPA treatments increased the auxin maximum domains in primary root tips, whereas only the highest 6-PP concentrations tested (175 and 200 μM) produced slightly decreased *DR5* expression in root tips (Fig. S5a–i). These data indicate that 6-PP did not induce auxin accumulation and/or response in primary root tips but increased the auxin response at early stages of LR development.

6-PP modulates the expression and distribution of auxin transporters in primary roots

Auxin is transported through the PIN family of proteins, which are expressed in a tissue-specific manner (Vieten *et al.*, 2005). To test whether 6-PP could regulate primary root growth and/or LR formation through differential expression of the PIN family of auxin transporters, we analyzed the pattern of PIN1, PIN2, PIN3 and PIN7 localization in primary roots and LRPs of seedlings expressing *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP*. In seedlings grown in medium lacking 6-PP, GFP fluorescence driven by PIN1, PIN3 and PIN7 was detected mainly in the stele of primary roots (Fig. 6a,i,m). By contrast, PIN2 expression was detected in the cortex and epidermal cells (Fig. 6e). In transgenic seedlings expressing GFP fusions with PIN1, PIN2 and PIN3 supplied with 75 μM 6-PP, the GFP fluorescence was significantly increased (Fig. 6b,f,j), whereas when treated with 150 μM 6-PP the opposite effect was observed for PIN1, PIN2 and PIN7 localization, as shown by decreased GFP florescence (Fig. 6c,g,o). In marked contrast to the other PIN transporters, PIN3 localization in response to 150 μM 6-PP still displayed a strong expression in the stele (Fig. 6k). These findings suggest that 6-PP affects the expression and distribution of the PIN auxin transporters in primary roots and that root responses to 6-PP did not occur in all tissues but rather showed clear preference for specific tissues and transport components.

Effect of 6-PP on primary and lateral root development of auxin- and ethylene-related *A. thaliana* mutants

Ethylene–auxin interactions regulate primary root growth and LR initiation and emergence in *A. thaliana* (Ivanchenko *et al.*, 2008). To further determine whether there is crosstalk between auxin and ethylene in controlling root responses to 6-PP, we analyzed the response of WT and *A. thaliana* triple, double or single mutants affected in genes related to auxin transport or response (*tir1afb2afb3*, *arf7arf19*, *axr1-3*, *aux1-7* and *eir1*) and ethylene response (*etr1*, *ein2* and *ein3*) to 6-PP treatments. To investigate the involvement of auxin in primary and lateral root responses to 6-PP, *A. thaliana* WT and mutant lines were grown in medium supplemented with the solvent only or with 150 μM 6-PP, and primary root growth and LR formation were analyzed at 10 dag. It was found that all five auxin-related mutants tested showed WT responses to 6-PP in terms of primary root growth

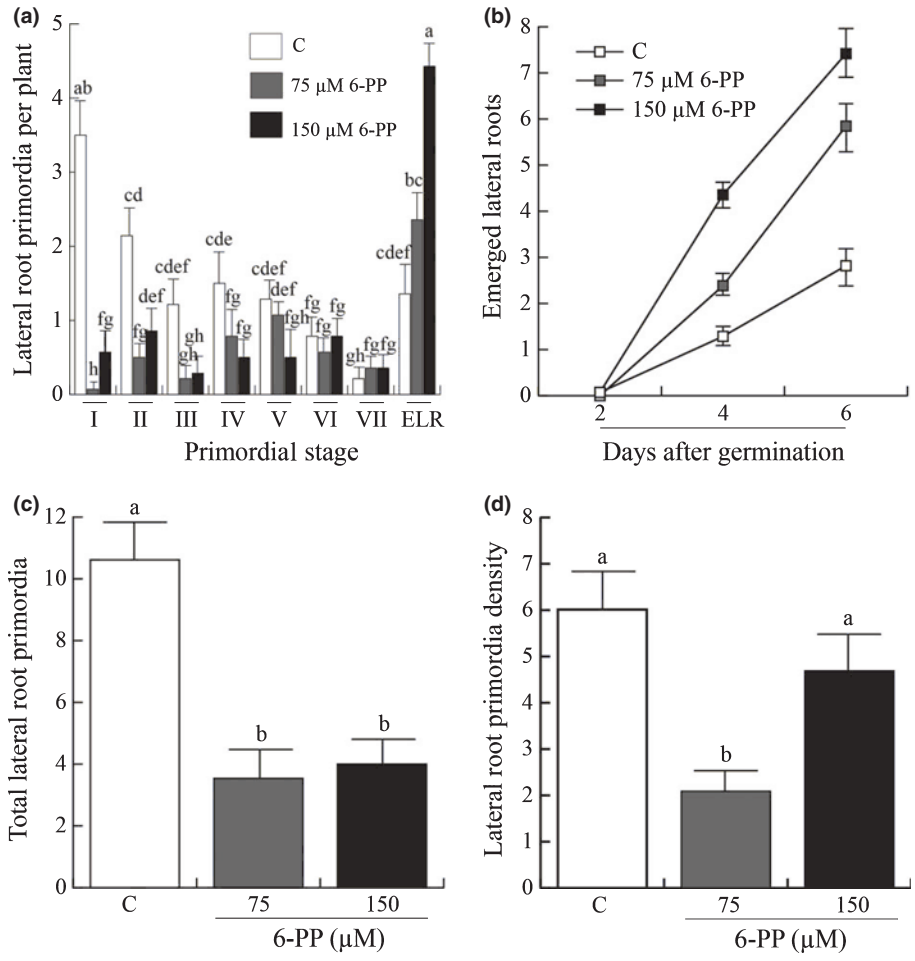

Fig. 4 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on lateral root (LR) development in *Arabidopsis thaliana*. Wild-type (Col-0) seedlings were germinated and grown for 2, 4 and 6 d on 0.2 × MS media supplemented with the solvent (control (C)), or 75 or 150 μM 6-PP. (a) Lateral root primordia (LRPs) per plant in 4-d-old seedlings. (b) Kinetics of emerged LRs in seedlings grown for 2, 4 and 6 d. (c) Total LRPs per plant. (d) LRP density in 4-d-old seedlings. Error bars represent ± SE for 15 *GUS*-stained seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

inhibition (Fig. 7a). By contrast, an induction of LR formation was lacking in *tir1afb2afb3*, *arf7arf19*, *axr1-3* and *aux1-7*, while *eir1* seedlings showed increased LR formation in response to 6-PP (Fig. 7b,c). To analyze possible auxin resistance in some selected auxin-related mutants, we performed an experiment comparing primary root growth of WT (Col-0), *axr1-3*, *aux1-7* and *arf7arf19* seedlings in medium supplied with concentrations of NPA or IAA that strongly repress primary root growth in the WT (Fig. S6). As expected, all three mutants tested had clear resistance to inhibition of primary root growth by either NPA or auxin (Fig. S6). These data, together with the observation that the auxin-related mutants *axr1-3*, *aux1-7* and *arf7arf19* sustain normal primary root growth inhibition in response to 6-PP (Fig. 7a), suggest that an auxin-independent pathway is responsible of sensing 6-PP in primary roots.

In opposition to auxin, ethylene has been found to repress LR formation (Lewis *et al.*, 2011). Therefore, we focused our analysis on root response to 6-PP, considering primary root growth. Interestingly, the *ein2* mutant was clearly resistant to primary root growth inhibition even at growth-repressing concentrations of 150 μM 6-PP (Fig. 8a). This resistance was confirmed in a dose-response curve of growth from 75 to 200 μM 6-PP (Fig. 8b, c). Together, these data indicate that auxin signaling components mediate the LR responses to 6-PP, while EIN2 is a crucial component mediating the primary root growth inhibition response to this fungal signal molecule.

6-PP fails to induce an ethylene response

The sustained primary root growth of *ein2* mutants under growth-repressing concentrations of 6-PP suggested the possibility that the ethylene response could be induced after treatment with 6-PP. This possibility was investigated by comparing the effects of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and 6-PP on the morphology of etiolated seedlings. After 4 d of incubation at 22°C in darkness, seedlings germinated on agar plates containing 0.2 × MS medium were readily distinguished from seedlings germinated in medium supplied with 2 μM ACC, exhibiting highly elongated hypocotyls and forming an apical hook at the terminal part of the shoot axis (Fig. 9a). Conversely, ACC-treated seedlings developed the so-called 'triple-response' consistent with its role as an ethylene precursor (Guzmán & Ecker, 1990), which consists of exaggerated tightening of the apical hook and swelling of the hypocotyl (Fig. 9b), and inhibition of root and hypocotyl elongation (Fig. 9s,t). ACC also induced root hair development in dark-grown seedlings (Fig. 9u,v). The visual features of the root–stem transition zone treated with ACC and 6-PP are shown in

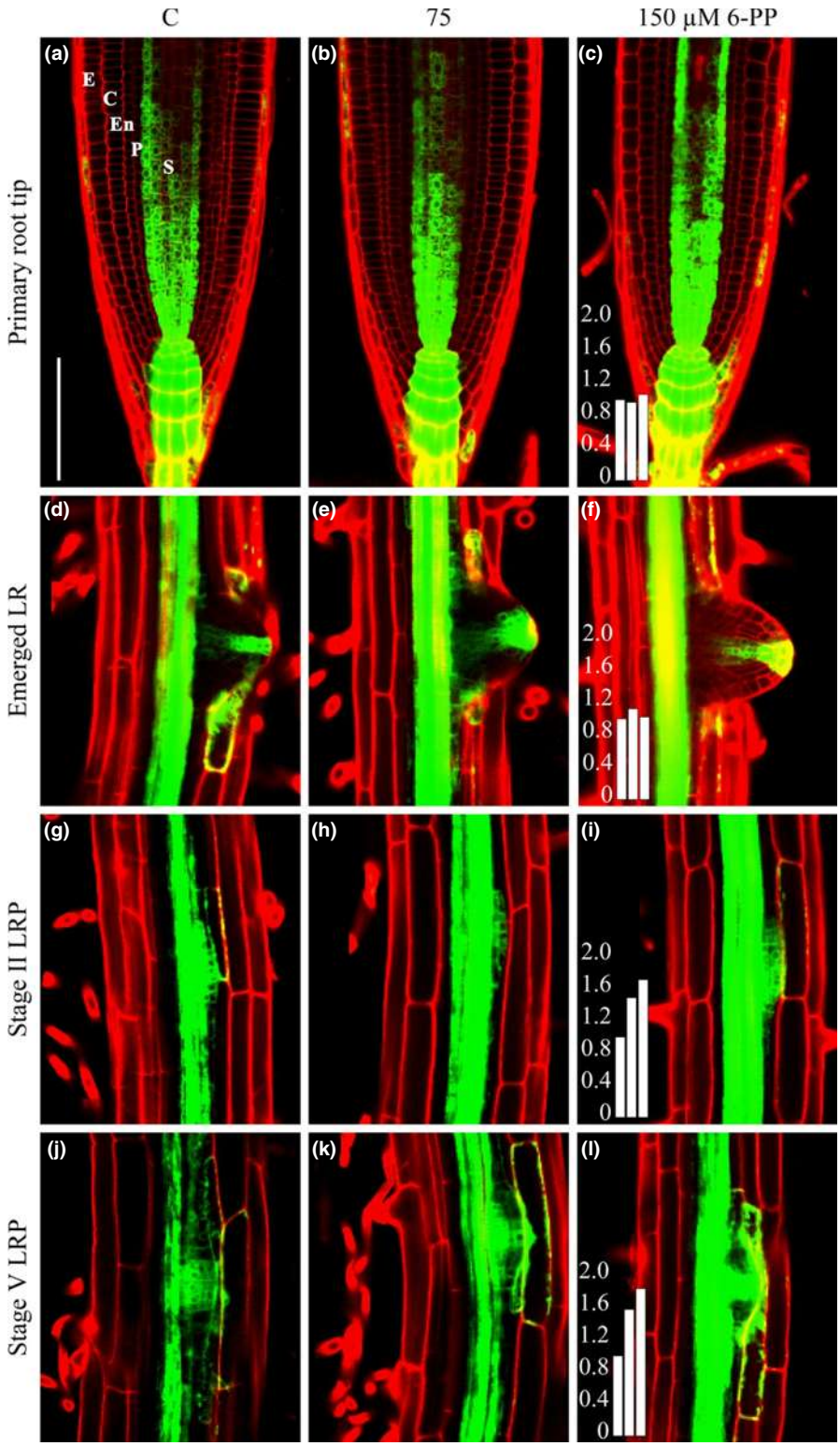

Fig. 5 6-pentyl-2*H*-pyran-2-one (6PP) modulates auxin-responsive gene expression in the lateral root formation zone. *Arabidopsis thaliana* transgenic *DR5: GFP* seedlings were grown in solvent added to 0.2 × MS medium (C), or supplemented with 75 or 150 µM 6-PP. Five days after germination, seedlings were stained with propidium iodide and analyzed by confocal microscopy. Micrographs show individuals representative of at least 15 seedlings. (a–c) Primary root tip; (d–f) emerged lateral roots; (g–i) stage II lateral root primordia; (j–l) stage V lateral root primordia. Note that 6-PP treatments increase *DR5: GFP* reporter expression in the lateral root formation zone, while it remains largely unchanged in root tips and emerged lateral roots. The graphs in (c, f, i, l) illustrate the differences in *DR5: GFP* expression between control (left bar) and 6-PP treatments (middle and right bars), assessed as relative fluorescence intensity. Bars, 100 µm.

Fig. 9(g–l), and those of the root tip in Fig. 9(m–r). Interestingly, 6-PP-treated seedlings did not develop the 'triple response' (Fig. 9c–f,s,t), and failed to form long root hairs at the differentiation zone of the primary roots (Fig. 9m–r,u,v). We also compared the growth of *A. thaliana* seedlings supplied with ACC or 6-PP and the ethylene inhibitor $AgNO_3$ simultaneously under 16h : 8 h, light : dark photoperiod conditions. Although both ACC and 6-PP were able to repress primary root growth, $AgNO_3$ specifically antagonized the ACC response, normalizing root growth without affecting the 6-PP response (Fig. S7). These data show that 6-PP did not induce an ethylene response in *A. thaliana* seedlings.

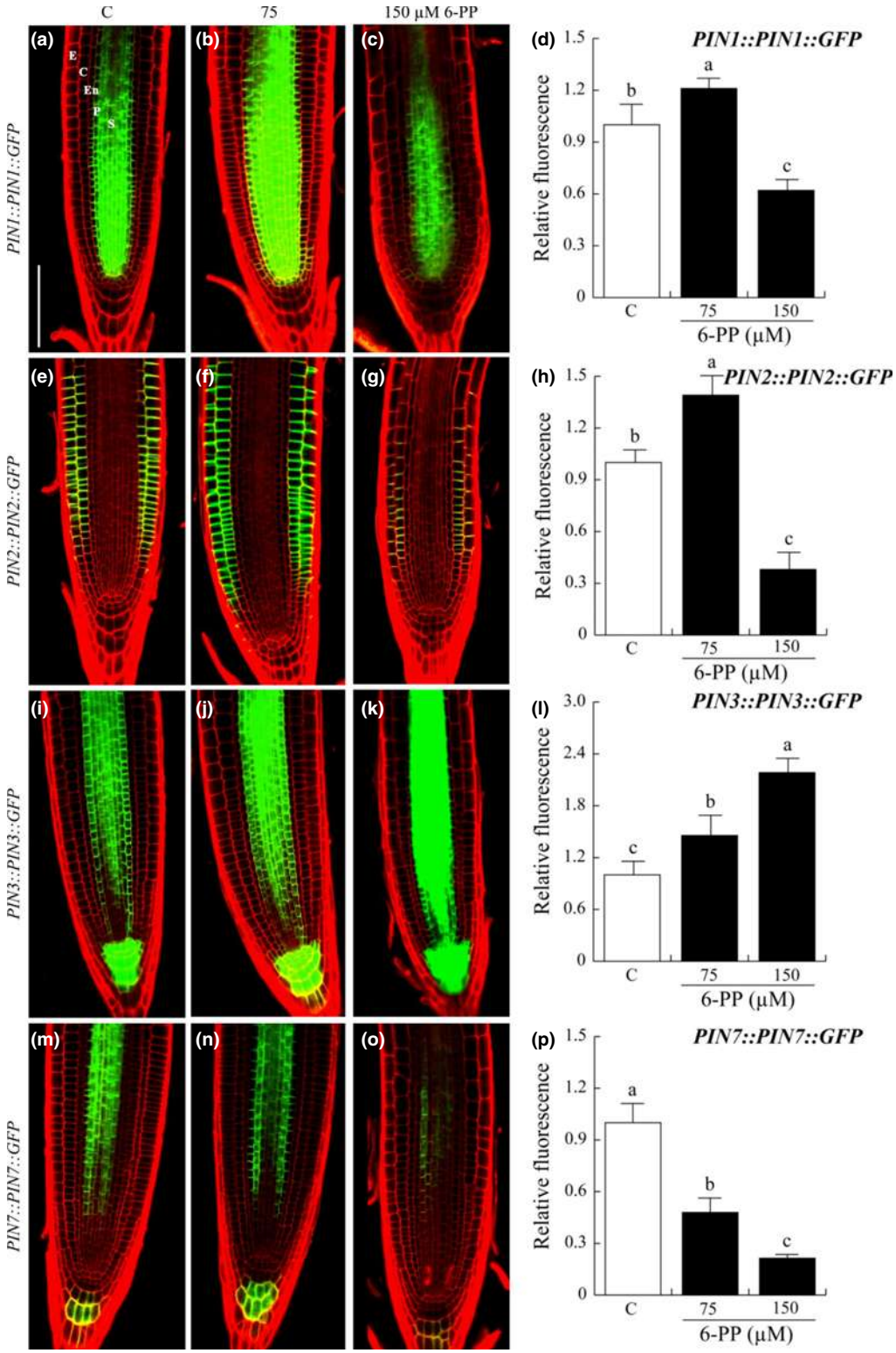

Fig. 6 Expression of auxin efflux transporters in response to 6-pentyl-2*H*-pyran-2-one (6PP) in primary roots. *Arabidopsis thaliana* transgenic *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* seedlings were grown in solvent (C), or 75 or 150 μM 6-PP supplemented medium. Five days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. Representative micrographs of primary root tips of (a–c) *PIN1::PIN1::GFP*, (e–g) *PIN2::PIN2::GFP*, (i–k) *PIN3::PIN3::GFP* and (m–o) *PIN7::PIN7::GFP* are shown ($n = 15$). Note that the increase of *PIN3::PIN3::GFP* expression is proportional to the increase in 6-PP treatments. Graphs in (d, h, l, p) illustrate differences in each reporter expression, assessed as relative fluorescence intensity. Values shown represent the means for 15 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). Bar, 100 μm. Letters in (a) are used to indicate cell files: E, epidermis; C, cortex; En, endodermis; P, pericycle; S, stele.

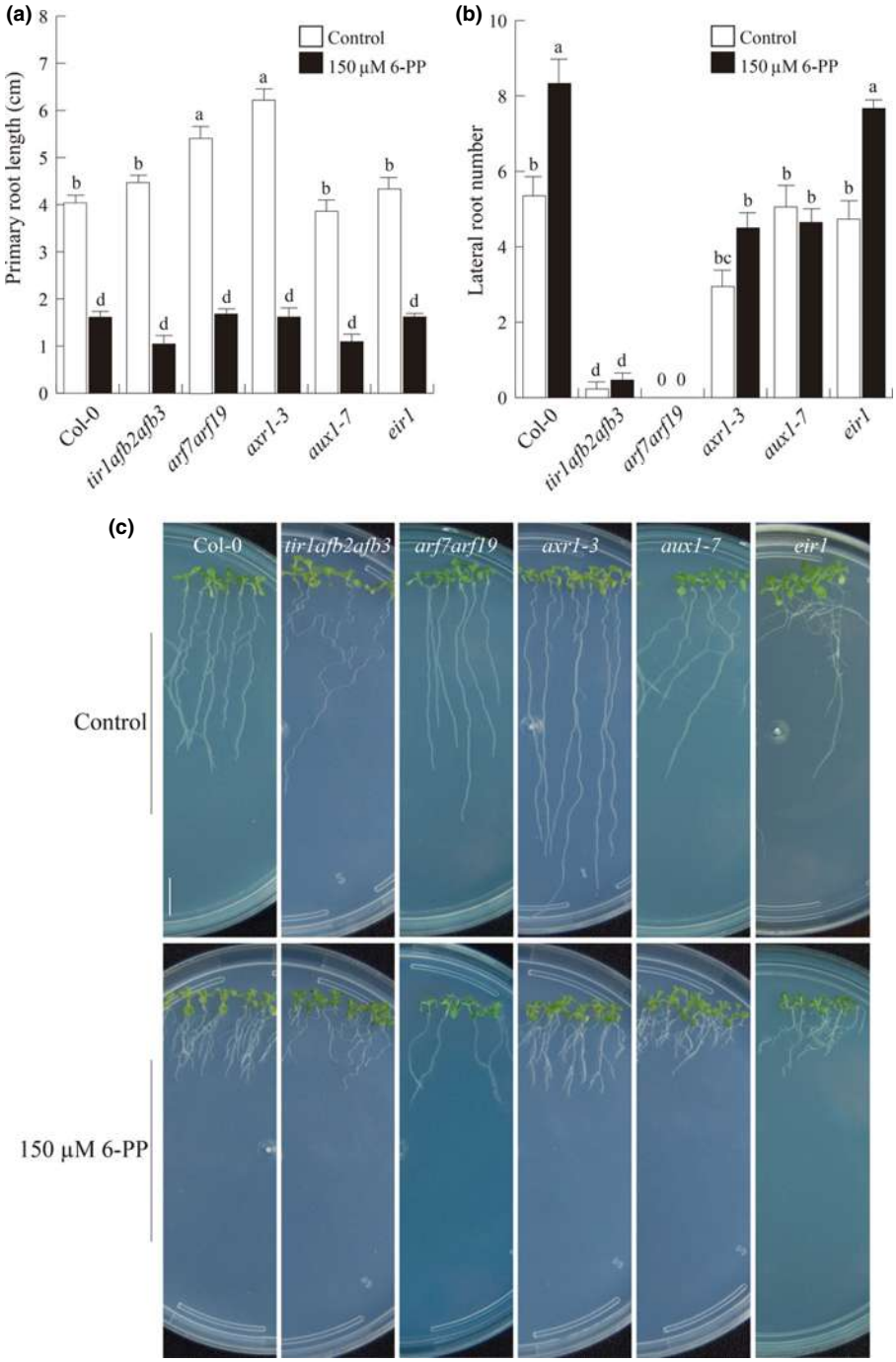

Fig. 7 6-pentyl-2*H*-pyran-2-one (6PP) requires components of auxin response and transport to modify *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* wild-type (Col-0) and *tir1/afb2/afb3*, *arf7-1/arf19-1*, *axr1-3*, *aux1-7* and *eir1*, triple, double or single mutant seedlings, respectively, were germinated and grown for 10 d in 0.2 × MS medium supplemented with the solvent (control) or 150 μM 6-PP. (a) Primary root length. (b) Lateral root number. (c) Representative photographs of *A. thaliana* seedlings grown in the indicated 6-PP treatment. Values shown represent the means of 15 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated twice with similar results. Bar, 1 cm.

Discussion

This study reveals a novel mechanism by which *T. atroviride* could promote plant growth and root branching via production of 6-PP. Recently, the production of auxins and auxin precursors has been reported from several *Trichoderma* species. In addition, over 180 secondary metabolites have been characterized to date, representing different classes of chemical compounds. These compounds can be classified as volatiles, diffusible compounds and peptaibols (Gams & Bissett, 1998; Reino *et al.*, 2008; Stoppacher *et al.*, 2010).

The current work builds on previous observations that fungal released volatiles increases biomass production and lateral root formation (Hung *et al.*, 2013; Contreras-Cornejo *et al.*, 2014b). *Trichoderma viride*, *T. harzianum*, and *T. koningii* are able to produce 6-PP, which plays a role in biocontrol of phytopathogens such as *Botrytis cinerea*, *Rhizoctonia solani*, and *Fusarium oxysporum*, and a strong relationship exists between the biosynthesis of this metabolite and the biocontrol ability of the producing strains (Scarselletti & Faull, 1994; Worasatit *et al.*, 1994). Interestingly, 6-PP may be involved in cross-kingdom signaling, as plants are able to respond to 6-PP by increasing growth

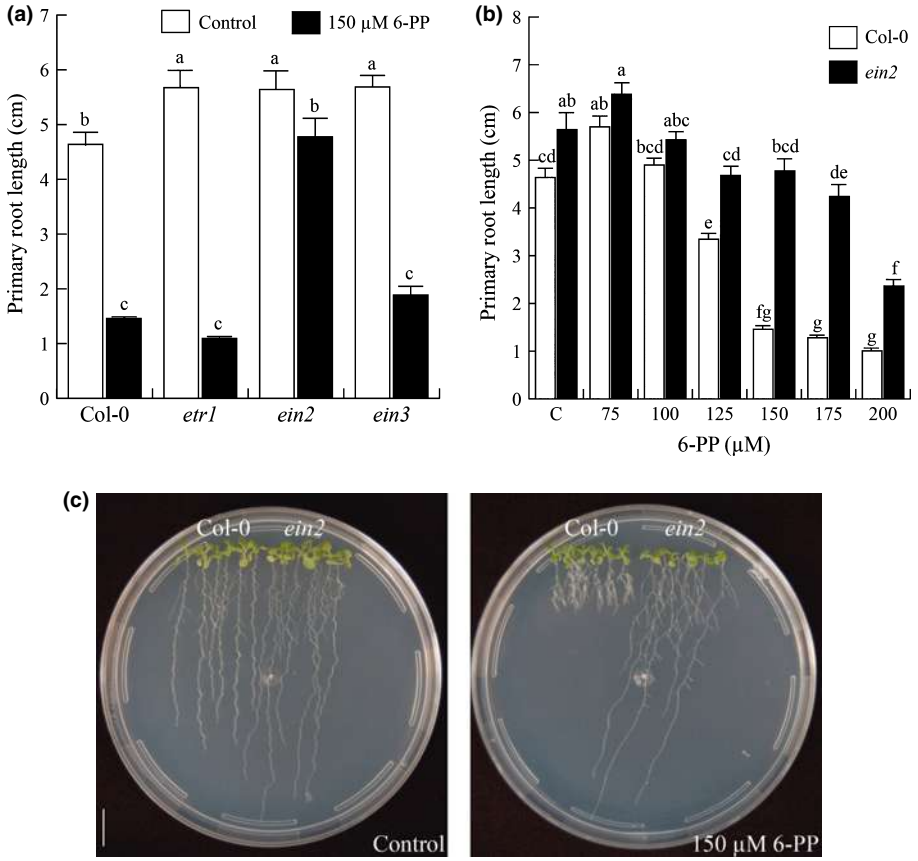

Fig. 8 *EIN2* is necessary for 6-pentyl-2*H*-pyran-2-one (6PP)-modulated primary root growth. *Arabidopsis thaliana* wild-type (Col-0) and *etr1-1*, *ein2-1*, *and ein3-1* ethylene-related mutant seedlings were germinated and grown for 10 d in 0.2 × MS medium supplemented with the solvent (control) or 150 μM 6-PP. (a) Primary root length. (b) Primary root growth of *ein2* mutants in response to increasing concentrations of 6-PP (c). Representative photographs of Col-0 and *ein2* seedlings grown side by side in the indicated 6-PP treatment. Values shown represent the means of 15 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated twice with similar results. Bar, 1 cm.

and producing more branched root systems (Vinale *et al.*, 2008). 6-PP is, to our knowledge, the first non-auxin-like natural molecule that has been found to induce LR formation and root hair development, but its mechanism of action has not been previously examined.

To understand the possible role of 6-PP in phytostimulation, we first monitored 6-PP production by *T. atroviride* as part of the blend of volatiles emitted by single fungal colonies alone or in interaction with *A. thaliana* seedlings. GC-MS analysis showed that the production of 6-PP was induced by the presence of plants, which indicates its possible role in *Trichoderma*–plant interactions. For instance, a recent report showed that tomato plants elicited the production of harzianic acid (HA) but negatively modulated the biosynthesis of its analog iso-HA, suggesting that different forms of the same metabolite have specific roles in the molecular mechanism regulating the *Trichoderma*–plant interaction (Vinale *et al.*, 2014). Very little is known about the mechanisms of 6-PP biosynthesis. Mutation in the G alpha subunit gene TGA1 of *T. atroviride* leads to decreased 6-PP production, continuous sporulation and elevated internal cAMP concentrations, which correlates with loss of mycoparasitic and antagonistic properties against *R. solani*, *B. cinerea*, and *Sclerotinia sclerotiorum* during direct confrontation (Reithner *et al.*, 2005). The transcription factor ThCTF1 also regulates the biosynthesis of 6-PP in *T. harzianum*. In *Thctf1* mutants, the yellow pigmentation and coconut aroma attributed to 6-PP production observed in the WT strain were affected, as was its antimicrobial activity (Rubio *et al.*, 2009). Although the interaction of *Trichoderma* strains defective in 6-PP production with plants remains to be investigated, one possibility is that such strains may still stimulate plant growth and LR formation, as these might be able to produce auxins; alternatively, the net effect on root branching may rather depend on the balance of auxin/6-PP production and release by *Trichoderma*. Our data clearly anticipate the existence of *Trichoderma* species and/or strains that promote growth without producing auxins, suggesting that 6-PP is another critical factor in fungal phytostimulation.

6-PP improved shoot and root growth and total biomass production of *A. thaliana* seedlings, and this was related to changes in root morphogenesis. LRs and root hairs are critical for water and nutrient acquisition and are important traits for plant adaptation to soil heterogeneity. The mechanism of LR formation is directly or indirectly related to primary root growth inhibition, which is mediated by the synergistic action of ethylene and auxin signaling (Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007; Strader *et al.*, 2010). Contreras-Cornejo *et al.* (2015) showed that the short root phenotype of mutants defective on CONSTITUTIVE TRIPLE RESPONSE 1 was probably caused by auxin being accumulated in primary root tips and that both auxin and ethylene signaling are important for *Trichoderma*-induced root hair and LR formation. LR development consists of two successive steps: LR initiation and LR emergence from the parent root, which are controlled by auxin fluxes mediated by

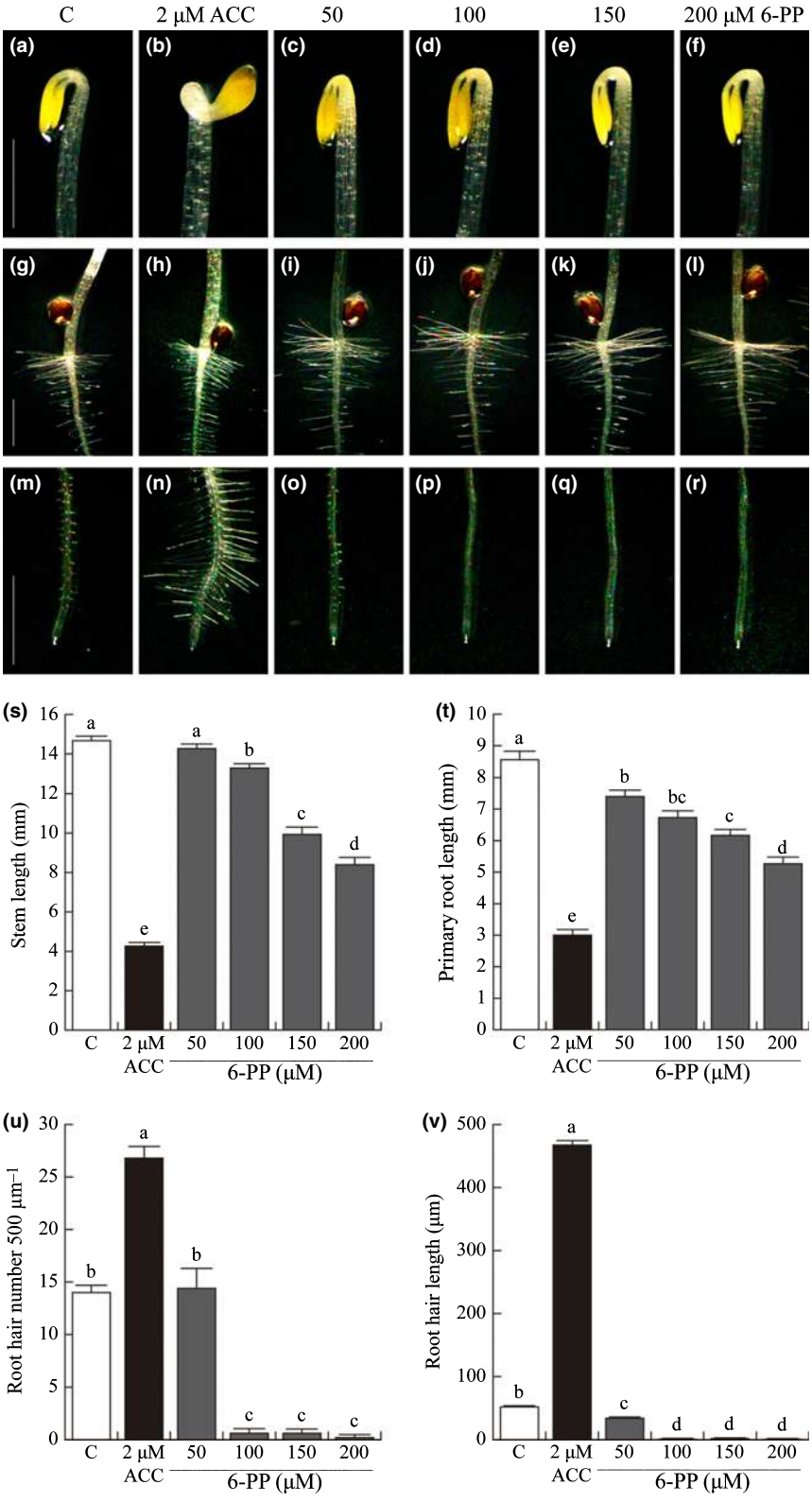

Fig. 9 6-pentyl-2*H*-pyran-2-one (6PP) does not induce ethylene responses in dark-grown *Arabidopsis thaliana* seedlings. Representative photographs of (a–f) stem, (g–l) root–shoot transition zone and (m–r) root tip are shown. Quantitative data are for (s) stem length, (t) primary root length, (u) root hair number at the root tip region and (v) root hair length at the root tip region. *Arabidopsis thaliana* Col-0 seedlings were grown in darkness for 4 d on 0.2 × MS supplemented with the indicated concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) or 6-PP. Note that only ACC is able to induce the pronounced apical hook on the tip of the stem (b), and root hair development, whereas 6PP inhibits root hair formation. Data shown are mean ± SD ($n = 30$ for stem and primary root length; 500 root hairs from 10 independent seedlings were counted and measured). This experiment was repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Bars, 1 mm. C, control.

PIN family membrane transporters (Zazimalova *et al.*, 2010). To further explore the mechanisms of auxin and ethylene crosstalk in response to 6-PP, we tested the effects of 6-PP concentrations that either promote (75 μM) or repress (150 μM) primary root growth on the expression of *DR5:GFP* and *DR5:VENUS* auxin-inducible markers. Interestingly, GFP fluorescence did not increase in the root tip in response to 6-PP treatment, consistent with an auxin-independent mechanism mediating the bioactivity of this compound. By contrast, we observed enhanced *DR5:GFP* fluorescence after 6-PP treatment in the LR-forming regions of

roots, particularly in the vascular tissue and during LR primordium development, which indicates an activation of auxin signaling during the LR initiation program. The structure/activity relationship of auxin signaling with small molecules has been extensively investigated. More than 200 natural or synthetic auxinic compounds have been identified, including the bacterial cyclodipeptides cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr), and cyclo (L-Pro-L-Phe). These small molecules possess weak auxin activity and were able to activate auxin-response gene markers in the *A. thaliana* root system (Ortiz-Castro *et al.*, 2011). An interesting possibility is that 6-PP could modulate auxin homeostasis in specific root regions, or, possibly, its positive effect in inducing LRP emergence is explained as an adaptive response to primary root growth inhibition.

IAA enters cells through the action of influx carriers such as AUXIN RESISTANT 1 (AUX1) and Like AUX (LAX1, 2 and 3) (Bennett *et al.*, 1996; Marchant *et al.*, 2002; Swarup *et al.*, 2008), and moves to adjacent cells via efflux proteins such as PIN FORMED 1 (PIN1) and ATP BINDING CASSETTE B 19/P-GLYCOPROTEIN 19/MULTIDRUG RESISTANT 1 (ABCB19/PGP19/MDR1) (Galweiler *et al.*, 1998; Noh *et al.*, 2001). Defects in AUX1, LAX3, PIN1, PIN2 and ABCB19 decrease initiation and/or elongation of LRs or negatively affect root gravitropism as a result of reduced auxin transport (Marchant *et al.*, 2002; Benkova *et al.*, 2003; Wu *et al.*, 2007; Swarup *et al.*, 2008). Changes in the abundance and localization of auxin transport proteins may define the growth of primary roots or the initiation of LRs (Raya-González *et al.*, 2014). Our finding that 6-PP increased auxin-induced gene expression in regions of LR initiation suggests that 6-PP affects root development by altering auxin distribution. Consistent with this idea, PIN1, PIN2 and PIN7-GFP fluorescence was increased or decreased after 6-PP treatment, respectively, indicating the possible role of PIN transporters in 6-PP root responses. At high 6-PP concentrations (i.e. 150 μM), localized depletion of the fluorescence of PIN1- and PIN7-GFP, normally found below the primary root meristem, was evidenced. These results suggest that 6-PP treatment increased PIN transporter expression at low doses, resulting in elevated auxin transport to the sites of LR initiation to drive LR growth, whereas higher concentrations repress primary root growth, probably blocking expression of PIN1 and PIN7. The increased LR branching associated with elevated expression of auxin transporters is not surprising, as recent studies have shown that auxin positively regulates PIN1 and PIN2 expression (Raya-González *et al.*, 2014).

To investigate whether the TIR1 family of auxin receptors and downstream signaling components are involved in *A. thaliana* responses to 6-PP, we evaluated primary root growth and LR formation in response to this metabolite in WT (Col-0) *A. thaliana* seedlings and in *tir1afb2afb3*, *arf7arf19*, *axr1-3*, *aux1-7* and *eir1* triple, double and single mutants, respectively. In solvent-treated WT seedlings, 6-PP decreased primary root length in WT and all five auxin-related mutants. Interestingly, the increase in LR formation observed in WT seedlings when treated with 6-PP was clearly reduced in *tir1afb2afb3*, *arf7arf19* and *aux1-7* mutants. Additional experiments testing primary root growth responses to 6-PP in WT and ethylene-related mutants *etr1*, *ein2* and *ein3* revealed that this compound similarly inhibited primary root growth in WT, *etr1* and *ein3* lines, whereas *ein2* was resistant to primary root growth inhibition by 6-PP, which was further confirmed in a kinetic experiment monitoring primary root growth in response to a wide range of 6-PP concentrations. Alterations in the response of dark-grown seedlings to ethylene (the 'triple response') have been used to characterize the ethylene signaling pathway in plants. In response to exogenously applied ACC, etiolated *A. thaliana* seedlings show inhibition of hypocotyl and root elongation, swelling of the hypocotyl, exaggerated tightening of the apical hook, and induced root hair development. Although ACC and 6-PP inhibit primary root growth, the phenotypes of seedlings treated with ACC or 6-PP are clearly different. In fact, 6-PP-treated seedlings did not develop the 'triple response' and failed to form long root hairs at the differentiation zone of primary roots. An additional experiment comparing the growth of *A. thaliana* seedlings supplied with ACC or 6-PP and the ethylene inhibitor $AgNO_3$ simultaneously showed that $AgNO_3$ specifically antagonized the ACC response, normalizing primary root growth without affecting the 6-PP response. These data show that 6-PP did not induce an ethylene response in *A. thaliana* seedlings and that *EIN2* is a specific and critical element mediating root responses to this fungal molecule.

These results showing the involvement of 6-PP in root development add to the emerging functions of fungal molecules in plants. Based on its growth-promoting activity and the involvement of *EIN2* in its signaling pathway, 6-PP can be regarded as a broad-spectrum molecule used to modulate both root growth and defense responses, and thus represents a novel compound enabling cross-kingdom communication. Manipulating 6-PP-dependent fungal–plant signaling and 6-PP biosynthesis in *Trichoderma* may be a promising strategy for development of fungal inoculants to enhance crop yields and plant protection in *A. thaliana* and crop plants.

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Author contributions

J.L-B., L.F.R-H., L.M-R. and A.G-V. planned and designed the research. A.G-V., A.M-B., J.R-G., L.F.R-H., S.B-O. performed experiments. A.G-V., A.M-B, E.M-P. S.B-O. and J.L-B analyzed data. A.G-V. and J.L-B. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 6-PP modifies *Arabidopsis thaliana* root system architecture.

Fig. S2 Effect of 6-PP on *Arabidopsis thaliana* cell viability.

Fig. S3 6-PP reduces the cell division zone in primary roots.

Fig. S4 Effects of 6-PP on *Arabidopsis thaliana* root hair development and epidermal cell length.

Fig. S5 Effect of 6-PP on auxin responsive gene expression in primary root tips of *Arabidopsis thaliana DR5:VENUS* seedlings.

Fig. S6 Effects of NPA and IAA on primary root growth of wildtype (Col-0) and auxin-related mutants.

Fig. S7 Effect of $AgNO_3$ on primary root growth inhibition induced by ACC or 6-PP.

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7. DISCUSIÓN Y CONCLUSIONES

Las plantas son organismos de vida sésil, las cuales responden a señales internas y externas que les permiten sobrevivir y adaptarse a las condiciones cambiantes del ambiente. El sistema radicular desempeña funciones adaptativas esenciales como la captación de agua y nutrientes, el anclaje al suelo y el establecimiento de interacciones bióticas y abióticas (Ortiz-Castro *et al*., 2009). Entre los microorganismos que habitan la rizósfera se encuentran los hongos del género *Trichoderma*, habitantes comunes de los suelos de diferentes ecosistemas y utilizados como bioinoculantes en la agricultura para el control de patógenos (Benítez *et al*., 2004).

Trichoderma produce y libera una amplia variedad de compuestos, a los cuales se les han asociado distintas funciones como antibióticos, inductores de respuestas de defensa y promotores del crecimiento vegetal (Vinale *et al*., 2008). *T. virens* produce ácido indol-3-acético (AIA), indol-3-acetaldehido (IAAld), indol-3-etanol (IEt) e indol-3-carboxaldehido (ICAld) que son los precursores del AIA y modifican el crecimiento y ramificación de la raíz (Contreras-Cornejo *et al*., 2009). Otro mecanismo fisiológico influenciado por *T. virens* y *T. atroviride,* es la modulación de la apertura de los estomas y el control de la transpiración de las plantas mediante la producción de ABA y la regulación de las fosfatasas ABI1 y ABI2, lo cual en conjunto con las auxinas, contribuyen al mejoramiento de la salud de las plantas bajo condiciones de estrés hídrico (Contreras-Cornejo *et al*., 2015b).

Un factor importante en la interacción *Trichoderma*-planta es el pH del suelo. *Trichoderma spp.* crece de mejor forma en condiciones ácidas y puede acidificar la rizósfera por la acción de H^+-ATPasas, un evento que es percibido por la raíz con la subsecuente activación de cascadas de señalización que modulan el crecimiento y la ramificación a través del factor de transcripción STOP1. Se ha sugerido que el transporte normal de auxinas es un factor importante en la adaptación de la raíz a la inoculación de *Trichoderma* y al consecuente cambio en el pH (Pelagio-Flores *et al*., 2017).

Otras sustancias que libera *Trichoderma* y que impactan en forma positiva en el desarrollo de la planta son los COVs. Uno de los primeros volátiles identificado fue la 6-PP, el compuesto volátil más abundante de algunas especies de *Trichoderma*. La 6-PP es un regulador del crecimiento en plantas de jitomate, canola y *A. thaliana*, la cual modifica la arquitectura de la raíz (Vinale *et al*., 2008; Kottb *et al*., 2015), lo que la convierte en una molécula clave en la interacción *Trichoderma*-planta. Sin embargo, antes de nuestras investigaciones, no se habían dilucidado los componentes moleculares y genéticos específicos que participan en la remodelación de la arquitectura radicular en respuesta a la 6-PP.

En un primer ensayo, fue posible confirmar que la 6-PP era producida como compuesto mayoritario por *T. atroviride* IMI 206040 en un medio de crecimiento vegetal como el de Murashige y Skoog (MS), (Murashige y Skoog, 1962), que es empleado para el crecimiento *in vitro* de *Arabidopsis* (Contreras-Cornejo *et al*., 2009; Contreras-Cornejo *et al*., 2014a). En reportes previos se había demostrado que las variaciones en el sustrato, temperatura y humedad repercuten en la emisión de la 6-PP (Cooney *et al*. 1997; Polizzi *et al*., 2011). En dos aislados de *T. harzianum* (T16 y T23), se encontró que sólo la cepa T23 fue capaz de producir la 6-PP (El-Hasan *et al*., 2007). Estos estudios sugieren la existencia de quimiotipos entre las distintas especies de *Trichoderma*.

En nuestro sistema de interacción, el perfil de COVs mostró variedad en los compuestos volátiles que se emiten, encontrando alcoholes, cetonas y la 6-PP, indicando que a pesar de la variación de sustrato y condiciones de crecimiento, este medio es adecuado para determinación de la 6-PP por *Trichoderma*. Desde su descubrimiento en la década de los 70s, se ha atribuido un papel multifacético a la 6-PP, por una parte actuando como compuesto antimicrobiano en contra de fitopatógenos como *Rhizoctonia solani*, *Botrytis cinerea* y especies de *Fusarium* (Reithner *et al*., 2005, El-Hasan *et al*., 2008) y como metabolito bioactivo en las plantas donde se ha demostrado su función en la inhibición del crecimiento de coleoptilos de trigo, de la germinación en lechuga y como inductor de crecimiento en jitomate y canola (Parker *et al*., 1997; Vinale *et al*., 2008). En este trabajo se cuantificó la emisión de la 6-PP de *T. atroviride* en interacción con *Arabidopsis* y

se observó un incremento en la concentración de la pirona, correlacionado además, con el efecto inductor en la ramificación de las raíces, lo que sugiere un papel clave de este volátil en el mecanismo molecular que regula la interacción *Trichoderma*-planta.

Un componente fundamental de la estructura radicular de las plantas son las raíces laterales, las cuales se forman después de la germinación y determinan la configuración tridimensional del sistema radicular, lo cual repercutirá en la captación de agua y nutrientes (Vilches-Barro y Maizel, 2015). Existe evidencia sólida sobre la inducción de la ramificación de las raíces de distintas plantas mediada por *Trichoderma* en sistemas axénicos o en el suelo (Chang *et al*., 1986; Yedidia *et al*., 2001; Adams *et al*., 2007, Contreras-Cornejo *et al*., 2009). La promoción del crecimiento de *Trichoderma* está mediada en parte por sus metabolitos secundarios. *T. virens* y *T. atroviride* producen AIA que es la principal auxina en plantas y sus compuestos precursores IAAld e ICAld y el derivado IEt, (Contreras-Cornejo *et al.* 2009., 2011), los cuales mostraron actividad dependiente de la concentración sobre diversos parámetros del crecimiento y desarrollo en *Arabidopsis*. Al incrementar la concentración de 6-PP en el medio de crecimiento se observaron cambios en el crecimiento de la raíz y la biomasa total de *Arabidopsis*, específicamente se observó un incremento en el número de raíces laterales por medio de la inducción de la emergencia de los primordios y acelerando su crecimiento. Se sabe que la formación de raíces laterales está relacionado con el acortamiento de la raíz primaria y este mecanismo está regulado por las rutas de señalización de los fitorreguladores auxinas y etileno (Muday *et al*., 2012). En un estudio reciente se observó que la mutante de la ruta del etileno *ctr1* (*constitutive triple response* 1), la cual tiene un fenotipo de raíz corta; presenta acumulación de auxinas en la punta de la raíz y que ambas vías de señalización son importantes para inducir pelos radiculares y para la formación de raíces laterales por *Trichoderma* (Contreras-Cornejo *et al*., 2015a).

En el proceso de formación de raíces laterales se han identificado gran variedad de moléculas microbianas que presentan actividad o estructura parecida a las auxinas. Tal es el caso de los ciclodipéptidos producidos por la bacteria

Pseudomona aeruginosa que tienen un efecto promotor en plantas de *A. thaliana* regulando la expresión de genes inducibles por auxinas (Ortiz-Castro *et al.,* 2011). Por otra parte, Vinale y col. (2008), observaron que a dosis bajas, la 6-PP promueve el crecimiento mientras que a dosis altas su efecto es inhibitorio, sugiriendo que la 6-PP puede actuar como un compuesto que es reconocido como una auxina.

Para evaluar el papel de la 6-PP en la regulación de la arquitectura radicular y su relación con la señalización de auxinas, se analizó la expresión del marcador de respuesta a auxinas *DR5:GFP* en la raíz primaria, primordios de raíces laterales y raíces laterales emergidas. En la punta de la raíz no se encontraron diferencias respecto al control en respuesta a la 6-PP, mientras que en la zona de formación de las raíces laterales se indujo la expresión del marcador, sugiriendo que la 6-PP modula el desarrollo de la raíz modificando la distribución de las auxinas, siendo crucial el transporte de auxinas para la formación de raíces laterales como se demostró al utilizar el inhibidor del transporte N-1-ácido naftiltalámico (NPA), el cual arresta el desarrollo de las raíces laterales, bloqueando las primeras divisiones (Casimiro *et al*., 2001).

La abundancia de las proteínas PIN se incrementó o disminuyó según los tratamientos de 6-PP aplicados. Estos resultados sugieren que: 1) las respuestas de la raíz a la 6-PP están mediadas por los transportadores de eflujo de auxinas, 2) en bajas concentraciones la 6-PP incrementa la expresión de algunos transportadores de la familia PIN, y 3) en altas concentraciones, la 6-PP, reprime el crecimiento de la raíz primaria, evento relacionado con una baja o nula expresión de PIN1 y PIN7. Al respecto, es pertinente mencionar que existen pocos estudios sobre la regulación del transporte de auxinas mediado por un microorganismo o por algún producto de su metabolismo.

En plantas de *Medicago truncatula* infectadas con *Sinorhizobium meliloti* se observó que la expresión de los transportadores MtLAX, MtPIN y MtABCB se modifica por la bacteria nodulante (Roy *et al*., 2017). Además, en una mutante resistente a la infección por *S. meliloti (dmi3*) la expresión de los genes relacionados al transporte de auxinas, así como el contenido de AIA, se

mantuvieron sin cambios durante la interacción, permitiendo a los autores concluir que el trasporte de auxinas es importante en la formación del nódulo en *M. truncatula* durante una fase temprana de infección por *S. meliloti* (Shen *et al*., 2015).

Mediante el estudio del efecto de un peptaibol, la Trichokonina VI (TK VI), sobre el desarrollo de la raíz de *Arabidopsis*, se reportó un detrimento en el crecimiento de la raíz primaria, causado por la inhibición de la división y elongación celular y una disminución en la actividad del nicho de células madre. Lo anterior correlaciona con el incremento en el contenido de auxinas en la punta de la raíz (Shi *et al*., 2016b). Esto indica que la homeostasis de auxinas juega un papel crucial en el establecimiento de interacciones con microorganismos. Específicamente, la 6-PP de *Trichoderma atroviride* influye en la distribución de los transportadores de auxinas PIN en la raíz primaria y esta respuesta ocurre de manera puntual y en ciertos tejidos, siendo este trabajo el primer reporte de una molécula bioactiva de hongos, la cual regula componentes específicos del transporte polar de auxinas en plantas.

Se ha reportado una interacción sinérgica entre las auxinas y el etileno en la regulación del crecimiento de la raíz primaria y el desarrollo de las raíces laterales (Rahman *et al*., 2001; Ivanchenko *et al*., 2008). Para determinar la interacción entre estas dos hormonas sobre la configuración de la arquitectura radicular en respuesta a la 6-PP, se analizó la respuesta de la línea silvestre Col-0 y las mutantes *tir1afb2afb3*, *arf7arf19*, *axr1-3*, *aux1-7* y *eir1* afectadas en genes de respuesta o transporte de auxinas y también en mutantes de la vía del etileno *etr1*, *ein2* y *ein3*. Se aplicó una concentración alta de 6-PP (150 µM) para evaluar el efecto tanto en la raíz primaria como en la formación de raíces laterales. En las líneas de respuesta a auxinas ocurrió una inhibición del crecimiento de la raíz primaria similar a la línea silvestre bajo tratamientos con la 6-PP, pero en las mutantes *tir1afb2afb3*, *arf7arf19*, *axr1-3*, *aux1-7* se observó una inducción nula en la formación de raíces laterales, indicando que la 6-PP requiere de la ruta de señalización de auxinas para evocar una respuesta en este proceso. En el caso del etileno se ha reportado que la aplicación del precursor ácido-1-carboxílico-1-

aminocliclopropano (ACC) induce la expresión de los transportadores de eflujo de auxinas PIN3 y PIN7, lo que resulta en cantidades elevadas de estos transportadores (Lewis *et al*., 2011). Debido a este antecedente, enfocamos nuestra atención a la respuesta en la raíz primaria de las mutantes de la ruta de señalización del etileno. Interesantemente, la mutante *ein2* mostró resistencia al acortamiento de la raíz primaria causado por una concentración alta de 6-PP, comprobando este efecto mediante una curva de crecimiento ante varias concentraciones de la molécula, por lo que *EIN2* es necesario y suficiente para la percepción de la 6-PP en la raíz primaria de *Arabidopsis*.

La participación de *EIN2* sugería que una vez percibida la 6-PP se podría activar una respuesta genérica al etileno, para lo cual se compararon los efectos de la adición del precursor de etileno ACC y la 6-PP. Se ha reportado que en plantas tratadas con ACC y crecidas en condiciones de obscuridad se induce la llamada “respuesta triple”, la cual se caracteriza por la formación de un hipocotílo grueso y corto, inhibición del crecimiento de la raíz primaria y una curvatura exagerada del gancho apical (Guzmán y Ecker, 1990). Nuestros resultados indican que las plantas tratadas con la 6-PP no desarrollan la respuesta triple. Otro efecto provocado por el etileno es la formación de pelos radiculares (Tanimoto *et al*., 1995), los tratamientos con la 6-PP, a diferencia del ACC, no incrementaron la formación ni el alargamiento de los pelos radiculares. Estos datos indican que la 6-PP no induce la biosíntesis local de etileno en *A. thaliana*, sino que EIN2 es un elemento crucial para regular las respuestas de la raíz a este compuesto volátil (Fig. 11).

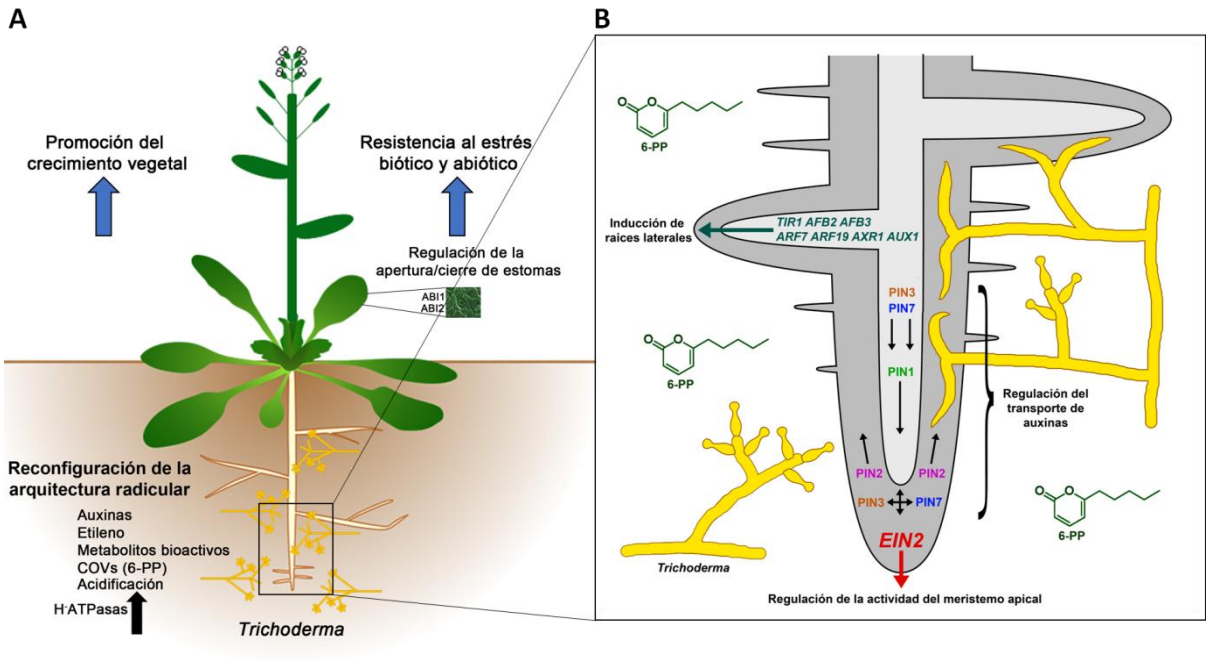

Figura 11. La 6-PP de *Trichoderma* regula procesos morfogenéticos en la raíz de *Arabidopsis*. A) En la interacción planta-*Trichoderma*, se producen diversos compuestos bioactivos como las auxinas, el etileno y los COVs como la 6-PP, que en conjunto con la acidificación mediada por H^+ATPasas influyen en la reconfiguración de la arquitectura radicular. B) *Trichoderma* produce la 6-PP que modifica la morfología de la raíz primaria mediante el elemento de la ruta de señalización de etileno EIN2 y regula el transporte de auxinas modulando la expresión de los transportadores PIN1, PIN2, PIN3 y PIN7. Además, induce la formación de raíces laterales aspecto que involucra a los receptores de auxinas TIR1AFB2 y AFB3, los factores transcripcionales ARF7 y ARF19 y los elementos de señalización AXR1 y AUX1.

La proteína EIN2 se localiza en la membrana del retículo endoplásmico en donde actúa como un regulador positivo de la señalización del etileno. Su secuencia muestra homología con proteínas transportadoras de metales de la familia Nramp (Alonso *et al*., 1999) y se ha sugerido su participación en cascadas de señalización reguladas por otras fitohormonas o estímulos (Bisson y Groth, 2011; Li *et al*., 2015b; Merchante *et al*., 2015). Las plantas mutadas en este gen, son insensibles a casi todos los aspectos de la respuesta a este volátil, por los que se vincula a esta proteína en el transporte de auxinas y la percepción de metabolitos de origen fúngico.

En conclusión el compuesto volátil 6-pentil-2H-piran-2-ona producido por *T. atroviride* regula programas de morfogénesis en la raíz de *A. thaliana* a través de la modulación específica y diferencial del transporte polar de auxinas mediado por

las proteínas de la familia PIN y el elemento de la ruta de señalización de etileno EIN2 es necesario para la respuesta en la raíz primaria. Se abren las perspectivas de que con el manejo de la producción de la 6-PP en *Trichoderma*, o de la respuesta de las plantas a este volátil, se puedan formular bioinoculantes que mejoren la salud y el crecimiento y desarrollo de las plantas de interés agrícola.

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9. APÉNDICE

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Artículos

Trichoderma: un hongo biofertilizante

Amira Garnica Vergara y Saraí Esparza Reynoso

Los registros fósiles muestran que la asociación entre las raíces y los hongos es tan antigua como las plantas terrestres. Hace millones de años, cuando las plantas evolucionaron en ambientes pantanosos, su colonización del suelo ocurrió gracias a la simbiosis establecida con diferentes especies de microorganismos, principalmente los hongos, cuyas hifas actúan como extensiones naturales del sistema radical. La liberación de sustancias ricas en carbono por la raíz, como azúcares, ácidos orgánicos, aminoácidos y otras substancias nutritivas permite la selección de microbiomas en un ambiente especial denominado rizósfera, diferente en propiedades y funcionamiento a otras regiones del suelo y cuyo estudio representa una de las grandes promesas para mejorar las prácticas agrícolas y la sustentabilidad del campo.

El protagonismo de *Trichoderma*

Entre los microorganismos que interactúan con la raíz de las plantas, encontramos a los hongos del género *Trichoderma*, los cuales se adaptan a una amplia variedad de condiciones ambientales y obtienen su energía a partir de los residuos de materia orgánica o material vegetal en descomposición. En años recientes, se han confirmado sus propiedades benéficas que potencian su uso como bioestimulante para los cultivos.

Trichoderma es un hongo filamentoso que pertenece al grupo de los ascomicetos, en los que

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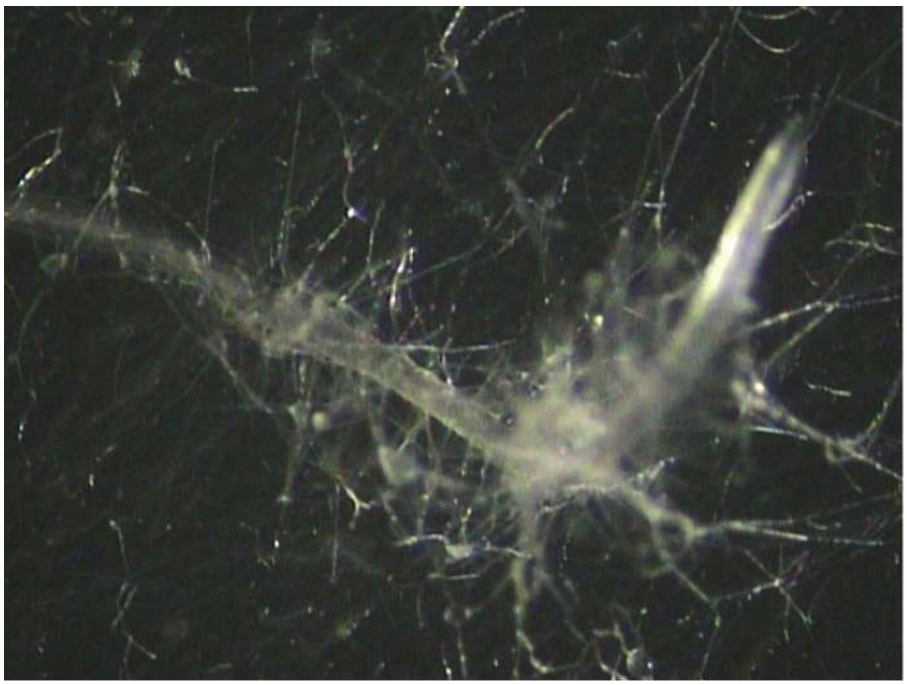
la mayoría de las especies no tienen un periodo sexual, simplemente producen esporas asexuales. Este hongo se caracteriza por predominar en los ecosistemas terrestres (suelos agrícolas, pastizales, bosques y desiertos) y acuáticos. Son muy diversos, pueden ser de vida libre en el suelo, oportunistas, simbiontes de plantas y micoparásitos. Además, pueden ser usados para producir un amplio rango de productos de interés comercial y agrícola. Algunas especies son utilizadas como agentes para el control de patógenos del suelo y de enfermedades vegetales y por sus habilidades de incrementar el crecimiento y desarrollo de plantas.



Colonización de *Trichoderma* en raíces

Colonización de *Trichoderma* en raíces

Entre las ventajas, destaca su inocuidad a los seres humanos, ya que no deja efectos residuales en el follaje, los frutos y las semillas, una diferencia fundamental al aplicarse fungicidas u otros agroquímicos. Además, posee un rápido crecimiento y desarrollo, puede proliferar en una amplia gama de suelos, es tolerante a condiciones ambientales extremas, puede tolerar altas concentraciones de agroquímicos y es capaz de parasitar, controlar y destruir hongos, nemátodos y otros fitopatógenos (patógenos de plantas).

-Es por estas propiedades que se le considera uno de los principales agentes para el control biológico en diferentes sistemas de producción-

Defensa en plantas

La colonización de la raíz por *Trichoderma* ejerce un efecto multifuncional en la biología de los cultivos como el maíz, el jitomate y la soya, por mencionar algunos. Por ejemplo, se incrementan las defensas y las plantas se hacen más resistentes a las enfermedades causadas por hongos y bacterias. Este fenómeno puede ser ocasionado por la inducción de compuestos químicos llamados fitoalexinas, los cuales se acumulan en altas concentraciones en la planta y ayudan a limitar la dispersión del patógeno o por la activación de rutas de señalización implicadas en defensa como la del ácido salicílico, ácido jasmónico o etileno.

¿Parásito de otros patógenos?

Además, *Trichoderma* ha desarrollado mecanismos para atacar y parasitar a otros organismos fitopatógenos y así, aprovechar una fuente nutricional adicional. Posee distintas formas de acción, como la producción de secreciones enzimáticas tóxicas, las cuales causan desintegración y muerte en hongos que habitan el suelo (micoparasitismo), la degradación de paredes celulares de hongos patogénicos (depredación), la producción de químicos volátiles y antibióticos antifúngicos que inhiben otros hongos (amensalismo), la colonización directa del hongo (predación) y la competencia por oxígeno, nutrientes y espacio en el suelo. ¡De verdad, su función en muy importante cuando coloniza las raíces de diversas plantas!

Inducción del crecimiento vegetal

El estímulo del crecimiento y desarrollo de las plantas por parte de *Trichoderma* ha sido conocido por muchos años. Muchas cepas que han sido aisladas y probadas en plantas, ya sea en condiciones de laboratorio o en suelos naturales de campo, incrementan el crecimiento de las raíces y esto repercute en el aumento de la productividad de las plantas.

En diversas investigaciones, los científicos han comprobado que la producción de fitohormonas como las auxinas y compuestos volátiles que libera *Trichoderma*, son los responsables de estimular la ramificación de la raíz, aumentando su capacidad para captar agua y nutrimentos minerales.

***Trichoderma* facilita la asimilación de fósforo en plantas**

Una problemática en la agricultura, es el bajo nivel de fósforo disponible en el suelo. El fosfato, principal forma asimilable del fósforo, es un macronutrimento esencial para el desarrollo y crecimiento vegetal; un constituyente necesario para la división celular, la fotosíntesis, la producción de proteínas y ácidos nucleicos; también para la fijación de nitrógeno, la biosíntesis de azúcares y almidones y otros procesos del metabolismo.

Pero la movilidad y concentración de fósforo en los suelos es muy baja, por lo que se requiere la aplicación de grandes cantidades de fertilizantes fosfatados, que además de ser muy costoso para la producción, propician efectos negativos para el ambiente. Se conoce que cerca del 90% del fosfato aplicado se precipita en formas insolubles con calcio y metales, como hierro y aluminio, que no pueden ser asimilados, limitando la producción de los cultivos a nivel mundial.

Ante esta situación, se reporta que *Trichoderma* es eficaz en la solubilización del fosfato a través de la producción de ácidos orgánicos que reducen el pH del suelo haciéndolo más biodisponible, la liberación de metabolitos quelantes y enzimas especializadas en la degradación de compuestos orgánicos de fósforo como las fosfatasas ácidas y alcalinas.

***Trichoderma*: un impulso para la aplicación de biofertilizantes**

La aplicación de microorganismos benéficos para las plantas implica la elaboración de biofertilizantes, es decir, vehículos en un medio líquido o sólido que usualmente contienen materia orgánica y microorganismos vivos o en un estado de "dormancia," que una vez en el suelo, colonizan las raíces promoviendo el crecimiento y el desarrollo vegetal. En general, se estima que un biofertilizante tiene un costo de sólo el 10% comparado con los fertilizantes químicos, lo que los convierte en productos rentables y útiles.

Si la aplicación de biofertilizantes representa un gran beneficio ¿por qué no se ha adoptado en México con tanto éxito?

Recientemente, la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) reportó que apoya 325 mil hectáreas con biofertilizantes, permitiendo un incremento en la producción de un 15%, además de un ahorro importante por la reducción en la importación de fertilizantes químicos. Sin embargo, aún existen limitaciones para el uso extensivo de los biofer-

tilizantes, derivadas de la falta de información sobre su efectividad, mecanismos de acción y reproducibilidad de sus efectos probióticos en diferentes ambientes y condiciones de cultivo.

Con base en lo anterior, un reto de las investigaciones actuales es el desarrollo de nuevos y mejores bioinoculantes, que se adapten con eficiencia a diversos tipos de suelo y condiciones ambientales locales, pero sobre todo debe existir una mayor vinculación entre la industria, los productores y los investigadores con el fin de colaborar en el desarrollo de sistemas de producción que incrementen la calidad de los bioinoculantes y garanticen la reproducibilidad de sus efectos en los cultivos.

Actualmente se conocen más de 200 especies de *Trichoderma*, las más comercializadas en cultivos agrícolas para el control biológico son *T. harzianum* (cepa T-22), *T. reesei.*, *T. viride* y *T. hamatum*, y pueden funcionar tanto para el control de enfermedades en hoja y tallo como de raíz. La forma más económica y extensa para emplear *Trichoderma* en la agricultura, consiste en el tratamiento de las semillas previo a la siembra, ya que este hongo es capaz de colonizar la superficie de la raíz a partir de las semillas tratadas. No obstante, existen tratamientos combinados para semillas y sustrato para asegurar que el inóculo permanezca viable en condiciones ambientales adversas y posteriormente se establezca como habitante normal de la rizósfera.

En el mercado existen diferentes formulaciones de *Trichoderma*, cuya presentación varía en forma granular o en polvo mojable o bien en presentación líquida. Estas formulaciones son realizadas con aislamientos o cepas específicas bajo un reglamento de control de calidad, que incluye la verificación de la pureza y la efectividad biológica mediante un proceso semi-industrial.

Para obtener la eficacia que necesita el agricultor se deben tomar en cuenta varios aspectos muy importantes: la procedencia del producto, la experiencia y confiabilidad de la empresa que lo produce, el respaldo técnico, la fecha de vencimiento, la presentación y las características específicas del producto como especie de Trichoderma, concentración, viabilidad, especificidad, dosificación y forma de aplicación, que garanticen su eficacia y efectividad.

Actualmente los principales fabricantes y comercializadores de productos de *Trichoderma* radican en Asia, Europa y Estados Unidos. En México, la producción actual de biofertilizantes se realiza por parte de empresas privadas e instituciones de investigación como el Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Pero, a pesar del desarrollo, distribución y aplicación de esta tecnología, aún no se contempla como una alternativa para la sustitución parcial de los fertilizantes minerales.

Por lo tanto, la difusión y el empleo de *Trichoderma* representa un alto potencial para la generación de biofertilizantes en beneficio de una agricultura orgánica, con alta capacidad productiva y menos repercusiones hacia el medio ambiente.

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¿POR QUÉ ESTUDIAR EL DESARROLLO REPRODUCTIVO DEL HONGO FILAMENTOSO *TRICHODERMA ATROVIRIDE*?

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Introducción

Trichoderma es un hongo saprófito que habita en diferentes ecosistemas; ha sido aislado comúnmente del suelo en diferentes países del mundo, también se ha encontrado sobre las raíces de las plantas, sobre cortezas en descomposición y sobre otros hongos. El interés científico en *Trichoderma*, se debe a que algunas especies actúan como agentes de biocontrol en la agricultura. Estos hongos protegen a las plantas contra el ataque de fitopatógenos, produciendo antibióticos o bien parasitando a otros hongos mediante la secreción de enzimas hidrolíticas tales como las quitinasas que le permiten penetrar la pared celular de los hongos fitopatógenos (Reithner et al., 2007). Una característica adicional de *Trichoderma* es que coloniza las plantas, estimula sus mecanismos de defensa y promueve su crecimiento mediante la producción de sustancias denominadas auxinas (Contreras-Cornejo et al., 2004).

Es debido a todas las bondades antes mencionadas que *Trichoderma* tiene una gran aplicación biotecnológica y hoy en día se desea ampliar el conocimiento acerca de qué factores ambientales son los que inducen su desarrollo reproductivo, como es que percibe el estímulo y como es que se da la señal al interior para dar inicio a la formación de espora con fines de dispersión y supervivencia por largo tiempo en condiciones de estrés.

Diferentes señales ambientales influyen en la transición de micelio a espora en *Trichoderma* pero ¿cómo las percibe?

La esporulación o conidiación es un proceso morfogenético común en los hongos filamentosos y consiste en la producción de esporas asexuales (Steyaert, et al*.,* 2010). En el ecosistema donde habita *Trichoderma*, existen distintas señales ambientales que desencadenan su reproducción asexual; tal es el caso de la escasez de nutrientes, el pH ambiental, la luz o algún tipo de lesión en el micelio (Steyaert et al., 2010).

Pero, ¿cómo es que *Trichoderma* percibe todos estos estímulos? Todas las células eucariotas tienen vías de señalización altamente conservadas, que les permiten reconocer, transmitir y procesar señales extracelulares todo con el fin de adaptarse al ambiente. Dichas vías están constituidas por una red de proteínas que interactúan entre sí para regular la transmisión de la señal a sus blancos intracelulares e integrar así respuestas altamente específicas al estímulo inicial. La cascada de señalización de las proteínas cinasas activadas por mitógenos (MAPK), tiene como principal función la transducción de señales desde la superficie celular hasta el núcleo, controlando así procesos tan vitales como la proliferación, la diferenciación y la muerte celular. En el caso de *Trichoderma*, este tipo de señalización está asociada con varios procesos fisiológicos esenciales como la esporulación, el crecimiento de hifas y el micoparasitismo (Reithner et al., 2007).

Trichoderma produce sustancias de origen lipídico denominadas oxilipinas que actúan como inductores químicos de la esporulación.

Diversos estudios indican que las bacterias producen y excretan sustancias químicas denominadas autoinductores, que actúan como moléculas señal; cuanto mayor sea la población, mayor será la concentración de autoinductores. Una vez alcanzada la concentración umbral, estas moléculas inducen a la población a cooperar en diversos comportamientos como la virulencia, producción de antibióticos, competencia y esporulación, entre otros. En el caso de hongos, este fenómeno no ha sido explorado a profundidad, pero al menos en *Candida albicans* se sugiere que una sustancia química denominada farnesol regula la transición de levadura a micelio (Hornby et al., 2001).

En *Trichoderma* se ha reportado que el 1-octen-3-ol y la 3-octanona podrían actuar como inductores de la esporulación. Estos compuestos provienen de la peroxidación de ácidos grasos insaturados y se denominan colectivamente como oxilipinas (Nemcovic et al., 2008).

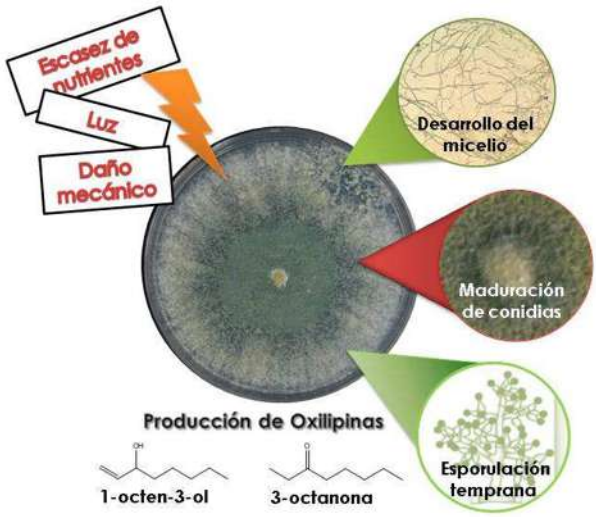

Figura 1. Diversos estímulos inducen la producción de oxilipinas en *Trichoderma atroviride*, tal como el 1-octen-3-ol y la 3-octanona que actúan como moléculas señal que afectan el desarrollo reproductivo del hongo.

Aportaciones al conocimiento durante la estancia en el verano nicolaita

Con el proyecto que se realizó durante el verano nicolaita, pudimos estudiar el desarrollo reproductivo de *T. atroviride* y cómo éste se afecta ante distintas condiciones de estrés. En la Figura 1, se observa una colonia de *T. atroviride*; la cual presenta una coloración típica en verde oscuro que indica madurez de las conidias. En el estudio se determinó que la escasez de nutrientes, la luz y el daño por herida, indujeron la producción de espora y cambios en la concentración de oxilipinas en el hongo. Mientras la luz induce la síntesis de estos compuestos, el daño por herida afecta negativamente su abundancia. Por otro lado, además del 1-octen-3-ol y la 3-octanona, se lograron identificar otras oxilipinas que no habían sido reportadas con anterioridad como la 2-heptanona, 2-nonanona, 3-octanol, 2-undecanona y 6-pentil-2*H*-piran-2-ona, cuyas concentraciones también variaron en cada tratamiento.

A la par, se estudió a la mutante Δ*tmk1*, que está afectada en un gen que codifica para una MAP cinasa. Esta mutante se caracteriza por un crecimiento más retardado y una coloración blanco-amarillenta de las conidias. Después de crecer a la mutante en condiciones de estrés, se observó que también se incrementó el número de esporas en cada tratamiento. Sin embargo, al medir los

niveles de oxilipinas en la mutante, se encontró que eran muy distintos a los de la especie silvestre. En presencia de luz, la concentración de 1-octen-3-ol se incrementó tres veces en comparación a la especie silvestre, mientras que la abundancia de la 2-heptanona, 3-octanona y 2-nonanona se disminuyó dos veces.

Estos resultados indican que son varias las oxilipinas las que actúan como señales químicas durante el desarrollo reproductivo del hongo y que en condiciones de estrés, la vía MAPK de *T. atroviride* dependiente de TMK1 es la encargada de regular su síntesis, afectando la transición de micelio a espora para garantizar la supervivencia y adaptación del hongo en ese ambiente.

Agradecimientos

Este proyecto fue aprobado por la Coordinación de la Investigación Científica (CIC-UMSNH) y el Consejo Nacional de Ciencia y Tecnología (CONACYT).

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Trichoderma Modulates Stomatal Aperture and Leaf Transpiration Through an Abscisic Acid-Dependent Mechanism in *Arabidopsis*

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Abstract *Trichoderma* species are widespread phytostimulant fungi that act through biocontrol of root pathogens, modulation of root architecture, and improving plant adaptation to biotic and abiotic stress. With the major challenge to better understand the contribution of *Trichoderma* symbionts to plant adaptation to climate changes and confer stress tolerance, we investigated the potential of *Trichoderma virens* and *Trichoderma 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 either *T. virens* or *T. atroviride*, and stomatal aperture and water loss were determined in leaves. *Arabidopsis* WT seedlings inoculated with these fungal species showed both decreased stomatal aperture and reduced water loss when compared with uninoculated seedlings. This effect was absent in *abi1-1* and *abi2-1* mutants. *T. virens* and *T. atroviride* induced the abscisic acid (ABA) inducible marker *abi4*:*uidA* and produced ABA under standard or saline growth conditions. These results show a novel facet of *Trichoderma*-produced metabolites in stomatic aperture and water-use efficiency of plants.

Keywords *Trichoderma* · *Arabidopsis* · Abscisic acid · Stomata · Transpiration

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Introduction

Abscisic acid (ABA) is an isoprenoid plant hormone with a role in many physiological processes during the plant life cycle, including vegetative development in response to various environmental stresses such as drought and high salinity conditions (Contreras-Cornejo and others 2014). In particular, salt stress induces accumulation of ABA, which regulates water balance by promoting stomatal closure in leaves. Stomata are natural microscopic pores surrounded by pairs of guard cells located on the leaf epidermis and in other aerial parts. Guard cells dynamically regulate the size of stomatal apertures and thereby control gas exchange, allowing entry of sufficient CO_2 for optimal photosynthesis (Brodribb and McAdam 2011). By opening and closing stomata, the guard cells control either water loss or water retention during transpiration (Allen and others 1999).

ABA signaling plays a major role in stomatal aperture. *Arabidopsis* ABA-insensitive mutants, *abi1* and *abi2,* do not close their stomata in response to exogenous ABA or drought stress (Roelfsema and Prins 1995; Pei and others 1997). The *abi1* and *abi2* loci encode semi-dominant mutations in two distinct type 2C protein phosphatases (Allen and others 1999). These proteins are thought to inhibit ABA signal transduction through binding to a putative substrate, thus preventing its activity (Merlot and Giraudat 1997; Sheen 1998). ABI4 belongs to the family of *AP2* (*APETALA 2*) transcription factors, and its loss of function in *abi4* mutants renders a sugar-insensitive phenotype (Finkelstein and others 1998). In addition to its role in sugar signaling, ABI4 is required for seed development and salt responses (Arroyo and others 2003).

Despite ABA being a major player in promoting abiotic stress resistance, little is known about its role in plant–fungi interactions. Recent evidence suggests that ABA

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plays an ambivalent role in plant defense responses to fungal pathogens, acting either as a positive or negative regulator of disease resistance by interfering at multiple levels with biotic stress signaling (Asselbergh and others 2008). Interestingly, ABA can be produced by filamentous fungi involved in the symbiotic or pathogenic interactions with plants suggesting its function during the establishment of these interactions (Siewers and others 2006).

Trichoderma species improve growth of plants and confer protection from pests and diseases (Contreras-Cornejo and others 2009, 2013). More recently, the protective effect of *Trichoderma* to improve salt tolerance in plants was shown. In that work, salinity repressed plant growth and root development of *Arabidopsis* seedlings by affecting auxin biosynthesis and/or signaling. Co-cultivation of *Arabidopsis* seedlings with *Trichoderma* improved salt tolerance by activating auxin signaling, which was related to the induction of lateral roots and root hairs and the elimination of Na^+ through root exudates (Contreras-Cornejo and others 2014). To investigate in more depth whether *Trichoderma* species can modulate plant adaptation to abiotic stress and its relationship to ABA signaling, we tested the effects of *Trichoderma virens* (Tv Gv29.8) and *Trichoderma atroviride* (IMI 206040) on stomatal apertures, water loss, and ABA-related signal transduction, and determined ABA biosynthesis in fungal cultures.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana Columbia-0 (Col-0) and Lansberg erecta (L*er*) wild-type lines, the transgenic line *abi4:uidA* (Arroyo and others 2003) and *abi1-1* and *abi2-1* mutants (Allen and others 1999) were used in this research. Seeds were disinfected with 96 % ethanol for 5 min and 20 % (v/v) bleach for 7 min, and after five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog basal salts mixture, Cat M5524: Sigma, St. Louis). Plates were placed vertically at a 65° angle to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants were grown at 24 °C in a growth chamber with a 16 h of light (200 μmol m^2 s^{-1}), 8 h dark photoperiod.

Fungal Growth and Plant Inoculation Experiments

The following fungal strains were used in this work: *T. virens* (Tv Gv29.8) and *T. atroviride* (IMI 206040). *T. virens* and *T. atroviride* were evaluated in vitro for their effects on water loss and stomatal opening. Spores (10^6) were placed 5 cm from the roots of *Arabidopsis* seedlings grown at the opposite ends of agar plates supplied with 0.2× Murashige and Skoog medium. Plates were arranged in a completely randomized design. The seedlings were cultured in a Percival AR95L growth chamber.

Plant Transpiration Measurements

Arabidopsis plants were grown on 0.2× MS medium and inoculated as mentioned above. Transpiration measurements on excised rosettes were monitored for 60 min. Water loss was determined by calculating the decrease in fresh weight of 20 rosettes per sample.

Stomatal Aperture Measurement

Arabidopsis seedlings inoculated with *Trichoderma* spp. for 5-days were collected and incubated in crystal violet for 2 h. At the end of this period, the seedlings were washed with distilled water and incubated in concentrated ethanol and heated for 10 min. After these procedures, seedlings were fixed in 50 % glycerol (v/v). The processed tissue was included in glass slips and sealed with commercial nail varnish. Stomatal apertures were measured using the image Tool software applied on pictures taken with an optical microscope (Optiphot-2, Nikon) fitted with a camera (Nikon coolpixs10). For each treatment, at least 100 stomata were measured.

Abscisic Acid Determination

ABA determinations were done from culture medium of *Trichoderma*. An inoculum of 10^6 conidia of *T. virens* or *T. atroviride* was added to 200 ml potato dextrose broth (Sigma), and grown for 3 days at 28 °C with shaking at 200 rpm. The fungal culture was filtered, and the pH of the supernatant adjusted to 3 using HCl. Acidic compounds in supernatant solutions were extracted three times with 500 ml of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen. ABA was derivatized with acetyl chloride in methanol (500 μl/2 ml), sonicated for 15 min and heated for 1 h at 75 °C. After cooling, the derivatized sample was evaporated and resuspended in 25 μl of ethyl acetate for GC–MS analysis. GC-SIM-MS and retention time were established for the ABA methyl ester (ABA-Me; *m/z* 134, 190 and 278 M^+, 15.51 min). *cis*, *trans*-ABA was purchased from Sigma and used as standard. The ABA identity was further confirmed by comparison with the pure standard, and the amounts were calculated by constructing a standard curve.

GC–MS Analysis

Samples were injected in an Agilent 6850 Series II gas chromatograph (GC) equipped with an Agilent MS detector

model 5973 and 30 m × 0.25 µm × 0.25 mm, 5 % phenyl methyl silicone capillary column (HP-5 MS). The operating conditions were 1 ml min^{-1} Helium as carrier gas, 300 °C detector temperature, and 250 °C injector temperature. The column was held for 5 min at 150 °C and programed at 5 °C min^{-1} to a 278 °C final temperature for 5 min.

Histochemical Analysis

Transgenic plants expressing *abi4:uidA* were co-cultivated with *Trichoderma* and incubated 10 h at 37 °C in GUS reaction buffer (0.5 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared as described by Contreras-Cornejo (2009). For each treatment, at least 15 transgenic plants were analyzed. The processed seedlings were included in glass slips and sealed with commercial nail varnish. For each treatment, a representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

Data Analysis

Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate analyses with a Tukey's post hoc test were used for testing differences in transpirational water loss and percentage of stomatic closure in *Arabidopsis* WT and mutants.

Results

Trichoderma Alters Stomatal Aperture and Transpiration in *Arabidopsis WT* Seedlings but not in ABA-Related Mutants

To analyze the effects of *Trichoderma* on transpiration, stomatal aperture was analyzed in 9-day-old WT *Arabidopsis* seedlings from the Columbia-0 (Col-0) ecotype grown on Petri plates containing agar-solidified 0.2× MS medium after 5 days of fungal co-cultivation. As previously reported (Contreras-Cornejo and others 2009), we found that *T. virens* and *T. atroviride* promoted shoot biomass accumulation and root branching. In addition, we observed that *T. virens* and *T. atroviride* induced 47 and 39 % stomatal closure, respectively, when compared with uninoculated seedlings that were considered as 100 % (Fig. 1a, b). The reduction of plant transpiration in inoculated seedlings represented as water loss compared with uninoculated seedlings indicated that *Trichoderma* regulates the water status in *Arabidopsis* (Fig. 1c). Stomatic aperture was then compared in *Arabidopsis* WT seedlings

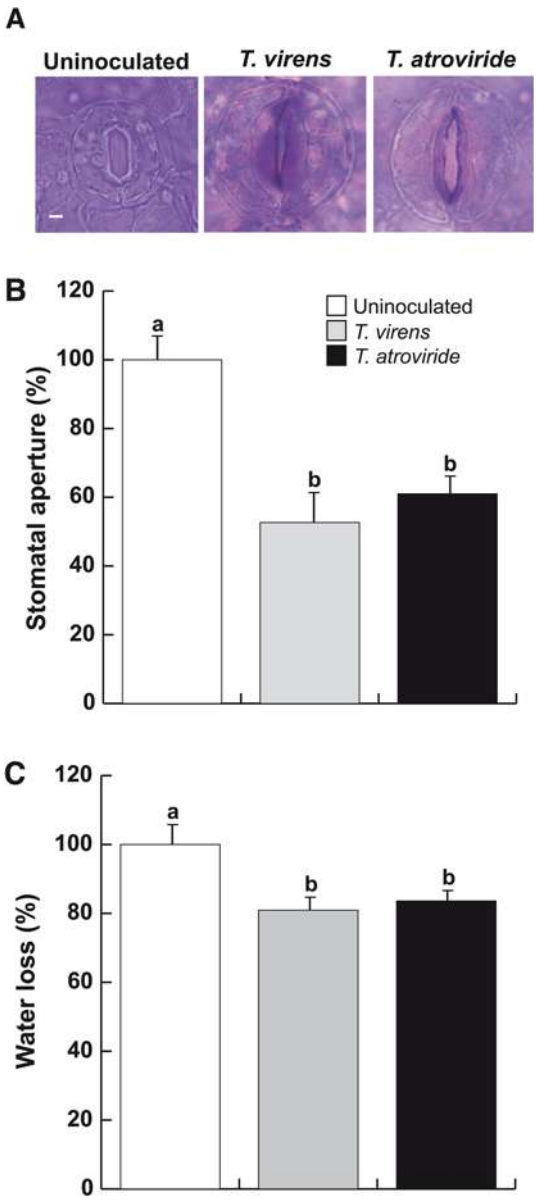

Fig. 1 *Trichoderma* regulates stomatal aperture and transpiration in *Arabidopsis thaliana* (Col-0) seedlings. **a** Representative images of stomata from seedlings inoculated with *Trichoderma* spp. *Bar* = 5 µm. **b** Effects of *Trichoderma* spp. on stomatal aperture and **c** effect of *Trichoderma* spp. on transpirational water loss represented as percentage. Values shown represent means of five groups of 20 seedlings ± SE. *Different letters* are used to indicate means that differ significantly ($P < 0.05$)

of the Landsberg erecta (Ler) ecotype and mutants defective in ABA signaling *abi1-1* and *abi2-1*, which are in the Ler genetic background. It was found that both fungal species induced stomatal closure in wild-type seedlings when compared with uninoculated seedlings (Fig. 2a–c). Interestingly, ABA-related mutants, *abi1-1* and *abi2-1,* did not show any change in stomatal closure in response to *Trichoderma* co-cultivation (Fig. 2d–j).

Trichoderma Induces the Expression of the Abscisic Acid Inducible *abi4:uidA* Marker in *Arabidopsis*

We next tested whether *Trichoderma* signals could alter the expression of *abi4:uidA* an ABA-regulated gene that encodes a transcriptional factor induced by salt and other abiotic stresses in *Arabidopsis*. Transgenic seedlings expressing *abi4:uidA* were germinated and grown on 0.2× MS medium. As *abi4:uidA* is expressed differentially during the seedling development (Arroyo and others 2003); this gene was analyzed at 5 days after germination. Seedlings expressing *abi4:uidA* were transferred to liquid medium containing the acidic extracts from *T. virens* or *T. atroviride,* incubated 3 h and then stained for GUS activity and cleared to visualize changes in GUS expression. We found that seedlings treated with the extracts of *Trichoderma* had an increased GUS expression when compared with the controls (Fig. 3a–c). These data suggest that *Trichoderma* activates the expression of ABA-related gene expression in *Arabidopsis*.

Trichoderma Produces Abscisic Acid

The effects observed in stomatal closure, water loss, and induced expression of *abi4:uidA* by *Trichoderma* open the possibility that these fungi could produce ABA. We conducted experiments aimed at identifying ABA from liquid cultures of *Trichoderma* determining this compound by GC–MS analysis under normal or saline growth conditions (Fig. 4). The production of ABA by *T. virens* and *T. atroviride* was determined in liquid medium with or without 100 mM NaCl, a salt concentration that drastically affects root branching in *Arabidopsis* (Contreras-Cornejo and others 2014). It was found that salt slightly increased ABA production in both fungal species from 0.11 ± 0.05 to 0.33 ± 0.1 ng ml^{-1} for *T. virens* and 0.15 ± 0.11 to

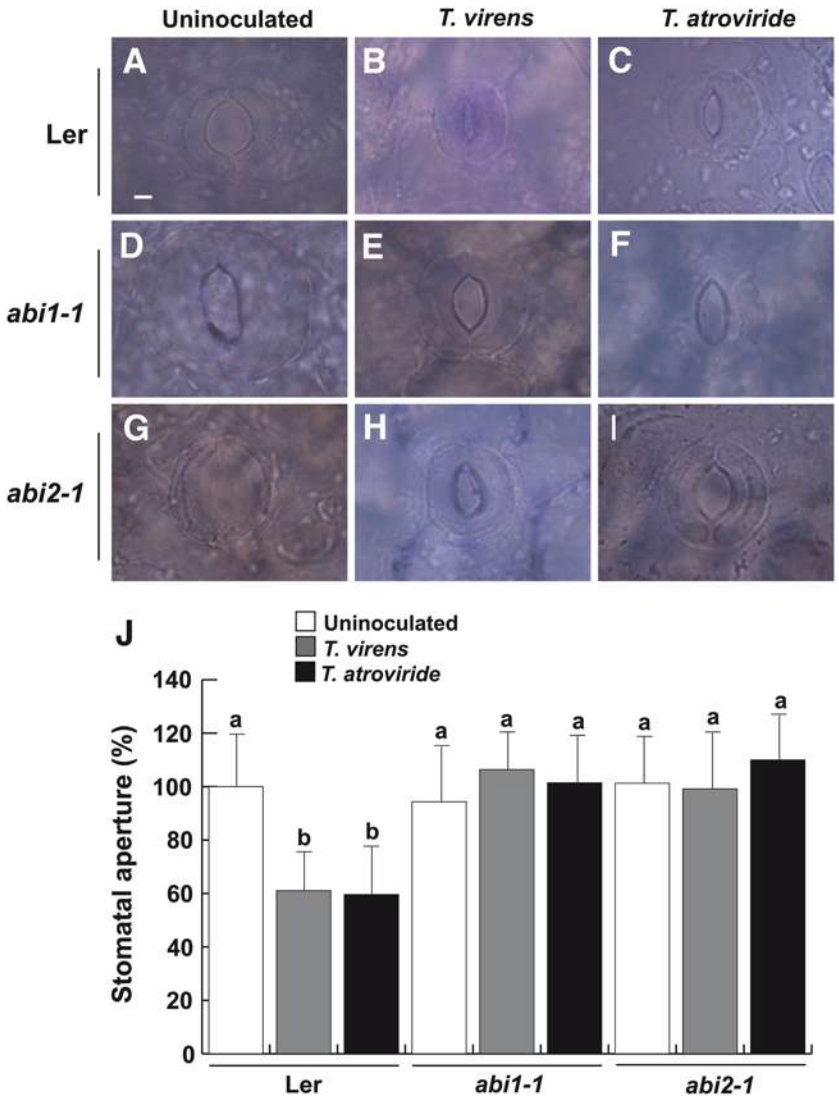

Fig. 2 Effect of *Trichoderma* on stomatal aperture in ABA-related mutants. **a**–**i** Representative images of stomata from *Arabidopsis* wild-type (Ler), *abi1-1* and *abi2-1* inoculated with *Trichoderma* spp. *Bar* = 5 µm. **j** Stomatal aperture. *Bars* show the mean ± SE of 100 measurements by treatment. *Different letters* are used to indicate the means that differ significantly ($P < 0.05$)

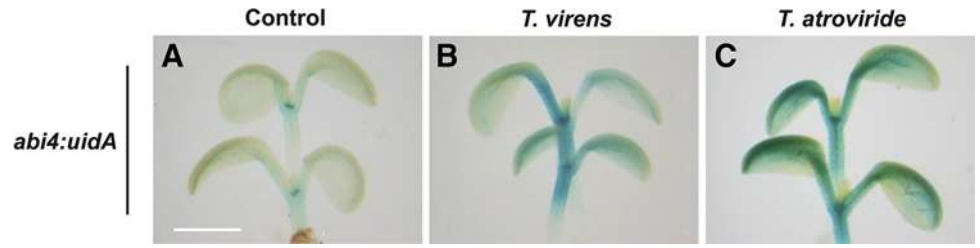

Fig. 3 Effect of *Trichoderma* on the expression of *abi4:uidA*. **a**–**c** Expression of *abi4:uidA* in 5-day-old transgenic seedlings. Notice that *T. virens* and *T. atroviride* induced significantly the β-glucuronidase activity in shoots. *Photographs* are representative individuals of at least 12 plants stained. *Scale bar* = 250 μm. The experiment was repeated two times with similar results

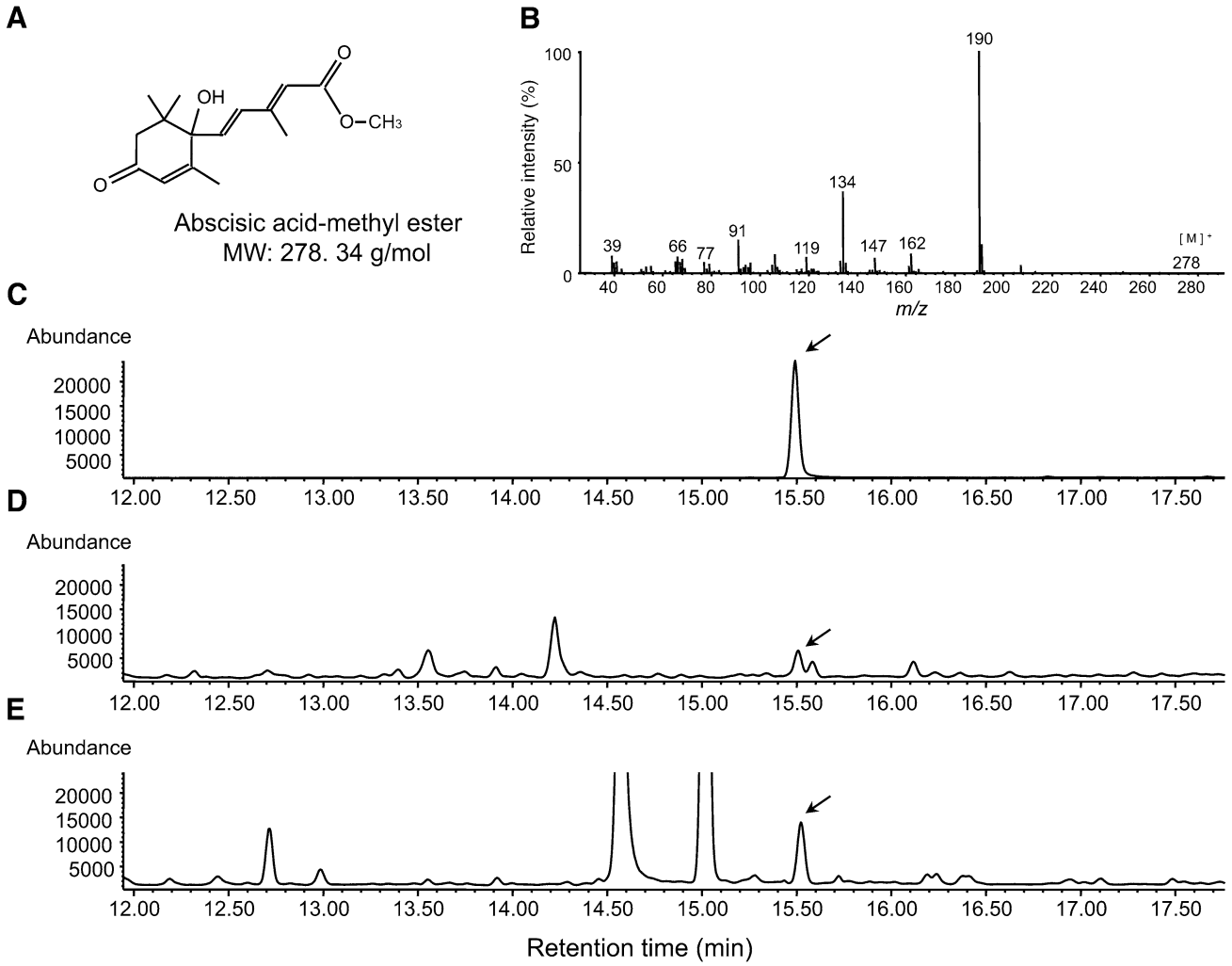

Fig. 4 Determination of ABA from *Trichoderma* spp. ABA was identified from acidic ethyl acetate from liquid culture medium of *Trichoderma* and analyzed by GC–MS. **a** Chemical structure of ABA-derivatized sample. **b** The 70-eV electron-impact full-scan mass spectra of ABA from pure standard. **c** Total ion chromatogram of ABA from pure standard. **d** Total ion chromatogram of ABA from *T. virens* and **e** from *T. atroviride*. The *arrows* show the presence of ABA. Determinations were done three times from independent samples

0.21 ± 0.009 ng ml^{-1} for *T. atroviride*. These data show that *Trichoderma* spp. are able to produce ABA when subjected to salt treatment, which could regulate the stomatal aperture to modulate water status of plants under salt stress.

Discussion

Salinity is one of the major environmental factors affecting plant growth and productivity as it influences membrane

organization, impairs nutrient and water acquisition, inhibits photosynthesis and protein synthesis, and increases production of reactive oxygen species (Contreras-Cornejo and others 2014). Salt tolerance mechanisms are coordinated by the action of many gene families that perform a variety of functions such as ion sequestration, osmotic adjustment, antioxidant defense, and control of water loss through stomata (Flors and others 2007). Adaptation to water stress results from an alteration in gene expression by up-regulation of the major ABA- and/or stress-responsive genes, like response to dehydration (RD), cold-responsive (COR), late embryogenesis-abundant (LEA)/dehydrin-like, and aquaporin genes (Seki and others 2001; Efetova and others 2007). One of the most rapid responses of plants to salt stress is the closure of stomata to prevent excessive water loss, as carbon and water flow between plants and the atmosphere is regulated by the opening and closing of stomatal pores (Brodribb and McAdam 2011). High turgor pressure deforms the guard cells to form an open pore, which allows rapid diffusion of atmospheric CO_2 through the epidermis into the photosynthetic tissues inside the leaf. Declining turgor causes the guard cells to close together, greatly reducing leaf water loss while also restricting entry of CO_2 for photosynthesis (Brodribb and McAdam 2011).

In this work, we found that *Arabidopsis* seedlings inoculated with *Trichoderma* have reduced transpiration when compared with uninoculated seedlings. The effect of *Trichoderma* on the water status could be part of a mechanism to control the water in transpiration in shoots, which may be useful in plants exposed to high temperatures, drought, cold stress, and/or salinity opening new avenues toward the use of *Trichoderma*-based inoculants (Fig. 1).

Guard cells use an extensive signal transduction pathway to regulate the aperture of stomata that facilitate gas exchange and transpirational water loss (Joshi-Saha and others 2011). Many lines of evidence strongly support a role for ABA in the regulation of stomatal aperture. ABA levels are regulated by a variety of environmental conditions (Efetova and others 2007). It has been reported that plant salt responses are triggered by increased levels of ABA (Achard and others 2006). A positive correlation between ABA accumulation and salt stress tolerance was found in *Trichoderma*-inoculated seedlings. *Arabidopsis* seedlings inoculated with *T. virens* or *T. atroviride* showed a two-fold increased ABA concentration in shoots when compared with axenically grown seedlings. ABA levels in uninoculated plants were six-fold higher in response to saline stress. Interestingly, plants subjected to 100 mM NaCl treatment and inoculated with *Trichoderma* spp. displayed similar levels of ABA compared with non-stressed plants (Contreras-Cornejo and others 2014). ABA may also be accumulated in roots and transported to leaves through the xylem, where it plays an important role in modulating stomatal responses (Allen and others 1999; Contreras-Cornejo and others 2014). An increase in ABA levels promotes the closing of stomata and inhibits the opening of closed stomata (Brodribb and McAdam 2011). Recently, it was reported that *T. hamatum* delayed drought-induced changes in stomatal conductance and net photosynthesis (Bae and others 2009). In this work, we found that *Trichoderma* spp. induced stomatal closure in *Arabidopsis* in a way that resembles the effects mediated by ABA.

A recent study suggests that ABI4 is a key factor that regulates primary seed dormancy by mediating the balance between ABA and GA biogenesis (Shu and others 2013). Seeds of the *Arabidopsis abi4* mutant that were subjected to short-term storage (one or two weeks) germinated more quickly than WT, whereas the ABA content of dry *abi4* seeds was remarkably lower than that of WT, but the amounts were comparable after stratification. The earliest events of the ABA signaling pathway occur through a central signaling module made up of three protein classes: PYR/RCARs, protein phosphatase 2Cs (PP2Cs), and SNF1-related protein kinase 2 s (SnRK2 s). Protein phosphatase 2C proteins called ABI1 and ABI2 have a central role in ABA response, as mutations in each gene affect all ABA responses (Santner and others 2009). *Arabidopsis abi1* and *abi2* mutants were isolated by selecting plants that grew well on a medium containing 10 μM ABA, and both mutations reduce seed dormancy and the sensitivity of germination to the inhibitory effects of ABA (Koornneef and others 1984; Finkelstein and Somerville 1990). Specifically, ABA does not close stomata of *abi1* and *abi2*, whereas increases in the extracellular calcium concentration cause a decrease in stomatal aperture in the WT, *abi1*, and *abi2* (Allen and others 1999). Our data are in agreement with those of Allen and coworkers (1999) by showing that WT, *abi1* and *abi2* seedlings have similar stomatal aperture when grown under standard growth conditions. However, we found that *abi1-1* and *abi2-1* had null response to *Trichoderma* in terms of stomatal closure induction (Fig. 2). Because ABA is produced in response to drought stress and mediates a reduction in stomatal aperture that prevents excessive evaporation-mediated water loss, the stomatal aperture alterations in *abi1* and *abi2* mutants would be more clearly evidenced under biotic or abiotic stress or under conditions that increase ABA levels and/or response.

The ABI4 transcription factor is known to be induced in response to ABA in developing seedlings, or in response to salt and sugar (Arroyo and others 2003; Finkelstein and others 2011). Alleles of ABI4 have been identified in salt stress screens (Gazzarrini and McCourt 2001). Our expression studies of the ABA-inducible marker *abi4:uidA* suggests that *Trichoderma* species increase the ABA

response in *Arabidopsis* likely through this transcription factor (Fig. 3).

In soil, microorganisms communicate with plants by exchanging chemical signals throughout the rhizosphere (Contreras-Cornejo and others 2013). Rhizospheric fungi are also exposed to abiotic stresses such as drought, cold stress, and salinity. Recently, it was reported that NaCl affects the growth of *T. virens* and *T. atroviride* but in a lower level compared to *Arabidopsis* seedlings (Contreras-Cornejo and others 2014). To determine the effect of salinity on ABA production, *T. virens* and *T. atroviride* were grown in liquid medium supplemented or not with 100 mM NaCl. We found that *Trichoderma* increase the production of ABA under salt stress (Fig. 4). Exogenous ABA is known to stimulate in vitro the growth and development of several saprophytic fungi such as *Aspergillus niger*, *Fusarium culmorum*, *Cylindrocarpon destructans*, *Schizophyllum commune*, *Monilia laxa*, *Monilia fructigena*, *Gloeosporium album*, *Botrytis cinerea*, and *Monilia fructigena* (Zielinska and Michniewicz 2001). Moreover, ABA is necessary for the proper formation of arbuscules, the key symbiotic interfaces of arbuscular mycorrhizal fungi and host cells, and for a sustained colonization of plant roots (Herrera-Medina and others 2007). Brotman and others (2013) performed genetic analyses in the root system of *Arabidopsis* inoculated with *T. asperelloides* T203. A group of biotic- and abiotic-responsive genes was clustered and the gene *ABI1* was increased 24 h post-inoculation (Brotman and others 2013). Presumably, the increase in plant growth and salt tolerance observed in previous research could be due to the combined activities of *Trichoderma* through the production and release of auxins, ABA, and possibly other metabolites. Because the stomata of *abi1-1* and *abi2-1* do not close in response to ABA, we suggest that phosphatases ABI1 and ABI2 could mediate the stomatal closure induced by these fungi.

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Trichoderma-Induced Acidification Is an Early Trigger for Changes in *Arabidopsis* Root Growth and Determines Fungal Phytostimulation

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Trichoderma spp. are common rhizosphere inhabitants widely used as biological control agents and their role as plant growth promoting fungi has been established. Although soil pH influences several fungal and plant functional traits such as growth and nutrition, little is known about its influence in rhizospheric or mutualistic interactions. The role of pH in the *Trichoderma–Arabidopsis* interaction was studied by determining primary root growth and lateral root formation, root meristem status and cell viability, quiescent center (QC) integrity, and auxin inducible gene expression. Primary root growth phenotypes in wild type seedlings and STOP1 mutants allowed identification of a putative root pH sensing pathway likely operating in plant–fungus recognition. Acidification by *Trichoderma* induced auxin redistribution within *Arabidopsis* columella root cap cells, causing root tip bending and growth inhibition. Root growth stoppage correlated with decreased cell division and with the loss of QC integrity and cell viability, which were reversed by buffering the medium. In addition, *stop1*, an *Arabidopsis* mutant sensitive to low pH, was oversensitive to *T. atroviride* primary root growth repression, providing genetic evidence that a pH root sensing mechanism reprograms root architecture during the interaction. Our results indicate that root sensing of pH mediates the interaction of *Trichoderma* with plants.

Keywords: plant growth, root development, symbiosis, soil pH, pH sensing, biocontrol

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INTRODUCTION

Plants are constantly exposed to biotic or abiotic stimuli and adjust their growth and developmental patterns to adapt and survive. Members of the fungal genus *Trichoderma* are frequently found in the rhizosphere, a narrow soil zone influenced by roots, where many species establish beneficial interactions with plants either antagonizing phytopathogens or directly influencing morphogenesis (Benítez et al., 2004; Harman et al., 2004; Harman, 2006; Druzhinina et al., 2011; Hermosa et al., 2012).

A complex chemical interaction is established between *Trichoderma* and their plant hosts comprising volatile and diffusible secondary metabolites, small peptides, and/or antibiotics, which influence root growth, branching and absorptive capacity (Samolski et al., 2012; López-Bucio et al., 2015). *T. virens* produces and releases auxinic compounds, including indole-3-ethanol (IET), indole-3-acetaldehyde (IALD), indole-3-carboxaldehyde (ICALD), and indole-3-acetic acid (IAA)

(Contreras-Cornejo et al., 2009), whereas *T. atroviride* and *T. asperellum* produce the volatile 6-pentyl-2*H*-pyran-2-one (6-PP), which modulates plant growth and root system architecture (Kottb et al., 2015; Garnica-Vergara et al., 2016). *T. atroviride* also produces ethylene, and ethylene-related mutants *etr1* and *ein2* show defective root-hair induction and enhanced primary-root growth inhibition when co-cultivated with this fungus (Contreras-Cornejo et al., 2015). Thus, auxin and ethylene (ET) signaling play a major role in the *Arabidopsis* root developmental response to *Trichoderma.* Furthermore, *Trichoderma* induces plant defense responses and improves crop performance under different stress conditions (Mastouri et al., 2010, 2012; Contreras-Cornejo et al., 2011, 2014a; Salas-Marina et al., 2011; Rawat et al., 2013; Hashem et al., 2014).

The rhizosphere is the region where plant roots, soil conditions, and microorganisms interact. While *Trichoderma* root colonization is often of benefit to plants, improves nutrition, and/or enhances the degradation of toxic chemicals, the mechanisms of phytostimulation remain mostly unknown. The rhizosphere physicochemical conditions are the major driving forces influencing microbe proliferation (Husson, 2013), and no other single chemical soil characteristic is more important in determining the success of plants and soil microbes than pH (Brady and Weil, 1999). Optimum pH for growth varies considerably among plants, but most cultivated species grow well on slightly acid or neutral soils, in which root cells function properly (Marschner, 1991; Brady and Weil, 2010; Shavrukov and Hirai, 2016). However, when soil pH becomes more acid (lower than 5.5), root growth is repressed and plant yield decreases, correlating with an increase in toxic levels of aluminum (Al^{3+}), manganese (Mn^{2+}), iron (Fe^{2+}), and protons (H^{+}), as well as decrease in the availability of phosphorous (P), calcium (Ca^{2+}), and magnesium (Mg^{2+}) (von Uexküll and Mutert, 1995; Kochian et al., 2004; Fan et al., 2016a; Shavrukov and Hirai, 2016). An acidic pH further inhibits root cell division and elongation, and compromises meristem cell viability (Koyama et al., 1995; Yokota and Ojima, 1995; Lager et al., 2010; Graças et al., 2016).

In fungi, pH is also an important factor that affects growth, development and competition (Alkan et al., 2013). Several pathogenic fungi acidify the pH of the growth media such as *Penicillium* sp., *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, and *Phomopsis mangiferae*; whereas *Colletotrichum* sp, *Alternaria alternata*, and *Fusarium oxysporum* alkalinize it, and this property is strongly involved in virulence regulation (Alkan et al., 2013; Prusky et al., 2016). *Trichoderma* spp. grows better in acidic conditions with an optimal growth at pH ranging from 4 to 6, and they can modify the pH of the rhizosphere (Trushina et al., 2013; Singh et al., 2014), but the consequences of fungal-mediated pH changes for root growth and development have not yet been analyzed.

Here, we hypothesized that acidification may play an important role in the configuration of root architecture and phytostimulation elicited by *Trichoderma*. Through detailed characterization of the effects of several *Trichoderma* species on *Arabidopsis* seedling growth, the fungal capacity to acidify the growth medium, the effects of low pH stress on root growth and plant development, as well as testing the responses of selected *Arabidopsis* mutants defective on pH sensing, we demonstrate the critical role of fungal acidification as an early response influencing root morphogenesis and plant growth. Moreover, since lateral root initiation started earlier or in parallel to root tip bending and root stoppage, we propose that a low pH independent program operates at the root pericycle to induce root branching.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All plants used in this study were in the *Arabidopsis thaliana* (L.) Heynh., Columbia (Col-0) background. Transgenic *Arabidopsis* lines *DR5::GFP* an auxin-inducible marker (Ottenschläger et al., 2003); *H2B::YFP* a cell viability marker (Boisnard-Lorig et al., 2001); *WOX5:GFP* a quiescent center (QC) marker in the root stem cell niche (SCN) (Sarkar et al., 2007); *CycB1:uidA* a marker of mitotic activity, expressed in the G2/M phase of the cell cycle (Colón-Carmona et al., 1999) previously characterized as well as the mutant *stop1* known to show a hypersensitive root response to low pH (salk_114108) obtained from the Nottingham Arabidopsis Stock Centre (NASC), were used for the different experiments. Seeds were surface-disinfected with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min, washed five times with distilled water, and stratified for 2 days at 4°C. Seeds were germinated and grown on agar plates containing 0.2X Murashige and Skoog (1962) medium (MS basal salts mixture, M524; PhytoTechnology), 0.6% sucrose (Sucrose: Ultrapure, MB Grade, 21938; USB Corporation) and 1% Agar (Agar, Micropropagation Grade, A111; PhytoTechnology) at pH 7. The suggested formulation is 4.3 $g.L^{-1}$ of salts for 1x medium; we used 0.9 $g.L^{-1}$, which we consider and refer to as 0.2X MS. For MES (2-(*N*-morpholino)ethanesulfonic acid) treatments it was included in the plant growth medium and the pH was adjusted to 7.0. All experiments were performed in an environmentally controlled growth room with a 16 h photoperiod (300 $\mu mol\ m^{-2}\ s^{-1}$ of light intensity), and 22°C.

Fungal Strain and Culture Conditions

Trichoderma atroviride IMI 206040 was propagated on potato dextrose agar (PDA; Difco), at 28°C for 5 days and then conidia were collected adding a small amount of sterile water into the Petri dishes and scraping the surface of the fungus. For the different experiments inoculation was carried out by placing a drop of a spore suspension containing 1×10^6 spores. In the interaction assays the *Trichoderma* inoculum was placed at 5 cm from *A. thaliana* primary roots germinated and grown for 5 days on agar plates containing 0.2X MS medium. The plates, which included 10 *A. thaliana* seedlings each, were arranged in a completely randomized design. After 3 and 5 days of co-cultivation, plant growth was determined. For acidification experiments *T. atroviride* was inoculated on plates containing MS 0.2X supplied or not with bromophenol blue (0.006%) and analyzed every 24 h for 4 days.

Analysis of Growth

Growth of primary roots was registered using a ruler. Lateral root number was determined by counting the lateral roots present in the primary root from the tip to the root/stem transition. Images were recorded using a digital camera (Nikon D3300, Osaka, Japan). The length of meristems was determined as the distance from the QC to the cell file where cells started to elongate and measured using IMAGEJ software (National Institute of Health, Bethesda, MD, United States). All experiments were repeated at least twice as indicated in the figure legends and data analyzed in the STATISTICA 10 software (Stat Soft Inc, 2011). Univariate and multivariate analyses with a Tukey's *post hoc* test were used for testing differences in the experiments. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

Trichoderma Soluble Metabolites Experiments

Trichoderma atroviride was inoculated on Petri plates containing MS 0.2X covered by a sterile cellophane sheet and incubated in darkness for the indicated times in the different experiments. The cellophane was removed together with the mycelium, then *Arabidopsis* seeds or seedlings were germinated or transferred, respectively, onto the plates where *Trichoderma* was pre-grown, and further grown for the indicated times.

Propidium Iodide Staining, GFP, and YFP Detection

For confocal microscopy, transgenic *A. thaliana* seedlings co-cultivated or not with *Trichoderma* were transferred from the growth medium to microscope slides with propidium iodide (20 μM), used as counterstain. All imaging was done using a Zeiss LSM 510 META inverted confocal microscope (Carl Zeiss, Germany) with either a 20X or 40X objective. GFP was excited with a 488 nm line of an Argon laser and propidium iodide (PI) with a 514 laser line. GFP emission was filtered with a BP 500–520 nm filter and PI emission was filtered with a LP 575 nm filter, or by using a confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan), with a 568-nm wavelength argon laser for excitation, and an emission window of 585–610 nm for propidium iodide and GFP or YFP fluorescence (488 nm excitation/505–550 nm emission, 514 nm excitation/527 nm emission, and 532 nm excitation/588 nm emission, respectively). Ten independent seedlings were analyzed per line, and treatment representative images were selected for figure construction.

Histochemical Analysis of GUS Expression

Histochemical β-glucuronidase (GUS) expression was evaluated by incubating the plant tissues in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M; pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100 with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37°C. Plants were cleared and fixed using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 15 transgenic plants were analyzed.

Determination of Developmental Stages of Lateral Root Primordia (LRP)

Lateral root primordia (LRP) were quantified 6 days after germination. Seedling roots were first cleared to enable LRP 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 eight to 10 'short' pericycle cells are formed). Stage II: the LRP are 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 are midway through the parent cortex. Stage VI: the LRP have passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appear to be just about to emerge from the parent root.

RESULTS

Early Root Responses during *Arabidopsis* Interaction with *Trichoderma*

To study the early responses of *Arabidopsis* seedlings to *Trichoderma*, we performed time-course experiments of *Arabidopsis* seedlings co-cultured with *T. atroviride*. We found that in the early stages of the interaction, from 24-to-60 h, primary root growth is unaffected (**Figure 1A**), and the meristem normally expresses *CyCB1:uidA*, a marker of mitotic activity, and the QC marker *WOX5:GFP*, whose corresponding WT protein is required to maintain the root SCN (**Figure 1B**). Nevertheless, *T. atroviride* clearly activated root branching at 60 h of the interaction (**Figure 1C**). The number of LRP per plant changed slightly at early stages of the interaction (24 and 48 h). However, such differences were no longer observed after 60 h (**Figure 1D**), suggesting that the differences in root branching could be due to an accelerated growth of LRP in response to *Trichoderma*. This was evidenced by analyzing the LRP developmental stages, where a decrease in the number of LRP still at the early stages of development, particularly those at stages III and IV, and an increase in the number of those more developed (stage VII) and emerged lateral roots (ELR), as early as 24 h of co-cultivation was observed (**Figure 1E**). These data suggest that *Trichoderma* increases root branching in *Arabidopsis* mainly by inducing the maturation of LRP and not *de novo* formation of LRP, as an early response that occurs independently of primary root growth inhibition.

Late Root Responses during Interaction with *Trichoderma*

Early root responses of *Arabidopsis* to *T. atroviride* did not evidenced any negative effect. In agreement with a previous report (Contreras-Cornejo et al., 2009), we observed that *T. atroviride* promoted growth and development of lateral roots. However, after a longer time of interaction (72–96 h), a primary

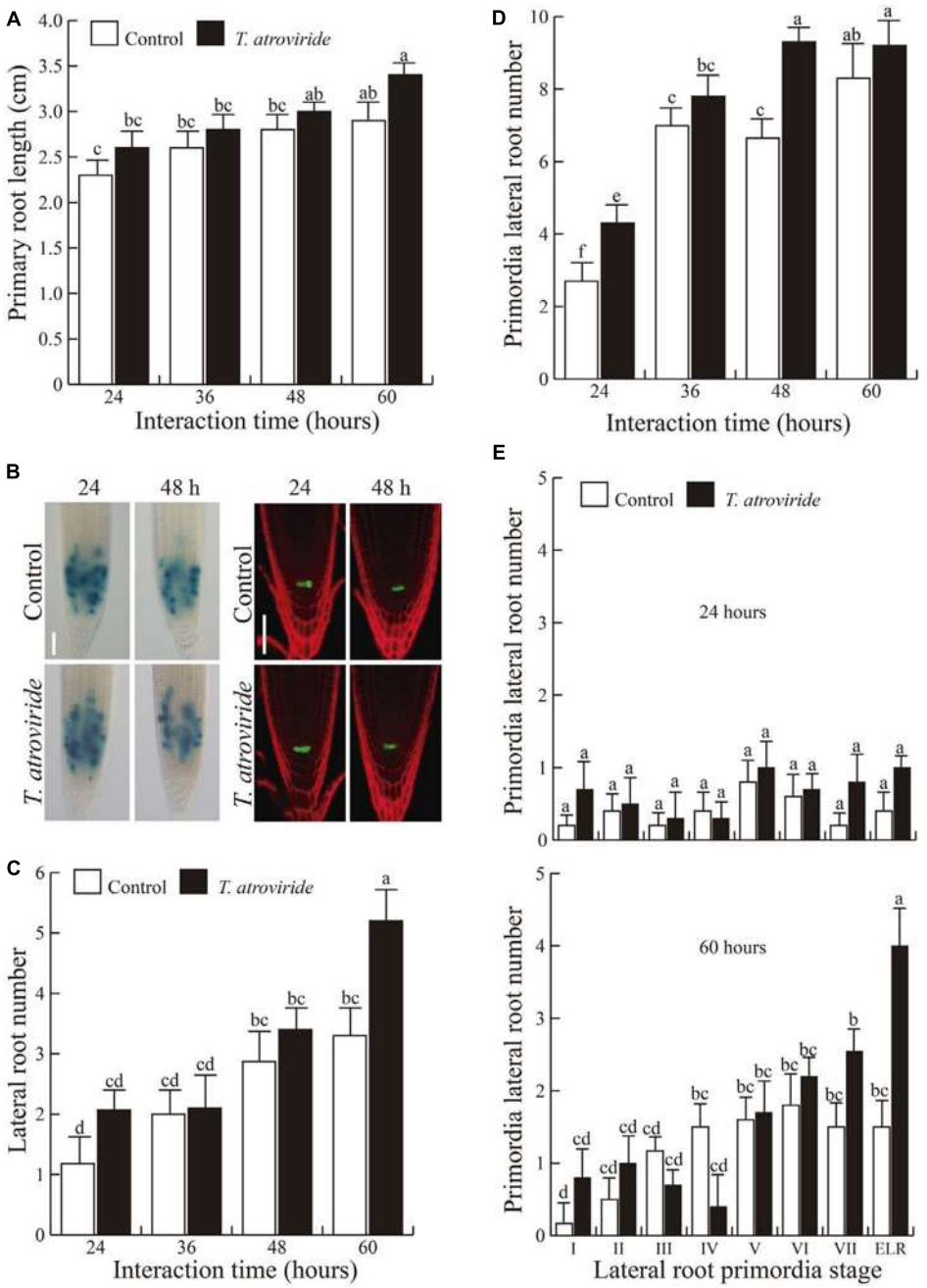

FIGURE 1 | Early responses of *Arabidopsis* to *Trichoderma atroviride*. *Arabidopsis* WT (Col-0) seeds were germinated and grown on agar solidified MS 0.2X medium. Four-day-old seedlings were inoculated with 1×10^6 spores of *T. atroviride* at the opposite side of where seeds were sown and analyzed at the indicated times. **(A)** Primary root length. **(B)** *CycB1:uidA* (left) and *WOX5:GFP* (right) expression after 24 and 48 h of *Trichoderma* inoculation. **(C)** Lateral root number per plant. **(D)** Total lateral root primordia (LRP) per plant. **(E)** Number of LRP per plant after 24 and 60 h of interaction. Stage I: LRP initiation (in the longitudinal plane, approximately eight to 10 'short' pericycle cells are formed). Stage II: the LRP are 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 are midway through the parent cortex. Stage VI: the LRP have passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appear to be just about to emerge from the parent root. Values shown represent means with SE of at least 30 seedlings. Different letters are used to indicate means that differ significantly ($P \geq 0.05$). Scale bars = 50 μm. The experiment was repeated three times with similar results.

root growth inhibitory effect could be appreciated. Hence, we characterized in detail this late root response and its relationship with lateral root formation.

Inoculation with *T. atroviride* shortened primary roots (**Figures 2A,C**) while increasing lateral root number (**Figures 2B,C**). Surprisingly, the root tips bent forming a

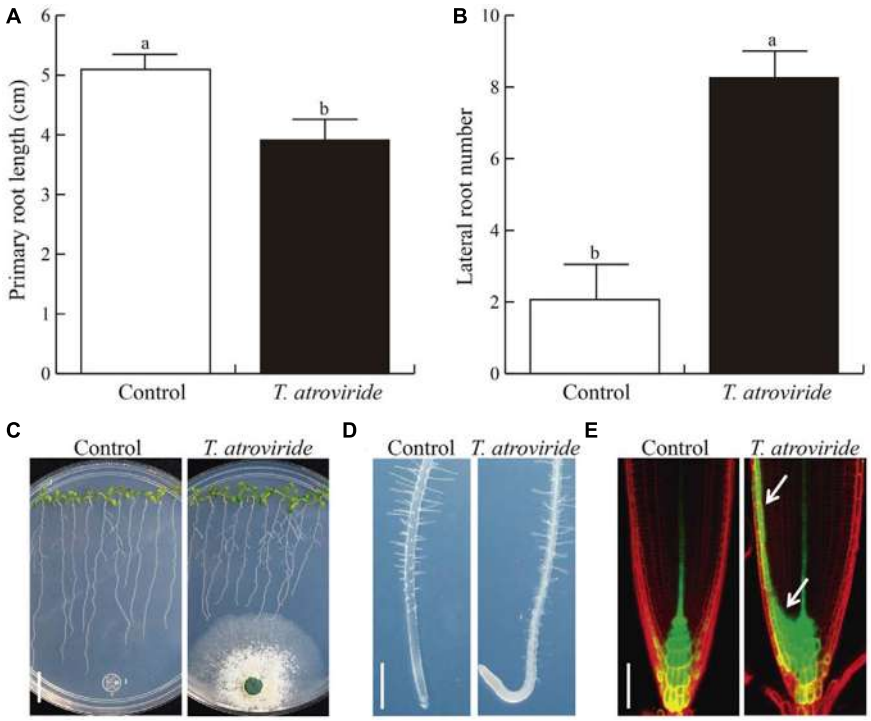

FIGURE 2 | Effects of *T. atroviride* inoculation on *Arabidopsis* root architecture. (A) Primary root length. **(B)** Lateral root number. **(C)** Representative photographs of *Arabidopsis* seedlings co-cultivated with *Trichoderma*. **(D)** Root tips of axenic or *Trichoderma* co-cultivated WT seedlings, and *DR5::GFP* seedlings **(E)**. White arrows show auxin redistribution. *Arabidopsis* seedlings were germinated and grown for 5 days on the surface of agar plates containing MS 0.2X medium and then inoculated with *T. atroviride* at the opposite side of the plate and grown for 4 more days. Different letters are used to indicate means that differ significantly ($P < 0.05$). Error bars represents SE. Scale bars in images **(C–E)** = 1 cm, 500 and 50 μm, respectively. The experiment was repeated three times with similar results.

hook. The latter event is followed by inhibition of primary root elongation, stopping at the place where hook formation occurred, before contacting the mycelium (**Figures 2C,D**). Unexpectedly, upon prolonged interaction these responses were accompanied by pigmentation and chlorosis of leaves (Supplementary Figure S1). To investigate whether the bending response was specific to *T. atroviride*, we analyzed the root response to different *Trichoderma* species (*T. asperellum*, *T. koningii*, and *T. harzianum*) and about 50 other native soil isolates. Interestingly, this response was similar, regardless of the *Trichoderma* species or isolate tested (representative images are shown in Supplementary Figure S2). These results suggest that a common signal released into the growth medium may be sensed by plants, thereof triggering the observed root responses.

To understand the signaling mechanisms involved in the elicited root bending response, and since *Trichoderma* was reported to produce auxins (Contreras-Cornejo et al., 2009), we analyzed the role of auxin signaling in this process. Like in the case of gravitational stimulation, an auxin- redistribution was observed in the root curvature response to *T. atroviride*, where auxins are redistributed within the root tip and accumulate on one side of the columella root cap cells, as indicated by the expression of the auxin-induced *DR5:GFP* marker (**Figure 2E**). This redistribution of auxin likely provokes a reduction of growth on one side of the root, which in turn could lead to the formation of the hook, or causes root growth reorientation. However, the observed redistribution of auxins did not explain the subsequent root growth inhibition, suggesting the involvement of additional fungal signals in root growth inhibition.

T. atroviride Has a Strong Capacity to Acidify the Growth Medium

Even though several studies have shown that pH is an important factor in fungal growth and development, little is known about the impact of fungal-mediated pH changes in root growth. Thus, we first determined the capacity of *T. atroviride* to modify the pH of the culture medium. For this purpose, we inoculated 1×10^6 spores in MS 0.2X medium supplemented with bromophenol blue, which is used as a pH indicator. We found that *T. atroviride* strongly acidifies the medium, which occurs at least in part through proton extrusion in a process that involves vanadate sensitive ATPases, since acidification was strongly reduced by adding increasing amounts of Sodium Orthovanadate (Vanadate, Na_3VO_4), a competitive inhibitor of plasma membrane ATPases (**Figure 3**). Therefore, media acidification by *Trichoderma* may explain the root bending response and thus, the primary root growth inhibition of *Arabidopsis* plants co-cultivated with *T. atroviride* described above.

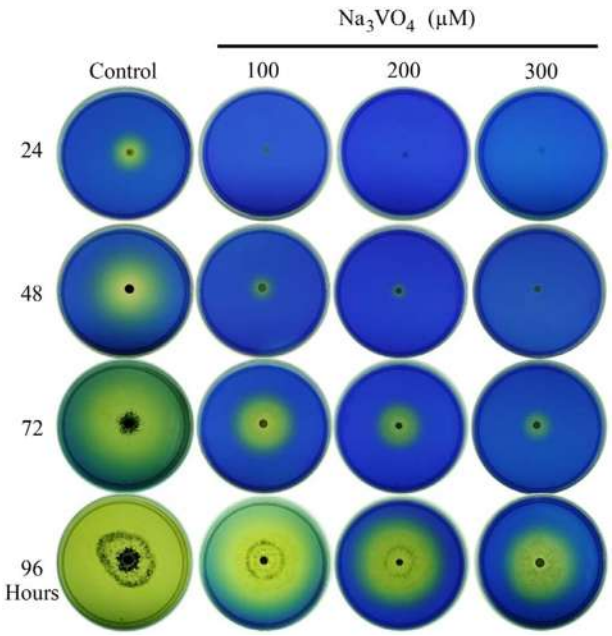

FIGURE 3 | *Trichoderma* induces acidification of the medium. *T. atroviride* was grown for the indicated times on MS 0.2X medium supplemented with bromophenol blue pH indicator and with or without vanadate at the indicated concentrations. Photographs show representative images of acidification by *Trichoderma*. The experiment was repeated twice with similar results.

Acidification Induced by *T. atroviride* Strongly Represses the *Arabidopsis* Growth and Development

To study the effect of *Trichoderma* induced-acidification on plant growth and development, *Arabidopsis* seeds or seedlings were sown or transferred, respectively, onto 0.2X MS growth medium where *Trichoderma* had grown. Interestingly, germination of *Arabidopsis* seeds sown on plates where *Trichoderma* had grown for 96 h was completely inhibited, in contrast with germination on control plates where all seeds germinated and seedlings developed normally (**Figure 4A**). Similar results were observed in *Arabidopsis* seedlings in transfer assays in which 4 days old *Arabidopsis* seedlings grown on MS 0.2X were transferred to control media or media where *T. atroviride* had been grown, and allowed to grow for 6 additional days. In this case, we observed that *Arabidopsis* primary root growth and overall plant development were inhibited in *Trichoderma*-treated media, as compared with the continuing growth observed for control plants (**Figures 4B,C**). Moreover, when the experiment was repeated allowing *Trichoderma* to grow on a cellophane sheet for 18, 24, 30, or 36 h, we observed a gradual plant response. Evident inhibition was observed when plants were transferred onto media where *Trichoderma* had grown for 30 h, and growth was completely inhibited six hours later (Supplementary Figure S3A). Similarly, *Arabidopsis* seed germination occurred when sown on media were *Trichoderma* had been grown for 30 h but growth stopped almost immediately, and no germination was observed by 36 h (Supplementary Figure S3B).

To determine if plant growth repression was indeed due to changes in the media conditions provoked by *Trichoderma*, we performed another experiment, in which *T. atroviride* was grown on un-buffered MS (0.2X) medium with initial pH 7 on a side of the plates for 48 h, time in which the growth medium has not been completely acidified by *Trichoderma*, and then *Arabidopsis* seeds were germinated and allowed to grow on the opposite side of the Petri dish. Interestingly, in seedlings that were grown under this condition, root growth orientation was affected, avoiding the area influenced by *Trichoderma*, compared with the normal vertical root growth of untreated seedlings (Supplementary Figure S3C). These results indicate that plants may sense the gradual changes in pH and adjust their root growth to escape from strongly acidic conditions.

The Growth Repressing Effects of *Trichoderma* on *Arabidopsis* are Associated with Acidification

All our findings on the root response of *Arabidopsis* to *Trichoderma*, were tightly correlated with the reported effects of low pH on plants (Koyama et al., 2001; Kang et al., 2013; Kobayashi et al., 2013). Thus, we evaluated the growth of plants in interaction with *Trichoderma* in pH-buffered media. Under these conditions, primary root growth of *Arabidopsis* seedlings co-cultivated with *Trichoderma* was not inhibited at all, as compared to control plants without *Trichoderma* (**Figure 5A**). Moreover, lateral root emergence was strongly stimulated, with 7–8-fold more lateral roots than in plants without *Trichoderma*, which were also much longer than those in the control (**Figures 5B,C**). Similar results were obtained in experiments in which *Arabidopsis* seeds were directly germinated (Supplementary Figure S4) or seedlings transferred (Supplementary Figure S5) to buffered medium (pH 7) where *Trichoderma* had been grown. Thus, buffering the medium eliminated the negative effects caused by *Trichoderma* on un-buffered media. In addition, we tested the toxic effects of low pH provoked by *T. atroviride* on *Arabidopsis* primary roots, to determine if it could be responsible for the observed growth inhibition. For this purpose, we analyzed cell viability by monitoring the expression of the *H2B::YFP* reporter construct (**Figure 6A**), which is specifically expressed in the nuclei of living cells (Boisnard-Lorig et al., 2001), and by using a vital staining with propidium iodide, by confocal microscopy. Further, as root growth is maintained by the SCN, which includes cells of the QC in the root apical meristem (van den Berg et al., 1997; Bennett and Scheres, 2010), we also followed the status of the QC by monitoring the expression of the reporter construct *WOX5:GFP* (**Figure 6B**). Finally, we analyzed the expression of the cell division marker *CyCB1:uidA* (**Figure 6C**), which is expressed only in cells in the G2/M transition of the cell cycle in the primary root meristem (Colón-Carmona et al., 1999). In all cases, the expression of each reporter construct was lost in the primary root meristem of plants grown on un-buffered medium in the presence of *Trichoderma,* correlating

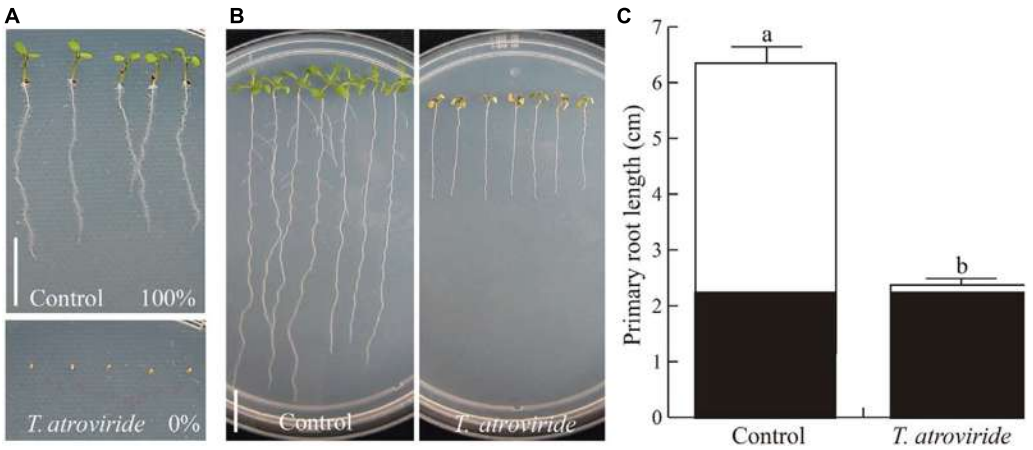

FIGURE 4 | Effects of acidification induced by *T. atroviride* on seed germination and growth of *Arabidopsis* plants. (A) Effect on germination. **(B)** Effect on growth of *Arabidopsis* seedlings. **(C)** Primary root length. In **(A)** *Arabidopsis* (Col-0) seeds were sown on medium MS 0.2X pH 7.0 (Control) or medium where *Trichoderma* had grown for 4 days (*T. atroviride*). In **(B)** *Arabidopsis* seedlings were first grown on control medium for 4 days and then transferred to the same growth conditions used in germination assays. Black bars in graph represent the root length at the time the plant was transferred and white bars the root length 6 days after transfer. Different letters indicate means that differ significantly ($P < 0.05$). Error bars represents SE. Scale bars = 1 cm. The experiment was repeated three times with similar results.

with primary root growth inhibition (**Figures 6A–C**) and a clear pH drop (**Figure 6D**). Together, these data indicate that media acidification by *T. atroviride* strongly affects cell viability and cell division in primary roots, consequently impairing meristem functionality.

Stop1 Response to *T. atroviride* Supports the Role of pH in the Plant–*Trichoderma* Communication

The *Arabidopsis* mutant *stop 1* (*sensitive to proton rhizotoxicity 1*) is well-known to be hypersensitive to low pH (Iuchi et al., 2007; Sawaki et al., 2009). Therefore, we evaluated whether *stop1* was also oversensitive to *T. atroviride* or not. Interestingly, in the presence of *Trichoderma*, the primary root of the *stop1* mutant stopped growing much earlier than WT *Arabidopsis* seedlings (**Figures 7A,B**), but root bending was not observed (**Figure 7B**). It is likely that the root tip of the *stop1* mutant could not bend or form a hook, because of its greater sensitivity to low pH. Indeed, the primary root tip of the mutant showed clear signs of deterioration. This sensitivity was more clearly confirmed in two independent experiments in which *T. atroviride* had been grown for 27 h on the plant growth media supplied or not with MES buffer (**Figure 8**). As shown in **Figure 8**, primary root growth of the *stop1* mutant was significantly reduced both at 10-dag (**Figure 8A**) and 3-days after transfer (dat) (**Figure 8B**). In both cases exposure to *T. atroviride* resulted in primary root growth similar to that observed at pH 4.7. In addition, we determined the effects of these treatments on cell division and elongation by measuring the primary root meristem size and length of fully developed cortical cells at the differentiation zone of 5 days old WT and *stop1 Arabidopsis* seedlings 48 h after transfer. Strong primary root growth inhibition of WT and *stop1* seedlings under low pH (4.7) and *T. atroviride* treatment correlated with smaller cortical cells and a smaller primary root meristem (Supplementary Figure S7). None of the negative effects caused by *Trichoderma* on the *stop1* mutant on un-buffered media was observed when media was buffered with MES (**Figure 8** and Supplementary Figure S6). The sensitivity of the *stop1* mutant to *Trichoderma* indicates that the transcription factor STOP1 is involved in mediating the *Arabidopsis* root responses to media acidification by *T. atroviride*.

DISCUSSION

The beneficial effects of *Trichoderma* on plants such as stimulation of growth, nutrient uptake, induction of defense responses, and indirectly due to its mycoparasitic activity have been widely documented (Altomare et al., 1999; Benítez et al., 2004; Harman et al., 2004; Shoresh et al., 2010; Contreras-Cornejo et al., 2013; López-Bucio et al., 2015). In this study, we show that *Arabidopsis* growth promotion is clearly observed during the early stages of the interaction and that acidification by *Trichoderma* plays an essential role in the *Trichoderma*-plant interaction.

Growth promotion effects of *Trichoderma*, reflected in root branching patterns and its correlation with plant biomass production have been studied to some extent but are not yet well understood. The participation of auxins in modulating these effects is supported by the induction of the expression of the auxin-inducible marker gene *DR5:uidA* in roots and shoots of WT plants and by the reduced response of *Arabidopsis* mutants affected in auxin transport or signaling to *Trichoderma virens* (Contreras-Cornejo et al., 2009). Although *Trichoderma* produces auxins and other diffusible compounds, which may modulate plant growth and development, the whole phytostimulation program may not solely or completely be

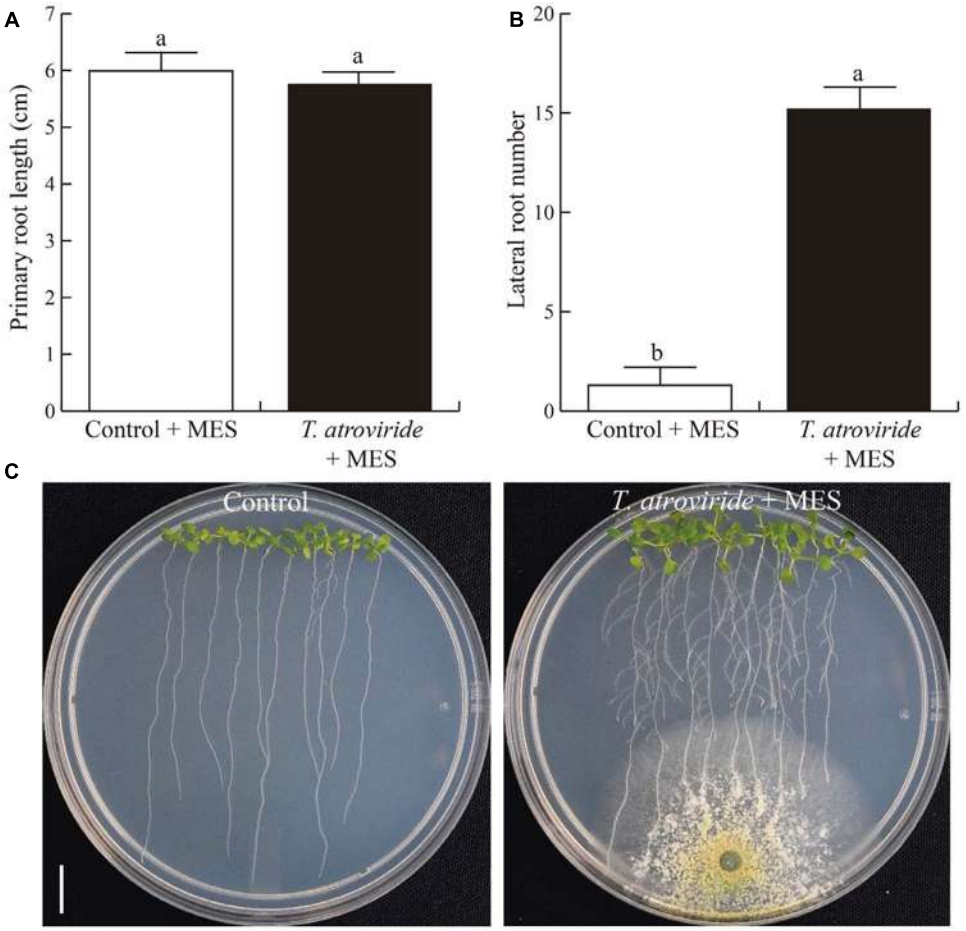

FIGURE 5 | Effects of *T. atroviride* inoculation on *Arabidopsis* growth under buffered medium. (A) Primary root length. **(B)** Lateral root number. **(C)** Representative photographs of *Arabidopsis* seedlings co-cultivated with *Trichoderma*. *Arabidopsis* seedlings were germinated and grown on MS 0.2X medium buffered with MES 0.12%, after 5 days *T. atroviride* was inoculated at the opposite side of the plate and grown for 4 additional days. Different letters are used to indicate means that differ significantly ($P < 0.05$). Error bars represents SE. Scale bar = 1 cm. The experiment was repeated three times with similar results.

explained by an auxinic mechanism, since IAA production is strain dependent and can be affected by diverse external stimuli (Nieto-Jacobo et al., 2017). Furthermore, some *Trichoderma* strains inhibit the auxinic root response of *Arabidopsis* primary roots (Nieto-Jacobo et al., 2017). In this regard, recent findings suggest that growth promotion induced by *Trichoderma* during the early plant–*Trichoderma* interaction stages could be attributed mostly to volatile organic compounds (VOCs) more than to the release of auxins or other diffusible compounds by the fungus. Exposure of plants to VOCs of different *Trichoderma* species have been found to stimulate plant growth, chlorophyll content, and plant size and biomass, which could be correlated with an enhanced soil exploratory capacity, better rooting and an enhanced capacity to take up nutrients and water (Hung et al., 2013; Contreras-Cornejo et al., 2014b; Lee et al., 2016; Nieto-Jacobo et al., 2017). It is noteworthy, that we found that longer interaction times with *Trichoderma* had clear detrimental effects on primary roots compared with untreated plants, such as root tip bending and growth inhibition, which correlate with anthocyanin-like pigmentation of leaves and the eventual development of leaf chlorosis. Anthocyanin production in leaves has been found to occur as consequence or parallel effect of plant defense induction in response to *Trichoderma* (Contreras-Cornejo et al., 2011). Based on our findings, we suggest that anthocyanin accumulation could be related with a plant response to acidification by *Trichoderma* rather than to the induction of the plant defense response.

Rhizosphere acidification by *Trichoderma* spp. particularly *Trichoderma harzianum* strain T-22, has been reported in a couple of studies, one of them performed by Altomare et al. (1999), which investigated the fungal capacity to solubilize, *in vitro,* insoluble or sparingly soluble minerals by acidification of the medium. In their report the authors indicated that *Trichoderma* acidified the medium, but concluded that acidification was not the major mechanism of solubilization of insoluble minerals. Similarly, Sofo et al. (2012) found that *T. harzianum* T22 acidified the growth medium and that this may account for its beneficial effects on plants under hostile growth

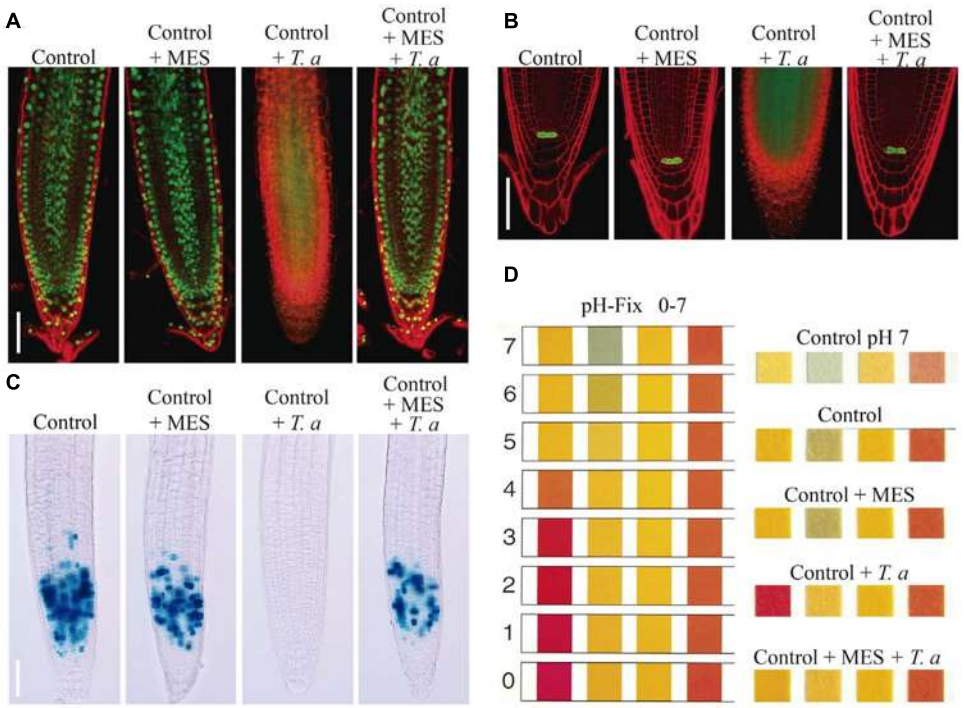

FIGURE 6 | Acidification induced by *T. atroviride* impairs the *Arabidopsis* root meristem functionality. (A) Expression of vital marker *H2B::YFP*. **(B)** Expression of the root quiescent center (QC) marker *WOX5:GFP*. **(C)** Expression of the cell division marker *CycB1:uidA*. **(D)** Estimated pH on the different growth conditions. Five-day-old seedlings of different transgenic lines used were transferred to the indicated treatments and analyzed 24 h later. Photographs show representative images of at least 10 seedlings analyzed per experiment. Scale bars = 50 μm. These experiments were repeated twice with similar results.

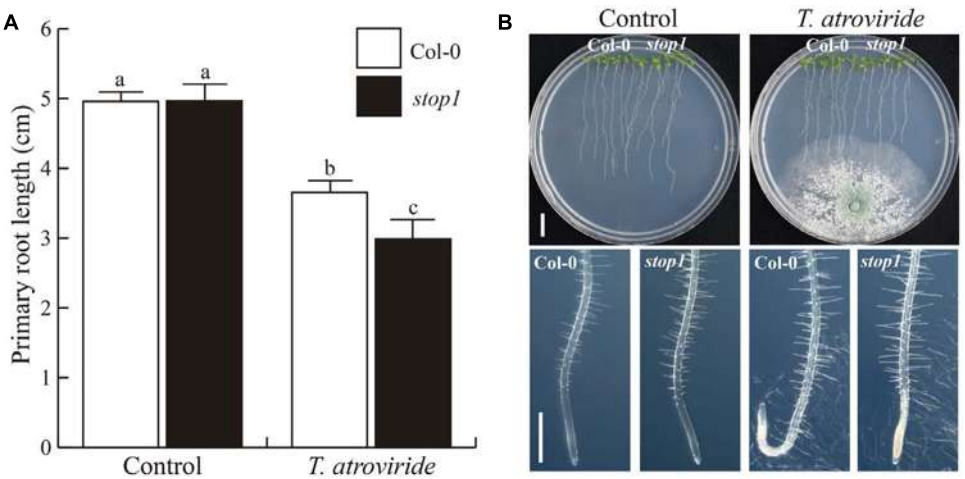

FIGURE 7 | Effect of *T. atroviride* on growth of *Arabidopsis* WT and *stop1* mutants. (A) Six-day-old *A. thaliana* WT seedlings and *stop1* mutant were co-cultivated with 1×10^6 spores of *T. atroviride*, by placing the fungus on the opposite side of the Petri plate, where seeds were sown. **(A)** Effect on primary root length. **(B)** Photographs of WT and *stop1* seedlings. Different letters indicate means statistically different at $P < 0.05$. Error bars represents SE. Scale bars = 1 cm and 500 μm. This experiment was repeated three times with similar results.

conditions. More recently, medium acidification by *T. atroviride* and *T. virens* was also observed in co-cultivation experiments with *Arabidopsis*, a phenomenon that was correlated with the plant promoting effects exerted by *Trichoderma* on *Arabidopsis* seedlings (Contreras-Cornejo et al., 2016).

It is known that plants can naturally acidify the rhizosphere to improve nutrient availability or uptake, and this may be particularly relevant in alkaline calcareous soils in which phosphate and iron are very limiting, but only a slight acidification can be beneficial for plant growth and development

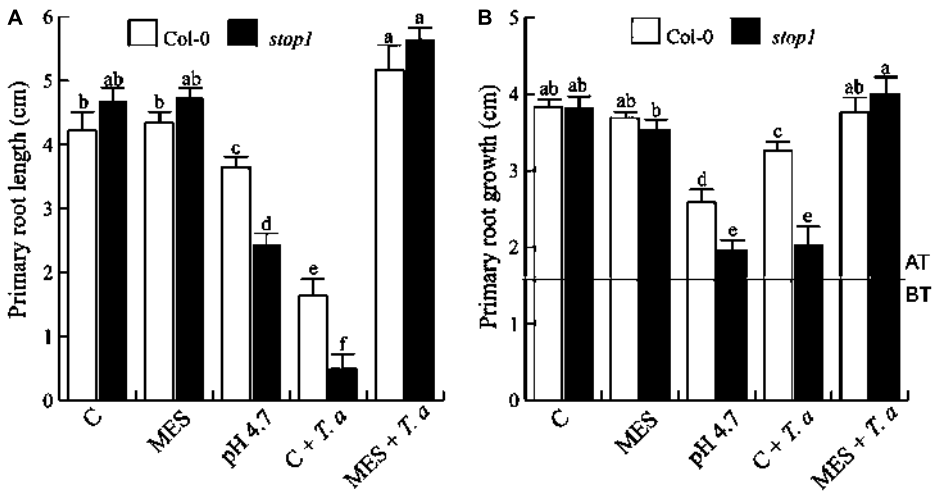

FIGURE 8 | Sensitivity of *stop1* to acidification induced by *T. atroviride*. **(A,B)** Primary root length. **(A)** Seeds were germinated and grown directly under the indicated conditions or **(B)** on a pH 7.0 medium for 4 days and then transferred to the indicated treatments and analyzed 3 days after transfer. BT and AT in graph **(B)** indicate the root growth before and after transfer, respectively. Different letters in graphic indicate means statistically different at $P < 0.05$. This experiment was repeated twice with similar results.

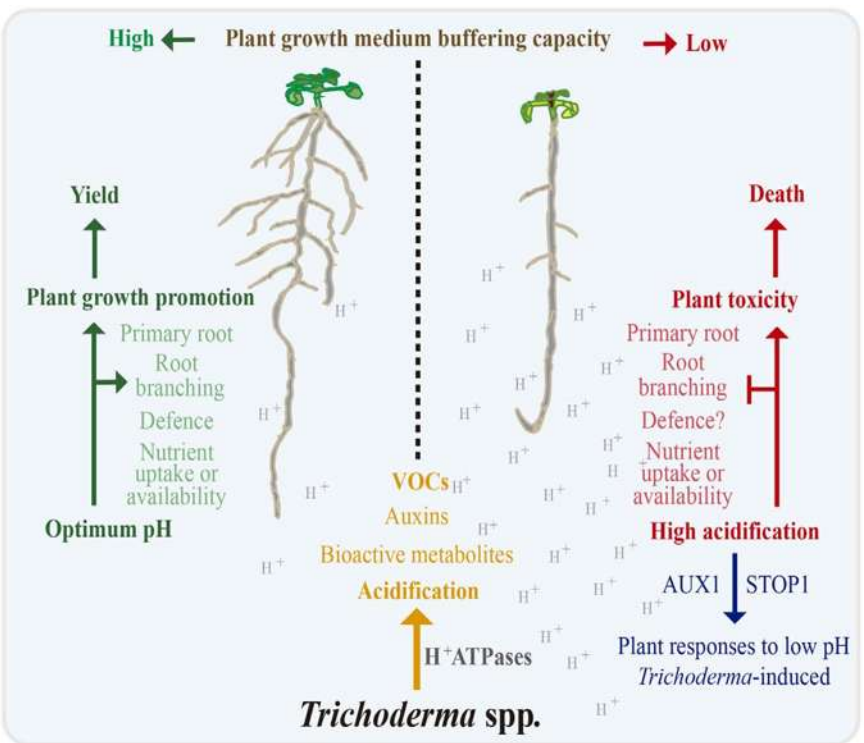

FIGURE 9 | Model for the interaction of *Trichoderma* with plants. Rhizosphere acidification by *Trichoderma* together with auxins, VOCs, and other bioactive molecules reconfigure root architecture and may promote or impair plant growth depending upon soil pH conditions. STOP1 and AUX1 likely act in a root pH sensing pathway to adapt the root system to the acidification likely via lateral root production.

in soils with neutral or slightly acid pHs (Marschner, 1991; Hinsinger et al., 2003), while strong acidification negatively affects plant growth. Here, we found that *T. atroviride* strongly acidified the medium, through a process mediated at least in part by H^+-ATPases, because the addition of sodium orthovanadate (Na_3VO_4), an ATPase inhibitor reduced acidification in a dose-dependent manner. Our data indicate that media acidification by *Trichoderma* has a clear detrimental effect on *Arabidopsis*,

affecting plant development, from seed germination to root and shoot growth. These findings are consistent with the effects of low pH reported in various studies, where acidity was found to affect *Arabidopsis* root growth (Koyama et al., 1995, 2001; Kang et al., 2013), and inhibit seed germination of different plants (Fan and Li, 1999; Zeng et al., 2005). Thus, the inhibitory effects observed on *Arabidopsis* seedlings in co-cultivation with *T. atroviride* may be explained by a high level of acidification. Furthermore, when the *Arabidopsis-Trichoderma* experiments were carried out under buffered conditions, a clear increase in growth promotion by *Trichoderma* was observed, and the negative effects (i.e., germination and growth inhibition) were negligible, restoring completely plant growth. These results correlated with the expression of different markers used to test root meristem functionality as well as with pH levels observed under the different growth conditions. Similar detrimental effects on *Arabidopsis* root growth were recently reported for certain *Trichoderma* strains (Nieto-Jacobo et al., 2017). Although, the authors associated this response with an impaired auxin signaling, in our view, root growth repression may rather be a consequence of loss of root meristem functionality caused by acidification.

Low pH stress is related with Al toxicity, because at low pH the highly toxic Al^{3+} severely affects plant growth. However, increasing evidence supports that toxic H^+ and Al^{3+} elicit different adaptive mechanisms in plants (Shavrukov and Hirai, 2016). In bacteria and fungi, different pH-signaling pathways have been identified (Arst and Peñalva, 2003; Yuan et al., 2008), but in plants no specific mechanisms for pH sensing have been reported. The transcription factor *STOP1* is one of the very few regulators of gene expression in plant responses to Al^{3+} and low pH stress (Iuchi et al., 2007; Sawaki et al., 2009). Accordingly, we found that the *Arabidopsis* mutant *stop1* showed higher root growth repression when co-cultivated with *Trichoderma* than WT seedlings, evidenced by a shorter primary root and absence of root bending or hook in the root tip. Such oversensitivity was confirmed in experiments where the WT and the *stop1* mutants were grown in medium supplemented with MES buffer, in which *Trichoderma* had been grown, supporting a role of pH as a signal in the interaction of *Trichoderma* with plants. Taken together, these results suggest that the pH status could be sensed by roots to activate a signaling cascade that modulates root growth and its orientation, and possibly, to activate lateral root initiation.

A recent report demonstrated that the inhibitory effects of low pH on root growth are mediated by an adaptive plant response rather than by a direct toxic effect of H^+, because direct root exposure to low pH, suppressed root growth and caused high cell death, while roots exposed gradually to the low pH stress stopped growth but maintained cell viability (Graças et al., 2016). Although the molecular components mediating the root responses to pH or their possible link with auxin signaling are currently unknown, the idea of pH as a signal in plant–microbe interactions is supported by global gene expression analyses, which showed that low pH alters the expression of genes related to auxin signaling, pathogen elicitors, and defense-associated hormones. It is possible that pH-sensing and Ca^{2+} signaling may be mediated by proton effects on inward rectifying K^+−channels or via a pH specific sensor (Lager et al., 2010). The fact that the *Arabidopsis aux1-7* mutant is hypersensitive to low pH, suggests an important role of auxin transport in root growth response to acidification (Inoue et al., 2016), and since the same mutant had a reduced response to *T. virens* inoculation in terms of shoot biomass production and lateral root development (Contreras-Cornejo et al., 2009), we propose that normal auxin transport is important for plant growth and root adaptation to low pH following *Trichoderma* inoculation. In addition, the nitrate transporters *NRT1.1* and *OsNRT2.3b* are involved in H^+ resistance in *Arabidopsis* and rice, respectively, a phenomenon that depends on their nitrate uptake activity (Fang et al., 2016; Fan et al., 2016b). The relationship of *Trichoderma* with nitrate nutrition of plants represents an interesting research avenue to follow.

CONCLUSION

This report provides compelling evidence that root sensing of pH mediates the interaction of *Trichoderma* with plants. Rhizosphere acidification by *Trichoderma* may influence the root developmental response to auxins, VOCs, and other bioactive molecules and stimulate or repress different plant processes in a plant specific manner and depending upon the soil characteristics (**Figure 9**). We have further identified STOP1 as a critical factor in mediating root adaptation to fungal-induced acidification, which might act in a pathway involving auxin signaling and transport, where AUX1 links acidity to growth responses and plant adaptation to H^+ stress. These data may help in the management of *Trichoderma* based strategies for crop improvement, and may aid in the identification of more efficient *Trichoderma*-strains for their use in bio-fertilizers or bio-inoculant formulation to increase plant growth and yield.

AUTHOR CONTRIBUTIONS

RP-F designed and performed experiments, collected, and interpreted data; AG-V performed experiments; SE-R performed experiments and provided technical support; JL-B and AH-E: designed experiments and contributed to data interpretation. RP-F, JL-B, and AH-E wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00822/full#supplementary-material

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Jasmonic Acid-Ethylene Crosstalk via *ETHYLENE INSENSITIVE 2* Reprograms *Arabidopsis* Root System Architecture Through Nitric Oxide Accumulation

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Abstract Plant growth and development are tightly regulated by phytohormones, including jasmonic acid (JA) and ethylene (ET), two canonical players in plant defense and in the control of root system architecture. Here, we show that JA inhibits primary root growth and promotes lateral root development while inducing nitric oxide (NO) accumulation in the wild-type (WT) primary root, but not in *jar1-1, coi1-1, myc2-1*, and *myc2-2 Arabidopsis* mutants defective in JA biosynthesis or response. NO-related mutants *nia1*/*nia2* and *Atnoa1* were indistinguishable in root architectural responses to JA when compared to WT seedlings, and the developmental changes were apparently unrelated to reactive oxygen species (ROS) accumulation. Root growth inhibition by the NO donor, sodium nitroprusside (SNP), was reduced in *coi1-1* mutants, and NO accumulation induced the expression of the downstream repressors *JAZ1* and *JAZ10* at the differentiation and/or meristematic root regions. Comparison of growth of WT, *ein2-1, jar1-1*, and *ein2-1*/*jar1-1* mutants further revealed a critical role of *ETHYLENE INSENSITIVE2* (*EIN2*) in mediating both JA and NO root sensing. Our results suggest that NO mediates JA signaling during the configuration of the *Arabidopsis* root system architecture and that *EIN2* plays a role in this developmental program.

Keywords *Arabidopsis* · Jasmonic acid · Nitric oxide · Root development · Phytohormones

Introduction

The fatty acid-derived plant hormone jasmonic acid regulates physiological and phenotypic plasticity as well as environmental adaptation. Besides being an inducer of plant defense, JA plays a key role in the configuration of the root system by inhibiting primary root growth and promoting lateral root formation (Staswick and others 1992; Chen and others 2011; Raya-González and others 2012).

Several molecular components are involved in JA-induced root architectural reprogramming. JA reduces root growth affecting both cell elongation and meristem activity, and represses the AP2-domain transcription factors PLETHORA1 (PLT1) and PLT2. This process requires the functioning of MYC2/JASMONATE INSENSITIVE1, a basic helix loop-helix transcription factor (Chen and others 2011; Gasperini and others 2015). In addition, the *Arabidopsis* F-box protein CORONATINE INSENSITIVE1 (COI1), which forms a functional E3 ubiquitin ligase SCF^{COI1} and acts as a JA receptor, plays a role in JA-induced lateral root formation (Xu and others 2002; Raya-González and others 2012).

The JASMONATE ZIM-DOMAIN (JAZ) proteins interact with TOPLESS (TPL) and NOVEL INTERACTOR OF JAZ (NINJA) to form a repressor complex that inhibits MYC2 activity in the absence of JA (Chini and others 2007; Thines and others 2007; Pauwels and others 2010). However, when the levels of JA increase and upon binding to it, COI1 targets the JAZ repressors for proteolytic degradation, allowing JA-responsive transcriptional activation (Thines and others 2007; Pauwels and others 2010). Regarding what is known on shoots, little is known

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about the cellular organization and regulation of the JAZ and other JA signaling components in roots.

Jasmonic acid and ethylene regulate a common set of plant responses to biotic stimuli and are frequently found to act in parallel. Both phytohormones are required for plant resistance to necrotrophic pathogens and defense-related gene expression (Xu and others 1994; Penninckx and others 1998; Lorenzo and others 2003), while acting as negative players in mutualistic plant–fungus symbiosis (Plett and others 2014). In roots, ethylene may also inhibit cell proliferating activity of meristems, because *CULLIN3* genes regulate cell division through the canonical ethylene signal transduction pathway that incorporates CONSTITUTIVE TRIPLE RESPONSE1, ETHYLENE INSENSITIVE2, and ETHYLENE INSENSITIVE3 as well as a phosphorelay pathway based on receptor histidine kinases and the type B cytokinin response regulators (Thomann and others 2009; Street and others 2015).

Some secondary metabolites, such as the plant alkamides, influence JA homeostasis. The alkamides are fatty acid amides naturally present in many plant families, and are structurally related to *N*-acyl-*L*-homoserine lactones (AHLs) from Gram-negative bacteria and to *N*-acylethanolamines (NAEs) from plants and mammals (Ramírez-Chávez and others 2004; Ortiz-Castro and others 2008; Blancaflor and others 2014; Greger 2016). Global analysis of gene expression in *Arabidopsis* seedlings in response to the strongly active alkamide *N*-isobutyl decanamide revealed an overrepresentation of genes encoding enzymes for JA biosynthesis, which occurred in parallel with JA and NO accumulation, indicating the possible link between these two plant signals (Méndez-Bravo and others 2011).

Nitric oxide (NO) is a free radical present in most plant organs, where it controls a wide range of environmental and physiological functions acting as a cellular messenger (Wendehenne and Hancock 2011). In *Arabidopsis* roots, NO reduces cell division and elongation (Fernández-Marcos and others 2012), and activates lateral root formation (Campos-Cuevas and others 2008; Méndez-Bravo and others 2010; Schlicht and others 2013). The precise role of NO and downstream targets in regulating the configuration of the root system and its relationship with jasmonic acid and ethylene signaling still awaits clarification. Therefore, a major current goal is to uncover new genetic elements integrating the hormonal plant response to NO sensing.

Here, we report that JA induces NO production in *Arabidopsis* primary roots in a *JAR1-, COI1-*, and *MYC2*-dependent manner, and activates *JAZ1* and *JAZ10* as downstream targets during root architecture configuration. Moreover, we identify *ETHYLENE INSENSITIVE2* as an important player in JA-induced primary root growth and NO accumulation. Our results suggest that NO mediates JA-ET crosstalk via EIN2 and that this interaction is important for root morphogenesis.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0), and mutant lines *coi1-1* (Feys and others 1994), *jar1-1* (Staswick and others 1992), *myc2-1* (SALK_061267), *myc2*-2 (SALK_017005), *ein2-1* (Guzmán and Ecker 1990), *rcd1* (SALK_116432), *nia1*/*nia2* (Wilkinson and Crawford 1993), and *Atnoa1* (Guo and others 2003) were used for the different experiments. Generation of *Arabidopsis ein2 jar1* double mutants was done by outcrossing *ein2-1* single mutants with *jar1-1* pollen to obtain the F1 progeny, and the corresponding plants were allowed to self-fertilize to recover the F2 generation. Homozygous *ein2 jar1* seedlings were identified upon the primary root resistance to both jasmonic acid and ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in primary root growth assays, and were propagated for at least three generations. *Arabidopsis* transgenic seedlings included *JAZ1*/*TIFY10A-GFP* (Grunewald and others 2009) and *JAZ10-GFP* (Pilliteri and others 2011).

Seeds were surface sterilized with 95% (v/v) ethanol for 4 min and 10% (v/v) bleach for 4 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, Catalogue No. M5524) was purchased from Sigma. 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-95 L) with a photoperiod of 16 h of light/8 h darkness, light intensity of 300 µmol, and temperature of 22 °C.

Selection of *Arabidopsis coi1-1* Homozygous Seedlings

For transfer experiments of WT and *coi1-1* seedlings, seeds were first sterilized and germinated on 0.2× MS medium as described above. For *coi1-1* mutant selection, 300 seeds from a *coi1-1*/*COI1* segregating population were screened for sustained primary root growth in agar-solidified MS 0.2× medium supplemented with 4 µM 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 roots were selected and transferred to plates with the different treatments.

Analysis of Growth

The *Arabidopsis* root system was analyzed with a stereoscopic microscope (Leica, MZ6). All lateral roots emerged from the parent root were observed and registered with the 3× objective. Primary root length was determined for each root using a ruler. Lateral root density was determined by dividing the lateral root number value by the primary root length for each seedling. For all experiments, the data were statistically analyzed using STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). 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 ($p < 0.05$).

Confocal Microscopy

NO was monitored by incubating *Arabidopsis* seedlings with 10 µM of the fluorescent probe DAF-2DA in 1 M Tris–HCl (pH 7.4). Living cells incorporate DAF-2DA, which subsequently is hydrolyzed by cytosolic esterases to release 4,5-diaminofluorescein (DAF-2), which reacts with NO to produce the fluorescent triazole derivate triazolofluorescein (DAF-2T). General ROS were visualized 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. JAZ1/TIFY10A-GFP and JAZ10-GFP seedlings were incubated with each fluorophore for 1 h in darkness, and washed three times for 20 min with fresh buffer. Fluorescence signals were detected using a confocal laser scanning microscope (Olympus FV1200), and monitored with an argon blue laser with an excitation line from 488 to 568 nm and an emission window from 585 to 610 nm. Micrographs acquired with the confocal microscope were analyzed in ImageJ software (http://rsbweb.nih.gov/ij/). For each treatment and line, fluorescence intensity was registered from six micrographs. Fluorescence intensity was quantified by determining green pixels in a defined area. An arbitrary unit value was obtained (AU = pixels µm^2) for each micrograph, and means were obtained from whole datasets.

Results

Jasmonic Acid Induces Nitric Oxide Accumulation in *Arabidopsis* Roots

As a first step to investigate the possible involvement of NO underlying JA regulation of root growth, we analyzed in situ levels and localization of NO using the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) in seedlings grown in medium supplemented with increasing concentrations of JA. As expected, JA modified *Arabidopsis* root system architecture, by inhibiting primary root growth and increasing lateral root density (number of lateral roots (LR)/cm) in a dose-dependent manner (Fig. 1a, b). The greatest JA concentration tested (8 µM) repressed root growth by 80%, whereas LR density was stimulated up to seven times respect to control conditions.

When primary roots of WT seedlings grown in medium lacking JA were loaded with DAF-2DA and analyzed by confocal microscope, NO was detected in several tissues and structures, including epidermal cells, root hairs, root elongation zone, root cap, and the quiescent center (QC) (Fig. 1c). Interestingly, the inhibition of root growth and enhanced LR formation in response to JA tightly correlated with the increased NO fluorescence in all these regions, and particularly at the primary root meristem (Fig. 1c). These data show that NO accumulates in *Arabidopsis* roots following JA application.

JA Biosynthesis and Signaling Components Mediate JA-Induced Root-Growth Inhibition and Nitric Oxide Accumulation in Primary Roots

Several molecular components involved in JA biosynthesis and response play a role in root architecture reprogramming (Chen and others 2011; Raya-González and others 2012). To evaluate at the genetic level the possible interaction between JA and NO on root system architecture, we tested root growth responses and changes in NO levels of *Arabidopsis* WT seedlings grown side by side with *jar1-1 or myc2-1* and *myc2-2* in media with or without JA. Supplementation of 1 and 4 µM JA to WT seedlings showed inhibition of primary root growth that was reduced in *jar1-1* or in two *myc2* mutant lines defective on two independent alleles (Figs. 2a, 3a). Besides primary root growth, *jar1-1, myc2-1*, and *myc2-2* mutants showed insensitivity to JA effects in promoting LR formation (Figs. 2b, 3b), indicating that JA requires an intact mechanism via JAR1 and MYC2 to modify root system architecture. Accordingly, JA could induce NO production in WT primary root tissues, but not in *jar1* or *myc2* mutants (Figs. 2c, 3c).

In another set of experiments, WT and homozygous *coi1-1* mutants were compared for primary root growth repression in medium supplemented with 4 µM JA. The strong resistance of the mutants to JA correlated with decreased levels of NO accumulation at the root tip (See Fig. S1 in Supplementary Information). Together, these data suggest that *JAR1, COI1*, and *MYC2* are required for NO production in primary roots in response to JA.

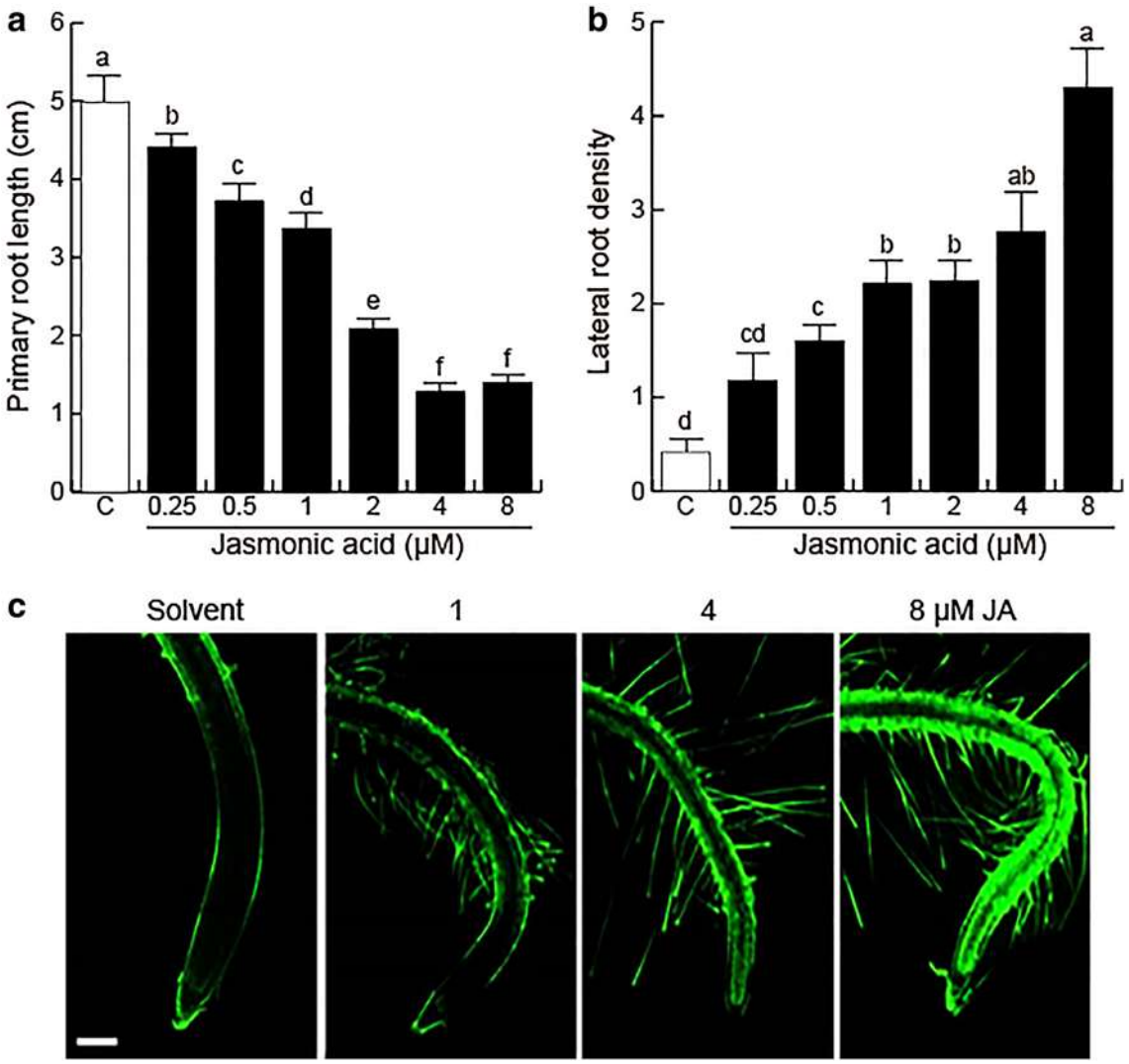

Fig. 1 Effects of JA on root architecture and nitric oxide accumulation in *Arabidopsis*. Primary root length (**a**) and lateral root density (**b**) were analyzed from 10-day-old WT (Col-0) seedlings germinated and grown on the indicated concentrations of JA. **c** NO detection on primary root tips of seedlings loaded with DAF-2DA and visualized by confocal microscope. Values shown in (**a**) and (**b**) represent the mean of 30 seedlings. Different letters are used to indicate means that differ significantly ($p < 0.05$). The experiment was repeated three times with similar results. *Scale bar* 100 μm

Jasmonic Acid-Induced Changes in Root Growth Occur Independently of *NIA1, NIA2*, and *AtNOA1*

NO levels in *Arabidopsis* are controlled by the activity of two nitrate reductases, encoded by the *NIA1* and *NIA2* genes, and the *NITRIC OXIDE ASSOCIATED1* (*AtNOA1*; Rockel and others 2002; Sudhamsu and others 2008). Given that JA modulates root development by inducing NO accumulation, we evaluated JA effects on *nia1*/*nia2*, and *Atnoa1* double and single mutants, respectively. Because *nia1*/*nia2* and *Atnoa1* mutants develop shorter primary roots with fewer lateral roots than WT seedlings in medium lacking JA, the growth repression and lateral root density were calculated as a percentage. In response to JA treatment, WT, *nia1*/*nia2*, and *Atnoa1* seedlings were indistinguishable regarding primary root growth inhibition or lateral root density (See Fig. S2 in Supplementary Information), suggesting that JA-regulated root architecture re-configuration operates independently of the *NIA1, NIA2*, and *AtNOA1*.

Nitric Oxide Modulates JAZ1 and JAZ10 Protein Levels in Roots

The JAZ proteins are induced by JA and act as downstream repressors of its own signaling pathway (Chini and others 2007; Thines and others 2007; Pauwels and others

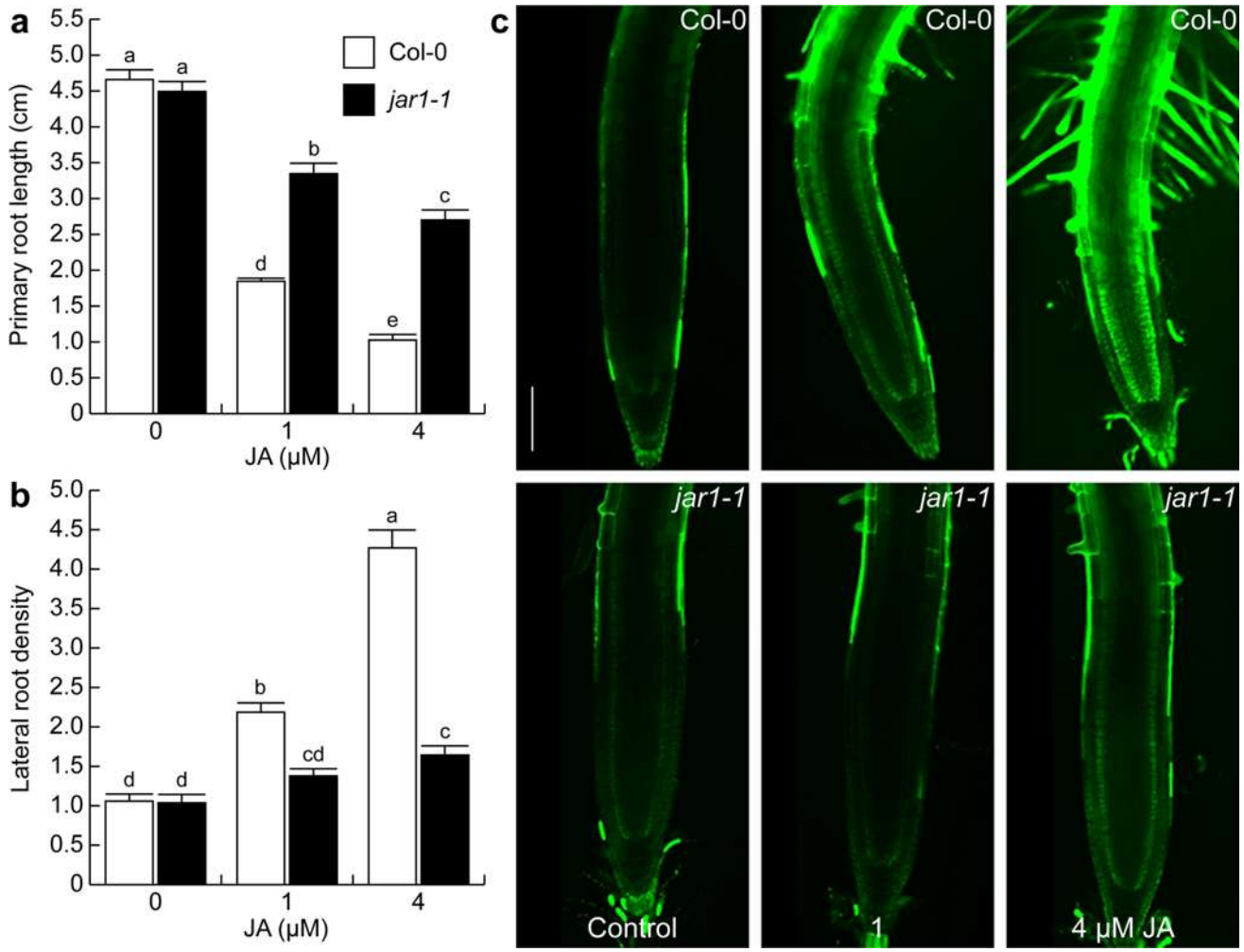

Fig. 2 Root response to jasmonic acid and nitric oxide accumulation in wild-type and *jar1-1 Arabidopsis* seedlings. WT and *jar1-1* seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, or with 1 and 4 μM JA for 10 days. **a** Primary root length and **b** lateral root density were recorded. Values shown represent the mean ± standard deviation ($n = 15$). *Different letters* indicate statistical differences following a Tukey test analysis ($p < 0.05$). **c** Representative micrographs of the detection of endogenous NO with 4,5-diaminofluorescein diacetate, which was determined in primary roots of at least six seedlings of each type and each growth condition mentioned above (*scale bar* 100 μm). The experiment was repeated twice with similar results

2010). To test the role of nitric oxide in regulating JAZ proteins in roots, we compared the expression pattern of *JAZ1/TIFY10A-GFP* (Grunewald and others 2009) and *JAZ10-GFP* (Chung and Howe 2009) in response to increasing concentrations of the NO-donor SNP. In agreement with previous results (Grunewald and others 2009), *JAZ1/TIFY10A-GFP* was localized in the nucleus of the vascular cylinder as discrete speckles or nuclear bodies (See Fig. S3 in Supplementary Information). NO clearly increased the number of cells with nuclear bodies expressing the construct in the vascular cylinder and cortex at the differentiation zone of the root, but not in primary root tips (See Fig. S3 in Supplementary Information). On the other hand, the analysis of *JAZ10-GFP* expression clearly indicated its induction in lateral root primordia and the protoxylem of primary root tips by NO (Fig. 4). Because *JAZ1* is inducible by JA (Grunewald and others 2009), the current data are consistent with a role of NO as mediator in the JA signaling pathway.

The Loss of *COI1* Function Decreases Nitric Oxide Accumulation and Response in Roots

If NO acts as a modulator of JA signaling, then we hypothesized that *Arabidopsis* mutants that are JA-resistant would also be resistant to NO in root growth and lateral root formation. To test this possibility, the primary root growth and root branching responses of WT and *coi1-1* mutants were

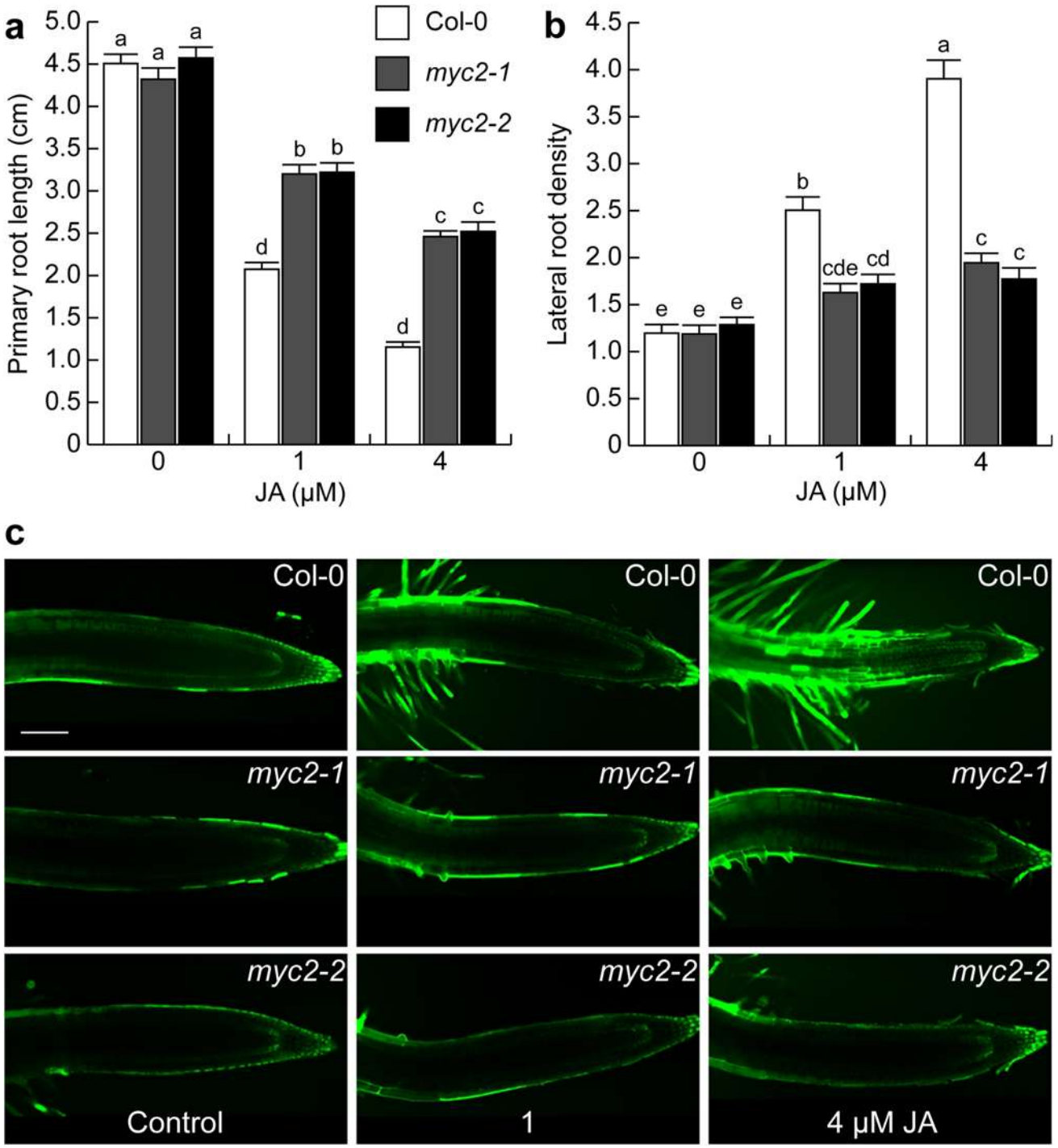

Fig. 3 Root response to jasmonic acid and nitric oxide accumulation in wild-type, *myc2-1*, and *myc2-2 Arabidopsis* seedlings. WT, *myc2-1*, and *myc2-2* seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, or with 1 and 4 μM JA for 10 days. **a** Primary root length and **b** lateral root density were recorded. Values shown represent the mean ± standard deviation ($n = 15$). Different letters indicate statistical differences following a Tukey test analysis ($p < 0.05$). **c** Representative micrographs of the detection of endogenous NO with 4,5-diaminofluorescein diacetate, which was determined in primary roots of at least six seedlings of each type and each growth condition mentioned above (*scale bar* 100 μm). The experiment was repeated twice with similar results

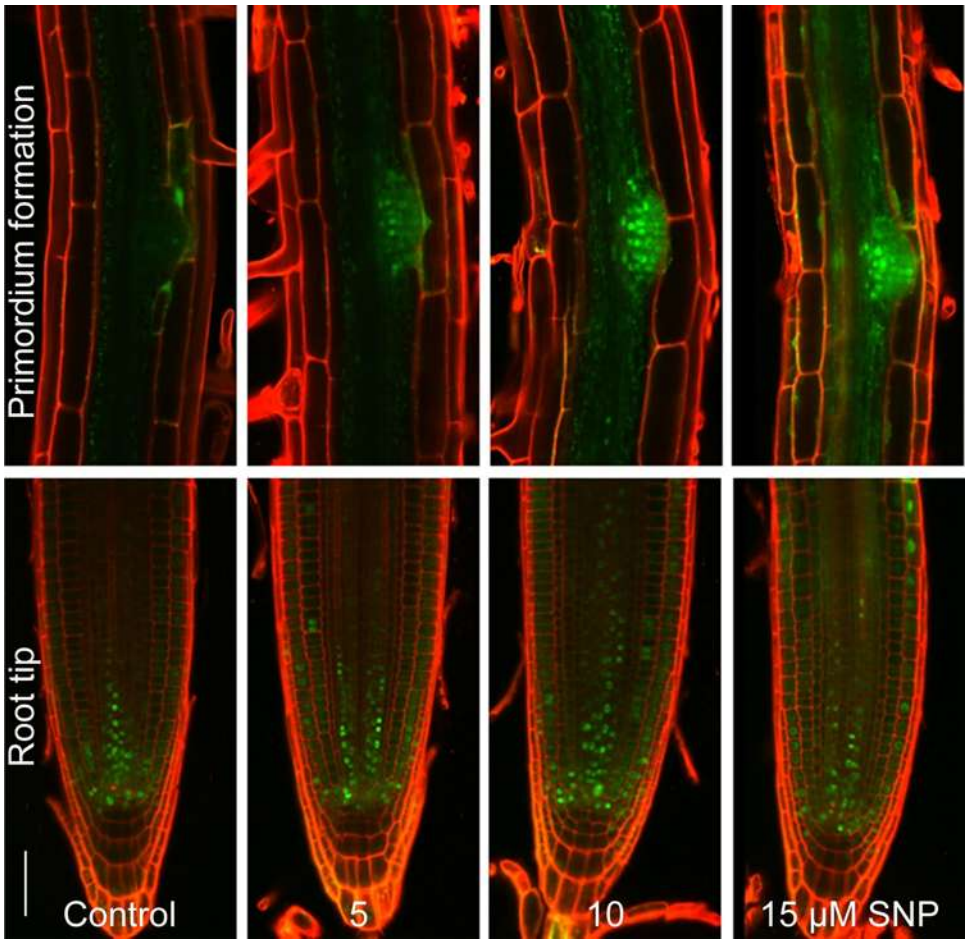

Fig. 4 Effect of nitric oxide donor sodium nitroprusside on *JAZ10* expression in *Arabidopsis* roots. Transgenic *Arabidopsis* seedlings harboring the *JAZ10-GFP* gene construct were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 5, 10, and 15 µM SNP for 10 days; at least six seedlings were incubated with propidium iodide, and confocal micrographs of expression pattern in two zones of primary root were taken (*scale bar* 100 µm). The experiment was repeated twice with similar results

compared in medium with or without SNP. Supplementation of 20 µM SNP repressed root growth while increasing lateral root density in WT plants, which correlated with NO fluorescence in primary root tips, and this response was reduced in *coi1-1* mutants (Fig. 5a–c). Thus, the NO response of *Arabidopsis* roots requires the JA receptor COI1.

ETHYLENE INSENSITIVE2 Plays a Role in Jasmonic Acid-Induced Primary-Root Growth and Nitric Oxide Accumulation and Sensitivity

Jasmonic acid and ethylene regulate a common set of plant responses to biotic stimuli and act in parallel to modulate gene expression (Lorenzo and others 2003; Plett and others 2014). Therefore, it could be possible that genetic elements of the ethylene signal transduction could be responsible for JA-induced repression of root growth. One of the most ethylene insensitive mutants identified to date is defective at *ETHYLENE INSENSITIVE2* (*EIN2*; Guzmán and Ecker 1990).

To determine possible synergic effects in response to JA among JA-related genes and ethylene signaling, comparisons of primary root growth and lateral root formation of WT, *ein2-1, jar1-1*, and *ein2-1/jar1-1* mutants were performed in response to 1 and 4 µM JA, and NO detection was performed in primary root tips. Interestingly, the *ein2-1* mutants were clearly resistant to the growth-repressing effects of JA and also in root branching promotion by this hormone, and this effect further increased in *ein2-1/jar1-1* double mutants (Fig. 6a–c). JA sensitivity in *ein2-1* and *jar1-1* single and double mutant combinations correlated with lower accumulation of NO in response to the JA treatments. These data reveal a novel and critical role of *EIN2* in JA-mediated NO accumulation in the *Arabidopsis* primary roots.

To test whether the strong JA resistance of *ein2-1/jar1-1* double mutants correlate with an altered NO sensitivity, the primary root growth response to SNP was compared in WT, *ein2-1, jar1-1,* and *ein2-1/jar1-1* single and double mutants. The data show a strong resistance to NO accumulation and response in both single and double mutants (Fig. 7a–c), implying a JA-ethylene crosstalk orchestrating NO biosynthesis and response during the configuration of the *Arabidopsis* root system.

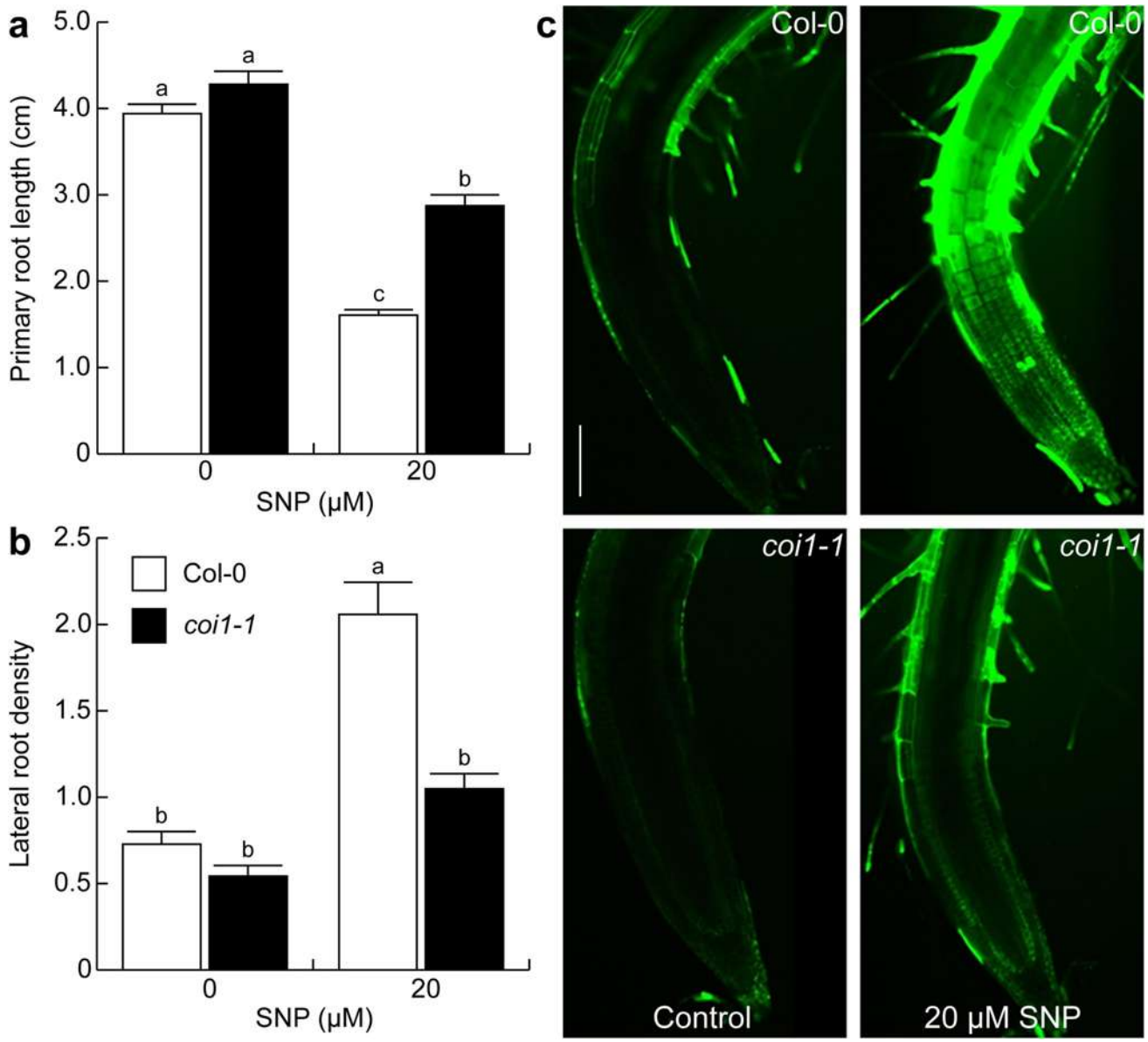

Fig. 5 Effect of nitric oxide donor sodium nitroprusside on root development and nitric oxide accumulation in wild-type and *coi1-1* seedlings. WT seeds were germinated and seedlings were grown on agar-solidified 0.2× MS media for 2 days, and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI1* segregating population in media supplemented with 4 μM JA 2 days after germination, and subsequently wild-type and *coi1-1* seedlings were transferred to agar-solidified 0.2× MS media supplied with the solvent or 20 μM SNP for 8 days. **a** Primary root length and **b** lateral root density. Values shown represent the mean ± standard deviation ($n = 15$). Different letters indicate statistical differences following a Tukey test analysis ($p < 0.05$). **c** Representative confocal micrographs of the detection of endogenous NO with 4,5-diaminofluorescein diacetate in primary roots ($n = 6$) (*scale bar* 100 μm). The experiment was repeated twice with similar results

Reactive Oxygen Species Did Not Act as Modulators During the JA-Induced Root Reprogramming

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 (Mittler and others 2011). To investigate if ROS could be part of the mechanism of primary root inhibition by JA, we monitored ROS accumulation in root tips using 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) and confocal imaging in WT and *coi1-1* plants treated with JA, SNP, and the herbicide methyl viologen (paraquat). Interestingly, *coi1-1 Arabidopsis* mutants showed higher ROS levels under standard

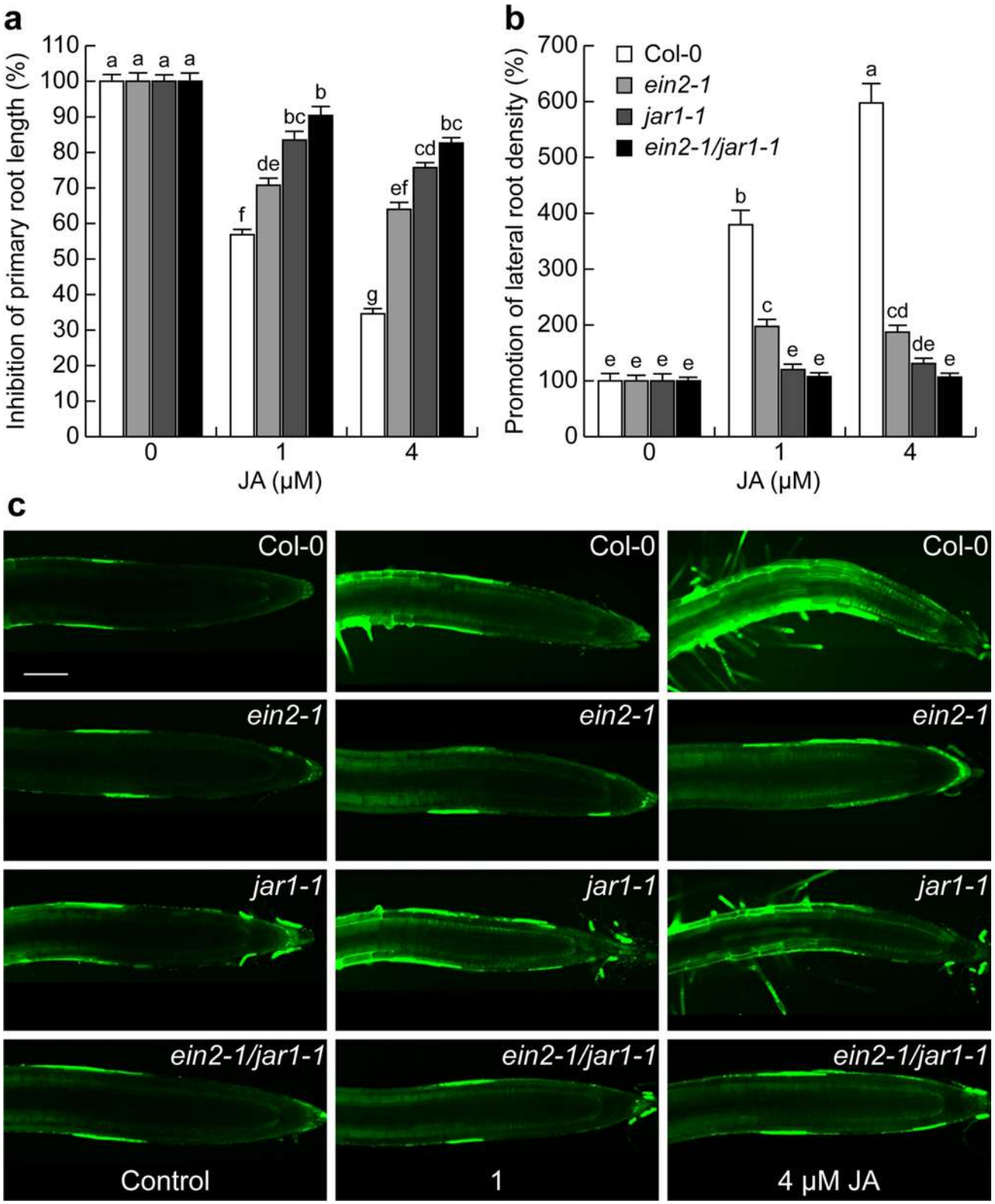

Fig. 6 Root response to jasmonic acid and nitric oxide accumulation in wild-type, *ein2-1, jar1-1*, and *ein2-1/jar1-1 Arabidopsis* seedlings. WT, single and double mutant seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 1 or 4 μM JA for 7 days. **a** Inhibition of primary root length (percentage) and **b** promotion of lateral root density (percentage) were recorded. Values shown represent the mean ± standard deviation ($n = 30$). *Different letters* indicate statistical differences following a Tukey test analysis ($p < 0.05$). **c** Representative confocal micrographs of the detection of endogenous NO with 4,5-diaminofluorescein diacetate, which was determined in primary roots of at least six seedlings (*scale bar* 100 μm). The experiment was repeated twice with similar results

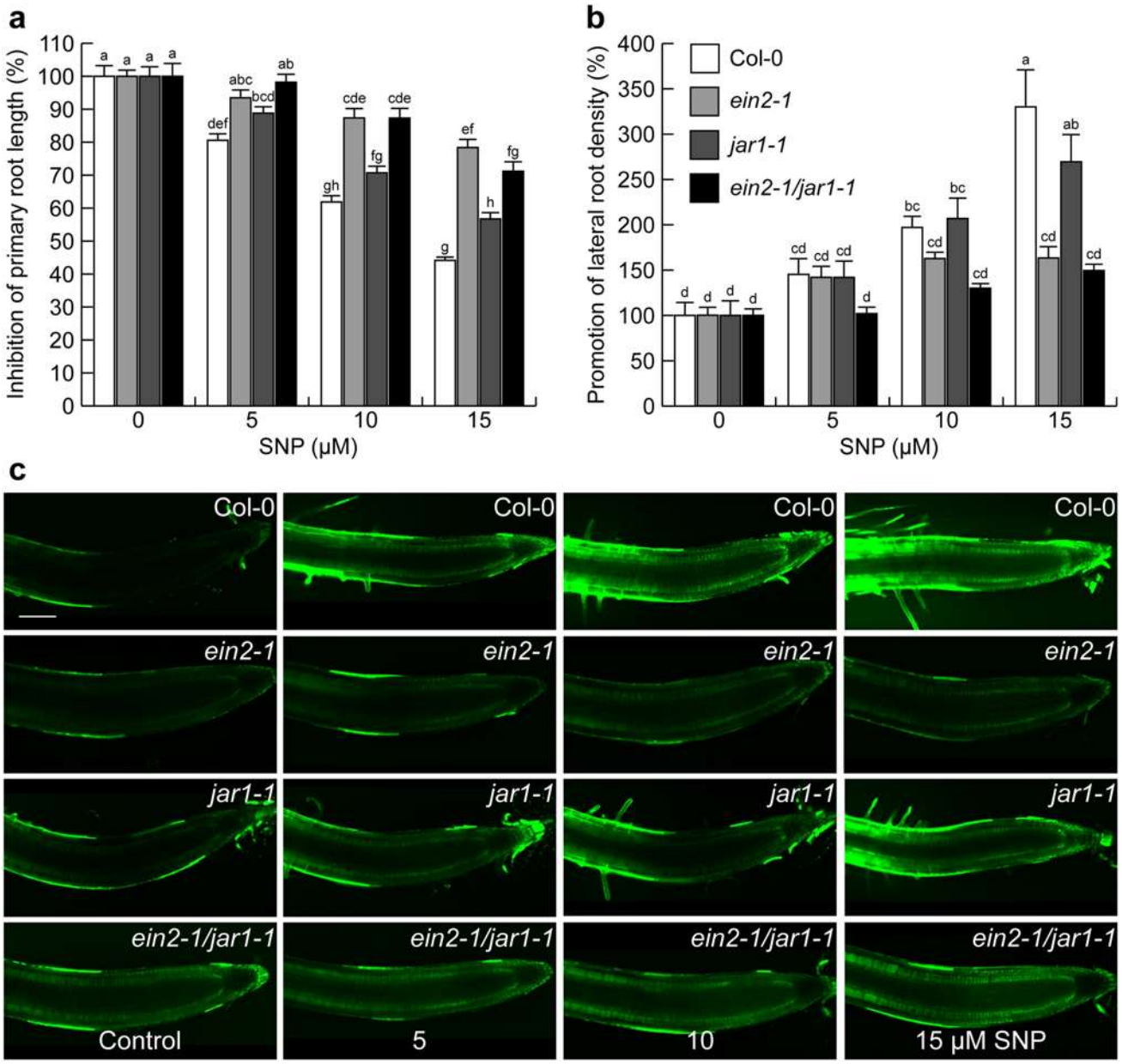

Fig. 7 Effect of nitric oxide donor sodium nitroprusside on root development and nitric oxide accumulation in wild-type, *ein2-1, jar1-1,* and *ein2-1/jar1-1* seedlings. WT, single and double mutant seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 5, 10, and 15 μM SNP for 7 days. **a** Inhibition of primary root length (percentage) and **b** promotion of lateral root density (percentage) were recorded. Values shown represent the mean ± standard deviation ($n = 15$). *Different letters* indicate statistical differences of a Tukey analysis with a value at $p < 0.05$. **c** Representative confocal micrographs of the detection of endogenous NO with 4,5-diaminofluorescein diacetate, which was determined in primary roots ($n = 6$) (*scale bar* 100 μm). The experiment was repeated twice with similar results

growth conditions and no further increases were evident in either the WT or the mutant in the JA treatment (Fig. 8a, b). Paraquat increased ROS levels in WT seedlings and in *coi1-1* mutants, suggesting that its accumulation within the root tip might not determine primary root growth inhibition by JA according to the resistance to JA of *coi1-1*.

We next compared primary root growth in response to JA of WT and *radical cell death (rcd1)* mutants, which are resistant to paraquat-induced ROS accumulation in primary roots (Pelagio-Flores and others 2016). The WT and *rcd1* seedlings displayed similar sensitivity to the primary root growth inhibition caused by JA (See Fig. S4 in

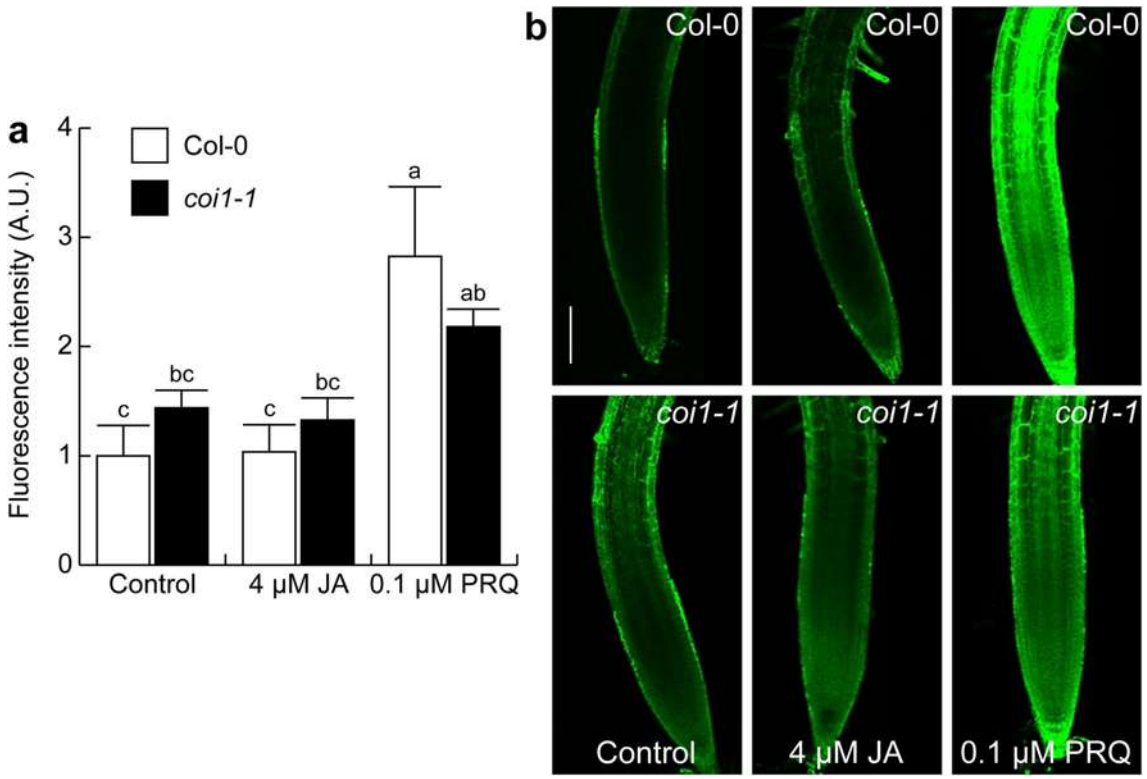

Fig. 8 Reactive oxygen species (ROS) accumulation in the primary root of wild-type and *coi1-1* mutants in response to jasmonic acid (JA), sodium nitroprusside (SNP), and paraquat (PRQ). Wild-type and *coi1-1* seedlings were germinated and grown on agar-solidified 0.2× MS media for two days, and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI1* segregating population in media supplemented with 4 µM JA two days after germination, and subsequently wild-type and *coi1-1* seedlings were transferred to agar-solidified 0.2× MS media supplied with the solvent, 4 µM JA, 20 µM SNP, or 0.1 µM PRQ for 8 days. **a** Fluorescence intensity was quantified using the IMAGEJ program in micrographs of the primary roots treated with 2′,7′-dichlorofluorescein diacetate (H2DCF-DA). Values shown represent the mean ± standard deviation ($n = 6$). *Different letters* indicate statistical differences of a Tukey analysis with a value at $p < 0.05$. **b** Representative confocal micrographs of the detection of endogenous ROS with H2DCF-DA mentioned above (*scale bar* 100 µm). The experiment was repeated twice with similar results

Supplementary Information). These results suggest that the primary root growth inhibition in response to JA is not mediated by an altered ROS homeostasis.

Discussion

Jasmonic acid orchestrates plant development and adaptation to biotic challenges through its regulated biosynthesis via JAR1 and closely related enzymes. JAR1 catalyzes the conjugation of isoleucine to JA, forming the bioactive jasmonoyl-isoleucine (JA-Ile) molecule, which upon recognition by COI1 results in the degradation of the JAZ repressors and the subsequent activation of MYC2-dependent transcriptional responses. This signal transduction pathway may influence the biosynthesis of other phytohormones and second messengers play important roles in hormonal crosstalk acting as modulators of gene expression (Lorenzo and others 2003; Mur and others 2008).

JA signaling has been thoroughly investigated in the shoot system, but scarce information is available about its importance for root organogenesis. Recently, it was reported that repetitive wounding of cotyledons transmits the shoot-to-root JA signal, which restricts root growth by inhibiting both cell proliferation and elongation (Gasperini and others 2015), an aspect that is mimicked either by application of nitric oxide or ethylene (Fernández-Marcos and others 2012; Street and others 2015). Indeed JA triggers NO accumulation in *Arabidopsis* under wounding stress and defense, whereas NO activates early JA signaling genes, indicating the existence of crosstalk between NO and JA signaling (Huang and others 2004; Xu and others 2005). The finding that JA induces NO levels in a concentration-dependent manner at the primary root tip indicates a repressing role on the activity of stem cells and/or the cell proliferation capacity, in agreement with its negative regulation of the AP2-domain transcription factors PLT1 and PLT2 (Chen and others 2011). Gel shift and

chromatin immunoprecipitation experiments revealed that MYC2 directly binds to the promoters of *PLT1* and *PLT2* and represses their expression (Chen and others 2011), which explains its critical role in root meristem activity and stem cell niche maintenance.

Whole-genome transcriptional profiling of *Arabidopsis thaliana* seedlings in response to *N*-isobutyl decanamide, a metabolite that improves root branching and defense responses increasing JA levels, revealed the induction of both JA-responsive and senescence-associated genes, and nitric oxide accumulation in roots and in leaves, and such responses were absent in the *coi1-1* mutants defective on the JA receptor (Méndez-Bravo and others 2011). Our current data extend these observations by showing that JA biosynthesis and signaling components including JAR1, COI1, and MYC2 mediate NO accumulation in primary roots in response to JA. In this regard, NO-related mutants *nia1*/*nia2* and *Atnoa1* were indistinguishable in root architectural responses to JA when compared to WT seedlings. This was somewhat surprising because the activity of the nitrate reductases encoded by the *NIA1* and *NIA2* genes are an important NO source to drive plant growth and development (Park and others 2011).

An alternate oxidative pathway for NO biosynthesis requiring a putative nitric oxide synthase (NOS)-like enzyme has been long proposed to act in plants. However, despite of years of research on this topic, little advancement has been achieved (Santolini and others 2017). Initial efforts to identify a plant NOS led to characterization of the *Atnoa1* mutant (Guo and others 2003), but strong evidence suggests that this protein did not function as a nitric oxide synthase but rather acts as a plastid-targeted GTPase, and that it might be required for ribosome function (Sudhamsu and others 2008). Here we show that *Atnoa1* is not involved in the root architectural response to JA, and thus additional efforts should be conducted towards identifying the genetic elements acting in NO sensing for hormonal or environmental configuration of root architecture.

NO interacts with several phytohormone pathways to orchestrate plant growth and defense and in an organ- and tissue-specific manner. Lateral root formation plays an important role in plant branching and absorptive capacity to take up nutrients and water. A relationship of JA-mediated NO production and an improved lateral root formation can be explained due to the positive function of NO activating root pericycle cells or via promoting auxin signaling in lateral root primordia (Schlicht and others 2013), because NO donors increase auxin-dependent gene expression and NO depletion blocks Aux/IAA protein degradation (Terrile and others 2012). Moreover, TIR1 S-nitrosylation enhances TIR1-Aux/IAA interaction, facilitating Aux/IAA degradation and subsequent activation of gene expression. Nitric oxide was found to induce the expression of *JAZ1* in the vascular cylinder and cortex at the differentiation zone of the root, but not in primary root tips, and enhanced *JAZ10* expression in lateral root primordia and the protoxylem of primary root tips. Thus, *JAZ1* and *JAZ10* have different expression domains in roots. Because *JAZ1* is inducible by JA (Grunewald and others 2009), the current data are consistent with a role of NO as mediator in the JA signaling pathway likely involved in lateral root formation and/or elongation.

Following the observation that the JA receptor mutant *coi1-1* was resistant to the repressing effects of NO-donor SNP in primary roots, we hypothesized that mutations conferring strong resistance to JA would render plants less sensitive to local accumulation of NO at the root tip. Because JA and ethylene have common targets for regulation of gene expression, we compared the WT primary root growth with that of *ein2-1* and *ein2-1*/ *jar1-1* mutants in response to either JA or NO-donor SNP. Interestingly, the loss of *EIN2* function renders plants resistant to JA and SNP for primary root growth stoppage. This suggests that EIN2 could be part of a NO sensing pathway not only because it mediates ethylene responsiveness in roots, but also because recent data demonstrated that NO coordinates responses throughout development based on targeted degradation of ethylene response factors (ERFs), which act as plant-specific transcriptional regulators (Gibbs and others 2014). Moreover, NO itself has been reported to induce ethylene production during the so-called hypersensitive response, which allows the plant to resist pathogen attack (Mur and others 2008).

In plants, both NO and reactive oxygen species (ROS) signaling occur through complex mechanisms and hormone response crosstalk with salicylic acid, jasmonic acid, and ethylene (Mittler and others 2011). Recently, the onset of nitrite-induced cell death was correlated with NO and H_2O_2 signaling and the decrease in the cellular level of antioxidants (Kasten and others 2016). Two sets of experiments indicated that ROS did not act as modulators during the JA-induced root reprogramming. First, paraquat-induced ROS accumulation in WT seedlings proceeds normally in *coi1* mutants, and second, *rcd1* mutants that are defective on the *RADICAL-INDUCED CELL DEATH1* (*RCD1*) gene were equally sensitive to the WT in JA-induced repression of root growth. *RCD1* encodes an ADP-ribosyl-transferase domain-containing protein involved in intracellular ROS generation by the herbicide paraquat and/or ultraviolet-B irradiation (Ahlfors and others 2004; Fujibe and others 2004). Therefore, although NO and ROS may have common targets in plant stress signaling, the root developmental responses triggered by JA actually depend more on NO accumulation linked to elements of the JA signal transduction pathway, which may act in concert with ethylene via the function of *ETHYLENE INSENSITIVE2*. Our findings underline the importance of NO in phytohormone signaling pathways and

highlight its role as a second messenger during the configuration of the root system architecture.

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