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“Propiedades y mecanismos de acción de fitoreguladores de defensa y autoinductores bacterianos sobre el desarrollo de la raíz de *Arabidopsis thaliana*”

Tesis que presenta
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La adversidad tiene el don de despertar talentos que en la prosperidad hubiesen permanecido durmiendo.

Horacio

Este trabajo fue realizado en el Laboratorio de Biología del Desarrollo Vegetal del Instituto de Investigaciones Químico Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo, bajo la dirección del D. C. José López Bucio y con la ayuda de los sinodales: el D. C. Eduardo Valencia Cantero, la D. C. Elda María Beltrán Peña, el D. C. Miguel Martínez Trujillo y el D. C. Lenin Sánchez Calderón. El recurso para el financiamiento de la parte experimental del trabajo y la beca de manutención (No. 249607) fue proporcionado por el Consejo Nacional de Ciencia y Tecnología (CONACYT).

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RESUMEN

El ciclo de vida de las plantas está modulado por un grupo heterogéneo de moléculas bioactivas conocidas como reguladores del crecimiento vegetal o fitohormonas. El ácido indol-3-acético, el ácido jasmónico, el etileno y el ácido abscísico, influyen desde la germinación de la semilla hasta la senescencia de la planta. En este contexto, el crecimiento y el desarrollo adecuados durante las primeras etapas posteriores a la germinación, integra una gama amplia de interacciones moleculares que son esenciales para la supervivencia y la transición desde la etapa de plántula hasta el estado adulto.

Recientemente, diversas moléculas como el óxido nítrico, el ácido fólico y las *N*-acil-homoserina lactonas (AHLs) han sido implicadas en la biología del desarrollo vegetal y se ha comenzado a dilucidar sus mecanismos de acción. En particular, el óxido nítrico actúa como un mensajero celular tanto en animales como en plantas y en estos últimos organismos se le ha vinculado con procesos de división, elongación y diferenciación del sistema radical. Por otra parte, el ácido fólico es un precursor del tetrahidrofolato, el cual actúa como cofactor en reacciones enzimáticas, en tanto que las AHLs coordinan la comunicación célula a célula en bacterias Gram negativas a través de un proceso llamado quorum sensing (QS). Dichos compuestos han sido implicados en el desarrollo de la raíz.

En este trabajo, se implementaron estrategias farmacológicas, genéticas y moleculares para estudiar la relación multifuncional del óxido nítrico, del ácido fólico y de la *N*-decanoil-*L*-homoserina lactona (C10-HL) con las rutas de señalización hormonal canónicas durante el desarrollo post-embrionario de la raíz de *Arabidopsis thaliana*. Los resultados obtenidos mostraron que el óxido nítrico es un mediador en la señalización del ácido jasmónico en un programa que requiere del gen *ETHYLENE INSENSITIVE 2* (*EIN2*) para la reconfiguración de la arquitectura del sistema radical de *Arabidopsis*. Adicionalmente, diversos ensayos indicaron que la modulación de la arquitectura de la raíz por el ácido fólico involucra la señalización de las auxinas (independientemente de la cinasa TOR) para regular los programas de división y elongación celular. En tanto que el producto del gen de *DECANAMIDE RESISTANT ROOT 1* (*DRR1*), previamente reportado para la percepción de las AHLs por la raíz de *Arabidopsis*, es un regulador negativo de la señalización del ácido abscísico, y actúa corriente arriba de los factores de transcripción ABI4 y ABI5 que participan en los programas del crecimiento de la raíz primaria y la germinación de la semilla.

Los resultados de este trabajo reflejan una coordinación muy fina en la regulación de la morfogénesis vegetal en la que las vías de transducción de señales de diversas fitohormonas se modifican por segundos mensajeros, vitaminas e incluso moléculas de origen bacteriano.

Palabras clave: *Arabidopsis thaliana*, *N*-decanoil-*L*-homoserina lactona, ácido abscísico, óxido nítrico, ácido jasmónico, etileno, ácido fólico, auxinas.

ABSTRACT

The life cycle of plants is highly modulated by a heterogeneous group of bioactive molecules known as plant growth regulators or phytohormones. Indole-3-acetic acid, jasmonic acid, ethylene, and abscisic acid control processes from seed germination to senescence. In this context, proper growth and development of a seedling during the first stages after seed germination integrate a wide range of molecular interactions that are essential for the survival and the transition from seedling stage to adult stage.

Currently, different compounds such as nitric oxide, folic acid and *N*-acyl-homoserine lactones (AHLs) have been implicated in plant development and their mechanisms of action have begun to be elucidated. In particular, nitric oxide acts as a signaling messenger in both animals and plants and in the latter organisms has been linked to processes of division, differentiation and development in the root system. On the other hand, folic acid is a precursor of tetrahydrofolate, which is an essential cofactor for enzymatic reactions, whereas AHLs coordinate cell-to-cell communication in Gram-negative bacteria through a process termed quorum sensing (QS). Such compounds have been implicated in the root development.

In this report, pharmacological, genetic and molecular strategies were used to study multifunctional relationship of nitric oxide, folic acid and *N*-decanoyl-*L*-homoserine lactone (C10-HL), with the canonical hormone signaling pathways during post-embryonic development of *Arabidopsis thaliana* root. Our results showed that nitric oxide mediates jasmonic acid signaling via interacting with the *ETHYLENE INSENSITIVE 2* (*EIN2*) gene functioning during the configuration of the *Arabidopsis* root system architecture. Additionally, several assays indicated that root system modulation by folic acid involves auxin signaling (acting independently of TOR kinase) to regulate cell division and elongation. Further experimental evidence suggests that the gene product of D*ECANAMIDE RESISTANT ROOT 1* (*DRR1*), previously reported to be necessary in AHL perception in the *Arabidopsis* root, is a negative regulator of ABA signaling probably acting upstream of transcription factors ABI4 and ABI5 that participate in primary root growth and seed germination.

The results of this work reflect a fine coordination in plant morphogenesis by which several phytohormone signaling pathways are modified by second messengers, vitamins and even molecules of bacterial origin.

Keywords: *Arabidopsis thaliana*, *N*-decanoyl-*L*-homoserine lactone, abscisic acid, nitric oxide, jasmonic acid, ethylene, folic acid, auxins.

1. INTRODUCCIÓN

El desarrollo post-embrionario de nuevos órganos es uno de los determinantes principales en la configuración del sistema radical, que posibilita el crecimiento óptimo de la planta debido a su papel en la captación de agua y nutrientes. Las condiciones ambientales en constante cambio demandan una enorme capacidad adaptativa, la cual se alcanza por la existencia de múltiples poblaciones de células proliferativas (meristemos) que permiten la formación de las estructuras necesarias para la supervivencia de estos organismos.

La coordinación de los procesos celulares depende de pequeñas moléculas conocidas como fitohormonas, que activan o reprimen la expresión de genes para que sus productos proteínicos realicen la función que se requiere por el organismo, y en última instancia determinan su fenotipo. Las fitohormonas canónicas incluyen a las auxinas, las citocininas, los brasinoesteroides, el etileno, el ácido abscísico, el ácido jasmónico y el ácido salicílico, las cuales son moléculas estructuralmente diversas. Los procesos que se llevan a cabo en los sistemas vegetales dependen de la gran cantidad de genes regulables por cada fitohormona a nivel de transcripción, de las múltiples combinaciones que se pueden conseguir entre elementos de sus rutas de señalización y de la interacción entre las diferentes vías hormonales.

Las interacciones entre fitohormonas pueden ocurrir a diferentes niveles incluyendo biosíntesis, transporte y respuesta, por lo que los mecanismos de transducción de señales se empiezan a considerar como redes y no como rutas lineales independientes. Adicionalmente, se ha comenzado a dilucidar el papel de nuevas moléculas que modulan el desarrollo vegetal, las cuales pueden ser endógenas o de origen microbiano y que han sido relacionadas con más de una vía de señalización hormonal vegetal. Con respecto a lo antes mencionado, evidencia experimental y varios reportes previos, muestran que el óxido nítrico, un importante mensajero de señalización en plantas y animales; el ácido fólico, una vitamina necesaria para el metabolismo celular; y la *N*-decanoil-homoserina lactona (C10-HL), un compuesto de quorum sensing de bacterias Gram negativas, regulan las redes de señalización durante el crecimiento y desarrollo vegetal, así como en la adaptación ante el ataque de patógenos.

2. ANTECEDENTES

2.1. La germinación de la semilla

La semilla es la estructura donde un embrión vegetal permanece latente y sobrevive hasta la germinación (Bewley, 1997; Koornneef *et al.*, 2002; Bentsink y Koornneef, 2008). El desarrollo de las semillas comprende dos fases principales: la formación del embrión y la maduración. La embriogénesis, inicia con un zigoto de una sola célula y termina en la etapa de corazón, cuando todas las estructuras del embrión han sido formadas (Mayer *et al.*, 1991), seguida por una fase de crecimiento durante la cual el embrión llena el saco que lo contiene (Goldberg *et al.*, 1994). Al final de la fase de crecimiento, la división celular se detiene (Fig. 1a) (Raz *et al.*, 2001). Subsecuentemente, la semilla, conteniendo el embrión de tamaño completo, acumula reservas de almidón y posteriormente ocurre la desecación. En este último punto la semilla se encuentra preparada para germinar una vez que supere la fase de latencia (Goldberg *et al.*, 1994).

La latencia (dormancia) se ha definido como la capacidad de una semilla viable para germinar bajo condiciones favorables (Bewley, 1997; Finch-Savage y Leubner-Metzger, 2006). El bloqueo de la germinación ha evolucionado de manera diferente entre especies con base en la adaptación al entorno prevaleciente, de modo que la germinación se produce cuando las condiciones para establecer una nueva generación de plantas son las más adecuadas (Hilhorst, 1995; Bewley, 1997; Li y Foley, 1997). La germinación comienza con la captación de agua por la semilla seca (imbibición) y termina cuando la radícula del embrión se extiende para penetrar las estructuras que lo rodean y finalmente emerge de las mismas (Fig. 1b) (Bewley, 1997; Bentsink y Koornneef, 2008). Los primeros signos de la germinación se reflejan en la activación de procesos moleculares esenciales, incluyendo la transcripción y la traducción, los cuales coordinan la división celular en los meristemos, seguido por la elongación celular y eventualmente con la protrusión de la radícula (Barrôco *et al.*, 2005; Masubelele *et al.*, 2005).

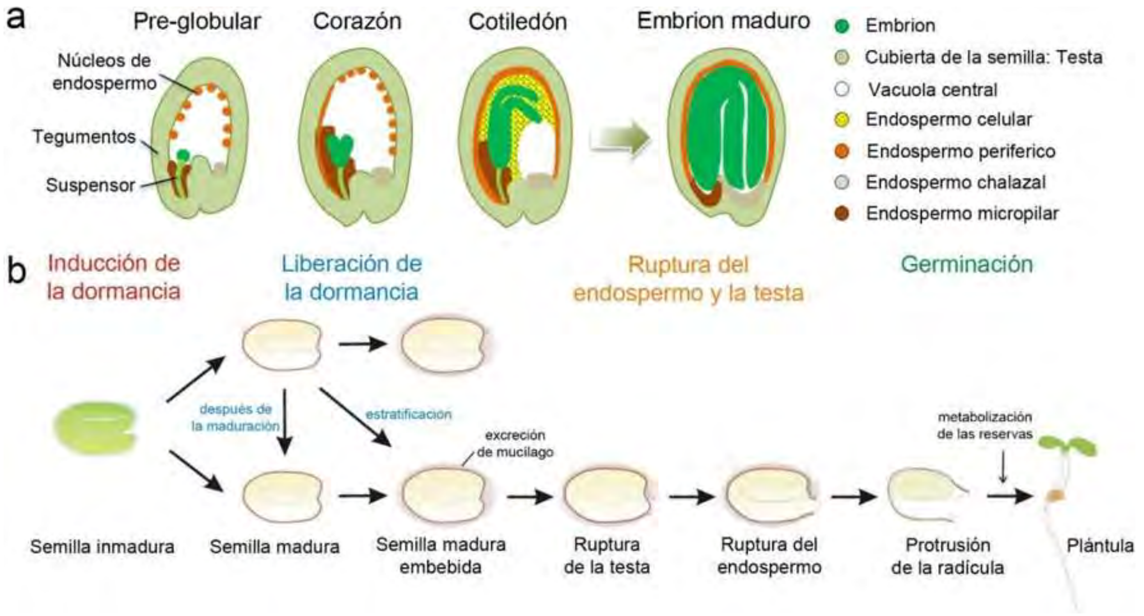


Figura 1. El Desarrollo y la germinación de la semilla de *Arabidopsis*. a) Representación esquemática del desarrollo de la semilla de *Arabidopsis*. La formación del endospermo se muestra a partir de la formación de "núcleos" colocados alrededor del citoplasma periférico, que origina al endospermo celular. Después, el volumen de la vacuola central disminuye progresivamente hasta desaparecer, en tanto que el endospermo es absorbido por el embrión en la semilla madura. Al final de la maduración, sólo quedan tres tipos de endospermo: de una sola célula, el micropilar que rodea a la radícula del embrión y el chalazal. b) Diagrama de la germinación de la semilla de *Arabidopsis*. Una semilla madura de *Arabidopsis* puede ser latente o no latente. La dormancia de la semilla puede ser interrumpida por la imbibición (estratificación), lo cual conducirá a la ruptura de la testa y el endospermo por la radícula (germinación visible). Adaptado de Bentsink y Koornneef (2008); Locascio y col. (2014).

2.2. La arquitectura del sistema radical de *Arabidopsis thaliana*

La arquitectura del sistema radical es la configuración espacial que adquiere la raíz en el suelo. Debido a que los recursos están heterogéneamente distribuidos, el despliegue espacial de las raíces determinará sustancialmente la capacidad de una planta para captar agua y nutrientes (Lynch, 1995). En este sentido, el crecimiento de la raíz primaria y la formación de las raíces laterales determinan la configuración tridimensional de la raíz (Jones y Ljung, 2012). En *Arabidopsis thaliana*, el sistema radical presenta una organización bastante simple, ya que se encuentra formado por una raíz primaria, de la cual emergen las raíces laterales y estas últimas también se ramifican en un proceso repetitivo. A continuación se describe con más detalle los dos componentes principales que conforman el sistema radical.

2.2.1. La raíz primaria

La formación de la raíz primaria ocurre desde la embriogénesis, cuando el embrión se polariza estableciendo los meristemos, los cuales son las estructuras proliferativas que darán origen al follaje en el extremo superior y a la raíz en el extremo inferior (Mansfield y Briarty, 1991). Una vez que el meristemo apical de la raíz se constituye durante la embriogénesis, se encuentra listo para proveer nuevas células para el crecimiento y el aumento en biomasa. El meristemo radicular contiene un grupo de células iniciales que rodean al centro quiescente (QC, por sus siglas en inglés), el cual presenta escasa actividad mitótica y mantiene el estado indiferenciado de las células iniciales (van den Berg *et al.,* 1997; Spradling *et al.,* 2001; Scheres, 2007).

La división de las células iniciales genera filas de una sola célula que se extienden a lo largo del eje longitudinal y forman capas de tejido distintivas. En consecuencia, cada tejido tiene un linaje celular distinto, sin embargo, es la posición y no el linaje lo que determina la identidad celular (van den Berg *et al.*, 1995; Kidner *et al.*, 2000). Las nuevas células producidas por las células iniciales progresan a través de tres fases de desarrollo en su camino a la madurez. En la zona meristemática, se dividen múltiples veces para generar un suministro constante de células que posteriormente se alargarán y diferenciarán. En la zona de elongación, las células pierden la habilidad para dividirse e incrementan su longitud. Finalmente, en la zona de diferenciación, las células adquieren sus funciones y características especializadas (Fig. 2a). Así, a partir de las células iniciales, se producen los tejidos del sistema vascular, la endodermis, el córtex, la epidermis, la cubierta lateral de la raíz y la columnela (Fig. 2b) (Ishikawa y Evans, 1995).

En la raíz de *Arabidopsis*, el tejido vascular está organizado dentro de un cilindro central o estela, el cual contiene al xilema (que transporta agua y nutrientes) y al floema (que transporta fotosintatos) interpuestos con células del procámbium no diferenciadas y una capa de periciclo circundante, mientras que los tejidos exteriores de la raíz exhiben simetría radial, la estela es bilateralmente simétrica (Fig. 2c) (Dolan *et al.*, 1993; Petricka *et al.*, 2012).

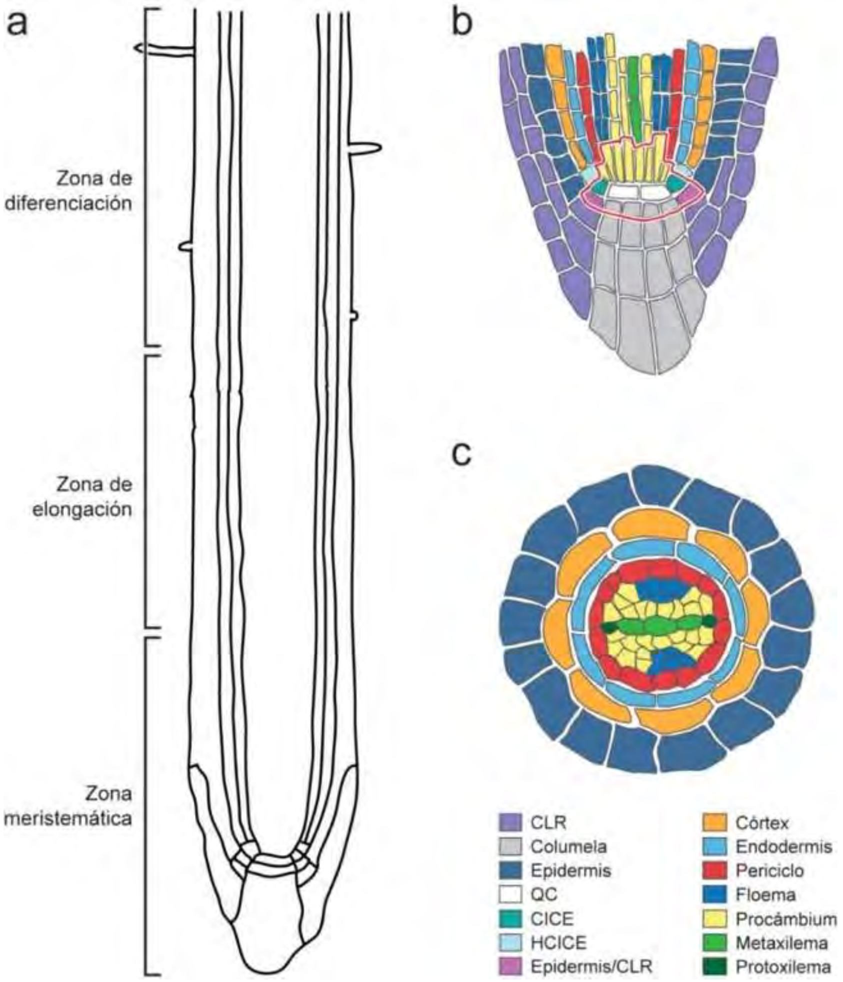


Figura 2. La raíz primaria de *Arabidopsis thaliana*. a) Las distintas zonas del desarrollo en la raíz primaria. La división celular ocurre en la zona meristemática, la expansión y elongación celular se lleva a cabo en la zona de elongación y la diferenciación celular (indicada por la formación de pelos radicales) se presenta en la zona de diferenciación. b) Organización del meristemo apical de la raíz. Los diferentes tipos de células se acomodan en filas celulares a través del eje longitudinal de la raíz. La región delimitada con rojo es el nicho de células fuente. c) Organización del tipo de células dentro de la estela. El diagrama muestra una sección transversal de la raíz. El patrón del tipo de células en la estela es bilateralmente simétrico: Un eje central de xilema es flanqueado por dos haces de floema. CLR, cubierta lateral de la raíz; QC, centro quiescente; CICE, células iniciales del córtex y la endodermis, HCICE, hijas de células iniciales del córtex y la endodermis; Epidermis/CLR, células iniciales de la cubierta de la raíz lateral y la epidermis. Modificado de Petricka y col. (2012).

Las capas celulares del córtex y endodermis provienen de las células iniciales del córtex/endodermis mediante un proceso regulado por los factores de transcripción SCARECROW (SCR) y SHORTROOT (SHR) (Fig. 2b-c) (Scheres *et al.*, 1995; Di Laurenzio *et al.,* 1996; Helariutta *et al.*, 2000). Debido a que en la punta de la raíz primaria se encuentra la estructura proliferativa, la cubierta lateral de la raíz y la columnela forman una capa protectora que se desprende continuamente. Junto con la epidermis, estos tejidos constituyen la superficie exterior de la raíz formando una envoltura cuyas células iniciales se dividen trasversalmente y su progenie experimenta una expansión celular rápida y posteriormente se diferencian y producen amiloplastos que contienen almidón, los cuales juegan un papel en la percepción de la gravedad (Dolan *et al.*, 1993; Petricka *et al.*, 2012).

Los factores de transcripción con dominio NAC llamados FEZ y SOMBRERO (SMB) participan en las divisiones transversales que incrementan el número de capas en los tejidos de la columnela. FEZ promueve la división celular en tanto que SMB reprime la expresión de FEZ (Fig. 2b-c) (Willemsen *et al.*, 2008). Finalmente, en la epidermis, una célula de este tejido en contacto con dos células del córtex se desarrollará en un pelo radicular, mientras que una célula epidérmica adyacente a una sola célula del córtex permanecerá como atricoblasto (Ishida *et al.*, 2008).

2.2.2. Las raíces laterales

Las raíces laterales se forman en la zona de diferenciación a partir de células del periciclo posicionadas adyacentemente a los polos del xilema. Un subgrupo de esas células se estimula para dividirse y forma un primordio de la raíz lateral (Blakely y Evans, 1979). El primer evento morfológico relacionado con la iniciación de un primordio ocurre cuando dos células fuente dentro de la misma fila experimentan divisiones transversales asimétricas polarizadas (Laskowski *et al.*, 1995; Casimiro *et al.*, 2001). Una serie idéntica de divisiones mitóticas ocurre en ambas células, formando una fila de aproximadamente 8 a 10 células “cortas”. Después de un periodo de expansión radial, ocurre una división longitudinal en las células centrales, originando un primordio con dos capas, lo cual está definido como etapa 2. Durante la etapa 3, la capa externa se divide longitudinalmente para formar una tercera capa. Una vez más, algunas células en los extremos de la segunda capa no se dividen, lo cual ocasiona que la estructura comience a tener forma de domo. Posteriormente, la capa interna se divide originando un primordio de cuatro capas que penetra el tejido endodérmico de la raíz parental donde se está formando el primordio durante la etapa 4. En las tres etapas siguientes, las divisiones transversales y longitudinales continúan y el primordio atraviesa los tejidos del córtex (etapa 5) y la epidermis (etapa 6) hasta que emerge de la raíz parental durante la etapa 7 (Fig. 3) (Malamy y Benfey, 1997; Casimiro *et al.*, 2003).

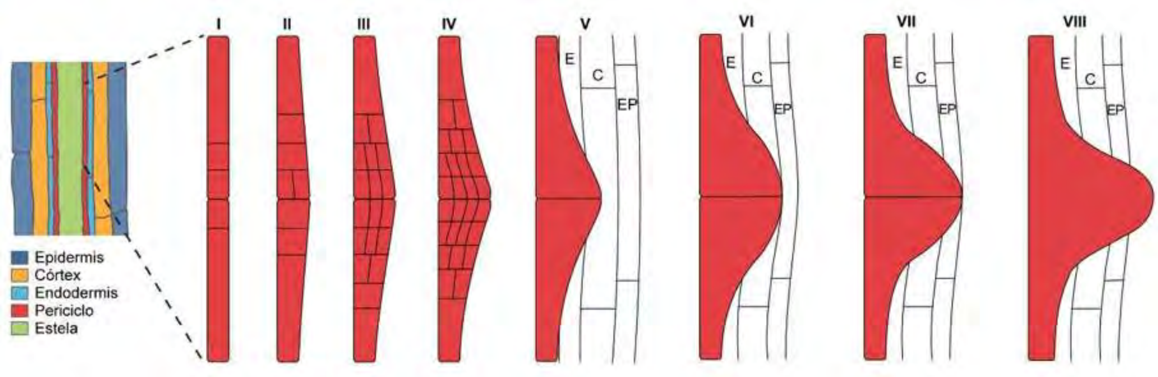


Figura 3. Desarrollo de la raíz lateral de *Arabidopsis*. Esquema de un segmento en la zona de diferenciación de la raíz primaria a través del eje longitudinal. Las raíces laterales se inician en la capa celular del periciclo (rojo), cuando divisiones celulares transversales asimétricas ocurren se origina un primordio de raíz lateral (etapa I), el cual experimentará una serie de divisiones periclinales avanzando por 6 etapas de desarrollo posteriores hasta su emergencia como una raíz lateral madura (etapa VIII). E, epidermis; C, córtex; E, endodermis. Adaptado de Casimiro y col. (2003) y Petricka y col. (2012).

2.3. Los reguladores del crecimiento vegetal o fitohormonas

Al igual que ocurre en animales, las plantas coordinan su crecimiento y desarrollo al nivel de tejidos y órganos mediante la emisión de moléculas de señalización extracelular que permiten la comunicación entre las células (Chow y McCourt, 2006). En consecuencia, las hormonas vegetales o fitohormonas también son llamadas reguladores del crecimiento vegetal debido a su característica principal de estimular programas de organogénesis. Sin embargo, también controlan respuestas de defensa, estrés y aspectos del metabolismo, la reproducción y el envejecimiento, es decir, virtualmente cada aspecto en el ciclo de vida de las plantas está bajo el control hormonal en algún grado (Gray, 2004).

Las auxinas, citocininas, brasinoesteroides, giberelinas, ácido abscísico, etileno, ácido jasmónico y ácido salicílico se consideran fitohormonas canónicas, debido a que sus mecanismos de síntesis, transporte, percepción y señalización son bien conocidos (Gray, 2004; Chow y McCourt, 2006; Santner *et al.*, 2009). Cabe mencionar que la actividad de cada hormona vegetal se determina por su disponibilidad, la cual se controla a nivel de su metabolismo y distribución, además de la eficiencia en su percepción y la posterior transducción de señales, por lo tanto, las alteraciones en alguno de estos eventos impactaran sobre la función hormonal (Vanstraelen y Benková, 2012).

En este contexto, las modificaciones de la actividad de una fitohormona por otra u otras distintas a ella generan un nivel adicional de complejidad en la regulación de procesos del desarrollo y proveen mecanismos interconectados (Kuppusamy *et al.*, 2009; Vanstraelen y Benková, 2012). Entonces, la comprensión de cómo se coordina el ciclo de vida de una planta por estas moléculas, requiere primero dilucidar cómo se perciben estas señales y la maquinaria celular que activan. A continuación se describen los mecanismos de señalización de las fitohormonas implicadas en este estudio.

2.3.1. Las auxinas

Las auxinas son fitohormonas indispensables para el crecimiento y desarrollo en las plantas, regulando procesos biológicos como la división y expansión celular, la formación de la raíz, el fototropismo, el gravitropismo, la dominancia apical, el desarrollo del fruto, y la partenocarpia. Las auxinas se sintetizan por tejidos que crecen activamente tales como el meristemo del follaje, los primordios de hoja, las hojas jóvenes en expansión, las semillas en desarrollo y los frutos (Wang e Irving, 2011).

Las auxinas son distribuidas en toda la planta a través de un sofisticado sistema de transporte célula a célula conocido como transporte polar de auxinas, donde las proteínas PIN funcionan como transportadores de eflujo (Vieten *et al.*, 2007). Para orquestar los distintos procesos en los que están implicadas, las auxinas emplean un mecanismo dependiente de una ruta proteolítica conservada (la ruta proteosoma-ubiquitina), en la cual las proteínas destinadas a la degradación son previamente etiquetadas con una pequeña proteína llamada ubiquitina. Las proteínas etiquetadas en esta forma son reconocidas por el proteosoma y finalmente son degradadas (Fig. 4).

La ubiquitina se conjuga covalentemente con diferentes sustratos proteínicos por la actividad secuencial de tres enzimas: la proteína activadora de la ubiquitina (E1), la que conjuga a la ubiquitina (E2) y la proteína ligasa de la ubiquitina (E3) (Pickart, 2001). Los complejos E3 mejor caracterizados están compuestos de las subunidades CUL1, RBX1, ASK1 y una proteína F-box, donde esta última determina la especificidad del reconocimiento molecular (Deshaies, 1999). El gen *TIR1* codifica una proteína F-box que interactúa directamente con los represores Aux/IAAs y dicha unión es incrementada por las auxinas (Gray *et al.*, 2001; Dharmasiri *et al.*, 2003; Kepinski y Leyser, 2004). Cabe mencionar que los genes *AFBs* codifican para proteínas F-box, de las cuales se conocen cinco con una función redundante a la de TIR1 (Parry *et al.*, 2009; Ljung, 2013). Por lo tanto, en presencia de las auxinas, las proteínas Aux/IAAs son degradadas por la ruta proteosoma-ubiquitina vía la familia TIR1/AFBs (Mockaitis y Estelle, 2008; Ljung, 2013). Un mecanismo que modula la actividad del complejo de degradación SCF$^{TIR1/AFBs}$, es la modificación de la subunidad CUL1 por RELATED TO UBIQUITIN (RUB), en un proceso denominado “rubilación” (Hotton y Callis, 2008). Cuando no son degradadas por el proteosoma, las represores Aux/IAAs se unen y bloquean a los factores de transcripción AUXIN RESPONSE FACTORs (ARFs) y reprimen la transcripción regulada por las auxinas con la ayuda del co-represor TOPLESS (TPL) (Fig. 4a) (Guilfoyle y Hagen, 2007; Szemenyei *et al.*, 2008). Entonces, la señalización de las auxinas ocurre cuando dichos compuestos permiten la interacción del complejo SCF$^{TIR1/AFBs}$ y los Aux/IAAs para que estas últimas sean marcadas con ubiquitina y posteriormente degradadas en el proteosoma, lo cual permite que los ARFs promuevan la transcripción de los genes de respuesta a las auxinas (Fig. 4b).

Adicionalmente, la cinasa TARGET OF RAPAMYCIN (TOR), un regulador maestro conservado evolutivamente entre levaduras, plantas, animales y humanos, el cual integra señales para promover el crecimiento y la proliferación celular, influye en programas morfogenéticos que involucran a las auxinas (Li *et al.*, 2017), por lo que es esencial esclarecer los posibles mecanismos de interacción entre la señalización de las auxinas y la cinasa TOR en el control del desarrollo vegetal.

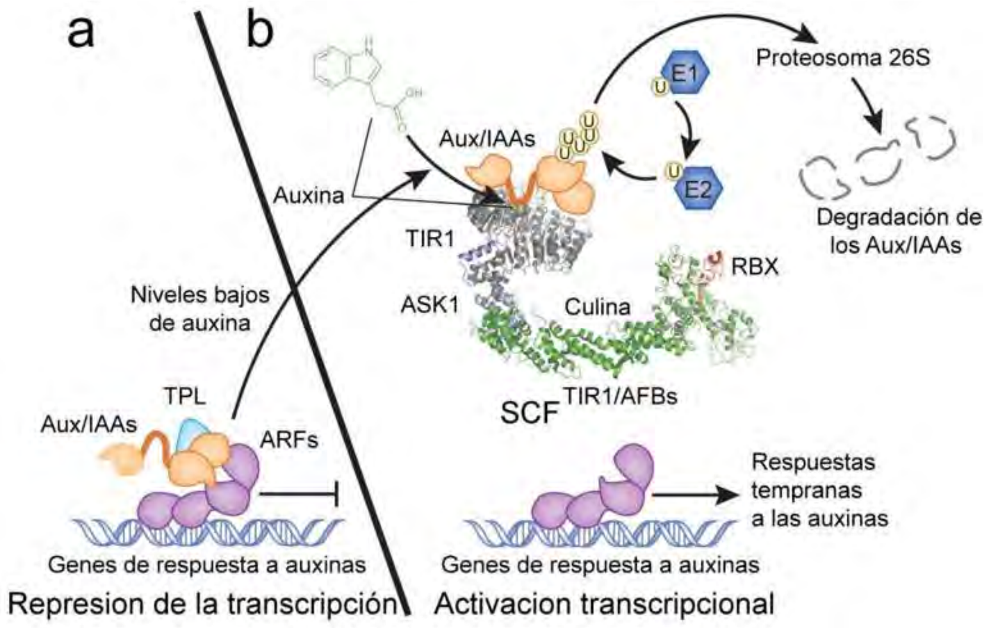


Figura 4. La ruta de señalización de auxinas. Los complejos $SCF^{TIR1/ARBs}$ se unen a la auxina y etiquetan a los represores Aux/IAA para su degradación. a) Niveles celulares bajos de auxinas. La transcripción de genes de respuesta a auxinas se encuentra reprimida por los factores Aux/IAAs. b) Niveles celulares de auxinas altos. La fitohormona se une a TIR1, permitiendo la interacción de este último con los Aux/IAAs para promover la ubiquitinación y subsecuente degradación de dichos represores, lo cual libera a los factores de transcripción ARFs para promover la transcripción de genes de respuesta a auxinas. Modificado de Santner y col. (2009).

2.3.2. El ácido jasmónico

Los jasmonatos forman una familia de oxilipinas derivada de la oxigenación enzimática de los ácidos grasos tri-insaturados de 16 y 18 carbonos (Wasternack y

Kombrink, 2010). El jasmonato más conocido es el ácido jasmónico (JA), el cual al conjugarse con aminoácidos hidrofóbicos produce ligandos bioactivos como la jasmonil-isoleucina (JA-Ile), por medio de una enzima codificada por el gen *JAR1* (Fig. 5) (Staswick y Tiryaki, 2004).

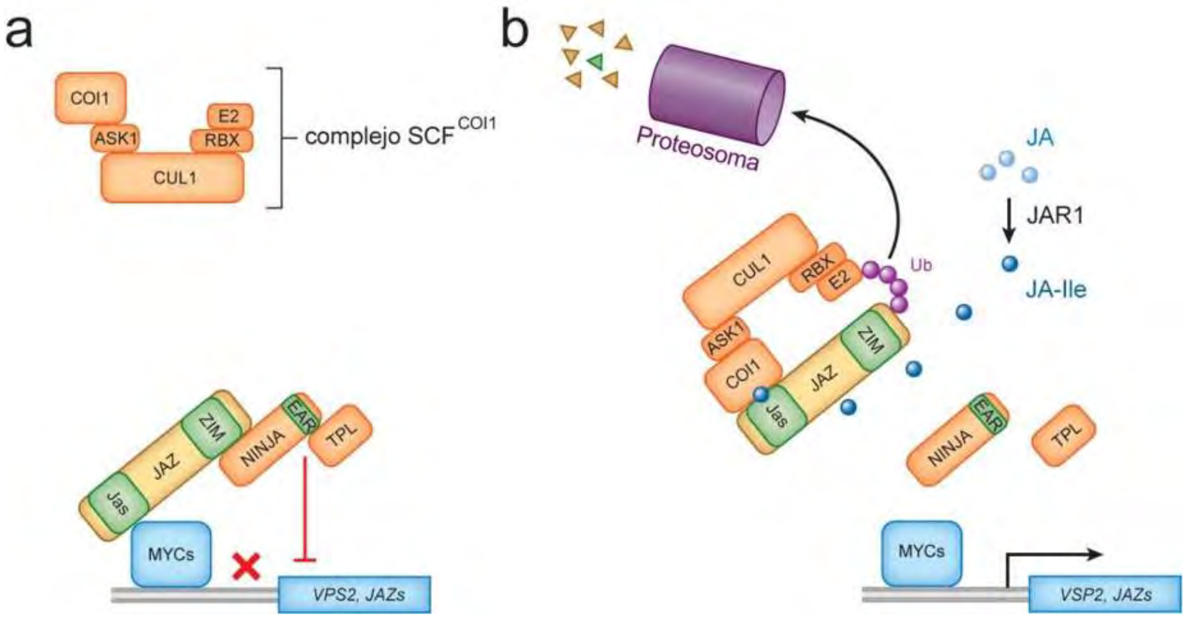


Figura 5. Transducción de señales del ácido jasmónico. a) Los elementos de la ruta de señalización en ausencia del ácido jasmónico (JA). Los represores JAZ en conjunto con NINJA y TPL bloquean a los factores de transcripción, en tanto que SCF^{COI1} se mantiene inactivo. b) La señalización en presencia del JA. El JA es transformado a jasmonoil-isoleucina (JA-Ile) por la enzima JAR1, entonces, JA-Ile activa a SCF^{COI1} para que este último promueva la degradación de los represores JAZ por el proteosoma vía ubiquitinación, mientras que los factores de transcripción (MYCs y otros relacionados) promueven la transcripción de genes de respuesta al JA (como *VSP2*, *JAZs*, etc.). Modificado de Pieterse y col. (2012).

Los jasmonatos desempeñan un papel esencial en la defensa de la planta, ya que controlan la expresión de un 67-85% de genes regulados por heridas o herbivoría en las hojas de *Arabidopsis* (Reymond *et al.,* 2004). También, dichos compuestos son mediadores importantes en las respuestas de defensa a patógenos necrotróficos (Kachroo y Kachroo, 2009) y orquestan diversos procesos del desarrollo vegetal, como la fertilidad masculina de las flores, el tamaño de los peciolos, de los pétalos y de las semillas, además de afectar la estructura de los vasos del xilema e inducir la ramificación de la raíz (von Malek *et al.*, 2002; Zhang y Turner, 2008; Farmer y Dubugnon, 2009; Brioudes *et al.*, 2009; Raya-González *et al.*, 2012). Los procesos antes mencionados requieren del funcionamiento del factor de transcripción con regiones hélice-vuelta-hélice MYC2/JASMONATE INSENSITIVE 1 (MYC2/JIN1) (Chen *et al.*, 2011; Gasperini *et al.*, 2015). En ausencia de los jasmonatos, la actividad de MYC2 es inhibida mediante un complejo represor formado por las proteínas JASMONATE ZIM-DOMAIN (JAZ), TOPLESS (TPL) y NOVEL INTERACTOR OF JAZ (NINJA) (Fig. 5a) (Chini *et al.*, 2007; Thines *et al.*, 2007; Pauwels *et al.*, 2010). No obstante, cuando los niveles de jasmonatos aumentan, la proteína F-box de *Arabidopsis* CORONATINE INSENSITIVE 1 (COI1), una E3 ubiquitina ligasa dentro del complejo SCF actúa como receptor de jasmonatos y su activación promueve la degradación proteolítica de los represores JAZ permitiendo la activación transcripcional de respuesta al JA, la cual es dependiente de MYC2 y otros factores de transcripción relacionados (Fig. 5b) (Thines *et al.*, 2007; Pauwels *et al.*, 2010).

2.3.3. El etileno

El etileno modula la germinación de la semilla, la morfología de la plántula, la maduración del fruto, la elongación del pelo radical, el envejecimiento de la hoja, la defensa y la tolerancia a factores bióticos y abióticos (Bleecker y Kende, 2000). Dichos procesos están coordinados mediante la unión de la fitohormona a sus receptores en forma de homodímeros, de los cuales se conocen cinco tipos, incluyendo a ETHYLENE RESPONSE 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2 y ETHYLENE INSENSITIVE 4 (EIN4) (Zhao y Guo, 2011). En ausencia de etileno, los receptores mantienen a la cinasa CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) en un estado activo, lo cual reprime las respuestas celulares (Fig. 6a) (Clark *et al.*, 1998). En presencia de etileno, el receptor se enciende (Hua y Meyerowitz, 1998), lo cual ocasiona que CTR1 se apague, por lo que es incapaz de fosforilar a ETHYLENE INSENSITIVE 2 (EIN2), permitiendo entonces el procesamiento proteolítico y translocación nuclear de este último (Qiao *et al.*, 2012; Ju *et al.*, 2012). Como resultado, EIN2 acciona una cascada transcripcional involucrando a los factores de transcripción ETHYLENE INSENSITIVE 3/ EIN3-like 1 (EIN3/EIL1) (Fig. 6b) (Chao *et al.*, 1997; Solano *et al.*, 1998).

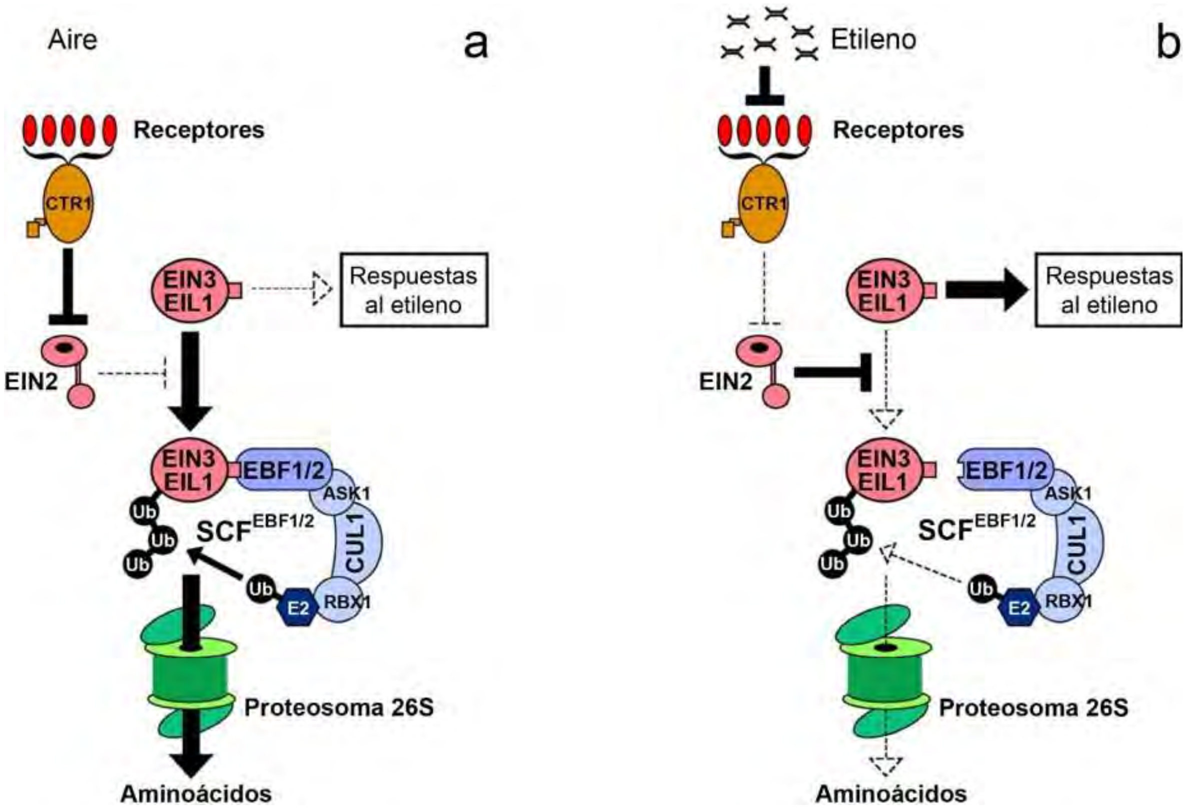


Figura 6. La ruta de señalización del etileno. a) En ausencia de etileno, la familia de receptores (ETR1, ETR2, ERS1, ERS2 y EIN4) activan al regulador negativo CTR1, lo cual apaga a EIN2, por lo que los factores de transcripción (EIN3 y EIL1) son degradados en el proteosoma 26S mediante el marcaje con ubiquitina (Ub) dependiente del complejo SCF$^{EBF1/2}$. b) En presencia de etileno, los receptores inactivan a CTR1, por lo que EIN2 bloquea la degradación de los factores de transcripción, donde estos últimos son capaces de promover la transcripción de genes y con esto las respuestas al etileno. Modificado de Binder y col. (2007).

EIN3 y EIL1 son regulados a nivel de proteína por los complejos SCF que contienen a las proteínas F-box EIN3-BINDING F-BOX PROTEIN 1 (EBF1) y EBF2 a través de una ruta de degradación proteica mediada por el proteosoma 26S (Guo y Ecker 2003; An *et al.*, 2010). A la fecha, se sabe que EIN2 modula la actividad de los complejos SCF$^{EBF1/2}$ y con esto la cantidad de factores de transcripción de respuesta al etileno pero se desconoce el mecanismo de acción en dicho proceso (Fig. 6) (An *et al.*, 2010; Guzmán y Ecker, 1990).

2.3.4. El ácido abscísico

El ácido abscísico (ABA) regula procesos importantes en las plantas, tales como el desarrollo de la semilla y el embrión, la tolerancia a la desecación y latencia, la germinación, el establecimiento de la plántula, el desarrollo vegetativo y la reproducción (Cutler *et al.*, 2010).

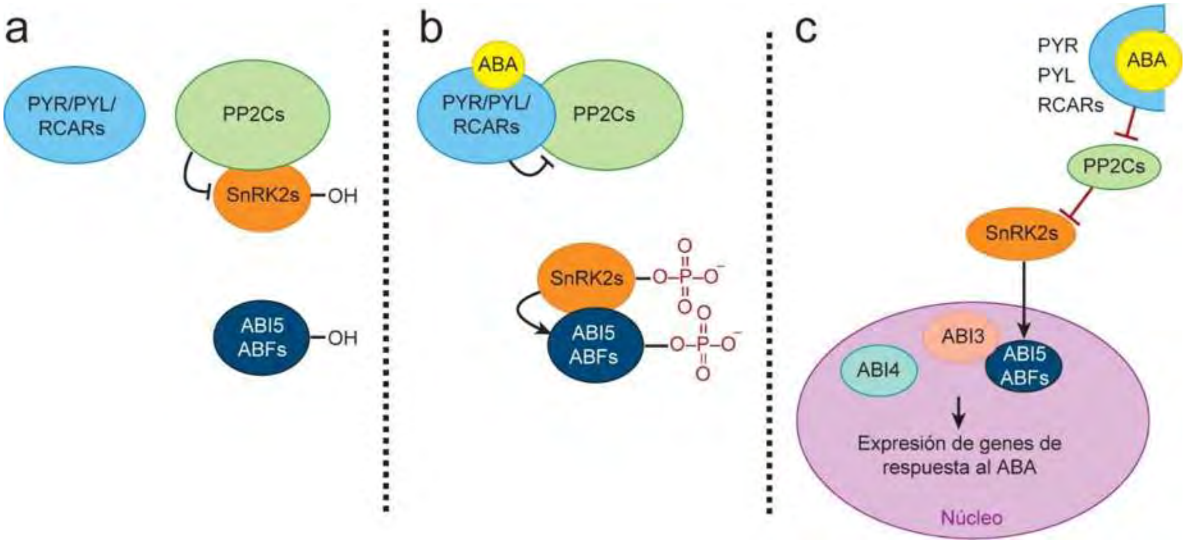


Figura 7. Transducción de señal del ácido abscísico. a) En ausencia del ácido abscísico, el receptor (PYR, PYLs o RCARs) se encuentra apagado. Las fosfatasas PP2Cs (como ABI1 y ABI2) quitan un grupo fosfato (desfosforilación) a las cinasas SnRK2s, lo cual mantiene inactivos a los factores de transcripción (ABI5 y ABFs). b) En presencia del ácido abscísico el receptor esta encendido, por lo que las PP2Cs se mantienen secuestradas por el receptor en una unión favorecida por el ácido abscísico, mientras que las SnRk2s fosforiladas son capaces de agregar un grupo fosfato a los factores de transcripción (ABA). c) Modelo de la ruta de señalización. El receptor unido al ABA bloquea a las PP2Cs impidiendo la inactivación de las SnRk2s, por lo que los factores de transcripción (FTs) pueden formar heterodímeros con ABI3 (otro tipo de FT) para promover la expresión de genes en un mecanismo dependiente ABI4 (un FT adicional implicado en la respuesta al ABA y azúcares). Adaptado de Cutler y col. (2010).

La ruta de señalización del ABA comienza cuando dicha hormona promueve la interacción de los receptores PYR/PYL/RCARs y las fosfatasas PP2Cs, resultando en la inactivación de estas últimas (Fig. 7a-b) (Ma *et al.,* 2009; Park *et al.,* 2009). Las PP2Cs inactivan a las cinasas SnRK2s por desfosforilación directa, por lo que la inactivación de las PP2Cs mediante los receptores permite a las cinasas fosforilar proteínas corriente abajo (Fujii *et al.*, 2007; Umezawa *et al.*, 2009; Vlad *et* *al.*, 2009; Umezawa *et al.*, 2010). En el núcleo, los blancos clave son los factores de transcripción de cierre de leucina básico (bZIP) ABSCISIC ACID INSENSITIVE 5 (ABI5) y los ABSCISIC ACID TRANSCRIPTION FACTORS (ABFs) relacionados a ABI5 (Fig. 7b). Los ABFs (incluido ABI5) fosforilados se unen como dímeros (con el factor de transcripción ABI3) a los elementos *cis* de genes de respuesta al ABA, para controlar la transcripción de estos últimos (Nakamura *et al.*, 2001; Furihata *et al.*, 2006; Raghavendra *et al.,* 2010). Adicionalmente a estos elementos de señalización, un factor de transcripción de tipo AP2 llamado ABSCISIC ACID INSENSITIVE 4 (ABI4) se une al promotor de genes de respuesta al ABA y activa su expresión (Fig. 7c) (Finkelstein *et al.,* 1998; Bossi *et al.,* 2009; Cutler *et al.,* 2010; Reeves *et al.,* 2011).

2.4. Nuevos reguladores del crecimiento vegetal

Desde su descubrimiento y hasta hace pocos años, la regulación de los procesos morfogenéticos vegetales había sido completamente atribuida a las moléculas de señalización clásicas, las fitohormonas. Del mismo modo, se había considerado que los metabolitos secundarios de las plantas no tenían un papel bioquímico vital en el proceso de construcción y mantenimiento celular, por lo que la investigación de estos compuestos se centró en sus propiedades medicinales y recreativas. Sin embargo, investigaciones recientes sugieren que los metabolitos secundarios tienen una función fundamental en la interacción de las plantas con el medio ambiente (López-Bucio *et al.*, 2006). Adicionalmente, diversos estudios muestran que las plantas al reconocer compuestos derivados de microorganismos ajustan su crecimiento, por lo que dicha señalización durante la interacción planta-microorganismo sugiere un papel regulador del crecimiento de las moléculas microbianas posiblemente afectando las rutas de señalización hormonal vegetal (Ortiz Castro *et al.,* 2009). A continuación se mencionan con mayor detalle tres tipos de compuestos que han comenzado a ganar importancia en el estudio de la biología del desarrollo vegetal; el óxido nítrico, los folatos (compuestos naturalmente producidos por las plantas) y las *N*-acil-homoserina lactonas (moléculas de origen bacteriano).

2.4.1. El óxido nítrico

El óxido nítrico (NO) es una molécula gaseosa bioactiva involucrada en diversos procesos morfogenéticos de las plantas, tales como la diferenciación del xilema, la muerte celular programada, el desarrollo de raíces adventicias y laterales, la floración, el cierre de estomas, el gravitropismo, la expansión de la hoja, la fotomorfogénesis y el envejecimiento (Beligni y Lamattina, 2001; Desikan *et al.*, 2002; Neill *et al.*, 2003; Correa-Aragunde *et al.*, 2006; Grün *et al.*, 2006; Méndez-Bravo *et al.*, 2010). Se han propuesto tres mecanismos de síntesis del NO en las plantas, incluyendo la oxidación de arginina a citrulina por la sintasa de óxido nítrico (NOS), un sistema de generación no enzimático y la reducción de nitrato vía nitrito por la nitrato reductasa (NR) (Bethke *et al.*, 2004).

En el primer caso, ningún gen o proteína con similitud funcional a la NOS de mamíferos ha sido encontrada en plantas, aunque existe evidencia farmacológica y ensayos con anticuerpos que sugieren la existencia de dicha enzima (Fig. 8) (Beligni y Lamattina, 2001). El intento más notable por aislar la NOS de plantas ocurrió en *Arabidopsis* cuando se identificó una enzima parecida a NOS con una secuencia similar a la de una proteína que había sido implicada en la síntesis de NO en el caracol *Helix pomatia*, la cual fue nombrada AtNOS1 (Guo *et al.*, 2003). Sin embargo, AtNOS1 no mostró actividad NOS siendo incapaz de unirse y oxidar la arginina a NO (Zemojtel *et al.*, 2006; Moreau *et al.*, 2008). Por lo tanto, AtNOS1 fue renombrada como AtNOA1 (un acrónimo del nombre en inglés *Arabidopsis thaliana NO-associated protein 1*) (Crawford *et al.*, 2006) y posteriormente se le vinculó con la familia de las GTPasas (cGTPase) (Moreau *et al.*, 2008; Sudhamsu *et al.*, 2008). De esta manera, la mutante *Atnoa1* de *Arabidopsis* ha sido ampliamente utilizada en la investigación del NO aún cuando no tiene relación funcional con la NOS hipotetizada de plantas, debido a que muestra niveles reducidos de NO durante el crecimiento vegetal, fertilidad, señalización hormonal, tolerancia a la sal y la respuesta a patógenos (Guo *et al.*, 2003; Zeidler *et al.*, 2004; Zhao *et al.*, 2007). En cuanto a la producción no enzimática de NO, el nitrito (NO_2^-) es protonado para formar ácido nitroso (HNO_2) en una reacción reversible que es favorecida en pH bajo, entonces, dos moléculas de HNO_2 interaccionan para originar NO, dióxido de nitrógeno (NO_2) y agua, donde el NO_2 también puede ser transformado a NO y una molécula de oxígeno (Fig. 8) (Yamasaki, 2000).

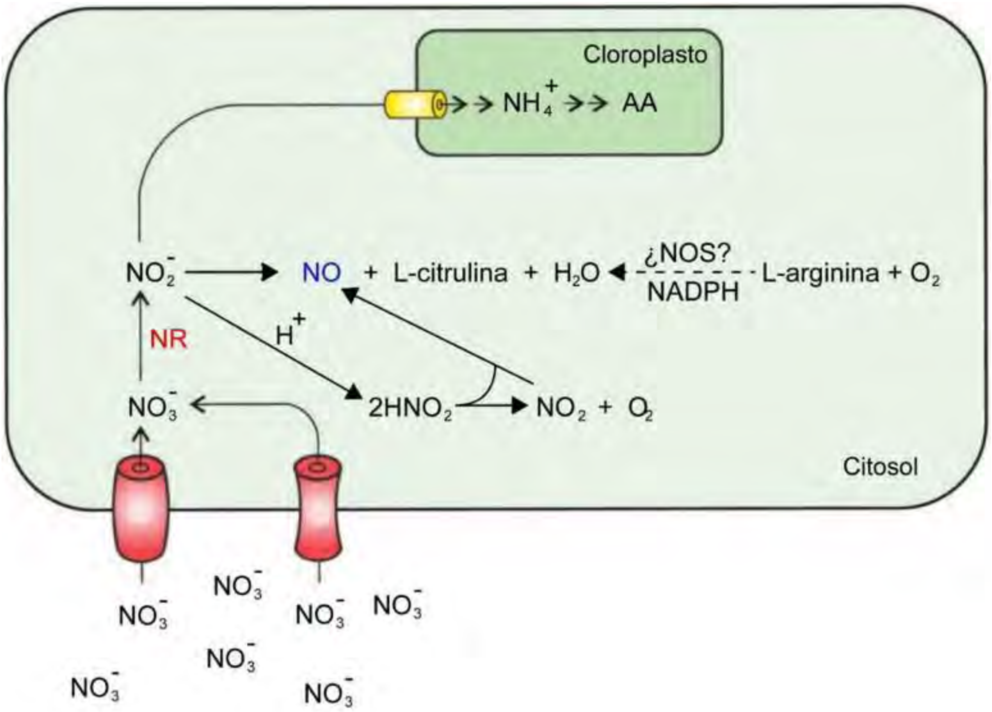


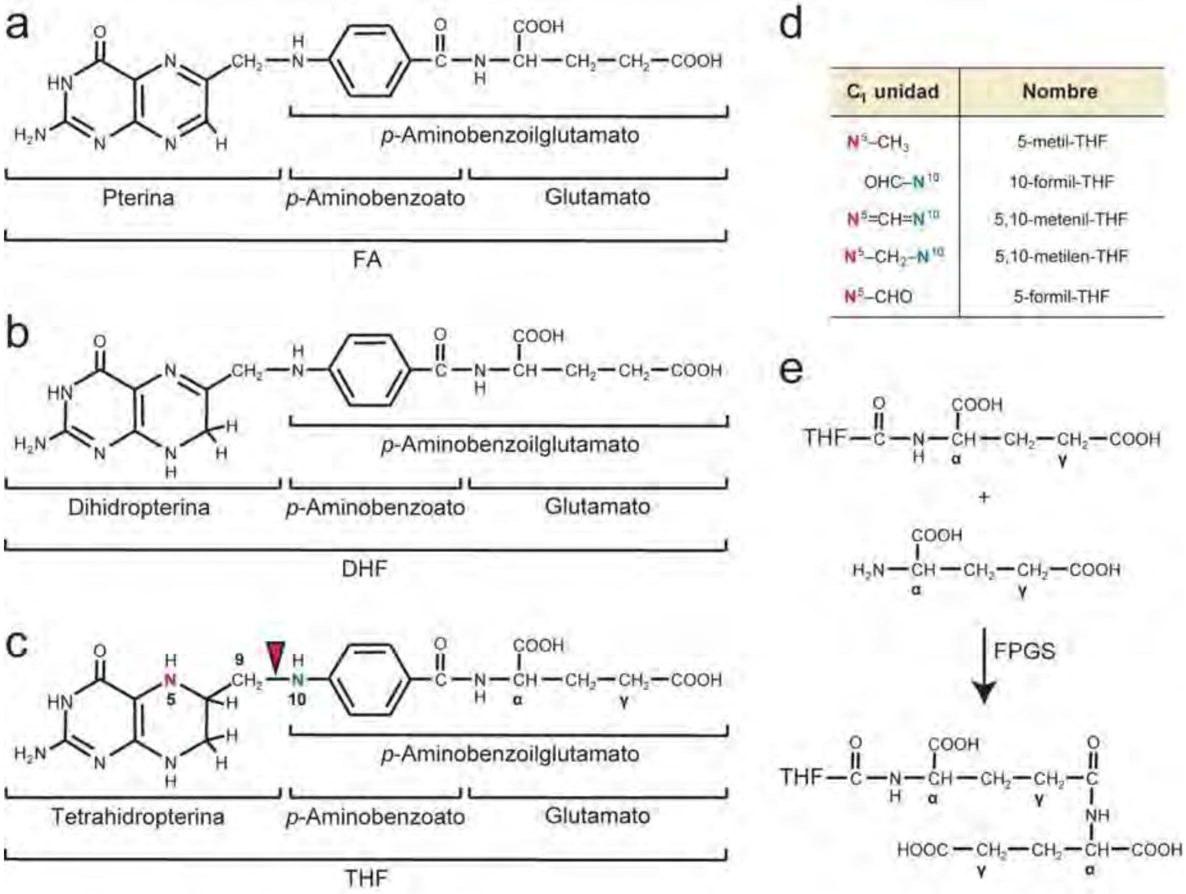
Figura 8. Modelo para la formación de óxido nítrico en la célula vegetal. El nitrato (NO_3^-) es captado por la célula mediante transportadores y transformado a nitrito (NO_2^-) por la nitrato reductasa (NR); entonces, el nitrito es reducido a óxido nítrico (NO) para permitir la transducción de señales en distintas rutas de señalización hormonal, o bien puede ser reducido a amonio (NH_4^+) para la formación de aminoácidos (AA). También, la producción de NO puede ocurrir por un mecanismo no enzimático dependiente protones (H^+) vía el ácido nitroso (HNO_2) y el dióxido de nitrógeno (NO_2). Adicionalmente, se propone un mecanismo oxidativo para la formación del NO, el cual posiblemente depende de una óxido nítrico sintasa (NOS) putativa y la coenzima NADPH. Las líneas continuas indican procesos demostrados y la punteada indica un proceso propuesto. Adaptado de Frungillo y col. (2014).

Agentes reductores como el ácido ascórbico y algunos compuestos fenólicos pueden acelerar la formación de NO, lo cual se demostró que ocurre en el apoplasto (Bethke *et al.*, 2004). Por otra parte, las enzimas NR están asociadas con la asimilación de nitrógeno, pero también pueden generar NO a partir de nitrito (Fig. 8) (Dean y Harper 1986; Rockel *et al.*, 2002). En relación a lo anterior, los genes *NIA1* y *NIA2* de *Arabidopsis thaliana* codifican para dos NRs capaces de reducir el nitrato permitiendo la asimilación de nitrógeno (N) y con esto un crecimiento adecuado de la planta (Wilkinson y Crawford, 1993). Las mutaciones con pérdida de la función en ambos genes ocasionan un mal funcionamiento celular, relacionada con una menor producción de la molécula gaseosa (Desikan *et al.*, 2002). Méndez-Bravo y col. (2010) establecieron la participación del NO durante la formación de raíces laterales mediada por la *N*-isobutil decanamida, un compuesto producido naturalmente por las plantas, implicado en la señalización del ácido jasmónico (JA) (Morquecho-Contreras *et al.*, 2010), por lo que es necesario establecer si existe una relación entre el JA y el NO durante la formación del sistema radical.

2.4.2. Los folatos

Los folatos (Vitamina B9) son intermediarios cruciales para un grupo de reacciones que involucran la transferencia de unidades de un carbono (C1), que están directamente involucrados en la síntesis de ácidos nucleicos, metionina, pantotenato, glicina y serina e indirectamente, a través de la S-adenosil metionina, en todas las reacciones de metilación (Rébeillé *et al.*, 2006). Las moléculas de folatos están formadas por tres partes: un anillo de pteridina, un *para* aminobenzoato (*p*-ABA) y una cola de uno o más *L*-glutamatos enlazados mediante sus grupos carboxilo (Fig. 9a-c). La poza de folatos en la célula contiene una mezcla de moléculas relacionadas, las cuales difieren en su estado de oxidación, en las C1 unidas y en la longitud de la cola de glutamato (Fig. 9d-e).

El tetrahidrofolato (THF) es el folato más reducido y la forma enzimáticamente activa, mientras que el ácido fólico (FA), una forma que no ocurre naturalmente, está completamente oxidada y por lo tanto es más estable (Blancquaert *et al.*, 2010). Debido a que el FA puede ser reducido, vía el dihidrofolato a THF, el primero puede ser empleado para satisfacer la necesidad de folatos para el metabolismo (Hanson y Gregory, 2011).



C_1 unidad	Nombre
N^5–CH_3	5-metil-THF
OHC–N^{10}	10-formil-THF
N^5=CH=N^{10}	5,10-metenil-THF
N^5–CH_2–N^{10}	5,10-metilen-THF
N^5–CHO	5-formil-THF

Figura 9. Estructura química y de los folatos. a) El ácido fólico (FA). La molécula químicamente más estable. b) El dihidrofolato (DHF), un intermediario metabólico. c) El tetrahidrofolato (THF), la molécula fisiológicamente activa. Los folatos (FA, DHF y THF) están constituidos por un anillo de pterina, para-aminobenzoato y glutamato, donde el estado de oxidación de la pterina determina el tipo de folato. d) Sustituyente del THF. Las unidades de un carbono (C1) transferidas en las reacciones enzimáticas. e) Formación de la cola de poliglutamato. La enzima folipoliglutamato sintetasa (FPGS) uniendo un residuo de glutamato (Glu) a una molécula de THF-Glu para formar THF-Glu-Glu. La punta de flecha roja en el panel “c” indica el enlace C9-C10 oxidativamente lábil del THF. Adaptado de Hanson y Gregory (2011).

La síntesis de folatos tiene un papel esencial para el funcionamiento apropiado del meristemo de la raíz. La mutación del gen *AtDFB* (*At5G05980*), que codifica la folipoliglutamato sintetasa (FPGS), la cual cataliza la unión de residuos de glutamato a la molécula de folato (Fig. 9e), interrumpe el crecimiento de la raíz primaria y este defecto fue atribuido a alteraciones en la identidad del centro quiescente (QC) (Srivastava *et al.*, 2011). La mutación en un segundo alelo (*mko2*) de *AtDFB* provocó que el meristemo de la raíz primaria se consumiera, lo cual correlacionó con cambios en el metabolismo de folatos (Reyes-Hernández *et al.*, 2014). Lo antes mencionado sugiere que los folatos juegan un papel crítico en la proliferación celular de la raíz.

2.4.3. Las *N*-acil-homoserina lactonas

Las bacterias se comunican usando señales químicas. Como ocurre en los eucariotas, la información suministrada por esas moléculas es crítica para sincronizar las actividades de grandes grupos de células mediante un proceso llamado quorum sensing (QS), que altera el comportamiento a escala poblacional. Las bacterias con sistemas de QS producen y liberan moléculas llamadas autoinductores cuya concentración externa aumenta en función del incremento en la densidad poblacional celular. Las bacterias detectan la acumulación de una concentración mínima estimulatoria de esos autoinductores y alteran su expresión genética, y por lo tanto su comportamiento (Fig. 10) (Waters y Bassler, 2005).

En particular, las bacterias Gram negativas utilizan a las *N*-acil-homoserina lactonas (AHLs) como moléculas de QS. Las AHLs contienen un anillo homoserina lactona conservado y una cadena lateral acilo unida por un enlace amida (N). Los grupos acilo de las AHLs van desde los 4 a 16 carbonos en longitud, pueden ser saturados o insaturados y con o sin un sustituyente en el carbono 3 (Waters y Bassler, 2005; Camilli y Bassler, 2006). Esas señales químicas son producidas por enzimas sintasas de AHLs, y son detectadas por una variedad amplia de factores de transcripción llamados “proteínas R” tales como LuxR o LasR y por una familia pequeña de sensores cinasas relacionados a LuxN (Duerkop *et al.*, 2007). La unión de la AHL a la mayoría de las proteínas R caracterizadas inicia la interacción de estas últimas con los promotores de genes para inducir o reprimir la transcripción (Pearson *et al.*, 1994; Parsek *et al.*, 1999; Churchill y Chen, 2011). La actividad específica de las diversas AHLs puede ser determinada por el anillo lactona, el grupo amida y la longitud de la cadena de ácido graso (Churchill y Chen, 2011; Lintz *et al.,* 2011).

La biosíntesis de las AHLs ha sido reportada en más de 90 especies bacterianas. Por ejemplo, *Pantoea stewartii* y *Agrobacterium tumefaciens* producen un solo tipo de AHL, 3-oxo-C6-HL y 3-oxo-C8-HL, respectivamente. En contraste, los genes *Bmal1* y *Bmal3* de *Burkholderia mallei* codifican para dos sintasas de AHLs, las cuales producen C8-HL y 3-hidroxi-C8-HL. En *P. aeruginosa*, han sido descritos dos sistemas de QS preponderantes basados en AHLs, estos son *las* y *rhl*, los cuales dependen del reconocimiento de la *N*-(3-oxododecanoil)-*L*-homoserina lactona (3-oxo-C12-AHL) y la *N*-butanoil-*L*-homoserina lactona (C4-HL) por los factores de transcripción LasR y RhlR respectivamente, los cuales activan cientos de genes (Churchill y Chen 2011).

Las AHLs se difunden libremente a través de las membranas celulares y se acumulan en el medio circundante a medida que aumenta la población bacteriana (Lee y Zhang, 2015). En la rizósfera, cantidades significativas de AHLs se acumulan y actúan como señales vegetales bioactivas. Por ejemplo, las AHLs de cadena mediana y larga reprimen el crecimiento de la raíz primaria de *Arabidopsis*, mientras que incrementan la formación de pelos radicales y raíces laterales de una forma dependiente de la concentración, dando lugar a sistemas radiculares más ramificados y con una mayor capacidad exploratoria (Fig. 10) (Ortiz Castro *et al.*, 2008; 2011).

Existen dos tipos de compuestos considerados metabolitos secundarios, que comparten similitud estructural con las AHLs: las alcamidas y las *N*-acil-etanolaminas (NAEs) (Fig. 10) (Blancaflor *et al.*, 2003; Ramírez-Chávez *et al.*, 2004). Una mutante recesiva de *Arabidopsis thaliana* llamada *decanamide resistant root 1* (*drr1*) fue identificada por Morquecho-Contreras y col. (2010), debido a su crecimiento continuo en medios suplementados con la *N*-isobutil decanamida o la *N*-decanoil-*L*-homoserina lactona. La caracterización de plántulas silvestres (WT) y mutantes *drr1* a lo largo del ciclo de vida completo, sugirió que la señalización del QS influye en los programas del desarrollo vegetal, en las transiciones de fase en la planta y en el envejecimiento (Morquecho-Contreras *et al.*, 2010).

Por otra parte, un análisis de líneas sobreexpresoras y mutantes del gen que codifica la hidrolasa de amidas de ácido graso de *Arabidopsis* (AtFAAH) indicó que las plantas tienen la maquinaria enzimática para metabolizar las AHLs (Ortiz-Castro *et al.*, 2008). Posteriormente, ensayos *in vitro* demostraron que AtFAAH degrada AHLs de diferente longitud de cadena lateral (Palmer *et al.*, 2014). La AtFAAH controla la señalización de ABA y de las NAEs durante la germinación de las semillas de *Arabidopsis* (Teaster *et al.*, 2007), por lo que resulta plausible que algunos componentes de la señalización del ABA puedan estar compartidos durante las respuestas de la semilla y la raíz a las señales del QS bacteriano.

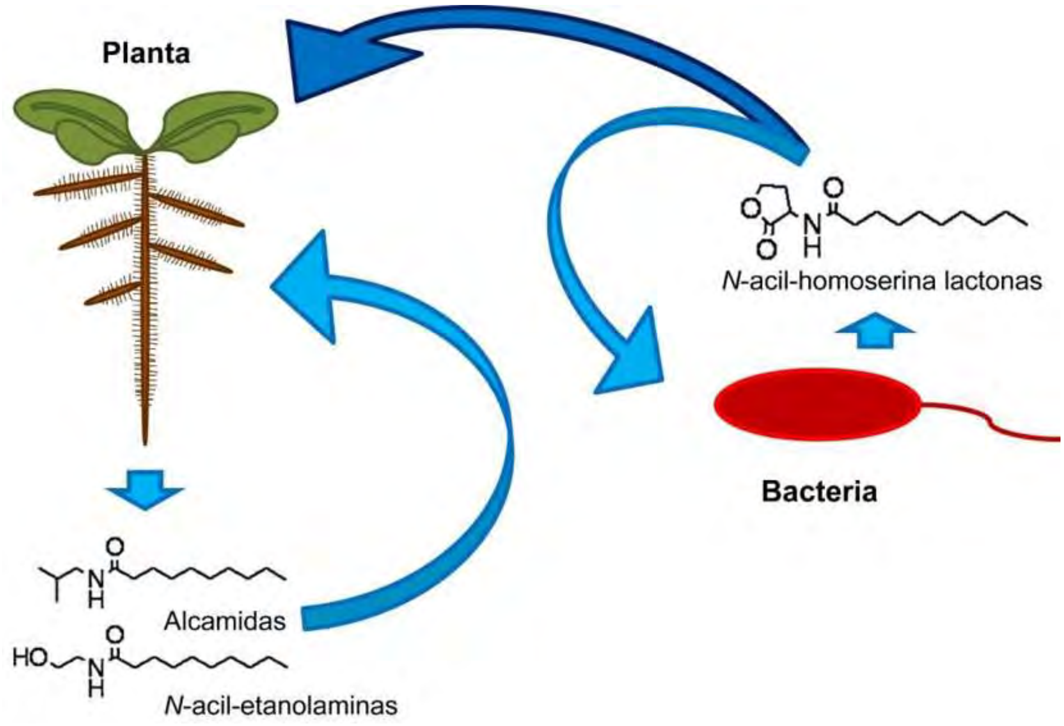


Figura 10. Interacción planta-bacteria dependiente de amidas *N*-sustituidas. Las plantas producen dos clases de compuestos que en principio eran considerados metabolitos secundarios y ahora se sabe que regulan su crecimiento y desarrollo, estos son las alcamidas y las *N*-acil-etanolamidas (NAEs). Por su parte, las bacterias producen y perciben señales químicas para coordinar su comportamiento poblacional, donde las *N*-acil-homoserina lactonas (AHLs) son específicas para bacterias Gram negativas. Investigaciones recientes sugieren que las AHLs alteran la fisiología vegetal, proponiendo un papel regulador en plantas para estos compuestos de origen bacteriano, el cual posiblemente depende de la similitud estructural con las alcamidas y las NAEs.

3. JUSTIFICACIÓN

El óxido nítrico es un mediador de eventos del desarrollo donde el ácido jasmónico y el etileno interactúan. En tanto que los folatos han demostrado ser indispensables para el crecimiento adecuado de la raíz, siendo este último modulado por la ruta de señalización de auxinas (AUXs). Adicionalmente, las *N*-acil-homoserina lactonas (AHLs) son compuestos producidos por bacterias Gram negativas con similitud estructural y funcional a las *N*-acil-etanolaminas de plantas implicadas en los procesos morfogenéticos y de germinación regulados por el ácido abscísico. Por lo antes mencionado, es posible que el óxido nítrico, el ácido fólico y la *N*-decanoil homoserina lactona, utilicen mecanismos dependientes de las rutas de señalización de las fitohormonas para llevar a cabo sus efectos en las plantas.

4. HIPÓTESIS

Los compuestos bioactivos óxido nítrico, ácido fólico y *N*-decanoil-L-homoserina lactona interactúan con las vías de señalización hormonal vegetal para reconfigurar el sistema radical de *Arabidopsis thaliana*.

5. OBJETIVOS

5.1. Objetivo general

Determinar el mecanismo de acción del óxido nítrico, el ácido fólico y la *N*-decanoil-L-homoserina lactona sobre el desarrollo post-embrionario de la raíz de *Arabidopsis thaliana*.

5.2. Objetivos particulares

1. Evaluar el papel del óxido nítrico en las vías de señalización del ácido jasmónico y el etileno.
2. Caracterizar el efecto de ácido fólico sobre desarrollo de la raíz y su relación con la ruta de señalización de auxinas.
3. Establecer la implicación de la ruta de señalización del ácido abscísico en la respuesta de la raíz a la *N*-decanoil-L-homoserina lactona.

6. RESULTADOS

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

6.2. Capítulo 1

Jasmonic acid-ethylene crosstalk via *ETHYLENE INSENSITIVE 2* reprograms *Arabidopsis* root system architecture through nitric oxide accumulation (Publicado en la revista *Journal of Plant Growth Regulation* con factor de impacto de 2.073).

6.3. Capítulo 2

Folic acid orchestrates root development linking cell elongation with auxin response and acts independently of the TARGET OF RAPAMYCIN signaling in *Arabidopsis thaliana* (Aceptado en la revista *Plant Science* con factor de impacto de 3.437).

6.3. Capítulo 3

Bacterial quorum-sensing signaling influences abscisic acid responsiveness in *Arabidopsis thaliana* (Sometido para su publicación en la revista *Journal of Plant Growth Regulation*).

J Plant Growth Regul
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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.

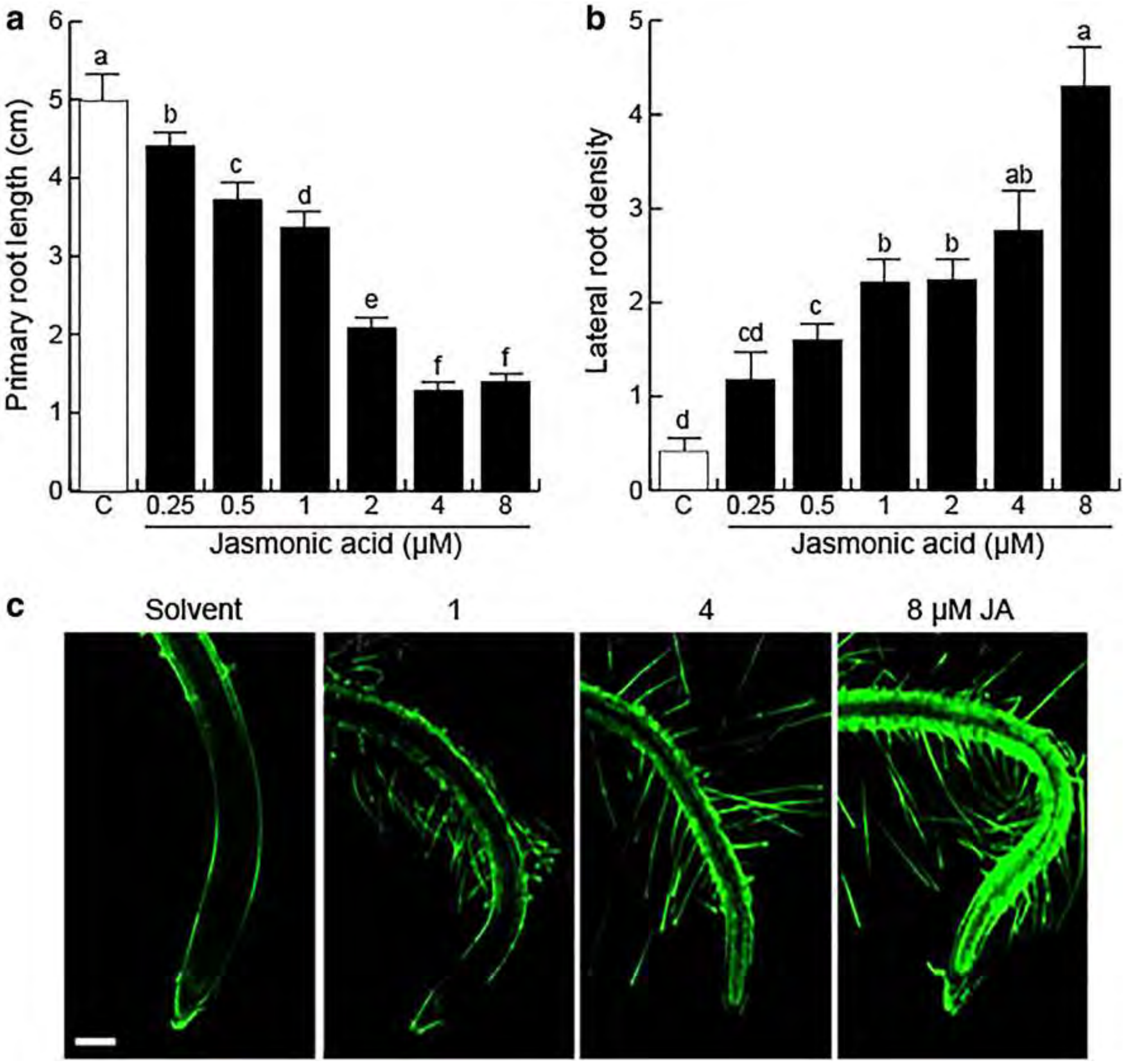

Fig. 1 Effects of JA on root architecture and nitric oxide accumulation in *Arabidopsis*. Primary root length (**a**) and lateral root density (**b**) were analyzed from 10-day-old WT (Col-0) seedlings germinated and grown on the indicated concentrations of JA. **c** NO detection on primary root tips 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

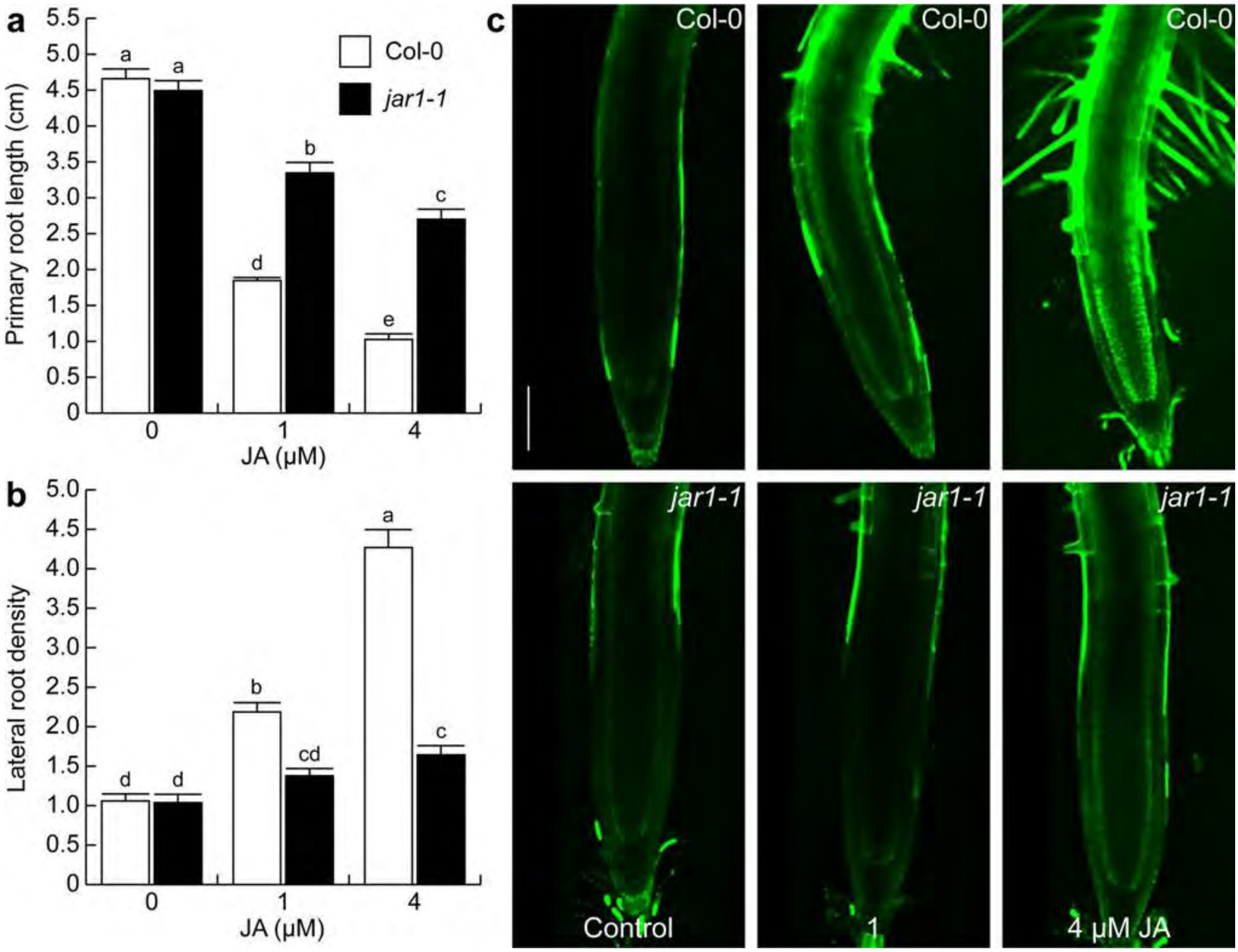

Fig. 2 Root response to jasmonic acid and nitric oxide accumulation in wild-type and *jar1-1 Arabidopsis* seedlings. WT and *jar1-1* seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, or with 1 and 4 μM JA for 10 days. **a** Primary root length and **b** lateral 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

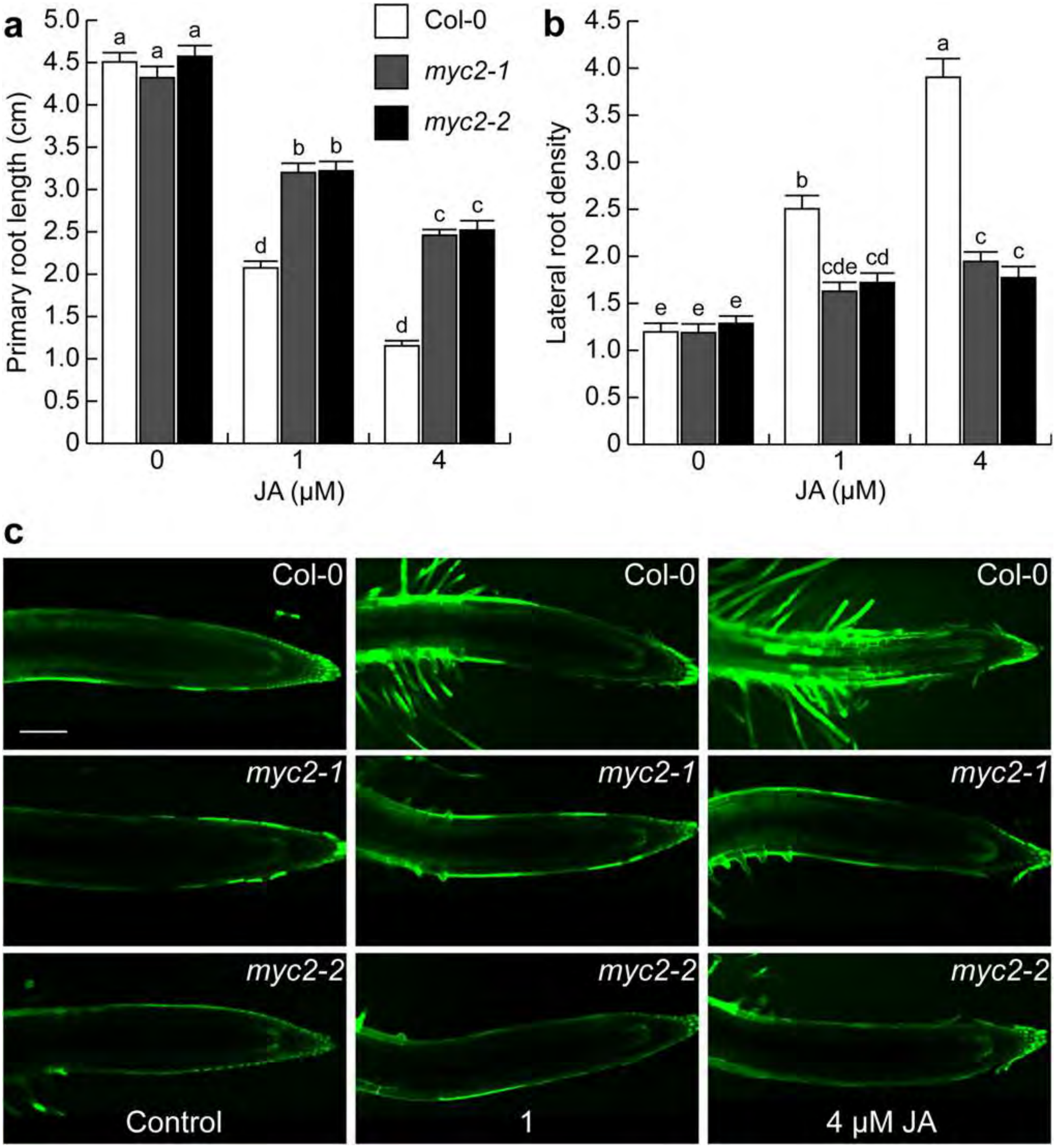

Fig. 3 Root response to jasmonic acid and nitric oxide accumulation in wild-type, *myc2-1*, and *myc2-2 Arabidopsis* seedlings. WT, *myc2-1*, and *myc2-2* seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, or with 1 and 4 µM JA for 10 days. **a** Primary root 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

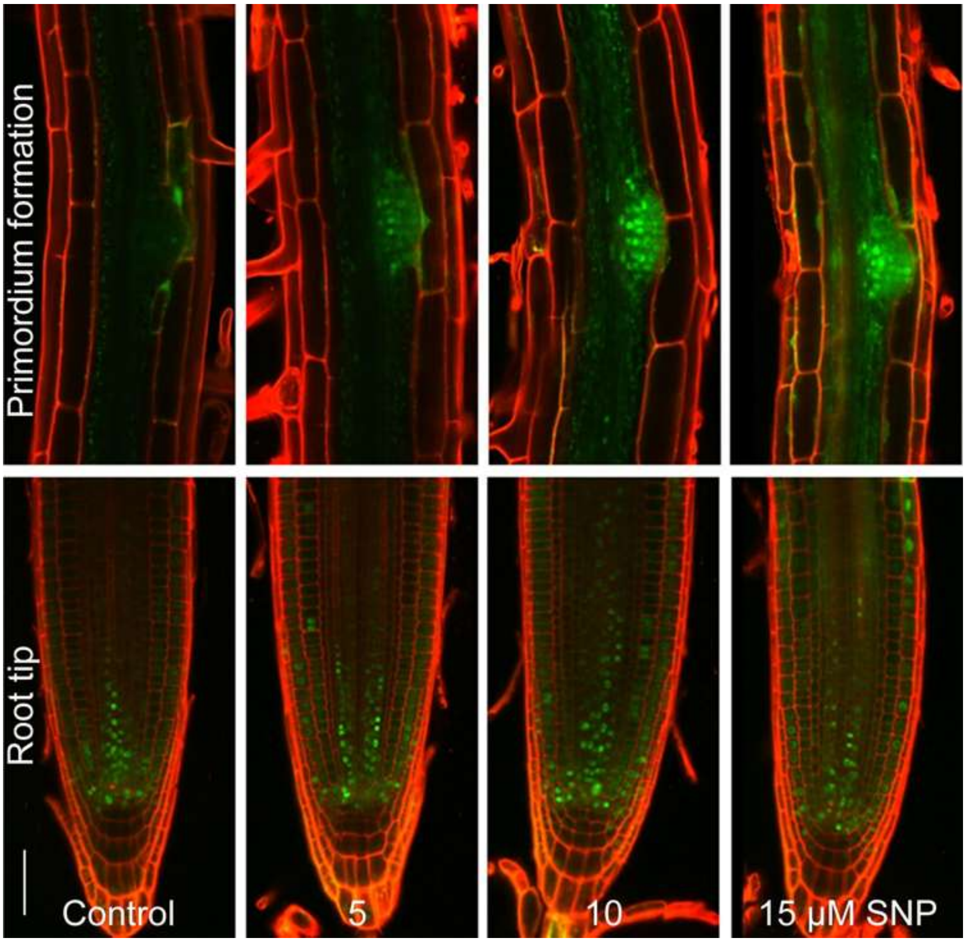

Fig. 4 Effect of nitric oxide donor sodium nitroprusside on *JAZ10* expression in *Arabidopsis* roots. Transgenic *Arabidopsis* seedlings harboring the *JAZ10-GFP* gene construct were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 5, 10, and 15 µM SNP for 10 days; at 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.

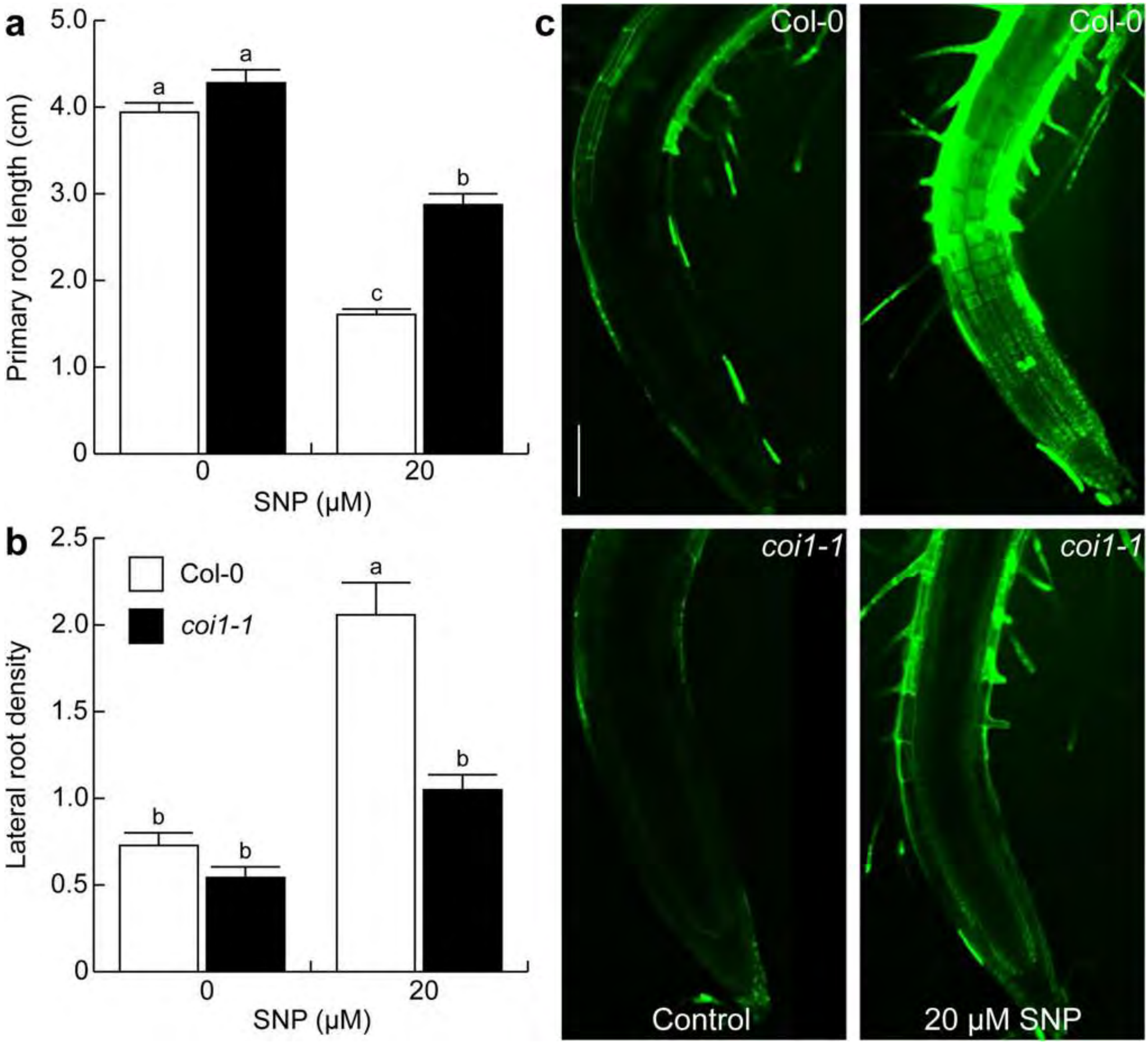

Fig. 5 Effect of nitric oxide donor sodium nitroprusside on root development and nitric oxide accumulation in wild-type and *coi1-1* seedlings. WT seeds were germinated and seedlings were grown on agar-solidified 0.2× MS media for 2 days, and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI1* 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

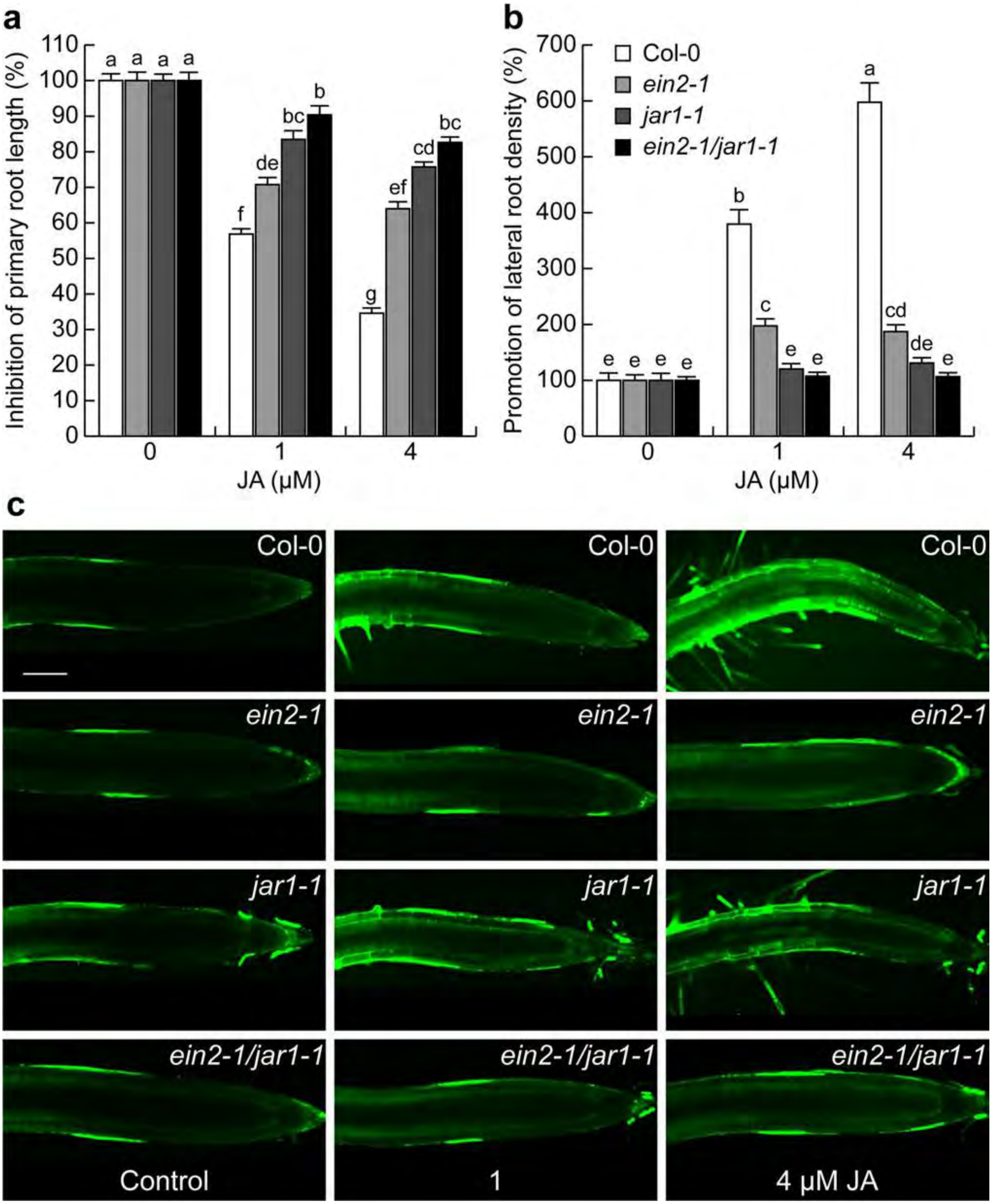

Fig. 6 Root response to jasmonic acid and nitric oxide accumulation in wild-type, *ein2-1, jar1-1*, and *ein2-1/jar1-1 Arabidopsis* seedlings. WT, single and double mutant seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 1 or 4 μM JA for 7 days. **a** Inhibition 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

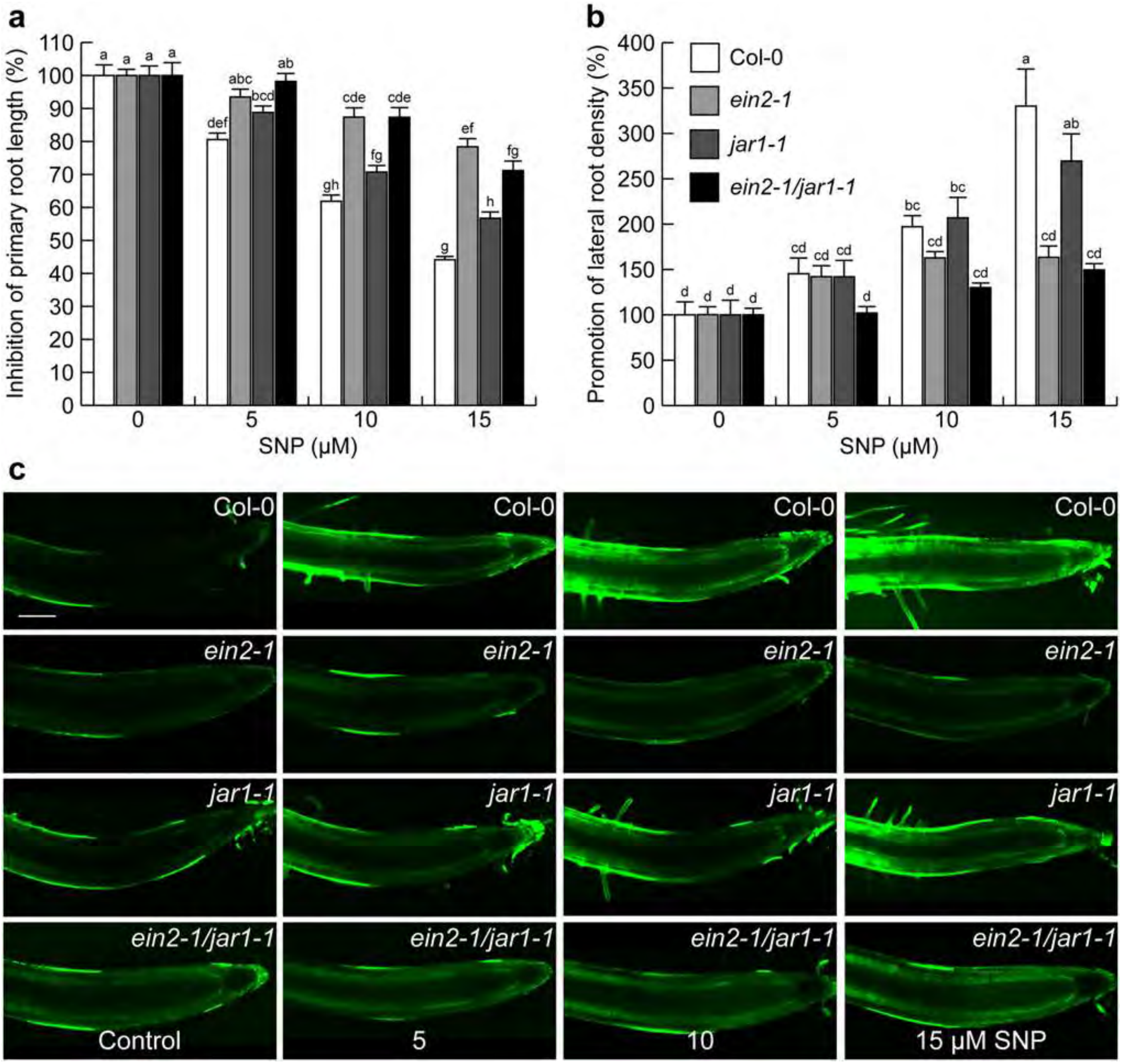

Fig. 7 Effect of nitric oxide donor sodium nitroprusside on root development and nitric oxide accumulation in wild-type, *ein2-1, jar1-1,* and *ein2-1/jar1-1* seedlings. WT, single and double mutant seedlings were germinated and grown on agar-solidified 0.2× MS media supplied with the solvent, 5, 10, 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

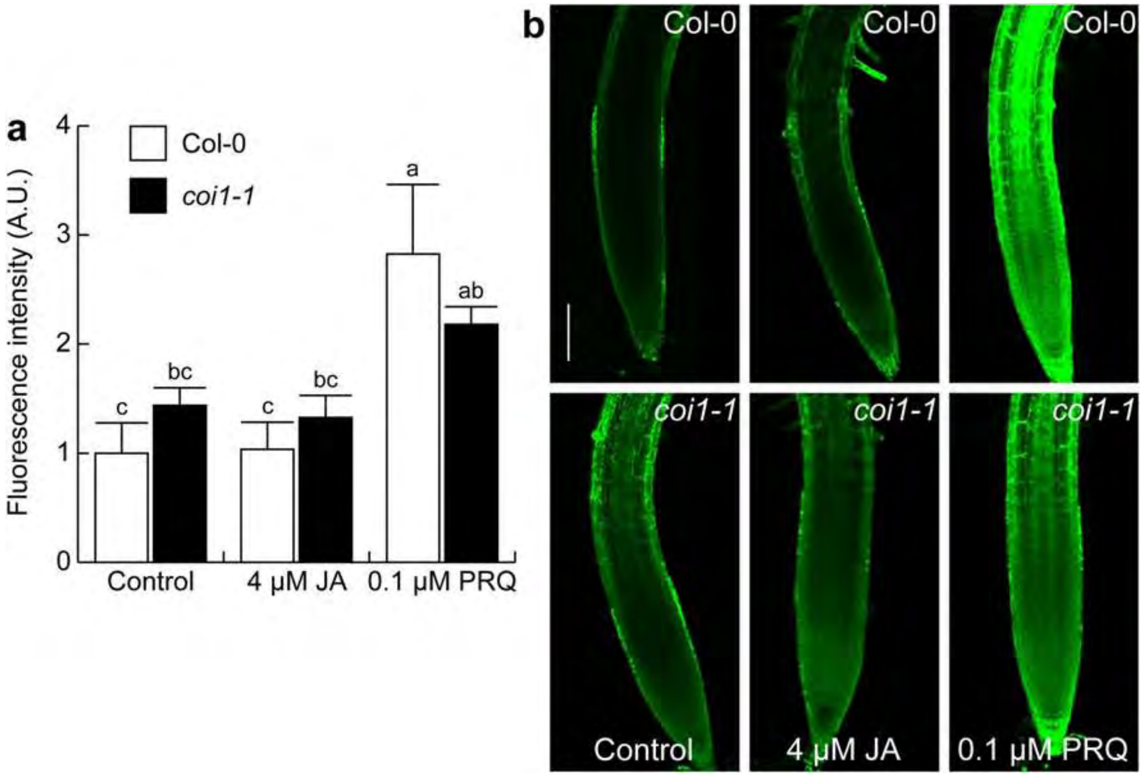

Fig. 8 Reactive oxygen species (ROS) accumulation in the primary root of wild-type and *coi1-1* mutants in response to jasmonic acid (JA), sodium nitroprusside (SNP), and paraquat (PRQ). Wild-type and *coi1-1* seedlings were germinated and grown on agar-solidified 0.2× MS media for two days, and homozygous *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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Folic acid orchestrates root development linking cell elongation with auxin response and acts independently of the *TARGET OF RAPAMYCIN* signaling in *Arabidopsis thaliana.*

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Highlights

- Pharmacological, genetic and confocal imaging data imply an important role of folic acid for the configuration of the *Arabidopsis* root system architecture.
- Folic acid induces cell proliferation but represses cell elongation in primary roots.
- Folic acid inhibits primary root growth and activates lateral root development through an auxin-mediated mechanism.
- Folic acid improves root branching in a TARGET OF RAPAMYCIN independent manner.

Abstract

Folic acid is a precursor of tetrahydrofolate (vitamin B9), which is an essential cofactor in most organisms, acting as a carrier for one-carbon units in enzymatic reactions. In this work, we employed pharmacological, genetic and confocal imaging strategies to unravel the signaling mechanism by which folic acid modulates root growth and development. Folic acid supplementation inhibits primary root elongation and induces lateral root formation in a concentration-dependent manner. An analysis of the expression of cell cycle genes *pCycD6;1:GFP* and *CycB1:uidA*, and cell expansion *Exp7:uidA* showed that folic acid promotes cell division but prevented cell elongation, and this correlated with altered expression of auxin-responsive *DR5:GFP* gene, and *PIN1::PIN1::GFP*, *PIN3::PIN3::GFP*, and *PIN7::PIN7::GFP* auxin transporters at the columella and vasculature of primary roots, whereas mutants defective in auxin signaling (*tir1/afb1/afb2* [receptors], *slr1* [repressor] and *arf7/arf19* [transcription factors]) were less sensitive to folic acid induced primary root shortening and lateral root proliferation. Comparison of growth of WT and *TARGET OF RAPAMYCIN* (*TOR)* antisense lines indicates that folic acid acts by an alternative mechanism to this central regulator. Thus, folic acid modulation of root architecture involves auxin and acts independently of the TOR kinase to influence basic cellular programs.

Keywords: Folic acid; vitamin B9; auxin, root growth, lateral roots; TOR kinase; transcription factors.

1. Introduction

The root is an essential plant organ that provides structural support to aerial tissues, and allows the uptake of water and nutrients. In *Arabidopsis thaliana*, which forms a taproot system, the primary root develops during embryogenesis and manifests an indeterminate developmental program during the life cycle. At different stages and in response to biotic or abiotic cues, lateral and adventitious roots are formed and extend the exploratory capacity of the primary root [1-3].

The root architecture is defined by the combination of morphogenetic events involving cell division, elongation and differentiation, which are largely responsive to the hormonal and energetic status [4]. The PINFORMED (PIN) family of efflux carriers controls auxin distribution within primary root tips and an auxin maximum established at the quiescent center and root meristem initials activates the PLETHORA (PLT), SHORT ROOT (SHR) and SCARECROW (SCR) transcription factors, which are essential for sustained growth [5-8]. On the other hand, a sugar-dependent activation of the TARGET OF RAPAMYCIN (TOR) kinase is required for meristem functioning (9-10). This pathway provides energy and metabolites through concerted communication between the shoot and proliferating root cells, but requires high local auxin concentrations to promote cell division (11).

Recently, folate (vitamin B9) synthesis was found to play an essential role for the proper functioning of root meristems. Mutation of the AtDFB (At5G05980) gene encoding folylpolyglutamate synthetase (FPGS), which catalyzes the attachment of glutamate residues to the folate molecule, disrupts primary root growth and this defect was attributed to alterations in quiescent center (QC) identity [12]. Indeed, mutation in a second allele (*mko2*) of AtDFB caused the root meristem to be consumed, which was correlated to changes in folate metabolism [13]. These results suggest that folates play a critical role in root cell proliferation.

Folates are crucial intermediates for a set of reactions that involve the transfer of single-carbon units (C1 metabolism). They are directly involved in the synthesis of nucleic acids, methionine, pantothenate, glycine and serine, and indirectly, through S-adenosyl methionine, in all methylation reactions [14]. Folate molecules consist of the following three moieties: a pteridine ring, a *para* aminobenzoate (*p*-ABA) ring and a tail of one or more *L*-glutamates linked through their γ-carboxyl groups. The folate pool of a cell is a mixture of related molecules, which differ in their oxidation state, in the attached one-carbon (C1) unit and in the length of the glutamate tail. Tetrahydrofolate (THF) is the most reduced folate and an enzymatically active form, whereas folic acid (FA), a form that does not occur naturally, is fully oxidized and hence, more stable [15]. Given that FA can be reduced via dihydrofolate to THF, it can be employed to satisfy the need of folate for metabolism [16].

Although genetic evidence has demonstrated that folate biosynthesis is important for cell division in meristems, the effects of folic acid application on root growth or lateral root formation have not been previously investigated. Moreover, since recent evidence suggests that both auxin and TOR may influence common morphogenetic programs [11], it is basic to clarify their possible interactions in controlling cell division and elongation and their role in mediating root growth responses to bioactive vitamins.

Here, we report that folic acid activates the expression of genes involved in cell cycle regulation in primary root meristems, but represses primary root growth by inhibiting cell elongation. Folic acid promoted maturation of lateral root primordia, which gave rise to the greater production of lateral roots in seedlings treated with this vitamin, and its effects on root architecture reconfiguration required the canonical auxin signaling pathway. Intriguingly, folic acid partly restored the growth and lateral root deficient phenotype in estradiol-inducible *tor-es1* seedlings. These data support the critical role of this vitamin in root organogenesis.

2. Materials and methods

2.1. Biological material and compounds with biological activity

Arabidopsis ecotype Columbia (Col-0), the transgenic lines *CycB1;1:uidA* [17], *pAtEXP7:GUS* [18], *pCYCD6;1:GFP* [19], *DR5:GFP* [20], *PIN1::PIN1::GFP* [21], *PIN3::PIN3::GFP* [22], *PIN7::PIN7::GFP* [6] as well as auxin signaling mutants *tir1 afb2 afb3* [23], *slr1* [24] and *arf7 arf19* [25]; in addition to an estradiol-inducible RNAi line called *tor-es1* [26] were used for all experiments. The compounds with biological activity used in this study are folic acid (AF, No. Cat. F8758) and estradiol (EST, No. Cat. E2758), which were purchased from Sigma-Aldrich.

2.2. Growth conditions

Seeds were surface sterilized with 95% (v/v) ethanol for 4 min and a commercial 10% (v/v) chloride solution (Cloralex®) for 4 min. After five washes with sterile distilled water, seeds were incubated in darkness at 4 °C for 48 h in sterile distilled water. Seeds were sown on plates containing 0.2X MS salts [27] supplemented with sucrose 0.6% (w/v) and phytagar 1% (w/v). pH was adjusted to 7.0 before the agar was added. Estradiol was dissolved in dimethyl sulfoxide (DMSO, purchased from Sigma-Aldrich) and folic acid in a 1:1 mixture of deionized water: DMSO. The compounds in solution were added to the liquid media (about 50 °C) at required concentration, and control media were supplemented with the greater volume of solvent used in the treatments. The plates with germinating seeds were incubated into a plant grown chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 μmol m^{-2} s^{-1}, and temperature of 22 ºC, in a vertical form to avoid root penetration in the medium. MS salts (MS basal salts mixture; catalog no. M5524) was purchased from Sigma. The suggested formulation is 4.3 g L^{-1} salts for a 1x concentration of medium; we used 0.9 g L^{-1}, which we consider and refer to as 0.2X MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology.

2.3. Analysis of plant growth

Ten days after germination, the primary root of the seedlings was measured with a ruler (PRL, primary root length); lateral root number (LRN) present in primary root from the apex to transition zone between root and stem was recorded using a stereomicroscope (Leica MZ6) at a 10X magnification; and the lateral root density (LRD) was calculated dividing LRN between PRL. The lateral root primordia (LRP) were classified and quantified according to their stages of development in seedlings 4 days after germination as reported [28]. The total fresh weight (TFW), shoot (SFW) and root (RFW) of 5 or 10 seedlings grown on the same plate was measured with an analytical balance (Ohaus) and three plates of each condition were evaluated. The differences between the data obtained from the same response (PRL, LRN, LRD, LRP, TFW, SFW and RFW) were statistically analyzed with STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). Univariate and multivariate analysis with a Tukey's post hoc test were performed. Different letters were used to indicate means that differ significantly ($\rho<0.05$).

2.4. Histochemical activity and phase contrast microscopy

Transgenic seedlings of four days after germination expressing *CycB1;1:uidA* and *pAtEXP7:GUS* were incubated in a microtiter plate with 0.1% X-Gluc (5-brome-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, overnight at 37 °C [29]. After removing X-Gluc solution, seedlings were incubated 60 minutes at 62 °C with 0.24 N HCl in 20% methanol (v/v). The solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 minutes at room temperature. Seedlings were dehydrated with ethanol treatments at 40%, 20% and 10% (v/v) for a 20-minutes period each, and fixed in 50% glycerol (v/v) [28]. The processed roots were included in glass slips, covered with coverslips and sealed with commercial nail varnish; subsequently they were observed and photographed in a phase contrast microscope (Nomarski optics, Leica DFC450 C). For each treatment, at least 10 transgenic plants were analyzed.

2.5. Propidium iodide staining and confocal microscopy

Transgenic seedlings of four days after germination expressing *pCYCD6;1:GFP*, and *DR5:GFP* were transferred from the growth medium to a solution of 10 mg/ml propidium iodide for 1 minute. The seedlings were rinsed in water and mounted with 50% glycerol on a slide and covered with a coverslip. 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, CFP or YFP fluorescence (488 nm excitation/505–550 nm emission, 439 nm excitation/476 nm emission and 514 nm excitation/527 nm emission, respectively) using a confocal microscope (Olympus FV1200) after which the two micrographs were merged to produce a final image. For each treatment and line, 10 independent seedlings were analyzed, and representative images were selected for figure construction.

2.6. Micrograph analysis

Micrographs were analyzed in ImageJ software (http://rsbweb.nih.gov./ij/). For each treatment and line, 10 micrographs were recorded, and meristem length, length of elongation zone, reporter-expressing cells and fluorescence intensity were registered. Expression was quantified by determining the pixels of interest in a defined area, then an arbitrary unit value was obtained (AU= pixels μm^2) for each micrograph, and means were obtained from whole data sets. AU for control conditions were given a value of 1, and those for FA treatments were adjusted relative to these.

3. Results

3.1. Folic acid modifies *Arabidopsis* root system architecture

To evaluate the role of folic acid in the root system architecture of *Arabidopsis* seedlings, increasing concentrations of the compound were added to the culture media. Folic acid inhibited primary root growth (Fig. 1a), and roughly doubled the number of lateral roots being formed in the primary root (Fig. 1b). An analysis of the number of lateral root number per centimeter of primary root, also referred as to lateral root density, showed that folic acid increases by five-fold this developmental parameter (Fig. 1c). The registration of total fresh weight in seedlings treated with the compound resulted in discrete changes in biomass accumulation between the control and the treatments applied (Fig. 1d). A more detailed analysis of the fresh weights showed an increase in shoot and a decrease in root biomass of treated seedlings with respect to seedlings treated with the solvent only (Fig. 1e-f). Representative images of seedlings clearly show the root architecture reprogramming in response to folic acid (Fig. 1g-i), implying that some hormonal and/or energetic signaling elements such as the TOR pathway may be involved in this response.

3.2. Folic acid regulates cell division and elongation in primary roots

Previous reports indicated the critical role of folic acid for root meristem maintenance [12, 13]. Thus, its repressing effect on root growth was somewhat unexpected. To understand how folic acid modifies primary root elongation, we determined the expression of the cell division marker constructs *pCYCD6;1:GFP* and *CycB1;1:uidA,* which are expressed during G1/S transition and in G2/M phase of the cell cycle, respectively. Interestingly, folic acid increased the number of cells expressing either *pCYCD6;1:GFP* (Fig. 2a, c) or *CycB1;1:uidA* (Fig. 2b, d) at the meristem.

To determine the possible impact of folic acid on cell elongation, an analysis was performed with seedlings expressing *pAtEXP7:GUS,* which functions as a marker of differentiated root epidermal cells. Histochemical staining of *pAtEXP7:GUS* seedlings allowed us to visualize the changes of the elongation zone of the root in response to folic acid supplementation, which drastically inhibited cell elongation (Fig. 3a). Concomitantly, the length of trichoblast cells at the root epidermis decreased as the folic acid concentration increased in the media (Fig. 3b). The increase of *pAtEXP7:GUS* expression in response to folic acid indicates that cell differentiation is progressing towards the root tip (Fig. 3c). These data show that folic acid represses primary root growth inhibiting cell elongation and promoting cell differentiation.

3.3. Folic acid causes auxin redistribution within primary roots

The primary root growth repressing effects of folic acid may be attributed to auxin accumulation at the root tip or to a decrease in the auxin maximum at the stem cell niche. To clarify between these possibilities, *DR5:GFP* expression was analyzed by confocal microscopy in primary root tips. As expected, *DR5:GFP* was located in the quiescent center and columella cells in seedlings grown in media lacking folic acid (Fig. 4a, c), whereas in response to folic acid, the expression was reduced in collumela and increased in root meristem initials (Fig. 4b, d). Folic acid supplementation increased fluorescence throughout the vascular tissue of the primary root, and particularly at the cell differentiation zone, where root hairs are formed (Fig. 4a, d). These data are consistent with folic acid causing auxin redistribution within the root meristematic/cell elongation region of primary roots, which may explain their contrasting effects on cell division and elongation.

3.4. Folic acid stimulates the maturation of lateral root primordia by an auxinic mechanism

To determine if folic acid contributes to lateral root initiation or maturation or to both developmental processes, we evaluated the changes in the type and number of lateral root primordia in primary roots. *Arabidopsis* seedlings expressing *DR5:GUS* were used to facilitate the analysis because it is expressed in these structures.
Quantitation of all primordia according to the seven stages of development described by [28] showed that in control condition, most primordia were in the early stages of development, while folic acid supplementation increased the transition to further developmental stages, and correlated with more lateral roots emerging from the primary root (Fig. 5a). However, the analysis of total primordia indicated that even when the compound induced the maturation of primordia, a decrease in the total number was also evident (Fig. 5b). Confocal microscopy imaging showed an increase of *DR5:GFP* expression along the vascular tissue of the primary root and in lateral root primordia following folic acid supplementation, and this expression was normalized in root tips of emerged lateral roots (Fig. 5c).

3.5. Folic acid alters expression of PIN auxin transporters in primary roots and during lateral root primordia development

Auxin transport occurs via the PIN family of auxin proteins, which are expressed in a tissue-specific manner in roots [6, 21, 22]. To analyze if folic acid effects on primary root growth correlate with an altered expression of PIN transporters, we analyzed the PIN1, PIN3 and PIN7 localization in seedlings expressing *PIN1::PIN1::GFP*, *PIN3::PIN3::GFP*, and *PIN7::PIN7::GFP*. In primary roots of seedlings grown in medium lacking folic acid, GFP fluorescence driven by PIN1 was detected mainly in the stele (Fig. 6a), while PIN3 and PIN7 were detected in both the stele and columella cell layers (Fig. 6c and e). In seedlings supplied with 125 µM folic acid, the GFP fluorescence was decreased in their particular expression domains (Fig. 6b, d, and f). In contrast, PIN1 and PIN3 localization in response to 125 µM folic acid clearly increased in lateral root primordia at most developmental stages (Fig. 7a and b). The expression patterns of the PIN proteins correlate with the diminished auxin level of the *DR5:GFP* construct in columella and with an enhanced auxin response in vascular tissue and lateral root primordia.

3.5. Auxin signaling mediates folic acid effects on root architecture

Because auxin signaling appeared to have a strong implication in the root architectural reconfiguration by folic acid, we evaluated the root growth response to the vitamin in *Arabidopsis* wild-type and auxin-related mutants *tir1 afb2 afb3* [23], *slr1* [24] and *arf7 arf19* [25], affected in genes encoding receptors, a repressor and transcription factors of the auxin signaling pathway, respectively. Wild-type and mutant seedlings were grown in media with or without folic acid. Although all three mutants analyzed shortened their primary roots in response to folic acid, a reduced response was evident in *slr1*, and *arf7 arf19* seedlings (Fig. 8a, d).

Regarding the formation of lateral roots, folic acid was unable to induce the formation of these structures in *arf7 arf19* and *slr1*, whilst *tir1 afb2 afb3* forms very few lateral roots and only in the greatest folic acid concentration assayed (Fig. 8b, c, d). Together, these data imply the canonical auxin signal pathway in mediating the effect of folic acid in root architecture configuration.

3.6. Folic acid induces growth and lateral root development in a TARGET OF RAPAMYCIN independent manner

Differential TOR activation and cell proliferation in *Arabidopsis* root and shoot meristems occurs in response to sucrose and glucose and is required for primary root growth and lateral root formation [9-11]. To understand the possible function of TOR in mediating folic acid bioactivity in roots, the growth of wild-type and estradiol-inducible *tor-es1* loss-of-function seedlings was compared in media supplemented with 0.07 µM estradiol, the minimum concentration required to achieve the most pronounced phenotype in *tor-es1* as determined in a growth-response concentration curve to several concentrations of estradiol (data not shown).

The media with or without estradiol was supplemented with increasing folic acid concentrations. Interestingly, whereas in *tor-es1* there was a strong inhibition of growth and development, which correlated with reduced primary root growth and lateral root formation when estradiol was added (Fig. 9a-g), folic acid restored partially the elongation of the primary root when the compound is supplied at 75 µM or greater concentration. Folic acid not only increased lateral root formation, but also normalized growth of *tor-es1* seedlings exposed to estradiol, since total plant biomass and root and shoot biomass was improved by estradiol in a dose-dependent manner (Fig. 9a-g). These data indicate that the effects of folic acid in plant growth and development likely occur independently of the TOR kinase.

4. Discussion

The function of vitamins is of great interest not only in animals but also in plants because of their redox chemistry and role as enzymatic cofactors, which are essential for metabolism [30, 31]. Vitamin B9, also called folic acid or folate, is a water-soluble vitamin with emerging and important roles in plant morphogenesis [12, 13]. Genetic evidence indicated that an altered folic acid biosynthesis caused the loss of the primary root meristem and induced cell differentiation but the effects of its pharmacological application to any plant species was unknown.

In this work, it was shown that folic acid supplementation alters root growth and development in *Arabidopsis thaliana* seedlings grown *in vitro*. Under the experimental conditions employed, the primary root grows indeterminately and the root meristem remains highly active providing the cells required for sustained root growth. However, contrary to our expectations, exogenously supplied folic acid inhibited primary root growth while promoting lateral root formation. An analysis of expression of cell cycle genes CYCB1 and CYCD6 as well as detailed cellular measurements indicated that primary root growth stoppage by folic acid likely occurs due to the inhibition of cell elongation rather than by an altered cell division, because the primary root meristems had more mitotically active cells and expressed *CycB1;1:uidA* and *pCYCD6;1:GFP* at higher levels. These results are in agreement with previous research [12, 13], which allowed the authors to suggest the essential role of folic acid for cell proliferation in meristems.

The data reported here demonstrate that cell elongation is strongly affected by folic acid, and thus the further changes in root system architecture may be explained not only by its promoting role on cell division, but by the balance between cell division and elongation of the cells, both of which are tightly connected with auxin signaling. Repression of root growth may be caused either by an increased accumulation of auxin at the root meristem or by an alternate cellular program that terminates cell division in meristems, giving rise to cell differentiation. This later program is also called “determinate”, because when it is activated, root growth cannot be longer sustained [32]. The analysis of *DR5:GFP* expression in *Arabidopsis* primary roots, indicated a shift of auxin-inducible expression from root cap initials to the root meristem, which correlated with root stoppage. Moreover, an increase in expression of this marker occurs in the vascular tissues across the differentiation zone of primary roots of folic acid-treated seedlings, which directly implies that more auxin is reaching the cell elongation region thus explaining cell growth repression as well as the activation of pericycle cells to divide and form lateral roots. This notion is also supported by the findings that folic acid differentially affects the expression and distribution of the PIN1, PIN3 and PIN7 auxin transporters in primary roots and during lateral root primordia development, which indicates that root responses to folic acid did not occur in all tissues in a similar manner, but rather showed clear preference for specific tissues and transport proteins, which are expressed in the columella, the vascular tissues and the pericycle.

Lateral root formation can be divided into two main phases: i) lateral root primordia initiation, which consists of various developmental stages, and ii) lateral root emergence from the parent primary root [28]. An analysis of the type and amount of lateral root primordia was performed to define if folic acid activates pericycle cells to give rise to more lateral root primordia, or if this vitamin activates the lateral root meristems to divide, and the lateral roots to elongate. Our data indicate that folic acid promotes basically lateral root maturation because increasing concentrations caused more primordia to advance towards later stages of development, while decreasing the total number of lateral root primordia in primary roots. It is possible that the generation of new primordia is carried out at a similar speed in seedlings grown in medium lacking folic acid and in folic acid-treated seedlings, but a faster emergence of these structures results in a smaller amount of them remained at earlier stages in primary roots of seedlings treated with the vitamin. Concomitantly, an analysis of the expression of auxin-inducible reporter *DR5:GFP*, and the distribution of PIN1 and PIN3 indicated that a greater activation of auxin signaling pathway occurred in vascular tissues and in lateral root primordia in response to folic acid, suggesting that the increased lateral root formation in response to this vitamin is also an auxin mediated developmental program.

Through detailed comparison of root growth and lateral root formation in wild-type and auxin-related mutants, it was found that *slr1* and *arf7 arf19* lines are partially resistant to the growth-repressing effects of folic acid in primary roots. The significant resistance observed in the mutants led us to propose that folic acid bioactivity requires directly or indirectly the *IAA14*, *ARF7* and *ARF19* protein products, which may function locally at the meristem and/or cell elongation regions. The *tir1 afb2 afb3* triple mutant did not show a differential behavior to the wild-type seedlings in primary root growth. This may be explained because the TIR1/AFB family of auxin receptors involves at least five members with potential redundant functions [23], and thus other functional AFB proteins might complement the response. Regarding lateral roots, it is notorious that auxin signaling pathway from the receptors (TIR1, AFB2 y AFB3), the repressor (IAA14) and transcriptional activators (ARF7 and ARF19) are necessary for lateral root development elicited by folic acid.

The growth of multicellular organisms is determined both by cell division and expansion, which are tightly linked with TOR signaling. The TOR kinase is a master regulator of growth, evolutionarily conserved among yeasts, plants, animals, and humans. This protein integrates nutrient and energy sensing to promote cell proliferation and growth. Very recent information points to the requirement of high levels of auxin for the TOR pathway to proceed, which differ between the shoot and root apical meristem [11]. To understand the relationship between the effects of folic acid with the TOR-dependent metabolic pathway, we assessed the folic acid response in the *Arabidopsis tor-es1* line, in which upon estradiol supplementation, TOR transcription is terminated. Concomitantly with the critical function of this kinase in plant growth and development, biomass production, root growth and lateral root formation were strongly inhibited following estradiol supplementation in *tor-es1* seedlings. Surprisingly, folic acid application normalized growth and restored lateral root formation in the TOR loss-of-function line, implying independent roles in plant developmental programs. We hypothesize that both TOR and folic acid may act upstream of auxin signaling, but through separate cellular targets. We cannot exclude the possibility that ethylene, cytokinins and other important root hormones, which affect cell elongation may act in concert to auxins to orchestrate the response to folic acid during root architecture reprogramming, this is certainly an ongoing research are to follow.

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Figure legends

Figure 1. Effects of folic acid on *Arabidopsis thaliana* root architecture and biomass production. Wild-type (Col-0) seedlings were germinated and grown on agar-solidified 0.2X MS media with or without folic acid for 10 days. (a) Primary root length, (b) lateral root number, (c) lateral root density, (d) total fresh weight, (e) shoot fresh weight and (f) root fresh weight were recorded. Representative images of agar plates with the seedlings supplemented with (G) 0, (H) 25 and (I) 125 μM folic acid (scale bar= 1 cm). Values shown represent the mean ± standard deviation (n= 30). Different letters indicate statistical differences of a Tukey analysis with a value at ρ<0.05. The experiment was repeated twice with similar results.

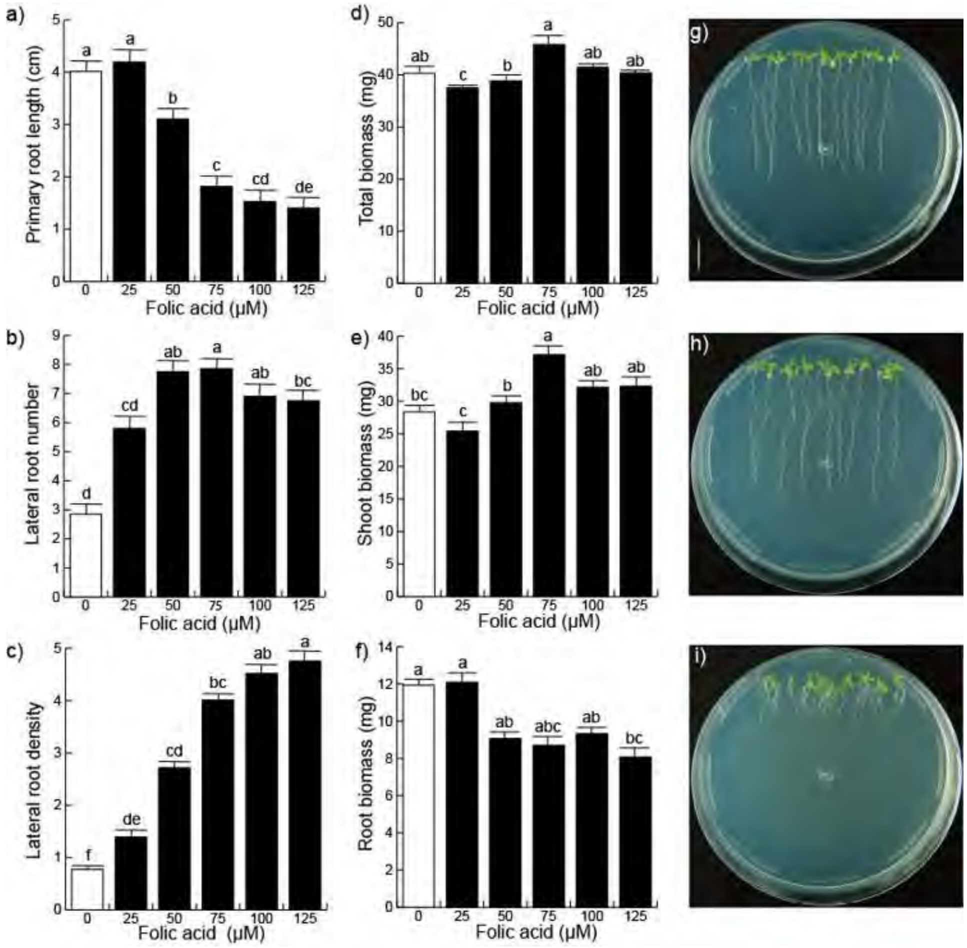


Figure 2. Folic acid induces *pCYCD6;1:GFP* and *CycB1;1:uidA*gene expression and cell proliferation in root meristems. Transgenic *Arabidopsis* seedlings expressing the cell cycle reporters were germinated and grown on agar-solidified 0.2X MS media with or without folic acid for 4 days; subsequently histochemical *CycB1;1:uidA* expression was analyzed and *pCYCD6;1::GFP* detection was performed by confocal microscopy. (a) Number of cells expressing *pCYCD6;1:GFP* or (b) *CycB1;1:uidA*. (c-d) Representative micrographs of roots expressing the cell cycle related genes. Values shown represent the mean ± standard deviation (n= 12). Different letters in graphs indicate statistical differences of a Tukey analysis with a value at ρ<0.05. The experiment was repeated twice with similar results.

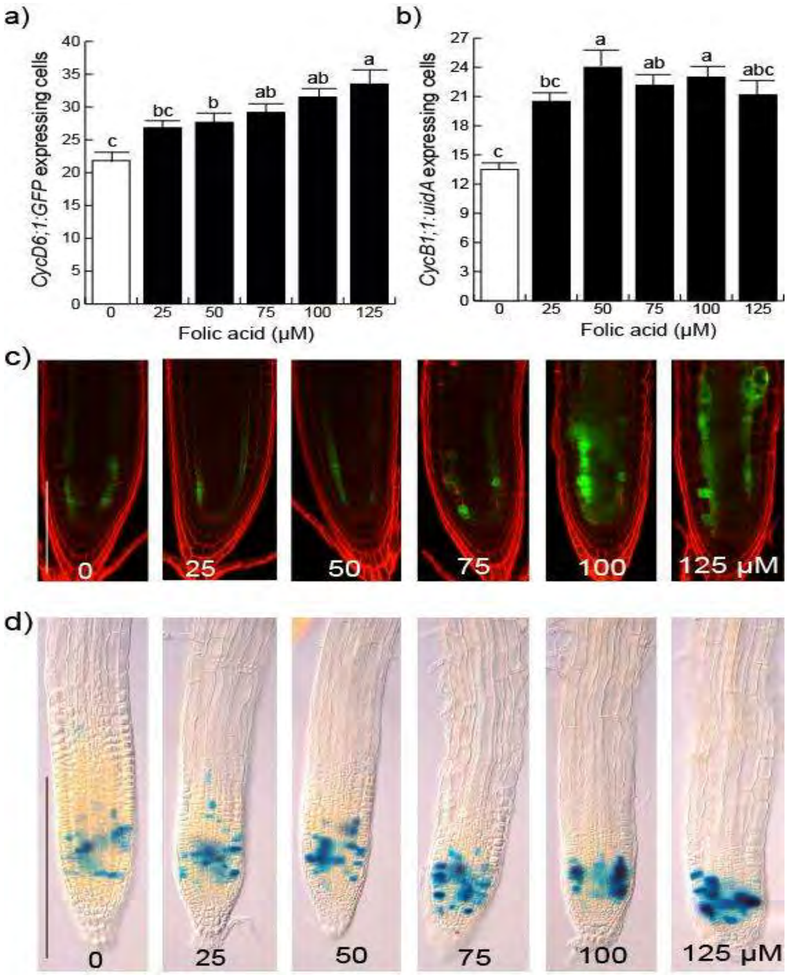


Figure 3. Folic acid represses cell elongation. Transgenic *Arabidopsis* seedlings expressing the *pAtEXP7:GUS* gene construct were germinated and grown on agar-solidified 0.2X MS media with or without folic acid for 4 days; subsequently processed for histochemical GUS activity, and imaging with Nomarski optics. (a) Elongation zone length and (b) trichoblast cell length was recorded. (c) Representative micrographs of solvent and folic acid-treated *pAtEXP7:GUS* roots. Values shown represent the mean ± standard deviation (n= 12). Different letters indicate statistical differences of a Tukey analysis with a value at ρ<0.05. The experiment was repeated twice with similar results.

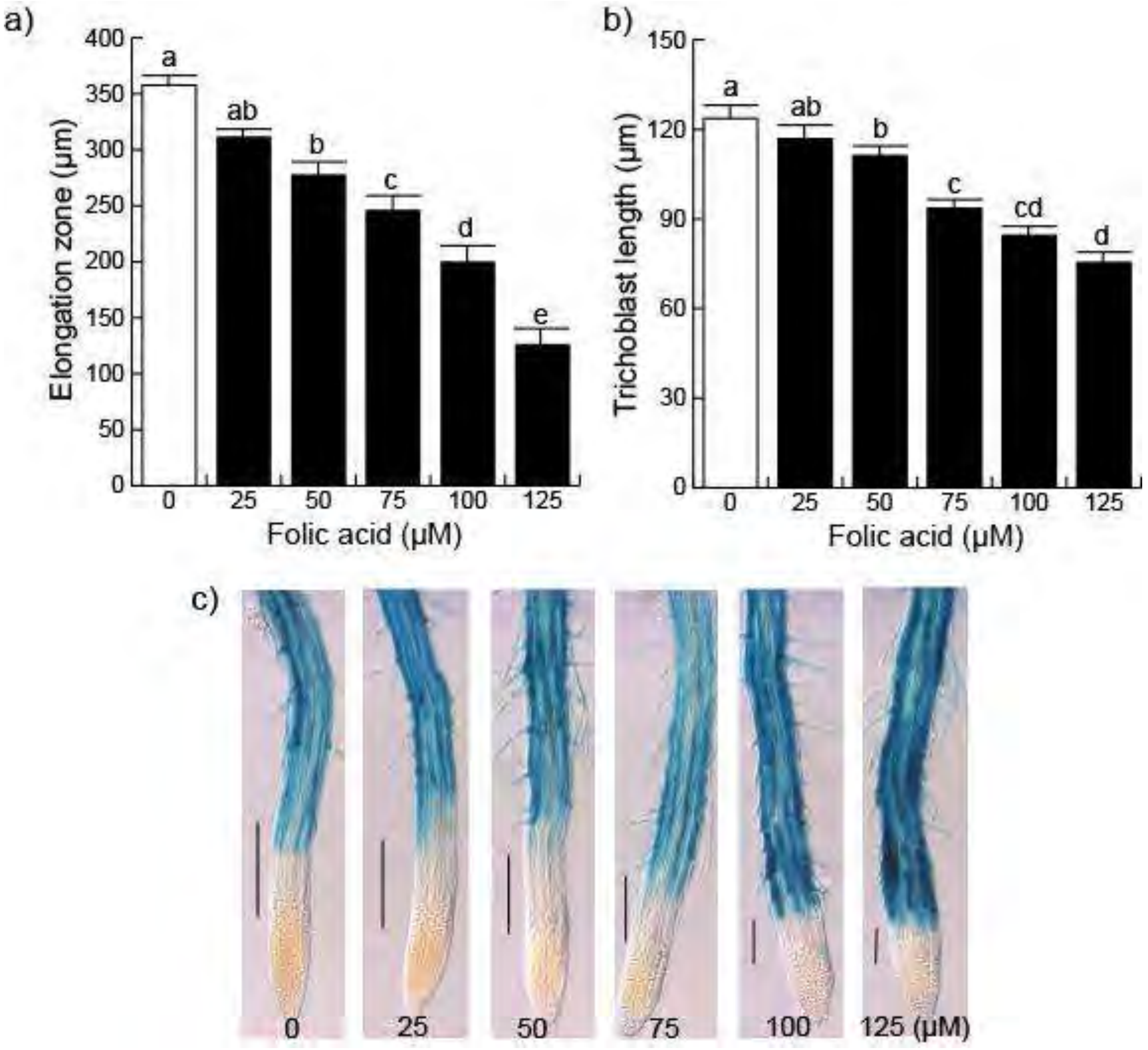


Figure 4. Folic acid causes changes in auxin redistribution in *Arabidopsis* primary roots. *Arabidopsis* seedlings expressing the *DR5:GFP* gene construct were germinated and grown on agar-solidified 0.2X MS media with or without folic acid for 4 days; subsequently transgenic lines were incubated with propidium iodide, and GFP fluorescence was visualized with a confocal microscope. The composite images of several fused micrographs show the expression pattern of auxin-response reporter *DR5:GFP* in roots of seedlings grown in media supplied with (a) the solvent, or (b) 125 µM FA (scale bar= 1cm). The panels "c" and "d" show magnifications of the root tip from seedlings in "a" and "b", respectively (scale bar= 100 µm). The experiment was repeated twice with similar results.

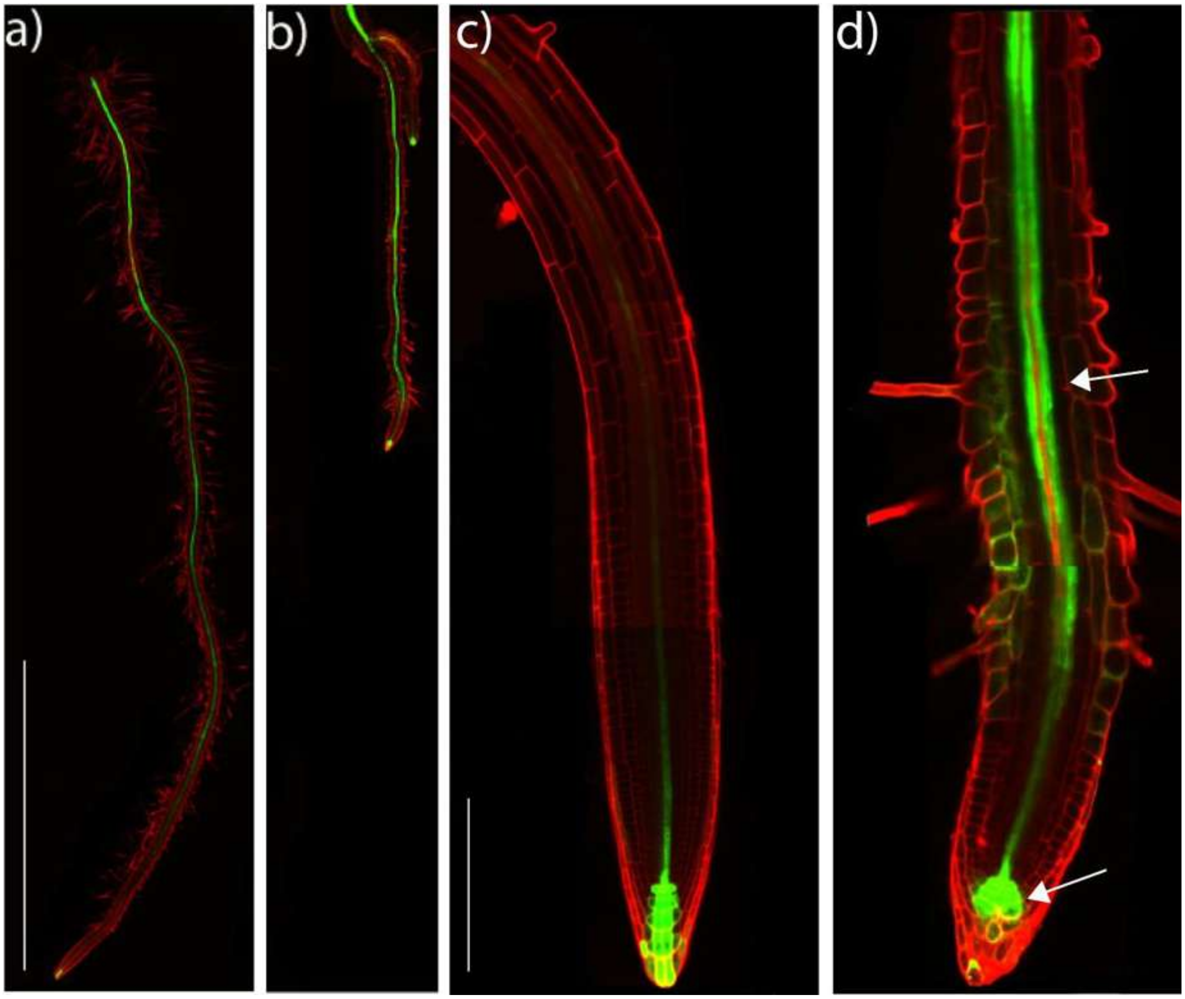


Figure 5. Folic acid induces lateral root development and the auxin response in vascular tissues and in lateral root primordia. *Arabidopsis DR5:GUS* and *DR5:GFP* seedlings were germinated and grown on agar-solidified 0.2X MS media with or without increasing concentrations of folic acid for 4 days; subsequently *DR5:GUS* seedlings were incubated with X-Gluc substrate, clarified and mounted to quantify lateral root primordia. At the same time *DR5:GFP* seedlings were stained with propidium iodide, and lateral root primordia visualized with a confocal microscope. (A) Stages of lateral root primordia and (B) total primordia were recorded from *DR5:GUS* seedlings. (C) Representative micrographs of primordia in similar stages of development (I, IV and VII) and an emerged lateral root (ELR) in *DR5:GFP* seedlings supplied with solvent only or the indicated folic acid treatments (scale bar 100 µm). Values shown represent the mean ± standard deviation (n= 12). Different letters indicate statistical differences of a Tukey analysis with a value at $\rho<0.05$. The experiment was repeated twice with similar results.

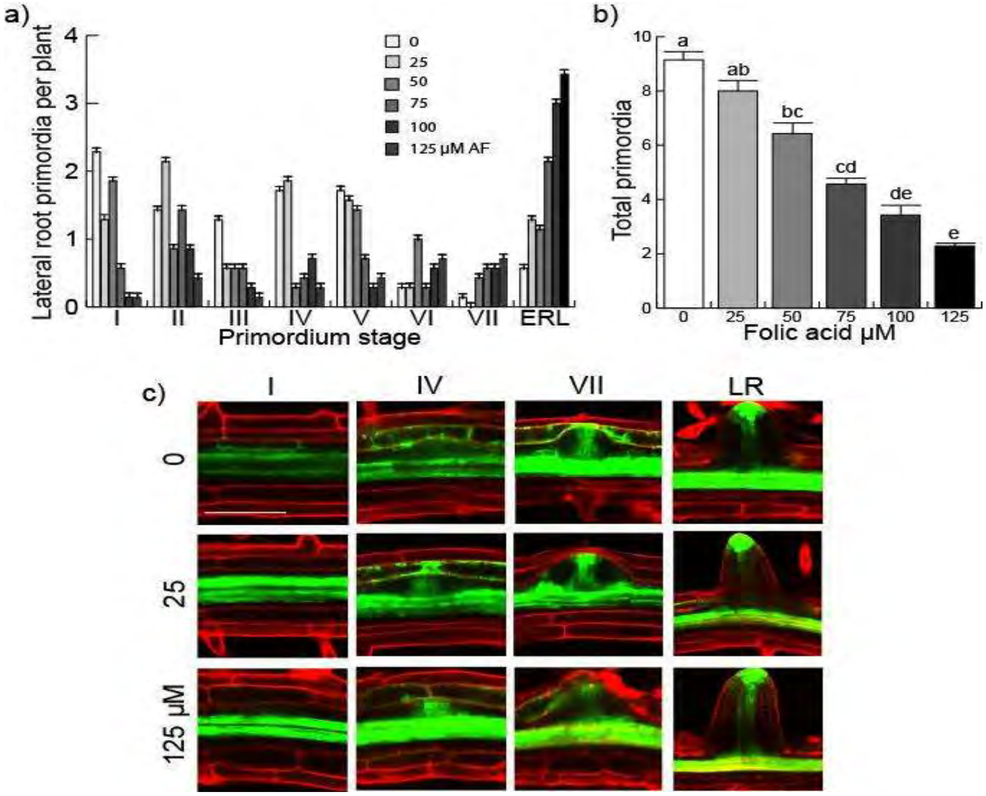


Figure 6. Folic acid (FA) decreases PIN1, PIN3 and PIN7 levels in primary roots. *Arabidopsis* seedlings expressing *PIN1::PIN1::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* were germinated and grown four days on agar-solidified 0.2X MS media with or without FA, incubated with propidium iodide, and GFP fluorescence was visualized with a confocal microscope. At least ten micrographs of primary roots expressing the constructions were taken for each growth condition anda representative image is shown (scale bar= 100 µm). The experiment was repeated twice with similar results.

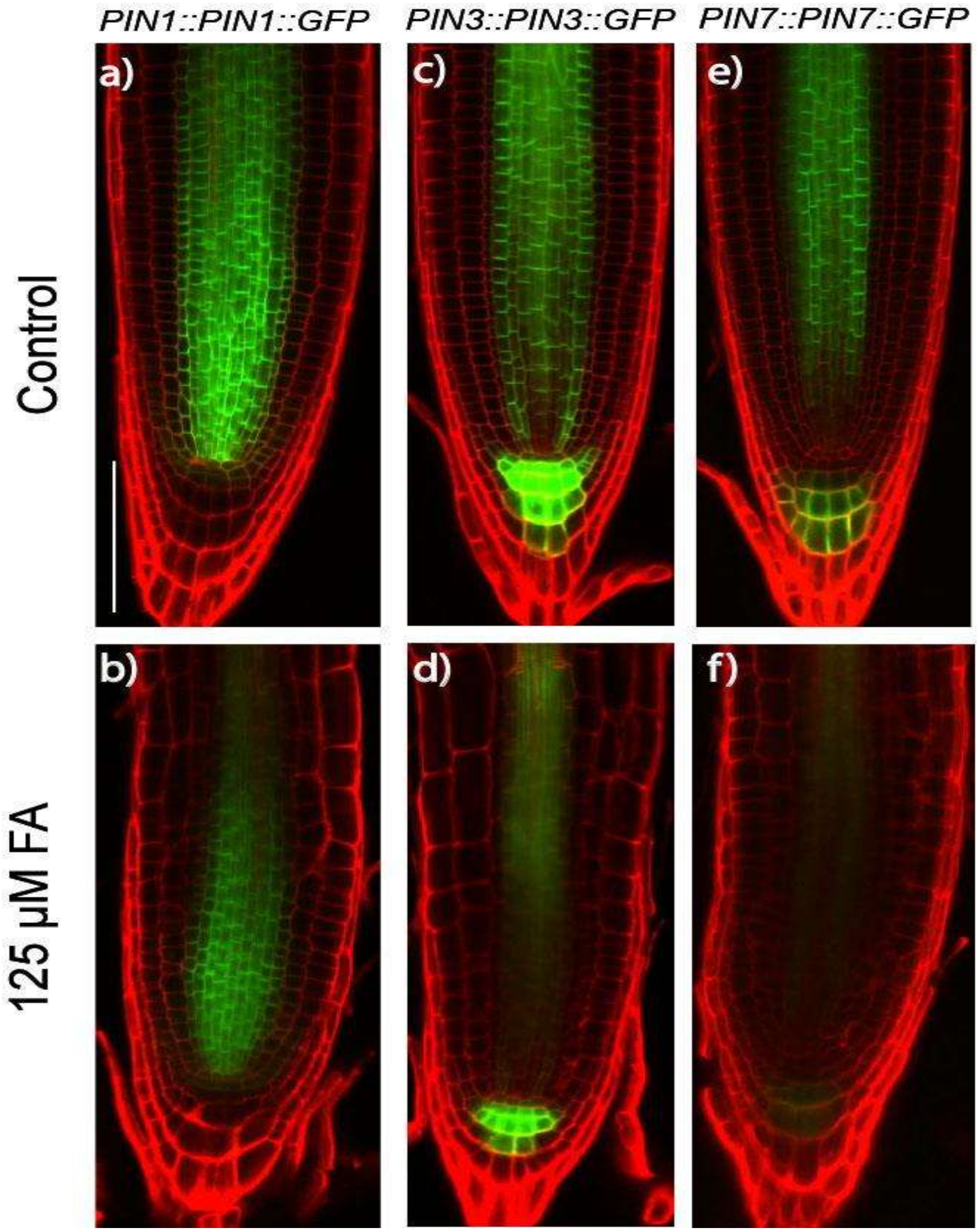


Figure 7. The levels of PIN1 and PIN3 auxin efflux transporters increase during the lateral root formation program in response to folic acid (FA). *Arabidopsis* seedlings expressing *PIN1::PIN1:GFP* and *PIN3::PIN3::GFP* were germinated and grown four days on agar-solidified 0.2X MS media with or without FA, incubated with propidium iodide, and GFP fluorescence was visualized with a confocal microscope. At least ten micrographs for each developmental stage were analyzed and representative images were chosen to construct the figure (scale bar= 100 µm). The experiment was repeated twice with similar results.

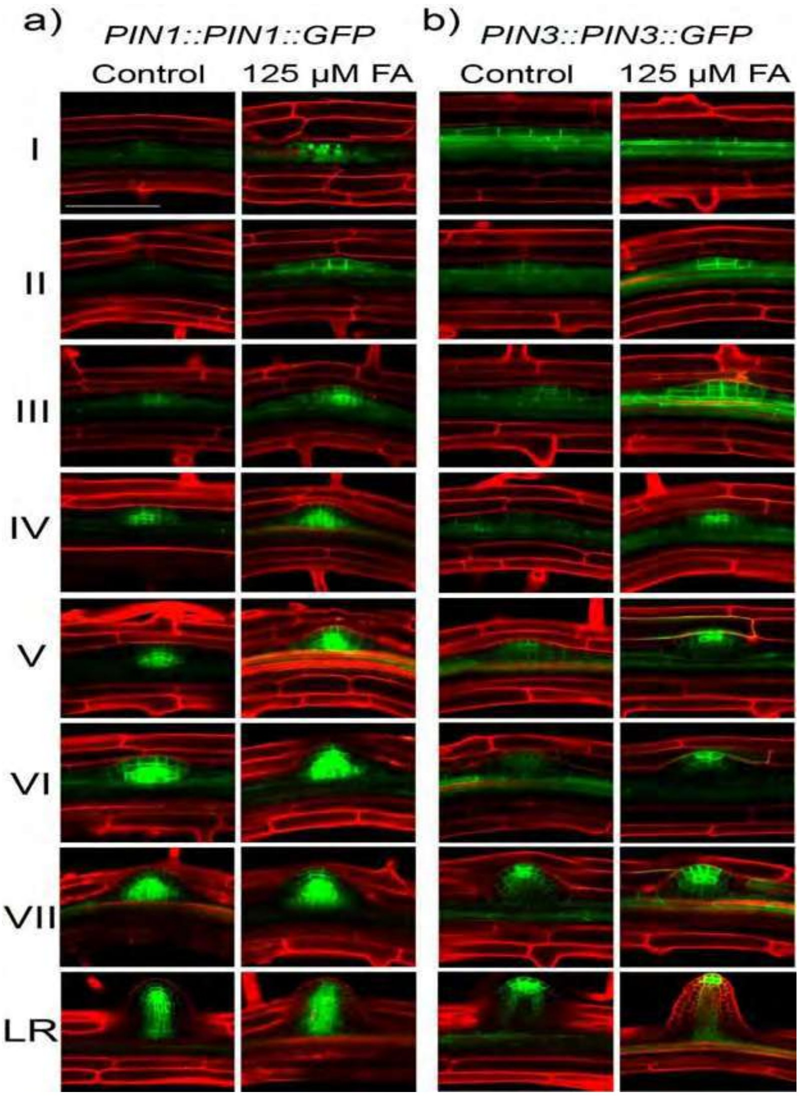


Figure 8. The effects of folic acid on *Arabidopsis* root architecture reconfiguration involves auxin signaling elements. Wild-type (Col-0) and mutant (*arf7 arf19*, *slr1* and *tir1 afb2 afb3*) seedlings were germinated and grown on agar-solidified 0.2X MS media with or without folic acid for 10 days. (a) Primary root length, (b) lateral root number and (c) lateral root density were recorded. (d) Representative images of plates (scale bar= 1 cm). Values shown represent the mean ± standard deviation (n= 15). Different letters indicate statistical differences of a Tukey analysis with a value at ρ<0.05. The experiment was repeated twice with similar results.

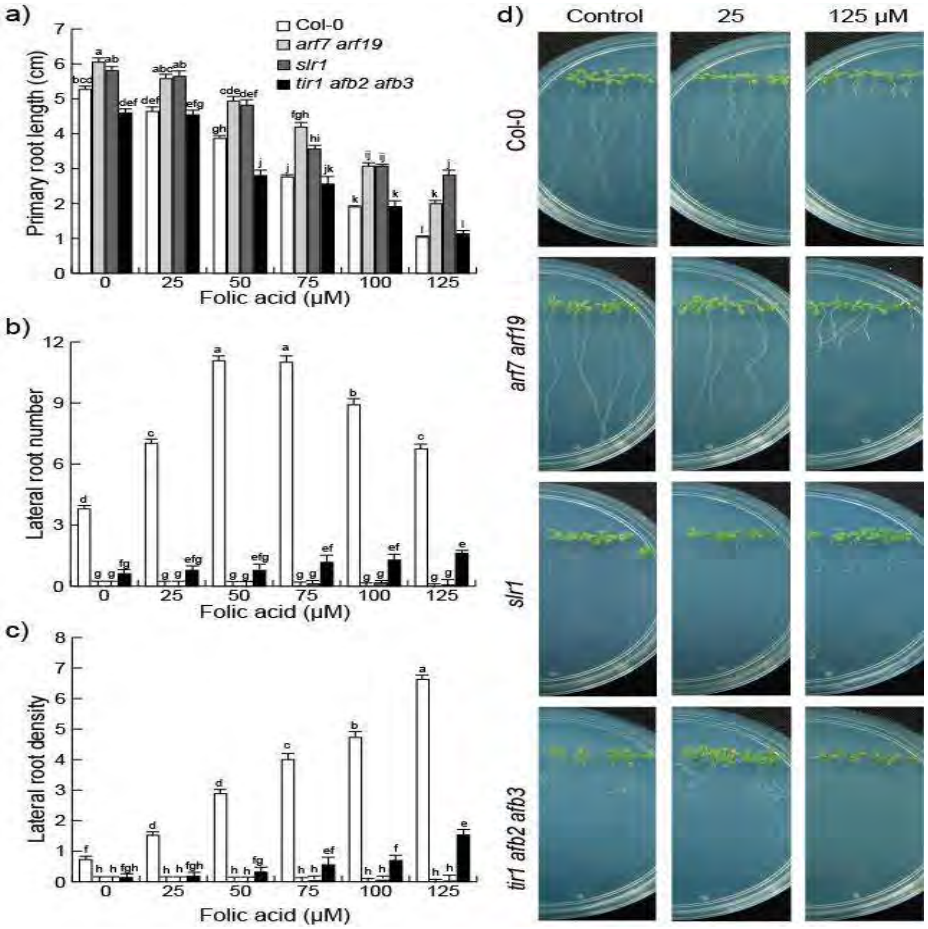
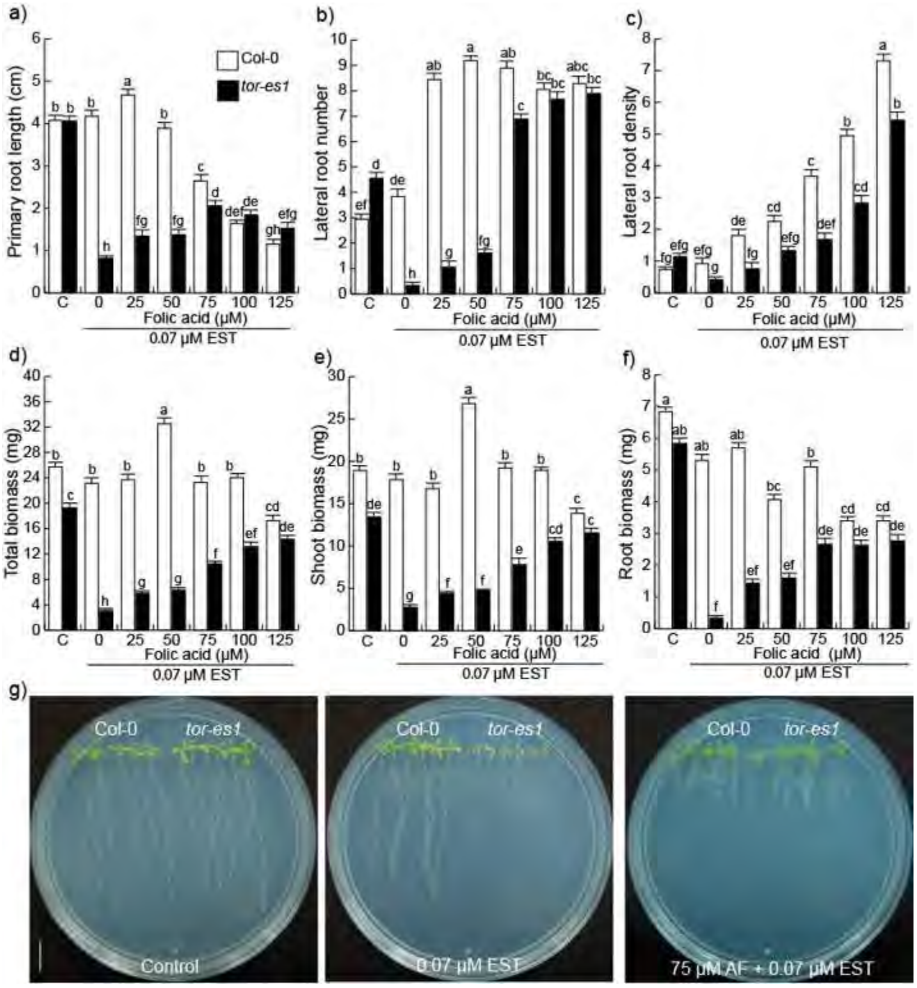


Figure 9. Folic acid modulation of root architecture is independent of TARGET OF RAPAMYCIN. Wild-type (Col-0) and *tor-es1* seedlings were germinated and grown on agar-solidified 0.2X MS media with estradiol and estradiol plus folic acid treatments for 10 days. (a) Primary root length, (b) lateral root number, (c) lateral root density, (d) total fresh weight, (e) shoot fresh weight and (f) root fresh weight were recorded. (g) Representative images of the plates (scale bar= 1 cm). Values shown represent the mean ± standard deviation (n= 15). Different letters indicate statistical differences of a Tukey analysis with a value at ρ<0.05. The experiment was repeated twice with similar results.



Bacterial quorum-sensing signaling influences abscisic acid responsiveness in Arabidopsis thaliana

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Abstract

N-acyl-*L*-homoserine lactones (AHLs) coordinate cell-to-cell communication in Gram-negative bacteria through a process termed quorum sensing (QS). In this report, we evaluated the primary root growth response to abscisic acid (ABA) in wild-type and *decanamide resistant1* (*drr1*) mutants, previously reported to be resistant to *N*-decanoyl-*L*-homoserine lactone (C10-HL). When compared to the WT, *drr1* mutant seedlings were oversensitive to ABA in primary root growth inhibition and this response was reversed in *drr1/abi5* double mutants. An analysis of expression of ARABIDOPSIS INSENSITIVE 4 (ABI4) showed an ABA inducible pattern in primary root tips, which was further increased in *drr1* mutant seedlings. Comparison of seed germination in WT, *drr1*, *abi5* and *drr1/abi5* lines showed contrasting germination percentages in *abi5* and *drr1,* which were normalized in *drr1/abi5* double mutants, thus positioning DRR1 and ABI5 in the same signaling pathway. Taken together, our results show that DRR1 is a negative regulator of ABA signaling probably acting upstream of the transcription factors, which influence ABA responsiveness in primary roots and seed germination.

Keywords: *Arabidopsis thaliana*, root development, abscisic acid, quorum sensing, alkamides, *N*-acyl-etanolamines, *N*-decanoyl-*L*-homoserine lactones.

Introduction

Plants and microorganisms communicate through chemical signaling. Plants biosynthesize a wide range of organic compounds including sugars, organic acids and vitamins, which can be released to the rhizosphere and are sensed as nutritional or regulatory cues by fungi and bacteria. On the other hand, microorganisms release phytohormones, small molecules or volatile compounds that directly or indirectly, influence plant immunity or regulate plant growth and morphogenesis (Ortiz-Castro and others 2009). Examples of bioactive molecules produced by bacteria that are perceived by roots are the *N*-acyl-*L*-homoserine lactones (AHLs). These compounds act as quorum-sensing signals in Gram negative bacteria and mediate pathogenic and symbiotic interactions with their host plants (Schikora and others 2016).

AHLs contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain. The acyl groups of naturally occurring AHLs range from 4 to 18 carbons in length, they can be saturated or unsaturated and with or without a C-3 substituent (Waters and Bassler 2005; Camilli and Bassler 2006). These chemical signals are produced by AHL synthase enzymes, and are detected by a wide variety of transcription factors called “R proteins”, such as LuxR or LasR, and by a small family of sensor kinases related to LuxN (Duerkop and others 2007). The binding of the AHL by most of the characterized R-proteins initiates their interaction with the promoters of genes to induce or repress transcription (Pearson and others 1994; Parsek and others 1999; Churchill and Chen 2011). The specific activity of the different AHLs can be determined by the lactone ring, the amide group and the fatty acid chain length (Churchill and Chen 2011; Lintz and others 2011).

AHL biosynthesis has been reported in more than 90 bacterial species. For example, *Pantoea stewartii* and *Agrobacterium tumefaciens* produce mainly 3-oxo-C6-HL or 3-oxo-C8-HL, respectively, by a single AHL synthase. In contrast, the *BmaI1* and *BmaI3* genes from *Burkholderia mallei* encode two AHL synthases, which produce C8-HL and 3- hydroxy-C8-HL. In *P. aeruginosa*, two preponderant AHL-based QS systems have been described, namely the *las* and *rhl* systems, which depend on recognition of *N*-(3-oxododecanoyl)-*L*-homoserine lactone (3-oxo-C12-AHL) and *N*-butanoyl-*L*-homoserine lactone

(C4-AHL) by the LasR and RhlR transcription factors, which specifically respond to the cognate molecules and activate hundreds of genes (Churchill and Chen 2011).

AHLs are neutral compounds that diffuse freely through cell membranes and accumulate in the surrounding medium as the bacterial population increases (Lee and Zhang 2015). In the rhizosphere, significant amounts of AHLs accumulate and act as bioactive plant signals. For instance, medium and long chained AHLs repress *Arabidopsis* primary root growth, while improving lateral root and root hair formation in a dose dependent manner, giving rise to more branched and exploratory root systems (Ortiz-Castro and others 2008; 2011).

Two major classes of compounds have been identified in plants that share structural similarity with AHLs, namely alkamides and *N*-acyl-ethanolamines (NAEs) (Blancaflor and others 2003; Ramírez-Chávez and others 2004). A recessive alkamide resistant mutant of *Arabidopsis* termed *decanamide resistant root 1* (*drr1*) was identified by Morquecho-Contreras and others (2010), due to its continued primary root growth in medium supplied with either the plant compound *N*-isobutyl decanamide or *N*-decanoyl-*L*-homoserine lactone, a bacterial QS signal. Characterization of WT and *drr1* plants along the entire life cycle, suggested that QS signaling influences plant developmental programs, plant phase transitions and senescence (Morquecho-Contreras and others 2010). An analysis of mutant and overexpressor lines for an *Arabidopsis* fatty acid amide hydrolase gene (AtFAAH) indicated that plants have the enzymatic machinery to metabolize AHLs (Ortiz-Castro and others 2008). Since AtFAAH was previously found to control abscisic acid (ABA) signaling and germination, and an intact ABA signaling pathway was required for NAE action (Teaster and others 2007), it is possible that some ABA components could be shared during the root response to bacterial QS signals.

ABA regulates important aspects of plant growth and development, such as embryo and seed development, seed desiccation tolerance and dormancy, germination, seedling establishment, vegetative development and reproduction (Cutler and others 2010). The ABA signaling pathway starts when ABA promotes the interaction of PYR/PYL/RCARs receptors and PP2Cs phosphatases, resulting in PP2C inactivation (Ma and others 2009; Park and others 2009). PP2Cs inactivate SnRK2s kinases by direct de-phosphorylation, PP2C inactivation by the receptors allows kinases to phosphorylate downstream proteins (Fujii and others 2007; Umezawa and others 2009; Vlad and others 2009; Umezawa and others 2010). In the nucleus, key targets are the basic leucine zipper transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5) and related ABSCISIC ACID TRANSCRIPTION FACTORS (ABFs). Phosphorylated ABFs bind as dimers to the ABA-responsive *cis*-element and in concert with other transcriptional regulators, control the ABA-responsive transcription (Nakamura and others 2001; Furihata and others 2006; Raghavendra and others 2010). ABSCISIC ACID INSENSITIVE 4 (ABI4), an AP2-type transcription factor binds directly to the promoter of ABA responsive genes and activates their expression (Finkelstein and others 1998; Bossi and others 2009; Cutler and others 2010; Reeves and others 2011).

To gain further insight into the mechanisms by which plants perceive bacterial QS sensing molecules, here we performed plant growth, genetic and gene expression analysis in *Arabidopsis* WT seedlings and *drr1* mutants, which demonstrates a C10-AHL/ABA signaling crosstalk in regulating primary root growth and seed germination.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana drr1 [Ws ecotype] (Morquecho-Contreras and others 2010), *abi5* [Ws ecotype] (Finkelstein 1994), *abi1* [Ler ecotype] (Koornneef and others 1984), *abi2* [Ler ecotype] (Koornneef and others 1984) and *abi3* [Ler ecotype] (Koornneef and others 1984) mutants and *ABI4:GUS* transgenic line [Col-0 ecotype] (Söderman and others 2006) were used for the experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 4 min and 10% (v/v) bleach for 4 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2x MS medium (Murashige and Skoog 1962). MS medium (MS basal salts mixture; catalog no. M5524) was purchased from Sigma. The suggested formulation is 4.3 g L^{-1} salts for a 1x concentration of medium; we used 0.9 g L^{-1}, which we consider and refer to as

0.2x MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant grown chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 μmol m^{-2} s^{-1}, and temperature of 22 °C.

Hormone treatments

For hormone crosstalk experiments, MS 0.2x nutrient medium was supplemented with either abscisic acid (ABA) or *N*-decanoyl-*L*-homoserine lactone (C10-HL). Dissolved compounds (Ethanol to C10-HL and dimethyl sulfoxide to ABA) were added to cooled (50 °C) molten medium and poured onto plates. Control plates were supplied with the greatest concentration of solvent used in the treatments. Chemicals were purchased from Sigma Co (USA).

Genetic analysis

The *ABI4:GUS* reporter was transferred into *drr1* mutants via manual pollination using pollen from *Arabidopsis ABI4:GUS* transgenic lines to fertilize the gynoecium of *drr1* flowers. The siliques developed from the crosses were labeled and subsequently, the seeds (F1) were collected, disinfected and screened to identify heterozygous seedling with normal root growth responses to C10-HL. F1 seedlings were selected, transferred to soil and allowed to self-fertilize to obtain the F2 generation and seeds from these plants were disinfected, germinated and allowed to grow over the surface of Petri plates containing agar-solidified 0.2x MS medium supplemented with C10-HL. As expected, homozygous *drr1* seedlings, whose roots are resistant to grow in medium supplied with 30 μM C10-HL, segregated in a 3:1 WT/*drr1* Mendelian proportion. *drr1* mutants harboring the *AB14:GUS* reporter were selected by staining the primary root tip with a GUS staining kit, and positive seedlings were then transferred to soil to grow and reproduce. Seeds from individual *drr1*/*ABI4:GUS* were harvested separately and each lot was analyzed again for GUS activity. *drr1* mutants with GUS activity were propagated once again to ensure that the progeny individuals were truly homozygous for the mutation and harbor the *ABI4:GUS* construct. To generate the *drr1/abi5* double mutant, a similar genetic strategy was employed. In this case, pollen from an *abi5* seedling was used to fertilize the gynoecium of *drr1* flowers. The heterozigous F1 progeny was recovered and allowed to self-fertilize. Seedlings from F2 populations were screened via a double selection procedure supplying 3 µM ABA and seedlings resistant to the ABA repressor effect were subsequently transferred to 100 µM kanamycin. The seedlings that survived this double selection were propagated again to ensure that the progeny was truly homozygous (Fig. S1). This selection scheme was possible because the mutation in DRR1 is caused by a T-DNA insertion, which contains a kanamycin resistance cassette, thus allowing seedlings to grow in medium supplemented with kanamycin (Krysan and others 1999; Morquecho-Contreras and others 2010).

Analysis of growth and statistical analysis

The growth of primary root was registered using a ruler and 15 individuals of each condition were evaluated for each treatment. The root fresh weight from 30 seedlings grown on the same plate was measured on an analytical scale (Ohaus) immediately after plant harvest and 3 plates of each condition were evaluated. Lateral roots of each seedling were quantified with a stereoscopic microscope (Leica MZ6) and lateral root density was calculated from lateral root number/primary root length. For all experiments with wild-type and mutant lines, the overall data were statistically analyzed using STATISTICA 10.0 Software (Dell StatSoft, Austin, Texas, USA). Univariate and multivariate analyses with a Tukey’s posthoc test were used for testing differences in the analyzed parameters. Different letters are used to indicate means that differ significantly ($\rho < 0.05$).

Histochemical analysis of GUS activity

Transgenic plants expressing the *GUS* reporter gene (Jefferson and others 1987) were stained in 0.1% 5-bromo-4-chlorium-3-indolyl-β-D-glucuronide (X-

Gluc) in phosphate buffer (NaH_2PO_4 and Na2HPO4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide overnight at 37 ^{o}C. Plants were cleared and fixed as described previously by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail varnish. Subsequently, plants were photographed using Nomarski optics on a Leica DFC450 C microscope. For each treatment, at least 10 transgenic plants were analyzed.

Seed germination assays

For germination assays, mature seeds from WT, *drr1*, *abi5* and *drr1/abi5* mutants were superficially disinfected and sown into Petri plates containing 0.2x MS-agar medium and incubated into a plant growth chamber with darkness conditions. Germination was registered at the time when radicle was completely emerged

Results

The *Arabidopsis decanamide resistant root 1* mutant is oversensitive to ABA

To evaluate the *drr1*-mutant response to ABA, we compared the growth of wild-type (Wassilewkija ecotype; Ws) and *decanamide resistant root 1* (*drr1*) mutants germinated and grown over the surface of agar plates containing 0.2x MS-agar medium supplemented with 0, 0.25, 0.5 and 1 μM ABA for 7 days, and the length of the primary root and root biomass were measured. In WT seedlings, ABA inhibits primary root growth (Fig. 1a) and decreases root biomass (Fig. 1b) in a dose-dependent manner. On the other hand, *drr1* mutants showed a greater response than wild-type seedlings to the repressor effect of ABA (Figs. 1a and b).

DRR1 negatively regulates ABA signaling by a mechanism involving *ABI5*

The ABA signaling pathway requires an ABI5 function (Finkelstein and Lynch 2000; Lopez-Molina and others 2001). The *Arabidopsis abi5* mutants, like many other ABA-insensitive mutants, was selected on the basis of ABA-resistant germination (Finkelstein 1994). To genetically test the specific role of ABA on primary root growth, ABA sensitivity of WT and *abi1, abi2, abi3* and *abi5* mutants was assessed on 0.2x MS-agar medium supplemented with increasing ABA concentrations. From this analysis, only the *abi5* mutants were able to sustain primary root growth in the ABA treatments performed (Fig. 2a-e). The above results motivated us to check whether *ABI5* could be in the same signaling pathway of *DRR1* since the corresponding mutants show opposite phenotypes to ABA in roots. Therefore, we genetically obtained homozygous *drr1/abi5* double mutants. The root response to ABA was evaluated in WT, single and the double mutants and a new, intermediate response between *drr1* and *abi5* was observed in *drr1/abi5* mutants for both primary root growth (Fig. 3a) and root biomass (Fig. 3b), which was different from the wild-type response in 0.5 and 1 μM ABA. These results indicate that ABI mediates root growth responses to ABA and that the mutation in DRR1 influences ABA responses likely acting upstream of ABI4.

ABI5 did not influence the root response to C10-HL

The *drr1* mutant was isolated because it shows resistance to primary root growth repression by C10-HL (Morquecho-Contreras and others 2010). Next, wild-type (Ws), *drr1*, *abi5* and *drr1/abi5* mutants were grown on 0.2x MS-agar medium supplemented with 0, 10, 20, 30 and 40 μM C10-HL. In these assays, WT and *abi5* seedlings shortened their primary roots in a dose-dependent manner in response to the QS signal, and no significant difference was observed between these two genotypes. However, when compared to the WT, *drr1* and *drr1/abi5* seedlings were able to sustain primary root growth in media supplied with 30 and 40 μM C10-HL (Fig. 4a). Lateral root formation increased in treatments with C10-HL in WT and *abi5* seedlings, and this response was also reduced in *drr1* and *drr1/abi5* seedlings (Fig. 4b). These results show that no additive effects are apparent in *drr1/abi5* seedlings in root response to the bacterial QS signal.

ABI4 expression is modulated by *DRR1* in response to abscisic acid

To determine the possible influence of *DRR1* in modulating the expression of the transcription factor *ABI4*, another critical element in the ABA signaling pathway, the *ABI4:GUS* marker was transferred into *drr1* seedlings by outcrossing. Then, we compared the *ABI4:GUS* expression pattern in 3-day-old seedlings grown in 0.2x MS-agar medium supplemented with ABA and found that in the WT, this reporter gene was mainly expressed in the root tip and increased its expression depending upon the ABA concentration (Fig. 5a-c). In *drr1* mutants, *ABI4:GUS* expression in solvent-treated seedlings was also located at the root tip (Fig. 4d), while in media with ABA, the expression was increased and extended (Fig. 5e-f). An analysis to evaluate changes in *ABI4:GUS* expression in MS 0.2x media supplemented with C10-HL showed that this QS signal did not alter the expression pattern of the reporter in WT or *drr1* seedlings when compared to the control condition (Fig. S2).

DRR1 influences seed germination acting in an ABI5 pathway

Arabidopsis mutants deficient in ABA biosynthesis or signaling show increased germination rates (León-Kloosterziel and others1996). To determine if the ABA oversensitive root responses of *drr1* mutants could be related to seed dormancy and to establish possible epistatic relations of AHL signaling with ABI5, we compared germination frequencies between WT, *drr1*, *abi5* and *drr1/abi5* seeds at 20, 40, 60 and 80 h in 0.2x MS-agar medium in darkness. WT seeds started germination 20 h after release of stratification and reached 100 % around 50 h (Fig. 6). The relative germination percentages were always *abi5* > *drr1/abi5* > wild type > *drr1,* this later attained 100 % around 60 h. These results indicate that the ABI5 mutation normalizes germination in *drr1* mutant seeds, thus positioning DRR1 and ABI5 in the same signaling pathway.

Discussion

Accumulating data reveal a symbiotic relationship between plants and bacteria via AHLs, which regulate root system architecture and influence plant fitness.

Ortiz-Castro and others (2008) investigated the root architectural changes of *Arabidopsis thaliana* following application of commercially available AHLs ranging from 4 to 14 carbons in length. Developmental changes elicited by C10-HL, the most active compound found to repress primary root growth, were related to inhibition of cell division and promotion of cell differentiation. Further, the isolation of *drr1 Arabidopsis* mutants resistant to C10-HL suggested that this QS signal accelerates developmental programs and senescence, since the mutants showed delayed flowering and increased their vegetative growth period (Morquecho-Contreras and others 2010).

The *in vivo* role of QS sensing for plant performance remains to be characterized, however, recent data about the interaction between the Gram negative plant growth promoting rhizobacteria (PGPR) *Burkholderia phytofirmans* PsJN and *Arabidopsis thaliana* throughout the entire plant life cycle, demonstrated that the bacteria instead accelerates flowering and shortened the vegetative growth period; these modifications correlated with the early up-regulation of flowering control genes (Poupin and others 2013). In addition, when *Arabidopsis* seedlings were inoculated with *Gluconacetobacter diazotrophicus*, a root endophyte, growth promotion was consistently observed for up to 50 days, which correlated with higher canopy photosynthesis, lower plant transpiration, and increased water-use efficiency, aspects related to ABA signaling (Rangel de Souza and others 2016). Thus, inoculations with bacteria that may produce and release AHLs could affect the whole life cycle of a plant, accelerating its growth rate and improving photosynthesis and water use efficiency, effects highly relevant for most crops. Our data indicate that at least in part, the influence of AHLs on plant functional processes may be related to its regulation of ABA signaling.

AHLs share structural chemical similarity with alkamides and *N*-acyl-ethanolamines (NAEs), compounds naturally produced by plants with interesting plant growth regulating properties. Although the molecular identity of the *DRR1* is at present unknown, its loss of function in *Arabidopsis* caused increased sensitivity to the repressor effects of ABA in root growth. This indicates that *DRR1* genetically regulates ABA signaling, influencing plant size and longevity according to the reported phenotype of *drr1* mutants grown in soil (Morquecho-Contreras and others 2010). We genetically defined that among

ABA-related genes the ABI5 transcription factor mediates the ABA repressing effects on primary root growth since the corresponding *abi5* mutant renders *Arabidopsis* roots resistant to pharmacological ABA application. *drr1/abi5* double mutants further revealed that DRR1 may act upstream of ABI5 in the ABA signaling pathway, since root ABA oversensitivity of *drr1* mutants is reversed in the *drr1/abi5* double mutant, the result is a phenotype similar to the wild-type rather than a phenotype like *abi5*.

Medium chained AHLs strongly induce lateral root formation. Since *drr1* seedlings exhibit greater response to ABA, which negatively regulates lateral root formation (De Smet and others 2003), then it is possible that the reported phenotype of few lateral roots in *drr1* would depend on an increased ABA responsiveness. However, our data show that in contrast to primary root growth, the formation of lateral roots does not necessarily involve ABI5 since the WT and *abi5* mutants behaved similarly, forming increasing numbers of lateral roots in response to C10-HL treatments and because the ABI5 loss-in-function mutation did not alter lateral root formation in response to C10-HL in *drr1/abi5* double mutants.

ABA signaling pathway influences several transcription factors that activate gene expression in a developmental and tissue specific context. Particularly, *ABI4* has been reported to regulate root system architecture by inhibiting lateral root formation (Shkolnik-Inbar and Bar-Zvi 2010). Our data are consistent with these previous findings since *ABI4:GUS* expression in roots increase following ABA treatment, and *drr1* mutants show an exacerbated *ABI4:GUS* expression when compared to wild-type seedlings, therefore, ABA oversensitivity of *drr1* mutants correlates with a greater expression of the ABA inducible transcription factor ABI4. Bossi and others (2009) reported that ABI4 is an essential activator of its own expression during development, acting in ABA signaling and in sugar responses besides inducing *ABI5* expression. Therefore, a higher *ABI4:GUS* expression would give rise to more transcription factors being recruiting to the ABA signaling pathway and this would be influenced by AHLs.

Arabidopsis thaliana exhibits seed dormancy, allowing the seeds in the natural environment to survive the dry summer period and germinate when the conditions are appropriate for growth and development. The degree of seed dormancy is reflected in the germination percentage and mutants deficient in the hormone ABA are among the most non-dormant, or instead, early germinating mutants in most plant species. Consistently, we found that the degree of dormancy and concurrently the rate of germination of the *abi5* mutants were higher than that of the wild-type. In contrast, the *drr1* mutants showed delayed germination. The *drr1/abi5* double mutants did not show an additive effect, but rather their seed germination phenotype was similar to the wild-type and different from the *abi5* or *drr1* single mutants. This also confirms the hypothesis that DRR1 and AB15 act in the same pathway.

In natural ecosystems, seeds germinate in the presence of a wide range of microorganisms and the soil microbiome may be crucial to germination and early plant growth. However, to the best of our knowledge, no studies have investigated the effects of AHL-producing bacteria on germinating seeds. This report shows that AHLs may be part of the signaling network determining not only seed dormancy but also other possible functional aspects related to ABA such as stress and senescence, the ecological roles of such molecular interactions remain to be investigated.

Acknowledgments

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Figure legends

Fig. 1. Abscisic acid effects on WT and *drr1* mutant roots. *Arabidopsis thaliana* seedlings were germinated and grown on 0.2x MS-agar medium with or without ABA for 7 days; (**a**) primary root length [PRL] and (**b**) root fresh weights were recorded. Representative photographs of (**c**) control condition and (**d**) 0.25 μM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (n = 15 to PRL; n = 3 to RFW). The experiment was replicated three times with similar results.

Fig. 2. The root response of WT, *abi1, abi2, abi3* and *abi5* mutants to ABA. *Arabidopsis thaliana* seedlings were germinated and grown on 0.2x MS-agar medium for 7 days; primary root length from (**a**) *abi1*, (**b**) *abi2*, (**c**) *abi3* and (**d**) *abi5* mutants in response to ABA were recorded. Representative photographs (**e**) control condition and (**f**) 0.5 μM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (*n* = 15). The experiment was replicated three times with similar results.

Fig. 3. Root response of WT, *drr1*, *abi5* and *drr1/abi5* mutants to abscisic acid. *Arabidopsis thaliana* seedlings were germinated and grown on 0.2x MS-agar medium for 7 days; (**a**) primary root length, and (**b**) root fresh weight in response to ABA were recorded. Representative photographs were taken from plants (Ws and *drr1, abi5* and *drr1/abi5*) grown at (**c**) control condition or (**d**) 0.25 μM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (*n* = 15 to PRL; *n* = 3 independent plates for RFW determinations). The experiment was replicated three times with similar results.

Fig. 4. Root response of WT, *drr1*, *abi5* and *drr1/abi5* mutants to *N*-decanoyl-*L*-homoserine lactone*. Arabidopsis thaliana* seedlings were germinated and grown on 0.2x MS-agar medium for 10 days; (**a**) primary root length and (**b**) lateral root density were recorded in response to C10-HL. Representative photographs from Ws, *drr1*, *abi5* and *drr1/abi5* grown on (**c**) control and (**d**) 40

μM C10-HL conditions (Scale bar = 1cm). Values shown represent the mean ± SD (*n* = 15). The experiment was replicated three times with similar results.

Fig. 5. The *ABI4:GUS* expression increases in *drr1* mutants in response to ABA. *β*-glucoronidase (GUS) staining from WT *ABI4:GUS* seedlings (**a-c**), or *drr1* mutants (**d-f**) grown on 0.2x MS-agar medium supplemented with the indicated concentrations of ABA 3 days after germination. Photographs show representative expression patterns of *ABI4:GUS* in primary root tip of representative individuals from at least 15 seedlings analyzed (Scale bar = 50 μm). The experiment was replicated three times with similar results.

Fig. 6. DRR1 interact with ABI5 on seed germination. Kinetics of germination percentage in WT, *drr1*, *abi5* and *drr1/abi5* mutants. Values shown represent the mean ± SD (n =100). The asterisks indicate statistically significant differences to the control at ρ = 0.05. The experiment was replicated three times with similar results.

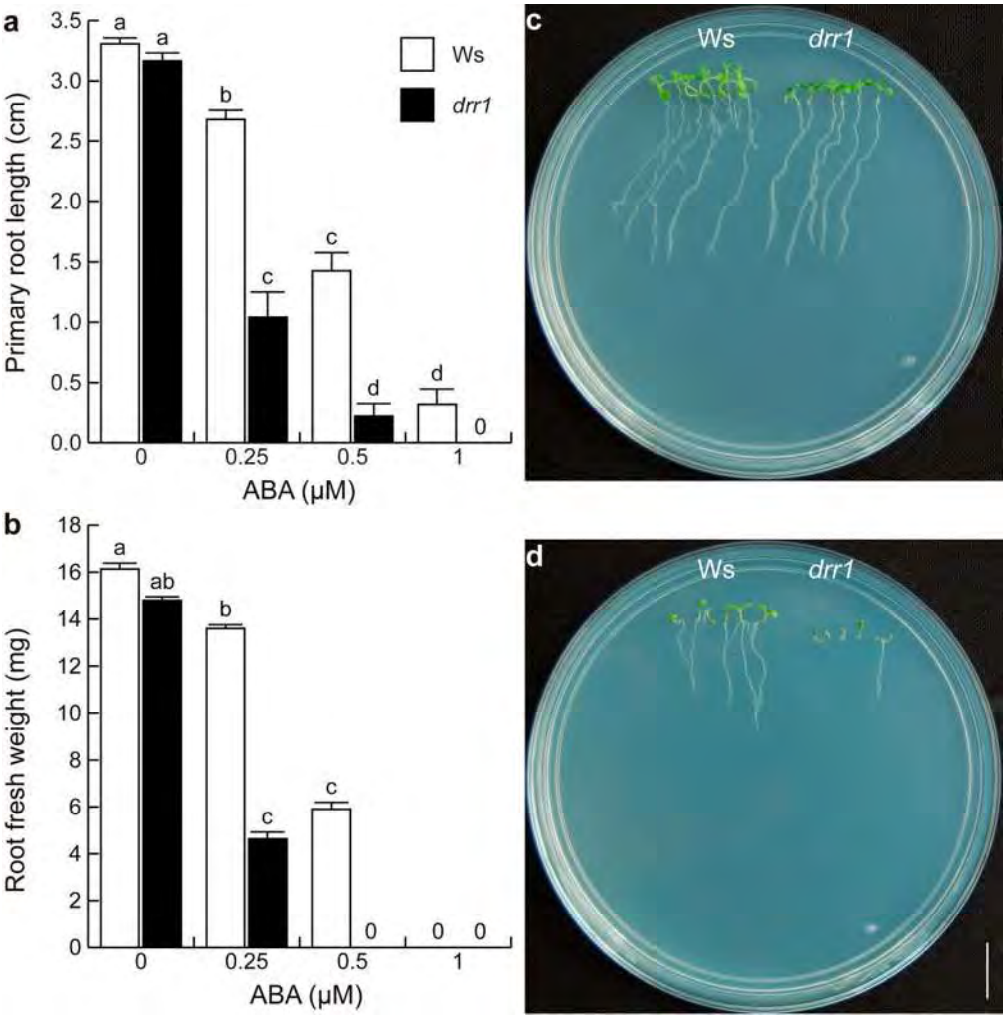

Fig. 1. Abscisic acid effects on WT and drr1 mutant roots. Arabidopsis thaliana seedlings were germinated and grown on 0.2x MS-agar medium with or without ABA for 7 days; (a) primary root length [PRL] and (b) root fresh weights were recorded. Representative photographs of (c) control condition and (d) 0.25 μM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (n = 15 to PRL; n = 3 to RFW). The experiment was replicated three times with similar results.

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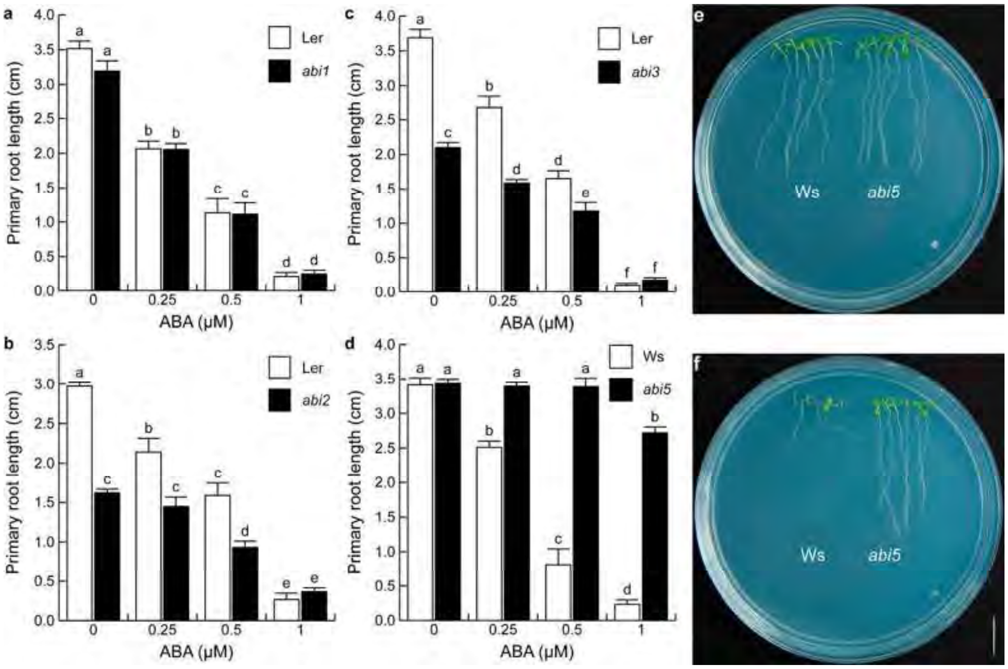

Fig. 2. The root response of WT, abi1, abi2, abi3 and abi5 mutants to ABA. Arabidopsis thaliana seedlings were germinated and grown on 0.2x MS-agar medium for 7 days; primary root length from (a) abi1, (b) abi2, (c) abi3 and (d) abi5 mutants in response to ABA were recorded. Representative photographs (e) control condition and (f) 0.5 µM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (n = 15). The experiment was replicated three times with similar results.

Figure 2

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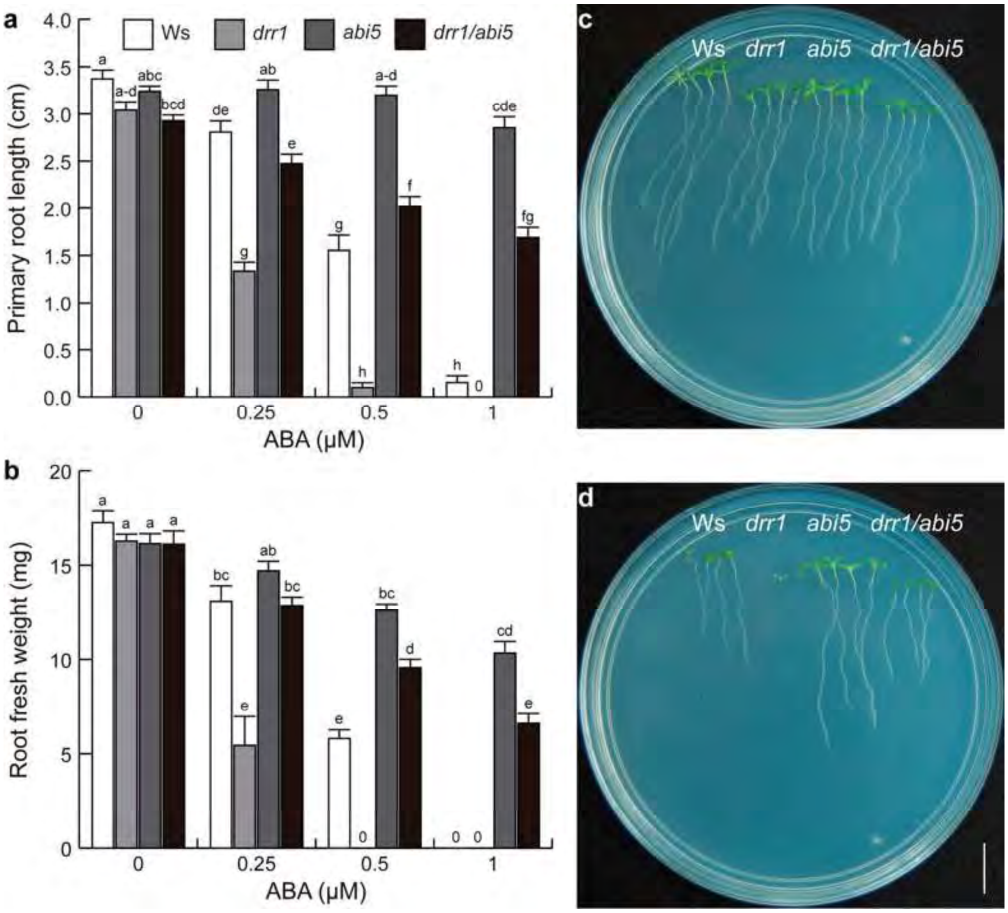

Fig. 3. Root response of WT, drr1, abi5 and drr1/abi5 mutants to abscisic acid. Arabidopsis thaliana seedlings were germinated and grown on 0.2x MS-agar medium for 7 days; (a) primary root length, and (b) root fresh weight in response to ABA were recorded. Representative photographs were taken from plants (Ws and drr1, abi5 and drr1/abi5) grown at (c) control condition or (d) 0.25 μM ABA (Scale bar = 1cm). Values shown represent the mean ± SD (n = 15 to PRL; n = 3 independent plates for RFW determinations). The experiment was replicated three times with similar results.

Figure 3

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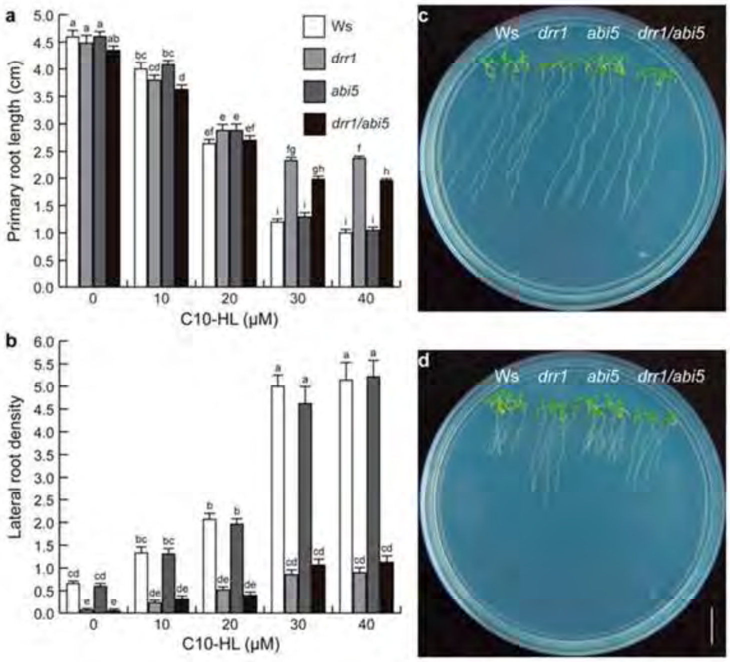

Fig. 4. Root response of WT, drr1, abi5 and drr1/abi5 mutants to N-decanoyl-L-homoserine lactone. Arabidopsis thaliana seedlings were germinated and grown on 0.2x MS-agar medium for 10 days; (a) primary root length and (b) lateral root density were recorded in response to C10-HL. Representative photographs from Ws, drr1, abi5 and drr1/abi5 grown on (c) control and (d) 40 µM C10-HL conditions (Scale bar = 1cm). Values shown represent the mean ± SD (n = 15). The experiment was replicated three times with similar results.

Figure 4

254x190mm (96 x 96 DPI)

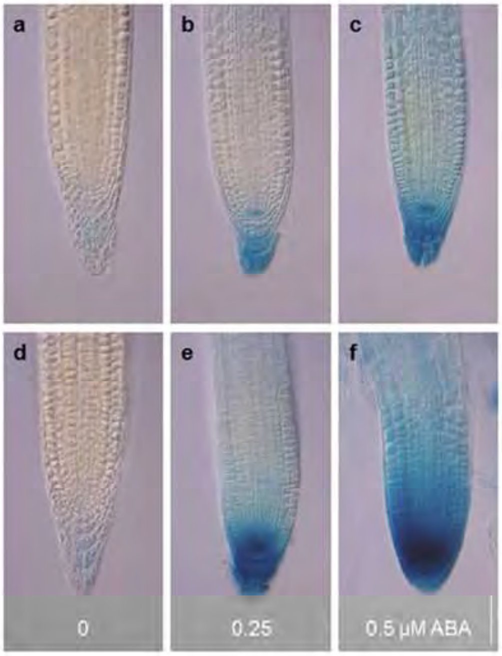

Fig. 5. The ABI4:GUS expression increases in drr1 mutants in response to ABA. β-glucoronidase (GUS) staining from WT ABI4:GUS seedlings (a-c), or drr1 mutants (d-f) grown on 0.2x MS-agar medium supplemented with the indicated concentrations of ABA 3 days after germination. Photographs show representative expression patterns of ABI4:GUS in primary root tip of representative individuals from at least 15 seedlings analyzed (Scale bar = 50 μm). The experiment was replicated three times with similar results.
Figure 5
254x190mm (96 x 96 DPI)

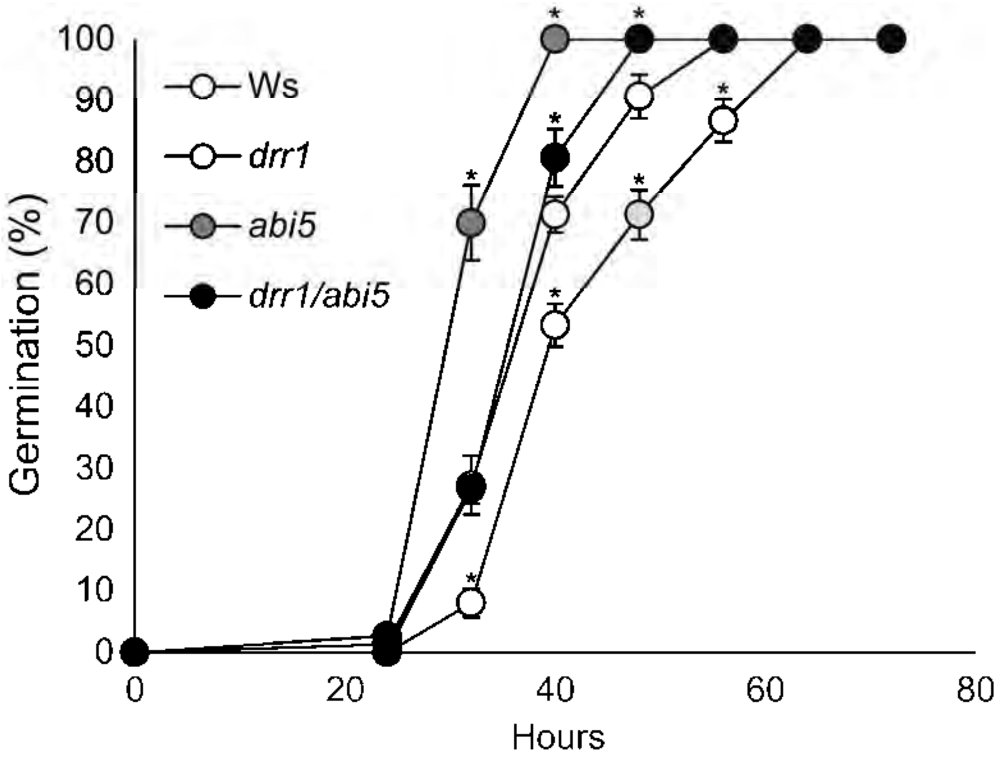

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Figure 6
90x68mm (600 x 600 DPI)

7. DISCUSIÓN Y CONCLUSIONES

La ruta de transducción de señales del ácido jasmónico puede alterar la biosíntesis de otras hormonas y segundos mensajeros con papeles importantes en la regulación cruzada del desarrollo y la defensa actuando como moduladores de la expresión de genes (Lorenzo *et al.*, 2003). Recientemente, se reportó que la herida repetitiva de los cotiledones transmite la señal del ácido jasmónico del follaje a la raíz, lo cual restringe el crecimiento radical al inhibir la proliferación y elongación celular (Gasperini *et al.*, 2015), un aspecto que también ocurre con la aplicación de óxido nítrico o etileno (Fernández-Marcos *et al.*, 2012; Street *et al.*, 2015). De hecho, el ácido jasmónico induce la acumulación de óxido nítrico en *Arabidopsis* bajo estrés y defensa por herida, mientras que el óxido nítrico activa los genes tempranos de la señalización del ácido jasmónico, lo que indica la existencia de una regulación cruzada en ambas vías (Huang *et al.*, 2004; Xu *et al.*, 2005).

El hallazgo de que el ácido jasmónico induce un incremento en los niveles de óxido nítrico en la punta de la raíz primaria indica un posible papel represor de la actividad de las células fuente y/o la capacidad de proliferación celular, de acuerdo con su regulación negativa de los factores de transcripción PLT1 y PLT2 (Chen *et al.*, 2011). Experimentos de inmunoprecipitación de cromatina y ensayos gel shift revelaron que el factor de transcripción MYC2 se une directamente a los promotores de PLT1 y PLT2, reprimiendo su expresión (Chen *et al.*, 2011). Un perfil transcripcional del genoma completo de *Arabidopsis thaliana* en respuesta a *N*-isobutil decanamida, un metabolito que incrementa la ramificación de la raíz y las respuestas de defensa aumentando los niveles de ácido jasmónico, reveló la inducción de genes asociados a la senescencia, además de la acumulación de óxido nítrico en raíces y hojas (Méndez-Bravo *et al.*, 2011). Los resultados del presente estudio extienden esas observaciones al mostrar que componentes de la biosíntesis y señalización del ácido jasmónico incluyendo JAR1, COI1 y MYC2 median la acumulación del óxido nítrico en la raíz primaria.

No obstante, las mutantes relacionadas al óxido nítrico *nia1 nia2* y *Atnoa1* fueron indistinguibles en las respuestas de arquitectura de la raíz al ácido jasmónico cuando se compararon con las plantas silvestres. Lo encontrado en la doble mutante fue algo sorprendente, porque la actividad de las nitrato reductasas (NRs) codificadas por los genes NIA1 y NIA2 es una importante fuente de óxido nítrico para impulsar el crecimiento y desarrollo vegetal (Park *et al.*, 2011), por lo que una ruta alterna a las NRs, como la hipotetizada ruta oxidativa dependiente de la sintasa de óxido nítrico (NOS) (Santolini *et al.*, 2017), podría permitir la respuesta en las mutantes dobles *nia1 nia2*, que manifiestan niveles disminuidos. En el caso de *Atnoa1*, nuestros datos sugieren que el gen *AtNOA1* no está involucrado en las respuestas de la arquitectura de la raíz al ácido jasmónico.

El óxido nítrico interactúa de una manera órgano y tejido específico con distintas rutas hormonales para orquestar el crecimiento y defensa vegetal. Una mayor formación de raíces laterales puede ser explicada debido a la función positiva del óxido nítrico activando a las células del periciclo de la raíz o vía la promoción de la señalización de auxinas en los primodios de raíces laterales (Schlicht *et al.*, 2013), debido a que los donadores de óxido nítrico incrementan la expresión de genes dependientes de auxinas mediante la degradación de los represores Aux/IAAs (Terrile *et al.*, 2012). Además, la S-nitrosilación del receptor nuclear de auxinas TIR1 aumenta la interacción de TIR1-Aux/IAA, facilitando la degradación de los Aux/IAAs y la subsecuente activación de la expresión de genes (Terrile *et al.*, 2012).

Por otra parte, se encontró que el óxido nítrico (NO) induce la expresión de *JAZ1* en el cilindro vascular y el córtex en la zona de diferenciación de la raíz, pero no en la punta de la raíz primaria, además el NO también incrementó la expresión de *JAZ10* en los primordios de raíz lateral y en el protoxilema de la raíz primaria. De esta manera, concluímos que *JAZ1* y *JAZ10* tienen dominios de expresión diferentes en la raíz. Debido a que *JAZ1* es inducible por ácido jasmónico (Grunewald *et al.*, 2009), nuestros resultados son consistentes con el papel del óxido nítrico como mediador en la ruta de señalización del ácido jasmónico, probablemente involucrado en la formación y/o elongación de raíces laterales.

Siguiendo la observación de que la mutante del receptor *coi1-1* fue resistente al efecto represor del SNP (donador de óxido nítrico) en las raíces primarias, hipotetizamos que las mutaciones que confieren resistencia al ácido jasmónico harían plantas menos sensibles a la acumulación de óxido nítrico. Debido a que el ácido jasmónico y el etileno tienen blancos comunes para la regulación de la expresión de genes, se comparó el crecimiento de la raíz primaria de plantas silvestres con el de las mutantes *ein2-1*, *ein2-1 jar1-1*. Interesantemente, la pérdida de la función de *EIN2* confiere a las plantas resistencia al ácido jasmónico y al SNP respecto a la inhibición del crecimiento de la raíz primaria. Esto sugiere que *EIN2* puede ser parte de una ruta de percepción de óxido nítrico, no solo porque interviene en la respuesta al etileno en la raíz, sino porque datos recientes demostraron su papel sobre la degradación dirigida de los factores de respuesta a etileno (ERFs), los cuales actúan como reguladores transcripcionales específicos en la planta (Gibbs *et al.*, 2014). Además, se ha reportado que el óxido nítrico por sí mismo induce la producción de etileno durante la así llamada respuesta hipersensible, la cual permite a las plantas resistir el ataque por patógenos (Mur *et al.*, 2008).

Recientemente, la muerte celular inducida por nitrito fue correlacionada con las señalizaciones del óxido nítrico y el H_2O_2 y la disminución en los niveles celulares de antioxidantes (Karsten *et al.*, 2016). Dos tipos de experimentos prueban que las ROS (incluido el H_2O_2) no actúan como moduladores durante la reprogramación de la raíz inducida por el ácido jasmónico. Primero, la acumulación de ROS inducida por paraquat en las plántulas silvestres ocurre normalmente en las mutantes *coi1-1*. Segundo, las mutantes *rcd1* que se encuentran afectadas en el gen *RADICAL-INDUCED CELL DEATH 1* (*RCD1*) fueron igualmente sensibles a la represión del crecimiento de la raíz primaria inducido por ácido jasmónico. *RCD1* codifica una proteína que contiene un dominio de ADP ribosil transferasa involucrado en la generación intracelular de ROS por el herbicida paraquat y/o la radiación ultravioleta B (Ahlfors *et al.*, 2004; Fujibe *et al.*, 2004). Por lo tanto, aunque el óxido nítrico y las especies reactivas de oxígeno pueden tener blancos comunes en la señalización por estrés vegetal, las respuestas sobre el desarrollo de la raíz detonadas por el ácido jasmónico dependen más de la acumulación de óxido nítrico, enlazando elementos de la ruta de transducción de señales del ácido jasmónico y del etileno vía la función de *ETHYLENE INSENSITIVE 2*. Los resultados de esta investigación subrayan la importancia del óxido nítrico en las rutas de señalización de fitohormonas y resaltan su papel como un segundo mensajero durante la configuración de la arquitectura del sistema radical.

Por otra parte, la función de las vitaminas es de gran interés no solo en animales sino también en plantas debido a su su química redox y su papel como cofactores de enzimas, los cuales son esenciales para el metabolismo. La vitamina B9, también conocida como ácido fólico (FA) o folato, es una vitamina hidrosoluble con papeles emergentes e importantes en la morfogénesis vegetal (Srivastava *et al.*, 2011; Reyes-Hernández *et al.*, 2014). Evidencia genética indicó que una biosíntesis alterada del ácido fólico causó la pérdida del meristemo de la raíz primaria e indujo la diferenciación celular, pero los efectos de su aplicación farmacológica a alguna especie de planta era desconocida.

En el presente estudio, se mostró que la suplementación de ácido fólico altera el crecimiento y desarrollo de la raíz en plántulas de *Arabidopsis thaliana* creciendo *in vitro*. Bajo las condiciones experimentales empleadas en esta investigación, la raíz primaria crece indeterminadamente y el meristemo de la raíz permanece altamente activo proveyendo las células requeridas para sostener el crecimiento de la raíz. Sin embargo, contrario a las expectativas, el ácido fólico suplementado exógenamente inhibió el crecimiento de la raíz primaria mientras que promueve la formación de raíces laterales.

El análisis de expresión de genes del ciclo celular CYCB1 y CYCB6 así como mediciones celulares detalladas, indicaron que la inhibición en el crecimiento de la raíz primaria probablemente ocurre debido a la inhibición de la elongación celular en lugar de una división celular alterada, debido a que los meristemos de la raíz primaria presentaron más células mitóticamente activas y expresaron *CycB1;1:uidA* y *pCYCD6;1:GFP* en niveles más altos. Esos resultados concuerdan con investigaciones previas (Srivastava *et al.*, 2011; Reyes-Hernández *et al.*, 2014). Los datos reportados aquí demuestran que la elongación celular está fuertemente afectada por el ácido fólico y de esta manera los cambios adicionales en la arquitectura del sistema radical pueden ser explicados no solo por su papel promotor sobre la división celular, sino por el balance entre la división y la elongación de las células, debido a que dicho balance está estrechamente conectado con la señalización de auxinas. El análisis de la expresión de *DR5:GFP* en la punta de la raíz primaria de *Arabidopsis* indicó un cambio en el patrón del marcador inducible por auxinas en los tratamientos con ácido fólico, el cual se localizó fuertemente en las células iniciales y el centro quiescente, correlacionando con el acortamiento de la raíz primaria. Además, un incremento en la expresión de este reportero de auxinas ocurrió en el tejido vascular a través de la zona de diferenciación de la raíz primaria, lo cual implica directamente que más auxinas están alcanzando la región de elongación y con esto, reprimen la expansión celular, además de activar las células del periciclo para dividirse y formar raíces laterales.

La formación de raíces laterales se puede dividir en dos fases principales: i) la iniciación de primordios de raíces laterales (LRP), la cual consiste de varias etapas del desarrollo; y ii) la emergencia a partir de la raíz primaria parental (Malamy y Benfey, 1997). Un análisis del tipo y cantidad de LRP indica que el ácido fólico promueve la maduración de las raíces laterales porque al incrementar la concentración del compuesto se presentaron más primordios avanzando hacia las últimas etapas del desarrollo, mientras que disminuyó su número total en la raíz primaria. Consecuentemente, el estudio de la expresión de *DR5:GFP* mostró la activación de la ruta de señalización de auxinas en los tejidos vasculares y los primordios de raíces laterales en respuesta al ácido fólico, sugiriendo que dicha ruta es la responsable de la maduración acelerada de dichas estructuras. A través de una comparación detallada del crecimiento de la raíz primaria y la formación de raíces laterales en plántulas silvestres y mutantes relacionadas a auxinas, se encontró que las líneas *slr1* y *arf7 arf19* son parcialmente resistentes a los efectos represores del crecimiento del FA en la raíz primaria. La resistencia significativa observada en las mutantes nos condujo a proponer que la bioactividad del ácido fólico requiere directa o indirectamente de los productos proteínicos de los genes

IAA14, *ARF7* y *ARF19*, los cuales pueden funcionar localmente en el meristemo y/o las regiones de elongación celular. La triple mutante *tir1 afb2 afb3* no mostró un comportamiento diferencial a las plántulas silvestres sobre el crecimiento de la raíz primaria, lo cual se puede explicar porque la familia TIR1/AFB de receptores de auxinas involucra al menos cinco miembros con funciones redundantes potenciales (Parry *et al.*, 2009), de esta manera, otras proteínas AFB funcionales pueden complementar la respuesta. Considerando el desarrollo de las raíces laterales, es notorio que la ruta de señalización de auxinas a partir de los receptores (TIR1 AFB2 AFB3) involucrando al módulo de represor/factor transcripcional (IAA14/ARF7 y ARF19), es necesaria para el desarrollo de raíces laterales inducido por el ácido fólico.

El crecimiento de organismos multicelulares se determina por los procesos de división y expansión celular, que están estrechamente vinculados con la señalización de TOR. La cinasa TOR es un regulador maestro del crecimiento, evolutivamente conservado entre levaduras, plantas, animales y humanos. Esta proteína integra señales energéticas y nutricionales para promover el crecimiento y la proliferación celular. Información reciente indica el requerimiento de niveles altos de auxinas para que la ruta TOR proceda, proceso que difiere entre los meristemos de la raíz y el follaje (Li *et al.*, 2017). Para entender la relación entre los efectos del ácido fólico con la ruta metabólica dependiente de TOR, se investigó la respuesta en plantas silvestres y la línea *tor-es1* de *Arabidopsis*, en la cual se bloquea la síntesis de la proteína TOR a nivel de mensajero una vez que se suplementa estradiol. Se encontró que la producción de biomasa, el crecimiento de la raíz primaria y la formación de raíces laterales fueron fuertemente inhibidas en los medios suplementados con estradiol. Sorprendentemente, la aplicación de ácido fólico normalizó el crecimiento y restauró la formación de raíces laterales en la *línea tor-es1*, por lo que hipotetizamos que TOR y el ácido fólico pueden actuar corriente abajo de la señalización de auxinas.

Las plantas y las bacterias se comunican a través de *N*-acil-homoserina lactonas (AHLs), donde estas últimas regulan la arquitectura del sistema radical e influyen en el crecimiento y desarrollo de las plantas. Ortiz-Castro y col. (2008) investigaron los cambios en la arquitectura radical de *Arabidopsis thaliana* ante la aplicación de AHLs comercialmente disponibles que van de 4 a 14 carbonos de longitud, encontrando que la *N*-decanoil-L-homoserina lactona (C10-HL) es el compuesto más activo reprimiendo el crecimiento de la raíz primaria, donde dicho fenómeno fue relacionado a la inhibición de la división celular y la promoción de la diferenciación de las células. Además, el aislamiento de las mutantes *drr1* de *Arabidopsis* resistentes a C10-HL sugirió que esta señal de quorum sensing (QS) acelera los programas de desarrollo y la senescencia, debido a que las mutantes mostraron floración tardía y aumentaron su período de crecimiento vegetativo (Morquecho-Contreras *et al.*, 2010).

El papel *in vivo* de la detección del quorum sensing para el funcionamiento vegetal es poco conocido, no obstante, datos recientes acerca de la interacción entre las rizobacterias promotoras del crecimiento vegetal (PGPR) Gram negativas *Burkholderia phytofirmans* PsJN y *Arabidopsis thaliana* durante el ciclo de vida de la planta, demostraron que las bacterias aceleraron la floración y redujeron el periodo de crecimiento vegetativo; estas modificaciones se correlacionaron con la inducción en la expresión de los genes de control de la floración (Poupin *et al.*, 2013). Adicionalmente, cuando las plántulas de *Arabidopsis* fueron inoculadas con *Gluconacetobacter diazotrophicus,* un endófito de raíz, la promoción del crecimiento pudo ser observada hasta por 50 días, lo cual correlacionó con una fotosíntesis más alta, menor transpiración vegetal y una incrementada eficiencia en el uso del agua, aspectos relacionados a la señalización del ácido abscísico (ABA) (Rangel de Souza *et al.*, 2016). De esta manera, la inoculación con bacterias que pueden producir y liberar AHLs podría afectar el ciclo de vida completo de una planta, acelerando su índice de crecimiento, mejorando la fotosíntesis y la eficiencia en el uso del agua, efectos altamente relevantes para la mayoría de los cultivos. Nuestros datos indican que al menos en parte, la influencia de las AHLs sobre los procesos funcionales de la planta pueden estar relacionados a su regulación de la señalización del ABA.

Las AHLs comparten similitud química estructural con las alcamidas y las *N*-acil-etanolaminas (NAEs), compuestos naturalmente producidos por las plantas con interesantes propiedades. Aunque la identidad molecular de DRR1 es actualmente desconocida, su pérdida de función en *Arabidopsis* causó sensibilidad incrementada a los efectos represores del ABA en el crecimiento de la raíz. Esto indica que DRR1 genéticamente regula la señalización del ABA, influenciando el tamaño y la longevidad de las plantas de acuerdo al fenotipo reportado de la mutante *drr1* crecida en suelo (Morquecho-Contreras *et al.*, 2010).

En este trabajo, definimos genéticamente que entre los genes relacionados con el ABA, *ABI5* (codificando para un factor de transcripción de la ruta de señalización del ABA) media el efecto represor del ABA sobre el crecimiento de la raíz primaria, ya que la mutante *abi5* correspondiente es resistente a la aplicación farmacológica del ABA. Las dobles mutantes *drr1/abi5* también revelaron que DRR1 puede actuar corriente arriba de ABI5 en la ruta de señalización del ABA, ya que la hipersensibilidad de la raíz al ABA de las mutantes *drr1* se revirtió en las dobles mutantes, resultando un fenotipo más parecido al de las plántulas silvestres en lugar de uno como el de *abi5*, proponiendo que además de ABI5, existen otros elementos de la ruta de señalización del ABA involucrados en el proceso.

Las AHLs de cadena media inducen fuertemente la formación de raíces laterales. Debido a que las plántulas *drr1* exhiben respuestas al ABA, y este último regula negativamente la formación de raíces laterales (De Smet *et al.*, 2003), entonces es posible que el fenotipo reportado de pocas raíces laterales en *drr1* dependiera sobre una sensibilidad incrementada al ABA. No obstante, nuestros datos muestran que a diferencia del crecimiento de la raíz primaria, la formación de raíces laterales no necesariamente involucra a ABI5 debido a que las plántulas silvestres y mutantes *abi5* se comportan similarmente, formando números incrementados de raíces laterales en respuesta a los tratamientos con C10-HL y porque la mutación con pérdida de la función en *ABI5* no altera la formación de raíces laterales en respuesta a la C10-HL en las dobles mutantes *drr1/abi5*.

La ruta de señalización del ABA influye sobre varios factores de transcripción que activan la expresión de genes en un contexto especifico de tejido y desarrollo.

Particularmente, ABI4 regula la arquitectura del sistema radical inhibiendo la formación de raíces laterales (Shkolnik-Inbar y Bar-Zvi, 2010). Nuestros datos son consistentes con resultados previos debido a que la expresión de *ABI4:GUS* en las raíces incrementó en tratamientos con ABA en plántulas silvestres, en tanto que las mutantes *drr1* mostraron una expresión de *ABI4:GUS* exacerbada, por lo tanto, la sensibilidad incrementada al ABA de *drr1* se correlaciona con una mayor expresión de *ABI4,* siendo este último el que origina al factor de transcripción ABI4. Bossi y colaboradores en (2009) reportaron que ABI4 es un activador esencial de la expresión del gen que lo codifica durante el desarrollo, actuando en la señalización del ABA y en las respuestas a azúcares además de inducir la expresión de *ABI5*. En este sentido, una expresión mayor de *ABI4:GUS* daría lugar a más factores de transcripción siendo reclutados para la ruta de señalización del ABA de forma dependiente de DRR1 y por lo tanto de las AHLs.

Arabidopsis thaliana exhibe latencia de la semilla, permitiendo a las semillas en el ambiente natural sobrevivir a las condiciones adversas y germinar cuando las circunstancias sean apropiadas. El grado de latencia de la semilla se refleja en el porcentaje de germinación de una población de semillas y las mutantes deficientes en la hormona ABA están entre las más no latentes, o dicho de otra forma, son mutantes de germinación temprana en la mayoría de las especies vegetales. Consistentemente, encontramos que la tasa de germinación de *abi5* fue mayor que la de las plántulas silvestres. En contraste, las mutantes *drr1* mostraron una germinación retrasada. Por su parte, las mutantes dobles *drr1/abi5* no mostraron un fenotipo como el de *abi5*, su germinación fue similar al tipo silvestre y diferente de las mutantes sencillas *abi5* o *drr1*. Esto confirma la hipótesis de que DRR1 y ABI5 actúan en la misma ruta.

En los ecosistemas naturales, las semillas germinan en presencia de una amplia gama de microorganismos y el microbioma del suelo puede ser crucial para la germinación y el crecimiento vegetal temprano. No obstante, según nuestro conocimiento, ningún estudio ha investigado los efectos de bacterias produciendo AHLs sobre la germinación. Este reporte muestra que las AHLs pueden ser parte de una red de señalización determinando no solo la latencia de la semilla sino también otros aspectos funcionales posibles relacionados al ABA tales como el estrés y la senescencia, por lo que los papeles ecológicos de tales interacciones moleculares ameritan investigaciones adicionales.

8. REFERENCIAS

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9. ADENDA

En este apartado se presentan las publicaciones que se originaron a partir de investigación adicional a este trabajo, además de una codirección de tesis de licenciatura.

9.1. Artículos publicados en revistas con arbitraje internacional

1. Raya-González J., Velázquez-Becerra C., **Barrera-Ortiz S.**, López-Bucio J., Valencia-Cantero E., (2017) *N,N*-dimethyl hexadecylamine and related amines regulate root morphogenesis via jasmonic acid signaling in *Arabidopsis thaliana*. Protoplasma 254: 1339-1410.
2. Garnica-Vergara A., **Barrera-Ortiz S**., Muñoz-Parra E., Raya-González J., Méndez-Bravo A., Macías-Rodríguez L., Ruiz-Herrera L. F., López-Bucio J., (2016) The volatile 6-pentyl-2H-pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and *ETHYLENE INSENSITIVE 2* functioning. New Phytologist 209: 1496-1512.

9.3. Artículos publicados en revistas con arbitraje nacional

1. **Barrera Ortiz S.**, López Bucio J., (2017) Una invasión (o colaboración) organizada en la Naturaleza. Boletín de Divulgación Científica K'ah ólaal (Conocer) Año 6 número 2: 15-18.

9.4. Codirección de tesis

1. Juan Angel Ayala Rodríguez. Universidad Michoacana de San Nicolás de Hidalgo. Facultad de Químico Farmacobiología. LICENCIATURA EN QUÍMICO FARMACOBIOLOGÍA. Título del trabajo: “Estudio del efecto del ácido fólico sobre el desarrollo de la raíz de *Arabidopsis thaliana*”. Fecha de titulación: 30 de mayo de 2017.

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ORIGINAL ARTICLE

N,*N*-dimethyl hexadecylamine and related amines regulate root morphogenesis via jasmonic acid signaling in *Arabidopsis thaliana*

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Abstract Plant growth-promoting rhizobacteria are natural inhabitants of roots, colonize diverse monocot and dicot species, and affect several functional traits such as root architecture, adaptation to adverse environments, and protect plants from pathogens. *N*,*N*-dimethyl-hexadecylamine (C16-DMA) is a rhizobacterial amino lipid that modulates the postembryonic development of several plants, likely as part of volatile blends. In this work, we evaluated the bioactivity of C16-DMA and other related *N*,*N*-dimethyl-amines with varied length and found that inhibition of primary root growth was related to the length of the acyl chain. C16-DMA inhibited primary root growth affecting cell division and elongation, while promoting lateral root formation and root hair growth and density in *Arabidopsis thaliana* (*Arabidopsis*) wild-type (WT) seedlings. Interestingly, C16-DMA induced the expression of the jasmonic acid (JA)-responsive gene marker *pLOX2:uidA*, while JA-related mutants *jar1*, *coi1-1*, and *myc2* affected on JA biosynthesis and perception, respectively, are compromised in C16-DMA responses. Comparison of auxin-regulated gene expression, root architectural changes in WT, and auxin-related mutants *aux1-7, tir1/afb2/afb3*, and *arf7-1/arf19-1* to C16-DMA shows that the C16-DMA effects occur independently of auxin signaling. Together, these results reveal a novel class of aminolipids modulating root organogenesis via crosstalk with the JA signaling pathway.

Keywords *N,N*-dimethyl-hexadecylamine · *Arabidopsis thaliana* · Auxin · Jasmonic acid · Root development

Introduction

When plants first colonized the land, intimate relationships with microorganisms enabled them to adapt and diversify (Beraldi-Campesi 2013). Currently, the plant microbiomes comprise thousands of bacterial and fungal species, which affect plant functional processes in many beneficial ways. Bacteria and plants recognize themselves via cross-kingdom signaling, in which bacterial quorum-sensing compounds, diffusible substances, and volatiles are emitted and perceived through complex molecular interactions (Hartmann and Schikora 2012; Mathesius et al. 2003; Ortiz-Castro et al. 2011).

N,*N*-dimethyl amines (DMAs) are amino-containing lipids, from which *N*,*N*-dimethyl-hexadecylamine (C16-DMA) was identified from a volatile organic compound (VOC) analysis from the plant growth-promoting actinobacteria *Arthrobacter agilis* UMCV2 (Velázquez-Becerra et al. 2011) (Fig. 1). C16-DMA has been found in the VOCs blend produced by different rhizobacteria, including *Bacillus subtilis* G8 (Liu et al. 2008), *Sinorhizobium meliloti* 1021 (Orozco-Mosqueda et al. 2013), and *Pseudomonas fluorescens* UM270 (Hernández-León et al. 2015), indicating that C16-DMA emission might be ubiquitous in several microorganisms. The current hypothesis is that DMAs could participate in cross-kingdom signaling while they can be directly perceived by plants to adjust functional and adaptive traits.

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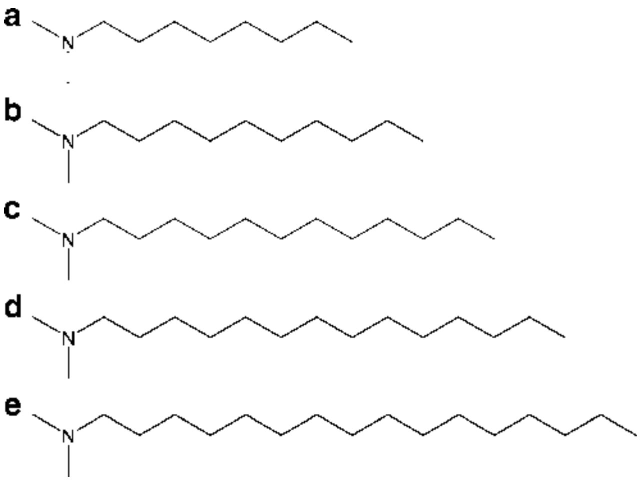

Fig. 1 Chemical structures of DMAs used in this study. **a** *N*,*N*-dimethyl-octamine (C8-DMA). **b** *N*,*N*-dimethyl-decilamine (C10-DMA). **c** *N*,*N*-dimethyl-dodecylamine (C12-DMA). **d** *N*,*N*-dimethyl-tetradecylamine (C14-DMA). **e** *N*,*N*-dimethyl-hexadecylamine (C16-DMA)

C16-DMA has been found to modulate growth and development in evolutionary distant plant species, such as *Medicago sativa*, *Sorghum bicolor*, and *Pinus devoniana*, affecting shoot biomass, stem length, chlorophyll production, and root system architecture (RSA) (Velázquez-Becerra et al. 2011; Valencia-Cantero et al. 2015; Castulo-Rubio et al. 2015). Interestingly, C16-DMA increased root ferric reductase (FRO) gene expression in leaves, suggesting an important role of this amine in modulating Fe nutritional status in aerial tissues (Castulo-Rubio et al. 2015). On the other hand, C16-DMA inhibits the growth of phythopathogens such as *Botrytis cinerea* and *Phytophthora cinnammomi*, which may lead to possible biotechnological applications consistent with their plant growth-promoting activity (Velázquez-Becerra et al. 2013).

Plants produce molecules with structural similarity to DMAs, including alkamides and *N*-acyl ethanolamines (NAEs). These compounds have been considered a novel class of plant growth regulators because of their wide distribution in plants and their potent biological activities (Chapman et al. 1998; López-Bucio et al. 2007; Morquecho-Contreras and López-Bucio 2007; Ortiz-Castro et al. 2010). Blancaflor et al. (2003) showed that application of micromolar concentrations of *N*-lauroylethanolamide (NAE12:0) to *Arabidopsis* seedlings inhibited primary root (PR) growth and stimulated lateral root formation. Like NAEs, plant alkamides can alter root and shoot development by modulating cell division and differentiation programs. Root growth, lateral and adventitious root formation, root hair production, and leaf development are regulated by alkamides via nitric oxide signaling involving the activity of the nitrate reductases NIA1 and NIA2, and several *Arabidopsis* mutants have been identified that define novel mechanisms of perception for these molecules (Ramírez-Chávez et al. 2004; López-Bucio et al. 2007; Campos-Cuevas et al. 2008; Méndez-Bravo et al. 2010; Morquecho-Contreras et al. 2010). Other amino compound-containing lipids are *N*-acyl-homoserine lactones (AHLs), which are produced by Gram negative bacteria as part of an intra and interspecific cell-cell communication mechanism (Keller and Surette 2006). Diverse gram negative bacteria produce different AHLs; these compounds contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain. The acyl groups of naturally occurring AHLs range from 4 to 18 carbons in length (Camilli and Bassler 2006), and drive effects on plants that vary in intensity with the length of the acyl group (Ortiz-Castro et al. 2008; Schikora et al. 2016). All these data show that the amino lipid family of bioactive natural compounds is very rich, and that many of these molecules may be acting for inter-kingdom communication.

Although diverse effects of C16-DMA on crop species have been reported, a detailed characterization of its signaling pathways is lacking, which make necessary the use of a plant species amenable to genetic analysis. The aim of this work was to explore the cellular basis of *Arabidopsis* root responses to C16-DMA and to compare the bioactivity of DMAs with varied chain length in regulating root growth. Our results provide physiological, genetic, and molecular evidence that C16-DMA modulate root development via crosstalk with jasmonic acid signaling.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 (wild-type), the transgenic *Arabidopsis* line *pLOX2:uidA* (Jensen et al. 2002), and mutant lines *coi1-1* (Feys et al. 1994), *jar1* (Staswick et al. 1992), *myc2* (SALK_017005), *aux1-7* (Picket et al. 1990), *tir1 afb2 afb3* (Parry et al. 2009), and *arf7 arf19* (Wilmoth et al. 2005) were used for the different experiments. Seeds were surface sterilized with 95 % (*v*/*v*) ethanol for 5 min and 20 % (*v*/*v*) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, 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 m^{-2} s^{-1}, and temperature of 22 °C.

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, 500 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 jasmonic acid (JA) by placing seeds on 100 cm^2 nutrient agar plates (20 seeds per plate). The seeds were distributed in two rows on the agar surface at a density of 1 seed/cm, stratified at 4 °C for 48 h, and then incubated at 22 °C. Putative JA-resistant mutants with long roots were selected and transferred to plates with the different treatments.

Generation of *coi1-1*/*pLOX2:uidA* line

For crossing the lines, *pLOX2:uidA* and *coi1-1*/COI1 seeds were disinfected and germinated on 0.2× MS medium. For *coi1-1* mutant selection, 100 seedlings from a *coi1-1*/COI1 segregating population were screened for sustained PR growth in agar solidified MS 0.2× medium supplemented with 4 μM jasmonic acid by placing seeds on nutrient agar plates (Fig. S2). The seeds were distributed in two rows on the agar surface, stratified at 4 °C for 48 h, and then incubated at 22 °C. Putative JA-resistant mutants with long PRs were selected, transferred to soil, and individual flowers were pollinated using pollen from soil grown *pLOX2:uidA* plants. F1 seedlings derived from the cross *coi1-1* × *pLOX2:uidA* showed WT response to JA treatment and were transferred to soil and allowed to self-fertilize. F2 seedlings were analyzed in MS 0.2× agar medium supplied with 4 μM JA. A typical 3:1 recessive segregation was observed for the wild-type/*coi1-1* phenotype, and homozygous *coi1*/*coi1* mutants sustained PR growth in JA supplied medium. Co-segregation of primary root growth resistance to JA and GUS expression was confirmed in F2 populations.

Chemicals

Jasmonic acid, *N*,*N*-dimethyl-octamine (C8-DMA), *N*,*N*-dimethyl-decilamine (C10-DMA), *N*,*N*-dimethyl-dodecylamine (C12-DMA), *N*,*N*-dimethyl-tetradecylamine (C14-DMA), and *N*,*N*-dimethyl-hexadecylamine (C16-DMA) were purchased from the Sigma company, kept at 4 °C, and dissolved in ethanol at the moment of use. In control seedlings, we added the solvents in equal amounts as present in the greatest concentration of each compound tested (Fig. 1).

Analysis of growth

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. Root hairs were measured in a 500-μm region from the PR tip. The average length of root hairs was determined upon measuring 100 hairs, taking as a reference the root protoxylematic plane to locate the radical hair base in the epidermic cell. Images were captured with a Samsung SCC 131A digital color camera adapted to the microscope. Primary root length was determined for each root using a ruler. Lateral root number was determined by counting the mature roots per seedling, and their corresponding density was determined by dividing the lateral root number value by the primary root length for each seedling. Root hair length was determined employing the Image J 1.49v software (http://imagej.nih.gov/ij). For transfer assays, the primary root length and lateral root number and density were determined from the tip to the marked site of the root tip when the transfer was made. For all experiments with WT (Col-0) seedlings, transgenic lines, and mutant lines, the overall data were statistically analyzed using the SPSS 10 program. Univariate and multivariate analyses with Tukey's post hoc test were used for testing differences in growth and root development responses. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Histochemical analysis

For histochemical analysis of GUS activity, *Arabidopsis* seedlings were stained and incubated overnight at 37 °C in a GUS reaction buffer (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained plants were cleared and fixed with 0.24 N HCl in 20 % methanol (*v*/*v*) and incubated for 60 min at 62 °C. The solution was substituted by 7 % NaOH (*w*/*v*) in 60 % ethanol (*v*/*v*) for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40, 20, and 10 % (*v*/*v*) for a 30-min period each, and fixed in 50 % glycerol (*v*/*v*). For each marker line and for each treatment, at least 15 transgenic plants were analyzed.

Results

DMAs modify *Arabidopsis* root system architecture

The rhizobacteria *Arthrobacter agilis* UMCV2 produces different DMAs with biological activity (Velázquez-Becerra et al. 2013) (Fig. 1). To investigate the mechanism of action of DMAs, we first evaluated the *Arabidopsis* root developmental response to DMAs with acyl side chain ranging from 8 to 16 carbons in length (Fig. 1). *Arabidopsis* seedlings were germinated and grown on 0.2× MS agar media supplemented with DMAs concentrations from 1 to 6 μM and primary root growth was recorded 10 days after germination (d.a.g.). We found that DMAs effects were strongly dependent on the acyl side length and the concentration supplied to the plant growth medium (Figs. 2a and S1). Small chain (C8-DMA) did not significantly affect primary root growth. Medium-chain (C10-DMA) showed a dose-dependent inhibitory effect on root growth, while larger chain (C12 to C16) DMAs showed the greatest biological activity (Figs. 2a and S1). Interestingly, 3 μM DMAs (C12-C16) caused more than 80 % reduction of primary root length and greater concentrations halted growth (Fig. 2a and S1). These results indicate that the acyl

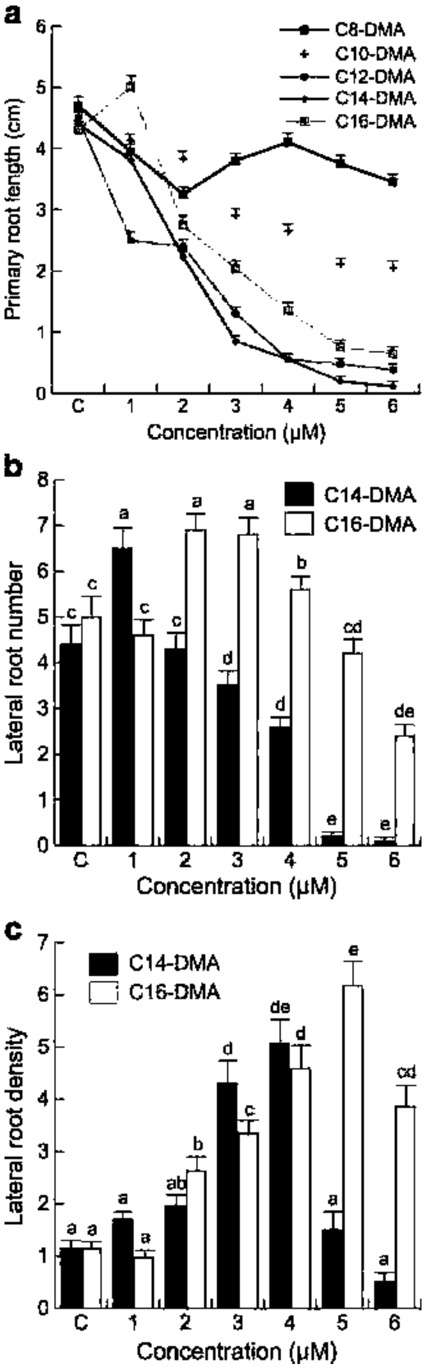

Fig. 2 Effect of DMAs on *Arabidopsis* root architecture. *Arabidopsis* seedlings were germinated and grown on MS 0.2× MS agar medium for 10 days and root phenotype was evaluated. **a** Primary root length in response to DMAs with varied chain length. **b** Lateral root number for C14 and C16-DMA. **c** Lateral root density (lateral root number/primary root length). *Error bars* represent SE from 30 seedlings. Note that C16 in low concentrations has a stimulatory effect on PR growth. *Different letters* indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results

side chain plays a key role in DMAs activity on plant development. The decrease of root length by DMAs treatments was accompanied by a shift on developmental patterning to form a highly branched, exploratory root system (Fig. S1). Further comparative analysis of C14-DMA and C16-DMA showed similar effects on *Arabidopsis* RSA (Fig. 2b), increasing lateral root number up 30 % at 2 μM (Fig. 2b), and promoting lateral root density by 600 % compared with control seedlings supplied with the solvent only (Fig. 2c). Taken together, these results indicate that DMAs regulate the *Arabidopsis* root morphogenesis program and reprograms root architectural traits.

C16-DMA regulates root hair growth

Root hairs are tip-growing extensions from root epidermal cells that play important roles in nutrient and water uptake and in plant-soil interactions. Given that C16-DMA is naturally produced by several rhizobacteria, including *Arthrobacter agilis* UMCV2, and promotes root development, we characterized further the C16-DMA root hair response. Thus, experiments were performed in which *Arabidopsis* WT (Col-0) seedlings were germinated and grown on the surface of agar plates containing different concentrations of C16-DMA. In the concentration interval (1–5 μM) tested, C16-DMA not only increased root hair length (Fig. 3a, c) but also modulated root hair density in a concentration-dependent manner (Fig. 3b, c). Increments close to 200 % in both parameters were observed with 3 μM C16-DMA.

To determine if the shorter primary root and increased root hair density in response to C16-DMA could be caused by diminished cell division and/or elongation, the expression of genes involved in cell division (*pCycB1:uidA*), and epidermal cell differentiation (*AtExp7:uidA*), was monitored along with detailed cell measurements in *Arabidopsis* roots grown under increasing C16-DMA supply. The expression domain of *pCycB1:uidA* and the number of meristematic cells expressing this mitotic marker decrease with C16-DMA concentration (Fig. 4a–c). *AtExp7:uidA* expression and trichoblast (epidermal cells that differentiate into root hairs) cell measurements further indicate that the primary root growth is inhibited because of an altered cell division and elongation program (Fig. 5a–c). Therefore, root hair density increases likely because C16-DMA drastically inhibits cell elongation. The notion that cell differentiation processes concomitantly occur at the root tip is substantiated not only because root hairs are forming close to the root meristem zone but also because xylem differentiation advances towards this region (Fig. 4c, asterisks).

C16-DMA responses involve the jasmonic acid signaling pathway

Several rhizobacteria may induce coordinated developmental and defense responses in plants, both actions are typically mediated by the phytohormone jasmonic acid (Wasternack 2007; Sun et al. 2009; Raya-González et al. 2012). Therefore, we asked if C16-DMA could activate JA-gene expression by

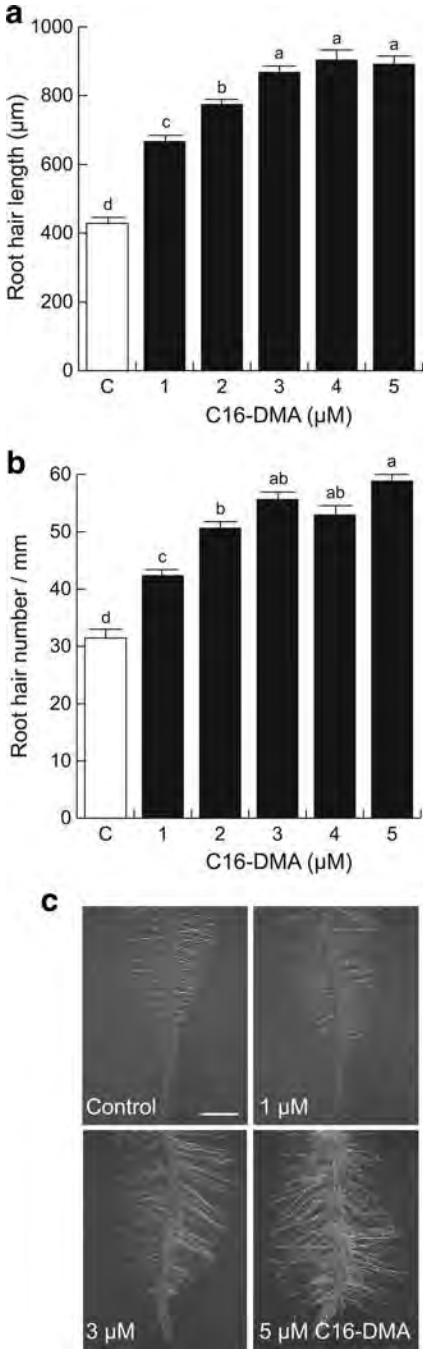

Fig. 3 C16-DMA regulates root hair growth. *Arabidopsis* seedlings were germinated and grown for 4 days on 0.2× MS agar medium with or without C16-DMA and root hair development was evaluated. **a** Root hair length. **b** Root hair number/mm of PR. **c** Representative photographs of root hairs formed at the PR tip region of 4-day-old *Arabidopsis* seedlings grown in the presence of the indicated concentrations of C16-DMA. *Error bars* represent SE from 100 root hairs. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. *Scale bar* = 200 µm

testing the effect of C16-DMA on the expression of the JA-responsive gene marker, *pLOX2:uidA*. *Arabidopsis* lines harboring the *pLOX2:uidA* gene construct (Jensen et al. 2002) were germinated and grown on 0.2× MS agar media supplemented with C16-DMA or JA. Ten days after germination, plants were stained for β-glucoronidase activity and cleared to show gene expression. As expected, 4 µM JA clearly induced *pLOX2:uidA* expression (Fig. 6). Interestingly, increased expression of this marker was observed throughout the shoot in C16-DMA-treated seedlings in a dose-dependent manner (Fig. 6). This suggests that C16-DMA activates JA-responsive gene expression.

The inhibitory effect of jasmonic acid on root growth has been well recognized and widely employed as a useful trait to identify jasmonate-related signaling components in *Arabidopsis*. To further determine if the JA pathway is involved in root system configuration in response to C16-DMA, we evaluated the C16-DMA effects in *Arabidopsis* WT (Col-0) and the *jar1* mutant, which is affected in JA biosynthesis. The C16-DMA effects on primary root growth and lateral root formation over a range of concentrations of this compound indicates that *jar1* mutants are resistant to root growth inhibition induced by C16-DMA (Fig. 7a, d). In wild-type plants, 3 µM C16-DMA inhibited 75 % root growth, whereas in *jar1* mutant, the inhibition was of about 15 % (Fig. 7a). When we analyzed lateral root induction, WT and *jar1* seedlings had a similar response to C16-DMA (Fig. 7b). However, clear differences were observed regarding lateral root density as a consequence of strong primary root growth repression (Fig. 7c). These results suggest that JAR1 is involved in C16-DMA responses on primary root growth.

C16-DMA requires COI1 and MYC2 to modulate root development

To determine if other jasmonic acid-signaling elements are involved in C16-DMA activity, we tested C16-DMA effects on *coi1-1* and *myc2* mutants, which are altered on JA perception. WT seedlings were germinated and grown for 4 days on 0.2× MS medium, while homozygous *coi1-1* mutant were selected from a *coi1-1/COI1* segregating population in medium supplemented with 4 µM JA (Fig. S2). Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates supplied or not with C16-DMA, and root architecture was analyzed 8 days after transfer. WT seedlings grown at 3 µM C16-DMA showed a discrete inhibition of root growth compared to control conditions, but *coi1-1* did not (Fig. 8a, b). However, at greater concentrations, the C16-DMA effects were indistinguishable between WT and *coi1-1* seedlings on root growth (Fig. 8a). As established above, C16-DMA increased lateral root formation in WT seedlings in a dose-dependent manner but this effect was strongly reduced in *coi1-1* mutants at all C16-DMA concentrations evaluated (Fig. 8b). At strongly

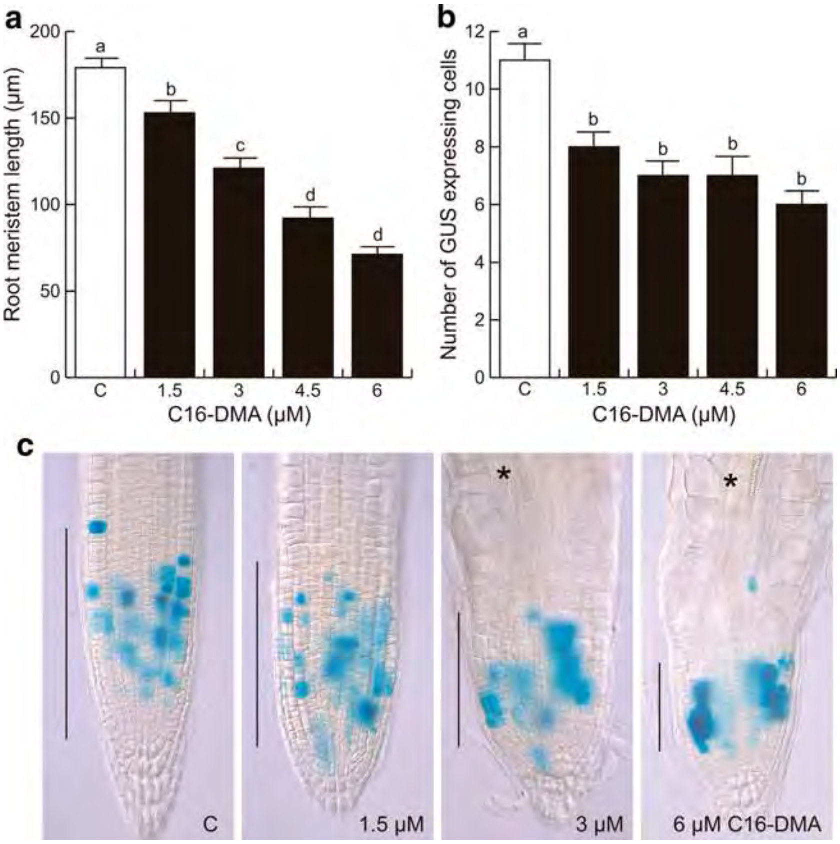

Fig. 4 C16-DMA alters cell division. **a** Root meristem length. **b** Number of *pCycB1:uidA* expressing cells. **c** Representative photographs of *pCycB1:uidA* expression in root meristems of 5-day-old seedlings in response to increasing C16-DMA concentrations. *Error bars* represent SE from 15 independent roots. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. *Scale bar* shows the *pCycB1:uidA* expression domain measured in panel (**a**)

growth-repressing concentrations of the compound, healthier shoots are evident in the mutants compared to the WT (Fig. S3). On the other side, root architectural comparisons between WT and *myc2* mutant seedlings revealed a slight resistance of the mutants to the bacterial amino lipid (Fig. S4).

To further clarify the role of COI1 in C16-DMA-induced jasmonic acid response in shoots, *pLOX2:uidA* seedlings were germinated and grown for 4 days on 0.2× MS medium, and homozygous *coi1-1/pLOX2:uidA* seedlings were selected from a *coi1-1/COI1* segregating population in medium supplemented with 4 µM JA. Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates supplied or not with C16-DMA. Eight days after transfer, plants were stained for β-glucoronidase activity and cleared to show gene expression. Under control conditions, *pLOX2:uidA* showed a basal expression in cotyledons and true leaves (Fig. 9a), which was increased in plants treated with C16-DMA in a dose-dependent manner (Fig. 9a–c). In the same way, *coi1-1/pLOX2:uidA* control plants showed a very slight *pLOX2:uidA* expression that also increased in response to C16-DMA, but in lower magnitude (Fig. 9d–f). Taken together, these results indicate that COI1 activates JA-responsive gene expression to modulate the root developmental response to C16-DMA.

C16-DMA controls root architecture via an auxin-independent mechanism

Auxin signaling is a major player for root development and some interactions with other phytohormones such as jasmonic acid have been described for regulation of primary and lateral root growth (Raya-González et al. 2012). To evaluate a possible interaction between C16-DMA and auxin signaling in root development, we performed analysis of auxin-regulated gene expression in *Arabidopsis* transgenic seedlings harboring the *DR5:uidA* gene marker and compared the root architectural changes of WT and auxin-related mutants defective in auxin transport, signaling, and/or perception, *aux1-7*, *arf7 arf19*, and *tir1 afb2 afb3*, single, double, and triple mutants, respectively, to C16-DMA. Seeds were germinated and grown for 10 days in 0.2× MS medium supplemented with or without C16-DMA, and the root system architecture analyzed. The analysis of auxin response using the *DR5:uidA* line did not show increased auxin responsiveness at the root tip by C16-DMA (Fig. 10a). Auxin-related mutants did not show differences on primary root length when compared to WT seedlings in response to 4.5 and 6 µM C16-DMA (Fig. 10b), while *tir1 afb2 afb3* mutants, which fail to produce lateral roots in control conditions (Dharmasiri et al. 2005; Wilmoth et al. 2005), were able to produce some roots under C16-DMA treatment. For instance, *aux1-7* mutants produced twofold more lateral

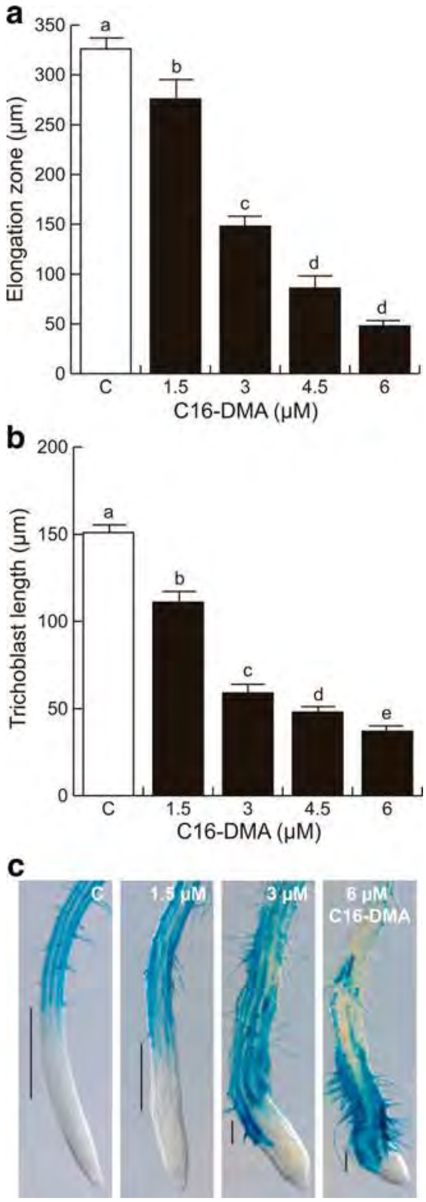

Fig. 5 C16-DMA inhibits cell elongation. **a** Length of the elongation zone of the primary root. **b** Length of trichoblast cells. **c** Representative photographs of *AtExp7:uidA* expression in root tips of 5-day-old seedlings in response to increasing C16-DMA concentrations. *Error bars* represent SE from 15 independent roots. *Different letters* indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. *Scale bar* shows the elongation zone domain of the primary root

roots than their respective control without the compound (Fig. 10c). These results indicate that C16-DMA acts independently of auxin signaling components to orchestrate root architectural changes in *Arabidopsis*.

Discussion

The repertoire of rhizobacterial compounds that coordinate plant functional processes is increasing, some of them are considered plant hormones itself, such as auxins or cytokinins, but other act as bacteria-bacteria signaling molecules that are recognized by roots and allow for adjustment of morphogenesis and defense (Ortiz-Castro et al. 2011; Raya-González et al. 2016). Although microorganisms release blends of VOCs, comprised by many different combinations of compounds, very few purified molecules have been found to possess clear biological activities in bacteria or their host. C16-DMA is a VOC emitted by rhizobacteria that modulate bacterial, fungal, and plant growth (Velázquez-Becerra et al. 2011, 2013).

The fact that compounds similar to DMAs have been identified as signal molecules for plants, such as the alkamides and NAEs, opens the possibility that some of the VOCs emitted could act as bioactive plant compound mimics. An in-depth understanding of how plants respond to plant growth-promoting rhizobacteria (PGPR) signals requires detailed analysis of the genetic and molecular programs underlying the phytostimulating effect and to define how the host hormonal and/or signaling pathways are modified by the emitted molecules. Here, we demonstrate that DMAs with C8 to C16 acyl chain length inhibit primary root growth (Figs. 1, 2, and S1), and that (C12-16) chained DMAs produced the strongest inhibitory effect (Figs. 1, 2, and S1). This was correlated with increasing lateral root numbers and density, which suggest that compromising root apical dominance may be a determinant to activate an alternate root branching program. From all compounds tested, only C16-DMA showed primary root growth promotion at low concentrations (Figs. 2 and S1).

These results indicate that diverse DMAs can be perceived likely via different mechanisms to alter root morphogenesis in plants and that their acyl-chain length plays a key role on their biological activity. Interestingly, small AHL quorum-sensing autoinducers from gram negative bacteria can improve *Arabidopsis* root growth at low concentrations (Von Rad et al. 2008; Zhao et al. 2015), while compounds of the same family but with larger acyl chains rather repress root growth (Ortiz-Castro et al. 2008). Consistent with their possible regulatory role, the bacterial AHLs affect plant gene expression and the proteome of *Arabidopsis* and *Medicago truncatula*, two plant species evolutionary distant (Mathesius et al. 2003).

C16-DMA was initially identified from the rhizobacterium *Arthrobacter agilis* UMCV2 as the most abundant DMA component in a VOCs blend analysis. Its possible role in plant-microbe signaling was evidenced since primary root growth, lateral root formation, and root hair development were modified in a dose-dependent manner, which further suggests that cell division and differentiation in root apical meristem as well as pericycle cell activity could be modulated by C16-DMA (Figs. 4 and 5). Root architecture can be modulated by several plant growth regulators, including auxin, cytokinins, ethylene, and jasmonic acid. Interestingly, C16-DMA

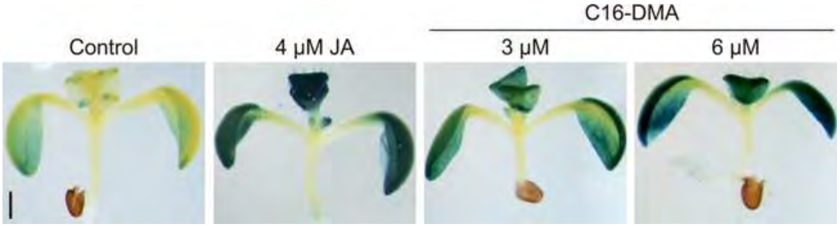

Fig. 6 C16-DMA induces JA-responsive gene marker expression *pLOX2:uidA*. *Arabidopsis* seedlings harboring *pLOX2:uidA* gene marker were grown and germinated for 10 days on MS 0.2× medium supplemented with indicate concentration of C16-DMA or JA (positive control). Plants were stained for β-glucoronidase activity and cleared to show gene expression. Note that C16-DMA induced a strong expression of *pLOX2:uidA* similar to JA treatment. Photographs show representative individuals from at least 15 stained plants. The experiment was repeated twice with similar results. *Scale bar* = 1 mm

induced the expression of the jasmonic acid-responsive gene marker, *pLOX2:uidA* (Fig. 6), suggesting that the JA signaling pathway is induced by the bacterial compound.

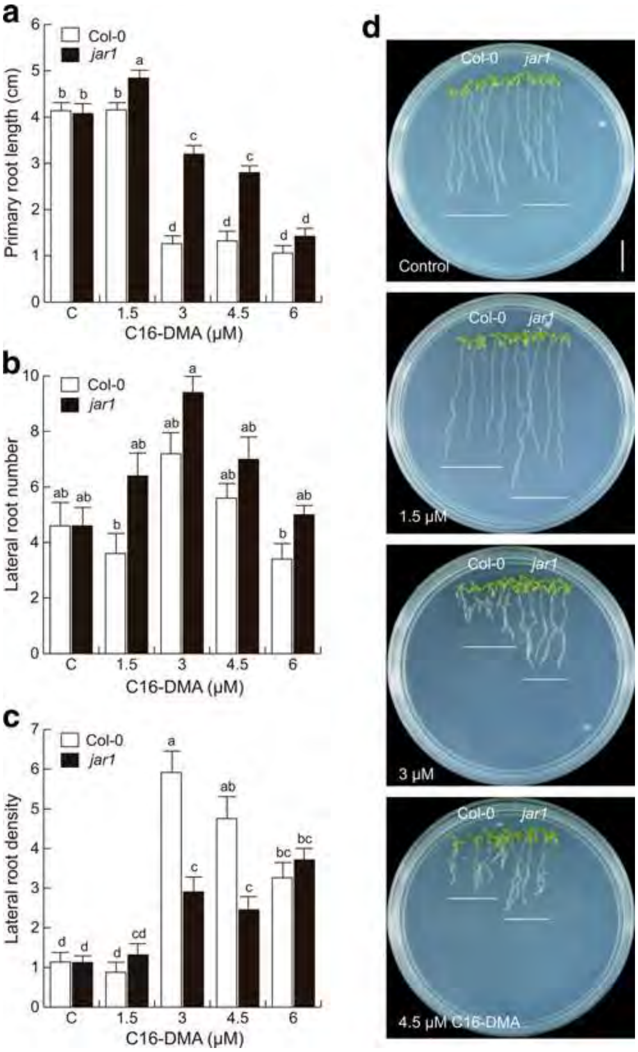

Fig. 7 Effect of C16-DMA on root architecture of *Arabidopsis* wild-type and JA-related mutant seedlings. *Arabidopsis* wild-type (Col-0) and *jar1* seedlings were germinated and grown side by side on MS 0.2× MS agar medium for 10 days and root development was evaluated. **a** Primary root length. **b** Lateral root number. **c** Lateral root density (lateral root number/primary root length). *Errors bar* represent SE from 30 seedlings. *Different letters* indicate statistical differences at $P < 0.05$. **d** Photographs of representative wild-type and *jar1* seedlings. Note that *jar1* showed resistance to C16-DMA on primary root growth. The experiment was repeated three times with similar results. *Scale bar* = 1 cm

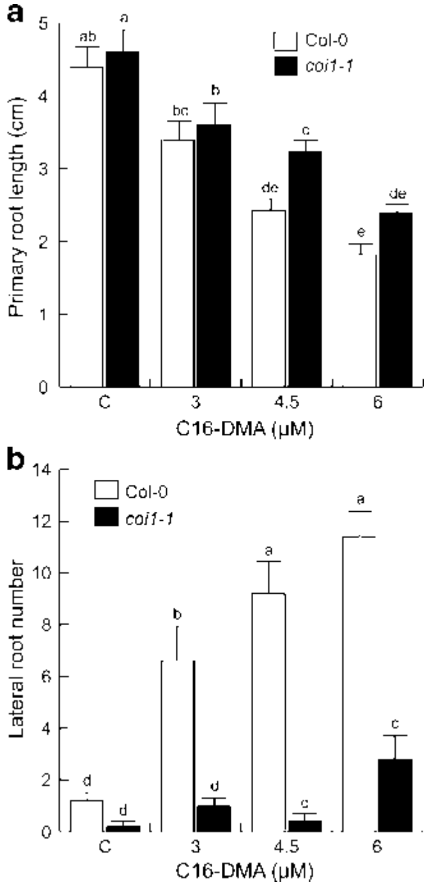

Fig. 8 Effect of C16-DMA on root development of wild-type and *coi1-1* seedlings. *Arabidopsis* wild-type seedlings were germinated and grown for 4 days on 0.2× MS medium, and homozygous *coi1-1* seedlings were selected from a *coi1-1/COI1* segregating population in medium supplemented with 4 μM JA. **a** Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates supplied or not with C16-DMA, and primary root length was measured 8 days after transfer (**a**). **b** Lateral root number. Note that C16-DMA failed to induce lateral root formation in *coi1-1* seedlings. Lateral root number was analyzed 8 days after transfer to C16-DMA. *Errors bar* represent SE from 20 seedlings. *Different letters* indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. *Scale bar* = 1 cm

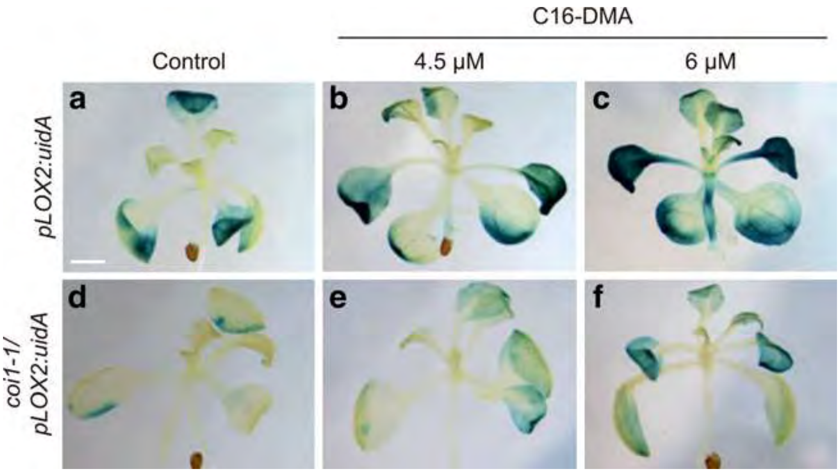

Fig. 9 C16-DMA requires COI1 to induce JA-responsive gene marker expression in *Arabidopsis*. Wild-type seeds harboring the *pLOX2:uidA* gene construct were germinated and seedlings were grown for 4 days on 0.2× MS medium, and homozygous *coi1-1/pLOX2:uidA* seedlings were selected from a *coi1-1/COI1* segregating population in medium supplemented with 4 μM JA. Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates supplied or not with C16-DMA. Eight days after transfer plants were stained for β-glucoronidase activity and cleared to show gene expression. Photographs show representative individuals from at least 15 stained plants. The experiment was repeated twice with similar results. *Scale bar* = 500 μm

Regarding the stimulatory effect on primary root growth by low concentrations of C16-DMA (Fig. 2a), no conclusive information is available to attribute this effect to JA signaling. To the best of our knowledge, no information has been published showing that JA stimulates root growth at low concentrations. However, since JA signaling shares components with the auxin pathway (i.e., *axr1*) and auxin directly controls root growth by promoting cell division and stem cell niche activity, we cannot discard the possibility that the growth-promoting effect on primary roots by low concentrations of C16-DMA could also operate via JA signaling.

To gain further insight into the JA-dependent responses to C16-DMA, JA-related mutants, *jar1* and *coi1-1*, were evaluated in response to C16-DMA and showed resistance to C16-DMA on root developmental programs (Figs. 7 and 8). The finding that *pLOX2:uidA* expression in *coi1-1* background was weaker in response to C16-DMA indicated that C16-DMA targets the JA pathway to modulate root development and possibly JA-dependent defense responses in *Arabidopsis* aerial parts, such as stems and leaves. Several beneficial microorganisms can induce an enhanced defensive capacity in plants to provide protection against a broad spectrum of pathogens and insect herbivores (Van der Ent et al. 2009). Given that C16-DMA induces JA-responsive gene expression in leaves, its potential use during plant challenges with foliar pathogens and/or herbivores is certainly promising.

Since the alkamides and NAEs have been shown to regulate root morphogenesis and defense responses via different hormonal mechanism such as cytokinins and abscisic acid signaling (López-Bucio et al. 2007; Teaster et al. 2007), we cannot exclude the possibility that any DMAs could also target these pathways.

Already published studies have investigated the implications of auxin signaling pathway in plant responses to PGPR. In fact, some PGPR can biosynthesize indole-3-acetic acid (IAA), a natural auxin, auxin precursors, or auxin signal mimics. Auxin signaling is a key phytohormone in plant root development, modulating root growth, lateral root emergence, and root hair formation (Santelia et al. 2005; Ortiz-Castro et al. 2011). However, plant hormones operate in a complex framework of interacting responses rather than through isolated linear pathways. This hormonal crosstalk network can be modulated by a multitude of signals from developmental or environmental origins. *Arabidopsis* mutants such as *axr1* are resistant to both jasmonic acid and auxin in primary root inhibition assays, indicating that the AXR1 protein is involved in auxin and JA-dependent signaling pathways (Tiryaki and Staswick 2002). Additional commonalities exist between perception mechanisms of jasmonates and auxin. They both use as receptor an SCF-type E3 ubiquitin ligase with a specific F-box protein for each hormone, COI1 for jasmonate and TIR1 or the closely related proteins AFB1, AFB2, and AFB3 for auxin (Santner et al. 2009). Taking into account this information, we compared the root growth response of WT and auxin-related mutants defective in auxin influx (*aux1-7*), signaling (*arf7 arf19*), and perception (*tir1 afb2 afb3*) in response to C16-DMA. We neither find an induction of the *DR5:uidA* marker nor clear differences on primary root length between WT seedlings and auxin-related mutants grown on media supplemented with C16-DMA (Fig. 10). Together, these results imply that C16-DMA modulates *Arabidopsis* root branching via an auxin-independent mechanism. Such data is in consonance with previous findings that jasmonic acid may alter *Arabidopsis* root development through auxin dependent or independent

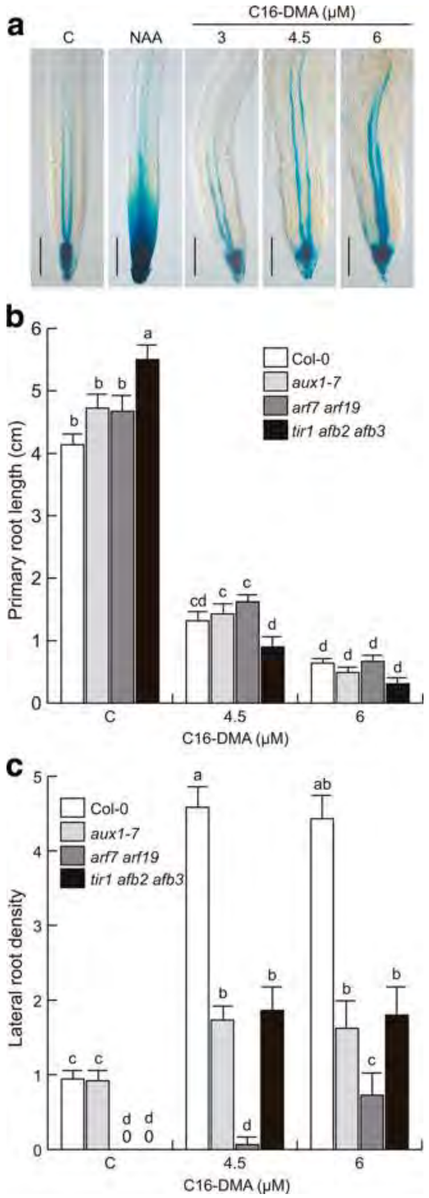

Fig. 10 Effect of C16-DMA on auxin response and root development of *Arabidopsis* WT and auxin-related mutants. **a** Representative photographs of *DR5:uidA* expression in root tips of 5-day-old seedlings in response to 60 nM napthtalene acetic acid (an auxin), or increasing C16-DMA concentrations. *Arabidopsis* wild-type (Col-0), *aux1-7*, *arf7 arf19*, and *tir1 afb2 afb3* single, double, and triple mutant seedlings, respectively, were germinated and grown for 10 days in 0.2× MS medium supplemented with or without C16-DMA and **b** primary root length or **c** lateral root density, recorded. *Errors bar* represent SE from 20 seedlings. *Different letters* indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. *Scale bar* = 100 μm

mechanisms (Raya-González et al. 2012). In another report, Ryu et al. (2003) showed that *Arabidopsis cre1*, a cytokinin receptor mutant, did not exhibit growth promotion when exposed to *Bacillus* spp. GB03, suggesting a role for the cytokinin signaling pathway in plant response to VOC emission by this strain. The fact that PGPR can modulate plant growth promotion using VOCs as elicitors of plant signaling pathways is very basic towards understanding the diversity and specificity of mechanisms responsible for phytostimulation.

In summary, our current work showed that (i) DMAs regulate *Arabidopsis* root development and their acyl chain length plays a key role for their biological activity; (ii) the rhizobacterial VOC, C16-DMA modulate primary root growth and lateral root formation; (iii) C16-DMA activates JA-responsive gene expression and modulates root development involving JAR1, COI1, and MYC2 elements; and (iv) C16-DMA operates independently of the canonical auxin signaling pathway. All these data strongly support the proposed hypothesis of DMAs as cross-kingdom signal molecules. We cannot exclude the possibility that C16-DMA could modulate shoot development and/or protect plants against leaf pathogens via the JA signaling and/or cytokinin signaling, and could further increase the plant capacity to access soil nutrients through improved root branching and direct nutrient assimilation effects such as the demonstrated iron uptake.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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New Phytologist

Research

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 $\mathrm{ml^{-1}}$ 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 $\mathrm{min^{-1}}$) 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 $\mathrm{min^{-1}}$ 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 $\mathrm{s^{-1}}$. 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 $\mathrm{cm^2}$) 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

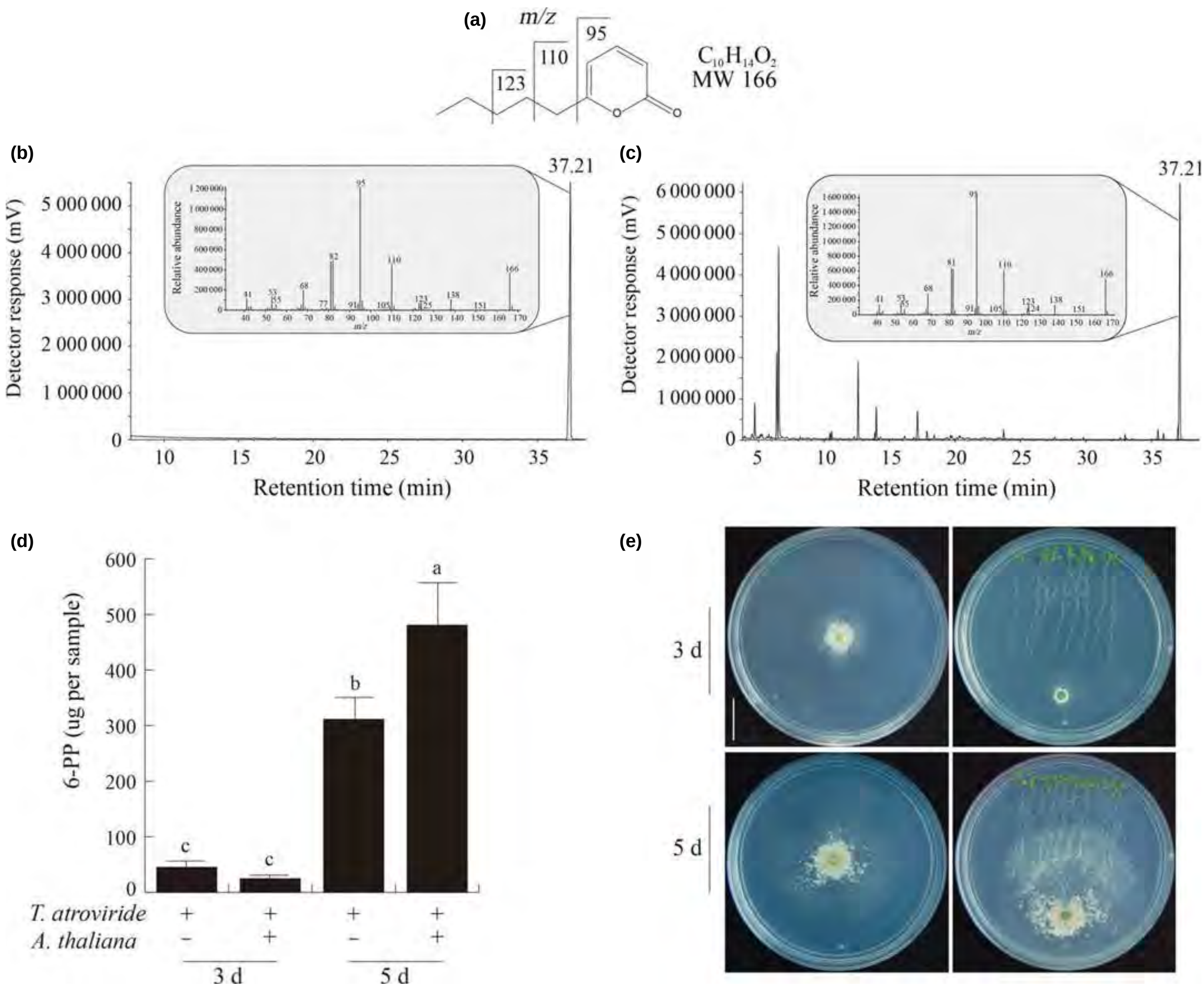

Fig. 1 Molecular characterization and production of 6-pentyl-2*H*-pyran-2-one (6-PP) by *Trichoderma atroviride* IMI 206040. (a) Chemical structure of 6-PP showing the major fragment ions (*m/z*) of electron ionization mass spectra. (b) Total ion chromatogram and mass spectra from commercial standard (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

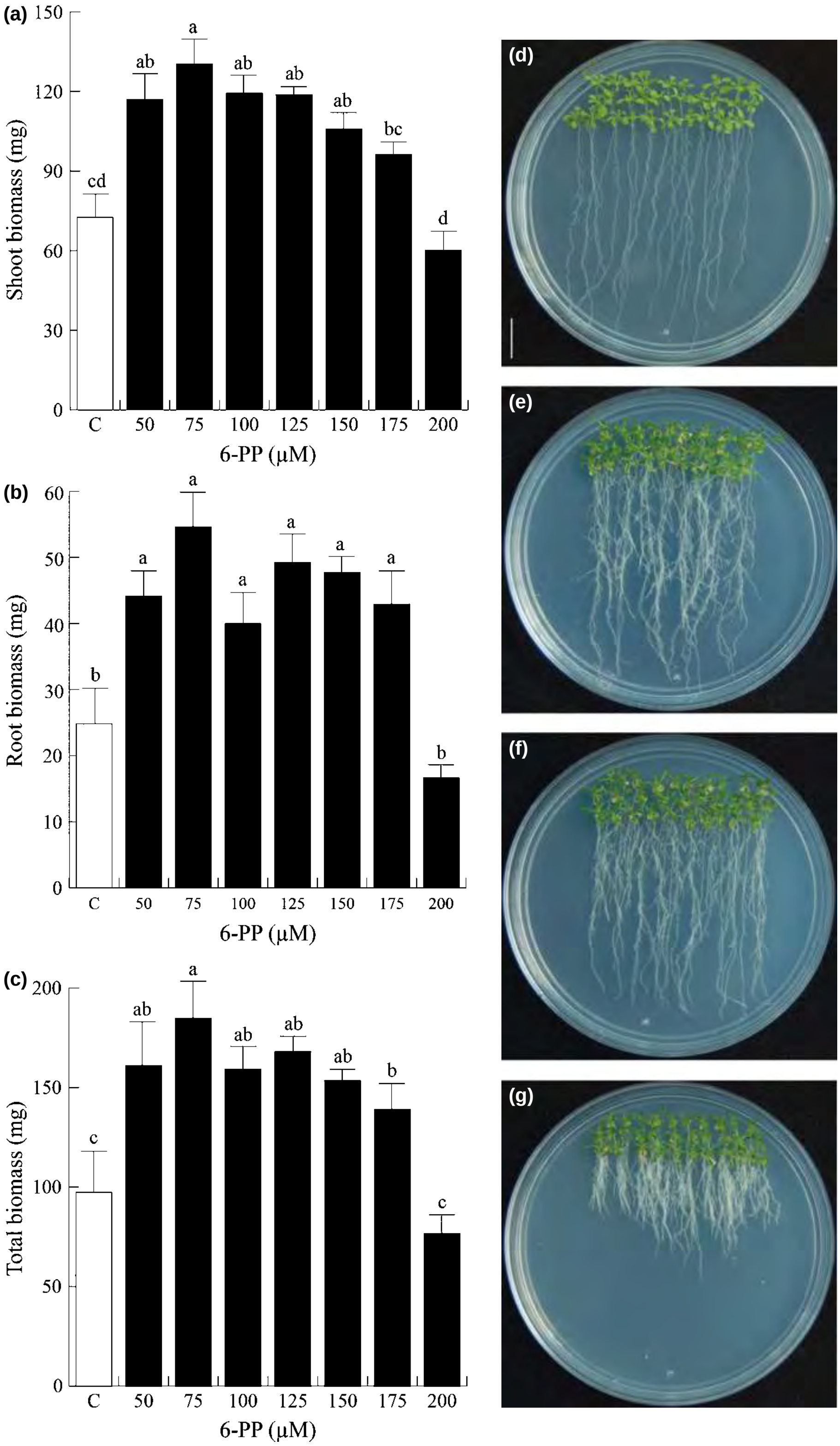

Fig. 2 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on plant biomass production. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 12 d under increasing 6-PP concentrations. (a) Shoot biomass. (b) Root biomass. (c) Total biomass. (d) Representative photographs of seedlings grown in (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). To further analyze possible changes in auxin accumulation and/or responsiveness in primary root tips caused by 6-PP, a detailed analysis

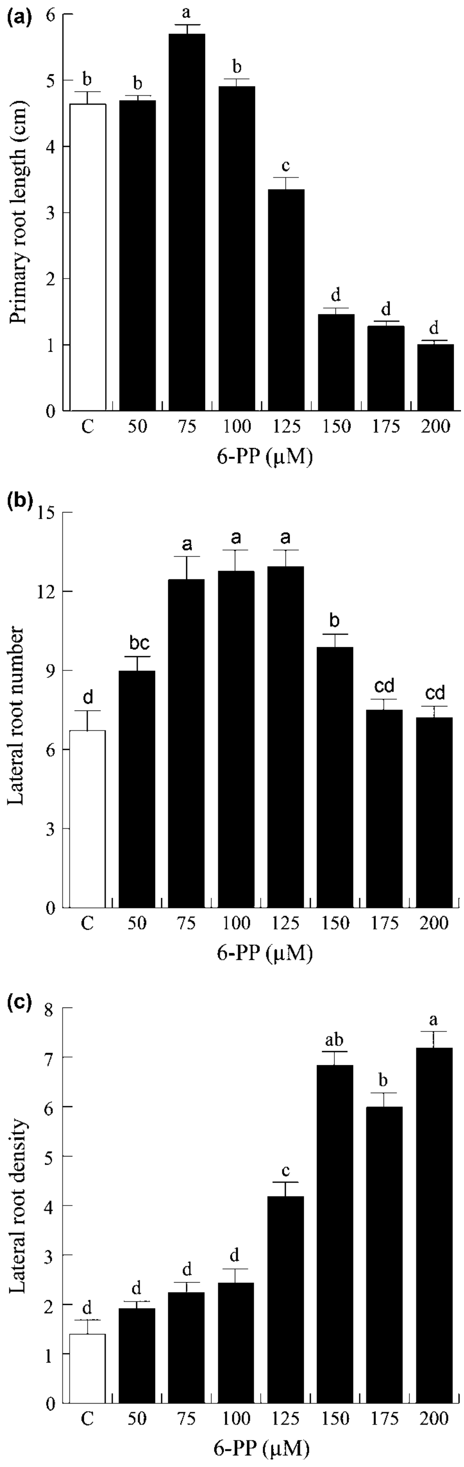

Fig. 3 6-pentyl-2*H*-pyran-2-one (6PP) regulates *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a) Primary root length. (b) Number of emerged lateral roots. (c) Lateral root density (number 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.

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 inhibition (Fig. 7a). By contrast, an induction of LR formation was lacking in *tir1afb2afb3*, *arf7arf19*, *axr1-3* and *aux1-7*, while *eir1*

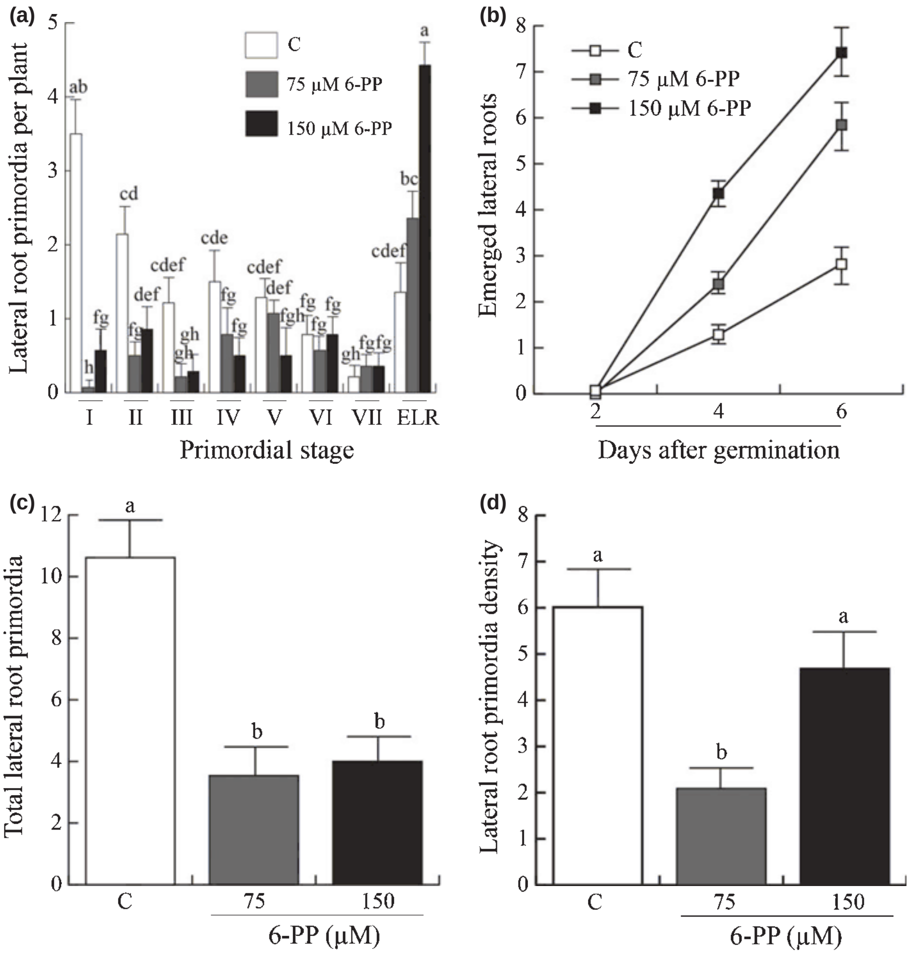

Fig. 4 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on lateral root (LR) development in *Arabidopsis thaliana*. Wild-type (Col-0) seedlings were germinated and grown for 2, 4 and 6 d on 0.2 × MS media supplemented with the solvent (control (C)), or 75 or 150 µM 6-PP. (a) Lateral root primordia (LRPs) per 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.

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. 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

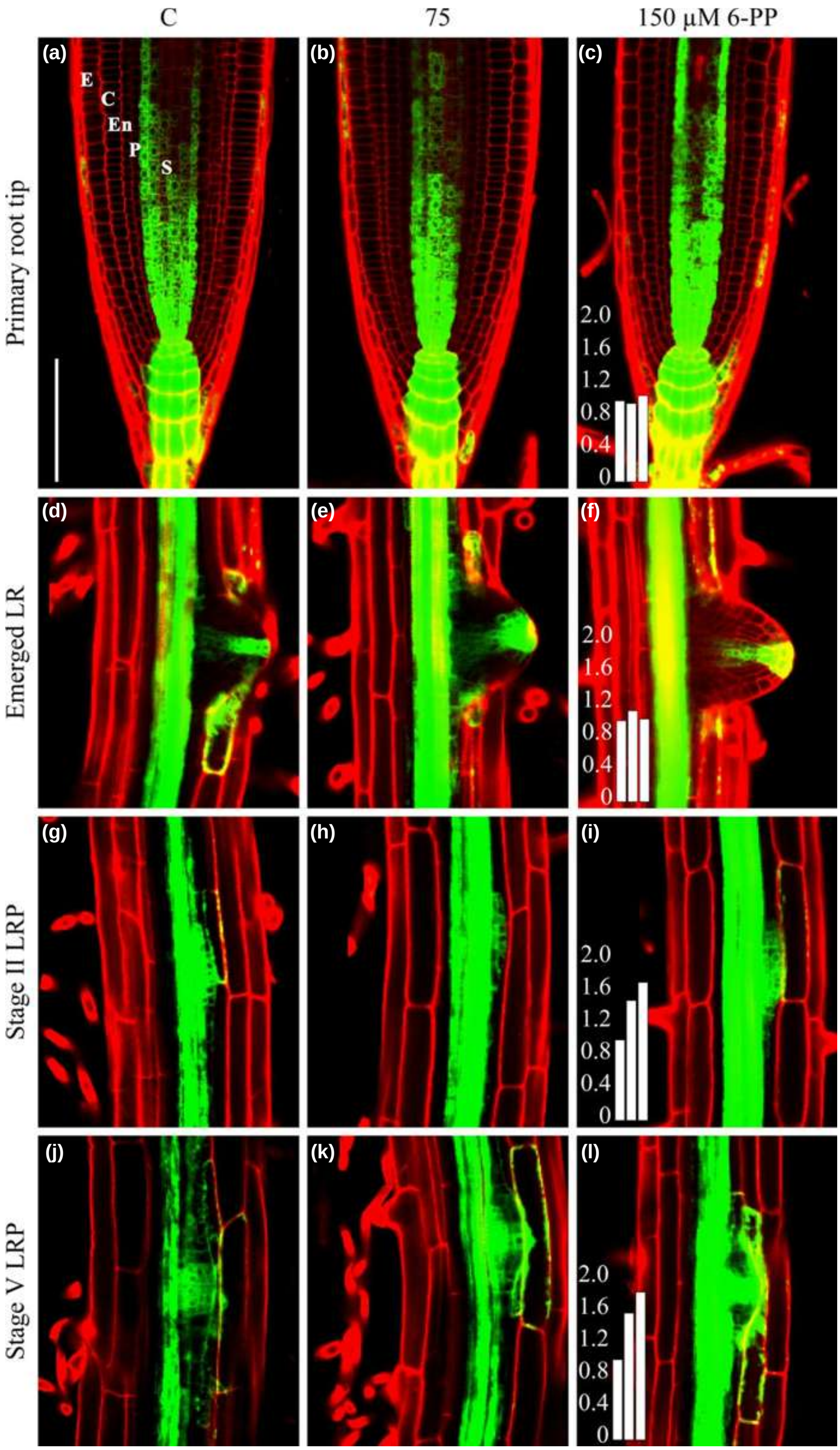

Fig. 5 6-pentyl-2*H*-pyran-2-one (6PP) modulates auxin-responsive gene expression in the lateral root formation zone. *Arabidopsis thaliana* transgenic *DR5: GFP* seedlings were grown in solvent added to 0.2 × MS medium (C), or supplemented with 75 or 150 μM 6-PP. Five days after germination, seedlings 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.

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.

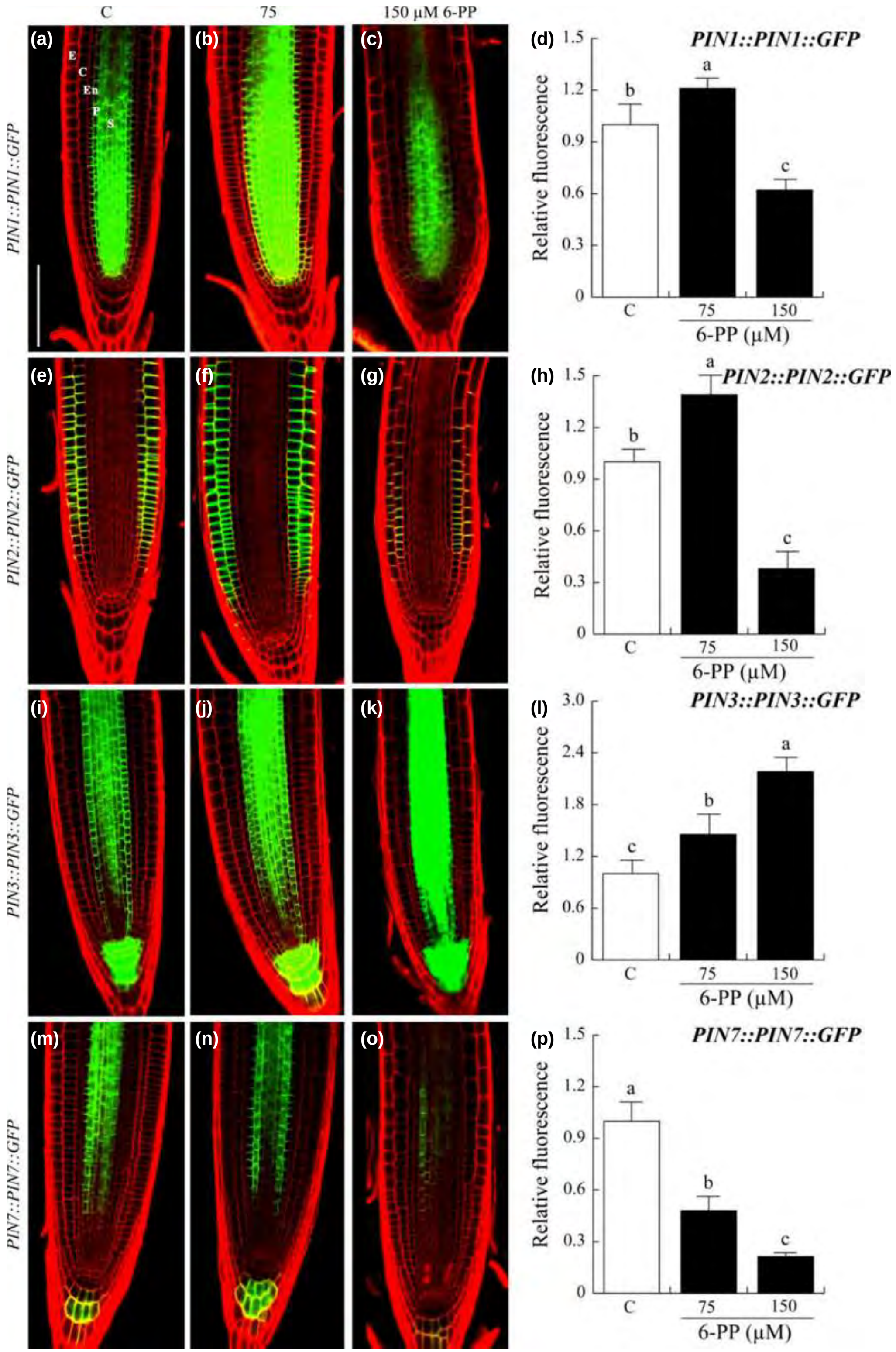

Fig. 6 Expression of auxin efflux transporters in response to 6-pentyl-2*H*-pyran-2-one (6PP) in primary roots. *Arabidopsis thaliana* transgenic *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* seedlings were grown in solvent (C), or 75 or 150 µM 6-PP supplemented medium. 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.

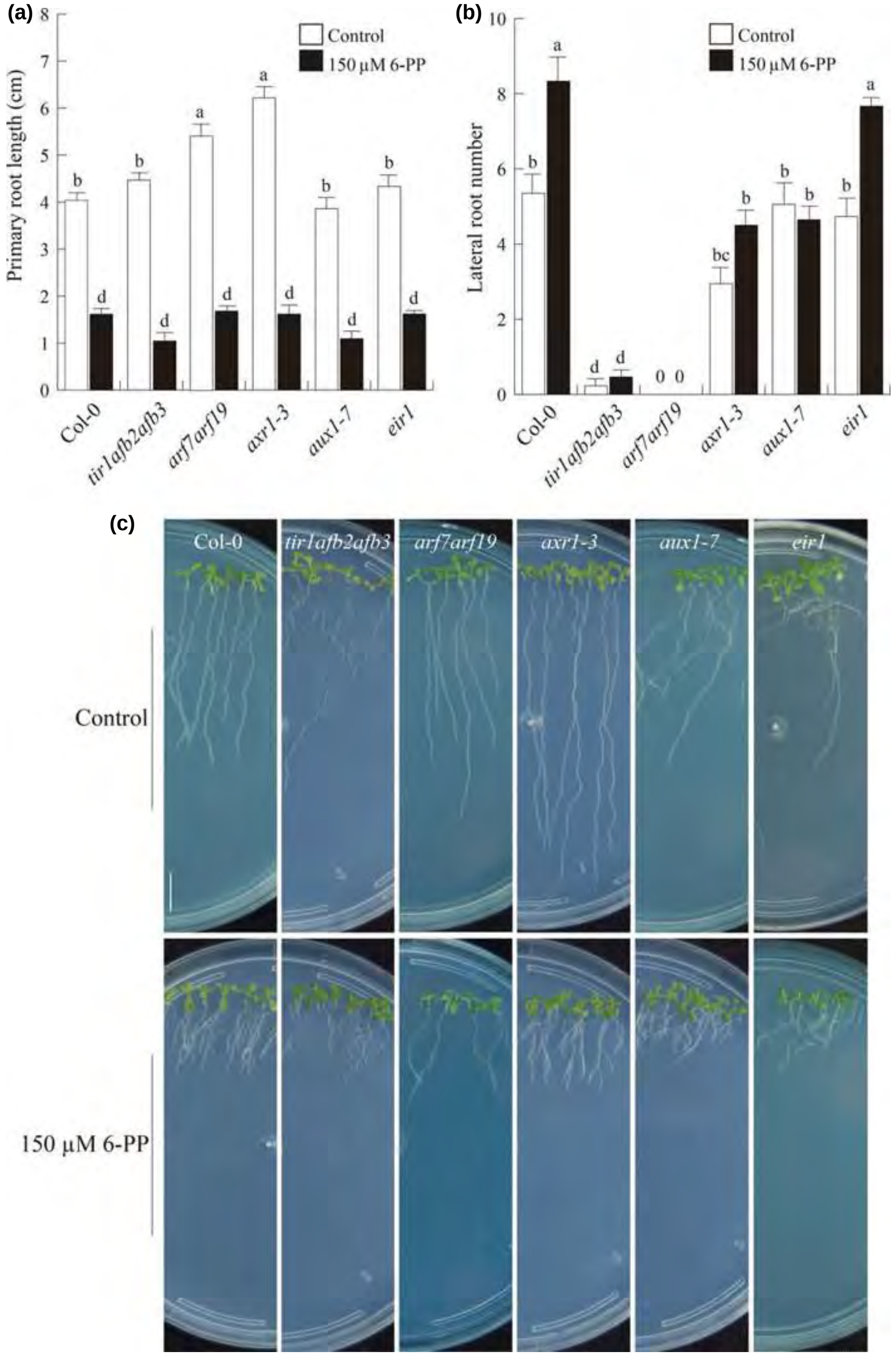

Fig. 7 6-pentyl-2*H*-pyran-2-one (6PP) requires components of auxin response and transport to modify *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* wild-type (Col-0) and *tir1/afb2/afb3*, *arf7-1/arf19-1*, *axr1-3*, *aux1-7* and *eir1*, triple, double or single mutant seedlings, 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

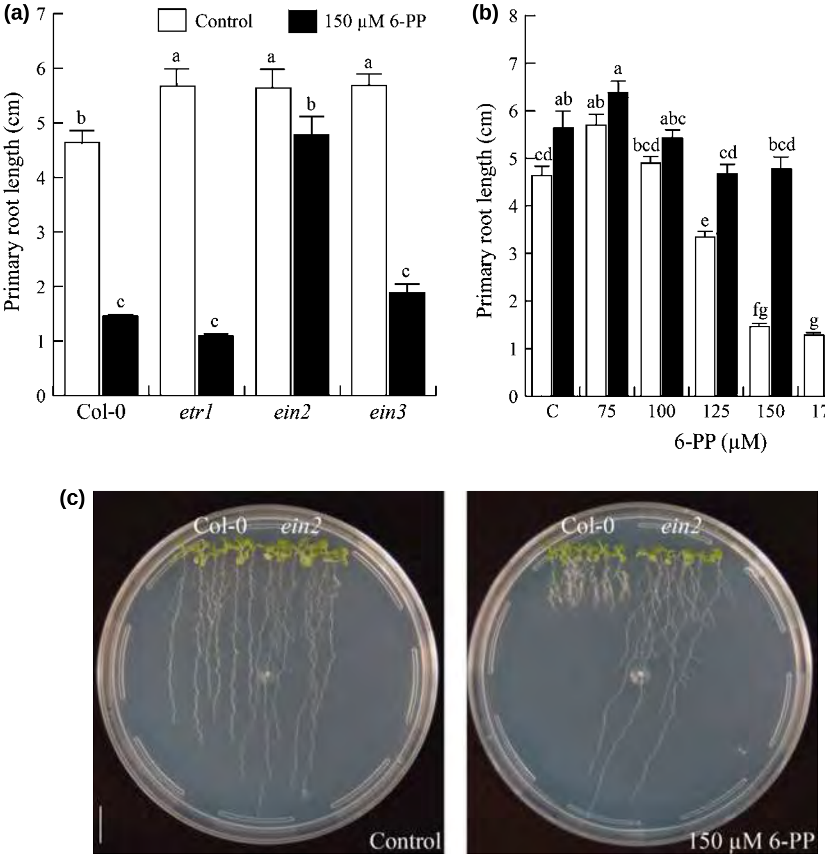

Fig. 8 *EIN2* is necessary for 6-pentyl-2*H*-pyran-2-one (6PP)-modulated primary root growth. *Arabidopsis thaliana* wild-type (Col-0) and *etr1-1*, *ein2-1*, *and ein3-1* ethylene-related mutant seedlings were germinated and grown for 10 d in 0.2 × MS medium supplemented with the solvent (control) or 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 PIN family membrane transporters (Zazimalova *et al.*, 2010). To

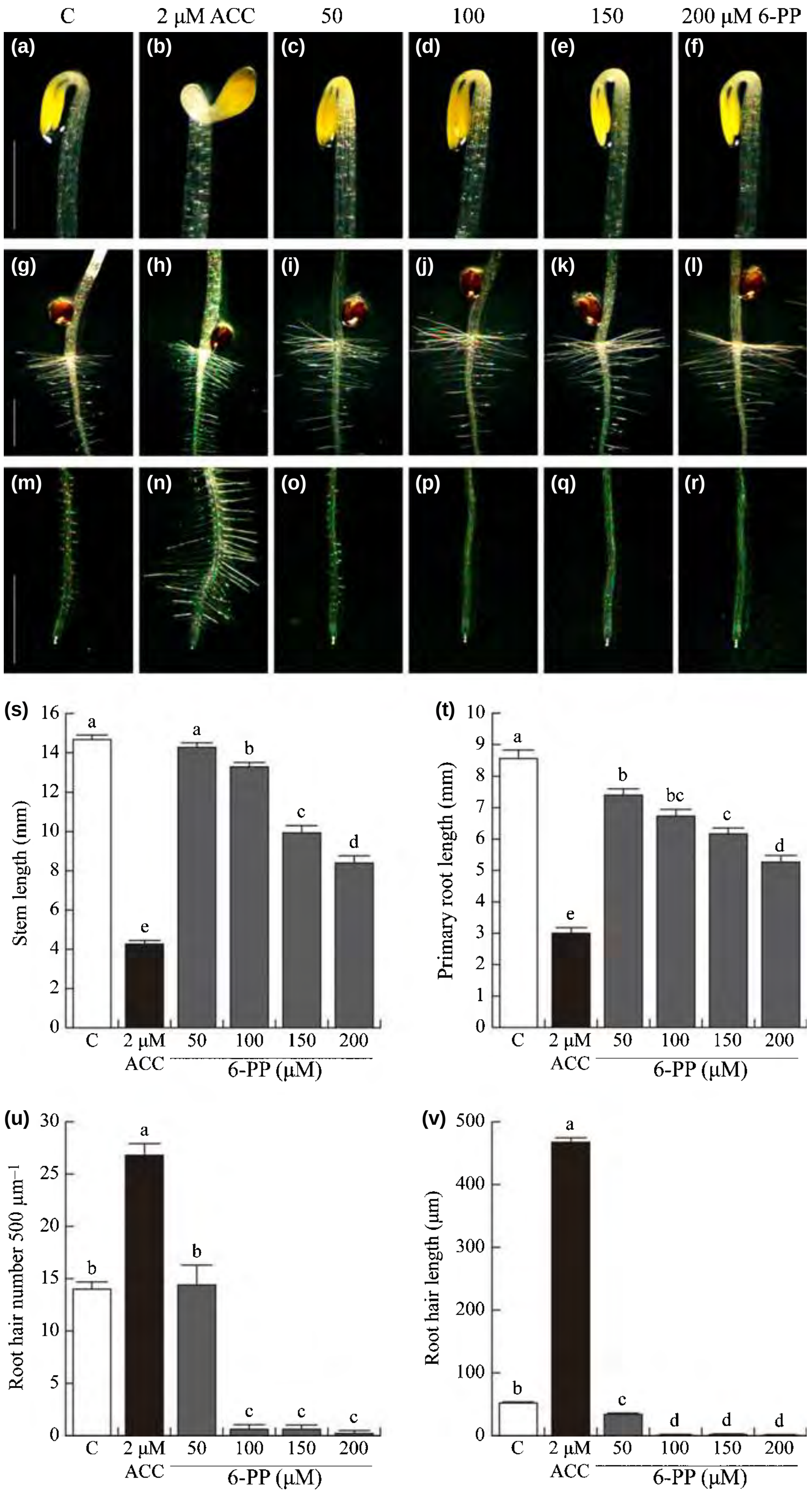

Fig. 9 6-pentyl-2*H*-pyran-2-one (6PP) does not induce ethylene responses in dark-grown *Arabidopsis thaliana* seedlings. Representative photographs of (a–f) stem, (g–l) root–shoot transition zone and (m–r) root tip are shown. Quantitative data are for (s) stem length, (t) primary root length, (u) root 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.

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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- All competition articles that are accepted after peer review will be published in *New Phytologist* and the Tansley medal winner selected by the judges from these final papers.

New Phytologist Supporting Information Figs S1–S7

Article title: 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

Authors: Amira Garnica-Vergara, Salvador Barrera-Ortiz, Edith Muñoz-Parra, Javier Raya-González, Alejandro Méndez-Bravo, Lourdes Macías-Rodríguez, León Francisco Ruiz-Herrera and José López-Bucio

Article acceptance date: 23 September 2015

The following Supporting Information is available for this article:

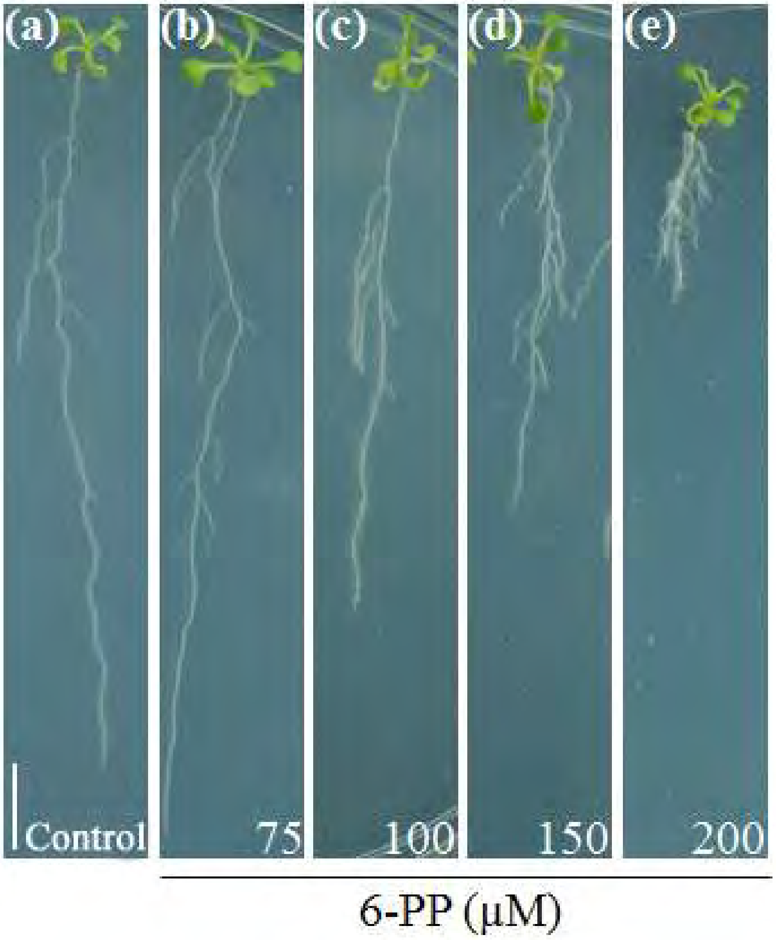

Fig. S1 6-PP modifies *Arabidopsis* root system architecture. *Arabidopsis* (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a–e) Photographs show representative individual seedlings. Bar, 1 cm.

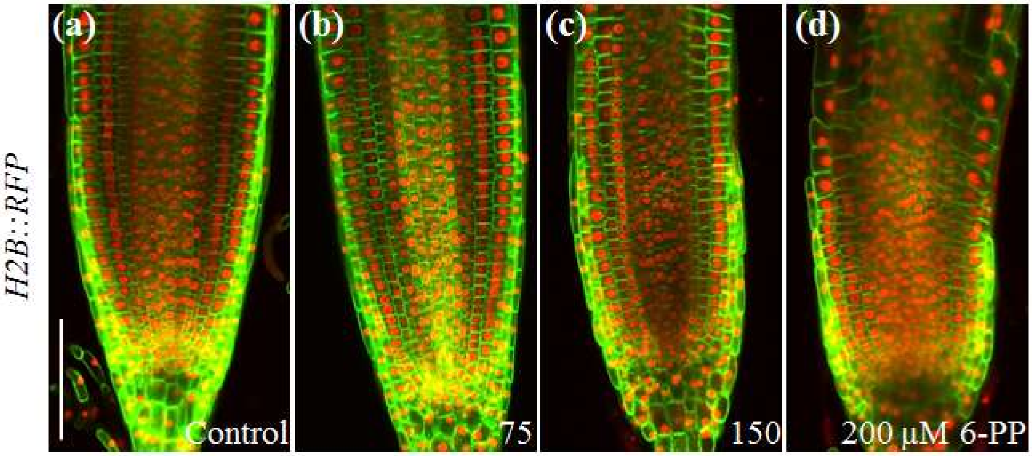

Fig. S2 Effect of 6-PP on *Arabidopsis* cell viability. *Arabidopsis H2B::RFP* expressing seedlings were grown for 10 d in media with the solvent only or supplied with increased concentrations of 6-PP. (a–d) Representative micrographs of primary root tips. Note that even under the highest concentration of 6-PP tested, root tip cell remain viable and show red intact nuclei. Bar, 100 µm.

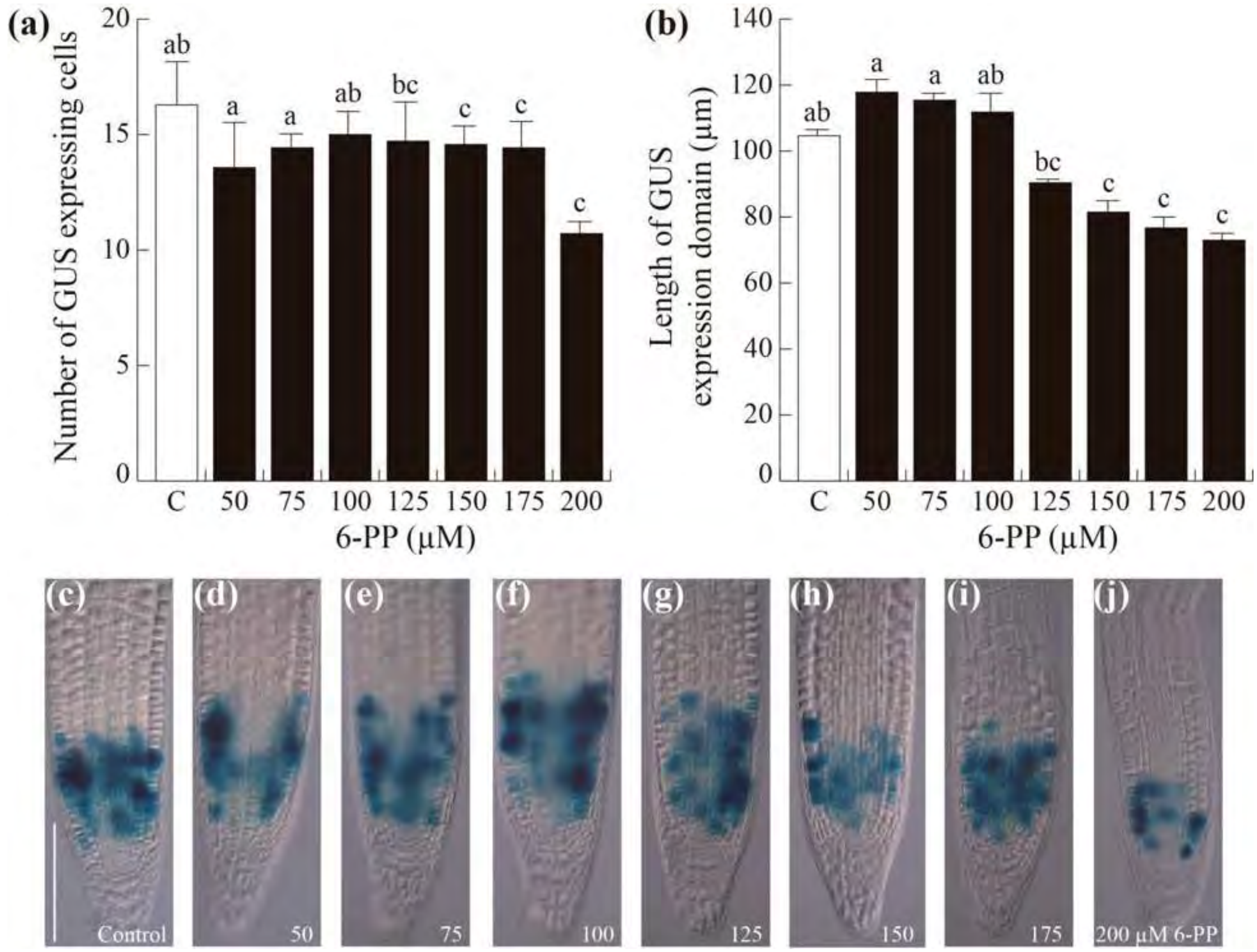

Fig. S3 6-PP reduces the cell division zone in primary roots. Transgenic *Arabidopsis CycB1:uidA* seedlings were germinated and grown for 5 d on 0.2 × MS medium supplied with increasing 6-PP concentrations. (a) Number of GUS expressing cells and (b) length of GUS expression domain were quantified. (c–j) Photographs showing representative individuals from 20 GUS-stained seedlings obtained from three independent plates. Error bars represent SE from 20 GUS-stained seedlings analyzed. Different letters indicate means statistically different at $P < 0.05$. The experiment was repeated two times with similar results. Bar, 100 μm.

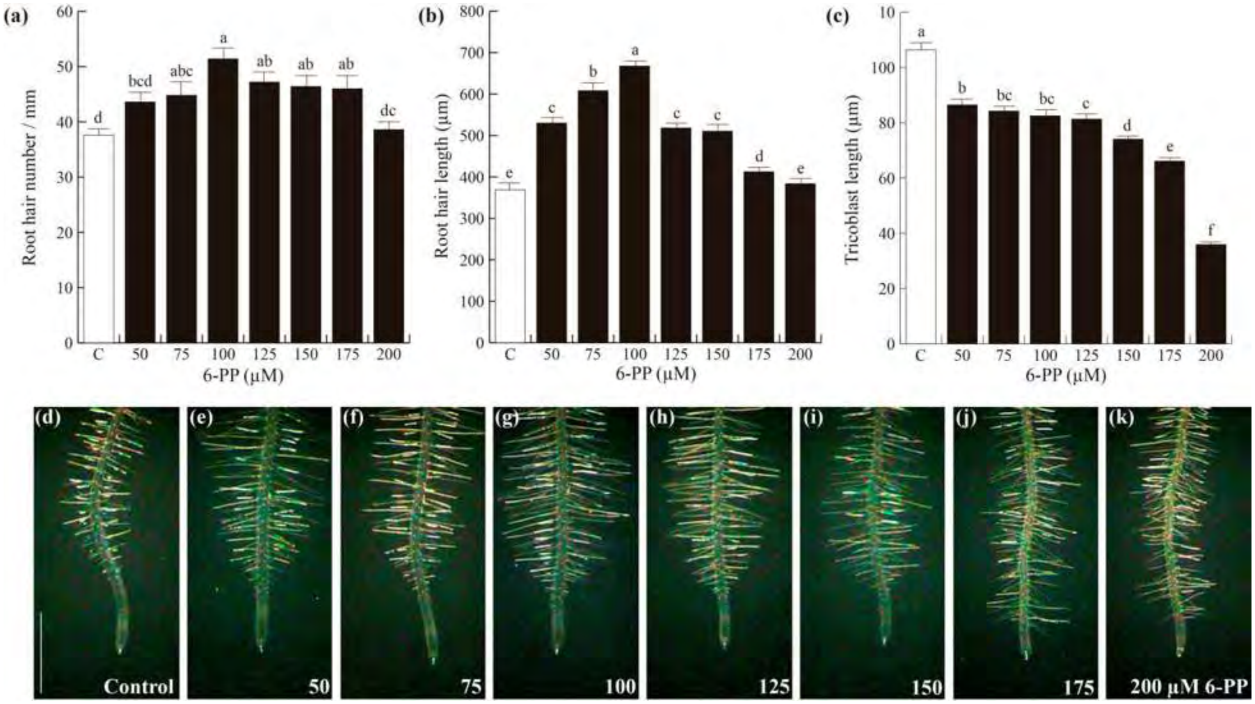

Fig. S4 Effects of 6-PP on *Arabidopsis* root hair development and epidermal cell length. (a) Root hair number, (b) root hair length, and (c) trichoblasts length. *Arabidopsis thaliana* seedlings were grown for 5 d on MS 0.2 × media with or without the indicated concentrations of 6-PP. Date points indicate mean ± SD ($n = 15$). (d–k) Representative photographs of root hairs formed at the primary root tip region of 5 d *Arabidopsis* seedlings supplied with the different 6-PP treatments. The results (a–c) show mean of 500 epidermal cells located at the root hair-forming zone of the primary root from 10 independent seedlings. This experiment was repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Bar, 1 mm.

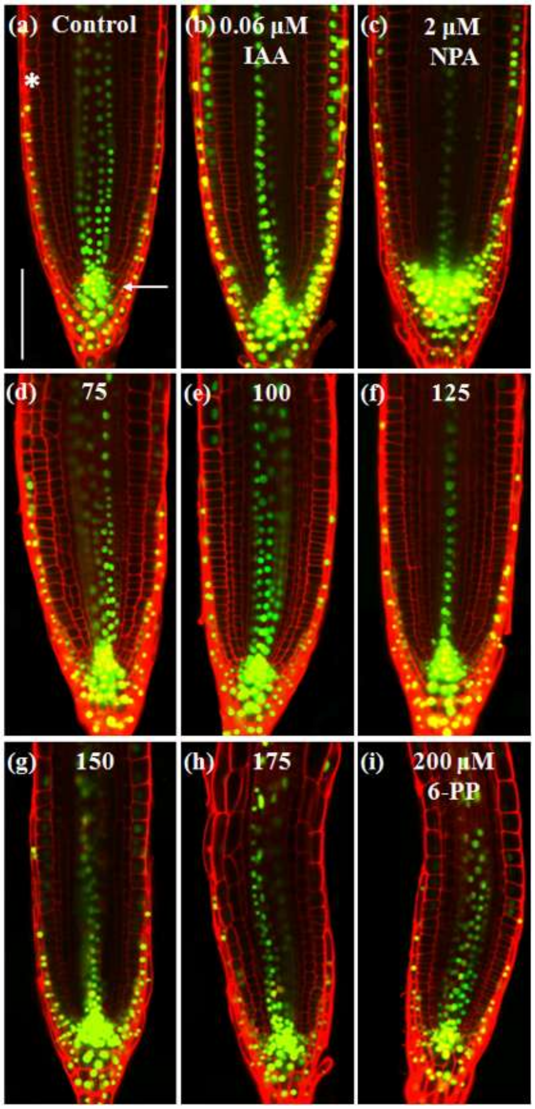

Fig. S5 Effect of 6-PP on auxin responsive gene expression in primary root tips of *Arabidopsis DR5:VENUS* seedlings. Seedlings were grown in MS 0.2 × media supplied with (a) solvent, (b) 0.06 µM IAA, (c) 2 µM NPA, (d) 75, (e) 100, (f) 125, (g) 150, (h) 175 and (i) 200 µM 6-PP. Five days after germination seedlings were stained with propidium iodide and analyzed by confocal microscopy. White asterisk and arrow in (a) indicate the epidermis and the area surrounding the quiescent center, respectively. This latter zone defines an auxin maximum response within the primary root tip. Note an induced expression by IAA or NPA treatments, whereas high concentrations of 6-PP decreased expression. Micrographs show representative individuals of at least 15 seedlings analyzed. Bar, 100 µm.

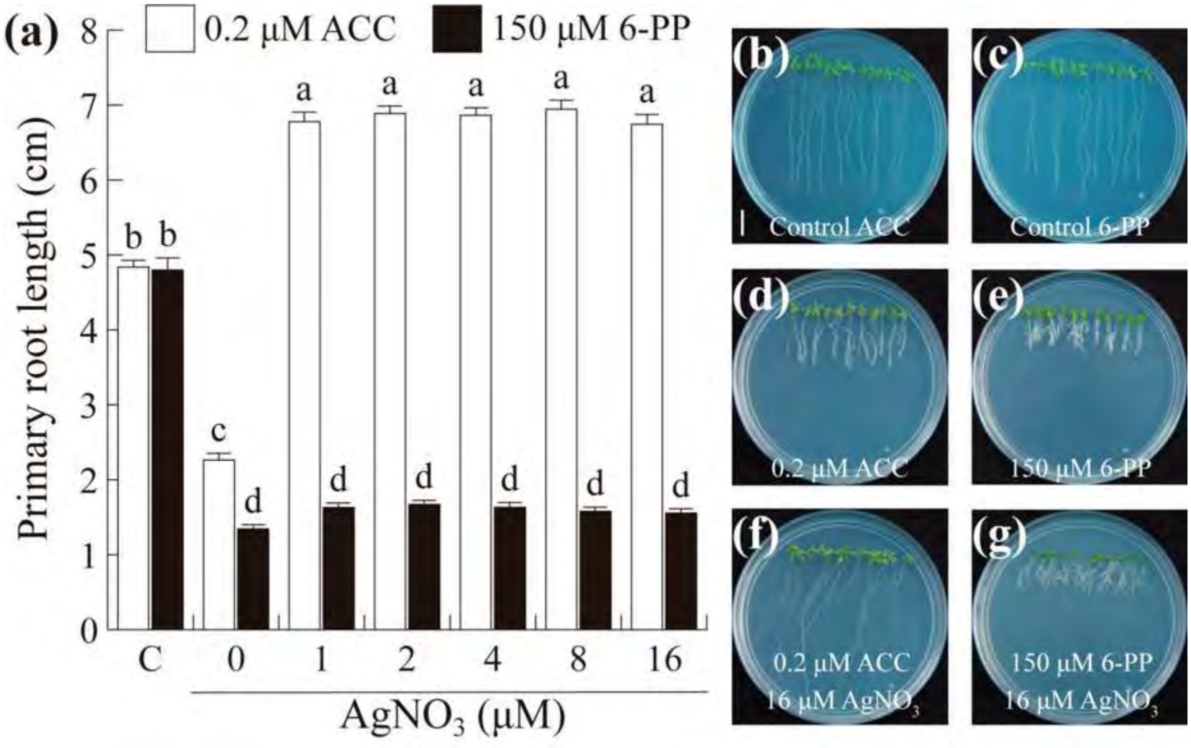

Fig. S7 Effect of $AgNO_3$ on primary root growth inhibition induced by ACC or 6-PP. *Arabidopsis thaliana* Col-0 seedlings were grown for 10 d on MS 0.2× medium supplemented with the indicated concentration of ACC or 6-PP with or without $AgNO_3$. Values shown represent the mean primary root length of 30 seedlings ± SD. Different letters indicate statistical differences at $P < 0.05$. Representative photographs of *Arabidopsis* seedlings grown in the indicated treatment .The experiment was repeated twice times with similar results. Bar, 1 cm.

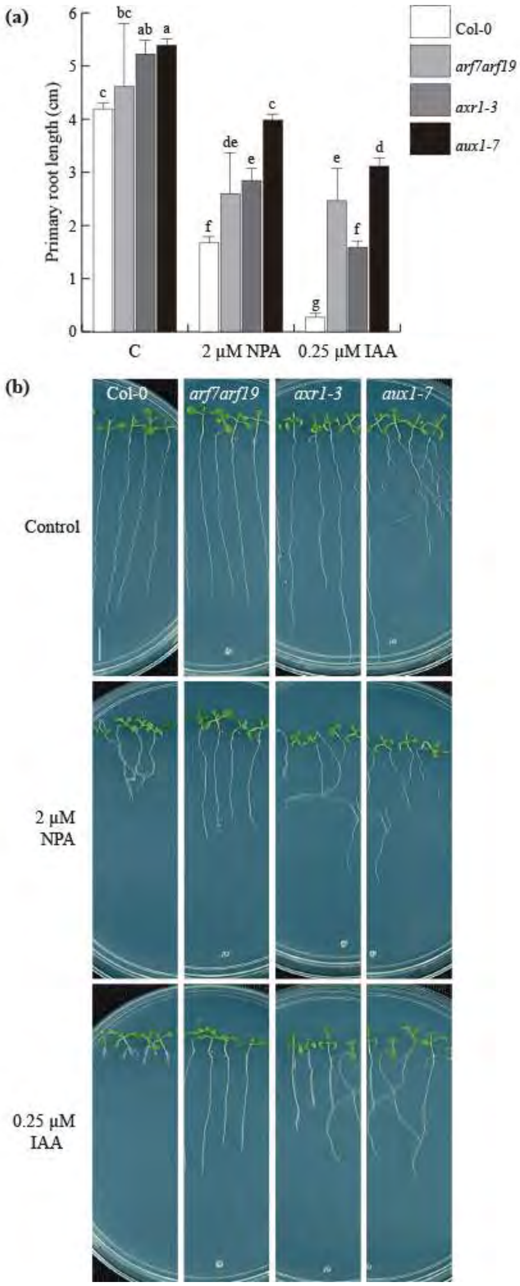

Fig. S6 Effects of NPA and IAA on primary root growth of wild-type (Col-0) and auxin-related mutants. *Arabidopsis thaliana* WT and *arf7arf19, axr1-3* and *aux1* mutant seedlings were grown for 10 d on MS 0.2× medium supplemented with the indicated concentration of NPA and IAA. Values shown represent the mean primary root length of 15 seedlings ± SD. Different letters indicate statistical differences at $P < 0.05$. Representative photographs of *Arabidopsis* seedlings grown in the indicated treatment .The experiment was repeated twice times with similar results. Bar, 1 cm.

Una invasión (o colaboración) organizada en la Naturaleza

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Relaciones tormentosas

Hoy en día es desafortunado escuchar en las noticias acerca de grupos criminales que afectan a la sociedad con asaltos, secuestros, extorsiones y tráfico de estupefacientes (por mencionar algunos delitos). El hecho de que estos delincuentes superen al poder de un estado, nos hace casi en automático pensar que esto depende del gran número de individuos que son y por supuesto de la gran cantidad de armamento con que cuentan (el cual llega a ser incluso más sofisticado que el de la policía, el ejército, o la marina). Esto es porque desde pequeños nos han enseñado en las clases de Historia, y en los cuentos y películas, que las guerras las ganan los que son más y cuentan con mejores armas. Aunque ser muchos y estar bien armados, es sin duda, una buena ventaja, esto de nada sirve si no hay una buena comunicación. Un ejemplo lo podemos ver en los seres más pequeños del planeta, las bacterias, las cuales se comunican para realizar una invasión en algún ser vivo (infección) causándole enfermedades que incluso pueden provocar la muerte o para colaborar en procesos que por el contrario, se podrían considerar altruistas.

Es mejor comunicarse

La comunicación que estos pequeños seres emplean se conoce como "quórum-sensing", un término adaptado del idioma inglés. Interesante es que en analogía con el lenguaje que empleamos los humanos, las "palabras" que utilizan las bacterias para señalizarse e incluso instruirse, son moléculas pequeñas o combinaciones de moléculas que transmiten un mensaje, el cual se recibe conduciendo a respuestas celulares que pueden ser individuales o poblacionales. Por estos efectos, las "palabras" de las bacterias se han llamado "autoinductores", cuando las bacterias son muchas en un lugar, producen la cantidad suficiente de una molécula que les permite a todas saber que tienen una función que realizar (Figura 1). Mediante este sistema de comunicación muy básico, se genera tal coordinación, que unos diminutos seres vivos, apenas visibles al micros-

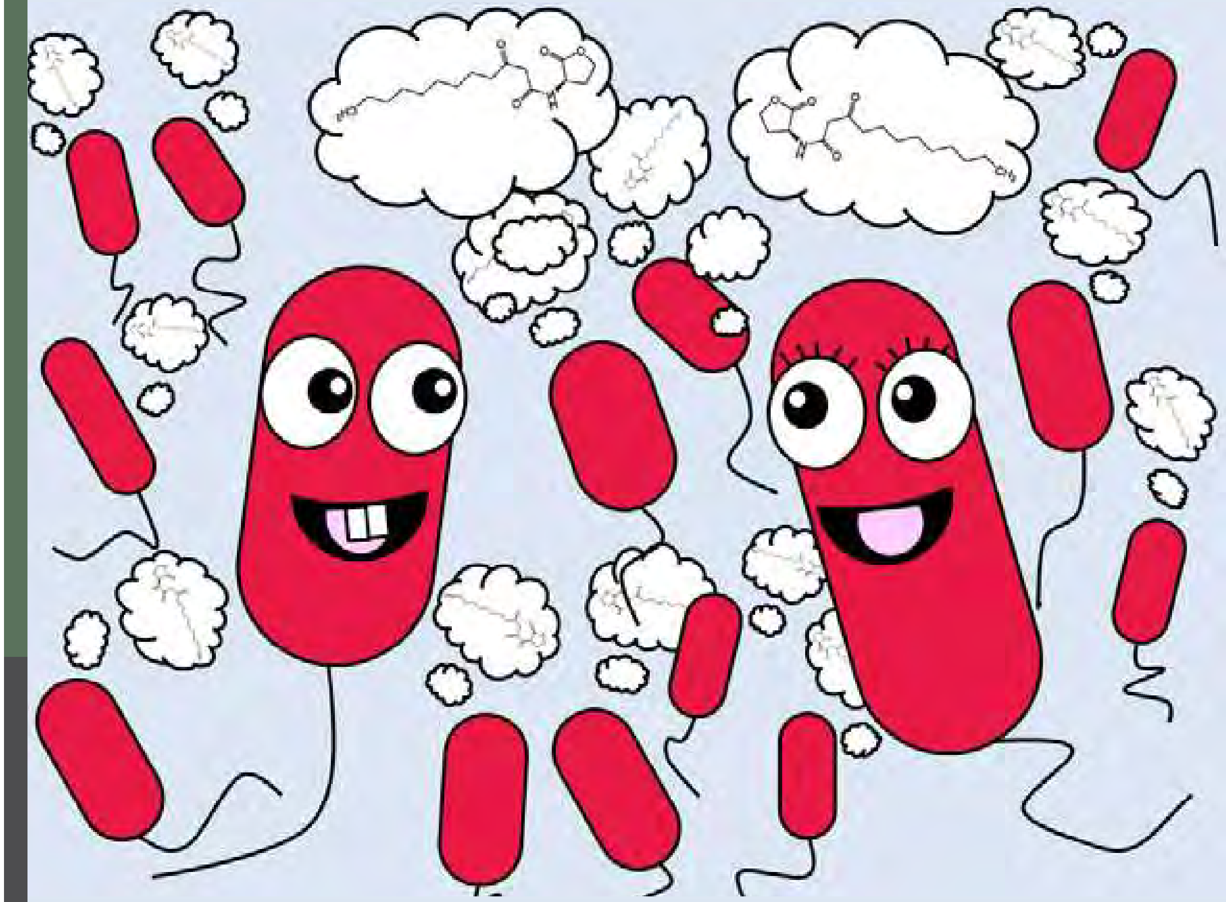


Figura 1. Las bacterias se comunican mediante la liberación de pequeñas moléculas en el ambiente.

copio, pueden colonizar a otro organismo miles de veces mayor y funcionalmente más complejo como un animal o una planta. De hecho, cada tipo de bacteria tiene su propio lenguaje, empleando moléculas específicas, pero también pueden tener moléculas genéricas para comunicarse con bacterias de otro tipo, ya que como ocurre en el caso de los seres humanos, existe una gran cantidad de idiomas en su mundo en miniatura.

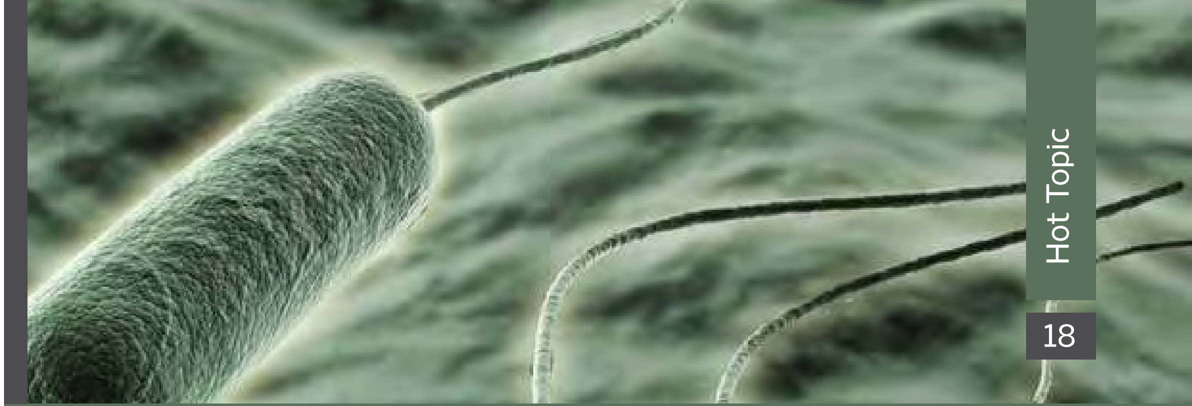
Impactos insospechados

La comunicación bacteriana les permite no solo causar infecciones, también posibilita establecer relaciones benéficas, por ejemplo, los órganos luminiscentes de calamares y peces marinos están poblados por millones de bacterias que viven en simbiosis y producen la luz que se emite. Ha causado sensación descubrir que cuando se corta la comunicación entre las bacterias, éstas pierden la capacidad de emitir luz y de asociarse con los calamares, ¡y de los calamares para atraer presas o una pareja!. Es evidente entonces que la clave para asociarse es establecer una buena estrategia.

Al comprender y descifrar el lenguaje químico de las bacterias, los científicos están abriendo un parte aguas para impedir que estas causen enfermedades y curarnos sin necesidad de ingerir antibióticos, que no solo eliminan a las bacterias que causan el padecimiento, sino en general acaban con todas, incluso aquellas que son benéficas para nosotros (por ejemplo, las de la flora intestinal). Cabe mencionar que con el tiempo, los antibióticos dejan de ser eficaces, ya que las bacterias cuentan con ingeniosos mecanismos para descomponerlos o eliminarlos de sus células. Otra posible aplicación es en la agricultura, donde se ha visto que algunas especies bacterianas se asocian con las raíces de las plantas como el frijol y el cacahuate, formando unas estructuras llamadas nódulos donde las bacterias viven y atrapan el nitrógeno del aire para convertirlo en un fertilizante (nitrato) que aprovecha la planta para crecer y producir frutos y semillas (Figura 2).

Figura 2. El crecimiento de las plantas como el maíz y el frijol depende de asociaciones con bacterias benéficas que viven en las raíces y aportan nutrientes.

Figura 3. Figura 3. Sistemas de cultivo para distinguir entre las bacterias que son dañinas de las que son benéficas. Arriba, plantas de Arabidopsis creciendo sin bacterias, en medio, plantas cuya raíz fue inoculada con bacterias dañinas (P. aeruginosa), abajo, plantas inoculadas con bacterias amigables (P. putida), que promueven el crecimiento. La flecha blanca indica la zona donde se encuentran las bacterias.



Domesticando bacterias

En el laboratorio, es posible domesticar a las bacterias para conocer si estas son amigables o dañinas. Desde tiempos de Luis Pasteur, se han utilizado animales para estudiar las consecuencias de una invasión bacteriana. Ahora es posible, gracias a una pequeña planta llamada Arabidopsis, que puede ser cultivada en los mismos ambientes donde se crecen bacterias, conocer la forma como se transmiten mensajes unas con otras y como su hospedero vegetal reacciona ante dicha comunicación (Figura 3). El desciframiento del lenguaje químico establecido nos ha permitido comprender que muchas bacterias nos ayudan mejorando el sistema inmune, en el funcionamiento de los órganos, así como en la adaptación a diferentes tipos de estrés. Después de todo, los mamíferos y las plantas no somos tan diferentes de esos diminutos seres que nos antecedieron en la evolución.

Conclusiones

Las plantas, los animales y los seres humanos, hemos aprendido a descifrar el lenguaje bacteriano, de tal forma que nuestras células pueden reconocer a las bacterias que son dañinas de las que son amigables, éstas últimas son la mayoría, y viven en nuestros cuerpos aportando nutrientes o estimulando a los tejidos y órganos para que realicen correctamente sus funciones. En respuesta, las plantas y los animales producen compuestos que instruyen a las bacterias para crecer o reproducirse, estableciéndose un diálogo para una coexistencia pacífica. Continuando con las analogías, parece que no siempre gana el que está mejor armado, sino el que se comunica mejor, tal vez los seres humanos podríamos seguir la estrategia de las bacterias para alcanzar una convivencia más armónica y pacífica.

Salvador Barrera Ortiz es Químico-Farmacobiólogo y Maestro en Ciencias con Especialidad en Biología Experimental. Actualmente cursa el Doctorado en Ciencias en el Instituto de Investigaciones Químico-Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo, en Morelia, Michoacán. Estudia las interacciones químicas entre las bacterias y las plantas. Ha publicado diversos artículos sobre el tema. Disfruta de correr, comer pizza y ver series de televisión. Está convencido de que la investigación científica es necesaria para el desarrollo de México y la educación es la clave para transformar al país.
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UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

FACULTAD DE QUÍMICO-FARMACOBIOLOGÍA

"2017, Año del Centenario de la Constitución y de la Universida
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DEPARTAMENTO: DIRECCION
NÚM. DE OFICIO: SAC/113/17
ASUNTO: CONSTANCIA

Morelia, Mich; 31 de mayo del 2017

A QUIEN CORRESPONDA:

Por medio de la presente se hace **CONSTAR** que el **M.C. SALVADOR BARRERA ORTÍZ,** participó como **CO-ASESOR** de Tesis con el nombre de "Estudio del efecto del ácido fólico sobre el desarrollo de la raíz de *Arabidopsis thaliana*." Efectuado el día 30 de mayo de 2017 del PQFB. **JUAN ANGEL AYALA RODRÍGUEZ,** egresado de esta Facultad.

Se extiende la presente para los fines que la persona interesada estime convenientes.

ATENTAMENTE

M.C. ROSA MARÍA TRUJILLO AGUIRRE
SECRETARIA ACADEMICA
FACULTAD DE QUIMICO FARMACOBIOLOGIA, UMSNH

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En la ciudad de Morelia, Michoacán, siendo las 09:00 horas del día 30 de mayo de 2017 se reunieron en las instalaciones de la Facultad de Químico Farmacobiología de la Universidad Michoacana de San Nicolás de Hidalgo, los C.C. sinodales MARTHA ESTRELLA GARCIA PEREZ, LEON FRANCISCO RUIZ HERRERA , ASDRUBAL AGUILERA MENDEZ, Presidenta y Vocales respectivamente del jurado designado para practicar el examen profesional para obtener el título de **Químico Farmacobiólogo,** concedido en oficio número 243300517082693 de fecha 22 de Mayo de 2017, expedido por la Dirección de Control Escolar de la propia Universidad a JUAN ANGEL AYALA RODRIGUEZ con matrícula 0848898G quien nació el día 1 de Octubre de 1993 en el municipio de Zacapu del estado de Michoacán y Clave Única de Registro de Población AARJ931001HMNYDN07, bajo la modalidad de Tesis presentando el trabajo titulado: "Estudio del efecto del ácido fólico sobre el desarrollo de la raíz de Arabidopsis thaliana", por lo que el día y hora señalados, el jurado procedió a practicar el EXAMEN ORDINARIO correspondiente, conforme a las disposiciones reglamentarias vigentes. Una vez concluida la evaluación, los integrantes de la mesa sinodal deliberaron sobre la capacidad e idoneidad del sustentante acordando **APROBARLO POR UNANIMIDAD DE VOTOS CON MENCIÓN HONORIFICA,** lo cual se comunicó al sustentante. Acto continuo, el Presidente del Jurado procedió a tomarle la protesta en los siguientes términos: *"¿Protesta conducirse con toda honradez y moralidad en el ejercicio de su profesión, teniendo siempre presente los elevados fines con los que la Universidad ha instituido esta carrera y de que la misma tiene un carácter eminentemente social; así como normar su conducta, de acuerdo a los principios filosóficos sustentados por esta Máxima Casa de Estudios en el desarrollo de sus actividades?"* contestando el sustentante *"SÍ PROTESTO".* Una vez hecho lo anterior se levanta la presente acta, siendo las 10:46 horas del día de su fecha, firmando en señal de legal y debida constancia de quienes en ella intervinieron, asistidos de ROSA MARÌA TRUJILLO AGUIRRE en su carácter de Secretaria de la dependencia en que se celebra el presente examen Profesional quien autoriza y da fe.

MARTHA ESTRELLA GARCIA PEREZ
Presidenta del Jurado

Lui Fco. Ru H.
LEON FRANCISCO RUIZ HERRERA
Vocal

ASDRUBAL AGUILERA MENDEZ
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EXAMEN PROFESIONAL

PRESENTADO POR

JUAN ANGEL AYALA RODRIGUEZ

PARA OBTENER EL TÍTULO DE

Químico Farmacobiólogo