UNIVERSIDAD MICHOACANA DE
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INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS
Laboratorio de Ecología Microbiana
Laboratorio de Biología del Desarrollo Vegetal

IMPACTO DE LAS INTERACCIONES ECOLÓGICAS Y LA NUTRICIÓN FÉRRICA EN EL CRECIMIENTO Y DESARROLLO DE *Arabidopsis thaliana*

TESIS

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Por este conducto nos permitimos comunicarle que después de haber revisado el manuscrito final de la Tesis Titulada: "Impacto de las interacciones ecológicas y la nutrición férrica en el crecimiento y desarrollo de *Arabidopsis thaliana*" presentado por la M. C. EDITH MUÑOZ PARRA con matrícula No. 0481949J, consideramos que reúne los requisitos suficientes para ser publicado y defendido en Examen de Grado de Doctora en Ciencias.

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RESUMEN

Las plantas perciben muchas señales ambientales que pueden modificar la expresión genética y por lo tanto su morfogénesis. Las interacciones ecológicas planta-planta y planta-microorganismo, así como la disponibilidad de nutrientes son una característica importante para la regulación del desarrollo vegetal. El hierro es un micronutriente esencial y su disponibilidad se encuentra frecuentemente limitada en los suelos, conduciendo a las plantas a la activación de respuestas adaptativas.

Arabidopsis posee un sistema de captación de hierro tipo Estrategia I; recientemente se ha descrito un nuevo mecanismo que permite la regulación de la homeostasis en la que participan los genes *BTS, PYE* e *ILR3*. Las rizobacterias influyen en la adquisición de hierro a través de la producción de compuestos volátiles (VOCs), pero las rutas de transducción de señales que participan en la percepción de dichas moléculas han sido poco estudiadas.

En este trabajo, reportamos que la densidad poblacional es un factor importante en el crecimiento y desarrollo de *Arabidopsis thaliana* y que la emisión de compuestos volátiles determinan las interacciones planta-planta. Por otra parte, evidenciamos que la rizobacteria promotora del crecimiento vegetal *Arthrobacter agilis* UMCV2 emite VOCs que modifican la arquitectura del sistema radicular, promoviendo el crecimiento y aumentando el contenido de clorofila, a través de los genes *ILR3* y *BTS*. Utilizando microscopía confocal, encontramos una correlación entre los efectos de los VOCs producidos por la cepa UMCV2 con un incremento en la expresión de los genes reporteros *ProBTS:GFP* y *ProPYE:GFP*.

La cepa UMCV2 de *A. agilis* produce el compuesto *N,N*-dimetil hexadecilamina (C16-DMA). El análisis del efecto de éste compuesto sobre el crecimiento vegetal, reveló un efecto dependiente de la concentración inhibiendo el crecimiento de la raíz primaria de *Arabidopsis* con la participación específica de *ILR3*; este efecto fue distinto al producido por el total de VOCs de la cepa UMCV2. Nuestros resultados indican que la regulación de la morfogénesis de la raíz implica la percepción de VOCs provenientes de otras plantas y microorganismos; además, las respuestas de *Arabidopsis thaliana* a los VOCs de la cepa UMCV2 de *A. agilis* involucran la participación de los genes de respuesta a la deficiencia de hierro *BTS* e *ILR3*.

PALABRAS CLAVE: *Arabidopsis thaliana, Arthrobacter agilis* UMCV2, Comunicación planta-planta, Comunicación planta-microorganismo

ABSTRACT

Plants perceive many environmental signals that modulate plant morphogenesis and gene expression. Ecological interactions, such as plant-plant or plant microbe relationships, as well as nutrient availability, are important traits for plant development. Iron is an essential micronutrient for plant development and its availability is frequently limited in soils, leading plants to activate iron deficiency responses.

Arabidopsis plants possess a Strategy I Fe-uptake system. Recently, a new homeostasis signaling mechanism has been described, where *BTS*, *PYE* and *ILR3* genes are important components. Some rhizobacteria can regulate plant growth triggering Fe-uptake systems using different mechanisms, such as production of volatile organic compounds (VOCs), but the signal transduction pathways mediating VOCs sensing are not fully understood.

In this work, we report that plant density is a critical factor for growth and development of *Arabidopsis thaliana* y and that VOCs emissions determine plant-plant interactions. On the other hand, plant growth promoting rhizobacteria *Arthrobacter agilis* UMCV2 modulate *Arabidopsis thaliana* growth and development through BTS and ILR3 activation via VOCs emission. We show that in a separate compartments system, VOCs emitted by *A. agilis* UMCV2 modified *Arabidopsis* root system architecture promoting plant growth, and increasing chlorophyll content. Using confocal microscopy and imaging, the effects of UMCV2 VOCs on root architecture were found to be correlated with *ProBTS:GFP* and *ProPYE:GFP* gene expression increased.

A. agilis UMCV2 produce *N,N*-dimethylhexadecylamine (C16-DMA). Analisys of C16.DMA on plant growth revealed a concentration-dependent primary root growth inhibition involving *Arabidopsis* ILR3 participation; this effect was different to that produced by the full UMCV2 VOCs. Our results indicate that root morphogenesis regulation integrate VOCs signals released both by plants and rhizobacteria; furthermore, A. thaliana responses to A. agilis UMCV2 VOCs involve the participation of iron deficiency responses genes, *BTS* and *ILR3*.

KEY WORDS: *Arabidopsis thaliana, Arthrobacter agilis* UMCV2, Plant-plant communication, Plant-microorganism communication.

1. INTRODUCCIÓN

Las plantas son organismos eucariontes, pluricelulares, con gran plasticidad fenotípica en respuesta a señales bióticas y abióticas; como productores primarios, desempeñan una función fundamental en la existencia de la vida en el planeta (Callaway *et al*., 2003; Valladares *et al*., 2007). Debido a ésta función en los ecosistemas, sus interacciones con el medio ambiente resultan de gran importancia. Entre estas interacciones destacan los factores que modulan su crecimiento y desarrollo, como son: la disponibilidad de agua, dióxido de carbono y minerales, la composición y estructura del suelo, la percepción de las comunidades bacterianas, de elementos volátiles y de los exudados radiculares propios y de otras plantas, así como la percepción de tropismos como luz y gravedad (Gersani *et al*., 1998; Kegge y Pierik, 2010).

Las interacciones que permiten la comunicación planta-planta y planta-microorganismo, pueden darse de manera directa como en el caso de la herbivoría, o indirecta durante el consumo individual de recursos que necesitan dos o más organismos (Novoplansky, 2009). Estas interacciones resultan en respuestas que van desde la simbiosis, pasando por el neutralismo y pueden llegar hasta la competencia por recursos como son los nutrientes minerales, el agua y la luz solar. Para mediar esta comunicación se involucran señales químicas, por ejemplo la producción de compuestos alelopáticos y liberación de sustancias tóxicas, tanto de las plantas como de los microorganismos, llevando así a una respuesta final de estimulación o supresión del desarrollo, particular o mutua (Ortiz-Castro *et al*., 2011; Kigathi *et al.*, 2013).

En especies de importancia económica, la competencia por recursos puede variar manipulando varios parámetros, entre ellos la densidad de individuos en un área determinada (Shennan, 2008). Se ha hecho relativamente poco trabajo para entender el efecto de la densidad poblacional sobre la regulación de la morfología y fisiología de las plantas, así como de los mecanismos que median estas respuestas.

Los reguladores de crecimiento son un grupo de moléculas pequeñas derivadas de diferentes vías metabólicas que funcionan como integradoras de información y cuyas vías de señalización con frecuencia interactúan con otras para regular la morfogénesis vegetal en respuestas a cambios ambientales, por ejemplo, la densidad poblacional (López-Bucio *et al.*, 2003). Un solo regulador de crecimiento puede afectar una amplia gama de aspectos celulares y procesos del desarrollo y a su vez un solo proceso puede ser regulado al mismo tiempo por múltiples vías de señalización (Gray, 2004). Se ha determinado que las auxinas (los reguladores del crecimiento vegetal más estudiados), participan modulando cada aspecto del crecimiento y desarrollo de la planta como son la organogénesis, la arquitectura general de la raíz y el follaje, el control de la abscisión, la división celular, el desarrollo reproductivo y la dominancia apical; en respuesta a diversos procesos como la densidad poblacional, variación en la cantidad de luz y precepción de la gravedad (Woodward y Bartel, 2005; Teale *et al*., 2006).

En nuestro grupo de trabajo, se caracterizó a la mutante *pft1-2* como sensible a bajas concentraciones de auxinas, esto evidenciado por la actividad de los marcadores de respuesta a auxinas *DR5:GFP* y *DR5:uidA*, lo que sugería que *PFT1/MED25*, podría estar involucrado en el transporte de auxinas, posiblemente mediando la regulación transcripcional y distribución del transportador de eflujo PIN1 (Raya-González *et al.,* 2014). Posteriormente, reportamos que el incremento en densidad poblacional vegetal, es un factor clave en la regulación de la arquitectura radicular, la morfogénesis general y la productividad final de *Arabidopsis.* Estas respuestas se correlacionan con una afectación en la expresión de genes de respuesta a auxinas, como *PIN1* y *PIN2.* Además se encontró que mutaciones en la subunidad *PFT1/MED25* del Complejo Mediador, provocan una insensibilidad al efecto del aumento de la densidad poblacional, durante el crecimiento radicular.

La percepción de organismos vecinos ha sido poco estudiada en plantas. El reconocimiento planta-planta puede ocurrir de dos formas: i) mediante un contacto directo o ii) a distancia, es decir sin necesidad de contacto físico entre los organismos (Masclaux *et al.*, 2012). Sin embargo, poco se conoce sobre los

mecanismos de reconocimiento planta-planta y las vías de señalización que éstos desencadenan. Reportes recientes indican que este reconocimiento puede ocurrir mediante la producción y percepción de moléculas bioactivas, difusibles o volátiles (Masclaux *et al.*, 2012).

En *Arabidopsis,* las respuestas adaptativas reportadas durante la interacción planta-planta están coordinadas por reguladores del crecimiento, como las auxinas y el etileno. Entre estas respuestas se encuentran la sincronía en el crecimiento y la regulación de procesos fisiológicos que modifican la arquitectura radicular, y producción de biomasa (Masclaux *et al.*, 2012). Aunque aún no se han reportado moléculas específicas que median esta percepción, nosotros reportamos el efecto de la percepción planta-planta en el crecimiento de *Arabidopsis* provocado por compuestos volátiles de manera dependiente de la densidad poblacional vegetal.

Las interacciones ecológicas de los organismos vegetales son complejas e involucran continuamente más de un estímulo. Una de las características importantes para el desarrollo de los organismos vegetales es la disponibilidad de nutrientes. Las respuestas a los parches de nutrientes en la rizosfera modulan la expresión de genes en la planta y con ello el desarrollo vegetal.

El hierro (Fe) es uno de los micronutrientes necesarios para el óptimo funcionamiento del metabolismo. Debido a su capacidad de ganar o perder electrones, los iones de Fe son participes importantes en los procesos metabólicos que mantienen la homeostasis celular, como son la cadena de transporte de electrones durante la fotosíntesis en cloroplastos y la cadena respiratoria en la mitocondria (Hell y Stephan, 2003; Vigani *et al*., 2013).

El mantenimiento de los niveles adecuados de hierro a nivel celular en los organismos, tiene implicaciones de importancia económica y de salud pública. Debido a que el contenido de hierro en las plantas varía desde 3 hasta 11 gramos por kilogramo de materia vegetal deshidratada, se convierten en una fuente importante para la nutrición animal y humana, al ser la ingesta vegetal el principal mecanismo de adquisición. La deficiencia de hierro desencadena anemia

ferropénica, uno de los desórdenes nutricionales más comunes en el mundo, que afecta a más de dos mil millones de personas.

La mayoría de los suelos cultivables presentan condiciones de pH alcalino, por lo que el hierro tiene una baja solubilidad, esto promueve respuestas adaptativas en las plantas (Morrisey y Guerinot, 2009). Las monocotiledóneas no gramíneas y dicotiledóneas como *Arabidopsis thaliana*, presentan respuestas a la deficiencia de hierro mediadas por un mecanismo conocido como Estrategia I (Hindt y Guerinot, 2012). Estas respuestas incluyen cambios morfológicos del sistema radicular, como una mayor formación de raíces laterales y pelos radiculares, área destinada a la reducción y adquisición de Fe (Muller y Schmidt, 2004; Martínez-Trujillo *et al*., 2014). En años recientes se ha descrito un segundo mecanismo que modula la homeostasis del hierro a través de la participación de factores transcripcionales miembros de la familia bHLH (**b**asic **H**elix-**L**oop-**H**elix), como son POPEYE (PYE) e IAA-LEUCINE RESISTANT 3 (ILR3) (Rampey *et al*., 2006; Long *et al*., 2010; Selote *et al*., 2015; Zhang *et al*., 2015).

Para poder adquirir el hierro de la rizosfera de manera más eficiente, las plantas han desarrollado relaciones estrechas con los microorganismos. Las rizobacterias promotoras del crecimiento vegetal o PGPR (***p****lant* ***g****rowth-****p****romoting* ***r****hizobacteria*) modifican la disponibilidad de nutrientes a través de cambios en el pH del medio, o mejoran su solubilidad y/o captación (Masalha *et al.*, 2000; Rroco *et al*., 2003; Pii *et al*., 2016). De esta forma al asociarse con las plantas mejoran su crecimiento mediante diversos mecanismos; algunos de éstos incluyen la interacción con compuestos volátiles o fitohormonas como el ácido jasmónico (Ortíz-Castro *et al*. 2008; Ortíz-Castro *et al*. 2011; Raya-González *et al*., 2017).

Arthrobacter agilis UMCV2, microorganismo perteneciente a las Actinobacterias, se relaciona con la raíz y promueve el crecimiento de *Phaseolus vulgaris, Medicago truncatula* y *Medicago sativa,* especies con sistemas de absorción de hierro tipo Estrategia I (Valencia-Cantero *et al*., 2007). En *Medicago sativa*, el compuesto volátil *N,N*-dimetil hexadecilamina (C16-DMA) producido por *A. agilis* UMCV2 es el responsable de la activación de la Estrategia I. Además, C16-DMA induce la expresión de genes de respuesta al ácido jasmónico y de esta

manera modula el desarrollo del sistema radicular (Velázquez-Becerra *et al*., 2011; Orozco-Mosqueda *et al*., 2013; Raya-González *et al*., 2017).

2. ANTECEDENTES

2.1. *Arabidopsis thaliana* como modelo de estudio

Arabidopsis thaliana es una especie dicotiledónea, miembro de la familia Brassicaceae (Cruciferae), que aunque no es una planta de importancia agrícola ni económica, ha sido el modelo de estudio más utilizado en genética, bioquímica y fisiología vegetal por más de 30 años. *Arabidopsis* se ha utilizado para dilucidar los posibles mecanismos moleculares de acción y/o funciones de una gran variedad de compuestos que produce.

Las principales características que la han posicionado como modelo de estudio por excelencia son sus requerimientos básicos como cualquier otro organismo autótrofo: luz, agua y nutrientes, un ciclo de vida entre seis y ocho semanas; producción de una gran cantidad de semillas, y un espacio pequeño para su crecimiento. Esta última característica permite su cultivo *in vitro* y en invernaderos. Además, se ha secuenciado su genoma, el cual consta de 120 megapares de bases, distribuidas en cinco cromosomas, que contienen alrededor de 25, 000 genes (Bennetzen, 2001).

Adicionalmente, la creación de semillas mutagenizadas ha permitido obtener una amplia colección de líneas mutantes y transgénicas. El genoma de *Arabidopsis* es relativamente pequeño, comparado con el de otras especies cultivables como el maíz (19 veces más grande), la cebada (39 veces más grande) o el trigo (128 veces más grande); los cuales presentan además, duplicación de genes por eventos de poliploidía (Bennetzen, 2002). Con la secuencia completa del genoma disponible, ahora es posible analizar la función de un gran número de genes que participan en diferentes rutas de señalización en los programas de crecimiento y desarrollo (Dinneny y Benfey, 2008).

2.2. Morfología de *Arabidopsis*

Los dos sistemas principales que presenta la morfología de una planta son: i) el aéreo, formado por tallo, hojas; y flores y frutos durante la etapa reproductiva; ii) y el radicular, que consta de una raíz primaria, raíces laterales, pelos radiculares y en ocasiones raíces adventicias (Fig. 1A).

En el sistema aéreo, primero emerge el hipocotilo y el o los cotiledones; posteriormente se forman las hojas verdaderas. Este sistema se encuentra adaptado para la realización de la fotosíntesis, principalmente en las hojas, cuyas células contienen cloroplastos, donde la clorofila (pigmento verde) se especializa en la absorción de luz. Durante la fotosíntesis, la planta utiliza la energía solar y el CO_2 que capta del ambiente, para oxidar el agua absorbida en las raíces. De esta manera libera O_2, y reduce CO_2 para formar trifosfato de adenosina (ATP), glucosa y otros carbohidratos que utilizará durante su desarrollo (Taiz y Zeiger, 2002) (Fig. 1A).

2.3. El sistema radicular

El sistema radicular capta agua y nutrientes del suelo y proporciona sostén a la parte aérea. Las raíces son órganos heterotróficos porque su nutrición depende de la fotosíntesis producida por las hojas; sin embargo, ésta última depende de la captación de agua y minerales absorbidos por la raíz. Adicionalmente el sistema radicular tiene una función de secreción de una gran cantidad de compuestos al ambiente que le rodea, denominado rizosfera (Narasimhan *et al*., 2003).

La raíz se diferencia del tallo por su estructura, el modo en que se forma y la falta de apéndices como yemas y hojas. La primera raíz derivada de la planta se conoce como radícula, la cual, se origina durante la germinación de la semilla y a partir de ella se forma la raíz primaria. Las raíces desarrolladas a partir de la raíz primaria se denominan raíces laterales o secundarias. Las raíces que crecen a partir de otras partes de la planta, como el tallo, se conocen como adventicias

(Scheres *et al*., 2002). Además, este sistema presenta pelos radiculares, que son proyecciones de células especializadas de la epidermis (tricoblastos) y que proporcionan a la planta una mayor superficie para la captación de agua y nutrientes (Fig. 1B).

Debido a que las plantas son sésiles, su estrategia para una mejor adaptación a los cambios ambientales es la plasticidad en los programas de desarrollo que modifican su arquitectura. De acuerdo a López-Bucio *et al*. (2003), se distinguen tres procesos principales que pueden afectar la arquitectura de la raíz: i) la división celular en los meristemos formando nuevas células, ii) la formación de raíces laterales aumentando la capacidad exploratoria y iii) la formación de pelos radiculares incrementando el área de absorción de agua y nutrientes. Estos procesos son particularmente sensibles a cambios internos y externos, tanto bióticos cómo abióticos. De igual forma en respuesta a estos estímulos, el sistema aéreo puede modificar el tamaño del hipocotilo, los peciolos, las hojas y los tallos (Franklin *et al*., 2003).

Particularmente, la raíz de *Arabidopsis* tiene características que la hacen un modelo ideal para estudiar procesos fisiológicos, entre ellas la simplicidad de su organización celular y la actividad del meristemo apical (Scheres y Wolkenfelt, 1998). Un número pequeño de células fuente genera todos los tipos celulares a través de divisiones en el meristemo apical de la raíz (RAM, *root apical meristem*), seguidas de una expansión en la zona de elongación y una posterior diferenciación celular en la zona de diferenciación. Debido a que el crecimiento de la raíz primaria es indeterminado, estos procesos son continuos (Scheres *et al*., 1994).

Dicho crecimiento ocurre en el RAM a través de la producción de células en dos direcciones. Una capa de tejido llamado cofia, que abarca el extremo distal de las raíces, protege la punta de la raíz a medida que crece a través del suelo y también percibe y procesa los estímulos ambientales, modulando la dirección del crecimiento en función de la gravedad (gravitropismo), la luz (fototropismo), obstáculos (tigmotropismo), gradientes de temperatura (termotropismo), humedad

(hidrotropismo), nutrientes y otras sustancias químicas (alelopatía o quimiotropismo) (Ishikawa y Evans, 1990; Okada y Shimura, 1990).

El sistema radicular es una estructura formada por diferentes tejidos celulares: la epidermis, el córtex, la endodermis, el periciclo y los haces vasculares (Fig. 1B). Las células que forman los diferentes tejidos se producen a partir de cuatro células iniciales localizadas en el ápice de la raíz (Dolan *et al*., 1993). Los linajes celulares se pueden reconocer por la formación de columnas o filas de células organizadas a lo largo del eje longitudinal (Schiefelbein *et al*., 1997). Internamente y en contacto con las células iniciales se encuentra un número pequeño de células llamado centro quiescente (QC, *quiescent center*), el cual presenta escasa actividad mitótica, y cuya función principal es mantener la organización de células adyacentes. A medida que va creciendo la raíz, la zona de división celular (zona meristemática) dará paso a una fase de expansión celular y al inicio de la zona de diferenciación (Fig. 1B). Una vez que incrementan su tamaño, las células se diferencian en su forma y función final, este proceso está evidenciado por la aparición de pelos radiculares (en la zona de diferenciación), que son células epidérmicas de la raíz primaria, especializadas en la captura de agua y nutrientes. Por otra parte, mediante eventos de división celular en el periciclo, en la zona de diferenciación de raíces laterales de la raíz primaria, se forman las raíces laterales, órganos que incrementan la superficie total de exploración del suelo y contribuyen a un mejor anclaje.

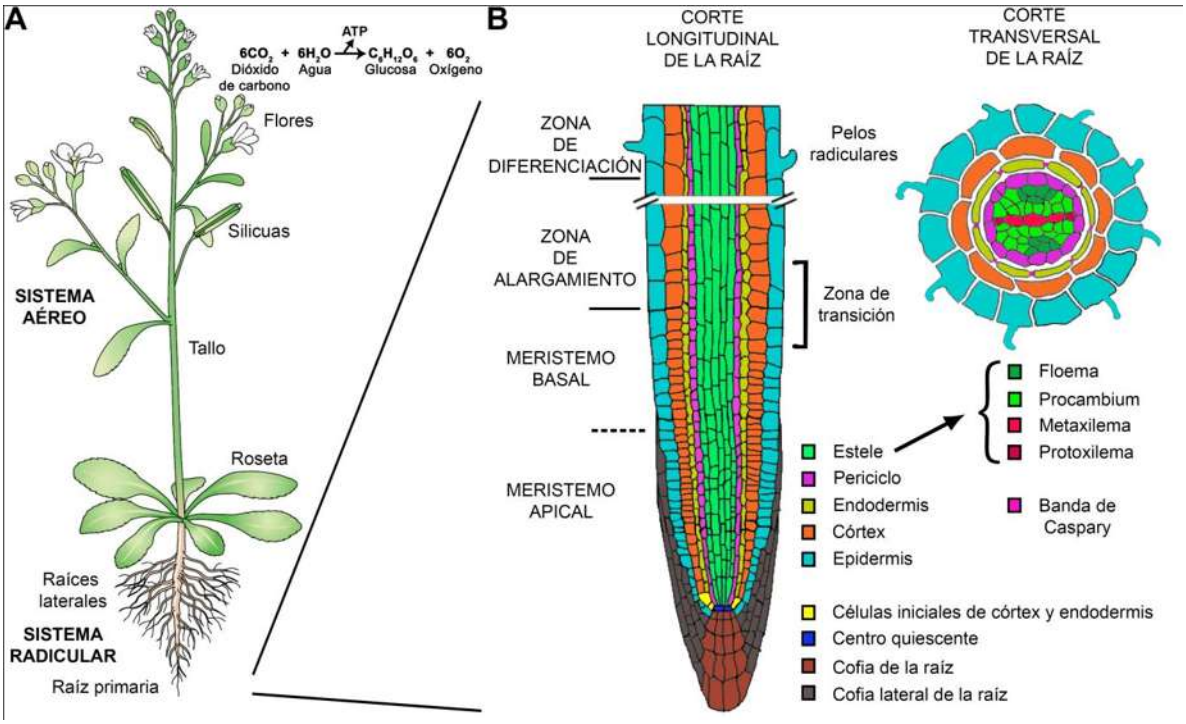


Figura 1. Morfología típica de *Arabidopsis*. A) Sistemas que conforman un organismo vegetal. El aéreo en donde se lleva a cabo la fotosíntesis, utilizando dióxido de carbono y agua para formar glucosa, oxígeno y ATP; este sistema incluye hojas, tallos, flores y frutos. El radicular consta de una raíz primaria, raíces laterales y pelos radiculares. **B)** Acercamiento de la punta de la raíz primaria donde se muestran sus tres zonas principales: meristemática (meristemo basal y apical), de elongación (alargamiento) y de diferenciación. Se puede observar el corte longitudinal y el transversal de la raíz. Los diferentes colores indican los tejidos vegetales (Modificada de Taiz y Zeiger, 2002; De Smet *et al*., 2015).

2.3.1. Raíces laterales

Después de que el patrón de crecimiento de la raíz primaria ha sido establecido y las capas celulares individuales han comenzado a diferenciarse, el aumento en biomasa está basado en eventos de ramificación mediante la formación de raíces laterales (RL). La mayor parte del sistema radicular está formado por RL, originadas a partir de la raíz primaria. El desarrollo de RL está controlado por diferentes factores incluyendo la concentración de nutrientes dentro de la planta y en el suelo (López-Bucio *et al.*, 2003). Por lo tanto, las plantas optimizan su desarrollo en función de la necesidad de absorción de agua y/o nutrientes o

percepción de cambios ambientales, modulando el desarrollo de las RL o de los pelos radiculares (Grierson *et al.*, 2014).

En *Arabidopsis*, las RL se originan a partir de células del periciclo, adyacentes a los polos del xilema; un subgrupo de esas células (denominado zona de oscilación) una vez estimuladas por un incremento de auxinas y la posterior activación del ciclo celular, se diferencian y proliferan formando un primordio de raíz lateral (PRL) (Dolan *et al.*, 1993, Petricka *et al*., 2012). Se ha sugerido que la formación de RL puede ocurrir a través de dos procesos: i) La estimulación de la diferenciación y proliferación en la capa del periciclo que formará el PRL (formación *de novo)* y; ii) La formación del meristemo de la raíz lateral en PRL ya existentes (Celenza *et al*., 1995; Laskowski *et al*., 1995).

Malamy y Benfey (1997), clasifican el desarrollo de las RL en las siguientes etapas: I) iniciación del primordio en un plano longitudinal con aproximadamente 8 divisiones a partir de una célula del periciclo. II) el primordio consta de dos capas celulares obtenidas por una división periclinal de la primera capa. III) se lleva a cabo otra división periclinal para dar lugar a la formación de una tercera capa de células. IV) el primordio consta de cuatro capas. V) el primordio alcanza el córtex. VI) el primordio forma un domo que alcanza la epidermis. VII) ocurre la emergencia de la raíz al romper la epidermis y formar una raíz lateral (Fig. 2).

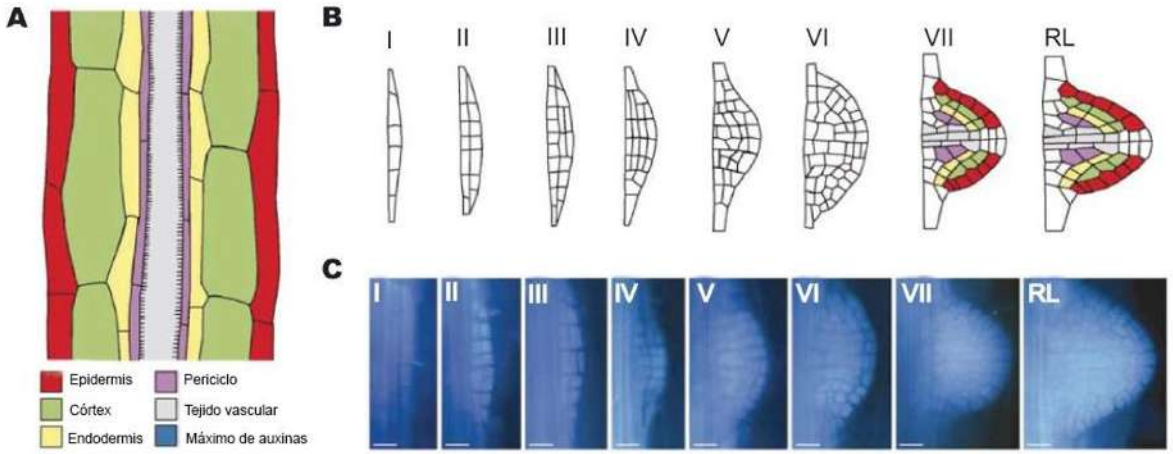


Figura 2. Cambios morfológicos durante el proceso de formación y desarrollo de raíces laterales de *Arabidopsis*. A) Las raíces laterales se originan de la raíz primaria a partir de células del pericicio. **B)** Esquema de los siete estados del desarrollo (Malamy y Benfey, 1997), y **C)** Imágenes representativas de dichos estadios. Las barras de escala representan 20 µm (Modificada de Péret *et al*., 2009).

2.4. Regulación del crecimiento vegetal en respuesta a las interacciones ecológicas

Uno de los principales temas de la sinecología, es la función que presentan las interacciones vegetales determinando la coexistencia y el desempeño de los organismos dentro de las comunidades (Dicke, 2009; Trewavas, 2009). Las interacciones entre organismos o especies, en donde la aptitud o adecuación de uno se ve modificada por la presencia de otro, puede ser de dos tipos: i) intraespecifica, entre individuos de la misma especie o ii) interespecifica, entre individuos de diferentes especies.

Las interacciones biológicas, incluyen estímulos bióticos y abióticos. La limitación de uno o varios recursos necesarios para la sobrevivencia (luz, agua, nutrientes o alimento, territorio, parejas de apareamiento y compuestos) conducen a respuestas adaptativas en los organismos, entre las que destacan: **i) el neutralismo,** cuando dos especies interactúan sin afectarse entre ellas; **ii) la simbiosis**, que es una relación obligada entre dos organismos o especies, que puede o no beneficiar a ambas y iii) **la competencia**, en la cual dos o más

organismos o especies se disputan uno o varios factores medioambientales necesarios (Novoplansky, 2009). Estas respuestas pueden ser intensas, débiles o imperceptibles debido a los requerimientos y las diferencias morfológicas de cada especie (Gurevitch *et al.* 1992); desencadenando un proceso selectivo en el que sobreviven sólo los organismos mejor adaptados (Biedrzycki y Bais, 2009).

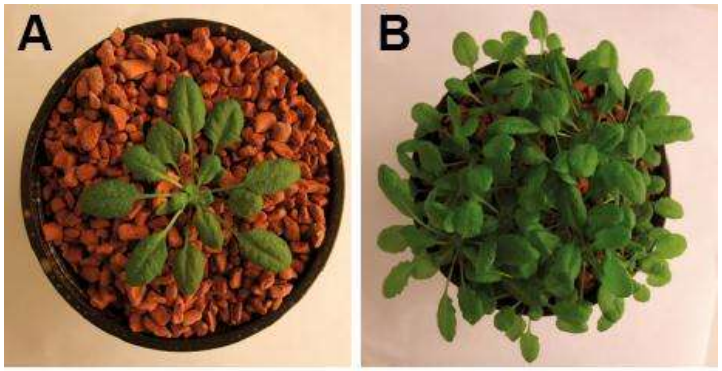
Debido al estilo de vida sésil de las plantas, éstas han perfeccionaron su capacidad de respuesta a un gran número de estímulos externos mediante la regulación genética de rutas metabólicas que funcionan a través de programas de señalización intracelular específicas que conducen al encendido o apagado de genes y de esta manera modifican su crecimiento y desarrollo, capacidad conocida como plasticidad fenotípica (Maathuis y Diatloff, 2013).

2.5. Densidad poblacional

Las investigaciones que se han enfocado en el fenómeno de competencia, la refieren como dependiente de la densidad poblacional, que es el número de individuos por unidad de área. Mientras mayor sea la densidad en una población y el recurso por el que compiten los organismos sea limitado, la probabilidad de relaciones intraespecíficas o interespecificas irá en aumento; y las señales percibidas inducirán respuestas de adaptación para un mejor aprovechamiento de recursos y así lograr la supervivencia.

Las adaptaciones descritas en respuesta a la densidad poblacional incluyen: el vigor de la planta en etapas tempranas (tamaño, altura, biomasa), el desarrollo del follaje (Barberi, 2002; Caton *et al.* 2003; Bertholdsson, 2005; Masclaux *et al*., 2012), la producción de compuestos químicos que influyen en el crecimiento, supervivencia o reproducción de organismos vecinos (alelopatía) (Bertholdsson, 2005; Ni y Zhang, 2005), la habilidad de consumir un recurso dado y/o la plasticidad del crecimiento en respuesta a la disponibilidad espacio-temporal de los recursos (Craine, 2005; Fargione y Tilman, 2006; Masclaux *et al*., 2012). Sin embargo, poco se sabe sobre las señales que inducen estos mecanismos y la

señalización genética que antecede estas respuestas (Masclaux *et al*., 2012) (Fig. 3).



C

	inducidos	reprimidos	total
Hoja	196	134	330
Raíz	158	99	257

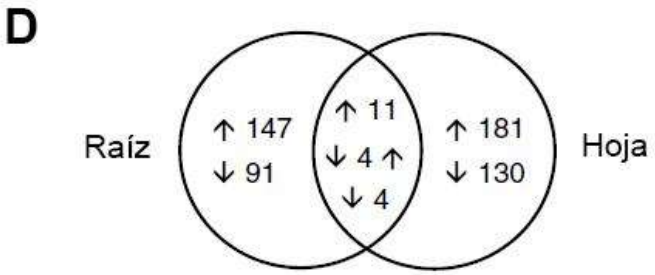


Figura 3. Genes expresados diferencialmente en respuesta a la competencia por densidad poblacional. Plantas de *Arabidopsis* crecieron en densidad de **A)** una o **B)** veinte en condiciones hidropónicas en macetas con perlas de barro cocido. Se realizaron ensayos de microarreglo de genoma completo para analizar el efecto de la competencia sobre la expresión génica. El número de genes regulados de manera diferencial en condiciones de competencia en hojas y raíz se presentan en el panel **C)**. **D)** El diagrama de Venn indica el número de genes que mostraron una expresión diferencial y cuya inducción ↑, o represión ↓ ocurrió en raíz, hoja o se traslapó en ambos. (Modificada de Masclaux *et al.*, 2012)

2.6. Nutrición mineral vegetal

Quizá la respuesta de los sistemas vegetales mejor estudiada, es la habilidad que presentan para responder a zonas del suelo ricas en nutrientes, mediante la proliferación de raíces laterales. Desde hace décadas, es objeto de investigación conocer los requerimientos nutricionales de especies vegetales, principalmente de importancia económica, que permitan su cultivo con mayor facilidad y mejoren la producción de biomasa y rendimiento final (Murashige y Skoog, 1962).

Es importante destacar que la abundancia o carencia de nutrientes también depende de la densidad poblacional que haga uso de ellos. Los efectos que provoca la carencia de nutrientes, pueden ser considerados también respuestas de competencia, aunque aún no se han descrito los procesos del desarrollo involucrados en dicha respuesta (Kegge y Pierik, 2010).

Las plantas requieren nutrientes minerales, además del oxígeno atmosférico y el agua adquirida del suelo. Éstos son elementos químicos esenciales para que la planta complete su ciclo de vida, y entre ellos no pueden reemplazarse, por lo que cada uno es indispensable (Barker y Pilbeam, 2007; Maathuis y Diatloff, 2013). Se ha demostrado la presencia de 60 elementos químicos en los tejidos vegetales, de los cuales 16 son considerados esenciales (Taiz y Zeiger, 2005; Rao *et al*., 2006; Barker y Pilbeam, 2007). Un grupo denominado “macronutrientes” son necesarios en cantidades relativamente altas (Maathuis, 2009) y comprenden nitrógeno, potasio, calcio, magnesio, fósforo y azufre. El segundo grupo, conocido como “micronutrientes” o “elementos traza”; son necesarios en concentraciones menores, e incluyen al cloro, cobre, manganeso, hierro, zinc, cobalto, molibdeno, níquel, sodio y silicio (Marschner, 2012) (Fig. 4).

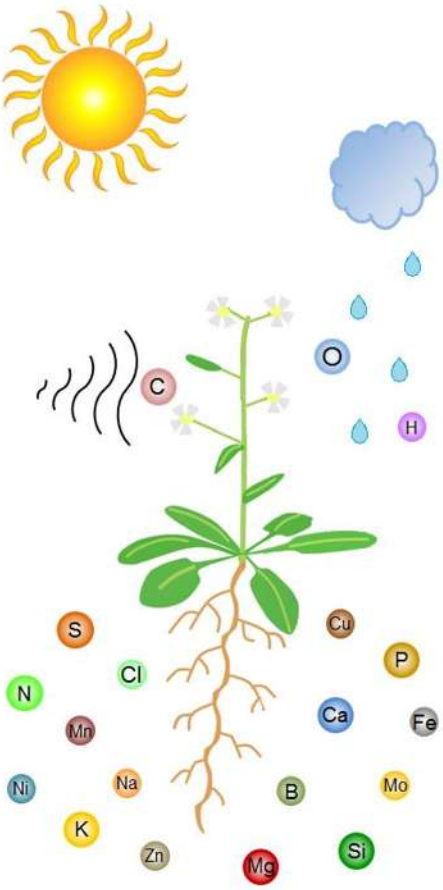


Figura 4. Nutrición mineral vegetal. Los principales nutrientes que requieren los organismos vegetales, provienen del aire y de la materia del suelo. Los macronutrientes: N, K, Ca, Mg, P y S; y los micronutrientes: Cl, Cu, Mn, Fe, Zn, Co, Mo, Ni, Na y Si, están representados como parte de la rizosfera y el aire.

En el suelo, las formas asimilables de los nutrientes minerales normalmente se encuentran presentes en bajas concentraciones, y esta disponibilidad se modifica de manera espacio-temporal, dependiendo de factores ambientales como la precipitación, la temperatura y el viento y/o factores fisicoquímicos como la erosión, el tipo de suelo y el pH (Maathuis y Diatloff, 2013). Las plantas enfrentan frecuentemente una heterogeneidad espacial para captar los nutrientes del suelo, y para mantener su crecimiento han desarrollado mecanismos adaptativos y flexibles que les permiten la adquisición y distribución de dichos elementos mediante la modificación de sus programas de crecimiento y desarrollo (Gross *et al*., 1995; Farley y Fitter, 1999; James *et al.*, 2003; Zhou *et al*. 2012).

Los mecanismos de asimilación de nutrientes, normalmente tienen varias fases. Por ejemplo, la toma de aniones (NO_3^-, PO_4^{3-}, SO_4^{2-}) y de la mayoría de los micronutrientes requiere de energía, lo cual ocurre acoplando su transporte al gradiente de protones (H^+). Por otro lado, los cationes (K^+, Ca^{2+}, Mg^{2+}, NH_4^+) entran mediante sistemas de transporte pasivo (canales iónicos) (Fig. 5). Las plantas han desarrollado adaptaciones morfológicas en respuesta a deficiencias o abundancias de nutrientes localizadas en el suelo (López-Bucio *et al.*, 2003), como la exudación de compuestos que permiten la liberación de los elementos de las partículas del suelo o los convierten a formas químicas asimilables, que entran a la raíz a través de las vías apoplastica y/o simplastica (Maathuis y Diatloff, 2013) (Fig. 6).

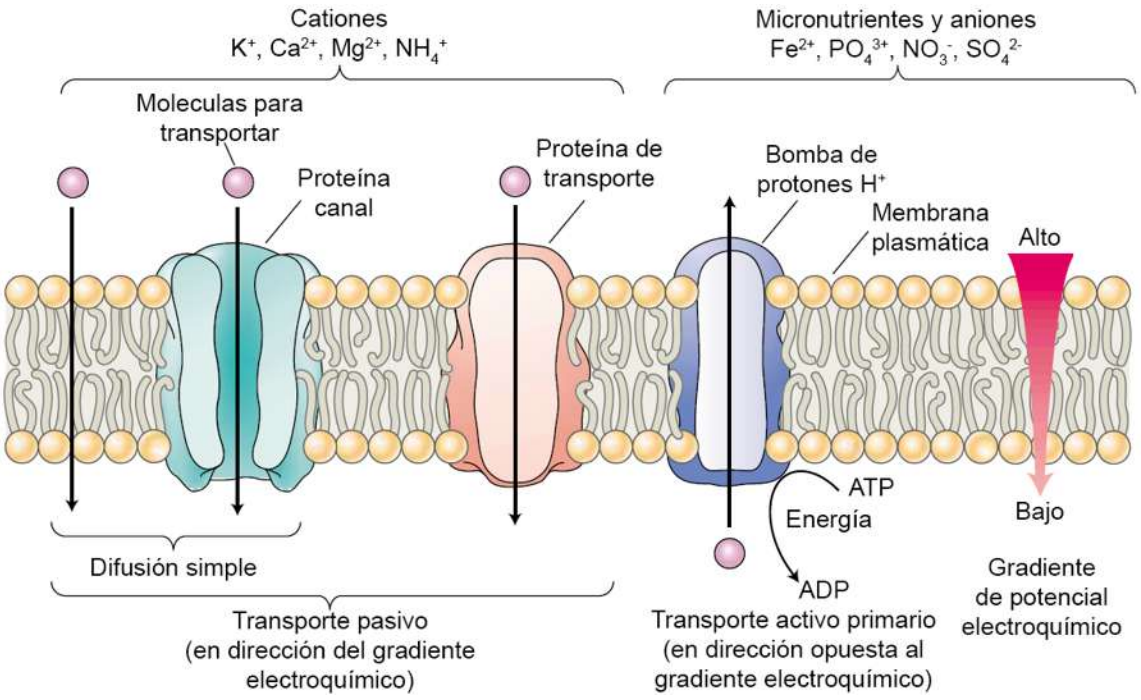


Figura 5. Tres clases de proteínas de transporte de membrana: canales transportadores y bombas. Los canales y los transportadores median el transporte pasivo de solutos a través de membrana por difusión simple o difusión facilitada en dirección del gradiente de potencial electroquímico de los solutos. Las proteínas de canal, actúan como poros en la membrana y su especificidad está determinada principalmente por las propiedades biofísicas del canal. Las proteínas de transporte unen a la molécula que será transportada en un lado de la membrana y la liberan al otro lado. El transporte activo primario se lleva a cabo mediante proteínas bomba que usan energía directa, usualmente hidrolizando ATP, para bombear solutos en contra del gradiente de potencial electroquímico. Modificado de Taiz y Zeiger, 2002)

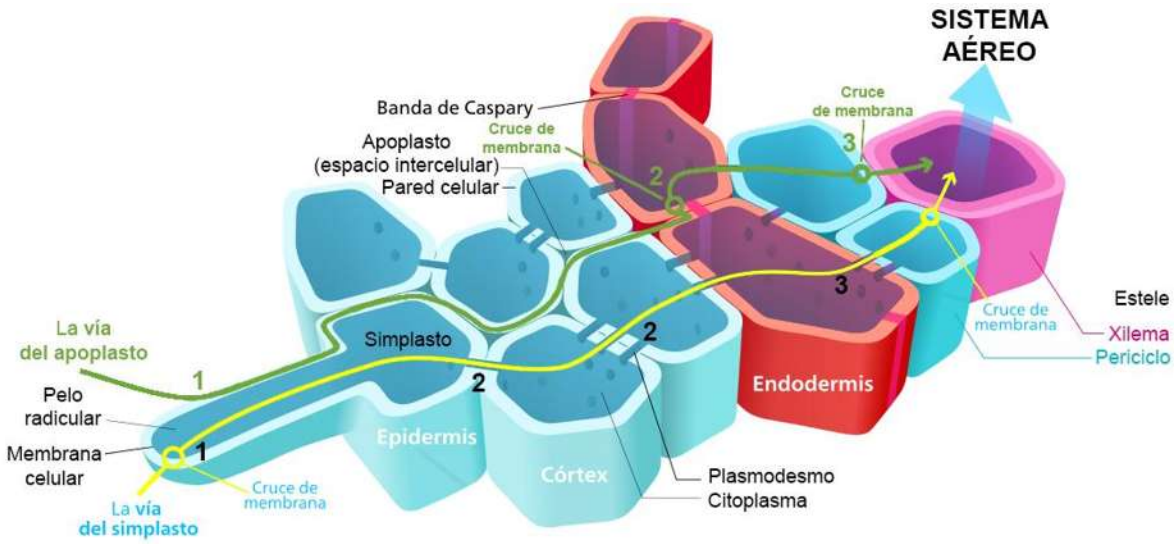


Figura 6. Mecanismos de absorción de agua y minerales en la raíz. En línea color verde, se encuentra marcada la vía del apoplasto: **1)** el agua y los minerales son absorbidos por las paredes hidrofilicas de la rizodermis y difunden a lo largo de las paredes celulares permeables en el córtex. **2)** Una vez que llegan a la banda de Caspary, esta barrera cerosa del apoplasto los obliga a cruzar una membrana celular para entrar a las células en la endodermis y filtrar los materiales antes de entrar a la estela o cilindro vascular. La solución filtrada es liberada por las células de la endodermis nuevamente al apoplasto y al otro lado de la banda de Caspary, de este modo **3)** atraviesa el periciclo y entra al xilema en donde transita a la parte aérea de la planta. En línea color amarilla, se marca la vía del simplasto: **1)** el agua y los minerales son filtrados inmediatamente mientras cruzan la membrana del pelo radicular para entrar al simplasto. **2)** Posteriormente, son movilizados célula-célula a través de los plasmodesmos hacía el cilindro vascular, **3)** evitando así la barrera de la banda de Caspary. (Tomado de Kelvinsong, 2016)

2.6.1. El hierro

El hierro es el cuarto elemento más común de la corteza terrestre, superado en abundancia únicamente por el oxígeno, el silicio y el aluminio; y contribuye con un 5% a la composición total de la tierra y con 3.8 % al peso total. Por su función y las cantidades requeridas en los tejidos vegetales, se le considera un micronutriente esencial (Tabla 1) (Maathuis y Diatloff, 2013)

Una de las principales características del Fe, es que en condiciones aerobias, neutras y alcalinas su solubilidad es muy baja. Se cree que hace

millones de años, cuando aparecieron los organismos fotosintéticos y comenzó la producción de oxígeno, esta nueva condición permitió la oxidación de hierro soluble Fe^{2+} (ferroso) a hierro insoluble Fe^{3+} (férrico) (Maathuis y Diatloff, 2013).

		Niveles en tejido vegetal			
Composición de la corteza terrestre (%)		Macronutrientes		Micronutrientes	
Oxígeno	46.6	Nitrógeno	100	Cloro	0.05
Silicio	27.7	Potasio	50	Hierro	0.03
Aluminio	8.1	Calcio	25	Boro	0.03
Hierro	5.0	Magnesio	10	Manganeso	0.02
Calcio	3.6	Fósforo	8	Zinc	0.007
Sodio	2.8	Azufre	5	Cobre	0.002
Potasio	2.6			Níquel	0.0004
Magnesio	2.1			Molibdeno	0.0001
Otros	1.5				

TABLA 1. Composición elemental de la corteza terrestre (%) y del tejido vegetal. La composición de la corteza terrestre está en proporción al peso con el que contribuye el elemento de un total de 100%. La proporción relativa típica de minerales encontrada en los tejidos vegetales, asumiendo que el nivel de nitrógeno es el 100% (Adaptado de Maathuis y Diatloff, 2013).

2.6.2. Estrategias para la captación de hierro en plantas

Las plantas adquieren el Fe principalmente de la rizosfera. En suelos aerobios o con pH mayor a 7, el hierro se encuentra mayoritariamente como óxido férrico insoluble. En suelos con bajo pH, el hierro se libera de los óxidos volviéndose disponible para su adquisición por la raíz (Marschner, 2012).

En el mundo, aproximadamente el 30% de los suelos cultivables tienen pH alcalino, lo que ocasiona que la solubilidad del Fe^{3+} sea demasiado baja, este es un problema particularmente importante en los suelos semiáridos del subtrópico (López-Bucio *et al*., 2000; Ramírez-Rodríguez *et al*., 2005). Además, algunas

especies de importancia económica como el arroz, son extremadamente susceptibles a la deficiencia de hierro (Takahashi *et al*., 2001). Por ello, se están realizando investigaciones enfocadas a dilucidar los mecanismos por los cuales estos organismos se adaptan a la escasez de hierro (Colangelo y Guerinot 2004; Dinneny *et al*., 2008).

Las plantas han desarrollado dos mecanismos que le permiten adquirir el hierro del suelo:

Estrategia I. La utilizan las monocotiledóneas no gramíneas y dicotiledóneas como *Arabidopsis thaliana*. Dicha estrategia se basa en la reducción del hierro para su posterior internalización (Fig 7A.). En respuesta a la deficiencia de hierro, una citocromo b5 reductasa 1 (CBR1) a través de un mecanismo desconocido, aumenta el contenido de ácidos grasos insaturados en la raíz (Oh *et al*., 2016), que permiten la activación de H^+-ATPasas de membrana en la epidermis de la raíz. Durante la hidrólisis del ATP, exportan protones al exterior de la célula, disminuyendo así el pH del suelo y solubilizando el Fe (Schmidt *et al*., 2003; Santi *et al*., 2005). En *Arabidopsis*, hay doce miembros de esta familia, de los cuales *AHA*2 y *AHA7* (**A**rabidopsis **H**+ -**A**TPase) son esenciales para una correcta respuesta a la deficiencia de hierro (Kim y Guerinot, 2007; Santi y Schmidt, 2009; Jeong *et al*., 2017). Posteriormente, la proteína ferríco-quelato-reductasa dependiente de NADPH, AtFRO2, reduce el Fe^{3+} a Fe^{2+}. Los electrones se transfieren del NADPH citosolico a través de grupos hemo cofactores, que se encuentran estabilizados por cuatro histidinas en el espacio transmembranal, hacia el hierro en la rizosfera (Finegold *et al*., 1996; Robinson *et al*., 1999; Connolly *et al*., 2003). En *Arabidopsis* se han descrito ocho proteínas que pertenecen a la familia de Fe (III) quelato reductasas (AtFROs), de las cuales FRO2 (FERRIC REDUCTION OXIDASE 2) es la responsable de la reducción de hierro a nivel de la superficie de la epidermis de la raíz (Wu *et al*., 2005).

Una vez reducido, el Fe^{2+} puede ser transportado al interior de las células epidérmicas a través del transportador de metal divalente AtIRT1 (Henriques *et al*., 2002; Vert *et al*., 2002). IRT1 también transporta Zn, Mn, Cd, Co y Ni (Eide *et al*., 1996; Korshunova *et al*., 1999; Schaaf *et al*., 2006). Vert *et al.* (2002) y Connolly *et*

al. (2003) reportaron que la pérdida de función de la mutante *irt1-1* disminuyó significativamente la acumulación de Fe, Mn, Zn y CO, lo que sugiere que AtIRT1 es el principal transportador de estos metales en condiciones de deficiencia de hierro.

Estrategia II. La utilizan las plantas gramíneas, que dependen de la adquisición de hierro quelado por sideróforos solubles con alta afinidad por el Fe^{3+} (Marschner, 2012). En respuesta a la deficiencia de hierro, las plantas sintetizan a partir de L-metionina, fitosideróforos derivados del ácido mugineíco, los cuales son liberados posteriormente desde la epidermis de la raíz (Negishi *et al*. 2002). El complejo resultante Fe^{3+}-fitosideróforo se transporta al interior de la epidermis por un sistema de alta afinidad (Curie *et al*., 2001) (Fig. 7B). Esta estrategia es menos sensible al pH de suelo y existe una correlación entre la cantidad de fitosideróforos liberados y la resistencia de las plantas a las condiciones de deficiencia de Fe (Nagasaka *et al*., 2009).

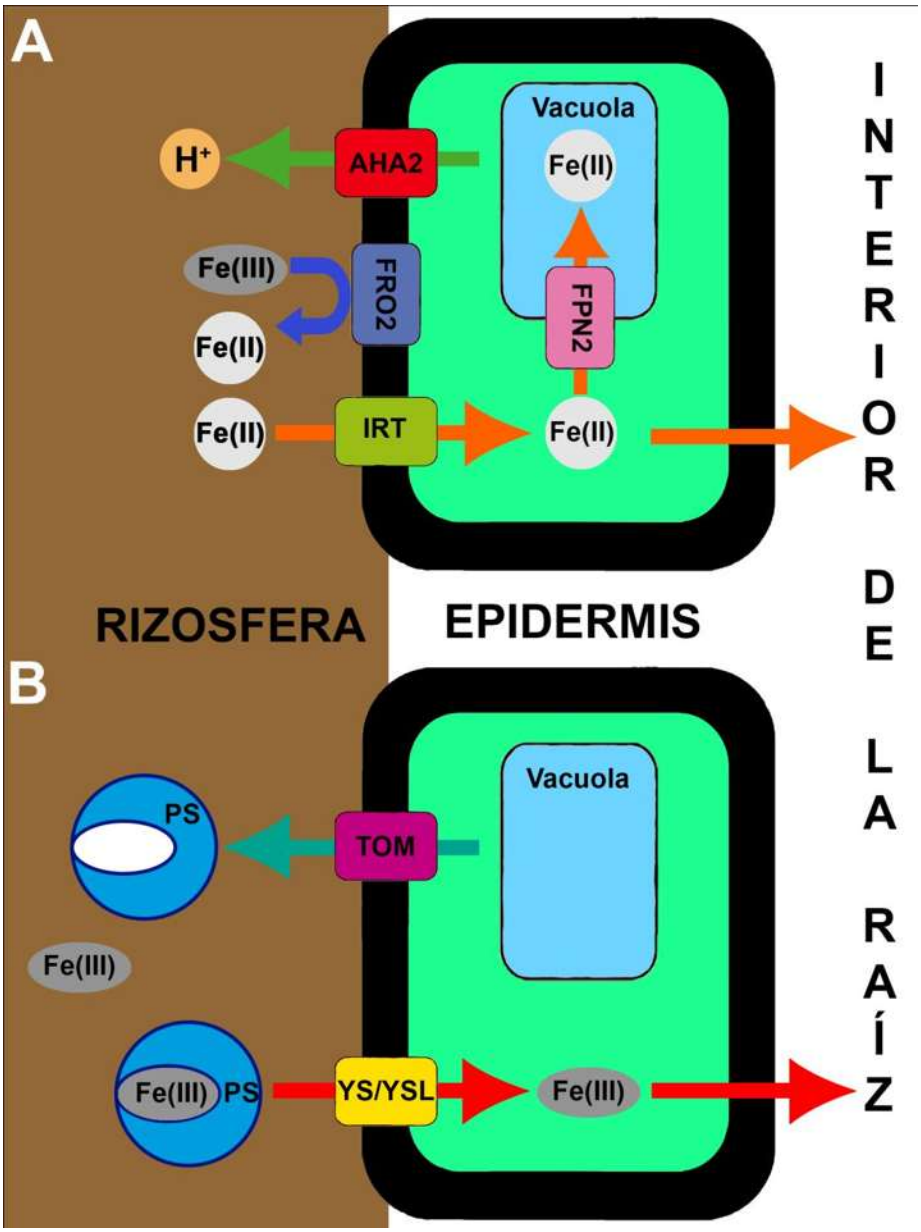


Figura 7. Estrategias para la captación de hierro en plantas. a) Reducción del hierro. En las células de la epidermis, las proteínas H^+-ATPasas (AHA2), exportan protones al exterior de la célula disminuyendo el pH del suelo y solubilizando el Fe. Posteriormente, la proteína férrico-quelato-reductasa (FRO2), reduce el Fe^{3+} a Fe^{2+}, el cual puede ser transportado al interior de las células epidérmicas a través del transportador IRT1. En el interior de la células, el Fe(II) es almacenado en la vacuola a través del transportador FPN2 o llevado a otros tejidos de la planta. **b)** Quelación del hierro. Las plantas sintetizan fitosideróforos que son liberados a la rizosfera desde la epidermis de la raíz a través de los transportadores de eflujo TOM (**t**ransporter **o**f **m**ugineic acid family phytosiderophores 1). En la rizosfera se forma el complejo Fe(III)-fitosideróforo que es de inmediato transportado al interior de la epidermis por un sistema de afinidad que involucra a las proteínas YS y YSL. (Modificado de Morrisey y Guerinot, 2009)

2.6.3. Transporte y almacenamiento de hierro en planta

Una vez que el hierro ha sido captado en la epidermis, debido a su potencial reactividad se une a quelantes o "chaperones" a partir de los cuales es transportado mediante las vías apoplástica y simplástica (Fig. 6). En las raíces, el hierro se almacena principalmente en el apoplasto, en forma de hidróxido de hierro, fosfato férrico o se une a componentes de la pared celular como la pectina y la hemicelulosa, los cuales son polisacáridos con alta carga negativa que participan como sitios de intercambio de cationes (Bienfait *et al*., 1985; Jin *et al*., 2007; Lei *et al.*, 2014) (Fig. 8). El hierro unido a la hemicelulosa es el reservorio de Fe más grande en la pared celular, el cual se ve disminuido en condiciones de deficiencia, mientras aumenta la abundancia de Fe soluble en raíces (Curie y Mari, 2016).

En el simplasto, el hierro es inmediatamente quelado para evitar que forme radicales hidroxilo. Su transporte ocurre a través del citoplasma interconectado entre las células, probablemente difundiendolo a través de un gradiente de concentración (Marschner, 2012). Cuando se ha transportado el Fe a la vasculatura, es quelado para evitar su precipitación. Se considera que para cada ambiente especifico existen quelantes que se unen al Fe; algunos ejemplos de ellos son el citrato que une al Fe^{3+} en el xilema a un pH de 5.5; y la nicotianamina que une indistintamente a Fe^{2+} y Fe^{3+} para prevenir su precipitación en el floema a un pH de 7.5 (von Wiren *et al*., 1999; Curie *et al*., 2009) (Figs. 8, 9).

En *Arabidopsis*, el citrato es internalizado al xilema mediante el transportador FRD3 (Fe-DEFICIENCY RELATED 3), el cual se expresa en la vasculatura de la raíz (Green y Rogers, 2004). Sin suficiente de citrato, el Fe no se moviliza de manera eficiente a través del xilema y por tanto no llega al follaje, sino que precipita en las paredes del apoplasto (Durrett *et al*., 2007). Para la movilización del Fe al interior y exterior del floema, se han propuesto ocho transportadores *Yellow Stripe Like* (YSL) en *Arabidopsis*, los cuales transportan el Fe unido a nicotianamina (Curie *et al.*, 2009).

El floema transporta el Fe hacia las semillas. Las semillas en desarrollo reciben Fe desde las raíces y hojas senescentes. El nivel de reciclaje de Fe del follaje a las semillas varía de acuerdo a cada especie (Morrissey y Guerinot, 2009). OPT3 (OLIGOPEPTIDE TRANSPORTER 3) es un miembro de la familia que incluye a las proteínas YSL y tiene una función esencial en el transporte de Fe a las semillas (Fig. 9).

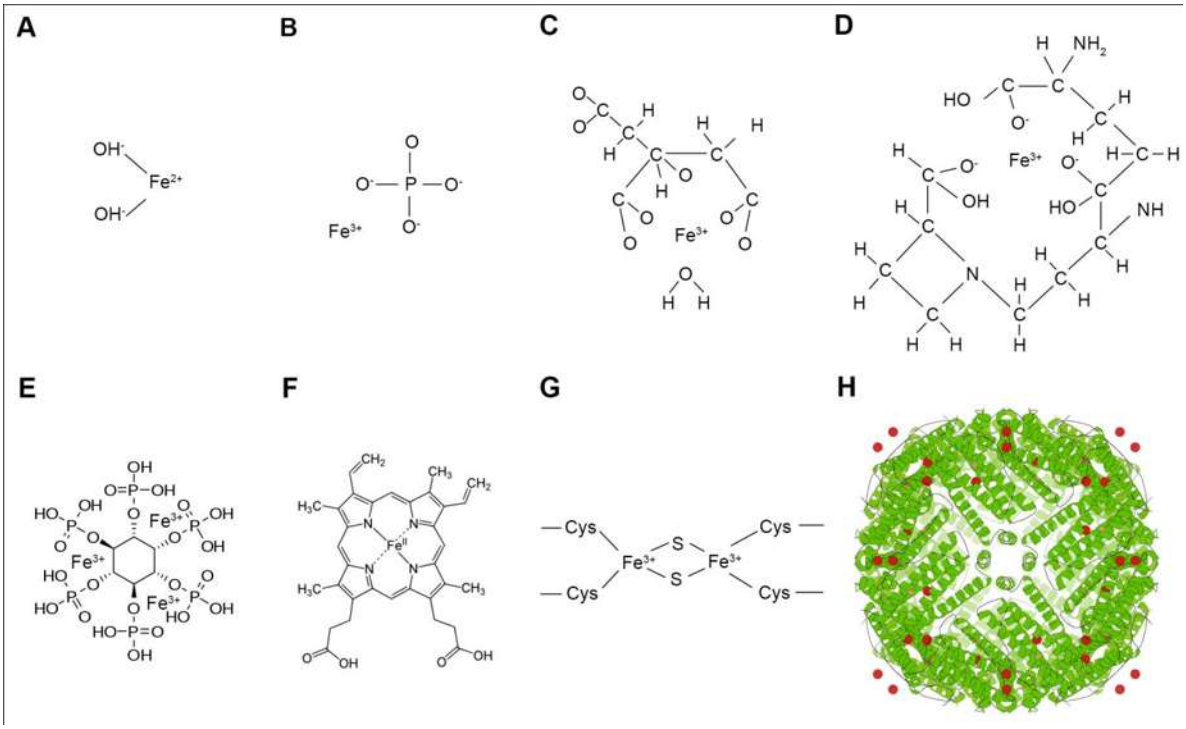


Figura 8. Moléculas quelantes de hierro. A) Hidróxido de hierro (II), **B)** fosfato férrico, **C)** complejo hierro-citrato, **D)** nicotianamina, **E)** fitato, **F)** grupo hemo, **G)** complejo hierro-azufre estabilizado con residuos de cisteína (Cys) y **H)** estructura terciaria de la ferritina en color verde y iones de Fe en color rojo. (Modificado de Chasteen y Harrison, 1999; von Wirén *et al.*, 1999)

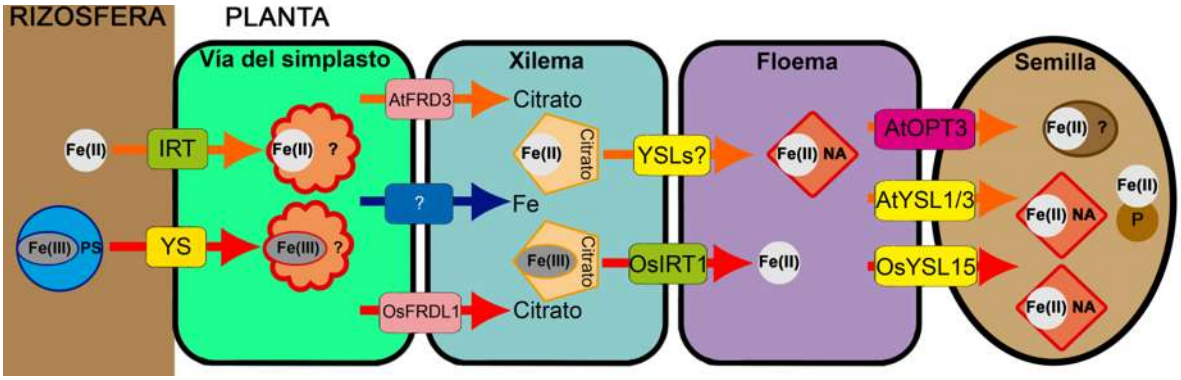


Figura 9. Transporte del hierro en plantas, desde la rizosfera hasta la semilla. En el exterior de la planta, el hierro se puede encontrar como Fe(II) o Fe(III). Las flechas anaranjadas indican el transporte del Fe(II), el cual ingresa a las células de la rizosfera por medio del transportador IRT, una vez en el simplasto se asume que se une a moléculas quelantes y de ahí es transportado al xilema. Las proteínas AtFRD3 y OsFRDL1 ingresan citrato al xilema donde éste se une al hierro, posteriormente el hierro es internalizado al floema posiblemente a través de proteínas YSLs. Una vez en el floema, el Fe(II) se une a nicotianamina y es transportado a la parte superior de la planta para finalmente ingresar a la semilla a través de transportadortes tipo OPT3 o YSL1/3. Las flechas rojas indican el transporte del Fe(III) en plantas tipo Estrategia 2; el complejo Fe(III)-PS ingresa al simplasto a través del transportador YS y posteriormente al xilema en donde se encuentra unido a citrato para posteriormente ser transportado al floema a través de IRT1. Una vez en el floema, el Fe(III) es reducido a Fe(II) y transportado a la parte superior de la planta en donde se almacena unido a quelantes como la nicotianamina o forma complejos Fe-P. (Modificado de Morrisey y Guerinot, 2009)

Se asume que el almacenamiento del hierro posiblemente ocurre en la vacuola de las células, unido a fitato o algún otro agente quelante (Morrisey y Guerinot, 2009) (Fig. 8). Las vacuolas acumulan el exceso de hierro y lo liberan al citosol cuando disminuye su disponibilidad en el suelo. En *Arabidopsis* se ha reportado a VIT1 como el transportador de ingreso de Fe^{2+} a la vacuola (Kim *et al*., 2006), así como a NRAMP3 y NRAMP4 como transportadores de salida (Lanquar *et al*., 2005) (Fig. 10).

El Fe se puede encontrar en los plastidios unido a ferritinas, proteínas de almacenamiento. En *Arabidopsis* se han encontrado cuatro genes que codifican para dicha proteína (Petit *et al*., 2001). Se ha sugerido que los plastidios tienen una función importante almacenando hierro, lo cual podría regular su transporte a larga distancia (Ravet *et al.*, 2009). Los cloroplastos contienen cerca del 90% del

Fe en las hojas (Terry y Abadia, 1986), en donde es requerido para la fotosíntesis, biosíntesis de grupos hemo, y formación de complejos hierro-azufre (Fe-S) (Jeong *et al*., 2008; Vigani *et al.*, 2013) (Figs. 8, 10).

En las mitocondrias, el hierro es requerido para la respiración, la biosíntesis de grupos hemo, y la formación de complejos Fe-S (Balk y Lobreaux, 2005; Vigani *et al*., 2013). La combinación de electrones y Fe libre, es altamente toxica; de modo que la regulación de su homeostasis en la mitocondria es vital. El único transportador mitocondrial de Fe reportado es el ATM, que se localiza en la membrana interna y se cree que permite la salida de agregados Fe-S desde la matriz mitocondrial (Kispal *et al*., 1997). Además, las proteínas ferritina y frataxina tienen una función esencial debido a que además de secuestrar el Fe, median su entrega durante la formación de los agregados Fe-S (Levi *et al*., 2001; Bencze *et al.*, 2006; Missirlis *et al*., 2006; Santambrogio *et al.*, 2007) (Fig. 10).

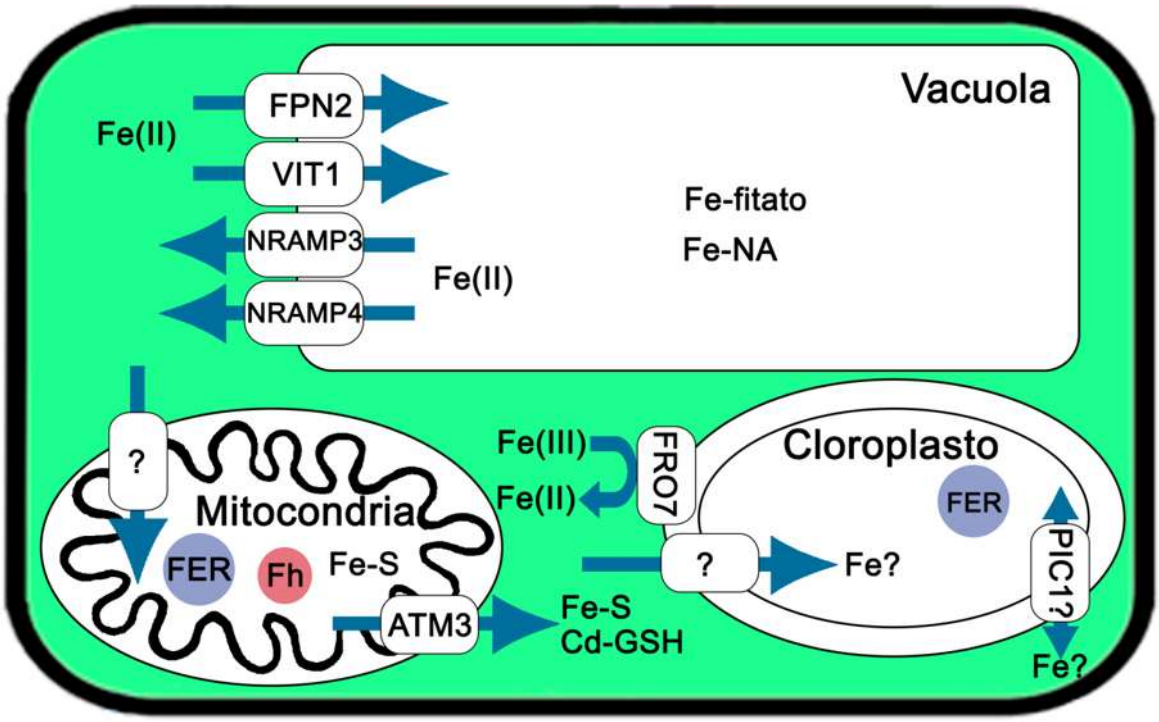


Figura 10. Transporte intracelular del hierro en plantas. El hierro es movilizado al interior de la vacuola por medio de los transportadores FPN2 y VIT1 (que se expresan en varios tejidos). Al interior de la vacuola, el hierro forma un complejo con fitato o nicotianamina (NA). Las proteínas NRAMP3 y NRAMP4 transportan el hierro al exterior de la vacuola principalmente en condiciones de deficiencia de hierro y durante el proceso de germinación. En la mitocondria, el Fe es "secuestrado" por la ferritina (FER) y la frataxina (Fh), principalmente para minimizar el estrés oxidativo. La Fh también tiene una función importante en el ensamblaje o la reparación de los agregados hierro-azufre (Fe-S). ATM3 permite el transporte de los agregados Fe-S al exterior de la matriz mitocondrial. Para ingresar al cloroplasto, el Fe (III) es reducido por la proteína férrico quelato reductasa FRO7 y posteriormente es internalizada por un transportador de Fe (II), posiblemente por medio del PIC1, localizado en la membrana interna. Al interior del cloroplasto, el hierro es incorporado a ferritina (FER). (Modificado de Morrisey y Guerinot, 2009)

2.5.4. Homeostasis del hierro

En *Arabidopsis thaliana*, los genes bHLH (**b**asic **H**elix-**L**oop-**H**elix) constituyen una de las familias de factores transcripcionales más amplias con 162 miembros, que se encuentran modulando diversas vías de señalización del metabolismo secundario, diferenciación celular y respuestas a factores ambientales, una de

ellas es la señalización en respuesta a la deficiencia de hierro (Heim *et al.*, 2003; Jones, 2004; Pires y Dolan, 2009; Zhang *et al*., 2015).

El dominio bHLH de las proteínas, está conformado por 50-60 aminoácidos y consta de dos regiones con funciones distintas: i) la región básica (**b**HLH) formada por alrededor de 15 aminoácidos que se localiza en el extremo N terminal y es la encargada de la unión al DNA y ii) la región HLH (b**HLH**), estructurada principalmente por residuos hidrofóbicos que forman dos hélices α separadas por una región de secuencia y longitud variables y que se localiza en el extremo C-terminal; la cual permite la dimerización proteíca (Heim *et al.,* 2003; Toledo-Ortíz *et al.*, 2003) (Fig. 11). Evidencias cristalográficas mostraron que la interacción entre dos regiones HLH lleva a la formación de homodímeros y/o heterodímeros, mientras que la región básica de cada uno se une a una mitad de la secuencia de reconocimiento del DNA conocida como caja E (5´-CANNTG-3´) (Heim *et al*., 2003; Toledo-Ortíz *et al*., 2003).

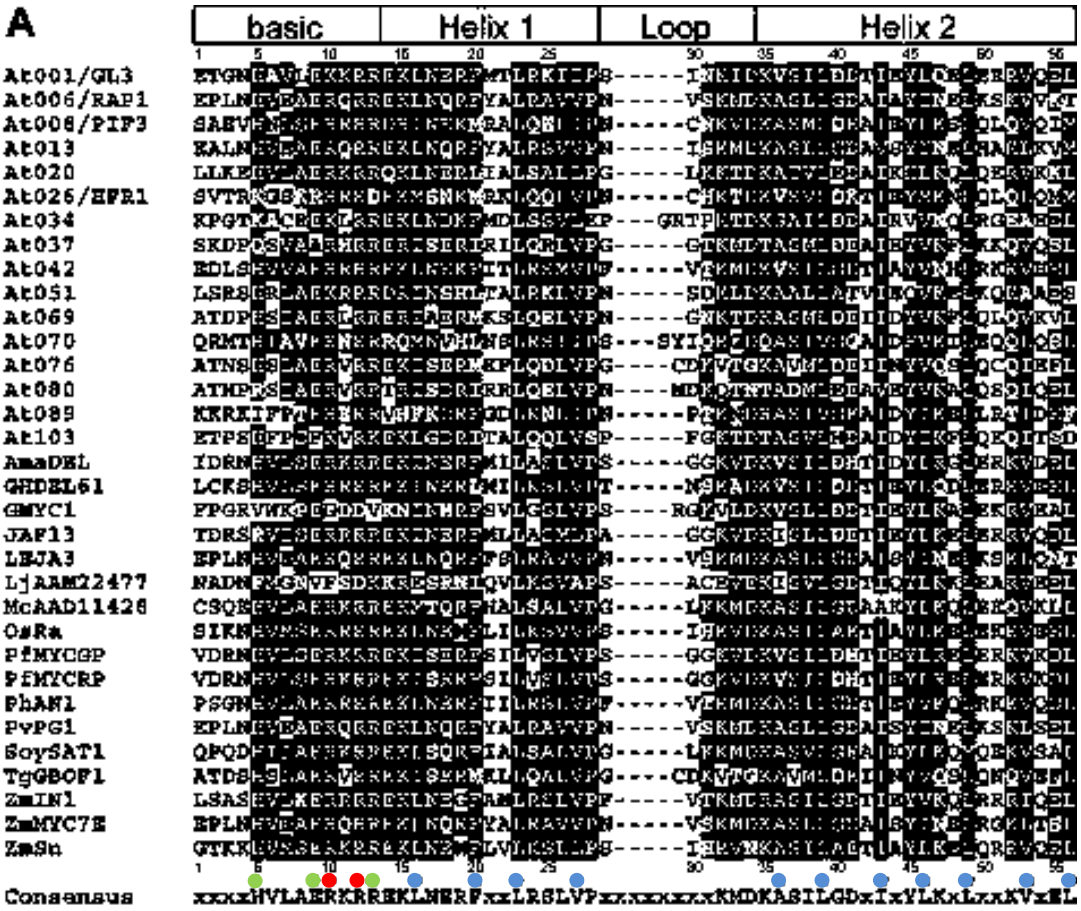


Figura 11. Dominio basic Helix-Loop-Helix en plantas. A) Alineamiento de algunos dominios bHLH en plantas. Los símbolos entre el alineamiento y la secuencia consenso indican residuos importantes para la unión a ADN o interacción proteína-proteína (puntos verdes indican aminoácidos que hacen contacto con bases nucleotídicas, puntos rojos indican aminoácidos que hacen contacto con el esqueleto del ADN y puntos azules indican residuos no polares que participan en interacciones proteína-proteína). En la parte superior se muestra el límite de las regiones b, H, L, H. En plantas, muchos dominios bHLH tienen una secuencia de residuos (H-E-R) en las posiciones 5, 9, 13 (marcadas con asteriscos) que son conservadas y en animales son la característica del grupo B de las proteínas bHLH. (Tomado de Heim *et al*., 2003)

La dimerización y el reconocimiento de diferentes cajas E, proporcionan un mecanismo a través del cual las proteínas bHLH, generan suficiente diversidad para regular una gran variedad de diferentes programas transcripcionales. En este contexto, estas proteínas podrían funcionar como reguladores negativos de otras de la misma familia, mediante la formación de heterodímeros incapaces de unirse al DNA (Heim *et al*., 2003; Toledo-Ortíz *et al*., 2003).

En condiciones de deficiencia de hierro, se incrementa la expresión de un miembro de la familia bHLH (Bauer *et al*., 2007), el factor transcripcional FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (*FIT*), el cual forma heterodímeros con las proteínas bHLH38 o bHLH39 (Yuan *et al*., 2008), y regula positivamente la expresión de *FRO2*, así como también la acumulación de IRT1 y de más del 40% de los genes inducibles en deficiencia de hierro (Eide *et al*., 1996; Robinson *et al*., 1999; Vert *et al*., 2002; Colangelo y Guerinot, 2004).

Long *et al*. (2010) reportaron una segunda vía de regulación de la homeostasis del hierro e identificaron dos genes clave del inicio de las respuestas a la deficiencia de hierro; uno de ellos al que denominaron *POPEYE* (*PYE*), codifica para una proteína bHLH. La línea mutante de *Arabidopsis* afectada en este gen mostró una respuesta hipersensible a la deficiencia nutrimental, con síntomas aumentados de clorosis en el follaje, inhibición del crecimiento de la raíz y pérdida de la capacidad de acidificación de la rizósfera (Fig. 12 A, B). El segundo gen denominado *BRUTUS* (*BTS*), fue identificado mediante el análisis *in silico* de la co-expresión de *PYE* con otros posibles genes blanco; en donde se muestra en una red de interacciones, probables genes que podrían estar regulando la homeostasis del hierro en respuesta a la deficiencia de éste nutriente (Fig. 12C). La línea mutante *bts1* mostró un fenotipo contrario al de *pye1*, sugiriendo funciones opuestas en la regulación de la homeostasis del Fe en condiciones de deficiencia (Fig. 12D). La construcción de las líneas reporteras *ProPYE:GFP* y *ProBTS:GFP* confirmó la expresión de estos genes en respuesta a la deficiencia de Fe, además de una localización en los mismos tejidos de la raíz, como son las células del periciclo (Long *et al*., 2010).

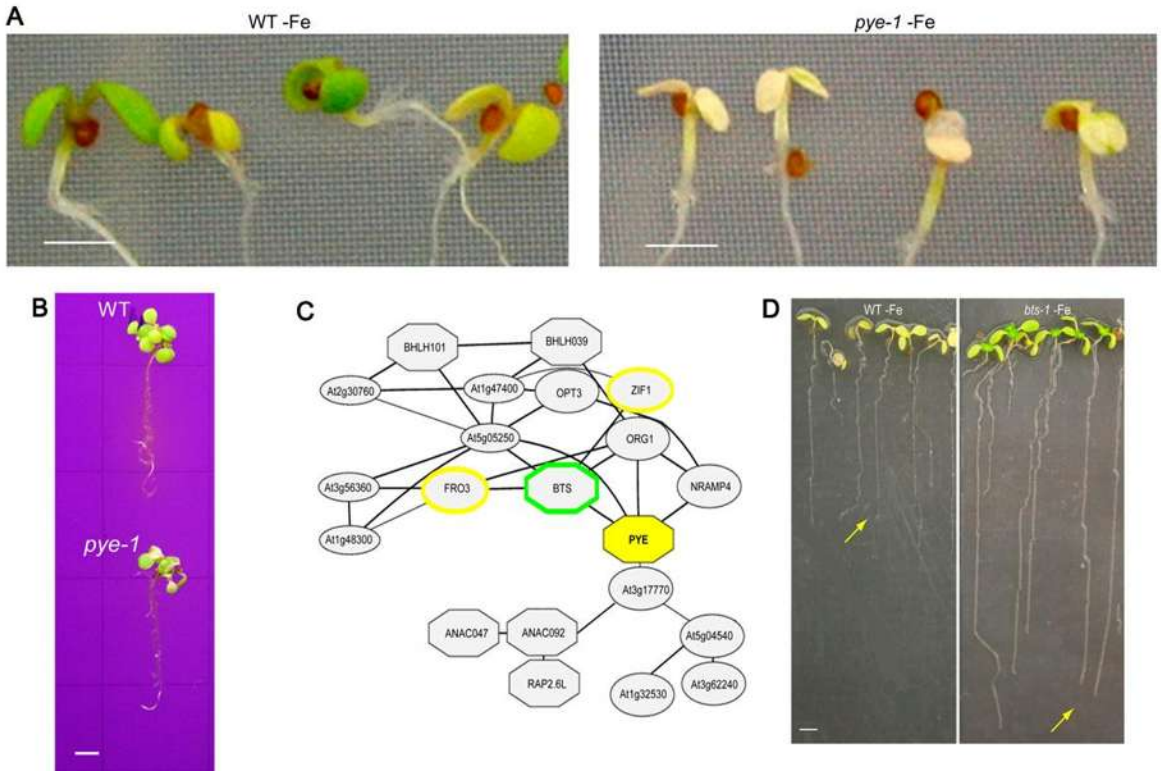


Figura 12. PYE y BTS tienen una función opuesta en la regulación de las respuestas a la deficiencia de hierro. A) Plantas del ecotipo silvestre (WT) y la mutante *pye1* crecidas siete días en medio con deficiencia de hierro. **B**) Acidificación de la rizosfera en plantas WT y *pye1* a los tres días después de la exposición a medio deficiente de hierro. Las plantas se transfirieron a medio que contiene purpura de bromocresol durante 24 horas para observar la acidificación evidenciada por el amarillamiento del medio alrededor de las raíces. La línea de escala indica un tamaño de 3 mm. **C**) Con base en un análisis *in silico* de la co-expresión de genes de *Arabidopsis* en deficiencia de hierro, se identificaron genes que son probables blanco directo de *PYE* y se presentan en la imagen como una red de probables interacciones proteicas. Los bloques octagonales indican proteínas que contienen dominios de unión a ADN. Las líneas amarillas indican blancos directos de PYE. La línea verde indica a la proteína BTS. **D**) Plantas de once días del ecotipo silvestre y la mutante *bts1* crecidas en medio con deficiencia de hierro. Las flechas amarillas indican las puntas de la raíz. La línea de escala indica un tamaño de 2 mm (Modificada de Long *et al.*, 2010).

En mamíferos, la proteína FBXL5 (F-BOX AND LEUCINE-RICH REPEAT PROTEIN 5) fue identificada como el sensor de hierro y en su estructura contiene un dominio hemeritrina (HHE) de unión a Fe y un dominio F-box para ubiquitinar y degradar a IRP2 (IRON REGULATORY PROTEIN2), la cual participa en la

regulación de la homeostasis de hierro (Salahudeen *et al.,* 2009; Vashisht *et al*., 2009). En *Arabidopsis* BTS posee un dominio HHE de unión a Fe^{2+}, un dominio RING que se ha descrito para proteínas tipo E3 ligasas (Selote *et al*., 2015). Se ha sugerido que BTS podría tener entonces dos funciones debido a los dominios en su estructura, la primera como regulador transcripcional y la segunda como E3 ubiquitina ligasa interactuando con proteínas blanco para causar su degradación. También se ha reportado que la mutante *bts1*, tiene la capacidad de acumular un exceso de Fe y la expresión de genes inducibles por deficiencia de hierro se ve significativamente incrementada. Debido a lo antes mencionado, se ha propuesto que BTS es un potencial sensor de hierro que regula negativamente la estabilidad de factores transcripcionales rio abajo, modulando así la adquisición de hierro en *Arabidopsis* (Zhang *et al*., 2015; Liang *et al*., 2017) (Fig. 13). Además se identificó una interacción proteína-proteína entre BTS e ILR3/bHLH105, esta última también participa en la homeostasis del hierro (Long *et al*., 2010; Rampey *et al*., 2006; Zhang *et al*., 2015).

El gen *ILR3* codifica para un factor de transcripción miembro de la familia bHLH. En *Arabidopsis*, las proteínas bHLH-ZIP, presentan el dominio bHLH seguido directamente por un motivo tipo zipper de leucina e incluyen a dos subgrupos dentro del grupo IV: i) ILR3/bHLH105, bHLH115, bHLH034, bHLH104 y ii) bHLH047, bHLH011, bHLH121. Aunque sólo estas siete proteínas tienen zippers de leucina en *Arabidopsis*, las proteínas bHLH-ZIP son muy comunes en animales (Buck y Atchley, 2003; Heim *et al*., 2003; Toledo-Ortiz *et al*. 2003).

Los miembros de la familia a la que pertenece ILR3 son ampliamente conservados en plantas con flores; se han encontrado ortólogos en arroz, álamo, tomate, soya, *Medicago*, uva y pino (Rampey *et al*., 2006). La presencia de homólogos bHLH-ZIP de ILR3 en monocotiledóneas y pino, sugiere la posibilidad de funciones distintas, ancestrales y evolutivamente conservadas en plantas con semillas.

En plantas de *Arabidopsis, ILR3* se expresa en varios tejidos de durante el desarrollo sugiriendo una función en múltiples etapas (Rampey *et al*., 2006). Además, la expresión en áreas donde también se expresan genes hidrolasas de

IAA-conjugado propone a ILR3 con una probable función reguladora de la actividad hidrolasa (Rampey *et al*., 2006). La mutante *ilr3-1* mantiene intactos los dominios bHLH-ZIP; esto, aunado a un incremento considerable en la acumulación de Fe, sugiere que la lesión *ilr3-1* confiere una ganancia de función (Rampey *et al.*, 2006). El fenotipo de *ilr3-1* en plantas crecidas bajo concentraciones normales de Fe, no presenta diferencias observables comparado con el ecotipo silvestre (Zhang *et al.*, 2015); y el análisis de la expresión de los genes *FIT, FRO2, IRT* y *Ib* reveló una regulación río debajo de *ILR3,* indicando una función positiva en las respuestas a la deficiencia de Fe (Zhang *et al*., 2015).

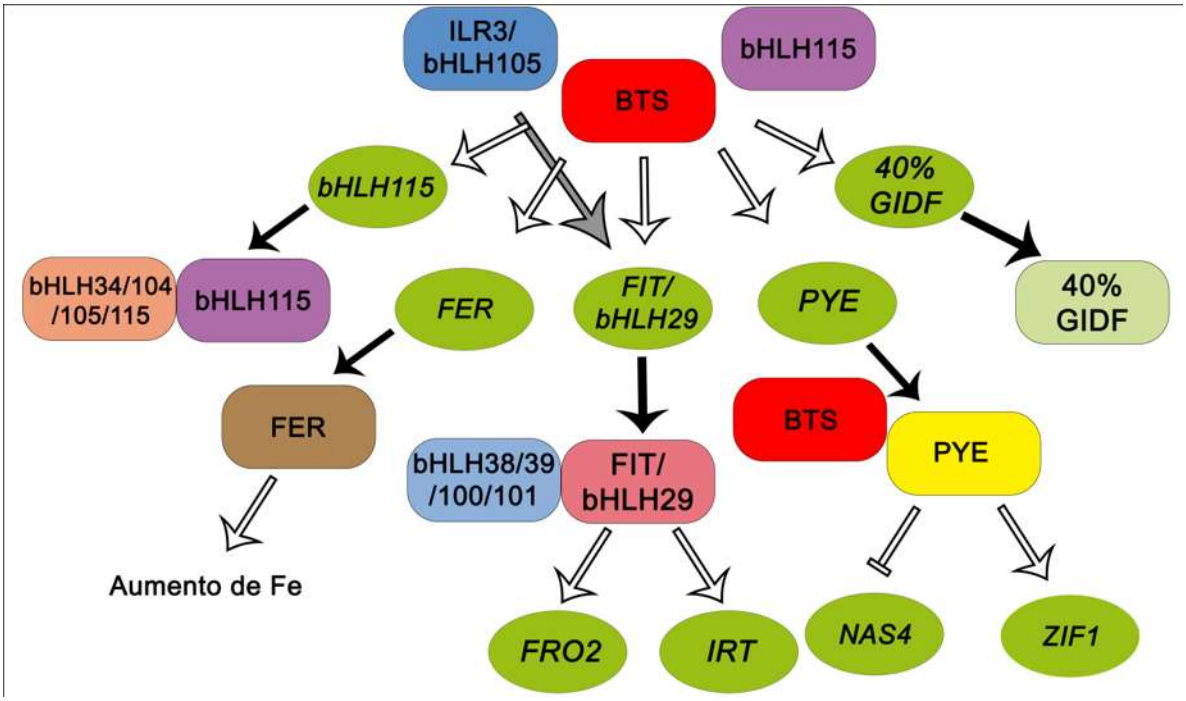


Figura 13. Genes activados por BTS. La proteína BTS puede formar heterodímeros con ILR3 o bHLH115. Reportes indican que puede activar la transcripción de *bHlH115*, que posteriormente puede homodimerizar con bHLH115 o heterodimerizar con bHLH34, bHLH104 o ILR3/bHLH105. BTS también induce la transcripción de Ferritina (*FER*) llevando así a un aumento en la acumulación de hierro. Puede también inducir la transcripción de *FIT*, que a su vez forma heterodímeros con bHLH38, bHLH39, bHLH100 y bHLH101 y de esta forma regula la transcripción de *FRO2* e *IRT*. Además BTS también regula al gen *PYE1,* que a su vez inhibe la transcripción de NAS4 y promueve la de ZIF1. Finalmente se ha reportado que regula la transcripción del 40% de los genes inducibles por deficiencia de hierro (GIDF) (Rampey *et al*., 2006; Long *et al*., 2010; Zhang *et al*., 2015).

2.6.5. Promotores inducibles por deficiencia de hierro

El uso de construcciones con promotores de genes de respuesta a rutas de transducción de señales en plantas, son útiles para monitorear la expresión de genes en diversas condiciones de crecimiento vegetal (Kovtun *et al*., 2000). Entre los reporteros ampliamente utilizados se encuentran la β-glucuronidasa (*uidA*) y la proteína verde fluorescente (GFP) (Jefferson *et al*., 1987; Ottenschaläger *et al.*, 2003).

En *Arabidopsis,* existen promotores de varios genes de respuesta a la deficiencia de hierro (como *PYE*, *BTS* e *ILR3*), unidos a genes reporteros. Para su construcción se utilizó una gran variedad de métodos genéticos y moleculares; por ejemplo, la transformación de plantas silvestres con construcciones génicas, que contienen la secuencia del promotor del gen *PYE* o del gen *BTS,* río arriba de la secuencia codificante para la GFP (*ProPYE:GFP, ProBTS:GFP*) (Long *et al*., 2010). Para confirmar los patrones de expresión de estas líneas, se realizó una inspección visual y caracterización molecular utilizando análisis de microarreglo (Long *et al*., 2010).

Para el caso de *ILR3*, una región reguladora de 1.8 kilobases (que van desde -1863 a -1 pares de bases, considerando el inicio de la transcripción ATG), se amplificaron y purificaron a partir de ADN del ecotipo silvestre usando la reacción en cadena de la polimerasa (PCR); posteriormente se clonaron en el vector pCR4-TOPO, y el plásmido fue secuenciado para verificar la ausencia de mutaciones derivadas del proceso de la PCR. El fragmento promotor de *ILR3* fue separado del plásmido mediante el uso de enzimas de restricción (*HindIII* y *BamHI*) y posteriormente se fue introducido por electroporación en la cepa *Agrobacterium tumefaciens* GV3101*.* Finalmente, por inmersión se transformaron plantas silvestres (Col-0) de *Arabidopsis*. Las líneas transformadas que llevaban la construcción del promotor del gen *ILR3* unido al gen codificante para la enzima β-glucuronidasa (GUS) crecieron en medio de selección (12 μg/mL de Kanamicina). Para confirmar la selección de líneas resistentes, se sembraron y crecieron (1-8

días) semillas resistentes a kanamicina, progenie de la generación filial 1 y fueron teñidas para observar la localización del gen reportero (Rampey *et al*., 2006).

Dichas construcciones son en herramientas poderosas para monitorear los eventos de expresión de estos genes de respuesta a la deficiencia de hierro en una amplia variedad de órganos y tejidos, durante las respuestas de crecimiento y desarrollo causadas por cambios en la disponibilidad de hierro (Rampey *et al*., 2006; Long *et al*., 2010) (Fig. 14).

Figura 14. Patrones de expresión de *PYE* y *BTS*. A) Inducción de *ProPYE:GFP* en células del periciclo después de 4 días en deficiencia de hierro. Las barras de escala indican 50 μm. **B)** Inducción de *ProBTS:GFP* en el periciclo después de 2 días de exposición a la deficiencia de hierro. Las barras de escala indican 100 μm en B1 y 50 μm en B2 y B3 (Modificada de Long *et al*., 2010).

2.7. Las bacterias y su relación con las plantas

Las raíces están rodeadas por una porción de suelo denominada rizosfera (Walker *et al.*, 2004; Bais *et al*., 2006), en la que la planta libera una amplia variedad de compuestos incluyendo azúcares, ácidos orgánicos y vitaminas, los cuales son utilizados como nutrientes o señales para los microorganismos como las bacterias (Rudrappa *et al*., 2008; Badri *et al*., 2009). La interacción de los organismos vegetales con microorganismos de la rizosfera es necesaria para el funcionamiento de los ecosistemas (Shi *et al*., 2016), ya que una gran variedad de bacterias prolifera en esta región (Smalla *et al*., 2006).

Las plantas pueden modular el microbioma de la rizósfera estimulando selectivamente la proliferación de microorganismos con características benéficas para su crecimiento. Se ha observado que las rizobacterias promotoras del crecimiento vegetal (**P***lant* ***G****rowth* ***P****romoting* ***R****hizobacteria*, PGPRs) pueden ejercer su efecto a través de mecanismos directos o indirectos. Los primeros incluyen la secreción de sustancias promotoras del crecimiento vegetal como auxinas, citocininas, giberelinas y compuestos orgánicos volátiles (VOCs, *volatile organic compounds*). Todas estas moléculas influyen en el desarrollo vegetal modulando los niveles de fitohormonas o estimulando el rápido establecimiento de las raíces, ya sea por la proliferación celular en la raíz primaria, la formación y emergencia de raíces laterales y/o raíces adventicias, y la formación de pelos radiculares, que incrementan el potencial exploratorio del sistema radicular (López-Bucio *et al.*, 2003; Ryu *et al*. 2004; Valencia-Cantero *et al*. 2007; Badri *et al*., 2009;). Los segundos incluyen la secreción de antibióticos, que incrementan el desarrollo de la planta al inhibir el crecimiento de microorganismos perjudiciales (Fig. 15).

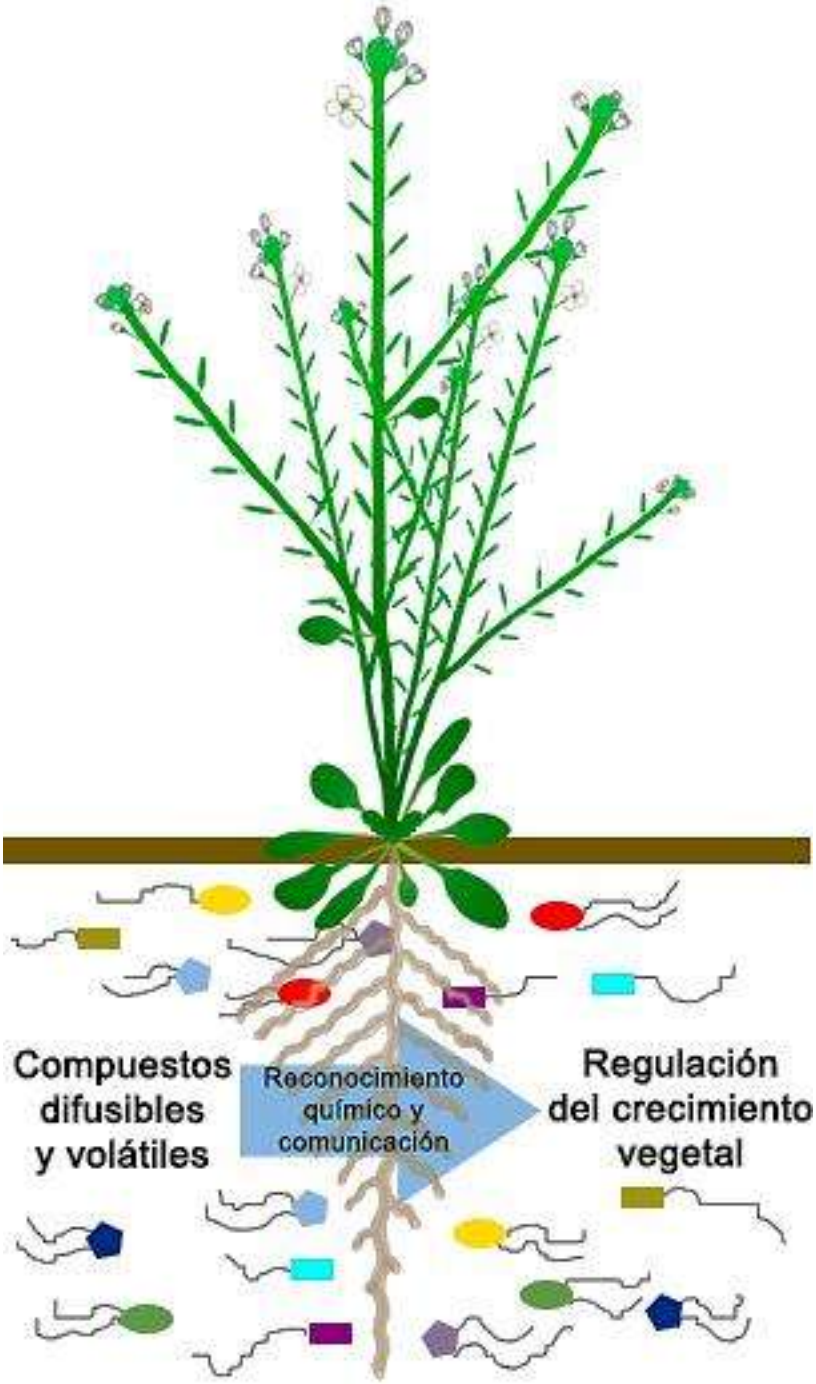


Figura 15. Regulación del crecimiento vegetal mediado por compuestos químicos liberados a la rizosfera. Los microorganismos y las plantas liberan a la rizosfera una variada cantidad de compuestos químicos que permiten una comunicación “informativa” sobre el ambiente, una vez que ocurre el reconocimiento químico, las plantas modifican sus procesos del desarrollo de manera dependiente de la señal percibida.

2.7.1. Adquisición de hierro en plantas, estimulada por la comunicación con *Arthrobacter agilis* UMCV2

Los organismos vegetales requieren mecanismos eficientes para adquirir hierro del suelo y cubrir sus requerimientos nutrimentales durante su desarrollo, evitando efectos tóxicos. Los microorganismos de la rizosfera pueden contribuir de manera importante a que las plantas tengan un mayor acceso al Fe rizosférico, a través de la suplementación del Fe necesario (Masalha *et al*., 2000; Rroco *et al*. 2003).

Algunas PGPRs pueden incrementar la adquisición de hierro a través de la reducción de Fe^{3+} a Fe^{2+} en la superficie de la raíz. Valencia-Cantero *et al*. (2007) aislaron la cepa *Arthrobacter agilis* UMCV2 proveniente de rizosfera de maíz (*Zea mays* L.) y la identificaron como una especie ferri-reductora con capacidad de incrementar la toma de Fe en plantas de frijol. Estudios posteriores mostraron que *Arthrobacter agilis* UMCV2 produce *N,N*-dimetil hexadecilamina, un VOC que modifica la arquitectura del sistema radicular en plantas de *Medicago sativa* (alfalfa), *M. truncatula*, y *A. thaliana* (Velazquez-Becerra *et al*., 2011; Orozco-Mosqueda *et al*., 2013; Velazquez-Becerra *et al.*, 2013; Raya-González *et al*., 2017) (Fig. 16). Orozco- Mosqueda *et al*. (2013) y Castulo-Rubio *et al*. (2015) mostraron que los VOCs de *A. agilis* UMCV2 tienen una función importante en la modulación del crecimiento vegetal en plantas de *M. truncatula* y *Sorghum bicolor*, mediante la regulación de la expresión de genes que participan en la captación de hierro (Fig. 16).

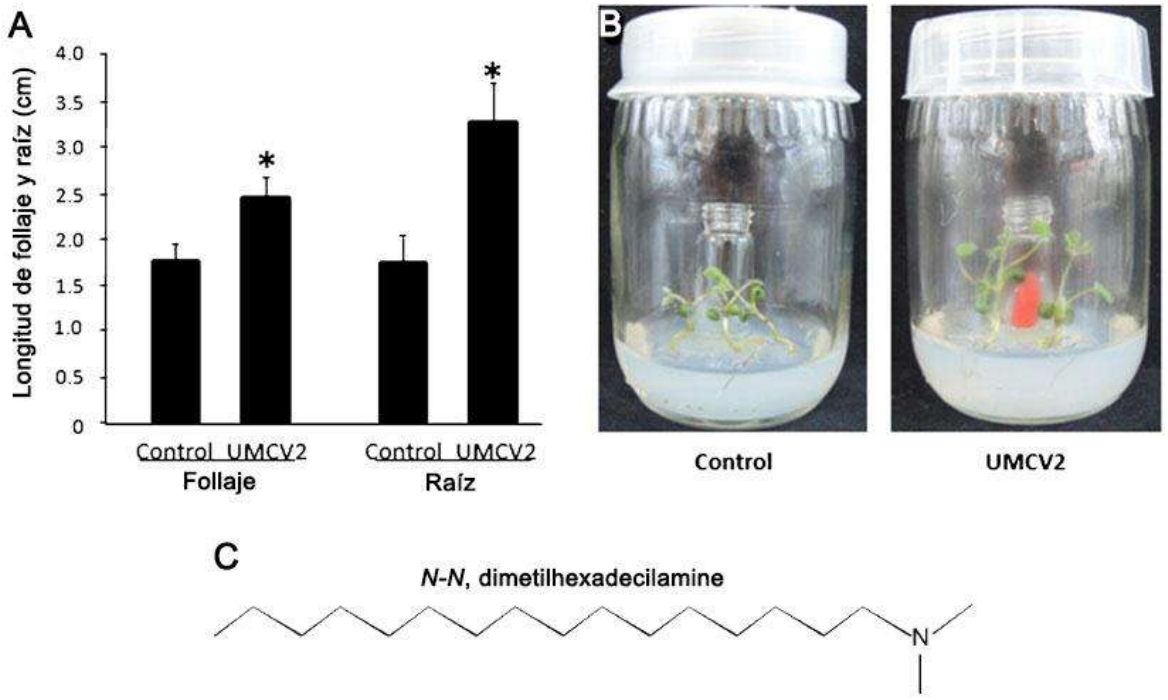


FIGURA 16. *Arthrobacter agilis* UMCV2 promueve el crecimiento de plantas de *Medicago truncatula*. Plantas de *M. truncatula* se colocaron en frascos de vidrio con medio MS. Después de cinco días, *A. agilis* UMCV2 se inoculó en viales con agar nutritivo y se incubaron por cinco días. **A)** Longitud de follaje y raíz. Los valores representan la media ± Error Estándar n=9. **B)** Fotografías representativas del sistema de interacción planta-bacteria, control e inoculado con *A. agilis* UMCV2, cinco días después de la inoculación. **C)** Estructura de la *N-N*,dimetilhexadecilamina producida por *A. agilis* UMCV2. (Modificada de Orozco-Mosqueda *et al*., 2013; Velazquez-Becerra *et al*., 2013)

3. JUSTIFICACIÓN

Las interacciones que se presentan entre los organismos en las comunidades naturales, se ven influenciadas por factores ambientales como la densidad de individuos, que hacen uso de los recursos minerales necesarios para su supervivencia. La regulación de los mecanismos genéticos de los organismos vegetales en respuesta a estímulos bióticos como son la comunicación planta-planta y planta-microorganismo, ocasiona cambios en las respuestas adaptativas que modifican la morfología de las plantas. Desde hace años se intenta elucidar cómo se llevan a cabo los procesos involucrados en estas respuestas, como forma de contribuir al mejor entendimiento del proceso de comunicación vegetal durante las interacciones ecológicas. Por lo tanto, analizar el efecto de la interacción planta-planta y planta-microorganismo utilizando como modelo de estudio a *Arabidopisis thaliana* y *Arthrobacter agilis* UMCV2, en condiciones óptimas de crecimiento y de estrés nutricional causado por deficiencia de hierro, aportará información valiosa sobre ecología molecular vegetal, y potenciales aplicaciones para incrementar la producción agrícola a través del uso de microorganismos para la adquisición más eficiente de nutrientes minerales.

4. HIPÓTESIS

Los compuestos volátiles producidos por las plantas y las bacterias modifican la arquitectura radicular y el crecimiento de *Arabidopsis thaliana* mediante mecanismos regulados por genes que participan en las respuestas a la deficiencia de hierro.

5. OBJETIVOS

5.1. General

Identificar genes de *Arabidopsis thaliana* que participan en las interacciones planta-planta y planta-bacteria, que modulan el crecimiento y la nutrición férrica.

5.2. Específicos

1. Estudiar el impacto de los compuestos volátiles emitidos por *Arabidopsis in vitro* y en suelo en densidades de siembra contrastantes.
2. Caracterizar el efecto de la interacción de los compuestos volátiles de *Arthrobacter agilis* UMCV2 sobre el crecimiento y desarrollo radicular de *Arabidopsis thaliana* en distinta disponibilidad de hierro.
3. Analizar la participación de los genes de respuesta a la deficiencia de hierro *PYE1*, *BTS1* e *ILR3* durante la interacción de *Arabidopsis thaliana* con los compuestos volátiles de *Arthrobacter agilis* UMCV2 en condiciones de disponibilidad contrastante de hierro.

RESULTADOS

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

CAPITULO I.

Muñoz-Parra E., Pelagio-Flores R., Raya-González J., Salmerón-Barrera G., Ruíz-Herrera LF., Valencia-Cantero E., López-Bucio J. 2017. Plant-plant interactions influence developmental phase transitions, grain productivity and root system architecture in *Arabidopsis* via auxin and *PFT1/MED25* signaling. *Plant, Cell and Environment* 40: 1887-1899. DOI: 10.1111/pce.12993.

CAPÍTULO II.

Muñoz-Parra E., Salmerón-Barrera G., Ruíz-Herrera LF., Valencia-Cantero E., López-Bucio J. 2017. Self-plant perception via long-distance signaling. *Plant Signaling and Behavior* 12: e1404218. DOI: 10.1080/15592324.2017.1404218.

CAPITULO III.

Muñoz-Parra E. 2018. Del hierro de las estrellas a nuestro cuerpo. Saber más 41: 25-27. www.sabermas.umich.mx/archivo/articulos/352-numero-41/641-del-hierro-de-las-estrellas-a-nuestro-cuerpo.html

CAPITULO IV.

Volatile compounds from *Arthrobacter agilis* UMCV2 regulates *Arabidopsis thaliana* development through iron-deficiency response genes BTS and ILR3 and dependently of iron availability.

CAPÍTULO I.

Plant, Cell & Environment

Plant, Cell and Environment (2017) **40**, 1887–1899 doi: 10.1111/pce.12993

Original Article

Plant–plant interactions influence developmental phase transitions, grain productivity and root system architecture in *Arabidopsis* via auxin and *PFT1/MED25* signalling

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ABSTRACT

Transcriptional regulation of gene expression influences plant growth, environmental interactions and plant–plant communication. Here, we report that population density is a key factor for plant productivity and a major root architectural determinant in *Arabidopsis thaliana*. When grown in soil at varied densities from 1 to 32 plants, high number of individuals decreased stem growth and accelerated senescence, which negatively correlated with total plant biomass and seed production at the completion of the life cycle. Root morphogenesis was also a major trait modulated by plant density, because an increasing number of individuals grown *in vitro* showed repression of primary root growth, lateral root formation and root hair development while affecting auxin-regulated gene expression and the levels of auxin transporters PIN1 and PIN2. We also found that mutation of the Mediator complex subunit PFT1/MED25 renders plants insensitive to high density-modulated root traits. Our results suggest that plant density is critical for phase transitions, productivity and root system architecture and reveal a role of Mediator in self-plant recognition.

Key-words: *Arabidopsis thaliana*; growth regulation; plant density; plant–plant communication; root architecture.

INTRODUCTION

Neighbour perception is a major survival trait in most species from bacteria to plants and animals (Callaway *et al.* 2003). High organismal density often reduces the availability of resources, which may affect growth, fitness and reproduction and may lead to competition or cooperation relationships. In bacteria, cell to cell communication is controlled via a quorum-sensing (QS)-mediated mechanism, where hormone-like signals known as auto-inducers are released into the medium, influence population gene expression and synchronize behavioural traits (Fuqua *et al.* 1996; Walters & Bassler 2005). Recent research suggests that animals and plants interpret QS signals and respond accordingly to the nature of the interacting bacteria. Plant perception of *Pseudomonas aeruginosa* QS signals induces the canonical auxin response pathway and influences root developmental plasticity, whereas animal cells interpret QS molecules to modulate inflammation or immunity (Ortiz-Castro *et al.* 2011; Grabiner *et al.* 2014). This information points to the existence of signalling mechanisms mediating intra or interspecific perception of neighbours in eukaryotic populations.

Plant density is a major trait controlling productivity in natural and agricultural ecosystems. Plants as sessile organisms cannot escape from depredation or environmental challenges and instead develop sophisticated phenotypic plasticity to efficiently capture light, or take up nutrients and water (Callaway *et al.* 2003; Kegge & Pierik 2010). Currently, how plants perceive other plants and the developmental consequences for growth under high or low densities remain as open questions. Masclaux *et al.* (2013) performed transcriptome analysis of intraspecific competition in *Arabidopsis*, which revealed organ-specific changes in gene expression related to transport processes, metabolism, nutrient acquisition and stress response pathways. This study also identified overregulated genes that correspond to salicylic acid (SA), abscisic acid (ABA) and methyl jasmonate (MJ) signalling, suggesting a complex hormonal crosstalk for biotic and abiotic stress adaptation. In another report, Misyura *et al.* (2014) showed that in rice, ethylene-regulated genes were differentially expressed in response to high plant density, which indicates that this gaseous phytohormone serves as a plant–plant communication signal.

The shade avoidance syndrome (SAS) that initiates upon changes in red:far red ratio of light reprograms shoot growth by adjusting the levels of plant growth regulators such as auxin, ethylene, gibberellins, brassinosteroids and jasmonates (Franklin *et al.* 2003; Pierik *et al.* 2009; Keuskamp *et al.* 2010; de Wit *et al.* 2012; Gommers *et al.* 2013; Leone *et al.* 2014; Crepy & Casal 2015). Root–root interactions have been characterized in some plant species including *Arabidopsis*, making clear that

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plants are able to distinguish their own roots and roots from neighbours and changes root number and length, which was hypothesized to occur through perception of secondary metabolites present in exudates (Krannitz & Caldwell 1995; Falik *et al.* 2003; Caffaro *et al.* 2013). Ethylene and jasmonates are strong candidates as the emitted signals from plants during competition because they are volatile organic compounds (VOCs), but currently neither genetic nor molecular evidence has been reported to explain the critical role of these or other phytohormones on inter or intraspecific plant plant recognition.

The Mediator complex is a large multiprotein complex conserved in all eukaryotes, from yeast to human. Mediator acts as a bridge between the RNA polymerase II complex and the transcription factors that control gene expression (Kim *et al.* 1994; Koleske & Young 1994) and, therefore, regulates a variety of physiological processes such as flowering time, hormone signalling pathways, as well as biotic and abiotic stress adaptation. The PHYTOCHROME AND FLOWERING TIME1 (PFT1)/MEDIATOR25 subunit was originally described as a regulator of shade avoidance, which promotes flowering in response to changes in light quality (Cerdan & Chory 2003). The *Arabidopsis med25* mutant also showed reduced transcription of a number of plant defence genes, particularly those responsive to the plant hormone jasmonic acid (JA) (Kidd *et al.* 2009), but increasing root responsiveness to auxin suggesting PFT1/MED25 as an important transcriptional regulator of root system architecture through auxin-related mechanisms (Raya-González *et al.* 2014). These findings suggest that PFT1/MED25 may orchestrate root/shoot sensing in response to environmental and adaptive cues.

To understand the role of plant plant communication on morphogenesis programs and the underlying molecular responses, in this work, we evaluated the effects of growth of *Arabidopsis* at different densities on developmental transitions, grain productivity and changes of root system architecture. Our data show that plant density is a major determinant for shoot and root development influencing auxin responses and acting via PFT1/MED25.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild-type Columbia-0 (Col-0), *ein2* (Guzman & Ecker 1990), *jar1* (Staswick *et al.* 1992), *pft1-1* (Cerdan & Chory 2003), *pft1-2* (SALK_129555) and *pft1-3* (SALK_059316) mutant plants, and *DR5:GFP* (Ottenschläger *et al.* 2003), *PIN1::PIN1::GFP* (Vieten *et al.* 2005) and *PIN2::PIN2::GFP* (Blilou *et al.* 2005) transgenic lines were used for the different experiments. Seeds were surface disinfected with 95% (v/v) ethanol for 5 min, 20% (v/v) bleach for 7 min and five washes in distilled water. Seeds were then stratified at 4 °C to promote and synchronize germination.

The seeds were germinated and grown on Petri plates containing agar solidified 0.2X MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalogue no. M5524) was purchased from Sigma. Phytagar (Micropropagation grade) was purchased from PhytoTechnology Laboratories. The plant number was established by placing 1-to-32 seeds in each plate following a logarithmic scale. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival Scientific AR95L) at 22 °C, with a 16 h light/8 h darkness photoperiod, light intensity of 100 μmol/m ^{2}/s $^{-1}$ and 80% humidity. For *in soil* analysis, 12 d after seed germination, seedlings were transferred to a soil mixture composed of organic matter (Shunshine), perlite and vermiculite in a 3:1:1 proportion, and transferred plants were again placed in the plant growth chamber to analyse and measure changes in developmental transitions during their life cycle.

Analysis of growth

Plant traits analysed during vegetative growth were rosette diameter, number and length of leaves and petioles, height of the main stem and stem number. Total yield was also analysed by counting fruit (silique) number per plant, number of seeds per silique and silique size, and thus the overall yield per plant was calculated. Seed length and width were also determined using a stereoscopic microscope (Leica, MZ6, Leica Microsystems, Wetzlar, Germany). Seed germination analysis was performed in a 72 h kinetics experiment, counting germination percentage of 100 seeds every 12 h.

Arabidopsis root system was analysed measuring primary root length using a rule. Lateral root number was determined counting the lateral roots present in the primary root, from the tip to the root/stem transition zone using a stereoscopic microscope, and lateral root density was determined dividing the lateral root number value by the primary root length for each seedling. Root hairs were measured in a 500 μm region from the primary root tip. The average length of root hairs was determined measuring 100 hairs for each treatment, taking as a reference the root protoxylem plane to locate the radical hair base in the epidermal cell.

For all experiments, the overall data were statistically analysed in the SPSS 10 program (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses with Tukey's post hoc test were used for testing the differences in growth and root developmental responses in all plants. Different letters are used to indicate the means that differ significantly ($P < 0.05$).

Determination of the developmental stages of lateral root primordia (LRP)

Lateral root primordia were quantified 7 d after germination. Seedling roots were first cleared to enable primordia to be visualized and counted at early stages of development. Each primordium was classified according to its stage of development (Malamy & Benfey 1997).

Propidium iodide staining and green fluorescent protein detection

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

Green fluorescent protein 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 centre, using the IMAGEJ software

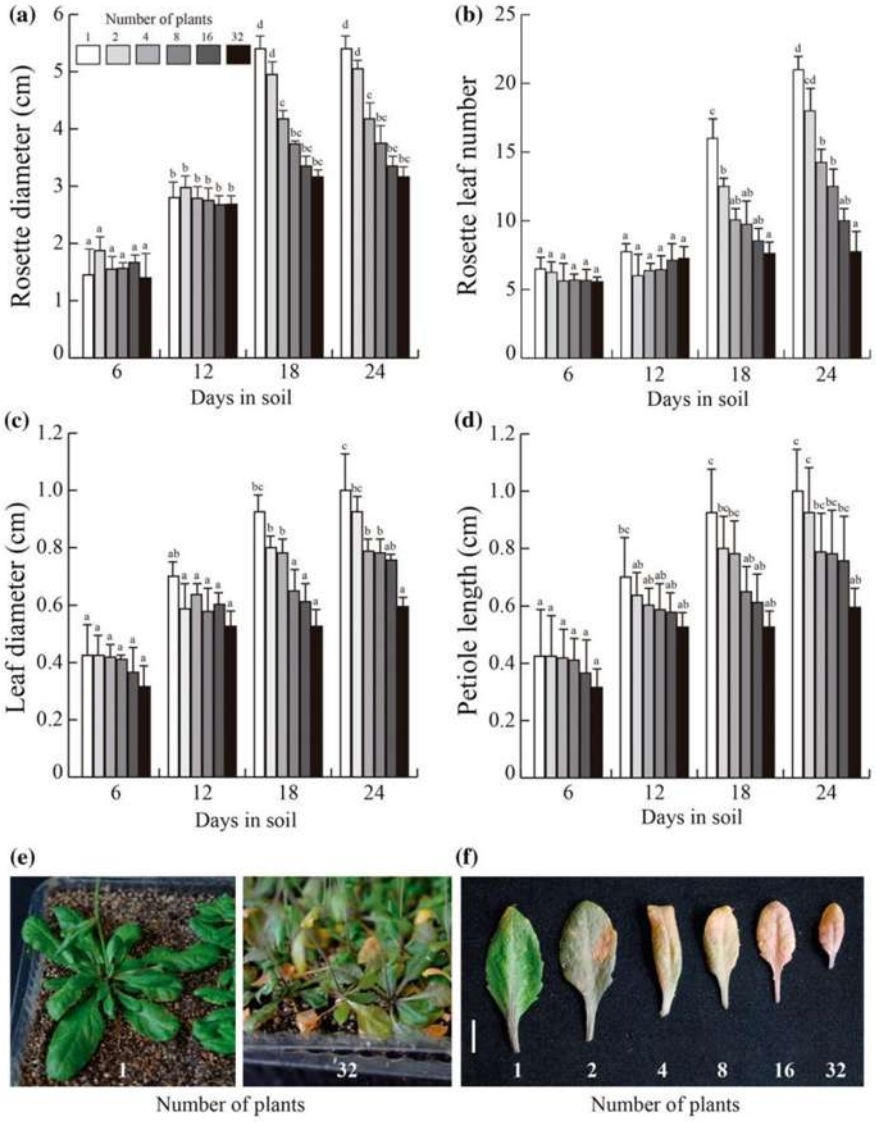

Figure 1. Plant density modulates vegetative growth. *Arabidopsis* seedlings were germinated and grown in 0.2X MS media and 12 d after germination were transferred to soil. (a) Rosette diameter. (b) Rosette leaf number. (c) Leaf diameter. (d) Petiole length. (e) Representative photographs of *Arabidopsis* rosettes of plants grown alone (1) or in a group of 32 plants 24 d after transfer to soil. (f) Representative *Arabidopsis* leaves 24 d after transplanting. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results. [Colour figure can be viewed at wileyonlinelibrary.com]

(http://rsbweb.nih.gov/ij/), in 10 micrographs per genotype and treatment. We then obtained an arbitrary unit value (AU = green pixels/μm) for each individual, and means were obtained from whole data sets. AU means for single plant treatments were given a value of 1, and those higher plant densities were adjusted relative to these, and are thus referred to in figures as relative fluorescence.

RESULTS

Plant density modulates shoot growth and development

Population size is a well-known factor modulating plant phenotypic plasticity when foraging for resources (Falik *et al.* 2003, 2006; Caffaro *et al.* 2013; Misyura *et al.* 2014). To

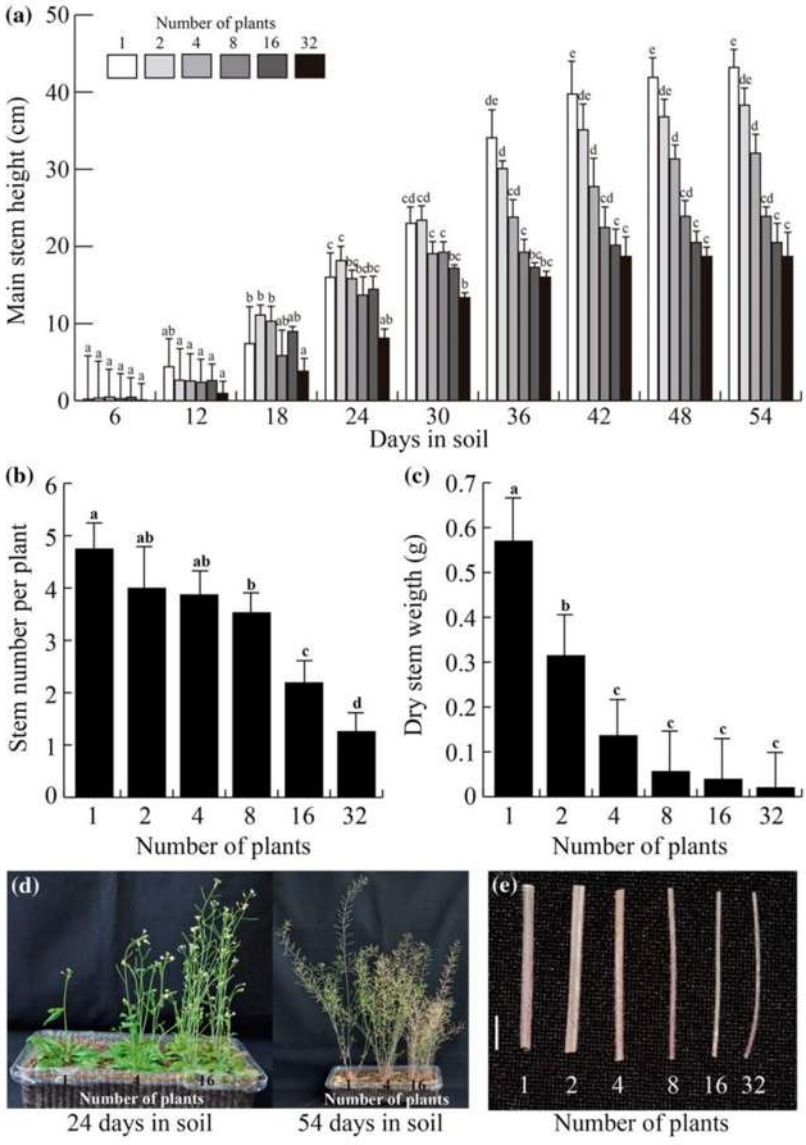

Figure 2. Plant density modulates reproductive development and stem formation. (a) Main stem height of *Arabidopsis* plants grown under different plant density. (b) Stem number per plant. (c) Dry stem weight. (d) Representative photographs showing the effect of density on reproductive and senescence developmental stages at 24 and 54 d in soil, at 1, 4 and 16 individuals. (e) Representative photographs of *Arabidopsis* stem sections. Scale bar represents 5 mm. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results. [Colour figure can be viewed at wileyonlinelibrary.com]

understand how population density affects *Arabidopsis* growth and development, seedlings were germinated and grown on 0.2X MS-agar-media at varying densities from 1-to-32 individuals per plate. Twelve days after germination the seedlings were transferred to soil containing boxes, at varying density, and grown to complete their life cycle. To define alterations in shoot system, time course measurements of rosette diameter, rosette leaf number, leaf diameter and petiole length were done at 6, 12, 18 and 24 d after transfer. We found that plants grown at low densities of one, two and four plants per box showed bigger rosette size, higher rosette leaf number and increased leaf diameter and petiole length than those grown at higher densities (Fig. 1a–f).

Plant density modulates stem height, stem number and senescence

To gain further insight into the plant density role on developmental phase transitions, we analysed its effects on stem development, stem height, stem number and width, and leaf senescence. The stem emergence time was similar at all plant densities from 1 to 32 plants (Fig. 2a). However, clear differences in stem growth were observed starting at 24 d in soil and during the remaining time until senescence. At day 54 after transfer, the main stem height for single plants was 43 cm, whereas in plants grown at a density of 32 individuals, the main stem was of 18 cm (Fig. 2a). Stem number was also modified by plant density, for instance, single plants produced between four and five stems, whereas under the highest plant densities only one main stem was produced (Fig. 2b), which correlated with a decrease in dry weight and width of stems under high density (Fig. 2c,e). It could be also observed that population density affected the activity of the shoot apical meristem and senescence in leaves, evidenced by reduced flower production and increased leaf yellowing after 42 d, this latter symptom appearing first in plants grown at high density (Figs 2d & S1).

Plant density determines fruit and seed production

The reproductive phase is an important stage during the life cycle of plants, determining progeny fitness for dispersion and population viability. In *Arabidopsis*, the reproductive stage starts with stem initiation followed by flower and fruit formation. To determine if plant density could affect fruit and seed production, we counted the number of siliques per plant and the number of seeds per silique at increasing density from 1 to 32 plants. In this experiment, a single plant was able to produce more than 400 siliques with approximately 52 seeds each, whereas in the highest density, plants produced in average 40 siliques with approximately 38 seeds each; this means that a single plant grown alone produced nearly 25 000 seeds, whereas plants at the highest density only produced around 1500 seeds (Fig. 3). These results indicate that seed production is a major trait modified by plant density.

To determine whether plant density affects seed quality, we analysed the size and germination of seeds produced by each

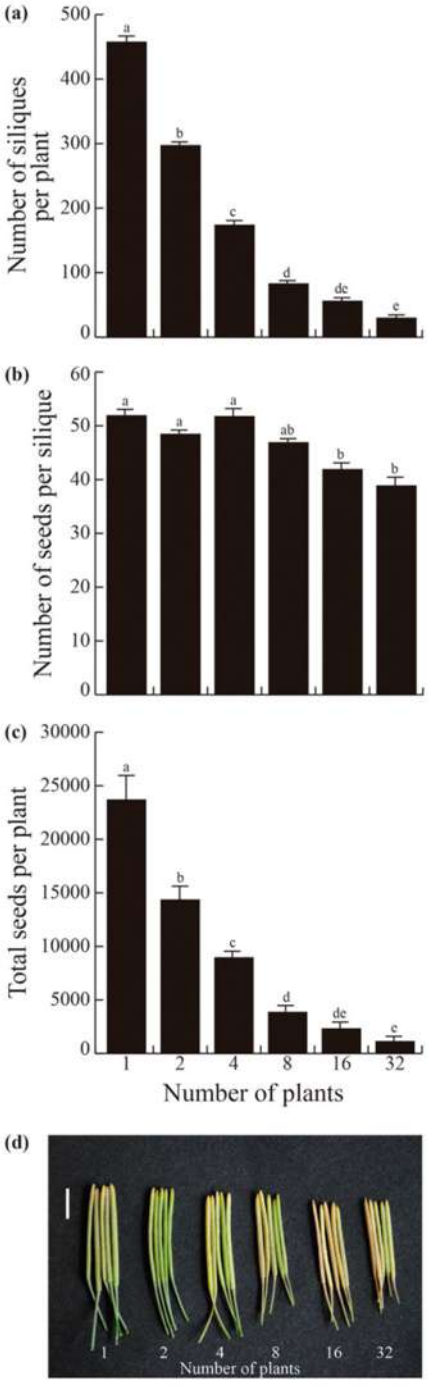

Figure 3. Plant density determines fruit and seed production. (a) Number of siliques per plant. (b) Number of seeds per silique. (c) Total seeds per plant. (d) Representative images of siliques taken from the middle part of stem in all treatments at 42 d in soil. Scale bar represents 5 mm. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results. [Colour figure can be viewed at wileyonlinelibrary.com]

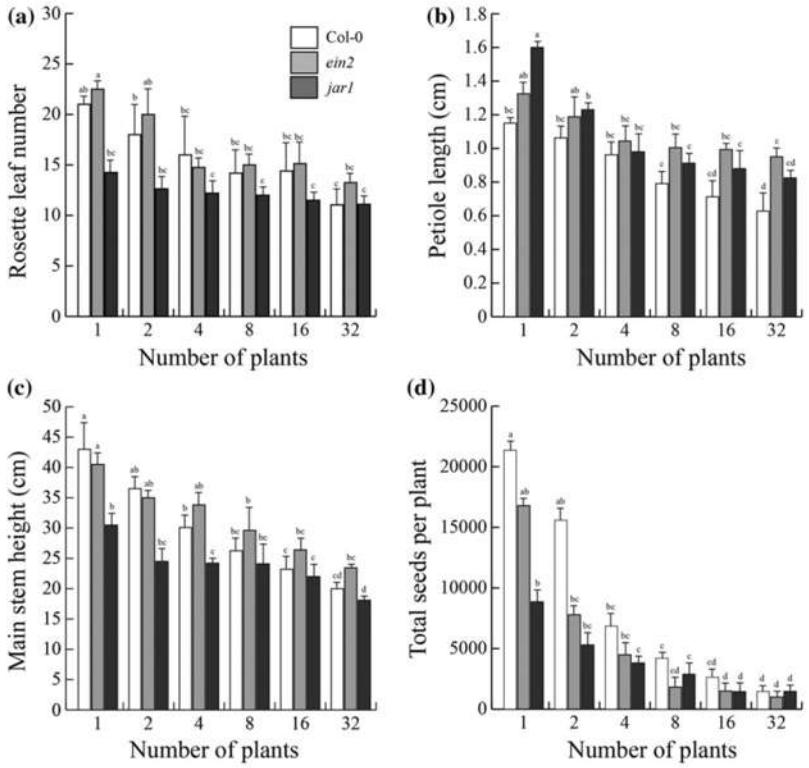

Figure 4. Plant density modulates vegetative and reproductive growth in *ein2* and *jar1 Arabidopsis* mutants. (a) Rosette leaf number and (b) Petiole length at 24 d in soil. (c) Main stem height at 54 d in soil. (d) Total seeds per plant at the end of life cycle. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

parent plant previously grown under varied density. Neither seed length nor seed width was affected by parent plant density in the progeny (Fig. S2). However, a detailed study of germination in a 72 h kinetic experiment indicated that parent density growth severely affected progeny germination because as parent density increased, seed germination decreased from 100% in the lowest density to 83% in the highest density (Fig. S2).

EIN2 and *JAR1* do not participate in plant density response in *Arabidopsis*

Previously, neighbour perception was suggested to be perceived via phytohormone signalling elements, mainly from ethylene and JA pathways. To test this, the growth of *Arabidopsis* WT seedlings was compared to that of mutants defective at the *ETHYLENE INSENSITIVE 2* (*ein2*) and *JASMONIC ACID RESISTANT 1* (*jar1*) loci, which are affected in ethylene and jasmonate signalling, respectively. Regarding rosette leaf number and petiole length, the mutant response to population density was indistinguishable from the WT (Fig. 4a,b). After 54 d of growth in soil both mutants reached maximum stem height (Fig. 4c), and although seed production varied in each mutant compared to the WT, the inhibitory effect on productivity caused by high density was similar in the WT and mutants analysed (Fig. 4d).

Plant density regulates root system architecture

The number of plants growing closely can be perceived by neighbour plants and modulate a subset of growth and development responses in the shoot system (Ballaré *et al.* 1988; Pierik *et al.* 2004, de Wit *et al.* 2012; Masclaux *et al.* 2013; Misyura *et al.* 2014). To determine if root developmental programs are sensitive to the number of plants grown together, we evaluated the consequences of increasing neighbour number of *A. thaliana* seedlings grown over the surface of agar plates on primary root length, lateral root number and lateral root density. We found that all these traits were affected by high plant density (Fig. 5a–c,f).

The lateral root primordia (LRP) stages at varied plant densities were classified according to Malamy and Benfey (1997). The total number of LRPs per seedling changed drastically in response to neighbour number (Fig. 5d). Moreover, LRP stage I, clearly decreased in high density-grown plants, but they increase in both stage IV and V, whereas the number of emerged lateral roots decreased in a density-dependent manner (Fig. 5e). These data suggest that plant

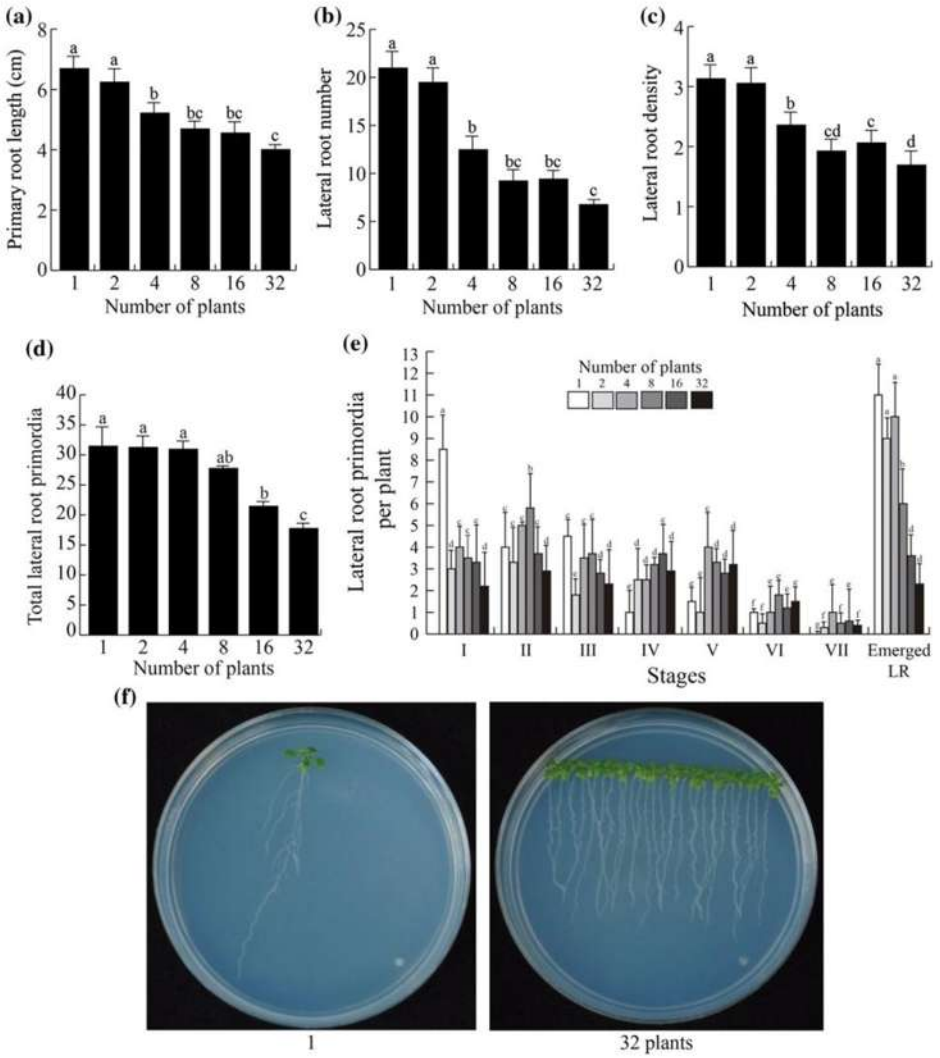

Figure 5. Plant density modulates *Arabidopsis* root system architecture *in vitro*. (a) Primary root length. (b) Lateral root number. (c) Lateral root density. (d) Total lateral root primordia. (e) Lateral root primordia per plant. (f) Representative photographs from single seedlings or 32 individuals grown together. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results. [Colour figure can be viewed at wileyonlinelibrary.com]

density affects several stages during the lateral root formation program.

We then analysed whether plant density could alter root hair morphogenesis. Root hair measurements at the maturation zone of the primary root (Fig. S3C, upper panel) show a small but significant effect of plant density repressing root hair growth (Fig. S3A). Quantification of the number of root hairs at the epidermis layer of the primary root comprising 500 μm in length indicates that plant density does not affect root hair initiation (Fig. S3B). Moreover, representative images show that the root hairs at the root tip develop normally regardless the number of plants grown together (Fig. S3C, lower panel).

Plant density modifies auxin response and PIN transporter levels in roots

The changes of root system architecture in response to plant density suggest that auxin, a phytohormone implicated in the control of root architectural configuration, could mediate these growth responses. Therefore, we evaluated *in vivo* the expression of the auxin-responsive gene marker *DR5:GFP* in transgenic *Arabidopsis* seedlings grown at varied population density by confocal microscopy. Figure 6 shows that *DR5:GFP* expression decreases in primary root tips and root transport tissues as plant density increases. Because auxin is directly transported in root tissues via PIN family proteins,

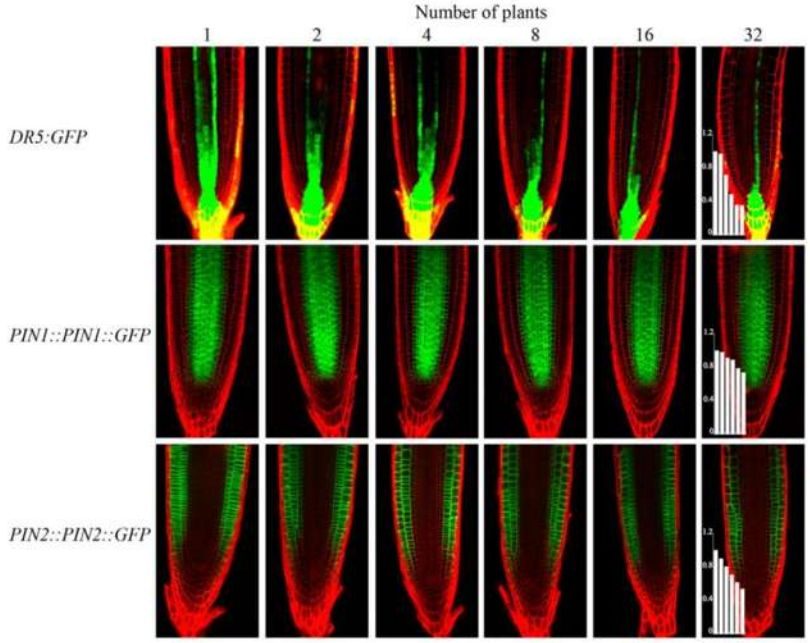

Figure 6. Plant density affects the auxin maximum in primary roots and the level of auxin transporters. *Arabidopsis* seedlings harbouring the *DR5:GFP, PIN1::PIN1::GFP* and *PIN2::PIN2::GFP* gene construct were germinated and grown at different plant densities for 7 d in agar-solidified 0.2X MS medium and photographs show representative individuals of at least 10 seedlings analysed by confocal microscopy. Note that seedlings grown at lower densities show stronger GFP reporter expression and seedlings grown at higher densities show weaker GFP reporter expression in roots. The graphs illustrate the differences in *DR5:GFP* expression between the single plant treatment (left bar) and higher plant densities (right bars), assessed as relative fluorescence intensity. [Colour figure can be viewed at wileyonlinelibrary.com]

which are tissue specific auxin efflux transporters in the root (Gälweiler *et al.* 1998; Müller *et al.* 1998; Wisniewska *et al.* 2006), we next analysed *PIN1::PIN1::GFP* and *PIN2::PIN2::GFP* expression in response to plant density. The results show both *PIN1::PIN1::GFP* and *PIN2::PIN2::GFP* detection diminished in a plant density dependent manner. Together, our data indicate that plant density modulates auxin transport-dependent regulation of root architecture.

Arabidopsis PFT1/MED25 plays a role in the root response to plant density

PFT1/MED25 links flowering time and auxin responsiveness of roots (Cerdan & Chory 2003; Raya-González *et al.* 2014), thus represent an interesting candidate as a plant–plant communication gene. To determine if PFT1/MED25 could modulate plant density responses, WT and *pft1-2* mutants were grown side by side at increasing densities from 1 to 32 individuals, and 10 d after germination root system architecture was analysed. *pft1-2* mutants showed resistance to the plant density effects, which repress primary root growth, lateral root number and lateral root density (Fig. 7). *pft1-2* seedlings developed a long primary root and increased number of lateral roots even at the highest neighbour number (Fig. 7). Two *PFT1* alleles (*pft1-1* and *pft1-3*) showed similar *pft1-2* phenotypes (Fig. S4), indicating the critical role of PFT1/MED25 in mediating plant–plant interactions that influence root architecture.

PFT1/MED25 mediates plant density changes, auxin response and PIN1 and PIN2 levels in roots

The previous observation of plant density modulating auxin response and PIN1 and PIN2 levels in roots, together with the contrasting plant density responses in *pft1-2* seedlings prompted us to compare *DR5:GFP, PIN1::PIN1::GFP* or *PIN2::PIN2::GFP* expression in WT and *pft1-2* seedlings. Firstly, seedlings harbouring these marker constructs were grown for 7 d on agar-solidified 0.2X MS medium at varying densities from 1 to 32 plants and then GFP was analysed *in vivo* by confocal microscopy. *DR5:GFP, PIN1::PIN1::GFP* or *PIN2::PIN2::GFP* expression decreased in WT seedlings in response to high plant density, but not in *pft1-2* mutants (Fig. 8). These findings suggest that plant density affects auxin responsiveness and auxin transporter levels in roots and that *PFT1* modulates root responses to plant density.

Auxin induces primary growth of *Arabidopsis* seedlings grown at high density

Because the auxin maximum at the root tip decreased in plants grown at high density, we investigated its effects on

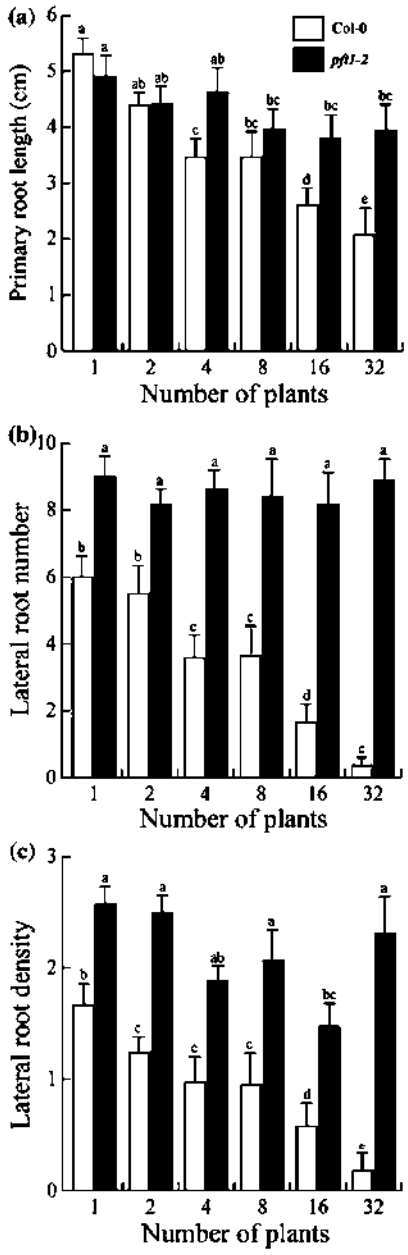

Figure 7. PFT1/MED25 mediates plant density effects on root system architecture. (a) Primary root length. (b) Lateral root number. (c) Lateral root density. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

the growth of *Arabidopsis* primary roots and expression of *DR5:GFP*. Interestingly, 10 nM IAA statistically increased root growth, and normalized *DR5:GFP* expression at primary root tips at a density of 32 seedlings per plate (Fig. 9a,b). It is possible that the phenotypic effects observed in plants grown at high density can be attributed to reduced local synthesis or distribution of auxin.

DISCUSSION

Population density influences resource competition impacting developmental processes such as vegetative growth and plant fitness and the genetic mechanisms underlying these responses are starting to be revealed (Caffaro *et al.* 2013; Masclaux *et al.* 2013; Misyura *et al.* 2014; Bou-Torrent *et al.* 2014). This study characterizes plant density as a triggering factor for plant phase transitions and productivity in *A. thaliana* and shows the critical role of PFT1/MED25 and auxin responsiveness for the configuration of root architecture under varied plant density.

Plant–plant interactions are driven by resource competition, which occurs when two or more individuals are grown together and the resources, mainly nutrients or light, taken up by one individual become limiting to the others, thereby decreasing its performance. Previous reports by Masclaux *et al.* (2013) and Misyura *et al.* (2014) showed that high plant density repress growth in *Arabidopsis* and rice plants. Our results extend these findings by showing that *Arabidopsis* plants changes developmental and growth programs throughout its life cycle that directly impacts plant productivity. Our data imply that either a growth repressing factor is accumulating or an essential element (s) is (are) becoming limiting for plants grown at high density, which directly affects cell division or elongation programs determining organ formation, biomass accumulation and grain productivity.

In our experiments, the plants were cultivated using a substrate with optimal nutrient supply similar to a high-nutrient soil environment, and were maintained into a growth chamber with a photoperiod of 16 h light/8 h darkness to avoid short day effects or the so-called shade avoidance response, a set of responses that plants display when they are subjected to the shade of another plant. In the highest density of individuals grown together (32 plants), the inhibitory effect in stem length is in opposition to the reported shade avoidance responses, which promotes stem elongation (Kebrom & Brutnell 2007; Keuskamp *et al.* 2010). Thus, light competition or shade effects are unlikely the signalling mechanisms responsible for growth repression under high density.

Considerable progress has been made in understanding the developmental and molecular mechanisms controlling root responses to low nutrient availability (López-Bucio *et al.* 2003; Ruiz-Herrera *et al.* 2015). The root architectural changes in plants grown in high density differ from the previously reported changes elicited by low phosphate or nitrate. Low Pi induces the consumption of the root apical meristem while promoting lateral root elongation and root hair differentiation (Sánchez-Calderón *et al.* 2005). In the case of low nitrate availability, the role of the root meristem in root growth remains unclear; however, lateral roots also elongate into nitrate-containing soil zones if the remaining root system is nitrogen deficient (Remans *et al.* 2006). Because we observed a general repressing effect in roots of plants grown at high density, including decreased primary root growth, lateral root

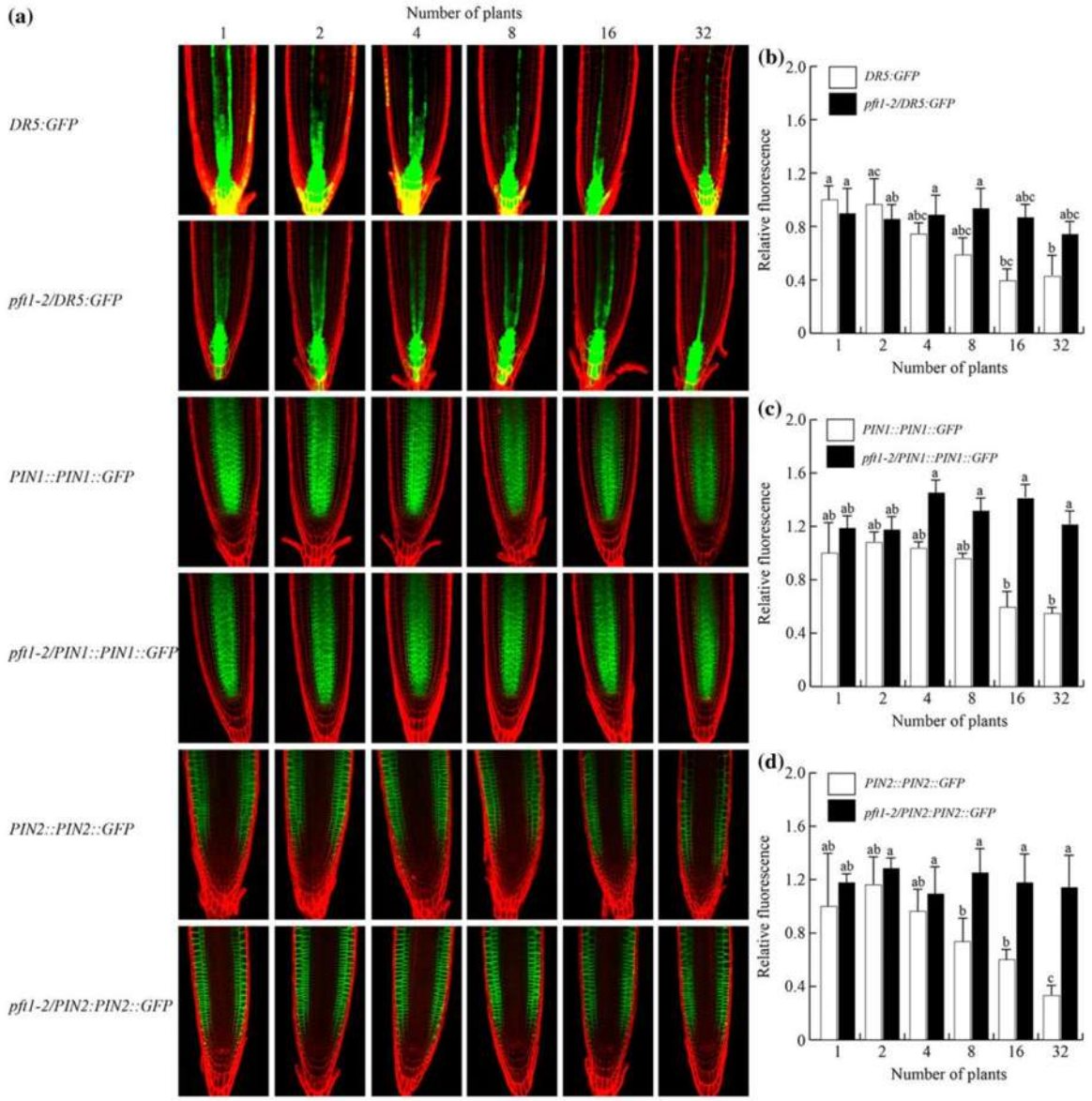

Figure 8. PFT1/MED25 mediates plant density effects on auxin response and auxin transporter levels in roots. *DR5:GFP, PIN1::PIN1:GFP* and *PIN2::PIN2::GFP* expression in WT and *pft1-2* seedlings. *DR5:GFP, pft1-2/DR5:GFP, PIN1::PIN1::GFP, pft1-2/PIN1::PIN1::GFP*, and *PIN2::PIN2::GFP, pft1-2/PIN2::PIN2::GFP* seeds were germinated and grown at varying neighbour number on agar-solidified 0.2X MS medium. Seven days after germination, the seedlings were stained with propidium iodide and analysed by confocal microscopy. Photographs show representative individuals of at least 10 seedlings analysed (a). The graphs (b–d) illustrate the differences in *DR5:GFP* expression among the varied plant densities, assessed as relative fluorescence intensity.

formation and root hair elongation that differ from the reported nitrogen and phosphorus deficiencies, we cannot assume that the inhibitory effects on growth and productivity of plants are caused by low nutrient availability. Moreover, if nutrients such as nitrogen or phosphorus became limiting for plant growth under the conditions tested, then the performance of an individual plant or some plants would be reduced if the neighbours take up these nutrients, and the best competing individuals should grow better than the others, but this is not the case. We consistently observed that all plants growing together either in soil or *in vitro* synchronize its growth and development, indicating that a signal (s) is (are) perceived that influence growth behaviour in the population, a response similar to QS signalling of bacteria (Walters & Bassler 2005).

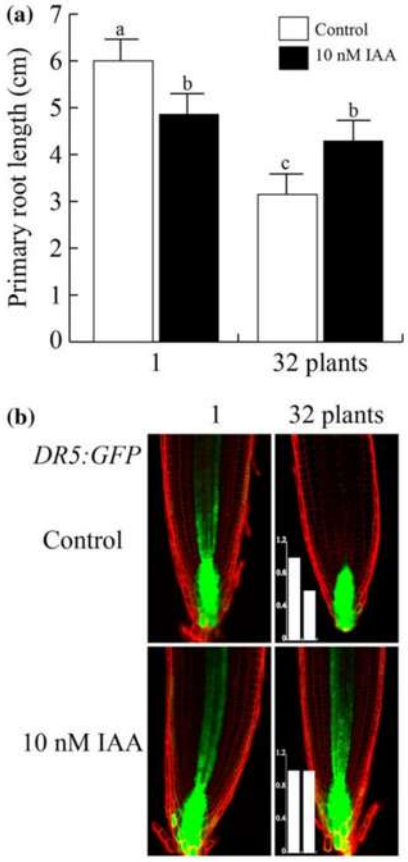

Figure 9. Auxin treatment improves growth and restores the auxin maximum in primary root tips at high density. *Arabidopsis* seedlings harbouring the *DR5:GFP* gene construct were germinated and grown at different plant densities for 10 d in agar-solidified 0.2X MS medium and primary root length (a) measured from 15 independent seedlings. Photographs in (b) show representative individuals of at least 10 seedlings analysed by confocal microscopy. The graphs illustrate the differences in *DR5:GFP* expression between the single plant treatment (left bar) and higher plant density (right bar), assessed as relative fluorescence intensity. [Colour figure can be viewed at wileyonlinelibrary.com]

Growth and developmental responses in plants are mediated by phytohormones, such as auxins, ethylene and jasmonates (Depuydt & Hardtke 2011). Genes participating in ethylene and jasmonic acid signalling pathways are induced in response to plant density in *Arabidopsis* and rice plants (Masclaux *et al.* 2013; Misyura *et al.* 2014). Our analysis of growth of ethylene and jasmonate-related mutants *ein2* and *jar1* indicates that plant density perception does not involve these genetic components, as the corresponding mutants showed similar growth inhibition to wild type plants under increasing neighbour number.

Our data revealed highly repression effects of neighbours on root morphogenetic processes, including the inhibition of primary root growth, lateral root formation and root hair elongation. Auxin plays an important role in root development, shoot development and root-shoot communication (Wolters & Jürgens 2009; Depuydt & Hardtke 2011; Leyser 2010). The findings that plant neighbour number affects the auxin maximum in the primary root as well as the detection of auxin transporters PIN1 and PIN2 reveal a novel facet of auxin in mediating plant–plant interactions. While low plant density have beneficial effects in *Arabidopsis* growth and productivity, which correlated with formation of robust root systems and may have a direct consequence in nutrient and water uptake, high plant density repress root development interfering with either local biosynthesis, transport and/or sensing of auxin. Thus, sensing of neighbours coordinates shoot patterns such as phyllotaxis, branching and stem initiation, which may be dependent on the multiple feedback loops in the auxin machinery, coupled with plant phase transitions that change the feedback relationships as patterning proceeds (Leyser 2010).

The reported phenotypes of *Arabidopsis pft1* mutants (Cerdan & Chory 2003; Raya-González *et al.* 2014) and the current data that auxin supplementation restores primary root growth and normalizes *DR5:GFP* expression in roots of plants grown at a high density suggest PFT1/MED25 as a signal node, linking shoot patterning through their requirement to access common auxin response/transport pathways to the configuration of the root system. However, at present, we cannot discard a role for root exudates in mediating root–root interactions, as plants release blends of molecules, which depend on plant size, photosynthetic activity and growth conditions, giving the potential to provide detailed chemical information to neighbours. Interactions between plant roots appear to be an important factor explaining the relation between population size and plant productivity.

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REFERENCES

Ballaré C.L., Sanchez R.A., Scopel A.L. & Ghersa C.M. (1988) Morphological responses of *Datura-ferox* L. seedlings to the presence of neighbors. Their relationships with canopy microclimate. *Oecologia* **76**, 288–293.

Blilou I., Xu J., Wildwater M., Willemsen V., Paponov I., Friml J., …, Scheres B. (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* **433**, 39–44.

Bou-Torrent J., Galstyan A., Gallemí M., Cifuentes-Esquivel N., Molina-Contreras M.J., Salla-Martret M., … Martínez-García J.F. (2014) Plant proximity perception dynamically modulates hormone levels and sensitivity in Arabidopsis. *Journal of Experimental Botany* **65**, 2937–2947.

Callaro M.M., Vivanco J.M., Botto J. & Rubio G. (2013) Root architecture of *Arabidopsis* is affected by competition with neighbouring plants. *Plant Growth Regulation* **70**, 141–147.

Callaway R.M., Pennings S.C. & Richards C.L. (2003) Phenotypic plasticity and interactions among plants. *Ecology* **84**, 1115–1128.

Cerdan P.D. & Chory J. (2003) Regulation of flowering time by light quality. *Nature* **423**, 881–885.

Crepy M.A. & Casal J.J. (2015) Photoreceptor-mediated kin recognition in plants. *New Phytologist* **205**, 329–338.

Depuydt S. & Hardtke C.S. (2011) Hormone signalling crosstalk in plant growth regulation. *Current Biology* **21**, R365–R373.

de Wit M., Kegge W., Evers J.B., Vergeer-van Eijk M.H., Gankema P., Voesenek L.A.C.J. & Pierik R. (2012) Plant neighbour detection through touching leaf tips precedes phytochrome signals. *Proceedings of the National Academy of Sciences USA* **109**, 14705–14710.

Falik O., Reides P., Gersani M. & Novoplansky A. (2003) Self/non-self discrimination in roots. *Journal of Ecology* **91**, 525–531.

Falik O., de Kroon H. & Novoplansky A. (2006) Physiologically mediated self/non-self root discrimination in *Trifolium repens* has mixed effects on plant performance. *Plant Signaling and Behavior* **1**, 116–121.

Franklin K.A., Praekelt U., Stoddart W.M., Billingham O.E., Halliday K.J. & Whitelam G.C. (2003) Phytochromes B, D and E act redundantly to control multiple physiological responses in *Arabidopsis*. *Plant Physiology* **131**, 1340–1346.

Fuqua C., Winans S.C. & Greenberg E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annual Review of Microbiology* **50**, 727–751.

Gälweiler L., Guan C., Müller A., Wisman E., Mendgen K., Yephremov A. & Palme K. (1998) Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226–2230.

Gommers C.M.M., Visser E.J.W., St Onge K.R., Voesenek L.A.C.J. & Pierik R. (2013) Shade tolerance: when growing tall is not an option. *Trends in Plant Science* **18**, 65–71.

Grabuter M.A., Fu Z., Wu T., Barry K.C., Schwarzer C. & Machen T.E. (2014) *Pseudomonas aeruginosa* quorum sensing molecule homoserine lactone modulates inflammatory signaling through PERK and eI-F2α. *Journal of Immunology* **193**, 1459–1467.

Guzman P. & Ecker J.R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell* **2**, 513–523.

Kebrom T.H. & Brutnell T.P. (2007) The molecular analysis of the shade avoidance syndrome in the grasses has begun. *Journal of Experimental Botany* **58**, 3079–3089.

Kegge W. & Pierik R. (2010) Biogenic volatile organic compounds and plant competition. *Trends in Plant Science* **15**, 126–132.

Keuskamp D.H., Pollmann S., Voesenek L.A.C.J., Peeters A.J. & Pierik R. (2010) Auxin transport through PIN FORMED 3 (PIN3) controls shade avoidance and fitness during competition. *Proceedings of the National Academy of Sciences USA* **107**, 22740–22744.

Kidd B.N., Edgar C.I., Kumar K.K., Aitken E.A., Schenk P.M., Manners J.M. & Kazan K. (2009) The Mediator complex subunit PFT1 is a key regulator of jasmonate-dependent defense in Arabidopsis. *The Plant Cell* **21**, 2237–2252.

Kim Y.J., Björklund S., Li Y., Sayre M.H. & Kornberg R.D. (1994) A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599–608.

Koleske A.J. & Young R.A. (1994) An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**, 466–469.

Krannitz P.G. & Caldwell M.M. (1995) Root growth responses of three Great Basin perennials to intra- and interspecific contact with other roots. *Flora* **190**, 161–167.

Leone M., Keller M.M., Cerrudo I. & Ballaré C.L. (2014) To grow or defend? Low red: far-red ratios reduce jasmonate sensitivity in Arabidopsis seedlings by promoting DELLA degradation and increasing JAZ10 stability. *New Phytologist* **204**, 355–367.

Leyser O. (2010) The power of auxin in plants. *Plant Physiology* **154**, 501–505.

López-Bucio J., Cruz-Ramírez A. & Herrera-Estrella L. (2003) The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology* **6**, 280–287.

Malamy J.E. & Benfey P.N. (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* **124**, 33–44.

Maslaux F.G., Bruessow F., Schweizer F., Gouhier-Darimont C., Keller L. & Reymond P. (2013) Transcriptome analysis of intraspecific competition in *Arabidopsis thaliana* reveals organ-specific signatures related to nutrient acquisition and general stress response pathways. *BMC Plant Biology* **12**, 227.

Misyura M., Guevara D., Subedi S., Hudson D., McNicholas P.D., Colasanti J. & Rothstein S.J. (2014) Nitrogen limitation and high density responses in rice suggest a role for ethylene under high density stress. *Genomics* **15**, 681–694.

Müller A., Guan C., Gälweiler L., Tänzler P., Huijser P., Marchant A., ... Palme K. (1998) AtPIN2 defines a locus of Arabidopsis for root gravitropism control. *The EMBO Journal* **17**, 6903–6911.

Ortiz-Castro R., Díaz-Pérez C., Martínez-Trujillo M., del Río R.E., Campos-García J. & López-Bucio J. (2011) Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proceedings of the National Academy of Sciences USA* **108**, 7253–7258.

Ottenschläger I., Wolff P., Wolverton C., Bhalerao R.P., Sandberg G., Ishikawa H., ..., Palme K. (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proceedings of the National Academy of Sciences USA* **100**, 2987–2991.

Pierik R., Whitelam G.C., Voesenek L.A.C.J., de Kroon H. & Visser E.J.W. (2004) Canopy studies on ethylene-insensitive tobacco identify ethylene as a novel element in blue light and plant–plant signalling. *Plant Journal* **38**, 310–319.

Pierik R., Djakovic-Petrovic T., Keuskamp D.H., de Wit M. & Voesenek L.A.C.J. (2009) Auxin and ethylene regulate elongation responses to neighbor proximity signal independent of gibberellin and DELLA proteins in Arabidopsis. *Plant Physiology* **149**, 1701–1712.

Raya-González J., Ortiz-Castro R., Ruiz-Herrera L.F., Kazan K. & López-Bucio J. (2014) PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 regulates lateral root formation via auxin signaling in Arabidopsis. *Plant Physiology* **165**, 880–894.

Remans T., Nacry P., Pervent M., Filleur S., Diatloff E., Mounier E., ..., Gojon A. (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proceedings of the National Academy of Sciences USA* **103**, 19206–19211.

Ruiz-Herrera L.F., Shane M.W. & López-Bucio J. (2015) Nutritional regulation of root development. *Wiley Interdisciplinary Reviews: Developmental Biology* **4**, 431–443.

Sánchez-Calderón L., López-Bucio J., Chacón-López A., Cruz-Ramírez A., Dubrovsky J. & Herrera-Estrella L. (2005) Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. *Plant Cell Physiology* **46**, 174–184.

Staswick P.E., Su W. & Howell S.H. (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences USA* **89**, 6837–6840.

Vieten A., Vanneste S., Wisniewska J., Benková E., Benjamins R., Beeckman T., ... Friml J. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**, 4521–4531.

Walters C.M. & Bassler B.L. (2005) Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* **21**, 319–346.

Wisniewska J., Xu J., Seifertová D., Brewer P.B., Ruzicka K., Blilou I., ... Friml J. (2006) Polar PIN localization directs auxin flow in plants. *Science* **312**, 883.

Wolters H. & Jürgens G. (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. *Nature Reviews Genetics* **10**, 305–317.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Plant density modulates shoot apical meristem activity and plant senescence. Representative photographs of mean stem tip and first stem leaf at varied plant densities 24 and 42 d after transfer to soil. Note that higher densities decrease flower primordia while accelerating leaf senescence. The experiment was repeated three times with similar results.

Figure S2. Growth of parents at increased plant density does not affect progeny seed size but decreases germination. (A)

Calfaro M.M., Vivanco J.M., Botto J. & Rubio G. (2013) Root architecture of *Arabidopsis* is affected by competition with neighbouring plants. *Plant Growth Regulation* **70**, 141–147.

Callaway R.M., Pennings S.C. & Richards C.L. (2003) Phenotypic plasticity and interactions among plants. *Ecology* **84**, 1115–1128.

Cerdan P.D. & Chory J. (2003) Regulation of flowering time by light quality. *Nature* **423**, 881–885.

Crepy M.A. & Casal J.J. (2015) Photoreceptor-mediated kin recognition in plants. *New Phytologist* **205**, 329–338.

Depuydt S. & Hardtke C.S. (2011) Hormone signalling crosstalk in plant growth regulation. *Current Biology* **21**, R365–R373.

de Wit M., Kegge W., Evers J.B., Vergeer-van Eijk M.H., Gankema P., Voesenek L.A.C.J. & Pierik R. (2012) Plant neighbour detection through touching leaf tips precedes phytochrome signals. *Proceedings of the National Academy of Sciences USA* **109**, 14705–14710.

Falik O., Reides P., Gersani M. & Novoplansky A. (2003) Self/non-self discrimination in roots. *Journal of Ecology* **91**, 525–531.

Falik O., de Kroon H. & Novoplansky A. (2006) Physiologically mediated self/non-self root discrimination in *Trifolium repens* has mixed effects on plant performance. *Plant Signaling and Behavior* **1**, 116–121.

Franklin K.A., Praekelt U., Stoddart W.M., Billingham O.E., Halliday K.J. & Whitelam G.C. (2003) Phytochromes B, D and E act redundantly to control multiple physiological responses in *Arabidopsis*. *Plant Physiology* **131**, 1340–1346.

Fuqua C., Winans S.C. & Greenberg E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annual Review of Microbiology* **50**, 727–751.

Gälweiler L., Guan C., Müller A., Wisman E., Mendgen K., Yephremov A. & Palme K. (1998) Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226–2230.

Gommers C.M.M., Visser E.J.W., St Onge K.R., Voesenek L.A.C.J. & Pierik R. (2013) Shade tolerance: when growing tall is not an option. *Trends in Plant Science* **18**, 65–71.

Grabuter M.A., Fu Z., Wu T., Barry K.C., Schwarzer C. & Machen T.E. (2014) *Pseudomonas aeruginosa* quorum sensing molecule homoserine lactone modulates inflammatory signaling through PERK and eI-F2α. *Journal of Immunology* **193**, 1459–1467.

Guzman P. & Ecker J.R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell* **2**, 513–523.

Kebrom T.H. & Brutnell T.P. (2007) The molecular analysis of the shade avoidance syndrome in the grasses has begun. *Journal of Experimental Botany* **58**, 3079–3089.

Kegge W. & Pierik R. (2010) Biogenic volatile organic compounds and plant competition. *Trends in Plant Science* **15**, 126–132.

Keuskamp D.H., Pollmann S., Voesenek L.A.C.J., Peeters A.J. & Pierik R. (2010) Auxin transport through PIN FORMED 3 (PIN3) controls shade avoidance and fitness during competition. *Proceedings of the National Academy of Sciences USA* **107**, 22740–22744.

Kidd B.N., Edgar C.I., Kumar K.K., Aitken E.A., Schenk P.M., Manners J.M. & Kazan K. (2009) The Mediator complex subunit PFT1 is a key regulator of jasmonate-dependent defense in Arabidopsis. *The Plant Cell* **21**, 2237–2252.

Kim Y.J., Björklund S., Li Y., Sayre M.H. & Kornberg R.D. (1994) A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599–608.

Koleske A.J. & Young R.A. (1994) An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**, 466–469.

Krannitz P.G. & Caldwell M.M. (1995) Root growth responses of three Great Basin perennials to intra- and interspecific contact with other roots. *Flora* **190**, 161–167.

Leone M., Keller M.M., Cerrudo I. & Ballaré C.L. (2014) To grow or defend? Low red: far-red ratios reduce jasmonate sensitivity in Arabidopsis seedlings by promoting DELLA degradation and increasing JAZ10 stability. *New Phytologist* **204**, 355–367.

Leyser O. (2010) The power of auxin in plants. *Plant Physiology* **154**, 501–505.

López-Bucio J., Cruz-Ramírez A. & Herrera-Estrella L. (2003) The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology* **6**, 280–287.

Malamy J.E. & Benfey P.N. (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* **124**, 33–44.

Maslaux F.G., Bruessow F., Schweizer F., Gouhier-Darimont C., Keller L. & Reymond P. (2013) Transcriptome analysis of intraspecific competition in *Arabidopsis thaliana* reveals organ-specific signatures related to nutrient acquisition and general stress response pathways. *BMC Plant Biology* **12**, 227.

Misyura M., Guevara D., Subedi S., Hudson D., McNicholas P.D., Colasanti J. & Rothstein S.J. (2014) Nitrogen limitation and high density responses in rice suggest a role for ethylene under high density stress. *Genomics* **15**, 681–694.

Müller A., Guan C., Gälweiler L., Tänzler P., Huijser P., Marchant A., ... Palme K. (1998) AtPIN2 defines a locus of Arabidopsis for root gravitropism control. *The EMBO Journal* **17**, 6903–6911.

Ortiz-Castro R., Díaz-Pérez C., Martínez-Trujillo M., del Río R.E., Campos-García J. & López-Bucio J. (2011) Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proceedings of the National Academy of Sciences USA* **108**, 7253–7258.

Ottenschläger I., Wolff P., Wolverton C., Bhalerao R.P., Sandberg G., Ishikawa H., ..., Palme K. (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proceedings of the National Academy of Sciences USA* **100**, 2987–2991.

Pierik R., Whitelam G.C., Voesenek L.A.C.J., de Kroon H. & Visser E.J.W. (2004) Canopy studies on ethylene-insensitive tobacco identify ethylene as a novel element in blue light and plant–plant signalling. *Plant Journal* **38**, 310–319.

Pierik R., Djakovic-Petrovic T., Keuskamp D.H., de Wit M. & Voesenek L.A.C.J. (2009) Auxin and ethylene regulate elongation responses to neighbor proximity signal independent of gibberellin and DELLA proteins in Arabidopsis. *Plant Physiology* **149**, 1701–1712.

Raya-González J., Ortiz-Castro R., Ruiz-Herrera L.F., Kazan K. & López-Bucio J. (2014) PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 regulates lateral root formation via auxin signaling in Arabidopsis. *Plant Physiology* **165**, 880–894.

Remans T., Nacry P., Pervent M., Filleur S., Diatloff E., Mounier E., ..., Gojon A. (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proceedings of the National Academy of Sciences USA* **103**, 19206–19211.

Ruiz-Herrera L.F., Shane M.W. & López-Bucio J. (2015) Nutritional regulation of root development. *Wiley Interdisciplinary Reviews: Developmental Biology* **4**, 431–444.

Sánchez-Calderón L., López-Bucio J., Chacón-López A., Cruz-Ramírez A., Dubrovsky J. & Herrera-Estrella L. (2005) Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. *Plant Cell Physiology* **46**, 174–184.

Staswick P.E., Su W. & Howell S.H. (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences USA* **89**, 6837–6840.

Vieten A., Vanneste S., Wisniewska J., Benková E., Benjamins R., Beeckman T., ... Friml J. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**, 4521–4531.

Walters C.M. & Bassler B.L. (2005) Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* **21**, 319–346.

Wisniewska J., Xu J., Seifertová D., Brewer P.B., Ruzicka K., Blilou I., ... Friml J. (2006) Polar PIN localization directs auxin flow in plants. *Science* **312**, 883.

Wolters H. & Jürgens G. (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. *Nature Reviews Genetics* **10**, 305–317.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Plant density modulates shoot apical meristem activity and plant senescence. Representative photographs of mean stem tip and first stem leaf at varied plant densities 24 and 42 d after transfer to soil. Note that higher densities decrease flower primordia while accelerating leaf senescence. The experiment was repeated three times with similar results.

Figure S2. Growth of parents at increased plant density does not affect progeny seed size but decreases germination. (A)

Seed length. (B) Seed width. (C) Germination percentage. (F) Representative photographs of seeds produced from plants at each treatment. Scale bar represents 500 μm. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

Figure S3. Plant density affects root hair growth. Root hair length (A) and number (B) at the epidermis layer of the primary root comprising 500 μm in length. (C) Representative photographs of root hairs at the maturation (upper panel) and root tip (lower panel) zone of the primary root as affected by plant density. Scale bar represents 500 μm. Error bars represent standard errors. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

Figure S4. *PFT1* alleles show resistance to plant density effects on root system architecture. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

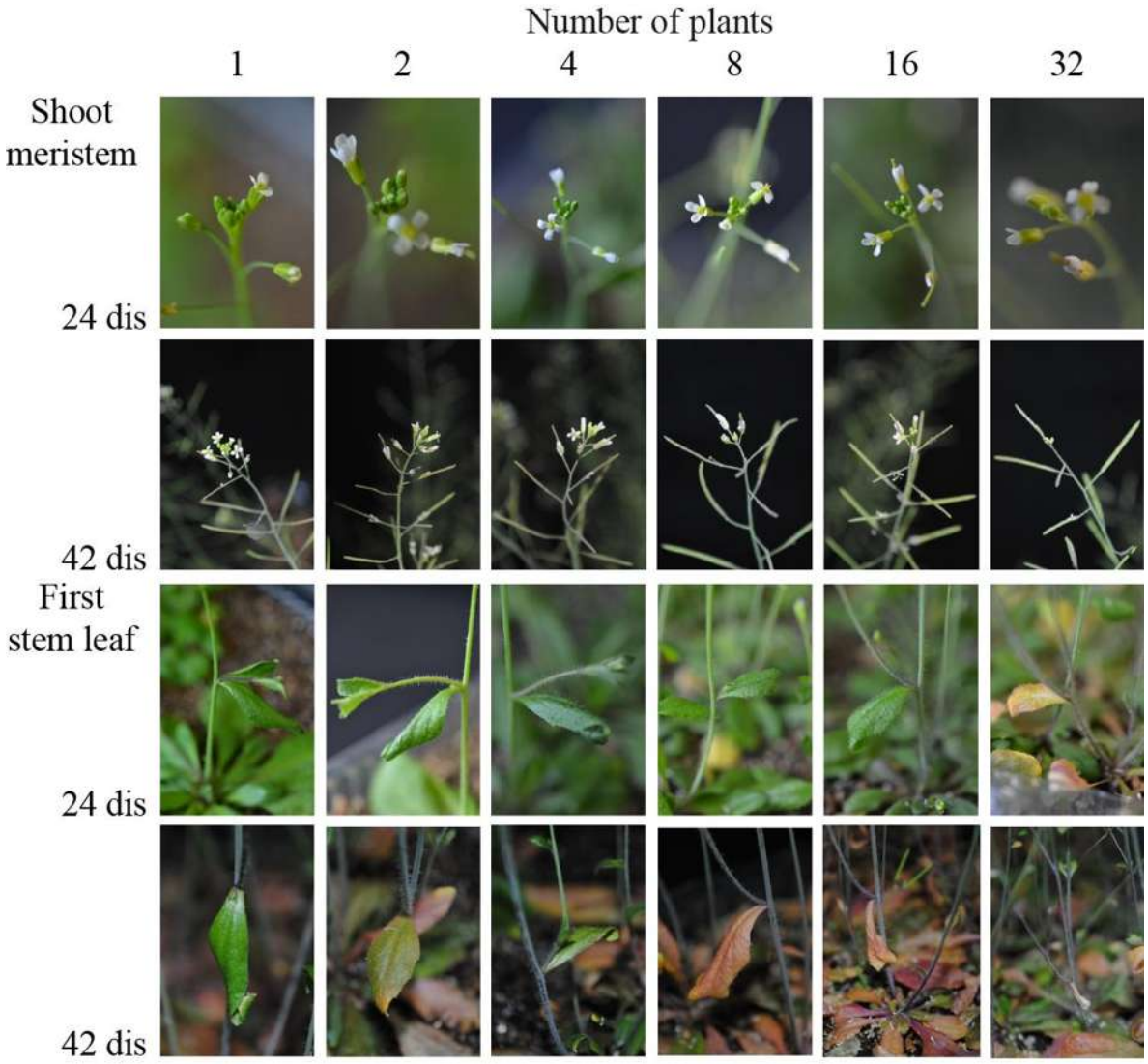
Number of plants
1
2
4
8
16
32
Shoot meristem
24 dis
42 dis
First stem leaf
24 dis
42 dis

Figure S1.

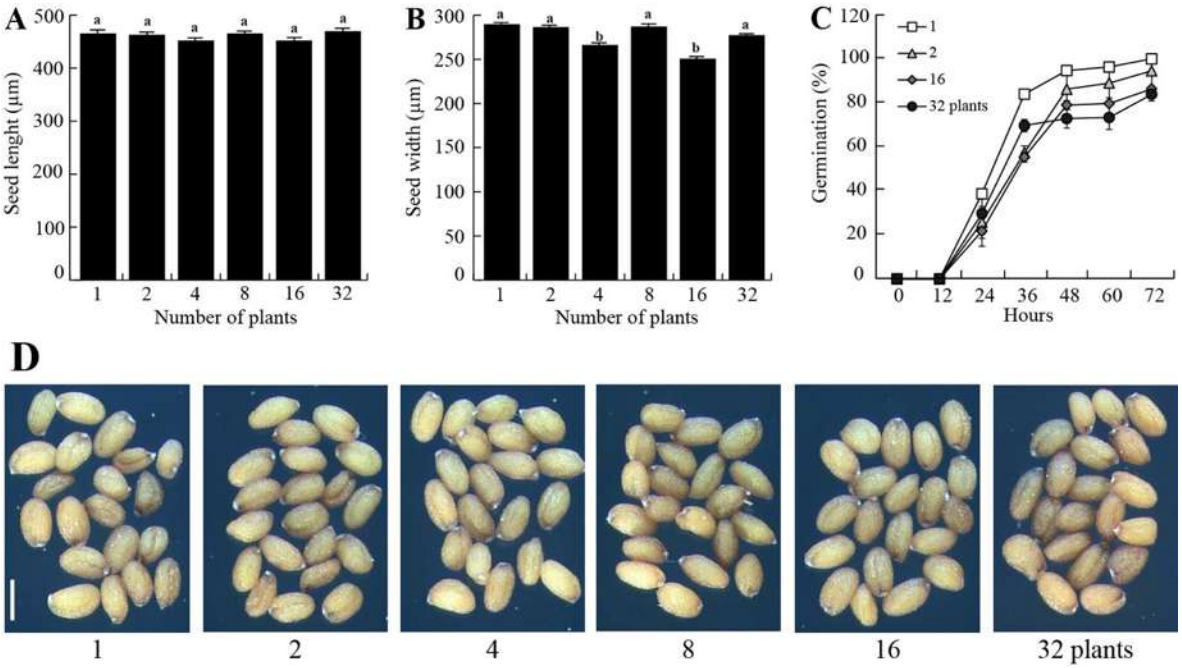
A
Seed lenght (µm)
Number of plants
B
Seed width (µm)
Number of plants
C
Germination (%)
Hours
1
2
16
32 plants
D
1
2
4
8
16
32 plants

Figure S2.

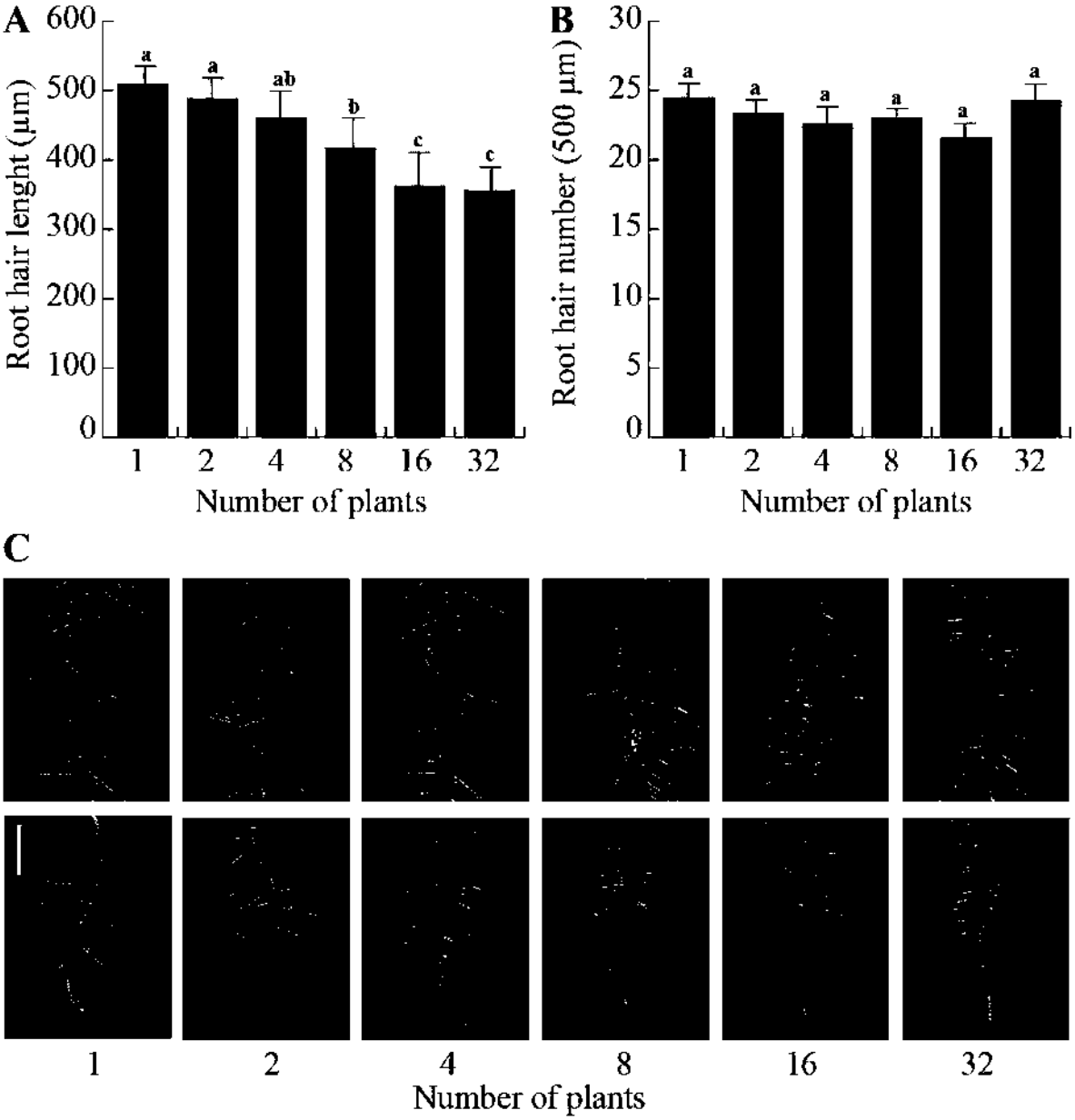
A
Root hair lenght (µm)
600
500
400
300
200
100
0
a
a
ab
b
c
c
1
2
4
8
16
32
Number of plants
B
Root hair number (500 µm)
30
25
20
15
10
5
0
a
a
a
a
a
a
1
2
4
8
16
32
Number of plants
C
1
2
4
8
16
32
Number of plants

Figure S3.

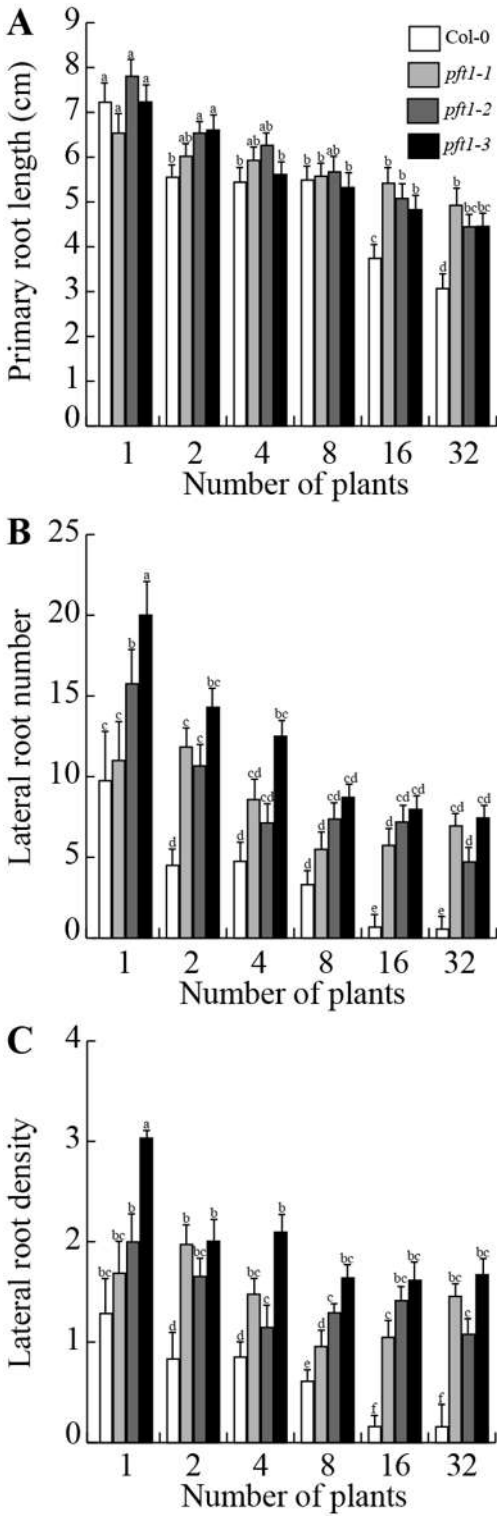
A
Primary root length (cm)
Col-0
pft1-1
pft1-2
pft1-3
Number of plants
B
Lateral root number
Number of plants
C
Lateral root density
Number of plants

Figure S4.

CAPÍTULO II.

Plant Signaling & Behavior

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Self-plant perception via long-distance signaling

Edith Muñoz-Parra, Guadalupe Salmerón Barrera, León Francisco Ruiz-Herrera, Eduardo Valencia-Cantero & José López-Bucio

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SHORT COMMUNICATION

Self-plant perception via long-distance signaling

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ABSTRACT

Plant growth and development are influenced by the interactions with other organisms including bacteria, fungi, herbivores and neighboring plants. Plant density influences the phase transitions during the entire life cycle and root architecture through a mechanism involving auxin and MEDIATOR 25 in *Arabidopsis thaliana*, but the nature of the signals that are perceived in response to increasing number of neighbors remains elusive. Here, we report that plant-plant perception can occur distantly, since root growth and auxin response in Arabidopsis seedlings grown at high plant density into half-divided Petri plates, decreased both primary root growth and lateral root formation in comparison with single plants grown alone, which correlates with reduced auxin response at the primary root tip. It is possible that a diffusible, yet unidentified volatile can be perceived by neighbors to synchronize physiological and developmental behavior.

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KEYWORDS
Self-plant perception; resources; competence; volatiles; auxin signaling; root architecture; plant productivity

Introduction

Population size changes the competitive behavior of plants by modifying gene expression and is a critical factor for plant phase transitions, productivity and root architecture.[1,2,3] An increasing number of plants grown together *in vitro* or in soil have a growth deleterious effect depending upon the size of the population, which correlates with an altered auxin-response in roots and decreased expression of auxin transporters PIN1 and PIN2 in a process linked to the MEDIATOR (MED) complex subunit PFT1/MED25.[3]

Plant-plant recognition mechanisms operate via local diffusible or air volatile bioactive molecules, which are naturally produced by most organisms including plants, bacteria or fungi and act as neighbor detection signals.[4-6] In Arabidopsis, volatile compounds (VCs) from microorganisms influence growth and development[7,8] while molecules from plants orchestrate adaptation to light deficiency, herbivory, pathogen attack, low nutrient availability and drives self-recognition.[4,6,9-12] These responses are coordinated by plant growth-regulators such as auxins, jasmonates and ethylene.[1,7,8] Auxins are involved in most developmental processes in plants and minor changes in auxin levels can modify root architecture modulating cell division, elongation and/or differentiation.[13,14]

Our previous observations[3] indicate that diffusible substances, either phytohormones or allelochemicals, or VCs released by plants grown at increasing densities might be perceived as signals for neighbor detection, in a similar way to the bacteria communication system termed quorum sensing (QS).[15] However, the precise mechanisms explaining this process are still unknown. Here, we show that plants are able to perceive the composition of neighbor populations located to some distance and synchronize growth and physiological processes.

Results

Long distance signaling determines plant-plant communication

To clarify whether plant neighbor density is perceived via local diffusible substances or through long distance signaling, the effect of growing a single Arabidopsis seedling on one side of a divided Petri plate containing agar-solidified Murashige and Skoog (MS) 0.2x medium, and an increasing number of seedlings grown together at the opposite side was analyzed 10 days after germination. Single seedlings grown alone (1/0) or in the vicinity of another plant (1/1) exhibited bigger roots and shoots than those grown in front of the greatest (1/16 and 1/32) density of individuals, which in turn developed shorter and less branched roots in a highly synchronized manner and transmitted the information to seedlings on the other side of the plate (Fig. 1). This suggests that plants can perceive themselves via long distance signaling.

The auxin response is sensitive to the environment of an increasing number of plant neighbors

The influence of an increasing number of neighbors on the root auxin response of single seedlings grown on opposite sides of divided Petri plates was tested via monitoring *DR5:GFP* expression by confocal microscopy and propidium

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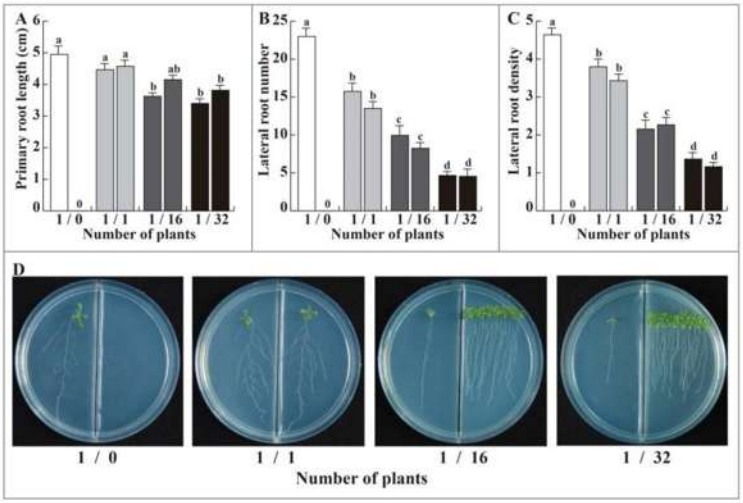

Figure 1. Population size modulates root growth in single neighboring plants grown at distance. (A) Primary root length (cm). (B) Lateral root number. (C) Lateral root density. (D) Representative photographs from four plates per treatment of 1/0, 1/1, 1/16 and 1/32 populations grown side to side. Note the growth synchronization of single plants (left side) grown next to variable neighbor numbers (right side). Error bars represent standard errors. Different letters indicate statistical differences at $p < 0.05$. The experiment was repeated three times with similar results.

iodide staining. In single plants grown alone (1/0) or in those in the vicinity of another plant (1/1), the auxin maximum revealed by GFP green fluorescence was located in collumela and protoxylem root tissues (Fig. 2A, B). However, in roots from single plants exposed to the influence of the greatest (1/32) density of individuals, GFP fluorescence slightly diminishes and this correlates with decreased root thickness (Fig. 2A–C), indicating that plants at the opposite side of plates synchronize its physiological response according to the number of neighbors.

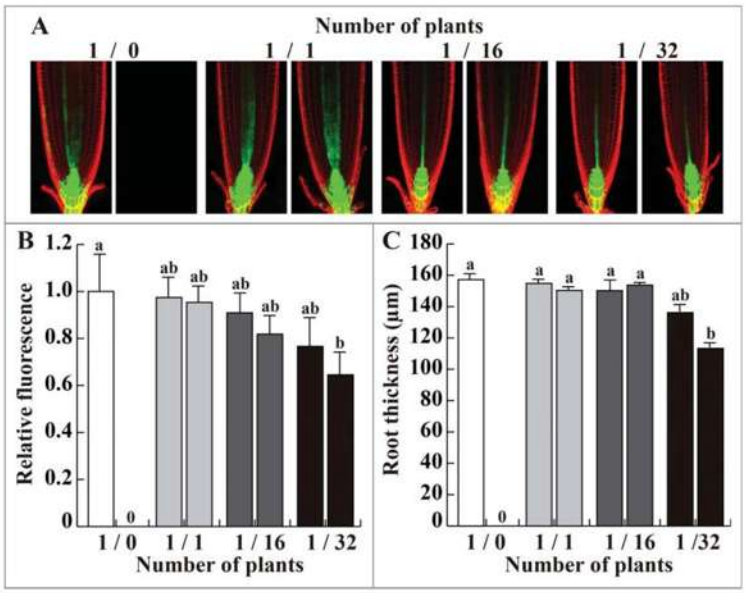

Figure 2. Plant density modulates *DR5:GFP* reporter gene expression in root tips of neighbor plants grown at distance. Arabidopsis seedlings harboring the *DR5:GFP* gene construct were germinated and grown side by side at 1/0, 1/1, 1/16 and 1/32 plant densities for 10 days. (A) Representative photographs of at least 5 seedlings analyzed by confocal microscopy. (B) The graph illustrates the differences in *DR5:GFP* expression among the treatments, assessed as relative fluorescence intensity. (C) Root tip thickness. Values shown represent the mean of 16 seedlings from five plates per treatment ± standard error. Different letters are used to indicate means that differ significantly ($p < 0.05$). The experiment was repeated three times with similar results.

Discussion

The field of plant-plant communication is just emerging and has many implications and possible direct applications for agriculture, since plant population size is an important determinant for biomass and grain productivity.[3] Little is known on how plant neighbors are perceived, and thus, this work contributes by showing that long distance signaling is important for self-recognition. Although the biochemical and/or molecular identity of the signals that are released by seedlings grown at high density remain unknown, it is tempting to hypothesize that VCs production and/or accumulation is modified depending upon the number of individuals grown together. These modifications in the environment are clearly perceived by closer neighbors but also by single plants located at some distance from the whole population to synchronize growth and physiological behavior (Fig. 3).

Additionally, as part of the volatile blends emitted by plants, the level of CO_2 available for photosynthesis may be another important factor for shoot and root biomass production, since sugars can act not only as energy and carbon resources, but also as regulatory signals for developmental processes.[16] Roots are highly energy demanding organs, and although the growth medium used in this research was supplemented with a low concentration of sucrose (0.6%), the availability of carbon in the shoot and its transport to the root may be required to strengthen root meristem activity and sustain cell production for prolific root growth. In this sense, very recent reports have evidenced the important role of the TARGET OF RAPAMYCIN (TOR) kinase to orchestrate sugar sensing, auxin biosynthesis and root growth and patterning.[17,18]

A few genes have just been identified that coordinate sugar-induced growth with auxin signaling. Among them, the MED12 and MED13 subunits are critical for the configuration of root architecture since the corresponding Arabidopsis mutants exhibit short primary roots with decreased meristem activity that correlate with low levels of auxin. Interestingly, sucrose supplementation reactivates both cell division and elongation in primary roots as well as auxin-responsive and stem cell niche gene expression in these mutants.[19] These data, together with the role attributed to MED25 in high density-modulated root traits,[3] provides strong support to MEDIATOR as a master regulator of self-plant recognition. Ongoing research should clarify the magnitude of the contribution of CO_2 and photosynthesis for growth and development at varied plant density, as well as the chemical identity of bioactive VCs released by plants, its purification and application will further determine if they act as a class of plant quorum sensing signals.

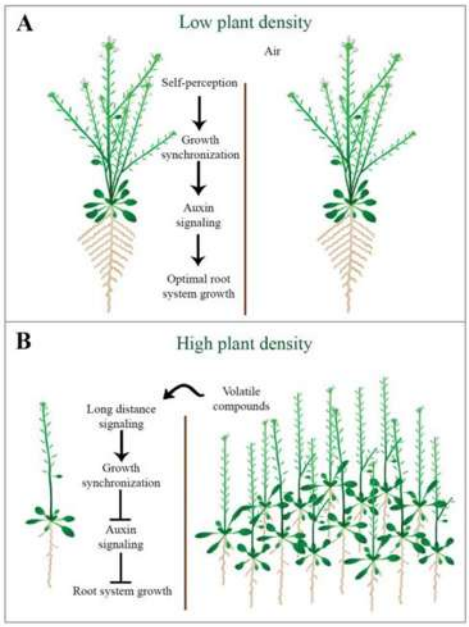

Figure 3. Model schematizing the Arabidopsis response to single or many neighbors. The model hypothesizes that VCs are strong candidates as the emitted signals. (A) At low plant densities, growth and development occurs optimally, since plants are plenty of resources and space while no deleterious or competing factors are perceived. (B) At high plant densities, increasing VCs production and/or accumulation causes growth synchronization among individuals, while repressing primary root growth and lateral root formation due to their antagonistic effects on auxin signaling. A less developed root system is compromised for water and nutrient acquisition and negatively impacts biomass and seed production.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L., Heynh.) wild-type Columbia-0 (Col-0) and *DR5:GFP*[20] transgenic lines were used for the different experiments. Seeds were surface disinfected with 95% (v/v) ethanol for 5 minutes, 20% (v/v) bleach for 7 minutes, and five washes in distilled sterilized water. Seeds were then stratified at 4°C for 48 hours. The seeds were germinated and grown on Petri plates containing agar solidified 0.2x MS medium supplemented with 0.6% sucrose. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Phytagar (Micropropagation grade) was purchased from PhytoTechnology Laboratories, Lenexa, KS, USA. The plant number was established by placing 1/0, 1/1, 1/16 and 1/32 seeds in each plate. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival Scientific AR95L, Perry, IA, USA) at 22°C, with a 16 h light/8 h darkness photoperiod, light intensity of 100 μmol/m^2/s and 80% humidity.

Analysis of growth

Growth of Arabidopsis primary root was measured using a rule. Lateral roots number was determined by counting the lateral roots present in the primary root, from the tip to the root/stem transition zone using a stereoscopic microscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany). Lateral root density was determined by dividing the lateral root number value by the primary root length.

For all experiments, the overall data were statistically analyzed in the SPSS 10 program (IBM Corp., Endicott, NY, USA).

Univariate and multivariate analyses with Tukey's post hoc test were used for testing the differences in growth and root developmental responses in all plants. Different letters are used to indicate the means that differ significantly ($P < 0.05$).

Propidium iodide staining and GFP detection

For fluorescent staining with propidium iodide, plants were transferred from the growth medium to 10 mg/mL^{-1} propidium iodide solution for 1 min. Seedlings were rinsed in water and mounted in 50% (v/v) glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to propidium iodide fluorescence, with a 568-nm excitation line and an emission window of 585 to 610 nm, and GFP emission, with a 500- to 523-nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan), after which the two images were merged to produce the final image. GFP fluorescence in primary root tips was quantified by determining the green pixels present in the root area using the IMAGE J software (http://rsweb.nih.gov/ij/) in at least 5 micrographs per treatment. We then obtained an arbitrary unit value (AU = green pixels) for each individual, and means were obtained from whole data sets. AU means for 1/0 treatment was given a value of 1, and those for other treatments were adjusted relative to these and are thus referred as relative fluorescence. The average root thickness was determined upon measuring thickness of primary root for at least five seedlings per treatment, taking as a reference the root protoxylem plane to locate the layer of epidermal cells using IMAGE J software.

Acknowledgments

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References

1. Misyura M, Guevara D, Subedi S, Hudson D, McNicholas PD, Colasanti J, Rothstein SJ. Nitrogen limitation and high density responses in rice suggest a role for ethylene under high density stress. BMC Genomics. 2014;15:681. doi:10.1186/1471-2164-15-681.
2. Masclaux FG, Bruessow F, Schweizer F, Gouhier-Darimont C, Keller L, Reymond P. Transcriptome analysis of intraspecific competition in *Arabidopsis thaliana* reveals organ-specific signatures related to nutrient acquisition and general stress response pathways. BMC Plant Biol. 2012;12:227. doi:10.1186/1471-2229-12-227.
3. Muñoz-Parra E, Pelagio-Flores R, Raya-González J, Salmerón-Barrera G, Ruíz-Herrera LF, Valencia-Cantero E, López-Bucio J. Plant-plant interactions influence developmental phase transitions, grain productivity and root system architecture in *Arabidopsis* via auxin and *PFT1/MED25* signalling. Plant Cell Environ. 2017;40:1887–99. doi:10.1111/pce.12993.
4. Pierik R, Visser EJW, De Kroon H, Voesenek LACJ. Ethylene is required in tobacco to successfully compete with proximate neighbours. Plant Cell Environ. 2003;26:1229–34. doi:10.1046/j.1365-3040.2003.01045.x.
5. Baldwin IT, Halitschke R, Paschold A, von Dahl CC, Preston CA. Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. Science. 2006;311:812–5. doi:10.1126/science.1118446.
6. Kiggathi RN, Wolfgang WW, Veit D, Gershenzon J, Unsicker SB. Plants suppress their emission of volatiles when growing with conspecifics. J Chem Ecol. 2013;39:537–45. doi:10.1007/s10886-013-0275-2.
7. Raya-González J., Velázquez-Becerra C, Barrera-Ortiz S, López-Bucio J, Valencia-Cantero E. *N,N*-dimethyl hexadecylamine and related amines regulate root morphogenesis via jasmonic acid signaling in *Arabidopsis thaliana*. Protoplasma. 2017;254:1399–410. doi:10.1007/s00709-016-1031-6.
8. Garnica-Vergara A, Barrera-Ortiz S, Muñoz-Parra E, Raya-González J, Méndez-Bravo A, Macías-Rodríguez L, Ruiz-Herrera LF, López-Bucio J. 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 Phytol. 2016;209:1496–512. doi:10.1111/nph.13725.
9. Kegge W, Weldegergis BT, Soler R, Vergeer-Van Eijk M, Dicke M, Voesenek LACJ, Pierik R. Canopy light cues affect emission of constitutive and methyl jasmonate-induced volatile organic compounds in *Arabidopsis thaliana*. New Phytol. 2013;200:861–74. doi:10.1111/nph.12407.
10. Truong DH, Delory BM, Vanderplanck M, Brostaux Y, Vandereycken A, Heuskin S, Delaplace P, Francis F, Lognay G. Temperature regimes and aphid density interactions differentially influence VOC emissions in Arabidopsis. Arthropod-Plant Interact. 2014;8:217–327. doi:10.1007/s11829-014-9311-6
11. Kishimoto K, Matsui K, Ozawa R, Takabayashi J. Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant Cell Physiol. 2005, 47:1093–102. doi:10.1093/pcp/pci122.
12. Asai N, Nishioka T, Takabayashi J, Furuichi T. Plant volatiles regulate the activities of Ca^{2+}-permeable channels and promote cytoplasmic calcium transients in Arabidopsis leaf cells. Plant Signal Behav. 2009, 4:294–300. doi:10.4161/psb.4.4.8275.
13. Zhao Y. Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol. 2010;61:49–64. doi:10.1146/annurev-arplant-042809-112308.
14. Depuydt S, Hardtke CS. Hormone signalling crosstalk in plant growth regulation. Curr Biol. 2011;21:R365−73. doi:10.1016/j.cub.2011.03.013.
15. Walters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 2005;21:319–46. doi:10.1146/annurev.cellbio.21.012704.131001.
16. Rolland F, Baena-Gonzalez E, Sheen J. Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol. 2006;57:675–709. doi:10.1146/annurev.arplant.57.032905.105441.
17. Li X, Cai W, Liu Y, Li H, Fu L, Liu Z, Xu L, Liu H, Xu T, Xiong Y. Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes. Proc Natl Acad Sci U S A. 2017;114:2765–70. doi:10.1073/pnas.1618782114.
18. Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J. Glucose-TOR signalling reprograms the transcriptome and activates meristems. Nature. 2013;496:181–6 doi:10.1038/nature12030.
19. Raya-González J, López-Bucio JS, Prado-Rodríguez JC, Ruiz-Herrera LF, Guevara-García AA, López−Bucio J. The MEDIATOR genes MED12 and MED13 control Arabidopsis root system configuration influencing sugar and auxin responses. Plant Mol Biol. 2017;95:141–56. doi:10.1007/s11103-017-0647-z.
20. Ottenschläger I, Wolff P, WolvertoN C, Bhalerao RP, SandberG G, Ishikawa H, Evans M, Palme K. Gravity-regulated differential auxin transport from columella to lateral root cap cells. Proc Natl Acad Sci U S A. 2003;100:2987–91. doi:10.1073/pnas.0437936100.

CAPÍTULO III.

ARTÍCULO

Del hierro de las estrellas a nuestro cuerpo

Edith Muñoz Parra

*La **M.C. Edith Muñoz Parra**, es estudiante del Programa Institucional de Doctorado en Ciencias Biológicas de la Opción en Biología Experimental, del Instituto de Investigaciones Químico Biológicas (Laboratorio de Ecología Microbiana) de la Universidad Michoacana de San Nicolás de Hidalgo.*

Todo comienza en una estrella

Las estrellas son cuerpos celestes compuestos fundamentalmente de hidrógeno, el cual por un proceso denominado fusión nuclear une los núcleos atómicos para formar otros elementos más pesados, de modo que a partir del hidrógeno se forman el resto de los átomos. El elemento más pesado que puede formarse en el interior de una estrella es el hierro, ya que el calor que se requiere para que se formen elementos más pesados no se alcanza de manera normal en el interior de la estrella.

Cuando el hidrógeno casi se ha agotado al convertirse en otros elementos, la estrella se expande y finalmente termina convirtiéndose en una supernova con una explosión que dura unos pocos segundos. Las condiciones de la explosión permiten que se formen elementos aún más pesados como la plata, el oro y el mercurio. Con esta explosión todos los elementos se

dispersan por el espacio hasta que nuevamente se unen formando nuevas estrellas o planetas como el que habitamos.

El hierro en los orígenes de la vida en la Tierra

En nuestro planeta, aproximadamente el 90% de la composición del núcleo terrestre es hierro, que se mantiene en estado líquido debido a la alta temperatura que se presenta a esa profundidad. Los depósitos de hierro líquido generan el geomagnetismo terrestre, determinando la localización de los polos magnéticos.

Pero el hierro no solamente se encuentra en el centro de la tierra, las teorías actuales sobre el origen de la vida en nuestro planeta, han propuesto que hace aproximadamente 3 900 millones de años, las sustancias que darían origen a los primeros organismos se encontraban en un "caldo primitivo", en donde el hierro era parte importante para conservar estos compuestos y finalmente se podrían formar las primeras células. Se sugiere que al paso de unos 1 500 millones de años, algunas bacterias (organismos unicelulares sin núcleo) adquirieron la capacidad de usar la energía proveniente de la luz para construir (sintetizar) sus propias moléculas orgánicas complejas, a partir de las moléculas inorgánicas simples que se encontraban a su disposición, es decir adquirieron la capacidad de realizar fotosíntesis.

Las bacterias con formas avanzadas de la fotosíntesis empezaron a utilizar la energía de la luz para romper moléculas de agua (H_2O) y quedarse con algunos de los electrones de esa molécula (poder reductor) que empleaban para la síntesis de sus propias biomoléculas, mientras que el oxígeno contenido en el agua fue liberado a la atmósfera donde empezó a acumularse. El aumento de oxígeno en la atmósfera creó las condiciones para la evolución de un segundo tipo de bacterias que utilizaban el oxígeno para oxidar compuestos orgánicos y obtener energía en un proceso que se denomina respiración aerobia.

Los estudios realizados a la fecha, indican que las bacterias con respiración aerobia se fusionaron con un tipo de organismo unicelular, que si bien era más complejo por ser eucariota (poseía núcleo), no tenía la habilidad de realizar la respiración aerobia, en esta esta fusión, la bacteria se trasformó en el orgánulo respirador de la célula eucariota (es decir la mitocondria), lo que permitiría después la evolución de organismos más avanzados como son los hongos y los animales, incluyendo a nuestra especie.

Posteriormente, este organismo eucariota primitivo, pero ya con mitocondria, habría de fusionarse con el otro tipo de bacteria (el de tipo fotosintético) y esto daría origen a los cloroplastos en organismos como algas y plantas. Con el surgimiento de los organismos vegetales se acentuó la acumulación de oxígeno en la atmósfera, hasta alcanzar su composición actual que permite el éxito de la vida que conocemos.

¿Y qué tiene que ver todo esto con el hierro?

Bueno, para realizar tanto la fotosíntesis como la respiración, las plantas y los animales requerimos que los electrones que tomamos de los compuestos que consumimos, puedan circular por varios sistemas dentro de las células y esto se puede llevar a cabo gracias al hierro. El hierro es un buen conductor de electrones en las células, ya que tiene la capacidad de tomar y donar fácilmente esos electrones en los lugares específicos requeridos, aquí opera eso de que fácil llega, fácil se va.

El hierro y las plantas

El hierro, además puede combinarse fácilmente con otros elementos formando compuestos de diversos tipos. En la mayoría de los suelos, el hierro no se encuentra en una forma en que las plantas puedan absorberlo directamente. En respuesta a este problema desarrollaron mecanismos adaptativos:

Primer mecanismo.- Por sí mismas, las plantas pueden acidificar el medio que rodea la raíz permitiendo que el hierro pueda cambiar a una forma en que su internalización a la planta sea posible.

Segundo mecanismo.- Permite la secreción de compuestos afines al hierro, que lo atrapan y ayudan a acarrearlo al interior de la planta sin tener un efecto tóxico.

Tercer mecanismo.- Es asociarse con microorganismos como son las conocidas "bacterias ferri-reductoras" que modifican el hierro convirtiéndolo a una forma disponible para la planta y se lo proporcionan fácilmente a cambio de habitar cerca de la raíz en donde la planta vierte compuestos orgánicos que le sirven de alimento.

El contenido de hierro en plantas varía desde 3 hasta 11 g por kilo de materia vegetal deshidratada, lo que convierte a las plantas en una importante fuente para la nutrición animal y humana. En altas cantidades el hierro puede ser muy tóxico, por lo que las células deben regular las concentraciones en su interior.

En plantas, la deficiencia de hierro ocasiona la aparición de una tonalidad amarilla de las hojas (clorosis) debido a que ya no es posible producir clorofila de manera óptima, también se presenta un acortamiento en el crecimiento del sistema foliar y radicular, además la acumulación de hierro en las semillas se vuelve escasa y éstas no serán capaces de germinar apropiadamente.

El hierro en nuestro organismo

Los organismos como los seres humanos, debemos ingerir hierro a través de la dieta diaria en cantidades adecuadas, nuestra principal fuente son las carnes rojas magras, verduras de hoja verde como espinacas y acelgas, legumbres como frijoles, lentejas y garbanzos, y frutos secos como nueces, pistaches y almendras. Una vez que el hierro es consumido en la ingesta, se absorbe en el intestino delgado, de donde es transferido directamente al torrente sanguíneo. Cada día se requieren aproximadamente mil millones de átomos de hierro para formar la hemoglobina necesaria para los nuevos eritrocitos en la sangre.

La deficiencia de hierro (anemia ferropénica) es uno de los desórdenes nutricionales más comunes en el mundo, afectando a más de dos mil millones de personas (aproximadamente el 30% de la población mundial), donde en particular riesgo se encuentran las mujeres embarazadas. La organización mundial de la salud advirtió que el porcentaje de mujeres embarazadas que presentan esta deficiencia varía de un 14% en países industrializados hasta un 75% en países en desarrollo, siendo así el único nutriente cuya deficiencia permanece en todo el mundo.

Nuestro cuerpo puede regular la absorción de hierro en el intestino, en respuesta a las cantidades disponibles de acuerdo a la ingesta diaria. Mientras en condiciones normales nuestro requerimiento se encuentra entre 1 y 8 mg diarios, las mujeres requieren 27 mg durante el embarazo y 10 mg durante la lactancia. En los niños recién nacidos, se ha observado que la alimentación basada en lactancia materna, permite una mayor absorción de hierro que cuando la alimentación es a base de formulaciones lácteas.

La ingesta de hierro es el principal tratamiento prescrito para tratar la anemia ferropénica, pero el hierro oral está compuesto principalmente por sales ferrosas con una baja y variable taza de absorción por lo cual se recomienda al menos consumir 80 mg de hierro elemental diarios, lo que equivale a una tableta de 250 mg. En el caso de anemia severa, la transfusión sanguínea ha sido el tratamiento más eficiente.

Lo que falta por saber

Aún con todo el conocimiento que se tiene en la actualidad sobre la importancia del hierro para la vida, falta conocer a fondo cuáles son los mecanismos que utilizamos los seres vivos para mantener niveles óptimos y de este modo también plantear estrategias que permitan mejorar el aprovechamiento de este mineral tanto en el reino vegetal como en el reino animal, sin ocasionar daños al ambiente y permitiendo una disminución de los efectos dañinos de su deficiencia en la salud de la población mundial.

«Lo que ahora sabes, es que el hierro que está en tu organismo, proviene de las estrellas»

SaberMás

Gómez-Caballero J.A. y Pantoja-Alor J. (2003). El origen de la vida desde un punto de vista geológico. http://www.jstor.org/stable/24920377

Juárez-Sans M. et al. (2007). Hierro en el sistema suelo-planta. http://hdl.handle.net/10045/1845

Organización Mundial de la Salud. 2017. Metas mundiales de nutrición 2025: Documento normativo sobre anemia. http://www.who.int/nutrition/publications/globaltargets2025_policybrief_anaemia/es/

CAPÍTULO IV.

Capítulo en revisión para su envío a la revista *Plant and Soil*

Volatile compounds from *Arthrobacter agilis* UMCV2 regulates *Arabidopsis thaliana* development through iron-deficiency response genes *BTS* and *ILR3,* and dependently of iron availability

ABSTRACT

Plants perceive many environmental signals that modulate plant morphogenesis and gene expression. Nutrient availability is an important trait for plant development. Iron (Fe) is an essential micronutrient for plant development and its availability is frequently limited in soils, leading plants to activate iron deficiency responses. *Arabidopsis* plants possess a Strategy I Fe-uptake system, recently had been described a new homeostasis signaling mechanism, where *BTS*, *PYE* and *ILR3* genes are important components. Some rhizobacteria can regulate plant growth triggering Fe-uptake systems using different mechanisms, such as production of volatile organic compounds (VOCs), but the signal transduction pathways mediating VOC sensing are not fully understood. In this work we report that plant growth promoting rhizobacteria *Arthrobacter agilis* UMCV2 modulate *Arabidopsis thaliana* growth and development trough BTS and ILR3 activation via VOCs emission. We show that in a separate compartments system, VOCs emitted by *A. agilis* UMCV2 modified *Arabidopsis* root system architecture promoting growth, and increase chlorophyll content. Using confocal microscopy and imaging, the effects of UMCV2 VOCs on root architecture were found to be correlated with *ProPYE:GFP* and *ProBTS:GFP* gene expression. We tested the effect of *N,N*-dimethylhexadecylamine (C16-DMA), produced by UMCV2 strain, on plant growth and found to be regulating root architecture, inhibiting primary root growth. C16-DMA induced a dose-dependent effect with specific participation of *ILR3*, but these effects are not comparable to those produced by the full UMCV2 VOCs. Our results indicate that root responses to UMCV2 VOCs involve the components of iron deficiency responses, *BTS* and *ILR3*.

INTRODUCTION

Iron (Fe) is a micronutrient, necessary for maintain cellular homeostasis during plant growth and development (Hell and Stephan, 2003). It plays a role in a number of essential biochemical reactions in the energetic metabolism of plant cells. Due to its ability to gain or lose electrons, is an important participant in respiratory and photosynthetic electron transport chains in mitochondria and chloroplast, respectively (Vigani *et al.*, 2013). Fe deficiency represents one of the main problems for crop production worldwide, although it is one of the most abundant elements on earth, it is found mostly as insoluble ferric oxide

or hydroxides in basic soil environments, resulting in low bioavailability (Samira *et al*., 2018). Because of this, plants have evolved different mechanisms to solubilize and absorb Fe from soil (Schmidt, 1999; Walker and Connolly, 2008; Palmer and Guerinot, 2009).

Nongraminaceous plants such as *Arabidopsis thaliana* respond to iron deficiency with the known reduction-based strategy (Strategy I) where, in response to iron deficiency, the bHLH transcription factor FIT forms heterodimers with other bHLH proteins and regulates expression of FRO2, an iron reductase that reduces Fe^{3+}, and also FIT regulates the accumulation of IRT1 protein, the transporter of reduced Fe^{2+} into the symplast (Bauer *et al.*, 2007; Yuan *et al.,* 2008; Walker and Connolly, 2008; Robinson *et al.,* 1999; Eide *et al.,* 1996; Vert *et al.*, 2002; Colangelo and Guerinot, 2004). After this, iron is bound to various chelators, transported to the vasculature, and relocated to tissues where Fe is needed. Two regulatory pathways controlled by bHLH transcription factors have been shown to mediate these responses in *Arabidopsis thaliana*. The first is the previously described FIT-regulated process. In the second, the bHLH transcription factor POPEYE (PYE) directly regulates the expression of genes involved in intercellular transport, mitochondrial ferric reduction, and other processes (Long *et al*., 2010). PYE physically interacts with PYE-like (PYEL) proteins including ILR3; and several PYEL proteins directly target PYE transcriptionally (Selote *et al*., 2015; Zhang *et al*., 2015; Li *et al*., 2016; Samira *et al*., 2018). PYE and PYEL proteins can physically interact, but only PYEL proteins such as ILR3 have been reported to directly interact with a third protein, BRUTUS (BTS). BTS, is induced transcriptionally in response to iron deprivation and *bts1* mutants show increased tolerance to iron deprivation, so that BTS and PYE/PYEL proteins act in opposing manners controlling the iron deficiency response (Long *et al*., 2010; Selote *et al*., 2015).

In order to have success acquiring Fe from the rhizosphere, plants have additionally developed close relationships with some important partners, microorganisms. Plant growth-promoting rhizobacteria (PGPR) are able to modify the availability of nutrients through changes in the environment (Pii *et al*., 2015; Terrazas *et al*., 2016) and also stimulate plant growth via different mechanisms, including volatile compounds (VOCs) interactions that modulate some plant growth regulators, including jasmonic acid (Ortíz-Castro *et al*. 2008; 2011; Spaepen *et al*. 2009; Raya-González *et al*., 2017). Different mechanisms have been suggested by which microbial species enhance Fe availability to plants, such as Fe movilization of soil iron via bacterial siderophores relese, direct dissimilatory Fe-reducing process, or the induction of ferric-chelate reductase activity in plant roots (Valencia-Cantero *et al.*, 2007; Zhao *et al.*, 2014; Pii *et al.*, 2015; Orozco-Mosqueda *et al*., 2013; Castulo-Rubio *et al*., 2015).

Some previous reports suggest that plant iron deficiency responses are related to plant growth and defense regulation mechanisms (Rampey *et al*., 2006). *A. agilis* UMCV2 is a rhizobacterium reported with capability to promote the growth of *Phaseollus vulgaris*, *Medicago truncatula*, and *Medicago sativa* (plants with Strategy I Fe-uptake systems), trough Fe reduction causing plants to uptake Fe (Valencia-Cantero *et al*., 2007). VOCs emitted by *A. agilis* UMCV2 triggered *M. sativa* growth via emission of the VOC *N,N*-dimethylhexadecylamine (C16-DMA) and induce expression of Strategy I Fe-uptake

system (Velázquez-Becerra *et al*., 2011; Orozco-Mosqueda *et al*., 2013). C16-DMA was also reported to activate the expression of Jasmonic acid-response gene and thus modulate root development (Raya-González *et al*., 2017).

Although some *A. agilis* UMCV2 VOCs effects, specifically those for C16-DMA have been already reported, its relation with iron signaling in *Arabidopsis* had not been investigated so far. In the present work, we use *Arabidopsis thaliana* plants as a Strategy I Fe-uptake model system. We tested the interaction of *A. agilis* UMCV2 VOCs-*A. thaliana* to dissect a plant mechanism and determine if C16-DMA was involved in the modulation of PYE system (a Fe-homeostasis system) in plants grown under varying Fe availabilities. The results confirm the PGPR effect of VOCs produced by *A. agilis* UMCV2, and also that this effect occurred trough BTS signaling but it is not induced by C16-DMA. Even though, C16-DMA does have an effect in plant growth regulation trough ILR3 participation.

MATERIALS AND METHODS

Biological material and growth conditions

A. thaliana wild-type Columbia-0 (Col-0), mutant plants *pye1*, *bts1, ilr3-1* and transgenic lines *ProPYE:GFP* and *ProBTS:GFP* (Long *et al*., 2010; Rampey *et al*., 2006) were used for the different experiments. Seeds were surface disinfected with 95% (v/v) ethanol for 5 min, 20% (v/v) bleach for 7 min and five washes in distilled water. Seeds were then stratified at 4 °C to promote and synchronize germination. The seeds were germinated and grown on Petri plates containing the varying treatments of iron availability in agar solidified 0.2 X MS medium. The tested treatments were prepared as follow, Control treatment was iron-sufficient media, a standard 0.2 X MS medium containing 20 μM of $FeSO_4 \bullet 7H_2O$. Iron deficient media, -Fe and -Fe+μM Ferrozine, are the same 0.2 X MS both without addition of $FeSO_4$, and the last carried Ferrozine as an iron chelator for iron trace amounts present in phytagar. The MS medium (Murashige and Skoog Basal Salts Mixture, catalogue no. M5524) was purchased from Sigma. Phytagar (Micropropagation grade) was purchased from PhytoTechnology Laboratories. Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt, catalogue no. P5338) was purchased from Sigma.

The PGPR strain *Arthrobacter agilis* UMCV2 was isolated from lightly acid soil (Valencia-Cantero *et al.*, 2007). The bacterium was grown on nutrient agar (NA) at 22°C. For UMCV2 VOCs interaction experiments, petri plates had a physical barrier for two compartments, where seeds were sown on a side and four days after germination, *A. agilis* UMCV2 was inoculated on the other side, allowing only VOCs interaction per six days and then seedlings were analyzed. For treatments with C16-DMA, the compound was added to the medium before plants were sown. Petri plates were placed in a plant growth chamber (Percival Scientific AR95L) at 22 °C, with a 16 h light/8 h darkness photoperiod, light intensity of 100 μmol/m^2 and 80% humidity. Petri plates were placed vertically at an angle of 65° to allow root growth along the agar surface and allow unimpeded aerial growth of the hypocotyls.

Analysis of growth

Arabidopsis root system was analyzed measuring primary root length using a rule. Lateral root number was determined counting the lateral roots present in the primary root, from the tip to the root/stem transition zone using a stereoscopic microscope, and lateral root density was determined dividing the lateral root number value by the primary root length for each seedling.

Determination of biomass and chlorophyll contents

For fresh weight measure, *Arabidopsis* seedlings were weighted using an analytical balance. To determinate chlorophyll content, Arabidopsis leaves were detached and weighed measured ten days after germination. Tissue was immediately immersed in aqueous ethanol (96% v/v) for 30 minutes in darkness. Absorbance readings of the solution were recorded at 648.6 and 664.2 nm. Total chlorophyll content was calculated as (5.24*A663.2 + 22.24*A648.6)/1000*(fresh weight of leaves); calculated values were reported as mg chlorophyll per g^{-1} fresh weight, as described by Lichtenthaler (1987).

Propidium iodide staining and green fluorescent protein detection

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

Data analyses

For all experiments, the overall data were statistically analyzed in the Statistica 10 program (StatSoft Inc., Tulsa, OK, USA). Univariate and multivariate analyses with Tukey's post hoc test were used for testing the differences in growth and root developmental responses in all plants. Different letters are used to indicate the means that differ significantly ($P < 0.05$).

RESULTS

Iron availability regulates root development, plant biomass and chlorophyll production

Nutrient availability is a well-known regulator of plant growth and development. Iron is an indispensable plant micronutrient and its homeostasis is needed to acquire optimal growth. To first characterize the effect of iron availability in *Arabidopsis* development, root system architecture, biomass and chlorophyll production were analyzed. *Arabidopsis* seeds were sown and grown for ten days on optimal iron conditions (20 μM) as Control treatment, and

on a series of treatments decreasing iron availability as an increase of Ferrozine was added from 25 to 150 μM. To determine if root developmental programs are sensitive to iron availability, we evaluated primary root length and lateral root number. We found that low iron availability significantly inhibit the primary root growth as Ferrozine concentrations increase (Fig. 1A) and also lateral root number increased as less iron is available (Fig. 1B). It has been reported that under low iron conditions, seedlings present lower chlorophyll content and produced less total biomass, therefore we analyzed this parameters for *Arabidopsis*. Ten days after germination, *Arabidopsis* seedlings were weight-measured and then treated for chlorophyll quantification. The obtained results indicate than both parameters decrease on an iron availability-dependent manner. Biomass in control conditions was of 20 mg per plant, while in plants grown in 150 μM Ferrozine treatment, biomass production was less than half. Moreover, while chlorophyll content was of 0.8 mg/g^{-1} FW in control, the treatment with less iron available show to produce only 0.2 mg/g^{-1} FW. These data suggest that iron availability affects several processes during seedling growth in *Arabidopsis*.

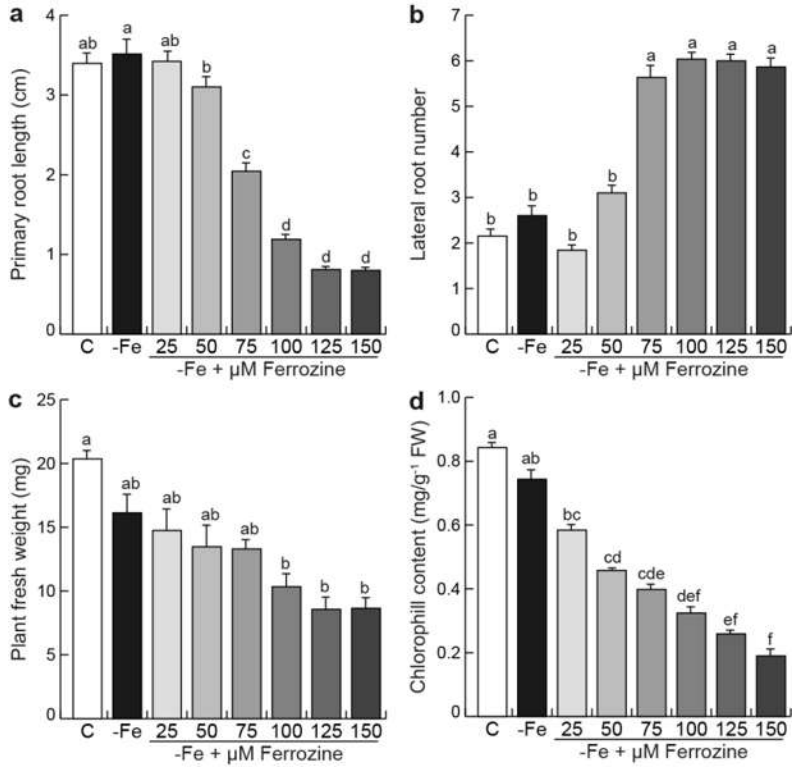

Figure 1. Iron availability modulates *Arabidopsis* root development, biomass and chlorophyll production. *Arabidopsis* Col-0 seedlings were grown in optimal iron conditions (Control), or Fe-deficient treatments induced by the increasing addition of ferrozine (chelator). Measures were realized ten days after germination. a) Primary root length, b) Lateral root number, c) Plant fresh weight and d) Chlorophyll content. Data show the mean ±SE for three groups of 15 seedlings. Error bars represent standard errors. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

Volatile compounds of *Arthrobacter agilis* UMCV2 regulates *Arabidopsis* development dependently of iron availability

To determine the effects of UMCV2 VOCs on the growth of *Arabidopsis* seedlings grown under different iron availability, we sown *Arabidopsis* seedlings in one side of one divided Petri plates containing 0.2x MS media in control, -Fe and -Fe + 100 µM Ferrozine treatments. Four days after germination *A. agilis* UMCV2 was inoculated on the other side of the plate to allow only volatile communication. Primary root length, lateral root number, biomass and chlorophyll production were determined six days after inoculation. The results showed that UMCV2 VOCs increased 50% the growth of primary root length, and also increased near twice lateral roots formation in control treatments (Fig. 2A, B), correlating to a 100% more biomass production and 50% chlorophyll content increase (Fig. 2 C, D). Interestingly, in -Fe treatments, even though UMCV2 VOCs showed a promoter effect, this was diminished compared to control treatment, and for the 100 µM Ferrozine treatment, UMCV2 VOCs were unable to promote any of the analyzed parameters (Fig. 2). This indicates that the growth and development promoter effect of *A. agilis* UMCV2 VOCs is dependent of iron availability in plant growth media.

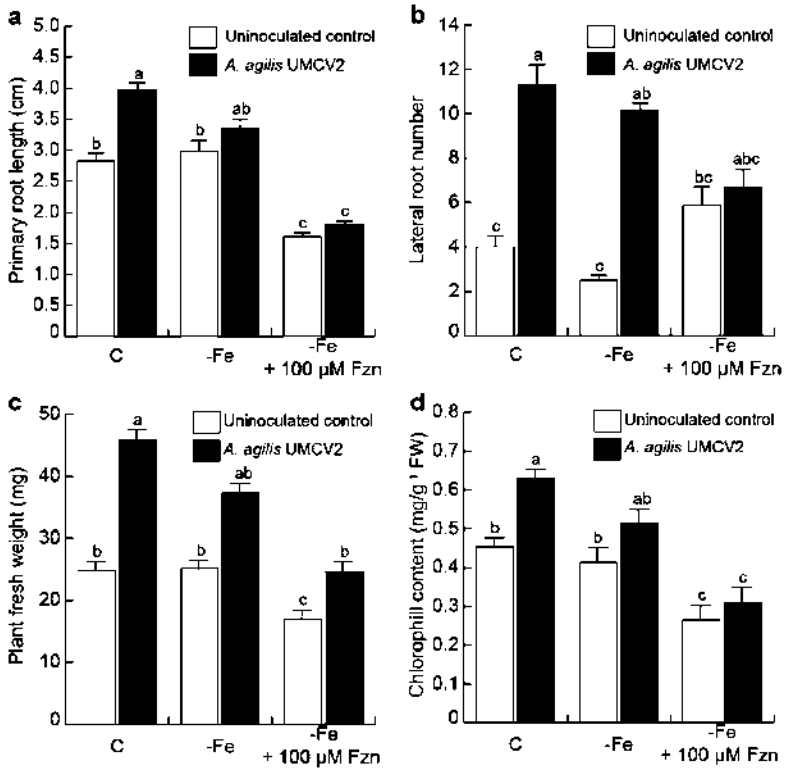

Figure 2. Volatile compounds produced by *A. agilis* UMCV2 increased Arabidopsis root growth, biomass and chlorophyll production dependently of iron availability. Arabidopsis Col-0 seedlings were grown in optimal iron conditions (Control), or Fe-deficient treatments. UMCV2 inoculation was made four days after germination and measures were realized six days after. a) Primary root length, b) Lateral root number, c) Plant fresh weight and d) Chlorophyll content. Data show the mean ±SE for three groups of 15 seedlings. Error bars represent standard errors. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

PYE, a transcription factor involved in responding to iron deficiency shows an expression induced by _A. agilis_ UMCV2 volatile compounds

It has been reported that plants exposed to iron deprivation showed PYE activation, a transcriptional factor that regulates iron homeostasis genes (Long *et al.*, 2010). To analyze if volatile compounds of *A. agilis* UMCV2 promote *Arabidopsis* development trough activating iron stress response genes, we next analyzed the transgenic line *ProPYE:GFP*. We sowed and grown *ProPYE:GFP* seeds in divided Petri plates under Control, -Fe and -Fe + 100 µM Ferrozine treatments. Four days after germination *A. agilis* UMCV2 was inoculated allowing only volatile communication, and six days after inoculation, seedlings were stained and analyzed by confocal microscopy. Figure 3 shows that, in uninoculated control *ProPYE:GFP* expression is not visible, but when iron deprivation is induced in growth media, expression levels increased and become visible in the root vasculature, columella root cap, and lateral root cap. Moreover, in treatments inoculated with *A. agilis* UMCV2, *ProPYE:GFP* expression was increased for all treatments, both iron sufficiency and deficiency, and also in the three analyzed tissues. These findings suggest that although *PYE* expression is stronger under iron deprivation conditions, bacterial VOCs form *A. agilis* UMCV2 had the capability to induce the expression of an iron stress gene in seedlings growing under optimal iron conditions and increase *PYE* expression in iron deprivation treatments compared to uninoculated controls.

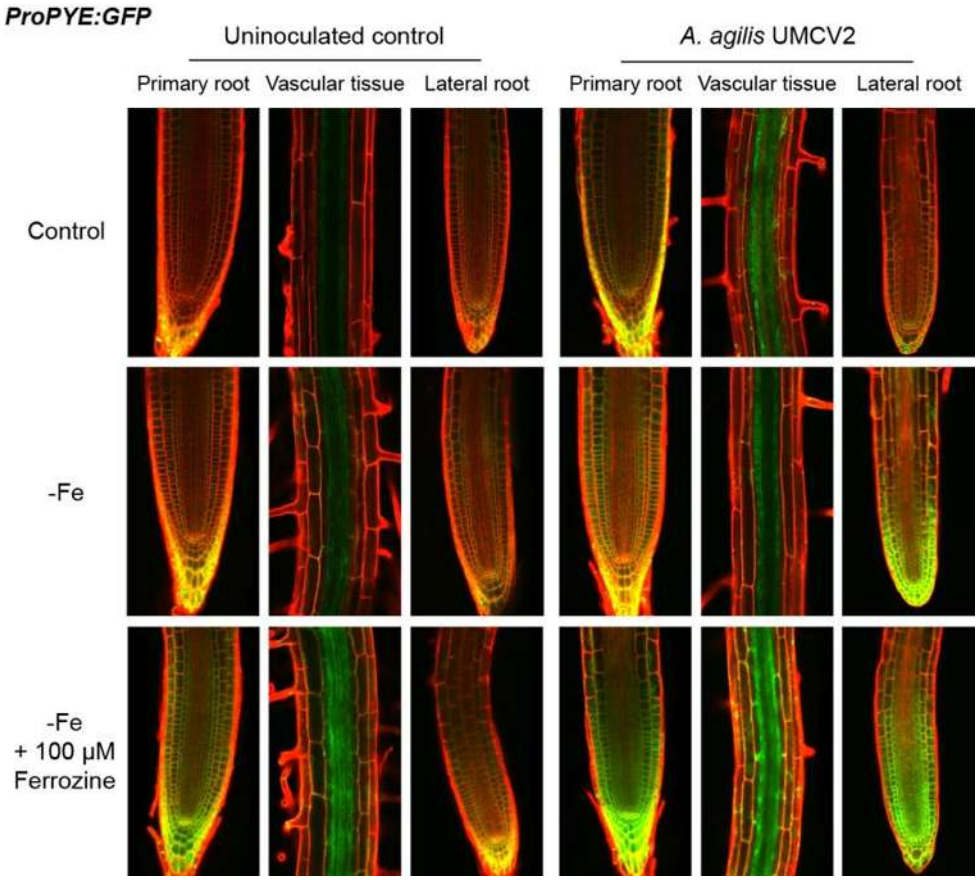

Figure 3. *A. agilis* UMCV2 volatile compounds modulate the expression of *ProPYE:GFP*, an iron deficiency-responsive gene in primary root system. *Arabidopsis thaliana* transgenic *ProPYE:GFP* seedlings were grown in optimal iron conditions (Control), or Fe-deficient treatments. UMCV2 inoculation was made four days after germination and six days later measure was performed. Seedlings were stained with propidium iodide and analyzed by confocal microscopy. Micrographs show individuals representative of at least 10 seedlings. Note that UMCV2 VOCs treatments increase *ProPYE*:GFP reporter expression in primary root, vascular tissue and lateral roots.

***BTS,* a *PYE*-opposite player in iron deficiency response is induced by *A. agilis* UMCV2 volatile compounds**

BRUTUS (BTS), a RING E3 ligase plays a major role in regulating iron-deficiency responses in *A. thaliana* (Long *et al*., 2010; Matthiadis and Long, 2016). Similar to *PYE*, *BTS* is upregulated by iron deficiency specifically in the pericycle (Long *et al.*, 2010), and it had been proposed that BTS acts upstream *PYE* (Zhang *et al*. 2015). For this reasons and our past results, we decide to evaluate *ProBTS:GFP* transgenic line under iron deficiency treatments in contact with UMCV2 VOCs. We sowed and grown *ProBTS:GFP* seeds in divided Petri plates containing Control growth media, -Fe and -Fe + 100 μM Ferrozine treatments. Four days after germination *A. agilis* UMCV2 was inoculated to allow only volatile communication, and six days after inoculation, seddings were stained and analyzed by confocal microscopy. The results obtained showed that under uninoculated tratments, *ProBTS:GFP* expression is induced mainly in vascular tissues, primary and lateral roots only under iron deprivation (Fig. 4). Interestingly, in treatments inoculated with *A. agilis* UMCV2, *ProBTS:GFP* expression was induced from control conditions in vascular tissue, to iron deficiency treatments in the three analyzed tissues (Fig. 4). Our finding suggests that *BTS* beside being induced by iron deprivation, its expression can also be induced by bacterial VOCs form *A. agilis* UMCV2. It is worth noting that UMCV2 VOCs can induce the activation of iron homeostasis genes in treatments with optimal iron quantities.

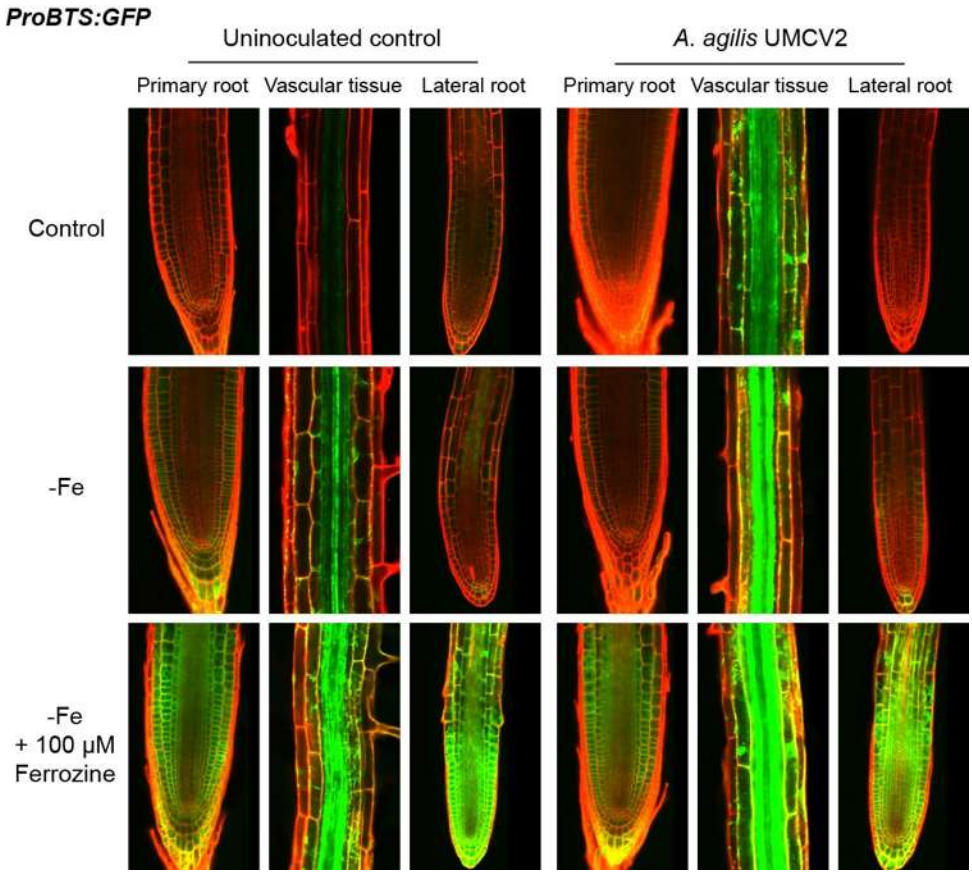

Figure 4. *A. agilis* UMCV2 volatile compounds modulate the expression of *ProBTS:GFP*, an iron deficiency-responsive gene in primary root system. *A. thaliana* transgenic *ProBTS:GFP* seedlings were grown in optimal iron conditions (Control), or Fe-deficient treatments. UMCV2 inoculation was made four days after germination and six days later measure was performed. Seedlings were stained with propidium iodide and analyzed by confocal microscopy. Micrographs show individuals representative of at least 10 seedlings. Note that UMCV2 VOCs treatments increase *ProBTS:GFP* reporter expression mainly in vascular tissue

Arabidopsis **development induced by volatile compounds of** ***A. agilis*** **UMCV2 is dependent of** ***BTS*** **participation**

To gain further insight into the role of *PYE* and *BTS* in response to *A. agilis* UMCV2 volatile compounds, we analyzed the response of wild type Columbia 0, and *A. thaliana* mutant lines *pye1* and *bts1*, affected in those genes related to iron deficiency responses. To investigate the involvement of UMCV2 VOCs in biomass production and root growth, *A. thaliana* WT and mutant lines *pye1* and *bts1* were grown on divided Petri plates containing control or iron deficient (-Fe + 100 µM Ferrozine) growth media. Four days after germination, *A. agilis* UMCV2 was inoculated and six days after inoculation plant fresh weight and primary root growth were analyzed. The obtained results showed that in control treatment volatile compounds from *A. agilis* UMCV2 induced biomass production and primary root growth in Col-0 and *pye1*, but not in *bts1*. Under Fe deprivation treatment, the opposite effect of *pye1* and *bts1* in response to low Fe availability appeared, *pye1* mutant

showed Fe-deficiency hypersensitivity evidenced by a reduced biomass production and a shortening in primary root growth compared to Col-0, while *bts1* mutant showed a phenotype of resistance to Fe deprivation, having bigger biomass production and primary root growth. Interestingly, UMCV2 VOCs were unable to induce any growth or developmental promotor effect on *bts1* mutant. Together, these results indicate that UMCV2 VOCs have a promotor effect during *Arabidopsis* growth and development and this is dependent of *BTS* participation (Fig. 5).

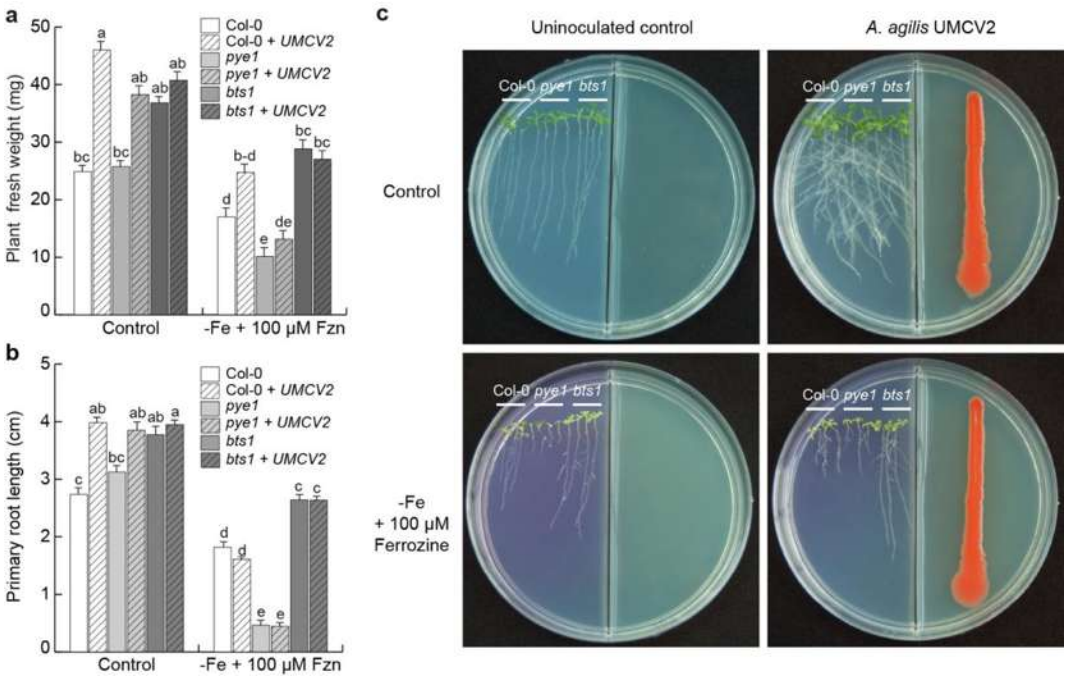

Figure 5. *A. agilis* UMCV2 volatile compounds requires BTS, an iron deficiency response gene to regulate *Arabidopsis thaliana* growth and development. Seedlings were germinated and grown under optimal iron conditions (Control), or Fe-deficient treatment. UMCV2 inoculation was made four days after germination and six days later measure was performed. a) Plant fresh weight, b) Primary root length, c) Representative photographs of seedlings grown in analyzed treatments. Photographs show representative plates; each treatment included three plates. Data from (a-b) show the mean ±SE for the three groups of 12 seedlings. Error bars represent standard errors. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

***N,N*-dimethylhexadecylamine, a major volatile produced by *A. agilis* UMCV2 is responsible for the promotor effect in growth and development in *Arabidopsis*.**

Previous works had shown that C16-DMA, a volatile compound emitted by *A. agilis* UMCV2, changes its abundance in presence of vegetal organisms like *Medicago truncatula* and induce some plant responses to iron deprivation (Orozco-Mosqueda *et al*., 2013). We wonder if C16-DMA was the VOC responsible for the previously observed plant responses. To analyzed *Arabidopsis* plants in response to C16-DMA, we sown and growth

Col-0, *pye1* and *bts1* plants in MS 0.2x media containing C16-DMA increasing concentrations. Ten days after germination, root system architecture was measured. The results indicate that for primary root growth, 2 μM of C16-DMA had a promotor effect, while 8 and 16 μM C16-DMA concentration causes a primary root growth inhibition compared to control seedlings (Fig. 6A). Interestingly, the mutants tasted did not show significant differences compared to WT in any of the analyzed concentrations (Fig. 6A). Lateral root number and lateral root density showed a dose-dependent effect to increase as C16-DMA concentration increases in WT and *pye1* seedlings, but for *bts1* this effect was opposite (Fig. 6B, C). Together, the results suggest that low concentrations of C16-DMA are responsible for the observed phenotype in primary root growth and C16-DMA might also be playing a role in the regulation of lateral root development though a mechanism involving *BTS* participation.

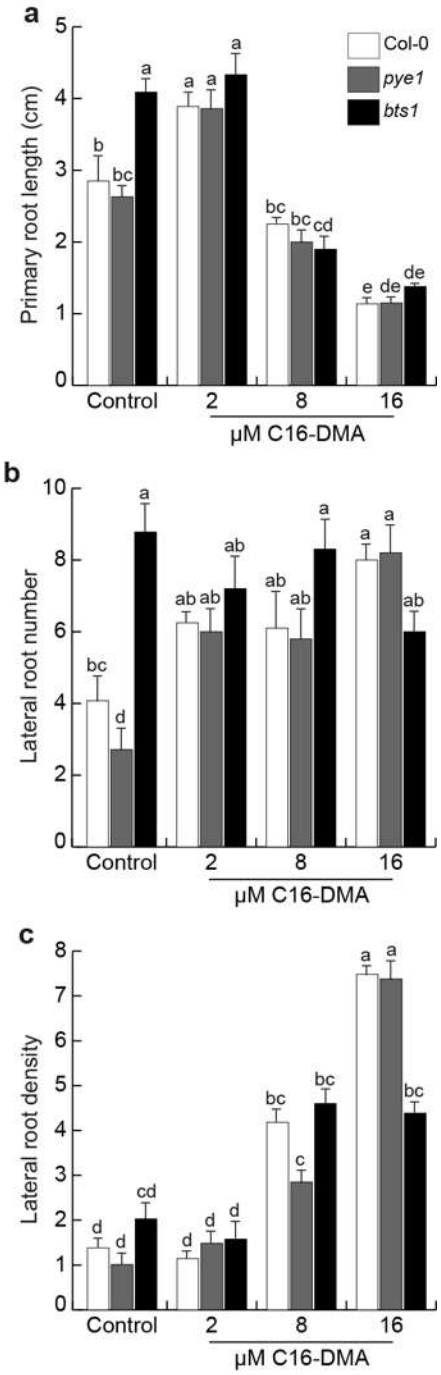

Figure 6. Effect of *N,N*-dimethylhexadecylamine on *A. thaliana* root architecture. *Arabidopsis* seedlings were germinated and grown on MS 0.2x MS agar medium supplemented with the solvent (Control) or C16-DMA concentrations for 10 days and root phenotype was evaluated. a) Primary root length, b) Lateral root number, c) Lateral root density (lateral root number/primary root length). Note that in primary root, 2 µM C16-DMA had a growth promotion effect and in high concentrations has an inhibitory effect, but no significant differences were observed in the analyzed mutants. Data show the mean ±SE from 12 seedlings. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

C16-DMA did not induce *ProPYE:GFP* nor *ProBTS:GFP* expression in primary root tips of *Arabidopsis* seedlings

To confirm our pasts results, we next analyzed the transgenic lines *ProPYE:GFP* and *ProBTS:GFP* in presence of 2 and 4 μM of C16-DMA, we used as positive control the treatment -Fe + 100 μM Ferrozine. Seedlings were analyzed seven days after germination by confocal microscopy and representative micrographs of the obtained results are shown in figure 7. The results shows that in control treatment there were no expression of the reporter genes, meanwhile in the positive control, plants shows the characteristic expression induced by iron deprivation for both reporter genes in primary root. Treatments with 2 and 4 μM of C16-DMA did not induce genes expression compared to its respective positive controls. It is important to point out that primary root morphology of plants treated with 4 μM of C16-DMA shows alterations on meristematic cell organization. Taken together, these results suggest that C16-DMA, a volatile compound produced by *A. agilis* UMCV2 is responsible for primary root growth promotor effect independently of *PYE* and *BTS*.

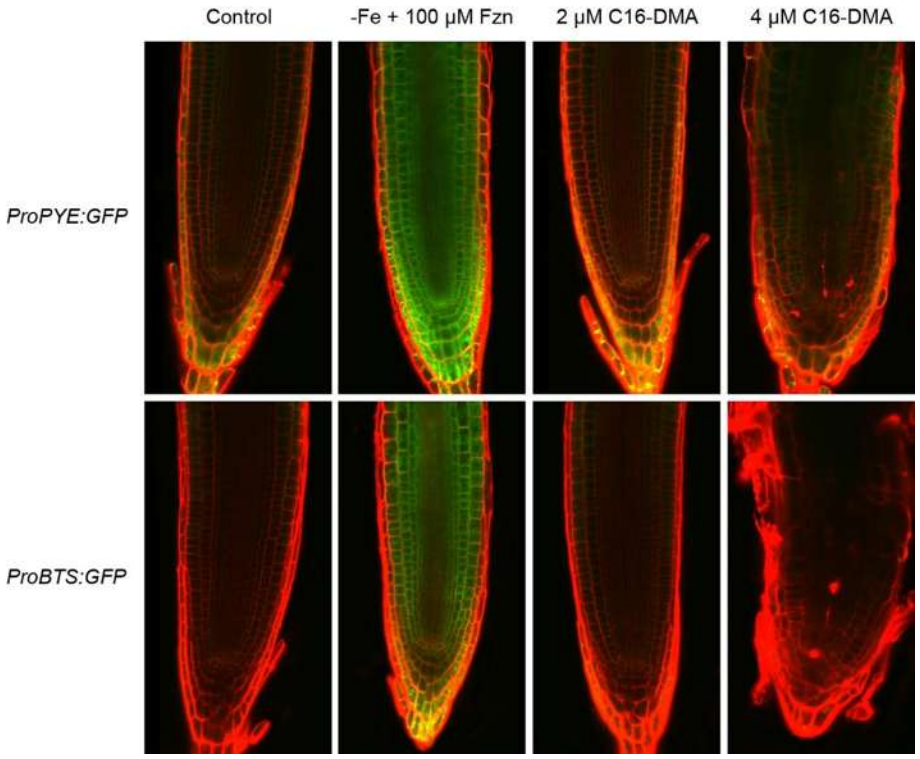

Figure 7. C16-DMA does not induce *ProPYE:GFP* nor *ProBTS:GFP* expression in primary root tips. *A. thaliana* transgenic *ProPYE:GFP* and *ProBTS:GFP* seedlings were grown in optimal iron conditions (Control), Fe-deficient treatments (-Fe+100 μM Fzn) as positive control, or C16-DMA treatments for seven days after germination. Seedlings were stained with propidium iodide and analyzed by confocal microscopy. Micrographs show individuals representative of at least 10 seedlings. Note that C16-DMA treatments does not increase *ProPYE:GFP* nor *ProBTS:GFP* reporter expression, but alters meristem cell organization in primary root tips. The experiment was repeated twice with similar results.

ILR3/bHLH105 **participates in root growth regulation of *Arabidopsis* in response to C16-DMA**

The transcription factor IAA-LEUCINE RESISTANT3 (ILR3) has been described to regulate iron deficiency responses in *Arabidopsis*, and also it has been suggested that interacts with POPEYE (PYE), a bHLH homolog and BTS, a putative E3 ligase. Due to our past results where *PYE1* and *BTS1* did not respond to C16-DMA, we wonder if *ILR3* might be implicated in response to this volatile compound produced by *A. agilis* UMCV2. To test this, we analyzed *ilr3-1* function gain mutant line form *Arabidopsis*, in treatments with iron deprivation, C16-DMA and a combination of iron deprivation treatment adding C16-DMA. Plants were grown in the different treatments and analyzed ten days after germination. The obtained results showed that in control treatment, *ilr3-1* had a small primary root compared to WT; under iron deprivation treatment, Col-0 seedlings showed the characteristic shortening in primary root while *ilr3-1* showed the reported phenotype of resistance to primary root inhibition; treatment with 8 µM C16-DMA causes hypersensitivity in *ilr3-1* mutant observed in the short size of primary root compared to Col-0 and finally, the combined treatment of iron deprivation and C16-DMA shows that in Col-0 seedlings seems to be a synergic effect causing the most significant primary root growth inhibition, but for *ilr3-1* mutant this effect did not occurred, because its primary roots had the same size as control plants (Fig. 8). These results suggest that *ILR3* plays a role in regulating root system growth of *Arabidopsis* plants in response to C16-DMA volatile compound.

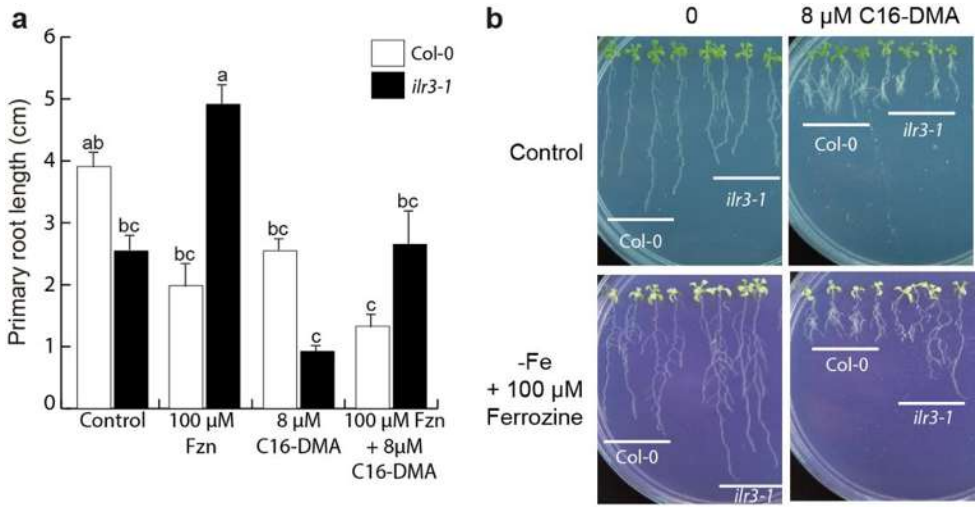

Figure 8. C16-DMA requires ILR3, a bHLH iron deficiency response gene to regulate primary root growth in *A. thaliana*. Seedlings were germinated and grown under optimal iron conditions (Control), or Fe-deficient treatment with or without 8 µM of C16-DMA supplemented. Ten days after germination, measure was performed. a) Primary root length, b) Representative photographs of Col-0 and *ilr3-1* mutant seedlings grown in analyzed treatments. Photographs are representative for three replicates each treatment. Note that ilr3-1 shows resistance to iron-deficiency root growth inhibition and hypersensitivity to C16-DMA treatment. Data from in panel a) show the mean ±SE for the three groups of 12 seedlings. Error bars represent standard errors. Different letters indicate statistical differences at P<0.05. The experiment was repeated three times with similar results.

DISCUSSION

Iron is one of the main microelements needed to acquire optimal plant development and functioning. In Arabidopsis, iron homeostasis is known to be controlled by bHLH family proteins (Long *et al*., 2010; Li *et al*., 2016; Samira *et al*., 2018; Cui *et al*., 2018), PYE transcription factor is responsible to trigger one main pathways responsible for iron deficiency responses (Long *et al*.,2010). Volatile organic compounds produced by plant growth promoting rhizobacterium (PGPR) have been reported to stimulate plant growth (Ryu *et al*., 2003; Park *et al*., 2015; Raza *et al*., 2016; Tahir *et al*., 2017). In previous works, our group reported that bacterial volatile compounds emmited by the Actinobacteria *A. agilis* UMCV2, can trigger growth promotion in Strategy I plants and also activate iron deficiency responses (Valencia-Cantero *et al*, 2007; Orozco-Mosqueda *et al*, 2013). However, is not completely clear which role are playing this responses in plant growth induction. In this study, we identified two Arabidopsis genes with a reported role in iron deficiency responses, PYE and ILR3 now functioning in plant growth promotion by volatile compounds of *A. agilis* UMCV2.

Iron availability modulate plant growth and development

Previous reports that suggest iron deprivation can be induced using iron chelators (Stookey, 1970). Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,3,4-triazine) function as an iron chelator and has been used for the pasts years to induce plant iron deficiency responses like root and vegetative growth inhibition in some plant species (Stookey, 1970; Valencia-Cantero *et al*., 2007; Long *et al*., 2010; Zhang *et al*., 2015). Reports using ferrozine in *Arabidopsis* showed that this compound induces plant chlorosis and root growth inhibition (Long *et al*., 2010; Zhang *et al*., 2015), both characteristic symptoms of iron deprivation. Our results nicely mesh with previous reports by showing that iron deprivation caused by ferrozine treatments inhibits primary root growth, induces lateral root formation and decreases biomass and chlorophyll production in a dose-dependent way in Arabidopsis seedlings (Fig. 1). Interestingly, higher ferrozine concentrations of up to 100 μM did not significantly affect primary root growth or lateral root formation (Fig. 1A, B), indicating a maximum inhibitory effect of iron deprivation toward regulating *Arabidopsis* growth and developmental processes like root system architecture and confirming the iron chelator role played by ferrozine in *Arabidopsis* growth media.

Arabidopsis growth and development can be modified by *A. agilis* UMCV2 VOCs

Once we were able to establish iron deprivation conditions by using ferrozine, and knowing that *A. agilis* UMCV2 volatile compounds were able to modulate plant growth in *Medicago truncatula* and *Sorgum bicolor* plants by activating some genes of iron deficiency response (Orozco-Mosqueda *et al*., 2013; Castulo-Rubio *et al*., 2015), we tested the hypothesis that volatile compounds from *A. agilis* UMCV2 could modified the growth of *Arabidopsis* plants. First, we determined *Arabidopsis* plant growth-promoting effect of *A. agilis* UMCV2 by volatile compounds interaction in an *in vitro* system. We used a separate compartment system to test the effect of *A. agilis* UMCV2 volatile compounds on the growth of

Arabidopsis Col-0 plants, and to avoid the effect of direct plant-bacteria contact or diffusible compounds. The analysis were performed after six days of VOCs interaction and it showed that plants grown under VOCs interaction, produced a significant increase in primary root growth and lateral root number under optimal iron conditions and this effect is lost as iron availability decreases (Fig. 2A, B). Simultaneously, volatile compounds of *A. agilis* UMCV2 induce a significant increase in plant fresh weight and chlorophyll content in control treatment but when iron deprivation exist, this effect is also loosed (Fig. 2C, D). These results suggest that volatile compounds of *A. agilis* UMCV2 are capable of regulate plant growth and development. Similar results had been obtained in systems with *Arabidopsis-Bacillus subtilis* GB03, *Medicago truncatula-Sinorhizobium melioti*, *Nicotiana tabacum-Pseudomonas fluorescens* SS101 and *Solanum lycopersicum-Bacillus subtilis* SYST2 (Xie *et al*., 2009; Orozco-Mosqueda *et al*., 2013; Park *et al*., 2015; Tahir *et al*., 2017).

A. agilis UMCV2 VOCs induce *Arabidopsis* growth and development involving BTS participation

To further explore the mechanisms of *Arabidopsis* in response to *A. agilis* UMCV2 volatile compounds in seedlings grown under different iron availability treatments, we tested the expression of *ProPYE:GFP* and *ProBTS:GFP* iron deficiency inducible markers. GFP florescence increased in treatments with iron deficiency, consistent with an iron-deficiency response mechanism (Long *et al*., 2010). Interestingly, we observed enhanced *ProPYE:GFP* and *ProBTS:GFP* fluorescence after *A. agilis* UMCV2 volatile compounds interaction in primary root tips, particularly in the vascular tissue and lateral root tips, which indicates an activation of PYE pathway during root growth and development (Figs. 3, 4). The iron deficiency responses activated by bacterial volatile compounds has been poorly investigated. For the past ten years, a few works revealed that microorganisms are capable of induce iron deficiency responses such as ferric reductase activity, rizosphere acidification, and expression of genes associated to those responses (Zhang *et al*., 2009; Orozco-Mosqueda *et al*., 2013; Castulo-Rubio *et al*., 2015; Montejano-Ramírez *et al*., 2018; Zhou *et al.*, 2018). The possibility that *A. agilis* UMCV2 volatile compounds could modulate iron deficiency response pathways in *Arabidopsis* to induce plant growth regulation, might be suggesting adaptive interkingdom communication evolutionary conserved.

In rice and mammals, HRZs and FBXL5 are the respective known iron sensors that negatively regulate iron acquisition (Salahudeen *et al*., 2009; Vashisht *et al*., 2009). In Arabidopsis, BTS is its homolog, Long *et al.* (2010) reported *bts1* mutant with tolerance to iron deficiency and Zhang *et al.* (2015) propose a model in which BTS may function as the Arabidopsis iron sensor by regulating the stability of downstream factors including PYE and ILR3. Changes in iron deficiency response genes correlate with root system architecture modulation (Valencia-Cantero *et al*., 2007; Long *et al*., 2010; Zhang *et al*., 2015). Our finding that *A. agilis* UMCV2 volatile compounds increase gene expression of iron deficiency response genes in Arabidopsis primary root suggests that UMCV2 VOC´s modifies root development by altering PYE pathway. Consistent with this hypothesis, *bts1*

mutant did not respond to UMCV2 VOCs, indicating the possible role of BTS in UMCV2 VOCs root responses. In the same treatments *pye1* mutant showed a normal response to UMCV2 VOCs in primary root (Fig. 5). These results suggest that UMCV2 VOCs increase in BTS expression, resulted in the activation of PYE pathway and drive to root growth regulation, although *PYE* was activated, *pye1* mutant results suggest BTS plays a main role in response to *A. agilis* UMCV2 VOCs by the probably activation of not only PYE, but some others downstream genes.

_N,N_- dimethylhexadecylamine, one main VOC produced by *A. agilis* UMCV2 have a distinct function than full VOCs emitted, and specifically needs ILR3 participation

N,N-dimethylhexadecylamine (C16-DMA) is an amino-containing lipid, identified from *A. agilis* UMCV2 (Velázquez-Becerra *et al*., 2011) and it has been found to be produced by some others rhizobacteria including genera of *Bacillus*, *Sinorhizobium* and *Pseudomonas* (Liu *et al*., 2008; Orozco-Mosqueda *et al*., 2013; Hernández-León *et al*., 2015). C16-DMA has been found to modulate growth and development in several plant species including *A. thaliana* 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; Raya-González *et al.*, 2017). Interestingly, in *Sorgum bicolor* plants, C16-DMA increased root ferric reductase (FRO) gene expression, suggesting an important role of this amine in modulating Fe nutritional status (Castulo-Rubio *et al*., 2015). Here, we demonstrate that at varying increasing concentrations, C16-DMA inhibits *Arabidopsis* primary root growth and that neither *PYE* nor *BTS* participate in this response (Fig. 6A). However, *BTS* participate in lateral root numbers formation, which suggest that specific root developmental programs are regulated by C16-DMA where *BTS* plays an important role.

To confirm whether *BTS* and *PYE* iron deficiency response genes are involved in *A. thaliana* responses to C16-DMA, we evaluated the expression of *ProPYE:GFP* and *ProBTS:GFP* in response to C16-DMA. No one of the analyzed *Arabidopsis* lines showed that GFP fluorescence increased after seven days of treatment (Fig. 7). The lack of GFP increase, associated with the primary root phenotype present in the higher C16-DMA concentration, which is quite different to that present in *A. agilis* UMCV2 VOCs interaction, supports our previous results, suggesting that C16-DMA is not the responsible VOC for Arabidopsis growth promotion observed in interaction with full VOCs pool. This was not entirely surprising, as recent studies have shown that C16-DMA regulates Arabidopsis root development and activates jasmonic acid responsive gene expression involving JAR1, COI1 and MYC2 elements (Raya-González *et al*., 2017). We cannot exclude the possibility that C16-DMA could modulate *Arabidopsis* development through the activation of some other iron deficiency response genes due to the connections between iron and jasmonic acid signaling (Martínez-Medina *et al*., 2017).

Another component in iron deficiency response signaling is *IAA-LEUCINE RESISTANT 3,* which encodes a basic helix-loop-helix (bHLH) leucine zipper transcription factor, bHLH105 that plays a role in metal ion-mediated auxin sensing (Rampey *et al*., 2006). bHLH proteins commonly form homodimers and heterodimers that interact with

downstream targets (Littlewood and Evan, 1998; Toledo-Ortíz *et al*., 2003). ILR3 was suggested to act as an intermediary, binding directly with BTS or PYE playing a role in iron-mediated auxin sensing in *Arabidopsi*s roots (Rampey *et al*., 2006; Long *et al*., 2010; Zhang *et al*. 2015). We were able to report here *ilr3-1*, a mutant with a gain of function to IAA-Leu sensitivity (conferred by its high mRNA expression levels), now playing a role in response to C16-DMA. Interestingly, the iron deficiency response of resistance presented in *ilr3-1* mutant was diminished in C16-DMA presence, revealing that the effect of this compound and iron signaling converge in ILR3 to regulate primary root growth in *Arabidopsis* seedlings. Based on these results, we suggest that C16-DMA acts trough specific target genes to regulate specific root developmental traits.

In conclusion, this work showed that *A. agilis* UMCV2 VOCs, modulated *A. thaliana* morphogenesis, promoted plant growth, and induced *PYE* and *BTS* expression, while C16-DMA induces *ILR3* and modified *ilr3-1* response to iron deprivation. As far as we know, this we are the first to relate components of an iron deficiency response mechanism with plant growth promotion in response to bacterial VOCs in *Arabidopsis thaliana*. We propose that *A. agilis* UMCV2 VOCs and C16-DMA produce a plant growth effect dependent of specific iron deficiency response genes induction. The utility of bacterial VOCs manipulation in plant physiology is a novel research field that will lead to future important applications in agricultural production.

REFERENCES

Bauer P, Ling H-Q, Guerinot ML. 2007. FIT, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in Arabidopsis. *Plant Physiology and Biochemistry* 45: 260-261. DOI: 10.1016/j.plaphy.2007.03.006

Castulo-Rubio DY, Alejandre-Ramírez N, Orozco-Mosqueda MC, Santoyo G, Macias-Rodríguez LI, Valencia-cantero E. (2015) Volatile organic compounds produced by the rhizobacterium *Arthrobacter agilis* UMCV2 modulate *Sorghum bicolor* (strategy II plant) morphogenesis and *SbFRO1* transcription in vitro. *Journal of Plant Growth Regulation* 34: 611-623. DOI: org/10.1007/s0034 4-015-9495-8

Colangelo EP, Guerinot ML. 2004. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *The Plant Cell* 16: 3400-3412. DOI: 10.1105/tpc.104.024315

Cui Y, Chen C-L, Cui M, Zhou W-J, Wu H-L, Ling H-Q. 2018. Four IVa bHLH transcription factors are novel interactors of FIT and mediate JA inhibition of iron uptake in *Arabidopsis*. *Molecular Plant* 11: 1166-1183. DOI: 10.1016/j.molp.2018.06.005

Eide D, Broderius M, Fett J, Guerinot ML. 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Science of the United States of America* 93: 5624-5628. DOI: 10.1073/pnas.93.11.5624

Hell R, Stephan UW. 2003. Iron uptake, trafficking and homeostasis in plants. *Planta* 216: 541-551. DOI: 10.1007/s00425-002-0920-4.

Hernández-León R, Rojas-Solís D, Contreras-Pérez M, Orozco-Mosqueda MC, Macías-Rodríguez LI, Reyes-de la Cruz H, Valencia-Cantero E, Santoyo G. 2015. Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains. *Biological Control* 81: 83-92. doi:10.1016/j.biocontrol.2014.11.011

Li X, Zhang H, Ai Q, Liang G, Yu D. 2016. Two bHLH transcription factors, bHLH34 and bHLH104, regulate iron homeostasis in *Arabidopsis thaliana. Plant Physiology* 170: 2478-2493. Doi: 10.1104/pp.15.01827

Littlewood T, Evan GI. 1998. Helix-Loop-Helix Transcription Factors, 3rd ed. (New York: Oxford University Press).

Liu W, Wei M, Bingyu Z, Feng L. 2008. Antifungal activities and components of VOCs produced by *Bacillus subtili*s G8. *Current Research in Bacteriology* 1: 28-34. DOI: 10.3923/crb.2008.28.34

Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN. 2010 The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. Plant Cell 22: 2219-2236. DOI: 10.1105/tpc.110.074096

Martínez-Medina A, VanWees SCM, Pieterse CMJ. 2017. Airborne signals from *Trichoderma* fungi stimulate iron uptake responses in roots resulting in priming of jasmonic acid-dependent defences in shoots of *Arabidopsis thaliana* and *Solanum lycopersicum*. *Plant Cell & Environment* 40: 2691-2705. DOI: 10.1111/pce.13016

Matthiadis A, Long TA. 2016. Further insight into BRUTUS domain composition and functionality. *Plant Signaling & Behavior* 11: e1204508. DOI: 10.1080/15592324.2016.1204508.

Montejano‐Ramírez V, Martínez‐Cámara R, García‐Pineda E, Valencia‐Cantero E. 2018. Rhizobacterium *Arthrobacter agilis* UMCV2 increases organ-specific expression of FRO genes in conjunction with genes associated with the systemic resistance pathways of *Medicago truncatula. Acta Physiologiae Plantarum* 40: 138 DOI: 10.1007/s11738-018-2712-x

Orozco-Mosqueda MC, Velázquez-Becerra C, Macías-Rodríquez LI, Santoyo G, Flores-Corez I, Alfaro-Cuevas R, Valencia-Cantero E. 2013. *Arthrobacter agilis* UMCV2 induces iron acquisition in *Medicago truncatula* (strategy I plant) in vitro via dimethylhexadecylamine emission. *Plant and Soil* 362: 51-66. DOI: 10.1007/s1110 4-012-1263-y

Ortiz-Castro R, Martínez-Trujillo M, López-Bucio J. 2008. N-acyl-Lhomoserine lactones: a class of bacterial quorum-sensing signals alter post-embryonic root development in *Arabidopsis thaliana. Plant Cell & Environment* 31: 1497-1509. DOI: 10.1111/j.1365-3040.2008.01863.x

Palmer CM, Guerinot ML. 2009. Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Natural Chemical Biology* 5: 333-340. DOI: 10.1038/nchembio.166.

Park YS, Dutta S, Ann M, Raaijmakers JM, Park K. 2015. Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochemical and Biophysical research communications* 461: 361-365. DOI: 10.1016/j.bbrc.2015.04.039

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S, Crecchio C. 2015. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertil of Soils* 51: 403-415. DOI: org/10.1007/s0037 4-015-0996-1

Rampey RA, Woodward AW, Hobbs BN, Tierney MP, Lahner B, Salt DE, Bartel B. 2006. An Arabidopsis basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. *Genetics* 174: 1841-1857. DOI: 10.1534/genetics.106.061044

Raya-González J, Velázquez-Becerra C, Barrera-Ortíz 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: 1399-1410. DOI: org/10.1007/s0070 9-016-1031-6

Raza W, Yousaf S, Rajer FU. 2016. Plant growth promoting activity of volatile organic compounds produced by biocontrol strains. *Science Letters* 4: 40-43.

Robinson NJ, Procter CM, Connolly EL, Guerinot ML. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* 397: 694-697. DOI: 10.1038/17800

Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Pare PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis. Plant Physiology* 134: 1017-1026. DOI: 10.1104/pp.103.026583

Salahudeen AA, Thompson JW, Ruiz JC, Ma HW, Kinch LN, Li Q, Grishin NV, Bruick RK. 2009. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* 326: 722-726. DOI: 10.1126/science.1176326

Samira R, Li B, Kliebenstein D, Li C, Davis E, Gillikin JW, Long TA. 2018. The bHLH transcription factor ILR3 modulates multiple stress responses in *Arabidopsis. Plant Molecular Biology* 97: 297-309 DOI: 10.1007/s11103-018-0735-8

Selote D, Samira R, Matthiadis A, Gillikin JW, Long TA. 2015. Iron-binding E3 ligase mediates iron response in plants by targeting basic helix-loop-helix transcription factors. *Plant Physiology* 167: 273-286. DOI: 10.1111/pce.13363

Schmidt W. 1999. Review: Mechanisms and regulation of reduction-based iron uptake in plants. *New Phytologist* 141: 1-26. DOI: 10.1046/j.1469-8137.1999.00331.x

Spaepen S, Das F, Luyten E, Michiels J, Vanderleyden J. 2009. Indole-3-acetic acid-regulated genes in *Rhizobium etli* CNPAF512. *FEMS Microbiology Letters* 291: 195-200. DOI: 10.1111/j.1574-6968.2008.01453.x.

Stookey LL. 1970. Ferrozine-a new spectrophotometric reagent for iron. *Analytical chemistry* 42: 779-781. DOI: 10.1021/ac60289a016

Tahir HAS, Gu Q, Wu H, Raza W, Hanif A, Wu L, Colman MV, Gao X. 2017. Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. *Frontiers in Microbiology* 8: 171. DOI: 10.3389/fmicb.2017.00171

Terrazas RA, Giles C, Paterson E, Robertson-Albertyn S, Cesco S, Mimmo T, Piill Y, Bulgarelli D. 2016. Plant-microbiota interactions as a driver of the mineral turnover in the rhizosphere. Advances in Applied Microbiology 95: 1-67. DOI: 10.1016/bs.aambs .2016.03.001

Toledo-Ortiz G, Huq E, Peter H. 2003. The *Arabidopsis* basic/Helix-Loop-Helix transcription factor family. *The Plant Cell* 15: 1749-1770. DOI: 10.1105/tpc.013839

Valencia-Cantero E, Hernández-Calderón E, Velázquez-Becerra C, López-Meza JE, Alfaro-Cuevas R, López-Bucio J. 2007 Role of dissimilatory fermentative Fe-reducing bacteria in Fe uptake by common bean (*Phaseolus vulgaris* L.) plants grown in alkaline soil. *Plant and Soil* 291: 263-273. DOI: org/10.1007/s11104-007-9191-y

Valencia-Cantero E, Flores-Cortez I, Ambriz-Parra J, Lopez-Albarran P, Velázquez-Becerra C. 2015. *Arthrobacter agilis* UMCV2 accelerates growth of *Pinus devoniana*. Phyton-Int J Exp Bot 84: 64-69.

Vashisht AA, Zumbrennen KB, Huang X, Powers DN, Durazo A, Sun D, Bhaskaran N, Persson A, Uhlen M, Sangfelt O, Spruck C, Leibold EA, Wohlschlegel JA. 2009. Control of iron homeostasis by an iron-regulated ubiquitin ligase. Science 326: 718-721. DOI: 10.1126/science.1176333

Velázquez-Becerra C, Macías-Rodríguez LI, López-Bucio J, Altamirano-Hernández J, Flores-Cortez I, Valencia-Cantero E. 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis *in vitro*. *Plant and Soil* 339: 329-340. DOI: 10.1007/s1110 4-010-0583-z

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat JF, Curie C. 2002. IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell* 14: 1223-1233. DOI: 10.1105/tpc.001388

Vigani G, Zocchi G, Bashir K, Philippar K, Briat JF. 2013. Signals from chloroplasts and mitochondria for Fe homeostasis regulation. *Trends in Plant Science* 18: 305-311. DOI:10.1016/j.tplants.2013.01.006

Walker EL, Connolly EL. 2008. Time to pump iron: Iron deficiency signaling mechanisms of higher plants. *Current Opinion in Plant Biology* 11: 530-535. DOI: 10.1016/j.pbi.2008.06.013

Xie X, Zhang H, Paré PW. 2009. Sustained growth promotion in *Arabidopsis* with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03). *Plant Signaling & Behavior* 4: 948-953. DOI: 10.4161/psb.4.10.9709

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling H-Q. 2008. FIT interacts with *AtbHLH38* and *AtbHLH39* in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis. Cell Research* 18: 385-397. DOI: 10.1038/cr.2008.26.

Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Paré PW. 2009. A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms *The Plant Journal* 58: 568-577. DOI: 10.1111/j.1365-313X.2009.03803.x

Zhang J, Liu B, Li M, Feng D, Jin H, Wang P, Liu J, Xiong F, Wang J, Wang HB. 2015 The bHLH transcription factor bHLH104 interacts with IAA-LEUCINE RESISTANT3 and modulates iron homeostasis in *Arabidopsis*. *The Plant Cell* 27: 787-805. Doi: 10.1105/tpc.114.132704

Zhao L, Wang F, Zhang Y, Zhang J. 2014. Involvement of *Trichoderma asperellum* strain T6 in regulating iron acquisition in plants. *Journal of Basic Microbiology* 54: S115–S124. DOI: org/10.1002/ jobm.20140 0148

Zhou C, Zhu L, Guo J, Xiao X, Ma Z, Wang J. 2018. *Bacillus subtilis* STU6 ameliorates iron deficiency in tomato by enhancement of polyamine-mediated iron remobilization. Journal of Agricultural and Food Chemistry. DOI: 10.1021/acs.jafc.8b05851

7. DISCUSIÓN GENERAL Y CONCLUSIONES

Uno de los principales enigmas en ecología de comunidades, es el papel que presentan las interacciones vegetales en determinar la coexistencia y desempeño de los individuos dentro de la comunidad. Los organismos vegetales debido a su carácter sésil, se encuentran bajo estímulos bióticos y abióticos, y las plantas tienen la capacidad de modificar su morfología y fisiología en respuesta a variaciones ambientales, particularmente en la búsqueda de recursos (Hodge, 2004, 2006; Valladares *et al*., 2007; Dicke, 2009; Trewavas, 2009).

En especies vegetales de importancia económica, se han llevado a cabo investigaciones sobre la búsqueda de variedades competitivas, es decir que presenten características que les permitan obtener un recurso dado con una menor inversión energética (Gibson *et al.,* 1999). Aunque, aún existe controversia al respecto, estas características incluyen la habilidad de consumir recursos y/o la plasticidad del crecimiento en respuesta a la disponibilidad espacio-temporal de los recursos (Craine, 2005; Fargione y Tilman, 2006). Sin embargo, aún no se encuentra completamente descrito cuáles serían los mecanismos que inducen estas características en respuesta a estímulos específicos.

Debido a la complejidad de las interacciones ecológicas que se presentan en las comunidades naturales activando variadas rutas de señalización; este trabajo, se enfocó en dos tipos de interacciones principales: i) interacción planta-planta, analizando el efecto de la densidad poblacional sobre el crecimiento y desarrollo de *Arabidopsis thaliana*, e ii) interacción planta-microorganismo utilizando a los modelos *A. thaliana* y *Arthrobacter agilis* UMCV2, en un sistema de disponibilidades contrastantes de nutrición férrica para la planta. Los resultados principales se discuten a continuación.

La densidad poblacional es un factor ecológico que modula la morfología vegetal y los procesos del desarrollo, sin embargo sus efectos han sido escasamente investigados. Se realizó el análisis de respuestas en programas del desarrollo mediados por auxinas, en plantas de *A. thaliana* crecidas en diferentes densidades de individuos tanto *in vitro* como en suelo.

Inicialmente, se analizó la morfología del crecimiento del sistema aéreo de la planta, para determinar si la densidad poblacional podría modular algún proceso fisiológico, que llevara a una respuesta en la plasticidad fenotípica de las plantas de manera dependiente del número de individuos, como ya se había reportado previamente (Masclaux *et al*., 2012). Para esto se analizaron plantas de *A. thaliana* crecidas en densidades crecientes de manera exponencial desde 1 hasta 32. Los resultados mostraron que, de manera dependiente de la densidad poblacional se presenta una disminución del tamaño de roseta en el número de hojas que la componen, el tamaño de las mismas y los peciolos que las sostienen. Lo anterior indica que la densidad poblacional modula el desarrollo del sistema vegetativo (Cap. I, Fig. 1), reduciendo así el área principal de la planta que se encarga de realizar la fotosíntesis y por tanto la producción de biomasa.

En cuanto al tiempo de emergencia de los tallos, que son los que dan lugar a las estructuras reproductivas, las flores y finalmente a los frutos, no se observó una diferencia significativa (Cap. I, Fig. 2A). Conforme pasa el tiempo, plantas en alta densidad llegan a una altura máxima antes que las que crecen en baja densidad y esta altura fue aproximadamente igual en todos los individuos del tratamiento (Cap. I, Fig. 2A, B). Junto con la aceleración de la etapa reproductiva en alta densidad, se presenta una aceleración en la senescencia (Cap I. Figs.1C, D; 2D y 3D), lo que coincide con la "decisión" de las plantas de detener su desarrollo vegetativo al iniciar el desarrollo reproductivo (Araki, 2001).

En el análisis sobre la producción de semillas se observó que la disminución en la producción por planta es drástica, dependiente de la densidad poblacional (Cap. I, Fig. 3). Desde el punto de vista ecológico, resulta interesante notar la importancia de este trabajo en la agricultura, donde organismos de la misma especie crecen de forma cercana en áreas definidas, induciendo así una relación intraespecifica. Este tipo de cultivos son el resultado de una selección antropogénica con la intención de obtener mayores rendimientos por área cultivada. Los mejoradores han realizado selecciones de variedades, en base a cambios positivos en la habilidad competitiva que permite una mayor producción de frutos (Weiner, 2003). Sin embargo, el desarrollo radicular no ha sido

considerado por los mejoradores, e indirectamente pudo haber llevado sin conocimiento alguno a una selección de variedades con reducida sensibilidad al reconocimiento de individuos vecinos. Adicionalmente a las implicaciones del reconocimiento de la raíz, el hecho de que las plantas respondan a la identificación de características específicas de sus vecinos podría contribuir al aumento en la habilidad competitiva, lo que resultaría en variedades con reducida capacidad de reconocimiento de vecinos como una característica que podría incrementar el rendimiento de los cultivos.

Por lo anterior, fue importante el análisis sobre la morfología de *Arabidopsis* en etapas tempranas, donde los organismos son más sensibles a los cambios en el ambiente (Knezevic *et al*., 2002; Seem *et al.,* 2003). Resultó entonces importante analizar la arquitectura radicular de *A. thaliana* bajo condiciones de crecimiento en diferente densidad de individuos. Los resultados indicaron que de manera dependiente del aumento en densidad poblacional, se presentó una disminución en el crecimiento de la raíz primaria, así como de la formación de raíces laterales y por tanto de la densidad de las mismas (Cap. I, Fig. 5). Dicho efecto inhibitorio sobre la arquitectura radicular podría deberse a: i) que las plantas estaban compitiendo por nutrientes del medio, CO_2 del ambiente o espacio en el sistema, o ii) que estaban autoregulando su crecimiento de forma similar al *quorum sensing* que se presenta en bacterias (Walters y Bassler, 2005).

Para analizar estas posibilidades se realizaron dos experimentos, el primero fue el análisis del sistema radicular en cajas divididas, para determinar si todas las variaciones en la densidad poblacional afectaban el crecimiento de plantas vecinas en baja densidad, sin contacto físico. Se observó de manera dependiente de la densidad poblacional una inhibición del crecimiento de plantas vecinas (Cap. II, Fig. 1), lo cual sugería que la disminución en el crecimiento de las plantas no se debía a una limitante de nutrientes, debido a que las crecidas en baja densidad no tenían ningún vecino que compitiera por espacio o nutrientes en el medio. Además, el análisis del presente estudio contrasta con el hecho de que los fenotipos reportados en deficiencia de nutrientes, incluían siempre la inhibición en el crecimiento de la raíz primaria, no así la formación de raíces laterales (López-

Bucio *et al*., 2002; Forde y Walch-Liu, 2009). En este estudio, los efectos de represión del desarrollo observados fueron en ambos procesos a la vez, descartando con esto que las plantas presenten esta respuesta por una deficiencia de nutrientes, los resultados contrastan también con trabajos previos en donde sugieren que las plantas responden al espacio en que crecen, más que a la cantidad de individuos que tengan a su alrededor (Schenk, 2006). Debido a que las plantas no tienen un contacto físico, este experimento también sugiere que la regulación del desarrollo puede estar mediada por compuestos volátiles. Algunos reportes sugieren que en especies como *Trifolium glomerata* y *Dactilus pratense* (Kigathi *et al*., 2013) la interferencia ocasionada por compuestos químicos entre las plantas altera las relaciones entre la densidad y el crecimiento vegetal (Weidenhamer *et al*. 1989; Sinkkonen, 2001).

Los análisis del sistema radicular mostraron una disminución en el número de raíces laterales de manera dependiente de la densidad poblacional, por lo que analizamos si tal fenómeno se debía a la formación *de novo* de primordios de raíces laterales o a una inhibición en la maduración de los primordios existentes de manera normal en las plantas. Los resultados obtenidos indicaron que en alta densidad, se observan pocos primordios en todas las etapas de maduración y las raíces laterales emergidas son menos, por lo que al analizar el número total de primordios concluimos que la densidad está afectando no solo la formación *de novo* debido a una deficiencia en la diferenciación celular, sino además afecta la maduración de los primordios ya existentes, sugiriendo que el proceso de división celular podría encontrarse afectado en alta densidad. Debido a que la división celular es un proceso modulado por auxinas, se analizó el efecto de la densidad poblacional sobre la expresión de la línea reportera *DR5:GFP* (Ottenschaläger *et al*., 2003), que permite analizar *in vivo* la expresión de la proteína verde fluorescente y de esta forma, la acumulación de auxinas en tejidos específicos de la raíz. El análisis mostró que la densidad poblacional permitió la acumulación de auxinas en la zona meristemática y de la cofia de la raíz, sugiriendo que la cantidad de auxinas podría ser menor en alta densidad (Cap. I, Fig. 6). También, el análisis anterior indicó que el transporte polar puede estar afectado debido a la

pérdida de expresión en el estele de la raíz. Para probar esto, se analizó la línea transgénica *PIN1::PIN1:GFP* (Benková *et al.,* 2003), que permite determinar en las células, la expresión específica del transportador de eflujo de auxinas, PIN1. Se observó que dicho transportador se encuentra en cantidades significativamente menores en alta densidad (Cap. I, Fig. 6). Los resultados anteriores, ponen en evidencia que la expresión de PIN1 se encuentra regulada por las cantidades de auxinas y no por el transporte de éstas, de manera dependiente de la densidad poblacional. Con todo lo anterior se confirma que la densidad poblacional regula procesos del desarrollo radicular de *A. thaliana* de manera dependiente de la señalización de auxinas.

A la fecha existe un gran número de mutantes de *Arabidopsis* afectadas en el transporte o la señalización de auxinas, que se caracterizan por su resistencia o sensibilidad a la inducción de los procesos regulados por auxinas. En nuestro grupo de trabajo, Raya-González *et al.* (2014) describieron que la mutante *pft1-2* presentaba un crecimiento mayor que el ecotipo silvestre Col-0, y una hipersensibilidad a bajas concentraciones de auxinas. Los autores sugirieron que PFT1 podría estar involucrado en el transporte de auxinas, mediando la regulación transcripcional y distribución de PIN1. Por estos antecedentes se decidió analizar a la mutante *pft1-2* en los tratamientos de densidad poblacional. Los resultados indicaron que *pft1-2* presenta resistencia al efecto de la densidad poblacional, aún en la densidad de 32 individuos, tanto en el crecimiento de la raíz primaria, como en el número y densidad de raíces laterales (Cap. I, Fig. 7). Esto sugiere que *PFT1* podría estar participando directamente en la regulación de la arquitectura radicular de *A. thaliana* (crecimiento de la raíz primaria y formación de raíces laterales) de manera dependiente de la densidad poblacional. En conjunto nuestros resultados de la interacción planta-planta, sugieren que el mecanismo de señalización en respuesta a la densidad poblacional involucra varias vías: una inducción mediada por la percepción de compuestos volátiles y mecanismos de respuesta que incluyen la señalización de auxinas, en donde participan específicamente el transportador de eflujo PIN1 y la subunidad del complejo mediador PFT1/MED25 (Fig. 17).

Dentro de las interacciones ecológicas, una de las respuestas mejor estudiada, es la habilidad que presentan las plantas para responder a zonas del suelo ricas o deficientes en nutrientes mediante la proliferación de raíces. Desde hace décadas, es objeto de investigación conocer los requerimientos de cada especie vegetal que permitan su cultivo con una mayor facilidad, mejorando la producción de biomasa y rendimiento final, principalmente en especies de importancia económica. En el campo de la investigación, también ha sido importante determinar los requerimientos nutricionales de los vegetales para facilitar su estudio (Murashige y Skoog, 1962).

Dentro de los micronutrientes esenciales, uno de los principales requeridos por las plantas es el hierro; este metal es necesario para el crecimiento vegetal ya que participa tanto en la fotosíntesis como en la cadena respiratoria, y es además importante para el consumo humano debido a que es adquirido por medio de las plantas. El hierro presenta una solubilidad limitada en la mayoría de los suelos, y debido a esto hay cantidades poco accesibles en la rizosfera. Las plantas han adquirido estrategias de adaptación para una mejor adquisición de Fe: la primera denominada Estrategia I, es utilizada por plantas dicotiledóneas, como *Arabidopsis* y se basa en la reducción de Fe al exterior de la raíz para posteriormente adquirirlo en forma asimilable (Hindt y Guerinot, 2012).

Algunos reportes muestran que las condiciones de deficiencia de hierro pueden inducirse utilizando compuestos quelantes en el medio de cultivo (Stookey, 1970). La ferrozina (3-(2-piridil)-5,6-bi(ácido 4-fenilsulfonico)-1,3,4-triazina) es el compuesto quelante más utilizado durante los últimos años para inducir en las plantas respuestas a la deficiencia de hierro entre las que se incluyen clorosis del follaje e inhibición del crecimiento radicular (Valencia-Cantero *et al*., 2007; Long *et al*., 2010; Zhang *et al*., 2015).

Para caracterizar el efecto de la disponibilidad de hierro sobre el crecimiento y desarrollo de *A. thaliana* se utilizaron semillas del ecotipo Columbia 0 en condiciones de distinta disponibilidad de hierro utilizando a la ferrozina (Fzn). Se observó que de manera dependiente de la deficiencia de hierro, las plantas presentan una disminución en la producción de biomasa y clorofila, mientras que

el sistema radicular presenta una inhibición del crecimiento de la raíz primaria y un aumento en el número de raíces laterales. Esto sugiere que la disponibilidad de hierro es una característica limitante durante el crecimiento del sistema radicular de *Arabidopsis thaliana*.

Los microorganismos que se asocian a la rizosfera, pueden mejorar el crecimiento de las plantas a través de la suplementación del Fe necesario para el crecimiento y la fotosíntesis (Masalha *et al*., 2000; Rroco *et al.*, 2003). Se han sugerido algunos mecanismos por los que las poblaciones microbianas incrementan la disponibilidad de Fe, incluyendo secreción de sideróforos bacterianos que movilizan el Fe del suelo a las raíces (Masalha *et al*., 2000; Rroco *et al*., 2003), o la transferencia de electrones de su metabolismo para procesos de reducción de Fe (Lovely, 1991), que es la característica de las llamadas bacterias ferrirreductoras entre las que se encuentran algunas especies de *Bacillus, Arthrobacter, Clostridium* y *Pseudomonas* (Lovley, 1993; Ottow y Glathe, 1971).

A. agilis UMCV2 es una bacteria Gram positiva, encontrada comúnmente en el suelo, es aerobia obligada, con forma de bacilo durante la fase de crecimiento exponencial y de coco durante la fase estacionaria. En nuestro grupo de trabajo, Valencia-Cantero *et al*. (2007) aislaron una cepa de *A. agilis* (UMCV2) que al ser inoculadas en *P. vulgaris* promueven el crecimiento vegetal. En trabajos posteriores se reportó que cultivos de *A. agilis* UMCV2 producen compuestos volátiles como la *N*-*N*, dimetil hexadecilamina, y que ésta participa en la activación de mecanismos de respuesta a estrés por deficiencia de Fe en plantas de *M. truncatula* y *S. bicolor*, incluyendo la acidificación del medio y la modulación de la expresión de genes relacionados a adquisición de hierro (genes FRO), lo que facilita la captación de Fe por las raíces y su distribución al sistema aéreo para usarse en procesos como la fotosíntesis (Velázquez-Becerra *et al*., 2011; Orozco-Mosqueda *et al*., 2013; Castulo-Rubio *et al*., 2015). Estos estudios son los primeros en tratar de dilucidar cómo se lleva a cabo la modulación de la acidificación de la rizosfera y la actividad vegetal ferrireductora mediada por compuestos bacterianos volátiles plenamente identificados.

Para entender el papel *in vivo* de la interacción de los compuestos volátiles de *A. agilis* UMCV2 en interacción con *A. thaliana* crecidas en distinta disponibilidad de hierro, en este trabajo exploramos genéticamente la participación de los VOCs producidos por *A. agilis* UMCV2 en el crecimiento y desarrollo vegetal. Para ello utilizamos un sistema de inoculación *in vitro* en cajas Petri divididas que permitieron únicamente una interacción vía compuestos volátiles de la cepa UMCV2 con el ecotipo silvestre Col-0 y las mutantes *pye1* y *bts1* afectadas en estos genes (Long *et al*., 2010; Rampey *et al*., 2006). Analizamos la producción de biomasa, clorofila y cambios en la arquitectura del sistema radicular tanto en condiciones óptimas de hierro como en deficiencia de éste. Se encontró que en condiciones control, la biomasa total y la producción de clorofila incrementan en plantas co-cultivadas con *A. agilis* UMCV2 (Cap. IV, Fig. 2), esto correlaciona con un incremento en el crecimiento de la raíz primaria y en la producción de raíces laterales, dependiente de la participación de *BTS,* ya que la mutante *bts1* al crecer en co-inoculación con *A. agilis* UMCV2 no presentó la estimulación del crecimiento en ninguno de los parámetros utilizados (Cap. IV, Fig. 5). Resulta relevante observar que la inoculación con UMCV2 no estimula los parámetros analizados en condiciones de deficiencia de hierro, lo que sugiere que el efecto promotor de los compuestos volátiles de *A. agilis* UMCV2 está ligado a la disponibilidad de hierro en que se encuentran las plantas (Cap. IV, Figs. 2, 5).

Nuestros resultados concuerdan con reportes previos, donde utilizando los sistemas de interacción *Arabidopsis-B. subtilis* GB03, *M. truncatula-S. melioti*, *N. tabacum-P. fluorescens* SS101 y *S. lycopersicum-B. subtilis* SYST2, se había sugerido que la interacción de los compuestos volátiles producidos por los organismos bacterianos tienen la capacidad de modular el crecimiento en plantas a través de la regulación de mecanismos como la fotosíntesis y la expresión de genes de respuesta a la deficiencia de hierro (Xie *et al*., 2009; Orozco-Mosqueda *et al*., 2013; Park *et al*., 2015; Tahir *et al.*, 2017).

Para explorar más a fondo el mecanismo por el cual *Arabidopsis* responde a los compuestos volátiles de *A. agilis* UMCV2, analizamos la expresión de los genes reporteros *ProPYE:GFP* y *ProBTS:GFP,* que son inducibles en deficiencia

de hierro (Long *et al*., 2010). De manera interesante, observamos un aumento en la fluorescencia en las puntas de la raíz en los tratamientos en interacción con los compuestos volátiles de *A. agilis* UMCV2 desde condiciones control, lo que indica la activación de la ruta de señalización que involucra a PYE y BTS (Cap. IV, Figs. 3, 4). Durante los últimos años, unos pocos trabajos han reportado que algunos microorganismos tienen la capacidad de inducir la activación de respuestas a la deficiencia de hierro como la actividad reductasa, la acidificación de la rizosfera y la expresión de los genes asociados a estas respuestas (Zhang *et al*., 2009; Orozco-Mosqueda *et al*., 2013; Castulo-Rubio *et al*., 2015; Montejano-Ramírez *et al*., 2018; Zhou *et al.*, 2018). Nuestros resultados sugieren que los compuestos volátiles de *A. agilis* UMCV2 pueden modular los mecanismos de respuesta a la deficiencia de hierro y con ello inducir una regulación del crecimiento vegetal, esto sugiere que puede existir una comunicación entre organismos de distintos reinos y ésta puede ser una característica conservada.

Las bacterias tienen la capacidad de producir distintos tipos de compuestos, en nuestro grupo de trabajo, se identificó que *A. agilis* UMCV2 produce *N,N*-dimetil hexadecilamina (C16-DMA), un lípido de cadena principal de 16 carbonos que contiene dos grupos metilo unidos a un nitrógeno en su estructura (Velázquez-Becerra *et al*., 2011). Se ha reportado que algunas otras rizobacterias incluyendo *Bacillus*, *Sinorhizobium* y *Pseudomonas* también lo producen (Liu *et al*., 2008; Orozco-Mosqueda *et al*., 2013; Hernández-León *et al*., 2015). C16-DMA modula el crecimiento y desarrollo de varias especies incluyendo *A. thaliana* donde regula la producción de biomasa, clorofila y la arquitectura del sistema radicular (Velázquez-Becerra *et al*., 2011; Valencia-Cantero *et al.*, 2015; Castulo-Rubio *et al*., 2015; Raya-González *et al*., 2017). Se encontró que en plantas de *S. bicolor*, C16-DMA aumenta la expresión de genes FRO (reductasas férricas) en la raíz, lo que sugiere que C16-DMA tiene un papel importante modulando el estatus nutricional del hierro (Castulo-Rubio *et al*., 2015). En este trabajo, nosotros investigamos el efecto de la C16-DMA sobre la regulación del crecimiento y desarrollo en *A. thaliana* y demostramos que un incremento en las concentraciones de C16-DMA inhibe el crecimiento de la raíz primaria y los genes *PYE* y *BTS* no inducen su

expresión en presencia de este compuesto, lo que excluye su participación en esta respuesta. Sin embargo, es importante mencionar que encontramos que BTS si participa durante la formación de raíces laterales, lo que sugiere que C16-DMA regula programas de desarrollo radicular específicos.

Un tercer componente caracterizado en respuesta a la deficiencia de hierro es *ILR3* (*IAA-LEUCINE RESISTANT 3*), gen que codifica para el factor transcripcional bHLH105, miembro de la familia bHLH (basic Helix-Loop-Helix) (Rampey *et al*., 2006). Las proteínas bHLH comúnmente forman homodímeros o heterodímeros que interactúan regulando genes blanco (Littlewood y Evan, 1998; Toledo-Ortíz *et al*., 2003). Se ha sugerido que ILR3 actúa como intermediario uniéndose directamente a BTS o PYE, teniendo un papel importante mediando la sensibilidad a auxinas inducida por hierro en la raíz de *Arabidopsis* (Rampey *et al*., 2006; Long *et al*., 2010; Zhang *et al*., 2015). Nosotros observamos que la mutante con ganancia de función *ilr3-1*, participa en respuesta a la C16-DMA. Resultó interesante que la respuesta característica de resistencia a la inhibición del crecimiento en condiciones de deficiencia de hierro que presenta esta mutante, se disminuyó en presencia de C16-DMA, sugiriendo que el efecto de este compuesto y la señalización de hierro podrían estar convergiendo en ILR3 para la regulación del crecimiento de la raíz primaria. En conjunto, nuestros resultados nos permiten sugerir que C16-DMA actúa a través de genes blanco, específicos para regular el desarrollo radicular.

La modificación del crecimiento vegetal en respuesta a la disponibilidad espacial y temporal de nutrientes involucra genes que participan en diversos procesos del desarrollo, incluyendo estrategias para una mejor adquisición y mantenimiento de la homeostasis celular de los mismos. Las cepas bacterianas que modifican la capacidad vegetal de asimilación de nutrientes y estimulan los procesos del desarrollo de las plantas, tienen un potencial poco explorado que podría representar una alternativa biotecnológica durante el cultivo de especies de importancia económica. Los resultados obtenidos de este trabajo muestran el papel de los compuestos volátiles de *A. agilis* UMCV2 en la modulación de programas del desarrollo de *A. thaliana*, sugiriendo la existencia de una regulación

muy específica en la comunicación entre organismos de diferentes reinos. Las respuestas a los VOCs en condiciones de distinta disponibilidad de hierro indican niveles de regulación complejos. La producción de compuestos volátiles bacterianos específicos podría estas asociada a la regulación de procesos del desarrollo vegetal específicos, lo que sugiere que la manipulación de cepas bacterianas como *A. agilis* UMCV2, así como de los compuestos volátiles que produce, podría llevar a importantes aplicaciones en biotecnología agrícola para mejorar la captación de agua y nutrientes minerales en las plantas.

Con el presente trabajo, se propone un modelo del impacto de las interacciones ecológicas planta-planta y planta-microorganismo sobre el crecimiento y desarrollo de *Arabidopsis*. En la Figura 17 panel A, podemos observar que conforme aumenta la densidad poblacional, se presenta una señalización de compuestos volátiles que lleva a la inhibición de varios procesos del desarrollo, incluyendo la el crecimiento del sistema radicular y del sistema aéreo a causa de afectaciones en los procesos de diferenciación y división celular, los cuales están regulados por auxinas. En el panel B se puede observar que durante la interacción *Arabidopsis* - *A. agilis*, la cepa bacteriana produce compuestos volátiles que estimulan el crecimiento de Arabidopsis con la participación de *BTS1.* Finalmente el en panel C, se observa que la respuesta de *Arabidopsis* al compuesto puro C16-DMA, producido por *A. agilis* UMCV2 involucra la participación de *ILR3*.

En conjunto podemos concluir que durante la interaccion *A. thaliana-A. thaliana* estimulada por compuestos volátiles, se induce la vías de señalización de las auxinas; mientras que durante las interacciones *A. thaliana-A. agilis* UMCV2 estimulada por compuestos volátiles, y *A. thaliana*-C16 DMA, se induce la activación de la vía de señalización de respuesta a la deficiencia de hierro.

Como perspectiva queda esclarecer si el mecanismo de inducción del crecimiento en Arabidopsis estimulado por los compuestos volátiles bacterianos incluye la percepción y adquisición de hierro. Además el análisis de la participación de subunidades del complejo mediador en las respuestas a condiciones de estés durante la nutrición férrica; así como la caracterización del

mantenimiento del nicho de células fuente en el meristemo radicular de *Arabidopsis* durante estrés por nutrición férrica.

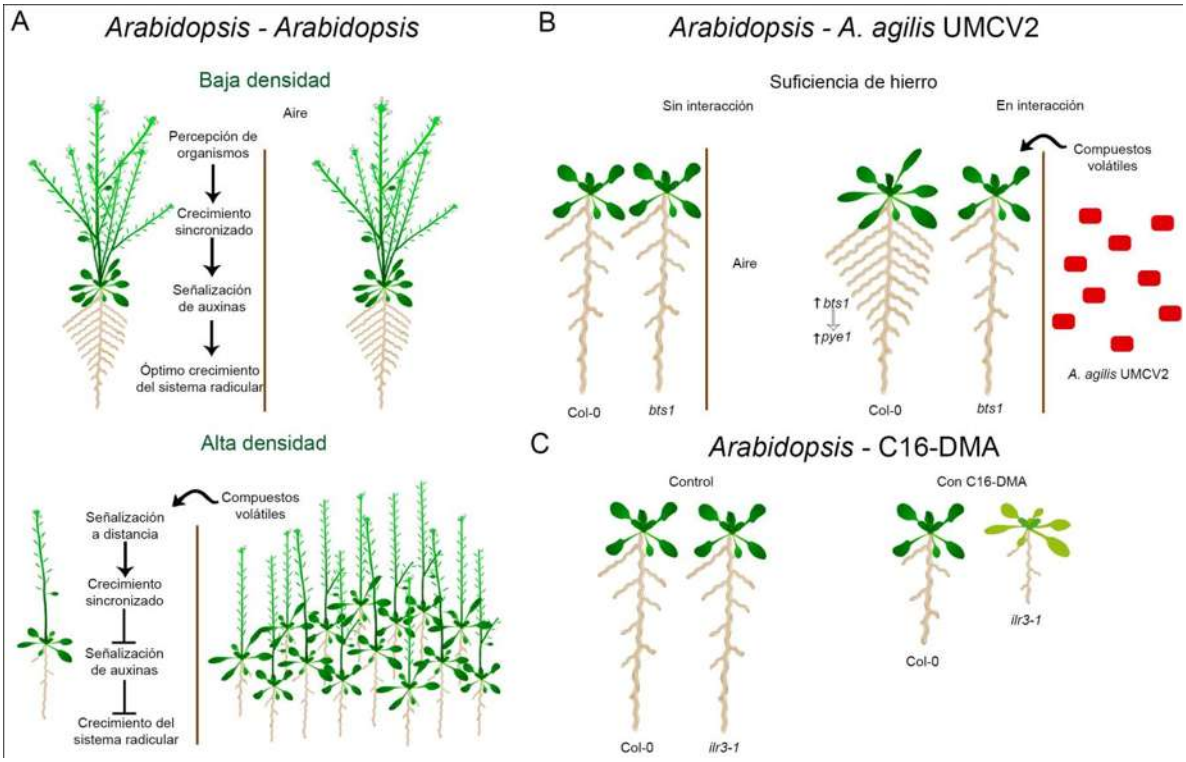

Figura 17. Modelo de las interacciones planta-planta y planta microorganismo mediadas por la percepción de compuestos volátiles. A) En baja densidad de individuos, las plantas presentan un crecimiento óptimo, debido a la escaza presencia de compuestos volátiles que modifiquen el crecimiento; mientras que en alta densidad de individuos se presenta una percepción de compuestos volátiles que regulan patrones del desarrollo sincronizando el crecimiento de plantas vecinas a través de la señalización de auxinas. **B)** Durante la interacción planta-microorganismo mediada por la totalidad de los compuestos volátiles de *A. agilis* UMCV2, se estimula el crecimiento y desarrollo de *Arabidopsis* por un mecanismo regulado por *BTS1* y *PYE1*. **C)** Las respuestas de *Arabidopsis* durante la interacción con C16-DMA, está regulada por *ILR3*, la mutante *ilr3-1* presenta hipersensibilidad al compuesto.

8. REFERENCIAS

Araki T. 2001. Transition from vegetative to reproductive phase. *Current Opinion in Plant Biology* 4: 63-68. DOI: 10.1016/S1369-5266(00)00137-0

Badri DV, Weir TL, van der Lelie D, Vivanco JM. 2009. Rhizosphere chemical dialogues: plant-microbe interactions. *Current Opinion in Biotechnology* 20: 642-650. DOI: 10.1016/j.copbio.2009.09.014

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology* 57: 233-266. DOI: 10.1146/annurev.arplant.57.032905.105159

Balk J, Lobréaux S. 2005. Biogenesis of iron-sulfur proteins in plants. *Trends Plant Sci*ence 10: 324-331. DOI: 10.1016/j.tplants.2005.05.002

Barberi P. 2002. Weed management in organic agriculture, are we addressing the right issues? *Weed Research* 42: 177-193. DOI: 10.1046/j.1365-3180.2002.00277.x

Barker AV, Pilbeam DJ. 2007. Handbook of plant nutrition. CRC Press. DOI: 10.1201/b18458

Bauer P, Ling H-Q, Guerinot ML. 2007. FIT, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in *Arabidopsis*. *Plant Physiology and Biochemistry* 45: 260-261. DOI: 10.1016/j.plaphy.2007.03.006

Bencze KZ, Kondapalli KC, Cook JD, McMahon S, Millán-Pacheco C, Pastor N, Stemmler TL. 2006. The structure and function of frataxin. *Critical Reviews in Biochemistry and Molecular Biology* 41: 269-291. DOI: 10.1080/10409230600846058

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115: 591-602. DOI: 0.1016/S0092-8674(03)00924-3

Bennetzen JL. 2001. *Arabidopsis* arrives. *Nature Genetics* 27: 3-5. DOI: 10.1038/83726

Bennetzen JL. 2002. The rice genome. Opening the door to comparative plant biology. *Science* 296: 60-63. DOI: 10.1126/science.1071402

Bertholdsson NO. 2005. Early vigour and allelopathy-two useful traits for enhanced barley and wheat competitiveness against weeds. *Weed Research* 45: 94-102. DOI: 10.1111/j.1365-3180.2004.00442.x

Biedrzycki ML, Bais HP. 2009. Root secretions: from genes and molecules to microbial associations. *Journal of Experimental Botany* 60: 1533-1534. DOI: 10.1093/jxb/erp105

Bienfait HF, Van Den Briel W, Mesland-Mul NT. 1985. Free space iron pools in roots. *Plant Physiology* 78: 596-600. DOI: 10.1104/pp.78.3.596

Buck MJ, Atchley WR. 2003. Phylogenetic analysis of plant basic Helix-Loop-Helix proteins. *Journal of Molecular Evolution* 56: 742. DOI: 10.1007/s00239-002-2449-3

Callaway RM, Pennings SC, Richards CL. 2003. Phenotypic plasticity and interactions among plants. *Ecology* 84: 1115-1128. DOI: 10.1890/0012-9658(2003)084[1115:PPAIAP]2.0.CO;2

Castulo-Rubio DY, Alejandre-Ramírez N, Orozco-Mosqueda MC, Santoyo G, Macias-Rodríguez LI, Valencia-cantero E. 2015. Volatile organic compounds produced by the

rhizobacterium *Arthrobacter agilis* UMCV2 modulate *Sorghum bicolor* (strategy II plant) morphogenesis and *SbFRO1* transcription in vitro. *Journal of Plant Growth Regulation* 34: 611-623. DOI: org/10.1007/s0034 4-015-9495-8

Caton BP, Cope AE, Mortimer M. 2003. Growth traits of diverse rice cultivars under severe competition, implications for screening for competitiveness. *Field Crops Research* 83: 157-172. DOI: 10.1016/S0378-4290(03)00072-8

Celenza Jr JL, Grisafi PL, Fink GR. 1995. A pathway for lateral root formation in *Arabidopsis thaliana. Genes & Development* 9: 2131-2142. DOI: 10.1101/gad.9.17.2131

Chasteen ND, Harrison PM. 1999. Mineralization in Ferritin: an efficient means of iron storage. *Journal of Structural Biology* 126: 182-194. DOI: 10.1006/jsbi.1999.4118

Colangelo EP, Guerinot ML. 2004. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *The Plant Cell* 16: 3400-3412. DOI: 10.1105/tpc.104.024315

Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML. 2003. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiology* 133: 1102-1110. DOI: 10.1104/pp.103.025122

Craine JM. 2005. Reconciling plant strategy theories of Grime and Tilman. *Journal of Ecology* 93: 1041-1052. DOI: 10.1111/j.1365-2745.2005.01043.x

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL. 2001. Maize *yellow stripe1* encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409: 346-349. DOI: 10.1038/35053080

Curie C, Cassin G, Couch D, Divol F, Higuchi K, Le Jean M, Misson J, Schikora A, Czernic P, Mari S. 2009. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of Botany* 103: 1-11. DOI: 10.1093/aob/mcn207

Curie C, Mari S. 2016. New routes for plant iron mining. *New Phytologist* 214: 521-525. DOI: 10.1111/nph.14364

De Smet S, Cuypers A, Vangronsveld J, Remans T. 2015. Gene networks involved in hormonal control of root development in *Arabidopsis thaliana*: a framework for studying its disturbance by metal stress. *International Journal of Molecular Science* 16: 19195-19224. DOI: 10.3390/ijms160819195

Dicke M. 2009. Behavioural and community ecology of plants that cry for help. *Plant, Cell and Environment* 32: 654-665. DOI: 10.1111/j.1365-3040.2008.01913.x

Dinneny JR, Benfey PN. 2008. Plant stem cell niches: standing the test of time. *Cell* 132: 553-557. DOI: 10.1016/j.cell.2008.02.001.

Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN. 2008. Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. *Science* 320: 942-945. DOI: 10.1126/science.1153795

Dolan L, Janmaat K, Willemsen V, Linstead P, Poethig S, Roberts K, Scheres B. 1993. Cellular organization of the *Arabidopsis thaliana* root. *Development* 119: 71-84. DOI: http://dev.biologists.org/content/119/1/71.long

Durrett TP, Gassmann W, Rogers EE. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology* 144: 197-205. DOI: 10.1104/pp.107.097162

Eide D, Broderius M, Fett J, Guerinot ML. 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Science of the United States of America* 93: 5624-5628. DOI: 10.1073/pnas.93.11.5624

Fargione J, Tilman D. 2006. Plant species traits and capacity for resource reduction predict yield and abundance under competition in nitrogen-limited grassland. *Functional Ecology* 20: 533-540. DOI: 10.1111/j.1365-2435.2006.01116.x

Farley RA, Fitter AH. 1999. Temporal and spatial variation in soil resources in a deciduous woodland. *Journal of Ecology* 87: 688-696. DOI: 10.1046/j.1365-2745.1999.00390.x

Fargione J, Tilman D. 2006. Plant species traits and capacity for resource reduction predict yield and abundance under competition in nitrogen-limited grassland*. Functional Ecology* 20: 533-540. DOI: 10.1111/j.1365-2435.2006.01116.x

Finegold AA, Shatwell KP, Segal AW, Klausner RD, Dancis A. 1996. Intramembrane Bis-Heme motif for transmembrane electron transport conserved in a *Yeast* iron reductase and the human NADPH oxidase. *The Journal of Biological Chemistry* 271: 31021–31024. DOI: 10.1074/jbc.271.49.31021

Forde BG, Walch-Liu P. 2009. Nitrate and glutamate as environmental cues for behavioural responses in plant roots. *Plant, Cell Environment* 32: 682-693. DOI: 10.1111/j.1365-3040.2008.01927.x

Franklin KA, Preaekelt U, Stoddart WM, Billingham OE, Halliday KJ, Whitelam GC. 2003. Phytochromes B, D and E act redundantly to control multiple physiological responses *in Arabidopsis. Plant Physiology* 131: 1340-1346. DOI: 10.1104/pp.102.015487

Gersani M, Abramsky Z, Falik O. 1998. Density-dependent habitat selection in plants. *Evolutionary Ecology* 12: 223-234. DOI: 10.1023/A:1006587813950

Gibson DJ, Conolly J, Hartnett DC, Weidenhamer JD. 1999. Designs for greenhouse studies of interactions between plants. *Journal of Ecology* 87:1-16. DOI: 10.1046/j.1365-2745.1999.00321.x

Gray WM. 2004. Hormonal regulation of plant growth and development. *PLoS Biology* 2: 1270-1273. DOI: 10.1371/journal.pbio.0020311

Green LS, Rogers EE. 2004. FRD3 controls iron localization in *Arabidopsis. Plant Physiology* 136: 2523-2531.DOI: 10.1104/pp.104.045633

Grierson C, Nielsen E, Ketelaar T, Schiefelbein J. 2014. Root Hairs. *The Arabidopsis Book* 12: e0172. DOI: 10.1199/tab.0172

Gross KL, Pregitzer KS, Burton AJ. 1995. Spatial variation in nitrogen availability in three successional plant communities. *Journal of Ecology* 83: 357-368. DOI: 10.2307/2261590

Gurevitch J, Morrow LL, Wallace A, Walsh JS. 1992. A meta-analysis of competition in field experiments. *The American Naturalist* 140 (4): 539-572. DOI: http://www.jstor.org/stable/2462913

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC. 2003. The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Molecular Biology Evolution* 20: 735-747. DOI: 10.1093/molbev/msg088

Hell R, Stephan UW. 2003. Iron uptake, trafficking and homeostasis in plants. *Planta* 216: 541-551. DOI: 10.1007/s00425-002-0920-4

Henriques R, Jásik J, Klein M, Martinoia E, Feller U, Schell J, Pais MS, Koncz C. 2002. Knock-out of *Arabidopsis* metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects. *Plant Molecular Biology* 50: 587. DOI: 10.1023/A:1019942200164

Hernández-León R, Rojas-Solís D, Contreras-Pérez M, Orozco-Mosqueda MC, Macías-Rodríguez LI, Reyes-de la Cruz H, Valencia-Cantero E, Santoyo G. 2015. Characterization of the antifungal and plant growth-promoting effectsof diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains. *Biological Control* 81: 83-92. DOI: 10.1016/j.biocontrol.2014.11.011

Hindt MN, Guerinot ML. 2012. Getting a sense for signals: Regulation of the plant iron deficiency response. *Biochimica et Biophysica Acta* 1823: 1521-1530. DOI: 10.1016/j.bbamcr.2012.03.010.

Hodge A. 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist* 162: 9-24. DOI: 10.1111/j.1469-8137.2004.01015.x

Hodge A. 2006. Plastic plants and patchy soils. *Journal of Experimental Botany* 57: 401-11. DOI: 10.1093/jxb/eri280

Ishikawa H, Evans ML. 1990. Gravity-induced changes in intracellular potentials in elongating cortical cells of mung bean roots. *Plant Cell Physiology* 31: 457-462. DOI: 10.1093/oxfordjournals.pcp.a077932

James SE, Pärtel M, Wilson SD, Peltzer DA. 2003. Temporal heterogeneity of soil moisture in grassland and forest. *Journal of Ecology* 91: 234-239. DOI: 10.1046/j.1365-2745.2003.00758.x

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The European Molecular Biology Organization Journal* 6: 3901-3907. DOI: 10.1002/j.1460-2075.1987.tb02730.x

Jeong J, Cohu C, Kerkeb L, Pilon M, Connolly EL, Guerinot ML. 2008. Chloroplast Fe(III) chelate reductase activity is essential for seedling viability under iron limiting conditions. *Proceedings of the National Academy of Science of the United States of America* 105: 10619-10624. DOI: 10.1073/pnas.0708367105.

Jeong J, Merkovich A, Clyne M, Connolly EL. 2017. Directing iron transport in dicots: regulation of iron acquisition and translocation. *Current Opinion in Plant Biology* 39: 106-113. DOI: 10.1016/j.pbi.2017.06.014

Jin CW, You GY, He YF, Tang C, Wu P, Zheng SJ. 2007. Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic Iron in red clover. *Plant Physiology* 144: 278-285. DOI: 10.1104/pp.107.095794

Jones S. 2004. An overview of the basic helix-loop-helix proteins. *Genome Biology* 5: 226. DOI: 10.1186/gb-2004-5-6-226.

Kegge W, Pierik R. 2010. Biogenic volatile organic compounds and plant competition. *Trends in Plant Science* 15: 126-132. DOI: 10.1016/j.tplants.2009.11.007

Kelvingsong. 2016. File: Into the stele.svg. Water and mineral uptake in roots. *Wikimedia Commons, the free media repository.* URL: commons.wikimedia.org/w/index.php?title=File:A_la_estela.svg&oldid=214548212

Kigathi RN, Weisser WW, Veit D, Gershenzon J, Unsicker SB. 2013. Plants suppress their emission of volatiles when growing with conspecifics. *Journal of Chemical Ecology* 39: 537-545. DOI: 10.1007/s10886-013-0275-2

Kim SA, Punshon T, Lanzirotti A, Li L, Alonso JM, Ecker JR, Kaplan J, Guerinot ML. 2006. Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1. *Science* 314: 1295-1298. DOI: 10.1126/science.1132563

Kim SA, Guerinot ML. 2007. Mining iron: iron uptake and transport in plants. *Federation of European Microbiological Societies Letters* 581: 2273-2280. DOI: 10.1016/j.febslet.2007.04.043

Kispal G, Csere P, Guiard B, Lill R. 1997. The ABC transporter Atm1p is required for mitochondrial iron homeostasis. *FEBS Letters* 418: 346-350. DOI: 10.1016/S0014-5793(97)01414-2

Knezevic SZ, Evans SP, Blankenship EE, Van Acker RC, Lindquist JL. 2002. Critical period for weed control, the concept and data analysis. Weed Science 50: 773-786. DOI: 10.1614/0043-1745(2002)050[0773:CPFWCT]2.0.CO;2

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB. 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Molecular Biology* 40: 37. DOI: 10.1023/A:1026438615520

Kovtun Y, Chiu WL, Tena G, Sheen J. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proceedings of the National Academy of Sciences of the United States of America* 97: 2940-2945. DOI: 10.1073/pnas.97.6.2940

Lanquar V, Lelièvre F, Bolte S, Hamès C, Alcon C, Neumann D, Vansuyt G, Curie C, Schröder A, Krämer U, Barbier-Brygoo H, Thomine S. 2005. Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO Journal* 24: 4041-4051. DOI: 10.1038/sj.emboj.7600864

Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM. 1995. Formation of lateral root meristems is a two stage process. *Development* 121: 3303-3310. DOI: http://dev.biologists.org/content/121/10/3303.long

Lei GJ, Zhu XF, Wang ZW, Dong F, Dong NY, Zheng SJ. 2014. Abscisic acid alleviates iron deficiency by promoting root iron reutilization and transport from root to shoot in *Arabidopsis*. *Plant, Cell and Environment* 37: 852-863. DOI: 10.1111/pce.12203

Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P, Drysdale J. 2001. A human mitochondrial ferritin encoded by an intronless gene. *The Journal of Biological Chemistry* 276: 24437-24440. DOI: 10.1074/jbc.C100141200

Liang G, Zhang H, Li X, Ai Q, Yu D. 2017. bHLH transcription factor bHLH115 regulates iron homeostasis in *Arabidopsis thaliana*. *Journal of Experimental Botany* 68: 1743-1755. DOI: 10.1093/jxb/erx043.

Littlewood T, Evan GI. 1998. Helix-Loop-Helix Transcription Factors, 3rd ed. (New York: Oxford University Press).

Liu W, Wei M, Bingyu Z, Feng L. 2008. Antifungal activities and components of VOCs produced by *Bacillus subtilis* G8. *Current Research in Bacteriology* 1: 28-34. DOI: 10.3923/crb.2008.28.34

Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN. 2010. The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. *The Plant Cell* 22: 2219-2236. DOI: 10.1105/tpc.110.074096

López-Bucio J, Nieto Jacobo MF, Ramírez Rodríguez V, Herrera Estrella L. 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. *Plant Science* 160: 1-13. DOI: 10.1016/S0168-9452(00)00347-2

López-Bucio J, Hernández-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J, Herrera-Estrella L. 2002. Phosphate availability alters architecture and causes changes in hormone sensitivity in the *Arabidopsis* root system. *Plant Physiology* 129: 244-256. DOI: 10.1104/pp.010934

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L. 2003. The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology* 6: 280-287. DOI: 10.1016/S1369-5266(03)00035-9

Lovley DR. 1991. Dissimilatory Fe(II) and Mn(IV) reduction. *Microbiological Reviews* 55: 259-287. DOI: 10.1016/S0065-2911(04)49005-5

Lovley DR. 1993. Dissimilatory metal reduction. *Annual Reviews in Microbiology* 47: 236-290. DOI: 10.1146/annurev.mi.47.100193.001403

Maathuis FJ. 2009. Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology* 12: 250-258. DOI: 10.1016/j.pbi.2009.04.003

Maathuis FJ, Diatloff E. 2013. Roles and functions of plant mineral nutrients. *Methods in Molecular Biology* 953: 1-21. DOI: 10.1007/978-1-62703-152-3_1.

Malamy JE, Benfey PN. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana. Development* 124: 33-44. DOI: http://dev.biologists.org/content/124/1/33.long

Marschner P. 2012. Mineral nutrition of higher plants. 3ra. ed. Academic Press, London, UK. DOI: 10.1016/C2009-0-63043-9

Martínez-Trujillo M, Méndez-Bravo A,·Ortiz-Castro R, Hernández-Madrigal F, Ibarra-Laclette E, Ruiz-Herrera LF. 2014. Chromate alters root system architecture and activates expression of genes involved in iron homeostasis and signaling in *Arabidopsis thaliana. Plant molecular biology* 86: 35-50. DOI: 10.1007/s11103-014-0210-0·

Masalha J, Kosegarten H, Elmaci O, Mengel K. 2000. The central role of microbial activity for iron acquisition in maize and sun flower. *Biology and Fertility of Soils* 30: 433-439. DOI: 10.1007/s003740050021

Masclaux FG, Bruessow F, Schweizer F, Gouhier-Darimont C, Keller L, Reymond P. 2012. Transcriptome analysis of intraspecific competition in *Arabidopsis thaliana* reveals organ-specific signatures related to nutrient acquisition and general stress response pathways. *BMC Plant Biology* 12: 227. DOI: 10.1186/1471-2229-12-227

Missirlis F, Holmberg S, Georgieva T, Dunkov BC, Rouault TA, Law JH. 2006. Characterization of mitochondrial ferritin in *Drosophila*. *Proceedings of the National Academy of Science of the United States of America* 103: 5893-5898. DOI: 10.1073/pnas.0601471103

Montejano−Ramírez V, Martínez−Cámara R, García−Pineda E, Valencia−Cantero E. 2018. Rhizobacterium *Arthrobacter agilis* UMCV2 increases organ-specific expression of FRO genes in conjunction with genes associated with the systemic resistance pathways of

Medicago truncatula. Acta Physiologiae Plantarum 40: 138. DOI: 10.1007/s11738-018-2712-x

Morrisey J, Guerinot ML. 2009. Iron uptake and transport in plants: the good, the bad and the ionome. *Chemical review* 109: 4553-4567. DOI: 10.1021/cr900112r

Muller M, Schmidt W. 2004. Environmentally induced plasticity of root hair development in *Arabidopsis. Plant physiology* 134: 409-419. DOI: 10.1104/pp.103.029066

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

Nagasaka S, Takahashi M, Nakanishi-Itai R, Bashir K, Nakanishi H, Mori S, Nishizawa NK. 2009. Time course analysis of gene expression over 24 hours in Fe-deficient barley roots. *Plant Molecular Biology* 69: 621-631. DOI: 10.1007/s11103-008-9443-0

Narasimhan K, Basheer C, Bajic VB, Swarup S. 2003. Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. *Plant Physiology* 132: 146-153. DOI: 10.1104/pp.102.016295

Negishi T, Nakanishi H, Yazaki J, Kishimoto N, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T, Kikuchi S, Mori S, Nishizawa NK. 2002. cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore secretion in Fe-deficient barley roots. *The Plant Journal* 30: 83-94. DOI: 10.1046/j.1365-313X.2002.01270.x

Ni HW, Zhang CX. 2005. Use of allelopathy for weed management in China-a review. *Allelopathy Journal* 15: 3-11.

Novoplansky A. 2009. Picking battles wisely: plant behaviour under competition. *Plant, Cell and Environment 3*2: 726–741. DOI: 10.1111/j.1365-3040.2009.01979.x

Oh YJ, Kim H, Seo SH, Hwang BG, Chang YS, Lee J, Lee DW, Sohn EJ, Lee SJ, Lee Y, Hwang I. 2016. Cytochrome *b*5 reductase 1 triggers serial reactions that lead to iron uptake in plants. *Molecular Plant* 9: 501-513. DOI: 10.1016/j.molp.2015.12.010.

Okada K, Shimura Y. 1990. Reversible root tip rotation in *Arabidopsis* seedlings induced by obstacle-touching stimulus. *Science* 250: 274-276. DOI: 10.1126/science.250.4978.274

Orozco-Mosqueda MC, Velázquez-Becerra C, Macías-Rodríquez LI, Santoyo G, Flores-Corez I, Alfaro-Cuevas R, Valencia-Cantero E. 2013. *Arthrobacter agilis* UMCV2 induces iron acquisition in *Medicago truncatula* (strategy I plant) in vitro via dimethylhexadecylamine emission. *Plant and Soil* 362: 51-66. DOI: 10.1007/s1110 4-012-1263-y

Ortíz-Castro R, Valencia-Cantero E, López-Bucio J. 2008. Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. *Plant Signaling & Behavior* 3: 263-265. DOI: 10.4161/psb.3.4.5204

Ortiz-Castro R, Díaz-Pérez C, Martínez-Trujillo M, del Río RE, Campos-García J, López-Bucio J. 2011. Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. Proceedings of the National Academy of Science of the United States of America 108: 7253-7258. DOI: 10.1073/pnas.1006740108

Ottenschläger I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K. 2003. Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proceedings of the National Academy of Sciences of the United States of America* 100: 2987-2991. DOI: 10.1073/pnas.0437936100

Ottow JCG, Glathe H. 1971. Isolation and identifcation of iron-reducing bacteria from gley soils. *Soil Biology and Biochemistry* 3: 43-55. DOI: 10.1016/0038-0717(71)90030-7

Park YS, Dutta S, Ann M, Raaijmakers JM, Park K. 2015. Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochemical and Biophysical research communications* 461: 361-365. DOI: 10.1016/j.bbrc.2015.04.039

Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplaze L, Beeckman T, Bennett MJ. 2009. *Arabidopsis* lateral root development: an emerging story. *Trends in Plant Science* 14: 399-408. DOI: 10.1016/j.tplants.2009.05.002

Petit JM, Briat JF, Lobréaux S. 2001. Structure and differential expression of the four members of the *Arabidopsis thaliana* ferritin gene family. *Biochemical Journal* 359: 575-582. DOI: 10.1042/bj3590575

Petricka JJ, Winter CM, Benfey PN. 2012. Control of *Arabidopsis* root development. *Annual Review of Plant Biology* 63: 563-590. DOI: 10.1146/annurev-arplant-042811-105501

Pii Y, Borruso L, Brusetti L, Crecchio C, Cesco S, Mimmo T. 2016. The interaction between iron nutrition, plant species and soil type shapes the rhizosphere microbiome. *Plant Physiology and Biochemistry* 99: 39-48. DOI: 10.1016/j.plaphy.2015.12.002

Pires N, Dolan L. 2009. Origin and diversification of basic-helix-loop-helix proteins in plants. *Molecular Biology and Evolution* 27: 862-874. DOI: 10.1093/molbev/msp288

Ramírez-Rodríguez V, López Bucio J, Herrera Estrella L. 2005. Adaptive responses in plants to non optimal soil pH. In: Jenks MA, Hasegawa PM (eds) Plant abiotic stress. Blackwell Plant Science Series, Oxford, UK, pp 145-170. DOI: 10.1002/9780470988503.ch6

Rampey RA, Woodward AW, Hobbs BN, Tierney MP, Lahner B, Salt DE, Bartel B. 2006. An *Arabidopsis* basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. *Genetics* 174: 1841-1857. DOI: 10.1534/genetics.106.061044

Rao KVM, Raghavendra AS, Reddy KJ. 2006. Physiology and molecular biology of stress tolerance in plants. Springer Science & Business Media DOI: https://www.springer.com/la/book/9781402042249

Ravet K, Touraine B, Boucherez J, Briat JF, Gaymard F, Cellier F. 2009. Ferritins control interaction between iron homeostasis and oxidative stress in *Arabidopsis*. *The Plant Journal* 57: 400-12. DOI: 10.1111/j.1365-313X.2008.03698.x.

Raya-González J, Ortiz-Castro R, Ruíz-Herrera LF, Kazan K, López-Bucio J. 2014. PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 regulates lateral root formation via auxin signaling in *Arabidopsis. Plant Physiology* 165: 1-15. DOI: 10.1104/pp.114.239806

Raya-González J, Velázquez-Becerra C, Barrera-Ortíz 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: 1399-1410. DOI: org/10.1007/s0070 9-016-1031-6

Robinson NJ, Procter CM, Connolly EL, Guerinot ML. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* 397: 694-697. DOI: 10.1038/17800

Rroco E, Kosegarten H, Harizaj F, Imani J, Mengel K. 2003. The importance of soil microbial activity for the supply of iron to sorghum and rape. *European Journal of Agronomy* 19: 487-493. DOI: 10.1016/S1161-0301(02)00185-5

Rudrappa T, Czymmek KJ, Paré PW, Bais HP. 2008. Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* 148: 1547-1556. DOI: 10.1104/pp.108.127613

Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Pare PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis. Plant Physiology* 134: 1017-1026. DOI: 10.1104/pp.103.026583

Salahudeen AA, Thompson JW, Ruiz JC, Ma HW, Kinch LN, Li Q, Grishin NV, Bruick RK. 2009. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* 326: 722-726. DOI: 10.1126/science.1176326

Santambrogio P, Biasiotto G, Sanvito F, Olivieri S, Arosio P, Levi S. 2007. Mitochondrial ferritin expression in adult mouse tissues. *The Journal of Histochemistry and Cytochemistry* 55: 1129-1137. DOI: 10.1369/jhc.7A7273.2007

Santi S, Cesco S, Varanini Z, Pinton R. 2005. Two plasma membrane H(+)-ATPase genes are differentially expressed in iron-deficient cucumber plants. *Plant Physiology and Biochemistry* 43: 287-292. DOI: 10.1016/j.plaphy.2005.02.007

Santi S, Schmidt W. 2009. Dissecting iron deficiency-induced proton extrusion in *Arabidopsis* roots. *New Phytologist* 183: 1072-1084. DOI: 10.1111/j.1469-8137.2009.02908.x

Schaaf G, Honsbein A, Meda AR, Kirchner S, Wipf D, von Wiren NJ. 2006. AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in *Arabidopsis thaliana* roots. *The Journal of Biological Chemistry* 281: 25532-25540. DOI: 10.1074/jbc.M601062200

Schenk HJ. 2006. Root competition: beyond resource depletion. *Journal of Ecology* 94: 725-739. DOI: 10.1111/j.1365-2745.2006.01124.x

Scheres B, Wolkenfelt H, Willemsen V, Terlouw M, Lawson E, Dean C, Weisbeek P. 1994. Embryonic origin of the *Arabidopsis* primary root and root meristem initials. *Development* 120: 2475-2487. DOI: http://dev.biologists.org/content/120/9/2475.short

Scheres B. y H. Wolkenfelt. 1998. The *Arabidopsis* root as a model to study plant development. *Plant Physiology and Biochemistry* 36: 21-32. DOI: 10.1016/S0981-9428(98)80088-0

Scheres B, Benfey P, Dolan L. 2002. Root development. *The Arabidopsis Book* 1: e0101. DOI: 10.1199/tab.0101

Schiefelbein JW, Masucci JD, Wang H. 1997. Building a root: the control of patterning and morphogenesis during root development. *The Plant Cell* 9: 1089-1098. DOI: 10.1105/tpc.9.7.1089

Schmidt W, Michalke W, Schikora A. 2003. Proton pumping by tomato roots. Effect of Fe deficiency and hormones on the activity and distribution of plasma membrane H+-ATPase in rhizodermal cells. *Plant Cell & Environment* 26: 361. DOI: 10.1046/j.1365-3040.2003.00967.x

Seem JE, Creamer NG, Monks DW. 2003. Critical weed-free period for 'Beauregard' sweetpotato (Ipomoea batatas). *Weed Technology* 17: 686- 695.DOI: 10.1614/WT02-089

Selote D, Samira R, Matthiadis A, Gillikin JW, Long TA. 2015. Iron-binding E3 ligase mediates iron response in plants by targeting basic helix-loop-helix transcription factors. *Plant Physiology* 167: 273-286. DOI: 10.1111/pce.13363

Shennan C. 2008. Biotic interations, ecological knowledge and agriculture. *Philosophical trasactions of the royal society B* 363: 717-739. DOI: 10.1098/rstb.2007.2180

Shi S, Nuccio EE, Shi ZJ, He Z, Zhou J, Firestone MK. 2016. The interconnected rhizosphere: High network complexity dominates rhizosphere assemblages. *Ecology Letters* 19: 926-936. DOI: 10.1111/ele.12630

Sinkkonen A. 2001. Density-dependent chemical interference - an extension of the biological response model. *Journal of Chemical Ecology* 27: 1513-1523. DOI: 10.1023/A:1010329612753

Smalla K, Sessitsch A, Hartmann A. 2006. The rhizosphere: 'soil compartment influenced by the root'. *FEMS Microbiology Ecology* 56: 165. DOI: 10.1111/j.1574-6941.2006.00148.x

Stookey LL. 1970. Ferrozine-a new spectrophotometric reagent for iron. *Analytical chemistry* 42: 779-781. DOI: 10.1021/ac60289a016

Taiz L, Zeiger E. 2002. Plant Physiology. 3rd Ed. Sinauer Associates Inc.. 690 pp. DOI: 10.1093/aob/mcg079

Tahir HAS, Gu Q, Wu H, Raza W, Hanif A, Wu L, Colman MV, Gao X. 2017. Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. *Frontiers in Microbiology* 8: 171. DOI: 10.3389/fmicb.2017.00171

Takahashi M, Nakanishi H, Kawasaki S, Nishizawa NK, Mori S. 2001. Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. *Nature Biotechnology* 19: 466-469. DOI: 10.1038/88143

Teale WD, Paponov IA, Palme K. 2006. Auxin in action: signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology* 7: 847-859. DOI: 10.1038/nrm2020

Terry N, Abadía J. 1986. Function of iron in chloroplasts. *Journal of Plant Nutrition* 9: 609-646. DOI: 10.1080/01904168609363470

Toledo-Ortiz G, Huq E, Peter H. 2003. The *Arabidopsis* basic/Helix-Loop-Helix transcription factor family. *The Plant Cell* 15: 1749-1770. DOI: 10.1105/tpc.013839

Trewavas A. 2009. What is plant behavior? *Plant, Cell and Envioronment* 32: 606-616. DOI: 10.1111/j.1365-3040.2009.01929.x

Valencia-Cantero E, Hernández-Calderón E, Velázquez-Becerra C, López-Meza JE, Alfaro-Cuevas R, López-Bucio J. 2007- Role of dissimilatory fermentative Fe-reducing bacteria in Fe uptake by common bean (*Phaseolus vulgaris* L.) plants grown in alkaline soil. *Plant and Soil* 291: 263-273. DOI: 10.1007/s11104-007-9191-y

Valencia-Cantero E, Flores-Cortez I, Ambriz-Parra J, Lopez-Albarran P, Velázquez-Becerra C. 2015. *Arthrobacter agilis* UMCV2 accelerates growth of *Pinus devoniana*. Phyton-Int J Exp Bot 84: 64-69. DOI: http://ppct.caicyt.gov.ar/index.php/phyton/article/view/8110

Valladares F, Gianoli E, Gómez JM. 2007. Ecological limits to plant phenotypic plasticity. *New Phytologist* 176: 749-763. DOI: 10.1111/j.1469-8137.2007.02275.x

Vashisht AA, Zumbrennen KB, Huang X, Powers DN, Durazo A, Sun D, Bhaskaran N, Persson A, Uhlen M, Sangfelt O, Spruck C, Leibold EA, Wohlschlegel JA. 2009. Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science* 326: 718-721. DOI: 10.1126/science.1176333

Velázquez-Becerra C, Macías-Rodríguez LI, López-Bucio J, Altamirano-Hernández J, Flores-Cortez I, Valencia-Cantero E. 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis *in vitro*. *Plant and Soil* 339: 329-340. DOI: 10.1007/s1110 4-010-0583-z

Velázquez-Becerra C, Macías-Rodríguez LI, López-Bucio J, Flores-Cortez I, Santoyo G, Hernández-Soberano C, Valencia-Cantero E. 2013. The rhizobacterium *Arthrobacter agilis* produces dimethylhexadecylamine, a compound that inhibits growth of phytopathogenic fungi *in vitro*. *Protoplasma* 250: 1251-1262. DOI: 10.1007/s00709-013-0506-y

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat JF, Curie C. 2002. IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell* 14: 1223-1233. DOI: 10.1105/tpc.001388

Vigani G, Zocchi G, Bashir K, Philippar K, Briat JF. 2013. Signals from chloroplasts and mitochondria for Fe homeostasis regulation. *Trends in Plant Science* 18: 305-311. DOI:10.1016/j.tplants.2013.01.006

von Wiren N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC. 1999. Nicotianamine chelates both Fe(III) and Fe(II). Implications for metal transport in plants. *Plant Physiol*ogy 119:1107-1114. DOI: 10.1104/pp.119.3.1107

Walker TS, Bais HP, Déziel E, Schweizer HP, Rahme LG, Fall R, Vivanco JM. 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiology* 134: 320-331. DOI: 10.1104/pp.103.027888

Walters CM, Bassler BL. 2005. *Quorum sensing:* cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* 21:319-346. DOI: 10.1146/annurev.cellbio.21.012704.131001

Weidenhamer JD, Hartnett DC, Romeo JT. 1989. Density-dependent phytotoxicity: distinguishing resource competition and allelopathic interference in plants. *Journal of Applied Ecology* 26: 613-624. DOI: 10.2307/2404086

Weiner J. 2003. Ecology-the science of agriculture in the 21st century. *The Journal of Agricultural Science* 141: 371-377. DOI: 10.1017/S0021859603003605

Woodward AW, Bartel B. 2005. Auxin: regulation, action, and interaction. *Annals of Botany* 95: 707-735. DOI: 10.1093/aob/mci083

Wu H, Li L, Du J, Yuan Y, Cheng X, Ling H-Q. 2005. Molecular and biochemical characterization of the Fe (III) chelate reductase gene family in *Arabidopsis thaliana*. *Plant and Cell Physiology* 46: 1505-1514. DOI: 10.1093/pcp/pci163

Xie X, Zhang H, Paré PW. 2009. Sustained growth promotion in *Arabidopsis* with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03). *Plant Signaling & Behavior* 4: 948-953. DOI: 10.4161/psb.4.10.9709

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling H-Q. 2008. FIT interacts with *AtbHLH38* and *AtbHLH39* in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis. Cell Research* 18: 385-397. DOI: 10.1038/cr.2008.26.

Zhang H, Sun Y, Xie X, Kim MS, Dowd SE, Paré PW. 2009. A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. *The Plant Journal* 58: 568-577. DOI: 10.1111/j.1365-313X.2009.03803.x

Zhang J, Liu B, Li M, Feng D, Jin H, Wang P, Liu J, Xiong F, Wang J, Wang HB. 2015. The bHLH transcription factor bHLH104 interacts with IAA-LEUCINE RESISTANT3 and modulates iron homeostasis in *Arabidopsis*. *The Plant Cell* 27: 787-805. DOI: 10.1105/tpc.114.132704

Zhou C, Zhu L, Guo J, Xiao X, Ma Z, Wang J. 2018. *Bacillus subtilis* STU6 ameliorates iron deficiency in tomato by enhancement of polyamine-mediated iron remobilization. *Journal of Agricultural and Food Chemistry*. DOI: 10.1021/acs.jafc.8b05851

Zhou J, Dong BC, Alpert P, Li HL, Zhang MX, Lei GC, Yu FH. 2012. Effects of soil nutrient heterogeneity on intraspecific competition in the invasive, clonal plant *Alternanthera philoxeroides. Annals of Botany* 109: 813-818. DOI: 10.1093/aob/mcr314

9. ADDENDA

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

Artículos publicados con arbitraje internacional:

1. Garnica-Vergara A., Barrera-Ortiz S., **Muñoz-Parra E.**, Raya-González J., Méndez-Bravo A., Macías-Rodríguez L., Ruíz-Herrera LF., 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. DOI: 10.1111/nph.13725

2. Mendoza-Vázquez MA, Espinoza-Madrigal RM, **Muñoz-Parra E**, Flores-García A, Huerta-Venegas PI, Ruiz-Herrera LF, Martínez-Pacheco M, López-Bucio J. 2019. Growth and development of *Arabidopsis thaliana* seedlings in interaction with fungi isolated from stem-end rot of avocado fruits. *Archives of Phytopathology and Plant Protection*. DOI: 10.1080/03235408.2018.1553473

Artículos publicados con arbitraje nacional

1. Pelagio-Flores R., **Muñoz-Parra E.**, Ortiz-Castro R., López-Bucio J. (2017). Regulación del desarrollo vegetal por indolaminas. *Biotecnología y sustentabilidad* 2 (1): 107-114. ISSN: 2448-7562

Capítulo de Libro

1. García-Cárdenas E., **Muñoz-Parra E.,** López-Bucio J, Beltrán-Peña E. 2019. Capítulo II. Las auxinas: hormonas que coordinan el crecimiento y desarrollo de las plantas y su interacción con el ambiente. En el libro Fronteras en la biología: señalización y comunicación de las plantas. ISBN: 978-607-542-028-8

Research

New Phytologist

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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Key words: 6-pentyl-2*H*-pyran-2-one (6-PP), auxin, ethylene (ET), phytostimulation, root development, *Trichoderma*.

Summary

- Plants interact with root microbes via chemical signaling, which modulates competence or symbiosis. Although several volatile organic compounds (VOCs) from fungi may affect plant growth and development, the signal transduction pathways mediating VOC sensing are not fully understood.
- 6-pentyl-2*H*-pyran-2-one (6-PP) is a major VOC biosynthesized by *Trichoderma* spp. which is probably involved in plant–fungus cross-kingdom signaling. Using microscopy and confocal imaging, the effects of 6-PP on root morphogenesis were found to be correlated with *DR5:GFP*, *DR5:VENUS*, *H2B::GFP*, *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* gene expression. A genetic screen for primary root growth resistance to 6-PP in wild-type seedlings and auxin- and ethylene-related mutants allowed identification of genes controlling root architectural responses to this metabolite.
- *Trichoderma atroviride* produced 6-PP, which promoted plant growth and regulated root architecture, inhibiting primary root growth and inducing lateral root formation. 6-PP modulated expression of *PIN* auxin-transport proteins in a specific and dose-dependent manner in primary roots. *TIR1*, *AFB2* and *AFB3* auxin receptors and *ARF7* and *ARF19* transcription factors influenced the lateral root response to 6-PP, whereas *EIN2* modulated 6-PP sensing in primary roots.
- These results indicate that root responses to 6-PP involve components of auxin transport and signaling and the ethylene-response modulator *EIN2*.

Introduction

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

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

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

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

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

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

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L., Heynh.) Columbia (Col-0) ecotype, the transgenic *A. thaliana* lines *DR5:GFP* (Ottenschläger *et al.*, 2003), *DR5:VENUS* (Brunoud *et al.*, 2012); *H2B::RFP* (Boisnard-Lorig *et al.*, 2001); *CycB1:uidA* (Colón-Carmona *et al.*, 1999); *PIN1::PIN1::GFP* (Benkova *et al.*, 2003), *PIN2::PIN2::GFP* (Blilou *et al.*, 2005), *PIN3::PIN3::GFP* (Žádníková *et al.*, 2010) and *PIN7::PIN7::GFP* (Blilou *et al.*, 2005) and the mutant lines *axr1-3* (Lincoln *et al.*, 1990), *aux1-7* (Picket *et al.*, 1990), *tir1/afb2/afb3* (Parry *et al.*, 2009), *arf7-1/arf19-1* (Wilmoth *et al.*, 2005), *eir1* (Roman *et al.*, 1995), *etr1* (Hua & Meyerowitz, 1998), *ein2* (Guzmán & Ecker, 1990), and *ein3* (Chao *et al.*, 1997) were used for the different experiments. Seeds were surface-sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2 × Murashige and Skoog (MS) medium (Murashige and Skoog basal salts mixture). The MS medium was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL (Grand Island, NY, USA). Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L; Percival Scientific, Perry, IA, USA), with a photoperiod of 16 h : 8 h, light : dark, a light intensity of 300 μmol m^2 s^{-1}, and a temperature of 22°C.

Fungal growth and plant inoculation experiments

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

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

Effect of 6-PP on plant growth and development

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

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

Propidium iodide staining and GFP, VENUS and RFP detection

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

Determination of developmental stages of lateral root primordia

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

Analysis of VOCs and 6-PP determinations

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

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

Data analyses

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

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

Results

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

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

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

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

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

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

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

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

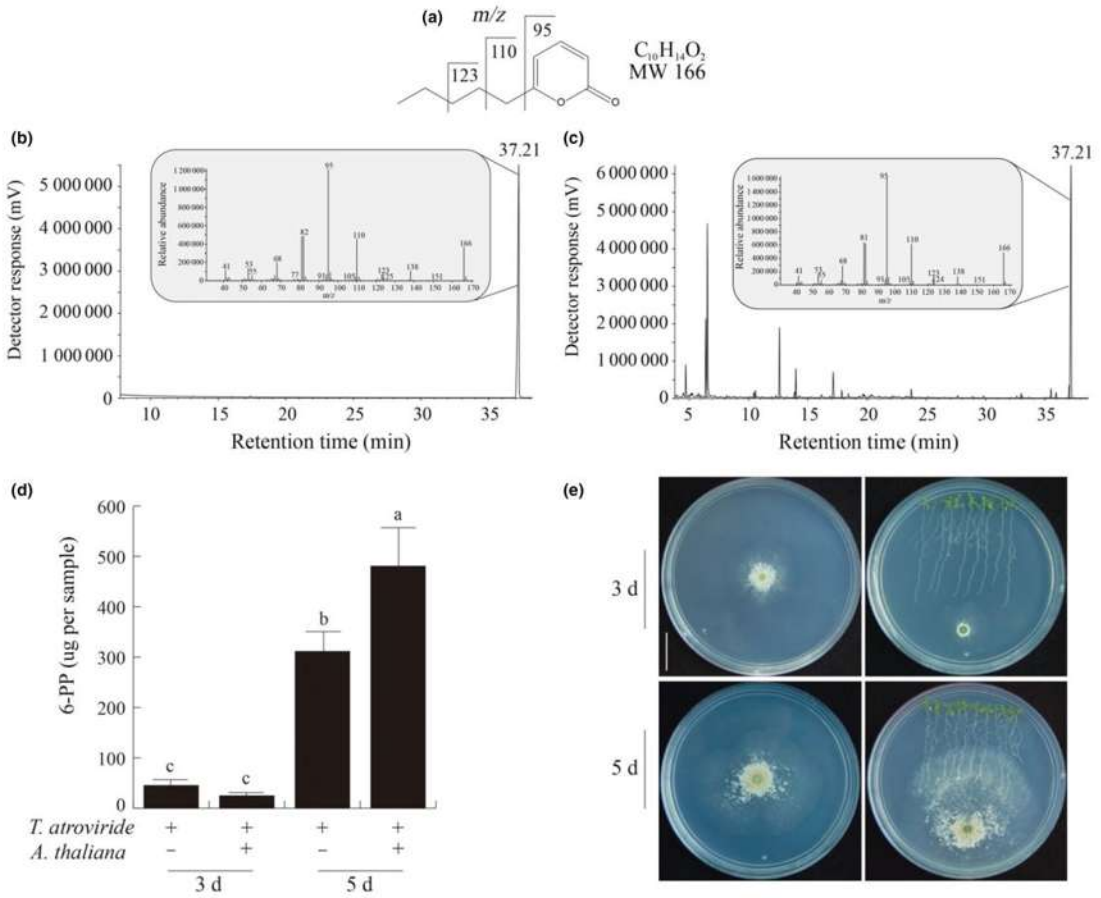

Fig. 1 Molecular characterization and production of 6-pentyl-2*H*-pyran-2-one (6-PP) by *Trichoderma atroviride* IMI 206040. (a) Chemical structure of 6-PP showing the major fragment ions (*m/z*) of electron ionization mass spectra. (b) Total ion chromatogram and mass spectra from commercial standard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

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

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

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

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

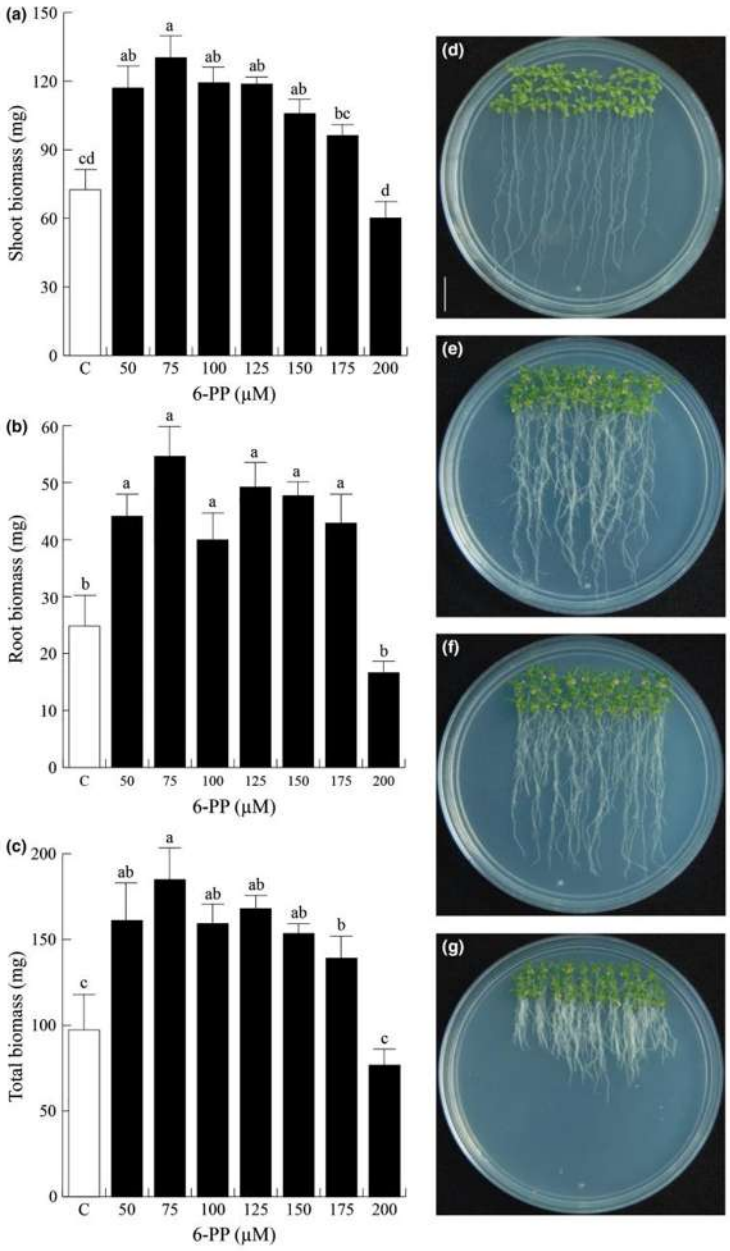

Fig. 2 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on plant biomass production. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 12 d under increasing 6-PP concentrations. (a) Shoot biomass. (b) Root biomass. (c) Total biomass. (d) Representative photographs of seedlings grown in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

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

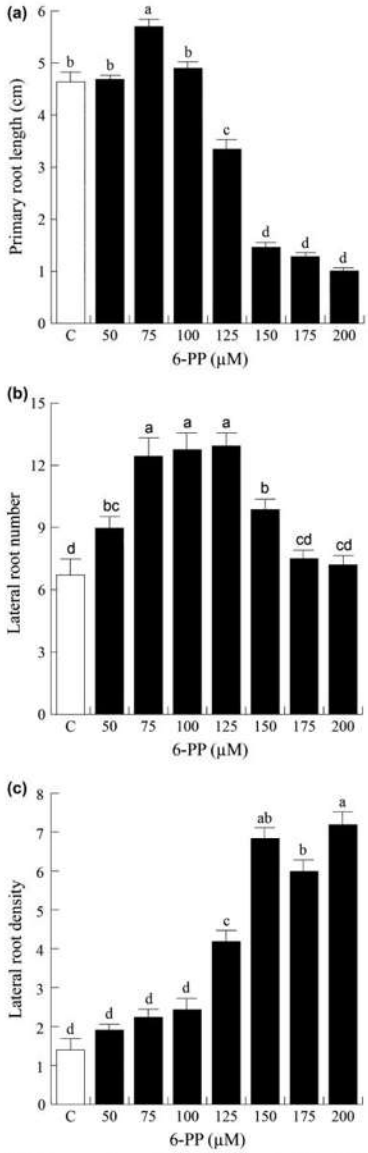

Fig. 3 6-pentyl-2*H*-pyran-2-one (6PP) regulates *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a) Primary root length. (b) Number of emerged lateral roots. (c) Lateral root density (number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

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

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

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

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

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

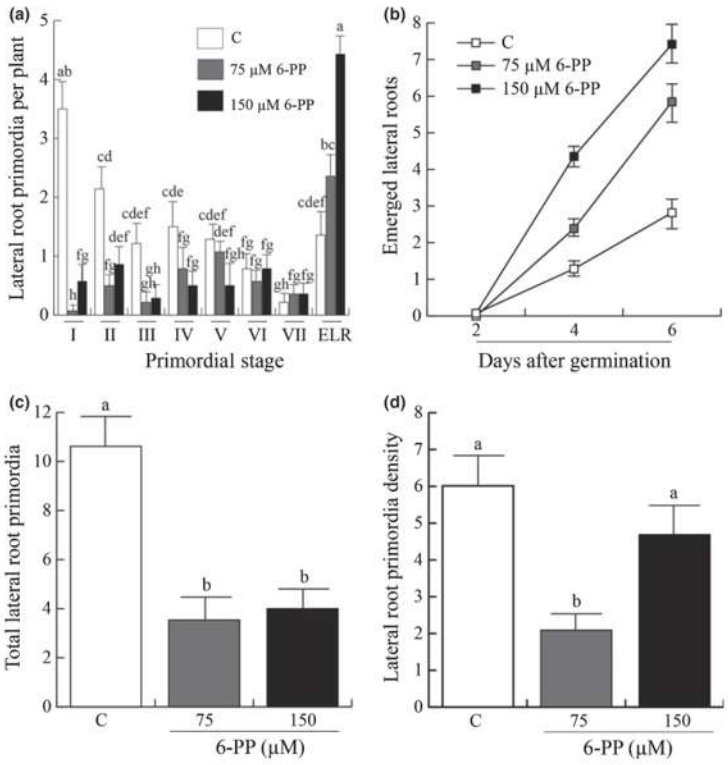

Fig. 4 Effect of 6-pentyl-2*H*-pyran-2-one (6PP) on lateral root (LR) development in *Arabidopsis thaliana*. Wild-type (Col-0) seedlings were germinated and grown for 2, 4 and 6 d on 0.2 × MS media supplemented with the solvent (control (C)), or 75 or 150 μM 6-PP. (a) Lateral root primordia (LRPs) per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

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

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

6-PP fails to induce an ethylene response

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

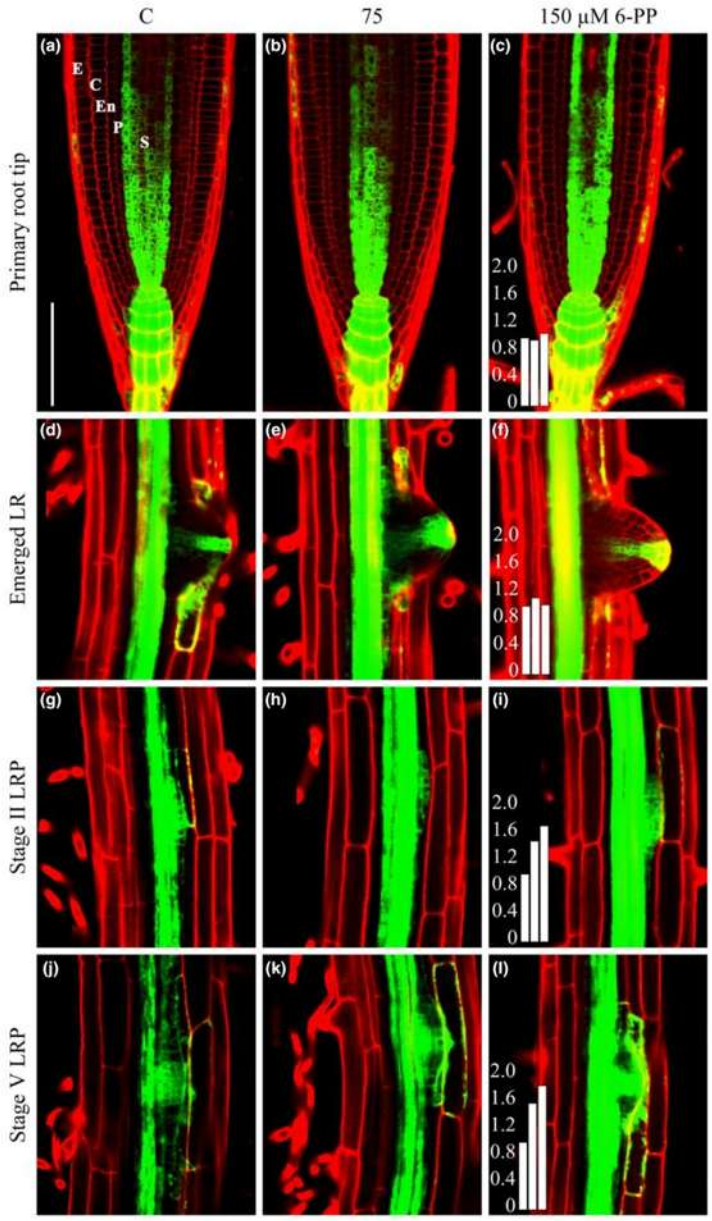

Fig. 5 6-pentyl-2*H*-pyran-2-one (6PP) modulates auxin-responsive gene expression in the lateral root formation zone. *Arabidopsis thaliana* transgenic *DR5: GFP* seedlings were grown in solvent added to 0.2 × MS medium (C), or supplemented with 75 or 150 μM 6-PP. Five days after germination, seedlings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

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

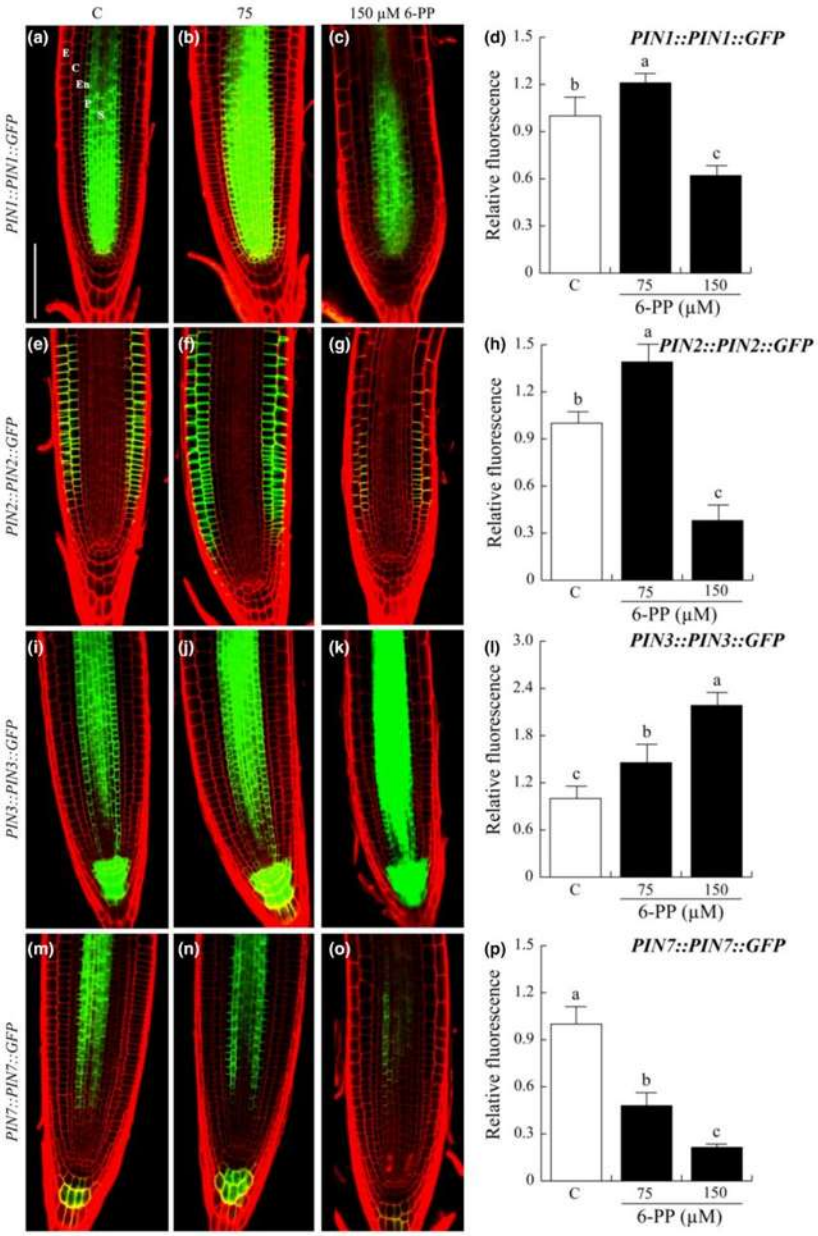

Fig. 6 Expression of auxin efflux transporters in response to 6-pentyl-2*H*-pyran-2-one (6PP) in primary roots. *Arabidopsis thaliana* transgenic *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* seedlings were grown in solvent (C), or 75 or 150 µM 6-PP supplemented medium.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

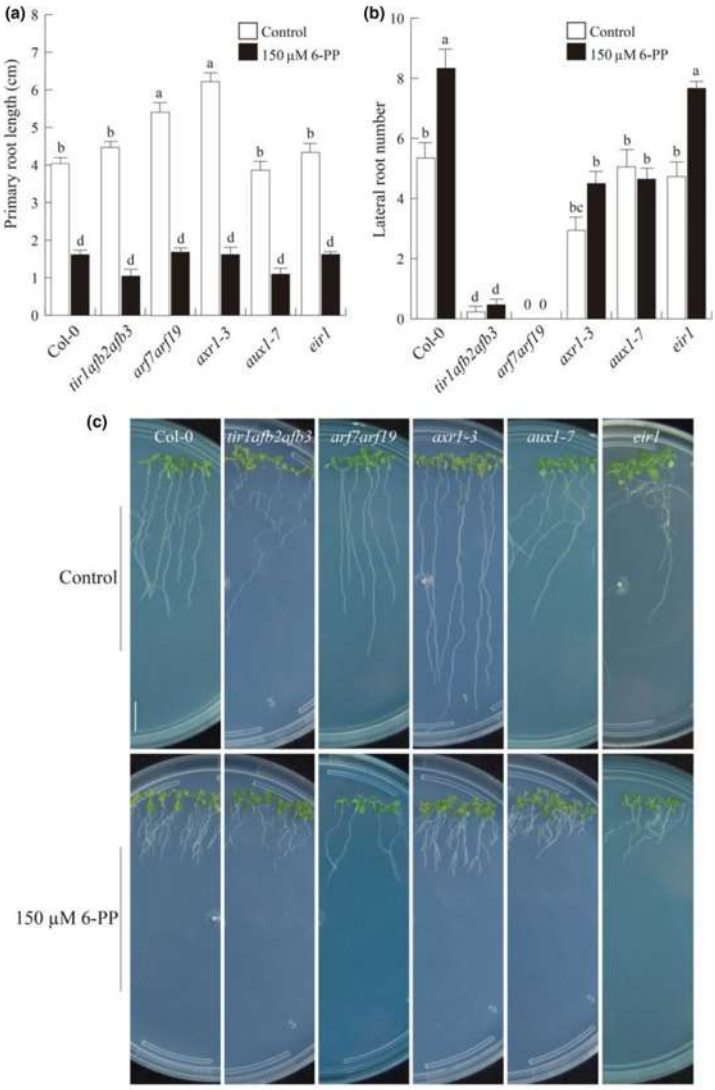

Fig. 7 6-pentyl-2*H*-pyran-2-one (6PP) requires components of auxin response and transport to modify *Arabidopsis thaliana* root system architecture. *Arabidopsis thaliana* wild-type (Col-0) and *tir1/afb2/afb3*, *arf7-1/arf19-1*, *axr1-3*, *aux1-7* and *eir1*, triple, double or single mutant seedlings,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

Discussion

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

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

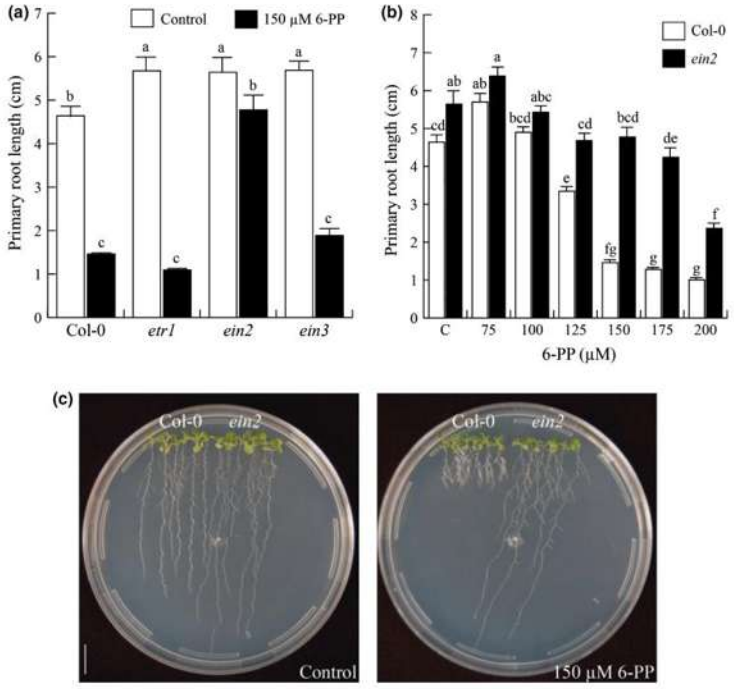

Fig. 8 *EIN2* is necessary for 6-pentyl-2*H*-pyran-2-one (6PP)-modulated primary root growth. *Arabidopsis thaliana* wild-type (Col-0) and *etr1-1*, *ein2-1*, *and ein3-1* ethylene-related mutant seedlings were germinated and grown for 10 d in 0.2 × MS medium supplemented with the solvent (control) or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

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

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

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

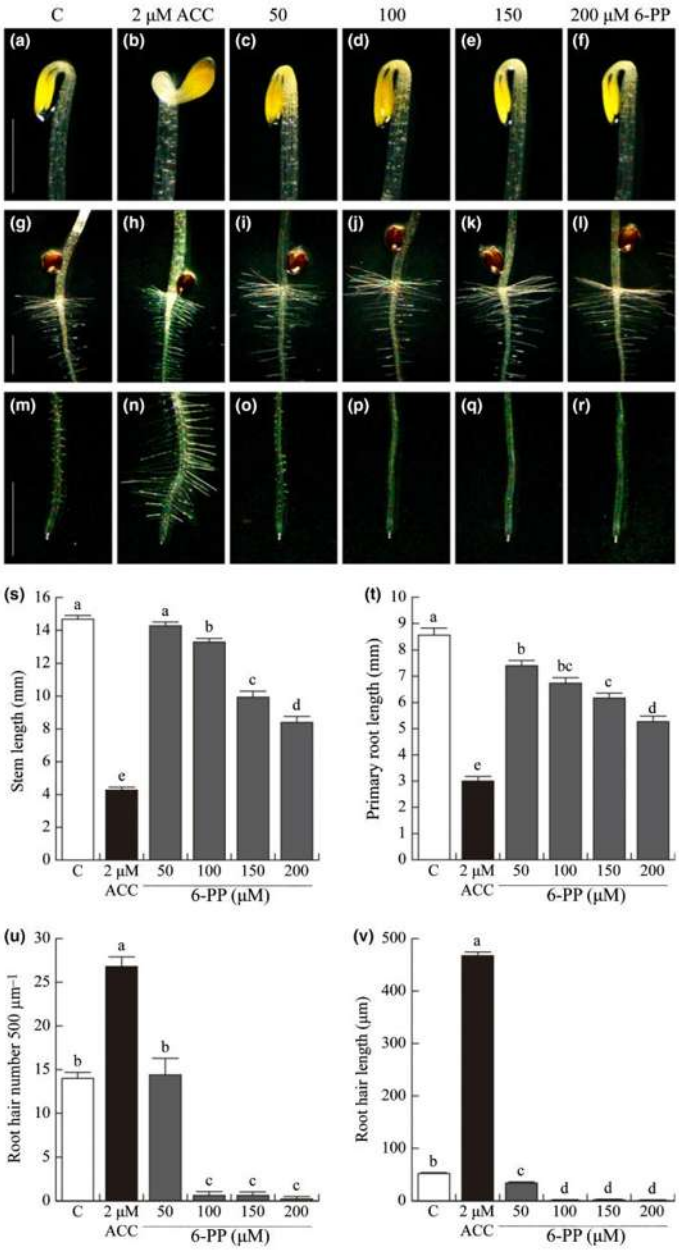

Fig. 9 6-pentyl-2*H*-pyran-2-one (6PP) does not induce ethylene responses in dark-grown *Arabidopsis thaliana* seedlings. Representative photographs of (a–f) stem, (g–l) root–shoot transition zone and (m–r) root tip are shown. Quantitative data are for (s) stem length, (t) primary root length, (u) root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

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

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

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

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

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

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Author contributions

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

References

Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**: 591–602.

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schulz B, Feldmann KA. 1996. Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science* **273**: 948–950.

Björkman T. 2004. Effect of *Trichoderma* colonization on auxin-mediated regulation of root elongation. *Plant Growth Regulation* **43**: 89–92.

Björkman T, Blanchard LM, Harman GE. 1998. Growth enhancement of *shrunken-2* sweet corn when colonized with *Trichoderma harzianum* 1295-22: effect of environmental stress. *Journal of the American Society for Horticultural Science* **123**: 35–40.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* **433**: 39–44.

Boisnard-Lorig C, Colon-Carmona A, Bauch M, Hodge S, Doerner P, Bancharel E, Dumas C, Haseloff J, Berger F. 2001. Dynamic analyses of the expression of the HISTONE:YFP fusion protein in Arabidopsis show that syncytial endosperm is divided in mitotic domains. *Plant Cell* **13**: 495–509.

Brunoud G, Wells DM, Oliva M, Learrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ *et al.* 2012. A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature* **482**: 103–106.

Chacón MR, Rodríguez-Galán O, Benítez T, Sousa S, Rey M, Llobell A, Delgado-Jarana J. 2007. Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum*. *International Microbiology* **10**: 19–27.

Chang YC, Baker R, Klefield O, Chet I. 1986. Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. *Plant Disease* **70**: 145–148.

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR. 1997. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* **27**: 1133–1144.

Colón-Carmona A, You R, Haimovitch-Gal T, Doermer P. 1999. Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. *The Plant Journal* **20**: 503–508.

Contreras-Cornejo HA, López-Bucio JS, Méndez-Bravo A, Macías-Rodríguez LI, Ramos-Vega M, Guevara-García A, López-Bucio J. 2015. *Trichoderma atroviride* alters *Arabidopsis* root-system architecture modulating the mitogen-activated protein kinase 6 activity through ethylene and auxin signaling pathways. *Molecular Plant–Microbe Interactions* **28**: 701–710.

Contreras-Cornejo HA, Macías-Rodríguez LI, Alfaro-Cuevas R, López-Bucio J. 2014a. *Trichoderma* spp. improve growth of *Arabidopsis* seedlings under salt stress through enhanced root development, osmolite production, and Na^+ elimination through root exudates. *Molecular Plant–Microbe Interactions* **27**: 503–514.

Contreras-Cornejo HA, Macías-Rodríguez L, Beltrán-Peña E, Herrera-Estrella A, López-Bucio J. 2011. *Trichoderma*-induced plant immunity likely involves both hormonal and camalexin-dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungi *Botrytis cinerea*. *Plant Signaling and Behavior* **6**: 1554–1563.

Contreras-Cornejo HA, Macías-Rodríguez LI, Cortés-Penagos C, López-Bucio J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiology* **149**: 1579–1592.

Contreras-Cornejo HA, Macías-Rodríguez LI, Herrera-Estrella A, López-Bucio J. 2014b. The 4-phosphopantetheinyl transferase of *Trichoderma virens* plays a role in plant protection against *Botrytis cinerea* through volatile organic compound emission. *Plant and Soil* **379**: 261–274.

Fukaki H, Okushima Y, Tasaka M. 2007. Auxin-mediated lateral root formation in higher plants. *International Review of Cytology* **256**: 111–137.

Galweiler L, Guan CH, Muller A, Wisman E, Mendgen K, Yephremov A, Palme K. 1998. Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* **282**: 2226–2230.

Gams W, Bissett J. 1998. Morphology and identification of *Trichoderma*. In: Harmann GE, Kubicek CP, eds. *Trichoderma and gliocladium*. London, UK: Taylor & Francis, 3–34.

Guzmán P, Ecker JR. 1990. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell* **2**: 513–523.

Harman GE. 2011. Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. *New Phytologist* **189**: 647–649.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* **2**: 43–56.

Hashem A, Allah EF, Alqarawib AA, Al Huqaila A, Egamberdieva D. 2014. Alleviation of abiotic salt stress in *Ochradenus baccatus* (Del.) by *Trichoderma hamatum* (Bonord.) Bainier. *Journal of Plant Interactions* **9**: 857–868.

Hua J, Meyerowitz E. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**: 262–271.

Hung R, Lee S, Bennett JW. 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. *Fungal Ecology* **6**: 19–26.

Ivanchenko MG, Muday GK, Dubrovsky JG. 2008. Ethylene–auxin interactions regulate lateral root initiation and emergence in *Arabidopsis thaliana*. *The Plant Journal* **55**: 335–347.

Jeleń H, Błaszczyk L, Chełkowski J, Rogowicz K, Strakowska J. 2014. Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species. *Mycological Progress* **13**: 589–600.

Keszler Á, Forgács E, Kótai L, Vizcaíno JA, Monte E, García-Acha I. 2000. Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography-mass spectrometry. *Journal of Chromatographic Science* **38**: 421–424.

Lee S, Hung R, Yap M, Bennett JW. 2015. Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. *Archives of Microbiology* **197**: 723–727.

Lewis DR, Negi S, Sukumar P, Muday GK. 2011. Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers. *Development* **138**: 3485–3495.

Lincoln C, Britton JH, Estelle M. 1990. Growth and development of the *axr1* mutant of *Arabidopsis*. *Plant Cell* **2**: 1071–1080.

Malamy JE, Benfey PN. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* **124**: 33–44.

Marchant A, Bhalerao R, Casimiro I, Eklof J, Casero PJ, Bennett M, Sandberg G. 2002. AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the *Arabidopsis* seedling. *Plant Cell* **14**: 589–597.

Martínez-Medina A, Alguacil M, Pascual JA, Van Wees SCM. 2014. Phytohormone profiles induced by *Trichoderma* isolates correspond with their biocontrol and plant growth-promoting activity on Melon plants. *Journal of Chemical Ecology* **40**: 804–815.

Mastouri F, Bjorkman T, Harman GE. 2010. Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. *Phytopathology* **100**: 1213–1221.

Mastouri F, Bjorkman T, Harman GE. 2012. *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Molecular Plant–Microbe Interactions* **25**: 1264–1271.

Mukherjee PK, Horwitz BA, Herrera-Estrella A, Schmoll M, Kenerley CM. 2013. *Trichoderma* research in the genome era. *Annual Review of Phytopathology* **51**: 105–129.

Noh B, Murphy AS, Spalding EP. 2001. Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. *Plant Cell* **13**: 2441–2454.

Ortiz-Castro R, Díaz-Pérez C, Martínez-Trujillo M, del Río R, Campos-García J, López-Bucio J. 2011. Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. *Proceedings of the National Academic of Sciences, USA* **108**: 7253–7258.

Ottenschläger I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K. 2003. Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proceedings of the National Academic of Sciences, USA* **100**: 2987–2991.

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechnera E, Grayd WM, Bennett M, Estelle M. 2009. Complex regulation of

the TIR1/AFB family of auxin receptors. *Proceedings of the National Academic of Sciences, USA* **106**: 22540–22545.

Pereira JL, Queiroz RML, Charneau S, Felix CR, Ricart CA, Lopes da Silva F, Stecca A, Steindorff C, Ulhoa CJ, Noronha EF. 2014. Analysis of *Phaseolus vulgaris* response to its association with *Trichoderma harzianum* (ALL-42) in the presence or absence of the phytopathogenic fungi *Rhizoctonia solani* and *Fusarium solani*. *PLoS ONE* **9**: e98234.

Picket FB, Wilson AK, Estelle M. 1990. The *aux1* mutation of Arabidopsis confers both auxin and ethylene resistance. *Plant Physiology* **94**: 1462–1466.

Polizzi V, Adams A, Picco AM, Adriaens E, Lenoir J, Van Peteghem C, De Saeger S, De Kimpe N. 2011. Influence of environmental conditions on production of volatiles by *Trichoderma atroviride* in relation with the sick building syndrome. *Building and Environment* **46**: 945–954.

Rawat L, Singh Y, Shukla N, Kumar J. 2013. Salinity tolerant *Trichoderma harzianum* reinforces NaCl tolerance and reduces population dynamics of *Fusarium oxysporum* f. sp. ciceri in chickpea (*Cicer arietinum* L.) under salt stress conditions. *Archives of Phytopathology and Plant Protection* **46**: 1442–1467.

Raya-González J, Ortiz-Castro R, Ruíz-Herrera LF, Kazan K, López-Bucio J. 2014. PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 regulates lateral root formation via auxin signaling in Arabidopsis. *Plant Physiology* **165**: 880–894.

Reino JL, Guerriero RF, Hernandez-Gala R, Collado IG. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochemistry Reviews* **7**: 89–123.

Reithner B, Brunner K, Schuhmacher R, Peissl I, Seidl V, Krska R, Zeilinger S. 2005. The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genetics and Biology* **42**: 749–760.

Roman G, Lubarsky B, Kieber JJ, Rotheneberg M, Ecker JR. 1995. Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. *Genetics* **139**: 1393–1409.

Rubio MB, Hermosa R, Reino JL, Collado IG, Monte E. 2009. Thctf1 transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2H-pyran-2-one production and antifungal activity. *Fungal Genetics and Biology* **46**: 17–27.

Rubio MB, Quijada NM, Pérez E, Domínguez S, Monte E, Hermosa R. 2014. Identifying beneficial qualities of *Trichoderma parareesei* for plants. *Applied and Environmental Microbiology* **80**: 1864–1873.

Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell* **19**: 2197–2212.

Samolski I, Rincón AM, Pinzón LM, Viterbo A, Monte E. 2012. The *qid74* gene from *Trichoderma harzianum* has a role in root architecture and plant biofertilization. *Microbiology* **158**: 129–138.

Scarselletti R, Faull JL. 1994. *In vitro* activity of 6-pentyl-a-pyrone, a metabolite of *Trichoderma harzianum*, in the inhibition of *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. lycopersici. *Mycological Research* **98**: 1207–1209.

Shoresh M, Harman GE. 2008. The molecular basis of shoot responses of maize 517 seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. *Plant Physiology* **147**: 2147–2163.

Siddiquee S, Cheong BE, Taslima K, Kausar H, Hasan MM. 2012. Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. *Journal of Chromatographic Science* **50**: 358–367.

Stepanova AN, Yun J, Likhacheva AV, Alonso JM. 2007. Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. *Plant Cell* **19**: 2169–2185.

Stoppacher N, Kluger B, Zeilinger S, Krska R, Schuhmacher R. 2010. Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. *Journal of Microbiological Methods* **81**: 187–193.

Strader LC, Chen GL, Bartel B. 2010. Ethylene directs auxin to control root cell expansion. *The Plant Journal* **64**: 874–884.

Studholme DJ, Harris B, LeCocq K, Winsbury R, Perer AV, Ryder L, Ward JL, Beale MH, Thornton CR, Grant M. 2013. Investigating the beneficial traits of *Trichoderma hamatum* GD12 for sustainable agriculture-insights from genomics. *Frontiers in Plant Science* **4**: 1–13.

Swarup K, Benkova E, Swarup R, Casimiro I, Peret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S *et al.* 2008. The auxin influx carrier LAX3 promotes lateral root emergence. *Nature Cell Biology* **10**: 946–954.

Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GT, Sandberg G, Bhalerao R, Ljung K, Bennett MJ. 2007. Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. *Plant Cell* **19**: 2186–2196.

Vargas WA, Crutcher FK, Kenerley CM. 2011. Functional characterization of a plant-like sucrose transporter from the beneficial fungus *Trichoderma virens*. Regulation of the symbiotic association with plants by sucrose metabolism inside the fungal cells. *New Phytologist* **189**: 777–789.

Vargas WA, Mandawe JC, Kenerley CM. 2009. Plant-derived sucrose is a key element in the symbiotic association between *Trichoderma virens* and maize plants. *Plant Physiology* **151**: 792–808.

Velázquez-Robledo R, Contreras-Cornejo HA, Macías-Rodríguez LI, Hernández-Morales A, Aguirre J, Casas-Flores S, López-Bucio J, Herrera-Estrella A. 2011. Role of the 4-phosphopantetheinyl transferase of *Trichoderma virens* in secondary metabolism and induction of plant defense responses. *Molecular Plant-Microbe Interactions* **24**: 1459–1471.

Vieten A, Vanneste S, Wiśniewska J, Benková E, Benjamins R, Beeckman T, Luschnig C, Friml J. 2005. Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**: 4521–4531.

Vinale F, Manganiello G, Nigro M, Mazzei P, Piccolo A, Pascale A, Ruocco M, Marra R, Lombardi N, Lanzuise S *et al.* 2014. A novel fungal metabolite with beneficial properties for agricultural applications. *Molecules* **19**: 9760–9772.

Vinale F, Sivasithamparam K, Ghisalberti E, Marra R, Barbetti M, Li H, Woo S, Lorito M. 2008. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological Molecular Plant Pathology* **72**: 80–86.

Wilmoth JC, Wang S, Tiwari SB, Joshi AD, Hage G, Guilfoyle TJ, Alonso JM, Ecker JR, Reed JW. 2005. NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. *Plant Journal* **43**: 118–130.

Worasatit N, Sivasithamparam K, Ghisalberti EL, Rowland C. 1994. Variation in pyrone production, pectic enzymes and control of rhizoctonia root rot of wheat among single-spore isolates of *Trichoderma koningii*. *Mycological Research* **98**: 1357–1363.

Wu G, Lewis DR, Spalding EP. 2007. Mutations in *Arabidopsis* multidrug resistance-like ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. *Plant Cell* **19**: 1826–1837.

Žádníková P, Petrášek J, Marhavý P, Raz V, Vandenbussche F, Ding Z, Schwarzerová K, Morita MT, Tasaka M, Hejátko J *et al.* 2010. Role of PIN-mediated auxin efflux in apical hook development of *Arabidopsis thaliana*. *Development* **137**: 607–617.

Zazimalova E, Murphy AS, Yang HB, Hoyerova K, Hosek P. 2010. Auxin transporters-why so many? *Cold Spring Harbor Perspectives in Biology* **2**: a0015.

Zhao L, Wang F, Zhang Y, Zhang J. 2014. Identifying beneficial qualities of *Trichoderma parareesei* for plant. *Journal of Basic Microbiology* **54**: S115–S124.

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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***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

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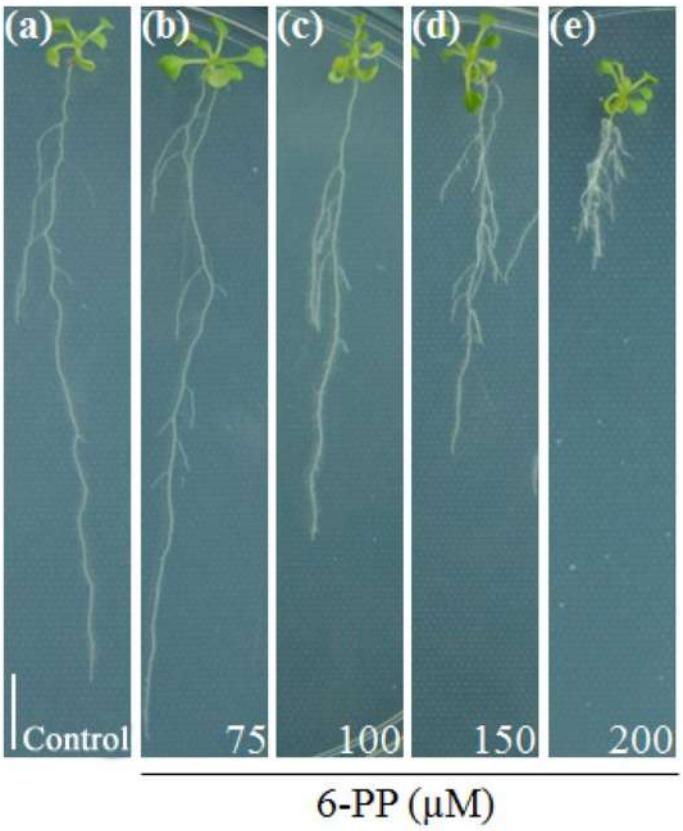

Fig. S1 6-PP modifies *Arabidopsis* root system architecture. *Arabidopsis* (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a–e) Photographs show representative individual seedlings. Bar, 1 cm.

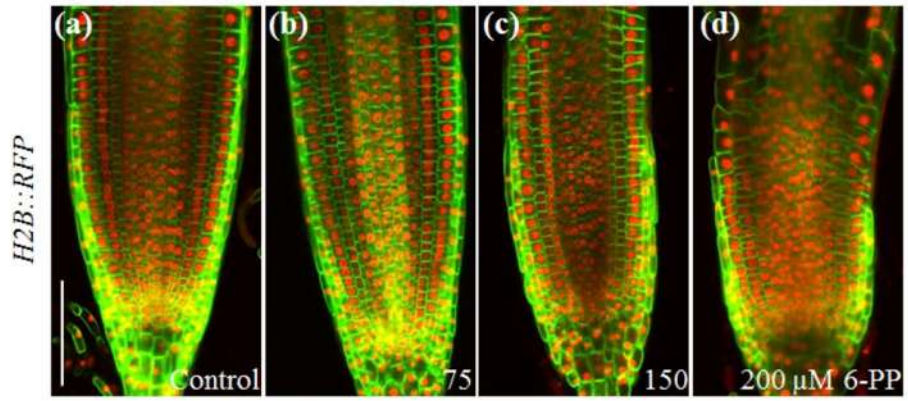

Fig. S2 Effect of 6-PP on *Arabidopsis* cell viability. *Arabidopsis H2B::RFP* expressing seedlings were grown for 10 d in media with the solvent only or supplied with increased concentrations of 6-PP. (a–d) Representative micrographs of primary root tips. Note that even under the highest concentration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

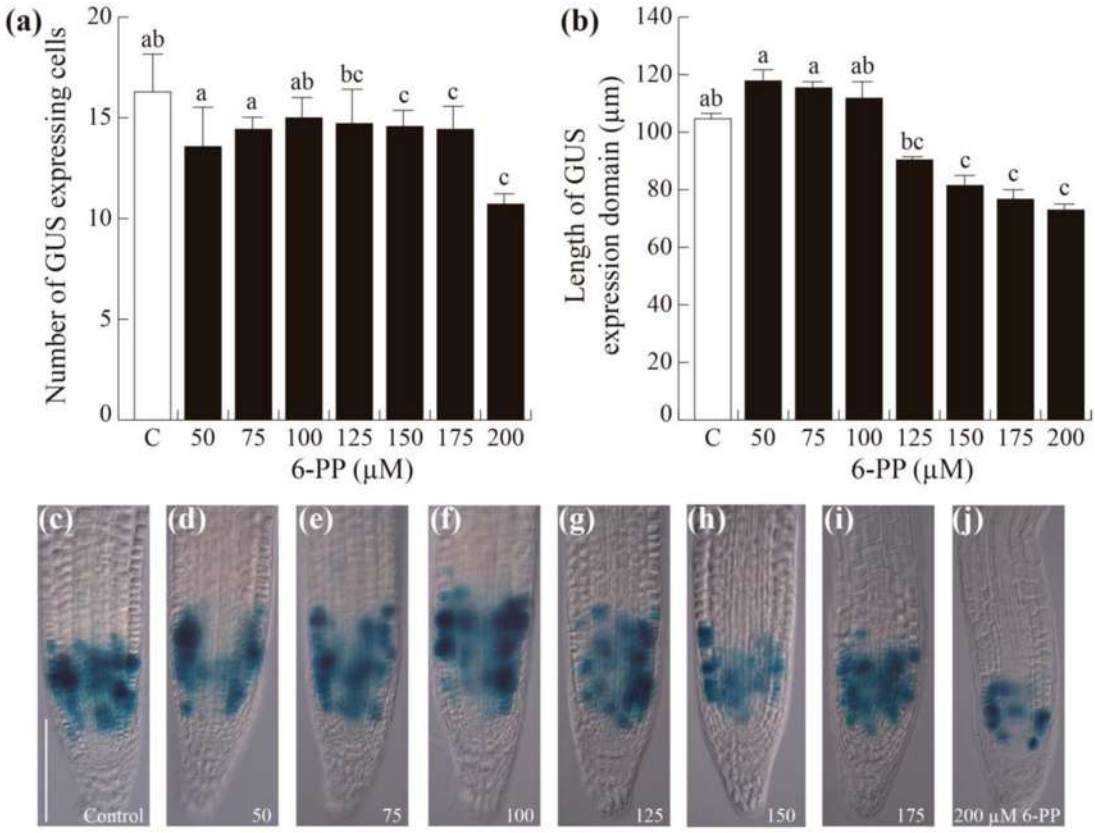

Fig. S3 6-PP reduces the cell division zone in primary roots. Transgenic *Arabidopsis CycB1:uidA* seedlings were germinated and grown for 5 d on 0.2 × MS medium supplied with increasing 6-PP concentrations. (a) Number of GUS expressing cells and (b) length of GUS expression domain were quantified. (c–j) 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.

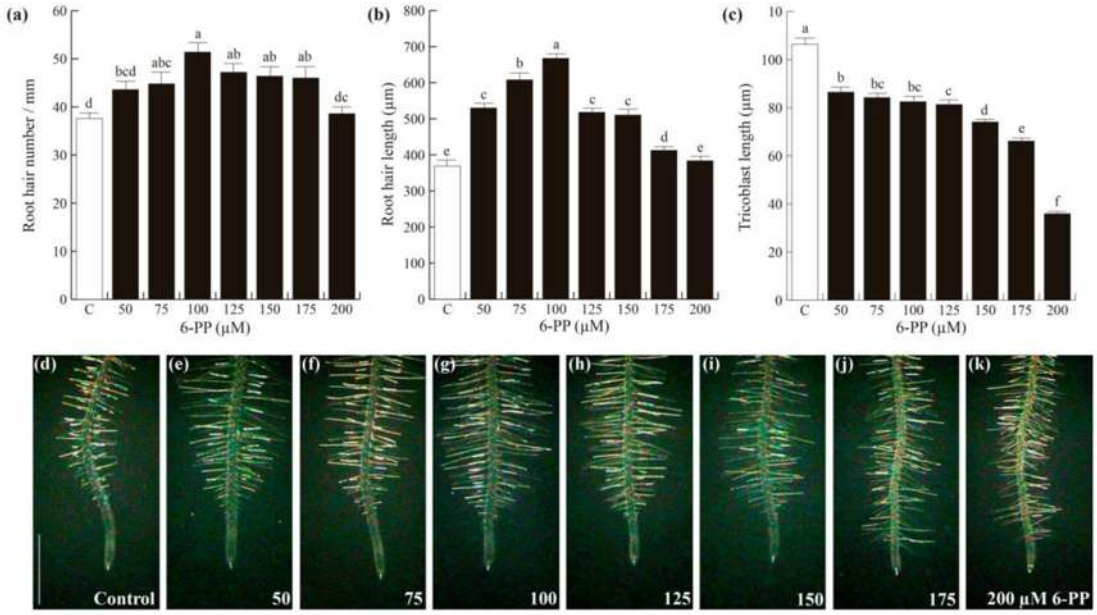

Fig. S4 Effects of 6-PP on *Arabidopsis* root hair development and epidermal cell length. (a) Root hair number, (b) root hair length, and (c) trichoblasts length. *Arabidopsis thaliana* seedlings were grown for 5 d on MS 0.2 × media with or without the indicated concentrations of 6-PP. Date points indicate 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.

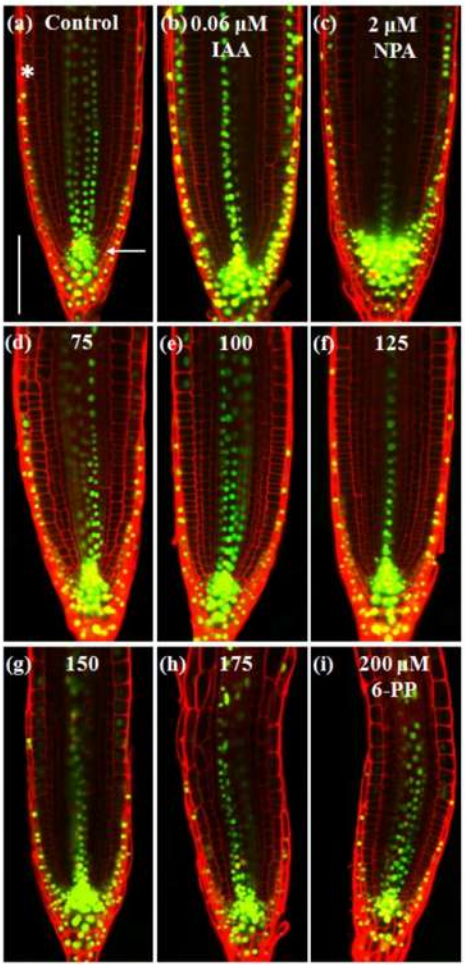

Fig. S5 Effect of 6-PP on auxin responsive gene expression in primary root tips of *Arabidopsis DR5:VENUS* seedlings. Seedlings were grown in MS 0.2 × media supplied with (a) solvent, (b) 0.06 µM IAA, (c) 2 µM NPA, (d) 75, (e) 100, (f) 125, (g) 150, (h) 175 and (i) 200 µM 6-PP. Five days after germination 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.

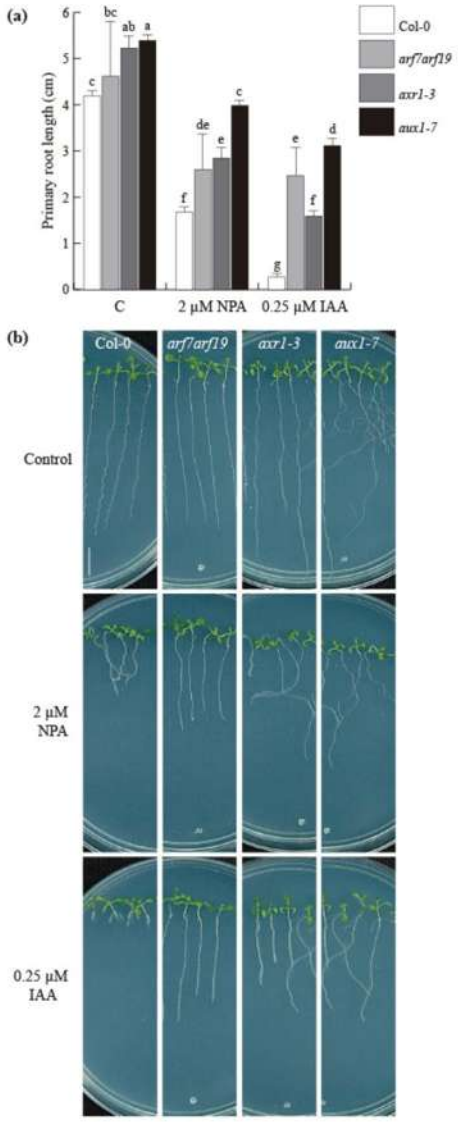

Fig. S6 Effects of NPA and IAA on primary root growth of wild-type (Col-0) and auxin-related mutants. *Arabidopsis thaliana* WT and *arf7arf19, axr1-3* and *aux1* mutant seedlings were grown for 10 d on MS 0.2× medium supplemented with the indicated concentration of NPA and IAA. Values shown represent 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.

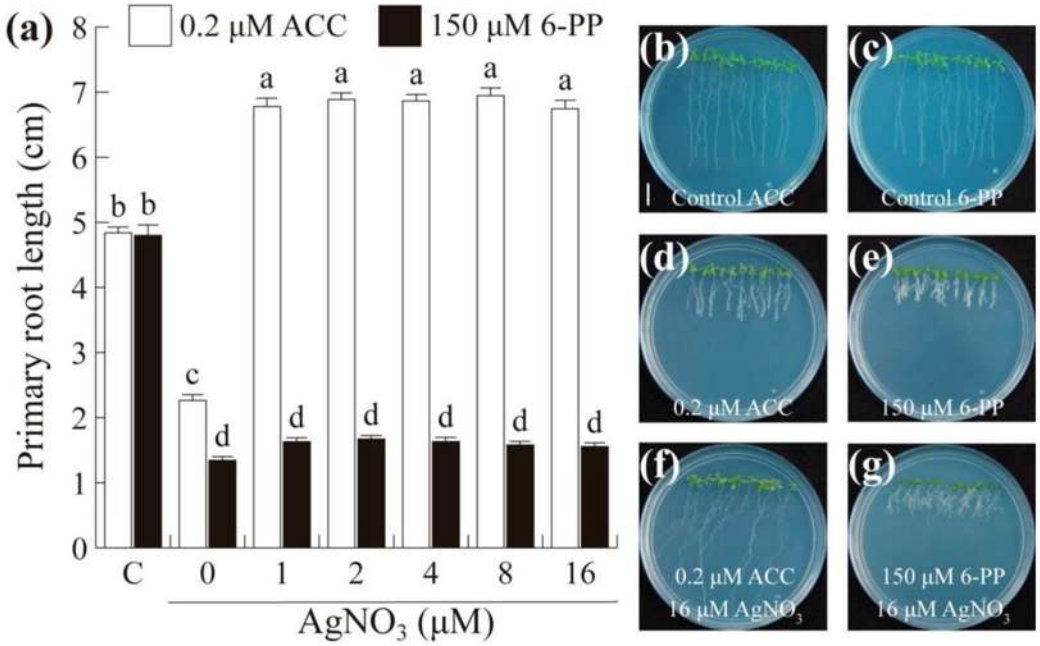

Fig. S7 Effect of $AgNO_3$ on primary root growth inhibition induced by ACC or 6-PP. *Arabidopsis thaliana* Col-0 seedlings were grown for 10 d on MS 0.2× medium supplemented with the indicated concentration of ACC or 6-PP with or without $AgNO_3$. Values shown represent the mean primary root length 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.

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Growth and development of *Arabidopsis thaliana* seedlings in interaction with fungi isolated from stem-end rot of avocado fruits

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ABSTRACT

The influence of *Phomopsis viticola*, *Diaporthe phaseolorum* and *Pseudofusicoccum stromaticum* isolated from avocado stem-end rot on growth of *Arabidopsis* seedlings was investigated. We found responses that are specific for each fungus, whereas *P. viticola* and *D. phaseolorum* strongly repressed root growth, *P. stromaticum* induced growth and branching, and enhanced shoot biomass production. Detailed microscopy and structural analyses revealed that the fungi did not affect cell division in root meristems but rather influenced cell elongation and differentiation, and these responses were related to changes in auxin responsiveness. *P. stromaticum* strongly acidifies the growth medium and this correlated with induction of the jasmonic acid responsive gene construct *LOX2:GUS* in leaves, whereas *P. viticola* and *D. phaseolorum* showed much reduced acidification properties. Taken together, our results show that fungal isolates from the stem-end of avocado fruits interact with *Arabidopsis* plants in highly diverse and contrasting manners influencing growth, patterning and defence.

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Stem-end rot; avocado; *Arabidopsis thaliana*; plant growth; root patterning; defense

1. Introduction

The plant disease stem-end rot of avocado fruits is caused by several fungi. It is characterised by a dark brown to black coloration halo, which further produces dark streaking of the water-conducting tissues, damages the edible mesocarp and reduces quality and economic value post-harvest. *Neofusicoccum*, *Phomopsis*, *Diaporthe* and *Colletotrichum* have been related to stem-end rot (Twizeyimana et al. 2013; Guarnaccia et al.

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2016). These fungi may be present on the avocado tree and grow into the stem of the fruit before harvest to develop stem-end rot upon tissue colonisation, afterwards the fungal pathogens proliferate taking up nutrients, which leads to the appearance of disease symptoms such as necrosis and black coloration of mesocarp, which reduces commercial acceptance (Hartill and Everett 2002).

In response to pathogen challenge, plants establish structural, biochemical and molecular responses to defend themselves, including cell wall reinforcement as well as production of protective metabolites such as phytoalexins that are toxic to pathogens and/or activate the so-called hypersensitive response. This later consists of the appearance of small necrotic patches around the infection site that restrict pathogen spread (Lam et al. 2001). In addition, an increased expression of pathogenesis-related (PR) proteins and defensins (PDFs) reinforces plant immunity (Narasimhan et al. 2001). The plant hormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) are the main defence regulators that orchestrate signal transduction pathways involving both nitric oxide (NO) and reactive oxygen species (ROS) as second messengers for regulation of genes encoding inducible defence proteins (Bolwell 1999; Robert-Seilaniantz et al. 2011).

Fungi are metabolically diverse and this enables their adaptation to diverse habitats. Success of pathogens in colonising plants depends on the release of toxic proteins and secondary metabolites collectively called "effectors", which can kill cells, suppress immunity or alter physiological responses via hormonal regulation (Kazan and Lyons 2014; Presti et al. 2015; Chanclud and Morel 2016; Patkar and Naqvi 2017). Moreover, several fungi change pH as consequence of metabolic activities or produce plant hormones such as cytokinins or auxins that are perceived by their hosts and affect growth, development or defence via modulating mitogen activated protein kinase (MAPK) signalling (Contreras-Cornejo et al. 2009; Contreras-Cornejo et al. 2015; Pelagio-Flores et al. 2017; Spallek et al. 2018). Thus, fungal metabolites, including effectors, are critical players in cross-kingdom relationships between plants and fungi.

Arabidopsis thaliana is a good model for plant-pathogen studies since more than three decades of research led to an extensive collection of mutants and overexpressing lines for genes related to hormone homeostasis. Moreover, an increasing number of lines are available, which express defence-inducible promoters fused to reporter genes encoding fluorescent proteins or enzymes that can be used to investigate in a tissue specific level the effects of selected fungal isolates, such as those related to stem-end rot disease (O'Connell et al. 2004; Andargie and Li 2016). Recently, *in vitro* growth systems have been developed to

investigate the early steps in the plant-fungus communication and recognition, allowing identification of auxin, JA and ET as critical components in the molecular dialogue established (Contreras-Cornejo et al. 2009; Splivallo et al. 2009; Casarrubia et al. 2016; Cordovez et al. 2017).

To enhance resistance of crops to pathogens, a better understanding of plant hormone homeostasis as well as the subsequent metabolic readjustments during the interaction. Although avocado stem-end rot is a tremendous problem in several countries (Twizeyimana et al. 2013; Guarnaccia et al. 2016), the mechanism is scarcely investigated. Advances in this field may help develop breeding strategies to decrease disease severity and improve post-harvest performance and quality.

Here, *in vitro* experiments were performed to test the effects of *Phomopsis viticola*, *Diaporthe phaseolorum* and *Pseudofusicoccum stromaticum*, isolated from stem-end avocado fruits in growth, development and defence-related gene expression in *Arabidopsis* seedlings. The data show a complex network of interactions, by which each fungus differentially modifies root architecture and cell patterning likely influencing auxin signalling. Moreover, we investigated the acidification capacity and induction of jasmonic acid-related *LOX2:GUS* in leaves following root exposure to all three fungal species, which showed the correlation between acidification of the medium and plant defence response in interaction with *P. stromaticum*.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana (Col-0) ecotype and transgenic plants that express the mitotic cyclin *CycB1,1:GFP* (Weimer et al. 2016), expansin protein *EXP7:GUS* (Cho and Cosgrove 2002), auxin-inducible construct *DR5:GFP* (Ottenschläger et al. 2003) and jasmonic acid-inducible *LOX2:GUS* (Jensen et al. 2002) were used in this study. Seeds were first disinfected with ethanol (95%) for 5 min and bleach (20%) containing sodium hypochlorite (NaOCl) as bioactive ingredient for 7 min, washed five times with distilled water, and stratified for 2 d at 4 °C. Seeds were sown on agar plates (9 cm diameter) supplied with 0.2X Murashige and Skoog medium (MS basal salts mixture, M524; PhytoTechnology), 0.6% sucrose (Sucrose: Ultrapure, MB Grade, 21938; USB Corporation) and 1% Agar (Agar, Micropropagation Grade, A111; PhytoTechnology) at pH 7. The suggested formulation is 4.3 g/L of salts for 1x medium; we used 0.9 g/L, which we consider and refer to as 0.2X MS. MES was included in the medium and the pH was adjusted to 7.0. The plates were included into a

Percival AR95L growth chamber, with 6 h light:8h darkness, 200 µmol m/s light and 22 °C of temperature. Seedlings were photographed using a Nikon SMZ1500 stereomicroscope equipped with a digital camera (Nikon SIGHT DS-Fi1c, Nikon Corporation, Tokyo, Japan).

2.2. Fungal strains and culture conditions

P. viticola 5480, *D. phaseolorum* 5185, *P. stromaticum* and *Trichoderma atroviride* IMI 206040 were isolated from pericarp of avocado fruits cv. "Hass" collected in the main avocado-producing area at the Michoacán state in México. The isolates were phenotypically and molecularly characterised and the disease symptoms were clearly demonstrated upon infection of fruits in vitro with *P. viticola*, suggesting it as a stem-end rot causal agent in avocado. Although it remains to be determined if *D. phaseolorum* and *P. stromaticum* also cause fruit decay symptoms, the fact that they were isolated from symptomatic fruits suggest that they might be related to the disease.

Fungal inoculum was propagated on potato dextrose agar (PDA; Difco), at 28 °C. One cm fragment of hyphae was placed at 4 cm from *A. thaliana* primary root tips germinated and grown for 5 days on agar plates containing 0.2X MS medium. Each plate, which included five *A. thaliana* seedlings were arranged in a completely randomised design and the experiment included at least three plates per treatment. After 4 d of co-cultivation, plant growth was determined. For acidification experiments the fungi were inoculated on plates containing 0.2X MS medium supplemented with bromophenol blue (0.006%) and analysed 4 d every 24 h.

2.3. Propidium iodide staining and GFP detection

A. thaliana seedlings expressing the GFP protein were grown axenically or in co-cultivation with stem end fungi, then taken up from the medium and included in microscope slides and covered by propidium iodide (PI, 20 µM) solution. GFP detection was done in a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan), with a 568-nm wavelength argon laser for excitation, and an emission window of 585–610 nm for propidium iodide and GFP fluorescence (488 nm excitation/505–550 nm emission, 514 nm excitation/527 nm emission, and 532 nm excitation/588 nm emission, respectively). Ten independent seedlings were analysed, and representative images were selected for figure construction.

2.4. Histochemical analysis of GUS expression

Seedlings expressing GUS protein were first grown for 5 d on the agar plates supplemented with MS 0.2X salts and the fungal inoculum was placed at two cm from the root tip and co-cultivated for a 4 d period. GUS analyses were done via incubation of seedlings in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M; pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100 with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37 °C. Plants were cleared and fixed using the procedure by Malamy and Benfey (1997). The analysis included 15 transgenic seedlings and representative photographs showing the blue colour indicative of GUS expression were taken.

2.5. Analysis of plant growth

The growth of the primary root was measured using a ruler. Lateral roots were counted from the tip to the root/stem transition in primary roots. Root hair images were taken using a digital camera (Nikon D3300 Osaka, Japan). Data were analysed using the STATISTICA 10 software (Stat Soft, Inc. 2011). Univariate and multivariate analyses with a Tukey's post hoc test helped to establish significant differences among the means. Different letters indicate means that statistically differ ($P \leq .05$).

3. Results

3.1. Effects of stem-end-related fungi on growth of Arabidopsis seedlings

Previous results showed that avocado fruits harvested from Michoacán state, México orchards showing stem-end rot symptoms were colonised with the fungi *P. viticola*, *D. phaseolorum* and *P. stromaticum* (Figure 1). To understand the effects that each fungus has on *A. thaliana* seedlings, biomass production and root growth patterns were analysed after 4 days of fungal co-cultivation in close proximity to the primary root. Shoot fresh weight, primary root length and lateral root number were quantified for 15 seedlings per treatment. While *P. viticola* and *D. phaseolorum* did not significantly affect shoot biomass, *P. stromaticum* almost duplicated the fresh weight, which correlated with a 5-five-fold increase in lateral root number (Figure 2(a–d)). In contrast, *P. viticola* and *D. phaseolorum* strongly repressed primary root growth (Figure 2(b,d)). These data show the dynamic responses of *Arabidopsis* to stem-end-related fungi, and the very contrasting influence of *P. viticola* and *P. stromaticum* on plant growth and development.

3.2. *Stem-end-related fungi* P. viticola *and* D. phaseolorum *increase root hair length*

Since *P. viticola* and *D. phaseolorum* strongly repressed primary root growth (Figure 2(b)), it was of interest to evaluate if the root

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Figure 1. Avocado stem-end rot. Representative images of avocado fruits developing the disease symptoms as ripening proceeds. The symptoms shown arise following infection with *P. viticola*.

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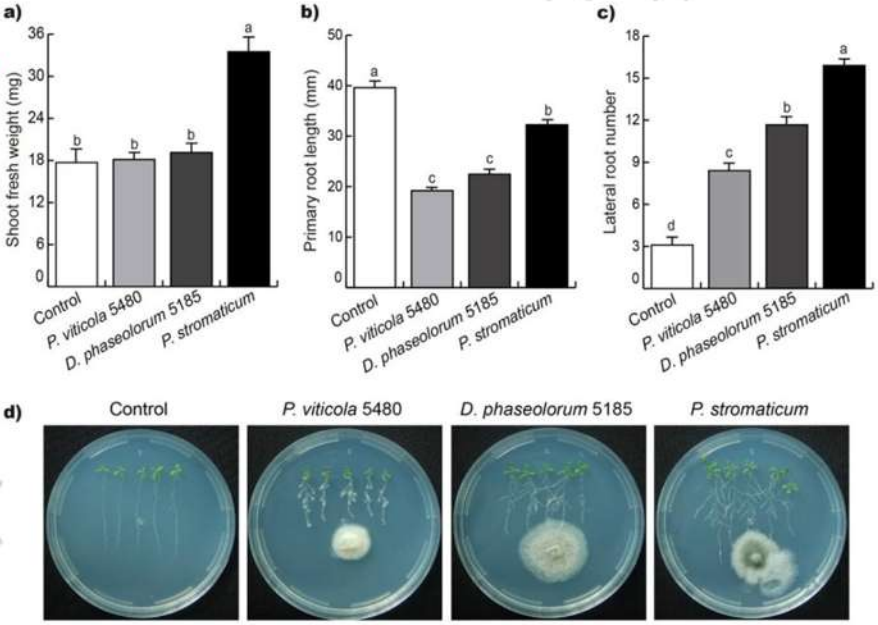

Figure 2. Effects of stem-end-related fungi co-cultivation on *Arabidopsis* shoot biomass and root architecture. (a) Shoot fresh weight; (b) primary root length; (c) lateral root number; and (d) representative photographs of *Arabidopsis* seedlings co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum*. *Arabidopsis* seedlings were germinated and grown for 5 d on the surface of agar plates containing MS 0.2X medium and then inoculated with fungal mycelium at two cm from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P < .05$), $n = 15$. Graph bars show the means ± SE. The experiment was repeated three times with similar results.

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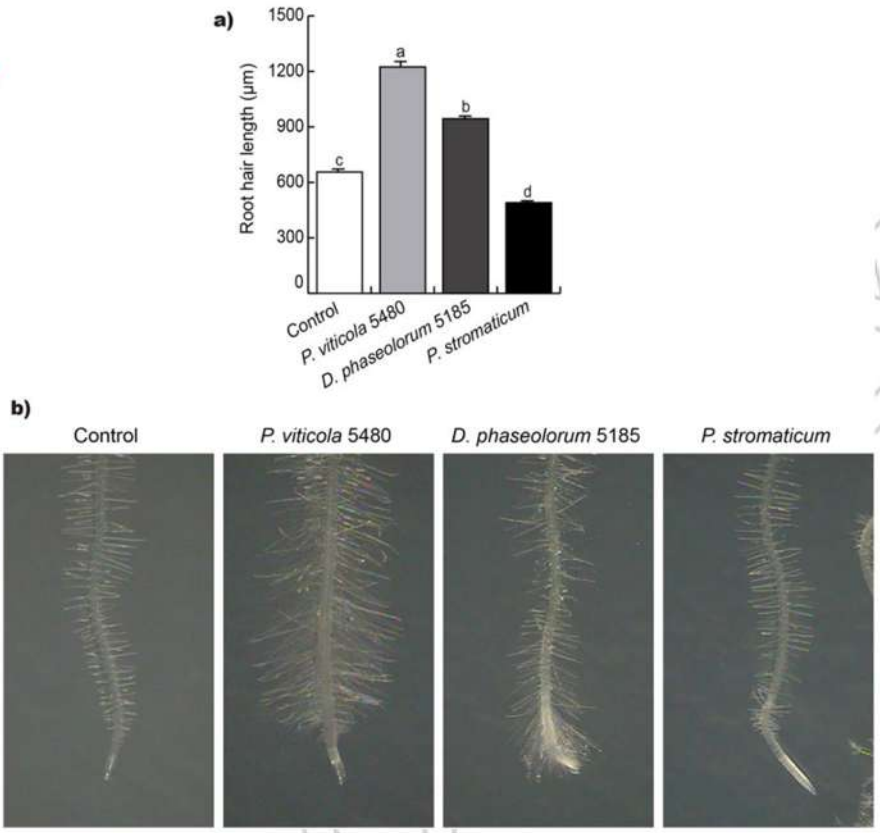

Figure 3. Effects of stem-end-related fungi co-cultivation on *Arabidopsis* root hair differentiation. (a) Root hair length and (b) representative photographs of root hairs developed on *Arabidopsis* primary roots grown under axenic conditions or co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum*. *Arabidopsis* seedlings were germinated and grown for 5 d on the surface of agar plates containing MS 0.2X medium and then inoculated with fungal mycelium at two cm from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P < .05$). Graph bars show the means ± SE. The experiment was repeated three times with similar results.

architectural adjustments elicited by these fungi, could influence epidermal cell differentiation. Root epidermal cells differentiate into root hairs, which are important for uptake of water and nutrients. We measured the length of 100 root hairs from primary roots of 15 independent *Arabidopsis* seedlings grown in axenic condition or co-cultivated with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. From all three fungi examined, *P. viticola* and *D. phaseolorum* could increase the length of root hairs, whereas *P. stromaticum* had the opposite effect, decreasing root hair growth (Figure 3(a,b)). These data show that root hairs develop as

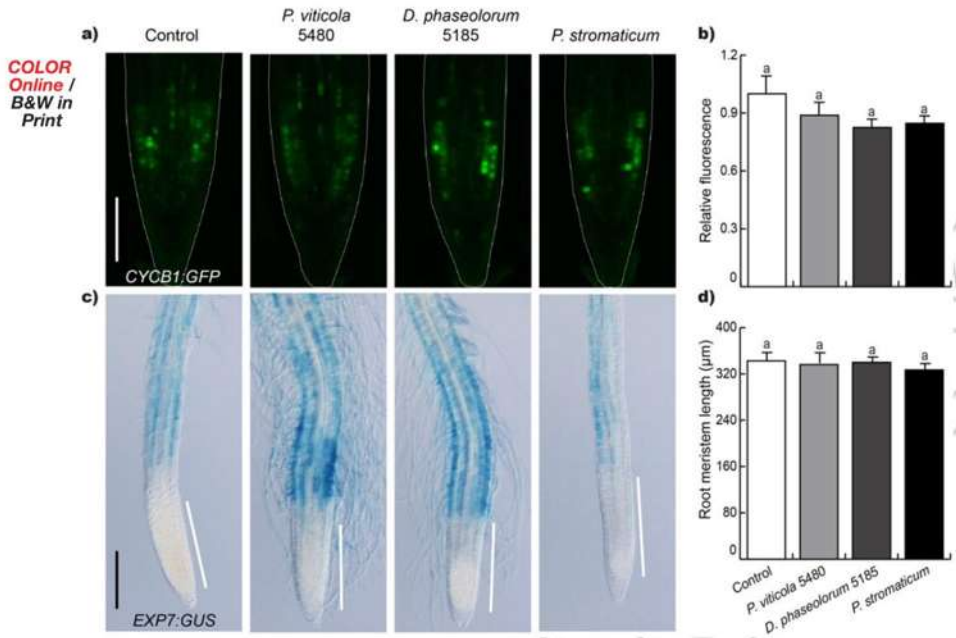

Figure 4. Effects of stem-end-related fungi co-cultivation on root cell division and differentiation. (a) Confocal microscopy images of expression of cell division marker *CycB1:GFP*; (b) cuantification of GFP fluorescence within primary root meristems; (c) expression of the cell differentiation marker *EXP7:GUS*; and (d) Length of the primary root meristem. Five-day-old *Arabidopsis* seedlings expressing the different genetic constructs were co-cultivated with *P. viticola 5480, D. phaseolorum 5185,* and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings and data represent the means of 15 seedlings. Scale bars = 100 µm. These analyses were performed twice with similar results. Note the increased expression of *EXP7:GUS* at the root hair forming zone of plants co-cultivated with *P. viticola* 5480 and *D. phaseolorum* 5185, which correlated with prolific growth of the cells.

primary root growth decreases and it occurs as a response to specific stem-end-related fungi.

3.3. P. viticola *and* D. phaseolorum *induce the expression of cell differentiation gene* EXP7:GUS

The expression of cell division and differentiation markers *CycB1,1:GFP* (Weimer et al. 2016) and *EXP7:GUS* (Cho and Cosgrove 2002), respectively, was assessed by growing plants under axenic conditions or in co-cultivation with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. Interestingly, no changes in *CycB1,1:GFP* already occur in meristems of primary roots from seedlings co-cultivated with the fungi (Figure 4(a,b)). However, these analyses revealed an increased expression of *EXP7:GUS* at the root hair forming zone of plants co-cultivated with *P.*

viticola and *D. phaseolorum* (Figure 4(c)), which correlated with prolific growth of these epidermal cells. Consistently with expression of CycB1,1:GFP, no significant changes were found in the length of the primary root meristem in axenic controls or seedlings inoculated with the fungi (Figure 4(d)). These data suggest that the inhibition of root growth is unlikely due to the production of toxins or virulence factors, since the root meristems remains viable (Figure 4(c), white lines) and instead it may be due to readjustments in cell growth and differentiation programs.

3.4. Effects of stem-end-related fungi on acidification of the growth medium

Stem-end pathogens can inhabit the unripe end or fruit cuticle, remain quiescent until ripening, and then proliferate. These fungal pathogens secrete organic acids or ammonia, which modify the host pH environment (Alkan et al. 2013; Prusky et al. 2016). Moreover, recent data demonstrated that fungi interacting with plants, such as *T. atroviride* induces acidification, which re-configurates *Arabidopsis* root architecture and this trait determines fungal phytostimulation (Pelagio-Flores et al. 2017). To assess whether *P. viticola*, *D. phaseolorum* or *P. stromaticum* could modify pH of the medium, equal amounts of fungal mycelia in MS 0.2X medium supplemented with bromophenol blue, which is used as a pH indicator. *P. stromaticum* strongly acidifies the growth medium, a property shared by *T. atroviride*. The growth repressing fungi *P. viticola* and *D. phaseolorum* showed much reduced acidification properties (Figure 5). Therefore, the amount of media acidification by stem-end-related fungi is species specific.

3.5. Effects of P. viticola and D. phaseolorum on plant growth in buffered media

To determine if the negative effects of *P. viticola* and *D. phaseolorum* on primary roots of *Arabidopsis* seedlings could be related to acidification, we evaluated the plant-fungal interactions in pH-buffered media. Under these conditions, *P. viticola* and *D. phaseolorum* did not increase shoot biomass production but strongly promoted lateral root formation (Figure 6(a,c)). Indeed, the growth repressing effects of these fungi on primary roots could not be attributed to acidification since both fungal isolates still repressed growth in pH-buffered media (Figure 6(b)). We conclude that acidification of the medium by stem-

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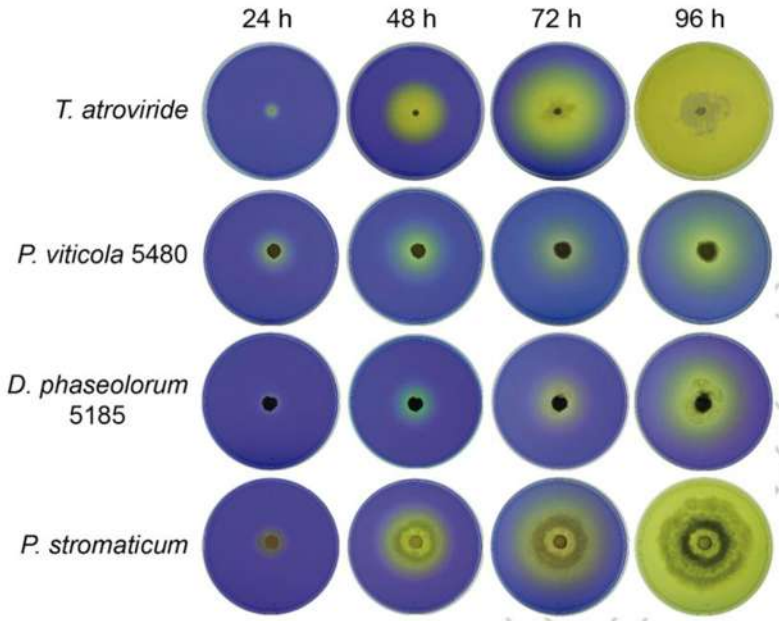

Figure 5. Effect of stem-end-related fungi and *T. atroviride* on acidification of plant growth medium. Stem-end-related fungi *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum*, and the plant symbiont *T. atroviride* were grown for the indicated times on 0.2x MS medium supplemented with bromophenol blue pH indicator. Photographs show representative images of plates in which yellow color indicates the degree of substrate acidification. Note the similar acidification effects of *T. atroviride* and *P. stromaticum*.

end fungi is unlikely related to their pathogenic or growth repressing effects on plants.

3.6. P. viticola *and* D. phaseolorum *induce local auxin response in primary roots*

The results that *P. viticola* and *D. phaseolorum* strongly repress root growth while inducing root hair development suggest the release of bioactive metabolite (s) into the growth medium, thus triggering the observed responses. Several fungi produce auxins (Contreras-Cornejo et al. 2009). Therefore, we analysed the changes in auxin-inducible gene marker *DR5:GFP* (Ottenschläger et al. 2003) in *Arabidopsis* primary roots in interaction with stem-end fungi. In axenically grown seedlings, auxins are distributed within the primary root tip in a narrow domain, which mostly extends following interaction with *P. viticola* and *D. phaseolorum* (Figure 7(a,b)). This increase in auxin response, at least in part, may explain the reduction of primary root growth and induction of root hairs caused by these fungi.

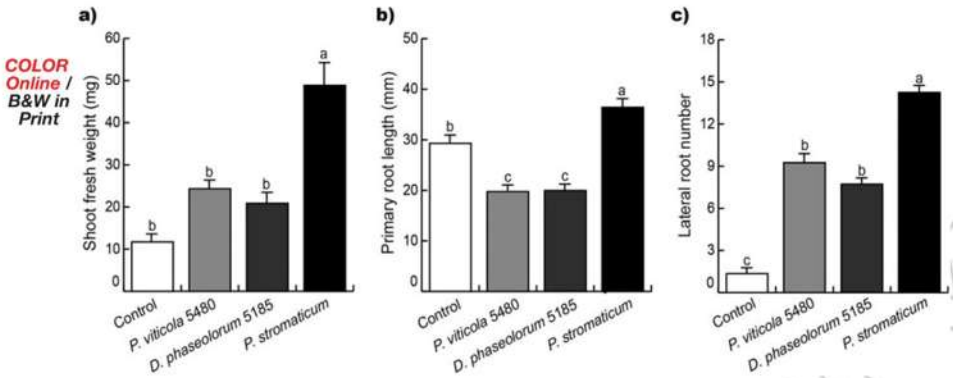

Figure 6. Effects of fungal co-cultivation on *Arabidopsis* shoot biomass and root development under buffered medium. (a) Shoot fresh weight, (b) primary root length, and (c) lateral root number. Fifteen *Arabidopsis* seedlings were germinated and grown on 0.2x MS medium buffered with MES 0.12%, after 5 days fungal mycelium was inoculated at 2 cm distance from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P<.05$). Error bars represents SE. The experiment was repeated three times with similar results.

3.7. Stem-end related fungi induce the jasmonic acid responsive gene LOX2:GUS

Several phytopathogens influence growth and defence responses via jasmonic acid synthesis and signal transduction (Guo et al. 2018). Therefore, we asked if the avocado stem-end fungi could modulate jasmonic acid-inducible gene expression by testing the effect of *Arabidopsis* co-cultivation with *P. viticola*, *D. phaseolorum* and *P. stromaticum* on the expression of the jasmonic acid-responsive gene marker *LOX2:GUS*. *Arabidopsis* seedlings expressing *LOX2:GUS* (Jensen et al. 2002) were co-cultivated with each fungus and after four days in interaction, plants were assessed for GUS activity and cleared to show gene expression in cotyledons and leaves, which is manifested in blue color. Interestingly, an enhanced GUS expression could be observed in petioles of cotyledons and in leaves of seedlings co-cultivated with *P. stromaticum* (Figure 8), which indicates that this fungus activates defense responses via jasmonic acid signalling.

4. Discussion

Stem end rot pathogens colonise climacteric fruits such as avocado, mango, as well as citrus fruits. In several countries avocado postharvest losses have been mainly attributed to fruit decay caused by consortia of latent fungi (Twizeyimana et al. 2013; Guarnaccia et al. 2016; Montealegre et al. 2016; Fuentes-Aragón et al. 2018). Many of these

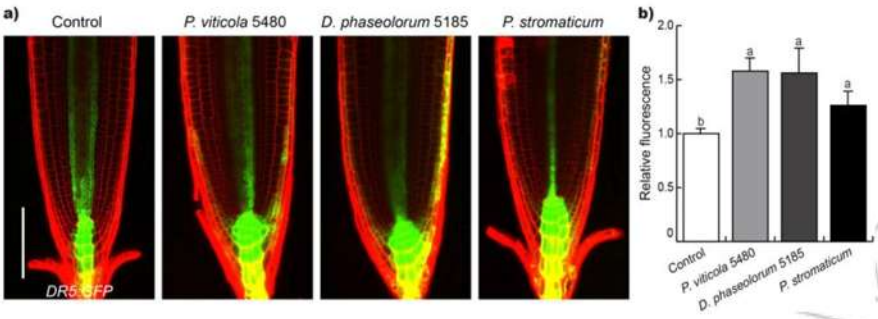

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Figure 7. Effects of stem-end-related fungi co-cultivation on auxin-inducible gene expression in primary roots. (a) Confocal microscopy images of expression of auxin-inducible *DR5:GFP* gene construct and (b) relative GFP fluorescence at the root tip. Five-day-old *Arabidopsis* seedlings expressing the construct were co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings and data were quantified for 15 seedlings. Scale bars = 100 μm. These analyses were performed twice with similar results. Note the increased expression at the columella region of plants co-cultivated with all three fungal species.

fungi are natural endophytic inhabitants of stem tissue and have been identified prior to inflorescence emergence (Johnson et al. 1992).

Although during pre-harvest the use of fungicides is a common practice to control stem end rot, mesocarp damage on fruits has still been observed postharvest in several countries. Many fungal species have been isolated from symptomatic fruits and some of them have been proven to be causal agents of avocado stem end rot, including *Pestalotiopsis clavispora*, *Neofusicoccum australe*, *Neofusicoccum parvum*, *Neofusicoccum luteum*, *Colletotrichum fructicola*, *Colletotrichum gloeosporioides* and *Phomopsis* sp. (Valencia et al. 2011; Twizeyimana et al. 2013; Guarnaccia et al. 2016; Montealegre et al. 2016; Fuentes-Aragón et al. 2018). Considering the lack of details into the biology of the causative agents, and that several fungi may act in consortia, very little is known about the early responses of a plant host in terms of development and defence responses that are activated during infection with stem-end rot pathogens.

The aim of this study was to characterise the effects of three fungal isolates, involved in this symptomatology and their possible relationship with development and defence in *A. thaliana*, a model plant amenable to genetic and molecular analyses. Every fungal species behaved differentially in their interaction with *Arabidopsis* seedlings. *P. viticola* strongly repressed primary root growth, which may be explained by the production of phytohormones, secondary metabolites and/or virulence factors, which accumulate in the growth medium as the fungal colony proliferates. Unexpectedly, *P. stromaticum* enhanced biomass production and

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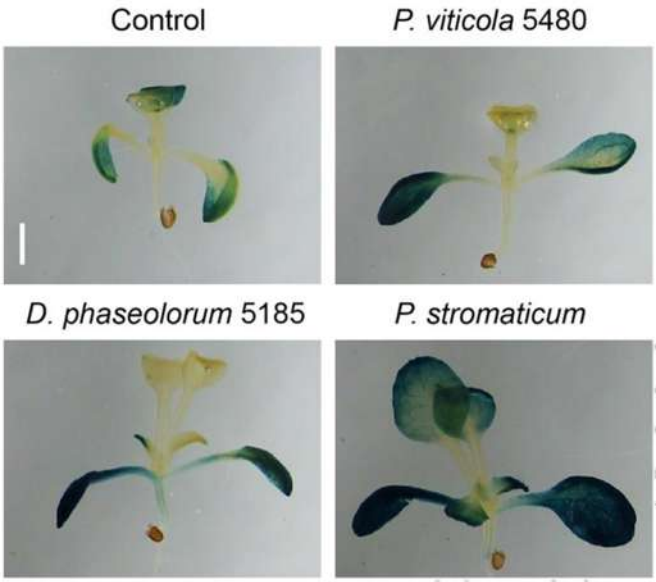

Figure 8. Effects of stem-end-related fungi co-cultivation on jasmonic acid-inducible gene expression in *Arabidopsis* shoots. Representative photographs showing expression of jasmonic acid inducible *LOX2:GUS* gene marker. Five-day-old *Arabidopsis* seedlings expressing the construct were grown under axenic conditions or co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings. These analyses were performed twice with similar results. Note the differential growth of leaves and the increased expression in shoots of plants co-cultivated with *P. stromaticum*.

promoted lateral root formation and growth, similar to the effects reported for plant symbionts such as *T. atroviride* (Contreras-Cornejo et al. 2009). These data were certainly unexpected, but reveal the metabolic diversity among stem-end rot fungi, and certainly it remains to be tested if *P. stromaticum* is a causal agent of avocado stem end.

The inhibition of primary root growth in plants co-cultivated with *P. viticola* and *D. phaseolorum* correlated with the differentiation and elongation of root hairs, which are epidermal cells specialised in water and nutrient acquisition. Similar root architectural adjustments have been reported for *Arabidopsis* seedlings exposed to environmental stress or in response to nutrient deprivation, for which the primary roots stop growing, lose their meristems and activate root hair growth (Sánchez-Calderón et al. 2005; Ruiz-Herrera et al. 2015). One possible explanation is that fungal metabolites are perceived at the root cap, a specialised sensory structure of the root, which transmits information to the cell division and elongation zones, changes growth and patterning to redirect growth to sites far away from the pathogen influence. In consonance

with the strong induction of root hair growth, *P. viticola* and *D. phaseolorum* enhanced expression of the cell differentiation marker *EXP7:GUS*. Previously, the expression of the *Arabidopsis EXP7* gene was tightly correlated to root hair initiation (Cho and Cosgrove 2002), thus, the positive regulation of this gene in the plant-fungi interactions helped us to establish how developmental, hormonal, and environmental factors orchestrate root growth and epidermal cell differentiation.

The metabolic diversity of stem-end rot fungi was also manifested when we compared their acidification capacity. Here, we found that both *P. stromaticum* and the plant beneficial fungus *T. atroviride* strongly acidified the medium, whereas *P. viticola* and *D. phaseolorum* showed much reduced acidification properties. These differences might depend on H^+-ATPase activity because treatment with the inhibitor sodium orthovanadate (Na_3VO_4), reduced acidification (Pelagio-Flores et al. 2017). The highly contrasting acidification capacities of all three stem-end rot fungi, indicates that pH lowering is unlikely linked to their developmental effects in *Arabidopsis*, but might play a role in the decay of avocado fruit when present as consortia.

Auxins repress root growth and concomitantly induce both lateral root and root hair formation (Du and Scheres 2018). Moreover, these compounds act as triggering factors for *EXP7* gene expression in *Arabidopsis* (Cho and Cosgrove 2002). Our analysis of expression of two auxin-inducible gene markers, namely *DR5:GFP* and *DR5:GUS* in *Arabidopsis* primary roots in interaction with stem-end fungi showed that *P. viticola* and *D. phaseolorum* increase the auxin response at the very root tip, which suggest that at least in part, local activation of the auxin signalling pathway may account for primary root inhibition and the induction of root hairs as a response to plant co-cultivation with the fungi. We cannot exclude the possibility that the fungi already produce auxins, auxin precursors or auxin signal mimics, with bioactive functions in the plant host.

The coordination of growth and defence occurs as a response to availability of resources, or under pathogen challenge, and requires prioritisation towards the stimuli encountered. Jasmonic acid is an important mediator of trade-offs and has profound implications for adaptation and success of plants. Indeed, it has been recently involved in the regulation of root system architecture (Raya-González et al. 2012). To determine possible interactions between defence and growth signalling pathways elicited by stem-end rot fungi, the expression of the jasmonic acid-responsive gene marker *LOX2:GUS* was assessed in *Arabidopsis* seedlings co-cultivated with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. Consistently with the activation of defence, an increased expression of

this marker was observed throughout the shoot of seedlings, regardless the fungal species co-cultivated in the vicinity of the root. These data show that stem-end fungi systemically activate defence responses in leaves via jasmonic acid signalling concomitantly with the growth and patterning changes elicited in roots.

In summary, the data presented here points to an extraordinary versatility in the manner by which stem-end rot pathogens interact with *Arabidopsis* seedlings. Understanding the molecular basis by which fungal metabolites balance growth–defence trade-offs in fruits as well as in model plants should provide a foundation to manage fungal-plant communication through chemical means, and for development of breeding strategies to protect avocado trees from stem-end rot or decrease the severity of symptoms in other climacteric fruits.

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Disclosure statement

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References

Alkan N, Espeso EA, Prusky D. 2013. Virulence regulation of phytopathogenic fungi by pH. Antioxid Redox Signal. 19(9):1012–1025.

Andargie M, Li J. 2016. *Arabidopsis thaliana*: A model host plant to study plant-pathogen interaction using rice false smut isolates of *Ustilaginoidea virens*. Front Plant Sci. 7:192.

Bolwell GP. 1999. Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol. 2(4):287–294.

Casarrubia S, Sapienza S, Fritz H, Daghino S, Rosenkranz M, Schnitzler JP, Martin F, Perotto S, Martino E. 2016. Ecologically different fungi affect *Arabidopsis* development: contribution of soluble and volatile compounds. PLoS One. 11(12):e0168236.

Chanclud E, Morel JB. 2016. Plant hormones: a fungal point of view. Mol Plant Pathol. 17(8):1289–1297.

Cho HT, Cosgrove DJ. 2002. Regulation of root hair initiation and expansin gene expression in *Arabidopsis*. Plant Cell. 14(12):3237–3253.

Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C, López-Bucio J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant. Physiol. 149:1579–1592.

Contreras-Cornejo HA, López-Bucio JS, Méndez-Bravo A, Macías-Rodríguez L, Ramos-Vega M, Guevara-García A, López-Bucio J. 2015. Mitogen-activated protein kinase 6 and ethylene and auxin signaling pathways are involved in *Arabidopsis* root-system architecture alterations by *Trichoderma atroviride*. Mol Plant Microbe Interact. 28(6): 701–710.

Cordovez V, Mommer L, Moisan K, Lucas-Barbosa D, Pierik R, Mumm R, Carrion VJ, Raaijmakers JM. 2017. Plant phenotypic and transcriptional changes induced by volatiles from the fungal root pathogen *Rhizoctonia solani*. Front Plant Sci. 8:1262.

Du Y, Scheres B. 2018. Lateral root formation and the multiple roles of auxin. J Exp Bot. 69(2):155–167.

Fuentes-Aragón D, Juárez-Vázquez SV, Vargas-Hernández M, Silva-Rojas HD. 2018. *Colletotrichum fructicola*, a member of *Colletotrichum gloeosporioides* sensu lato, is the causal agent of anthracnose and soft rot in avocado fruits cv. "Hass". Mycobiology.

Guarnaccia V, Vitale A, Cirvilleri G, Aiello D, Susca A, Epifani F, Perrone G, Polizzi G. 2016. Characterisation and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. Eur J Plant Pathol. 146(4):963–976.

Guo Q, Major IT, Howe GA. 2018. Resolution of growth-defense conflict: mechanistic insights from jasmonate signaling. Curr Opin Plant Biol. 44:72–81.

Hartill WF, Everett KR. 2002. Inoculum sources and infection pathways of pathogens causing stem-end rots of 'Hass' avocados. New Zealand J Crop Hort Sci. 30(4): 249–260.

Jensen AB, Raventos D, Mundy J. 2002. Fusion genetic analysis of jasmonate-signalling mutants in Arabidopsis. Plant J. 29(5):595–606.

Johnson GI, Mead AJ, Cooke AW, Dean JR. 1992. Mango stem end rot pathogens – fruit infection by endophytic colonisation of the inflorescence and pedicel. Ann Appl Biol. 120(2):225–234.

Kazan K, Lyons R. 2014. Intervention of phytohormone pathways by pathogen effectors. Plant Cell. 26(6):2285–2309.

Lam E, Kato N, Lawton M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. Nature. 411(6839):848–853.

Malamy JE, Benfey PN. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development. 124(1):33–44.

Montealegre JR, Ramírez M, Riquelme D, Armengol J, León M, Pérez LM. 2016. First report of *Neofusicoccum australe* in Chile causing avocado stem-end rot. Plant Dis. 100(12):2532.

Narasimhan ML, Damsz B, Coca MA, Ibeas JI, Yun D-J, Pardo JM, Hasegawa PM, Bressan RA. 2001. A plant defense response effector induces microbial apoptosis. Mol Cell. 8(4):921–930.

O'Connell R, Herbert C, Sreenivasaprasad S, Khatib M, Esquerré-Tugayé MT, Dumas B. 2004. A novel *Arabidopsis-Colletotrichum* pathosystem for the molecular dissection of plant-fungal interactions. Mol Plant MPMI. 17:272–282.

Ottenschläger I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K. 2003. Gravity-regulated differential auxin transport from columella to lateral root cap cells. Proc Natl Acad Sci USA. 100(5):2987–2991.

Patkar RN, Naqvi NI. 2017. Fungal manipulation of hormone-regulated plant defense. PLoS Pathog. 13(6):e1006334.

Pelagio-Flores R, Esparza-Reynoso S, Garnica-Vergara A, López-Bucio J, Herrera-Estrella A. 2017. *Trichoderma*-induced acidification is an early trigger for changes in *Arabidopsis* root growth and determines fungal phytostimulation. Frontiers Plant Sci. 8(822).

Q3 Presti L, Lanver D, Schweizer G, Tanaka S, Liang L, Tollot M, Zuccaro A, Reissmann S, Kahmann R. 2015. Fungal effectors and plant susceptibility. Annu Rev Plant Biol. 66: 513–545.

Prusky D, Barad S, Ment D, Bi F. 2016. The pH modulation by fungal secreted molecules: a mechanism affecting pathogenicity by postharvest pathogens. Isr J Plant Sci. 63(1):22–30.

Raya-González J, Pelagio-Flores R, López-Bucio J. 2012. The jasmonate receptor COI1 plays a role in jasmonate-induced lateral root formation and lateral root positioning in *Arabidopsis thaliana*. J Plant Physiol. 169(14):1348–1358.

Robert-Seilaniantz A, Grant M, Jones JD. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu Rev Phytopathol. 49: 317–343.

Ruiz-Herrera LF, Shane MW, López-Bucio J. 2015. Nutritional regulation of root development. Wiley Interdiscip Rev Dev Biol. 4(4):431–443.

Sánchez-Calderón L, López-Bucio J, Chacón-López A, Cruz-Ramírez A, Nieto-Jacobo MF, Dubrovsky JG, Herrera-Estrella L. 2005. Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. Plant Cell Physiol. 46(1):174–184.

Spallek T, Gan P, Kadota Y, Shirasu K. 2018. Same tune, different song-cytokinins as virulence factors in plant-pathogen interactions? Curr Opin Plant Biol. 44:82–87.

Splivallo R, Fischer U, Göbel C, Feussner I, Karlovsky P. 2009. Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiol. 150(4): 2018–2029.

Twizeyimana M, Förster H, McDonald V, Wang DH, Adaskaveg JE, Eskalen A. 2013. Identification and pathogenicity of fungal pathogens associated with stem-end rot of avocado in California. Plant Dis. 97(12):1580–1584.

Valencia AL, Torres R, Latorre BA. 2011. First report of *Pestalotiopsis clavispora* and *Pestalotiopsis* spp. causing postharvest stem end rot of avocado in Chile. Plant Dis. 95(4):492.

Weimer AK, Biedermann S, Harashima H, Roodbarkelari F, Takahashi N, Foreman J, Guan Y, Pochon G, Heese M, Van Damme D, et al. 2016. The plant-specific CDKB1-CYCB1 complex mediates homologous recombination repair in *Arabidopsis*. Embo J. 35(19):2068–2086.

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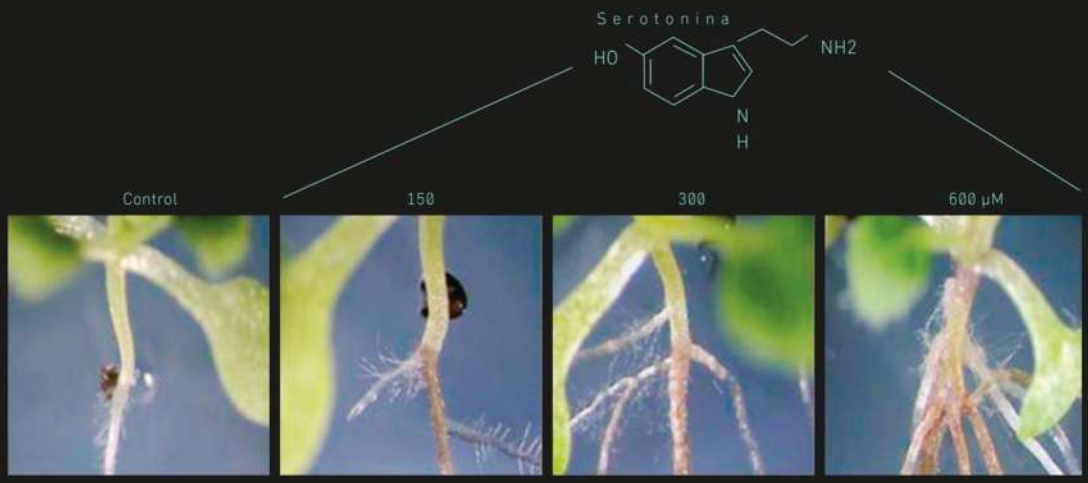

In vitro

REGULACIÓN DEL DESARROLLO VEGETAL POR INDOLAMINAS

REGULATION OF PLANT DEVELOPMENT BY INDOLEAMINES

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RESUMEN

Las indolaminas serotonina y melatonina, un neurotransmisor y hormona de mamíferos respectivamente, las cuales participan en la regulación de importantes procesos fisiológicos en dichos organismos, son también compuestos naturales de plantas. Debido a su similitud estructural con el ácido indol acético (AIA) la principal auxina, estudios previos sugerían una actividad similar entre ellos. En este estudio se caracterizaron los efectos de la serotonina y melatonina sobre el crecimiento y desarrollo en plantas de *Arabidopsis thaliana*. La serotonina causó efectos inhibitorios sobre el crecimiento de la raíz primaria, el crecimiento de raíces laterales y en el desarrollo de pelos radiculares pero estimuló fuertemente la formación de raíces adventicias de una manera dosis dependiente. Por el contrario, la melatonina promovió la formación de raíces laterales y adventicias e inhibió mínimamente el crecimiento de la raíz primaria. Además se investigó el papel de la señalización por auxinas en las alteraciones del desarrollo observadas por serotonina y melatonina en las plantas utilizando las construcciones *DR5:uidA, BA3:uidA* y *HS::AXR3NT-GUS* como marcadores de la respuesta auxínica. Interesantemente, las dos indolaminas no activan la expresión de genes de respuesta a auxinas, por el contrario, la serotonina inhibió fuertemente la expresión de *DR5:uidAyBA3:uidA*. Nuestros resultados muestran que la serotonina y melatonina tienen efectos diferentes sobre la arquitectura del sistema radicular de *Arabidopsis thaliana* y que la serotonina puede actuar como un inhibidor natural de la vía de las auxinas en las plantas.

Palabras clave: ***Arabidopsis thaliana,*** **serotonina, melatonina, arquitectura de la raíz.**

ABSTRACT

The indoleamines serotonin and melatonin, a neurotransmitter and hormone, respectively, regulate important physiological processes in animals and are commonly present in plants. Due to their structural similarity with indole-3-acetic acid (IAA), the main auxin, previous studies suggested the indoleamines could possess an auxin like-activity. In this report, the effects of serotonin and melatonin on growth and development of *Arabidopsis thaliana* seedlings were characterized. Serotonin showed growth repressing effects on primary and lateral roots, and root hairs while promoting adventitious root development. In contrast, melatonin increased lateral and adventitious root formation without inhibiting primary root growth. Moreover, the auxin bioactivity of serotonin and melatonin was tested using *DR5:uidA, BA3:uidA* and *HS::AXR3NT-GUS* gene constructs. The indoleamines did not induce expression of these genes, but serotonin strongly inhibited *DR5:uidA* and *BA3:uidA*. These results indicate that serotonin and melatonin have different effects on *Arabidopsis* root architecture and that serotonin may act as a natural auxin inhibitor in plants.

Keywords: *Arabidopsis thaliana*, serotonin, melatonin, root architecture.

INTRODUCCIÓN

Las plantas sintetizan y utilizan una gran variedad de compuestos para coordinar su crecimiento y desarrollo durante todo el ciclo de vida. Uno de los principales grupos de reguladores del crecimiento son las auxinas, las cuales participan en una gran variedad de procesos del desarrollo (Woodward y Bartel, 2005). La principal auxina de las plantas es el ácido-3-indolacético (AIA). Aunque el AIA se encuentra dentro de los derivados del triptófano mejor caracterizados, se han reportado altos niveles de compuestos relacionados al AIA en las plantas, como son la serotonina y la melatonina. Estos compuestos participan en múltiples funciones en neurotransmisión, hormonales y mitogenicas en células animales (Hardelan *et al*., 2006). En plantas, la serotonina y la melatonina se han encontrado en una gran variedad de especies (Ramakrishna *et al*., 2011; Arnao, 2014). Sin embargo, los procesos en los que participan han sido poco estudiados y actualmente se desconocen sus mecanismos de acción. Para profundizar en el estudio de los efectos y posibles mecanismos de señalización de la serotonina y melatonina en las plantas, en este trabajo se caracterizaron sus efectos sobre el desarrollo y las respuestas mediadas por auxinas en *Arabidopsis thaliana*.

MATERIALES Y MÉTODOS

En este trabajo, se utilizó como modelo a *Arabidopsis thaliana* del ecotipo Columbia Col-0, además de las líneas transgénicas *HS::AXR3NT-GUS* (Gray *et al*., 2001), *DR5:uidA* (Ulmasov *et al*., 1997), y *BA3:uidA* (Oono *et al*., 1998). Las semillas fueron desinfectadas superficialmente con etanol al 96% (v/v) por 5 min, hipoclorito de sodio al 20% (v/v) agitando por 7 min y lavando con agua destilada estéril, las semillas fueron germinadas y crecidas sobre placas de agar que contiene medio MS 0.2X suplementado con diferentes concentraciones de serotonina, melatonina o auxinas, colocadas en una cámara de crecimiento con condiciones controladas (Percival AR-95L), con un fotoperiodo de 16 horas luz/ 8 horas obscuridad, hasta el momento del análisis y registro de cada experimento.

RESULTADOS Y DISCUSIÓN

La serotonina y la melatonina afectaron la arquitectura del sistema radicular, de manera diferencial. Por un lado la serotonina inhibe el crecimiento de la raíz primaria y la formación de raíces laterales, pero favorece la formación de raíces adventicias de manera dependiente de su concentraciónen en el medio (Fig. 1).

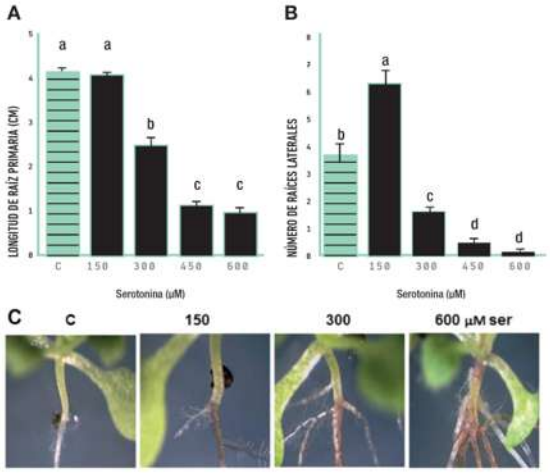

FIGURA 1.

Efecto de la serotonina sobre la arquitectura de la raíz y la formación de raíces adventicias.

La serotonina también inhibió el desarrollo de los pelos radiculares (Fig. 2). Lo anterior evidencia la capacidad de la serotonina para modular la arquitectura de la raíz de las plantas así como su alta actividad organogénica.

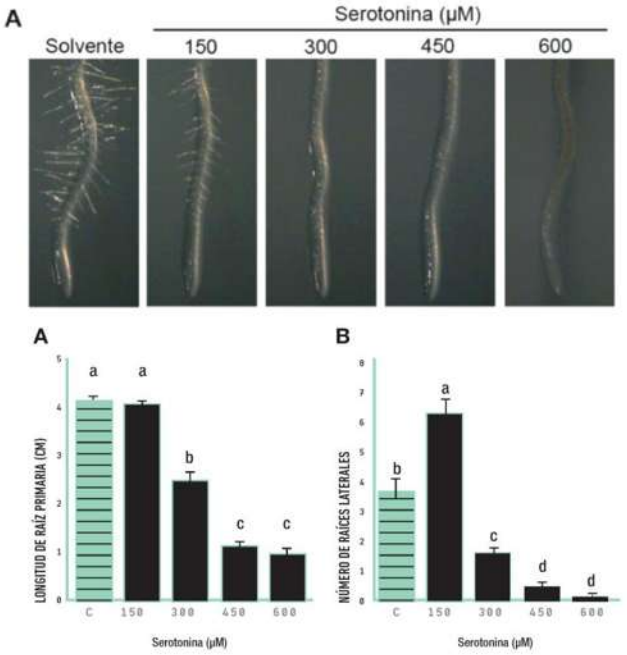

FIGURA 2.
Efecto de la serotonina sobre el desarrollo de pelos radiculares.

Para determinar si los efectos de la serotonina sobre la arquitectura de la raíz en *Arabidopsis thaliana* coincidían con la acumulación de este compuesto en las plantas, se cuantificó la serotonina en muestras de raíz y follaje, encontrando que *Arabidopsis* produce serotonina en pequeñas cantidades, las cuales se incrementan en medio suplementado con este compuesto, tanto en raíz como en follaje (Fig. 3) lo cual se correlaciona con los efectos observados (Pelagio-Flores *et al*., 2011).

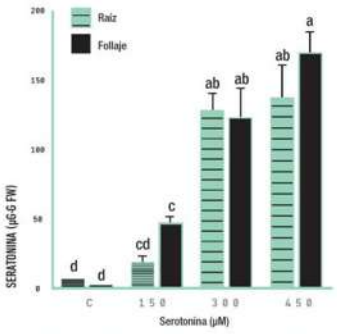

FIGURA 3.
Cuantificación de serotonina en plantas de *Arabidopsis thaliana*.

Por otro lado y a diferencia de lo observado con serotonina, la melatonina afectó la arquitectura del sistema radicular, estimulando la formación de raíces laterales sin inhibir significativamente el crecimiento de la raíz primaria (Fig.4).

Además de inducir también la formación de raíces adventicias a partir de explantes de tallo (Fig. 5A y B), sin comprometer significativamente el desarrollo de los pelos radiculares (Fig. 4C), mostrándose con esto que la melatonina funciona como una molécula benéfica para las plantas, al aumentar la ramificación del sistema radicular.

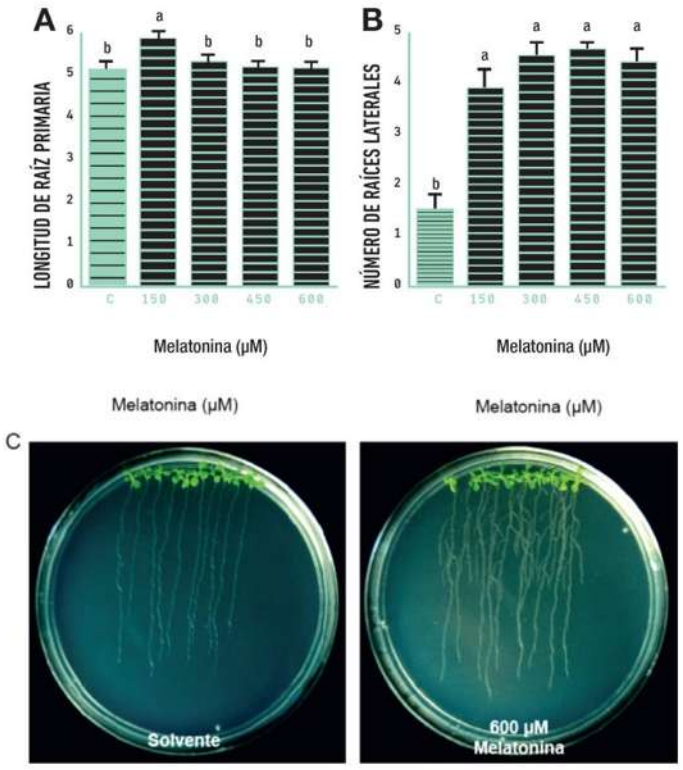

FIGURA 4.

Efecto de la melatonina sobre la arquitectura de la raíz.

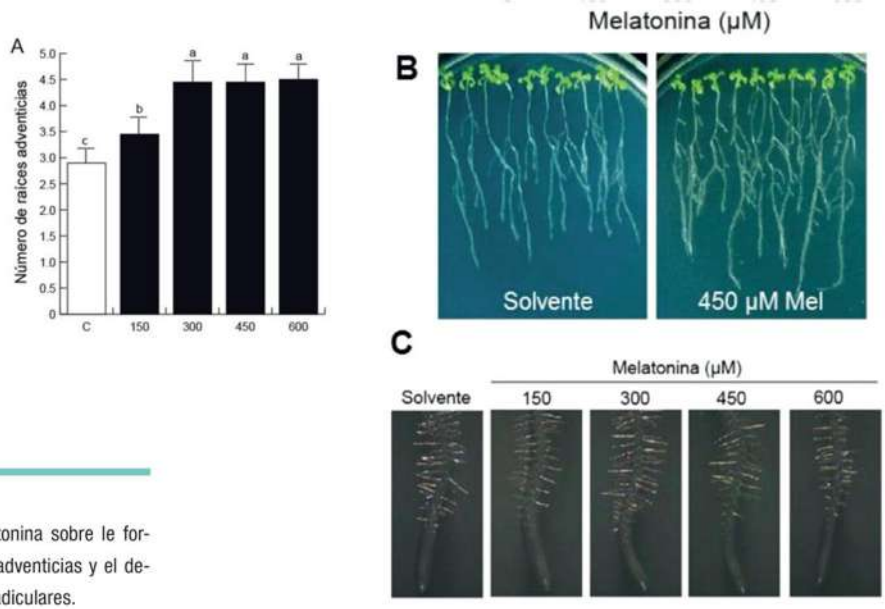

FIGURA 5.

Efecto de la melatonina sobre le formación de raíces adventicias y el desarrollo de pelos radiculares.

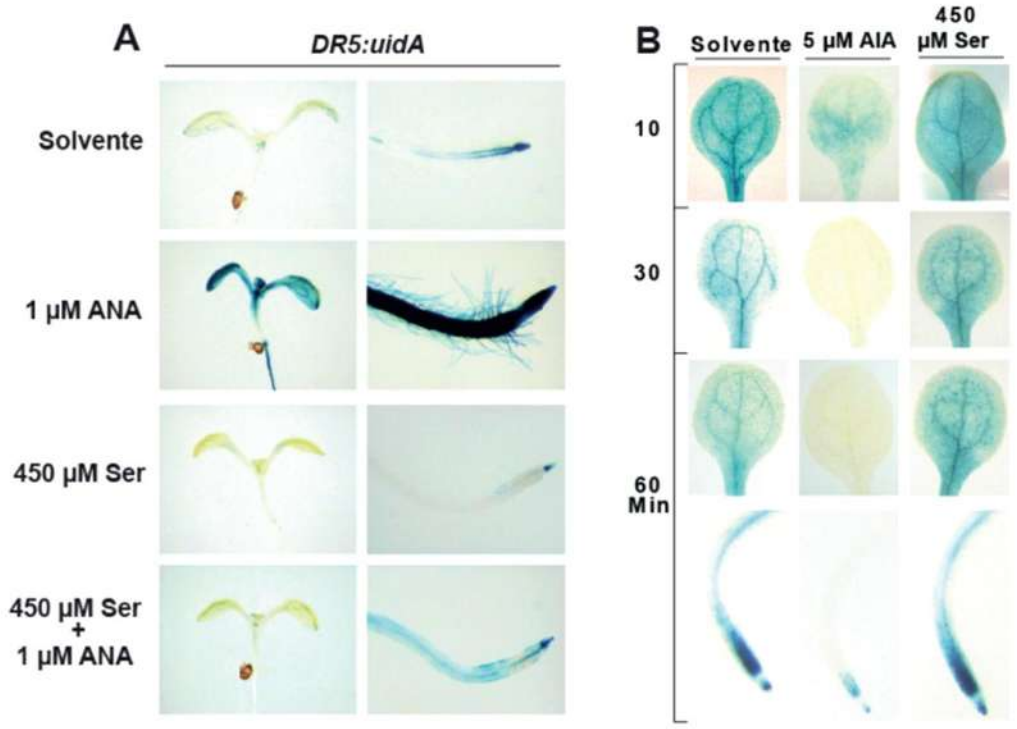

FIGURA 6.

Efecto de la serotonina sobre la expresión de genes regulados por auxinas.

Para determinar si tanto la serotonina como la melatonina podían actuar en una ruta de señalización relacionada a auxinas, se determinó su efecto sobre la expresión de los genes marcadores *DR5:uidA* y *BA3:uidA* inducibles por auxinas. En plantas transgénicas que expresan el marcador *DR5:uidA,* la expresión del gen reportero se localiza principalmente en los meristemos de la raíz y el follaje, mientras que plantas tratadas con 1 µM de la auxina sintética ANA, su expresión se presenta en toda la planta. En contraste, en las plantas tratadas con serotonina, el gen reportero disminuye fuertemente su expresión, aún bajo tratamiento con auxinas (Fig. 6A), este efecto fue similar en las plantas transgénicas *BA3:uidA*. Para complementar el análisis de los efectos de la serotonina sobre la vía auxínica, se analizó su efecto sobre la degradación de uno de los represores de la respuesta a auxinas *AXR3/IAA17* usando la fusión *HS::AXR3NT-GUS* el cual es degradado en respuesta a auxinas. Plantas de esta línea fueron tratadas con AIA y/o serotonina, mostrando la degradación del represor por el AIA a los pocos minutos del tratamiento, sin embargo, la serotonina no indujo la degradación de este represor aún después de 60 minutos de tratamiento (Fig. 6B). Estos resultados muestran que la serotonina reprime la expresión de los genes inducibles por auxinas y no induce la degradación de AXR3/IAA17 sugiriendo que la serotonina puede actuar como un inhibidor natural de auxinas.

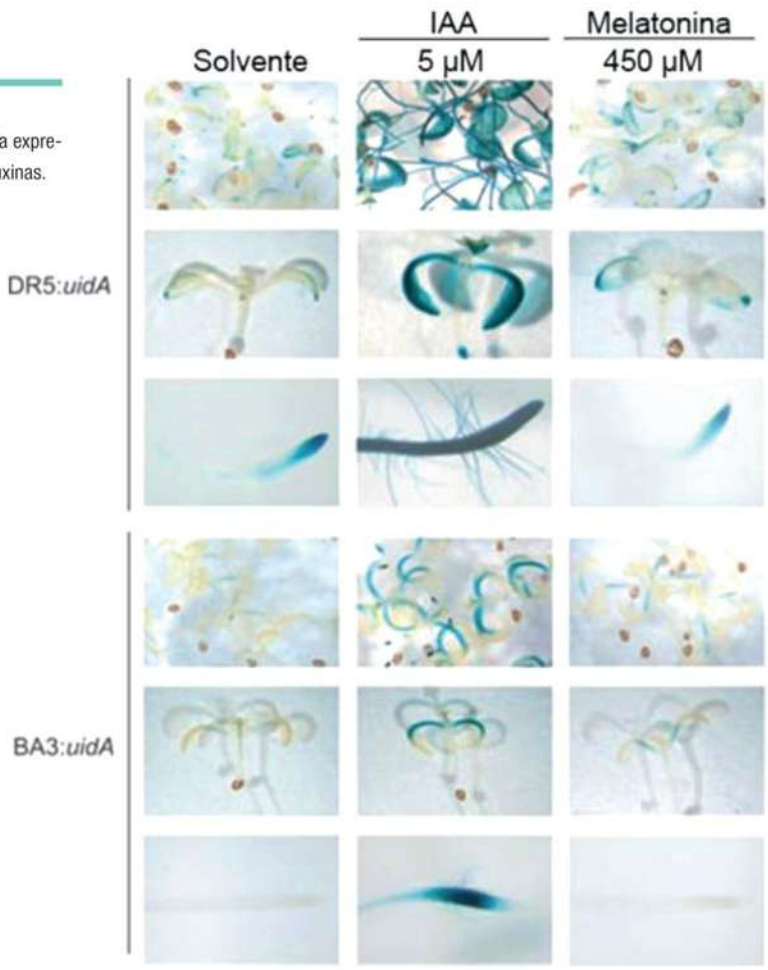

Figura 7.

Efecto de la melatonina sobre la expresión de genes regulados por auxinas.

De manera similar nuestros resultados mostraron que la melatonina no induce la expresión de *DR5:uidA* y *BA3:uidA* (Fig. 7), ni activa la degradación de *HS::AXR3NT-GUS* como lo hacen los compuestos con actividad auxínica, indicando que los procesos del desarrollo modulados por la melatonina ocurren mediante un mecanismo distinto de la señalización por auxinas (Pelagio-Flores *et al*., 2012).

CONCLUSIONES

La serotonina y la melatonina son compuestos naturales de plantas que tienen la capacidad de regular la arquitectura de la raíz de *Arabidopsis thaliana* de manera diferencial, dotando a la planta de un mayor número de estructuras como son las raíces laterales y

adventicias que bajo determinadas condiciones pueden favorecer su supervivencia. Se concluye además que ambos compuestos actúan de manera independiente a la ruta de señalización de las auxinas y más específicamente que la serotonina actúa como un represor de esta vía.

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REFERENCIAS

ARNAO, M.B. (2014). Phytomelatonin: Discovery, Content, and Role in Plants. Advances in Botany, Volume 2014, ID 815769, 1-11.

GRAY, W.M., Kepinski, S., Rouse, D., Leyser O., Estelle, M. (2001) Auxin regulates SCF-TIR1-dependent degradation of AUX/IAA proteins. Nature, 414: 271-276.

HARDELAND, R., Pandi-Perumal, S.R., Cardinali, D.P. (2006). Molecules in focus: melatonin. Int J Biochem Cell Biol ,38: 313-316.

OONO, Y., Chen, Q.G., Overvoorde, P.J., Kohler, C., Theologis, A. (1998). Age mutants of Arabidopsis exhibit altered auxin-regulated gene expression. Plant Cell, 10:1649-1662.

PELAGIO-FLORES, R., Ortiz-Castro, R., Méndez-Bravo, A., Macıas-Rodrıguez,L.,

LOPEZ-BUCIO, J. (2011). Serotonin, a tryptophan-derived signal conserved in plants and animals, regulates root system architecture probably acting as a natural auxin inhibitor in Arabidopsis thaliana. Plant Cell Physiol ,52:490–508.

PELAGIO-FLORES, R., Muñoz-Parra, E., Ortiz-Castro, R., López-Bucio, J. (2012). Melatonin regulates Arabidopsis root system architecture likely acting independently of auxin signalling. Journal of Pineal Research, 53:279–288.

RAMAKRISHNA, A., Giridhar,P., Ravishankar G. A. (2011). Phytoserotonin: a review. Plant Signaling and Behavior, 6:800–809.

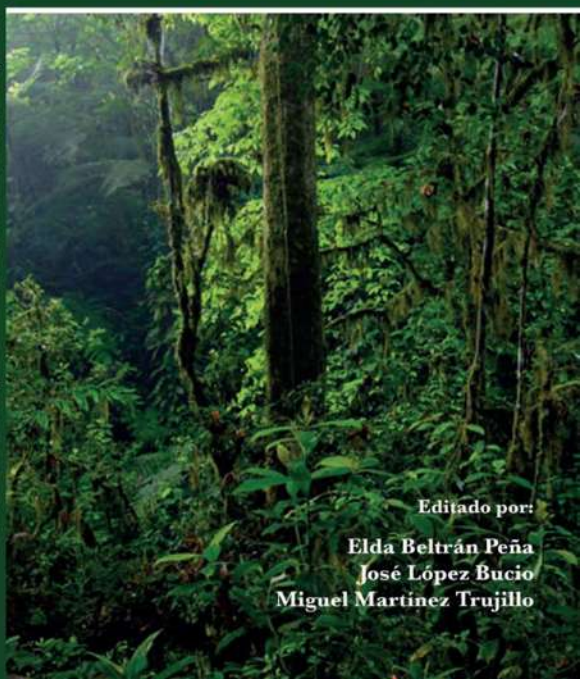
ULMASOV, T., Murfett, J., Hagen, G., Guilfoyle,T. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell, 9: 1963-1971.

WOODWARD, A.W., Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann Bot, 95: 707-735.

... Los tres primeros capítulos de este libro presentan una revisión actualizada de las vías de señalización de las principales fitohormonas, haciendo un énfasis especial en las auxinas, ya que éstas participan prácticamente en la regulación de todos los procesos del desarrollo vegetal, desde la embriogénesis hasta la senescencia. Es tal su importancia, que en el capítulo tres se analiza su antagonismo con las citocininas para regular el mantenimiento de los meristemos de la raíz y de los brotes apicales, y permitir el crecimiento y desarrollo continuo de las plantas.

Fronteras en la biología: señalización y comunicación de las plantas

FRONTERAS EN LA BIOLOGÍA:
SEÑALIZACIÓN Y COMUNICACIÓN DE LAS PLANTAS



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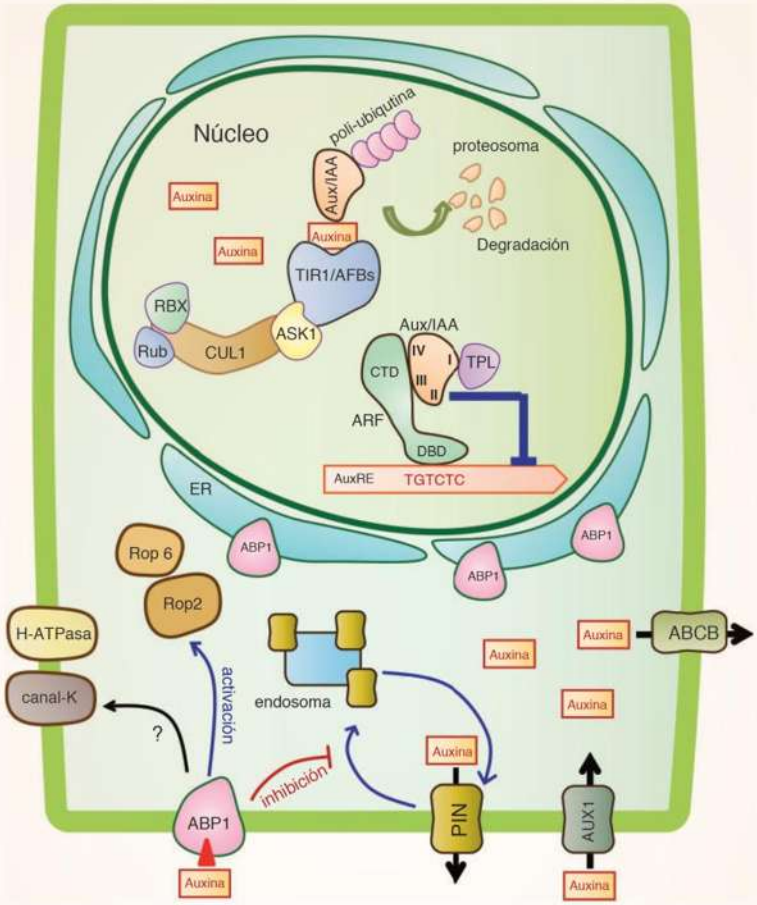

CAPÍTULO 2

Las auxinas: hormonas que coordinan el crecimiento y desarrollo de las plantas y su interacción con el ambiente

Las auxinas: hormonas que coordinan el crecimiento y desarrollo de las plantas y su interacción con el ambiente

Elizabeth García Cárdenas, Edith Muñoz Parra, José López Bucio y Elda Beltrán Peña

Las plantas son organismos multicelulares sésiles que responden a estímulos ambientales con una gran plasticidad fenotípica, modificando así la arquitectura de sus raíces y del follaje para optimizar los recursos disponibles en su ambiente. Las auxinas son los reguladores del crecimiento vegetal más estudiados, debido a que controlan las diferentes etapas del ciclo de vida incluyendo la embriogénesis, los tropismos, la organogénesis de la raíz y del follaje, la resistencia al ataque de patógenos, así como la senescencia. La auxina natural más abundante es el ácido indol-3-acético (AIA), molécula que se produce en los brotes, en los meristemos y en los ápices de las hojas y se transporta a diferentes tejidos por proteínas acarreadoras localizadas en la membrana plasmática. En este artículo se discute la información más relevante sobre los procesos del crecimiento y desarrollo de las plantas en los que participan las auxinas, así como sus mecanismos de transporte e interacciones a nivel molecular.

Introducción

Las plantas son organismos autótrofos, es decir, fabrican sus propios alimentos a partir de la luz del sol, agua y nutrimentos minerales que toman del suelo. Aunque tradicionalmente se les ha diferenciado de los animales por su naturaleza sésil, cada vez resulta más evidente su enorme capacidad para adaptarse a las fluctuaciones en las condiciones ambientales cambiando su morfología. Esta propiedad se conoce como plasticidad fenotípica, e incluye la recepción, decodificación e integración de señales que provienen de factores abióticos y bióticos como la intensidad lumínica, la disponibilidad de agua y nutrientes, los cambios en la temperatura, las condiciones fisicoquímicas del suelo y la presencia de microorganismos (Vanneste y Friml, 2013; Satbhai *et al.*, 2015). Diversos compuestos también conocidos como fitohormonas, entre los que se incluye a las citocininas, los brasinoesteroides, el etileno, el ácido salicílico, el ácido abscísico, las estrigolactonas, el ácido jasmónico, las giberelinas y las auxinas coordinan las respuestas de los tejidos a las señales del ambiente con los procesos de crecimiento y desarrollo subyacentes (Depuydt y Hardtke, 2011). Las auxinas desempeñan un papel preponderante en la organogénesis (que va desde la germinación hasta la senescencia), ya que promueven la formación de las raíces laterales, el mantenimiento de los meristemos apical y radicular, la formación de yemas axilares y de los tejidos vasculares, los tropismos y el desarrollo del fruto. El concepto clásico de regulación hormonal implica que una molécula se sintetiza en un órgano desde donde se moviliza a otros tejidos dentro del organismo, estimulando una respuesta celular. En el caso de las auxinas, éstas se sintetizan en los meristemos y hojas jóvenes, y se transportan a zonas adyacentes o distantes mediante proteínas presentes en las membranas de las células.

La respuesta celular a las auxinas ocurre mediante su percepción por un grupo de proteínas que actúan como receptores, que están localizadas en el núcleo de las células. Sí, en el núcleo, este hallazgo fue sorpresivo porque en la mayor parte de los organismos, los receptores de señales típicamente se localizan en la membrana plasmática. Los receptores de auxinas forman parte de un complejo cuya función consiste en modificar a otras proteínas (las Aux/IAA, represoras de los factores de transcripción ARF) mediante la adición de residuos de ubiquitina; este marcaje permite un recambio activo al degradarse las Aux/IAA por el proteosoma, un complejo enzimático ubicuo en eucariontes. Particularmente relevante es el recambio de los factores de transcripción que determinan el fenotipo, las respuestas ambientales y la expresión de genes. Cuando los factores de transcripción ARF quedan libres, se unen a los promotores de los genes cuyos productos regulan el crecimiento y desarrollo vegetal, modulando la división, elongación y diferenciación (Ortiz-Castro *et al.*, 2009).

Las auxinas naturales principales son el ácido indol-3-acético (AIA) y el ácido indol-3-butírico (AIB) (Korasick *et al.*, 2013). También existen diversos compuestos sintéticos con actividad auxínica que por sus propiedades se han usado tradicionalmente como herbicidas en la agricultura y horticultura, algunos ejemplos son el ácido 2,4-diclorofenoxiacético (2,4-D), el ácido 2,4,5-triclorofenoxiacético (2,4,5-T), el ácido 4-amino-3,5,6-tricloro-2-picolínico (picloram) y el ácido 3,6-dicloro-2-metoxibenzoico (dicamba) (Ljung, 2013). Las aplicaciones inherentes del conocimiento sobre el funcionamiento de las auxinas van desde la posibilidad de cultivar plantas o algunas de sus partes *in vitro*, hasta la formulación de agroquímicos con impacto en el manejo de los cultivos.

1. Homeostasis de las auxinas: biosíntesis, conjugación y degradación

Las auxinas son moléculas pequeñas y móviles, que se acumulan en los tejidos vegetales en respuesta a una amplia variedad de estímulos. Se han descrito dos rutas de biosíntesis del AIA, una dependiente del triptófano y otra independiente de este aminoácido. La primera vía involucra varios intermediarios como la indol-3-acetamida (IAM), el ácido indol-3-pirúvico (IPA), la indol-3-acetaldoxima (IAOX), y la triptamina (TAM). La vía independiente de triptófano no ha sido completamente dilucidada aunque se propone como precursor un compuesto indólico (Finet y Jaillais, 2012). Recientemente, se sugirió que una proteína que sintetiza indol, de localización citosólica, es clave en la vía independiente de triptófano, y tiene un papel preponderante en la embriogénesis de las plantas (Wang *et al.*, 2015). La inactivación mediante conjugación es una forma de regulación de los niveles de AIA, debido a que altas concentraciones de auxinas son tóxicas para la planta, lo que explica que diferentes compuestos se puedan usar como herbicidas. Las formas conjugadas del AIA se clasifican en tres grupos: i) de bajo peso molecular, incluye la adición de azúcares, ii) de bajo peso molecular, en cuyo caso la auxina se modifica con la adición de aminoácidos y iii) de alto peso molecular, lo cual ocurre mediante la adición de péptidos. La vía más conocida en la conjugación del AIA con aminoácidos involucra a la familia de genes *GRETCHEN HAGEN* (*GH3*) que codifican amido sintasas y amido hidrolasas (Kramer y Ackelsberg, 2015). Los genes *GH3* se encuentran ampliamente distribuidos en el reino vegetal y las mutaciones con ganancia o pérdida de función de

estos genes afectan el crecimiento y desarrollo, como sucede en la mutante con ganancia de función *ydk1-D*, que presenta una reducción en la raíz primaria, en el número de raíces laterales y en la dominancia apical (Westfall *et al.*, 2010). El AIB y el éster metil inactivo del AIA (MeAIA) pueden almacenarse e interconvertirse en auxinas activas (Korasick *et al.*, 2013). La familia de genes *ILR1/ILL*, que codifican para hidrolasas de aminoácidos, posibilitan el catabolismo de algunos conjugados, con lo que se mantiene un balance en los niveles de auxinas conjugadas y libres (Kramer y Ackelsberg, 2015). La inactivación irreversible de los conjugados con azúcares se lleva a cabo mediante oxidación cuando los niveles de auxinas se incrementan. Los metabolitos que son producto de dicha degradación se han identificado como el ácido 2-oxoindol-3-acético (oxAIA) y glucosa (oxAIA-Glu) (Ljung, 2013).

2. Distribución de las auxinas

El transporte de auxinas en las plantas se lleva a cabo mediante dos vías distintas: i) el AIA que se distribuye hacia zonas lejanas de los sitios de biosíntesis se mueve a través del floema con el flujo de fotosintatos, y ii) el transporte polar de auxinas (TPA) se lleva a cabo entre tejidos, célula a célula y ocurre por la acción de proteínas membranales de entrada y salida (Petrásek y Friml, 2009). Los acarreadores de entrada pertenecen a la familia AUX1/LIKE AUX1 (AUX1/LAX) y se identificaron con la caracterización de una mutante agravitrópica llamada *auxin resistant 1* (*aux1*) de *Arabidopsis* (Taiz y Zeiger, 2002). Por otra parte, la salida de auxinas de las células está mediada por proteínas transportadoras de la familia PIN (PIN-FORMED), que contienen de 10 a 12 segmentos transmembranales separados por una región hidrofílica. En *Arabidopsis thaliana* se han reportado ocho proteínas PIN, las PIN 1, 3, 4 y 7 son importantes durante la organogénesis y en las respuestas gravitrópicas de la raíz (Barbosa y Schwechheimer, 2014; Adamowski y Friml, 2015). A diferencia de las PIN de la membrana plasmática, las PIN 5, 6 y 8 se localizan en el retículo endoplásmico, junto con los transportadores denominados LIKE PIN (PILS) (Barbez *et al.*, 2012). Los métodos para estudiar la localización de las PIN y la determinación de las concentraciones endógenas de auxinas durante los procesos del desarrollo se basan en el uso de inhibidores del transporte de entrada a las células como el ácido 1-naftoxiacético (1-NOA), el ácido 2-naftoxiacético (2-NOA), el ácido 3-cloro-4-hidroxifenil acético (CHPAA) y la cromosaponina natural; así como de inhibidores de salida, entre los que se incluyen moléculas sintéticas como el ácido 1-naftilftalámico (NPA), ácido 2,3,5-triiodobenzoico (TIBA) y algunos flavonoides naturales (Hosek *et al.*, 2012). Los flavonoides son un grupo de compuestos polifenólicos que resultan del metabolismo secundario de las plantas y que al modular el transporte de auxinas afectan los tropismos (Petrásek y Friml, 2009). Recientemente, se ha descrito otra familia de transportadores de salida de auxinas de las células denominada ABCB/MDR/PGP, que transportan diferentes tipos de moléculas y cuya sobreproducción confiere resistencia a drogas o toxinas; aquí se incluye a las proteínas ABCB 1, 4, 19, 15 y 21 involucradas en la formación de pelos radiculares, de raíces adventicias y el fototropismo (Retzer *et al.*, 2014). Las PGPs pertenecen a una subfamilia de los transportadores ABCB que se descubrieron inicialmente en líneas cancerígenas de mamíferos, donde su sobreexpresión confería resistencia a fármacos (Cho y Cho, 2013). En las plantas, se sugirió que dichos transportadores funcionan como bombas de eflujo con acción inespecífica, después se les atribuyó un papel en el transporte de auxinas en monocotiledóneas y dicotiledóneas. Como un ejemplo de la importancia de este grupo de proteínas, se resalta el hecho que la mutante *atpgp1*, deficiente en el transportador PGP1 presenta reducción en el desarrollo de la raíz, transporte de auxinas disminuido y pérdida del gravitropismo (Geisler y Murphy, 2006). El modelo vigente sugiere que las proteínas PIN se distribuyen desde organelos internos como el retículo endoplásmico hacia la membrana dentro de vesículas secretoras (Finet y Jaillais, 2012). La brefeldina A (BFA) es una toxina que inhibe el flujo de las vesículas y promueve así la acumulación del transportador PIN1 en compartimentos internos. Las proteínas ARF-GEF GNOM controlan el tráfico vesicular y en consecuencia el transporte de auxinas, causando diferentes anormalidades en el desarrollo. Por ejemplo, las mutantes *gnom* muestran defectos desde la embriogénesis que conducen a la malformación de órganos (Adamowski y Friml, 2015). Existen otras proteínas que regulan la distribución vesicular de los transportadores como la cinasa PINOID (PID/WAG) y la fosfatasa PP2A, necesarias para re-direccionar el flujo de auxinas (**Fig. 1**).

3. Señalización de las auxinas

La percepción de las auxinas inicia con su unión al receptor TIR1 (TRANSPORT INHIBITOR RESPONSE1), localizado en el núcleo de las células. Estas moléculas actúan como un "pegamento molecular" y posibilitan la interacción del receptor con otras proteínas (Guilfoyle, 2007). En particular, TIR1 es un co-receptor asociado con otras proteínas para formar un complejo denominado SKP1-Cul1-F-Box ($SCF^{TIR1/AFB}$) (Calderón *et al.*, 2012). Cuando los niveles de auxinas son bajos, las proteínas represoras

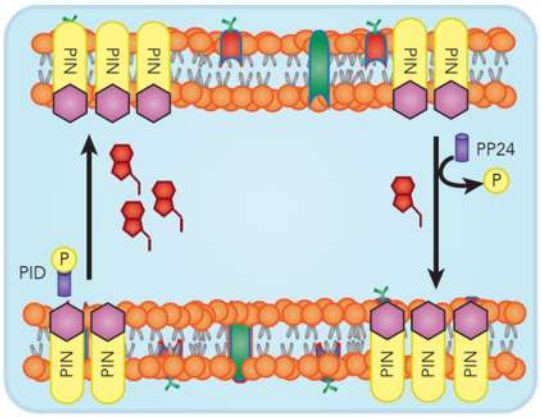

Figura 1. Localización de los transportadores de las auxinas de la familia PIN. El transporte de dichas proteínas desde los sitios de biosíntesis en el retículo endoplásmico hacia la membrana plasmática está mediado por reacciones de fosforilación que regulan la localización del transportador y por lo tanto la concentración intracelular de las auxinas.

Aux/IAA forman multímeros con los factores de transcripción de respuesta a auxinas denominados ARFs (AUXIN RESPONSE FACTORS) mediante interacciones proteína-proteína, bloqueando así la transcripción de genes de respuesta a auxinas. Al aumentar la concentración de auxinas se promueve la ubiquitinación del represor Aux/IAA y entonces ocurre su degradación por el proteosoma 26S, dejando los ARF libres para la activación de los promotores de los genes de respuesta a auxinas. Interesantemente, se encontró un dominio de dimerización (DD) en los ARFs que al unirse a la secuencia AuxREs de los promotores forman dímeros para activar la expresión de genes correspondiente a las familias *GRETCHEN HAGEN* (*GH3*), *Small Auxin Up Regulated* (*SAUR*) y *Aux/IAA* (Tian *et al.*, 2002). Recientemente se reportó que los componentes en la señalización de auxinas también pueden presentar modificaciones pos-traduccionales que afectan la vía. La nitración de TIR1 conduce a la degradación de los represores Aux/IAA, en tanto que los ARFs pueden ser fosforilados por la cinasa BIN2 (BRASSINOSTEROID-INSENSITIVE2) suprimiendo con ello su interacción con los represores Aux/IAA y promoviendo entonces la transcripción de los genes de respuesta a auxinas (Wang y Estelle, 2014) (**Fig. 2**). A nivel transcripcional, el co-receptor TIR1/AFB-Aux/IAA controla la vía de señalización auxínica, sin embargo, existen procesos que requieren respuestas rápidas a las auxinas que no involucran la transcripción *de novo* de los genes (Vanneste y Friml, 2013). ABP1 es una proteína de 22 kDa que contiene un péptido señal de retención en el retículo endoplásmico; estudios iniciales sugirieron un papel esencial para esta proteína en casi todos los aspectos del desarrollo de las plantas, controlando la división y expansión celular (Sauer y Kleine, 2011). Sin embargo, un estudio controversial publicado por Gao *et al.* (2015), mostró que ABP1 no era un componente en la señalización de auxinas ni tampoco jugaba un papel importante en la regulación del desarrollo, debido a que las mutantes *abp1* presentaban un fenotipo normal. En conclusión, parece ser que las investigaciones de la función de ABP1 han regresado al siglo XX, por lo que valdría la pena revisar la hipótesis propuesta desde la década de los 60's de que los transportadores PIN podrían ser los receptores de las auxinas (Strader y Zhao, 2016).

4. Eventos del crecimiento y desarrollo vegetal regulados por auxinas

4.1. Embriogénesis

La formación del embrión se lleva a cabo por divisiones anticlinales del cigoto fertilizado. Desde esta etapa, la distribución de auxinas en forma de un gradiente apical-basal en la hipófisis conduce a la formación del meristemo radicular. En el estadio de corazón del embrión, la acumulación de auxinas se presenta mayoritariamente en los sitios de formación de los cotiledones, donde se diferencian grupos de células para formar el meristemo del follaje (Wendrich y Weijers, 2013). Durante la germinación, la primera estructura en salir de la testa de la semilla es la radícula, en la que ya se distinguen tres zonas bien definidas: meristemática, de elongación y de diferenciación. En el meristemo, las células se encuentran en división constante para posteriormente crecer y aumentar de volumen. En la zona de diferenciación se forman los pelos radiculares a partir de los tricoblastos de la epidermis y las raíces laterales que emergen de algunas células del pericíclo (Satbhal *et al.*, 2015).

Las raíces laterales permiten al sistema radical una mejor exploración del suelo para hacer más eficiente la captación de agua y nutrientes y son componentes clave para la adaptación de las plantas a los cambios en el ambiente (Lavenus *et al.*, 2013). En el desarrollo de estos órganos, el transporte de auxinas mediado por los transportadores PIN1 y PIN3 es necesario para regular el reflujo de auxinas entre la epidermis y el pericíclo. Cascada abajo en la señalización, el factor de transcripción (ARF5)/MONOPTEROS (MP) participa en la formación de las raíces laterales y activa la expresión de PIN1 a través de una retroalimentación positiva (Tian *et al.*, 2014).

4.2. Tropismos

Las auxinas están involucradas en la orientación del crecimiento de las plantas a estímulos de luz

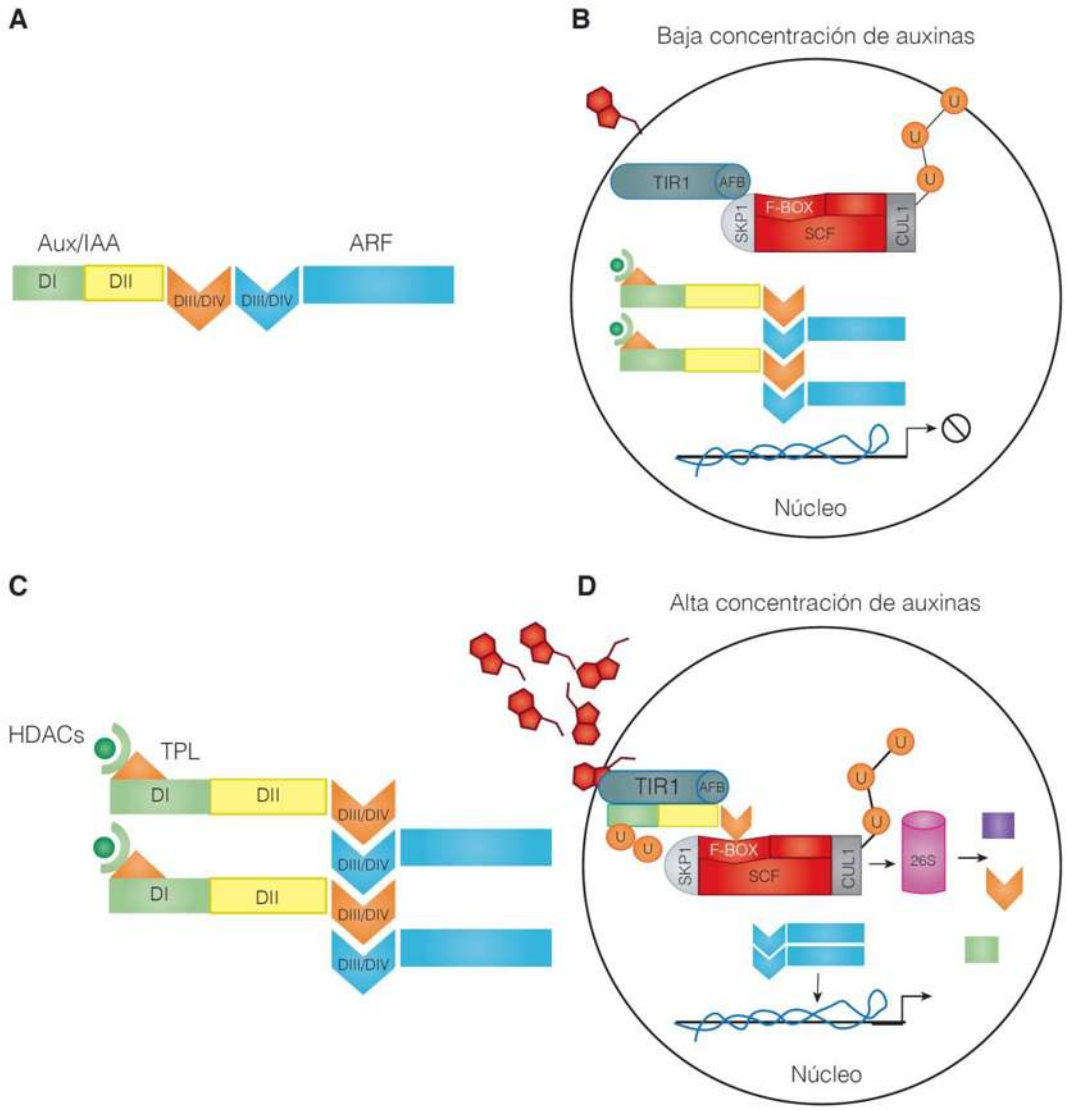

Figura 2. Señalización de las auxinas. **(A)** Interacción del represor Aux/IAA y el activador transcripcional ARF a través del dominio DIII/DIV. **(B)** Las auxinas, en bajas concentraciones, no son percibidas por el receptor nuclear TIR1/AFB/Aux-IAA. **(C)** El represor forma un complejo multimérico con el activador que interactúa por el dominio I con el co-represor TOPLESS (TPL), constituido por desacetilasas de histonas (HDACs) lo que resulta en la represión de la transcripción de genes de respuesta a auxinas. **(D)** El co-receptor percibe una alta concentración de auxinas y permite el acoplamiento y ubiquitinación del represor para su degradación en el proteosoma 26S, el activador queda libre y se dimeriza para unirse a los elementos AuxRE de los promotores para activar la transcripción de genes de respuesta a auxinas.

(fototropismo), tacto (tigmotropismo) y gravedad (gravitropismo) (Taiz y Zeiger, 2002). La gravedad es percibida por la raíz en las células de la cofia que contienen almidón, es ahí donde PIN3 se localiza después de dicha percepción. El flujo auxínico se dirige entonces desde la punta de la raíz hacia la zona de elongación. PIN2 y AUX1 son requeridos en esta ruta de transporte. Esto se dedujo al observar que las mutantes de *Arabidopsis* deficientes en cada uno de estos genes forman raíces agravitrópicas. Por otra parte, se ha propuesto que los transportadores ABCB podrían estar implicados en la respuesta a la gravedad, ya que las mutantes *abcb4*, *abcb19* y *abcb1* son agravitrópicas (Petrásek y Friml, 2009). El calcio

participa como un mensajero intracelular durante el gravitropismo inducido por auxinas, debido a que su acumulación colocaliza en la misma dirección del flujo auxínico conducido por PIN3 y PIN7 (Vanneste y Friml, 2013).

4.3. Funcionamiento de los meristemos

Para el mantenimiento del meristemo la tasa de diferenciación debe ser igual a la generación de nuevas células. Poco después de la germinación, el meristemo de la raíz alcanza un tamaño estable que se conserva en el tiempo, permitiendo el crecimiento indeterminado. El aumento de los niveles de la proteína SHY2, inducible por auxinas y su interacción con diversos reguladores del crecimiento como las citocininas, estrigolactonas, ácido abscísico, y brasinoesteroides permite el balance entre la división y la diferenciación celular requerido para el crecimiento y funcionalidad de la raíz (Pacifici *et al.*, 2015).

La formación de órganos a partir del meristemo apical del brote involucra a las auxinas (Vermoux *et al.*, 2010). Este meristemo apical está formado por tres capas, un centro organizacional (OC) rodeado por un grupo de células fuente con poca actividad proliferativa ubicado en la base de la zona central (CZ). También se reportan una zona basal/medular (RZ) y otra periférica (PZ) donde los nuevos órganos laterales se forman, incluyendo las yemas axilares y los primordios de hojas. La función que tienen las auxinas en la iniciación de primordios de raíces laterales y/o de las hojas depende de la localización de los transportadores PIN1 y AUX/LAX (Kalve *et al.*, 2014). Las auxinas también se coordinan con la señalización por citocininas a través de uno de los complejos que regulan el ciclo celular, CYCD/CDK, que al fosforilar a la proteína represora RB (RETINOBLASTOMA), libera al factor de transcripción E2F y entonces se induce la progresión de la fase G1 a la S (síntesis de DNA) del ciclo celular (Schaller *et al.*, 2015).

4.4. Interacción de la vía auxínica con el complejo MEDIADOR

La transcripción es un proceso catalizado por diversas proteínas que funcionan en sincronía. En eucariontes, las principales proteínas implicadas son la enzima ARN Polimerasa II, los Factores de Transcripción Generales (GTFs por sus siglas en inglés 'General Transcription Factors'), así como activadores y represores que actúan en *trans*, y el Complejo MEDIADOR (MED) (Kim *et al.*, 1994; Koleske y Young, 1994; Malik *et al.*, 2005). Desde su descubrimiento en *Saccharomyces cerevisiae*, las proteínas MED se han encontrado en casi todos los eucariontes (Boube *et al.*, 2002; Bourbon *et al.*, 2004; Bourbon, 2008). En *Arabidopsis thaliana*, se conservan 21 subunidades incluyendo las del dominio catalítico formado por MED12, MED13 y CDK8 (Wang y Chen, 2004; Backstrom *et al.*, 2007; Gillmor *et al.*, 2010; Ito *et al.*, 2011). Además de controlar el desarrollo de la planta, se ha demostrado que las subunidades MED modulan las respuestas a estrés biótico y abiótico, la floración y las vías de señalización hormonal, o contribuyen en funciones esenciales de los núcleos de las células como, la actividad de la helicasa del ADN, así como el empalme de intrones y exones del ARN mensajero denominado "splicing" (**Fig. 3**). Con los análisis de las mutantes afectadas en MED14, se ha evidenciado la participación del Complejo Mediador en la organización del tamaño de órganos vegetales y en la diferenciación celular, ya que dichas mutaciones ocasionan una desorganización del meristemo apical del brote (Autran *et al.*, 2002, Sundaravelpandian *et al.*, 2012). Además de MED14, las mutantes del módulo cinasa *med12*, *med13* y *cdk8* muestran fenotipos de diferenciación celular alterada durante el desarrollo (Wang y Chen, 2004; Gillmor *et al.*, 2010; Ito *et al.*, 2011) y se ven afectadas en la transición del embrión de la etapa globular al estadio de corazón (Gillmor *et al.*, 2010). El efecto de la mutación en *MED13* sobre la diferenciación celular ha sido explicado por una respuesta defectuosa a las auxinas, cuyo flujo direccional mediado por PIN1 es visiblemente anormal en la zona de división celular y en los cotiledones en desarrollo (Ito *et al.*, 2011). El gen *MED25*, también conocido como *PHYTOCHROME AND FLOWERING TIME1 (PFT1)*, fue descrito como un regulador del proceso de adaptación conocido como Síndrome de Evasión de la Sombra (SAS por sus siglas en inglés 'Shade Avoidance Syndrome') que promueve la floración en respuesta a cambios en la calidad de la luz (Cerdan y Chory, 2003) y podría estar relacionado directamente a una respuesta auxínica a través de la participación de PIN3 (Keuskamp *et al.*, 2010). El reporte más reciente de la participación de PFT1/MED25 en el desarrollo de las plantas mostró que la pérdida de su función estimula el crecimiento de la raíz primaria y raíces laterales, así como la formación *de novo* de raíces laterales y adventicias; por el contrario, y coincidentemente la sobreexpresión de PFT1/MED25 redujo estas respuestas, lo que sugiere que PFT1/MED25 es un elemento importante en la proliferación celular del meristemo y en el control del tamaño celular en la raíz primaria y las raíces laterales (Raya-González *et al.*, 2014). Estos autores, también demostraron que PFT1/MED25 regula negativamente el transporte de auxinas a través de la modulación de la expresión y distribución del transportador PIN1, y la expresión de genes de respuesta a auxinas en toda la planta (Raya-González *et al.*, 2014).

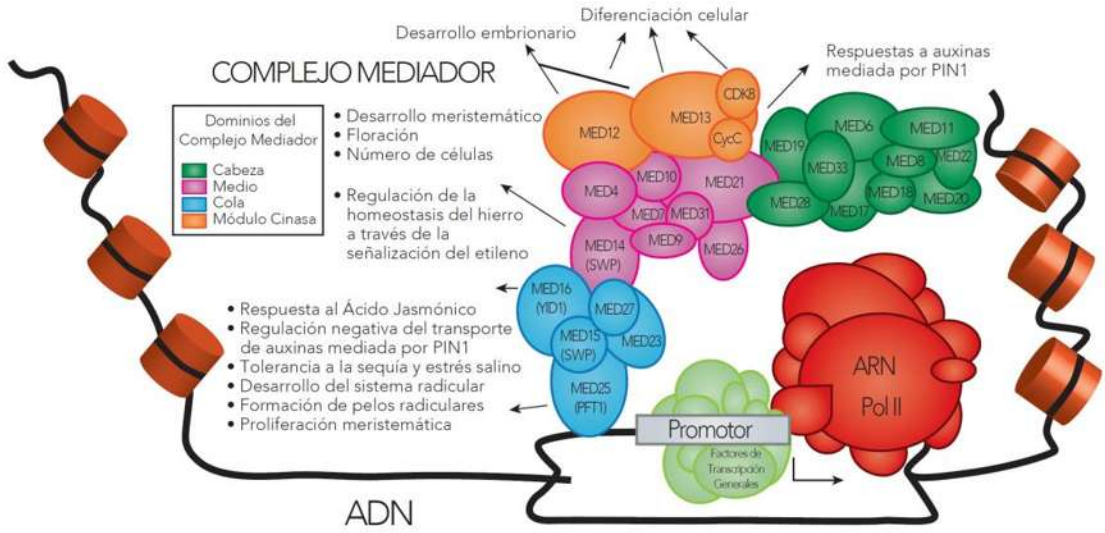

Figura 3. El Complejo MEDIADOR como componente importante de la maquinaria transcripcional eucariota. Algunas subunidades específicas son importantes para la regulación de diversos procesos del desarrollo vegetal. En particular, la subunidad MED25 participa en la respuesta auxínica y en los procesos celulares subyacentes, como la formación de los pelos radiculares y de las raíces laterales.

Conclusiones

Las plantas pueden cambiar la arquitectura del follaje y de la raíz ante las fluctuaciones en las condiciones ambientales aumentando o disminuyendo las concentraciones endógenas de los reguladores del crecimiento vegetal como las auxinas. El ácido indol-3-acético puede ser producido por plantas y por diversos microorganismos que forman parte del microbioma vegetal, por lo que su participación en la interacción entre organismos de diferentes reinos es un área de investigación activa. La homeostasis de las auxinas en los tejidos vegetales involucra aspectos de biosíntesis, transporte, transducción de señales y metabolismo, cada uno de los cuáles resulta esencial para el desarrollo de las plantas. Con el advenimiento de técnicas de secuenciación masiva de transcritos y de identificación de proteínas y metabolitos a gran escala, de abre un panorama amplio para dilucidar la forma como las auxinas interactúan con otras fitohormonas para coordinar los programas de organogénesis que determinan la forma y función de los vegetales y su adaptación al ambiente.

Referencias

Adamowski M, Friml J (2015) PIN-dependent auxin transport: action, regulation, and evolution. Plant Cell 27: 20-32.

Autran D, Jonak C, Belcram K, Beemster GT, Kronenberger J, Grandjean O, Inzé D, Traas J (2002) Cell numbers and leaf development in *Arabidopsis*: a functional analysis of the STRUWWELPETER gene. EMBO J 21: 6036-6049.

Backstrom S, Elfving N, Nilsson R, Wingsle G, Bjorklund S (2007) Purification of a plant mediator from *Arabidopsis thaliana* identifies PFT1 as the Med25 subunit. Mol Cell 26: 717-729.

Barbez E, Kubes M, Rolcik J, Beziat C, Pencik A, Wang B, Rosquete MR, Zhu J, Dobrev PI, Lee Y, Zazimalova E, Petrasek J, Geisler M, Friml J, Kleine- Vehn J (2012) A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. Nature 485: 119–122.

Barbosa CRI, Schwechheimer C (2014) Dynamic control of auxin transport-dependent growth by AGCVIII protein kinases. Curr Opin Plant Biol 22: 108-115.

Boube M, Joulia L, Cribbs DL, Bourbon HM (2002) Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell 110: 143-151.

Bourbon HM (2008) Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional Mediator complex. Nucleic Acids Res 36: 3993-4008.

Bourbon H.M., Aguilera A., Ansari A.Z., Asturias F.J., Berk A.J., Bjorklund S., Blackwell T.K., Borggrefe T, Carey M, Carlson M, Conaway JW, Conaway RC, Emmons SW, Fondell JD, Freedman LP, Fukasawa T, Gustafsson CM, Han M, He X, Herman PK, Hinnebusch AG, Holmberg S, Holstege FC, Jaehning JA, Kim YJ, Kuras L, Leutz A, Lis JT, Meisterernest M, Naar AM, Nasmyth K, Parvin JD, Ptashne M, Reinberg D, Ronne H, Sadowski I, Sakurai H, Sipiczki M, Sternberg PW, Stillman DJ, Strich

R, Struhl K, Svejstrup JQ, Tuck S, Winston F, Roeder RG, Kornberg RD (2004) A unified nomenclature for protein subunits of Mediator complexes linking transcriptional regulators to RNA polymerase II. Mol Cell 14: 553–557.

Calderón-Villalobos LI, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M (2012) A combinatorial TIR1/AFB-Aux co-receptor system for differential sensing of auxin. Nat Chem Biol 8: 477-485.

Cerdan PD, Chory J (2003) Regulation of flowering time by light quality. Nature 423: 881-885.

Cho M, Cho TH (2013) The function of ABCB transporters in auxin transport. Plant Signal Behav 8: e22990.

Depuydt S, Hardtke SC (2011) Hormone signalling crosstalk in plant growth regulation. Current Biol 21: 365-373.

Finet C, Jaillais Y (2012) AUXOLOGY: When auxin meets plant evo-devo. Dev Biol 369: 19-31.

Flanagan PM, Kelleher RJ, Feaver WJ, Lue NF, LaPointe JW, Kornberg RD (1990) Resolution of factors required for the initiation of transcription by yeast RNA polymerase II. J Biol Chem 265: 11105–11107.

Gao Y, Zhang Y, Zhang D, Dai X, Estelle M, Zhao Y (2015) Auxing binding protein 1 (ABP1) is not required for either auxing signaling or *Arabidopsis* development. Proc Natl Acad Sci 112: 2275-2280.

Geisler M, Murphy SA (2006) The ABC of auxin transport: The role of p-glycoproteins in plant development. FEBS Lett 580: 1094-1102.

Gillmor CS, Park MY, Smith MR, Pepitone R, Kerstetter RA, Poethig RS (2010) The MED12-MED13 module of Mediator regulates the timing of embryo patterning in Arabidopsis. Development 137: 113-122.

Guilfoyle T (2007) Sticking with auxin. Nature 446: 621-622.

Hosek P, Kubes M, Lanková M, Dobrev PI, Klima P, Kohoutová M, Petrásek J, Hoyerová K, Jirina M, Zazimalová EM (2012). Auxin transport at cellular level: new insights supported by mathematical modelling. J Exp Bot 63: 3815-3828.

Ito J, Sono T, Tasaka M, Furutani M (2011) MACCHI-BOU 2 is required for early embryo patterning and cotyledon organogenesis in *Arabidopsis*. Plant Cell Physiol 52: 539-552.

Kalve S, De Vos D, Beemster TSG (2014) Leaf development: a cellular perspective. Frontiers Plant Sci 5: 362 1-25.

Keuskamp DH, Pollmann S, Voesenek LACJ, Peeters AJM, Pierik R (2010) Auxin transport through PIN-FORMED 3 (PIN3) controls shade avoidance and fitness during competition. Proc Natl Acad Sci 107: 22740-22744.

Kim YJ, Björklund S, Li Y, Sayre MH., Kornberg RD (1994) A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell 77: 599-608.

Koleske AJ, Young RA (1994) An RNA polymerase II holoenzyme responsive to activators. Nature 368: 466-469.

Korasick AD, Enders AT, Strader CL (2013) Auxin biosynthesis and storage forms. J Exp Bot 64: 2541-2555.

Kramer ME, Ackelsberg ME (2015) Auxin metabolism rates and implications for plant development. Front Plant Sci 6: 150.

Lavenus J, Goh T, Roberts I, Guyomarc S, Lucas MI, De Smet I, Fukaki H, Beeckman T, Bennett M, Laplaze L (2013) Lateral root development in *Arabidopsis*: fifty shades of auxin. Trends Plant Sci 1059: 1-9.

Ljung K (2013) Auxin metabolism and homeostasis during plant development. Development 140: 943-950.

Malik S, Baek HJ, Wu W, Roeder RG (2005) Structural and functional characterization of PC2 and RNA polymerase II-associated subpopulations of metazoan Mediator. Mol Cell Biol 25: 2117-2129.

Ortiz-Castro R, Contreras-Cornejo HA, Macías-Rodríguez L, López-Bucio J (2009) The role of microbial signals in plant growth and development. Plant Signal Behav 4: 701-712.

Pacifici E, Polverari L, Sabatini S (2015) Plant hormone cross talk: the pivot of root growth. J Exp Bot 66: 1113-1121.

Petrásek J, Friml J (2009) Auxin transport routes in plant development. Development 136: 2675-2688.

Raya-González J, Ortiz-Castro R, Ruíz-Herrera LF, Kazan K, López-Bucio J (2014) PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 regulates lateral root formation via auxin signaling in *Arabidopsis*. Plant Physiol. 165: 880-894.

Retzer K, Butt H, Korbei B (2014) The far side of auxin signaling: fundamental cellular activities and their contribution to a defined growth response in plants. Protoplasma 251: 731-746.

Satbhal BS, Ristova D, Busch W (2015) Underground tuning: quantitative regulation of root growth. J Exp Bot 66: 1099-1112.

Sauer M, Kleine VJ (2011) AUXING BINDING PROTEIN1: The outsider. Plant Cell 23: 2033-2043.

Schaller EG, Bishoop A, Kieber JJ (2015) The yin-yang of hormones: cytokinin and auxin interactions in plant development. Plant Cell 27: 44-63.

Strader LC, Zhao Y (2016) Auxin perception and downstream events. Curr Opin Plant Biol 33: 8–14

Sundaravelpandian K, Chandrika NN, Schmidt W (2012) PFT1, a transcriptional Mediator complex subunit, controls root hair differentiation through reactive oxygen species (ROS) distribution in Arabidopsis. New Phytol 197: 151-161.

Taiz L, Zeiger E (2002) Plant Physiol. 3rd. Ed. 690 pp.

Tian H, De Smet I, Ding Z (2014) Shaping a root system: regulating lateral versus primary root growth. Trends Plant Sci 1137: 1-6.

Tian Q, Uhlir JN, Reed WJ (2002) Arabidopsis SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell 14: 301-319.

Vanneste S, Friml J (2013) Calcium: The missing link in auxin action. Plant 2: 650-675.

Vermoux T, Besnard F, Traas J (2010) Auxin at the shoot apical meristem. Cold Spring Harbor Perspective Biology 2: a001487.

Wang B, Chu J, Yu T, Xu Q, Sun X, Yuan J, Xiong G, Wang G, Wang Y, Li J (2015) Tryptophan-independent auxin biosynthesis contributes to early embryogenesis in *Arabidopsis*. Proc Natl Acad Sci 112: 15: 4821-4826.

Wang R, Estelle M (2014) Diversity and specificity: auxin perception and signaling through the TIR/AFB pathway. Curr Opin Plant Biol 21: 51-58.

Wang W, Chen X (2004) HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in *Arabidopsis*. Development 131: 3147-3156.

Wendrich JR, Möller BK, Uddin B, Radoeva T, Lokerse AS, De Rybel B, Weijers D (2015) A set of domain-specific markers in the *Arabidopsis* embryo. Plant Reprod 28:153-160.

Wendrich RJ, Weijers D (2013) The *Arabidopsis* embryo as a miniature morphogenesis model. New Phytol 199: 14-25.

Westfall SC, Herrmann J, Chen Q, Wang S, Jez MJ (2010) Modulating plant hormones by enzyme action. The GH3 family of acyl acid amido synthetases. Plant Signal Behav 5: 1607-1612.