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"Participación de PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 en el desarrollo de la raíz y la respuesta a auxinas en *Arabidopsis thaliana*"

Tesis que presenta:

Mtro. en Ciencias. Javier Raya González

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Asesor de Tesis:

Dr en Ciencias José López Bucio

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1. RESUMEN

El sistema radicular de las plantas desempeña un papel importante en la absorción de agua y nutrientes, la interacción con microorganismos de la rizósfera y la tolerancia al estrés. El complejo Mediador es un complejo multiproteínico conservado en organismos eucariontes, el cual participa como un adaptador entre los factores de transcripción y la RNA polimerasa II. Mutaciones con pérdida de función en *PHYTOCHROME AND FLOWERING TIME1*/*MEDIATOR25* (*PFT1/MED25*), un componente del complejo Mediador, incrementan el crecimiento de la raíz primaria y las raíces laterales, y la formación de raíces laterales y adventicias, afectando la división y la elongación celular. De manera opuesta, la sobreexpresión de *PFT1/MED25* reduce estas respuestas, indicando que PFT1/MED25 es un importante elemento de la proliferación celular meristemática y del control del tamaño celular tanto en raíz primaria como raíces laterales. El estudio de marcadores de genes de respuesta a auxinas, indicaron que PFT1/MED25 regula negativamente la expresión de genes de respuesta y transporte de auxinas, evidenciado por un incremento y una disminución de los marcadores asociados a auxinas *PIN-FORMED1* (*PIN1*)*::PIN1::GFP* (proteína verde fluorescente), *DR5:GFP*, *DR5:uidA* y *BA3:uidA* en mutantes *pft1-2* y plantas *35S:PFT1,* respectivamente. Sin embargo, no se encontró ningún cambio en los niveles endógenos de auxinas en plantas mutantes *pft1-2* o en plantas *35S:PFT1*. El análisis detallado de la actividad de *DR5:uidA*, *DR5:GFP* y *BA3:uidA* en respuesta al ácido indol-3-acético, al ácido naftalen acético y al inhibidor del transporte polar de auxinas, el ácido N-naftiltalámico indicó que PFT1/MED25 regula principalmente el transporte y la respuesta a auxinas. En conjunto, estos resultados sugieren la participación de PFT1/MED25 en la regulación de la arquitectura del sistema radicular a través de un mecanismo dependiente de la señalización por auxinas. Este estudio abre nuevas perspectivas de investigación para determinar la participación del complejo Mediador en los diferentes programas de desarrollo de la raíz, así como en respuesta a múltiples señales ambientales.

Palabras clave: *Arabidopsis thaliana*, raíces laterales, complejo Mediador, auxinas, ácido jasmónico.

2. ABSTRACT

The root system architecture is a major determinant of water and nutrient acquisition, interaction with rhizosphere microorganisms, and stress tolerance in plants. The Mediator complex is a conserved multiprotein complex, which acts as an adaptor between transcription factors and the RNA polymerase II. Loss-of-function mutations in *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*)/*MED25*, a component of Mediator complex, increase primary and lateral root growth as well as lateral and adventitious root formation, by affecting cell division and elongation. In contrast, *PFT1*/*MED25* overexpression reduces these responses, suggesting that PFT1/MED25 is an important element of meristematic cell proliferation and cell size control in both lateral and primary roots. Analysis of auxin-responsive gene expression markers showed that PFT1/MED25 negatively regulates auxin transport and response gene expression, as evidenced by increased and decreased expression of the auxin-related reporters *PIN-FORMED1 (PIN1)::PIN1::GFP* (for green fluorescent protein), *DR5:GFP*, *DR5:uidA*, and *BA3:uidA* in *pft1-2* mutants and *35S:PFT1* seedlings, respectively. However, no alterations in endogenous auxin levels could be found in *pft1-2* mutants or in *35S:PFT1*-overexpressing seedlings. Detailed analysis of *DR5:GFP*, *DR5:uidA*, and *BA3:uidA* activity in wild-type, *pft1-2*, and *35S:PFT1* seedlings in response to indole-3-acetic acid, naphthaleneacetic acid, and the polar auxin transport inhibitor 1-*N*-naphthylphthalamic acid indicated that PFT1/MED25 principally regulates auxin transport and response. Taken together, these results provide compelling physiological, molecular and genetic evidence for a role for PFT1/MED25 as an important transcriptional regulator of root system architecture through auxin-related mechanisms in *Arabidopsis*. This work opens a research field to determinate the participation of Mediator complex subunits on root morphogenetic programs as well as multiples environmental cues.

3. INTRODUCCIÓN

Las raíces de las plantas son órganos que típicamente se encuentran debajo de la superficie del suelo, en donde crecen y responden a una amplia variedad de barreras y estímulos ambientales. Las raíces no solo proporcionan apoyo estructural a la parte aérea de las plantas, sino además, establecen interacciones bióticas y abióticas con la rizósfera y modulan la adquisición de agua y nutrientes, los cuales son vitales para su crecimiento. Así, el crecimiento global de las plantas depende de un óptimo crecimiento, desarrollo y funcionamiento de la raíz. Por lo tanto, es de suma importancia estudiar y entender los mecanismos, fisiológicos, celulares y moleculares involucrados en los procesos que son regulados por las raíces. La mayoría de las especies de plantas, poseen un sistema radicular altamente complejo, por lo que la caracterización de los mecanismos básicos de su desarrollo son difíciles de abordar. Gran parte del conocimiento que se tiene hasta la fecha sobre el desarrollo de la raíz, ha sido mediante el uso del organismo modelo *Arabidopsis thaliana*. La raíz de *Arabidopsis* posee una organización celular sencilla y puede crecer bajo condiciones de laboratorio, lo cual facilita su análisis. El sistema radicular de *Arabidopsis* está formado por una raíz primaria, raíces laterales, raíces adventicias y pelos radiculares. El desarrollo de la raíz primaria inicia durante la embriogénesis a través de un grupo de células embrionarias llamado hipófisis. Posteriormente, este grupo de células formarán el centro quiescente (QC, por sus singlas en inglés), el cual activará procesos de división celular para dar lugar al meristemo de la raíz primaria. De manera opuesta, la iniciación de raíces laterales es un proceso post-embrionario, el cual consiste en la activación de un grupo determinado de células del pericicIo, las cuales se dividen asimétricamente para formar primordios de raíces laterales (PRL), quienes mediante eventos de divisiones celulares periclinales y anticlinales formaran las raíces laterales (RL) (Malamy y Benfey, 1997).

El desarrollo de las plantas está regulado por factores fisiológicos internos y señales ambientales, los cuales regulan la división, la expansión, la diferenciación

y la muerte celular. Para controlar su desarrollo, las plantas producen compuestos de diferente identidad química, llamados reguladores del desarrollo vegetal o fitohormonas, los cuales están involucrados en los diferentes procesos celulares (Tabla 1). Se sabe que las auxinas participan prácticamente en cada aspecto del desarrollo de las plantas, incluyendo, la regulación del sistema radicular. La auxina principal es el ácido indol acético (AIA), la cual desempeña una función esencial en los procesos de formación de raíces laterales (Casimiro et al., 2003; De Smet et al., 2006; Fukaki et al., 2007). La aplicación de AIA o auxinas sintéticas como el ácido naftalen acético (ANA) estimulan la formación de RL, mientras que tratamientos con inhibidores del transporte polar de auxinas como el ácido N-naftiltalámico (NPA, por sus siglas en inglés) las bloquean (Casmiro et al., 2001). Los sitios de biosíntesis de auxinas proveen del regulador a todos los tejidos, su transporte genera un gradiente o acumulación local y finalmente la percepción o respuesta afectará el desarrollo de las plantas. A distancias cortas, las auxinas son transportadas célula-célula mediante transportadores de influjo y eflujo (Friml, 2003). Los transportadores de eflujo de auxinas PIN, dirigen el transporte requerido para mantener un máximo de auxinas en el desarrollo de la raíz. Se sabe que alteraciones sobre la expresión de los *PINs* pueden afectar la actividad meristemática de la raíz, la diferenciación del tejido vascular, las respuestas a gravedad y la organogénesis de la raíz (Blilou et al., 2005).

Una vez que las señales son percibidas y procesadas por las plantas se activa una cascada de transducción de señales que culmina con el proceso de transcripción. Durante la transcripción, un gran número de proteínas participan, las cuales deben de funcionar en sincronía. En eucariontes, las proteínas principales involucradas son: la holoenzima RNA polimerasa II (Pol II), los factores transcripcionales generales (GTFs, por sus siglas en inglés), los factores trancripcionales de unión a ADN, y recientemente, se identificó el complejo Mediador. El complejo Mediador está formado por varias subunidades proteínicas ubicuas en la mayoría de los eucariontes, el cual transmite diferentes señales a través de factores transcripcionales hacia la RNA polimerasa II para modular la expresión génica. Se ha reportado que subunidades del complejo Mediador están

involucradas en respuestas asociadas a procesos de desarrollo, de estrés abiótico y de defensa. Sin embargo, hasta hace poco se desconocía el papel del complejo Mediador en los programas del desarrollo de la raíz así como los mecanismos celulares y moleculares por los cuales actúa (Raya-González et al., 2014).

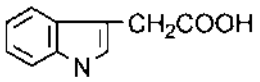
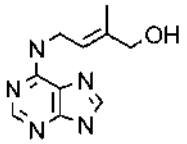
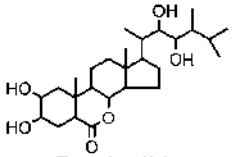
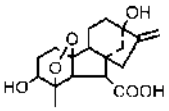
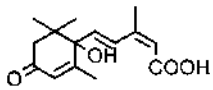
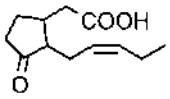
4. ANTECEDENTES

4.1. *Arabidopsis thaliana* como modelo de estudio.

Gran parte de los avances que actualmente existen en el conocimiento acerca de los mecanismos fisiológicos, celulares, bioquímicos y moleculares que controlan el desarrollo de las plantas, se han realizado utilizando al modelo biológico *Arabidopsis thaliana*. *Arabidopsis* es una planta que pertenece a la familia *Brasicaceae,* la cual tiene numerosas ventajas. En primer lugar es pequeña (30 cm) y fácil de manipular en el laboratorio, además, es posible generar mutantes en un tiempo corto (6-8 semanas). Existen bancos de semilla dedicados a generar mutantes de adquisición por catálogo mediante el tratamiento con organismos biológicos, sustancias químicas o radiaciones. *Arabidopsis* posee alrededor de 27,500 genes, que en conjunto comprende un genoma pequeño comparado con el de otras angiospermas, por lo que facilita los métodos de clonación para aislar los genes definidos por las mutaciones.

El crecimiento de la raíz primaria, la formación de raíces laterales, el crecimiento y desarrollo de los pelos radiculares, la elongación de las células y la actividad del meristemo apical son marcadores biológicos sencillos de analizar pero eficientes en la identificación de nuevos mecanismos de señalización en plantas (Scheres y Wolkenfelt, 1998).

Tabla 1. Función de los principales reguladores del desarrollo vegetal (Modificado de Morquecho-Contreras y López-Bucio, 2007).

Reguladores del crecimiento	Estructura química	Funciones en la planta
Auxinas	 Ácido indol acético	Regulan respuestas tróficas a la luz y gravedad, la arquitectura general de la raíz y follaje, el desarrollo vascular y el crecimiento en cultivo de tejidos.
Citocininas	 Cinetina	Regulan la división celular, senescencia de la hoja. Controlan la proliferación de las células madre en los meristemos, y regulación de la actividad auxínica.
Brasinoesteroides	 Brasinolido	Regulan la elongación y diferenciación celular, la germinación de la semilla y la tolerancia al estres.
Etileno	 Etileno	Regula la senescencia de hojas y flores, la maduración de frutos y desencadena respuestas a factores de estrés biótico y abiótico.
Giberelinas	 Ácido giberélico	Regula el crecimiento, la germinación de la semilla, promueve la floración y el crecimiento del fruto.
Ácido abscícico	 Ácido abscícico	Regula la dormancia y maduración de la semilla, tolerancia a sequia, respuestas a estres abiótico y controla la apertura de estomas.
Ácido jasmónico	 Ácido jasmónico	Regula respuestas de defensa a hongos necrótrofos, respuestas anti-hervivoría. Esta involucrado en procesos de fertilidad y el desarrollo del sistema radicular.

4.2. Desarrollo de la raíz de *Arabidopsis*.

Desde una perspectiva del desarrollo, la raíz de *Arabidopsis* representa un modelo sencillo y adecuado para las investigaciones sobre procesos

morfogenéticos en las plantas. Un número pequeño de células madre generan todos los tipos celulares a través de divisiones estereotípicas seguidas de una expansión y diferenciación celular. Debido a que el crecimiento de la raíz de Arabidopsis es indeterminado, estos procesos son continuos. El crecimiento de la raíz primaria ocurre en el meristemo apical de la raíz (RAM, por sus siglas en inglés). El RAM produce células en dos direcciones, produce una capa de tejido llamado cofia, que abarca el extremo distal de las raíces. La cofia protege a la punta de la raíz a medida que crece a través del suelo. Este tejido percibe y procesa los estímulos ambientales y modula la dirección del crecimiento de la raíz en función de la gravedad (gravitropismo), luz (fototropismo), obstáculos (tigmotropismo), gradientes de temperatura (termotropismo), humedad (hidrotropismo), nutrientes y otras sustancias químicas (quimiotropismo) (Hasenstein y Evans 1988; Ishikawa y Evans 1990; Okada y Shimura, 1990). Durante el desarrollo de la raíz, la planta activa procesos de muerte celular programada para inducir la separación de células de la cofia (Hawes et al., 2000; Driouich et al., 2013). Estas células corresponden a células del borde de la raíz, y su producción, entre otros factores, correlaciona con asociaciones micorrízicas dentro de la raíz. Las especies de plantas que son altamente propensas a ser colonizadas, liberan a la rizósfera un gran número de células del borde de la raíz, a diferencia de aquellas con menor probabilidad. Diversos estudios indican que el número de células del borde de la raíz se incrementa en respuesta a múltiples estímulos, incluyendo patógenos, dióxido de carbono, metales, tipo de suelo, y metabolitos secundarios (Zhao et al., 2000; Cannesan et al., 2011; Hawes et al., 2012). Además, las células del borde de la raíz son capaces de atraer o repeler microorganismos patógenos, incluyendo nematodos, bacterias, y oomicetos (Cannesan et al., 2011; Hawes et al., 2012). Lo que indica que la cofia posee una gran versatilidad para responder a las diferentes señales ambientales.

El RAM también produce células que contribuyen a la estructura de la raíz. El sistema radicular es una estructura formada por diferentes tejidos celulares, la epidermis, el córtex, la endodermis, el periciclo y los haces vasculares (Fig. 1). Las células que forman los diferentes tejidos se producen a partir de cuatro células

madre (células iniciales) localizadas en el ápice de la raíz (Dolan et al., 1993). Internamente y en contacto con las células iniciales se encuentra un número pequeño de células llamado QC (Fig. 1). Diferentes reguladores clave de la identidad del QC han sido caracterizados (Di Laurenzio et al., 1996; Aida et al., 2004; Sarkar et al., 2007). Uno de ellos es el factor de transcripción WUSCHEL-RELATED HOMEOBOX5 (WOX5) el cual es necesario para mantener el estado indiferenciado de las células cercanas al QC (Sarkar et al., 2007). Otros elementos importantes que controlan la identidad de QC y la actividad de meristemo apical de la raíz son: SHORT-ROOT (SHR), SCARECROW (SCR) y PLETHORA1 y 2 (PLT1 y 2) (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004). SHR y SCR son factores de transcripción requeridos para el desarrollo del patrón radial de la raíz. SHR es expresado en las células de la estela y puede moverse hacia las células vecinas. Para el caso de SCR, los dominios de expresión son la endodermis y el QC (Helariutta et al., 2000). La pérdida de función de SHR o SCR resulta en el desarrollo de una raíz primaria corta, la cual es deficiente en mantener la actividad del mersitemo y del QC, indicando que la vía de señalización dependiente de SHR/SCR regula la identidad del QC y la actividad de las células del nicho. Además, se sabe que los genes PLTs son reguladores importantes del desarrollo de la raíz (Aida et al., 2004). Alteraciones en la expresión de PLT1 y PLT2 afecta los marcadores del QC, el desarrollo de los pelos radiculares y el desarrollo de la cofia, indicando que se requiere una correcto funcionamiento de PLTs para mantener la actividad del QC. El QC presenta poca actividad mitótica, sin embargo, su función principal es la de mantener la organización de las células adyacentes. A medida que va creciendo la raíz, la zona de división celular (zona meristemática) dará paso a una fase de expansión celular que marca el final de la zona meristemática y el inicio de la zona de elongación (Fig. 1). 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, células epidérmicas especializadas en la captura de agua y nutrientes. Por otra parte, mediante eventos de división celular en el periciclo, se forman las raíces laterales, órganos que incrementan la superficie

total de exploración del suelo y contribuyen con un mejor anclaje. A pesar del conocimiento que se tiene de los procesos morfológicos que se llevan a cabo en la raíz, poco se sabe acerca de los mecanismos celulares y moleculares que participan en la regulación del sistema radicular de las plantas.

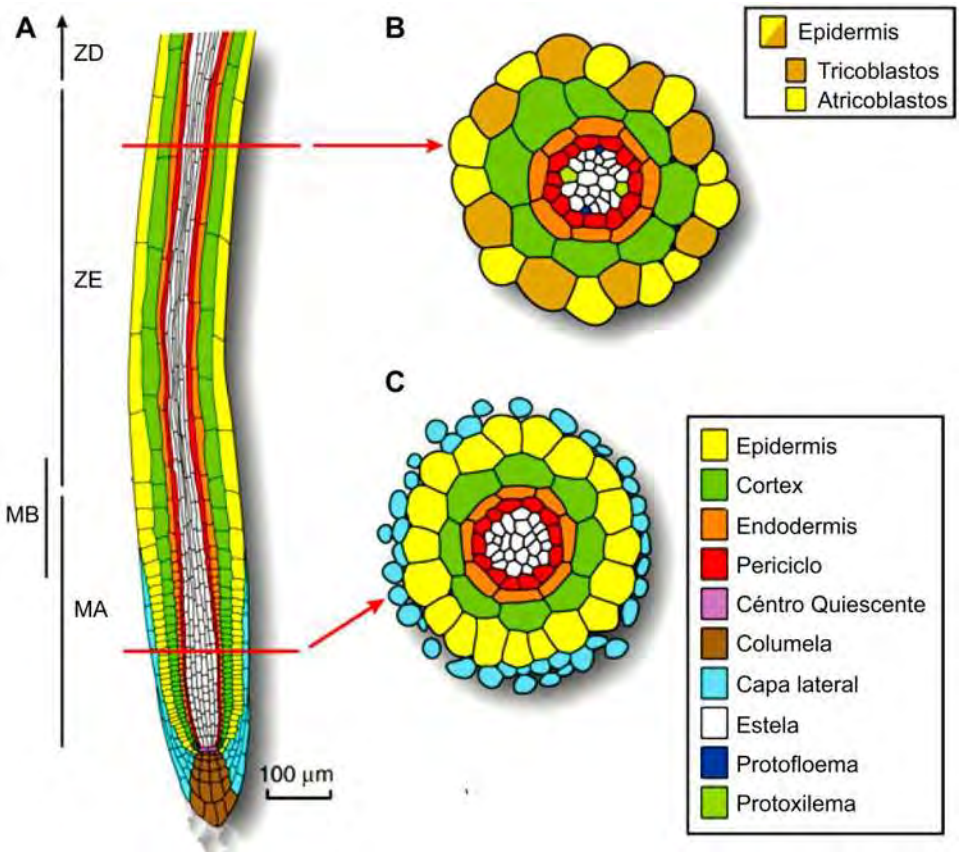


Figura 1. Esquema representativo de la estructura celular de la raíz de *Arabidopsis thaliana*. (A) El ápice de la raíz de *Arabidopsis* consiste de tres diferentes zonas de crecimiento. En orden a partir de la punta del meristemo apical, la zona de división celular activa; la zona de elongación (ZE) celular, en donde las células crecen rápidamente; y la zona de diferenciación (ZD), en donde las células crecen lentamente y son diferenciadas en estructuras, como los pelos radiculares. A nivel de tejido, la raíz de *Arabidopsis* está compuesta de capas de células arregladas concéntricamente (de afuera hacia adentro), la epidermis, el cortex, la endodermis, el periciclo y los tejidos vasculares de la estela. (B) Corte transversal de la raíz en la zona de elongación muestra la organización circunferencial y radial de la raíz. La posición de las filas celulares de los tricoblastos y atricoblastos es indicado. (D) Corte transversal de una zona inmadura de la raíz mostrando la organización celular de las filas de células. (MA) Mersitemo apical. (MB) Meristemo Basal (Modificado de Overvoorde et al., 2010).

4.3. Raíces laterales.

La formación de la raíz ocurre mediante organogenesis *de novo*, siendo las raíces laterales la principal determinante del sistema radicular. Generalmente, la formación de raíces laterales inicia cerca del meristemo apical de la raíz primaria y emergen en la zona de diferenciación (Figs. 2 y 3). El desarrollo de una raíz lateral inicia a partir de células del periciclo, las cuales experimentan divisiones celulares asimétricas altamente coordinadas para generar diversidad celular y formación de tejidos celulares. El conocimiento acerca de los mecanismos regulatorios en la formación de raíces laterales ha incrementado considerablemente (De Smet et al., 2006; Péret et al., 2009). En *Arabidopsis*, a partir de análisis celulares basados en marcadores, se ha revelado que las raíces laterales se desarrollan a partir de células fundadoras del periciclo, las cuales son re-activadas y un primordio de raíz lateral es iniciado (De Smet et al., 2012). Las células fundadoras toman lugar en el meristemo basal, en donde existe oscilación del flujo de auxinas y destino celular (De Smet et al., 2007; Moreno-Risueno et al., 2010; Laskowski, 2013; Van Norman et al., 2013). Posteriormente, las células fundadoras son sometidas a divisiones asimétricas altamente coordinadas para dar lugar a un PRL. Divisiones celulares periclinales subsecuentes formarán un PRL en forma de domo, el cual eventualmente emerge de la raíz primaria parental (Fig. 3) (Malamy y Benfey, 1997; Himanen et al., 2002; Benková y Bielach, 2010; Dastidar et al., 2012; Smith y De Smet, 2012). En *Arabidopsis*, las raíces laterales pueden desarrollarse en cualquier posición respecto a los polos del xilema, pero nunca se desarrollan en posición de los polos del floema (Beeckman et al., 2001; De Smet et al., 2006; Parizot et al., 2008). Esta fuerte asociación con el polo del xilema es apoyada por el fenotipo de la mutante *lonesome highway* (*lhw*), la cual presenta sólo un polo del xilema y forma raíces laterales de forma unilateral (Parizot et al., 2008). Sin embargo, en la mutante *wooden leg* (*wol*), la cual carece de floema, se forman raíces laterales de manera indistinta a la silvestre (Parizot et al., 2008). Hasta la fecha, no es claro como los polos del xilema y el floema determinan las células que formaran raíces laterales. No obstante, las células del periciclo de ambos

polos presentan diferencias en cuanto a tamaño, estructura, proteínas específicas y la expresión de genes (De Smet et al., 2006; Parizot et al., 2008).

La iniciación de raíces laterales esta correlacionada con la curvatura de la raíz. Las raíces laterales se forman en el lado convexo de la curva de la raíz primaria, aun cuando esta se haya realizado de manera manual, explicando el patrón de formación de raíces lateral izquierda-derecha (De Smet et al., 2007; Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008; Raya-González et al., 2012). Los giros de la raíz primaria y el subsecuente patrón de formación de raíces laterales sugiere que fuerzas mecánicas actúan sobre las células del periciclo y/o células vecinas en respuesta a estímulos ambientales, que son determinantes en la iniciación de las raíces laterales (Laskowski et al., 2008; Richter et al., 2009; Raya-González et al., 2012). El estudio de la inducción de raíces laterales en respuesta a un estímulo mecánico sobre la raíz primaria nos da una idea de las respuestas que experimenta la planta a medida que su sistema radicular se desarrolla en el ambiente (Raya-González et al., 2012).

La formación de raíces laterales está regulada por una red compleja de factores moleculares y diferentes reguladores del desarrollo vegetal (Nibau et al., 2008). La vía de señalización de las auxinas tiene una función esencial para la activación de las células del periciclo, la iniciación de divisiones celulares, la formación y el desarrollo del PRL y la emergencia de una raíz lateral (De Smet, 2012). En contraste a las auxinas, la citocininas reprimen la formación de raíces laterales mediante el bloqueo de la iniciación *de novo* del PRL e indirectamente afectando el transporte polar de auxinas (Laplaze et al., 2007; Osmont et al., 2007; Bielach et al., 2012). El ácido abscísico (ABA) actúa como un antagonista durante la iniciación de las raíces laterales (Signora et al., 2001; De Smet et al., 2003; Fukaki y Tasaka, 2009; Guo et al., 2009). Además del papel directo en la iniciación de raíces laterales, el ABA regula el balance entre las auxinas y las citocininas y así inhibe indirectamente la formación de raíces laterales (Shkolnik-Inbar y Bar-Zvi, 2010; Guo et al., 2012). El etileno regula la respuesta a auxinas y reprime la formación de raíces laterales (Vandenbussche y Van Der Straten, 2007; Negi et al., 2008, 2010; Lewis et al., 2011; Muday et al., 2012). Para el caso del ácido

jasmónico (AJ), se ha encontrado que promueve la formación de raíces laterales y modula el posicionamiento de estas a través de su receptor COI1 a través de un mecanismo dependiente a la señalización por auxinas (Sun et al., 2009; Raya-González et al., 2012). El ácido giberélico, los brasinoesteroides y las estrigolactonas afectan la formación de raíces laterales interactuando con la sensibilidad y el transporte de auxinas (Bao et al., 2004; Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Otras moléculas como el óxido nítrico, las *N*-acyl-L-homoserinas lactonas (AHL) y las alcamidas, las cuales se han considerado como nuevos reguladores del desarrollo, promueven la formación de raíces laterales a través de mecanismos dependientes e independientes a la señalización por auxinas (López-Bucio et al., 2007; Ortíz-Castro et al., 2008; Méndez-Bravo et al., 2010; Morquecho-Contreras et al., 2010).

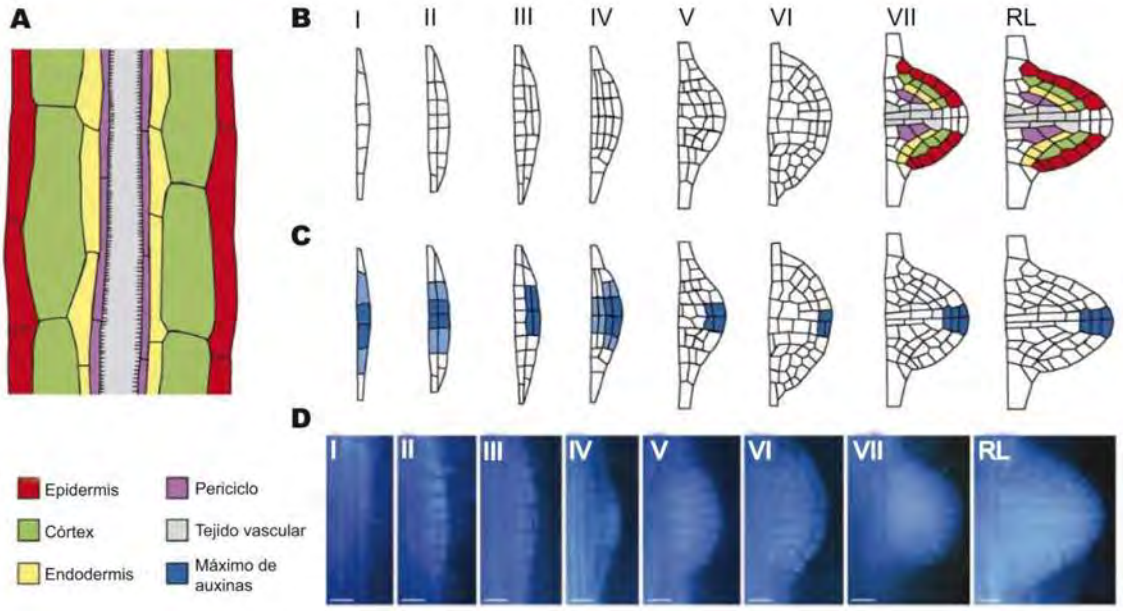


Figura 2. Cambios morfológicos durante el proceso de formación y desarrollo de raíces laterales. Las raíces laterales se originan de la raíz primaria a partir de células del pericliclo (A). Los siete estados del desarrollo de PRL (en números romanos [Malamy y Benfey, 1997]) se muestran en (B) así como el establecimiento del máximo de auxinas, demostrado por el marcador *DR5:uidA* (gradiente azul). [D]. Las barras de escala representan 20 µm (Modificado de Péret et al., 2009).

4.4. Los pelos radiculares.

Los pelos radiculares son extensiones cilíndricas de las células epidérmicas de la raíz, los cuales son esenciales en la toma de agua y nutrientes, en la interacción con organismos de la rizósfera y en el anclaje de la planta. En *Arabidopsis*, los pelos radiculares tienen aproximadamente 10 µm de diámetro y pueden llegar a medir 1 mm o más de longitud. Estas estructuras han atraído mucha atención debido a que proveen numerosas ventajas para estudios de desarrollo, biología y fisiología celular (Schiefelbein y Somerville, 1990). La presencia de los pelos radiculares en la superficie de la raíz los hace fácil de ser visualizados y accesibles para diferentes manipulaciones experimentales. Es importante mencionar que los pelos radiculares no son esenciales para la viabilidad de las plantas, lo cual permite el análisis de todo tipo de mutantes que estén alteradas en el desarrollo y la función de estas estructuras.

Durante el desarrollo de la raíz primaria surgen dos tipos celulares en la epidermis, las células que forman pelos radiculares (tricoblastos, células H) y las células que no forman pelos (atricoblastos, células N) (Fig. 3). La epidermis de la raíz de *Arabidopsis* se produce a partir de un grupo de 16 células iniciales durante la embriogénesis (Dolan et al., 1994; Scheres et al., 1994). En las células hijas ocurren divisiones transversales en la región meristemática de la raíz y esas divisiones sirven para generar células adicionales dentro de la misma fila (Baum y Rost, 1996). La epidermis de la raíz de *Arabidopsis* es similar a otros miembros de la familia de las *Brassicaceae*, ya que presenta un patrón dependiente de la posición de las células H y N. Las células que forman los pelos radiculares se encuentran espaciadas intercelularmente entre dos capas de células corticales, mientras que las células N están presentes sobre una sola capa cortical (Fig. 3). Debido a que la raíz primaria de *Arabidopsis* consistentemente posee 8 filas de células corticales, hay 8 filas de células H y aproximadamente 10-14 filas N (Dolan et al., 1994; Galway et al., 1994). La simple correlación entre la posición celular y la diferenciación del tipo celular implica que existen eventos de comunicación

célula-célula, los cuales son críticos para el establecimiento de la identidad celular en la epidermis de la raíz.

Diversos estudios fisiológicos, genéticos y moleculares han identificado a un grupo de genes asociados a la regulación de la diferenciación celular epidérmica. Mutaciones en *WEREWOLF (WER)*, *TRANSPARENT TESTA GLABRA (TTG)*, *GLABRA3 (GL3)/ENHANCER OF GLABRA (EGL3)*, y *GL2* causan la formación de pelos radiculares en cada una de las filas epidérmicas, lo que implica que *WER, TTG, GL3/EGL3* y *GL2* promueven la diferenciación de las células N y/o reprimen la formación de los pelos radiculares (Galaway et al., 1994; Di-Cristina et al., 1996; Masucci y Schiefelbein, 1996; Lee y Schiefelbein, 1999; Bernhardt et al., 2003).

WER codifica un factor de transcripción tipo MYB de la clase R2-R3 (Lee y Schiefelbein, 1999). *TTG* codifica una proteína pequeña con dominios WD40 repetidos. TTG es capaz de interactuar físicamente con los activadores de transcripción hélice-vuelta-hélice (bHLH) GL3 y EGL3 (Bernhardt et al., 2003). GL3 y EGL3 también interactúan físicamente con WER (Bernhardt et al., 2003; Song et al., 2011). *GL2* codifica un factor de transcripción expresado preferencialmente en células N dentro de la zona mersitemática y de elongación celular (Masucci y Schiefelbein, 1996). La expresión de *GL2* es influenciada por WER, GL3/EGL3 y TTG (Fig. 3).

Otros genes como *CAPRICE* (*CPC*) regulan el destino celular de la epidermis de una manera diferente. La mutante *cpc* produce un número reducido de pelos radiculares (Wada et al., 1997). Se ha demostrado que la mutante *gl2* es epistática a *cpc*, lo que sugiere que CPC participa en la vía WER/TTG/GL3/EGL3/GL2 como regulador negativo de GL2. Interesantemente, CPC es capaz de moverse de una célula a otra durante el desarrollo de la raíz (Kurata et al., 2005; Kang et al., 2013) (Fig. 3). Además de CPC, diferentes proteínas tipo MYB como son TRIPTYCHON (TRY) y ENHANCER OF TRY and CPC1 han sido reportadas por presentar redundancia funcional parcial (Schellman et al., 2002; Kirik et al., 2004; Simon et al., 2007; Serna, 2008; Wang et al., 2010).

El gen *SCRAMBLED* (*SCM*) difiere de los genes mencionados previamente, debido a que la mutación altera la distribución de las filas de células H y N (Kwak

et al., 2005). SCM codifica un receptor con actividad de cinasa y dominios ricos en leucina (LRR-RLK), la cual puede estar regulando la especificidad del tipo celular y por lo tanto el destino celular. Toda esta información indica que el destino celular de las células H y N así como la formación y desarrollo de los pelos radiculares está regulado estrictamente por una red compleja de factores de transcripción (Fig. 3).

Resultados de numerosos experimentos farmacológicos y genéticos indican que las auxinas y el etileno promueven la formación y el desarrollo de los pelos radiculares. Tratamientos con el precursor de etileno, 1-amino-cyclopropano-1-ácido carboxílico (ACC, por sus siglas en inglés) induce la formación de pelos radiculares ectópicos (Tanimoto et al., 1995). Además, mutaciones en *CONSTITUTIVE TRIPLE REPONSE* (*CTR1*), el cual regula negativamente la vía de señalización del etileno, también lleva a la formación de pelos radiculares ectópicos (Dolan et al., 1994; Ikeda et al., 2009). Se sabe que la señalización por auxinas está implicada en el desarrollo de pelos radiculares. Al igual que el ACC, la aplicación de la auxina AIA promueve la formación de los pelos radiculares y mutaciones en genes asociados a la señalización por auxinas *AUXIN RESISTANT2* y *3* (*AXR2* y *3*), *SOLITARY ROOT* (*SLR*) afectan el desarrollo de los pelos radiculares (Mizra et al., 1984; Wilson et al., 1990; Leyser et al., 1996). Mutaciones en *ROOT HAIR DEFECTIVE6* (*RHD6*), un factor de transcripción bHLH, afecta la formación de pelos radiculares, pero su fenotipo puede ser restaurado por la aplicación de ACC o AIA en el medio de crecimiento (Masucci y Schiefelbein, 1994). Además del etileno y las auxinas, otras hormonas pueden afectar la formación de pelos radicular. La aplicación de GR24, una estrigolactona sintética, incrementa la longitud de los pelos radiculares, posiblemente modulando la expansión celular regulada por auxinas (Kapulnik et al., 2011). Finalmente, la aplicación de AJ promueve el crecimiento de los pelos radiculares de una manera dependiente de la dosis, interactuando con las vías de señalización de etileno y auxinas (Zhu et al., 2006). Lo que indica que la diferenciación celular epidérmica puede estar influenciada por diferentes vías hormonales y por múltiples señales ambientales.

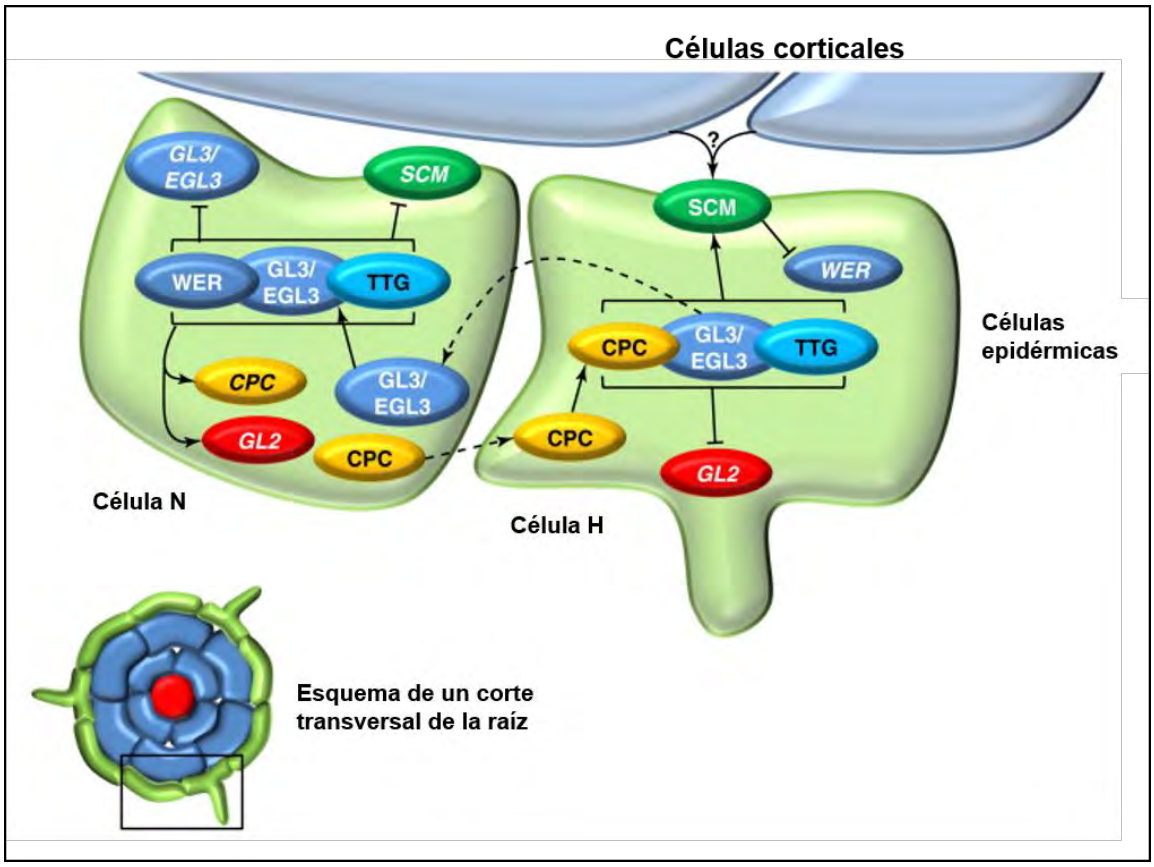


Fig. 3. La diferenciación de los pelos radiculares es dependiente de una comunicación intercelular. Una señal producida por las células corticales es detectada por el receptor SCRAMBLED (SCM). En células H, esta señal a través de SCM regula negativamente la expresión del factor de transcripción *WEREWOLF* (*WER*) . En células N, el gen *WER* no es expresado y la proteína WER interactúa con GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), y TRANSPARENT TESTA GLABRA (TTG), las cuales regulan positivamente la expresión de *CAPRICE* (*CPC*) y *GLABRA2* (*GL2*). EL movimiento de CPC a partir de células N y H previene la formación del complejo WER-GL3-EGL3-TTG1 en el futuro tricoblasto y consecuentemente, conduce a la represión de *GL2*. Además de su regulación genética, GL2 es también dependiente de modificaciones epigenéticas, tal como la acetilación de K9 y de histonas H3. EL movimiento de GL3 a partir de las células N y H es necesario para mantener la formación del complejo WER-GL3-EGL3-TTG1 debido a que la expresión de GL3 y EGL3 es regulada negativamente por el complejo WER-GL3-EGL3-TTG1 en células N (Modificado de Libault et al., 2010).

4.5. Los reguladores del desarrollo vegetal.

EL crecimiento y desarrollo vegetal involucra la integración de varias señales ambientales y endógenas, las cuales junto con el programa genético intrínseco determinan la forma de la planta (Gray, 2004). Diferentes moléculas orgánicas pequeñas denominadas fitohormonas o reguladores del crecimiento son responsables de cada aspecto del desarrollo vegetal, desde la embriogénesis

hasta la senescencia. El control del desarrollo se realiza modulando la división, la expansión, la diferenciación y la muerte celular. De esta manera se regulan diferentes procesos morfogenéticos incluyendo la germinación de la semilla, la configuración de la arquitectura de la planta, la floración, la maduración de frutos y el desprendimiento de las hojas (Bishopp et al., 2006).

En los últimos años, se ha generado una gran cantidad de información acerca del conocimiento en la biología vegetal, incluyendo nuevos descubrimientos en las áreas de la biosíntesis, el transporte, la percepción y la respuesta a hormonas. En general, los reguladores del crecimiento vegetal están presentes en baja concentración y pueden actuar localmente, cerca del sitio de síntesis, o incluso en tejidos distantes. La lista de los regulares del crecimiento ha incrementado, en donde se incluyen a las auxinas, las citocininas, las giberelinas, ácido abscisico, el etileno, los brasinoesteroides, el ácido salicílico, el ácido jasmónico, las *N*-acil etenolaminas, el glutamato, el óxido nítrico y algunos lípidos como las alcamidas y las AHLs (López-Bucio et al., 2007; Morquecho-Contreras y López-Bucio, 2007; Santner et al., 2009; Méndez-Bravo et al., 2010; Morquecho-Contreras et al., 2010). Prácticamente, cada aspecto del desarrollo de las plantas es regulado por los diferentes reguladores del crecimiento. Se sabe que un programa del desarrollo puede ser influenciado por uno o por múltiples reguladores del crecimiento, lo que nos habla de la gran versatilidad de estos compuestos para controlar la morfogénesis y las respuestas a estrés biótico y abiótico en la plantas.

4.6. Las auxinas.

La palabra auxina que en griego significa “crecer” se usa como un nombre genérico para un grupo de moléculas importantes en las plantas, las cuales también están presentes en humanos, animales y microorganismos. El AIA es la auxina predominante en las plantas y es una fitohormona bien documentada capaz de modular un gran número de programas del desarrollo.

En general, los efectos de las auxinas dependen de la concentración, ya que en concentraciones bajas promueven el crecimiento induciendo la elongación de hipocotilos, de tallos y raíces, en tanto que en concentraciones elevadas los efectos son opuestos inhibiendo la elongación celular. Además del AIA, existen otras auxinas naturales y sintéticas capaces de regular diversos procesos del desarrollo.

Las auxinas son de gran importancia en el desarrollo de la raíz, incluyendo la formación y emergencia de raíces laterales, la organización del mersitemo y la respuesta gravitrópica. La biosíntesis de auxinas ocurre tanto en la parte aérea de la planta como en la raíz, principalmente en las regiones meristemáticas y en aquellas con crecimiento activo como las hojas jóvenes, cotiledones y en los primordios de las raíces, siendo dichos tejidos los de mayor capacidad biosintética de este compuesto. Estudios genéticos y bioquímicos indican que el AIA es sintetizado a partir de dos vías metabólicas, la dependiente y la independiente de triptófano (Trp) (Korasick et al., 2013; Tivendale et al., 2014). Los principales precursores del AIA son: el ácido indol-3-pirúvico (IPyA, por sus siglas en inglés), el indol-3-acetaldoxima (IAOx), el indol-3-acetonitrilo (IAN), el indol-3-acetamida (IAM), y el indol-3-acetaldehído (IAAld). La vía del IPyA es considerada la principal vía de biosíntesis de auxinas (Zhao, 2012). La familia de las enzimas TRIPTOFANO AMINOTRANSFERASAS DE ARABIDOPSIS (TAA) convierte el triptófano en IPyA (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009), y la familia de las enzimas YUCCA (YUC) convierte el IPyA en AIA (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Dai et al., 2013), creando una conversión sencilla en dos pasos de Trp a AIA. La vía dependiente de IAOx es principalmente activada en la producción de compuesto de defensa y sólo una pequeña porción es convertida a AIA (Zhao et al., 2002; Sugawara et al., 2009). Se ha sugerido un menor papel para la vía IAOx en la producción de AIA. Sin embargo, las mutantes *superrot1* y *superrot2* presentan una hiperacumulación de IAOx resultando en un incremento de los niveles de AIA (Mikkelsen et al., 2004), sugiriendo que al menos, bajo determinadas condiciones, la via del IAOx es importante para la producción de AIA.

Existen diversas formas de almacenamiento de AIA inactivo, incluyendo conjugados de AIA, los cuales pueden ser rápidamente convertidos a AIA libre para regular la homeóstasis de auxinas (Korasick et al., 2013). Estas formas de almacenamiento de auxinas pueden influir en la sensibilidad, el transporte y la acumulación de auxinas (Cohen y Bandurski, 1982) y a su vez regular diferentes programas del desarrollo, como son: la elongación del hipocotilo, la expansión de los pelos radiculares, la formación de raíces laterales, la actividad del meristemo de la raíz, entre otros (Strader et al., 2010, 2011). Esto indica que estas formas de almacenamiento de auxinas son necesarias para un correcto desarrollo vegetal bajo condiciones específicas de crecimiento. Por lo tanto, se requiere un mayor esfuerzo para entender las condiciones en que en las plantas se activa la biosíntesis de auxinas *de novo* o su conversión a las formas de almacenamiento que incrementan o disminuyen los niveles de auxina libre.

Una vez que las auxinas son producidas, son movilizadas hacia sus sitios blancos a través de dos vías principales: la primera de transporte rápido, desde los tejidos jóvenes del follaje hacia los tejidos de demanda en la raíz. Este tipo de distribución de auxinas se realiza a través del floema (Friml, 2003). La otra vía implica el transporte lento, importante en la distribución de auxinas a distancias más cortas. En este último, el transporte ocurre célula a célula y requiere de proteínas especializadas. Una característica de este tipo de transporte es que su direccionalidad está estrictamente controlada dentro de un tejido mediante la localización diferencial de transportadores membranales.

El AIA al ser un ácido débil puede encontrarse en forma protonada o no protonada dependiendo del pH. El pH apoplástico es ~5.5 favoreciendo que las moléculas del AIA se encuentren en su forma disociada (AIA^-) en un ~83%, y en su forma protonada (AIAH) en un ~17% (Zazímalova et al., 2010). Esta última forma se puede difundir libremente hacia el interior de la célula (difusión pasiva) o a través del transportador de entrada AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) (Zazímalova et al., 2010). Sin embargo, una vez dentro de la célula el cambio de pH del medio favorece la forma disociada del AIA^- casi en su totalidad, por lo que las moléculas en dicha forma, requieren de un mecanismo de transporte

activo a través de múltiples proteínas de las familias PIN-FORMED (PIN) y ATP-BINDING CASSETTE SUBFAMILY (ABCB, por sus siglas en inglés) (Peer et al., 2011) (Fig. 4). La diversidad de los fenotipos mostrados por la mutantes afectadas en los diferentes miembros de la familia AUX/LAX revelan distintas funciones para esos transportadores. La actividad de AUX1 es necesaria para las respuestas a gravedad (Bennett et al., 1996; Marchant et al., 1999), el desarrollo de los pelos radiculares (Grebe et al., 2002; Jones et al., 2009) y la filotaxis (Reinhardt et al., 2003; Bainbridge et al., 2008). La mutante *lax3* presenta una menor emergencia de raíces laterales, mayor número de primordios y una represión en la expresión de enzimas que modifican la pared celular (Swarup et al., 2008), sugiriendo que LAX3 modifica la pared celular en respuesta a auxinas, para la separación de las células corticales y la emergencia de las raíces laterales. Esto indica que los transportadores de influjo de auxinas son críticos para el transporte polar de auxinas y son esenciales en diversos aspectos del desarrollo vegetal.

Los miembros de la familia PIN y ABCB regulan el eflujo de auxinas (Fig. 4). Las proteínas PIN son transportadores de auxinas controladas por un gradiente y son específicas de las plantas. Cada una de las 8 proteínas PIN presentan una localización distinta y específica. En los tejidos de la raíz, PIN1 se expresa en células de la estela, endodermis y del pericíclo (Friml et al., 2002b; Blakeslee et al., 2007). PIN2 es localizado en células corticales y epidérmicas (Muller et al., 1998; Blakeslee et al., 2007) y en la capa lateral de la raíz (Friml, 2003), mientras que PIN3 está presente en el pericíclo y la columela (Friml et al., 2002a). PIN4 se expresa en el mersitemo de la raíz (Friml et al., 2002b) y PIN7 en la estela y columela (Blilou et al., 2005). Los patrones de expresión específicos y la localización polar de las proteínas PIN son necesarios para una distribución diferencial de auxinas y así se regulan los diferentes programas del desarrollo vegetal (Zazimalova et al., 2010).

La localización polar de las proteínas PIN requiere de endocitosis y el reciclado selectivo hacia la membrana plasmática (Geldner et al., 2001; Kleine-Vehn y Friml, 2008). EL reciclado de los PIN permite una rápida relocalización en respuesta a señales ambientales o de desarrollo (Lofke et al., 2013). Por su parte

los transportadores ABCB, incluyendo ABCB1, ABCB4, ABCB19, y ABCB21 regulan el eflujo de AIA (Geisler et al., 2005; Lewis et al., 2007) (Fig. 4). El transportador ABCB4 se localiza en la membrana plasmática y compartimentos endomembranales de la raíz (Terasaka et al., 2005; Cho et al., 2007). ABCB4 y ABCB21 pueden funcionar tanto como transportadores de eflujo e influjo, dependiendo de las concentraciones de auxinas internas (Yang y Murphy, 2009; Kamimoto et al., 2012; Kubes et al., 2012).

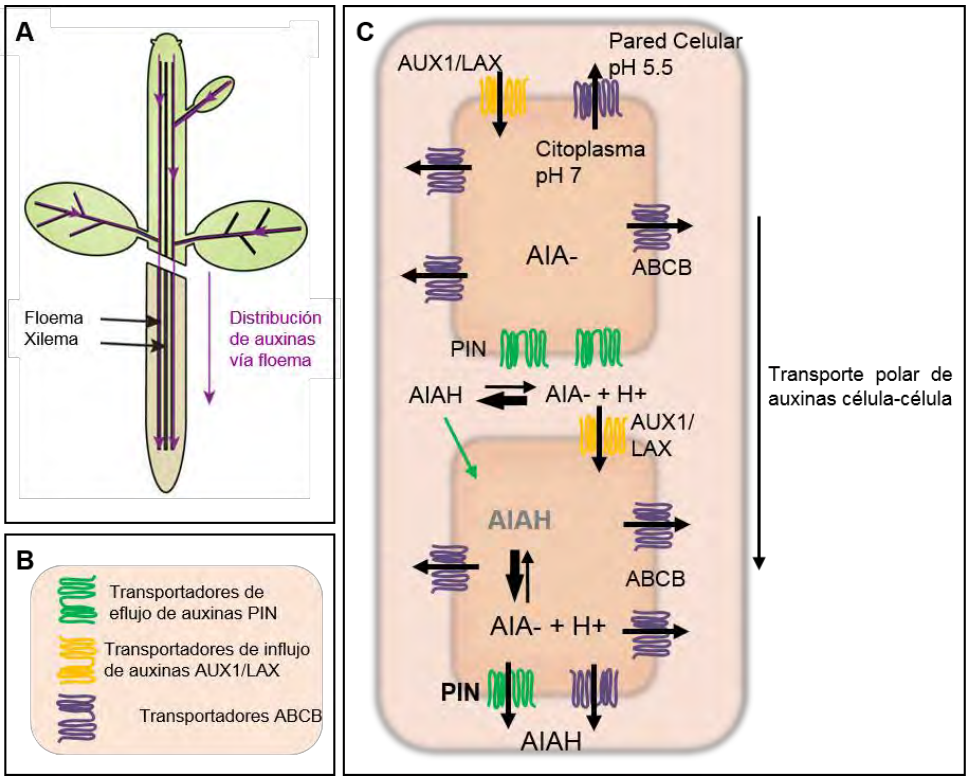


Figura 4. Transporte de auxinas. Las axinas pueden ser movilizadas de sus sitios de biosíntesis a través de dos mecanismos. (A) Distribución de auxinas vía floema a partir de sitios fuente (hojas jóvenes y yemas florales) hacia la raíz. (B y C) Modelo quimiosmótico del transporte polar de auxinas basado en la diferencia de pH entre el apoplasto (pH 5.5) y el citoplasma (pH 7). La auxina protonada (AIAH) puede difundir libremente a través de la membrana plasmática lipídica o ser transportada por los transportadores de influjo AUX1/LAX al interior de la célula. En el citosol, el AIAH es disociado y atrapado en el interior de célula en su forma desprotonada (AIA^-). El AIA^- puede salir de la célula por la acción de los transportadores de eflujo tipo ABCB o PIN. La localización celular polar de los transportadores determina la direccionalidad del flujo de auxinas intercelular (Modificado de Robert y Friml, 2009).

Una vez que el AIA es sintetizado y movilizado hacia sus sitios blanco, su percepción puede ser controlada por dos vías de señalización independientes: (1) A través de la vía dependiente de TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) y (2) a través de AUXIN BINDING PROTEIN1 (ABP1). Aunque la vía de TIR1 está bien documentada (Chapman y Estelle, 2009), poco se sabe acerca de la vía de ABP1.

La vía de señalización dependiente de TIR1/AFB controla la respuesta transcripcional a las auxinas. En concentraciones bajas de auxinas, las proteínas AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) reprimen la actividad de los factores de transcripción AUXIN RESPONSE FACTOR (ARF) (Chapman y Estelle, 2009). Cuando los niveles de auxinas se incrementan, la proteína F-box TIR1/AFB interactúa con una proteína AUX/IAA para forma el co-receptor de auxinas y unir auxinas (Dharmasiri et al., 2005; Kepinski y Leyser, 2005; Chapman y Estelle, 2009). La proteína F-box TIR1/AFB participa en un complejo ubiquitin ligasa Skp1-Cullin-F-box (SCF) E3 para ubiquitinar a las proteínas represoras AUX/IAA y ser degradadas vía proteosoma 26S (Fig. 5). Esta degradación de AUX/IAA permite que los ARF activen la transcripción de genes de respuesta a auxinas (Ramos et al., 2001; Zenser et al., 2001; Dreher et al., 2006). Esta interacción ligando-receptor permite una cascada transduccional muy corta para facilitar una rápida respuesta transcripcional de respuesta a auxinas (Fig. 5).

En *Arabidopsis* existen 29 proteinas AUX/IAA y seis TIR/AFBs que pueden dimerizarse para formar co-receptores de auxinas. Interesantemente, diferentes combinaciones de AUX/IAA-TIR1/AFB presentan diferentes afinidades por una u otra auxina, incluyendo tanto naturales como sintéticas (Calderón-Villaobos et al., 2012; Lee et al., 2014; Shimizu-Mitao y Kakimoto, 2014). Esta diferencia de afinidades, probablemente, es un mecanismo por el cual las auxinas regulan una amplia variedad de las respuestas de la planta y los diferentes aspectos del desarrollo. Por otra parte, *Arabidopsis* presenta 22 proteinas ARF, de las cuales 5 activan la transcripción, mientras que el resto actúan como represores transcripcionales (Guilfoyle y Hagen, 2007). Estudios recientes sugieren que los ARFs y los AUX/IAA pueden multimerizarse, más que interactuar en una

dimerización sencilla (Korasick et al., 2014; Nanao et al., 2014), sugiriendo que la formación del complejo ARF-AUX/IAA puede incorporar otras proteínas ARF y AUX/IAA diferentes para regular finamente las respuesta a auxinas.

ABP1 ha sido estudiado desde los 70s (Hertel et al., 1972), pero hasta hace poco fue aceptado como receptor de auxina. En esta vía, la auxina es percibida fuera de la membrana plasmática por ABP1 (Shi y Yang, 2011; Sauer et al., 2013). ABP1 está anclada a la membrana plasmática e interactúa con el C-TERMINAL de la proteína PEPTIDE-BINDING PROTEIN (CBP1) (Shimomura, 2006) y esta a su vez interactúa con la proteína TRANSMEMBRANE KINASE (TMK) de una manera dependiente de auxinas (Xu et al., 2014). ABP1 y las auxinas son requeridos para la activación de dos proteínas RHO-LIKE GTPASES OF PLANTS (ROP), ROP2 y ROP6 (Xu et al., 2010). Sin embargo, el mecanismo exacto entre la unión de ABP1 con las auxinas, la actividad de TMK y la activación de ROP no han sido dilucidados.

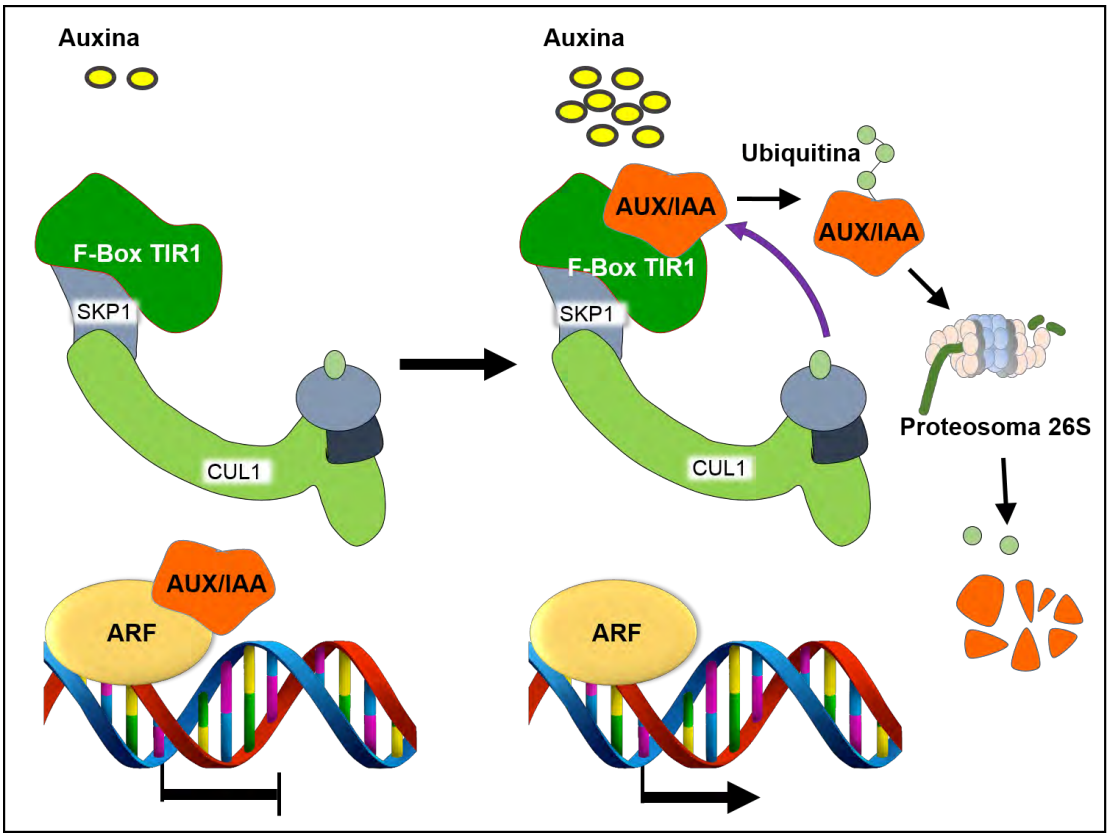


Figura 5. Modelo para la activación transcripcional mediado por auxinas. En bajas concentraciones de auxinas, los factores de transcripción AUXIN RESPONSE FACTORS (ARF) unidos a los promotores de los elementos de genes de respuesta a auxinas, se encuentran formando un complejo con las proteínas AUX/IAA. La actividad del promotor está reprimida por la actividad de las proteínas AUX/IAA y co-represores transcripcionales. En altas concentraciones de auxinas, el AIA se une al complejo SCF^{TIR1}, esto incrementa la afinidad para la unión de los AUX/IAA al complejo SCF^{TIR1} y así activar el proceso de ubiquitinación. Al ser degradadas las proteínas AUX/IAA por el proteosoma 26S, permite que los ARF se encuentren libres para la expresión de genes de respuesta a auxinas.

4.7. El ácido jasmónico (AJ).

A lo largo de su ciclo de vida, las plantas se encuentran constantemente sometidas a estrés biótico y abiótico. Una de las fitohormonas encargadas de regular este tipo de respuestas es el AJ. El AJ y su derivado volátil metil jasmonato son considerados las formas activas debido a sus efectos fisiológicos y su abundancia en las plantas (Santino et al., 2013).

La biosíntesis del AJ es un proceso enzimático, el cual se lleva a cabo en tres diferentes compartimentos celulares, el plástido, el peroxisoma y el citosol. El

primer paso en la síntesis de AJ ocurre en el plástido mediante la acción de una fosfolipasa para la liberación del ácido α-linoleico a partir de fosfolípidos de la membrana. Posteriormente, el ácido α-linoleico es oxidado por la acción de la 13-lipoxigenasa (13-LOX) generando el 13-hydroperoxy (Vick y Zimmerman 1983; Bell y Mullet 1993, Bannenberg et al., 2009). EL genoma de *Arabidopsis* codifica para seis lipoxigenasas, de las cuales, tres de ellas (LOX2, LOX3, y LOX4) han sido asociadas con la producción de AJ (Bell y Mullet 1993; Caldelari et al., 2011). En el plástido, el OPDA (ácido cis-(+)-12-oxofitodienoico es convertido a AJ a través de varios ciclos de β-oxidación mediante reacciones enzimáticas de oxidación, hidratación y tiólisis. Posteriormente, en el citosol el AJ sufre diferentes modificaciones para formar las diferentes formas activas.

El sistema de ubiquitin-proteosoma es el regulador central del censado y señalización de esta fitohormona. Similar al de auxinas, éste consiste de un complejo SCF, el cual funciona como una E3 ubiquitin ligasa, en donde la proteína F-box CORONATINE INSENSITIVE (COI1) reconoce una proteína blanco la cual es ubiquitinada y posteriormente degradada por el proteosoma. Las proteínas reconocidas como blancos por el complejo SCF^{COI1} son miembros de la familia JASMONATE ZIM DOMAIN (JAZ). La degradación de JAZ permite la liberación de los factores transcripcionales como MYC2, el cual se une a elementos en promotores de genes de respuesta a AJ para iniciar la transcripción (Fig. 6). Como componentes finales en la vía de señalización, se encuentran los co-represores TOPLESS (TPL) y proteínas relacionadas a TPL, que interactúan con la proteína de andamiaje “NOVEL INTERACTOR OF JAZ” (NINJA) (Pauwels et al., 2010) (Fig. 6).

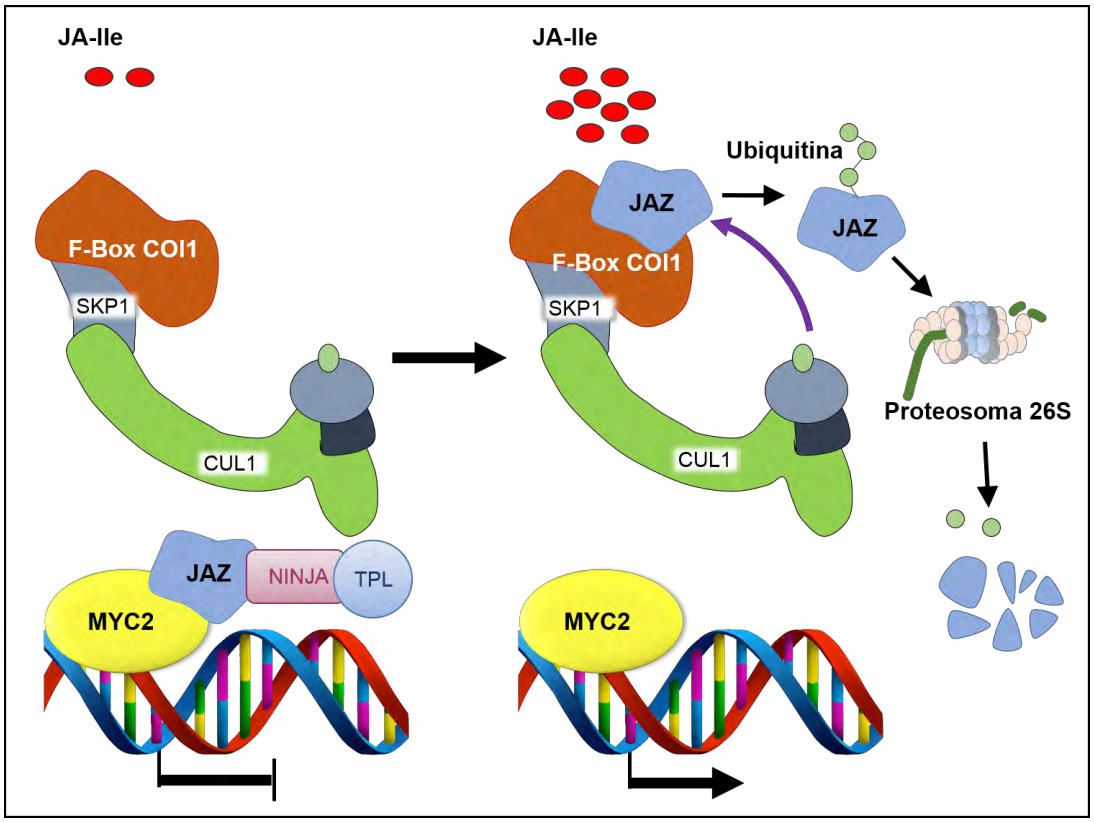


Figura 6. Activación transcripcional mediada por ácido jasmónico (AJ). En baja concentración de la forma activa JA-Ile (jasmonoil-isoleucina), los represores JAZ se unen a los factores de transcripción tipo MYC2 y son reclutados co-represores, incluyendo NINJA y TOPLESS (TPL) para reprimir la expresión de genes de respuesta a AJ. Por otro lado, en alta concentración de JA-Ile, la unión de JA-Ile con el componente E3 ubiquitin ligasa SCF^{COI1} facilita la unión de complejo JAZ-NINJA-TPL. Como resultado las proteínas JAZ son ubiquitinidas y subsecuentemente degradas en el proteosoma 26S, permitiendo la liberación de los FT tipo MYC2 y ocurre la activación de la transcripción de genes de respuesta a AJ.

EL AJ tiene un papel central en el desarrollo y la reproducción de las plantas (Creelman y Mullet, 1997; Wasternack, 2007; Browse, 2009). Algunos de los procesos fisiológicos en donde el AJ ha sido involucrado son: desarrollo floral, inducción de senescencia, inhibición del crecimiento, maduración de frutos, formación de tricomas y desarrollo de pelos radiculares (Santino et al., 2013). El AJ también ha sido descrito como un regulador importante de la morfogénesis de la raíz. En *Arabidopsis*, la aplicación de AJ inhibe el crecimiento de la raíz primaria, afectando tanto la división como la elongación celular (Staswick et al., 1992; Feys et al., 1994; Yan et al., 2007; Zhang y Turner, 2008; Raya-González et

al., 2012) (Fig. 7). EL AJ promueve la formación de raíces laterales directamente induciendo la expresión del gen *ANTRANILATE SYNTASE1* (*ASA1*) y la acumulación de proteínas PIN2 (Sun et al., 2009, 2011). En nuestro grupo de trabajo encontramos que el AJ inhibe el crecimiento de la raíz primaria y la formación de raíces laterales de una manera dependiente de la concentración (Fig. 7). Ésta respuesta fue acompañada por un incremento en la formación de primordios de raíces laterales. Además, determinamos que el receptor del AJ, COI1 está implicado en la formación de RL en respuesta a jasmonatos, en el posicionamiento de las RL y la emergencia de RL en respuesta a estímulos mecánicos (Raya-González et al., 2012) (Fig. 7). Esto indica que el AJ además de ser un factor clave en la activación de respuestas de defensa, también es un regulador importante en diferentes programas del desarrollo vegetal.

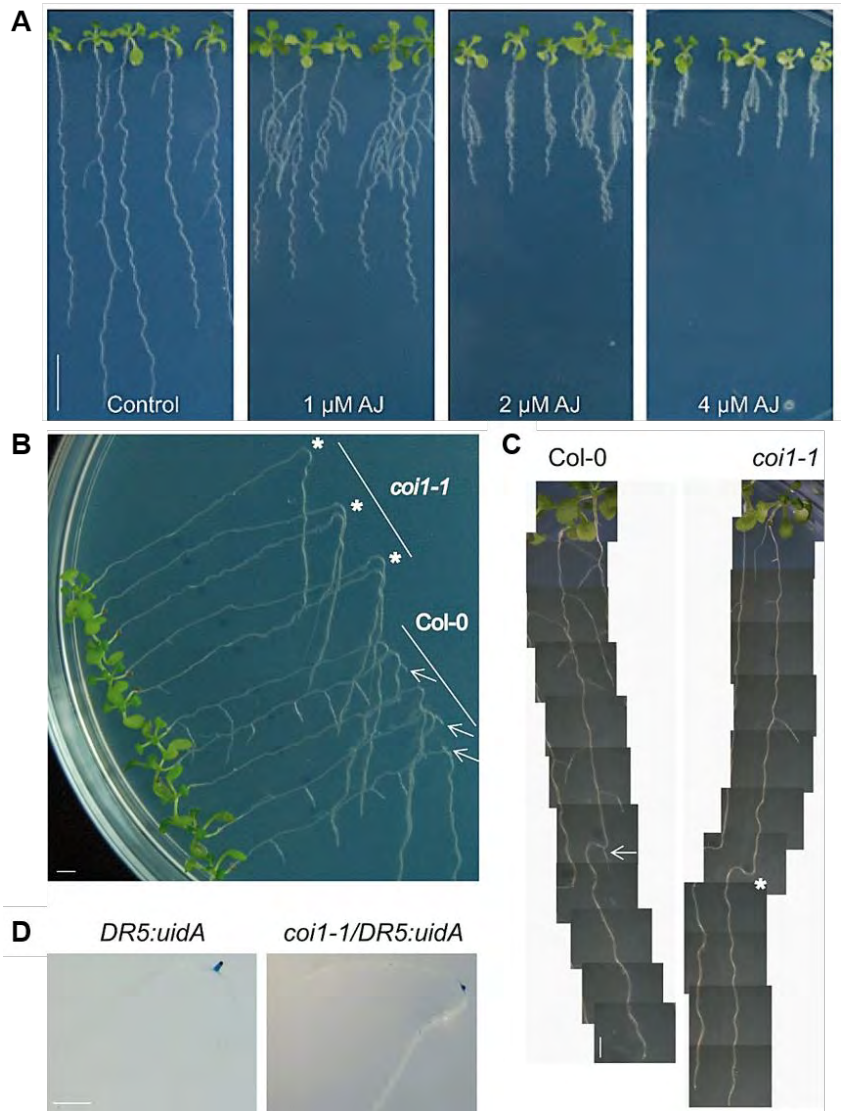


Figura 7. Participación del AJ y su receptor COI1 en la arquitectura del sistema radicular de *Arabidopsis.* (A) El AJ afecta el crecimiento de la raíz primaria y promueve la formación de raíces laterales. (B) Desarrollo de raíces laterales en plántulas silvestres Col-0 y mutantes *coi1-1* afectadas en el receptor de AJ, en respuesta a un estímulo gravitatorio. (C) Raíces laterales emergidas después de un estímulo mecánico. La raíz primaria de Col-0 y *coi1-1* fueron dobladas y se dejaron crecer por 12 h. (D) Desarrollo de un PRL después de un estímulo gravitatorio en plántulas *DR5:uidA* y *coi1-1/DR5:uidA*. Las flechas indican desarrollo de una RL y asterisco (*) marca una ausencia de RL (Modificado de Raya-González et al., 2012).

4.8. El complejo Mediador.

EL proceso de transcripción es un proceso que es llevado a cabo por un gran número de proteínas actuando de manera sincronizada. En eucariontes, las

proteínas involucradas en la transcripción son la Pol II, los GTFs, los factores de transcripción (TFs, por sus siglas en inglés) de unión a ADN y el complejo Mediador (Med). Este último es un complejo multiproteínico integrado por hasta 30 subunidades, cuya función predicha es actuar como puente entre la Pol II y los TFs de unión a ADN presentes dentro de la célula (Kim et al., 1994; Koleske y Young, 1994). Mediante la unión distal entre los activadores/represores, los GTFs y la Pol II al promotor de los genes blanco, Med modula de manera específica y controlada las señales celulares para iniciar la transcripción (Malik y Roeder, 2005) (Fig. 8).

El descubrimiento del complejo Mediador ocurrió mediante estudios de sistemas de transcripción *in vitro*, en donde la actividad transcripcional únicamente fue restaurada por la adición de un cultivo celular crudo, pero no por la adición de cualquiera de los componentes previamente conocidos del sistema transcripcional (Flanagan et al., 1991). Posteriormente, se aisló un complejo conformado de aproximadamente 20 proteínas el cual fue necesario para restablecer la actividad transcripcional y nombrado como el complejo Mediador (Kim et al., 1994). Desde su descubrimiento en *Saccharomyces cerevisiae*, las proteínas que conforman el complejo Mediador han sido identificadas en casi todos los eucariontes (Boube et al., 2002; Bourbon et al., 2004; Bourbon, 2008). Estructuralmente, el complejo Mediador está integrado por cuatro dominios conservados, incluyendo un dominio cinasa (Dotson et al., 2000). El complejo Mediador es un regulador global de la expresión de genes, y como tal, es considerado como un GTF (Ansari et al., 2009; Takagi y Kornberg, 2006). Sin embargo, lo que distingue al complejo Mediador de otros GTFs (con la posible excepción de TFIID) es su alto grado de flexibilidad estructural y su composición diversa de proteínas (Malik y Roeder, 2010). Consistente con su capacidad para activar la transcripción, se ha establecido que el Mediador es el principal sitio de unión de los TFs de unión a ADN dentro del complejo de iniciación de la transcripción (TIC, por sus siglas en inglés) (Borggrefe y Yue, 2011). Evidencia experimental indica que el Mediador activa la transcripción, en parte, actuando como un puente entre los TFs de unión a ADN, la RNA Pol II, los GTFs y otros componentes del complejo transcripcional (Fig. 8).

Debido a esto, el Mediador puede ser requerido para la transcripción de todos los genes. Dado el papel fundamental del Mediador en la transcripción génica, se ha propuesto que alteraciones a nivel de transcripción o de función de las subunidades individuales del Mediador podrían llevar a defectos en la expresión global de genes y afectar diversos programas esenciales del desarrollo. Se sabe que diversas subunidades del Mediador son esenciales para un correcto crecimiento y desarrollo en organismos como *Drosophila*, pez zebra, ratón y *Caenorhabditis elegans* (Tabla 2).

Tabla 2. Efectos de mutaciones en subunidades del complejo Mediador (Modificada de Hentges, 2011).

Subunidad	Organismo	Fenotipo mutante
MED1	Ratón	Insuficiencia placentaI, hemorragias y necrósis en hígado, y crecimiento retardado.
MED6	*Drosophila*	Letalidad en estado larvario.
MED12	*C. elegans*	Alteraciones en la división celular.
MED12	Pez zebra	Defectos el en desarrollo del cerebro, e hígado. Deficit en la diferenciación celular neuronal, defectos cardiovasculares, y desarrollo anormal del oído.
MED12	Ratón	Desregulación de genes Nanog en células del tallo embrionario, defectos en el desarrollo del sistema cardiaco.
MED12	*Arabidopsis*	Alteración en el desarrollo embrionario y en la división celular, defectos en el desarrollo de hojas y morfogénesis.
MED13	*C. elegans*	Letalidad embrional.
MED13	*Arabidopsis*	Alteración en el desarrollo embrionario y en la división celular, defectos en el desarrollo de hojas y morfogénesis.
MED14	Pez zebra	Defecto en células de la retina.
MED15	*Drosophila*	Pérdida de las venas y reducción en el tamaño de las alas.
MED21	Ratón	Arresto en el desarrollo del blastocito.
MED23	*C.elegans*	Letalidad en el estado larvario.
MED23	Ratón	Defectos en el sistema circulatorio.
MED24	Pez zebra	Alteración en el sistema nervisoso.
MED24	Ratón	Hipoplasia cardiaca, defectos en el desarrollo vascular y desarrollo celular.
MED28	Células NIH3T3 y C2C12	Defectos en la expresión de genes del músculo liso.
MED31	Ratón	Alteración en la proliferación celular y retraso en el desarrollo.

La participación del Mediador en el crecimiento y desarrollo vegetal se demostró inicialmente a partir del análisis de la mutante *med14* (Autran et al., 2002). MED14/STRUWWELEPETER (SWP) regula el desarrollo del meristemo y

controla la duración de la proliferación celular. Los genes *CENTER CITY* (*CCT*) y *GRAND CENTRAL CITY* codifican las subunidades MEDIATOR12/MED12 y MED13, respectivamente. Mutaciones en *MED12/CCT* y *MED13/GCT* afectan el desarrollo embrionario (Gillmor et al., 2010). Además, MED13 fue identificado como un regulador de PINOID (PID) y designado como MACCHI-BOU2 (MAB2) (Ito et al., 2011). Mutaciones en MED13/MAB2 conllevan al desarrollo de cotiledones aberrantes como resultado de un defecto en la respuesta auxínica. Así mismo, mutantes *med21* homocigotas presentan letalidad embrionaria (Dhawan et al., 2009). Recientemente, se reportó que mutantes afectadas en MED18 desarrollan sépalos, pétalos y estambres anormales, posiblemente debido a una expresión reducida o ectópica de *AGAMOUS* (*AG*), *APETALA1* (*AP1*) y *PISTILLATA* (*PI*) (Zheng et al., 2013).

MED25/PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 (PFT1/MED25) es una subunidad que está involucrada en diversos procesos biológicos. MED25 regula el desarrollo de órganos florales modulando la proliferación y la expansión celular (Xu y Li, 2012), el crecimiento y formación de pelos radiculares a través de la regulación en la producción de especies reactivas de oxígeno (ROS, por sus siglas en inglés) (Sundaravelpandian et al., 2013). Un estudio reciente reveló que MED25 está involucrado en el crecimiento de la raíz primaria y la formación de raíces laterales (Raya-González et al., 2014). El transporte y la respuesta a auxinas están incrementadas en las raíces de las mutantes *pft1/med25*, sugiriendo un mecanismo regulado por auxinas mediado por MED25 (Raya-González et al., 2014). MED8 también fue reportado como un elemento asociado a la diferenciación de los pelos radiculares. Sin embargo, no mostró ninguna participación en la regulación de la arquitectura radicular (Sundaravelpandian et al., 2013; Raya-González et al., 2014).

Al igual que en procesos del desarrollo, se sabe que el complejo Mediador tiene un papel importante en respuestas de defensa. Mutantes afectadas en MED25 presentan susceptibilidad al ataque de hongos necrótrofos, pero muestran resistencia a hongos hemibiótrofos que infectan la raíz, esto debido a una desregulación de la vía de señalización del AJ (Kidd et al., 2009). Diversos

estudios han reportado una interacción directa entre MED25 y los factores de transcripción involucrados en la respuesta a AJ, MYC2 y ETHYLENE REPONSE FACTOR1 (ERF1), revelando una vía de respuestas de defensa mediada por MED25 (Ou et al., 2011; Chen et al., 2012). De manera similar, mutantes en MED18 y MED21 son susceptibles a hongos necrótrofos (Dhawan et al., 2009; Lai et al., 2014). Otras subunidades como MED14, MED15 y MED16 regulan la expresión de genes de defensa dependientes de la vía de señalización del ácido salicílico (AS) (Canet et al., 2012; Wathugala et al., 2012; Zhang et al., 2013).

Recientemente, se reportó que las subunidades del módulo cinasa del Mediador, incluyendo MED12, MED13, CDK8 y CycC regulan las respuestas de defensa (Zhu et al., 2014). CDK8 afecta respuestas de defensa mediadas por AJ. Mutantes en MED12, MED13 y CycC son suceptibles o resistentes a diferentes patógenos.

Las plantas perciben e integran diferentes señales ambientales, como temperatura, sequía, salinidad e intensidad de luz, por diferentes vías regulatorias. MED16 es una subunidad que integra múltiples señales de estrés abiótico y está involucrada en la tolerancia al estrés por frío y en la homeostasis del hierro (Knight et al., 2009; Yang et al., 2014). MED16 interactúa con MED25 para regular la homeostasis de hierro, ya que ambas subunidades controlan la expresión de genes que son activados en respuesta a la deficiencia como IRON REGULATED TRANSPORT1/*IRT1* y *FERRIC CHELATE REDUCTASE DEFECTIVE2*/*FRO2*. Además, se ha mostrado que MED25 regula respuestas a estrés salino y de sequía (Elfving et al., 2011).

Estos resultados indican que cada una de la subunidades del complejo Mediador pueden estar participando en diferentes procesos. Es de esperarse que subunidades individuales del Mediador reconozcan y respondan a un subgrupo de los ~1500 factores transcripcionales presentes en el genoma de *Arabidopsis*. Por lo tanto, determinar cuáles factores transcripcionales interactúan con cada subunidad será un avance importante en la biología molecular de las plantas.

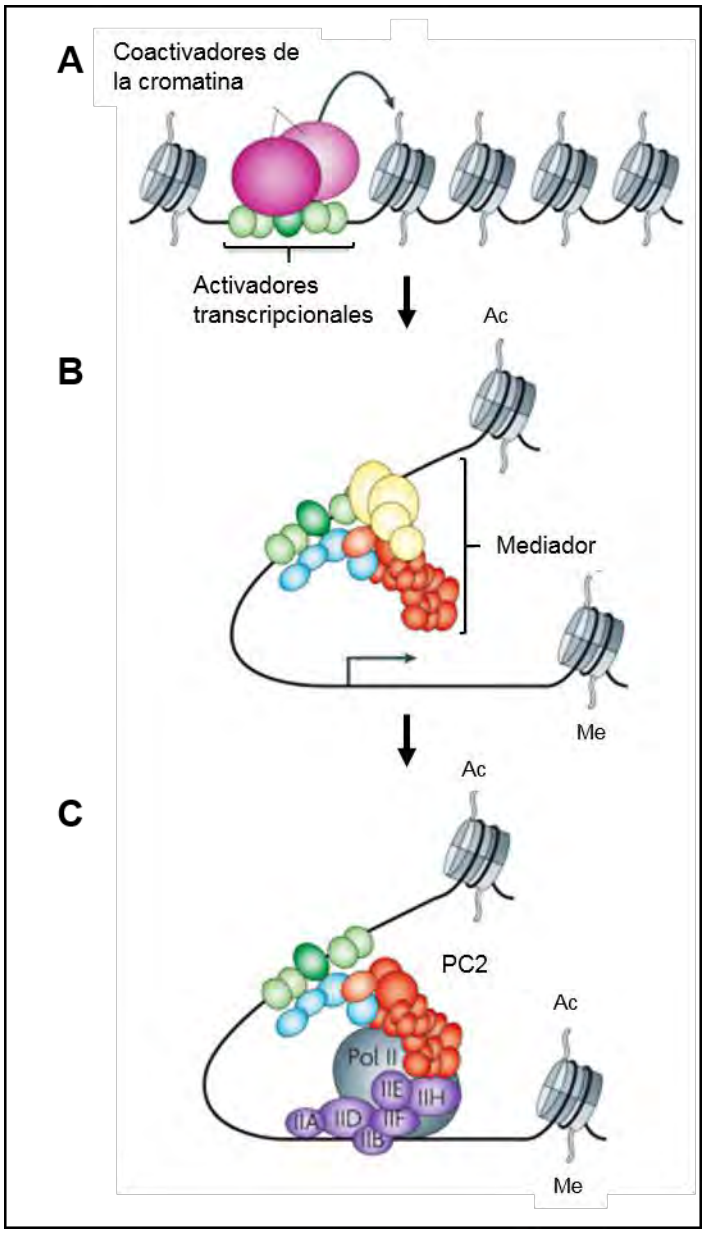


Figura 8. **Papel del complejo Mediador en la transcripción**. La activación transcripcional involucra múltiples pasos. (A) La vía de activación es iniciada por uno o más activadores transcripcionales que se unen a sus sitios en regiones regulatorias del gen. Estos factores reclutan una serie de co-activadores de la cromatina, los cuales pueden modificar covalentemente los nucleosomas en residuos específicos de histonas. (B) La cromatina sufre modificaciones covalentes mediadas por acetilación (Ac) y metilación (Me). Posteriormente, los activadores reclutan al complejo Mediador, el cual consiste de un núcleo y un módulo cinasa. (C) El complejo de pre-iniciación de la transcripción es ensamblado y consiste de varios GTFs (TFIIA, TFIIB, TFIID, TFIIE, y TFIIH) y la Pol II y la iniciación de la transcripción requiere del complejo Mediador re-estructurado, que resulta de la pérdida del módulo cinasa. Una vez que la transcripción es iniciada, el complejo transcripcional, incluyendo el Mediador, se mantienen en el promotor del gen para facilitar rondas de transcripción (Modificado de Malik y Roeder, 2010).

5. JUSTIFICACIÓN

Las plantas responden y se adaptan a las diferentes señales ambientales a través de un estricto control de la transcripción. Se ha considerado al complejo Mediador como un factor de transcripción general. PFT1/MED25, una subunidad del complejo Mediador está involucrada en procesos asociados a respuestas a defensa y en diferentes programas del desarrollo vegetal. Sin embargo, se desconoce su participación en la regulación de la arquitectura radicular y la relación con las diferentes vías hormonales (ej. auxinas), así como los mecanismos celulares y moleculares por los cuales actúa. El estudio de PFT1/MED25 mediante el uso de líneas mutantes y transgénicas de *Arabidopsis thaliana* permitirá entender la función de este elemento en dichos procesos.

6. HIPÓTESIS

La subunidad PFT1/MED25 regulan el desarrollo de la raíz a través de la señalización por auxinas en *Arabidopsis thaliana.*

7. OBJETIVOS

7.1. Objetivo general.

Determinar la participación de PFT1/MED25 en el desarrollo de la raíz y la respuesta a auxinas en *Arabidopsis thaliana*.

7.2. Objetivos específicos

1. Caracterizar el efecto de la mutación y sobre-expresión de la subunidad PFT1/MED25 sobre el sistema radicular de *Arabidopsis thaliana*.

2. Analizar el efecto de diferentes reguladores del desarrollo vegetal sobre las diferentes líneas mutantes y transgénicas afectadas en PFT1/MED25 de *Arabidopsis*.

3. Evaluar la expresión de marcadores de genes involucrados en la señalización, transporte, y respuesta a auxinas y marcadores del ciclo celular en plantas mutantes y transgénicas afectadas en PFT1/MED25 de *Arabidopsis*.

4. Determinar los niveles de AIA en plántulas silvestres, mutantes y transgénicas afectadas en PFT1/MED25.

8. RESULTADOS

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

8.1. CAPÍTULO I

Raya-González, J., Ortiz-Castro, R., Ruíz-Herrera, L.F., 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.

8.2. CAPÍTULO II

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* 169:1348-1358.

PHYTOCHROME AND FLOWERING TIME1/MEDIATOR25 Regulates Lateral Root Formation via Auxin Signaling in Arabidopsis[1][C][W]

Javier Raya-González, Randy Ortiz-Castro, León Francisco Ruíz-Herrera, Kemal Kazan, and José López-Bucio*

Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Ciudad Universitaria, CP 58030 Morelia, Michoacan, Mexico (J.R.-G., R.O.-C., L.F.R.-H., J.L.-B.); and Commonwealth Scientific and Industrial Research Organization Plant Industry, Queensland Bioscience Precinct, St. Lucia, Queensland 4067, Australia (K.K.)

Root system architecture is a major determinant of water and nutrient acquisition as well as stress tolerance in plants. The Mediator complex is a conserved multiprotein complex that acts as a universal adaptor between transcription factors and the RNA polymerase II. In this article, we characterize possible roles of the MEDIATOR8 (MED8) and MED25 subunits of the plant Mediator complex in the regulation of root system architecture in Arabidopsis (*Arabidopsis thaliana*). We found that loss-of-function mutations in PHYTOCHROME AND FLOWERING TIME1 (PFT1)/MED25 increase primary and lateral root growth as well as lateral and adventitious root formation. In contrast, *PFT1/MED25* overexpression reduces these responses, suggesting that PFT1/MED25 is an important element of meristematic cell proliferation and cell size control in both lateral and primary roots. PFT1/MED25 negatively regulates auxin transport and response gene expression in most parts of the plant, as evidenced by increased and decreased expression of the auxin-related reporters *PIN-FORMED1* (*PIN1*)::*PIN1*::*GFP* (for green fluorescent protein), *DR5*:*GFP*, *DR5*:*uidA*, and *BA3*:*uidA* in *pft1-2* mutants and in *35S*:*PFT1* seedlings, respectively. No alterations in endogenous auxin levels could be found in *pft1-2* mutants or in *35S*:*PFT1*-overexpressing seedlings. However, detailed analyses of *DR5*:*GFP* and *DR5*:*uidA* activity in wild-type, *pft1-2*, and *35S*:*PFT1* seedlings in response to indole-3-acetic acid, naphthaleneacetic acid, and the polar auxin transport inhibitor 1-*N*-naphthylphthalamic acid indicated that PFT1/MED25 principally regulates auxin transport and response. These results provide compelling evidence for a new role for PFT1/MED25 as an important transcriptional regulator of root system architecture through auxin-related mechanisms in Arabidopsis.

The indeterminate growth of the plant root system through continuous cell division and elongation processes can be profoundly affected by nutrient and water availability as well as various stress conditions, such as extreme temperatures, drought, and/or salt stress (López-Bucio et al., 2003; Malamy, 2005). Therefore, the plasticity of the root system is of critical importance for the plant to compete for resources and adapt to constantly changing growth conditions. The Arabidopsis (*Arabidopsis thaliana*) root system architecture, which consists of a primary root, lateral roots, and root hairs, determines the exploratory ability of roots in the soil. Lateral roots initiate from a few pericycle cells that acquire the attributes of founder cells and then divide asymmetrically to give rise to lateral root primordia (LRP), which continue to grow and eventually emerge from the primary root (Dubrovsky et al., 2000, 2008). Finally, the new apical meristem is established and takes over the control of the growth of mature lateral roots (Malamy and Benfey, 1997).

The phytohormone auxin (indole-3-acetic acid [IAA]) plays an important role during all stages of lateral root formation (Casimiro et al., 2003; De Smet et al., 2006; Fukaki et al., 2007). Application of IAA or synthetic auxins such as naphthaleneacetic acid (NAA) stimulates lateral root formation (Celenza et al., 1995), whereas treatment with polar auxin transport inhibitors prevents lateral root initiation (Casimiro et al., 2001; Himanen et al., 2002). Consistently, Arabidopsis mutants with increased auxin levels, such as *rooty* and its alleles *aberrant lateral root formation1* and *superroot1*, have increased numbers of lateral roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants such as *AUXIN RESISTANT1* (*aux1*), *AUXIN RESPONSE1* (*axr1*), *DARK OVEREXPRESSION OF CAB1* (*doc1*), *SOLITARY ROOT1*, and *AUXIN-RESPONSE FACTORS7/19*, with defective auxin transport, perception, and/or signaling, show reduced lateral root formation (Lincoln et al., 1990; Gil et al., 2001; Swarup et al., 2001;

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* Address correspondence to jbucio@umich.mx.

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

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Fukaki et al., 2002). Auxin is unique among plant hormones in being actively and directionally transported from the place of synthesis in young apical parts to distant tissues. The auxin-efflux regulators PIN-FORMED (PIN) are crucial for auxin distribution throughout the plant. PIN proteins have been shown to mediate various developmental processes. For instance, vascular tissue and flower development is regulated by PIN1 (Gälweiler et al., 1998), tropisms are regulated by PIN2 and PIN3 (Friml et al., 2002b), and the patterning of the root is regulated by PIN4 (Friml et al., 2002a).

Auxin can interact with other plant hormones to orchestrate root development. Recently, it has been found that CORONATINE-INSENSITIVE1 (COI1), the jasmonic acid (JA) receptor, is required to mediate lateral root formation in response to JA, a canonical defense signal, thus indicating the participation of downstream signaling components integrating the responses of two or more plant hormones into plant organogenesis (Raya-González et al., 2012).

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 myriad transcription factors present within the cell (Kim et al., 1994; Koleske and Young, 1994). Mediator fine-tunes diverse regulatory inputs and presents a balanced output to the RNA polymerase II to initiate transcription by binding to distal activators/repressors as well as general transcription factors at the promoter site (Malik and Roeder, 2005). In *Saccharomyces cerevisiae*, Mediator is composed of 25 subunits, of which 22 are at least partially conserved among eukaryotes (Boube et al., 2002; Bourbon et al., 2004). Specific Mediator subunits are required for growth and development in organisms such as *Drosophila melanogaster*, zebrafish, mouse, and *Caenorhabditis elegans* (Lehner et al., 2006; Wang et al., 2006; Loncle et al., 2007; Jiang et al., 2010).

Plant growth is regulated by both cell number and cell size, which in turn are controlled by coordinated cell proliferation and expansion events during organogenesis (Mizukami, 2001; Sugimoto-Shirasu and Roberts, 2003). Recently, the participation of the Mediator complex in plant organ size control and cell differentiation was evidenced through the identification of 21 conserved and six putative plant-specific Mediator subunits in Arabidopsis and the analysis of Arabidopsis mutants defective in MEDIATOR14 (MED14) and MED25 (Autran et al., 2002; Xu and Li, 2011; Sundaravelpandian et al., 2013). Prior to its identification as Mediator subunit 14, STRUWWELPETER was found to regulate cell number and shoot meristem development (Autran et al., 2002). Arabidopsis MED25 was originally identified as PHYTOCHROME AND FLOWERING TIME1 (PFT1), a nuclear protein acting in a photoreceptor pathway that induces flowering in response to suboptimal light conditions (Cerdán and Chory, 2003). In addition, Arabidopsis mutants compromised in the kinase module of the Mediator complex, MED12, MED13, and CYCLIN-DEPENDENT KINASE8, all show developmental phenotypes due to altered cell differentiation (Wang and Chen, 2004; Gillmor et al., 2010; Ito et al., 2011). The *med12* and *med13* mutants are affected in the transition of embryos from globular to heart stage, due to a delay in the expression of *KANADI1* and *KANADI2* transcription factors during early development (Gillmor et al., 2010). The effect of the *med13* mutation on cell differentiation has also been explained by a defective response to the hormone auxin (Ito et al., 2011). Even though MED8 and MED25 play important roles in JA signaling, stress responses, and plant development such as root hair formation and flowering time, at present, possible roles of these and other Mediator complex subunits on root system architecture and auxin signaling are unknown.

In this study, we tested the possible participation of MED8 and PFT1/MED25 subunits of Mediator in root development and auxin signaling in Arabidopsis. While *med8* mutants did not show any evident alteration of root architecture, the *pft1* mutants had increased primary root growth and root branching. Analysis of the cell cycle marker *CycB1*:*uidA*, cell size measurements, auxin transport, and auxin-responsive reporter gene expression in *pft1-2* and *35S*:*PFT1* seedlings before and after treatments with IAA, NAA, and the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) further indicated that the Mediator subunit PFT1/MED25 acts as a negative regulator of cell proliferation, lateral root formation, auxin transport, and auxin-responsive gene expression in Arabidopsis.

RESULTS

The PFT1/MED25 Subunit of Mediator Regulates Root Architecture in Arabidopsis

To determine the participation of the two subunits of Mediator, MED8 and PFT1/MED25, as regulators of Arabidopsis root architecture, we compared root growth phenotypes of the wild type (Columbia-0 [Col-0]), *pft1-2* and *med8* single mutants, the *pft1 med8* double mutant, as well as *pft1-1* mutants that were transformed with a genomic copy of *PFT1* (G1 complementation line [*gPFT1*]; Cerdán and Chory, 2003) and *PFT1/MED25* overexpression seedlings (*35S*:*PFT1*). The seedlings were grown on agar-solidified 0.2× Murashige and Skoog (MS) medium, and primary root lengths and lateral root numbers and lengths were quantified 8 d after germination. We found that *pft1-2* and *pft1 med8* seedlings had longer primary roots than wild-type seedlings, whereas *med8* and *gPFT1* plants were not affected in primary root growth (Fig. 1A). In contrast, *35S*:*PFT1* plants had shorter primary roots than wild-type plants (Fig. 1A). Interestingly, *pft1-2* and *pft1 med8* seedlings showed nearly 2-fold increases in lateral root numbers and lateral root lengths when compared with wild-type, *gPFT1*, and *med8* seedlings (Fig. 1, B–D). In contrast, *35S*:*PFT1* seedlings showed fewer and shorter lateral roots than wild-type and *gPFT1* seedlings (Fig. 1, B–D). An increase in primary root growth and lateral root formation in *pft1-1*, *pft1-2*, and *pft1-3* mutants relative to the wild type was confirmed in additional experiments (Supplemental

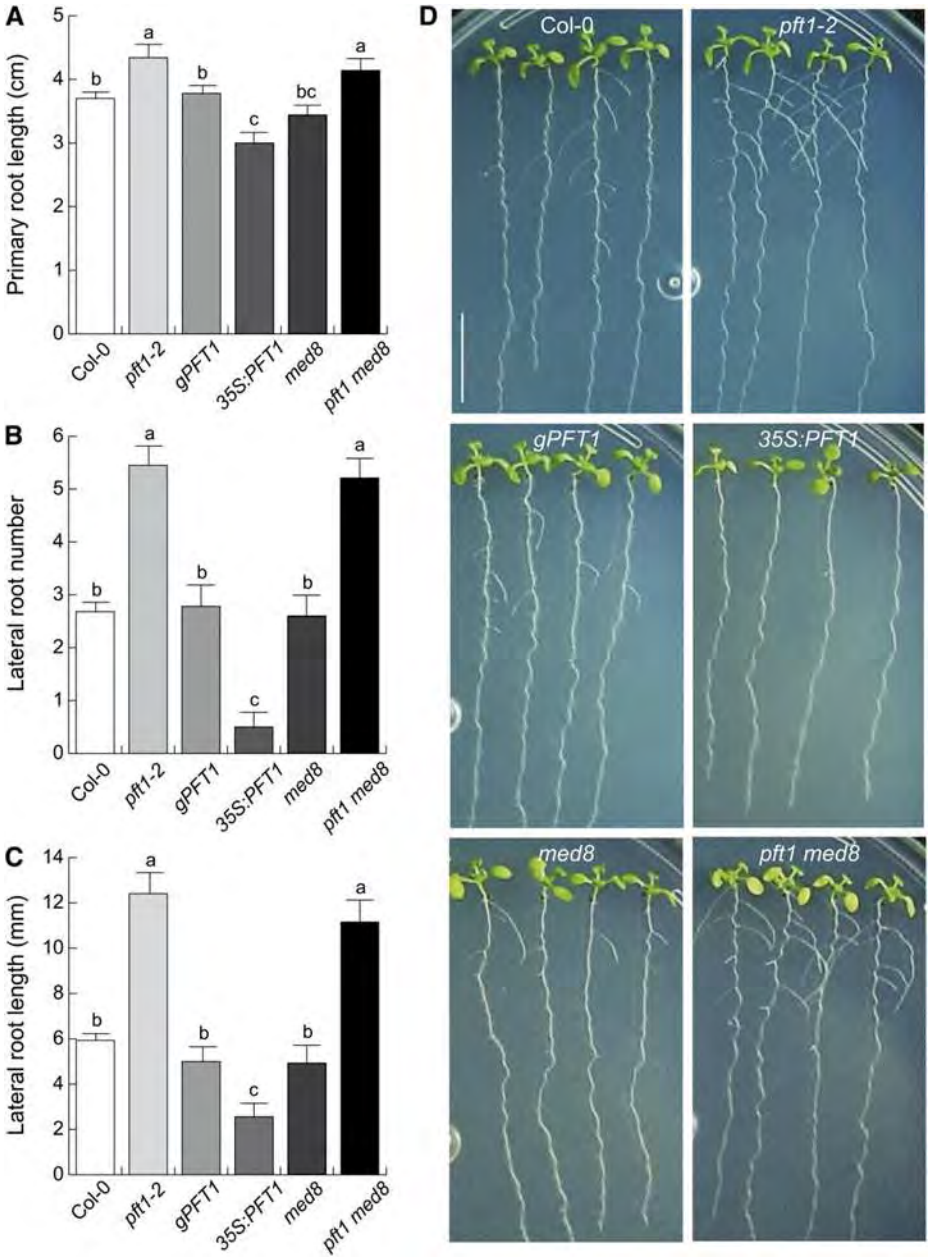

Figure 1. PFT1/MED25 regulates root system architecture in Arabidopsis. Wild-type (Col-0), *pft1-2*, *gPFT1*, *35S:PFT1*, *med8*, and *pft1 med8* seeds were germinated and seedlings were grown for 8 d on agar-solidified 0.2× MS medium. A, Primary root length. B, Lateral root number. C, Lateral root length. Root traits were scored as indicated in "Materials and Methods." Error bars represent SE from 30 seedlings. Different letters indicate statistical differences at $P < 0.05$. D, Photographs of representative wild-type (Col-0), *pft1-2*, *gPFT1*, *35S:PFT1*, *med8*, and *pft1 med8* seedlings. Note that an opposite response is seen in lateral root formation and lengths between the *pft1-2* mutant and *PFT1*-overexpressing seedlings. The experiment was repeated twice with similar results. Bar = 1 cm. [See online article for color version of this figure.]

Fig. S1). Together, these results indicate that PFT1/MED25 regulates root system architecture in Arabidopsis.

Adventitious roots originate from stem tissue and provide an increased ability for the plant to explore soil for water and nutrients. To evaluate the participation of PFT1/MED25 and MED8 in adventitious root formation, we obtained stem explants from wild-type, *pft1-2*, *35S:PFT1*, *med8*, and *pft1 med8* seedlings grown in dark conditions. These explants were cultured for 5 d over the surface of petri plates containing agar-solidified 0.2× MS medium, and both first- and second-order adventitious roots were quantified. In these experiments, *pft1-2* and *pft1 med8* seedlings had 2- and 5-fold increases in first- and second-order adventitious root numbers, respectively, when compared with wild-type seedlings, while the formation of second-order adventitious roots was drastically reduced in *35S:PFT1* seedlings (Supplemental Fig. S2). These results suggest that PFT1/MED25 also regulates processes associated with adventitious root development in Arabidopsis.

PFT1/MED25 Controls Cell Division and Elongation

Primary root growth depends on two basic processes: cell division in the root apical meristem and elongation of divided cells that subsequently leave the root meristem (Blilou et al., 2002). Because root architecture was altered in *pft1* mutants, we next explored the role of PFT1/

MED25 on cell division and elongation of primary and lateral roots. For this aim, we crossed *pft1-2* and *35S:PFT1* plants with Arabidopsis plants expressing the cell cycle marker *CycB1:uidA* (Colón-Carmona et al., 1999), which monitors cell cycle progression in the root meristem. We found that *pft1-2/CycB1:uidA* root meristems contain 40% more dividing cells than wild-type meristems, as revealed by the increased numbers of blue spots in the GUS expression domain of *pft1-2/CycB1:uidA* primary root meristems (Fig. 2, A and D), which were also larger than *CycB1:uidA* and *35S:PFT1/CycB1:uidA* root meristems (Fig. 2B). In contrast, *35S:PFT1/CycB1:uidA* seedlings had fewer dividing cells and smaller root meristems than *CycB1:uidA* seedlings (Fig. 2, A, B, and D). Contrasting expression of *CycB1:uidA* was also observed in emerged lateral roots of *pft1-2/CycB1:uidA* and *35S:PFT1/CycB1:uidA* seedlings (Supplemental Fig. S3). To determine the involvement of PFT1/MED25 in cell elongation, we measured fully developed cortical cells of the primary and lateral roots of *CycB1:uidA*, *pft1-2/CycB1:uidA*, and *35S:PFT1/CycB1:uidA* seedlings and found that cortical cells of *pft1-2/CycB1:uidA* seedlings were, on average, 15% longer than those of *CycB1:uidA* seedlings (Fig. 2C; Supplemental Fig. S3). In contrast, cortical cells of *35S:PFT1/CycB1:uidA* seedlings were significantly shorter than those of *pft1-2/CycB1:uidA* seedlings (Fig. 2C). Taken together, these data indicate that PFT1/MED25 acts as a repressor of cell division and elongation during primary and lateral root growth in Arabidopsis.

PFT1/MED25 Regulates LRP Development through Auxin Signaling

To understand the role played by PFT1/MED25 during lateral root formation and its possible relationship with auxin signaling, which regulates this organogenesis process, we analyzed LRP originating from the primary roots of wild-type, *pft1-2*, and *35S:PFT1* seedlings, according to Malamy and Benfey (1997). In 7-d-old *pft1-2* roots, the number of stage I and II LRP were 2-fold higher than in wild-type roots (Fig. 3), suggesting that *pft1-2* primary roots are more branched because they produce more de novo LRP from pericycle cells. Given that lateral root formation is a process regulated by auxin signaling, we then evaluated the expression of the auxin-responsive marker *DR5:uidA* during LRP development in *DR5:uidA*, *pft1-2/DR5:uidA*, and *35S:PFT1/DR5:uidA* seedlings. *DR5:uidA* expression in *pft1-2/DR5:uidA* seedlings was stronger at all LRP developmental stages and in mature lateral roots than in *DR5:uidA* and *35S:PFT1/ DR5:uidA* seedlings (Supplemental Figs. S4 and S5). These data suggest that PFT1/MED25 modulates lateral root formation by regulating auxin signaling during LRP development.

PFT1/MED25 Regulates Auxin-Responsive Reporter Gene Expression in Roots and Shoots

Auxin signaling has been implicated in many development processes in both root and shoot systems. To determine whether PFT1/MED25-mediated alterations in auxin responses could also occur in other plant tissues, we evaluated reporter gene activity in wild-type as well as *pft1-2* and *35S:PFT1* seedlings harboring the *DR5:GFP*, *DR5:uidA*, or *BA3:uidA* gene construct. First, seedlings harboring the *DR5:GFP* construct were grown for 7 d on agar-solidified 0.2× MS medium with or without 60 nM IAA, and different parts of the plants, including root tips, vascular tissues, and root/shoot transition zones, were

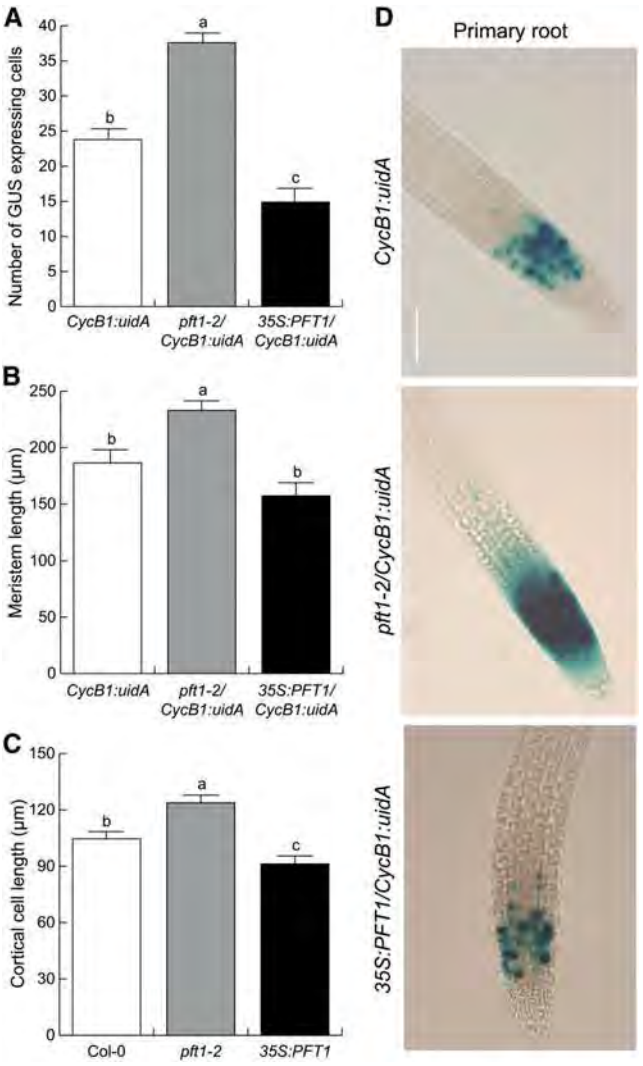

Figure 2. PFT1/MED25 represses cell division and elongation in primary root apical meristems. Wild-type (Col-0), *pft1-2*, and *35S:PFT1* Arabidopsis seeds harboring the *CycB1:uidA* gene construct were germinated and seedlings were grown for 7 d on agar-solidified 0.2× MS medium. A, Number of GUS-positive spots per root meristem. B and C, Meristem length (B) and cortical cell length (C) were scored as indicated in "Materials and Methods." D, Primary roots of young seedlings were stained for GUS activity and cleared to show the expression of *CycB1:uidA*. Photographs show representative individuals from 15 GUS-stained seedlings. Error bars represent SE from 15 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. [See online article for color version of this figure.]

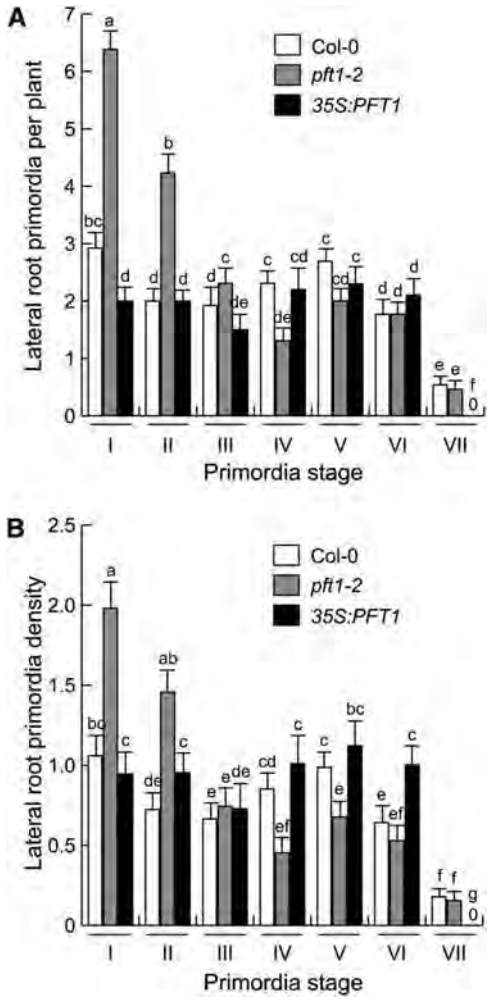

Figure 3. PFT1/MED25 modulates LRP formation. Wild-type (Col-0), *pft1-2*, and *35S:PFT1* Arabidopsis seeds were germinated and seedlings were grown for 7 d on agar-solidified 0.2× MS medium and the development of root primordia was evaluated. A, LRP per plant. B, LRP density (LRP per cm). LRP stages were recorded according to Malamy and Benfey (1997). Error bars represent SE from 15 GUS-stained seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

analyzed by confocal microscopy. GFP expression was higher in *pft1-2/DR5:GFP* but lower in *35S:PFT1/DR5:GFP* in all three regions analyzed (Fig. 4). Exogenous auxin further induced *GFP* expression in *pft1-2/DR5:GFP* and *DR5:GFP*, while no such induction was evident in *35S:PFT1/DR5:GFP* seedling roots (Fig. 4). We next analyzed the *DR5:uidA*-driven GUS activity in cotyledons, young leaves, shoot meristems, the stem/root transition zone, and the primary root tip. GUS activity was present in the primary root tip region and in leaves of untreated *DR5:uidA* seedlings (Supplemental Fig. S6). However, an increase in GUS activity was evident in most tissues of *pft1-2/DR5:GUS* seedlings, including cotyledons, petioles, the stem/root transition zone, lateral roots, and primary root tips. In contrast, GUS activity was much lower in *35S:PFT1* seedlings than in *DR5:GUS* and particularly in *pft1-2/DR5:GUS* in all tissues analyzed (Supplemental Fig. S6).

PFT1/MED25 Regulates the Auxin Response

The observation of contrasting root growth phenotypes resulting from the loss and gain of PFT1/MED25 function, together with the contrasting expression of auxin reporter genes in *pft1-2* and *35S:PFT1* seedlings, suggest a role for PFT1/MED25 in the auxin signaling pathway. As IAA is a major regulator of root architecture, PFT1/MED25-mediated effects in root development could be due to potential alterations in auxin biosynthesis, auxin transport, and/or auxin response. To determine whether *pft1* mutants could overaccumulate auxin, we first measured free IAA levels in wild-type, *pft1-2*, and *35S:PFT1* whole seedlings by gas chromatography-mass spectrometry. No significant differences in auxin accumulation were observed between wild-type, *pft1-2*, and *35S:PFT1* seedlings (Supplemental Fig. S7), suggesting that PFT1/MED25 is not a regulator of auxin biosynthesis.

To assess whether the observed root phenotypes could be due to changes in auxin-responsive gene expression, we performed experiments with wild-type, *pft1-2*, and *35S:PFT1* seedlings harboring the *DR5:uidA* or *BA3:uidA* gene construct after treatments with IAA. Arabidopsis seedlings were grown for 7 d on 0.2× MS medium solidified with agar and then transferred to liquid 0.2× MS medium supplemented with either the solvent only or different concentrations of IAA and incubated for 8 h at 22°C. In solvent-treated *DR5:uidA* seedlings, GUS expression was present in leaves and primary roots (Fig. 5). As expected, *DR5:uidA* seedlings treated with IAA showed a dose-dependent increase in GUS activity (Fig. 5). In contrast, the GUS activity in response to IAA was higher and lower in *pft1-2/DR5:uidA* and *35S:PFT1/DR5:uidA* seedlings, respectively, than in *DR5:uidA* (Fig. 5).

We further evaluated the IAA response in wild-type, *pft1-2*, and *35S:PFT1* seedlings harboring the *BA3:uidA* marker. We found that this marker is strongly expressed in petioles and vascular tissues of *pft1-2/BA3:uidA* seedlings under standard growth conditions (Supplemental Fig. S8). In response to IAA, *pft1-2/BA3:uidA* even showed a stronger GUS activity than the wild type in petioles, vascular tissues, and primary root elongation zones (Supplemental Fig. S8). In contrast, *35S:PFT1/BA3:uidA* seedlings showed a weaker GUS activity than *BA3:uidA* in petioles and primary roots both in the absence of any treatment and in response to IAA (Supplemental Fig. S8). These results indicate that PFT1/MED25 modulates the auxin response in Arabidopsis.

PFT1/MED25 Affects Lateral Root Formation in Response to Auxin

Auxin has been shown to inhibit the elongation of the primary root and to stimulate lateral root formation, whereas auxin transport inhibitors (e.g. NPA) antagonize

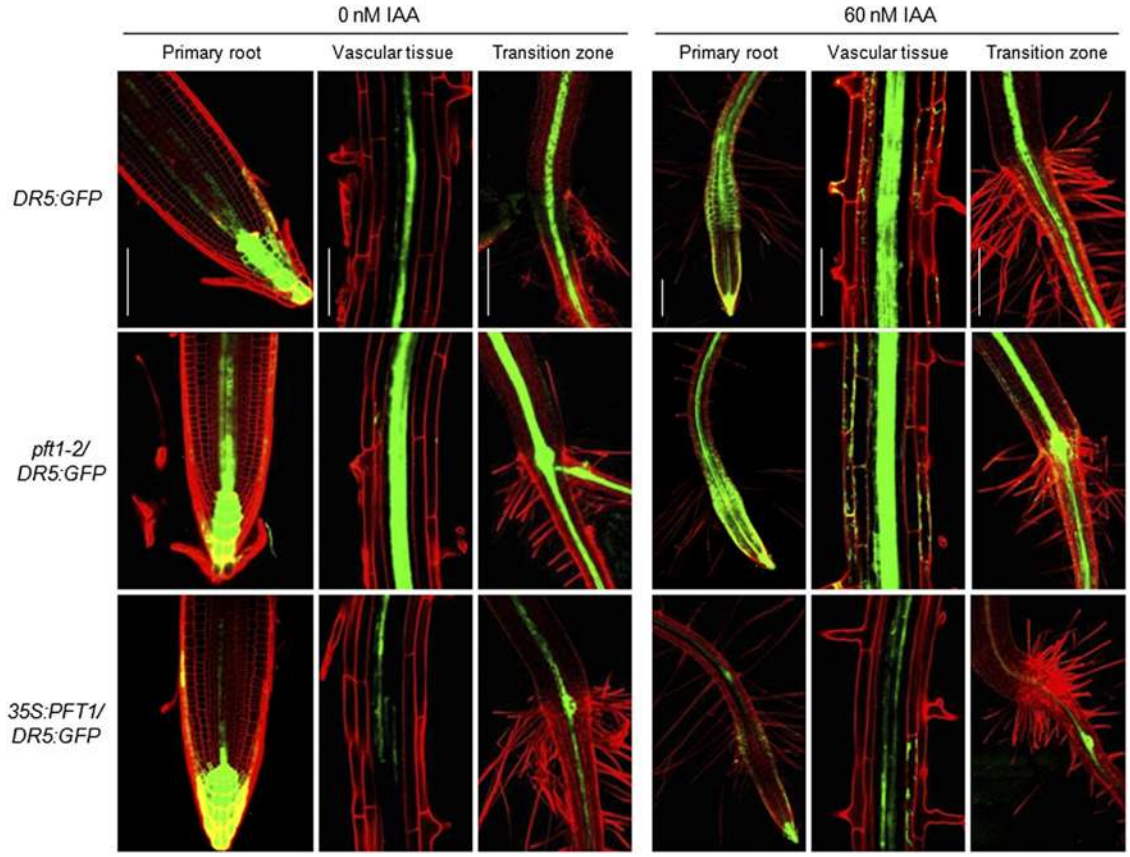

Figure 4. PFT1 modulates auxin-responsive gene expression. Wild-type, *pft1-2*, and *35S:PFT1* seeds harboring the *DR5:GFP* gene construct were germinated and seedlings were grown for 7 d in agar-solidified 0.2× MS medium and then transferred for 8 h to liquid medium supplemented with or without IAA. Photographs show representative individuals of at least 15 seedlings analyzed by confocal microscopy. Note that *pft1-2* and *35S:PFT1* seedlings show stronger and weaker *DR5:GFP* reporter expression, respectively, than wild-type seedlings in all regions analyzed. Bars = 100 μm. [See online article for color version of this figure.]

lateral root formation (Blakely et al., 1988; Muday and Haworth, 1994; Casimiro et al., 2001). To determine whether auxin transport is an important determinant of the root developmental changes mediated by PFT1/MED25, we evaluated root architectural responses of wild-type, *pft1-2*, and *35S:PFT1* seedlings grown on petri plates containing 0.2× MS medium supplied with low concentrations of NAA, a synthetic auxin that enters the root cells via diffusion. Primary root growth was similarly inhibited in wild-type and *pft1-2* seedlings in response to 5 to 30 nM NAA (Fig. 6A). Interestingly, *pft1-2* seedlings showed an increased response to NAA, as evidenced by 2- to 3-fold increases in the number and density of lateral roots in all evaluated NAA concentrations (Fig. 6, B–D). In contrast, in response to NAA, *35S:PFT1* seedlings produced lower numbers and density of lateral roots than wild-type and *pft1-2* seedlings (Fig. 6, B–D). When the lateral root data are normalized to the value obtained in the untreated control for each genotype, the fold increases in lateral root numbers appear clearly different in all three genotypes, with a nearly 2-fold higher relative lateral root formation in *pft1-2* seedlings than in the wild type (Fig. 6C). Together, these observations suggest that PFT1/MED25 regulates pericycle cells to divide in response to auxin.

PFT1/MED25 Regulates the Expression and Distribution of the Auxin Transporter PIN1

Auxin positively influences the PIN family of auxin transporters in a tissue-specific manner through an AUX/IAA-dependent signaling pathway (Vieten et al., 2005). PIN1 and PIN2 play important roles in lateral root formation and auxin-mediated gravitropism, respectively (Benková et al., 2003). To test whether PFT1/MED25 could regulate primary root growth and/or lateral root formation through differential expression of PIN1 or PIN2, we analyzed the spatial pattern of PIN1 and PIN2 localization in wild-type, *pft1-2*, and *35S:PFT1* seedlings. In primary roots of seedlings expressing *PIN1::PIN1:GFP* (Vieten et al., 2005), the GFP fluorescence was detected in the stele and endodermis cells (Fig. 7A). In *pft1-2* primary roots, the GFP fluorescence was stronger than in *PIN1::PIN1:GFP* and extended toward the root differentiation zone (Fig. 7B), while in *35S:PFT1* seedlings, GFP fluorescence was weaker than in *PIN1::PIN1:GFP* and

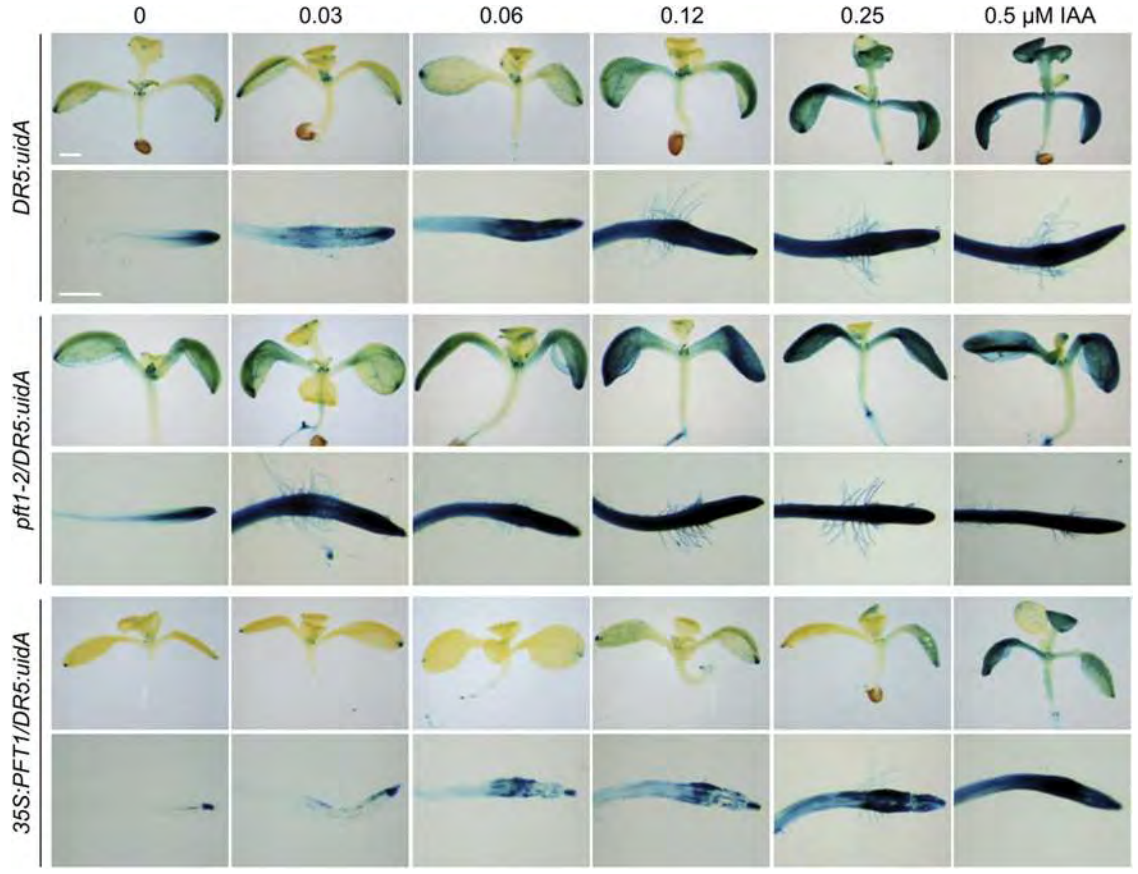

Figure 5. Effects of IAA on auxin-responsive reporter gene expression in wild-type, *pft1-2*, and *35S:PFT1* seedlings. Wild-type, *pft1-2*, and *35S:PFT1* seeds harboring the *DR5:uidA* gene construct were germinated and seedlings were grown for 7 d in agar-solidified 0.2× MS medium and then transferred for 8 h to liquid medium supplemented with or without IAA. Photographs show representative individuals of at least 15 GUS-stained seedlings analyzed. Note that *pft1-2* and *35S:PFT1* seedlings show stronger and weaker GUS activity, respectively, than wild-type seedlings. Bars = 200 μm. [See online article for color version of this figure.]

pft1-2/PIN1::*PIN1*:*GFP* and remained somewhat restricted (Fig. 7C). An analysis of PIN1 localization during lateral root initiation showed that in wild-type plants, stage V and VII primordia displayed the typical localization of PIN1 in most external cell layers, while increased and decreased PIN1 expression was evident in stage V and VII primordia of *pft1-2/PIN1*::*PIN1*:*GFP* and *35S*:*PFT1/PIN1*::*PIN1*:*GFP* seedlings, respectively (Fig. 7, D–I). In contrast to the differential expression of PIN1 in *pft1*, PIN2 expression was similarly detected in cortex and epidermal cells of wild-type, *pft1-2*, and *35S*:*PFT1* primary roots (Supplemental Fig. S9). These findings suggest that PFT1/MED25 specifically regulates the expression and distribution of the PIN1 auxin transporter.

PFT1/MED25 Modulates the Response of Root Architecture to NPA

To further analyze the participation of PFT1/MED25 in auxin transport, we evaluated the effects of the polar auxin transport inhibitor NPA on root architecture in wild-type, *pft1-2*, and *35S*:*PFT1* seedlings. Arabidopsis seedlings were grown side by side for 8 d on agar plates containing 0.2× MS medium supplied either with 0.25 to 4 μM NPA or without NPA.

NPA at 4 μM inhibited primary root growth approximately 60% in wild-type seedlings when the data for root lengths were normalized to the values obtained in their untreated counterparts. Relative to the response we observed in wild-type seedlings, *pft1-2* seedlings showed reduced responses to 2 and 4 μM NPA (Fig. 8A), while *35S*:*PFT1* seedlings had a wild-type-like response (Fig. 8A). As expected, NPA dramatically inhibited lateral root formation in wild-type seedlings (Fig. 8, B–D). However, lateral root formation in *pft1-2* seedlings was less sensitive while in *35S*:*PFT1* seedlings it was more sensitive to NPA than in the wild type. Together, these contrasting responses of *pft1-2* and *35S*:*PFT1* seedlings to an IAA efflux inhibitor in terms of both primary root growth and lateral root formation suggest a role for PFT1/MED25 in modulating auxin transport and response.

Root Architectural Responses of *pft1* and *coi1* Mutants to JA

Recent reports have shown that PFT1/MED25 is required for JA-mediated defense gene expression (Kidd

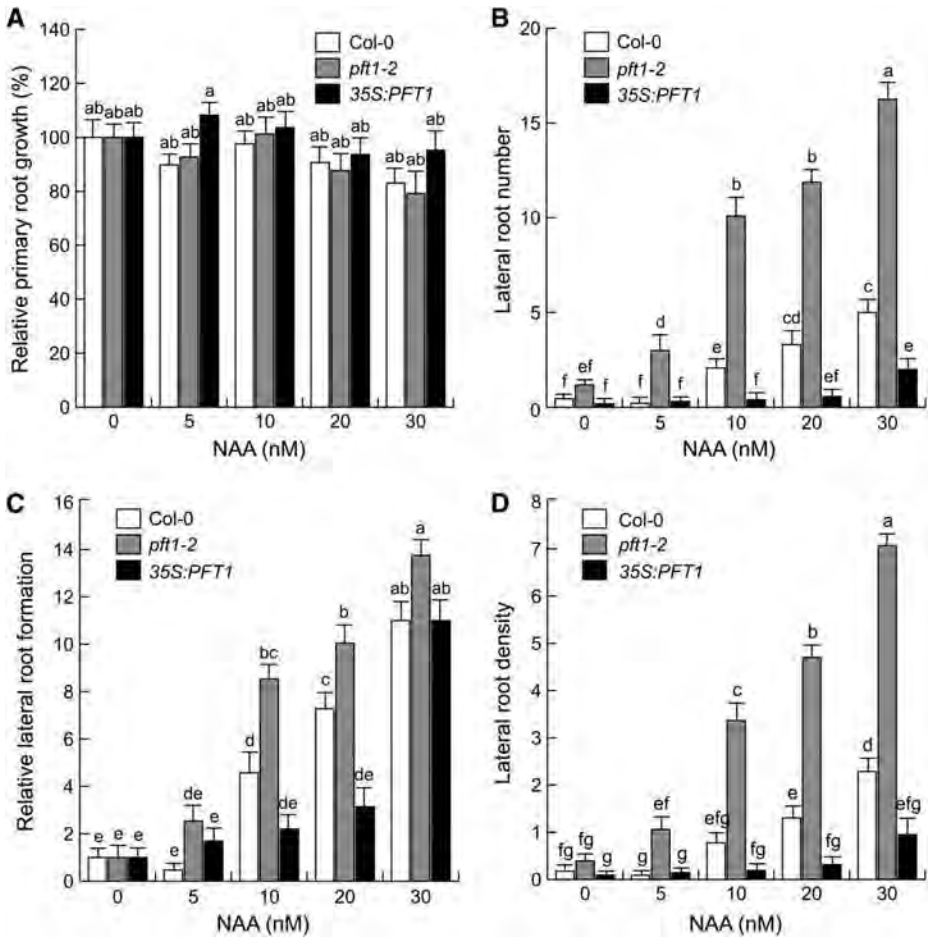

Figure 6. Effects of NAA on root system architecture of wild-type (Col-0), *pft1-2*, and *35S:PFT1* seedlings. Arabidopsis seeds were germinated and seedlings were grown for 7 d with or without NAA. A, Relative primary root growth. B, Lateral root number. C, Relative lateral root formation (fold induction). D, Lateral root density. Primary root lengths at 0 nM NAA were 26 mm for Col-0, 30 mm for *pft1-2*, and 19 mm for *35S:PFT1*. Error bars represent SE from 15 seedlings. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

et al., 2009). Because JA affects both auxin signaling and root architecture (Raya-González et al., 2012), it was important to test the function of PFT1/MED25 in JA-mediated root architecture regulation. Primary root growth and lateral root formation were analyzed in wild-type, *pft1-2*, and *coi1-1* seedlings grown side by side. In these experiments, *pft1-2* showed a wild-type-like phenotype in its relative response to JA inhibition of primary root growth (Fig. 9, A and B). Also, a similar increase in lateral root numbers to wild-type seedlings in response to 4 μM JA was evident in *pft1-2* seedlings (Fig. 9, C–E). In contrast, as expected, *coi1-1* seedlings were highly resistant to primary root inhibition and lateral root promotion by JA (Fig. 9, B and E). Thus, these data show that PFT1/MED25 likely acts independently of the jasmonate receptor COI1 and JA to regulate lateral root formation in Arabidopsis.

DISCUSSION

PFT1/MED25 Plays a Role in Root Development

The root system, which plays an important role in anchoring the plant to the soil and in water and nutrient acquisition, exhibits an amazing architectural diversity manifested through changes in root hair, lateral root, and adventitious root formation and primary root growth (López-Bucio et al., 2003; Nibau et al., 2008). Engineering of the root architecture of crop plants can be of value for increasing plant stress tolerance, but this requires a thorough understanding of complex and interacting endogenous and exogenous factors that control individual aspects of root system configuration.

In this study, we investigated possible roles of MED8 and PFT1/MED25 Mediator subunits as regulators of the root system architecture of Arabidopsis seedlings. To the best of our knowledge, the Mediator complex has not been implicated so far in lateral root development and auxin signaling, despite its requirement as an essential component of gene transcription in all eukaryotes. Our results show that while *med8* single mutants had root architecture similar to wild-type seedlings, the loss and gain of function of PFT1/MED25 showed opposite effects on lateral and adventitious root development and on primary root growth, indicating that PFT1/MED25 functions as a key modulator of cellular processes that control root architecture configuration.

The root phenotypes observed in *pft1-2* and *35S:PFT1* seedlings correlated with changes in *CycB1:uidA* expression and cell expansion in primary and lateral roots (Fig. 2; Supplemental Fig. S3). Therefore, it is plausible that PFT1/MED25 regulates root development by inhibiting cell division and expansion in the roots. Through microarray

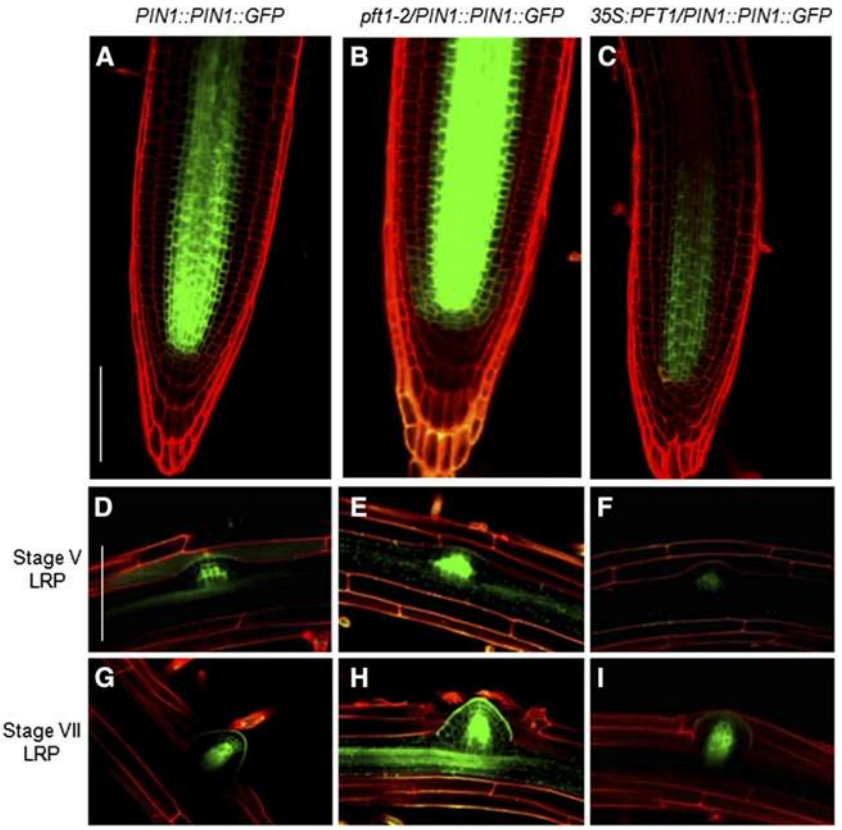

Figure 7. *PIN1* expression in wild-type, *pft1-2*, and *35S:PFT1* seedlings. *PIN1::PIN1::GFP*, *pft1-2/PIN1::PIN1::GFP*, and *35S:PFT1/PIN1::PIN1::GFP* seeds were germinated and seedlings were grown on agar-solidified 0.2× MS medium. Seven days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. A to C, Primary root apical meristems. D to F, Stage V LRP. G to I, Stage VII LRP. Representative photographs of primary roots and LRP are shown (n = 10). Note the increase and decrease of *PIN1::PIN1::GFP* in *pft1-2* and *35S:PFT1* backgrounds, respectively. Bars = 100 μm. [See online article for color version of this figure.]

analyses conducted on wild-type and *pft1* roots, Sundaravelpandian et al. (2013) found that the genes implicated in growth- and cell cycle-associated processes were differentially expressed between *pft1-2* and wild-type seedlings. For instance, the genes associated with the indole butyric acid-related processes were found to be differentially expressed in *pft1* roots. In the light of our results, this is of particular interest, as genetic evidence in Arabidopsis suggests that indole-3-butyric acid converted into IAA by peroxisomal β-oxidation plays an important role in root hair formation and other developmental events by regulating cell expansion (Strader et al., 2010). Recently, Xu and Li (2011), through the use of transgenic Arabidopsis plants harboring the *MED25* promoter::*GUS* fusion (*pMED25::GUS*) construct, showed that *MED25* is expressed throughout the plant. The expression of *PFT1/MED25* in roots is particularly relevant to our findings, which suggest that both cell proliferation and elongation are affected by PFT1/MED25 in the Arabidopsis root system. Also, given that PFT1/MED25 was first described as a positive regulator of shade avoidance and later as a regulator of basal defense and abiotic stress responses (Cerdán and Chory, 2003; Bäckström et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011; Chen et al., 2012), it seems likely that PFT1/MED25 represents a molecular node for the integration of distinct environmental and developmental cues.

The PFT1/MED25 protein is highly conserved across diverse eukaryotes. Remarkably, MED25 in other eukaryotes is also associated with several developmental processes. For example, the RNA interference-mediated suppression of *MED25* expression in *D. melanogaster* results in the failure of extension of some axons that affect the embryonic nervous system (Koizumi et al., 2007). In zebrafish, morpholino-mediated knockdown of *MED25* induces palatal malformation, suggesting an important role for MED25 in cartilage development (Nakamura et al., 2011). Remarkably, through the characterization of mutants and complemented and overexpressing lines of PFT1/MED25, we obtained evidence in this study that PFT1/MED25 functions in the development of organs and tissues ubiquitous to plants, such as lateral and adventitious roots.

PFT1/MED25 Is a Negative Regulator of LRP Development

Lateral root formation is initiated when the pericycle cells respond to auxin and acquire the status of founder cells and, through subsequent asymmetric cell divisions, give rise to new LRP (Boerjan et al., 1995; Malamy and Benfey, 1997; Dubrovsky et al., 2008). The loss of PFT1/MED25 function leads to the formation of increased numbers of LRP, particularly in stages I and II. In contrast, the overexpression of *PFT1/MED25* resulted in decreased primordium formation (Fig. 3). These results indicate that PFT1/MED25 modulates root branching in Arabidopsis by inducing the de novo formation of LRP from pericycle cells. Kidd et al. (2009) showed that *pft1* seedlings form more rosette leaves than wild-type plants. In plants, lateral

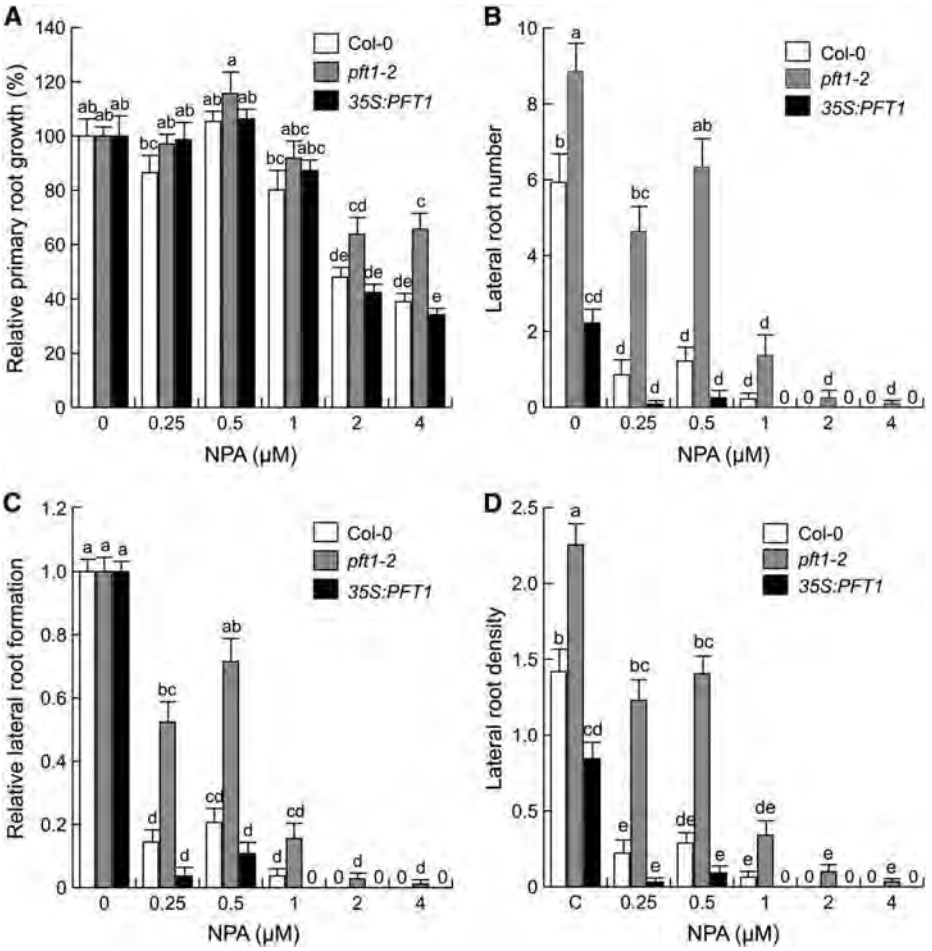

Figure 8. Effects of NPA on root architecture in wild-type (Col-0), *pft1-2*, and *35S:PFT1* seedlings. Arabidopsis seeds were germinated and seedlings were grown for 8 d with or without NPA. A, Relative primary root growth. B, Lateral root number. C, Relative lateral root formation. D, Lateral root density. Primary root lengths at 0 μM NPA were 38 mm for Col-0, 40 mm for *pft1-2*, and 27 mm for *35S:PFT1*. Error bars represent SE from 15 seedlings. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results.

root formation and leaf development are both regulated by auxin. Increased and reduced lateral root formation in *pft1-2* and *35S:PFT1* seedlings, respectively, were correlated with alterations in *DR5:uidA*-driven reporter gene activity detected in all stages of LRP and emerged lateral roots (Supplemental Fig. S4). Benková et al. (2003) showed that auxin accumulates at developing primordia. Subsequently, an auxin gradient is established with its maximum at the tip of the forming lateral root, in which the auxin transporter PIN1 plays an important role (Benková et al., 2003). Therefore, both increased distribution of auxin from producing cells and efficient transport by PIN1 may explain why the *pft1-2* mutant shows accelerated lateral root formation and greater proliferative capacity in pericycle cells than wild-type seedlings. Our results indicate that PFT1/MED25 negatively regulates lateral root initiation and development, probably by modulating an initial step required for the establishment of an auxin response maximum in lateral root founder cells.

PFT1 Modulates Auxin-Inducible Gene Expression

The plant growth hormone auxin has been implicated in regulating many developmental and cellular processes by altering basic patterns of gene expression (Kende and Zeevaart, 1997). The availability of well-established auxin markers such as *DR5:GFP*, *DR5:uidA*, and *BA3:uidA* provides important genetic tools to study the involvement of auxin signaling in plant development, as different markers show different sensitivities to endogenous and applied auxins. Importantly, we found differential expression of all three markers, *DR5:GFP*, *DR5:uidA*, and *BA3:uidA*, in *pft1-2* and *35S:PFT1* seedlings (Fig. 4; Supplemental Figs. S5 and S6). These results indicate that PFT1/MED25 might regulate either auxin distribution and/or response. PFT1/MED25 was initially identified as a nuclear protein that acts in the phytochrome B pathway that induces flowering in response to suboptimal light conditions. *pft1* mutants display defects in hypocotyl elongation under both red and far-red light conditions (Cerdán and Chory, 2003). The involvement of auxin in photomorphogenesis, including the shade avoidance response, has long been known (Shinkle et al., 1998; Steindler et al., 1999; Gil et al., 2001). It is noteworthy that *axr1*, *doc1/tir3*, and other auxin-related mutants with altered responses to light and shade avoidance manifest important alterations in root system architecture, including root hair and lateral root formation (Lincoln et al., 1990; Pitts et al., 1998; López-Bucio et al.,

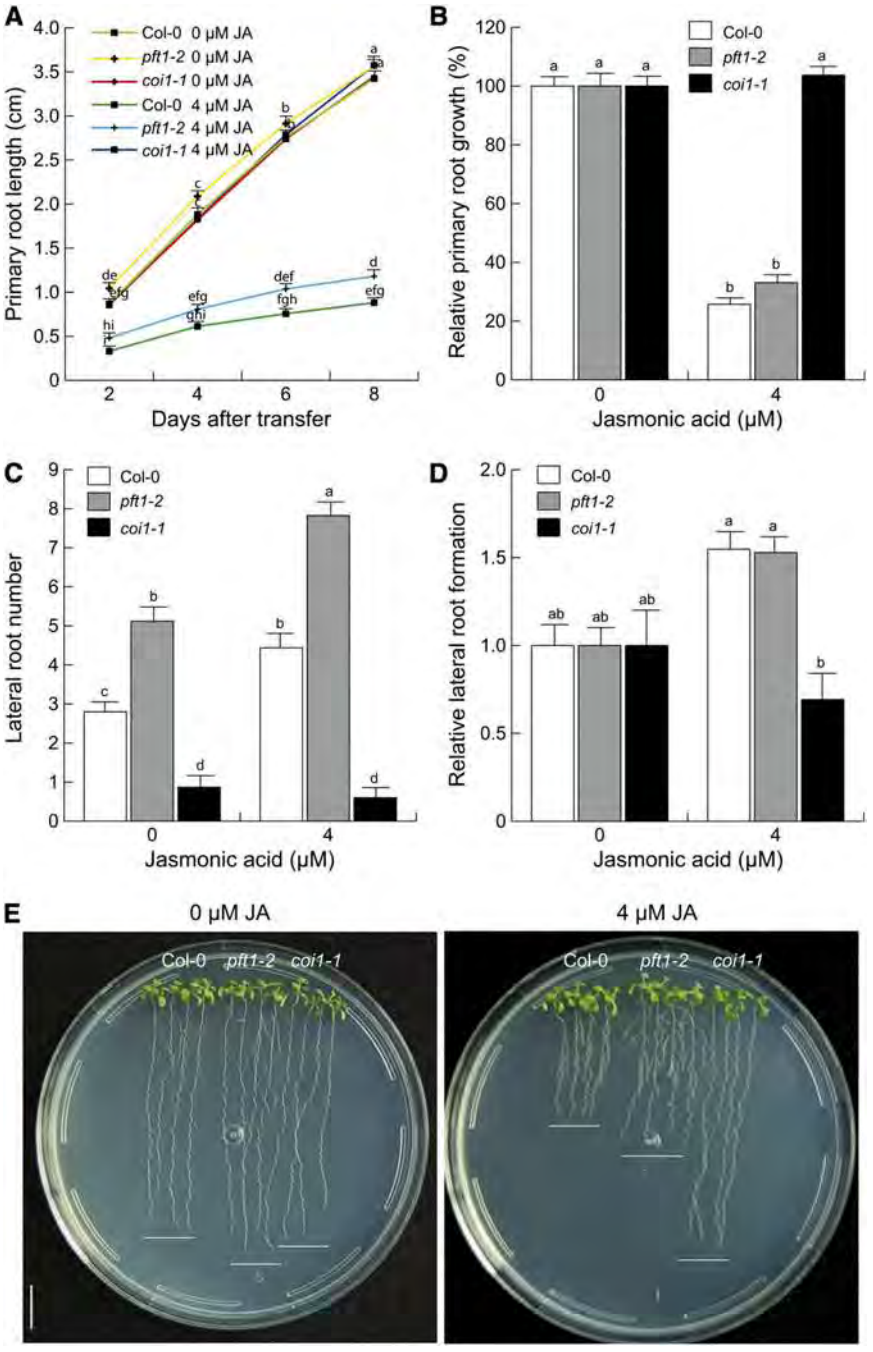

Figure 9. Effects of JA on root development of wild-type (Col-0), *pft1-2*, and *coi1-1* seedlings. Wild-type and *pft1-2* seeds were germinated and seedlings were grown for 4 d on 0.2× MS medium, and homozygous *coi1-1* seedlings were selected from a *coi1-1/COI1* segregating population in medium supplemented with 4 μM JA. A, Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates supplied or not with 4 μM JA, and primary root growth was measured every 2 d. B, Relative primary root growth in response to 4 μM JA. C, Lateral root number. D, Relative lateral root formation. Error bars represent SE from 15 seedlings. Different letters indicate statistical differences at $P < 0.05$. E, Photographs of representative wild-type, *pft1-2*, and *coi1-1* seedlings illustrating the phenotype in response to JA. Relative primary root growth and lateral root formation were analyzed 8 d after transfer to JA. The experiment was repeated three times with similar results. Bar = 1 cm. [See online article for color version of this figure.]

2005). The expression of *AXR1* is localized to zones of active cell division and elongation and in epidermal cells that differentiate the root hairs. This expression pattern is correlated with a defect in root hair elongation observed in *axr1* mutant seedlings (Pitts et al., 1998; del Pozo et al., 2002). Therefore, it is possible that the altered auxin responses observed in the *pft1* mutant could contribute to developmental phenotypes such as altered flowering time, response to light quality, and root development.

Through an analysis of the microarray data reported by Kidd et al. (2009), we identified several auxin-associated genes, such as *ANTHRANILATE SYNTHASE1* (*ASA1*), *TRYPTOPHAN SYNTHASE BETA-SUBUNIT2*, *CHORISMATE MUTASE3*, several auxin-responsive *GH3* family genes, *IAA17*, *AUX/IAA*, an amino acid permease, and a gene similar to *AUX1* that were differentially expressed between wild-type and *pft1* seedlings. Although our analyses did not show any alteration in free auxin levels in the *pft1-2* mutants (Supplemental Fig. S7), the gene expression data are overall in agreement with the detailed experiments reported here, with the plants expressing well-established auxin reporters in *pft1* and *35S:PFT1* backgrounds.

The phytohormone JA is a crucial component of the plant defense signaling system. JA and its metabolites,

collectively called jasmonates, are lipid-derived signals produced during defense responses against insects and pathogens but also under exposure to ozone, UV light, wounding, and other abiotic stresses (Wasternack, 2007). Reduction in root growth and carbon allocation patterns in several plant species upon mechanical wounding or by herbivory was ascribed to JA. In Arabidopsis, treatment with jasmonates strongly inhibits primary root growth and promotes lateral root formation (Sun et al., 2009; Raya-González et al., 2012). JA promoted lateral root formation through auxin biosynthesis and transport by directly inducing the auxin biosynthesis gene *ASA1* (Sun et al., 2009). Emerging evidence suggests that jasmonate and auxin signaling contain many common components (for review, see Cuéllar Pérez and Goossens, 2013). However, the exact cellular/tissue responses to jasmonates during Arabidopsis root system remodeling are currently not understood. Given the involvement of PFT1/MED25 in the regulation of both JA (Kidd et al., 2009) and auxin responses (this study), it was important to test the function of this protein in JA-mediated root architecture. A comparison of the root architecture of wild-type, *pft1-2*, and *coi1-1* seedlings indicates that the increased lateral root formation in *pft1-2* is likely independent of COI1 and JA signaling, in which a loss-of-function mutation in COI1 renders the plants highly resistant to JA in both primary root growth inhibition and lateral root formation (Fig. 9).

It is worth noting that auxin itself positively feeds back on *PIN* gene expression in a tissue-specific manner through an AUX/IAA-dependent signaling pathway. Vieten et al. (2005) suggested a positive effect of IAA on *PIN1* expression. Our data suggest that both auxin response and transport rather than auxin accumulation might be important factors involved in root system remodeling in *pft1* mutants. Indeed, an analysis of the spatial pattern and abundance of PIN1 localization in the wild type, *pft1-2*, and *35S:PFT1* revealed an increased GFP fluorescence in the stele and endodermal cells of *pft1-2* primary roots (Fig. 7, A–C) and in LRP (Fig. 7, D–I). In contrast, PIN2 was detected only in the cortex and epidermal cells in a similar manner in wild-type, *pft1-2*, and *35S:PFT1* primary roots (Supplemental Fig. S9). These findings suggest that PFT1/MED25 regulates the expression and distribution of the PIN1 auxin transporter and may explain why *pft1* seedlings show an amplified response to exogenous auxin based on enhanced auxin-responsive gene expression and associated root developmental phenotypes. These results are also informative in explaining why *pft1* seedlings are more resistant than the wild type and *35S:PFT1* to NPA-mediated inhibition of lateral root formation.

PFT1 Regulates Lateral Root and Root Hair Formation through Different Mechanisms

Auxin is important for a multitude of physiological processes and regulates plant development through its biosynthesis and transport. The ability of plant cells to respond to this phytohormone in an appropriate manner is also critical for auxin-mediated plant development (Okushima et al., 2007). Our analysis by gas chromatography-mass spectrometry revealed that PFT1/MED25 is unlikely to be involved in general auxin biosynthesis (Supplemental Fig. S7). The activity of the *DR5:GFP* and *DR5:uidA* markers may not necessarily reflect global auxin levels but the sensitivity of tissues to IAA or other auxins (Benková et al., 2003). Our results suggest that auxin distribution and/or response could be involved in the activation of auxin-inducible genes involved in lateral root formation modulated by PFT1/MED25. Detailed analysis of *DR5:uidA* and *BA3:uidA* in the wild type, *pft1-2*, and *35S:PFT1* in response to IAA revealed that *pft1-2* and *35S:PFT1* were more sensitive and resistant, respectively, to auxin (Fig. 5; Supplemental Fig. S8). This indicates that PFT1/MED25 modulates the auxin response rather than auxin biosynthesis and auxin transport through the regulation of the PIN1 auxin transporter. This hypothesis is supported by the finding that *pft1-2* and *35S:PFT1* seedlings had opposite responses to NAA during lateral root formation (Fig. 6). The increased and reduced NAA responses shown by *pft1-2* and *35S:PFT1* seedlings, respectively, suggest that PFT1/MED25 is a key element that controls pericycle cell activation during lateral root formation, which is modulated by the auxin signaling pathway. Similarly, *35S:PFT1* seedlings showed developmental alterations and auxin-responsive gene expression consistent with a decreased auxin transport that correlates with their increased sensitivity to NPA (Figs. 4–6 and 8). This suggests that PFT1/MED25 might be involved in auxin transport, possibly by mediating the transcriptional regulation and/or distribution of PIN1 (Fig. 7).

In a recent work, Sundaravelpandian et al. (2013) reported that PFT1/MED25 controls root hair differentiation through reactive oxygen species distribution. Both *pft1-2* and *pft1-3* Arabidopsis mutants showed a short-root-hair phenotype that was correlated with perturbations in hydrogen peroxide and superoxide distribution. Supply of hydrogen peroxide or potassium cyanide rescued the *pft1* mutant phenotype, indicating that PFT1/MED25 regulates root hair differentiation through reactive oxygen species. The short-root-hair phenotype of *pft1* mutants could be reproduced in our research. Furthermore, we also found that *35S:PFT1* shows the opposite phenotype, with root hairs longer than wild-type and *pft1* seedlings (Supplemental Fig. S10). Considering the positive effect of auxin on root hair growth (Pitts et al., 1998), the short-root-hair phenotype of *pft1* mutants indicates that the role of PFT1/MED25 in epidermal cell differentiation most likely occurs through an auxin-independent mechanism.

In conclusion, our results have shown that (1) gain and loss of PFT1/MED25 function lead to opposite responses in primary root growth and lateral and adventitious root development; (2) PFT1/MED25 negatively regulates cell division and elongation processes that are important in modulating the configuration of the root system; (3)

PFT1/MED25 regulates auxin-responsive gene expression during LRP development; and (4) PFT1/MED25 modulates auxin responses to endogenous and supplied auxin and in lateral root formation, which seems to be independent from JA signaling. Emerging evidence indicates that PFT1/MED25 plays multiple roles in a number of essential plant processes, including light signaling and flowering time (Cerdán and Chory, 2003; Wollenberg et al., 2008; Iñigo et al., 2012; Klose et al., 2012), JA-mediated pathogen defense (Kidd et al., 2009), organ growth (Xu and Li, 2011), abiotic stress (Elfving et al., 2011), and JA and abscisic acid signaling (Çevik et al., 2012; Chen et al., 2012). This is consistent with the expectation that individual Mediator subunits recognize and respond to a subset of the approximately 1,500 transcription factors present in the Arabidopsis genome. Therefore, determining which transcription factor(s) interacts with PFT1/MED25 to coordinate auxin responses in pericycle cells should provide important information about the roles of PFT1/MED25 and the Mediator complex in root morphogenesis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0, the transgenic Arabidopsis lines *35S:PFT1* (Cerdán and Chory, 2003), *CycB1:uidA* (Colón-Carmona et al., 1999), *DR5:uidA* (Ulmasov et al., 1997), *DR5:GFP* (Ottenschläger et al., 2003), *BA3:uidA* (Oono et al., 1998), *PIN1::PIN1::GFP* (Benková et al., 2003), and *PIN2::PIN2::GFP* (Blilou et al., 2005), and the mutant lines *pft1-1* (Cerdán and Chory, 2003), *pft1-2* (SALK_129555), *pft1-3* (SALK_059316), *med8* (SALK_092406), *pft1 med8* (Kidd et al., 2009), and *coi1-1* (Feys et al., 1994) were used for the experiments reported here. To generate the wild type, *pft1-2*, and *35S:PFT1* lines expressing auxin reporter gene constructs, crosses were made between the respective lines, and the lines homozygous for both loci were used in subsequent experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture; catalog no. M5524) was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h of light/8 h of darkness, light intensity of 300 μmol m^{-2} s^{-1}, and temperature of 22°C.

Chemicals

IAA, NAA, and NPA were purchased from Sigma and dissolved in dimethyl sulfoxide. In control treatments, the solvents were used in equal amounts as present in the greatest concentration of each compound tested.

Analysis of Growth

Arabidopsis root system and primary root meristem integrity were analyzed with a stereoscopic microscope (Leica MZ6). All lateral roots emerged from the primary root and observed with the 3× objective were taken into account for lateral root number data. Images were captured with a Samsung SCC 131-A digital color camera adapted to the microscope. Primary root length was determined for each root using a ruler. Lateral root number was determined by counting the lateral roots per seedling, and lateral root density was determined by dividing the lateral root number value by the primary root length values for each analyzed seedling. For all experiments with wild-type and mutant lines, the overall data were statistically analyzed using the SPSS 10 program. Univariate and multivariate analyses with Tukey's posthoc test were used for testing the differences in growth and root development responses. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Determination of the Developmental Stages of LRP

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

Histochemical Analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37°C in a GUS reaction buffer (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained plants were cleared and fixed with 0.24 N HCl in 20% (v/v) methanol and incubated for 60 min at 62°C. The solution was substituted by 7% (w/v) NaOH in 60% (v/v) ethanol for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40%, 20%, and 10% (v/v) for a 24-h period each and fixed in 50% (v/v) glycerol. The processed roots were placed on glass slides and sealed with commercial nail varnish. For each marker line and each treatment, at least 15 transgenic plants were analyzed.

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 both 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.

Free IAA Determination

The determination of IAA was from whole plants grown on agar-solidified 0.2× MS medium for 10 d, and free IAA was quantified as described by Edlund et al. (1995).

Supplemental Data

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

Supplemental Fig. S1. PFT1/MED25 mutations affect root architecture.

Supplemental Fig. S2. PFT1/MED25 modulates adventitious root development.

Supplemental Fig. S3. PFT1/MED25 represses cell division and elongation in lateral roots.

Supplemental Fig. S4. PFT1/MED25 regulates auxin-responsive gene expression in young roots.

Supplemental Fig. S5. PFT1/MED25 modulates auxin responses in whole plants.

Supplemental Fig. S6. PFT1/MED25 is involved in auxin responses in roots and shoots.

Supplemental Fig. S7. IAA contents in Col-0, *pft1-2*, and *35S:PFT1* seedlings.

Supplemental Fig. S8. PFT1/MED25 modulates auxin sensitivity.

Supplemental Fig. S9. PFT1/MED25 regulates PIN2 auxin transporter.

Supplemental Fig. S10. PFT1/MED25 is involved in root hair formation.

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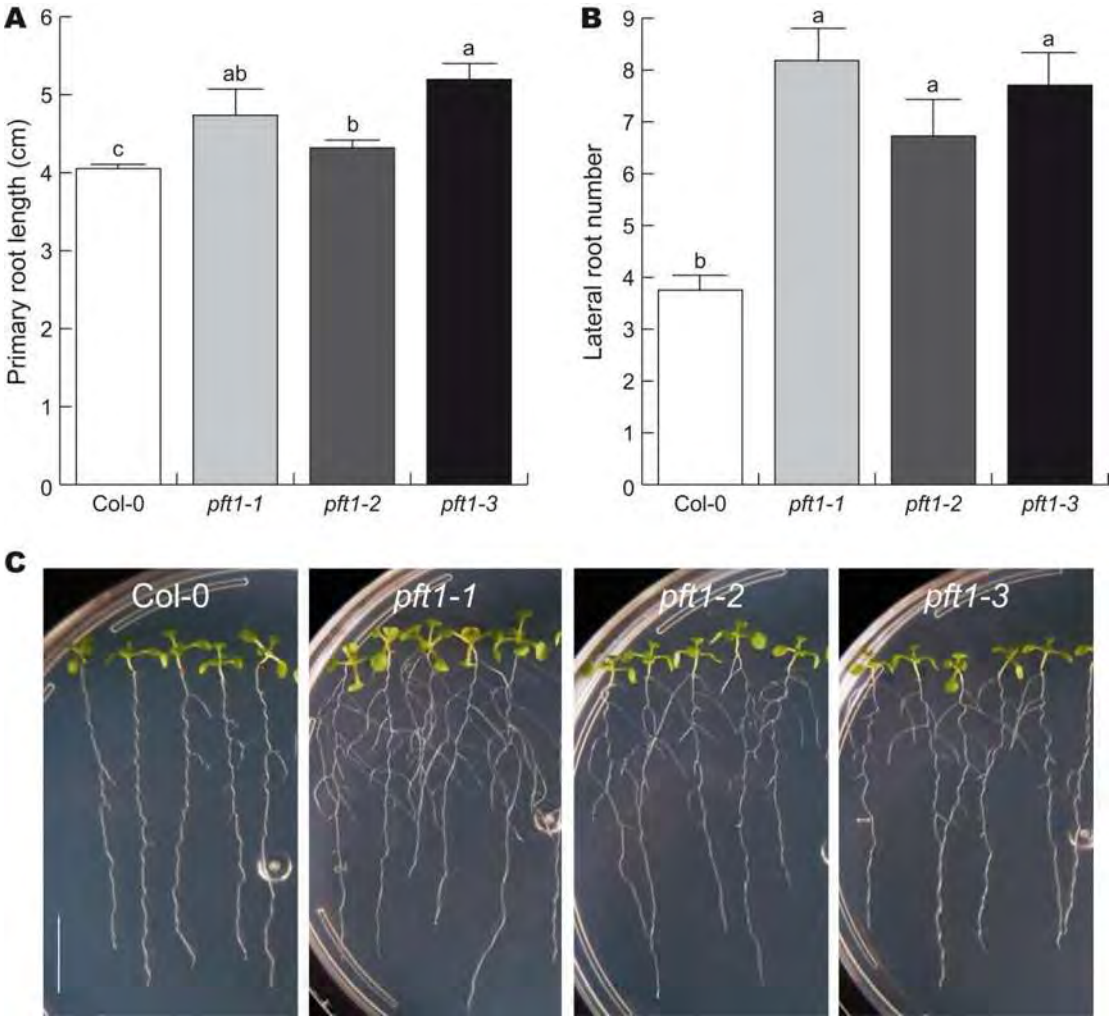
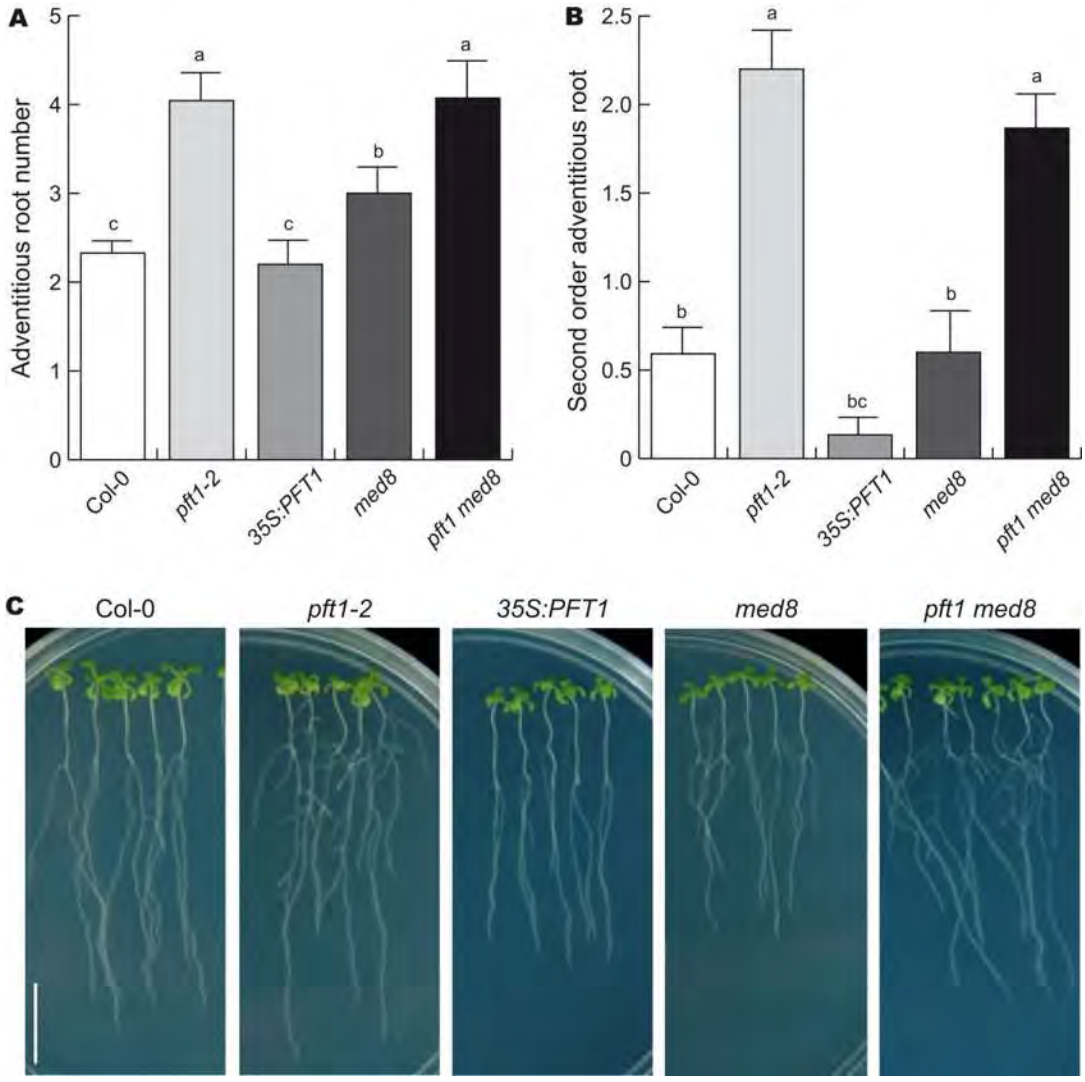
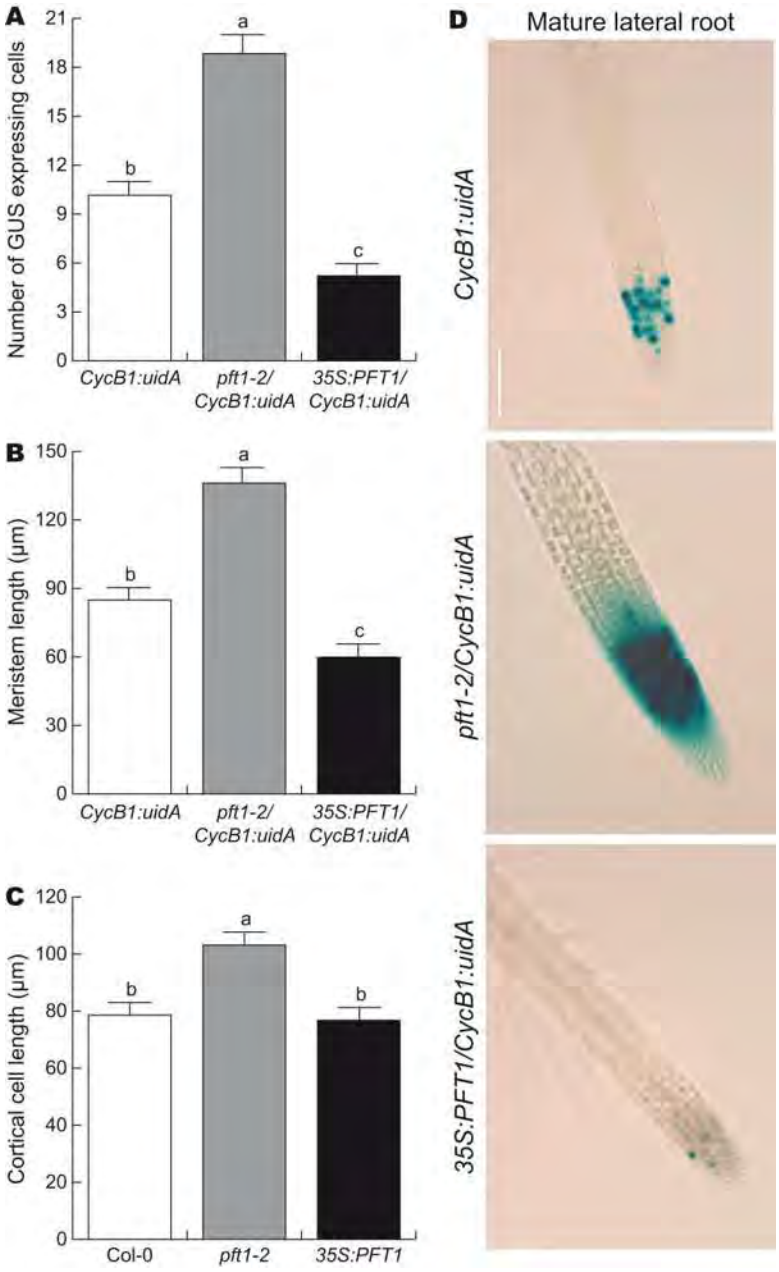


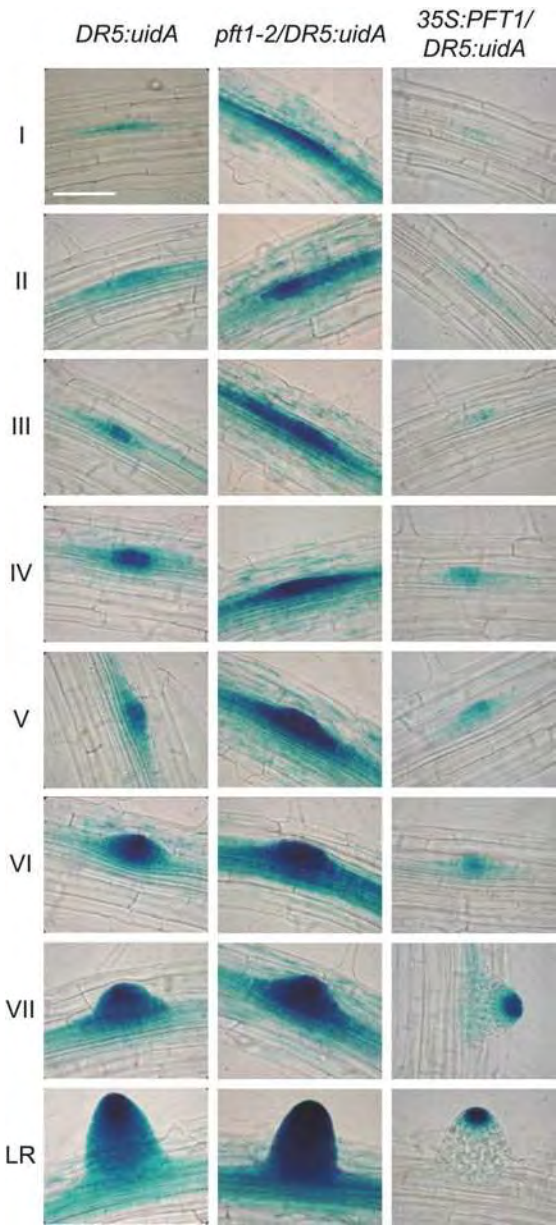
Supplemental Fig S1. PFT1 mutations affect root architecture in Arabidopsis. WT (Col-0), *pft1-1*, *pft1-2* and *pft1-3* Arabidopsis seedlings were germinated and grown for 8 days on agar solidified 0.2X MS medium. (A) Primary root length. (B) Lateral root number. Error bars represent standard errors from 15 seedlings. Different letters indicate statistical differences at $P<0.05$. (C) Photographs of representative WT (Col-0), *pft1-1*, *pft1-2* and *pft1-3* seedlings are shown. The experiment was repeated three times with similar results. Scale bar = 1 cm.



Supplemental Fig. S2. PFT1/MED25 is involved in adventitious root development in Arabidopsis. WT (Col-0), *pft1-2*, *35S:PFT1*, *med8* and *pft1 med8* Arabidopsis seedlings were germinated and grown in darkness for 5 d on the surface of agar plates containing 0.2X MS medium and hypocotyls explants were obtained. Hypocotyls explants were transferred to 0.2X MS medium and 6 d after the transfer adventitious root formation recorded. (A) Adventitious root numbers. (B) Second order adventitious root numbers. Error bars represent standard errors from 15 seedlings. Different letters indicate statistical differences at $P<0.05$. (C) Photographs of representative WT (Col-0), *pft1-2*, *35S:PFT1*, *med8* and *pft1 med8* seedlings. The experiment was repeated two times with similar results. Scale bar = 1 cm.

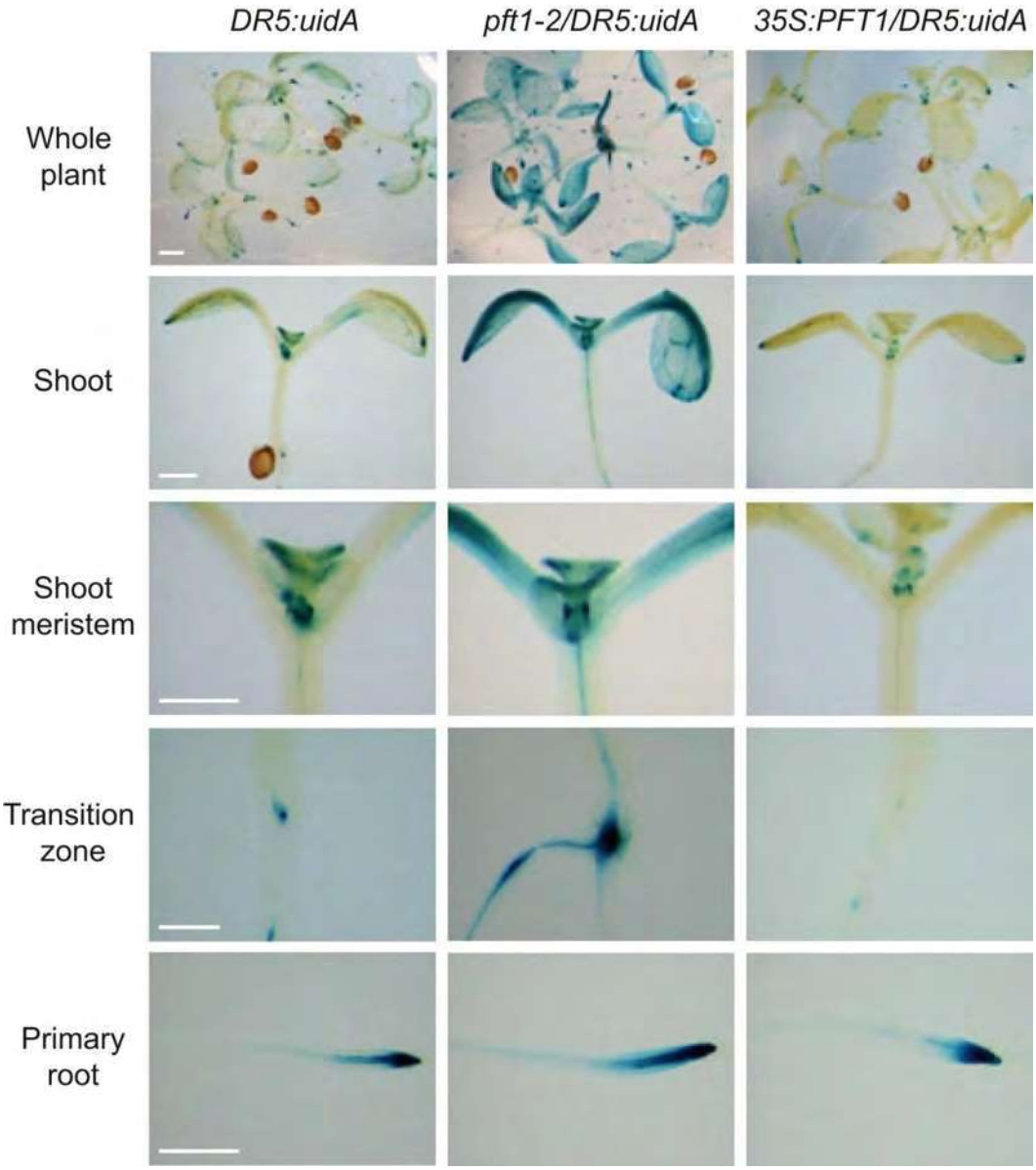


Supplemental Fig. S3. PFT1/MED25 represses cell division and elongation in lateral root meristems. WT (Col-0), *pft1-2* and *35S:PFT1* Arabidopsis seedlings harboring the *CycB1:uidA* gene construct were germinated and grown for 7 days on agar solidified 0.2X MS medium. (A) Number of GUS positive spots/root meristem. (B) Meristem lengths and (C) Cortical cell lengths were scored. (D) Lateral roots were stained for GUS activity and cleared to show the expression of *CycB1:uidA*. Photographs show representative individuals from at least 15 GUS-stained seedlings. Error bars represent standard errors from 15 GUS-stained seedlings analyzed. Different letters indicate means statistically different ($P<0.05$). The experiment was repeated two times with similar results. Scale bar =100 µm.

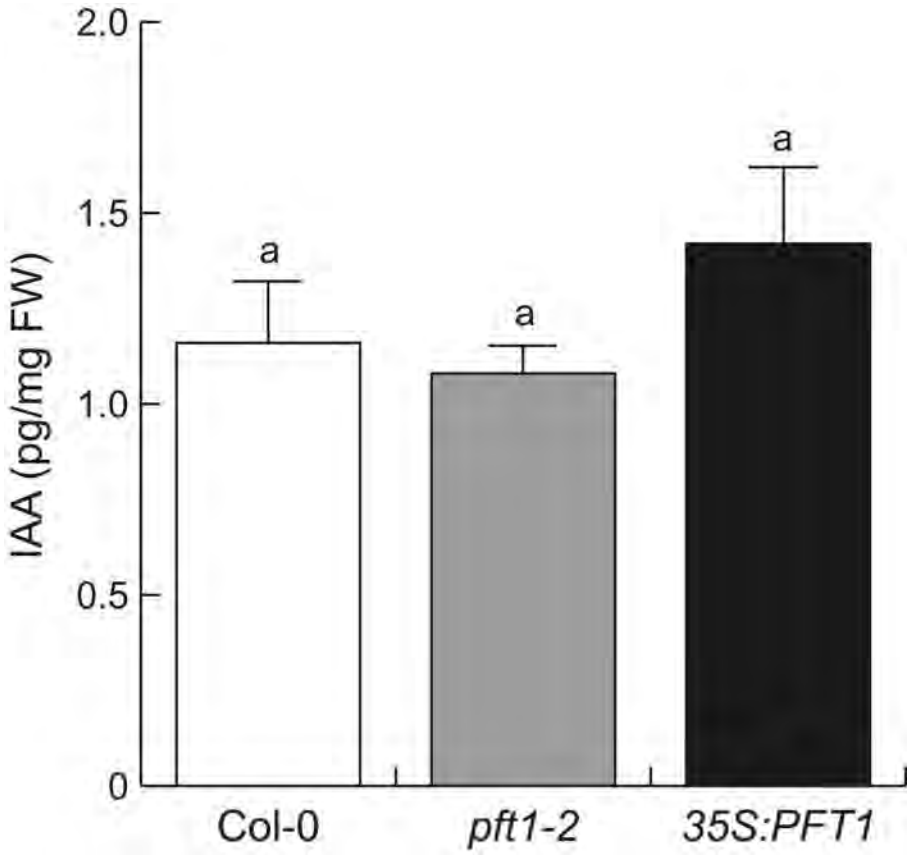


Supplemental Fig. S4. *DR5:uidA* expression in lateral root primordial (LRP) in WT, *pft1-2* and *35S:PFT1* seedlings. Arabidopsis seedlings were grown 7 d on agar solidified 0.2X MS medium. The LRPs from *DR5:uidA* expressing WT, *pft1-2*, and *35S:PFT1* seedlings stained twelve hours for GUS activity are shown. LRP stages were recorded according to Malamy and Benfey (1997). Note that GUS activity from *DR5:uidA* is stronger in LRPs and emerged lateral roots from *pft1-2* than WT, whereas an opposite response is seen in *35S:PFT1* primordia The photographs show representative individuals of at least 15-GUS stained seedlings analyzed. The experiment was repeated twice with similar results. LR= Lateral root. Scale bar = 100 μm.

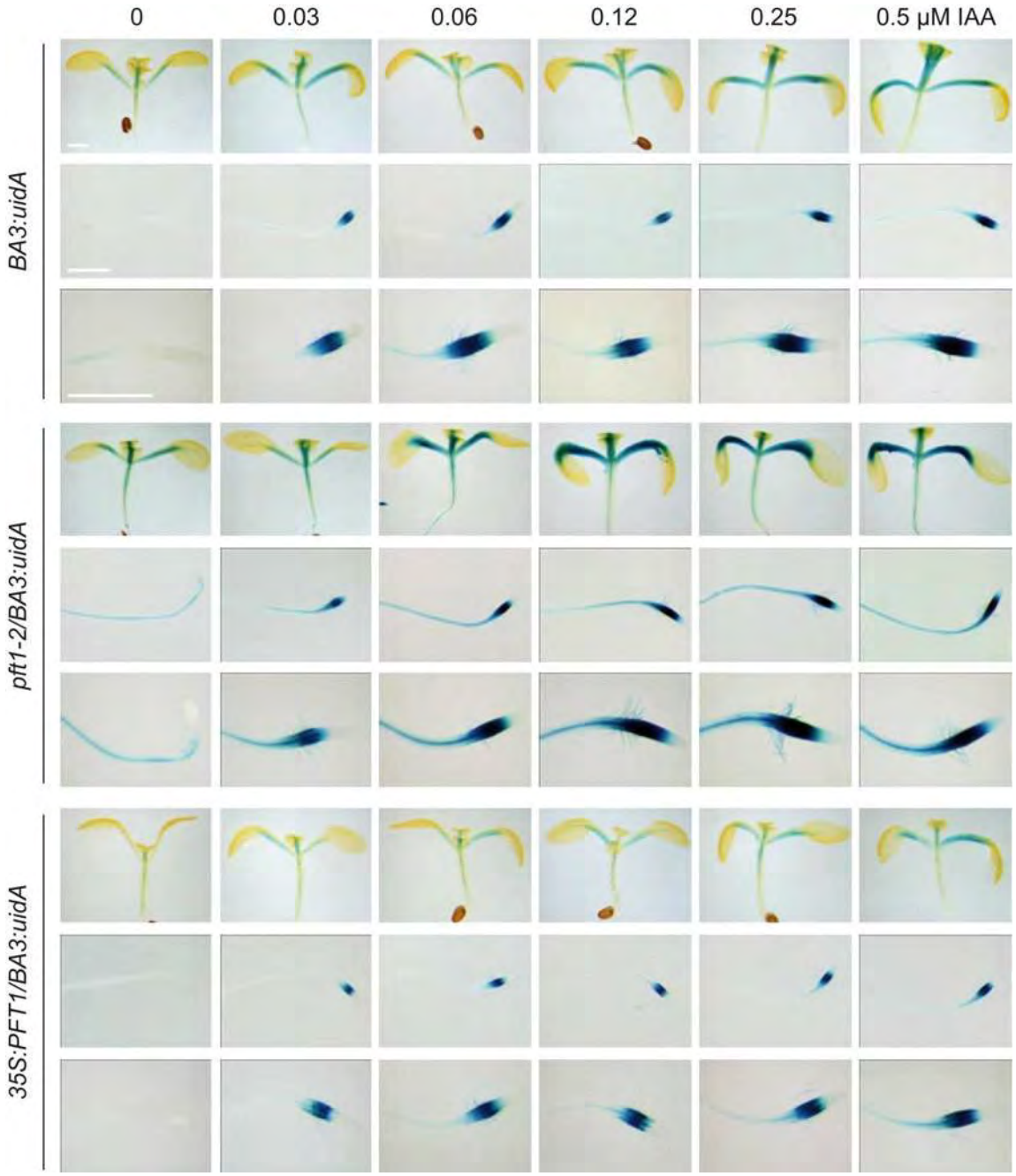
Supplemental Fig. S5. *DR5:uidA* expression in WT, *pft1-2* and *35S:PFT1* whole seedlings. *DR5:uidA*, *pft1-2/DR5:uidA* and *35S:PFT1/DR5:uidA* Arabidopsis seedlings grown for 9 days on agar plates containing 0.2X MS medium were stained twelve hours for GUS. Photographs are representative individuals of at least 15 GUS stained seedlings. Note opposite response between *pft1-2* and *35S:PFT1* seedlings on *DR5:uidA* expression and LRP formation. The experiment was repeated three times with similar results.



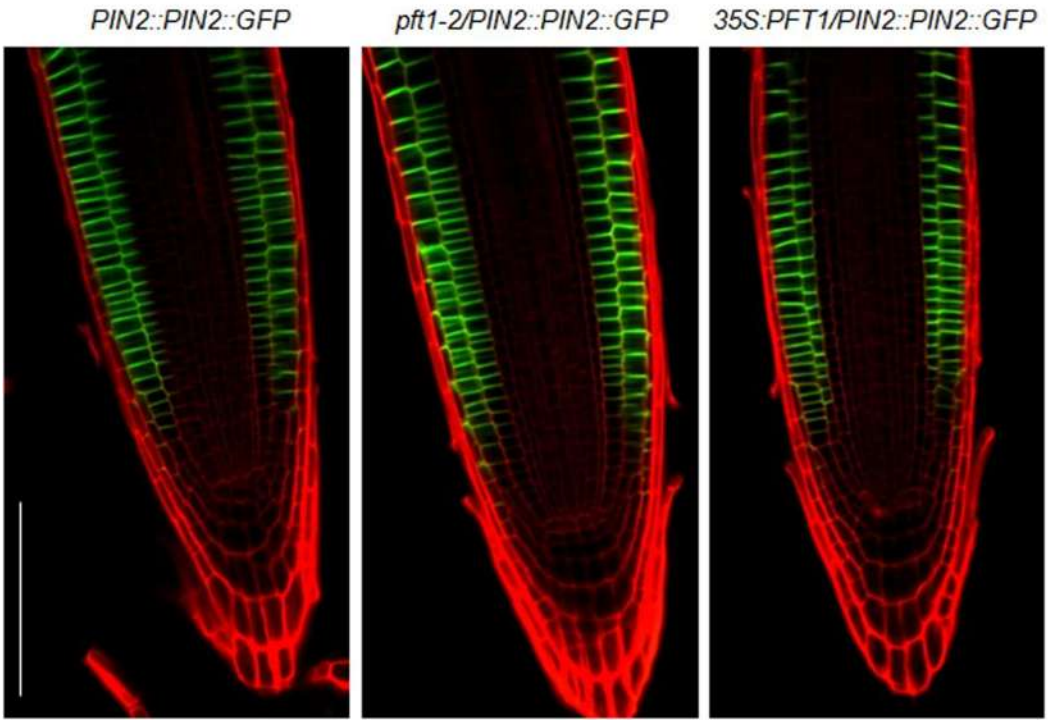
Supplemental Fig. S6. Auxin-regulated reporter gene expression in WT, *pft1-2* and *35S:PFT1* seedlings. *DR5:uidA*, *pft1-2/DR5:uidA* and *35S:PFT1/DR5:uidA* Arabidopsis seedlings were grown for 7 days on agar solidified 0.2X MS medium and stained twelve hours for GUS. Photographs show representative individuals of at least 15 GUS-stained seedlings analyzed. Note that *pft1-2* and *35S:PFT1* seedlings show stronger and weaker GUS activity, respectively, than WT seedlings. Scale bar = 200 μm.



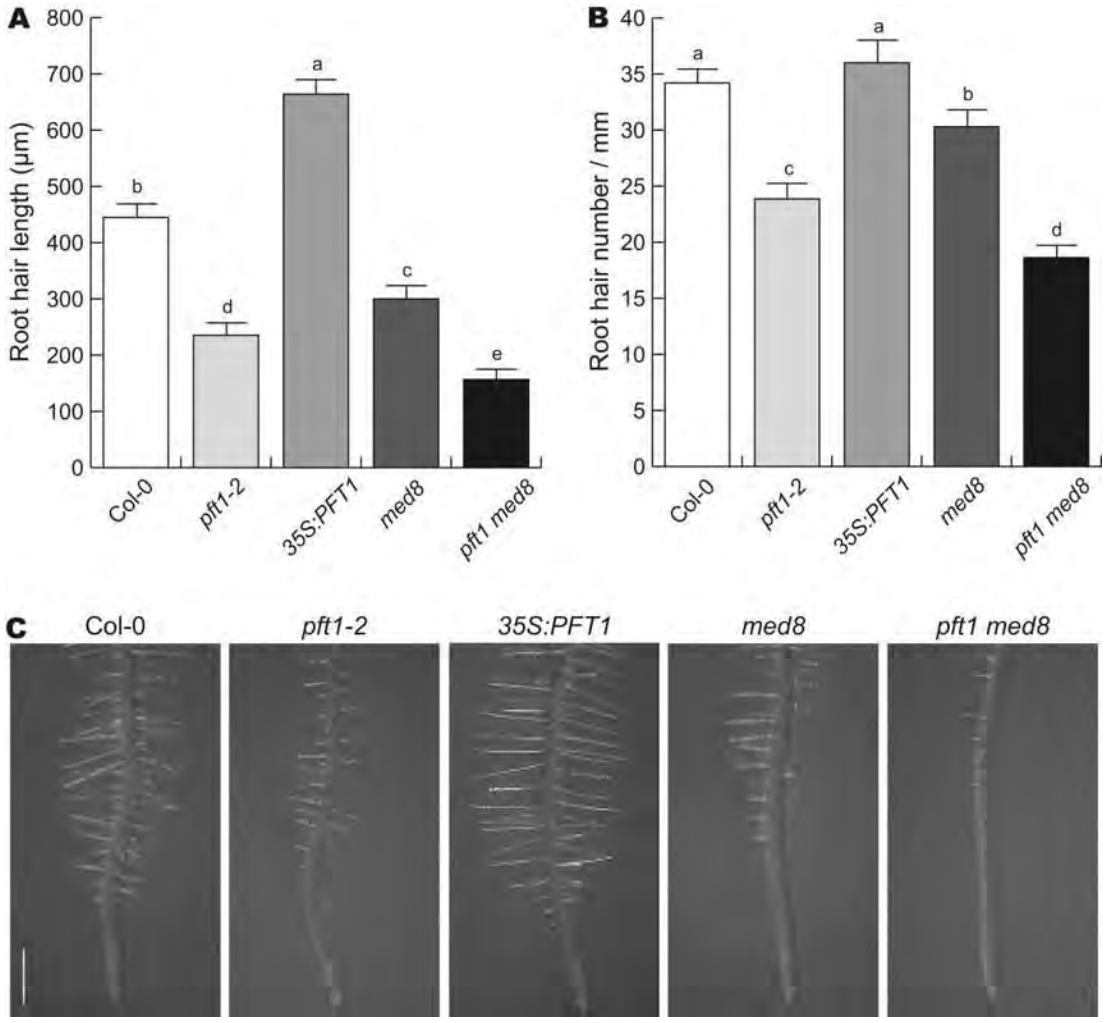
Supplemental Fig. S7. Determination of IAA content from WT (Col-0), *pft1-2* and *35S:PFT1* Arabidopsis seedlings by GC-MS. Arabidopsis seedlings were germinated and grown for 10 days in 0.2X MS medium and harvested for IAA quantification as explained in Materials and Methods.



Supplemental Fig. S8. Effects of IAA on auxin-responsive reporter gene expression in WT, *pft1-2* and *35S:PFT1* Arabidopsis seedlings. WT, *pft1-2* and *35S:PFT1* seedlings harboring the *BA3:uidA* gene construct were germinated and grown for 7 d in agar-solidified 0.2X MS medium and then transferred for 8 h to liquid medium either without IAA or supplemented with increasing IAA concentrations before stained for GUS activity. Photographs are representative individuals of at least 15 GUS-stained seedlings. Scale bar = 200 µm.



Supplemental Fig. S9. *PIN2* expression in WT, *pft1-2* and *35S:PFT1* seedlings. *PIN2::PIN2::GFP*, *pft1-2/PIN2::PIN2::GFP* and *35S:PFT1/PIN2::PIN2::GFP* were germinated and grown on agar solidified 0.2X MS medium. Seven days after germination the seedlings were stained with PI and analyzed by confocal microscopy to visualize the *PIN2* expression in primary root apical meristem. Representative photographs of primary roots from 10 seedlings analyzed. Scale bar = 100 µm.



Supplemental Fig. S10. PFT1/MED25 is involved in root hair formation in Arabidopsis. WT (Col-0), *pft1-2*, *35S:PFT1*, *med8* and *pft1 med8* Arabidopsis seedlings were germinated and grown for 4 days on agar solidified 0.2X MS medium. (A) Root hair length (B) Root hair number/mm, (C) Representative photographs of root hairs in WT and Mediator mutants. Data from A and B are from 50 root hairs analyzed from the primary roots of 10 independent seedlings. The experiment was repeated three times with similar results. Scale bar = 200 µm.

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

Javier Raya-González, Ramón Pelagio-Flores, José López-Bucio*

Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio A-1', CP 58030 Morelia, Michoacán, Mexico

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ABSTRACT

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

Introduction

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

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

Little is known about the mechanisms that control LR development. However, accumulating evidence indicates that LR initiation, the establishment of the meristem and LR emergence are regulated independently (Fukaki and Tasaka, 2009). The plant hormone auxin (indole-3-acetic acid [IAA]) is an important long- and short-distance signal that controls multiple developmental processes in the RS through changes in cell division, elongation and/or differentiation (Chapman and Estelle, 2009; Vanneste and Friml, 2009; Kieffer et al., 2010). IAA plays an important role during each stage of LR formation (De Smet et al., 2006; Fukaki et al., 2007; Dubrovsky et al., 2008; Fukaki and Tasaka, 2009). Application of IAA or synthetic auxins such as 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid stimulates LR formation (Celenza et al., 1995; Woodward and Bartel, 2005), whereas polar auxin transport inhibitors such as N-(1-naphthyl)-phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid prevent LR formation (Casimiro et al., 2001; Himanen et al., 2002). Consistently, *Arabidopsis* mutants with increased auxin levels, such as *rooty* and its alleles *aberrant lateral root formation1* and *superroot1*, have increased number of LRs (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants defective on auxin transport, perception, or signaling, including *aux1*, *axr1*, *tir3*, *slr* and *arf7*/*arf19*, show reduced LR

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

* Corresponding author.
E-mail addresses: javierrayagonzalez@gmail.com (J. Raya-González), pelagio1085@hotmail.com (R. Pelagio-Flores), jbucio@umich.mx (J. López-Bucio).

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

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

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

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

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

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana* Col-0), the transgenic *Arabidopsis* lines *CycB1:uidA* (Colón-Carmona et al., 1999), *DR5:uidA* (Ulmasov et al., 1997), *BA3:uidA* (Oono et al., 1998), *pLOX2:uidA* (Jensen et al., 2002) and mutant lines, *coi1-1* (Feys et al., 1994), *axr1-3* (Lincoln et al., 1990), *aux1-7* (Picket et al., 1990), *axr2-1* (Timpte et al., 1994), *axr4-1* (Hobbie and Estelle, 1995), *tir1/afb2/afb3* (Parry et al., 2009), *arf7-1/arf19-1* (Wilmoth et al., 2005), *slr* (Fukaki et al., 2002) and *eir1-3* (Luschnig et al., 1998), were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h of light/8 h darkness, light intensity of 300 μmol/m^{-2}/s^{-1}, and temperature of 22 °C.

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

Chemicals

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

Analysis of growth

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

Determination of developmental stages of lateral root primordia (LRP)

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

Histochemical analysis

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

Results

JA regulates Arabidopsis RS architecture

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

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

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

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

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

coi1-1 mutants are defective in LR positioning

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

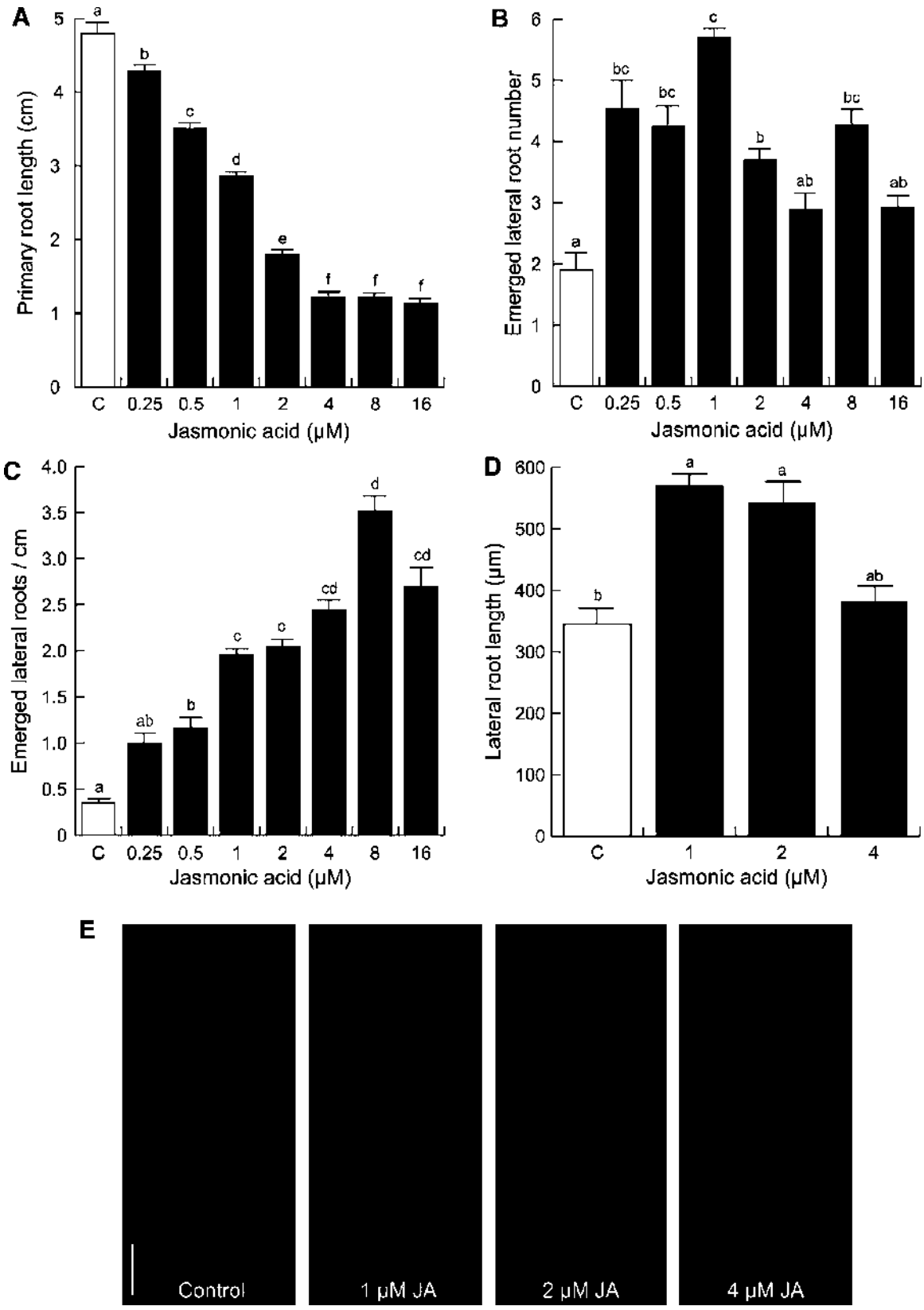

Fig. 1. Effect of JA on *Arabidopsis* RSA. *Arabidopsis* (Col-0) seedlings were germinated and grown for 12 d under increased JA concentrations. (A) PR length. (B) Number of emerged LRs. (C) LR density (number of emerged LRs per cm). (D) Length of emerged LRs. (E) Representative photographs of *Arabidopsis*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

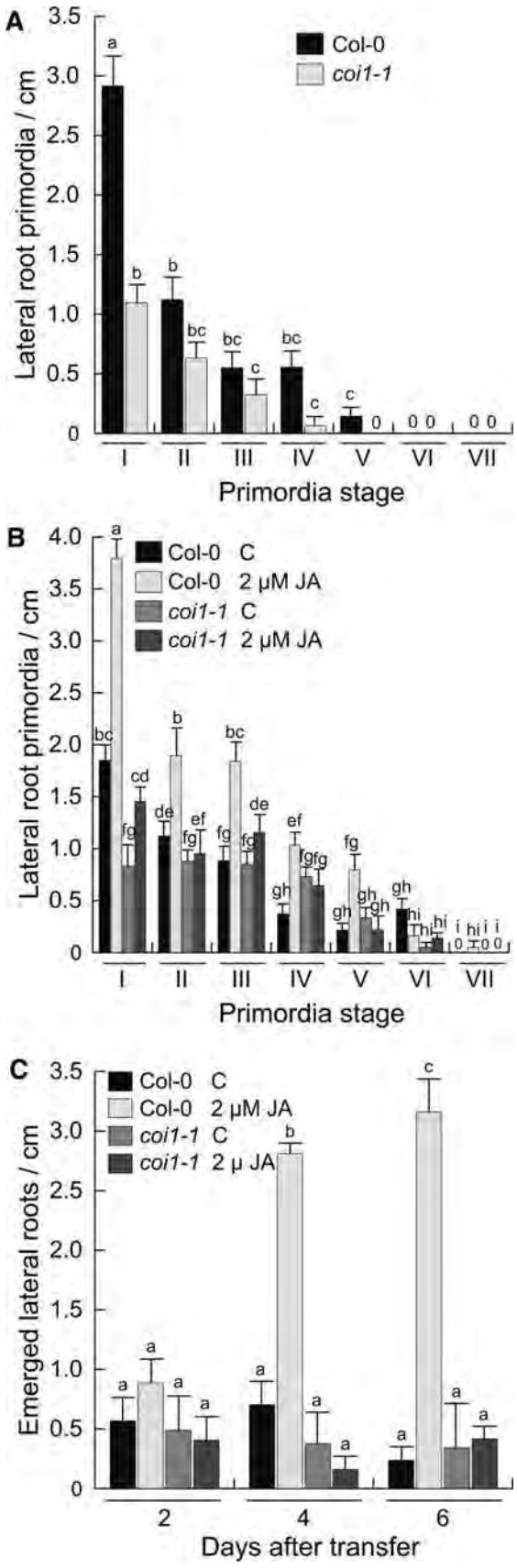

Fig. 2. Effect of JA on LR development in WT and *coi1-1* seedlings. WT seedlings were germinated and grown for 4d on 0.2× MS medium and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI* segregating population in medium supplemented with 4 µM JA. (A) LRP density in four-day-old WT and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

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

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

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

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

coi1-1 mutants show normal auxin-responsive gene expression

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

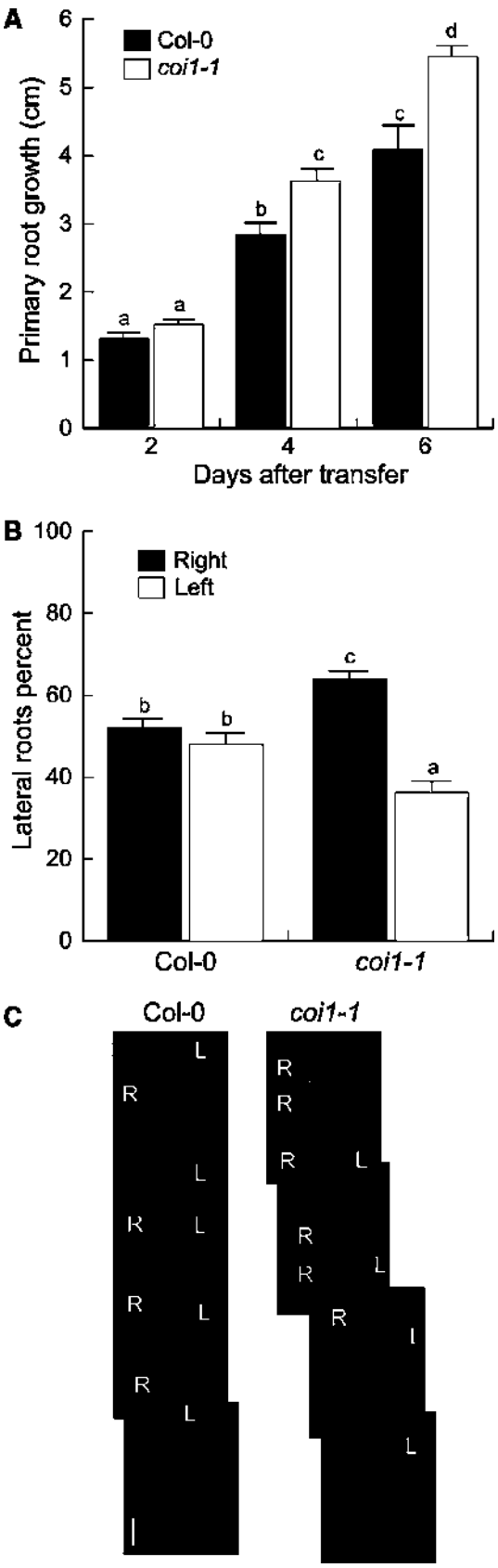

Fig. 3. Effects of JA on LR positioning in WT and *coi1-1* seedlings. WT seedlings were germinated and grown for 4 d on 0.2× MS medium and homozygous *coi1-1* seedlings were selected from a *coi1-1*/*COI* segregating population in medium supplemented with 4 μM JA. Four-day-old seedlings were transferred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

for 12 h at 22 °C. As previously reported (Ulmasov et al., 1997), in solvent-treated *DR5:uidA* seedlings, GUS expression was absent from cotyledons and leaves and was expressed primarily in the root tip region (Fig. 5A and D). *DR5:uidA* seedlings treated with a concentration of 1 μM IAA showed strong GUS activity throughout the plant (Fig. 5B and E), whereas seedlings treated with 4 μM JA showed a GUS activity similar to solvent-treated plants (Fig. 5C and F). *coi1-1*/*DR5:uidA* seedlings showed a similar GUS expression pattern both in shoots and in roots (Fig. 5G–L). Untreated *BA3:uidA* seedlings did not show detectable levels of GUS activity (Fig. 5M and P), similarly to results reported by Oono et al. (1998), whereas when treated with 1 μM IAA, they showed GUS expression mainly in the petioles of the cotyledons and in the root elongation zone (Fig. 5N and Q). GUS expression in *BA3:uidA* seedlings treated with JA was undetectable (Fig. 5O and R). This pattern of expression remained unchanged in *coi1-1*/*BA3:uidA* seedlings (Fig. 5S–X). As a further control, we tested GUS expression in WT and *coi1-1 Arabidopsis* lines harboring the *pLOX2:uidA* gene construct, which has been shown to be activated by JA (Jensen et al., 2002). As expected, JA clearly induced *pLOX2:uidA* expression in shoots in WT but not in *coi1-1* seedlings (Fig. S3). These results indicate that JA did not affect the general auxin-response in WT or in *coi1* mutants.

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

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

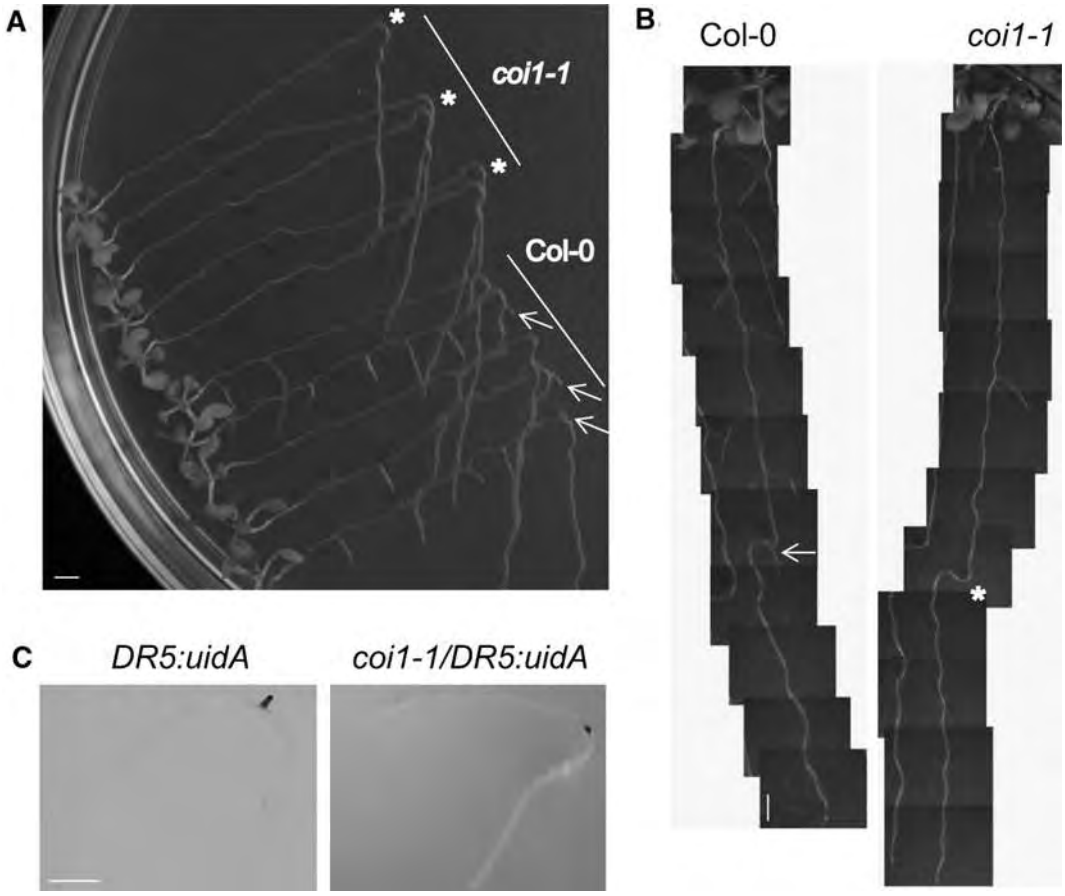

Fig. 4. LR formation after a change of gravity vector and root bending in WT and *coi1-1* mutants seedlings. (A) LR development after gravistimulation for 96 h; Ten-day-old WT and *coi1-1* seedlings grown on agar plates were rotated through 135°. (B) LRs emerged in six-day old WT and *coi1-1* seedlings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

Discussion

JA induces changes in RS architecture in Arabidopsis

The RS shares with the shoot the basic body plants and the pathways that are essential for organogenesis and growth (Veit, 2004). The site of LR initiation seems to depend on correct auxin transport to perycicle cells in the PR (Dubrovsky et al., 2000; López-Bucio et al., 2005), whereas the final architecture of the roots is coordinated by hormonally regulated processes that affect cell division, elongation and differentiation (Casson and Lindsey, 2003). Although IAA is considered the major plant growth-regulating substance underlying RSA adjustment, the discovery of novel signals such as jasmonates affecting PR growth and LR formation has been a recent goal in plant biology. In a previous report, we determined an important role of JA in LR development in our analysis of the *decanamide root resistant1* (*drr1*) *Arabidopsis* mutant (Morquecho-Contreras et al., 2010). The *drr1* mutant was isolated in a screen for identifying *Arabidopsis* mutants that fail to inhibit PR growth when grown under a high concentration of *N*-isobutyl decanamide, a plant alkamide very active in modulating RSA. Detailed characterization of LRP development in WT and *drr1* mutants revealed that *DRR1* is required at an early stage of pericycle cell activation to form LRP. When grown both *in vitro* and in soil *drr1* mutants showed dramatically increased longevity and reduced hormone and age dependent senescence, which were related to reduced LR formation when exposed to stimulatory concentrations of JA. These results provided genetic evidence indicating that alkamides can be perceived by plants to modulate RSA and senescence-related processes possibly by interacting with JA signaling and that JA is an important signal for LR development (Sun et al., 2009; Morquecho-Contreras et al., 2010).

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

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

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

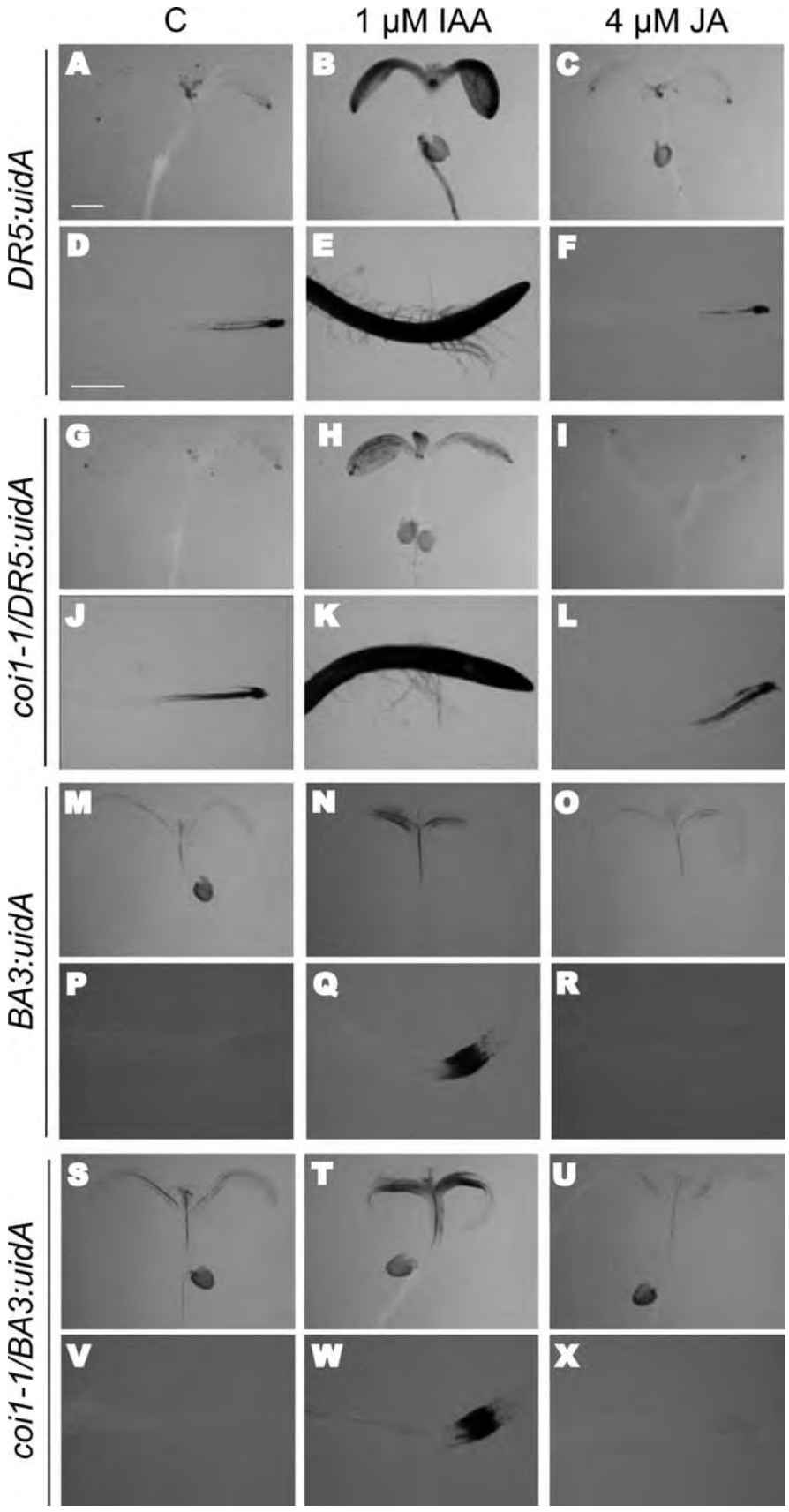

Fig. 5. Effect of JA and IAA on auxin-inducible gene expression in WT and *coi1-1* seedlings. (A-L) 12-h GUS staining *DR5:uidA* and *coi1-1*|*DR5:uidA Arabidopsis* seedlings that were grown for 7 d on agar plates containing 0.2× MS medium supplemented with the solvent (control) or 1 μM IAA or 4 μM JA.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

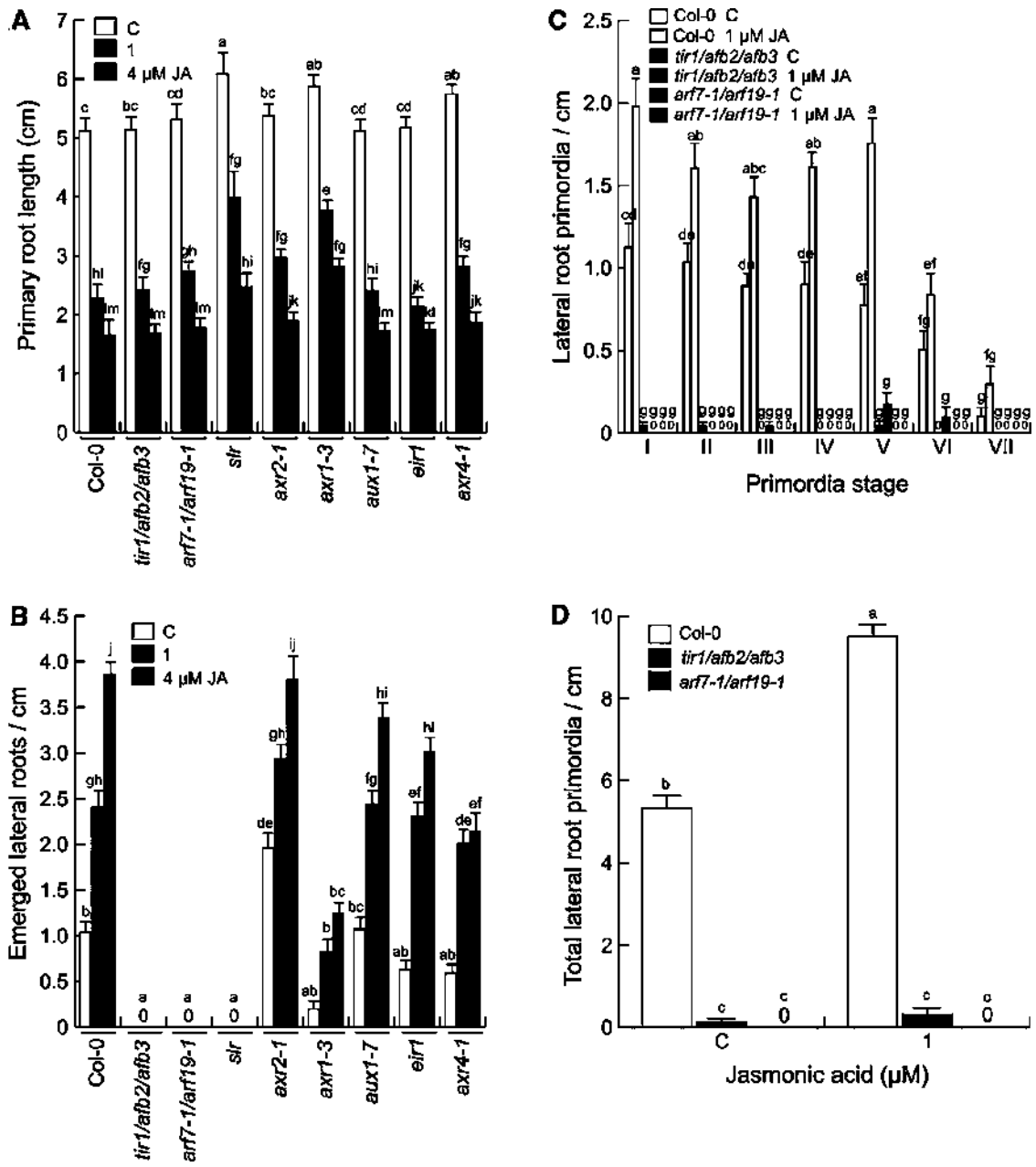

Fig. 6. Effects of JA on PR growth and LR development in WT seedlings and auxin-related mutants. *Arabidopsis* WT and *tir1/afb2/afb3*; *arf7-1/arf19-1*, *slr*, *axr2-1*, *axr1-3*, *aux1-7*, *eir1*, and *axr4-1* triple, double or single mutant seedlings, respectively, were germinated and grown for 12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

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

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

COI1 is involved in LR growth on bends

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

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

JA require a canonical auxin signaling pathway for inducing LR development

Auxin is perceived by direct binding to the TRANSPORT INHIBITOR RESPONSE1 (TIR1) protein, a member of a small family of F-box proteins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). This interaction accelerates the Skp1, Cdc53/Cullin1, F-box protein ubiquitin ligase-catalyzed degradation of Aux/IAA repressor proteins, allowing de-repression of auxin regulated genes by auxin response transcription factors ARFs (Gray, 2004). To determine whether the TIR1 family of auxin receptors and ARFs are involved in *Arabidopsis* responses to JA, we analyzed PR growth and LR formation in response to JA in WT (Col-0) *Arabidopsis* seedlings, in *tir1*/*afb2*/*afb3* triple mutants in *arf7-1*/*arf19-1* double mutants, and in *slr*, *axr2-1*, *axr1-3*, *aux1-7*, *eir1* and *axr4-1* single mutants, which are well known for their resistance to auxin in PR growth. Seedlings from all mutant lines showed similar inhibition in PR growth by JA treatment, with exception of *slr* and *axr1-3*, which showed significant resistance when compared to WT seedlings (Fig. 6A and Fig. S4). Interestingly, the increase in both LR and LRP formation observed in WT seedlings when treated with JA was clearly reduced in *tir1*/*afb2*/*afb3*, *arf7-1*/*arf19-1* and *slr* mutants (Fig. 6A–D and Fig. S4).These results clearly show the dependence of an intact auxin signaling pathway for JA-induced LR formation.

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

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Appendix A. Supplementary data

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

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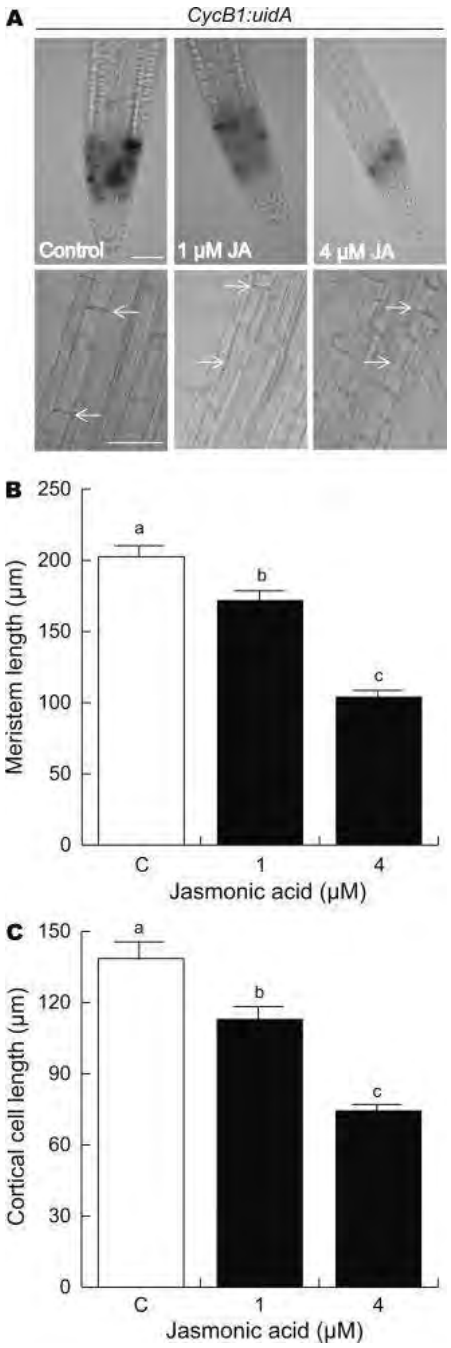

Fig. S1.
Effects of JA on cell division and elongation. *CycB1:uidA A. thaliana* seedlings were grown 12 d on 0.2× MS medium supplemented with the indicated concentrations of JA. (A) Plants were stained for GUS activity and cleared to show gene expression of *CycB1:uidA* and cell size. (B) Meristem length. (C) Cortical cell length. Data points represent the mean ± SD (n = 30). Photographs show representative individuals from at least 15 stained plants. The experiment was repeated twice with similar results. Different letters indicate means statistically different ($P < 0.05$). Scale bar = 100 μm.

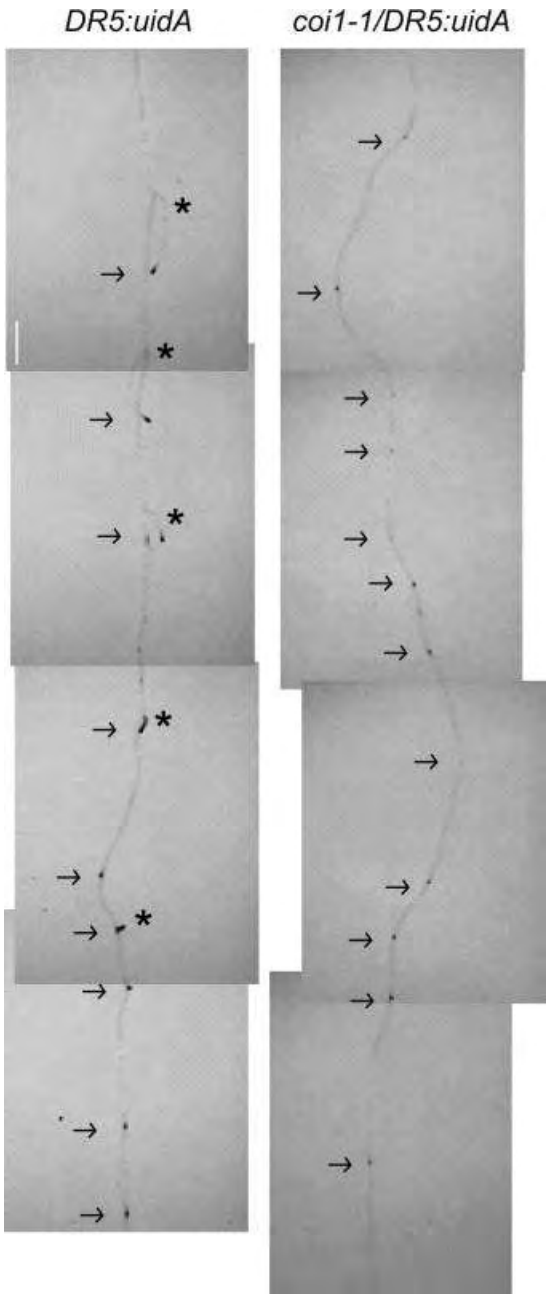

Fig. S2.
LRP development in *DR5:uidA* and *coi1-1*/*DR5:uidA* seedlings. Seedlings were germinated and grown for 4 d on 0.2× MS medium and homozygous *coi1-1*/*DR5:uidA* seedlings were selected from a *coi1-1*/*COI* segregating population obtained from outcrossing the *coi1-1* mutant with a *DR5:uidA* plant in medium supplemented with 4 μM JA. Four-day-old seedlings were transferred and grown side by side over the surface of 0.2× MS agar plates and stained for GUS activity. Photographs of *DR5:uidA* and *coi1-1*/*DR5:uidA* seedlings illustrating the formation of LRP and mature LRs four days after transfer. Photographs were taken to individual seedlings from a total of 20 seedlings that were stained. Scale bar = 300 μm.

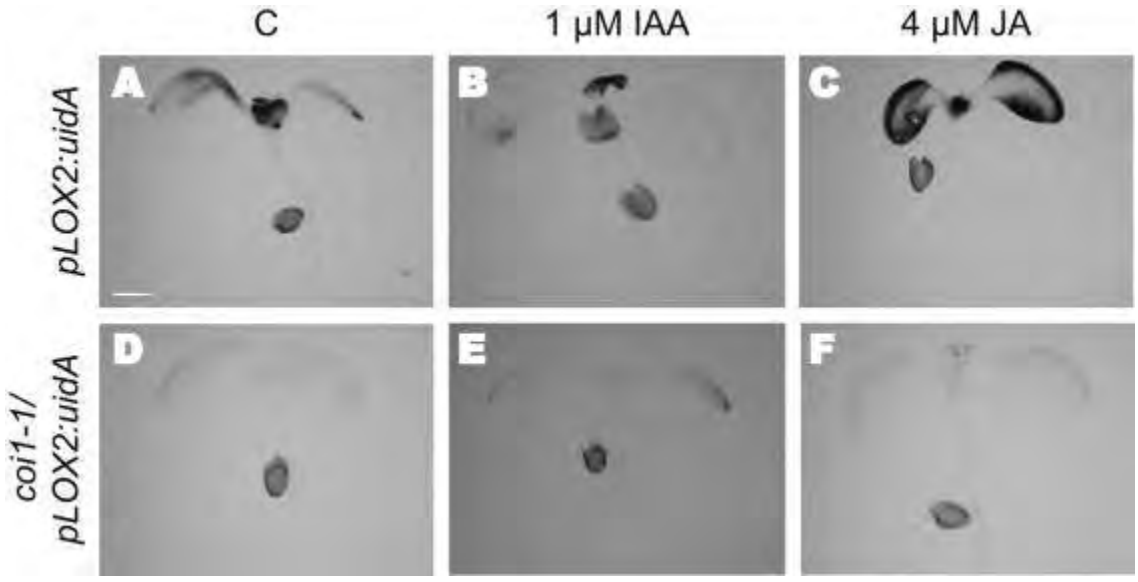

Fig. S3.
Effect of JA and IAA on JA-inducible gene expression in WT and *coi1-1* seedlings. Twelve-hour GUS staining of *pLOX2:uidA* and *coi1-1*/*pLOX2:uidA Arabidopsis* seedlings that were grown for 7 d on agar plates containing 0.2× MS medium supplemented with the solvent (control) or 1 μM IAA or 4 μM JA. Photographs are representative individuals of at least 15 stained seedlings. The experiment was repeated three times with similar results. Scale bar = 200 μm.

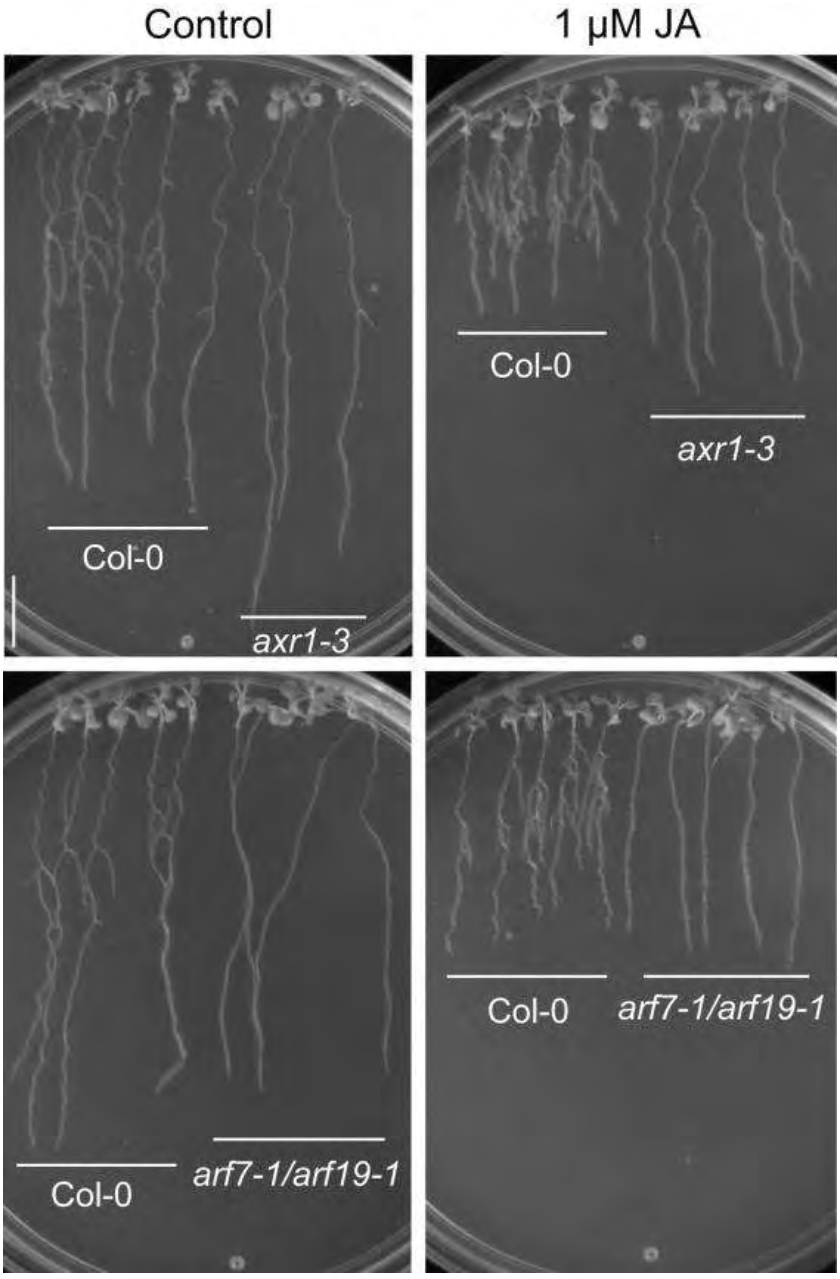

Fig. S4.
Effects of JA on RSA in WT *Arabidopsis* (Col-0) seedlings and auxin-related mutants *axr1-3* and *arf7-1*/*arf19-1*. WT and mutant seedlings were germinated and grown side by side for 12 d in 0.2× MS medium supplemented with the solvent (control) or 1 μM JA and representative photographs from at least 5 independent plates are shown. The experiment was repeated two times with similar results. Scale bar = 1 cm.

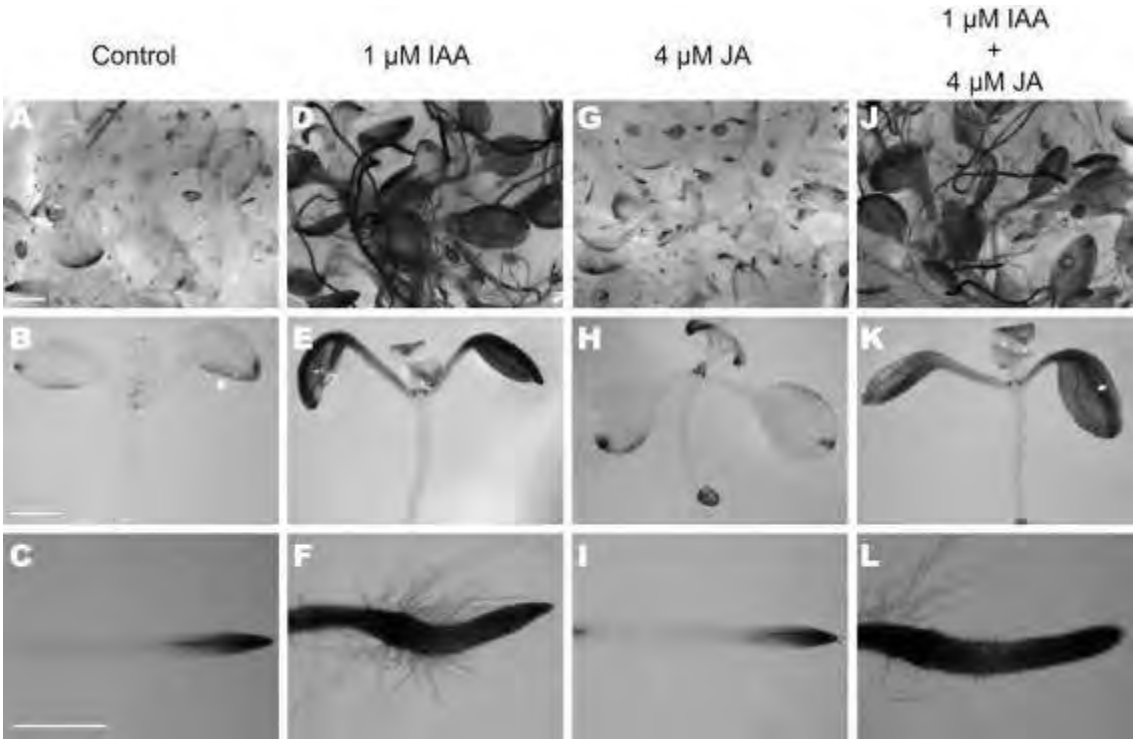

Fig. S5.
Effect of JA and IAA on auxin-inducible gene expression. Twelve-hour GUS staining of *DR5:uidA Arabidopsis* seedlings that were grown for 7 d on agar plates containing 0.2× MS medium supplemented with the solvent (control), 1 μM IAA, 4 μM JA or a combination of IAA and JA. Photographs are representative individuals of at least 15 stained seedlings. The experiment was repeated three times with similar results. Scale bar = 200 μm.

9. DISCUSIÓN, CONCLUSIONES Y PERSPECTIVAS

El crecimiento de las plantas depende en gran medida del funcionamiento de su sistema radicular, ya que las raíces no sólo proporcionan apoyo estructural a la parte aérea, sino además, establecen interacciones bióticas en la rizósfera y modulan la adquisición de agua y nutrientes (López-Bucio et al., 2003). Las plantas regulan la arquitectura del sistema radicular a través de cambios en el crecimiento de la raíz primaria, la formación de raíces laterales y adventicias y la formación y crecimiento de los pelos radiculares, mediados por programas de división y elongación celular (Fig. 1 y 2) (Nibau et al., 2008). Estudiar los mecanismos celulares y moleculares responsables del desarrollo radicular en respuesta a las diferentes señales ambientales, permitirá conocer la compleja interacción que ocurre entre los factores endógenos y exógenos que controlan aspectos individuales de la arquitectura radicular.

Los diferentes aspectos del desarrollo de las plantas se encuentran regulados por fitohormonas, las cuales a través de mecanismos de señalización activan/reprimen la expresión de genes. Recientemente, en diferentes organismos eucariontes, incluyendo plantas, se encontró un componente adicional de la maquinaria transcripcional, el complejo Mediador (Fig. 8). Existen reportes en donde se ha mostrado la participación de subunidades del complejo Mediador en diferentes procesos del desarrollo y respuestas de defensa en plantas (Kidd et al., 2011). Sin embargo, a pesar de la importancia del Mediador en la regulación de la expresión génica se desconoce su participación en el desarrollo de la raíz y la interacción con los diferentes reguladores del desarrollo vegetal. Mediante análisis de líneas mutantes, líneas transgénicas y líneas reporteras, se estudió la participación de diferentes subunidades del Mediador en la configuración del sistema radicular, así como la interacción con diferentes vías hormonales en *Arabidopsis thaliana*. Evidencia experimental indica que mutantes afectadas en MED8 no presentan alteraciones en el sistema radicular, mientras que la pérdida y ganancia de función de PFT1/MED25 causan efectos opuestos en el desarrollo de raíces laterales y adventicias, así como en el crecimiento de la raíz primaria y el

crecimiento y desarrollo de los pelos radiculares (Raya-González et al., 2014). Estos datos indican que PFT1/MED25 funciona como modulador clave de procesos celulares que controlan la configuración del sistema radicular. El fenotipo de la raíz observado en plantas *pft1* y *35S:PFT1* correlaciona con cambios en la expresión del marcador del ciclo celular *CycB1:uidA* y la expansión celular en raíz primaria y raíces laterales (Raya-González et al., 2014). Análisis de la expresión global de genes realizados en plantas silvestres y mutantes *pft1* arrojaron genes diferencialmente regulados, involucrados en el crecimiento y el ciclo celular (Sundaravelpandian et al., 2013). Esto sugiere que PFT1/MED25 regula el desarrollo de la raíz modulando procesos de división y elongación celular. Inicialmente, PFT1/MED25 fue descrito como un regulador positivo de respuestas de evasión por sombra, y posteriormente, como un regulador basal de respuestas de defensa y estrés abiótico (Cerdán y Chory, 2003; Bäckström et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011; Chen et al., 2012), sugiriendo que actúa como un nodo molecular para la integración de diferentes señales ambientales y de desarrollo.

El gen MED25 está altamente conservado en todos los organismos eucariontes. En otros eucariontes, se ha descrito que MED25 tiene un papel muy importante en procesos de desarrollo y defensa. La represión de MED25 en *D. melanogaster* afecta la extensión de axones del sistema nervioso central (Koizumi et al., 2007). Mutantes en MED25 provocan mal formaciones en el pez zebra (Nakamura et al., 2011). En humanos, se ha descrito que MED25 puede interactuar con el activador transcripcional del virus del herpes symplex VP16, actuando como una barrera para la infección y propagación viral (Mittler et al., 2003). En nuestro trabajo, través de la caracterización de líneas mutantes y sobreexpresoras de PFT1/MED25, se obtuvo evidencia de que PFT1/MED25 modula respuestas de desarrollo en plantas.

La formación de raíces laterales inicia cuando células del periciclo responden a un máximo de auxinas, adquiriendo el estado de células fundadoras, las cuales a través de divisiones celulares subsecuentes formarán un primordio (Boerjan et al., 1995; Malamy y Benfey, 1997; Dubrovsky et al., 2008). La ganancia y pérdida de

función de PFT1/MED25 causan fenotipos opuestos en la formación de primordios, particularmente en los estados I y II. Estos datos indican que PFT1/MED25 regula la inducción *de novo* de PRL a partir de células del pericliclo. El análisis del marcador de genes de respuesta a auxinas, *DR5:uidA*, sobre el desarrollo de PRL, mostró que las líneas mutantes y sobreexpresoras *pft1-2*, y *35S:PFT1* presentaron una expresión diferencial con respecto a la línea tipo silvestre (Raya-González et al., 2014). Benková et al. (2003) encontró que las auxinas se acumulan en el PRL. Posteriormente, un máximo de auxina mediado principalmente por el transportador PIN1, es establecido en la punta del primordio para inducir el crecimiento de la raíz lateral. Por lo tanto, la expresión diferencial de los marcadores de respuesta y transporte a auxinas las líneas *pft1-2* y *35S:PFT1* explican el fenotipo en la formación de raíces laterales. Estos resultados indican que PFT1/MED25 puede regular la respuesta y/o la distribución de auxinas.

PFT1/MED25 fue inicialmente identificado como una proteína nuclear que participa en la vía dependiente de fitocromo B para inducir la floración en respuesta a condiciones de luz sub-óptimas. Las mutantes *pft1* presentan defectos en la elongación del hipocotilo en respuesta a deficiencia de luz roja y roja-lejana (Cerdán y Chory, 2003). La participación de las auxinas en los procesos de fotomorfogénesis y evasión por sombra ha sido bien documentada (Shinkle et al., 1998; Steindler et al., 1999; Gil et al., 2001). Se sabe que mutantes como *axr1*, *doc1/tir1*, asociadas a la señalización por auxinas, están afectadas en la respuesta a luz y evasión por sombra, pero además presentan alteraciones en su sistema radicular, incluyendo la formación de raíces laterales y el desarrollo de los pelos radiculares (Lincoln et al., 1990; Pitts et al., 1998; López-Bucio et al., 2005). Posiblemente, la respuesta auxínica alterada en la mutantes *pft1-2* contribuya a su fenotipo en el desarrollo de la raíz. Mediante un análisis de la expresión global de genes reportado por Kidd et al (2009), se identificaron diversos genes diferencialmente regulados en la mutante *pft1-2* asociados a la respuesta auxínica. Algunos de ellos como *ANTHRANILATE SINTASE* (*ASA1*), *TRYPTOPHAN SYNTHASE BETA SUBUNIT*, *CHORISMATE MUTASE3*, son genes de respuesta

temprana a auxinas de la familia GH3, IAA17, AUX/IAA, entre otros. Aun cuando no fueron observadas alteraciones en los niveles de AIA en las líneas *pft1-2* y *35S:PFT1*, los datos son consistentes con la expresión de genes y la respuesta de los diferentes marcadores de auxinas en *pft1-2* y *35:PFT1* (Raya-González et al., 2014). Esto indica que la respuesta y el transporte a auxinas, más que la acumulación del AIA, pueden ser los factores responsables que afectan el sistema radicular en las mutantes *pft1*. Con base en lo anterior, el análisis espacial y temporal *in vivo* del transportador de eflujo de auxinas, *PIN1::PIN1::GFP* en plántulas tipo silvestre, *pft1-2* y *35S:PFT1*, revelaron un incremento en la fluorescencia de GFP en células de la estela y endodermis de la raíz de *pft1-2*. Estas observaciones, sugieren que PFT1/MED25 regulan la expresión y distribución del transportador de auxinas PIN1, lo cual explica porque la mutante *pft1* muestra una respuesta amplificada a auxinas exógenas (Raya-González et al., 2014).

La actividad de los marcadores *DR5:uidA* y *DR5:GFP* permiten monitorear la sensibilidad de los tejidos a AIA y otras auxinas, lo cual no necesariamente correlaciona con los niveles de auxinas (Benková et al., 2003). El análisis de la expresión de *DR5:uidA*, *DR5:GFP* y *BA3:uidA* en plantas tipo silvestre, *pft1-2* y *35S:PFT1* en respuesta al AIA, revelaron que las plantas *pft1-2* y *35S:PFT1* fueron más sensibles o resistentes, respectivamente, a auxinas (Raya-González et al., 2014). Estos datos son consistentes con la respuesta de *pft1-2* y *35S:PFT1* al efecto del ANA en la formación de raíces laterales (Raya-González et al., 2014). La hipersensibilidad y resistencia mostrada por *pft1-2* y *35S:PFT1*, respectivamente, al efecto del ANA, indican que PFT1/MED25 es un regulador clave que controla la activación del pericicloo para la formación de raíces laterales, el cual es modulado vía señalización por auxinas. En concordancia con nuestros resultados, Sundaravelpandian et al. (2013) reportó que PFT1/MED25 controla la diferenciación de los pelos radiculares a través de la distribución de las especies reactivas de oxígeno. Plantas mutantes *pft1-2* presentan pelos radiculares más cortos y menos abundantes que la línea silvestre, mientras que *35S:PFT1* presenta un fenotipo opuesto (Raya-González et al., 2014). Esto indica que PFT1

regula de manera positiva la formación de pelos radiculares, posiblemente a través de un mecanismo independiente a la señalización por auxinas.

El AJ es un regulador del crecimiento esencial en la activación de respuestas de defensa en las plantas. Se ha reportado que el AJ y sus metabolitos, llamados jasmonatos, no sólo se sintetizan durante respuestas de defensa ante el ataque por patógenos, insectos y herbívoros, sino que también en respuestas a estrés abiótico (Waasternack, 2007). En *Arabidopsis*, tratamientos con jasmonatos, inhiben el crecimiento de la raíz primaria, promueven la formación de raíces laterales y de pelos radiculares, afectando tanto la división como la elongación celular (Sun et al., 2009; Raya-González et al., 2012; Raya-González et al., 2014). Se ha reportado que el AJ puede regular el desarrollo de la raíz a través de mecanismos dependientes e independientes a la señalización por auxinas. La formación de raíces laterales inducida por el AJ, involucra la participación de auxinas, a través del gen asociado a la biosíntesis de auxinas *ASA1* (Sun et al., 2009; Raya-González et al., 2012). Sin embargo, para el caso del crecimiento de la raíz primaria, probablemente, el AJ actúa de manera independiente (Raya-González et al., 2012). Existe evidencia que indica que el mecanismo de señalización de auxinas y AJ comparten algunos de sus elementos (Cuéllar Pérez y Goosens, 2013). Esto sugiere que las respuestas de auxinas y AJ a nivel transcripcional pueden estar reguladas por componentes en común. PFT1/MED25 participa en la señalización de AJ y auxinas (Kidd et al., 2009; Raya-González et al., 2014), sugiriendo que PFT1/MED25 puede actuar como nodo para la integración de estas dos vías de respuesta a hormonas. El análisis del efecto del AJ sobre el sistema radicular en plantas silvestres y mutantes *pft1-2* y *coi1-1* indica que PFT1/MED25 es independiente de COI1 y la señalización por AJ en el crecimiento de la raíz primaria y la formación de raíces laterales.

En conclusión, los datos presentados muestran que: (i) la pérdida y ganancia de función de PFT1/MED25 induce respuestas opuestas sobre el crecimiento de la raíz primaria y la formación de raíces laterales y adventicias; (ii) PFT1/MED25 modula la configuración del sistema radicular, regulando de manera negativa la división y la elongación celular; (iii) PFT1/MED25 regula la expresión

de genes de respuesta a auxinas en el desarrollo de PRL; (iv) PFT1/MED25 regula respuestas dependientes de auxinas endógenas y exógenas en la formación de raíces laterales; (v) PFT1/MED25 regula el desarrollo de la raíz a través de un mecanismo independiente a la señalización por AJ.

Con base a las conclusiones resulta claro que PFT1/MED25 está involucrado en múltiples procesos de desarrollo y de respuesta a estrés biótico y abiótico, a través de integrar e interactuar con diversos factores transcripcionales para responder a las diferentes señales celulares. Esto es consistente, ya que el genoma de Arabidopsis codifica para ~1500 factores transcripcionales, los cuales responden a las diferentes señales a través de la interacción con subunidades específicas del complejo Mediador. Por lo tanto, una perspectiva de este trabajo sería determinar los genes blanco de PFT1/MED25, lo que indudablemente generará información valiosa acerca de su función sobre el desarrollo de la raíz.

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11. APÉNDICE

En este apartado se presentan las publicaciones derivadas de la colaboración con diferentes grupos de trabajo durante el desarrollo de la tesis.

11.1. Méndez-Bravo, A., Calderón-Vázquez, C., Ibarra-Laclette, E., **Raya-González, J.,** Ramírez-Chávez, E., Molina-Torres, J., Guevara-García, A.A., López-Bucio, J., Herrera-Estrella. (2011). Alkamides activate jasmonic acid biosythesis and signaling pathways and confer resitance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Plos One* 6:e27251.

11.2. López-Bucio, J.S., Dubrovsky, J., **Raya-González, J.,** Ugartechea-Chirino, Y., López-Bucio, J., de Luna-Valdez, L.A., Ramos-Vega, M., León, P., Guevara-García, A.A. (2014). Arabidopsis thaliana mitogen-activated protein kinase 6 is involved in seed formation and modulation of primary and lateral root development. *J Exp Bot* 65:169-183.

11.3. Garnica-Vergara, A., Barrera-Ortiz, S., Muñoz-Parra, E., **Raya-González, J.,** Macías-Rodríguez, L., Ruíz-Herrera L., López-Bucio, J. The volatile 6-n-pentyl-2H-pyran-2-one from Trichoderma atroviride regulates root morphogenesis via auxin signaling and ETHYLENE INSENSITIVE 2 functioning. *New Phytol.* Submitted.

11.4. López-García, C.M., **Raya-González, J.,** López-Bucio, J.S., Guevara-García, A.A., López-Bucio, J. ALTERED MERSITEM PROGRAM 1 is involved in seed coat, root hair and trichome development in Arabidopsis. *Plant Mol Biol*. Submitted.

11.5. López-Bucio, J.S., López-Bucio, J., **Raya-González, J.,** Ramos-Vega, M., León, P., Guevara-García, A. Mitogen activated protein kinase 6 and MAP kinase phosphatase 1 mediate Arabidopsis root responses to glutamate. *Plant J*. Submitted.

11.6. Garnica-Vergara, A., **Raya-González, J.,** López-Bucio, J., Beltrán-Peña, E. (2012). Señalización del ácido jasmónico y el óxido nítrico durante el desarrollo de *Arabidopsis thaliana*. *Ciencia Nicolaita* 55:28-41.

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Alkamides Activate Jasmonic Acid Biosynthesis and Signaling Pathways and Confer Resistance to *Botrytis cinerea* in *Arabidopsis thaliana*

Alfonso Méndez-Bravo[1,2], Carlos Calderón-Vázquez[2,5], Enrique Ibarra-Laclette[2], Javier Raya-González[1], Enrique Ramírez-Chávez[3], Jorge Molina-Torres[3], Angel A. Guevara-García[4], José López-Bucio[1]*, Luis Herrera-Estrella[2]*

1 Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México, **2** Laboratorio Nacional de Genómica para la Biodiversidad, Cinvestav Irapuato, Irapuato, Guanajuato, México, **3** Departamento de Biotecnología y Bioquímica, Unidad Irapuato, Cinvestav, Irapuato, Guanajuato, México, **4** Instituto de Biotecnología-UNAM, Cuernavaca, Morelos, México, **5** Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-IPN, Guasave, Sinaloa, México

Abstract

Alkamides are fatty acid amides of wide distribution in plants, structurally related to *N*-acyl-L-homoserine lactones (AHLs) from Gram-negative bacteria and to *N*- acylethanolamines (NAEs) from plants and mammals. Global analysis of gene expression changes in *Arabidopsis thaliana* in response to *N*-isobutyl decanamide, the most highly active alkamide identified to date, revealed an overrepresentation of defense-responsive transcriptional networks. In particular, genes encoding enzymes for jasmonic acid (JA) biosynthesis increased their expression, which occurred in parallel with JA, nitric oxide (NO) and H_2O_2 accumulation. The activity of the alkamide to confer resistance against the necrotizing fungus *Botrytis cinerea* was tested by inoculating *Arabidopsis* detached leaves with conidiospores and evaluating disease symptoms and fungal proliferation. *N*-isobutyl decanamide application significantly reduced necrosis caused by the pathogen and inhibited fungal proliferation. *Arabidopsis* mutants *jar1* and *coi1* altered in JA signaling and a MAP kinase mutant (*mpk6*), unlike salicylic acid- (SA) related mutant *eds16/sid2-1*, were unable to defend from fungal attack even when *N*-isobutyl decanamide was supplied, indicating that alkamides could modulate some necrotrophic-associated defense responses through JA-dependent and MPK6-regulated signaling pathways. Our results suggest a role of alkamides in plant immunity induction.

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* E-mail: jbucio@umich.mx (JL-B); lherrera@ira.cinvestav.mx (LH-E)

Introduction

Plants continuously respond to abiotic and biotic stress by adjusting their metabolism and activating diverse intracellular and systemic responses. Biotic stress induced by pathogens triggers complex signaling cascades regulated by hormones once an invader has been detected. Three main phytohormones have been classically recognized as essential components of responses triggered by pathogens, namely salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Hormonal-dependent pathways result in the expression of defense-related genes such as those encoding pathogenesis-related (PR) proteins, and the production of antimicrobial secondary metabolites [1]. These responses are assisted by reactive molecules, such as nitric oxide (NO) and reactive oxygen species (ROS) that function both, as signaling components of transcriptional and metabolic readjustment and as antimicrobial substances [2]. Lifestyle of pathogens largely determines the effectiveness of a plant-induced response to combat the pathogen challenge. The SA-dependent signaling pathway is often considered to be effective against pathogens that derive nutrients from living hosts cells (biotrophs), and JA/ET pathways against pathogens that derive nutrients from dead cells (necrotrophs), although, the persistence of defense responses and the disease outcome are determined by complex networks of interactions between multiple hormone signaling pathways [3].

Lipids have a key role in maintaining the fluidity and structural integrity of all cell membranes. Additionally, lipids and various fatty acid derivatives have been described to act as signaling molecules in response to diverse environmental cues [4]. Structural features of fatty acids, such as the chain length and their unsaturation degree, determine their function and biological activity by altering membrane lipid composition [5]. Exogenous and endogenous mono- and poly-unsatured fatty acids (PUFAs) alter plant gene expression and metabolism, thus impacting the plant-microbe and plant-herbivore interactions [5,6]. For instance, alterations in enzymatic machinery that regulates production of cellular unsaturated fatty acids alter the SA- and JA-mediated defense signaling. A reduction in the endogenous levels of oleic

acid (18:1) caused by mutation of a gene encoding STEAROYL-ACYL CARRIER PROTEIN-DESATURASE, increases the expression level of *PR* genes in a SA-dependent way, but at the same time, reduces expression of a subset of JA-dependent response genes and decreases resistance to *Botryris cinerea* in *Arabidopsis* [7]. A kind of PUFAs that impact plant defense responses are the eicosapolyenoic acids, which are produced and released by several species of oomycete plant pathogens. Specifically, exogenous application of arachidonic acid (20:4) to *Arabidopsis* and tomato (*Solanum lycopersicum*) plants induces expression of general-stress responsive genes, increases endogenous JA levels and confers resistance against the necrotrophic fungi *Botrytis cinerea* [8]. Based on this evidence, it has been proposed that lipids and their derivates have transorganismal signaling activity, and that their dependent pathways are conserved throughout the evolutive history of organisms [8].

Commonly, the full range of biological effects triggered by PUFA signals, are carried out by their metabolism into more potent substances, the oxylipins. Oxylipins are a diverse class of lipid metabolites that include fatty acid hydroxiperoxides, hydroxy-, oxo-, or keto-fatty acids, volatile aldehydes, or mostly, jasmonates (JAs) [9]. JAs act as regulatory molecules in metabolic and developmental processes, as well as in defense responses [10,11,12]. They rapidly accumulate by wounding, insect attack and necrotrophic pathogen infection [13]. JA is synthesized through a series of reactions involving lipoxygenases (LOXs), allene oxide synthases (AOSs), allene oxide cyclases (AOCs) and 12-oxophytodienoate reductases (OPRs). Then, JA is further modified to produce JAs, for example, as conjugates with various lipophilic amino acids such as isoleucine (Ile) produced by a jasmonate amino acid synthetase, encoded by *JASMONATE RESISTANT1* (*JAR1*). The JA signal (JA-Ile) is perceived by an intracellular receptor, the F-box protein COI1, which plays a key role in JA signaling [14], and is required for the majority of the JA-mediated responses described to date, such as fertility, secondary metabolite biosynthesis, pest and pathogen resistance, and wound responses [15]. COI1 is an E3 ubiquitin ligase that catalyzes the ubiquitination of proteins destined to degradation via the proteosome-mediated pathway. COI1 activates a signal transduction pathway that culminates in the transcriptional activation or repression of JA-responsive genes. The *coi1* mutant is resistant to JAs and to the *Pseudomonas syringae* toxin coronatine. The essential role of JAs in plant immunity is also evidenced by JA-related mutant phenotypes, for example both *jar1* and *coi1* show an enhanced susceptibility to necrotrophic pathogens [16,17]. In addition, protein phosphorylation and dephosphorylation have important roles in JA signaling. The mitogen-activated protein kinase (MAPK) cascade, which is one of the major signal transduction pathways in plants, as well as other eukaryotes, has been found to be regulated by JA to modulate JA-dependent gene expression [17]. In Arabidopsis, three MAPKs (MPK3, MPK4 and MPK6) have been implicated in defense against pathogens [18,19,20]. MPK6 functions as substrate of at least four MAPK kinases (MKK2, MKK3, MKK4 and MKK5) in response to different stimuli, including developmental, microbial or environmental cues. Once phosphorylated, MPK6 activates several transcriptional regulators, such as members of the WRKY, MYC and ERF gene families. Particularly, but not exclusively, the MKK3-MPK6 cascade is activated in response to JA and both, positively and negatively regulates the expression of JA-related genes [17,21]. Concordantly, the *MKK3*-knockout mutant *mkk3* and *coi1* had an altered activation of MPK6 in response to JA. Moreover, mutations in *MPK6* compromise the accumulation of antifungal phytotoxin camalexin in response to infection with *Botrytis cinerea* [17,22].

An additional class of lipids conserved among different kingdoms with signaling functions in plants is the fatty acid amides group, including the plant-, fungal- or animal-produced *N*-acylamides (alkamides), *N*-acylethanolamines (NAEs), and the *N*-acyl-L-homoserine lactones synthesized by Gram-negative bacteria. Compounds representative of these three classes of lipids have been shown to modulate seedling development in *Arabidopsis* and to affect plant biomass production in a dose-dependent way, indicating a strong biological activity [23,24,25]. NAEs are compounds with aminoalcohol linked as an amide to the fatty acid, which accumulate in seeds of higher plants, including cotton, corn, soybean, tomato, pea and *Arabidopsis*, and decrease during germination [26]. Pioneering research on fatty acid amides showed that NAE production in plants is associated to defense responses. NAE 14:0 accumulates in tobacco leaves treated with fungal elicitors, and exogenous application of this NAE is able to induce expression of defense-related genes [27]. Moreover, the ectopic overexpression of a plant fatty acid amide hydrolase (FAAH), an enzyme that degrades NAEs, renders *Arabidopsis* plants more susceptible to both host and non-host bacterial pathogens [28]. *N*-acyl-L-homoserine lactones (AHLs) are structural analogs of NAEs and alkamides, that are produced by Gram negative bacteria and participate in the cell-to-cell communication process commonly referred to as quorum-sensing (QS). Interestingly, plants have the genetic machinery to perceive and respond to AHLs. The presence of AHL-producing bacteria in the rhizosphere of tomato induces SA- and JA-dependent defense responses, conferring resistance to the fungal pathogen *Alternaria alternata* [29]. Moreover, the application of purified AHLs to *Medicago truncatula* and *Arabidopsis* plants results in differential transcriptional changes in roots and shoots, affecting expression of genes potentially involved in immune responses and development [30,31]. Interestingly, FAAH knockouts and overexpressors *Arabidopsis* lines are more sensitive and tolerant, respectively, to the root inhibitory effects of AHLs, in a similar fashion to their response to exogenous NAEs and alkamides, while an alkamide resistant mutant termed *decanamide resistant root 1* (*drr1*) showed decreased root responses to alkamides and AHLs [25,32].

Alkamides comprise over 200 related compounds and they have been found in several plant families: Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, and Solanaceae, reviewed in [33,34]. Some traditional medicinal plants produce these secondary metabolites during their life cycle in response to several stress conditions to mediate, among other processes, plant chemical defense against plant competitors or microbial and herbivorous predators [35]. Several species from the genus *Echinacea* accumulate unsaturated alkamides ranging from 12 to 18 carbon atoms in response to JAs [36,37]. These unsatured alkamides are also active in mammals; they activate immune responses in alveolar macrophages from rats, in concert with a sustained production of NO, a canonical messenger in plant and animal defense responses [38,39]. Alkamides have also been identified in insects, such as *N*-linolenoyl-L-glutamine, present in oral secretions of the tobacco hornworm (*Manduca sexta*), which is able to elicit defensive responses in plants by inducing volatile chemicals that attract predators and parasites of the attacker [40]. The wound-induced JA production is amplified by application of these oral secretions in *Nicotiana attenuata* leaves, indicating a reciprocal crosstalk between JAs- and alkamides-related signal pathways [41]. To date, however, there is no direct evidence as to whether alkamides can switch JA production and its transcriptional targets.

The short-chain alkamide affinin from the "gold-root" *Heliopsis longipes* has been reported to have antimicrobial activity inhibiting

in vitro growth of some plant microbial pathogens, including bacteria and fungi [35]. To explore the structure-activity relationships of alkamides, we previously evaluated the root developmental responses of *Arabidopsis* seedlings to application of a group of affinin-derived natural and/or synthetic fatty acid amides with similar chain length [42]. We found that *N*-isobutyl decanamide, a C:10 saturated alkamide that is naturally produced in *Acmella radicans* [43] and *Cissampelos glaberrima* [44], was the most active compound in inhibiting primary root growth and stimulating lateral root formation. Interestingly, root developmental alterations induced by *N*-isobutyl decanamide were related to a sustained increase in nitric oxide (NO) production and required the activity of the *DRR1* protein [45].

To further understand the molecular responses to fatty acid amides, in this work we performed whole-genome transcriptional profiling of *Arabidopsis thaliana* seedlings in response to *N*-isobutyl decanamide. Our results show the activation of defense-related gene expression, concomitant to an increase in JA accumulation and in the expression of JA-responsive and senescence-associated genes. Moreover, *N*-isobutyl decanamide application to mature *Arabidopsis* leaves conferred resistance against fungal necrotizing pathogen *Botrytis cinerea* in a process involving JA-dependent signaling.

Results

Transcriptomic profiling of *Arabidopsis* in response to *N*-isobutyl decanamide

To characterize at the transcriptional level the molecular responses of *Arabidopsis* to *N*-isobutyl decanamide, Col-0 WT seedlings were germinated and grown for 6 d on 0.2× MS medium and then transferred to fresh medium supplied with or without 60 μM of *N*-isobutyl decanamide to directly compare their effect on whole-genome transcriptional profile after 1, 3, 7 and 14 d of treatment (Figure S1) employing a two-channel long-oligonucleotide microarray platform (see Methods).

According to a stringency level of FDR 0.05 (fold change ≥2), a total of 1,281 genes showed differential expression in at least one of the four sampled time points. The complete list of differentially expressed genes is provided in Table S1. Among differentially expressed genes, 727 were found to be up-regulated and 554 down-regulated by *N*-isobutyl decanamide (Figure 1A). Only 22 from the 727 induced genes and 33 down-regulated genes were common to all time points evaluated (Figure 1B). Of these overlapping genes, highest expression values were reached on the seventh day of *N*-isobutyl decanamide treatment (Figure 1C). Analysis of expression patterns by agglomerative hierarchical clustering showed that the number of differentially regulated genes increased from day 1 to day 7 after treatment and then decreased at day 14 (Figure 1C).

In addition to the statistical methods described (see Materials and Methods), validation of microarray data was achieved by real-time quantitative PCR (qRT-PCR) of 15 randomly chosen genes, including up- and down-regulated genes. These experiments were carried out using RNA extracted from an independent batch of control and treated plants than those used for microarray analysis experiments. qRT-PCR gene expression profiles obtained for the analyzed loci were quite consistent with those generated by the microarray analysis (Figure S2).

Functional categories of genes up-regulated by *N*-isobutyl decanamide

Differentially expressed genes were classified into functional categories according to the Munich Information Center for Protein Sequences classification (MIPS) using the FunCat database [46]. The categories "Metabolism" (290 genes), "Storage protein" (16 genes), "Cellular transport, transport facilities and transport routes" (161 genes), "Cell rescue, defense and virulence" (189 genes), "Interaction with the environment" (165 genes), "Systemic interaction with environment" (92 genes), "Cell fate" (31 genes) and "Biogenesis of cellular components" (90 genes) were identified as significantly over-represented MIPS categories among *N*-isobutyl decanamide responsive genes (Table S2). Most of these genes belong to defense- and stress-related categories, including subcategories belonging to the "Metabolism" set such as, 'Metabolism of glutamate, polyamines, nitrogen and related groups, chitin and others polysaccharides, and secondary metabolism' (Table S2).

When we performed functional categorization per day of treatment, we found that the highest percentage of genes in every category was represented at the seventh day (Figure 2A). Two remarkable over-represented categories identified were "Cell rescue, defense and virulence" and "Systemic interaction with environment" (Figure 2A). Detailed analyses of these two categories showed significant overrepresentation of the 'stress response', 'disease, virulence and defense', 'detoxification', 'plant/fungal specific systemic sensing and response' and 'animal systemic sensing and response' subcategories (Figure 2B). Within these subcategories, we found 70 genes involved in oxygen and radical detoxification, 75 genes involved in hormone-related responses (auxin, ethylene, cytokinin and abscisic acid), and particularly, genes encoding enzymes involved in JA synthesis and associated responses (Table S2; Figure 3). Additional differentially regulated genes encoded proteins related to biotic stress, including different secreted pathogenesis-related proteins (PR) such as chitinases and glucanases (At4g07820, At2g19990, At2g14610, At3g57260, At3g04720, At1g75040, At2g19970, At2g14580, At4g33720 and At2g14580) (Table S1). Overrepresentation of biotic stress-related categories can be appreciated more clearly in the functional categorization of *N*-isobutyl decanamide-induced genes (Figure 2C). These results suggest that alkamides are likely involved in triggering defense-associated responses in *Arabidopsis*.

General defense responses but not salicylic acid biosynthesis are activated by *N*-isobutyl decanamide

Because *N*-isobutyl decanamide increased the transcript level of a wide class of PR genes, we examined its effect on the production of salicylic acid (SA) and signaling molecules related to local and systemic responses in defense processes. SA is a phenolic hormone whose activity is required to successfully respond against several different invading pathogens [47] and their biosynthesis succeeds in association with changes in redox homeostasis producing reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) [48]. In turn, SA and H_2O_2 release is accompanied by another reactive signalling molecule, nitric oxide (NO). Whole-transcriptional profiling regulated by *N*-isobutyl decanamide showed that *PATHOGENESIS-RELATED1* (*PR1*, At2g14610), a marker for SA signaling, and overall defense responses [49,50,51] increased its transcript level by 7.5-fold at day 7 (Table S1). However, none of the genes encoding enzymes related to SA biosynthesis were significantly up-regulated. Moreover, *N*-isobutyl decanamide did not appear to significantly affect the overall SA content despite an observed induction of the *PR1:GUS* reporter-gene expression (Figure 3A &B), suggesting that *N*-isobutyl decanamide-mediated gene expression of *PR1* occurred independently of SA accumulation.

It is well documented that some stress-associated molecules, such as ROS, play signaling roles as second messengers in

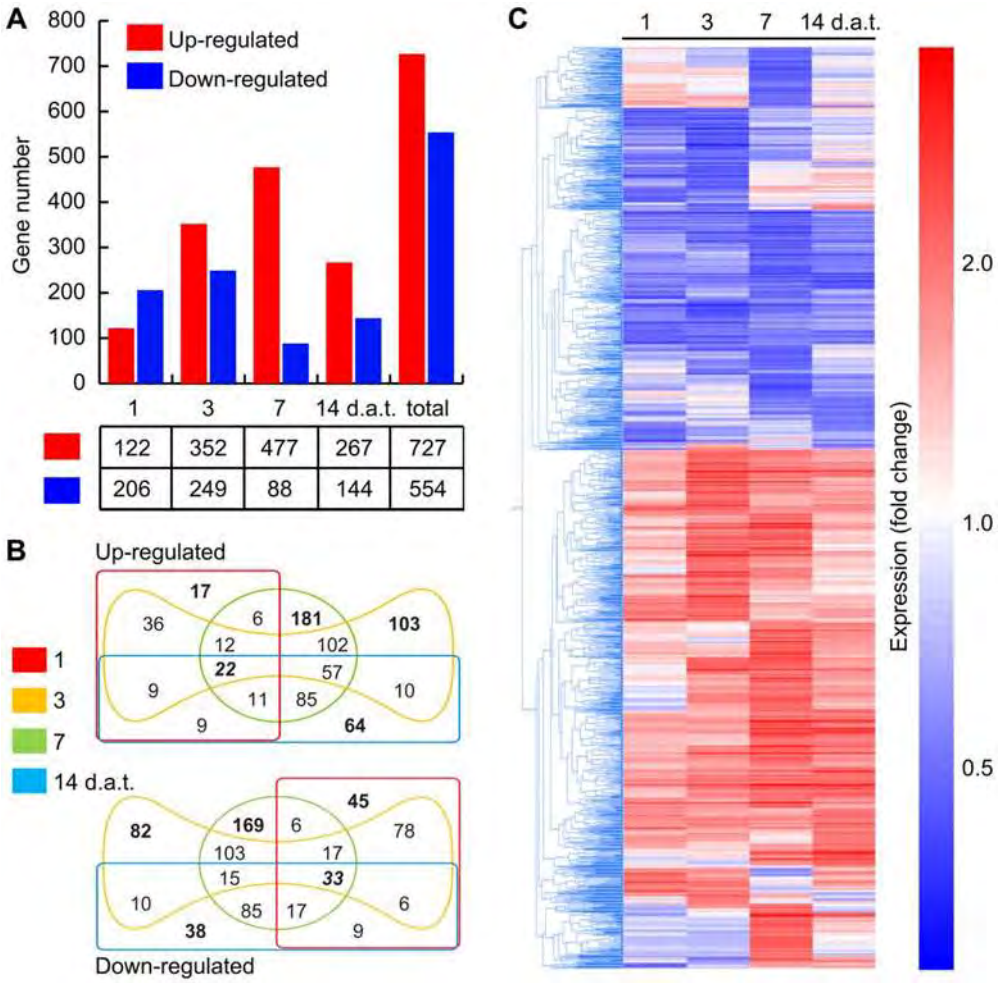

Figure 1. Overview of *N*-isobutyl decanamide responsive genes in *Arabidopsis* seedlings. Number of genes (vertical axis) Up-regulated (red) and Down-regulated (blue) by *N*-isobutyl-decanamide treatment at 1, 3, 7, and 14 d.a.t. (A). Edwards-Venn diagrams showing common or distinct responsive genes identified at every time evaluated (B). The number of genes up- or down-regulated in a single condition is shown in bold letters. The number of genes regulated at all sampled-times are shown in bold italic font. Agglomerative hierarchical clustering of differentially expressed genes at every sampled times (C). Clustering was performed using the Smooth correlation and average linkage clustering in GeneSpring GX 7.3.1 software (Agilent Technologies®). Blue color indicates Down-regulated, red Up-regulated and white unchanged values, as shown on the color scale at the right side of the figure.
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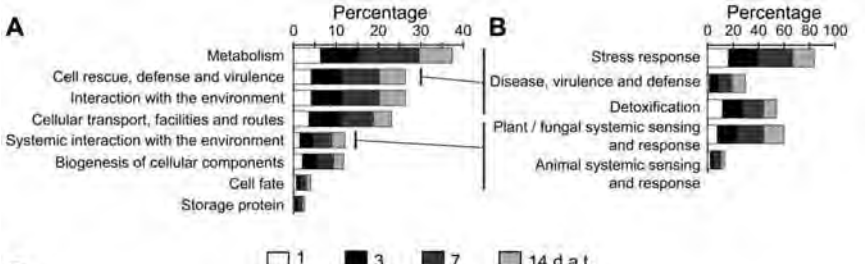
developmental and defense process. Among the *N*-isobutyl decanamide differentially expressed genes, at least 70 belonging to the functional group "oxygen and radical detoxification" were regulated by alkamide treatment (Table S2), having their highest expression level at days 3 and 7 after transfer. Up-regulated genes included ten peroxidases (At4g08780, At5g06730, At4g08770, At5g06720, At5g58390, At5g05340, At2g18150, At3g49960, At3g49110 and At2g18140), two thioredoxin H-type *TH8* and *TH7* (At1g69880, At1g59730) and a glutaredoxin (At5g40370), two glutathione peroxidases *ATGPX4* and *ATGPX6* (At2g48150, At4g11600), five FAD-binding oxidoreductases (At1g26410, At1g26380, At1g26390, At1g26400 and At1g26420), the catalase *CAT3* (At1g20620) and *HYDROPEROXIDE LYASE1* (At4g15440) (Table S1 & S2). Given this overrepresentation, we decided to explore whether ROS accumulation coincided with the increase in transcript level of the group of oxygen and radical detoxification genes. We detected hydrogen peroxide (H_2O_2) production in situ in *Arabidopsis* seedlings that were transferred for 7 d from MS 0.2× medium to a medium containing *N*-isobutyl decanamide. At this stage the seedlings were treated with 3,3-diaminobenzidine (DAB), which in the presence of peroxidases polymerizes as soon as it comes into contact with H_2O_2, forming a brown precipitate. Leaves from *N*-isobutyl decanamide-treated seedlings clearly showed an increase in H_2O_2 (Figure 3C) and NO production (Figure 3D) when compared to solvent-treated seedlings. Overall, these results suggest that general defense-associated responses elicited by *N*-isobutyl decanamide appear to be related to both hormonal and oxidative stress response.

Endogenous levels of JA and their corresponding transcripts are induced by *N*-isobutyl decanamide

Virtually all genes encoding for biosynthetic enzymes for JA production were regulated by *N*-isobutyl decanamide (Figure 4A). Canonical JA-dependent inducible genes involved in JA signaling and response pathways showed predominantly induction profiles (Figure 4C). Among them *CORONATINE-INDUCED3* (*CORI3*, At4g23600), *NAC DOMAIN-CONTAINING PROTEIN81* (*ATAF2/ ANAC081*, At5g08790), *JASMONATE-ZIM-DOMAIN* (*JAS1/ JAZ10*, At5g13210), *ETHYLENE RESPONSE FACTOR2* (*ERF2*,



C

Functional category	Absolute counts	P-Value
METABOLISM	167	3.79E-05
amino acid metabolism	15	2.96E-02
nitrogen, sulfur and selenium metabolism	11	2.96E-03
C-compound and carbohydrate metabolism	71	9.88E-06
secondary metabolism	44	3.86E-15
ENERGY	15	1.81E-01
metabolism of energy reserves	4	4.55E-02
STORAGE PROTEIN	11	6.80E-07
storage facilitating proteins	11	6.80E-07
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	178	1.00E+00
polysaccharide binding	1	7.43E-02
lipid binding	13	6.64E-05
metal binding	44	3.21E-02
complex cofactor/cosubstrate/vitamine binding	6	1.02E-01
oxygen binding	15	1.07E-03
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	79	1.28E-02
transported compounds (substrates)	70	1.05E-03
CELL RESCUE, DEFENSE AND VIRULENCE	121	3.81E-32
stress response	69	6.63E-18
disease, virulence and defense	36	3.08E-09
detoxification	33	6.58E-14
INTERACTION WITH THE ENVIRONMENT	98	3.95E-15
cellular sensing and response to external stimulus	96	4.26E-17
SYSTEMIC INTERACTION WITH THE ENVIRONMENT	64	4.40E-17
plant / fungal specific systemic sensing and response	57	7.73E-15
animal specific systemic sensing and response	15	8.91E-08
BIOGENESIS OF CELLULAR COMPONENTS	54	1.27E-02
cell wall	15	3.19E-03
eukaryotic plasma membrane	1	9.78E-02
nucleus	37	2.86E-02
prokaryotic cytoplasmic membrane	1	2.54E-02
prokaryotic cell envelope structures	5	5.30E-04
SUBCELLULAR LOCALIZATION	251	9.09E-01
cell wall	27	1.87E-05
extracellular / secretion proteins	13	1.38E-06

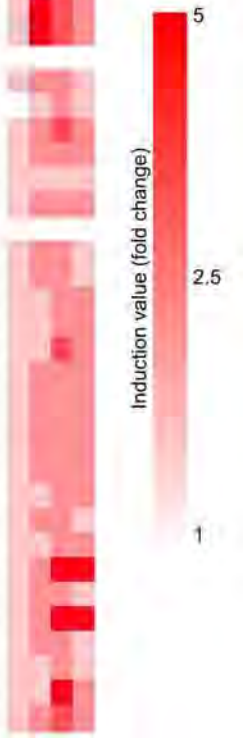


Figure 2. Functional classification of *Arabidopsis* genes differentially expressed in response to *N*-isobutyl decanamide treatment. Categorization of 1,281 genes regulated by treatment was obtained according to the Munich Information Center for Protein Sequences classification (MIPS) classification using FunCat database (http://mips.helmholtz-muenchen.de/proj/funcatDB/) and *Arabidopsis* annotation. Statistically significant categories were identified by using a hypergeometric method and Bonferroni correction with a cutoff of p-value >0.05 (A). MIPS defense-related subcategories significantly represented (B). Percentages relate to total differentially regulated genes per sampled times. The average transcriptional change of all induced genes in overrepresented functional categories was also calculated along time of treatment (C).
doi:10.1371/journal.pone.0027251.g002

At5g47220), *VEGETATIVE STORAGE PROTEIN2* (*VSP2*, At5g24770), *LIPID TRANSFER PROTEINS* (*LTP3*, *LTP2* and *LTP*, At5g59320, At2g38530, and At4g12490, respectively) and the *SENESCENCE-ASSOCIATED GENE13* (*SAG13*, At2g29350) sustained high expression values through our temporal kinetic experiment. The genes *CORI1* (At1g19670), *JAZ8* (At1g30135), *ERF4* (At3g15210) and *PHYTOALEXINE DEFICIENT3* (*PAD3*, At3g26830) showed increased expression from basal levels to induced expression levels. Four *PLANT DEFENSIN* genes *PDF1.2a*, *PDF1.1*, *PDF1.2b* and *PDF1.2c* (At5g44420, At1g75830, At2g26020, At5g44430) and a chitinase (*BASIC CHITINASE*, At3g12500) ranged from repression to induction values (Figure 4B). However, all of them, in a similar way to the genes of the JA biosynthetic pathway, were overexpressed at day 7 after alkamide treatment (Figure 4A &B).

To determine whether increase in the transcript level of JA-related genes correlated with changes in endogenous levels of JA, we quantified, by gas chromatography coupled to mass spectrom-

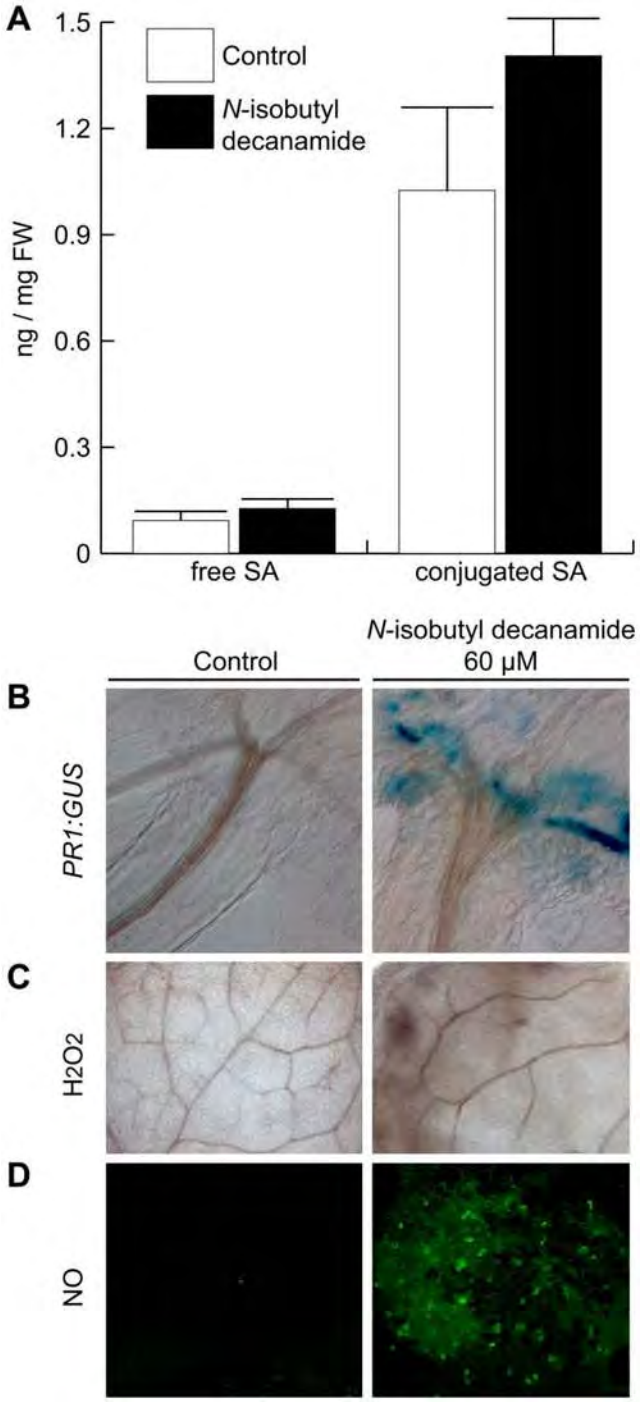


Figure 3. Effects of *N*-isobutyl decanamide on defense-related metabolite production and *PR1* expression. *Arabidopsis* seedlings were grown for 6 days on *N*-isobutyl decanamide-free medium and transferred to control plates with or without *N*-isobutyl decanamide for 7 additional days. Salicylic acid (SA) accumulation was determined by measuring free and conjugated SA by GC-MS (A). Benzoic acid was used as internal standard, data are means of three independent experiments ± SD. Transgenic *Arabidopsis* line carrying *PR1:GUS* was stained for *GUS* expression (B). Detection of hydrogen peroxide (H_2O_2) was made by staining leaves from control and treated seedlings with DAB (C). Images were captured with a Nomarski microscope. Nitric oxide (NO) from leaves of control and treated seedlings was detected by analyzing fluorescent signal of DAF-2DA with a confocal microscope (D). All photographs are representative individuals from at least 9 seedlings analyzed.
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etry (GC-MS), JA accumulation in seedlings treated with the solvent or 60 µM *N*-isobutyl decanamide . A nearly two-fold increase in JA level was observed in response to alkamide treatment (Figure 5A), indicating that the effects of this alkamide on gene expression might be mediated, at least in part, by increasing JA production in the plant. To gain further insight into the transcriptional activation of JA-dependent responses to *N*-isobutyl decanamide, transgenic seedlings containing a chimeric gene in which the *LOX2* promoter is fused to the *GUS* reporter gene (*LOX2:GUS*) were treated with 30 and 60 µM *N*-isobutyl decanamide, and GUS histochemical analysis performed in 7 d-old seedlings. Interestingly, increased expression of this marker was observed throughout the shoot in alkamide-treated seedlings in a dose-dependent manner (Figure 5B).

Because a concentration of 30 µM *N*-isobutyl decanamide was able to induce *LOX2* expression, we evaluate the expression levels of JA-responsive transcripts by qRT-PCR in seedlings treated for 7 days with this alkamide concentration. Among the biosynthetic genes, we focused on genes encoding the *LOX2* and *LOX3* lipoxigenases, *ALLENE OXIDE SYNTHASE* (*AOS*) and *ALLENE OXIDE CYCLASE2* (*AOC2*) enzymes, and *OPDA REDUCTASE3* (*OPR3*). We also examined the expression levels of the JA-inducible genes *JAZ8*, *VSP2* and *ERF2*. All tested genes were induced by *N*-isobutyl decanamide application more strongly than JA itself (Figure 5C). It has been reported that JA-responsive gene expression occurs in a short time after JA perception [52]. For this reason, the JA insensitive mutant *coronatine insensitive1* (*coi1-1*) was employed as negative control to determine if an intact JA signaling pathway was required for the long-term (7 days) response. As shown in Figure 5C, most tested genes either did not respond or were repressed in response to JA or to *N*-isobutyl decanamide treatment in the *coi1-1* mutant.

Additionally, we evaluated local activity of *N*-isobutyl decanamide to induce transcriptional activation of defense-related genes in fully developed leaves, which were excised and incubated for 24 h in media supplemented with 30 µM *N*-isobutyl decanamide. Alkamide-treated leaves showed increased expression of the *LOX2:GUS* reporter gene in a dose-dependent manner as compared to untreated controls (Figure S3A). Moreover, the relative expression level of *OPR3*, *VSP2* and *PAD3* genes was at least two-fold higher in treated wild-type leaves than in the controls (Figure S3B). These results show that at least some transcriptional networks modulated by *N*-isobutyl decanamide are active even in detached tissue.

N-isobutyl-decanamide confers resistance to fungal necrotizing pathogen *Botrytis cinerea*

JA accumulation and JA-responsive gene expression analyses suggest that *N*-isobutyl decanamide may function as a potential defense-inducing factor. To determine whether *N*-isobutyl decanamide could effectively activate defense mechanisms that lead to pathogen resistance, we tested the responses of leaves from 20 d-old *Arabidopsis* plants to the necrotrophic pathogen *Botrytis cinerea*. In these experiments, again, fully developed leaves were transferred 24 h to agar plates supplied with 30 µM *N*-isobutyl decanamide or with the solvent as control. A 10 µl droplet of *B. cinerea* spores was inoculated on the leaf surface and disease symptoms evaluated 3, 4 and 5 days after inoculation (d.a.i.). In leaves transferred to control medium and inoculated for 3 days, the fungus induced necrotic lesions in over 90% of inoculated leaves (Figure 6A), whereas in *N*-isobutyl decanamide leaves treated only 10% presented necrotic lesions (Figure 6A). Four d.a.i., it was found that 100% of the control leaves showed necrotic lesions, whereas in *N*-isobutyl decanamide-treated leaves, around

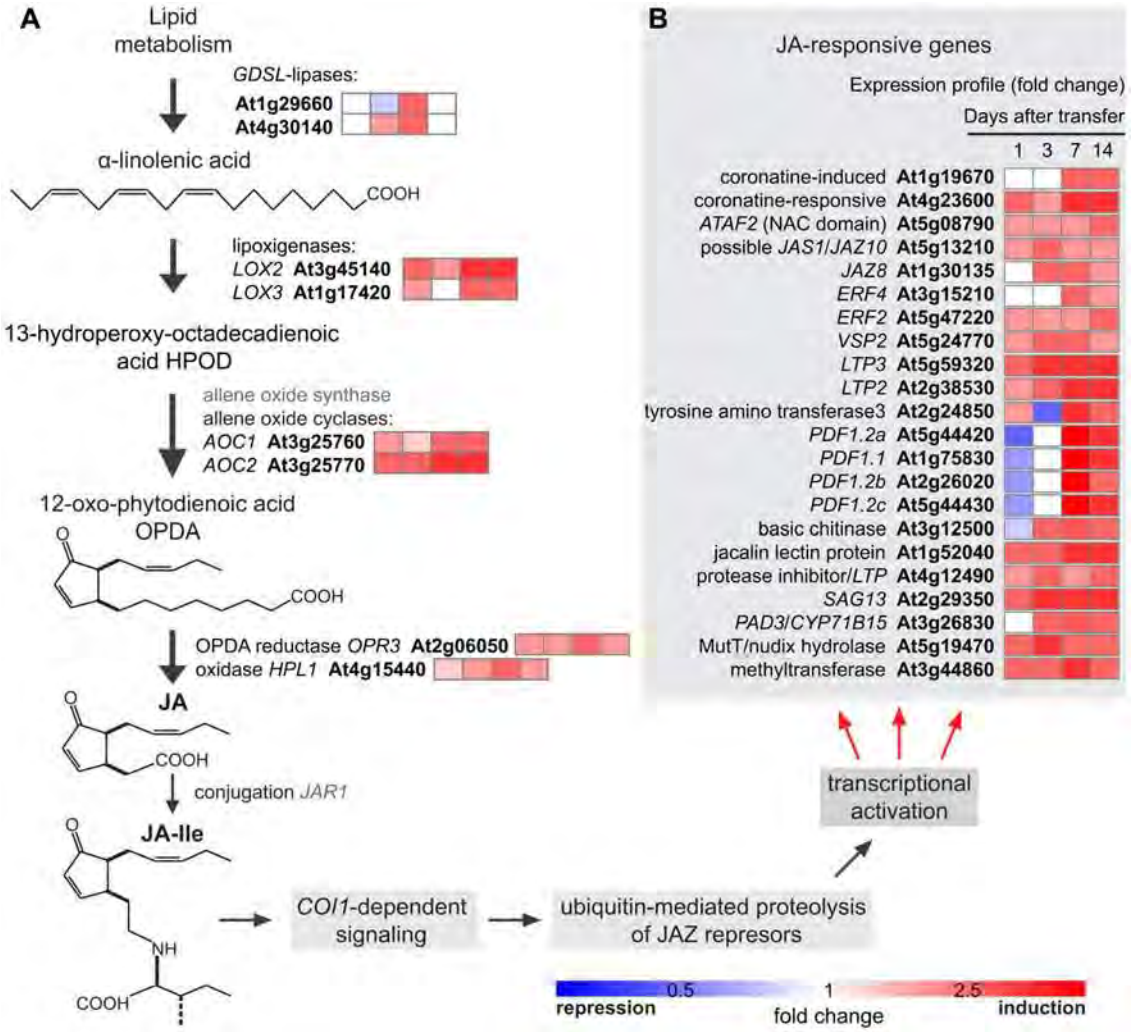

Figure 4. JA-related pathways are transcriptionally induced by *N*-isobutyl decanamide. Simplified representation of JA biosynthetic pathway, genes and metabolites are illustrated (A). Fold-change values of JA-biosynthetic and –responsive pathway genes differentially expressed with *N*-isobutyl decanamide treatment (A, B). Data from microarray expression profiles are shown in the color scale from blue to red.
doi:10.1371/journal.pone.0027251.g004

15% and 60% of infected leaves showed necrotic lesions at fourth and fifth d.a.i., respectively. It is important to note that lesions in control leaves five d.a.i. were of about 6 mm in diameter, whereas in alkamide-treated leaves, the lesions had a diameter between 0.8 and 1.5 mm (Figure 6B). Visual inspection showed that after 5-d of inoculation, solvent-treated leaves inoculated with the pathogens presented generalized necrotic lesions spanning half or more the surface of the leaf, while *N*-isobutyl decanamide-treated leaves manifested significantly reduced symptoms (Figure 6C). We monitored hyphal growth of the pathogens by direct microscopic observation of stained mycelium in infected leaves. We found that disease symptoms in solvent-treated leaves at day 3 after inoculation were accompanied by prolific mycelium growth. In contrast, *N*-isobutyl decanamide treatment inhibited fungal growth over leaf surfaces, as compared to the control (Figure 6C). On the basis of these findings, it can be concluded that *N*-isobutyl decanamide treatment renders enhanced resistance to *B. cinerea* in *Arabidopsis* leaves.

To determinate if the reduced leaf damage and fungal growth inhibition observed in alkamide-treated leaves could be the result of a direct toxic effect of *N*-isobutyl decanamide on the fungal pathogen tested, we evaluated the antifungal activity of *N*-isobutyl decanamide on *B. cinerea* mycelial growth by inoculating mycelia disks on Petri plates containing PDA media supplemented with the solvent or with increasing concentrations of alkamide. Although 120 μM *N*-isobutyl decanamide inhibited mycelium growth by approximately 15%, any lower concentration had no significant effect (Figure S4). Indicating that plant defense responses elicited by *N*-isobutyl decanamide, and no an antifungal activity were responsible of pathogen proliferation over inoculated leaves.

JA signaling is required for the *N*-isobutyl decanamide-induced resistance to *B. cinerea*

To test whether JA signaling is involved in the *N*-isobutyl decanamide-induced increased resistance to necrotrophic fungal infection of *Arabidopsis* leaves, we evaluated the responses of *Arabidopsis* JA-related mutants *jasmonic acid resistant1* (*jar1*), *coronatine insensitive1* (*coi1-1*), a mutant defective at the *MITOGEN-ACTIVATED PROTEIN KINASE6* (*MPK6*) locus, which has been found to be critical in defense responses to *B. cinerea* [22], and the SA-related mutant *enhanced disease symptoms16* (*eds16/sid2-1*). Fully developed leaves from Col-0 wild-type (WT) and mutant plants were pre-

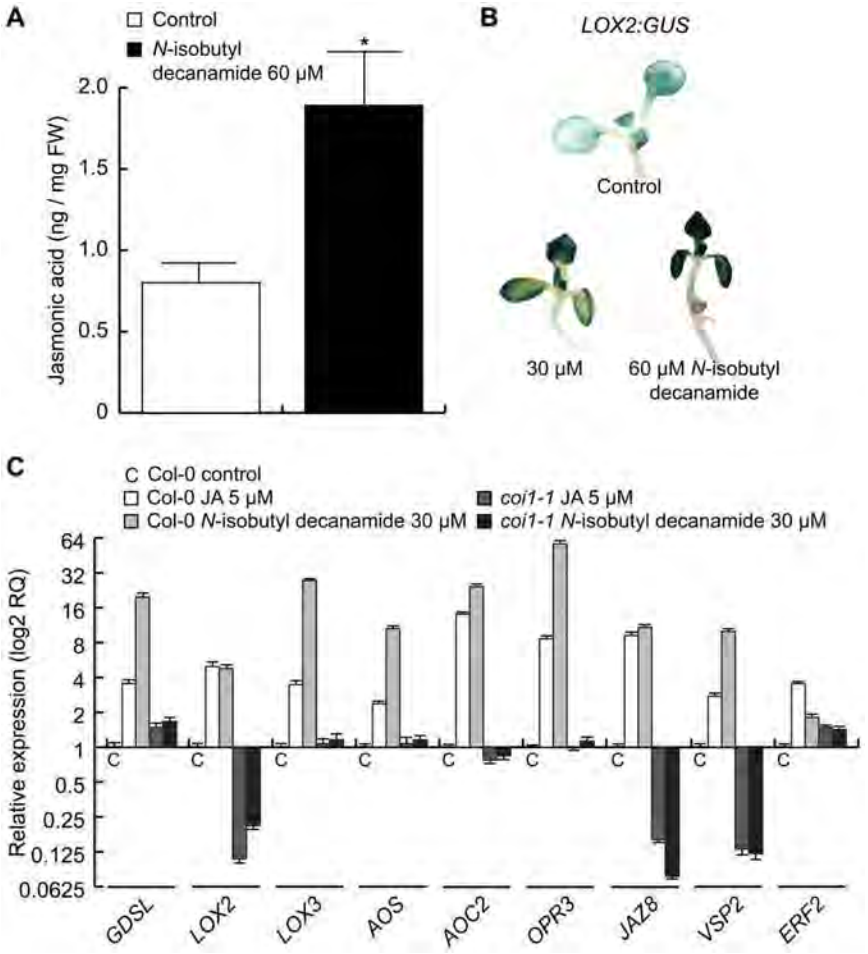

Figure 5. Levels of JA and their corresponding transcripts are enhanced by *N*-isobutyl decanamide. *N*-isobutyl decanamide-dependent accumulation of JA was determined by GC-MS from three biological replicate samples (A), data are means of three independent experiments ± SD, asterisks denote a significant difference from control seedlings ($P \leq 0.05$). Transgenic *Arabidopsis* seedlings expressing *GUS* under the regulation of the JA-induced *LOX2* promoter (*LOX2:GUS*) were grown for 7 days on solidified medium supplied with the solvent (control) or with 30 and 60 µM *N*-isobutyl decanamide, and then stained for *GUS* expression (B). Quantitative real-time PCR (qRT-PCR) analysis of nine JA-responsive genes using C_T value of *ACT2/7* as internal expression reference (C). Relative expression values were normalized with endogenous levels from each transcript in Col-0 control seedlings. Bars represent ± SE from three independent biological replicates, and there were four technical replicates for qRT-PCR assay.
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incubated for 24 hours with 30 µM *N*-isobutyl decanamide or with the solvent as control and then, inoculated with a droplet of 5×10^5 spores/ml *B. cinerea* spores on the surface. WT and *eds16* leaves showed decreased disease symptoms when treated with *N*-isobutyl decanamide, unlikely *jar1*, *mpk6* and *coi1-1* leaves, which presented symptoms similar to solvent-treated controls, and, therefore, were not responsive to the alkamide-activated resistance (Figure 7A & B). Similarly, fungal growth, assessed by quantitative PCR amplification of *Actin A* DNA of *B. cinerea* [53], was significantly enhanced in the *jar1*, *mpk6* and *coi1-1* mutant leaves, but restricted in the WT and *eds16* with the *N*-isobutyl decanamide treatment (Figure 7C).

In order to qualitatively analyze the damage caused by the pathogen, infection was estimated by recording a range of severity of disease symptoms (from no symptoms, to severe tissue maceration). For this purpose, we placed leaves from WT and mutant plants on 0.7% agar plates supplied with solvent or *N*-isobutyl decanamide for 24 hours. Then, leaves were immersed into *B. cinerea* spores solution and transferred to *N*-isobutyl decanamide-free agar plates. As shown in Figure 7D, three d.a.i., lesion severity was higher in the *jar1*, *mpk6* and *coi1-1* mutants than in WT leaves, even when they received *N*-isobutyl decanamide pre-treatment. As expected in the WT, disease symptoms decreased when the alkamide was supplied. Unlike JA-related mutants, *eds16/sid2-1* leaves displayed reduced injuries with *N*-isobutyl decanamide pretreatment, as compared to those observed for the corresponding untreated controls (Figure 7D).

All together, these results show that *N*-isobutyl decanamide-induced resistance to *B. cinerea* in *Arabidopsis* WT leaves is due to the induction of defense programs that require an intact JA signaling pathway.

Discussion

Lipids, besides being important structural molecules in living systems, function as modulators of a multitude of signal transduction pathways evoked by environmental and developmental stimuli. Alkamides belong to a novel class of lipid signals that regulate morphological processes in plants [23]. Recent findings provided evidence of a widespread distribution of structurally related lipid amide signals in evolutionary distant organisms, including the animal, fungal and plant-produced NAEs and the bacterial quorum-sensing AHL regulators [54,33].

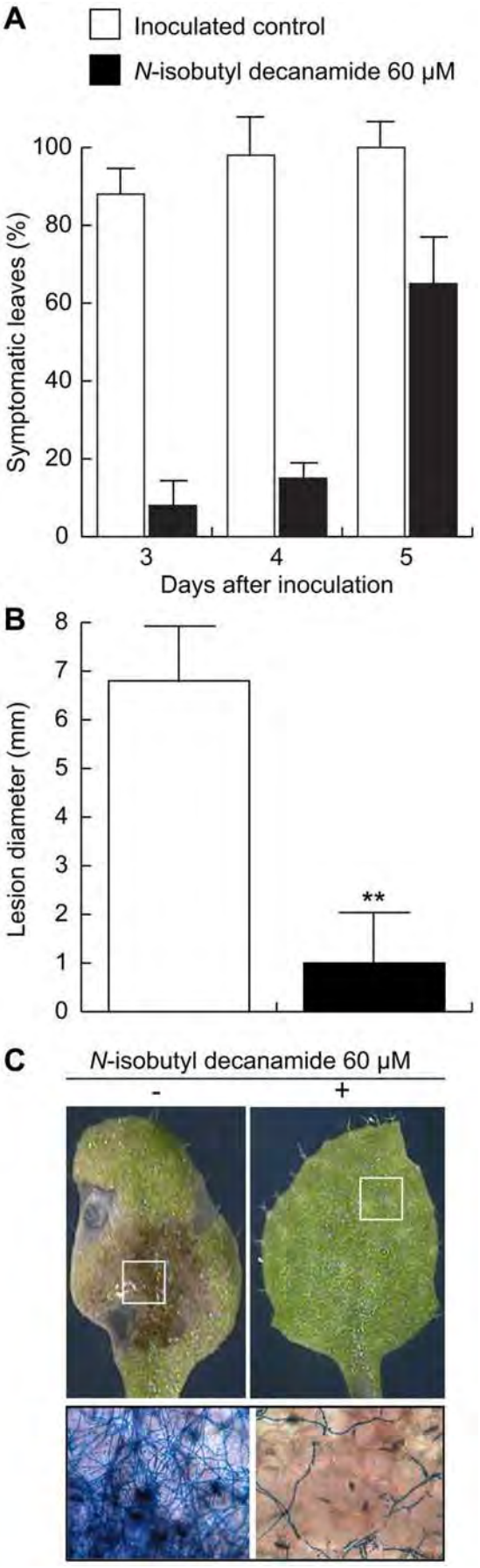

Figure 6. *N*-isobutyl decanamide confers protection against *Botrytis cinerea* attack. Leaves from 20 day-old plants grown in soil were pre-incubated 24 h on solvent (control, white squares), or 30 µM *N*-isobutyl decanamide containing plates (black squares), transferred to decanamide-free plates and then inoculated with a 10 µl droplet of *B. cinerea* spores (5×10^5 conidiospores/ml). The percentage of leaves with necrotic symptoms at 3, 4 and 5 days after inoculation was determined (A). Bars mean ± SE of 30 inoculated leaves from two independent experiments. Tissue damage caused by *B. cinerea* was measured at 5 days after inoculation (B). Data points represent average lesion size ± SE from 30 independent leaves, asterisks denote a significant difference from control leaves ($P\leq0.05$) as determined by *t* test. Representative inoculated leaves at 5 days after inoculation were imaged (C, top panels) and trypan blue-stained, inoculation sites are shown (C, bottom panels).
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The central idea of this work was to explore the transcriptional responses in *Arabidopsis* to alkamides and ascertain its relevance in activating long-term defense responses. Toward this goal, we performed a global transcription profile of *Arabidopsis* response to *N*-isobutyl decanamide (Figure S1).

Our results establish that exogenous application of *N*-isobutyl decanamide triggers profound physiological changes in *Arabidopsis*, with activation of developmental and defense- and stress-related genes (Table S1; Figures 2 & 3). Interestingly, treatment with *N*-isobutyl decanamide resulted in an increase in the endogenous levels of JA (Figure 5), a lipid phytohormone known to be central in activation of plant defense responses to a range of biotic challengers, including herbivores, insects, necrotrophic fungi and oomycetes [55,56,57]. These results are consistent with previous reports showing that NAEs induce LOXs activity and JA accumulation [58,59]. NAEs comprise a group of bioactive signaling lipids naturally present from fungi to plants to mammals that share structural and functional relationship with alkamides. In mammals, anandamide (NAE 20:4) acts as an endogenous ligand for cannabinoid receptors and plays different physiological roles including the modulation of neurotransmission in the central nervous system [60], synchronization of embryo development [61] and vasodilation [62]. Interestingly, arachidonic acid (AA, 20:4) a precursor of anandamide in mammals has been shown to posses important signaling roles in plant stress and defense networks trough production of JA and the activation of JA-dependent transcripts [8]. These results indicate that lipid signals biochemically related to alkamides and NAEs could regulate the same or similar signaling pathways.

Previously, Teaster and coworkers [63] conducted microarray analyses to identify transcriptional targets of plant NAE 12:0 in 4 d-old *Arabidopsis* seedlings. We found a set of 171 differentially expressed genes by *N*-isobutyl decanamide, whose expression was also reported as regulated by NAEs, including ABA-responsive genes (At3g02480, At5g53820) and germin-like genes (At5g38910, At5g39550, At5g39180, At5g39110, At5g39190) (Table S1). Our results indicate that although important differences in plant age, concentrations of compounds and time of exposure already exists when comparing our expression analysis results with those reported for NAE 12:0, common genes were found to be up-regulated by the two compounds, indicating commonalities in the transcriptional responses elicited by NAE 12:0 and *N*-isobutyl decanamide. One of the first indications that plant fatty acid amides indeed participate in plant-pathogen interactions was the observation that NAEs accumulated in the growth media of tobacco suspension cells and leaves after application of the fungal elicitor xylanase. Indeed, exogenous NAE application triggered the expression of the *PHENYLALANINE AMMONIA LYASE* gene (*PAL*), which has been implicated in plant defense against

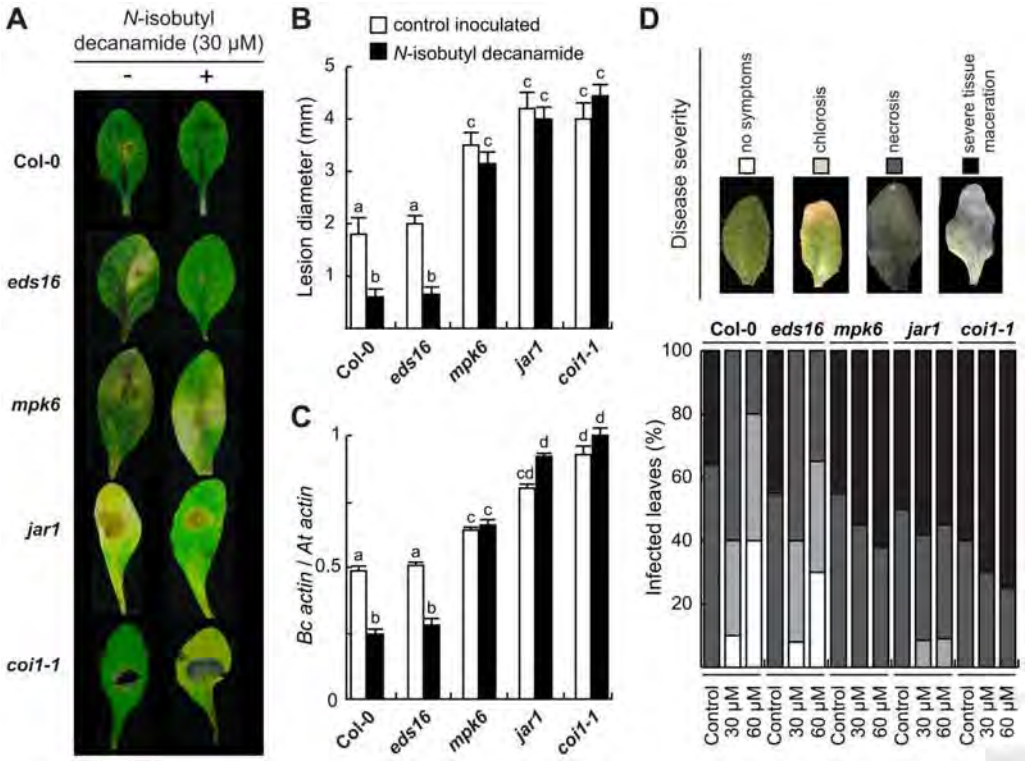

Figure 7. Effect of JA-related mutations on disease resistance response induced by *N*-isobutyl decanamide. (A–C) Disease symptoms on detached 20 day-old leaves at 4 days after drop inoculated with a 5 µl droplet as described in the legend for Figure 6 from wild-type (Col-0) plants, JA-related mutants *jar1*, *coi1-1* and *mpk6*, and the SA-deficient mutant *eds16/sid2-1*. Images show necrotic lesions (A), mean lesion size (B) and, in (C), fungal growth. The data show the qPCR amplification of *B. cinerea ActinA* relative to the *Arabidopsis ACT2/7* gene. (D) Leaves from 20 day-old wild-type and mutant plants were pre-incubated 24 h on solvent (control), 30 or 60 µM *N*-isobutyl decanamide containing plates, dipped into a *B. cinerea* inoculum of 5×10^5 spores/ml, transferred to decanamide-free plates and then incubated. Disease symptoms were scored 3 days post-inoculation, graphical representation of disease rating (upper panel) caused in leaves was determined as percentage of leaves showing no symptoms (white bars), chlorosis (grey bars), necrosis (dark grey bars), or severe tissue maceration (black bars). Data values represent one of two independent experiments that gave similar results, 15 leaves were employed per treatment in each assay.
doi:10.1371/journal.pone.0027251.g007

pathogens [64,27]. Moreover, the ectopic overexpression of FAAH, a NAE-metabolizing enzyme, renders *Arabidopsis* seedlings more susceptible to both host and non-host bacterial pathogens [28,65]. However, it remains to be determined whether NAE application can actually confer improved resistance of plants to pathogens.

Upon *N*-isobutyl decanamide treatment, several JA-related genes such as *PDFs* (At2g43510, At5g44420, At1g75830, At2g26010), *VSP2* (At5g24770), *JAZ10* and *JAZ8* (At5g13210, At1g30135), were induced, with a maximum at day 7 after alkamide treatment (Figure 4), which correlates with the up-regulation of several genes encoding enzymes involved in JA biosynthesis and with a two-fold increased JA level (Figure 4). Similar long-term gene induction patterns and JA increase have been described in *Medicago truncatula* plants inoculated with the pathogenic soilborne fungus *Phymatotrichopsis omnivora*, which has a very broad host range and infects almost 2,000 dicotyledonous species. Transcriptomic analysis of this interaction provided evidence that JA production is sustained and prolonged, inducing expression of genes encoding for LOXs, AOC2, OPR3, OPR5, OPR12, and wound-inducible serine proteinase inhibitors (PII) at 3 and 5 days after inoculation [66]. We show that *N*-isobutyl decanamide treatment conferred protection against *B. cinerea* attack to *Arabidopsis* leaves (Figure 6). In contrast to wild-type and the SA-related mutant *eds16/sid2-1*, all three *jar1*, *coi1* and *mpk6* *Arabidopsis* mutants, whose gene products are involved in JA sensitivity and signaling, failed to resist *B. cinerea* attack when *N*-isobutyl decanamide was supplied (Figure 7), suggesting that *N*-isobutyl decanamide-conferred resistance to necrotrophic fungi requires an intact JA signaling pathway.

Responses of plants to necrotrophic pathogens involve multiple intermediates in signal transduction and anti-microbial responses, which includes nitric oxide (NO) and reactive oxygen species (ROS) [67]. The production and accumulation of reactive oxygen species ROS, primarily superoxide (O_2-) and hydrogen peroxide (H_2O_2), during the course of a plant-pathogen interaction has long been recognized. Evidence suggests that the oxidative burst and the cognate redox signaling engaged subsequently, may play a central role in the integration of a diverse array of plant defense responses [68,69]. One well studied effect of oxidative burst is the induction of hypersensitive response (HR) mechanism, where the tissue at the infection site dies and in turn confines the pathogen growth preventing its spreading [70]. In our microarray analysis, we identified several potential components of the ROS signaling pathway, including scavenging enzymes catalases and ascorbate peroxidases, as well as at least 20 cytochrome P450 genes, including the antifungal gene *CYP71B15/PAD3*, which plays a key role in camalexin production and resistance against necrotrophic pathogens [71] (Figure 4B & Table S1). Activation of these genes correlated with accumulation of hydrogen peroxide (H_2O_2) and NO in *N*-isobutyl decanamide-treated leaves (Figure 3). We propose that alkamides might influence plant-pathogen interac-

tions by affecting the level of other lipids or by modulating the levels of second messengers involved in signal transduction to these lipids such as Ca^{2+}, NO, and/or ROS. In a developmental context, the relationship between NO and alkamides pathways in *Arabidopsis* was recently investigated, mitotic activation of pericycle cells from seedlings roots induced by *N*-isobutyl decanamide occurred in parallel and in a dependent way to NO synthesis [45]. However, whether NO mediates the defense responses to alkamides remains to be clarified.

Our previous research revealed a genetic interaction of alkamides and senescence responses mediated by the *DRR1* locus in *Arabidopsis* [32]. Leaf senescence is a metabolic active process controlled by a genetic program [72,73]. Interestingly, ultrastructural changes in senescing cells are accompanied by production of several metabolites that may influence interactions with other organisms. For example, antimicrobial compounds often accumulate in senescing tissues, preventing diseases [74]. In agreement with this, many senescence genes are transcriptionally up-regulated by *N*-isobutyl decanamide, i.e. *PR* genes, *SAG* genes (At2g29350, At4g17670, At5g47060), a member of TCP family (At5g40070), and JA-related genes. It is tempting to speculate that *N*-isobutyl decanamide can be recognized as a senescence-induced signal, thus influencing developmental and defense programs involving JA signaling and possibly other additional signaling/metabolic pathways regulated by NO, ROS and/or MAPK messengers.

N-isobutyl decanamide share structural similarity to *N*-decanoyl homoserine lactone (C:10 AHL) [25], a member of the bacterial quorum-sensing signals, which have been found to alter root development and activate defense responses in different plant species [30,29,25]. An interesting hypothesis is that small lipid signaling based on plant fatty acid amides and/or AHLs might be part of an ancestral inter-kingdom communication system between plants and their associated bacteria. Our data thus expand the repertoire of signaling lipid molecules known to trigger plant defenses and provide evidence that alkamides interact with the JA pathway. The use of alkamides and bacterially produced fatty amides in pathogen resistance by acting as defense elicitors in plants shows great potential towards application of these compounds to combat pathogen pests.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 was used for all experiments unless indicated otherwise. Col-0, transgenic *LOX2:GUS* [75] and *PR1:GUS*, and mutants *jar1* [16], *mpk6* [76], *coi1-1* [77] and *eds16/sid2-1* [78] seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded growth of the hypocotyl into the air. For plant growth, we used a plant growth cabinet (Percival Scientific AR95L, Perry, IA), with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 300 μmol/m-^{2}/s-^{1} and temperature of 22°C. After grown for 6 days, plants were transferred to control or *N*-isobutyl decanamide containing solid MS medium for different times.

Homozygous *coi1-1* seedlings were selected by screening a heterozygous population in agar solidified MS medium supplied with 5 μM JA (Sigma Chemical Co., St. Louis), seedlings resistant to root inhibition were transferred to soil baskets and leaves from 20 d-old were detached for *in vitro* pathogenicity assays.

Synthesis of *N*-isobutyl decanamide

N-isobutyl-decanamide was obtained by catalytic reduction of affinin, the most abundant alkamide present in *Heliopsis longipes* (Gray) Blake (Asteraceae) roots as described before [23].

Experimental design and microarray platform

For microarray analyses a dye balanced modified loop design was implemented. Four biological replicates representing each sampling point were obtained by pooling in a 1:1 proportion shoot and root purified RNA from 120 randomly chosen seedlings. This experiment involved a total of sixteen sets of microarray hybridizations, including direct and dye swap comparisons between treatments as well as across time points for the same treatment. This design allowed us to determine differences in gene expression between *N*-isobutyl decanamide-treated and control seedlings, and whether the differences were time dependent. The *Arabidopsis* Oligonucleotide Array version 3.0 from The Arizona University was used to carry out this study. Array annotation and composition are available at http://ag.arizona.edu/microarray. RNA isolation, fluorescent labeling of probes, slide hybridization and washing were performed as described previously in [79]. Slides were scanned with an Axon GenePix 4100 scanner at a resolution of 10 μm adjusting the laser and gain parameters to obtain similar levels of fluorescence intensity in both channels. Spot intensities were quantified using Axon GenePix Pro 5.1 image analysis software.

All microarray data is MIAME compliant and the raw data has been deposited in the Gene Expression Omnibus database (GEO), accession number GSE12107, as detailed on the MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html.

Normalization and data analysis

Raw data were imported into the R 2.2.1 software (http://www.R-project.org). Background correction was done using the method "substract" whereas normalization of the signal intensities within slides was carried out using the "printtiploess" method [80] using the LIMMA package (www.bioconductor.org). Normalized data were log2 transformed and then fitted into mixed model ANOVAs [81] using the Mixed procedure (SAS 9.0 software, SAS Institute Inc., Cary, NC, USA) with two sequenced linear models considering as fixed effects the dye, time, *N*-isobutyl-decanamide treatment and time × *N*-isobutyl-decanamide treatment. Array and array × dye were considered as random effects. The type 3 F-tests and p-values of the time × *N*-isobutyl-decanamide treatment and *N*-isobutyl-decanamide treatment were also carried out. Model terms were explored and significance levels for those terms were adjusted for by the False Discovery Rate (FDR) method [82]. Estimates of the expression differences were calculated using the mixed model. Based on these statistical analyses, the spots with tests with an FDR less or equal to 5% and with changes in signal intensity between *N*-isobutyl decanamide treatment and control seedlings of 2.0-fold or higher were considered as differentially expressed.

Expression analysis by qRT-PCR

Total RNAs were isolated from *Arabidopsis* plants using TRIzol reagent (Invitrogen). Primer design (Tm, 60–65°C) was performed using Primer Express Software, Version 3 (Applied Biosystems); full sequences from each primer are shown in Table S3. cDNA templates for PCR amplification were prepared from all samples by using reverse specific primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Each reaction contained cDNA template from

~30 µg total RNA, 1× SYBR Green PCR Master Mix (Applied Biosystems) and 500 nM forward and reverse primers. Real-time PCR was performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems) under the following thermal cycling conditions: 10 min at 95°C followed by a total of 40 cycles of 30 s at 95°C, 30 s at 60°C and 40 s at 72°C. For qRT-PCR, relative transcript abundance was calculated and normalized with respect to *ACTIN2/7* to minimize variation in cDNA template levels, with the solvent-treated (control) and control Col-0 samples acting as calibrators (for microarrays validation assay for and JA responsive genes assay respectively). Data shown represent mean values obtained from at least three independent amplification reactions; the SE of the C_Ts averaged 0.1, demonstrating the high precision of the assays. All calculations and analyses were performed using 7500 Software v2.0.1 (Applied biosystems) and the $2^{-\Delta\Delta CT}$ method [83]. Amplification efficiency for the primer sets was determined by amplification of cDNA dilution series (1:5). The values obtained not change significantly between different cDNA smaples, and were always higher than 0.90. Specificity of the RT-PCR products was followed by a melting curve analysis with continual fluorescence data acquisition during the 65–95°C melt.

Analysis of JA levels

250 mg of freshly harvested plant tissues were chilled in liquid nitrogen and JA extraction was performed as in [84] using dihydrojasmonate as internal standar, derivatized with chloroform/*N*,*N*-diisoprpyl-etylamine 1:1. In order to analyze the samples by GC/MS the extract was added with 10 µl of PFBr and 200 µl of cloroform: *N*,*N*-diisopropilethilamine (1:1) then incubated at 65°C for 1 h. When cooled, the solvent was evaporated to dryness and resuspended in 100 µl methanol. Samples were analyzed in a gas chromatograph (Agilent Technologies 7890A) equipped with a capillary column J&W DB-1 (60 m×250 µm×0.25 µm) coupled to a mass selective detector (Agilent 5973 Series MSD). Using an autosampler 7683B Series. 2 µl of the sample was injected in a splitless way. Operating conditions were: injector temperature 250°C; the oven temperature was programmed as: initial temperature 150°C for 3 min then increasing at the rate of 4°C per min to a final temperature of 280°C maintained for 20 min. Helium was used as carrier gas with a constant flow of 1 ml/min. The MS was set to scan from 40 to 600 uam in Synchronous SIM/Scan mode for selectively monitor the following ions for jasmonic acid derivative: 141, 181, 390, and 392. MS temperatures were: Source 230°C, MS Quadrupole 150°C.

Microscope Analyzes

For histochemical analysis of transgenic lines *LOX2:GUS* and *PR1:GUS*, 7 d-old transgenic seedlings expressing these marker constructs were incubated at 37 °C in a GUS reaction buffer (0.5 mg/ml of 5-bromo-4-chloro-3-indolyl-B-D-glucuronide in 100 mM sodium phosphate, pH 7.0). The stained seedlings were cleared by the method of Malamy and Benfey [85]. For each treatment, at least 9 transgenic plants were analyzed. A representative plant was chosen for each treatment and photographed using the Nomarski optics on a Leica DMR microscope.

H_2O_2 production was detected by the endogenous peroxidase-dependent staining procedure using 3,3-diaminobenzidine (DAB) uptake [86]. Control, 15 and 30 µM *N*-isobutyl decanamide-treated 7 d seedlings were placed in a solution of 1 mg mL^{-1} DAB, pH 3.8, and incubated in dark for 2 h. Subsequently, were immersed in boiling 96% (v/v) ethanol for 10 min and then stored in 96% (v/v) ethanol. For each treatment, at least 9 treated-seedlings were analyzed. A representative plant was chosen for each treatment. H_2O_2 production was visualized as a reddish-brown precipitated coloration and photographed using the Nomarski optics on a Leica DMR microscope.

Nitric Oxide (NO) was monitored by incubating *Arabidopsis* seedlings with 10 µM of the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) [87] in 0.1 M Tris–HCl (pH 7.4). Treated seedlings were incubated for 2 h in the dark, and washed three times for 20 min with fresh buffer. Fluorescence signals from at least 9 treated and control leaves were detected using a confocal laser scanning microscope (model BX50, Olympus), and monitored with an argon blue laser with an excitation line from 488 to 568 nm and an emission window from 585 to 610 nm.

Fungal growth and plant inoculation

Pathogenesis assays were modified from [88]. *Botrytis cinerea* was grown on agar PDA medium (PhytoTechnology) for 7–12 days at 22° C in darkness. Spores were collected with distilled water. Col-0 superficially sterilized seeds were germinated and grown in MS-agar medium into 100 ml flasks with transparent lid. At 20 days after germination, rosette leaves were placed in Petri dishes with 60 µM of *N*-isobutyl decanamide containing medium or medium supplied with the solvent. Inoculation was performed by placing a 5 µl drop of a suspension of 5×10^5 conidiospores/ml on the surface of leaves. The samples were incubated at 22°C and analyzed at a further 3, 4 and 5 d period after inoculation. Susceptibility was evaluated by microscopic observation of necrotic symptoms under a dissecting microscope (Leica MZ6) connected to a digital color camera (Samsung SCC-131A). The percent of necrotic leaves was scored for 30 independent inoculated leaves. The disease symptoms on inoculated leaves and fungal growth over leaves was estimated by trypan blue staining and further cleared with chloral hydrate and the extension of necrotic lesions (lesion diameter) measured at 4 d after inoculation. For mutant inoculation, leaves from soil grown adult plants were incubated in agar solution supplied with solvent (ethanol) or *N*-isobutyl decanamide during 24 h prior to inoculation by leaves immersion into solution of 5×10^5 conidiospores/ml.

Supporting Information

Figure S1 Experimental design for microarray analysis. 6 day-old *Arabidopsis* Col-0 seedlings were grown on *N*-isobutyl decanamide-free medium and then transferred to control medium (A) supplied with the solvent, or to 60 µM *N*-isobutyl decanamide-containing medium (B). Pictures were taken 14 days after transfer (d.a.t.). Modified loop design including 4 independent replicates evaluated at 1, 3, 7, and 14 d.a.t. (C). A total of 16 slides were employed. Each replicate was conformed by at least 120 transferred seedlings, which were harvested from four independent plates.
(TIF)

Figure S2 Validation of microarray results via qRT-PCR. Quantitative real-time PCR analysis was performed for 15 genes in *Arabidopsis* (Col-0) seedlings, under the same conditions used for microarray analysis (1, 3, 7 and 14 days of treatment with 60 µM *N*-isobutyl decanamide). Fold-change (control to *N*-isobutyl-decanamide) expression for the indicated selected genes in a log2 scale is shown. Expression ratios obtained by microarray experiments (A). Estimates of the differences of expression levels were calculated using the mixed model as described in methods. Expression ratios obtained by qRT-PCR (B). RQ (relative

quantification number) was obtained from the equation $2^{\Delta\Delta C_T}$ where $\Delta\Delta C_T$ represents ΔC_T(control) - ΔC_T(*N*-isobutyl decanamide 60 μM). Each C_T was previously normalized using the expression levels of *ACT2/7* as internal reference. Expression levels were obtained from four independent replicates, every set of oligonucleotides had an efficiency greater than 99%. Standar deviations were less than 0.1 arbitrary units.
(TIF)

Figure S3 Local induction of defense genes by *N*-isobutyl decanamide on detached leaves. Leaves from 20 day-old transgenic *LOX2:GUS* or WT (Col-0) plants grown in soil were detached and incubated 24 h on solvent (control, white squares), or 30 μM *N*-isobutyl decanamide containing plates (black squares), transferred to decanamide-free plates and then analized. (A) Dose-response assay with leaves from transgenic *Arabidopsis* line carrying *LOX2:GUS* were stained for *GUS* expression 24 h after transference to agar plates. (B) qRT-PCR analysis of the JA-responsive genes *OPR3* and *VSP2*, and the camalexin biosynthetic marker *PAD3* using C_T value of *ACT2/7* as internal expression reference. Relative expression values were normalized with endogenous levels from each transcript in Col-0 control seedlings. Bars represent ± SE from three independent biological replicates from 30 leaves each one, and from four technical replicates for the assay.
(TIF)

Figure S4 Effect of *N*-isobutyl decanamide on *Botrytis cinerea* mycelial growth. *B. cinerea* mycelium excised from a solid culture in Petri dishes was transferred to potato dextrose agar dishes supplemented with *N*-isobutyl decanamide at the concentrations indicated. Radial growth of the fungus was measured 24, 48 and 72 h after inoculation (A). Data means average radial growth from three independent samples ± SD; no statistical differences were found at any concentration tested. Mycelial growth at 72 h after inoculation on solvent-containing media (Control) and 120 μM *N*-isobutyl decanamide-supplied media (B). Fungicide Techto 60 was employed at 1 mg/ml as fungal growth inhibition control to compare with the highest concentration of *N*-isobutyl decanamide in divided Petri dishes (C).
(TIF)

Table S1 Full list of genes differentially expressed by *N*-isobutyl decanamide in *Arabidopsis*.
(PDF)

Table S2 Functional categories over-represented in *N*-isobutyl decanamide responsive genes.
(PDF)

Table S3 Sequences of oligonucleotides used as PCR primers for quantitative expression analysis.
(PDF)

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Author Contributions

Conceived and designed the experiments: JLB AMB CCV EIL LHE. Performed the experiments: AMB EIL ERC CCV JRG. Analyzed the data: AMB LHE JLB EIL CCV AGG ERC. Contributed reagents/materials/analysis tools: LHE JLB AGG JMT. Wrote the paper: AMB LHE JLB.

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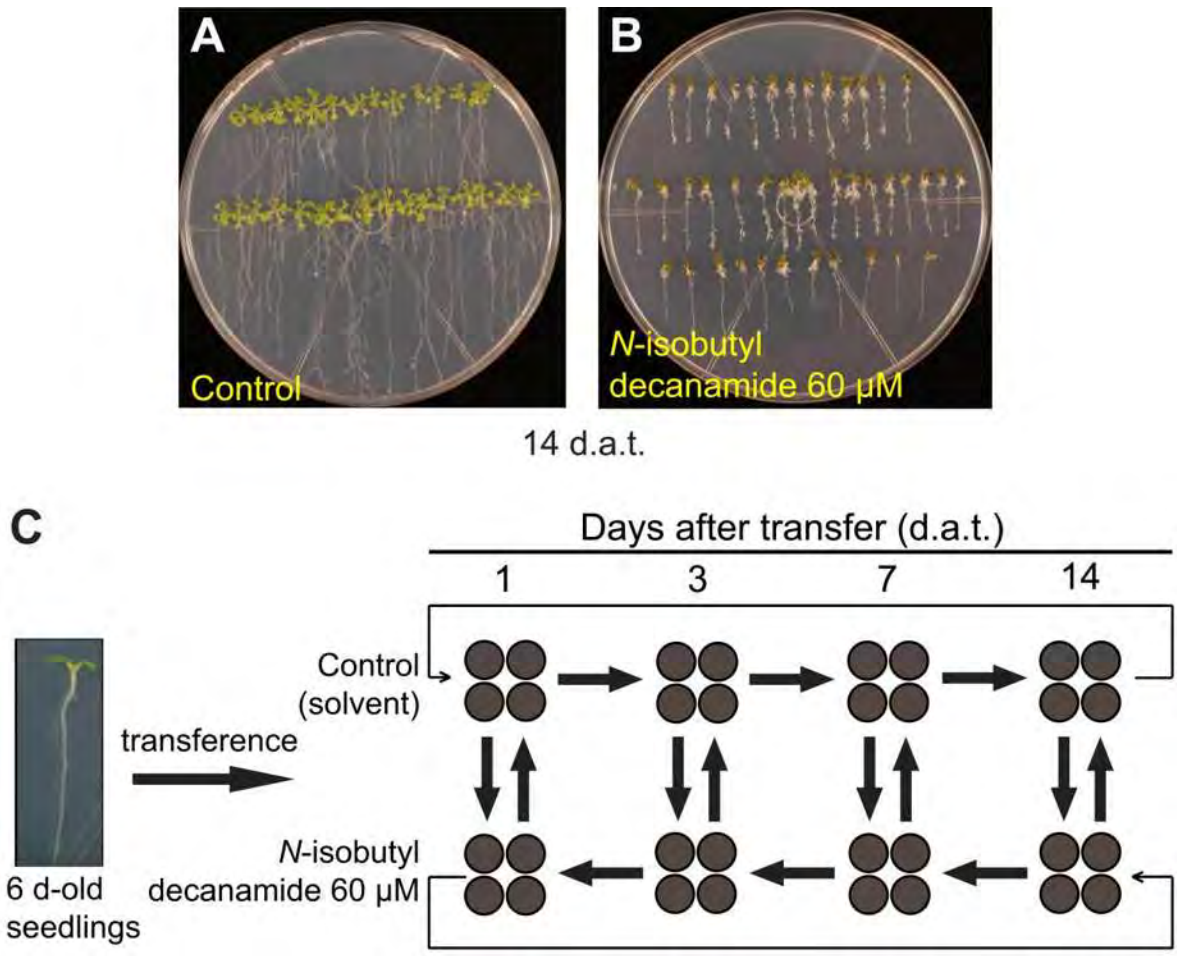
A
Control
B
N-isobutyl
decanamide 60 µM
14 d.a.t.
C
Days after transfer (d.a.t.)
1
3
7
14
Control
(solvent)
transference
N-isobutyl
decanamide 60 µM
6 d-old
seedlings

Figure S1

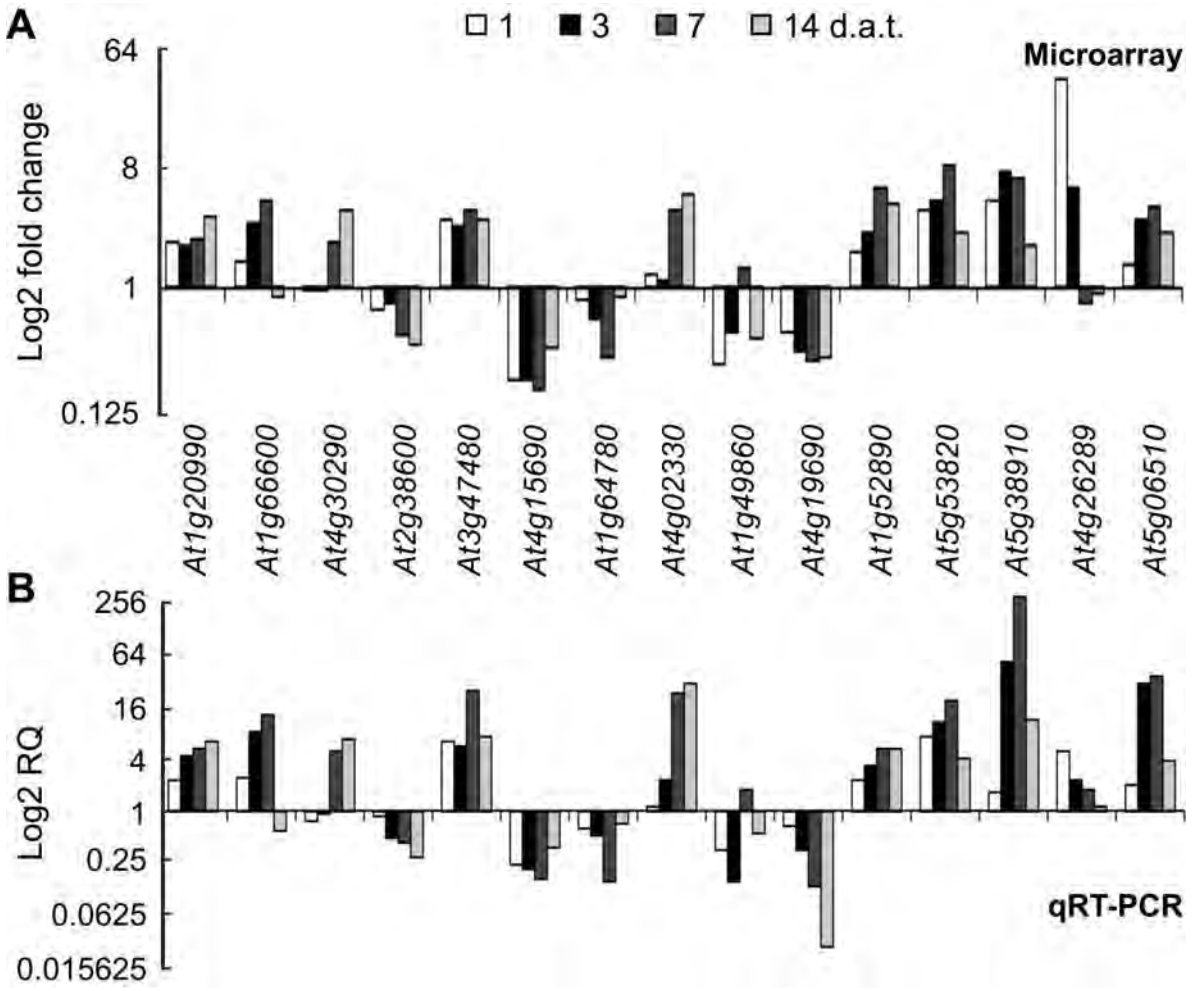
A
1
3
7
14 d.a.t.
Microarray
Log2 fold change
64
8
1
0.125
At1g20990
At1g66600
At4g30290
At2g38600
At3g47480
At4g15690
At1g64780
At4g02330
At1g49860
At4g19690
At1g52890
At5g53820
At5g38910
At4g26289
At5g06510
B
Log2 RQ
256
64
16
4
1
0.25
0.0625
0.015625
qRT-PCR

Figure S2

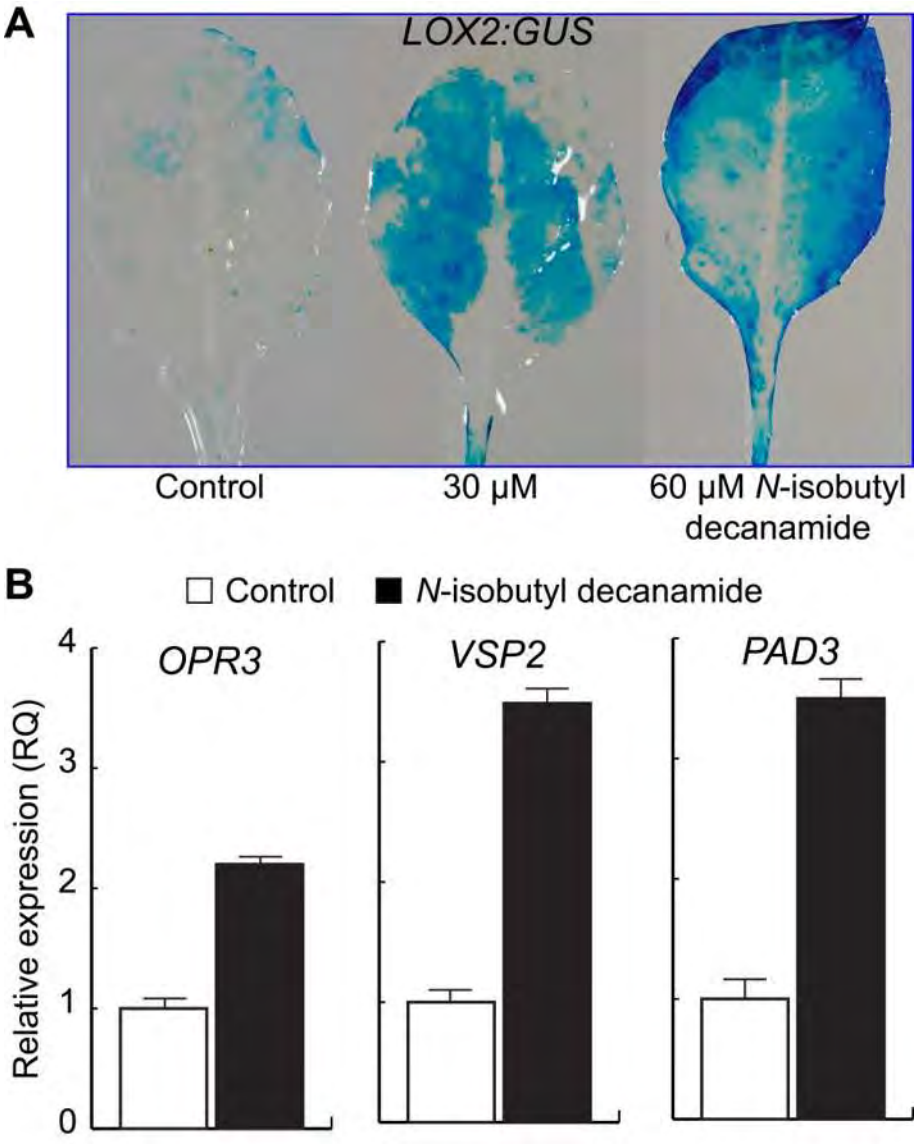
A
LOX2:GUS
Control
30 µM
60 µM N-isobutyl decanamide
B
Control
N-isobutyl decanamide
OPR3
VSP2
PAD3
Relative expression (RQ)
0
1
2
3
4

Figure S3

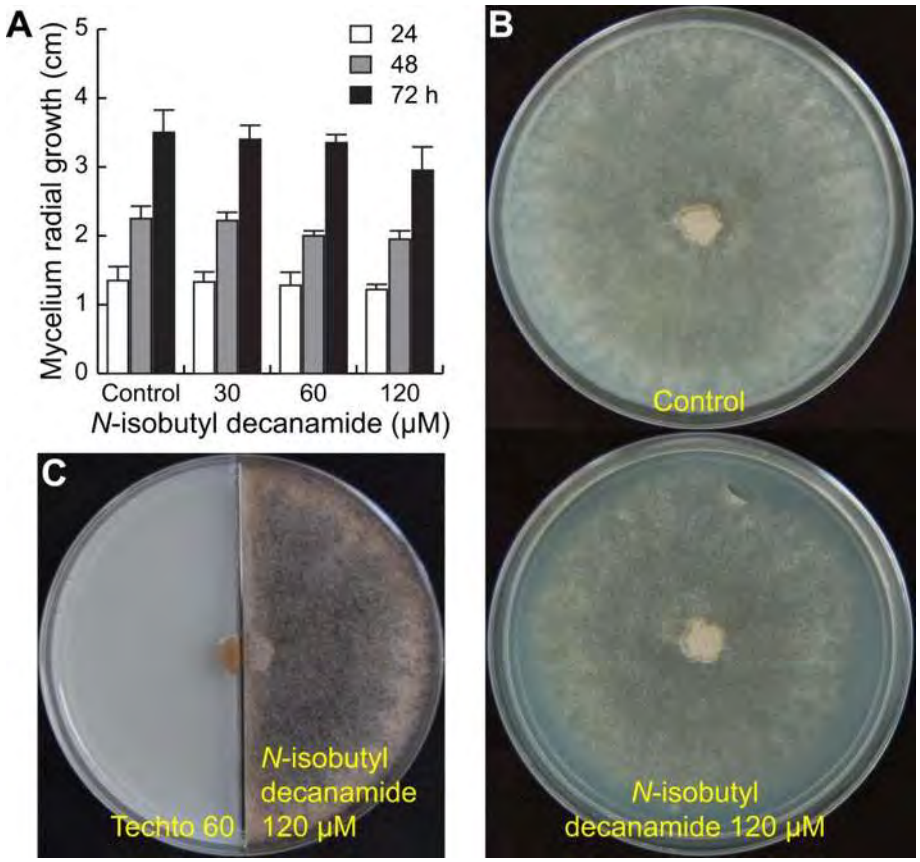
A
Mycelium radial growth (cm)
0
1
2
3
4
5
24
48
72 h
Control
30
60
120
N-isobutyl decanamide (μM)
B
Control
C
Techto 60
N-isobutyl decanamide 120 μM
N-isobutyl decanamide 120 μM

Figure S4

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RESEARCH PAPER

Arabidopsis thaliana mitogen-activated protein kinase 6 is involved in seed formation and modulation of primary and lateral root development

J. S. López-Bucio[1], J. G. Dubrovsky[1], J. Raya-González[2], Y. Ugartechea-Chirino[3], J. López-Bucio[2], L. A. de Luna-Valdez[1], M. Ramos-Vega[1], P. León[1] and A. A. Guevara-García[1,*]

[1] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, 62250 Cuernavaca, Morelos, México
[2] Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio A-1´, CP 58030 Morelia, Michoacán, México
[3] Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 3er circuito exterior SN, Del. Coyoacán, México D.F. 04510, México

* To whom correspondence should be addressed. E-mail: aguevara@ibt.unam.mx

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Abstract

Mitogen-activated protein kinase (MAPKs) cascades are signal transduction modules highly conserved in all eukaryotes regulating various aspects of plant biology, including stress responses and developmental programmes. In this study, we characterized the role of MAPK 6 (MPK6) in *Arabidopsis* embryo development and in post-embryonic root system architecture. We found that the *mpk6* mutation caused altered embryo development giving rise to three seed phenotypes that, post-germination, correlated with alterations in root architecture. In the smaller seed class, mutant seedlings failed to develop the primary root, possibly as a result of an earlier defect in the division of the hypophysis cell during embryo development, but they had the capacity to develop adventitious roots to complete their life cycle. In the larger class, the MPK6 loss of function did not cause any evident alteration in seed morphology, but the embryo and the mature seed were bigger than the wild type. Seedlings developed from these bigger seeds were characterized by a primary root longer than that of the wild type, accompanied by significantly increased lateral root initiation and more and longer root hairs. Apparently, the increment in primary root growth resulted from an enhanced cell production and cell elongation. Our data demonstrated that MPK6 plays an important role during embryo development and acts as a repressor of primary and lateral root development.

Key words: *Arabidopsis*, embryo development, MAP kinases, MPK6, plant signalling, root development.

Introduction

Mitogen-activated protein kinase (MAPK) cascades are signal transduction modules that are highly conserved in eukaryotes (Zhang *et al.*, 2006). A MAPK module consists of at least three kinases: a MPKKK, a MPKK, and a MPK, which activate downstream targets by phosphorylation. The last kinase of the module, a MPK, is able to phosphorylate several substrates, including transcription factors, to regulate gene expression (Andreasson and Ellis, 2009). MAPKs are known regulators of biotic and abiotic stress responses, hormone perception, and developmental programmes (Colcombet and Hirt, 2008; Suarez-Rodriguez *et al.*, 2010).

Abbreviations: CPR, cell production rate; DAG, days after germination; IAA, indole-3-acetic acid; LR, lateral root; LRP, lateral root primordium; MAPK, mitogen-activated protein kinase; MPK6, *Arabidopsis thaliana* mitogen-activated protein kinase 6; NO, nitric oxide; PD, proliferation domain; PR, primary root; RAM, root apical meristem; RH, root hair; TD, transition domain.

The *Arabidopsis* genome encodes 20 different MPKs (MAPK Group, 2002), from which MPK3, MPK4, and MPK6 play important roles both in stress and developmental responses (Colcombet and Hirt, 2008). In particular, MPK6 has been found to participate in bacterial and fungal resistance (Nuhse *et al.*, 2000; Asai *et al.*, 2002; Menke *et al.*, 2004; Wan *et al.*, 2004; Zhou *et al.*, 2004; Zhang *et al.*, 2007), in mutualistic interactions (Vadassery *et al.*, 2009), in priming of stress (Beckers *et al.*, 2009), and in regulation of plant architecture (Bush and Krysan, 2007; Müller *et al.*, 2010).

Functional redundancy is common among MAPKs. Particularly, MPK3 and MPK6 participate in biotic and abiotic stress resistance as well as in developmental processes (Lee and Ellis, 2007; Hord *et al.*, 2008; Lampard *et al.*, 2009; Liu *et al.*, 2010). MPK4/MPK6 and even MPK3/MPK4/MPK6 have been shown to act redundantly in osmotic, touch, wounding, and defence responses (Ichimura *et al.*, 2000; Droillard *et al.*, 2004; Meszaros *et al.*, 2006; Brader *et al.*, 2007). MPKs are proposed to act through common downstream targets and upstream activators (Feilner *et al.*, 2005; Merkouropoulos *et al.*, 2008; Andreasson and Ellis, 2009; Popescu *et al.*, 2009), but the interaction of these pathways is poorly understood. The MPK6 loss-of-function mutant displays alterations in the embryo and early root development, indicating that, at least for these processes, the function of this kinase cannot be substituted by any other MPK (Bush and Krysan, 2007; Müller *et al.*, 2010; Wang *et al.*, 2010).

The first evidence demonstrating that MPK6 (and/or MPK3) is involved in embryo development was reported by Wang *et al.* (2007), who showed that *mpk3*$^{-/-}$ *mpk6*$^{-/-}$ double mutants die at the embryo stage and a viable double mutant (*mpk6*$^{-/-}$ MPK3RNAi) is developmentally arrested at the cotyledon stage. In a different study, Bush and Krysan (2007) reported that several development programmes are influenced by MPK6. In that work, it was observed that *mpk6* null mutant alleles had defects in anther and embryo development, and displayed reduced male fertility. The observed *mpk6* phenotypes display variable penetrance, probably influenced by the growth conditions. Additionally, mutations in the *MPK6* gene have been linked to protrusion of the embryo detected in about 7% of the seeds from an *mpk6* homozygous population (Bush and Krysan, 2007).

Post-embryonic root development is regulated by multiple plant hormones, nutrient availability, and environmental signals (Fukaki and Tasaka, 2009; López-Bucio *et al.*, 2003). The primary root (PR) originates from the embryo and gives rise to many lateral roots (LRs) during vegetative growth, and each of these will produce more LRs. The quantity and placement of these structures among other factors determine the root system architecture (RSA), and this in turn plays a major role in determining whether a plant will survive in a particular environment (Casimiro *et al.*, 2003; Dubrovsky and Forde, 2012). A further adaptation to increase water and nutrient absorption is performed by root hairs (RHs). These are long tubular-shaped epidermal cell extensions covering roots and increase their total absorptive surface (Datta *et al.*, 2011). Auxin (indole-3-acetic acid, IAA) is recognized as the key hormone controlling both RSA and RH development, whereas cytokinins are regulators of PR growth and LR formation (Fukaki and Tasaka, 2009; De Smet *et al.*, 2012).

Current challenges are focused on determining the signalling events for which cell identity regulators are connected with hormone receptors to coordinate stress and development responses. Recently, MPK6 was proposed to be involved in early root development, possibly through altering cell division plane control and modulating the production of second messengers, such as nitric oxide (NO) in response to hydrogen peroxide (Müller *et al.*, 2010; Wang *et al.*, 2010). It was observed that *mpk6-2* and *mpk6-3* mutants produced more and longer LRs than wild-type seedlings after application of a NO donor or H_2O_2 (Wang *et al.*, 2010). However, the hormonal responses underlying these root alterations and the role of MPK6 in these processes are still unknown. Thus, independent data support the participation of MPK6 in both shoot and root development, but no relationship has been established between embryo and root phenotypes in *mpk6* mutants, nor the impact of earlier root development alterations in the configuration of post-embryonic root architecture.

In this study, we provided physiological and molecular evidence that seedlings defective in two independent *mpk6* mutant alleles showed three distinct classes of seed phenotype, which correlated with alterations in cell division and elongation processes that affected root architecture. These alterations were independent of MPK3. These data indicate that MPK6 is an essential component of early signalling processes linked to proper embryo development and maintenance of *Arabidopsis* RSA.

Materials and methods

Additional details are available in Supplementary Methods at *JXB* online.

Plant material and growth conditions

Arabidopsis thaliana Heyhn wild-type and mutant plant lines were in the Columbia-0 (Col-0) ecotype. *MPK6* (At2g43790) T-DNA insertion lines (SALK_073907 and SALK_127507) were obtained from the Salk T-DNA collection (Alonso *et al.*, 2003) and provided by TAIR (http://arabidopsis.org). Both mutant lines were described previously as *mpk6-2* and *mpk6-3* (Liu and Zhang, 2004). The *MPK3* T-DNA insertion line (SALK_151594), was kindly donated by Dr Shuqun Zhang from Missouri University, USA (Wang *et al.*, 2007). The transgenic line *ABI4*::*GUS* (Söderman *et al.*, 2000) was kindly provided by Dr Ruth Finkelstein from the University of California, USA. This marker gene was introduced into the *mpk6-2* background by crossing homozygous plants. Surface-sterilized seeds were incubated at 4 °C for 3 d to break dormancy and then grown on agar (0.8%, w/v, Bacto™ Agar, BD Difco, Sparks, MD, USA) solidified 0.2× MS medium (Caisson, Laboratories, Noth Logan, UT, USA) with 1% (w/v) sucrose. Kinetin and IAA were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and added to the medium at the indicated concentration. Seedlings were grown on vertically oriented Petri dishes maintained in growth chambers at 21 °C under a 16:8 h light:darkness photoperiod under 105 µmol m^{-2} s^{-1} light intensity. For seed production, plants were grown in Metro-Mix 200 (Grace Sierra, Milpitas, CA, USA) in a growth room at 23 °C under a 16/8 h photoperiod and a light intensity of 230 µmol m^{-2} s^{-1}.

Embryo analysis

Wild-type and *mpk6-2* mutant embryos were processed as described previously (Ugartechea-Chirino *et al.*, 2010). Briefly, ovules were dissected from the silique and punched with a needle in order to favour contact between the embryos and the staining solutions. Embryos were fixed for 1–7 d with 50%, v/v, methanol, 10%, v/v, acetic acid. They were rinsed and incubated at room temperature for 30–45 min in 1% periodic acid. After a second rinse, they were incubated for 2 h in pseudo-Schiff's reagent (1.9 g of sodium metabisulfite in 97 ml of H_2O and 3 ml of 5 M HCl with 0.1 mg ml^{-1} of propidium iododide). Embryos were rinsed again and transferred to a drop of chloral hydrate (80 g of chloral hydrate in 30 ml of H_2O) on a microscope slide. Excess chloral hydrate was removed, and the embryos were mounted in Hoyer's solution (30 g of gum arabic, 200 g of chloral hydrate, 20 g of glycerol in 50 ml of H_2O). Mounted embryos were cleared in Hoyer's solution for at least a week before confocal imaging.

Seed size analysis

Dry seeds were measured individually using ImageJ (http://rsb.info.nih.gov/ij) with a set scale tool to establish a 1 mm reference on a micrometer image taken with a Nikon SMZ1500 stereomicroscope equipped with a digital SIGHT DS-Fi1c camera. Seed stereomicroscope images were then analysed with ImageJ using the 1 mm reference. Seed weight was obtained by weighting a batch of 100 seeds placed in Eppendorf tubes in an analytical balance.

Growth analysis

Photographs of representative seedlings were taken with an EOS REBEL XSi digital camera (Canon, Tokyo, Japan). The growth of PRs was registered using a ruler. LR number was determined counting all LRs emerged from the PRs under the Nikon SMZ1500 stereomicroscope. LR density, LR primordium (LRP) density, length of cortical cells, LR initiation index, length of root apical meristem (RAM), length of proliferation domain (PD), length of transition domain (TD), and number of cells in the PD (NC_{PD}) were determined on cleared roots as described previously (Dubrovsky *et al.*, 2009; Dubrovsky and Forde, 2012; Ivanov and Dubrovsky, 2013). Position of the most distal (rootward) LRP and the most distal LR as well the number of LRPs in the LR formation and the branching zones was determined on cleared root preparations under a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany), equipped with differential interferential contrast optics. Cortical cell length was determined for 10 cells per root on cleared preparations using an ocular micrometer. Images of RHs and etiolated seedling images were taken under a Nikon SMZ1500 stereomicroscope equipped with a digital SIGHT DS-Fi1c camera. RH density (number of RHs mm^{-1}) and RH length were determined from roots mounted in H_2O on microscope slides and observed under a Zeiss Axiovert 200M microscope. Cell production rate (CPR) was calculated with the equation $CPR = Vl_e^{-1}$, where V (μm h^{-1}) is the rate of root growth during the last 24 h before the termination of the experiment and l_e (μm) is the length of fully elongated cortical cells, whereas cell-cycle duration (T, hours) was calculated with the equation $T = (NC_{PD} \times l_e \times \ln 2)/V^{-1}$ in accordance with Ivanov and Dubrovsky (1997). This method is applicable to steady-state growing roots. One condition of steady-state growing roots is a linear increase in the root length. We analysed root growth during the last 24 h in seedling samples 5 and 8 d after germination (DAG) and found that at both time points the growth in both the mutant and the wild-type was stabilized (see Results). Another condition was a constant number of cells in the meristem (Ivanov and Dubrovsky, 1997). As the transition domain of the RAM has not been defined previously, the number of meristematic cells in the cited work corresponds to the NC_{PD} in the current study. To verify if the NC_{PD} was constant during the analysed time periods, we estimated this parameter in samples at t_0 (24 h prior to termination of the experiments) and found no statistical differences in the NC_{PD} within the same genotype at t_0 and at final time points. This preliminary analysis permitted us to apply the above equation for estimation of average cell-cycle time in the PD. Criteria for defining the PD and TD have been described (Ivanov and Dubrovsky, 2013). Briefly, the PD comprises cells that maintain proliferation activity and the TD comprises cells that have very low probability of cell proliferation but grow at the same rate as cells in the PD and have not yet started rapid elongation. As no marker lines have yet been proposed to identify these domains, we determined the domains based on relative changes in the cell lengths analysed on cleared root preparations. In the PD, the cell length commonly varies no more than 2-fold, and in the TD cells are longer than the longest cells in the PD. The distance from the quiescent centre to the point where a cortical cell becomes longer than the longest cell in the PD was considered to be the border between the PD and the TD. In the elongation zone, the cell length starts steadily to increase simultaneously in all tissues. The point where this increase can be observed was defined as the distal (rootward) border between the TD and elongation zone. All measurements were done with an ocular micrometer.

All experimental data were analysed statistically with SigmaPlot 11 (Systat Software, San Jose, CA, USA). Student's *t*-test or Tukey's post-hoc test were used for testing differences in growth and root developmental responses, as indicated. The number of independent experiments in each case is indicated in the corresponding figure legend.

Results

Mutation of the MPK6 *gene causes three distinct and stable seed phenotypes*

Through a careful phenotypic analysis of two independent *mpk6* T-DNA insertion null mutant lines (SALK_073907 and SALK_127507) (Supplementary Fig. S1A at *JXB* online), we corroborated that the protruding embryo phenotype, previously described by Bush and Krysan (2007), was present in the homozygous seed populations from both mutant alleles. Closer inspection of the seeds from these mutants showed three segregating phenotypically distinctive classes. In the larger class (~70%, *mpk6wb*/*w*ild-type-like *b*igger seeds) the lack of MPK6 did not cause any evident alteration in seed morphology, but the seeds were significantly bigger than those from wild-type plants (Figs 1C and 2). The second class (~23%, *mpk6rs*/raisin-like *s*eed phenotype) included seeds with rough coats (Fig. 1D). Finally, the smaller class (~7%, *mpk6bs*/*b*urst *s*eed phenotype) included seeds with protruding embryos from the seed coat (Fig. 1E). It is important to point out that the rough coat phenotype was not uniform, as we observed some seeds that looked more affected than others (Fig. 1D). However, in this study, all of them were pooled together within the same class. In contrast to the heterogeneous phenotype of the rough coat seeds, the other two seed phenotypes were clearly recognized. To determine whether all three *mpk6* seed phenotypes were linked to the *MPK6* mutation, we performed crosses between a homozygous *mpk6-2* mutant with pollen from wild-type (Col-0) plants. In the F1 progeny of these crosses, no phenotypic alterations were evident. Interestingly, in seedlings from all three different seed classes obtained from *mpk6* homozygous mutant populations, MPK6 activity was absent, and this was observed consistently in at least three subsequent generations of homozygous *mpk6*

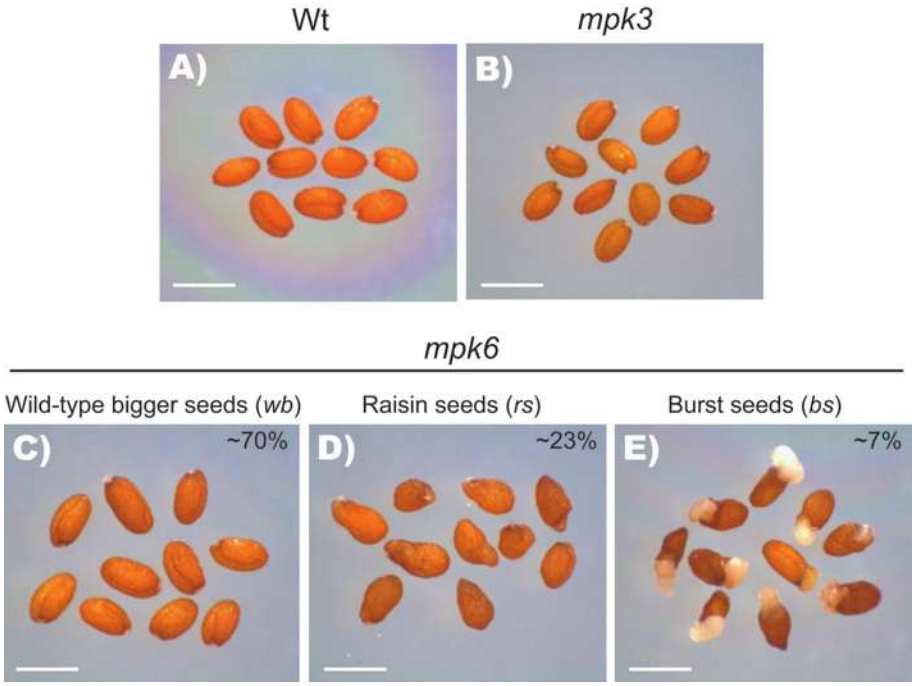

Fig. 1. *MPK6* mutation causes three different seed phenotypes. (A, B) Seeds from wild-type plants (Wt, Col-0) (A) and *mpk3* mutant (B) are shown for comparison to stable and distinguishable *mpk6* mutant seed phenotypes. (C) Seeds resembling the wild type but with a bigger seed phenotype (*mpk6wb*). (D) Seeds displaying a rough coat raisin-like seed phenotype (*mpk6rs*). (E) Embryos protruding from the seed coat burst seed phenotype (*mpk6bs*), described previously by Bush and Krysan (2007). For the pictures, seeds from each class were pooled, but the proportion of each phenotype, obtained from 1000 analysed *mpk6* seeds through several generations, is indicated. Bars, 500 μm.

seedlings from the referred seed phenotypes (Supplementary Fig. S1B).

For a better inspection of seed structure, a *pABI4::GUS* transgene encoding β-glucuronidase (GUS), expressed in embryos (Bossi *et al.*, 2009; Söderman *et al.*, 2000), was introduced into the *mpk6-2* homozygous line. We found that in homozygous *mpk6 pABI4::GUS* F3 populations, all three seed phenotypes were present (Supplementary Fig. S2, at *JXB* online). Previous studies have demonstrated redundancy between MPK6 and MPK3 (Lee and Ellis, 2007; Hord *et al.*, 2008; Lampard *et al.*, 2009; Liu *et al.*, 2010). However, a null *mpk3* mutant allele (SALK_100651) did not display any distinguishable seed phenotype when grown side by side with wild-type or *mpk6* seedlings (Figs 1B and 2), nor was the exacerbated MPK3 activity on *mpk6* seedlings (Supplementary Fig. S1B) able to compensate the *mpk6* phenotypes. Therefore, we concluded that the defects observed in seed morphology were caused specifically by a mutation in *MPK6* and they were apparently independent of *MPK3*.

mpk6 *seed phenotypes are linked to root developmental alterations*

To analyse whether the observed alterations in *mpk6* seeds affected post-germination development, we compared the early seedling growth of wild-type and *mpk6* homozygous mutant populations. Initially, we included *mpk3* seeds in our analysis, but we did not find any phenotypic alteration in *mpk3* mutant seeds or root seedlings (data not shown). Inspection of seedlings at 2 DAG demonstrated that it was possible to differentiate three different root phenotypes within the *mpk6* seedlings. Around 70% of the population analysed displayed PRs of greater length than WT seedlings (*l*onger *r*oot; *mpk6lr*). Additionally, roughly 20% of the seedlings displayed *s*hort *r*oots (*mpk6sr*), whereas around 10% of the seedlings did not develop PRs (*m*inus *r*oots; *mpk6mr*) (Fig. 3A, B). Although a previous report has already documented defects in root formation in the *mpk6-2* (SALK_073907) mutant (Müller *et al.*, 2010), no further analysis of these morphological alterations in the root architecture or their relationship with earlier embryonic alterations was performed. Interestingly, the proportion of each of the three root phenotypes correlated with those proportions observed from the seed phenotypes described previously (Figs 1C–E and 3A), suggesting that they may be related.

To analyse if *mpk6* mutant seed phenotypes had any effect on the post-germination development, the different classes of seed from this mutant were separated and germinated independently. The root morphology from each seed population was then compared with that of wild-type seedlings. Surprisingly, a high proportion of the *mpk6bs* seeds germinated *in vitro*, indicating that, in spite of the protrusion from the seed coat, these embryos were viable (Fig. 3C). However, around 80% of these germinated seedlings failed to develop PRs and most of them died a few days after germination. Those seedlings that survived all developed adventitious roots (Fig. 3C, inset) and were able to complete their life cycle and produce seeds. The progeny from these *mpk6bs* seedlings segregated again

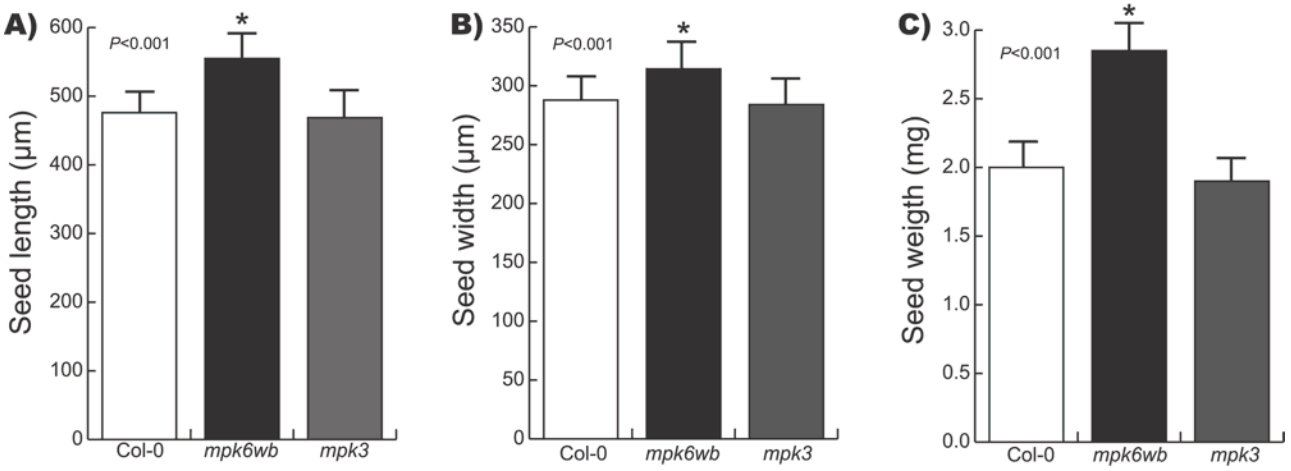

Fig. 2. *mpk6* mutant produces seeds bigger than the wild-type. The *mpk6* seeds were longer (A), wider (B), and heavier (C) than wild-type (Col-0) and *mpk3* seeds. Error bars represent the mean ±standard error (SE) from 500 seedlings analysed at each line. Asterisk indicates Student's *t*-test statistically significant differences at *P* values indicated.

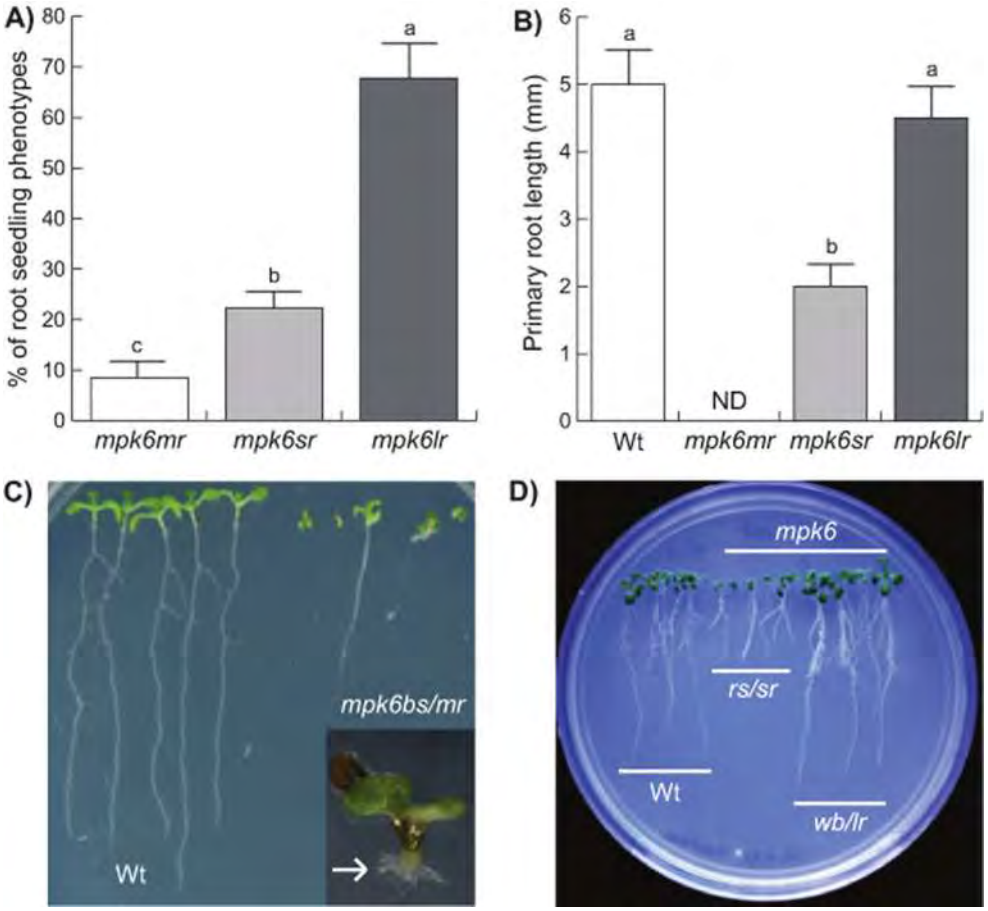

Fig. 3. *mpk6* mutant displays three different root phenotypes. The *mpk6* mutant displayed three root phenotypes each related to the seed morphology: seedlings lacking PR (minus root; *mpk6mr*), seedlings with short roots (*mpk6sr*), and seedlings with PR longer than wild-type root (*mpk6lr*). (A) Proportion of 6 DAG seedlings in each *mpk6* root mutant class.(B) PR length in 2 DAG wild-type (Wt) and the three *mpk6* root mutant classes seedlings. Notice that in this developmental stage the root length of the later *mpk6lr* phenotype is similar to that of the wild-type. Error bars represent the mean±SE from data obtained from three independent experiments, each performed with 120 (A) or 100 (B) seedlings. Different letters on the bars indicate Tukey's post-hoc test statistical difference at $P \leq 0.001$. (C) Seedlings lacking PR (minus root) developed from *mpk6bs* seeds (*mpk6bs/mr*); some of these seedlings were able to produce adventitious roots (white arrow). (D) Seeds at 6 DAG *mpk6rs* (*rs/sr*) and *mpk6wb* (*wb/lr*) develop shorter and longer PRs compared with the wild-type roots.

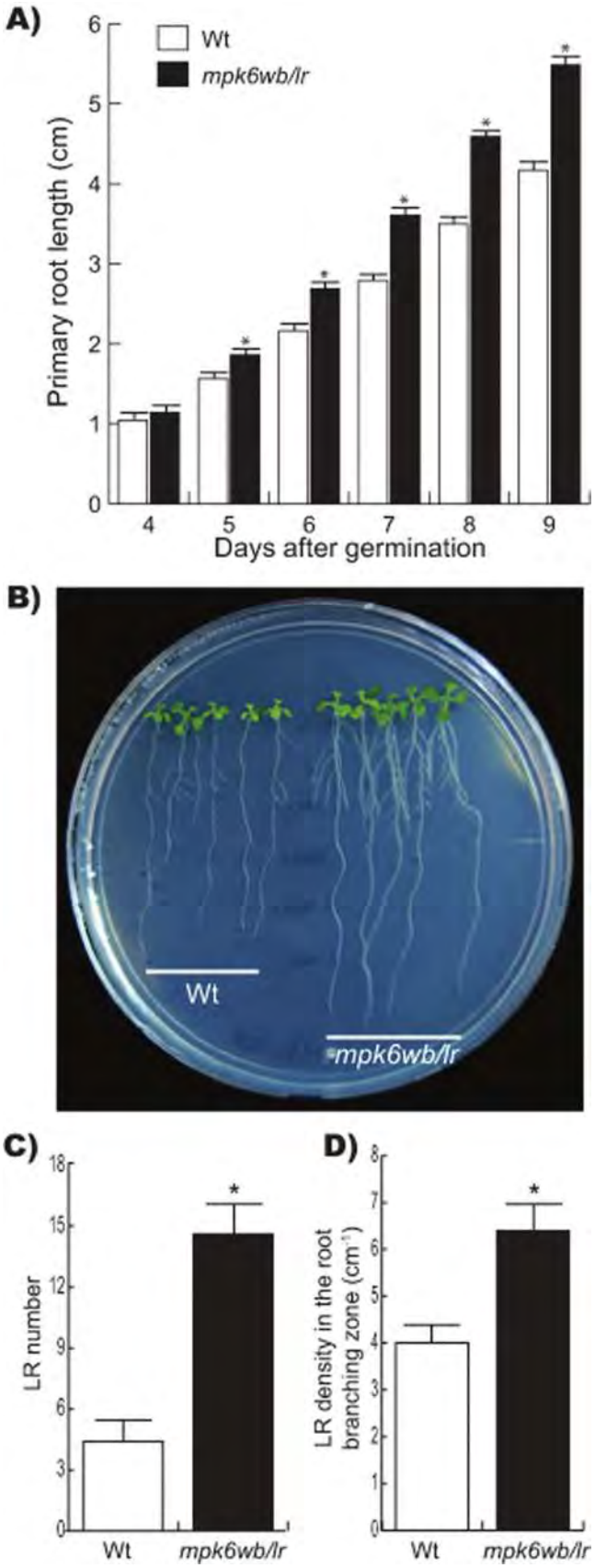

Fig. 4. *mpk6* mutant has altered primary root development, more LRs and higher LR density. (A) Primary root length changes with time. Starting from 5 DAG, statistically significant differences were observed between wild-type (Wt) and *mpk6wb/lr* PR length. Error bars represent the mean ±SE from 30 seedlings analysed at each indicated DAG. The experiment was repeated three times with similar results. Asterisk indicates Student's *t*-test statistically significant differences at $P \leq 0.001$. (B) Representative photograph of wild-type and *mpk6wb/lr* 8 DAG seedlings. Notice that *mpk6wb/lr* seedlings had longer PRs, and more and longer LRs than the wild-type seedlings. (C, D) LR number (C) and LR density (D) were obtained from the root branching zone of wild-type and *mpk6wb/lr* mutant. Error bars in (C) represent the mean ±SE from 60 seedlings analysed at 8 DAG from three independent experiments and in (D) represents the mean ±SE from 12 seedlings at 6 DAG from two independent experiments. Asterisk indicates Student's *t*-test statistically significant differences at $P \leq 0.001$.

into all three seed phenotypes shown in Fig. 1 with similar proportions (data not show). Marked differences were also observed in root development of the seedlings derived from the *mpk6wb* and *mpk6rs* seed types when compared with wild-type seedlings. Seedlings at 6 DAG derived from *mpk6rs* displayed shorter roots than the wild-type, and those derived from *mpk6wb* had longer roots (Fig. 3D). In particular, analysing the rate of growth of the PR of *mpk6wb/lr*, we found that, starting from 5 DAG, it was greater in the mutant than in wild-type seedlings (Fig. 4A). The data described so far clearly demonstrated that MPK6 plays an important role in root development and that these root phenotypes are linked with particular seed phenotypes. Besides a longer root, *mpk6wb/lr* seedlings grown *in vitro* also clearly developed more LRs (Fig. 4B). We next decided to explore the participation of MPK6 in LR development, quantifying the number of LRs and LR density in *mpk6wb/lr* and wild-type plants. These analyses demonstrated that *mpk6wb/lr* seedlings contained a higher number of LRs and a greater LR density in the root branching zone (Fig. 4C, D). These data indicated that MPK6 acts as a negative regulator of LR formation.

mpk6 *mutant has embryo development defects*

The longer root phenotype of the *mpk6wb/lr* mutant could be a result of differences in germination time compared with that of wild-type. We found that this was not the case, as both wild-type and *mpk6wb* seeds had similar germination times (data not shown) and similar root length during the first days after germination (Figs 3B and 4A). Thus an alternative hypothesis is that the short-root phenotype and the inability to form PRs are associated with defects during embryo development and do not represent alterations in the vegetative root developmental programme. To test this idea, we analysed the morphology of a total of 239 *mpk6* embryos representing all stages of embryonic development from two independent experiments (Fig. 5). During early embryogenesis, the suspensor uppermost cell is recruited to the embryo proper and acquires hypophyseal identity (Jürgens, 2001). Mutant lines defective in generating this cell lineage often produce rootless seedlings (Peris *et al.*, 2010; Jeong *et al.*, 2011). Microscopic analyses of early *mpk6* embryos showed ectopic divisions in the suspensor at the time when

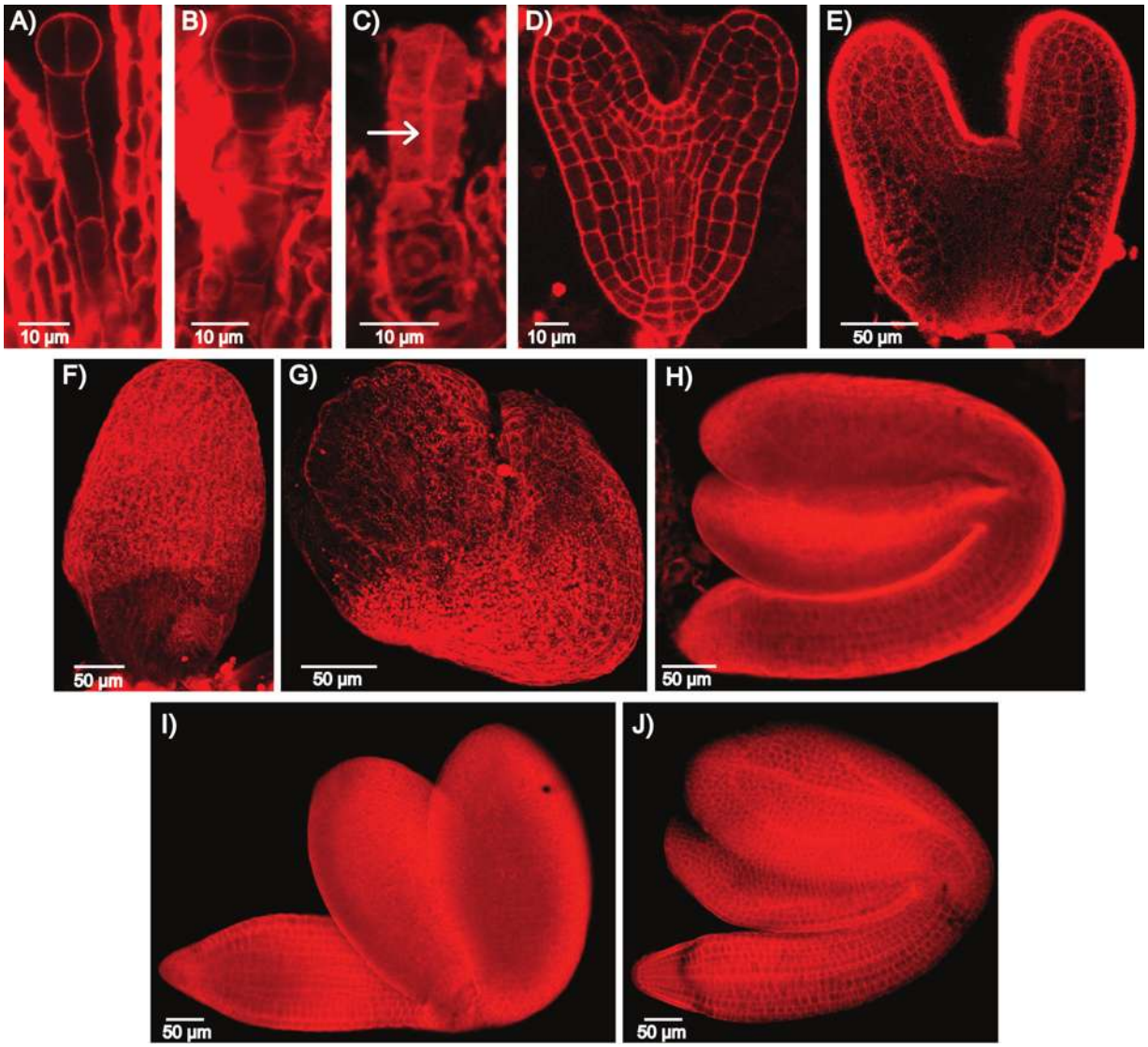

Fig. 5. *mpk6* cell organization is affected throughout embryonic development. Cell organization in wild-type (Col-0) and *mpk6* embryos. (A, B) Representative two- to four-cell (A) and eight-cell (B) wild-type embryos during their first three rounds of cell divisions. (C) Two-cell *mpk6* embryo showing ectopic periclinal cell division in the uppermost suspensor cell (arrow). This embryo failed to establish the transversal cell division plane necessary to generate an eight-cell embryo proper. (D) Wild-type heart-stage embryo. (E–G) Immature *mpk6* embryos showing arrested development at the heart (E), torpedo (F), or bent cotyledon (G) stages. (H) *mpk6* embryo with complete embryonic organogenesis at the bent cotyledon stage. (I, J) Representative *mpk6* (I) and wild-type (J) embryos at mature stage. Propidium iodide pseudo-Schiff staining of the cell wall (red) was carried out according to Ugartechea-Chirino *et al.* (2010). Bars are as indicated.

the hypophyseal cell should be specified. Seven out of 23 embryos observed between the one-cell and globular stages had ectopic divisions either in the suspensor or in the embryo proper (Fig. 5C). Later during development, 24% of the embryos were arrested at the heart stage embryo and did not proceed to develop hypocotyl and root (Fig. 5E), while 18% showed arrested development but developed hypocotyl and root (Fig. 5F–G) and 71% achieved complete embryonic organogenesis by the bent cotyledon stage (Fig. 5H). Interestingly, at the mature stage, *mpk6* embryos seemed to be bigger than the wild-type (Fig. 5I, J). Considering that *mpk6* developed several short siliques, with few seeds and many abortion events (Supplementary Fig. S3 at *JXB* online), we estimated that the frequency of these *mpk6* embryo developmental patterns correlated roughly with the frequencies observed for the burst, the raisin-like and the bigger phenotypes of mature seeds, respectively.

MPK6 is involved in the control of RSA

RSA is an important trait determining plant productivity. At present, little is known about the intrinsic mechanisms that control root growth and branching. To analyse the role that MPK6 has over RSA, we performed experiments to compare PR growth, LR formation, and RH development in wild-type and *mpk6* mutants. As they apparently do not have embryo alterations that can affect the post-germination development, to do this analysis we used only the big seed phenotype that

was also associated with long roots (*mpk6wb/lr*). LR number and length were important determinants of RSA and both were affected in the *mpk6wb/lr* mutant (Fig. 4C, D). LRs develop from the parent root through the specification of pericycle founder cells. After activation, these cells undergo repeated rounds of cell division leading to the formation of a LRP that eventually emerges as a new LR (Laskowski *et al.*, 1995; Malamy and Benfey, 1997; Dubrovsky *et al.*, 2001). A strict analysis of LR development must take into account all LR initiation events (Dubrovsky and Forde, 2012). Thus, the densities of all LR initiation events (LR and LRP) in the branching zone and in the branching zone plus LR formation zone (the latter comprises the root portion from the most rootward primordium to the most rootward emerged root) were also analysed. As shown in Fig. 6A, B, both the LR and LRP densities were significantly higher in the *mpk6wb/lr* mutant than in wild-type roots. As the fully elongated cortical cells in the *mpk6wb/lr* mutant could be longer than those from the wild type, the cell length in the LR formation zone (Fig. 6C) and the LR initiation index (Fig. 6D) were also evaluated. The latter parameter permits evaluation of LR initiation on a cellular basis and estimates the number of LR initiation events per root portion comprising 100 cortical cells of average length in a file (Dubrovsky *et al.*, 2009). This analysis also confirmed that LR initiation was significantly higher in the *mpk6wb/lr* mutant compared with wild-type seedlings. These data together strongly supported the conclusion that MPK6 acts as a negative regulator of LR initiation in *Arabidopsis*.

RHs differentiate from the root epidermal cells in a cell-position-dependent manner, increasing the total surface of roots (Tominaga-Wada *et al.*, 2011). Based on our previous results, we were interested to determine the effect of the *MPK6* mutation on RH differentiation. As shown in Fig. 7, the total number of RHs was significantly increased in the mutant compared with wild-type seedlings (Fig. 7A, B). These data indicated that the loss of function of MPK6 resulted in more and longer RHs. Moreover, we also found that the length of RHs in two different zones of the PR (2–3 and 5–7 mm root portions from the root tip) was also increased in the *mpk6wb/lr* mutant (Fig. 7C). These data also showed an important role of MPK6 in the differentiation and subsequent growth of RHs.

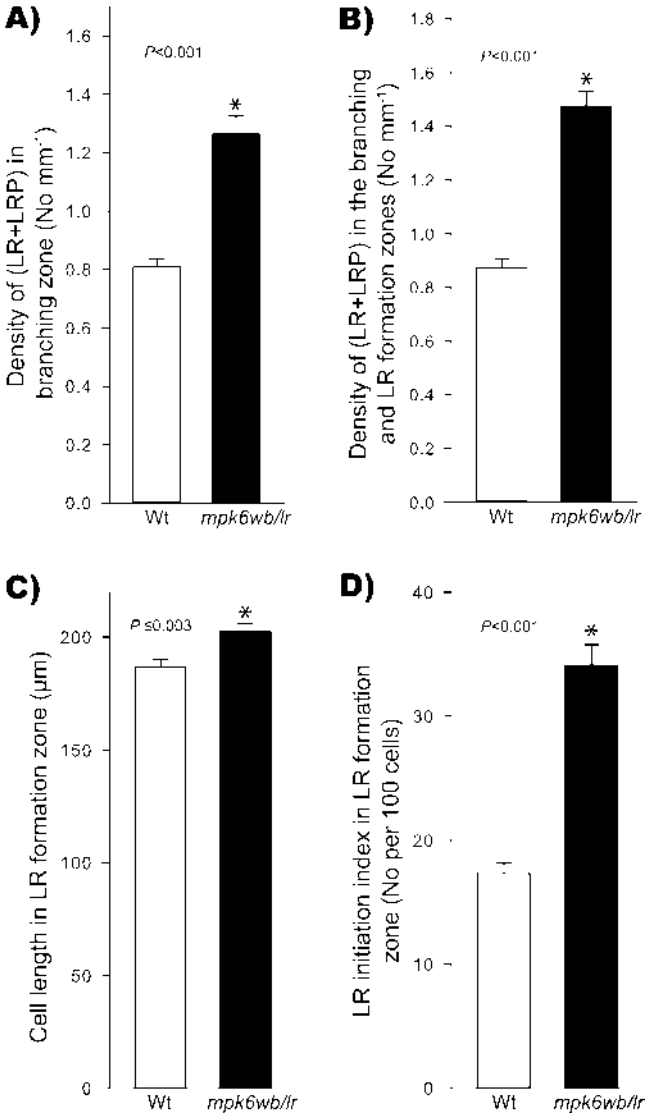

Fig. 6. The *mpk6wb/lr* mutant has increased LR initiation. LR formation in wild-type (Wt) and *mpk6wb/lr* seedlings. (A) Density of LRs and LRP in the primary root branching zone. (B) Density of LRs and LRP in branching and in LR formation zones. (C) Cortical cell length in the LR formation zone. For each individual root, 10 fully elongated cortical cells were measured. (D) LR initiation index in the LR formation zone. Error bars represent mean ±SE of 23 roots from two independent experiments. Asterisks mark Student's *t*-test significant differences at the *P* values indicated.

mpk6 *primary root growth alterations are multifactorial*

Cell division, elongation, and differentiation are closely linked cellular processes that determine PR growth. The RAM comprises two different zones: the PD, where high cell proliferation activity and a relatively slow growth takes place, and the TD, where cell proliferation probability is low but cell growth is maintained at a similar level to that found in the PD (Ivanov and Dubrovsky, 2013). After cells leave the RAM, they enter into the elongation zone, where rapid cell elongation takes place. To elucidate the contribution of cell division and elongation to the longer root phenotype of the *mpk6wb/lr* mutant, a detailed morphometric analysis of both PD and TD was conducted on 5 DAG plants. We observed that, while the size of the TD in the *mpk6wb/lr* mutant was 45% greater than that in the wild-type, the PD was 9% greater in the *mpk6wb/lr* mutant (Table 1). We also found that the number of cells, the rate of root growth, the fully elongated cell length, and cell production were also increased in *mpk6wb/lr* PRs compared with those of wild-type (Table 1 and Fig. 8A–C). A significant decrease (13%) in cell-cycle duration over time (5–8 DAG) was found in mutant seedlings (Student's *t*-test at $P \leq 0.001$), whereas in the wild type, no changes in cell-cycle duration were found during the same period (Fig. 8D). These results indicated that both cell production and cell elongation have a significant impact on the accelerated root growth found in the *mpk6wb/lr* mutant.

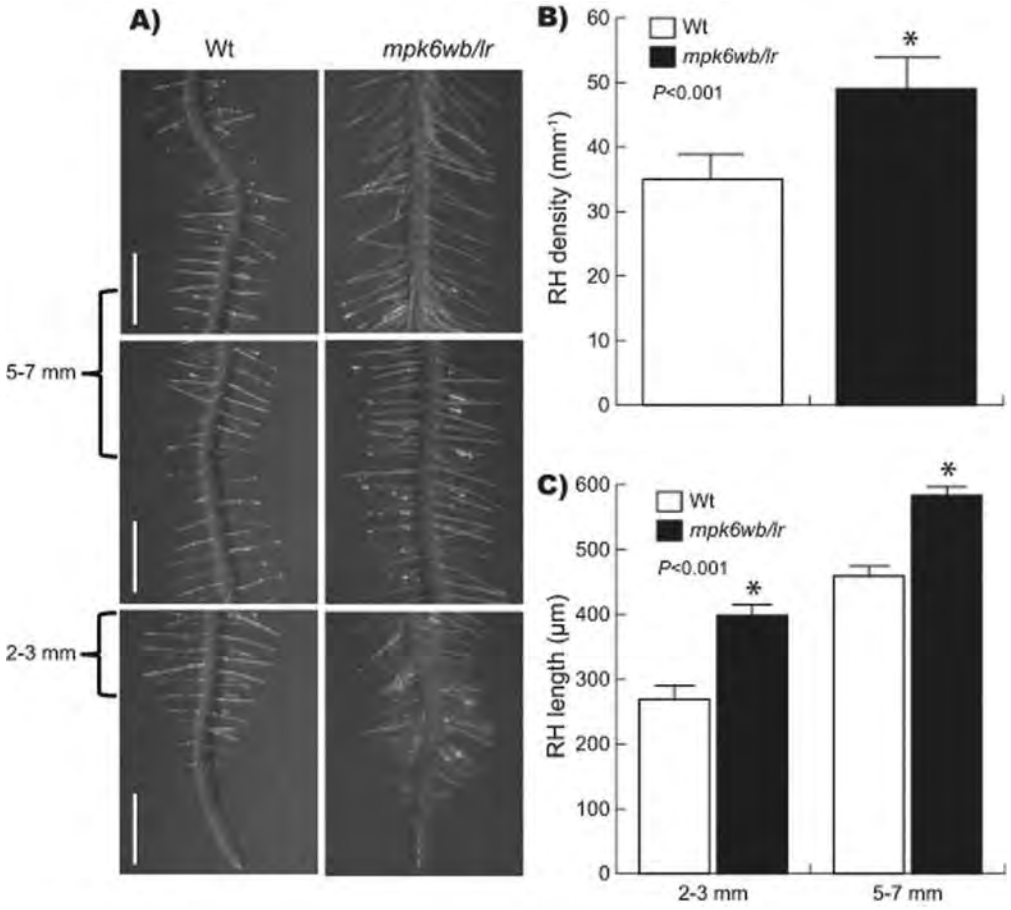

Fig. 7. The *mpk6wb/lr* mutant develops more and longer RHs. (A) RHs formed along ~1 cm from the tip of the PR from representative 6 DAG seedlings. Bars, 1 mm). (B) The RH density in 6 DAG seedlings from 2–3 and 5–7 mm root segments measured from the root tip. (C) Comparative quantification of RH length in the same root segments as in (B). Error bars represent the mean ±SE from 30 seedlings in three independent experiments. Asterisks mark Student's *t*-test significant differences at the *P* values indicated.

Table 1. *Wild-type (Col-0) and mpk6wb/lr RAM comparative analysis*

Genotype	RAM length (µm)	Difference (%)	TD length (µm)	Difference (%)	PD length (µm)	Difference (%)	NC_{PD}	Difference (%)
Col-0	355±39	–	142±20	–	213±19	–	37.3±2.7	–
mpk6wb/lr	438±47*	23	206±18*	45	232±29**	9	45.7±4.6*	22

Combined data were used from two independent experiments (n=24).
*Student's *t*-test significant differences at $P<0.001$.
**Student's *t*-test significant differences at $P \leq 0.019$.

Discussion

MPK6 is essential for embryogenesis and root development

The central role that MAPK signalling has over different aspects of plant development is well accepted (Andreasson and Ellis, 2009; Suarez-Rodriguez *et al.*, 2010). However, the dissection of the particular function of each of the MAPK proteins has been difficult due to the partial redundancy among them. Using a genetic strategy, MPK6 has been associated with pathogen resistance (Menke *et al.*, 2004) and anther, inflorescence, embryo, and root development (Bush and Krysan, 2007; Müller *et al.*, 2010; Wang *et al.*, 2010). In particular, with respect to embryo and root development, previous analysis focused on embryo protruding seeds (Bush and Krysan, 2007), short-root seedlings (Müller *et al.*, 2010), and LR development in response to NO treatment (Wang *et al.*, 2010). In this work, we performed a detailed analysis of seed morphology and its correlation with root development in *mpk6* mutants. Three phenotypic classes of seed were identified in the progeny of homozygous *mpk6* plants, including seeds with a normal appearance but bigger than wild-type seeds, seeds with rough coats, and seeds with protruding embryos, each giving rise to seedlings with totally different root growth and development patterns (Figs 1–4). A previous work reported that the *mpk6* mutant displayed a

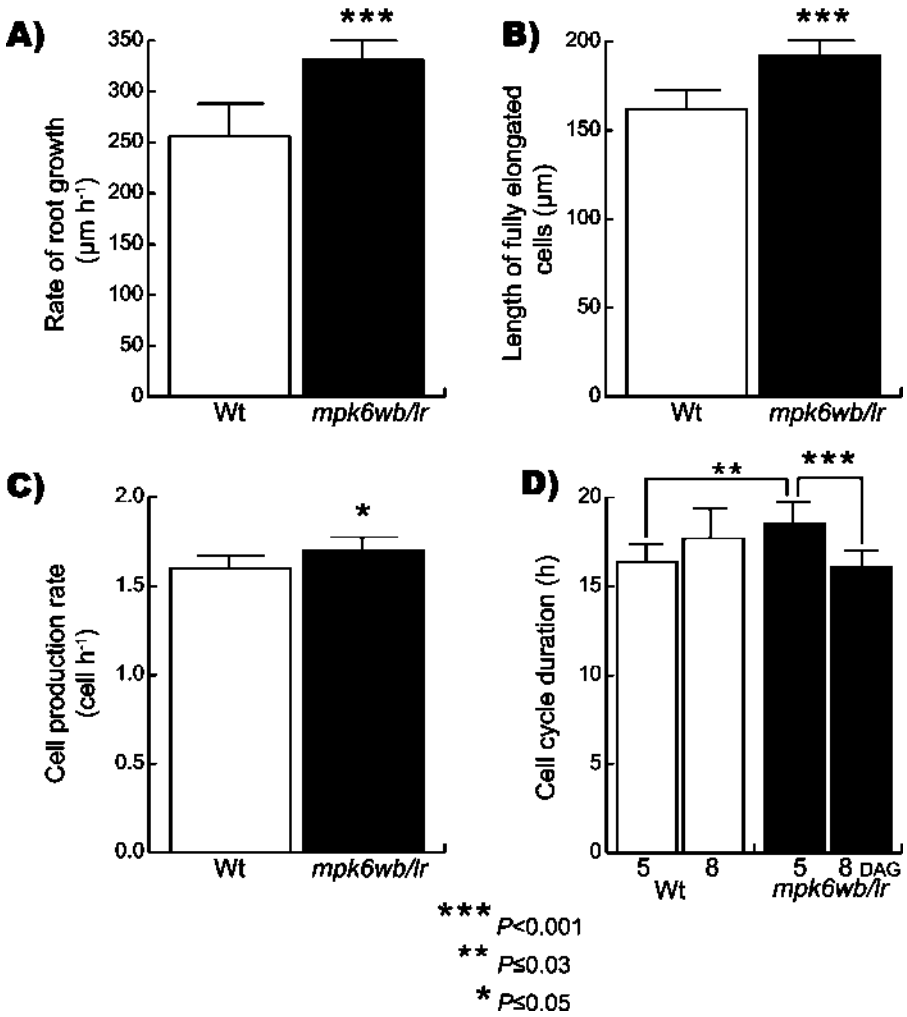

Fig. 8. Quantitative analysis of the wild-type (Wt) and *mpk6wb/lr* primary root growth and development. (A–C) Comparison between wild-type and *mpk6wb/lr* of root growth rate (A), length of fully elongated cells (B) and cell production rate (C) from 5 DAG seedlings. Error bars represent the mean ±SE from 24 seedlings in two independent experiments. (D) Cell-cycle duration (*T*) in wild-type and *mpk6wb/lr* at 5 and 8 DAG. In 5 DAG seedlings, *T* is the mean ±SE from 24 roots in two independent experiments. In 8 DAG seedlings, *T* is the mean ±SE from 12 roots in one experiment. Asterisks indicate Student's *t*-test significant differences at the *P* values indicated.

reduced fertility phenotype with variable penetrance depending on growth conditions, but the environmental variable affecting that phenotype remained to be determined (Bush and Krysan, 2007). However, the seed and root phenotypes reported here were reproducible in at least four generations of the progenies of homozygous *MPK6* from two independent null alleles (SALK_073907 and SALK_127507) grown under conditions of 21 °C, long days (16/8 h light/dark), 105 µmol m^{-2} s^{-1} of light intensity, and 45–60% of relative humidity at ~1580 m above sea level.

Between 500 and 1000 *Arabidopsis* loci have been related to embryo-defective phenotypes (Meinke *et al.*, 2009), and several of these include members of the MAPK cascade. For example, mutations in the *MPKKK4* (*YDA*) protein kinase gene cause defects in embryo development (http://www.seedgenes.org). Since the identification of *YDA* as a gene required for embryonic cell fates, it has been suggested that a MAPK signalling pathway is involved in *Arabidopsis* embryogenesis (Lukowitz *et al.*, 2004). Interestingly, the *yda/emb71* ethyl methanesulfonate heterozygous mutant, affected in the *YDA* gene (At1g63700), displays a similar embryo-protruding phenotype to that observed in *mpk6* (Lukowitz *et al.*, 2004; Meinke *et al.*, 2009). *MPKKK4* and *MPK6* are components of a common MAPK cascade involved in regulation of the embryo (Bush and Krysan, 2007) and in stomata developmental programmes (Wang *et al.*, 2007). The *yda* mutant has defects in hypophysis development similar to that observed in *mpk6bs* mutants (Fig. 5) (Lukowitz *et al.*, 2004; Meinke *et al.*, 2009). The phenotypes observed during early embryogenesis suggest that MPK6 acts as a repressor of cell proliferation involved in the establishment of embryonic polarity (Fig. 5C). This MPK6 role seems to be maintained throughout development because the *mpk6* mature embryos that achieved complete organogenesis were larger than their wild-type counterparts (Fig. 5I, J). The molecular and cellular mechanisms regulating seed development and size are complex (Sun *et al.*, 2010). Potential targets (transcription factors) and activators (leucine-rich repeat receptor kinases),

but not MAPKs were previously involved in that process (Sun *et al.*, 2010). The data from this study further confirm that both YDA and MPK6 are components of a MAPK cascade involved in the regulation of the embryo developmental programme, as already proposed (Bush and Krysan, 2007). The components acting up- and downstream of these MAPKs remain to be identified. In addition, our data support the suggestion that the failure to form a PR and the short-root phenotypes are consequences of *mpk6* mutant embryo development defects. Therefore, without considering the pleiotropic effects caused by embryo defects, the PR phenotype that can be directly associated with the loss of *MPK6* function is a long PR. Notably, this *mpk6* long PR was observed previously (Takahashi *et al.*, 2007), and in the *Arabidopsis* Hormone Database (http://ahd.cbi.pku.edu.cn) *MPK6* is included as one of the 79 genes related to a long-root phenotype.

Previous analysis made on *mpk6* short-root seedlings showed that the loss of *MPK6* function resulted in a slight but significant reduction in the number of LRs, suggesting that MPK6 is a positive regulator of LR formation (Müller *et al.*, 2010). In contrast, the data shown here using only *mpk6* mutants germinated from seeds without embryo damage demonstrated that the longer PRs had increased numbers of LRs and RHs (Figs 4 and 7). These apparent contradictory results could be explained from the different seed classes produced in the *mpk6* mutant progenies. These observations highlight how critical is to perform detailed analyses of the phenotypes associated with a gene mutation in different organs and under strictly controlled growth conditions.

MPK6 is a negative regulator of primary root growth

The comparisons of the RAM TDs and PDs and the fully elongated cell lengths between wild-type and *mpk6* mutant roots revealed that both cell division and elongation are altered in PRs of *mpk6* mutant (Table 1 and Fig. 8). Moreover, the number of cells in the *mpk6wb/lr* PD was also higher compared with that in the wild type (Fig. 6) and correlated with lower cell-cycle duration in this mutant (Fig. 8), supporting an important role of MPK6 in controlling cell proliferation and suggesting that its loss of function has a direct consequence in the long-root phenotype. For more than a decade, experimental evidence has supported the involvement of MAPKs in the regulation of cell-cycle progression in yeast (Gustin *et al.*, 1998; Strickfaden *et al.*, 2007), animals (Aliaga *et al.*, 1999; Rodríguez *et al.*, 2010), and plants (Calderini *et al.*, 1998; Jonak *et al.*, 2002; Suarez-Rodriguez *et al.*, 2010).

Regulation of plant cell division and growth is associated with microtubule reorganization, which is assisted with the action of microtubule-associated proteins. Previous reports have shown that some microtubule-associated proteins are regulated by reversible phosphorylation through MAPK cascades (Komis *et al*, 2011). For example, a MAPK from *M. sativa* (MKK3) is indispensable for spindle microtubule reorganization during mitosis (Bögre *et al.*, 1999) and the *Arabidopsis* MPK4 has been shown to be essential for the correct organization of microtubules through the phosphorylation of microtubule-associated protein 65-1 (Beck *et al.*, 2010). Additionally, the *Arabidopsis* MPK18 has been demonstrated to interact physically with a dual-specificity MAPK phosphatase (PROPYZAMIDE HYPERSENSITIVE 1/PHS1) to conform to a reversible phosphorylation/dephosphorylation switch that regulates cortical microtubule formation (Walia *et al.*, 2009). Phosphorylation of a MAP3K (NPK1) by cyclin-dependent kinases has been proposed to be critical for the appropriate cytokinesis progression in *Arabidopsis* (Sasabea *et al.*, 2011). Expression of *MPK6* has been shown to be strong in both the RAM PD and TD, specifically during the pre-prophase band and in the phragmoplast, where it controls cell division plane specification (Müller *et al.*, 2010). Epigenetic modifications like methylation or deacetylation of histones have also been suggested to regulate root development (Fukaki *et al.*, 2006; Krichevsky *et al.*, 2009). Interestingly, in animal systems, MAPK mediates histone phosphorylation, which in turns drives acetylation of histone H3, impacting on gene transcription (Clayton *et al.*, 2000). It remains to be addressed whether a similar regulatory mechanism operates in plant systems.

MPK6 regulates LR initiation

RSA is determined primarily by the spatio-temporal regulation of lateral root initiation events (Bielach *et al.*, 2012). Mutants having increased number of LRs are relatively infrequent compared with those with reduced number of LRs (De Smet *et al.*, 2006), although an increased number of LRs does not necessary indicate an increase in LR initiation (Dubrovsky and Forde, 2012). The participation of MAPK cascades in LR formation was documented by the phenotypes observed in mutants of MPK4 and its upstream activator MEKK1-1, both displaying from a slight to a severe reduction in LR density (Nakagami *et al.*, 2006; Su *et al.*, 2007). Previous studies have also shown that MPK3 and MPK6 are activated in response to the same signals as MEKK1/MPK4, supporting a possible role of these kinases in the LR development programme (Ichimura *et al.*, 2006; Suarez-Rodriguez *et al.*, 2007). However, our results demonstrated that the role of MPK6 in LR development is opposite to that of MPK4 (and MEKK1). The observation that the *mpk6wb/lr* long PR phenotype is accompanied by an increase in LR initiation (Figs 4C, D and 6), fully demonstrated that MPK6 acts as a negative regulator of LR initiation. The clearest examples of increased LR initiation are the mutants related to auxin homeostasis and signalling (Zhao, 2010). CEGENDUO, a subunit of SCF E3 ligase, has a negative role in auxin-mediated LR formation (Dong *et al.*, 2006). MAPK cascades have been found to directly or indirectly affect auxin signalling (Mockaitis and Howell, 2000; Takahashi *et al.*, 2007), which could alter LR formation. Cytokinin is a negative regulator of LR initiation. Decreased endogenous cytokinin concentration in protoxylem-adjacent pericycle cells results in increased LR initiation; in contrast, when the cytokinin concentration in these cells is increased, LR initiation is repressed (Laplaze *et al.*, 2007). In this context, the *mpk6* mutant shows a phenotype of decreased

endogenous cytokinin content. Altogether, these findings highlight the complexity of the MAPK cascades in root morphogenesis. However, an increase in cell production in the PR meristem and increased LR initiation in *mpk6wb/lr* both indicate a link between cell proliferation and its regulation by MPK6. Stress and development responses are tightly coordinated by MPKs, but their interaction is still poorly understood. As few research studies have focused on the interplay between development and environmental stresses, our findings highlight the power of studying root processes in terms of unravelling MPK signalling interactions.

MPK6 is important for RH formation

Our data also demonstrated that MPK6 is a negative regulator of RH development, as its mutation resulted in an increase in the number and size of RHs (Fig. 7). Multiple cellular factors regulate RH growth and development, including vesicle exocytosis, calcium (Ca^{2+}) homeostasis, reactive oxygen species and cytoskeleton modifications (Cardenas, 2009). Ca^{2+} is a universal second messenger, which, through interactions with Ca^{2+} sensor proteins, performs important roles in plant cell signalling (Batistič and Kudla, 2012). These sensor proteins include calmodulins, calmodulin-like proteins, Ca^{2+}-dependent protein kinases, calcineurin B-like proteins, and their interacting kinases, among others. Several genes implicated in RH differentiation have been identified; one of them, *OXIDATIVE SIGNAL INDUCIBLE 1* (*OXI1*) from *Arabidopsis*, is required for MPK6 activation by reactive oxygen species (Rentel *et al.*, 2004). The MPKKK1 (MEKK1) also has been involved in reactive oxygen species homeostasis (Nakagami *et al.*, 2006) and apparently, together with MKK2 and MPK4/MPK6, constitutes a MAPK cascade that participates in several stress responses (Ichimura *et al.*, 2000). In alfalfa, stress-induced MAPK (SIMK), an *Arabidopsis MPK6* orthologue, performs an important role in RH tip growth (Šamaj *et al.*, 2002). The alfalfa SIMK protein seems to be a positive regulator of RH growth, as treatment of plants with the MAPK inhibitor UO126 resulted in aberrant RHs, whereas the overexpression of SIMK in tobacco induced a rapid growth of these cells (Šamaj *et al.*, 2002). These results contrast with our observations of the function of MPK6 and highlight a specific role of each member of the MAPK cascade in a particular developmental process.

Possible role of MPK6 in hormone responses

As the precise mechanism underlying the root developmental alterations in *mpk6* seedlings is still unknown, we hypothesized that *mpk6wb/lr* root architectural phenotypes might result from altered responses to auxins or cytokinins, as these hormones control RSA (Perilli *et al.*, 2012). Thus, to determine whether MPK6 could affect PR responses to auxins or cytokinin, we evaluated the PR growth of wild-type and *mpk6wb/lr* mutant seedlings in response to the exogenous addition of IAA and kinetin. We observed that, at low concentrations of IAA (0.03–0.125 µM) and kinetin (0.25–2 µM), *mpk6wb/lr* was slightly insensitive and slightly hypersensitive, respectively, to the inhibitory effects of these hormones on PR growth. However, these differences were not clear at higher concentrations (0.25–0.5 µM IAA and 4–16 µM kinetin) of both hormones (Supplementary Fig. S4 at *JXB* online). These assays showed that *mpk6wb/lr* PR is not insensitive to the exogenous addition of these two plant growth regulators, suggesting that the observed root length differences in the *mpk6* mutant cannot be explained by different sensitivities to auxin or cytokinins. This observation agrees with the finding that the root growth-inhibition response to several hormones of the MPKKK mutant *yda*, which acts upstream of MPK6, is normal (Lukowitz *et al.*, 2004).

In summary, the results presented here indicate that MPK6 is a negative regulator of at least three developmental programmes in the root, namely PR growth, LR formation, and RH development, which probably occurs through regulation of cell division and elongation processes. Understanding the signalling events regulated by MPK6 activity during root development will ultimately require identification of the up- and downstream components, as well as the signal (or combination of signals) turning on and off phosphorylation of the MAPK cascade and impacting on RAM behaviour.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Methods.

Supplementary Figure S1. *mpk6* is a null mutant.

Supplementary Figure S2. *mpk6* seed phenotypes are stable.

Supplementary Figure S3. *mpk6* siliques are shorter than wild type and contain many aborted seeds.

Supplementary Figure S4. Effect of auxin and cytokinins on primary root growth.

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***Arabidopsis thaliana* MAP kinase 6 mutation causes three different seed phenotypes correlated with alterations in cellular processes affecting root architecture**

López-Bucio J. S., Dubrovsky, J. G., Raya-González J., Ugartechea-Chirino, Y., LópezBucio J., de Luna-Valdés, L. A., Ramos-Vega, M., León, P. and Guevara-García, A. A[1].

Supplementary Methods

Plant genotyping

Total RNA was isolated using TRIZOL® (Invitrogen™, Carisbad, CA) from freezing tissue and the first-strand cDNA was synthesized using 3 µg of RNA in a volume of 30 µL containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 2 mM deoxynucleotide triphosphate mixture, 5 mM oligo(dT) primer, 10 units of RNase inhibitor (Invitrogen™), and 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen™) for 1 h at 42 ºC. To inactivate the enzyme the samples were incubated at 92 ºC by 10 min. One microliter of the first-strand reaction was used for the genotypic analysis of the plant lines under study. The amplification reaction was performed containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 mM of each primer, 10 mM Tris-HCl, pH 8.8 at 25 ºC, 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase (MBI/Fermentas, Hannover, MD). Thirty cycles of amplification were performed in a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA), each having 30 s of denaturation at 94 ºC, 40 s of annealing at 56 ºC, and 1 min of extension at 72 ºC. The specific set of primers for each amplification reaction was as follows: 5'-CTTCCGCCGTAAAAGC-3' (forward) and 5'-GTCCCAGCACCACAGG-3' (reverse) for *UBI6* (At2g47110), 5'-ATCTTTATGGAGCTTATG-3' (forward) 5'-CCGTATGTTGGATTGAG-3' (reverse) for *MPK3* (At3g45640) and 5'-CGAGTCACTTCTGAGAG-3' (forward) and 5'-TTGCTGATATTCTGG-3' (reverse) for *MPK6* (At2g43790). PCR products were analyzed on 1% agarose gels.

Protein extraction and kinase assays

Seedlings were ground in liquid nitrogen, homogenized in buffer containing 250 mM sorbitol, 50 mM HEPES-BTP (pH 7.8), 10 mM NaF, 5 mM DTT, 1 mM EDTA, 1 mM KCl, 1 mM Na_3VO_4, 1 mM PMSF and 40 µg/ml of protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), then centrifuged at 12,000 x g 20 min at 4ºC. The crude extracts were stored at –70ºC. Protein concentration in the extracts was estimated with Bradford protein assay kit (Bio-Rad, Hercules, CA) using BSA as a standard. The in-gel kinase assay was performed as previously described (Zhang & Klessig 1997). Briefly, 50µg of protein extracted from plant tissue were fractionated on a 10% SDS-polyacrylamide gel containing 0.25 mg/ml myelin basic protein (MBP, Sigma) as substrate for the kinases. After electrophoresis, the gel was washed three times with 25 mM Tris (pH 7.5); 0.5 mM DTT; 0.1 mMNa3VO4; 5mM NaF; 0.5 mg/ml BSA; 0.1% (vol/vol) Triton X-100 for 30 min each at room temperature. Proteins in the gel were then renatured by incubating the gel in 25 mM Tris (pH 7.5); 1 mM DTT; 0.1 mM Na_3VO_4 and 5 mM NaF at 4°C overnight, with three changes of buffer. The kinase reactions were then carried out by incubating the gel in 30 ml of buffer containing 25 mM Tris (pH 7.5); 2 mM EGTA; 12 mM $MgCl_2$; 1mM DTT; 0.1 mM Na_3VO_4; 200 nM ATP, and 50µCi of [γ-^{32}P]ATP (>4,000 Ci_mmol; 1 Ci = 37 GBq), for 60 min at room temperature. To

[1] aguevara@ibt.unam.mx

remove free ^{32}P, the gel was extensively washed at room temperature with several changes of 5% (wt/vol) trichloroacetic acid and 1% (wt/vol) NaPPi until ^{32}P-radioactivity in the wash solution was barely detectable. The gel was dried under vacuum on Whatman 3MM paper and used to expose a Kodak XAR-5 film. Prestained size markers (Bio-Rad, Hercules CA) were used to calculate the size of kinases. As loading control 20µg of proteins from the same extracts used for kinase assays were fractionated in a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue dye.

β-glucuronidase histochemical activity

Expression of the *GUS* reporter gene (Jefferson *et al.,* 1987) was detected by incubating *Arabidopsis* imbibed seeds or *in vitro* grown seedlings, in histochemical assay buffer (100 mM NaH_2PO_4 and Na_2HPO_4 mix, pH 7; 0.5 mM potassium ferrocyanide; 0.5 mM potassium ferricyanide; Na_2EDTA pH 8.0; 0.1% Triton X-100 and 0.1% 5-bromo-4-chlorium-3-indolyl-b-D-glucuronic acid), at 37 ºC during the indicated time. Chlorophyll was removed with 70% ethanol for several hours. Seedlings were cleared according to Malamy and Benfey (1997) with some modifications (Dubrovsky *et al.*, 2006), and then mounted in 50% glycerol on microscope slides. Histochemically stained or unstained seeds and seedlings were analyzed using stereoscopic (Nikon SMZ1500) and transmission (Nikon EclipseE600) microscopes equipped with a digital camera (Nikon SIGHT DS-Fi1c, Nikon Corporation, Tokyo, Japan).

Figure Legends

Supplemental Figure S1. *mpk6* is a null mutant.

A) Genotyping of the plants under study. RT-PCR analysis of RNAs from wild-type (Wt, Col-0), *mpk3-1* (SALK_151594), *mpk6-2* (SALK_073907) and *mpk6-3* (SALK_127507) lines, previously characterized (Liu and Zhang, 2004; Wang *et al.*, 2007). The gene fragment amplified in each case is indicated. UBI6 corresponds to the ubiquitin-6 (AT2G47110) *Arabidopsis* gene. All the PCRs were made with the same cDNA from the indicated plant lines. B) In-gel kinase assays were conducted with total protein extracts from wild-type (Wt/Col-0), F1 progeny of *mpk6* x Col-0, F2 seedlings from three seed phenotypes (*mpk6bs*, *mpk6rs*, *mpk6wb*) and *mpk3*, 6DAG seedlings. Notice that whereas MPK6 activity is evident on heterozygous F1 progeny, no MPK6 activity could be detected on *mpk6* seedling with seed phenotypes described here. A Coomassie Brilliant Blue Gel (CBBG) is shown as loading control.

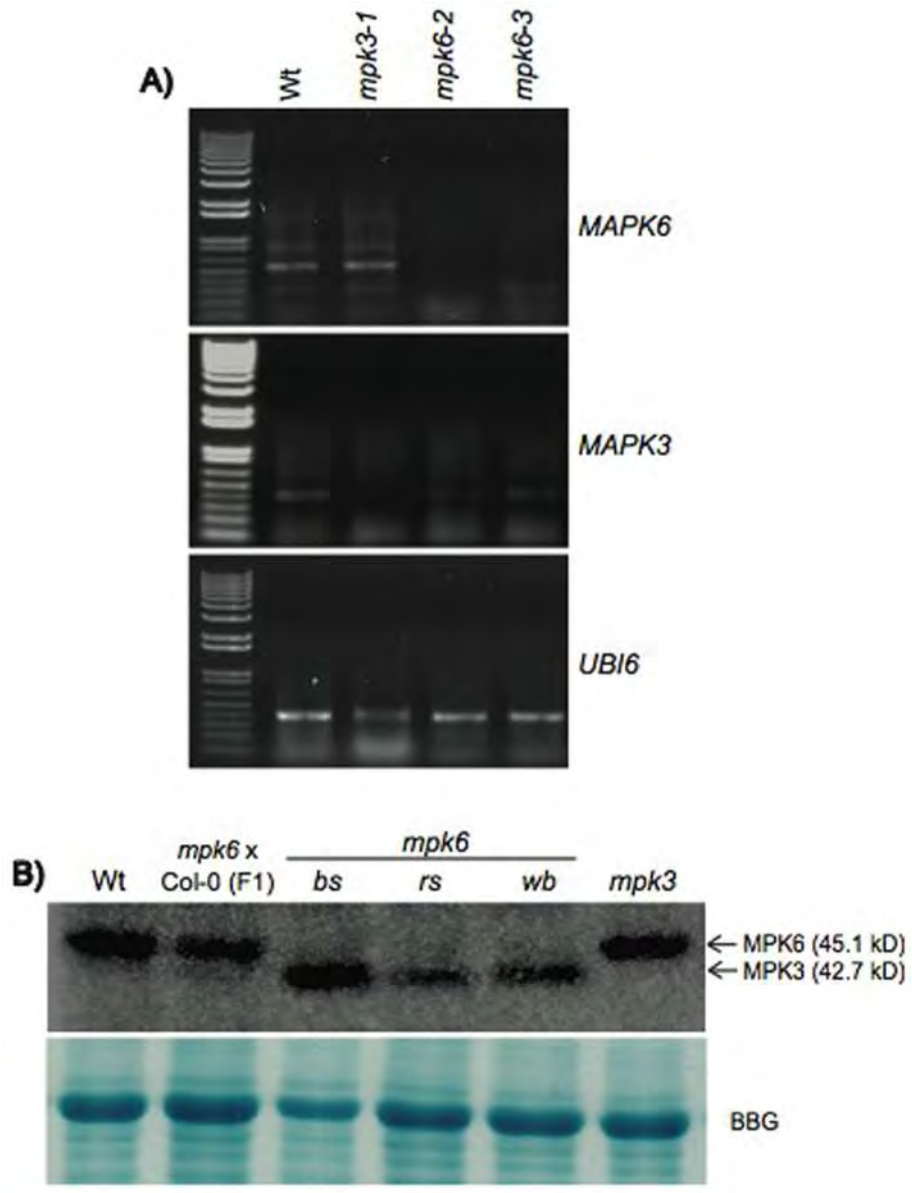
Supplemental Figure S2. *mpk6* seed phenotypes are stable.

A) Representative *mpk6* x *pABI4::GUS* silique containing the three *mpk6* classes of seeds (wb/wild-type bigger; rs/raisin-like and bs/burst seed) is shown. B) Segregating seed phenotypes and histochemical GUS activity on the F3 *mpk6* x *pABI4::GUS* seed protruding embryos are shown. In the picture the GUS positive burst-seeds are over represented, but each phenotype was present in a proportion similar to that observed in the homozygous *mpk6* mutant line (~70, ~23 and ~7%, respectively). Scale bars = 1 mm.

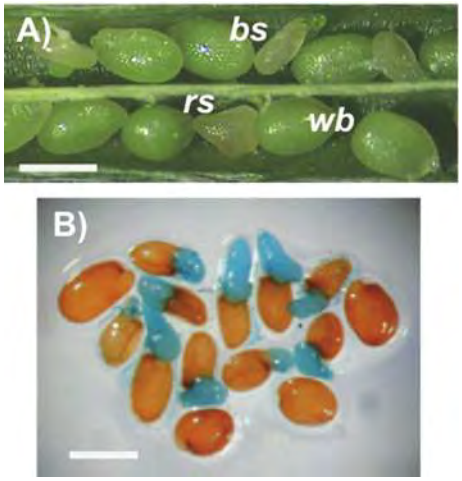
Supplemental Figure S3. *mpk6* siliques are shorter than wild-type and contain many aborted seeds. Comparison between wild-type (Wt, Col-0) and *mpk6* homozygous line of: A) The length of siliques, B) The number of seeds per silique and C) Seeds aborted per silique. The Error bars represent SE from 10 siliques along the stem of 5 independent plants (n= 50 for each). Asterisk marks Student's t-test significant differences at P indicated. D) Representative photograph of wild-type (Wt, Col-0) and *mpk6* siliques. An abortion event, apparently frequent on *mpk6* siliques, is highlighting (arrow). Scale bars = 1 mm.

Supplemental Figure S4. Effect of auxin and cytokinins on primary root growth
Primary root length inhibition caused by the indicated concentrations of: A) Idol-3Acetic Acid (IAA) and B) kinetin over wild-type (Wt, Col-0) and *mpk6wb/lr* seedlings**.** Data were recorded from 8 DAG seedlings growing in media supplemented with the corresponding hormone. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition. Values are mean ± Standard Error (n = 15). Different letters represent Tukey's post-hoc test significant differences ($P \leq 0.05$). The experiment was repeated three times with similar results**.**

Supplementary Figures



Supplemental Figure S1.



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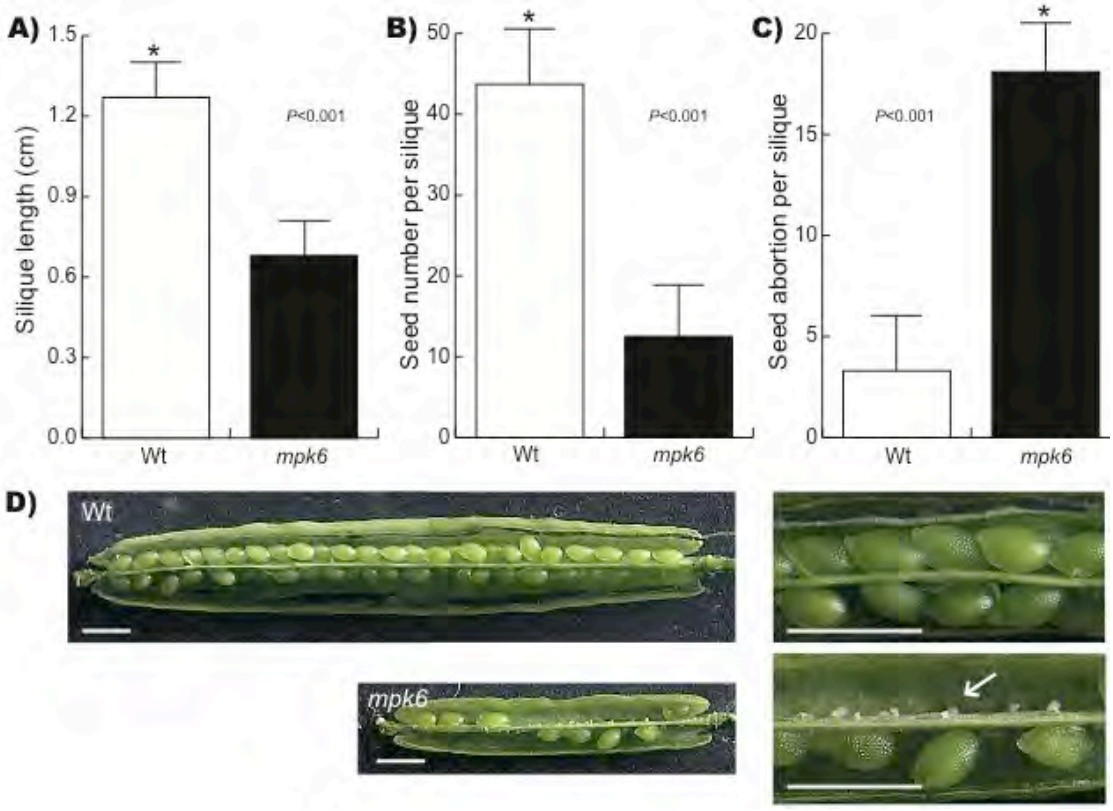

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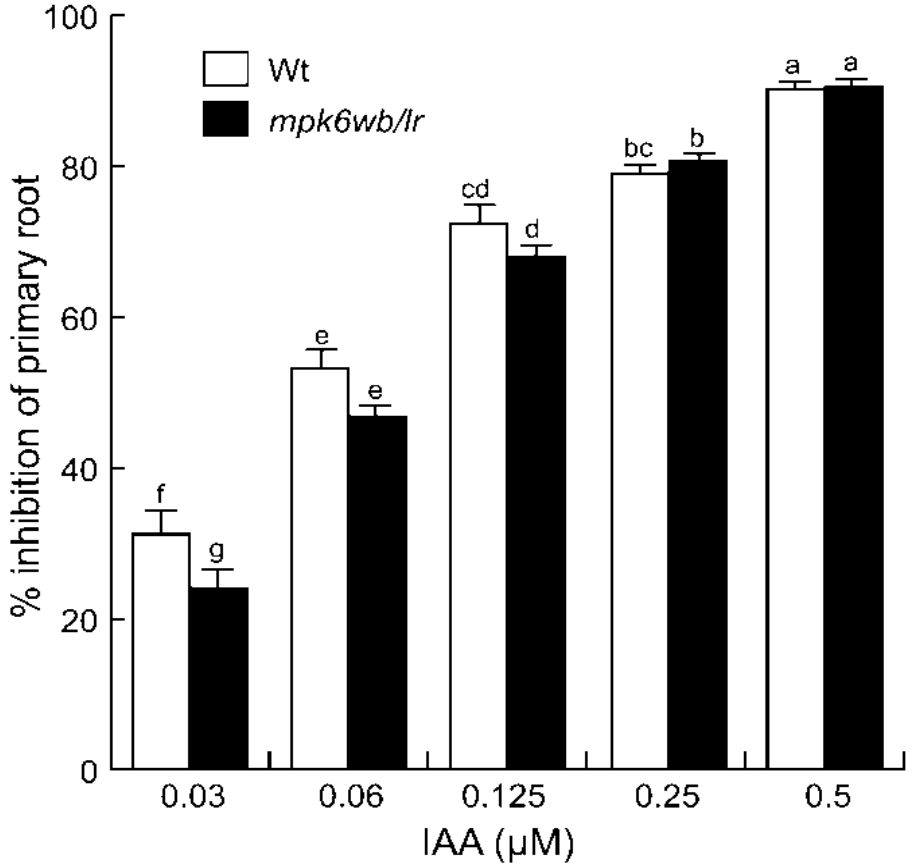

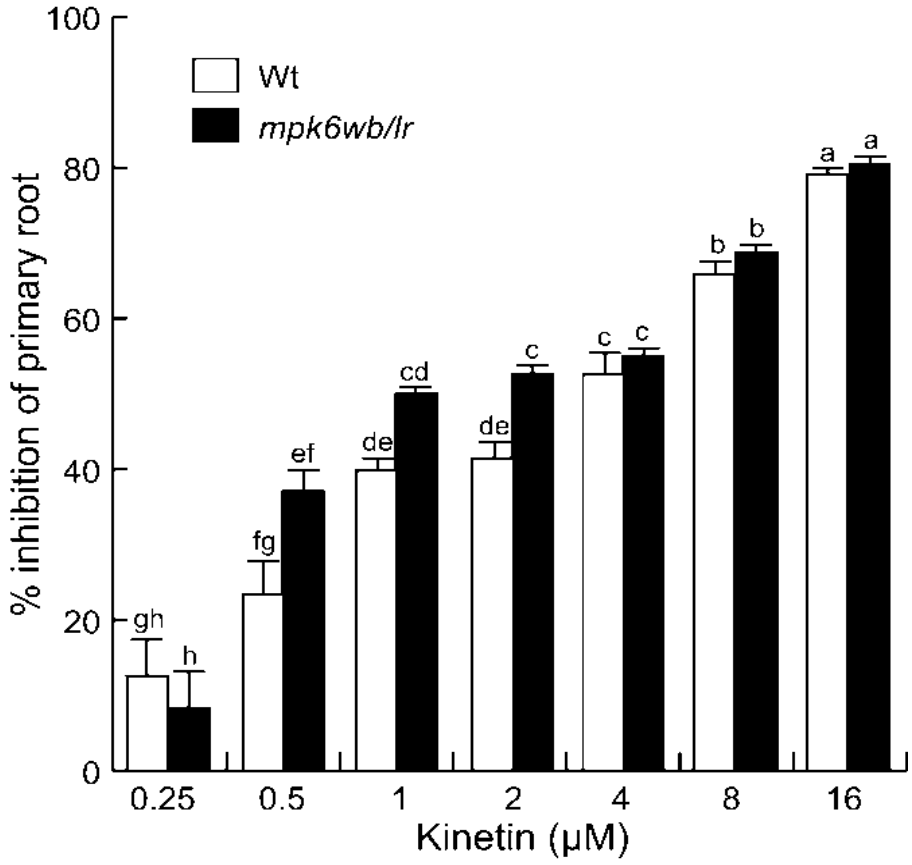

Supplemental Figure S4.

New Phytologist

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

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Full names of authors:

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

Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030, Morelia, Michoacán, México.

*Correspondence: José López-Bucio. Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México. Telephone 5 443 3265788, fax: (443) 3265788. E-mail: jbucio@umich.mx.

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Summary

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

6-pentyl-2H-pyran-2-one (6-PP), is a major VOC biosynthesized by *Trichoderma* spp. likely involved in plant-fungus cross-kingdom signaling. Using microscopy and confocal imaging, the effects of 6-PP on root morphogenesis were correlated with *DR5:GFP*, *PIN1:GFP*, *PIN2:GFP*, *PIN3:GFP* and *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*.

Running title: Fungal-plant cross-kingdom signaling via 6-pentyl-2H-pyran-2-one.

Keywords: *Trichoderma*, auxin, ethylene, root development, phytostimulation, 6-pentyl-2H-pyran-2-one.

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 (Mukherjee *et al.,* 2013; Harman *et al.,* 2004; Harman, 2011). Amongst their different attributes, *Trichoderma* spp. benefit agricultural activities acting as biofungicides, in bioremediation of soils contaminated with metals or chemical wastes, or eliciting plant development and defense (Chang *et al.,* 1986; Bjorkman *et al.,* 1998; Bjorkman, 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, biomass production or elicit developmental programs via regulation of gene expression (Chacón *et al.,* 2007; Shoresh & Harman, 2008; Vargas *et al.,* 2009, 2011, Harman, 2011; Rubio *et al.,* 2012; Studholme *et al.,* 2013; Pereira *et al.,* 2014; Martínez-Medina *et al.,* 2014).

T. virens and *T. 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 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 and coworkers (2009). Fungal colonization of *Arabidopsis* roots induced the expression of the auxin-inducible gene marker *DR5:GUS* 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 all identified *Trichoderma* auxins to *Arabidopsis* seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (mM range). In particular, application of indole-3-carboxaldehyde inhibited primary root growth, induced adventitious root formation and increased 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* not only in phystostimulation 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*., 2014b; 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). Cocultivation of *Arabidopsis* roots with *T. atroviride* modulated lateral root growth and root hair formation and increased MPK6 activity, likely depending of ethylene (ET) and auxin signaling. It was also found that ET, IAA and IAAld produced by the fungus induced MPK6 activity, while auxin-inducible *DR5:GUS* gene expression was concomitantly enhanced in *Arabidopsis* 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 MAP kinase (MAPK) kinase kinase. Detailed analysis of root hair and lateral root responses to *T. atroviride* in *Arabidopsis* WT seedlings and ethylene-related mutants *etr1*, *ein2* and *ein3* showed that the effect of ET on root morphogenesis was apparently mediated by an auxin-ethylene crosstalk involving MPK6, which fine-tunes seedling growth and development in response to *Trichoderma* (Contreras-Cornejo 2015). In consequence, MPK6, and its MAP kinase associated cascade, likely 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 include volatile and non-volatile substances such as sesquiterpenes, 6-*n*-pentyl-2*H*-pyran-2-one (6-PP), gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Vinale *et al.,* 2008; Reino *et al.,* 2008). Exposure of *Arabidopsis* seedlings to 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). This information suggests that some *Trichoderma* metabolites may be interpreted by

plants as trans-kingdom 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 emerges as a plant bioactive metabolite (Vinale *et al.,* 2008), it is important to uncover molecular components specific to root architecture remodeling and its relationship with plant genetic programs. Here, we show that *Trichoderma atroviride* produces 6-PP, whose levels increase in co-cultivation with *Arabidopsis* seedling. Supplying *Arabidopsis* seedlings with 6-PP enhanced shoot and root biomass production in a dose dependent manner and improved root branching. 6-PP induced an auxin response in primary root tips and in young lateral root primordia and differentially modulates 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 an specific component in the ethylene pathway in root architectural remodeling and highlights the complex network of signaling molecules involved in fungal-plant interaction.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 ecotype, the transgenic *Arabidopsis* lines *DR5:GFP* (Ottenschläger *et al.,* 2003), *PIN1::PIN1::GFP* (Benková *et al.,* 2003), *PIN2::PIN2::GFP* (Blilouet *et al.,* 2005), *PIN3::PIN3::GFP* (Žádníková *et al.,* 2003), *PIN7::PIN7::GFP* (Blilouet *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), *ein3* (Chao *et al.,* 1997), were used for the different

147 experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20%
148 (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and
149 grown on agar plates containing 0.2x MS medium. The MS medium (Murashige and
150 Skoog Basal Salts Mixture), was purchased from Sigma. Phytagar (commercial grade),
151 was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to
152 allow root growth along the agar surface and unimpeded aerial growth of the
153 hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L), with a
154 photoperiod of 16 h of light/8 h of darkness, light intensity of 300 µmol m^2 s^{-1}, and
155 temperature of 22 °C.
156
157 Fungal growth and plant inoculation experiments
158
159 *Trichoderma atroviride* (formerly *T. harzianum*) IMI 206040 was used. An inoculum of
160 1 x 10^6 spores was placed at 5 cm from *Arabidopsis* primary roots germinated and
161 grown 4 days on agar plates containing MS 0.2x medium. The plates, which included
162 10 *Arabidopsis* seedlings each, were arranged in a completely randomized design into a
163 Percival AR95L growth chamber. After 3 and 5-days of co-cultivation, determinations
164 of 6-PP accumulation and plant growth were done.
165
166 Effect of 6-PP on plant growth and development
167
168 6-PP was purchased from Sigma, and dissolved in ethanol. To investigate whether 6-PP
169 could have an effect on *Arabidopsis* growth, the compound was supplied at different
170 doses (0, 50, 75, 100, 125, 150, 175, and 200 µM), to the plant growth medium. In
171 control seedlings, we added ethanol in equal amounts as present in the greatest
172 concentration of the compound tested. The Petri plates with 30 plants under different
173 treatments were placed on a Percival AR95L growth chamber for 10 days to estimate
174 biomass production.
175 *Arabidopsis* root system and primary root (PR) meristem integrity were analyzed with a
176 stereoscopic microscope (Leica, MZ6 Leica, Microsystems). All lateral roots (LRs)
177 emerged from the PR were counted at 30x magnification. Images were taken with a
178 Samsung SCC 131-A digital color camera adapted to the microscope and processed
179 with the Zeiss Axio Vision 4AC software (Carl Zeiss). PR length was determined for

each root using a ruler. LR density was determined by dividing the LR number value by the PR length values for each analyzed seedling.

Propidium iodide staining and GFP detection

For confocal microscopy, solvent- or 6-PP-treated transgenic *Arabidopsis* 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. The same sample was recorded separately at wavelengths specific to both propidium iodide fluorescence, with a 568 nm excitation line and an emission window of 585-610 nm, and GFP emission, with a 500-523 nm emission filter (488 nm excitation line and emission detected at 505-550 nm), using a confocal microscope (Olympus FV1000), after which the two images were merged to produce the final image.

Determination of developmental stages of lateral root primordia (LRP)

LRP were quantified 6d after germination. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follow, Stage I: LRP initiation. In the longitudinal plane, approximately 8–10 'short' pericycle cells are formed. Stage II: the LRP is divided into two layers by a periclinal division. Stage III: the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV: an LRP with four cell layers. Stage V: the LRP is midway through the parent cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appears to be just about to emerge from the parent root.

Analysis of VOCs and 6-PP determinations

The VOCs released by *T. atroviride* were analyzed in Petri dishes containing 0.2x MS medium with a SPME technique and gas chromatography-mass spectrometry (GC-MS). The compounds were collected for 1 h with a blue SPME fiber (PDMS/DVB) (Supelco,

Inc., Bellafonte, PA, U.S.A.) and desorbed at 180 °C for 30 s in the injector port of a gas chromatograph (Agilent 7890B; Agilent, Foster City, CA, U.S.A.), equipped with a MS detector 5977A from Agilent and Mass Hunter Workstation Software for data acquisition and processing. A free fatty acid-phase capillary column (HP-FFAP) (30 m x 0.25 mm I.D., film thickness of 0.25 µm) was used. Operating conditions used helium as the carrier gas (1 ml/min), detector temperature of 250 °C. The column was held for 1 min at 60 °C, and then programmed to rise at a rate of 3 °C per minute 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 was 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 comparison of retention time 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 days of growth and during the interaction *T. atroviride – A. thaliana*, we constructed an external calibration curve using 6-PP standard following a similar method established by Polizzi *et al*. (2011). A diluted solution of 6-PP in ethanol was prepared. Petri dishes were filled with 0.2x 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 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 the SPSS 10 program. Univariate and multivariate analyses with Tukey's post hoc test were used for testing differences in growth and root development responses. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Results

6-PP is the most abundant compound within the VOCs profile of *Trichoderma atroviride*

Previous reports have shown the VOCs 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; Siddique *et al.,* 2012; Jelén *et al.,* 2014; Lee *et al.,* 2015). All this research identified the compound 6-PP within the corresponding VOCs profile. To assess the possible roles of 6-PP during interaction of *T. atroviride* with plants, in this study we analyzed the VOCs emitted from *T. atroviride* from 5d fungal colonies growth in Petri plates supplied with MS 0.2x-agar solidified medium. This medium was chosen because it is commonly used for *Arabidopsis* growth and the effects of plants on 6-PP could then be evaluated. Table 1 shows that 6-PP is the major compound within VOCs 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 with GC-MS analysis is denoted as 2-pyrone, with an alkyl group at the 6-position (Fig. 1a). The identification of 6-PP was made by comparison with retention time (37.21 min) and mass spectra from standard (Fig. 1b) with those obtained from *T. atroviride* colonies (Fig. 1c).

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 or at 3 and 5 days of direct interaction with *Arabidopsis* 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 increases by 40% as compared to the level registered 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 and promote root branching in *Arabidopsis* seedlings

To investigate the plant growth-regulating activity of 6-PP, we tested the effects of increasing, low micromolar doses of this compound in *Arabidopsis* (ecotype Columbia,

Col-0) seedlings, germinated and grown on Petri plates containing agar-solidified 0.2x Murashige and Skoog (MS) medium. The seedlings were treated with ethanol (control treatment) or with 50-200 µM 6-PP dissolved in ethanol. After 10 days of growth in medium supplied with 50-to-175 µM 6-PP, a roughly two-fold increase in shoot, root and total plant biomass was observed (Fig. 2a–c). In contrast, the greatest concentration (200 µM) of the compound did not increase biomass accumulation (Fig. 2a-c). Representative photographs of plates illustrating the biostimulation potential of 6-PP are shown in Fig. 2d-g. Noteworthy, 6-PP treatments increased both lateral root number and density in a dose-dependent manner, while an inhibition of primary root growth was determined from 125 µM onwards (Fig. 3a-c). We next analyzed the stages of lateral root primordium (LRP) development affected by 6-PP by quantifying the number of stage I-to-VII LRP originating from primary roots of 6 d.a.g seedlings treated with the solvent or with 150 µM 6-PP, this latter treatment strongly increases 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 describes young LRPs were significantly decreased in 6-PP-treated seedlings (Fig. 4a). In contrast, emerged LR number was induced 2 or 3-fold by 6-PP in seedlings at 4 and 6 d.a.g, respectively (Fig. 4b). The total number of LRP per seedling decreased in response to 6-PP treatments (Fig. 4c), whereas the LRP density, did not significantly differ among treatments (Fig. 4d). These data indicate that 6-PP likely increases lateral root branching by inducing the emergence of pre-formed LRP from pericycle cells and accelerating the growth of lateral roots.

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

Lateral root development is tightly correlated with auxin signaling (Fukaki *et al.,* 2007). To understand the role played by 6-PP in 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, lateral root primordia and emerging lateral roots in transgenic *Arabidopsis* seedlings expressing this marker and exposed to 75 and 150 µM 6-PP. *DR5::GFP* expression was slightly increased in primary root tips at 150 µM or higher 6-PP concentrations (Fig. 5a-c), which coincided with decreased root growth (Fig. 3a). Concomitantly, an analysis of *DR5::GFP* expression at stage II and V lateral root primordia and in emerging lateral roots showed an enhanced auxin-

inducible expression in the vasculature of primary roots and in developing primordia (Fig. 5d-i), as well as in emerging lateral root tips (Fig. 5j-l). These data indicate that 6-PP affects auxin signaling during primary and lateral root development.

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

Auxin is transported through the PIN family of auxin 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 lateral root formation through differential expression of PIN family of auxin transporters, we analyzed the pattern of PIN1, PIN2, PIN3 and PIN7 localization in primary roots and lateral root primordia 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, g and j). In contrast, PIN2 expression was detected in the cortex and epidermal cells (Fig. 6d). In transgenic seedlings expressing all these four markers supplied with 75 μM 6-PP the GFP fluorescence was strongly increased (Fig. 6b, e, h and k), whereas when treated with 150 μM 6-PP the opposite effect was observed for PIN1, PIN 2 and PIN7 expression as shown by decreased GFP florescence (Fig. 6c, f and l). In marked difference with the other PIN transporters, PIN3 localization in response to 150 μM 6-PP still displayed a strong expression in the stele (Fig. 6i). 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 *Arabidopsis* mutants

Ethylene-auxin interactions regulate lateral root initiation and emergence in *Arabidopsis* (Ivanchenko *et al.,* 2008). To further define whether there is a crosstalk between auxin and ethylene in controlling root responses to 6-PP, we analyzed the response of WT and *Arabidopsis* 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 test the involvement of auxin in primary

and lateral root response to 6-PP, *Arabidopsis* WT and mutant lines were grown in 0.2x MS medium supplemented with 150 µM 6-PP and primary root growth and lateral root formation analyzed 10 days after germination. It was found that all five auxin-related mutants tested showed WT responses to 6-PP in terms of primary root growth inhibition (Fig. 7a). In contrast, an induction of lateral root formation was lacking in *tir1afb2afb3*, *arf7arf19*, *axr1-3*, *aux1-7*, while *eir1* seedlings showed increased lateral root formation in response to 6-PP (Fig. 7b and c).

In opposition to auxin, ethylene has been found to repress lateral root formation (Lewis *et al.,* 2011). Therefore, we focused our analysis of 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 lateral root responses to 6-PP while EIN2 is a crucial component mediating the primary root growth inhibition to this fungal signal molecule.

Discussion

This study uncovers a novel mechanism by which *Trichoderma 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 up to date, representing different classes of chemical compounds. These compounds can be classified as volatiles, diffusible compounds and peptaibols (Gams & Bisset, 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.,* 2014c). *T. viride*, *T. harzianum*, and *T. koningii* are able to produce 6-PP (6-pentyl-α-pyrone), which plays a role in biocontrol of phytopathogens such as *B. cinerea*, *R. solani*, and *F. oxysporum* and a strong relationship exists between the biosynthesis of this metabolite and the biocontrol ability of the producing strains (Scarselletti and 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 increasing growth and producing more branched root systems (Vinale *et al.,* 2008). 6-PP is to our

knowledge the first non-auxin-like natural molecule that induces lateral root formation, 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 *Trichoderma atroviride* as part of the blend of volatiles emitted by single fungal colonies alone or in interaction with *Arabidopsis* 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 analogue 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 a G alpha subunit gene TGA1 of *Trichoderma atroviride* leads to decreased 6-PP production, continuous sporulation and elevated internal cAMP levels, which correlates with loss of mycoparasitic and antagonistic properties against *Rhizoctonia solani*, *Botrytis 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*. *Thctf1* mutants affected the yellow pigmentation and coconut aroma attributed to 6-PP production observed in the wild-type strain and affected its antimicrobial activity (Rubio *et al.,* 2009). Although the interaction of *Trichoderma* strains defective of 6-PP production with plants remains to be investigated, one possibility is that such strains may still stimulate plant growth and lateral root 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, being 6-PP another critical factor in phytostimulation.

6-PP clearly improved shoot and root growth and total biomass production of *Arabidopsis* seedlings, which was related to changes in root morphogenesis. Lateral root formation is a critical factor for water and nutrient acquisition and is an essential trait for plant adaptation to soil heterogeneity. The mechanism of lateral root 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; Strader *et al.,* 2010; Swarup *et al.,* 2007). Contreras-Cornejo *et al.* (2015) showed that the short root phenotype of mutants defective on CONSTITUTIVE TRIPLE RESPONSE 1 was likely caused by auxin being accumulated in primary root tips and that both auxin and ethylene signaling are important for *Trichoderma*-induced root hair and lateral root formation. Lateral root development consists of two successive steps: lateral root initiation and lateral root emergence from the parent root, which are controlled by auxin fluxes mediated by 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* auxin-inducible marker. Interestingly, GFP fluorescence slightly increased in the root tip in response to 6-PP treatment, consistent with an auxin-like activity of this compound. Similarly, we observed enhanced *DR5:GFP* fluorescence after 6-PP treatment in the lateral root forming regions of roots, particularly in the vascular tissue and during lateral root primordium development, which further indicates an activation of auxin signaling during the lateral root 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 *Arabidopsis* root system (Ortiz-Castro *et al.,* 2011). An interesting possibility is that 6-PP could interact with the TIR1 auxin receptor, or with receptors from the same gene family, thus acting as auxin signal mimics.

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 lateral roots or negatively affect root gravitropism due to reduced auxin transport (Benkova *et al.,* 2003; Marchant *et al.,* 2002; Swarup *et al.,* 2008; Wu *et al.,* 2007). Changes in the abundance and localization of auxin transport proteins may define the growth of primary roots or the initiation of lateral roots (Raya-

González *et al.,* 2014). Our results that 6-PP increased auxin-induced gene expression in the root apex, and in regions of lateral root initiation suggest that 6-PP affects root development by altering auxin distribution. Consistently 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 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 lateral root initiation to drive lateral root growth, whereas higher concentrations repress primary root growth likely blocking expression of PIN1 and PIN7. The increased lateral root 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-Gonzαlez *et al.,* 2014).

To analyze whether the TIR1 family of auxin receptors and downstream signaling components are involved in *Arabidopsis* responses to 6-PP, we evaluated primary root growth and lateral root formation in response to this metabolite in WT (Col-0) *Arabidopsis* 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. These results showing the involvement of 6-PP in root development add to the emerging functions of fungal molecules in plants. Based on their auxin-like 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 represent 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 *Arabidopsis* and crop plants.

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Figure legends

Fig. 1 Molecular characterization and production of 6-pentyl-2H-pyran-2-one (6-PP) by *T. 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 VOCs from the fungus, indicating the presence of 6-PP at Rt of 37.21 min. 6-PP was identified by comparison of mass spectra according with the NIST 2011 library and from commercial standard. (d) Estimation of 6-PP content in *T. atroviride* and *A. thaliana – T. atroviride* interaction system. (e) Representative photographs of the fungal colonies 3 and 5 d of growth and during the interaction with plants. Scale bar = 1 cm.

Fig. 2 Effect of 6-n-pentyl-6H-pyran-2-one (6PP) on plant biomass production. *Arabidopsis* (Col-0) seedings 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 0.2x MS medium or (e) in 0.2x MS medium supplemented with 75, (f) 125, and (g) 175 µM 6-PP. Photographs show representative plates, each treatment included three plates. Data from a-c show means ± SD from 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. Scale bar = 1 cm.

Fig. 3 6-PP regulates *Arabidopsis* root system architecture. *Arabidopsis* (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 per cm). Values represent the means of 30 seedlings ± SD. Different letters represent means statistically different ($P < 0.05$). The experiment was repeated three times with similar results. Scale bar = 1 cm.

Fig. 4 Effect of 6-PP on LR development in *Arabidopsis.* WT (Col-0) seedlings were germinated and grown for 2, 4 and 6d on 0.2x MS media supplemented with the solvent (control) or 150 µM 6-PP. (a) LRP per plant in four-day-old seedlings. (b) Kinetics of emerged LR in seedlings grown during 2, 4 and 6 days. (c) Total LRP per plant. (d) LRP density in four-day-old seedlings. Error bars represent SE from 15 *GUS*-stained seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.

Fig. 5 6-PP modulates auxin-responsive gene expression in the lateral root formation zone. *DR5:GFP* seeds were germinated and grown on agar-solidified 0.2x MS medium or supplemented with 75 or 150 µM 6-PP. Five days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. Photographs show representative individuals of at least 10 seedlings. (a-c) Primary root apical meristems. (d-f) Stage II LRP. (g-i) Stage V LRP, (j-l) Emerged LR. Note that 6-PP treatments increase *DR5:GFP* reporter expression in the lateral root formation zone. Bars = 100 µm.

Fig. 6 Expression of auxin efflux transporters in response to 6-PP in primary roots. *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN3::PIN3::GFP* and *PIN7::PIN7::GFP* seedlings were germinated and grown in media with the solvent only or supplied with 75 or 150 µM 6-PP. Four days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. (a-c), Primary root of *PIN1::PIN1::GFP*, (d-e) *PIN2::PIN2::GFP*, (g-i) *PIN3::PIN3::GFP* and (j-l) *PIN7::PIN7::GFP*. Representative photographs of primary roots are shown (n = 10).

Note the increase of *PIN3::PIN3::GFP* expression is proportional to the increase of 6-PP treatments. Bars = 100 µm.

Fig. 7 6-PP requires components of auxin response and transport to modify *Arabidopsis* root system architecture. *Arabidopsis* WT 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 0.2x MS medium supplemented with the solvent (control) or 150 µM 6-PP. (a) PR length. (b) LR number. (c) Representative photographs of *Arabidopsis* seedlings grown in the indicated 6-PP treatment. Values shown represent the means of 15 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results.

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

Table 1. Volatile organic compounds produced by *Trichoderma atroviride* 5d of growth in 0.2x MS medium and analyzed by SPME-GC-MS.

Compounds	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 m.w. sesquiterpene)	0.86 ± 0.08
Unknown (a 204 m.w. sesquiterpene)	1.71 ± 0.18
Unknown (a 204 m.w. sesquiterpene)	1.07 ± 0.09
6-Pentyl-2H-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 ± standard errors of the sum of three independent determinations.

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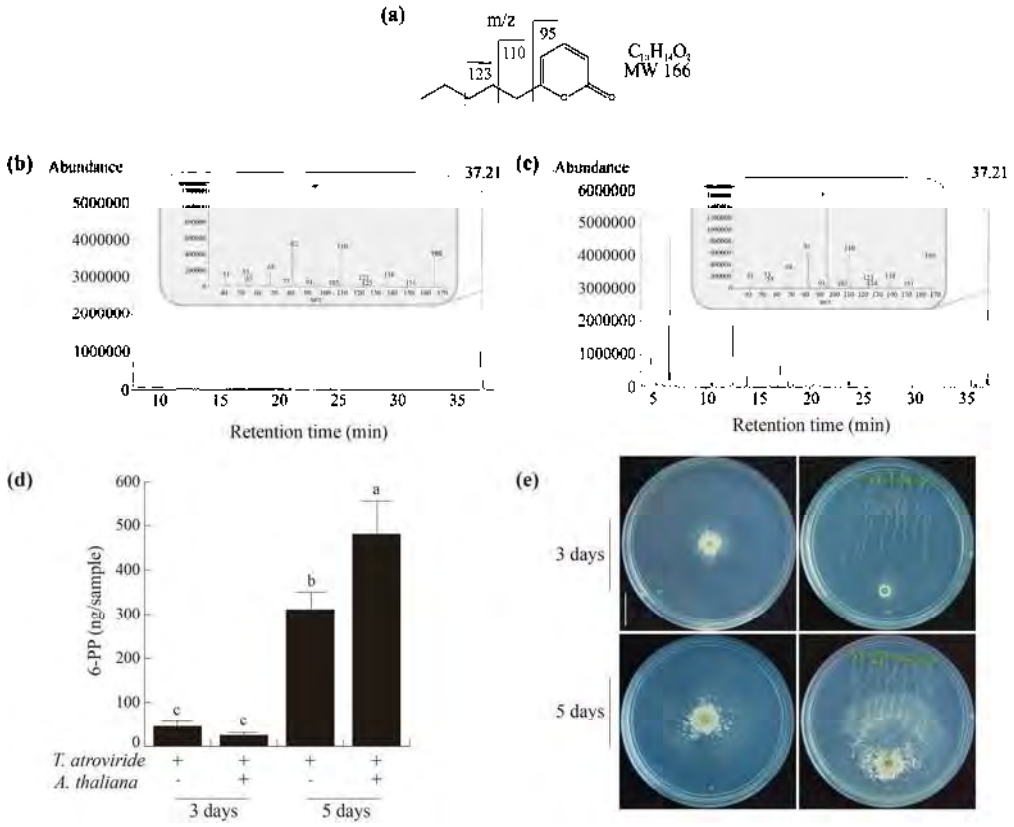

Fig. 1 Molecular characterization and production of 6-pentyl-2H-pyran-2-one (6-PP) by T. atroviride IMI 206040. (a) Chemical structure of 6-PP showing the major fragment ions (m/z) of electron ionization mass spectra. (b) Total ion chromatogram and mass spectra from commercial standard (6-PP, Rt = 37.21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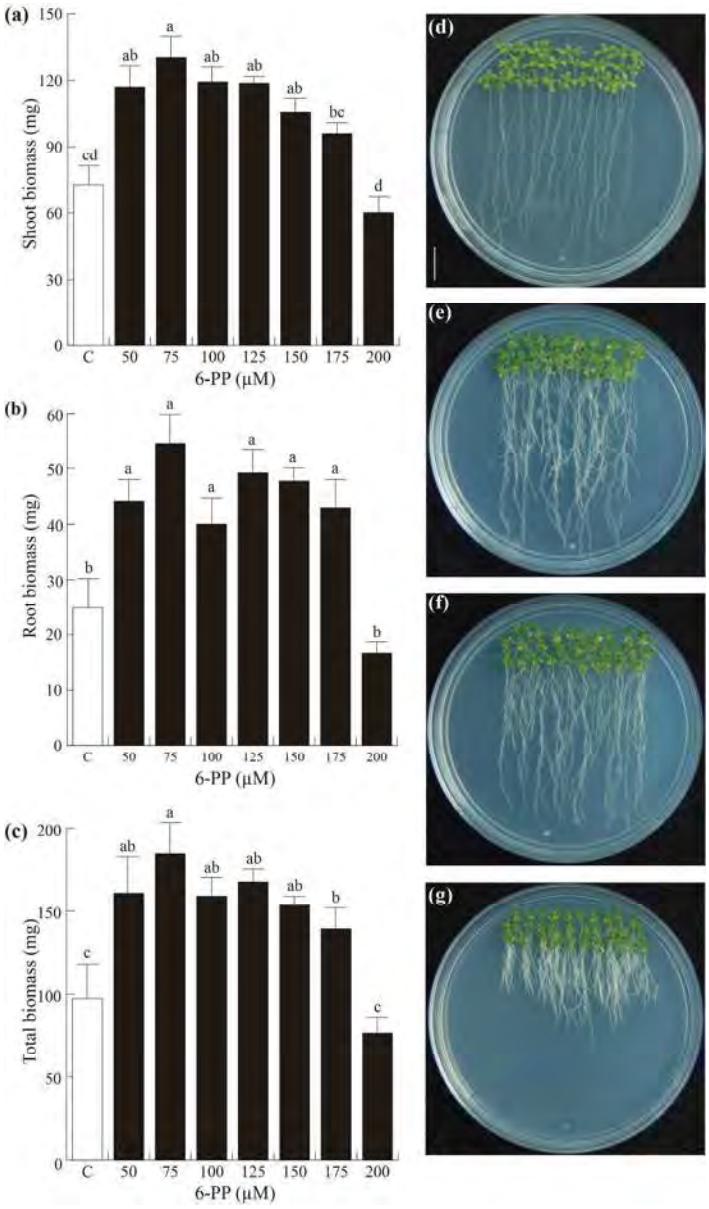

Fig. 2 Effect of 6-n-pentyl-6H-pyran-2-one (6PP) on plant biomass production. Arabidopsis (Col-0) seedings were germinated and grown for 12 d under increasing 6-PP concentrations. (a) Shoot biomass. (b) Root biomass. (c) Total biomass. (d) Representative photographs of seedlings grown in 0.2x MS medium or (e) in 0.2x MS medium supplemented with 75, (f) 125, and (g) 175 μM 6-PP. Photographs show representative plates, each treatment included three plates. Data from a-c show means ± SD from 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. Scale bar = 1 cm.
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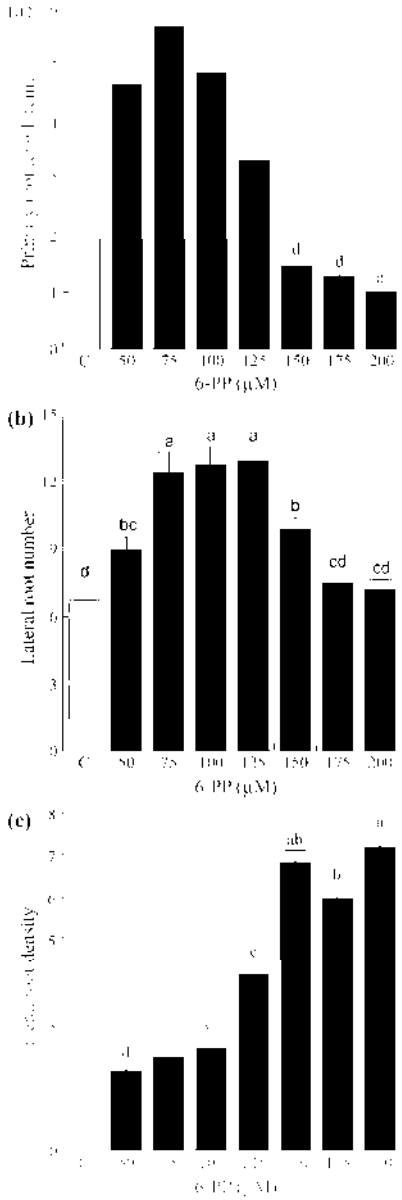

Fig. 3 6-PP regulates Arabidopsis root system architecture. Arabidopsis (Col-0) seedlings were germinated and grown for 10 d under increased 6-PP concentrations. (a) Primary root length. (b) Number of emerged lateral roots. (c) Lateral root density (number of emerged lateral roots per cm). Values represent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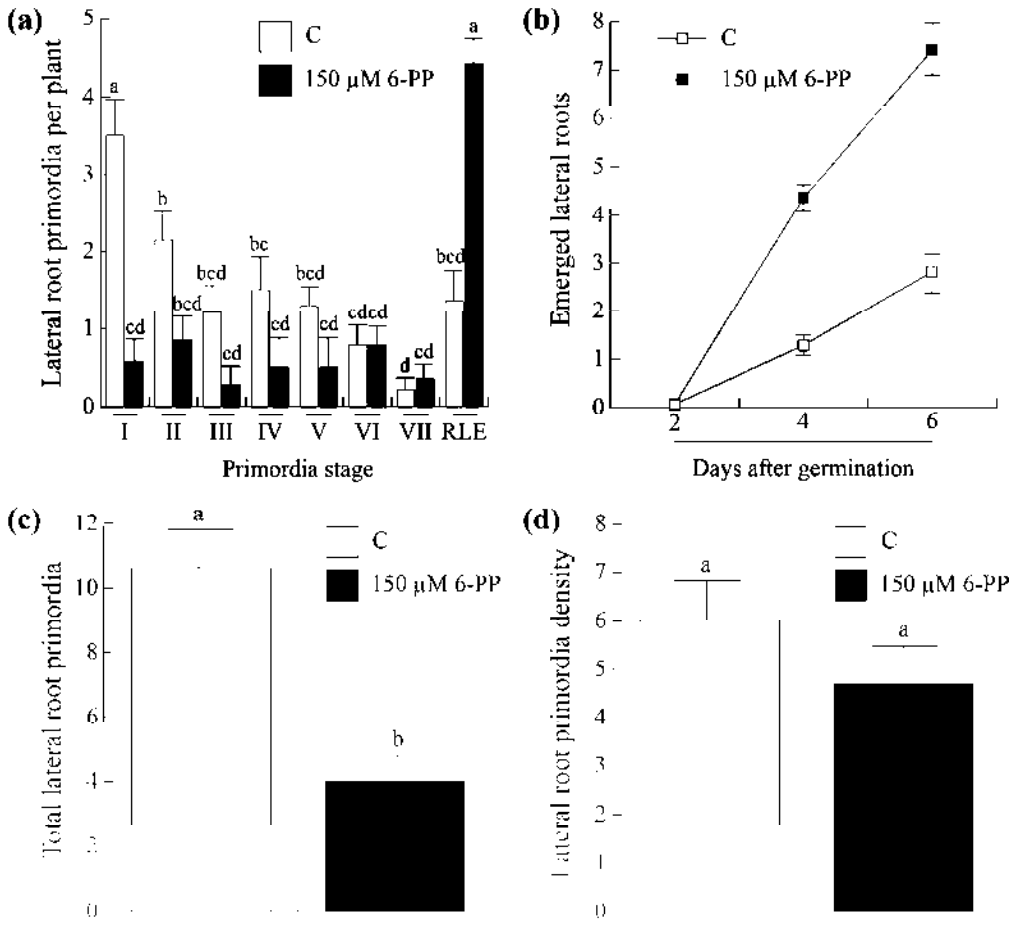

Fig. 4 Effect of 6-PP on LR development in Arabidopsis. WT (Col-0) seedlings were germinated and grown for 2, 4 and 6d on 0.2x MS media supplemented with the solvent (control) or 150 µM 6-PP. (a) LRP per plant in four-day-old seedlings. (b) Kinetics of emerged LR in seedlings grown during 2, 4 and 6 days. (c) Total LRP per plant. (d) LRP density in four-day-old seedlings. Error bars represent SE from 15 GUS-stained seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated two times with similar results.
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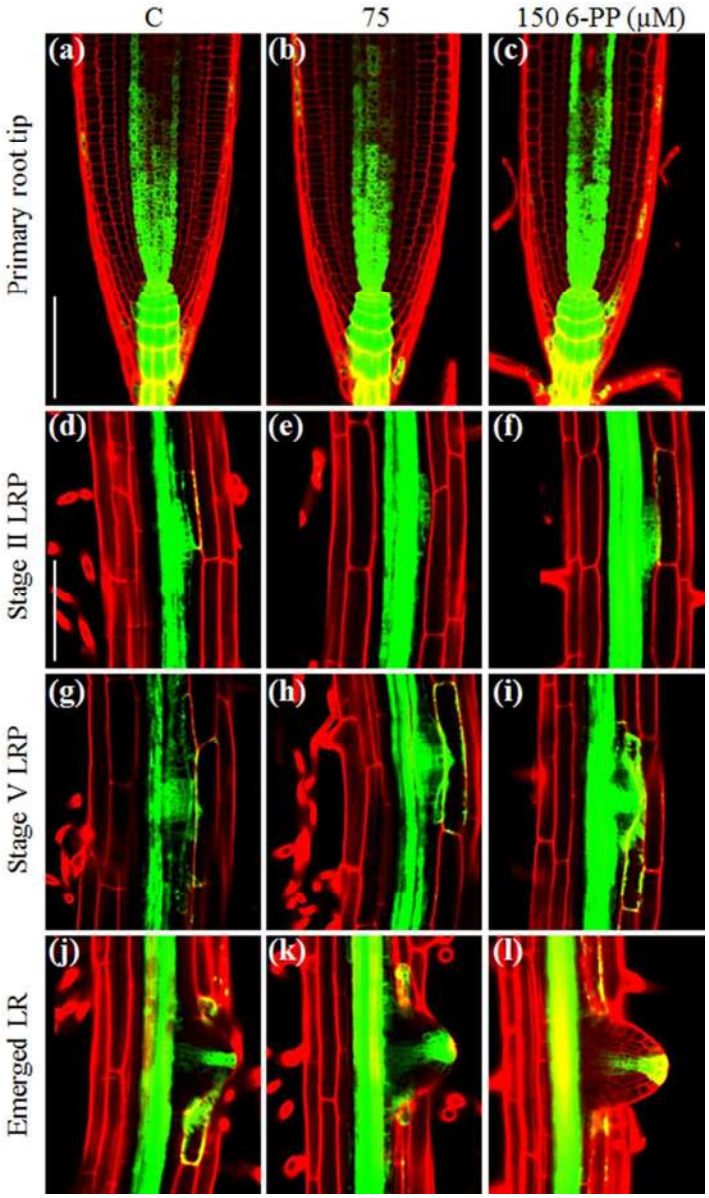

Fig. 5 6-PP modulates auxin-responsive gene expression in the lateral root formation zone. DR5:GFP seeds were germinated and grown on agar-solidified 0.2x MS medium or supplemented with 75 or 150 µM 6-PP. Five days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. Photographs show representative individuals of at least 10 seedlings. (a-c) Primary root apical meristems. (d-f) Stage II LRP. (g-i) Stage V LRP, (j-l) Emerged LR. Note that 6-PP treatments increase DR5:GFP reporter expression in the lateral root formation zone. Bars = 100 µm.
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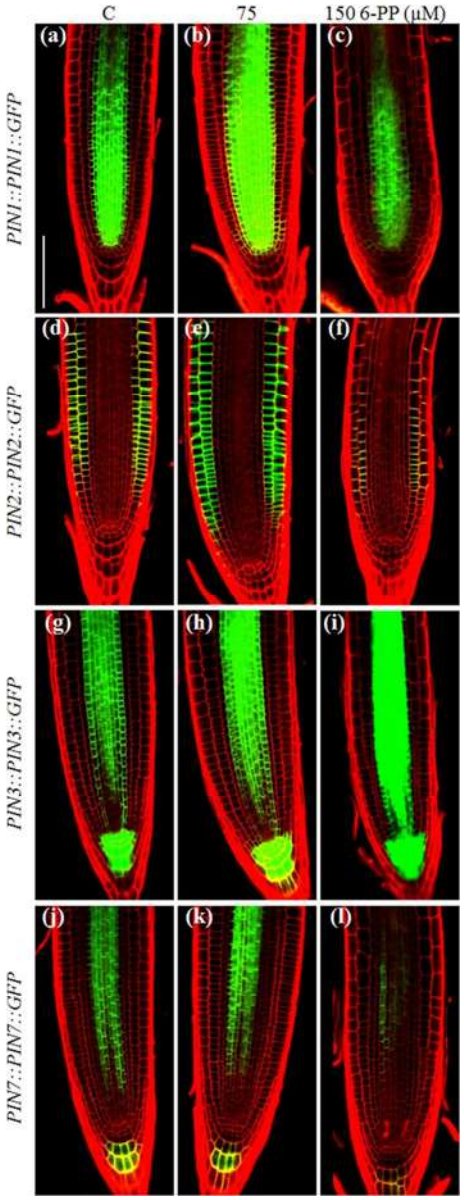

Fig. 6 Expression of auxin efflux transporters in response to 6-PP in primary roots. PIN1::PIN1::GFP, PIN2::PIN2::GFP, PIN3::PIN3::GFP and PIN7::PIN7::GFP seedlings were germinated and grown in media with the solvent only or supplied with 75 or 150 μM 6-PP. Four days after germination, the seedlings were stained with propidium iodide and analyzed by confocal microscopy. (a-c), Primary root of PIN1::PIN1::GFP, (d-e) PIN2::PIN2::GFP, (g-i) PIN3::PIN3::GFP and (j-l) PIN7::PIN7::GFP. Representative photographs of primary roots are shown (n = 10). Note the increase of PIN3::PIN3::GFP expression is proportional to the increase of 6-PP treatments. Bars = 100 μm.
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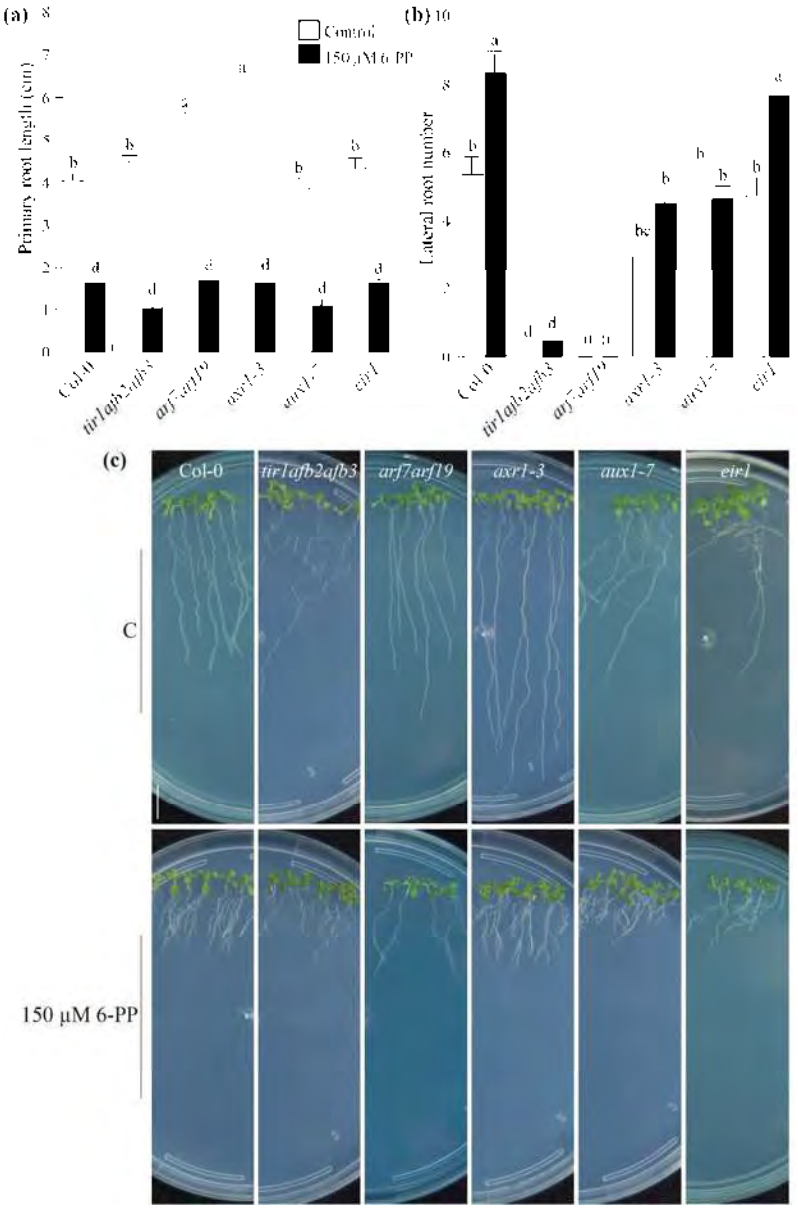

Fig. 7 6-PP requires components of auxin response and transport to modify Arabidopsis root system architecture. Arabidopsis WT and tir1/afb2/afb3, arf7-1/arf19-1, axr1-3, aux1-7 and eir1, triple, double or single mutant seedlings, respectively, were germinated and grown for 10 d in 0.2x MS medium supplemented with the solvent (control) or 150 µM 6-PP. (a) PR length. (b) LR number. (c) Representative photographs of Arabidopsis seedlings grown in the indicated 6-PP treatment. Values shown represent the means of 15 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results.
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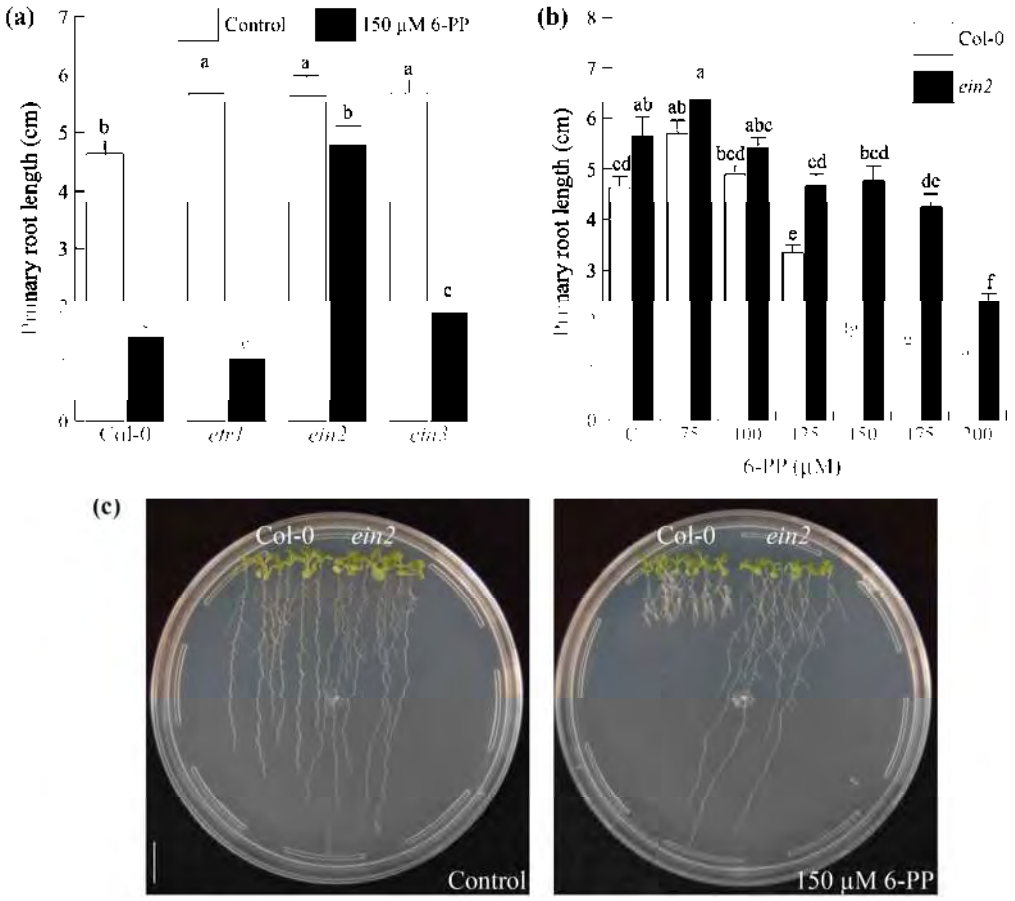

Fig. 8 EIN2 is necessary for 6-PP-modulated primary root growth. Arabidopsis WT and etr1-1, ein2-1, and ein3-1 ethylene related mutant seedlings, were germinated and grown for 10 d in 0.2x MS medium supplemented with the solvent (control) or 150 µM 6-PP. (a) PR length. (b) Primary root growth of ein2 mutants in response to increasing concentrations of 6-PP (c). Representative photographs of seedlings grown (Col-0 and ein2 grown side by side) in the indicated 6-PP treatment. Values shown represent the means of 15 seedlings ± SD. Different letters indicate means statistically different ($P < 0.05$). The experiment was repeated twice with similar results.
246x219mm (300 x 300 DPI)

Plant Molecular Biology

ALTERED MERISTEM PROGRAM 1 is involved in seed coat, root hair and trichome development in Arabidopsis.

--Manuscript Draft--

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Corresponding Author:	José López-Bucio, Ph.D. MEXICO
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	José López-Bucio, Ph.D.
First Author Secondary Information:	
Order of Authors:	José López-Bucio, Ph.D.
	Claudia Marina López-García
	Javier Raya-González
	Jesús Salvador López-Bucio
	Angel Arturo Guevara-García
Order of Authors Secondary Information:	
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Abstract:	During plant development, cells interpret positional information and translate it into patterned cell differentiation. The epidermis differentiates into several types of specialized cells, giving rise to the seed coat in the embryo, root hairs in roots and trichomes in shoots. ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a glutamate carboxypeptidase involved in embryo development, plant growth and phytohormone homeostasis. Here, we show that AMP1 plays a pleiotropic role in epidermal tissue differentiation. AMP1 mutants defective in two independent alleles (amp1-10 and amp1-20), show increased frequency of embryo abortion, low seed production and retarded germination. They also display four distinct and stable seed phenotypes defined as "regular", "raisin", "irregular" and "burst" seeds, which are related to an altered seed coat differentiation program. We further analyzed the trichome and root hair phenotypes of wild-type and all four amp1-10 seed classes and found that amp1 seedlings produce less trichomes per leaf, and short or bifurcated root hairs in primary roots. Our data suggest that AMP1 is necessary for the normal seed coat and embryo establishment during seed development and plays an important role in epidermal cell differentiation in roots and leaves.
Suggested Reviewers:	Tamara Western McGill University tamara.western@mcgill.ca Prof. Western is an expert in seed biology
	Maarten Koornneef Max Planck Institute for Plant Breeding Research

	koornneef@mpipz.mpg.de Prof. Koornneef is an expert in seed development and plant morphogenesis.
	George Haughn University of British Columbia george.haughn@ubc.ca Prof. Haughn is an expert in seed biology and plant morphogenesis.
	Beverley Glover University of Cambridge bjg26@cam.ac.uk Prof. Glover is an expert in epidermal differentiation.

Full title: *ALTERED MERISTEM PROGRAM 1* is involved in seed coat, root hair and trichome development in *Arabidopsis*

Running title: Role of *AMP1* in epidermal cell differentiation

Full names of authors: Claudia Marina López-García[1], Javier Raya-González[1], Jesús Salvador López-Bucio[2], Ángel Arturo Guevara-García[2], and José López-Bucio[1]*

[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030, Morelia, Michoacán, México.

[2]Instituto de Biotecnología-UNAM. Av. Universidad No. 2001, Col. Chamilpa, Cuernavaca, Morelos, México.

*__Author to whom correspondence should be addressed__.

José López-Bucio. Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México. Telephone 5 443 3265788, fax: (443) 3265788, E-mail adress: jbucio@umich.mx

Abstract

During plant development, cells interpret positional information and translate it into patterned cell differentiation. The epidermis differentiates into several types of specialized cells, giving rise to the seed coat in the embryo, root hairs in roots and trichomes in shoots. *ALTERED MERISTEM PROGRAM 1 (AMP1)* encodes a glutamate carboxypeptidase involved in embryo development, plant growth and phytohormone homeostasis. Here, we show that AMP1 plays a pleiotropic role in epidermal tissue differentiation. *AMP1* mutants defective in two independent alleles (*amp1-10* and *amp1-20*), show increased frequency of embryo abortion, low seed production and retarded germination. They also display four distinct and stable seed phenotypes defined as “regular”, “raisin”, “irregular” and “burst” seeds, which are related to an altered seed coat differentiation program. We further analyzed the trichome and root hair phenotypes of wild-type and all four *amp1-10* seed classes and found that *amp1* seedlings produce less trichomes per leaf, and short or bifurcated root hairs in primary roots. Our data suggest that AMP1 is necessary for the normal seed coat and embryo establishment during seed development and plays an important role in epidermal cell differentiation in roots and leaves.

Keywords: *Arabidopsis*, cell differentiation, seed coat, root hairs, trichomes, development

Abbreviations

ABA	Abscisic acid
ABI	ABSCISIC ACID INSENSITIVE
CK	Cytokinins
ET	Ethylene
GA	Gibberellins
JA	Jasmonic acid
NO	Nitric oxide
PA	protoanthocyanidin
WT	Wild-type

Introduction

Plant form and function require specialization of cells through acquisition of distinct morphological, biochemical and physiological properties. The epidermis is the protective outer layer of clonally related cells covering all plant organs at most developmental stages. It is composed of cells that differentiate in adaptively significant frequencies and patterns (Glover 2000; Javelle et al. 2011). The embryo seed coat and seedling epidermis arise from the outer cell layer during embryogenesis. The leaf and stem epidermis originate after germination from the shoot apical meristem, while the root epidermis develops from the root apical meristem. Epidermal differentiated cells include stomatal guard cells, which allow gas exchange, trichomes that protect the aerial parts from herbivores, and root hairs that increase the root surface area for water and nutrient uptake (Ishida et al. 2008; Casson and Hetherington 2010; Bruex et al. 2012).

Seeds ensure the propagation of angiosperms via embryo protection and geographical dispersion. Seed development initiates after a double fertilization event that gives rise to the three major components of the seed, i) the embryo, which comes from egg cell fertilization, ii) the endosperm, which comes from central cell fertilization and provides supply of nutrients to the developing embryo, and iii) the seed coat, which originates from maternal integuments and act as a mechanical and protective barrier (Haughn and Chaudhury 2005; Dekkers et al. 2013). After fertilization, cell division begins yielding an apical and a larger basal cell, the apical cell continues dividing to finally produce the hypocotyl, the shoot meristem and cotyledons. The basal cell divides horizontally producing the suspensor, whose hypophyseal cell participates in

the formation of the embryonic root meristem (Breuninger et al. 2008; Lau et al. 2012; Locascio et al. 2014). The final seed size, weight and form are coordinately determined by endosperm development, growth of the embryo and differentiation of the integuments (Berger et al. 2006; Zhou et al. 2009). The proper embryo development depends on an adequate provision of sucrose and nutrients by the endosperm and seed coat; if endosperm development fails or transport of sucrose via the seed coat is affected, embryo development does not proceed normally (Hehenberger et al. 2012; Lafon and Köhler 2014; Chen et al. 2015). Genetic analysis suggests that the endosperm produces a signal that initiates the differentiation of the integuments to produce the seed coat (Berger et al. 2006; Ingouff et al. 2006; Figueiredo and Köhler 2014). Few loci have been implicated in the development of the seed coat in *Arabidopsis*, including *APETALA 2* (*AP2*) and *TRANPARENT TESTA GLABRA 1* (*TTG1*) which regulates elongation of seed coat cells, protoanthocyanidin (PA) and mucilage biosynthesis (Debeaujon et al. 2000; Orozco-Arroyo et al. 2015). Moreover, *TTG1* and *AP2* loss of function causes limited and prolonged cell proliferation of endosperm, respectively, indicating an endosperm-seed coat cross-talk during seed development (Koornneef 1981; Koornneef et al. 1982; Jofuku et al. 1994; Figueiredo and Köhler 2014).

Following germination, the plant epidermis plays important structural and adaptive roles. In primary and lateral roots, epidermal cells differentiate into two cell types following a file-specific program, root-hair cells, also named trichoblast or H (Hairy) cells, and non-hair cells, termed atrichoblasts or N (Non hair) cells. Trichoblasts are commonly located between two cortical cells and are thought to control root hair determination and differentiation via differential

signal exchange (Rerie et al. 1994; Masucci et al. 1996; Ishida et al. 2008; Libault et al. 2010). TTG1 and GLABRA 2 (*GL2)* transcription factors are important regulators of root hair specification controlling the expression of genes that modify the cell wall composition, such as *CELLULOSE SYNTHASE 5 (CESA5)* and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* 17 (XTH17) (Tominaga-Wada et al. 2009; Libault et al. 2010). After root hair specification, several processes including cytoskeleton dynamics, vesicle trafficking and ion and metabolite exchange regulate the polarized growth of root hairs (Rerie et al. 1994; Masucci et al. 1996; Ishida et al. 2008; Libault et al. 2010). In leaves, some epidermal cells differentiate as trichomes, which act in defense against herbivores, in protection against UV light, and in production of protective chemicals (Hülskamp et al. 1994; Pattanaik et al. 2014). In *Arabidopsis,* trichome cells are three branched structures, whose specification depends on a signaling network involving GLABRA 3 (GL3), GLABRA 1 (GL1) and *TTG1* (Pattanaik et al. 2014).

Plant growth regulators control epidermal cell differentiation thoroughly. Auxin, ethylene (ET), cytokinin (CK), abscisic acid (ABA) and gibberellin (GA) signaling pathways play crucial roles in seed development, dormancy and germination. Phytohormones regulate both root hair and trichome development via changes in expression of genes encoding transcription factors, enzymes and structural components, such as cellulases and expansins required to achieve the final morphology and function of epidermal cells (Hülskamp et al. 1994; Ishida et al. 2008; Pattanaik et al. 2014). Although seed coat differentiation, root hair and trichome development rely on the expression of a common set of genes, very little is known about the specific role of each gene product in generic cell

patterning specification. Mutations that affect the correct differentiation of generic epidermal cells usually leads to lethality, while defects in specialized epidermal cell types often interfere with plant growth and／or development without causing lethality (Javelle et al. 2011). This may explain why little is known about the molecular mechanisms sustaining the differentiation of generic epidermal cells during embryogenesis, while an increasing body of information of the control of trichome, stomata and root hair development is accumulating (Ishida et al. 2008; Casson and Hetherington 2010; Bruex et al. 2012).

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes an endoplasmic reticulum membrane-localized glutamate carboxypeptidase involved in several developmental processes in plants. Mutations in *Arabidopsis AMP1* gene cause large-scale alterations in phenotype including abnormal embryo development, altered number of cotyledons, de-etiolation in dark grown seedlings, increased leaf initiation, dwarfing, earlier flowering, semi-sterility and increased cell proliferation (Chaudhury et al. 1993; Mordhorst et al. 1998; Nogué et al. 2000a, b; Vidaurre et al. 2007; Kong et al. 2015). These alterations have been related to increased levels of endogenous CKs, along with altered ABA and nitric oxide signaling and cell cycle gene expression (Griffiths et al. 2011; Arc et al. 2013; Shi et al. 2013 a,b). In this study, we provide evidence that *AMP1* has a key role in the development of three different epidermal cell types, namely the seed coat, root hairs and trichomes, suggesting a primary role of this glutamate carboxypeptidase not only in coordinating embryo development to the formation of the seed coat, but also in post-embryonic epidermal cell differentiation programs.

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes Columbia (Col-0), Landsberg erecta (Ler) and Wassilewskija (Ws), and the single, double and triple mutant lines *amp1-10* (SALK_021406), *amp1-20* (SALK_138749), *cre1-12* (Inoue et al. 2001), *ahk2-2* (Ueguchi et al. 2001), *ahk3-3* (Ueguchi et al. 2001), *det2-1* (Chory et al. 1991), *rpn12* (Smalle et al. 2002), *ein2* (Guzman and Ecker 1990), *ein3* (Chao et al. 1997), *etr1* (Bleecker et al. 1988), *eto3* (Woeste et al. 1999), *ctr1* (Kieber et al. 1993), *Atnoa1* (Guo et al. 2003), *nia1nia2* (Wilkinson and Crawford 1993), *jar1* (Tiryaki and Staswick 2002), *pft1-2* (Raya-González et al. 2014), *aux1-7* (Pickett et al. 1990), *axr2-1* (Wilson et al. 1990), *slr1* (Fukaki et al. 2002), *arf7arf19* (Okushima et al. 2007), *tir1afb2afb3* (Parry et al. 2009), *abi1* (Leung et al. 1997), *abi2* (Leung et al. 1997), *abi3* (Nambara et al. 1992), *abi4* (Finkelstein et al. 1998), *abi5* (Finkelstein 1994) and *drr1* (Morquecho-Contreras et al. 2010) were used for the experiments reported here.

Seeds were surface sterilized with ethanol 95%, sodium hypochlorite 20% for 5 and 7 minutes, respectively, and then washed five times with 1 ml sterile distilled water and incubated for 7 days at 4 ºC. The seeds were plated into 0.2X solidified MS medium containing MS basal salts (Murashige and Skoog Basal Salts Mixture, Sigma-Aldrich, St Louis MO), 1% agar (Phytagar Gibco-BRL) and 1% sucrose (Sigma-Aldrich, St Louis MO) and incubated in a plant growth chamber (Percival AR-95L) at 22 ºC with a photoperiod of 16h light/ 8h darkness under light intensity of 105 µmol/m^{-2}/s^{-1}.

Seed coat analysis

The seed phenotype analysis was performed using a stereoscopic microscope MZ6 (Leica Microsystems). Seed phenotypes of *amp1* mutant were classified and photographed with a camera adapted to the microscope (Cyber-shot DSC-S75; Sony Electronics), and the seed length and width were determined with ImageJ program (Wayne Rasband National Institutes of Health, USA). For the analysis of the seed coat, dry WT and *amp1-10* seeds were covered with a copper layer and analyzed and photographed using a Jeol JSM-7600F field emission scanning electron microscope, equipped with a Bruker X-Flash6/30 camera.

Seed germination assays

For germination assays, mature seeds from WT and the four *amp1-10* mutant classes were sterilized and plated into 0.2X MS medium and/or the same medium supplemented with 1 µM GA, and incubated in a plant growth chamber to register germination at the time when radicle was completely emerged.

Trichome, root hair and silique analyses

Arabidopsis WT and *amp1-10* mutant seeds were surface disinfected and plated as described above and then grown into a plant growth chamber (Percival AR-95L). Root hairs were analyzed from primary roots of 7 day-old seedlings and trichomes were counted and photographed from 14 day-old

leaves. For embryo development analysis, WT and *amp1* mutant seedlings were grown in Petri plates for 12 days and then transferred to soil until fruit production. 10 siliques were dissected with a needle and the developing seeds and embryos photographed with a stereoscopic microscope (MZ6, Leica Microsystems), and with a DM5000B differential contrast microscope, respectively.

Mucilage detection

Dry seeds of *Arabidopsis* WT and *amp1* mutants were incubated in 1 ml 50 mM EDTA at 1000 rpm during 2 h, and then incubated in agitation in 0.01% (w/v) Ruthenium Red at 1000 rpm during 1 h. Stained seeds were mounted on glass slides and analyzed and photographed with an optic microscope DM5000B (Leica Microsystems) using Differential Interference Contrast (DIC) microscopy.

Embryo analysis

Arabidopsis WT and *amp1* embryos were dissected from the silique using a needle and then incubated for two days in Hoyer´s solution (prepared as described in Seed Genes Project database; http://www.seedgenes.org). After clearing, the seeds were analyzed and photographed by DIC microscopy in a DM5000B optic microscope (Leica Microsystems).

Results and discussion

AMP1 mutation affects fruit size and seed production driving *Arabidopsis* plants to produce four phenotypically distinctive seed classes

Seed development is an important agricultural and adaptive trait. An analysis of *Arabidopsis* WT (Col-0) and *amp1-10* mutants grown in soil showed that *amp1* siliques were 65% shorter than WT, which correlated with decreased seed production and ovule fertilization (Supplementary Fig. S1). To analyze seed viability, we obtained several seed pools from individual homozygous *amp1-10* and *amp1-20* mutants and compared with WT seeds. Surprisingly, both *amp1* mutant alleles exhibited four segregating phenotypically distinctive seed classes not previously described for these mutants (Fig. 1a-e and Supplementary Fig. S2), including seeds that we defined as “regular”, “raisin”, “irregular” and “burst”. In the first class (~80% *amp1-10*/regular seeds), the loss-of-function of AMP1 caused slight alterations in seed morphology (Fig. 1b and Supplementary Figs. S2-S3). The second class (~8% *amp1-10*/raisin seeds) included seeds with rough coats (Fig. 1c and Supplementary Figs. S2, S3). The third class (~10% *amp1-10*/irregular seeds) had alterations in shape, seed coat structure and pigmentation (Fig. 1d and Supplementary Figs. S2, S3), whereas the smaller class (~2% *amp1-10*/burst seeds phenotype) included seeds with protruding embryos (Fig. 1e and Supplementary Figs. S2, S3). An AMP1 overexpressing line AMP1 OX2 showed normal seed phenotypes both in form and size whereas *amp1-10* mutants developed smaller and thinner seeds (Supplementary Figs. S3, S4). These data show that although the loss of

function in AMP1 affects seed form and size higher AMP1 levels did not disrupt normal seed morphogenesis.

Since most phytohormones are known to participate in seed development, we tested whether the seed phenotype of *amp1* mutants could be observed in *Arabidopsis* mutants affected in auxin, ET, CK, ABA, nitric oxide (NO) and jasmonic acid (JA) signaling pathways. Dry seed screening, considering form, width and length, of *cre1-12*, *ahk2-2*, *ahk3-3*, *det2-1*, *rpn12*, *ein2*, *ein3*, *etr1*, *eto3*, *ctr1*, *Atnoa1*, *nia1nia2*, *jar1*, *pft1-2*, *sag13*, *aux1-7*, *axr2-1*, *slr1*, *arf7arf19*, *tir1afb2afb3*, *abi1*, *abi2*, *abi3*, *abi4*, *abi5* and *drr1,* homone-related mutants, revealed that the *abi5* and *tir1afb2afb3* single and triple mutants, respectively, were longer than WT seeds (Supplementary Fig. S5). However, none of the above mentioned mutants showed the seed phenotypes already found in the *amp1* mutants.

AMP1 seeds show defects in seed coat structure and mucilage production

The outer cell layer of *Arabidopsis* seeds consists of polygonal structures that form a donut-shaped pocket with a volcano-shaped central elevation known as columella, which results from deposition of mucilage between the primary cell wall and protoplasm (Windsor et al. 2000; Volodymyr and Borisjuk 2014). To determine if *AMP1* modulates seed morphology through modifications on seed coat cells, we performed an ultra-structure scanning electron microscopy analysis on mature WT and *amp1-10* seeds. This analysis revealed the characteristic polygonal cells with thickened radial cell walls and well-defined columella in WT seeds (Fig. 2a, b, c). In contrast, the seed coat of the four

279 different *amp1-10* seed classes exhibited unusual epidermal cells (Fig. 2d-o).
280 Although no significant differences in size were evident, “regular” and “raisin”
281 seeds showed more robust columellas than the WT (Fig. 2f, i, p, q), whereas
282 “burst” seeds had an abnormal organization of seed coat cells, lacking well-
283 defined cell walls and columellas (Fig. 2m, n, o, p, q).
284 Mucilage mainly consists of pectin, which contains large amounts of
285 galacturonic acid and rhamnose, and low amounts of monosaccharides such as
286 arabinose, galactose, glucose, xylose and mannose forming complex
287 polysaccharides (Voiniciuc et al. 2015). Seed mucilage content can be
288 estimated via ruthenium red staining, which reveals the acidic biopolymers
289 found in the seed coat (Hanke and Northcote 1975). A ruthenium red staining
290 assays on WT seeds revealed inner and outer domains, the inner domain was
291 defined by a characteristic magenta color, which radiates out from the mucilage
292 pocket, whereas the outer mucilage domain is defined by an unstained halo
293 surrounding the inner layer (Fig. 3a, b ,c). The staining assay in *amp1-10*
294 “regular” and “raisin” seeds showed similar staining patterns as in WT seeds
295 (Fig. 3d-i), but in the “irregular” and “burst” seeds, regions without mucilage
296 staining could be observed (Fig. 3j-o), indicating a direct link between *amp1*
297 seed phenotypes and the proper formation of the mucilage capsule during seed
298 development.
299
300 **Relationship between seed coat and embryo development in *Arabidopsis***
301 ***amp1* mutants**
302
303 Some *amp1* embryos are affected during globular stage, producing new organs

304 with cotyledon identity via ectopic cell divisions (Vidaurre et al. 2007). This was
305 further confirmed through an analysis of embryo development in which *amp1-10*
306 mutants with “regular” seed coats commonly developed embryos with three
307 cotyledons (Fig. 4a-s). These abnormal embryos were able to fill the seed and
308 the development of the seed proceeded normally, giving rise to testas with
309 phenotype similar to the WT (Fig. 4b-j, l-t). A more detailed microscopic analysis
310 of mature *amp1-10* seeds, revealed that “irregular” seeds contained embryos
311 arrested at early stages during their development (Fig. 5e-h). Other “irregular”
312 seeds apparently lacked embryos (Fig. 5c, d), while “burst” seeds often included
313 cell masses protruding from the seed coat (Supplementary Fig. S6). In most
314 cases, an altered embryo development in *amp1-10* mutants correlated with
315 defective seed testas (Fig. 5c-j).
316
317 **Altered seed shape in *amp1* mutants correlates with viability**
318
319 *AMP1* has been involved in seed dormancy, germination and ABA responses
320 (Griffits et al. 2011; Shi et al. 2013b). To determine if the seed coat alterations
321 of *amp1* mutants could be related to viability, we compared germination
322 frequencies between WT and *amp1* seeds. WT seeds started germination 24 h
323 after sown and reached 100% around 32 h. On the other hand, *amp1* seeds
324 with “regular” phenotype germinated earlier than the WT, but showed
325 decreased viability as about 25% of the seeds did not germinate (Fig. 6).
326 “Raisin”, “irregular” and “burst” *amp1* seeds also germinated earlier than WT,
327 but only around 40%, 15% and 8% of seeds, respectively were able to
328 germinate. Since gibberellic acid (GA) induced germination (Ullah et al. 2002;

329 Holdsworth et al. 2008), we tested if GA addition could normalize germination in
330 *amp1* seeds. Indeed, GA promoted germination of both WT and *amp1* “regular”
331 seeds but failed to restore germination in “raisin”, “irregular” and “burst” a*mp1*
332 seeds (Fig. 6). These results indicate that *amp1* seeds have decreased viability,
333 which is apparently related to altered seed coat and embryo development.
334
335 ***AMP1* is involved in post-embryonic shoot and root epidermal cell**
336 **differentiation**
337
338 The plant epidermis plays important roles during post-embryonic development.
339 Trichomes are specialized epidermal cells formed on leaf and stems that protect
340 the aerial parts from herbivores. Root hairs also are specialized epidermal cells
341 developed in primary and lateral roots that play a critical role in water and
342 nutrient acquisition (Javelle et al. 2011).To determine if *AMP1* could have a
343 function in differentiation of root and shoot epidermal cells, we compared root
344 hair and trichome formation in 7 and 14 day-old WT and *amp1* seedlings,
345 respectively. Compared with WT (Col-0), *amp1* seedlings germinated from
346 *“*regular” and “raisin” seeds produced 50% less trichomes per leaf (Fig. 7a-k).
347 Trichome production was even lower (75%) in seedlings from “irregular” and
348 “burst” seeds (Fig. 7k). Additionally, *amp1* produced abnormal trichomes, which
349 fail to branch (Fig. 7l). When comparing root hair development in WT and *amp1*
350 seedlings, no alterations in root hair density were evident, but interestingly, the
351 root hair length decreased in *amp1* seedlings (Fig. 8a-o) and some of these root
352 hairs were abnormally bifurcated (Fig. 8k-m, p). These results suggest that

353 *AMP1* plays an important role in epidermal cell differentiation, not only during
354 seed coat development, but also in trichome and root hair differentiation.
355
356 **Discussion**
357
358 Genes controlling cell differentiation are important for coordinating the activities
359 of specialized tissues and organs, mutations in these genes can cause large-
360 scale changes in the structure of an organism. Mutation of *AMP1* leads to
361 several defects in embryo development, germination, photomorphogenesis,
362 shoot apical meristem, flowering and hormonal responses (Chaudhury et al.
363 1993; Helliwell et al. 2001; Saibo et al. 2007; Vidaurre et al. 2007; Griffiths et al.
364 2011; Shi et al. 2013b). In this work, we performed a detailed analysis of seed
365 morphology in two *amp1* mutant alleles (*amp1-10* and *amp1-20*) and
366 established a correlation between defective embryo development, seed coat
367 structure and viability. Four phenotypic seed classes were identified in the
368 progeny of homozygous *amp1* seedlings, including seeds with “regular” (WT)
369 appearance along with seeds showing rough or very irregular coats and with
370 protruding embryos. The alterations in seed structure and embryo development
371 were reproducible in at least four generations of homozygous *AMP1* plants and
372 could be traced back to early seed development in siliques from *amp1* plants
373 showing the reported phenotypes of altered shoot development and early
374 flowering (Chaudhury et al. 1993; Vidaurre et al. 2007; Griffiths et al. 2011; Shi
375 et al. 2013b). Thus, we conclude that *amp1* seed phenotypes are genetically
376 stable and apparently they are related to the previously reported embryo
377 defects in this mutant.

Arabidopsis seed development is controlled by several genes, including *FUSCA 3* (*FUS3*), *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *LEAFY COTYLEDON 1* and 2 (*LEC1, 2*), *AP2*, *TTG1* and *GL2*. All four *abi3*, *lec1*, *lec2*, and *fus3* mutants are severely affected in seed maturation (Bäumlein et al. 1994; Parcy et al. 1997; Nambara et al. 1995; Braybrook et al. 2006; Chiu et al. 2012; Koornneef 1981; Koornneef et al. 1982; Jofuku et al. 1994). To the best of our knowledge, none of these mutants display the *amp1* seed coat phenotypes described here. Scanning electron microscopy analysis of epidermis of *amp1* seeds revealed that “raisin”, “irregular” and “burst” classes of *amp1* seeds had a deformed surface, which is phenotypically similar to the *wrinkled 1* (*wri1)* mutant defective in an APETALA 2/ETHYLENE RESPONSIVE ELEMENT BINDING (AP2/EREB) transcription factor involved in seed storage metabolism (Focks and Benning 1998; Cernac and Benning 2004). Any possible relationship between *WRI1* and *AMP1* cannot be excluded based on the similarity of seed phenotypes. Another interesting connection between defective embryo development, seed coat specification and sugar transport was recently revealed from characterization of *Arabidopsis* mutants defective on sucrose transporters SWEET11, 12, and 15. The corresponding mutants exhibited specific tissue and temporal expression patterns in developing seeds, and a *sweet11;12;15* triple mutant showed severe seed defects, including retarded embryo development, reduced seed weight, and reduced starch and lipid content that result in a “wrinkled” seed phenotype similar to that of *amp1* “raisin” or “irregular” seeds. In *sweet11;12;15* triple mutant, starch accumulates in the seed coat but not in the embryo, implicating SWEET mediated sucrose efflux in the transfer of sugars from seed coat to embryo. An open possibility waiting to be demonstrated is that *AMP1*

403 could regulate SWEET family sucrose transporters for sugar portioning to
404 embryos and in this way affect the seed developmental program.
405 Previous research documented that mutation in the *MITOGEN ACTIVATED*
406 *PROTEIN KINASE 6(MPK6)* affects seed morphology and embryo development
407 leading to formation of three distinct seed phenotypes including rough seed
408 coats and seeds with protruding embryos, which correlate with defects in
409 seedling root development (López-Bucio et al. 2014). Here, taking into account
410 previous findings by Chaudhury et al. 1993, and our analysis of embryo
411 development in WT and *amp1-10* mutants, we propose that the absence of
412 embryos in some “irregular” seeds of *amp1* is likely explained because of a
413 failure in the fertilization process of egg and/or central cell. Abortion events
414 were observed in *amp1* siliques, particularly from “irregular” seeds, as these
415 contained embryos with retarded growth or lacked an embryo. In contrast, the
416 analysis of less affected “regular” *amp1* seeds confirmed that this mutation
417 causes an exaggerated activity of the shoot apical meristem, yielding embryos
418 with three cotyledons likely due to CK overproduction (Chaudhury et al. 1993;
419 Nogué et al. 2000a).
420 The closest *AMP1* homologous protein is a human glutamate carboxypeptidase
421 II (GCPII), which is up-regulated in many tumors but its role in cancer
422 development or the cell cycle is currently unknown (Hlouchova et al. 2012). It is
423 possible that the abnormal divisions during embryo development in *amp1*
424 embryos arose through changes in the proteins controlling the plant cell cycle,
425 but this hypothesis needs to be verified experimentally. The *Arabidopsis* mature
426 seed coat consists of epidermal cells that produce mucilage and its proper
427 development is important to seed dispersion, water retention and embryo

428 protection (Arsovski et al. 2010; Haughn and Western 2012). During
429 germination, the mucilage is hydrated to form a gelatinous capsule composed
430 by two layers: an inner one strongly adhered to the coat and another water-
431 soluble. Using electronic scanning microscopy and ruthenium red staining, we
432 determined that *amp1* seed coats are defective not only in epidermal cell
433 structure, but also in formation of mucilage pocket. *amp1* "irregular" and "burst"
434 phenotypical classes have areas of mucilage pocket thinner than the WT, and
435 in some seed areas the mucilage layers are missing. Interestingly, the outer
436 layer increased its size in "regular" *amp1* seeds suggesting that *AMP1* is
437 important for correct differentiation of the seed coat. Mutations on MUM genes
438 cause defects in both mucilage production and chemical seed composition
439 (Western et al. 2001). In particular, the MUM4 gene is necessary for columella
440 formation. MUM4 encodes an enzyme implicated in rhamnose biosynthesis and
441 is thought to be regulated by *GL2* and *TTG* (Western et al. 2004). One
442 interesting possibility arising from our previous and current data is that *AMP1*
443 could regulate the expression and/or activity of master transcription factors
444 regulating seed coat development or genes acting downstream such as MUM4.
445 The similar seed phenotypes caused by mutations in *MUM4*, *AMP1* and *MPK6*
446 raises the significant question about the identity of the growth regulator
447 mediating both seed coat and embryo development defects. To address this
448 question, we compared the seed phenotypes of WT, *amp1* and 27 hormone-
449 related mutants. None of these mutants showed the *amp1* seed phenotypes
450 described here, suggesting that *AMP1* controls seed development
451 independently of the classical hormonal pathways, which evidently regulate
452 other aspects of seed development, dormancy or germination. Our observations

453 further suggest a possible genetic link with MAPK signaling. Mutations in the
454 MPKKK4 (YDA) protein kinase gene cause defects in embryo development
455 resulting in protruding embryos similar to those observed in *mpk6* and *amp1*
456 mutants (Lukowitz et al. 2004). Besides, *MPKKK4* and *MPK6* are components
457 of a common MAPK cascade involved in regulation of the embryo (Bush and
458 Krysan 2007), stomata (Wang et al. 2007) and root hair development (López-
459 Bucio et al. 2014), indicating their important role in more generic epidermal
460 developmental programs.
461 Germination begins with water uptake by the seed and proceeds to radicle
462 emergence through the epidermis. The embryo, seed coat and endosperm
463 coordinately regulate seed dormancy and germination, independently or
464 synergistically depending of the plant species (Bewley 1997). In a previous
465 report, treatments with GA improved by 60% *amp1* germination (Griffits et al.
466 2011). The data from “regular” *amp1* seeds are in agreement with this previous
467 report. However, GA failed to normalize seed germination of most *amp1* seeds
468 with “raisin”, “irregular” and “burst” phenotypes, suggesting that the seed coat
469 defects in these mutants likely occur independently of GA signaling and that
470 seed germination did not proceed because the embryos failed to develop.
471 In *Arabidopsis*, some common genes are involved in the production of seed
472 mucilage, root hair and trichome development, including *TTG1* and *GL2*
473 (Walker et al. 1999; Rerie et al. 1994). *ttg1* and *gl2* mutants lack the mucilage
474 pocket in the seed coat, moreover, the trichomes and root hairs are defective in
475 these mutants. This prompted us to investigate whether *amp1* mutants could
476 have any developmental alteration during trichome and root hair development.
477 The *amp1* mutants had reduced numbers of trichomes on leaves, which were

shorter and less branched than the WT. In contrast, when compared to WT seedlings, *amp1* mutants developed short, abnormally branched root hairs. Together, these observations support a pleiotropic function of *AMP1* on generic epidermal cell differentiation programs. The opposite phenotypes of trichomes and root hairs observed in *amp1* mutants can be explained because epidermal differentiation processes in leaves and roots are controlled by the same sets of genes but in opposite way. One gene cassette regulates trichome cell fate; another cassette regulates root hair organization. In the leaf epidermis *TTG*, *GL2* and an upstream myb family factor (*GL1* or *CAPRICE*, *CPC*) induce trichome differentiation. In roots, *TTG*, *GL2* and *CPC* are used to block root hair differentiation (Benfey 1999; Schiefelbein 2003). Lin and Schiefelbein (2001) found that the *GL2* expression starts in the protoderm stage during embryo development and concluded that the cell pattern of trichomes and root hairs are established early during embryogenesis. Indeed, *GL2* and *TTG1* are required for both mucilage synthesis and columella formation. From these evidences, it is tempting to speculate that *AMP1* could regulate *GL2* and/or *TTG* expression impacting the generic epidermal processes during the embryogenesis and post-embryonic development.

In summary, our results suggest that *AMP1* serve in the differentiation of epidermal cells from the embryo, root and leaf. Future studies to examine *AMP1* gene expression and function in *Arabidopsis* mutants with seed coat and epidermal phenotypes such as *yoda* and *mpk6* could shed much light on MAPK signaling involved in generic epidermal patterning.

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871 **Figure legends**

872

873 **Fig. 1.** *AMP1* mutation causes four different seed phenotypes. Seeds from wild-
874 type plants (Col-0) are shown in (**a**). (**b-e**) *amp1-10* mutant seeds that were
875 collected from individual homozygous plants and separated according to the
876 seed phenotype using a dissection microscope: (**b**) Seeds with phenotype
877 similar to WT ("regular"). (**c**) Seeds resembling raisins ("raisin"). (**d**) Seeds with
878 clearly collapsed coats ("irregular"). (**e**) Seeds with embryos protruding from the
879 seed coat ("burst"). Scale bar= 500 μm.

880

881 **Fig. 2.** *AMP1* mutation affects seed coat structure. Dry seeds were covered with
882 a copper layer and observed with a scanning electron microscopy. (**a-c**) Seed
883 phenotype of *Arabidopsis* WT (Col-0). Note hexagonal epidermal cells with
884 thickened radial cell walls and volcano-shaped columella at the center of each
885 cell. (**c**) Close-up of WT seed coat. *amp1* mutant seed photographs with
886 "regular" (**d-f**), "raisin" (**g-i**), "irregular" (**j-l**) and "burst" (**m-o**) phenotypes.
887 Raisin, irregular and burst *amp1* phenotypes show altered seed coat shape and
888 columella structure. (**p**) Diameter of mucilage pocket. (**q**) Columella area
889 (measured from electronic micrographs using the image J program). Error bars
890 represent SD from 30 seeds analyzed. Different letters indicate statistical
891 differences at $P<0.05$. This analysis was performed from three individual *amp1*
892 seed harvests with similar results.

893

894 **Fig. 3.** The mucilage capsule of seeds is altered in *amp1* mutant. *Arabidopsis*
895 WT and *amp1-10* mutant seeds were staining with ruthenium red and observed
896 in a Leica DM5000B microscope. The mucilage capsule from WT (Col-0) seeds
897 shows the characteristic outer and inner domains, the inner region stained in
898 magenta color radiates out from the mucilage pocket and the outer, unstained
899 mucilage domain covers the inner layer. The coat of “irregular” and “burst”
900 *amp1* seeds show decreased mucilage capsule width in some areas of the
901 seed coat. Scale bars indicated in (**a-c**), are the same for all photographs in a
902 column.
903
904 **Fig. 4.** *amp1* embryos are affected in development. *Arabidopsis* WT (Col-0)
905 embryos at different developmental stages are shown: globular (**a**); pre-heart
906 (**c**); heart (**e**), cotyledon (**g**) and blend (**i**) stages. *amp1-10* mutant embryos are
907 shown in (**k**), (**m**), (**o**), (**q**) and (**s**) panels. WT and *amp1-10* embryos in the
908 same file were taken from siliques at a similar stage of development. To the
909 right of each embryo picture a close up of the corresponding seed coat is
910 shown. All the pictures were taken using Nomarsky optics. Scale bars indicated
911 in (**a**), (**b**), (**k**) and (**l**) are for all pictures within the same column.
912
913 **Fig. 5.** Halted embryo development and defective seed testa in *amp1* mutants
914 appear to be related. *Arabidopsis* WT (Col-0) and *amp1* seeds were taken from
915 the same silique and clarified with chloral hydrate. WT embryos and their
916 corresponding seed coats are shown (Col-0; **a, b**), *amp1-10* mutant (**c-i**
917 embryos; **d-j** seed coats). Note that defective embryo development in *amp1-10*

"irregular" seeds seems to be related to an altered seed testa. Scale bar indicated in (**a**) is the same in all the pictures.

Fig. 6. Seed germination in WT *amp1* mutants. *Arabidopsis* WT (Col-0) and *amp1* seeds were collected from individual plants. *amp1* seeds were grouped according to the different seed phenotypes: Seeds were sown on 0.2X MS medium or the same medium supplemented with 1 µM gibberelic acid (GA). Beginning at 16 hours, the percentage of germination (radicle protrusion) was evaluated every 4 h during 16 h. Note the germination percentages of all seed classes of *amp1* mutant are lower and that GA fails to rescue the germination of "irregular" and "burst" seeds.

Fig. 7. Trichome development is affected in *amp1* mutant. Trichomes of *Arabidopsis* WT (Col-0) and *amp1* seedlings germinated and grown on 0.2X MS medium were analyzed using a stereomicroscope. Seedling (**a, c, e, g, i**) and leaf close up (**b, d, f, h, j**) phenotypes of WT (**a,.b**) and *amp1* mutant (**c-j**), are shown. Scale bars shown in (**a, b**) are the same for the corresponding column. Arrows in (**d, h**) are used to show abnormal trichomes. Note that besides the evident reduction in the number of trichome per leaf (**k**), an abnormal trichome branching (without branches or less branched than WT) is observed in all *amp1* seedling classes (**l**). Error bars represent SD from 10 leaves analyzed. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

Fig. 8. Root hair development is affected in *amp1* mutant. Root hairs of *Arabidopsis* WT (Col-0) and *amp1* mutant seedlings were scored 7 days after germination from a primary root using a stereomicroscope (**a-j**) or mounted on slides and visualized with the Nomarsky optics (**k-m**), then measured with image J program (http://imagej.nih.gov/ij/). Root hairs from WT (**a, b**), *amp1* "regular" seeds (**c, d**), "raisin" seeds (**e, f**), "irregular" seeds (**g, h**), or "burst" seeds (**i, j**), are shown. Arrows in (**d, f**, **j**) are used to show small (**f**) and branched root hairs (**d, j**). Representative photographs of WT (**k**) and small (**l**), and branched root hairs (**m**) from *amp1* mutant seedlings using Nomarsky optics, are shown. Root hair density (number of root hairs/mm of root), length and percentage of normal, small and branched root hairs from each *amp1* seed classes are plotted in (**n, o**, **p**) panels, respectively. Error bars represent SD from 10 plants analyzed. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

Figure 1
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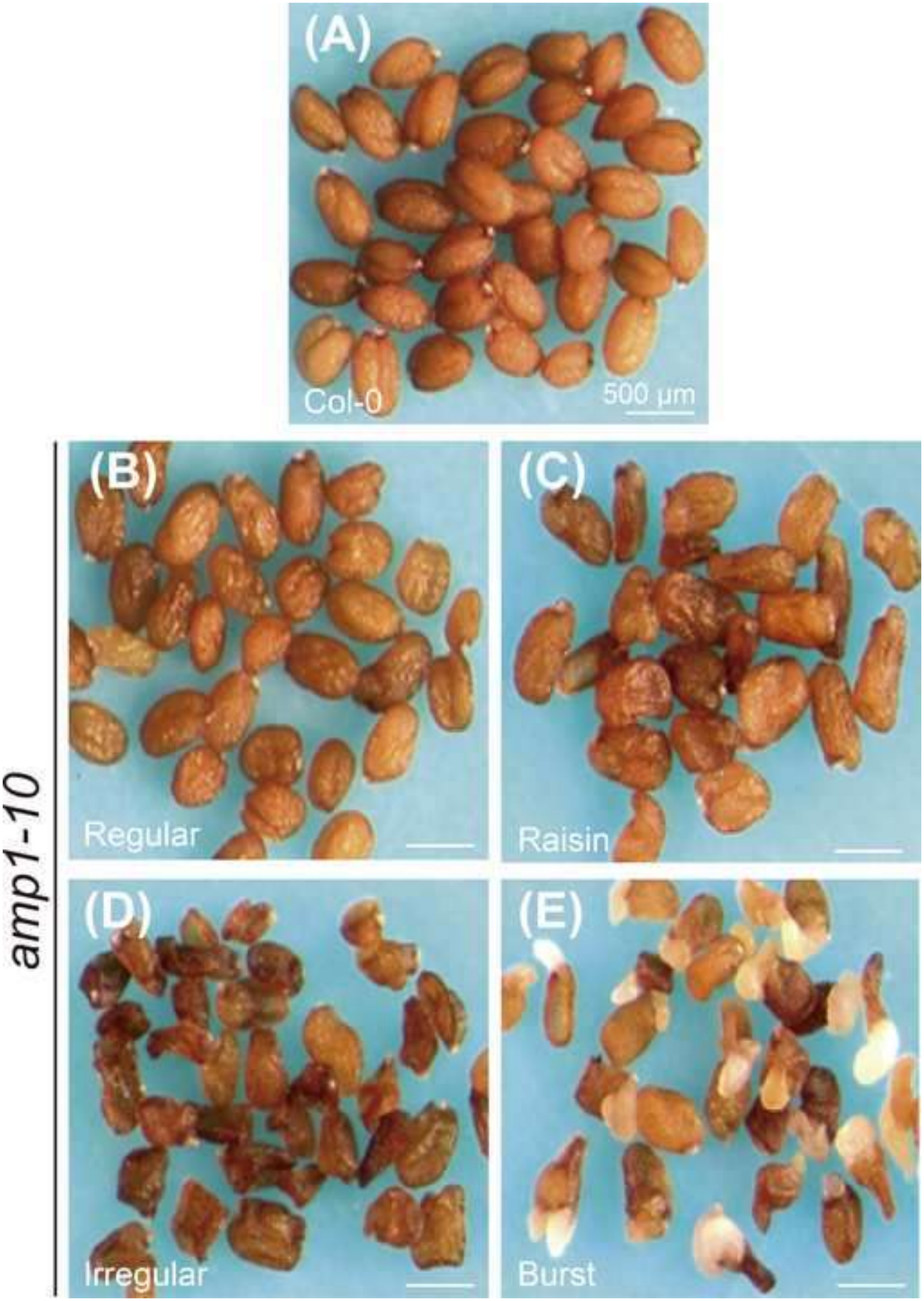

Figure 2

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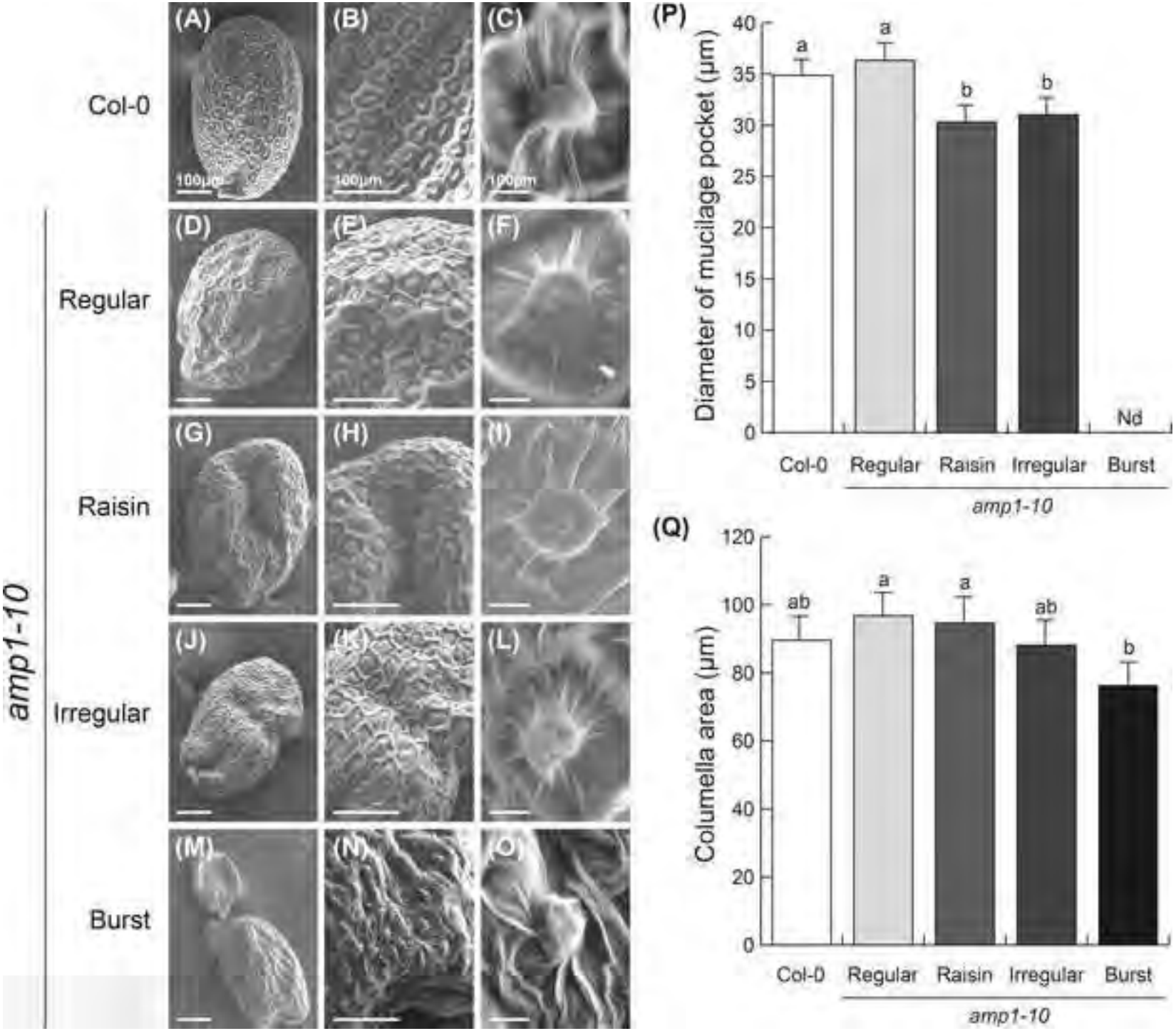

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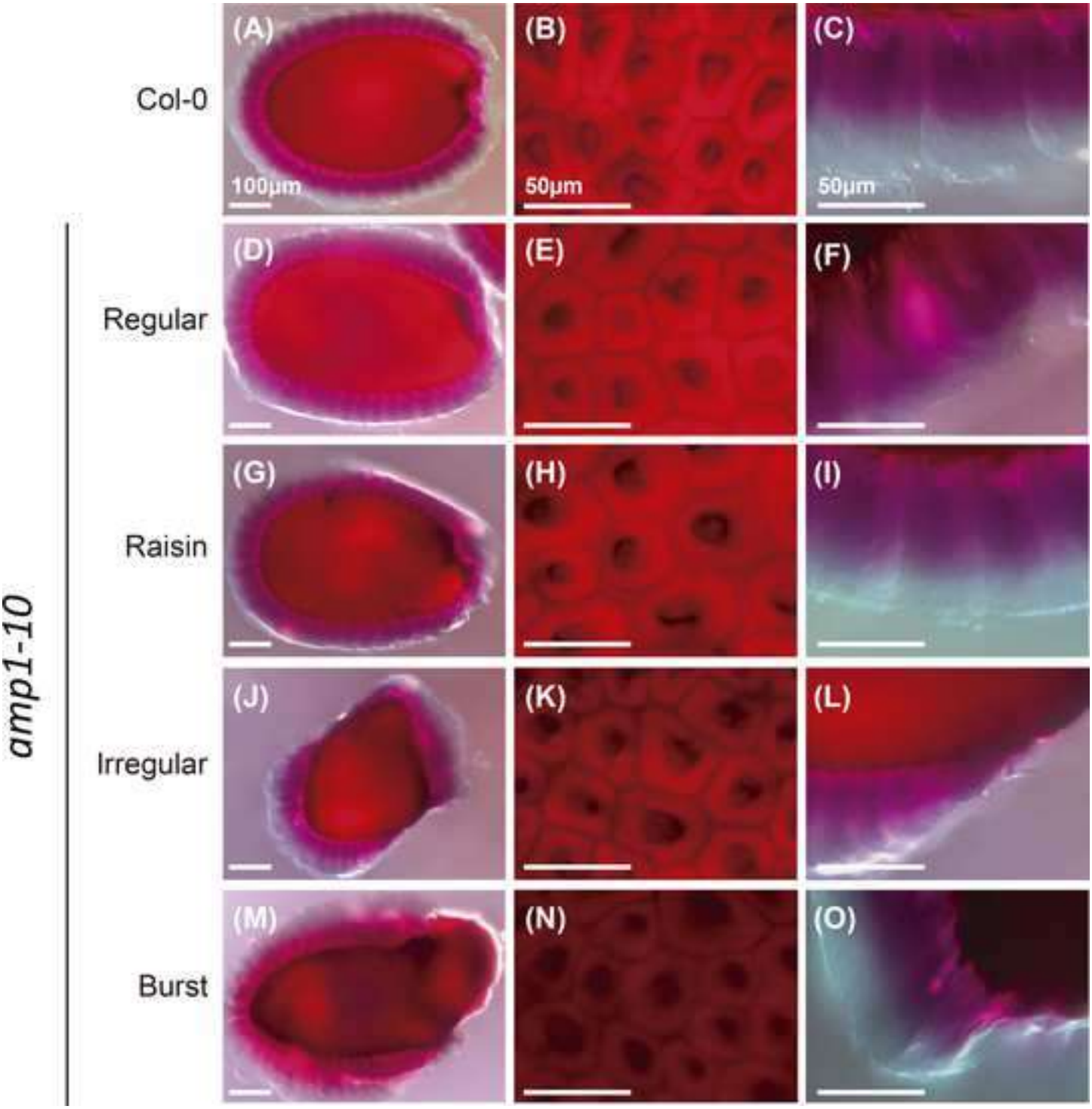

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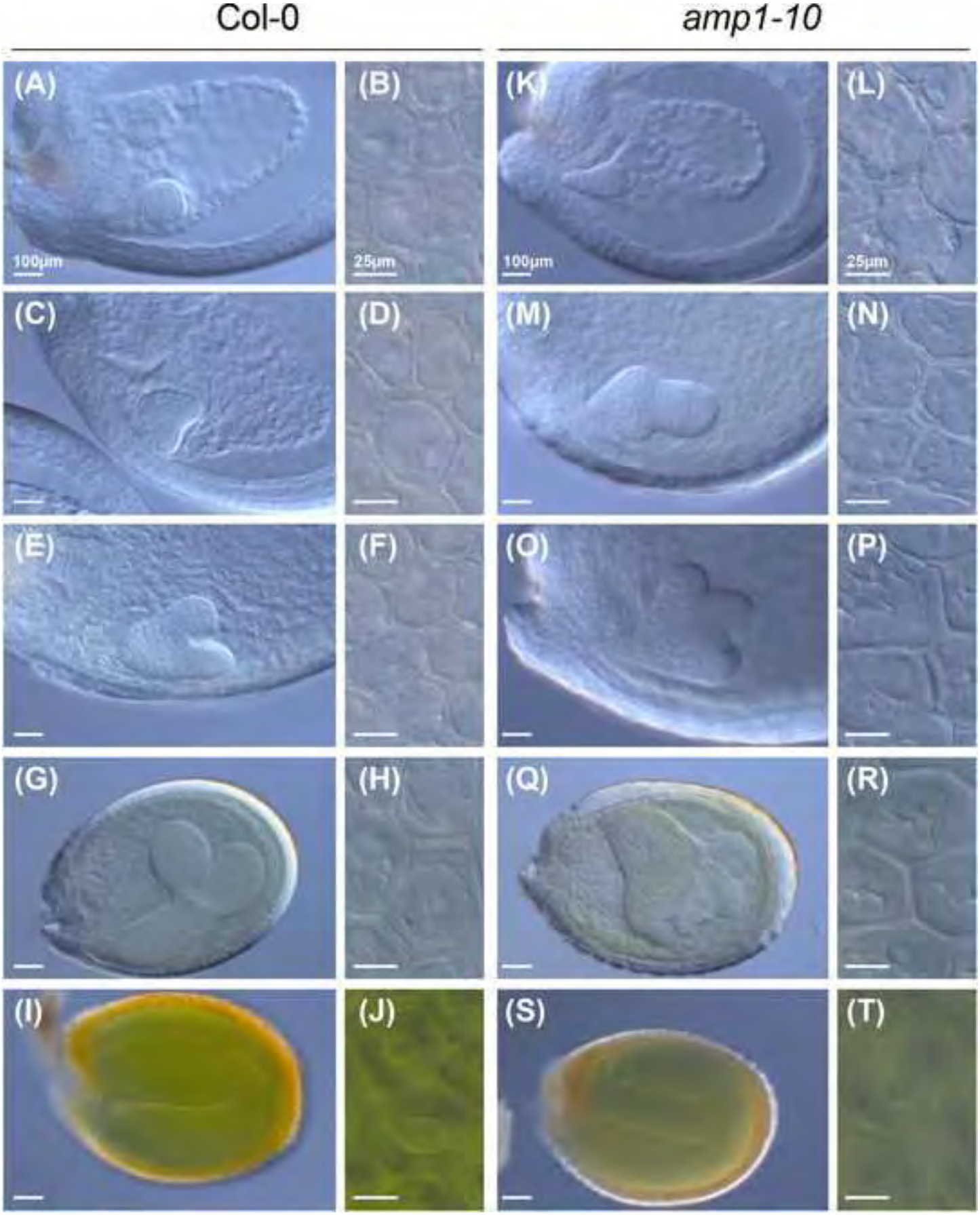

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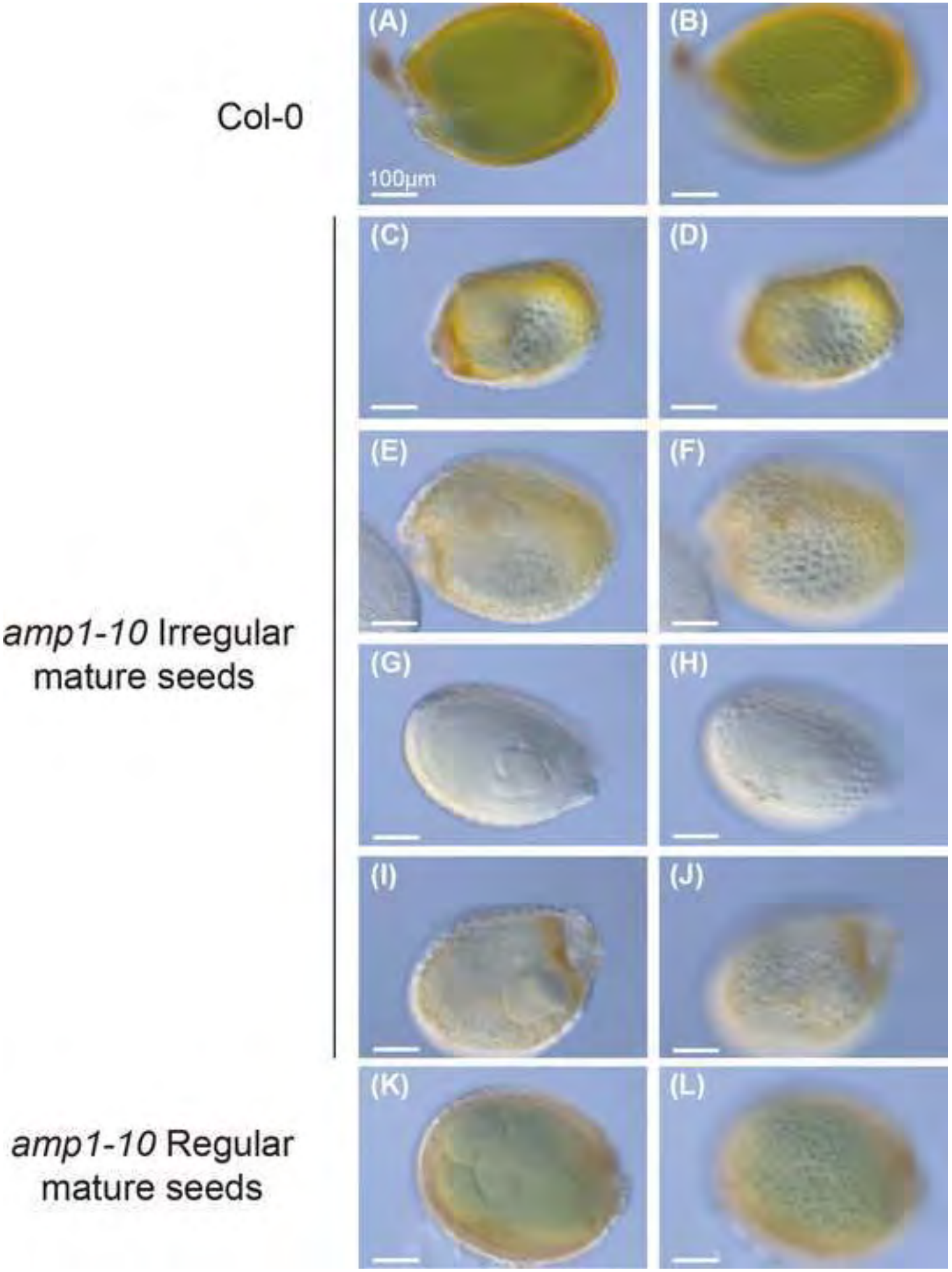

Figure 6
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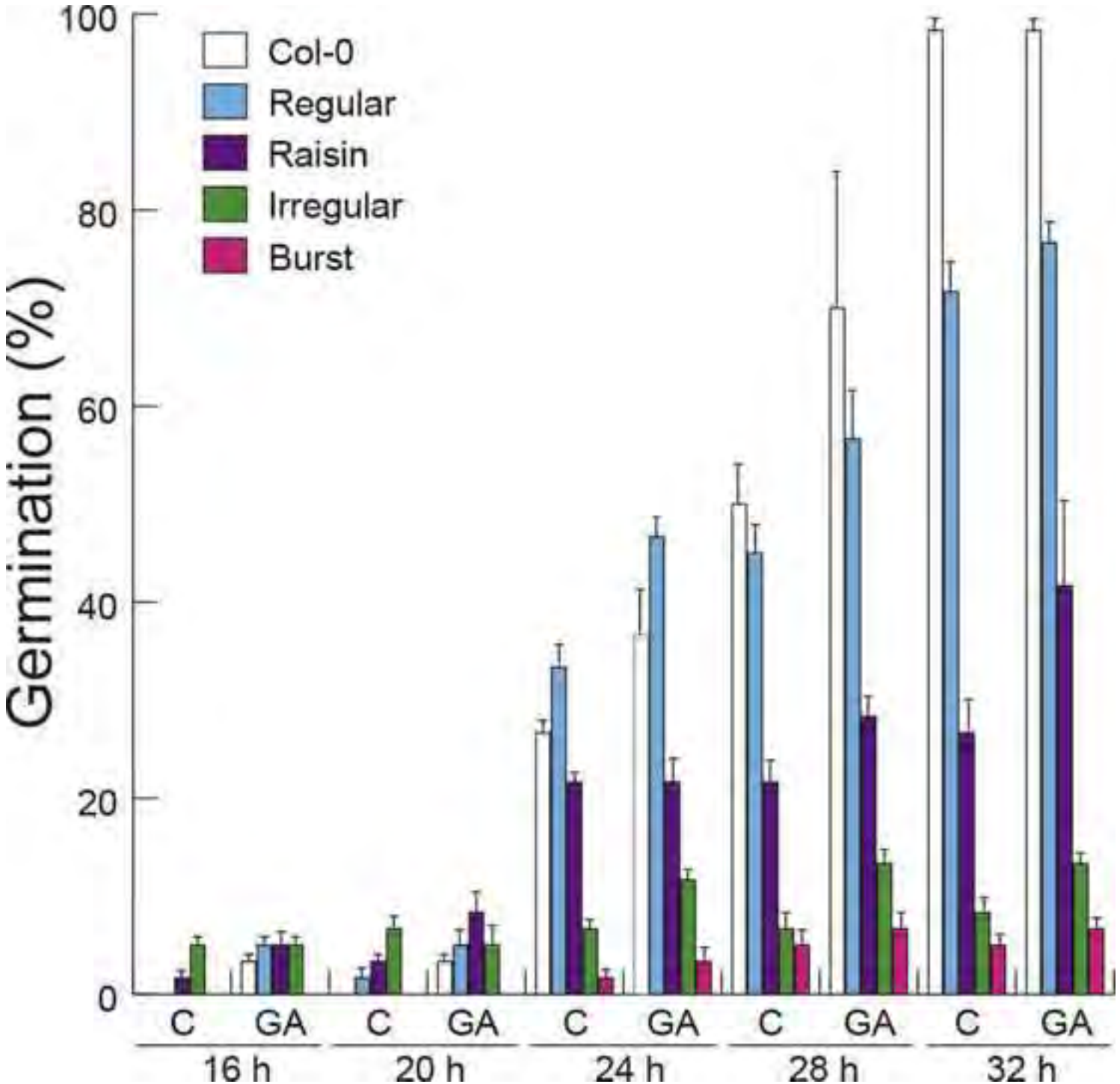

Figure 7
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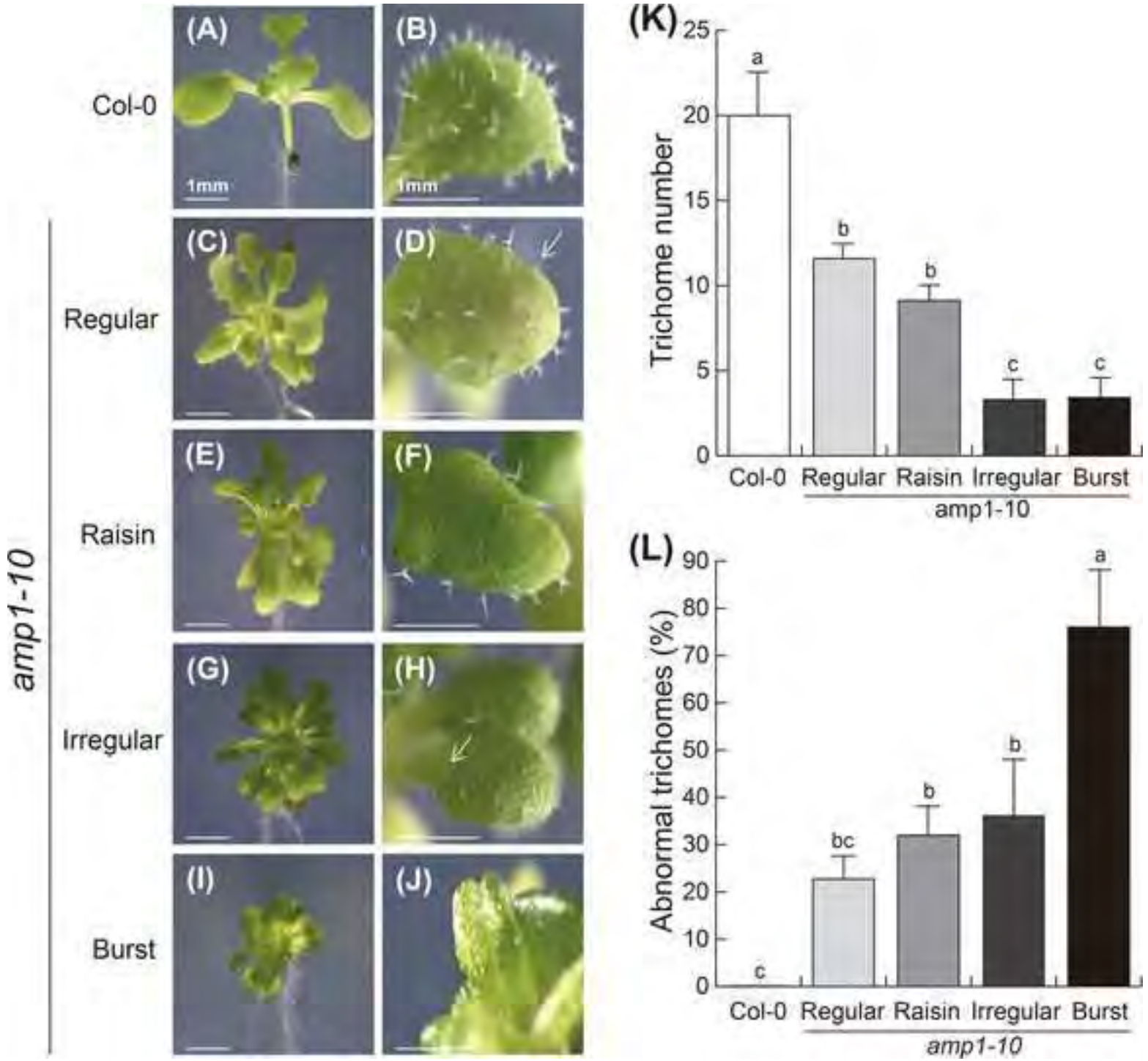

Figure 8
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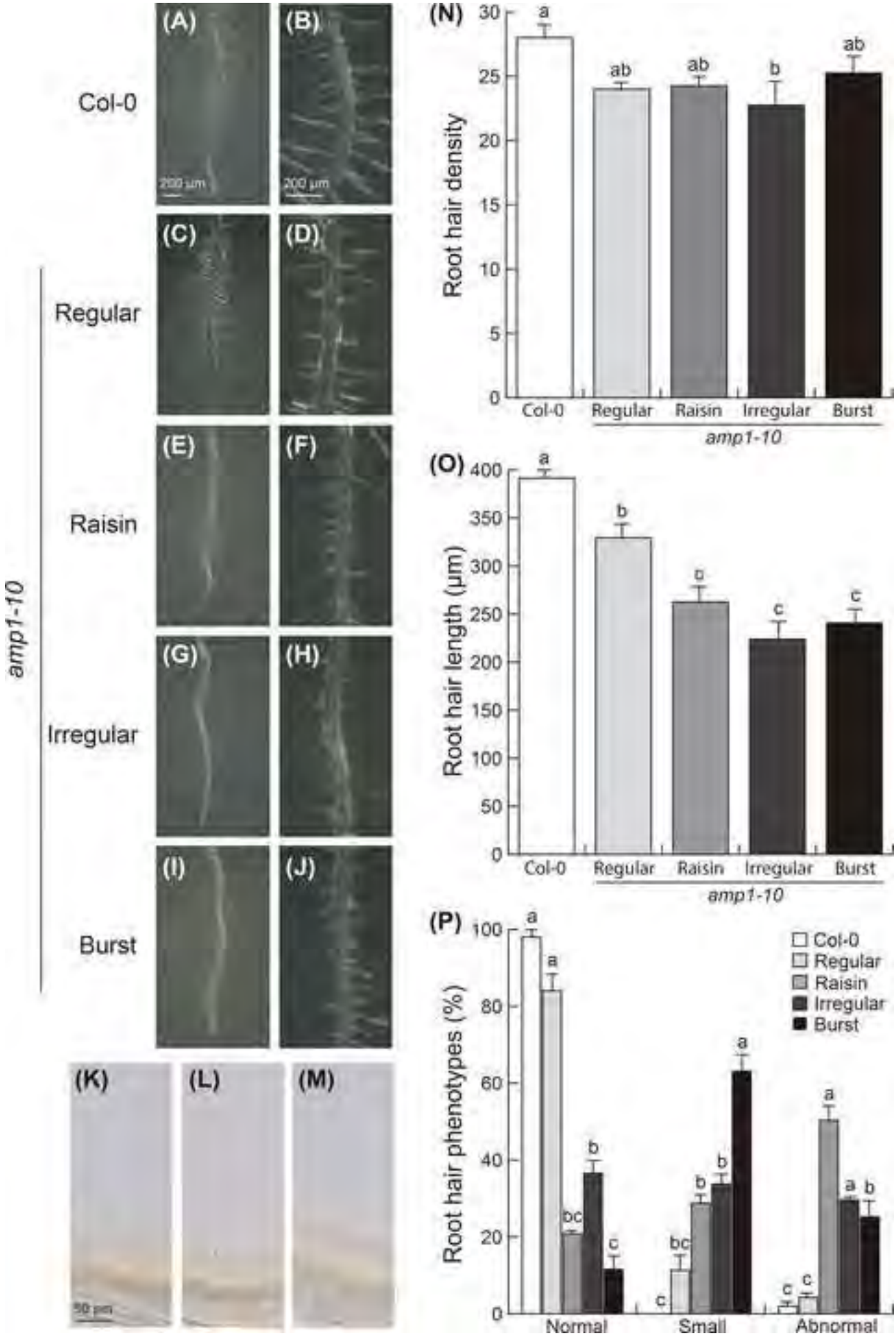

Supplementary material

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Full title: *ALTERED MERISTEM PROGRAM 1* is involved in seed coat, root hair and trichome development in *Arabidopsis*

Running title: Role of *AMP1* in epidermal cell differentiation

Full names of authors: Claudia Marina López-García[1], Javier Raya-González[1], Jesús Salvador López-Bucio[2], Ángel Arturo Guevara-García[2], and José López-Bucio[1]*

[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030, Morelia, Michoacán, México.

[2]Instituto de Biotecnología-UNAM. Av. Universidad No. 2001, Col. Chamilpa, Cuernavaca, Morelos, México.

[*]**Author to whom correspondence should be addressed**.

José López-Bucio. Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México. Telephone 5 443 3265788, fax: (443) 3265788, E-mail adress: jbucio@umich.mx

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Supplementary material: 6 supplementary figures grouped into a single PDF file.

Cover letter

Professor Wilhelm Gruissem

Editor in Chief

Plant Molecular Biology

Dear Prof. Gruissem:

Please find enclosed our manuscript entitled “ALTERED MERISTEM PROGRAM 1 is involved in seed coat, root hair and trichome development in Arabidopsis” by López-García et al., that we would like to be considered for publication in Plant Molecular Biology.

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a glutamate carboxypeptidase previously found to regulate embryo development, plant growth and phytohormone responses. In this study, we provide evidence that AMP1 has a key role in the development of three different epidermal cell types, namely the seed coat, root hairs and trichomes, suggesting its primary role not only in coordinating embryo development to the formation of the seed coat, but also in post-embryonic epidermal cell differentiation programs. A detailed characterization of *amp1* mutants defective in two independent alleles (*amp1-10* and *amp1-20*), show increased frequency of embryo abortion, low seed production and retarded germination. They also display four distinct seed phenotypes related to an altered seed coat differentiation program. We further analyzed the trichome and root hair phenotypes of wild-type and all four *amp1-10* seed classes and found that *amp1* seedlings produce less trichomes per leaf, and short or bifurcated root hairs in primary roots. Our data suggest that AMP1 is necessary for the normal seed coat and embryo establishment during seed development and plays an important role in epidermal cell differentiation in roots and leaves.

We kindly propose Professors Hong Ma and James H. Murray as reviewers of this manuscript. We think our research provides novel and interesting information regarding the role of AMP1 in plant morphogenesis, and therefore merits publication in Plant Molecular Biology.

Thank you so much for your kind attention.

Respectfully,

José López-Bucio

Abstract

During plant development, cells interpret positional information and translate it into patterned cell differentiation. The epidermis differentiates into several types of specialized cells, giving rise to the seed coat in the embryo, root hairs in roots and trichomes in shoots. *ALTERED MERISTEM PROGRAM 1 (AMP1)* encodes a glutamate carboxypeptidase involved in embryo development, plant growth and phytohormone homeostasis. Here, we show that AMP1 plays a pleiotropic role in epidermal tissue differentiation. *AMP1* mutants defective in two independent alleles (*amp1-10* and *amp1-20*), show increased frequency of embryo abortion, low seed production and retarded germination. They also display four distinct and stable seed phenotypes defined as “regular”, “raisin”, “irregular” and “burst” seeds, which are related to an altered seed coat differentiation program. We further analyzed the trichome and root hair phenotypes of wild-type and all four *amp1-10* seed classes and found that *amp1* seedlings produce less trichomes per leaf, and short or bifurcated root hairs in primary roots. Our data suggest that AMP1 is necessary for the normal seed coat and embryo establishment during seed development and plays an important role in epidermal cell differentiation in roots and leaves.

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Mitogen Activated Protein Kinase 6 and MAP Kinase Phosphatase 1 mediate Arabidopsis root responses to glutamate

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Key Words:	Arabidopsis, Glutamate, MPK6, MKP1, Root development, Gravitropism

Mitogen Activated Protein Kinase 6 and MAP Kinase Phosphatase 1 mediate *Arabidopsis* root responses to glutamate

López-Bucio, J. S.[1a], López-Bucio, J.[2a], Raya-González, J.[2b], Ramos-Vega, M.[1b], León, P.[1c] and Guevara-García, A. A.[1d]

[1]Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, 62250 Cuernavaca, Morelos, México.

[2]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio A-1', CP 58030 Morelia, Michoacán, México.

[1a] lopbucio@ibt.unam.mx

[2a] jbucio@umich.mx

[2b] javierrayagonzalez@gmail.com

[1c] mramos@ibt.unam.mx

[1c] patricia@ibt.unam.mx

[1d] aguevara@ibt.unam.mx

Running title:

Role of MPK6 and MKP1 in glutamate signaling

Summary

The Root System Architecture (RSA) of a plant is regulated by extrinsic and intrinsic signals, plays an essential role in plant adaptation to biotic and abiotic factors. Glutamate (L-Glu) affects root gravitropism, primary root growth and lateral root formation, which are important RSA determinants. However, the underlying genetic mechanisms involved in this modulation are poorly understood. Mitogen-activated protein kinases (MAPKs) are key signaling modules that respond to various extracellular stimuli that regulate developmental programs. Even so, it is the protein-reversible phosphorylation, catalyzed by kinases and phosphatases that ultimately provide the regulatory framework underlying most biological processes. Here, we employ a combination of genetic and biochemical strategies to show that Mitogen Activated Protein Kinase 6 (MPK6) and MAP Kinase Phosphatase 1 (MKP1) are involved in the control of RSA alterations triggered by L-Glu. We used gel-based phosphorylation assays to reveal a rapid and dose-dependent effect of L-Glu on MPK3 and MPK6 activities in wild-type *Arabidopsis* seedlings. Moreover, we found that the loss-of-function of MPK6 results in a decreased primary root growth inhibition and gravitropic response to L-Glu. These defects are related to an altered root cap structure and statocyte accumulation in the columella cells. In contrast, the loss-of-function of MKP1 results in the opposite phenotypes. Our data highlight the role of MKP1 and MPK6 as components of a MAPK signaling pathway, controlling root and gravitropic responses to glutamate.

Key words: *Arabidopsis*, glutamate, MPK6, MKP1, root development, gravitropism.

Significance statement

- The function and components of glutamate signaling pathways in plants have just begun to be elucidated. Here, we use a combination of genetic and biochemical strategies to demonstrate that the kinase MPK6 and the phosphatase MKP1 control root architectural and gravitropic responses to glutamate.

INTRODUCTION

Root System Architecture (RSA), which plays an essential role in plants' capture of mineral nutrients and water, is highly regulated by extrinsic and intrinsic factors such as hormones, nutrients and environmental signals (Kerk et al. 2002; López-Bucio et al. 2003; Malamy 2005; Fukaki and Tasaka 2009). Although nitrate is the principal source of nitrogen for plants and its concentration limits root growth and development, other organic forms of N, represented mainly by amino acids, also contribute to plant nutrition and impact the RSA (Forde and Clarkson 1999; Zhang and Forde 2000; Forde and Lea 2007; Zhang et al. 2007). Recent evidence has demonstrated that Glutamate (L-Glu), which is best known for its role as a neurotransmitter in the mammalian nervous system, is an emerging signaling molecule in plants (Walch-Liu et al. 2006b; Dawe et al. 2014; Forde 2014a). The supply of L-Glu to *Arabidopsis thaliana* seedlings alters the RSA by inhibiting primary root (PR) growth and promoting lateral root (LR) formation, which results in a shorter and more branched root system (Walch-Liu et al. 2006a; Forde 2014b). PR growth inhibition has been related to an agravitropic response, decreased root cell proliferation and differentiation of cells at the root tip (Forde et al. 2013). The search for molecular signaling components involved in glutamate-mediated responses in plants has resulted in the identification of a family of glutamate-related receptors (GLR) that are apparently involved in transducing the glutamate signal for root developmental responses (Kim et al. 2001; Lacombe et al. 2001; Kang and Turano 2003; Kang et al. 2004; Kang et al. 2006; Forde and Roberts 2014). In rice, the single *Osglr3.1* knockout mutant displays a short root phenotype caused by an arrest in cell division and the death of root meristem cells (Li et al. 2006). In contrast, *Arabidopsis* mutations of the AtGLR3.2 and AtGLR3.4

genes resulted in an increased number of LR primordia, supporting the idea that these factors negatively regulate LR initiation (Vincill et al. 2013). A limited root agravitropic response was recently reported for the *atglr3.3* single mutant, indicating a possible role of the AtGLR3.3 in mediating root gravity perception (Miller et al. 2010). These data show that some members of the GLR family play important non-redundant roles in the control of root development.

In mammals, signal transduction downstream of the GLR receptors involves protein phosphorylation cascades (Wang et al. 2007b). A recent report showed that an evolutionarily conserved MAP3K in *Arabidopsis* (MEKK1) plays a key role in glutamate signaling, eliciting changes in the RSA (Forde et al. 2013). Thus, these data provide compelling evidence for the existence of a glutamate signaling pathway analogous to that of animals' systems (Tapken et al. 2013). However, additional elements participating in the plant glutamate sensing and signaling pathway still remain to be uncovered.

Mitogen Activated Protein Kinases (MAPKs) are key signaling modules that mediate responses to various extracellular stimuli and regulate diverse plant developmental programs (Asai et al. 2002; Bush and Krysan 2007; Colcombet and Hirt 2008; Fiil et al. 2009). MPKs are the terminal components of the MAPK modules, comprising three kinases (MP3K/MP2K/MPK) that follow a sequential activation by phosphorylation. MPK6 is one of the twenty encoded MPKs in the *Arabidopsis* genome (MAPK Group 2002; Suárez-Rodriguez et al. 2010). MAP kinases have overlapping substrates and upstream activators, resulting in functional redundancy at different levels (Andreasson and Ellis 2009). In particular, MPK3 and MPK6 act redundantly in processes such as ethylene biosynthesis, pathogen signaling and stomata development (Asai et al. 2002; Liu

and Zhang 2004; Djamei et al. 2007; Kanaoka et al. 2008; Lampard et al. 2008). However, in some cases the activity of MPK6 cannot be compensated by any other MPKs, as suggested by profound alterations in embryo and root development displayed by the Arabidopsis *mpk6* mutant (Bush and Krysan 2007; López-Bucio et al. 2014). Mutations in the MPK6 gene cause three different seed phenotypes that correlate with alterations in RSA (López-Bucio et al. 2014). A small proportion (~7%) of *mpk6* mutant seedlings failed to develop the PR, possibly as a result of an earlier defect in the division of the hypophysis during embryo development. Another small (though larger) group (~23%) had a short primary root, while the most prevalent group (~70%) had a longer primary root and an increased LR formation when compared to wild-type (WT) seedlings (López-Bucio et al. 2014).

Although MAP kinases regulate numerous developmental processes, it is the protein reversible phosphorylation, catalyzed by these kinases and their phosphatase partners, which finally provides the regulatory framework responsible for most biological processes (Bartels et al. 2010). Several phosphatases are able to inactivate various components of MAPK cascades (Keyse 2008; Bartels et al. 2009). Biochemical and genetic studies have revealed that the MKP1, a dual specificity serine-threonine and tyrosine phosphatase, physically and genetically interacts with MPK6 and MPK3 to regulate their activity *in vivo* (Ulm et al. 2001; Ulm et al. 2002), thus representing a good candidate mediating RSA via MPK6 dephosphorylation.

In this report, by comparing the gravitropic response and RSA of the *mpk6* and *mkp1* mutants with those of WT in response to glutamate, we provide physiological and molecular evidence that the activities of these kinase and phosphatase are involved in the control of root architecture alterations triggered

by glutamate. Our data suggest that MKP1 and MPK6 are components of a glutamate signaling pathway controlling both root gravitropism and RSA.

RESULTS

L-Glu inhibits primary root growth and affects root gravitropism of *Arabidopsis* WT seedlings

To confirm previous reports of the role of glutamate in modulating root developmental responses (Walch-Liu et al. 2006a; Forde and Walch-Liu 2009), and to establish an experimental system for further analysis of glutamate responses, *Arabidopsis* WT seedlings (Col-0) were grown on an agar-solidified MS media supplemented with different L-Glu concentrations. A dose-dependent effect on PR growth was observed seven days after germination (DAG) in seedlings in treatments of 100 to 600 µM L-Glu. The L-Glu caused between 15 and 60% inhibition on PR growth (Figure 1a). Additionally, the supply of 400 µM of L-Glu results in a clear increase of PR curvature (skewing), probably as a result of defects in the PR gravity response (Figures 1b & c).

L-Glu induces MPK6 and MPK3 activities

To determine the possible role of MAPKs in modulating root developmental responses to glutamate, an *in-gel* phosphorylation assay was used to determine changes in the myelin basic protein phosphorylation activity in WT seedlings grown in media supplemented with increasing L-Glu concentrations. Interestingly, a dose-response effect of L-Glu was evidenced, with a maximum activity of both MPK3 and MPK6 at 400 µM Glu, which decreased afterwards (Fig. 2a). To evaluate the temporality of the L-Glu effect on the activity of MPKs activities, 10 DAG WT

seedlings were grown on basal media and then transferred to liquid basal media either without or supplemented with 200 or 400 µM L-Glu for 30 min. As shown in Figure 2b, exposure of seedlings to L-Glu for a short time period was sufficient to induce MPK6 and MPK3 activities. As MKP1 has been demonstrated to be a regulator of MPK6 activity (Ulm et al. 2002), we next explored if L-Glu inductive effect is affected by the loss-of-function of this phosphatase. Short time induction experiments (30 min) showed that MPK6 and MPK3 activities were induced by L-Glu in *mkp1* mutant seedlings (Fig. 2c). Taken together, these results show that the activity of MPK6 and MPK3 are induced by L-Glu, therefore they might be involved in the signaling pathway that controls plant responses to this amino acid.

Root developmental responses to L-Glu in *mpk3*, *mpk6* and *mkp1* mutants

The prevalent *mpk6* mutants have been shown to have a longer PR than WT seedlings (López-Bucio et al. 2014) and previous work has shown redundant functionality between MPK6 and MPK3 in different plant developmental and stress responses (Hord et al. 2008; Wang et al. 2008; Beckers et al. 2009; Liu et al. 2010; González Besteiro et al. 2011; Meng et al. 2013). In addition, we observed that the *mkp1* mutant in Col-0 background (*mkp1*/Col-0) (Bartels et al. 2009) shows clear root phenotypes (Figure 2a/Control (C) condition; Supplemental Figures S1a and Supporting Figures S4a and S4b/Control (C) conditions).

Thus, considering the well-supported MPK3/MPK6 functional redundancy, and the contrasting *mpk6* and *mkp1* root phenotypes, we analyzed in more detail the effects of glutamate over root development in those cognate mutants. In a first set of experiments, WT, *mpk6* and *mkp1* seedlings were germinated and grown in basal MS media for four days, then five seedlings from each genotype were

transferred either to the same basal MS media or to MS media supplied with increasing L-Glu concentrations (100, 200 and 400 µM). The PR growth and the percentage of PR growth inhibition of each condition was evaluated seven Days After Transfer (DAT). Our data shows that L-Glu has a clear dose-dependent inhibitory effect on the PR growth in both *mkp1* and WT seedlings resulting in a PR growth inhibition of 80% and 45%, in the presence of 400 µM of L-Glu, respectively (Fig 3a and 3b). In contrast, when compared to *mkp1* and WT seedlings, the *mpk6* mutant displays a clear insensitivity to L-Glu.

A representative picture of plants grown on basal media shows that the *mpk6* PR is longer than the wild-type PR, whereas the roots of *mkp1* seedlings, even if curved to the left side of the plate, were shorter than the WT (Figure 3a and Supporting Figures S1a & S1c). In these pictures, it is evident that the differential sensitivity of *mkp1* and *mpk6* mutants to 400 µM of L-Glu (Supporting Figures S1b & S1d). The results of these experiments support the idea that MPK6 and MKP1 are components of the glutamate signaling pathway involved in PR growth and root gravitropism.

As the *mpk6* mutant is in Columbia (Col-0) background, an additional experiment was performed using an *mkp1* mutant allele introgressed into Col-0 ecotype (Bartels et al. 2009). Since different *Arabidopsis* ecotypes display natural variation in their L-Glu sensitivity (Walch-Liu et al. 2006b), and some *mpk1* phenotypes are exclusively apparent in the Col-0 accession, but not in the *mkp1* original Wassilewskija (Ws) ecotype (Bartels et al. 2009), we also tested the L-Glu sensitivity of the *mkp1* in the Ws genetic background (*mkp1*/Ws). In the Supporting Figure S2, it can be appreciated that in comparison with its WT background the *mkp1*/Ws mutant PR displays a skewing phenotype similarly to

the one observed in the same allele in the Col-0 background (Fig. 3a) as well as a hypersensitivity to L-Glu for PR growth inhibition and root apical meristem (RAM) damage. From these observations, we concluded that the effects of L-Glu on the *mkp1* mutant are genetically stable.

We next performed experiments to compare the root responses of WT and *mpk3* mutant seedlings to glutamate. Although MPK3 activity is induced in the presence of L-Glu (Fig. 3), *mpk3* mutant seedlings are slightly more sensitive to the L-Glu-induced PR growth inhibition and showed similar gravitropic response than WT seedlings (Supporting Figure S3). Therefore, we conclude that MPK6 most likely functions independently of MPK3 activity to modulate the PR responses to Glu.

Glutamate affects root gravity responses in *mpk6* and *mkp1* mutants

Gravity has major effects on both the form and overall length of the root system, resulting in a differential growth. It has been reported that glutamate is perceived specifically at the primary root tip inhibiting mitotic activity in the RAM, where gravity is sensed and where MPK6 has recently been demonstrated to play important functions (Morita, 2010; López-Bucio et al. 2014). To investigate the possible roles of MPK6 and MKP1 on root gravity responses to glutamate, WT, *mpk1* and *mpk6* seedlings were germinated and grown for seven days in basal MS media on vertically oriented Petri dishes. Then, four seedlings of each plate were transferred to the same basal MS media or MS media supplemented with 400 µM L-Glu, and grown during two additional days after 90° rotation of the plate. The curvature response followed by the root growth in each condition was registered (Figure 4a). In these gravity response assays, we found that the root curvature of the *mpk1* and *mpk6* mutants in the presence of L-Glu are clearly contrasting. In the

WT seedling the presence of 400 µM L-Glu changed the curvature from 89°±2 to 111°±3, confirming that L-Glu affects the root gravitropic response. In contrast, in the same conditions *mpk6* mutants did not significantly change the growth angle, from 82°±1 in the absence to 88°±1 in presence of L-Glu. On the other hand, *mpk1* mutant seedlings exposed to L-Glu resulted in changes of the curvature from 99°±2 to 127°±1, indicating an L-Glu hypersensitivity (Figure 4b). Hypersensitivity of *mkp1* to L-Glu is also supported by the pronounced curvature of its PR in the presence of L-Glu, even in the absence of rotation (Supporting Figure S4). Together, these observations indicate that the increase in root curvature response caused by L-Glu depend, at least partially, on protein phosphorylation/dephosphorylation events mediated by MPK6 and MKP1 proteins.

Sedimentation of the amyloplast is an early key step in root gravitropic response (Wolverton et al. 2011). Given the contrasting performance of *mkp1* and *mpk6* root mutants in the presence of L-Glu, we decided to analyze their root cap and statocyte structures in response to 200 and 400 µM L-Glu. Figure 5 shows that whereas 400 µM Glu treatment only has a slight effect on root cap structure and statocyte accumulation in *mpk6* root tips, it clearly damages root cap structure and decreases statocyte accumulation in *mkp1* seedlings. Notably, the effects of L-Glu on statocyte accumulation in WT seedlings, is lighter than those on *mkp1*, but stronger than those on *mpk6*. Interestingly, root exposure to low L-Glu concentrations (100 µM), which inhibits only around 10% of the PR growth (Figure 1a), clearly affected the curvature response in the *mkp1* seedlings, whose roots exhibit an affected gravitropic growth with only a slight effect over statocyte integrity (Figures 6a and 6b). This L-Glu concentration has only mild effects on WT

and *mpk6* seedlings. These observations support the notion that statocyte functionality and root cap structure are key components of the L-Glu effects on gravitropism and growth of PR, and that these effects involve MPK6 and MKP1 functions.

DISCUSSION

The results presented in this report establish a role of the MPK6 and MKP1 proteins in regulating PR growth and root gravitropic responses to glutamate. It was shown that the presence of this amino acid causes a rapid dose-dependent induction of the activity of MPK3 and MPK6, suggesting that both kinases participate in the glutamate signaling pathway. Even though MPK3 and MPK6 activities were induced by L-Glu, we observed that the primary roots of *mpk3* mutants are slightly oversensitive to L-Glu treatment when compared to WT plants, whereas the primary roots of *mpk6* mutants are clearly insensitive to glutamate effects. These data favor the idea that MPK6 activity plays a positive role for the primary root responses to glutamate, while MPK3 activity may be a negative factor in mediating this response. As recent data demonstrated that the kinase activity of MEKK1 is dispensable for glutamate root architectural responses (Forde et al. 2013) a future path of this research field should be to focus on identifying the MPKKs and MPKKKs working upstream of MPK6.

MPK6 is a target of the MKP1 phosphatase, thus it was possible that the mutation in this latter gene should result in an over-activation of MPK6. Interestingly, the *mkp1* mutant had the opposite root phenotype of the *mpk6* mutant; that is, a shorter primary root and the production of lesser lateral roots and hairy roots than WT plants. Also, *mkp1* is more sensitive to L-Glu treatments, all phenotypes

being consistent with an increased MPK6 activity. However, our *in-gel* kinase assays demonstrated that this is not exactly the case. The *mkp1* mutants preserve a basal MPK6 activity, which increases with L-Glu supply. Therefore, the high sensitivity of the *mkp1* mutants to L-Glu cannot be directly associated with an increased MPK6 activity. Since MAPK cascades are part of complex signaling networks, the participation of other phosphatases still to be identified involved in the control of plant response to glutamate via MAPK signaling cannot be dismissed (Xu and Zhang 2015). Interestingly, using an inactive version of *MEKK*1, it was demonstrated that the kinase activity of this enzyme is not required for glutamate signaling (Forde et al. 2013). Since MEKK1 activity is necessary for the response to pathogens (Rasmussen et al. 2012), it was then speculated that both pathogen and glutamate responses could be acting through the same signaling pathway (Forde et al. 2013). In the case of pathogen responses, it was demonstrated that a flagellin elicitor peptide (flg22) treatment results in a MPK3, MPK4 and MPK6 induction, while MPK4 induction is dependent of MPKK1 activity, the induction of MPK3 and MPK6 is independent of this MAP3K activity, as registered in a *mekk1* mutant background (Rasmussen et al. 2012). The activity of an iGluR (ionotropic glutamate receptor)-like channel controlling MAMP (microbe-associated molecular patterns)-triggered by Ca^{2+} influx, was associated with a low-level glutamate-mediated induction of MPK3, MPK4 and MPK6 kinases (Kwaaitaal et al. 2011). Altogether these observations support the involvement of a MAPK cascade (including MPK6) in the plant glutamate signaling pathway.

The accumulated evidence supports the proposal that glutamate-mediated root response may include glutamate receptors, MEKK1, and MPK6 and MKP1, but the way they interact, as well as the role of other yet unknown factors remains to be

investigated in the future. A possible factor involved in this signaling pathway is the Protein Tyrosine Phosphatase1 (PTP1), which has been reported to physically interact with MPK6 and works in coordination with MKP1 to repress plant defense responses (Gupta and Luan 2003; Bartels et al. 2009;). Another candidate is the Toll Interleukin 1 Receptor/Nucleotide Binding/Leucine Rich Repeat (TIR-NB-LRR) receptor-like resistance gene homolog Suppressor of NPR1-1, Constitutive1 (SNC1), which has been identified as a natural modifier of MPK1 (Li et al. 2007; Bartels et al. 2009;)

The importance of gravity on plant growth has been recognized for centuries, as already described by Charles Darwin, who even recognized the root cap as an essential element for root gravitropism (Chen et al. 1999). Currently, the biochemical signaling pathway and the molecular mechanism behind root gravity responses remain to be defined. It is tempting to speculate that the inhibitory effect that L-Glu has on primary root growth could be related to altered gravitropic responses. Thus, taking advantage of the contrasting phenotypes between *mkp1* (hypogravitropic) and *mpk6* (hypergravitropic) mutants, we investigated this hypothesis. Genetic and molecular evidences support two hypotheses about the sensing mechanism of gravitropism: the starch-statolith hypothesis, which propose that amyloplasts in the gravity-sensing cells (statocytes) function as statoliths determining the gravity vector and triggering the intracellular signaling cascade controlling gravitropic responses. In contrast, the Cholodney–Went hypothesis postulates that gravity induces a lateral auxin transport resulting in an asymmetrical auxin distribution that controls organ bending (Driss-Ecole et al. 2003; Morita and Tasaka 2004; Hashiguchi et al. 2013; Rigas et al. 2013). As statocytes are key players in gravity sensing, we focused our analysis on the

statocyte content of root columella cells and performed analysis of root gravity responses to L-Glu, clearly affecting the gravitropic response (Fig. 4), and this was related to statocyte/statolit integrity (Figs. 5 & S2). L-Glu effects in gravitropism were less and more pronounced on the *mpk6* and *mkp1* mutants, respectively. The *mpk6* mutant was hypergravitropic and insensitive to L-Glu inhibition of PR growth whereas *mkp1* mutants, which are hypogravitropic under standard growth conditions, showed an exacerbated agravitropism in response to L-Glu, including disruption of statocyte/statolit structure (Fig. 5). Our observations suggest that MPK6 is a positive modulator of the L-Glu gravitropic effect. In contrast, MKP1 enzymatic activity could be acting as a negative modulator of the gravitropic response directed by L-Glu. Besides, our results also support the existence of an alternative mechanism of gravity sensing, independent of starch and probably located outside the root cap (Morita 2010; Baldwin et al. 2013), as a concentration of L-Glu having very little effect on statocyte/statolit integrity is still capable of altering gravitropism, which is especially noticeable in the *mkp1* mutants (Fig. 6). Several factors, including the cytoskeleton and the auxin and Ca^{++} intracellular levels, have been related to gravitropism (Morita 2010; Hashiguchi et al. 2013; Toyota and Gilroy 2013), but to the best of our knowledge this is the first time that glutamate sensing via MKP1 and MPK6 activities have been associated with this process. The precise function of each of these factors, as well as the possible crosstalk between them, remains to be determined in the future. It is interesting that MAP kinase modules and their related phosphatases have been reported to participate in the signaling pathway of several factors controlling gravitropism (Bartels et al. 2010), but no interaction has been established between these different signaling pathways to control such processes.

Another interesting observation from our analysis is the fact that the statocytes in the *mpk6* mutant are bigger in size than the WT plants. As it has been reported that conditions that result in larger amyloplasts are associated with greater gravisensitivity (Vitha et al. 2007), it is very suggestive that this statocyte structure may be responsible for the hypergravitropic behavior of the *mpk6* mutant.

The changes in root architecture induced by glutamate might be explained by a possible interaction between glutamate and auxin signaling. The auxin transporter mutant *aux1-7* is slightly insensitive to L-Glu (Maher and Martindale 1980; Walch-Liu et al. 2006b), but conversely two mutants on the *AXR1* locus resulted in L-Glu hypersensitive (Lincoln et al. 1990; Walch-Liu et al. 2006b), while other auxin responsive mutants tested did not show alterations in L-Glu responses Walch-Liu et al. 2006b). As clearly auxin relocalization mediated by auxin efflux carriers (Brunoud et al. 2012) is involved in differential cell elongation required to gravibending (Kleine-Vehn et al. 2010; Baldwin et al. 2013), a process in which, according to the results presented here, the glutamate, the kinase MPK6 and the phosphatase MKP1 also participate, the possible relationship between these elements deserves further analysis

The signaling pathway behind the plant responses to glutamate is just beginning to be elucidated, therefore, the identification of the genes and proteins mediating L-Glu sensing, such as MPK6 and MKP1, represent an important advance in understanding small molecule and amino acid signaling in plants.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana Heyhn Columbia-0 (Col-0) and Wassilewskija (Ws) wild-type

ecotypes were used as controls. The *mpk6-2* line (SALK_073907) in Col-0 background (Liu and Zhang 2004) was obtained from the Salk T-DNA collection (Alonso et al. 2003). *mpk3* mutant T-DNA insertion line (SALK_151594) in Col-0 background (Wang et al. 2007a), was kindly donated by Dr. Shuqun Zhang from Missouri University. The *mkp1* mutant in the Ws ecotype (Ulm et al. 2001) was kindly donated by Dr. Scott C. Peck from Missouri University. The *mkp1* mutant introgressed into the Col-0 ecotype (Bartels et al. 2009) was kindly donated by Dr. Marina A. González Basteiro from the Dr. Roman Ulm Laboratory (University of Geneva, Switzerland). All seed were surface sterilized and incubated at 4ºC for three days to break dormancy, then grown on agar (0.8% w/v Bacto™ Agar BD DIFCO, Sparks, MD) solidified 0.2X MS medium (Caisson, Laboratories, Inc., North Logan, UT) with 1% (w/v) sucrose. L-Glutamic Acid was purchased from Sigma (Sigma-Aldrich Corporation St. Louis, MO) and added to the medium at the indicated concentration. Seedlings were grown on vertically oriented Petri dishes maintained in growth chambers at 21°C under a 16:8 h light:darkness photoperiod with 105 µmol m^{-2} s^{-1} light intensity.

For gravitropism experiments, seedlings were germinated and grown for seven days on vertically oriented Petri dishes as previously described, then the front root growth was marked and the Petri dishes were rotated 90º and maintained under the same growing conditions. The angle of root growth was registered two days later with a protractor.

In-Gel Kinase Assay

Seedlings were ground in liquid nitrogen and homogenized in extraction buffer (250 mM sorbitol, 50 mM HEPES-BTP pH 7.8, 10 mM NaF, 5 mM DTT, 1 mM EDTA,

1 mM KCl, 1 mM Na3VO4, 1 mM PMSF and 40 µg/ml of protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN]), then centrifuged at 12,000 x g 20 min at 4ºC. Protein concentration in the extracts was estimated with Bradford protein assay kit (Bio-Rad, Hercules, CA) using BSA as a standard. The in-gel kinase assay was performed as previously described (Zhang and Klessig 1997). Briefly, 50 µg of protein extracted from plant tissue were fractionated onto a 10% SDS-PAGE gel containing 0.25 mg/ml myelin basic protein (MBP, Sigma), as substrate for the kinases. After electrophoresis, the gel was washed three times with 25 mM Tris pH 7.5; 0.5 mM DTT; 0.1 mM Na_3VO_4; 5 mM NaF; 0.5 mg/ml BSA; 0.1% (v/v) Triton X-100 for 30 min each at room temperature. Proteins in the gel were renatured by incubating the gel in 25 mM Tris pH 7.5; 1 mM DTT; 0.1 mM $Na_3VO_4$and 5 mM NaF at 4°C overnight. The kinase reactions were carried out by incubating the gel in 30 ml of kinase buffer (25 mM Tris pH 7.5; 2 mM EGTA; 12 mM MgCl2; 1 mM DTT; 0.1 mM Na_3VO_4; 200 nM ATP, and 50 µCi of [γ-^{32}P]ATP [>4,000 Ci_mmol; 1 Ci = 37 GBq]), for 60 min at room temperature. To remove free ^{32}P, the gel was washed at room temperature with several changes of 5% (w/v) trichloroacetic acid and 1% (w/v) NaPPi until the ^{32}P-radioactivity in the wash solution was barely detectable. The gel was dried and used to expose a Kodak XAR-5 film. Pre-stained size markers (Bio-Rad, Hercules CA) were used to calculate the molecular weight of the kinases. As a loading control, 20µg of proteins from the same extracts used for kinase assays were fractionated in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue dye.

Statocyte staining

To analyze the statocyte structure the roots were fixed overnight with 4% (v/v)

paraformaldehyde (Sigma-Aldrich) in 0.025 M phosphate buffer pH 7.2, then treated with Lugol's solution (1:2 w/w, iodine:potassium iodide, pH 4.0-4.5) by 10 seconds and quickly rinsed with water. Finally, the roots were mounted in water on microscope slides and immediately analyzed using a transmission microscope (Nikon EclipseE600) equipped with a digital camera (Nikon SIGHT DS-Fi1c, Nikon Corporation, Tokyo, Japan).

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SUPPORTING INFORMATION

Supporting Figure S1. **Glutamate primary root growth inhibition and skewing on *mkp1* and *mpk6* mutants.**

Supporting Figure S2. **Effects of Glutamate on *mkp1*/Ws mutant.**

Supporting Figure S3. **Effects of glutamate on *mpk3* mutant.**

Supporting Figure S4. **Glutamate drives skewing.**

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FIGURES

Figure Legends

Figure 1. Glutamate affects *Arabidopsis* primary root development. a) Primary root length inhibition caused by L-Glu. Data were obtained from 7-DAG seedlings growing in MS media supplemented with the indicated L-Glu concentrations. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition. Values are the mean ± Standard Error (n = 15) of three biological independent experiments. Different letters represent Tukey's post-hoc test significant differences ($P \leq 0.05$). In b and c, representative 7-DAG seedlings growing in basal MS media (Control) or MS basal media supplemented with 400 µM L-Glu, are shown. Notice that besides root growth inhibition, L-Glu treatment drives waiving and skewing.

Figure 2. Glutamate induces MPK3 and MPK6 activites. *In-gel* kinase assays were conducted with 30 µg of total protein extracts from seedlings under the following conditions: (a) 7-day-old wild-type (Col-0), germinated and grown on MS basal media supplemented with the indicated L-Glu concentration. c) wild-type (Col-0) 7-days old seedling grown on MS basal media, and transferred to the

indicated L-Glu concentration for 30 minutes. In a and b, *mpk3* and *mpk6* are total protein extracts from 7-day-old seedlings germinated and grown on MS basal media. (d) 7 day-old *mkp1* seedlings grown on MS basal media and transferred to the indicated L-Glu concentration for 30 minutes. Coomassie Brilliant Blue stained gels (BB) are shown as loading controls for each assay. The gels are representative of three biologically independent experiments with similar results.

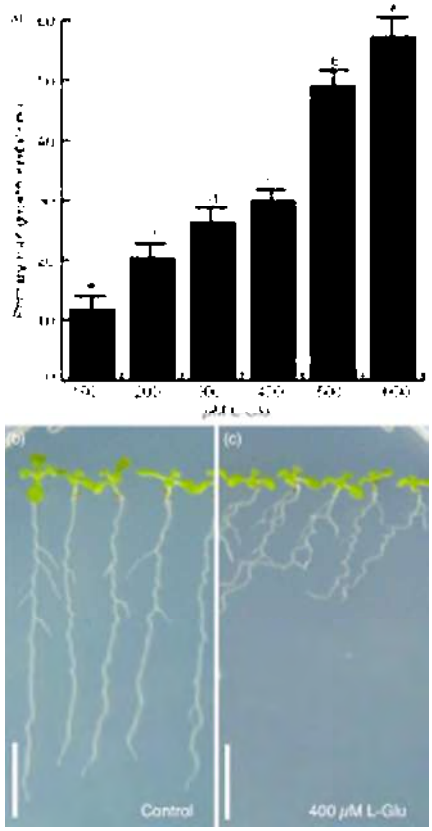
Figure 3. Primary root responses to glutamate are altered in *mkp1* and *mpk6* mutants. Seeds from wild-type (Col-0), *mpk6 and mkp1* mutants were germinated and grown for 5 days on MS basal media, and then transferred to same media supplemented with the indicated L-Glu concentrations. Primary root length (a) and root growth inhibition (b) were measured and calculated 5 days after transfer. Values are the mean ± Standard Error (n = 30) of three independent replicates. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition (Control in b). Different letters represent Tukey's post-hoc test significant differences ($P \leq 0.05$).

Figure 4. Glutamate affects gravitropic response. Wild-type (Col-0), *mpk6* and *mkp1* mutants seedlings subjected to gravitropism stimuli, as described in Materials and Methods, on MS basal media (Control) and MS basal media supplemented with 400 µM L-Glu. a) Representative pictures of wild-type and mutants seedlings behavior in each condition at the end of the experiment. Arrows indicate the representative angle formed in each plant line after gravistimulation. b) The schemes shown at the bottom of each picture represent

the angle of root growth in relation to the gravity vector of the indicated plant line for each experimental condition. The angle marked in each case is the average ± Standard Error of 16 roots for each seedling type from tree independent experiments.

Figure 5. Glutamate affects statocyte structure. Wild-type (Col-0), *mpk6* and *mkp1* mutants seedlings were germinated and grown side by side during 7-days on MS basal media (C) or MS basal media supplemented with 200 or 400 μM L-Glu. After treatment seedlings were collected and their roots were stained with Lugol's solution to analyze the statocyte structure. Representative photographs of root tips of different plant lines in each experimental condition are showed. The experiment was repeated three times with similar results (n=15). Scale bars = 100 μm.

Figure 6. *mkp1* mutant is hypersensitive to glutamate. Wild-type (Col-0), *mpk6* and *mkp1* mutants seedlings were germinated and grown side by side during 7-days on MS basal media supplemented with 100 μM L-Glu. Following the treatment the seedlings were collected and their roots stained with Lugol solution to analyze the statocyte structure. Representative photographs at 10X (left) and 40X (right) magnifications of root tips from indicated seedling in each condition are showed (a). The experiment was repeated three times with similar results (n=15). In (b), a picture of a representative Petri dish of the described experiment is shown. Scale bar =100 μm.



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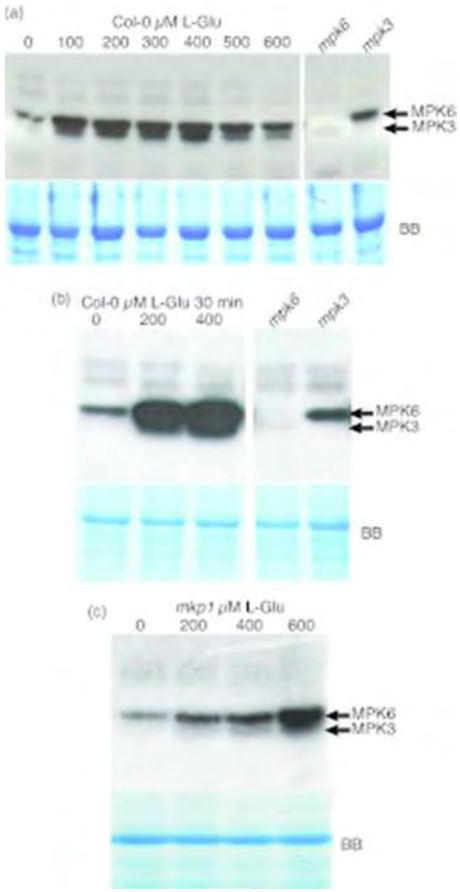

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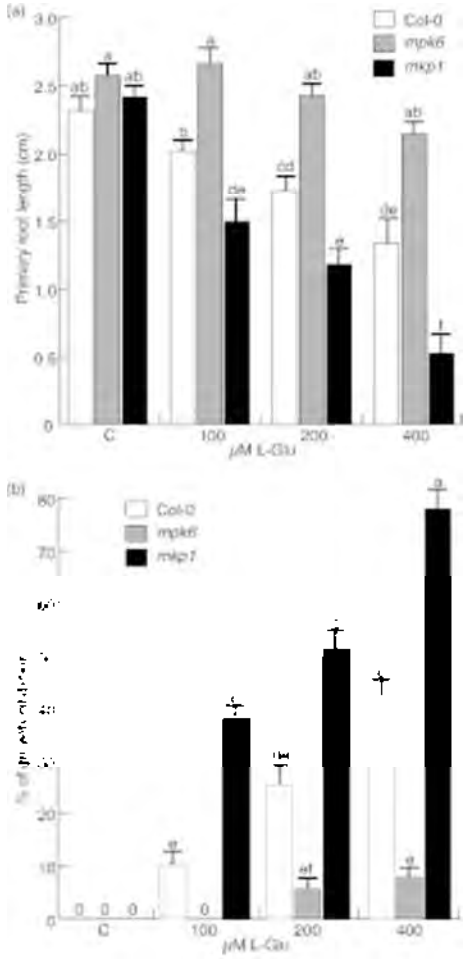


Figure 3. Primary root responses to glutamate are altered in mkp1 and mpk6 mutants. Seeds from wild-type (Col-0), mpk6 and mkp1 mutants were germinated and grown for 5 days on MS basal media, and then transferred to same media supplemented with the indicated L-Glu concentrations. Primary root length (a) and root growth inhibition (b) were measured and calculated 5 days after transfer. Values are the mean ± Standard Error (n = 30) of three independent replicates. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition (Control in b). Different letters represent Tukey's post-hoc test significant differences (P≤0.05).
80x168mm (72 x 72 DPI)

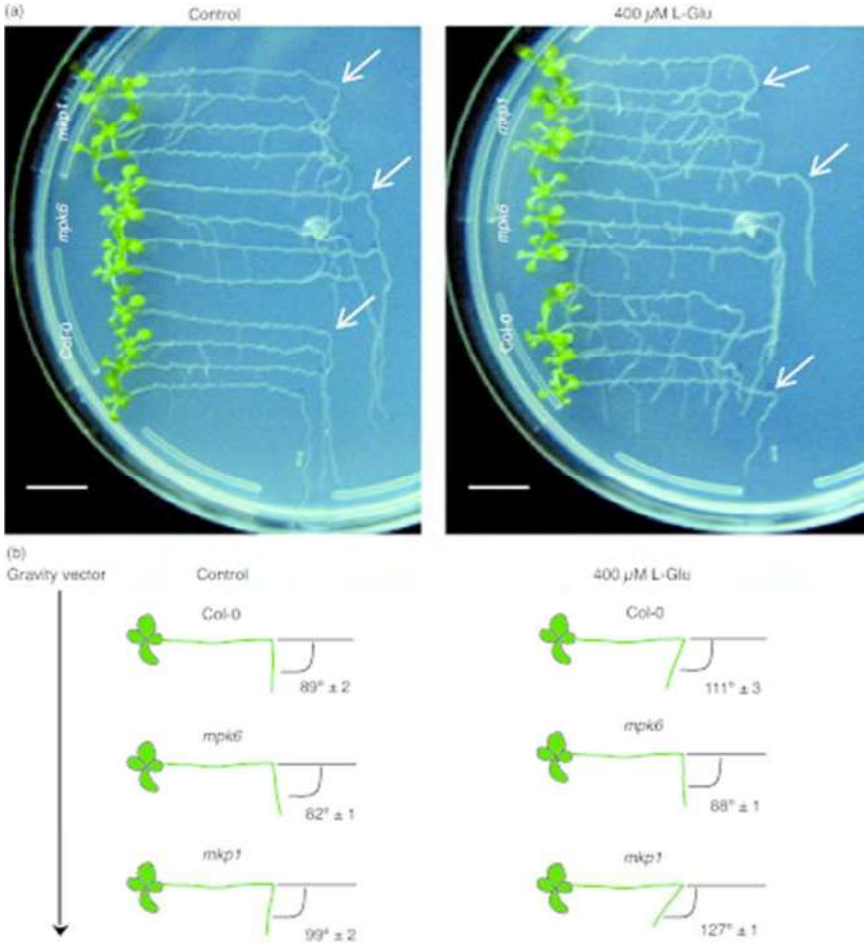


Figure 4. Glutamate affects gravitropic response. Wild-type (Col-0), mpk6 and mkp1 mutants seedlings subjected to gravitropism stimuli, as described in Materials and Methods, on MS basal media (Control) and MS basal media supplemented with 400 μM L-Glu. a) Representative pictures of wild-type and mutants seedlings behavior in each condition at the end of the experiment. Arrows indicate the representative angle formed in each plant line after gravistimulation. b) The schemes shown at the bottom of each picture represent the angle of root growth in relation to the gravity vector of the indicated plant line for each experimental condition. The angle marked in each case is the average ± Standard Error of 16 roots for each seedling type from tree independent experiments.
151x166mm (72 x 72 DPI)

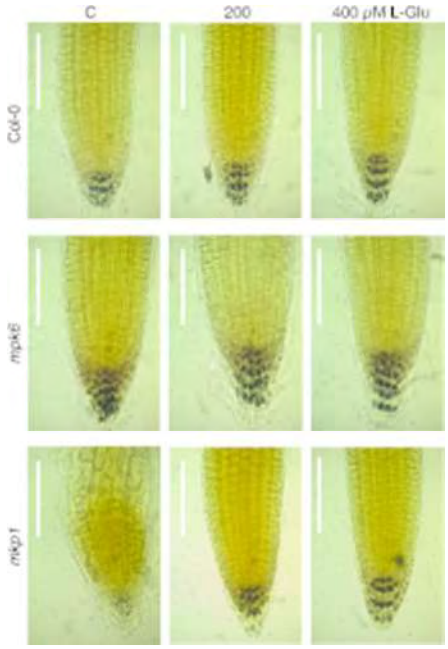


Figure 5. Glutamate affects statocyte structure. Wild-type (Col-0), mpk6 and mkp1 mutants seedlings were germinated and grown side by side during 7-days on MS basal media (C) or MS basal media supplemented with 200 or 400 μM L-Glu. After treatment seedlings were collected and their roots were stained with Lugol's solution to analyze the statocyte structure. Representative photographs of root tips of different plant lines in each experimental condition are showed. The experiment was repeated three times with similar results (n=15). Scale bars = 100 μm.
76x112mm (72 x 72 DPI)

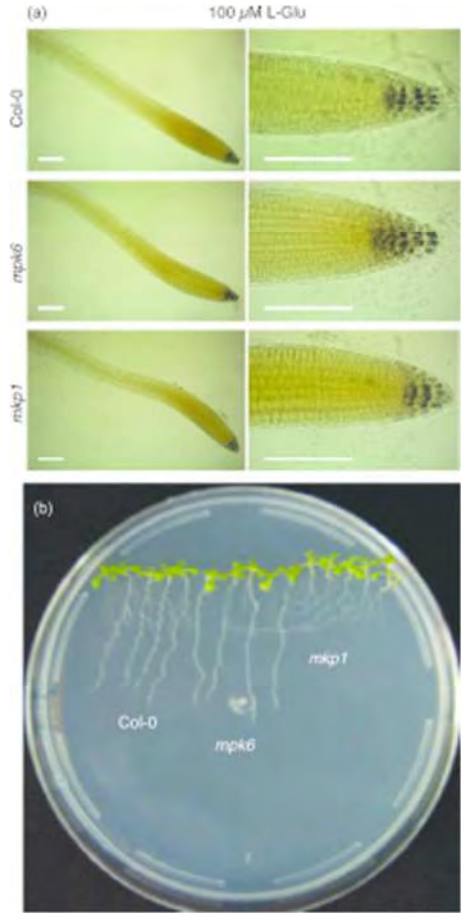
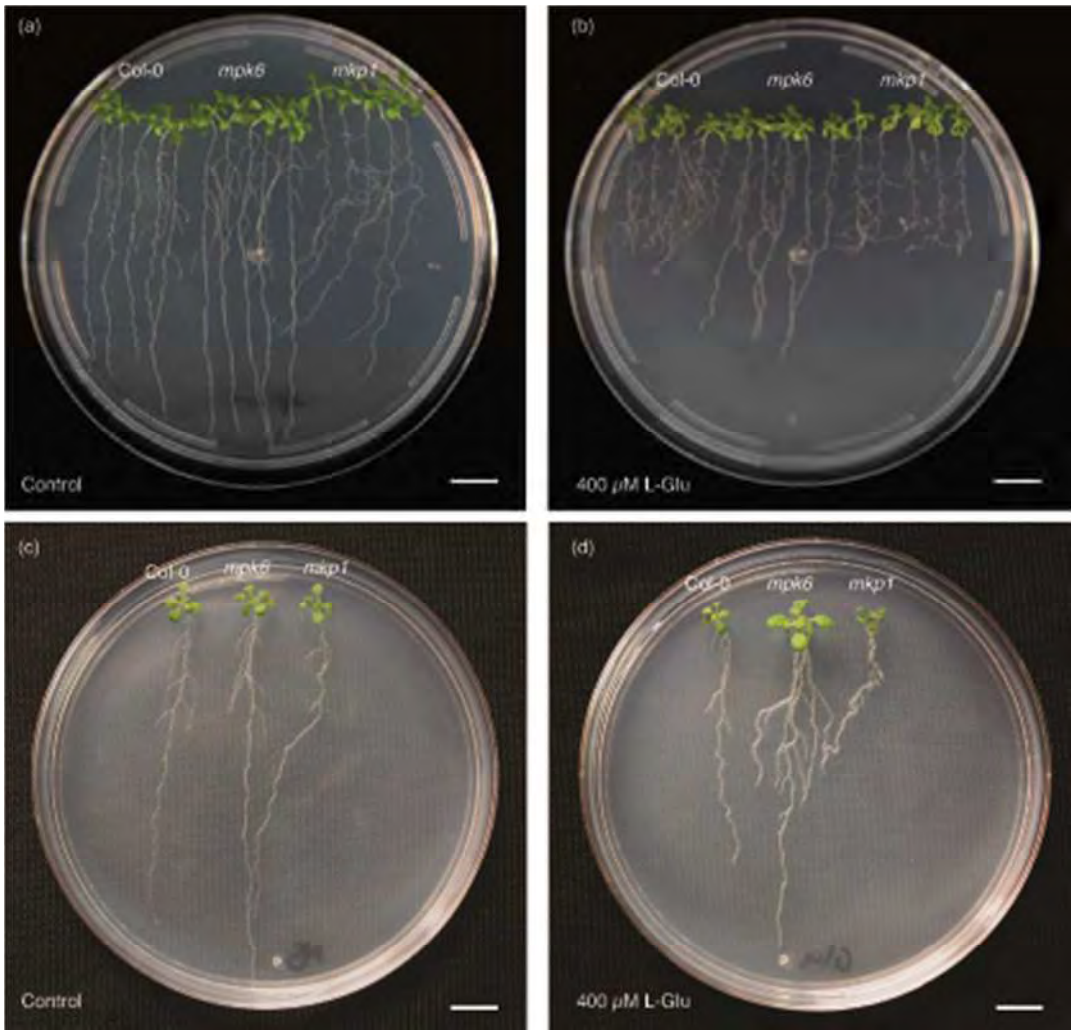


Figure 6. mkp1 mutant is hypersensitive to glutamate. Wild-type (Col-0), mpk6 and mkp1 mutants seedlings were germinated and grown side by side during 7-days on MS basal media supplemented with 100 µM L-Glu. Following the treatment the seedlings were collected and their roots stained with Lugol solution to analyze the statocyte structure. Representative photographs at 10X (left) and 40X (right) magnifications of root tips from indicated seedling in each condition are showed (a). The experiment was repeated three times with similar results (n=15). In (b), a picture of a representative Petri dish of the described experiment is shown. Scale bar =100 µm.
79x159mm (72 x 72 DPI)

Mitogen Activated Protein Kinase 6 and MAP Kinase Phosphatase 1 are involved in the *Arabidopsis* root responses to glutamate.

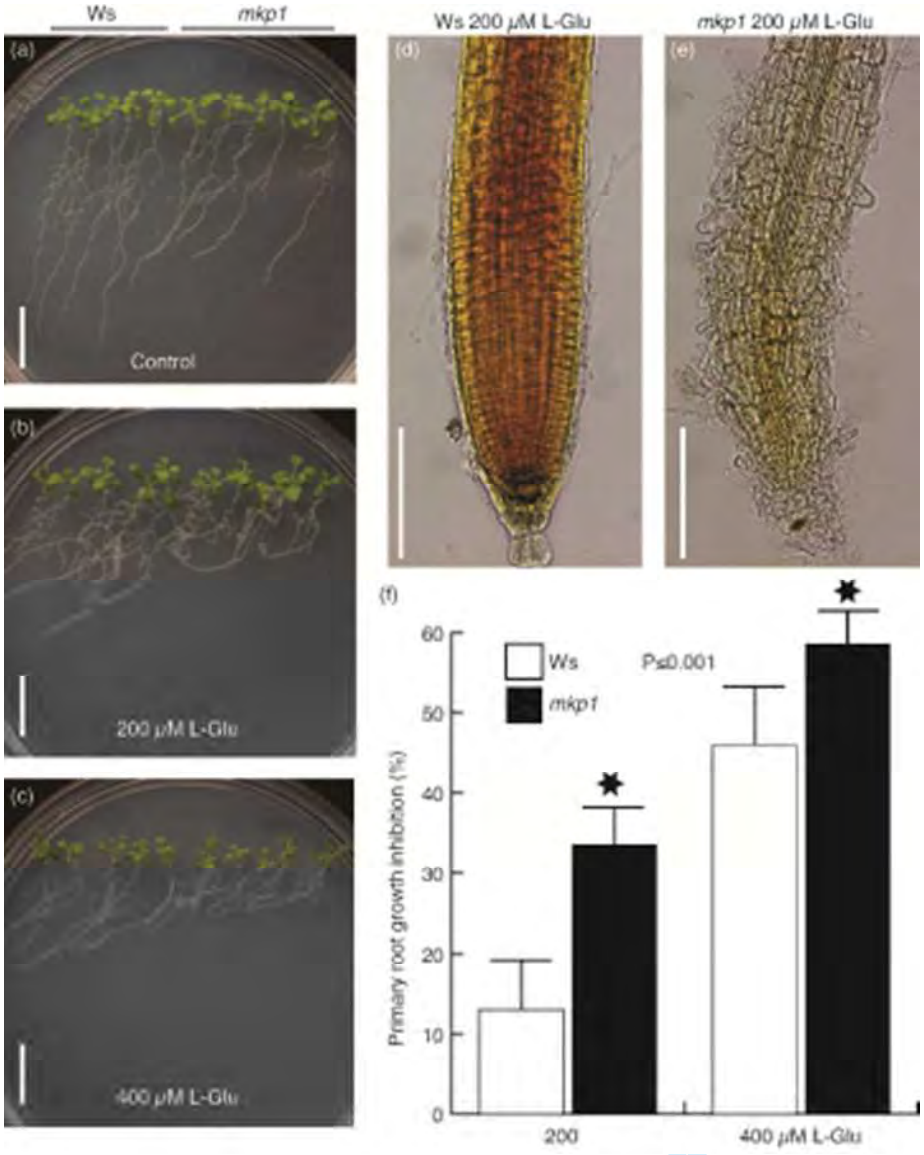
López-Bucio, J. S., López-Bucio, J., Raya-González, J., Ramos-Vega, M., León, P. and Guevara-García, A. A.[1]

Supporting Information

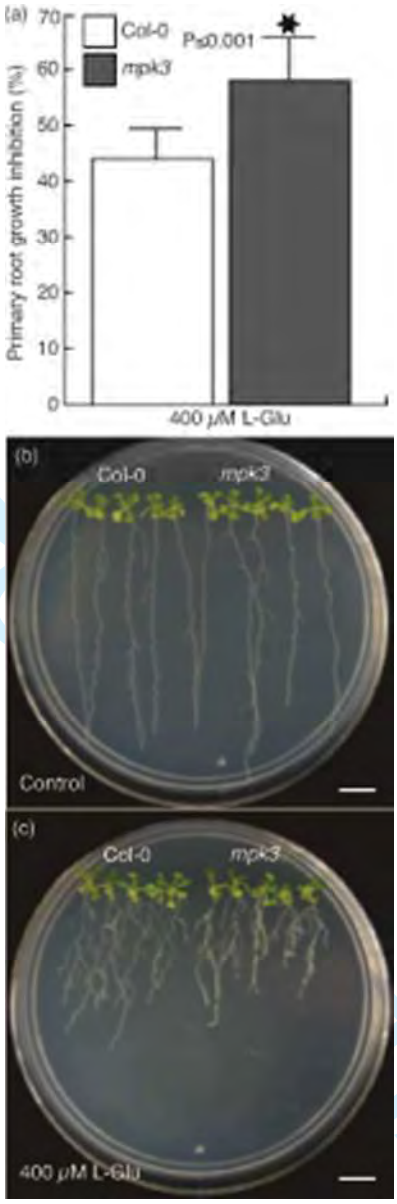


Supporting Figure S1. Glutamate primary root growth inhibition and skewing on *mkp1* and *mpk6* mutants. Pictures correspond to representative Petri dishes with 10-DAG seedlings growing in basal MS media (Control) (a and c)) and MS basal media supplemented whit 400 μM L-Glu (b and d). The experiment was repeated three times with similar results. Notice that besides root growth inhibition L-Glu treatment drives waiving and skewing of wild-type (Col-0) and *mkp1* mutant, but *mpk6* is less sensitive to these L-Glu effects. Moreover, in control conditions it is clear that the roots of *mpk6* and *mkp1* mutants are longer and shorter that wild type (Col-0), respectively.

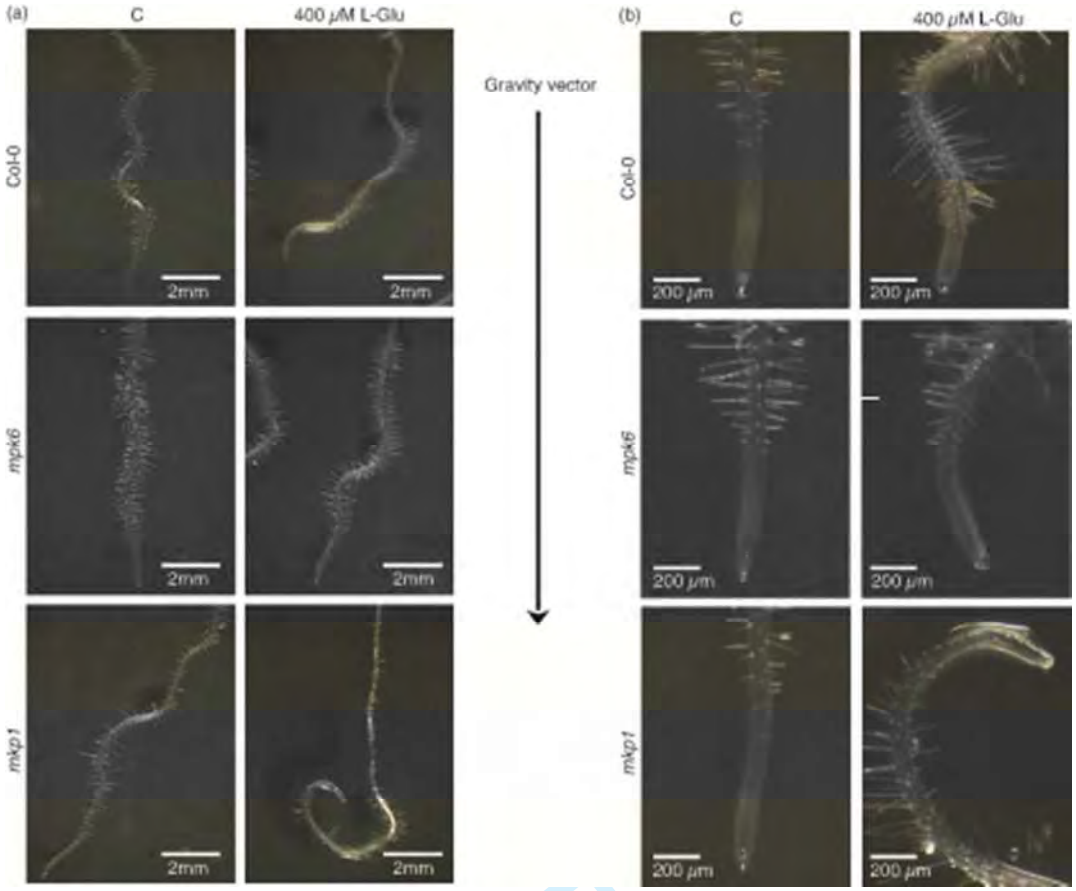
[1] Corresponding author: aguevara@ibt.unam.mx



Supporting Figure S2. Effects of Glutamate on *mkp1*/Ws mutant. In a, b and c representative Petri dishes with 10-DAG *mkp1*/Ws (Wassilewskija genetic Backgroud) mutant and wild-type (Ws) seedlings growing side by side in basal MS media and MS basal media supplemented whit 200 and 400 µM L-Glu, respectively, are showed, Scale bars= 1cm. In d and e representative stereo microscopy photographs of root tips of wild-type (Ws) and *mkp1*/Ws 7-DAG seedlings, respectively, grown in MS basal media supplemented whit 200 µM L-Glu and stained with Lugol's solution are showed. Scale bars= 1µm. Notice the dramatic effect that L-Glu have over the *mkp1*/Ws root apical meristem in general and over statocyte structure in particular. In f, a comparison between root growth inhibitions caused by 200 and 400 µM L-Glu on wild-type (Ws) and *mkp1*/Ws mutant is showed. Data were recorded from 7-DAG seedlings growing in media supplemented with the indicated L-Glu concentration. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition. Values are mean ± Standard Error (n = 15). Star marks indicate Student's t-test significant differences at $P \leq 0.001$.



Supporting Figure S3. Effects of glutamate on *mpk3* mutant. a) Comparison between root growth inhibitions caused by 400 µM L-Glu on wild-type (Col-0) and *mpk3* homozygous line. Data were recorded from 10-DAG seedlings growing in media supplemented with 400 µM L-Glu. The percentages of root growth inhibition were calculated taken PR length of seedlings growing in basal medium as 0% of inhibition. Values are mean ± Standard Error (n = 15). Star marks Student's t-test significant differences at *P*=<0.001. In (b) and (c) representative Petri dishes with 7-DAG wild-type (Col-0) and *mpk3* mutant seedlings growing side by side in basal MS media and MS basal media supplemented whit 400 µM L-Glu, respectively, are showed.



Supporting Figure S4. Glutamate drives skewing. *mpk6* and *mkp1* mutants and wild-type (Col-0) seedlings were germinated and grown side by side during 7-days on MS basal media (C) and MS basal media supplemented with 400 µM L-Glu in Petri dishes maintained in vertical position. Representative stereomicroscopy photographs of primary root of the indicated plant lines at 0.63 X (a) and 2.5 X (b) magnifications in each condition are showed. The gravity vector (arrow) is showed as reference. Scale bars are indicated.

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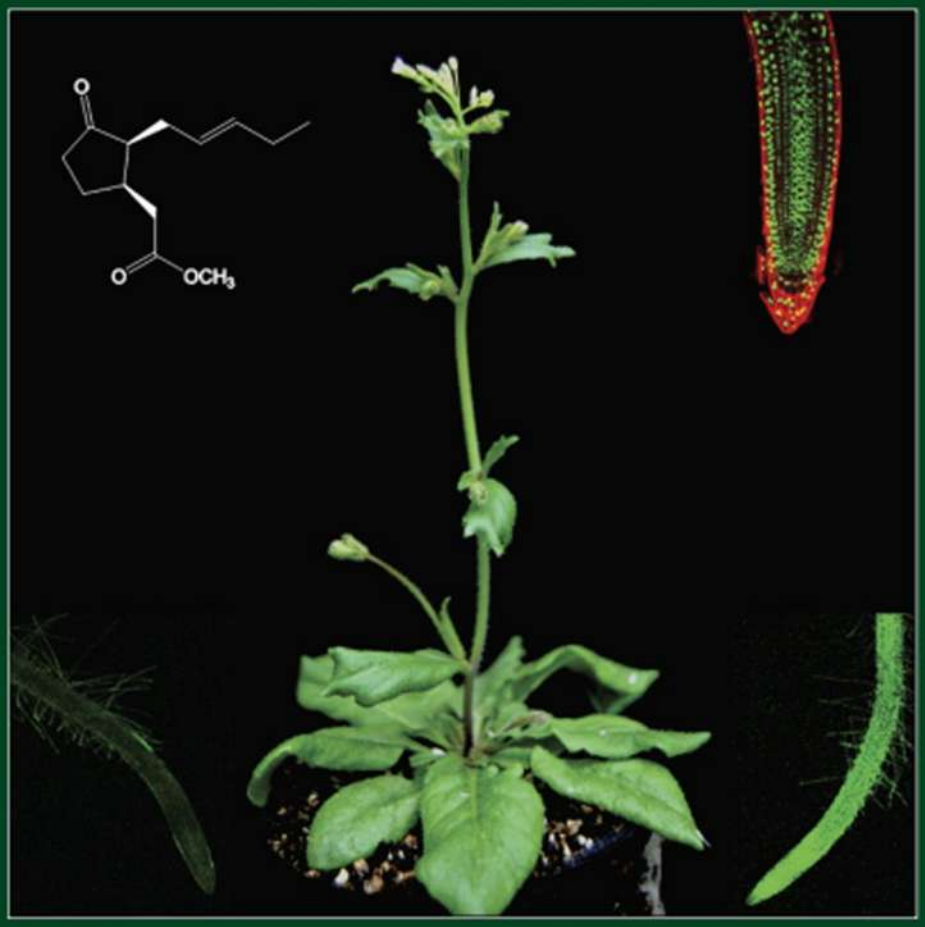

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Señalización del ácido jasmónico y el óxido nítrico durante el desarrollo de *Arabidopsis thaliana*

Amira Garnica Vergara, Javier Raya González, José López Bucio y Elda Beltrán Peña.

Instituto de Investigaciones Químico Biológicas, UMSNH.

Resumen

Los jasmonatos son compuestos lipídicos de las plantas que median respuestas de defensa y también participan en el desarrollo, la reproducción y el metabolismo. Recientemente, se ha involucrado al ácido jasmónico (AJ) en la modulación de la arquitectura de la raíz. Sin embargo, su mecanismo de acción aún se desconoce. En las hojas en respuesta a una herida se induce la acumulación de óxido nítrico (ON), lo que sugiere que la producción de dicha molécula podría estar conectada con la señalización del AJ. El ON se encuentra presente en las plantas participando como mensajero en diferentes procesos fisiológicos y del desarrollo que incluye la muerte celular programada, la inducción de genes de defensa y la regulación de la arquitectura radicular. Nuestras investigaciones han demostrado que el ON participa en la regulación del sistema radicular en *Arabidopsis thaliana*. En esta revisión se discute el uso de *A. thaliana* como modelo y el potencial que presenta para el aislamiento y caracterización de mutantes involucradas en la señalización por AJ y ON para entender los mecanismos de acción de estas moléculas y la interacción entre ellas en las plantas.

Palabras clave: ácido jasmónico (AJ), óxido nítrico (ON), *Arabidopsis thaliana*.

Abstract

***Jasmonic acid and nitric oxide signaling during development of* Arabidopsis thaliana**

Jasmonates are lipid compounds of plants that mediate defense responses, development, reproduction, and metabolism. Recently, jasmonic acid (JA) has been involved to act in the modulation of root architecture. However, its mechanism of action remains unknown. In leaves, leaf damage by herbivory or injury causes accumulation of nitric oxide (NO), indicating that NO may participate in defense responses associated with JA. ON is present in plants acting as a second messenger in various physiological and developmental processes including programmed cell death, induction of defense genes and root growth. Our research has shown that NO participates in the regulation of root system architecture in *Arabidopsis thaliana*. In this review, we discuss the importance of using *A. thaliana* as a model research and the identification of mutants defective in the processes regulated by JA and NO towards a better understanding of the mechanisms of action of these molecules and their interaction in plants.

Key words: Jasmonic acid (JA), nitric oxide (NO), *Arabidopsis thaliana*.

Introducción

Las plantas son organismos sésiles que se adaptan y responden a los cambios ambientales mediante la producción de un gran número de compuestos químicos tanto difusibles como volátiles. Entre dichos metabolitos, las hormonas vegetales juegan un papel importante en la fisiología de la planta (Avanci et al., 2010). Los jasmonatos son fitorreguladores que modulan respuestas de estrés y del desarrollo (Li et al., 2004; Buchanan-Wollaston et al., 2005; Glazebrook 2005; Howe y Jander, 2008; Browse 2009) Esto ocurre mediante una reprogramación de la expresión génica a gran escala (Mandaokar et al., 2006). Sin embargo, no se han determinado los mecanismos fisiológicos y celulares por los cuales los jasmonatos controlan dichos procesos. Recientemente, se determinó la participación del óxido nítrico (ON) en la respuesta del AJ al estrés por heridas (Wünsche et al., 2011). El ON es un segundo mensajero involucrado en diferentes procesos celulares y fisiológicos en las plantas, incluyendo el cierre de estomas (Neill et al., 2002), la floración (He et al., 2004) y la germinación (Beligni y Lamattina, 2000). Además, el ON modula la expresión de genes involucrados en las vías de señalización de otras hormonas. En *A. thaliana* se ha observado que el ON regula la expresión de genes que participan en la síntesis y respuesta a jasmonatos (Orozco-Cardenas y Ryan, 2002). Sin embargo se desconocen las posibles interacciones entre los jasmonatos y el ON en otros programas de desarrollo, por ejemplo, durante el desarrollo de la raíz.

Jasmonatos

El ácido jasmónico y sus metabolitos se conocen colectivamente como jasmonatos. Estas son moléculas importantes en la señalización por efecto del estrés biótico y abiótico. Los jasmonatos pertenecen a una familia de oxilipinas, ácidos grasos con uno o más átomos de oxígeno derivados de la oxigenación enzimática de ácidos grasos tri-insaturados de 16 y 18 carbonos. El jasmonato mejor caracterizado es el ácido jasmónico (AJ) el cual puede ser modificado por conjugación y metilación y se ha reportado que juega un papel crucial en la reproducción (Avanci et al., 2010). El metil jasmonato (MeJA) es un compuesto volátil que media la comunicación intracelular y entre diferentes plantas, modulando respuestas de defensa. Por otra parte, el jasmonoil-isoleucina (JA-Ile) es la forma activa de la molécula implicada en la señalización de los jasmonatos en hojas y posiblemente en las flores de *Arabidopsis* (Acosta y Farmer, 2010) (Fig. 1).

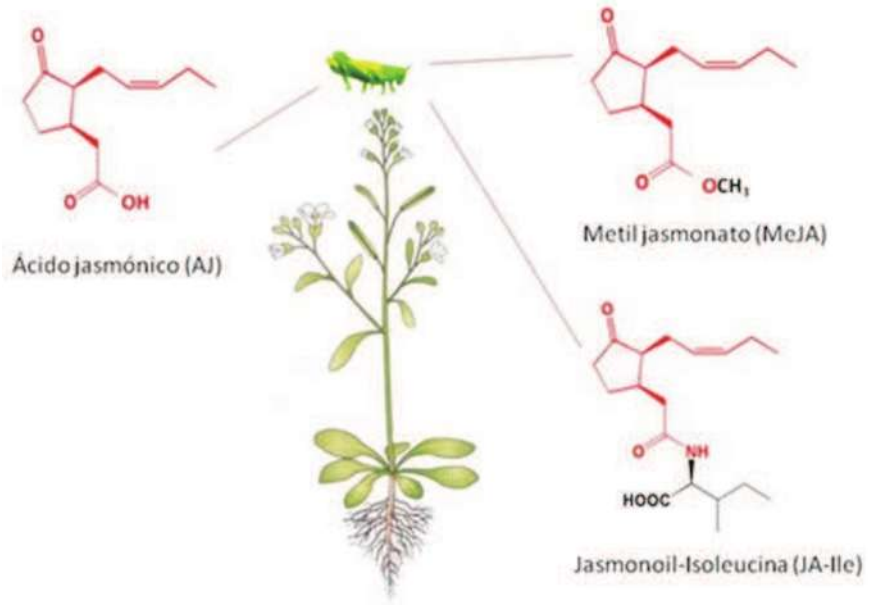

Figura 1.- Estructura de los jasmonatos y su función en las plantas. El metil jasmonato es un compuesto volátil que participa en señales entre plantas en respuesta a herbivoría, mientras que el jasmonoil-Isoleucina y el ácido jasmónico son los compuestos que activan la inmunidad vegetal contra insectos y regulan el desarrollo.

Biosíntesis de los jasmonatos

La síntesis de jasmonatos ocurre principalmente en las hojas, donde los ácidos grasos son liberados de la membrana plasmática de los plástidos por acción de enzimas fosfolipasas y desaturasas. El ácido graso sufre reacciones de oxigenación, oxidación y ciclación

por medio de una lipoxigenasa (LOX2), la aleno óxido sintasa (AOS) y la aleno óxido ciclasa (AOC), respectivamente. Así se forma el ácido 12-oxo-fitodienóico (OPDA), principal precursor de los jasmonatos, el cual se exporta hacia el peroxisoma, único orgánulo donde ocurre la ß-oxidación, reduciéndose por la enzima ácido oxo-fitodienóico reductasa 3 (OPR3). Posteriormente, este compuesto es sometido a tres ciclos de ß-oxidación para producir el ácido jasmónico (AJ). El AJ es exportado al citosol por un mecanismo desconocido donde ocurren las diferentes reacciones metabólicas que darán origen a los distintos jasmonatos (Wasternack 2007) (Fig. 2). Entre las principales transformaciones del AJ se encuentra la metilación en el carbono 1 (C1), para producir el compuesto volátil metil-jasmonato (MeJA), el cual está involucrado en la señalización entre plantas. Otra modificación relevante es la conjugación del grupo carboxilo al aminoácido isoleucina para formar el jasmonoil-isoleucina (JA-Ile). En *A. thaliana*, el JA-Ile se sintetiza por una enzima codificada por el gen *JAR1*. Las mutantes en el locus *jar1* se caracterizan por una respuesta disminuida al AJ (Zhang y Turner, 2008). Estos resultados indican que el JA-Ile es una señal primaria importante para las respuestas de defensa y desarrollo.

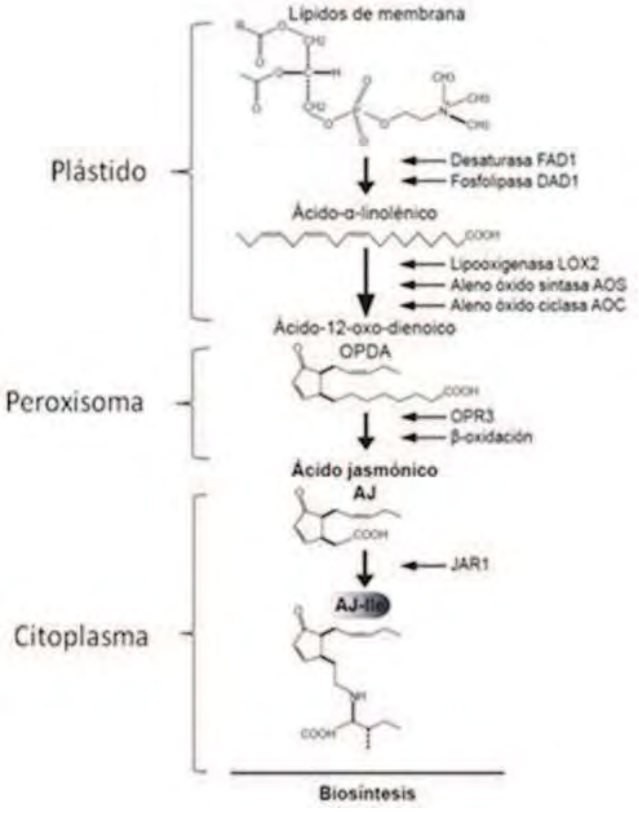

Figura 2.- Vía de síntesis de los jasmonatos. La síntesis de los jasmonatos ocurre a partir del ácido α-linolénico. Se muestran las principales enzimas de la vía (Modificada de Browse 2009).

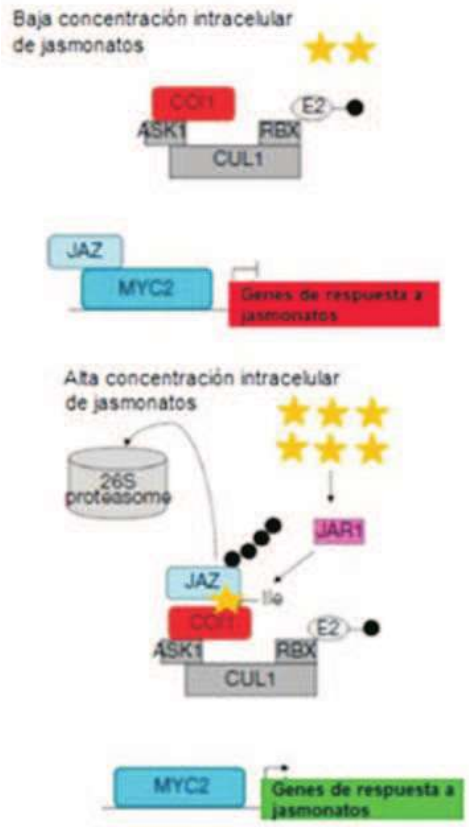

Figura 3.- El complejo SCF^{COI1} es requerido para la señalización de jasmonatos. El complejo SCF^{COI1} está formado por la proteína de la familia SKP1 ASK1, la proteína de la familia de las CULINAS CUL1 y la de la caja F COI1. La unión de jasmonoil-Isoleucina (JA-Ile) al receptor CORONATINE-INSENSITIVE1 (COI1) promueve la unión de los represores de jasmonatos ZIM-Domain (JAZ) y su ubiquitinación, resultando así en la liberación del factor de transcripción MYC2 y la transcripción de genes de respuesta a jasmonatos (Modificado de Santner y Estelle, 2009).

Señalización de los jasmonatos

Los jasmonatos y las auxinas, a pesar de ser compuestos químicamente diferentes, comparten algunos elementos en las vías de señalización para su percepción y respuesta en las células (Santner y Estelle, 2009). TIR1 es una proteína tipo F-box que funciona como uno de los receptores en la señalización de auxinas. Existen cerca de 700 genes que codifican para proteínas tipo F-box en *A. thaliana*. La mutante de *A. thaliana* denominada *coronatine-insensitive1* (*coi1*) permitió definir el papel de los jasmonatos en el crecimiento de la planta (Feys et al., 1994). La coronatina es una fitotoxina relacionada estructural y biológicamente con el Ja-Ile. La mutante *coi1* antes mencionada es resistente tanto a la coronatina como al MeJA y su principal característica es la esterilidad masculina. COI1 codifica para una proteína tipo F-box estrechamente relacionada con TIR1, lo que sugiere que al igual que los represores auxínicos ampliamente estudiados, la respuesta a los jasmonatos implicaría la degradación de un grupo de represores de los jasmonatos conocidos como JAZ que poseen el dominio ZIM dependiente de SCF^{COI1} (Santner y Estelle, 2009). El complejo multi-

proteico SCF pertenece al tipo *E3 ubiquitina ligasa* que media la ubiquitinación de proteínas para su degradación por el proteosoma 26S. Dicho complejo contiene varias proteínas F-box que se unen directamente con el blanco de la ubiquitinación. COI1 se une a las proteínas JAZ, que actúan como reguladores negativos de la transcripción de genes de respuesta a jasmonatos, las cuales interactúan con el factor de transcripción tipo bHLH (hélice-vuelta-hélice básica) MYC2 interfiriendo con la función de este activador transcripcional (Chini et al., 2007). Una gran cantidad de genes JAZ son auto regulados en respuesta a jasmonatos, indicando un mecanismo de retroalimentación negativa. Análisis bioquímicos han mostrado que la coronatina se une al complejo COI1-JAZ con alta afinidad, donde el jasmonato conjugado a isoleucina (JA-Ile) es la molécula activa (Fonseca et al., 2009). Por lo tanto, el complejo SCF^{COI1} sirve como receptor para el JA-Ile para estabilizar la interacción entre la proteína tipo F-box y su sustrato, lo cual permite la degradación del represor JAZ y la liberación del factor de transcripción MYC2, ocurriendo entonces la activación de los genes de respuesta a jasmonatos (Fig. 3) (Katsir et al., 2008).

Funciones de los jasmonatos

Inmunidad vegetal

La señalización por jasmonatos es esencial para la activación de las respuestas de defensa en plantas en contra de insectos y herbívoros (Browse 2005). La síntesis de jasmonatos se activa por herbivoría, que comúnmente daña a las hojas y otros tejidos, así como por heridas causadas por un daño mecánico. Esto ocurre mediante la inducción de cientos de genes que codifican para proteínas para la síntesis y percepción de los jasmonatos, así como proteínas implicadas en el flujo de iones y en la adaptación al estrés en general (Reymond et al., 2000). La producción de jasmonatos induce la formación de tricomas en hojas, los cuales confieren protección a la lamina foliar (Yan et al., 2007; Yoshida et al., 2009). Otra función esencial de los jasmonatos es en la activación de la inmunidad en contra de patógenos que se alimentan de tejidos muertos como es el caso de algunos hongos necrotróficos (Browse 2009), o de ciertas bacterias que causan enfermedades a la planta (Gutjahr y Paszkowski, 2009).

Regulación del desarrollo

Los jasmonatos AJ y MeJA son esenciales para la maduración del polen, la elongación de los filamentos de los estambres y la apertura de los estambres que permite la liberación del polen (Delker et al., 2006). Existe una evidencia clara de lo anterior, sustentada en el fenotipo de las flores en las mutantes de *Arabidopsis* afectadas en la síntesis de AJ y en el receptor de jasmonatos COI1, que muestran esterilidad masculina. La mutante *coi1* es incapaz de producir polen viable (von Malek et al., 2002). Otro proceso importante del desarrollo en la que participan los jasmonatos es la regulación de la arquitectura de la raíz. El tratamiento de plantas de *A. thaliana* con AJ causa el acortamiento de la raíz primaria y promueve

la formación de raíces laterales. Adicionalmente, se ha observado que las mutantes afectadas en los componentes de la síntesis y de la transducción de señales dependientes de COI1 presentan una reducción en el crecimiento de la raíz primaria (Wasternack 2007). La participación del MeJA en la formación de raíces laterales, está relacionada con la biosíntesis de auxinas dependiente del gen *ANTHRANILATE SYNTHASE* α*1* (*ASA1*) y la atenuación en el transporte de auxinas al disminuir los niveles de expresión de los transportadores de eflujo de auxinas PIN1 y PIN2 (Sun et al., 2009). Otra actividad biológica de los jasmonatos es la inducción de la senescencia (Schommer et al., 2008),la cual se atribuye a la regulación de genes que codifican para proteínas de mantenimiento de los cloroplastos y de síntesis de carbohidratos (Wasternack 2007).

Óxido nítrico

El óxido nítrico (ON) es un radical libre debido a que contiene un electrón desapareado que se sintetiza en los animales por una familia de enzimas denominadas óxido nítrico sintasas (NOS) a partir de L-arginina. El ON tiene una gran importancia biológica en animales en la neurotransmisión, la vasodilatación, la respuesta inmune y en la regulación del consumo de oxígeno en la mitocondria (Stuehr et al., 2004).En años recientes, el ON se ha implicado en la señalización en plantas durante la xilogénesis, la apoptosis, la defensa contra patógenos, la floración, el cierre de los estomas y el gravitropismo (Neill et al., 2003). También se ha reportado que el ON participa en la modulación de la expresión de genes que codifican proteínas para la biosíntesis de AJ y para la interacción con otras rutas hormonales incluyendo las del ácido abscísico, citocininas y etileno (Orozco-Cardenas y Ryan, 2002; Correa-Aragunde et al., 2006), para la fotosíntesis y la apoptosis (Polverari et al., 2003).

Síntesis de óxido nítrico

Las vías de síntesis del óxido nítrico pueden ser clasificadas como oxidativas o reductoras. La producción de ON por la nitrato reductasa es una reacción reductora; mientras que las que ocurren a partir de L-arginina o poliaminas son rutas oxidativas (Kapuganti et al., 2010). La vía reductora conduce a la producción de ON dependiente de nitrito (NO_2) como sustrato primario, el cual es producido a partir del nitrato (NO_3) por la nitrato reductasa (NR). La NR se encuentra en el citosol. El fenotipo de la doble mutante nia1 nia2 de Arabidopsis muestra que la NR está involucrada en la apertura estomática, la activación de enzimas antioxidantes, la señalización por patógenos, el desarrollo floral, el estrés osmótico, así como la formación de raíces laterales inducida por auxinas (Benamar et al., 2008; Kolbert et al., 2008). También se ha documentado la producción de ON por una proteína membranal (NiNOR) y por la reducción del nitrito en la membrana interna de la mitocondria probablemente vía la citocromo c oxidasa y/o reductasa (Kapuganti et al., 2010). Las vías oxidativas de producción del ON comprenden una actividad parecida a la de la óxido nítrico sintasa (NOS), la cual podría utilizar como substrato poliaminas e hidroxilaminas. Esta actividad se ha descrito en cloroplastos y peroxisomas y es importante para el desarrollo de la raíz y la

embriogénesis, toxicidad por cadmio y estrés por drogas (Jasid et al., 2006; Tun et al., 2006; Corpas et al., 2009) (Fig. 4).

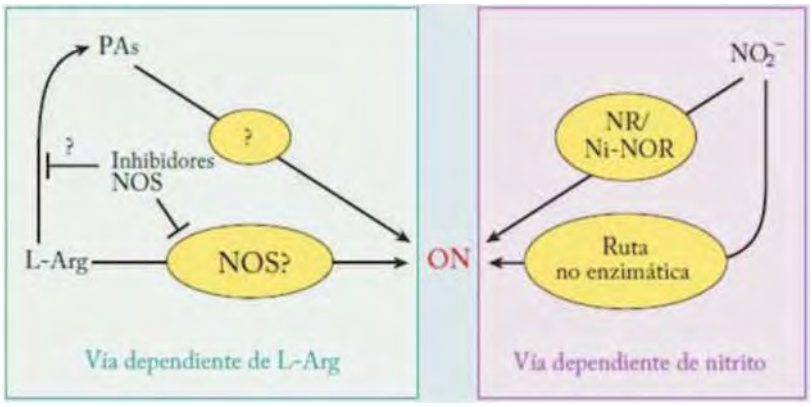

Figura 4.- Biosíntesis de ON en plantas. El ON puede ser sintetizado por dos vías, la vía dependiente de nitrito que involucra una ruta enzimática en la que las nitrato reductasa (NR) y la reductasa de nitrito a ON (NiNOR) catalizan la síntesis del ON y otra ruta no enzimática que comprende reacciones químicas de reducción. La vía aún no caracterizada dependiente de L-arginina implica la participación de una enzima similar a la óxido nítrico sintasa (NOS) y otra ruta no descrita que utiliza poliaminas (PAs) como sustrato (Modificada de Besson-Bard et al., 2008).

Percepción del ON y transducción de la señal en plantas

Aunque hasta la fecha no se ha identificado ningún receptor de ON, por su naturaleza, este radical es muy reactivo y puede interactuar con numerosas proteínas. En células animales, la guanilil ciclasa soluble (sGC) tiene un papel fundamental en la señalización de ON, donde este último se une al dominio hemo de la sGC estimulando la liberación del monofostato de guanosina cíclico (cGMP) que a su vez activa distintos blancos celulares. En las plantas, estudios farmacológicos usando inhibidores del ON como el 2-fenil-4,4,5,5-tetrametil imidazol-1-oxi-3-óxido (PTIO) o el 2-(4-carboxifenil)-4,4,5,5 -tetrametilbimidazol- 1- oxi- 3-óxido (cPTIO) han implicado al cGMP y a la señalización por ácido abscísico (ABA) río abajo del ON en células guardia de los estomas (Neill et al., 2003). El ON induce un incremento de cGMP y activa cascadas de señalización que modulan la expresión génica (Grün et al., 2006). Una molécula esencial río abajo del cGMP es la ADP ribosa cíclico (cADPR). En células animales, la cADPR estimula la liberación de Ca^{2+} intracelular, activando al receptor rianodina (RYR) de los canales de calcio y es posible que un mecanismo de señalización similar ocurra en plantas. En tabaco, el cADPR eleva la expresión genética de la fenilalanina amonio liasa (PAL) y la proteína 1 relacionada con patogénesis (PR-1), que es sensible a los inhibidores del RYR. Los dos genes antes mencionados también son regulados por el ON y los antagonistas de cADPR reducen la expresión de PR-1. El ON causa un incremento en los niveles de Ca^{2+} libre, por lo tanto podría actuar a través del cGMP, cADPR y Ca^{2+} para promover el cierre de estomas inducido por ABA. La inhibición por cPTIO durante este pro-

ceso inducido por ABA lleva a la inactivación de los canales de K^+ dependientes de Ca^{2+} y a la activación de los canales de Cl^-, lo que implicaría fuertemente al ON y el Ca^{2+} en la cascada de señalización por ABA (Neill et al., 2008). En animales, la S^-nitrosilación es un mecanismo de modificación post-traduccional que regula numerosos procesos de señalización y vías metabólicas. En plantas, se ha reportado que la S-nitrosilación de proteínas juega un papel importante en la señalización del ON. En extractos de cultivos de células de *Arabidopsis* tratados con glutatión S-nitrosilado (GSNO), se han aislado-proteínas que contienen grupos S-nitrosilados (S-ON) que están involucradas con el estrés oxidativo, señalización, estructura celular y metabolismo (Lindermayr et al., 2005). También es posible que el ON interactúe nitrando residuos de tirosina a través de especies reactivas de nitrógeno como el anión peroxinitrito ($ONOO^-$) y el dióxido de nitrógeno (NO_2) que se forman durante el metabolismo del ON en presencia de especies reactivas de oxígeno y centros de metales de transición. La nitración de tirosina de proteínas endógenas ha sido demostrada en líneas antisentido de tabaco para la nitrato reductasa (NiR) donde se observaron cantidades incrementadas de ON (Morot-Gaudry-Talarmain et al., 2002). Estudios recientes mostraron la participación de la proteína cinasa 6 activada por mitógenos (MAPK6) en la señalización por ON, donde se encontró que la MAPK6 y la nitrato reductasa (NIA2), interactúan *in vitro* e *in vivo*. En dicha interacción MAPK6 fosforila a NIA2, aumentando la producción de ON, lo que finalmente condujo a cambios morfológicos en el sistema radicular de *Arabidopsis* (Wang et al., 2010).

Interacción de los jasmonatos con el ON

Diferentes evidencias han implicado al ON como un modulador en respuestas a heridas y/o estrés mecánico (Garces et al., 2001). En jitomate, la generación de H_2O_2 inducido por herida inhibió la producción de ON y la expresión de genes para la síntesis de ácido jasmónico. Por otra parte, se ha reportado que el ON tiene un papel fundamental en la regulación de la expresión de genes de defensa inducidos por herida durante la patogénesis (Orozco-Cardenas y Ryan, 2002). Estudios realizados en hojas de *Arabidopsis* mostraron que en respuesta a heridas y a tratamientos con AJ, se incrementó la concentración de ON, lo que proporcionó una evidencia del posible entrecruzamiento entre el AJ y el ON (Huang et al., 2004). Actualmente se sabe que tanto el AJ como el ON están involucrados en el desarrollo de la arquitectura de la raíz de *Arabidopsis thaliana* acortando la longitud de la raíz primaria e incrementando el número de raíces laterales (Campos-Cuevas et al., 2007; Sun et al., 2009; Méndez-Bravo et al., 2010). Sin embargo, se desconoce la interacción entre estas moléculas de señalización en la regulación de la arquitectura radicular de *Arabidopsis*, por lo cual sería de gran interés obtener información al respecto debido a que ambas moléculas regulan procesos similares en el desarrollo de la raíz.

Conclusiones

Los jasmonatos son hormonas ampliamente estudiadas principalmente por su participación en la inmunidad vegetal, en la adaptación al estrés biótico y abiótico y como reguladores de diversos aspectos del crecimiento y desarrollo de las plantas (Santner y Estelle, 2009). Como se ha evidenciado, el ON interactúa con el AJ en las respuestas de defensa y por heridas. Con las mutantes de *A. thaliana* como herramienta nuestro grupo reportó la participación de los genes *NIA1* y *NIA2* durante la formación de raíces laterales inducida por alcamidas (Méndez-Bravo et al., 2010). El aislamiento y caracterización de la mutante *decanamide resistant root1* (*drr1*), reveló posteriormente que el AJ interactúa con las alcamidas en los procesos de senescencia y desarrollo de la raíz y que esta mutante es resistente a la inhibición del crecimiento de la raíz primaria por el ON (Méndez-Bravo et al., 2010). Estos resultados muestran el enorme potencial que tiene *A. thaliana* para investigaciones en las vías de señalización donde se cuenta con información escasa. Actualmente estamos investigando las posibles interacciones entre el ON y el AJ sobre la arquitectura radicular de *Arabidopsis thaliana*, evaluando la participación del óxido nítrico en la vía de señalización de jasmonatos mediante herramientas genéticas y farmacológicas que nos permitirán esclarecer los mecanismos celulares y moleculares implicados en la interacción del AJ y el ON.

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