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Efecto de la alcamida *N*-isobutil decanamida en la expresión global de genes de *Arabidopsis thaliana*

Tesis que para obtener el grado de Doctor en Ciencias Biológicas

presenta

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CONTENIDO

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I. RESUMEN

A lo largo de su ciclo de vida, las plantas continuamente modifican sus patrones de crecimiento y desarrollo en respuesta a los cambios ambientales. Estas respuestas implican modificaciones a nivel de expresión de genes, síntesis y de actividad de proteínas, de actividad metabólica, y de producción y transporte hormonal. El reconocimiento de otros seres vivos, principalmente de patógenos o simbiontes es un detonante de reajustes importantes a nivel bioquímico y fisiológico en las plantas. La activación de las respuestas de defensa en las plantas implica el refuerzo de la pared celular, la producción de compuestos antimicrobianos y la expresión de proteínas relacionadas con la patogénesis. Las alcamidas pertenecen a un grupo de lípidos bioactivos de plantas que regulan en diferentes procesos del desarrollo vegetal. La *N*-isobutil decanamida es una alcamida de longitud corta en su cadena lipofílica (10 carbonos, sin instauraciones) proveniente de la reducción catalítica de la afinina, que es a su vez un componente abundante de la raíz de *Heliopsis longipes*. En estudios previos se ha reportado que la aplicación exógena en concentraciones micromolares de *N*-isobutil decanamida a cultivos *in vitro* de *Arabidopsis thaliana* (Arabidopsis), causa alteraciones drásticas en el desarrollo, inhibiendo el crecimiento de la raíz primaria, induciendo la formación de raíces laterales y adventicias, y provocando la formación de estructuras indiferenciadas de aspecto calloso sobre las hojas.

Se realizó un análisis global de los cambios en la expresión génica de Arabidopsis en respuesta a la *N*-isobutil decanamida. El transcriptoma obtenido por estos estudios reveló que por lo menos 181 genes relacionados con respuestas de defensa y estrés incrementaron sus niveles de expresión. En particular, los genes que codifican para enzimas involucradas en la biosíntesis del ácido jasmónico (AJ). Dicha inducción se produjo en paralelo con la acumulación de AJ, óxido nítrico (ON) y peróxido de hidrógeno (H_2O_2) en las plantas tratadas. La activación de las respuestas antes mencionadas fueron suficientes para conferir resistencia en hojas de Arabidopsis contra el ataque de los hongos necrótrófos *Botrytis cinerea* y *Alternaria brassicicola*. Mutantes de Arabidopsis alteradas en las vías de señalización del AJ fueron incapaces de defenderse de manera exitosa contra el ataque de *B. cinerea* en tratamientos con la *N*-isobutil decanamida, lo que sugiere que las alcamidas modulan respuestas de defensa, a través del control de la biosíntesis y señalización del AJ.

Por otra parte, para determinar una posible interacción entre las alcamidas, el ON y el AJ en la regulación del desarrollo Arabidopsis, se comparó el efecto de la aplicación exógena de AJ a plantas mutantes resistentes al AJ (*jar1* y *coi1-1*) y a la *N*-isobutil decanamida (*drr1*). La aplicación de AJ y de concentraciones micromolares de *N*-isobutil decanamida y de donadores químicos de ON, redujeron de manera similar el crecimiento de la raíz primaria y promovieron la formación de raíces laterales en plantas tipo silvestre. Sin embargo, ni las plantas mutantes afectadas en el receptor de AJ (*coi1-1*), ni las mutantes *drr1* presentaron inducción en la formación de raíces laterales *de novo* en respuesta al AJ. Interesantemente, tampoco los donadores químicos de ON promovieron la iniciación de raíces laterales en las plantas mutantes *drr1*. Estos resultados, en conjunto, sugieren que algunos elementos necesarios en la señalización regulada por las alcamidas, dependen del AJ y del ON.

II. SUMMARY

Plants activate defense responses against pathogens through cell wall reinforcement, production of antimicrobial compounds, and the expression of pathogenesis-related proteins. Alkamides belong to a class of bioactive lipids of wide distribution in plants that regulate development in *Arabidopsis thaliana* (Arabidopsis). A global analysis on gene expression changes in response to the alkamide *N*-isobutyl decanamide revealed at least 181 up-regulated genes related to defense and stress responses. In particular, increased expression was observed on genes encoding enzymes for jasmonic acid (JA) biosynthesis. Gene induction occurred in parallel with an increment in defense and stress-related metabolites, such as JA, nitric oxide (NO) and hydrogen peroxide (H_2O_2) in treated seedlings. The role of the alkamide to confer resistance against necrotizing fungi *Botrytis cinerea* and *Alternaria brassicicola* was tested by inoculating Arabidopsis leaves with fungal spores and evaluating disease symptoms and fungal proliferation. *N*-isobutyl decanamide application significantly reduced necrosis caused by the pathogens and inhibited fungal proliferation in WT leaves. Arabidopsis mutants altered in JA signaling were unable to defend from fungal attack even when *N*-isobutyl decanamide was supplied in the medium, indicating that alkamides modulate necrotrophic-associated defense responses through JA-dependent signaling pathways. Our results thus reveal a novel function of alkamides inducing plant immunity through directly modulating JA biosynthesis.

To more in deep investigate the mechanism by which alkamides regulate Arabidopsis root development, and to establish a possible interaction with NO and JA-dependent signaling pathways, we compared morphogenic effects of JA application to Arabidopsis WT and mutant seedlings affected in the JA receptor (*coi1-1*) or in *N*-isobutyl decanamide responses (*drr1*). JA caused root developmental alterations in a similar way to *N*-isobutyl decanamide and to chemical donors of NO, by inhibiting primary root growth and stimulating lateral root formation in WT seedlings. However, both *coi1-1* and *drr1* mutants seedlings showed reduced lateral root formation when exposed to stimulatory concentrations of JA. In addition to JA failure to induce lateral roots, the *drr1* mutant was less sensitive both in primary root growth inhibition and lateral root formation induced by NO donors than WT seedlings. Together, our results suggest a role for JA and NO signaling pathways in mediating root morphogenetic responses to alkamides in Arabidopsis.

III. INTRODUCCIÓN

A lo largo del su ciclo de vida de las plantas, por su naturaleza sésil, están permanentemente expuestas a condiciones ambientales cambiantes y frecuentemente desfavorables, por lo que para crecer, desarrollarse y reproducirse, han desarrollado sofisticados mecanismos que les permiten adaptarse a su entorno biótico y abiótico. Estos mecanismos son regulados por un grupo amplio de fitohormonas que incluyen a las auxinas, las citocininas, el etileno, el ácido abscísico y el ácido jasmónico. Las fitohormonas funcionan en las células vegetales como iniciadores de cascadas de señalización que determinan diferentes patrones de expresión de los genes requeridos para mantener y extender espacial y temporalmente tales respuestas (Cow y McCourt, 2009). En los últimos años, se ha evidenciado que las plantas producen también otros compuestos con función reguladora inicialmente caracterizados en los animales. Estos compuestos incluyen a las *N*-acil etanolaminas, el glutamato y el óxido nítrico, entre otros. En particular, el óxido nítrico es una molécula gaseosa ampliamente reconocida como un segundo mensajero capaz de integrar diversas señales hormonales, que le permiten a las plantas responder eficazmente ante diferentes condiciones activando simultáneamente múltiples rutas de señalización (Lamattina et al, 2003).

3.1 Respuestas de defensa inducibles

El contacto de las plantas con microorganismos patógenos las ha llevado a desarrollar sistemas de defensa inducibles que contrarrestan el daño causado por patógenos. Ante la invasión del tejido vegetal por un microorganismo, la respuesta de defensa inducible más temprana, a nivel local, es la activación de una vía de transducción que conlleva a la muerte celular controlada, conocida también como “respuesta hipersensible”. La respuesta hipersensible es un fenómeno que restringe la zona de invasión del microorganismo, aislando el área infectada mediante el refuerzo mecánico de las paredes celulares de células circundantes y secretando compuestos que son tóxicos para el patógeno, o bien funcionando como segundos mensajeros en las células vegetales (fitoalexinas, péptidos pequeños conocidos como defensinas, glucanasas, quitinasas, especies reactivas del oxígeno y óxido nítrico) (López et al, 2008). Sin embargo, estos mecanismos no siempre resultan eficaces, tal es el caso de fitopatógenos necrótrofos, es

decir, aquellos que provocan la muerte celular secretando enzimas digestivas que le permiten utilizar como fuente energética los restos de tejido vegetal necrosado. Contra este tipo de patógenos, la respuesta hipersensible puede o no llevarse a cabo, pero se requiere una segunda fase de respuestas, que sean generalizadas en la planta: las respuestas de defensa sistémica (Penninckx et al, 1996). Las respuestas de defensa sistémica preparan a los tejidos y órganos distantes para defenderse de un proceso infeccioso. Tales reacciones comprenden la producción de toxinas antimicrobianas (como las fitoalexinas) y el reforzamiento estructural de paredes celulares en el resto de tejidos. La señalización sistémica consta de diversas reacciones y procesos complejos en las que intervienen el metabolismo secundario y los patrones bioquímicos de los lípidos de membrana (Conrath et al, 2006). Los lípidos de membrana son precursores moleculares de diferentes compuestos con funciones importantes a nivel inter e intracelular, como es el caso del ácido fosfatídico y los jasmonatos (Meijer y Munnik, 2003) (Figura 1).

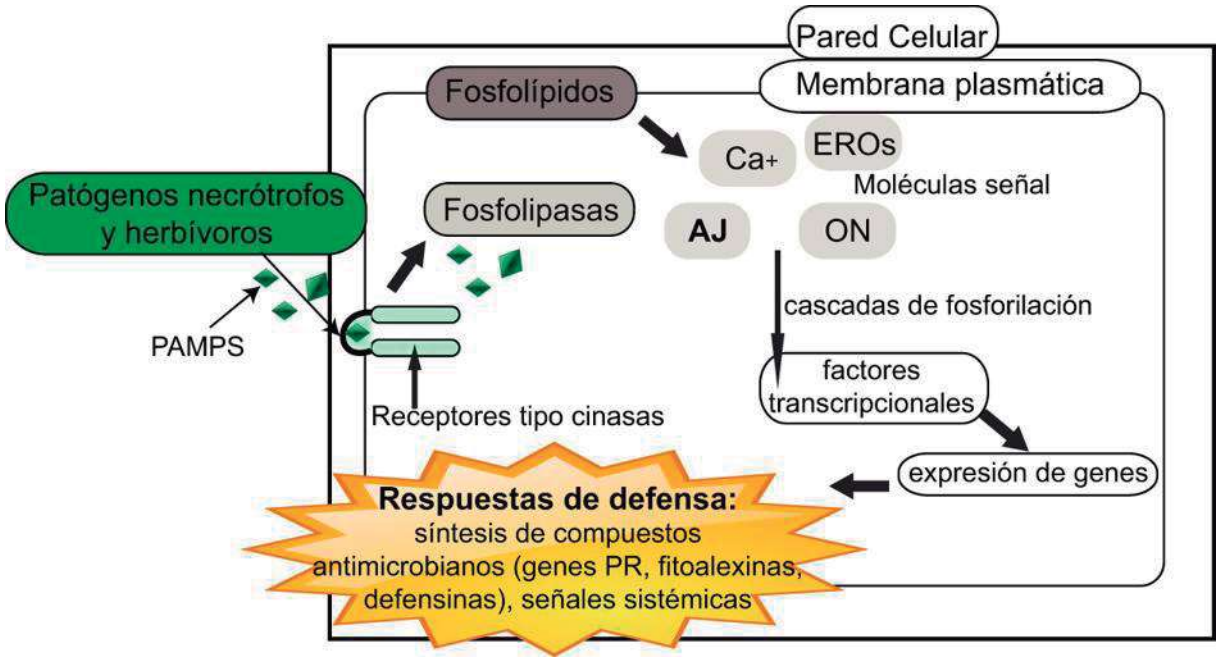

Figura 1. Respuestas de defensa inducidas dependientes del ácido jasmónico. Algunos compuestos y derivados del patógeno que son percibidos como patrones moleculares asociados a patógenos (PAMPS) inician cascadas de señalización dependientes o no de receptores membranales. Ácido jasmónico (AJ), calcio intracelular (Ca^{+}), especies reactivas de oxígeno (EROs), óxido nítrico (ON).

3.2 El ácido jasmónico

Los jasmonatos son fitohormonas lipídicas sintetizadas a partir de los ácidos grasos linoleico y linolénico que regulan una gran cantidad de procesos fisiológicos asociados a respuestas de defensa y desarrollo. Entre las respuestas de estrés que regulan están las heridas, la exposición a ozono, la sequía y el ataque por hongos necrotróficos patógenos. Entre los procesos de desarrollo en los que participan están el crecimiento de la raíz, la maduración de frutos, programas de senescencia y el desarrollo del polen (Penninckx et al, 1996; Creelman y Mullet, 1997; Gális et al, 2009; Berger, 2002).

La ruta biosintética de los jasmonatos (ruta de los octadecanoides; Figura 2), comienza con la liberación de fosfolípidos de membrana de los cloroplastos por la actividad de enzimas fosfolipasas. Así, los ácidos grasos linoleico y linolénico son liberados por al menos tres desaturasas de ácidos grasos. Posteriormente, los ácidos grasos son oxigenados por la lipoxigenasa LOX2, formándose el ácido epoxilinolénico, que a su vez es transformado en ácido 12-oxo fitodienoico por las aleno-óxido sintasa y aleno-óxido ciclasa (AOS y AOC). El ácido 12-oxo fitodienoico es reclutado durante tres ciclos de β -oxidación produciendo finalmente el ácido jasmónico, el jasmonato más abundante. El ácido jasmónico puede adoptar otras formas químicas con actividad biológica importante, como es el caso del metil-jasmonato (MeJA) y su forma conjugada con el aminoácido isoleucina, este paso de transformación es catalizado por la conjugasa JAR1 (Shan et al, 2007).

El ácido jasmónico es un inductor indispensable de las respuestas de defensa necesarias para contrarrestar el ataque por patógenos (Mueller, 1997; Berger, 2002; Turner et al, 2002). Esto se ha demostrado a través del aislamiento de mutantes de *Arabidopsis thaliana* afectadas en algún paso de la ruta biosintética de los jasmonatos, como es el caso de la mutante *jar1* (*methyl-jasmonate resistant1*; Staswick et al, 1992), que presenta un fenotipo de hipersensibilidad a los patógenos necrotróficos *Botrytis cinerea* y *Alternaria brassicicola*. Además, se han identificado mutantes afectadas en la percepción y respuesta (señalización) al ácido jasmónico, como es *coi1* (*coronatine insensitive1*). Esta mutante muestra una pérdida completa de sensibilidad a la hormona en ensayos de inhibición del crecimiento de la raíz y de inducción de raíces laterales mediados por el ácido jasmónico (Feys et al, 1994). El gen COI1

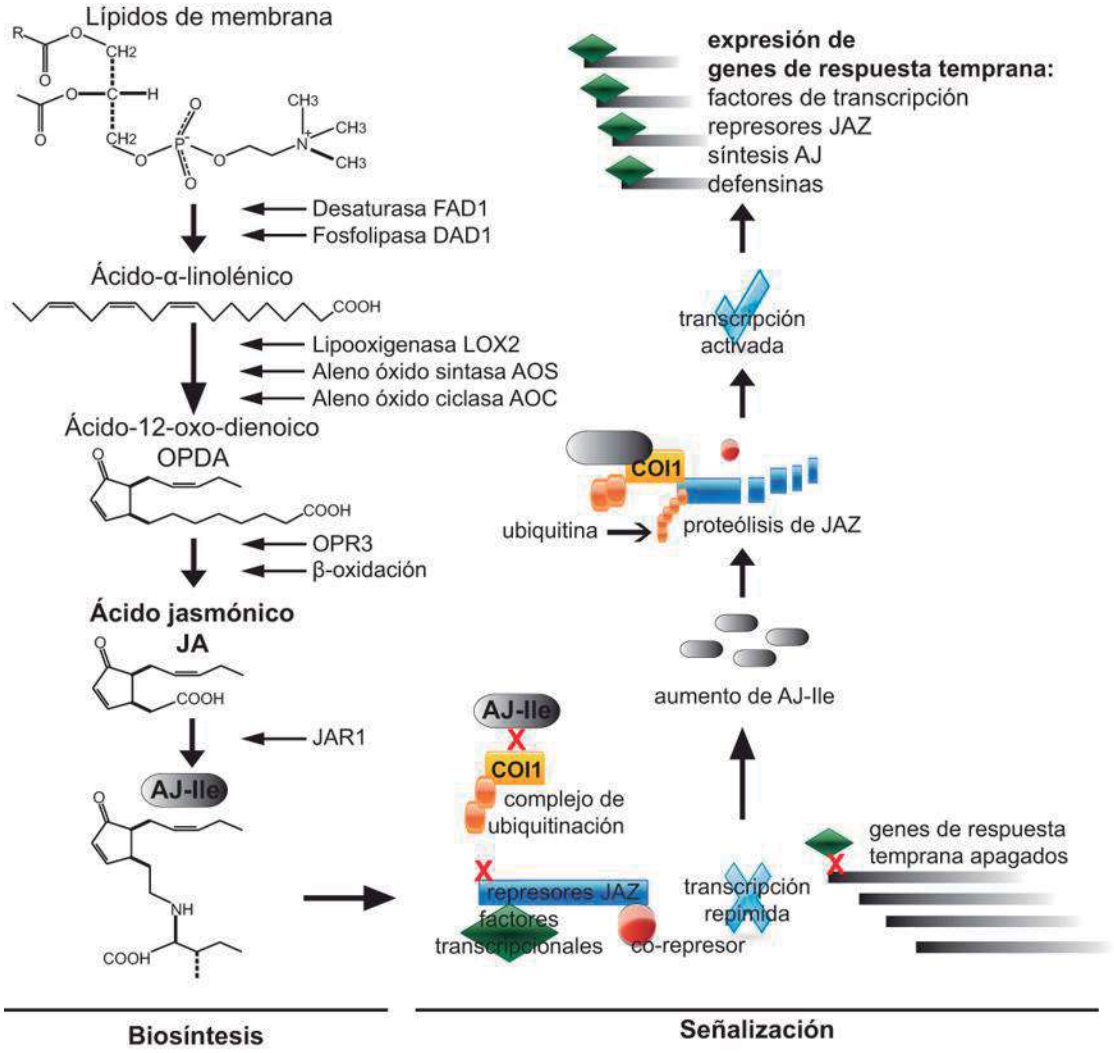

Figura 2. Ruta biosintética y de señalización del ácido jasmónico. En la parte izquierda se ilustra la estructura química los intermediarios de la vía de síntesis, de manera simplificada se indican las enzimas que catalizan cada reacción. Del lado derecho se esquematiza la degradación de los represores transcripcionales JAZ (*jasmonic acid ZIM domain*) tras la unión del conjugado ácido jasmónico-isoleucina (AJ-Ile) con el receptor COI1 (modificado de Berger, 2002).

codifica una proteína ubiqitin-ligasa E3, de tipo SCF y funciona como receptor intracelular del ácido jasmónico (Xie et al, 1998; Yan et al, 2009). La unión de la forma activa de jasmónico (AJ-isoleusina) al complejo SCF-COI1 induce la degradación de los represores transcripcionales JAZ, permitiendo así la expresión de genes de respuesta temprana y tardía al ácido jasmónico, entre los que se encuentran factores transcripcionales, enzimas de síntesis de jasmónico, proteínas de almacenamiento y los propios genes codificantes para proteínas JAZ (Figura 2). Otra mutante afectada en la vía de señalización del ácido jasmónico, y que además

presenta un fenotipo de susceptibilidad exacerbada a *B. cinerea* es la mutante *mpk6*- (Asai et al, 2006). El gen MPK6 codifica una proteína MAP cinasa cuya actividad de fosforilación es necesaria para activar factores transcripcionales inductores de la expresión sistémica de genes de respuesta al ácido jasmónico (Bethke et al, 2008; Takahashi et al, 2007).

De tal manera que las mutantes afectadas en la síntesis y respuestas al ácido jasmónico constituyen una herramienta importante para estudiar mecanismos de defensa asociados a las rutas reguladas por los jasmonatos.

3.3 Las alcamidas, un grupo nuevo de lípidos con funciones reguladoras en las plantas

Otra clase de moléculas señal conservadas en grupos biológicos distantes entre sí, son las *N*-acil amidas (alcamidas). Las alcamidas pertenecen a un grupo de lípidos bioactivos de amplia distribución en las plantas, se han detectado en concentraciones altas (milimolares) en tejidos de plantas medicinales y han sido identificadas en diferentes extractos vegetales con efectos terapéuticos, alelopáticos y antimicrobianos (López-Bucio et al, 2006). Sin embargo, hasta hace poco, se desconocía si pudieran estar implicadas en la regulación de procesos morfogénicos de las plantas. Sus efectos en los programas del desarrollo vegetal se han estudiado mediante la aplicación exógena de alcamidas purificadas o sintetizadas a cultivos *in vitro* de *Arabidopsis thaliana*. Entre los procesos regulados por las alcamidas están la alteración de la estructura meristemática en la raíz primaria, la estimulación de la división y diferenciación celular, evidenciadas por la formación de órganos de novo en tejidos foliares y radiculares (Figura 3), y por la presencia de tricoblastos, que son células diferenciadas en la epidermis de la raíz, conocidas como pelos radiculares (López-Bucio et al, 2007). Además inducen la proliferación de raíces adventicias en explantes de tallos de Arabidopsis (Campos-Cuevas et al, 2008).

A partir de análisis de actividad biológica de diferentes alcamidas sobre procesos de desarrollo, se determinó que la *N*-isobutil decanamida, que posee una cadena lipídica de 10 carbonos sin instauraciones y un grupo isobutilo (Figura 3), es la molécula con mayor actividad biológica de las alcamidas conocidas (López-Bucio et al, 2007), lo que la convierte

en una importante herramienta farmacológica para conocer cuáles son las rutas de señalización reguladas por la alcamidas.

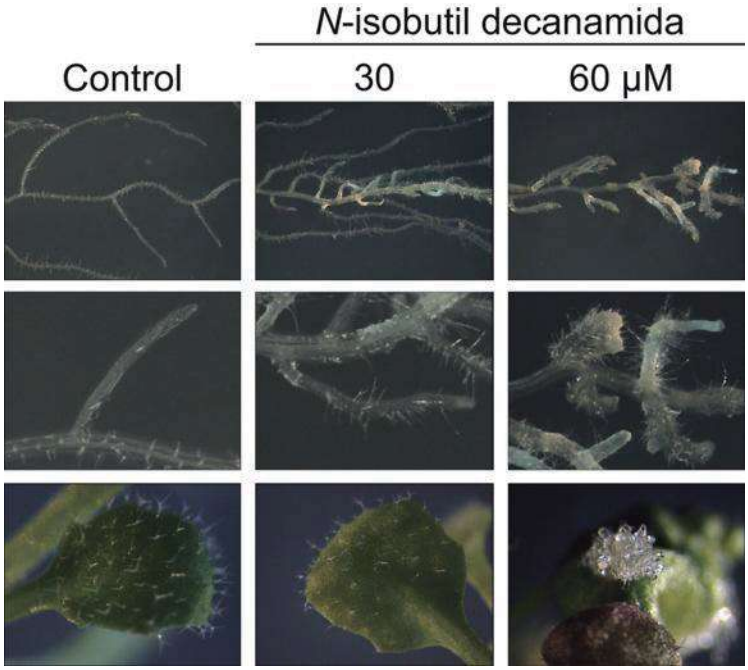

Figura 3. Efectos de la *N*-isobutil decanamida sobre el desarrollo de *Arabidopsis thaliana*. Se muestran plántulas de 14 días de edad crecidas *in vitro* en cajas de Petri con medio MS 0.2X en condiciones control (columna izquierda), o adicionado con la alcamida en la concentración indicada (columna central y derecha). Las imágenes muestran (de arriba hacia abajo) la región apical de la raíz primaria, un acercamiento de raíces laterales jóvenes y de láminas foliares con tricomas. Nótese la cantidad de raíces laterales en la misma zona analizada con la aplicación de la *N*-isobutil decanamida, la formación de pelos radiculares en estas raíces y la formación de callos sobre los peciolos. (Datos no publicados).

Por otra parte, se sabe que las bacterias Gram negativas producen compuestos con una estructura química similar a la de las alcamidas vegetales (Figura 4), las *N*-acil homoserina lactonas (AHLs), que regulan en las poblaciones bacterianas procesos de señalización, participan en el control de la proliferación celular y en procesos de simbiosis y patogénesis con eucariontes. La interacción de microorganismos productores de AHLs con las plantas induce la activación de respuestas de defensa, mediadas por el ácido salicílico y el ácido jasmónico, las cuales confieren resistencia a plantas contra el ataque por patógenos necrotróficos (Schuhegger et al, 2006).

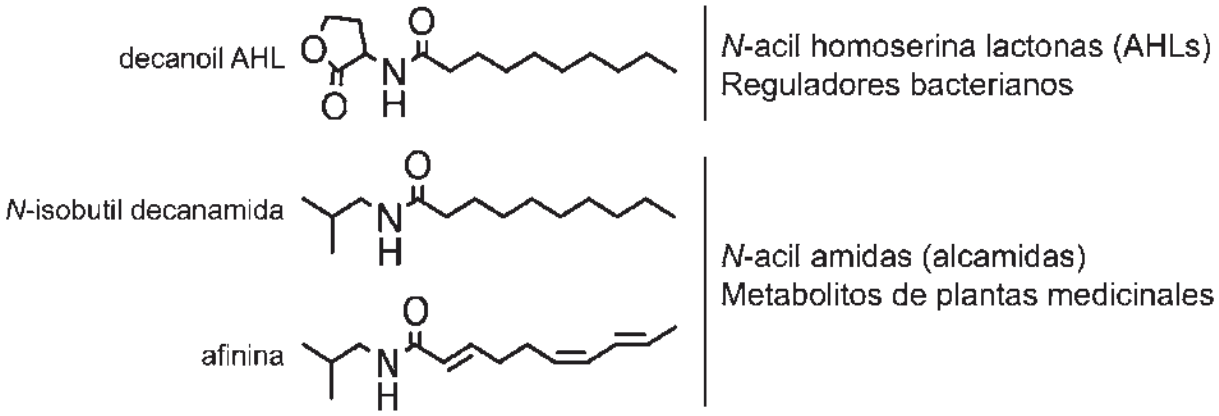

Figura 4. Similitud estructural de las *N*-acil homoserina lactonas (AHLs) bacterianas con algunas alcamidas de plantas (modificado de Ortíz-Castro et al, 2009).

Resulta muy interesante que las AHLs poseen también la capacidad de regular el desarrollo de *Arabidopsis thaliana* de manera similar a las alcamidas vegetales (Ortiz-Castro et al, 2008). Sin embargo, hasta antes de comenzar el presente trabajo de investigación, se desconocía si, además de regular el desarrollo de las plantas, las alcamidas vegetales pudiesen regular algún otro tipo de procesos, tales como la inducción de programas de defensa y la síntesis de reguladores endógenos que participan en la señalización tanto en procesos de desarrollo como de respuestas a patógenos.

3.4 El óxido nítrico en las plantas

En la naturaleza existen moléculas ubicuas de estructura sencilla con la capacidad de regular e integrar diversas rutas de señalización. Tal es el caso del óxido nítrico, una pequeña molécula gaseosa, de carga neutra, que posee una gran afinidad hacia metales de transición (Fe, Cu, Zn) y reacciona rápidamente con el radical superóxido (O_2-) y con el oxígeno molecular (O_2). El óxido nítrico es soluble tanto en ambientes hidrofóbicos como hidrofílicos. Sus características químicas y físicas le confieren la propiedad de atravesar fácilmente las membranas biológicas y en consecuencia de difundirse a través de las membranas con relativa facilidad. Su vida media es corta pero su gran reactividad química le confiere las características de un efector biológico potente (Besson-Bard et al, 2008).

El óxido nítrico regula la actividad y expresión de proteínas y enzimas que actúan en eventos de señalización celular involucrados en las respuestas fisiológicas de las plantas a diversos estímulos endógenos y exógenos (Leshem et al, 1998). Funciona en las células vegetales integrando y mediando diversas señales hormonales, sus inductores incluyen a las auxinas (Pagnussat et al, 2002) y a las citocininas (Tun et al, 2008), pero además existen otros inductores que son sintetizados en situaciones de estrés biótico y abiótico, como el ácido abscícico, los jasmonatos, el ácido salicílico y las especies reactivas del oxígeno (Besson-Bard et al, 2008). Por estudios de expresión global de genes en Arabidopsis se ha determinado que el óxido nítrico regula la expresión de numerosos genes que codifican proteínas relacionadas para las respuestas de defensa, metabolismo, detoxificación celular, transporte, homeostasis de hierro, señalización y floración (Figura 5) (Parani et al, 2004; Polverari et al, 2003).

En las plantas existen dos vías enzimáticas principales en la biosíntesis de óxido nítrico, la primera que, a través de alguna sintasa de óxido nítrico (NOS) utiliza al aminoácido $_L$-arginina (Arg) y al O_2 para generar citrulina y óxido nítrico. La segunda vía es dependiente de las enzimas con actividad de nitrato reductasa (NR) (Lamattina et al, 2003). La vía dependiente de NOS ha sido reportada en diferentes especies de plantas, chícharo, arroz y maíz, sin embargo, no se ha logrado identificar al gen codificante para tal enzima. Hace algunos años, Guo et al., (2003) reportaron el aislamiento de una enzima de Arabidopsis con actividad NOS denominada inicialmente AtNOS. La mutante en el gen que codifica para esta enzima, *Atnos1* se caracteriza por tener niveles reducidos de óxido nítrico en diferentes etapas del desarrollo y, en consecuencia, presenta diversas anomalías en su capacidad reproductiva, en sus mecanismos de señalización dependientes de algunas hormonas y en la tolerancia a factores de estrés biótico y abiótico como son las respuestas a patógenos y a estrés salino (Guo et al, 2003; Zeidler et al, 2004; Zhao et al, 2007). A pesar de tal evidencia experimental, estudios más precisos acerca de la estructura y función molecular de esta enzima han demostrado que es incapaz de unirse y oxidar a la Arg. El gen AtNOS1 en realidad codifica para una hidrolasa de GTP que regula procesos de biogénesis ribosomal en mitocondrias y cloroplastos, y los niveles bajos de óxido nítrico de la mutante *Atnos1* parecen ser consecuencia indirecta de anomalías metabólicas, y no porque esté afectada sintetizar óxido nítrico a partir de Arg (Zemojtel et al, 2006; Moreau et al, 2008). Por lo antes mencionado, su nombre AtNOS1 fue sustituido por el

de proteína “asociada a óxido nítrico” (AtNOA1, por sus siglas en inglés) (Crawford et al, 2006), y en la actualidad la mutante *Atnoa1* es utilizada como modelo de señalización alterada en respuesta al óxido nítrico (Gas et al, 2009).

La ruta dependiente de NR ha se ha descrito en algunas plantas leguminosas y en Arabidopsis. Las enzimas NR normalmente reducen el nitrato (NO_3-) a nitrito (NO_2-), pero también poseen una actividad intrínseca a través de la cual, en condiciones en que el NO_2- se acumula a concentraciones micromolares, son capaces de reducir el NO_2- a óxido nítrico. *Arabidopsis thaliana* contiene en su genoma dos genes que codifican para nitrato reductasas, los productos de estos genes, las proteínas NIA1 y NIA2 tienen una identidad en su secuencia de 83.5 % y varían únicamente en su región amino-terminal, por lo que se considera que tienen funciones redundantes. La importancia de la actividad de nitrato reductasas en la generación de óxido nítrico ha sido descrita en Arabidopsis con una doble mutante *nia1*, *nia2* que presenta deficiencias en la síntesis de óxido nítrico durante el cierre estomático en tejido fotosintético por acumulación de ácido abscísico y en condiciones de estrés por deficiencia de nitrógeno (Desikan et al, 2002).

A pesar de que existen rutas adicionales en la generación de óxido nítrico que involucran a enzimas como las peroxidasas, xantina oxidoreductasas y miembros de la familia del citocromo P450, estas no han sido bien caracterizadas (Lamattina et al, 2003), por lo que las mutantes *Atnoa1* y la doble mutante *nia1*, *nia2* son la herramienta genética más utilizada en el estudio de procesos fisiológicos en los que participa el óxido nítrico.

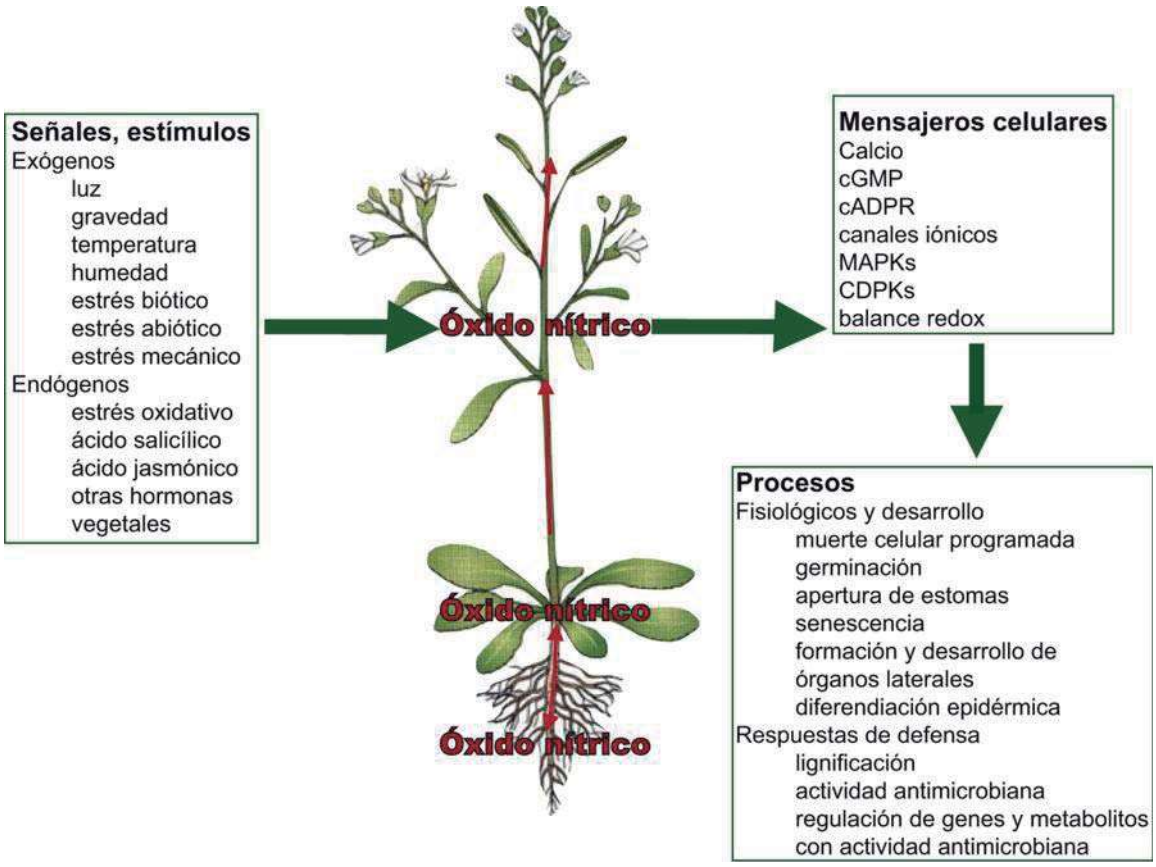

Figura 5. Procesos de crecimiento, desarrollo y adaptación de las plantas regulados por el óxido nítrico. Monofosfato de guanosina cíclico (cGMP), difosfato de adenosina ribosilado cíclico (cADPR), proteinas cinasas dependientes de mitógenos (MAPKs), proteínas cinasas dependientes de calcio (CDPKs).

IV. JUSTIFICACIÓN

Las alcamidas inducen alteraciones en diferentes procesos del desarrollo de las plantas y poseen similitud estructural con otras moléculas lipídicas que regulan tanto procesos del desarrollo como de defensa, las *N*-acil homoserina lactonas. Por tanto, resulta importante la caracterización molecular de sus vías de acción a través de un estudio global de expresión génica, utilizando a la *N*-isobutil-decanamida como molécula inductora para caracterizar el transcriptoma de *A. thaliana*. Estos análisis permitirán determinar si las alcamidas regulan respuestas de defensa, y las posibles rutas con las éstas interactúan.

V. HIPÓTESIS

La alcamida *N*-isobutil decanamida induce la expresión de genes y la síntesis de metabolitos que participan en la regulación de procesos del desarrollo y de defensa de *Arabidopsis thaliana*.

VI. OBJETIVOS

OBJETIVO GENERAL

Estudiar los cambios en la expresión génica global de *Arabidopsis thaliana* en respuesta a la *N*-isobutil decanamida.

OBJETIVOS ESPECÍFICOS

1. Caracterizar el transcriptoma de *Arabidopsis thaliana* que es regulado por la *N*-isobutil-decanamida.

2. Analizar los patrones de expresión de genes regulados por la *N*-isobutil decanamida.

3. Determinar si la *N*-isobutil decanamida induce respuestas de defensa en *Arabidopsis thaliana*, y conocer sus posibles blancos de señalización mediante el uso de mutantes hipersensibles al ataque por patógenos.

4. Evaluar posibles interacciones de las respuestas inducidas por la *N*-isobutil decanamida con la vía de señalización del óxido nítrico.

VII. RESULTADOS

Los principales resultados generados durante la realización del presente proyecto se presentan en los Capítulos I y II. El primer capítulo corresponde a un manuscrito en el que se reporta el efecto de la *N*-isobutil decanamida en la expresión global de genes de Arabidopsis y la inducción de respuestas de defensa, a través de la ruta del ácido jasmónico. Este artículo fue sometido a revisión en la revista "*Molecular Plant-Microbe Interactions*", y aunque no fue aceptado en la versión revisada, los comentarios sugeridos por el comité revisor han sido incorporados en el capítulo que se presenta en este trabajo, y esperamos obtener su aprobación definitiva en los meses próximos.

En el Capítulo II se describe la participación del óxido nítrico en procesos del desarrollo regulados por la *N*-isobutil decanamida en Arabidopsis, particularmente la inducción de raíces laterales. Este trabajo está publicado "en prensa" en la revista "*Plant and Cell Physiology*", la cual tiene un factor de impacto de 4.0 en el índice del *Information Sciences Institute*.

En los cuatro anexos finales se encuentran algunos resultados que fueron incluídos en 3 artículos publicados por nuestro grupo de trabajo y en un capítulo publicado en el libro "*Plant Developmental Biology - Biotechnological Perspectives*".

CAPÍTULO I

Molecular Plant-Microbe Interactions

Alkamide-modulated gene expression activates defence responses in Arabidopsis and confers resistance to necrotizing fungal pathogens through JA biosynthesis and signalling

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1

Full title: Alkamide-modulated gene expression activates defence responses in Arabidopsis and confers resistance to necrotizing fungal pathogens through JA biosynthesis and signalling.

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ABSTRACT

Alkamides belong to a class of bioactive lipids of wide distribution in plants, which are structurally related to *N*-acyl-L-homoserine lactones, a class of quorum-sensing signals from Gram negative bacteria. Global analysis of gene expression changes in *Arabidopsis thaliana* in response to *N*-isobutyl decanamide, the most highly active alkamide identified to date, revealed at least 181 up-regulated genes related to defence and stress responses. In particular, genes encoding enzymes for jasmonic acid (JA) biosynthesis increased their expression, which occurred in parallel with JA accumulation. The activity of the alkamide to confer resistance against necrotizing fungi *Botrytis cinerea* and *Alternaria brassicicola* was tested by inoculating *Arabidopsis* leaves with fungal spores and evaluating disease symptoms and fungal proliferation. *N*-isobutyl decanamide application significantly reduced necrosis caused by the pathogens and inhibited fungal proliferation in WT leaves, which was independent of the effects of *N*-isobutyl decanamide at directly inhibiting fungal growth. *Arabidopsis* mutants *jar1* and *coi1* altered in JA signaling and a MAP kinase mutant (*mpk6*) were unable to defend from fungal attack even when *N*-isobutyl decanamide was supplied, indicating that alkamides modulate necrotrophic-associated defence responses through JA-dependent and MPK6 modulated signaling pathways. Our results reveal a novel function of alkamides inducing plant immunity through directly affecting JA biosynthesis and signaling.

INTRODUCTION

Plants continuously respond to abiotic and biotic stress by adjusting their metabolism and activating diverse intracellular signaling responses. In particular, plant architecture modifications in response to physical or chemical stimuli or the activation of pathogen-specific defence mechanisms upon microbial infection permits plant survival and reproduction. The plant immune system is activated by perception of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by host encoded pattern recognition receptors. Successful pathogens secrete effectors that suppress PAMP triggered immunity inducing disease, as a counter defence strategy, plants may recognise a given effector, either directly or indirectly resulting in disease resistance (Jones and Dangl 2006; Bari and Jones 2009). The activation of immunity triggered by effectors, coupled to sustained and systemic production of antimicrobial and signaling molecules restricts pathogen growth and allows plant survival. Classic signal molecules such as jasmonic acid (JA), ethylene, salicylic acid (SA), abscisic acid (ABA), and more recently auxins, have been found to participate in defence responses against pathogens (Eulgem 2005; Kepinsky 2006). Interestingly, signaling networks triggered by these plant hormones show crosstalk and are often complex involving multiple interactions.

A novel group of fatty acid amides from different kingdoms, including the plant-produced alkamides and *N*-acylethanolamines (NAEs), and *N*-acyl-L-homoserine lactones from bacteria have been recently shown to alter plant metabolism and development. Alkamides comprise over 200 related compounds, which have been found in as many as 10 plant families: Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, and Solanaceae (for review López-Bucio et al. 2006; Morquecho-Contreras and López-Bucio 2007). Plant-produced alkamides have been found to alter root and shoot system architecture in *Arabidopsis* by affecting cell division and differentiation processes (Ramírez-Chávez et al. 2004; López-Bucio et al. 2007; Campos-Cuevas et al. 2008). The *Heliopsis longipes*-produced alkamide affinin have been reported to have antimicrobial activity inhibiting *In vitro* growth of some plant microbial pathogens, including bacteria and fungi (Molina-Torres et al. 2004).

An important function of NAEs in regulating physiological processes is supported by their wide distribution in plants, selective accumulation and rapid metabolism in response to

developmental transitions and by the identification of a fatty acid amide hydrolase (FAAH) enzyme involved in NAE degradation (Chapman et al. 1999; Wang et al. 2006). In addition to developmental roles in *Arabidopsis thaliana* (Blancaflor et al. 2003), NAEs have been associated to some defence related processes. For instance, exogenous application of NAE 14:0 induce the expression of phenylalanine ammonia lyase (*PAL2*), a key enzyme in phenylpropanoid metabolism in a similar way to that shown by fungal elicitors in both cell suspensions and in leaves of tobacco (Tripathy et al. 1999). Moreover, over expression of *AtFAAH* compromises innate immunity in *Arabidopsis* (Kang et al. 2008).

Many Gram negative bacteria produce alkamide-related fatty acid signals termed *N*-acyl-L-homoserine lactones (AHLs). These compounds participate in cell-to-cell communication that is usually referred to as quorum-sensing (QS), and are freely diffused through the bacterial membrane distributing within the rhizosphere (Pearson et al. 1994; Scott et al. 2006; Schuhegger et al. 2006). Plants are able to perceive AHLs, the presence of AHL-producing bacteria in the rhizosphere of tomato induces SA- and JA-dependent defence responses, conferring resistance to the fungal pathogen *Alternaria alternata* (Schuegger et al. 2006). The application of purified AHLs to *Medicago truncatula* and *Arabidopsis thaliana* plants results in differential transcriptional changes in roots and shoots, affecting expression of genes potentially involved in immune response and development (Mathesius et al. 2003; Von Rad et al. 2008). The *Arabidopsis* root system architecture is also regulated by AHLs. *in vitro*-cultivated seedlings responded to AHLs by affecting their primary root growth, lateral root formation and root hair development (Ortíz-Castro et al. 2008).

Crosstalk between alkamide and AHL signalling at the level of root development could be recently demonstrated. An *Arabidopsis* mutant line termed *decanamide resistant root* (*drr1*), which is resistant to primary root growth inhibition by *N*-isobutyl decanamide, also showed reduced sensitivity to C10-AHL application, suggesting that there may exist a conserved genetic mechanism in plants to perceive bacterial and plant fatty acid amides. When grown in soil, *drr1* mutants showed a dramatically increased longevity, which correlated with altered and developmental responses to jasmonic acid (JA) (Morquecho-Contreras et al. In press). The *drr1* phenotype provided the first genetic evidence indicating that alkamide and AHL responses in plants may involve JA signalling.

JA and its biologically active derivatives (jasmonates; JAs) are fatty acid-derived signals that

act as regulatory molecules in metabolic and developmental processes, as well as in defence responses (Berger 2002; Devoto et al. 2005). JAs rapidly accumulate by wounding, insect attack, and necrotrophic pathogen infection (Turner et al. 2002). JA biosynthesis starts in chloroplasts, where α-linolenic acid is released from membrane phospholipids by the action of phospholipases. α-linolenic acid is first converted to 13-hydroperoxy linolenic acid (13-HPOT) and then to 12-OPDA in a series of reactions catalyzed by 13-lipoxygenase (*LOX2*), allene oxide synthase (*AOS*), and allene oxide cyclase (*AOC*), respectively. 12-OPDA is subsequently reduced by 12-OXOPHYTODIENOATE REDUCTASE3 (*OPR3*) to 3-oxo-2-(2´-pentenyl)-cyclopentane-1-octanoic acid, which then undergoes three cycles of ß-oxidation in the peroxisomes to produce JA. In the cytosol, JA is further modified to produce various JAs. For instance, it is converted to volatile oxylipin MeJA by a JA methyl transferase or conjugated into several amino acids by the JAR1 amino acid synthetase, to give rise, among others, to (+)-7-iso-JA (reviewed by Wasternack 2007). Following synthesis, JAs may be perceived by an intracellular receptor protein encoded by *COI1* (*coronatine-insensitive 1*) that is an F-box component of an SCF (SKIP–CULLIN–F-box) complex (Yan et al. 2009). These complexes are multiprotein E3 ubiquitin ligases catalysing the ubiquitination of proteins destined to proteasomal degradation, which activates a signal transduction pathway that culminates in the transcriptional activation or repression of JA-responsive genes. The *coi1* mutant displays defects in many JA-dependent functions, such as fertility, secondary metabolite biosynthesis, pest and pathogen resistance, and wound responses, and shows resistance to JAs and to the *Pseudomonas syringae* toxin coronatine (reviewed by Kazan and Manners 2008). The essential role of JAs in plant immunity is also evidenced by JA-related mutants, such as *jar1*, which shows an enhanced susceptibility to necrotrophic pathogens (Staswick et al. 1992; Takahashi et al. 2007). In addition, a JA-activated mitogen-activated protein kinase (MAPK) cascade, in which *MPK6* is involved, has been found to modulate JA-dependent gene expression (Takahashi et al. 2007; Hu et al. 2009).

Chemical communication in the rhizosphere between plants and soil microorganisms is often mediated by root-secreted compounds. Although plant fatty acid amides have been studied in the context of plant developmental processes, little is know about their potential role in plant-microbe interactions. In this work, we performed global transcription profiling to more deeply investigate the *Arabidopsis* molecular responses to alkamides in *Arabidopsis* by testing the

effects of *N*-isobutyl decanamide on gene expression. Our results show that many defence-related genes are induced by *N*-isobutyl decanamide. We further found that in alkamide-treated *A. thaliana* seedlings the level of JA increases, which correlates with enhanced and sustained expression of JA-responsive and senescence-associated genes. *N*-isobutyl decanamide application to mature *Arabidopsis* leaves conferred resistance against fungal necrotizing pathogens *Botrytis cinerea* and *Alternaria brassicicola* in a process involving JA and MPK6 signaling.

RESULTS

Transcript profile of *Arabidopsis* plants in response to *N*-isobutyl decanamide.

To determine the transcript profile of *Arabidopsis* seedlings in response to *N*-isobutyl decanamide, *Arabidopsis* WT (Col-0) seedlings were germinated and grown for 6 d on MS 0.2x medium and then transferred to the same medium supplied with or without 60 μM of *N*-isobutyl decanamide and gene expression analyzed at further 1, 3, 7 and 14 d periods. Total RNA extractions were prepared from shoots and roots and mixed in a 1:1 relation to avoid possible effects of shoot or root weight on overall gene expression. A long oligonucleotide microarray platform containing 31,000 spots, which represent 25,240 *Arabidopsis* genes and contains 850 negative controls, 92 commercial controls and 96 blanks was employed (www.ag.arizona.edu/microarray). In our analyses, we chose a 2-fold change in expression level compared with the control as a minimum threshold to consider a gene as responsive to *N*-isobutyl decanamide. We also tested temporal differences in gene expression between *N*-isobutyl decanamide treatment and controls in a time course manner (Fig. 1A).

According to a stringency level of FDR 0.05 (fold ± 2), a total of 1,281 genes showed differential expression in at least one of the four sampled time points (Table S1). 727 genes were found to be up-regulated and 554 down-regulated in response to *N*-isobutyl decanamide treatment in at least one of the time points tested. The number of differentially expressed genes increased from day 1 to day 7 after treatment (dat) and then decreased at day 14 (Fig. 1B).

A hierarchical clustering analysis allowed organization of *N*-isobutyl decanamide-regulated genes into five clusters or sets depending of gene expression patterns: permanently repressed genes (set1); genes with expression values oscillating from highly repressed to basal or

induced levels at 7 and 14 dat (set2); induced genes at 1 and 3 dat (set4); permanently induced genes with expression levels further increased at 7 and 14 dat (set3), and genes specifically induced at 7 dat (set5) (Fig. 1C). Functional classification of differentially expressed genes was done using both the Gene Onthology database from the TAIR 8.0 web page (http://www.arabidopsis.org) and with the MapMan program (Thimm et al. 2004). Among the annotated genes, putative regulatory genes accounted for about 26% of the *N*-isobutyl-decanamide up-regulated and 25% of down-regulated genes (Fig. 2A, B). These genes were classified as transcriptional regulators, genes involved in protein modification and degradation, cell cycle, hormone responses, and development. In addition, genes involved in cell wall synthesis and modification accounted for 3 and 7% of up- and down-regulated genes, respectively, and genes related to lipid metabolism accounted for about 3% of the transcripts. Interestingly, stress- and defence-related genes accounted for about 25% of the total number of genes up-regulated by *N*-isobutyl decanamide, of which at least 62 transcripts correspond to JA-responsive genes (Fig. 2A, Table S1).

Quantitative-real time reverse transcription-polymerase chain reaction analysis of representative genes.

In addition to the statistical methods described (see Materials and Methods), validation of microarray data was achieved by expression analyses of 15 randomly chosen genes, which included at least one gene from each cluster set presented in Fig. 2 (for a list, see Table S3), by quantitative real-time RT-PCR (Q-RT-PCR). The validated genes included up- and down-regulated genes identified in the microarray analysis. These Q-RT-PCR experiments were carried out using RNA extracted from an independent batch of control and treated plants to that used for the microarray analysis. The expression profile obtained for the analyzed genes using Q-RT-PCR were quite consistent with those determined from the hybridization signals generated by the microarray analysis (Fig. 3A, B). However, some important quantitative differences were observed since the level of induction or repression was significantly higher when determined by Q-RT-PCR than from the array data; for instance, some genes that showed less than 10-fold change in the array experiment showed a 100-fold change by Q-RT-PCR. Nevertheless, the increased and decreased expression patterns of the microarray analysis were well supported by Q-RT-PCR analyses.

194

195 ***N*-isobutyl decanamide activates transcription of defence-related genes.**

196 In order to clarify the temporal effect of *N*-isobutyl decanamide on gene expression by
197 functional categories, we analyzed the microarray-expression data profiles of the 50 most
198 induced genes at the different time points (Table S2). We found that the most highly
199 represented category was related to disease resistance and stress responses. Among these
200 genes with high levels of differential expression we found several genes potentially involved
201 in cell wall modifications codifying for a pectinesterase (At4g02330) and a xyloglucosyl
202 transferase (At4g30290), respectively. Moreover, the stress- and defence-related genes
203 comprised 47 genes (Table S1 and S2), which were induced from 3 to 14 d after treatment
204 including genes related to responses to oxidative burst such as a germin-like gene
205 (At5g39150), two peroxidases (At5g06730 and At4g08780), JA-related genes such as five
206 plant defensin-like genes (At5g44420, At1g75830, At2g26020, At5g44430 and At5g44430),
207 jacalin-lectin (At1g52040) and a senescence-associated gene (At2g29350), mostly induced 3
208 and 7 d after treatment, and lipoxygenase 2 (*LOX2*) [At3g45140].

209

210 ***N*-isobutyl decanamide induces accumulation of JA and activates the expression of JA-**
211 **related genes.**

212 Since the greatest group of differentially expressed genes in response to *N*-isobutyl
213 decanamide treatment is related to defence mechanisms and many of these genes are induced
214 in response to JA, it was relevant to determine whether alkamides could increase the
215 accumulation of JA. With this aim, the levels of JA were quantified in seedlings treated or
216 untreated with 60 μM *N*-isobutyl-decanamide. JA was determined by gas chromatography
217 coupled to a mass spectrometer detector (see materials and methods). A nearly two-fold
218 increase in JA level was observed in response to alkamide treatment (Fig. 4A), indicating that
219 some effects of this alkamide on gene expression might be mediated, at least in part, by JA
220 accumulating in the plant.

221 In agreement with an increased level of JA in *N*-isobutyl decanamide treated seedlings, the
222 array data shows that genes that have been previously shown to be JA-responsive are
223 differentially regulated by this alkamide. For instance, genes encoding *LOX2* (At3g45140),
224 *SAG13* (At2g29350), MutT/nudix (At5g19470), lipid transfer 3 protein (At5g59320) and

PDF1.2a (At5g44420) were highly induced 7 d after transfer of seedlings to *N*-isobutyl decanamide medium (Fig. 4B). To confirm that *LOX2* expression is activated by *N*-isobutyl decanamide at the transcriptional level, transgenic *A. thaliana* seedlings containing a chimeric gene in which the *LOX2* promoter is fused to the *GUS* reporter gene (*LOX2:GUS*) were treated with 60 μM *N*-isobutyl decanamide, and GUS histochemical determinations performed in 10 d-old seedlings. As previously reported (Schommer et al. 2008), control seedlings did not show *LOX2:GUS* expression in roots or shoots (Fig. 4C). Interestingly, increased expression of this marker was observed throughout the shoot system in alkamide-treated seedlings (Fig. 4C).

_N_-isobutyl-decanamide confers resistance to fungal necrotizing pathogens *Botrytis cinerea* and *Alternaria brassicicola*.

The results from the microarray experiments, JA accumulation and JA-responsive gene expression analyses suggest that *N*-isobutyl decanamide may function as a potential defence-inducing factor. To study whether *N*-isobutyl decanamide could effectively activate defence mechanisms that lead to pathogen resistance, we tested the responses of leaves from 20 d-old *Arabidopsis* plants to necrotrophic pathogens *Alternaria brassicicola* and *Botrytis cinerea,* which provoke spreading necrotic lesions on leaves. In these experiments, fully developed leaves from 20 d-old plants were transferred to medium supplied with 60 μM *N*-isobutyl decanamide or with the solvent as control. *A. brassicicola* and *B. cinerea* spores were inoculated on the leaf surface and disease symptoms evaluated 3, 4 and 5 days after inoculation (dai). In leaves transferred to control medium and inoculated for 3d, both pathogenic fungi were found to induce necrotic lesions in over 90% of inoculated leaves (Fig. 5A, B). In contrast, in leaves treated with *N*-isobutyl decanamide for 3d, only 30% of leaves presented necrotic lesions caused by *A. brassicicola* and 10% by *B. cinerea* infection (Fig. 5A, B). At 4 dai it was found that 100% of the control leaves showed necrotic lesions for both pathogens, whereas in *N*-isobutyl decanamide-treated leaves, 60% and 85% of *A. brassicicola* infected leaves and, only 15% and 60% of *B. cinerea* infected leaves showed necrotic lesions at 4 and 5 dai, respectively. We also noted that 3 dai lesions in control leaves were of about 6 mm in diameter, whereas in *N*-isobutyl decanamide-treated leaves lesions had a diameter between 0.8 and 1.5 mm (Fig. 5C, D). Visual inspection showed that control leaves inoculated

with the pathogens showed generalized necrotic lesions spanning half or more the surface of the leaf at 5 dai, while alkamide-treated leaves presented reduced symptoms (Fig. 5E).

Next, we monitored the growth of the pathogens by direct microscopic observation of stained mycelium in infected leaves. We found that disease symptoms in control leaves at day 3 after inoculation with *A. brassicicola* were accompanied by prolific mycelium growth, which correlated with increased damage of leaf tissue. Interestingly, *N*-isobutyl-decanamide treatment inhibited fungal growth, which was related to decreased degradation of leaf tissue (Fig. 5E). A similar protective effect could be observed in alkamide-treated leaves inoculated with *B. cinerea*, which showed decreased fungal growth and disease symptoms on leaves. On the basis of these findings, it can be concluded that *N*-isobutyl decanamide treatment renders enhanced resistance to necrotizing fungi.

_N_-isobutyl decanamide did not directly affect the growth of fungal plant pathogens *in vitro*.

A previous report indicates that affinin inhibits the growth of some plant microbial pathogens, including bacteria and fungi (Molina-Torres et al. 2004). Therefore, it was possible that the reduced leaf damage and fungal growth inhibition observed in alkamide treated leaves could be the result of a direct toxic or growth inhibition effect of *N*-isobutyl decanamide on the fungal pathogen tested. To test this possibility, the antifungal activity of *N*-isobutyl decanamide on *B. cinerea* was investigated by inoculating mycelia disks on Petri plates containing PDA media supplemented with the solvent or with increasing concentrations of *N*-isobutyl decanamide. Even at a 120 µM *N*-isobutyl decanamide concentration, no significant inhibition in mycelium growth was observed at 3 days after inoculation (Fig. 6A, B). The fungicide Techto 60 was used as a positive control showing a very clear inhibition of growth on *B. cinerea* (Fig. 6C). In a second set of experiments, we tested the effect of immersing filter paper on solvent, Techto 60 or *N*-isobutyl decanamide solutions and placing it symmetrically on PDA plates inoculated with the fungi to visualize mycelium growth in the proximity of each filter containing compound. Fig. 6D shows that Techto 60 markedly inhibited fungal growth, while 30 or 60 µM *N*-isobutyl decanamide concentrations did not affect growth of the pathogen.

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

To test whether JA signaling is involved in the response of *Arabidopsis* leaves to necrotrophic fungi in treatments with *N*-isobutyl decanamide, we evaluated the responses of *Arabidopsis* JA-related mutants (*jar1* and *coi1*), or a mutant defective at the *AtMAPK6* locus, which has been found to be critical in defence responses to *B. cinerea* (Ren et al. 2008). We placed adult leaves from wild-type (Col-0) and mutant plants on 0.7 % agar plates supplied with solvent or *N*-isobutyl decanamide for 24 hours. Then, leaves were transferred to *N*-isobutyl decanamide free agar, and inoculated with *B. cinerea* spores. Susceptibility to fungal infection was estimated by recording a range of disease symptoms (from no symptoms, to severe tissue maceration). As shown in Fig. 7A, At 3 dai, the disease symptoms severity were higher in the *jar1*, *mpk6*- and *coi1* mutants than in Col-0, even when they received *N*-isobutyl decanamide pre-treatment, whereas the Col-0 disease symptoms decreased when alkamide was supplied (Fig. 7A, B). These results indicate that *N*-isobutyl decanamide-induced resistance in *Arabidopsis* wild-type leaves is due to an induction of defence programs through JA-dependent signaling and that apparently all three tested *jar1*, *coi1* and *mpk6* gene products seem to be involved in the build-up of that response.

DISCUSSION

Alkamides belong to a novel class of lipid signals that modulate developmental processes in plants (Ramírez-Chávez et al. 2004; López-Bucio et al. 2007). Recent findings, however, provided evidence of a widespread distribution of structurally related signal lipid amides in evolutionary distant organisms (Dudler and Eberl 2006; López-Bucio et al. 2006). *N*-isobutyl decanamide share structural similarity to *N*-decanoyl homoserine lactone (C-10 HL) (Fig. S1), a member of the *N*-acyl homoserine lactone (AHL) class of bacterial quorum-sensing signals, which have been found to alter root development and activate defence responses in different plant species (Mathesius et al. 2003; Schuegger et al. 2006; Ortíz-Castro et al. 2008). AHLs comprise a class of bacterial signals important for population communication. These compounds contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain (Parsek and Greenberg, 2000). The acyl groups of naturally occurring AHLs range from 4 to 18 carbons in length; they can be saturated or insaturated and with or without a C-3 substituent (Waters and Bassler 2005; Camilli and Bassler 2006). Experimental evidence indicates that bacteria commonly associated to plants are capable to produce a variety of

318 AHLs (Cha et al. 1998; Elasri et al. 2001; Khmel et al. 2002; D´Angelo-Picard et al. 2005).
319 Our recent work has shown that *N*-isobutyl decanamide and C10-HL can be perceived by a
320 common signaling mechanism in *Arabidopsis thaliana*, which requires an intact *DRR1* locus
321 (Morquecho-Contreras et al. 2009). An interesting hypothesis is that small lipid signaling
322 based on plant fatty acid amides and/or AHLs might be part of an ancestral inter-kingdom
323 communication system between plants and their associated bacteria.
324 Gene expression profiling through the use of microarrays has been recognized as a powerful
325 approach to obtain a global view on gene expression and physiological processes involved in
326 response to a particular stimulus. Using a microarray platform, we performed a global analysis
327 of changes in transcript accumulation in response to *N*-isobutyl decanamide. We found a set of
328 genes induced by *N*-isobutyl decanamide, whose expression was also reported to be activated
329 by NAE 12:0 (Teaster et al., 2007). These include ABA-responsive genes (At3g02480,
330 At5g53820) and germin-like genes (At5g38910, At5g39550, At5g39180, At5g39110,
331 At5g39190). Previously, Teaster and coworkers (2007) conducted microarray analyses to
332 elucidate transcriptional targets of NAE 12:0 in 4-d-old Arabidopsis seedlings. Transcriptional
333 profiling revealed elevated transcripts for a number of ABA-responsive genes and genes
334 typically enriched in desiccated seeds. The levels of *ABI3* transcripts were inversely associated
335 with NAE-modulated growth. Overexpression of the *Arabidopsis* NAE degrading enzyme
336 fatty acid amide hydrolase (*AtFAAH*) resulted in seedlings that were hypersensitive to ABA,
337 whereas the ABA insensitive mutants *abi1-1*, *abi2-1*, and *abi3-1*, exhibited reduced sensitivity
338 to NAE. These data suggested that NAE metabolism is important for certain ABA-regulated
339 processes involved in early seedling growth. Our results indicate that although important
340 differences in plant age, concentrations of compounds and time of exposure already exists
341 when comparing our expression analysis results using *N*-isobutyl decanamide with those
342 reported for NAE 12:0, common genes were found to be up-regulated by the two compounds,
343 indicating similarities in the transcriptional responses elicited by NAE 12:0 and an alkamide.
344 However, important differences in the number and functional categories of represented genes
345 were found, for instance, transcriptional regulators and genes potentially involved in growth
346 and developmental processes and in defense responses were consistently up-regulated in
347 response to *N*-isobutyl decanamide treatment, suggesting an important role of this compound
348 in remodelling plant architecture and in adaptation to biotic stress (Table S1).

Our data support our previous findings suggesting that alkamides and JA signaling interact to modulate plant responses (Morquecho-Contreras et al. 2009). Several JA-related genes such as plant defensin-like (At2g43510, At5g44420, At1g75830, At2g26010), jacalin-lectin (At1g52040), and tropinone reductase (At2g29350), were mostly induced at 7 and 14 d after transfer to alkamide treatment (Table S1), which correlates with increased levels of JA in plants (Fig. 4). One of the first indications that plant fatty acid amides indeed participate in plant-pathogen interactions was the observation that alkamide-related NAEs accumulated in the growth media of tobacco suspension cells and leaves within 10 minutes of applying the fungal elicitor, xylanase. Indeed exogenous NAE application triggered the expression of phenylalanine ammonia lyase (PAL), which has been implicated in plant defence against pathogens (Chapman et al. 1998; Tripathy et al. 1999). Moreover, the ectopic overexpression of AtFAAH, renders Arabidopsis seedlings more susceptible to both host and non-host bacterial pathogens. By using the same FAAH mutant and overexpressor lines reported by Wang and associates (2006), we found that manipulation of fatty acid amide hydrolase activity in Arabidopsis also modified the sensitivity to C10-HL in terms of primary root growth and biomass production, suggesting that plants can metabolize both NAEs and bacterial AHLs (Ortíz-Castro et al. 2008).

Although the aforementioned studies with NAEs and AHLs provided correlative evidence linking defence related gene transcription to fatty acid amides (Mathesius et al. 2003; Von Rad et al. 2008, Teaster et al. 2007) direct evidence demonstrating that treatment with these compounds could confer improved resistance of plants to disease causing pathogens was not available. We show that *N*-isobutyl decanamide was effective against two different fungal necrotizing pathogens *Botritys cinerea* and *Alternaria brassicicola* (Fig. 5). In contrast to wild-type, all three *jar1, coi1* and *mpk6 Arabidopsis* mutants, whose gene products are involved in JA sensitivity and signalling, failed to resist *B. cinerea* attack when *N*-isobutyl decanamide was used as defence elicitor (Fig. 7), indicating that *N*-isobutyl decanamide-conferred resistance to necrotrophic fungi requires an intact JA signaling pathway.

In addition to small fatty acid amides, several other lipids have been shown to affect plant responses to pathogens including very long chain fatty acids, phosphatidic acid (PA) and sphingolipids. Alkamides might influence plant-pathogen interactions by modulating the levels of other lipids such as PA by affecting the activity of enzymes involved in their

biosynthesis or by affecting the levels of second messengers involved in signal transduction to these lipids such as Ca2+, nitric oxide, and/or reactive oxygen species (ROS). The relationship between NO and alkamides was previously investigated in *A. thaliana* shoot explants. It was shown that *N*-isobutyl decanamide is able to induce NO accumulation in different stages of development of the explants. NO was detected by confocal microscopic analysis and its level increased with alkamide treatment (Campos-Cuevas et al. 2008). Whether NO mediates the defense responses to alkamides and AHLs remain to be investigated.

The production and accumulation or 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 defence responses (Alvarez et al., 1998; Grant and Loake, 2000). In our microarray analysis, we have identified several potential components of the ROS signaling pathway, including H_2O_2-scavenging enzymes catalases, ascorbate peroxidases and at least 20 cytochrome P450 proteins, including the antifungal *CYP71B15/PAD3*, which plays a key role in phytoalexin production (Tables 1 and S1). Activation of these genes correlated with accumulation of hydrogen peroxide in *N*-isobutyl decanamide-treated roots (Data not shown). In addition, regulatory genes encoding kinases, ankyrin repeat-containing proteins, members of the TGA and OBF family of bZIP transcription factors and WRKY transcription factors were induced in response to *N*-isobutyl decanamide treatment that could play a role in ROS and defence mediated signaling (Eulgem, 2005). WRKY transcription factors have been reported to be involved in transcriptional regulation during plant immune responses (Eulgem, 2006).

Taken together, our results show that *N*-isobutyl decanamide is a strong activator of defence responses, which may confer immunity against fungal pathogens in *Arabidopsis thaliana* seedlings. A role of alkamides in pathogen resistance by acting as defence elicitors in plants shows great potential towards application of alkamide-derived compounds to combat pathogen pests.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions.

A. thaliana ecotype Col-0 was used for all experiments unless indicated otherwise. Col-0, transgenic *LOX2:GUS* (Schommer et al. 2008) and mutants *jar1* (Staswick et al. 1992), *mpk6* (Bush and Krysan 2007), and *coi1* (Xie et al. 1998) 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 0.2x 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^{o}C. After growth for 6 days after germination, plants were transferred to control or *N*-isobutyl-decanamide containing solid MS medium for different times prior to RNA extraction.

Homozygous *coi1* was selected by screening a heterozygous population in solid MS medium supplied with 50 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 (Ramirez-Chávez *et al*., 2004).

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. The array contains about 31,000 individual spots on one slide, which putatively contains all *Arabidopsis* genes. Array annotation and composition is available at www.ag.arizona.edu/microarray.

RNA isolation, labelling, hybridization and image processing.

Total RNA was isolated from shoots or roots using the Trizol reagent (Invitrogen) and purified with the RNeasy kit (Qiagen) following the manufacturer's instructions. Purified total RNA from roots and shoots was then mixed and labeled according to the protocols recommended at www.maizearray.org. Briefly: For each treatment by time combination, four biological replicates were used for probe synthesis. For each sample, 1.5 μg total RNA were amplified in the presence of aminoallyl-dUTP (Ambion) using the Aminoallyl Message Amp II kit (Ambion). Resulting amplified RNA probes were further labeled with fluorescent Cy3 and Cy5 dyes (Amersham). The fluorescent dye-labeled probes were then purified using RNeasy columns (Qiagen). For hybridization, probes were mixed, concentrated by precipitation and resuspended in the hybridization solution (50% formamide, 5X SSC, 0.1% SDS, 0.4 μg/ l tRNA and 0.2 μg/ l Salmon Sperm DNA) for 14h. Slides were washed 5 min in each of the following solutions: a) 2x SSC, 0.1% SDS/42°C, b) 0.1x SSC/RT and two final washes with 0.05x SSC/RT carried out.

Slides were scanned with an Axon GenePix 4000 B 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 6.0 image analysis software. The mean of the signals and the median of backgrounds were used for further analysis. The design, protocols and microarray data can be found at the Gene Expression Omnibus database.

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 (Yang *et al*., 2002) using the LIMMA package (Smyth et al. 2005, www.bioconductor.org). Normalized data were log2 transformed and then fitted into mixed model ANOVAs (Wolfinger et al. 2001; Gibson and Wolfinger 2004) 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 (Benjamini and Hochberg 1995). 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.

Real-Time Quantitative RT-PCR (Q-RT-PCR).

Genes whose expression was considered as regulated by *N*-isobutyl decanamide treatment in the microarray analysis were selected with the aims of validating the expression patterns found and also to gain further biological information. Genes known to be defence regulated were also included. Primer design (Tm, 60-65ºC) was performed according the guidelines recommended in the Primer Express Software, Version 3 (Applied Biosystems) using as template the original target sequences from which the oligonucleotides printed in the array were designed. Oligonucleotide sequences for Q-RT-PCR are shown in Table S3. Total RNA for Q-RT-PCR was isolated from 1g frozen root tissue based on the protocol of the Trizol reagent (Invitrogen) and further purified using Qiagen RNeasy columns according to manufacturer's protocol (Qiagen). cDNA was first synthesized using 10 μg total RNA with SuperscriptIII reverse transcriptase (Invitrogen), according to manufacturer's instructions and used for performing Q-RT-PCR (7500 Real Time PCR System, Applied Biosystems). QRT-PCR of *ACTIN 1-7 (ACT)* was performed for normalization. SYBR Green PCR Master Mix was used for the PCRs according to the manufacturer's protocol. Gene expression was normalized to that of the control *ACT* gene by 1 subtracting the CT value of *ACT* from the CT value of the gene of interest. Control to *N*-isobutyl decanamide treatment average expression ratios were obtained from the equation $(1+E)^{\Delta\Delta C_T}$ where $\Delta\Delta C_T$ represents ΔC_T(control) - ΔC_T(*N*-isobutyl-decanamide 60 μM), and E is the PCR efficiency according to protocol reported by (Czechowski et al. 2004).

Analysis of JA levels.

250 mg of freshly weighted (FW) plant tissues were chilled in liquid nitrogen and JA extraction was performed as Pluskota et al. (2007) using dihydrojasmonate as internal standar, derivatizating with chloroform/*N*,*N*-diisoprpyl-etylamine 1:1. In order analyze the samples by GC/MS the extract was added with 10ul of PFBr and 200ul of cloroform : *N*,*N*-

503 diisopropilethilamine (1:1) then incubated at 65^{o}C for 1h. When cool y the solvent was
504 evaporated to dryness and resuspended in 100 µl methanol.
505 Samples were analyzed in a gas chromatograph (Agilent Technologies 7890A) equipped with
506 a capillary column J&W DB-1 (60 m x 250 µm x 0.25 µm) coupled to a mass selective
507 detector (Agilent 5973 Series MSD). Using an autosampler 7683B Series. 2µl of the sample
508 was injected in a splitless way. Operating conditions were: injector temperature 250^{o}C; the
509 oven temperature was programmed as: initial temperature 150^{o}C for 3 min then increasing at
510 the rate of 4^{o}C min^{-1} to a final temperature of 280^{o}C maintained for 20 min. Helium was used
511 as carrier gas with a constant flow of 1 ml min^{-1}. The MS was set to scan from 40 to 600 uam
512 in Synchronous SIM/Scan mode for selectively monitor the following ions for jasmonic acid
513 derivative: 141, 181, 390, and 392. MS temperatures were: Source 230^{o}C, MS Quadrupole
514 150^{o}C.
515 **Histochemical analysis.**
516 For histochemical analysis of transgenic line *LOX2:GUS*, 7 d-old transgenic seedlings
517 expressing this marker construct were incubated at 37 °C in a GUS reaction buffer (0.5 mg/ml
518 of 5-bromo-4-chloro-3-indolyl-B-D-glucuronide in 100 mM sodium phosphate, pH 7). The
519 stained seedlings were cleared by the method of Malamy and Benfey (1997). For each
520 treatment, at least 9 transgenic plants were analyzed. A representative plant was chosen for
521 each treatment and photographed using the Nomarski optics on a Leika DMR microscope.
522 **Fungal growth and inoculation.**
523 Pathogenesis assays were modified from Zimmerli et al. (2001). *Botrytis cinerea* and
524 *Alternaria brassicicola* were grown on agar PDA medium (PhytoTechnology) for 7-12 days at
525 22º C in darkness. Spores were collected with distilled water. Col-0 superficially sterilized
526 seeds were germinated and grown in MS-agar medium into 100 ml flasks with transparent lid.
527 At 20 days after germination, rosette leaves were placed in Petri dishes with 60 µM of *N*-
528 isobutyl-decanamide containing medium or medium supplied with the solvent. Inoculation
529 was performed by placing a 5 µl drop of a suspension of 5 x 10^{5} conidiospores ml^{-1} on the
530 surface of leaves. The samples were incubated at 22 ºC and analyzed at a further 3, 4 and 5 d
531 period after inoculation. Susceptibility to *B. cinerea* and *A. brassicicola* was evaluated by
532 microscopic observation of necrotic symptoms under a dissecting microscope (Leyca MZ6)
533 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 the growth of *B. cinerea* and *A. brassicicola* on 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 JA mutants inoculation, leaves from soil grown adult plants were incubated in agar solution supplied with solvent (et-OH) or *N*-isobutyl decanamide during 24 hours prior to inoculation in *N*-isobutyl decanamide-containing agar solution.

***B. cinerea* growth inhibition assays.**

Mycelia from *B. cinerea* were excised from a fresh culture plate with solid

PDA medium and transferred to the centre of new Petri dishes (diameter 9 cm) containing *N*-isobutyl decanamide at concentrations 0 (control), 60 and 120 µM. Radial growth of the fungus was measured 24, 48 and 72 h after fungus deposition (data not shown) and visually registered at 72 h from three independent plates for each condition. Fungicide techto 60 was added at a 2 mg ml^{-1} concentration.

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SUPPLEMENTARY MATERIAL

Table S1. Full list of *N*-isobutyl decanamide-regulated genes.

Table S2. The 50 most up-regulated genes by *N*-isobutyl decanamide.

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

Figure S1. Comparative structure between *N*-isobutyl decanamide and C-10 *N*-acyl homoserine lactone. Top, *N*-isobutyl decanamide; bottom, *N*-decanoyl homoserine lactone. Notice the presence of a lactone ring in *N*-decanoyl homoserine lactone.

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728

728

Table 1 Jasmonic acid-related genes regulated by *N*-isobutyl decanamide in *Arabidopsis*.

		Fold change at days after transference			
		1	3	7	14
JA synthesis					
At2g26560	Phospholipase 2A	1.59	1.58	2.98	2.09
At3g45140	LOX2	2.23	1.94	5.20	4.25
At1g17420	LOX3	1.65	1.12	2.49	1.92
At3g25760	AOC1	1.45	1.32	2.08	2.26
At3g25770	AOC2	3.01	3.10	5.00	4.61
At2g06050	OPR3	1.50	1.48	2.91	1.93
At4g15440	HPL1	1.22	1.77	2.18	1.86
JA signaling and response					
At1g19670	COI1	1.18	1.07	2.94	3.78
At4g23600	Coronatine-responsive	2.23	1.94	5.62	5.72
At5g08790	NAC domain containing protein 81	1.80	1.63	1.28	2.02
At5g13210	Similar to JAS1	1,53	2.94	1.51	1.84
At5g59320	LTP3	2.37	5.83	9.91	5.66
At2g38530	LTP2	1.60	2.17	4.02	5.00
At5g24770	VSP2	1.31	2.00	2.27	1.29
At2g24850	Tyrosine aminotransferase 3	1.35	0.32	6.41	3.19
At5g44420	PDF1.2[a]	0.47	0.99	12.58	5.45
At1g75830	PDF1.1	0.53	0.91	14.64	5.03
At2g26020	PDF1.2b	0.56	0.91	11.18	3.06
At5g44430	PDF1.2c	0.70	0.91	15.51	4.18
At3g12500	Basic chitinase	0.79	2.11	3.06	2.05
At4g14060	Major latex protein-related	0.86	4.99	2.63	1.17
At1g52040	Jacalin lectin protein	1.25	2.41	9.92	7.98
At5g39150	GLP6	0.95	7.05	5.06	3.62
At4g12490	Protease inhibitor/LTP	1.59	3.67	1.81	1.38
At2g29350	SAG13	3.03	4.29	8.05	6.57
At3g26830	PAD3/cytochrome P450 (CYP71B15)	1.04	2.68	3.85	2.52
At2g42540	Cold-responsive (cor15a)/LEA	2.48	2.89	3.70	2.17
At5g19470	MutT/nudix kinase)	3.44	4.94	3.11	1.35
At1g66700	Carboxyl methyltransferase/defense-related	1.54	3.81	2.63	2.72
At3g44860	Carboxyl methyltransferase	2.03	1.21	6.30	2.53

729

730

730 **FIGURE LEGENDS**

731 **Figure 1.** Experimental design and expression patterns analysis of genes differentially
732 regulated by *N*-isobutyl-decanamide.
733 (A) Modified loop design including 4 independent replicates evaluated at 1, 3, 7, and 14 days
734 after transfer (dat) of *Arabidopsis* seedlings to solvent-supplemented medium or to medium
735 containing 60 μM *N*-isobutyl decanamide. A total of 16 slides were employed. Each replicate
736 was conformed by 120 transferred seedlings, which were harvested from 3 independent plates.
737 (B) Up-regulated (red) and down-regulated (blue) gene number (vertical axis) by *N*-isobutyl-
738 decanamide in *A. thaliana* seedlings at 1, 3, 7, and 14 dat; (C) Hierarchical cluster analysis of
739 the expression patterns of 1,281 differentially genes (arrayed along the vertical axis) at
740 different dat to medium containing 60 μM *N*-isobutyl-decanamide (horizontal axis).
741 Normalized signal intensities are color coded according to the scale at bottom, and genes are
742 grouped according to similarities in expression profiles in a standard correlation tree analysis.
743 Sets represent cluster groups of genes with similar expression patterns.
744 **Figure 2.** Functional classification of *N*-isobutyl decanamide-regulated genes.
745 *N*-isobutyl decanamide regulated differentially the expression level of 1,281 genes in
746 *Arabidopsis*. These genes were classified by function and grouped as up-regulated (A) or
747 down-regulated (B) based on a two-fold level expression change.
748 **Figure 3.** Validation of microarray results via Q-RT-PCR.
749 Quantitative real-time PCR analysis was performed for 15 genes in *Arabidopsis* (Col-0)
750 seedlings, under the same conditions used for microarray analysis (1, 3, 7 and 14 d of
751 treatment with 60 μM *N*-isobutyl decanamide). Fold-change (control to *N*-isobutyl-
752 decanamide) expression for the indicated selected genes in a log2 scale is shown. (A)
753 Expression ratios obtained by microarray experiments. Estimates of the differences of
754 expression levels were calculated using the mixed model as described in methods. All
755 indicated genes were selected to corroborate the expression patterns obtained in the microarray
756 analysis and also within the threshold limits (FDR 5% and fold change ±2 in any sampled time
757 point); (B) expression ratios obtained by Q-RT-PCR. RQ (relative quantification number) was
758 obtained from the equation $2^{\Delta\Delta C}{}_T$ where $\Delta\Delta\, C_T$ represents ΔC_T(control) - $\Delta\, C_T$(*N*-isobutyl-
759 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 replicate samples. SD was minor than 0.5.

Figure 4. Induction of jasmonic acid signaling in response to *N*-isobutyl decanamide.

(A) *N*-isobutyl decanamide-dependent accumulation of JA as determined by GC-MS from three biological replicate samples. (B) Quantitative real-time PCR analysis of five JA-related genes, using C_T value of *ACT2/7* as internal expression reference; (C) a transgenic *Arabidopsis* line expressing *GUS* reporter gene under the regulation of the JA-induced *LOX2* promoter was grown for 7 d in 0.2x MS medium supplied with the solvent or with 30 and 60 µM *N*-isobutyl decanamide and then stained for *GUS* expression.

Figure 5. *N*-isobutyl-decanamide confers protection against fungal necrotizing pathogens.

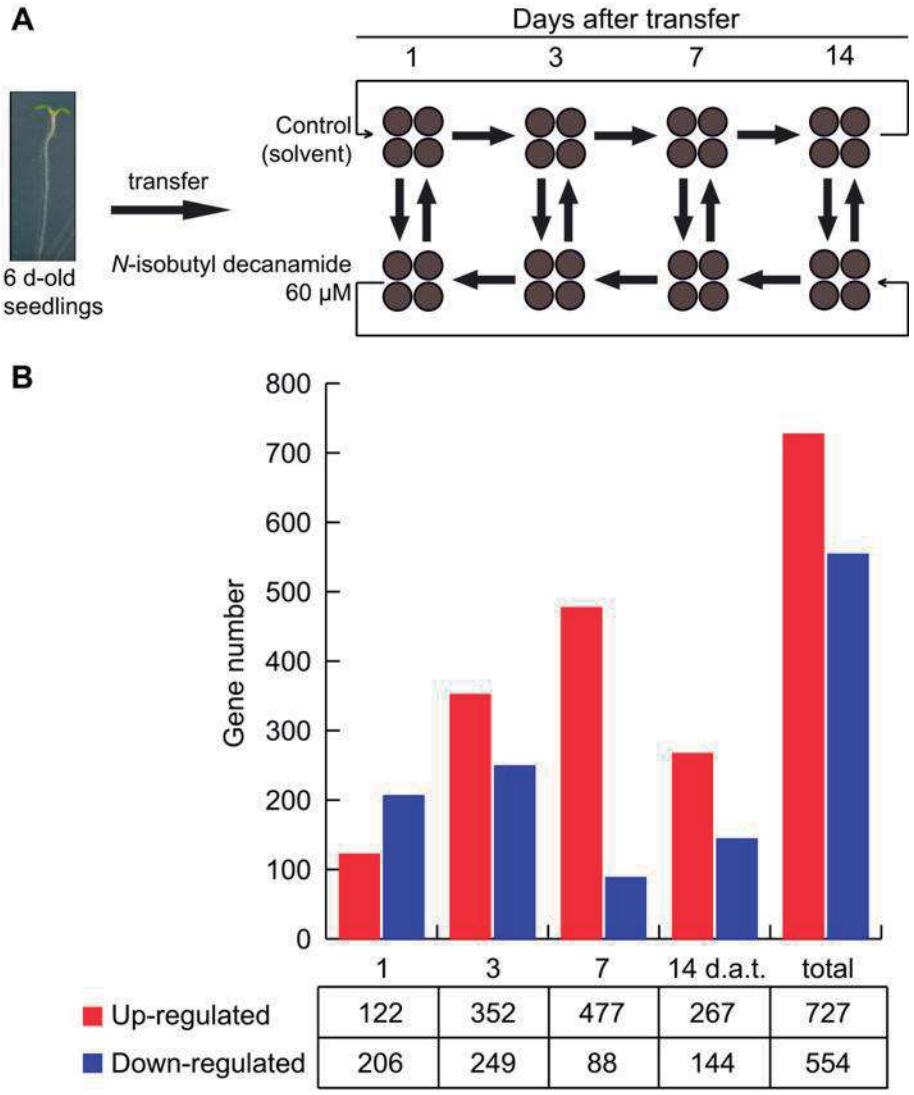
Leaves from 20 d-old *A. thaliana* plants grown in vitro were detached and placed in Petri dishes containing MS 0.2X medium supplied with the solvent (white) or in MS 0.2X medium containing 60 µM *N*-isobutyl decanamide (black). A minimal of 12 leaves were mock (water) treated or inoculated with 5 µl of 1 X 10^5 *Alternaria brassicicola* and *Botrytis cinerea* spores per ml solution/per leaf, depositing the inoculum on the leaf surface. Percent of leaves with conspicuous necrotizing symptoms at 3, 4 and 5 days after inoculation (dai) in response to *A. brassicicola* (A), or *B. cinerea* (B) is shown. (C, D) Lesion diameter in diseased leaves was determined by measuring the major axis of the necrotic area at 3d after inoculation**.** (E) Representative leaves showing visible symptoms of *A. brassicicola* and *B. cinerea* infection at day 5 after treatment. Leaves were stained with trypan blue and photographed 3d after infection with the fungal pathogens. Blue staining is indicative of cellular damage. Notice reduced fungal proliferation in *N*-isobutyl decanamide treated leaves.

Figure 6. Effect of *N*-isobutyl decanamide in *B. cinerea* mycelial growth.

B. cinerea mycelium excised from a solid culture in Petri dishes was transferred to the center of PDA supplied Petri plates to evaluate radial growth of the fungus in solvent (A), or *N*-isobutyl decanamide 120 µM treatment (B). Fungicide Techto 60 was employed at 1 mg/ml as fungal growth inhibition control (C). Hyphal structures growing on PDA medium supplied with filter paper disks imbibed with solvent, Techto 60, or decanamide solutions (D), f letters indicate position of filter paper disks.

Figure 7. Effect of *N*-isobutyl decanamide in pathogen responses in WT, JA-related and *mpk6* *Arabidopsis* mutants.

Leaves from 20 d-old plants grown in soil were pre-incubated 24 h on solvent, 30 or 60 µM *N*-isobutyl decanamide containing plates, transferred to decanamide-free plates and then inoculated with *B. cinerea*. The graphical representation of disease symptoms caused by *B. cinerea* in leaves from WT plants, JA-related mutants *jar1* and *coi1* and *mpk6* 3 days after inoculation with a $5x10^5$ spores ml^{-1} suspension The disease rating (upper panel) is represented in the graph as percent 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.



800

801 **Figure 1**

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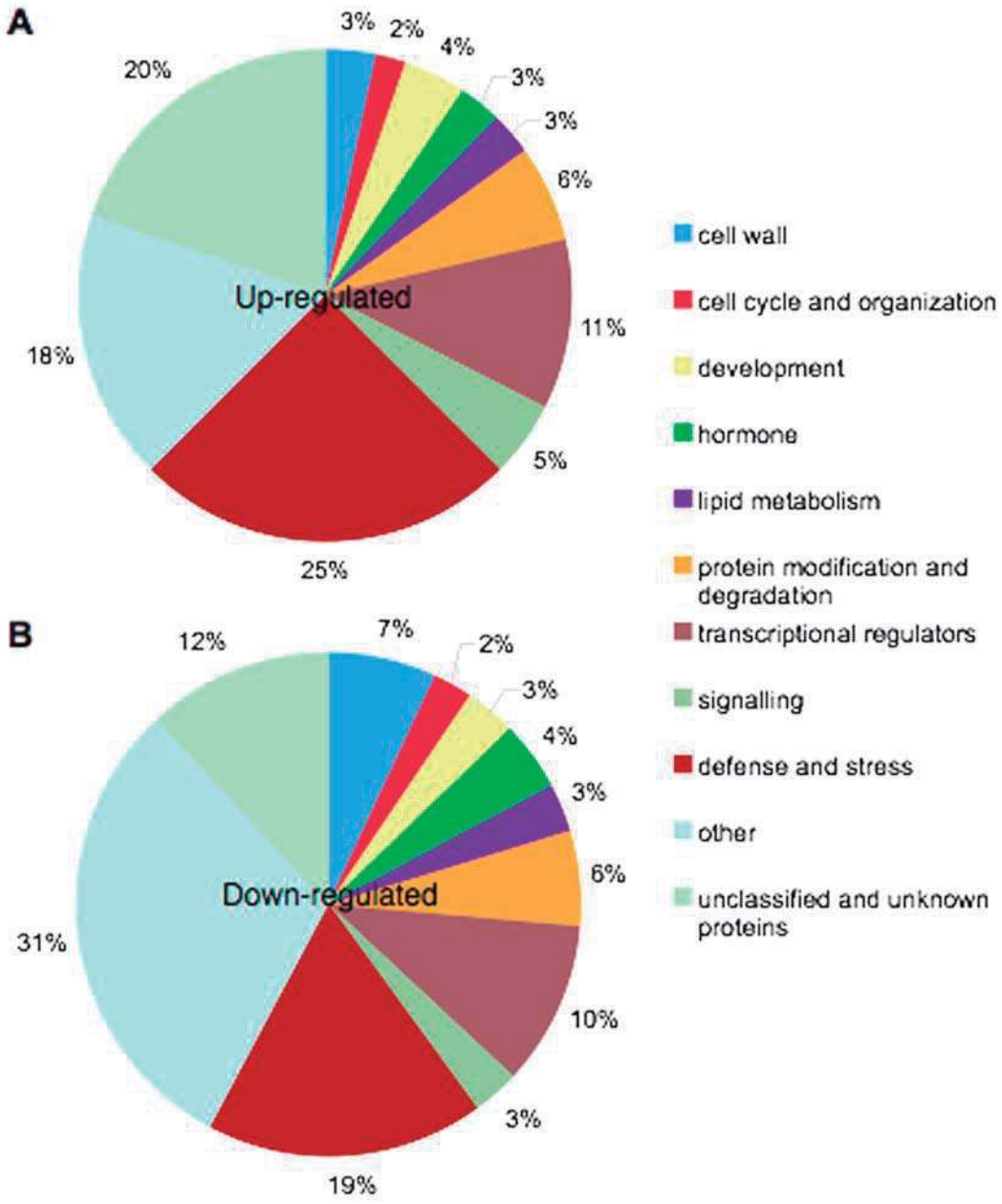

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803 **Figure 2**

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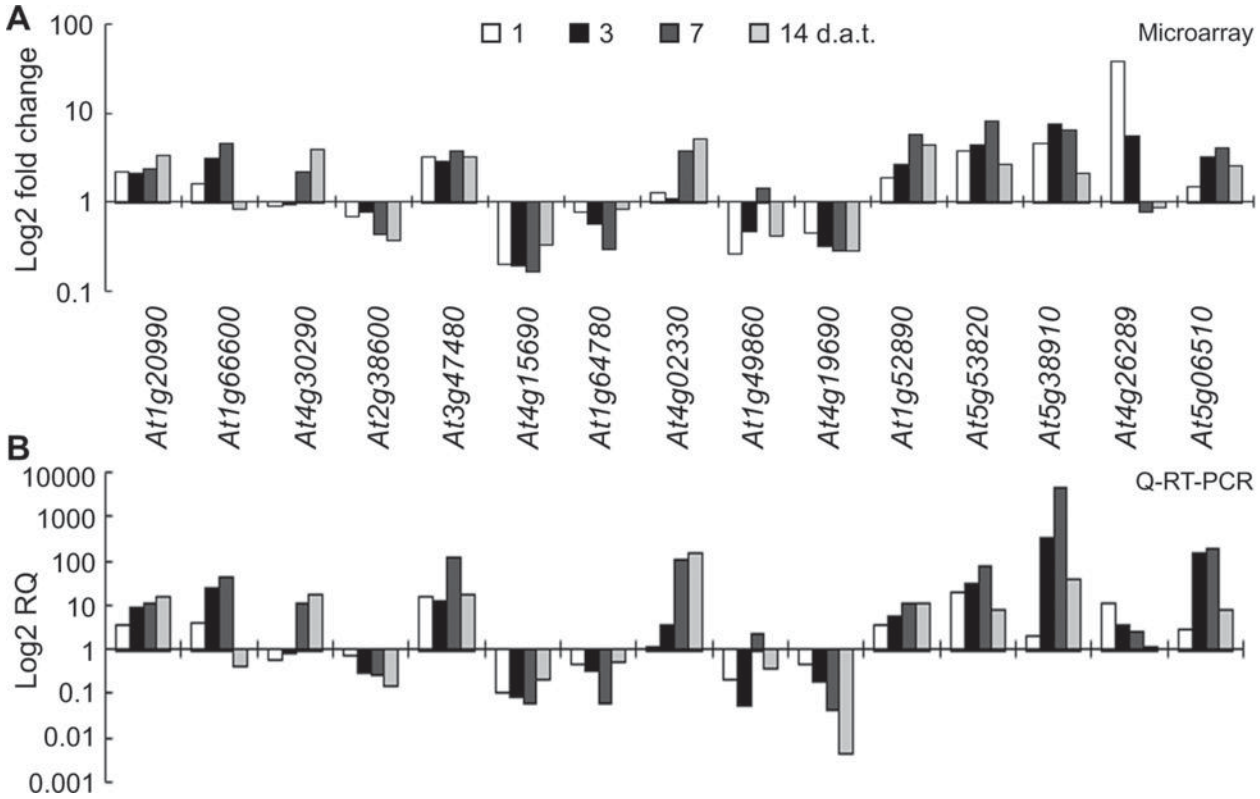

A
Log2 fold change
100
10
1
0.1
1
3
7
14 d.a.t.
Microarray
At1g20990
At1g66600
At4g30290
At2g38600
At3g47480
At4g15690
At1g64780
At4g02330
At1g49860
At4g19690
At1g52890
At5g53820
At5g38910
At4g26289
At5g06510
B
Log2 RQ
10000
1000
100
10
1
0.1
0.01
0.001
Q-RT-PCR

Figure 3

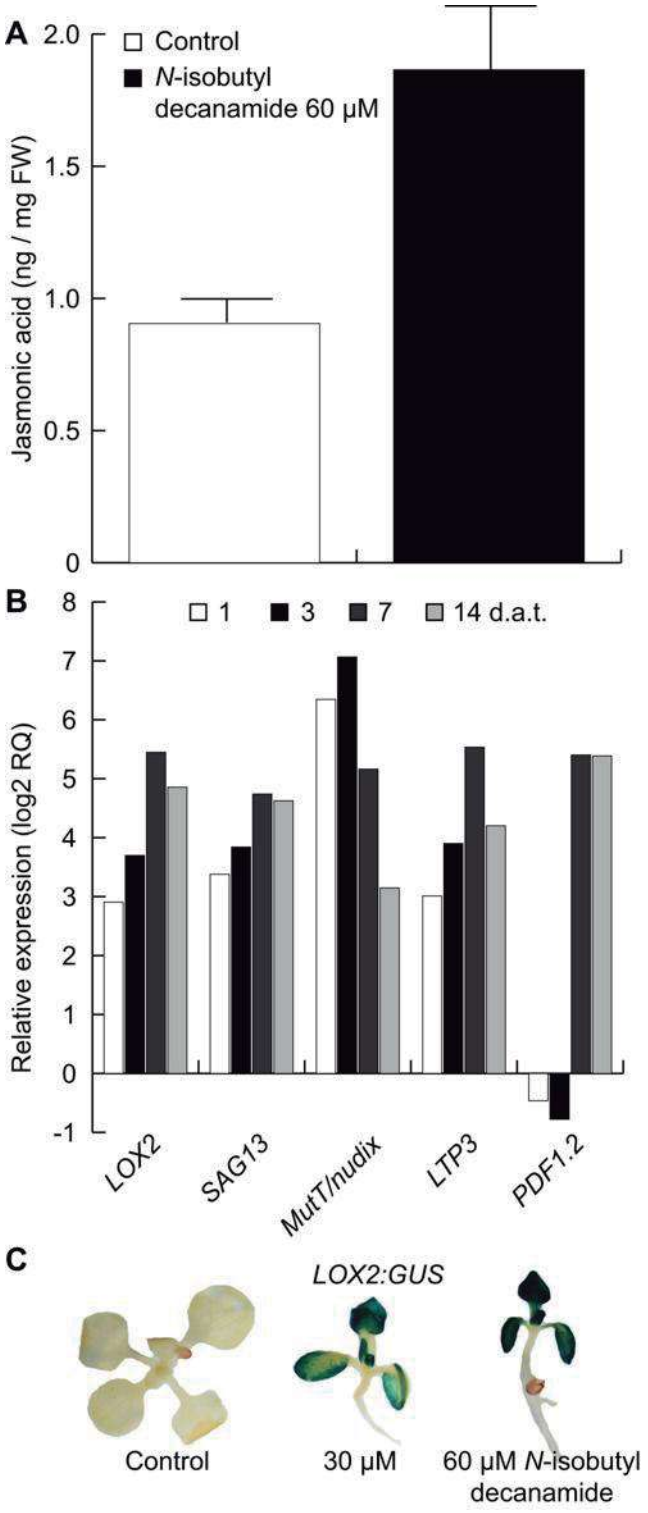
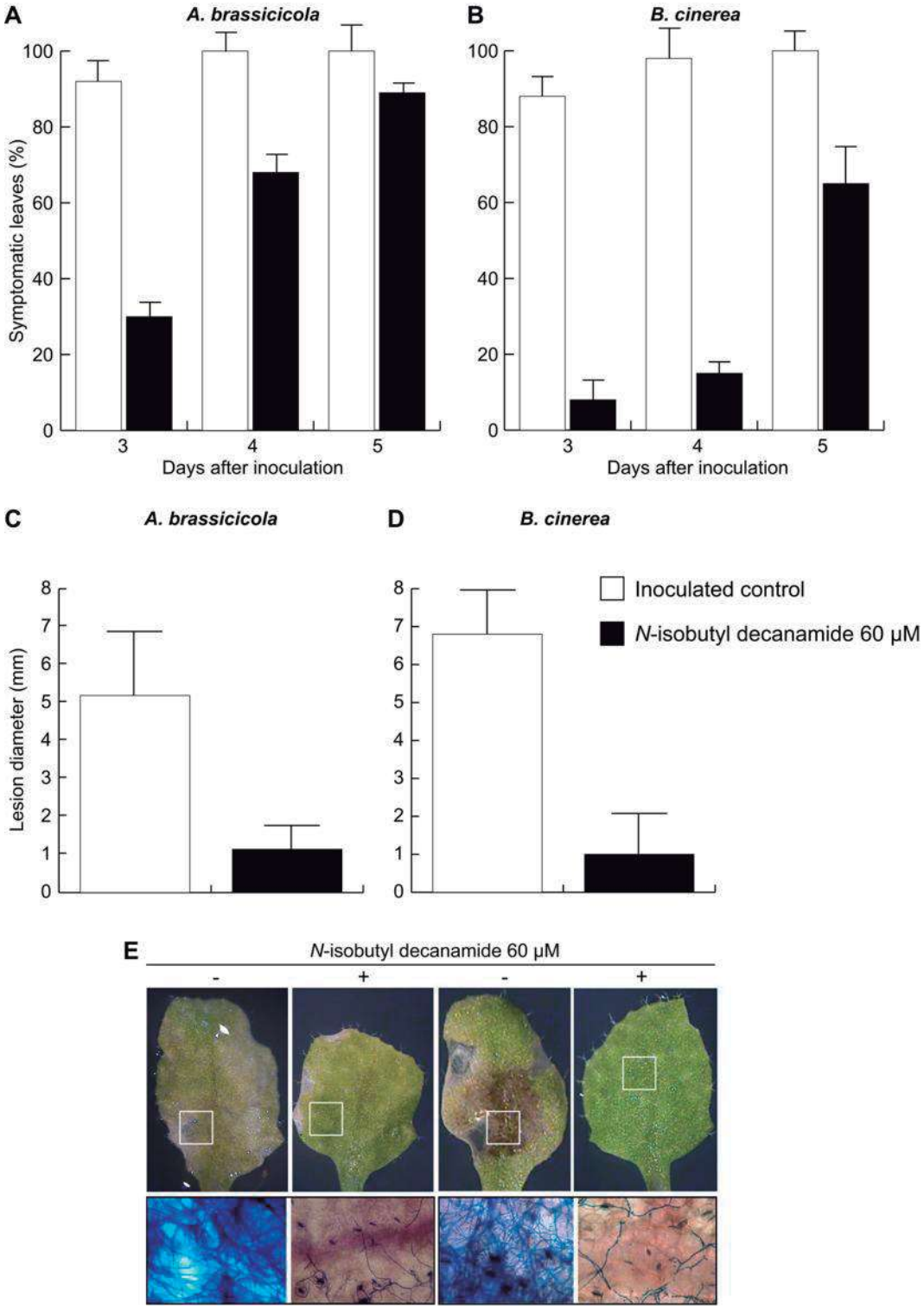

A
Control
N-isobutyl decanamide 60 µM
Jasmonic acid (ng / mg FW)
2.0
1.5
1.0
0.5
0
B
1
3
7
14 d.a.t.
Relative expression (log2 RQ)
8
7
6
5
4
3
2
1
0
-1
LOX2
SAG13
MutT/nudix
LTP3
PDF1.2
C
LOX2:GUS
Control
30 µM
60 µM N-isobutyl decanamide

Figure 4



808

809 **Figure 5**

810

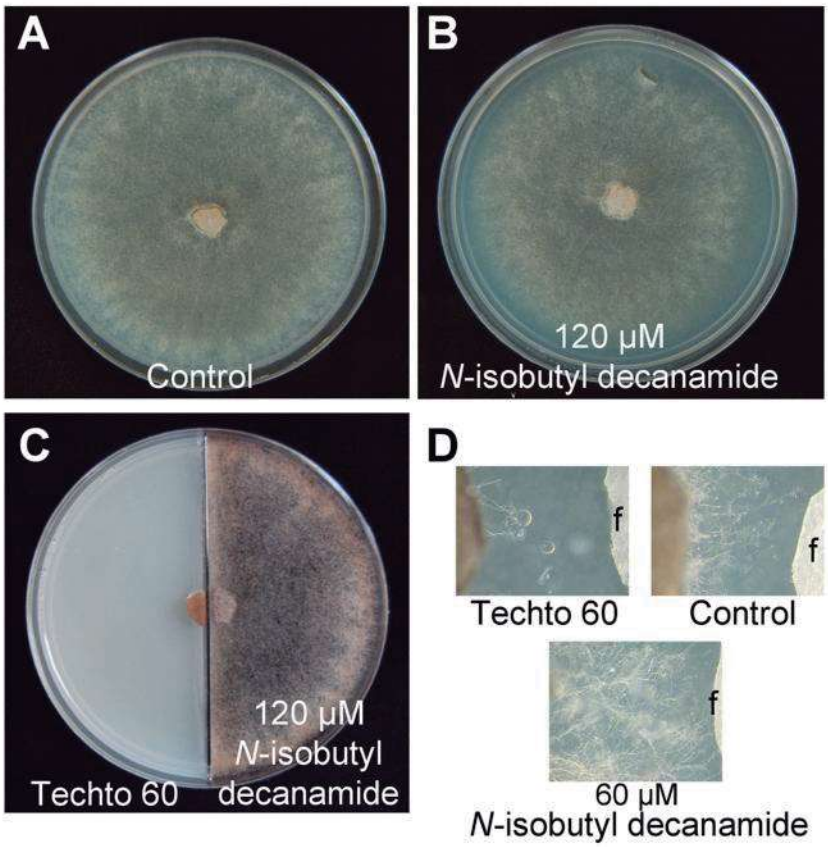

A
Control
B
120 µM
N-isobutyl decanamide
C
Techto 60
120 µM
N-isobutyl
decanamide
D
f
Techto 60
f
Control
f
60 µM
N-isobutyl decanamide

Figure 6

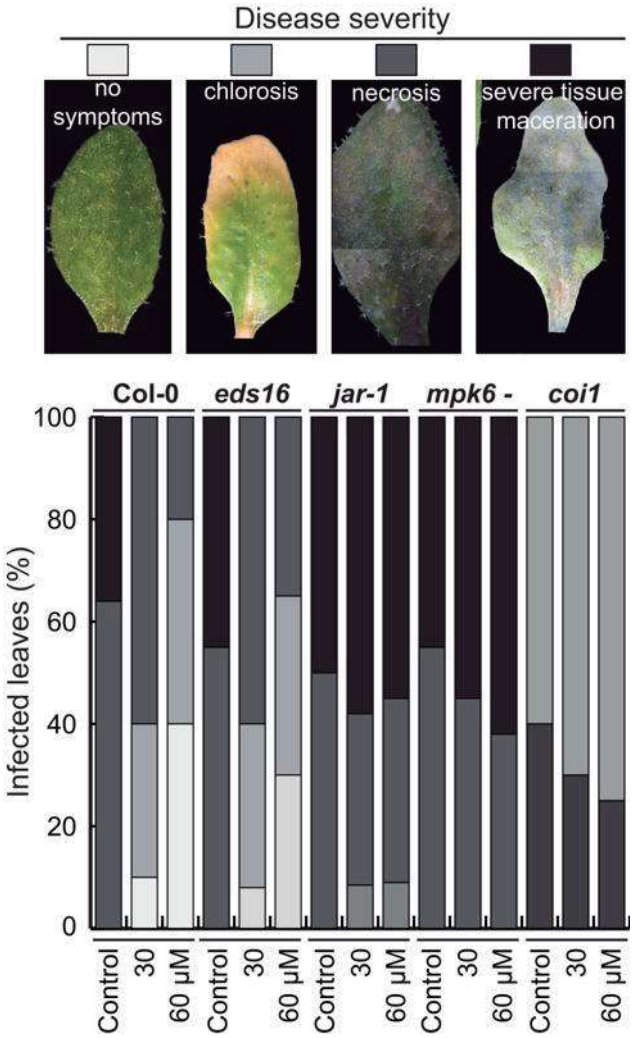
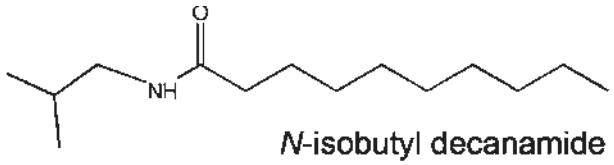
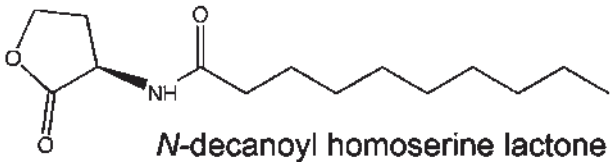

Disease severity
no symptoms
chlorosis
necrosis
severe tissue maceration
Col-0
eds16
jar-1
mpk6 -
coi1
Infected leaves (%)
100
80
60
40
20
0
Control
30
60 µM

Figure 7

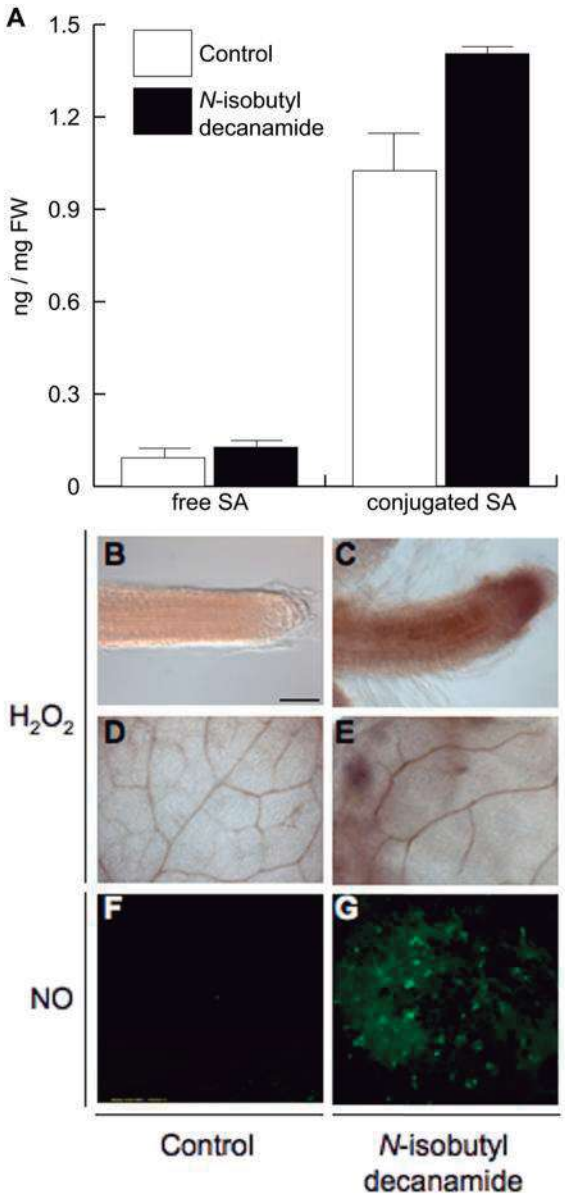


N-isobutyl decanamide



N-decanoyl homoserine lactone

Supplementary Figure S1



Supplementary Figure S2

CAPÍTULO II

Q1

PCP
PLANT & CELL PHYSIOLOGY

Nitric Oxide is Involved in Alkamide-Induced Lateral Root Development in Arabidopsis

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Regular Paper

Alkamides are small bioactive lipid signals with a wide distribution in plants. In this report, the role of nitric oxide (NO) in the alterations induced by *N*-isobutyl decanamide on the Arabidopsis (*Arabidopsis thaliana*) root system architecture (RSA) was investigated. We first compared the effects of *N*-isobutyl decanamide and NO donors sodium nitropruside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) on root morphogenetic processes. Both *N*-isobutyl decanamide and NO donors modulated RSA in a similar way and in a dose-dependent manner, inhibiting primary root growth and promoting lateral root primordia (LRP) formation. RSA alterations induced by *N*-isobutyl decanamide correlated with NO accumulation in the primary root tip and in developing lateral roots. Morphogenetic effects of *N*-isobutyl decanamide decreased when NO scavengers were supplied to alkamide-treated seedlings. *N*-Isobutyl decanamide-regulated root architectural changes were also investigated in mutants defective in NO biosynthesis, *nia1 nia2*, and NO signalling, *Atnoa1*, and in the alkamide-resistant mutant *drr1*. The *nia1 nia2* and *Atnoa1* mutants were indistinguishable in primary root growth inhibition by the alkamide when compared with wild-type (WT) seedlings, but showed reduced lateral root responses. The *drr1* mutant was less sensitive in both primary root growth inhibition and LRP induction by NO donors than WT seedlings. Detailed *DR5:uidA* and *BA3:uidA* marker analysis showed that *N*-isobutyl decanamide and its interacting signals jasmonic acid and NO act downstream or independently of auxin-responsive gene expression to promote LRP formation. Our results provide compelling evidence that NO is an intermediate in alkamide signaling mediating RSA adjustment in Arabidopsis.

Keywords: *N*-Acyl-L-homoserine lactones • Alkamides • *Arabidopsis thaliana* • Nitric oxide • Root development.

Abbreviations: AHL, *N*-acyl-L-homoserine lactone; cPTIO, 2-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF, 4,5-diaminofluorescein; DMSO, dimethylsulfoxide; GUS, β-glucuronidase; JA, jasmonic acid; LR, lateral root; LRP, lateral root primordia; MB, methylene blue; MS, Murashige and Skoog; NAE, *N*-acyl ethanolamine; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PI, propidium iodide; QC, quiescent center; RSA, root system architecture; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; WT, wild type; YFP, yellow fluorescent protein.

Introduction

Coordination of signaling pathways responding to physiological and environmental changes has a central role in plant growth and development. A group of fatty acid amides including alkamides, *N*-acyl ethanolamines (NAEs) and *N*-acyl-L-homoserine lactones (AHLs) have emerged as important modulators of developmental process (López-Bucio et al. 2006, Ortíz-Castro et al. 2008, Morquecho-Contreras et al. 2010). Plant and bacterial fatty acid amides have been shown to alter root and shoot system architecture in Arabidopsis (*Arabidopsis thaliana*) and to affect biomass production in a dose-dependent way (Blancaflor et al. 2003, Ramírez-Chávez et al. 2004, Campos-Cuevas et al. 2008, Ortíz-Castro et al. 2008). Rapid establishment of roots in response to alkamide treatment, whether by proliferation of lateral roots (LRs) or adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients. Although application of fatty acid amides to crops may be advantageous, their agricultural potential may increase if the mechanisms of action of these compounds were known.

As a first step in exploring the structure–activity relationships of NAEs and alkamides, López-Bucio and co-workers (2007) reported the root architectural responses of Arabidopsis seedlings to natural and synthetic compounds. From a group of similar chain length alkamides and NAEs, *N*-isobutyl decanamide, a C10 saturated alkamide that is naturally produced in *Acmella radicans* (Ríos-Chávez et al. 2003) and

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Cissampelos glaberrima (Laurerio-Rosario et al. 1996), was identified as the most active compound in inhibiting primary root growth and stimulating LR formation. *N*-Isobutyl decanamide was also found to induce adventitious root formation both in shoot explants and in intact Arabidopsis seedlings. Stimulation of adventitious root branching was related to an increase in nitric oxide (NO) levels in sites of adventitious root initiation, suggesting a role for NO in alkamide-regulated development processes (Campos-Cuevas et al. 2008).

NO is a highly active gaseous molecule involved in diverse morphogenetic and developmental processes such as xylem differentiation, programmed cell death, LR and adventitious root development, flowering, stomatal closure, gravitropism, leaf expansion, photomorphogenesis and senescence (Beligni and Lamattina 2001, Desikan et al. 2002, Neill et al. 2003, Correa-Aragunde et al. 2004, Grün et al. 2006). NO participates as an integrative signal of diverse endogenous plant hormones including ABA-regulated stomatal closure (Desikan et al. 2002), auxin-mediated LR formation (Pagnussat et al. 2002), jasmonic acid (JA) and cytokinin signaling (Huang et al. 2004, Tun et al. 2008).

In animals, NO is produced by NO synthase (NOS). The sources of NO synthesis in plants include reduction of nitrite by nitrate reductase (NR), oxidation from arginine to citrulline by NOS, and a non-enzymatic NO generation system (Bethke et al. 2004). Although evidence for arginine-dependent NO synthesis has accumulated, no gene or protein with functional similarity to mammalian-type NOS have been found in plants. Guo et al. (2003) identified a NOS-like enzyme from Arabidopsis (*AtNOS1*) with a sequence similar to that of a protein that has been implicated in NO synthesis in the snail *Helix pomatia*. However, the AtNOS1 protein did not show NOS activity, since it was unable to bind and oxidize arginine to NO (Zemojtel et al. 2006, Moreau et al. 2008). Therefore, *AtNOS1* was renamed *AtNOA1* for *NO-associated protein 1* (Crawford et al. 2006). Experimental evidence has shown that AtNOA1 is a member of the circularly permuted GTPase family (cGTPase) (Moreau et al. 2008). The Arabidopsis *Atnoa1* mutant has been widely used in NO research; it shows reduced levels of NO during plant growth, fertility, hormonal signaling, salt tolerance and plant pathogen responses (Guo et al. 2003, Zeidler et al. 2004, Zhao et al. 2007).

Another source of plant NO results from the activity of the enzyme NR. This enzyme is usually associated with nitrogen assimilation, but can also generate NO from nitrite in an NAD(P)H-dependent reaction (Dean and Harper 1986, Rockel et al. 2002). Such activity can be inhibited by tungstate, which has been used to assess the involvement of NR in potential signaling pathways (Bright et al. 2006). NR is a molybdenum-containing enzyme, and it is assumed that tungstate in some way competitively interferes within the metal-binding site of the enzyme. Arabidopsis has two *NR* genes, *NIA1* and *NIA2*, encoding proteins that are very similar (83.5% identical at the amino acid level), but which differ in a number of specific sequence regions, e.g. the N-terminal region. Using the double mutant *nia1 nia2*, it has been shown that NR is involved in ABA-induced NO generation in guard cells (Desikan et al. 2002).

Although our previous research has shown that alkamides induce NO accumulation during adventitious root formation in Arabidopsis shoot explants (Campos-Cuevas et al. 2008), it is not known whether NO modulates other factors important for root architectural responses such as primary root growth and LR formation. In addition, it is unknown whether NO stimulates LR development by activating pericycle cells to produce more lateral root primordia (LRP) or by stimulating LRP outgrowth to increase the number of mature LRs.

In our previous research, we identified an alkamide-resistant mutant termed *decanamide resistant root 1* (*drr1*), which shows reduced sensitivity to primary root growth inhibition by *N*-isobutyl decanamide (Morquecho-Contreras et al. 2010). The *drr1* line represents a powerful biological tool to explore a potential interaction between alkamides and NO in plant signaling. Here, we show that *N*-isobutyl decanamide induces NO accumulation in meristematic and proliferating root regions in Arabidopsis seedlings, and that exogenous application of the NO donors sodium nitropruside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) mimics root system architechture (RSA) responses to alkamides. We explored at the genetic level the involvement of *NIA1*, *NIA2* and *AtNOA1* in mediating the RSA responses to *N*-isobutyl decanamide and show that the corresponding genes are important factors in LR formation under alkamide treatment. Moreover, our comparative RSA analysis in wild-type (WT) and *drr1* mutants in response to NO donor treatment provided information indicating that *DRR1* is involved in NO-mediated LR induction. Taken together, our results show that NO may act in mediating root developmental responses to alkamides in *A. thaliana*.

Results

N-Isobutyl decanamide induces NO accumulation in both primary and lateral roots

To determine whether NO could be involved in root architectural responses to alkamides, we analyzed the presence of NO in the Arabidopsis root system by using the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) in seedlings germinated and grown for 7 d on 0.2× Murashige and Skoog (MS) medium, supplied with different concentrations of *N*-isobutyl decanamide. Living cells incorporate DAF-2DA, which subsequently is hydrolyzed by cytosolic esterases to release 4, 5-diaminofluorescein (DAF-2), which reacts with NO to produce the fluorescent triazole derivative triazolofluorescein (DAF-2T) (Kojima et al. 1998). As previously reported (López-Bucio et al. 2007), this alkamide was found to inhibit primary root growth and increase both LR number and density (number of LRs cm^{-1}) in a dose-dependent way (**Fig. 1A–C**). We also calculated the LR density induced by the alkamide in the LR formation zone, which comprises the root portion from

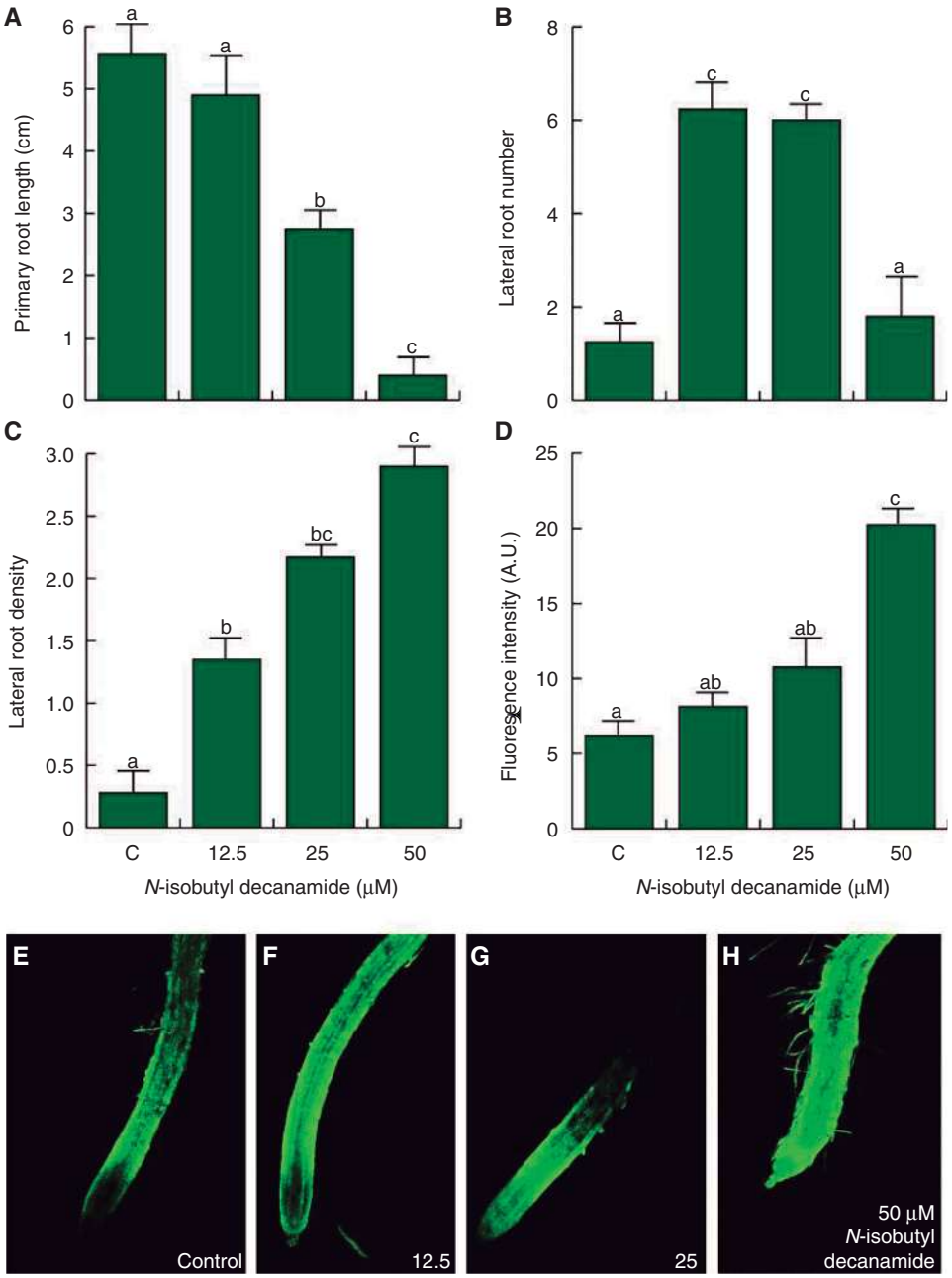

Fig. 1 Effects of *N*-isobutyl decanamide on root architecture and NO accumulation in Arabidopsis seedlings. (A) Primary root length, (B) total number of emerged lateral roots and (C) lateral root density (lateral root number per cm) from 7-day-old WT (Col-0) seedlings grown on the indicated concentration of *N*-isobutyl decanamide. (D) DAF-2T fluorescence signals from roots ($n = 10$) were quantified using the ImageJ program. The graph is expressed in arbitrary units. (E–H) Detection of endogenous NO on primary root tips in 7-day-old seedlings with DAF-2DA. Values shown in A, B and C represent the mean of 30 seedlings ± SE. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results. Photographs are representative individuals of at least 10 seedlings analyzed; bars represents the SD.

the root base to the youngest (most distal) emerged LR as reported by Dubrovsky et al. (2009). In control seedlings the LR formation zone comprised 10 mm in length; a similar length was recorded in Arabidopsis seedlings treated with 12.5 μM *N*-isobutyl decanamide. Treatments with 25 and 50 μM *N*-isobutyl decanamide decreased the LR formation zone length by 45 and 75%, respectively (**Supplementary Fig. S1**). In contrast, all three alkamide treatments clearly increased LR

density (**Supplementary Fig. S1**) confirming the stimulatory effect of the alkamide on LR formation. When Arabidopsis seedlings grown in medium with *N*-isobutyl decanamide were loaded with DAF-2DA, specific green fluorescence could be detected by means of confocal microscopy. Interestingly, reduced primary root growth and enhanced LR formation in response to the alkamide correlated with enhanced DAF-2T fluorescence detected in the meristematic region of the primary root tip (**Fig. 1D–H**). In agreement with the strong primary root growth inhibition observed in seedlings treated with 25 and 50 µM *N*-isobutyl decanamide, differentiation processes were induced as revealed by root hair formation close to the primary root tip. Fluorescence was clearly seen in the differentiation region of the primary root, in which root hair formation takes place (**Fig. 1H**). This suggests that increased fluorescence in root tips in response to alkamide treatment probably occurs by NO accumulating in this region.

NO donors mimic the root architectural responses of Arabidopsis seedlings to N-isobutyl decanamide

A widely used strategy to investigate NO signaling in plant development involves the pharmacological application of NO donors such as SNP or SNAP to emulate NO production (Planchet and Kaiser 2006). To determine whether NO donors could alter RSA in Arabidopsis in a similar way to *N*-isobutyl decanamide, Arabidopsis WT (Col-0) seedlings were germinated and grown in medium with increasing concentrations of SNP or SNAP, and primary root growth and LR density were determined in 7-day-old seedlings. A dose-dependent primary root growth inhibition was found in seedlings treated with SNP when compared with solvent-treated seedlings (**Fig. 2A**). The number of emerged LRs per seedling increased significantly with 10 and 20 µM ~~*N*-isobutyl decanamide~~ treatments (**Fig. 2B**), while LR density increased with SNP treatments (**Fig. 2C**). The overall RSA of SNP-treated seedlings changed depending on the SNP concentration from a long primary root with a low number of LRs to a short and more branched root system (**Fig. 2B, C**).

SNP is a potentially toxic NO donor, since it produces other volatiles such as HCN. Therefore, we determined whether a second NO donor SNAP could alter Arabidopsis RSA. Similarly to SNP, exogenous application of SNAP modulated primary root growth and LR formation albeit at higher concentrations than SNP (**Supplementary Fig. S2**). These results indicate that the root morphogenetic effects of NO donors are likely to be due to NO and not HCN accumulation in root tissues.

NO donor SNP affects the stage distribution of LRP and increases LRP density

Although reported information has shown the positive role of NO donors in stimulating LR growth in different plant species (Correa-Aragunde et al. 2004, Correa-Aragunde et al. 2006, Wang et al. 2010), it is uncertain whether NO acts by stimulating LRP growth, inducing de novo formation of LRP or

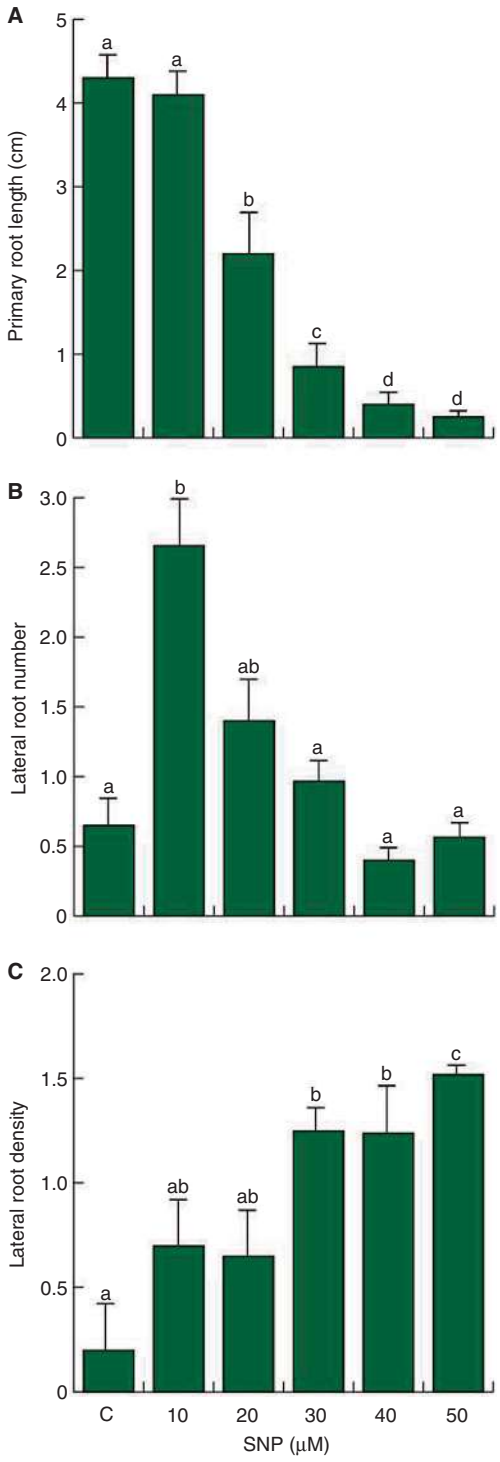

Fig. 2 Effects of NO donor SNP on Arabidopsis root system architecture. WT (Col-0) seedlings were grown for 7 d on agar plates supplied with the NO donor SNP at the indicated concentrations. Primary root length (A), lateral root number (B) and lateral root density (C) determined for a total of 30 seedlings. Data in A and B show the mean ± SD of 30 plants analysed. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

modulating both of these processes. We therefore investigated the stages of LRP development affected by SNP. LRP were quantified 7 d after germination in plants treated with the solvent or with SNP concentrations of 20, 25 and 30 µM, which inhibit 50% primary root growth and induce LR formation (**Fig. 2**). Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. LRP were classified according to their stage of development as reported by Malamy and Benfey (1997). We found that the stage distribution of LRP was affected by treatment with the NO donor. In particular, LRP stages I–V, which refer to developing LRP at early stages of development, were significantly increased in SNP-treated seedlings (**Fig. 3A**). The total number of LRP cm^{-1} was significantly increased in response to SNP treatments (**Fig. 3B**). We also analyzed LR initiation induced by an NO donor using the method described by Dubrovsky et al. (2009), which calculates LRP density in the primary root zone between the youngest LRP and the most distal emerged LR (LRP formation zone). Normalizing data with the length of 100 cortical cells, we determined the LR initiation index, which defines how many LRP are formed along a parent root portion. We found that although SNP inhibits root growth and cortical cell length, SNP treatments induce both LRP density and LR initiation index (**Supplementary Fig. S3**). These data suggest that NO can promote root branching in Arabidopsis by inducing de novo formation of LRP from pericycle cells.

NO inhibits primary root growth without compromising cell viability

To determine the effects of NO accumulation in response to SNP application on cell viability, cell division and differentiation processes in root tips, we analyzed the expression of the vital cell nuclei marker *AtHistH2B:YFP* (Boisnard-Lorig et al. 2001) by confocal laser scanning microscopy in seedlings stained with propidium iodide (PI), a fluorochrome that freely diffuses through dead or damaged cells (Kirik et al. 2001, Cruz-Ramírez et al. 2004). Treatments from 20 to 60 µM SNP were found to increase NO accumulation in root tips (**Fig. 4A–D**), inducing differentiation processes at the root tip region of primary roots, as revealed by root hair formation at the zone previously occupied by the meristem (**Fig. 4C, D**) and in LRs (**Fig. 4E–H**). Visualization of *AtHistH2B:YFP* in the nuclei of cells in 7-day-old SNP-treated roots indicated that the cells in the primary root meristem were viable; in these cells, PI was unable to penetrate (**Fig. 4I–L**). These results suggest that the effects of NO on root development involve changes in cellular processes without affecting cell viability or integrity.

Endogenous NO depletion disrupt root architectural alterations induced by *N*-isobutyl decanamide in Arabidopsis

A pharmacological approach was employed to determinate the role of NO in *N*-isobutyl decanamide-induced RSA modulation. We tested the effects of *N*-isobutyl decanamide on root development and NO accumulation in the presence of the NO scavenger 2-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Arabidopsis WT (Col-0) seedlings were germinated and grown for 5 d on 0.2× MS medium and then transferred to the same fresh medium supplied with the solvent (control), with 100 µM cPTIO, 25 µM *N*-isobutyl decanamide or *N*-isobutyl decanamide plus 100 µM cPTIO.

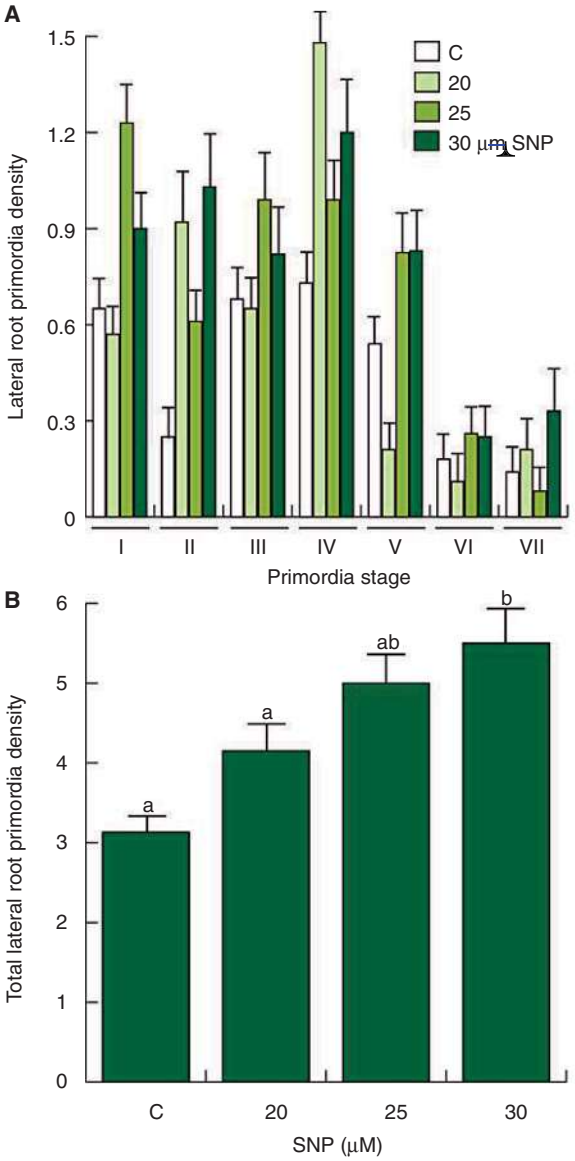

Fig. 3 Effects of NO on LRP development. Arabidopsis Col-0 seedlings were grown for 7 d on agar plates supplied with the solvent (Control, black bars) or with the NO donor sodium nitroprusside (SNP) at 20, 25 and 30 µM (color bars) (A). Data are presented for LRP developmental stages, and total LRP density (B). LRP stages were recorded according to Malamy and Benfey (1997). Values shown represent the mean of 15 seedlings ± SD. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

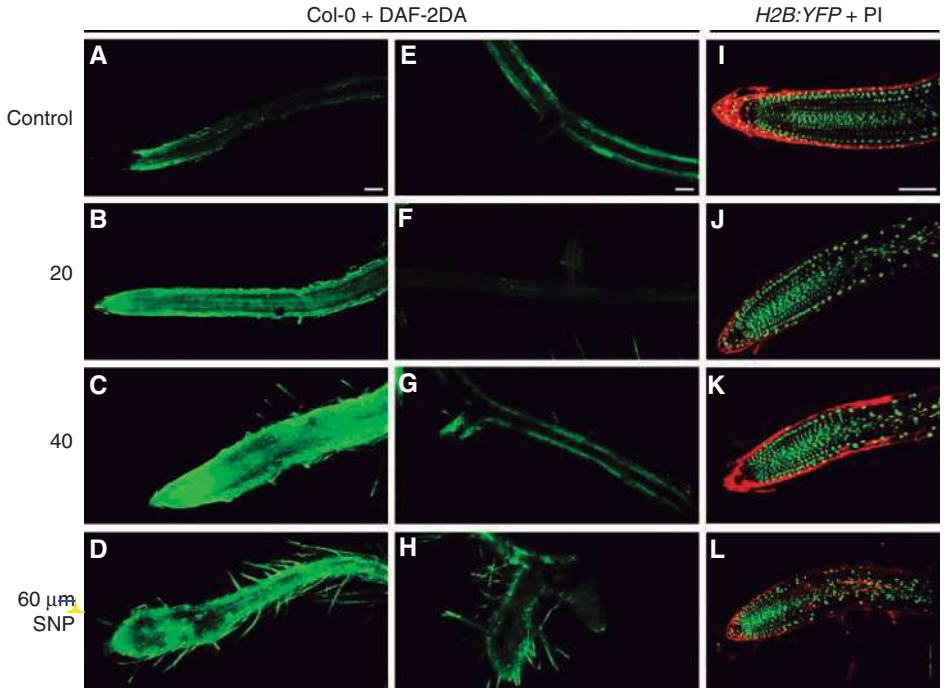

Fig. 4 Effects of SNP on NO accumulation, meristem cell viability and differentiation processes. WT (Col-0) seedlings were grown for 7 d on agar plates supplied with SNP at the indicated concentrations and then stained with DAF-2DA to reveal NO accumulation in primary root tips (A–D), and on emerged lateral roots (E–H). *AtHistH2B:YFP* seedlings were grown under the same conditions during 7 d and were stained with PI to determine cell structure and viability (I–L). A total of 10 seedlings were analyzed for each treatment condition and representative photographs were taken. The experiment was repeated three times with similar results.

RSA changes induced by the alkamide were quantified 3 d after transfer. It was found that seedlings transferred from 0.2× MS medium to solvent- or cPTIO-containing media showed similar primary root growth, while seedlings transferred to *N*-isobutyl decanamide showed decreased growth (**Fig. 5A, B**). cPTIO supply partially restored primary root growth to alkamide-treated seedlings (**Fig. 5A, B**), suggesting that NO is involved in primary root growth modulation by *N*-isobutyl decanamide.

As a method to determine the role of NO in alkamide-induced LR development, we quantified LRP specifically formed in response to alkamide treatment at the distal region of the primary root in a transfer assay. As shown in **Fig. 5C**, control or cPTIO-treated seedlings did not form any visible LRP in this root region. In contrast, the number of LRP dramatically increased in seedlings transferred to 25 µM *N*-isobutyl decanamide. This enhanced LRP formation and emergence of LRs was abolished when cPTIO was supplied in the medium together with the alkamide (**Fig. 5C, D**), suggesting that endogenous NO is involved in LRP formation triggered by *N*-isobutyl decanamide. Similar results were observed when we antagonized the effects of *N*-isobutyl decanamide with another NO scavenger, methylene blue (MB). MB was able to decrease NO accumulation in the root tip of *N*-isobutyl decanamide-treated seedlings, and partially reversed the primary root growth inhibition (**Supplementary Fig. S4**).

In addition to primary root growth, cell division and differentiation parameters were analyzed to determine the role of NO in cellular responses to *N*-isobutyl decanamide. We analyzed the expression of the cell cycle marker *CycB1:uidA* in transgenic seedlings transferred from 0.2× MS medium to medium containing 25 µM *N*-isobutyl decanamide with or without 100 µM cPTIO for a further 4 d growth period. The proliferative capacity of *N*-isobutyl decanamide-treated primary root meristems was restored by addition of cPTIO as revealed by the presence of *GUS* (β-glucuronidase)-expressing cells (**Supplementary Fig. S5**). As an indication of differentiation processes occurring at the root tip region, we also measured both the length from the quiescent center (QC) to the first root hair-containing cell at the root epidermis and the length from the QC to protoxylem cells. In seedlings that were transferred from 0.2× MS medium to medium containing *N*-isobutyl decanamide, dramatic reductions in the distance from the QC to the first root hair and from the QC to protoxylem cells were observed (**Supplementary Fig. S5**). These differentiation markers were modified in *N*-isobutyl decanamide-treated seedlings when cPTIO was supplied to the growth medium (**Supplementary Fig. S5**), indicating that cPTIO restores meristematic activity in roots of *N*-isobutyl decanamide-treated Arabidopsis seedlings, and that pharmacological reduction in NO levels decreases the effects of the alkamide on differentiation processes.

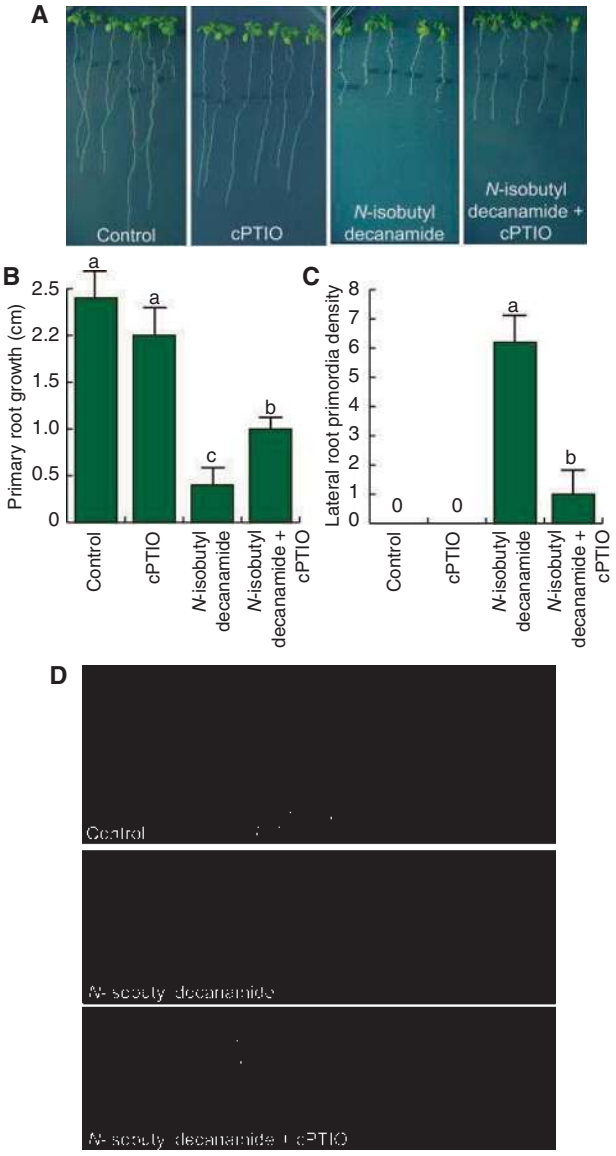

Fig. 5 Effects of endogenous NO depletion on *N*-isobutyl decanamide-modulated root system architecture. Five-day-old Arabidopsis (Col-0) seedlings were transferred for 3 d to plates supplied with the solvent (control), with 100 μM of the NO scavenger cPTIO, with 25 μM *N*-isobutyl decanamide or with *N*-isobutyl decanamide plus cPTIO. (A) Visible effects of treatments on primary root growth after transfer. (B) Primary root growth and (C) lateral root primordia density after transfer. (D) Stereoscopic magnification from the growing root tip after transfer; note the decrease in small emerged lateral roots when cPTIO was supplied with the alkamide. Photographs shown in A are representative of at least three plates analyzed ($n = 15$). The experiment was repeated twice with similar results.

Involvement of NO-related genes in Arabidopsis responses to ~~N~~-isobutyl decanamide

To explore the contribution to NO biosynthesis of NR activity, i.e. *NIA1* and *NIA2* (Wilkinson and Crawford 1993), and *AtNOA1* (Guo et al. 2003), in Arabidopsis responses to *N*-isobutyl decanamide, we compared the RSA of WT plants, the *Atnoa1* single mutant and *nia1 nia2* double mutants when grown under 30 μM SNP or *N*-isobutyl decanamide concentrations. In these experiments, WT (Col-0), *Atnoa1* and *nia1 nia2* seeds were germinated and grown for 5 d on 0.2× MS agar plates, and then transferred onto 0.2× MS-containing plates supplied with the solvent (control), or the above indicated concentrations of SNP and *N*-isobutyl decanamide. Four days after transfer, primary root length and LR number were quantified. In medium lacking SNP and *N*-isobutyl decanamide, mutant lines *nia1 nia2* and *Atnoa1* sustained reduced primary root growth when compared with WT plants. This primary root growth defect could not be rescued by exogenous application of SNP or *N*-isobutyl decanamide (**Fig. 6A**). Moreover, we found that both mutant lines sustained similar sensitivity to SNP and the alkamide in terms of primary root growth inhibition (**Fig. 6A**), indicating that primary root growth inhibition caused by SNP and *N*-isobutyl decanamide might be independent of NO production by NRs and *AtNOA1*. Interestingly, the two NO-related mutants showed reduced LR responses to SNP or *N*-isobutyl decanamide (**Fig. 6B, C**), indicating that *NIA1*, *NIA2* and *AtNOA1* could participate in alkamide-modulated LR induction.

The alkamide-resistant mutant ~~drr1~~ displays reduced sensitivity to NO donors

To investigate a potential interaction between alkamide and NO signaling at the level of the alkamide-related *DRR1* locus, we evaluated biomass production and RSA responses of WT (Ws ecotype) and *drr1* seedlings to exogenous application of SNP. Developmental alterations induced by SNP treatment were accompanied by a dose-dependent reduction in both shoot and root fresh weight in WT seedlings, while *drr1* seedlings were less affected by SNP (**Supplementary Fig. S6**). A 60 μM SNP treatment dramatically inhibited leaf formation in WT seedlings, but showed no or little inhibition in the mutants (**Supplementary Fig. S6**).

The SNP dose–response of WT and *drr1* seedlings in RSA modification was also determined. Both WT and *drr1* primary root growth was inhibited by increased concentrations of SNP. However, *drr1* seedlings showed a significantly reduced inhibition at most SNP concentrations tested when compared with WT seedlings (**Fig. 7A**). SNP concentrations from 30 to 50 μM increased the number of emerged mature LRs in WT seedlings by around 60–80% (**Fig. 7B, C**). As previously reported by Morquecho-Contreras et al. (2010), in solvent-treated medium, LR formation in *drr1* was significantly reduced compared with WT seedlings. Interestingly, *drr1* mutants failed to produce increased numbers of LRs when exposed to stimulatory concentrations of SNP (**Fig. 7B, C**).

To understand further the role played by *DRR1* in LR formation in response to SNP, we determined LRP originating from the primary root at 7 d after germination in plants grown in 0.2× MS agar medium supplied with the solvent only or with 30 μM SNP. In solvent-treated WT plants, LRP induction at an

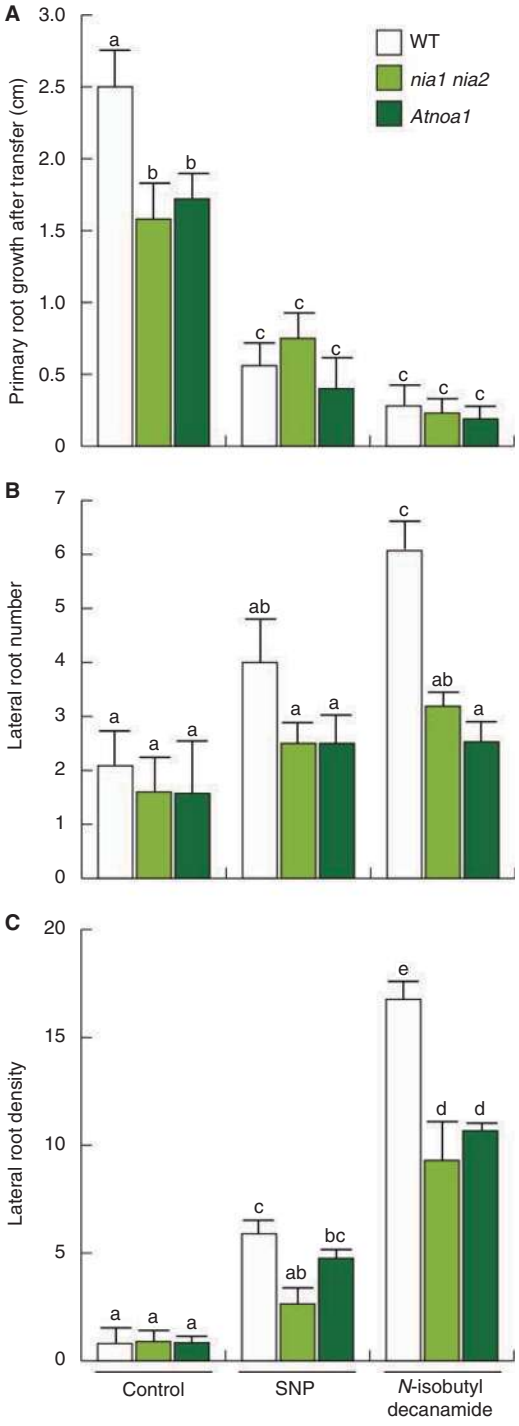

Fig. 6 Effects of *N*-isobutyl decanamide on root system architecture in Arabidopsis NO-related mutants. Wild-type (Col-0), *nia1 nia2* and *Atnoa1* seedlings were germinated and grown for 5 d on 0.2× MS agar medium and transferred to plates supplied with the solvent (control), with 30 μM of either the NO donor SNP or *N*-isobutyl decanamide. Four days after transfer, primary root growth (A) and emerged lateral roots (B and C) were determined for a total of 15 seedlings. Different letters are used to indicate means that differ significantly ($P<0.05$). The experiment was repeated twice with similar results.

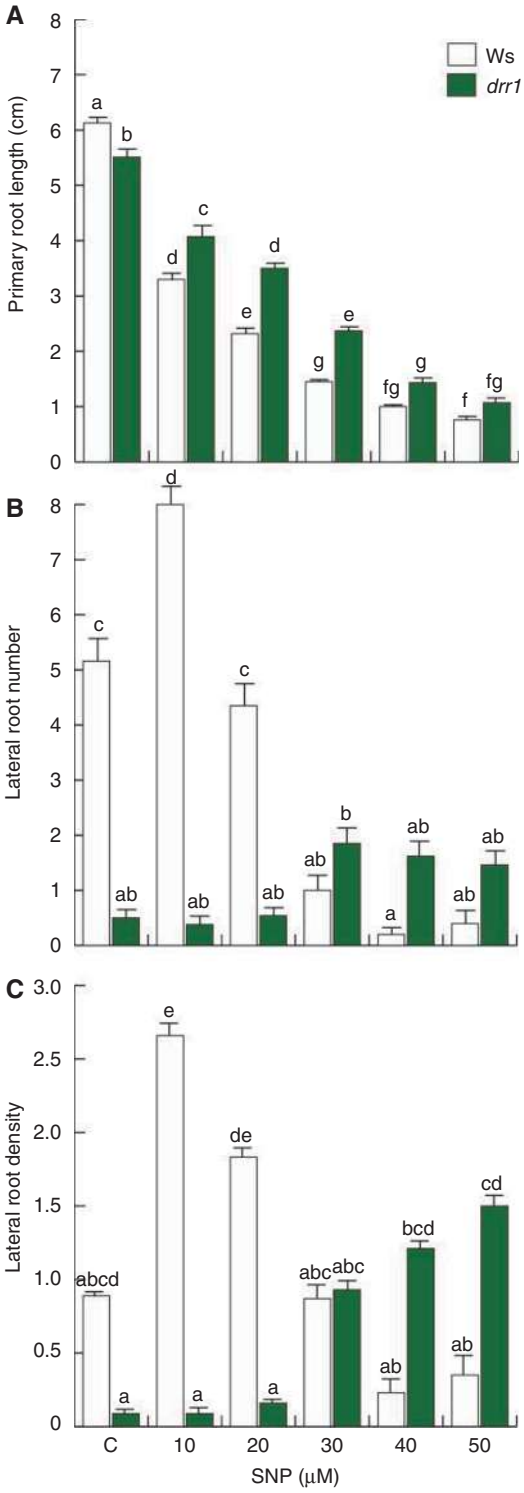

Fig. 7 Effects of SNP on root system architecture of WT and *drr1* seedlings. (A) Primary root length. (B) Number of emerged lateral roots per plant. (C) Lateral root density expressed as the number of lateral roots per cm. Data were recorded at 10 d after germination. Values shown are the mean ± SD ($n=20$). Different letters represent statistically different means ($P<0.05$). The experiment was repeated three times with similar results.

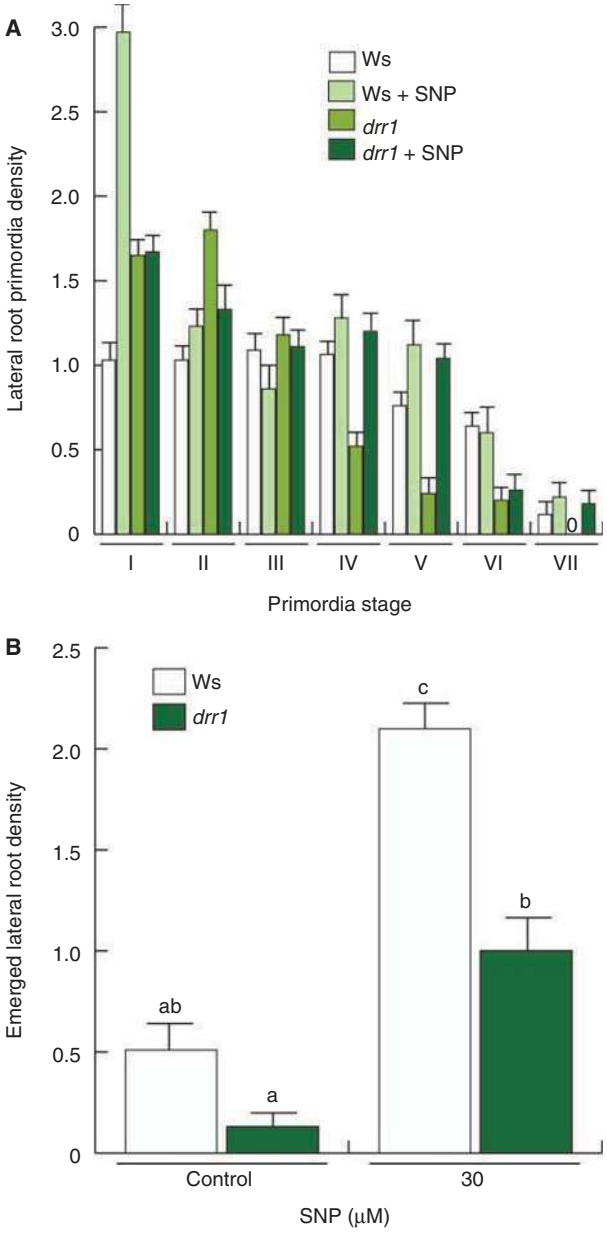

Fig. 8 Effects of SNP on WT and *drr1* lateral root development. (A) LRP stage distribution in 7-day-old primary roots grown on medium supplied with the solvent only or with 30 μM SNP. (B) Emerged lateral root density in the same experiment. WT and *drr1* seedlings were cleared and the number and stage of LRP recorded according to Malamy and Benfey (1997). Values shown are the mean ± SD ($n = 15$). Different letters represent statistically different means ($P < 0.05$). This analysis was repeated twice with similar results.

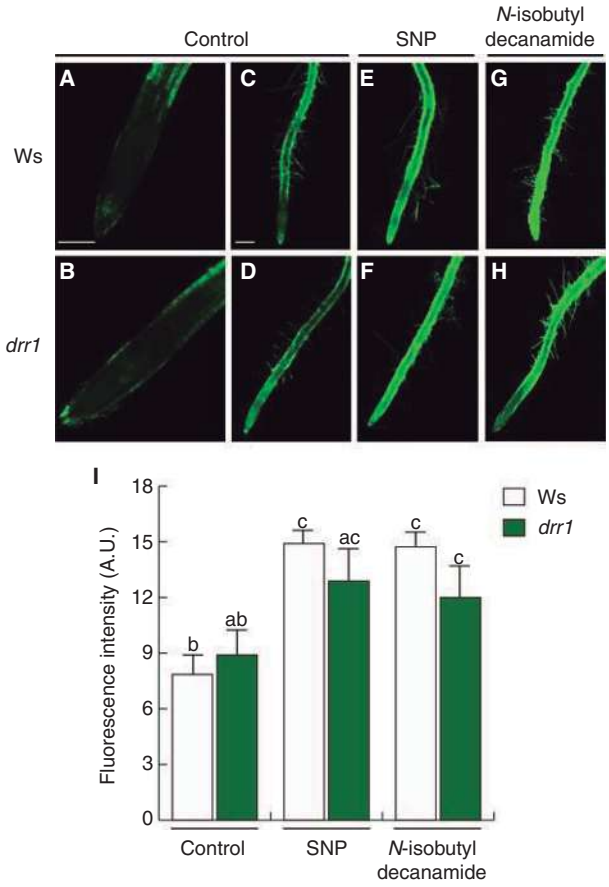

Fig. 9 NO endogenous levels in WT (Ws ecotype) and *drr1*. Seven-day-old seedlings were stained with DAF-2DA after growth in solvent (control, A–D), 30 μM SNP (E and F) or 30 μM *N*-isobutyl decanamide (G and H). Representative images were captured by laser confocal microscopy. (I) DAF-2T fluorescence signals from roots ($n = 10$) were quantified using the ImageJ program. The graph is expressed in arbitrary units.

early developmental stage (stage 1) was increased by 300% by SNP. Interestingly, SNP did not increase LRP density at early developmental stages in *drr1* plants (**Fig. 8A, B**). Although SNP treatment significantly increased total LRP density in *drr1* mutants, the induction never reached the level observed in WT seedlings (**Fig. 8B**), indicating that *drr1* was less sensitive to de novo formation of LRP induced by an NO donor. A similar effect was observed when LRP and emerged LRs mm^{-1} were calculated as described by Dubrovsky et al. (2009) (**Supplementary Fig. S7**). Furthermore, our data show that SNP modifies RSA probably by inducing more pericycle cells to form stage I LRP and by accelerating the emergence of LRP from the primary root to form mature LRs. To determine whether reduced alkamide and NO responses in the *drr1* could be caused by defective accumulation of NO or NO perception, the level of NO in Arabidopsis WT and *drr1* seedlings was determined by DAF-2DA and confocal microscopy. **Fig. 9** shows that treatment with 30 μM SNP or *N*-isobutyl decanamide similarly increased fluorescence in primary roots, indicative of NO accumulation. Taken together, our data show that mutations in *drr1* interfere with earlier stages of LRP formation in response to NO, probably affecting a signaling component rather than blocking NO accumulation in the plant.

Alkamide, JA, and NO act downstream or independently of auxin signaling to promote LR formation

The plant hormone JA plays an important role in LR development (Sun et al. 2009). While JA application led to increased LR numbers in WT seedlings, it failed to activate LR formation in *drr1*, suggesting an alkamide–JA cross-talk in LR development (Morquecho-Contreras et al. 2010). Auxin-induced LR formation is another well-studied, NO-mediated process (Correa-Aragunde et al. 2004, Correa-Aragunde et al. 2006). To test whether alkamides, JA and NO could modulate auxin-inducible gene expression and in this way stimulate LR formation, we compared the effect of these signals with that of IAA on LRP formation in Arabidopsis transgenic plants expressing the auxin-responsive markers *DR5:uidA* (Ulmasov et al. 1997) and *BA3:uidA* (Oono et al. 1998). We found that 0.125 µM IAA or 30 µM JA, *N*-isobutyl decanamide or SNP similarly inhibited primary root growth in both *DR5:uidA* and *BA3:uidA* seedlings (**Fig. 10A**, and data not shown). LRP stage determination showed that all four compounds IAA, *N*-isobutyl decanamide, JA and SNP were able to induce LRP formation in a similar way (**Fig. 10B**). Interestingly, while a 0.125 µM IAA treatment clearly induced auxin-responsive gene expression, 30 µM JA, *N*-isobutyl decanamide or SNP failed to elicit *DR5:uidA* and *BA3:uidA* (**Fig. 10C**). These data suggest that alkamides and interacting partners JA and NO act downstream or independently of auxin-responsive gene expression to promote LRP formation.

Discussion

Plants display considerable root developmental plasticity in response to biotic and abiotic factors. This developmental plasticity is an integral part of adaptive strategies for overcoming their immobility and allows plants to place their resource-capturing organs in close contact with vital resources such as nutrients and water, which vary significantly in both quantity and spatial distribution in natural environments. Root length, LR density and root hair formation become important for nutrient and water transport to the plant (López-Bucio et al. 2003, Malamy 2005). Although IAA is considered the major plant growth-regulating substance underlying RSA adjustment, the discovery of novel signal molecules such as alkamides, JA and NO, which are involved in the intricate network that triggers RSA, has been a recent goal in plant biology. Post-embryonic root development relies on the activity of the primary root meristem, established during embryogenesis, and the continued production of new meristems to form LRs. IAA promotes root branching through the dedifferentiation of pericycle cells to form LR meristems. Although a variety of components of auxin transport and signal transduction have been identified, the molecular mechanisms underlying the initiation of new LR meristems in response to other promoting molecules such as alkamides, NAEs, AHLs and their interacting partners is poorly understood (Nibau et al. 2008, Ortíz-Castro et al. 2008, Sun et al. 2009, Morquecho-Contreras et al. 2010).

In this work we investigated the role of NO in RSA modulation by *N*-isobutyl decanamide in the model plant *A. thaliana*. Much of the work with NO signaling has involved the pharmacological application of NO donors such as SNP to emulate NO production, NO scavengers such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) or cPTIO to remove the signal (Goldstein et al. 2003), and detection methods that employ dyes such as DAF-2DA, which fluoresces when it binds to the N_2O_3 formed by the reaction of NO with NO_2 (Wilson et al. 2008). By using these different strategies, the roles of NO in plants have been elucidated in numerous physiological processes throughout the entire plant life cycle. We used the cell-permeable diacetate DAF-2DA as an NO fluorescence indicator. Upon NO production, DAF-2 is nitrosated to give the highly fluorescing and relatively stable DAF-2T. We found that *N*-isobutyl decanamide treatments from 12.5 to 50 µM induced an intense DAF-2T fluorescence in root tips, which was related to primary root growth inhibition and increased LR formation (**Fig. 1**). Two NO donors, SNP and SNAP, were found to mimic the effects of *N*-isobutyl decanamide on RSA (**Figs. 2, 3**, and **Supplementary Figs S2, S3**). In our assays, SNP was more active than SNAP at inhibiting primary root growth; this effect could be explained because SNP produces other volatiles such as HCN. However, HCN ions inhibited both primary root growth and LR development (**Supplementary Fig. S8**), indicating that at least the stimulation of LR development by SNP occurs independently of HCN. These data not only confirm the signaling role played by NO on LR development as previously reported (Pagnussat et al. 2002, Correa-Aragunde et al. 2004, Correa-Aragunde et al. 2006), but also suggest that NO is involved in root responses to alkamides by acting as a second messenger, as supported by the findings that SNP treatment indeed increases DAF-2T fluorescence (**Fig. 4**) and because depletion of endogenous NO resulted in decreased *N*-isobutyl decanamide responses in terms of LR formation (**Fig. 5**, and **Supplementary Figs. S4, S5**).

To date, a limited number of Arabidopsis NO-related mutants have been reported, including *Atnoa1*, which is defective in a cGTPase, and *nia1 nia2*, which is defective in NRs. In medium lacking SNP and *N*-isobutyl decanamide, both mutant lines *nia1 nia2* and *Atnoa1* sustained reduced primary root growth when compared with WT plants. This primary root growth defect could not be rescued by exogenous application of SNP or *N*-isobutyl decanamide (**Fig. 6A**). Moreover, we found that both mutant lines sustained similar sensitivity to SNP and the alkamide in terms of primary root growth inhibition (**Fig. 6A**), indicating that primary growth inhibition caused by SNP and *N*-isobutyl decanamide occurs independently of NO production by NIA1, NIA2 and AtNOA1. Interestingly, *Atnoa1* and *nia1 nia2* mutants showed reduced LR formation in response to *N*-isobutyl decanamide treatments when compared with WT seedlings, suggesting that these genes

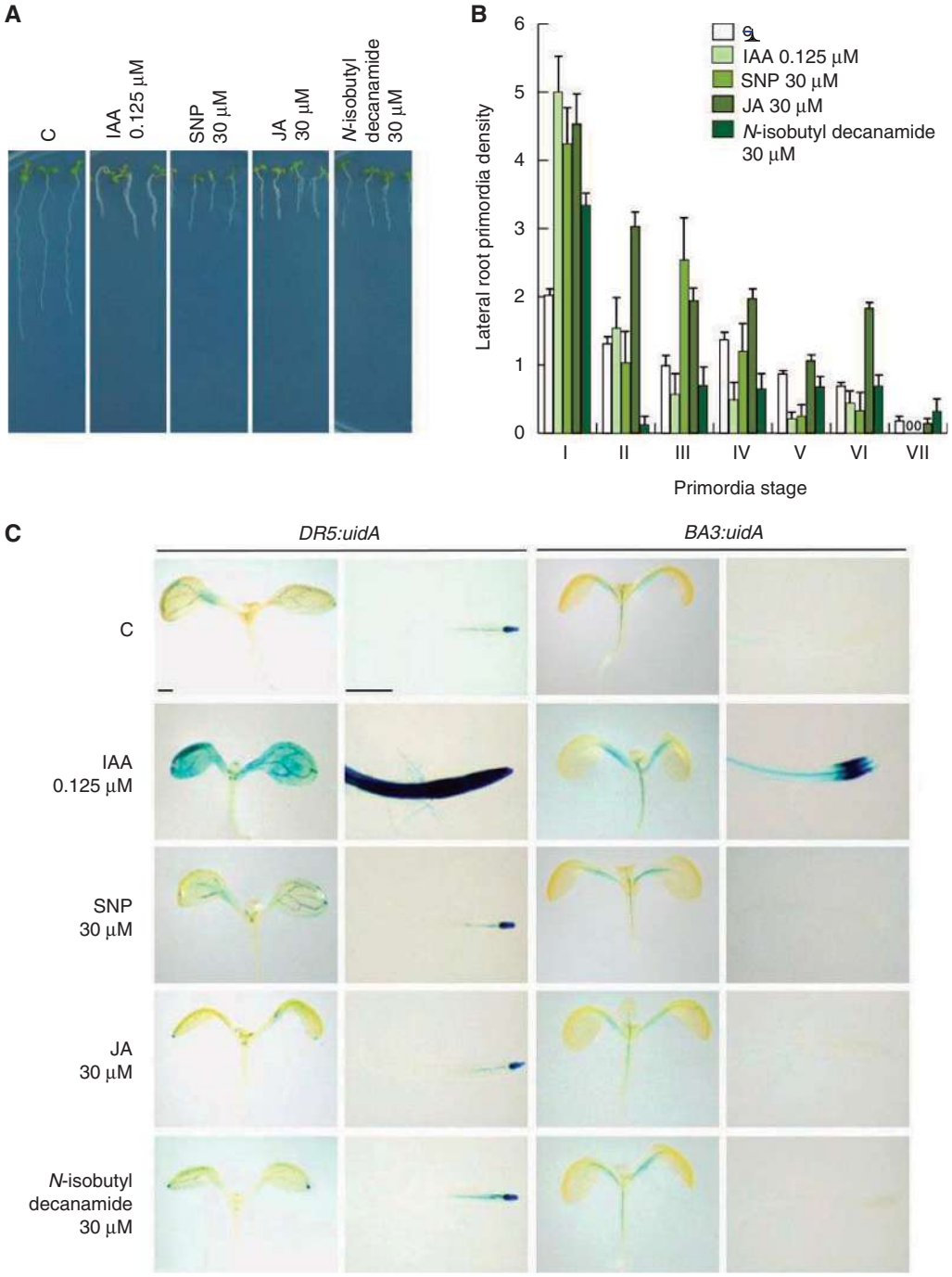

Fig. 10 Comparative effect of IAA, SNP, *N*-isobutyl decanamide and JA on RSA and auxin-inducible gene expression. (A) Seven-day old Col-0 Arabidopsis seedlings grown on agar plates with the indicated concentrations of compounds. (B) Effect of compounds on LRP stages. (C) Twelve hour GUS staining of *DR5:uidA* and *BA3:uidA* Arabidopsis seedlings grown for 6 d on agar plates containing 0.2× MS medium and then transferred for 24 h to liquid 0.2× MS medium supplied with 0.125 µM IAA or 30 µM SNP, JA and *N*-isobutyl decanamide. Values shown in B are the mean ± SD ($n = 15$). Photographs in C are representative individuals of at least 15 stained seedlings. The experiment was repeated twice with similar results.

play a role in pericycle cell activation in response to the alkamide (**Fig. 6B, C**). These results suggest that different NO sources are involved in promoting LR development with potentially redundant functions. It has been recently suggested that the decreased NO production in the *Atnoa1* mutant might result from pleiotropic effects of defective plastids (Flores-Pérez et al. 2008, Gas et al. 2009) rather than by a direct role on NO synthesis. Our results are consistent with this

hypothesis by showing that neither primary root growth nor LR development can reach normal levels after SNP or alkamide induction in *Atnoa1*. It is therefore possible that multiple NO sources are involved in producing NO in response to alkamide stimuli, which would explain the effects of the compounds inducing callus formation on leaves (López-Bucio et al. 2007), an effect reminiscent of altered cytokinin levels, and their auxin-like effects involved in RSA modulation.

Our previous research provided genetic evidence that alkamide and AHL perception are under genetic control in Arabidopsis, which was achieved by the isolation and characterization of the *N*-isobutyl decanamide-resistant mutant *drr1* (Morquecho-Contreras et al. 2010). Under normal growth conditions *drr1* plants show normal primary root growth but reduced LR formation, indicating that small fatty acid amide signaling is important for lateral root development. IAA and 1-naphthaleneacetic acid treatment similarly induced lateral root formation in WT and *drr1* plants, indicating that the mutant is not inherently defective on LRP initiation, but rather shows retardation in the establishment and emergence of LR meristems. Interestingly, our current analysis shows that *drr1* is less sensitive to SNP in terms of primary root growth inhibition and LR formation than WT plants (**Fig. 7**, and **Supplementary Fig. S7**). Moreover, the mutant was unable to activate de novo formation of LRP in response to SNP, indicating that NO is a mediator of alkamides in pericycle cell induction (**Fig. 8**). DAF-2DA fluorescence determination in WT and *drr1* seedlings in response to SNP or *N*-isobutyl decanamide treatments showed strong fluorescence in both WT and *drr1* primary roots (**Fig. 9**). These results indicate that *drr1* is not inherently defective in NO accumulation induced by the alkamide. It is tempting to speculate that *DRR1* plays a signaling role mediating the effects of NO on LR formation.

The effects of *N*-isobutyl decanamide treatment on Arabidopsis RSA is reminiscent of plants treated with auxins. Pagnussat et al. (2002) and Correa-Aragunde et al. (2004) provided evidence of the involvement of NO during auxin-induced lateral and adventitious root formation in plants. However, our previous results showed that auxins might not be involved in the root architectural responses of Arabidopsis to alkamides. This hypothesis was mainly based on two lines of evidence: expression studies of the auxin-inducible marker *DR5:uidA* and the WT induction of lateral and adventitious roots in the auxin-related mutants *axr2-1*, *aux1-7*, *eir1-1* and *axr4-1* when grown in a stimulating concentration of alkamides (Ramírez-Chávez et al. 2004, Campos-Cuevas et al. 2008). Our present results show that: (i) NO inhibits primary root growth and stimulates LR development in Arabidopsis seedlings; (ii) *N*-isobutyl decanamide promotion of LR development can be prevented by scavenging NO; (iii) NO-related mutants *Atnoa1* and *nia1 nia2* are compromised in LR responses to *N*-isobutyl decanamide; (iv) *drr1* mutants are less sensitive to SNP in primary root growth and LR development; and (v) SNP application did not induce *DR5:uidA* and *BA3:uidA* gene expression. Since previous research suggested that

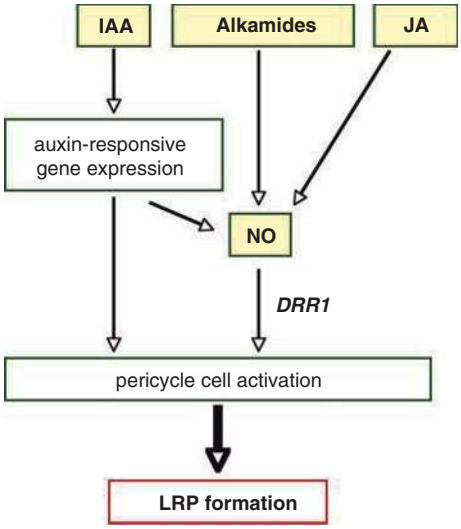

Fig. 11 Role of NO in lateral root formation in responses to different plant signals. IAA activates auxin-responsive gene expression (i.e. *DR5:uidA*, *BA3:uidA*) in pericycle cells to promote LRP initiation. The NO donor SNP, *N*-isobutyl decanamide and JA failed to induce auxin-responsive gene markers (**Fig. 10**); therefore, NO probably acts downstream of auxin signaling to promote lateral root formation. *DRR1* may act as a messenger in alkamide- and JA-induced pericycle cell activation modulating NO root responses.

alkamide and JA signaling interact at the level of the *DRR1* locus to modulate LRP formation in plants (Morquecho-Contreras et al. 2010), all this novel evidence provides compelling support for a role for NO as an integrator of different signaling pathways, which promote LR development, including auxins, alkamides and JA (**Fig. 11**). Our model supports the hypothesis that NO acts downstream of auxin signaling to modulate cell cycle gene expression in pericycle cells either by acting as a second messenger modulating transcription of cell cycle genes or by direct binding to regulatory and/or structural proteins responsible for cell cycle transition, affecting their function by nitration or nitrosylation. We cannot exclude the possibility that NO may also play a role as an integrator of morphogenetic responses to alkamides in green tissues or during plant senescence, since the *drr1* mutants show a dramatically extended life span (Morquecho-Contreras et al. 2010). We anticipate that cell tissue specificity and the amplitude of the NO signal induced by a different plant growth regulator may determine the particular development outcome involved in determination of plant architecture.

Materials and Methods

Plant material and growth conditions

Arabidopsis wild-type and transgenic seedlings expressing the histone *H2B:YFP* (Boisnard-Lorig et al. 2001), both of the ecotype Columbia (Col-0), were used. For *drr1* mutant analysis, Wassilewskija (Ws) ecotype seeds were employed as WT control.

Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min, and stratified for 3 d at 4°C. Later, they were germinated and grown on 0.8% agar plates supplied with 0.2× MS salts. 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), with a photoperiod of 16 h light, 8 h darkness, light intensity of 300 µmol/m^{-2}/s^{-1} and temperature of 22°C.

For transfer experiments, WT Col-0, transgenic *CycB1:uidA* (Colón-Carmona et al. 1999), *nia1 nia2* double mutant (Wilkinson and Crawford 1993) and *Atnoa1* knockout (Guo et al. 2003) seeds were first sterilized and germinated on 0.2× MS medium as described above, and, after 5 d of further growth, seedlings were transferred to control [dimethylsulfoxide (DMSO)- or ethanol-containing medium], or to *N*-isobutyl decanamide-, cPTIO- or SNP-containing 0.2× MS medium for a 4 d additional period prior of analysis.

Chemicals

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 (Ramirez-Chávez et al. 2004). The NO donors SNP and SNAP, and NO scavengers MB and cPTIO were purchased from Sigma. All reagents were dissolved in DMSO and used at the indicated concentrations. In control seedlings, we added the solvents in equal amounts as present in the greatest concentration of compound tested.

Analysis of growth

Arabidopsis root system and primary root meristem integrity were analyzed with a stereoscopic microscope (Leica, MZ6). All LRs emerged from the primary root 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. Primary root 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 primary length values for each analyzed seedling. For transference assays, the LRs were counted from the tip to the marked site of primary root length when the transfer was made. Root differentiation patterns were analyzed by measuring the length from the QC to the first root hair, to protoxylem initiation and to the first LR in semi-permanent preparations of cleared seedlings using a compound microscope (Leica CME) at ×20 or ×40 magnifications. The same preparations were used for LRP analysis. For all presented data, the mean ± SD was calculated from at least 30 seedlings. For all experiments with WT and mutant lines, the overall data were statistically analyzed using the SPSS 10 program. Univariate and multivariate analyses with a Tukey's post-hoc test were used for testing differences in growth and root developmental responses. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Determination of developmental stages of LRP

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

Confocal microscopy

NO detection. NO was monitored by incubating Arabidopsis seedlings with 10 µM of the fluorescent probe DAF-2DA (Kojima et al. 1998) 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 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. Fluorescence signals were quantified by counting pixel number in the green channel by employing ImageJ software.

PI staining and yellow fluorescent protein (YFP) detection. For fluorescent staining with PI, recently collected plants with intact root systems were transferred to a solution of 10 mg ml^{-1} PI for 3 min. Seedlings were rinsed in water and mounted in 50% glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568 nm excitation line and a emission window of 585–610 nm, and *YFP* emission with a 505–550 nm bandpass emission filter (488 nm excitation line), after which the two images were merged to produce the final image.

Histochemical analysis

For histochemical analysis of GUS activity, transgenic *CycB1:uidA DR5:uidA* and *BA3:uidA* Arabidopsis seedlings were immersed in GUS reaction buffer (0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7) and incubated for 14 h at 37°C. The stained seedlings were cleared by the method of Malamy and Benfey (1997). For each treatment at least 10 transgenic plants were analyzed. A representative plant was chosen for each treatment and photographed using the Leica CME compound microscope.

Supplementary data

Supplementary data are available at PCP online.

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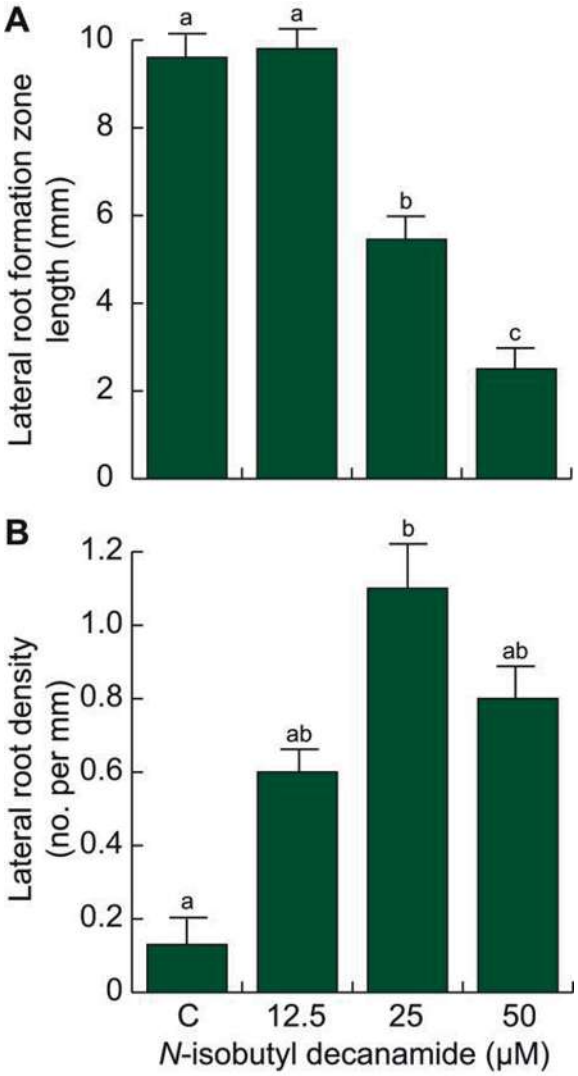
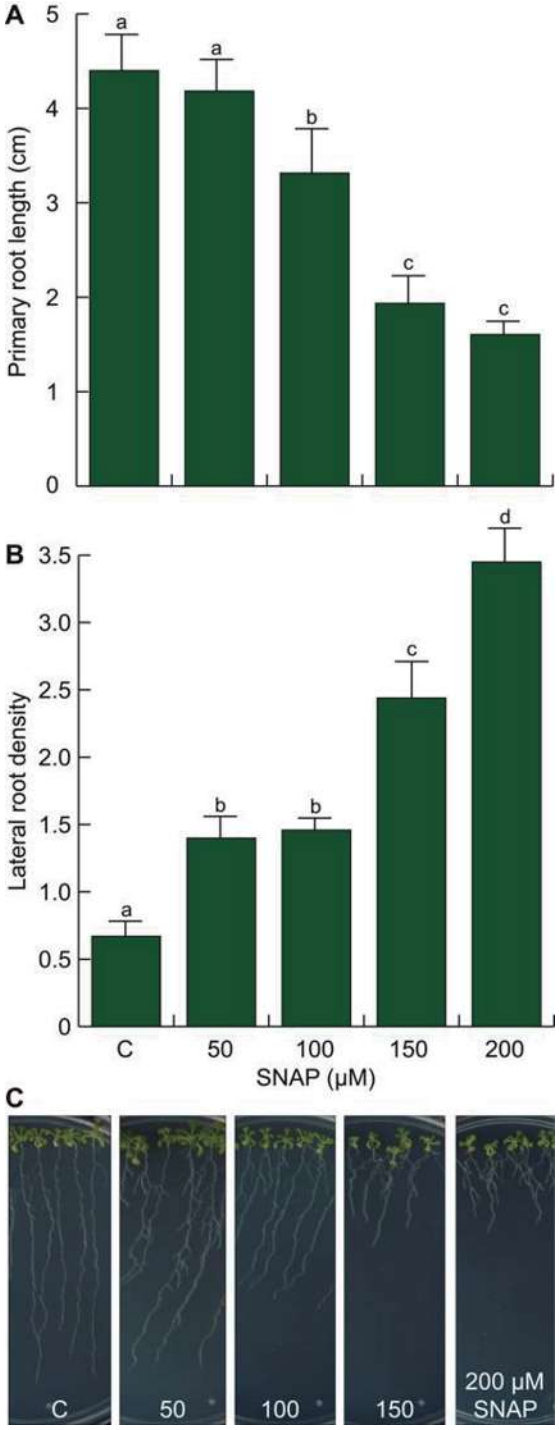
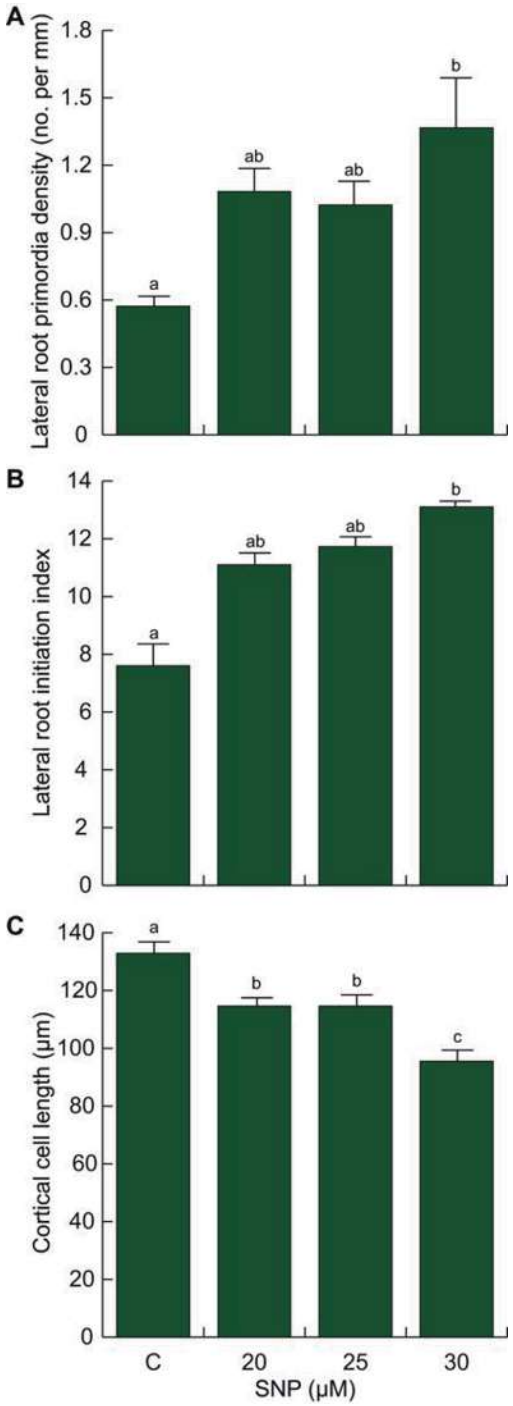


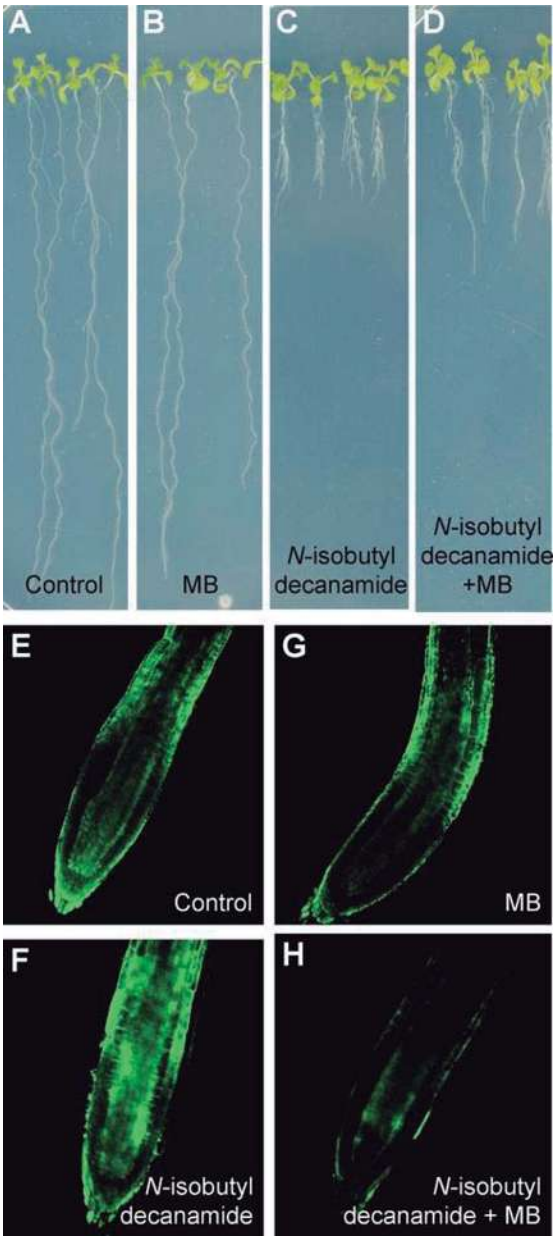
Supplemental Fig. S1. Lateral root emergence induced by *N*-isobutyl decanamide in 7 d-old seedlings. This analysis was performed considering the lateral root formation zone length (A), which comprises the root portion between the root base and the youngest (most-distal) emerged lateral root. Emerged lateral root density was calculated into this zone (B). Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results. Bars represent the mean ± SE



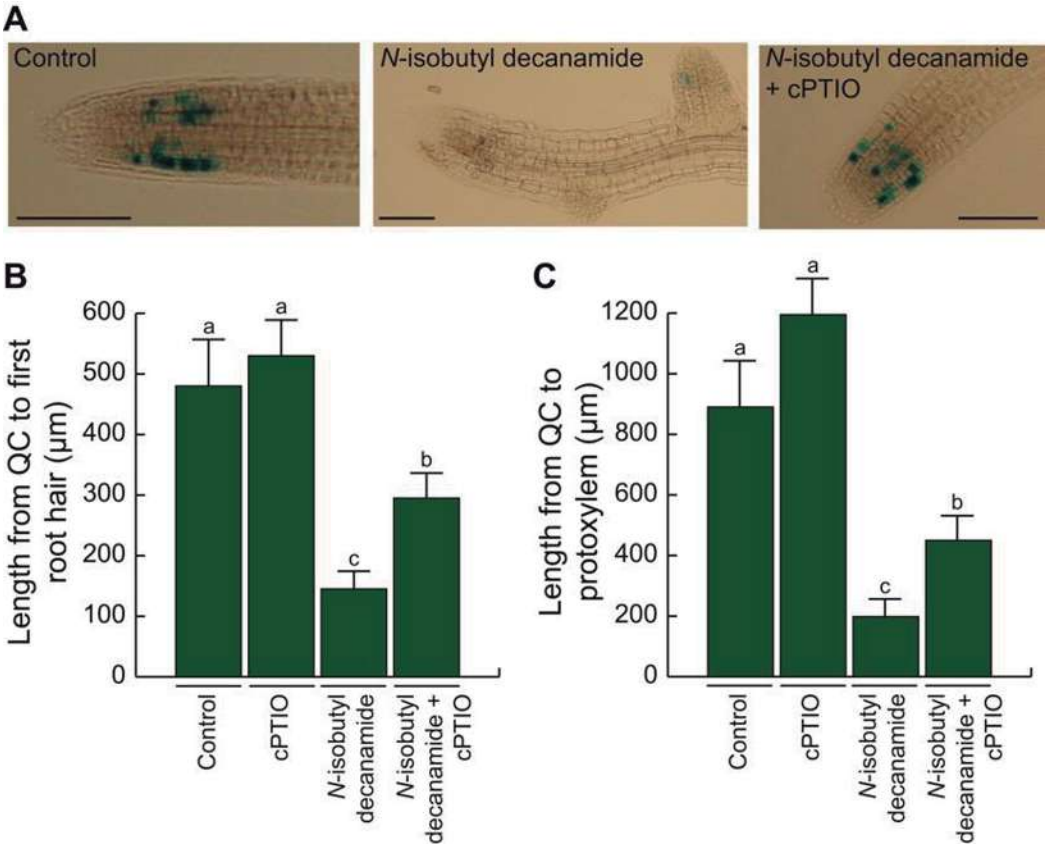
Supplemental Fig. S2 Effects of NO donor SNAP on Arabidopsis root system architecture. WT (Col-0) seedlings were grown for 7-d on agar plates supplied with NO donor SNAP at the indicated concentrations and the length of the primary root (A), lateral root density (B), and whole developmental effects (C) determined for a total of 30 seedlings ± SE. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.



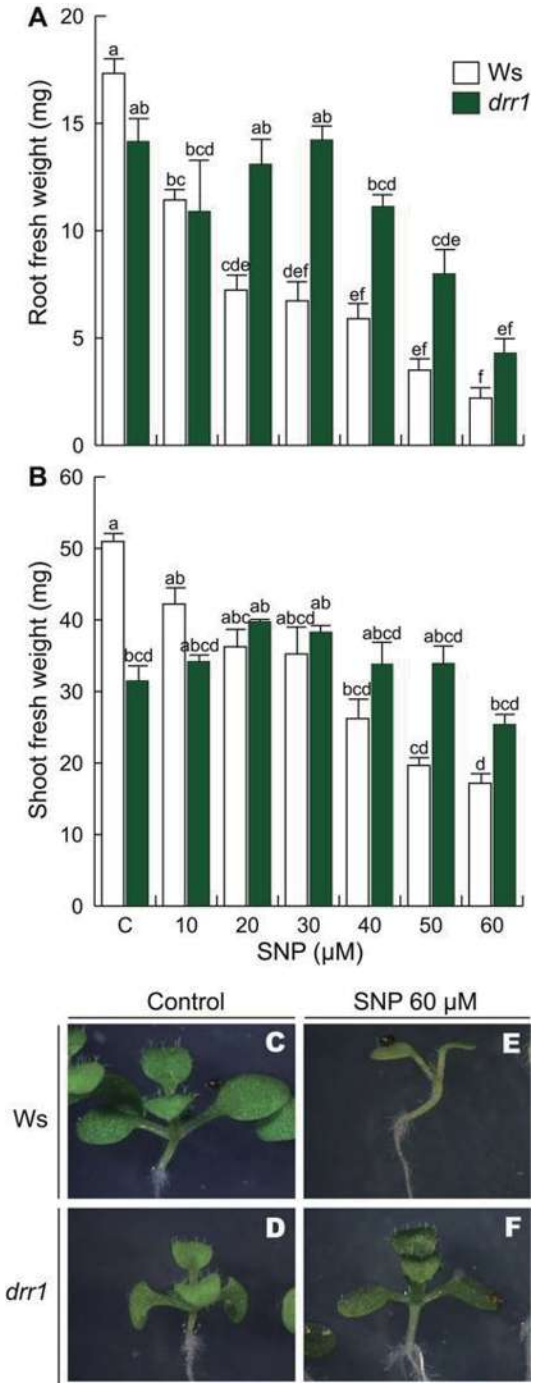
Supplemental Fig. S3 Lateral root initiation induced by NO-donor SNP. (A) Lateral root primordium density, (B) lateral root initiation index, and (C) fully elongated cortical cell length in Col-0 7-d old seedlings. Measurements were done accordingly to Dubrovsky et al. (2009). Different letters indicate statistically significant differences when analyzed by one-way ANOVA and a multiple comparison using Tukey's test at $P < 0.05$ from at least 10 plants per treatment.



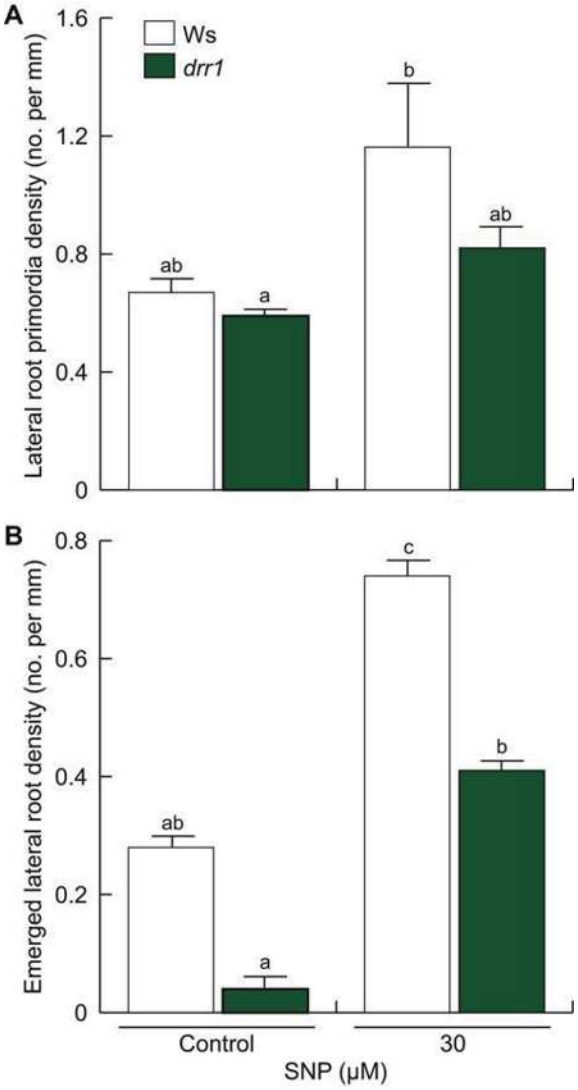
Supplemental Fig. S4 Effect of nitric oxide scavenger methylene blue on root architectural responses to *N*-isobutyl decanamide and NO accumulation. Representative photographs of *Arabidopsis* WT (Col-0) seedlings grown with or without *N*-isobutyl decanamide in the presence or absence of methylene blue (MB). (A) Solvent grown seedlings. (B) Seedlings treated with 1 µM MB, 25 µM *N*-isobutyl decanamide (C), or 25 µM *N*-isobutyl decanamide plus 1 µM MB (D). NO detection on primary root meristems was revealed by staining with DAF-2DA in control (E), and 25 µM decanamide (F), 1µM MB (G) or 25 µM decanamide plus 1 µM MB (H) treated seedlings. A total of 10 seedlings per treatment were analysed by confocal microscopy and representative photographs were taken. The experiment was repeated twice with similar results.



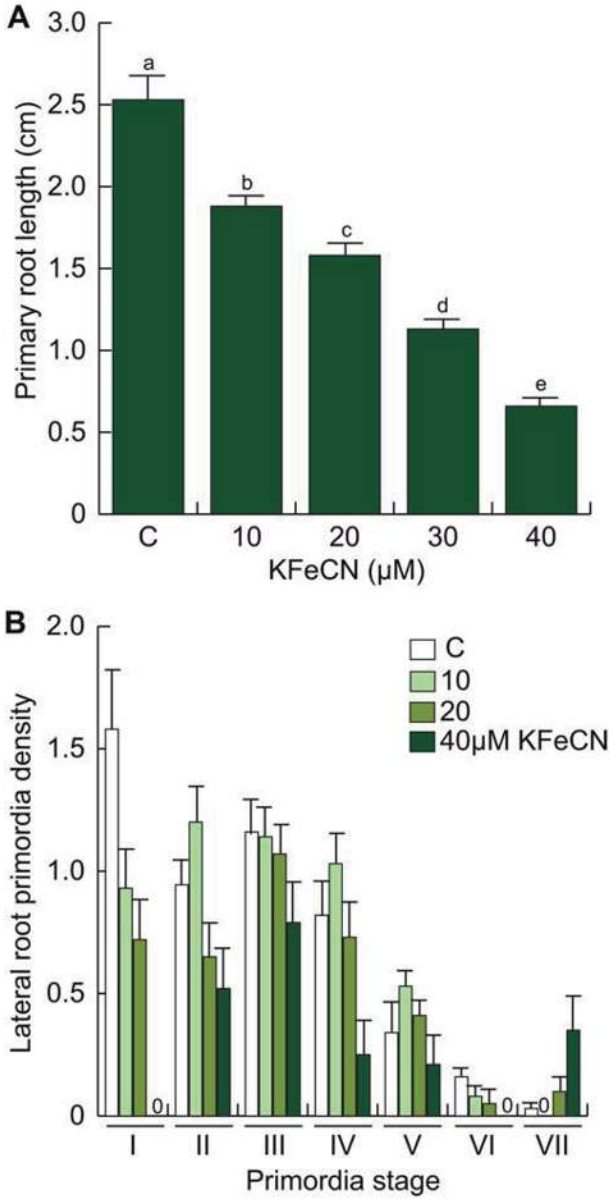
Supplemental Fig. S5 Effects of NO depletion on cell division and differentiation in response to *N*-isobutyl decanamide. Arabidopsis *CycB1:uidA* seedlings were grown for 5 d on MS 0.2X medium and then transferred to plates supplied with 24 μM *N*-isobutyl decanamide or *N*-isobutyl decanamide plus cPTIO for an additional 4 d period. At this stage, seedlings were stained for GUS activity and cleared to determine mitotic activity. (A) *CycB1:uidA* expression in the primary root meristem. (B) Length from quiescent centre (QC) to first root hair, and (C) length from quiescent centre to the first protoxylem cell. Values shown represent the mean of 30 seedlings ± SD. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.



Supplemental Fig. S6 Resistance to growth inhibition induced by SNP in *drr1* mutant seedlings. WT and *drr1* seedlings were grown side by side on 0.2X MS agar medium supplied with the solvent or indicated SNP concentrations. (A, B) root and shoot biomass production from 60 seedlings at 10 d after germination. (C-F) visual effects of SNP on shoot growth in WT and *drr1* plants. Different letters (A and B) are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results. Bars represent the mean ± SD.



Supplemental Fig. S7 Induction of lateral root initiation events by SNP in WT (Ws) and *drr1* seedlings. Lateral root primordial density was estimated in the zone between most-distal LRP and the youngest (most-distal) emerged lateral root (A). Lateral root density was determined by analyzing the root portion from the root base to the youngest (most-distal) emerged lateral root. Letters indicates statistical differences between treatments from at least 15 plants. Bars represent the means ± SE.



Supplemental Fig. S8 Effects of KFeCN on Arabidopsis root system architecture. WT (Col-0) seedlings were grown for 7-d on agar plates supplied with KFeCN at the indicated concentrations and the length of the primary root (A), and lateral root primordia density (B) were determined for a total of 15 seedlings ± SD. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

VIII. DISCUSIÓN GENERAL

Los lípidos constituyen una importante reserva de biomoléculas, que además de su valor energético y estructural como componentes primarios de las membranas celulares, están implicados en la regulación de diversos procesos fisiológicos vitales en diferentes grupos de organismos. En los mamíferos, los procesos que regulan incluyen la proliferación celular, muerte celular programada, señalización intercelular y respuestas inmunológicas (Wymann y Schneiter, 2008). Un ejemplo bien estudiado es el de los esfingolípidos (ceramida y esfingosina), que poseen propiedades pro-apoptóticas y antimitóticas (Taha et al, 2006). Sus homólogos moleculares de plantas, las ceramidas, esfingosinas y el ácido fosfatídico, participan en la regulación del crecimiento y desarrollo vegetal, así como en respuestas de estrés biótico y abiótico. Su biosíntesis está regulada por sistemas enzimáticos finamente regulados, que constan de fosfolipasas, cinasas y fosfatasas, sin embargo, a diferencia de los grupos animales, en plantas es escaso el conocimiento acerca de los blancos moleculares y los mecanismos de acción de estas moléculas señal (Wang, 2004).

Las alcamidas de origen vegetal poseen similitud estructural a los esfingolípidos producidos por una gran variedad de organismos incluyendo líquenes, hongos y plantas vasculares. Estos compuestos son acumulados en grandes cantidades por una gran variedad de plantas con uso medicinal tradicional (López-Bucio et al, 2006). Por ejemplo, las alcamidas producidas por las especies *Echinacea purpurea*, *Echinacea angustifolia* y *Heliopsis longipes* se han identificado como los principales compuestos activos en sus extractos lipídicos. Estas alcamidas sintetizadas por plantas del género *Echinacea* activan cascadas de señalización en células animales a través de su unión con receptores de tipo proteínas G (CB1 y CB2). Los receptores CB1 y CB2 regulan el sistema de señalización dependiente de endocanabinoides (anandamida o sus análogos vegetales, principalmente el ácido araquidónico). La activación de los receptores a endocanabinoides en animales regula diversos procesos del sistema nervioso central y del sistema inmune, e involucran la producción de óxido nítrico y la activación de proteínas MAP cinasas, señales que, entre otras funciones, inducen la síntesis de más moléculas lipídicas con propiedades señalizadoras, como esteroides y prostaglandinas (Raduner et al, 2006). Sin embargo, se desconoce si las vías de señalización endógenas

reguladas por las alcamidas de origen vegetal involucran también al óxido nítrico y a las cascadas de MAP cinasas.

Con la finalidad de caracterizar y obtener un panorama general de la regulación de genes y rutas dependientes de otros reguladores del desarrollo en respuesta a la *N*-isobutil decanamida, se realizó un análisis del transcriptoma en plántulas de Arabidopsis a diferentes tiempos de exposición (1, 3, 7 y 14 días de tratamiento), utilizando hibridaciones del RNA vegetal con microarreglos de oligonucleótidos largos de DNA (Capítulo I). De los ≈25000 genes de Arabidopsis representados en el microarreglo, 1281 tuvieron niveles de expresión diferencial en respuesta al tratamiento con la *N*-isobutil decanamida, en cualquiera de los tiempos evaluados. 727 genes fueron inducidos (al menos 2 veces con respecto al control) y 554 genes reprimidos (0.5 o menos veces respecto al control). De los 727 genes inducidos, un 22 % correspondió a genes relacionados al desarrollo, señalización y crecimiento. Sorprendentemente, cerca del 25 % de los genes inducidos fueron genes de defensa y estrés, sugiriendo una posible dualidad en los procesos de regulación de las alcamidas: 1) reajustes en los programas del desarrollo (identidad y proliferación celular), y 2) respuestas a condiciones de estrés biótico. La lista completa de los genes regulados por la *N*-isobutil decanamida puede consultarse en la base de datos pública del *Gene Expression Omnibus* (http://www.ncbi.nlm.nih.gov/geo/) con la clave **GSE12107**. La lista incluye a todos los genes que codifican a las enzimas reguladoras de la biosíntesis del ácido jasmónico (Figura 2 Introducción), los genes de las lipoxigenasas LOX2 y LOX3, de la aleno óxido sintasa (AOS1), de las aleno óxido ciclasas (AOC1 y AOC2), y la óxido reductasa OPR3 (Cuadro 1, Capítulo I pag. 43).

Para validar funcionalmente los estudios de expresión global de genes y determinar a nivel metabólico si la inducción de los genes de síntesis del ácido jasmónico en respuesta a la *N*-isobutil decanamida repercute en la acumulación del compuesto, los niveles de ácido jasmónico endógeno de las plantas tratadas se cuantificaron por cromatografía de gases acoplada a espectometría de masas (GC-MS). Los resultados mostraron que a los 7 días después de la inducción con la *N*-isobutil decanamida, la concentración de ácido jasmónico se incrementó hasta dos veces, en comparación con las plantas control.

Los resultados antes descritos sugerían una posible participación de las alcamidas en la regulación de respuestas a patógenos. Esta hipótesis fue probada realizando ensayos *in vitro* de patogeneicidad con hojas de plantas de Arabidopsis transferidas a medios con, o sin *N*-isobutil decanamida a una concentración de 60 μM e inoculadas con esporas de los hongos necrotróficos *Alternaria brassicicola* y *Botrytis cinerea*. A los 3 días después de la infección (ddi) un 70 % de las hojas tratadas con *N*-isobutil decanamida fueron resistentes a los daños causados por *A. brassicicola* y 90 % a los de *B. cinerea*. Aún a los 5 ddi, el tamaño de las lesiones en hojas tratadas fue de 4 y 5 veces menor en comparación a los controles con los patógenos. Tinciones de las hojas con azul de tripano revelaron una escasa proliferación de los hongos en hojas tratadas con la alcamida en comparación con sus controles, indicando que las alcamidas activan en las plantas respuestas de defensa útiles para contrarrestar el ataque por hongos patógenos. Estos resultados respaldan la hipótesis propuesta en el presente trabajo y son consecuentes con las propiedades inmunoestimuladoras reportadas en plantas de jitomate inoculadas con bacterias productoras de *N*-acil homoserina lactonas (AHLs; Schuhegger et al, 2006), posibles análogos moleculares de las alcamidas vegetales. Las AHLs regulan, entre otros procesos, la inducción de sistemas de virulencia, patogénicas o simbióticas en las comunidades bacterianas, en función de su densidad poblacional (Alfano y Collmer, 1996). Debido a la similitud estructural entre las AHLs y las alcamidas que hiperacumulan algunas plantas en sus raíces, se abre la posibilidad de estudiar los efectos de las alcamidas en procesos de proliferación bacteriana, así como su posible participación en interacciones simbióticas y patogénicas entre plantas y microorganismos.

Además de los genes de la ruta de síntesis del ácido jasmónico, la *N*-isobutil decanamida indujo la expresión de una gran cantidad de reguladores de las rutas de señalización y respuesta al ácido jasmónico (ver Figura 2 Introducción). Algunos de los componentes de esta ruta de señalización se han identificado mediante el uso de escrutinios genéticos en Arabidopsis, buscando plantas que muestran una respuesta alterada al ácido jasmónico, a sus derivados o a algún análogo, como la toxina bacteriana coronatina (Turner et al, 2002). Entre las líneas mutantes insensibles al ácido jasmónico identificadas en Arabidopsis destaca *coi1* (*coronatine insensitive1*). Esta mutante muestra una pérdida completa de sensibilidad a la hormona en ensayos de inhibición del crecimiento de la raíz y acumulación de antocianinas

mediados por jasmonatos (Feys et al, 1994). El gen COI1 codifica una proteína con un dominio F-box, y es considerada como el receptor intracelular de jasmonatos (Yan et al, 2009). Las proteínas con dominio F-box participan en la formación de complejos ubiquitin-ligasa E3 de tipo SCF con proteínas específicas, cuya degradación es requerida como un mecanismo de regulación en la señalización dependiente del ácido jasmónico (Devoto et al, 2002). Los genes relacionados a estos mecanismos y que están inducidos en respuesta a la *N*-isobutil decanamida son COI1, cinco proteínas tipo F-Box, y tres miembros del complejo SCF (SKP1, 11, SKP1, 12 y RUB-conjugating1), lo que sugiere que las rutas de señalización dependientes del ácido jasmónico son requeridas en las respuestas de las células vegetales a las alcamidas, y quizás a las AHLs bacterianas. La dependencia de esta ruta en las respuestas de defensa inducidas por la *N*-isobutil decanamida se confirmó mediante el uso de dos mutantes de Arabidopsis afectadas en la percepción y respuesta al ácido jasmónico (*mpk6*- y *jar1*; Takahashi et al, 2007; Staswick et al, 1992). En conjunto, nuestros resultados indican que las alcamidas inducen la inmunidad vegetal a través de la regulación de los niveles de ácido jasmónico y de sus rutas de señalización (para una discusión más completa, revisar Capítulo I).

Por otra parte, el óxido nítrico es un metabolito secundario que es producido en respuesta al ácido jasmónico y a diversas señales de estrés (Asai y Yoshioka, 2009). Esta producción de óxido nítrico induce la expresión de los genes codificantes para las enzimas AOS, LOX2, OPR3, retroalimentando la ruta de señalización de los jasmonatos (Huang et al, 2004).

Para complementar los estudios de expresión génica en respuesta a la *N*-isobutil decanamida y profundizar en sus vías de señalización, se analizó la participación del óxido nítrico en diferentes procesos de morfogénesis regulados por *N*-isobutil decanamida. Estos procesos incluyeron la formación y desarrollo de raíces adventicias en explantes de *A. thaliana* (Campos-Cuevas et al, 2008. Anexo 2), la regulación de la actividad meristemática, la activación mitótica del periciclo en la formación de raíces laterales, y la estimulación de eventos de diferenciación celular (Capítulo II).

Estos resultados en conjunto, permitieron entender de qué manera participa el óxido nítrico en la regulación del desarrollo de la raíz de Arabidopsis y su inducción en los procesos regulados por la *N*-isobutil decanamida. La aplicación exógena de donadores químicos de óxido nítrico alteró la actividad meristemática de la raíz primaria de Arabidopsis y estimuló el desarrollo de raíces laterales, de manera similar que las alcamidas, sugiriendo que el óxido nítrico puede participar como un intermediario en las respuestas a las alcamidas, de hecho, los efectos en el desarrollo de la raíz inducidos por la *N*-isobutil decanamida son atenuados cuando se elimina al óxido nítrico endógeno mediante la adición de inhibidores o atrapadores químicos de agentes oxidantes. Las mutantes afectadas en la señalización (*Atnoa1*) y en la síntesis (*nia1, nia2*) de óxido nítrico están también alteradas en sus respuestas a la *N*-isobutil decanamida, demostrando a nivel genético la participación de este mensajero en la señalización dependiente de las alcamidas.

Finalmente, los resultados descritos se validaron genéticamente mediante el análisis en las respuestas al ácido jasmónico y al óxido nítrico en una mutante insensible a las alcamidas vegetales recientemente reportada por nuestro grupo de trabajo (*drr1*, Anexo 3). Esta mutante está afectada en algún elemento necesario para el desarrollo normal de raíces laterales en Arabidopsis, y en concordancia con nuestros hallazgos, es incapaz de incrementar el desarrollo de órganos laterales en la raíz en respuesta al ácido jasmónico y al óxido nítrico, pero responde de manera normal a otros reguladores del crecimiento como las auxinas. Lo que sitúa al gen DRR1, en un modelo hipotético, como un elemento que regula una vía de señalización en respuesta al óxido nítrico y al ácido jasmónico (Figura 6).

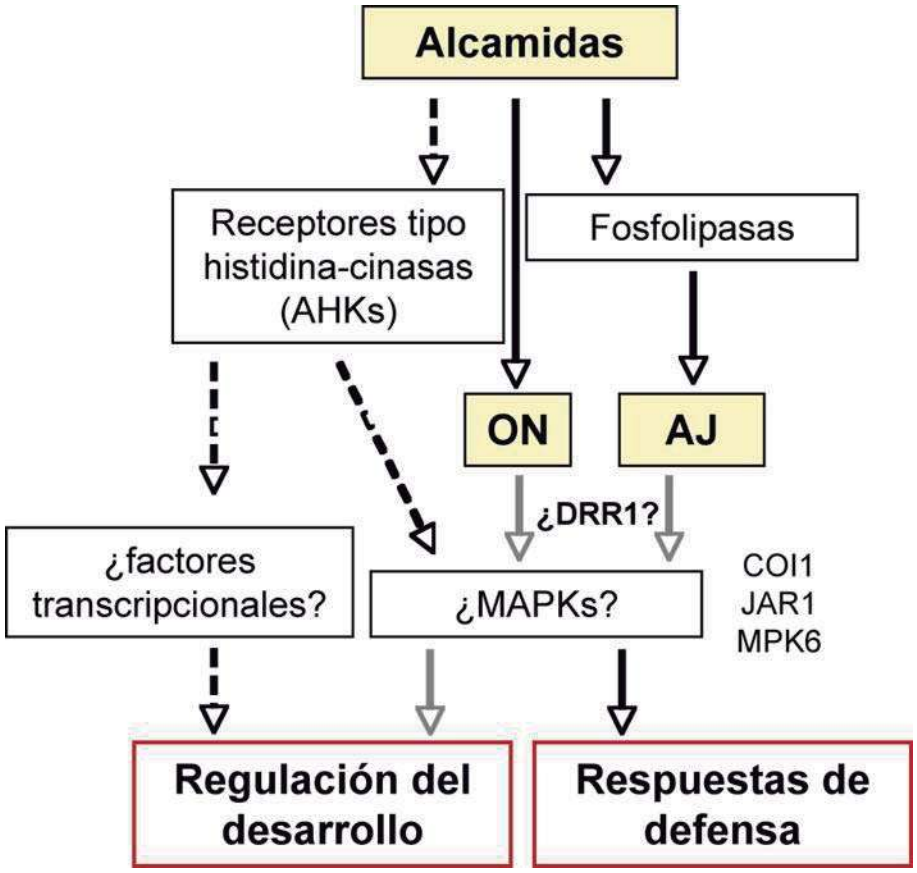


Figura 6. Modelo propuesto de las vías de señalización reguladas por las alcamidas. La percepción de la alcamida requiere la presencia de receptores tipo histidina-cinasas (AHKs. Ver Anexo 1) que han sido reportados como receptores de citocininas y regulan la actividad de factores transcripcionales, en un sistema similar al de dos componentes en bacterias productoras de AHLs. La aplicación de *N*-isobutil decanamida induce la expresión de fosfolipasas (At2g26560, At3g03530, At1g52700; ver la lista completa del microarreglo GEO GSE12107) y la síntesis tanto de óxido nítrico (ON) como de ácido jasmónico (AJ). Las cascadas de fosforilación dependientes de MAP cinasas (MAPKs) pueden actuar como reguladores transcripcionales de genes que participan en procesos de desarrollo y en respuestas de defensa. Las flechas continuas en color negro indican resultados descritos en este trabajo, las flechas negras discontinuas corresponden a resultados reportados en otros trabajos, las flechas grises indican posibles interacciones.

IX. CONCLUSIONES Y PERSPECTIVAS

1. Las alcamidas vegetales, al igual que sus homólogos moleculares de origen bacteriano, las *N*-acil homoserina lactonas, regulan procesos del desarrollo y respuestas de defensa en las plantas.

2. La *N*-isobutil decanamida induce la expresión de genes reguladores de respuestas de defensa. El máximo de expresión (d-7) coincide con la acumulación de compuestos involucrados en dichos procesos, óxido nítrico, peróxido de hidrógeno y ácido jasmónico.

3. El encendido de estas rutas de señalización le confieren a Arabidopsis la capacidad de resistir al ataque de hongos patógenos.

4. Las respuestas de defensa reguladas por esta alcamida en procesos de patogénesis requieren las rutas de señalización dependientes del ácido jasmónico.

5. Los efectos morfogénicos de la alcamida *N*-isobutil decanamida son dependientes del aumento en los niveles endógenos de óxido nítrico en Arabidopsis.

Como perspectiva principal surgida de este trabajo, está el determinar la identidad del gen codificante para DRR1 y caracterizar sus respuestas ante patógenos.

X. REFERENCIAS COMPLEMENTARIAS

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XI. ANEXOS

En esta sección se presentan cuatro publicaciones que contienen resultados generados durante la realización del presente trabajo y no están descritos en los Capítulos I y II, a continuación se enlistan las citas correspondientes.

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Cytokinin Receptors Are Involved in Alkamide Regulation of Root and Shoot Development in Arabidopsis[1][C][OA]

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Alkamides and *N*-acilethanolamides are a class of lipid compounds related to animal endocannabinoids of wide distribution in plants. We investigated the structural features required for alkamides to regulate plant development by comparing the root responses of Arabidopsis (*Arabidopsis thaliana*) seedlings to a range of natural and synthetic compounds. The length of the acyl chain and the amide moiety were found to play a crucial role in their biological activity. From the different compounds tested, *N*-isobutyl decanamide, a small saturated alkamide, was found to be the most active in regulating primary root growth and lateral root formation. Proliferative-promoting activity of alkamide treatment was evidenced by formation of callus-like structures in primary roots, ectopic blades along petioles of rosette leaves, and disorganized tumorous tissue originating from the leaf lamina. Ectopic organ formation by *N*-isobutyl decanamide treatment was related to altered expression of the cell division marker *CycB1*:*uidA* and an enhanced expression of the cytokinin-inducible marker *ARR5*:*uidA* both in roots and in shoots. The involvement of cytokinins in mediating the observed activity of alkamides was tested using Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors *CRE1*, *AHK2*, and *AHK3*. The triple cytokinin receptor mutant was insensitive to *N*-isobutyl decanamide treatment, showing absence of callus-like structures in roots, the lack of lateral root proliferation, and absence of ectopic outgrowths in leaves under elevated levels of this alkamide. Taken together our results suggest that alkamides and *N*-acylethanolamides may belong to a class of endogenous signaling compounds that interact with a cytokinin-signaling pathway to control meristematic activity and differentiation processes during plant development.

Most organisms are known to contain in their inner and outer membranes amphipatic lipids based on one or two amino acids linked to a fatty acid through an amide bond. Thus, they have structural similarity to ceramides. *N*-acylethanolamides (NAEs) represent compounds with aminoalcohol linked as an amide to the fatty acid. They were reported as constituents of soy (*Glycine max*) lecithin and peanut (*Arachis hypogaea*) meal and as antiinflammatory agents, but now they have been identified in a variety of seeds and plant tissues (Chapman et al., 1999; Chapman, 2004). Anandamide, the main ligand of the cannabinoid receptor in mammals regulates many cellular and developmental responses including the modulation of neurotransmission in the central nervous system, synchronization of embryo development, and vasodilation (for review, see Pertwee, 2006). It appears that NAE signaling has important biological functions in plants, such as germination (Wang et al., 2006; Teaster et al., 2007), defense responses (Chapman et al., 1998), and root development (Blancaflor et al., 2003), but research is still at a relatively early stage.

Alkamides comprise over 200 related compounds that have been found in as many as 10 plant families: Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, and Solanaceae. Species containing high levels of alkamides are found in the Asteraceae, Piperaceae, and Rutaceae (Christensen and Lam, 1991; Kashiwada et al., 1997; Parmar et al., 1997). Two reports indicated that alkamides, a class of plant-produced

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amino compound-containing lipids, structurally related to NAEs, are also able to play a signaling role in plants. Amidenin, a nonsubstituted alkamide isolated from the actinomycete fungus *Amycolatopsis* sp., was shown to stimulate growth of rice (*Oryza sativa*) seedlings (Kanbe et al., 1993) and *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide (affinin), an alkamide present in the roots of *Heliopsis longipes* was reported to alter the growth and development of the Arabidopsis (*Arabidopsis thaliana*) root system (Ramírez-Chávez et al., 2004). Developmental alterations induced by affinin included primary root growth inhibition, enhanced formation and elongation of lateral roots, and increased root hair growth. Two affinin-derived alkamides (*N*-isobutyl-2*E*-decenamide and *N*-isobutyl-decanamide) were found to be even more active than affinin in stimulating root hair growth (Ramírez-Chávez et al., 2004). The effects of alkamides on Arabidopsis were found to be independent of auxin signaling as revealed by normal primary root growth response of auxin-resistant mutants to alkamides and because of the inability of alkamides to affect the expression of auxin-inducible gene markers. This information led to the proposal that NAEs and alkamides might belong to a class of endogenous lipid signals that regulate plant development (López-Bucio et al., 2006; Morquecho-Contreras and López-Bucio, 2007). However, it is as yet unknown whether altering the synthesis or signaling pathways of other plant hormones such as cytokinins could mediate the biological activity of alkamides.

In this study, as a first step in exploring the structure-activity relationships of NAEs and alkamides, we quantified the root growth response of Arabidopsis seedlings to natural and synthetic compounds. From a group of similar chain length NAEs and alkamides, we identified *N*-isobutyl decanamide, an alkamide that is naturally produced in *Acmella radicans* (Rios-Chávez et al., 2003) and *Cissampelos graberrima* (Laurerio-Rosario et al., 1996), as the most active compound in inhibiting primary root growth and stimulating lateral root formation. We show that this compound regulates many aspects of plant development by altering cell division and differentiation processes. In leaves, exogenous application of *N*-isobutyl decanamide was found to alter cell fate determination, leading to the production of ectopic blades along leaf petioles and vigorous outgrowths in the leaf lamina. In the root, these effects were accompanied with developmental transitions from lateral roots to callus-like structures. The involvement of cytokinins in mediating the observed activity of alkamides was tested using a suite of cytokinin-signaling Arabidopsis mutants lacking one, two, or three of the genes encoding the putative cytokinin receptors *CYTOKININ RESPONSE1* (*CRE1*), *ARABIDOPSIS HISTIDINE KINASE2* (*AHK2*), and *AHK3*. Our results suggest that alkamides may function as partial mimics of some endogenous lipid mediators whose action requires a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes.

RESULTS

Structure-Activity Relationships for Small Chain NAEs and Alkamides with Effects in Root Development

The mechanism by which NAEs and alkamides exert their developmental effects in plants is unknown but the finding that small chain NAEs and alkamides were active in inhibiting primary root growth and promoting lateral root development suggests a common signaling mechanism for these two classes of compounds (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). To further investigate the structural features required for an alkamide to regulate root development in Arabidopsis seedlings, three compounds of similar acyl chain length but different saturation degree were evaluated at concentrations ranging from 3.5 to 112 μM, namely affinin, *N*-isobutyl decanamide, and NAE10:0 (Fig. 1). The three different compounds were found to inhibit primary root growth (Fig. 2A) and to promote lateral root formation (Fig. 2, B and C). Higher concentrations of NAE10:0 were required to inhibit lateral root formation and of affinin to promote lateral root formation. Interestingly, *N*-isobutyl decanamide was found to be the most active compound, showing a 50% primary root growth inhibition and a 4-fold increase in the number of lateral roots at 14 μM. We also found a dramatic increase in lateral root density (lateral root number per centimeter of primary root) from 7 to 56 μM of this compound.

To determine whether the amino residue and the length of the acyl chain are important for the observed *N*-isobutyl decanamide activity, we compared the effects of low micromolar concentrations of *N*-isobutyl decanamide on primary root growth and lateral root

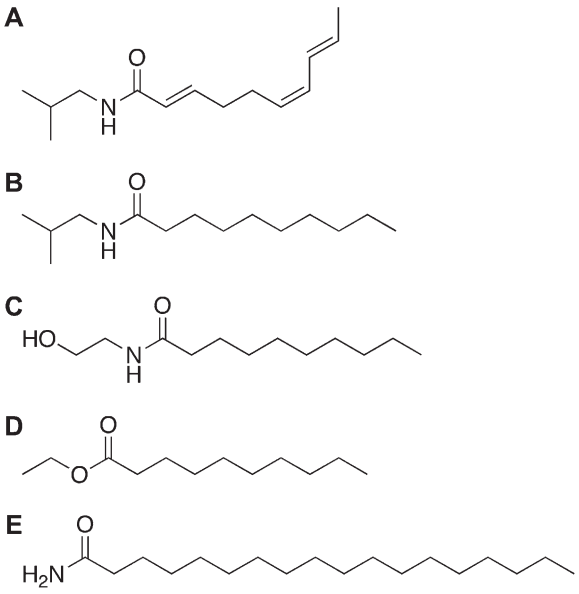

Figure 1. Structure of NAEs and alkamides used in this study. A, Affinin. B, *N*-isobutyl decanamide. C, NAE10:0. D, Decanoic acid ethyl ester. E, Octadecanamide.

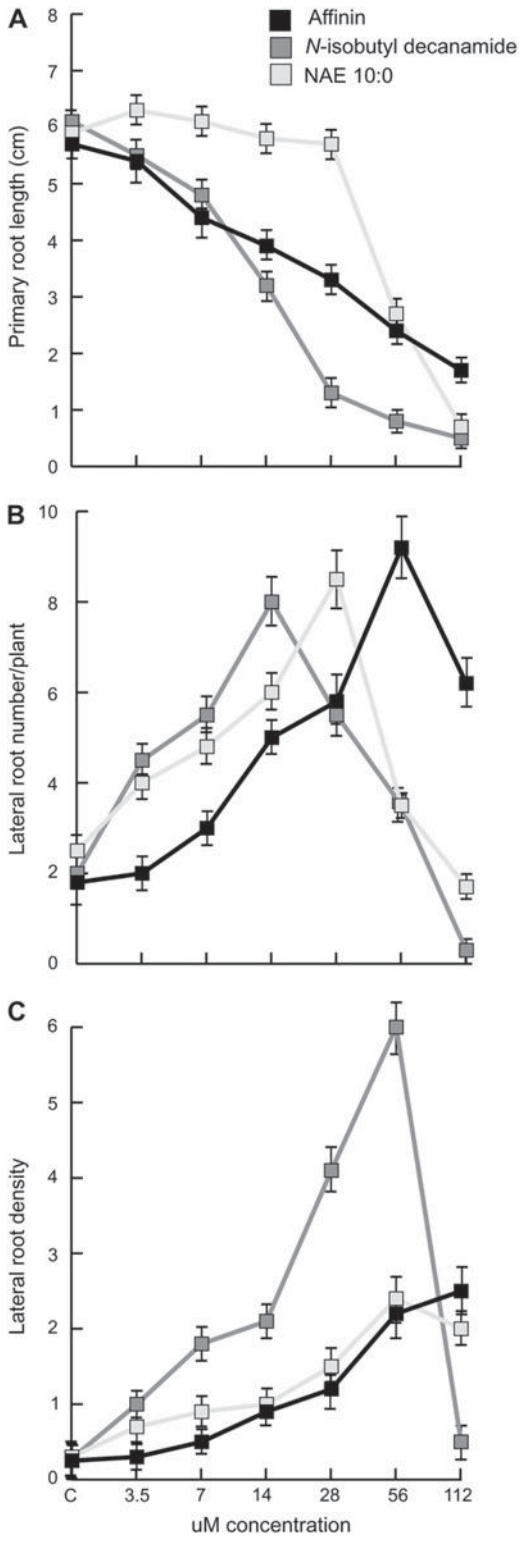

Figure 2. Structure activity for different NAE/alkamides with similar chain length. Arabidopsis plants were grown for 12 d on agar plates supplied with the different compounds at the indicated concentrations and the length of the primary root (A), lateral root number per plant (B), and lateral root density (lateral roots/cm; C) determined for a total of 30 seedlings.

formation with those of octadecanamide, a long chain amide and decanoic acid ethyl ester, a 10 carbon lipid compound lacking the amide moiety (for chemical structures see Fig. 1). Neither octadecanamide nor decanoic acid ethyl ester was found to inhibit primary root growth or to promote lateral root formation as shown for *N*-isobutyl decanamide (Fig. 3, A and B). These results indicate that both the amino residue and the length of the acyl chain appear to be important for alkamide activity to regulate root system architecture in Arabidopsis seedlings.

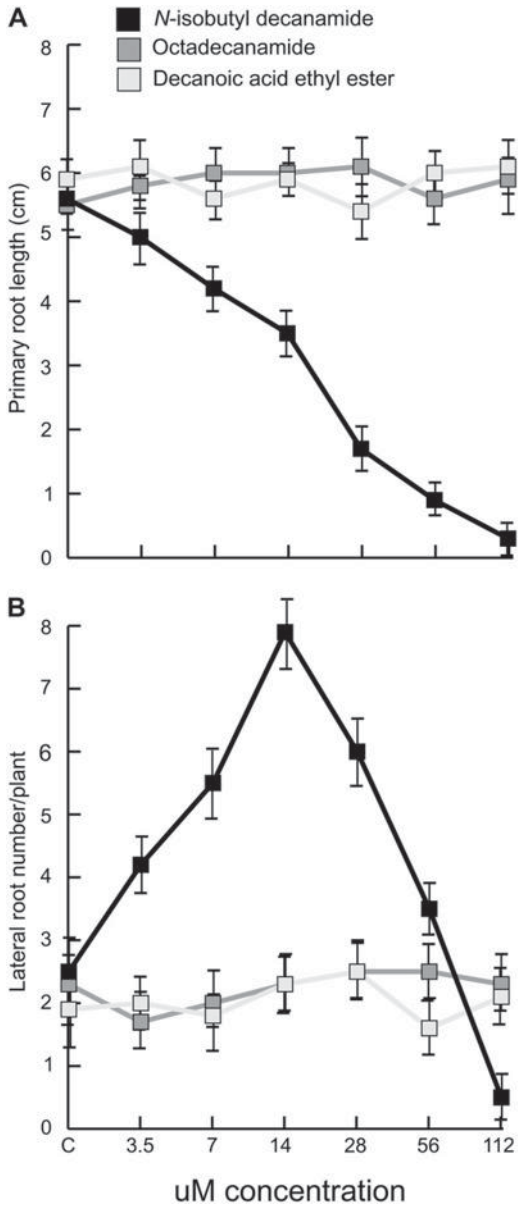

Figure 3. Comparative effects of *N*-isobutyl decanamide, decanoic acid ethyl ester, and octadecanamide in primary and lateral root development. Arabidopsis plants were grown for 12 d on agar plates supplied with the different compounds at the indicated concentrations and the length of the primary root (A) and lateral root number per plant (B) determined for a total of 30 seedlings.

Effect of *N*-Isobutyl Decanamide on Lateral Root Development

Previous work showed that affinin, the most abundant alkamide present in the roots of *H. longipes* regulates several traits during root system development in Arabidopsis and that *N*-lauroylethanolamine was able to regulate lateral root growth depending on its concentration in the medium (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). To investigate the effects of *N*-isobutyl decanamide on lateral root development, Arabidopsis seedlings were treated for 14 d with different concentrations of this molecule and lateral root length and morphology examined. *N*-isobutyl decanamide concentrations from 3.5 to 28 μM showed a dose-dependent lateral root growth promotion (Fig. 4, A–E). In contrast, 40% to 80% reduction in lateral root elongation was observed at higher concentrations of *N*-isobutyl decanamide (56–112 μM, see Fig. 4A). Interestingly, *N*-isobutyl decanamide at 112 μM produced a dramatic morphological effect in which the formation of callus-like structures instead of normal lateral roots was observed (Fig. 4, F and G).

Effects of *N*-Isobutyl Decanamide on Shoot Development

The formation of callus-like structures in primary roots suggested that *N*-isobutyl-decanamide could play an important role in cell division. To investigate whether this alkamide could affect shoot development, Arabidopsis Columbia-0 (Col-0) seedlings were grown for 18 d on 0.2× Murashige and Skoog agar medium containing different concentrations of this compound. It was found that Arabidopsis seedlings respond to exogenous *N*-isobutyl decanamide in a dose-dependent manner. In plants grown on 28 μM, ectopic organogenesis was observed on the adaxial side of petioles of rosette leaves (Fig. 5, A–F). Ectopic organs resembled leaf blades, as revealed by trichome development on their surfaces (Fig. 5, D–F). Plants exposed to 56 μM *N*-isobutyl decanamide were smaller than solvent treated controls, with shorter, thicker petioles and dark green leaves, which often formed callus-like structures over their surfaces (Fig. 5, G–I). Treatment with 112 μM *N*-isobutyl decanamide severely impaired growth and plants had a compact rosette with round leaves and short petioles (Fig. 5J). The most severely affected plants showed fleshy and chlorotic cotyledons (Fig. 5K) and occasionally, the entire shoot appeared to be arrested in growth (Fig. 5L). Moreover, a dose-dependent increase in the number of plants with callus-like structures on leaves was evident starting at 14 μM and reaching 100% at 112 μM *N*-isobutyl decanamide (Fig. 6). None of the described alterations were observed in a large number of solvent-treated control plants or in plants exposed to concentrations of 3.5 to 7 μM *N*-isobutyl decanamide that were examined during these experiments (Fig. 6).

Effect of *N*-Isobutyl Decanamide on *CycB1*:*uidA* Expression in Roots and in Shoots

To determine whether the neoplasic effects of *N*-isobutyl decanamide on shoots were due to an effect of this compound on cell division, we examined the expression of the *CycB1*:*uidA* reporter gene in Arabidopsis seedlings subjected to treatment with different concentrations of *N*-isobutyl decanamide. The *CycB1*:*uidA* fusion protein is a good cell marker of proliferative activity since it is expressed only in cells in the G2/M phase and is destroyed rapidly when cells passed through mitosis (Colón-Carmona et al., 1999). In the roots of transgenic *CycB1*:*uidA* plants grown in medium lacking *N*-isobutyl decanamide, cell divisions

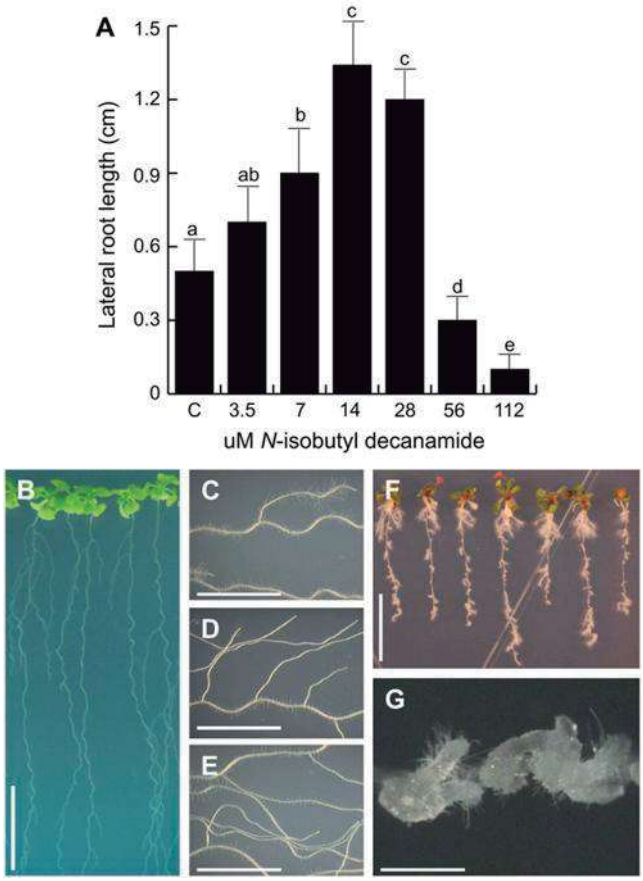

Figure 4. Effect of *N*-isobutyl decanamide on lateral root development. Wild-type (Col-0) seedlings were grown under increasing *N*-isobutyl decanamide concentrations on vertically oriented agar plates. Twelve days after germination, the lateral root length was determined. A, Mean lateral root length. Representative photograph of wild-type seedlings grown in *N*-isobutyl decanamide free medium supplied with the solvent (B). Close up showing a representative lateral root grown in *N*-isobutyl decanamide free medium (C). Lateral roots forming on 7 and 14 μM *N*-isobutyl decanamide treatments, respectively (D and E). Plants treated with 56 μM *N*-isobutyl decanamide (F). Close up from F (G). Values shown in A represent the mean of 100 lateral roots scored from 20 independent seedlings ±SD. Scale bars = 500 μm. [See online article for color version of this figure.]

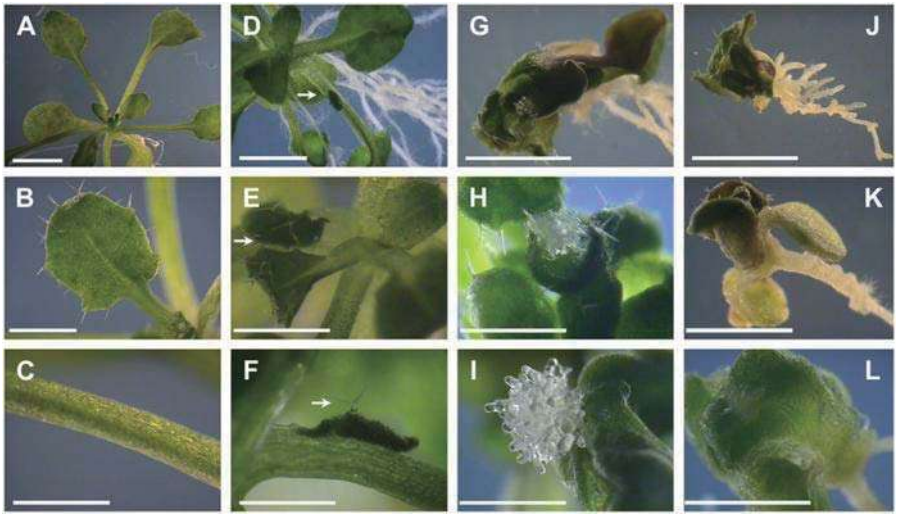

Figure 5. Effects of *N*-isobutyl decanamide on Arabidopsis shoot development. Wild-type Col-0 seedlings were grown for 18 d under varied *N*-isobutyl decanamide concentrations on vertically oriented agar plates. Control plants at the rosette stage of development (A). Close up of a developing leaf blade showing formation of trichomes (B). Magnification of a petiole (C). Plant treated with 28 μM *N*-isobutyl decanamide showing an ectopic outgrowth on petiole (arrow; D). Close up of petioles with ectopic blades (E and F). Note the presence of trichomes on these blades (arrows). Plants treated with 56 μM *N*-isobutyl decanamide (G–I). Formation of callus-like structures over the surface of leaves (H and I). Plants treated with 112 μM *N*-isobutyl decanamide (J–L). Note the reduction in length of petioles and inhibited growth of leaves. Scale bars in A to L = 500 μm. [See online article for color version of this figure.]

were clearly visible in the region comprising the root apical meristem (RAM; Fig. 7A). In the aerial part of the plant, cells expressing *CycB1*:*uidA* were restricted to the shoot apical meristem and to the base of developing leaves (Fig. 7B). In young seedlings (7 d after germination) treated with 56 μM *N*-isobutyl decanamide, cell divisions were also mainly confined to the RAM and shoot apical meristem (data not shown). However, later in development (18 d after germination) cell proliferation in the RAM decreased (Fig. 7C) and patches of GUS expression were preferentially seen in the shoot over a wider region in rosette leaves than in control seedlings (Fig. 7D), showing that cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. In callus formed on leaf blades, clusters of cells expressing GUS could be observed, indicating mitotic activity in these tissues (Fig. 7, E and F).

N-Isobutyl Decanamide Activates Transcription of the Cytokinin Reporter *ARR5*:*uidA*

Cytokinins are a class of phytohormones involved in various physiological responses, including cell division, root hair growth, chloroplast development, and shoot formation (Howell et al., 2003; Kakimoto, 2003). To test the possibility that *N*-isobutyl decanamide could affect cytokinin signal transduction, we examined transgenic plants harboring a fusion of the ARR5 promoter region to the GUS reporter sequence (*ARR5*:*uidA*). This cytokinin-inducible marker has been shown to be sensitive, highly specific for cytokinins, and reflects the expression of the corresponding resident gene (D'Agostino et al., 2000; Romanov et al., 2002). In control plants, the most prominent *ARR5*:*uidA* expression was detected in the shoot meristem region (Fig. 8A) and in the cap of primary roots (Fig. 8B). Moderate staining was also present in the vasculature of the hypocotyl (Fig. 8C), but not in the median

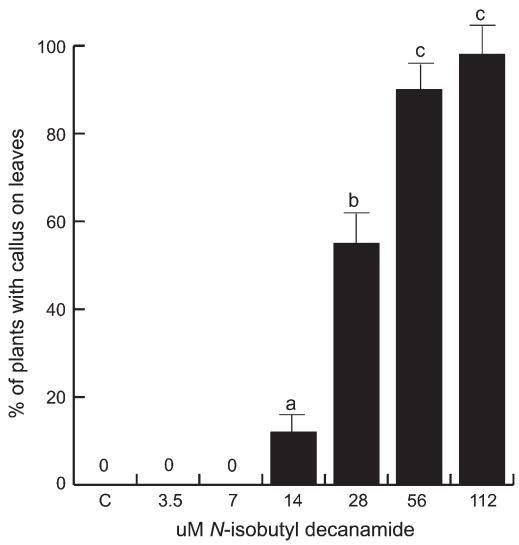

Figure 6. Effect of *N*-isobutyl decanamide on callus-like structure formation on leaves. Wild-type (Col-0) seedlings were grown under increasing *N*-isobutyl decanamide concentrations on vertically oriented agar plates. Eighteen days after germination, the number of plants showing callus-like structures on leaves was scored. Values shown represent the mean of 200 seedlings ± SD.

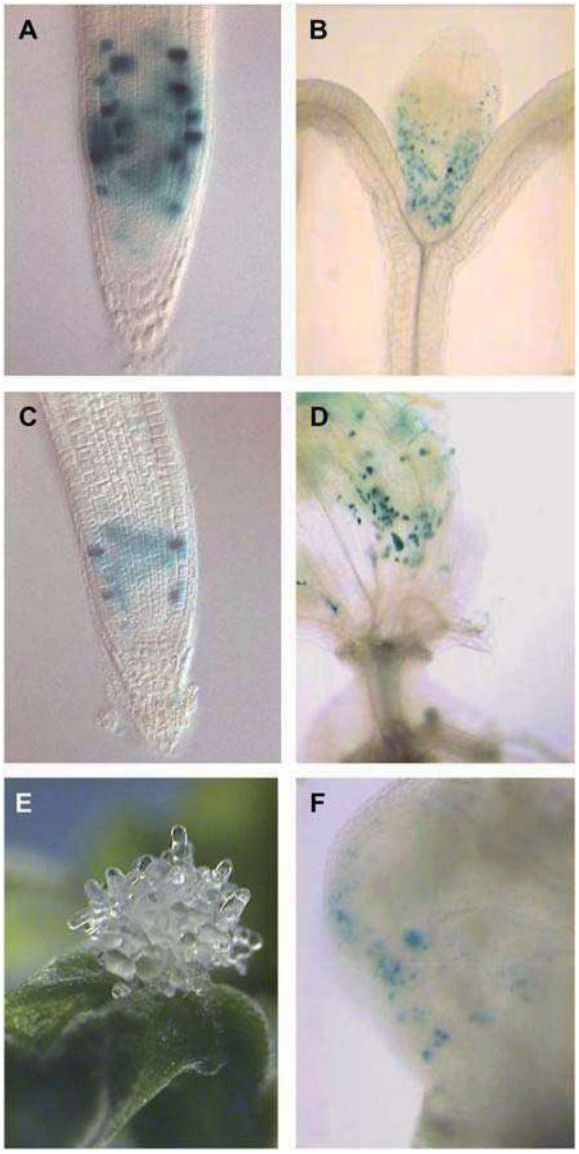

Figure 7. Effects of *N*-isobutyl decanamide on *CycB1*:*uidA* expression. In control plants GUS expression is found in the RAM (A) and young leaves (B). In plants grown in 56 μM *N*-isobutyl decanamide reduced GUS expression is detected in the RAM (C), whereas GUS expression is localized to extended regions in leaves (D) and in callus-like structures (E and F). [See online article for color version of this figure.]

region of the primary root (Fig. 8D). Treatment of 5 μM benzyl aminopurine increased GUS staining in the primary root tip, but did not significantly affect GUS expression in the cotyledons, in the root-shoot junction, or in the median region of the primary root (Fig. 8, E–H). GUS activity was further increased in the whole plant by treatment of 20 μM benzyl aminopurine (Fig. 8, I–L), confirming the cytokinin-inducible expression previously reported for this construct (D'Agostino et al., 2000). In plants grown for 6 d in 28 μM *N*-isobutyl decanamide, a localized induction of *ARR5*:*uidA* expression in cotyledons (Fig. 8M), in primary root tips (Fig. 8N), in adventitious roots (Fig. 8O), and in lateral root primordia (Fig. 8P, arrowheads) was clearly observed. It is important to note that induction of *ARR5*:*uidA* by *N*-isobutyl decanamide followed a different induction pattern when compared to the effects caused by cytokinin treatment. For instance, growth of plants under treatment of 20 μM benzyl aminopurine induced a constitutive expression of the *ARR5*:*uidA* marker throughout the root system (Fig. 8, J–L), whereas growth of plants under treatment of 28 μM *N*-isobutyl decanamide failed to induce this marker expression in the central region of the root and in the root-shoot junction (Fig. 8, O and P). Interestingly, *N*-isobutyl decanamide treatment of 28 μM strongly induced *ARR5*:*uidA* in proliferating tissues such as adventitious roots and lateral root primordia (Fig. 8, O and P). Quantitation of GUS activity by fluorometry showed that *ARR5*::*uidA* plants grown in treatment of 20 μM benzyl aminopurine had an increase of 68.5% in GUS activity when compared to untreated plants (data not shown), whereas plants grown under treatment of 28 μM *N*-isobutyl decanamide showed a small but statistically significant increase in GUS activity of 15.6% when compared to untreated plants (data not shown). These results suggest a role for decanamide in regulating the localized expression of a cytokinin primary-response gene.

Cell Proliferative Responses to *N*-Isobutyl Decanamide Are Impaired in Cytokinin-Signaling Mutants

The *ARR5*:*uidA* induction by exogenous *N*-isobutyl decanamide indicated a possible cross talk between alkamides and cytokinins. To investigate whether alkamide action could involve the cytokinin signaling pathway, we evaluated the effects of exogenously supplied *N*-isobutyl decanamide on the growth and development of Arabidopsis loss-of-function mutants lacking one, two, or three of the genes encoding cytokinin receptors (*CRE1*, *AHK2*, and *AHK3*; Higuchi et al., 2004; Nishimura et al., 2004). Callus-like structure formation on leaves was chosen as a developmental marker to test the effect of a high concentration (56 μM) of *N*-isobutyl decanamide on wild type (Col-0) and cytokinin response mutant seedlings. Addition of exogenous alkamide to the growth medium induced the formation of callus-like structures on wild-type plants (Fig. 9, A–C). In contrast, the *cre1-12* or *ahk3-3* single mutants exhibited 20% to 30% reduced sensitivity to *N*-isobutyl decanamide in terms of callus formation. Increased resistance to the effect of *N*-isobutyl decanamide was seen in the *ahk2-2* single mutant and in double mutant combinations involving *ahk2-2* (Fig. 9A). The triple mutant *cre1-12 ahk2-2 ahk3-3* showed the most altered responses in this essay, in which callus formation induced by *N*-isobutyl decanamide was totally impaired (Fig. 9A). Detailed phenotypical analysis revealed that the extent of callus-like structure proliferation was indeed very reduced in double

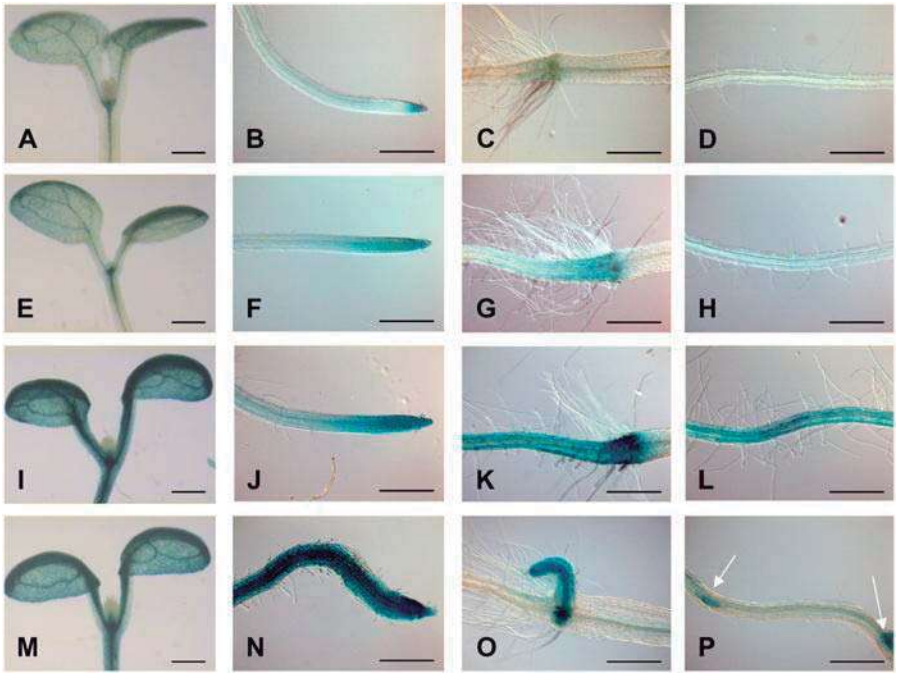

Figure 8. Effect of *N*-isobutyl decanamide on cytokinin-regulated gene expression. Six hours of GUS staining of *ARR5*:*uidA* seedlings grown for 6 d in medium without cytokinin (A–D), under 5 μM benzyl aminopurine (E–H), under 20 μM benzyl aminopurine (I–L), and with treatment of 28 μM *N*-isobutyl decanamide (M–P). Photographs are representative individuals of at least 20 plants stained. Scale bars = 300 μm. [See online article for color version of this figure.]

mutants involving *ahk2-2*, such as *ahk2-2 ahk3-3* (Fig. 9, D and E) and in the *cre1-12 ahk2-2 ahk3-3* triple mutant (Fig. 9, F and G). In the root, the formation of callus-like structures was also absent in *cre1-12 ahk2-2 ahk3-3* triple mutant (Fig. 9, H–K). These results suggest that CRE1, AHK2, and AHK3 have redundant function in callus induction by exogenous *N*-isobutyl decanamide and that the three cytokinin receptors are required for normal proliferative responses at least in terms of callus-like structure formation.

Effect of *N*-Isobutyl Decanamide on Primary Root Growth and Lateral Root Formation in Cytokinin Signaling Mutants

The reduction in callus-like structure formation in leaves of the cytokinin receptor mutants in response to *N*-isobutyl decanamide evidenced the involvement of cytokinin signaling in alkamide responses. To further define whether cytokinin mutants were also less sensitive to *N*-isobutyl decanamide effects of primary root growth and lateral root formation, the effects of 14 and 28 μM *N*-isobutyl decanamide on

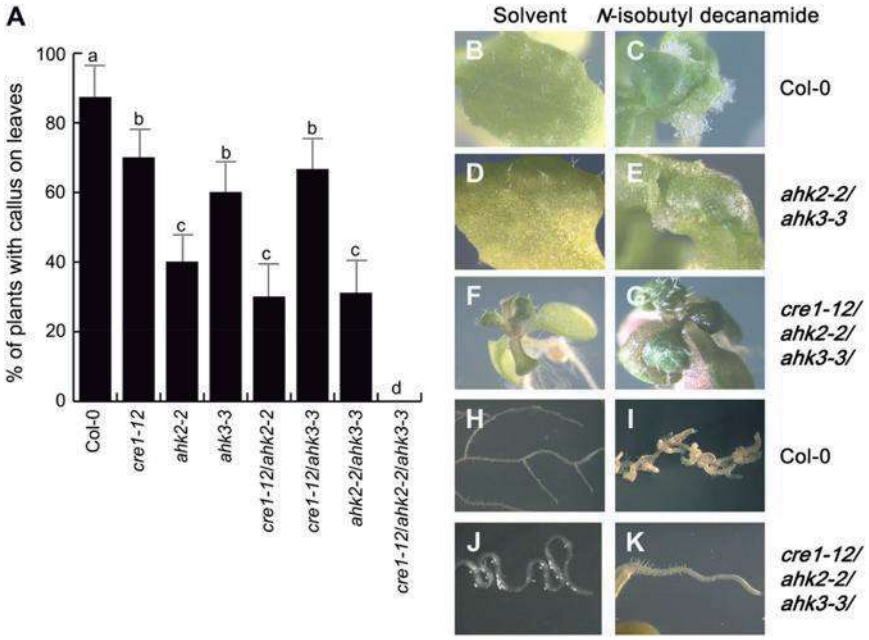

Figure 9. Effects of *N*-isobutyl decanamide on callus-like structure formation on leaves in wild-type (Col-0) and cytokinin response mutant seedlings. Seedlings were grown for 18 d on nutrient media with or without 56 μM *N*-isobutyl decanamide on vertically oriented agar plates. Plants showing callus-like structures on leaf surfaces were scored positive (n = 90). The experiment was repeated twice with similar results. [See online article for color version of this figure.]

primary root growth and lateral root density of single, double, and triple cytokinin receptor knockouts were tested. As shown in Figure 10, treatment with 28 μM *N*-isobutyl decanamide caused 86% inhibition in primary root growth in wild-type (Col-0) plants compared with solvent-treated control plants. In media lacking *N*-isobutyl decanamide, all seven mutant lines *cre1-12*, *ahk2-2*, *ahk3-3*, *cre1-12 ahk2-2*, *cre1-12 ahk3-3*, *ahk2-2 ahk3-3*, and *cre1-12 ahk2-2 ahk3-3* showed smaller primary roots compared to wild-type plants (Fig. 10A). Growth was severely impaired in the triple mutant, giving rise to dwarf plants with small roots. When mutant plants were grown in the presence of 14 or 28 μM *N*-isobutyl decanamide an inhibition in root elongation was observed depending on the alkamide treatment (Fig. 10A). In medium lacking *N*-isobutyl decanamide, all single and double cytokinin receptor mutants showed normal lateral root formation, with statistically similar lateral root densities compared to wild-type plants (Fig. 10B). Furthermore, we observed a 4- to 9-fold increase in lateral root density in 14 and 28 μM *N*-isobutyl decanamide treatments, respectively, in wild-type, single, and double mutants (Fig. 10B). Interestingly, *cre1-12 ahk2-2 ahk3-3* mutant plants showed no lateral root induction in *N*-isobutyl decanamide treatments, indicating an important role for cytokinins in pericycle cell activation in response to this alkamide.

DISCUSSION

N-Isobutyl Decanamide Activates Developmental Transitions in Roots

The root system originates from a primary root that develops during embryogenesis. This primary root produces lateral roots that increase its exploratory capacity. The root system shares with the shoot the basic body plans and the pathways that are essential for organogenesis and growth (Veit, 2004). Recent studies have addressed the role that hormones, such as auxin and cytokinin, play in root system development. The site of lateral root initiation seems to depend on correct auxin transport to pericycle cells in the primary root (Dubrovsky et al., 2000; López-Bucio et al., 2005), whereas the final architecture of the root is coordinated by hormonally regulated processes that affect cell division and cellular differentiation (Casson and Lindsey, 2003). Recently two groups of single chain amides have been reported to alter several aspects of root development, alkamides, and NAEs (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). Blancaflor et al. (2003) evaluated the effects of micromolar concentrations of *N*-lauroylethanolamine on early root development of Arabidopsis. In young seedlings, a 50 μM concentration of this molecule was found to inhibit root elongation, increase radial swelling of root tips, and alter root hair development. Older seedlings showed increased lateral root formation. These developmental effects were related to altered cell division, endomembrane organization, and vesicle trafficking, suggesting that *N*-lauroylethanolamine may play a role in these fundamental processes (Blancaflor et al., 2003). Similarly, Ramírez-Chávez et al. (2004) reported that affinin, the most abundant alkamide in the roots of *H. longipes*, regulates Arabidopsis root system architecture in a dose-dependent manner. In this work, we extend these previous findings by showing that *N*-isobutyl decanamide, a saturated alkamide obtained by affinin reduction, alters both primary root growth and lateral root formation. These effects were similar to those for NAE10:0 and affinin (Fig. 2), suggesting that alkamides and NAEs may comprise a

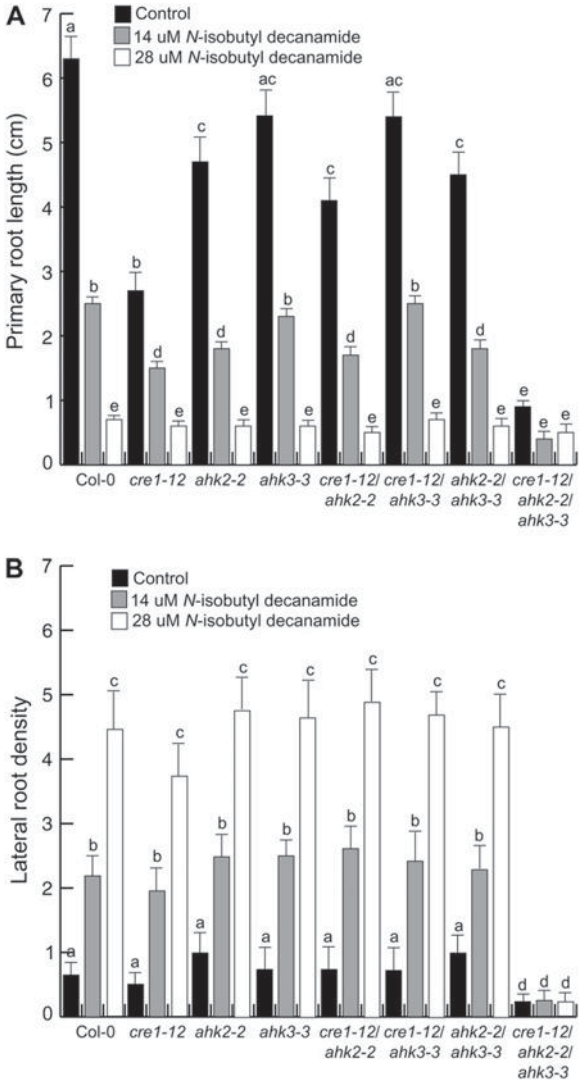

Figure 10. Effects of *N*-isobutyl decanamide on primary root growth and lateral root formation in wild-type (Col-0) and cytokinin response mutant seedlings. Seedlings were grown for 12 d on nutrient media with or without the indicated *N*-isobutyl decanamide concentrations on vertically oriented agar plates and the length of the primary root (A) and lateral root density (lateral roots/cm; B) determined for a total of 30 seedlings. Values shown represent the mean ± SD. The experiment was repeated twice with similar results.

new group of plant growth regulating substances, which may act through common signaling mechanisms to modify root development. Interestingly, both the amide moiety and the length of the acyl chain were found to be essential for biological activity of exogenous applied amides, since octadecanamide and decanoic acid ethyl ester failed to inhibit primary root growth or to stimulate lateral root formation (Fig. 3). We cannot exclude the possibility that the longer acyl chain in octadecanamide results in a lower permeability and that this could partially mask the activity for this compound.

N-Isobutyl Decanamide Regulates Cell Proliferation in Arabidopsis

Leaf initiation at the shoot apical meristem involves a balance between cell proliferation and commitment to make primordia. Arabidopsis has a typical simple leaf, which consists of a petiole and a blade. The developmental control of petioles is presumed to be important in the effective capture of light by ensuring that the leaf blades do not overlap. To produce this leaf shape, the cells on the proximal side of the leaf differentiate into petioles without producing blades or other organs (Ha et al., 2003). One of the most conspicuous effects of *N*-isobutyl decanamide on shoot development was the ectopic induction of outgrowths along the leaf petioles. These outgrowths resembled a leaf blade in that they showed the presence of trichomes on the adaxial side (Fig. 5). These observations suggest that following treatment with *N*-isobutyl decanamide, the petiole cells do not undergo correct developmental specification and can be diverted toward other developmental fates or that this alkamide is capable of reprogramming petiole cells to initiate the de novo formation of organs in differentiated cells.

Cell division normally ceases during leaf development (Donnelly et al., 1999; De Veylder et al., 2002) and is not observed in mature organs. Increased concentrations of *N*-isobutyl decanamide in the growth medium were found to induce the production of callus-like structures in roots (Fig. 4) and on leaves (Fig. 5). Moreover, in the epidermis of mature leaves and in callus-like structures of alkamide-treated plants, clusters of cells expressing *CycB1*:*uidA* were observed (Fig. 7), GUS staining being present over a wider region than in nontreated plants, suggesting that cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. Taken together, these results suggest that alkamides alter several aspects of plant morphogenesis through the control of meristematic activity.

Alkamides and NAEs: Evidence for Endocannabinoid Signaling in Plant Development?

Alkamides are structurally related to sphingolipids such as ceramide and sphingosine (Ng and Hetherington, 2001; Ramírez-Chávez et al., 2004), and to NAEs, of which the latter are likely produced by hydrolysis of the membrane phospholipid *N*-acylphosphatidylethanolamine by phospholipase D (Chapman, 2000; López-Bucio et al., 2006). In animals, this reaction is part of the endocannabinoid-signaling pathway, which regulates a variety of physiological processes, including cell proliferation, neurotransmission, and embryo development (Howlett and Mukhopadhyay, 2000; Wilson and Nicoll, 2002). In plants, NAEs are present in different tissues, being quite abundant in dessicated seeds, where their levels decline during seed imbibition and germination (Chapman et al., 1999). In response to pathogen elicitors, *N*-acylphosphatidylethanolamine is hydrolized by phospholipase D, and medium chain, saturated NAEs are released by plant cells where they act as lipid mediators to modulate ion flux and activate defense gene expression (Chapman et al., 1998). Although the biosynthesis pathway for alkamides is at present unknown, the structural similarity of these molecules with NAEs and sphingolipids suggests that molecules derived from the hydrolysis of membrane lipids can act as signaling molecules for plant development. However, it remains to be determined whether NAEs and/or sphingolipids can also modify shoot development as shown in this work for *N*-isobutyl decanamide, and whether alkamides such as affinin and *N*-isobutyl decanamide can activate defense mechanisms in plant cells as it occurs for NAEs and sphingolipids.

N-Isobutyl Decanamide Effects on Cell Proliferation Require Normal Cytokinin Signaling

The ability of alkamides and NAEs to regulate organ development in Arabidopsis seedlings is compelling evidence for the possibility that these molecules act as regulators of morphogenesis. Thus, the possibility was raised that alkamides could interact with plant hormones such as auxin or cytokinins to regulate plant cell division.

Our previous work showed that the effects of alkamides on root growth are likely independent on auxin action (Ramírez-Chávez et al., 2004). However, the finding that *N*-isobutyl decanamide activates the expression of the cytokinin primary-response gene fusion *ARR5*:*uidA* suggests that cytokinin signaling is probably involved in the alterations in cell proliferation and differentiation caused by alkamides. Cytokinins are purine derivatives that promote and maintain plant cell division in cultures and are also involved in various differentiation processes including shoot formation, primary root growth, and callus formation (Grayburn et al., 1982; Werner et al., 2001, 2003; Catterou et al., 2002; Higuchi et al., 2004). Three sensor His kinases, CRE1/AHK4/WOL, AHK2, and AHK3 have been shown to act as cytokinin receptors (Kakimoto, 2003). These receptors activate the expression of several response regulators in a cytokinin-dependent manner (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Further downstream, cytokinin signaling stimulates the G1/S transition of the cell cycle, which

has been proposed to be mediated by the transcriptional induction of the *CYCD3* gene that encodes a D-type cyclin (Riou-Khamlichi et al., 1999). We observed that alkamides induce cell proliferation as observed by the formation of callus-like structure in shoots and the increased expression of the *CycB1*:*uidA* gene marker.

When grown on soil, none of the single cytokinin receptor mutants (*cre1-12*, *ahk2-2*, *ahk3-3*) exhibited significant defective phenotype. The *ahk2-2 ahk3-3* double mutants had smaller leaves and shorter stems than did the wild-type plants. All single and double mutants produced apparently normal flowers that yielded viable seeds. The *cre1-12 ahk2-2 ahk3-3* triple mutants showed a dwarf phenotype with reduced growth. Occasionally, the triple mutants produced an influorescence with nonfunctional flowers, which failed to produce seeds (data not shown). These data suggest that cytokinin receptors play an important role in plant growth and development. The complete lack of *N*-isobutyl decanamide responses in the triple cytokinin receptor mutant *cre1-12 ahk2-2 ahk3-3*, in terms of lateral roots or callus-like structure proliferation (Figs. 9 and 10) and the absence of ectopic blades on petioles and callus on mature leaves, suggest that cytokinin receptors are necessary for normal cellular responses to alkamides. There are several scenarios that could explain the alkamide-cytokinin interaction; one in which alkamide treatment induces cytokinin biosynthesis and in this way alters plant development. However, since there are no reports that exogenous treatment with cytokinins alone is able to induce lateral roots or callus-like structure formation in leaves in a similar way to *N*-isobutyl decanamide, makes this possibility unlikely. Another possibility is that the cytokinin receptors are direct targets of alkamides and that this interaction starts the signaling cascade that activates gene expression responsible for cellular responses. A third scenario is that alkamides could alter cytokinin sensitivity in specific tissues by either modulating the level of the cytokinin receptor or their activity by direct or indirect interaction with an as yet undiscovered alkamide receptor. However, since the effects of treatments with *N*-isobutyl decanamide differ from those produced by exogenous cytokinin, it is likely that alkamide action involves additional signaling pathways yet to be discovered. These different scenarios are currently under investigation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 and the transgenic lines *ARR5*:*uidA* (D'Agostino et al., 2000) and *CycB1*:*uidA* (Colón-Carmona et al., 1999) were used for all experiments unless indicated otherwise. The cytokinin receptor mutants *cre1-12*, *ahk2-2tk*, *ahk3-3*, *cre1-12 ahk2-2tk*, *cre1-12 ahk3-3*, *ahk2-2tk ahk3-3*, and *cre1-12 ahk2-2tk ahk3-3* were previously described (Higuchi et al., 2004; Mähönen et al., 2006).

Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× Murashige and Skoog medium, pH 5.7, 0.5% (w/v) Suc, and 1% (w/v) agar. The basic medium contained 2.0 mM NH_4NO_3, 1.9 mM KNO_3, 0.3 mM $CaCl_2$ $2H_2O$, 0.15 mM $MgSO_4$ $7H_2O$, 5 μM KI, 25 μM H_3BO_3, 0.1 mM $MnSO_4$ H_2O, 0.3 mM $ZnSO_4$ $7H_2O$, 1 μM Na_2Mo0_4 $2H_2O$, 0.1 μM $CuSO_4$ $5H_2O$, 0.1 μM $CoCl_2$ $6H_2O$, 0.1 mM $FeSO_4$ $7H_2O$, 0.1 mM Na_2EDTA $2H_2O$, inositol (10 mg L^{-1}), and Gly (0.2 mg L^{-1}). 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) 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 to 24°C. After growth for different periods as indicated, plants were cleared according to the method by Malamy and Benfey (1997) and lateral roots counted using a 40× objective on a Leica DMR microscope.

Isolation of *cre1-12 ahk2-2tk ahk3-3* Triple Mutants

In agar medium homozygous *cre1-12 ahk2-2tk ahk3-3* triple mutants develop short primary roots and can be easily distinguished from *cre1-12/cre1-12 ahk2-2tk/ahk2-2tk ahk3-3/AHK3* (heterozygous for *ahk3-3*) or *cre1-12/cre1-12 ahk2-2tk/ahk2-2tk*. To select for the triple mutants, a pool of seeds produced by a *cre1-12/cre1-12 ahk2-2tk/ahk2-2tk ahk3-3/AHK3* plant were sterilized and sown on agar plates. After 10 d, seedlings with short primary roots were selected and transferred to plates with the different decanamide concentrations for a further 10 to 15 d growth period. Separately we have examined the genotypes of at least 50 plants with short roots, and all were confirmed to be triple homozygotes. For triple mutant selection, 500 seeds from this *cre1-12/cre1-12 ahk2-2tk/ahk2-2tk ahk3-3/AHK3* segregating population were screened for reduced primary root growth 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 mutants with short primary roots were selected and transferred to plates with the different *N*-isobutyl decanamide treatments for a further 10 to 15 d growth period.

Synthesis of Alkamides and NAEs

Affinin was purified from *Heliopsis longipes* (Gray) Blake (Asteraceae) plants collected at Xichú, Sierra Gorda of Guanajuato State, central México, and *N*-isobutyl decanamide was obtained from affinin by catalytic reduction as described before (Ramírez-Chávez et al., 2004). Octadecanamide was purchased from Sigma Aldrich. NAE10:0 was synthesized from the acyl chlorides in ethanolamine (Tripathy et al., 1999), at room temperature, in a reaction mixture of 25 mg of decanoylchloride, 2.5 mL of dichloromethane, and 2.5 mL of ethanolamine (Sigma-Aldrich) for 15 min with gentle swirling. The reaction was stopped with 10 mL of bidistilled water and washed twice with an equal volume of bidistilled water (MilliQUF Plus). The NAE was then collected in the organic layer and the dichloromethane was evaporated under N_2 gas stream. The product was resuspended in anhydrous ethanol and purity determined by gas chromatography-mass spectroscopy.

Histochemical Analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings 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). The stained seedlings were cleared by the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen for each decanamide or cytokinin treatment and photographed using the Nomarski optics on a Leica DMR microscope.

Data Analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All lateral roots emerged from the primary one and observed at the 3× objective were taken into account for lateral root number data. Primary root length was determined for each root using a ruler. For all experiments, the overall data was statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with a Tukey's Post Hoc test were used for testing differences in primary root length, lateral root number, and lateral root density under NAE and alkamide treatments in wild-type and mutant plants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

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Tissue culture of *Arabidopsis thaliana* explants reveals a stimulatory effect of alkamides on adventitious root formation and nitric oxide accumulation

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Abstract

Alkamides and *N*-acylethanolamines represent a new class of lipid compounds related to animal endocannabinoids, which regulate different aspects of plant morphogenesis. To elucidate further the plant regenerative properties of alkamides and their role on plant development, we used an *in vitro* system to cultivate *Arabidopsis thaliana* explants under varied concentrations of *N*-isobutyl decanamide and *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide (affinin). Cultivation of explants that harbor the shoot apical meristem on MS 0.2× medium lacking alkamides resulted in formation of mature plants with fully developed shoot and root systems. On the contrary, explants obtained from stems or primary roots resulted in development of two main classes of regenerative structures: adventitious roots and lateral roots, depending on the source of explants. *N*-Isobutyl decanamide treatments showed a dose-dependent effect on adventitious and lateral root formation from stem and primary root explants, respectively, and in growth of regenerated plants. The stimulatory effect of *N*-isobutyl decanamide and affinin on adventitious root formation was further confirmed in *A. thaliana* seedlings. Although the effects of alkamides were similar to those produced by auxins on adventitious root development, the ability of shoot explants to respond to alkamides was found to be independent of auxin signaling. Furthermore, we show that *N*-isobutyl decanamide is able to induce nitric oxide accumulation in sites of adventitious root proliferation. Our results suggest a role for alkamides in regulating adventitious root development, probably operating through the NO signal transduction pathway.

Keywords: *Arabidopsis thaliana*; Alkamides; Root architecture; Adventitious roots; Nitric oxide

1. Introduction

Plants produce compounds of different chemical identity that mediate a range of cellular functions including auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids and ethylene. These compounds are considered as plant hormones because of their widespread occurrence in plants and their diverse physiological roles [1].

In the past 5 years, it has become evident that plants use – along with the above mentioned classical hormones – a diverse array of small molecules for extra- and intracellular signaling. Of particular interest are the plant lipids because, like their mammalian counterparts, they act not only as structural components of membranes but also as signaling molecules that regulate developmental and adaptive responses to environmental stimuli [2,3]. *N*-Acylethanolamides (NAEs) comprise a group of lipids, which are produced from the hydrolysis of *N*-acylphosphatidylethanolamide (NAPE), a minor lipid constituent of cell membranes, by phospholipase D (PLD) [4]. There is information indicating that NAEs might be involved in diverse physiological processes, including seed germination, pathogenesis, regulation of root architecture and response to herbivory [5–7]. NAE related compounds include alkamides (*N*-alkyl amides), which have been found to alter root development and to regulate cell division and differentiation processes in *Arabidopsis thaliana* [8,9].

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Two reports indicate that alkamides may act as plant growth regulating substances. Kanbe et al. showed that amidenin, a non-substituted alkamide isolated from the actinomycete *Amycolatopsis* sp., promoted the growth of rice (*Oryza sativa*) plants at concentrations of 0.6 and 1.8×10^{-5} M and inhibited growth at concentration of 6×10^{-5} M [10]. More recently, our group evaluated the effects of affinin, an alkamide produced in *Heliopsis longipes* roots, and its reduced amides in the growth and development of *A. thaliana* seedlings [8]. Together with a general plant growth promoting effect, affinin showed a dose-dependent effect in primary root growth, increased growth of root hairs and lateral roots at concentration of 2.8×10^{-5} M, and decreased lateral root growth at higher concentrations. The repressing effect of affinin on primary root growth correlated with an inhibition in proliferative activity in the primary root meristem and reduced cell elongation [8]. These observations, and the known regulatory functions of NAEs and alkamides in animal systems, indicate that the participation of alkamides is likely to be demonstrated for many cellular and morphogenetic processes in plants [9].

In this study, we report a further characterization of the plant regenerative properties of two alkamides, namely *N*-isobutyl decanamide, and *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide. The role of these compounds in plant development was investigated by cultivating explants from *A. thaliana* etiolated plants under varied concentrations of exogenously applied alkamides. We observed differential effects of alkamide treatment depending on the source of the explants that included increased growth of regenerated plants and a stimulation of adventitious root formation, a process no previously reported to be regulated for this class of signaling molecules. The role of auxin in mediating the adventitious root proliferating activity of alkamides was tested using the auxin responsive marker gene *DR5:uidA* and *axr2-1*, *eir1-1*, *aux1-7*, and *axr4-1* auxin-resistant *A. thaliana* mutants. In addition, nitric oxide (NO) accumulation was detected by confocal microscopic analysis in cultured shoot explants. All together, our results suggest a novel role for alkamides in the regulation of adventitious root development, acting independently of auxin, and probably operating in the NO signal transduction pathway.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis (Col-0 ecotype) WT and *DR5:uidA* [11] transgenic plants were used for the different experiments and as the source of explants. *Arabidopsis* mutant *eir1* [12] was kindly provided by Dr. Plinio Guzmán (Departamento de Ingeniería Genética, Centro de Investigación y Estudios Avanzados, Irapuato, Gto. México). *aux1-7* [13], *axr4-1* [14], and *axr2* [15] were kindly provided by Dr. Claire Grierson (School of Biological Sciences, University of Bristol, Bristol, UK). Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. MS medium (Murashige and Skoog basal salts mixture, Cat. M5524) was purchased from Sigma. The suggested formulation is 4.3 g/l of salts for a 1× concentration of medium, we used 0.9 g/l, which we consider and refer as MS 0.2×. This medium lacks aminoacids and vitamins. *N*-Isobutyl decanamide and affinin were added to cooled (50 °C) molten medium and poured into plates. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, USA). Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival Scientific AR95L) with a photoperiod of 16 h of light, 8 h darkness, light intensity of 200 μmol m^2 s^{-1} and temperature of 22 °C. For dark grown plants, seeds were sown on the surface of agar plates and the plates covered by four layers of aluminum foil. Plants were included in the growth chamber for 7 d until development of long hypocotyls. Etiolated seedlings were selected on the basis of the continuous growth of the stem that ensures a suitable source of plant tissue, and the developmentally inhibited growth mode of the root and shoot systems. These combined properties represent a good experimental system to evaluate the growth promoting and regenerating properties of alkamides.

2.2. Synthesis of alkamides

Affinin was purified from *H. longipes* (Gray) Blake (Asteraceae) plants collected at Xichú, Sierra Gorda of Guanajuato State, central México, and *N*-isobutyl-decanamide was obtained from affinin by catalytic reduction as described before [8].

2.3. Analysis of growth

Lateral root number was determined by counting the lateral roots present in explants, since these explants were obtained from primary roots, the lateral roots were counted from the tip to the cutting end. Fresh weight of the root and the shoot was determined with an Ohaus analytical balance with a 0.0001 g precision value. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in WT and mutant plants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

2.4. Microscopy

The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6). Total lateral roots were counted at a 30× magnification. The primordial phases of lateral and adventitious roots were analyzed in semi-permanent preparations of cleared roots using a composed microscope (Leica CME) at 100× or 400× magnifications. Images were captured with a SAMSUNG digital color camera SCC 131-A adapted to the microscope.

2.5. Imaging of endogenous nitric oxide

NO was monitored by incubating *A. thaliana* explants with 10 μM of the fluorescent probe 4, 5-diaminofluorescein diacetate (DAF-2 DA, Sigma, USA) in 0.1 M Tris–HCl (pH 7.4). The treated explants were incubated 2 h in dark, and washed three times for 20 min with fresh buffer. Fluorescence signals were detected using a confocal laser scanning microscope (model BX50; Olympus, Japan), 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.

3. Results

3.1. N-Isobutyl decanamide promotes adventitious root development from hypocotyl explants

To evaluate the effects *N*-isobutyl decanamide in organ formation from plant tissues, we used dark grown *A. thaliana* seedlings as a source of root and shoot explants. Fig. 1A shows a representative *Arabidopsis* seedling grown for 7 d in dark conditions. The seedlings exhibited etiolated growth with elongated hypocotyls topped by closed, under-developed cotyledons (Fig. 1A). The primary roots sustained moderate growth, sufficient to ensure the availability of root explants. Fig. 1B illustrates the way in which the different explants were obtained. Type I explants included the shoot apical meristem and a 0.7 cm of the stem region, type II explants included the remaining 0.7–1.0 cm stem region, whereas type III explants comprised the most distal 1.0 cm region from the primary root including the root apical meristem (Fig. 1B). Samples of the three different classes of explants were cultured for 12 d in Petri plates containing agar-MS 0.2× medium supplied with varied concentrations of *N*-isobutyl decanamide. Fig. 2 shows the effects of alkamide treatments on plant regeneration from explants. For type I explants, cutting of hypocotyls was found to promote adventitious rooting. These explants developed fully expanded leaves at later times, which proceed normally in their development (Fig. 2A). Interestingly, both the length and number of adventitious roots were modified in the explants in response to *N*-isobutyl decanamide treatments (Fig. 2B–E). For type II explants, although events of adventitious root initiation proximal to the cutting ends were frequently observed, the effect of alkamide treatment was less evident and no significant differences were recorded for adventitious root formation (Fig. 2F–J). For type III explants, *N*-isobutyl decanamide increased lateral root formation and root tip swelling (Fig. 2K–O).

From 20 type I explants analyzed, concentrations of 24–48 μM *N*-isobutyl decanamide showed a roughly twofold increase in adventitious root number ($C = 3.7 \pm 0.7$; 24 μM = 7.2 ± 0.4; 36 μM = 7.5 ± 0.3; 48 μM = 6.4 ± 0.5) (Fig. 3A). On the contrary, treatments of 24–60 μM *N*-isobutyl decanamide had an inhibitory effect on adventitious root growth (Fig. 3B).

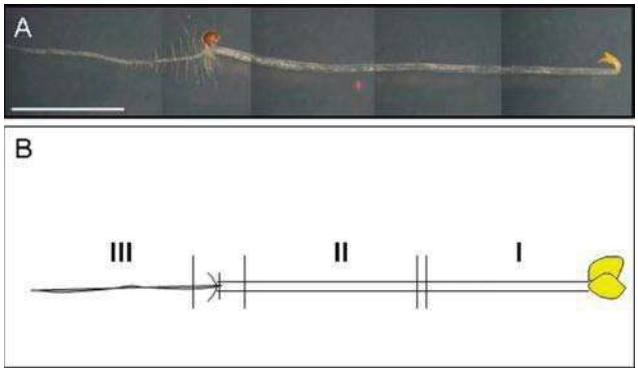

Fig. 1. Dark grown *A. thaliana* plants as a source of explants. *A. thaliana* (Col-0) seeds were sown in 0.2× MS agar medium and grown for 7 d under dark conditions. (A) Representative 7 d seedling used as an explant source. (B) Distribution of explants from seedling regions. Scale bar = 0.5 cm.

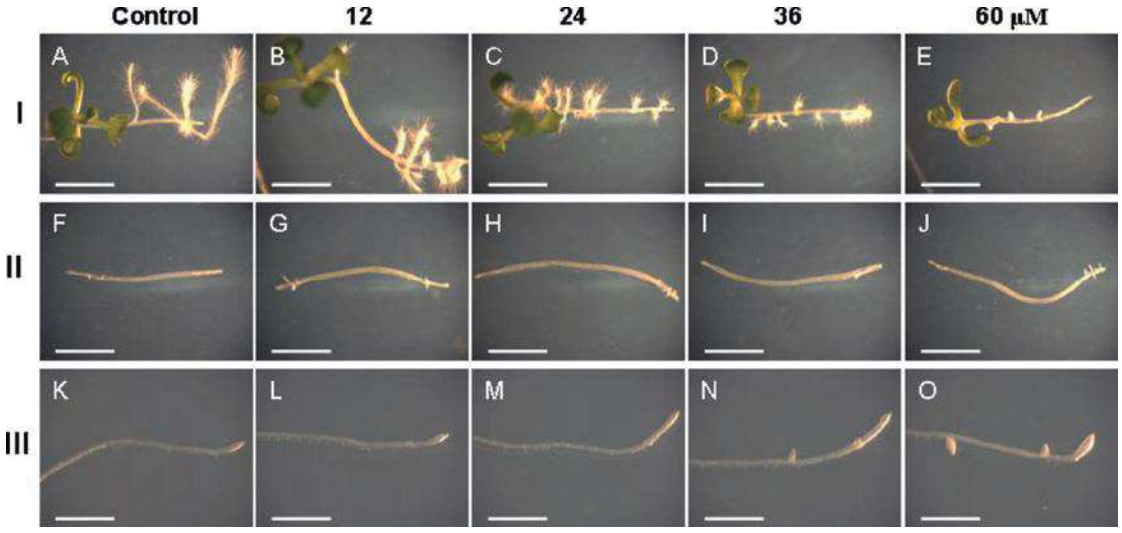

Fig. 2. Growth and developmental responses of *A. thaliana* explants to *N*-isobutyl decanamide. Representative photographs were taken from plants grown for 12 d under the indicated alkamide concentrations. Photographs were taken at 1.5× magnification in a Leica MZ6 stereoscopic microscope. Scale bars = 0.5 cm.

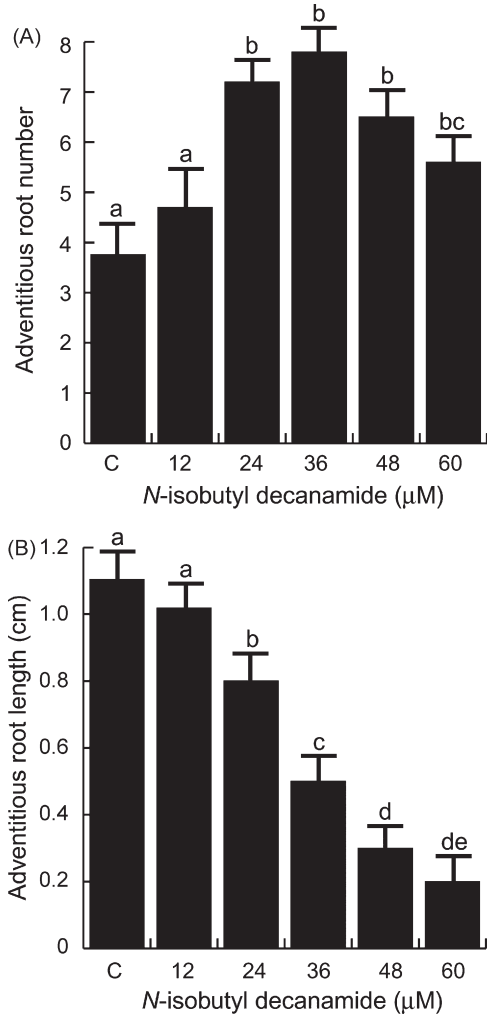

Fig. 3. Effect of *N*-isobutyl decanamide on adventitious root development from type I explants. *A. thaliana* (Col-0) hypocotyl explants were transferred to MS 0.2× medium containing the indicated alkamide concentrations. Plants were grown for a further 14 d period and the number (A) and length (B) of adventitious root recorded. Values shown represent the mean ± S.D. ($n = 30$). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P < 0.05$.

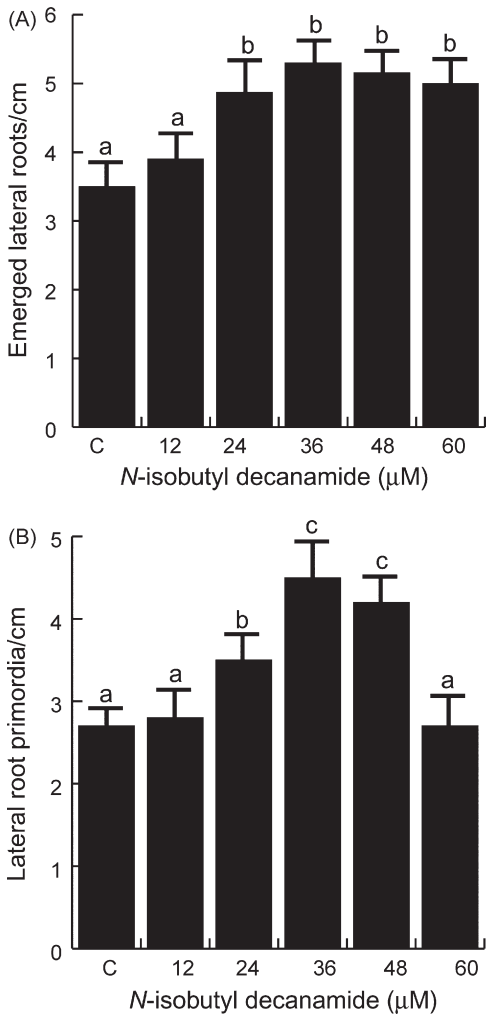

Fig. 4. Effect of *N*-isobutyl decanamide on lateral root development from type III explants. *A. thaliana* (Col-0) primary root explants were transferred to MS 0.2× medium containing the indicated alkamide concentrations. Plants were grown for a further 14 d period and the number of emerged roots (A) and total LRP (B) recorded. Values shown represent the mean ± S.D. ($n = 30$). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P < 0.05$.

3.2. N-Isobutyl decanamide promotes lateral root development in explants from primary roots

We evaluated the role on *N*-isobutyl decanamide in lateral root formation by quantifying the number of emerged lateral roots in type III explants. This compound increased significantly lateral root number at concentration of 24 μM and higher concentrations (Fig. 4A). Lateral roots were often located close to the root tip (Fig. 2K–O). To define whether this increased number of lateral roots in response to alkamide treatment could be due to a stimulation of the emergence of pre-existing lateral root primordia (LRP) or to the de novo formation of additional primordia, we counted the LRP from type III explants that were first cleared to visualize LRP. Fig. 4B shows the density of total LRP recorded at day 12 after transfer of explants to *N*-isobutyl decanamide treatments. It was found that explants treated with 36 and 48 μM *N*-isobutyl decanamide formed 67 and 56% more LRP than solvent-treated controls (Fig. 4B).

3.3. N-Isobutyl decanamide increases growth of regenerated A. thaliana plants from hypocotyl explants

As shown in Fig. 2, from the three different classes of explants used in this study, only type I explants were able to regenerate leaves and adventitious roots, which progressing in development produced fully mature shoot and root systems. Next, we monitored the growth progress of these regenerated plants at later stages of development and evaluated the effects of *N*-isobutyl decanamide treatment in this process. A dose–response growth effect was observed in plants subjected to *N*-isobutyl decanamide treatment with promoting or repressing effects depending on alkamide concentration (Fig. 5A–E). At 27 d of growth, plants were harvested and excised at the root/

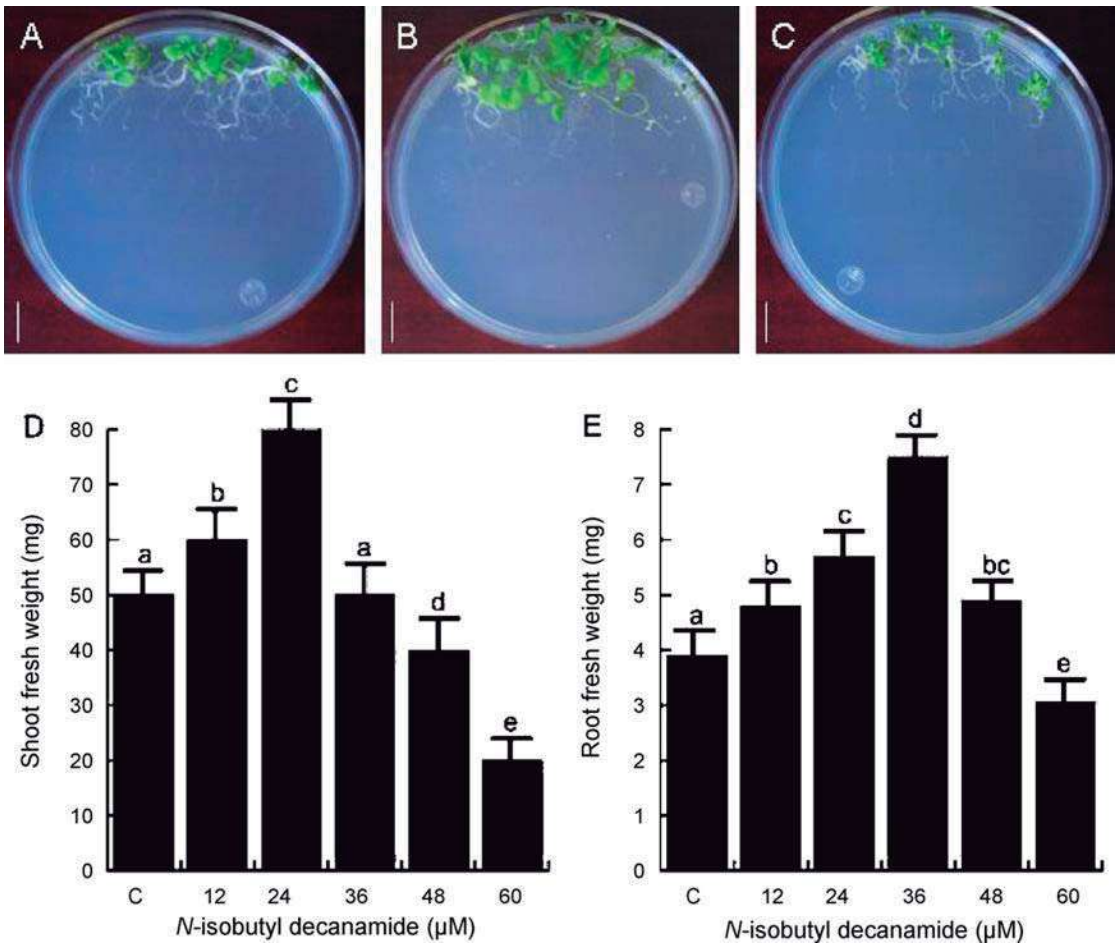

Fig. 5. Effect of *N*-isobutyl decanamide on growth of regenerated *A. thaliana* plants. Type I *A. thaliana* explants were obtained from 7 d dark-grown plants, transferred to MS 0.2× media containing the indicated *N*-isobutyl decanamide concentrations and grown for a further 27 d period. Representative pictures were taken at the end of this period. (A) Control, (B) 24, (C) 60 μM *N*-isobutyl decanamide. Plants were excised at the root/shoot junction and the shoot (D) and root fresh weight (E) determined on an analytical scale for groups of 10 plants. Values shown represent the mean ± SD ($n = 30$). Each experiment consisted of three independent plates with ten explants each. The experiment was repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Scale bars = 1 cm.

shoot junction to quantify root and shoot biomass. Treatments of 12 and 24 μM *N*-isobutyl decanamide significantly increased shoot fresh weight when compared to control plants, while concentrations of 48 and 60 μM decreased shoot biomass (Fig. 5D). Interestingly, the root system showed a different sensitivity to the shoot in its response to *N*-isobutyl decanamide. For instance, treatments of 12–36 μM *N*-isobutyl decanamide had a gradual increasing effect in root fresh weight when compared to the control and no significant decrease was recorded at 60 μM *N*-isobutyl decanamide, which was the greatest concentration tested of this compound (Fig. 5E).

3.4. Alkamides promote adventitious root formation on A. thaliana seedlings

Because a very clear increase of adventitious root number was registered in *A. thaliana* type I explants in response to *N*-isobutyl decanamide treatments, the effect of two alkamides, *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide (affinin) and *N*-isobutyl decanamide, in adventitious root development was also monitored in seedlings that were germinated and grown for 18 d in agar plates with MS 0.2× medium supplied with increasing concentrations of the compounds. A general promoting effect of *N*-isobutyl decanamide treatment in adventitious root number was observed starting at a concentration of 36 μM. The most clear stimulating effect was recorded at higher concentrations (48 and 60 μM), for which a four- to sixfold increase in adventitious root number was recorded (Fig. 6A). The opposite effect was observed for adventitious root length (data not shown). Affinin was also found to increase adventitious root number in *A. thaliana* seedlings albeit at concentrations higher than 40 μM (Fig. 6B).

3.5. Effect of alkamides on auxin-inducible gene expression in shoot explants

The observed effect of alkamides on adventitious root formation is similar to that described for auxin in most plant species, including *A. thaliana*. Auxins such as indole-3-acetic acid (IAA) and 1-napththalene acetic acid (NAA) increase adventitious root formation in nanomolar or low micromolar concentrations [16,17]. To test whether affinin and *N*-isobutyl

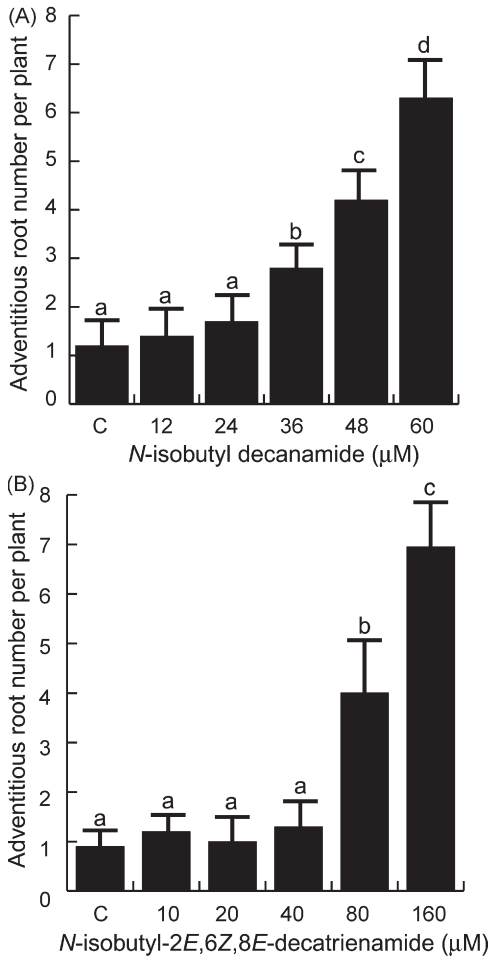

Fig. 6. Effect of alkamides on adventitious root development in *A. thaliana* (Col-0) seedlings. Plants were germinated and grown for 14 d under the indicated alkamide concentrations. (A) Number of adventitious roots per plant in *N*-isobutyl decanamide treatments. (B) Number of adventitious roots per plant in *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide (affinin) treatments. Values shown represent the mean ± S.D. ($n = 30$). The experiment was replicated two times with similar results. Different letters indicate statistical differences at $P < 0.05$.

decanamide could alter auxin-regulated gene expression in shoot explants, and in this way promote adventitious root formation, we conducted analyses of the expression of the β-glucuronidase (GUS) reporter gene in explants from *Arabidopsis* transgenic seedlings harboring the *DR5:uidA* gene construct. This reporter line has been useful in studying auxin-regulated gene expression in *Arabidopsis* [11]. Fig. 7 shows histochemical staining for transgenic *DR5:uidA* explants that were transferred to NAA, affinin or *N*-isobutyl decanamide treatment and grown for 12 d. In solvent-treated control explants, *DR5:uidA* expression is absent from the hypocotyl section and is primarily located in the columella of developing adventitious roots (Fig. 7A and B). *DR5:uidA* explants grown in a concentration of 1 μM IAA or 0.5 μM NAA showed GUS activity throughout the stem and developing adventitious roots (Fig. 7C–F). In contrast, the pattern of GUS expression in *DR5:uidA* explants treated with a concentration of affinin or *N*-isobutyl decanamide that promotes adventitious root formation remained similar to that observed in solvent-treated controls (Fig. 7G–J).

3.6. Alkamides induce adventitious root formation in auxin-signaling mutants

The result from *DR5:uidA* reporter expression analyses suggested that alkamide effects on adventitious root formation could not be related to auxin-altered responsive gene expression. To explore at the genetic level any potential role of the auxin-signaling pathway in mediating adventitious root formation induced by alkamides, we tested the effect of 24 μM *N*-isobutyl decanamide on adventitious root formation of the *aux1-7*, *eir1-1*, *axr2-1* and *axr4-1* auxin mutants. As shown in Fig. 8, *N*-isobutyl decanamide treatment induced a two- to threefold increase in adventitious root number in WT explants compared with solvent-treated controls. In media lacking *N*-isobutyl decanamide, explants from *axr2-1*, *aux1-7* and *axr4-1* mutants displayed a small, not significant reduction in adventitious root number compared to WT explants. When explants from all four auxin-resistant mutants were cultured in medium with 24 μM *N*-isobutyl decanamide, an increase in adventitious root formation was observed (Fig. 8). From these results, we conclude that explants from the auxin mutants are unaffected in *N*-isobutyl decanamide regulation of adventitious root formation.

3.7. N-Isobutyl decanamide increases endogenous nitric oxide in cultured A. thaliana shoot explants

Nitric oxide (NO) has been considered a signal involved in adventitious root development [18,19]. To further assess whether *N*-isobutyl decanamide could increase NO production and in this way promote adventitious root formation, we analyzed the presence of NO in explants by using the fluorescent probe DAF-2 DA. Living cells incorporate DAF-2 DA and subsequently, it is hydrolyzed by cytosolic esterases to release DAF-2, which reacts with NO to produce the fluorescent triazole derivative DAF-2T [20]. When *A. thaliana* hypocotyl explants transferred for 36 h to MS 0.2× medium were loaded with DAF-2 DA, specific green fluorescence was observed in pericycle tissue close to the cutting zone (Fig. 9A and B). In explants that were transferred to 36 μM *N*-isobutyl decanamide a very clear increase in fluorescence could be detected (Fig. 9C and D). In explants that were cultured for 14 d in *N*-isobutyl decanamide free medium, NO was absent from the stem and could be detected in developing adventitious roots (Fig. 9E and F). Interestingly, in *N*-isobutyl decanamide treated explants, increased formation of adventitious roots correlated with NO-specific fluorescence detected by the probe in explants, and thereafter, with increased NO levels within the stem and developing adventitious roots (Fig. 9G and H).

4. Discussion

In this study we evaluated the effects of *N*-isobutyl decanamide, a natural alkamide, in the morphogenetic responses of explants obtained from hypocotyls and roots of

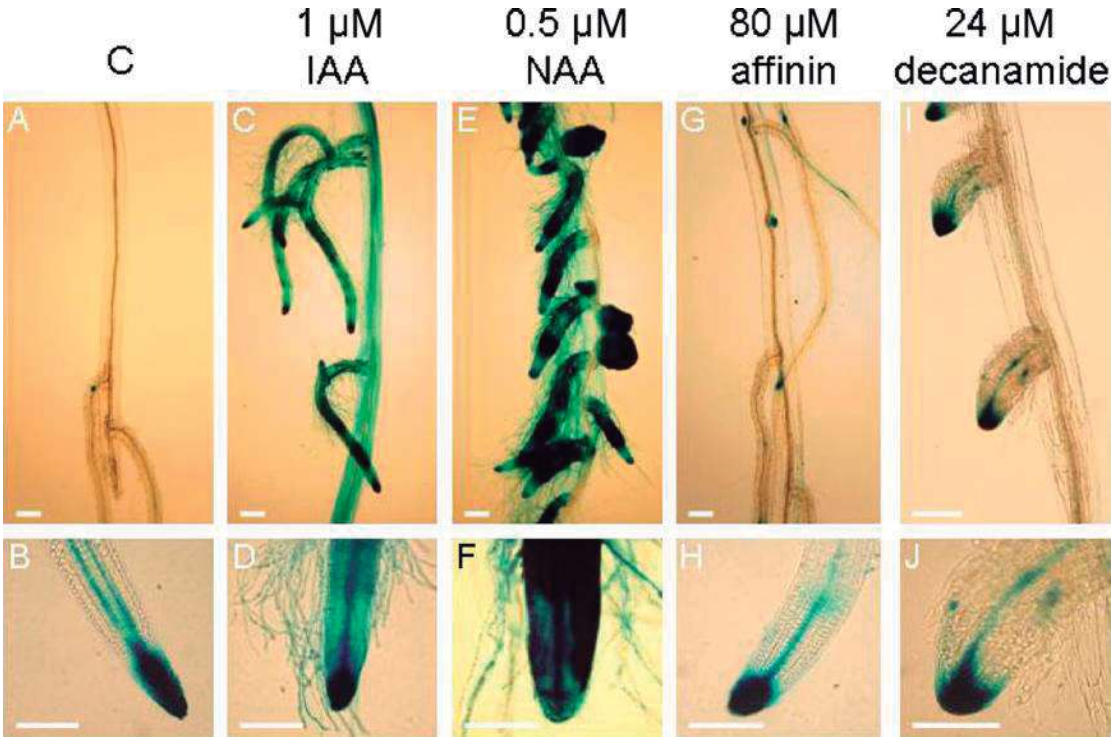

Fig. 7. Effect of alkamides on auxin-regulated gene expression. (A and B) Twelve hours of GUS staining of *DR5:uidA* type I explants grown for 14 d in medium without auxin, (C and D) under 1 μM IAA, (E and F) 0.5 μM NAA, (G and H) 80 μM affinin or (I and J) 24 μM *N*-isobutyl decanamide. Photographs are representative individual of at least 10 plants stained. Scale bar = 100 μm.

dark grown *A. thaliana* seedlings. *N*-isobutyl decanamide was chosen from the alkamide group because of their saturation grade and medium length of the fatty acid chain, a characteristic shared by the most active NAEs [5–7,9].

Our results confirmed previous findings suggesting that alkamides posses plant growth promoting properties and report the effects for this class of compounds in the regulation of adventitious root formation. Previously, Ramírez-Chávez et al. showed that affinin, the major alkamide present in *H. longipes* roots increased the number of lateral roots in *A. thaliana* seedlings. This effect was attributed to the dual activity of this compound to induce pericycle cells to divide and form new LRP, and to stimulate the emergence of existing LRP [8]. The effects of *N*-isobutyl decanamide in promoting lateral root emergence and increasing the number of total LRP in primary root explants (Figs. 2–4) suggest that both affinin and *N*-isobutyl decanamide may share common signaling mechanisms in regulating lateral root development. In later stages of development, *N*-isobutyl decanamide increased both shoot and root weights in plants regenerated from shoot explants, suggesting a positive effect of this alkamide in plant biomass production from tissue-cultured plants (Fig. 5).

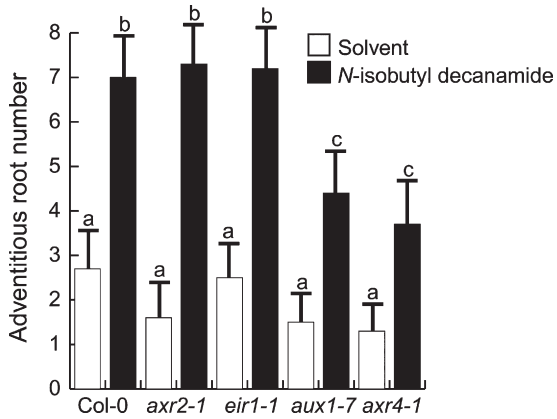

Fig. 8. Adventitious root response of wild-type (Col-0) and auxin-resistant mutants to *N*-isobutyl decanamide. Type I explants were obtained from all plant genotypes and grown for 14 d on MS 0.2× medium with or without 24 μM *N*-isobutyl decanamide. Values shown represent the mean adventitious root number per explant ($n = 30 \pm$ S.D.). Different letters are used to indicate means that differ significantly at the 0.05 level.

Our results show that both *N*-isobutyl decanamide and affinin increased the number of adventitious roots in *A. thaliana* seedlings (Fig. 6). This suggests that alkamides can mimic the effect of an endogenous compound that play some role in the generation and development of adventitious roots. Adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones and environmental factors such as light and wounding. Lateral and adventitious roots are formed post embryonically. While lateral roots typically form from the root pericycle, adventitious roots form naturally from stem tissue [21–23]. The molecular mechanisms by which adventitious root formation is regulated are still poorly understood. Two candidate pathways emerge as potential targets of the alkamide signal: (i) the classic auxin pathway; accumulating pieces of physiological and genetic evidence have demonstrated a critical role of the plant regulator auxin, which is supplied by shoot tissues through the polar transport system, in the initiation of adventitious roots [16,17],

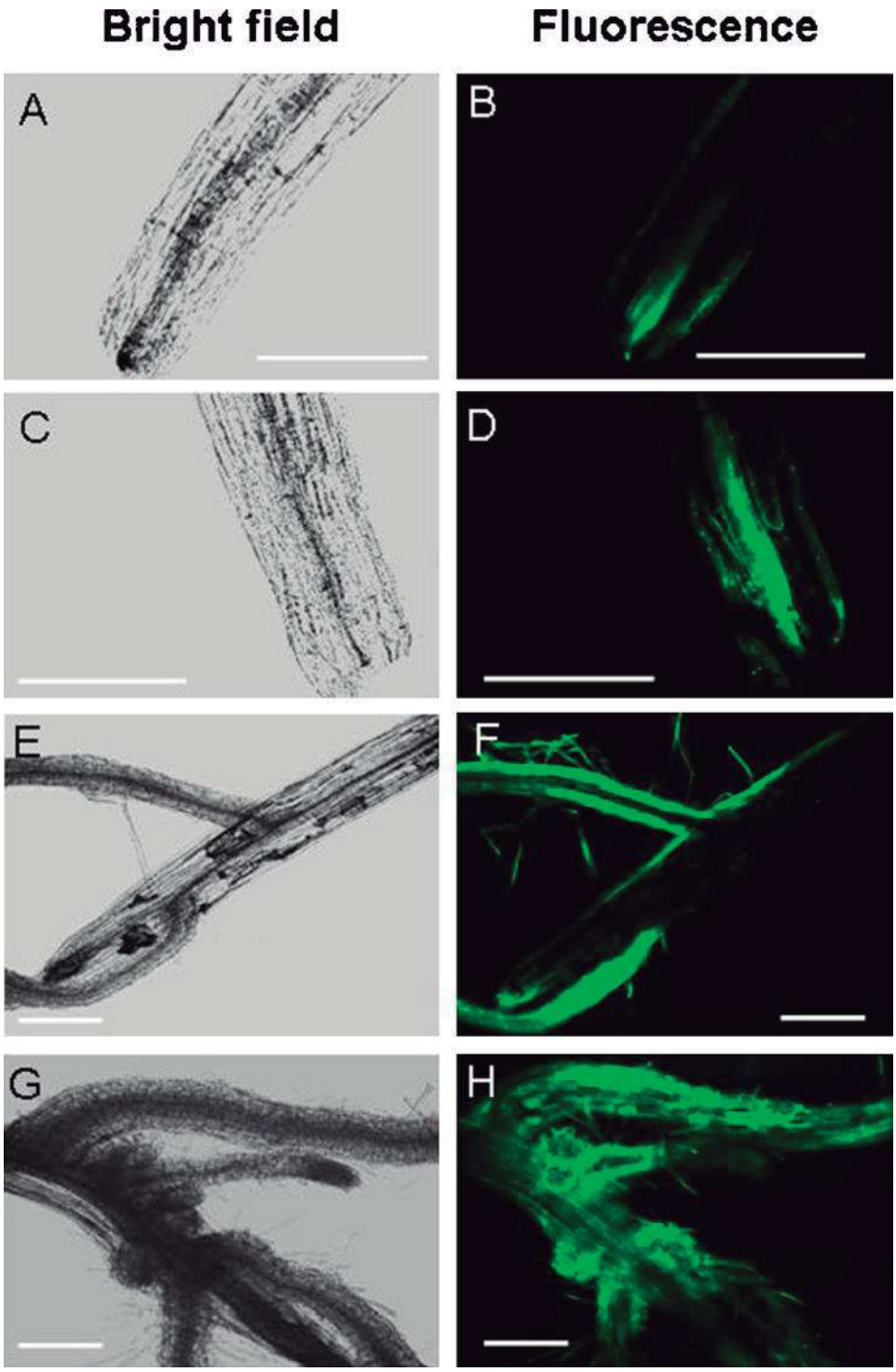

Fig. 9. Effect of *N*-isobutyl decanamide on NO accumulation in *A. thaliana* (Col-0) explants. (A and B) NO accumulation in type I explants grown for 36 h in medium without alkamide, or (C and D) under 36 μM *N*-isobutyl decanamide. (E and F) NO accumulation in explants grown for 14 d in medium without alkamide, or (G and H) under 36 μM *N*-isobutyl decanamide. Photographs are representative individuals of at least 10 DAF-DA loaded explants. Scale bars = 200 μm.

or (ii) the nitric oxide (NO) response route; NO is a diffusible multifunctional molecule involved in numerous physiological processes in plants [24]. It was recently found to play a critical role in adventitious root formation acting downstream of the auxin pathway [18,19]. The target molecules of both auxin and NO in the shoot response to generate adventitious roots are still unknown.

Our results show that auxin might be not involved in the root architectural responses of *A. thaliana* seedlings to alkamides. This hypothesis is mainly based on two lines of evidence: expression studies of the auxin-inducible marker *DR5:uidA* and the WT induction of adventitious roots in the auxin-resistant mutants *axr2-1*, *aux1-7*, *eir1-1* and *axr4-1* when grown in a stimulating concentration of *N*-isobutyl decanamide. In these

experiments, treatment with 1 μM IAA or 0.5 μM NAA induced *DR5:uid* expression in shoot explants, whereas concentrations up to 24 μM of *N*-isobutyl decanamide or 80 μM affinin failed to induce this auxin-inducible gene marker (Fig. 7). The adventitious root response of all four tested auxin-resistant mutants was equally sensitive to affinin than WT plants (Fig. 8), indicating that the root developmental effects induced by alkamides are not mediated by the known auxin-signaling pathway.

The relationship between NO and alkamides was also investigated in *A. thaliana* shoot explants. We showed that *N*-isobutyl decanamide is able to induce NO accumulation in different stages of development of the explants. NO was detected by confocal microscopic analysis in the sites of adventitious root formation and its level increased with alkamide treatment (Fig. 9). Whether NO mediates the adventitious root response and other morphogenetic responses of plants to alkamides remain to be investigated.

Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species for which it is difficult to induce rooting [25]. It would be also of interest to evaluate the rooting efficiency of different alkamides in horticulturally important species. We are currently involved in the isolation of alkamide-resistant *A. thaliana* mutants to clarify the genetic mechanisms involved in alkamide perception and their interactions with other plant signals.

Acknowledgements

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Characterization of *drr1*, an Alkamide-Resistant Mutant of Arabidopsis, Reveals an Important Role for Small Lipid Amides in Lateral Root Development and Plant Senescence[1][C][W][OA]

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Alkamides belong to a class of small lipid signals of wide distribution in plants, which are structurally related to the bacterial quorum-sensing signals *N*-acyl-L-homoserine lactones. Arabidopsis (*Arabidopsis thaliana*) seedlings display a number of root developmental responses to alkamides, including primary root growth inhibition and greater formation of lateral roots. To gain insight into the regulatory mechanisms by which these compounds alter plant development, we performed a mutant screen for identifying Arabidopsis mutants that fail to inhibit primary root growth when grown under a high concentration of *N*-isobutyl decanamide. A recessive *N*-isobutyl decanamide-resistant mutant (*decanamide resistant root* [*drr1*]) was isolated because of its continued primary root growth and reduced lateral root formation in response to this alkamide. Detailed characterization of lateral root primordia development in the wild type and *drr1* mutants revealed that *DRR1* is required at an early stage of pericycle cell activation to form lateral root primordia in response to both *N*-isobutyl decanamide and *N*-decanoyl-L-homoserine lactone, a highly active bacterial quorum-sensing signal. Exogenously supplied auxin similarly inhibited primary root growth and promoted lateral root formation in wild-type and *drr1* seedlings, suggesting that alkamides and auxin act by different mechanisms to alter root system architecture. 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 lateral root formation when exposed to stimulatory concentrations of jasmonic acid. Taken together, our results provide genetic evidence indicating that alkamides and *N*-acyl-L-homoserine lactones can be perceived by plants to modulate root architecture and senescence-related processes possibly by interacting with jasmonic acid signaling.

Plant growth and development require the integration of a variety of environmental and endogenous signals, which together with the intrinsic genetic program determine plant form and longevity. Lipids have long been recognized as signals that have the capacity to trigger profound physiological responses. In animals, ceramides and sphingosines are lipids that have proapoptotic and antiproliferative actions (Wymann and Schneiter, 2008). In plants, ceramides, sphingosines, and phosphatidic acid are involved in mediating plant growth, development, and responses to environmental stimuli (Worrall et al., 2003; Wang, 2004).

In the past few years, additional small lipids have been found to acts as plant signals, including alkamides and *N*-acyl-ethanolamines (NAEs). Alkamides comprise at least 200 amides with varied acyl chain lengths and saturation grades (for review, see López-Bucio et al., 2006; Morquecho-Contreras and López-Bucio, 2007). These compounds have been found to alter root and shoot system architecture in Arabidopsis (*Arabidopsis thaliana*; Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008). NAEs represent compounds with aminoalcohol linked as an amide to the fatty acid. They are likely produced from the hydrolysis of *N*-acyl-phosphatidylethanolamines, a minor constituent of cell membranes, by phospholipase D (Chapman, 2004). NAEs have been found to accumulate in seeds of some higher plants, including cotton (*Gossypium hirsutum*), corn (*Zea mays*), Arabidopsis, soybean (*Glycine max*), tomato (*Solanum lycopersicum*), and pea (*Pisum sativum*), and their levels decline during germination (Wang et al., 2006).

Many gram-negative bacteria produce alkamide-related substances termed *N*-acyl-L-homoserine lactones (AHLs). These compounds participate in

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cell-to-cell signaling that is usually referred to as quorum sensing (Pearson et al., 1994). The AHL signals contain a conserved HL ring and an *N*-linked acyl side chain. The acyl-chain moiety of naturally occurring AHLs can differ in length and substitution at position C3, which is either unmodified or carries an oxo or hydroxyl group (Pearson et al., 1994; Parsek et al., 1999). These molecules are freely diffused through the bacterial membrane, which is to some extent dependent upon the length of the acyl side chain and the nature of any C3 substitutions and distribute within the rhizosphere (Pearson et al., 1999; Schuhegger et al., 2006; Scott et al., 2006). Evidence has accumulated indicating that plants are able to perceive AHLs. The application of AHLs to *Medicago truncatula* and Arabidopsis plants resulted in differential transcriptional changes in roots and shoots, affecting the expression of genes potentially involved in development (Mathesius et al., 2003; Von Rad et al., 2008). Ortíz-Castro et al. (2008) evaluated Arabidopsis growth responses to a variety of saturated AHLs ranging from four to 14 carbons in length, focusing on alterations in postembryonic root development. The compounds affected primary root growth, lateral root (LR) formation, and root hair development. While this information clearly indicates that plants are able to sense a variety of small lipid signals, including alkamides, NAEs, and AHLs, which modulate root architecture, the genetic mechanisms involved in signal perception to these compounds are unknown.

The Arabidopsis root system is an excellent model to dissect the genetic and developmental processes that determine plant architecture. It mainly consists of an embryonic primary root and postembryonic developed LRs (López-Bucio et al., 2005). LR formation is influenced by a wide range of environmental cues, such as nutrients and water availability in the soil (López-Bucio et al., 2003; Malamy, 2005; Nibau et al., 2008). The plasticity of LR formation is of critical importance, allowing plants to compete for resources and adapt to constantly changing growth conditions. LRs originate from pericycle founder cells located opposite to xylem poles, which undergo several rounds of anticlinal divisions to create a single-layered primordium composed of up to 10 small cells of equal length (termed stage I; Dolan et al., 1993; Malamy and Benfey, 1997; Dubrovsky et al., 2001). Further anticlinal and periclinal divisions create a dome-shaped primordium (spanning stages III–VII), which eventually emerges from the parental root (Malamy and Benfey, 1997; Casimiro et al., 2003; Péret et al., 2009).

The phytohormone auxin (indole-3-acetic acid [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 (NAA) stimulates LR formation (Celenza et al., 1995; Woodward and Bartel, 2005), whereas polar auxin transport inhibitors such as *N*-(1-naphthyl)-phthalamic acid 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 numbers of LRs (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants with defective auxin transport, perception, or signaling, including *aux1*, *axr1*, and *tir3/doc1/big*, show reduced LR formation (Lincoln et al., 1990; Gil et al., 2001; Swarup et al., 2001). In contrast to auxin, less is known about the action of alkamides, AHLs, and other related small lipid signals on LR formation and whole plant development.

To identify the genetic components responsible for the root architectural responses to alkamides, we performed a visual screening for Arabidopsis mutants that under high *N*-isobutyl decanamide concentration do not manifest primary root growth reduction. We isolated an *N*-isobutyl decanamide-resistant mutant (*decanamide resistant root* [*drr1*]) defective in a single recessive trait. Detailed cellular and developmental studies of wild-type and *drr1* plants indicate that *drr1* mutants show resistance to primary root growth inhibition and LR growth promotion induced by both an alkamide (*N*-isobutyl decanamide) and a bacterial quorum-sensing signal (*N*-decanoyl-L-homoserine lactone [C10-AHL]). We further show that *DRR1* is a crucial component of the regulation of plant senescence, which likely links alkamide and jasmonic acid (JA) in modulating plant longevity and LR development.

RESULTS

Isolation of *drr1*, an Arabidopsis Mutant with Altered Primary Root Growth Response to *N*-Isobutyl Decanamide

From a group of similar chain length alkamides and NAEs, López-Bucio and coworkers (2007) identified *N*-isobutyl decanamide, a C10 saturated alkamide that is naturally produced in *Acmella radicans* (Ríos-Chávez et al., 2003) and *Cissampelos glaberrima* (Laurerio-Rosario et al., 1996), as the most active compound in inhibiting primary root growth and stimulating LR formation in Arabidopsis.

To investigate the genetic basis of plant responses to alkamides, we screened 25,000 lines from T-DNA insertion mutant collections (Krysan et al., 1999) by inspecting the root architecture of plants grown over the surface of 0.2× Murashige and Skoog (MS) agar plates supplied with 30 μM *N*-isobutyl decanamide. A mutant line was isolated that, in contrast to the wild type, was able to sustain primary root growth under this inhibitory concentration of the alkamide (Fig. 1A). The mutant was backcrossed to wild-type plants (Wassilewskija [Ws] ecotype) three times prior to detailed phenotypical analysis. In F2 progeny from these crosses, in plants grown in medium supplied

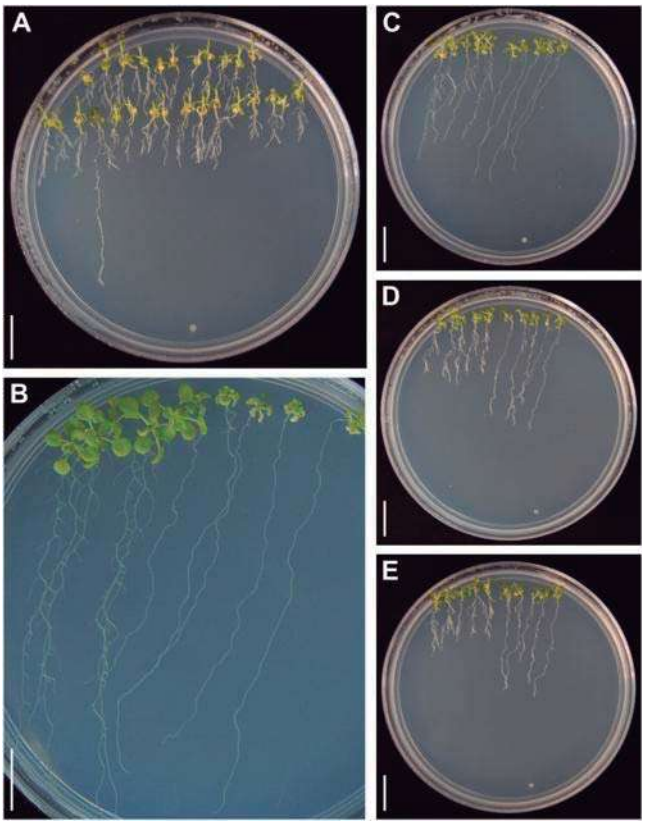

Figure 1. Genetic screen and phenotypic characterization of *drr1* mutants. A, Photograph of an agar plate supplied with 30 μM *N*-isobutyl decanamide showing a putative *drr1* mutant with long primary root. B, Five 14-d-old wild-type (Ws) and *drr1* seedlings grown side by side on the surface of agar plates containing 0.2× MS medium lacking *N*-isobutyl decanamide. C to E, Photographs of agar plates supplied with 20 μM (C), 25 μM (D), or 30 μM (E) *N*-isobutyl decanamide, showing five wild-type (left) and *drr1* (right) plants grown side by side. Photographs in B and C are representative individuals of four plates per treatment. Bars = 1 cm. [See online article for color version of this figure.]

with 30 μM *N*-isobutyl decanamide, the line segregated the mutant phenotype in a 1:3 ratio (Table I). These results indicate that the primary root growth resistance to the alkamide resulted from a recessive single-gene mutation. We named this locus *drr1*. To further study the developmental alterations induced by *N*-isobutyl decanamide in wild-type and *drr1* plants, we grew ecotype Ws and *drr1* plants side by side on vertically oriented agar plates with varied alkamide contents. Wild-type plants grown in 0.2× MS agar medium without *N*-isobutyl decanamide showed a typical root system, consisting of a long primary root with many LRs forming in a gradient from the root/shoot junction to the primary root tip (Fig. 1B). In the same medium, *drr1* mutants developed a long primary root lacking visible LRs (Fig. 1B), thus indicating that *DRR1* is important for normal LR development under normal growth conditions. In wild-type plants treated with 20, 25, or 30 μM *N*-isobutyl decanamide, there was a dose-dependent inhibitory effect of the alkamide on primary root growth, which correlates with an increase in LR formation. In these plants, multiple LRs developed, giving rise to a highly exploratory root system with different architecture from that observed in plants grown in medium without *N*-isobutyl decanamide (Fig. 1, C–E). In contrast, alkamide-treated *drr1* mutants showed longer primary roots and reduced LR formation when compared with wild-type plants in most concentrations of *N*-isobutyl decanamide tested (Fig. 1, C–E).

drr1 Mediates the Root Architecture Responses of Arabidopsis to *N*-Isobutyl Decanamide

To more clearly define the alterations in the root architectural response to *N*-isobutyl decanamide caused by mutation in *DRR1*, we performed temporal and single-point measurements of primary root length, LR number per plant, and LR density in wild-type and *drr1* mutants treated with varied concentrations of *N*-isobutyl decanamide. Primary root growth was similar in wild-type and *drr1* plants in concentrations of up to 15 μM *N*-isobutyl decanamide, while in concentrations of 20, 25, and 30 μM of this compound, *drr1* primary roots were significantly longer that wild-type plants (Fig. 2A). *N*-Isobutyl decanamide increased the number of emerged LRs in wild-type plants, while *drr1* plants were resistant to this effect (Fig. 2B). The density of emerged LRs dramatically increased in response to alkamide treatment in wild-type plants, but the mutants again showed reduced responses. The most contrasting responses between wild-type and *drr1* plants were observed in 25 μM *N*-isobutyl decanamide, in which wild-type plants showed a highly branched root system harboring second- and third-order LRs (Fig. 1, C–E), with a 6-fold increased density of LRs (Fig. 2C). In this alkamide concentration, *drr1* mutant plants produced less than 15% of the LRs observed in wild-type plants. Interestingly, although *drr1* mutants produced

Table I. *Segregation ratio of progeny resulting from crosses between drr1 mutant and wild-type seedlings*

Generation	Phenotype of Progeny		Ratio Obtained, Wild Type:*drr1*	Ratio Tested, Wild Type:*drr1*	χ^{2a}
	Many LRs (Wild Type)	Few LRs (*drr1*)			
F1	128	0			
F2	730	260	2.81:1	3:1	0.84

[a]With one degree of freedom and a critical value of 5%, the hypothesis is accepted if the χ^2 is smaller than 3.841.

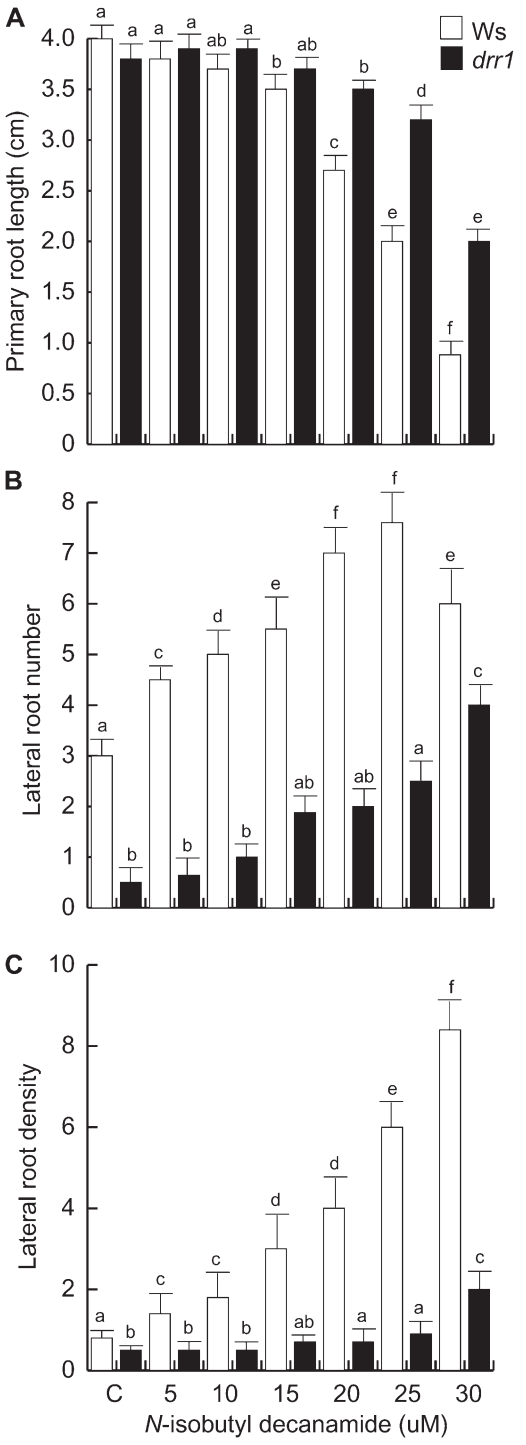

Figure 2. Effects of *N*-isobutyl decanamide on the root system architecture of wild-type (Ws) and *drr1* plants. A, Primary root length. B, Number of emerged LRs per plant. C, LR density expressed as the number of LRs per centimeter. Data were recorded at 12 d after germination. Values shown are means ± SD ($n = 20$). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

consistently fewer LRs compared with the wild type in most *N*-isobutyl decanamide treatments, exposure to 30 μM *N*-isobutyl decanamide caused an 8-fold increase in LR number and a 2-fold increase in LR density (Fig. 2, B and C), indicating that the mutants are not completely insensitive to the alkamide.

drr1 Mutants Are Resistant to Inhibitory Effects of *N*-Isobutyl Decanamide on Cell Division in Primary Root Meristems

An important factor determining primary root growth reduction in wild-type seedlings grown in high *N*-isobutyl decanamide concentrations is the reduction in cell division in the root meristem (López-Bucio et al., 2007). To analyze the cell division responses of *drr1* mutants to alkamide treatment, we crossed *drr1* with a transgenic plant harboring the *CycB1*:*uidA* construct, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al., 1999). *CycB1*:*uidA* seedlings and *drr1* seedlings were grown in 0.2× MS agar medium supplied with the solvent or with 20, 25, and 30 μM *N*-isobutyl decanamide. In both wild-type *CycB1*:*uidA* and *drr1* mutant seedlings supplied with the solvent only, a patchy pattern of single cells expressing *CycB1*:*uidA* was observed in the primary root meristem (Fig. 3, A and B). In wild-type plants subjected to treatment with 30 μM *N*-isobutyl decanamide, GUS expression in the primary root tip decreased and root hairs were formed in close proximity to the root meristem (Fig. 3C). Interestingly, *CycB1*:*uidA* expression in the primary root apex of *drr1* seedlings treated with the alkamide was not as much inhibited as in the wild type, and their root meristems were anatomically similar to those of solvent only-treated seedlings (Fig. 3D). Root hair formation close to the root meristem was not observed in *drr1* seedlings treated with the alkamide (Fig. 3D). Next, we quantified the length of the primary root meristems in wild-type and *drr1* plants at 4 and 12 d after germination. At these developmental stages, increased concentrations of *N*-isobutyl decanamide decreased the length of the meristem in wild-type plants, while *drr1* mutants were resistant to this effect (Fig. 3E).

drr1 Is Defective in *N*-Isobutyl Decanamide-Induced LR Primordia Development

LR formation is a major determinant of root system architecture. Next, we investigated the effects of *N*-isobutyl decanamide on lateral root primordia (LRP) development and LR emergence in wild-type and *drr1* plants. LRP originating in the primary root and emerged LRs were counted at 6 d after germination in plants grown in 0.2× MS agar medium supplied with the solvent only or with 30 μM *N*-isobutyl decanamide. The developmental stage of each LRP was classified according to Malamy and Benfey (1997; see "Materials and Methods"). In solvent-treated wild-type plants,

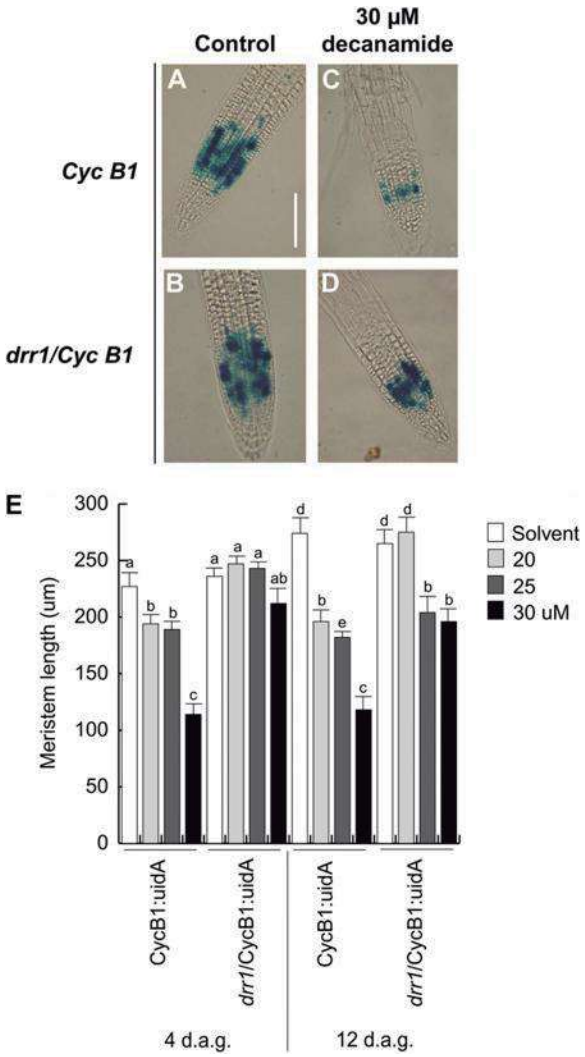

Figure 3. *CycB1*:*uidA* expression in transgenic wild-type and *drr1* seedlings. Twelve-hour GUS staining is shown for *CycB1*:*uidA* primary roots in wild-type and *drr1* Arabidopsis seedlings grown on agar-solidified 0.2× MS medium with or without *N*-isobutyl decanamide. A and B, Solvent-treated seedlings. C and D, Plants supplied with 30 μM *N*-isobutyl decanamide. E, Meristem length. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Bar = 100 μm. d.a.g., Days after germination. [See online article for color version of this figure.]

most LRP remained at an early developmental stage (stage I). Interestingly, *N*-isobutyl decanamide treatment increased both the number of LRP at stage I and the density of emerged LRs (Fig. 4). Solvent-treated *drr1* mutants showed similar LRP density to wild-type plants (Fig. 4A) but dramatically decreased density of emerged LRs (Fig. 4B), indicating that the mutant is not inherently defective in LRP initiation but rather shows a retardation in the maturation of LRP. When treated with *N*-isobutyl decanamide, *drr1* mutant seedlings did not show an increase in stage I LRP or in LRP emergence observed in wild-type plants (Fig. 4), indicating the *DRR1* locus is involved in alkamide responses in the pericycle and during LRP development. Although alkamide treatment significantly increased the density of emerged LRs in *drr1* mutants, *drr1* always showed lower LR density than wild-type plants in the different growth conditions (Fig. 4B). These results indicate that *N*-isobutyl decanamide modifies root system architecture both by inducing more pericycle cells to form stage I LRP and by accelerating the emergence of LRP from the primary root to form mature LRs. Mutations in *drr1* interfere with both of these processes.

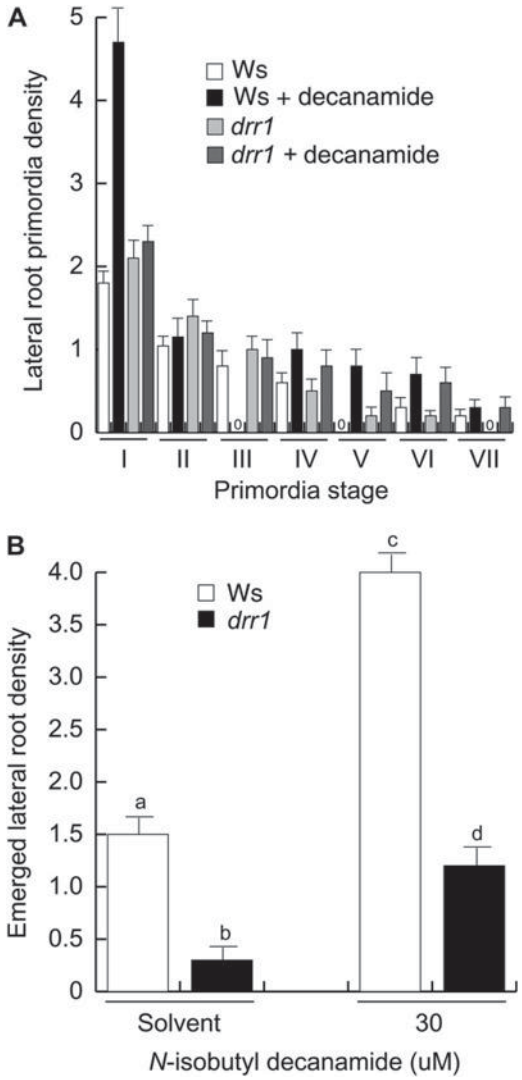

Figure 4. Effects of *N*-isobutyl decanamide on wild-type (Ws) and *drr1* LR development. A, LRP stage distribution in 6-d-old primary roots grown on medium supplied with the solvent only or with 30 μM *N*-isobutyl decanamide (indicated as decanamide). B, Emerged LR density in the same experiment. Wild-type and *drr1* seedlings were cleared, and the number and stage of LRP were recorded according to Malamy and Benfey (1997). Values shown are means ± SD (n = 15). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results.

drr1 Is Defective in Root Architectural Responses to C10-AHL, a Quorum-Sensing Signal from Bacteria

Previous studies documented that AHLs, a class of alkamide-related quorum-sensing signals from bacteria, modulate root system architecture in Arabidopsis (Ortíz-Castro et al., 2008; Von Rad et al., 2008). To determine if *DRR1* is involved in AHL responses, we tested the primary root growth and LR responses of *drr1* seedlings to C10-AHL over a range of concentrations of this compound as compared with wild-type plants. *drr1* had a level of resistance to primary root growth inhibition by C10-AHL over most concentrations tested (Fig. 5A). At 30 μM C10-AHL in wild-type plants, about 60% inhibition of growth occurred, whereas in the mutant, it was about 30%. As previously reported (Ortíz-Castro et al., 2008), C10-AHL stimulated LR formation (Fig. 5B). A dose-dependent effect increasing LR density was observed (Fig. 5C), confirming the positive role of AHLs in LR induction. In contrast, *drr1* mutants showed reduced LR formation when compared with wild-type seedlings over most concentrations of C10-AHL tested (Fig. 5, B and C).

drr1 Shows Normal Auxin Responses

Several auxin-related mutants have been characterized in screens for primary root growth resistance to inhibitory amounts of IAA, which display alterations in LR formation (Rogg et al., 2001; Swarup et al., 2001; Fukaki et al., 2002). To determine if *drr1* operates in a genetically defined auxin pathway, wild-type Arabidopsis seedlings (ecotype Columbia [Col-0] and Ws), *drr1* seedlings, and the auxin-related mutants *aux1-7* and *axr2* were evaluated in primary root growth response assays to IAA. First, to confirm the auxin resistance of auxin-related mutant lines, homozygous *aux1-7* and *axr2-1* seedlings were screened for resistance to IAA based on primary root growth. In these experiments, *aux1-7* and *axr2* were resistant to the inhibition of primary root elongation by IAA when compared with wild-type Col-0 seedlings (Fig. 6A). These mutants also failed to form abundant root hairs at the root tip region in response to increasing IAA concentration in the medium, a phenotype associated with increased auxin resistance (Fig. 6B). In contrast, the auxin response in *drr1* mutants was equally sensitive to IAA than the wild-type (Ws ecotype) both in primary root growth assays (Fig. 6A) and toward induction of root hair formation close to the root tip (Fig. 6B). Because *drr1* mutants showed normal root responses to IAA, we conclude that auxin signaling is unaffected in the mutant.

To better understand the role played by auxin in LR formation in wild-type and *drr1* plants, we tested the effects of NAA to activate LR formation in a transfer assay. In these experiments, wild-type and *drr1* plants were first germinated and grown for 7 d in 0.2× MS agar medium. At day 7 after germination, plants were

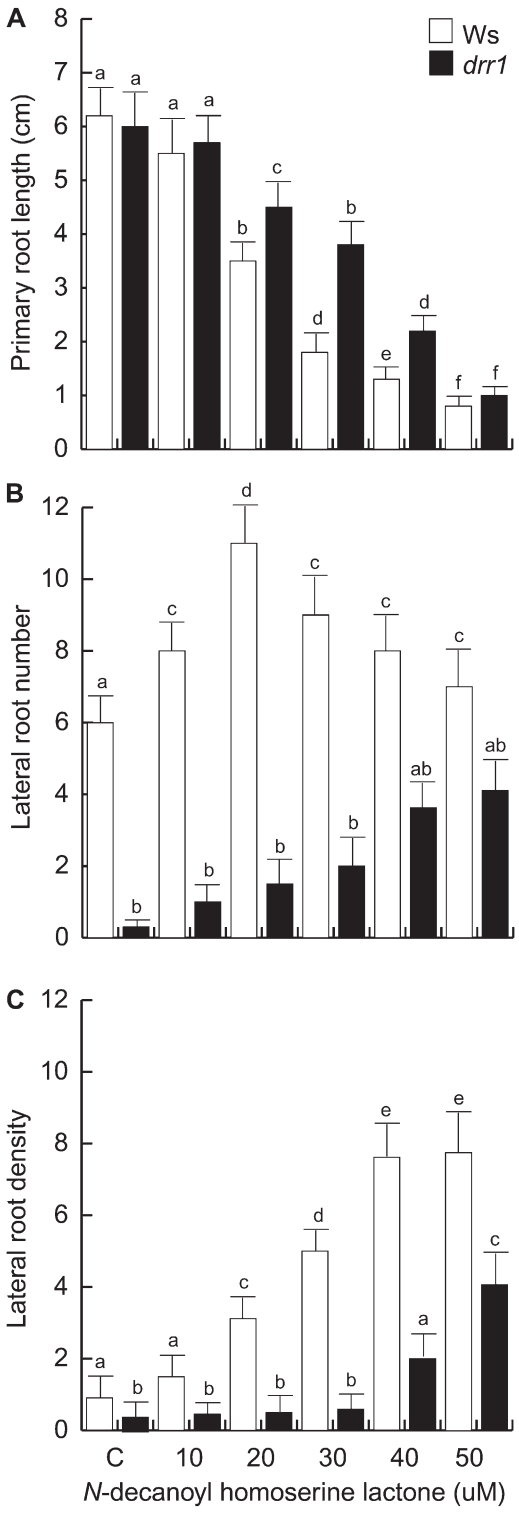

Figure 5. Effects of C10-AHL on the root system architecture of wild-type (Ws) and *drr1* plants. A, Primary root length. B, Number of emerged LRs per plant. C, LR density expressed as the number of LRs per centimeter. Data were recorded at 14 d after germination. Values shown are means ± SD (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

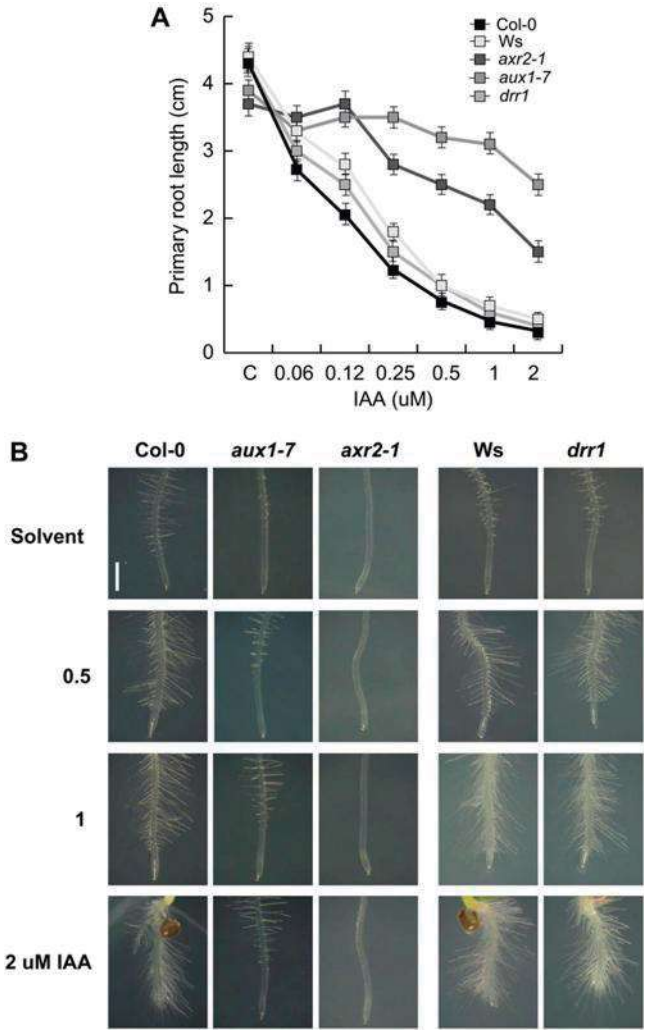

Figure 6. Auxin responses in wild-type and *drr1* seedling roots. A, Primary root growth in 12-d-old primary roots of wild-type (Col-0 and Ws), *axr2-1* and *aux1-7* auxin-resistant mutants, and *drr1* Arabidopsis mutants grown on medium supplied with the solvent only or with varied IAA concentrations. B, Morphology of root tips of wild-type and mutant lines exposed to IAA. Seedlings were photographed at 7 d after germination using a digital camera connected to a dissecting microscope. Values shown in A are means ± SD ($n = 30$). The experiment was repeated twice with similar results. [See online article for color version of this figure.]

transferred to 0.2× MS liquid medium supplied with the solvent or varied concentrations of NAA for an additional 4-d period. At this stage, the number and density of LRs were determined. As shown in Figure 7, NAA treatment caused a dose-response effect in LR formation (Fig. 7A), which was similar between wild-type and *drr1* plants. Both wild-type and *drr1* plants produced highly branched root systems with normal LR growth (Fig. 7, B–I). These results indicate that *drr1* seedlings are not inherently defective in pericycle cell activation to form LRs and are able to correctly sense and respond to auxins.

drr1 Mutants Show Extended Longevity

To study the role of *DRR1* in plant growth and development, we compared the phenotype of wild-type and homozygous *drr1* plants of the same age that were first germinated and grown for 10 d on 0.2× MS agar medium and then transferred to soil. Wild-type and *drr1* plants were grown side by side during their entire life cycle. The young and adult phenotypes of plants are shown in Figure 8. During the first 28 d after transfer, a general delay in the growth of *drr1* mutants was observed, as illustrated by their delay in stem formation (Figs. 8, A and B, and 9A) and significantly decreased rosette size during early stages of vegetative growth (Fig. 9B). At 28 d after transfer, the rosette leaves of wild-type plants had already turned yellow and stem growth ceased, but *drr1* leaves remained

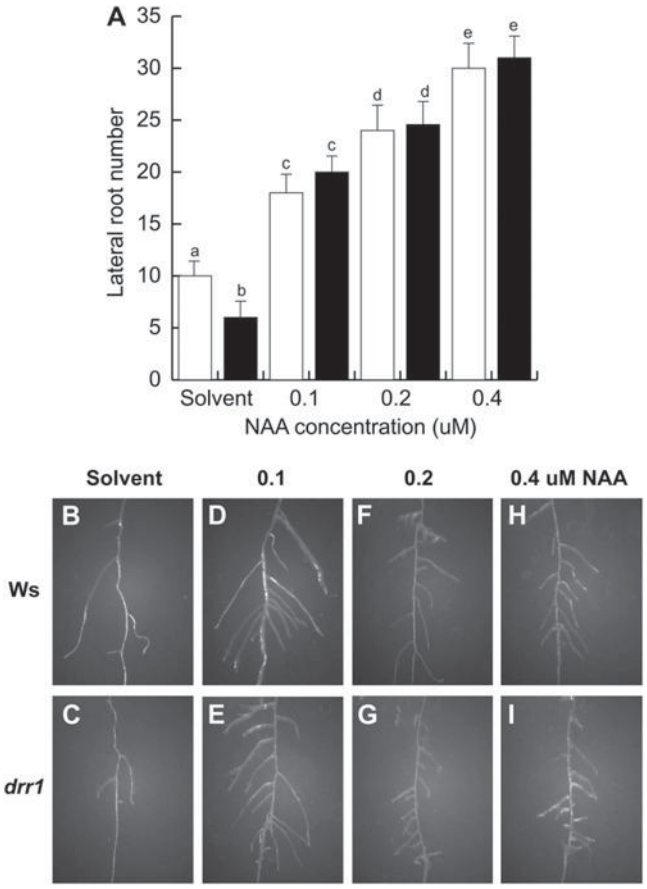

Figure 7. Auxin restoration of LR development in *drr1* plants. A, Total LR number per plant in 11-d-old wild-type (Ws) and *drr1* plants that were first grown for 7 d in 0.2× MS agar medium and then transferred to 0.2× MS liquid medium supplied with the solvent or with varied concentrations of NAA for an additional 4-d growth period. B to I, Representative photographs of wild-type and *drr1* LRs in plants exposed to NAA. Values shown in A are means ± SD ($n = 15$). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results. [See online article for color version of this figure.]

Figure 8. Phenotypes of wild-type and *drr1* plants grown in soil. A to D, Phenotypes of wild-type (Ws; left) and *drr1* (right) plants grown side by side at 14, 28, 56, or 84 d after transfer to soil. E, Closeup of rosette leaves at 84 d after transfer. Plants were grown with a 16-h-light/8-h-dark cycle at 22°C in a growth chamber. The retarded leaf senescence in *drr1* was related to the retarded emergence of floral stems and flowering time. [See online article for color version of this figure.]

green, and 7 d later, the stems just started to be formed (Figs. 8, A–C, and 9, A and B). At 35 d after transfer, wild-type leaves had turned completely yellow and showed signs of death with drying (Fig. 8C). In contrast, the *drr1* mutant leaves retained a significant amount of chlorophyll and maintained the integrity of the leaf shape (Fig. 8C). The extension of leaf longevity at a whole plant level dramatically increased in *drr1* mutants with time. Delayed flowering was accompanied by the generation of new leaves, increased rosette size, and greater stem length in *drr1* mutants when compared with wild-type plants (Figs. 8, D and E, and 9, A–C). *drr1* sustained chlorophyll production for a longer time period (Fig. 9D). In addition, the shoot architecture of *drr1* mutants was different from that observed in wild-type seedlings, producing only one primary stem with reduced branches, which suggests increased apical dominance in the mutants (Fig. 9, E and F). Aside from the delayed senescence and altered shoot architecture, *drr1* mutants produced fertile flowers that yielded fruits with fully viable seeds (Fig. 8D). We determined that the longevity in *drr1* mutants was extended by approximately 2-fold when compared with wild-type plants, which correlates with a 3- to 4-fold increase in the number of visible leaves and overall increased plant size (Fig. 9, A–C).

The *drr1* Mutant Shows Delayed Senescence Symptoms in JA- and Alkamide-Induced Senescence

Leaf senescence is modulated by JA (Schommer et al., 2008). Therefore, the possibility was open that the *drr1* mutant could be deficient in the JA-induced senescence program. We compared the effects of JA and *N*-isobutyl decanamide in wild-type and *drr1* plants in a senescence-induced assay for detached leaves (Fig. 10). In this assay, after 6 d of incubation in water, wild-type detached leaves gradually lost chlorophyll content (Fig. 10, A and C). A deficient senescence program for detached *drr1* plants incubated in water was evident (Fig. 10, B and D). In response to treatments with JA and *N*-isobutyl decanamide, wild-type leaves showed severe senescence symptoms that were reduced in *drr1* mutants (Fig. 10, E–I). Taking together the increased *drr1* longevity in soil and the delaying response to hormone-induced senescence, we conclude that *DRR1* plays an important role in the senescence process modulated by JA and *N*-isobutyl decanamide as well as in age-dependent senescence.

drr1 Is Altered in Jasmonate-Mediated LR Induction

Jasmonates are signals involved in root system architecture modulation (Wasternack, 2007). The in-

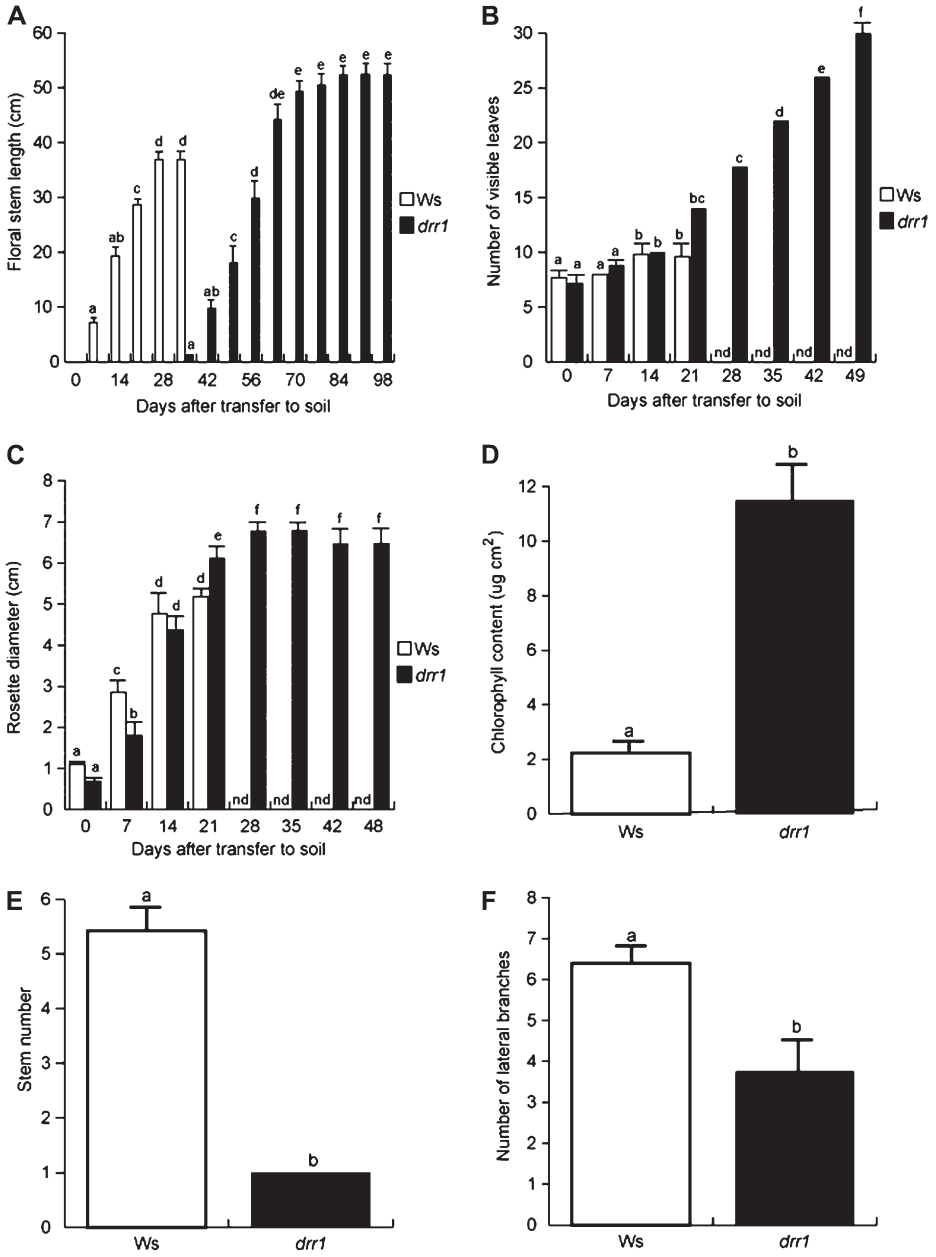

Figure 9. Age-dependent senescence symptoms and other developmental traits of wild-type (Ws) and *drr1* plants grown in soil. A, Age-dependent stem size. B, Rosette diameter. C, Number of visible rosette leaves. D, Chlorophyll content in rosette leaves at 28 d after transfer to soil. E, Stem number. F, Number of stem branches. Plants were grown with a 16-h-light/8-h-dark cycle at 22°C in a growth chamber, and developmental traits were monitored during their entire life cycle. Values shown are means ± SD ($n = 18$). Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results. nd, Not determined.

hibitory effect of methyl jasmonate (MeJA) on primary root growth has been well recognized and widely employed as a useful trait to identify jasmonate-related mutants in Arabidopsis. Recently, it has been reported that MeJA also promotes LR formation (Sun et al., 2009). The MeJA dose response of *drr1* in primary root growth and LR formation was compared with *jar1*, a MeJA-insensitive mutant, and wild-type seedlings of the Ws and Col-0 ecotypes; the Col-0 ecotype provided the genetic background for *jar1* and therefore was included as an additional control. When compared with Ws and Col-0 plants, the *jar1* mutant showed strong resistance to MeJA-induced primary root growth inhibition over most concentrations tested (Fig. 11A). The primary root growth inhibition in *drr1* was essentially the same as in Ws seedlings (Fig. 11A). Interestingly, MeJA, at concentrations of 30 to 45 μM, increased emerged LRs in wild-type seedlings and in *jar1* mutants by 70% to 150% (Fig. 11B; Supplemental Fig. S1). In the absence of MeJA, LR formation in *drr1* was significantly reduced compared with wild-type and *jar1* plants. However, *drr1* mutants failed to produce increased numbers of LRs when grown on medium containing a range of concentrations of MeJA (Fig. 11B; Supplemental Fig. S1). Our data reveal that *drr1* encodes a novel genetic locus modulating the effects of MeJA on LR formation.

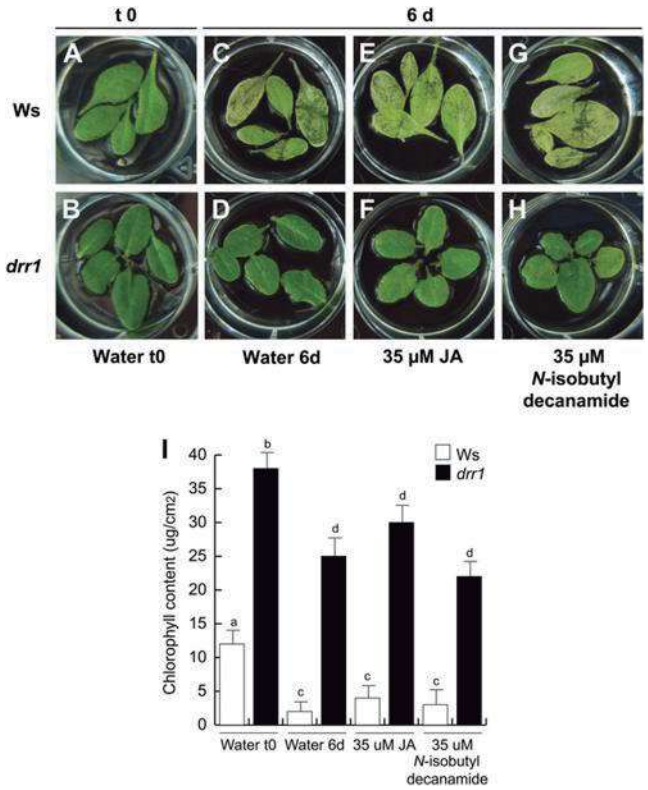

Figure 10. Hormone-dependent senescence symptoms in the *drr1* mutant. Detached leaves of wild-type (Ws) and *drr1* plants were incubated in 2-mL water solutions supplied with the indicated concentrations of compounds. The plates were included in a growth chamber (Percival ARR95L) under dark conditions, and representative photographs of leaves subjected to the different treatments were taken 6 d later (A–H) and chlorophyll determination was performed (I). The experiment included at least three independent samples of five leaves each and was replicated three times with similar results. [See online article for color version of this figure.]

DISCUSSION

drr1 Mutants Define a Locus Involved in Root Architectural Responses to Both Alkamides and AHLs

This report describes the identification and characterization of an Arabidopsis mutant that was defective in its root response to *N*-isobutyl decanamide but with additional characteristics, which suggest that alkamides play a role in plant longevity. Our characterization of root architectural responses in the wild type and *drr1* to *N*-isobutyl decanamide provided insights into the genetic mechanisms mediating the responses to alkamides. While *N*-isobutyl decanamide inhibited primary root growth and promoted LR formation in wild-type seedlings, resistance to the repressive effect of this alkamide on primary root growth and the failure to increase LR formation typified the *drr1* phenotype (Figs. 1 and 2).

Detailed cellular analysis of wild-type and *drr1* plants showed that the mutants sustained almost normal root meristematic activity when grown under inhibitory concentrations of *N*-isobutyl decanamide, as revealed by cell counts and *CycB1:uidA* expression in the primary root meristem (Fig. 3). Interestingly, the typical increase in LR primordia initiation and LR emergence observed in wild-type plants treated with the alkamide was reduced in *drr1* (Fig. 4). Several types of reported experimental evidence suggested that conditions that reduce primary root meristematic activity, including destruction of the primary root meristem by cell ablation and physical decapitation of the root tip, elicit an increase in LR number (Tsugeki and Fedoroff, 1999). Our findings that *drr1* mutants grown in medium lacking alkamides sustain normal primary root growth but reduced LR formation (Figs. 1 and 2) indicate that LR proliferation in response to *N*-isobutyl decanamide is not a direct consequence of

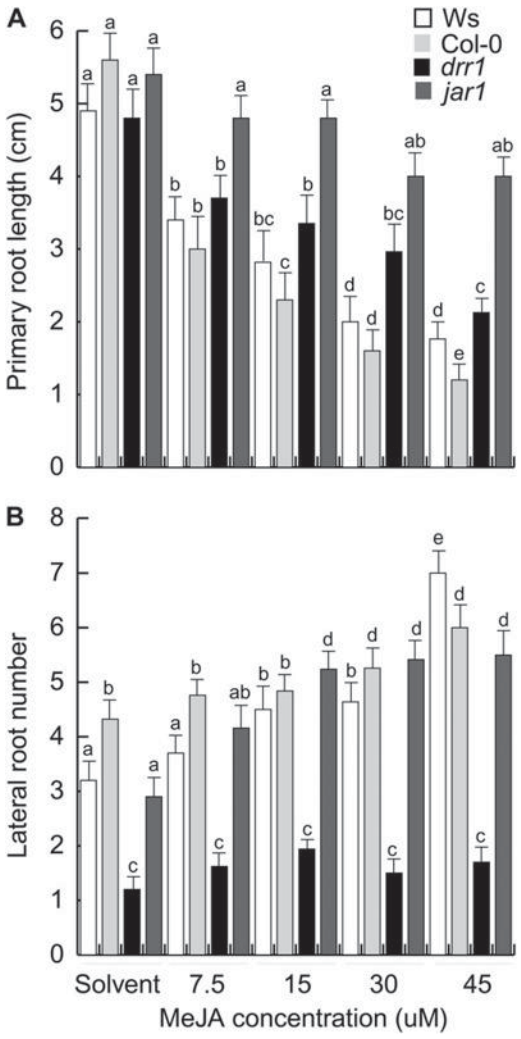

Figure 11. Effects of JA on primary root growth and LR development in wild-type (Ws and Col-0) and mutant (*drr1* and *jar1*) lines. A, Primary root length. B, Number of emerged LRs per plant. Data were recorded at 12 d after germination. Values shown are means ± SD (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

primary root growth inhibition but rather suggest a positive effect of the alkamide on pericycle cells to produce more LRP (Fig. 4).

Many bacterial species use small molecule signaling to communicate with each other and to coordinate their growth activities, a process commonly referred to as quorum sensing (Taga and Bassler, 2003; Reading and Sperandio, 2006). Diverse gram-negative bacteria produce AHLs, and these compounds contain a conserved HL ring and an *N*-linked acyl side chain. Our previous work indicated that saturated medium (C8–C14)-chained AHL compounds showed a dose-dependent effect on root architecture, inhibiting primary root growth and promoting LR formation (Ortíz-Castro et al., 2008). In this work, we used the *drr1* mutant to determine whether alkamides and AHLs could be perceived by similar genetic mechanisms. We show that C10-AHL inhibited primary root growth and promoted LR formation in Arabidopsis wild-type seedlings (Fig. 5). *drr1* mutants showed reduced sensitivity to both *N*-isobutyl decanamide and C10-AHL, indicating a potential genetic interaction in plant responses to alkamides and AHLs in roots. These results also suggest that plants have evolved the capacity to sense AHLs in order to activate developmental responses.

Several reports indicate that bacteria commonly associated with plants are capable to produce a variety of AHLs (Cha et al., 1998; Elasri et al., 2001; Khmel et al., 2002; D'Angelo-Picard et al., 2005). Several strains of *Pseudomonas* have been studied for their ability to colonize plant-related niches, such as the rhizosphere (i.e. *P. aeruginosa*, *P. fluorescens*, and *P. putida*), where they can act as plant growth-promoting rhizobacteria by antagonizing plant-deleterious microorganisms and through the production of traits that directly influence plant disease resistance and growth (Venturi, 2006). The rhizospheric *P. putida* plant beneficial strains WCS358 and IsoF produce 3-oxo-C_{12}-AHL, whereas in the rhizosphere-colonizing biocontrol *P. fluorescens* strain $F_1$13, the production of three AHL molecules, including C10-AHL, has been reported (Laue et al., 2000; Venturi, 2006). Interestingly, C10-AHL and C12-AHL seem to be also produced in the nitrogen-fixing bacterial symbiont *Sinorhizobium meliloti* (Marketon et al., 2002; Teplitski et al., 2003). The marked resistance of *drr1* to C10-AHL on root development opens new possibilities to identify novel genetic determinants involved in plant-bacteria interactions. Furthermore, the *drr1* mutant can be used as a tool to identify novel plant growth-promoting bacterial strains, which could modulate root system architecture through AHL production.

Two recent reports suggested that auxin signaling might be involved in plant responses to AHLs (Mathesius et al., 2003; Von Rad et al., 2008). Auxins are signaling molecules that regulate the asymmetric pericycle cell divisions and thereby influence the patterning of newly initiated LRP. Thus, the possibility was open that altered auxin responses could be responsible for reduced LR formation in *drr1* mutants. Our results, however, showed that *drr1* mutants are not resistant to IAA or NAA treatments in terms of primary root growth inhibition or LR formation (Figs. 6 and 7), indicating that *DRR1* might not be directly connected to the auxin response pathway to modulate plant growth and development. These results are in agreement with our previous research showing that both alkamides and AHLs modulate root system architecture, likely through auxin-independent signaling mechanisms (Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008; Ortíz-Castro et al., 2008).

DRR1 Plays a Role in Senescence-Related Processes

Senescence is a developmental process that limits the longevity of an organism. Genetic studies of longevity mutants have also suggested that some common mechanisms, such as alterations in energy metabolism and oxidative damage, might play a role in determining life span in animals as divergent as nematodes, *Drosophila*, and mammals (Lin et al., 1998; Parkes et al., 1998). Plants also undergo a distinctive senescence process at the organ and/or organism level. A number of studies have provided evidence suggesting that leaf senescence is an active process controlled by a genetic program (Woo et al., 2001, 2002, Schommer et al., 2008). However, our understanding of how senescence and longevity are controlled at the whole plant level remains quite limited. Our results suggest that LR development and age-dependent plant senescence are directly connected through *DRR1*. Obvious alterations were seen in *drr1* plants grown in soil under long days (16-h-light/8-h-dark conditions). In *drr1* plants, leaf senescence was delayed by about 4 to 5 weeks when compared with wild-type plants (Figs. 8 and 9). The extended longevity of leaves was related to an extended growth period as well as to slower onset and/or progression of senescence after the maturation stage. The reduced growth observed at early stages of development in *drr1* mutants opens the possibility that it may contribute to extended longevity. Consistent with this hypothesis, we observed that reduced LR formation was not caused by the failure of the pericycle to produce these structures but by the retarded development of LRP to emerge from the primary root (Fig. 4). In this way, the *drr1* mutation shows a senescence character that differs from the delayed leaf senescence phenotype observed in the *oresara* (*ore*) and *teosinte branched/cycloidea/PCF* (*tcp*) mutants described previously (Woo et al., 2001, 2002; Schommer et al., 2008). In *ore* mutants, the retarded senescence phenotype seems to be specifically observed in leaves. For instance, the leaf longevity in *ore9-1* was extended only by about 27%, without affecting other developmental traits such as flowering time and/or plant size (Woo et al., 2001). To our knowledge, no LR phenotypes have been reported for leaf senescence mutants such as the *ore* and *tcp* lines. Interestingly, the *drr1* mutants also show that

plants that bolt and senesce late produce more leaves and increase in size (Figs. 8 and 9), which could lead to potential agricultural applications. Together, our findings suggest that *DRR1* may function normally as a positive regulator of senescence in Arabidopsis, limiting longevity at the whole plant level. Because the *drr1* mutation affects a wide variety of age-dependent developmental and senescence responses (Fig. 10), *DRR1* may function upstream in the regulatory cascade of senescence pathways.

drr1 Mutants Reveal Cross Talk between Alkamides and Jasmonate in LR Formation

Cross-resistance of mutants to multiple hormones is well documented (Wilson et al., 1990; Hobbie and Estelle, 1994; Tiryaki and Staswick, 2002) and suggests that the action of hormones is coordinated by common intermediates or modulators. Several phytohormones are involved in leaf senescence, including ethylene, cytokinin, and JA (Schommer et al., 2008). High concentrations of *N*-isobutyl decanamide have been found to induce callus formation in leaves and in roots (López-Bucio et al., 2007). Although not explicitly tested here, preliminary information shows that *drr1* plants are also resistant to callus formation (data not shown). The proliferative growth activity elicited by *N*-isobutyl decanamide on callus formation in leaves and LR formation in roots was previously shown to be decreased or even absent in Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors *CRE1*, *AHK2*, and *AHK3* (López-Bucio et al., 2007). The triple cytokinin receptor mutant *cre1-12/ahk2-2/ahk3-3* was particularly insensitive to high alkamide concentrations in terms of developmental alterations, indicating that *N*-isobutyl decanamide requires, at least in part, a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes. However, the primary root growth response of the *drr1* mutants to kinetin, a highly active cytokinin in modulating root development, was similar to that observed in wild-type plants (Supplemental Fig. S2A), indicating that *drr1* is not resistant to root inhibition by cytokinin. However, we cannot exclude the possibility that cross talk between alkamide and cytokinin responses may account for the increased longevity and/or reduced senescence of *drr1* mutants. Abscisic acid and ethylene are two growth regulators also involved in senescence; *DRR1* mutation rendered the *drr1* seedlings more sensitive to the primary root growth inhibitory effect of low abscisic acid concentrations than wild-type seedlings (Supplemental Fig. S2B), whereas the ethylene precursor 1-aminocyclopropane-1-carboxylic acid similarly inhibited growth (Supplemental Fig. S2C).

The plant hormone JA plays a key role in the environmental stress responses and developmental processes of plants. A recent report has revealed an important role of JA in LR development. In such work (Sun et al., 2009), it was shown that exogenous MeJA promotes LR formation in Arabidopsis wild-type plants but not in *anthranilate synthase1* mutants, leading to the proposal that localized auxin biosynthesis in response to jasmonate could be important for fine-tuned modulation of LR formation. Our detailed morphological comparison among wild-type, *drr1*, and *jar1* plants indicated that, when grown on JA-free medium, LR development in *drr1* was significantly lower than in wild-type or *jar1* plants. Interestingly, while JA application led to increased LR numbers in wild-type and *jar1* plants, it failed to activate LR formation in *drr1* (Fig. 11; Supplemental Fig. S1). Comparison of the primary root response to JA also showed that *drr1* behaves essentially different from *jar1*, which was very insensitive to primary root growth inhibition by JA but responded similarly to wild-type plants in LR induction by this compound. Therefore, *drr1* shows alkamide resistance in terms of primary and LR growth, whereas it has resistance to jasmonate in LR formation only. Taking into account these results, it is tempting to speculate that further cross talk of alkamide signaling with phytohormones such as cytokinins or jasmonates might vary in different tissues or in a developmental context, possibly explaining why *drr1* mutants exhibit no defects in primary root growth inhibition assays to these phytohormones.

In summary, we have provided evidence that alkamide and AHL signaling are under genetic control in Arabidopsis and that normal responses to these signals are important for plant development. Elucidation of the genetic identity of the *DRR1* product is critical to understand the molecular mechanisms underlying the distinct effects of these and other small lipid signals on root architecture adjustment and their role in plant longevity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type plants (Col-0 and/or Ws ecotypes), the transgenic line *CyCB1:uidA* (Colón-Carmona et al., 1999), and the mutant lines *jar1* (Tiryaki and Staswick, 2002), *axr2-1* (Timpte et al., 1994), and *aux1-7* (Picket et al., 1990) were used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog, 1962). MS medium (MS basal salts mixture; catalog no. M5524) was purchased from Sigma. The suggested formulation is 4.3 g L^{-1} salts for a 1× concentration of medium; we used 0.9 g L^{-1}, which we consider and refer to as 0.2× MS. This medium lacks amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology. Plants were placed in a plant grown chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 μmol m^{-2} s^{-1}, and temperature of 22°C.

Mutant Isolation Procedure

T-DNA lines (Ws; Krysan et al., 1999) were provided by the Ohio Arabidopsis Seed Stock Center. Seeds were surface sterilized and plated on 0.2× MS medium supplied with 30 μM *N*-isobutyl decanamide. A number of approximately 25,000 T-DNA lines were screened for reduced LR formation

by placing seeds on nutrient agar plates (20–25 seeds per plate). The seeds were distributed in two rows on the agar surface at a density of one seed per centimeter, stratified at 4°C for 48 h, and then incubated at 22°C. Fourteen days after germination, *N*-isobutyl decanamide-treated plants have a short primary root and a large number of LRs are formed. Putative mutants with long primary roots and a reduced number of LRs were selected, transferred to soil, and allowed to self-fertilize. Homozygous M3 seeds were rescreened for sustained primary root growth in medium supplied with 30 μM *N*-isobutyl decanamide, transferred to soil, and backcrossed three times to the wild type (Ws) to remove unlinked mutations.

Genetic Analysis of *drr1* Mutants

To determine the segregation pattern of the *drr1* phenotype, 990 F2 seedlings derived from the cross *drr1* × Ws were analyzed in MS 0.2× agar medium supplied with 30 μM *N*-isobutyl decanamide. A typical 3:1 recessive segregation was observed for the wild-type/*drr1* phenotype. Cosegregation of primary root growth resistance and increased longevity was further confirmed in *drr1* seedlings grown in soil.

Hormone Treatments

For all experiments, MS 0.2× nutrient medium was supplemented with *N*-isobutyl decanamide, C10-AHL, or the indicated phytohormones. Ethanol-dissolved compounds were added to cooled (50°C) molten medium and poured onto plates. Control plates were supplied with the greatest concentration of solvent used in the treatments. For hormone-induced senescence, leaves at 22 d after leaf emergence were detached and floated on sterilized water in the presence or absence of 35 μM JA or 35 μM *N*-isobutyl decanamide for 6 d. All treatments were performed at 22°C under dark conditions. Chemicals were purchased from Sigma Chemical.

Analysis of Growth and Statistical Analysis

Growth of primary roots was registered using a rule. LR number and LR density were determined by counting the LRs present in the primary root from the tip to the root/stem transition. LR density was determined by dividing the LR number by the primary root length and expressed as LR density per centimeter. The length of the meristem was determined as the distance between the quiescent center and the cell file where cells started to elongate. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with a Tukey's posthoc test were used for testing differences in growth and root developmental responses in wild-type and mutant lines. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Determination of Developmental Stages of LRP

LRP were quantified at day 4 after germination. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LR primordium was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follows. Stage I, LRP initiation; in the longitudinal plane, approximately eight to 10 "short" pericycle cells are formed. Stage II, the formed LR primordium is divided into two layers by a periclinal division. Stage III, the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV, LR primordium with four cell layers. Stage V, the LR primordium is midway through the parent cortex. Stage VI, the LR primordium has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII, the LR primordium appears to be just about to emerge from the parent root.

Chlorophyll Determination

We used leaves from wild-type (Ws) and *drr1* plants germinated and grown on 0.2× MS medium and then transferred to soil for 35 d. Wild-type leaves were yellowed as a result of age-dependent senescence; *drr1* leaves remained green at this stage. We used a hand-held chlorophyll meter (CCM-200; Opti-Sciences) to calculate a chlorophyll content index based on absorbance measurements at 660 and 940 nm on 15 independent leaves. Five separate measurements with the hand-held meter were made on each leaf. Chlorophyll content was finally determined as described previously (Richardson et al., 2002).

Histochemical Analysis of GUS Activity

Transgenic plants that express the *uidA* reporter gene (Jefferson et al., 1987) were stained in 0.1% 5-bromo-4-chlorium-3-indolyl-β-D-glucuronide in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M, pH 7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37 °C. Plants were cleared and fixed as described previously by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.

Microscopy

The Arabidopsis root system was analyzed with a stereoscopic microscope (MZ6; Leica Microsystems). Total LRs were counted at 30× magnification. Primary root meristems were analyzed in semipermanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus; Carl Zeiss) at 100× or 400× magnification. Images were captured with a Cyber-shot DSC-S75 digital camera (Sony Electronics) adapted to the microscope and processed with the Axio Vision 4AC software (Carl Zeiss).

Supplemental Data

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

Supplemental Figure S1. *drr1* shows defective LR formation in response to JA treatment.

Supplemental Figure S2. Effects of kinetin, abscisic acid, and ethylene on primary root growth in wild-type and *drr1* plants.

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