Universidad Michoacana de San Nicolás de Hidalgo

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Caracterización de los efectos de la inoculación de *Trichoderma* spp. sobre plantas de *Arabidopsis thaliana* crecidas en estrés salino

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Doctor en Ciencias en Biología Experimental

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Maestro en Ciencias en Biología Experimental
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P R E S E N T E

Por este conducto nos permitimos comunicarle que después de haber revisado el manuscrito final de la Tesis Titulada: " Caracterización de los efectos de la inoculación de *Trichoderma* spp. sobre plantas de *Arabidopsis thaliana* crecidas en estrés salino" presentado por el M.C HEXON ANGEL CONTRERAS CORNEJO, consideramos que reúne los requisitos suficientes para ser publicado y defendido en Examen de Grado de Doctor en Ciencias.

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A T E N T A M E N T E

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EL PRESENTE TRABAJO FUE REALIZADO EN LOS LABORATORIOS DE FISIOLOGÍA DEL DESARROLLO VEGETAL Y BIOQUÍMICA ECOLÓGICA DEL INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS, DEPENDIENTE DE LA UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO, BAJO LA DIRECCIÓN DEL DOCTOR JOSÉ LÓPEZ BUCIO Y CODIRECCIÓN DE LA DOCTORA LOURDES MACÍAS RODRÍGUEZ.

RECONOCIMIENTOS

ESTE TRABAJO FUE APOYADO POR EL CONSEJO NACIONAL DE CIENCIA Y TECNOLOGÍA (CONACYT) Y EL CONSEJO DE LA INVESTIGACIÓN CIENTÍFICA DE LA UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO.

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DEDICATORIA

A mis padres y hermanos.

En el campo de la observación el azar favorece sólo a las mentes preparadas.

-Louis Pasteur.

Para quienes dedican su vida a una carrera científica, nada es más gratificante que aumentar el número de sus descubrimientos: Pero cuando los resultados de sus observaciones quedan demostrados por su aplicación práctica, su gozo no tiene límites.

-Louis Pasteur.

El microbio no es nada, el ambiente lo es todo.

-Louis Pasteur.

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RESUMEN

Los hongos del género *Trichoderma* se encuentran distribuidos ampliamente en diferentes ecosistemas y forman estrechas asociaciones con las raíces de las plantas, lo cual induce cambios en el metabolismo y la expresión de genes en ambos organismos. Estos hongos producen un gran número de metabolitos secundarios. Por ejemplo, *Trichoderma atroviride* IMI 206040 y *Trichoderma virens* Gv. 29-8 producen ácido indol-3-acético (AIA), indol-3-acetaldehído (IAAld), indol-3-etanol (IEt) e indol-3-carboxaldehído (ICAld). Por otro lado, la salinidad es un problema importante para la agricultura moderna, por lo que en este trabajo, fue evaluado el efecto de *Trichoderma* spp. sobre el crecimiento vegetal bajo estrés salino. Se encontró que *T. virens* y *T. atroviride* inducen tolerancia a la salinidad en plantas de *Arabidopsis thaliana*. Se estudio el efecto de diferentes concentraciones de cloruro de sodio (NaCl) sobre la formación de biomasa foliar y el desarrollo de sistema radicular de *A. thaliana*. La sal reprimió de manera dosis dependiente el crecimiento vegetal y el desarrollo de la raíz. El análisis en la línea silvestre Col-0 y las mutantes *eir1*, *aux1-7*, *arf7arf19* y *tir1afb2afb3* afectadas en el transporte y señalización de las auxinas mostraron que esta hormona juega un papel importante en la tolerancia a la salinidad. Interesantemente, *Trichoderma* spp. promovió el crecimiento de *A. thaliana* en condiciones normales y de salinidad, lo cual correlacionó con la inducción de raíces laterales y pelos radiculares. Las plántulas de *A. thaliana* inoculadas y crecidas bajo estrés salino, incrementaron los niveles de ácido abscísico (ABA), L-prolina y ácido ascórbico (AA) y se estimuló la eliminación de Na^+ mediante los exudados radiculares. Estos datos muestran un papel importante para la señalización de las auxinas y el desarrollo del sistema radicular en la tolerancia al estrés salino en *A. thaliana*. Los aportes de este trabajo muestran que el uso de *Trichoderma* para la formulación de bioinoculantes y su uso en la agricultura es una estrategia biotecnológica para contrarrestar el estrés abiótico en cultivos.

Palabras clave: *Trichoderma*, *Arabidopsis*, sal, auxinas, ABA.

ABSTRACT

Fungi of the genus *Trichoderma* are widely distributed in different ecosystems and form close associations with plant roots, which induce changes in metabolism and gene expression in both organisms. These fungi produce a large number of secondary metabolites. For example, *Trichoderma atroviride* IMI 206040 and *Trichoderma virens* Gv. 29-8 produce indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), indole-3-ethanol (IEt) and indole-3-carboxaldehyde (ICAld). Moreover, salinity is a major problem for modern agriculture, so in this work the effect of *Trichoderma* spp. on plant growth under salt stress was evaluated. It was found that *T. atroviride* and *T. virens* induce salinity tolerance in *Arabidopsis thaliana* seedlings. The effect of different concentrations of sodium chloride (NaCl) on foliar biomass and development of *A. thaliana* root system was studied. Salt suppressed plant growth and root development in a dose-dependent manner. The analysis in the wild type line Col-0 and the mutants affected in auxin signaling and transport *eir1*, *aux1-7*, *arf7arf19* and *tir1afb2afb3* showed that this hormone plays a role in plant tolerance to salinity. Interestingly, *Trichoderma* spp. promoted *A. thaliana* growth in normal and saline conditions, which correlated with the induction of lateral roots and root hairs. *A. thaliana* seedlings inoculated and grown under salt stress, increased levels of abscisic acid (ABA), L-proline and ascorbic acid (AA) and elimination of Na^+ through roots exudates was stimulated. These data show an important role for auxin signaling and root system development in salt stress tolerance in *A. thaliana*. The contributions of this work show that the use of *Trichoderma* in the formulation of bioinoculants and its use in agriculture is a promising biotechnological strategy to counter abiotic stress in crops.

Keywords: *Trichoderma*, *Arabidopsis*, salt, auxins, ABA.

1. INTRODUCCIÓN

En la rizósfera, ocurre una amplia comunicación entre las plantas y las poblaciones microbianas a través del intercambio de diferentes clases de compuestos producidos por los microorganismos y las plantas. Este "diálogo molecular" determinará el resultado final de la interacción, que puede ir desde el parasitismo a la simbiosis. Por lo general, estas interacciones implican procesos celulares estrictamente coordinados (Pozo *et al.* 2005; Ortiz-Castro *et al.* 2009; Bednarek y Osbourn 2009).

Los hongos filamentosos del género *Trichoderma* son habitantes comunes de la rizósfera. Han sido ampliamente estudiados por su capacidad de producir antibióticos, parasitar a otros hongos (micoparasitismo) y competir por nutrientes con otros microorganismos (Harman *et al.* 2004). Se conoce desde hace años que las especies de *Trichoderma* promueven el crecimiento y mejoran la productividad vegetal tanto en sistemas axénicos como en el campo (Chang *et al.* 1986; Yedidia *et al.* 2001; Adams *et al.* 2007; Contreras-Cornejo *et al.* 2009).

En su ambiente natural, *Trichoderma* participa también en el control de las enfermedades de las plantas ocasionadas por microorganismos patógenos (Contreras-Cornejo *et al.* 2011, 2014a, b, c). Los reportes sobre las respuestas de defensa inducidas por estos hongos sobre las plantas, muestran que activan las rutas reguladas por el ácido salicílico (AS), ácido jasmónico (AJ) y etileno (ET), lo cual dependerá de la cantidad de inóculo y del modelo vegetal estudiado (Segarra *et al.* 2007; Salas-Marina *et al.* 2011; Mukherjee *et al.* 2012a; Contreras-Cornejo *et al.* 2011, 2014a).

Por otro lado, las plantas también están expuestas al estrés abiótico causado por el frío, el déficit por agua, la radiación del sol, la contaminación del aire, el calor y la salinidad (Vickers *et al.* 2009). Sin embargo, se desconocen en gran manera los efectos de *Trichoderma* y los mecanismos moleculares que estos hongos podrían activar en las plantas para promover el crecimiento en condiciones de estrés salino. La salinidad afecta severamente la productividad de las plantas y se calcula que >800 millones de hectáreas de la tierra cultivable es afectada por la salinidad, lo cual equivale al 6% del suelo de la

superficie terrestre (Munns 2005). Debido a la demanda de alimentos de origen vegetal en el mundo y considerando a la salinidad como un problema agrícola, es importante generar alternativas para afrontar este problema utilizando bioinoculantes.

En este trabajo se utilizó un sistema *in vitro* de interacción para investigar las respuestas fisiológicas y los mecanismos moleculares que *Trichoderma* regula en las plantas para inducir efectos benéficos. Se utilizó *A. thaliana* como modelo vegetal porque posee un ciclo de vida corto, es fácil de manipular en el laboratorio y se ha aislado y caracterizado un gran número de mutantes y líneas transgénicas que son una herramienta útil. Las plántulas fueron cocultivadas con los hongos en condiciones normales y de salinidad. Mediante técnicas de bioquímica analítica se analizó el perfil de metabolitos secundarios difusibles y volátiles de *Trichoderma* spp. que podrían estar involucrados durante la interacción regulando los procesos de desarrollo y respuestas de defensa. Los resultados obtenidos muestran que *Trichoderma* spp. promueve el crecimiento de las plantas mediante un mecanismo dependiente de auxinas y que esta señalización juega un papel clave en la adaptación de las plantas al estrés salino (Contreras-Cornejo *et al.* 2009; Contreras-Cornejo *et al.* 2014b). Además, los compuestos volátiles de *T. virens* inducen tolerancia al estrés salino y activan respuestas de defensa (Contreras-Cornejo *et al.* 2014a, b, c; Macías-Rodríguez *et al.* 2015). Se encontró también que *Trichoderma* spp. regula la transpiración de las hojas modulando la apertura de los estomas lo cual resulta benéfico para las plantas bajo estrés salino porque retardaría la deshidratación (Bae *et al.* 2009; Contreras-Cornejo *et al.* 2015a). En conjunto, estos resultados muestran el potencial de *Trichoderma* spp. para proteger a las plantas del estrés biótico y abiótico (Contreras-Cornejo *et al.* 2011, 2013, 2014b, c).

2. ANTECEDENTES

2.1. El sistema radicular y sus exudados

El sistema radicular se refiere a la parte de la planta que la sujeta en el suelo y se compone de órganos formados *de novo* con patrones de crecimiento y desarrollo complejos (Caldwell 1994). El patrón de enraizamiento está determinado por el tipo de planta, la estructura del suelo y las interacciones entre ambos (Uren 2000). El sistema radicular se encuentra formado por la raíz primaria, a partir de la cual surgen directamente las raíces laterales. Una de las estructuras más pequeñas de la raíz son los pelos radiculares que son células diferenciadas de la epidermis (López-Bucio *et al.* 2005). Aunque el sistema radicular suele ser expansivo, las raíces de una planta rara vez interfieren con las de otra, esto se debe en parte a la heterogeneidad de la estructura del suelo lo cual resulta en la separación espacial de las raíces (Young 1998). El sistema radicular es importante porque participa en la captación de agua y los nutrientes necesarios para el desarrollo, crecimiento y productividad. Además de estas funciones principales, las raíces también sirven como sitio para la síntesis de compuestos reguladores del crecimiento como las auxinas (Contreras-Cornejo *et al.* 2009).

Todas las raíces tienen la capacidad de exudar compuestos de bajo y alto peso molecular en la rizósfera en respuesta al estrés biótico o abiótico (Bertin *et al.* 2003). La exudación radicular incluye la liberación de compuestos orgánicos, iones, oxígeno, enzimas, mucílago y agua, y también participa en la regulación de los procesos metabólicos internos de la planta, como la respiración y externos, como la adquisición de nutrientes (Uren 2000). De manera general, los metabolitos exudados por la raíz son transportados a través de la membrana celular y son secretados en la rizósfera circundante. Este proceso representa un gasto energético y una pérdida considerable de carbono en la planta, debido a esto el perfil de metabolitos exudados varía con el tipo de suelo, la edad, el estado fisiológico de la planta, y la disponibilidad de nutrientes (Brimecombe *et al.* 2001; Bais *et al*. 2006).

En la rizósfera ocurren diferentes tipos de interacciones entre las raíces de las plantas y los microorganismos del suelo (Hirsch *et al.* 2003). Sin embargo,

como las raíces se encuentran bajo el suelo, diversos procesos interesantes son desapercibidos, en particular, el papel de las señales químicas entre las plantas y los hongos. En la última década, han sido caracterizados varios metabolitos de los exudados radiculares y su función en las interacciones planta-planta, planta-bacteria, planta-hongo, planta-insecto, etc. (Hirsch *et al.* 2003). Por ejemplo, Akiyama y colaboradores (2005) encontraron que *Lotus japonicus* libera sesquiterpenos que funcionan como moléculas señal en la comunicación con el hongo micorrícico *Gigaspora margarita* para que se efectúe la colonización radicular.

2.2. Género de hongos *Trichoderma*

Trichoderma es un género de hongos de reproducción asexual y se encuentra presente en casi todos los tipos de suelo. Por ejemplo, los suelos tropicales y templados contienen ~10^1-10^3 propágulos viables por gramo (Harman *et al.* 2004; Druzhinina *et al.* 2011). Algunas especies compiten con otros microorganismos por nutrientes y espacio en el suelo (Elad 1996; Harman 2000). Estos hongos pueden parasitar a otros hongos, fenómeno conocido como micoparasitismo. Las especies de *Trichoderma* muestran un alto nivel de diversidad genética y pueden ser usadas para producir un amplio rango de productos de interés comercial y ecológico (Kubicek *et al.* 2011). Se ha mostrado que algunas especies son productoras prolíficas de enzimas que degradan celulosa y quitina, aunque también producen otras enzimas y un gran número de metabolitos secundarios (Harman *et al.* 2012). Más de 350 compuestos con actividades antibióticas se han descrito para este género (Reino *et al*. 2008; Mukherjee *et al.* 2012b; Crutcher *et al*. 2013). En su medio natural, estos hongos son capaces de colonizar las raíces de las plantas y materiales herbáceos. Diversas especies han sido reconocidas como agentes para el control de enfermedades de las plantas y por su habilidad para promover el crecimiento y desarrollo vegetal. La taxonomía de estos hongos ha sido revisada minuciosamente y especies nuevas han sido identificadas (Druzhinina *et al.* 2011).

2.3. Interacción de *Trichoderma* con las raíces de las plantas

Las especies de *Trichoderma* comúnmente viven en los suelos y las raíces de las plantas. Descubrimientos recientes muestran que son oportunistas, simbiontes avirulentos de plantas (Harman *et al.* 2004). Algunas especies establecen colonizaciones duraderas y robustas sobre los tejidos radiculares, llegando incluso a penetrar la epidermis y la corteza, siendo en este aspecto semejantes a los hongos micorrícicos (Brotman *et al.* 2008). Estos hongos crecen intracelularmente en los tejidos externos de la raíz e inducen la acumulación de compuestos fenólicos en el sitio colonizado, esta reacción limita que *Trichoderma* penetre más en el tejido radicular (Varma *et al.* 1999). Varias especies producen y liberan una amplia variedad de compuestos que inducen respuestas de defensa local y sistémica en diferentes modelos vegetales, explicando con esto su avirulencia (Velázquez-Robledo *et al.* 2011; Hermosa *et al.* 2012; Contreras-Cornejo *et al.* 2014a). Estas asociaciones raíz-microorganismo, causan importantes cambios en el proteoma y metabolismo de la planta. La colonización de *Trichoderma* también estimula el crecimiento y desarrollo de la raíz, repercutiendo en la captación y utilización de nutrientes, la productividad postcosecha y resistencia a diferentes tipos de estrés (Fig. 1) (Harman *et al.* 2004; Contreras-Cornejo *et al.* 2009).

En el suelo, diversas especies de *Trichoderma* pueden competir contra hongos fitopatógenos por los exudados de las semillas en proceso de germinación, dichos exudados son utilizados para la nutrición de los propágulos (Howell 2002). *Trichoderma* spp. tiene la capacidad de inhibir y degradar pectinasas y otras enzimas que son esenciales para los hongos fitopatógenos como *Botrytis cinerea* para penetrar las superficies de las hojas (Zimand *et al.* 1996). Los efectos directos de *Trichoderma* sobre las plantas son diversos y recientemente se han considerado como las bases por las cuales *Trichoderma* promueve el crecimiento y desarrollo de las plantas (Contreras-Cornejo *et al.* 2009). Investigaciones sobre estos temas han generado un amplio cuerpo de conocimientos, incluyendo la caracterización de los reguladores del crecimiento, el esclarecimiento del papel de las auxinas fúngicas en la adaptación de plantas al estrés salino, la inducción de la resistencia sistémica mediante compuestos volátiles y la participación de la enzima 4-fosfopanteteinil

transferasa de *T. virens* en la producción de metabolitos secundarios y la interacción con las plantas (Contreras-Cornejo *et al.* 2009, 2011, 2014a, c).

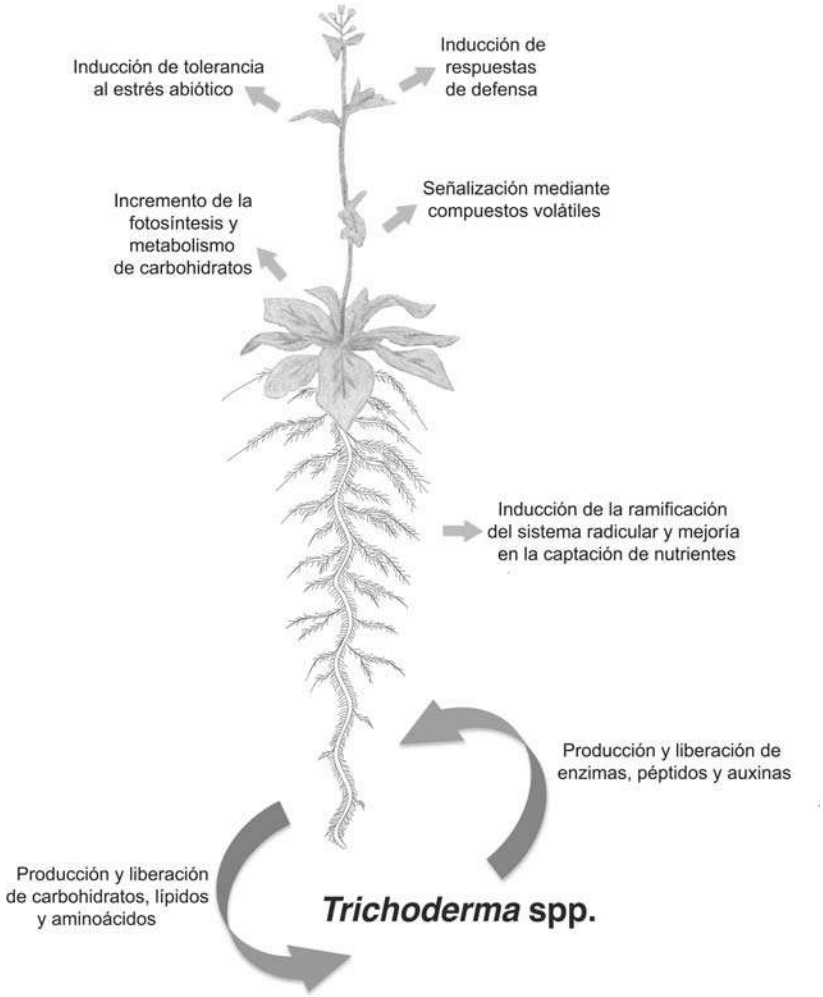

Figura 1. Efectos de *Trichoderma* spp. sobre las plantas. Los procesos mencionados en la figura, se han reportado en diferentes vegetales como *Arabidopsis thaliana*, maíz (*Zea mays*), jitomate (*Lycopersicon esculentum*), pepino (*Cucumis sativus*), etc. Imagen tomada del Capítulo: Promotion of plant growth and the induction of systemic defence by *Trichoderma*: Physiology, genetics and gene expression. Pages: 175-196 por Contreras-Cornejo *et al.* (2013). En el libro *Trichoderma* biology and applications. Ed. CABI.London, UK.

2.4. Efectos de *Trichoderma* sobre las plantas

2.4.1. Estimulación del crecimiento

Los microorganismos de la rizósfera inducen la biosíntesis de hormonas y respuestas de defensa en las plantas. La inoculación de las raíces de las plantas con diferentes especies de *Trichoderma* conduce a cambios favorables en el desarrollo del sistema radicular lo que permite mayor captación de agua y de nutrientes; como consecuencia, las plantas inoculadas con *Trichoderma* son más robustas y verdes (Chang *et al.* 1986; López-Bucio *et al.* 2003; Harman *et al.* 2004). Por ejemplo, en plantas de maíz se encontró que las raíces inoculadas con *Trichoderma* son más ramificadas y presentan pelos radiculares más grandes en comparación a las raíces sin inocular (Harman 2000; Shoresh *et al.* 2010). Efectos similares fueron observados en *Cistus incanus*, una planta huésped del hongo ectomicorrícico *Tuber melanopsorum* y también en *A. thaliana*, lo que evidencia que los microorganismos de la rizósfera ejercen efectos directos sobre el sistema radicular de las plantas (Splivallo *et al.* 2009).

Una proteína tipo hidrofobina de *T. harzianum* (T-22) también promueve el crecimiento vegetal lo cual sugiere que compuestos proteínicos pueden regular estos procesos (Shoresh *et al.* 2010). En investigaciones recientes se reportó que plántulas de *A. thaliana* inoculadas con *T. virens* acumularon mayor cantidad de biomasa foliar que las no inoculadas y este efecto se correlacionó con un incremento en el número de raíces laterales (Fig. 2) (Contreras-Cornejo *et al.* 2009, 2011). Los cambios en la morfología de las raíces se han atribuido a la actividad de las auxinas y compuestos indólicos. Los hongos *Pisolithus tinctorius* y *Piriformospora indica* promueven la acumulación de biomasa y ramificación del sistema radicular mediante la producción de sustancias reguladoras del crecimiento (Frankenberger y Poth 1987; Contreras-Cornejo *et al.* 2015a,b). Recientemente, se encontró que *T. virens* produce ácido indol-3-acético (AIA), indol-3-acetaldehído (IAAld), indol-3-etanol (IEt) e indol-3-carboxaldehído (ICAld) que son los precursores del AIA. Cuando se adicionó L-triptófano (Trp) al medio de cultivo del hongo, se incrementó la producción de estos compuestos (Contreras-Cornejo *et al.* 2009, 2011). En las plantas, varias

rutas para la síntesis de AIA a partir del Trp han sido propuestas: I. la ruta del ácido indol-3-pirúvico (AIP), II. del indol-3-acetamida, III. de la triptamina y IV. del indol-3-acetaldoxima (Woodward y Bartel 2005). En microorganismos la principal ruta para la producción de auxinas es: Trp→IPA→IAAld→IAA, misma que también ha sido reportada en plantas (Koga 1995; Stepanova *et al.* 2008; Contreras-Cornejo *et al.* 2009). El ICAld también se encuentra en *A. thaliana* y otras plantas brasicáceas. Este compuesto indujo la formación de raíces adventicias en *A. thaliana* (Contreras-Cornejo *et al.* 2011). Estos datos muestran que la gran cantidad de compuestos indólicos que produce *Trichoderma* spp. son reconocidos por las plantas y actúan como señales, que dependiendo de los estímulos ambientales determinarán el efecto final de estos hongos sobre el desarrollo.

Se ha observado que el efecto benéfico de *Trichoderma* spp. sobre las plantas depende de la especie fúngica y el genotipo de las plantas (Tucci *et al.* 2011). Estos hongos también solubilizan el fósforo y micronutrientes como hierro, cobre, zinc y manganeso que se encuentran de forma insoluble en el suelo (Altomare *et al.* 1999; Yedidia *et al.* 2001; Tucci *et al.* 2011). *Trichoderma* spp. también incrementa la captación y eficiencia del nitrógeno (Harman *et al.* 2004; Sherameti *et al.* 2005; Rai *et al.* 2008). Este efecto fue observado primero en experimentos de campo utilizando plantas de maíz crecidas de semillas inoculadas con *T. harzianum*. Las semillas inoculadas que fueron sembradas en condiciones de escaso nitrógeno desarrollaron plantas con un color verde más oscuro (Harman 2000). Interesantemente, se encontró que las plantas inoculadas con *T. harzianum* utilizaron de 40 a 50% menos fertilizante que las plantas no inoculadas (Harman *et al.* 2004). Esta propiedad de *T. harzianum* es explotada en los Estados Unidos, donde aproximadamente 0.3 millones de hectáreas de trigo son sembradas con semillas tratadas con *Trichoderma* spp. (Harman 2000, 2004).

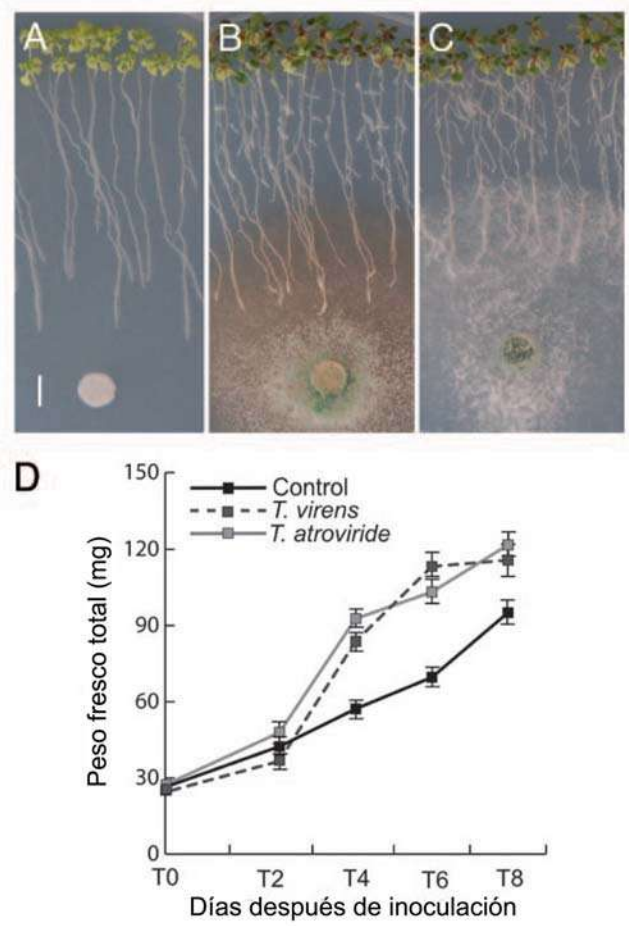

Figura 2. Efectos de *Trichoderma* sobre el crecimiento de *A. thaliana*. Las semillas de *A. thaliana* del ecotipo Columbia-0 fueron desinfectadas y crecidas por 4 días después de la geminación en medio Murashige y Skoog (MS) al 0.2X. Al término de este periodo fueron inoculadas con 10^6 esporas de *Trichoderma*. Se muestran imágenes representativas de cada tratamiento 6 días después de la inoculación. **A,** Patrón de crecimiento de las plantas control tratadas con agua. **B,** Efecto de *T. virens* y **C,** *T. atroviride* sobre *A. thaliana*. Nótese que ambas especies inducen el desarrollo de raíces laterales comparado con el tratamiento control. **D,** Efecto de *Trichoderma* sobre la acumulación de biomasa total en diferentes etapas de la interacción. Imagen tomada del artículo de Contreras-Cornejo *et al.* (2011) *Plant Signal. Behav.* 6, 1554-1563.

2.4.2. Respuestas de defensa

Las plantas poseen diversos mecanismos de defensa inducibles para la protección contra el ataque de microorganismos fitopatógenos (Dixon 2001). Un ejemplo de ello es la resistencia sistémica adquirida (RSA), que se activa después de la infección por patógenos necrótrofos y generalmente implica respuestas dependientes del AS pero no del ET (Lawton *et al.* 1995; Ryals *et al.* 1996). Algunos hongos y bacterias de la rizósfera benéficas para las plantas también pueden inducir resistencia sistémica dependiente del AJ, AS y/o ET (van Loon *et al.* 1998; Contreras-Cornejo *et al.* 2014a). Investigaciones sobre las respuestas de defensa de las plantas muestran una alta similitud a las activadas en los mamíferos. Por un lado, en las plantas, los productos de los genes de resistencia (R) que reconocen proteínas específicas de virulencia de los patógenos (Avr) tienen motivos en común en sus receptores con el sistema de los mamíferos y *Drosophila* (La Camera *et al.* 2004). Los productos de los genes *Avr* han sido bien identificados en una gran variedad de microorganismos patógenos de plantas. Generalmente son elicitores, que dependiendo de las especies de hongos y bacterias así como de los sistemas vegetales, inducen respuestas de defensa (Backer *et al.* 1997). El análisis del proteoma de *T. harzianum* ha permitido la identificación de genes homólogos a *Avr4* y *Avr9* de *Cladosporium fulvum* (Harman *et al.* 2004; Woo *et al*. 2006).

Por otro lado, se ha propuesto que la percepción de elicitores que activan respuestas de defensa en las plantas se debe a la interacción y reconocimiento de los patrones de reconocimiento molecular de los microorganismos (ej. carbohidratos, proteínas, lípidos, etc.) (Boller y Yang-He 2009; Danna *et al.* 2011). Esto indica que la inmunidad innata y los procesos de reconocimiento del microorganismo con la planta están conservados. Sin embargo, algunas de las respuestas de defensa activadas en las células vegetales ante microorganismos son específicas de plantas debido a que el análisis del trascriptoma de plantas de *A. thaliana* inoculadas con algunos microorganismos muestra una reprogramación transcripcional (Truman *et al.* 2007). Entre diversos genes cuya expresión cambia en las células infectadas están los de respuesta a patogénesis *(PATHOGENESIS-RELATED; PR)*; los cuales están asociados con el desarrollo de la RSA que codifican para

proteínas antimicrobianas. Las proteínas PR tienen actividades deletéreas en contra de componentes estructurales de los microorganismos patógenos. Algunos miembros de las familias PR-1 y PR-5 interactúan con la membrana plasmática de los hongos, mientras otras PRs con actividad de β-1, 3-glucanasa o quitinasa degradan la pared celular de los hongos.

La actividad de una gran cantidad de proteínas involucradas en el metabolismo secundario son también afectadas tras la infección de microorganismos patógenos, induciendo cambios en el contenido de los metabolitos de las plantas (Brotman *et al.* 2012). En particular, en las respuestas de defensa de *A. thaliana*, tabaco y otras plantas infectadas por distintos microorganismos, los productos del metabolismo de las oxilipinas y fenilpropanoides son activados y están involucrados en diferentes niveles de los mecanismos de defensa (La Camera *et al.* 2004; Truman *et al.* 2007; Brotman *et al.* 2012). Las fitooxilipinas se sintetizan enzimáticamente a partir de los ácidos grasos poliinsaturados a través de tres vías principalmente: I. la ruta de las lipoxigenasas, la cual ha sido la única ruta enzimática de oxidación; II. a través de citocromos P450 que se localizan en el retículo endoplasmático que catalizan la ω-hidroxilación de ácidos grasos, y III. la participación de una α-dioxigenasa que muestra similitud con las cicloxigenasas que catalizan la α-oxigenación de los ácidos grasos (La Camera *et al.* 2004).

De manera general, las fitoalexinas participan restringiendo la proliferación de los microorganismos patógenos (Darvill y Albersheim *et al.* 1984; Rogers *et al.* 1996). Se encontró que en *A. thaliana* la producción de camalexina es inducida por distintos microorganismos, incluyendo patógenos y se mostró que su síntesis depende del indol-3-acetaldoxima (Glawischning *et al.* 2004). En *A. thaliana*, *T. virens*, *T. atroviride* y el ICAld incrementaron los niveles de camalexina (Contreras-Cornejo *et al.* 2011). Según Zook y Hammerschmidt (1997), esta fitoalexina se produce por la reacción de condensación del ICAld con la L-cisteína. A la fecha, se conocen tres clases de compuestos que son producidos por *Trichoderma* y que inducen resistencia en las plantas: I. proteínas con actividad enzimática u otras funciones, II. homólogos de proteínas codificadas por genes de avirulencia y III. oligosacáridos de bajo peso molecular que son liberados de la pared celular del hongo (Harman *et al.*

2004; Woo *et al.* 2006). Un grupo diferente de metabolitos que inducen respuestas de defensa en contra de patógenos son los péptidos de origen fúngico. Los péptidos producidos por *Trichoderma*, son compuestos lineales de cadena corta (≤ 20 residuos) sintetizados por una péptido sintasa no ribosomal (Viterbo *et al.* 2007; Vargas *et al.* 2008; Velázquez-Robledo *et al.* 2011). Se ha mostrado que estos péptidos poseen actividad antimicrobiana (Schirmböck *et al.* 1994; Rebuffat *et al.* 2000; Chugh y Wallance 2001; Szekeres *et al.* 2005; Chanikul *et al.* 2008; Velázquez-Robledo *et al.* 2011).

Djonović y colaboradores (2006, 2007) reportaron que *T. virens* produce un péptido pequeño denominado Sm1 (Small protein en inglés), que en plantas de algodón y maíz induce respuestas de defensa dependientes del AJ. Sm1 también indujo la acumulación de peróxido de hidrógeno en los cotiledones de algodón. Estos datos concuerdan con la evidencia de la participación del AJ y el ET en las respuestas de defensa de plantas de pepino (*Cucumis sativus*) activadas con *T. asperellum* T203 (Shoresh *et al.* 2005). En ese mismo trabajo, estudios de la reacción en cadena de la polimerasa en tiempo real mostraron que T203 induce cambios en la expresión del gen *LOX1 (LIPOXYGENASE 1)* el cual codifica para una enzima implicada en la síntesis del AJ (Shoresh *et al.* 2005). En *A. thaliana*, *T. virens* indujo la expresión de los genes *PR-1a::GUS* activado por AS y *LOX2::GUS* activado por AJ (Velázquez-Robledo *et al.* 2011) (Fig. 3). Otro gen regulado por *T. virens* en plantas de maíz es el que codifica para la fenilalanina amonio liasa (PAL) (Djonović *et al.* 2007; Lamba *et al.* 2008). PAL1 puede activar el mecanismo de señalización mediado por AJ/ET. Existe evidencia que *T. viride* induce respuestas de defensa en las plantas por la actividad de una xilanasa de 22-kDa responsable de la emisión de ET (Fuchs *et al.* 1989; Lotan y Fluhr 1990; Martínez *et al.* 2001). Interesantemente, esta proteína cuando se introduce en los peciolos de las hojas induce respuestas de defensa local y es translocada a través del sistema vascular de plantas de tabaco. De manera semejante, las celulasas y proteínas de pared celular similares a las expansinas (ej. la “swollenin” en inglés) secretadas por distintas especies de *Trichoderma* spp. inducen respuestas de defensa (Brotman *et al.* 2008; Shoresh *et al.* 2010).

Se han generado algunas cepas de *Trichoderma* transformadas con sistemas reporteros basados en la proteína verde fluorescente o en actividades

enzimáticas específicas (glucosa oxidasa) bajo el control de promotores implicados en el biocontrol (Harman *et al.* 2004). Esto ha permitido el aislamiento y caracterización de moléculas bioactivas que son liberadas por la acción de las enzimas degradadoras de pared celular de los hongos patógenos o de las plantas mismas. Estas moléculas pueden ser producidas durante múltiples interacciones que ocurren en la naturaleza entre *Trichoderma*, hongos patógenos y las raíces de las plantas (Kubicek *et al.* 2001).

La protección de las plantas por parte de *Trichoderma* al ataque de microorganismos patógenos se ha estudiado con cierto detalle. Por ejemplo, *T. asperellum* puede colonizar el sistema radicular de plantas de pepino e inducir respuestas de defensa efectivas en contra del ataque de *Pseudomonas syringae* pv. lachrymans (*Psl*) en la parte foliar. Durante este proceso el contenido de AS no varió entre las plantas inoculadas y las no tratadas, este dato resulta interesante ya que *Psl* por si misma eleva las concentraciones de salicilatos (Shoresh *et al.* 2005).

En *A. thaliana* se encontró que *T. virens* y *T. atroviride* incrementaron los niveles del AS y AJ (Contreras-Cornejo *et al.* 2011). Cuando las plántulas fueron inoculadas con el hongo patógeno *Botrytis cinerea* se encontró que este hongo causó la muerte de ~85% de las plantas. Sin embargo, las plantas tratadas previamente con *Trichoderma* fueron menos afectadas. La mutación del gen *PPT1* que codifica para la enzima 4-fosfopanteteinil transferasa en *T. virens*, afecta la capacidad del hongo para inducir respuestas de defensa dependientes del AS, la acumulación de camalexina y conferir resistencia en contra de *B. cinerea* (Velázquez-Robledo *et al.* 2011). Estos datos muestran que las especies de *Trichoderma* regulan múltiples mecanismos para activar respuestas de defensa y conferir resistencia en las plantas al ataque de microorganismos patógenos.

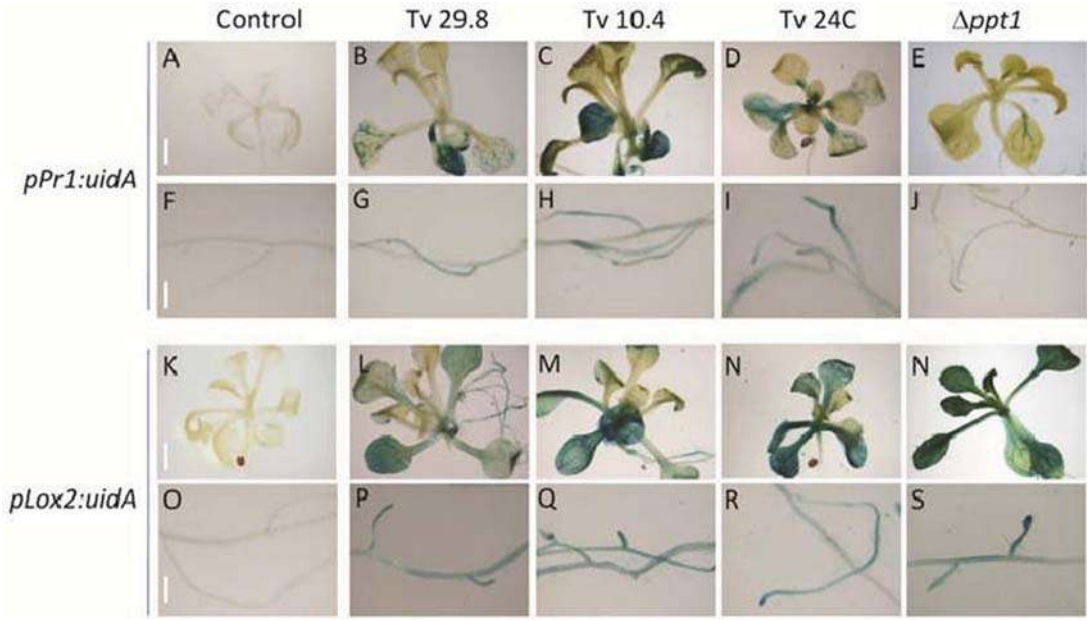

Figura 3. Efecto de *T. virens* sobre la expresión de los genes de defensa *PR-1a::GUS* activado por AS y *LOX2::GUS* por AJ. Las plantas transgénicas que expresan estos marcadores fueron crecidas y geminadas en medio MS 0.2X por 4 días e inoculadas con 10^6 esporas. En el caso de las plantas inoculadas con la mutante auxotrófa a lisina (L-Lys) Δ*ppt1* la interacción se llevó a cabo en medio MS 0.2X suplementado con 300 μM Lys. La determinación de la expresión de cada gen se realizó 6 días después de la inoculación con cada cepa (Tv29.8, Tv10.4 y Tv24C). El incremento de la expresión del gen reportero está indicado por el precipitado azul que evidencia la actividad de la β-glucuronidasa (GUS). **A-J,** Cambios en la expresión del gen *PR-1a::GUS* y **K-S,** en el gen *LOX2::GUS*. Imagen tomada del artículo de Velázquez-Robledo *et al.* (2011) *Mol. Plant-Microbe Interact.* 24, 1459-1471.

2.4.3. Señalización mediante compuestos volátiles

Las plantas son organismos que poseen una extraordinaria red de mecanismos de protección ante el estrés. Uno de los principales es la activación de la ruta de los isoprenos volátiles (Tholl *et al.* 2006; Vickers *et al.* 2009). Es conocido que los isoprenos y otros compuestos orgánicos volátiles están implicados en interacciones bióticas y bajo estrés abiótico (Ping y Boland 2004; Zhang *et al.* 2007; Yang *et al.* 2008; Contreras-Cornejo *et al.* 2014c; Macías-Rodríguez *et al.* 2015). Se ha reportado que *T. atroviride* produce una amplia gama de sustancias volátiles como alcoholes, cetonas, ésteres y compuestos de ocho carbonos, y en el caso de *T. virens* que produce un gran número de sesquiterpenos (Fig. 4) (Stoppacher *et al.* 2010; Contreras-Cornejo *et al.* 2014c). Los resultados del presente estudio muestran que plántulas de *A. thaliana* crecidas en estrés salino expuestas a los volátiles de *T. virens* fueron menos afectadas en los procesos de desarrollo que las plantas no inoculadas (Macías-Rodríguez *et al.* 2015). Este efecto se correlacionó con la disminución en la acumulación de peróxido de hidrógeno. También se observó que los volátiles de *T. virens* inducen la expresión del gen *LOX2::GUS* (Contreras-Cornejo *et al.* 2014c).

Engelberth y colaboradores (2001) mostraron que el péptido alamenticina de *T. viride* induce la emisión de ET, AJ y volátiles implicados en la ruta de señalización ocatadecanoica de plantas. Esta última ruta es útil para la síntesis de AJ a partir del ácido octadecanoico (AO) y otros ácidos grasos (Howe *et al.* 1996). Varios metabolitos que son intermediarios entre el AO y el AJ o conjugados del AJ como el metil jasmonato funcionan como moléculas señal en respuestas de defensa (Blechert *et al.* 1995; Howe *et al.* 1996).

Estos datos muestran que algunos compuestos volátiles de *Trichoderma* spp. también pueden ser moléculas señalizadoras para inducir respuestas de defensa y promover el crecimiento vegetal (Contreras-Cornejo *et al.* 2014c; Macías-Rodríguez *et al.* 2015). Por otro lado, algunos compuestos volátiles de *Trichoderma* han sido implicados en el fenómeno del micoparasitismo (Vinale *et al.* 2008). Recientemente, se reportó que la presencia de la micotoxina ácido fusárico de *Fusarium oxysporum* afectó el perfil de compuestos volátiles producidos por *T. atroviride* (Stoppacher *et al.* 2010). Es claro que los

compuestos volátiles están directamente implicados en las complejas interacciones entre *Trichoderma* y los demás organismos (plantas y microorganismos). El aporte en el conocimiento de la actividad biológica de algunos metabolitos volátiles ha ayudado a comprender la función de *Trichoderma* en la rizósfera.

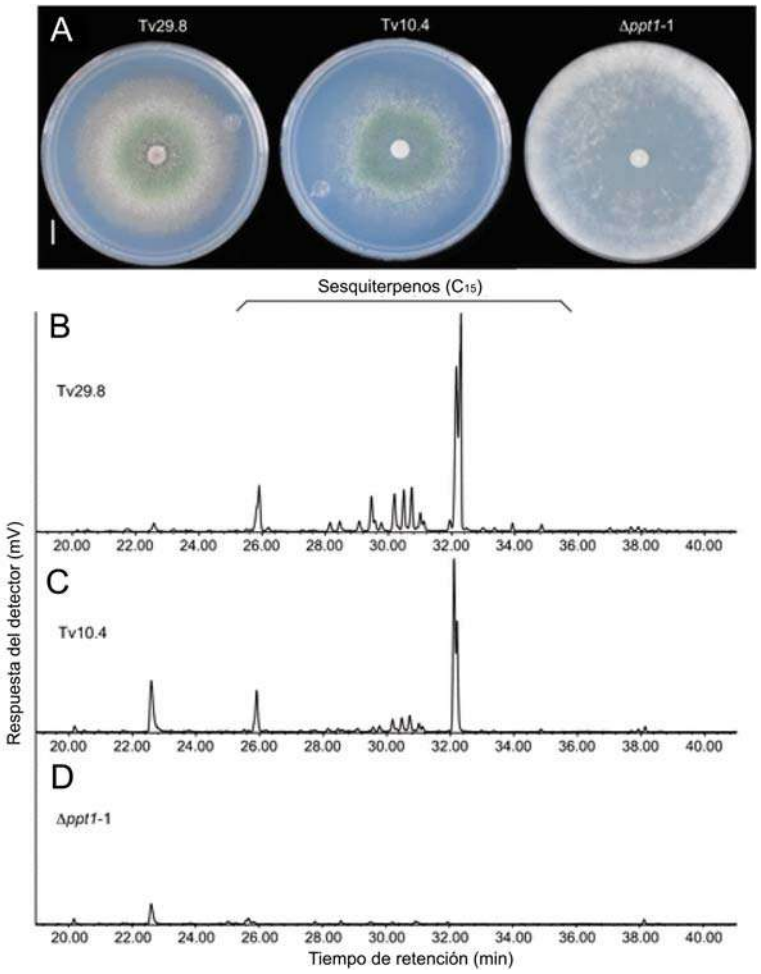

Figura 4. Perfil de compuestos orgánicos volátiles de las cepas Tv29.8, Tv10.4 y la mutante Δ*ppt1* de *T. virens*. **A,** Imágenes representativas del crecimiento de *T. virens* después de 5 días de un inoculo de 10^6 esporas sobre medio MS 0.2X. **B-D,** Cromatogramas representativos de los metabolitos volátiles de *T. virens*. La línea superior indica la presencia de sesquiterpenos. Nótese que la mutación del gen *PPT1* (*PHOSPHOPANTETHEINYL TRANSFERASE 1*) afectó la producción es estos compuestos. Imagen tomada del artículo de Contreras-Cornejo *et al.* (2014) *Plant Soil* 379, 261-274.

2.5. Adaptación al estrés abiótico

2.5.1. Estrés salino

La salinización del suelo es un problema de la agricultura que se está extendiendo por todo el mundo. Se calcula que el ~7% de la superficie total de la tierra es salina y aproximadamente un tercio de la tierra cultivable en el mundo se somete a la salinización secundaria, fenómeno que resulta de la acumulación de Na^+ en el suelo que anteriormente formaba parte del fertilizante químico (Shabala y Cuin 2008; Hariadi *et al.* 2011). Bajo estrés salino, las plantas experimentan deshidratación, deficiencias nutricionales, disfunción de la membrana y estrés oxidativo, lo que conduce a daños en los tejidos o senescencia temprana (Fig. 5) (Essah *et al.* 2003; Katori *et al.* 2010). Para evitar la acumulación tóxica de sodio Na^+ en el follaje, las plantas no captan más del 3% de Na^+ de la rizósfera.

En condiciones de salinidad algunos mecanismos para la adaptación se activan (Zhang *et al.* 2008; Jiang *et al.* 2013a). La tolerancia a la salinidad implica múltiples mecanismos fisiológicos y bioquímicos: por ejemplo, se regula la homeostasis de Na^+, se induce la expresión de genes de respuesta a deshidratación, se activan cascadas de señalización mediadas por proteínas cinasas activadas por mitógenos, se producen y acumulan osmolitos y sustancias antioxidantes (Xiong *et al.* 2002a,b; Zhu 2003; Seki *et al.* 2003; Mehlmer *et al.* 2010).

Por otra parte, las plantas reducen la pérdida de agua mediante el cierre de los estomas y maximizan la captación de agua para resistir al estrés osmótico. Además, minimizan los efectos deletéreos del estrés iónico causado por el Na^+ mediante la eliminación de este ión a través de los estomas de la epidermis de las hojas o a través de su acumulación en las vacuolas (Blumwald 2000; Munns y Tester 2008). *A. thaliana* es un excelente modelo para evaluar *in vitro* el efecto de la sal sobre diferentes parámetros del crecimiento y desarrollo sobre la zona área y el sistema radicular.

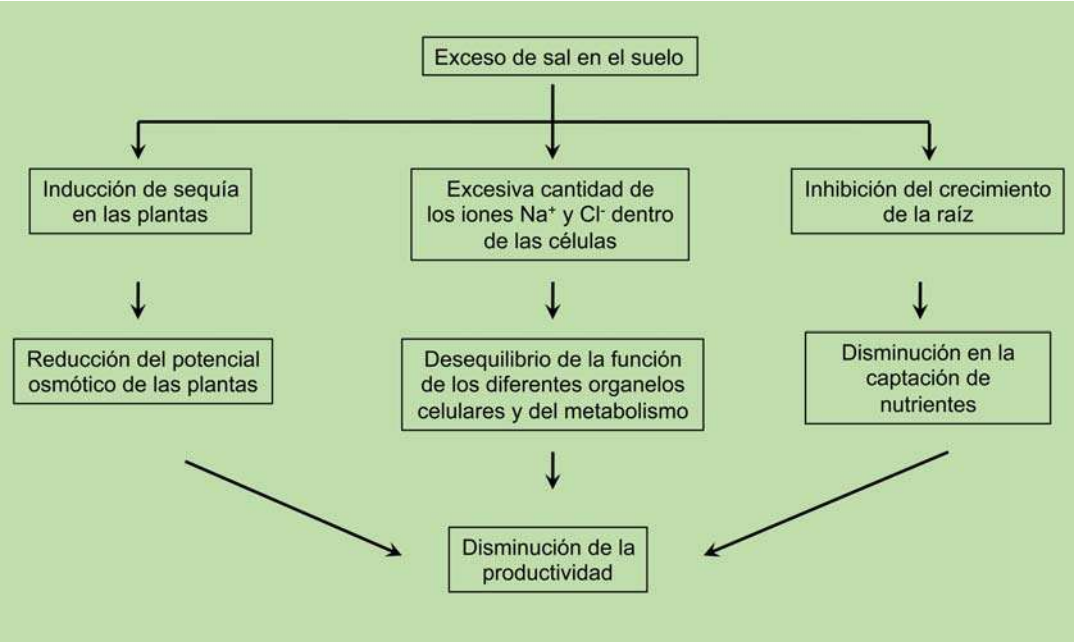

Figura 5. Efectos negativos del exceso de sal en el suelo. Esta imagen muestra que el ión Na^+ puede afectar en diferentes niveles a las plantas. Por ejemplo, si se inhibe el crecimiento del sistema radicular las plantas tendrán limitada capacidad exploratoria en el suelo lo cual limita la captación de agua y de nutrientes.

En la figura 6 se muestran los efectos de concentraciones crecientes de NaCl sobre plántulas de *A. thaliana*. Estos datos muestran que la sal afecta el crecimiento de las plantas debido a que se reprime el desarrollo del follaje y el sistema radicular de manera dosis dependiente. También se observa que a partir de 100 mM NaCl se induce una respuesta agravitrópica en la raíz denominada "halotropismo" (Galvan-Ampudia *et al.* 2013). Dos de los efectos más severos que induce la sal sobre el sistema radicular son: la inhibición de la formación de raíces laterales y pelos radiculares (Fig. 7). A la fecha, poco se conoce sobre la respuesta del sistema radicular a la sal. Sin embargo, varios reportes han descrito que el estrés osmótico reduce la elongación de la raíz en plantas de maíz (Zolla *et al.* 2010).

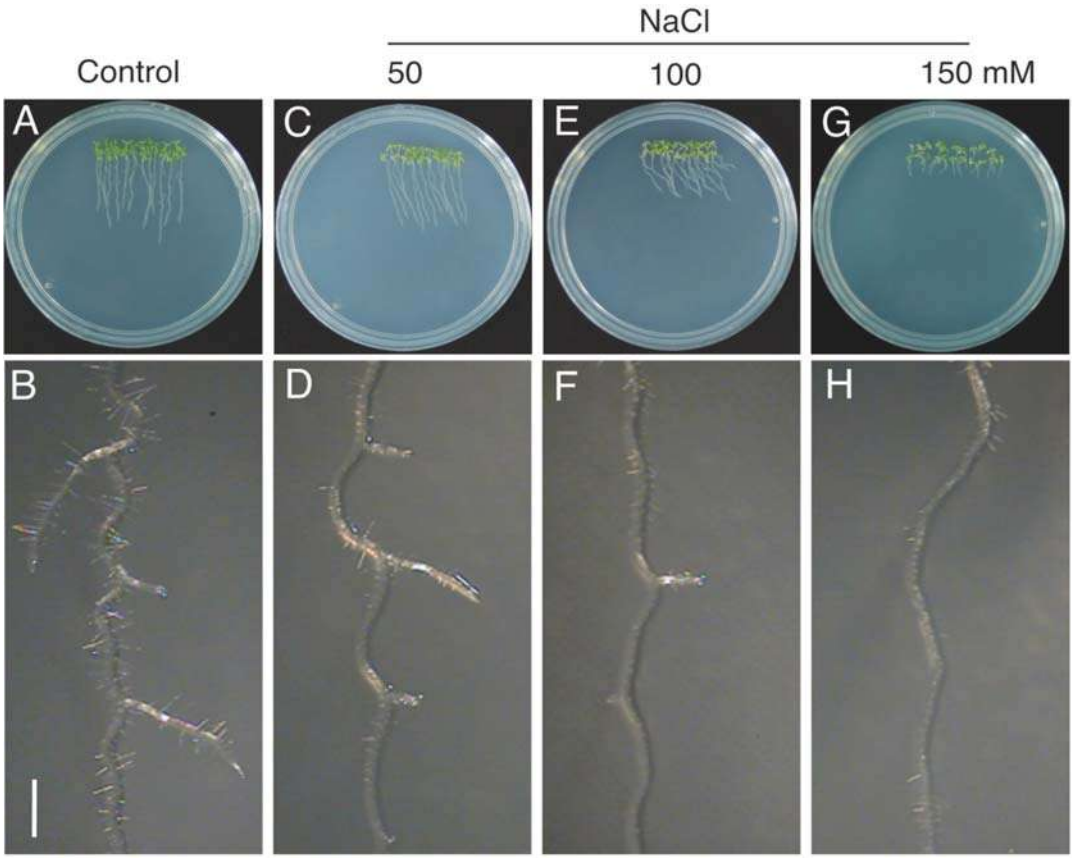

Figura 6. Efecto de la sal (NaCl) sobre el crecimiento de *A. thaliana*. Las semillas de *A. thaliana* del ecotipo Columbia-0 fueron desinfectadas y crecidas por 9 días después de la geminación en medio Murashige y Skoog (MS) al 0.2X. Se muestran imágenes representativas de cada tratamiento. **A,** Patrón de crecimiento de las plantas control en placas Petri y **B,** Imagen de una zona de la raíz primaria que muestra la formación de raíces laterales y pelos radiculares. **C-H,** Efecto de diferentes concentraciones de sal. Barra 500 μm. Nótese que la sal reprime el crecimiento de la raíz primaria y la formación de raíces laterales y pelos radiculares. El experimento de hizo tres veces con una n= 60 plantas.

En el suelo, las plantas perciben, transportan y/o discriminan los diferentes iones para su óptimo crecimiento. A continuación se describen los principales mecanismos que en conjunto inducen tolerancia al estrés salino.

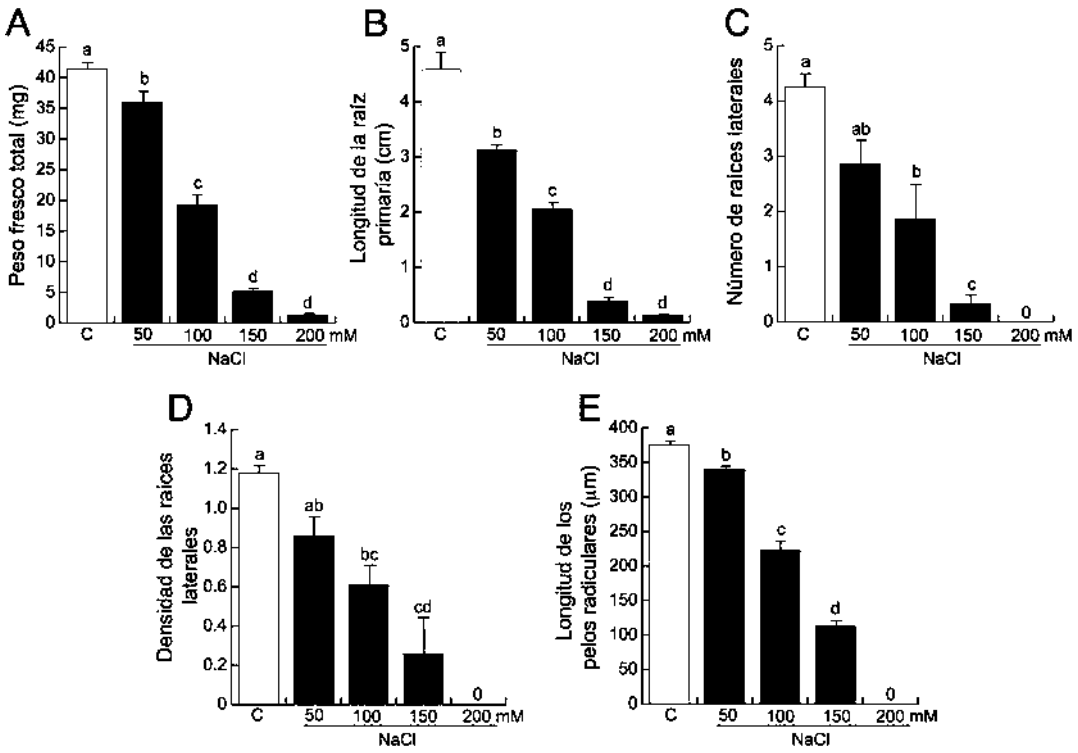

Figura 7. Efecto de la sal sobre el crecimiento de *A. thaliana*. Las semillas de *A. thaliana* del ecotipo Columbia-0 fueron desinfectadas, germinadas y crecidas por 9 días en medio Murashige y Skoog (MS) al 0.2X suplementado con distintas concentraciones de NaCl. Al término de este periodo las variables del desarrollo fueron evaluadas. Los histogramas muestran el **A**, Peso fresco total. **B,** Longitud de la raíz primaria. **C,** Número de raíces laterales. **D,** Densidad de las raíces laterales. **E,** Longitud de los pelos radiculares. Nótese que conforme se incrementa la concentración de sal en el medio, se afectan todas las variables analizadas. Imagen tomada del artículo de Contreras-Cornejo *et al.* (2014) *Mol. Plant Microbe-Interact.* 27, 503-514.

2.5.2. Mecanismos en las plantas para detectar el estrés salino

Los mecanismos de respuesta al estrés abiótico en las plantas han sido dilucidados mediante el análisis de un gran número de genes que se expresan durante la sequía (estrés hídrico), la salinidad y el frío (Oono *et al.* 2003). Los productos de los genes inducibles por el estrés se clasifican en dos grupos: I. los que participan directamente en la protección al estrés abiótico y II. los que regulan la expresión génica y la transducción de señales en respuesta al estrés (Bray 1997; Shinozaki y Yamaguchi-Shinozaki 1997; Thomashow 1999; Hasegawa *et al.* 2000). La figura 8 muestra el mecanismo de señalización nuclear mediado por ABA, la principal hormona vegetal implicada en activar mecanismos de resistencia bajo estrés biótico y/o abiótico.

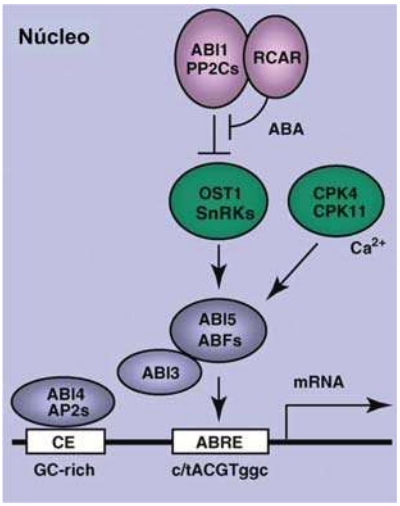

Figura 8. Mecanismo de señalización regulado por el ABA en el núcleo. El receptor del ABA está formado por el complejo heteromérico de la fosfatasa PP2C (PROTEIN PHOSPHATASE TYPE 2C; ej. ABSCISIC ACID INSENSITIVE 1 [ABI1] presentado en color rosa fucsia) y la proteína RCAR (REGULATORY COMPONENT OF ABA RECEPTOR) de unión al ABA. Las fosfatasas PP2Cs inhiben la actividad de las proteínas cinasas SnRKs (SUCROSE-NONFERMENTING KINASE1-RELATED PROTEIN KINASES; ej. OST1 en color verde), y posiblemente de cinasas dependientes de Ca^{2+}. En presencia de ABA, la actividad de las fosfatasa del receptor (PP2Cs) se inhibe. En consecuencia, las proteínas cinasas son liberadas de la inhibición de las PP2Cs, lo que induce su actividad fosforilando componentes clave de la ruta del ABA como factores transcripcionales bZIP (basic leucine zipper) como ABI5 (ABSCISIC ACID INSENSITIVE 5) (en color morado). Imagen tomada de la revisión de Raghavendra *et al.* (2010) *Trends Plant Sci.* 15, 395-401.

La alta concentración de Na^+ en el suelo induce estrés hiperosmótico y iónico en las raíces (Deinlein *et al.* 2014). Por lo general, las plantas enfrentan estos efectos negativos evitando el estrés salino modificando la plasticidad de las raíces o activando mecanismos de tolerancia (Galvan-Ampudia *et al.* 2013). Estos mecanismos son activados debido a que las plantas tienen la capacidad de percibir el ión Na^+. Los sensores hiperosmóticos están estrechamente acoplados a los canales de Ca^{2+} debido a que las plantas incrementan rápidamente la concentración citosólica de Ca^{2+} a los pocos segundos de ser expuestas al NaCl o manitol (Knight *et al.* 1997). Las especies reactivas de oxígeno (ERO) también se han relacionado como segundos mensajeros al estrés salino y la respuesta hiperosmótica (Jiang *et al.* 2013b). Río abajo de la señalización del Ca^{+2}, pueden ser activadas las proteínas cinasas dependientes de calcio y las proteínas tipo calcineurina B (CDPKs y CBLs por sus siglas en inglés, respectivamente). Estas cinasas transducen las señales de estrés y activan factores transcripcionales. Algunos de estos últimos pueden ser activados directamente por Ca^{2+} o por calmodulinas (Pandey *et al.* 2013). También existen mecanismos de percepción de Na^+ y estrés osmótico independientes de Ca^{2+} (Fig. 9).

Los factores transcripcionales son elementos importantes para unir la percepción de sal con los mecanismos de tolerancia. Algunas familias de factores transcripcionales se activan diferencialmente en respuesta a elevada salinidad e incluyen: BASIC LEUCINE ZIPPER, WRKY, APETALA2/ETHYLENE RESPONSE FACTOR, MYB, BASIC HELIX-LOOP-HELIX y NAC, denominadas así debido a que estas proteínas en su región N-terminal contienen un dominio altamente conservado (NAM, ATAF1,2 y CUC2) el cual forma una estructura hélice-giro-hélice que se une específicamente al sito blanco del ADN (Aida *et al.* 1997; Kasuga 1999; Tran *et al.* 2004; Yang *et al.* 2009; Jiang *et al.* 2009; Jiang y Deyholos 2009; Golldack *et al.* 2011; Cui *et al.* 2013). Estos factores transcripcionales regulan la expresión de varios genes que inducen la adaptación de las plantas al estrés salino (Fig. 9). En respuesta a la alta salinidad, ocurren cambios transcripcionales aproximadamente 3 horas después del estímulo (Geng *et al.* 2013). Para mitigar la disminución del potencial de agua que resulta de la salinidad, los factores transcripcionales activan la expresión de genes que codifican para transportadores de iones

como el K^+ (Geng *et al.*2013). Algunas hormonas vegetales pueden regular algunos factores transcripcionales como los WRKY o NAC (Dinneny *et al.* 2008; Geng *et al.* 2013).

He y colaboradores (2005) mostraron que el gen *AtNAC2* codifica para un factor de transcripción en *A. thaliana*, que se localiza en el núcleo y es altamente expresado en las raíces y las flores. Además de la inducción por salinidad, la expresión de *AtNAC2* también puede ser estimulada por el ABA, el ácido 1-aminociclopropano-1-carboxílico (precursor del ET) y el ácido naftaleno-acético (compuesto con actividad de auxina). Análisis moleculares y genéticos en las mutantes de etileno *eto1-1*, *etr1-1*, *ein2-1* y del receptor de auxinas *tir1-1* revelaron que la respuesta a la salinidad mediada por NAC2 está relacionada con la señalización de las vías de etileno y auxinas. Interesantemente, la sobreexpresión de *AtNAC2* en las plantas de *A. thaliana* resultó en la promoción del desarrollo de las raíces laterales. Estos resultados indican que AtNAC2 puede ser un factor de transcripción que incorpora los estímulos ambientales y endógenos en el proceso de desarrollo de las raíces laterales de las plantas.

El ET es una hormona volátil que controla diversos aspectos del desarrollo como la formación de raíces laterales y pelos radiculares. Sin embargo, también regula varias respuestas de estrés abiótico (Achard *et al.* 2006; Zhang *et al.* 2011). Por ejemplo, la modulación de las respuestas a la salinidad son controladas por el componente ETR1 (ETHYLENE TRIPLE RESPONSE 1), CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), EIN2 (ETHYILENE INSENSITIVE 2) y EIN3. Se encontró que la mutante *ein3-1* es hipersensible a la sal respecto al ecotipo silvestre Col-0. Por otro lado, se mostró que durante el estrés salino, se incrementa la acumulación de EIN3 en la doble mutante *ebf1ebf2* (Zhang *et al.* 2011). Estos datos sugieren que EIN3 es un importante regulador de la respuesta al estrés salino.

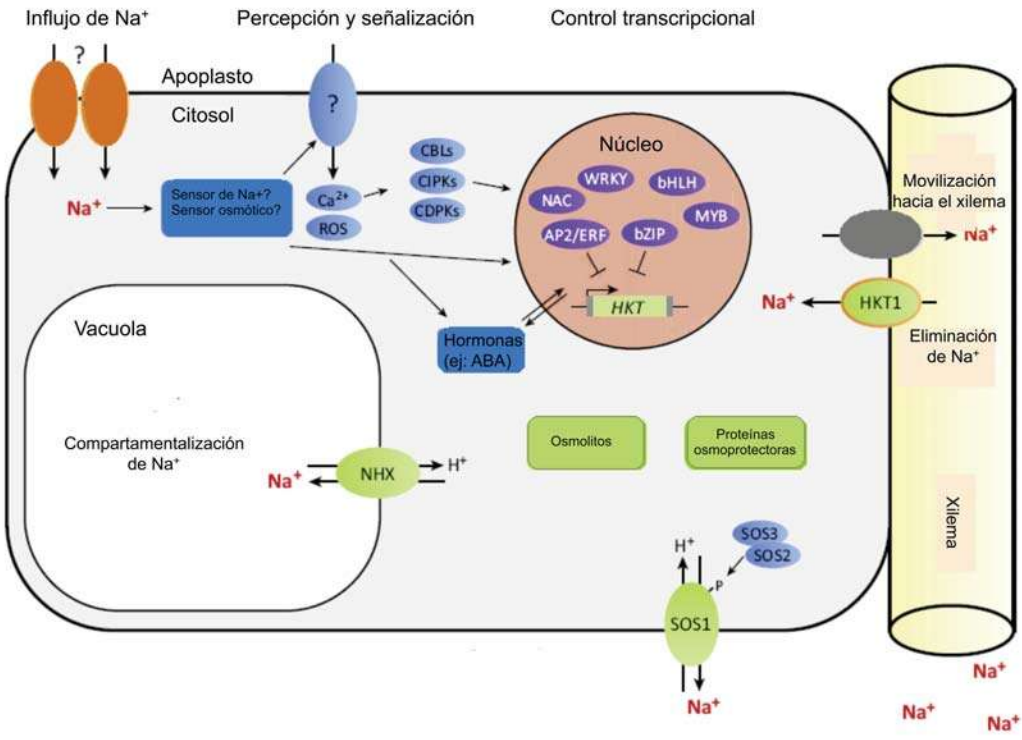

Figura 9. Mecanismos que permiten la tolerancia al estrés salino en las células de la raíz. El ión Na^+ (en rojo) entra en la célula mediante los transportadores de membrana NSCCs (NONSELECTIVE CATION CHANELS; óvalos naranjas). Dentro de la célula, el Na^+ puede ser percibido por un mecanismo no identificado. Posteriormente, se incrementa la concentración de Ca^{2+}, se producen ERO y se activa la señalización mediada por hormonas. Las proteínas CBL (CALCINEURIN B-LIKE PROTEINS), CIPKs (CBL-INTERACTING PROTEIN KINASES) y CDPKs (CALCIUM-DEPENDENT PROTEIN KINASES) son componentes de la vía de señalización mediada por Ca^{2+} (los componentes activados por Ca^{2+} se marcan en azul). Las proteínas cinasas de unión a calcio CBLs y CIPKs al activarse, afectan la transcripción (las familias de factores transcripcionales en el núcleo se indican de morado). Los factores transcripcionales AP2/ERF (APETALA2/ETHYLENE RESPONSE FACTOR) y bZIP regulan negativamente la expresión del gen *HKT* (*HIGH-AFFINITY* K^+ *TRANSPORTER*). Todas estas rutas de señalización temprana conducen a la activación de mecanismos de detoxificación celular. Los mecanismos mediados por HKT, NHX (Na^+/H^+ exchanger) y SOS (SALT OVERLAY SENSITIVE) son algunos de ellos. El mecanismo SOS inicia cuando se incrementan los niveles de Ca^{2+} en el citoplasma. El Ca^{2+} se une a la proteína SOS3. Los cambios conformacionales en esta proteína hacen que forme un dímero e interaccione de manera física con la proteína cinasa SOS2. Entonces la proteína SOS2 del dímero SOS3-SOS2 fosforila a SOS1 que es el transportador de Na^+, el cual se recluta en las vacuolas. Existe evidencia que el Na^+ también es expulsado de la planta a través de los exudados radiculares o mediante los estomas de las plantas. Imagen tomada del artículo de Deinlein *et al.* (2014) *Trends Plant Sci.* 19, 371-9.

2.5.3. Respuesta de la raíz y modificación hormonal al estrés salino

Tras la percepción del estrés salino las plantas activan mecanismos para la recuperación del crecimiento y la adaptación, ambos correlacionan con cambios en los niveles de hormonas de la planta como el ABA, ET, auxinas, jasmonatos, ácido giberélico y brasinoesteroides (Jiang *et al.* 2013a). Uno de los marcadores fisiológicos del desarrollo vegetal es la arquitectura radicular, la cual desempeña funciones indispensables, como la absorción de nutrientes y agua, de anclaje en el suelo y la interacción con microorganismos (López-Bucio *et al.* 2003, 2005). En consecuencia, el desarrollo del sistema radicular es central para que la planta alcance un crecimiento óptimo y contribuye directamente en los niveles de rendimiento obtenidos en los cultivos. El impacto de la raíz en el crecimiento de la planta se ha hecho evidente, no sólo en plantas modelo como *A. thaliana*, *Medicago truncatula* y *Lotus japonicus*, sino también en cultivos de importancia agrícola como el trigo (*Triticum aestivum*), arroz (*Oryza sativa*), y maíz (Hochholdinger y Tuberosa 2009; Coudert *et al.* 2010).

Una forma de minimizar el impacto negativo de los factores bióticos y abióticos sobre el rendimiento, consiste en alterar la arquitectura de la raíz. Los aspectos básicos de la arquitectura de las raíces implican crecimiento de la raíz primaria, formación de raíces laterales y adventicias. El sistema radicular está cubierto por pelos radiculares, una clase de células epidérmicas diferenciadas que aumentan aún más el potencial exploratorio. La raíz primaria se origina en el embrión y produce un gran número de raíces laterales durante el crecimiento vegetativo, y cada una de ellas a su vez producirá más raíces (Malamy y Benfey 1997; Casimiro *et al.* 2003; López-Bucio *et al.* 2005).

La arquitectura del sistema radicular es modificada por el nivel de auxinas endógenas o en respuesta a factores ambientales que incrementan sus concentraciones en las plantas o afectan la sensibilidad de las mismas, en la figura 10 se describe el mecanismo de señalización de las auxinas (Himanen *et al.* 2002; López-Bucio *et al.* 2003; Pérez-Torres *et al.* 2008). Las auxinas también han sido implicadas en respuesta al estrés salino. En *A. thaliana*, la sal induce la expresión de los genes *NIT1* y *NIT2*, que codifican para nitrilasas involucradas en la biosíntesis de AIA (Bao y Li 2002). Además, Wang y

colaboradores (2009) reportaron que la exposición a altas concentraciones de sal reprime la iniciación y la organogénesis de raíces laterales. Cuando se evaluó el efecto de 150 mM de NaCl sobre la expresión del gen *PIN2::GFP* que codifica para un transportador de eflujo de auxinas fusionado con la proteína reportera verde fluorescente, se encontró que la sal afecta la expresión de dicho gen y que su regulación se correlacióna con el halotropismo (Fig. 11). Estos resultados sugieren que la homeostasis de auxinas es importante para la adaptación y el desarrollo del sistema radicular bajo estrés salino.

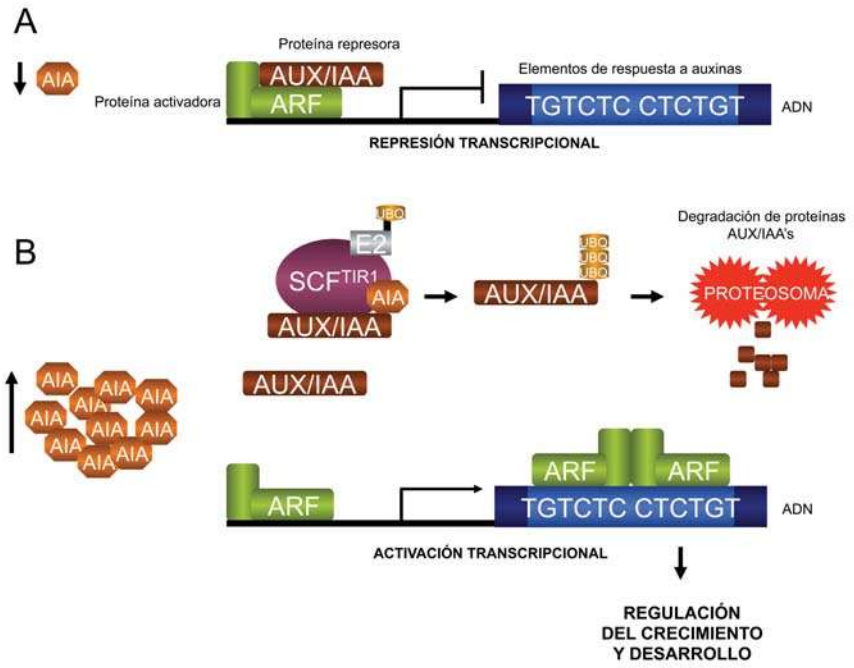

Figura 10. Mecanismo de señalización regulado por el AIA. Las auxinas se unen específicamente al receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1). Las AUX/IAA son proteínas de respuesta temprana que se unen a los factores de respuesta a auxinas (ARFs). Se han reportado 23 ARFs en *A. thaliana* que funcionan como factores de transcripción que se unen a los promotores de los genes de respuesta a auxinas, constituidos por varias configuraciones del motivo TGTCNC, mediando su transcripción. La evidencia experimental sugiere que diferentes ARFs pueden activar o reprimir la transcripción de los genes de respuesta a auxinas. Las proteínas AUX/IAA son degradadas por el proteosoma 26S después de ser ubiquitinadas en presencia de auxinas. Esta reacción es catalizada por el complejo $SCF^{TIR1}E2$. La influencia de las auxinas sobre la regulación mediada por los ARFs se establece principalmente por la concentración de auxinas. **A,** En ausencia de auxinas las proteínas Aux/IAA bloquean a los ARF. **B,** Cuando las auxinas están presentes, se degradan las proteínas Aux/IAA, lo que libera a los factores de transcripción ARF permitiendo la expresión de los genes de respuesta a auxinas, de los cuales se obtiene una respuesta celular (Modificada por Martínez de la Cruz E de Gray *et al.* 2001, 2004; Kieffer *et al.* 2010).

La ruta de señalización mediada por auxinas es uno de los blancos que afectan la salinidad, debido a que el NaCl disminuye la producción de biomasa, el crecimiento de la raíz y la formación de raíces laterales en las plantas de *A. thaliana* (Fig. 7) (Contreras-Cornejo *et al.* 2014b).

El análisis del crecimiento y de la respuesta del sistema radicular a la sal, de la línea silvestre Col-0 y las mutantes afectadas en algunos componentes de la ruta de las auxinas como *eir1/pin2* (transportador de eflujo), *aux1-7* (transportador de influjo), *arf7arf19* (factores transcripcionales) y *tir1afb2afb3* (receptores nucleares), apoya la hipótesis que para activar la tolerancia a la salinidad se requiere intacta la vía de las auxinas (Contreras-Cornejo *et al.* 2014b).

Recientemente, se determinó el perfil hormonal de una línea de maíz resistente a la sal y otra sensible y se encontraron diferencias significativas en las hojas y raíces. En respuesta a la salinidad, el maíz resistente aumentó los niveles de ácido indol-butírico en hojas y se mantuvo la concentración de AIA en las raíces (Zörb *et al.* 2013). Estos cambios adaptativos a nivel hormonal activaron la expresión de una β-expansina, lo cual permitió mantener de manera progresiva el crecimiento de la línea de maíz resistente a la salinidad. Por otra parte, las concentraciones de ABA aumentaron significativamente en las hojas de maíz resistente a la sal lo cual contribuye a la acidificación del apoplasto que, a su vez, es un prerrequisito para el crecimiento (Zörb *et al.* 2013).

Mediante el halotropismo, las plantas tratan de eludir la salinidad al alterar la dirección del crecimiento de la raíz. Galvan-Ampudia y colaboradores (2013) reportaron que el cambio de la dirección de la raíz se debe a la redistribución de auxinas en la punta, la cual es regulada por el transportador PIN2, y que la salinidad indujo la actividad de la fosfolipasa D, lo que estimuló la endocitosis de PIN2 en el sitio de la raíz en contacto con el sito salino, este efecto no se indujo por el estrés osmótico (Galvan-Ampudia *et al.* 2013). Explorar más a fondo los mecanismos moleculares detrás del halotropismo puede mejorar nuestra comprensión de las estrategias de la tolerancia a la salinidad de las plantas. A pesar de que varios componentes de respuesta al estrés salino en plantas se han identificado en los últimos años, existen importantes interrogantes que esperan respuesta como la caracterización del posible

sensor de Na^+, los componentes moleculares que conectan la percepción de Na^+con los segundos mensajeros, etc.

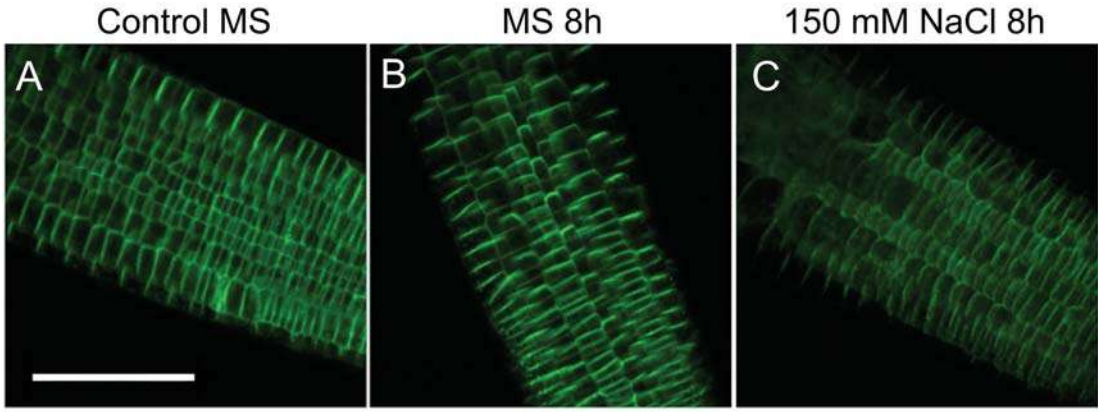

Figura 11. Efecto de la sal y la gravedad sobre la expresión de gen *PIN2::GFP* implicado en el transporte de eflujo de auxinas de *A. thaliana*. Imágenes representativas de una sección de la raíz analizada por microscopia confocal. **A,** Patrón de expresión en las plantas control creciendo en condiciones normales. **B y C,** Expresión de *PIN2::GFP* de plántulas transferidas en medio MS sin y con 150 mM NaCl respectivamente y reorientadas 90º durante 8 horas. Nótese que en las plantas control reorientadas 90º se disminuye ligeramente la expresión de *PIN2::GFP* y que el tratamiento con sal afecta severamente la expresión de dicho gen. Imagen tomada y modificada del artículo de Sun *et al.* (2008) *Plant Physiol.* 146, 178-188.

2.5.4. Defensa antioxidante en respuesta al estrés salino

Las especies reactivas de oxígeno (ERO) generadas durante el estrés salino causan importantes daños en componentes celulares como lípidos, proteínas y ácidos nucleicos (Abogadallah 2010). Las ERO más comunes son: el oxígeno singlete ($^1O_2$, atómico), peróxido de hidrógeno (H_2O_2), anión superóxido (O^-_2) y radicales hidroxilo ($OH^\bullet$). Las plantas sometidas a estrés salino muestran respuestas moleculares complejas, que incluyen componentes enzimáticos y no enzimáticos como: la síntesis de proteínas de estrés, compuestos antioxidantes y osmolitos (Zhu 2001). Estos componentes de respuesta antioxidativa tienen la función de desintoxicar las células de las ERO.

Se han descrito varias enzimas que participan en la eliminación de ERO en condiciones normales y durante el estrés oxidativo inducido por salinidad como: la glutatión peroxidasa (GPX), superóxido dismutasa (SOD), catalasa (CAT), ascorbato peroxidasa (APX), dehidroascorbato reductasa (DHAR) y glutatión reductasa (GR) (Zhu 2001; Waller *et al.* 2005). Por ejemplo, en condiciones de salinidad, las enzimas antioxidativas SOD, APX y GR fueron inducidas en altos niveles en plantas de maíz y GR, SOD, POD y CAT en girasol (Rios-Gonzalez *et al.* 2002; Abogadallah 2010). Los osmolitos manitol, fructanos, trehalosa, ononitol, prolina, glicina betaína y ectoína probablemente también participan en la eliminación de ERO (Zhu 2001). El ácido ascórbico (AA, vitamina C), α-tocoferol y carotenoides son importantes moléculas antioxidantes. La mutante *vtc1* de *A. thaliana* tiene bajo contenido de ascorbato, baja actividad de las enzimas monodehidroascorbato reductasa (MDAR) y DHAR, y altos niveles de H_2O_2, indicando esto que el ascorbato funciona como eliminador de ERO en condiciones de salinidad (Huang *et al.* 2005). Athar y colaboradores (2008) atenuaron con ácido ascórbico el estrés oxidativo inducido por estrés salino en plantas de trigo. El α-tocoferol se localiza en la membrana celular y es clave en la eliminación de ERO que afectan los lípidos (Munné-Bosh *et al.* 1999, 2005). Los carotenoides reaccionan con el $^1O_2$ y minimizan su formación al recibir el exceso de energía de la clorofila excitada (Siefermann-Harms 1987). Sin embargo, la participación de los carotenoides en la eliminación de ERO no se conoce a detalle debido a que su contenido disminuye en las plantas sometidas a salinidad (Parida 2005).

2.5.5. Importancia de los osmolitos

La acumulación de osmolitos orgánicos, tales como la prolina, glicina betaína, trehalosa y poliaminas desempeñan un papel clave para el mantenimiento del potencial osmótico intracelular de las plantas y para la prevención de efectos nocivos del estrés por salinidad (Deinlein *et al.* 2014). La síntesis de osmolitos y el posterior reordenamiento metabólico es fundamental para la tolerancia a la salinidad. Análisis moleculares han mostrado que el estrés abiótico estimula la síntesis de prolina, mientras que tras la recuperación de las plantas se potencia su degradación (Székely *et al.* 2008; Sharma y Verslues 2010). Durante la fase de recuperación, la prolina puede funcionar como una molécula señal en procesos de desarrollo regulando la proliferación celular, la muerte celular y la expresión de genes de recuperación de estrés abiótico (Szabados y Savoure 2010). El análisis del perfil transcripcional de plantas de *A. thaliana* crecidas en condiciones normales y posteriormente sometidas a deshidratación durante 2 y/o 5 horas y después rehidratadas, mostró que un tercio de los genes inducibles por rehidratación son inducidos por prolina (Oono *et al.* 2003; Szabados y Savoure 2010). En *A. thaliana*, se han identificado por lo menos 21 genes que son inducidos durante la rehidratación y la prolina. Dichos genes poseen en su promotor una región conservada que es específica para factores transcripcionales tipo bZIP (Szabados y Savoure 2010). Oono y colaboradores (2003) reportaron que la secuencia ACTCAT es común en la región del promotor de los genes inducidos por hipoosmolaridad y por la prolina. En este mismo estudio, entre 152 genes inducidos por rehidratación, 58 genes poseen la secuencia ACTCAT y codifican para factores de transcripción, proteínas cinasas, de transferencia de lípidos y LEA (late-embryogenesis-abundant protein). La tabla 1 muestra algunos de los genes que son inducidos tras la recuperación al estrés hídrico y por la prolina.

En *A. thaliana*, la mutante knockout del gen *p5cs1*, que codifica para una sintetasa conocida como D-1-pirrolina-5-carboxilato de etilo, afecta la síntesis de prolina que debería ocurrir tras el estrés salino (Székely *et al.* 2008). Se ha sugerido que la prolina juega un papel crucial en el ajuste osmótico; sin embargo, existen evidencias que muestran que dicho aminoácido actúa como eliminador del oxígeno reactivo, tampón redox o chaperona molecular para la

estabilización de proteínas y la estructura de la membrana bajo condiciones de estrés (Ashraf y Foolad 2007; Verbruggen y Hermans 2008). Similar a la prolina, la glicina betaína es un compuesto sintetizado por varias familias de plantas para equilibrar el potencial osmótico que inducen algunos iones bajo salinidad. La glicina betaína es un soluto implicado en la protección de las principales enzimas y la estructura de las membranas (Raza *et al.* 2007; Guinn *et al.* 2011). La trehalosa es un disacárido no reductor que se encuentra ampliamente en la naturaleza (Fernandez *et al.* 2010). En las bacterias, hongos e insectos la trehalosa tiene función de carbohidrato de almacenamiento y protección contra las tensiones que ocurren en la célula durante el estrés abiótico (Schluepmann *et al.* 2003, 2012; Paul *et al.* 2008; Lunn *et al.* 2014). En plantas sometidas a estrés salino se ha encontrado que la trehalosa funciona como regulador del equilibrio iónico. Yang y colaboradores (2014) encontraron que en *A. thaliana* la trehalosa antagonizó los daños inducidos por las ERO. Además, este carbohidrato fue capaz de restringir el transporte de Na^+ hacia las hojas lo cual correlacionó con la tolerancia a la salinidad.

Las poliaminas son policationes orgánicos con dos o más grupos amino primarios. Las diamina putrescina, la triamina espermidina y la tetraamina espermidina son comunes en los organismos vivos. Las poliaminas están involucradas en varios procesos celulares fundamentales, como la transcripción, modificación del ARN, síntesis de proteínas y la modulación de la actividad de las enzimas (Tabor y Tabor 1999). Las bacterias termófilas contienen poliaminas inusuales de cadena larga y algunas ramificadas, que están implicadas en la estabilización de ADN y ARN a altas temperaturas (Oshima 2007). La poliamina más simple, la putrescina, se deriva directamente de la ornitina por la ornitina descarboxilasa (ODC) o de la arginina a través de varios pasos catalizados por la arginina decarboxilasa (ADC) (Hanfrey *et al.* 2001). Las poliaminas en las plantas se localizan principalmente en los tejidos en crecimiento y están implicadas en el control de la división celular, la embriogénesis, la formación de las raíces, el desarrollo y maduración del fruto, y las respuestas al estrés biótico y/o abiótico (Kumar *et al.* 1997). La ADC es una enzima clave en la síntesis de putrescina en las plantas. El genoma de *A. thaliana* posee dos genes que codifican ADC: *ADC1* y *ADC2*. En contraste a *ADC1* que se expresa constitutivamente en todos los tejidos, *ADC2* se expresa

en condiciones de sequía (Soyka y Heyer 1999). Un alelo de la mutante del gen *ADC2* fue más sensible al estrés salino que la línea silvestre (Urano *et al.* 2004). Esta mutante tiene bajos niveles de putrescina, pero no de espermidina o espermina, y la tolerancia al estrés salino se restableció cuando se le aplicó putrescina de manera exógena (Takahashi y Kakehi 2010).

Tabla 1. Lista de genes de *A. thaliana* inducibles por rehidratación y prolina que poseen el motivo ACTCAT en el promotor.

Gen	**Categoría funcional**	**Descripción**	**Posición del motivo ACTCAT**	**Motivo ACTCAT**
ProDH	Catabolismo	Prolina deshidrogenasa	-719 a -714	CCACTCATCC
(=RAFL05-17-E01)	-	-	-591 a -586 -327 a 332	TGACTCATCC GGACTCATTG
RAFL07-10-K05	Proteína relacionada con pared celular	Xiloglucan endo-1, 4-β-D-glucanasa XTR-7	-364 a -369 -180 a -185 -87 a -92	AAACTCATCA TTACTCATAT AAACTCATAC
RAFL09-07-E15	Proteína relacionada con pared celular	Xilosidasa	-724 a -729 -689 a 694	AGACTCATTT AGACTCATTT
RAFL04-15-O22	Metabolismo	Glutamato deshidrogenasa 2	-634 a -639	TGACTCATTT
RAFL07-15-D01	Proteína desconocida	Proteína inducida por fosfato tipo phi-1	-810 a -815	CTACTCATGG
RAFL08-09-D04	Proteína desconocida	-	-775 a -770 -520 a -525	TTACTCATAA GGACTCATAT
RAFL05-19-C02	Proteína desconocida	Proteína de imbibición	-165 a -170	CAACTCATTA
RAFL05-01-A07	Proteína desconocida	-	-536 a -541	TTACTCATTC
RAFL11-11-M23	Proteína desconocida	Homóloga de la proteína pEARLI 1	-683 a -688 -304 a -299	CAACTCATCA GAACTCATGA

La expresión de estos genes ocurrió después de la hidratación de plantas deshidratadas durante 2 horas (Oono *et al.* 2003).

2.5.6. Efecto de los microorganismos rizosféricos sobre las plantas en estrés salino

El uso de microorganismos benéficos de la rizósfera es una estrategia para la agricultura debido a que mejoran el crecimiento y productividad de las plantas en diferentes condiciones. Por ejemplo, se mostró que el hongo basidiomiceto endofítico de raíz *P. indica* indujo la tolerancia de la cebada a altas concentraciones de sal (100-300 mM NaCl). Además, la colonización de la raíz por *P. indica* aumentó el crecimiento de las plantas y atenuó la peroxidación de lípidos inducida por NaCl en las hojas de cebada del cultivar 'Ingrid' sensible a la sal. La inoculación de *P. indica* también incrementó significativamente la cantidad de ácido ascórbico y la actividad de la enzima dehidroascorbato reductasa en las raíces (Waller *et al.* 2005). Echeverria y colaboradores (2008) reportaron que 150 mM de NaCl afectó severamente el desarrollo del sistema radicular de *Lotus glaber*. Sin embargo, las plantas tratadas con sal e inoculadas con el hongo micorrícico *Glomus intraradices* desarrollaron un sistema radicular ramificado similar a las plantas crecidas sin estrés salino.

La capacidad de las plantas para adaptarse a condiciones de estrés a menudo parece depender de su asociación con los microorganismos. Existen reportes que muestran que las señales hormonales producidas por los microorganismos rizosféricos mejoran la ramificación de la raíz en *A. thaliana* con un impacto dramático en la producción de biomasa (Ortiz-Castro *et al.* 2009). Por ejemplo, los microorganismos asociados a las plantas podrían influir en el incremento de auxinas endógenas *in planta* (Contreras-Cornejo *et al.* 2009; Felten *et al.* 2012; Hilbert *et al.* 2012).

Está claro que algunas especies de *Trichoderma* tienen una influencia directa en la salud de la planta y el desarrollo mediante la producción de reguladores del crecimiento. También se conoce que estos hongos pueden conferir tolerancia al estrés salino (Brotman *et al.* 2013). La adaptación a la salinidad se ha correlacionado con la acumulación de prolina en plantas de trigo (Rawat *et al.* 2011). Sin embargo, poco se sabe acerca del papel de las auxinas en la interacción de *Trichoderma* spp. con las plantas bajo estrés salino.

3. JUSTIFICACIÓN

Varios microorganismos rizosféricos pueden ayudar a las plantas a hacer frente al estrés salino mediante diferentes mecanismos. El uso de *Trichoderma* en los sistemas agrícolas está cobrando mayor interés. Sin embargo, no se conoce el efecto de estos hongos sobre el crecimiento vegetal en condiciones de estrés salino por lo que es importante esclarecer la respuesta de *A. thaliana* y *Trichoderma* spp. a la salinidad y el impacto del cocultivo en estas condiciones.

4. HIPÓTESIS

Trichoderma virens Gv. 29-8 y *Trichoderma atroviride* IMI 206040 inducen resistencia a la salinidad en *A. thaliana* aumentando la plasticidad del sistema radicular y modificando la respuesta auxínica.

5. OBJETIVOS

5.1. Objetivo general

Caracterizar las respuestas de *A. thaliana* al estrés salino y el impacto de *T. virens* y *T. atroviride* en el crecimiento vegetal en condiciones de salinidad.

5.2. Objetivos particulares

1. Caracterizar los efectos de la salinidad en el crecimiento de *Trichoderma* y *A. thaliana*.
2. Evaluar los efectos de la salinidad sobre la arquitectura de la raíz y la respuesta a auxinas.
3. Determinar los efectos de la inoculación con *Trichoderma* en la tolerancia a salinidad en plantas de *A. thaliana*.
4. Identificar los compuestos que produce *Trichoderma* spp. potencialmente involucrados en la tolerancia a salinidad en *A. thaliana*.

6. RESULTADOS

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e-Xtra*

6.1. CAPÍTULO I.

Trichoderma spp. Improve Growth of *Arabidopsis* Seedlings Under Salt Stress Through Enhanced Root Development, Osmolite Production, and Na^+ Elimination Through Root Exudates

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Salt stress is an important constraint to world agriculture. Here, we report on the potential of *Trichoderma virens* and *T. atroviride* to induce tolerance to salt in *Arabidopsis* seedlings. We first characterized the effect of several salt concentrations on shoot biomass production and root architecture of *Arabidopsis* seedlings. We found that salt repressed plant growth and root development in a dose-dependent manner by blocking auxin signaling. Analysis of the wild type and *eir1*, *aux1-7*, *arf7arf19*, and *tir1abf2abf19* auxin-related mutants revealed a key role for indole-3-acetic acid (IAA) signaling in mediating salt tolerance. We also found that *T. virens* (Tv29.8) and *T. atroviride* (IMI 206040) promoted plant growth in both normal and saline conditions, which was related to the induction of lateral roots and root hairs through auxin signaling. *Arabidopsis* seedlings grown under saline conditions inoculated with *Trichoderma* spp. showed increased levels of abscissic acid, L-proline, and ascorbic acid, and enhanced elimination of Na^+ through root exudates. Our data show the critical role of auxin signaling and root architecture to salt tolerance in *Arabidopsis* and suggest that these fungi may enhance the plant IAA level as well as the antioxidant and osmoprotective status of plants under salt stress.

Salinity is a major environmental factor affecting crop production. Up to 7% of the total land surface is saline and approximately one-third of the world's irrigated land is subjected to secondary-induced salinization (Hariadi et al. 2011; Shabala and Cuin 2008). Under salt stress, plants experience dehydration, nutrient deficiencies, membrane dysfunction, and oxidative stress, which lead to tissue damage or early senescence (Essah et al. 2003; Katori et al. 2010).

To avoid accumulation of toxic sodium (Na^+) levels in shoots, plants must take up no more than 3% of the Na^+ present in the rhizosphere, and many adaptive mechanisms are then activated (Zhang et al. 2008a). Salt tolerance is a complex trait that involves multiple physiological and biochemical mechanisms: for example, the salt-overly sensitive pathway, which regulates ionic homeostasis; the inducer of the CBF (C-repeat/dehydration-responsive element binding factor) expression and dehydration-responsive element–binding pathway that controls the expression of dehydration response element and C repeat-containing genes; and the mitogen-activated protein kinase cascade that regulates the generation of osmolytes and antioxidants with protective functions (Mehlmer et al. 2010; Seki et al. 2003; Xiong et al. 2002a and b; Zhu 2003).

The root system performs indispensable plant functions such as uptake of nutrients and water, anchorage in the soil, and interaction with symbiotic microorganisms (López-Bucio et al. 2003, 2005). Consequently, root system development is central for the plant to reach optimal growth and directly contributes to the levels of yield obtained in crops. The impact of the root on plant growth has become apparent not only in model plants such as *Arabidopsis thaliana*, *Medicago truncatula*, and *Lotus japonicus* but also in important crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Coudert et al. 2010; Hochholdinger and Tuberosa 2009). One way to minimize the negative impact of biotic and abiotic factors on yield is to manipulate root system architecture (RSA). The basic aspects of root architecture involve primary root growth and lateral and adventitious root formation. Branching structures are covered by root hairs (RH), a class of differentiated epidermal cells that further increase the exploratory potential. The primary root originates in the embryo and produces many lateral roots (LR) during vegetative growth, and each of these will produce more LR (Casimiro et al. 2003; López-Bucio et al. 2005; Malamy and Benfey 1997).

RSA is modified by the endogenous auxin level or in response to environmental factors that increase the auxin pool in the plant or affect auxin sensitivity (Himanen et al. 2002; López-Bucio et al. 2003; Pérez-Torres et al. 2008). In *Arabidopsis*, salt stress induces nitrilase genes *NIT1* and *NIT2,* which are involved in indole-3-acetic acid (IAA) biosynthesis (Bao and Li 2002). In addition, Wang and associates (2009) reported that high salt exposure suppresses LR initiation and organogenesis, which correlated with the concomitant reduction of expression of the auxin-inducible reporter *DR5::GUS* in primary root tips. These results suggest that auxin homeostasis is important for adaptive root system development under salt stress; however, it still remains to be determined whether auxin biosynthesis, transport, or sensitivity is the key target of salt stress. Other-

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*The *e*-Xtra logo stands for "electronic extra" and indicates that two supplementary figures are published online and that Figures 1, 3, 6, and 7 appear in color online.

wise, the ability of plants to adapt to stress conditions often appears to depend on their association with microbes. Recent reports have shown that auxin-like signals produced from rhizosphere microorganisms could improve root branching in *Arabidopsis*, with a dramatic impact in plant biomass production (Ortiz-Castro et al. 2011). The potential of plant-associated microorganisms to produce IAA, auxin precursors, or auxin signal mimics represents a means to influence the endogenous auxin pool of the host (Contreras-Cornejo et al. 2009; Felten et al. 2012; Hilbert et al. 2012). However, little is known about the implication of this hormone in symbiosis or plant tolerance to stress.

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi, and compete with deleterious plant microorganisms (Harman et al. 2004). Until recently, these traits were considered to be the basis for how *Trichoderma* spp. exert beneficial effects on plant growth and development. However, it is clear that certain species also have substantial direct influence on plant health and development by producing phytohormones, activating defense responses, or conferring stress tolerance in plants (Brotman et al. 2013; Contreras-Cornejo et al. 2009, 2011, 2013; Velázquez-Robledo et al. 2011). *Trichoderma* spp. allowed the accumulation of proline in wheat plants under salt stress condition (Rawat et al. 2011), and microarray analysis of *Arabidopsis* and cucumber roots exposed to salt stress and inoculated with *Trichoderma* spp. revealed an increased expression of genes related to salt-tolerance, osmoprotection processes, and ascorbic acid (AA) production (Brotman et al. 2013).

Our previous research showed that *Trichoderma virens* and *T. atroviride* produce IAA and the indolic compounds indole-3-ethanol, indole-3-acetaldehyde, and indole-3-carboxaldehyde as part of their metabolism (Contreras-Cornejo et al. 2009, 2011). It was found that mutations in genes involved in auxin transport or signaling (*AUX1*, *BIG*, *EIR1*, and *AXR1*) reduced the growth-promoting and root developmental effects of *Trichoderma* spp. inoculation. Colonization of plant roots by fungal hyphae activated *DR5:uidA* expression, which correlated with an increased cell proliferation in LR tips. Application of all three indolic compounds produced by the fungus to

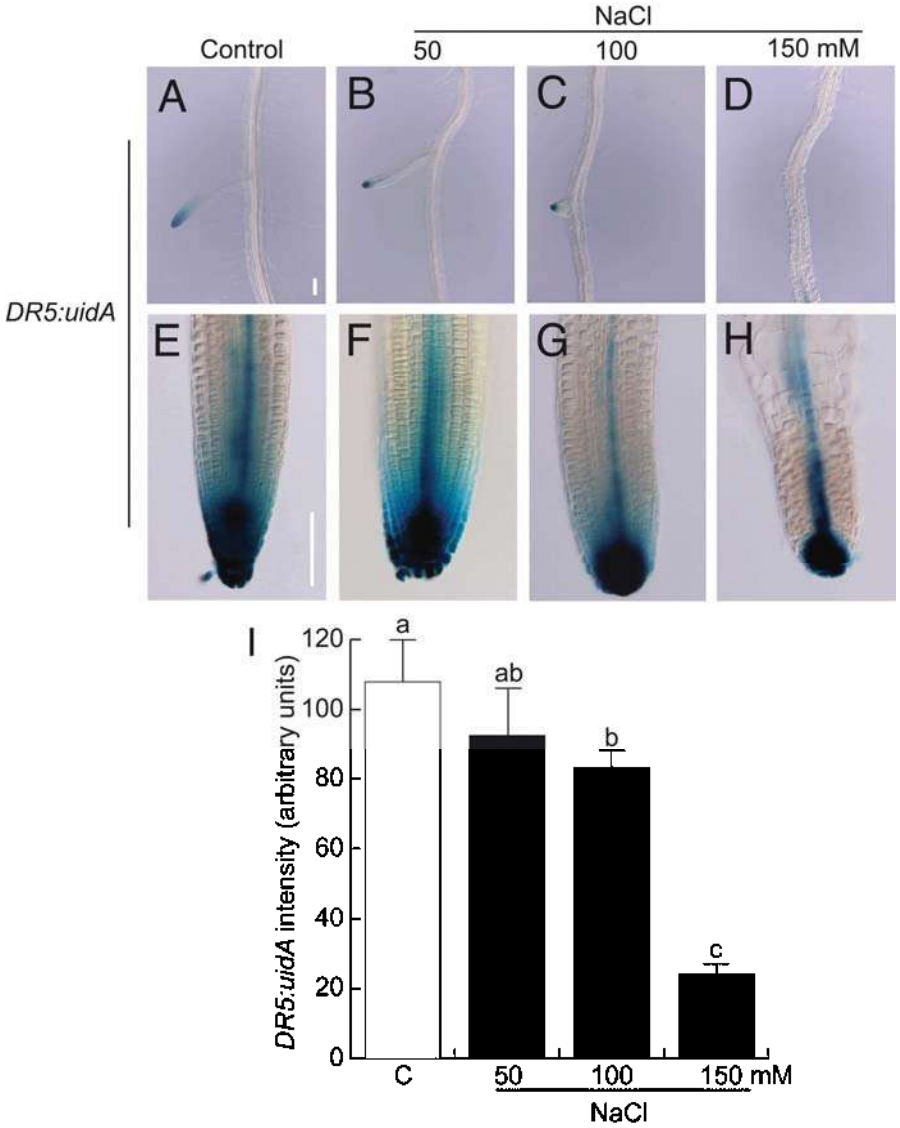

Fig. 1. Effect of NaCl on *DR5:uidA* expression. Representative photographs of **A** to **D,** lateral roots or **E** to **H,** primary root tips from transgenic *Arabidopsis* seedlings expressing the *DR5:uidA* auxin-inducible gene marker, which were grown on media with increasing concentrations of NaCl. **I,** Quantitative analysis of β-glucuronidase expression in primary root tips using the image J program. Photographs are representative individuals of at least 15 plants stained. Scale bar = 1 mm. The experiment was repeated two times with similar results.

Arabidopsis seedlings showed a dose-dependent effect on biomass production and in activation of *DR5:uidA* expression.

In this report, we show that salt affects plant biomass production and reduces root growth, LR formation, and RH development by decreasing auxin responsiveness. Cocultivation of plant roots with *T. virens* or *T. atroviride* normalized root development, likely because these fungi provide IAA to plants. Moreover, *Trichoderma* spp. improved the antioxidative and osmoprotective capacity and increased growth. Our results reveal that, by enhancing LR and RH development through sustained auxin production, *Trichoderma* spp. may affect plant performance and yield under saline conditions.

RESULTS

Salinity affects root architecture and decreases auxin responsiveness in *Arabidopsis* seedlings.

A common negative effect of salinity is reduced root growth and decreased plant biomass (Achard et al. 2006). Little is known about specific root architectural responses to salt and the contribution of auxin signaling in the different traits responsible for root adaptation to this stress. To evaluate the effect of salt on *Arabidopsis* growth and development, wild-type (WT) (Col-0) seedlings were germinated and grown for 9 days on vertically oriented agar plates containing 0.2× Murashige and Skoog (MS) medium supplied with increasing concentrations of salt (50 to 200 mM NaCl). It was found that increasing salt concentration in the medium affected total fresh weight, primary root length, LR number and density, and RH length in a dose-dependent manner (Supplementary Fig. S1A to E). These effects were more evident in seedlings grown in 100 to 150 mM or higher salt concentrations, which drastically decreased plant growth and biomass production. At 150 mM NaCl, LR and RH formation was totally blocked (Supplementary Fig. S2). These results show that NaCl affects fundamental cellular processes responsible for the configuration of RSA.

To test whether the repression of root branching by NaCl could be associated with changes in auxin accumulation or

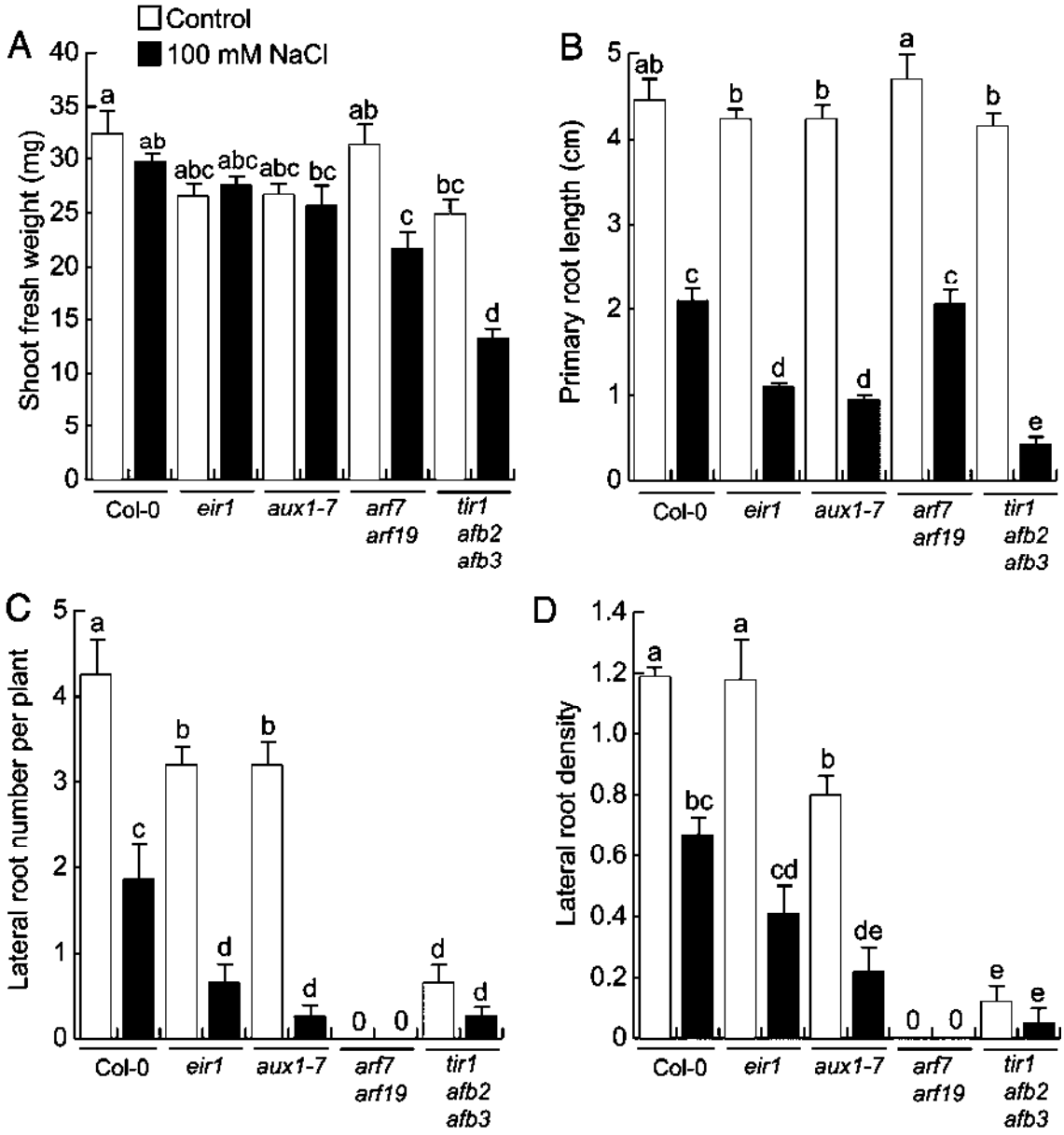

Fig. 2. Effect of NaCl on *Arabidopsis* wild type and mutants with defects in auxin transport or signaling. **A,** Shoot fresh weight. **B,** Primary root length (n = 15). **C,** Lateral root number per plant (n = 15). **D,** Lateral root density (n = 15). Error bars represent the standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

response, expression of the β-glucuronidase (GUS) reporter gene driven by the auxin-sensitive *DR5* promoter (Dubrovsky et al. 2008; Ulmasov et al. 1997) was examined in primary roots and LR. It was found that 50 to 150 mM NaCl affected the expression of *DR5:uidA* in primary roots and LR (Fig. 1A to H), which correlated with decreased root branching. The intensity of *DR5:uidA* gene expression was determined from images of primary root tips, which were processed using the Image J software and the data expressed as arbitrary units (Fig. 1I).These data show that salt inhibits auxin-inducible gene expression in a dose-dependent manner in primary root tips.

Arabidopsis mutants defective on auxin receptors are oversensitive to salt treatments.

Previous work showed that *Arabidopsis* mutants with defects in auxin influx (*aux1-7*) or efflux (*pin2*) were slightly oversensitive to salt stress, indicating that auxin transport might play a role in root system remodeling under salt stress (Wang et al. 2009). To further characterize the contribution of auxin transport and response in plant salt tolerance and its relationship with root adaptive traits, we compared the growth and development of WT and *Arabidopsis* mutants, including *eir1-1* and *aux1-7*, defective on auxin transporters, and *arf7arf19* and *tir1afb2afb3*, defective in transcription factors or receptors involved in auxin response, respectively. When grown under 100 mM NaCl, it was found that the *eir1-1* and *aux1-7* mutants showed a slight decrease of shoot fresh weight under salt treatment in a manner similar to WT seedlings. Interestingly, the *arf7arf19* and *tir1afb2afb3* mutants were clearly oversensitive to this salt treatment, with a 35 and 50% inhibition in shoot fresh weight, respectively (Fig. 2A). Salt treatment repressed approximately 55 to 60% of primary root growth, LR number per plant, and LR density in WT seedlings; whereas, in *eir1*, *aux1-7*, and *tir1afb2afb3*, these root architectural traits were more severely affected (Fig. 2B to D). The fact that the *tir1afb2afb3* triple mutant, which is defective in three putative auxin receptors of the TIR1 family, shows salt oversensitivity in biomass production and primary root growth suggests that auxin signaling (and not only auxin transport) is an important target of salinity.

T. virens and *T. atroviride* show differential growth and produce auxin under salt stress.

High salinity may affect plants, their microbial partners, and the outcome of the plant-microbe interaction. We next determined the effect of salinity on growth of *T. virens* and *T. atroviride*. In all, 10^6 conidia from each species were germinated and grown for 5 days on agar plates containing 0.2× MS medium supplied with increased concentrations of salt (50 to 300 mM NaCl). It was found that salt affected the growth of both *Trichoderma* spp. in a dose-dependent manner (Fig. 3A). However, both fungal strains tolerated 150 mM NaCl well (Fig. 3B and C) and differential effects were observed with the 300 mM salt treatment, in which the growth of *T. virens* and *T. atroviride* was repressed 27 and 74%, respectively. These results show that *T. virens* tolerates higher NaCl levels than *T. atroviride* and may represent a very promising agent to test salt responses in plant-fungus interactions.

Trichoderma spp. release auxin and auxin precursors as part of their metabolism (Contreras-Cornejo et al. 2009); thus, it was important to know whether salinity could affect auxin production in these fungi. The production of IAA by *T. virens* and *T. atroviride* was determined in liquid medium with or without 100 mM NaCl, a salt concentration that drastically affects LR formation in *Arabidopsis*. It was found that salt slightly increased IAA production in *T. virens* from 4.21 to 5.88 ng/ml, whereas in *T. atroviride*, a higher and sustained auxin production from 7.65 to 7.64 ng/ml was registered under both normal and saline growth conditions. These data show that *Trichoderma* spp. are able to produce auxin when subjected to salt treatment, which could benefit plant growth under salt stress.

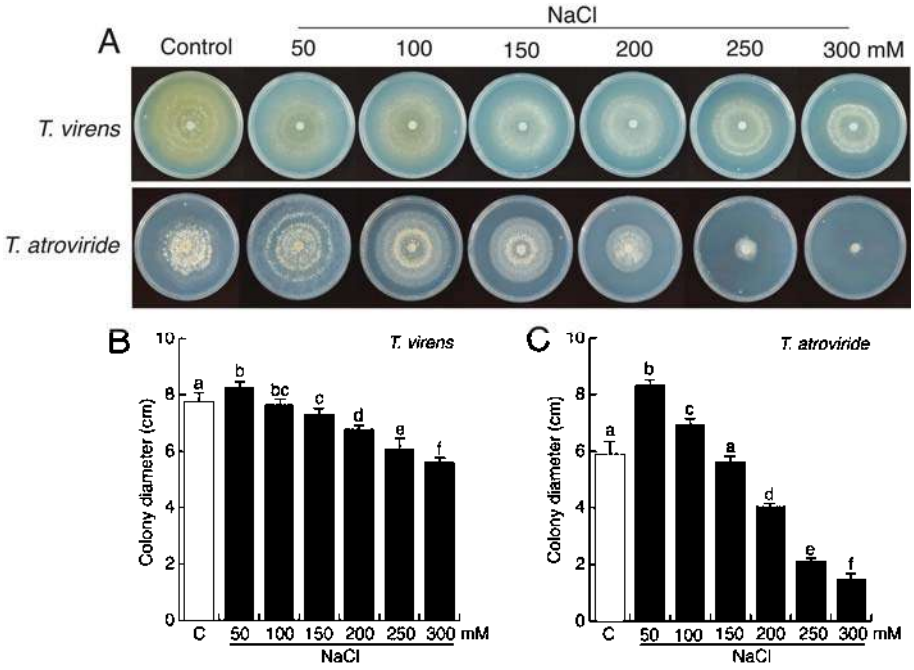

Fig. 3. Effect of NaCl on *Trichoderma* spp. growth. To determine the effect of NaCl on fungi, 1×10^6 spores from *Trichoderma* spp. were used to inoculate 0.2× Murashige and Skoog medium with or without NaCl. **A,** Representative photographs showing the aspect of the colonies of the strains grown at 24°C and photographed after 5 days. **B,** Kinetic of *Trichoderma virens* growth determined by measuring colony diameter. **C,** Kinetic of *T. atroviride* growth. Bars represent the means ± standard deviation, based on two independent experiments with three petri dishes each. Different letters represent means statistically different at the 0.05 level.

***Trichoderma* spp. promote growth and confer salt tolerance in *Arabidopsis*.**

To investigate whether *Trichoderma* spp. could confer salt tolerance to plants, *Arabidopsis* (Col-0) seedlings were germinated and grown on petri plates containing agar-solidified 0.2× MS medium with or without 100 mM NaCl. At 4 days after germination, the seedlings were treated with sterilized water (control treatment) or with 10^6 conidia of *T. virens* or *T. atroviride*. Fungal spores were placed at a 5-cm distance from the primary root tip to test the possibility that auxins released by the fungal colony could reach the root system and affect growth and development.

Plants inoculated with *T. virens* or *T. atroviride* showed enhanced shoot growth when compared with control seedlings when grown in medium with or without 100 mM salt. The dif-

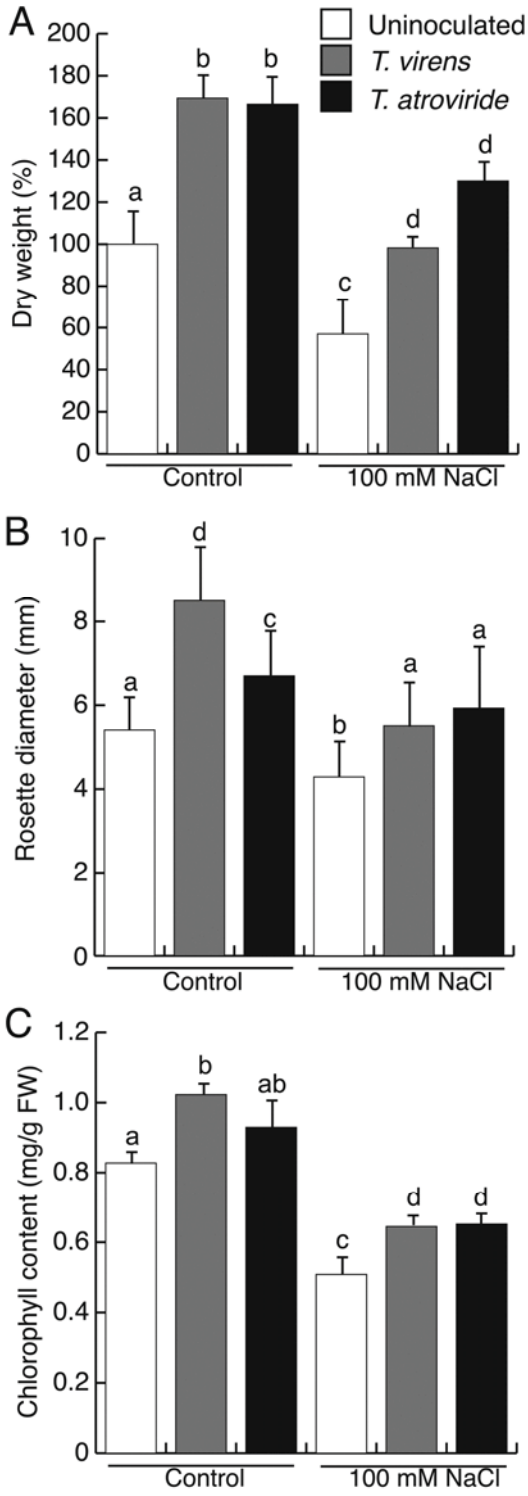

Fig. 4. *Trichoderma* spp. confer salt tolerance in *Arabidopsis*. **A,** Effect of *Trichoderma* spp. on total biomass accumulation represented as dry weight. **B,** Shoot diameter ($n = 60$). **C,** Total chlorophyll content. Rosettes were excised after 5 days of inoculation and chlorophyll content was determined. Values shown represent means of six groups of 20 seedlings ± standard error. Asterisks are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

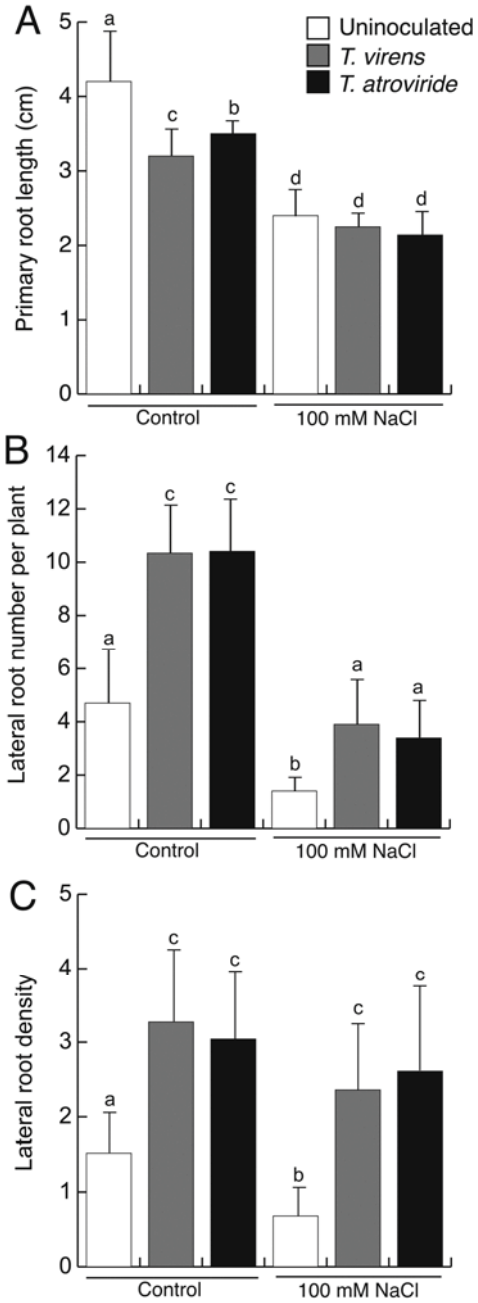

Fig. 5. Effects of *Trichoderma* spp. on root architecture of *Arabidopsis* seedlings grown under salinity. Seedlings were germinated and grown for 5 days on the surface of agar plates containing 0.2× Murashige and Skoog medium. Plates were inoculated with *Trichoderma virens* or *T. atroviride* at a distance of 5 cm from the primary root tip and grown for an additional 5-day period. **A,** Primary root length. **B,** Lateral root number per plant. **C,** Lateral root density. Bars represent the means ± standard deviation, based on three independent experiments with 60 seedlings each. Different letters represent means statistically different at the 0.05 level.

ference in dry weight and rosette diameter clearly demonstrated the beneficial effects of these fungi under salt stress (Fig. 4A and B). Saline stress also had a negative effect on chlorophyll content. The content of chlorophyll in plants grown with 100 mM NaCl decreased by 38.56% when compared with the control treatment. However, the chlorophyll level increased by 15.66% in plants inoculated with *T. virens* and 16.87% by *T. atroviride* (Fig. 4C). These results show the beneficial effects of *Trichoderma* spp. to confer salt tolerance.

Trichoderma spp. improve root-system architecture of *Arabidopsis* seedlings grown under saline conditions.

The mechanisms by which plants incorporate microbial signals into root-system development under saline stress are poorly understood. We performed bioassays to determine whether *Trichoderma* spp. could affect root plasticity in *Arabidopsis* grown under salt stress. We found that seedlings grown in normal conditions or with 100 mM NaCl co-cultivated with *T. virens* or *T. atroviride* developed a more branched root system. There were slight differences in primary root growth in *Trichoderma* spp.-inoculated seedlings when compared with axenically grown seedlings. Salt stress repressed by 50% primary root growth in control or inoculated seedlings (Fig. 5A). As expected, the LR number and density (LR number per centimeter) were reduced by 100 mM salt treatment. Interestingly, *Trichoderma* spp. normalized LR formation, increasing both LR number and density to the levels shown in uninoculated seedlings grown without salt (Fig. 5B and C). These results show that *Trichoderma* spp. can normalize root branching in *Arabidopsis* under salt stress.

RH are specialized tubular structures formed from differentiated epidermal cells of roots called trichoblasts. To investigate the effect of *Trichoderma* spp. on RH formation under elevated salinity, *Arabidopsis* seedlings were germinated and grown for 4 days on 0.2× MS with or without salt; after this period, seedlings were treated with water as a control or inoculated with *Trichoderma* spp. and grown for 5 additional days. Both RH length and density decreased significantly with 100 mM NaCl (Fig. 6G and H). Importantly, *Trichoderma* spp. increased the RH length and density in *Arabidopsis* grown in normal or saline conditions when compared with their respective controls (Fig. 6A to H). These data show that *Trichoderma* spp. can promote RH length and density.

Trichoderma spp. increase auxin-inducible gene expression in *Arabidopsis* under salt stress.

The results described above strongly suggest that *Trichoderma* spp. induce LR formation under salt stress, likely providing auxin to plants. Because auxin triggers various developmental effects through the activation of auxin-responsive

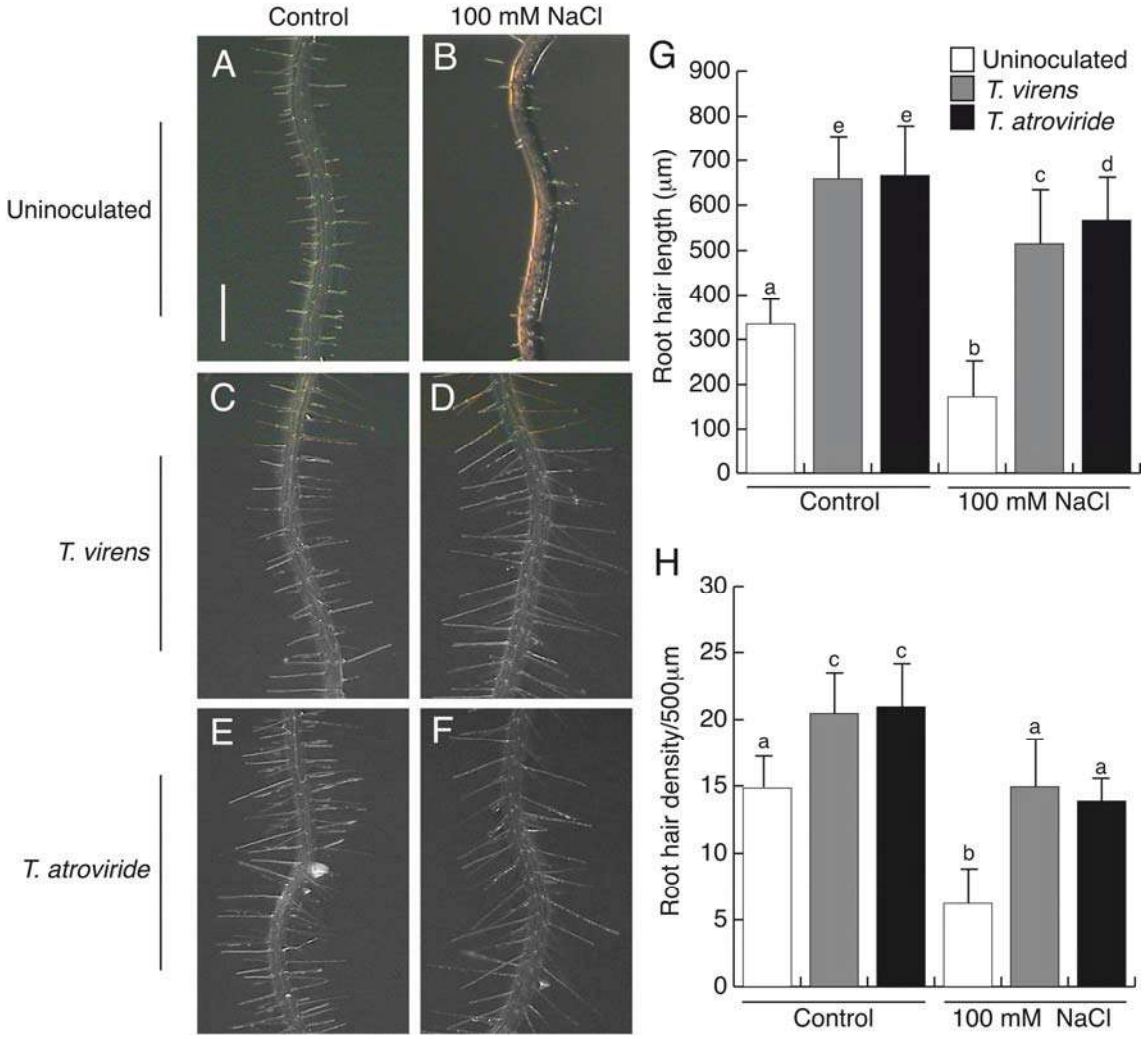

Fig. 6. Effect of *Trichoderma* spp. on root-hair development in *Arabidopsis* seedlings grown under salt stress. **A** to **F,** Representative photographs of root hairs from seedlings grown in normal or saline conditions and inoculated with *Trichoderma* spp. Bar = 500 µm. **G,** Length of root hairs formed in the primary root and **H,** root-hair density. Values shown represent the mean of 100 root hairs ± standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$).

genes, we evaluated the expression of the auxin-responsive marker gene *DR5:uidA*. *T. virens* and *T. atroviride* increased the expression of *DR5:uidA* in LR and primary root tips in nonsaline conditions (Fig. 7A to F). Although expression of *DR5:uidA* was reduced in plants treated with 100 mM NaCl, both *Trichoderma* spp. clearly induced the expression of *DR5:uidA* in LR and primary root tips under elevated salinity (Fig. 7G to L), which correlated with an improved growth of LR. An analysis of the *DR5:uidA* expression domain in primary root tips processed using the Image J software showed that *T. virens* and *T. atroviride* increased auxin-responsive gene expression by 40% (Fig. 7M). These results show that *Trichoderma* spp. increase auxin-responsive gene expression under saline stress, which positively affect LR development.

***Trichoderma* spp. increase abscissic acid, L-proline, AA content, and salt exudation.**

To determine the biochemical and metabolic events that occur in *Arabidopsis* during the interaction with *Trichoderma* spp. under saline or normal conditions, we quantified the amounts of metabolites related to salt stress. Abscissic acid (ABA) is considered the universal plant stress hormone (Verslues and Zhu 2005; Wasilewska et al. 2008). *Arabidopsis* seedlings inoculated with *T. virens* or *T. atroviride* showed a twofold increased ABA concentration in shoots when compared with axenically grown seedlings. ABA levels in uninoculated plants were sixfold increased in response to saline stress. Interestingly, plants subjected to 100 mM NaCl treatment and inoculated with *Trichoderma* spp. displayed similar levels of ABA compared with nonstressed plants (Fig. 8A). L-proline (L-Pro) is accumulated in response to salinity, and its accumulation frequently correlates with tolerance to drought or salt stress in plants (Ben et al. 2008; Parida et al. 2008). Saline stress induced twofold the amount of L-Pro in uninoculated seedlings when compared with nonstressed seedlings, and a further increase in L-Pro was evident in *Arabidopsis* seedlings inoculated with *Trichoderma* spp. (Fig. 8B). Several reports have suggested that AA has a crucial function as antioxidant. *Arabidopsis* seedlings inoculated with *T. virens* grown under nonsaline conditions showed increased AA levels when compared with uninoculated seedlings. No changes were found in seedlings inoculated with *T. atroviride*. In contrast, the AA amounts in *Arabidopsis* under salt-stress inoculated with *Trichoderma* spp. increased further when compared with the saline control (Fig. 8C).

Excessive Na^+ accumulated in plant cells is the primary cause of inhibition of plant growth (Ghoulam et al. 2002; Ungar 1996). Because detoxification mechanisms would prevent salt toxicity, we determined whether *Trichoderma* spp. inoculation could affect the levels of Na^+ in root exudates. Seedlings were grown in normal or saline conditions and inoculated with *Trichoderma* spp. by 5 days. Then, seedlings were transferred to falcon tubes containing tridistilled water. Root exudates were collected 2 days later, samples were filtered and Na^+ was determined by atomic absorption spectrophotometry. In plants grown in medium without salt, no significant differences in Na^+ exudation were found among treatments. However, plants grown under salinity increased exudation of Na^+ by roots, with a clearly enhanced amount of Na^+ being exuded in *Trichoderma* spp.-inoculated plants (Fig. 8D). These data show that *Arabidopsis* seedlings inoculated with *Trichoderma* spp. are better adapted to cope with salt stress, likely by increasing plant vigor, osmolite and antioxidant production, and salt exudation.

DISCUSSION

Plants synthesize and require a variety of signals to adjust growth and development throughout their life cycle. Auxins,

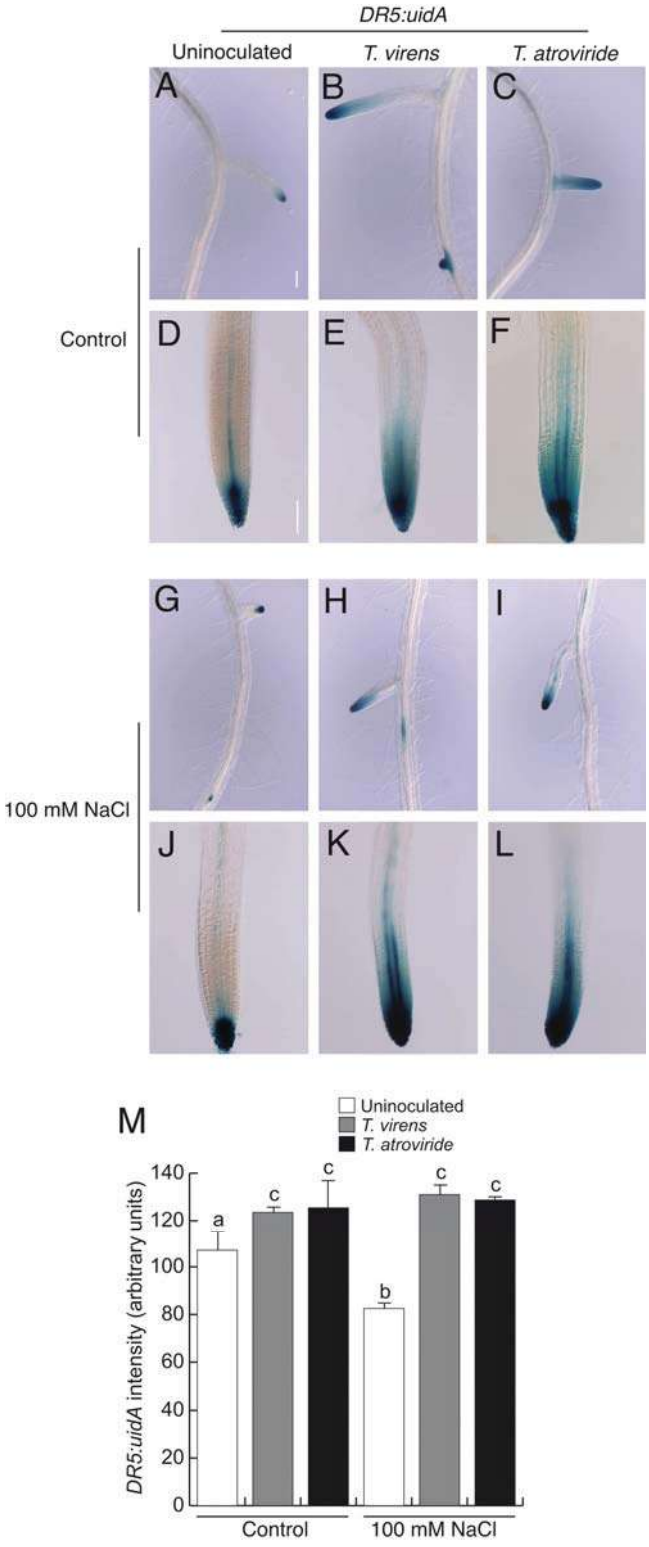

Fig. 7. Effect of *Trichoderma* spp. on the expression of *DR5:uidA* in normal or saline growth conditions. *Arabidopsis* transgenic seedlings were grown by 4 days on agar plates containing 0.2× Murashige and Skoog medium with or without 100 mM NaCl and inoculated with *Trichoderma* spp. by 5 days. **A to F,** Representative images from *DR5.uidA* seedlings uninoculated or inoculated with *Trichoderma* spp. in normal conditions. **G** to **L,** Photographs from seedlings uninoculated or inoculated with *Trichoderma* spp. under elevated salinity. **M,** Quantitative analysis of β-glucuronidase expression in primary root tips using the image J program. Photographs are representative individuals of at least 15 plants stained. Scale bar = 100 μm. The experiment was repeated three times with similar results.

including IAA, comprise a group of tryptophan-derived signals which are involved in most aspects of plant development (Woodward and Bartel 2005) These compounds exert a strong biological activity at very low concentrations in both in vivo and in vitro systems. Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms, and signal perception through a family of auxin transporters, transcription factors, and IAA receptors (Leyser 2006; Ljung et al. 2002; Mockaitis and Estelle 2008). Auxins have also been implicated in an abiotic stress response (Bao and Li 2002). Recent breeding improvements in terms of salt resistance of maize have led to a genotype with improved growth under saline conditions. By comparing this salt-resistant hybrid with a sensitive hybrid, it was possible to show differences in hormone concentrations in expanding leaves and roots. In response to salinity, the salt-resistant maize significantly increased indole-butyric acid concentrations in leaves and maintained IAA concentration in roots (Zörb et al. 2013). These hormonal adaptations were suggested to activate β-expansin gene expression to maintain growth of resistant maize hybrids under salt stress. Moreover, ABA concentrations significantly increased in resistant maize leaves under salt stress, which may contribute to acidifying the apoplast which, in turn, is a prerequisite for growth (Zörb et al. 2013). In consonance with this information, it was reported that auxin transport is required for remodeling RSA under salt stress because *aux1-7* and *pin2 Arabidopsis* mutants showed slight oversensitivity to salt exposure and because salt treatments repress expression of the auxin efflux carrier *PIN2*, and led to a stable reduction in PIN2 protein abundance (Galvan-Ampudia and Testerink 2011; Wang et al. 2009).

Despite this available information, little is known about the relationship between salinity stress and auxin levels in plants and the role of auxin in alleviating salt stress. Our data further extend these previous observations by showing that auxin responsiveness is an important target of salinity, because NaCl treatments decrease biomass production, root growth, and LR formation in *Arabidopsis* seedlings, which correlates with a dose-dependent reduction of auxin-inducible gene expression (Fig. 1). The genetic analysis of growth and root architectural responses of the WT and *eir1*, *aux1-7*, *arf7arf19*, and *tir1abf2abf19* auxin-related mutants further supports the hypothesis that an intact auxin signaling pathway and not only auxin transport is required for salt tolerance, because *arf7arf19* and *tir1abf2abf19* were the most sensitive of the mutants tested regarding salt repression of growth (Fig. 2).

The use of plant symbionts is a promising strategy for agriculture sustainability because they improve plant health under different conditions. For instance, the root endophytic basidiomycete *Piriformospora indica* increased resistance against biotic stress and tolerance to abiotic stress in many plants. Root

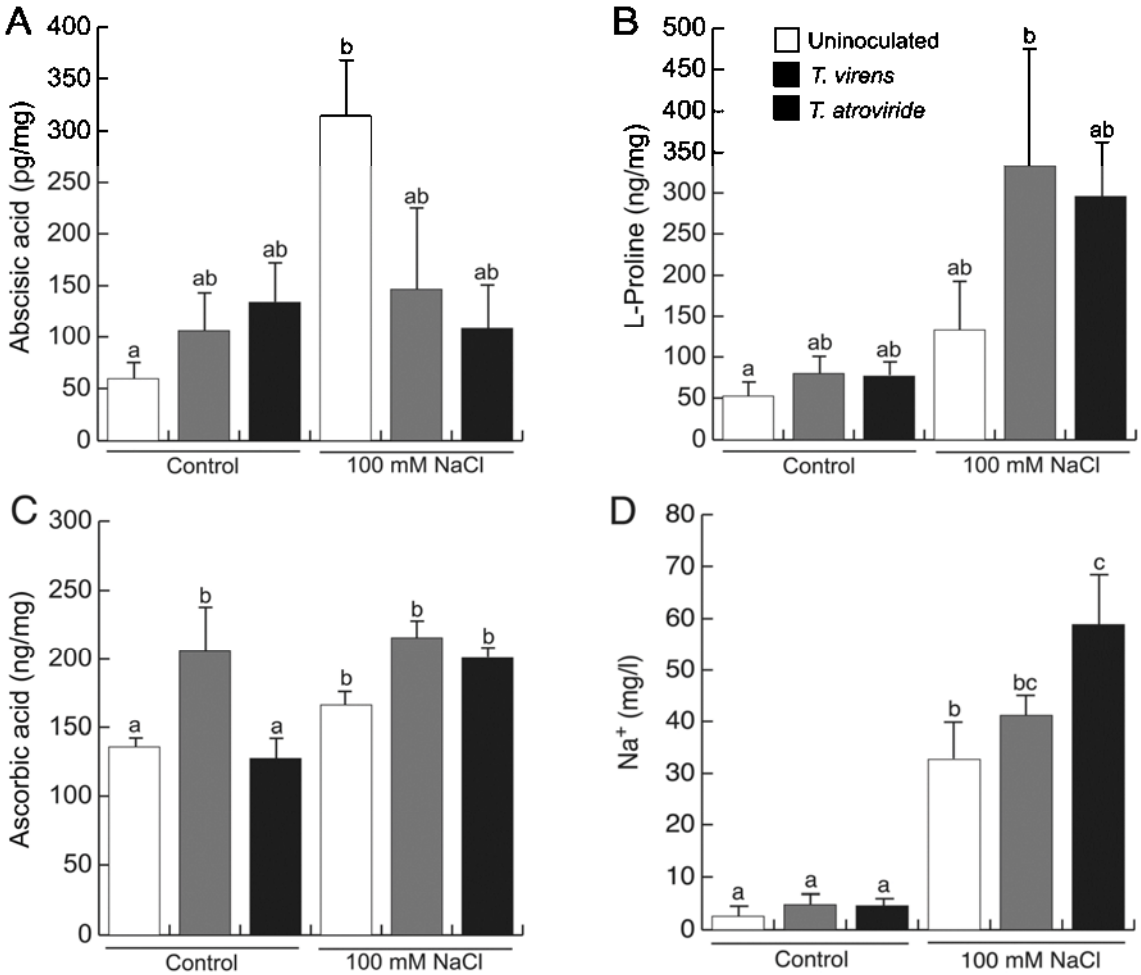

Fig. 8. Effects of *Trichoderma* spp. on biochemical changes and Na^+ elimination through root exudates**.** Metabolite determination was performed after 5 days of fungal inoculation. **A,** Endogenous content of *cis*, *trans*-abscisic acid (ABA). **B,** Content of free L-proline. **C,** Content of ascorbic acid. **D,** Root exudates were collected by 2 days from 9-day-old *Arabidopsis* maintained hydroponically. Aqueous Na^+ was determined by atomic absorption spectrophotometry ($n = 3$). Error bars represent the standard error. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated four times with similar results.

colonization by *P. indica* increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux, and fatty acid desaturation in leaves of the salt-sensitive barley 'Ingrid'. In addition, *P. indica* significantly elevated the amount of AA and increased the activities of antioxidant enzymes in barley roots under salt stress conditions. Likewise, a sustained upregulation of the antioxidative system was demonstrated in NaCl-treated roots of the salt-tolerant barley 'California Mariout' (Waller et al. 2005). These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

T. virens and *T. atroviride* were found to produce IAA and auxin-related substances, which may normalize root growth under salinity stress. *Arabidopsis* and cucumber (*Cucumis sativus* L.) plants treated with *Trichoderma* spp. prior to salt stress show significantly improved seed germination (Brotman et al. 2013). In addition, *Trichoderma* spp. modulated the expression of several genes related to osmo-protection and general oxidative stress in roots of both plant species. The MDAR gene coding for monodehydroascorbate reductase was significantly upregulated and, accordingly, the pool of reduced AA was increased in *Trichoderma* spp.-treated plants. Therefore, it was interesting to examine whether *T. virens* or *T. atroviride* could contribute to salt tolerance in *Arabidopsis* seedlings via auxin production or through other mechanisms. We found that both *Trichoderma* spp. tested were able to sustain prolific growth at 150 mM NaCl. However, a differential salt response was found because, at greater salt concentrations, *T. virens* was more halotolerant than *T. atroviride* (Fig. 3). Importantly, *T. virens* and *T. atroviride* were able to produce IAA when grown in medium supplied with 100 mM NaCl or even greater IAA levels than those quantified in medium without salt, suggesting their great potential as plant growth-promoting fungi to cope with salinity in crops.

In *Arabidopsis* seedlings cocultivated with *T. virens* or *T. atroviride*, there were an increased number of LR, RH, and biomass accumulation (Figs. 4 to 6). RH development is a useful marker of differentiation processes that take place in the root (López-Bucio et al. 2005). It has been reported that NaCl treatment inhibits RH growth (Halperin et al. 2003). In contrast, *Trichoderma* spp. increased both RH number and length (Fig. 6). These data suggest that root plasticity could be part of a mechanism by which *Trichoderma* spp. can confer salt tolerance to plants. In *Arabidopsis* and other plant species, exogenous auxin application can induce both LR and RH formation (Laskowski et al. 1995); therefore, we speculated that, by providing auxins, *Trichoderma* spp. could restore auxin homeostasis and, consequently, growth and development could be normalized when grown under stressing salt levels. To verify this, the effect of cocultivation with *Trichoderma* spp. on *DR5:uidA* expression was tested under salinity. It was found that *T. virens* or *T. atroviride* could increase the expression of *DR5:uidA* in LR and that more of these structures were formed in response to fungal interaction (Fig. 7). Therefore, it is tempting to speculate that auxins derived from *Trichoderma* spp. may be important to sustain root developmental programs under saline stress. A key role for IAA in plant salt tolerance induced by fungi has started to be revealed. For example, Redman and associates (2011) reported that *Fusarium culmorum* and *Curvularia protuberata* enhanced growth of rice plants under salinity. Similarly, the endophytic fungi *Phoma glomerata* and *Penicillium* sp. significantly increased plant biomass of cucumber under saline stress (Waqas et al. 2012). A *Streptomyces* sp. isolate increased the growth and development of wheat plants in normal and saline conditions. This isolate also produced IAA in axenic conditions, which increased when salt was added (Sadeghi et al. 2012). Thus, plant-growth-promoting fungi may be advantageous to plants grown under salt stress by producing auxins.

Salt tolerance is a complex trait involving the coordinated action of many gene families that perform a variety of functions such as control of water loss through stomata, ion sequestration, metabolic and osmotic adjustments, and antioxidative protection (Abogadallah 2010). For example, under severe saline stress, reactive oxygen species (ROS) production can damage cell components (Mittler 2002). The osmoprotectant L-Pro is accumulated in many plant species in response to drought and salinity, and its accumulation frequently correlates with stress tolerance. This amino acid functions as a scavenger of hydroxyl radicals, controlling redox homeostasis (Ben et al. 2008; Fabro et al. 2004). Another potent antioxidant molecule is AA, which detoxifies ROS, particularly hydrogen peroxide (Smirnoff 2000). Here, we show that *Trichoderma* spp.-induced plant salt tolerance in *Arabidopsis* is correlated with increases in L-Pro and AA (Fig. 8). Roots secrete many substances which include ions, free oxygen and water, enzymes, mucilage, and a diverse array of carbon-containing primary and secondary metabolites (Bais et al. 2006). Root exudates are transported across the cellular membrane and secreted into the surrounding rhizosphere. Na^+ toxicity is considered one of the most important factors limiting root growth (Ghoulam et al. 2002; Ungar 1996) and, therefore, it is expected that exclusion or detoxification mechanisms might be integral to plant adaptation to salinity. We tested whether *Trichoderma* spp. induced the Na^+ elimination through root exudates as part of a plant detoxification mechanism. The content of Na^+ exudated from roots grown in normal conditions was similar among treatments (Fig. 8D). In contrast, uninoculated seedlings grown under salinity exuded an enhanced amount of Na^+ when compared with nonstressed plants. Importantly, the Na^+ exuded by roots was increased by 25.76 and 79.65% when cocultivated with *T. virens* or *T. atroviride,* respectively. These effects may allow plants to better tolerate excess Na^+ levels. One possibility to explain the increased production of osmolite, antioxidants, and Na^+ exclusion is that the beneficial effects of *Trichoderma* spp. in roots through increasing the auxin pool improves plant health and activates metabolic or transport processes, which lead to an improved capacity to react or adapt to salt stress.

In conclusion, our research demonstrates the beneficial role of *Trichoderma* spp. to improve saline stress tolerance in *Arabidopsis*. Our data reveal a novel facet of auxins produced by fungi in promoting plant health, which may lead to potential applications in agriculture.

MATERIALS AND METHODS

Plant material and growth conditions.

A. thaliana was used in this work. The transgenic and mutant lines were derived from the parental *Arabidopsis* ecotype Columbia-0 (Col-0). The lines *aux1-7* (Picket et al. 1990), *eir1-1* (Roman et al. 1995), *arf7arf19* (Okushima et al. 2007), and *tir1afb2afb3* (Dharmasiri et al. 2005) are auxin-related mutants defective on auxin transporters AUX1 or PIN2 or affected in the transcription factors ARF7 and ARF19 or auxin receptors TIR1, AFB2, and AFB3, respectively. *DR5:uidA* is an auxin responsive marker (Ulmasov et al. 1997). Seed were surface sterilized with ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes in distilled water, seed were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog basal salts mixture, catalog number M5524; Sigma-Aldrich, St. Louis). Plates were placed vertically at a 65° angle to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants

were grown at 24°C in a chamber with a photoperiod of 16 h of light (200 μmol m^2 s^{-1}) and 8 h of darkness.

Fungal growth and plant inoculation experiments.

The following fungal strains were used in this work: *T. virens* Gv29.8 and *T. atroviride* (formerly *T. harzianum*) IMI 206040. Fungal strains were grown in 0.2× MS medium with (50 to 300 mM NaCl) or without salt (catalog number 11830-031; Gibco BRL, Bethesda, MD, U.S.A.). *T. virens* and *T. atroviride* were evaluated in vitro for their ability to promote salt tolerance in *Arabidopsis*. Fungal spore densities of 10^6 spores were inoculated on 0.2× MS medium with 100 mM NaCl or without salt by placing the spores at 5 cm in the opposite ends of agar plates containing 4-day-old germinated *Arabidopsis* seedlings (10 seedlings/plate). Plates were arranged in a completely randomized design. The seedlings were cultivated in a Percival AR95L growth chamber.

Quantification of shoot and root growth.

For shoot diameter quantification, seedlings were photographed 9 days after the treatments using a stereoscopic microscope (Leica MZ6; Leica Microsystems, Ryswyk, The Netherlands). Measurements were determined by using the Image J software. Growth of primary roots was registered using a ruler. LR numbers were determined by counting the LR present in the primary root. LR densities were determined by dividing the LR number by the primary root length. RH were measured in a 500-μm region at a 1-cm distance from the primary root tip. The average length of RH was determined by measuring 100 hairs for each root, taking as a reference the root protoxylematic plane to locate the radical hair base in the epidermal cell.

Chlorophyll content measurement.

Chlorophyll content was determined from excised *Arabidopsis* shoots 5 days after fungal inoculation. Shoots were homogenized in 1 ml of 80% aqueous acetone. Chlorophyll from different samples was extracted by 48 h in darkness at –20°C. Supernatant readings were taken at 647 and 663 nm. Total chlorophyll content was calculated as (7.15 × A663) + (18.71 × A647) divided by 1,000 × shoot fresh weight and was reported as milligrams of chlorophyll per gram of fresh weight, as described by Zhang and associates (2008b).

Root exudate collection and Na^+ content measurement.

Arabidopsis WT (Col-0) plants were grown as described above and root exudates from 40 seedlings per sample were collected. Five days after fungal inoculation, seedlings were transferred to falcon tubes containing tri-distilled water supplemented with 0.2× sucrose and placed at 24°C in a chamber with a photoperiod of 16 h of light (200 μmol m^2 s^{-1}) and 8 h of darkness. Root exudates were collected 2 days later. Each treatment consisted of three replicates and each replicate consisted of a total volume of 4 ml of exudate. The collected root exudates were filtered using nylon filters (Econofilter 25/0.45 μm; Agilent Technologies Netherlands BV, Amsterdam) to remove root-border cells. After filtration, the exudates were stored at –72°C for further analyses. Next, aqueous Na^+ was determined by atomic absorption spectrophotometry (Model AAnalyst 200; Perkin Elmer, Norwalk, CT, U.S.A.).

L-Pro determination.

L-Pro extraction and determination were performed in *Arabidopsis* (ecotype Col-0) seedlings at 5 days after inoculation. For sample preparation, plant tissues were frozen and ground in liquid N_2. Approximately 250 mg of ground tissue was placed in an Eppendorf tube. Tissue was homogenized with 1 ml of 0.7% (vol/vol) concentrated HCl in methanol and shaken for 5 min. Samples were centrifuged at 11,500 rpm for 3 min, and supernatants were collected and evaporated under a stream of gaseous nitrogen. L-Pro was derivatized with acetyl chloride in methanol (500 μl per 2 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the methylated sample was evaporated and added to acetic anhydride (1.5 ml) and dichloromethane (1 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and redissolved in 50 μl of methanol for gas chromatography mass spectrometry (GC-MS) analysis. GC-selected ion-monitoring mass spectrometry (GC-SIM-MS) and retention time were established for N-acetyl-proline methyl ester (*m/z* 70, 112 and 171 M^+, 4.62 min), respectively. L-Pro (Merck, Amsterdam) was derived and used as pure standard. To estimate the amount of L-pro in *Arabidopsis* seedlings, we constructed a standard curve.

ABA and AA determinations.

ABA and AA were extracted and determined in *Arabidopsis* (Col-0) 5 days after *Trichoderma* spp. inoculation. Plant tissues were frozen and ground in liquid N_2. Approximately 200 mg of ground tissue was placed in an Eppendorf tube, homogenized with 500 μl of isopropanol/H_2O/concentrated HCl (2:1:0.002, vol/vol), and shaken for 30 s. Samples were centrifuged at 11,500 rpm for 3 min, and supernatants were collected and subjected to ABA or AA extraction with 300 μl of dichloromethane. ABA was derivatized with acetyl chloride in methanol (500 μl per 2 ml), sonicated for 15 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25 μl of ethyl acetate for GC-MS analysis. GC-SIM-MS and retention time were established for *cis*, *trans*-ABA methyl ester (ABA-ME; *m/z* 134, 190, and 278 M^+, 15.51 min); *cis*, *trans*-ABA was purchased from Sigma-Aldrich and used as standard.

AA was derivatized with acetic anhydride and dichloromethane (1.5 ml per 1.0 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and redissolved in 25 μl of ethyl acetate for GC-MS analysis. GC-SIM-MS and retention time were established for AA and 2, 3, 5, 6-tetra-acetyl ester (*m/z* 200 and 344 M^+, 14.79 min) respectively. L(+) AA was obtained from Merck, derivatized, and used as standard.

The identity of each compound was further confirmed by comparison with the pure standard and, to estimate the amount, we constructed an independent standard curve.

IAA determination and GC-MS analysis.

For the production of IAA, an active inoculum of 1×10^6 spores of *T. virens* or *T. atroviride* was added to 200 ml of potato dextrose broth (Fluka Analytical; Sigma-Aldrich) and grown for 3 days at 24°C, with shaking at 180 rpm. To evaluate the effect of salt supply on IAA production, the medium was supplemented or not with NaCl at a concentration of 100 mM. The fungal cultures were filtered and the supernatant was adjusted to pH 3 using 1 N HCl. IAA in supernatant solution was extracted two times with 500 ml of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen and then diluted in 1 ml of ethyl acetate. IAA was methyl esterified with 500 ml of acetyl chloride in 2 ml of dry methanol, sonicated for 15 min, and heated at 75°C for 1 h. IAA content was determined as described previously (Contreras-Cornejo et al. 2009).

Samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and 30-m by 0.2-μm by 0.25-mm, 5% phenyl methyl silicone capillary column (HP-5 MS). The operating conditions used

were helium at 1 ml/min^{-1} as carrier gas, 300°C detector temperature, and 250°C injector temperature. The column was held for 5 min at 150°C and programmed at 5°C min^{-1} to a 278°C final temperature for 5 min.

Histochemical analysis and GUS expression measurements.

Transgenic plants that expressed the *uidA* reporter gene (Jefferson et al. 1987) were incubated 10 h at 37°C in GUS reaction buffer (5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 0.5 mg/ml in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each treatment, at least 15 transgenic plants were analyzed. The processed seedlings were included in glass slips and sealed with commercial nail varnish. For each treatment, a representative plant was chosen and photographed using a Leica MZ6 stereomicroscope.

GUS expression was determined in primary root tips of 9-day-old *Arabidopsis* seedlings grown, stained, and cleared as described above. Fifteen obtained images were processed using the Image J software and the data expressed as arbitrary units.

Data analysis.

Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Multivariate analyzes with a Tukey's post hoc test was used for testing differences in the fresh and dry weight, shoot length, chlorophyll content, ABA, L-Pro, AA, aqueous Na^+, and RSA. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

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

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

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

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Introduction

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

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

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

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

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

Materials and Methods

Plant Material and Growth Conditions

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

Fungal Growth and Plant Inoculation Experiments

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

Plant Transpiration Measurements

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

Stomatal Aperture Measurement

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

Abscisic Acid Determination

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

GC–MS Analysis

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

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

Histochemical Analysis

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

Data Analysis

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

Results

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

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

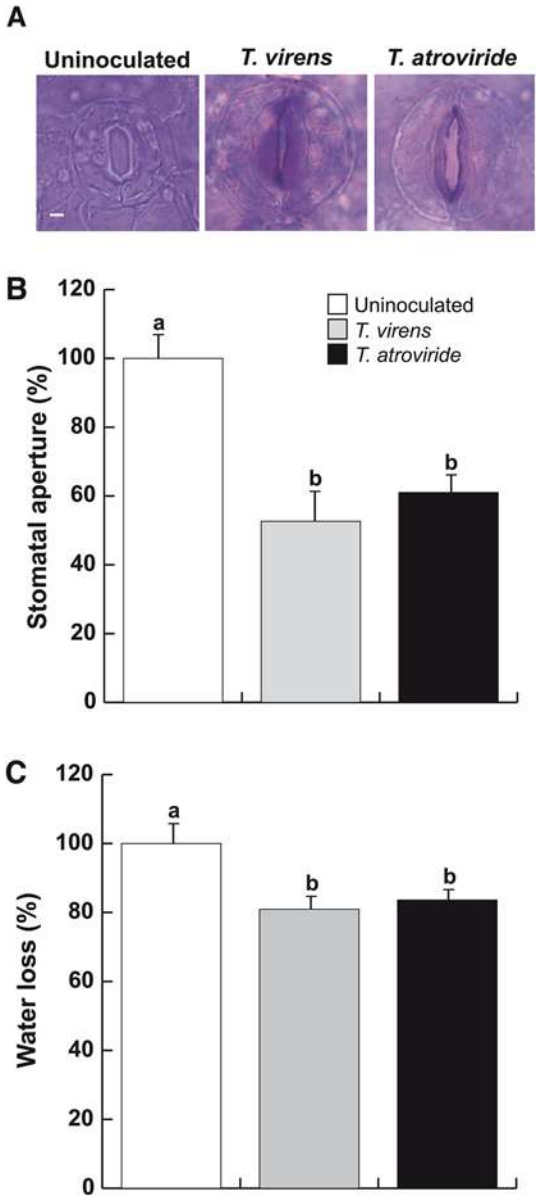

Fig. 1 *Trichoderma* regulates stomatal aperture and transpiration in *Arabidopsis thaliana* (Col-0) seedlings. **a** Representative images of stomata from seedlings inoculated with *Trichoderma* spp. *Bar* = 5 µm. **b** Effects of *Trichoderma* spp. on stomatal aperture and **c** effect of *Trichoderma*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

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

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

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

Trichoderma Produces Abscisic Acid

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

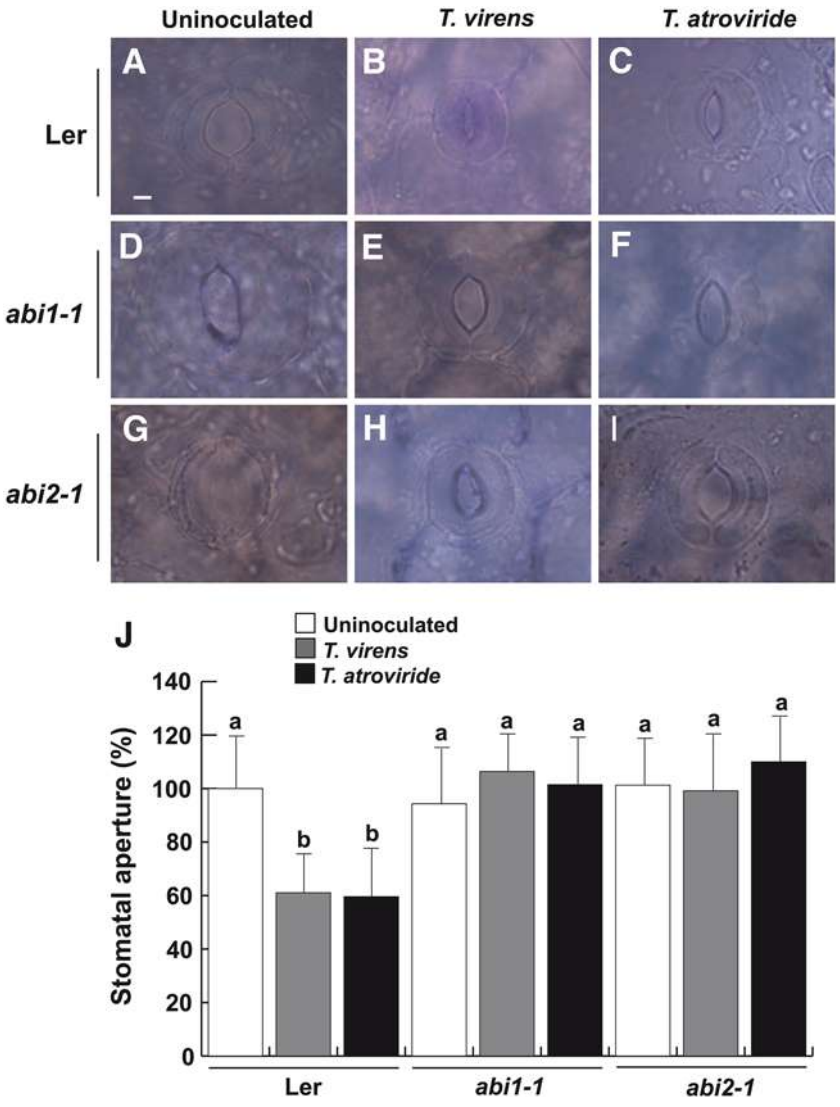

Fig. 2 Effect of *Trichoderma* on stomatal aperture in ABA-related mutants. **a**–**i** Representative images of stomata from *Arabidopsis* wild-type (Ler), *abi1-1* and *abi2-1* inoculated with *Trichoderma* spp. *Bar* = 5 μm. **j** Stomatal aperture. *Bars* show the mean ± SE of 100 measurements by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

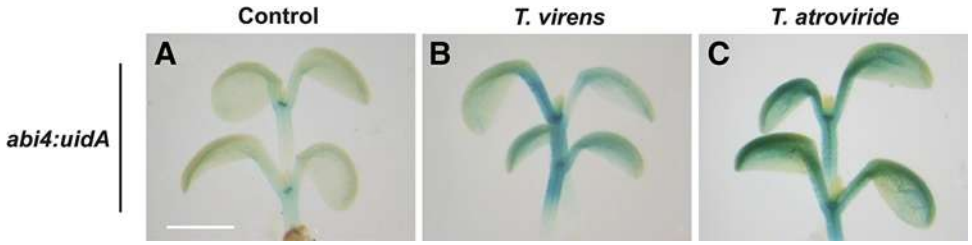

Fig. 3 Effect of *Trichoderma* on the expression of *abi4:uidA*. **a**–**c** Expression of *abi4:uidA* in 5-day-old transgenic seedlings. Notice that *T. virens* and *T. atroviride* induced significantly the β-glucuronidase activity in shoots. *Photographs* are representative individuals of at least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

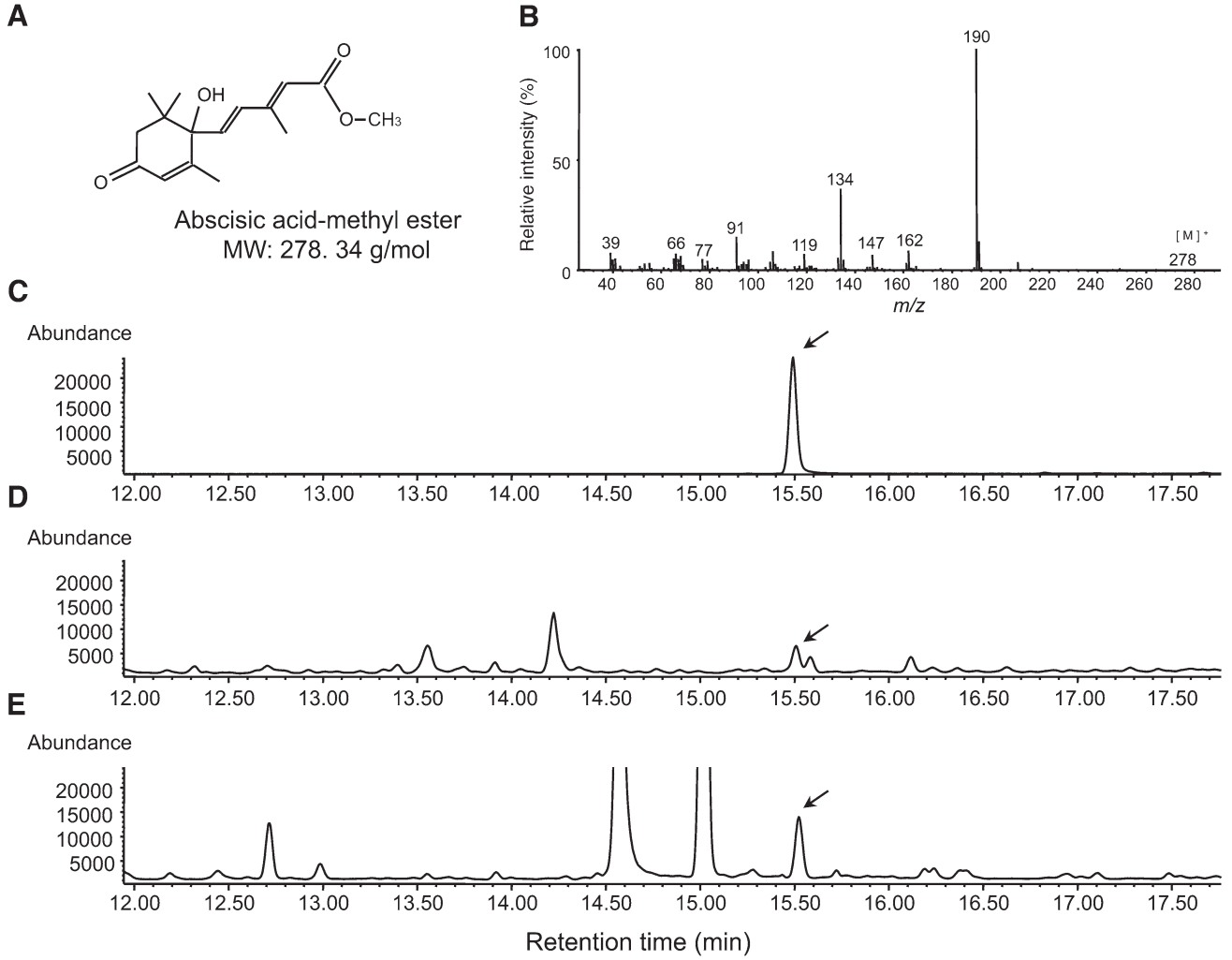

Fig. 4 Determination of ABA from *Trichoderma* spp. ABA was identified from acidic ethyl acetate from liquid culture medium of *Trichoderma* and analyzed by GC–MS. **a** Chemical structure of ABA-derivatized sample. **b** The 70-eV electron-impact full-scan mass spectra of ABA from pure standard. **c**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

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

Discussion

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

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

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

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

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

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

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

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

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REGULAR ARTICLE

6.3. CAPÍTULO III.
The 4-phosphopantetheinyl transferase of *Trichoderma virens* plays a role in plant protection against *Botrytis cinerea* through volatile organic compound emission

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Abstract
Aims This work was conducted to examine the effects of volatile organic compounds (VOCs) from *Trichoderma virens* and the *4-phosphopantetheinyl transferase 1* (TvPPT1) mutant in growth promotion and induction of defense responses of *Arabidopsis thaliana* seedlings using a co-cultivation system in vitro.
Methods The contribution of VOCs to plant development and immunity was assessed by comparing the effectiveness of WT and Δ*ppt1* mutant strains of *T. virens* in the formation of lateral roots and protection conferred against *Botrytis cinerea*. VOCs released by *T. virens* and Δ*ppt1* mutant were compared by gas chromatography–mass spectrometry.
Results Plants exposed to volatiles from WT *T. virens* showed 2-fold increase in fresh weight when compared to axenically-grown seedlings, which correlated with increased root branching and enhanced expression of the jasmonic acid-responsive marker *pLox2:uidA* as well as accumulation of jasmonic acid and hydrogen peroxide. *T. virens* produced a series of hydrocarbon terpenes, including the sesquiterpenes β-caryophyllene, (−)-β-elemene, germacrene D, τ-cadinene, δ-cadinene, α-amorphene, and τ-selinene and the monoterpenes β-myrcene, *trans*-β-ocimene, and *cis*-β-ocimene that were absent in TvPPT1 mutant.
Conclusions Our results indicate that *T. virens* VOCs elicit both development and defense programs and that PPT1 plays an important role in biosynthesis of terpenes and plant protection against *B. cinerea*.

Keywords *Trichoderma* · *Arabidopsis* · Root development · Plant immunity · Volatiles

Abbreviations

FPP	Farnesyl pyrophosphate
GC-SIM-MS	Gas chromatography-selected ion monitoring mass spectrometry
JA	Jasmonic acid
MVA	Mevalonic acid
MEP	2-C-methyl-D-erythritol 4-phosphate
NRPS	Nonribosomal peptide synthase
OA	Orto-anisic acid
PKS	Poliketide synthase
POD	Peroxidase
PPT1	4-phosphopantetheinyl transferase 1
ROS	Reactive oxygen species

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SA	Salicylic acid
VOCs	Volatile organic compounds

Introduction

Volatile organic compounds (VOCs) are the metabolites that plants and microorganisms release into the air. The quantities released by plants are not negligible and several adaptive functions including protection against abiotic stress, herbivores, pathogens and/or competitors have been suggested (Rohloff and Bones 2005; Kaiser 2006; Pichersky et al. 2006). Recent information indicates that microbial VOCs may regulate plant growth and morphogenesis (Ryu et al. 2003; Gutiérrez-Luna et al. 2010) and activate plant immunity (Ryu et al. 2004). This opens the possibility that certain plant beneficial fungi, in the absence of physical contact or in close proximity with roots may stimulate growth or elicit plant adaptive traits via VOCs emission.

Free-living fungi of the genus *Trichoderma* are very common in different soils and root ecosystems. Some *Trichoderma* strains colonize the rhizosphere and can modulate plant growth either directly or indirectly. Direct mechanisms of plant growth promotion by *T. virens* may involve the release of indole-3-acetic acid (IAA) and related indolic compounds with auxin activity, which promote root hair and lateral root development, thus increasing the total absorptive capacity of the root system (Contreras-Cornejo et al. 2009). Indirect mechanisms involve the protection of plants against pathogens by activating immunity mediated by the canonical defense signals jasmonic acid (JA) and salicylic acid (SA), which depends on the amount of conidia inoculated or through its biocontrol properties (Shoresh et al. 2010; Contreras-Cornejo et al. 2011).

Studies about the biological role of *Trichoderma* VOCs on plants have begun to emerge. Vinale et al. (2008), reported that 6-*n*-pentyl-6*H*-pyran-2-one (6PP) produced by *T. atroviride* stimulates the growth of tomato and canola seedlings and induces systemic defense responses in plants against *Botrytis cinerea* and *Leptosphaeria maculans*. In addition, Hung et al. (2013) observed that the pool of VOCs produced by *T. viride* promotes *Arabidopsis* growth increasing lateral root formation, plant height and flowering.

The wealth of VOCs in *Trichoderma* is apparently species-dependent and changes with substrate composition (Wheatley et al. 1997; Reino et al. 2008; Stoppacher et al. 2010; Crutcher et al. 2013). The ecological role and the genetics of the biosynthesis of many of these compounds remain to be investigated. However, a recent report indicates that the putative terpene cyclase *vir4* in *T. virens* participates in the synthesis of terpenoids (Crutcher et al. 2013), compounds with several roles in fungi and plants (Rolf and Wolf-Rainer 2012; Tholl 2006).

Non-ribosomal peptides (NRPs) or polyketides are metabolites with important biological functions. The key enzymes needed for their production belong to the family of polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) that are generally known to be post-translationally modified by PPTases such as *4-phosphopantetheinyl transferase1* (PPT1) (Márquez-Fernández et al. 2007; Wiemann et al. 2012). PPTases are involved in conidiation and sexual mating recognition possibly by activating PKS- and/or NRPS-derived metabolites that could act as diffusible signals (Velázquez-Robledo et al. 2011; Wiemann et al. 2012). In particular, PPT1 from *Trichoderma virens* (TvPPT1) has an important role in antibiosis and induction of SA and camalexin-dependent plant defense responses (Velázquez-Robledo et al. 2011).

Reactive oxygen species (ROS) are important signaling molecules in all living organisms as a consequence of aerobic life (Dickinson and Chang 2011). During plant-pathogen or elicitor recognition, ROS (i. e. hydrogen peroxide; H_2O_2) levels increase and trigger early defense responses (Dixon et al. 1994; Apel and Hirt 2004; Dickinson and Chang 2011). The role of ROS in a given plant-pathogen interaction will depend on the sensitivity of the pathogen to the concentration of ROS present, as well as the amount of H_2O_2 produced, which depends on several factors including the nature of the elicitor, the plant species, and/or developmental stages (Shetty et al. 2008). In a previous work, we observed that physical contact of *Trichoderma* with roots induces the accumulation of H_2O_2 in *Arabidopsis* (Contreras-Cornejo et al. 2011). Current challenges are to decipher how root signaling is connected with defense responses in leaves and which factors and processes are implied in such long distance communication.

In this study we performed research to determine the role of TvPPT1 in the regulation of VOCs emission by *T. virens* and clarify if these compounds participate in plant growth stimulation and plant protection against the necrotrophic pathogen *Botrytis cinerea*.

Materials and methods

Plant material and growth conditions

Arabidopsis transgenic lines were derived from the parental *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). *Arabidopsis* transgenic lines included a JA-inducible *pLox2:uidA* line (Schommer et al. 2008) and the SA-inducible *pPr-1a:uidA* line (Shah et al. 1997). 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 Petri plates or divided Petri (I-plates) plates containing 0.8 % agar, 0.2X MS medium (Murashige and Skoog basal salts mixture, Cat. M5524: Sigma, St. Louis), and 0.6 % sucrose. Plates were placed vertically at a 65° angle to allow root growth along the agar surface, and unimpeded aerial growth of the hypocotyls. Plants were grown at 24 °C in chamber with a 16 h light (200 μmol m^2 s^{-1}), 8 h darkness photoperiod.

For JA and SA treatments, *pLox2:uidA* and *pPr-1a:uidA* seedlings grown on 0.2X MS medium for 9 days in agar solidified 0.2X MS medium were transferred to the same liquid medium supplied with 200 μM JA, SA or DMSO as solvent control for 24 h. After this time, histochemical analysis of GUS activity was performed.

Fungal growth and plant inoculation experiments

This research used *Trichoderma virens* Tv29.8, Tv10.4 and *Δppt1* mutant strains. To determine the effects of VOCs from *T. virens* on plant growth and defense responses, an inoculum of 1×10^6 spores was placed at the opposite half of the divided-dishes containing 4-days-old germinated *Arabidopsis* seedlings (20 seedlings per plate). The plates contained agar-solidified 0.2X MS medium. After 5-days of co-cultivation, all the analyses were done. Plates were arranged in a completely randomized design. The seedlings were cultured for different time periods in a Percival AR95L growth chamber.

Determination of H_2O_2 production

For the quantification of H_2O_2, whole plant tissue (400 mg) was crushed in liquid nitrogen. The samples were homogenized in 1 ml of distillated water and shaken for 30 s. The homogenate was stirred on ice for 1 h and centrifuged at 12, 000*g* for 10 min. The supernatant was used for assays of peroxidase (POD; Svalheim and Robertsen 1990). Soluble POD activity was analyzed by following the formation of tetraguayacol in a Beckman DU 7400 spectrophotometer (Beckman Instruments, Fullerton, CA). Each reaction mixture (1 ml) consisted of 10 μl enzyme extract and 990 μl guaiacol solution containing 0.05 % guaiacol (v/v) in 25 mM sodium phosphate buffer pH 7.0 and 0.125 % H_2O_2 (v/v). The reaction was incubated by 15 min in darkness. POD activity in the extracts was measured as an increase in absorbance at 450 nm. Finally, H_2O_2 determinations were performed essentially as described by García-Pineda et al. (2010). The experiment was repeated four times with similar results.

Histochemical analysis

For histochemical analysis of GUS activity, *Arabidopsis* seedlings were incubated 12 to 14 h at 37 °C in a GUS reaction buffer (0.5 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 15 transgenic plants were analyzed. A representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

SA and JA extraction and measurement

The SA and JA extraction and determination were performed in *Arabidopsis thaliana* (ecotype Col-0) seedlings 5-days after *T. virens* VOCs exposure. For sample preparation, whole plant tissues were frozen and ground in liquid nitrogen. 100 mg of each sample was placed in a polypropylene microtube, homogenized with 700 μl isopropanol/H_2O/concentrated HCl (2:1:0.002, v/v), and supplemented with 300 ng of orto-anisic acid (OA; Sigma) as internal standard for SA, and shaken for 30 s. The tubes were centrifuged at 11, 500 r.p.m. for 3 min. The supernatants were collected and subjected to SA and JA extraction with 500 μl of dichloromethane. SA and JA were derivatized with acetyl chloride in cold methanol (500 μl/2 ml), sonicated for 15 min and heated for 1 h at 75 °C. After cooling, the derivatized samples were evaporated and resuspended in 20 μl of methanol for GC-MS analysis. Gas chromatography-selected ion monitoring mass spectrometry (GC-SIM-MS) and retention time was established for SA-ME (*m/z* 120 and

m/z 152 [M^+], 2.41 min), OA-ME (*m/z* 135 and *m/z* 166 [M^+], 3.39 min) and for JA-ME (*m/z* 83 and *m/z* 224 [M^+], 7.92 min. JA was quantified by comparison with a standard curve obtained by using purified Me-JA (Sigma). GC-MS analysis was performed using a gas chromatograph (Agilent 6850 Series II; Agilent, Foster City, CA, U.S.A), equipped with an Agilent MS detector model 5973 and HP-5MS capillary column (5 % phenyl methyl silicone, 30 m×0.25 mm I.D., film thickness of 0.25 μm). Operating conditions used 1 ml min^{-1} helium as carrier gas, detector temperature of 300 °C, and injector temperature of 250 °C. The column was held for 5 min at 150 °C and programmed at 5 °C min^{-1} to a final temperature of 278 °C for 5 min.

GC-MS analysis of volatile compounds

The VOCs released by *T. virens* Tv29.8, Tv10.4, and *Δppt1*-1 mutant were analyzed in 0.2X MS medium and by SPME and gas chromatography–mass spectrometry (GC-MS). The volatiles were collected for 60 min using a blue SPME fiber (PDMS/DVB) (Supelco, Inc., Bellafonte, PA, U.S.A) and desorbed for 30 s in the injector port of the gas chromatograph equipped with a MS detector and a HP-FFAP capillary column (free fatty acid-phase, 25 m× 0.32 mm I.D., film thickness of 0.25 μm). Operating conditions used helium as carrier gas (1 ml/min), detector temperature of 250°C, and injector temperature of 180 °C. The column was held for 5 min at 40°C, and the temperature was programmed to rise at a ratio of 3°C per min to a final temperature of 220°C, which was maintained for 5 min. Mass spectra were recorded in the scan mode at 70 eV. All VOCs were tentatively identified by the use of a combination of NIST 2.0 mass spectra database search and deconvolution software (AMDIS v.2.0). Linear retention indices were calculated according to the Kovats method using a mixture of normal paraffin C_6-C_{20} as external references. Retention indices were compared with those from accessible scientific literature.

Bioassays for *Trichoderma* VOCs-induced resistance against *B. cinerea*

To test plant protection conferred by the VOCs emitted by *T. virens* against *B. cinerea* in divided Petri dishes, 10-days-old *Arabidopsis* seedlings were co-cultivated with a fungal colony established in the opposite side of the plate and allowed to grow for 3-days to elicit defense responses. After this period *Arabidopsis* shoots were inoculated with mycelium of *B. cinerea.* Induced disease resistance in plants was evaluated 3-days after pathogen inoculation. *Arabidopsis* seedlings exhibiting leaves with soft rot symptoms were determined by visual inspections. The percentage of dead plants was determined 3-days after pathogen inoculation for a total of 40–60 plants. Plants were grown at 24 °C in a chamber with a 16 h light (200 μmol m2 s-1), 8 h dark photoperiod.

Data analysis

All experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyzes with a Tukey's post hoc test were used for testing differences in the different experiments. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

Results

Volatiles from *Trichoderma virens* stimulate growth of *Arabidopsis* seedlings

Rhizospheric microorganisms release hormones, hormone-like substances, small molecules or VOCs, which may activate plant immunity or regulate plant morphogenesis and growth (Ryu et al. 2004; López-Bucio et al. 2007; Gutiérrez-Luna et al. 2010). Little is known about the role of VOCs in *Trichoderma*-plant interactions. Therefore, we tested the effects of VOCs produced by *T. virens* on *Arabidopsis* seedlings grown in vitro. *Arabidopsis* seedlings (ecotype Columbia-0; Col-0) were grown 4-days on a side of divided Petri plates supplied with MS 0.2X-agar medium and after this period, *T. virens* (Tv29.8 and 10.4) or Δ*ppt1*-1 mutant was inoculated on the opposite side of the plate (Fig. 1a–d). Plants were co-cultivated with each fungal colony and growth and development registered 5-days later. It was found that WT *T. virens* and Δ*ppt1*-1 mutant increased roughly 2-fold the fresh weight of seedlings compared with axenically-grown seedlings (Fig. 1a-d, h). This effect was accompanied with alterations in root system architecture increasing the formation and growth of lateral roots, without affecting primary root

growth (Fig. 1e–g). Since plants and *Trichoderma* were cultivated separately on opposite sides of plates, our data indicate that fungal VOCs can modulate plant growth and developmental programs and that Δ*ppt1*-1 mutant is still able to promote plant growth.

VOCs released by *T. virens* activate plant immunity

Recognition of a microbe by the plant may induce the expression of genes necessary to defend against the challenge or to make more amenable the interaction. To determine whether the blend of VOCs released by *T. virens* could boost plant immunity, we monitored the expression of defense-related genes that were up-regulated by SA and JA during direct interaction of *Arabidopsis* roots with *T. virens* mycelium (Contreras-Cornejo et al. 2011). In these experiments, we used transgenic *Arabidopsis* lines expressing β-glucuronidase (*uidA,* GUS) fusions to the *Pr-1a* promoter, which is activated by SA (Shah et al. 1997) and the *Lox2* promoter, activated by JA (Schommer et al. 2008). The positive chemical controls of SA and JA significantly induced GUS activity in each transgenic line. It was found that the exposure of transgenic *Arabidopsis* seedlings expressing the *pPr-1a:uidA* to VOCs from *T. virens* did not cause an increase in GUS activity when compared with axenically-grown seedlings (Fig. 2a). Interestingly, 5-days after co-cultivation with *T. virens*, the JA-activated marker *pLox2:uidA* increased its expression when compared with its respective controls (Fig. 2a). An additional experiment was performed to determine the induction of *Pr-1a* and *Lox2* promoters in response to inoculation with Δ*ppt1*-1 mutant. No significant changes in GUS expression were registered in plants cocultivated with Δ*ppt1*-1 colony when compared to WT *T. virens* (Supplementary Fig. 1). Our results suggest that *T. virens* emits VOCs that activate JA-dependent signaling mechanism in *Arabidopsis* and that this property is unaltered in Δ*ppt1*-1 mutant.

Several microbe-associated molecular patterns (MAMPs) have been reported in *Trichoderma* that trigger rapidly and transiently plant defense responses (Hermosa et al. 2012), so we asked whether the VOCs from *T. virens* could act in signaling events and whether plants could perceive them by quantifying H_2O_2 accumulation in roots of *Arabidopsis* seedlings exposed 5 days to VOCs from the fungal colony. It was found that the presence of the fungus in one side of the divided Petri plate induced accumulation of H_2O_2 in plants

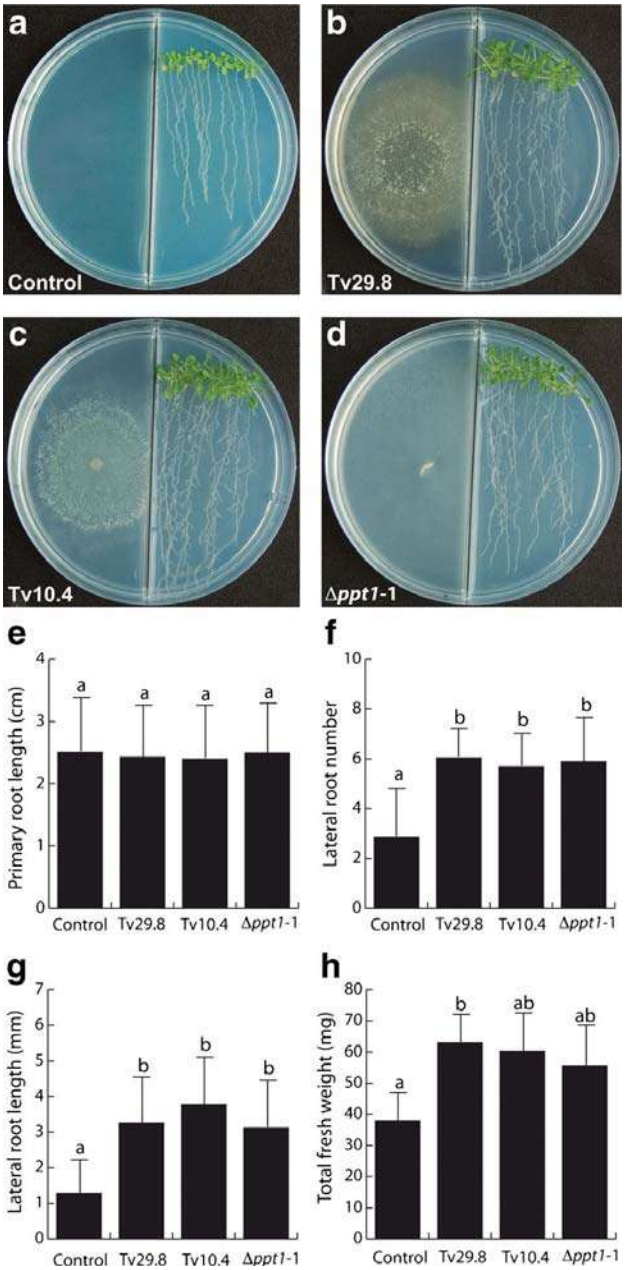

Fig. 1 Effect of VOCs emitted by *T. virens* WT and Δ*ppt1*-1 mutant on *Arabidopsis* growth and root development. **a**–**d** Photographs of 15-days-old *Arabidopsis* (Col-0) seedlings grown on the surface of agar plates containing 0.2X MS medium. **a** Seedlings were treated with sterilized water at day 10 and photographed 5-days later. **b** and **c** Representative photograph of *Arabidopsis* seedlings that were inoculated with *T. virens* Tv29.8 and Tv10.4 at the opposite side of the Petri plates at 10-days after germination and grown for a further 5-days period. **d** Photograph of *Arabidopsis* seedlings inoculated with the mutant Δ*ppt1*-1. **e** Primary root growth, (**f**) lateral root number per plant, (**g**) lateral root length from *Arabidopsis* seedlings exposed to volatiles from *T. virens* strains Tv29.8, Tv10.4 and the Δ*ppt1*-1 mutant. *Bars* show the mean ± SD of 30 *Arabidopsis* seedlings. The experiment was done twice. **h** Effects of fungal inoculation on total biomass production. Different *letters* are used to indicate means that differ significantly ($P < 0.05$)

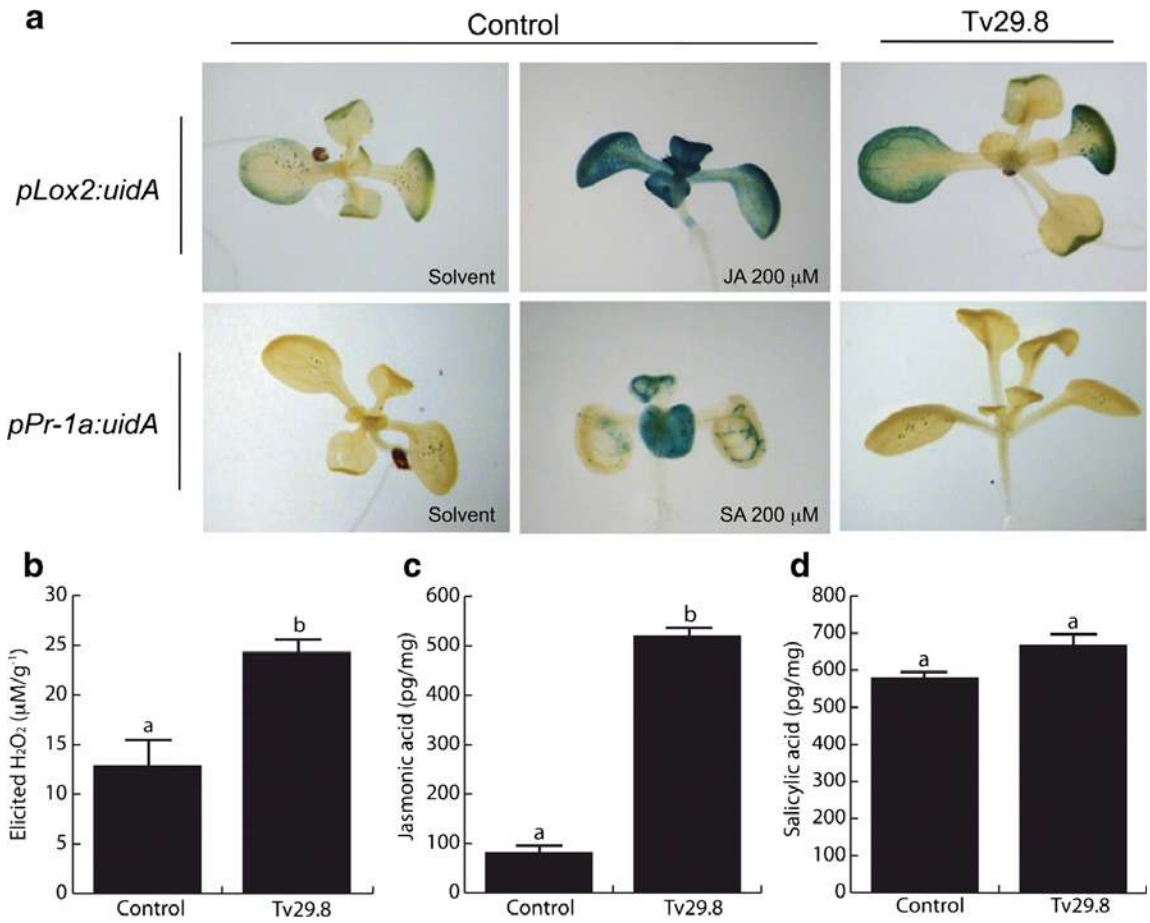

Fig. 2 Effect of *T. virens* VOCs on induction of plant immunity. **a** Analysis of expression of the JA responsive gene marker *pLox2:uidA* and the SA responsive gene marker *pPr-1a:uidA*. GUS expression in *Arabidopsis* seedlings was determined after 5-days of co-cultivation with *T. virens*, or grown for 9 days in agar solidified 0.2X MS medium and then transferred to the same liquid medium supplied with 200 μM JA, SA or DMSO as solvent control for 24 h. After this time, seedlings were cleared to show GUS expression. *Photographs* show representative individuals of at least 15 stained seedlings. The experiment was repeated twice with similar results. **b** Effect of VOCs emitted by *T. virens* on H_2O_2 accumulation in *Arabidopsis*. Effect of *T. virens* VOCs on JA and SA accumulation in *Arabidopsis*. *Arabidopsis* (Col-0) seedlings were germinated for 4-days on 0.2X MS medium, and then co-cultivated with *T. virens* in divided Petri plates for 5 days. **c** Free JA or **d** SA. Four independent determinations were done by treatment. *Error bars* represent the SE. Different *letters* are used to indicate means that differ significantly ($P < 0.05$)

grown in the opposite side of the plate (Fig. 2b). We next quantified JA and SA in wild-type *Arabidopsis* (Col-0) seedlings after 5 days of co-cultivation with *T. virens* in divided Petri plates. The levels of JA in *Arabidopsis* exposed to *T. virens* increased 6-fold when compared with the control (Fig. 2c). In contrast, the levels of SA were not increased in response to *T. virens* VOCs (Fig. 2d). These results suggest that VOCs from *T. virens* can be sensed by plants and activate H_2O_2 and JA accumulation.

T. virens release a wealth of terpenes

Fungi produce a number of VOCs comprising aliphatic and aromatic hydrocarbons, esters, ketones, aldehydes, alcohols, monoterpenes, sesquiterpenes and diterpenes, which may be involved in the elicitation of growth or defense of plants (Kramer and Abraham 2011). To determine the profile of VOCs released by *T. virens* and *Arabidopsis*, we performed chemical analyses to characterize the blend of VOCs produced by *Arabidopsis* itself or by a colony of *T. virens*. The determination of metabolites was done by gas chromatography–mass spectrometry (GC-MS) by introducing a blue SPME fiber into sealed Petri plates containing *Arabidopsis* seedlings or *T. virens* colony after 5-days of growth. We previously standardized the method to collect microbial VOCs and the optimal time was 60 min. The VOCs identified were classified as major and minor compounds

according to their relative abundances and in comparison of the obtained mass spectra with those existing in the NIST database, which were corroborated with the calculated retention index (Table 1). It was found that *Arabidopsis* VOCs were mainly constituted by acetophenone (29.90 %), 6-methyl-2-heptanol (16.59 %) and six different terpenes whose peak areas comprised 41.23 % (Table 1). On the other hand, nearly 75 % of all compounds identified in the headspace of *T. virens* colony corresponded to sesquiterpenes, whose sum of normalized areas represented more than 95 % of the total normalized area (Table 1). Some of these compounds were β-caryophyllene (10.13 %), (−)-β-elemene (10.45 %), germacrene D (12.01 %), τ-cadinene (10.37 %), α-amorphene (6.84 %), δ-cadinene (22.15 %), and τ-selinene (10.17 %). Interestingly, δ-cadinene has been classified as a phytoalexin, and the sesquiterpenoids, α-amorphene, τ-muurolene, and α-muurolene and their tertiary alcohols, the cadinols belong to cadinene-type compounds (Wu et al. 2005) (Table 1). The compounds shown in Table 1 designated as unknown presented high similarities with sesquiterpenic structures (C_{15}) because their mass spectrum showed the *m/z* 105, *m/z* 134, *m/z* 161 and a tentative molecular weight of 204 [M^+]. These results show that *T. virens* produces sesquiterpenes in high amounts.

The 4-phosphopantetheinyl transferase of *T. virens* plays a role in VOC production

Mutation of PPT1 in *T. virens* resulted in absence of polyketides and non-ribosomal peptides (NRPS), indicating a critical role of this enzyme in fungal metabolism (Velázquez-Robledo et al. 2011). Therefore, we quantified the production of volatile organic compounds in Δ*ppt1*-1 mutant and parental strains *T. virens* Tv29.8 and Tv10.4. All three different strains sustained prolific mycelial growth, while the reduced sporulation phenotype already reported for Δ*ppt1*-1 mutant could be appreciated (Fig. 3a). Analysis of the VOC emissions using SPME GC-MS indicated similar VOC profiles emitted by Tv29.8 and Tv10.4 that were different to the compounds released by the agar-solidified 0.2X MS culture medium under axenic conditions (Fig. 3b and c; Supplementary Tables 1 and 2), but it clearly showed a reduced production of VOCs in the Δ*ppt1*-1 mutant when compared to the parental and WT strains (Fig. 3d). A detailed analysis of the compounds revealed that the mutation affected dramatically the number and amount of VOCs, including terpenes (Table 2). These data show that PPT1 is required for the production of volatile compounds in *T. virens*.

Role of VOCs emitted by *T. virens* in activation of plant immunity

To determine whether the sesquiterpenes emitted by WT strains but not Δ*ppt1*-1 could activate plant immunity, we tested the responses of *Arabidopsis* seedlings exposed to VOCs from *T. virens* Tv29.8, Tv10.4 and Δ*ppt1*-1 strains to disease caused by the necrotrophic fungus *Botrytis cinerea*. In these experiments, *B. cinerea* mycelium was inoculated on the surface of leaves in control seedlings or seedlings exposed to *T. virens* VOCs in divided Petri plates, and the percentage of plants with deleterious symptoms determined after 2 days of exposure. *Arabidopsis* plants showed chlorotic foliage as the fungus proliferated over plant tissues and died 3 days later. As plants died the fungal mycelium expanded over the surface of the medium in the Petri plate (Fig. 4a–e). *T. virens* WT strains clearly decreased the chlorosis symptoms induced by *B. cinerea* in leaves, while Δ*ppt1*-1 showed significantly less protection (Fig. 4f). In control plants, *B. cinerea* caused death in 80 % of inoculated seedlings. In contrast, in plants exposed to Tv10.4 and T.v.29.8 VOCs, only 10 % and 15 %, respectively, were damaged by *B. cinerea* infection, while 55 % of plants exposed to the Δ*ppt1*-1 VOCs were dead (Fig. 4g). These data show that PPT1 participates, at least in part, in the regulation of sesquiterpene emission, which is necessary for plant protection against *B. cinerea*.

Discussion

Trichoderma species can be found in different ecosystems interacting with a number of microbes and plants. In consequence *Trichoderma* has developed different mechanisms for communication with other organisms. Of the thousands of different metabolites that plants and microbes produce, VOC emissions form a cloud around the producing organism and may have different functions. We started our work by testing the hypothesis that *T. virens*, a fungus typified as beneficial to plants, could

Table 1 Volatile organic compounds produced by *A. thaliana* (Col-0) or *T. virens* (Tv29.8) and detected by SPME-GC-MS

Compounds	IK (Rt)[A]	IK[B]	*A. thaliana*	*T. virens*
			Normalized amount of major VOCs (%)	
Terpinolene	1268	1269	12.30±0.91[a]	0.03±0.02[b]
6-Methyl-2-heptanol	1381	-	16.59±3.73	nd
β-Caryophyllene	1570	1594	nd	10.13±4.68
(−)-β-Elemene	1571	-	nd	10.45±4.87
Acetophenone	1646	1659	29.90±1.01	nd
Germacrene D	1670	1772	nd	12.01±1.98
τ-Cadinene	1737	-	nd	10.37±1.51
α-Amorphene	1745	-	nd	6.84±0.94
δ-Cadinene	1748	1765	nd	22.15±3.05
τ-Selinene	(44.02)	-	nd	10.17±1.67
			Normalized amount of minor VOCs (%)	
Camphene	1064	1097	5.02±2.58	nd
3-Carene	1137	1130	7.30±1.79	nd
β-Myrcene	1160	1160	nd	0.86±0.32
β-Phellandrene	1187	1194	3.15±2.56	0.03±0.01
Eucalyptol	1191	1243	7.19±0.38	nd
trans-β-Ocimene	1227	1229	nd	0.14±0.01
β-Terpinene	1231	-	6.27±0.45	nd
cis-β-Ocimene	1244	1290	nd	0.10±0.01
Methyl (2*E*)- 4,4-dimethyl-2-pentenoate	1315	-	nd	0.06±0.01
Sorbic acid	1346	-	nd	0.06±0.03
α-Cubebene	1441	1354	nd	0.05±0.02
δ-Elemene	1457	-	nd	0.11±0.01
Acetic acid	1466	1465	8.34±0.61[a]	0.09±0.09[b]
β-Gurjunene	1471	-	nd	0.09±0.07
Copaene	1472	1489	nd	0.10±0.02
β-Bourbonene	1497	-	nd	0.18±0.05
α-Gurjunene	1504	-	nd	0.55±0.10
3-Amino-4-pyrazolecarbonitrile	1512	-	nd	0.18±0.07
β-Cubebene	1521	-	nd	0.09±0.03
Linalool	1553	1548	nd	0.08±0.01
(+)-Aromadendrene	1580	-	nd	0.21±0.08
τ-Gurjunene	1606	-	nd	2.15±0.57
α-Caryophyllene	1631	-	nd	0.27±0.02
2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7 octahydronaphthalene	1650	-	nd	0.30±0.21
τ-Muurolene	1658	-	nd	0.23±0.12
β-Farnesene	1669	-	nd	0.48±0.14
3-Furan methanol	1673	-	3.95±2.49	nd
(+)-Ledene	1683	-	nd	0.32±0.24
α-Selinene	1684	1729	nd	2.81±0.52
β-Selinene	1695	-	nd	1.46±0.54
α-Muurolene	1704	-	nd	0.56±0.46
Bicyclogermacrene	1709	-	nd	4.06±0.73

Table 1 (continued)

Compounds	IK (Rt)[A]	IK[B]	*A. thaliana*	*T. virens*
3,4-Dimethylbenzylalcohol	1718	-	nd	t
1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a,hexahydronaphthalene	1764	-	nd	0.10±0.05
(−)-Calamanene	1774	1840	nd	0.32±0.10
Benzyl alcohol	1838	-	nd	t
α-Calacorene	1871	-	nd	0.19±0.19
Palustrol	1896	-	nd	0.88±0.27
Unknown (a 204 m.w. sesquiterpene)	1904	-	nd	0.06±0.07
.tau.-Muurolol	(46.88)	-	nd	0.25±0.11
Unknown (a 204 m.w. sesquiterpene)	(47.37)	-	nd	0.38±0.38
α-Cadinol	(47.79)	-	nd	0.08±0.05

Compounds were tentatively identified on the basis of NIST library searches

Mean values ± standard errors of the sum of three independent determinations. Statistical analysis was performed from the individual compounds. Means with same letter (a-b) are not significantly different ($P \leq 0.05$; Tukey)

nd not detected, *t* traces

[A] Kovats indices (Retention time in min) on capillary FFAP column

[B] Kovats Index available in the literature

release volatiles, which may be detected by neighboring plants and reinterpreted as instructions to adjust growth and/or defense. Although many different reports have evidenced that *Trichoderma* may activate plant immunity as well as developmental responses through different mechanisms, it is at present unknown whether the release of VOCs by fungal cells play a role in any of these processes.

To investigate whether the reduced production of VOCs in loss of function of PPT1 in *T. virens* could affect the induction of lateral roots, plants were co-cultivated with Tv29.8, Tv10.4 and Δ*ppt1* colonies in divided Petri plates and the root system analyzed 5 days later. It was found that all three strains of *T. virens* increased by 2-fold the number of lateral roots per plant and the length of lateral roots compared with axenically-grown seedlings, indicating that Δ*ppt1* mutant still emits VOCs that affect root development.

Our results show that *T. virens* stimulates *Arabidopsis* growth and root development even though the fungal colony was proliferating in the opposite side of the plate. Fungal emissions did not affect primary root growth, but for instance the formation of lateral roots and the growth of these structures were clearly induced, indicating that VOCs promote root branching. It is possible that the increase in biomass accumulation in seedlings exposed to VOCs from *T. virens* or Δ*ppt1* mutant is due to an increase in nutrient uptake through an increased root absorptive capacity.

When plant cells interact with potential pathogens, they often produce hormones that activate signaling cascades such as salicylic and/or jasmonic acid, which increase immunity through changes in gene expression. The phytohormone jasmonic acid is a crucial component of the plant defense signaling system. JA and its metabolites, collectively called jasmonates, are lipid-derived signals produced during defense responses against insects and pathogens (Liechti and Farmer 2002; Ellis et al. 2002; Diaz et al. 2003; El Oirdi et al. 2011). Interestingly, the VOCs from *T. virens* caused an induction of *pLox2:uidA* a JA-responsive marker and increased JA accumulation. *LOX2* encodes a chloroplast-targeted lipoxygenase thought to be involved in the biosynthesis of JA from linolenic acid (Jensen et al. 2002). JA is known to trigger induction of proteinase inhibitors and polyphenol oxidases in tomato (*Lycopersicon esculentum*; Fidantsef et al. 1999) and induce phytoalexin accumulation in bean and barley (Weidhase et al. 1987; Croft et al. 1993). The biochemical basis for this rapid defense response has been elucidated mainly by applying elicitors derived from pathogens to plant cell suspension cultures.

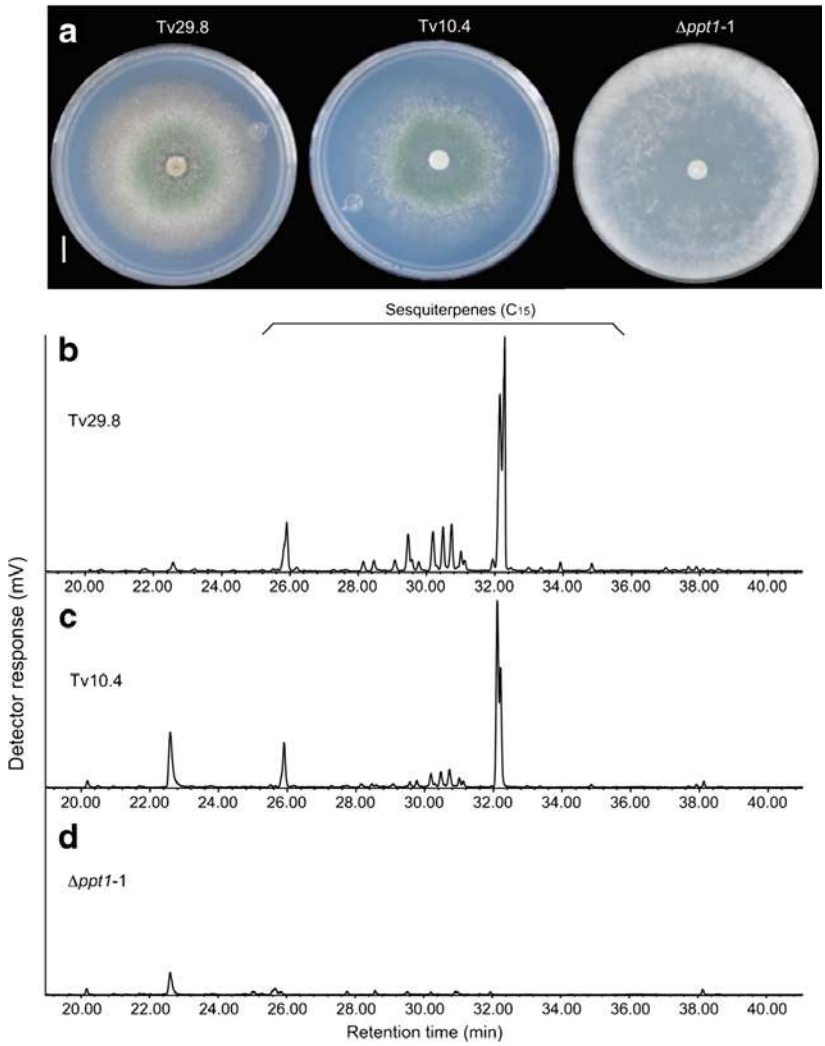

Fig. 3 Chromatographic profiles of volatiles from *T. virens* strains Tv29.8, Tv10.4 and the Δ*ppt1*-1 mutant. **a** Phenotype of colonies. **b**–**d** The compounds identified include, β-Caryophyllene, (−)-β-Elemene, Germacrene D, τ-Cadinene, α-Amorphene, δ-Cadinene and τ-Selinene as major compounds in Tv29.8 and Tv10.4. The line on the peaks of the chromatogram designates the sesquiterpenoid compounds

Moreover, the production of ROS is an important signature of activated defense responses. The major source for ROS appears to be an NAD(P)H-oxidase system that is associated with the plasma membrane (Kauss et al. 1999). This enzyme complex is directly linked to the elicitor signaling cascade and reduces molecular oxygen to $O_2^{\cdot-}$, which is rapidly dismutated to the more stable H_2O_2. We found that *T. virens* VOCs increased the levels of H_2O_2 in seedlings, indicating that airborne signals emitted by the fungus can be perceived by plants and early signaling events that modulate plant immunity are then activated. However, Hung et al. (2013) did not found accumulation of ROS in *Arabidopsis* leaves after exposure to *Trichoderma viride* VOCs. The reason of this discrepancy is not clear, but may be due to the different experimental systems used and the quantification methods employed in the research.

In filamentous fungi, diverse polyketide synthases (PKSs) and/or nonribosomal peptide synthetases (NRPSs) participate in the biosynthesis of secondary metabolites such as pigments, antibiotics, siderophores, and mycotoxins (Márquez-Fernández et al. 2007; Velázquez-Robledo et al. 2011). It has been reported that the Δ*ppt1*-1 mutant of *T. virens* is unable to produce conidial pigments and non-ribosomal peptides (Velázquez-Robledo et al. 2011). In this work, we determined the VOCs profile of *T. virens* Tv10.4 and Δ*ppt1*-1, parental and mutant strains, respectively, to determine whether PPT1 may function in volatile emissions. GC-MS analyses revealed that *Arabidopsis* seedlings release acetophenone, 6-methyl-2-heptanol (16.5 %) and six different terpenes, while *T. virens* emits a wealth of sesquiterpenes including β-caryophyllene, (−)-β-elemene, germacrene D, τ-cadinene, δ- cadinene, α-amorphene, and τ-selinene. Previously, Crutcher

Table 2 Volatile organic compounds produced by *T. virens* Δ*ppt1*-1 mutant detected by SPME-GC-MS

Compounds	IK (Rt)[A]	Δ*ppt1*-1
β-Phellandrene	1187	33.03±3.34
Methyl (2*E*)- 4,4-dimethyl-2-pentenoate	1315	6.14±1.13
Sorbic acid	1346	27.61±6.90
1-Octen-3-ol	1450	2.40±0.23
Acetic acid	1466	2.69±1.02
3,4-Dimethylpent-2-en-1-ol	1473	7.53±1.22
p-Menth-3-ene	1555	4.49±1.44
β-Cedrene	1566	5.49±0.96
Phenylethylalcohol	1891	10.61±0.51

Compounds were tentatively identified on the basis of NIST library searches

Mean values ± standard errors of the sum of three independent determinations

nd not detected

[A] Kovats indices (Retention time in min) on capillary FFAP column

et al. (2013) reported that *T. virens* 10.4 produces many terpenoids when the fungus is grown in PDA as culture medium. Here we determined the VOCs profile in 0.2X MS media because it was our interest to evaluate if the growth medium could affect the blend of terpenes. Sesquiterpenes and the tertiary alcohols of the δ-cadinene-type are often associated with fungal antagonism (Wu et al. 2005). Since *T. virens* but not the growth medium released many of these cadinene-type compounds, our data are very supportive of its role as biocontrol agents. Strobel et al. (2011), when characterizing the gases of *Phoma* sp., an endophytic of *Larrea tridentata* identified a mixture of VOCs with antifungal properties, including sesquiterpenoids, some alcohols and several reduced naphthalene derivatives. Some of the test organisms with the greatest sensitivity to the *Phoma* sp. VOCs were *Verticillium*, *Ceratocystis*, *Cercospora* and *Sclerotinia* while those being the least sensitive were *Trichoderma*, *Colletotrichum* and *Aspergillus*.

The changes in VOC production during the interaction, suggest that the blend of volatiles might protect the plant either directly or indirectly against the attack of pathogen microbes such as *B. cinerea*. In agreement with this possibility, metabolite analysis showed that exposure of *Arabidopsis* for 2 h to ocimene increased tissue levels of MeJA, whereas in a longer exposure (24 h), a terpene synthase and an expansin (EXP 11) were induced, which suggest a regulation loop in production of terpenoids by other volatiles (Godard et al. 2008). As shown in Table 1, *T. virens* produced β-myrcene, *trans*-β-ocimene, and *cis*-β-ocimene in low abundance. An interesting observation is that *Trichoderma* may induce defense responses in plants through the JA signaling pathway, because the exogenous application of JA in *Zea mays* stimulated the emission of sesquiterpenes (Schmelz et al. 2001), and it was recently demonstrated that terpene VOCs are biosynthesized from deuterated primary precursors after MeJA elicitation in *Achyranthes bidentata* (Tamogami et al. 2013), suggesting the direct interaction of JA with terpene synthesis. Therefore, it is tempting to speculate that *Trichoderma* can modulate the synthesis of terpenes in *Arabidopsis* by an increase in the synthesis of JA, which should be confirmed by using JA-impaired mutants or pharmacological approaches.

The SPME GC-MS analysis revealed that the mutation of *PPT1-1* affects the production of a number of terpenes in Δ*ppt1-1* mutant when compared with Tv10.4, the parental strain. The failure of the Δ*ppt1-1* mutant to produce terpenes suggests that PPTase is needed to activate terpene synthases. In *Fusarium fujikuroi* the deletion of Ff*ppt1* affected not only the biosynthesis of the PKS and PKS/NRPS but also terpene-derived secondary metabolites and the expression of genes coding for the respective key enzymes. The deletion of Ff*ppt1* in strain C-1995 led to a significant increase in the content of *ent*-kaurene and acorenol (Wiemann et al. 2012).

Interestingly, *Arabidopsis* seedlings exposed to the Δ*ppt1-1* VOCs showed decreased resistance against the necrotrophic fungus *Botrytis cinerea* when compared to those exposed to the WT strains, suggesting that terpenes produced by WT strains Tv29.8 and Tv10.4 may protect plants from the pathogen. To test the possibility that the fungal VOCs could have a direct effect on the growth of *B. cinerea*, the effect of VOCs from *T. virens* Tv29.8 and Δ*ppt1-1* mutant on *B. cinerea* growth was determined. These data show a modest, yet statistically significant inhibition of *B. cinerea* growth both by WT *T. virens* and Δ*ppt1-1* mutant (Table 3), indicating that VOCs still being produced in the mutant might inhibit *B. cinerea* growth.

Since the data on root development show similar response among the strains tested, we hypothesize that volatiles are still being produced by the Δ*ppt1* mutant,

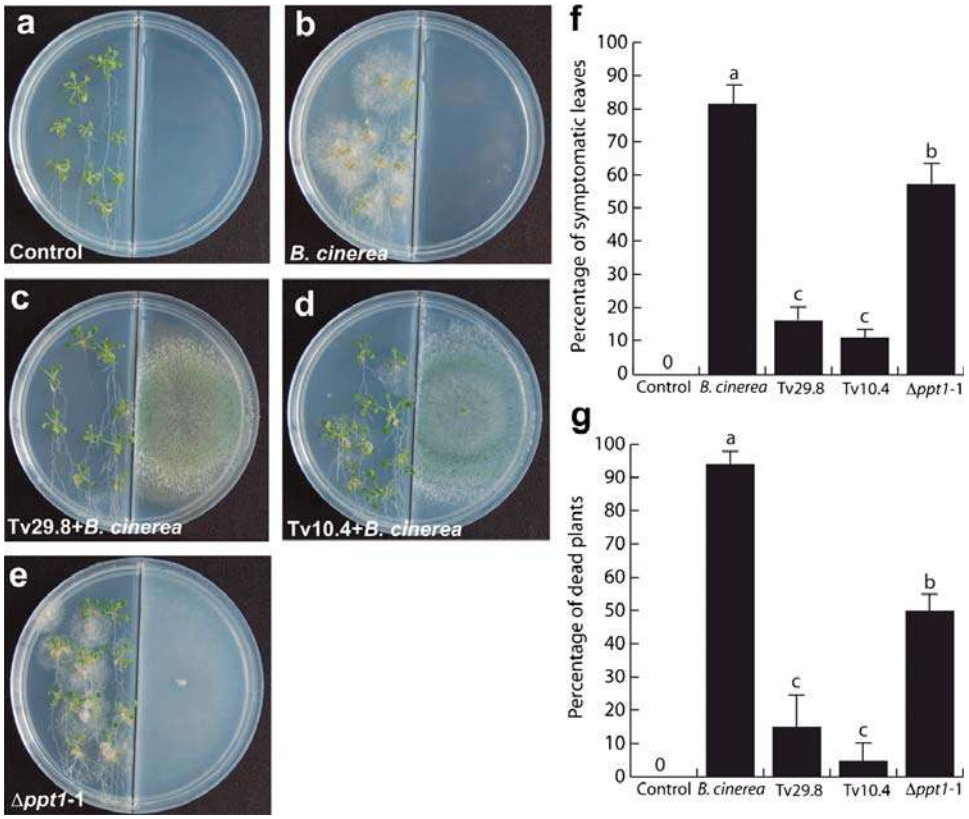

Fig. 4 Effect of VOCs from *T. virens* on disease resistance of *Arabidopsis* seedlings against *Botrytis cinerea*. **a**–**e** Representative photographs of 16-days old *Arabidopsis* seedlings inoculated with *T. virens* by 3-days and then infected with the plant pathogen *B. cinerea* by an additional period of 3-days. **f** Percentage of chlorotic leaves per plant 48 h after *B. cinerea* inoculation. **g** Percentage of dead plants 3-days after *B. cinerea* infection. Notice that VOCs from Tv29.8 and Tv10.4 reduce the infection severity of *Arabidopsis* seedlings and also restrict *B. cinerea* growth in the culture medium but these effects are not equally observed in seedlings inoculated with the mutant Δ*ppt1*-1. *Bars* show the mean ± SE of 20 *Arabidopsis* seedlings. Different *letters* are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated two times with similar results

although the blend is certainly different to that of the WT. The majority of these compounds are not sesquiterpenes, yet they affect root development as the blend produced by WT. Therefore, we conclude that PPT1-regulated sesquiterpene emissions play a particular role in protection against *B. cinerea*. Since we did not expose the roots or shoots to specific compounds, determining which VOCs are responsible for the root branching effects or the resistance against *B. cinerea* in leaves cannot be derived from the presented data. It also remains to be determined whether the VOCs profile of *A. thaliana*, *T. virens* or during the interaction changes, as early volatile formation may be important for development and induction of systemic responses in the plant.

Table 3 Effect of VOCs from *T. virens* Tv29.8 and *Δppt1-1* mutant on *B. cinerea* growth. *B. cinerea* mycelium was inoculated on 0.2X MS medium at opposite sides of divided Petri dishes containing WT *T. virens* or *Δppt1-1* colonies. The plates were incubated 96 h at 21ºC in darkness and the percent of *B. cinerea* growth determined for 6 independent plates

	Treatment		
	Control	Tv29.8	Δ*ppt1*-1
Growth (%)	100 ± 2.97 [a]	88.19 ± 1.12 [b]	94.93 ± 2.42 [ab]

Different letters denote statistically significant differences ($P \leq 0.05$; Tukey)

Recent research has shown a role of CO_2 in phytostimulation by microorganisms (Kai and Piechulla 2009). Although we cannot exclude the possibility of an influence of CO_2 in plant growth promotion by *T. virens*, we believe that it is the particular composition of VOCs released what determines the plant response. For instance, the 0.2X MS medium used in our experiments contains sucrose as a carbon source and the optimal conditions for the growth of plants are evidenced by the prolific growth of the primary root. We

did not see a further increase in primary root growth in seedlings cocultivated with *Trichoderma*. Moreover, the particular effect of fungal cocultivation in lateral root induction and in activation of plant immunity suggests that it is the composition of the volatile blend what affects plant growth or defense. This possibility is also supported by the data obtained with the Δ*ppt1-1* mutant, which has prolific growth in the conditions tested but differs in its ability to protect *Arabidopsis* plants against *B. cinerea* when compared to WT *T. virens*. The production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. A screen of 42 rhizobacterial strains for volatile-mediated effects on *Arabidopsis thaliana* showed that all 42 strains tested showed significant volatile-mediated plant growth modulation, with effects ranging from plant death to a six-fold increase in plant biomass (Blom et al. 2011). Therefore, we speculate that plant growth in soil is largely influenced by the microbial composition of the rhizosphere and that VOCs emissions represent an important way of signaling between microbes and plants. Our results not only expand the number of known and predicted secondary metabolites produced by *T. virens* but also show the critical role of the PPT1 enzyme in the biosynthesis of terpenes with potential antimicrobial activities.

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7. DISCUSIÓN

El crecimiento de las plantas es el resultado de la percepción e integración de los estímulos ambientales y programas de desarrollo endógenos que regulan la división y expansión celular en los diferentes tejidos (Contreras-Cornejo *et al.* 2015a,b). Por ejemplo, la formación de las raíces laterales y pelos radiculares es regulada por la percepción de "señales fúngicas" y la modulación de la proteína cinasa activada por mitógenos 6 en *A. thaliana* (Contreras-Cornejo *et al.* datos no publicados). Estos eventos fisiológicos pueden ser alterados por el estrés biótico o abiótico (Ortiz-Castro *et al.* 2009). En condiciones ambientales, las raíces de las plantas crecen en asociación con un gran número de bacterias y hongos (Fig. 1). Estas interacciones pueden ser neutrales (sin efecto aparente entre ambos organismos), patogénicas o benéficas (Contreras-Cornejo *et al.* 2013, 2014a). Los microorganismos benéficos que promueven el crecimiento vegetal, representan una estrategia alternativa al uso de pesticidas y fertilizantes para contrarrestar los efectos negativos que induce el estrés abiótico. El estrés causado por el exceso de sal en el suelo, afecta severamente el crecimiento y desarrollo de las plantas, principalmente de dos maneras: I. induce estrés osmótico, lo que repercute negativamente en la captación de agua y II. el exceso del ión Na^+ en la célula altera un gran número de procesos metabólicos (Fig. 5) (Abogadallah *et al.* 2010). Es importante resaltar que la tolerancia a la salinidad depende del tipo de planta, la concentración de sal en el suelo y del tiempo de exposición del sistema radicular al estrés (Hasegawa *et al.* 2000; Zhu 2002; Munns y Tester 2008). El estrés salino también puede afectar las interacciones de las plantas con los microorganismos (Gal-Hemed *et al.* 2011).

En condiciones normales, varios hongos rizosféricos inducen la ramificación del sistema radicular, lo cual repercute directamente en la captación de agua y nutrientes, el incremento de biomasa, la fotosíntesis y la productividad (Fig. 1) (Contreras-Cornejo *et al.* 2013, 2015a). Mediante análisis químicos se ha encontrado que los hongos promotores del crecimiento vegetal producen auxinas, ET, citocininas, giberelinas y ABA (Contreras-Cornejo *et al.* 2015a,b).

Trichoderma es un habitante común de la rizósfera y ha sido estudiado ampliamente por su capacidad de producir antibióticos, parasitar otros hongos

y competir con microorganismos fitopatógenos (Harman *et al.* 2004). Hasta hace poco, estos mecanismos indirectos, en conjunto eran considerados como la base de *Trichoderma* spp. para ejercer efectos benéficos sobre el crecimiento y desarrollo de las plantas. Sin embargo, algunas especies ejercen una influencia sobre las plantas mediante mecanismos directos que afectan el crecimiento y desarrollo. Estos fenómenos involucran la producción de sustancias reguladoras del crecimiento, la activación de respuestas de defensa dependientes del AJ, AS y/o ET y cambios a nivel bioquímico y sobre el sistema radicular para conferir adaptación al estrés abiótico (Figs. 1 a 4) (Velázquez-Robledo *et al.* 2011; Contreras-Cornejo *et al.* 2009, 2011, 2013, 2014a, b, c, 2015a,b; Brotman *et al.* 2013).

Por ejemplo, estudios realizados en este trabajo muestran que *T. virens* y *T. atroviride* promueven el crecimiento de *A. thaliana*. Este fenómeno implica la acumulación de biomasa foliar, la inducción de raíces laterales y pelos radiculares (Contreras-Cornejo *et al.* 2009, 2011, 2014b). Los análisis químicos de los metabolitos secundarios mostraron que ambas especies producen AIA que es la principal auxina en plantas que regula el crecimiento y desarrollo vegetal, y sus compuestos precursores: IEt, IAAld e ICAld (Contreras-Cornejo *et al.* 2009, 2011). Experimentos de inoculación *in vitro* de las mutantes en los genes implicados en el transporte de AIA o su señalización (*AUX1*, *BIG*, *EIR1* y *AXR1*) de *A. thaliana,* mostraron ser resistentes al efecto promotor del crecimiento de *Trichoderma* spp. (Contreras-Cornejo *et al.* 2009). Experimentos farmacológicos para evaluar la actividad biológica de los compuestos indólicos mencionados anteriormente, mostraron un efecto dependiente de la concentración sobre los parámetros del desarrollo. Se encontró que el AIA y el IAAld inducen la formación de biomasa foliar, raíces laterales y pelos radiculares. Mientras que el ICAld promueve la formación de raíces adventicias (Contreras-Cornejo *et al.* 2009, 2011).

Para evaluar el potencial de *T. virens* y *T. atroviride* para inducir tolerancia al estrés salino en *A. thaliana*, ambos organismos fueron cocultivados *in vitro* en condiciones sin sal y con elevada salinidad. Para establecer estas condiciones, primero se evaluó el efecto de un rango de concentraciones de NaCl (50-200 mM) sobre las plantas y (50-300 mM) para *Trichoderma* spp. Se encontró que *T. virens* y *T. atroviride* tienen diferente respuesta a la sal. Sin embargo ambas

especies fueron tolerantes a 100 mM (Contreras-Cornejo *et al.* 2014b). En el caso de *A. thaliana*, se encontró que 100 mM de sal reprime aproximadamente el 50% del crecimiento global (Figs. 6 y 7). Debido a los efectos de 100 mM sobre la planta y el hongo, esta fue la concentración de sal utilizada en los experimentos de interacción (Contreras-Cornejo *et al.* 2014b). Como se observó anteriormente en condiciones sin sal, las plántulas de *A. thaliana* inoculadas con *Trichoderma* spp. incrementaron la acumulación de biomasa foliar, el número de raíces laterales y pelos radiculares (Contreras-Cornejo *et al.* 2009). Consistente con los resultados descritos por Halperin y colaboradores (2003), la sal inhibió la formación y crecimiento de pelos radiculares (Fig. 7). Por el contrario, *Trichoderma* spp. incrementó el número y longitud de los pelos radiculares. Cuando se evaluaron las concentraciones de AIA en los hongos crecidos sin sal y con 100 mM NaCl, se encontró que *T. virens* incrementó ligeramente sus niveles de 4.21 a 5.88 ng/ml y que *T. atroviride* produjo más elevadas concentraciones y mantuvo sus niveles de 7.65 a 7.64 ng/ml sin sal y con sal respectivamente. Debido a que *Trichoderma* spp. produce auxinas y estas son sensadas por las raíces, es lógico argumentar que la diferenciación de las células epidérmicas de la raíz para formar pelos radiculares implica un mecanismo dependiente de auxinas (Contreras-Cornejo *et al.* 2009, 2014b).

En *A. thaliana* y otras especies de plantas, la aplicación de auxinas puede inducir tanto la formación de raíces laterales y pelos radiculares (Laskowski *et al.* 1995). Para determinar si el efecto negativo de la sal sobre el crecimiento y desarrollo de *A. thaliana* es consecuencia de la modificación del transporte y señalización de las auxinas, se evaluó el crecimiento y la respuesta de las raíces de la línea silvestre Col-0 y las mutantes *eir1/pin2*, *aux1-7*, *arf7arf19* y *tir1afb2afb3* a la sal, y se encontró que 100 mM de NaCl afectó el crecimiento de la raíz primaria y la formación de raíces laterales de las mutantes *eir1/pin2*, *aux1-7* y *tir1afb2afb3* (Contreras-Cornejo *et al.* 2014b). De manera semejante, Sun y colaboradores (2008) encontraron que 150 mM afecta la expresión del gen *PIN2* en *A. thaliana* (Fig. 11). Estos datos apoyan la hipótesis que para activar la tolerancia a la salinidad se requiere intacta la vía de las auxinas (Contreras-Cornejo *et al.* 2014b). Posiblemente los efectos negativos de la sal en la modificación de la señalización de las auxinas se debe a que la sobreproducción de ERO bajo estrés salino modifica la síntesis, conjugación,

transporte y señalización de esta hormona (Bartoli *et al.* 2013). Interesantemente, las ERO funcionan como mediadores de la transducción de la señal auxínica para controlar la respuesta gravitrópica de la raíz (Joo *et al.* 2001).

Bajo estrés salino, *Trichoderma* spp. podría restaurar la homeostasis de auxinas y en consecuencia promover el crecimiento. Para investigar esta hipótesis, se evaluó el efecto de *Trichoderma* spp. sobre la expresión del gen *DR5::GUS* en condiciones de elevada salinidad. Interesantemente se encontró que *Trichoderma* spp. aumentó la expresión del gen *DR5::GUS* en la punta de la raíz primaria y las raíces laterales y que nuevas raíces se formaron en respuesta a la interacción con estos hongos (Contreras-Cornejo *et al.* 2014b). Por lo tanto, es factible especular que las auxinas liberadas por *Trichoderma* spp. pueden ser importantes para mantener los programas de desarrollo de las raíces bajo estrés salino (Fig. 12). El papel clave para el AIA en la tolerancia a la salinidad de las plantas inducida por otros hongos se ha mostrado también recientemente. Por ejemplo, Redman y colaboradores (2011) reportaron que *Fusarium culmorum* y *Curvularia protuberata* mejoraron el crecimiento de plantas de arroz en condiciones de salinidad. Del mismo modo, *Phoma glomerata* y *Penicillium* sp. incrementaron significativamente la biomasa de plantas de pepino bajo estrés salino (Waqas *et al.* 2012).

Por otro lado, Macías-Rodríguez y colaboradores (2015) mostraron que plántulas de *A. thaliana* crecidas en 100 mM NaCl y expuestas a los compuestos volátiles de *T. virens*, acumularon mayor cantidad de biomasa total y clorofila y que se ramificó el sistema radicular comparado con las plantas no expuestas. El análisis químico por cromatografía de gases acoplado a espectrometría de masas de los compuestos volátiles, reveló que *T. virens* produce una gran cantidad de terpenos cuyos compuestos mayoritarios son: el β-cariofileno, (-)-β-elemeno, germacreno D, τ-cadineno, α-amorfeno, δ-cadineno y τ-selineno (Contreras-Cornejo *et al.* 2014c). Estos compuestos no fueron detectados en la mutante Δ*ppt1*-1 de *T. virens* afectada en la 4-fosfopanteteinil transferasa (Fig. 4). Esta proteína pertenece a una superfamilia de enzimas necesarias para la síntesis de ácidos grasos, aminoácidos, policétidos y péptidos no ribosomales (Velázquez-Robledo *et al.* 2011).

La tolerancia a la sal implica la unificación de varios mecanismos complejos que involucran la acción coordinada de familias de genes que realizan una variedad de funciones tales como el control de la pérdida de agua a través de los estomas, el secuestro de iones, ajustes metabólicos y osmóticos y la protección al daño oxidativo por parte de agentes antioxidantes como el ácido ascórbico (Abogadallah 2010). Las respuestas de tolerancia a la salinidad que activan las plantas involucran en parte la señalización del ABA (Achard *et al.* 2006). En este trabajo se encontró que *T. virens* y *T. atroviride* regulan la transpiración de las hojas de *A. thaliana* y que producen ABA, el cual regula la apertura de los estomas mediante un mecanismo dependiente de las fosfatasas ABI1 y ABI2 (Contreras-Cornejo *et al.* 2015a). Este escenario resulta útil para las plantas por dos razones: I. después de la división celular en los órganos en crecimiento, las células necesitan agua para expandirse por lo que se requiere controlar el contenido y la salida de agua. II. el estrés salino induce deshidratación en las plantas, por lo que se requiere controlar el potencial hídrico regulando la apertura estomática.

Por otra parte, se conoce que bajo estrés salino grave, las ERO pueden dañar los componentes celulares (Mittler 2002). Uno de los procesos que participan en la tolerancia al estrés salino es la desintoxicación de las ERO. El compuesto osmoprotector prolina se acumula en diversas especies de plantas en respuesta a la sequía y la salinidad, y su acumulación se correlaciona frecuentemente con la tolerancia al estrés. Este aminoácido funciona como eliminador de radicales hidroxilo y el control de la homeostasis redox (Fabro *et al.* 2004; Ben *et al.* 2008).

El AA es una molécula con múltiples funciones fisiológicas en animales y plantas. Este compuesto es bien conocido que funciona como antioxidante que desintoxica la célula de ERO, especialmente del H_2O_2 (Smirnoff 2000). En condiciones óptimas de crecimiento, la producción de ERO en la célula es baja, sin embargo, aumenta bajo condiciones ambientales adversas, como la sequía, la salinidad, los contaminantes del aire, o el ataque por patógenos (Mukherjee *et al.* 2010). Debido a que las ERO pueden causar daño celular, su producción y procesos de detoxificación son controlados por varios mecanismos protectores que incluyen la actividad de enzimas antioxidativas y componentes no enzimáticos como compuestos antioxidantes y osmolitos (Niyogi 2000;

Mittler 2002). La tolerancia a la salinidad en *A. thaliana* inducida por *Trichoderma* spp. se correlacionó con incrementos del osmolito prolina y el agente antioxidante AA. Estos cambios a nivel bioquímico inducidos por los hongos, proporciona una gran ventaja a las plantas para mitigar los efectos negativos de la sal (Contreras-Cornejo *et al.* 2014b).

También se ha reportado que el estrés salino induce rápidamente la acumulación de Na^+ y que afecta la homeostasis de los iones Ca^{2+}, K^+ y NO^-_3. En el citosol, el K^+ es esencial para la activación de un gran número de enzimas, por ejemplo: las requeridas para la síntesis de piruvato y la traducción del ARN (Maathuis 2006). Debido a las similitudes fisicoquímicas entre el Na^+ y el K^+, las células tienden a sustituir el K^+ por el Na^+ en sus sitios de unión, lo que origina una disfunción a nivel celular. Estos efectos negativos desencadenan en las plantas varios mecanismos para regular el exceso de Na^+ (Maathuis 2006). Las raíces exudan un gran número de sustancias que incluyen: iones, enzimas, mucílago y metabolitos primarios y secundarios (Bais *et al.* 2006). Los exudados de la raíz se transportan a través de la membrana celular y son liberados en la rizósfera circundante. La toxicidad del Na^+ se considera uno de los factores más importantes que limitan el crecimiento de raíces y por lo tanto, se considera que la eliminación de Na^+ mediante los exudados radiculares puede ser parte integral de un mecanismo de adaptación a la salinidad (Ungar 1996; Ghoulam *et al.* 2002). En este trabajo se mostró que *Trichoderma* spp. activa la eliminación de Na^+ a través de los exudados de la raíz como parte de un mecanismo de desintoxicación en *A. thaliana*. El contenido de Na^+ exudado por las raíces crecidas en condiciones normales fue similar entre tratamientos (Contreras-Cornejo *et al.* 2014b). En contraste, las plantas inoculadas con *Trichoderma* spp. crecidas bajo salinidad exudaron mayor cantidad Na^+ en comparación con las plantas no inoculadas. Es importante destacar que el Na^+ exudado por las raíces se incrementó 25.76 y 79.65%, cuando se inocularon las plantas con *T. virens* o *T. atroviride*, respectivamente. En el caso de los hongos micorrícicos, se ha observado que bajo estrés salino, promueven el crecimiento vegetal al incrementar la captación de los minerales esenciales y disminuyen la del Na^+ (Hall 2002; Li *et al.* 2012). Por ejemplo, *Scleroderma bermudense* incrementa los niveles de

fósforo y K^+ pero disminuye las concentraciones de Na^+ y Cl^- en las plantas *Coccoloba uvifera* (Li *et al.* 2012).

En conclusión, la regulación de la transpiración de las hojas mediante el cierre de los estomas dependiente de un mecanismo regulado por ABA, la activación de la ruta auxínica, la modificación del sistema radicular, los incrementos del contenido de prolina y AA y la eliminación de Na^+ mediante los exudados radiculares proveen en conjunto capacidad a las plantas para adaptarse al estrés salino (Fig. 12). Por lo tanto, los hongos promotores del crecimiento vegetal pueden representar una alternativa de uso sobre plantas que se cultivan en suelos salinos.

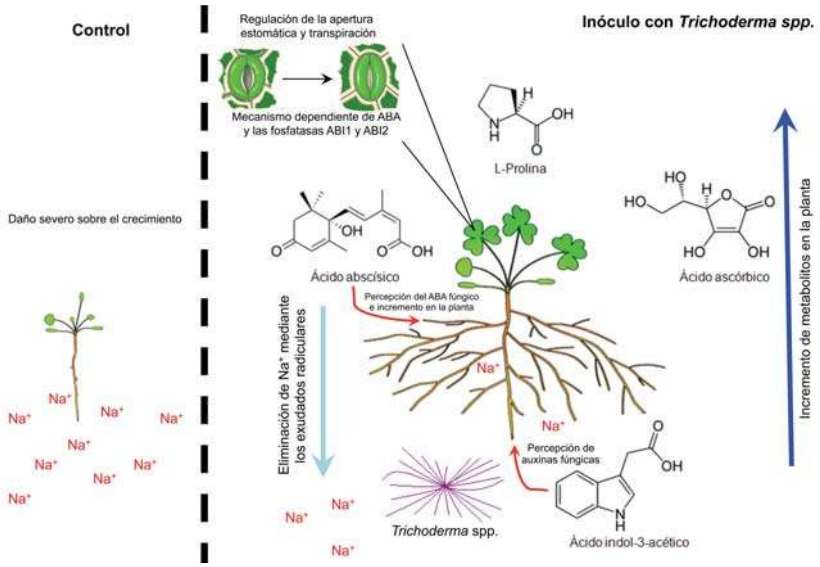

Figura 12. Mecanismos de tolerancia al estrés salino activados por *Trichoderma* spp. Cuando las plantas se encuentran bajo condiciones de salinidad se afectan severamente los procesos metabólicos, la expresión de genes y las respuestas fisiológicas al medio ambiente. Datos derivados de este trabajo muestran que *Trichoderma* puede producir auxinas y ABA y liberarlos al medio de interacción con las plantas. Las raíces de las plantas perciben las señales hormonales y reajustan el crecimiento bajo estrés salino. En particular, uno de los efectos benéficos de *Trichoderma* spp. es la modulación del sistema radicular lo que implica la formación de raíces laterales y pelos radiculares cuyo desarrollo y crecimiento son regulados por auxinas. Por otro lado, estos hongos inducen en las plantas incrementos en el contenido endógeno de ABA el cual puede regular el cierre de estomas para controlar la pérdida de agua, el contenido de osmolitos como la prolina y de ácido ascórbico que funciona como antioxidante. De manera alternativa se induce la eliminación del ión Na^+ mediante los exudados radiculares. Todos estos efectos en conjunto pueden contribuir a la adaptación y sobrevivencia de las plantas al estrés salino.

8. CONCLUSIONES

En este trabajo se estudió el efecto de *T. virens* y *T. atroviride* sobre el crecimiento de *A. thaliana* en condiciones de salinidad. Se encontró que la sal afecta el crecimiento global (desarrollo de la parte foliar, el crecimiento de la raíz primaria, formación de raíces laterales y pelos radiculares). Cuando se estudió a nivel molecular el blanco de acción del ión Na^+ se encontró que afecta la señalización de las auxinas debido a la hipersensibilidad de las mutantes *eir1*/*pin2*, *aux1-7* y *tir1afb2afb3* de *A. thaliana*. Sin embargo, nuestros datos muestran que *Trichoderma* spp. es tolerante a 100 mM NaCl (concentración de sal que disminuye el 50% del crecimiento de *A. thaliana*). Cuando las plantas de *A. thaliana* se cocultivaron con *Trichoderma* spp. se encontró que los hongos inducen la formación de biomasa total e incrementan el contenido de clorofila. Los efectos benéficos de *Trichoderma* también se observaron sobre el sistema radicular debido a que las plantas inoculadas formaron un mayor número de raíces laterales y pelos radiculares lo cual puede favorecer la captación de agua y nutrientes bajo estrés salino. Ambas especies de *Trichoderma* producen auxinas y su nivel se mantiene constante en condiciones normales y de salinidad, lo que resulta útil para las plantas sometidas también al estrés. En análisis de la expresión del gen *DR5::GUS* de respuesta a auxinas disminuyó en las plantas no inoculadas en condiciones de salinidad. Sin embargo, la expresión de *DR5::GUS* se incrementó en la punta de la raíz primaria y raíces laterales de las plantas bajo estrés salino inoculadas. Estos datos sugieren que las auxinas tienen un papel clave en el desarrollo de las plantas y en la tolerancia al estrés salino. A nivel bioquímico, se encontró que *Trichoderma* spp. induce la acumulación de osmolitos, compuestos antioxidantes y que activa la eliminación de Na^+ mediante los exudados radiculares.

Por otro lado, un mecanismo alternativo para inducir tolerancia al estrés salino es el regulado por el ABA que es un compuesto isoprenoide, el cual modula la apertura de los estomas para controlar la liberación de agua y gases. El cierre de los estomas en la epidermis de las hojas durante la interacción con *Trichoderma* spp. involucra la participación de las fosfatasas ABI1 y ABI2. Estos efectos son consecuencia de la percepción del ABA de origen fúngico

por parte de las células de la raíz. En conjunto, las respuestas fisiológicas de las plantas a *Trichoderma* spp. resultan en ventaja para *A. thaliana* bajo estrés salino, debido a que este tipo de estrés abiótico ocasiona deshidratación, y al estar cerrados los estomas, se evita la pérdida de agua y se mantiene la planta hidratada.

Los volátiles de *T. virens* también están involucrados en inducir tolerancia al estrés salino. Se encontró que las plantas expuestas a los volátiles de esta especie formaron mayor cantidad de biomasa total y se incrementó el contenido de clorofila. Estos compuestos activaron mecanismos desconocidos sobre el sistema radicular para inducir su desarrollo. Los análisis químicos de los volátiles de *T. virens* mostraron la presencia de un gran número de sesquiterpenos que son considerados también como sustancias antioxidantes. En conclusión, esto datos muestran que *Trichoderma* spp. podría ser utilizado como bioinoculante en el campo para promover el crecimiento vegetal e inducir tolerancia al estrés salino.

9. PERSPECTIVAS

Los datos presentados en este trabajo muestran una función importante de *Trichoderma* spp. en la rizósfera. Sin embargo, los efectos de estos hongos sobre las plantas se desconoce en gran manera. Por lo que resultaría útil estudiar el papel de los compuestos volátiles de *Trichoderma* spp. sobre las plantas. Los conocimientos teóricos de los efectos de estos hongos sobre los diferentes modelos vegetales podrían permitir mejorar la aplicación de *Trichoderma* spp. en los suelos cultivables.

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11. APÉNDICE

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11.1
Review

The role of microbial signals in plant growth and development

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Plant growth and development involves a tight coordination of the spatial and temporal organization of cell division, cell expansion and cell differentiation. Orchestration of these events requires the exchange of signaling molecules between the root and shoot, which can be affected by both biotic and abiotic factors. The interactions that occur between plants and their associated microorganisms have long been of interest, as knowledge of these processes could lead to the development of novel agricultural applications. Plants produce a wide range of organic compounds including sugars, organic acids and vitamins, which can be used as nutrients or signals by microbial populations. On the other hand, microorganisms release phytohormones, small molecules or volatile compounds, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis. In this review, we focus on recent developments in the identification of signals from free-living bacteria and fungi that interact with plants in a beneficial way. Evidence has accumulated indicating that classic plant signals such as auxins and cytokinins can be produced by microorganisms to efficiently colonize the root and modulate root system architecture. Other classes of signals, including *N*-acyl-L-homoserine lactones, which are used by bacteria for cell-to-cell communication, can be perceived by plants to modulate gene expression, metabolism and growth. Finally, we discuss the role played by volatile organic compounds released by certain plant growth-promoting rhizobacteria in plant immunity and developmental processes. The picture that emerges is one in which plants and microbes communicate themselves through transkingdom signaling systems involving classic and novel signals.

Introduction

Plants are sessile, multicellular organisms, which rely on developmental and metabolic changes for growth. At least three well defined parts can be recognized in the developing plant, (1) the root, the below-ground part of the plant, which provides anchorage and plays an important role in water and nutrient uptake from the soil, (2) the stem, which performs essential functions as a supporting structure for the leaves and as a conduit for water and nutrients moving from one part of the plant to another, and (3) the shoot, which produces leaves, flowers and fruits that enables efficient light capture and provides a means for reproduction and seed dispersal. The anatomical configuration of the root, stem and shoot systems to build a plant is known as plant architecture. Plant architecture is a crucial factor in the agronomic success of crops and is a vital consideration for plant breeders.[1,2]

The general developmental pattern in plants depends on indeterminate growth and iterative organogenesis, characterized by continued cell division in the meristematic regions. The shoot apical meristem represents a source of undifferentiated cells that divide and contribute to the new leaf primordia during vegetative growth, and to inflorescence and floral meristems during the reproductive phase of the life cycle. At the other end of the plant, the root apical meristem provides new cells, which add to the preexisting files extending back into the mature root.[3,4] Behind the root and shoot meristems, the newly added cells expand before fully differentiating, in the maturation zone, into the varied cell types that build the plant.[5]

The root system displays considerable plasticity in its morphology and physiology in response to variability within its environment. By contrast to the primary root that is formed during embryogenesis, lateral and adventitious roots are formed postembryonically. Lateral roots are formed typically from the pericycle, as a consequence of the activation of the cell cycle in specific groups of so-called 'founder cells', which undergo a series of periclinal and anticlinal divisions to generate a meristem de novo.[6] Activation of pericycle cells for lateral root initiation might take place in the basal meristem and correlated with elevated auxin sensitivity in this part of the root.[6,7] Once the developing lateral root primordium has formed its own meristem, this new meristem produces cells that expand and push the new root tip through the ground and epidermal layers to the outside.[8-10]

Extensive communication occurs between plants and microorganisms during different stages of plant development in which signaling molecules from the two partners play an important role. Fungal and bacterial species are able to detect the plant host and initiate their colonization strategies in the rhizosphere by

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producing canonical plant growth-regulating substances such as auxins or cytokinins. On the other hand, plants are able to recognize microbe-derived compounds and adjust their defense and growth responses according to the type of microorganism encountered. This molecular dialogue will determine the final outcome of the relationship, ranging from pathogenesis to symbiosis, usually through highly coordinated cellular proceses.[11] Bacterial and fungal phytopathogens are not restricted to infecting aerial or root tissues exclusively, as such, communication between the shoot and root can confer a survival advantage to the plant and potentially limit or prevent diseases. For instance, beneficial soil bacteria and fungi can confer immunity against a wide range of foliar diseases by activating plant defenses, thereby reducing a plant's susceptibility to pathogen attack.[12] For many years, this was considered the basis by which beneficial microorganisms could increase plant yield when inoculated in crops, however, it is increasingly appreciated that classic and novel microbial signals may also directly participate on plant morphogenesis.

Signaling during plant-pathogen interactions has been a central topic in phytopathology for many years, whereas more recent efforts are being made to discover the signals involved in plant communication with non pathogenic microbes, especially those that enhance plant productivity. Among the latter, nitrogen-fixing bacteria belonging to Rhizobium, as well as mycorrhizal fungi, which are able to establish endosymbiosis with plants, have already been extensively reviewed[13-17] and will therefore not be further discussed here. Our aim is rather to discuss recent findings about the signals involved in the interaction of plants with free-living, beneficial microbes including filamentous fungi of the genus Trichoderma and plant growth promoting rhizobacteria (PGPR), for which important recent discoveries have been made on their chemical communication, the biological processes they sustain and the benefits to plants involved in these interactions.

Plants: Contribution to Microbes

Diverse compounds released by different parts of the root system create an unique environment in the surrounding soil, which is known as the rhizosphere. These compounds are collectively termed as root exudates and belong to three main classes: (1) Low-molecular weigth, (2) High-molecular weigth and (3) Volatile organic compounds (VOCs). Low-molecular weigth compounds represent the main portion of exudates and consist of sugars, amino acids, organic acids, phenolics, vitamins and various secondary metabolites. High-molecular weigth compounds consist of mucilage and proteins, while carbon dioxide, certain secondary metabolites, alcohols and aldehydes constitute volatiles.[18,19] Different plant species contain many common constituents of each of these categories but may vary in their amounts and time of release by the root. Several factors such as temperature, light, age and soil type can affect the nature and timing of exudation.[19-21] In plants grown under low phosphate availability or in the presence of toxic concentration of aluminum, the exudation of organic acids such as oxalic acid, malic acid and citric acid is particularly increased.[22-24] These compounds may act as signals for microbial attraction such as malate (see below), or be used as carbon sources for microbial nutrition.

The physical, biochemical and ecologic characteristics of the rhizosphere are defined by the balance between different compounds released, timing of release and any unique substances that are produced constitutively or in an inducible manner. It is estimated that between 20 to 40% of all photosynthetically fixed carbon is eventually transferred to the rhizosphere. This high cost may be borne by the plant owing to the significant influence the rhizosphere exerts on plant health by affecting proceses such as nutrient and water uptake and establishment of beneficial interactions with soil microbial populations.[11,19]

The types of microorganisms within a rhizosphere include bacteria, fungi, actinomycetes and algae. Microbial populations react to the exudates released by plant roots, their numbers can vary by as much as 10–100-fold in the rhizosphere from those found in the soil.[25] Microorganisms and their products also affect the roots in a variety of positive, negative and neutral ways.[26,27] The rhizosphere is therefore a dynamic system in which interactions and communication between the root and microorganisms play an important role in continuing to maintain plant growth and productivity. Managing the rhizosphere may represent an important area for biotechnology improvement with the aim of boosting the intrinsic yield and biomass production with a minimum input of water, fertilizers and agrochemicals. This can be achieved by inoculating rhizospheres with selected beneficial microorganisms or by engineering plants to modify the nature and level of exudate compounds (Fig. 1).

The role of root exudates as signaling molecules has been recently addressed by Rudrappa and associates, who showed that root-secreted malic acid recruits the beneficial soil bacteria *Bacillus subtilis* to the root and this interaction plays a role in plant protection against the foliar pathogen *Pseudomonas syringae*.[28] Similarly, in tobacco and alfalfa plants genetically engineered to overproduce citric or malic acid, an increased colonization by mycorrhizal fungi and rhizobacteria have been reported, which highlights the role played by organic acids in plant-microbe interactions.[29,30] These results also suggest that manipulation of organic acid biosynthesis and exudation in transgenic plants may represent an attractive technology to modify the rhizosphere with potential novel applications in agriculture.

Elicitors are molecules involved in plant defense responses, many of them are directly derived from beneficial or pathogenic microbes.[31] Roots of elicited plants exude an array of compounds not detected in the exudates of non-elicited plants. Exogenous application of defense signalling molecules, such as salicylic acid, methyl jasmonate and nitric oxide induces the accumulation of a wide range of secondary metabolites including indole glucosinolates, phytoalexins and alkamides, which may play a role in communication with microbial populations.[32-34] The demonstration that roots selectively secrete organic compounds to effectively signal bacteria and fungi establishes a regulatory role of plant metabolites in recruitment of beneficial microbes. In addition, the effects of elicitors and other microbe-derived molecules on root exudation underscore the breadth and sophistication of plant microbe interactions.

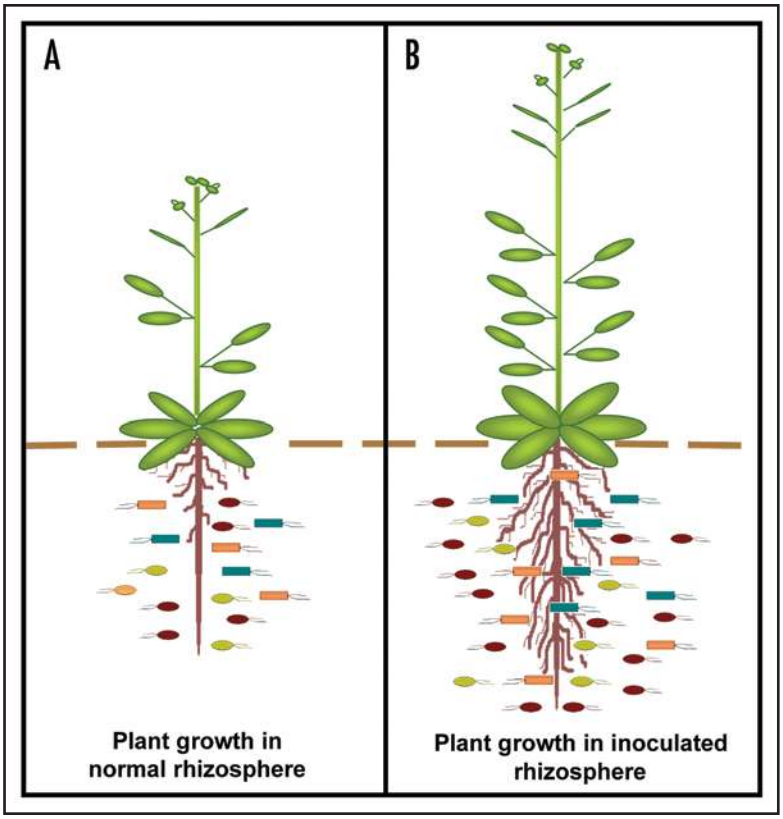

Figure 1. Rhizosphere modification improves plant productivity. Inoculation with plant beneficial microorganisms offers many advantages to crops, including enhanced rooting, activation of immunity and improved plant yield.

Microbes: Contribution to Plants

The release of carbon compounds from plants into the rhizosphere increases microbial biomass and activity. Free-living microbes including filamentous fungi of the genus Trichoderma and a variety of plant growth-promoting rhizobacteria (PGPR) are able to suppress soil-borne plant pathogens and to stimulate plant growth by different direct or indirect mechanisms, such as production of phytohormones, mycoparasitism and competence with plant pathogens, decomposition and mineralization of organic mater and enhancing the bioavailability of mineral nutrients such as phosphorus and iron.[35] Furthermore, microorganisms may also contribute to plant immunity by producing elicitor molecules. In the course of their life, plants are exposed to many potential pathogens. To counter these attacks, they have evolved a large set of defense responses. These defenses include pre-existing physical and chemical barriers, as well as inducible responses that are activated after pathogen perception.[36] This recognition step can be achieved by the means of molecules common to many classes of microbes known as microbe-associated molecular patterns (MAMPs).[31] MAMPs, also known as general elicitors in plants are involved in non-specific immunity and the associated resistance is effective against a broad range of pathogens.[37] MAMPs belong to different families including proteins, glycans and lipids. Certain beneficial bacteria produce rhamnolipids, which are novel MAMPs conferring resistance to pathogenic fungi in grapevine.[38]

Although not directly economically important, the model plant *Arabidopsis thaliana* offers a number of experimental advantages over crop species, including its small size, short life cycle, and the suitability to be grown under axenic conditions. The adoption of this model in plant-microbe interactions research has increased our knowledge about the molecular and physiological mechanisms by which microbial inoculation modulates growth and development (Fig. 2).

Next, we discuss more in depth the molecular mechanisms involved in plant growth promotion by two classes of beneficial microorganisms, namely Trichoderma fungi and plant growth promoting rhizobacteria as a means to show the importance of free-living microbes that proliferate in close proximity to plant roots.

Free-Living Beneficial Fungi

Fungi have the advantage over bacterial inoculants in that they are generally more effective at spreading through the soil and rhizosphere. The mechanisms involved in plant growth promotion by fungi are competition with fungal pathogens, antibiotic production and elicitation of defense responses.[39] In addition, many plant beneficial fungi are able to parasitize spores, sclerotia or hyphae of pathogenic fungi, resulting in biocontrol. Mycoparasitism is initiated by host sensing, which is generally followed by direct growth towards it, recognition, penetration and degradation. Production of a number of degradative enzymes, including chitinases, proteases and glucanases is involved in the biocontrol process.[39]

Trichoderma species belong to a class of free-living fungi beneficial to plants that are common in the rhizosphere. In addition to their mycoparasitic capabilities, many Trichoderma strains are able to colonize and grow in association with plant roots and significantly increase plant growth and development. Colonization by Trichoderma very rarely is detrimental to the plant or results in a pathogenic interaction.[39] In contrast, root colonization by Trichoderma frequently is associated with induction of both local and systemic resistance, which depend on the production of a protein elicitor by the fungus designated *Sm1* (*small protein 1*). *Sm1* lacks toxic activity to plants and microbes. Instead, native, purified *Sm1*, triggers production of reactive oxygen species in rice and cotton seedlings, and induces the expression of defense-related genes both locally and systemically.[40] The beneficial effects of Trichoderma on plant growth and development may also depend on more direct mechanisms as a recent report has shown that certain species including *T. virens* and *T. atroviride* can produce indole-3-acetic acid (IAA) and other auxin-related compounds. In Arabidopsis, normal auxin perception is a prerequisite for growth enhancement when inoculated with *T. virens*.[41]

Plant Growth-Promoting Rhizobacteria (PGPR)

PGPR are natural rhizosphere-inhabiting bacteria, which belong to diverse genera such as Pseudomonas and Bacillus species. These microorganisms have been isolated from a wide variety of wild and

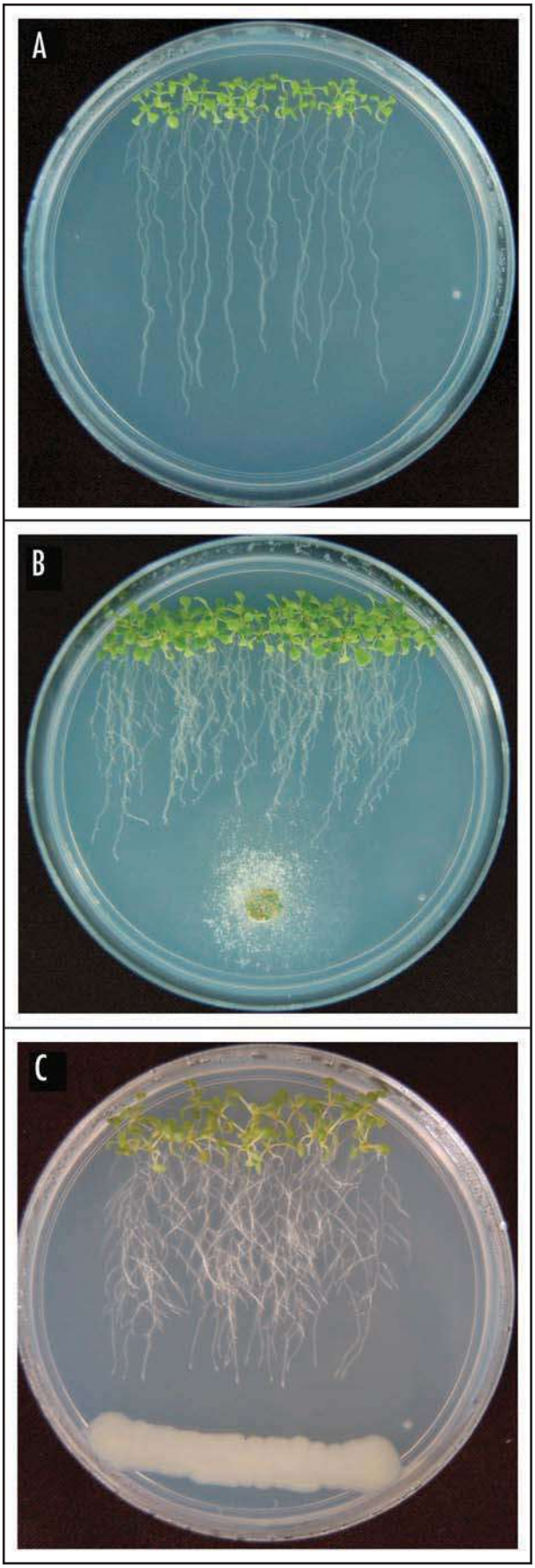

Figure 2. Use of *Arabidopsis thaliana* for research in plant-microbe interactions. Representative photographs showing uninoculated Arabidopsis seedlings grown in a 0.2x Murashige and Skoog medium (A), inoculated with *Trichoderma atroviride* (B), or with *Bacillus megaterium* (C). Note the elicitation of shoot growth by both the fungus and bacterium and the formation of branched root systems.

cultivated plant species such as Arabidopsis, barley, rice, canola and bean.[42] PGPR are used as inoculants for biofertilization, phytoestimulation and biocontrol. The general effect of PGPR is an increased growth and productivity of plants. Their contribution can be exerted through different mechanisms including root system architecture modulation and increased shoot growth by production of phytohormones such as auxins and cytokinins. Other indirect mechanisms include the effects of products such as antibiotics and hydrogen cyanide, which promote plant growth by inhibiting the growth of deleterious microorganisms in the rhizosphere. PGPR can induce defense programs such as systemic acquired resistance and induced systemic resistance, thus reducing phytotoxic microbial communities. They also can elicit induced systemic tolerance (IST) to abiotic stress.[43-45]

Pseudomonas comprises a genus of ubiquitous Gram-negative bacteria that can live in several environmental niches such as the rhizosphere. Although a few Pseudomonas are studied for their roles as plant pathogens (i.e., *Pseudomonas syringae*), there are many species such as *P. fluorescens*, *P. putida*, *P. aeaureofasciens* and *P. chloraphis*, which may act as plant beneficial bacteria by antagonizing plant pathogens and through the production of traits that directly influence plant disease resistance and growth.[46] *Pseudomonas fluorescens* strain FII3 was isolated from the root hairs of sugar beet and found to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG). The growth of plant pathogenic fungi *Pythium ultimum*, *Phoma beta*, *Rhizopus stolonifera* and *Fusarium oxisporum* were found to be inhibited by strain F113 in vitro due to the production of DAPG.[47] This strain was also found to protect sugar beet seedlings from damping-off in soil infested by Pythium.[48] Pseudomonas strains, therefore, have considerable potential as biocontrol agents.

Recent information indicates that different Pseudomonas may also regulate plant development by production of autoinducer signals, namely *N*-acyl-L-homoserine lactones.[49] Free-living bacteria belonging to the Azospirillum and Bacillus genuses can also promote growth of plants by production of auxins or cytokinins.

Microbial Signals Involved in Plant Growth and Development

A wide range of microorganisms found in the rhizhosphere are able to produce substances that regulate plant growth and development. Bacterial and fungal production of phytohormones such as auxins and cytokinins can affect cell proliferation in the shoot leading to tumorous growth as in the case of *Agrobacterium tumefaciens* or *Ustilago maydis* infection, or modify root system architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake. Therefore, the balance between auxin-to-cytokinin and the site of hormone

accumulation in the plant may determine whether a microbial interaction may be beneficial or detrimental. In the last five years, additional signals from microbes have been found to play a role in plant morphogenetic processes, including the above mentioned *N*-acyl-L-homoserine lactones (AHLs) and volatile organic compounds (VOCs). AHLs belong to a class of bacterial quorum-sensing signals from Gram negative bacteria such as Pseudomonas. These compounds enable bacterial cells to regulate gene expression depending on population density. Very recently, it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots and modulate defense and cell growth responses. In a similar way, bacterial volatiles such as acetoin and 2,3-butanediol produced by certain PGPR can be used for plant-bacteria communication, and as a plant growth promotion triggers.

Auxins and Cytokinins

Auxins and cytokinins interact in the control of many important developmental processes in plants, particularly in apical dominance, and root and shoot development. The balance between auxin and cytokinin is a key regulator of in vitro organogenesis. Exposing callus cultures to a high auxin-to-cytokinin ratio results in root formation, whereas a low ratio of these hormones promotes shoot development. Many experiments have demonstrated the existence of synergistic, antagonistic or additive interactions between auxins and cytokinins, suggesting complex signal interactions involved in the modulation of root and shoot architecture.[50,51]

Although both auxins and cytokinins can be produced in roots and shoots, the production of these signals does not occur randomly but is regulated by the location of the producing tissues, the developmental stage of the plant and environmental growth conditions such as light and temperature. Young shoot organs such as the first true leaves and developing primary and lateral roots are important sites of IAA production.[52-54] This can be visualized in transgenic Arabidopsis seedlings expressing the auxin-response marker *DR5:GUS*,[55] while the root cap is the major site of cytokinin synthesis.[56] From the tissues involved in hormone production, the signals move through specific transport systems and different mechanisms to regulate plant growth and development, IAA can be transported from the shoot to the root through the vascular tissue. In addition to long distance auxin transport, local transport of IAA along and across tissues is important for auxin localization in small groups of cells, for example in an emerging lateral root primordium or in the root cap during gravitropism. This can be achieved by the action of specific influx and efflux transporter systems. Auxin importers include members of the amino acid permease family *AUX1* (*auxin resistant 1*), *LAX* (*like-aux1*) and *PGP4*, this later belong to the *MDR/PGP* (*multidrug resistanse/P-glycoprotein*).[57-59] Auxin is exported by transporters of the *PIN* (*pin-formed*) and *PGP* families, including *PIN1* to *PIN7*, *PGP1* and *PGP19* in Arabidopsis.[60,61] Conversely, the root cap produces cytokinins, which appear to regulate primary root growth and gravitropism.[56] Free bioactive cytokinins can be visualized by the expression of *ARR5:GUS*, which consists of the cytokinin-activated promoter of the response regulator *ARR5* fused to the β-glucoronidase reporter gene.[62] Transgenic Arabidopsis seedlings expressing this construct react to cytokinins in a concentration dependent manner.[62] The use of *ARR5:GUS* expressing seedlings shows that the cap of the primary root produces elevated concentrations of free bioactive cytokinins. Root synthesized cytokinins in Arabidopsis are distributed to the shoot by the transpiration stream.[63]

Auxins

The architecture of the root system is modified by the endogenous auxin level and by environmental stimuli such as the availability of water and mineral nutrients.[64-66] Therefore, the beneficial activities of rhizosphere microorganisms can be related to the production of auxins or to the solubilization of nutrients as well, which may affect the initiation of lateral roots, the growth of lateral roots or both developmental processes, giving rise to root systems with increased exploratory capacity.

Diverse bacterial species produce auxins as part of their metabolism including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or their precursors.[67,68] Evidences indicating that IAA is a positive regulator of plant growth comes from the analysis of mutants that overproduce it, such as *super root* and *yucca*, which have long hypocotyls and increased numbers of lateral roots and root hairs,[69,70] and the positive effect of IAA application on growth of excised stems and hypocotyls and of auxin analogs in intact Arabidopsis seedlings.[71] Plant growth-promoting activity of certain microorganisms has been related to the production of auxins. Loper and Schroth (1986) found that 12 of 14 PGPR isolates produced IAA in culture filtrates and there was a significant relationship between IAA production, decreased root elongation, and increased shoot-to-root ratios in sugar beet seedlings.[72] A positive correlation between auxin production and growth-promoting activity of diverse PGPR has been also reported in *Brassica juncea* and wheat.[73,74] Auxins are quantitatively the most abundant phytohormones secreted by Azospirillum species, and it is generally agreed that auxin production is the major factor responsible for the stimulation of root system development and growth promotion by this bacterium.[68]

Many fungal species also produce auxins. Recent findings about the role of fungal-produced IAA in different plant-fungus interacting systems open the possibility that fungi may use IAA and related compounds to interact with plants as part of its colonization strategy, leading to plant growth stimulation and modification of basal plant defense mechanisms.[41,75] In maize (*Zea mays*) and *Arabidopsis thaliana*, Trichoderma inoculation affected root system architecture, which was related to increased yield of plants. Reported developmental effects include increased lateral root formation and root hair growth.[41,76,77]

The signaling mechanisms by which *Trichoderma virens* promote growth and development were further investigated in *Arabidopsis thaliana* by Contreras-Cornejo and associates (2009). It was found that mutations in genes involved in auxin transport or signaling, *AUX1*, *BIG*, *EIR1* and *AXR1*, reduced the growth-promoting and root developmental effects of Trichoderma inoculation.[41] When grown under axenic conditions *T. virens* produced IAA and the IAA-related substances indole-3-acetaldehyde (IAAld)

and indole-3-ethanol (IEt). Interestingly, application of all three compounds to Arabidopsis seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (mM range). These results indicate that the effects of inoculation with auxin-producing fungi in plants under natural conditions may depend on the type and concentration of auxins produced by the fungi.

Cytokinins

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. Plants continuously use cytokinins to maintain the pools of totipotent stem cells in their shoot and root meristems.[78,79] Endogenous cytokinin overproduction in transgenic plants causes pleiotropic phenotypic alterations including cytokinin-auxotrophic growth of calli in vitro.[78] Analysis of cytokinin-overproducing and cytokinin-deficient mutants has confirmed a stimulatory role for these compounds in the regulation of cell division activity in the shoot meristem and young leaves.[80-82] Recent data indicate regulatory interactions between cytokinins and alkamides, these later compounds belong to a novel class of plant signals reported to affect both shoot and root system architecture in plants.[83]

The positive effect of cytokinins on growth at the whole plant level has been demonstrated by the identification of genes involved in cytokinin perception and signaling. Three sensor histidine kinases, *CRE1/AHK4/WOL*, *AHK2*, *AHK3* have been shown to act as cytokinin receptors.[84] These receptors activate the expression of several response regulators in a cytokinin-dependent manner.[85,86] Further downstream, cytokinin signaling stimulates the G_1/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.[87] The cytokinin receptors play redundant functions in transducing the signal to downstream factors. When grown on soil, none of the single cytokinin receptor mutants of Arabidopsis (*cre1-12*, *ahk2-2*, *ahk3-3*) exhibited significant defective phenotype. However, 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. Interestingly, the *cre1-12 ahk2-2 ahk3-3* triple mutants showed a dwarf phenotype with reduced root and shoot growth and smaller meristems. These mutants also produced inflorescences with nonfunctional flowers, which failed to produce seeds.[83,88] These data suggest that cytokinin receptors are important for plant viability and normal growth.

Cytokinins can be produced by microorganisms. Their production by PGPR has been well documented and correlated with increased growth of plants.[89-91] Until recently, little was known on the genetic basis and signal transduction components that mediate the beneficial effects of these PGPR. A recent report has provided important information on the role played by cytokinin receptors in plant growth promotion by *Bacillus megaterium* rhizobacteria. *B. megaterium* UMCV1 strain was initially isolated from the rhizosphere of bean (*Phaseolus vulgaris* L.) plants. Inoculation with this bacterium was found to promote biomass production of *Arabidopsis thaliana* and bean plants in vitro and in soil.[44] This effect was related to altered root system architecture in inoculated plants, with an inhibition in primary root growth followed by an increase in lateral root formation and root hair length (Fig. 2). The effects of bacterial inoculation on plant growth and development were found to be independent of auxin- and ethylene-signaling as revealed by normal responses of auxin resistant mutants *aux1-7*, *axr4-1* and *eir1* and ethylene-response mutants *etr1* and *ein2*, and the failure to activate the expression of auxin-reporter markers.[44]

The involvement of cytokinin signaling in mediating plant growth promotion by *Bacillus megaterium* in plants was further investigated using *A. thaliana* mutants lacking one, two or three of the cytokinin receptors and *RPN12*, a gene involved in cytokinin signaling acting downstream of the receptors. It was found that growth promotion was reduced in *AHK2-2* single and double mutant combinations and in *RPN12*. Furthermore, growth promotion and lateral root induction was completely abolished in the *cre1-12 ahk2-2 ahk3-3* triple mutant, indicating the importance of cytokinin perception in the plant's response to *B. megaterium*.[92]

It is expected that in the coming years novel molecular components involved in the interaction between plants and PGPR will be discovered with the continued use of Arabidopsis as a model system. It is also expected that the knowledge gained on the role of auxin and cytokinin in plant-microbe interactions will open new avenues to use microbial strains with a capability to produce these phytohormones for plant improvement under controlled and field conditions.

N-acyl-L-homoserine Lactones

Many bacteria regulate diverse cellular processes in concert with their population size—a process commonly referred to as quorum-sensing (QS).[93] Bacterial cell-to-cell communication utilizes small diffusible signals, which the bacteria both produce and perceive. The bacteria couple gene expression to population density by eliciting a response only when the signaling reaches a critical threshold. The population as a whole is thus able to modify its behavior as a single unit. In Gram-negative bacteria, the quorum-sensing signals most commonly used are *N*-acyl-L-homoserine lactones (AHLs). It is now apparent that AHLs are used for regulating diverse behaviors in rhizosphere inhabiting bacteria and that plants may produce their own metabolites, which may interfere with quorum-sensing signaling.

AHLs are composed of a homoserine lactone residue linked to an acyl-side chain. The specificity derives from the length of the acyl chain (4–18 carbon atoms), substitution at the C3 position and saturation level within the acyl chain.[94-96] AHLs can be broadly classified as long, medium or short-chained depending on whether their acyl moiety consists of more than eight, between eight-to-twelve or less than twelve carbon atoms, respectively.[49,97] These molecules are freely diffused through the bacterial membrane and distribute within the rhizosphere.[98-100]

Examples of AHL-regulated gene systems are diverse and include virulence, bioluminescense, sporulation, swarming,

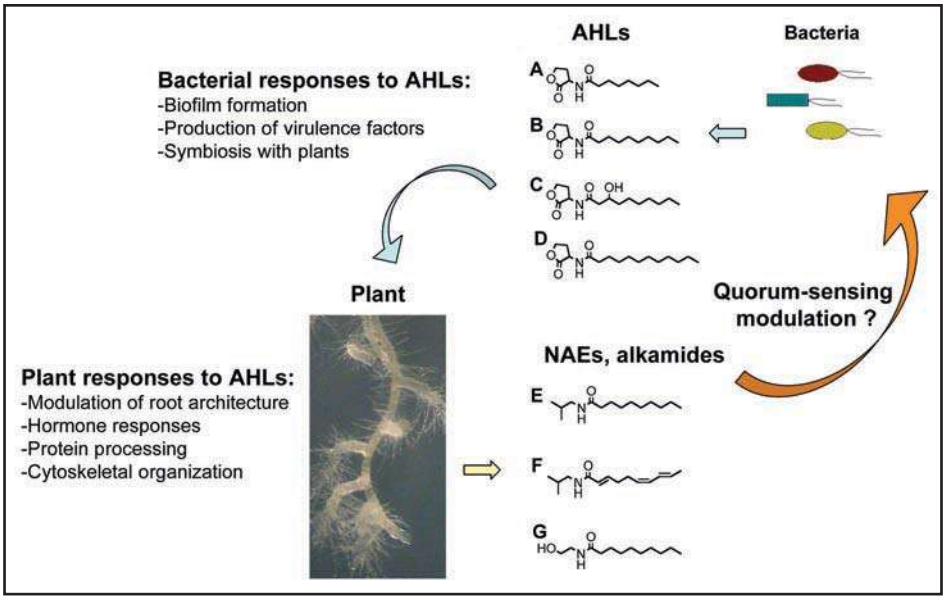

Figure 3. Communication between plants and bacteria mediated by AHLs and plant-produced signals. (A) *N*-octanoyl-homoserine lactone (C8-HL), (B) *N*-decanoyl-homoserine lactone (C10-HL), (C) *N*-3-oxo-decanoyl-homoserine lactone, (D) *N*-dodecanoyl-homoserine lactone (C12-HL), (E) *N*-isobutil decanamide, (F) *N*-isobutyl-*2E,6Z,8E*-decatrienamide (Affinin), (G) *N*-ethanol decanamide (NAE 10:0). The bacteria produce AHLs, which modulate root system architecture and the plant produces NAEs and alkamides, which may interfere with bacterial quorum-sensing.

siderophore production, antibiotic biosynthesis and plasmid conjugal transfer.[101,102] AHLs orchestrate important processes of many beneficial rhizosphere colonizing bacteria. For example: deletion of the gene *pco1* responsible for the production of the AHLs 3-oxo-C6-HL and 3-oxo-C8-HL in *Pseudomonas fluorescens* 2P24 caused the mutant to be defective in biofilm formation, colonization of wheat rhizosphere and biocontrol ability against wheat take-all, while complementation of *pco1* restored the biocontrol activity to the wild-type level.[103] While bacteria make use of AHLs for signaling, until recently it was unknown if plants functionally respond to these same signals. The first work showing that plants are able to perceive AHLs was published by Mathesius and coworkers (2003), they found that in *Medicago truncatula* plants grown axenically, application of nanomolar concentrations of two different AHL types, 3-oxo-C12-HL and 3-oxo-C16:1-HL caused significant changes in the accumulation of over 150 proteins.[104] These proteins were found to have functions in plant defense, stress response, energetic and metabolic activities, transcriptional regulation, protein processing, cytoskeletal activities and hormone responses.[104] These results were further confirmed by microarray expression analysis in *Arabidopsis thaliana*.[105]

The presence of AHL-producing bacteria in the rhizosphere of tomato induced the salicylic acid and ethylene-dependent defense responses, which play an important role in the activation of systemic resistance in plants and conferred protection against the fungal pathogen *Alternaria alternata*.[100] Furthermore, AHLs were found to be taken up by plants in a process dependent on the length of the acyl chain.[105,106] Application of a homoserine lactone, a breakdown product of AHL by means of soil bacteria, to bean roots leads to an increase in stomatal conductance and transpiration in shoots. This in turn is beneficial for both the plant and the bacteria through an increase in mineral nutrient uptake.[107]

Plants produce substances that mimic AHLs, at least 10 chromatographically separable active compounds can be detected in root exudates of *Medicago truncatula*.[108] These plant compounds can affect QS responses in bacteria indicating that plants produce AHL signal mimics.[108,109] Some of these signals may belong to the *N*-acylethanolamines (NAEs) and alkamide groups, which are structurally similar to AHLs. NAEs are naturally produced by plants and accumulate in desiccated seeds. Their synthesis can also be stimulated in leaves in response to elicitor treatment.[110] Alkamides are present in at least 20 plant families and preferentially accumulate in certain medicinal plants such as *Echinacea angustifolia* and *Heliopsis longipes*.[111,112] Exogenous application of NAEs and alkamides to Arabidopsis seedlings were found to alter root system architecture and shoot development in a dose-dependent way, indicating that these signals may play a role in plant morphogenetic processes.[83,113-115] Thus, the possibility is open the NAEs and alkamides can be used by plants to interfere with bacterial QS (Fig. 3).

In two recent reports, the root developmental responses of Arabidopsis seedlings to exogenous application of AHLs were presented.[49,105] It was found that low micromolar concentrations of C4-HL and C6-HL increased growth of roots, while C10-HL decreased growth of roots and rosettes.[105] In this way, Ortíz-Castro and coworkers (2008) performed a detailed analysis of root architectural responses to a variety of AHLs ranging form 4 to 14 carbons. The compounds affected primary root growth, lateral root formation and root hair development in Arabidopsis seedlings. From the different AHLs evaluated, C10-HL was found to be the most active compound in altering root architecture. Developmental changes altered by C10-HL were related to changes in cell division, cell elongation and cell differentiation and its mechanism of action was found to be independent of auxin signaling.[49] Interestingly, mutant and overexpresor lines of Arabidopsis with altered levels of the enzyme fatty acid amide hydrolase, which play a role in NAE metabolism in plants,[116] sustained differential growth and developmental responses to C10-HL, indicating that this enzyme may play a role in AHL degradation under natural conditions.

Several plant species have been genetically transformed to express enzymes involved in AHL synthesis or degradation. Tobacco and potato plants expressing the *yenI* gene coding for the enzyme AHL synthase from *Yersinia enterocolitica*, responsible for short-chain AHL production as well as tobacco and tomato plants engineered

to produce the AHL synthase *LasI* from *Pseudomonas aeruginosa*, responsible for the synthesis of medium-chain AHLs have been obtained.[97,117-119] In these plants, the AHLs produced diffused freely across the plastid and plasma membranes and were released to the rhizosphere,[97] where they have the potential to affect bacterial processes regulated by such molecules.

The information above discussed suggests that organisms from different kingdoms, namely plants and bacteria, have acquired mechanisms to sense and respond to each other's signaling molecules. We further propose that in the rhizosphere, AHLs, NAEs and alkamides are excellent candidates for mediating this interkingdom communication. By increasing our understanding on this communication, it could then be possible to develop novel strategies to increase sustained plant production.

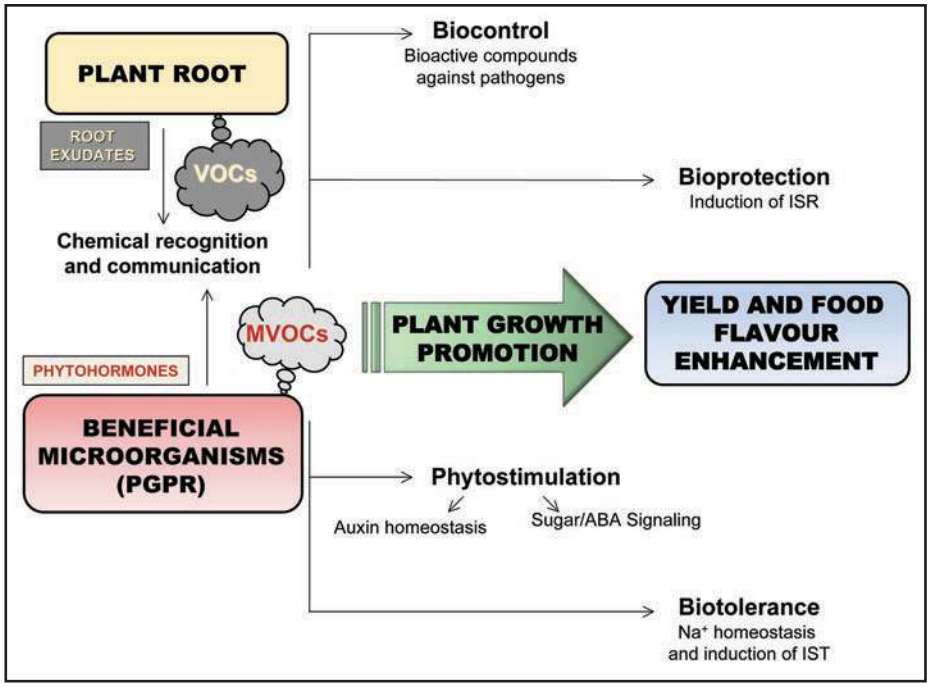

Figure 4. Mechanisms involved in volatile organic compound modulation of plant growth. Microorganisms produce VOCs, which can be sensed by plants to alter morphogenesis or activate defense and stress-related responses.

Volatile Organic Compounds

Volatile organic compounds (VOCs) are defined as compounds that have high enough vapor pressures under normal conditions to significantly vaporize and enter the atmosphere. This class of chemicals includes compounds of low molecular weight (<300 g/mol^{-1}), such as alcohols, aldehydes, ketones and hydrocarbons.[120,121]

VOCs are efficient mediators for communication acting universally as attractants, repellents or warning signals in organisms from all kingdoms. Mammals can detect at least 10,000 different VOCs as odors from different sources, which act as matting signals, alert signals and sensory molecules. How the olfactory system can distinguish so many VOCs was a longstanding mystery until 1991, when Richard Axel and Linda Buck identified a gene family that encodes about 1,000 different types of olfactory receptors in the mouse, and a smaller number, about 350 in humans.[122] These findings provided important information on the mechanism of VOC perception, for which Axel and Buck were awarded the Nobel Prize in Physiology or Medicine in 2004.

It is increasingly appreciated that sensory experiences based on VOCs represent a beautifully orchestrated response to a wide range of stimuli not only in animals, but also in plants and microbes. The diversity, distribution and function of these compounds are starting to be revealed.[121] Choudhary and coworkers (2008) have shown that VOCs are actively produced and used as a sophisticated "language" by plants to pursue communication with other organisms.[123] In fact, due to the volatility properties of this kind of compounds it is tempting to speculate that roots emit volatiles to be sensed quickly and effectively by other organisms such as microbes in order to establish a communication. On this way, Steeghs and coworkers (2004) explored the Arabidopsis rhizosphere for VOC emission and their induction by biotic stresses.[124] They found alcohols, aldehydes, acids, ketones, esters and terpenes, which can be produced constitutively or induced specifically as a result of different positive or negative interactions with microorganisms.

Recent studies have been conducted in order to clarify the role of microbial VOCs during positive plant-microbe interactions. The ecological functions of bacterial volatiles are not understood in detail, but several functions such as communication, defense and plant growth-promotion have been suggested.[125] Available information indicates that the diversity of bacterial volatiles has a comparable complexity to that known for plants. Recent investigations using gas chromatography (GC) and mass spectrometry (MS) illustrate the splendid capacity of bacteria to produce a wealth of VOCs and the appearance of a characteristic volatile profile or compound is attributable to the specific metabolism or metabolic pathway(s) that are active in the bacteria, which can vary depending on the growth media and growth conditions; more information on this topic has been reported by Schulz and Dickschat, (2007), who made an excellent work in summarizing most known bacterial compounds reported to be released by bacteria, their occurrence and biosynthesis.[18]

In most of the mechanisms that PGPR use to interact with plants, VOC emission has a crucial participation (Fig. 4). The role of VOCs on antibiosis and the biocontrol of plant pathogens is the mechanism that has received most attention in the last decade, as the finding that certain volatiles having antifungal properties determine to a large extent the biocontrol performance of many rhizobacteria.[120,126-128] There are numerous reports showing that volatiles produced by bacteria such as ammonia, butyrolactonas, HCN, phenazine-1-carboxylic acid, alcohols, among others, may

have activity in vivo in different fungal species.[126-128] The effects of these volatiles on fungi range from mycelium growth inhibition and promotion to the stimulation or reduction of sporulation. Therefore, volatiles can be used for communication between bacteria and their eukaryotic neighbours. Kai et al. (2009) discussed PGPR species that produce bioactive volatiles with activity in fungi.[125] It was shown that the volatiles from any one bacterial strain do not cause the same effect or the same degree of response in all fungi; rather the responses depend on the specific fungal-bacteria combination.[125]

The ability of PGPR VOCs to act as bioprotectants via induced systemic resistance has been demonstrated. ISR occurs when the plant's defense mechanism is stimulated and primed to resist infection by pathogens,[129] and recently it has been reported that PGPR volatiles may play a key role in this process.[129] For example, volatiles secreted by *Bacillus subtilis* GB03 and *B. amyloquefaciens* IN937a were able to activate an ISR pathway in Arabidopsis seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora*. The majority of bacteria that activate ISR appear to do so via a SA-independent pathway involving jasmonate and ethylene signals. VOCs from strain IN937a triggered ISR through an ethylene-independent signaling pathway, whereas VOCs from strain GB03 appear to operate through an ethylene-dependent, albeit independent of the salicylic acid or jasmonic acid signaling pathways. This finding provided new insight into the role of VOCs as initiators of defense responses in plants.

The fact that PGPR VOCs emitted by *B. subtilis* GB03 can trigger different hormonal signaling networks in *A. thaliana*, involving cytokinins, brassinosteroids, auxin, salicylic acid and gibberellins,[130-133] opens new expectations on the role of volatiles during plant-microorganism relationship with regard to plant development. To investigate how PGPR VOCs trigger growth promotion in *A. thaliana*, Zhang et al. (2007) examined mRNA levels of Arabidopsis seedlings exposed to volatiles of *B. subtilis* GB03 using oligonucleotide microarrays. In screening over 26,000 protein-coded transcripts, a group of approximately 600 differentially expressed genes related to cell wall modifications, primary and secondary metabolism, stress responses and auxin homeostasis were identified.[132] These data for the first time implicate VOCs as modulators of auxin homeostasis and cell expansion and suggest that VOCs can directly impact pathways involved in plant morphogenesis. More recently, the same group demonstrated that *B. subtilis* GB03 VOCs augment photosynthesis capacity by increasing photosynthetic efficiency and chlorophyll content in Arabidopsis through the modulation of endogenous sugar/ABA signaling.[133] This strain produces different kinds of volatiles such as short-chained alcohols, aldehydes, acids, esters, ketones, hydrocarbons and sulphur-containing compounds.[134] The specific roles of these VOCs in plant signaling and the mechanism of perception by plants merit further research.

The term "induced-systemic tolerance" (IST) was recently proposed for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress.[43,45,135] The role of VOCs emitted from *B. subtilis* GB03 on IST to salt stress (100 mM NaCl) in Arabidopsis was evaluated by Zhang et al. (2008b). The authors observed that VOCs concurrently downregulated *HKT1* (*High-affinity K^+ transporter1*) expression in roots, but upregulated it in shoots, resulting in lower Na^+ accumulation throughout the plant.[135]

Studies on the effect that PGPR VOCs have on secondary metabolite production in plants such as accumulation of aroma compounds for flavor enhancement in agronomic crops have begun to emerge,[136] so the addition of highly active but cheap compounds to plants for increasing plant productivity and immunity, induction of IST, and enhance food flavor, might represent a novel and promising strategy in agriculture.

Concluding Remarks

Plants and microorganisms have coexisted for million of years. Plants maintain a complex interaction with their rhizospheric populations, which is crucial for nutrient assimilation, development and activation of defense mechanisms. These mutually beneficial associations are possible because plants and microorganisms can communicate with each other through various signaling mechanisms.

In this review, we have considered four major classes of signals that participate in the interactions that occur between plants and beneficial microorganisms, auxins, cytokinins, AHLs and VOCs. It can be generally appreciated that plants are able to sense and respond to rhizosphere-inhabiting bacterial and fungal populations and their products. Aside from the discovery of different types of chemical communication for interkingdom signaling, a challenge for the future is to begin to address the possibility that there is a significant specific communication. A further challenge is to determine the role played by phytohormones and other plant-derived metabolites such as NAEs, alkamides and VOCs in the physiology of microorganisms. In addition, the mechanisms of NAE, alkamide and AHL perception in plants remains as a mystery. In *Vibrio harveyi*, an AHL called autoinducer-1 (AI-1) acts as a species-specific quorum-sensing signal, which activates a two-component signaling system.[137,139] Plants possess two-component signaling systems underlying the regulation of growth and development in response to cytokinins and ethylene.[140] In this regard, recent research has revealed an important interaction between alkamides and cytokinin signaling at the level of cytokinin receptors.[83] Whether small lipid amides act as ligands of cytokinin receptors remains to be determined, however, the possibility is open that AHLs, NAEs and alkamides could regulate plant development by modulate two-component signaling systems.

Studies of transkingdom signaling between plants and bacteria based on small lipid signals (i.e., AHLs) are just beginning to reveal its diverse roles in healthy plants and the field of plant-microbe interactions will undoubtedly provide good examples of the molecular mechanisms involved in the interaction. Exploring further the interactions by means of global gene expression analyses and proteomic strategies along with the identification of plant mutants defective on signal perception/transduction should help increase our knowledge on the mechanisms used by plants to communicate with beneficial microbial populations.

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11.2.

Chapter 23

Fungal biomolecules in plant growth promotion

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23.1 Interactions of fungi with plants

Plant growth and development involves the integration of many environmental and endogenous signals, which, together with the intrinsic genetic programme, determine plant form. Every aspect of plant morphogenesis is under hormonal control, and phytohormones such as auxins, cytokinins (CKs), gibberellins (GAs) and ethylene, which are endogenously produced, represent key elements for the regulation of cell division, elongation and differentiation (López-Bucio et al., 2003; Negi et al., 2008). Roots provide shoots with a secure supply of nutrients and water as well as anchorage and support (Contreras-Cornejo et al., 2009; Ortiz-Castro et al., 2009). Fluctuations in soil conditions such as water or nutrient availability modify phytohormone synthesis, transport and/or sensitivity, leading to changes in root system architecture (RSA) (López-Bucio et al., 2003). In many plant species, RSA alterations are associated with adaptive strategies to cope with these limitations. Symbiotic associations with soil microorganisms are widespread and currently represent promising alternatives to overcome the negative effects of environmental stress on plant productivity with additional benefits through enhancement of growth and plant immunity.

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Free-living, saprotrophic fungi access carbon supplies by decomposing organic materials or having access to decaying organic matter. By contrast, symbiotic fungi satisfy carbon needs through intimate associations with host species, which can be mutualistic when the interaction confers benefits to host plants (Harman et al., 2004a; Fitze et al., 2005; Maeda et al., 2006; Moran, 2006; Harman, 2011; Harrison, 2012). Symbiotic associations vary in the cell-to-cell mechanisms involved in the recognition process, in the evolutionary history of the relationship and in the genomic features of the microorganism and the plant (Moran, 2006).

This chapter focuses on recent advancements in biochemical and molecular mechanisms of plant–fungus interaction and discusses the remarkable diversity of the active biomolecules produced by fungal species. The role of the biomolecules in plant signalling and the available information of their role in fungal physiology are also discussed.

23.1.1 Mycorrhizal fungi

The most widespread mutualistic symbiosis is the arbuscular mycorrhizal (AM) interaction formed between fungi of the phylum *Glomeromycota* and roots of 70–90% of terrestrial plants. The AM symbiosis is

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ancient and appeared more than 400 million years ago (Parniske, 2008). Molecular phylogenies and phenotypical analyses have shown the diversity of mycorrhizal fungi (Redecker and Raab, 2006).

AM fungi (AMF) colonize root cortical cells to obtain carbon from the plant while assisting with acquisition of mineral nutrients and water from the soil (Harrison, 2012). This activity is efficiently performed by the extensive extraradical mycelium of the fungal symbionts. Fungal spores are prevalent in the soil as spores, and following germination, the hyphal germ tubes grow through the soil in search of a host root (Maillet et al., 2011). Once contact with root epidermis is established, the fungus forms an appressorium on the root surface and enters inside root tissues where it develops extensively branched hyphae named arbuscules within the inner cortical cells. As each arbuscule develops, symbiotic phosphate (Pi) transport between fungal mycelium and root cells is enhanced. Increasing evidence shows that fungus and plant roots start to recognize each other long before the appearance of the first colonization structures on the root epidermis (Requena et al., 2007). It seems that the exchange of signals between plants and fungi initiates with secretion of strigolactones from roots, which are sensed to stimulate spore germination and hyphal branching (Akiyama et al., 2005; Besserer et al., 2006, 2008). Strigolactones are a group of terpenoid lactones consisting of a tricyclic ABC lactone and a methylbutenolide coupled with an enol ether bond that interact with a putative fungal receptor as truncation of the A and AB rings in the tricyclic ABC lactone of strigolactones resulted in a drastic reduction in hyphal branching activity (Akiyama et al., 2005, 2010).

In response to strigolactones, AMF synthesize lipochitooligosaccharides (LCOs), called Myc factors (Maillet et al., 2011), which act as diffusible signals important for the mycorrhization process. An interesting effect of Myc factors is the induction of lateral roots; apparently, root branching occurs without inhibition of primary roots or modifications of root geotropism, making this type of plant response quite different from that reported for auxins (Oláh et al., 2005). Myc factors belong to the same chemical family as the Nod factors produced by rhizobia, the symbiotic bacteria that elicit the formation of nitrogen-fixing nodules on their leguminous hosts (van Rhijn et al., 1997; Catoira et al., 2000; Oláh et al., 2005; Op den Camp et al., 2011). The genetic control of Nod factor transduction is performed by four *nod* genes, *DMI1* (for doesn't make infection), *DMI2*, *DMI3* and *NSP* (for noduling signalling pathway), which are involved in the control of host specificity, infection and nodulation (Catoira et al., 2000). It is noteworthy that three of these genes, *DMI1*, *DMI2* and *DMI3*, are also required for the mycorrhization process (Catoira et al., 2000; Oláh et al., 2005), indicating that the symbiotic pathways activated by both bacterial and fungal symbionts share central components of the signalling pathway that trigger their symbiotic programmes (Oláh et al., 2005; Markmann et al., 2008; Op den Camp et al., 2011).

Another mutualistic symbiosis between filamentous fungi of the Basidiomycota, Ascomycota or Zygomycota and tree roots is ectomycorrhiza (ECM) (Horton and Bruns, 2001; Wiemken and Boller, 2002; Deveau et al., 2008). Ectomycorrhizal fungi do not penetrate their host's cell walls. Instead, they form a hyphal sheath, or mantle, covering the root and a dense hyphal network called Hartig net, surrounding the plant cells within the root cortex (Acioli-Santos et al., 2008; Deveau et al., 2008). In forest ecosystems, these fungi are spread to cover areas often several square meters, implying that mycelia can extend for considerable distances and persist for several years (Nehls, 2008). Studies conducted to investigate changes in the expression of fungal genes during the pre-infection and proliferative stages on root tissues suggest the role of auxins in early stages of ECM development (Menotta et al., 2004; Acioli-Santos et al., 2008; Flores-Monterroso et al., 2013). The presence of membrane transporters that allows the uptake and translocation of nutrients and carbohydrates from the soil through the fungus and then to the plant is a hallmark of both classes of mycorrhizal fungi (Müller et al., 2007; Deveau et al., 2008; Fajardo-López et al., 2008; Avolio et al., 2012; García et al., 2013). The fungal sheath and Hartig net have different functions – the former acts as a site for storage of nutrients and carbohydrates and the latter serves as an interface between plant and fungus where cells are adapted to the exchange of plant-derived carbohydrates and fungal-derived nutrients (Nehls, 2008).

23.1.1.1 Endophytes

The term *endophyte* (from the Greek *endo*, within, and *phyton*, plant) is used for any bacterial or fungal microorganism, which colonizes internal tissues of the host plant, without apparent symptoms of disease (Tan and

Zou, 2001). In general, two major groups of endophytic fungi have been recognized reflecting differences in evolutionary relatedness, taxonomy, plant hosts and ecological function: the clavicipitaceous endophytes (C-endophytes or class 1), which colonize shoots and rhizomes of some grasses, and the nonclavicipitaceous endophytes (NC-endophytes that comprise classes 2–4), isolated from the shoots and/or roots from asymptomatic tissues of nonvascular plants, ferns, conifers and angiosperms (Rodríguez et al., 2009). Class 4 endophytic fungi colonize only roots and a nice example is *Piriformospora indica*, which is considered as a model root endophyte that colonizes the roots of several plant species, including *Arabidopsis thaliana* (Stein et al., 2008). Similarly, some *Trichoderma* strains that colonize roots have been considered as endophytic plant symbionts. A particular case is *Trichoderma hamatum*, which was isolated from a pod of *Theobroma gileri* and promoted cacao growth (Evans et al., 2003).

Endophytes can enhance the hosts' uptake of nutrients (Li et al., 2012) or improve adaptation to environmental stresses (Lorito et al., 2010; Mastouri et al., 2010; Gal-Hemed et al., 2011). Ek-Ramos et al. (2013) reported a positive effect of endophytes on plant growth including higher rates of germination and rooting and increased tissue biomass and seed production under adverse conditions. Interestingly, biochemical changes have been reported in grasses colonized by endophytic fungi. For example, *Neotyphodium lolii* modified phenolic compound production and volatile organic compound (VOC) emission on perennial ryegrass infected by *Fusarium poae*, which improve the host plant's immunity and resistance to stress factors (Pańka et al., 2013).

Plant endophytic fungi have been recognized as an important and novel source of natural bioactive compounds with their potential applications in agriculture, medicine and food industry. Since the anticancer drug Taxol found in *Taxomyces andreanae*, many investigations have been focused in endophytes as potential producers of novel and biologically active compounds (Das et al., 2012; Aly et al., 2013).

23.1.1.2 Free-living fungi

The rhizosphere is one of preferred habitats for fungi. *Trichoderma* can colonize the epidermis and outer cortex, causing substantial changes in plant metabolism (Segarra et al., 2007; Contreras-Cornejo et al., 2011). *Trichoderma* colonization implies the ability to adhere and recognize plant roots, penetrate inside plant tissues and withstand toxic metabolites produced by the plants in response to invasion by foreign organisms (Benítez et al., 2004). Important attributes of *Trichoderma* are the antagonistic activity against plant pathogens through mycoparasitism, production of antibiotics and competition (Elad, 2000; Benítez et al., 2004; Harman et al., 2004a; Reino et al., 2008; John et al., 2010; Lorito et al., 2010; Gal-Hemed et al., 2011).

Fusarium equiseti GF19-1 is also a beneficial fungus that promotes plant growth after establishing a symbiotic relationship with the host plant. *F. equiseti* GF19-1 was isolated from the rhizosphere of zoysiagrass and is also very effective as a biocontrol agent in suppressing crown and root rot in tomatoes and inducing systemic resistance in cucumbers (Kojima et al., 2013). *Penicillium simplicissimum* collected from the rhizosphere of the zoysiagrass *Zoysia tenuifolia* is considered as a plant growth-promoting fungus (Shimizu et al., 2013). Another free-living fungus identified as *Aspergillus ustus* by ITS sequencing was isolated from potato plants. Experiments of inoculation of *Arabidopsis* seedlings evidenced that *A. ustus* promotes plant growth in a way that resembles the effect induced by auxins (Salas-Marina et al., 2011). Jogaiah et al. (2013) isolated and characterized 79 PGPF from rhizosphere soil; among them, 9 strains revealed saprophytic ability, root colonization, phosphate solubilization, indole-3-acetic acid (IAA) production and plant growth promotion. Furthermore, seed priming with four PGPF exhibited early seedling emergence and enhanced vigour of a tomato cultivar susceptible to bacterial disease compared to untreated controls.

23.2 Effects of fungal colonization in plants

The rhizosphere provides carbon sources for a vast number of microorganisms, since plants can release a large part of their photosynthetically fixed carbon as root exudates. Exudates are chemically diverse and include amino acids, amides, sugars, organic acids and phenols, as well as a wide variety of secondary metabolites, polysaccharides and proteins of higher molecular mass, which may act as messengers that communicate and initiate biological and physical interactions between roots and their associate microbes

(Wenke et al., 2010). The dominance of certain microbial species and suppression of others may be important for plant success; indeed, the microbial associations may pass through various successions (Pandey et al., 2001). In this way, the rhizosphere should be considered a dynamic place in which different microbial traits may contribute to adaptation and other environmental factors such as temperature and pH determine together with the plant genotype the microbial diversity and the ecosystem sustainability (Pandey et al., 2001; Sule and Oyeyiola, 2012).

Co-inoculation of plants with selected beneficial fungi may be a promising biotechnological approach for boosting plant growth and also for remediation of soils. Next, we highlight some successful examples in which inoculation with fungi favoured crop production and the common biochemical mechanisms that fungi used to improve the uptake of nutrients from soil.

23.2.1 Growth promotion, grain production and other beneficial traits

Fungi may affect the physiology and ecology of their host plants in multiple ways. Endophytes have been applied to a wide range of agricultural species for the purpose of growth enhancement, including increased seed emergence, plant weight, crop yield and disease control (Kloepper et al., 1980; Hyakumachi, 1994). Inoculation experiments have shown that *P. indica* has growth-promoting effects on maize (*Zea mays* L.), tobacco (*Nicotiana tabacum* L.), bacopa (*Bacopa monnieri* L.), parsley (*Petroselinum crispum* L.) and poplar (*Populus tremula* L.) (Varma et al., 1999). The effect of *P. indica* on the medicinal plant *Coleus forskohlii* was investigated under field conditions. *P. indica* promoted inflorescence development, lateral root formation (LRF) and chlorophyll accumulation (Das et al., 2012). *Penicillium funiculosum* LHL06 inoculated in soybean plants also increased biomass production in normal or abiotic stress conditions (Khan and Lee, 2013).

Trichoderma strains promoted plant growth, increased nutrient availability, improved crop production and enhanced resistance to pathogens (Elad, 2000; Yedidia et al., 2003; Harman et al., 2004a; Shoresh et al., 2005; Korolev et al., 2008; Vinale et al., 2008). Tomato and *Arabidopsis* roots inoculated with *Trichoderma virens* resulted in greater root colonization, and this process was correlated with an increase in fresh weight (Contreras-Cornejo et al., 2011; Velázquez-Robledo et al., 2011). The colonization of cacao seedlings by *T. hamatum* isolate DIS 219b enhanced seedling growth and improved adaptation of cacao to drought stress in leaves at the molecular, physiological and phenotypic levels, an effect with potential practical applications for management of this tropical crop (Bae et al., 2009). *Trichoderma harzianum* increased the shoot fresh weight in melon plants (*Cucumis melo* cv. Giotto) by 20% when compared with uninoculated plants (Martínez-Medina et al., 2011). Recently, the impact of *Trichoderma viride* on soybean growth and protection against *Fusarium oxysporum* f. sp. adzuki and *Pythium arrhenomanes* was investigated. *T. viride* enhanced growth of shoot and root systems and fruit yield after 12 weeks of growth (John et al., 2010). The impact of *Trichoderma* sp. on white maize crop was positive, yielding plants more robust and greener (Harman, 2011). Similarly, in the interaction between *Trichoderma harzianum* (T-22) and maize, root and shoot growth were increased in both sterilized and non-sterilized soils and in the presence of soil fungicides indicating the wide spectrum, beneficial activities of these fungi (Harman et al., 2004a, 2004b).

23.2.2 Regulation of RSA

Fungal colonization affects root architecture through modifying root hair and LRF. Morphological changes of the root have been attributed to production of auxin (IAA) and other biomolecules. Fungal species such as *Pisolithus tinctorius*, *Trichoderma* sp., *Piriformospora indica* and *Tuber* sp. are capable of enhancing plant biomass production and promoting lateral root growth through hormonal-dependent mechanisms and/or are able to produce biomolecules active in plants or some analogues of phytohormones (Frankenberger and Poth, 1987; Contreras-Cornejo et al., 2009). Ascomycete fungi, predominantly of the genus *Tuber* (i.e. *Tuber borchii* and *Tuber melanosporum*), which form association with roots of trees induce alterations in root morphology such as primary root shortening, LRF and root hair stimulation in the host *Cistus incanus* and the non-host *A. thaliana* (Felten et al., 2009; Splivallo et al., 2009).

The effect of *T. virens* on plant growth promotion correlated with induction of lateral roots and root hairs. Analyses by gas chromatography–mass spectrometry of diffusible secondary metabolites produced

by *T. virens* revealed the presence of auxin-like compounds identified as IAA, indole-3-ethanol, indole-3-acethaldehyde and indole-3-carboxaldehyde. Interestingly, these indolic compounds applied exogenously induced root responses that mimic the effect of *T. virens* on *Arabidopsis* (Contreras-Cornejo et al., 2009, 2011).

23.3 Fungal biomolecules active in plants

The use of beneficial fungi as an integral component of agricultural practices is continuously increasing. Fungi can be found in almost all sorts of habitats interacting with crops and with other organisms. As a consequence, fungi have developed a number of strategies for protection and communication with plants (Rohlfs and Churchill, 2011). Like plants, fungi synthesize an enormous diversity of secondary metabolites, which might be involved in communication with above- and belowground plant parts (Wenke et al., 2010). The term 'secondary metabolite' includes a heterogeneous group of chemically different natural compounds with a limited molecular weight less than 3000 Da. Secondary metabolites derived from fungi comprise aliphatic and aromatic hydrocarbons, organic acids, esters, ketones, aldehydes, alcohols and mono-, sesqui- and diterpenes (Contreras-Cornejo et al., 2009; Kramer and Abraham, 2012). Depending on the chemical properties of the signal molecules involved in the interaction, these compounds can be either volatiles or non-volatiles (Contreras-Cornejo et al., 2009; Splivallo et al., 2009). These compounds in plants can mediate defence against predators, parasites and diseases and may be produced for competition between species and to facilitate reproductive processes (Stoppacher et al., 2010). In most plant–fungus associations studied, the interaction results in increased hormone synthesis or accumulation in plant tissues. So, the anatomical and morphological changes in plant roots inoculated with fungal strains may be influenced by plant growth-regulating substances secreted by fungi, which could directly affect plant metabolism or modulate phytohormone production or degradation in plant parts (Figure 23.1). Thus, fungal biomolecules are critical for plant development and for the interaction of plants with fungi (León-Morcillo et al., 2012).

23.3.1 Auxins

Auxins were discovered almost a century ago as plant hormones that coordinate many growth and behavioural processes during the plant's life cycle, including embryogenesis of roots and stems, apical dominance and tropisms such as photo- and gravitropism (Rao et al., 2010; Qi et al., 2012). IAA is the most widely distributed auxin in plants. The chemical features that are recognized in IAA are the aromatic indole ring with a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring, and the carboxylic acid group; both features fit perfectly in the auxin-binding pocket of TIR1 receptor (Figure 23.2a). Other derivatives of indole and benzene (Figure 23.2b) or naphthalene have auxin activity, suggesting that a flat, hydrophobic ring system and the carboxyl group are important for auxin perception. In addition, novel information reveals that some microbial compounds including bacterial cyclodipeptides may interact with the TIR1 auxin receptor, revealing that TIR1 has a highly promiscuous auxin-binding pocket (Ortíz-Castro et al., 2011).

Recent studies in *Saccharomyces cerevisiae* and *Candida albicans* indicate that IAA also controls physiological processes in fungi (Prusty et al., 2004), suggesting that auxin is a reciprocal signal, which is important from an ecological perspective but also from a physiological standpoint in both plants and fungi. IAA is synthesized via tryptophan (TRP)-dependent and TRP-independent pathways, although the major route for its production is the TRP-dependent pathway. Three TRP-dependent pathways have been identified, known as the indole-3-acetamide (IAM), indole-3-pyruvate (IPA) and TRP side-chain oxidase pathways (Dimkpa et al., 2012). The major IAA-biosynthesis pathway in *Colletotrichum gloeosporioides* is the IAM pathway, as indicated by accumulation of IAM in culture media and detection of enzymatic activities of the IAM pathway enzymes in fungal protein extracts (Robinson et al., 1998; Maor et al., 2004). In maize and soybean, an increase in auxin levels after inoculation of roots with AMF has been reported (Kaldorf and Ludwig-Müller, 2000; Fitze et al., 2005; Meixner et al., 2005). In maize, indole-3-butyric acid (IBA) is increased, but IAA remained unaltered (Schmitz et al., 1991; Ludwing-Müller et al., 1997; Kaldorf and Ludwig-Müller, 2000). In soybean, IAA levels were higher in roots with AMF than in

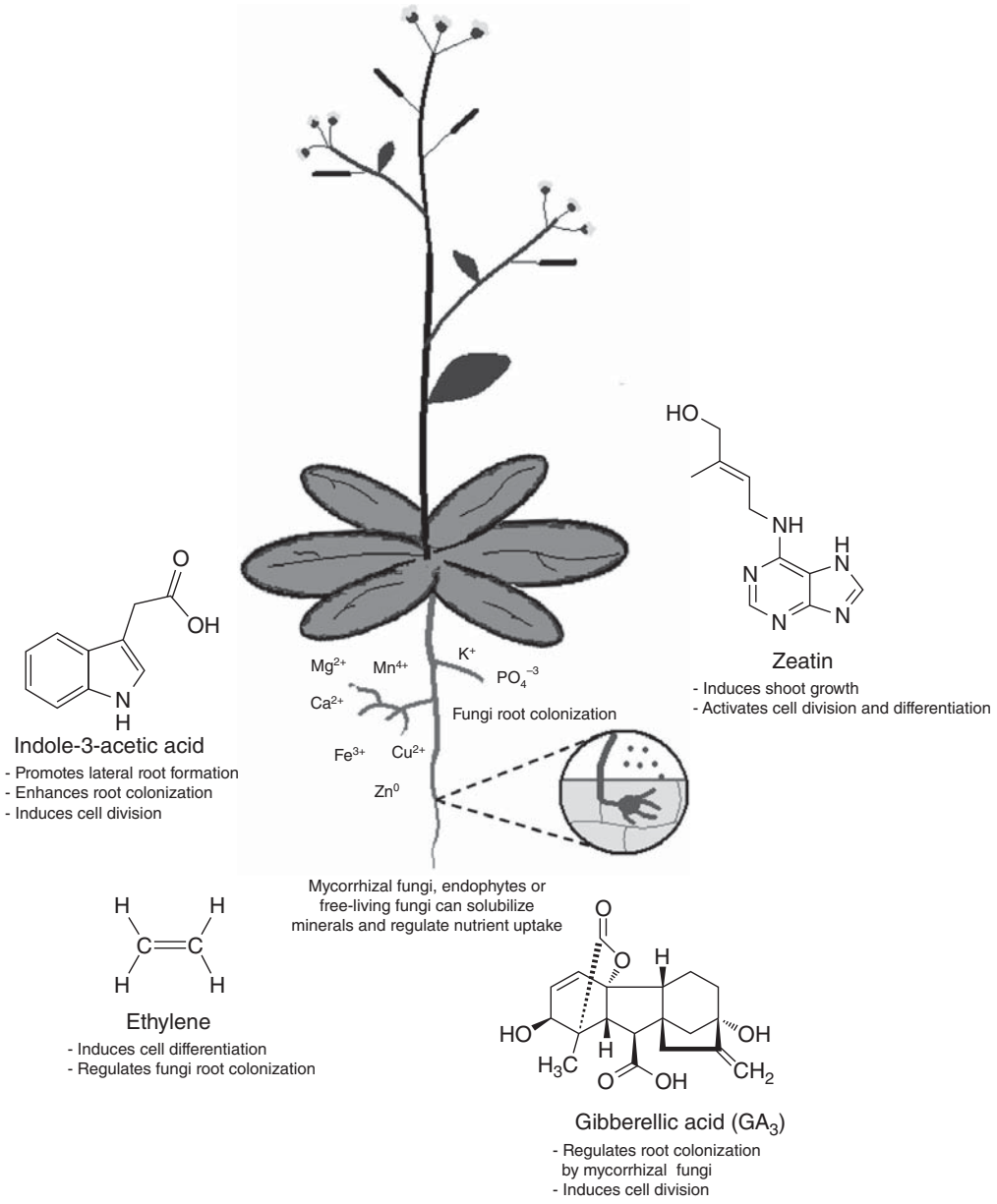

Figure 23.1 Fungal biomolecules with activity in plants. Auxin (IAA), ethylene (ET), cytokinins (CKs) and gibberellins (GAs) can be produced by mycorrhizal fungi, endophytes or free-living fungi. These compounds have key roles in regulating several aspects of plant growth and development and in establishing an efficient association with their host plants that impact in plant health and productivity

controls (Meixner et al., 2005). However, no changes in IAA levels were observed in leek or tomato (Torelli et al., 2000; Shaul-Keinan et al., 2002). IBA may facilitate the colonization of the plant host by increasing the number of lateral roots, which are preferential colonization sites for the fungi during early growth phases (Kaldorf and Ludwing-Müller, 2000).

Combined application of IAA and ET induced root responses equivalent to the presence of truffle mycelium in both the host *Cistus incanus* and the non-host plant *Arabidopsis* (Splivallo et al., 2009). On the other hand, it has been reported that *Pisolithus tinctorius* is capable of producing IAA, which can promote Douglas fir (*Pseudotsuga menziesii*) growth when provided

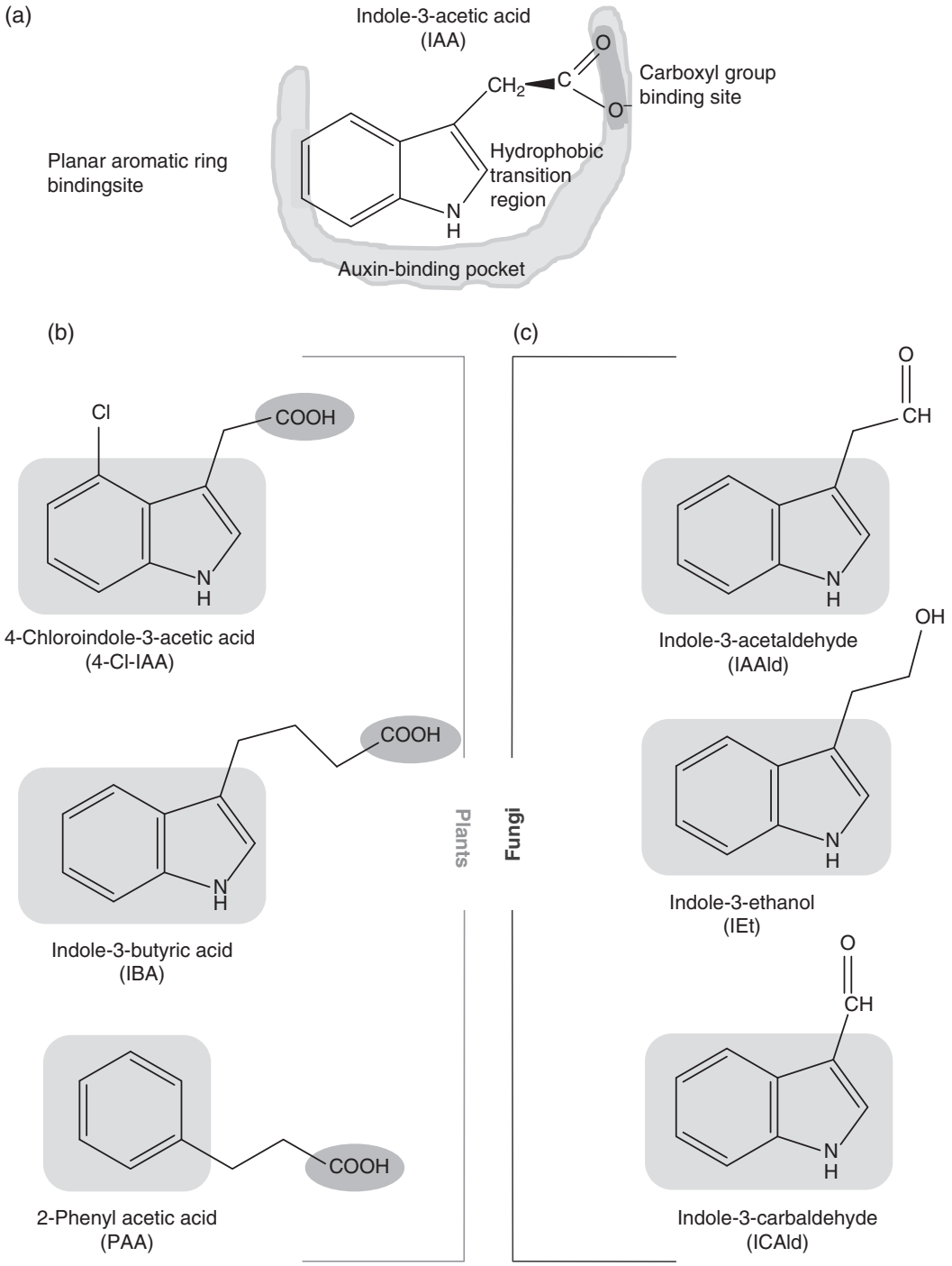

Figure 23.2 Auxin-like compounds with activity in plants and fungi. (a) Chemical structure of indole-3-acetic acid highlighting the features that are recognized as critical for auxin activity. (b) Naturally occurring auxins in plants. (c) Indole derivatives found in *Trichoderma* fungi with different capacity in plant growth stimulation

with TRP as the auxin precursor in the nanogram-to-microgram range per kilogram of soil (Frankenberger and Poth, 1987). Similarly, *Phoma glomerata* and *Penicillium* sp. promoted rice and cucumber growth, and this effect was correlated also with the production of IAA (Waqas et al., 2012). Felten et al. (2009) reported that the ectomycorrhizal fungus *Laccaria bicolor* stimulates LRF in poplar (*Populus tremula* × *Populus alba*).

LRF was correlated with increased auxin levels in root apices. Blocking auxin transport in roots with 1-naphthylphthalamic acid inhibited lateral root development and auxin accumulation. An oligoarray-based transcript profile of poplar roots exposed to molecules released by *L. bicolor* revealed the differential expression of 2945 genes, including several components of polar auxin transport (*PtaPIN* and *PtaAUX* genes), auxin conjugation (*PtaGH3* genes) and auxin signalling (*PtaIAA* genes). In *Pinus pinaster*, *Pp-GH3.16* encodes a polypeptide sharing extensive homologies with GH3 proteins of different plants. In *Arabidopsis*, the *GH3* gene encodes an auxin-conjugating enzyme that plays a role in stress responses by modulating endogenous levels of active auxin through a negative feedback regulation (Park et al., 2007). In the *P. pinaster* and ectomycorrhizal associations, the gene *Pp-GH3.16* was upregulated by auxins but downregulated following inoculation with both fungal species (Reddy et al., 2006).

During the interaction of *Glomus intraradices* with tomato (*Solanum lycopersicum*) roots, mycorrhization of the auxin-resistant mutant diageotropica (*dgt*) and a mutant with hyperactive polar auxin transport named polycotyledon (*pct*) showed contrasting responses. While *G. intraradices* stimulated presymbiotic root branching in *pct*, no effects were observed in *dgt* roots (Hanlon and Coenen, 2011). These results provided genetic evidence that auxin signalling within host roots is required for the early stages of mycorrhization.

Piriformospora indica improves the growth of many plant species including *Arabidopsis thaliana* through auxin production (Sirrenberg et al., 2007). Hilbert et al. (2012) showed that the TRP aminotransferase gene (*Tam1*) from *P. indica* (*piTam1*) is induced during the biotrophic phase. Interestingly, *P. indica* strains in which the *piTam1* gene was silenced via RNA interference were compromised in IAA production and displayed reduced colonization of barley (*Hordeum vulgare*) roots in the biotrophic phase. Thus, production of IAA might be important at certain stages of host colonization in some species but not in others, depending on host plant, the colonization and lifestyle of the fungus. Studies of fluoroindole-resistant mutants of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* that overproduce TRP and IAA support the idea that fungal IAA controls major anatomical features of ectomycorrhizae including enhanced LRF and fungal branching, but IAA overproduction also induced an abnormal proliferation of the intercellular network of hyphae and intracellular penetration of hyphae (Gay et al., 1994; Gea et al., 1994; Ditengou and Lapeyrie, 2000).

23.3.2 CKs

CKs are compounds that were characterized based on their ability to promote cytokinesis or cell division in plants. The major site of CK biosynthesis is the root, specially the mitotically active root tip (Kakimoto, 2003; Hirose et al., 2008). Plant CKs are a group of N^6-substituted adenine derivatives or aminopurines (Figure 23.3), and like other phytohormones, CKs may pleiotropically affect vascular development, sink/source relationships, apical dominance, stress tolerance and leaf senescence (Werner et al., 2001; Kakimoto, 2003; Huynh et al., 2005; Hirose et al., 2008).

CK metabolism can be affected by pathogens or symbionts to withdraw plant resources for their own benefit. An example of this is the implication of CKs in the formation of green and metabolically active areas in otherwise yellow senescent leaves known as 'green islands' after pathogen or insect attack. In these sites, nutrients are redirected towards the infection place and host cell death is delayed (Giron et al., 2013). A large array of natural and synthetic compounds fitting into definition of CKs have been identified including adenine and phenylurea derivatives. All naturally occurring CKs are derived from adenine substituted by a side chain at N^6-position – examples are shown in Figure 23.3a–c.

The physiological effects of a variety of CKs have been well documented in higher plants, but information on their occurrence and function in other biological systems is limited. CKs also regulate different biological aspects in other organisms, like biomass productivity in soil fungi (Ghazala and Memoona, 2009) or sporulation in amoeba (Anjard and Loomis, 2008). Many bacteria and fungi that are intimately associated with higher plants produce CKs and/or cause the plant cells to synthesize them, inducing tissue division forming special structures, such as nodules (for nodulating rhizobia) or mycorrhizas (for AMF), in which the microorganism can reside in a mutualistic relationship with the plant, where CKs appear to act as multifunctional regulators (Giron et al., 2013). For example, the fungi *Piriformospora indica* promoted *Arabidopsis* growth, and the molecular mechanism activated by this fungus involved the CK signalling. Substantial

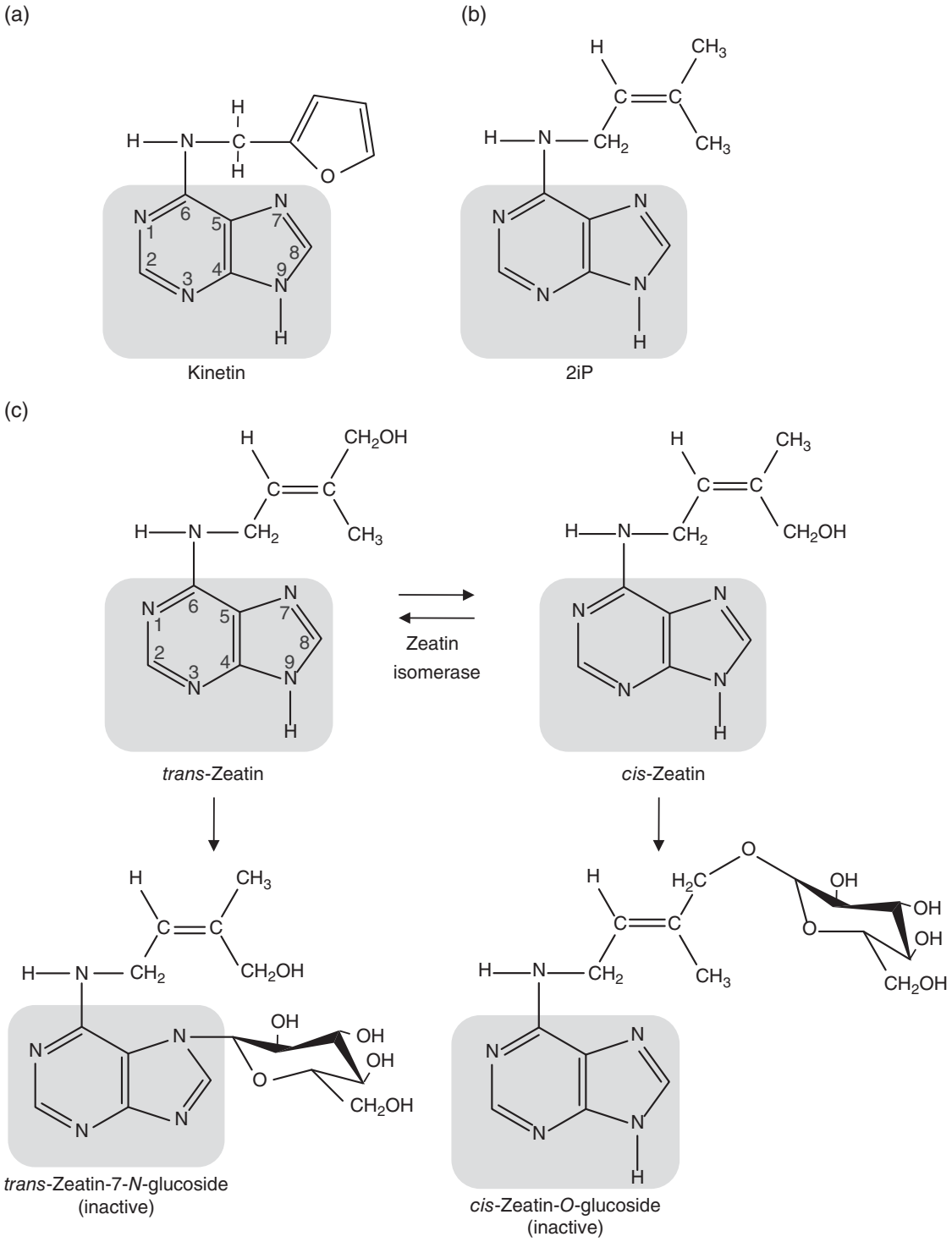

Figure 23.3 Chemical structure of cytokinins. (a) Kinetin is the first and best-known cytokinin; it is a by-product of the heat-induced degradation of DNA, in which deoxyribose sugar of adenosine is converted to a furfuryl ring and shifted from the 9-position to the 6-position on the adenine ring (gray square). (b) Isopentenyl adenine (2iP), a substituted aminopurine isolated from plants and microorganisms that is active as cytokinin. (c) Zeatin is the most abundant natural cytokinin; it is also an aminopurine, but the side chain of zeatin has a double bond, it can exist in either the *trans* or *cis* configuration, and both can be interconverted by the enzyme zeatin isomerase. These cytokinins are inactive forms if they are conjugated with sugar moieties at the 9-position of the adenine ring (*N*-glucosides) or at the N^6-isoprenoid side chain (*O*-glucosides); the enzymatic conversion to the free zeatin base makes them very active

amounts of *cis*-CKs were accumulated in *P. indica*-colonized *Arabidopsis* roots, and the double-receptor combination *CRE1/AHK2* was necessary for full responsiveness to *P. indica* (Vadassery et al., 2008).

Ustilago maydis is a basidiomycete fungus that infects maize (*Zea mays*), leading to common smut of corn characterized by the production of tumours in susceptible aboveground tissues; according to Bruce et al. (2011), the fungus produces substantially higher CK levels than those measured in plant tissue, and also six different forms of CK were found in liquid media where five of them were either *cis*-isomers or iP forms. In addition, the amounts of free base CKs were much lower than the conjugated ones, but all forms appear to be involved in the infection process.

23.3.3 Ethylene

The plant hormone ET is the simplest alkene (C_2H_4) which plays numerous roles in the development and environmental responses of the plant such as leaf and flower abscission, adventitious and LRF, ripening of fruits and biotic and abiotic stresses (Clark et al. 1999; Negi et al., 2008; Martínez et al., 2013). In plants, ET perception and signalling occur via a complex pathway with several regulatory circuits (Kendrick and Chang, 2008). ET is perceived by ET-binding receptors, such as ETHYLENE RESPONSE 1 (ETR1) and ETHYLENE RECEPTOR SUBFAMILY 1 (ERS1). The activation of these receptors causes repression of CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), which in turn permits ETHYLENE-INSENSITIVE 2 (EIN2) to induce certain transcriptional changes relaying the ET signal to the transcription factors EIN3 and ETHYLENE-INSENSITIVE 3-LIKE (EILs). EIN3 activates ETHYLENE RESPONSE FACTOR 1 (ERF1), inducing the expression of ET-responsive genes (Sánchez-Rodríguez et al., 2010).

Two pathways of ET biosynthesis are known to operate in microorganisms, which involve 2-oxo-4-methylthiobutyrate (OMTB) or 2-oxoglutarate (Cristescu et al., 2002). The OMTB pathway presumably operates in *Pseudomonas syringae*, the yeast *Saccharomyces cerevisiae* and the fungi *Penicillium digitatum* and *Botrytis cinerea* (Nagahama et al., 1994; Weingart et al., 1999; Cristescu et al., 2002). Splivallo et al. (2009) showed that *T. borchii* produced ET from L-methionine via α-keto-γ-(methylthio) butyric acid (KMBA), which is degraded to ET by light. Several recent studies have shown the significance of ET in the establishment and development of plant–fungus interactions. A mechanism by which fungi could lower ethylene level in the host plant to establish the association involves the enzymatic activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (AcdS) that catalyses the degradation of ACC in α-ketobutyrate and ammonia. AcdS activity and/or gene has been found in many microorganisms, including the fungi *Penicillium citrinum* and *Trichoderma asperellum* T203 and the yeast *Hansenula saturnus* (Minami et al., 1998; Jia et al., 1999; Viterbo et al., 2010).

P. indica colonizes mono- and dicotyledonous plants, including barley (*Hordeum vulgare*) and *Arabidopsis*, in which the fungus increases yield and adaptation to abiotic and biotic stress (Varma et al., 1999; Peškan-Berghöfer et al., 2004; Waller et al., 2005). Global gene expression studies in barley colonized by *P. indica* showed that this basidiomycete regulated differentially several ET synthesis and signalling components and induced ACC synthesis, which could be an efficient strategy to initiate/reinforce the association and to improve plant health and yield (Khatabi et al., 2012). In *Arabidopsis*, nine genes encoding ACC synthases (*ACS1*, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, *ACS9* and *ACS11*) have been identified (Tsuchisaka and Theologis, 2004). Analyses of the *Arabidopsis* reporter plants that express β-glucuronidase (GUS) fusions with promoters of the genes *ACS1* and *ACS8* showed a strong GUS activation at 7 days after inoculation at the root tip region of *P. indica*-colonized plants (Khatabi et al., 2012). Thus, regulation of ethylene biosynthesis may be an important target of fungal endophytes.

23.3.4 Gibberellins

GAs are ubiquitous natural plant hormones that participate in plant growth promotion by triggering stem elongation, seed germination, flowering, formation of fruits and senescence (Yamaguchi, 2008; Hamayun et al., 2009). GAs belong to a large family of diterpenoids that are based on the tetracyclic *ent*-gibberellane skeletal structure. Although GAs possess very important physiological roles in plants, the first GA identified was in the rice pathogenic fungus *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi*), where it was typified as a 'phytotoxic' metabolite

(Bömke and Tudzynski, 2009). This fungus is able to produce at least 27 different GAs (MacMillan, 2002), including GA_4, the most bioactive in *Arabidopsis* plants (Figure 23.4). Currently, more than 130 derivatives have been identified from various plants and fungi. Specific GAs enhance the interaction of GA-Insensitive Dwarf 1 (GID1) receptor with DELLA proteins (Hirano et al., 2008), which are key components of GA signalling in plants. Biologically active GAs are not hydroxylated at C-2 (Bömke and Tudzynski, 2009), since hydroxylation at this position is critical for inactivation of GA *in planta*; bioactive GAs possess a carboxyl group at C-6, a hydroxyl at C-3 in the β-orientation and a γ-lactone ring (Figure 23.4).

GAs are biosynthesized in fungi through the mevalonate (MVA) pathway, and two important steps are involved in this process, the formation of *ent*-kaurene by soluble cyclase and the oxidation reactions of this precursor to GA_{12} by microsomal cytochrome P450 monooxygenases (Kawaide, 2006). Besides *G. fujikuroi*, other fungi that produce GAs are *Sphaceloma manihoticola* and some strains belonging to *Phaeosphaeria* sp. (MacMillan, 2002; Bömke and Tudzynski, 2009). All of them have in common the presence of a GA biosynthetic gene cluster, which consists of four P450 monooxygenase-encoding genes (*P450-1* to *P450-4*) and a desaturase gene. However, differences in the fungal pathways and in the final products have been reported (Bömke and Tudzynski, 2009).

In plants, a very efficient mechanism for maintaining an optimal GA level is the inactivation of biologically active GAs by several ways that include the transcriptional upregulation of GA2ox genes, epoxidation of the 16,17-double bond of GAs by a cytochrome P450 monooxygenases (Zhu et al., 2006) or methylation by GA methyl transferases (Varbanova et al., 2007). Interestingly, GA_3 from *F. fujikuroi* is degraded much slower than other GAs, which helps to explain the symptoms observed in the disease caused by this fungus.

GA levels/activity also plays a role in modulating plant growth in response to abiotic stresses. Drought and salinity make plants change the root-to-shoot ratio through an orchestrated balance of phytohormones among different organs, affecting plant performance under stress conditions and in consequence their tolerance to stress. GA-deficient and GA-insensitive *Arabidopsis* mutants exhibit higher tolerance to salt stress (Achard et al., 2006), and also, a transgenic tomato overexpressing the *Arabidopsis* GA METHYL TRANSFERASE 1 (*AtGAMT1*) gene that encodes an enzyme that catalyses the methylation of active GAs to generate inactive GA methyl esters promoted water-deficit tolerance (Nir et al., 2013), suggesting that inhibition of GA levels/activity promotes tolerance to abiotic stress indirectly via suppression of growth (Magome et al., 2004; Achard et al., 2006; Nir et al., 2013). Currently, limited information is available to understand the role of phytohormones secreted by fungi during symbiosis in plants that are under stress. However, biochemical analysis of cultures from the endophytic fungi *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 revealed the presence of IAA and various GAs (GA_1, GA_3, GA_4 and GA_7) in several amounts, and both endophytes enhance the growth in Dongjin-beyo rice cultivar and in GA-deficient dwarf mutant *Waito-C* under salinity and drought stress, suggesting the potential of endophytes conferring tolerance against abiotic stress (Waqas et al., 2012). *Fusarium* and *Penicillium* species were isolated from six halophytes (*Artemisia fukudo* Makino, *Carex scabrifolia* Steud., *Kochia scoparia* var. littorea Makino, *Phragmites communis* Trin., *Suaeda australis* and *Suaeda japonica* Makino), and one of them, the strain *Penicillium* sp. Sj-2-2, produced bioactive GAs that correlated with an improved growth of *Waito-C* rice, highlighting the importance of endophytes as well as the role of GAs in plant development under salt stress environment (You et al., 2012). GAs play a key role in regulating arbuscule formation in pea roots and during root colonization by *P. indica*. In these processes, DELLA proteins and the GA receptor GID1 play an important role (Schäfer et al., 2009; Foo et al., 2013).

23.4 Conclusion

Associations between fungi and plants are ubiquitously present in natural and agricultural ecosystems. The growth patterns of roots in fungal associations are different from those of free-living roots. Therefore, the particular phenotype, architecture and immunity of most plant species cannot be understood without considering the contribution of fungi and their products. Several fungi can confer benefits to plants due to increased root branching capacity that in turn improves nutrient uptake, shoot biomass, photosynthesis and yield. Fungal secondary metabolites with plant growth-regulating activity such as auxins, ET, CKs and GAs have been characterized through combinations of biochemical analytical and physiological

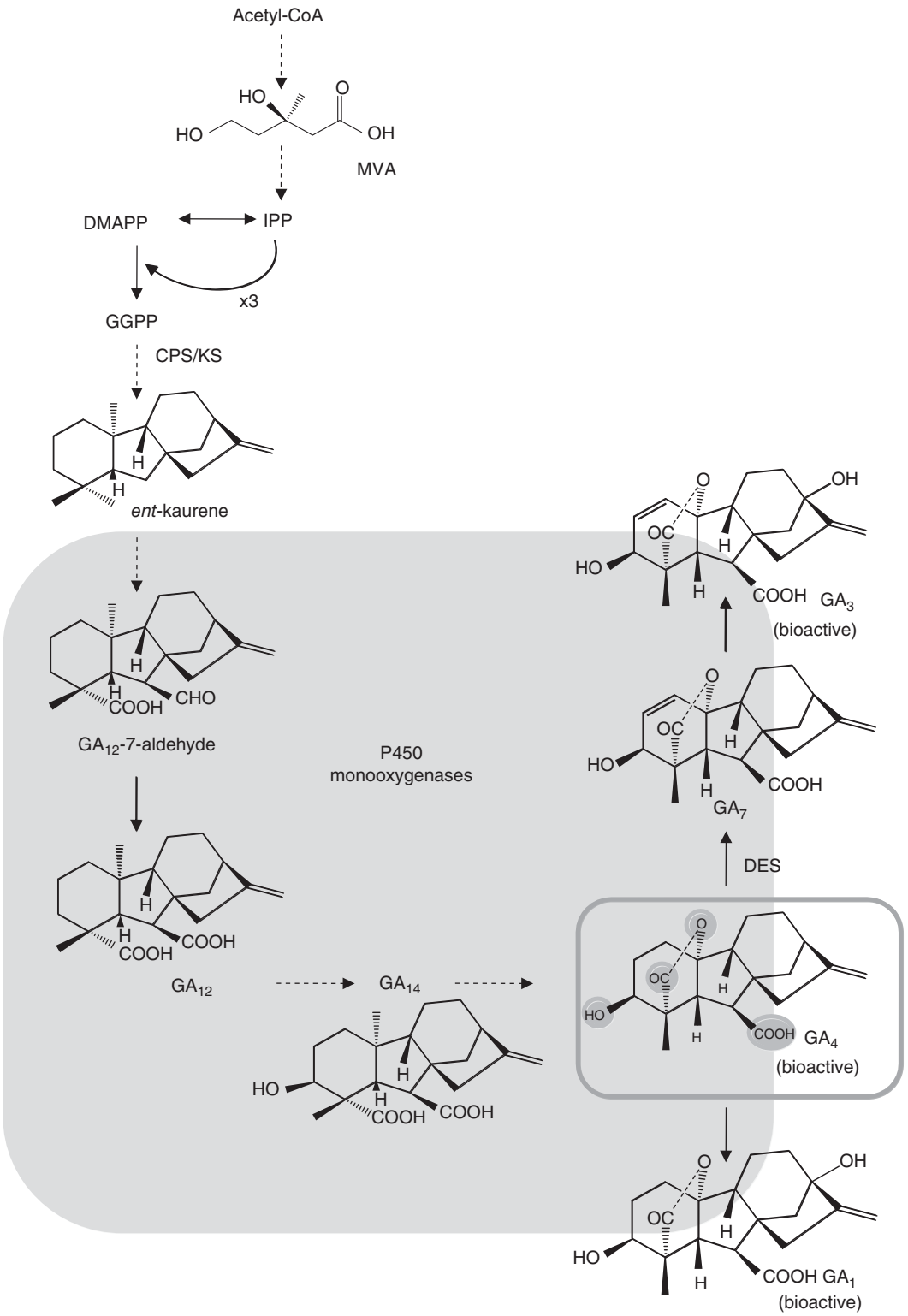

Figure 23.4 The gibberellin (GA) biosynthesis pathway in *Gibberella fujikuroi*. GA synthesis starts from acetyl-CoA via MVA pathway. The isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are produced as the basic building blocks for the generation of geranylgeranyl diphosphate (GGPP), which is cycled to *ent*-kaurene through the bifunctional cyclase *ent*-kaurene synthase (KS) with activities of both *ent*-copalyl diphosphate synthase (CPS) and KS. Oxidation and hydroxylation reactions from *ent*-kaurene to GA_4 and GA_1 are catalysed by cytochrome P450 monooxygenases (grey square). The enzyme desaturase (DES) acts at C-1,2 in GA_4 to form GA_7, which in turn is transformed to GA_3 by a 13-hydroxilation. The end products of the biosynthesis are GA_1 and GA_3. Dotted lines indicate several steps. The common features in bioactive GAs are highlighted in green in GA_4

approaches. Because the metabolites produced by fungi are extremely diverse in structure and function, they certainly represent a source to design novel compounds for practical application in agriculture and several fungal species are promising towards formulation of bioinoculants. Of particular relevance are the compounds with proved hormonal role in plants such as auxins, CKs and GAs, which are produced in significant amounts and whose structures vary depending upon the fungal strain. Deciphering the function of these metabolites in fungal physiology and in the several stages of root colonization deserves further attention. Once the regulatory signals are perceived by root cells, signalling cascades are activated that induce responses in distant plant tissues such as leaves and stems. This may involve long-distance transport/signalling mechanisms that are started to be revealed. Moreover, many fungal species can inhabit plant tissues as endophytes, further increasing the complexity of the interactions. With the advent of the genomic revolution, it is expected that much detailed information will accumulate in the coming years to help unravel the genes and proteins that modulate fungal plant communication and biosynthesis of signalling molecules.

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11.3.

Chapter 7

Recent advancements on the role of volatile organic compounds from fungi

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7.1 Definition and classification of VOCs

VOCs are a large group of carbon-based chemicals with a molecular weight lesser than 300 g/mol, which easily evaporate at room temperature and are able to diffuse through the air and soil (Morath et al., 2012). Organisms from all kingdoms produce VOCs for intra- and inter-species communication. Fungi are not the exception; they release a large spectrum of VOCs including alcohols, aldehydes, aromatic compounds, esters, furans, ketones, terpenes as well as nitrogen- and sulphur-containing compounds, which are derived from both primary and secondary metabolism (Figure 7.1). The variability in the volatile constituents within single fungal species has been well documented and can be attributed to many factors, including genotype, developmental stages and growth conditions (Brodhagen et al., 2008; Miyazawa et al., 2008; Splivallo et al., 2012).

Fungal VOCs may be considered as 'biomarkers' for specific species and growth stages, being useful for comparison of strains or detection of food off-flavours caused by fungi, and recently, several research avenues have increased our understanding about the ecological role of these compounds during the interaction with microbes and plants as well as in multilevel interactions (Table 7.1).

7.2 Chemotaxonomy of fungal VOCs

In recent years, the technology for quantifying VOCs has experienced a big progress, allowing the establishment of chemotaxonomy in fungi due to qualitative and quantitative differences in fungal-emitted VOC profiles. Chemotyping based on VOC emission is a strategy that is still used today. Recently, Müller et al. (2013) analysed the compounds produced from Ectomycorrhizae (*Cenococcum geophilum*, *Laccaria*

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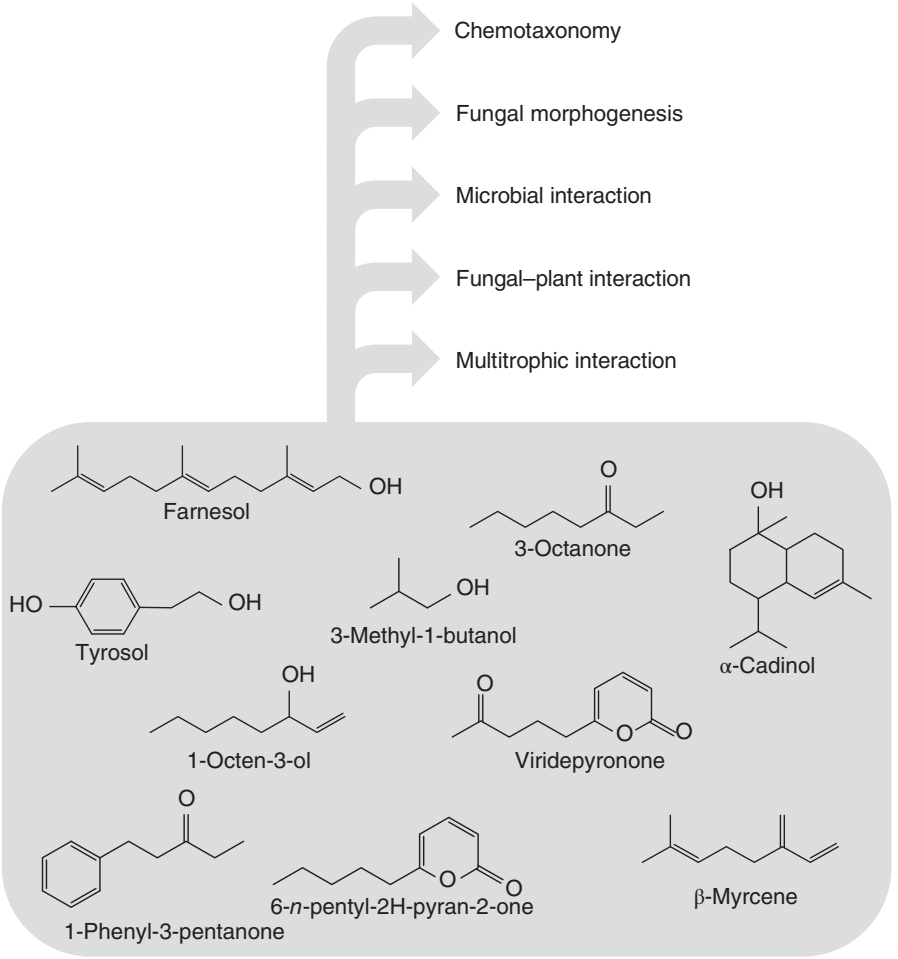

Figure 7.1 Chemical structures from selected VOCs produced by fungi. Fungi produce a wealth of VOCs useful for chemotaxonomy. The diversity of VOCs includes alcohols, lactones, hydrocarbons and terpenes, among others. In addition many of these VOCs have biologically dynamic roles. New findings reveal that one compound is involved in more than one function, like 1-octen-3-ol, which affect fungal morphogenesis and microbial, plant or multitrophic interactions

bicolor, *Paxillus involutus*-MAJ and *Paxillus involutus*-NAU) and pathogenic (*Armillaria mellea*, *Pholiota squarrosa* and *Verticillium longisporum*) and saprophytic (*Stropharia rugosoannulata* and *Trichoderma viride*) fungal species. These different ecological groups could be predicted with probabilities of 90–99%, whereas for the individual species, the probabilities to be grouped into beneficial, pathogen or saprophytic varied between 55 and 83%. Additional studies carried out by Splivallo et al. (2012) determined a volatile fingerprint of 223 fruiting bodies of truffles *Tuber uncinatum* morphotypes *T. uncinatum* Chatin and *Triticum aestivum* Vittad collected over 4 years from seven European countries. These analyses revealed that regardless of the geographical origin of the truffles, the aroma variability was caused by variations in eight-carbon-containing volatiles (C_8-VOCs) such as 1-octen-3-ol, suggesting that the production of these compounds is under metabolic control.

VOCs profiling has been useful to distinguish health and disease in plants interacting with pathogenic fungi (Jelen et al., 2005; Vikram et al., 2006; Ibrahim et al., 2011). In these studies, VOC profiles from healthy plants and the fungal pathogen were determined separately and then compared to those obtained from infected plants. The analysis of tomato fruits infected with three different fungal pathogens indicated that nonane, 1,2,3-trimethylbenzene, tetracyclo[3.3.1.0(2,8).0(4,6)]-non-2-ene, tricyclo[5.2.1.0-2.6]decane, tetrahydronaphthalene, 4-phenyl but-3-ene-1-yne, 1,8-dimethyl naphthalene, butylated hydroxytoluene, pentadecanecarboxylic acid and (6*Z*, 9*Z*)-6,9-pentadecadien-1-ol were unique to

Table 7.1 VOCs produced by fungi and their ecological functions

Compound	Fungus	Function	Reference
Acids			
Propanoic acid	*Muscodor crispans*	Antimicrobial	Mitchell et al. (2010)
Isobutyric acid	*Muscodor albus*	Controls conidia germination of plant pathogen fungi	Braun et al. (2012)
Methacrylic acid	*Phoma* sp. GS8-3	Plant growth-promoting effect on tobacco	Naznin et al. (2013)
Esters			
3-Methylbutyl propanoate	*Tuber melanosporum*	Serves as a pheromone to attract bees	Splivallo et al. (2007)
Ketones			
3-Octanone	*Trichoderma atroviride*	Induces conidiation in *Trichoderma* spp.	Stoppacher et al. (2010)
Acetophenone	*Tuber borchii* and *Tuber melanosporum*	Acts in oviposition aggregation pheromone to the female desert locust *Schistocerca gregaria*	Rai et al. (1997), Splivallo et al. (2007)
Aldehydes			
Hexanal	*Tuber melanosporum*	Disrupts the egg-laying behaviour of *Phthorimaea operculella*	Splivallo et al. (2007), Anfora et al. (2014)
trans-2-Octenal	*Tuber indicum*	Induces accumulation of hydrogen peroxide (H_2O_2) in *Arabidopsis*	Splivallo et al. (2007)
Octanal	*Tuber indicum*	Reduces the *Phthorimaea operculella* infestation rate when used under storage conditions	Splivallo et al. (2007), Anfora el al. (2014)
Alcohols			
2,3-Butanediol	*Tuber borchii*	Promotes plant growth	Splivallo et al. (2007)
1-Octen-3-ol	*Trichoderma atroviride*	Induces conidiation in the fungus and defence responses in *Arabidopsis*	Nemcovic et al. (2008), Kishimoto et al. (2007)
3-Octanol	*Trichoderma atroviride*	Induces conidiation	Nemcovic et al. (2008), Stoppacher et al. (2010)
Benzyl alcohol	*Trichoderma aureoviride*	Exerts insect-attractive properties in the Brassicaceae family	Bruce et al. (2000)
Terpenes			
α-Pinene	*Penicillium chrysogenum*	Attracts the pine weevil	Wilkins et al. (2000)
β-Myrcene	*Trichoderma virens*	Blocks the expression of a monooxygenase involved in the production of aflatoxin in *Aspergillus flavus*	De-Oliveira et al. (1997), Crutcher et al. (2013)
Limonene	*Aspergillus flavus*	Upregulates genes involved in the antioxidant system and the regeneration of NADPH in *Saccharomyces cerevisiae*	Liu et al. (2013)
(+)-3-Carene	*Penicillium roqueforti*	Attracts the pine weevil	Azeem et al. (2013)
β-Farnesene	*Fusarium sambucinum*	Acts as an alarm pheromone in aphids and is a repellent against herbivores	Kunert et al. (2005)
β-Caryophyllene	*Fusarium oxysporum*	Attracts nematodes, which prey on insect larvae, and promotes growth in lettuce	Rasmann et al. (2005), Minerdi et al. (2009)
Thujopsene	*Penicillium decumbens*	Inhibits fungal growth	Polizzi et al. (2011)

(*Continued*)

Table 7.1 (*Continued*)

Compound	Fungus	Function	Reference
α-Cadinol	*Fomitopsis pinicola*	Anti-fungal activity against the ascomycetous pathogens *Rhizoctonia solani* and *Fusarium oxysporum* and acts as repellent against termites	Rösecke et al. (2000), Chang et al. (2001, 2008)
Farnesol	*Candida albicans*	Acts as a quorum-sensing signal	Langford et al. (2009), Morales and Hogan (2010)
Others			
6-*n*-Pentyl-6*H*-pyran-2-one	*Trichoderma atroviride*	Auxin-like activity and elicitor of defence responses	Vinale et al. (2008)
Cinnamene	*Penicillium caseifulvum*	The attractive odour of freshly cut pine twigs for both sexes of pine weevil	Azeem et al. (2013)

Aspergillus flavus, while *Aspergillus niger* produced 2-(3-hydroxy 1-2-nitrocyclohexyl)-1-phenylethanone, oxalic acid, isobutyl pentyl ester, 1-methylene-1*H*-indene and (1*E*)-1-ethylidene-1*H*-indene compounds, and *Fusarium oxysporum* produced 1,2-dimethylbenzene, isopropylbenzene, methyl 14-methylpentadecanoate and methyl *cis*-octadec-11-enoate compounds (Ibrahim et al., 2011). This study showed that VOCs can be used as biomarkers to detect a pathogen at an early stage of disease progression. Similarly, VOC analysis has been done to identify potential human pathogens such as *Aspergillus fumigatus*, causal agent of aspergillosis and *Epicoccum nigrum*, which might be responsible of sinusitis. These results also showed that VOCs from *E. nigrum* differ from those of *A. fumigatus,* indicating specificity in the emissions (Di Cagno et al., 2009; Ulanowska et al., 2011; Bazemore et al., 2012).

7.3 Role of VOCs in fungal growth and development

Microorganisms monitor their population density by releasing signalling molecules also called auto-inducers to which they respond. In bacteria, the auto-inducers represent a class of quorum-sensing signals, and in Gram-negative bacteria these compounds mainly belong to *N*-acyl-homoserine lactones, while in Gram-positive bacteria they are usually modified peptides. After reaching a concentration level, these molecules induce the population to cooperate in diverse ways and establish common cellular behaviours such as bioluminescence, antibiotic production, virulence, biofilm formation, competence and sporulation (Chen et al., 2004; Ortíz-Castro et al., 2008; Ortíz-Castro et al., 2011). Some bacteria release VOCs including ethylene, allyl alcohol, trimethylamine and benzaldehyde that act as fungistatic compounds inhibiting germination or growth of fungal hyphae in soil (Garbeva et al., 2011). Furthermore, quorum sensing has been described in the dimorphic fungus *Candida albicans*, in which small molecules such as farnesol (Hornby et al., 2001) and tyrosol (Chen et al., 2004) inhibit or promote the morphological transition from yeast to the filamentous stage depending on environmental conditions.

Oxylipins are fatty acid polyunsaturated secondary metabolites derived from lipid peroxidation, which are involved in regulation of developmental processes and environmental responses acting as signals for intra- and intercellular communication in fungi, plants and animals. In fungi, the 1-octen-3-ol and its analogues 3-octanol and 3-octanone are the most studied oxylipins, which are able to induce sporulation and conidiation (Calvo et al., 2002). These C_8-VOCs have been detected from cultures of *Trichoderma*, and it has been suggested that the specificity of the cell response to particular C_8-VOCs implies the presence of membrane receptors that could transmit the VOC signal into the conidiation pathways (Nemcovic et al., 2008; Steyaert et al., 2010).

Other fungal hydroxylated oleic, linoleic, and linolenic acid-derived oxylipins are collectively called precocious sexual inducer (*psi*) factors including *psi*Aα, *psi*Aαβ and *psi*Aαγ; *psi*Bα, *psi*Bβ and *psi*Bγ; and *psi*Cα, *psi*Cβ and *psi*Cγ. The proportions of *psi*A, *psi*B and *psi*C are proposed to alter the ratio of sexual–asexual

reproduction in *Aspergillus nidulans*, as well as mycotoxin synthesis (Tsitsigiannis et al., 2005; Brodhagen et al., 2008). The analysis of the *A. nidulans* genome has led to the identification of three fatty acid oxygenases (PpoA, PpoB and PpoC) predicted to produce *psi* factors because the deletion of PpoB (Δ*ppoB*) decreased the production of oleic acid-derived oxylipins and increased the ratio of asexual to sexual reproduction (Tsitsigiannis et al., 2005). Phylogenetic analyses showed that *Ppo* genes are present in saprophytic and pathogenic Ascomycetes and Basidiomycetes, suggesting a conserved role for Ppo enzymes in the life cycle of fungi (Tsitsigiannis et al., 2005). In organisms from other kingdoms such as plants and animals, oxylipins are important for developmental and defence processes, and their signal transduction pathways have been thoroughly investigated. Interestingly, plant-derived oxylipins may affect sporulation and mycotoxin production, and vice versa, and fungal oxylipins are involved in plant lipoxygenase (*LOX*) gene expression changes, altering plant oxylipin production, leading to possible alterations in the fungus-host interaction (Brodhagen et al., 2008). It is possible that in analogy to oxylipin perception in mammalian cells (Obinata et al., 2012), presumably, oxylipins are perceived in fungi through G-protein-coupled receptors (GPCR) and that internal or external stimuli may induce the synthesis *de novo* of *psi* factors with biological activities (Tsitsigiannis and Keller, 2007).

In zygomycetes such as *Phycomyces* and *Mortierella* where the zygophores meet within the substrate layer, diffusible signals that belong to the trisporoids, a family of C_{18} or C_{19} isoprenoid compounds, regulate recognition between mating partners during early sexual morphogenesis, and although trisporoids are apparently produced by particular species, they may elicit different responses in various interacting organisms, thus representing true signalling molecules (Schimek and Wöstemeyer, 2009).

7.4 Fungal VOCs in microbial interactions

Soil-borne fungi survive in a highly competitive environment under limitation of carbon sources (Owen et al., 2007; Wenke et al., 2010). Antagonism between species of naturally competing fungi has been observed in virtually every ecosystem. Common strategies in this process are the production of secondary metabolites with antimicrobial activity either of volatile or diffusible nature. Examples of VOCs with antimicrobial properties produced by fungi are ketones (Nishino et al., 2013), alcohols (Ting et al., 2010; Singh et al., 2011) and terpenes, whose stereo configurations influence their bioactivity (Angioni et al., 2003; Wu et al., 2005). Fungi from Ascomycota and Basidiomycota families have been found to produce sesquiterpenes (Hynes et al., 2007; Agger et al., 2009; Minerdi et al., 2009; Rolf and Wolf-Rainer, 2012; Crutcher et al., 2013). C_8-alcohols isolated from oyster mushroom (*Pleurotus ostreatus*) exhibit antibacterial activity against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhimurium* (Beltran-Garcia et al., 1997).

Trichoderma fungi are well documented as biocontrol agents that reduce the negative effects of plant pathogens (Shoresh et al., 2010). Mycoparasitism is apparently an ancestral trait and the ability to parasitize and kill other fungi has been the major driving force behind commercial success of *Trichoderma* as biofungicides. These fungi produce a great number of VOCs (i.e. pyrones, sesquiterpenes) and non-volatile secondary metabolites (i.e. peptaibols) with antibiotic activity (Reino et al., 2008; Amin et al., 2010). The identification of 6PP (6-pentyl-2*H*-pyran-2-one) as a major aroma constituent of *Trichoderma viride* was reported by Collins and Halim (1972). Later on, the production of pyrone-like metabolites was related to the effectiveness of certain *Trichoderma harzianum* isolates against *Gaeumannomyces graminis* var. *tritici* (Ghisalberti et al., 1990) and Cooney and Lauren (1998) reported an induction of 300–700% in the biosynthesis on this compound by the presence of *Botrytis cinerea*, suggesting a potential antagonistic function for 6PP.

7.5 VOCs in fungal–plant interactions

The complex signalling network between plants and fungi has been extensively studied over the past 20 years, and VOCs have been included in the increasingly growing list of signals important for interkingdom communication. Steeghs et al. (2004) showed that VOCs are constitutively emitted by *Arabidopsis* roots and that they are induced in response to the presence of beneficial or pathogenic microorganisms. This suggests that the VOCs emitted by roots play a

decisive role in the establishment of plant–microbe interaction since the volatility of these compounds allows them to be quickly and effectively perceived by neighbouring organisms.

Beneficial and pathogen soil microorganisms may induce changes in plant VOC emissions during their interaction. Beneficial microbes activate induced systemic resistance and prime plants against pathogen attack (Ryu et al., 2004) and even may affect behaviour of pollinators (Barber et al., 2013) (Figure 7.2). On the other hand, fungal pathogens may damage plants or attract deterrent organisms, as in the case of *Fusarium* spp., which induce VOC emission from maize (*Zea mays*) plants including green leaf volatiles (GLVs), terpenes and shikimic acid pathway derivatives that attract herbivores (Piesik et al., 2011).

Activation of plant defence by different *Trichoderma* strains involves the production of defence-related metabolites such as phytoalexins or induction of pathogenesis-related (PR) proteins (Contreras-Cornejo et al., 2011). To investigate the involvement of secondary metabolites in the induction of ISR during

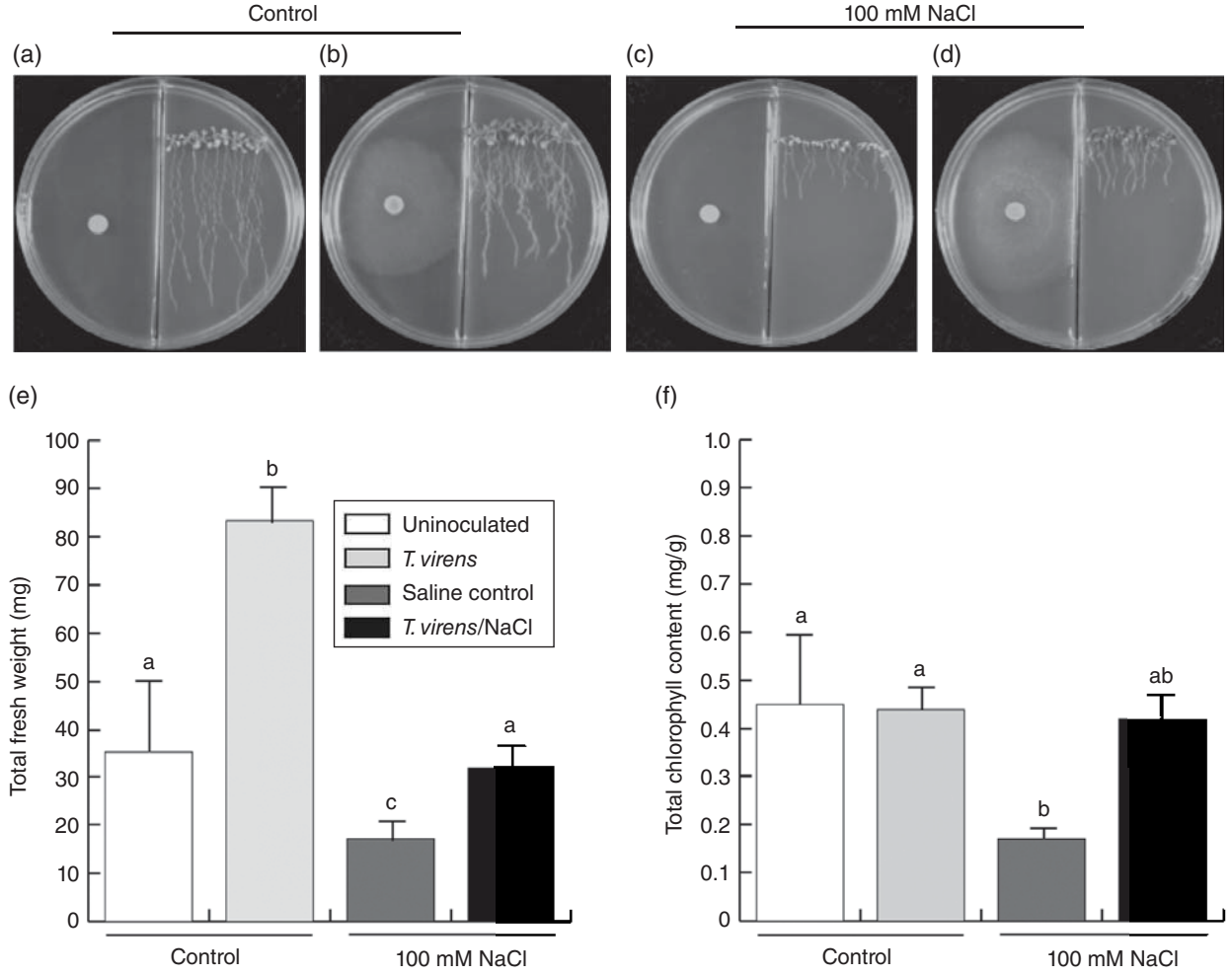

Figure 7.2 Effect of VOCs from *Trichoderma virens* on *Arabidopsis thaliana* grown under normal or saline conditions. (a) Photograph of 9-day-old *Arabidopsis* (Col-0) seedlings grown on the surface of agar plates containing 0.2× Murashige and Skoog medium. (b) Representative photograph of seedlings inoculated with *T. virens* at the opposite side of a Petri plate 4 days after germination and co-cultured for a further 5-day period. (c) Photograph of *Arabidopsis* seedlings grown under elevated salinity (100 mM NaCl) 9 days after germination. (d) Effects of fungal inoculation on seedlings. (e) Total fresh weight. (f) Total chlorophyll content. Data from (e) and (f) show means±SD from three groups of 10 seedlings that were harvested from the medium. Different letters represent means statistically different at the 0.05 level (*See insert for color representation of the figure*)

the *Trichoderma*–plant interaction, Vinale et al. (2008) evaluated the ISR-inducing ability of the volatile compound 6-PP isolated from *Trichoderma atroviride* P1 culture filtrates. In tomato (*Lycopersicum esculentum*) and canola (*Brassica napus*) seedlings inoculated with the pathogens *Botrytis cinerea* or *Leptosphaeria maculans*, respectively, a reduction of disease symptoms was observed when treated with the purified metabolite, which correlated with induced expression of the salicylic acid-responsive gene *PR-1*.

The role of microbial VOCs in the promotion of plant growth has been studied in *Arabidopsis* plants co-cultivated with plant growth-promoting rhizobacteria (PGPR). PGPR emit various VOCs including HCN and other compounds with antimicrobial activity to protect against invaders (Chaurasia et al., 2005; Grosch et al., 2005; Kai et al., 2009) or that stimulate developmental processes (Ryu et al., 2003; Gutiérrez-Luna et al., 2010; Velázquez-Becerra et al., 2011). Furthermore, VOCs from *T. viride* modulate *Arabidopsis* growth resulting in an increase of shoot and root biomass and chlorophyll content in leaves (Hung et al., 2012). Ryu et al. (2003, 2004) and Zhang et al. (2007) reported that 2,3-butanediol and acetoin belong to a new category of signalling molecules in plant–PGPR interaction and both compounds have growth-stimulating effects in *Arabidopsis*. A mixture of 2-methyl-propanol, 3-methyl-butanol, methacrylic acid and isobutyl acetate (30:60:7:3) extracted from the plant growth-promoting fungus *Phoma* sp. GS8-3 increased the biomass of tobacco plants (Naznin et al., 2013). These molecules modulate classical hormone pathways stimulating plant growth or inducing ISR. Gutiérrez-Luna et al. (2010) noted that *Arabidopsis* plants respond differentially to VOCs emitted by different rhizobacteria, which caused changes in root architecture depending on the bacterial strain. This suggests that VOCs of microbial origin can modulate plant development programs such as rhizogenesis, root growth, and lateral and root hair formation.

Ethylene (ET) is a gaseous unsaturated hydrocarbon and a classic plant growth regulator that is produced by a wide variety of soil-borne microorganisms. In the early stage of *Tuber borchii*–plant interaction, the mycelium released ET and induced lateral root and root hair formation in the host *Cistus incanus* and the non-host *Arabidopsis* (Splivallo et al., 2009). Exogenous application of the ET precursor 1-aminocyclopropane-1-carboxylic acid partially mimicked the effects of the fungus in roots. Experiments of inoculation of the *Arabidopsis* double mutant *aux1-7ein2* showed reduced sensitivity to fungus-induced root branching, indicating an important role of auxin/ET signalling. The ecological advantages for soil microorganisms in inducing changes in root anatomy remain to be clarified. However, the previously mentioned reports add on the ecological gain that root structure modification confers to microorganisms. It is expected that increasing the length of the primary root and the number and/or length of lateral roots could increase colonization and reinforce the symbiosis (López-Bucio et al., 2007).

Recent reports have focused on the effect of fungal VOCs on defence responses or plant growth. VOCs from *T. viride*, *Trichoderma virens* and *T. atroviride* stimulate growth of *Arabidopsis* seedlings and induce root hair and lateral root development (Figure 7.2a and b). Moreover, when grown under salt stress conditions (100 mM NaCl) and/or exposed to fungal VOCs, *Arabidopsis* seedlings were healthier than salt-exposed plants; concomitantly, total fresh weight and chlorophyll content were higher in plants elicited with fungal VOCs (Figure 7.2c–f). These data show that *Trichoderma* may protect plants against salinity via VOC emission, in agreement with previous studies in which *Trichoderma* conferred resistance to a wide range of adverse environmental conditions (Rawat et al., 2011; Brotman et al., 2013). Elucidating the mechanisms and signalling pathways that are involved in this fungal-induced stress tolerance to plants and clarifying whether the VOCs of *Trichoderma* modulate auxin homeostasis to achieve high levels of salt tolerance merit further research.

Using microarray technology, Godard et al. (2008) observed that in *Arabidopsis* exposure to myrcene volatiles or to a blend of ocimene volatiles consisting of *trans*-β-ocimene, *cis*-β-ocimene and allo-ocimene increased the abundance of several hundred transcripts. Many of the monoterpene-induced transcripts were annotated as either transcription factors or as stress or defence genes including those of the octadecanoid pathway. On the contrary, the oxylipin 1-octen-3-ol activated defence genes and the production of hydrogen peroxide and conferred resistance against *Botrytis cinerea* (Kishimoto et al., 2007; Splivallo et al., 2007). On the other hand, it seems clear that fungi are able to perceive plant oxylipin precursors such as linolenic and hydroperoxy linolenic acids, which can induce mycotoxigenic or sporogenic effects in *A. nidulans*, *A. flavus* and *Aspergillus parasiticus*.

Maize oxylipin (ZmLOX3) could restore conidiation in the conidia-deficient *A. nidulans* mutant *ppoAC*, evidencing a fungus–plant oxylipin-mediated cross-talk (Calvo et al., 1999; Brodhagen et al., 2008; Christensen and Kolomiets, 2011).

7.6 Fungal VOCs in multitrophic interactions

Plants are the primary producers of ecosystems. As sessile organisms, they are exposed to numerous biotic and abiotic factors, which alter growth and development and impact grain and fruit production. Plants have developed adaptive mechanisms that include communication with other plants and resident microorganisms. Through VOC emission, plants can attract pollinators, seed dispersers or natural enemies of herbivores or activate chemical barriers against insects and pathogenic microorganisms. Moreover, VOCs may further act as warning signals to neighbouring plants, which activate their own defence mechanisms (Dicke et al., 2003; Gershenzon, 2007).

Several beneficial fungi can help plants to cope with stress through promotion of plant growth and activation of defence (Figure 7.3). Some regulatory elements for modulation of the induced response include Ca^{2+}, ion fluxes, jasmonic acid (JA), ET and reactive oxygen species (Wu and Baldwin, 2010). Various microbial or pathogen-associated molecular patterns (MAMPs, PAMPs) are able to induce plant defence responses (Pineda et al., 2010); similarly, herbivore-associated molecular patterns (HAMPs) may initiate a hypersensitive response (Wu and Baldwin, 2010). Interest in aboveground-belowground plant-mediated interactions has increased in recent years. Several lines of evidence suggest that microorganisms can be mediators of interactions among pollinators, herbivores and plants. Enhanced plant growth due to an improved nutrient status translates into increased food supply for insects and herbivores, whereas beneficial microbes can facilitate the regeneration of shoot tissues after leaf damage by consumers (Pineda et al., 2010). In addition, ISR can be triggered by beneficial microorganisms eliciting the JA and ET responses in the plant. Plants under herbivore attack emit complex blends of VOCs that attract the natural enemies of herbivores, and the JA-signalling pathway is the most important signalling pathway for VOC emission. Therefore, plant defences through ISR against herbivores partially overlap with that of microbes.

VOCs can be mediators of multitrophic interactions between communities above and below the soil in which fungi are important components (Figure 7.3). A few studies began to emerge on this topic, which revealed the importance of fungal symbiosis in plants by altering interactions with other community members. The presence of fungi can strongly influence the metabolism of their host plants (Fontana et al., 2009; Barber et al., 2013; Estrada et al., 2013). Fontana et al. (2009) showed that colonization of arbuscular mycorrhizal fungi (AMF) allows the attraction of herbivore enemies of *Plantago lanceolata* by the stimulation of the synthesis of GLVs, thereby attracting herbivore predators. More recently, Barber et al. (2013) indicated that while mycorrhizal treatment had no effect on plant biomass or floral traits, AMF significantly affected leaf nutrient content, pollinator behaviour and herbivore attack. Thus, the magnitude of AMF colonization of roots impacts on plant–insect interactions, which depends on both the insect and the AMF species that colonizes the plant.

Fungal endophytes, a kind of symbionts that live inside plant tissue without causing signs of disease, have been found to change leaf chemistry. For instance, Estrada et al. (2013) observed that the leaf-cutting ant species *Atta colombica* prefer harvesting leaves from *Cucumis sativus* containing relative lower densities of the endophyte *Colletotrichum tropicale* and the chemical composition analyses from the leaves of colonized plants revealed changes in compounds with low volatility released after wounding, thus influencing foraging by ants when choosing between plants with low or high endophyte loads. Although this study did not discard that the physical properties of the leaves or compounds with high molecular weight also change with endophyte colonization and contribute to the observed foraging patterns, it opens a new and exciting avenue towards understanding the ecological role of fungal endophytes.

7.7 Concluding remarks

Volatile compounds are fundamental in chemical communication in organisms from all kingdoms and domains of life, acting as attractive, repulsive and alert signals. Fungi can produce blends or unique sets of VOCs and employ multiple biochemical pathways for

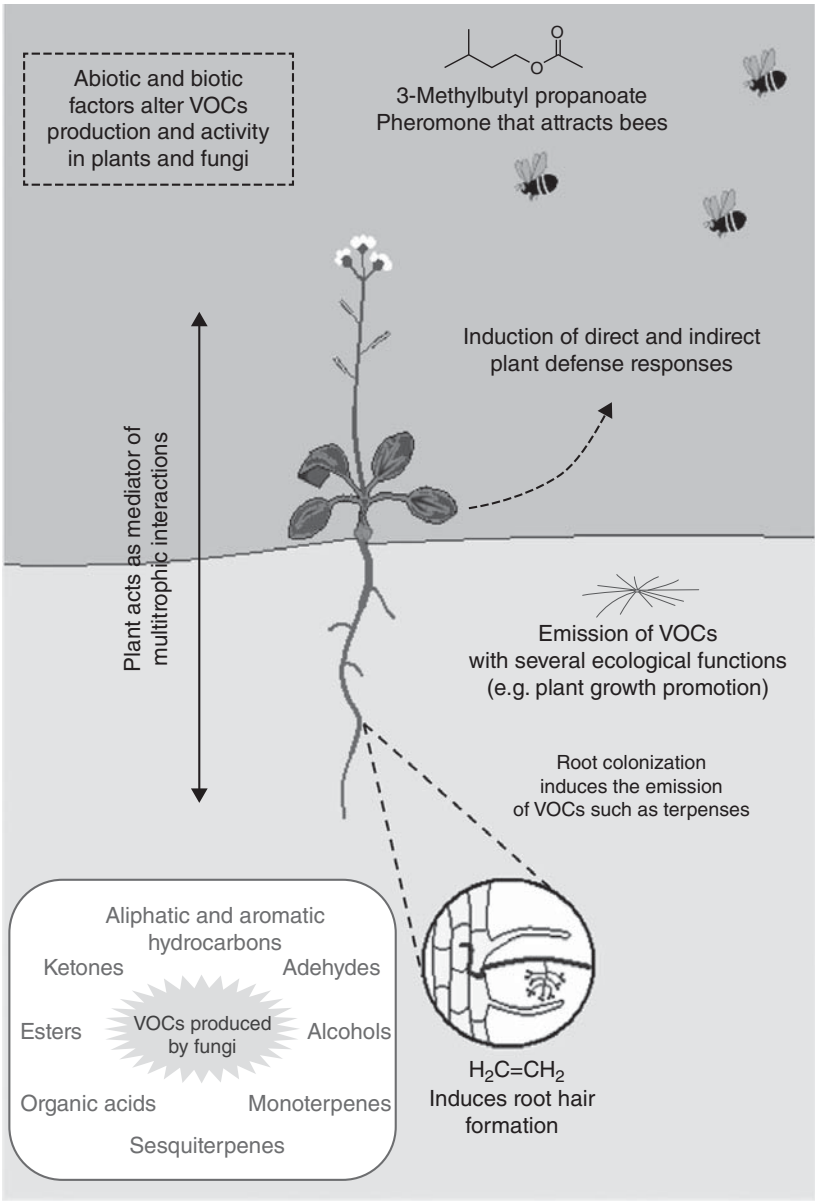

Figure 7.3 Fungi and host plants emit VOCs with diverse biological functions. Different biotic and abiotic factors may induce *de novo* biosynthesis of VOCs. The cell type, growth and developmental stages as well as temperature, light, nutrients and biotic stimuli influence the biosynthesis of VOCs. During fungus–plant interaction, several biochemical and physiological changes occur in the plant that may affect interactions with aboveground insects including herbivores or pollinators

their synthesis. In the plant partners, regulation of emission is fully orchestrated, which allows for successful adaptation and survival. The biochemical pathways and enzymes involved in the generation of VOCs that are induced when a plant is exposed to beneficial or harmful organisms impact not only the plant physiology but also the ecological interactions with their fungal symbionts. Fungal hyphae are highly sensitive to VOCs, and then developmental processes such as sporulation are modified. In an ecological context, it is tempting to speculate that exposure to VOCs released by roots either healthy or damaged might result in activation of mycoparasitic responses or root colonization by beneficial fungi. Although the VOC-mediated interplay of fungi with other organisms is not well understood, highlights about *Trichoderma*–plant

interactions have shown that particular VOCs such as 6PP can interfere with cellular processes in fungi and plants.

From an adaptive viewpoint, there are several advantages for plants interacting with beneficial fungi, which may confer protection to roots against soil-borne pathogens or may directly boost developmental programs by producing bioactive signals such as VOCs. Therefore, there is currently great interest in determining the selection and discrimination mechanisms that allow the plant to modify its root architecture due to colonization with beneficial fungi such as *Trichoderma*, *Mycorrhizae* or *Piriformospora indica*. VOCs may act as recognition signals such as the already reported MAMPs. This hypothesis is strengthened by the fact that each microorganism has apparently its own chemical fingerprint to which plants respond in specific ways. Determining the VOCs that act as biomarkers of certain fungal species would not only aid in the identification and classification of new fungal strains but, together with genome sequencing projects, would also provide new information on the biosynthesis and signalling mechanisms of VOCs. Further research on the biochemistry of these metabolites may also be of interest for applications of fungi in agriculture and industry and for the discovery of novel bioactive substances of broad biotechnological interest.

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11.4

Trichoderma

Biology and Applications

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Foreword

Trichoderma spp. have emerged as a group of fungi with immense impact on human welfare. The literature of these remarkable fungi expands daily, demanding that a timely and exclusive volume be conceived and brought to fruition as a benchmark publication on the biology and application of *Trichoderma*. Prasun Mukherjee and colleagues have presented such a prestigious collection of authoritative chapters on *Trichoderma*. The last such effort was made by Gary Harman and Christian Kubicek in 1998, and the face of *Trichoderma* research has changed considerably in the past 15 years, especially with the advancements in genetics and genomics. The current volume is an update on the advances in *Trichoderma* research, covering most of the aspects related to the biology, genetics, genomics and applications of *Trichoderma* in human welfare. The book starts with an introductory chapter by Mukherjee *et al*. (Chapter 1), which provides an overview of *Trichoderma* and its applications, and ends with an update on the negative impact of these fungi on human health (Hatvani *et al*., Chapter 17). All the chapters are written by authorities in the field with vast experience in their respective areas. The chapter on taxonomy (Atanasova *et al*., Chapter 2) covers the molecular phylogeny of 200 *Trichoderma* spp., which is the first treatise of its kind, and will certainly prove to be very useful in exploration and exploitation of *Trichoderma* spp. The chapter on *in vitro* sexual development (Schmoll, Chapter 4) is again a unique compilation of a recent development in the field and will provide guidance for applications of this novel breeding tool in strain improvement of these fungi. In place of a chapter on secondary metabolism in general, this book has two related chapters addressing this subject (Chapter 6 on volatile metabolites and Chapter 15 on metabolites from marine-derived *Trichoderma*), which demonstrates the novelty and importance of these compounds. The section on *Trichoderma*–plant interactions (Section II) will be of special interest to readers because the scope is broad and illustrates many of the recent developments in this rapidly unfolding and intensively interrogated field of *Trichoderma* research. All the chapters in this section represent an advanced treatise on this topic, providing rich and insightful text regarding the physiology, biochemistry and genetics of interactions of these beneficial fungi with plants (Chapters 8–12). The book also revisits some of the 'traditional' topics, but viewing them in new perspectives that reveal the applications of *Trichoderma* from plant health management (Chapter 14), to biofuels (Chapter 13) and cell factories (Chapter 16). Similarly, the much discussed topics on light response and asexual sporulation (Chapters 3 and 5) are enriched with new and absorbing information and details (especially related to the genetics and

genomics) that will help readers comprehend and understand these processes that have the potential to lead to more effective and economical formulation products. Overall, this book is a treat to all those involved in R&D activities dealing with *Trichoderma* and will prove to be an invaluable tool in furthering basic understanding as well as the commercial success of these economically important fungi.

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Preface

After decades of improvement of agriculture and chemicals production towards industrialized processes, environmental issues and sustainability needs initiated movement for a greener industry and agriculture. Several principles are needed to make agriculture sustainable. One of these is to use natural biological control, where possible. The idea to use members of the genus *Trichoderma* to control plant pathogens can be traced back to before the middle of the past century, but the past decade has seen a qualitative leap in the tools and approaches for understanding these beneficial fungi and their interaction with plants. In addition, however, the industrial production of chemicals and enzymes has started to shift towards biotechnological processes, in many cases applying filamentous fungi as work horses. Here also, *Trichoderma* is a major organism, especially with the research focus on second-generation biofuels.

The landmark achievement since the last monograph on *Trichoderma* is, without doubt, the publication of the genomes of three species. Other paradigm shifts are almost as important. One is the realization that induction of resistance in the plant rivals direct killing of pathogenic fungi as a mechanism for control of disease. Another is the ability to do genetic crosses in *Trichoderma* species that were thought to lack a sexual cycle under laboratory-defined conditions. Methods for engineering of strains have been optimized. Next-generation sequencing is becoming the best way to follow gene expression, as well as to identify the genes corresponding to classical mutant phenotypes.

The concept of this book grew, in part, from our participation in the genome projects. Sequencing shone a spotlight on the genus *Trichoderma*, the most important members of which from the biotechnological point of view are often hidden underground in the rhizosphere or within plants as endophtyes. We were further encouraged by our dialogue with an international community of researchers who focus in every aspect from molecular genetics to field applications. The chapters in this volume address basic biology, morphogenesis in response to light and other signals, genetics, interactions with plants and secondary metabolites, just to mention a few of the topics. The collection of diverse approaches should serve as a link between genomes and biology. Moreover, we trust that having this information, critically reviewed and within easy reach, will encourage the connections that start new research. Finally, we hope that the unfolding of the story of *Trichoderma* research will provide an enjoyable path through the myriad of biotechnological and genetic details.

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10 Promotion of Plant Growth and the Induction of Systemic Defence by *Trichoderma*: Physiology, Genetics and Gene Expression

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10.1 Introduction

Plants are essential to human life and to most organisms as well. They produce food, fuel, fibre, medicines and materials for humans and are integral to most ecosystems. Plant growth and development are greatly affected by environmental stresses such as drought, salinity, nutrient deficiency and adverse temperatures. Owing to climate changes these challenges are becoming even more intensified. Pathogens can also have a severe impact on plant health, decreasing agricultural production. For the past 50 years, the major challenge of providing sufficient food for the increasing human population has been facilitated by the application of high inputs of chemical fertilizers containing nitrogen (N), phosphorus (P) and potassium (K), which, together with advances in crop and agricultural techniques focusing on shoot biomass and seed yield, has resulted in increasing productivity (González *et al.,* 2009; Xing and Zhang, 2010). Current production methods based on high amounts of nutrients are not only costly but also lead to several environmental and health problems (Conway and Pretty, 1988). Additionally, in crops such as wheat and maize, intensive arable cultivation is no longer sustainable because it often leads to soil degradation (Loneragan, 1997). In this scenario, research with plants and microbes will be central in finding alternative methods to cope with the threat of food shortage.

There is a huge diversity of microorganisms that colonize plant roots and some of them play beneficial functions in biocontrol, protecting hosts from pests and diseases and promoting plant growth by releasing hormones or hormone-like signals (Ortiz-Castro *et al.,* 2009; Harman *et al.,* 2011; Berendsen *et al.,* 2012). A number of fungi are known to proliferate in the rhizosphere, the part of the soil that receives the influence of plant roots, or even penetrate plant tissues without causing disease. These include endo- and ecto-mycorrhizas, binucleate *Rhizoctonia* spp., *Piriformospora indica* and *Trichoderma* spp. (Waller *et al.,* 2005; Shoresh *et al.,* 2010; Harman *et al.,* 2011). Some of these organisms were initially appreciated because of their biocontrol properties antagonizing root pathogens

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and protecting plants from diseases, but recent studies have demonstrated that they may possess additional attributes for application in agriculture.

Small amounts of *Trichoderma* spp. supplied as bioinoculants may confer significant advantages on a wide variety of crops, including both monocotyledons and dicotyledons. These include systemic resistance to diseases through an induction of jasmonic acid and salicylic acid signalling (Segarra *et al.,* 2007; Contreras-Cornejo *et al.,* 2011; Salas-Marina *et al.,* 2011), improved adaptation to abiotic stress including drought, salt and temperature (Mastouri *et al.,* 2010), enhanced nutrient solubility (Yedidia *et al.,* 2001; Rudresh *et al.,* 2005; Azarmi *et al.,* 2011) and regulation of root system architecture (Björkman, 2004; Contreras-Cornejo *et al.,* 2009; Samolski *et al.,* 2012).

The relevance of the root system has often been overlooked in plant-breeding programmes aimed at increasing food supply. Nevertheless, the root system has indispensable functions in the plant such as the uptake of nutrients and water, anchorage in the soil and interaction with symbiotic microorganisms (López-Bucio *et al.,* 2003, 2005). Consequently, root system development is central for the plant to reach optimal growth and directly contributes to the levels of yield obtained in crops. The impact of the root on plant growth has become apparent not only in model plants such as *Arabidopsis thaliana, Medicago truncatula* and *Lotus japonicus* but also in important crops such as wheat (*Triticum aestivum*), rice (*Oriza sativa*) and maize (*Zea mays*) (Hochholdinger and Tuberosa, 2009; Coudert *et al.,* 2010). One way to minimize the negative impact of biotic and abiotic factors on yield is to manipulate root system architecture (RSA) towards a distribution of roots in the soil that optimizes water and nutrient uptake. It is now established that most of the genetic variation for RSA is driven by a suite of genetic and hormonal factors on the plant and is modulated by its interactions with microorganisms (Den Herder *et al.,* 2010; Berendsen *et al.,* 2012).

In the past 10 years, the role of genetic factors, plant hormones and nutrients on root biomass, root branching and root absorptive capacity has been studied in detail in various plant species (López-Bucio *et al.,* 2003, 2005; De Dorlodot *et al.,* 2007; Hochholdinger and Tuberosa, 2009; Coudert *et al.,* 2010). Many plant symbionts detect signals derived from roots for colonization, and use plant carbon sources such as organic acids, amino acids and sucrose for nutrition. Indeed, root colonization represents increased sink strength, thus providing an additional level of complexity in modulating plant growth through sugar distribution. Uptake, exchange and competition for sugar at plant–fungus membranes seem to be essential in determining the outcome of the *Trichoderma*–maize interactions (Vargas *et al.,* 2009) and we may think that both plants and fungi benefit from an increased root absorptive capacity, thus providing a new avenue to explore towards potential agricultural applications. The available data highlight the need for a better comprehension of cellular and molecular mechanisms involved in signalling exchange between fungi and their host plants.

10.2 The Rhizosphere and Plant Fitness

The capacity of plants to survive adverse conditions and reach reproductive maturity critically depends on their ability to continuously adapt to changes in the environment. Plants have therefore evolved an array of intricate regulatory mechanisms that involve the generation of signalling molecules mediating the activation of adaptive responses: in particular, the activation of pathogen-specific defence mechanisms upon infection, as well as the acquisition of architectural and physiological adjustments that permits survival, development and reproduction (Ortiz-Castro *et al.,* 2009).

Many complex interactions between plants and microorganisms occur at the rhizosphere, the soil zone in close contact with roots. The root system performs the essential activities of providing water, nutrients and physical support to the plant. The primary root originates in the embryo and produces many lateral roots during vegetative growth, and each of these will produce more lateral roots. The quantity and placement of these structures determine the architecture of the root

system, and this in turn plays a major role in determining whether a plant will survive in a particular climate or environment (Malamy and Benfey, 1997; Casimiro *et al.,* 2003; López-Bucio *et al.,* 2005). During the post-embryonic development of plants, new axes of growth emerge from shoot tissues through adventitious organogenesis. This is particularly important in crops such as maize, in which adventitious root formation provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or after injury (Hochholdinger and Tuberosa, 2009). Although lateral roots typically form from lateral root primordia initiated on the primary root pericycle, adventitious roots form naturally from stem tissue. Lateral and adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones such as auxin, and environmental factors such as light and nutrient deprivation (Casimiro *et al.,* 2003; López-Bucio *et al.,* 2003; Péret *et al.,* 2009).

A further adaptation to take in water and nutrients is performed by root hairs. These are long tubular-shaped outgrowths from root epidermal cells. In *Arabidopsis*, root hairs are approximately 10 μm in diameter and can grow to be 1 mm or more in length. A single rye (*Secale cereale* L.) plant may have 14 billion root hairs that provide 400 m^2 of surface area (Datta, 2011). Along with the vast increase in the root absorptive capacity and the root diameter conferred by root hairs, they are generally thought to aid plants to interact with microbes. This has been particularly demonstrated in the *Rhizobium*–legume symbiosis, in which a root hair forms a channel allowing penetration of the bacteria into the root tissues to form N-fixing nodules (Marx, 2004). Root hairs play an important role in the uptake of sparingly soluble nutrients that have low diffusion in the soil, such as phosphate. Because they have a small radius, root hairs explore a larger volume of soil per unit of surface area than thicker lateral roots. Root hairs also play a role in modulating the properties and composition of the rhizosphere because they exude high quantities of organic compounds, including organic acids, amino acids, sugars, proteins, mucilage, phenolics and secondary metabolites. In *Sorghum* spp. exudates seem to be produced solely by root hairs (Czarnota *et al.,* 2003) and exudation is positively correlated with root hair number and density (Yan *et al.,* 2004).

Root exudates perform diverse functions in the rhizosphere including mineral weathering, mobilization of nutrients, metal detoxification and growth inhibition of pathogenic bacteria, invertebrate herbivores or neighbouring plants (Badri and Vivanco, 2009). Some compounds such as organic acids can act as chemotactic signals to attract symbiotic fungi and bacteria (Rudrappa *et al.,* 2008), whereas sugar plays a fundamental role in interactions with mycorrhizal fungi and *Trichoderma* (Vargas *et al.,* 2009, 2011).

Microorganisms and plants emit signalling molecules for communication. Plants are able to recognize microbe-derived compounds and adjust their defence and growth responses according to the type of microorganism encountered. This molecular dialogue will determine the final outcome of the relationship, ranging from pathogenesis to symbiosis, usually through highly coordinated cellular processes (Ortiz-Castro *et al.,* 2009). Regarding their positive effects on plant growth, many rhizobacterial or fungal species that elicit plant biomass production or increase crop performance have been used as biofertilizers. Plant-growth-promoting rhizobacteria (PGPR) are natural rhizosphere-inhabiting bacteria that belong to diverse genera such as *Pseudomonas* and *Bacillus* species (Soleimani *et al.,* 2005). The general effect of PGPR is an increased growth and productivity of plants. Their contribution can be exerted through different mechanisms including modulation of root system architecture and increased biomass production through the release of phytohormones such as auxins or cytokinins (Lugtenberg *et al.,* 2002; López-Bucio *et al.,* 2007; Ortíz-Castro *et al.,* 2009). Besides, several fungi such as mycorrhizas, *Piriformospora indica* and *Trichoderma* spp. can interact with plants in many beneficial ways, some of which resemble those of PGPR.

Below, we present and discuss recent information on the mechanisms of growth promotion by the biocontrol agents of the *Trichoderma* genus.

10.3 Beneficial Effects of *Trichoderma* on Plants

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi and compete with deleterious plant microorganisms (Harman *et al.,* 2004a). Until recently, these traits were considered to be the basis for how *Trichoderma* exert beneficial effects on plant growth and development. It is clear, however, that certain strains also have substantial direct influence on plant development and crop productivity (Fig. 10.1) (Harman, 2006, 2011).

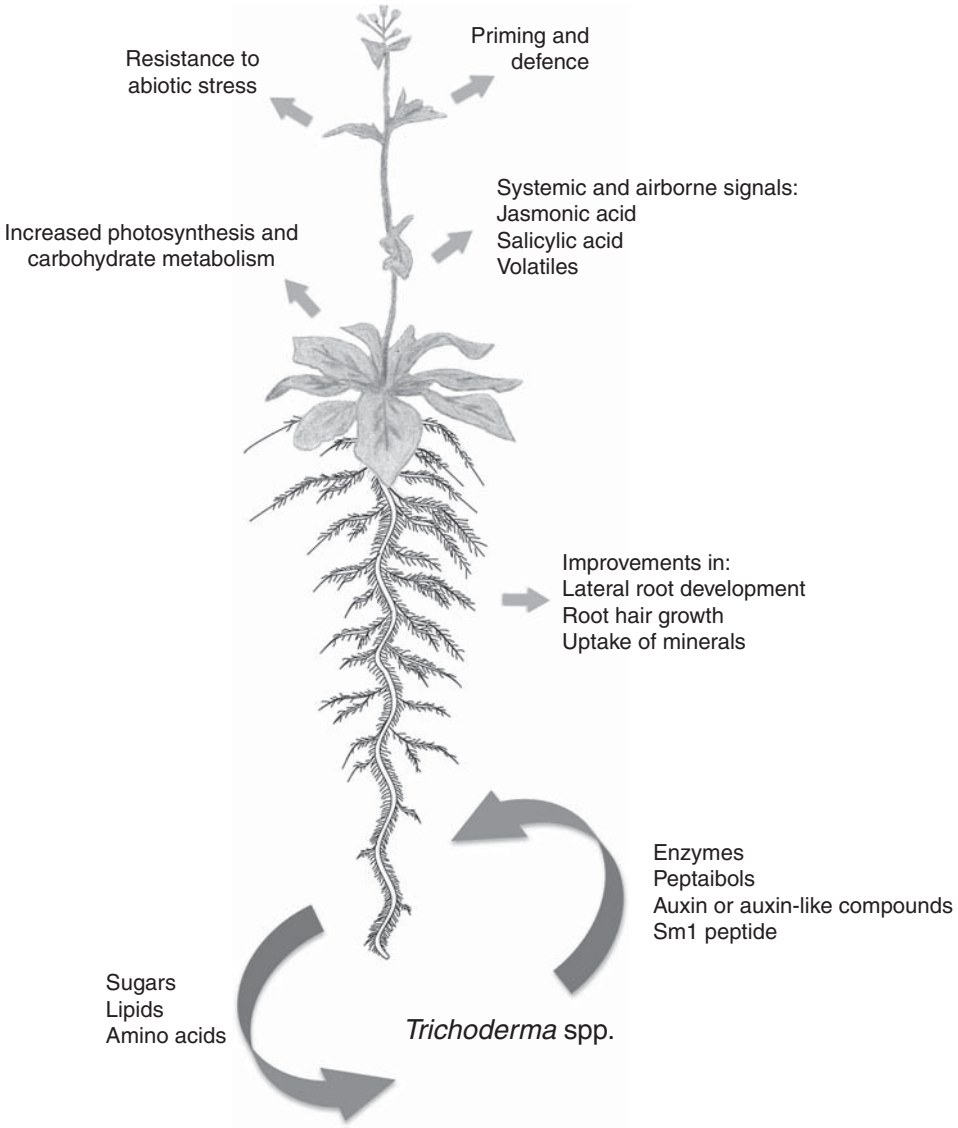

Fig. 10.1. Promotion of plant growth by *Trichoderma*. Root-derived nutrient sources such as sugars, lipids, organic acids and amino acids attract fungal symbionts, which colonize the root system and increase its absorptive capacity through root hair and lateral root production. Root colonization triggers the fungal and/or plant emission of diffusible signals such as jasmonic acid, salicylic acid and volatiles that increase photosynthesis, activate defence responses and confer protection against abiotic stress in distant parts of the plant.

10.3.1 Regulation of plant growth and development

Trichoderma enhancement of plant growth has been known for decades and can occur in axenic systems or in soil. Early reports of the effects of *Trichoderma* spp. on floricultural and horticultural crops such as cucumber, periwinkle and chrysanthemum indicated that these fungi impact on seed germination, flowering and vegetative growth (Chang *et al.,* 1986). It was reported that cucumber seedlings grown in soil amended with *Trichoderma harzianum* propagules sustain a 30% increase in seedling emergence 8 days after sowing. Three weeks later, these plants exhibited a 95% and 75% increase in root area and cumulative root length, respectively, and substantial increases in dry weight (80%), shoot length (45%) and leaf area (80%) were registered (Yedidia *et al.,* 2001). This report showed the correlation between increased root growth and shoot biomass production, which has been confirmed in maize plants (Bjorkman *et al.,* 1998; Harman *et al.,* 2004b; Vargas *et al.,* 2009).

In a study comparing the effects of *T. harzianum* Rifai 1295-22 (also known as 'T22') and commercial formulations of ectomycorrhizal fungi in the establishment and growth of crack willow (*Salix fragilis*), Adams *et al.* (2007) found that after 5 weeks of growth in soil, tree saplings grown with *T. harzianum* T22 produced shoots and roots that were 40% longer than those of the controls and shoots that were 20% longer than those of saplings grown with ectomycorrhiza. Moreover, *T. harzianum* T22 saplings produced more than double the dry biomass of controls and more than 50% extra biomass than the ectomycorrhiza-treated saplings. These results highlight the potential of *Trichoderma* for establishment and early growth of trees in forest plantations. More recently, Tucci *et al.* (2011) showed that genetic variability among wild and cultivated tomato lines affected the outcome of the interaction with *Trichoderma atroviride* and *T. harzianum*. The beneficial response, which included enhanced growth and systemic resistance against the necrotrophic fungus *Botrytis cinerea*, was evident for some, but not all, of the tested lines. At least in one case (line M82), treatment with *Trichoderma* had no effect or was even detrimental for plant growth. In contrast, Azarmi *et al.* (2011) reported the beneficial effects of three *Trichoderma* isolates including *T. harzianum* isolate T969, *T. harzianum* isolate T447 and *Trichoderma* sp. isolate T in tomato seedling vigour. *Trichoderma* spore suspension was supplied either directly to seeds or to nursery soil with *Trichoderma*-fortified wheat. Seed germination rate was not affected by *Trichoderma* application, but shoot height, shoot diameter, shoot fresh and dry weight as well as root fresh and dry weight in tomato seedlings were increased when sown in *Trichoderma* sp. T and *T. harzianum* T969 fortified soil. Plants grown on soil amended with *Trichoderma* sp. T and *T. harzianum* T969 also had marked increases in leaf number, leaf area and chlorophyll content (Azarmi *et al.,* 2011).

The interaction between *T. harzianum* CECT 2413 strain and the tomato-root system was also studied during the early stages of root colonization by the fungus. When *T. harzianum* conidia were inoculated into the liquid medium of hydroponically grown tomato plants, profuse adhesion of hyphae to the roots as well as colonization of the root epidermis and cortex was observed. Confocal microscopy of a *T. harzianum* transformant that expressed the green fluorescent protein (GFP) revealed intercellular hyphal growth and the formation of plant-induced papilla-like hyphal tips. Analysis of the *T. harzianum*–tomato interaction in soil indicated that the contact between the fungus and roots persisted over a long period of time (Chacón *et al.,* 2007).

Arabidopsis thaliana has been established as an excellent model system to study the genetic and physiological mechanisms of *Trichoderma*–plant interactions and the influence of fungi on the basic elements of root architecture and adaptation to the environment. This has been possible because of the vast knowledge gained from plant developmental programmes, the availability of mutants and gene reporter lines, the small size of the plant and the ability to test the interaction under axenic conditions (Contreras-Cornejo *et al.,* 2009, 2011). *Trichoderma virens* and *T. atroviride* were found to promote *Arabidopsis* seedling growth with significant increases in root and shoot biomass production. Promotion of plant growth elicited by *Trichoderma* correlated

with prolific formation of lateral roots in wild-type plants of the Columbia-0 (Col-0), Wassilewskija (Ws) and Landsberg erecta (Ler) ecotypes (Fig. 10.2).

In order to detect any potential deleterious effect of *T. virens* or *T. atroviride* in *Arabidopsis*, a separate study was conducted to test temporal responses of plants to varied concentrations of conidia (Contreras-Cornejo *et al.*, 2011).

Fig. 10.2. *Trichoderma virens* and *Trichoderma atroviride* promote root branching in *Arabidopsis* seedlings. Photographs of *Arabidopsis* seedlings (ecotype Landsberg erecta, Ler) grown in a 0.2 × Murashige and Skoog medium and co-cultivated with *T. virens* or *T. atroviride*. Notice the great stimulatory effect of the fungi on lateral root formation.

Total plant biomass in control or inoculated seedlings was determined every two days after inoculation (dai). During the first two days, no significant differences were observed in biomass production between control plants and plants co-cultivated with *T. virens* or *T. atroviride*. However, from 4 to 8 dai, a 40% increase in total fresh weight was evident in *Trichoderma* co-cultivated plants. In this work, enhanced lateral root proliferation was a typical response of *Arabidopsis* roots colonized by the mycelia of *T. virens* or *T. atroviride*, and no deleterious symptoms such as chlorosis or necrosis could be observed in leaves. Interestingly, co-cultivation with *Trichoderma* increased anthocyanin production in leaves and the plants were more robust and greener, probably as a result of enhanced nutrient efficiency (Contreras-Cornejo *et al.*, 2011). Similar effects have been described in maize plants grown under field conditions (Harman *et al.*, 2011).

All the above-described information shows the potential of *Trichoderma* spp. stimulating the growth in a wide variety of plant families. Once the interaction with roots has been established, the growth-promoting effects to plants can last for the entire life cycle of the plant because the fungus continues to colonize the root system. The ability of *Trichoderma* to induce developmental changes in plants, resulting in improved root systems, may provide a competitive advantage through different mechanisms.

10.3.2 Contributions to plant nutrition

Plant growth and biomass production requires an adequate supply of nutrients, which act as structural components of cells or play roles in metabolism. Sixteen chemical elements are known to be important for plant growth and reproduction. They are divided into two main groups: macronutrients and micronutrients. Although both groups of elements are essential for plants to complete their life cycles, macronutrients such as N, P and K are required in greater amounts. In the soil–plant interface, both macronutrients and micronutrients undergo a complex dynamic equilibrium of

solubilization, uptake and transport that is greatly influenced by the soil pH and rhizospheric microorganisms. Certain nutrients such as N and P can directly acts as signals that alter post-embryonic root development, modifying primary and lateral root growth and root hair formation (López-Bucio *et al.,* 2003).

Roots interact with diverse populations of soil microorganisms, which have significant implication for growth and nutrition. Soil nutrients are transferred towards the root surface through the rhizosphere or, in the case of roots associated with mycorrizal fungi, through the fungal hyphae, prior to acquisition (Richardson *et al.,* 2009). Most plant species improve their mineral nutrition with the help of beneficial microorganisms such as fungi and bacteria, some of which are important in N fixation, P solubilization and micronutrient uptake (Tallapragada and Gudini, 2011). *Trichoderma* species may enhance nutrient uptake either by modifying RSA or through the exudation of substances that increase nutrient availability.

The potential of the biocontrol agent *T. harzianum* strain T-203 (later on identified as *Trichoderma asperellum* and recently as *Trichoderma asperelloides*) to induce a growth response in cucumber plants was correlated with improvements in the nutrition of plants. An increase of 90% and 30% in P and Fe concentration, respectively, was observed in *T. harzianum*-inoculated plants. An increased growth response was apparent as early as 5 days post-inoculation, resulting in an increase in root and shoot biomass production with a concomitant elevation in the concentration of Cu, P, Fe, Zn, Mn and Na in inoculated roots. In the shoots of these plants, the concentration of Zn, P and Mn increased by 25, 30 and 70%, respectively (Yedidia *et al.,* 2001). *T. harzianum* 1295-22 was reported to increase the solubility of P and several micronutrients such as Fe, Mn and Zn in a liquid sucrose–yeast extract medium *in vitro* (Altomare *et al.,* 1999). Rudresh *et al.* (2005) reported the ability of nine isolates of *Trichoderma* spp. to solubilize insoluble phosphate as compared with an efficient phosphate-solubilizing bacterium *Bacillus megaterium* subsp. *phospaticum* PB that was used as the reference strain. All nine *Trichoderma* isolates were found to solubilize tricalcium phosphate to various extents. *T. viride* (TV 97), *T. virens* (PDBCTVs 12), and *T. virens* (PDBCTVs 13) solubilized 70% of that solubilized by the reference bacterial strain. Pot culture and field evaluations further demonstrated that *T. harzianum* (PDBCTH 10), *T. viride* (TV 97), and *T. virens* (PDBCTVs 12) increased P uptake in chickpea (*Cicer arietinum* L.) plants supplied with rock phosphate as P source, which correlated with growth and yield parameters. *T. harzianum* retained its P solubilizing potential at varying concentrations of cadmium, indicating that *Trichoderma* may provide advantages to plants even in soils polluted with heavy metals (Rawat and Tewari, 2011). In another study, *T. harzianum* isolate T969, increased the concentrations of Ca^{2+}, Mg^{2+}, P and K compared with the control, with positive effects on shoot height, shoot diameter, and shoot fresh and dry weights in tomato seedlings (Azarmi *et al.,* 2011).

The use of high quantities of chemical fertilizers in agriculture causes pollution of soils and water bodies. Thus, a major goal of biotechnology is to develop novel strategies to optimize fertilizer use. With this aim, Molla *et al.* (2012) tested the ability of *Trichoderma* spp. to increase growth of tomato plants when supplied together with fertilizer. It was found that supplementation of fertilizer with *Trichoderma* enhanced plant production by 50% compared with a standard dose of NPK macronutrients, minimizing the use of fertilizer and their potential negative effects in the environment. A recent application in the field came from manipulation by genetic means of the *T. harzianum qid74* gene, which encodes a cysteine-rich cell-wall protein (Samolski *et al.,* 2012). Microscopic observations revealed more and longer root hairs in cucumber plants treated with the *qid74*-overexpressing strains and fewer and shorter hairs in roots treated with *qid74*-disrupted transformants, compared with those observed in plants inoculated with the wild-type strain. Modifications in root architecture induced by *qid74* increased the total absorptive surface, facilitating nutrient uptake and translocation of nutrients to the shoots, resulting in increased plant biomass through an efficient use of NPK and micronutrients. The nutrient uptake improvements in

plants conferred by *Trichoderma* spp. present the opportunity for more sustainable agricultural practices with a high yield, low cost and less polluting effects to fulfill the current demand for plant-derived products.

10.3.3 Induction of defence responses

It is generally believed that plants activate defence responses upon pathogen or insect attack. This means that plants save energy under enemy-free conditions and could invest photosynthetically fixed carbon in growth and reproduction. Interestingly, some types of soil can suppress the symptoms of plant diseases. Research has shown that the observed increased resistance in these plants is the result of the presence of rhizosphere microorganisms, including bacterial and fungal species, which exert their protective effect by directly inhibiting the growth of pathogens or by means of the activation of a part of the plant's immune system (Pieterse *et al.,* 2009). Plants possess various inducible defence mechanisms for protection against pathogens. An example of this is systemic acquired resistance (SAR), which is activated by a wide range of pathogens, especially those that cause tissue necrosis (Ryals *et al.,* 1996). Similarly, colonization of plant roots by certain non-pathogenic rhizobacteria can activate induced systemic resistance (ISR) in the host plant (Van Loon *et al.,* 1998; Conrath, 2011). Both pathogen-induced SAR and rhizobacteria-mediated ISR are effective against different types of pathogens, and are typically characterized by a restriction of pathogen growth and a suppression of disease development compared with primary infected, non-induced plants.

The signalling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR differ. Whereas SAR requires endogenous accumulation of salicylic acid (SA), the signalling pathway controlling ISR functions independently of SA and requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene (Pieterse *et al.,* 2009). Additionally, it has been established that accumulation of phytoalexins and other low molecular weight antimicrobial metabolites is integral to plant protection (Glawishnig, 2007). The chemical structures of phytoalexins vary among different plant families and include flavonoids, terpenoids and indoles. According to the classical vision, SA and JA play antagonistic relationships during defence responses. However, this traditional view of ISR seems to be more complex (Niu *et al.,* 2011), a notion that is confirmed by recent information on *Trichoderma*–plant interactions (Segarra *et al.,* 2007; Korolev *et al.,* 2008; Contreras-Cornejo *et al.,* 2011).

Several pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), microbe effectors or wound stimuli can initiate a stereotypical defence response, which involves the so-called priming of cells, both in tissues exposed to the stimuli and also in distant parts of the plant. Priming is defined as the physiological state that enables plants to respond to a stimulus in a very efficient way, in a more rapid and robust manner than non-primed plants. Priming has been found to be crucial in various types of systemic plant immunity, including SAR and ISR (Conrath, 2011). Like plant beneficial rhizobacteria, *Trichoderma* can induce priming for enhanced defence in plants (Alfano *et al.,* 2007; Mathys *et al.,* 2012). The mechanisms underlying this process are starting to be revealed (Segarra *et al.,* 2007; Contreras-Cornejo *et al.,* 2011; Mathys *et al.,* 2012).

The characterization of two kinds of ISR elicitors secreted by *T. virens* Gv29-8 has been described. Peptides with antimicrobial activity termed peptaibols have ISR effects and systemically induce defences in cucumber leaves (Viterbo *et al.,* 2007). The second ISR elicitor is the extracellular small protein Sm1, the gene expression of which was up-regulated in the presence of cotton plants (Djonovic *et al.,* 2006). Further *in vivo* studies, using reverse genetic analyses, demonstrated that expression of *SM1* is essential for triggering ISR in maize plants and providing protection against the foliar pathogen *Colletotrichum graminicola* (Djonovic *et al.,* 2007). In maize, the metabolic pathways that lead to the establishment of Sm1-mediated ISR involve the signalling networks associated with SA, green leafy volatiles and JA metabolism and

seem to be independent of pathogenesis-related proteins (Djonovic *et al.,* 2007).

Several recent reports have confirmed that the primed state of plants inoculated with *Trichoderma* is modulated by an intricate network of signalling pathways. Treatment with *Trichoderma hamatum* T382 primes *Arabidopsis* plants, resulting in an accelerated activation of the defence response against *B. cinerea* (Mathys *et al.,* 2012). Normalized microarray data were used to identify genes that were differentially expressed during priming, which were classified as involved in the plant-type hypersensitive response, as responsive to chitin and as defence-related genes responsive to SA and abscisic acid. Priming was also characterized by anthocyanin production and the stimulation of the transport of a variety of compounds in the plant such as phospholipids and ions (Mathys *et al.,* 2012).

The determination of plant growth regulators involved in the primed state induced by *Trichoderma* has confirmed the production of the phytohormones JA and SA in leaves. In the first hours of interaction between cucumber plant roots and *Trichoderma asperellum* strain T34, SA and JA levels and peroxidase activity increased in the cotyledons to different degrees, depending on the applied concentration of fungi (Segarra *et al.,* 2007). During co-cultivation of *Arabidopsis* roots with *T. virens* or *T. atroviride*, an induction of hydrogen peroxide, SA and JA was observed in leaves, which correlated with induction of pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA* (Contreras-Cornejo *et al.,* 2011). It was also found that both *T. virens* and *T. atroviride* increased accumulation of camalexin, a characteristic phytoalexin of *Arabidopsis*, in plants. All these combined responses seem to contribute to the *Trichoderma*-conferred resistance to *B. cinerea* because *Arabidopsis* mutants defective in genes from the respective pathways are compromised in the protection conferred by the biocontrol agents (Mathys *et al.,* 2012). Interestingly, co-cultivation of *Arabidopsis* seedlings with *T. virens* mutants defective in the 4-phosphopantetheinyl transferase 1 gene (*PPT1*) compromised the SA and camalexin responses, resulting in decreased protection against the pathogen (Velázquez-Robledo *et al.,* 2011). These data are in agreement with the gene expression data of Mathys and coworkers (2012), who observed a close similarity between *Trichoderma*-induced priming and SAR.

10.4 The Auxins from *Trichoderma*: Comparison with Other Plant Symbionts

Plants synthesize and use a variety of signals to adjust growth and development throughout their life cycle. Auxins, including indole-3-acetic acid (IAA), comprise a group of tryptophan (Trp)-derived signals that are involved in most aspects of plant development (Woodward and Bartel, 2005). Extensive studies over the past decade have investigated the factors involved in the regulation of plant morphogenesis by auxins. These compounds exert a strong biological activity at very low concentrations in both *in vivo* and *in vitro* systems and are essential for the maintenance of physiological and morphogenetic processes including gravity and light responses, root hair development, lateral root, adventitious root and shoot system development (Woodward and Bartel, 2005). Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms, and signal perception through a family of IAA receptors (Ljung *et al.,* 2002; Leyser, 2006; Mockaitis and Estelle, 2008).

Although the role of auxin signalling in symbiosis between plants and fungi still remains controversial, genetic evidences indicating that IAA is a positive regulator of plant growth comes from the analysis of *Arabidopsis* mutants that overproduce it, such as *super root* and *yucca*, which have long hypocotyls and increased numbers of lateral roots and root hairs (Boerjan *et al.,* 1995). Moreover, the positive effect of IAA application on growth of excised stems and hypocotyls and of auxin analogues in intact *Arabidopsis* seedlings has been described (Zhao *et al.,* 2001). The architecture of the root system is modified by the

endogenous auxin level and environmental stimuli that increases the auxin pool in the plant and/or affect auxin sensitivity such as temperature and the availability of water and mineral nutrients (Himanen *et al.,* 2002; López-Bucio *et al.,* 2003; Pérez-Torres *et al.,* 2008). A recent report has further shown that auxin-like signals produced from rhizosphere microorganisms could increase the exploratory capacity of the root system in *Arabidopsis* with a dramatic impact in plant biomass production (Ortiz-Castro *et al.,* 2011).

The potential of plant-associated microorganisms to produce free IAA, as already reported for *Trichoderma* spp., represents a means to influence the endogenous auxin pool of the host (Contreras-Cornejo *et al.,* 2009; Felten *et al.,* 2012; Hilbert *et al.,* 2012). Little is known, however, about the implication of this hormone in symbiosis. Auxin has often been suggested to play a role in the crosstalk between plant and fungal signalling during ectomycorrhizal establishment and in the colonization of barley roots by *Piriformospora indica* (Felten *et al.,* 2009, 2012; Hilbert *et al.,* 2012).

The early phase of the interaction between tree roots and ectomycorrhizal fungi, prior to symbiosis establishment, is accompanied by stimulation of lateral root development. For instance, plant inoculation with an IAA-overproducing strain of the ectomycorrhizal fungus *Hebeloma cylindrosporum* resulted in a faster and deeper colonization of the root compared with the wild-type strain and in a faster transcriptional response in the plant (Tranvan *et al.,* 2000). Another ectomycorrhizal fungus, *Laccaria bicolor*, increased lateral root development in poplar (*Populus tremula* × *Populus alba*) and *Arabidopsis*, which correlated with an increase in auxin accumulation at root apices. Blocking plant polar auxin transport with 1-naphthylphthalamic acid inhibited lateral root development and auxin accumulation. An oligoarray-based transcript profile of poplar roots exposed to molecules released by *L. bicolor* revealed the differential expression of 2945 genes, including several components of polar auxin transport (*PtaPIN* and *PtaAUX* genes), auxin conjugation (*PtaGH3* genes), and auxin signalling (*PtaIAA* genes). Transcripts of *PtaPIN9*, the homologue of *Arabidopsis AtPIN2*, and several *PtaIAAs* accumulated specifically during the early interaction phase (Felten *et al.,* 2009). These results reveal a critical role for auxin in root interactions with ectomycorrhiza.

Piriformospora indica, a newly described cultivable endophyte that colonizes roots, has been found to promote plant growth during its symbiotic relationship with a wide variety of plants (Waller *et al.,* 2005). *P. indica* can produce the phytohormones IAA and indole-3-lactate (ILA) through the intermediate indole-3-pyruvic acid (IPA). Time-course transcriptional analyses after exposure to tryptophan identified the tryptophan aminotransferase (*piTam1*) gene as a key player. *P. indica* strains in which the *piTam1* gene was silenced via an RNA interference (RNAi) approach were compromised in IAA and ILA production and displayed reduced colonization of barley (*Hordeum vulgare*) roots, but the elicitation of growth was not affected (Hilbert *et al.,* 2012).

Trichoderma species produce auxins as part of their metabolism including IAA and its precursors indole-3-ethanol, indole-3-acetaldehyde and indole-3-carboxaldehyde (Fig. 10.3; Contreras-Cornejo *et al.,* 2009, 2011).

The role of auxin signalling in *Trichoderma*–plant interactions was investigated in detail in *Arabidopsis thaliana* by Contreras-Cornejo and coworkers (2009). It was found that mutations in genes involved in auxin transport or signalling, *AUX1, BIG, EIR1* and *AXR1*, reduced the growth-promoting and root-developmental effects of *Trichoderma* inoculation. Colonization of plant roots by fungal hyphae activated the auxin-inducible reporter *DR5:uidA*, which correlated with an increased cell proliferation in primary and lateral root tips. Interestingly, the application of all three identified indolic compounds to *Arabidopsis* seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nM range) but repressing growth at higher concentrations (μM range). Furthermore, *T. virens* also produced indole-3-carboxaldehyde (ICAld), a compound related to IAA metabolism probably involved in camalexin biosynthesis (Fig. 10.3;

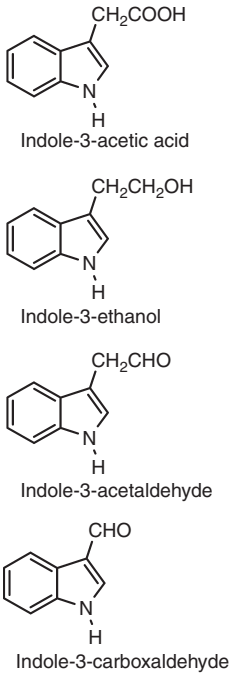

Fig. 10.3. Indolic compounds produced by *T. virens*. The chemical structures of all four compounds identified in *T. virens* cultures are shown. The levels of indoles increase when the culture medium is supplied with tryptophan (Trp), indicating that they probably derive from Trp metabolism.

Contreras-Cornejo *et al.*, 2011). The supply of ICAld to *Arabidopsis* seedlings inhibited primary root growth, induced adventitious root formation and increased camalexin levels (Contreras-Cornejo *et al.*, 2011).

At different stages of their life cycle, fungi release specific volatile organic compounds (VOCs) in order to interact with particular organisms (Splivallo *et al.*, 2011). *T. atroviride* produced at least 25 different VOCs including alcohols, ketones, alkanes, furanes, pyrones (mainly the bioactive 6-pentyl-alpha-pyrone), monoterpenes and sesquiterpenes (Stoppacher *et al.*, 2010). Vinale and co-workers (2008) reported an auxin-like effect in etiolated pea stems treated with harzianolide and 6-pentyl-α-pyrone, common metabolites produced by *Trichoderma* strains (Vinale *et al.*, 2008; Hermosa *et al.*, 2012). Certain VOCs from rhizobacteria could affect auxin biosynthesis and transport and have been proposed as candidate signals for plant growth promotion by PGPR (Zhang *et al.*, 2007). The possibility that VOCs from *Trichoderma* spp. could affect auxin homeostasis in plants remains to be determined.

The reported findings about the role of fungal-produced IAA in different plant–fungus interacting systems presents the possibility that fungi may use IAA and related compounds to communicate with plants as part of its colonization strategy, leading to plant growth stimulation and modification of basal plant defence mechanisms (Prusty *et al.*, 2004; Kazan and Manners, 2009). We speculate that the effects of inoculation with *Trichoderma* in plants under natural conditions may depend on the type and concentration of auxins produced by the fungi as well as the production of volatiles or auxin signal mimics. Perhaps auxin signalling may also play a role in colonization of roots by *Trichoderma* as already described for mycorrhizal fungi and *P. indica*. Confirmation of this hypothesis requires further experimentation.

10.5 Genes Regulated in the *Trichoderma*–Plant Interaction

With the advent of the genomics era, it has been possible to analyse the fungal and plant genes that are regulated in fungi during the interaction with plants as well as the plant genes responsive to root colonization by Trichoderma (Table 10.1).

In the following section, we will summarize the role played by some relevant genes in the plant–fungi interactions.

10.5.1 *Trichoderma* genes

To study the molecular mechanisms underlying the fungal ability to colonize the roots of tomato, the *T. harzianum* transcriptome was analysed during the early stages of the plant–fungus interaction. The expression of fungal genes related to redox reactions, lipid metabolism, detoxification and sugar or amino-acid transport increased in *T. harzianum* colonized roots (Chacón *et al.*, 2007). In another report, gene expression analysis of *T. harzianum* in the presence of tomato plants, chitin or glucose was performed through a high-density

Table 10.1. Genes involved in *Trichoderma*–plant interactions.

Fungal genes			
Gene name	Interaction	Function	References
Endopolygalacturonase 1 (*Thpg1*)	*T. harzianum*–Tomato	Plant cell wall-degrading enzyme involved in root colonization and defence responses	Morán-Diez *et al.,* 2009
Invertase (*TvInv*)	*T. virens*–Maize.	Sugar metabolism	Vargas *et al.,* 2009
Cysteine-rich cell wall protein (*qid74*)	*T. harzianum*–Tomato	Adherence to hydrophobic surfaces and cell protection	Samolski *et al.,* 2012
1-Aminocyclopropane-1-carboxylate deaminase (*Tas-acdS*)	*T. asperellum T203*–Canola	Cleaves 1-aminocyclopropane-1-carboxylate to produce ethylene	Viterbo *et al.,* 2010
Small protein (*SM1/Epl1*)	*T. virens*–Cotton, maize *T. atroviride*–Maize	Induction of defence responses	Djonović *et al.,* 2006; Vargas *et al.,* 2008
4'-Phosphopantetheinyl transferase (*PPT1*)	*T. virens–Arabidopsis*	Activates enzymes involved in primary and secondary metabolism	Velázquez-Robledo *et al.,* 2011
Xylanase (*TvX/EIX*)	*T. viride*–Tobacco	β-1,4-endoxylanase, elicits plant defence responses and emission of ethylene	Sharon *et al.,* 1993
Swollenin (*TasSwo*)	*T. asperellum*–Cucumber	Involved in defence responses	Brotman *et al.,* 2008
Plant genes			
Transcription factor MYB77 (*MYB77*)	*T. asperelloides* T203–*Arabidopsis*	Modulates plant responses to auxin	Brotman *et al.,* 2012
Auxin responsive (*IAA29*)	*T. harzianum–Arabidopsis*	Member of the family of auxin repressors Aux/IAA	Morán-Diez *et al.,* 2012
Hookless 1 (*HLS1*)	*T. asperelloides* T203–*Arabidopsis*	Auxin signalling and cell growth	Brotman *et al.,* 2012
Root hair deficient (*RHD6*)	*T. virens–Arabidopsis.*	Cell differentiation	Contreras-Cornejo *et al.,* 2009
High indolic glucosinolate or transcription factor MYB51 (*HIG1/MYB51*)	*T. asperelloides* T203–*Arabidopsis*	Regulator of indolic glucosinolate biosynthesis	Brotman *et al.,* 2012
Transcription factor WRKY40 (*WRKY40*)	*T. asperelloides* T203–*Arabidopsis.*	Transcription factor induced by pathogens	Brotman *et al.,* 2012
Pathogenesis-related 1 (*PR-1*)	*T. virens*/*T. atroviride–Arabidopsis*	Defence responses (encodes a defensin)	Contreras-Cornejo *et al.,* 2011
Pathogenesis-related 2 (*PR-2*)	*T. asperellum* T203–Cucumber	β-1,3-glucanase.	Shoresh *et al.,* 2005
Chitinase (*EgCHI1/2/3*)	*T. harzianum*–Oil palm.	Hydrolizes glycosidic bonds in chitin	Naher *et al.,* 2012
Pathogenesis related 3 (*PR-3*)	*T. asperellum* T203–Cucumber.	Chitinase	Shoresh *et al.,* 2005

Lipoxygenase 1 (*LOX1)*	*T. asperellum* T203–Cucumber.	Biosynthesis of JA	Shoresh *et al.,* 2005
Lipoxygenase 2 (*LOX2*)	*T. virens*/*T. Atroviride–Arabidopsis*	Biosynthesis of JA	Contreras-Cornejo *et al.,* 2011
Phenylalanine ammonia lyase (*PAL*)	*T. asperellum* T-203–Cucumber *T. virens*–Maize	Biosynthesis of SA	Yedidia *et al.,* 2003; Shoresh *et al.,* 2005, 2008; Mukherjee *et al.,* 2012
Glutathione S-transferase (*GST*)	*T. harzianum* T22–Maize	Cell detoxification	Shoresh *et al.,* 2008
Hydroxyperoxide lyase (*HPL*)	*T. asperellum* T-203–Cucumber	Production of antimicrobial and wound-related C_6-volatiles	Yedidia *et al.,* 2003
Chitinase (*ChiB*).	*T. stromaticum*–Cacao	Chitinase	De Souza *et al.,* 2008
Endo-1,4-β-glucanase (*Glu-1*)	*T. stromaticum*–Cacao	Endo-1,4-β-glucanase	De Souza *et al.,* 2008
Mitogen activated protein kinase 3 (*MAPK3*)	*T. hamatum*–Cacao	Signal transduction cascades that regulate defence responses and abiotic stress resistance	Bae *et al.,* 2009
Trichoderma-induced MAPK (*TIPK*)	*T. asperellum*–Cucumber	Defence and wound responses	Shoresh *et al.,* 2006
Ethylene-overproducing 3 (*ETO3*)	*T. asperellum* T203–Cucumber	Ethylene biosynthesis	Brotman *et al.,* 2012
Ethylene receptor 1 (*ETR1*)	*T. asperellum* T203–Cucumber; *T. asperelloides* T203–*Arabidopsis*	Defence responses	Shoresh *et al.,* 2005; Brotman *et al.,* 2012
Constitutive triple response 1 (*CTR1*)	*T. asperellum* T203–Cucumber	Defence responses	Shoresh *et al.,* 2005
Calcineurin B-like proteins-interacting protein kinase (*OsCIPK14/15*)	*T. viride*–Rice	Recognition of microbe-associated molecular patterns.	Kurusu *et al.,* 2010
Stearoyl–acyl carrier protein desaturase (*SAD1/2*)	*T. harzianum*–Oil palm	Regulates cellular polyunsaturated fatty acid content	Alizadeh *et al.,* 2011
Lipid transferase protein 4 (*LTP4*)	*T. asperelloides–Arabidopsis*	Lipid transferase involved in resistance induced by *Trichoderma*	Brotman *et al.,* 2011
R gene (*HR4*)	*T. atroviride–Arabidopsis*	Recognition of specific microbe factors as signals of invasion	Saenz-Mata and Jimenez-Bremont, 2012

oligonucleotide microarray analysis. The results revealed 1617 probe sets showing differential expression in *T. harzianum* mycelium under at least one of the culture conditions tested as compared with one another. Hierarchical clustering and heat map representation showed that the expression patterns obtained in glucose medium clustered separately from the expression patterns observed in the presence of tomato plants and chitin. Interestingly, some up-regulated transcripts were predicted to encode proteins related to *Trichoderma*–plant interactions, such as Sm1/Elp1, proteases P6281 and PRA1, enchochitinase CHIT42, or QID74 protein. In this study, previously uncharacterized genes were also identified, including those responsible for the possible biosynthesis of nitric oxide, xenobiotic detoxification, mycelium development, and others related to the formation of infection structures (Samolski *et al.,* 2009).

By using *in vitro* and *in vivo* assays with *T. harzianum* CECT 2413 (T34), *T. virens* Gv29-8 (T87) and *T. hamatum* IMI 224801 (T7), Rubio *et al.* (2012) showed that these strains affected the growth and development of lateral roots in tomato plants in different ways, with beneficial effects reported for strains T7 and T34. After 20 h of incubation in the presence of tomato plants, using a high-density oligonucleotide microarray, the authors showed that carbohydrate metabolism was the most significantly over-represented process commonly observed in the three *Trichoderma* strains with an induction of the chitin degradation enzymes *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and chitinase. Strains T7 and T34, both of which stimulate plant development, were found to enhance hexokinase activity and the transcription of genes encoding a 40S ribosomal protein and a P23 tumour protein orthologue.

As mentioned earlier, sugars exuded by roots into the rhizosphere are crucial nutrient sources for the symbiotic association between *Trichoderma* and plants (Vargas *et al*., 2009). By using several bioinformatics tools, two genes likely to be involved in the uptake of sucrose by *T. virens*, an intracellular invertase (TvInv) and a plant-like sucrose transporter (TvSut), were recently identified. Genetic, biochemical and physiological studies were conducted to characterize the role of sucrose on invertase activity in the fungus and in the interactions with maize plants (Vargas *et al.,* 2009). The loss-of-function on *tvsut* caused a detrimental effect on fungal growth when sucrose was the sole source of carbon in the medium, and also affected the expression of genes involved in the symbiotic association (Vargas *et al.,* 2011). These results show that *T. virens* contains genes for sucrose uptake and metabolism, which play an important role during early stages of root colonization. These exciting results provide new insights into the mechanisms and roles of fungal genes in the *Trichoderma*–plant interaction; it might be of further interest to investigate the contribution of nitric oxide released by fungal hyphae to root growth because recent information suggests that it affects primary root growth and induces lateral root formation (Fernández-Marcos *et al.,* 2011; Méndez-Bravo *et al.,* 2011).

10.5.2 Plant genes

Genes and proteins regulated by *Trichoderma* have been discovered and characterized in *Arabidopsis thaliana,* tomato, maize, cacao, chilli pepper and oil palm plants. The first evidence for auxin signalling in mediating the observed developmental alterations by *T. virens* inoculation in plants was inferred from tests using the auxin-responsive marker constructs *DR5:uidA, BA3:uidA* and *HS::AXR3NT-GUS* and the analysis of *aux1-7, doc1, eir1* and *axr1* auxin-related mutants of *Arabidopsis*. The *aux1-7* mutant is defective at the AUX1 locus, which encodes an auxin influx facilitator participating in both acropetal and basipetal auxin transport at the root tip (Swarup *et al.,* 2001); *doc1* is a mutant allele of *BIG*, which encodes a protein important for the correct location of certain auxin transport proteins (Gil *et al.,* 2001), whereas *eir1* encodes the auxin transporter AtPIN2 (Luschnig *et al.,* 1998). Five days after plants were inoculated, *T. virens* increased (by 62%) shoot fresh weight in wild-type seedlings when compared with uninoculated seedlings. In contrast, all four mutant lines, *aux1-7, doc1, eir1* and *axr1-3*, showed decreased or null responses in growth

promotion by the fungus. Interestingly, it was found that *T. virens* induced up to a fourfold increase in lateral root number when compared with axenically grown plants, a reduction in lateral root formation when compared with inoculated wild-type plants was observed for *aux1-7* and *axr1-3* inoculated seedlings, and no lateral root induction was registered for uninoculated or inoculated *doc1* seedlings. These results support the hypothesis that both normal auxin transport and response are important for the effects of *T. virens* on plant growth and lateral root development (Contreras-Cornejo *et al.*, 2009).

Plants have large collections of so-called resistance proteins that recognize specific microbe factors as signals of invasion. One of these proteins is coded by the *Arabidopsis thaliana HR4* gene in the Col-0 ecotype that is homologous to *RPW8* genes present in the Ms-0 ecotype. In a recent study, Saenz-Mata and Jiménez-Bremont (2012) investigated the expression patterns of the *HR4* gene in *Arabidopsis* seedlings interacting with *T. atroviride*. It was observed that the induction of the *HR4* gene mainly occurred at 96 h post-inoculation, at a time when the fungus directly interacted with roots. To examine the effect of phytohormones involved in biotic stress signalling on the *HR4* gene, 15-day-old *Arabidopsis* (Col-0) seedlings were sprayed with the ethylene donor ethephon, SA and methyl jasmonate (MeJA) and harvested at 1, 3 and 24 h after spraying. Ethephon treatment induced the *HR4* gene at 1 and 3 h by about threefold, and this induction was maintained at 24 h. For SA and MeJA treatment, a strong initial induction (about tenfold at 1 h) of this gene was observed. The *HR4* gene was also differentially regulated in interactions with the beneficial bacterium *Pseudomonas fluorescens* and the pathogenic bacterium *Pseudomonas syringae*. Although the functional relevance of the *HR4* gene or its homologues in the *Trichoderma*–plant interaction still needs to be investigated by mutant and transgenic means, these results indicate that *HR4* and *RPW8* genes could play a role in the establishment of *Arabidopsis* interactions with beneficial microbes.

The molecular basis of the ISR in *A. thaliana* by *T. hamatum* T382 against the phytopathogen *B. cinerea* B05-10 was unravelled by microarray analysis both before and after pathogen inoculation (Mathys *et al.*, 2012). In general, the defence responses elicited by *Trichoderma* alone were similar to those activated after SAR, the systemic defence response that is triggered in plants upon pathogen infection leading to increased resistance toward additional infections. Root colonization with *T. hamatum* T382 primed the plant, resulting in an accelerated activation of the defence response genes against *B. cinerea*, which were dependent upon SA and JA signalling and the phenylpropanoid pathway. The involvement of different defence-related pathways identified in this transcriptomic study was validated using phenotypic analysis of *A. thaliana* disease signalling mutants related pathways including *npr1, sid2* and NahG for the SA pathway, *ein2* and *etr1* for the ethylene pathway and *myc2* for the JA pathway, or in defence-related mechanisms such as *tt, chs* and *f3h*, all carrying mutations in the phenylpropanoid pathway. The suppressive effect on *B. cinerea* disease, as observed earlier in wild-type *Arabidopsis* plants pre-treated with *T. hamatum* T382, was not detected in most of these mutants, indicating that the corresponding genes and pathways play an important role in this interaction. Indeed, mutants corresponding to key genes in SA- or JA-mediated signalling, or anthocyanin production did not display the *T. hamatum* T382-induced ISR against *B. cinerea*.

A major challenge of studying model plants, such as *Arabidopsis*, is transferring the knowledge and new tools to crop species; transcriptomic and proteomic approaches have proven to be effective toward this goal. The proteome and transcriptome of plants change in response to root colonization by *Trichoderma*, indicating that these fungi reprogram the expression of plant genes. Alfano and co-workers (2007) showed that root colonization by *T. hamatum* T382 protected plants against bacterial spot of tomato (*Xanthomonas euvesicatoria* 110c). To gain insight into the mechanism by which *T. hamatum* T382 induced resistance in tomato, microarrays were used to determine its effect on the expression pattern of 15925 genes in leaves just before inoculation with the pathogen. *T. hamatum* T382

modulated the expression of genes in leaves and 45 genes were found to be differentially expressed with functions associated with biotic or abiotic stress, as well as RNA, DNA and protein metabolism. Four extensin and extensin-like proteins were induced. This work showed that *T. hamatum* T382 actively induces systemic changes in plant leaves and disease resistance through systemic modulation of the expression of stress- and metabolism-associated genes.

Endophytic *Trichoderma* isolates collected in tropical environments have been evaluated for changes in gene expression in cacao (*Theobroma cacao)* and chilli pepper (*Capsicum annuum*). During the interaction between cacao seedlings and four endophytic *Trichoderma* isolates, *Trichoderma ovalisporum*-DIS 70a, *T. hamatum*-DIS 219b, *T. harzianum*-DIS 219f and *Trichoderma* sp.-DIS 172ai, seven cacao genes were induced during root colonization. These included putative genes for ornithine decarboxylase (P1), GST-like proteins (P4), zinc finger protein (P13), wound-induced protein (P26), EF-calcium-binding protein (P29), carbohydrate oxidase (P59) and an unknown protein (U4). Two plant expressed sequence tags (ESTs), extensin-like protein (P12) and major intrinsic protein (P31), were repressed owing to colonization. The plant gene expression profile was dependent on the *Trichoderma* isolate colonizing the cacao seedling (Bailey *et al.,* 2006). Six additional endophytic isolates were tested for induced resistance capabilities in pepper. The isolates induced defence reactions and conferred protection against *P. capsici. Trichoderma* endophytic colonization induced multiple lipid transferase protein (LTP)-like family members. The timing and intensity of induction varied between isolates. Expression of *CaLTP-N*, encoding a LTP-like protein in pepper, in *Nicotiana benthamiana* leaves reduced disease development in response to *P. nicotianae* inoculation, suggesting LTPs are functional components of resistance induced by *Trichoderma* species (Bae *et al.,* 2011). An additional LTP (*LTP4*) was regulated during the systemic defence response of *A. thaliana* plants to the leaf pathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) mediated by the beneficial fungus *T. asperelloides* T203. Among the defence-related genes affected by T203, *LTP4* was up-regulated, whereas the *WRKY40* transcription factor, known to contribute to *Arabidopsis* susceptibility to bacterial infection, showed reduced expression (Brotman *et al.,* 2011).

These data and other recent discoveries demonstrate that fatty acid metabolism pathways play significant roles in pathogen defence in addition to phytohormone-mediated defence pathways (Christensen and Kolomiets, 2011). A key regulator in the fatty acid biosynthetic pathway is stearoyl–acyl carrier protein desaturase (SAD). Plant SAD is known to regulate cellular polyunsaturated fatty acid content. This enzyme also catalyzes conversion of saturated stearic acid (18:0) to monounsaturated oleic acid (18:1) and plays essential roles in maintenance of biological membrane structure, and synthesis of storage lipids and signalling molecules. Alizadeh and coworkers (2011) investigated the effects of *T. harzianum* in *SAD1* and *SAD2* gene expression in the oil palm (*Elaeis guineensis*), which is one of the most profitable oil-bearing crops. In *T. harzianum* inoculated seedlings, the expression levels of *SAD1* and *SAD2* increased gradually and were stronger in roots than in leaves, which was consistent with the protection conferred by this fungus against the pathogen *Ganoderma boninense*.

Proteomic approaches using two-dimensional gel electrophoresis and mass spectrometry have provided additional information on the protein profiles modulated by *Trichoderma* in plants, particularly focusing on systemic changes. In the first hours of interaction between cucumber roots and *T. asperellum* strain T34, SA and JA levels and peroxidase activity increased in the cotyledons to different degrees depending on the applied concentration of the fungi. These effects correlated with changes in 28 proteins, 17 of which were up-regulated while 11 were down-regulated. Proteins involved in reactive oxygen species (ROS) scavenging, stress response, isoprenoid and ethylene biosynthesis, and in photosynthesis, photorespiration and carbohydrate metabolism were differentially regulated by *Trichoderma* (Segarra *et al.,* 2007). Another study was conducted to investigate changes in the proteome of maize leaves induced by a seed treatment, and subsequent root colonization by *T. harzianum* T22 (Shoresh *et al.,* 2010).

A large portion of the up-regulated proteins have putative functions in carbohydrate metabolism, photosynthesis, stress and defence responses. Other processes that were up-regulated were amino acid metabolism, cell wall metabolism and genetic information processing. Up-regulation of carbohydrate metabolism, stress response and plant defence correspond well with the enhanced growth response and induced resistance conferred by the *Trichoderma* inoculation.

10.6 Concluding Remarks

Trichoderma-based bioinoculants are increasingly used in agriculture, with several hundred formulations available as registered products worldwide. Several strategies have been applied to identify the genes and signals involved in the interactions of *Trichoderma* with plants. Proteome and genome analysis in crops as well as genetic analysis in the model plant *Arabidopsis* have greatly enhanced our knowledge on the signalling pathways by which these biocontrol agents promote plant growth and activate defence responses. Using these different approaches, a variety of novel genes and gene products have been identified, including enzymes that allow the fungus to metabolize plant-derived sugars, elicitors of induced resistance and plant proteins specifically induced by *Trichoderma*. The *Trichoderma*–plant interaction can be viewed as a mycorrhiza-like system in several respects: (i) it depends on carbon sources supplied by plants; (ii) it requires physical contact and possibly internal proliferation of the fungus in plant tissues; (iii) it involves the exchange of IAA and auxin-related signals; (iv) it improves plant nutrition increasing N, P, K and micronutrient content, thus boosting photosynthesis and carbon metabolism; and (v) it activates defence responses through JA-, SA- and phytoalexin-dependent mechanisms.

The crosstalk between hormones at both physiological and molecular levels is receiving increasing importance, bringing a new understanding of how they are able to act either antagonistically or synergistically in a tissue-specific fashion to influence plant growth and defence responses. The hormonal crosstalk in the plant induced by *Trichoderma* is dynamic and the expression of growth and defence-related genes of the auxin, JA/ethylene and/or SA pathways may overlap, depending on the *Trichoderma* strains and the amount of inoculum, the plant species, the developmental stage of the plant and the timing of the interaction. Therefore, there is a need for more studies aimed at testing the functional relevance of genes and proteins whose expression is modulated during the interaction both in the plant and the fungi, as well as characterizing the phenotypes of loss-of-function mutants and overexpressing lines during *Trichoderma*–plant interactions in the presence of pathogens and/or different types of abiotic stresses. The use of *Trichoderma* mutants impaired in the production of volatiles or secondary metabolites will be a powerful tool to establish the ecological roles of these signals.

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11.5.
Trichoderma virens, a Plant Beneficial Fungus, Enhances Biomass Production and Promotes Lateral Root Growth through an Auxin-Dependent Mechanism in Arabidopsis[1][C][W][OA]

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Trichoderma species belong to a class of free-living fungi beneficial to plants that are common in the rhizosphere. We investigated the role of auxin in regulating the growth and development of Arabidopsis (*Arabidopsis thaliana*) seedlings in response to inoculation with *Trichoderma virens* and *Trichoderma atroviride* by developing a plant-fungus interaction system. Wild-type Arabidopsis seedlings inoculated with either *T. virens* or *T. atroviride* showed characteristic auxin-related phenotypes, including increased biomass production and stimulated lateral root development. Mutations in genes involved in auxin transport or signaling, *AUX1*, *BIG*, *EIR1*, and *AXR1*, were found to reduce the growth-promoting and root developmental effects of *T. virens* inoculation. When grown under axenic conditions, *T. virens* produced the auxin-related compounds indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-ethanol. A comparative analysis of all three indolic compounds provided detailed information about the structure-activity relationship based on their efficacy at modulating root system architecture, activation of auxin-regulated gene expression, and rescue of the root hair-defective phenotype of the *rhd6* auxin response Arabidopsis mutant. Our results highlight the important role of auxin signaling for plant growth promotion by *T. virens*.

Plant growth is affected by a plethora of environmental factors, including light, temperature, nutrients, and microorganisms. The region around the root, the rhizosphere, is relatively rich in nutrients, because as much as 40% of plant photosynthesis products can be lost from the roots (Bais et al., 2006). Consequently, the rhizosphere supports large microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

Trichoderma species are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi, and compete with deleterious plant microorganisms (Harman et al., 2004a). Until recently, these traits were considered to be the basis for how *Trichoderma* exert beneficial effects on plant growth and development. However, it is becoming increasingly clear that certain strains also have substantial direct influence on plant development and crop productivity (Harman, 2006). *Trichoderma* enhancement of plant growth has been known for many years and can occur in axenic systems or in soils (Chang et al., 1986; Yedidia et al., 2001; Adams et al., 2007).

In maize (*Zea mays*) plants, *Trichoderma* inoculation affected root system architecture, which was related to increased yield of plants. Reported effects include enhanced root biomass production and increased root hair development (Bjorkman et al., 1998; Harman et al., 2004b). The root system is important for plant fitness because it provides anchorage, contributes to water use efficiency, and facilitates the acquisition of mineral nutrients from the soil (López-Bucio et al., 2005a). Many lines of evidence strongly support a role for auxin in the regulation of root system architecture. Application of natural and synthetic auxins increases lateral root and root hair development, whereas auxin transport inhibitors reduce root branching (Reed et al., 1998; Casimiro et al., 2001). The auxin-resistant mutants *axr1* and *axr2* produce fewer lateral roots than wild-type plants (Estelle and Somerville, 1987). Conversely, increased formation of lateral roots has been observed in Arabidopsis (*Arabidopsis thaliana*) mutants with elevated auxin content, including the *rooty* mu-

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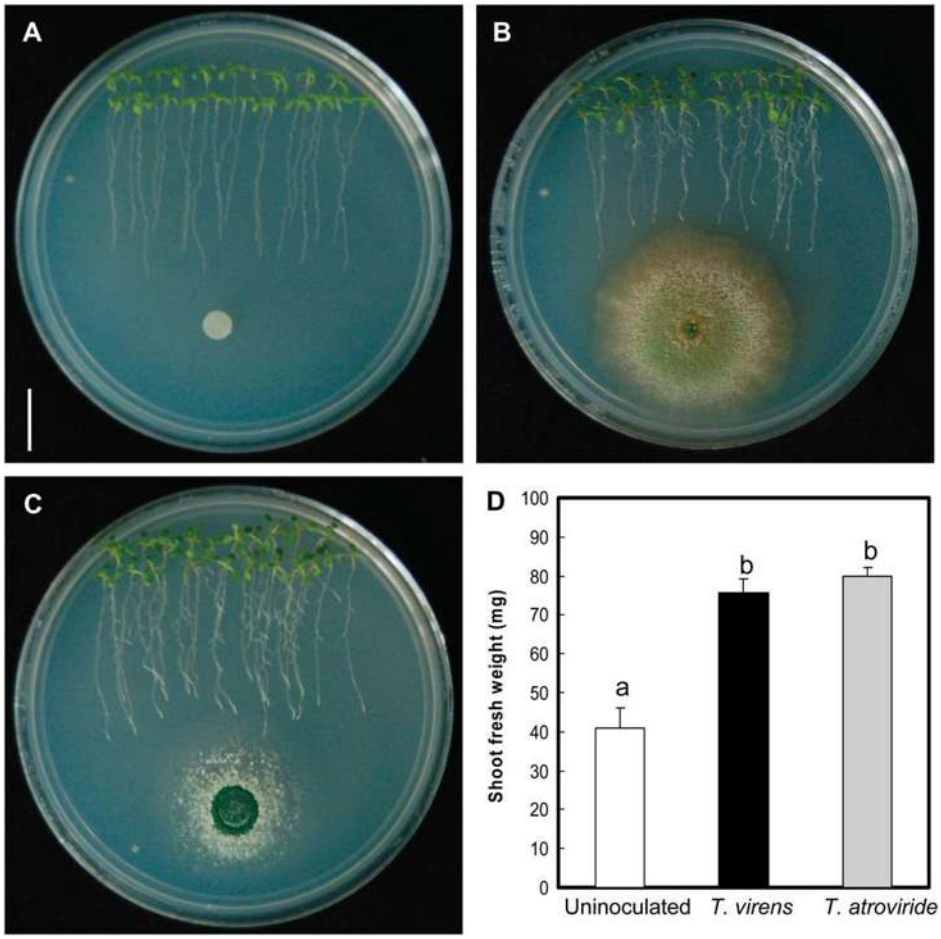

Figure 1. Effects of *T. virens* and *T. atroviride* inoculation on the growth of Arabidopsis seedlings. A, Photograph of 9-d-old Arabidopsis (Col-0) seedlings grown on the surface of agar plates containing 0.2× MS medium. Seedlings were treated with sterilized water at day 4 and photographed 5 d later. Bar = 1 cm. B, Representative photograph of Arabidopsis seedlings that were inoculated with *T. virens* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 5-d period. C, Photograph of Arabidopsis seedlings inoculated with *T. atroviride* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 5-d period. D, Effects of fungal inoculation on shoot biomass production. Photographs show representative individuals of four plates per treatment. Data from D show means ± SD from three groups of 10 seedlings that were recovered from the medium, excised at the root/shoot junction, and weighed on an analytical scale. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results. [See online article for color version of this figure.]

tant and its alleles *alf1* and *superroot1* (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). Additional mutants with auxin-related phenotypes include *aux1*, *doc1*, and *eir1*. The *aux1-7* mutant is defective at the *AUX1* locus, which encodes an auxin influx facilitator participating in both acropetal and basipetal auxin transport at the root tip (Swarup et al., 2001). *doc1* is a mutant allele of *BIG*, which encodes a protein important for the correct location of certain auxin transport proteins (Gil et al., 2001), whereas *EIR1* encodes the auxin transporter *AtPIN2* (Luschnig et al., 1998). It has been determined that auxin deprivation keeps pericycle cells in G1 phase and readdition promotes the G1-S transition of the cell cycle, thus promoting lateral root initiation (Himanen et al., 2002). Despite auxin being a major player in root growth regulation, little is known about its role in plant growth promotion by fungi.

To elucidate the signaling mechanisms by which *Trichoderma* species promote plant growth and development, we evaluated the Arabidopsis response to inoculation with two *Trichoderma* species, *Trichoderma atroviride* (formerly known as *Trichoderma harzianum*) and *Trichoderma virens*. The two fungal species were found to promote Arabidopsis seedling growth under axenic conditions. Plant growth promotion elicited by these fungi correlated with prolific formation of lateral roots. A role for auxin signaling in mediating the observed developmental alterations by *T. virens* inoculation in plants was inferred from tests using the auxin-responsive marker constructs *DR5:uidA*, *BA3:uidA*, and *HS::AXR3NT-GUS* and the analysis of *aux1-7*, *doc1*, *eir1*, and *axr1* auxin-related mutants of Arabidopsis. We further show that *T. virens* is able to produce the indolic compounds indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and indole-3-ethanol (IEt), which may play roles in mediating plant growth promotion by this fungus.

RESULTS

T. atroviride and *T. virens* Promote Growth and Development of Arabidopsis Seedlings

To study the plant growth-promoting activity of *T. atroviride* and *T. virens*, we used Arabidopsis as a model. Arabidopsis (ecotype Columbia [Col-0]) seedlings were germinated and grown for a 4-d period on petri plates containing agar-solidified 0.2× Murashige and Skoog (MS) medium. At day 4 after germination, the seedlings were treated with distilled sterilized water (control treatment) or with 10^6 spores of each

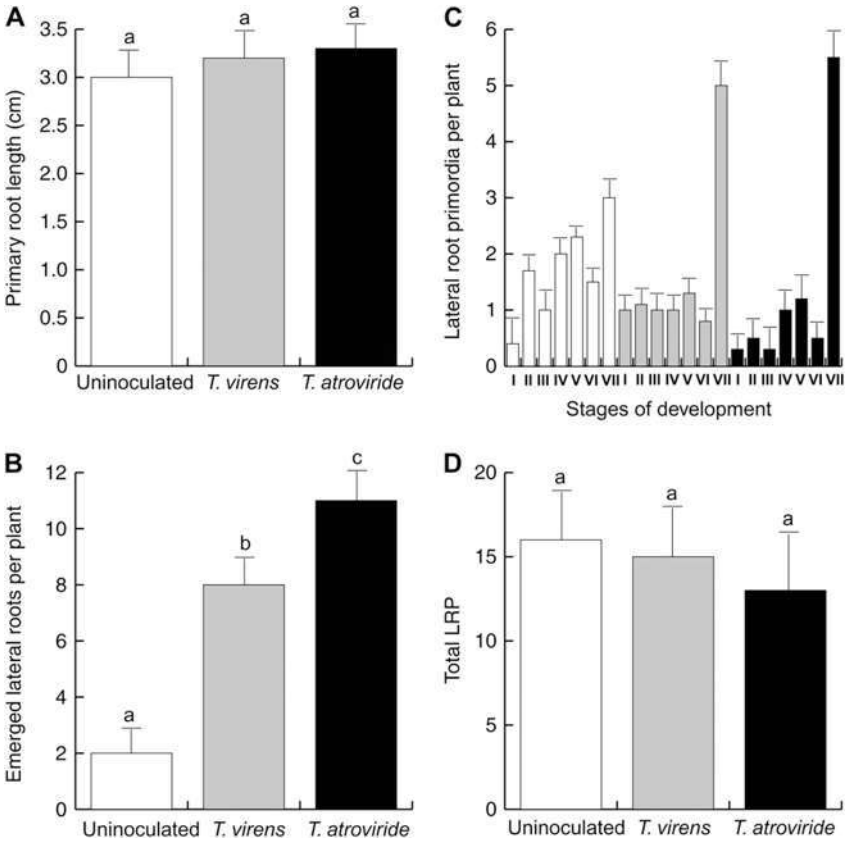

Figure 2. Effects of *Trichoderma* inoculation on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 4 d on the surface of agar plates containing 0.2× MS medium. Half of the plates were inoculated with *T. virens* or *T. atroviride* at a distance of 5 cm from the primary root tip and grown for an additional 5-d period. A, Primary root length. B, Lateral root number per plant. C, Stage number of lateral root primordia per plant. D, Total lateral root primordia per plant. Values shown are means ± SD (n = 30). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

fungal species dissolved in water. Fungal spores were placed at a 5-cm distance from the primary root tip to test the possibility that diffusible fungal compounds could affect plant growth and development. After 5 d of growth in the presence of *T. atroviride* or *T. virens*, increases in shoot and root growth were observed (Fig. 1, A–C). Interestingly, fungal inoculation stimulated lateral root formation (Fig. 1, A–C) and increased shoot biomass production (Fig. 1D), indicating a beneficial effect of inoculation on plant growth and development.

T. atroviride and *T. virens* Alter Root System Architecture in Arabidopsis

To more closely analyze the effects of *Trichoderma* on plant development, primary root length and number of emerged lateral roots were determined in 9-d-old Arabidopsis seedlings grown on petri plates containing agar-solidified 0.2× MS medium after 5 d of fungal inoculation. No significant effects of inoculation with *T. atroviride* or *T. virens* were observed for primary root growth (Fig. 2A). However, a 4- to 6-fold increase in lateral root number was observed in seedlings inoculated with each fungus (Fig. 2B). The effect of *Trichoderma* at increasing the number of lateral roots could be due to the stimulation of lateral root growth or to the de novo formation of lateral root primordia (LRP) by activation of pericycle cells. To distinguish between these two possibilities, LRP were quantified at day 5 after fungal inoculation. 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). We found that the stage distribution of LRP was affected by inoculation with *T. atroviride* or *T. virens*. In particular, stage VII LRP, which belongs to developing LRP with fully active meristems, was significantly increased in *T. atroviride*- and *T. virens*-inoculated seedlings (Fig. 2C). The total number of LRP per plant was similar between uninoculated and *Trichoderma*-inoculated seedlings (Fig. 2D). These data suggest that *Trichoderma* can promote root branching in Arabidopsis by inducing lateral root growth rather than by increasing de novo formation of LRP.

T. virens Alters Auxin-Inducible Gene Expression in Arabidopsis

The observed effect of *Trichoderma* in promoting lateral root development is similar to that described for auxins in plants (Casimiro et al., 2001). We next tested whether *T. virens* could alter auxin-regulated gene expression in Arabidopsis by inoculating *DR5*:*uidA* transgenic seedlings with this fungus. The *DR5*:*uidA* line has been used to study auxin-regulated gene expression in Arabidopsis (Ulmasov et al., 1997). *DR5*:

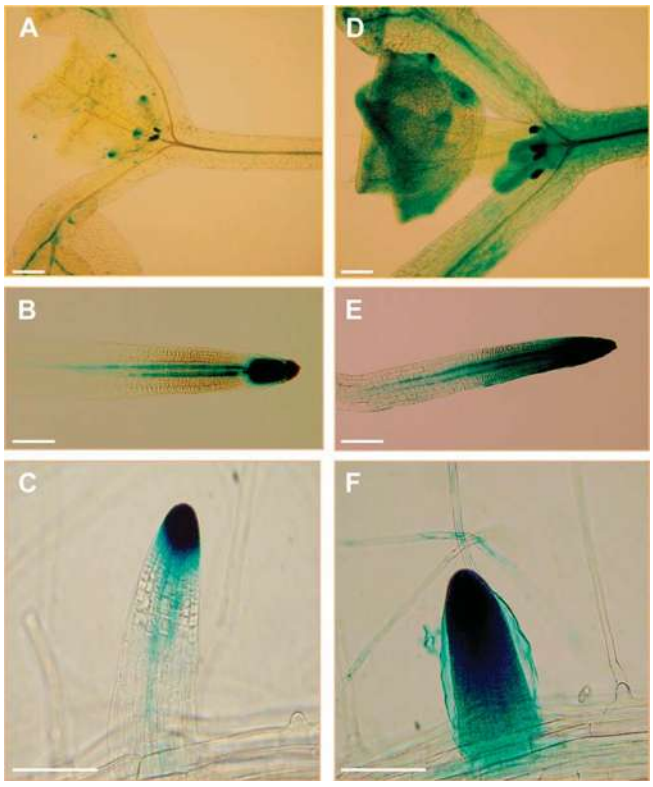

Figure 3. Effects of *T. virens* inoculation on auxin-regulated gene expression. Twelve-hour GUS staining of *DR5*:*uidA* primary roots of Arabidopsis seedlings grown for 4 d on agar-solidified 0.2× MS medium. A to C, Uninoculated seedlings. D to F, *T. virens*-inoculated seedlings. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Bars = 100 μm. [See online article for color version of this figure.]

uidA seedlings were germinated and grown for 4 d on petri plates containing agar-solidified 0.2× MS medium and then inoculated with *T. virens* at 5 cm from the primary root tip. After an additional 5-d growth period, *DR5*:*uidA* seedlings were stained for GUS activity and further cleared to visualize changes in GUS expression. Although no significant effect of fungal inoculation was observed for primary root growth for wild-type (Fig. 2A) and *DR5*:*uidA* (data not shown) seedlings, an increase in GUS expression could be detected in shoots (Fig. 3, A and D), primary root tips (Fig. 3, B and E), and developing lateral roots (Fig. 3, C and F) from *T. virens*-inoculated seedlings when compared with uninoculated seedlings. These data suggest that *T. virens* inoculation increases auxin-regulated gene expression.

Effects of *T. virens* Inoculation on Growth and Lateral Root Development of Auxin-Related Arabidopsis Mutants

Next, we evaluated the effects of *T. virens* inoculation on growth of Arabidopsis wild-type seedlings and mutants defective in auxin transport (*aux1-7*, *doc1*, and *eir1*) or auxin response (*axr1-3*). Five days after plants were inoculated, *T. virens* increased by 62% shoot fresh weight in wild-type seedlings when compared with uninoculated seedlings. In contrast, all four mutant lines, *aux1-7*, *doc1*, *eir1*, and *axr1-3*, showed decreased or null responses in growth promotion by the fungus (Fig. 4A). We also quantified lateral root number in the wild type and all above mentioned mutants. It was found that *T. virens* inoculation induced up to a 4-fold increase in lateral root number when compared with uninoculated plants. Interestingly, a reduction in lateral root formation when compared with inoculated wild-type plants was observed for *aux1-7* and *axr1-3*

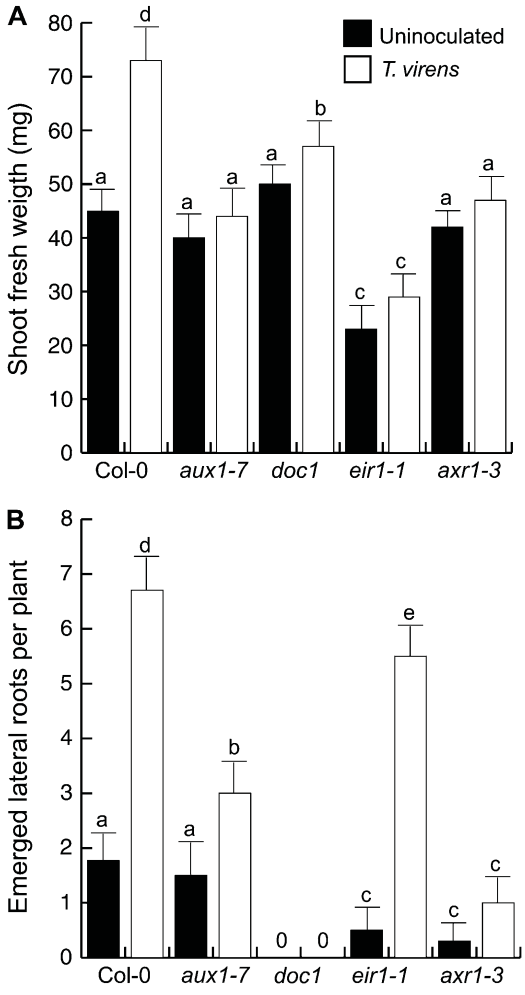

Figure 4. Effects of *T. virens* inoculation on biomass production and lateral root development in wild-type Arabidopsis (Col-0) and auxin-related mutants. Plant material was harvested 5 d after fungal inoculation. Shoots were excised at the root/shoot junction, and the fresh weight was determined on an analytical balance. A, Shoot fresh weight. B, Lateral root number per plant. Values shown represent means of four groups of 10 seedlings ± SD. Lateral roots were quantified for 30 seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

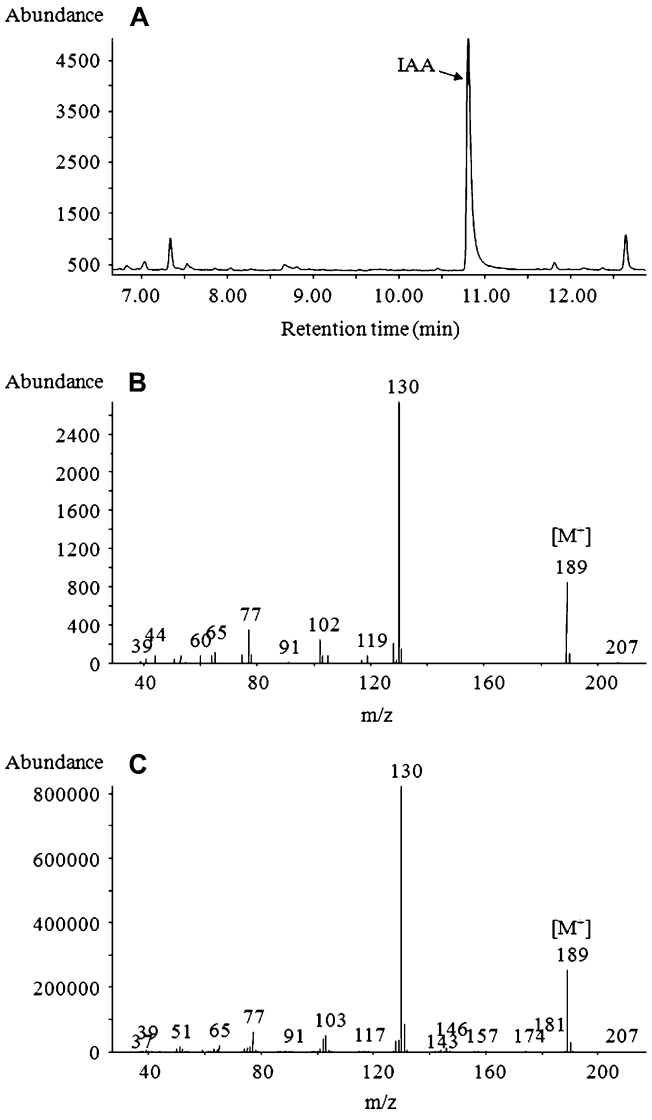

Figure 5. Determination of IAA from derivatized samples from *T. virens* growth medium by GC-MS. A, Total ion chromatogram of IAA from acidic ethyl acetate extract obtained from 1 L of culture medium of *T. virens*. B and C, The 70-eV electron-impact full-scan mass spectra from *m/z* 50 to 500 of IAA methyl ester identified in the extract (B) and the methylated IAA standard (C). Determinations were done from at least five independent samples.

inoculated seedlings, and no lateral root induction was registered for uninoculated or inoculated *doc1* seedlings (Fig. 4B). These results indicate that both normal auxin transport and response are important for promoting the effects of *T. virens* on plant growth and lateral root development.

T. virens Produces IAA, IAAld, and IEt

The induced expression of *DR5*:*uidA* by *T. virens* and the decreased response of auxin-related Arabidopsis mutants to fungal inoculation opens the possibility that the fungus could produce IAA or other auxin-like compounds. We conducted experiments aimed at identifying IAA or IAA-related substances by growing *T. virens* on liquid cultures and determining indolic compounds from the supernatant by gas chromatography-mass spectrometry (GC-MS) analysis. We determined the actual (no Trp addition) and potential (100 mg L^{-1} Trp) production of indolic compounds produced by *T. virens* from either derivatized or underivatized samples from the growth medium. When derivatized samples were analyzed by GC-MS, we identified IAA (Fig. 5), which increases up to 17-fold in concentration in *T. virens* growth medium supplied with Trp (Table I). When underivatized samples from *T. virens* growth medium without Trp were analyzed for indolic compounds, the presence of IEt (retention time = 9.97 min) and IAAld (retention time = 8.83 min) was found (Fig. 6). The production of IEt was enhanced upon Trp addition, while a small yet significant increase in IAAld production was also detected in Trp-supplied cultures (Table I). IAA could not be further detected from underivatized samples.

IAAld Activates Auxin-Inducible Gene Expression

To determine if IAAld and IEt act in an auxin-related signaling pathway, we conducted analyses of the expression of the auxin-inducible *DR5*:*uidA* and *BA3*:*uidA* gene markers. Figure 7 shows histochemical staining for transgenic *DR5*:*uidA* and *BA3*:*uidA* seedlings that were grown for 6 d under IAA, IAAld, or IEt treatment. As reported previously (Ulmasov et al., 1997), in untreated control plants, *DR5*:*uidA* is absent from cotyledons and leaves and expressed primarily in the root tip region (Fig. 7, A and E). *DR5*:*uidA* seedlings grown at a concentration of 2 μM IAA showed GUS activity in the cotyledons and the primary root (Fig. 7, B and F). The pattern of GUS expression in *DR5*:*uidA* seedlings treated with 4 μM IAAld remained similar to that observed for IAA-treated plants (Fig. 7, C and G). In contrast, up to a 64 μM concentration of IEt showed a modest increase in expression of this marker (Fig. 7, D and H), indicating different auxin-related activity for the compounds. Untreated *BA3*:*uidA* plants did not show detectable levels of GUS activity (Fig. 7, I and M), whereas when treated with 2 μM IAA, they showed GUS expression mainly in petioles of cotyledons (Fig. 7J) and in the root elongation zone (Fig. 7N). GUS expression in plants treated with IAAld was clearly observed in the same regions as in IAA-treated seedlings (Fig. 7, K and O). IEt failed to activate *BA3*:*uidA* expression (Fig. 7, L and P). These results show that IAAld, IEt, and IAA treatments can differentially activate the expression of auxin-inducible gene markers.

IAAld Enhances Aux/IAA Protein Degradation

Auxin promotes the degradation of Aux/IAA repressor proteins via the ubiquitin-proteasome pathway and thereby induces primary auxin-responsive

Table I. *Quantification of auxin-like compounds from T. virens*

T. virens was inoculated in 1 L of nutrient solution with or without 100 mg of L-Trp, and determinations were performed by GC-MS after 3 d of growth. Data shown are means ± SE for samples from three independent cultures ($n = 3$). Different letters represent means statistically different at the 0.05 level.

Compound	Retention Time	Concentration	
		(−) L-Trp	(+) L-Trp
	min	$\mu g\ L^{-1}$	
IAAld	8.83	59.4 ± 4.47[a]	70.15 ± 3.78[b]
IEt	9.97	72.33 ± 1.41[a]	141.88 ± 4.85[b]
IAA	10.81	13.48 ± 0.97[a]	233.64 ± 3.06[b]

gene expression (Gray et al., 2001). To address the effect of IAA, IAAld, and IEt on auxin-mediated degradation of Aux/IAA proteins, we examined the effects of these compounds on Aux/IAA stability using the Arabidopsis *HS::AXR3NT-GUS* line, in which a translational fusion between domains I and II of AXR3 and the GUS reporter protein is expressed under the control of a heat shock promoter (Gray et al., 2001). Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37°C for 2 h and further treated with 5 μM IAA, IAAld, or IEt for 5, 10, 20, and 60 min. Treatment with IAA or IAAld showed enhanced degradation of the fusion protein in a similar way, but IEt failed to induce degradation of the fusion protein even after 60 min of treatment (Fig. 8, A–P). Our data indicate that IAAld likely acts in an auxin-mediated signaling pathway, either by direct binding to an auxin receptor or by its conversion to IAA, which rapidly destabilizes the AXR3 protein.

IAAld and IEt Differentially Regulate Arabidopsis Root System Architecture

To determine more closely the effects of IAAld and IEt on the architecture of the Arabidopsis root system, wild-type Arabidopsis seedlings were germinated and grown on vertically oriented agar plates containing 0.2× MS medium supplied with IAAld or IEt concentrations ranging from 0.25 to 8 μM. Under these conditions, primary root length, number of lateral roots, and lateral root density were quantified. After 10 d of growth, it was observed that concentrations of IAAld greater than 1 μM inhibited primary root growth in a dose-dependent way (Fig. 9A). It was observed that IAAld-treated Arabidopsis seedlings produced a highly branched root system with abundant lateral roots. A roughly 2-fold increase in lateral root number per plant was found at concentrations of IAAld from 0.25 to 2 μM when compared with solvent-treated

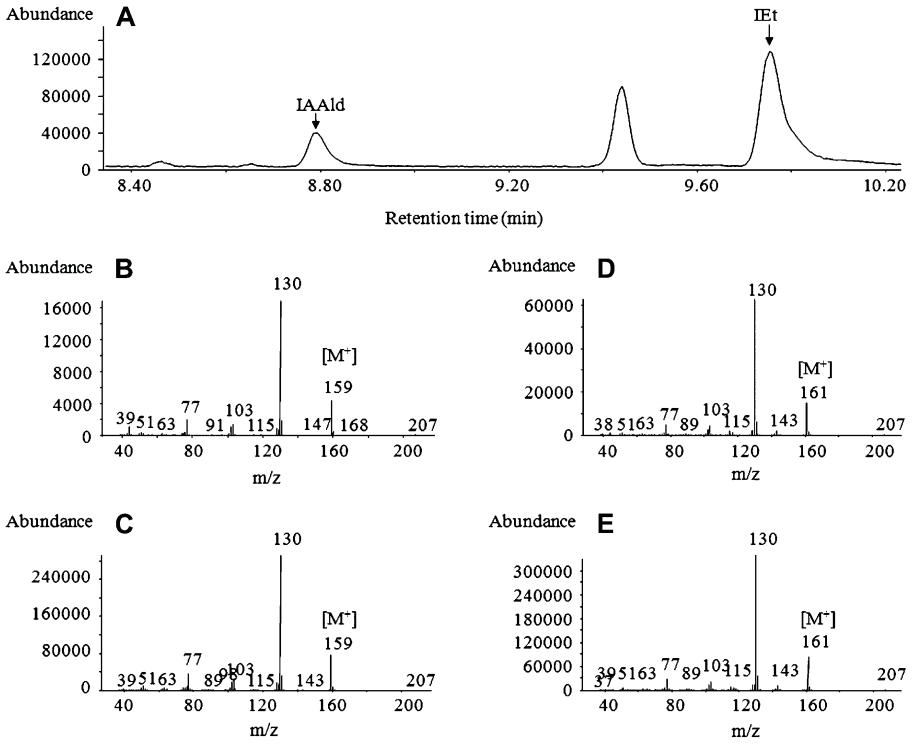

Figure 6. Determination of indolic compounds from underivatized samples from *T. virens* growth medium by GC-MS. A, Total ion chromatogram of IAAld and IEt from neutral ethyl acetate extract obtained from 1 L of culture medium of *T. virens*. B to E, The 70-eV electron-impact full-scan mass spectra from *m/z* 50 to 500 of IAAld identified in the extract (B), the standard IAAld (C), IEt identified in the extract (D), and the standard IEt (E). Determinations were done from at least five independent samples.

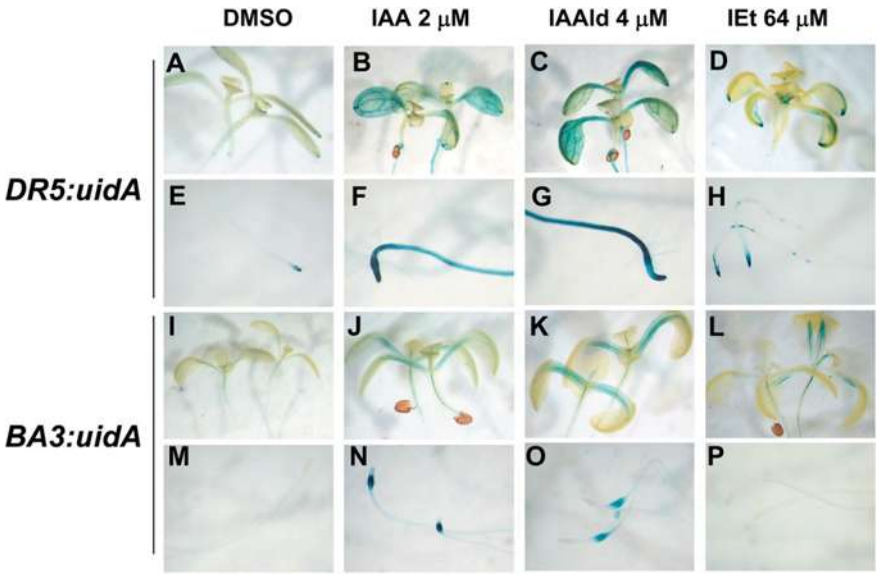

Figure 7. Effects of indolic compounds produced by *T. virens* on auxin-regulated gene expression. A to H, Twelve-hour GUS staining of *DR5:uidA* Arabidopsis seedlings grown for 6 d on agar plates containing 0.2× MS medium (A and E) and on medium supplied with 2 μM IAA (B and F), 4 μM IAAld (C and G), or 64 μM IEt (D and H). Notice the increase in GUS expression in shoots and roots in the treatments with IAAld. I to P, Twelve-hour GUS staining of *BA3:uidA* Arabidopsis seedlings grown for 6 d on agar plates containing 0.2× MS medium (I and M) and on medium supplied with 2 μM IAA (J and N), 4 μM IAAld (K and O), or 64 μM IEt (L and P). Notice the increase in GUS expression in the root elongation region in the treatments with IAA or IAAld. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. DMSO, Dimethyl sulfoxide. [See online article for color version of this figure.]

control seedlings (Fig. 9B). The density of lateral roots was also calculated by dividing the number of lateral roots by the length of the primary root to normalize for the effects of IAAld on primary root length. Lateral root density increased over 2-fold in plants treated with IAAld when compared with untreated seedlings (Fig. 9C). This increase in lateral root density was due to a stimulatory effect of IAAld on both LRP formation and lateral root emergence (Supplemental Fig. S1).

Interestingly, after 12 d of growth, IEt showed modest activity at inhibiting primary root growth (Fig. 10A) and failed to increase lateral root formation even when supplied at concentrations up to 64 μM (Fig. 10B). Lateral root density significantly increased only at 64 μM IEt concentration in the medium (Fig. 10C), indicating that this compound acts at high concentrations to activate pericycle cells. These results show that IAAld and IEt have different activity in Arabidopsis root system architecture modulation and that the effects of fungal inoculation on root development are likely due to a combined effect of all three indolic compounds, IAA, IAAld, and IEt, produced by the fungus.

IAAld Rescues the Root Hair-Defective Phenotype of the Auxin-Related *rhd6* Arabidopsis Mutant

Arabidopsis root hairs are a good system in which to study cell differentiation and morphogenesis in plants. The study of their development is also of great interest because of their putative function in water and nutrient uptake. Several auxin-related mutations have been found to alter root hair development (Parker et al., 2000). Of particular interest is the *rhd6* mutant, which is defective in root hair initiation and has been shown previously to be rescued by auxin (Masucci and Schiefelbein, 1994). We used the *rhd6* mutant as a tool to probe the mechanism of IAAld action. We compared the root hair response of Arabidopsis wild-type seedlings and *rhd6* mutants with IAA and IAAld treatments at day 5 after germination. As shown in Figure 10, treatments with 0.5 μM IAA or 4 μM IAAld stimulated root hair elongation and increased root hair formation at the primary root tip region in Arabidopsis wild-type seedlings (Fig. 11, A–C). *rhd6* mutant seedlings grown in medium without auxin were completely devoid of root hairs (Fig. 11D). Interestingly, both IAA and IAAld were found to rescue the *rhd6* root hair-defective phenotype (Fig. 11, E and F). The root hairs produced in each of these experiments exhibited normal growth and morphology. These results imply that the application of IAAld can suppress the root hair formation defects of *rhd6*.

IAA and IAAld Alter Arabidopsis Biomass Production in a Dose-Dependent Way

The fact that *T. virens*-enhanced shoot biomass production was dependent on auxin transport/signaling prompted us to determine whether exogenous auxin

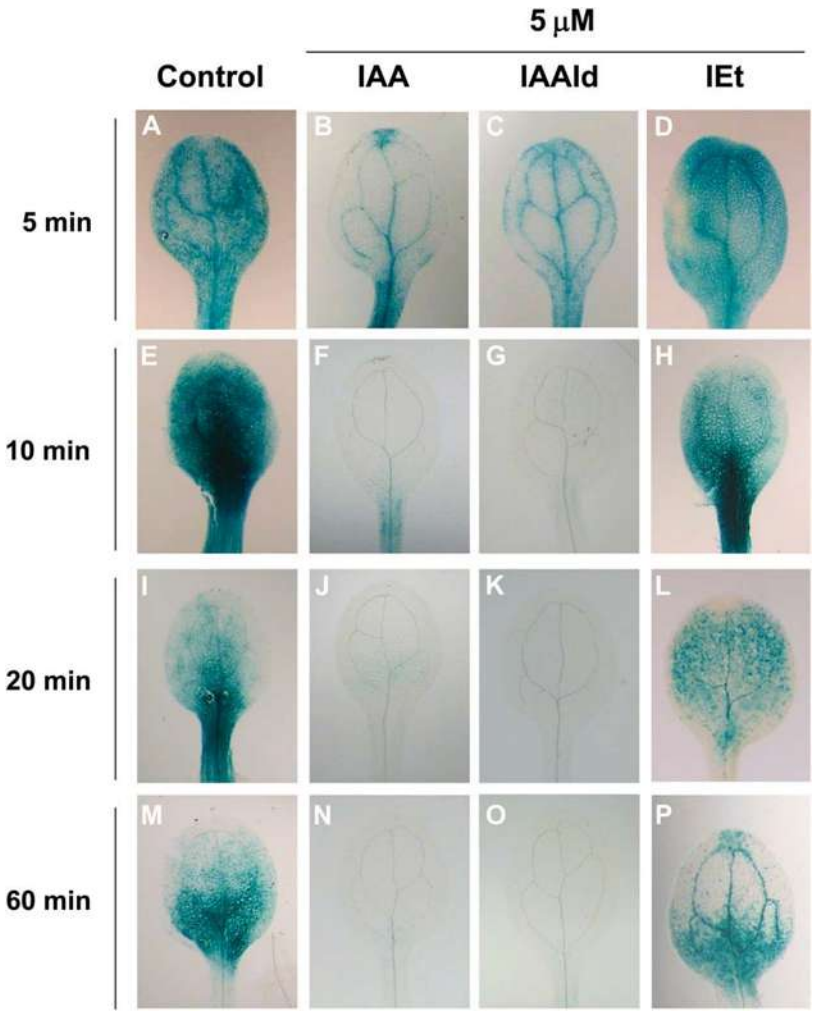

Figure 8. Analysis of Aux/IAA stability with *HS*::*AXR3NT-GUS* fusions. Wild-type seedlings expressing the *HS*::*AXR3NT-GUS* constructs were heat shocked at 37°C for 2 h. After heat induction, the seedlings were treated with IAA, IAAld, or IEt for different time periods at the indicated concentrations and stained overnight for GUS activity. Notice the degradation of the fusion protein by either IAA or IAAld. A to P, Representative photographs of cotyledons (n = 10 stained seedlings). Similar results were obtained in two independent experiments. [See online article for color version of this figure.]

application could increase the growth of Arabidopsis seedlings. We quantified root, shoot, and total fresh weight of plants grown under varied concentrations of IAA or IAAld. Treatments of 15 to 60 nM IAA significantly increased root, shoot, and total fresh weight when compared with control plants, while concentrations of 120 to 960 nM did not affect or decreased biomass production (Fig. 12). Similar dose-dependent effects on growth were observed for IAAld-treated plants, albeit at greater concentrations than IAA (Supplemental Fig. S2).

To further define whether the effects of IAAld are mediated by auxin transport/signaling, we performed experiments to investigate the resistance of auxin-related mutants to exogenous application of IAAld. A commonly used developmental marker for auxin responses is primary root growth. Therefore, we grew wild-type plants and the auxin-related mutants *aux1-7*, *doc1*, *eir1-1*, and *axr1-3* in medium with or without 8 μM IAAld, a concentration that inhibits root growth. Our results show that *aux1-7*, *eir1-1*, and *axr1-3* are indeed very resistant to IAAld and sustained primary root growth in an IAAld concentration that drastically inhibits growth in wild-type plants (Supplemental Fig. S3). Thus, we conclude that both auxin transport and response are important for root developmental responses to IAAld.

DISCUSSION

T. virens Promotes Arabidopsis Growth and Development through an Auxin-Dependent Mechanism

Trichoderma species are naturally occurring soil fungi that colonize roots and stimulate plant growth. Such fungi have been applied to a wide range of plant species for the purpose of growth enhancement, with a positive effect on plant weight, crop yields, and disease control. Their agricultural use could be expanded if the mechanisms of growth enhancement were known. A number of mechanisms for plant growth promotion by *Trichoderma* have been proposed (Harman et al., 2004a). Among these, fungal interaction with auxin

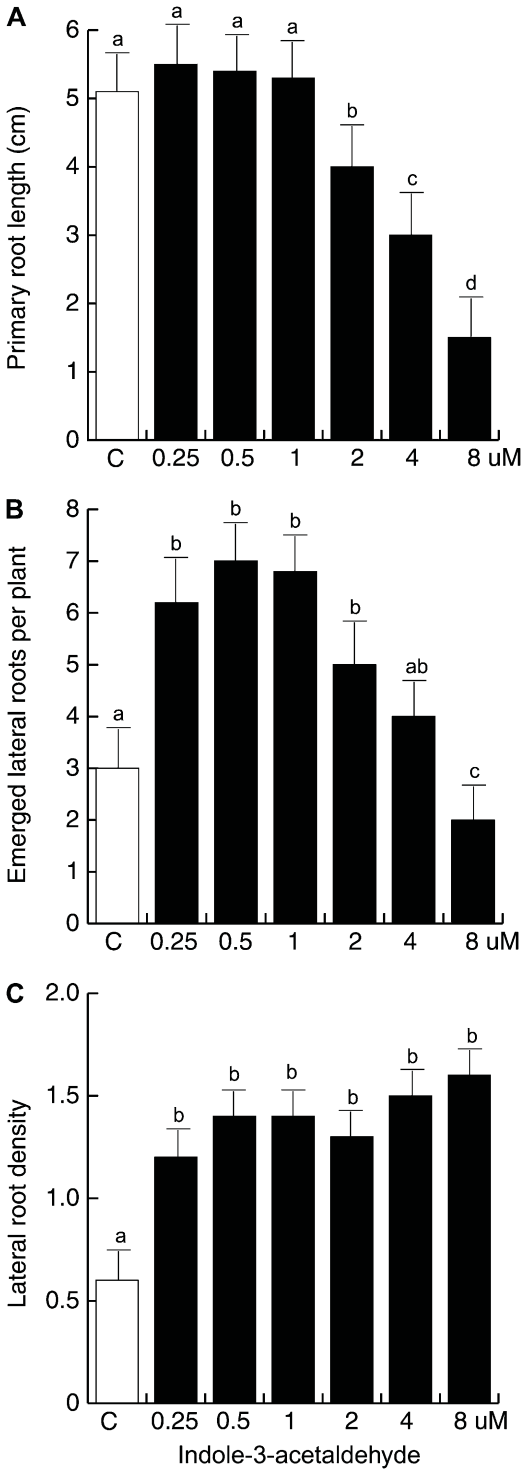

Figure 9. Effects of IAAld on Arabidopsis root architecture. Wild-type Col-0 seedlings were grown for 10 d under increasing IAAld concentrations on vertically oriented agar plates. Data are given for the length of the primary root (A), lateral root number (B), and lateral root density (C). Values shown represent means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

signaling has not been examined, despite auxin being a central plant growth-regulating substance.

It was noticeable that inoculation with *Trichoderma* affected lateral root development in Arabidopsis wild-type plants in a way that suggests that the effects are mediated by auxin (Figs. 1 and 2). IAA is a molecule that is synthesized by plants and a few microbes (Woodward and Bartel, 2005). In plants IAA plays a key role in root and shoot development. The hormone moves from one part of the plant to another by specific transporter systems that involve auxin importer (*AUX1*) and efflux (*PIN1-7*) proteins. IAA is a key regulator of lateral root development and root hair development (Casimiro et al., 2001). Expression studies of the auxin-inducible marker *DR5:uidA* suggested that *T. virens* inoculation increases the auxin response in Arabidopsis seedlings (Fig. 3). To further elucidate some of the aspects of auxin transport/perception involved in the Arabidopsis response to *T. virens*, we analyzed the growth and development of Arabidopsis mutants with defects in the auxin signal transduction pathway. We found that the auxin transport mutants (*aux1-7*, *eir1*, and *doc1*) have a reduced response to the fungus in terms of growth promotion (Fig. 4A) and lateral root development (Fig. 4B). In particular, the *doc1* mutant, which shows defects in lateral root initiation that can be complemented by nutrient deficiency (López-Bucio et al., 2005b), showed null induction of lateral roots when inoculated with *T. virens* (Fig. 4B). These results indicate that normal auxin transport is important for plant responses to *T. virens*. The finding that the auxin-resistant *axr1-3* mutant also shows a reduced response to inoculation suggests that the corresponding wild-type gene is required in Arabidopsis for increased growth and lateral root formation when inoculated with the fungus (Fig. 4). *AXR1* encodes a protein related to the ubiquitin-activating enzyme E1 (Leyser et al., 1993). These results indicate that plant growth promotion by *T. virens* operates through the classical auxin response pathway.

T. virens Produces IAA, IAAld, and IEt

In this study, we determined the presence of IAA (Fig. 5) and of two substances structurally related to IAA, namely IAAld and IEt, in *T. virens* growth medium (Fig. 6). When Trp was added to the growth medium of *T. virens*, an increased production of all three metabolites was evident (Table I). Although it is widely accepted that plants use several pathways to synthesize IAA, none of the pathways are yet defined to the level of knowing each relevant gene, enzyme, and intermediate. Several Trp-dependent pathways have been proposed: the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide pathway, the tryptamine pathway, and the indole-3-acetaldoxime pathway (Woodward and Bartel, 2005). The IPA pathway (Trp → IPA → IAAld → IAA) is important in some IAA-

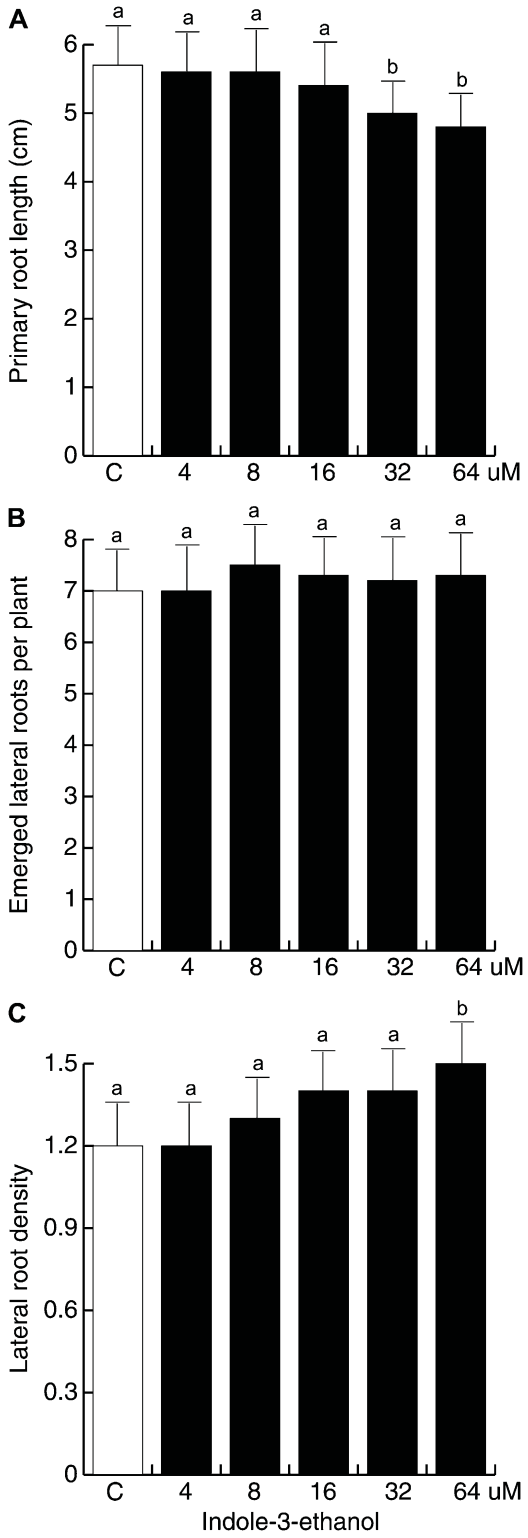

Figure 10. Effects of IEt on Arabidopsis root architecture. Wild-type Col-0 seedlings were grown for 12 d under increasing IEt concentrations on vertically oriented agar plates. Data are given for the length of the primary root (A), lateral root number (B), and lateral root density (C). Values shown represent means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

synthesizing microorganisms (Koga, 1995), and recently it was demonstrated that it operates in plants as well (Stepanova et al., 2008; Tao et al., 2008). The final enzyme in the proposed IPA pathway is an IAAld-specific aldehyde oxidase (AAO1) that has increased activity in the IAA-overproducing *superroot1* mutant (Seo et al., 1998). The identification of Arabidopsis AAO1 suggests that plant- and microbe-produced IAAld can be used to produce IAA in plants. Several lines of evidence support the view that the rate of auxin biosynthesis is subject to regulation, with several IAA precursors acting as storage compounds. IAAld can be converted to IEt by an indole acetaldehyde reductase enzyme. This enzyme has been characterized in cucumber (*Cucumis sativus*) seedlings, where it plays an important role in auxin biosynthesis (Brown and Purves, 1980). Both IAAld and IEt occur naturally in plants (Purves and Brown, 1978; Magnus et al., 1982), which suggests that these compounds can act as flexible storage pools for IAA. Although IEt does show a modest auxin-like activity in activating the auxin-regulated gene markers *DR5*:*uidA* and *BA3*:*uidA* (Fig. 7), conversion of IEt to IAA has been already demonstrated in cucumber seedling shoots (Rayle and Purves, 1967).

Relatively little information is available on IAA biosynthesis in fungi. Production of IAA through the IPA pathway was identified in the fungus *Colletotrichum acutum* (Chung et al., 2003). HPLC analysis and chromogenic stains after a fluorescence thin-layer chromatography separation unambiguously identified IAA, IEt, IAAld, and IPA from cultures supplemented with Trp. Interestingly, increasing Trp concentrations drastically increased the levels of IEt but not IAA (Chung et al., 2003). It has been suggested that in this case IEt may be the end product of Trp metabolism rather than a side product of the IPA pathway. In contrast, our results show that IAA levels dramatically increase in Trp-supplied cultures of *T. virens* (Table I); it is tempting to speculate that Trp supply to *T. virens* cultures increases IAA accumulation as a direct product of its metabolism.

IAAld Shows Auxin-Like Activity in Arabidopsis

To maximize the capability of an organ to expand or elongate, or to establish a particular developmental program such as lateral root formation, plants have evolved mechanisms tightly coupled to the perception of biotic and abiotic stimuli. Many of the plant responses to environmental factors are mediated by phytohormones, such as auxin.

IAA has been found to be the typical auxin in plants, mainly evaluated by cell elongation tests in hypocotyls and primary root growth responses (Woodward and Bartel, 2005). However, the chemical space, which encompasses the term "auxin," is actually not easily achieved, since many compounds were found to exhibit an auxin-like activity in several different bioassays (Ferro et al., 2007). Our compar-

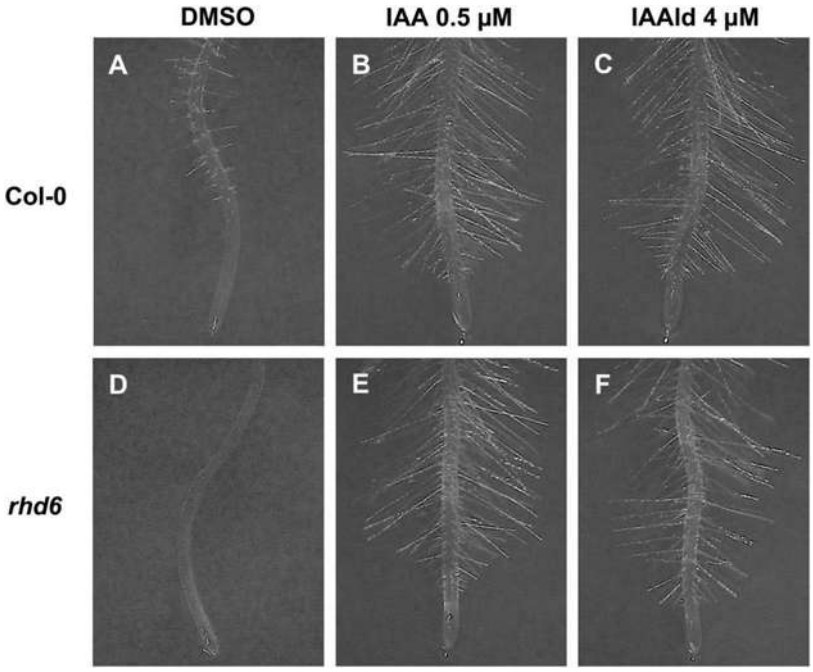

Figure 11. IAAld rescues the *rhd6* mutant phenotype. A, Wild-type Col-0 Arabidopsis root with normal root hair formation. B and C, Root hair formation in response to IAA (B) or IAAld (C) treatment. D, A typical *rhd6* Arabidopsis mutant root showing a reduction in root hair formation. E and F, Formation of root hairs in *rhd6* roots in response to IAA (E) or IAAld (F) treatment. The experiment was repeated three times with similar results. DMSO, Dimethyl sulfoxide. [See online article for color version of this figure.]

ative analysis of auxin activity for IAA, IAAld, and IEt (Fig. 7) identified IAAld, an IAA precursor in the IPA pathway, as an active auxin. Three additional lines of evidence indicate that IAAld acts as an auxin: (1) the effect of the compound on Aux/IAA stability using the Arabidopsis *HS*::*AXR3NT-GUS* line; (2) the regulation of root system architecture by its exogenous application to the seedlings; and (3) the rescue of the root hair-defective phenotype of the *rhd6* mutant of Arabidopsis when exogenously supplied to the growth medium. Treatment with IAA or IAAld showed enhanced degradation of the fusion protein *HS*::*AXR3NT-GUS* in a similar way, but IEt failed to induce degradation of the fusion protein even after 60 min of treatment (Fig. 8). These data indicate that IAAld likely acts in an auxin-mediated signaling pathway. Interestingly, exogenously supplied IEt was found to inhibit primary root growth and to increase lateral root density at a 64 μM concentration (Fig. 10), a much higher concentration than that required for IAA or IAAld to affect the same developmental traits. Compelling evidence that IAAld shows auxin-like activity came from the analysis of the root hair response in wild-type and *rhd6* mutant seedlings to this compound. The reported association between auxin and the *rhd6* mutation indicated that the *RHD6* gene product could be used as a tool to probe the mechanism of action of auxin-like compounds (Masucci and Schiefelbein, 1994). Treatment with IAAld was found to rescue the root hair phenotype of the *rhd6* mutant in a similar way to that of IAA (Fig. 11). Inoculation with *T. virens* or application of *T. virens* extracts also induced normal formation of root hairs in the *rhd6* mutant (data not shown), suggesting that developmental effects of fungal inoculation in Arabidopsis likely occur by the production of an auxin, presumably IAA or IAAld.

Role of Auxin Signals in *Trichoderma*-Plant Interactions

The importance of auxins for plant development has been long recognized, and redundancy for IAA biosynthesis is widespread in plants and among plant-associated microorganisms. Accumulation of auxins or increased responses to auxins might lead to diverse outcomes on the plant side, varying from pathogenesis to growth promotion. *T. virens* and *T. atroviride* were found to stimulate the growth of Arabidopsis plants in vitro (Fig. 1), suggesting that these fungi likely act as plant growth-promoting microorganisms. It was previously reported that *Trichoderma* was able to colonize the entire root system of maize plants and to persist for the entire lifespan of this crop (Harman et al., 2004b). Fungal colonization stimulated plant growth by factors including increased root size and rooting depth, which aid in nutrient uptake (Yedidia et al., 2001; Harman et al., 2004a).

To further investigate whether IAA and IAAld produced by *T. virens* could have a positive effect on Arabidopsis growth, we quantified biomass production in plants treated with varied concentrations of these compounds. Both compounds showed a dose-dependent effect on growth by increasing biomass production in small amounts but repressing growth at higher concentrations (Fig. 12; Supplemental Fig. S2). Thus, the effect of inoculation with *Trichoderma* strains in plants under natural conditions may depend

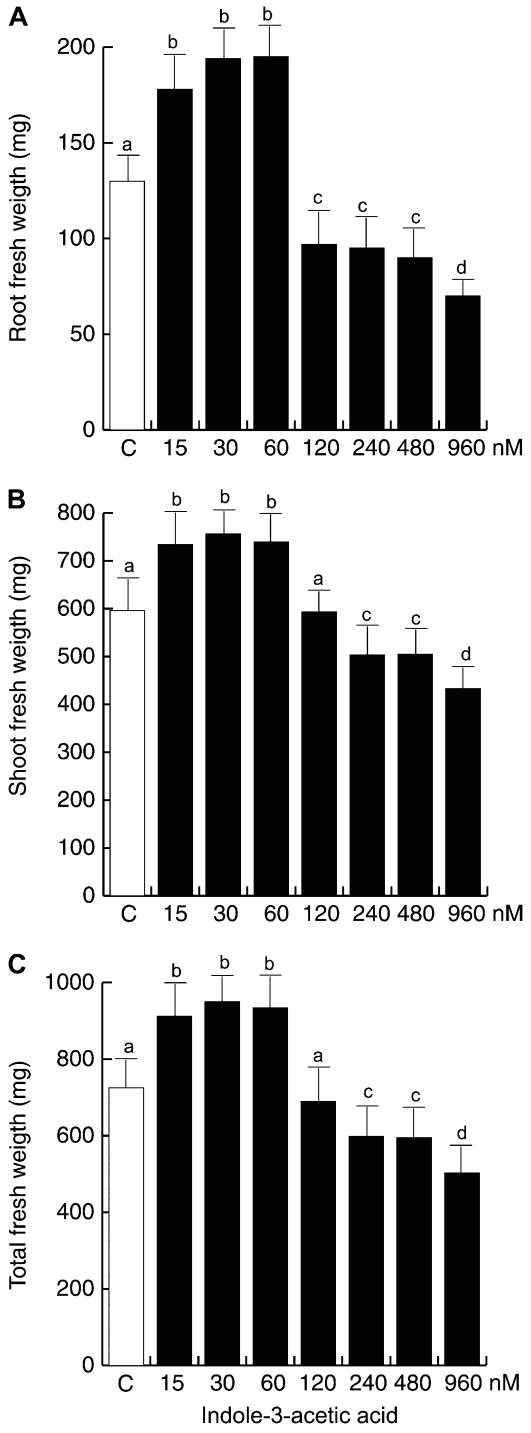

Figure 12. Effects of IAA on Arabidopsis biomass production. Wild-type Col-0 seedlings were grown for 14 d under increasing IAA concentrations on vertically oriented agar plates. Data are given for the mean root fresh weight (A), shoot fresh weight (B), and total fresh weight (C). Plants were excised at the root/shoot junction, and fresh weights were determined on an analytical scale for four groups of 25 plants. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

on the type and concentration of auxins being produced by the fungi.

Little is known about the molecular determinants involved in the interaction of *T. virens* with plants. We hypothesize that auxin production by this fungus promotes the interaction with roots by circumvention of basal plant defense mechanisms, as recently reported by Navarro et al. (2006), who showed that repression of auxin signaling restricts *Pseudomonas syringae* growth, implicating auxin in disease susceptibility and RNA-mediated suppression of auxin signaling in resistance. The fungus can also produce auxins as part of its colonization strategy, as published information indicates that fungus-produced IAA induces adhesion and filamentation of *Saccharomyces cerevisiae* (Prusty et al., 2004).

Although we cannot exclude the possibility that IAAld could be converted to IAA and in this way exert its biological action, the concerted action of all three indolic compounds identified may account for the plant growth-promoting properties of *T. virens* (Fig. 13). In the plant partner, alteration in lateral root formation may provide a greater root surface area for fungal colonization. In turn, increased absorptive surface by branched roots may increase water and nutrient uptake capacity of plants. It is tempting to speculate that production of auxins by *Trichoderma* may benefit plant hosts by initiating or reinforcing symbiotic behaviors with fungal partners in the rhizosphere.

The data presented in this work suggest an important role for auxin signaling in plant growth regulation by *T. virens*. Our results show great promise for the use of *Trichoderma* species as inoculants for plant improvement under controlled and field conditions.

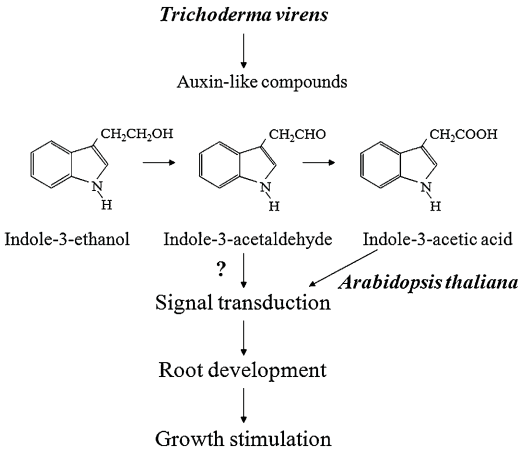

Figure 13. Arabidopsis growth responses to *T. virens* and their regulation. *T. virens* induces lateral root proliferation and enhances biomass accumulation by production of IAA and IAAld. IAAld can be converted to IAA by plant enzymes or can directly regulate auxin-inducible gene expression, possibly by interacting with auxin receptors. IEt did not show clear auxin-like activity, but it can act as a storage form for other active indolic compounds such as IAAld or IAA.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* Col-0), the Arabidopsis transgenic lines *HS*::*AXR3NT-GUS* (Gray et al., 2001), *DR5*:*uidA* (Ulmasov et al., 1997), and *BA3*:*uidA* (Oono et al., 1998), and the mutant lines *eir1-1* (Roman et al., 1995), *doc1* (Li et al., 1994), *axr1-3* (Lincoln et al., 1990), *aux1-7* (Picket et al., 1990), and *rhd6* (Masucci and Schiefelbein, 1994) were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h of light/8 h darkness, light intensity of 300 μmol m^2 s^{-1}, and temperature of 22°C.

Fungal Growth and Indolic Compound Determinations

The following strains were used in this work: *Trichoderma virens* Gv. 29-8 and *Trichoderma atroviride* (formerly *Trichoderma harzianum*) IMI 206040. The strains of *Trichoderma* were grown and maintained on potato dextrose agar medium (Difco).

For the production of indolic compounds, an active inoculum of 1×10^6 spores of *T. virens* was added to 1 L of potato dextrose broth (Difco) and grown for 3 d at 28°C with shaking at 200 rpm. To evaluate the effect of Trp supply on indolic compounds, the medium was supplemented with L-Trp (Merck) at a concentration of 100 mg L^{-1}. For IAAld and IEt determinations, the fungal culture was filtered and the supernatant was adjusted to pH 7 using 2 N NaOH. Indolic compounds in supernatant solutions were extracted three times with 1 L of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen and then diluted in 1 mL of ethyl acetate.

For IAA determination, the fungal culture was filtered and the supernatant was adjusted to pH 3 using 1 N HCl. IAA from supernatant solutions was extracted three times with 1 L of ethyl acetate, and the extracts were combined, evaporated to dryness under a stream of nitrogen, and diluted in 1 mL of ethyl acetate. IAA was methyl esterified with 600 μL of acetyl chloride in 2 mL of dry methanol, sonicated for 15 min, and heated at 75°C for 1 h. The IAA methyl ester was evaporated under a stream of nitrogen and redissolved in 1 mL of ethyl acetate. The sample was diluted 1:10 (v/v) without L-Trp in the medium and 1:100 (v/v) with L-Trp before GC-MS analysis.

The indolic compounds were analyzed in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and a 30-m × 0.2-μm × 0.25-mm, 5% phenyl methyl silicone capillary column (HP-5 MS). Operating conditions used 1 mL min^{-1} helium as carrier gas, detector temperature of 300°C, and injector temperature of 250°C. The volume of the injected sample was 1 μL. The column was held for 3 min at 80°C and programmed at 6°C min^{-1} to a final temperature of 230°C for 5 min. Indolic compounds were identified by comparison with a mass spectra library (National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health; Chem Station; Hewlett-Packard). The identities of the indolic compounds were further confirmed by comparison of the retention time in the fungal extract with samples of the pure IAAld, IEt, and IAA standards (Sigma). A selected ion monitoring analysis was used to verify the presence of these indolic compounds in the samples. The molecular ions were monitored after electron impact ionization (70 eV). For IAAld, mass-to-charge ratios (*m/z*) were *m/z* 144, *m/z* 116, and *m/z* 89; for IEt, they were *m/z* 161, *m/z* 130, *m/z* 103, and *m/z* 77; and for IAA methyl ester, they were *m/z* 189, *m/z* 130, *m/z* 103, and *m/z* 77. To estimate the amount of compounds produced by *T. virens*, we constructed individual calibration curves for all three standards using concentrations from 40 to 400 μg for IAAld, 30 to 300 μg for IEt, and 0.5 to 5 μg for IAA.

Inoculation Experiments

T. virens and *T. atroviride* were evaluated in vitro for their plant growth-promoting ability using the Arabidopsis Col-0 ecotype. Fungal spore densities of 1×10^6 spores were inoculated by placing the spores at the opposite ends of agar plates containing 4-d-old germinated Arabidopsis seedlings (10 seedlings per plate). Plates were sealed with Parafilm and arranged in a completely randomized design. The seedlings were cultured for different time periods in a Percival AR95L growth chamber. Plants were sectioned at the root/shoot interface to quantify shoot weight. The fresh weight was measured on an analytical scale immediately after plant harvest, stem and root lengths were measured with a ruler, and lateral roots were counted and measured with a dissection microscope.

Determination of Developmental Stages of LRP

LRP were quantified at day 5 after fungal inoculation. 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 to 10 "short" pericycle cells are formed. Stage II, the formed LRP is divided into two layers by a periclinal division. Stage III, the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV, LRP with four cell layers. Stage V, the LRP is midway through the parent cortex. Stage VI, the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII, the LRP appears to be just about to emerge from the parent root.

Histochemical Analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37°C in a GUS reaction buffer (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using 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 and photographed using Nomarski optics on a Leica DMR microscope.

Aux/IAA Protein Degradation Assay

Six-day-old *HS*::*AXR3NT-GUS* Arabidopsis transgenic seedlings were incubated on liquid 0.2× MS medium for 2 h at 37°C, followed by transfer of the seedlings into liquid 0.2× MS medium supplied with the different indolic compounds for 5, 10, 20, or 60 min at 22°C. The seedlings were washed with fresh 0.2× MS medium and, 12 to 14 h later, histochemically stained for GUS activity.

Data Analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All lateral roots emerging from the primary root and observed under the 3× objective were taken into account for lateral root number data. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with Tukey's posthoc test were used for testing differences in growth and root developmental responses in wild-type and mutant plants. In the figures, different letters are used to indicate means that differ significantly ($P < 0.05$).

Supplemental Data

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

Supplemental Figure S1. Effects of IAAld on Arabidopsis lateral root development.

Supplemental Figure S2. Effects of IAAld on Arabidopsis biomass production.

Supplemental Figure S3. Effects of IAAld on primary root growth in wild-type (Col-0) plants and auxin-related Arabidopsis mutants.

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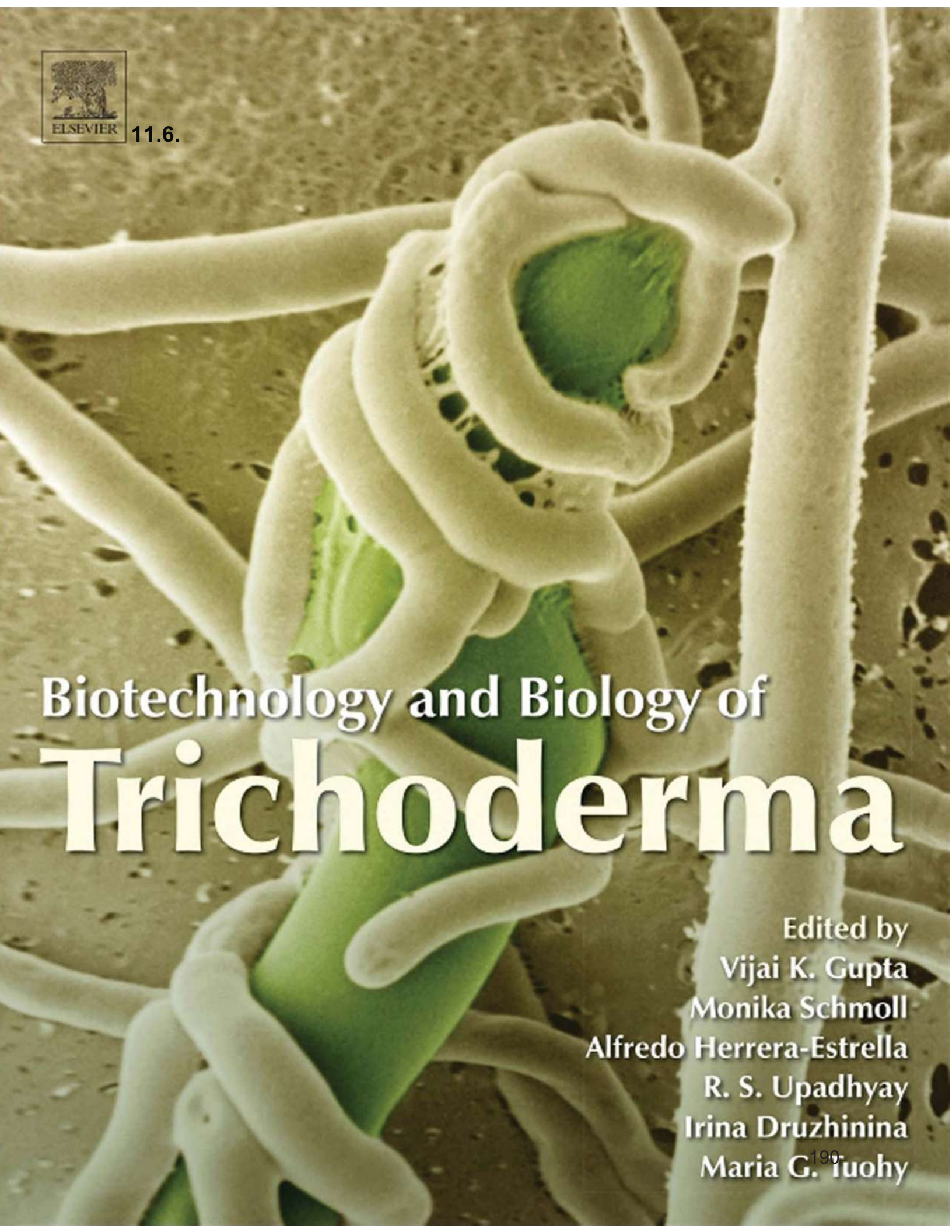
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BIOTECHNOLOGY AND BIOLOGY OF TRICHODERMA

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Preface

A growing world population and the increased energy consumption caused by a higher standard of living pose a challenge on current efforts to sustain a healthy environment and counteract climate change in the future. Replacing the limited resource of fossil oil and related products with renewable, carbon dioxide-neutral resources requires a considerate strategy, as also renewable biomass is not an unlimited resource. In order to achieve a sustainable economy, the delicate balance between use of biomass/land for food production and for use in industry and as an energy resource has to be kept. Species of the genus *Trichoderma* can play a significant role in the strategy for a sustainable future and this book summarizes the capabilities these fungi offer.

On the one hand, the metabolic capacities of *Trichoderma* are of central importance for breakdown of plant cell walls into small compounds that can be utilized by yeast not only for bioethanol production, but also as building blocks for chemical synthesis. With its potent cellulase system and its versatility for heterologous proteins, which facilitates complementation of this system with efficient enzymes from other organisms, *Trichoderma reesei* has become one of the cornerstones for second-generation biofuel production. Several chapters of this book provide an overview of the enzyme system of *Trichoderma* and its optimization for efficient utilization and conversion of lignocellulosic material. Additionally, novel and established tools for enhancing cellulase production are discussed. However, besides production of second-generation biofuels from plant material, industrial use of *Trichoderma* also extends to production of silver nanoparticles and applications in beer and wine industry as well as in textile industry.

Trichoderma also serves as a versatile host for expression of heterologous proteins and a broad array of tools are available for modification of the genome of this fungus for improvement of its production capacity. Chapters on heterologous protein production with *Trichoderma*, secretion and industrial strain improvement provide an overview of the use of this fungus as a cell factory in biotechnology. The enzyme systems of *Trichoderma* have even been used for bioremediation, which is a further important contribution to environmental sustainability. Other products of potential relevance for industry are the secondary metabolites produced by *Trichoderma* spp. as well as metabolic byproducts with interesting physiological or chemical functions.

While *T. reesei* serves as a workhorse for industrial enzyme production, other species of the genus are used for plant protection in agriculture. Thereby, these fungi play an important role in establishing this important and delicate balance between food production and the use of biomass for energy production and chemical industry. Efficient and sustainable use of biomass requires protection of energy plants and food crops from pathogens in order to guarantee that biomass as a limited resource can fulfill the need of both society and industry. Different species of *Trichoderma* act positively on plant growth and resistance of plants against disease. The chapters of this book include a thorough summary on mechanisms and application of biocontrol, the enhancing effect on plant immunity and mycoparasitism.

Considering the huge potential of *Trichoderma* for use in agriculture and industry, exploration of natural isolates of the genus is warranted to further increase the genomic resources to be exploited. Screening the biodiversity of different habitats and the ecophysiology of *Trichoderma* in a genomic perspective as well as analysis of this diversity delivers important insights into the promises the genus *Trichoderma* holds for the future.

In summary, this book gives a detailed overview of the field of industrial and agricultural use as well as the research with *Trichoderma* from industrial enzyme production to strain improvement to biocontrol and diversity.

Editors

Foreword

Trichoderma exists probably since at least 100 millions of years, but it entered the scientific spotlight only in the late seventies of the last century, when the first oil shock prompted governments to look for alternatives for fossil fuel. Researchers at the US military laboratories at Natick, Massachusetts, then remembered to possess a culture of a green fungus that had been destroying all the cotton material (tents, belts, clothes) of the US soldiers during the Second World War in the South Pacific at Guadalcanal (Solomon Islands), and who was subsequently demonstrated to have exceptional cellulolytic abilities. This fungus, like any other *Trichoderma* isolate at that time, was then named "*T. viride*" because the genus was believed to consist only of a single species. It was later re-identified as "*T. reesei*" (in honor of one of the researchers exploring its cellulolytic properties, Elwyn T. Reese), for a few years misnamed as *T. longibrachiatum*, and finally found out to be the asexual form of a very common tropical ascomycete, *Hypocrea jecorina*. The interest in this organism was of outmost importance to the *Trichoderma* community in general, because it challenged researchers to develop a whole toolbox of molecular genetics techniques for its manipulation, finally culminating in the sequencing of the genome of the original isolate QM6a and several of its cellulase-producing mutants, which comprise an invaluable aid to study this organism.

While *Trichoderma* is consequently known to many people only as the organism that makes cellulases, a parallel world of *Trichoderma* started to develop in 1932 when R. Weindling published the mycoparasitic abilities of *Trichoderma* "lignorum" (an illegitimate name) on plant pathogenic fungi. This biocontrol ability is due to the profound ability of *Trichoderma* to parasitize or even prey on other fungi, which today is known to be the innate nature of the whole genus. Weindling's findings formed the basis for a multitude of studies on the potential use of various *Trichoderma* spp. for the biological control of plant pathogenic fungi, resulting in the commercialization of some of them. The cellulase and the biocontrol researchers long formed two isolated communities with little information exchange, but this improved once the need for exchange of molecular genetics research techniques became apparent. Most recently the genomes of several *Trichoderma* biocontrol species have been sequenced, and two of them (*T. atroviride*, *T. virens*) have been published.

Yet *Trichoderma* offers much more to science: its species are among the most frequent mitosporic fungi commonly detected in cultivation-based surveys. They can be isolated from an innumerable diversity of natural and artificial substrata, particularly also from materials infested with xenobiotics, demonstrating their high opportunistic potential and adaptability to various ecological conditions. Consequently, it has broad impacts on mankind: one of the most stimulating recent findings is that some *Trichoderma* spp. occur or can occur as symptomless associates of plant-endophytes, thereby stimulating plant growth, delaying the onset of drought stress and preventing attacks of pathogens. Yet, there are also negative impacts of *Trichoderma* on mankind: in a clinical context, a pair of genetically related species (*T. longibrachiatum* and *T. orientale*) have been shown to occur as opportunistic pathogens of immunocompromised humans, and several *Trichoderma* spp. can occur as indoor molds, although their effect on human health is less severe than that of other fungal species. Finally, some species like *T. aggressivum, T. pleuroticola*, *T. pleurotum*, and *T. mienum* have turned their mycoparasitic abilities against commercial mushrooms like *Agaricus* and *Pleurotus*, thereby causing large economic losses.

All these properties make *Trichoderma* one of the most versatile and intriguing fungal genus, which still offers numerous aspects to be dealt with in more detail. This book has been initiated to describe the current stage of knowledge on *Trichoderma* from various perspectives, thereby outlining also those areas where further progress is needed.

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CHAPTER

36

Enhanced Plant Immunity Using *Trichoderma*

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OUTLINE

INTRODUCTION

The roots of plants grow in close association with numerous bacterial and fungal species, and these relationships can be neutral, pathogenic or beneficial. Several species of *Trichoderma* are considered symbiotic as they help in biocontrol of root pathogens, promote growth and development and induce plant defense responses by several mechanisms (Druzhinina et al., 2011). Four levels of interactions have been described in the phytostimulation process elicited by *Trichoderma*: (1) communication based on release of Volatile Organic Compounds (VOCs) (Vinale et al., 2008; Hung et al., 2013), (2) production of auxin and precursors of auxins that are detected by plants to modulate root architecture (Contreras-Cornejo et al., 2009), (3) activation of local and systemic responses through physical contact between hyphae and root epidermis or by releasing elicitors (Contreras-Cornejo et al., 2011; Brotman et al., 2012; Mathys et al., 2012), and (4) proliferation of mycelium in internal parts of the plant (Contreras-Cornejo et al., 2011; Velázquez-Robledo et al., 2011) (Fig. 36.1).

Currently there is an increasing effort to unravel the molecular mechanisms by which plants sense *Trichoderma* and how these fungi induce plant immunity. This chapter focuses on recent developments in the signal recognition, perception, and transduction mechanisms underlying *Trichoderma*-induced plant defense responses.

MECHANISMS OF PLANT PROTECTION BY MICROBES

Over the course of evolution, plants have developed very sensitive mechanisms through which they can perceive and respond to biotic stimuli, changing their physiology and morphology to ensure survival. Therefore, an array of signaling and response elements must already be organized to rapidly coordinate external stimuli from symbionts and pathogens with developmental traits to avoid an invasion and a subsequent damage or disease. Some of these defense mechanisms are preformed and provide physical and chemical barriers (Hückelhoven, 2007; Hématy et al., 2009); others are induced only after

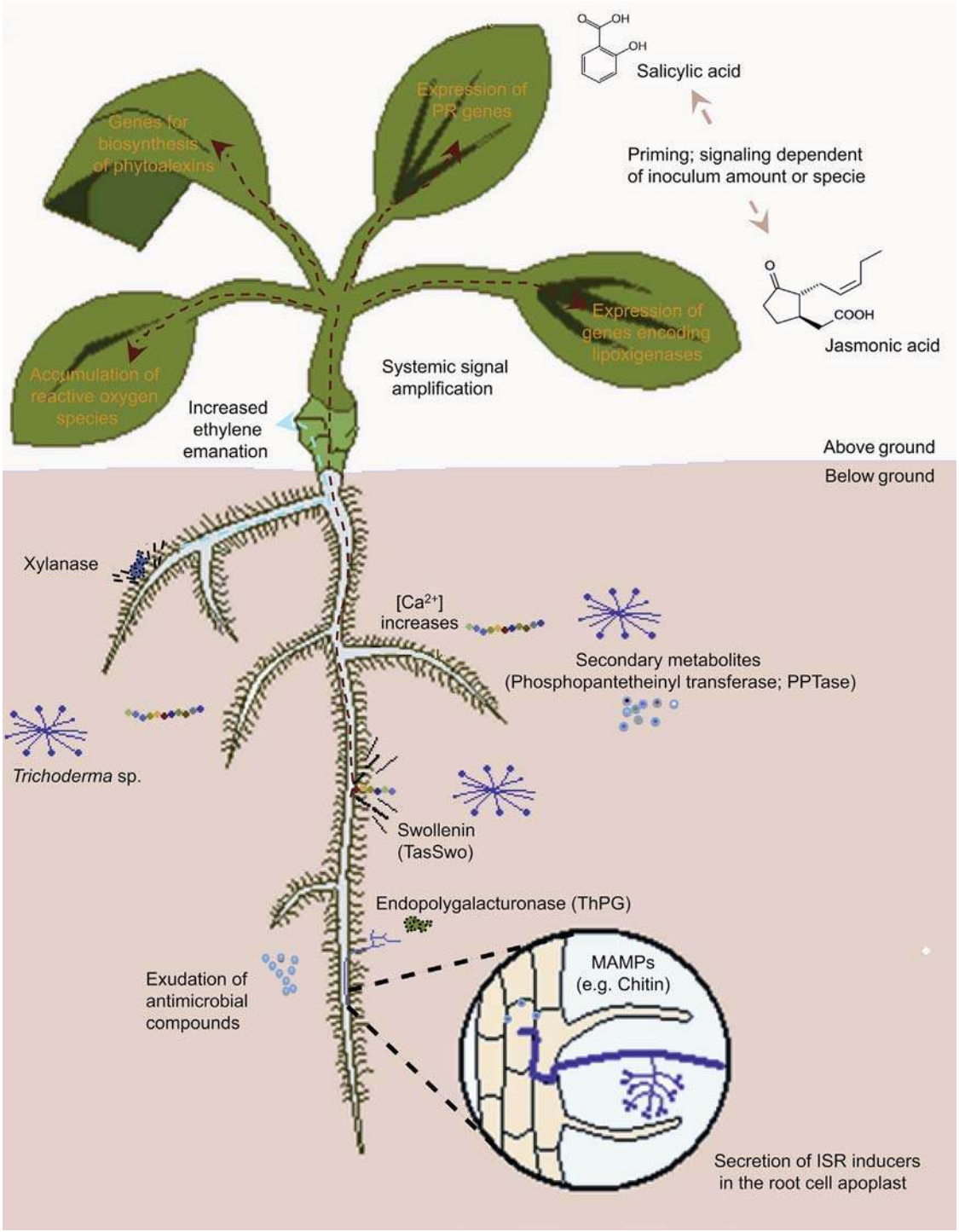

FIGURE 36.1 A simplified model for plant defense responses induced by *Trichoderma*. Host recognition of *Trichoderma* elicitors (MAMPs) such as xylanase, ThPG, TasSwo initiates early signaling events such as protein phosphorylation/dephosphorylation, ion fluxes and oxidative burst. Subsequent events imply biosynthesis of phytohormones such as SA, JA and ET, the production of antimicrobial compounds and induction of plant defense genes such as *LOX2* and *PR1*. Mutation of PPTase in *T. virens* affects defense responses induced by this fungus through the SA pathway. (For color version of this figure, the reader is referred to the online version of this book.)

perception of microbe-derived molecules (Koornneef and Pieterse, 2008; Zamioudis and Pieterse 2012).

The root surface and the rhizosphere, the soil volume influenced by the presence of roots, are niches rich in nutrients, which may attract a myriad of free living species of bacteria, fungi and protozoa that compete for these nutrients and coexist in a complex ecological environment (Raaijmakers et al., 2009). Once microorganisms are attracted, the next step to define the resulting interaction as neutral, pathogenic or beneficial to plants is the

recognition process, which can occur through the emission of VOCs, by diffusible molecules or through physical interactions (Ryu et al., 2004; Göhre and Robatzek, 2008; Nürnberger and Kemmerling, 2009; Hung et al., 2013).

Microbes produce several molecules that plants specifically recognize and in consequence activate an innate immune response. If the molecules are associated with beneficial microbes then are designed as microbe-associated molecular patterns (MAMPs), in contrary to those related to pathogens, which are called pathogen-associated molecular patterns (PAMPs) (Göhre and Robatzek, 2008; Gimenez-Ibanez et al., 2009; Nürnberger and Kemmerling, 2009). Flagellin, chitin, ergosterol, glycoproteins and lipopolysaccharides are examples of the prototypical PAMPs that are recognized by plant resistance (R) proteins, resulting in an enhanced immune response called Effector-Triggered Immunity (ETI; Jones and Dangl, 2006; Chisholm et al., 2006).

Early responses in plant defense involve changes in cytosolic calcium (Ca^{2+}) levels, in the generation of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), and reactions as protein phosphorylation and activation of phospholipases and also GTP-binding proteins (Levine et al., 1994; Felix et al., 1999; Pedley and Martin, 2005; Zipfel and Felix, 2005; Naoumkina et al., 2007; Van Loon et al., 2008).

In addition to eliciting primary defense responses, signals from microbes may be amplified through the generation of plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). SA and JA can be methylated (SA-Me and JA-Me, respectively), and then become volatiles that are potent inducers of genes for protection of root and aboveground tissues (Heil and Ton, 2008; Park et al., 2007; Pieterse et al., 2009; Shah, 2009). SA is generally related with biotroph organism resistance, whereas JA and ET are generally associated with resistance to necrotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005; Truman et al., 2007).

Resistance that is induced in response to local attack is often extended systematically in organs that are not suffering any damage. The vasculature provides an important conduit for translocation of diffusible signals that contribute to root-shoot communication thus enabling immunity in distal plant parts (Van Bel and Gaupels, 2004).

Two kinds of induced resistance have been defined in plants on the basis of differences in their stimulation: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is triggered by pathogens causing limited infection, such as those causing hypersensitive necrosis (Durrant and Dong, 2004), and is characterized by an early increase in the synthesis of SA (Ryals et al., 1996; Sticher et al., 1997). On the other hand, ISR is activated through JA and ET when plants are stimulated and primed by beneficial microbes to resist infection by pathogens (Van Loon et al., 1998, Van Loon, 2007; Van Wees et al., 2008; Truman et al., 2007).

The protection mechanism is primed by the expression of defense genes and also by the production of chemical compounds called phytoalexins that include flavonoids, phenols, glucosinolates, terpens and alkaloids with a broad spectrum of antimicrobial functions. These compounds can be synthesized as result or in parallel to hormonal responses (Dixon et al., 2002; Ahuja et al., 2012).

Systemic Acquired Resistance

Salicylic acid appears to be a critical regulator to the induction of SAR. SA mediates the activation of a large set of pathogenesis-related (*PR*) genes, which may encode for proteins with antimicrobial activity (Durrant and Dong, 2004; Van Loon et al., 2008). The activation of late defense genes by SA, such as *PR-1*, involves the participation of the nonexpressor of PR genes1 (NPR1) protein. This protein acts as co-activator of transcription factors that recognize *as-1*-like elements in the *PR-1* promoter and in promoters of other genes involved in protection of plants (Uquillas et al., 2004). *Arabidopsis* mutants defective in the NPR1 protein (*npr1*) are unable to express the *PR-1*, *PR-2* and *PR-5* genes and fail to develop resistance against pathogens in response to SA or active analogs of SA (Cao et al., 1994). NPR1 has also been implicated in JA/ET-dependent ISR (Dong, 2004; Pieterse and Van Loon, 2004). For instance, *Arabidopsis npr1* mutants are blocked in their ability to express ISR upon colonization of the roots by the beneficial bacterium *Pseudomonas putida* WCS417r (Pieterse et al., 1998). Research performed by Verberne et al. (2003) demonstrated that in tobacco ET perception is required for the onset of SA-dependent SAR that is triggered upon infection by tobacco mosaic virus.

Induced Systemic Resistance

Selected strains of Plant Growth Promoting Rhizobacteria (PGPR) and Plant Growth Promoting Fungi (PGPF) contribute to plant immunity inducing ISR. Some PGPF including mycorrhizal fungi and nonpathogenic strains of *Fusarium oxysporum*, *Trichoderma* sp., *Penicillium* sp. GP16-2, *Pythium oligandrum*, *Piriformospora indica* and related *Sebacinales* can reduce disease in above ground plant parts through the induction of ISR (Schafer et al., 2009; Van der Ent et al., 2009; Van Loon et al., 1998). This type of resistance occurs when the plant defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon, 1997). A signal transduction pathway for ISR triggered by PGPR shows that the mechanism is dependent on JA, ET and NPR1, but independent of SA (Ryu et al., 2004). The interaction between JA and ET signaling may be sometimes synergistic as in the regulation of the *Arabidopsis* plant defensin gene *PDF1.2*, which requires concomitant activation of the JA and ET response pathways (Penninckx et al., 1998).

Although most studies on the beneficial role of microbes inducing resistance, point to a role for JA and ET in the regulation of the induced immune response (Van der Ent et al., 2009), several examples of PGPR and PGPF that trigger the SA-dependent SAR response have been documented (Contreras-Cornejo et al., 2011). SAR and ISR are similar in that they both confer a broad-spectrum disease resistance. However, they seem to be regulated by different signal transduction pathways and the resulting protection.

Chemical Defenses

In parallel or as a result or hormone activated defense, biosynthesis of phytoalexins contributes to plant immunity (Dixon et al., 2002; Ahuja et al., 2012). These phytochemicals are broad-spectrum antagonists and rapidly accumulate in areas of pathogen infection, affecting the growth and development of pathogens at infection sites (Hain et al., 1993; Kuc, 1995). Rice produces 15 phytoalexins, including momilactones A (MA) and B (MB), phytocassanes A to E (PA to PE), oryzalexins A to F, and oryzalexin S, and sakuranetin a flavonoid phytoalexin (Hasegawa et al., 2010). These compounds quickly accumulate and exhibit antibiotic activity to inhibit the invasion of the rice-blast pathogens *Magnaporthe grisea* and *Rhizoctonia solani* (Kuc, 1995; Koga et al., 1995; Dillon et al., 1997). Slight phytoalexin production is present in healthy leaves of rice and *Arabidopsis* under normal growth conditions, but there is an increase in levels in both susceptible and resistant plants in response to attack by pathogens, including the bacterium *Pseudomonas syringae*, the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* and the blast fungus *M. grisea* (Koga et al., 1995; Nafisi et al., 2007). In contrast, more highly and rapidly accumulated phytoalexins, such as MA, MB, and PA to PE, contribute to the resistance to blast fungus in resistant rice, compared with the delayed induction of phytoalexin biosynthesis in susceptible rice plants (Umemura et al., 2003; Kim et al., 2009; Hasegawa et al., 2010). The accumulation of camalexin in *Arabidopsis* in response to *Trichoderma* was recently reported indicating a further level of plant protection by these beneficial fungi.

TRICHODERMA-INDUCED IMMUNITY

Electron microscopy of ultrathin sections from *Trichoderma*-treated roots revealed penetration of hyphae into the epidermis and outer cortex. The colonization of roots by *Trichoderma* leads to a state of induced resistance (Yedidia et al., 1999). The defense responses elicited are complex and depending of the amount of inoculum may involve the canonical defense hormones SA and JA, as well as biosynthesis of camalexin, the major phytoalexin in *Arabidopsis* (Contreras-Cornejo et al., 2011). Therefore, the mechanisms by which *Trichoderma* interacts with roots are important to decipher the aspects of plant immunity and may open new ways to protect plants from pathogens.

Sensing the Fungus

Generally, plants sense fungi by recognition of the MAMPs ergosterol and chitin oligomers released from fungal cell walls; this process occurs through the LysM receptor proteins CERK1 and CEBiP identified in *Arabidopsis* and rice, respectively (Gimenez-Ibanez et al., 2009). In addition, there might be specificity in the recognition process. The first recognized MAMP from *Trichoderma* was an ET-inducing xylanase (Xyn2/Eix) produced by *Trichoderma viride,* which acts as a potent elicitor of plant defense in tobacco and tomato (Sharon et al., 1993). Other elicitors from *Trichoderma virens* include cellulases, swollenin TasSwo, endopolygalacturonase ThPG1, alamethicin (ALA), 18mer peptaibols and a small cysteine-rich protein termed Sm1, which is a member of the cerato-platanin family (Sharon et al., 1993; Engelberth et al., 2001; Martinez et al., 2001; Djonovic et al., 2006; Viterbo et al., 2007; Vargas et al., 2008; Brotman et al., 2008; Morán-Diez et al., 2009; Hermosa et al., 2012), the molecular mechanisms by which these elicitors are recognized by plants is an area of active research.

Currently, various elicitors from *Trichoderma* have been reported to trigger fluxes of Ca^{+2} and increase the accumulation of ROS (Hanson and Howell, 2004; Navazio et al., 2007). The changes of cytosolic Ca^{2+} induced by MAMPs plays a pivotal role in innate immunity in plants. Calcineurin B-like proteins (CBLs) act as Ca^{2+} sensors to activate specific protein kinases, CBL-interacting protein kinases (CIPKs). Two CIPKs from rice (*Oryza sativa*) identified as OsCIPK14 and OsCIPK15, were rapidly induced by chitooligosaccharides and xylanase from *T. viride* (Kurusu et al., 2010). Interestingly, TvX/EIX-induced cell death was enhanced in rice plants that overexpress OsCIPK15 (Kurusu et al., 2010). These data suggest that OsCIPK14/15 play a crucial role in the defenses induced by MAMP in rice.

Production of ROS via consumption of oxygen in a so-called oxidative burst is one of the earliest cellular responses following microbe recognition. Recently, it was reported an increase in ROS in cotton treated with the Sm1 elicitor from *T. virens* and in *Arabidopsis* roots inoculated with the same fungus (Djonovic et al., 2006; Contreras-Cornejo et al., 2011). Current challenges imply deciphering the local and systemic effects of ROS produced locally in roots during *Trichoderma* interaction and how ROS are produced following the perception of MAMPs to trigger immune responses. It would be important to understand which genes act downstream

of ROS signaling and how root immunity differs from defense reactions in the leaf.

MAPK (mitogen-activated protein kinases) signal transduction cascades mediate various aspects of plant biology, including stress responses and developmental programs (Meskiene and Hirt, 2000). Activation of MPK3 and MPK6 by ectopic expression of constitutively active MAPKK led to hypersensitive responses and camalexin biosynthesis (Ren et al., 2002; Takahashi et al., 2007). Phytoalexin production is compromised in *mpk3* and *mpk6 Arabidopsis* mutants (Ren et al., 2008; Mao et al., 2011). Recently, it was reported that PAMP treatment causes rapid and transient activation of MAPK cascade (Ishihama and Yoshioka, 2012).

In cucumber, a MAPK that is activated in response to *Trichoderma asperellum* was identified and referred to as *Trichoderma*-induced MAPK (TIPK). This protein is homologous to MPK3 from *Arabidopsis*. TIPK is activated by pathogen challenge and application of JA or SA or inhibitors of JA and ET, suggesting that TIPK operates through JA/ET signaling pathways (Shoresh et al., 2006). In cacao plants inoculated with *Trichoderma hamatum*, or during the *T. asperellum*-cucumber interaction, the expression of a homolog of *MPK3* was also increased (Shoresh et al., 2006; Bae et al., 2009).

Hormone Signaling

Different studies indicate that induction of pathogen resistance by *Trichoderma* may be associated in part to marked metabolic changes in the host, including enhanced production of JA, SA and ET (Shoresh et al., 2005; Segarra et al., 2007). Interestingly, expression of JA or SA-responsive genes or hormone content is modulated by the amount of conidia or time of interaction (Segarra et al., 2007; Gallou et al., 2009; Contreras-Cornejo et al., 2011). In plants inoculated with *Trichoderma* the content of JA rapidly increases (Segarra et al., 2007; Martínez-Medina et al., 2010). In *Arabidopsis* the accumulation of JA was accompanied by the systemic expression of *LOX2* that encodes for a lipoxygenase involved in JA-biosynthesis (Contreras-Cornejo et al., 2011). ISR triggered by *Trichoderma harzianum* T39 was blocked in JA and ET signaling mutants of *Arabidopsis* (Korolev et al., 2008). Application of silver thiosulfate and diethyldithiocarbamate, which block the action of ET and the synthesis of JA, respectively, reduced *T. asperellum* T203-mediated ISR against *P. syringae* pv. *lachrymans*, indicating a role for JA/ET-dependent signaling in ISR in cucumber (Shoresh et al., 2005). Colonization of maize roots by *T. virens* also induced the expression of JA biosynthetic genes (Mukherjee et al., 2012).

The recognition of *Trichoderma* by the plant triggers SA production and induction of the plant immune marker *PR-1*, which is regulated by SA (Segarra et al., 2007; Contreras-Cornejo et al., 2011). Interestingly, mutation of *phosphopantetheinyl transferase1* gene *(PPT1-1)* in *T. virens* affected the accumulation of SA and the expression of *PR-1* in *Arabidopsis* seedlings inoculated with this fungus, resulting in decreased protection against *B. cinerea* (Velázquez-Robledo et al., 2011).

Modulation of Gene Expression

Interaction of plants with *Trichoderma* results in rapid and systemic induction of defense-related genes (Alfano et al., 2007; Contreras-Cornejo et al., 2011; Salas-Marina et al., 2011; Brotman et al., 2012; Mathys et al., 2012). Recent studies using microarray analysis in *Arabidopsis* and tomato increased our understanding of the physiological and biochemical changes that occur in the host plant. In *Arabidopsis*, it was shown that after 24 h of colonization with *T. harzianum* T34, some genes related to SA or JA signaling were downregulated. This would facilitate root colonization by the symbiotic fungus at earlier times (Morán-Diez et al., 2012). Systemic response might be a consequence of root colonization, being the first contact of *Trichoderma* with the plant through the root epidermis an important point of control of the interaction.

Changes in expression of genes regulated by JA and ET have been revealed in several plant species inoculated with *Trichoderma*. For example, *T. asperellum* modulates the local and systemic expression of *LOX1*, *PR-2*, *PR-3*, *ETR1* and *CTR1* in cucumber (Shoresh et al., 2005). The *Arabidopsis* mutants *ein2-1*, *eto2*, *eto3* and *npr1-5* inoculated with *T. harzianum* and challenged with *B. cinerea* were increasingly affected by this pathogen, suggesting that the resistance induced by *T. harzianum* against *B. cinerea* is dependent on JA/ET pathways (Korolev et al., 2008). In *Arabidopsis*, the expression profile of a set of SAR and ISR-related genes has been assessed. It was reported that *Trichoderma asperelloides* induce changes in the expression of the transcription factor MYB51 involved in regulation of glucosinolate biosynthesis, the transcription factor WRKY40 induced by pathogens, *ETO3*, which plays a role in ET biosynthesis and *LTP4* a lipid protein transferase (Brotman et al., 2012). In seedlings that were inoculated with *Trichoderma atroviride*, changes in the expression of *PAD3* gene that encodes the last enzyme involved in the synthesis of camalexin were reported (Salas-Marina et al., 2011). Several studies indicate that root colonization by *Trichoderma* results in increased levels of defense-related enzymes in plants, including peroxidases, chitinases and β-1-3-glucanase. For example, in oil-palm the chitinases *EgCHI1/2/3* were upregulated when inoculated with *T. harzianum* (Naher et al., 2012). A similar effect was reported for the *ChiB* and *Glu-1* genes in the interaction *Trichoderma stromaticum*–cacao (De Souza et al., 2008). During the *T. asperellum*–cucumber interaction the *HPL* gene, which

is activated by the production of antimicrobial compounds was upregulated (Yedidia et al., 2003). PAL is a key enzyme for the synthesis of SA regulated by the JA/ET signaling pathway (Shah, 2003; Djonovic et al., 2007). PAL provides precursors for the formation of an array of antimicrobial compounds (Dixon et al., 2002). It has been reported the upregulation of *PAL* in cucumber plants inoculated with *T. asperellum* and maize inoculated with *T. virens* (Yedidia et al., 2003; Shoresh et al., 2005; Djonovic et al., 2007; Mukherjee et al., 2012).

Most of the above mentioned studies indicate *Trichoderma*-induced defense and the protection conferred to pathogens is associated with the transcript accumulation of genes regulated by SA, JA and ET, as well as genes that encode transcriptional factors and genes involved in biosynthesis of antimicrobial compounds. The mechanisms by which these beneficial microbes activate the host's immune response not only share intriguing similarities but also display crucial differences with those that are commonly observed in interactions with other types of symbiotic microorganisms such as mycorrhizal fungi and PGPR (Gallou et al., 2009).

Chemical Communication and Defense

Trichoderma produces a number of VOCs that may have protective functions in plants. VOCs have been found to participate in different biological processes such as biocontrol or communication between microorganisms and their living environment and seem to play a key role in mycoparasitism of *Trichoderma* as well as in its interaction with plants (Vinale et al., 2008). *Trichoderma atroviride* grown in axenic conditions produced a blend of terpenes such as α-phellandrene, β-phellandrene, α-bergamotene and α-farnesene (Stoppacher et al., 2010). Cellulysin from *T. viride* triggers the emission of VOCs such as (3Z)-hexenyl acetate, β-ocimene, linalool, 4,8-dimethylnona-l,3,7-triene, indole and *cis*-jasmone involved in the octadecanoid signaling pathway (Piel et al., 1997). Alamethicin from *T. viride* is an ion channel-forming peptide that can induce VOC emission and increase endogenous levels of JA and SA in plants (Engelberth et al., 2001; Bruinsma et al., 2009). Eight-carbon VOCs such as 1-octen-3-ol, 3-octanol and 3-octanone act as signaling molecules regulating development and mediates intercolony communication in fungi (Nemcovic et al., 2008). Production of two compounds identified as harzianolide and 6-n-pentyl-6*H*-pyran-2-one (6PP) by *Trichoderma* spp. has been correlated with the plant resistance against *B. cinerea* in tomato plants. Interestingly, 6PP purified from *T. atroviride* induced the expression of *PR-1* in canola (Vinale et al., 2008).

Since biosynthesis of phytoalexins requires the activity of numerous enzymes, highly coordinated signaling events must be required in the elicited cells to establish successfully this type of defense response (Dixon and Paiva, 1995; Grayer and Kokubun, 2001; Yang et al., 2004). In *Arabidopsis*, previous studies have implicated camalexin in fungal response (Chassot et al., 2008). The indole ring of camalexin is derived from tryptophan and early biosynthetic steps are shared with other indolic compounds, such as indole glucosinolates (Sanchez-Vallet et al., 2010; Ahuja et al., 2012). *Trichoderma virens* and *T. atroviride* induce the accumulation of camalexin in *Arabidopsis*. *Trichoderma virens* also produces indole-3-carboxaldehyde (ICAld) a tryptophan-derived compound with activity in plant development (Contreras-Cornejo et al., 2011). A number of aldehydes possess the ability to react with Cys to form the corresponding thiazolidinecarboxylic acid (Zook and Hammerschmidt, 1997). It is plausible that the ICAld reacts with Cys to eventually form camalexin *in planta*.

Disruption of pathogen genes that encode enzymes known to detoxify phytoalexins can lead to loss of pathogenicity. In fungi, 4-phosphopantetheinyl transferases (PPTases) activate enzymes involved in primary and secondary metabolism. *Arabidopsis* seedlings inoculated with the Δ*ppt1-1* mutant of *T. virens* compromised the camalexin response, resulting in decreased protection against *B. cinerea* (Velázquez-Robledo et al., 2011). Better knowledge of the mode of action of phytoalexins and information about regulation of their biosynthesis in specific tissues and at specific developmental stages is required to improve the uses of *Trichoderma.*

PLANT PROTECTION CONFERRED BY *TRICHODERMA*

Trichoderma improved innate immunity in cucumber, cotton, maize, potato, tomato, cacao, melon, and *Arabidopsis* by all above mentioned mechanisms (Yedidia et al., 1999; Djonović et al., 2006; Brotman et al., 2009; Gallou et al., 2009; Morán-Diez et al., 2009; Salas-Marina et al., 2011; Tucci et al., 2011). For example, the colonization of maize roots by either *T. harzianum* T22 or *T. virens* enhanced plant growth and suppressed the soilborne disease caused by *Colletotrichum graminicola* (Harman et al., 2004; Djonovic et al., 2007). Similarly, when *Arabidopsis* seedlings were inoculated with *B. cinerea*, a number of plants developed necrotic lesions and died after 3 days, in contrast, when the seedlings were inoculated with either *T. virens* or *T. atroviride* and then challenged with *B. cinerea*, fewer plants were damaged and less necrotic lesions were observed in leaves (Contreras-Cornejo et al., 2011). In cucumber plants inoculated with *T. asperellum* T34 and then challenged with *P. syringae* pv. *lachrymans*, multiplication of *P. syringae* in the cotyledons was restricted when compared to uninoculated controls (Segarra et al., 2007). In melon plants, significant changes in hormonal content occurred as a consequence of the

interaction between the plant, the pathogen *F. oxysporum* and/or *T. harzianum*. Attack by *F. oxysporum* activated a defense response in the plant, mediated by SA, JA, ET, and abscisic acid, similar to that induced by *T. harzianum*, which was able to attenuate the SA-mediated response (Martínez-Medina et al., 2010). These data suggest that the induction of plant basal resistance and the attenuation of the hormonal responses caused by *F. oxysporum* are both mechanisms by which *T. harzianum* can control *F. oxysporum* wilt in plants.

In a very recent study by Alizadeh and associates (2013), the effectiveness of *T. harzianum* Tr6 and *Pseudomonas* sp. Ps14 on the efficacy of induced resistance was investigated in cucumber and *Arabidopsis.* Both biological control agents were isolated from the rhizosphere of cucumber and tested as a single application or in combination for their abilities to elicit induced resistance in cucumber against *F. oxysporum* f. sp. *radicis cucumerinum* and in *Arabidopsis* against *B. cinerea*. The combination of Tr6 and Ps14 induced a significantly higher level of resistance in cucumber, which was associated with the primed expression of a set of defense-related genes upon challenge with *Fusarium*. In *Arabidopsis* both Ps14 and Tr6 triggered ISR against *B. cinerea* but their combination did not show enhanced effects. In the induced systemic resistance-defective *Arabidopsis* mutant *myb72*, none of the treatments protected against *B. cinerea*, whereas in the SA-impaired mutant *sid2* all treatments were effective. Taken together, these results indicate that in *Arabidopsis* Ps14 and Tr6 activate the same signaling pathway and thus have no enhanced effect in combination. The enhanced protection in cucumber by the combination is most likely due to activation of different signaling pathways by the two biocontrol agents.

CONCLUSIONS

Plants interact with a myriad of microbial pathogens or symbionts with different lifestyles and colonization strategies. In the past few years, there has been a significant progress in understanding immunity activated by beneficial fungi. Plant inoculation with *Trichoderma* results in a state of improved resistance both locally and systemically. Communication and colonization of plant roots by *Trichoderma* can induce defense responses and confer protection against plant pathogens. This response may be activated by fungal elicitors and involve the accumulation of hormones such as SA, JA or ET in plant tissues. The pattern of altered gene expression observed in several plants inoculated with *Trichoderma* suggests a complex signal transduction network involving genetic cross talk. The degree and characteristics of the plant defense mechanism seem to be strain dependent and is associated to the accumulation of antimicrobial compounds such as VOCs and phytoalexins.

In the first stages of interaction with plants, MAMPs from *Trichoderma* may be perceived by receptors to activate signaling pathways and host responses. The characterization of two specific ISR elicitors secreted by *T. virens* Gv29-8 has been reported. Peptides with antimicrobial activity termed peptaibols have ISR effects and systemically induce defenses in cucumber leaves (Viterbo et al., 2007). Another ISR elicitor is the extracellular small protein Sm1, whose gene expression was upregulated in the presence of cotton plants (Djonovic et al., 2006). Further in vivo studies, using reverse genetic analyses, demonstrated that expression of *SM1* is essential for triggering ISR in maize plants and providing protection against the foliar pathogen *C. graminicola* (Djonovic et al., 2007). A reaction similar to ISR or SAR may occur later in the plant amplifying the magnitude of the response through priming. Priming is defined as the physiological state that enables plants to respond to a stimulus in a very efficient way, in a more rapid and robust manner than nonprimed plants. Priming has been found to be critical in various types of systemic plant immunity, including SAR and ISR. Like plant beneficial rhizobacteria, *Trichoderma* can induce priming for enhanced defense in plants (Alfano et al., 2007; Mathys et al., 2012). The mechanisms underlying this process are starting to be revealed (Segarra et al., 2007; Contreras-Cornejo et al., 2011; Mathys et al., 2012). Currently, there is a need for more studies aimed at testing the functional relevance of genes and proteins whose expression is modulated during the interaction both in the plant and the fungi, as well as characterizing the phenotypes of loss-of-function mutants and overexpressing lines during *Trichoderma*–plant interactions in the presence of pathogens. Current challenges are to decipher how root signaling is connected with defense responses in leaves and which genes and processes are implied in such long distant communication.

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11.7. Trichoderma-induced plant immunity likely involves both hormonal- and camalexin-dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungus *Botrytis cinerea*

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Key words: Arabidopsis, Trichoderma, phytostimulation, defense responses, jasmonic acid, salicylic acid, camalexin

Abbreviations: WT, wild type; dai, days after inoculation; JA, jasmonic acid; SA, salicylic acid; ET, ethylene; ROS, reactive oxygen species; SAR, systemic acquired resistance; ISR, induced systemic resistance; PR, pathogenesis-related proteins; PAL, phenylalanine ammonia-lyase; ICAld, indole-3-carboxaldehyde; Cys, cysteine; GUS, β-glucuronidase; GC-MS, gas chromatography-mass spectrometry

Filamentous fungi belonging to the genus Trichoderma have long been recognized as agents for the biocontrol of plant diseases. In this work, we investigated the mechanisms involved in the defense responses of *Arabidopsis thaliana* seedlings elicited by co-culture with *Trichoderma virens* and *Trichoderma atroviride*. Interaction of plant roots with fungal mycelium induced growth and defense responses, indicating that both processes are not inherently antagonist. Expression studies of the pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA* in response to *T. virens* or *T. atroviride* provided evidence that the defense signaling pathway activated by these fungi involves salicylic acid (SA) and/or jasmonic acid (JA) depending on the amount of conidia inoculated. Moreover, we found that Arabidopsis seedlings colonized by Trichoderma accumulated hydrogen peroxide and camalexin in leaves. When grown under axenic conditions, *T. virens* produced indole-3-carboxaldehyde (ICAld) a tryptophan-derived compound with activity in plant development. In Arabidopsis seedlings whose roots are in contact with *T. virens* or *T. atroviride*, and challenged with *Botrytis cinerea* in leaves, disease severity was significantly reduced compared with axenically grown seedlings. Our results indicate that the defense responses elicited by Trichoderma in Arabidopsis are complex and involve the canonical defense hormones SA and JA as well as camalexin, which may be important factors in boosting plant immunity.

Introduction

Plants are in continuous interaction with a plethora of microorganisms, including pathogens and symbionts. In the rhizosphere, extensive communication occurs between plants and their associated microbes by exchange of different classes of microbial and plant compounds. This molecular dialog will determine the final outcome of the relationship, usually through highly coordinated cellular processes.[1]

Filamentous fungi of the genus Trichoderma are common rhizosphere inhabitants. They have been widely studied due to their capacity to produce antibiotics, parasitize other fungi or compete with deleterious plant microorganisms.[2] It has been known for many years that Trichoderma species enhance plant growth and productivity in axenic systems and in soil.[3-5] In *Arabidopsis thaliana*, growth promotion by *T. virens* correlated with increased root biomass production and enhanced lateral root formation, which was attributed to the production of auxin signals by this fungus.[6] Trichoderma also induces plant defense responses. Co-cultivation of cucumber plants with *T. harzianum* increased production of peroxidase and chitinase, while *T. asperellum* T34 conferred protection against *Pseudomonas syringe* pv. *Lachrymans* through regulation of proteins involved in adaptation to stress, isoprenoid and ethylene biosynthesis, photorespiration and carbohydrate metabolism.[7,8] Nevertheless, the signaling mechanisms by which Trichoderma species activate plant immunity remain uncertain.

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Plants possess various inducible defense mechanisms for protection against pathogen attack. An example of this is systemic acquired resistance (SAR), which is activated by a wide range of pathogens, especially (but not only) those that cause tissue necrosis.[9] Similarly, colonization of plant roots by certain non-pathogenic rhizobacteria can induce systemic resistance (ISR) in the host plant.[10] Both pathogen-induced SAR and rhizobacteria-mediated ISR are effective against different types of pathogens, and are typically characterized by a restriction of pathogen growth and a suppression of disease development compared with primary infected, non-induced plants. However, the signaling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR differ. Whereas SAR requires endogenous accumulation of salicylic acid (SA), the signaling pathway controlling ISR functions independently of SA and requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene.[10-12] Additionally, it has been established that accumulation of phytoalexins and other low molecular weight antimicrobial metabolites is integral to plant protection. The chemical structures of phytoalexins vary among different plant families and include flavonoids, terpenoids and indoles.[13]

The antimicrobial properties of phytoalexins, which have been extensively investigated, suggest their potential function in the host defense machinery. However, only recently they have been found to make an important contribution to plant defense against particular pathogens.[14] Mutations that cause reduced phytoalexin levels can lead to increased susceptibility of plants to pathogens such as *Botrytis cinerea*.[15-17] The main phytoalexin detected in Arabidopsis is an indole derivative called camalexin (3-thiazol-2'-yl-indole).[17-20] Camalexin accumulates in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringe* and after inoculation with the fungus *Cochliobolus carbonum* and is able to inhibit bacterial and fungal growth.[19,21,22]

The indole ring of camalexin is produced from Trp through at least three CYP (cytochrome P450) steps. Trp is converted to indole-3-acetonitrile (IAN) by CYP79B2/CYP79B3 and CYP71A13. Conversion of Cys(IAN) to dihydrocamalexic acid and subsequently to camalexin is catalyzed by CYP71B15.[23,24] Based on the *pad3* mutant phenotype CYP71B15 has been involved in camalexin biosynthesis by converting cysteine-indole-3-acetonitrile to camalexin.[24-26] Alternatively, camalexin biosynthesis may proceed through condensation of indole-3-carboxaldehyde (ICAld) with cysteine (Cys) followed by cyclization and decarboxylation.[27]

B. cinerea is the causal agent of gray mold and causes rot symptoms in more than 200 different plant species, including Arabidopsis. An intact ethylene/JA signaling pathway is thought to be necessary for resistance to necrotrophic pathogens, such as *B. cinerea* and *E. carotovora*, whereas the SA signaling pathway is believed to mediate resistance to biotrophic pathogens. However, Arabidopsis defense responses against *B. cinerea* may also involve camalexin induction.[16] While SA or JA induce many defense responses, these signaling compounds are apparently not essential for camalexin production.[17] It is possibly that several input signals are integrated to trigger camalexin biosynthesis. Based on this information, it is tempting to speculate that biotic interactions capable of regulating multiple defense responses may increase the fitness of plants when challenged with pathogens.

In this report, we used an in vitro Trichoderma-Arabidopsis interaction system,[6] to explore some of the signaling networks involved in defense responses triggered by *T. virens* and *T. atroviride* in plants. Our data show that co-cultivation of plant roots with these fungi induces hydrogen peroxide, SA and JA accumulation, which correlates with induction of pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA.* We also found that both *T. virens* and *T. atroviride* increased camalexin accumulation in plants and conferred resistance to *B. cinerea*. Taken together, our results show the simultaneous involvement of hormonal signaling and camalexin biosynthesis in Trichoderma-induced plant immunity.

Results

Co-cultivation of Arabidopsis roots with Trichoderma stimulates lateral root development and plant biomass production. To evaluate the growth response of plants during physical contact with Trichoderma, we performed bioassays in which WT *Arabidopsis thaliana* ecotype Columbia (Col-0) seedlings were germinated and grown for 4 d on Petri plates, then, a drop of distilled water (control treatment) or a spore suspension from *T. virens* or *T. atroviride* was deposited at the opposite side of the plates. Six days after inoculation (dai), physical contact between the mycelium and the primary root tips could be observed, and two days later the fungi covered roughly 30% of the root system (**Fig. 1A–C**). In order to detect any potential deleterious effect of Trichoderma, total plant biomass in control or inoculated seedlings was determined every two days. During the first two days, no significant differences were observed in biomass production between control plants and plants co-cultivated with *T. virens* or *T. atroviride* (**Fig. 1D**). However, from 4 to 8 dai, a 40% increase in total fresh weight was evident in Trichoderma co-cultivated plants. Enhanced lateral root proliferation was a typical response of Arabidopsis roots colonized by the mycelia of *T. virens* or *T. atroviride*, and no deleterious symptoms such as chlorosis or necrosis could be observed in leaves (**Fig. 1A–C**). Interestingly, co-cultivation with Trichoderma increased anthocyanin production in leaves (**Figs. 1A–C and S1**), which might occur as a consequence/parallel effect of defense induction.

Trichoderma induces hydrogen peroxide accumulation in Arabidopsis. The production of reactive oxygen species (i.e., hydrogen peroxide, H_2O_2) is an early response in plant-pathogen or elicitor recognition.[31,49] In Arabidopsis seedlings at 6 d of co-cultivation H_2O_2 production was detected by DAB staining. The appearance of a brownish-red precipitate in plant tissues indicates hydrogen peroxide accumulation via polymerization with 3,3'-diaminobenzidine (**Fig. 2A–E**). *T. atroviride* induced accumulation of H_2O_2 in leaves (**Fig. 2B**), in the primary root and mature lateral roots (**Fig. 2D**) and in meristems of young lateral roots (**Fig. 2E**) compared with the respective controls (**Fig. 2A and C**). H_2O_2 induction was much weaker in leaves than in roots (**Fig. 2A–E**).

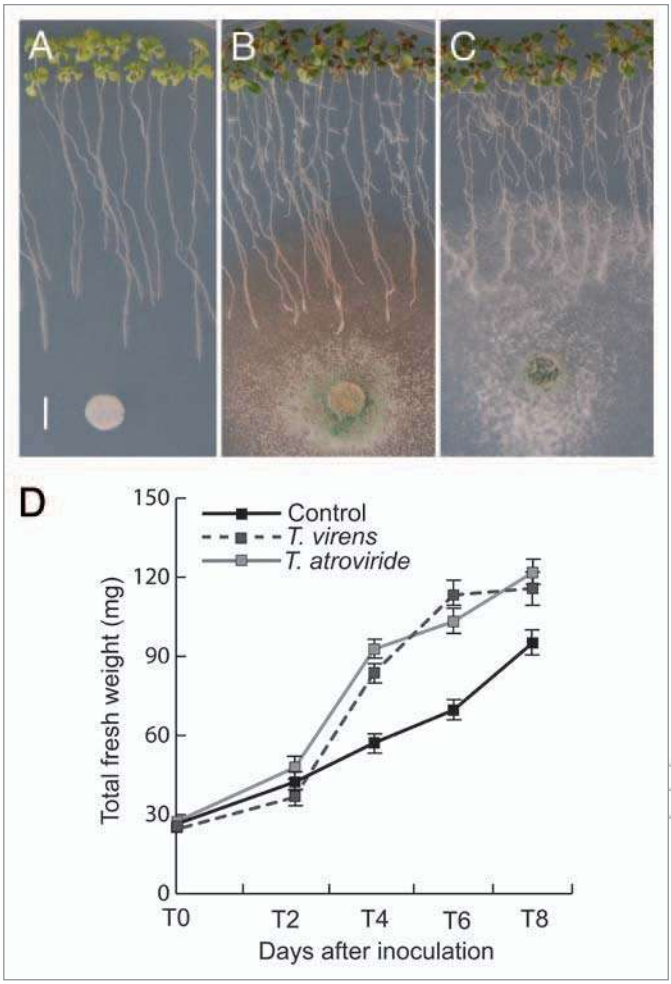


Figure 1. Effect of Trichoderma on Arabidopsis growth. Photographs of 10-d-old Arabidopsis (Col-0) seedlings grown on the surface of agar plates containing 0.2x MS medium. (A) Seedlings were treated with sterilized distilled water at day 4 and photographed 6 d later. Bar = 5 mm. (B) Representative photograph of Arabidopsis seedlings that were inoculated with *T. virens* at a distance of 5 cm from the root tip 4 d after germination and grown for a further 6 d period. (C) Photograph of Arabidopsis seedlings inoculated with *T. atroviride* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 6-d period. (D) Effects of Trichoderma on Arabidopsis biomass production. Mean ± SD values were plotted at the indicated days in the kinetic experiment (n = 30). The experiment was repeated twice with similar results.

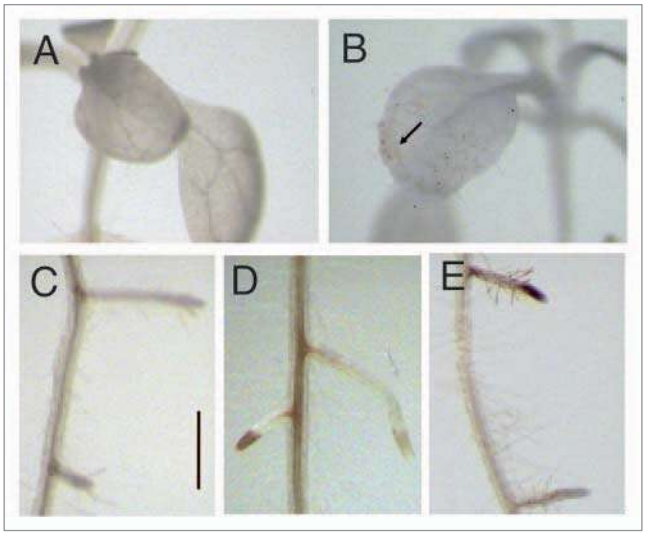


Figure 2. Effect of Trichoderma on H_2O_2 accumulation in Arabidopsis. Representative photographs of leaves (A) and roots (C) from axenically-grown (control) seedlings or from seedlings co-cultivated with *T. atroviride* for 6 d (B, D and E). Arabidopsis seedlings were treated with a solution of 3,3'-diaminobenzidine (DAB). In the presence of H_2O_2, DAB polymerizes, forming a dark red-brown coloration in plant tissues. Microscopy was performed using a Leica MZ6 Stereomicroscope. Bar = 1 mm. Arrow shows the spots of accumulated H_2O_2. Photographs show representative individuals of at least 15 seedlings stained with DAB. The experiment was repeated twice with similar results.

Trichoderma induces hormone-dependent defense responses in Arabidopsis. Infection of plants with pathogens or colonization of roots with certain beneficial microbes causes the induction of defense responses, which may depend on plant production of the alarm signals SA, JA and/or ET.[12] However, the defense signaling pathways that are activated in Arabidopsis upon exposure to Trichoderma remain unknown. To examine whether root colonization by Trichoderma involved altered SA or JA responses, we monitored expression of selected marker genes that are upregulated by these hormones. We used transgenic Arabidopsis lines expressing β-glucuronidase (*uidA*, GUS) fusions to the *Pr1a* promoter, which is activated by SA,[28] and the *Lox2* promoter, activated by JA.[29] Arabidopsis transgenic seedlings expressing each of these markers were co-cultivated with either *T. virens* or *T. atroviride* and the roots allowed to be colonized by the fungi. Axenically-grown seedlings did not show *pPr1a:uidA* expression in shoots at different time points in a time-course experiment (**Fig. 3A–E**). Interestingly, during pre-contact or physical contact between the roots and mycelium *pPr1a:uidA* expression was activated in shoots of plants co-cultivated with both *T. virens* and *T. atroviride* (**Fig. 3F–O**). The time required for the activation of the *Pr1* promoter depended on the fungal species and amount of conidia inoculated. *pPr1a:uidA* expression in plants co-cultivated with 10^6 conidia of *T. virens* significantly increased at 4-dai and continued to increase gradually to a maximum at 8 d. Changes in GUS activity by *T. atroviride* were evident at 6-dai and reached a maximum at 8 dai. In both cases, this response was apparently delayed when less conidia (10^3) were inoculated and was detectable only at 8 dai in cotyledons and older leaves (**Fig. 3J and O**). SA clearly induced changes in *pPr1a:uidA* expression (**Fig. 3P**). In contrast, the JA-activated marker *pLox2:uidA* was better induced by low density of Trichoderma at 8-dai (**Fig. 4A–P**). These data suggest that SA and JA are likely involved in Arabidopsis responses to Trichoderma inoculation.

Interaction between Arabidopsis roots and Trichoderma induces SA and JA accumulation in leaves. To determine whether the changes in the expression of *pPr1a:uidA* and *pLox2:uidA* markers were associated with changes in endogenous SA and/or JA content in plants co-cultivated with Trichoderma, free SA and JA were quantified in WT Arabidopsis (Col-0) plants at 8 dai. A 3- to 4-fold increase in the accumulation of SA was observed in plants inoculated with Trichoderma (10^6 spores)

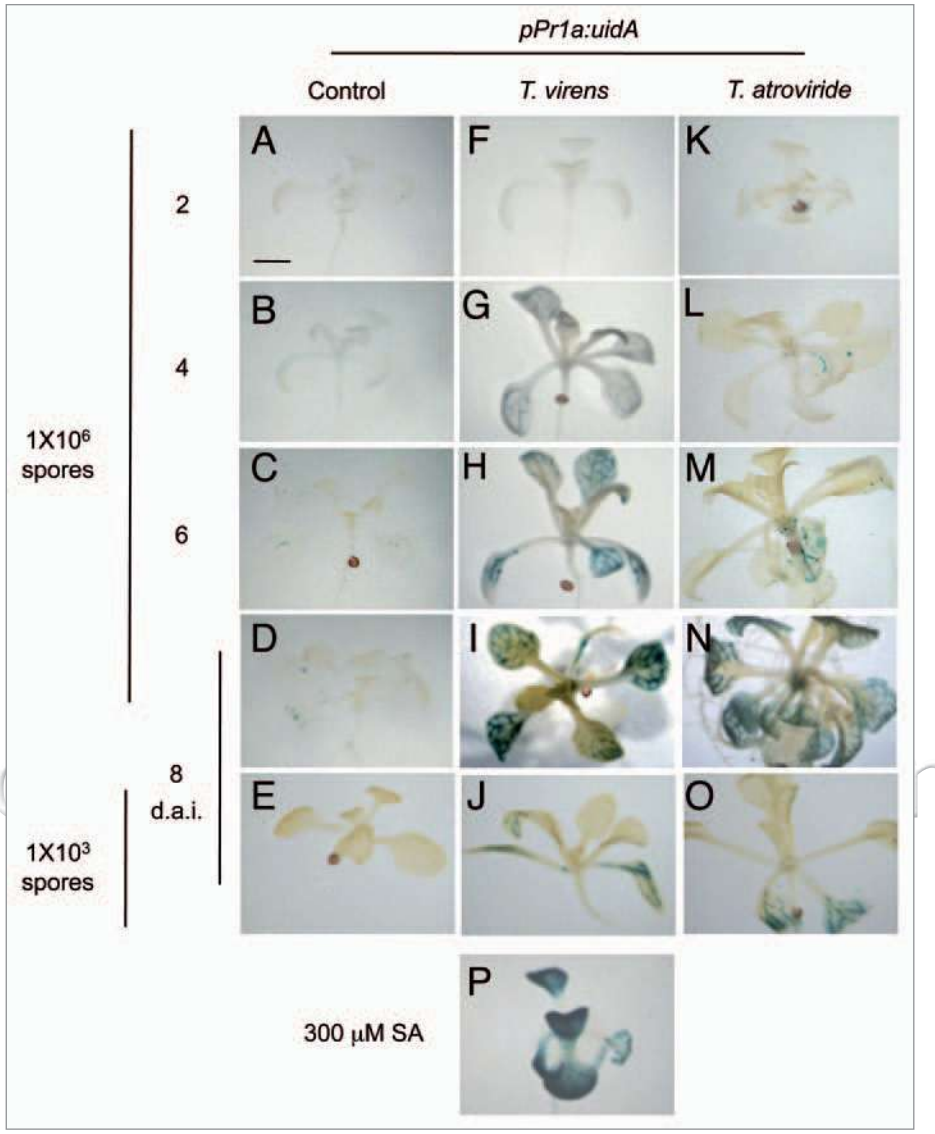


Figure 3. Effect of *T. virens* and *T. atroviride* on expression of the pathogen-related response gene marker *pPr1a:uidA*. In a time-course plant-fungus co-cultivation experiment GUS expression in seedlings was determined every two days after inoculation. SA was used as a positive control and dimethyl sulfoxide served as negative control. Bar = 1 mm. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

when compared with axenically-grown seedlings, with slightly higher levels upon treatment with *T. atroviride* (**Fig. 5A**). The levels of JA dramatically increased in plants co-cultivated with *T. virens* and to a lesser extent with *T. atroviride* (**Fig. 5B**). These data indicate that *T. virens* and *T. atroviride* can induce both SA and JA accumulation.

Trichoderma induces camalexin accumulation in Arabidopsis and produces indole-3-carboxaldehyde. The main phytoalexin that accumulates in Arabidopsis after infection by fungi or bacteria is 3-thiazol-2'-yl-indole (camalexin). To investigate whether Trichoderma inoculation could increase camalexin production, we determined camalexin levels in axenically-grown seedlings, seedlings treated with $AgNO_3$, a well-known inducer of camalexin, or in seedlings at 8-dai with *T. virens* or *T. atroviride*. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that seedlings co-cultivated with *T. atroviride* as well as plants treated with $AgNO_3$ dramatically increased (15-fold) camalexin accumulation when compared with control seedlings. Co-cultivation with *T. virens* also resulted in strongly increased camalexin levels (**Fig. 6**). Previously, we determined the production of several indolic compounds by Trichoderma, including indole-3-acetic acid, indole-3-acetaldehyde and indole-3-ethanol.[6] Further analysis of the metabolites produced by Trichoderma by GC-MS revealed the presence of indole-3-carboxaldehyde (ICAld, retention time 10.79 min, *m/z* 144) (**Fig. 7A–C**), a compound previously reported by Zook and Hammerschmidt (1997), as a camalexin reactant.[27] When *A. thaliana* was grown in the presence of ICAld, the production of camalexin increased significantly (**Fig. 7D**), indicating that this compound is likely involved in camalexin biosynthesis. Supply of

L-Trp to *T. virens* culture medium increased ICAld accumulation in culture supernatants (**Fig. S2**).

Trichoderma confers resistance to *Botrytis cinerea*. Camalexin is essential for resistance to *B. cinerea* in Arabidopsis.[16,17] The results from SA and JA accumulation, *pPr1a:uidA* and *pLox2:uidA* expression, and camalexin accumulation suggest that Trichoderma may act as potential defense-inducing fungus. To study whether Trichoderma could effectively activate defense mechanisms that lead to pathogen resistance, we tested the responses of 12-d-old Arabidopsis plants grown axenically or co-cultivated with *T. virens* or *T. atroviride* and further inoculated with the necrotrophic pathogen *Botrytis cinerea,* which causes spreading necrotic lesions on leaves. In these experiments, *B. cinerea* conidia were inoculated over the leaf surfaces and disease symptoms evaluated 3 d after inoculation with the pathogen. In control plants *B. cinerea* was found to induce necrotic lesions in over 82% of inoculated leaves (**Fig. 8A**). In contrast, in plants colonized by *T. virens* or *T. atroviride*, only 22% and 25% of leaves, respectively, presented necrotic lesions caused by *B. cinerea* infection (**Fig. 8A**). Five days after pathogen inoculation, it was found that *B. cinerea* caused death in about 70% of control plants; whereas only 10% of Trichoderma colonized plants were dead at this stage (**Fig. 8B**).

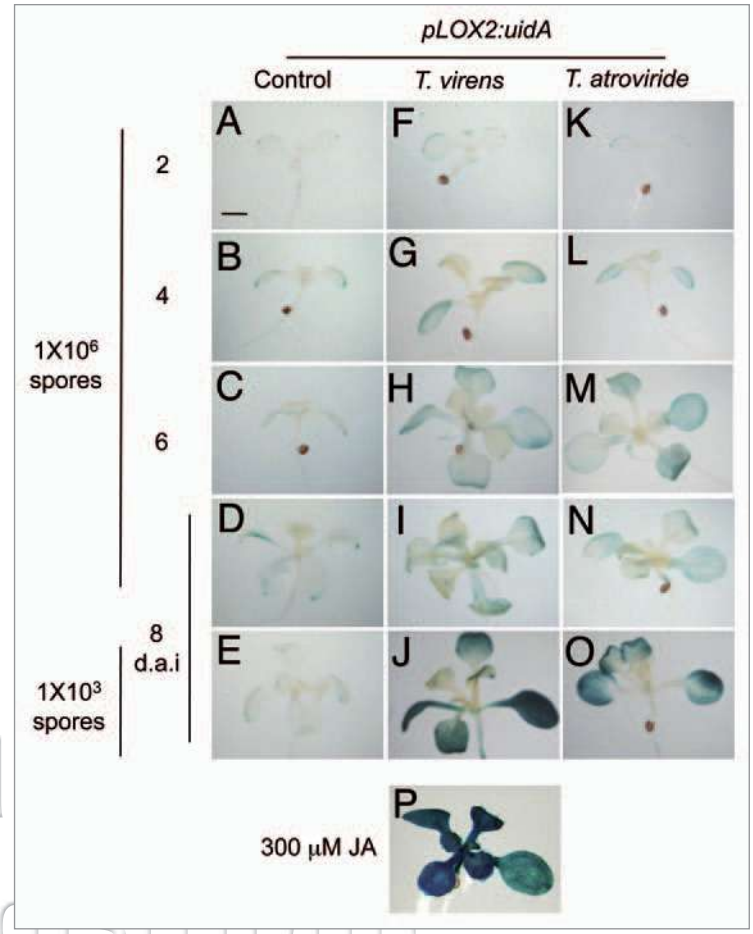


Figure 4. Effects of *T. virens* and *T. atroviride* on expression of the JA responsive gene marker *pLox2:uidA*. GUS expression in seedlings was determined every 2 d after inoculation in a time-course plant-fungus co-cultivation experiment. JA was used as a positive control and dimethyl sulfoxide served as negative control. Bar = 1 mm. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

Discussion

Fungi are an enormous resource of structurally diverse metabolites, which may affect interactions with plants and other organisms. Deciphering the signaling processes that mediate these interactions represents a continuous challenge. Although not directly economically important, the model plant *Arabidopsis thaliana* offers a number of experimental advantages over crop species, including its small size, short life cycle, and the suitability to be grown under axenic conditions. The adoption of an Arabidopsis-Trichoderma model has increased our knowledge about the molecular and physiological mechanisms by which Trichoderma modulate plant growth and development.[6]

Here we show that colonization of Arabidopsis roots by *T. virens* or *T. atroviride* sustained increased plant biomass production at 8 dai (**Fig. 1**). Root colonization by these fungi was associated to induction of lateral root development and anthocyanin accumulation in leaves (**Figs. 1 and S1**). Anthocyanin accumulation was highly dependent on the amount of conidia of Trichoderma applied. Importantly, we did not observe chlorosis or necrosis symptoms in leaves of seedlings co-cultivated with either *T. virens* or *T. atroviride* indicating that high fungal colonization in roots is not detrimental to plant growth and development.

We found that Arabidopsis roots-colonized by Trichoderma accumulated H_2O_2 (**Fig. 2**), indicating that Trichoderma triggers plant defense responses through reactive oxygen species (ROS). Interactions between plants and microbes involve recognition and signaling events that are distinct for different microbial elicitors. These microbial elicitors may belong to pathogen-or microbe-associated molecular patterns (PAMPs or MAMPs).[30] At the cellular level, the result of pathogen recognition often becomes apparent as a necrosis localized at the site of attack, called the hypersensitive response, which is accompanied by cellular changes such as an oxidative burst leading to the release of ROS, a rise in salicylic acid levels, and the induction of defense genes such as those coding for pathogenesis-related (*PR*) proteins.[31-33] The contribution of H_2O_2 in plant protection by Trichoderma and its relationship with SA and JA pathways remains to be investigated.

Certain Trichoderma species have been previously found to elicit physical or chemical changes related to plant defense by activating ISR.[34,35] ISR elicited by Trichoderma may suppress plant diseases caused by a range of phytopathogens in both greenhouse and field conditions. In this study, we used transgenic Arabidopsis lines expressing β-glucuronidase (GUS) fusions to elucidate the signal transduction pathway by which *T. virens* and *T. atroviride* elicit plant defense responses. In our experiments, pre-contact or direct physical contact between the Arabidopsis

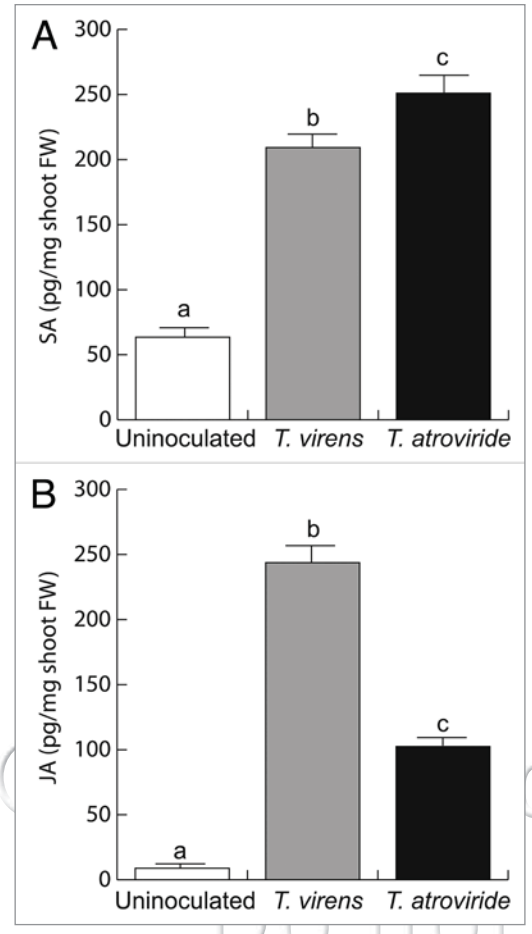
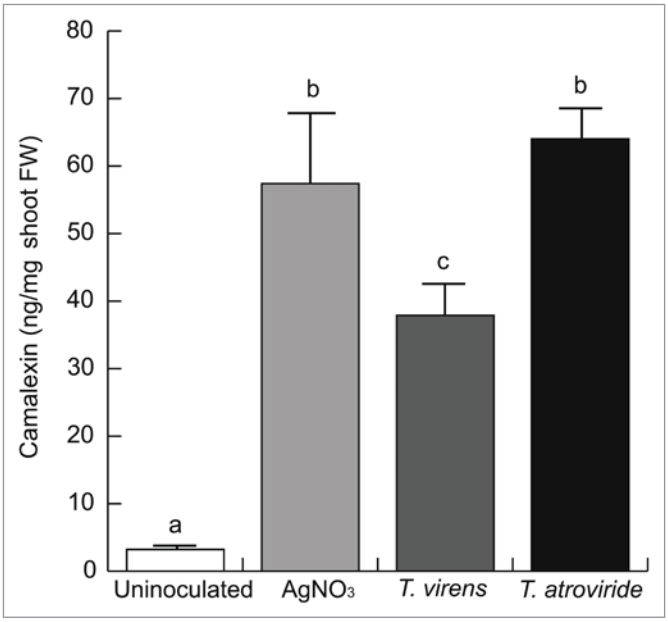


Figure 5. Effect of Trichoderma co-cultivation on SA and JA accumulation in Arabidopsis leaves. (A) Free SA or (B) JA at 8 d of co-cultivation with either *T. virens* or *T. atroviride*. Error bars represent the SE. Different letters are used to indicate means that differ significantly ($p < 0.05$). The experiment was repeated three times with similar results.



root system and Trichoderma stimulated *pPr1a:uidA* expression and SA accumulation in plants (**Figs. 3 and 5A**). The concerted action of *PR* proteins, some of which display antimicrobial activity may act restricting pathogen growth. Low fungal concentration was also found to induce *pLox2:uidA* and JA accumulation (**Figs. 4 and 5B**). Both SA and JA changes have been previously described in cucumber plants inoculated with *Trichoderma asperellum* strain T34,[36] indicating that hormonal defense protection of plants is widespread among Trichoderma species. These results are in agreement with previous reports that *T. virens* induces the expression of *PAL* in maize through a JA-dependent response.[34,35] Moreover, our data suggest that SA and JA are involved in the signal transduction events mediating Trichoderma induced defense responses and are even consistent with the SA/JA antagonism. Similar defense activation patterns have been previously identified in Arabidopsis seedlings inoculated with plant growth promoting rhizobacteria.[37-39]

Antimicrobial compounds can be produced during normal plant growth and are usually stored in specialized organs or tissues such as trichomes, oil glands or epidermal cell layers.[40] Arabidopsis is a member of the Brassicaceae family, which is known for the production of various indolic constituents, including several indolic glucosinolates and closely related indolyl thiazoles, including camalexin and 6-methoxycamalexin.[40-42] Camalexin production can be elicited by bacterial and fungal phytopathogens and possess antimicrobial activity. In this work, we showed that Arabidopsis seedlings colonized with *T. virens* or *T. atroviride* accumulate greater levels of camalexin than axenically-grown plants (**Fig. 6**). Interestingly, we found that *T. virens* also produces ICAld, a compound related to indole-3-acetic acid metabolism likely involved in camalexin biosynthesis.[43] ICAld concentration in *T. virens* liquid extracts increased when Trp was supplied in the culture medium (**Fig. S2**), suggesting that Trp might be a precursor for ICAld. A number of aldehydes possess the ability to react with Cys to form the corresponding thiazolidinecarboxylic acid.[27] It has been reported that the synthesis of camalexin may proceed through the condensation of indole-3-carboxaldehyde with cysteine followed by a two-step oxidation and decarboxylation.[44] We found that application of ICAld increased camalexin accumulation in Arabidopsis seedlings (**Fig. 7**). These results indicate that Trichoderma species can induce both SA- and JA-mediated defense responses and camalexin accumulation. Hormone-dependent signaling and camalexin accumulation are considered independent or complimentary mechanisms involved in plant immunity. The results that *T. virens* produces ICAld suggest that plants might use this compound for camalexin production by either direct or indirect means. The activity of ICAld on plant signaling was evidenced

Figure 6. Effect of Trichoderma co-cultivation on camalexin accumulation in Arabidopsis. Camalexin levels were determined in leaves of axenically-grown WT Arabidopsis seedlings, in seedlings treated with 5 mM $AgNO_3$, or co-cultivated 8 d with *T. virens* or *T. atroviride*. Bars show the mean ± SD of five independent biological replicates. Each replicate included 20 seedlings. Different letters are used to indicate means that treatments differ significantly ($p < 0.05$). The experiment was repeated twice with similar results.

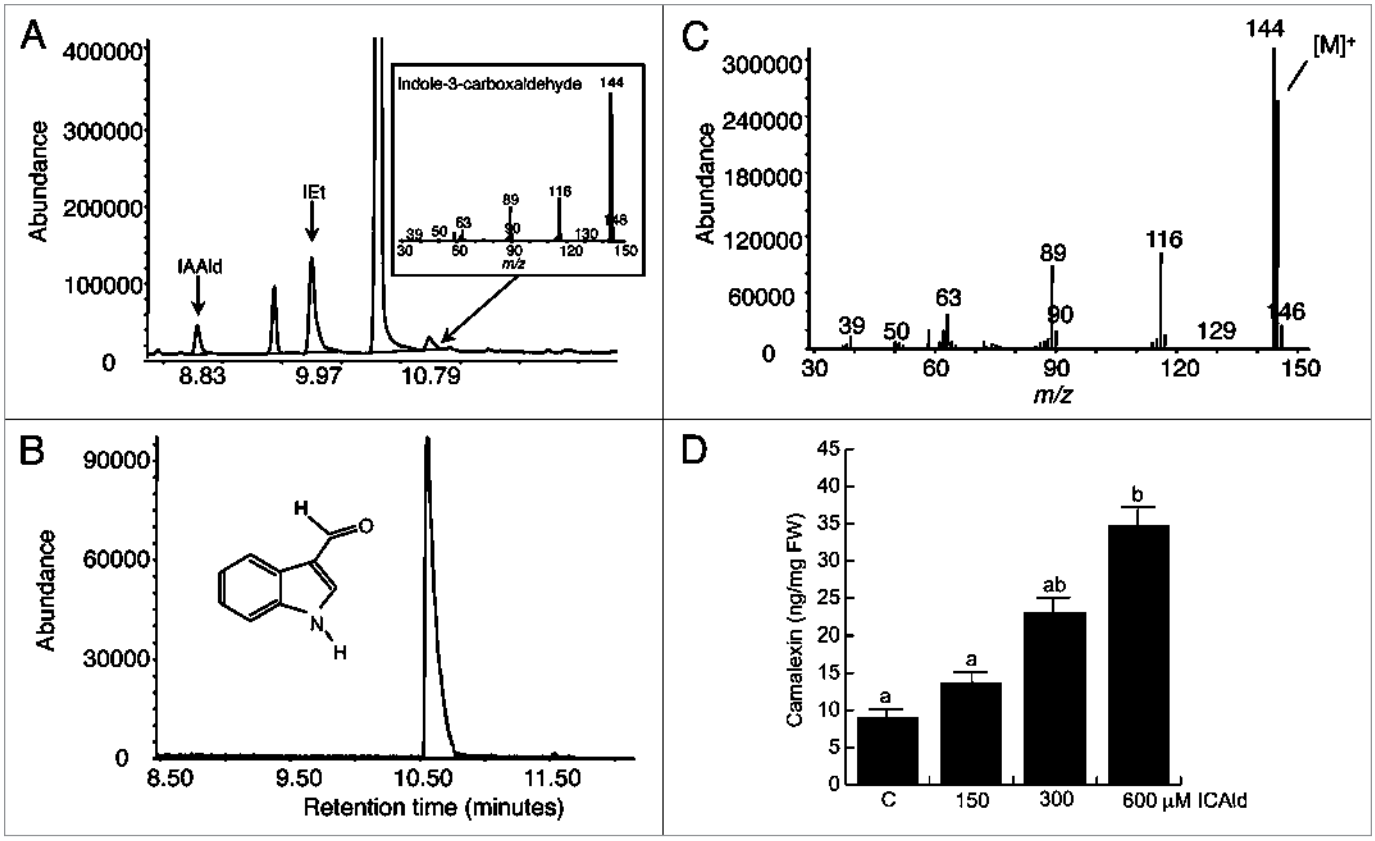


Figure 7. Identification of indole-3-carboxaldehyde from underivatized samples from *T. virens* growth medium by GC-MS. (A) chromatogram from neutral ethyl acetate extract obtained from 1 l of culture medium of *T. virens*, arrows indicate the presence of different indolic compounds, indole-3-acetaldehyde (IAAld), indole-3-ethanol (IEt) and indole-3-carboxaldehyde (ICAld). (B) ICAld standard. (C) the 70-eV electron-impact full-scan mass spectra from *m/z* 50 to 500 of ICAld standard. (D) Indole-3-carboxaldehyde increases camalexin biosynthesis. Induction of camalexin in shoots of Arabidopsis after treatment with 300 µM ICAld for 72 h. Bars shown in (D) represent the mean ± SD of three independent replicates. Each replicate included 20 seedlings. Different letters are used to indicate means that differ significantly ($p < 0.05$). The experiment was repeated twice with similar results.

monitoring primary root growth and adventitious root formation, which were clearly altered by ICAld supply to the medium (**Fig. S3**). The combination of such complex defense and/or plant growth regulating mechanisms may account for better performance of plants inoculated with Trichoderma.

In accordance with its involvement in activating defense-signaling pathways, *T. virens* and *T. atroviride* were found to be effective against the fungal necrotizing pathogen *B. cinerea,* a gray mold that causes rot symptoms in more than 200 different plant species. Our results of disease susceptibility showed that colonization of Arabidopsis roots by *T. virens* or *T. atroviride* reduced disease symptoms and plant death caused by this pathogen in leaves (**Fig. 8**). The correlation found between defense gene expression, H_2O_2 induction, SA and JA accumulation, camalexin production and reduced disease symptoms in Arabidopsis colonized by Trichoderma, suggests that the combined activation of these defense pathways might be important to confer plant immunity against a fungal necrotizing pathogen. The characterization of two ISR elicitors secreted by *T. virens* was recently described. Peptides with antimicrobial activity termed peptaibols, which are produced by *T. virens* were shown to have ISR elicitor effects, and they systemically induced defense in cucumber leaves.[45] The second ISR elicitor of *T. virens* is an extracellular small protein (Sm1), which is overproduced in the presence of cotton plants.[34,46] In maize, the metabolic pathways that lead to the establishment of Sm1-mediated ISR involves the signaling networks associated with salicylic acid, green leaf volatiles and jasmonic acid metabolism.[34,35,46] Our results are in agreement with these findings by showing that Trichoderma species can confer protection in Arabidopsis seedlings against *B. cinerea*, possibly by producing diffusible signals prior or during direct contact with roots.

We conclude that Trichoderma species are capable of regulating multiple defense responses. In addition to the defense gene induction and hormone biosynthesis, we now provide chemical evidence supporting a role for Trichoderma in phytoalexin induction, an important defense response less characterized. The combined effect of these responses may ultimately determine the role played by particular Trichoderma isolates in conferring disease resistance to crops.

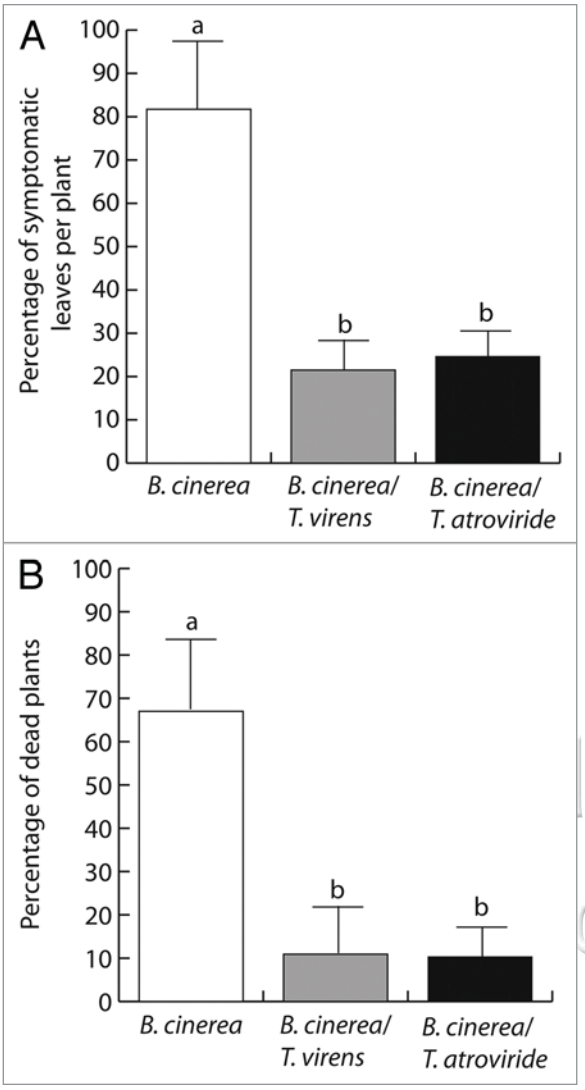


Figure 8. Trichoderma confers protection against *Botrytis cinerea* in Arabidopsis seedlings. Twelve-day-old axenically-grown *A. thaliana* seedlings or seedlings co-cultivated 3-d with *T. virens* or *T. atroviride* in vitro were placed in Petri dishes containing 0.2x MS medium and mock (water) treated or inoculated with 5 μl of 1 x 10^6 *Botrytis cinerea* spores per ml solution/per leaf, depositing the inoculum on leaf surfaces. The number of symptomatic leaves per plant (A) and dead plants (B) are shown. Bars show the mean ± SD of 30 Arabidopsis seedlings. Different letters are used to indicate means that treatments differ significantly ($p < 0.05$). The experiment was repeated three times with similar results.

Materials and Methods

Plant material and growth conditions. All mutant and transgenic lines were derived from parental Arabidopsis ecotype Columbia-0 (Col-0). Arabidopsis transgenic lines used in this work expressed a JA-inducible *lipoxygenase2* (At3g45140) or the *pathogenesis-related1* (At2g14610) gene promoters fused to the *uidA* reporter gene, which are referred as *pLox2:uidA*[29] and *pPr1a:uidA*,[28] respectively. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) household bleach (6% NaOCl) for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2x MS medium (Murashige and Skoog basal salts mixture, Cat M5524: Sigma, St. Louis). Plates were placed vertically at a 65 degrees angle to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants were grown at 24°C in a growth chamber with a 16 h light (200 $\mu molm^2s^{-1}$)/8 h darkness photoperiod.

Fungal growth and plant co-cultivation experiments. The following fungal strains were used in this work: *Trichoderma virens* Gv.29-8 and *Trichoderma atroviride* (Formerly *Trichoderma harzianum*) IMI 206040. *T. virens* and *T. atroviride* were evaluated in vitro for their ability to elicit defense responses in Arabidopsis. Fungal spore densities of ~1 x 10^6 or 1 x 10^3 spores were inoculated by placing the spores at 5 cm in the opposite ends of agar plates containing 4-d-old germinated Arabidopsis seedlings (10 seedlings per plate). Plates were arranged in a completely randomized design. The seedlings were cultured for different time periods in a Percival AR95L growth chamber. The percentages of primary roots colonized by Trichoderma were determined with a ruler by measuring the primary root length and the surface covered by fungal hyphae.

Histochemical analysis. For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated 12 to 14 h at 37°C in a GUS reaction buffer (0.5 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997).[47] For each marker line and for each treatment, at least 15 transgenic plants were analyzed. A representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

Determination of H_2O_2 production. The production of H_2O_2 in Arabidopsis seedlings co-cultivated with Trichoderma was determined at 6 dai. The seedlings were included in 1 mg/ml solution of 3,3'-diaminobenzidine (DAB; Sigma), incubated 2 h, fixed and cleared in alcoholic solution. In presence of H_2O_2, DAB polymerizes, forming a dark red-brown precipitate staining. After these procedures the seedlings were examined for the production of H_2O_2 by microscopy. For each treatment at least 15 plants were analyzed. A representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

Anthocyanin determination. Anthocyanin content was determined in WT Arabidopsis (Col-0) seedlings 6 d after Trichoderma inoculation. 100 mg leaf samples were placed in an Eppendorf tube containing 1 ml of 0.1% HCl in methanol for 48 h at 4°C. After this period, the methanol extracts were analyzed in a spectrophotometer at 530 nm. The amount of anthocyanin in the extracts was reported as described by Pirie and Mullins (1976).[48]

SA and JA extraction and measurements. SA and JA extraction and determinations were performed in *Arabidopsis thaliana* (ecotype Col-0) shoots at 8 d after Trichoderma inoculation. For sample preparation, plants were sectioned at the root/shoot interface. Plant tissues were frozen and ground in liquid N_2. Three hundred milligrams ground tissue was placed in an eppendorf tube, homogenized with 500 μl isopropanol/H_2O/concentrated HCl (2:1:0.002, v/v), and 200 ng orto-anisic acid (OA; Sigma) added to serve as internal standard for SA and shaken for

30 sec. Samples were centrifuged at 11,500 rpm for 3 min, supernatants were collected and subjected to SA extraction with 200 µl of dichloromethane. SA and JA were derivatized with acetyl chloride in methanol (1 ml/250 µl), sonicated for 15 min and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25 µl of ethyl acetate for GC-MS analysis. Gas chromatography-selected ion monitoring mass spectrometry (GC-SIM-MS) and retention time were established for SA-ME (2.3 min, *m/z* 152), OA-ME (3.2 min, *m/z* 166) and (7.5 min, *m/z* 224) respectively. JA was quantified by comparison with a standard curve obtained by using purified Me-JA (Sigma).

Camalexin determination. Camalexin was extracted from leaves of WT Arabidopsis seedlings 8 d after *T. virens* or *T. atroviride* inoculation. As a positive control for camalexin induction, 12-d-old plants were treated with 5 mM $AgNO_3$ for 12 h, or 300 µM indole-3-carboxaldehyde (Sigma) for 72 h. Camalexin levels were determined as described by Glazebrook and Ausubel (1994),[21] and GC-MS analysis performed. For GC-MS analysis 100 mg per sample of shoot material were submerged in 800 µl of methanol and kept at 80°C for 20 min. The supernatant was transferred to a vial, evaporated under a stream of nitrogen and redissolved in 10 µl of methanol, and injected 2 µl for GC-SIM-MS analysis. The ions with *m/z* 58, 142 and 200 were monitored. Camalexin (Rt 18.0 min) was quantified by comparison with a standard curve obtained by using purified camalexin kindly provided by Prof. J. Glazebrook (University of Minnesota) and dissolved in methanol for chemical analysis.

Identification and quantification of indole-3-carboxaldehyde. For ICAld determination, an inoculum of 1 x 10^6 conidia of *T. virens* was added to 1 l potato dextrose broth (Sigma), and grown for 3 d at 28°C with shaking at 200 rpm. To evaluate the effect of Trp supply on ICAld accumulation, the medium was supplemented with L-Trp (Merck) at a concentration of 100 mg/l. For ICAld determinations, the fungal culture was filtered and the pH of the supernatant adjusted to 7 using 2 N NaOH. Indolic compounds in supernatant filtrate were extracted three times with 1 l of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen, taken up and diluted 1:10 (v/v) without L-Trp in the medium and 1:100 (v/v) with L-Trp before GC-MS analysis. ICAld was identified by comparison with mass spectra from the library (NIST/EPA/NIH, "Chem Station" Agilent Technologies Rev. D.04.00 2002). The identity of ICAld was further confirmed by comparison of retention time in the fungal extract with samples of the pure ICAld (Sigma). The molecular ion was monitored after electron impact ionization (70 eV). ICAld, *m/z* 144. To estimate the amount of ICAld produced by *T. virens*, we constructed a standard curve.

Mass spectrometry analysis. Identification and determination of all compounds was performed using a gas chromatography-mass spectrometry system. Samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and 30-mm x 0.2 mm x 0.25 mm, 5% phenyl methyl silicone capillary column (HP-5 MS). The operating conditions used were: 1 ml min^{-1} helium as carrier gas, 300°C detector temperature and 250°C injector temperature. The column was held for 5 min at 150°C and programmed at 5°C min^{-1} to a 278°C final temperature for 5 min.

Bioassays for Trichoderma-induced resistance against *B. cinerea*. To test plant protection conferred by *T. virens* and *T. atroviride* against *B. cinerea,* Arabidopsis seedlings were inoculated with a fungal density of ~1 x 10^6 spores by placing the spores at 1 cm at the opposite ends of agar plates containing 9-d-old germinated Arabidopsis seedlings (10 seedlings per plate). Trichoderma was allowed to grow for 3-d to elicit defense responses by the physical contact of the mycelium with the root system. *B. cinerea* was grown on PDA medium for 12 d, at this time, the conidia were collected and resuspended in sterilized distilled water. Arabidopsis shoots were then inoculated with ~1 x 10^6 conidia of *B. cinerea* and leaves exhibiting soft rot symptoms were determined by visual inspection 3 d later. Numbers of symptomatic leaves per seedling were counted as a measure of disease severity. The percentage of dead plants was determined 5 d after pathogen inoculation for a total of 30 plants. Plants were grown at 24°C in a chamber with a 16 h light (200 µmol m^2 s^{-1})/8 h darkness photoperiod.

Data analysis. Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyzes with a Tukey's post hoc test were used for testing differences in the biochemical analysis for SA, JA, ICAld, camalexin and anthocyanin measurements, number of lesions and percentage of dead plants in WT Arabidopsis. Different letters are used to indicate means that differ significantly ($p \leq 0.05$).

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Note

Supplemental materials can be found at:
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11.8.
Role of the 4-Phosphopantetheinyl Transferase of *Trichoderma virens* in Secondary Metabolism and Induction of Plant Defense Responses

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Trichoderma virens **is a ubiquitous soil fungus successfully used in biological control due to its efficient colonization of plant roots. In fungi, 4-phosphopantetheinyl transferases (PPTases) activate enzymes involved in primary and secondary metabolism. Therefore, we cloned the PPTase gene *ppt1* from *T. virens* and generated PPTase-deficient (Δ*ppt1*) and overexpressing strains to investigate the role of this enzyme in biocontrol and induction of plant defense responses. The Δ*ppt1* mutants were auxotrophic for lysine, produced nonpigmented conidia, and were unable to synthesize nonribosomal peptides. Although spore germination was severely compromised under both low and high iron availability, mycelial growth occurred faster than the wild type, and the mutants were able to efficiently colonize plant roots. The Δ*ppt1* mutants were unable of inhibiting growth of phytopathogenic fungi in vitro. *Arabidopsis thaliana* seedlings co-cultivated with wild-type *T. virens* showed increased expression of *pPr1a:uidA* and *pLox2:uidA* markers, which correlated with enhanced accumulation of salicylic acid (SA), jasmonic acid, camalexin, and resistance to *Botrytis cinerea*. Co-cultivation of *A. thaliana* seedlings with Δ*ppt1* mutants compromised the SA and camalexin responses, resulting in decreased protection against the pathogen. Our data reveal an important role of *T. virens* PPT1 in antibiosis and induction of SA and camalexin-dependent plant defense responses.**

Phosphopantetheinyl transferases (PPTases) belong to a superfamily of enzymes found in prokaryotes and eukaryotes which are required for the synthesis of a wide range of compounds, including fatty acids, amino acids, polyketides, and nonribosomal peptides. PPTases activate carrier proteins in specific biosynthetic pathways by the transfer of a phosphopantetheinyl moiety to an invariant serine residue. PPTases catalyze the nucleophilic attack of the hydroxyl side chain of the conserved carrier protein serine residue on the 5′-β-pyrophosphate linkage of CoA. This causes the transfer of the phosphopantetheinyl moiety of CoA to the side chain of a conserved serine, converting the carrier protein from an inactive apo-form to an active holo-form (Lambalot et al. 1996; Walsh et al. 1997).

The PPT superfamily has been divided into two paralogous groups that correspond to substrate specificity. The first is the AcpS family that comprises homotrimers of 120 to 140 amino acids that are involved in fatty acid biosynthesis in bacteria and fungi (Allen et al. 2011; Hiltunen et al. 2010). Members of the second family such as Sfp are monomeric enzymes of 220 to 240 amino acids, which participate in the synthesis of secondary metabolites by activating nonribosomal peptide synthases (NRPS) and polyketide synthases (PKS) in both bacteria and eukaryotes (Lambalot et al. 1996). This family includes PPTases involved in cyanobacterial heterocyst differentiation, fungal lysine biosynthesis, β-alanine conjugation, hybrid peptide synthase/polyketide synthase complexes, and other enzymes with an as-yet-unidentified function (Copp and Neilan 2006). A third group of PPTases is characterized by being an integral part of fatty acid synthases in eukaryotes (Mootz et al. 2001).

The importance of PPTases in the synthesis of antibiotics and siderophores has been widely demonstrated in prokaryotes such as *Escherichia coli* and *Pseudomonas syringae* (Flugel et al. 2000; Lambalot et al. 1996; Seidle et al. 2006). Many microorganisms possess multiple PPTases. The genome of *Bacillus subtilis* encodes Sfp for surfactin biosynthesis, and AcpS, involved in siderophore synthesis (Mootz et al. 2001; Ollinger et al. 2006). In comparison, the genome of *E. coli* encodes three PPTases: AcpS and EntD, for synthesis of fatty acids and the siderophore enterobactin; and YhhU, an uncharacterized PPTase (Flugel et al. 2000, Lambalot et al. 1996). These enzymes act in distinct pathways and display contrasting specificity for carrier proteins. The Sfp-like EntD is unable to complement an AcpS mutant of *E. coli*. In contrast, the *B. subtilis* Sfp displays a remarkable range of carrier protein substrates (Gehring et al. 1998; Keating and Walsh 1999; Marahiel et al. 1997). When an AcpS-like PPTase is not present in an organism, the Sfp-like PPT will act in both primary and secondary metabolic pathways, displaying a preference for the carrier proteins of fatty acid synthesis (FAS) (Finking et al. 2002). In the case of the fungal antagonistic bacteria *B. subtilis*, a PPTase

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was found to be involved in the synthesis of antibiotics such as surfactin, which promotes induced systemic resistance (ISR) in common bean and tomato (Ongena et al. 2007). Currently, PPTases of the Sfp family have been studied in bacteria pathogenic to humans or plants, or in model systems. In the case of microorganisms used in biological control in agriculture, one of the few cases in which their function has been studied is that of *P. luminescens* (Chiche et al. 2001). This bacterium is associated with an entomopathogenic nematode, and its corresponding PPTase was found to be essential for the synthesis of toxins that kill the insect.

In fungi, there is still limited information about the role of PPTases. A single multifunctional PPTase has been described in *Aspergillus nidulans* (Keszenman-Pereyra et al. 2003; Márquez-Fernández et al. 2007; Oberegger et al. 2003). Similar findings were reported in *A. fumigatus* (Neville et al. 2005), *Penicillium chyrisogenum* (García-Estrada et al. 2008), and the plant pathogens *Colletotrichum graminicola* and *Magnaporthe oryzea* (Horbach et al. 2009). However, recently, a mitochondrial PPTasa (PptB) of the AcpS type, specific for the mitochondrial acyl carrier protein AcpA, was reported in *A. fumigatus* (Allen et al. 2011). These studies also demonstrated that PPTases are necessary for synthesis of lysine and many secondary metabolites, including antibiotics, siderophores, and pigments.

The genus *Trichoderma* comprises a large number of filamentous fungi of wide distribution in agricultural ecosystems, which are well characterized in terms of production of polyketides and nonribosomal peptides (Reino et al. 2007). A single NRPS can produce up to three distinct peptaibols. Accordingly, Neuhof and co-workers (2007) analyzed 34 phylogenetically related *Trichoderma* strains, identifying 58 different classes of peptaibols. Several species of the genus *Trichoderma* are necrotrophic mycoparasites of phytopathogenic fungi and are widely used in biological control of diseases in agriculturally important crops. Moreover, some *Trichoderma* isolates are capable of activating plant defense responses (Yedidia et al. 1999, 2003).

Plants possess various inducible defense mechanisms for protection against pathogen attack. An example of this is systemic acquired resistance (SAR), which is activated after infection by necrotizing pathogens (Ryals et al. 1996). Similarly, colonization of plant roots by certain nonpathogenic rhizobacteria can produce ISR in the host plant (van Loon et al. 1998). ISR is a plant-mediated mechanism similar to SAR which is initiated at the root and extends up to the shoot, conferring protection against different types of plant pathogens. Induction of ISR depends, in part, on phytohormone signaling mediated by jasmonic acid (JA) and ethylene (ET) (Glazebrook 2005). Accumulation of antimicrobial metabolites is integral to plant protection. In *Arabidopsis thaliana*, accumulation of the phytoalexin camalexin was found in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringae* (Glazebrook and Ausubel 1994; Tsuji et al. 1992) and after inoculation with the fungus *Cochliobolus carbonum* (Glazebrook et al. 1997). In vitro studies demonstrated that camalexin inhibits bacterial and fungal growth (Ferrari et al. 2003; Jejelowo et al. 1991; Rogers et al. 1996; Tsuji et al. 1992).

There is a wide range of hormone-like factors that affect the responses of plants to *Trichoderma* spp. In cucumber (*Cucumis sativus*) and maize (*Zea mays*), the main signaling pathway by which *Trichoderma virens* induces systemic resistance involves JA and ET (Djonovi et al. 2007; Yedidia et al. 2003). Both salicylic acid (SA) and JA changes have been previously described in cucumber plants inoculated with *T. asperellum* T34 (Segarra et al. 2007), indicating that hormonal defense protection of plants is widespread among *Trichoderma* spp. By using an *Arabidopsis–Trichoderma* co-cultivation system, we analyzed the response of pathogenesis-related reporter genes to *T. virens* or *T. atroviride,* which provided evidence that the defense signaling pathway activated by these fungi involves SA and JA (Salas-Marina et al. 2011). Interestingly, accumulating evidence suggests that the 18-mer peptaibols are critical for the chemical communication between *Trichoderma* spp. and the plant (Brotman et al. 2009; Viterbo et al. 2007). In fact, the peptaibol alameticin produced by *T. viride* sprayed on *Phaseolus lunatus* plants activates ISR, resulting in the production of defense compounds against herbivores (Engelberth et al. 2000). In a more recent study, cucumber plants co-cultivated with *T. virens* strains disrupted in the NRPS encoding gene *tex1* showed a significant reduction in systemic resistance against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*, and reduced production of phenolic compounds with inhibitory activity against this bacterium (Viterbo et al. 2007).

Here, we report the characterization of *T. virens* mutants defective in the *ppt1* gene and show that the corresponding protein is required for the synthesis of lysine, peptaibols, pigments, and siderophores. The Δ*ppt1* mutants showed a dramatically reduced capacity to inhibit the growth of phytopathogenic fungi in vitro. In addition, decreased activation of ISR in *A. thaliana* was observed when plants were grown in association with *T. virens* lacking PPTase PPT1, which correlated with a reduction in SA and camalexin levels in plants. Surprisingly, the production of JA and activation of JA-dependent *pLox2:uidA* were still observed in plants co-cultivated with *T. virens* Δ*ppt1* mutants, even though the mutants failed to produce polyketides and nonribosomal peptides. Our results reveal a key role of *T. virens* PPTase in antibiosis, and the specific induction of SA and camalexin-dependent plant defense responses.

RESULTS

T. virens ppt1 mutants produce nonpigmented conidia, show enhanced growth, and require supplemental iron to germinate.

Bioinformatics analysis of the sequenced genomes of members of the genus *Trichoderma* revealed that there are three sequences with similarity to PPTases in the genome of *T. virens* (protein ID 194983 of the Sfp type, protein ID 203026 of the AcpS type, and protein ID 48659 of the fatty acid synthesis type I), as well as those of *T. atroviride* (protein ID 52102 of the Sfp type, protein ID 286047 of the AcpS type, and protein ID 85662 of the fatty acid synthesis type I), whereas that of *T. reesei* has only two PPTases (protein ID 56081 of the AcpS type and protein ID 48788 of the fatty acid synthesis type I). Analysis of the deduced protein sequence of the *T. virens* gene (*ppt1*), corresponding to the PPTase of the Sfp type (ID 194983) indicated that it contains the conserved motifs FNVTHQ (102 to 107), VAIGTD (123 to 128), and WCLREAYVK (188 to 196) characteristic of PPTases. To determine the functional role of the *T. virens* phosphopantetheinyl transferase, the *ppt*1 gene was replaced by the *T. virens arg2* gene in the arginine auxotrophic strain Tv10.4 of *T. virens* (Supplementary Fig. 1). Gene replacement was confirmed by polymerase chain reaction (PCR) and Southern blot. A null mutant was then retransformed with the wild-type *ppt1* gene to verify that all observed phenotypes were due to the replacement of the gene. Mutant complementation was confirmed by Southern blot, showing the integration of multiple copies of the gene in the complemented strains (data not shown), and the complemented strain included in all subsequent analyses. To determine if PPT1 is a limiting factor for the activation of PKS and NRPS in *T. virens*, overexpressing strains were generated by transformation of the wild-type strain using a plasmid containing the cod-

ing sequence of *ppt1* fused to the constitutive promoter of the *T. reesei* pyruvate kinase gene. Integration of the construct was confirmed in two transformants by Southern blot (Supplementary Fig. 2). Analysis of the expression of *ppt1* in these transformants (OEppt1-4 and OEppt1-6) revealed high levels of expression, whereas no mRNA could be detected in the wild-type strain (Supplementary Fig. 3). Expression of the gene in the wild-type strain (Tv29.8), the parental strain used to generate the mutant (Tv10.4), and the retransformed strain (TvC-24) used throughout our studies was confirmed by reverse-transcription PCR (data not shown).

The evaluation of growth and conidiation of the mutant (Δ*ppt1*-1*)* and complemented (TvC-24) strains in comparison with the wild-type (Tv29.8) and parental (Tv10.4) strains was carried out (Fig. 1A and B). A fluffy phenotype was observed in the gene-replacement mutant, which unexpectedly had a marked increase in radial growth (93%) compared with the wild type (Fig. 1A and C). In addition, the mutant produced white conidia, lacking the characteristic green pigment of the wild type (Fig. 1A), suggesting that the mutation likely affected the activation of the polyketide synthase responsible for the synthesis of this pigment. As expected, these phenotypes disappeared in the complemented strains, which showed no difference in growth or conidiation when compared with the wild-type strain (Fig. 1B and C). All Δ*ppt1* mutants obtained behaved similarly (data not shown).

Growth of the wild-type and mutant strains in minimal medium supplemented with iron revealed a clear reduction in conidial germination, which was even more drastically reduced in the absence of iron, suggesting an important role of siderophores in the iron capture mechanism necessary for germination of conidia (Fig. 2A and B). To determine whether the mutation also affected iron capture during active growth, the mutant was evaluated in minimal medium with or without iron and without the addition of siderophores. In contrast to germination of conidia, the mutant grew without any apparent problem without iron, although the mycelium was scarce and growth was arrested after 72 h (Fig. 2C). In medium supplemented with iron, the mutant grew normally and produced abundant mycelium (Fig. 2D).

The *ppt1* mutants show decreased antagonistic activity against phytopathogens.

To better understand the role played by secondary metabolites in the control of phytopathogenic fungi by *T. virens*, we evaluated the inhibitory activity of small molecules produced by the different *Trichoderma* strains generated on the growth of seven phytopathogenic fungi (*Alternaria solani, Fusarium* spp.*, Fusarium oxysporum, Phytophthora capsici, Rhizoctonia solani, Sclerotium rolfsii,* and *S. cepivorum*). The growth of all pathogens was completely inhibited when grown in media where the wild type, overexpressing, or complemented *Trichoderma* strains had been pregrown, with the exception of *S. rolfsii*, which only showed a delay in growth. In contrast, the growth of pathogens in medium where the Δ*ppt1* strain had been pregrown was unaffected, except in the cases of *Fusarium* spp. and *F. oxysporum*, which grew but whose colonies clearly showed less dense mycelial mats (Fig. 3A). All seven phytopathogens grew normally in the medium without *Trichoderma* metabolites. These data suggested that the lack of activation of PKS and NRPS, responsible for the production of most secondary metabolites by *Trichoderma* spp., severely affects the capacity of *T. virens* to inhibit the growth of phytopathogenic fungi. To confirm that the loss of this capacity was due to the lack of antibiotic production, mycelial extracts of *Trichoderma* spp. grown in liquid medium were analyzed by mass spectrometry using electrospray ionization quantitative time-of-flight (ESI-QTOF). In the cases of parental Tv10.4 (Fig. 3B) and wild-type Tv29.8 strains (data not shown), three classes of peptaibols were identified by mass spectrometry analysis, including those previously reported: 11, 14, and 18 mer (Viterbo et al. 2007). In contrast, the Δ*ppt1* mutant did not produce any of these metabolites (Fig. 3C), confirming that mutation of the corresponding gene severely affects the synthesis of peptaibols, thus decreasing the potential of *Trichoderma* spp. to inhibit the growth of other fungi.

***T. virens* Δ*ppt1* mutants are capable of colonizing plant roots.**

It has previously been demonstrated that *Trichoderma* spp. colonize plant roots. Following root penetration, the exchange of bioactive compounds controls the endophytic proliferation of the fungus (Chacón et al. 2007; Yedidia et al. 1999). Although it has been shown that some secondary metabolites may potentiate systemic induced response, it is not known if they play a role in the plant–*Trichoderma* communication. For this reason, the colonization of *Solanum lycopersicum* roots by wild-type *T. virens* and Δ*ppt1* mutants in vitro and their permanence in the root system were analyzed. Using vital staining of fungal hyphae and confocal microscopy, we detected the presence of all tested strains of *T. virens* Tv10.4, Tv29.8, and Δ*ppt1*-1 in the root epidermis after 48 h of plant-fungus interaction. At this time, all strains were present in the first cell layers of the root (Fig. 4B to D). Twenty-four hours later, the strains Tv10.4, Tv29.8, and TvC-24 colonized the root system without extending to the aerial part of the plant, whereas the Δ*ppt1* mutant colonized the root system but also invaded the stem of the plant (Supplementary Fig. 4). After 72 h of the interaction, plants were transferred to soil and grown for 15 additional days. At the end of this period, persistence of

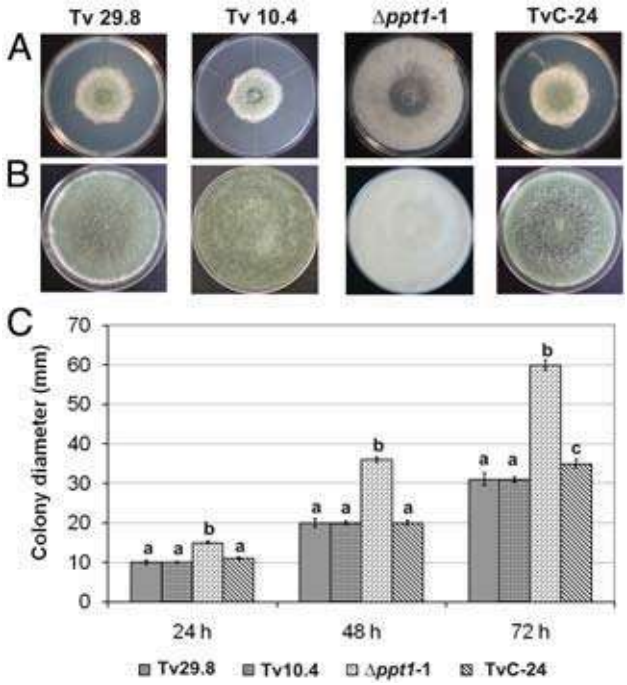

Fig. 1. *Trichoderma virens* mutants in *ppt1* are altered in growth and conidiation. Representative photographs showing the aspect of the colonies of the strains photographed after **A,** 72 or **B,** 120 h of growth at 28°C in potato dextrose agar (PDA). Names on top of columns indicate the *T. virens* strain evaluated. **C,** Kinetics of growth of the indicated strains in PDA as determined by measuring colony diameter. Values shown represent the mean of three different plates ± standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

Trichoderma spp. in the roots was evaluated by recovering them from surface-sterilized root fragments in selective media (Fig. 4E to H). Even though the gene replacement mutant (Δ*ppt1*-1) and the parental strain (Tv10.4) were auxotrophic for lysine and arginine, respectively, they could survive in the root system. The identity of the recovered strains was confirmed by PCR using oligonucleotides *ppt1-G* and *ppt1-H* that allowed the distinction between the wild type and the gene replacement mutant (data not shown).

Nonribosomal peptides and polyketides play a minor role in seed protection by *Trichoderma* spp.

The role of antibiotics in fungal antagonism by *Trichoderma* spp. has been well established in vitro, and synergism with cell wall–degrading enzymes has also been observed (Schirmböck et al. 1994). In spite of the efforts made to understand the role of antibiotics in the interaction with the plant (Tijerino et al. 2011; Vinale et al. 2006), their role in vivo remains poorly understood. Therefore, we next investigated the role of nonribosomal peptides and polyketides from *T. virens* in seed protection through the analysis of the protection conferred by wild-type *T. virens* and Δ*ppt1* mutants to *S. lycopersicum* seeds exposed to a substrate infested with *R. solani*. By measuring seed germination, we determined that, without *Trichoderma* spp., only 45% of *S. lycopersicum* seeds germinated in the presence of *R. solani*. Seed treated with *T. virens* Tv29.8 and Tv10.4 behaved similarly, showing 65% germination. A contrasting result was obtained when seeds were treated with the gene-replacement mutant, which apparently were more efficiently protected, showing 77% germination (Fig. 5A). Axenic seeds or *T. virens* wild-type and Δ*ppt1*-1–treated seeds showed a roughly 80% germination in medium without *R. solani* (Fig. 5B). These data suggest that antibiotics of the NRP and polyketide type do not play a significant role in seed protection against *R. solani*.

T. virens* Δ*ppt1* mutant is defective in induction of SA-dependent defense responses in *Arabidopsis thaliana.

The defense signaling pathways that are activated in *A. thaliana* upon exposure to *Trichoderma* spp. involve both SA and JA (Salas-Marina et al. 2011; Segarra et al. 2007). To examine whether mutation in *ppt1* could affect the SA or JA responses, we monitored expression of selected marker genes that are upregulated by these hormones. We used *A. thaliana* transgenic lines expressing β-glucuronidase (GUS) (*uidA*) fusions to the promoters of *Pr1a*, a gene activated by SA (Shah et al. 1997) and *Lox2*, a gene activated by JA (Schommer et al. 2008). *A. thaliana* transgenic seedlings carrying each of these markers were co-cultivated with the different *T. virens* strains. Axenically grown seedlings did not express *pPr1a:uidA* (Fig. 6A, K, F, and O). *pPr1a:uidA* expression was activated in both shoots and roots of plants inoculated with the wild-type strain (Tv29.8), the parental strain (Tv10.4), and the complemented strain TvC-24 (Fig. 6B to D and G to I). In sharp contrast, co-cultivation of this reporter line with the *T. virens* Δ*ppt1*-1 mutant failed to activate GUS expression (Fig. 6E and J). Surprisingly, the JA-activated marker *pLox2:uidA* was expressed at a similar level in plants co-cultivated with wild-type *T. virens* or the Δ*ppt1 mutant* strains (Fig. 6L to N and P to S). These data suggest that *T. virens* PPT1-dependent secondary metabolites are specifically involved in triggering a subset of *A. thaliana* responses mediated by SA.

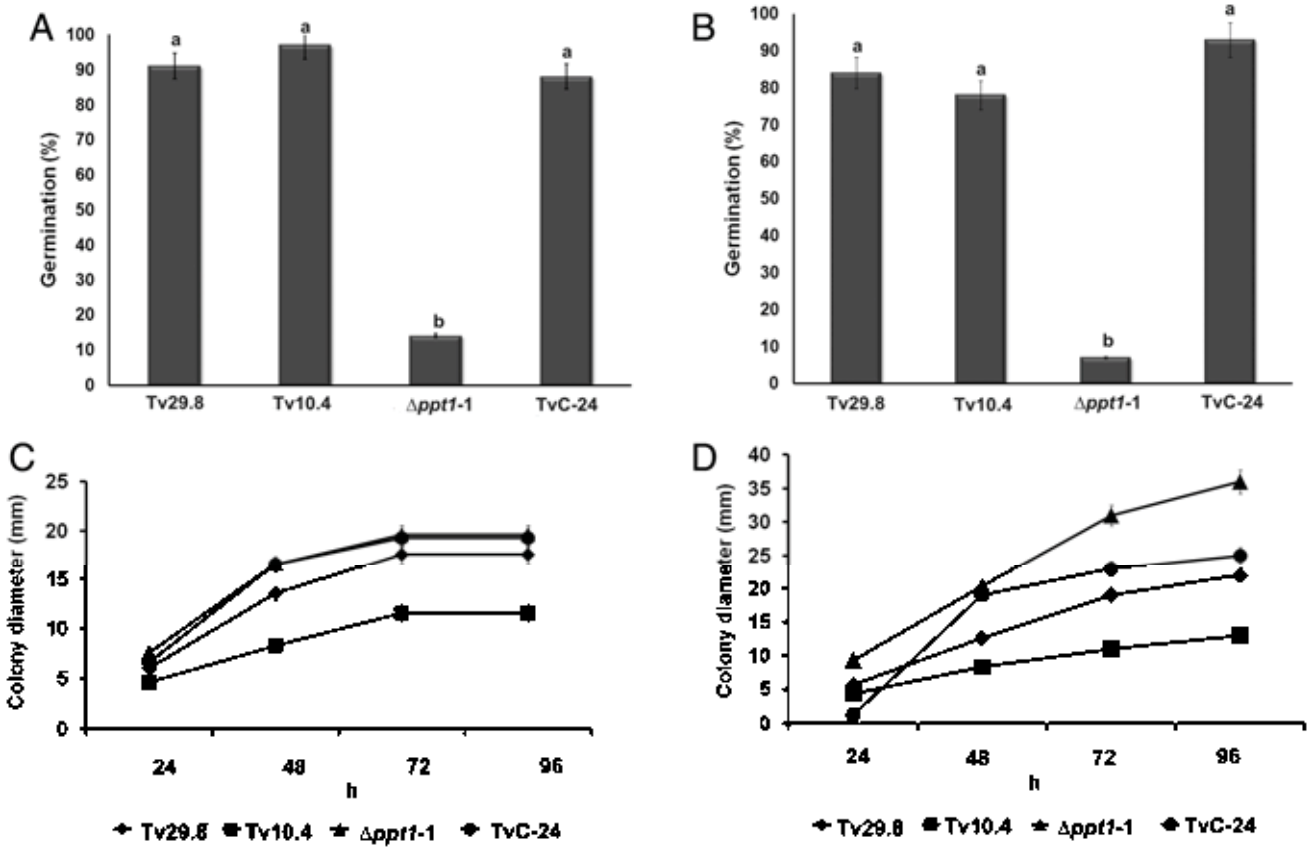

Fig. 2. The Δ*ppt1* mutant of *Trichoderma virens* is affected in germination of conidia. Germination of conidia of the indicated *T. virens* strains in VMS medium (Viterbo et al. 2007) **A,** with or **B,** without iron. Mycelial growth of the indicated *T. virens* strains in Grimm-Allen medium **C,** with or **D,** without iron. **A** to **D,** Bars indicate standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

Interaction between *A. thaliana* roots and *T. virens* Δ*ppt1* mutant fail to induce SA accumulation in leaves.

To determine whether the changes in the expression of *pPr-1a:uidA* and *pLox2:uidA* markers were associated with changes in endogenous SA or JA content in plants co-cultivated with *Trichoderma* spp., free SA and JA were measured in wild-type *A. thaliana* (Col-0) plants co-cultivated with wild-type *T. virens* or Δ*ppt1* mutants by gas chromatography–mass spectrometry (GC-MS). A fourfold increased accumulation in SA was observed in treatments with wild-type *Trichoderma* spp. when compared with axenically grown seedlings (Fig. 7A). The levels of SA dramatically decreased in plants co-cultivated with the Δ*ppt1*-1 mutant (Fig. 7A). In contrast, JA determinations clearly showed a three- to fourfold increase in JA in plants that were co-cultivated with either wild-type *T. virens* or Δ*ppt1*-1 mutant strains (Fig. 7B).

Trichoderma* Δ*ppt1* mutants fail to induce camalexin accumulation in *A. thaliana.

To investigate whether deletion of *ppt1* could affect camalexin production, axenically grown plants, or plants colonized with wild-type *T. virens* or the Δ*ppt1-1* mutant were used for camalexin determinations. GC-MS analysis revealed that *A. thaliana* seedlings interacting with wild-type *T. virens* increased camalexin levels by three- to fourfold when compared with axenically grown plants (Fig. 8). This effect was highly reduced in plants co-cultivated with Δ*ppt*1 mutants (Fig. 8).

Deletion of *ppt1* compromises *T. virens* protection against the necrotizing pathogen *Botrytis cinerea.*

To determine whether the alterations in SA and camalexin-dependent responses observed in *A. thaliana* seedlings exposed to Δ*ppt1* mutants could affect pathogen resistance, we tested the responses of leaves from 12-day-old *Arabidopsis* plants whose roots had been colonized or not with wild-type *T. virens* or Δ*ppt1* mutant and inoculated with the necrotrophic pathogen *Botrytis cinerea,* which causes spreading necrotic lesions on leaves. In these experiments, *B. cinerea* spores were inoculated on the leaf surface and disease symptoms evaluated 3 and 5 days later. In control plants, *B. cinerea* was found to induce necrotic lesions in approximately 55% of inoculated leaves (Fig. 9A). In contrast, in plants colonized by the *T. virens* wild type, only 29% presented necrotic lesions caused by *B. cinerea* infection (Fig. 9A), whereas plants co-cultivated with the Δ*ppt1* mutant showed levels of necrotic lesions similar to the control plants (Fig. 9A). Furthermore, *B. cinerea* caused death

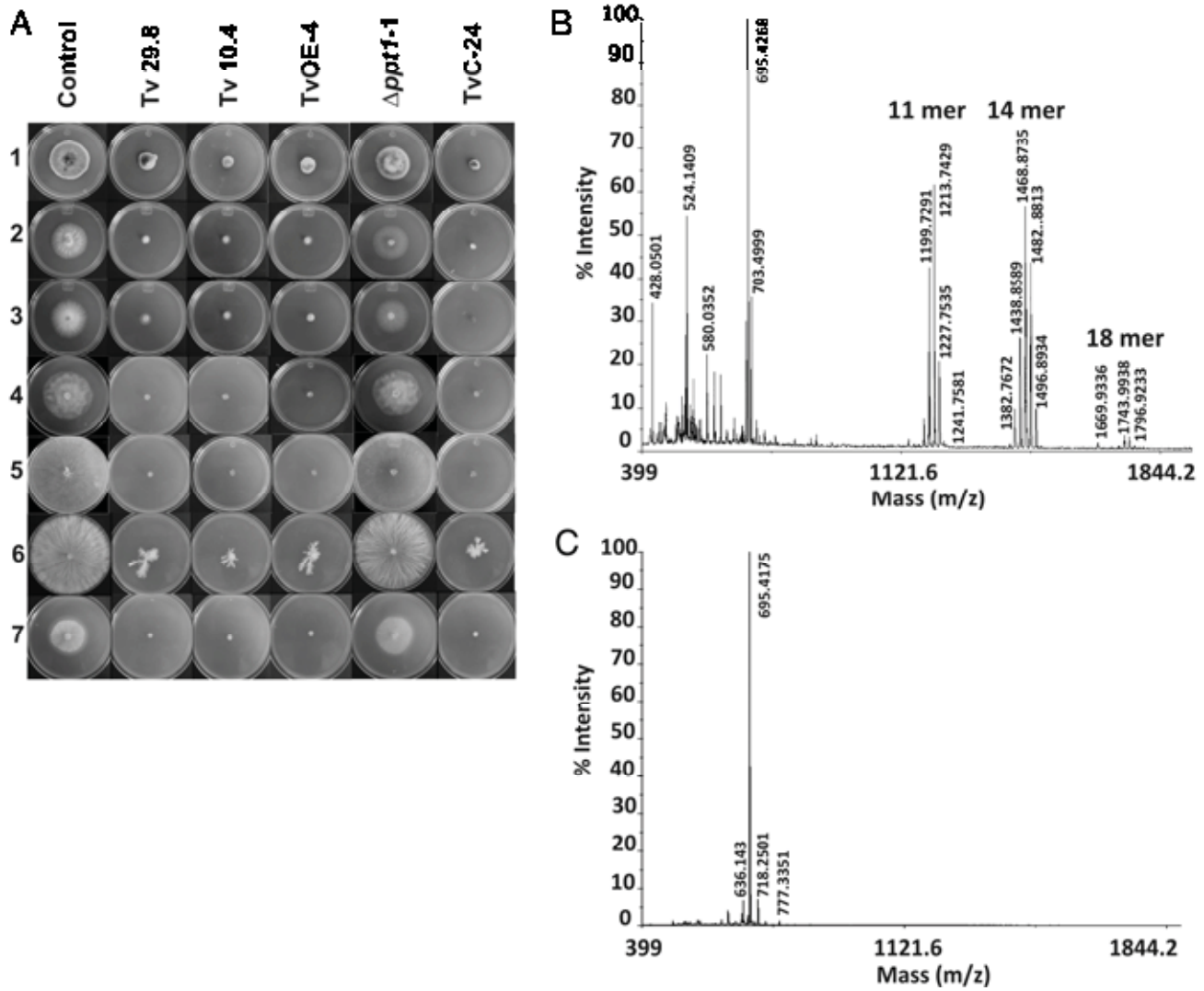

Fig. 3. Effect of antibiotics from *Trichoderma virens* on phytopathogenic fungi and production of peptaibols. **A,** Growth of phytopathogens on plates where *Trichoderma* had been pregrown photographed after 48 h of growth. Row 1, *Alternaria solani*; row 2, *Fusarium* spp.; row 3, *Fusarium oxysporum*; row 4, *Phytophthora capsici*; row 5, *Rhizoctonia solani*; row 6, *Sclerotium rolfsii*; row 7, *S. cepivorum*. Names on top of each column indicate the *T. virens* strain evaluated. Detection of peptaibols by mass spectrometry in extracts from strains **B,** Tv 10.4 and **C,** Δ*ppt1*-1.

in approximately 90% of control plants, in contrast to only 25% death in plants colonized by the wild-type *Trichoderma* strain, whereas the *ppt*1 mutant failed to confer protection (Fig. 9B).

DISCUSSION

Phosphopantetheinyl transferases compose a class of ubiquitous enzymes found in filamentous fungi, which are necessary for primary metabolism due to their role in activating the α-aminoadipato reductase and in secondary metabolism for the activation of polyketide synthases and NRPS. Here, we report the role of an Sfp-class phosphopantetheinyl transferase from *T. virens* in fungal physiology, biocontrol, and plant interaction.

We observed that Δ*ppt*1 mutants of *T. virens* had an accelerated radial growth of vegetative hyphae and delayed production of conidia. Conidia were produced only when growth of aerial hyphae decreased. A similar alteration in vegetative growth and a sharp decrease in conidiation were reported in the *cfwA/npgA* mutant of *Aspergillus nidulans*, which was attributed to a delay in hyphal branching (Márquez-Fernández et al. 2007). Increased growth was also observed in the conditional mutant *npgA/cfwA* from *A. nidulans,* which produced more mycelial mass than the wild type in liquid culture (Keszenman-Pereyra et al. 2003). We hypothesize that the enhanced mycelium growth and decreased conidiation in the *T. virens* Δ*ppt1* mutant is due to the lack of production of secondary metabolites, because growth of the mutant in media with such metabolites excreted by the wild type were sufficient to revert this phenotype, as well as the fluffy aspect of the colony. A plausible explanation is that the peptaibols produced by *Trichoderma* spp. can affect its own plasma membrane functions, and that the lack of production of these metabolites by the mutant potentiates growth, leading to the production of more aerial mycelium. Although it has never been reported that *T. virens*'s own antibiotics could affect its growth or conidiation, Howell and Stipanovic (1983) reported that a virtidiol overproducing mutant of *T. virens* (formerly *Gliocladium virens*) grew more slowly than the parental strain.

When grown in media with low or high iron concentration, conidia of the Δ*ppt1* mutant showed low germination index. In this respect, trihydroxamates such as ferricrocin are essential to store iron intracellularly, which aids spore germination in iron-poor media, as demonstrated for *sidC* in *A. fumigatus* (Schrettl et al. 2007), *A. nidulans* (Wallner et al. 2009), and *Neurospora crassa* (Berthold et al. 1987; Charlang and Williamst

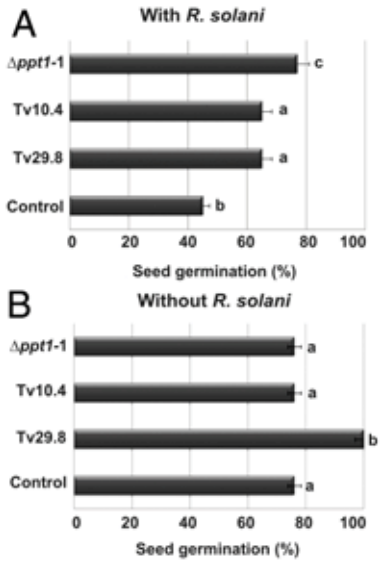

Fig. 5. Protection of *Solanum lycopersicum* seeds by *Trichoderma* spp. **A,** Effect of inoculation of the indicated *Trichoderma virens* strains on germination of seeds in soil infested with *Rhizoctonia solani.* **B,** Effect of inoculation of *T. virens* on germination of seeds in sterile soil. **A** and **B,** Control corresponds to uninoculated seed. Bars indicate standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

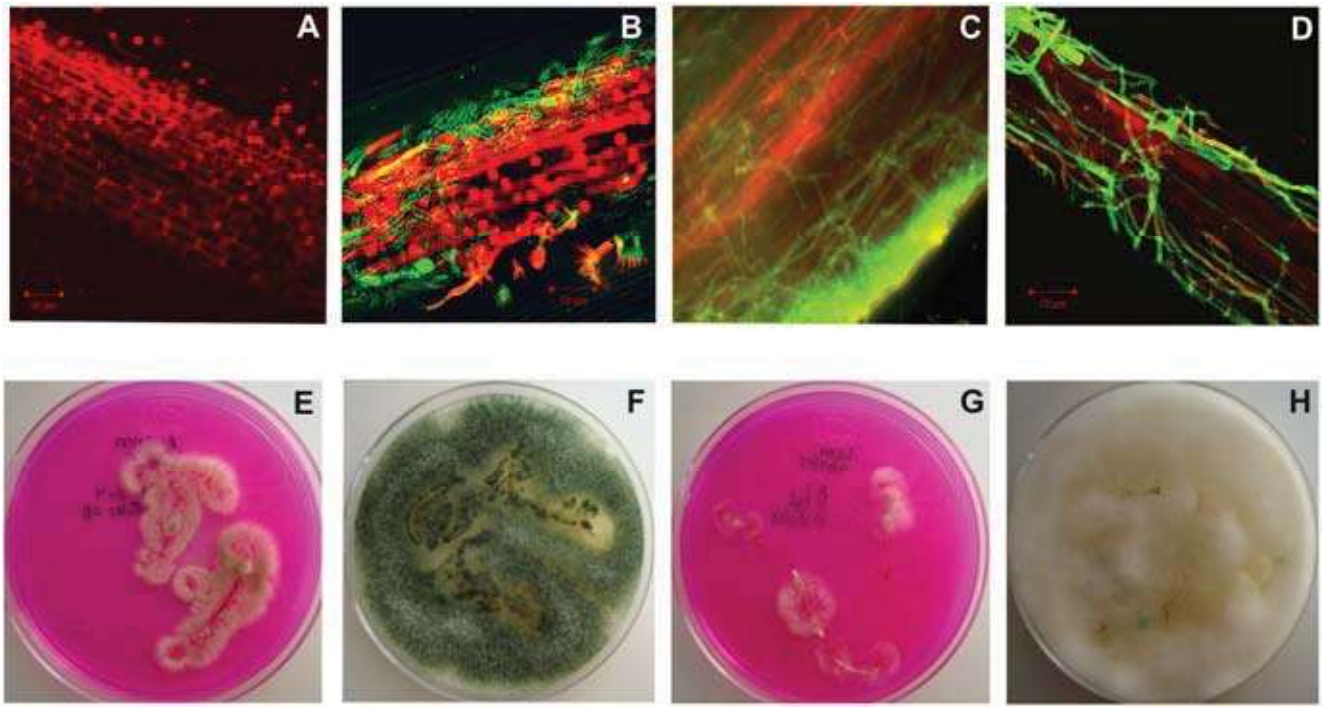

Fig. 4. Colonization of *Solanum lycopersicum roots*. Top: Confocal microscopy representative photographs of **A,** roots without *Trichoderma* spp. or roots colonized by **B,** Tv 29.8; **C,** Tv10.4; or **D,** Δ*ppt1*-1 strains. Bottom: *Trichoderma* strains recovered from root fragments; **E,** Δ*ppt1*-1 and **G,** Tv10.4 recovered and grown in Rose Bengal selective medium; **F,** Δ*ppt1*-1 and **H,** Tv10.4 strains recovered and grown in potato dextrose agar.

1977). On the other hand, when mycelial growth of the null mutant of *T. virens* was analyzed under iron-limiting conditions, it grew at approximately the same rate as observed for the wild-type strain, although mycelium was scarce. Similar results were also observed in *cfwA* and *PPT1* mutants in *A. nidulans* and *Colletotrichum graminicola*, respectively (Horbach et al. 2009; Oberegger et al. 2003). The observed phenotype might be due to the lack of cis/trans fusarinine and dimerum acid, which trap extracellular iron, and might be substituted by the reductive iron assimilation (RIA) pathway, a two-step process that involves the extracellular reduction of Fe^{3+} to Fe^{2+} followed by high-affinity uptake of Fe_2. In this sense, several orthologous genes to the three components of RIA (Fre1, FetC, and FtrA) were found in the *T. virens* genome.

One of the main attributes of the genus *Trichoderma* is the production of secondary metabolites that inhibit the growth of or kill phytopathogenic fungi and some gram-positive bacteria in vitro (Fravel 1988; Neuhof et al. 2007; Reino et al. 2007; Wiest et al. 2002). Among the main antibiotics produced by *T. virens*, peptaibols have deserved more attention. Peptaibols of class 11, 14, and 18 mer were produced by the wild-type strain of *T. virens* as well as the strain used for transformation (Tv10.4), which correspond to those classified as short (11 to 16 amino acids) and long (18 to 20 amino acids) sequences. These peptaibols belong to the TvA class, which is a mix of 11-amino-acid peptides similar to Harzianines HB, and class TvBI Trichorzins type, with 18 residues (Wiest et al. 2002). These peptaibols were reported in the study of the function of the *T. virens* 29.8 NRPS encoding gene *tex1*, where the corresponding mutants failed to produce 18-, 14-, and 11-amino-acid residue peptides peptaibols (Viterbo et al. 2007; Wiest et al. 2002). Our results showed that the Δ*ppt1*-1 mutant did not produce peptaibols, thus indicating that the NRPS that participate in their synthesis require PPT1 for their activation.

The role of nonribosomal peptides and polyketides in antagonism by the different *T. virens* strains analyzed here was evaluated through their effects on the growth of several phytopathogenic fungi. Overexpression of *ppt1* did not result in increased inhibitory capacity. In contrast, Δ*ppt1* mutants showed strongly decreased antagonistic activity against phytopathogens, suggesting that normal PPT1 function is an important factor in biocontrol by *T. virens,* and that higher levels of PPT1 do not seem to result in increased antagonistic activity by *T. virens.*

An important application of microbial antagonists is seed protection, which allows plant germination in pathogen-infested soils. However, the role of antibiotics produced by *Trichoderma* spp. in plant protection in soil has remained speculative. The fact that the Δ*ppt1*-1 mutant of *T. virens* conferred greater protection to seed of *S. lycopersicum* in soil infested with *R. solani* than the wild-type and parental strains indicates that nonribosomal peptide and polyketide antibiotics play a minor role in seed protection by *T. virens*. The higher protection observed with the Δ*ppt1*-1 mutant could be due to the fact that the mutant grows faster, increasing its competition capacity, although we cannot discard the possibility that other antimicrobial compounds are overproduced by *Trichoderma* spp. in the absence of active NRPS or PKS, which might aid in seed protection. Our findings suggest that hydrolytic enzymes and mycoparasitism are more relevant than antibiotics in the control of *R. solani* during seed protection. A similar observation was made in the case of a *T. virens* mutant that did not produce gliotoxin but remained efficient in the protection of plants against infection by *R. solani* (Howell and Stipanovic 1995).

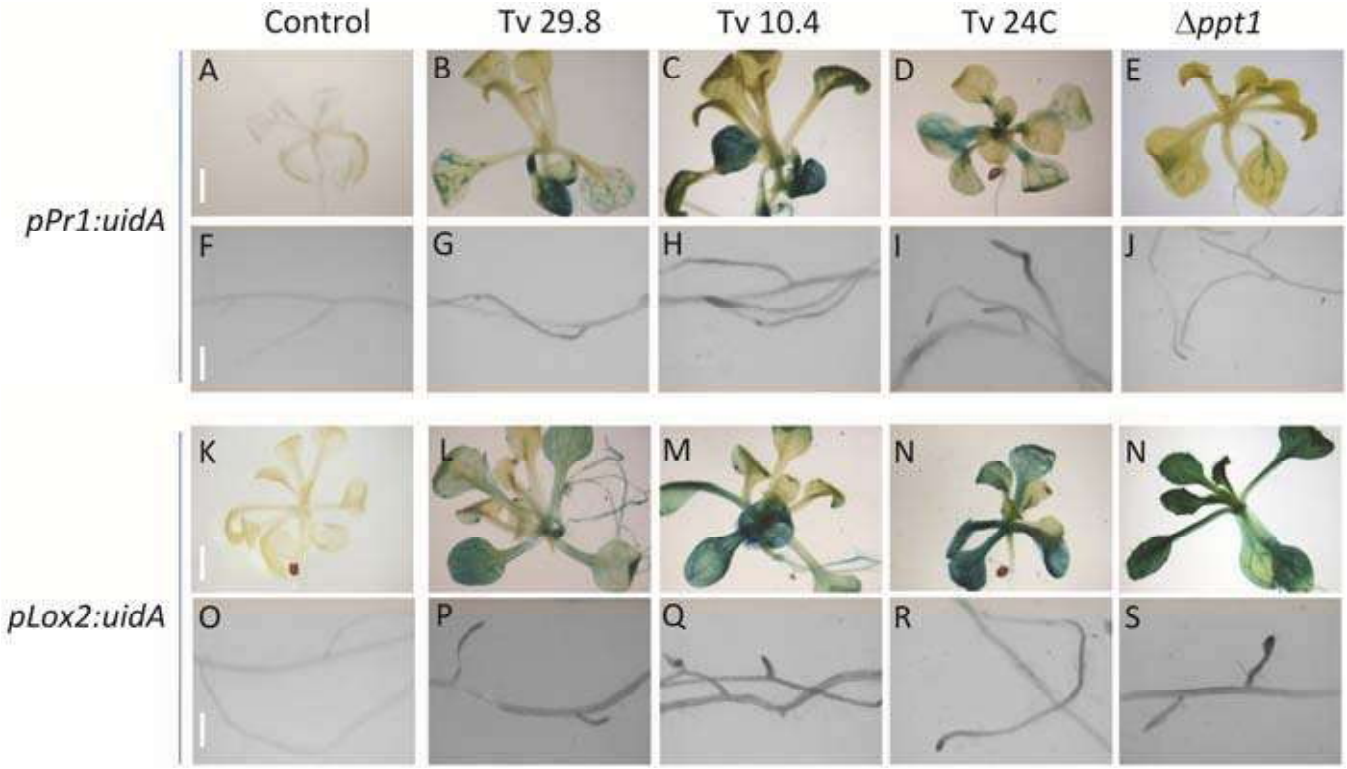

Fig. 6. Effect of *Trichoderma virens* on expression of the pathogen-related response markers *pPr1a:uidA* and *pLox2:uidA*. Transgenic *Arabidopsis thaliana* seedlings carrying the marker constructs were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, transferred to 0.2× Murashige and Skoog medium supplemented with 300 μM Lys, and then co-cultivated with the *T. virens* strains indicated on top. β-Glucuronidase (GUS) expression in seedlings was determined 6 days after transfer. **A** to **J,** Plants carrying the *pPr1a*:*uidA* construct. **K** to **S,** Plants carrying the *pLox2*:*uidA* construct. **A, F, K,** and **O,** Axenically grown plants. **A** to **E** and **K** to **N,** GUS expression in aerial parts of the plant. **F** to **J** and **O** to **S,** GUS expression in roots. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

As in other fungi, *T. virens* Δ*ppt1* mutants are auxotrophic to lysine. This represented an obstacle in evaluating the role of PPT1 in biocontrol. To overcome this obstacle, we analyzed the possibility of allowing colonization of the root system of *S. lycopersicum* plantlets in vitro and then transferring them into soil. The root system was colonized after 48 h and the fungus remained in plants up to 3 weeks. This is explained by the fact that root exudates of *S. lycopersicum* contain essential amino acids such as lysine, arginine, aspartic acid, and glutamic acid, among others, that provide the requirements for growth (Simons et al. 1997).

Some natural isolates of *Trichoderma* spp. have been found to colonize not only plant roots but also other parts of the plant, as in the case of *Theobroma cacao,* colonizing roots, stem, leaves, and even seed (Bailey et al. 2006). By using an in vitro system, we showed that the Δ*ppt1*-1 mutant colonized the root system as well as the wild-type strain. Interestingly, after a longer period of time, the mutant continues growing, invading the stem and leaves, which did not occur when the plant interacts with the wild-type strain. This may be interpreted as either a miscommunication effect or as a simple defect in the interaction due to the accelerated growth of the mutant.

Jasmonate and ET mediate the main defense responses of plants during infection by necrotrophic fungal pathogens, whereas SA-dependent responses and SAR were initially not predicted to play a role (Bent 2006). In the interaction of *Trichoderma* spp. with common bean (*Phaseolus vulgaris*), tomato (*S. lycopersicum*), and *A. thaliana*, the fungus promotes accumulation of PR proteins (Salas-Marina et al. 2011; Woo et al. 2006). In agreement with these findings, we found that wild-type *T. virens* activated *pPr1a*:*uidA* and induced a fourfold increase in the levels of SA. These responses were absent in plants co-cultivated with the Δ*ppt1*-1 mutant. Notably, the levels of JA and the expression of the JA-induced marker *pLox2*:*uidA* remained similarly induced by the wild type and the Δ*ppt1*-1 mutant, suggesting a specific role for PPT1 function during SAR.

Antimicrobial compounds can be produced as part of normal plant growth and in response to pathogens. Camalexin production has been found to be elicited by bacterial and fungal phytopathogens and possess antimicrobial activity (Glazebrook and

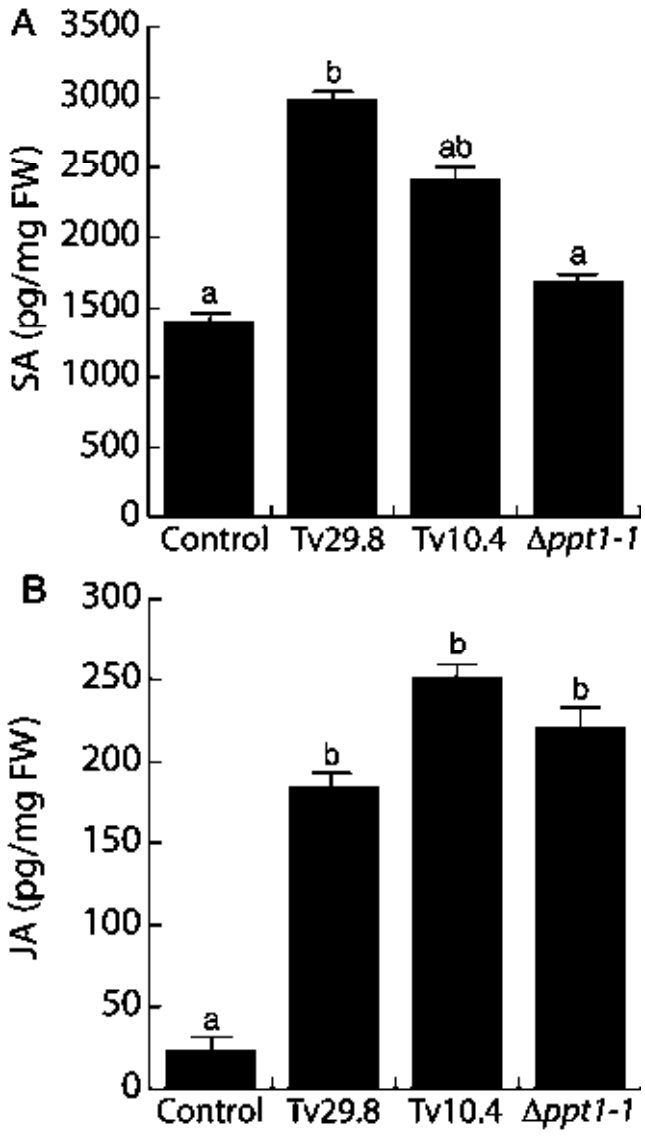

Fig. 7. Effect of *Trichoderma virens* on salicylic acid (SA) and jasmonic acid (JA) accumulation in *Arabidopsis thaliana*. Wild-type *A. thaliana* (Col-0) seedlings were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, then co-cultivated with the indicated *T. virens* strains in 0.2× Murashige and Skoog medium supplemented with 300 μM Lys for 8 additional days. Free **A,** SA or **B,** JA in *A. thaliana* shoots. Control corresponds to axenically grown seedlings. Error bars represent the standard error. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

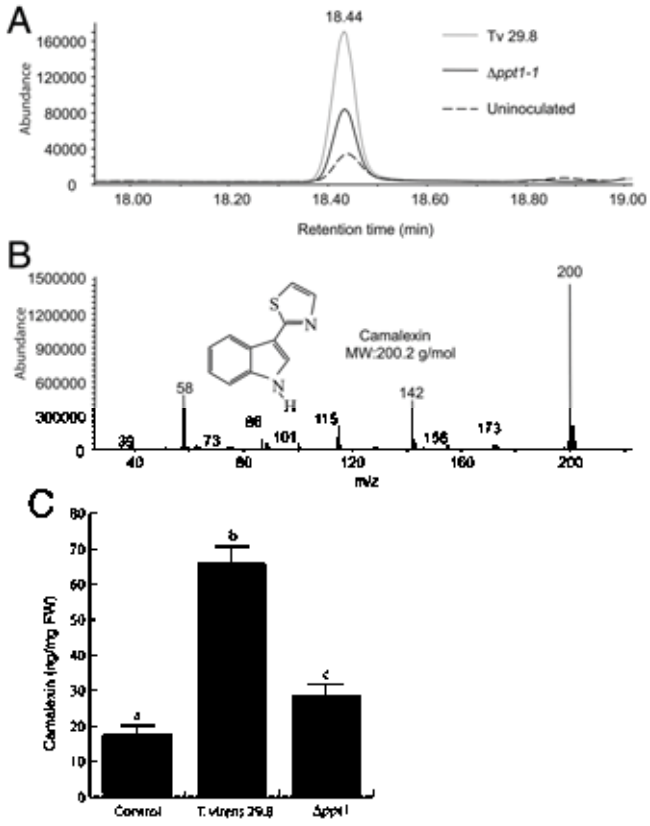

Fig. 8. Effect of *Trichoderma virens* inoculation on camalexin accumulation in *Arabidopsis thaliana*. Wild-type *A. thaliana* (Col-0) seedlings were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, transferred to 0.2× Murashige and Skoog medium supplemented with 300 μM Lys, and then co-cultivated with the indicated *T. virens* strains for 8 additional days. **A,** Representative chromatogram showing camalexin levels in leaves of wild-type *A. thaliana* seedlings. Control corresponds to axenically grown-seedlings. **B,** Mass spectra from camalexin standard. **C,** Camalexin quantification from *Arabidopsis* leaves. The bars show the mean ± standard deviation of three independent biological replicates. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Ausubel 1994; Ferrari et al. 2003; Thomma et al. 1999). Here, we showed that *A. thaliana* seedlings colonized with wild-type *T. virens* accumulated higher levels of camalexin than axenically grown seedlings. This response was compromised in plants co-cultivated with the Δ*ppt1*-1 mutant.

In agreement with the role of PPT1 in activating defense-signaling pathways, a *Trichoderma* mutant lacking PPT1 failed to confer protection against the fungal necrotizing pathogen *B. cinerea*. In this regard, it has been previously shown that *Arabidopsis* resistance to *B. cinerea* involves SA and camalexin (Ferrari et al. 2003). *Arabidopsis pad2-1* and *pad3-1* mutants, which accumulate low levels of camalexin, are highly susceptible to *B. cinerea*, and purified camalexin inhibits growth of this pathogen in a dose-dependent manner (Ferrari et al. 2003; Glazebrook et al. 1997). The relation found between reduced SA and camalexin production and much lower protection in plants colonized by the *T. virens* Δ*ppt1*-1 mutant confirmed that the combined activation of SA-dependent pathways and camalexin production are important to confer plant immunity against a fungal necrotizing pathogen. Based on these analyses, we conclude that *T. virens* is capable of regulating multiple defense responses. In addition to the defense gene induction and hormone biosynthesis, we now provide chemical evidence supporting a role for *ppt1* from *T. virens* in phytoalexin induction, another important defense response.

In conclusion, we have shown the important role of the 4-phosphopantetheinyl transferase PPT1 from *T. virens* in secondary metabolism and plant defense responses. Among the several mechanisms activated in *A. thaliana* by *T. virens* to confer immunity against *B. cinerea*, there is a role of *ppt1* in a resistance mechanism involving SA and camalexin production.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *T. virens* wild-type strain Tv29.8 and an arginine auxotroph (Tv10.4) derived from it were used in this study (Baek and Kenerly 1998). *T. virens* strains were grown in potato dextrose agar (PDA) medium (Difco Laboratories, Detroit) or in Vogel's minimal medium supplemented with 2 mM arginine. Pathogenic strains of *R. solani*, *F. oxysporum*, *Fusarium* spp., *Alternaria solani*, *Sclerotium rolfsii*, *S. cepivorum*, and *Phytophthora capsici* were grown in PDA medium (Difco Laboratories). *T. virens* Δ*ppt*1 mutants were grown in Vogel's minimal medium supplemented with 10 mM lysine or 50% siderophore-containing conditioned medium. To prepare siderophore-containing medium, 1×10^9 conidia from strain Tv29.8 were used to inoculate Vogel's liquid minimal medium without iron (Marquéz-Fernández et al. 2007).

Cloning and overexpression of *ppt1*.

Based on the *T. virens* genome sequence, forward primer *F_orf_ppt1* and reverse primer *R_orf_ppt1* (Table 1) were designed and used to amplify by PCR the 4-phosphopantetheinyl transferase-encoding gene (*ppt1*) from the wild-type strain Tv29.8 using genomic DNA as template and the following conditions: an initial cycle of 95°C for 3 min; 30 cycles of

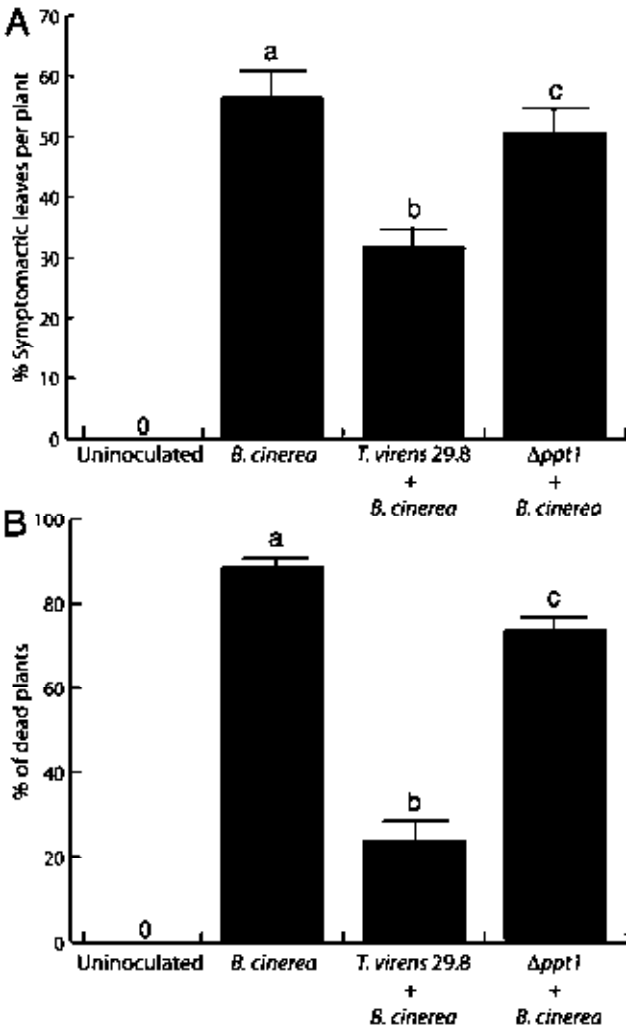

Fig. 9. Protection against *Botrytis cinerea* in *Arabidopsis thaliana* seedlings is compromised in Δ*ppt1* mutants. **A,** *A. thaliana* plants (12 days old) grown axenically or co-cultivated for 6 days with the indicated *Trichoderma virens* strains in vitro were placed in petri dishes containing 0.2× Murashige and Skoog medium, and the shoot was treated with sterilized deionized water or inoculated with 1×10^6 *B. cinerea* spores, distributing the inoculum over the leaf surface. Each treatment was applied to 30 plants. **A,** Number of symptomatic leaves per plant was scored 3 days post-infection and **B,** the percentage of dead plants was scored 5 days post-infection. Bars show the mean ± standard deviation of 30 *A. thaliana* seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Table 1. List of primers of *Trichoderma virens*

Name	Sequence	Temperature (°C)	Size (bp)[a]
F_orf_ppt1	5′-TCGCAGCCATGGCTCAGCCCA-3′	60.8	987
R_orf_ppt1	5′-AATCGCCATCTCACTCGCCCAGC-3′	…	…
ppt1_A	5′-GAGACGCAGTAGGTTCTTTC-3′	60.8	1,006
ppt1_B	5′-CGCCGTGGGGCTCTGTATCGACAAGCTGGCTGCAGCGATCATTTATATGTCG-3′	…	…
ppt1_C	5′-CGCAACGAATAATCCCTCCCTGTAGAAGGCAAGATCACCGACAAGTTGGTCG-3′	60.8	857
ppt1_D	5′-TAAGCATGGGGCGCCACAGTTGC-3′	…	…
ppt1_E	5′-TTGATATCGAGCCCCCAGGCG-3′	53.8	2,929
ppt1_F	5′-GGAATTGGGTCTGGCACG-3′	…	…
ppt1_G	5′-CGCTTTAAACGGGCGTCTGTTC-3′	65	4,792
ppt1_H	5′-GGGGACTTTGAGCTGAAGTGGATG-3′	…	…

[a] Size of the amplified fragment using the corresponding pair of oligonucleotides.

94°C for 45 s, 60.8°C for 45 s, and 72°C for 1 min; and a final cycle of 72°C for 7 min. The PCR product was cloned into PCR 2.1 TOPO (Invitrogen, Carlsbad, CA, U.S.A.). To generate the overexpression plasmid, one of the clones was selected and plasmid DNA was digested with *Eco*RI and subsequently ligated into plasmid pUE08 (Esquivel-Naranjo and Herrera-Estrella 2007). DNA of the resulting plasmid (pOEppt) was used in polyethylene glycol–mediated transformation of protoplasts of the Tv29.8 strain, as described previously (Baek and Kenerly 1998). Hygromycin-resistant transformants were subjected to three consecutive monosporic passes. Southern blot analysis was used to confirm plasmid integration, using as a probe a fragment of the *ppt1* gene containing the complete open reading frame.

Replacement of the phosphopantetheinyl transferase gene.

Gene *ppt1* was replaced in strain Tv10.4 by the *T. virens arg*2 gene using the double-joint PCR procedure described by Yu and associates (2004). In a first round of PCR, 5′ forward primer *ppt1_A*, 5′ reverse primer *ppt1_B*, 3′ forward primer *ppt1_C,* and 3′ reverse primer *ppt1_D* (Table 1) were used to generate the upstream and downstream regions flanking the *ppt1* gene. In addition, to generate the marker cassette, the following primers were used: arg2 forward primer *ppt1_E*, and arg2 reverse primer *ppt1_F* (Table 1). In the third and final round of amplification, we used the nested forward primer *ppt1_G* and reverse primer *ppt1_H* (Table 1). The final product was used to transform protoplasts. Selection of transformants was carried out in Vogel's minimal medium. For the selection of transformants, it was considered that, initially, transformants could require lysine and siderophores, which were added to the medium for the recovery of transformed protoplasts. All arginine prototrophs were subjected to three rounds of monosporic culture. Gene replacement was confirmed by PCR using primers *ppt1_G* and *ppt1_H* (Table 1). Putative gene replacement mutants were then confirmed by Southern blot analysis, using as probe a 1-kb fragment of the 5′ upstream region covering up to the translation start codon of the *ppt1* gene.

Complementation of the null mutant.

The complete *ppt1* gene, including the 5′ and 3′ flanking regions, was amplified by PCR with oligonucleotides 5′ forward primer *ppr1_A* and 3′ reverse primer *ppt1_D* (Table 1) using genomic DNA of the wild-type strain. The PCR conditions were an initial cycle at 95°C for 3 min; 30 cycles of 45 s at 94°C, 3 min at 55.7°C, and 1 min at 72°C; followed by a final cycle at 72°C for 7 min. The PCR product was cloned into TOPO PCR 2.1 (Invitrogen). The resulting plasmid, named ppt1-complement (7 kb), was used in co-transformation experiments using, as selectable plasmid, pCB1004 (FGSC), which carries a hygromycin resistance cassette, and protoplasts of the gene replacement mutant (Δ*ppt1-1*). Hygromycin-resistant transformants were then transferred to medium without lysine and siderophores and were subjected to three rounds of monosporic culture. These transformants were designated *T. virens* complemented (TvC). Confirmation of transformation was carried out by PCR amplification of the *ppt1* gene followed by Southern blot analysis

Phenotypic analysis of mutants.

Overexpressing strains, gene-replacement mutants, complemented strains, and Tv29.8 and Tv10.4; were grown in the dark on PDA, and radial growth of the colonies measured at 24, 48, and 72 h. To determine the effect of the manipulation of the *ppt1* gene in the production and development of conidiophores, all strains were grown in Vogel's medium and incubated at 28°C for 5 days with white light illumination. For analysis of conidial germination, the medium used was Grimm-Allen (Vittone 2008) with and without ferric chloride and ferrous sulfate.

Antibiosis.

For antibiosis tests, all strains were inoculated in plates containing PDA covered by a sterile cellophane membrane and incubated for 48 h in total darkness. The cellophane was removed together with the mycelium, an agar disk carrying mycelium of the indicated fungus placed on the antagonist-free medium, and the plates further incubated for 48 h in total darkness.

Determination of peptaibols.

T. virens strains were inoculated in VMS medium as described by Viterbo and associates (2007), with some modifications. In this case, fermentation was allowed to proceed for 9 days with constant agitation and sucrose as carbon source to 1.5%. Mycelium was harvested and lyophilized. In total, 250 µl of mixture A (methanol, water, an acetonitrile in a 1:1:1 ratio) or mixture B (5% acetonitrile and 0.1% formic acid) was added to 5 mg of dry mycelia. The suspension was homogenized for 15 min in vortex, with subsequent sonication for 15 min. The mixture was then centrifuged at 12,000 rpm for 15 min and the supernatant collected and concentrated in a vacuum evaporation system to a final volume of 50 µl. The extract (10 µl) was taken and passed through a C18 micro-column (Zip Tip, Millipore, Bedford, MA, U.S.A.) as recommended by the supplier, and eluted with 5 µl of a mixture of solvents (60% acetonitrile and 1% formic acid), for ESI-QTOF analysis.

Root colonization.

Solanum lycopersicum var. Rio Grande (Emerald) seeds were surface disinfected and germinated on water agar (1.5%) in a climate chamber at 25°C for 3 days with a photoperiod of 16 h of light and 8 h of darkness. Germinating seeds were then transferred to 150-mm-diameter petri dishes (three per plate), containing 0.5% Murashige and Skoog medium (Murashige and Skoog basal salts mixture; Sigma-Aldrich, St. Louis) and 0.5% agar, placed at a 65-degree angle to prevent root burial into the medium. Seelings were incubated at 25°C until the development of two true leaves. A disk of mycelium of strains Tv29.8, Tv10.4, Δ*ppt1*, and TvC was placed near the root area. Media were supplemented with 10 mM lysine or 2 mM arginine in the case of the Δ*ppt1* mutant and the Tv10.4 strain, respectively. The root system overgrown by *Trichoderma* spp. was collected with the help of a scalpel. Samples were placed in two dyes, one that stains the nuclei of root cells (propidium iodide to 20 µg/ml) and one that stains chitin in fungal cell wall (WGA Alexa Flour 488; 10 µg/ml) (Invitrogen). Samples were visualized at the National Institute of Neurobiology Campus Juriquilla, Mexico, in a Nikon Eclipse E-600 PCM 2000 confocal microscope. Images were obtained with the ×40 objective.

Isolation of *T. virens* from roots of *S. lycopersicum*.

According to the methodology described above, seedlings once colonized in vitro by *Trichoderma* spp. were transplanted into sterile soil, and allowed to grow for an additional 15-day period. The roots were then collected, fragmented, and placed on Rose Bengal selective medium for the isolation of *Trichoderma* spp. (Ahmed et al. 1999). In the case of roots colonized by auxotrophic strains, lysine or arginine was added to the medium. Root fragments were incubated at 28°C until the appearance of colonies. Genomic DNA was extracted from each of the recovered colonies, as reported by Raeder and Broda (1985), to detect the strains, and analyzed by PCR using primers *ppt1_G* and *ppt1_H* (Table 1).

Seed protection assays.

One-liter polypropyline pots containing sterile soil were inoculated with *R. solani* according to Brunner and associates (2005). Seeds were surface sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) sodium hypochlorite for 7 min, followed by five washes in distilled water. Ten *S. lycopersicum* seeds were placed per pot, with three replicates per treatment. Four discs of mycelium of the indicated *T. virens* strain were added near the area where the seeds were sown. Seeds were incubated in a growth chamber at 28°C until the emergence of two true leaves. The results were validated with analysis of variance statistical analysis with a Tukey-Kramer multiple comparison test ($\alpha = 0.05$), using the Statistical Analysis and Graphics software package (version NCSS 2007).

***Arabidopsis* co-cultivation experiments.**

A. thaliana Columbia-0 (Col-0) ecotype wild type, a transgenic line carrying a JA-inducible *pLox2:uidA* construct (Schommer et al. 2008), and a transgenic line carrying an SA-inducible *pPr1a:uidA* construct (Shah et al. 1997) were used throughout this work. Seeds were surface sterilized as described above, germinated, and grown on agar plates containing 0.2× Murashige and Skoog medium. Plates were settled 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 Percival AR95L growth chamber with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 200 µmol m^2 s^{-1}, and temperature of 24°C.

***Arabidopsis* inoculation experiments.**

The *T. virens* 29.8 and Δ*ppt1*-1 mutant strains were evaluated in vitro for their ability to elicit defense responses in *A. thaliana*. Fungal spore densities of 1×10^6 conidia were inoculated by placing a drop of a spore suspension at 4 cm in the opposite ends of agar plates containing 4-day-old germinated *Arabidopsis* seedlings (10 seedlings per plate). Plates were arranged in a completely randomized design. The seedlings were cultured for six additional days in a Percival AR95L growth chamber. The percentages of primary roots colonized by *T. virens* were determined with a ruler by measuring the primary root length and the surface covered by fungal hyphae. In the fungal co-cultivation experiments, the Murashige and Skoog 0.2× medium was supplemented with 300 µM lysine to allow fungal growth.

Histochemical analysis.

For histochemical analysis of GUS activity, *Arabidopsis* seedlings were incubated 12 to 14 h at 37°C in a GUS reaction buffer (5-bromo-4-chloro-3-indolyl-b-D-glucuronide at 0.5 mg/ml in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 20 transgenic plants were analyzed. A representative plant was chosen and photographed using a Leica MZ6 stereomicroscope.

SA and JA extraction and measurement.

The SA and JA extraction and determination were performed in *A. thaliana* (ecotype Col-0) shoots at 8 days after co-cultivation with *Trichoderma* spp. in vitro. For sample preparation, plants were sectioned at the root/shoot interface. Plant tissues were frozen and ground in liquid nitrogen. Each sample (300 mg) was placed in a polypropylene microtube, homogenized with 500 µl of isopropanol/H_2O/concentrated HCl (2:1:0.002, vol/vol), supplemented with 200 ng of orto-anisic acid (OA) (Sigma-Aldrich) as internal standard for SA, and shaken for 30 s. The tubes were centrifuged at 11,500 rpm for 3 min. The supernatants were collected and subjected to SA extraction with 200 µl of dichloromethane. SA and JA were derivatized with acetyl chloride in methanol (1 ml per 250 µl), sonicated for 15 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25 µl of ethyl acetate for GC-MS analysis. A retention time and selected ion monitoring (SIM) were established for SA-methyl ester (ME) (2.3 min, *m/z* 152, respectively), OA-ME (3.2 min, *m/z* 166), and JA-ME (7.5 min, *m/z* 224). JA was quantified by comparison with a standard curve obtained by using purified methyl jasmonate (Sigma-Aldrich).

Camalexin determination.

Camalexin was extracted from leaves of wild-type *A. thaliana* seedlings 8 days after *T. virens* inoculation. Camalexin levels were determined as described by Glazebrook and Ausubel (1994) and GC-MS analysis performed. For GC-MS analysis, 100 mg per sample of shoot material was submerged in 800 µl of methanol and kept at 80°C for 20 min. The supernatant was transferred to a vial, evaporated under a stream of nitrogen, and redissolved in 10 µl of methanol for GC-SIM-MS analysis. The volume of injected sample was 2 µl. The molecular ions with *m/z* 58, 142, and 200 $[M]^+$ were monitored to verify the presence of camalexin in the sample. Camalexin (retention time 18.44 min) was quantified with a standard curve, using purified camalexin provided by J. Glazebrook (University of Minnesota) and dissolved in 100 µl of methanol for chemical analysis.

MS analysis.

Identification and determination of all investigated compounds were performed using a GC-MS system. The samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and a 5% phenyl methyl silicone capillary column, 30 m by 0.2 mm by 0.25 mm (HP-5 MS). Operating conditions used helium 1 ml min^{-1} as carrier gas, detector temperature of 300°C, and injector temperature of 250°C. The column was held for 3 min at 80°C and programmed at 6°C min^{-1} to a final temperature of 230°C for 5 min.

Bioassays for *Trichoderma* spp.-induced resistance against *B. cinerea*.

To test plant protection conferred by the wild-type and Δ*ppt1 T. virens* strains against *B. cinerea, A. thaliana* seedlings were inoculated with a fungal density of approximately 1×10^6 spores by placing the spores at 1 cm at the opposite ends of agar plates containing 12-day-old germinated seedlings (10 seedlings per plate). *Trichoderma* spp. were co-cultivated for 6 days with the plant to elicit defense responses by the physical contact of the mycelium with the root system. *A. thaliana* shoots were inoculated with a density of *B. cinerea* spores of 1×10^6, distributing drops of the inoculum over leaf surfaces. Induced disease resistance in *A. thaliana* seedlings was evaluated 3 or 5 days after pathogen inoculation by scoring symptomatic leaves or percentage of dead plants, respectively, for a total of 30 plants. Plants were placed in a plant-growth chamber with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 200 µmol m^2 s^{-1}, and temperature of 24°C.

Data analysis.

Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyzes with a Tukey's post hoc test were used for testing differences in the biochemical analysis for SA, ICAld, camalexin measurements, number of lesions, and percentage of dead plants in wild-type *A. thaliana*. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Joint Genome Institute *T. virens* genome webpage:
genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html