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Análisis Molecular de la Interacción Microorganismo Benéfico-Planta en dos Patosistemas: *Trichoderma*-Arabidopsis y *Trichoderma*-Tomate

Tesis que presenta

Miguel Ángel Salas Marina

Para obtener el grado de

Doctor en Ciencias en Biología Molecular

Director de la Tesis:

Dr. J. Sergio Casas Flores

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Constancia de aprobación de la tesis

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RESUMEN

Las plagas y las enfermedades de las plantas, se encuentran entre los principales factores que contribuyen a la pérdida de cultivos. El abuso en la utilización de compuestos químicos para el control de estas enfermedades, ha favorecido que los patógenos desarrollen resistencia a estos compuestos. Para reducir el impacto en el uso de los fungicidas, los microorganismos antagónicos de los fitopatógenos representan una alternativa viable para la supresión de las enfermedades.

El presente trabajo de investigación se enfocó en estudiar el dialogo molecular entre plantas modelo y hongos promotores del crecimiento de las plantas. Utilizando cepas transformantes de *Trichoderma atroviride* que expresan a la proteína verde fluorescente demostramos que este hongo es capaz de penetrar y colonizar las raíces de *Arabidopsis thaliana,* cuyo efecto se vio reflejado en la promoción del crecimiento de la planta, así como en la inducción de la resistencia sistémica contra los fitopatógenos *Pseudomonas syringae* y *Botrytis cinerea*. Así mismo, demostramos que la colonización de *Arabidopsis* por *T. atroviride* indujo la expresión de genes relacionados con la resistencia sistémica adquirida (SAR), la resistencia sistémica inducida (SIR) y con la síntesis de camalexina.

En un segundo capítulo, se analizó el papel de los genes *sm*-1 de *T. atroviride* y *T. virens* en la inducción de la respuesta sistémica en plantas de tomate. El gen *sm*-1 codifica para la proteína SM-1, una molécula inductora de la respuesta sistémica en plantas. En el presente trabajo demostramos que las cepas de *T. atroviride* y *T. virens* que sobreexpresan el gen *sm*-1 (OE) indujeron mayor resistencia sistémica en plantas de tomate contra *Alternaria solani*, *B. cinerea* y *P. syringae* comparadas con aquellas plantas inoculadas con las cepas silvestres (WT) o con las cepas mutantes nulas (KO). Así mismo, las plantas de tomate tratadas con las cepas OEs presentaron mayores niveles de inducción de genes relacionados con la SAR y la SIR, al compararlas con sus respectivas cepas WT o KO.

En este trabajo demostramos por primera vez que *Trichoderma* spp. indujo un grupo de genes relacionados con la SIR y la SAR en plantas de tomate y *Arabidopsis*.

Palabras clave: resistencia sistémica, patógenos, camalexina

ABSTRACT

Plants pests and diseases are among the most important factors that produce economic lost on important crops. The intensive uses of pesticides to control diseases have provoked an increased resistance of microorganisms against such chemicals. To reduce the use of pesticides, the beneficial microorganisms show an alternative to control the plant diseases.

This research was focused on the study of the molecular communication between model plants and plant growth promoting fungi. By means of a *T. atroviride* transformant that constitutively express the green fluorescent protein we demonstrated that this fungus is able to penetrate and colonize the *Arabidopsis* roots and that such effect on root colonization allow to an increased in plant growth. In addition, we showed that inoculated of plants with *Trichoderma* increased their resistance against the foliar pathogens *Pseudomonas syringae* and *Botrytis cinerea*. Furthermore, we showed that the *Arabidopsis* colonization by *Trichoderma* induced the level expression of a set genes related to the systemic acquired resistance, induced systemic resistance and camalexin synthesis.

In a second chapter, we analyzed the role of the *sm*-1 genes of *T. atroviride* and *T. virens* on the induction of systemic resistance against phytopathogens during their interaction with tomato seedlings. The *sm*-1 gen encodes for the SM-1 protein, which has been classified as an elicitor of the systemic resistance in plants. Here, we showed that tomato plants inoculated with the *T. atroviride* and *T. virens sm*-1-overexpressing strains increased their resistance against the foliar pathogens *Alternaria solani*, *B. cinerea* y *P. syringae* when compared with the mocked plants or seedlings inoculated with the wild type (wt) or knockout strains (KO). Besides, we found that inoculated plants with the over-expressing strains showed higher transcription levels of the defense related genes to SAR and ISR pathways, when compared with respective WT or KO.

This is the first report showing that *Trichoderma* induces SAR and ISR defense related genes in tomato and *Arabidopsis* plants and that SM-1 is an elicitor of such responses in tomato seedlings

Key words: systemic resistance, pathogens, camalexin

INTRODUCCIÓN

Las plantas son organismos sésiles que constantemente están interactuando con microorganismos, donde muchos de estos pueden invadir el interior de la planta penetrando directamente la superficie de la hoja, de la raíz, o a través de heridas o de aperturas naturales como los estomas; esto indica que desde que las plantas comenzaron a colonizar la tierra han estado en contacto con los microorganismos y juntos han co-evolucionado (Gehrig *et al*., 1996), de esta manera todos los organismos vivientes evolucionan continuamente adquiriendo diversas habilidades adaptativas que son requeridas para sobrevivir dentro de su medio ambiente. La condición de inmovilidad de las plantas y la falta de un sistema inmune adaptativo como el de los humanos, las han llevado a desarrollar una inmunidad específica de defensa para reconocer agentes invasores y minimizar el daño provocado por estos (Ausubel, 2005; Jones y Dangl, 2006). La habilidad para detectar e inducir una respuesta de defensa contra un microorganismo patógeno ha llevado a descubrir que las plantas poseen diversos mecanismos de defensa constitutivos e inducibles contra diversos patógenos como virus, bacterias, hongos, nemátodos, insectos, entre otros (Heath, 2000; Pieterse *et al.,* 2009).

Los bacterias, hongos y oomycetos patógenos que atacan a las plantas pueden ser divididos en tres clases: necrotróficos (los que matan al hospedero y se alimentan de él), biotróficos (estos requieren vivir en el hospedero para completar su ciclo de vida) (Dangl y Jones, 2001; Glazebrook, 2005) y el tercer grupo incluye a los hemibiotróficos, los cuales una parte de su ciclo de vida se desarrollan como biotrófos para posteriormente pasar a ser necrótrofos (Tons *et al*., 2009).

Durante la interacción planta-microorganismo, la primera línea de defensa de las plantas son las barreras estructurales preformadas constituídas por compuestos de la pared celular, tales como la celulosa, la hemicelulosa, ligninas y pectinas (Hammond-kosack y Jones, 1996; Dangl y Jones, 2001). Ante esta barrera, los hongos y las bacterias patógenos secretan enzimas que degradan la pared celular de las plantas como las pectinasas, xilanasas y celulasas, que además de su actividad hidrólitica, se han descrito por parte de las plantas como moléculas inductoras del sistema de defensa mejor conocidas como patrones moleculares asociados a microbios y a patógenos (MAMPs y PAMPs por sus siglas en ingles) (Calderón *et al*., 1994; Avni *et al*., 1994). Los PAMPs y MAMPs son reconocidos por las plantas a través de receptores que tienen un patrón de reconocimiento (PRRs por sus siglas en ingles) y estos desencadenan una cascada de señalización que lleva a una respuesta basal conocida como una inmunidad inducida por PAMPs (PTI por sus siglas en ingles) (Pieterse *et al.,* 2009; Uchida y Tasaska, 2010). Sin embargo, hay patógenos que son capaces de suprimir esta primera linea de defensa a través de la inyección de efectores que suprimen la respuesta PTI y producen una infección (Uchida y Tasaska, 2010), ante esta situación las plantas presentan otras proteínas que tienen un sitio de unión a nucleótidos y un dominio rico en repetidos de leucinas (NB-LRRs por sus siglas en ingles) que reconocen estos efectores e inducen una respuesta de defensa más fuerte que las PTI, frecuentemente induciendo la respuesta hipersensible HR y esta se caracteriza por una necrosis local en el sitio de infección (Mur *et al.,* 2008). Cuando una planta llega a ser infectada por un patógeno biotrófico, desarrolla una resistencia contra patógenos conocida como resistencia sistémica adquirida (SAR,

por sus siglas en Inglés), cuya hormona señalizadora es el ácido salicílico (AS) (Dong, 2001; Durrant y Dong, 2004). Otro tipo de respuesta mediada por patógenos necrotróficos, por bacterias y hongos promotores del crecimiento, es conocida como respuesta sistémica inducida (ISR, por sus siglas en Inglés), cuyas moléculas señalizadoras son las hormonas como el ácido jasmónico (AJ) y el etileno (ET) (Pieterse *et al*.,1998).

En el grupo de microorganismos capaces de colonizar e inducir los sistemas de defensa en las plantas, se encuentra un grupo de rizobacterias y hongos promotores del crecimiento de las plantas (por sus siglas en Inglés: PGPR y PGPF respectivamente). Para las bacterias promotoras del crecimiento se han descrito las pertenecientes a los generos *Frankia*, *Streptomyces*, *Bacillus*, *Pseudomonas* entre otros. Respecto a los hongos, se han reportado los géneros *Trichoderma*, *Penicillium*, *Fusarium*, *Phoma* y *Phytium* (Glick, 1995, Larkin y Fravel, 1999; Koike *et al*., 2001). El control de los fitopatógenos por PGPR y PGPF puede involucrar la producción de enzimas antimicrobianas, antibiosis, micoparasitismo y la inducción de la SIR (Shivanna *et al*., 1996; Whipps, 2001). La inducción de la ISR por estos microorganismos se debe a que pueden producir MAMPs o PAMPs tales como: oligosacáridos, péptidos, lipopolisacáridos, sideróforos, flagelinas, elicitinas y micotoxinas (Hahn, 1996; Keller *et al*., 1996; De Meyer y Hofte, 1997; Dow *et al*., 2000; Hennin *et al*., 2001; Pieterse *et al.,* 2009). Adicionalmente, algunos de estos hongos y bacterias promotoras del crecimiento mejoran los cultivos debido a que incrementan la absorción de nitrógeno, solubilizan los fosfatos, modifican el crecimiento y desarrollo de las plantas debido a que contrarrestan a los patógenos de suelo, secretan fitohormonas como auxinas, citocininas y giberelinas, y pueden

modificar la homeostasis de estas hormonas dentro de la planta (Patten y Glick, 1996; Lugtenberg y Kamilova, 2009).

Debido a la importancia que tienen los microorganismos promotores del crecimiento durante su interacción con las plantas, surge la inquietud de estudiar microorganismos ya descritos que causan este efecto, así como identificar nuevos hongos y bacterias que promuevan el crecimiento e induzcan los sistemas de defensa en las plantas. La presente tesis estuvo enfocada en estudiar el diálogo molecular de los hongos promotores del crecimiento de las planta; *Trichoderma atroviride* y *T. virens* durante su interacción con las plantas modelo; *Arabidopsis thaliana* y tomate (*Lycopersicum esculentum*) y por lo tanto los objetivos fueron los siguientes.

OBJETIVOS

OBJETIVO GENERAL

Estudiar el diálogo molecular del hongo *Trichoderma spp.* con las plantas modelo *Arabidopsis thaliana* y Solanum *lycopersicum*.

OBJETIVOS ESPECIFICOS DEL CAPITULO 1

1. Generar cepas transformantes de *T. atroviride* que expresen la proteína verde fluorescente para evaluar el proceso de colonización.

2. Determinar si la colonización de raíces de *Arabidopsis* por *T. atroviride* promueve el crecimiento de las plantas.

3. Determinar si la colonización de raíces de *Arabidopsis* por *T. atroviride* induce resistencia sistémica contra hongos y bacterias patógenas

4. Determinar si la colonización por *T. atroviride* induce la expresión de genes de defensa de las vías de señalización ISR, SAR y síntesis de fitoalexinas en plantas de *Arabidopsis*

OBJETIVOS ESPECIFICOS DEL CAPITULO 2

1. Generar cepas que expresen diferentes niveles del gen *sm-1* (OE) en los hongos *T. atroviride* y *T. virens*
2. Generar cepas mutantes del gen *sm-1* (KO) en los hongos *T. virens y T. atroviride.*
3. Evaluar el efecto ”protector” de las cepas transformantes y mutantes de *Trichoderma* en plantas de tomate contra *Pseudomonas syringae, Botrytis cinerea* y *Alternaria solani.*
4. Determinar el efecto de las cepas OE y KO en la inducción de genes de defensa en plantas de tomate.

REVISIÓN DE LITERATURA

Microorganismos que promueven el crecimiento e inducen el sistema de defensa de las plantas

Las hormonas de las plantas (auxinas, citocininas, giberelinas, etileno, etc.) son un grupo de substancias orgánicas producidas de manera natural, que actúan a bajas concentraciones y regulan procesos fisiológicos tales como: el crecimiento, la diferenciación, el desarrollo y otros procesos como la apertura y el cierre de estomas (Benfey, 2002; Bari y Jones, 2009). El concepto de hormona fue definido como una sustancia que es sintetizada en una parte y que puede tener un efecto *in situ* o en una región alejada de su sitio de síntesis (Pieterse *et al*., 2009). La síntesis de las hormonas de las plantas puede ser localizada o puede ocurrir en un amplio rango de tejidos o células dentro de un tejido (Pieterse *et al.,* 2009; Kazan y Manners, 2009).

Las hormonas funcionan en una red organizada entre las señales ambientales y fisiológicas y las rutas de estas respuestas pueden ser disparadas por cambios en los niveles de estas hormonas variando dramáticamente a través de los tejidos y de la edad de la planta formando gradientes que son un componente central de su acción (Pieterse *et al*., 2009). Se infiere que estos cambios se deben a que las plantas han adquirido rutas regulatorias de considerable plasticidad, redundantes y adaptativas para mantener los niveles de cada una de las hormonas en respuesta a los cambios ambientales y fisiológicos. Este fenómeno es conocido como homeostasis hormonal y se lleva a cabo específicamente en los procesos de biosíntesis, inactivación, transporte e inter-conversión que regulan los niveles de estas hormonas (Benfey, 2002; Berleth *et al.,* 2000).

Los cambios fisiológicos de las plantas no son únicamente modificados por las hormonas producidas por ellas mismas, sino también, por microorganismos colonizadores de las raíces y productores de sustancias que estimulan el crecimiento de las plantas en ausencia de patógenos. El mejor ejemplo es la hormona auxina producida por bacterias promotoras del crecimiento de las plantas (Lugtenberg y Kamilova, 2009). Algunas bacterias también producen otras hormonas volátiles que estimulan el crecimiento como la pirrolquinolina quinona (PQQ) (Lugtenberg y Kamilova, 2009). Estas hormonas producidas por las bacterias son sintetizadas a partir de los exudados del aminoácido triptófano de la raíz de la planta. Es importante mencionar que la concentración del exudado entre plantas no es la misma (Lugtenberg y Kamilova, 2009). Se ha estimado que cerca del 80 % de las bacterias del suelo tienen la capacidad de producir IAA y estas pueden ser patógenas y no patógenas (Glick *et al*., 1999). Entre estas bacterias tenemos a *Agrobacterium tumefasciens, Pseudomonas syringae* pv. *savastanoi*, *Pseudomonas fluorescens* y *Azotobacter paspali* (Patten y Glick, 2002). Además de las bacterias también se han reportado hongos que producen auxinas, entre estos tenemos a: *Rhizopus, Phycomyces, Pythium, allomyces arbuscula, Absidia ramosa, Taprina, Ceratocystis, Nectria, Giberella, Aspergillus, Penicillium, Saccharomyces, Ustilago, Puccinia, Agaricus, Fusarium* (Bowen y Rovira, 1999); recientemente se ha reportado que hongos del género *Trichoderma* también las producen (Contreras *et al*., 2008).

Otras hormonas que son sintetizadas y secretadas por los microorganismos durante la interacción con plantas son las citocininas y las giberelinas. Entre las

bacterias que producen estas hormonas se encuentran: *Corynebacterium fascinas*, *A. tumefaciens*, *Azospirillum, Burkholderia, Erwinia, Xanthomonas, Arthrobacter, Methylobacterium, Rhizobium*, *Bacillus, Acetobacter* y *Pseudomonas*. Los hongos micorrizicos son: *Rhizopogon roseolus*, *Boletus edulis* var. *pinicolus* y también otros hongos como, *Taphrina*, *Nectria galligena*, *Exobasidium*, *Gibberella fujikuroi, Phaeospheria* sp. *Sphaceloma* sp. *Monilia fructicola, Mesophilicum* y *Pisolithus tinctorius*. (Greene, 1980; Macmillan, 2002; Garcias de Salomone *et al*., 2005; Yamaguchi, 2008).

Además de los microorganismos benéficos, también existen aquellos microorganismos que al interactuar con las plantas causan enfermedades de muchos cultivos agrícolas. En forma particular, los patógenos de suelo (*Phytium*, *Phytophthora*, *Botrytis*, *Rhizoctonia* y *Fusarium)* causan importantes pérdidas económicas (Harman *et al.,* 2004; Benítez *et al.,* 2004). Además, el efecto de algnunos patógenos como *Fusarium* spp. y *Asperigillus* spp. no solo se han reportado en campo sino también en alimentos almacenados (Benítez *et al*., 2004).

El uso de compuestos químicos ha sido utilizado para el control de las enfermedades de las plantas (control químico), pero el abuso en el uso de estos compuestos ha favorecido que los patógenos desarrollen resistencia a los pesticidas. Desafortunadamente existen pesticidas de muy amplio espectro que no solo afectan a los patógenos, sino también a microorganismos benéficos de las plantas. Para reducir el impacto del uso de los fungicidas, el utilizar microorganismos antagónicos de los fitopatógenos de las plantas (control biológico) son una alternativa para la supresión de las enfermedades, evitando el

uso de los fungicidas (Tjamos *et al*., 1992; Monte, 2001). Entre los microorganismos para usar como una alternativa encontramos hongos del genero *Trichoderma* spp.

Trichoderma spp.

La mayoría de las cepas pertenecientes a este género han sido clasificadas como hongos imperfectos debido a que no se les conoce un estado sexual. Sin embargo, algunas especies de *Trichoderma* son morfológicamente similares al anamorfo *Hypocrea* y su espaciador de secuencia intergenica ribosomal (ITS) ha revelado su proximidad taxonómica (Monte, 2001). Las especies más comunes de *Trichoderma* usadas en el control biológico son *T. virens, T. viride, T. atroviride* y *T*. *harzianum* (Grondona *et al*., 1997). El éxito de las cepas de *Trichoderma* como agentes de control biológico se debe a la capacidad que presentan para modificar la rizosfera, a la actividad micoparasítica que tienen contra fitopatógenos, a la eficiencia en promover el crecimiento de las plantas e inducir mecanismos de defensa. También tienen una alta capacidad reproductiva, ya que crecen tanto en suelos ácidos, como alcalinos, tienen habilidad para sobrevivir bajo condiciones desfavorables y presentan una alta eficiencia en la utilización de los nutrientes (Harman *et al.,* 2004).

Micoparasitismo y antibiosis

El micoparasitismo se define como el ataque directo de un hongo a otro, en este proceso *Trichoderma* crece hacia el huésped, se adhiere través de los carbohidratos de la pared celular que se unen a las lectinas de los patógenos y

forma estructuras tipo apresorio, las cuales sirven para penetrar al hospedero (Harman *et al*., 2004; Benítez *et al*., 2004), los pasos siguientes son degradación de la la pared celular del hospedero por la acción de enzimas líticas producidas por *Trichoderma* como: quitinasas, glucanasas y proteasas (Howell, 2003; Harman *et al*., 2004).

La investigación sobre las rutas de señalización responsables del proceso de micoparasitismo de *Trichoderma,* ha conducido al aislamiento de componentes claves de las rutas de señalización del cAMP y las MAP cinasas, donde las MAP cinasas regulan negativamente la expresión de genes relacionados con el micoparasitismo (Mendoza *et al.,* 2003) y las proteinas G (G-α) mediadas por cAMP controlan la síntesis de enzimas extracelulares, la producción de antibióticos y el enrollamiento de las hifas hospedero (Omero *et al*., 1999). Por otro lado, cepas sobreexpresantes del gen de la subunidad α de la proteína G (*tga1)* en *T. atroviride,* presentaron un incremento en el enrollamiento de las hifas y en el micoparasitismo contra *R. solani* (Rocha-Ramírez *et al*., 2002). Por el contrario, cepas mutantes del gen *tga3* de la subunidad α de la proteina G fueron avirulentas contra *R. solani* (Zeilinger *et al*., 2005). Otro gen involucrado en el micoparasitismo es *vel1* de *T. virens*, ya que recientemente se reportó que este gen es un regulador maestro que controla la expresión de genes involucrados en la síntesis de antibióticos, en el proceso de micoparasitismo y de la inducción del sistema de defensa en plantas de algodón (Mukherjee y Kenerley, 2010).

Antibiosis

La antibiosis ocurre durante la interacción entre *Trichoderma* y el patógeno, proceso que involucra la producción por *Trichoderma* de compuestos difusibles de bajo peso molecular o antibióticos que inhiben el crecimiento de otros microorganismos. Muchas cepas de *Trichoderma* producen metabolitos tóxicos volátiles y no volátiles que inhiben el crecimiento de los microorganismos patógenos; entre estos metabolitos tenemos el ácido harziánico, alamethicinas, tricholinas, peptaiboles, antibioticos (gliovirina y gliotoxina), 6-pentil-α-pirona, massoilactona, viridina, gliovirina, glisopreninas, ácido heptelídico entre otros (Benítez *et al*., 2004). En este sentido, cepas de *T. virens* (GV-P) que sobreproducen al antibiótico gliovirina presentaron igual control que la silvestre, pero cepas mutantes en la síntesis de gliovirina fueron deficientes en controlar al oomiceto *Phytium ultimun* (Chet *et al*.,1997). Por otro lado, mutantes de *T. virens* (G22, G151) deficientes en la producción del antibiotico gliotoxina fueron tan eficiente como la cepa parental en controlar al hongo patogeno *Phytium* (Wilhite *et al.,* 2004; Howell y Stipanovic**,** 1995). También se ha observado en ensayos *in vitro* que la combinación de enzimas hidrolíticas y antibióticos (endoquitinasa y gliotoxina, o endoquitinasa y petaiboles) resulta en un mayor nivel de antagonismo que el obtenido para cada uno de los mecanismos de forma independiente (Howell, 2003). Una mutante de *T. harzianum* que expresa altos niveles de enzimas extracelulares y de α-pirona presentó un mejor control contra *R. solani in vitro* comparada con la cepa silvestre (Rey *et al*., 2001).

Modificación de la rizosfera

Un mecanismo que ha atraído la atención de los investigadores en años recientes es la competencia por colonizar la rizosfera, ya que un buen agente de biocontrol es el que pueda colonizar la rizosfera de las plantas y que además sea un buen competidor por espacio y nutrientres comparado con los otros microorganismos del suelo. Las especies de *Trichoderma* se adhieren al suelo o a las semillas tratadas, donde el hongo crece fácilmente conforme el sistema radicular de las plantas tratadas se van desarrollando (Harman, 2000; Howell *et al*., 2000). Este efecto se puede observar colocando fragmentos de raíces esterilizados de plantas que previamente fueron inoculadas con *Trichoderma* sobre medio agar y después de un periodo de incubación se puede observar el crecimiento del hongo emergiendo de todas las partes de las raíces. Algunas especies de *Trichoderma* pueden colonizar las raíces de manera localizada, todo el sistema radicular o en algunos caso toda la planta como la hace *T. stromaticum* (Metcalf y Wilson, 2001; Evans *et al*., 2003). Durante este proceso de colonización, *Trichoderma* sufre cambios morfológicos parecidos a los observados durante el micoparasitismo, pero estas estructuras de colonización son usualmente limitadas a las primeras o segundas capas celulares de la raíz (Yedidia *et al*., 2000).

La habilidad de *Trichoderma* para colonizar y controlar a los patógenos, se debe en gran parte a su gran versatilidad y dinámica para crecer en un amplio espectro de pH, esto se debe a que este hongo puede modificar su ambiente externo acidificando el sustrato donde crece a un pH adecuado para que su maquinaria enzimática funcionen mejor (Benitez *et al*., 2004). En este sentido, los niveles transcripcionales de varias proteasas, glucanasas, proteínas de pared celular y

transportadores de glucosa tanto de *Trichoderma* como de algunos patogenos son controlados por pH (Prusky y Yakoby, 2003). De esta manera la modificación del pH externo determina la habilidad de *Trichoderma* o del patógeno para colonizar e invadir a su hospedero. Un sistema de respuesta sensible a pH que probablemente evolucionó para propiciar al hongo, un mejor ambiente de crecimiento, es el regulado por la proteína PACC, un activador transcripcional de genes de respuesta alcalina y represor de genes de respuesta a condiciones ácidas (Arst *et al*., 2003). La regulación de genes por estos factores de transcripción han sido identificados en muchos hongos incluyendo *Trichoderma*. En *T. harzianum* la cepa mutante del gen *pac1* (cepa R13) en condiciones de pH 5.5 crecieron mas lentas y fueron incapaces de sobrecrecer a hongos patogenos como *R. solani, R. meloni* y *P. citrophthora*, mientras que la cepa complementada con el gen (cepa P2.32) fue mas eficiente en parasitar a estos patogenos (Moreno *et al*., 2007).

En general se ha encontrado que *Trichoderma* es muy resistente a una variedad de toxinas y compuestos xenobióticos, incluyendo antibióticos producidos por otros microorganismos, compuestos antimicrobianos de la planta y a fungicidas químicos (Harman *et al*., 1996). Las bases moleculares para que *Trichoderma* sea más resistente que los patógenos a ambientes toxicos se debe a que *Trichoderma* presenta transportadores con “casset” de unión a ATP (ABC). Cepas mutantes de *T. atroviride* en el gen *Taabc2* que codifica para un transportador ABC fueron muy susceptibles a la presencia de fungicidas, de fuentes de carbono como quitina y glucosa y a toxinas de *B. cinerea, R. solani* y *P. ultimum* presentando un fenotipo de lento crecimiento (Ruocco *et al.,* 2008). Por otro lado, se identificó que la

proteína TASHYD1 de *T. asperellum* está involucrada en la colonización de la raíz ya que cepas sobreexpresantes de este gen no fueron afectadas en su actividad micoparasítica contra *R. solani,* ni en su habilidad para colonizar, pero cepas mutantes de este gen fueron menos capaces para adherirse a las raíces y a la colonización de las mismas (Viterbo y Chet, 2006).

En el año 2008 Brotman y colaboradores, caracterizaron el gen *tasswo* de *T. asperellum* que codifica para una proteína tipo expansina llamada "swollenina", donde se observo que cepas sobreexpresantes o cepas mutantes presentaron una mayor o menor grado de colonización de las raíces de plantas de pepino respectivamente (Brotman *et al.,* 2008). Por otro lado, cepas mutantes de la proteina ThPG1 (endopoligalacturonasa) crecieron más lentas sobre medio suplementado con pectina y redujeron su capacidad de colonizar las raíces de plantas de tomate (Moran *et al*., 2009).

Incremento del desarrollo de las raíces y crecimiento de la planta

La colonización de las raíces por cepas de *Trichoderma* frecuentemente incrementan el crecimiento y desarrollo de las raíces, productividad de los cultivos, resistencia a estrés abiótico y la absorción y uso de nutrientes (Harman *et al*., 2004; Yedidia *et al*., 2001). Estas caracteristicas de que *Trichoderma* pueda mejorar los cultivo se debe a que *Trichoderma* acidifica su nicho secretando ácidos orgánicos como, el ácido glucónico, el ácido cítrico o ácido fumárico (Gómez y de la Torre, 1994) y estos ácidos orgánicos son capaces de solubilizar fosfatos, micronutrientes y cationes minerales incluyendo fierro, manganeso y

magnesio, que la hacen mas disponible a las plantas (Harman *et al*., 2004).

La productividad de los cultivos pueden incrementar hasta un 300 % en experimentos en invernadero después de que las semillas o las plantas son inoculadas con *T. hamatum* o *T. koningii* (Howell, 2000; Benítez *et al.,* 2004). Por otro lado, cuando se realizó un experimento de interacción *Trichoderma*-semilla pero con la diferencia de que entre las semillas y el hongo se colocó una membrana de celofan se observo un efecto positivo en la germinación, este experimento indicó que *Trichoderma* produce factores que incrementan la velocidad de germinación de la semillas (Benítez *et al.,* 2004). Sin embargo, hay muy pocos reportes sobre cepas de *Trichoderma* que produzcan factores de crecimiento que hayan sido detectados e identificados en el laboratorio (auxinas, citocininas y etileno) (Arora *et al*., 1992). Cepas de *Trichoderma* que producen moléculas tipo citocininas (Zeatina) y giberelina (AG3) o moléculas relacionadas a AG3, han sido recientemente detectadas y la producción de estos compuestos podrían mejorar la biofertilización (Osiewacz, 2002; Benítez *et al*., 2004). Recientemente, Contreras y colaboradores en el 2009, demostraron que *T. virens* promueve el crecimiento de plántulas de *Arabidopsis* a través de la producción de compuestos relacionados a auxinas incluyendo, el acido indole-3-acetico, el indole-3-acetaldehido y el indole-3-etanol. Un análisis comparativo de estas moléculas tipo auxinas presentaron su eficiencia en promover el crecimiento y modular la arquitectura del sistema radicular y la activación de genes regulados por auxinas en *Arabidopsis* (Contreras *et al*., 2009).

Resistencia Sistémica Inducida (ISR) por *Trichoderma* spp.

La ISR inducida por *Trichoderma* ha sido pobremente estudiada comparada con la ISR inducida por bacterias ya que en *Trichoderma* se han estudiado mas los efectos de micoparasitismo y antibiosis. La primera demostración de ISR por este hongo se observó en plantas de frijol inoculadas con *T. harzianum* T-39 que presentaron mayor resistencia contra los patógenos, *B. cinerea* y *Colletotrichum lindemuthianum* (Bigirimana *et al*., 1997). Posteriormente, varios grupos de investigación extendieron sus descubrimientos usando diferentes especies de *Trichoderma*, en interaccion tanto en plantas monodicotiledones como en dicotiledoneas y utilizando diferentes patogenos foliares (De Meyer *et al*., 1998). Sin embargo, la ISR inducida por *Trichoderma* es más difícil estudiarla contra patógenos que causan enfermedades en las semillas y raíces, debido a que ambos tipos de organismos tanto los de biocontrol como los patógenos se encuentran en el mismo sitio (Harman *et al*., 2004). Uno de los ejemplos de la inducción de ISR se observó utilizando a *T. harzianum,* donde esta cepa proporcionó control tanto espacial como distal del punto de aplicación, en campos de tomate infectados de manera natural por *Alternaría solani* y el daño fue reducido sobre el follaje debido a la aplicación de *Trichoderma,* 100 días antes de la infestación. Aun se desconoce cuanto dura la ISR por este *Trichoderma*, sin embargo la inducción de la resistencia puede durar mientras el hongo crece junto con la raíz de la planta (Harman *et al*., 2004).

En estudios moleculares reportan que la activación de la respuesta de defensa inducida por *Trichoderma* durante la colonización de plantas de pepino, estuvo asociado con el incremento de la actividad enzimática de quitinasas y peroxidasas

en las plantas, asi como también presentaron mayor expresión en los genes fenilalanina ammonio liasa (PAL) e hidroxiperoxido liasa (HPL) tanto en raíces como en hojas (Yedidia *et al*., 2000, 2003). La inducción de la resistencia sistémica por *Trichoderma* ha sido reportada para plantas monocotiledóneas y dicotiledóneas, esta respuesta involucra el reconocimiento del hongo por la planta y la inducción de la resistencia sistémica inducida (ISR) es mediada por las fitohormonas acido jasmonico AJ y etileno ET; esta respuesta es la más cercana análogamente a la resistencia sistémica inducida por rizobacterias figura 1 (Baker *et al*., 2003; Van Loon, 2007). Además, se conoce que *Trichoderma* induce la expresión de genes PR que codifican para proteínas relacionados a patogenecidad, genes que su respuesta es mediada el acido salicílico (AS) y ésta respuesta ha sido conocida como resistencia sistémica adquirida (SAR), respuesta que también es inducida por patógenos biotróficos (Martínez *et al*., 2001). Sin embargo, recientemente se descubrió que hay una convergencia entre las rutas de respuesta SAR y ISR en el factor de transcripción NPR1 debido a que este en un co-activador transcripcional de genes PR dependiente de AS y ademas reprime la inducción de ISR atraves del acido salicilico (Koornneef y Pieterse, 2008). También hay evidencias que sugieren que durante la interacción entre la planta-*Trichoderma* hay un diálogo molecular generado por moléculas producidas por la planta y/o del microorganismo (MAMPs), en el cual *Trichoderma* induce la ISR dependiente de NPR1 para el priming de los genes de defensa como sucede con la respuesta inducida por rizobacterias como se ilustra en la figura 1 (Pozo *et al*., 2005, Van Wees *et al*., 2008). En este sentido plantas de pepino y algodón tratadas con cultivos filtrados de *Trichoderma* incrementaron las síntesis de

terpenoides y la inducción de genes de defensa en dichas plantas (Yedidia *et al*., 2000; Howell *et al*., 2000).

Hasta la fecha un gran número de inductores del sistema de defensa en plantas han sido caracterizados: proteínas con actividad enzimáticas, proteínas homólogos a Avr y compuestos de bajo peso molecular (Bailey, 1991, Baker, 1997). En *Trichoderma* se descubrieron que las proteínas (xilanasas y glucanasas) ademas de su actividad enzimática, también fueron descritas como inductores de la expresión de genes PR y a la producción de fitoalexinas en varias plantas (Calderón *et al.,* 1993; Martínez, 2001), También se han identificado las proteínas de los genes Avr en una gran variedad de hongos y bacterias patógenos de plantas, estos inductores regularmente funcionan de manera especifica planta-patovariedad y son capaces de inducir la respuesta hipersensible y de inducir genes relacionados a defensa (Harman *et al*., 2004). En un análisis proteómico de *T. harzianum* T-22 se identificaron proteínas que son homólogas a Avr4 y Avr9 de *Cladosporium fulvum*; y también se ha encontrado que *T. atroviride* (P1) produce proteínas similares (Harman *et al*., 2004).

Recientemente Djonovic y colaboradores identificaron y caracterizaron la proteína SM-1 de *T. virens*, la cual es producida y secretada por el hongo en la etapas tempranas de la interacción planta-patógeno, sugiriendo que esta proteína tiene un papel de señalización durante esta interacción. Por otro lado, la proteína SM-1 purificada eficientemente indujo la respuesta de defensa en plantas de algodón tanto de manera local, como sistémica contra el patógeno foliar *Colletotrichum* spp; la actividad protectora de SM-1 se asoció con la acumulación de especies reactivas de oxigeno (ROS), compuestos fenólicos y un incremento en los niveles

de transcripción de genes regulados por AS, así como genes involucrados en la biosíntesis de sesquiterpenos y fitoalexinas (Djonovic *et al*., 2006).

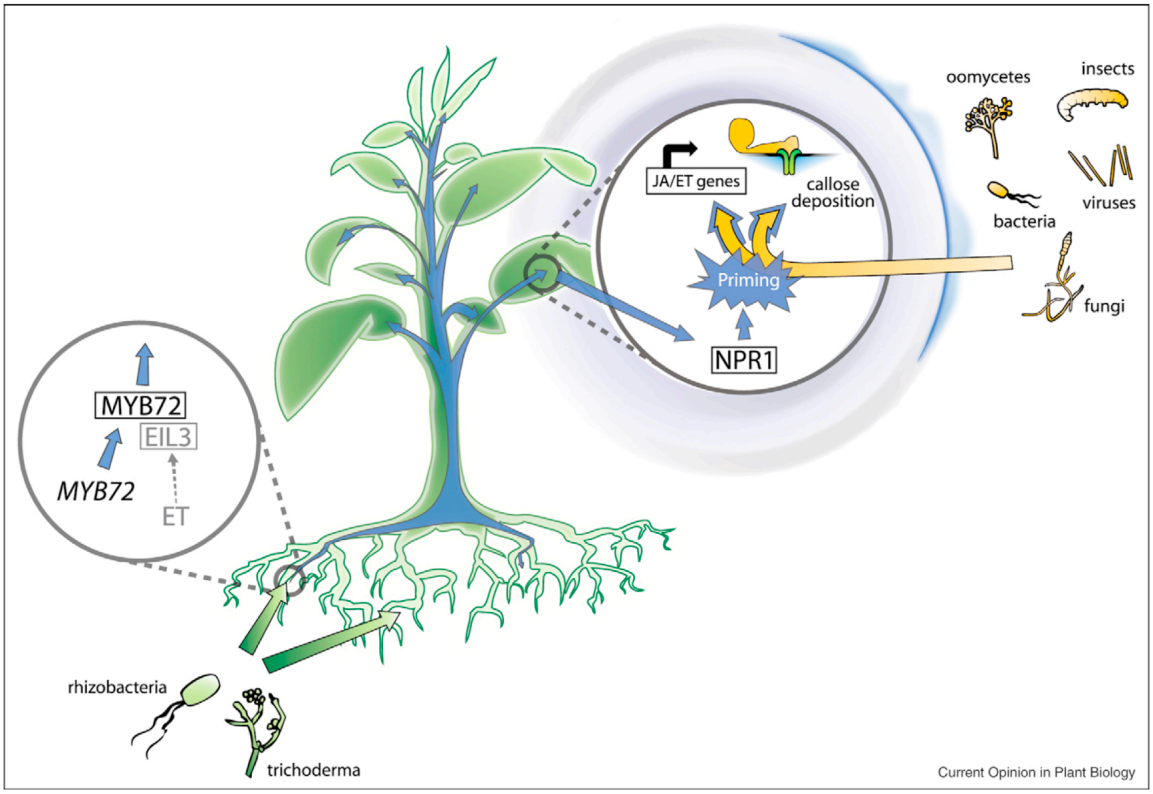

Figura 1. Respuesta sistemica inducida por bacterias y hongos benéficos. El reconocimiento de MAMPs por los microorganismos benéficos que colonizan las raíces tales como *Pseudomonas fluorescens* WCS417 o "*T. asperellum* (T34)", por parte de la planta, conduce a una activacion local de genes que codifican para factores de transcripcion MYB72 en las raíces. Subsecuentemente, MYB72 interactúa con un factor de transcripción EILs e inducen la ISR en las hojas donde la cascada de señalización requiere NPR1, la inducción de ISR, esta asociada con el "priming" del incremento de expresión de genes que responde a acido jasmonico y a etileno. Y el ataque de los patogenos activa la respuesta de defensa en las plantas que ya habia sido "primed" por ISR. (figura tomada de Van wees *et*

al. 2008).

Mecanismos de defensa de las plantas

Las plantas en sus ambientes naturales están sujetas al ataque de una amplia variedad de microorganismos patógenos e insectos y en respuesta a estos las plantas expresan numerosos mecanismos de defensa para evitar la infección por el patógeno (Uchida y Tasaka, 2010). Los mecanismos moleculares de las respuestas de defensa de las plantas son muy complejos, pero se sabe que las respuestas inician con el reconocimiento del patógeno (Mur *et al.,* 2008). La producción de ciertas moleculas como PAMPs y efectores por parte del patógeno conduce al reconocimiento por las plantas que tienen proteinas que reconocen a estos efectores (genes R) como se ilustra en la figura 2, este reconocimiento resulta en una rápida activación de las respuestas de defensa y como consecuencia la limitación del avance del patógeno (esta respuesta se conoce como interaccion incompatible). La resistencia de las plantas mediada por los genes R es usualmente acompañada por un estallido oxidativo, que consiste en una rápida producción de Especies Reactivas de Oxigeno (ERO). La producción de ERO es requerida para otro componente de la respuesta hipersensible (HR), un tipo de muerte celular programada que evita que el patógeno tenga acceso al agua y nutrientes, y que además pueda esparcirse a otras zonas de la planta (Glazebrook, 2005, Van Wees *et al*., 2008).

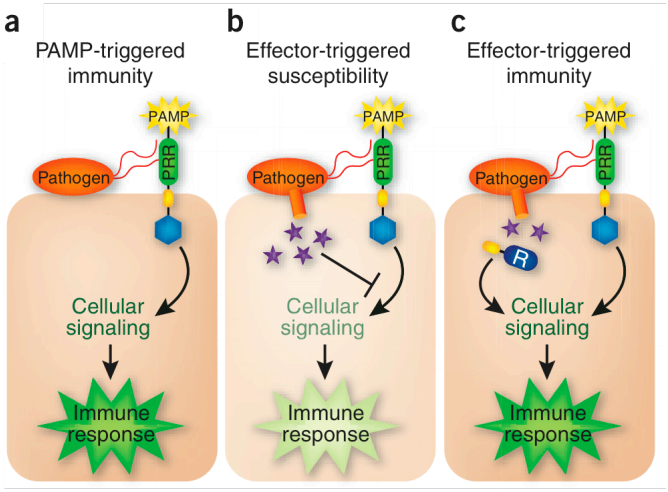

Figura 2. Representación esquematica simplificada del sistema immune en las plantas a). cuando un patógeno ataca produce un patron molecular asociado al patogeno (PAMPs), activa en la planta un receptor que reconoce al patron (PRRs) resultando en cascada de señales rio abajo que conduce a la immunidad disparada por PAMPs (PTI). b). patogenos virulentos han adquirido efectores (estrellas) que reprime la immunidad por PAMPs, resultando en una suceptibilidad disparada por el efector. c). En este caso las plantas han adquirido resitencia proteinas (R) que reconocen a los efectores especificos del patogeno resultando en una respuesta de immunidad secundaria disparada por el efector.
(figura tomada de Pieterse *et al*., 2009).

Resistencia gen por gen.

Durante el transcurso de la evolución, las plantas han adquirido la habilidad para reconocer y responder a moléculas de un patógeno especifico conduciendo a una activación rápida de respuesta de defensa. Este fenómeno fue observado durante

la interacción entre un patógeno portando un solo gen dominante (genes de avirulencia) que son reconocidos por genes de resistencia dominantes de las plantas (genes R), conduciendo a la interacción gen por gen (Bent y Mackey, 2007). Los patógenos que son reconocidos por esta vía y que fallan en proliferar en la planta son llamados patógenos avirulentos y el hospedero es resistente, por lo tanto tenemos una interacción incompatible. En ausencia de reconocimiento gen por gen, debido a la ausencia del gen R del hospedero, el patógeno es virulento y el hospedero susceptible, lo cual conlleva a una interacción compatible (Bent y Mackey, 2007). Hay dos tipos de respuestas que dependen directamente de la resistencia gen por gen. Una es la rápida producción de intermediarios de especies reactivas de oxigeno (IERO) llamada respuesta oxidativa, (superoxido (O_2^-) oxido nitrico (NO) y peroxido de hidrogeno (H_2O_2). Estas pueden tener un efecto antimicrobiano directo, y también sirven como señal para la activación de otras respuestas de defensa. La citotoxicidad y la naturaleza reactiva de (O_2^-) requiere que sus concentraciones celulares sean controladas y equilibrada por la inducción de enzimas antioxidantes, tales como glutatión S-transferasa y glutatión peroxidasa (Kombrink y Schmelzer, 2001). La segunda respuesta es una forma de muerte celular programada conocida como respuesta hipersensible (HR).

Durante los últimos 10 años muchos genes R y de avirulencia han sido identificados (Belkhadir *et al*., 2004). Dentro de los genes R tenemos a los que pertenecen al grupo mas grande de proteinas que son ricas en repitidos de lisinas que ademas tiene un sition de unio a un nucleotido (NBS-LRR) (Belkhadir *et al*., 2004). En el caso de las bacterias patógenas muchos de los genes de avirulencia codifican para efectores tipo III como los producidos por *P. syringae* (*avrPto* y

avrPphB) y contribuyen en la virulencia en aquellos hospederos que no tienen el gen R (Bent y Mackey, 2007). Durante la interaccion planta-microorganismo se han identificado unas cuantas proteínas donde la interacción con su receptor puede ser directa o indirecta. La primera evidencia reportada de interacción directa entre proteínas NBS-LRR y el efector de un patógeno corresponde al gen R de arroz denominado *pi-ta* que confiere resistencia al patógeno *Magnaporthe grisea* que expresa el efector AVR-pita, esta interacción fue confirmada con ensayos de dos híbridos (Jia *et al*., 2000). Estudios sobre los *locus* de resistencia L de lino mostraron que la proteína L interactúa directamente con las variantes del efector AvrL del hongo patógeno *Melampsora lini*, agente causal de la roya del lino (Dodds *et al*., 2006). Por otro lado, durante las interacciones indirectas se ha determinado que las proteínas efectoras AVRRpm1 y AVRB de la bacteria patógena *P. syringae,* son detectadas por la proteína RPM1 (NBS-LRR) de *Arabidopsis* (Innes *et al.,* 1993). Sin embargo, otro efector de *P. siryngae* AVRRpt2 es detectado por la proteína RPS2 (NBS-LRR) (Mindrinos *et al.,* 1994). Otro ejemplo de un mecanismo de reconocimiento indirecto se refiere al de las proteínas de *Arabidopsis* RPS5 y PBS1 en la detección del efector AVRPphB de *P. syringae*, en donde RPS5 es una proteína de la planta tipo NBS-LRR mientras que PBS1 es una proteína cinasa con sustrato desconocido (De young e Innes, 2006).

Repuesta Hipersensible (HR)

Se ha sugerido que la respuesta hipersensible HR es una forma de muerte celular programada (MCP) en plantas, además se ha visto que hay una similitud entre la

HR y la apoptosis en mamíferos, así como también hay muchas diferencias (Greenberg, 1996). Las células muertas debido a la HR presentan algunos cambios morfológicos como los de la apoptosis en mamíferos: en la HR se detiene el flujo citoplasmatico, hay una condensación de la cromatina, cortes del ADN en los nucleosomas se forman vesiculas que contienen fragmentos de ADN, se activan proteasas y las celulas vecinas de la HR inducen autofagia (Mur *et al*., 2008).

En plantas de papa que portan un gen de resistencia R conocido, la muerte celular por respuesta hipersensible parece ser la mayor respuesta de defensa a la infección de *P. infestans*. La HR es siempre observada en plantas resistentes y esto ocurre rápidamente, usualmente 24 h después de la inoculación, resultando en la muerte de una o tres células y esto es frecuentemente restringido por la epidermis (Freytag *et al*., 1994). Por otro lado en plantas de papas susceptibles, las células epidérmicas penetradas por el patógeno ocasionalmente muestran las características de HR, tales como granulación, oscurecimiento del citoplasma, engrosamiento de la pared celular, autoflorescencia bajo luz UV y núcleos condesados cerca del sitio de penetración (Freytag *et al*., 1994; Vleeshouwers, 2000).

Resistencia Sistémica Adquirida (SAR)

En la respuesta HR durante e inmediatamente después de esta respuesta, se activan señales dependientes del acido salicílico (AS), la cual inducirá la SAR. La inducción de la SAR puede ser dada por exponer la planta a un patógeno

virulento, no virulento o con químicos sintéticos tales como ácido salicílico, ácido 2,6-dichloro-isonicotonico (INA) o ácido 1,2,3 benzothidiazol S-metil ester-7-carbotioco (BTH). Cualquier interrupción en la habilidad de las plantas para acumular AS resulta en la pérdida de la expresión de genes relacionados a patogénesis (PRs) y de la atenuación de la respuesta SAR, cuando estos son confrontados con patógenos (Vallad y Goodman, 2004).

La importancia de la acumulación de AS para la expresión de la SAR fue demostrada utilizando plantas transgénicas de *Arabidopsis* NahG, estas plantas expresan el gen nahG de *Pseudomonas putida* que codifica para una salicilato hidroxilasa que hace a la planta incapaz de acumular AS, ya que esta enzima lo degrada a catecol y como consecuencia éstas plantas no presentaron respuesta SAR, pero la aplicación exógena de AS o de algunos de sus análogos BTH o INA rescata el fenotipo para la expresión de la SAR (Verhagen *et al*., 2006).

Plantas mutantes deficientes en la producción de AS, como *sid1* y *sid2* (también llamadas *eds5* y *eds16*) son incapaces de inducir la SAR después de la infección con patógenos biotróficos, lo cual indica que el AS es necesario para la inducción de la SAR (Shulaev *et al.,* 1995). En plantas transgénicas portando genes que codifican para enzimas que incrementan la síntesis de AS, o mutaciones de genes para ganar resistencia en *Arabidopsis* tales como *cpr1*, *cpr5* y *cpr6* en el cual todas contienen niveles constitutivos altos de AS, en estas plantas la expresión de genes de defensa relacionados a patogénesis es permanente y el fenotipo es de enanismo y estas lucen raquiticas (Goellner y Conrath, 2008). Con estos resultados los autores concluyeron que el óptimo funcionamiento de la planta se da en ciertos niveles de hormonas que balanceen el funcionamiento fisiológico y la

respuesta de defensa (Heidel *et al*., 2004).

Por otro lado, se descubrio que para la transducción sistemica de la señal del AS para la expresión de los genes PRs, se requiere de la proteína regulatoria NPR1 (de sus siglas en Inglés: expresión nula de genes PRs). En este sentido plantas mutantes *npr1* que acumulan niveles normales de SA después del ataque de patógenos, son incapaces inducir genes PR y respuesta SAR, y el fenotipo de SAR no se recupera ni con aplicación de AS o con su análogo INA. Estos resultados indican que la proteína NPR1 está actuando río abajo del AS en la transducción de la señal de la ruta de la SAR (Cao *et al*., 1994). Cuando los niveles de AS son bajos, NPR1 se encuentra en forma de oligómeros en el citoplasma. Cuando los niveles de AS incrementan, los oligómeros de NPR1 se disocian en monómeros al romperse los puentes disulfuro que las mantienen unidas. Estos monómeros entran al núcleo e interactúan con los factores de transcripción de la familia TGA como se ilustra en la figura 3 (Xiang *et al.,* 1997; Beckers y Spoel, 2006). Estos factores de transcripción se caracterizan por tener una región básica de unión de ADN y por tener un dominio cierre de leucina para su dimerización (Xiang *et al.,* 1997). Los factores de transcripción TGA 2, 5 y 6 son requeridos para la expresión del gen PR-1 mediada por AS. El factor de transcripción WRKY70 es también requerido para la expresión del gen PR-1 en respuesta a una infección. La expresión de WRKY70 es inducida por SA y es dependiente de NPR1, sin embargo, la interacción entre NPR1 y WRKY70 no ha sido reportada (Koornneef y Pieterse, 2008). En su conjunto, los mecanismos de transducción de señales mencionados, lleva a la inducción de los genes de defensa marcadores de la SAR.

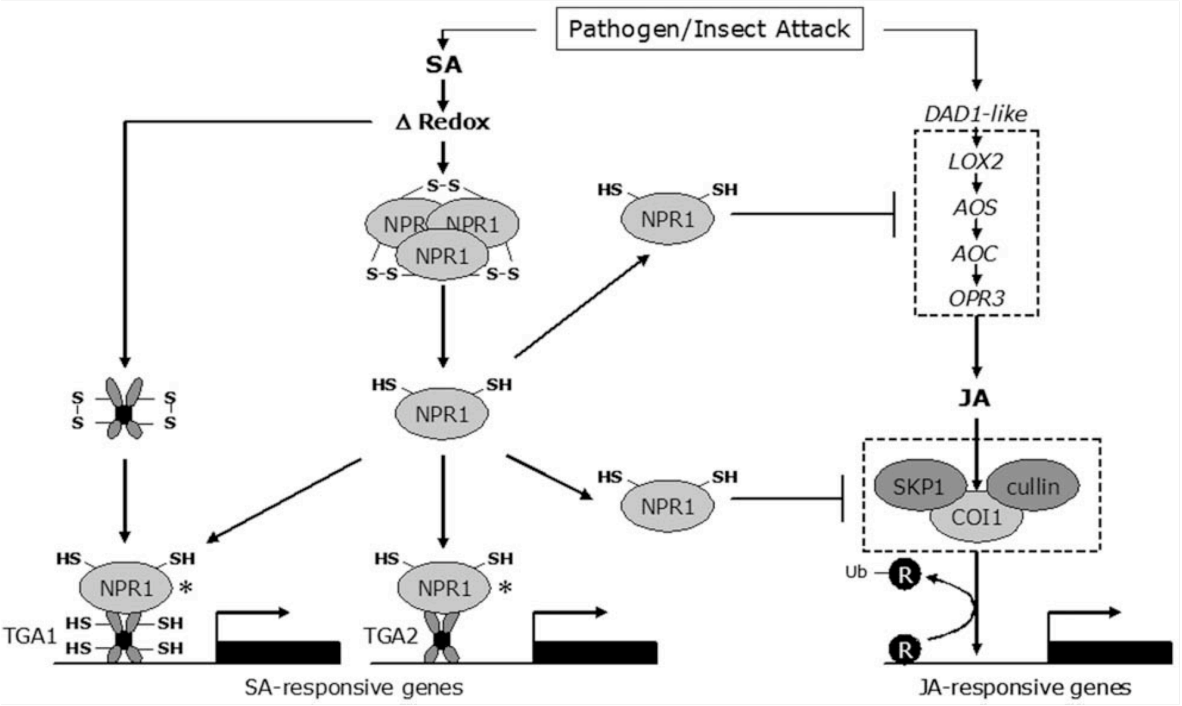

Figura 3. Representación esquemática del papel de NPR1 inducido por ácido salícílico AS y represión de la ruta dependiente del ácido jasmónico AJ. El ácido salícílico cambia el potencial redox lo cual promueve que los puentes disulfuros de los oligomeros de NPR1 se disocien en Monoceros, los cuales se translocan al nucleo e interactúan con los factores de transcripcion TGA en inducen los genes de defensa dependiente de AS. Por otro lado, lo monomeros de NPR1 que se encuentran en el citoplasma inhiben la transcripción de genes de la biosintesis de AJ (figura tomada de Beckers y Spoel, 2006).

Proteinas relacionadas a patogenecidad PRs

Las PRs son proteinas que presentan actividad antimicrobiana *in vitro* y su acumulación en las plantas esta relacionada con respuestas de defensa aunque es importante mencionar que para algunas PRs no se le ha caracterizado un papel

funcional directo en el mecanismo de defensa como se ilustra en la tabal 1 (Sels *et al.,* 2008). Las PRs han sido descrita en muchas especies de plantas pertenecientes a varias familias (Edreva, 2005; Sels *et al.,* 2008) y la inducción de las PRs pueden ser dependientes de acido salicilico o de acido jasmonio/etileno, dentro de las PRs dependientes de AS en la respuesta SAR, tenemos a las PR1, PR2, PR3, PR4 y PR8 tabla 1 (Van Loon *et al.,* 2006; Sels *et al.,* 2008).

Tabla 1. Clasificacion de las proteinas PRs segun su propiedad enzimática (tabla tomada de Sels *et al.,* 2008).

Familia	**Tipo de miembro**	**Tamaño (kDa)**	**Propiedades**	**Sustrato microbial**
PR-1	Tabaco PR-1a	15	Antifunfica	Desconocido
PR-2	Tabaco PR-2	30	β-1,3-glucanasa	β-1,3-glucana
PR-3	Tabaco P, Q	25-30	Quitinasa (clase I,II, IV,V,VI,VI)	Quitina
PR-4	Tabaco R	15-20	Quitinasa clase I y II	Quitina
PR-5	Tabaco S	25	Tipo taumatina	Membrana
PR-6	Tomate inhibidor I	8	Inhibidor de proteasa	Desconocido
PR-7	Tomate P_{69}	75	Endoproteasa	Desconocido
PR-8	Pepino quitinasa	28	Quitinasa clase III	Quitina
PR-9	Tabaco peroxidada formando lignina	35	Peroxidasa	Desconocido
PR-10	Perejil “PR1”	17	Tipo ribonucleasa	Desconocido
PR-11	Tabaco quitinasa	40	Quitinasa clase I	Quitina
PR-12	Rabano Rs-AFP3	5	Defensina	Membrana
PR-13	Arabidopsis THI2.1	5	Tionina	Membrana
PR-14	Barley LTP4	9	Proteina que tranfiere	Membrana

			lipidos	
PR-15	Barley OxOa germina	20	oxidasa Oxolato	Desconocido
PR-16	Barley OxOLP	20	Tipo oxidasa oxolato	Desconocido
PR-17	Tabaco PRp27	27	Desconocida	Desconocido

Resistencia Sistémica Inducida (ISR)

Para conocer la funcionalidad de la ISR durante la defensa contra patogenos se han generado plantas mutantes que están afectadas en su respuesta a acido jasmonico (AJ) o etileno (ET), donde se ha observado que estas plantas mutantes presentan niveles normales de la respuesta SAR dependiente de los patógenos, llevando a la conclusión que la ISR es inducida de manera independiente de la SAR y trabajando con mutantes Nahg de *Arabidosis* que no acumulan AS, se observo que estas plantas presentaron respuesta de la ISR cuando se inocularon con la rizobacteria *Pseudomonas fluorescens* que coloniza la raíz, de esta manera se sabe que AJ y ET regulan esta ruta de defensa y los genes marcadores de estas dos hormonas son *hel* (proteina tipo heveina), *chiB* (endoquitinasa basica), *pdf1*.2 (defensina), mientras que otros transcritos inducidos solo por AJ son *Atvsp* (proteína de almacenamiento vegetativo), *lox1* (lipoxigenasa 1) y *pal1* (fenilalanina ammonio liasa) (Pieterse *et al*., 2001).

El ataque a las plantas por patógenos necrotróficos e insectos herbívoros disparan la producción de una gran diversidad de moléculas como ácidos grasos oxigenados llamado (oxilipinas), las cuales pueden ser potentes reguladores en

señales de defensa (Beckers y Spoel, 2006). Especialmente las oxilipinas, también conocidas como jasmonatos, inducen muchas respuestas de defensa como la respuesta a herida causada por insectos y en la respuesta inducida por rizobacterias (Beckers y Spoel, 2006). Interesantemente, la molécula ácido jasmonico y otros jasmonatos generan señales específicas dependiendo del tipo de estrés. Además de jugar un papel en el sistema de defensa de las plantas, el AJ participa en muchos procesos fisiológicos del desarrollo, tales como: maduración del polen, desarrollo de flores y frutos, regulan el almacenaje en los tejidos de reserva como tubérculos y semillas, además participan en procesos como fotosíntesis, senescencia y crecimiento de las raíces (Reymond *et al.,* 2000; Creelman y Mulpuri, 2002).

La síntesis de jasmonatos ocurre a través de la ruta de los octadecanoides e inicia con la liberación de ácido linoleico de la membrana del cloroplasto donde ésta molécula es procesada por una fosfolipasa. Es importante mencionar que en esta ruta de síntesis participan otras enzimas como la lipoxigenasa (LOX2), aleno oxido sintasa (AOS), aleno oxido ciclasa (AOC) y una reductasa (OPR3), todas ellas inducidas por AJ, heridas y enfermedades. Estas enzimas involucradas en la síntesis de AJ son muy abundantes en las hojas de *Arabidopsis,* sin embargo, la acumulación de AJ se observa solo después de un estrés biótico o abiótico (Beckers y Spoel, 2006).

Las señales dependientes de AJ proceden a través del incremento en la síntesis de AJ en respuesta al ataque de patógenos y consecuentemente incrementa la expresión de genes marcadores de esta ruta como es *pdf1*.2. También es importante mencionar que la ruta de señales de AJ puede ser complejo, ya que

algunos genes regulados por AJ también pueden ser regulados por etileno ET. Por ejemplo en el caso del gen *pdf1*.2 es inducido por estas dos hormonas. En contraste, el ET no es requerido para la expresión de genes regulados por AJ como *vsp1* (Norman-Setterblad *et al*., 2000).

Utilizando mutantes en la respuesta a JA (jar1-1), y ET (etr1-1), demostraron que la respuesta mediada por rizobacterias es dependiente de estas dos hormonas (Pieterse *et al*., 2001). Por otro lado, la protección inducida por metil jasmonato fue bloqueada en las plantas mutantes jar1-1, etr1-1 y npr1-1 y la protección inducida por 1-aminocyclopropano-1 carboxylato (ACC), precursor del ET, fue afectada en plantas etr1-1 y npr1-1, pero no en las mutantes jar1-1. Estos resultados indicaron que npr1-1 regula la expresión de genes de la SAR y reprime la respuesta ISR, y que hay un "cross-talk" entre estas dos rutas y que la inducción de la SAR es antogonica de la ruta SIR como se ilustra en la figura 4 (Pieterse *et al*., 2001; Pieterse *et al.,* 2009).

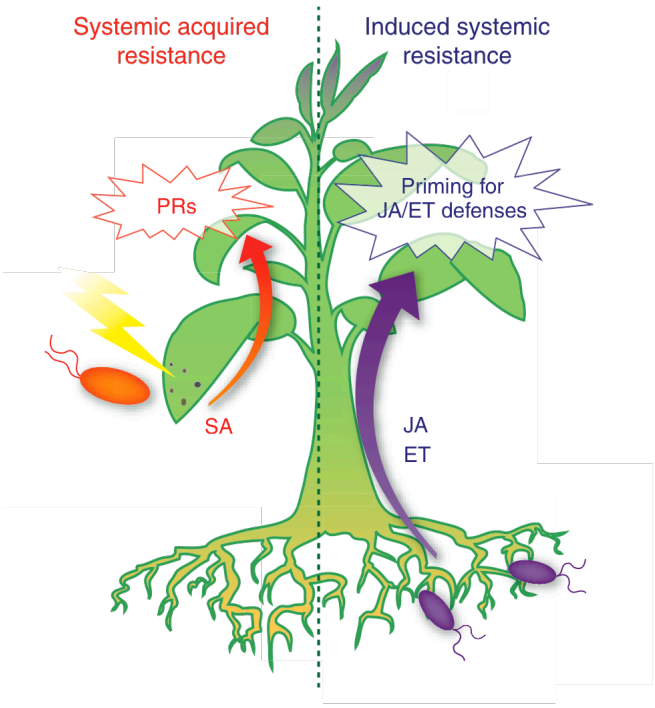

Figura 4**. Representación esquematica de las respuestas sistémica en las plantas SAR y ISR.** La respuesta SAR es inducida después de la infeccion del patogeno donde una señal se mueve atraves del sistema vascular que activa a los genes en los tejidos distantes de la infeccion. El acido salicilico es una molecula esencial para la SAR ya que activa genes relacionados a patogenecidad que tienen propiedades antimicrobianas. La respuesta SIR es activada por microorganismos beneficos que colonizan la raices de las plantas y esta es regulada por las hormonas AJ y ET, donde estas hormonas activan genes marcadores dependiente de AJ/ET como *pdf1*.2, *lox1* y *hel*. (figura tomada de Pieterse *et al.,* 2009).

Tomate como modelo de estudio

En México el tomate es cultivado en casi todas los estados del país, sin embargo, los de mayor importancia por su superficie sembrada y por su rendimiento por hectárea son: Sinaloa, Michoacán, Baja California, San Luis Potosí, Baja California sur, Jalisco, Zacatecas (www.sagarpa.gob.mx). Estas regiones productoras reducen su producción por enfermedades causadas por hongos y bacterias (*A. solani. R. solani*. *F. oxysporum, Phytium* spp. *Xantomonas vesicatoria, Rhalstonia solanacearum, Clavibacter michiganensis, Peudomonas syringae,* etc.) (Kazan y Manners, 2009; Jones *et al.,* 2004).

Para el control de las enfermedades causadas por estos patógenos se ha utilizado el control químico, pero el abuso en el uso de estos pesticidas ha llevado al desarrollo de resistencia de estos patógenos a los compuestos químicos (Benítez *et al*., 2004). Debido a la importancia economica de este cultivo, a la disposición de secuencias de su genoma y a las problemáticas fitosanitarias para su producción, lo hacen un buen modelo para medir el efecto de aquellos microorganismos benéficos capacez de promover el crecimiento y que funcionen como agentes de control biológico (Monte, 2001).

***Arabidopsis thaliana* como modelo de estudio**

Arabidopsis thaliana es un miembro que pertenece a la familia de la mostaza (crucifera o brasicacea) con una distribución geográfica muy amplia a través de Europa, Asia y Norte America. Muchos diferentes ecotipos han sido colectados de poblaciones naturales y se encuentran disponibles para su análisis experimental, aunque solo los ecotipos Columbia y Landsberg son aceptados como estándares

para estudios genéticos y moleculares (Koornneef *et al.,* 2004). Esta planta anual tiene un ciclo de vida de seis semanas, durante este tiempo se llevan acabo todas sus etapas fonológicas como la germinación de sus semillas, formación de la roseta de la planta, crecimiento del tallo principal, floración y maduración de las semillas (Meinke *et al.,* 1998).

El genoma de *Arabidopsis* ya ha sido secuenciado y esta organizado en 5 cromosomas y contiene 20,000 genes aproximadamente, este avance tecnológico a llevado a las grupos científicos a tomarlo como un modelo genético y molecular (Meinke *et al.,* 1998). Existen diferentes herramientas para el estudio de esta planta modelo incluyendo técnicas de mutagénesis insercional, mutagénesis química, métodos eficientes de obtención de cruzas, métodos de transformación genetica, grandes colecciones de mutantes con diversos fenotipos y una variedad de mapas cromosómicos de genes mutados y marcadores moleculares (Koornneef *et al*., 2004). Es importante mencionar que la mutagénesis insercional de ADN (T-DNA) mediado por *Agrobacterium tumefasciens* ha llegado a ser usado rutinariamente entre los métodos de transformación de estas plantas (Koornneef *et al*., 2004).

Para el estudio de interacción planta-microorganismo, tanto para patógenos como para microorganismos benéficos se han utilizado mutantes de *Arabidopsis* afectadas en la producción o en la regulación de algunos compuestos de señalización como AS, AJ y ET, donde el incremento o la disminución de los sintomas causados por un patógeno en éstas mutantes ha indicado el papel clave de la señalización hormonal en los mecanismos de defensa reguladas por estas hormonas (Van Loon *et al.,* 2006).

Por otro lado las hormonas de plantas como las auxinas, giberelinas y brasinosteroides aunque son considerados como reguladores del crecimiento de las plantas, también se han visto asociados con la defensa de las plantas (Kazan y Maners, 2009), esto debido a que los microorganismos patógenos o benéficos producen estas hormonas o pueden afectar la homeostasis de las hormonas de la planta durante la interacción (Kazan y Maners, 2009; Lugtenberg y Kamilova, 2009).

RESULTADOS

Capitulo 1

Colonization of *Arabidopsis* roots by *Trichoderma atroviride* promotes growth and enhances protection against bacterial and fungal pathogens through SA and JA pathways

En esta primera parte del trabajo se estudió la colonización de raíces de plantas de *Arabidopsis* por el hongo *Trichoderma atroviride* y su efecto en el crecimiento y en la resistencia sistémica contra patógenos foliares. La inoculación de plantas de *Arabidopsis* con *T. atroviride* promovió el crecimiento y estuvo asociada a la colonización de las raíces por el hongo. Para observar la colonización de la raíz de la planta por *Trichoderma* se generó una cepa transformante del hongo que expresa constitutivamente la proteína verde fluorescente, la cual fue inoculada en raíces de *Arabidopsis* (*in Vitro*) y se incubaron los co-cultivos por 72 y 96 h. Por microscopia confocal se observó la penetración y colonización de la raíz por el hongo. También evaluamos si la colonización de las raíces por *Trichoderma* inducía la resistencia sistémica en estas plantas, encontrando que las plantas inoculadas fueron mas resistente contra el patógeno biotrofico *Pseudomonas syringae* y contra el patógeno necrotrofico *Botrytis cinerea*. Debido a los resultados obtenidos de supresión de la enfermedad con los patógenos nos preguntamos si esta resistencia se debía a una inducción de genes relacionados con los sistemas de defensa. Para responder esto se inocularon *in vitro* plántulas de *Arabidopsis* con conidias de *T. atroviride* y se mantuvieron en interacción por 72 y 96 h, tiempo en el cual se evaluó la expresión de genes marcadores de la respuesta sistémica inducida (SIR), de la respuesta sistémica adquirida (SAR) y de la vía de síntesis de la fitoalexina (camalexina) y se observó que *T. atroviride* es capaz de inducir la expresión de estos genes marcadores. Hasta donde sabemos, este el primer trabajo donde se reporta que la colonización de raíces de *Arabidopsis* por *T. atroviride* induce la co-expresión de genes involucrados en la respuesta sistémica adquirida, en la respuesta sistémica inducida y en la síntesis de camalexina.

Colonization of *Arabidopsis thaliana* roots by *Trichoderma atroviride* promotes growth and enhances protection against bacterial and fungal pathogens through salicylic acid, jasmonic acid, and camalexin pathways

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Running title: *T. atroviride* induces SA and JA pathways simultaneously in *Arabidopsis*

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Key words: *Trichoderma*, plant–fungus interaction, *Arabidopsis*, gene expression, salicylic acid, jasmonic acid, systemic resistance.

ABSTRACT

Trichoderma spp. are common soil fungi widely used as biocontrol agents due to their capacity to produce antibiotics, induce systemic resistance in plants and parasitize phytopathogenic fungi of major agricultural importance. In this study we investigated whether colonization of *Arabidopsis thaliana* seedlings by *Trichoderma atroviride* affected plant growth and development. Our results showed that *T. atroviride* promotes systemic growth in *Arabidopsis*. In addition, we tested whether colonization of *Arabidopsis* roots by *T. atroviride* can induce systemic protection against foliar pathogens. We determined that *Arabidopsis* inoculation with *T. atroviride* provided systemic protection to the leaves inoculated with bacterial and fungal pathogens with different life styles. To investigate the possible pathway involved in the systemic resistance induced by *T. atroviride*, we assessed the expression of salicylic acid (SA), jasmonic acid (JA), and camalexin related genes in *Arabidopsis*. Interestingly, *T. atroviride* induced an overlapped expression of defense-related genes of SA and JA pathways, and of the gene involved in the synthesis of the main antimicrobial phytoalexin, camalexin, both locally and systemically. To our knowledge this is the first report where colonization of *Arabidopsis* roots by *Trichoderma atroviride* induce the expression of SA and JA pathways simultaneously to confer resistance against biotrophic and necrotrophic phytopathogens.

INTRODUCTION

Plants have developed sophisticated defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. When under attack, a plant is capable of enhancing its resistance, a condition often referred to as induced, or acquired, resistance. Acquired disease resistance is thought to involve an enhancement of basal resistance (Van Loon 2007; Ton *et al.*, 2002). In this response, salicylic acid (SA) plays a crucial role in plant defense and is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens, as well as the establishment of Systemic Acquired Resistance (SAR) (Grant & Lamb 2006). Mutants that are affected by the accumulation of SA or are insensitive to SA show enhanced susceptibility to biotrophic and hemi-biotrophic pathogens. Recently, it has been shown that, methyl salicylate, which is induced upon pathogen infection, acts as a mobile inducer of SAR in tobacco (Park *et al*. 2007). SA levels increase in pathogen-challenged tissues of plants and exogenous applications result in the induction of pathogenesis-related (PR) genes and enhanced resistance to a broad range of pathogens (Bari & Jones 2009).

In contrast to SAR, jasmonic acid (JA) and ethylene (ET) mediate the induced systemic response (ISR), this resistance is usually associated with defense against necrotrophic pathogens and herbivorous insects (Bari & Jones 2009). In this response, several JA-dependent genes that encode pathogenesis-related proteins, including plant defensin1.2 (PDF1.2), thionin2.1 (THI2.1), hevein-like protein (HEL), and chitinase B (CHIB), are commonly used to monitor JA/ET-dependent defense responses (Reymond & Farmer 1998).

Although, SA and JA/ET defense pathways are mutually antagonistic, evidences of synergistic interactions have also been reported mediated by the transcription factor NPR1

(Non-expressor of PR genes 1). The NPR1 protein is an important transcriptional co-activator of SA-responsive PR genes; NPR1 is also a key regulator in SA-mediated suppression of JA signaling. Furthermore, NPR1 has been implicated in several other JA/ET-dependent defense responses, including beneficial rhizobacteria-mediated ISR (Van Wees *et al*. 2008) and JA/ET-dependent resistance against the soil-borne fungus *Verticillium longisporum* (Pieterse *et al*. 2009); however, the pathway involved in the response to beneficial microorganisms has not been elucidated.

Trichoderma spp. are common soil fungi widely used as biocontrol agents against plant pathogens of major agricultural importance (Harman *et al*. 2004; Chet & Inbar 1994; Fravel 2005). The biocontrol mechanism exerted by *Trichoderma* is comprised by different mechanisms, including the production of antibiotics, competition for space and nutrients with other rhizosphere microorganisms, as well as the direct attack of phytopathogenic fungi by means of mycoparasitism (Benítez *et al*. 2004).

In addition, some *Trichoderma* rhizosphere-competent strains can colonize either the root surfaces or the entire plant, a process that has been shown to bestow significant beneficial effects to plants, such as root growth, plant growth enhancement, and increases in productivity (Yedidia *et al*. 1999; Bailey *et al*. 2006). Moreover, root growth induced by *Trichoderma* increases nutrient uptake, drought and soil packing tolerance, and fosters germination and vigor of the seeds (Harman *et al*. 2004; Howell 2003). Furthermore, *Trichoderma* promotes plant growth by the production of phytohormones and auxin-related compounds (Contreras *et al*. 2009).

Activation of plant defense responses by *Trichoderma* has been reported. During root colonization, *Trichoderma* induces the defense system in cucumber, by increasing chitinase and peroxidase activity in leaves and roots (Yedidia *et al*. 2000). This response involves

recognition of the fungus through the SIR pathway. This response is the closest analogue of induced resistance activated by rhizobacteria (Baker *et al*. 2003; Van Loon 2007; Segarra *et al*. 2006). In addition, *Trichoderma* induces the expression of PR genes whose response is mediated by SA, this response is known as SAR, which is also triggered by necrotizing pathogens (Martinez *et al*. 2001).

In this work we studied the *T. atroviride-Arabidopsis* interaction and its implication on growth, induction of systemic protection against biotrophic and necrotrophic pathogens, as well as on the induction of defense related genes mediated by SA, JA/ET, and the synthesis of camalexin in *Arabidopsis* seedlings.

MATERIALS AND METHODS

Organisms and growth conditions

Arabidopsis thaliana ecotype Col-0 was used for this study. *Arabidopsis* seeds were sterilized with a 10% (v/v) sodium hypochlorite solution for 10 min and washed three times with sterile distilled water, then seeds were germinated and grown on agar plates containing MS medium (Murashige & Skoog 1962).

Fungi strains *Trichoderma atroviride* IMI206040 and the *T. atroviride* transformant *pki1*::*gfp* TaGFP22 and *Botrytis cinerea* were grown at 28 ºC on potato dextrose agar (PDA) (DIFCO) for six days and conidia were collected with sterile distilled water and adjusted to a concentration of 1×10^6 conidia ml^{-1}. The bacterium *Pseudomonas syrigae pv* tomato DC 3000 was grown at 28 ºC on Kings B medium (King *et al*. 1954).

Plant-growth promotion assay

Arabidopsis seeds were grown on 0.3X MS medium and, four days after germination,

seedlings were transplanted to flowerpots containing peat moss as substrate (LAMBERT^{TM}), and inoculated with 20 µl of 1 × 10^6 spores ml^{-1} of *T. atroviride.* After 24 h, flowerpots were irrigated with MS (0.3X) to allow the fungus to colonize the rhizosphere. Six days later, plants were watered with the nutritive solution HUMIFERT (Cosmocel) at doses of 3 ml $liter^{-1}$ of water. After twenty days, *Trichoderma* treated plants were removed from the containers and the roots were washed with sterile distilled water. The entire plant length was measured with a ruler and weighed with an analytical balance. Then, plants were air-dried at 70 °C for 72 h to further measure the dry weight. Each treatment consisted of 15 plants, and the experiment was repeated three times.

Root colonization by *T. atroviride* assay

The pHYG-GFP vector carrying the *gfp* gene from *Aequorea victoria* under the control of the constitutive promoter *pki1* (pyruvate kinase) of *T. reesei* (Casas-Flores *et al.* 2006; Zeilinger *et al.* 1999) was used for the transformation of *T. atroviride* protoplast, as described by Baek and Kenerley (1998). Several transformants that expressed *gfp* gene and showed similar morphological characteristics when compared with the wild type strain were selected. The TaGFP22 transformant was chosen for the colonization assay. Seven days old *Arabidopsis* plants were inoculated with the TaGFP22 and roots were visualized at 48 and 72 h using an inverted Zeiss Laser Scanning Confocal Microscope (LSCM) (Zeiss LSM-510 META). GFP expression was imaged with Argon-2 laser, Abs/Em 488/515-530 nm. Confocal images were captured using LSM-510 software (version 3.2; Carl Zeiss) and evaluated with an LSM-510 Image Examiner (version 3.2).

Protection assay against fungal and bacterial phytopathogens induced by *T. atroviride*

Arabidopsis plants used for protection assays were treated as described for plant growth promotion experiments. After 15 days, 3 leaves from each plant were inoculated with 10 µl of a suspension of *Pseudomonas siryngae* grown at a DO=0.2, or with 10 µl of a suspension of 1×10^6 conidia ml^{-1} of *Botrytis cinerea*. The lesion area was evaluated seven days post-pathogen inoculation. Percentage of leaves damage was calculated obtaining the total leaf area and the total damaged leaf area, the ratio between these values gave the percentage of damaged area. Each treatment consisted of 10 plants, and the experiment was repeated three times with similar results.

Expression analysis of *Arabidopsis* defense related genes

Twenty-day-old *Arabidopsis* plants were grown on Petri dishes and inoculated in between the roots (3 cm) with 15 µl of a suspension of 1×10^6 conidia ml^{-1} of *T. atroviride*, allowing the interaction for 72 and 96 h. Mocked plants were included as control. *Arabidopsis* roots and leaves were harvested, separated and frozen in liquid nitrogen at the indicated times. Total RNA was extracted using the Concert RNA extraction solution (Invitrogen) as described by the manufacturer. Expression of plant defense-related genes was assessed by quantitative real-time RT-PCR. The *Arabidospsis* gene specific primer pairs were designed with primer express 3.0 program (Applied Biosystems) based on sequences available in GenBank database (Table 1). Total RNA was DNase-treated using rDNase I (Ambion), and 2 µg of total RNA was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen). The qRT-PCR reaction was performed using the kit Fast Syber Green Master Mix (Applied Biosystems) with 1 ng of cDNA. Experiments were performed using an Abiprism 7500 fast Real-Time PCR system (Applied Biosystems)

following the conditions suggested by the manufacturer. The absence of primer-dimmers was confirmed in reactions without cDNA. The experiments were independently repeated two times and each reaction was performed in triplicate using a relative quantification analysis. The expression of each specific gene was normalized versus the reference control with the formula ΔΔCT.

RESULTS

T. atroviride promotes growth of *Arabidopsis* seedlings

With the aim of closely analyzing the interaction of *Trichoderma* with plants, we decided to work with the pathosystem *Arabidopsis thaliana*-*Trichoderma atroviride*, whose members are used as model systems for genetic and molecular studies due to their simplicity. To assess this analysis, four- day-old *Arabidopsis* seedlings were root inoculated with *T. atroviride* and allowed to interact for 20 days. We observed that *Arabidospsis* treated seedlings were bigger than the untreated control plants (Fig 1A and 1B). Clearly, there was an increase in foliar area and plant growth (Fig 1C). We also observed an increase in plant biomass, which was measured as fresh (Fig 1D) and dry weight (Fig 1E). Fresh weight almost doubled in treated plants compared with control untreated seedlings, while dry weight results of treated plants was one third higher than that of the untreated control. These results indicate a beneficial effect on *Arabidopsis* growth and development by the inoculation of roots with *T. atroviride*.

T. atroviride colonize *Arabidopsis* roots

To know if the *T. atroviride* effect on *Arabidopsis* growth was associated with colonization of roots, a colonization assay was performed by inoculating *in vitro* the *Arabidopsis* roots

and allowing them to interact for 48 and 72 h. Briefly, plugs of actively growing mycelium of *T. atroviride* at the indicated times were taken from the co-culture and washed with sodium hypochlorite for 5 min and placed on Petri dishes containing fresh MS medium. Roots and shoots of *Arabidopsis* seedlings were also washed with sodium hypochlorite for 5 min and placed on a Petri dish with MS medium. No growth of the fungus was observed on plates where the plugs were washed and placed, whereas an actively growing mycelium was observed emerging from the *Arabidopsis* roots (data not shown). To further study the *Arabidopsis* root colonization by *Trichoderma*, several GFP-expressing transformants of *T. atroviride* were generated. Seven-day-old *Arabidopsis* seedlings were inoculated with 10 µl of a 1×10^6 conidia ml^{-1} of TaGFP22-expressing transformant. Roots of seedlings were collected and analyzed by LSCM at 72 and 96 h of fungus-plant interaction. The epidermis, cortex, and vessels of the root cells were intact or only minimally altered. After 48 h of co-culture, hyphae had entered the roots and grown in the intercellular space of the epidermis (Fig. 2A-C). The green fluorescent hyphae entered into the epidermal cells. In some cases, the elongated zone of the hyphae showed structures similar to an appressorium (Fig. 2A-C). Extensive colonization of the root surface was observed even at the root tip (Fig. 2D-F). Together, these results showed that the fungus is able to colonize *Arabidopsis* roots but not the aerial parts and that the fungus forms appressorium-like structures in the plant epidermis.

T. atroviride **colonization induces resistance against foliar plant pathogens in** ***Arabidopsis***

To test whether *T. atroviride* colonization provides protection against fungal and bacterial pathogens, we conducted assays using the biotrophic bacteria *Pseudomonas syringae* and

the necrotrophic fungus *Botrytis cinerea*. Four- day-old *Arabidopsis* seedlings were inoculated with a suspension of 1×10^6 conidia ml^{-1} of *T. atroviride* and placed in flowerpots. Mocked plants were included as control. After 15 days, 3 leaves from each plant were inoculated with 10 µl of a suspension of *Pseudomonas siryngae* grown at a DO=0.2, or with 10 µl of a suspension of 1×10^6 conidia ml^{-1} of *Botrytis cinerea.* After eight days of plant-pathogen interaction, the control plants, not treated with *T. atroviride* but inoculated with *P. syringae*, showed the typical bacterial speck disease provoked by this pathogen (Figure 3A). In contrast, plants inoculated with *T. atroviride* showed reduced lesion area compared with the mocked plants. In the case of the *Trichoderma-Arabidopsis* protection assays against *B. cinerea*, a marked reduction in lesion area was observed on leaves of treated seedlings compared with mocked control plants (Figure 3B). The protection effect was better against *Pseudomonas syringae* compared with *Botrytis cinerea*, and, in fact, almost no lesion development could be observed in leaves of plants treated with *T. atroviride* and infected with *P. syringae* (Fig. 3A and B). Based on these results, we can conclude that colonization by *T. atroviride* induces systemic resistance in *Arabidopsis* against foliar pathogens with different lifestyle.

T. atroviride induces the expression of _Arabidopsis_ SA, JA, and camalexin defense-related genes both locally and systemically

Our protection assays showed that *T. atroviride* enables *Arabidopsis* to counteract pathogens with different life styles, which trigger different resistance response pathways. It is well known that biotrophic pathogens, such as *P. syringae*, trigger the salicylic acid pathway, whereas *B. cinerea*, a necrotrophic microorganism, triggers the JA/ET pathway. With the aim of exploring the possible pathway involved in the protection against such

pathogens in *Arabidopsis*, we assessed the expression profiles of SA and JA/ET defense-related genes, as well as of the gene involved in the synthesis of the antimicrobial compound camalexin at different times post *Trichoderma* inoculation (72 and 96 h). Mocked plants were included as control. Expression of defense-related genes was first examined locally at the site of colonization (roots) at 72 and 96 h after *Trichoderma* inoculation (Figure 4A). Figure 4A shows that *PR1*, was not induced at 72 h, whereas *PR2*, *PDF1.2, LOX1*, and *ATPCA* were slightly induced as compared with mocked plants. The gene *PAD3* that encodes for the last enzyme involved in the synthesis of camalixin was induced fifty-times higher as assessed at 72 h. Expression of PR1, PR2, PDF1.2, and PAD3 were up-regulated in roots at 96 h post-inoculation, whereas LOX1 and ATPCA underwent no significant changes. To analyze the induction of systemic defense response in *Arabidopsis*, mRNA levels of defense-related genes were analyzed in leaves. Expression of PR1 and PDF1.2 was not induced in leaves at 72 h. Figure 4B shows that PR2, LOX1, and ATPCA were slightly up-regulated at 72 h post-inoculation with *T. atroviride*, whereas PAD3 was induced almost 175-fold at the same time (Figure 4B). At 96 h post-treatment, all genes reached their maximum level of expression, excluding the ATPCA gene whose levels decreased compared with 72 h. Together, these data indicate that *T. atroviride* activated systemic and local expression of SA and JA/ET defense-related genes, as well as the gene encoding the last enzyme involved in the synthesis of the antimicrobial camalexin in *Arabidopsis*. Contrasting with other reports, our work clearly demonstrated that *T. atroviride* induces the simultaneous expression of SA- and JA- related genes in *Arabidopsis*.

DISCUSSION

It has been demonstrated that plant growth promotion by *Trichoderma* spp. is dependent on either root colonization or colonization of the entire plant (Baker 1989; Chacón *et al*. 2007; Harman 2000; Kleifeld & Chet 1992; Lindsey *et al*. 1967); however, for *Arabidopsis* there are only a couple of reports (Korolev *et al.* 2008; Segarra *et al.* 2009). In this work, we showed that, *T. atroviride* promotes growth in *Arabidopsis* when applied to roots, revealing that growth enhancement might depend on root colonization. In this sense, it has been suggested that the mechanism involved in growth promotion could be due to root colonization and the ability of *Trichoderma* to provide nutrients and phytohormones (Contreras *et al*. 2009, Harman 2000) or by changing the internal phytohormone homeostasis in the plant. To study closely the *T. atroviride-Arabidopsis* interaction, root colonization experiments were carried out. We generated a *T. atroviride* GFP-expressing transformant to visualize the fungus-plant interaction. Indeed, we observed through LSCM that the fungus is able to colonize *Arabidopsis* roots, the intercellular space of the epidermis forming appressoriun-like structures. This is in agreement with previous studies, where *T. harzianum* hyphae grew and branched directly towards their host plant. It was also demonstrated that, during those interactions, *Trichoderma* induced systemic resistance on those plants (Yedidia *et al*. 1999; Chacón *et al*. 2007). *Arabidopsis* root colonization by *T. atroviride* resulted in an increase in biomass of the entire plant, demonstrating that the effect on seedling growth is systemic; this asseveration was confirmed because we were unable to recover the fungus from the aerial parts of the plant. Taken together these results, it can be concluded that *T. atroviride* is able to colonize *Arabidopsis* roots and promote growth systemically, as described for other *Trichoderma* species.

The effect of plant growth promoting rhizobacteria on the induction of plant systemic

resistance is well known, however the effect of plant growth promoting fungi has been just recently launched. The *Trichoderma* research community has focused its efforts mainly on the study of the mechanisms of mycoparasitism and antibiosis, devoting less attention to the induction of systemic resistance induced by *Trichoderma* (Harman *et al*. 2004). In this work, we showed that *T. atroviride* induced protection in *Arabidopsis* against both the biotrophic bacteria *Pseudomonas syringae* and the necrotrophic fungus *Botrytis cinerea*. In our pathosystem, the pathogens were applied on leaves, which ensure the spatial separation of the pathogen from *T. atroviride,* which secretes antibiotics and has mycoparasitic activity. Our results showed that the systemic resistance induced by *T. atroviride* was slightly higher against the biotrophic pathogen *P. syringae* than that obtained for *B. cinerea*. These results are in agreement with the work of Yedida *et al*. (2003), where the application of strain *T. asperellum* T-203 to cucumber roots, after infection with *Pseudomonas syringae* pv. lachrymans, reduced considerably the diseased plants, furthermore there was production of antifungal compounds in leaves. De Meyer *et al.* (1998) demonstrated that inoculation of strain *T. harzianum* T-39 on bean roots reduced considerably the lesion area provoked by *Botrytis cinerea* (Zimand *et al*. 1996). Based on the aforementioned, we can conclude that suppression of diseases development is systemic and is due to neither mycoparasitic activity nor production of antimicrobial molecules by the fungus. As mentioned above, *Trichoderma*-induced resistance was slightly better against the biotropic pathogen *P. syringae*, which suggests that an overlapping of SA and JA/ET pathways could be the main reason of such pathogen growth suppression in *Arabidopsis*. In this sense, activation of the SA pathway by *P. syringae* similarly suppresses JA signaling and renders infected leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel *et al*. 2007). Although many reports describe an

antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well. Recently, the effect of co-treatment with various concentrations of SA and JA were assessed in tobacco and *Arabidopsis,* finding a transient synergistic enhancement in the expression of genes associated with either JA (PDF1.2 and Thi1.2) or SA (PR1) signaling when both signals were applied at low (typically 10–100 mM) concentrations (Mur *et al*. 2006). Antagonism was observed at more prolonged treatment times or at higher concentrations (Mur *et al*. 2006). A possible explanation for these results could be that colonization of *Arabidopsis* roots by *Trichoderma* turns on the SA and JA pathways simultaneously; subsequently the increase in SA could be suppressing partially the JA pathway, allowing the plant to be more resistant against the biotrophic pathogen as compared with the necrotrophic one.

A number of investigations have reported the induction of systemic resistance for several plants including *Arabidopsis* (Beckers & Spoel 2006; Pieterse *et al*. 2009). Pharmacological analysis using specific inhibitors of JA/ET pathways on *Trichoderma asperellum*-cucumber interaction showed that these signal transduction pathways are involved in the protective effect conferred by *T. asperellum* against *P. syringae* pv lachrymans. Accumulation analysis of SA in roots and leaves of cucumber treated with *Trichoderma* did not show differences when compared with non-inoculated plants. Expression analysis of JA/ET-regulated genes also showed that *T. asperellum* modulates the local and systemic expression of these genes in cucumber (Shoresh *et al*. 2005). Korolev et al. (2007) showed that *Trichoderma*-induced resistance against *B. cinerea* is dependent on JA/ET pathways, by using mutants impaired in such transduction pathways. Later, it was demonstrated that the defense pathways induced by *T. asperellum* and the

beneficial bacteria *Pseudomonas* spp. are very similar and both of them are independent from SA but require NPR1 and MYB72 (Segarra *et al.* 2009).

With the aim of closely studying the possible signal transduction pathway involved in the induction of systemic resistance against pathogens with different lifestyles in *Arabidopsis*, we assessed the expression profile of a set of defense-related genes at 72 and 96 h post-inoculation with *T. atroviride*. In our study, almost all genes were induced by *T. atroviride* both locally and systemically, achieving their maximum expression in both, roots and leaves, at 96 h post-inoculation. Our work showed increased expression of PR genes at 96 h post treatment with *Trichoderma* both locally and systemically. The β-1-3-glucanase (PR2)-encoding gene is highly induced in leaves in response to inoculation with *T. atroviride*. Several studies have indicated that root colonization by *Trichoderma* strains results in increased levels of defense-related enzymes in plants, including peroxidases, chitinases, β-1-3-glucanase (Howell *et al*. 2000; Yedidia *et al*. 1999, 2003; Harman *et al*. 2004). The expression levels of *ATPCA* were not significantly affected in roots at 72 h, but increased at 96 h. When measured in leaves, *ATPCA* increased almost 3-fold and decreased 2-fold at 96 h as compared with 72 h post-inoculation. Peroxidases accumulate as a response to reactive oxygen species (ROS) generation provoked by pathogen attack; the increase in enzyme activities in leaves suggests a systemic defense response to the presence of *Trichoderma* in the rhizosphere (Yedidia *et al*. 1999). Expression levels of JA and of pathogen-induced genes, *PDF1.2* and *LOX1*, were different at both times. *PDF1.2* was up regulated at 96 h post-inoculation with *Trichoderma* in both roots and leaves, whereas *LOX1* underwent no significant changes in roots, but reached four- times the level of expression in leaves. Expression analysis of the *PAD3* gene that encodes the last enzyme involved in the synthesis of the antimicrobial compound, camalexin, showed an up-

regulation in both roots and leaves after treatment of *Arabidopsis* with *T. atroviride*. Camalexin is a phytoalexin, whose members are low-molecular-weight compounds that have antimicrobial activity and are produced by plants in response to attack by pathogens (Paxton, 1981). Yedidia *et al.* (1999) showed that *T. asperellum* might activate metabolic pathways in cucumber, leading to the systemic accumulation of phytoalexins. In addition, *Arabidopsis PAD* mutants loose their ability to restrict the growth of bacterial pathogens (Glazebrook & Ausubel 1994). Accumulation of PR proteins in response to *Trichoderma* has been described in several plants. In contrast with the work of Segarra et al. (2009), who suggest that JA/ET pathways are responsible for the systemic resistance in Arabidospis induced by *T. asperellum*, we observed an overlap of SAR and SIR gene expression. Thus, the systemic resistance induced by *T. atroviride* root colonization seems to have similarities and differences with that of *T. asperellum* and *P. fluorescens* WCS417r, but appear to be distinct from either of them. These reports and our findings suggest that *Trichoderma* induces an overlapping in the expression of SA- and JA/ET-dependent genes, oxidative burst and the synthesis of camalexin-related genes both locally and systemically to suppress pathogen growth in *Arabidopsis*.

In conclusion, we demonstrated that inoculation of *Arabidopsis* roots with *T. atroviride* promotes growth and development of *Arabidopsis* seedlings, and systemically inhibits proliferation of *P. syringae* and *B. cinerea*. The reduction in *Arabidopsis* foliar damage appeared to be associated with simultaneous transcript accumulation of SA, JA/ET defense-related genes, as well as with the expression of genes involved in the oxidative burst and with the synthesis and accumulation of the antimicrobial compound camalexin in *Arabidopsis*.

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Figure Legends

Figure 1. Effect of *T. atroviride* colonization on plant growth of *Arabidopsis*. A and B, *Arabidopsis* plant growth of not inoculated and inoculated with *Trichoderma,* respectively. C, lenght of plants, D, Fresh weight, E, Dry weight. Grey bars show *T. atroviride*-untreated seedlings, while black bars represent *T. atroviride*-treated seedlings.

Figure 2. Confocal images of *T. atroviride* pkil::gfp transformant and *Arabidopsis* co-cultures. A, B, and C show the growth of the green fluorescent hyphae entering into the epidermal cells (arrows indicate the aspersoria-like structure). In some cases, the elongated zone of the hyphae showed structures similar to an appressorium indicated by the arrow. D, E and F, show the extensive colonization in the root tip surface (arrows show the hyphae penetrating the root tip).

Figure 3. Effect of *T. atroviride* on induced systemic resistance in *Arabidopsis* seedlings against the phytopathogens*, B. cinerea* and *P. syringae*. The graphs illustrate the levels of systemic disease protection observed against *P. syringae* (A) or *B. cinerea* (B). Grey bars show *T. atroviride*-untreated seedlings, while black bars represent *T. atroviride*-treated seedlings.

Figure 4. Quantitative expression analysis of defense-related genes in *Arabidopsis* seedlings inoculated with *T. atroviride*. Total RNA from roots and leaves of *Arabidopsis* plants inoculated with *T. atroviride*, was subjected to qRT-PCR to quantify six genes related to different plant defense pathways: *PR1* and *PR2* (SAR), *PAD3* (synthesis of camalexin), *ATPCA* (oxidative burst), and *PDF1.2* and *LOX1*(SIR).

Figure 1.

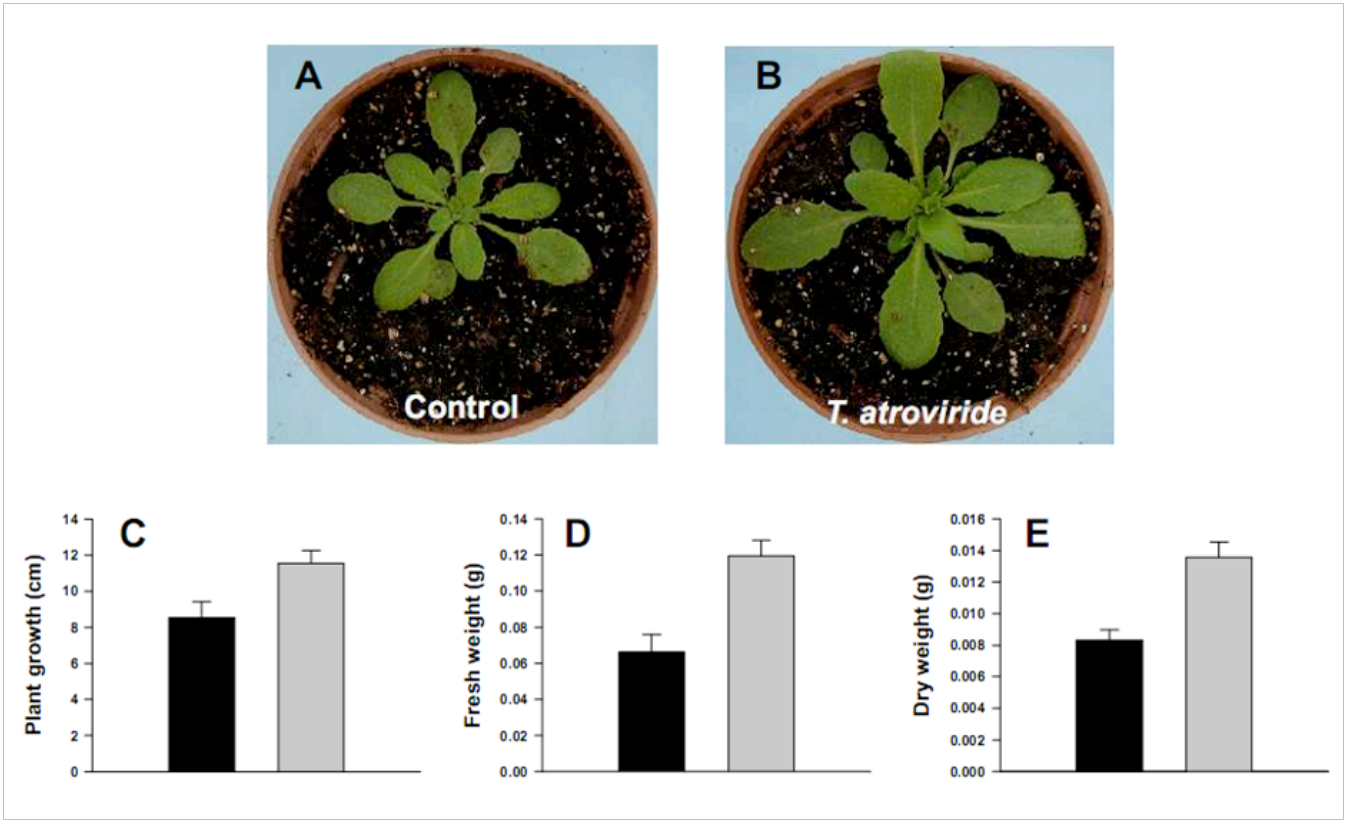
A
Control
B
T. atroviride
C
Plant growth (cm)
0
2
4
6
8
10
12
14
D
Fresh weight (g)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
E
Dry weight (g)
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0.014
0.016

Figure 2.

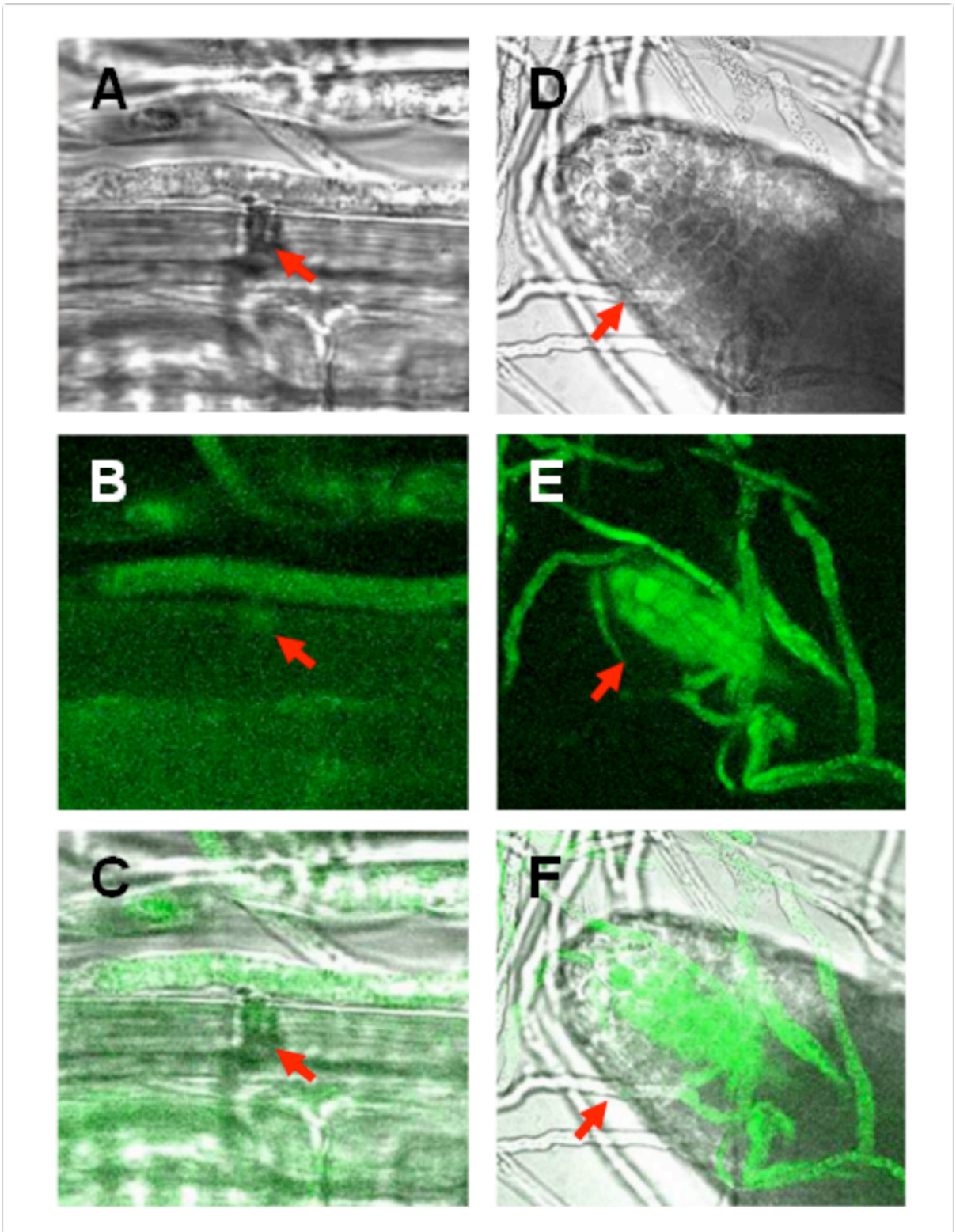

Figure 3.

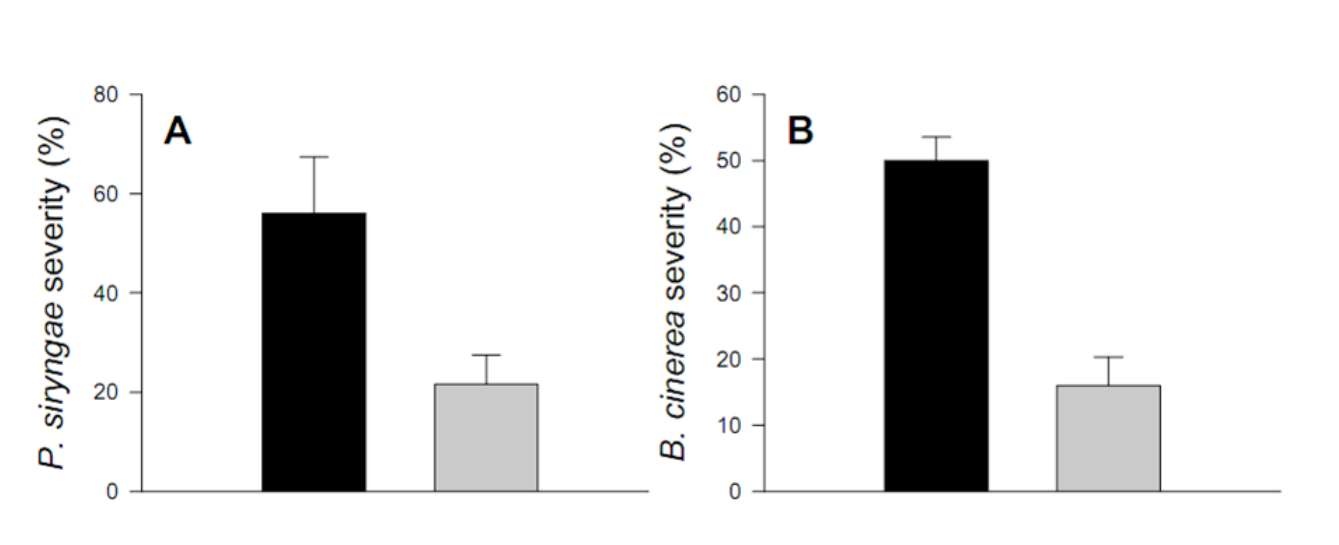

Figure 4.

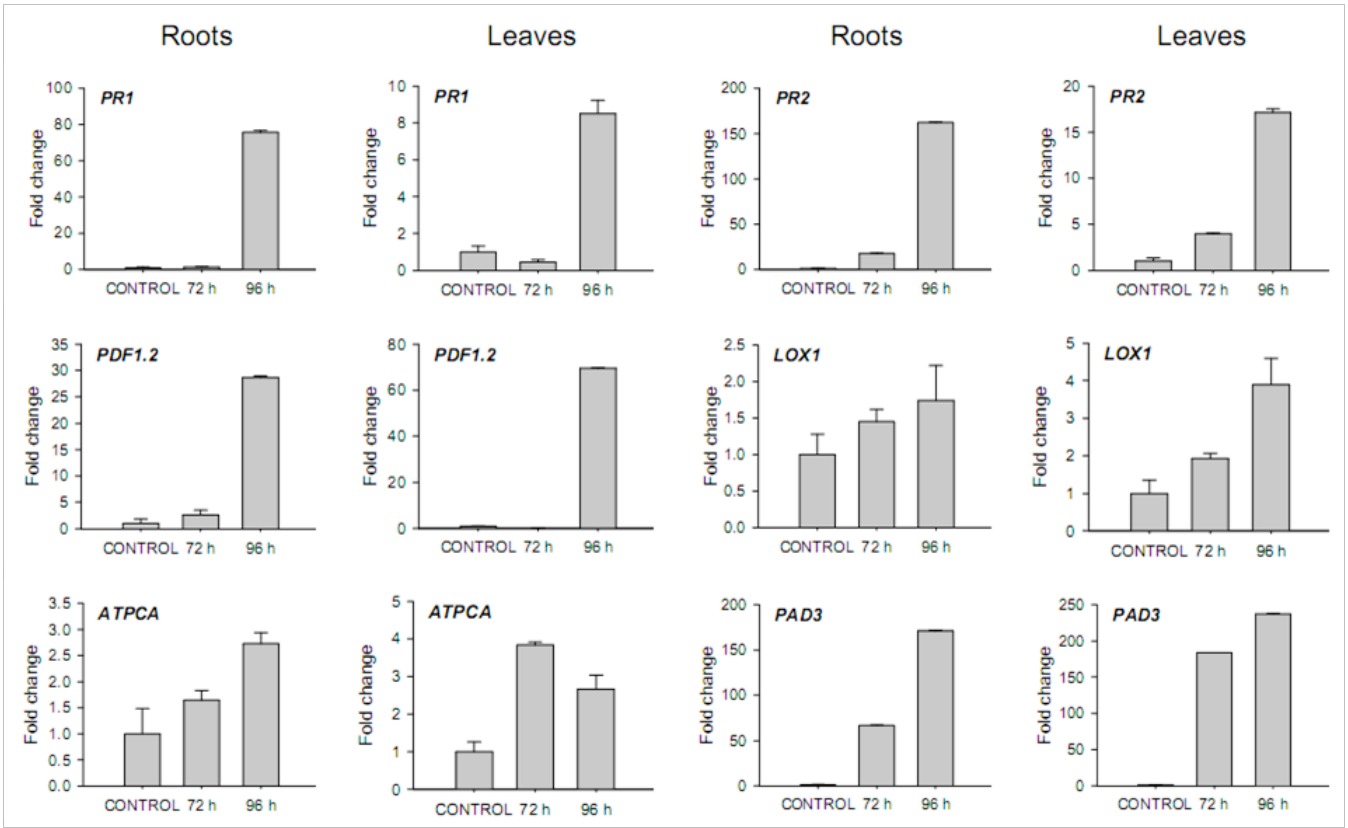

Table 1. Oligonucleotides used in this study for qRT-PCR analysis of defense-related genes in *Arabidopsis*.

Primer name	Sequence 5' to 3'	Gene amplified (GenBank Accession number)
PR1-f	atctaagggttcacaaccaggcac	
PR1-r	tgcctcttagttgttctgcgtagc	pathogenesis-related gene 1, (M90508)
PR2-f	aggagcttagcctcaccacc	
PR2-r	gaggatgagctcgatgtcagag	Beta-1,3-glucanase, (NM_115586.2)
LOX1-f	agacgttccaggccatggcag	
LOX1-r	cttgggtaaggatactcctgtg	Lipoxygenase 1, (NM_104376.2)
ATPCA-f	agacgttccaggccatggcag	
ATPCA-r	ggagagcgcaacaagatcag	Peroxidase, (NM_114770.2)
PAD3-f	cgatggagatgctctcaagttc	
PAD3-r	gtctccttgaccacgagc	Phytoalexin deficient 3, (NM_113595.3)
PDF1.2-f	cacccttatcttcgctgctc	
PDF1.2-r	ggaagacatagttgcatgatcc	Defensin, (NM_123809.3)
Actin-f	gactcagatcatgtttgagacc	
Actin-r	catgtaacctctctcggtaagg	ACTIN 8, (NM_103814.3)

Capitulo 2

Over-expression of *sm-1* in *Trichoderma atroviride* enhances plant defense response in tomato plants

Se sabe que la inducción de los sistemas de defensa en las plantas inducida por bacterias promotoras del crecimiento se debe a que estos microorganismos presentan patrones moleculares asociados a patogenicidad (PAMPs) o porque producen moléculas efectoras. En este sentido se ha reportado que hongos la especie *Trichoderma* producen a la proteína llamada SM-1 (small proteína 1), un inductor del sistema de defensa en plantas de algodón y de maíz. En esta investigación se caracterizó el papel de la proteína SM-1 de *T. atroviride* y de *T. virens* en la inducción de la respuesta de defensa sistémica en plantas de tomate. Para responder estas preguntas generamos cepas sobre-expresantes (OE) y KO del gen *sm*-1 en ambas especies. Las cepas mutantes y sobre-expresantes se co-cultivaron con plantas de tomate para evaluar su efecto en la supresión de patógenos foliares. En este trabajo observamos que las plantas inoculadas con las cepas OE fueron mas resistentes contra los patógenos foliares *P. syringae, B. cinerea* y *Alternaria solani*, comparada con las cepas KO y silvestre. Una vez obtenido estos resultados evaluamos *in vitro* la eficiencia de estas cepas para inducir la respuesta de genes relacionados con los sistemas de defensa a las 72 h de interacción. Cuando las plantas se inocularon con las cepas OE de *T. atroviride*, los genes glucanasa y peroxidasa fueron sobre-expresados tanto en raíces como en hojas. En la interacción *T. virens*-tomate encontramos que los genes de quitinasa y glucanasa fueron inducidos con la cepa OE, mientras que en hojas solo la peroxidasa fue sobrexpresada, comparado con las plantas que se inocularon con la cepa WT y KO, así como las no inoculadas. posiblemente la mejor eficiencia de las cepas OE en inducir protección sistémica contra patógenos se relaciona con mayores niveles de transcritos de algunos genes de defensa de la planta inducidos por la sobre-expresión de sm-1.

Over-expression of *sm-1* in *Trichoderma atroviride* enhances plant defense response in tomato plants

Running title: *Trichoderma spp.* SM-1 induces tomato defense response

Byline: plant-microbe interaction team

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Abstract

The SM-1 protein is produced and secreted by fungi belonging to the genus *Trichoderma*. It has been reported that this protein induces the defense system in cotton and maize plants. In this work, we evaluated the effect of the disruption and

over-expression of *sm-1* in *T. atroviride* during the interaction with tomato plants. We found that *sm-1* over-expressing strains increased the protection against foliar pathogens compared to the knockout and wild type strains. Additionally, we compared the levels of systemic protection induced by *T. atroviride* SM-1 over-expressing strains with their *T. virens* equivalents in tomato. *T. atroviride* strains induced more protection against *Alternaria solani* (76%)*, Botrytis cinerea* (74%) and *Pseudomonas syringae* (62%), while that *T. virens* over-expressing strains protected 52, 62 and 56 % respectively. In addition, we evaluated and compared the induction of the defense system related genes in tomato seedlings inoculated with the different strains from both species of *Trichoderma*, finding that SM-1 is an inducer molecules of defense genes in tomato. Ours data suggest that SM-1 is involved in the induction of defense response system by inducing the defense related genes in tomato.

Introduction

Fungi from the genus *Trichoderma* spp. are free-living organisms commonly found in soil or colonizing root surface of plants. *Trichoderma* has been widely used in biological control due to the production of antibiotics and mycoparasitic activity against plant phytopathogens, such as *Rhizoctonia solani*, *Fusarium oxisporum* and *Verticillium dahliae* (Harman *et al*., 2004; Chet and Inbar, 1994; Fravel, 2005). In addition,*Trichoderma* rhizosphere-competent strains can colonize either the root surfaces or the entire plant, a process that has been shown to have significant beneficial effects to plants such as root growth, plant growth enhancement and

increases in productivity (Yedidia *et al.,* 1999; Bailey *et al.,* 2006; Harman *et al.,* 2004, Howell, 2003). Recently, activation of plant defense responses by *Trichoderma* has been reported. Tomato plants treated with *Trichoderma hamatum* 382 and *Trichoderma harzianum* T22 were more tolerant to the bacterial phytopathogen *Xanthomonas euvesicatoria* 110c and to the fungus *Alternaria solani*, respectively, and the provided control was both spatially and temporally distant from the point of application and the disease resistance was accomplished by systemic modulation of the expression of stress genes (Alfano *et al*., 2007; Hanson and Howell, 2004). During root colonization, *Trichoderma* induced the defense system in cucumber, observed as an increase in chitinases and peroxidase activity in roots and leaves (Yedidia *et al*., 2000). The induction of systemic resistance by *Trichoderma* has been reported for both monocotyledoneous and dicotyledoneous plants (Harman *et al.,* 2004). This response involves recognition of the fungus by the systemic induced resistance (SIR), which is mediated by the phytohormones jasmonic acid (JA) and ethylene (ET). Such response is the closest analogue of induced resistance activated by rhizobacteria (Baker *et al*., 2003, Van Loon, 2007). In addition, *Trichoderma* induces the expression of *Pathogenesis-Related Protein* (PR) genes whose response is mediated by salicylic acid (SA), this response has been known as the systemic acquired resistance (SAR), which is also triggered by necrotizing pathogens (Martinez *et al.,* 2001). However, there is a cross-talk between the SAR and SIR pathways, mediated by the NPR1 transcription factor (Koornneef and Pieterse 2008). Indeed, there is evidence suggesting that during the interaction a molecular dialogue between plant and microbe takes place, which is provoked by

molecules produced by both the plant and/or the microorganism (Pozo *et al.,* 2005). In this sense, cucumber and cotton plants treated with *Trichoderma* or with the addition of culture filtrates of *Trichoderma* trigger the synthesis of terpenoids in the plant, and the expression of defense genes (Yedidia *et al*., 2000, Howell *et al.,* 2000). A number of elicitors of the plant defense system have been characterized as well as proteins with enzymatic activity, Avr homologues, oligosaccharides and low molecular weight compounds (Bailey, 1991, Baker, 1997). In *Trichoderma*, only proteins with enzymatic activity (xylanase and glucanase) had been described as elicitors, these proteins induce the expression of PR proteins, hypersensitive response and production of phytoalexins in several plants (Martinez, 2001, Calderon *et al*., 1993). Recently, the SM-1 protein from *T. virens* was identified and characterized*;* this is produced and secreted by the fungus at the early stages of the plant–*Trichoderma* interaction, suggesting a signaling role of SM-1 during this relationship. Indeed, the purified SM-1 protein efficiently elicited plant defense response locally and systemically against the cotton foliar pathogen *Colletotrichum* sp. the protective activity of SM-1 was associated with the accumulation of reactive oxygen species (ROS) and phenolic compounds, and increased levels of transcription of the defense genes regulated by SA and JA/ET, as well as genes involved in the biosynthesis of sesquiterpenoid phytoalexins (Djonovic *et al.,* 2006). Djonovic *et al.* (2007), reported that *sm-1* induces systemic protection in maize leaves inoculated with *Colletotrichum graminicola*, this protection was associated with the induction of JA and green leaf volatile-biosynthetic genes. In a recent study, Brotman and coworkers characterized a *T. asperellum* gene (*TasSwo*) encodes for an expansin-like protein (swollenin) involved in root colonization of

cucumber (Brotman *et al*., 2008). Furthermore, a swollenin synthetic 36mer peptide from the N-terminal carbohydrate binding module family 1 domain induced local defense response in root and leaves, and local protection against *Botrytis cinerea* and *Pseudomonas syringae* pv. lachrymans (Brotman *et al.,* 2008).

In this work we describe the systemic protection in tomato plants inoculated with *T. atroviride sm-1* over-expressing strains. An interesting comparison between *T. atroviride* and *T. virens* SM1 overexpression or disrupted strains was carried out. We also evaluated the protection against *Alternaria solani, Botrytis cinerea* and *Pseudomonas syringae.* Furthermore, expression analysis of tomato defense related genes, when the tomato plants interacted with the transformants *Trichoderma* strains is presented.

MATERIALS AND METHODS

Fungal and bacterial strains

T. virens Gv29-8, *T. atroviride* IMI206040, *Rhizoctonia solani* AG3 and *Sclerotium rolfsii* were used throughout this study. *Botrytis cinerea* and *Alternaria solani* strains were isolated from a tomato field at San Luis Potosi, Mexico, and identified by PCR amplification of rDNA 18S using the oligonucleotides ITS1 and ITS4 (White *et al.,* 1990). Fungal strains were routinely maintained on potato dextrose agar (PDA) (Difco, Franklin lakes, NJ, USA), hygromycin B (Invitrogen) was added at 100 μg/ml when it was necessary. *Pseudomonas syrigae pv* tomato DC 3000 was provided by Dr. Ariel Alvarez (CINVESTAV-Irapuato, Mexico), this strain was

grown on Kings B medium (King *et al*., 1954). *Escherichia coli* Top 10 F' was used for DNA manipulations, this strain was grown in LB medium routinely and antibiotic carbenicillin 100 μg/ml were added when it was necessary (Sambrook *et al*., 2001). Tomato seeds variety (EL CID F1 (Harris Moran Seed Company) were grown on Murashige and Skoog (1962) solid medium at 1X or in soil-less germination mix (Lambert Peat Moss Inc.).

Generation of *sm-1* over-expression and disruption constructs

For the over-expression constructs we isolated the *sm-1* gene from *T. atroviride* using the forward Tasm1OE-f and the reverse Tasm1OE-r primers (Table 1) including the *Xba*I and *Nsi*I restriction sites, respectively, to further clone the amplicon at the indicated restriction sites on pHyg-GFP vector (Casas-Flores *et al*., 2006). For *T. virens* we used the forward primer Tvsm1OE-f and reverse primer Tvsm1OE-r (Table 1) included the same restriction sites to further clone the PCR product in the pHyg-GFP vector.

DNA from *T. atroviride* and *T. virens* was extracted by using the method described by Raeder and Broda (Raeder and Broda, 1989). DNA from each fungus was used as template to amplify the *sm-1* gene by PCR using the primers previously described. The PCR products were cloned into pGEM-T-easy (Promega) and verified by sequencing. Then, the *sm-1* clones were double digested with *Xba*I and *Nsi*I restriction enzymes and subcloned in the pGFP-Hyg vector on the corresponding restriction sites under regulation of the pyruvate kinase gene (pki) promoter from *T. reesei*. The constructs were used to transform protoplast from *T. atroviride* and *T. virens* with their corresponding constructs.

To obtain the *sm-1* deletion construct, we took advantage of the Double Join-PCR technique (Yu *et al*., 2004). In, the first round of PCR we amplified the hygromycin phosphotransferase gene (*hph*) (primers hph-f and hph-r) and 1.5 kb of the 5' region flanking the *sm-1* gene (Tasm1KO5'-f and Tasm1KO5'-r to *T. atroviride* and Tvsm1KO5'-f and Tvsm1KO5'-r to *T. virens*) and 3' regions flanking the *sm-1* gene (Tasm1KO3'-f and Tasm1KO3'-r to *T. atroviride* and Tvsm1KO3'-f and Tvsm1KO3'-r to *T. virens*). (see table 1). The reverse primer designed for the up stream *sm-1* flanking region includes 30 pb that overlaps with the 5´ from *hph*, while the forward primer for the downstream *sm-1* flanking region overlaps with 30 pb with the 3´ from the hph gene. Then, we performed a fusion PCR by mixing the corresponding *sm-1* flanking regions with the *hph* amplicon obtaining the gene replacing constructs, products of the second round of PCR were eletrophoresed in an agarose gel and the expected band was purified and cloned in pGEM-T-easy (Promega). Clones of both fungi were used as template to amplify the constructions using their corresponding 5´and 3´ *sm-1* ORF flanking primers. The amplicons were used to transform protoplast from *T. atroviride* and *T. virens* with their corresponding constructs.

Transformation and selection of transformants

T. atroviride and *T. virens* WT strains were transformed with *sm-1* over-expression and disruption constructs by using the protoplast technique (Baek and Kenerley, 1998). Stable colonies resistant to hygromycin were selected for consecutive transfer of a single colony to PDA medium plus hygromycin 100 µg/ml. In order to verify the *sm-1* gene replacement a couple of primers flanking the complete

construct were designed on the genomes of *T. atroviride* and *T. virens* (table 1) to be used in combination with a couple of complementary primers designed on the *hph* gene. For *T. atroviride sm-1* gene replacement, a 202 bp upstream TaKO-f and a 299 bp downstream TaKO-r primers were designed. For the *T. virens sm-1* deletion verification, a 246 bp upstream 5' primer TvKO-f and a 137 bp downstream from the 3' primer TvKO-r from the construct were designed and used for PCR reaction in combination with the couple of primers designed for the *hph* gene. Strains from *T. atroviride* whose PCR product were 3.15 Kb to 5' region and 3.3 Kb to 3' region and strains from *T. virens* whose PCR product were of 3.16 Kb to 5' region and 3 Kb to 3' region were used to further analyze the *sm-1* expression.

Growth and mycoparasitic phenotypes of the transformants

Selected strains were compared with their respective WT strains for colony morphology, radial growth and sporulation. Agar plugs from actively growing colonies were inoculated in the center of PDA plates. Plates were placed in the darkness at 28°C, and after five days, plates were visually inspected for production of conidia, and morphology of the colony.

Mycoparasitic activity of the transformants were analyzed and compared to the WT activity. Agar plugs from actively growing colonies were inoculated on the edge of the plates while in the opposite edge were inoculated with *R. solani* or *S. rolfsii.* These confrontations were placed in the darkness at 28°C and were analyzed after seven days of interaction. Mycoparasitic activity of transformants and WT strains

was visually inspected with the aim to evaluate their capacity to overgrow and to stop the growth of phytopathogenic fungi.

RT-PCR analysis of the *sm-1* over-expressing and disruption candidate strains

sm-1 expression of *T. atroviride* and *T. virens* WT, over-expression and gene replacement strains were assessed by semi quantitative reverse transcriptase analysis (sqRT-PCR). Conidia from the different strains were used to inoculate PDA medium overlaid with a sheet of cellophane. The inoculated plates were incubated at 28°C, after three days mycelia was harvested and total RNA was extracted with TRIzol Reagent (Invitrogen) as described by the manufacturer. Then, 2 μg of total RNA was treated with rDNase I (Ambion) and reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen). The synthesized cDNAs were used as template to amplify the *sm-1* gene. Actin gene was used as loading control. PCR amplicons were electrophoresed, stained with ethidium bromide and photographed.

Expression analysis of transformants strains by SDS-PAGE

For this experiment 100 ml of Vogel medium (Vogel, 1956) supplemented with sucrose 1.5 % was inoculated with a conidial suspension of the transformants strains of *T. atroviride* and *T. virens* to final concentration of 10^6 conidia ml^{-1}. The inoculated mediums were incubated in a rotary shaker at 200 rpm to 28 °C for 6 days. After the incubation the liquid mediums were filtered through a 0.45-μm filter

(Millipore). Proteins were precipitated by 80 % ammonium sulfate (Fermont). Pellets were resuspended in 10mM Tris, pH 7.8, and dialyzed against the same buffer (8 kDa, SPECTRUM). Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Proteins were subjebted to SDS-PAGE using 40 µg and stained with Coomassie Brilliant Blue.

Protection assays in tomato plants against *A. solani, B. cinerea* and *P. syringae*

Cultures of *A. solani* and *B. cinerea* were grown for seven days on PDA at 28°C with a 12 h photoperiod. Conidia were harvested and suspended in distilled water. Conidia were counted by using a hematocytometer and the spore suspension was adjusted to 10^6 and 10^5 conidia ml^{-1} for *B. cinerea* and for *A. solani*, respectively. *P. syringae* was grown in Kings B medium at 200 rpm for 48 h at 28°C and the suspension was adjusted to OD= 0.2. Break-Thru, (Goldsmidt Chemical Corporation) was added to a final concentration of 0.1% as surfactant agent.

Tomato seeds were inoculated with 15 µl of 10^6 conidia ml^{-1} of *T. atroviride* WT, OE 1.1, OE 2.1, OE 3.1 and KO9. *T. virens* WT, OE 2.1, OE2.2, OE6.2 and KO2. Control (non-treated seeds) and treated seeds were planted in pots (10.6 x 8 cm) containing germination mix (Lambert Peat Moss Inc). Twenty-four h later seeds were irrigated with MS (0.3X), to allow the fungi to colonize the rhyzosphere. Six days later, plants were watered with the nutritive solution HUMIFERT (Cosmocel) to doses of 3 ml $liter^{-1}$ in water. A total of 8 plants were used for each treatment.

Fifteen days later treated and no treated plants with the *Trichoderma* strains were inoculated with *B. cinerea, A. solani* and *P. syringae*, respectively. Three leaves from each plant were inoculated with 10 μl of the pathogen solution on the adaxial side, away from mid vein of the leaf. Inoculated plants were placed in the greenhouse under controlled conditions and everyday were irrigated to increase relative humidity. Eight days post-incubation leaf damage area was evaluated. Percentage of leaves damage was calculated obtaining the total leaf area and the total leaf area damaged and the ratio between these values gave the percentage of damaged area. For each treatment, we used 8 plants, from each plant three leaves were inoculated with the pathogen. Each experiment was repeated three times. Experimental data were subjected to analysis of variance, with values $P < 0.0001$, LSD range test $\alpha < 0.05$ considered significant.

Expression analysis of tomato defense related genes

Expression of defense related genes was analyzed in tomato seedlings (roots and leaves) grown *in vitro* with or without *T. atroviride* WT, OE2.1 and KO9, or with *T. virens* WT, OE2.2 and KO2, respectively. Fourteen days old plants were inoculated with 15 μl of 10^6 conidia ml^{-1} with the different strains as mentioned before, co-cultures were allowed to interact for 72 h. Tomato roots and leaves were harvested, separated and frozen in liquid nitrogen at the indicated times. Total RNA was extracted by using the Concert RNA extraction solution (Invitrogen) as described in the protocol provided by the manufacturer.

Expression of plant defense related genes was assessed by quantitative real-time RT-PCR. The tomato gene specific primer pairs were designed with primer express 3.0 program (Applied Biosystems) based on sequences available in GenBank database (see table 1), namely: Chit (chitinase, gi|19190), Gluc (glucanase, gi|498925), Pod (peroxidase, gi|1161565), Hmgr (3-hydroxy-3-methylglutaryl CoA reductase, gi|16304119) and actin as an internal control (gil1498365). Total RNA was DNase-treated using rDNase I (Ambion). Then, 2 μg of total RNA was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen). The qRT-PCR reaction was performed using the kit Fast Syber Green Master Mix (Applied Biosystems) and 1 ng of cDNA. The experiments were performed using the Abiprism 7500 fast Real-Time PCR system (Applied Biosystems) with the conditions suggested by the manufacturer. The absence of primer-dimers was confirmed in reactions without RNA. The experiments were independently repeated two times and each reaction was performed in triplicate using a relative quantification analysis. The expression of each specific gene was normalized versus the control reference with the formula $\Delta\Delta$CT.

Results

Generation of *sm-1* deletion and over-expression strains

In order to elucidate the role of *T. atroviride* SM-1 to induce systemic resistance in tomato plants, we generated *sm-1*-deletion (KO) and over-expressing (OE) strains. With the aim of comparing the behavior of *T. atroviride* against *T. virens* during the interaction with tomato, the corresponding *sm-1* KO and OE strains from *T. virens*

were also generated. For *sm-1* gene disruption in both *Trichoderma* species, the Tasm1KO and Tvsm1KO deletion constructs with hygromycin phosphotransferase gene were obtained using the double-joint PCR strategy (Yu *et al*., 2004). Once we obtained these constructs, *T. atroviride and T. virens* wild type strains were transformed as described by Baek and Kenerley (1998). Disruption of *sm-1* was confirmed by PCR using an upstream forward primer and a downstream reverse primer designed on the *T. atroviride* (primers TaKO-f and TaKO-r) and *T. virens* (primers TvKO-f and TvKO-r) chromosome flanking the region used for the disruption constructs, combined with a couple of complementary primers designed on the hygromycin phosphotransferase gene (Fig. 1A). Figure 1B and 1C show the expected 3,152 and 3,322 bp amplicons for six *T. atroviride* candidates (KO1, KO2, KO5, KO8, KO9 and KO11). For *T. virens* a total of five stable transformants were tested for gene disruption observing the expected 3,167 and 3,036 pb amplicons in two of the five candidates (KO2 and KO5) (Fig. 1D and 1E). *Trichoderma* genomic DNA from the wild type strains was used as negative control.

In order to generate the SM-1 over-expressing strains, the expression vector pGFP-Hyg (Casas-Flores *et al.,* 2006) was used to clone the *sm-1* gene driven by the constitutive Pyruvate Kinase (pki) promoter (Zeilinger *et al.,* 1999). Ten potential over-expression (OEs) transformants of *T*. *atroviride* and *T*. *virens* were selected on the basis of their hygromycin resistance and by their similar growth and sporulation phenotype when compared to the WT strains. Both OE and KO selected strains of *T. atroviride* and *T. virens* did not show notorious changes in growth rate, colony appearance and pigmentation during sporulation. Only the *T*. *atroviride* OE1.1 strain, showed a slow growth rate when compared to the WT

strain (data not shown). We selected three mitotically stable transformants for *T. atroviride* (OE1.1, OE2.1 and OE3.1) and three for *T. virens* (OE2.1, OE2.1 and OE6.2). All strains were assayed for expression analysis of *sm-1.*

Growth and mycoparasitic activity of the transformants strains

Growth phenotype of *T. atroviride* and *T. virens* transformant strains was analyzed on PDA and compared wit their respective WT strain. We did not found differences in color, growth and conidiation of the evaluated strains (data not shown).

Mycoparasitic activity of the transformants were analyzed and compared to the WT strain activity. Agar plugs from actively growing colonies were inoculated on the edge of the plates while in the opposite edge were inoculated with *R. solani* or *S. rolfsii.* We found that all transformants strains of *T. atroviride* and *T. virens* were no affected in their activity of mycoparasitism during the genetic manipulation and the *sm-1* gene is not involve in this fungus ability because all the strains were able to overgrow and stop effectively the grow of *R. solani* (Fig. 2). Similar results were obtained when the confrontation was made against *Sclerotium rolfsii* (data not shown).

Level expression of the *sm-1* gene in the OE and KO strains

Mycelia from 72 h old grown colonies from WT, OE and KO strains were scraped and collected for total RNA extraction in order to synthesize cDNA to evaluate *sm-1* expression. Semi-quantitative gene expression for *sm-1* was performed for the selected over-expressing strains of *T. atroviride* OE1.1, OE2.1 and OE3.1 (Fig. 3A), and for *T. virens* OE2.1, OE2.2 and OE6.2 (Fig. 3B). Two of the *T. atroviride*

OE strains showed higher *sm-1* transcript levels when compared to the WT, except for OE1.1 that showed the same levels as WT strain (Fig. 3A). The three *T. virens* OE strains, showed slightly higher levels of *sm-1* when compared with the WT strain (Fig. 3B). The RT-PCR analysis showed no transcripts for *sm-1* gene in any of the KO strain (*T. atroviride* KO9 and *T. virens* KO2, Fig. 3A and 3B, respectively). Transformant strains were grown in Vogel minimal medium in order to evaluate their production level of SM-1 protein. The cultures were filtered; precipitated, dialyzed and the proteins were subjected to SDS-PAGE. In this experiment we found that over-expression strains of *T. atroviride* and *T. virens* produced more protein compared with WT strains control and the KO strains of both species the SM-1 protein was absent (data not shown).

Induced systemic resistance against foliar pathogens in tomato plants

In order to investigate the ability of the *Trichoderma* strains to protect systemically to tomato seedlings against the fungus phytopathogens *A. solani, B. cinerea* and the bacterial phytopathogen *P. syringae*. Tomato seeds were inoculated with *T. atroviride* KO, OE and WT strains. Fifteen days post-inoculation, tomato leaves were inoculated with fungal spores or with a bacterial suspension. Disease lesion was evaluated 8 days post-inoculation of the pathogens. We observed that plants inoculated with over-expressing strains from *T. atroviride* gave more protection compared to WT, the best strain was OE2.1, followed by OE3.1 and OE1.1 respectively, while plants treated with the KO *T. atroviride* strain presented major damage when inoculated with *B. cinerea or A. solani* compared to the WT strain, however never showed the same damage as non-treated plants (Fig. 4A-C).

Interestingly, plants treated with the *T. atroviride* KO strains, post-inoculated with *P. syringae*, showed less damage than those treated with the WT strain (Fig. 4A-C). The same experiment was conducted for the *T. virens* OE, KO and WT strains, we found that over-expressing strains showed more protection against foliar pathogen *A. solani, B. cinerea* and *P. syringae* when compared with the WT, while inoculated plants with KO strain presented major lesion than WT strain. The non-treated plants showed more damaged compared with treated plants (Fig. 5A-C). These results suggest that the SM-1 protein is involved in the plant defense against foliar plant pathogens.

Tomato defense related genes expression during the interaction with the OE, KO and WT strains

In order to determine the tomato molecular response when it was inoculated with the OE and KO strains, the expression profiles of defense related genes were measured by quantitative RT-PCR. A set of four pairs of primers of genes related to different plant defense pathways were designed on the basis of sequences available in the GenBank databases to be amplified in *Lycopersicum esculentum*: Chit (Chitinase: gi|19190) and Gluc (Glucanase: gi|498925) (PR proteins), Pod (Peroxidase: gi|1161565) (related to oxidative burst and hypersensitive reactions), and Hmgr (3-hydroxy-3-methylglutaryl CoA reductase: gi|16304119) (terpenoid phytoalexin pathway), see table 1. Our results showed that the expression of these defense related genes were induced locally and systemically. During the *T. atroviride*-tomato interaction, glucanase, chitinase y peroxidase genes were induced and up-regulated in roots of inoculated plants with OE compared with WT

and KO strains and the untreated seedlings (Fig. 6A). Furthermore, in the leaves the glucanase, peroxidase genes were up-regulated with OE strain but interestingly the chitinase was up-regulated by KO strain followed by the WT and the OE inoculated seedlings respectively (Fig. 6B). The HMGR gene was not induced neither roots nor leaves (Fig 6B). These results can be an explanation why plants inoculated with OE strains presented less damage when these were inoculated with the foliar pathogens compared with the inoculated WT and KO plants respectively.

For the *T. virens*-tomato interaction we detected highest level of chitinase and glucanase transcripts in roots when plants were inoculated with OE and high levels of peroxidase in KO inoculated plants while HMGR only was induced by WT (Fig. 7A). Expression analysis of defense related genes in tomato leaves showed that glucanase and chitinase genes were up-regulated when plants were inoculated with WT and KO strains while peroxidase was up-regulated with OE and KO strains (Fig. 7B) and the HMGR gene in the leaves only was induced by WT and KO Plants (Fig. 7B).

DISCUSSION

T. atroviride induces resistance against pathogens

It is well known that *Trichoderma* species induce diseases resistance in several plants, however, the *Trichoderma* research community has focused its efforts mainly in the study of the mechanisms of mycoparasitism and antibiosis, while the molecular mechanisms of the resistance in plants induced by *Trichoderma* have been poorly studied (Harman *et al*., 2004). In a previous work, it was reported that

purified *sm-1* protein from *T. virens* induced plant defense response and provided high levels of systemic resistance in cotton plants against the foliar pathogen *Colletotrichum* spp. (Djonovic *et al*., 2006). During the development of this work, the Charles Kenerley group reported the molecular analysis of *sm-1* over-expressing and knockout strains and their effect on maize seedlings, observing a relevant role of *sm-1* in the plant defense response and protection against *Colletotrichum graminicola*. It was also demonstrated that this protection was accompanied by the induction of JA and volatile-biosynthetic genes, while those dependent of SA pathway did not suffered substantial changes (Djonovic *et al*., 2007). With the aim of determining if the *T. atroviride* and *T. virens sm-1* protein induces protection against bacterial and fungal aerial phytopathogens with different lifestyle in tomato, we generated the *sm-1* deletion and over-expressing strains of both fungal species. In this study we showed that *T. atroviride* and *T. virens* induced systemic resistance in tomato seedlings against both the necrotrophic fungal pathogens *A. solani,* and *B. cinerea*, and against the biotrophic bacterial pathogen *P. syringae*. In a comparative analysis between *T. atroviride* and *T. virens* we observed different level of protection in plants inoculated with the OE strains or even among them when compared with the KO or the WT, Those OE strains that produce more protein, but not necessarily produced more transcript, protected better the tomato plants against the three pathogens tested. The *T. atroviride* and *T. virens* WT strains treated plants showed different level of suppression with the different pathogens tested, however the *T. atroviride* OE strains protected better the tomato plants against the tested pathogens, protruding the OE2.1. These results suggest that *T. atroviride sm-1* is an elicitor of induced

systemic response in this organism. Tomato plants treated with the deletion strains showed less protection than those plants treated with the WT and OE strains, but never reached the damage observed on the non-treated control. Interestingly, plants treated with *T. atroviride* KO9 and post-inoculated with *P. syringae*, showed higher levels of protection than the WT strain. Comparing our results with those obtained by Djonovic *et al*. (2007), we observed similar results, confirming that *sm-1* plays important roles on inducing systemic response in plants. In addition, our results showed that in some cases that *T. atroviride* and *T. virens sm-1* deletion strains induced the same levels of protection as the WT strain against *A. solani* and *B. cinerea* respectively. Interestingly, the KO9 induced better the systemic response against *P. syringae.* These results could be explained by the existence of others elicitors in *Trichoderma* including cellulases, xylanases, endopolygalacturonases, expansin like proteins or peptaibols, produced by *Trichoderma* species. It has been described that these proteins act as elicitors of local and systemic plant defense response against several bacterial and fungal pathogens by inducing the expression of defense related genes (Martínez *et al*., 2001; Ron *et al.,* 2000; Viterbo *et al*., 2007; Morán *et al*., 2009; Brotman *et al.,* 2008). Looking through the *T. atroviride* and *T. virens* genomes we found near 15 similar proteins to *sm-1*. Taken together our results we hypothesizes that such hypothetical elicitors could be compensating the absence of *sm-1* in *Trichoderma* KO strains, further analysis on these proteins will help to elucidate their roles on the induction of plant defense system against pathogens. Generation of double and triple mutants in *Trichoderma* species will help to elucidate the role of proteins on the induction of plant defense response, as well as the mechanism of protection

conferred by *Trichoderma* to plants. Further *in vivo* studies of the effect of SM-1 in several plants could reveal its mode of action in the plant defense response.

During plant invasion by a pathogen a plethora of biochemical and genetic events occur. An increase in endogenous salicylic acid (SA) and the synthesis of pathogenesis-related (PR) proteins is one of the most common responses triggered in plants following an infection with inducing microorganisms (Durrant and Dong, 2004; Van Loon and Van Strien, 1999). The local and systemic resistance induced by *T. harzianum* in cucumber has been attributed to the penetration and colonization of plant root epidermis which is accompanied by an increased in the enzymatic activity of peroxidase and chitinase which are involved in JA/ET and SA response respectively (Yedidia *et al*., 1999). Here, we are reporting that *T. atroviride* and *T. virens* are able of induced systemic disease resistance accompanied by increased levels of expression of defense-related genes. Chitinase and glucanase (PR protein) mRNAs genes were up-regulated in the roots and leaves of the plants inoculated with *T. atroviride* OE strain, compared with plants inoculated with the WT. Interestingly, transcription levels of PR genes were higher in tomato seedlings when inoculated with *T. atroviride* and *T. virens* KO strains compared with the WT strain. In agree with our protection data against phytopathogens, these results suggest that these genes are involved in the systemic resistance in tomato mediated by *T. atroviride* and *T. virens* SM-1 protein.

An alternate pathway in the plant response to pathogens is mediated by JA, which is characterized by the production of a cascade of oxidative enzymes (peroxidases, polyphenol oxidases and lipoxygenases) and the accumulation of low-molecular weight compounds known as phytoalexins (Choudhary *et al.,* 2007). In this sense a

gene encoding to a peroxidase from plants was reported to be involved in the response to pathogen mediated by JA/ET whose activity has been related to resistance responses, including lignifications and suberization, cross-linking of cell wall proteins, generation of reactive oxygen species and the synthesis of phytoalexins, these last show antifungal activity themselves (Quiroga *et al*., 2000; Bolwell and Wojtaszek, 1997; Caruso *et al*., 2001). In this study, peroxidase was both local and systemically induced when tomato plants were treated with the different *T. atroviride* and *T. virens*, however the higher transcripts in roots and leaves was induced by OE of *T. atroviride*, this result it is in agreement with that reported for cucumber and cotton plants inoculated with *T. harzianum* or *T. virens* respectively (Shoresh, *et al*., 2005; Djonovic *et al*., 2006). These results suggest that high levels of *T. atroviride sm-1* preferentially induce the expression of JA/ET genes compared with the *T. virens sm-1*, such asseveration is supported by our results where the *T. atroviride* OE treated plants presented less foliar damage than tose plants inoculated with *T. virens* OE.

In our study, the HMRG gene was induced both locally and systemically in tomato by *T. virens* WT and OE strains, while tomato KO treated plants repress the expression of this gene, suggesting that SM-1 protein is responsible of such induction. In contrast, this gene was induced in roots by *T. virens* WT strain but it was not induced by OE y KO. Expression of this gene in leaves was observed in plants inoculated with WT and KO strains. In this sense it was reported that Hmg 1 was strongly induced in potato tissue by wounding, but the wound induction was strongly suppressed by treatment of the tissue with the fungal elicitor arachidonic acid or by inoculation with an incompatible or compatible race of the pathogen

oomycete *Phytophthora infestans* (Choi *et al*., 1992). The hmg2 and hmg3 mRNAs also accumulated in response to wounding, but in contrast to hmg 1, these mRNAs were strongly enhanced by arachidonic acid or inoculation of the pathogens (Choi *et al*., 1992).

We found that induced systemic resistance by *T. atroviride* and *T. virens* was trough both pathway SA and JA. In base to these results we can hypothesized that *Trichoderma* initially is recognized by the plant as necrotrophic microorganisms, triggering the SA response until the root colonization to further establish a relationship as symbiont through the JA/ET pathway as rhizobacteria does during their interaction with plants. Kolarev and coworkers (Korolev *et al*., 2008) showed that *T. harzianum* Rifai T39 induce systemic resistance in *Arabidopsis* through ethylene/jasmonic acid against *B. cinerea*, they also demonstrated that the salicylic acid pathway is not involved in conferring resistance to this pathogen. Our results suggest and overlapping of the different analyzed pathway in this work, to suppress foliar pathogens.

Taking into account all data together we can conclude that the induction of defense response mechanisms in plants by *Trichoderma* with the consequently response to pathogens would depend of the species involved during the tripartite interaction. In summary, this research provides genetic evidence of the role of SM-1 in *T. atroviride* and *T. virens* to induce protection against foliar pathogens with different life styles in tomato. In addition, our results suggest that there exist more that one pathway used by *Trichoderma* to induce the systemic resistance in plants. In addition, the expression of JA/ET, SA and phytoalexin genes suggest an overlapping in these pathways to suppress the infection and dissemination of

necrotrophic and biotrophic phytopathogens in tomato. To our knowledge this is the first report involving directly the SM-1 protein in to the induction of systemic resistance to tomato plants.

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49.

FIGURE LEGENDS

Figure 1. PCR analysis for confirmation of *sm-1* gene replacement in *T. atroviride* and *T. virens* genomes. A). Schematic representation of *sm-1* endogenous gene

(upper panel) and the disruption construct (lower panel) for *T. atroviride*. B) Total DNA from *T. atroviride* TaKO1, TaKO2, TaKO5, TaKO8, TaKO9 and TaKO11 was used for PCR analysis. Total DNA from *T. virens* TvKO2, TvKO3, TvKO4, TvKO5 and TvKO6 were subjected to PCR. A homologous double recombination event produced 3.15 kb and 3.16 kb amplicons for the 5´ flanking region, using the corresponding forward primer 1 (TaKO-f) designed on *T. atroviride* and (TvKO-f) *T. virens* genome, respectively, in combination with the reverse primer 2 designed on the *hph* gene (hph-r). The expected amplicons sizes for 3´flanking region were 3.3 and 3.0 kb for *T. atroviride* and *T. virens* respectively using the forward primer 3 designed on the *hph* gene (hph-f) in combination with their respective reverse primer 4 designed on *T. atroviride* (TaKO-r) and *T. virens* (TvKO-r) genomes. Wild type total DNA from *T. atroviride* and *T. virens* was used as negative control in PCR reactions.

Figure 2. Mycoparasitic activity of the transformants strains of *T. atroviride* or *T. virens* against *R. solani.* To this confrontation agar plugs from actively growing colonies of *Trichoderma* strains were inoculated on the edge of the plates (right side) while in the opposite edge (left side) were inoculated with *R. solani.* The placed in the darkness at 28°C and were analyzed after seven days of interaction.

Figure 3. *sm-1* Semiquantitative RT-PCR analysis of WT, OE and KO strains. Total RNA was extracted from mycelia grown on PDA for 72 h of *T. atroviride* over-expression and deletion mutants (OE1.1, OE2.1, OE3.1 and KO9 strains) (A) and *T. virens* over-expression and disruptants (OE2.1, OE2.2, OE6.2 and KO2) (B). *sm-1* specific primers were used to measure the expression level of *sm-1* gene. Actin gene amplification products were used as loading controls. Wild type strains

were used as reference for *sm-1* gene expression.

Figure 4. Effect of *T. atroviride* WT, OE and KO strains on induced systemic resistance in tomato seedlings against the phytopathogens *A. solani, B. cinerea* and *P. syringae*. The graphs illustrate the levels of systemic disease protection observed in each treatment. Tomato seeds were inoculated with 15 μl of 10^6 conidia ml^{-1} of WT, OE and KO strains from *T. atroviride* and were inoculated 15 days post-inoculation with the plant pathogens *B. cinerea* (A), *A. solani* (B) and *P. syringae* (C). Foliar damage was evaluated 8 days post inoculation with the pathogens, taking the lesion area of three inoculated leaves per plant from a total of 8 plants. Each bar represents an average of three independent experiments given as arbitrary units. Letter indicates statistically significant differences (analysis of variance, $P < 0.0001$, LSD range test $\alpha < 0.05$).

Figure 5. Effect of *T. virens* WT, OE and KO strains on induced systemic resistance in tomato seedlings against the plant pathogens *A. solani, B. cinerea* and *P. syringae*. The graphs illustrate the levels of systemic disease protection observed in each treatment. Tomato seeds were inoculated with 15 μl of 10^6 conidia ml^{-1} of WT, OE and KO strains from *T. virens* and were inoculated after 15 days with *B. cinerea* (A), *A. solani* (B) and *P. syringae* (C). Foliar damage was evaluated 8 days post inoculation of the pathogens taking the lesion area of three inoculated leaves per plant of a total of 8 plants. Each bar represents an average of three independent experiments given as arbitrary units. Letter indicates statistically significant differences (analysis of variance, $P < 0.0001$, LSD range test $\alpha < 0.05$).

Figure 6. Quantitative expression analysis of defense-related genes in tomato seedlings inoculated with *T. atroviride* WT, OE and KO strains. Total RNA from roots and leaves of fourteen days old tomato plants inoculated with 10 μl of 10^6 conidia ml^{-1} of *T. atroviride* WT, OE2.1, KO9 strains was extracted after 72 h post-inoculation, and subject to quantitative real-time RT-PCR assays. Expression profile of four defense related genes of *Lycopersicum esculentum* from different signal transduction pathways was assessed by using specific primers: Chitinase and Glucanase (Pathogenesis Related proteins), Peroxidase (related to oxidative burst and hypersensitive reactions), and Hmgr (terpenoid phytoalexin pathway). Actin gene was used as an internal control.

Figure 7. Expression analysis of defense-related genes in tomato seedlings inoculated with *T. virens* WT, OE and KO strains. Total RNA from roots (A) and leaves (B) of fourteen days old tomato plants inoculated with10 μl of 10^6 conidia ml^{-1} of *T. virens* OE2.2, KO2 strains along with the wild type (WT) was extracted after 72 h post-inoculation, and subject to quantitative real-time RT-PCR analysis. Expression profile of four defense related genes of *Lycopersicum esculentum* from different signal transduction pathways was assessed by using specific primers: Chitinase and Glucanase (Pathogenesis Related proteins), Peroxidase (related to oxidative burst and hypersensitive reactions), and Hmgr (terpenoid phytoalexin pathway). Actin gene was used as an internal control.

Figure 1.

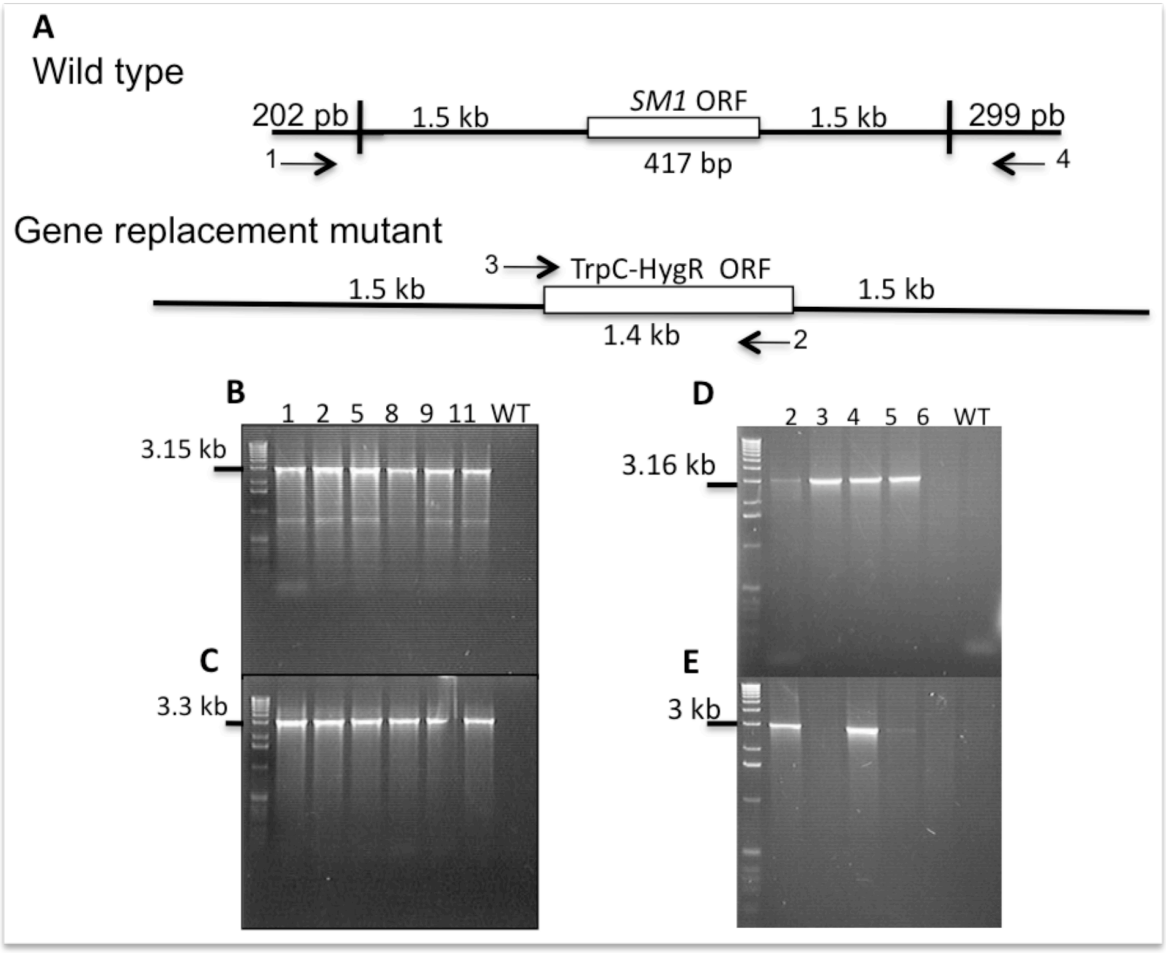
A
Wild type
202 pb
1.5 kb
SM1 ORF
1.5 kb
299 pb
1
417 bp
4
Gene replacement mutant
3
TrpC-HygR ORF
1.5 kb
1.5 kb
1.4 kb
2
B
1 2 5 8 9 11 WT
3.15 kb
C
3.3 kb
D
2 3 4 5 6 WT
3.16 kb
E
3 kb

Figure 2

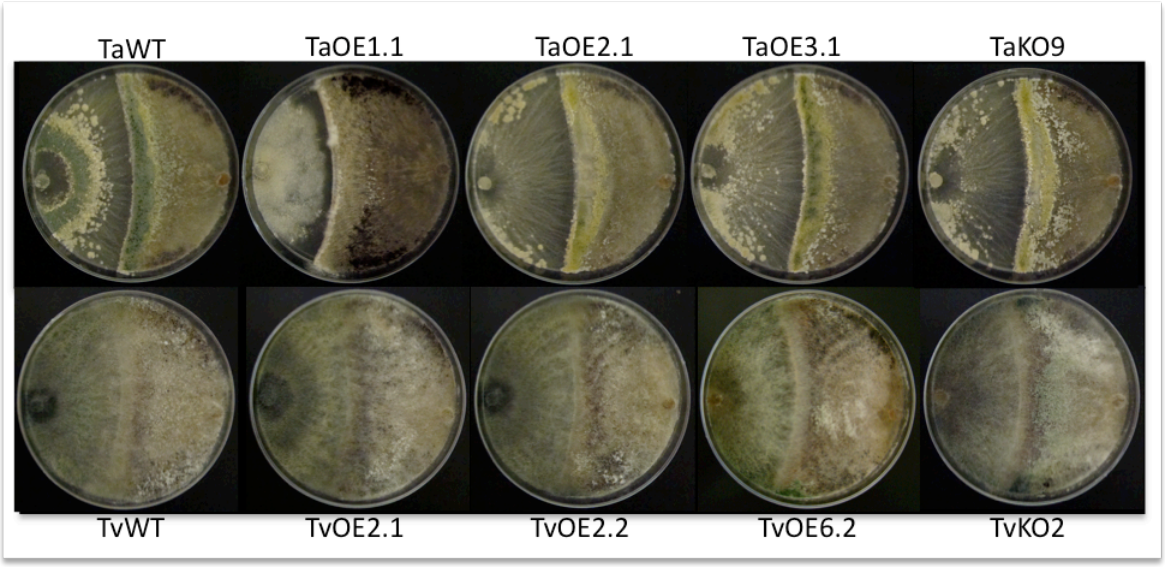

Figure 3.

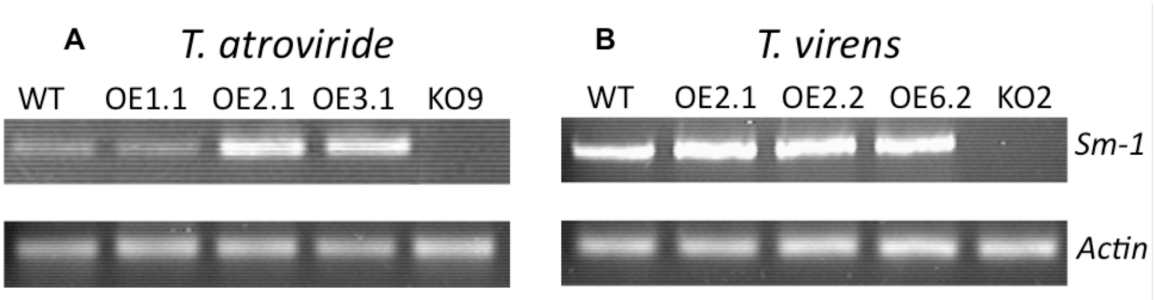

Figure 4.

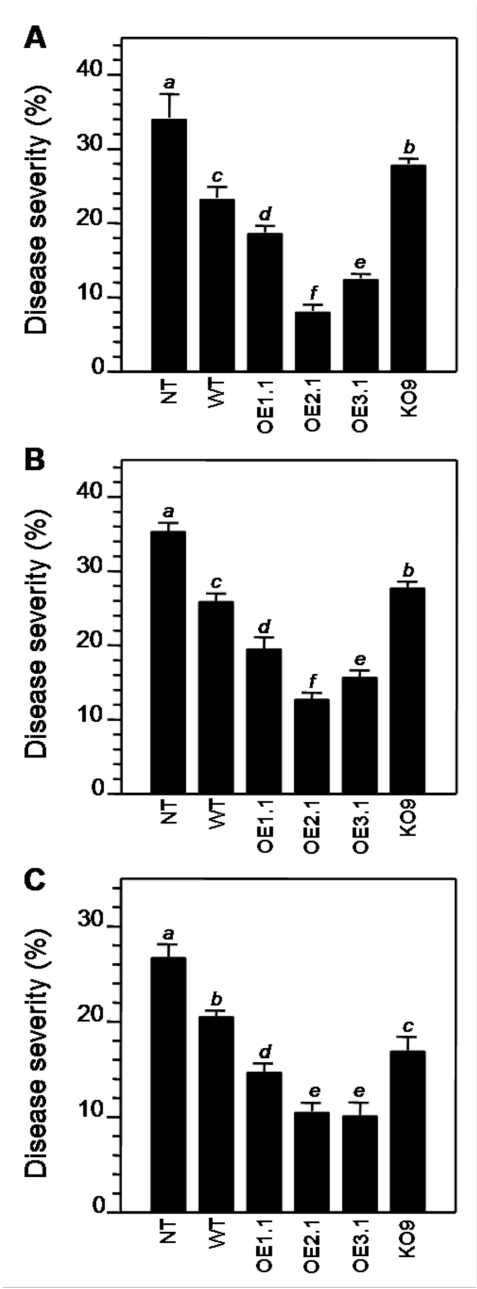

Figure 5.

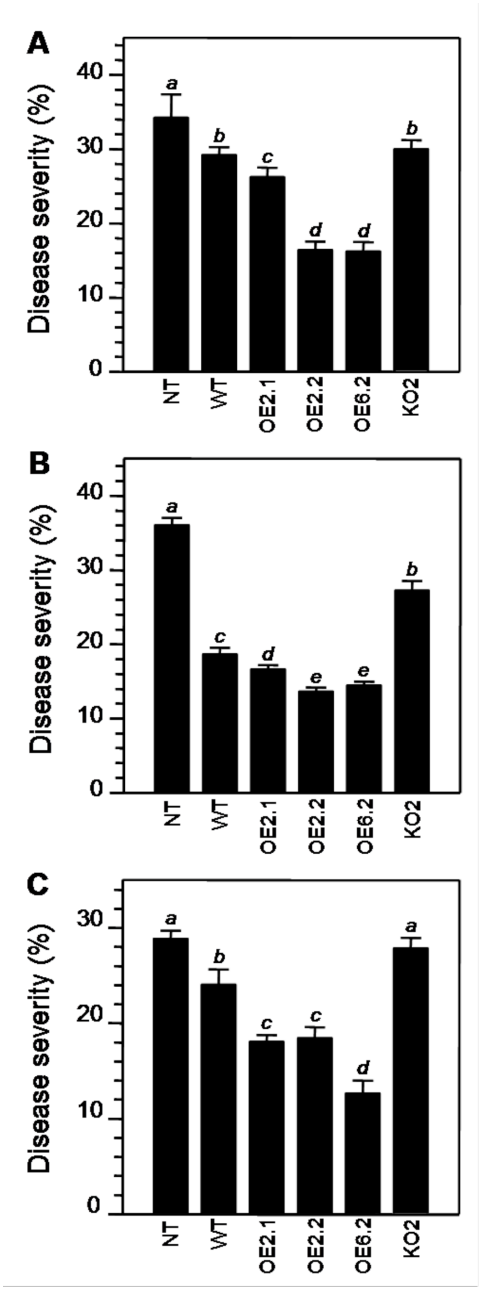

Figure 6

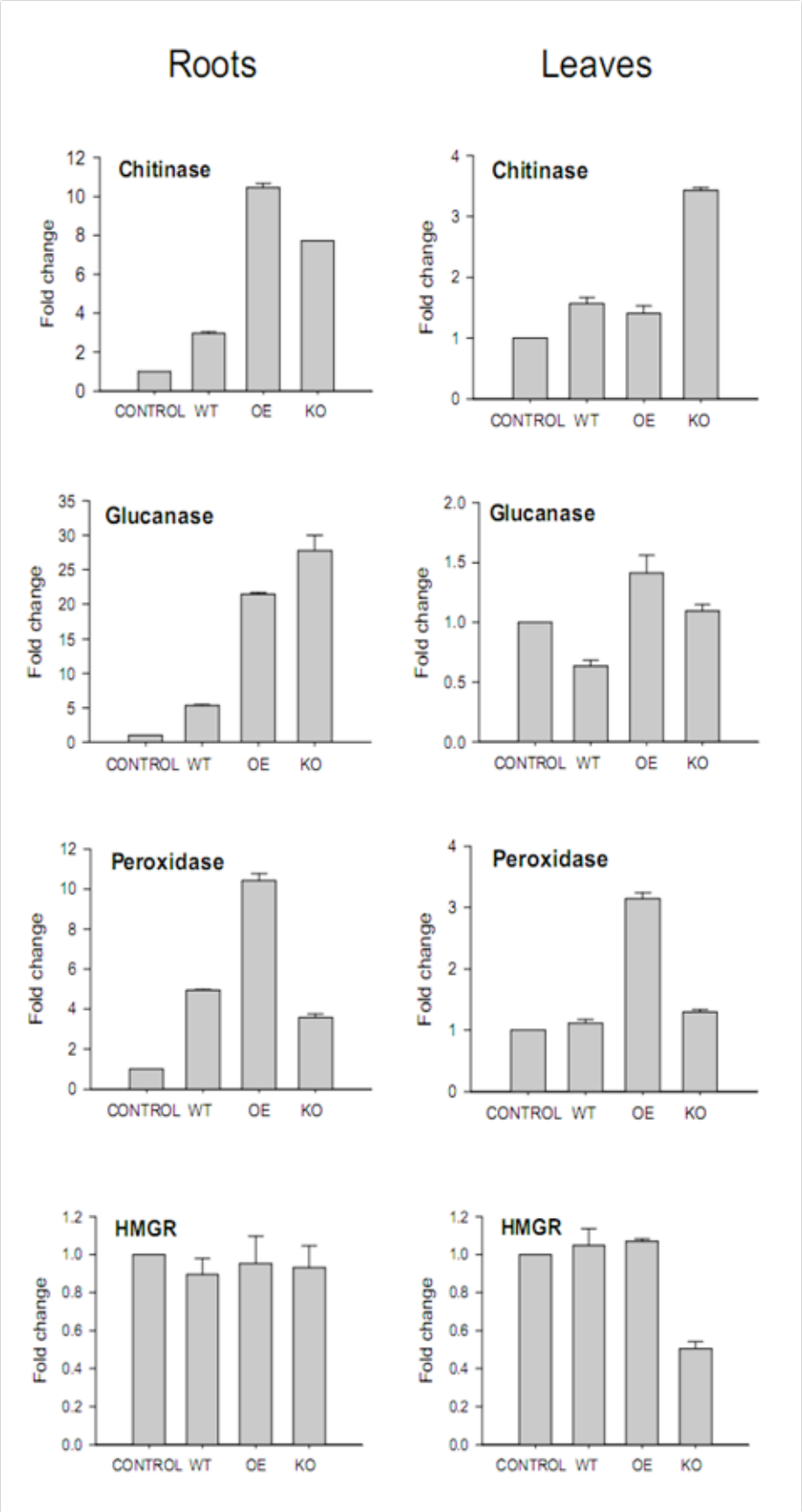
Roots
Leaves
Chitinase
Glucanase
Peroxidase
HMGR
Fold change
CONTROL WT OE KO

Figure 7.

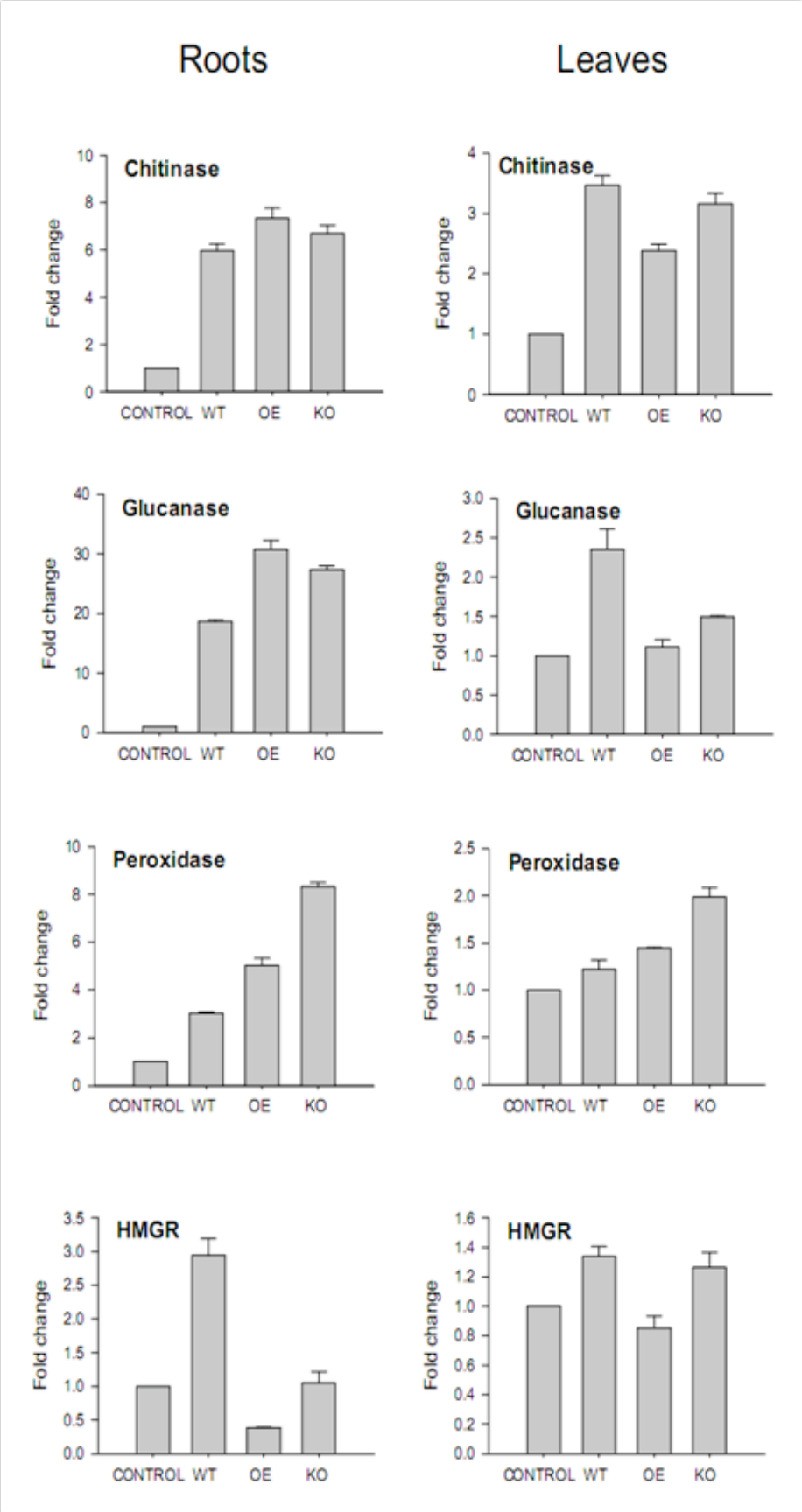
Roots
Leaves
Chitinase
Fold change
CONTROL WT OE KO
Glucanase
Peroxidase
HMGR

Table 1. Primers used in this research to make constructions, used in RT-PCR and in qRT-PCR analysis.

Primer name	Sequence 5' to 3'	Gene amplified
Tasm1OE-f	GCT CTA GAA TGC AAC TGT CCA ACA TCT TCA CTC	
Tasm1OE-r	CCA ATG CAT TTA GAG ACC GCA GTT CTT AAC AGG	Sm1 gene of *T. atroviride*
Tvsm1OE-f	GCT CTA GAA TGC AGT TCT CCA GCC TCT TCA AG	
Tvsm1OE-r	CCA ATG CAT TTA GAG GCC GCA GTT GCT CAC AGC	Sm1 gene of *T. virens*
Tasm1KO5'-f	CGGGATCCGCACTGGGTAGATGCTGGTCTG	5' region of the sm1 gene of
Tasm1KO5'-r	CTCCTTCAATATCAGTTAACGTCGATCCTGAGTAGTGAAGCGAATGTGCTG	*T. atroviride*
Tasm1KO3'-f	CAGCACTCGTCCGAGGGCAAAGGAATAGCGGAGCAATGTAAGCAGATCGAC	3' region of the sm1 gene of
Tasm1KO3'-r	CCGCTCGAGCCTTACTGCAAAGGGTCTGGATGC	*T. atroviride*
hph-f	GATCGACGTTAACTGATATTGAAGGAG	hygromycin
hph-r	CTATTCCTTTGCCCTCGGACGAGTGCTG	phosphotransferase gene
Tvsm1KO5'-f	GCTCTAGAACAATGCCGGTAGTACACCGTTCG	5' region of the sm1 gene of
Tvsm1KO5'-r	CTCCTTCAATATCAGTTAACGTCGATCGGGTACAGCAAACTGACTCGTCAC	T. virens
Tvsm1KO3'-f	CAGCACTCGTCCGAGGGCAAAGGAATAGCGACCAGTAAACCGCCATTCATCG	5' region of the sm1 gene of
Tvsm1KO3'-r	CCGCTCGAGGGACTTGTCGAATTTCCCATCTCG	T. virens
TaKO-f	AGCCAAGGACATGGATGCGCTGTC	Complete construction
TaKO-r	CGATCCATCCGTCCACTTGTGG	integrated in the genome of *T. atroviride*
TvKO-f	AAATGCCGGCTCCTAAGCCCTG	Complete construction
TvKO-r	ACGTTCCGTCCAACGGATGACGAC	integrated in the genome of *T. virens*
CHIT-f	GCCCAAACTTCCCATGAAACT	Pathogen induced chitinase
CHIT-r	CCATGCGTATGGTCCATCTG	Pathogen induced
GLUC-f	GAGAGCGGTTGGCCTTCA	glucanase
GLUC-r	AAGTCCTCGCGTTGTCAATAGAG	Pathogen induced HMG-
HMGR-f	AACTGAGGTTGCTGCTCTTGTG	CoA reductase
HMGR-r	CCATGGCTGACCCAGTAAGG	Pathogen induced
POD-f	GGAACCTCAGCATGCCAATT	peroxidase
POD-r	GGGCCACCAGTGGAGTTG	*Licopersicum esculentum*
ACT-f	CCTCACCGAGAGAGGTTACATGT	actina (TOM51)
ACT-r	CATGTCGCGGACAATTTCC	

[21] Número de solicitud: MX/a/2008/005316 [22] Fecha de presentación: 23/04/2008
[71] Solicitante(s): INSTITUTO NACIONAL DE NEUROLOGIA Y NEUROCIRUGIA MANUEL VELASCO SUAREZ; Insurgentes Sur 3877, Col. La Fama, 14269, TLALPAN, Distrito Federal
[72] Inventor(es): JULIO EVERARDO SOTELO MORALES; MARIA GUADALUPE PALENCIA HERNANDEZ; Insurgentes Sur 3877, Col. La Fama, 14269, TLALPAN, Distrito Federal
[74] Agente: JAIME GABRIEL NARVAEZ GENIZ; Insurgentes Sur 3877, Col. La Fama, 14269, TLALPAN, Distrito Federal
[30] Prioridad (es):
[51] Clasificación: *A61K31/00* **(2006-01)** *A61K31/33* **(2006-01)** *A61K31/395* **(2006-01)** *A61K31/396* **(2006-01)** *A61K31/4015* **(2006-01)** *A61K31/404* **(2006-01)** *C07D247/00* **(2006-01)**
[54] Título: USO DE LA TALIDOMIDA COMO INHIBIDOR DE LAS CRISIS EPILEPTICAS.
[57] Resumen: La talidomida fue originalmente sintetizada y probada como sedante, hipnótico y antiemético; sin embargo, después de observar su efecto teratogénico se abandonó la idea de usarlo para enfermedades neurológicas y psiquiátricas La epilepsia es una enfermedad neurológica muy frecuente en México por tal motivo y por el efecto sedante de la talidomida se estudió el efecto potencial anticonvulsivante de la talidomida: Se probaron diferentes dosis de talidomida en las crisis mioclónicas y tónico clónico generalizadas inducidas con 50 mg/kg ó 70 mg/kg de penüleritetrazol (PTZ); el efecto anticonvulsivante de la talidomida también se comparó con al efecto del ácido valpróico. Las crisis y la latencia fueron individualmente registradas. La talidomida a bajas dosis (5-10 mg/kg) pi-eviso las crisis en todos los animales tratados con 50 mg/kg de PTZ también, de una manera dosis-dependiente, la talidomida inhibió las crisis en ratas expuestas a altas dosis de PTZ (70 mg/kg); la talidomida mostró una actividad anticonvulsivante similar a la del ácido valpróico. La talidomida es un anticonvulsivante efectivo y se aseguran más estudios sobre el potencial antiepileptogénico de ésta substancia

[21] Número de solicitud: MX/a/2008/005324 [22] Fecha de presentación: 24/04/2008
[71] Solicitante(s): FORTUNATO MERLOS RUEDA; Lino Merino No. 389, Col. Juan Escutia, 09100, Distrito Federal
[72] Inventor(es): FORTUNATO MERLOS RUEDA; ELIO SANTIAGO CASTILLO HERACLEO; JESÚS ZAMUDIO GUERRERO; ODÍN MORENO AMEZCUA; Lino Merino No. 389, Col. Juan Escutia, 09100, Distrito Federal
[74] Agente: ELIO SANTIAGO CASTILLO HERACLEO; Mirasoles No. 11, Col. Los Cerrillos, 16780, Distrito Federal
[30] Prioridad (es):
[51] Clasificación: *F25B25/00* **(2006-01)** *F24F3/00* **(2006-01)**
[54] Título: SISTEMA DE ENFRIAMIENTO AUTOMATICO- PARA NAVES INDUSTRIALES Y/O TECHUMBRES METALICAS.
[57] Resumen: La invención se refiere a un Sistema de Enfriamiento Automático para reducir la temperatura interna, originada por radiación solar, en naves industriales y/o construcciones con techumbre metálica. El sistema está basado en una mezcla cuya doble función es la de enfriar la techumbre y protegerla de la oxidación; varias sondas resistivas de temperatura, conectadas a la unidad de control automático son instaladas al interior de la nave, las cuales permiten que el sistema arranque o para de forma automática; la mezcla se impulsa mediante una bomba y se distribuye a lo largo del techo a través de una red de tubería de irrigación permitiendo que la mezcla entre en contacto con la techumbre llevándose acabo la transferencia de calor al bajar por gravedad a través de ella hasta un canalón, y conducida mediante una red de tubería de bajada al tanque enchaquetado mismo que está diseñado para bajar la temperatura de la mezcla, conservar la misma e infiltra los lodos y basura; un sensor de lluvia enviará una señal a la unidad de control automático para conducir el agua de lluvia al drenaje y no al tanque enchaquetado de almacenamiento, acción que se realiza a través de una válvula desviadora.

[21] Número de solicitud: MX/a/2008/005371 [22] Fecha de presentación: 25/04/2008
[71] Solicitante(s): INSTITUTO POTOSINO DE INVESTIGACION CIENTIFICA Y TECNOLOGICA, A.C.; Camino a la Presa San José 2055, Col. Lomas 4ª sección, 78216, SAN LUIS POTOSI, San Luis Potosí
[72] Inventor(es): ALFREDO HERIBERTO HERRERA ESTRELLA; J. SERGIO CASAS FLORES; GERARDO RAFAEL ARGÜELLO ASTORGA; MIGUEL ÁNGEL SALAS MARINA; Paseo de la Fundación Nº 1088, Col. Villas de Irapuato, 36670, Irapuato, Guanajuato
[74] Agente: NORMA ISABEL GARCÍA CALDERÓN; Prolongación Comegidora Norte 1088 planta baja, Col. Arboledas, 76140, QUERETARO, Queretaro
[30] Prioridad (es):
[51] Clasificación: *C12N15/00* **(2006-01)**
[54] Título: CEPAS TRANSFORMANTES DEL HONGO MICOPARASITO TRICHODERMA SPP. PROMOTORAS DE CRECIMIENTO Y RESISTENCIA A ENFERMEDADES FUNGICAS Y BACTERIANAS EN PLANTAS SOLANACEAS, COMPOSICIONES QUE LAS CONTIENEN, PROCEDIMIENTO DE APLICACION Y USO DE LAS MISMAS.
[57] Resumen: La presente invención describe y reclama cepas transformantes novedosas del hongo Trichoderma ssp., capaces de promover el crecimiento y la resistencia a fitopatógenos en plantas de interés agronómico de una manera significativa en comparación con las cepas convencionales. La utilización de estas cepas disminuyen considerablemente el uso abonos y de pesticidas químicos cuya fabricación y uso dañan el medio ambiente y la salud humana.

[21] Número de solicitud: MX/a/2008/005376 [22] Fecha de presentación: 25/04/2008
[71] Solicitante(s): VICTOR HUGO DIAZ; Arquímedes 31 Interior 23 A, Col. Chapultepec Polanco, 11560, MIGUEL HIDALGO, Distrito Federal
[72] Inventor(es): VICTOR HUGO DIAZ; Arquímedes 31 Interior 23 A, Col. Chapultepec Polanco, 11560, MIGUEL HIDALGO, Distrito Federal
[74] Agente: RAFAEL JIMÉNEZ CAMACHO; Arquímedes 31 Interior 23 A, Col. Chapultepec Polanco, 11560, MIGUEL HIDALGO, Distrito Federal
[30] Prioridad (es):
[51] Clasificación: *A23L1/00* **(2006-01)**
[54] Título: COMPOSICION LA CUAL INCLUYE SUPEROXIDO DISMUTASA Y CACTUS NOPAL PARA DISMINUIR Y PREVENIR LA RESACA.
[57] Resumen: La invención se trata de una composición para prevenir, proteger minimizar y acelerar la recuperación de los síntomas típicos asociados con la ingesta de alcohol, conocido comúnmente como resaca.

[21] Número de solicitud: MX/a/2008/005412 [22] Fecha de presentación: 25/04/2008
[71] Solicitante(s): JAVIER ESCAMILLA SALDAÑA; 20 de Noviembre No. 12, Col. Col. Centro, 99000, FRESNILLO, Zacatecas
[72] Inventor(es): JAVIER ESCAMILLA SALDAÑA; 20 de Noviembre No. 12, Col. Col. Centro, 99000, FRESNILLO, Zacatecas
[74] Agente:
[30] Prioridad (es):
[51] Clasificación: *C12N15/00* **(2006-01)**
[54] Título: USO DE LA VACUNA SABIN O VACUNA TRIVALENTE DE POLIOVIRUS ATENUADOS PARA EL TRATAMIENTO DE HERPES ZOSTER
[57] Resumen: El tratamiento que propongo para tratar la enfermedad del Herpes zoster en todas sus presentaciones y en todos los estadios de su evolución, tiene la ventaja que se administra en dosis única y la remisión total se obtiene en un periodo máximo de 8 o 10 días. No deja secuelas ni presenta reacciones adversas.Las pruebas realizadas fueron en pacientes tanto del sexo femenino como masculino de diferentes edades, y en todos los casos no ha habido reacción adversa de ningún tipo. Los medicamentos y tratamientos actuales para tratar esta enfermedad se caracterizan porque los períodos de recuperación son más dolorosos, largos en tiempo y costosos.

DISCUSIÓN GENERAL Y CONCLUSIONES

Discusión general

En este trabajo de investigación se planteo estudiar el dialogo molecular entre plantas modelo y hongos promotores del crecimiento de las plantas. En primera instancia se estudio el efecto de la colonización de *Trichoderma atroviride* en plantas de *Arabidopsis thaliana*. También se estudió la interrupción y sobreexpresión del gen *sm*-1 que codifica para una proteína inductora del sistema de defensa SM-1 (small proteín 1), en los hongos *T. atroviride* y *T. virens*, así como el efecto de estas cepas mutantes y transformantes durante su interacción con plantas de tomate (*Lycopersicum esculentum*). A continuación, presentamos y discutimos de manera breve los resultados obtenidos.

En este trabajo reportamos que la colonización de la raíz de plántulas de *Arabidopsis* por *T. atroviride* induce el crecimiento. Con la finalidad de demostrar la colonización de las raíces de *Arabidopsis*, se generaron cepas transformantes que expresan el gen de la proteína verde fluorescente GFP de *Aquarea victoria* y mediante microscopia confocal observamos la penetración, la colonización de los tejidos de la raíz y la formación de estructura tipo apresorio de *T. atroviride* en las plantas de *Arabidopsis*. Con lo que respecta a este resultados, en otros trabajos se ha sugerido que el mecanismo involucrado en la promoción del crecimiento podría ser debido a la colonización de las raíces de las plantas por *Trichoderma* y a la habilidad de este hongo para proporcionar nutrientes y hormonas a las plantas (Harman, 2000; Contreras *et al*., 2009). Por otro lado, encontramos que la colonización de las raíces de *Arabidopsis* por *T. atroviride* trajo como resultado

que las plantas presentaran resistencia sistémica contra los patógenos foliares *P. syringae* y *B. cinerea*. Cabe mencionar que la habilidad de *Trichoderma* para inducir protección sistémica contra patógenos foliares ha sido pobremente estudiada, ya que todos los esfuerzos de investigación han sido orientados a estudiar los procesos de micoparasitismo (Harman *et al.,* 2004). En algunos trabajos ya se ha observado que la aplicación de *T. asperellum* torna a las plantas de pepino a ser más resistentes contra *P. syringae* pv. *lachrymans* (Yedidia *et al*., 2003). Para conocer la respuesta molecular de la protección sistémica inducida por *Trichoderma* contra patógenos foliares, evaluamos la expresión de genes de defensa en *Arabidopsis* involucrados en diferentes rutas de señalización, donde encontramos que *T. atroviride* fue capaz de inducir la expresión de genes marcadores de las diferentes rutas SAR, SIR, HR y síntesis de la fitoalexina (camalexina). Una posible explicación de esta respuesta es que ya se ha reportado que aunque las rutas de señalización SAR y SIR son antagónicas, pero se ha visto que cuando las hormonas señalizadores AS y AJ se aplican en cantidades pequeñas inducen una sinergia en la respuesta de las rutas SAR y SIR (Mur *et al*., 2006). Las respuestas de estas vías de señalización en la planta inducida por *Trichoderma* puede deberse a que durante la interacción hay un diálogo molecular entre la planta y el microorganismo, provocado por moléculas producidas por ambos, la planta y el microorganismo (Pozo *et al.,* 2005). En este sentido, se reportó que la proteína SM-1 de *T. virens* es un inductor del sistema de defensa y además induce protección sistémica contra *Colletotrichum* sp. en plantas de algodón (Djonovic *et al*., 2006). En ese mismo año también se reporto que *T. atroviride* produce la proteína EPL1, ortóloga a SM-1 de *T. virens*, ambas

clasificadas como inductores del sistema de defensa (Seidl *et al*., 2006). Con la finalidad de caracterizar y comparar la funcionalidad de este gen en ambas especies, en este trabajo se generaron cepas sobreexpresantes y mutantes de dicho gen tanto en *T. atroviride* como en *T. virens* y se evaluó el efecto de estas cepas en plantas de tomate. Al comparar las cepas de ambas especies de *Trichoderma* que sobreexpresan más al gen *sm*-1 encontramos que la cepa de *T. atroviride* OE2.1 indujo mayor nivel de protección contra *A. solani*, *B. cinerea* y *P. syringae,* mientras que la protección inducida por la cepa de *T. virens* OE2.2 fue menor. Por otro lado, las plantas que presentaron mayor daño fueron las que se inocularon con las cepas mutantes, comparadas con las que se inocularon con las sobreexpresantes y silvestres, pero el nivel de daño nunca alcanzo el observado en las plantas control (no inoculadas). Con estos resultados se muestra que las cepas sobreexpresantes de *T. atroviride* fueron mas eficientes en inducir la respuesta sistémica en plántulas de tomate contra hongos y bacterias patógenos foliares y englobando estos datos se puede sugerir que la vía de señalización en la cual participa la proteína SM-1 no es la única responsable en inducir protección sistémica en las plantas de tomate. Al realizar estudios sobre los posibles mecanismos moleculares involucrados en la respuesta sistémica inducida por *Trichoderma*, se encontró que durante la interacción *T. atroviride*-tomate los genes de quitinasa en raíces y glucanasa en hojas (PRs) presentaron mayores niveles de inducción en plantas inoculadas con la cepa OE. Este mismo efecto se observo con la peroxidasa (respuesta hipersensible) tanto en raíces como en hojas, comparado con las plantas que se inocularon con las cepas WT y KO.

Por otro lado, en la interacción *T. virens*-tomate todos los genes evaluados fueron inducidos en raíces y hojas, aunque algunos genes como la quitinasa y glucanasa (PRs) presentaron mayores niveles de inducción en tejido de raíz cuando las plantas se inocularon con la cepa OE comparado con las plantas que se inocularon con las cepas WT y KO. En este sentido se ha reportado que la colonización por *T. harzianum* a plantas de pepino induce respuesta local y sistémica de genes de quitinasas y peroxidasas, que están involucradas en las respuesta de defensa (Yedidia *et al*., 1999)

Estos resultados pueden explicar porque, las plantas inoculadas con las cepas sobreexpresantes presentaron menor nivel de daño cuando se inocularon con los patógenos comparado con las plantas que se inocularon con las cepas WT y KO. Este ultimo resultado concuerda con lo reportado por Djonovic donde cepas sobreexpresante del gen *sm*-1 en *T. virens* proporcionaron mayor protección sistémica contra patógenos foliares en plantas de maíz (Djonovic *et al.,* 2007). Los resultados obtenidos en este trabajo dieron perspectivas para seguir estudiando la interacción de la proteína SM-1 con genes de las plantas que responden a la presencia de la proteína, esto con la finalidad de identificar genes blanco de la planta que respondan a la presencia de este inductor.

Conclusiones

5. *T. atroviride* promueve el crecimiento de *Arabidopsis thaliana* .

6. La colonización de raíces de *Arabidopsis* por *T. atroviride* induce protección sistémica contra hongos y bacterias patógenas.

7. *T. atroviride* induce la expresión de genes de defensa de las vías de señalización SIR, SAR y síntesis de fitoalexinas.

8. Las cepas OE del gen *sm-1* de *T. atroviride* y *T. virens* indujeron mayor protección sistémica contra los patógenos foliares y mayor nivel de inducción de algunos de los genes de defensa.

9. Las plantas inoculadas con las cepas KO presentaron un nivel de inducción de los genes evaluados pero nunca se comportaron como las plantas no inoculadas

10. La vía de señalización que induce la proteína SM-1 no es la única responsable de inducir el sistema de defensa en las plantas.

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ANEXO

The plant growth-promoting fungus *Aspergillus ustus* promotes growth and induces resistance against different life style pathogens in *Arabidopsis thaliana*

The plant growth-promoting fungus *Aspergillus ustus* promotes growth and induces resistance against different life style pathogens in *Arabidopsis thaliana*

Running head: *A. ustus,* Arabidopisis growth and pathogen resistance

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Abstract

In their natural setting, plants have to interact with beneficial or deleterious microorganisms. To deal with pathogens, plants have evolved sophisticated mechanisms including constitutive and induced defense mechanisms. Phytohormones play important roles in plant growth and development, as well as in the systemic response induced by beneficial and pathogen microorganisms. In this work, we identified an *Aspergillus ustus* isolate that promotes growth and induces developmental changes in *Solanum tuberosum* and *Arabidopsis thaliana*. *A. ustus* inoculation on *A. thaliana* and *S. tuberosum* roots induced an increase in shoot and root growth, lateral-root and root hair number. Assays performed on Arabidopsis lines to measure reporter gene expression of auxin-induced/repressed or cell cycle controlled genes (*DR5* and *CycB1*, respectively) showed enhanced GUS activity, when compared with mock-inoculated seedlings. The root hair initiation phenotype of *Arabidopsis rhd6* mutant was also rescued by co-cultivation with the fungus. To determine the contribution of phytohormone signaling pathways in the effect elicited by *A. ustus*, we evaluated the response of a collection of hormone mutants of *Arabidopsis* defective in auxin, ethylene, cytokinin, or abscisic acid signaling, respectively, to the inoculation with this fungus. All mutant lines inoculated with *A. ustus* showed increased biomass production, suggesting that these genes are not required to respond to this fungus. In addition, *A. ustus* induced systemic resistance against the necrotrophic fungus *Botrytis cinerea* and hemibiotrophic bacteria *Pseudomonas syringae,* probably through the induction of the expression of salicylic acid, jasmonic acid/ethylene, and camalexin defense related genes in *Arabidopsis*.

Keywords: *Aspergillus*, plant–growth promoting fungus, *Arabidopsis*, gene

expression, salicylic acid, jasmonic acid, systemic resistance.

Introduction

In their natural setting, plants have to deal with a whole range of environmental changes that determine plant growth and development. Hormones and many endogenous signals regulate plant growth and development, which in combination with the genetic information determine the plant's shape (development) (Benfey, 2002). Auxins and cytokinins regulate cell division and expansion, lateral root development, and apical dominance (Benfey, 2002; Sakakibara, 2006). Gibberellins (GB) and brassinosteroids (BS) promote germination, stem elongation, flowering, and regulate photomorphogenesis (Pieterse *et al*., 2009). Abscisic acid (ABA) is involved in several stress signaling pathways and promotes seed dormancy (Asselbergh *et al*., 2008).

It is also well known that the phytohormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play important roles in the induced defense responses (Kazan *et al.,* 2009). Furthermore, ABA, brassinosteroids, GB, and auxins have also been reported to play important roles in plant defense response, but their involvement has been poorly studied (Navarro *et al*., 2006; Wang *et al*., 2007; Walters and McRoberts, 2006).

Perception of microorganisms by plants is highly coordinated through cellular processes that determine the final outcome of the relationship, ranging from parasitism to mutualism (Nimchuk *et al*., 2003; Bais *et al.,* 2004; Pozo *et al.,* 2005). With the aim to defend from pathogen attack, plants have evolved constitutive and inducible resistance mechanisms (Verhagen *et al.,* 2006). During recognition of a

pathogen, plants activate the hypersensitive response (HR); a localized mechanism characterized by the production of reactive oxygen species and programmed cell death where the plants prevent invasion and spread of pathogens (Dicke and Hilker, 2003). After a localized response by exposure to a pathogen, plants trigger another response known as systemic acquired resistance (SAR), this can be either local or systemic and is associated with the synthesis of pathogenesis-related proteins (PR), which is dependent of endogenous SA accumulation (Durrant and Dong, 2004; Bostock, 2005; Glazebrook, 2005).

Induction of defense responses is not only provoked by pathogens, it might result also from the colonization of plant roots by some plant growth-promoting rhizobacteria (PGPR). This colonization results in the induction of a response called induced systemic resistance (ISR) (Van Loon *et al.,* 1998; Pieterse, *et al.,* 2003). JA and ET regulate the ISR, which is effective against a broad range of phytopathogens (Van Loon *et al*., 1998). Recently, it has been demonstrated that there is a cross talk between SA and JA/ET signaling pathways modulated through the function of NPR1 protein (non-expressor of PR-genes 1) (Dong, 2001). Furthermore, a transcript-profiling analysis showed a high number of co-induced or co-repressed genes by SA and JA (Schenk *et al.,* 2000; Glazebrook *et al.,* 2003). These studies illustrate the complexity of the interaction among plant defense pathways and support the flexibility of the plant defense response to fine-tune the appropriate mechanisms (Beckers and Spoel, 2006).

In addition to PGPR, plant growth-promoting fungi (PGPF) can also provide protection against pathogens not only by the production of phytohormones, but through the production of molecules that affect hormone homeostasis within the

plants (Patten and Glick, 1996; Furukawa *et al*., 1996). A phytotoxin, called coronatine (COR) and produced by *P. syrinagae*, affects JA homeostasis, induces host gene transcription and physiological changes related to auxin signaling (Bender *et al*., 1999; Staswick 2008; Uppalapati *et al*., 2005).

Several studies have demonstrated that PGPF induce systemic protection against phytopathogens (Hossain *et al*., 2007; Hossain *et al*., 2008). Similarly, species from the genus *Trichoderma* are able to induce plant growth and plant resistance against pathogens (Harman *et al*., 2004). The production of auxins by *Trichoderma* and the addition of exogenous auxin to *Arabidopsis* plants lead to a modification of the shape of plant architecture and to a substantial increase in disease symptoms development during inoculation with *P. syringae* pv tomato (Chen *et al*., 2007; Contreras *et al*., 2008).

As mentioned, plant pathogens and beneficial microorganisms are able to induce multiple plant signals mediated by elicitor molecules and hormones or by interfering with the plant defense system by distorting the plant hormonal networks (Spoel and Dong, 2008). Here, we are reporting the effects of the plant growth promoting fungus *Aspergillus ustus* on growth and development of *Arabidopsis thaliana,* as well as the induction of systemic resistance against biotrophic and necrotrophic phytopathogens. We are also reporting local and systemic induction of JA/ET, SA defense related genes and of that involved in the synthesis of the main phytoalexin, camalexin, in this model plant.

Materials and methods

Biological material and growth conditions

Potato plants, *Arabidopsis thaliana* ecotype Col-0*,* transgenic lines *CycB1;1:uidA* (Colón-Carmona *et al*., 1999), *DR5:uidA* (Ulmasov *et al*., 1997), and mutant lines *etr1-3* (Hua and Meyerowitz, 1998), *eir1-1* (Luschnig *et al*., 1998), *ahk2-2* (Nishimura *et al*., 2004), *ahk3-3* (Higuchi *et al*., 2004), *axr4-1* (Hobbie and Estelle, 1995), *aux1-7* (Pickett *et al*., 1997), *rhd6* (Masucci and Schiefelbein, 1994), and *abi4-1* (Finkelstein, 1994) were used in the different experiments. *Arabidopsis* seeds were sterilized in 95% (vol/vol) ethanol for 5 min and washed twice in distilled water. Seeds were germinated and grown on agar plates containing 1x MS medium (Murashige and Skoog, 1962). Plates were placed vertically in an angle of 70 degrees to allow root growth along agar surface and to allow aerial grow of the hypocotyls. Plants were placed in a plant-growth chamber with a photoperiod of 16 h light, 8 h of darkness and temperature of 24°C.

Potato (*Solanum tuberosum*) plants were grown for 20 days on MS medium and transferred to 4" pots containing peat moss as substrate (LAMBERT^{TM}). Potato plants were treated with 1 × 10^6 spores ml^{-1} of *Aspergillus ustus* or *Paecilomyces fumosoroseus* (control*)*. Treated and untreated plants were irrigated once per week with 0.3X MS medium, during six weeks. Each experiment included 20 plants per treatment and it was conducted twice. After six weeks, the effect on plant growth by the fungi was evaluated on roots and stems. Potato seedlings were kindly provided by Dr. Alberto Flores (Antonio Narro Agrarian Autonomous University).

Pseudomonas psyringae pv tomato (*Pst)* was kindly provided by Dr. Ariel Alvarez (CINVESTAV-Irapuato, Mexico). *P. syringae* was routinely grown on solid Kings B medium. For bacterial suspension it was grown on liquid Kings medium (King *et al*., 1954) until reaching an OD of 0.2. *Botrytis cinerea* was isolated from a tomato field in

San Luis Potosi, Mexico, and was routinely grown for 7 days on PDA (Difco). Conidia were scraped and collected from Petri dishes with distilled water, counted in a hemocytometer and adjusted to 1×10^6 conidia ml^{-1}. *Aspergillus ustus* was isolated from axenic tissue potato cultures and was routinely grown on PDA for 10 days, spores were scrapped and collected with sterile distilled water, counted in a hemocytometer and adjusted to 1×10^6 conidia ml^{-1} in sterile distilled water.

Isolation and molecular characterization of *Aspergillus ustus*

A. ustus plug was placed on a Petri dish overlaid with sterile cellophane, incubated for 7 days at 28ºC and the mycelia were scraped from the surface of the cellophane and frozen in liquid nitrogen for DNA extraction. Total DNA extraction was done as described by Rader and Broda (1989). Total DNA was used to amplify the Intergenic Transcribed Region (ITS) from rDNA 18S using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). Amplicons were cloned in pGEM-Teasy (Promega) and sequenced by Sanger reaction in an AB sequencer. Obtained sequences were compared against nonredundat NCBI database, using the Blast algorithm (Altschul *et al*., 1990). A 100% identity was found between sequences of *A. ustus* and sequences of different strains of *A. ustus* in the NCBI database.

Plant growth promotion experiment

A. ustus was evaluated *in vitro* for its plant growth-promotion ability on all the lines used in this work. Ten-day-old seedlings were grown on agar plates containing

1x MS medium (5 seedlings per plate) and inoculated by placing three plugs of *A. ustus* at 3 cm from *A. thaliana* roots (*Col 0* and mutant lines). The interaction was evaluated after 8 days. Each experiment consisted of 25 plants and was repeated twice.

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 of 5-bromo-4-chloro-3-indolil-β-D-glucoronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Jefferson *et al.* (1987). For each marker line and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen and photographed, using a digital camera connected to a microscope using the Motic Images plus 2.0 ML software.

Plant protection conferred by *A. ustus* against bacterial and fungal phytopathogens

Five-day-old *Arabidopsis* seedlings grown in MS medium were inoculated with *A. ustus* and planted in pots containing peat moss as substrate; after 17 days, the plants were inoculated with 10 l of a suspension of *Pst* OD_{600}= 0.2 or with 10 l of 1 × 10^6 spores ml^{-1} suspension of *B. cinerea*. To the suspension of bacterial or fungal spores, Break-Thru® was added to a final concentration of 0.1% as surfactant agent (Goldsmith Chemical Corporation). Then, two leaves of ten different plants were inoculated. Plants were placed in the greenhouse; infected leaves were watered for 5 days to increase relative humidity. Seven days later, disease severity was measured

for each plant, percentage of leaves damage was calculated obtaining the total leaf area and the total leaf area damaged. Then, the ratio between these values gave the percentage of damaged area. Each treatment, consisted of 10 plants, and the experiment was repeated two times with similar results.

RT-PCR analysis

Fourteen-day-old *Arabidopsis* Col-0 plants grown in MS medium were inoculated with a 1 × 10^6 spores ml^{-1} suspension from *A. ustus*. Co-cultures were placed in a plant-growth chamber with a photoperiod of 16 h light, 8 h darkness, and temperature of 24°C and allowed to interact for 48, 72, and 96 h. At the indicated times, roots and leaves were separated, placed in a mortar, frozen in liquid nitrogen, and RNA was isolated using concert plant RNA reagent (Invitrogen) as described by the manufacturer. Total extracted RNA was DNAse treated using rDNAse I (Ambion). Total RNA (2 g) was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen). cDNA was used as a template for PCR reactions in 25 l, performed with 1 U of Taq DNA Polymerase (Invitrogen). The gene-specific primer pairs used were *PR1* (pathogenesis-related gene 1, M90508) (F-ATCTAAGGGTTCACAACCAGGCAC, R-TGCCTCTTAGTTGTTCTGCGTAGC), *PR2* (beta-1,3-glucanase, NM_115586.2) (F-AGGAGCTTAGCCTCACCACC, R-GAGGATGAGCTCGATGTCAGAG), *LOX1* (Lipoxygenase 1, NM_104376.2) (F-AGACGTTCCAGGCCATGGCAG, R-CTTGGGTAAGGATACTCCTGTG), *ATPCA*, (peroxidase, NM_114770.2) (F-CCAAGAACCGTTTCATGCG, R-GGAGAGCGCAACAAGATCAG), *PAD3* (phytoalexin deficient 3, NM_113595.3) (F-CGATGGAGATGCTCTCAAGTTC, R-GTCTCCTTGACCACGAGC), *PDF1.2*

(defensin, NM_123809.3) (F-CACCCTTATCTTCGCTGCTC, R-GGAAGACATAGTTGCATGATCC) and control actin (ACTIN 8, NM_103814.3) (F-GACTCAGATCATGTTTGAGACC, R-CATGTAACCTCTCTCGGTAAGG). To define the optimal number of PCR cycles for linear amplification of genes, a range of amplifications was established. Subsequently, PCR products were electrophoresed, stained in ethidium bromide, and photographed.

Results

Aspergillus ustus induced potato plant growth

A plant growth promoting fungus was isolated from potato plants that was identified by molecular techniques as *Apergillus ustus*. Total DNA from the isolated fungus was used as template for a PCR reaction using the ITS1 and ITS4 primers to amplify the 18S rDNA (White *et al.,* 1990). Three clones of the amplified product were sequenced and compared using the Blast algorithm, obtaining a 100% of identity with reported sequences of *Aspergillus ustus* (Lucero *et al*., 2008). To investigate whether the fungus identified as *Asperigillus ustus* induces growth of potato, 20-day-old potato plants were inoculated with fungal conidia or with a conidial suspension of the entomopathogenic fungus *Paecilomyces fumosoroseus* included as control. Six weeks later, the effect of *A. ustus* on potato plants was evaluated. Figure 1A shows that potato seedlings treated with *A. ustus* grew more than those treated with *P. fumosoroseus* or untreated control plants. A similar behavior was observed when fresh (Figure 1B) or dry plant weight was measured (Figure 1C). These results clearly show that this fungus, identified as *Aspergillus ustus,* is a plant-growth promoting fungus.

Aspergillus ustus induced growth and root architecture changes in *Arabidosis thaliana*

To closely investigate the role of *A. ustus* in plant growth and development, 10-day-old seedlings from wild type *A. thaliana* line Col-0 were inoculated with three plugs of *A. ustus* at 3 cm from the root tips. After eight days, the *A. ustus* effect on *A. thaliana* seedlings was visually determined, observing marked growth modifications in both the shoot and roots when compared with the control seedlings (Figure 2A). To further quantify the effect of *A. ustus* on *Arabidopsis* roots and shoots, we quantified the lateral root number and the root hairs number in the WT Col-0 line (Figures 2B and 2C, respectively). Root and shoot fresh weights were also determined (Figures 2D and 2E, respectively). Plant root length was also measured, finding that those plants inoculated with *A. ustus* showed shorter roots than control plants (data not shown). We also assessed the same experiment on a group of *A. thaliana* transgenic lines, including a cyclin *CycB1* marker and the *DR5* auxin marker, observing similar results as those obtained with the Col-0 WT line (data not shown).

The lines tested showed between 2 to 3 times more lateral root numbers than the group of non-inoculated plants, which presented an average of 10 lateral roots per plant (Figure 2B). Concerning the root hairs number, the inoculated plants showed a significant increase as compared to the untreated plants (Figure 2C). In addition, *A. ustus* promoted shoot and root biomass production (Figures 2E and 2F, respectively). Based on these results, we can conclude that *A. ustus* is a plant-growth promoting fungus that alters root architecture and promotes biomass

production in *A. thaliana*.

Aspergillus ustus induced expression of the cell cycle marker *CycB1*::uidA in *A. thaliana* root tips and meristems

Based on our results of *A. ustus* induction of *A. thaliana* growth promotion and root architecture changes, we decided to use an *Arabidopsis* line that carries a fusion of the cyclin *CycB1* promoter to the GUS reporter gene to investigate if the fungus promotes cell division in this plant. Thus, 10-day-old *CycB1*,1::*uid*A *Arabidopsis* seedlings were inoculated with three *A. ustus* plugs at 3 cm from the root tips. After 8 days post-inoculation, seedlings were stained for GUS activity to further analyze plants under the microscope. A basal expression of GUS activity was observed in the root primary tips of the mocked *CycB1*,1::*uid*A plants (Figure 3A), whereas the *A. ustus*-inoculated plants showed an enhanced GUS activity (Figure 3B). Interestingly, the inoculated plants showed a notorious thickening of the root when compared to the mocked plants (Figures 3A and 3B, respectively). Microscopic analysis of GUS activity in the lateral root meristems from inoculated plants showed more activity (Figure 3D) than control plants (Figure 3C). Based on these results, we can conclude that *A. ustus* promoted growth through a cyclin based mechanism. Based on our results in which *A. ustus* induced formation of lateral roots, an increase in root hairs number, shortening of roots, and cell division, we hypothesize that this behavior could be due to the presence of auxin or ethylene-like molecules secreted by the fungus.

A. ustus rescued the altered root hair initiation phenotype of *Arabidopsis rhd6*

mutant

Inoculation of cyclin marker *CycB1*,1::*uid*A transgenic line with *A. ustus* induced the activity of the GUS reporter gene, indicating a cell division activity promoted by the fungus on *Arabidopsis*. Until now, our results clearly show a plant development as described for the action of auxins or ethylene. Taking into account these results, we decided to test a mutant affected in the root hair initiation, whose phenotype is recued by the addition of exogenous indole acetic acid (IAA) or by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The *rhd6* mutant has been used as a tool to study the mechanism of IAA or ET action (Masucci and Schiefelbein, 1994; Cornejo *et al*., 2009). Figure 4 shows that inoculation of *rhd6* plants with *A. ustus* rescued their root hair initiation phenotype. The upper panel shows a comparison between the *rhd6* untreated plants against the *A. ustus* inoculated plants; the second one depicts around 12 lateral roots against 2 from the mocked plants (lower panel). The middle panel shows a representative magnification of the apical root of non-treated and treated seedlings, where the mocked plants presented no root hairs, while the WT phenotype was rescued in *rhd6* seedlings treated with *A. ustus.* These results support our hypothesis that an auxin-like mechanism is working during *A. ustus-Arabidopsis* interaction to promote growth and change the root architecture in *Arabidopsis*.

Aspergillus ustus* induced the *DR5* auxin marker in *A. thaliana

Until now, our results clearly point to a plant development described for the action of auxins or ethylene. In order to closely elucidate the participation of an auxin-like molecule during the interaction of *Arabidopsis* with *A. ustus*, we further

analyzed the interaction between them by inoculating 10-day-old *DR5::uidA* plants growing in MS 1X medium with three plugs from *A. ustus*. The *DR5::uidA* transgenic line has been used to monitor auxin regulated gene expression in several works (Wang and Jiayang, 2008; Kazan and Manners, 2009). After eight days of interaction, *DR5::uidA* plants were stained for GUS activity and clarified to later analyze them under the microscope. Interestingly, *A. ustus* inoculation enhanced the GUS activity in the root tips as compared to mocked plants (Figures 5A and 5B). We also analyzed the lateral root primordia in several stages, observing the same effect of *A. ustus* on these regions from seedlings (Figures 5C-5F). We did not observe an enhancement of GUS activity in the meristematic zone from leaves (Figures 5G and 5H). These results strengthen our hypothesis about the involvement of an auxin-dependent mechanism in plant growth and root architecture changes in *A. thaliana* mediated by *A. ustus.*

Inoculation of *Arabidopsis* mutants insensitive or resistant to several hormones responded to the secreted molecules by the fungus

To understand better the growth effect on *A. thaliana* induced by *A. ustus,* we took advantage of some existing mutants in auxin, ethylene, or abscicic acid signaling. Briefly, we placed to grow on MS medium a set of mutants, including the ethylene *etr1-3*, *eir1-1*, cytokinin receptors *hk2-2*, *ahk3-3*, the abscisic acid *abi4-1*, and mutants resistant to auxins, *Aux1-7*, *Axr4-1*. Figure 6A shows that the whole set of mutant lines responded to the presence of the fungus, the lateral root number average for the mocked seedlings was near 10 per plant, whereas inoculated plants showed from 25 (*etr1-3*, *hk2-2*) (more than the double) up to 45 lateral roots per

plant (Fig. 6A). Concerning the root hairs number, the mocked seedlings showed an average of 10 to 15, whereas the treated plants presented from 25 to 40 more root hairs than the control plants, except the *Aux1-7* and *Axr4-1* whose hair number reached 110 and 120 root hairs per plant, respectively (Fig. 6B). We also measured the effect of *A. ustus* in plant growth by weighing the shoot and root fresh weights. The root and shoot fresh weights were lesser in non-treated mutants (Fig. 6C and 6D) than that reached by the non-treated wild type plants (Figure 1B). The *hk2-2*, *ahk3-3* mutants did not show as good a response as the other mutant lines, while the cytokinin mutants presented 2 mg per plant in non-inoculated plants, *eir1-1* and *etr1-3* weighed from 3 to 5 mg. The *Arabidopsis* line that showed the best response was the mutant *abi4-1* reaching 4 mg for mocked plants vs. 9 mg for treated plants (Fig. 6C and 6D). Our results indicate that these genes are not required to respond to *A. ustus*.

Aspergillus ustus conferred resistance to _A. thaliana_ against different lifestyle pathogens

It has been described that several beneficial microorganisms induce plant defense response against phytopathogens. To assess the possible protection induced by *A. ustus* to *A. thaliana* against phytopathogens, *Arabidopsis* seedlings were inoculated on the leaves either with a suspension of the biotrophic bacteria *Pseudomonas syringae* or with spores of the necrotrophic fungus *Botritis cinerea*. The interaction was allowed for 8 days and the foliar damage was quantified. Figure 7A shows that the mocked leaves were 60% affected by the bacterial phytopathogen *P. syringae*, whereas the *A. ustus*-pretreated plants showed 20% of foliar damage.

The inoculation of *Arabidopsis* seedlings with *A. ustus* reduced considerably the foliar damage induced by *Pseudomonas*. Concerning the *Arabidopsis* seedlings that were not inoculated with *A. ustus* and inoculated with *B. cinerea*, (Fig. 7B) the foliar damage almost reached a 75% whereas the *A. ustus*- pretreated plants diminished their damage around 40%. The foliar damage in non-inoculated plants with *A. ustus* was almost the same when the plants were inoculated with the bacteria; however, the protection against the fungus was much lesser than that conferred against *P. syringae*. From these results, we can conclude that the plant growth-promoting fungus *A. ustus* confers protection to *A. thaliana* against fungal and bacterial phytopathogens with different life style.

Aspergillus ustus induced local and systemic expression of genes associatedwith the plant defense system in _A. thaliana_

To understand better the mechanism by which *A. ustus* induces resistance against fungal and bacterial pathogens in *Arabidopsis,* the expression of a set of defense related genes to SA, JA and camalexin were measured at 48, 72, and 96 h post-inoculation of *Arabidopsis* with *A. ustus*. Expression of *PR1* and *PR2* SA related genes was clearly induced locally at all the tested times (roots) (Figure 8A); however, in leaves only the *PR2* gene was induced (Figure 8B). The ET/JA selected genes *PDF1.2* and *LOX1*, as well as *PAD3* and *ATPCA* involved in the synthesis of camalexin and oxidative burst respectively, were locally and systemically induced at all the tested times (Figures 8A and 8B). These results indicate that *A. ustus* induces protection against fungal and bacterial pathogens through SAR, SIR, and the synthesis of the main phytoalexin (camalexin) in *Arabidopsis thaliana*.

Discussion

Plant growth promoting rhizobacteria (PGPR) have been extensively studied concerning their capability to induce biomass production and systemic resistance to plants **(**Lugtenberg and Kamilova, 2009). However, plant growth promoting fungi (PGPF) have been less studied. Fungi described in the literature as PGPF include several species from the Ascomycota, such as *Penicillium*, *Trichoderma*, *Fusariumn* and *Phoma*. Genera from the oomycetes include *Phythium* and *Phytophtora*. Almost all these PGPF have been classified as hypovirulent or non-pathogenic strains of plants (Bent, 2006).

The fungus *Aspergillus ustus* has been isolated from aseptically cultured grass *Bouteloua eriopoda* (black grama) and from the shrub *Atriplex canescens* (fourwing saltbush). *A. ustus* was classified as endophyte to these plants (Lucero *et al*., 2006). Barrow and Osuna (2002) described that this fungus assists with phosphorus uptake in *A. canescens* and it can propagate vegetatively or sexually in soil. By culturing potato plants in aseptically cultures, we isolated and identified molecularly a fungus denominated *A. ustus*, which promotes growth of potato plants. To better understand the effect of *A. ustus* on plant growth promotion, *Arabidopsis* plants Col-0 were tested in presence or absence of *A. ustus*, observing a positive effect on growth when plants were inoculated with the fungus. In this work, we demonstrate that *A. ustus* is able to enhance growth on *Arabidopsis thaliana* and potato seedlings, which is in agreement with the data reported by Barrow and Osuna, where they described an increased shoot and root biomass in *Atriplex canescens* (Pursh) Nutt. In addition, these authors observed that *A. ustus* colonized

the root of *A. canescens* (Pursh) Nutt, opposite to these results we did not observe colonization of the *Arabidopsis* roots by *A. ustus* (data not shown). Our results demonstrate clearly that root colonization is not necessary for *A. ustus* promotion of *Arabidopsis* plant growth. Inoculation of *Arabidopsis* with *A. ustus* affected the root system by inhibiting primary root growth, an increasing the lateral root number, lateral root growth, and root hairs length. These effects provoked by the fungus on *Arabidopsis* suggest the action of phytohormones. In this sense, many microorganisms have been reported to produce and secrete auxins, cytokinins, and gibberellins to the medium (Costacurta and Vanderleyden, 1995; Patten and Glick, 2002). Based on these results we conclude that *A. ustus* could be classified as an endophyte for some grass plants and as a PGPF for potato, *A. thaliana,* and grass. We hypothesize that *A. ustus* might be producing and secreting hormone-like molecules to the medium.

It is well known that phytohormones play an essential role in plant cell cycle mainly at the transcription level (Stals and Inzé, 2001). Cytokinins are phytohormones produced in roots and shoots that play important roles regulating the cell cycle, growth, and development in plants. Cytokinins are mainly synthesized in plant root tips (Aloni *et al*., 2005). Cytokinins regulate negatively growth and development in roots (Werner *et al*., 2003), while regulating positively growth and development in shoots (Howell *et al*., 2003). To detect cell cycle activity at the transcriptional level induced by the inoculation of plants with *A. ustus*, we used the *Arabidopsis* marker line for cell division *CYCB1,1::uidA*, whose promoter activity correlates well with the mRNA localization (Ferreira *et al.,* 1994a, 1994b). We observed an enhanced activity of *CycB1* promoter in tip roots and root meristems,

which correlates well with those effects induced by the application of auxin-like molecules (Himanen *et al.,* 2002; Sorin *et al.,* 2005). Based on these results, we conclude that *A. ustus* promotes cell division in *Arabidopsis*.

Cytokinins and IAA show antagonistic roles in root development; auxin promotes lateral root formations (Malamy and Benfey, 1997; Zhang and Hasenstein, 1999; Casimiro et al., 2001; Guo et al., 2005; Woodward and Bartel, 2005) and adventitious roots (Falasca et al., 2004; Sorin et al., 2005), whereas CK inhibits root formation (Torrey, 1986; Zhang and Hasenstein, 1999; Lloret and Casero, 2002). In this work, we found that inoculation with *A. ustus* of *Arabidopsis* cytokinin receptor mutants *ahk2-2* and *ahk3-3* resulted in recovery of the mutant phenotypes. We determined a high number of roots, root hairs, and an increase in fresh weight, suggesting that this fungus could be producing and secreting to the medium auxin- and cytokinin-like molecules. We determined that proteins encoded by *ahk2-2* and *ahk3-3* genes are not necessary to respond to the secreted molecules by *A. ustus*.

Many microorganisms are known to produce phytohormones to stimulate plant growth and development. The beneficial fungi *Trichoderma virens* and *Tuber borchii* (Contreras *et al*., 2009; Splivallo *et al*., 2009) produce auxin-like molecules, which promote root architecture changes and growth of *Arabidopsis* seedlings. In addition to the increase in the *Arabidopsis* biomass, we observed that *A. ustus* enhanced the production of lateral root number and root hairs number, which has been related with the action of auxin in roots (Benfey, 2002; Sakakibara, 2006). To test the possible role of an auxin-like molecule secreted by *A. ustus* to promote the increased lateral root number and root hairs number in *A. thaliana*, we used the well-characterized marker lines for auxin responsiveness (*DR5*::*uidA*; Ulmasov et al.,1997). The DR5

promoters are highly responsive to auxins; the promoter activity is a reflection of endogenous auxin levels (Peret *et al*., 2009; Negi *et al*., 2008) or of the exogenously application of auxin (Himanen *et al.,* 2002; Wang *et al*., 2007). Our results with the *DR5::GUS* transgenic lines showed induction of *DR5::GUS* expression in primary root tips, supporting our hypothesis about the production of an auxin-like molecule by *A. ustus*. Indeed, we observed a higher expression of DR5::GUS in lateral root primordia, which suggests that this effect could be due to an endogenous auxin effect.

The *rhd6* mutant can be used as a tool to screen ethylene or auxin-like molecules activity due to its defects on the reduction in the number of root hairs and the overall basal shift in the site of root hair emergence. Similar alterations have been described also in roots of the auxin-, ethylene-, abscisic acid *abi4-1*- resistant mutant *axr2* and the ethylene resistant mutant *etr1*. All three mutant phenotypes are rescued when auxins or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid are applied to the medium (Masucci and Schiefelbein, 1994). Our interaction experiments with the *aux1-7*, *Axr4-1, eir1-1*, *etr1-3,* and *rhd6* mutants, inoculated with *A. ustus*, showed that this fungus is able to rescue their phenotypes; the *DR5::uidA* marker indicated the same. These results suggest that these genes are not required to respond to *A. ustus* and that the fungus could be secreting molecules that act like IAA or ET through a different pathway.

Several pathogenic and non-pathogenic fungi produce hormones, such as auxins, gibberellins, or cytokinins (Costacurta and Vanderleyden, 1995; Patten and Glick, 2002), being these the three major groups of plant growth-promoting hormones, with an important role in growth regulation and development (Kazan and

Manners, 2009). The ethylene, abscisic acid, and auxin mutants used in this work responded to *A*. *ustus* inoculation by inhibiting root growth and lateral root formation. These results suggest that these genes are not directly involved in root architecture changes and biomass production promoted by this fungus.

It is well known that several plant beneficial microorganisms, including bacteria and fungi, play important roles in plant growth and health. Several direct and indirect mechanisms have been ascribed to these microorganisms to enhance stress tolerance, promote growth, and provide disease resistance to plants. PGPF can use more than one mechanism to control plant pathogens including competition, antibiosis, predation, mycoparasitism; triggering the systemic induced resistance (Hossain et al., 2007; Hossain et al., 2008, Harman et al., 2004). During plant microbe interaction, phytohormones play an important role in plant growth and defense, these hormones can be self-synthesized by plants or by their associated microorganisms. The roles of SA, JA, and ET in plant defense have been well established (Kazan and Manners, 2009). Plants activate distinct defense responses depending on the life style of the pathogen. Salicylic acid induces defense against biotrophic pathogens, whereas JA/ET activates defense against necrotrophic attackers. On the other hand, it has been demonstrated that cross-talk between these defense signaling pathways optimizes the response against a single pathogen (Spoel *et al*., 2003 Spoel *et al.,* 2007). In this work, we demonstrated that plants inoculated with *A. ustus* were more resistant to the necrotrophic fungus *B. cinerea* and to the hemibiotrophic bacteria *P. syringae* as compared with untreated seedlings, being more relevant the protection conferred to *Arabidopsis* by *A. ustus* against *P. syringae.* We also demonstrated that the PGPF *A. ustus* induces the

expression of genes related to the SA and JA/ET pathways and of the gene involved in the synthesis of camalexin, the main phytoalexin produced in *Arabidopsis* to counteract pathogens. JA signaling genes were induced both locally and systemically, whereas SA-induced genes were barely induced locally. These results demonstrate that *A. ustus* is able to induce SA and JA/ET and the synthesis of camalexin pathways, which could explain the enhanced systemic, induced resistance against *P. syringae* and *B. cinerea*. Based on our results, we hypothesize that *A. ustus* produces and secretes molecules to the medium and these molecules activate the systemic resistance in *Arabidopsis* against phytopathogens.

In conclusion, *A. ustus* is able to promote growth of *Arabidopsis* and it is also able to rescue the phenotype of a set of mutants affected in different hormone response pathways. Therefore, the tested wild type gene products are not necessary to respond to the secreted *A. ustus* molecules. *A. ustus* induced the expression of cyclin and auxin markers, as well as the expression of JA, SA, and synthesis of camalexin. These related genes involved in the systemic resistance without colonizing the *Arabidospis* roots, allowed us to hypothesize that the overlapping of genes related to these pathways is responsible of the systemic resistance against biotrophic and necrotrophic phytopathogens in *Arabidospis*.

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Figure legends

Figure 1. Effect of *A. ustus* inoculation on potato (*Solanum tuberosum)* plant growth. A, 20-day-old potato seedlings non-inoculated (C) or inoculated with either *A. ustus* (Au) or P. fumosoroseus (Pf). The promotion of plant growth was quantified 6 weeks later (B), Fresh weight (C), Dry weight. Data are expressed as mean values ± standard error of the mean. Letters indicate results of ANOVA and Tukey test (a-c) $p \leq 0.005$.

Figure 2. Effect of *A. ustus* inoculation on plant growth of *Arabidopsis thaliana* Col-0. A, 10-day-old *Arabidopsis* seedlings non-inoculated or inoculated with *A. ustus.* B, lateral root number and C, root hair number. D, root fresh weight and E, shoot fresh weight. Open bars show untreated seedlings while solid bars show the treated plants. Data are expressed as mean values ± standard error of the mean. Asterisks

indicate results of ANOVA test $p \leq 0.005$.

Figure 3. Effect of *A. ustus* inoculation on cell differentiation and division in *Arabidopsis* primary roots. A, expression of cell division marker *CycB1,1:uidA* in primary root tip and C, in the primary-root meristem of a representative mocked plant. B, expression of *CycB1,1:uidA* in primary root tip and D, primary root meristem of inoculated plants.

Figure 4. *Aspergillus ustus* rescued the root hair initiation phenotype of the *rhd6* mutant. The upper panel shows 6-day-old untreated and treated *rhd6* seedlings with *A. ustus*; the middle panel shows the absence and formation of root hairs in *rhd6* mutants in response to water and *A. ustus* inoculation, respectively. Effect of *A. ustus* inoculations on *rhd6* root architecture system (lower panel). Open bars show untreated seedlings while solid bars show the treated plants. Data are expressed as mean values ± standard error of the mean. Asterisk indicates results of ANOVA test $p \leq 0.005$.

Figure 5. Effect of *A. ustus* inoculation on the *DR5:uidA* auxin marker line. A, primary root tip; C, lateral root; E, root meristem; and G, leave meristem from untreated plants. B, primary root tip; D, lateral root; F, root meristem; and H, leave meristem from inoculated plants.

Figure 6. Effect of *A. ustus* inoculation on plant growth of ethylene-insensitive mutants *etr1-3*, *eir1-1*, cytokinin receptors mutants *hk2-2*, *ahk3-3*, abscisic acid

insensitive mutant *abi4-1*, and mutants resistant to auxins *Aux1-7*, *Axr4-1*. A, lateral root number. B, root hair number; C, root fresh weight; and D, shoot fresh weight. Open bars show untreated seedlings while solid bars show the treated plants. Data are expressed as mean values ± standard error of the mean. Asterisks indicate results of ANOVA test $p \leq 0.005$.

Figure 7. Effect of *A.ustus* WT on induced systemic resistance in *Arabidopsis* seedlings against the phytopathogens, *B. cinerea* and *P. syringae*. The graphs illustrate the levels of systemic disease protection observed in *Arabidopsis* seedlings inoculated with *P. syringae* (A) or *B. cinerea* (B). Open bars show untreated seedlings while solid bars show the treated plants. Data are expressed as mean values ± standard error of the mean. Asterisks indicate results of ANOVA test $p \leq 0.005$.

Figure 8. RT-PCR expression analysis of defense-related genes in *Arabidopsis* seedlings inoculated with *A. ustus*. Total RNA from roots (A) and leaves (B) of 20-day-old *Arabidopsis* plants inoculated with *A. ustus* was extracted after 48, 72, and 96 h post-inoculation. Six genes related to different plant defense pathways were selected: *PR1* and *PR2* (SAR), *PAD3* (synthesis of antimicrobial phytoalexin, camalexin), *ATPCA* (related to oxidative burst and hypersensitive reactions), and *PDF1.2* and *LOX1* (SIR). *ACT* was used as loading control.

Figure 1

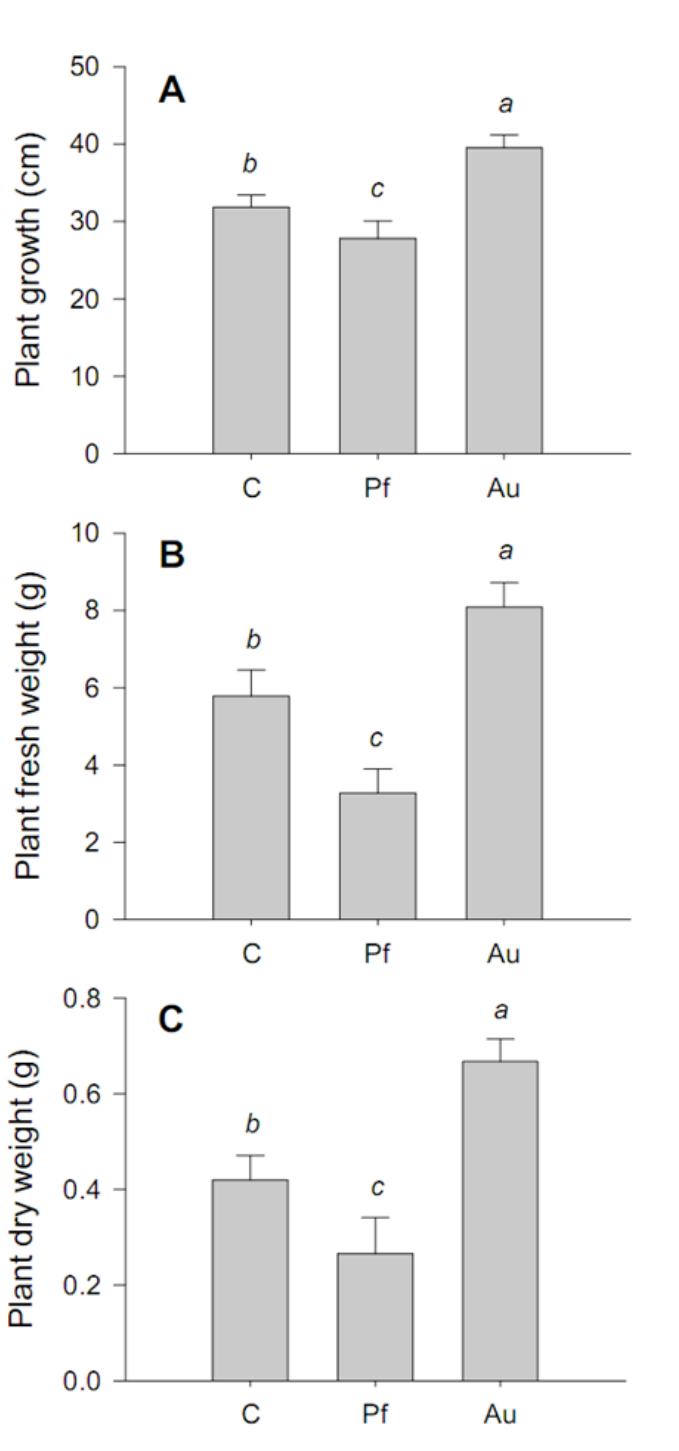

A
Plant growth (cm)
50
40
30
20
10
0
b
c
a
C
Pf
Au
B
Plant fresh weight (g)
10
8
6
4
2
0
b
c
a
C
Pf
Au
C
Plant dry weight (g)
0.8
0.6
0.4
0.2
0.0
b
c
a
C
Pf
Au

Figure 2

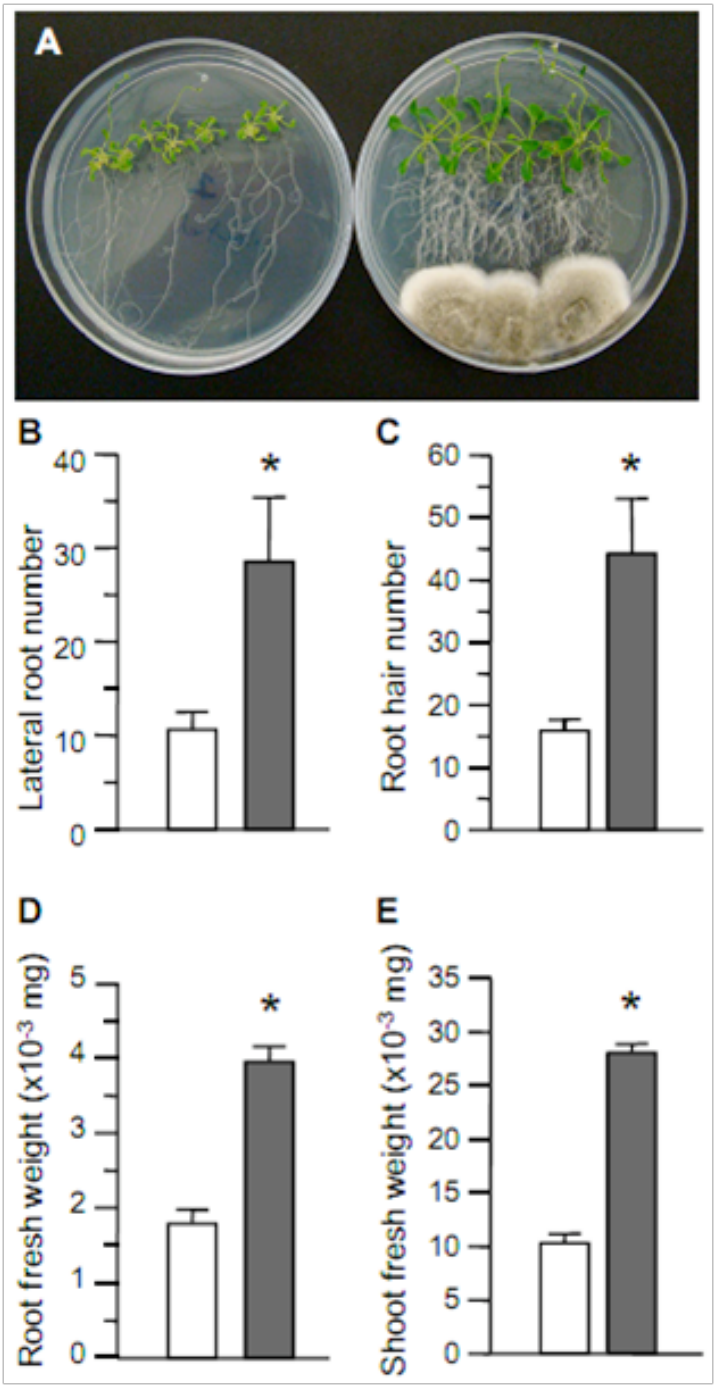

A
B
Lateral root number
40
30
20
10
0
*
C
Root hair number
60
50
40
30
20
10
0
*
D
Root fresh weight (x10-3 mg)
5
4
3
2
1
0
*
E
Shoot fresh weight (x10-3 mg)
35
30
25
20
15
10
5
0
*

Figure 3

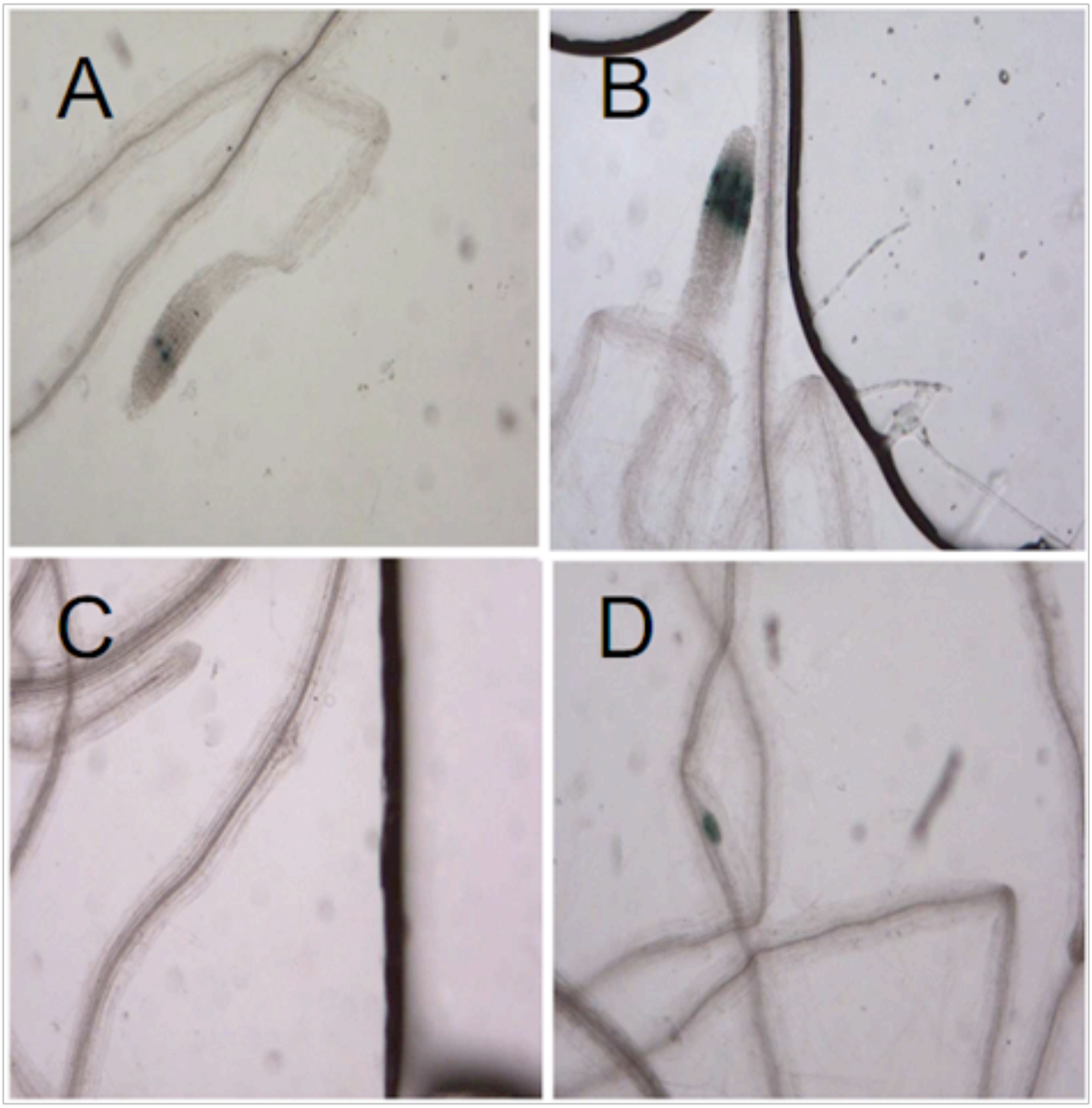


Figure 4

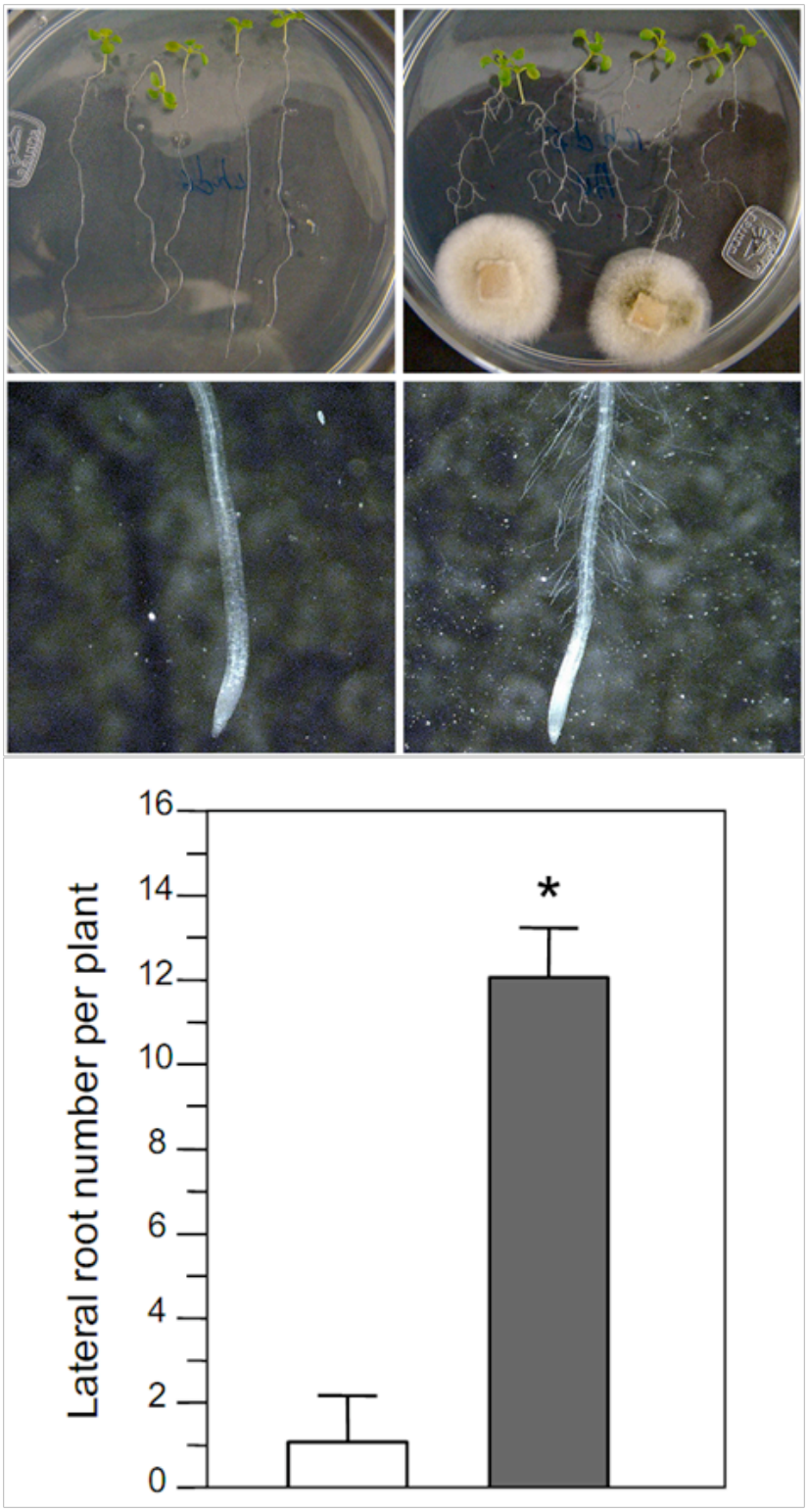


Figure 5

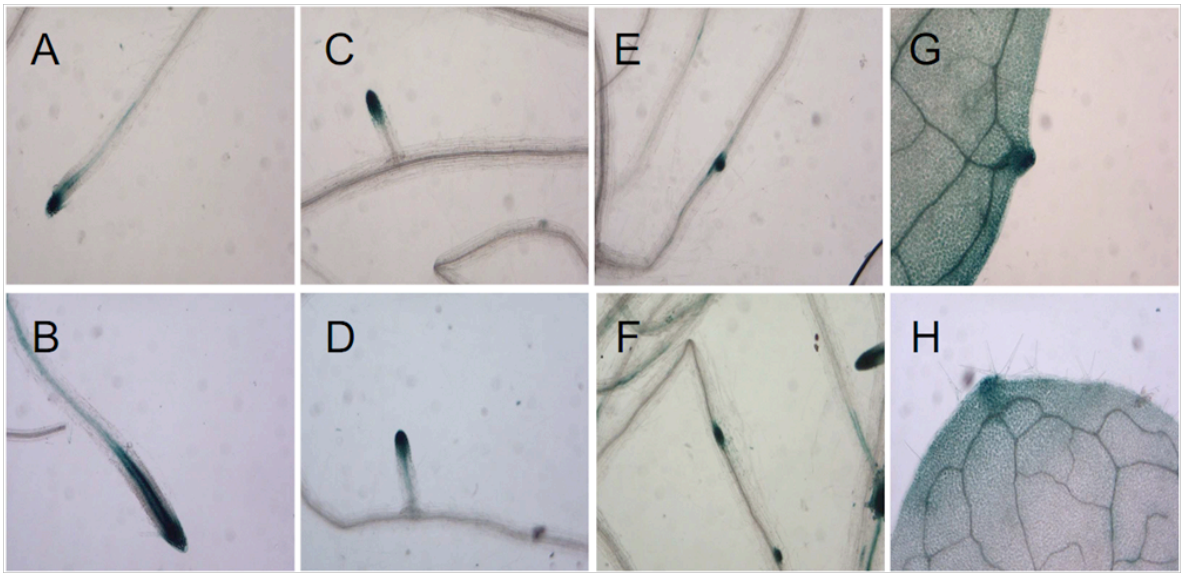


Figure 6

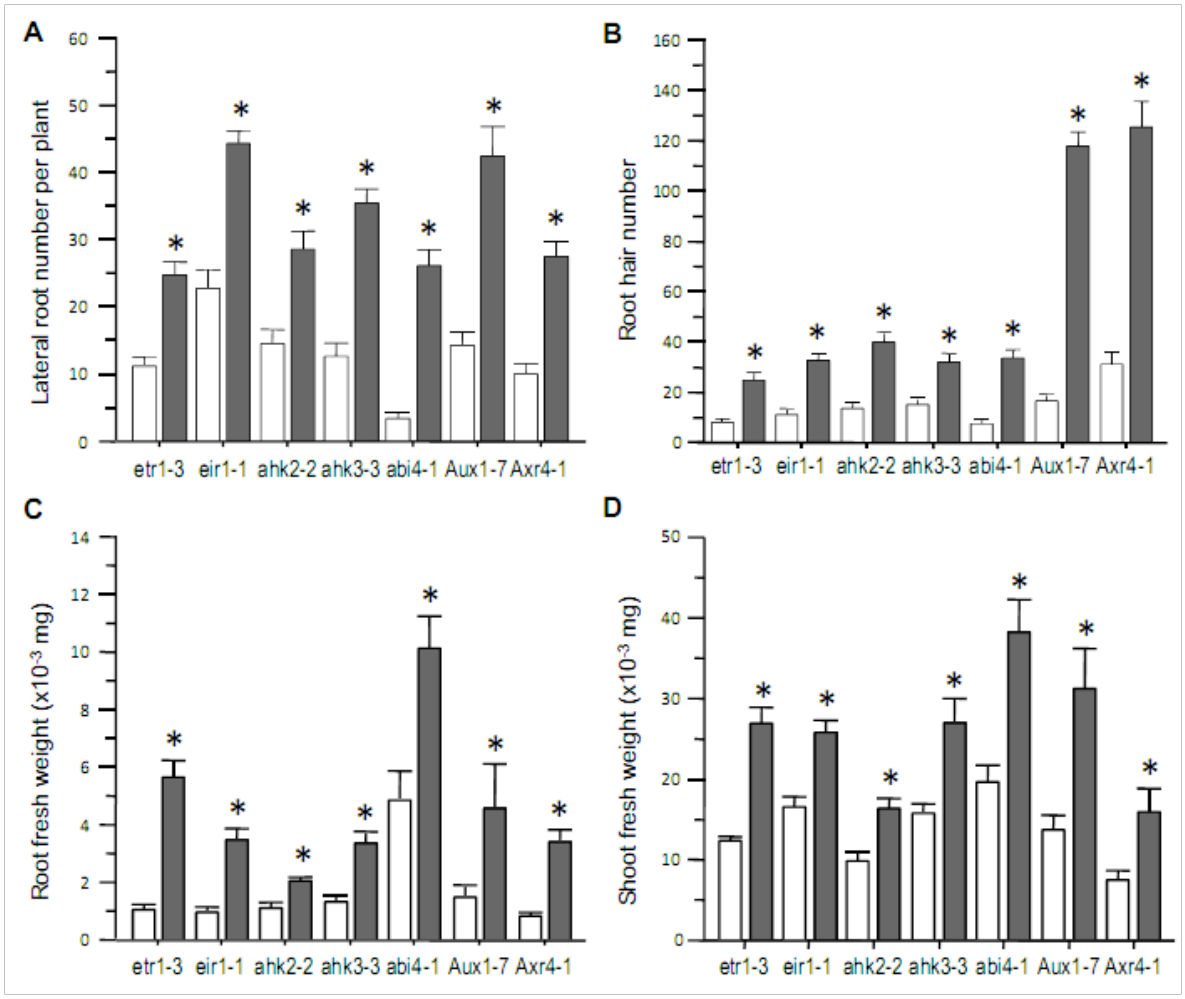

A
Lateral root number per plant
0
10
20
30
40
50
60
etr1-3 eir1-1 ahk2-2 ahk3-3 abi4-1 Aux1-7 Axr4-1
B
Root hair number
0
20
40
60
80
100
120
140
160
etr1-3 eir1-1 ahk2-2 ahk3-3 abi4-1 Aux1-7 Axr4-1
C
Root fresh weight (x10-3 mg)
0
2
4
6
8
10
12
14
etr1-3 eir1-1 ahk2-2 ahk3-3 abi4-1 Aux1-7 Axr4-1
D
Shoot fresh weight (x10-3 mg)
0
10
20
30
40
50
etr1-3 eir1-1 ahk2-2 ahk3-3 abi4-1 Aux1-7 Axr4-1

Figure 7

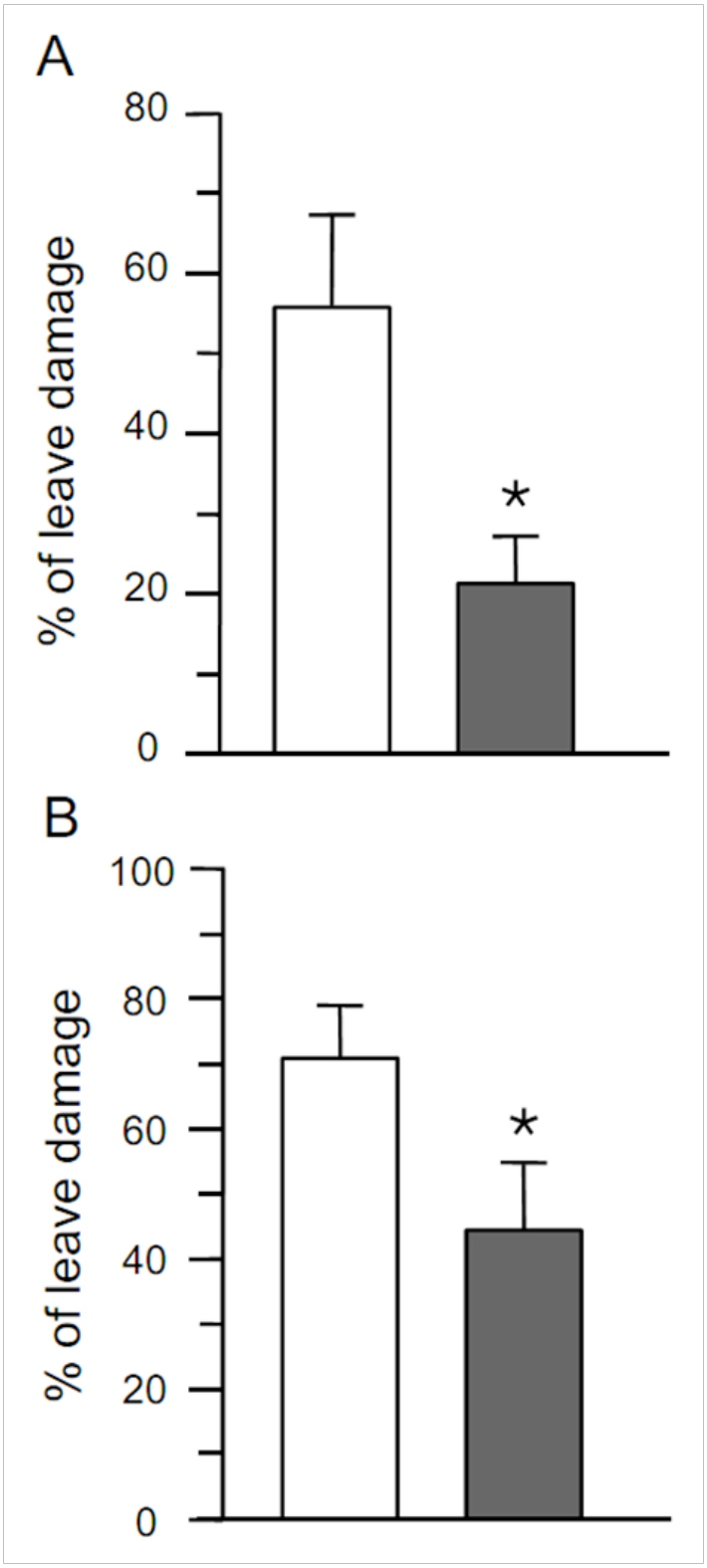
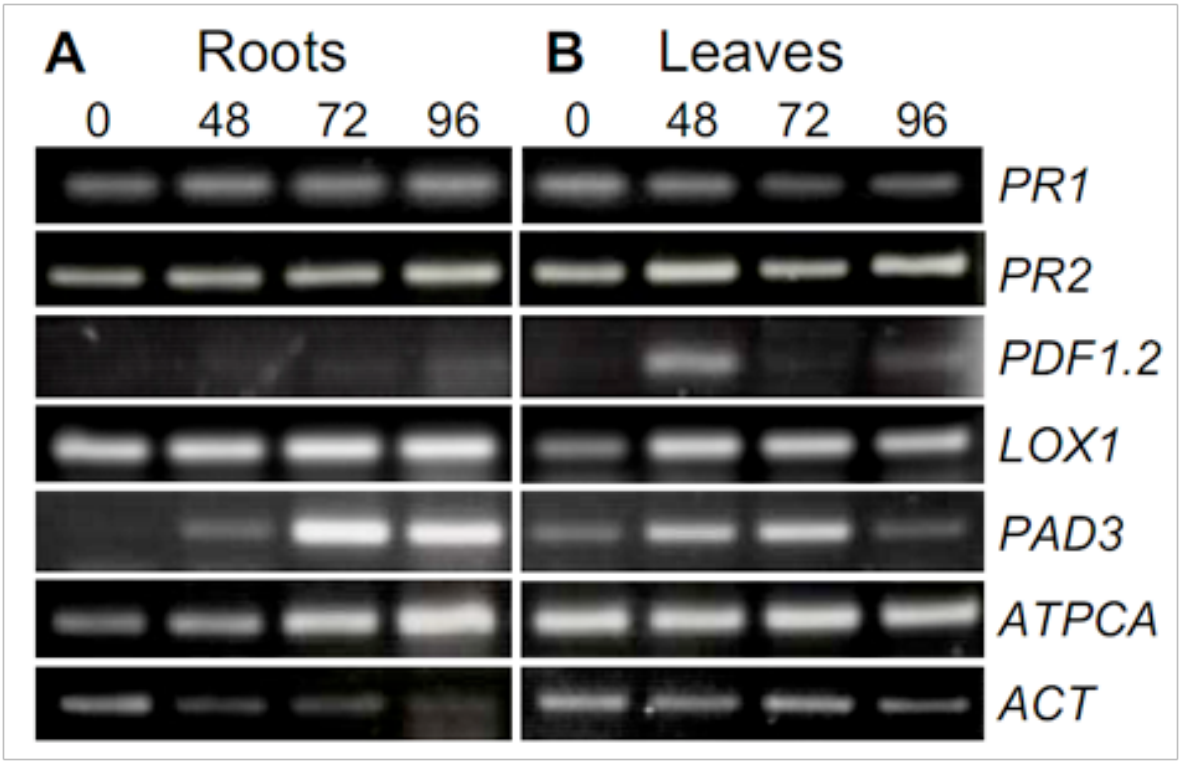


Figure 8


A
Roots
0
48
72
96
B
Leaves
0
48
72
96
PR1
PR2
PDF1.2
LOX1
PAD3
ATPCA
ACT

MATERIALES Y METODOS (CAPTULO 2)

Cepas de hongos y bacterias

En este trabajo se utilizaron las cepas silvestres de los hongos de *Trichoderma virens* Gv29-8, *Trichoderma atroviride* IMI206040, *Rhizoctonia solani* AG3 y *Sclerotium rolfsii*. Tambien se utilizaron los hongos *Botrytis cinerea* y *Alternaria solani,* los cuales fueron aislados de campos de cultivo de tomate en San Luis Potosí, México y fueron identificados amplificando por PCR la región espaciadora interna que se transcribe (de sus siglas en Inglés: ITS). Las cepas fúngicas fueron crecidas en papa dextrosa agar (PDA) (Difco, Franklin lakes, NJ, USA), cuando fue necesario, se agregó higromicina al medio a una concentración final de 100 µg/ml. Con respecto a las cepas bacterianas, se utilizó a *Pseudomonas syringae pv* tomate DC 3000, proporcionada por el Dr. Ariel Álvarez del CINVESTAV-Irapuato, México, esta cepa se creció en medio Kings B (King *et al*., 1954). La cepa de *Escherichia coli* Top 10 F' se utilizó para la manipulación genetica durante la generación de las construcciones, esta se creció en medio LB y se agregó antibiótico carbenicilina a 100 µg/ml cuando fue necesario (Sambrook *et al.,* 2001).

Plantas

Las semillas de tomate que se utilizaron en los experimentos de interacción planta- *Trichoderma*-patógeno fueron: EL CID F1 (Harris Moran Seed Company; semillas hibridas y certificadas libres de patógenos) y fueron crecidas en medio sólido MS 1X (Murashige y Skoog) o en sustrato peat moss (Lambert peat moss

Inc.).

Generación de las construcciones para las cepas sobreexpresantes (SE) y mutantes del gen *sm-1* (KO)

Para la generación de las construcciones para obtener las cepas SE, primeramente se aíslo el gen *sm*-1 de *T. atroviride* usando los oligos sentido Tasm1OE-f y reverso Tasm1OE-r (tabla 1; capitulo 2) y para aislar el gen *sm*-1 de *T. virens* se utilizaron los oligos sentido Tvsm1OE-f y reverso Tvsm1OE-r (tabla 1) en ambos pares de oligos se incluyeron los sitios de restricción de XbaI y NsiI respectivamente, el producto de PCR amplificado se clonó en el vector pGFP- Hyg donde el gen quedó bajo la regulación del promotor de la piruvato cinasa (pKi) y con el terminador de la celobiohidrolasa (cbh). Adicionalemnte, este vector tiene el gen de la higromicina fosfotransferasa (*hph*) de *E. Coli* bajo el control del promotor *trpC* de *A. nidulans* (Casas-Flores *et al*., 2006)

El ADN de ambas especies de *Trichoderma* se extrajo utilizando el método descrito por Raeder y Broda (1989), el ADN de cada una de las cepas se utilizó como templado para amplificar por PCR el gen *sm*-1 usando los oligos previamente descritos. El producto de PCR se clonó en el vector pGEM-T-easy (promega) y este se verificó por secuenciación. Las clonas elegidas fueron digeridas con las enzimas *Xba* I y *Nsi* I y el fragmento liberado fue subclonado en los sitios de restricción correspondiente en el vector pGFP-Hyg, quedando el gen *sm*-1 bajo la regulación del promotor de la piruvato cinasa (pki) de *Trichoderma reesei* (Zeilinger *et al.,* 1999).

Las construcciones para generar las cepas mutantes del gen *sm*-1, se obtuvieron

mediante la técnica Double joint-PCR (Yu *et al*., 2004), con pequeñas modificaciones; en la primera ronda de PCR se amplificó el gen de la higromicina fosfotransferasa (*hph*) y los fragmentos de 1.5 kb de las regiones 5' y 3' que flanquean al gen *sm*-1 de *T. atroviride* y *T. virens* usando los primers correspondientes para cada construcción y para cada cepa (Tabla 1; anexo 1). El primer reverso diseñado para amplificar la region 5' que flanquea al gen incluye 30 pb de sm-1 y 30 pb de la region 5' de hph, mientras que el primer sentido de la región 3' que flanquea el gen sm-1, incluye 30 pb de este e incluye 30 pb de la región 3' del gen hph (ver figura 1 del capitulo 1). Se realizó una PCR de fusión mezclando los productos de PCR de las regiones que flanquean al gen *sm*-1 junto con el fragmento de hph obteniendo así la construcción para reemplazar el gen *sm*-1, el producto de PCR se corrió por electroforesis en un gel de agarosa al 1% y la banda esperada se purificó y se clonó en el vector pGEM-T-easy (promega). Las clonas positivas para la construcción de ambos hongos se utilizaron como templado para amplificar las construcciones completas usando los oligos que flanquean cada una de las construcciones (tabla 1). El producto de PCR se uso para transformar protoplastos de *T. atroviride* y *T. virens*.

Transformación y selección de las cepas transformantes

Protoplástos de las cepas silvestre de *T. atroviride* y *T. virens* se transformaron con las construcciones sobreexpresantes y mutantes como describieron Baek y Kenerley (1998). Las colonias estables resistentes a higromicina se seleccionaron transfiriendo de manera consecutiva una sola colonia del hongo a PDA con 100 µg/ml de higromicina. Para verificar el reemplazo génico de *sm*-1 se diseñaron un

par de primers sobre el genoma de los *Trichoderma* y que flanqueán las construcciones. Para el reemplazo génico de *sm*-1 en *T. atroviride*, los oligos fueron diseñados 202 pb río arriba TaKO-f y 299 pb río debajo de la construcción TaKO-r. Para *T*. *virens* los oligos se diseñaron 246 pb río arriba TvKO-f y 137 pb río debajo TvKO-r de donde se integro la construcción y estos se utilizaron en combinación con el par de primers diseñados sobre el gen hph (tabla 1). Para las cepas de *T. atroviride* se esperaban productos de PCR de 3.15 kb para la región 5' y de 3.3 kb en la región 3' mientras que, para las cepas de *T. virens* se esperaba un producto de 3.16 kb para la región 5' y de 3.0 kb para la región 3'.

Análisis de tipo Southern de las cepas transformantes

Se extrajo el ADN total de micelio de *T. atroviride* y *T. virens* tanto de las cepas WT, como las cepas SE y KO, siguiendo el protocolo descrito por Raeder y Broda (1989). El análisis tipo Southern se realizó usando el Gene Images Alkphos Direct Labelling and Detection System (Amersham) siguiendo las recomendaciones del fabricante.

Análisis de RT-PCR del gen *sm*-1 en las cepas candidatas sobreexpresantes y mutantes

El análisis de la expresión del mensajero del gen *sm*-1 de las cepas silvestres, sobreexpresantes y mutantes de *T. atroviride* y *T. virens* se analizaron por RT-PCR semicuantitativa. Conidias de las diferentes cepas se inocularon en cajas de PDA que contenía un celofan. Las cajas inoculadas se incubaron por tres días a 28°C, después de este tiempo se colectó el micelio, se molió en un mortero en

presencia nitrógeno líquido y se extrajo el RNA total utilizando el reactivo TRIzol (Invitrogen), de acuerdo a las recomendaciones del fabricante. Posteriormente 2 μg de RNA total se trató con rDNase I (Ambion) y la reacción de transcripción reversa se realizó con la transcriptasa SuperScript II (Invitrogen). El cADN sintetizado se utilizó para amplificar el gen *sm*-1 utilizando los primers correspondientes (Tabla 1; capitulo 2). Como control de carga se utilizó el gen de actina. Para obtener el numero de ciclos optimos de la PCR para una amplificación lineal de los genes se realizo un rango de amplificaciones a varios números de ciclos utilizando el de 25 ciclos. Los productos de PCR fueron separado en un gel de agarosa al 1% , teñidos con bromuro de etidio y la foto se tomó en un fotodocumentador.

Análisis de expresión de la proteína SM1 en las cepas transformantes

Para este experimento se inocularon 100 ml de medio líquido Vogels (Vogels, 1956), suplementado con 1.5 % de sacarasa (VMS) con una suspencion de 10^6 conidia ml^{-1} de cada una de las cepas y se incubaron a 28 °C con agitacion a 200 rpm por 6 días. El cultivo se filtró con papel para cromatografía 3MM (whatman) y posteriormente con una membrana de 0.45 μm (Millipore), el cultivo obtenido se precipitó con sulfato de amonio al 80 % (Fermont) y el botón se resuspendió con pequeñas alicuotas de Tris 10 mM, pH 7.8, y se dializó con el mismo buffer utilizando una membrana de 8 kDa (SPECTRUM), el liquido dializado se concentró en medio de liofilización y se resuspendió en 40 μl de Tris 10 mM, pH 7.8. Finalmente, la concentración de las proteínas fue determinado por el método de

Bradford (Bio-Rad), y se corrieron 40 µg de proteina en un gel de SDS-PAGE y para su visualización se tiño con azul de Coomassie y la foto se tomó en un fotodocumentador.

Crecimiento y actividad micoparasitica de las cepas transformantes

Las cepas mutantes y sobreexpresantes seleccionadas se compararon con sus respectivas cepa silvestre, para evaluar la morfología de la colonia, crecimiento radial y esporulación. Explantes de agar de las colonias de los hongos fueron colocadas en el centro de una caja de Petri y fueron incubadas por 5 días a 28°C, con un fotoperiodo de 12 h para permitir la esporulación, después de la incubación las cajas se inspeccionaron visualmente para evaluar la morfología, conidiación y color de la colonia.

La actividad micoparasítica de las cepas transformantes fueron evaluadas y comparadas con las cepas silvestre. Explantes de agar de crecimiento activo de las cepas transformantes se colocó en un extremo de la caja y el otro extremo de la misma se colocó a *Rhizoctonia* solani o *Sclerotium rolfsii*. Las confrontaciones se incubaron en la oscuridad a 28°C y la interacción se evalúo después de 7 días de incubación. La actividad micoparasítica de las cepas transformantes y sus respectivas cepas silvestres fue evaluada en su capacidad para sobrecrecer y detener el crecimiento del hongo patógeno.

Ensayo de protección inducida por las cepas de *Trichoderma* contra *A. solani, B. cinerea* and *P. syringae* en plantas de tomate

Para este experimento se utilizaron los hongos fitopatógenos *A. solani* y *B. cinerea*, los cuales se crecieron sobre PDA por siete días a 28°C con un fotoperiodo de 12 h, después de este tiempo de incubación las esporas se cosecharon y se resuspendieron en agua destilada y las conidias se contaron en un hematocitómetro. Las suspensiones de esporas se ajustaron a 10^6 y 10^5 conidias ml^{-1} para *B. cinerea* y para *A. solani*, respectivamente. La bacteria fitopatógena *P. syringae* se creció en medio Kings B a 200 rpm por 48 h a 28°C y la suspensión se ajustó a una densidad óptica OD=0.2, a esta solución se le agrego el surfactante Break-Thru (Goldsmidt Chemical Corporation) a una concentración de 0.1 % para facilitar la penetración de la hoja por el patógeno.

Las semillas de tomate fueron inoculadas con 15 μl de conidias a una concentración de 10^6 conidia ml^{-1} de las cepas *T. atroviride* WT, OE 1.1, OE 2.1, OE 3.1, KO9 y de las cepas *T. virens* WT, OE 2.1, OE2.2, OE6.2 y KO2. Semillas no tratadas se utilizaron como control. Las semillas tratadas con las esporas de las diferentes cepas de *Trichoderma* se sembraron en un maceta de 10.6 x 8 cm con sustrato peat moss (Lambert Peat Moss Inc). Después de 24 h las plántulas fueron irrigadas con medio MS al 0.3X para acelerar el proceso de colonización de la rizosfera por *Trichoderma*. Seis días después, las plantas fueron irrigadas con la solución nutritiva HUMIFERT (Cosmocel) a una dosis de 3 ml por $litro^{-1}$ de agua. Para cada tratamiento se utilizaron 8 plantas y los experimentos se repitieron al menos dos veces.

Una vez que las plantas tratadas cumplieron 22 días de edad, se inocularon con *B. cinerea*, *A. solani* y *P. syringae*, respectivamente. La inoculación se realizó en tres

hojas por planta, en la zona del haz con 10 µl de la suspensión de los patógenos. Las plantas inoculadas se colocaron en el invernadero bajo condiciones controladas y se regaron todos los días para incrementar la humedad relativa y favorecer la invasión por el patógeno, ocho días posteriores de la inoculación, se evalúo el área dañada de las hojas. El porcentaje del área dañada de las hojas se obtuvo calculando la relación entre el área dañado y el área total de la hoja. El experimento se repitió tres veces y los resultados fueron sometidos a un análisis de varianza con valor de $P< 0.0001$, y a una prueba de LSD $\alpha< 0.05$.

Análisis de la expresión de genes relacionados con defensa

La expresión de genes relacionados con defensa se analizó en raíces y en hojas de plantas de 14 día de edad crecidas *in vitro* e inoculadas con *T. atroviride* WT, OE2.1 y KO9, *T*. *virens* WT, OE2.2 y KO2, respectivamente. Como control se incluyeron plantas inoculadas con agua bidestilada estéril. Plantas de 14 días de edad fueron inoculadas con 15 μl of 10^6 conidias ml^{-1} a 4 cm de la raíz con las diferentes cepas y fueron co-incubadas por 72 hrs. A las 72 de interacción *in vitro*, las hojas y raíces fueron colectadas por separado y se molieron en presencia de nitrógeno liquido y se procedió a la extracción de RNA total con el kit de extracción RNA Concert (Invitrogen).

La expresión de los genes de defensa de tomate fueron evaluados por qRT-PCR. Los oligos utilizados en esta reacción se diseñaron con el programa primer Express 3.0, tomando como molde la secuencia de cada uno de los genes de tomate que se utilizaron como marcadores que se encuentran disponible en la

base de datos (GenBank) (Tabla 1; capitulo 2) y estos genes marcadores fueron; Chit (quitinasa, gi|19190) Gluc (glucanasa, gi|498925) Pod (peroxidasa, gi|1161565) Hmgr (3-hydroxy-3-methylglutaryl CoA reductasa, gi|16304119) y actina se utilizo como control interno (gil1498365). Del RNA total extraído 2 µg se trato con rDNase I (Ambion), y enseguida fue sometido a retrotranscripción con la enzima transcriptasa reversa SuperScript II (Invitrogen). La mezcla de reaccion de la PCR tiempo real se realizó siguiendo las instrucciones del kit Fast Syber Green Master Mix (Applied Biosystem). Para las condiciones de amplificación y de disociación se utilizaron las recomendadas para el equipo Abiprism 7500 Fast Real-Time PCR system (Appied Biosystems). Para le reacción se uso 1 ng de cDNA para cada par de primers de los distintos genes. Los experimentos se repitieron dos veces de manera independiente y cada reacción se realizó por triplicado. La expresión de cada uno de los genes se normalizo con respecto al control con la formula $\Delta\Delta$CT.

Ensayo de promoción del crecimiento de plantas de tomate

Las semillas de tomate fueron inoculadas con las diferentes cepas de *T. atroviride* WT. OE1.1, OE2.1, OE3.1 y KO9, *T. virens* WT, OE2.1, OE2.2, OE6.2 y KO2, como se describió para los ensayos de protección contra patógenos. 22 días después del tratamiento con las cepas de *Trichoderma*, las plantas fueron removidos de las macetas y las raíces fueron lavadas con agua bidestilada, se midió la longitud de las mismas considerando la hoja más alta hasta la raíz. Posteriormente, se determinó el peso fresco, y después fueron deshidratadas en una estufa a 70 °C por 72 hrs y el peso seco fue determinado. Cada tratamiento

consistió de 15 plantas y el experimento se repitió tres veces. Los datos experimentales se sometieron a un análisis de varianza de $P< 0.0001$ y a una comparación de medias LSD $\alpha< 0.05$.

MATERIALES Y METODOS (ANEXO 1)

Material biológico y condiciones de crecimiento

En este trabajo para los diferentes experimentos se utilizaron las plantas de *Arabidopsis thaliana* ecotipo col-0, líneas transformantes *CycB1;1:uidA* (Colón-Carmona *et al*., 1999), *DR5:uidA* (Ulmasov *et al*., 1997) y las líneas mutantes etr1-3 (Hua and Meyerowitz, 1998)), eir 1-1 (Luschnig *et al*., 1998), ahk2-2 (Nishimura *et al*., 2004)), ahk3-3 (Higuchi *et al*., 2004), axr4-1 (Hobbie and Estelle, 1995), aux1-7 (Pickett *et al*., 1990), rhd6 (Masucci and Schiefelbein, 1994) and abi4-1 (Finkelstein, 1994). Las semillas de cada una de las líneas se esterilizaron con etanol al 95 % y se lavaron con agua destilada estéril dos veces. La semillas se colocaron en cajas petri que contenía medio agar-MS al 1x (Murashige and Skoog, 1962). Las cajas petri se colocaron en un angulo de 70 grados para permitir que las plántulas crecieran por la superficie del medio, esto fue en una cámara bioclimática con un fotoperiodo de 16 horas luz y 8 horas oscuridad, a una temperatura de 24°C.

Las plantas de papas (*Solanum tuberosum*) que se utilizaron se crecieron por 20 días en medio MS y posteriormente se transplantaron a macetas de 4 pulgadas que contenía sustrato peat moss ((Lambert peat moss Inc.).

La bacteria *Pseudomonas syringae* pv tomato fue proporcionada por el Dr. Ariel Álvarez (CINVESTAV-Irapuato, Mexico) y se creció en medio sólido o liquido Kings

B, para el medio liquido se crecieron hasta alcanzar una densidad óptica OD=0.2. *Botrytis cinerea* fue aislado de campos de cultivo de tomate en San Luis Potosí, México y se creció en medio PDA (Difco) por siete días. *Aspergillus ustus y Paecilomyces* fumosoroseus se crecieron en medio PDA por 10 días.

Aislamiento y caracterización molecular de *Aspergillus ustus*

Este hongo primeramente se aíslo de cultivos axenicos de papa, que se contaminaban con mucha frecuencia y las plantas contaminadas presentaban un fenotipo de mayor crecimiento comparada con las no contaminadas. Un explante de crecimiento micelial fue colocado en una caja petri que tenia una película de celofán y se incubo por 7 días a 28 C, después de la incubación el micelio se cosecho y se molió en nitrógeno liquido para la extracción del ADN. El material genético ADN se extrajo utilizando el método descrito por Rader y Broda, (1989). El ADN total fue usado como templado para amplificar la región intergenica transcrita del ribosoma rDNA 18S utilizando los primers (ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ y ITS4 5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). Los fragmentos amplificados se clonaron en el vector pGEM-T-easy (Promega) y se secuenciaron por el método de Sanger en un secuenciador AB. Las secuencias obtenidas fueron comparadas contra la base de datos del NCBI, utilizando el algoritmo Blast (Altschul *et al*., 1990). En el alineamiento se obtuvo una identidad del 100 % con la secuencia de la base de datos del hongo *Aspergillus ustus*.

Ensayo de promoción de crecimiento inducido por *A. ustus*

Las esporas de *Aspergillus ustus y Paecilomyces* fumosoroseus se colectaron, se cuantificaron en un hematocitometro y se ajustaron a una suspensión de $1X10^6$ esporas mL con agua destilada estéril.

Primeramente se evalúo el efecto de *A. ustus* sobre plantas de papa de donde fue aislado. Para esto las plantas de 20 días de edad que habían sido transplantadas a macetas se inocularon con $1X10^6$ esporas mL de *A. ustus* y con *P. fumosoroseus* hongo entomapatógeno utilizado como control. Las plantas tratadas y no tratadas se irrigaron una vez por semana con MS al 0.3X, durante seis semanas. Cada experimento tuvo 20 plantas por tratamiento y este experimento se realizo por duplicado. Después de seis semanas de la inoculación, el efecto del crecimiento en las plantas inducida por el hongo fue evaluado sobre raíces y tallos de las plantas de papa. Las plántulas de papa fueron donadas por el Dr. Alberto Flores Olivas (Universidad Autónoma Agraria Antonio Narro).

El efecto de *A. ustus* sobre la promoción de crecimiento de las plantas también fue evaluado de manera in *vitro* en plantas de *Arabidopsis*. Plantas de 10 días de edad crecidas sobre medio MS al 1X (cinco plantas por cajas) se inocularon con tres explantes de micelio activo a 2 cm de las raíces de *Arabidopsis* (Col-0 y líneas mutantes) y el efecto de la interacción se evalúo después de 8 días, por cada experimento se utilizaron 25 plantas.

Análisis histoquímico

Para el análisis histoquímico de la actividad de GUS, las plantas de *Arabidopsis* se incubaron en el buffer de reacción de toda la noche a 37 °C (0.5 mg/ml of 5-bromo-4-chloro-3-indolil-β-D-glucoronide en 100 mM de fosfato de sodio, PH 7). Después de la incubación las plantas fueron desteñidas utilizando el método

descrito por Jefferson *et al*. (1987). Para cada línea de *Arabidopsis* y para cada tratamiento se analizaron 10 plantas transgénicas. De las plantas analizadas se escogió una representativa y se fotografío usando una cámara digital conectada a un microscopio con el sofware motic images plus 2.0 ML.

Ensayo de protección contra bacterias y hongos Fitopatógenos inducida por *A. ustus*

La semillas de *Arabidopsis* se inocularon con esporas de *A. ustus* y se sembraron en macetas que contenían peat moss, después de 17 días las plantas se inocularon con 10 µl de una suspensión de *Pseudomonas* DO_{600}=0.2, o con 10 µl de $1x10^6$ esporas de *B. cinerea*. La suspensión de la bacteria o de las esporas del hongo se le añadió Break-Thru a una concentracion de 0.1% (Goldsmith Chemical Corporation).

En este experimento se inocularon dos hojas de cada una de las plantas, se utilizaron 10 plantas por tratamiento, las plantas se colocaron en el invernadero y estas se regaron por cinco días para incrementar la humedad relativa. Después de los cinco días se evalúo la severidad de la enfermedad en las plantas, esta se obtuvo midiendo el área total de la hoja y el área total dañado de los síntomas necróticos y se saco una proporción de daño reportándose en porcentaje.

Análisis de RT-PCR de genes de defensa inducidos por *A. ustus*

Las plantas de *Arabidopsis* Col-0 se crecieron por 14 días en medio MS y se inocularon con 10 µl de una suspensión de $1X10^6$ esporas de *A. ustus*. Las interacciones se incubaron en una cámara de crecimiento con un fotoperiodo de 16 horas luz y 8 horas oscuridad a una temperatura de 24 °C y la interacción se dejo por 48, 72 y 96 h. después de cada uno de los tiempos indicados las raíces y

hojas se separaron, colocándolo en un mortero y se molió con nitrógeno liquido, el tejido molido se utilizo para la extracción de ARN usando el reactivo Concert Plant RNA Reagent (Invitrogen) siguiendo el protocolo del fabricante. El ARN total extraído se trato con DNasa usando rDNase I (Ambion). 2 µg del ARN total se retro transcribió con la enzima transcriptasa reversa SuperScript II (Invitrogen). El cDNA generado se uso como templado para las reacciones de PCR en volumen final de 25 µl con 1U de la ADN taq polimerasa (Invitrogen).

Los primers específicos de los genes evaluados en la reacción de PCR fueron PR1 (gen relacionado a patogénesis 1; M90508) (F-atctaagggttcacaaccaggcac, R-tgcctcttagttgttctgcgtagc), PR2 (beta-1,3-glucanasa, NM_115586.2) (F-aggagcttagcctcaccacc, R-gaggatgagctcgatgtcagag), LOX (Lipoxigenasa 1, NM_104376.2) (F-agacgttccaggccatggcag, R-cttgggtaaggatactcctgtg), PER, (peroxidasa, NM_114770.2) (F-ccaagaaccgtttcatgcg, R-ggagagcgcaacaagatcag), PHY (deficiente en fitoalexina 3, NM_113595.3) (F-cgatggagatgctctcaagttc, R-gtctccttgaccacgagc) , DEF (defensina**,** NM_123809.3) (F-cacccttatcttcgctgctc, R-ggaagacatagttgcatgatcc) y control interno el gen de actina (Actina 8, NM_103814.3) (F-gactcagatcatgtttgagacc, R-catgtaacctctctcggtaagg). Para definir el numero optimo de ciclos de amplificación de los genes en la reacción de PCR un rango de amplificaciones fueron realizadas. Subsecuentemente los productos de PCR fueron separados en un gel de agarosa (electroforesis), teñido con bromuro de etidio y la foto se tomó en un fotodocumentador.