TESIS DOCTORAL

Papel de los jasmonatos y prosistemina en el establecimiento y desarrollo de una simbiosis micorrízica arbuscular

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Role of jasmonates and prosystemin on the stablishment and development of an arbuscular mycorrhizal symbiosis

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Granada, 15 de Febrero de 2013

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Doctorando

Índice.

INTERÉS DEL TRABAJO Y OBJETIVOS

Aproximadamente el 80% de las plantas terrestres, incluyendo especies de cultivo agrícola y hortícola, son capaces de establecer asociaciones mutualistas con hongos del suelo del phylum Glomeromycota. La capacidad de establecer una simbiosis micorrízica arbuscular (AM) es considerado como un paso clave en la evolución de las plantas terrestres. Como resultado de su amplia distribución, la simbiosis AM tiene un impacto global en la nutrición vegetal de fósforo y en el ciclo del carbono. La simbiosis AM es una endosimbiosis, es decir, una simbiosis donde un organismo vive dentro de las células de otro. El establecimiento de la simbiosis AM requiere, por lo tanto, que las células de la raíz hospedadora sufran modificaciones estructurales y funcionales significativas para acomodar al hongo simbionte. Para obtener una simbiosis funcional, mutuamente beneficiosa, es necesario un alto grado de coordinación entre los dos simbiontes. Estudios genéticos, moleculares y celulares han revelado que la simbiosis funcional parece ocurrir al final de una serie de puntos de control regulados por la planta en la que diferentes clases de fitohormonas juegan un papel esencial. Las fitohormonas son pequeñas moléculas esenciales para la regulación del crecimiento de las plantas, desarrollo, reproducción y supervivencia. También pueden actuar como moléculas de señal durante la interacción de las plantas con microorganismos, incluyendo patógenos y simbiontes mutualistas. Por lo tanto no es sorprendente que estas pequeñas moléculas esten involucradas en el desarrollo de la simbiosis AM, que implica el reconocimiento mutuo y un control preciso de ajustes morfológicos y fisiológicos en el hospedador. Por otra parte, cambios en la homeostasis hormonal pueden ser la base de algunos de los beneficios de la simbiosis, como la mejora de la resistencia del estrés en las plantas micorrizadas. En los últimos años se han utilizado herramientas moleculares, genéticas y genómicas para desvelar el papel de las diferentes hormonas durante la simbiosis AM. Entre ellos, el ácido jasmónico (JA), el etileno (ET), el ácido salicílico (SA) y el ácido abscísico (ABA) participan en la regulación de las respuestas de defensa de la planta en interacciones con microorganismos, incluyendo a los hongos micorrícicos. Más recientemente, las estrigolactonas, clasificadas como una nueva clase de fitohormonas, han surgido como nuevas moléculas implicadas en las interacciones planta-microorganismo. Además el JA y sus

derivados, conocidos colectivamente como jasmonatos, tienen un papel muy importante en estas interacciones, incluyendo la simbiosis AM. Sin embargo, los datos experimentales sobre el papel de jasmonatos y su patrón de acumulación durante la simbiosis AM son muy controvertidos, probablemente debido al uso de plantas, hongos y sistemas experimentales distintos. Hay considerables lagunas en nuestro conocimiento sobre cómo la planta y los hongos micorrízicos arbusculares contribuyen a la regulación de la producción de hormonas durante la simbiosis, y cómo éstas fitohormonas regulan las diferentes etapas y procesos de la simbiosis. Por lo tanto, ***el objetivo general de esta Tesis Doctoral es el estudio de las principales hormonas reguladoras de la respuesta de defensa de la planta involucradas en el establecimiento y desarrollo de una simbiosis AM, con especial énfasis en el papel de los jasmonatos y la hormona péptidica sistemina implicada en diferentes rutas de señalización.***

Para estudiar este objetivo general, hemos definido los siguientes objetivos específicos:

1. Evaluar la modulación de las principales rutas hormonales implicadas en la señalización de la respuesta de defensa de plantas, durante una simbiosis micorrízica arbuscular funcional.
2. Determinar el grado de conservación en la modulación de la señalización de la respuesta de defensa asociados a la simbiosis entre diferentes plantas hospedadoras y hongos micorrízicos arbusculares.
3. Investigar el papel de los jasmonatos y la (pro) sistemina como reguladores del establecimiento y desarrollo de una simbiosis micorrízica arbuscular.
4. Caracterizar el patrón de regulación de la prosistemina en la respuesta inmune de la planta frente diferentes hongos del suelo.

INTEREST OF THE STUDY AND AIMS

About 80% of all terrestrial plants, including most agricultural and horticultural crop species, are able to establish mutualistic associations with soil fungi from the phylum Glomeromycota. The ability to form arbuscular mycorrhizal (AM) symbiosis occurred early in the plant lineage and it is considered to be a key step in the evolution of terrestrial plants. As a result of its widespread distribution, AM symbiosis has a global impact on plant phosphorus nutrition and on the carbon cycle.

AM symbiosis is an endosymbiosis, that is, a symbiosis where one organism lives within the cells of another. The establishment of AM symbiosis requires, therefore, the host root cells to undergo significant structural and functional modifications to accommodate the fungal partner. A high degree of coordination between the two partners is required to achieve a functional, mutually beneficial symbiosis. Genetic, molecular, and cellular approaches reveals that functional symbiosis appears to occur at the end of a series of plant-controlled checkpoints in which different classes of phytohormones play an essential role. Phytohormones are small molecules that are essential for the regulation of plant growth, development, reproduction and survival. They can also act as signal molecules during plants interaction with microbes, including both pathogens and mutualistic symbionts. It is therefore not surprising that these small molecules are involved in the AM symbiosis development, which involves mutual recognition and precise control of morphological and physiological adjustments in the host. Moreover, changes in the hormone homeostasis may underlie some of the benefits of the symbiosis, as the improvement of plant stress resistance.

In recent years, molecular, genetic and genomic tools have been used to unravel the role the different hormones during AM symbiosis. Among them, jasmonic acid (JA), ethylene (ET), salicylic acid (SA) and abscisic acid (ABA) are known to be involved in fine-tuning plant responses in plant microbe interactions, including those with mycorrhizal fungi. More recently, strigolactones, recently classified as phytohormones, have emerged as new players on the battle field as well.

Special attention has received JA and its derivatives, collectively known as jasmonates, since they are believed to play a major role in plant microbe interactions, including the AM symbiosis. However, experimental data on the role of jasmonates and their

accumulation pattern during mycorrhizal symbioses is highly controversial, probably due to the use of different plant, fungi and experimental systems.

There are still considerable gaps in our knowledge on how both partners in the symbiosis contribute to the regulation of hormone production and how plant hormones regulate the different stages and processes of the symbiosis.

Therefore, the overall objective of this Doctoral Thesis is the study of ***the main hormone-regulated defense signaling pathways involved in the establishment and development of arbuscular mycorrhizal symbioses, with special emphasis in the role of jasmonates and the peptide hormone systemin*.**

To reach this main objective, we have defined the following secondary objectives:

1. To evaluate the modulation of the main hormone regulated defense signalling network in the host plant associated to a functional mycorrhizal symbiosis.

2. To determine the degree of conservation of the changes in defense signalling associated to symbioses among different host plant-mycorrhizal fungus combinations.

3. To investigate the role of jasmonates and (pro) systemin as regulators of the establishment and functioning of the arbuscular mycorrhizal symbiosis.

4. To characterize the regulation pattern of prosystemin in the plant immune response to soil fungi.

INTRODUCCIÓN

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1. Interacciones entre las plantas y los organismos del medio.

Las plantas se encuentran en un continuo intercambio de información con el medio que les rodea y gracias a esto, son capaces de adaptarse a distintas condiciones ambientales y reaccionar frente a una gran cantidad de organismos con los que interactúan. Las distintas interacciones de la planta con los organismos del medio son de vital importancia ya que pueden afectar a la salud y crecimiento de la planta. Dichas interacciones se pueden dar tanto en la parte aérea de la planta como en la raíz (Fig. 1).

A la raíz tradicionalmente se le han atribuido únicamente las funciones de anclaje al suelo y captación de agua y nutrientes, pero en los últimos años se ha visto que juega un papel fundamental en el intercambio de moléculas señal con el medio, regulando multitud de interacciones en el suelo. Desde el punto de vista de la planta, estas interacciones pueden ser de carácter beneficioso o perjudicial, debiendo la planta determinar el tipo de organismo con el que se enfrenta y adecuar su respuesta a dicha interacción.

Entre las interacciones perjudiciales son muchos los organismos que pueden causar daños mecánicos en la planta, como los nemátodos y los insectos fitófagos entre otros. Pero además, hay una gran cantidad de microorganismos patogénicos (hongos y bacterias, entre otros) capaces de provocar enfermedades en las plantas. Teniendo en cuenta su estilo de vida, los microorganismos patógenos se pueden dividir en necrótrofos y biótrofos (Berger et al., 2007). Necrótrofos son aquellos microorganismos que destruyen los tejidos de la planta huésped, a menudo mediante la producción de toxinas, y luego asimilan el contenido celular de manera saprofita. Algunos ejemplos de microorganismos necrótrofos son los hongos patógenos *Botrytis cinerea* y *Fusarium oxysporum* (Stone, 2001). Sin embargo, los necrótrofos no son muy frecuentes en la naturaleza. La mayoría de los microorganismos han evolucionado para vivir conjuntamente con el organismo que han infectado. Estos microorganismos se denominan biótrofos, y requieren de la planta huésped viva para completar su ciclo de vida (Schulze-Lefert y Panstruga, 2001). Los microorganismos patógenos biótrofos

obtienen nutrientes de las células metabólicamente activas de la planta huésped, comúnmente a través de estructuras especializadas que penetran en las células de la planta huésped. Aunque la planta huésped sigue viva, su crecimiento puede ser impedido. Un típico ejemplo de microorganismo biótrofo es el hongo patógeno *Hyaloperonospora* spp. Muchos patógenos vegetales pueden además mostrar ambos estilos de vida, dependiendo de la etapa de su ciclo de vida presentando una fase inicial biotrófica y en estadios avanzados necrotrófica, como por ejemplo hongos del genero *Colletotrichum* y oomicetos como *Phytophthora infestans.* Estos se denominan hemibiótrofos.

Para defenderse del ataque de estos patógenos, las plantas poseen una variedad de mecanismos constitutivos de defensa que incluyen barreras estructurales (por ejemplo, presencia de capas gruesas de cutícula y tricomas, deposición de ceras, entre otros) (Łaźniewska et al., 2012), así como la producción metabolitos con capacidad antimicrobiana (como en el caso de las plantas cianogénicas, poseen cantidades considerables de compuestos relacionados con el ácido cianhídrico) (Wittstock y Gershenzon, 2002). Estos mecanismos constitutivos suponen un primer nivel de protección pre-invasivo, para prevenir o atenuar la invasión de los posibles atacantes y generalmente no involucran una respuesta activa del hospedante ante la presencia del patógeno. A pesar de la gran diversidad de defensas constitutivas que las plantas poseen, muchos microorganismos han desarrollado la capacidad de romper esta primera línea de defensa de la planta. Para limitar la infección de patógenos e insectos, las plantas poseen además un amplio espectro de mecanismos de defensa inducibles, que a diferencia de la defensa constitutiva, conlleva cambios claros en el metabolismo provocados por la expresión diferencial de genes (Jones y Dangl, 2006). Por lo tanto, para que ocurra la inducción de la defensa, es necesaria la mediación de sistemas de reconocimiento específico, mediante los cuales la planta reconoce la presencia del patógeno.

Además en la naturaleza se dan un gran número de asociaciones beneficiosas entre las plantas y determinados grupos de microorganismos del suelo tales como los hongos micorrícicos y otros hongos promotores del crecimiento vegetal, las rizobacterias, los rizobios y otros organismos rizosféricos. Estas interacciones ejercen efectos positivos sobre el crecimiento y la supervivencia de las plantas a través de diferentes

mecanismos, tanto directos como indirectos mediante la alteración de la fisiología de la planta. Estos organismos beneficiosos son inicialmente reconocidos por el sistema inmune de las plantas como potenciales invasores, por lo tanto para poder establecer una interacción mutualista con su huésped, estos microorganismos beneficiosos han desarrollado diferentes estrategias para interferir con el sistema inmune de las plantas (Zamioudis y Pieterse, 2012).

Tras el reconocimiento mutuo entre la planta y el microorganismo, se inducen diferentes cascadas de señalización en la planta que acabaran con una modulación del sistema inmune para adecuar la respuesta de defensa de la planta al tipo de interacción que se está dando (De Vos et al., 2005; Reinhardt, 2007). Cabe destacar que todo este proceso es dinámico y que puede verse alterado por cualquier variación que ocurra en alguno de los participantes en este sistema.

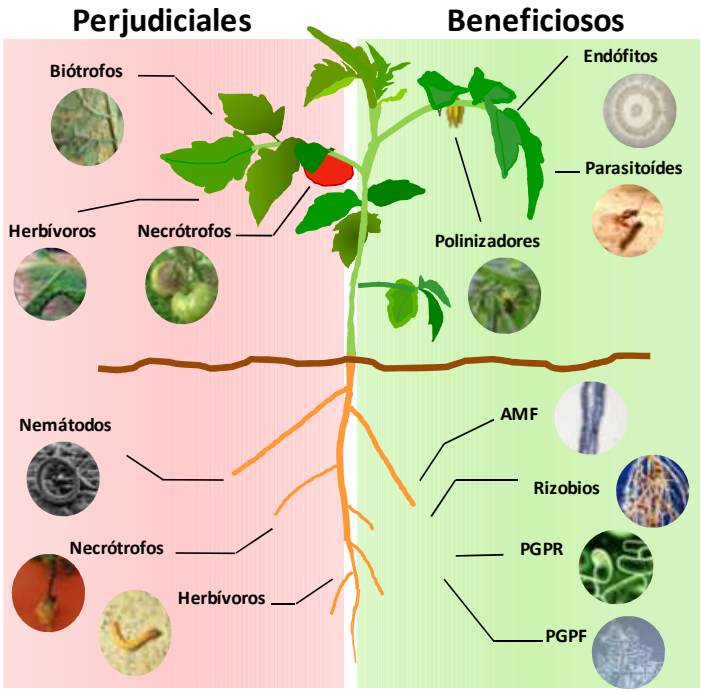

Figura 1. Esquema general interacciones planta-organismos. Las plantas interaccionan con multitud de organismos, algunos potencialmente perjudiciales para ella como bacterias, hongos patógenos e insectos herbívoros. Por otro lado la planta también interacciona con organismos beneficiosos como bacterias, hongos promotores del crecimiento vegetal, agentes de control biológico e insectos polinizadores.

2. Sistema inmune de la planta

Las plantas poseen un sistema de defensa sofisticado, que de manera similar al sistema inmune de los animales, es capaz de reconocer determinadas moléculas o señales y responder activando una respuesta inmune adaptada al organismo atacante (Jones y Dangl, 2006; Howe y Jander, 2008). Para poder combatir de manera eficiente el ataque de patógenos o insectos por un lado, y permitir el establecimiento de asociaciones beneficiosas por otro, las plantas han desarrollado diferentes estrategias para reconocer diferentes tipos de interacciones bióticas y responder activando respuestas específicas que le permitan protegerse frente a los agresores y sacar provecho de las interacciones beneficiosas (Pieterse y Dicke, 2002; Zamioudis y Pieterse, 2012).

Defensa basal y defensa específica de huésped

El sistema inmune de las plantas está compuesto por dos líneas de defensa frente a los organismos patogénicos presentes en el medio (Jones y Dangl, 2006). El primer nivel del sistema inmune de la planta es conocido como **defensa basal** y depende de la presencia de los receptores de transmembrana denominados **PRRs** (del inglés "*Pattern Recognition Receptors*"), que son capaces de reconocer ciertos patrones moleculares asociados a posibles agresores. Estos PRRs son receptores ricos en leucina y pueden tener un dominio kinasa (**LRR-RKs**, del inglés "*Leucine-rich Repeat Receptor kinases*") o bien tener la capacidad de interaccionar con proteínas que tienen ese dominio kinasa. Los PRRs son capaces de reconocer patrones moleculares asociados a microorganismos, conservados en la mayoría de los grupos microbianos y conocidos como **MAMPs**, del inglés "*Microbe-Associated Molecular Patterns*"), o PAMPs, del inglés "Pathogen associated Molecular Patterns", los cuales tradicionalmente se denominaban **elicitores** por ser capaces de desencadenar la activación de los mecanismos de defensa de la planta. Entre los MAMPs se encuentran moléculas con funciones básicas para el microorganismo, como son los lipopolisacáridos (**LPS**), peptidoglucanos y los oligómeros de quitina y glucanos, componentes mayoritarios de la pared celular y membrana externa de bacterias y hongos, respectivamente, y la flagelina, principal componente del flagelo bacteriano (Newman et al., 2007, Spoel y Dong, 2012, Schwessinger y Roland, 2012). La inmunidad inducida en la planta tras la percepción de estas moléculas se denomina **PTI** (del inglés "*PAMP-Triggered*

Immunity"). Muchos de estos PAMPs son moléculas esenciales para la vida de estos microorganismos patógenos y no pueden ser suprimidos o atenuados, lo que proporciona a la planta una ventaja evolutiva para poder percibir el ataque de estos patógenos. Tras la detección de los PAMPs por el sistema inmune, se activa en la planta una respuesta de defensa para protegerse del organismo atacante. Esta respuesta incluye entre otros procesos la fortificación de la pared celular, la síntesis de callosa y la acumulación de diversos compuestos con actividad antimicrobiana como las fitoalexinas y las **proteínas PR** (del inglés "*Pathogenesis-Related protein*") (Fig. 2). De manera similar, la planta puede reconocer insectos potencialmente perjudiciales a través de la percepción de patrones moleculares llamados **HAMPs** (del inglés "*Herbivore-Associated Molecular Patterns"*) que proceden de las secreciones del insecto o de compuestos que se modifican mediante su alimentación (Howe y Jander, 2008; Wu y Baldwin, 2012).

La planta además de reconocer estos elicitores exógenos (MAMPs/PAMPs/HAMPs) es capaz de detectar elicitores endógenos, liberados de forma mecánica o enzimática por la propia planta tras el ataque de un patógeno. Estas moléculas se denominan **DAMPs** (del inglés "*Damage-Associated Molecular Patterns*") (Boller y Felix, 2009; Heil, 2009) y son comunes tras el ataque de diversos patógenos. La detección de estos DAMPs por la propia planta supone una vía alternativa de defensa muy eficaz en el caso de que el microorganismo sea capaz de modificar o enmascarar sus PAMPs.

Como en una carrera armamentística, algunos patógenos han desarrollado la capacidad de producir una serie de efectores que bloquean la señalización de la planta evitando esta primera línea de defensa (PTI) dando lugar a una susceptibilidad de la planta hospedadora frente al patógeno (**ETS**, del inglés "*effector-triggered susceptibility*") (Jones y Dangl, 2006; Chisholm et al., 2006). En respuesta, algunas plantas han desarrollado la capacidad de reconocer estos efectores mediante unas proteínas (proteínas R) y desencadenar así una segunda respuesta de defensa. Esta segunda línea de defensa del sistema inmune de la planta se denomina **defensa específica de huésped o ETI** (del inglés "*Effector-Triggered Immunity*"), (Fig. 2). Este tipo de defensa, también conocida como **resistencia gen a gen** depende del cultivar de planta huésped y cepa del patógeno atacante. La resistencia a patógenos mediada por las proteínas R solo es efectiva frente a patógenos que pueden crecer en tejidos vivos

(biótrofos o hemibiótrofos) y no frente a necrótrofos (Ayers et al., 1976). Las proteínas R son capaces de detectar diferentes efectores liberados por los microorganismos patógenos, tras lo que se activa en la planta una respuesta de defensa similar a la PTI que incluye entre otras respuestas la formación de callosa/lignina o producción de proteínas PR. Aunque activan respuestas de defensa similares en la planta, la defensa específica de huésped es más rápida, intensa y duradera en el tiempo, siendo por tanto más eficaz que la defensa basal (Fig. 2). Cuando estas proteínas R reconocen los efectores liberados por el patógeno normalmente se produce en la planta una respuesta hipersensible o **HR** (del inglés "*Hypersensitive Response*") que desencadena la muerte celular programada o **PCD** (del inglés "*Programmed Cell-Death*") en el lugar de la infección, confinando al patógeno en una zona concreta (Dangl et al., 1996; de Wit, 1997; Hatsugai et al., 2009; Spoel y Dong, 2012).

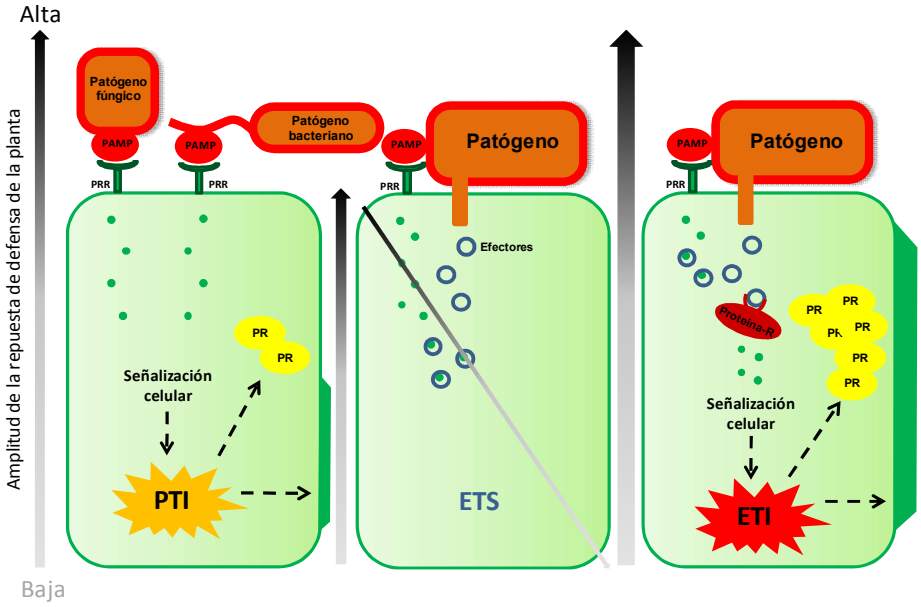

Figura 2. Modelo 'zig-zag' que ilustra la intensidad de la respuesta del sistema inmune de la planta.
En una primera fase la planta detecta patrones moleculares asociados a patógenos (PAMPs) mediante los receptores de reconocimiento de patrones (PRRs), activando la respuesta inmune asociada a PAMPs (PTI). En una segunda fase los patógenos liberan efectores al interior de la célula interfiriendo con la PTI resultando una susceptibilidad de la planta al patógeno (ETS). En la tercera fase los efectores son reconocidos por proteínas-R de la planta, activando un respuesta del sistema inmune mucho más intensa que la PTI (ETI). Figura adaptada de Jones y Dangl, 2006.

Resistencia sistémica inducida y adquirida

Una vez que las defensas de la planta se activan las plantas pueden desarrollar una mayor resistencia a un nuevo ataque. Este fenómeno se conoce como **resistencia inducida** y comúnmente actúa no solo de manera local, sino que también se activa de manera sistémica en partes alejadas de donde se ha producido la infección, para proteger los tejidos de la planta que no han sido dañados. Dependiendo del tipo de estimulo que actúa como inductor, y de los mecanismos responsables de la resistencia alcanzada se habla de distintos tipos de resistencia inducida:

El primer tipo de resistencia inducida descrito fue el derivado de la interacción de la planta con un patógeno avirulento o de baja virulencia, que hace a la planta más resistente al ataque posterior de patógenos más virulentos. Esta respuesta de defensa se denomina **SAR** (del inglés "*Systemic Acquired Resistance"*), suele ser de larga duración, y es capaz de proteger a la planta frente a un amplio rango de diferentes patógenos tales como hongos, bacterias o virus (Durrant y Dong, 2004; Vlot et al., 2009). La activación de la resistencia SAR, puede producirse tras la activación de las respuestas ETI o PTI y se caracteriza por la activación de un conjunto de **genes PR** (del inglés "*Pathogenesis Related"*), muchos de los cuales codifican para proteínas con actividad antimicrobiana (Van Loon et al., 2006). La activación de SAR se asocia además con niveles elevados de la hormona **SA** en el lugar de la infección y a menudo también de manera sistémica en partes distales de la planta. El uso de líneas mutantes y plantas transgénicas afectadas en la capacidad de producir SA, ha permitido establecer el papel clave de esta hormona en la activación de la respuesta SAR. Diferentes estudios han demostrado que estas plantas, afectadas en la producción de SA son incapaces de desarrollar la respuesta SAR, y no muestran una activación de genes PR tras la infección de un patógeno, lo que indica que el SA es un intermediario necesario para la señalización de esta ruta (Durrant y Dong, 2004).

Determinados microorganismos beneficiosos del suelo como los hongos micorrícicos arbusculares (**AMF**, del inglés "*Arbuscular Mycorrhizal Fungi*"), las rizobacterias promotoras del crecimiento de las plantas y los hongos beneficiosos pertenecientes al género *Trichoderma*, son capaces de inducir un tipo de inmunidad similar a la inducida por los patógenos avirulentos, llamada **ISR** (del inglés "*Induced Systemic Resistance"*) (Pieterse et al., 1996, 1998; Van Loon et al., 1998; Pozo y Azcón Aguilar, 2007; Segarra

et al., 2008). La respuesta ISR, al igual que la SAR es una resistencia sistémica, duradera y de amplio espectro que puede conferir protección frente a bacterias, hongos virus e incluso insectos. A diferencia de la respuesta SAR, la activación de ISR por microorganismos beneficiosos es independiente de la hormona SA, y no conlleva cambios importantes en la activación de genes *PR* (Van Wees et al., 2008; Van der Ent et al., 2009). La activación de esta respuesta depende sin embargo de las rutas reguladas por las hormonas ET y JA. Estudios recientes han demostrado que la respuesta ISR requiere igualmente de la proteína reguladora NPR1 y que los factores de transcripción MYC2 y MYB72 desempeñan un papel clave en los procesos tempranos de señalización de la respuesta ISR (Pieterse et al., 1998; Van Wees et al., 2000; Pozo et al., 2008; Van der Ent et al., 2008).

La activación de ISR por microorganismos beneficiosos no conlleva una inducción directa de los mecanismos de defensa de la planta, lo que supondría considerables costes energéticos, sino que está asociada con un aumento de la capacidad de la planta para activar sus mecanismos de defensa tras el ataque de un patógeno, proceso llamado "**priming**" por analogía al proceso descrito con este nombre en el sistema inmune de mamíferos (Pozo et al., 2008; Van der Ent et al., 2008; Van der Ent et al., 2009). La inducción de resistencia mediante *priming* tiene un coste mínimo sobre el crecimiento de la planta o la cosecha final, ya que la respuesta defensiva se desencadena solo en caso del reconocimiento de un posible atacante.

2.1 Señalización en el sistema inmune de plantas

En las plantas, al igual que en los animales, el **Ca^{2+}** juega un papel importante como mensajero secundario en las distintas cascadas de señalización que ocurren en respuesta a cualquier estrés, tanto abiótico o biótico. En los procesos de señalización temprana que ocurren durante una interacción planta-microorganismo, la pérdida de la homeostasis de Ca^{2+} es clave para el reconocimiento del microorganismo y por lo tanto para la activación de la respuesta inmune de la planta (Dangl et al., 1996; Grant et al., 2000; Lecourieux et al., 2006; Ma y Berkowitz, 2007). Una de las primeras respuestas fisiológicas de la planta durante la interacción con un microorganismo es un cambio en el flujo de iones a través de la membrana plasmática de la planta. Este

cambio en el flujo de iones se basa en un influjo de H^+ y Ca^{2+} acompañado de un eflujo de K^+, acidificando el citoplasma y alcalinizando el apoplasto (Zhao et al., 2005; García-Brugger et al., 2006) (Fig. 3). Diferentes estudios han demostrado que tras el reconocimiento de los PAMPs durante una interacción planta-patógeno, se produce una entrada de Ca^{2+} al citoplasma, que es clave para todos los procesos de señalización relacionados con la respuesta de defensa de la planta.

De manera similar trabajos recientes han mostrado **picos de Ca^{2+}** (en inglés "***calcium spiking***"), tanto a nivel citoplasmático como nuclear, durante la etapa presimbiótica de interacciones beneficiosas como son la simbiosis micorrícica arbuscular (**AM**, del inglés "a*rbuscular mycorrhizal*") (Kosuta et al., 2008; Chabaud et al., 2010) o la nodulación por *Rhizobium* (Miwa, 2006). Estos resultados ponen de manifiesto el papel de esta molécula de señalización durante la interacción de la planta con diferentes tipos de microorganismos tanto patógenos como beneficiosos. Dependiendo de la duración, intensidad y localización de este incremento de Ca^{2+}, se activarán distintos procesos de señalización en la planta, permitiendo una respuesta de defensa adecuada al tipo de microorganismo que este interaccionando con la planta (Harper y Harmon, 2005; Charpentier et al., 2008; McAinsh y Pittman, 2009).

La planta es capaz de percibir e interpretar estas distintas alteraciones de Ca^{2+}. Estas señales son interpretadas principalmente mediante dos rutas: la ruta dependiente de las proteínas kinasas dependientes de calcio (**CDPKs**, del inglés "*calcium-dependent protein kinases***"**) y la ruta dependiente de las proteínas kinasas activadas por mitógeno (**MAPKs**, del inglés "*mitogen-activated protein kinases*") (Wurzinger et al., 2011). CDPKs y MAPKs son componentes centrales de la inmunidad innata de la planta (Meszaros et al., 2006; García-Brugger et al., 2006, Coca y San Segundo, 2010). Entre los mecanismos de defensa que controlan estas dos rutas destacan la biosíntesis de algunas fitohormonas relacionadas con la defensa de la planta, expresión de genes de defensa, producción de especies reactivas de oxígeno, acumulación de oxido nítrico (**NO**, del inglés "*Nitric oxide*") y como consecuencia la PCD (Wurzinger, et al., 2011). Algunos estudios han mostrado una interconexión o "**crosstalk**" entre CDPKs y MAPKs frente a estreses bióticos (Ludwig et al., 2005), sin embargo estudios más recientes muestran una total independencia entre ambas rutas durante la respuesta inmune de la planta (Boudsocq et al., 2010).

Cuando la planta se enfrenta a algún estrés abiótico o biótico se produce en la planta una acumulación de especies reactivas de oxígeno (**ROS**, del inglés "*Reactive Oxygen Species*") dando lugar a la llamada explosión oxidativa (Alscher y Hess, 1993; Arora et al., 2002; Bhattacharjee, 2012) (Fig. 3). Las principales ROS que participan en los procesos de señalización asociados a la respuesta inmune de la planta son el radical superóxido (O_2^-), el peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (OH) (Bhattacharjee, 2010; Gill y Tuteja, 2010). La célula vegetal en condiciones normales produce bajos niveles de ROS que se acumulan en las mitocondrias y cloroplastos como producto del metabolismo aeróbico. Para evitar los daños oxidativos y mantener los niveles adecuados de ROS en la célula, las plantas han desarrollado distintos mecanismos enzimáticos o no enzimáticos para regular la biosíntesis y degradación de estas moléculas. Las ROS presentan una doble función en los mecanismos de defensa de la planta, ya que pueden actuar directamente sobre los patógenos como compuestos antimicrobianos o indirectamente, participando en la señalización de la respuesta inmune de la planta, regulando la inducción de las respuestas defensivas (Quan et al., 2008). Durante una interacción no compatible con un patógeno, la planta produce grandes cantidades de ROS, específicamente H_2O_2 (Levine et al., 1994), que es liberado al apoplasto cerca del lugar de penetración del patógeno (Apel y Hirt, 2004). Esta acumulación de H_2O_2 provoca un aumento en la síntesis de SA y la inducción de algunos genes relacionados con la defensa de la planta, como *PR-1* (Chen et al., 1993; Desikan et al., 1998). Además el H_2O_2 parece tener un papel clave en la PCD que ocurre durante la fase HR tras el ataque de un patógeno (Pennell y Lamb, 1997). La acumulación de H_2O_2 en la planta tras la interacción con un patógeno provoca un estrés oxidativo, que da origen a un aumento de Ca^{2+} citosólico (Hirt, 2000) y la posterior activación de las CDPKs y/o MAPKs (Alveraz y Lamb, 1997). Toda esta cascada de señalización terminara con la PCD y la confinación del patógeno.

El **NO** es otra molécula de señalización clave durante las primeras etapas de la interacción entre las plantas y los microorganismos patógenos debido principalmente a su implicación en la PCD que ocurre durante la HR. Es una molécula gaseosa que participa en numerosos procesos de señalización tanto en plantas como en animales (Wendehenne et al., 2001; Besson-Bard et al., 2008). En las plantas, el NO participa en procesos como la germinación, el cierre estomático, la senescencia y la defensa frente

a estreses tanto abióticos como bióticos (Hong et al., 2008; Wilson et al., 2008; Leitner et al., 2009). Cuando la planta interacciona con un patógeno, tanto durante una interacción incompatible con un biótrofo como en interacciones compatibles con necrótrofos, se produce una rápida producción de NO en la planta (Delledonne et al., 1998; Van Baarlen et al., 2004) a través de la actividad de la enzima óxido nítrico sintasa dependiente de Arg (Delledonne et al., 1998; Huang y Knopp, 1998; Zhang et al., 2003). Se ha demostrado además un incremento en la producción de NO en la planta en respuesta a ciertos elicitores basales como los LPS, activadores de la respuesta inmune de la planta (Zeidler et al. 2004). Muchos de los procesos en los que el NO tiene un papel clave, son regulados a través de la interacción con otras pequeñas biomoléculas. Por ejemplo, el NO y el H_2O_2 actúan de forma conjunta en la PCD que se da en la HR de la respuesta inmune de la planta frente a un patógeno (Delledonne et al., 2001). Lin et al., 2012 observó que la aplicación de H_2O_2 induce la producción de NO en la planta, confirmando la interconexión entre ambas moléculas.

El NO además parece jugar un papel clave como molécula señal durante la interacción de la planta con microorganismos beneficiosos como *Rhizobium* o los AMF. Durante las primeras etapas de la interacción con *Rhizobium* (Shimoda et al. 2005; Nagata et al., 2008; del Giudice et al., 2011) así como en etapas posteriores (Baudouin et al., 2006; Pii et al., 2007; Horchani et al., 2011) se produce una acumulación de NO en la planta, que parece ser necesaria para el establecimiento de esta simbiosis. De manera similar, en trabajos más recientes se ha detectado una acumulación de NO en la planta en respuesta a exudados procedentes de AMF (Calcagno et al., 2012; Martínez-Medina et al., unpublished), sugiriendo un papel clave de esta molécula en procesos de señalización durante la interacción de la planta con hongos micorrícicos. Por tanto el NO parece determinante en la regulación de la respuesta de la planta a microorganismos regulando tanto interacciones patogénicas como mutualistas (Leitner et al., 2009; Wang y Ruby, 2011).

Las evidencias experimentales revelan la interconexión o diálogo molecular ("crosstalk") entre las rutas en las que intervienen las moléculas señal descritas como H_2O_2, Ca^{2+} y NO (González et al., 2012). Esta estrecha relación entre las moléculas de señalización durante la respuesta de la planta frente a diversos estreses pone de manifiesto la enorme complejidad de las cascadas de señalización que regulan los

mecanismos de defensa. Estos elementos, además de regular eventos muy tempranos en las respuestas del sistema inmune también contribuyen a la regulación de la biosíntesis de las fitohormonas relacionadas con defensa, descritas en el apartado 2.2. Estas juegan un papel fundamental en la coordinación de la respuesta a desencadenar para que sea apropiada y eficaz frente al tipo de agresor con el que la planta se enfrenta.

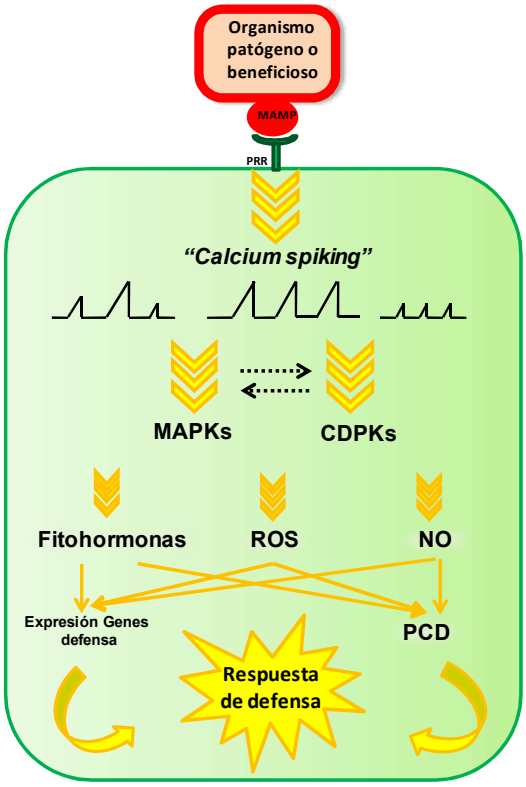

Figura 3. Modelo general de señalización en la respuesta de defensa de la planta frente a un microorganismo. La planta detecta patrones moleculares asociados a microorganismos (MAMPs) mediante sus receptores de reconocimiento de patrones (PRRs), produciéndose un aumento de calcio citosólico en la célula ("Calcium spiking"). Dependiendo del microorganismo que interaccione con la planta el patrón de calcio será distinto, siendo procesado por proteínas kinasas activadas por mitógeno (MAPKs) o por proteínas kinasas dependientes de calcio (CDPKs). Ambas rutas regulan la producción de moléculas muy importantes que participan en procesos clave para la defensa de la planta, como son la expresión de genes de defensa y la muerte celular programada (PCD). Entre estas moléculas están las especies reactivas de oxígeno (ROS) y óxido nítrico (NO) y las fitohormonas implicadas en la defensa de la planta

2.2 Implicación de las fitohormonas en la inmunidad de la planta

En los apartados anteriores se ha descrito el papel de diversas moléculas y procesos de señalización que tienen un papel clave durante la respuesta inmune de la planta. Aguas abajo de de esta cascada de señalización determinadas fitohormonas cobran un papel esencial en la modulación del sistema de defensa de la planta, traduciendo estos primeros eventos de señalización en la activación de la respuesta de defensa apropiada. Entre todos los grupos de fitohormonas descritos, incluyendo auxinas, giberelinas (Gas), citoquininas, brasinosteroides, estrigolactonas, ácido abscísico (ABA), etileno (ET), ácido salicílico (SA) y ácido jasmónico (JA), éstas últimas se reconocen como las principales hormonas de defensa en las plantas (Browse, 2009: Vlot et al., 2009, Pieterse et al., 2009). A pesar de ello son múltiples las evidencias que demuestran que otras hormonas como el ET (Van Loon et al., 2006), ABA (Ton et al., 2009) Gas (Walters y McRoberts, 2006) y brasinoesteroídes (Nakashita et al., 2003) tienen también un papel importante en la modulación del sistema inmune.

Tras el ataque de un patógeno o insecto, se producen cambios en la concentración endógena o en la sensibilidad a estas hormonas en la planta que median la activación de diferentes respuestas de defensa. Dependiendo del tipo de agresor la planta varía la proporción y tiempo de aparición de las distintas hormonas lo que permite priorizar una u otra vía de señalización (de Vos et al., 2005, Pieterse et al., 2009). En general, los patógenos biótrofos parecen ser más sensibles a las respuestas reguladas por SA, relacionadas con la muerte celular programada, mientras que lo necrótrofos y los insectos suelen ser sensibles a las respuestas dependientes de JA y de ET. Puesto que la inducción de los mecanismos de defensa lleva frecuentemente asociados importantes costes a nivel de crecimiento y desarrollo para la planta (Walters y Heil, 2007) es de vital importancia la activación de los mecanismos más eficaces para un agresor particular. A este respecto, las diferentes rutas de señalización que son reguladas por estas hormonas interaccionan entre sí de manera sinérgica o antagónica, formando una compleja red de interacciones o "*crosstalk*", que proporciona a la planta una gran capacidad para ajustar su respuesta de defensa al invasor de una manera más eficiente (Pieterse et al., 2009).

2.2.1 Etileno (ET)

El ET es una fitohormona pequeña y muy difusible que juega un papel crítico en diversos procesos de desarrollo de la planta como son la germinación de las semillas, maduración del fruto, abcisión y senescencia (Abeles et al., 1992; Klee, 2004). Además es una hormona importante en la respuesta de la planta frente a distintos estreses bióticos y abióticos. Durante estreses bióticos el ET es un modulador importante de la respuesta inmune de la planta, pudiendo actuar de forma sinérgica o antagónica con respecto a la respuesta de defensa de la planta. Este papel dual sobre la respuesta inmune de la planta es debido a su implicación en el "crosstalk" que existe entre las rutas hormonales del SA y JA, las cuales tienen un papel clave para la defensa de la planta (Leon-Reyes et al., 2009, 2010; Sato et al., 2010). Se considera que el ET junto con el JA regulan fundamentalmente las respuestas frente a patógenos necrótrofos, induciendo entre otros genes codificantes para isoformas básicas de proteínas PR como glucanasas o quitinasa.

Las plantas sintetizan esta hormona vegetal a partir de la metionina en tres reacciones enzimáticas, las cuales están reguladas por numerosos factores. La enzima limitante en esta biosíntesis es la ácido 1-aminociclopropano-carboxílico sintetasa (ACS) (Kende, 1993), la cual cataliza la conversión de S-Adenosil metionina (SAM) a 1-aminociclopropano-carboxílico (ACC). EL ACC será finalmente transformado en ET por una ACC oxidasa (ACO).

2.2.2 Ácido abscísico (ABA)

El ABA es una fitohormona implicada en la regulación de diversos procesos de desarrollo en la planta como son la geminación, embriogénesis y senescencia de la hoja. Además, juega un papel importante en la respuesta de defensa de la planta frente a estreses de tipo abiótico, en concreto frente a salinidad y sequia (Tuteja, 2007; Wasilewska et al., 2008; Ruiz-Lozano et al., 2009; Aroca et al., 2012a) ya que participa en la activación de genes que codifican para proteínas, tales como las **dehidrinas**, que regulan el balance hídrico de la planta. Además el ABA es clave en la regulación del cierre estomático, mecanismo por el cual la planta puede responder frente a condiciones de sequia o salinidad elevada (Schroeder et al., 2001). En el proceso de

biosíntesis de esta hormona destaca la la enzima 9-cis-epoxicarotenoide dioxigenasa 1 (**NCED1**, del inglés "*9-cis-epoxycarotenoid dioxigenase 1*"), que cataliza la oxidación de los 9-cis-epoxycarotenoides. Esta enzima regula la biosíntesis del ABA cuando la planta es sometida a distintos estreses (Schwartz et al., 1997; Tan et al., 1997; Qin y Zeevaart, 1999).

Por otro lado, en recientes estudios se ha observado que el ABA actúa modulando la respuesta inmune de la planta frente a estreses bióticos (Adie et al., 2007; Hernández; Asselbergh et al., 2008; Ton et al., 2009; Cao et al., 2011). El papel del ABA en la regulación de la respuesta inmune de la planta es muy complejo y depende en gran medida de su inter-relación con otras rutas hormonales implicadas en la respuesta de defensa de la planta (Spoel y Dong, 2008; Grant y Jones, 2009). El ABA interacciona generalmente de forma antagonista con las rutas hormonales dependientes de SA y JA/ET (Anderson et al., 2004; Mauch-Mani and Mauch, 2005; Robert-Seilaniantz et al., 2007; Yasuda et al., 2008; de Torres-Zabala et al., 2009). Debido a esta interacción antagonista, el ABA es considerado una fitohormona capaz de comprometer la respuesta inmune de la planta (Asselbergh et al., 2008). De hecho numerosos estudios muestran un efecto negativo del ABA sobre la respuesta inmune de la planta frente a necrótrofos y biótrofos o hemibiótrofos (Adie et al., 2007; de Torres-Zabala et al., 2007; Goritschnig et al., 2008; Fan et al., 2009; Jiang et al., 2010). Estudios realizados con líneas mutantes que mostraban comprometida la síntesis de esta hormona, han demostrado una mayor resistencia al ataque de estos patógenos, mientras que el aporte exógeno de la hormona conlleva una mayor susceptibilidad de las plantas a la infección. Además, ciertos patógenos presentan la capacidad de producir ABA (Assante et al., 1977; Dörffling et al., 1984; Jiang et al., 2010), e incluso modular los niveles de esta hormona en la planta infectada (Kettner y Dörffling, 1995). Debido a que no hay evidencias de que el ABA tenga un papel en la fisiología de estos patógenos, parece que éstos usan la maquinaria de biosíntesis de ABA para promover el proceso de infección.

Aunque son numerosos los trabajos que muestran el efecto negativo del ABA sobre el sistema inmune de la planta, hay algunos trabajos en los que se muestra un efecto positivo del ABA sobre la respuesta de defensa de la planta, principalmente frente a microorganismos de de tipo viral o necrótrofo (Ton y Mauch-Mani, 2005; Mauch-Mani

y Mauch 2005; Adie et al., 2007; Vleesschauwer et al., 2010). Aunque se desconocen los mecanismos moleculares por los que el ABA actúa como regulador positivo de las respuestas de defensa a nivel pre-infectivo, el ABA regula el cierre estomático de la planta, regulando en cierta medida las entrada del patógeno (Melotto et al., 2006; Zenget al. 2010). Además el ABA tiene un papel importante en el proceso de deposición de callosa en la hoja durante el ataque de un biótrofo, lo que posibilita que el patógeno quede encapsulado, limitando la invasión (Ton y Mauch-Mani, 2004; Ton et al., 2005; Kaliff et al., 2007; Luna et al., 2011).

2.2.3 Ácido salicílico (SA)

El SA es una fitohormona con un importante papel en la señalización durante la respuesta de defensa de la planta (Vlot et al., 2009). La ruta regulada por esta hormona es efectiva principalmente frente a patógenos biótrofos (Glazebrook, 2005) ya que es el principal regulador de la muerte celular programada. El SA es un compuesto fenólico que puede ser sintetizado a partir de un metabolito primario, mediante dos rutas enzimáticas distintas. Una de esas rutas está asociada a la fenilalanina amonio liasa (**PAL**, del inglés "*Phenylalanina Ammonia Lyase*") y otra al isocorismato sintasa (ICS/SID2, del inglés "*Isochorismate Synthase*") (Garcion y Métraux, 2006, Dempsey et al., 2011). La biosíntesis de SA comienza durante la PTI o ETI tras el reconocimiento de los PAMPs o efectores del patógeno (Mishina y Zeier, 2007). El aumento transitorio en los niveles de Ca^{2+} citoplasmático, inducido por los patógenos, es clave en las etapas previas a la biosíntesis de SA (Due et al., 2009). La proteína reguladora NPR1 tiene un papel importante en la transducción de la señalización dependiente de SA. NPR1 tras ser activado por SA, actúa como un co-activador transcripcional de una batería de genes *PR*, para frenar el ataque del patógeno (Dong, 2004; Moore et al., 2011). Entre estos genes, la activación de ***PR-1a***, codificante para la forma ácida de la proteína de actividad antimicrobiana PR1 está ampliamente descrita, y por ello este gen es usado como marcador de la activación de las respuestas reguladas por SA.

Una vez que la ruta hormonal regulada por el SA se activa en el lugar de la infección tras el ataque de un patógeno, frecuentemente se activa de manera sistémica una

respuesta similar en partes distales de la planta, para proteger los tejidos no dañados frente a nuevas invasiones del patógeno (SAR, vista en el apartado 2). La SAR suele ser de larga duración, y es capaz de proteger a la planta frente a un amplio espectro de diferentes patógenos tales como hongos, bacterias o virus. (Vlot et al., 2009). Además el SA puede ser metilado pasando a Metil-SA (MeSA), el cual es un compuesto muy volátil que puede ser emitido por la planta tras el ataque de un insecto (Ament et al., 2004) para atraer a parasitoides como mecanismo de defensa indirecto. La conversión de SA en MeSA está regulada por jasmonatos (Ament et al., 2004).

2.2.4 Ácido jasmónico (JA)

El JA y sus derivados, metabolitos relacionados estructuralmente con el JA, conocidos colectivamente como **jasmonatos** (JAs), son componentes lipídicos que se sintetizan a través de la ruta de biosíntesis de las oxilipinas (Gfeller et al., 2010). La ruta de biosíntesis de las oxilipinas, y en concreto del JA está ampliamente descrita (Fig. 4) y muchas de las enzimas que participan en este proceso están bien caracterizadas mediante estudios bioquímicos, genéticos y estructurales (Wasternack, 2007; Schaller y Stintzi, 2009; Wasternack y Kombrink, 2010; Kombrink, 2012). La biosíntesis de JA comienza con la liberación de ácido α-linolénico (18:3) de las membranas lipídicas de los cloroplastos. El ácido α-linolénico liberado es oxidado por la 13-lipoxigenasa (**LOX**) pasando a "*13(S)-hidroperoxy-octadecatrienoic acid*" [13(S)-HPTO], el cual puede ser metabolizado para formar diferentes clases de oxilipinas (Feussner y Wasternack, 2002; Mosblech et al., 2009). El primer paso clave en la biosíntesis del JA, es el paso por el que el [13(S)-HPTO] es enzimáticamente catalizado hasta [12,13(S)-EOT] ("*12,13(S)-epoxy-octadecatrienoic acid*"), mediante la enzima **AOS** (del inglés "allene oxide synthase") (Park et al., 2002; von Malek et al., 2002). Sobre este epóxido alélico producido por la AOS, actúa la enzima **AOC** (del inglés "*allene oxide ciclase*") dando lugar al "*(9S, 13S)-12-oxo-phytodienoic acid*" o **OPDA**. El OPDA es el primer producto de esta ruta biológicamente activo (Stenzel et al., 2003; Wasternack, 2007). Posteriormente el OPDA pasa del cloroplasto a los peroxisomas, donde la biosíntesis continua hasta obtener el **(+)-7-iso-JA**. Además de las epimerizaciones, el JA también está sujeto a diversas modificaciones enzimáticas originando numerosos metabolitos a

partir del JA (Wasternack, 2007; Göbel y Feussner, 2009; Koo y Howe, 2012). Esos metabolitos incluyen el JA conjugado con aminoácidos, tales como el **JA-Ile** (del inglés "*jasmonoyl-L-isoleucine*") (Fonseca et al., 2009), que es la forma natural más activa de JA. Además de la isoleucina aparecen otros aminoácidos (Leu, Val, Tyr, Trp) y moléculas (ACC, del inglés *"aminocyclopropane carboxylic acid*") conjugadas con el JA (Staswick y Tiryaki, 2004; Kramell et al., 2005; Staswick, 2009). El JA también puede ser metilado o glucosilado dando lugar a **MeJA** (Gfeller et al., 2010) o JAG (Kourtchenko et al., 2007). En síntesis, el desarrollo de nuevas técnicas analíticas, más sensibles, han puesto de manifiesto la existencia de una gran variedad de JAs (Glauser et al., 2008; Göbel y Feussner, 2009; Glauser et al., 2010). La interconversión entre distintas formas parece constituir un mecanismo para la activación-desactivación y regulación de esta ruta de señalización (Miersch et al., 2008)

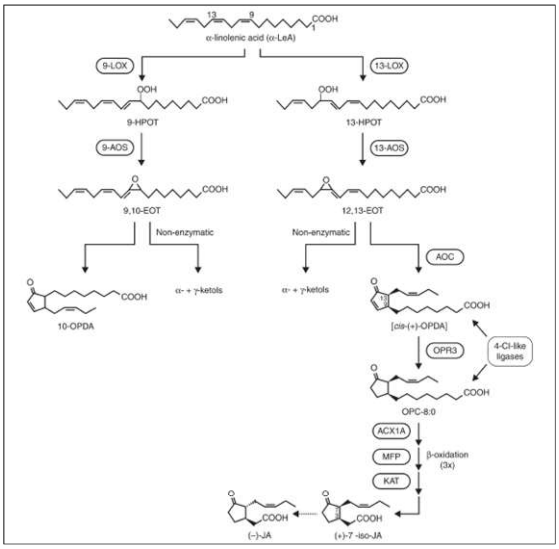

Figura 4. Esquema metabólico de la ruta biosintética de las oxilipinas incluyendo las ramas de la 9- y 13- lipoxigenasa (9-LOX y 13-LOX) y biosíntesis de jasmonatos (Wasternack, 2007).

La presencia de numerosos tipos de JAs con actividad biológica indica la gran variedad de procesos biológicos en los que estas moléculas están implicados, relacionados tanto con defensa como con desarrollo (Miersch et al., 2008; Wasternack, 2007, Hause et al., 2009). Una de las principales funciones de los JAs, es su importante papel en la señalización de la planta frente a estreses abióticos y bióticos. A diferencia del SA, los JAs suelen ser efectivos para la defensa de la planta frente a necrótrofos e insectos. Tras una herida local o herbivoría, se activa su ruta de biosíntesis provocando un

aumento local en los niveles de JA (Stenzel et al., 2003; Narváez-Vásquez y Ryan, 2004; De Vos et al., 2005). Además, el JA actúa como una señal sistémica regulando la expresión de genes que codifican para **inhibidores de proteasas** (***PIs***, del inglés "*Protease Inhibitors*") y otros componentes con efectos negativos para los herbívoros (Halitschke y Baldwin, 2004; Howe, 2004; Pieterse et al., 2006), en zonas distales al lugar donde se ha producido el ataque, protegiendo a la planta frente a nuevos ataques por herbívoros. Los JAs regulan también la producción de compuestos volátiles en respuesta al ataque de insectos herbívoros que atraen a enemigos naturales del herbívoro, regulando por tanto las llamadas defensas indirectas. Además el JA está relacionado con resistencia a patógenos necrótrofos y hemibiótrofos como se ha demostrado con el uso de mutantes afectados en su biosíntesis o percepción (Thaler et al., 2004). El JA está relacionado con el ET en la activación de la defensa en plantas, y en algunos casos sus cadenas de señalización se solapan, regulando ambas la síntesis de compuestos antimicrobianos como las defensinas y tioninas e isoformas básicas de numerosas proteínas PR (Glazebrook, 2005).

Además de su función en la respuesta de defensa, los JAs están implicados en multitud de **procesos relacionados con el desarrollo** de la planta (Wasternack, 2007) (Tabla 1). Uno de los primeros efectos detectados del JA sobre el desarrollo fue una **disminución en el crecimiento de la raíz** (Dathe et al., 1981; Staswicket al., 1992). Posteriores estudios mediante el uso de líneas mutantes de plantas con niveles de JA alterados, confirmaron el efecto negativo del JA sobre el crecimiento de la raíz (Ellis et al., 2002; Browse, 2005; Wasternack, 2006). Otra de las primeras funciones biológicas descritas para los JAs fue el efecto promotor que tiene sobre la **senescencia** de la planta, el último estadio en el desarrollo de la planta (Ueda y Kato, 1980; Parthier, 1990). Esta etapa se caracteriza por una alta transcripción de genes que codifican para proteínas implicadas en la relación fuente/sumidero, fotosíntesis, metabolismo, proteólisis y defensa de la planta (Wasternack y Hause, 2002; Wasternack, 2004). El papel del JA en la senescencia está ligado a una disminución de proteínas codificadas por genes fotosintéticos y un aumento en la expresión de genes implicados en la defensa de la planta frente a estreses de carácter biótico y abiótico (Wasternack, 2004, 2006). Algunos estudios han mostrado un "*crosstalk*" entre diversas hormonas para regular la senescencia (Lin y Wu, 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al.,

2006). Independientemente de este "*crosstalk*", se ha descrito un papel directo del JA sobre la senescencia de la hoja (He et al., 2002). Además, la **formación del tubérculo** es inducida por el ácido tuberónico (12-OH-JA), el cual es un derivado hidroxilado del JA (Yoshihara et al., 1989).

En las plantas, los **procesos cinéticos mediados por contacto** son iniciados por órganos táctiles, siendo muchos de estos procesos dependientes de JA (Falkenstein et al., 1991; Weiler, 1997). En concreto, diversos estudios revelan que el OPDA es más activo que el JA en estos procesos, observando un aumento de OPDA tras el contacto con la planta (Stelmach et al., 1998; Blechert et al., 1999). Otros derivados del JA como es el 12-OH-JA-O-glucosido, también parecen estar involucrados en estos procesos kinéticos de la planta (Nakamura et al., 2006). El **desarrollo floral** de la planta está también muy relacionado con la biosíntesis y señalización del JA. En *Arabidopsis*, mutantes que presentan alterada la ruta de biosíntesis y señalización del JA muestran alteraciones en el fenotipo floral, siendo todos ellos estériles con un desarrollo incompleto de las anteras o filamentos (Turner et al., 2002; Delker et al., 2006). Además, el JA y otras oxilipinas tienen un papel específico en la producción de la proteína AOC en los óvulos de las flores de plantas de tomate (Hause et al., 2000).

Proceso	Molécula	Efecto
Crecimiento raíz	JA/JA-Ile	Inhibición
Germinación semilla	JA	Inhibición
Formación tubérculo	12-OH-JA	Inducción
Movimientos táctiles	OPDA	Estímulo
Formación tricomas	JA	Inducción
Senescencia	JA	Estímulo
Dehiscencia	JA	Inducción
Desarrollo anteras	JA	Inducción
Desarrollo órganos femeninos	JA	Inducción
Elongación filamento	JA	Inducción

Tabla 1. Implicación de los jasmonatos en los procesos de desarrollo de la planta (adaptada de Wasternack, 2007).

2.3 Péptidos endógenos en la inmunidad de la planta

La respuesta de defensa de la planta no solo es inducida por la invasión de organismos y/o el reconocimiento de algunos patrones moleculares característicos (MAMPs, PAMPs, DAMPs o HAMPs), sino que además, tras una lesión o infección determinadas moléculas endógenas de la planta son sintetizadas/procesadas y reconocidas por la propia planta como una señal de alarma, participando activamente en la respuesta de defensa (Yamaguchi y Huffaker, 2011). Además, elicitores endógenos como ROS, oligosacáridos o fragmentos de proteínas son liberados tras la degradación de las paredes celulares vegetales y la muerte celular (Albersheim y Anderson, 1971; Chai y Doke, 1987; Pearce et al., 1991, 2001). La planta no solo es capaz de reconocer estas moléculas a nivel local, sino que también puede amplificar la respuesta de defensa mediante la generación de estas mismas moléculas, mediante una actividad enzimática específica. Entre estas moléculas endógenas capaces de elicitar la respuesta de defensa de la planta, cabe destacar los péptidos endógenos, como es el caso de la sistemina, que se describe en detalle en el apartado 2.3.1. Estos péptidos suelen encontrarse inactivos en forma de precursores (propéptido), pasando a su forma activa (péptido) mediante un proceso de catálisis no descrito hasta el momento. Los genes que codifican para los precursores de estos elicitores peptídicos son inducidos en respuesta a un estrés biótico o a un tratamiento con elicitores (Pearce et al., 1991, 2001; Huffaker et al., 2006, 2011). En la actualidad hay 5 familias descritas de este tipo de péptidos y están agrupadas en 3 grupos atendiendo a la estructura de su proteína precursora, que es la que determina los mecanismos por los cuales el péptido será procesado y liberado a su lugar de actuación (Yamaguchi y Huffaker, 2011). Estas familias de péptidos no muestran una secuencia conservada que las haga fácilmente identificables, pero sí inducen una respuesta de defensa en la planta muy similar. Esta respuesta de defensa conservada incluye la inducción de genes que codifican para proteínas tales como defensinas, quitinasas o inhibidores de proteasas, las cuales tienen carácter antimicrobiano y/o actúan contra la herbivoría (Huffaker et al., 2006; Pearce et al., 2007; Pearce et al., 2010; Huffaker et al., 2011). Estas familias de péptidos también regulan la producción de metabolitos secundarios relacionados con la defensa de la planta, tales como los benzoxazinoides en maíz (Huffaker et al., 2011)

o los terpenos en tomate, judía y tabaco (Schmelz et al., 2006; Heiling et al., 2010; Degenhardt et al., 2011).

Los péptidos de estas familias establecen además una retroalimentación positiva o "*positive feed-back*" mediante pequeñas moléculas relacionadas con las fitohormonas, induciendo la expresión de genes que codifican para enzimas implicadas en la biosíntesis de el JA, ET y/o SA en sus distintas formas. Por tanto, estas fitohormonas son acumuladas en la planta en respuesta a la elicitación provocada por los péptidos endógenos (Howe et al., 1996; Heitz et al., 1997; Schmelz et al., 2006; Narváez-Vásquez et al., 2007; Krol et al., 2010; Pearce et al., 2010; Huffaker et al., 2011). Estudios mediante el uso de líneas mutantes con estas rutas hormonales alteradas indican que las rutas de señalización de estas hormonas deben estar intactas para que se produzca la respuesta de defensa elicitada por péptidos (Howe et al., 1996; Narváez-Vásquez et al., 2007; Degenhardt et al., 2010;).

La alcalinización extracelular, el aumento intracelular de Ca^{2}+, la producción de ROS y la activación de las MAPKs son otros de los procesos que también se encuentran conservados durante la señalización que ocurre tras la elicitación de la planta por parte de estos péptidos (Fig. 5) (Qi et al., 2010; Orozco-Cárdenas et al., 2001; Huffaker et al., 2006; Kandoth et al., 2007;). Todos estos mecanismos de señalización son también típicos de la respuesta de defensa de la planta frente a los MAMPs (Zipfel et al., 2001, 2006). Además de estas similitudes, los receptores que median la percepción de los MAMPs (PRRs) y los receptores que median la percepción de los elicitores peptídicos endógenos (**PEPRs**, del inglés "*Peptides receptor*") muestran una estructura similar, con un dominio kinasa y ricos en leucina (LRR-RKs) (Zipfel et al., 2001; Lee et al., 2009; Yamaguchi et al., 2010). Además los PRRs y PEPRs pueden formar un complejo con el mismo coreceptor ("**BAK1**, del inglés "*BRI1-associated kinase 1*"), requerido para la señalización elicitada por MAMPs y péptidos endógenos (Fig. 5) (Heese et al., 2007; Postel et al., 2010; Schulze et al., 2010). Esta inducción de la respuesta inmune por parte de los péptidos endógenos, está conservada en monocotiledóneas y dicotiledóneas, las cuales divergieron evolutivamente hace 150 millones de años (Chaw et al., 2004), sugiriendo que estos péptidos pueden actuar como mediadores en las primeras fases de la respuesta inmune basal de la planta (siendo análogos funcionales de las citokininas en mamíferos).

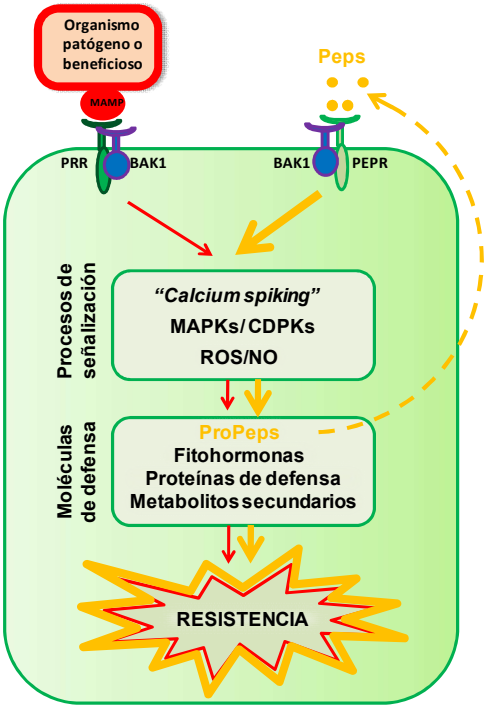

Fig. 5. Papel de los propéptidos en la respuesta inmune de la planta. La planta reconoce microorganismos mediante la unión de patrones moleculares característicos (MAMPs), a receptores que reconocen estos patrones (PRRs). A continuación comienza una cascada de señalización incluyendo picos de calcio citosólico ("calcium spiking"), activación de las rutas dependientes de proteínas kinasas (MAPKs y CDPKs) y producción de especies reactivas de oxígeno y nitrógeno (ROS y NO). Como consecuencia de esta señalización se inducen multitud de moléculas claves para la defensa de la planta, incluyendo propéptidos de señalización que son procesados hasta su forma peptídica activa. Estos péptidos son capaces de unirse a receptores específicos (PEPRs) que activan una cascada similar amplificando todo el proceso de señalización de la respuesta inmune, confiriendo mayor resistencia a la planta. Los receptores PRRs y PEPRs son receptores transmembrana con estructuras muy similares y ambos trabajan junto a otro receptor transmembrana denominado BAK1 (adaptado de Yamaguchi y Huffaker, 2011).

2.3.1 Sistemina

La sistemina (**Sys)** es uno de los péptidos endógenos de las plantas mejor estudiado hasta el momento (Ryan y Pearce, 1998), aunque solo se encuentra en un reducido grupo de plantas pertenecientes a la familia de las solanáceas. La Sys fue la primera hormona peptídica descrita (Pearce et al., 1991). Esta hormona peptídica está relacionada con la señalización y la activación de genes de defensa en la planta como respuesta a herida o herbivoría (Ryan y Pearce, 2003). La Sys es un oligopéptido

compuesto por 18 aminoácidos con una secuencia palindrómica central flanqueada por dos prolinas a ambos lados (Fig. 6).

AVQSKPPSKRDPPKMQTD

XX**Q**XBPPXBBXPPBX**Q**XX

Figura 6. Secuencia aminoacídica de la sistemina. En esta figura se muestra la secuencia de la sistemina con naturaleza palindrómica. Las dobles prolinas se muestran remarcadas en rojo, los aminoácidos básicos en azul y las glutaminas en negro (adaptada de Pearce, 2011).

La Sys se encuentra localizada en el extremo carbono terminal de una proteína citosólica precursora de 200 aminoácidos, denominada prosistemina (**PS**) (McGurl et al., 1992; Ryan y Pearce, 1998) (Fig. 7). El gen que codifica para la PS contiene 11 exones y 10 intrones, de los cuales el último es el que codifica para la Sys (McGurl y Ryan, 1995). Dentro del gen de la PS aparecen 5 repeticiones de un mismo dominio, lo cual sugiere que el gen de la PS se originó mediante procesos de duplicación y elongación. La función de estas regiones repetidas en la secuencia de la PS así como su posible papel en diferentes procesos de señalización todavía es desconocida.

1 MGTPSYDIKNKGDDMQEEPKVKLHHEKGGDEKEKIIEKETPSQDINNKDTISSYVLRDDTQEIPK

66 MEHEEGGYVKEKIVEKETISQYIIKIEGDDDAQEKLKVEYEEEEYEKEKIVEKETPSQDINNKGD

131 DAQEKPKVEHEEGDDKETPSQDIIKMEGEGALEITKVVCEKIIVREDL**AVQSKPPSKRDPPKMQTD**

197 NNKL

Figura 7. Proteína precursora de la sistemina de tomate. La secuencia de la sistemina (18 aminoácidos) está en el carbono terminal de la prosistemina (200 aminoácidos), subrayada y remarcada en negro. Cinco repeticiones imperfectas (subrayadas) sugieren que la PS se originó debido a fenómenos de duplicación y elongación (adaptada de Pearce, 2011).

La PS no presenta ninguna secuencia señal, por lo que no es sintetizada a través de la ruta secretora de la planta. La PS se encuentra en el citosol de la célula y tras un estrés ambiental, del tipo herida o ataque por un insecto, es procesada proteolíticamente hasta Sys. La Sys es transportada fuera de la célula (apoplasto), por un mecanismo todavía no descrito, donde interactúa con su receptor de membrana (Fig. 8). Aunque

este es el mecanismo más aceptado, todavía no se descarta que directamente tras el estrés ambiental, la PS sea liberada al apoplasto y una vez allí procesada hasta Sys. En cualquiera de los dos casos, tras una herida o herbivoría la PS es procesada proteolíticamente hasta Sys. La Sys una vez que se une a su receptor de membrana, inicia una cascada de señalización que concluye con la producción de diversos componentes de defensa para la planta, tales como PIs (Ryan et al., 2002) (Fig. 8). El receptor de membrana de la PS se denomina SR160, debido a su peso molecular. El receptor SR160 comparte muchas de sus características con algunos receptores para brasinosteroides, siendo la secuencia del SR160 idéntica a la del receptor BRI1 (Scheer y Ryan, 2002). En trabajos posteriores se ha demostrado que ambos receptores son diferentes, presentando cada uno de ellos funciones específicas (Holton et al., 2007; Lanfermeijer et al., 2008). Además en trabajos recientes, se ha observado que la PS puede unirse al receptor BRI1 pero esta unión no activa la ruta de señalización característica de la Sys (Malinowski et al., 2009). Estos datos refuerzan la idea propuesta en anteriores estudios, de que la percepción de la Sys está localizada en los tejidos vasculares y que las células del mesófilo son insensibles a la percepción de la Sys (Hind et al., 2010; Malinowski et al., 2009). Por tanto aunque la secuencia del receptor SR160 es homóloga a la del receptor BRI1, ambos receptores se expresan en diferentes tipos celulares para poder llevar a cabo sus diferentes funciones de señalización en la planta.

La importancia de la PS en la respuesta de defensa de la planta fue demostrada por primera vez mediante el uso de plantas transgénicas de tabaco (McGurl et al., 1994). Las plantas de tabaco que sobreexpresaban PS producían más inhibidores de proteasas (PIs) entre otros componentes de defensa y en consecuencia estas plantas eran más resistentes a la herbivoría por larvas de *Manduca sexta* en comparación con plantas de tabaco silvestres (McGurl et al., 1994). En cambio, las plantas de tabaco con los niveles de PS reducidos mostraban menor cantidad de PIs y otros componentes de defensa, siendo más susceptibles a la herbivoría en comparación con las plantas silvestres (Orozco-Cardenas et al., 1993). Todas estas observaciones demuestran el importante papel de la PS en la respuesta de defensa de la planta frente a herida y herbivoría.

La percepción de la sistemina lleva a la activación de la ruta de biosíntesis de los JAs, dando lugar por un lado a la transcripción de genes implicados en defensa (PIs) y por otro lado a un aumento en la propia biosíntesis de PS ya que el gen codificante para PS es inducible por JA, poniendo de manifiesto una retroalimentación positiva entre JAs y PS (Fig. 8). La unión de la Sys con su receptor de membrana SR160 provoca la activación de una cascada de señalización que comienza con una alcalinización del medio extracelular debido a la inhibición de una H-ATPasa de la membrana plasmática (Felix y Boller, 1995; Moyen et al., 1996; Schaller y Oecking, 1999). Además, de forma independiente a este fenómeno, comienza una cascada de señalización mediada por las MAPKs (Higgins et al., 2007). Esta cascada de señalización provoca la activación de fosfolipasas que liberan ácido linolénico de las membranas celulares (Narváez-Vásquez et al., 1999) iniciando así la ruta de biosíntesis del JA (descrita en el apartado 2.2.4). En esta ruta de biosíntesis aparecen algunas moléculas, como el OPDA y el JA, que actúan como potentes inductores de genes de defensa en la planta (Doares et al., 1995; Weiler, 1997). Las proteínas codificadas por estos genes de defensa se pueden clasificar principalmente en 4 grupos: **1)** Proteínas de defensa (PIs y Cystatin, entre otros), cuya función es disminuir la digestibilidad de las hojas ingeridas por el herbívoro, siendo esta la finalidad de los productos de la ruta de biosíntesis del JA. **2)** Proteasas, siendo la más común la leucina-aminopeptidasa A (LAP-A). *LapA* es el gen que codifica para esta proteína, siendo un gen de respuesta tardía y con un patrón de expresión similar al de los genes *PIs*. **3)** Componentes de la ruta de señalización del JA y **4)** otras proteínas con funciones desconocidas en la defensa de la planta. Recientemente se ha descrito que la sobreexpresión de PS induce la producción de volátiles que atraen a insectos parasitoides de los herbívoros siendo este un mecanismo de defensa indirecto para la planta (Corrado et al., 2007).

Los primeros estudios sobre el papel de la PS en la respuesta de defensa de la planta parecían indicar un papel importante de este péptido como molécula de señalización sistémica, activando mecanismos de defensa en la planta en zonas distales del daño o estimulo. Esta hipótesis se basaba en la capacidad de la PS de viajar a través del floema (Pearce et al. 1991; Narváez-Vásquez et al. 1994; Lucas y Lee, 2004). Estudios con Sys marcada (“*14C-systemin”*) mostraron un movimiento de la Sys desde las hojas con herida hasta zonas distales sin herida, coincidiendo con un aumento en la producción

de PIs en estas zonas distales (Narváez-Vásquez et al., 1995). Otros estudios pusieron de manifiesto además que la PS es sintetizada y almacenada en los vasos vasculares de peciolos y tallo de la planta (Wasternack et al., 2006), lo que proporciona una ventaja para ser liberada tras una herida. Sin embargo, también se ha encontrado producción de JA en estos vasos vasculares, lo que permite que el JA sea también liberado la floema y transportado tras el ataque de un insecto (Narváez-Vásquez y Ryan, 2004; Wasternack et al., 2006). Trabajos posteriores han demostrado que la señal que actúa a larga distancia es el JA u otro componente relacionado con él (Wasternack et al., 2006). El uso de injertos ha permitido demostrar que la Sys no es necesaria en los tejidos para la producción de PIs, aunque si es clave para la producción de la señal sistémica en el lugar donde se ha producido la herida (Lee y Howe, 2003). Estudios recientes sugieren que la Sys amplifica la producción de JA, que actúa a su vez como señal sistémica frente a una herida, en concreto el Ile-JA parece ser la señal sistémica que viaja hasta zonas distales de la herida para activar la defensa (Schilmiller y Howe, 2005). Cuando los niveles de JA aumentan comienza una retroalimentación positiva, mediante la cual el propio JA aumenta la biosíntesis de PS, produciéndose como resultado final más JA transportable para la respuesta sistémica de la planta (Bergey et al., 1996; Stratmann, 2003; Schilmiller y Howe, 2005).

Sorprendentemente la secuencia de la PS solo se encuentra en un reducido subgrupo de la familia de las solanáceas (tomate, patata, boniatos, pimiento, entre otros) (Constabel et al., 1998). Debido al importante papel que tiene la PS para la respuesta de defensa de la planta, es lógico pensar que en otras familias de plantas existan mecanismos de señalización similares al descrito en este apartado, mediado por péptidos similares a la PS.

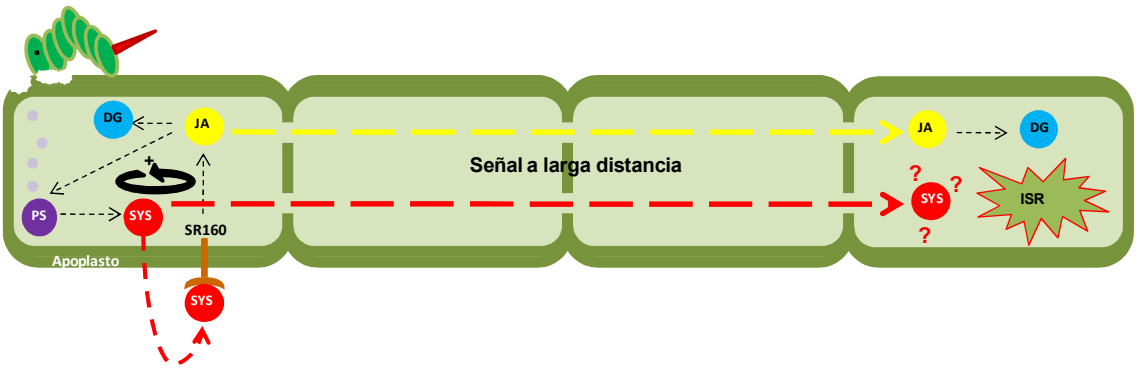

Figura 8. Función de la sistemina en la respuesta de defensa de la planta frente a hebivoría. Tras un proceso de herbivoría o herida comienza una cascada de señalización que provoca un aumento en la biosíntesis de prosistemina (PS), pasando esta a su forma activa, sistemina (Sys). La Sys es liberada al apoplasto donde se une a su receptor transmembrana SR160, activando toda la ruta de biosíntesis del jasmonato (JA). El JA induce la expresión de genes de defensa, desarrollando una respuesta de defensa local en la planta. Además el JA induce la biosíntesis de PS, creándose un bucle positivo entre JA-PS. Por tanto la Sys aumenta el nivel de JA a nivel local. Cuando la concentración de JA es suficiente, este viaja hasta zonas distales de la herida, induciendo la expresión de genes de defensa y por tanto, induciendo una resistencia sistémica (ISR) en la planta. La Sys también es capaz de viajar hasta zonas distales de la herida, pero su función en este aspecto todavía es desconocida.

2.3.2 Homólogos de la prosistemina

Además de la PS, las plantas de tomate poseen otras tres moléculas relacionadas funcionalmente con ella denominadas glicopéptidos ricos en hidroxiprolina (**TomHypSYS I, II y III**). Estos glicopéptidos son residuos de 15-20 aminoácidos que son sintetizados a partir de una sola proteína precursora, ProHypSYS, independiente de la PS (revisados en Pearce, 2011). La ProHypSYS es sintetizada con un péptido señal para su secreción y está localizada en la pared celular de la planta (Narváez-Vásquez et al. 2005).

En otras especies pertenecientes a distintas familias se han descrito otros péptidos similares a la PS. Por ejemplo, en la planta modelo *Arabidospsis thaliana* se ha identificado un nuevo péptido señal relacionado con la defensa de la planta e inducible por herida (**AtPep1**) (Huffaker et al., 2006), el cual, al igual que la Sys, es originado a partir de una molécula precursora (AtProPep1) mediante diversos procesos de proteólisis. AtPep1 forma parte de una pequeña familia de péptidos involucrados en la respuesta de inmunidad innata frente a patógenos. A partir del gen *AtPROPEP1* se ha

encontrado un ortólogo en maíz denominado *ZmPROPEP1*. Este propéptido es el precursor del péptido **ZmPep1**, el cual es funcionalmente homologo al péptido AtPep1, transcribiéndose en respuesta a JA e infecciones por patógenos y activando distintos componentes de la inmunidad innata de la planta (Huffaker et al., 2011). Posteriormente en soja también se ha identificado un ortólogo de los propéptidos arriba mencionados, GmProPep914 (Yamaguchi et al., 2011). El propéptido GmProPep914 es transcrito en respuesta a MeJA al igual que la PS. Una vez transcrito, mediante proteólisis se origina su forma activa, péptido **GmPep914**, que consta de tan solo 8 aminoácidos, haciendo de él, el péptido señal relacionado con defensa en plantas más pequeño descrito hasta la fecha. GmPep914 regula la expresión de genes de defensa como algunos implicados en la biosíntesis de fitoalexinas y enzimas hidrolíticas (Yamaguchi et al., 2011).

3. Modulación del sistema inmune de la planta por organismos beneficiosos

Los microorganismos beneficiosos del suelo son inicialmente reconocidos por el sistema inmune de la planta como posibles agresores. De manera similar a la interacción con un patógeno, el sistema inmune de la planta es capaz de percibir la presencia de éstos microorganismos a través de la percepción de sus MAMPs, y activar así una respuesta de defensa denominada MTI (del inglés, "*MAMP-triggered immunity"*) (Soto et al., 2009; Zamioudis y Pieterse, 2012). Esto resulta generalmente en la activación de las diferentes cascadas de señalización mencionadas en los apartados anteriores. Por lo tanto, para poder establecer esta interacción con las raíces de las plantas, los microorganismos beneficiosos tienen que enfrentarse a esta respuesta inmune que se activa tras la percepción de sus MAMPs. Determinados microorganismos beneficiosos han desarrollado diferentes estrategias para reducir la estimulación del sistema inmune de las plantas y suprimir o reducir así la activación de la respuesta MTI.

Para evitar la detección por parte del sistema inmune de la planta huésped, algunos beneficiosos son capaces de minimizar el reconocimiento de sus MAMPs. La flagelina es el principal componente del flagelo de las bacterias. Esta proteína estructural es

reconocida como un MAMP por el sistema inmune de la mayoría de las plantas (Boller y Felix, 2009), tras lo cual se inicia una cascada de señalización que conduce a la activación de un programa de defensa contra las bacterias invasoras. La actividad inductora de la molécula está localizada en un epítopo altamente conservado de 22 aminoácidos en el dominio N-terminal, denominado flg22 (Heese et al., 2007; Zipfel, 2008). Sin embargo, la flagelina que forma parte del flagelo de los simbiontes mutualistas del género *Rhizobium* difieren del resto ya que esta no posee el epítopo flg22 en su secuencia, lo que proporciona a estos beneficiosos la ventaja evolutiva de reducir la estimulación del sistema inmune de la planta huésped (Lopez-Gomez et al., 2011). Otro ejemplo es la bacteria promotora del crecimiento *Pseudomonas brassicacearum*, que puede presentar en la rizosfera dos variaciones morfológicas diferentes denominadas fase I y fase II (Achouak et al., 2004). Durante la fase I la bacteria se encuentra principalmente en las partes basales de la raíz y produce cantidades muy reducidas de flagelina con respecto a la fase II, que se encuentra principalmente en las raíces secundarias y los pelos radicales. Parece ser que una vez que la bacteria ha colonizado nuevos nichos es capaz de pasar a la fase I con el fin de evitar el reconocimiento de su flagelina y reducir por lo tanto la estimulación del sistema inmune de la planta (Achouak et al., 2004). Además de los MAMPs, determinados grupos de microorganismos pueden ser detectados mediante los DAMPs. Interesantemente en el genoma de los hongos ectomicorrícicos *Laccaria bicolor* y *Tuber melanosporum* está muy limitada la presencia de ciertas familias de genes relacionados con la degradación de la pared celular de la planta y que por lo tanto podrían inducir la respuesta inmune de la planta (Martin et al., 2008, 2010).

Además de evitar la estimulación del sistema inmune de la planta, ciertos microorganismos beneficiosos son capaces de suprimir de manera activa la respuesta MTI que se induce en la planta. Durante los primeros estadios de la interacción, la planta reacciona a la presencia del beneficioso de manera similar a la invasión por un patógeno activando una respuesta inmune que incluye entre otros la producción de especies reactivas de oxígeno, la alcalinización del medio extracelular, un incremento de los niveles de calcio citoplasmático y la expresión de batería de genes de defensa. Sin embargo, en estadios posteriores de la interacción algunos microorganismos beneficiosos son capaces de suprimir activamente esta respuesta de de defensa para

poder colonizar de manera efectiva la raíz de la planta (Kapulnik et al., 1996; Liu et al., 2003; El Yahyaoui et al., 2004; Kouchi et al., 2004; Lohar et al., 2006; Heller et al., 2008; Moreau et al., 2011;). Por ejemplo la rizobacteria promotora del crecimiento *Pseudomonas fuorescens* WCS417 es capaz de suprimir esta respuesta MTI mediante la secreción de determinados efectores de bajo peso molecular en el apoplasto de la planta (Millet et al., 2012). El proceso de establecimiento de las asociaciones mutualistas requiere por lo tanto un proceso complejo de reconocimiento y una coordinación entre las respuestas de la planta y del beneficioso (Zamioudis y Pieterse, 2012).

Este dialogo molecular que ocurre entre la planta y el mutualista es especialmente complejo durante las asociaciones simbióticas endofíticas que se dan entre las plantas y determinados microorganismos del suelo como los AMF o *Rhizobium*. Estas asociaciones implican, tras un reconocimiento mutuo la "invasión" por parte del microorganismo de los tejidos de la planta huésped, y resultan en la formación de estructuras especializadas llamadas nódulos en el caso de *Rhizobium* y arbúsculos en el caso de los AMF. La evasión del sistema inmune de la planta huésped por parte del simbionte es por lo tanto crucial para el establecimiento de estas interacciones tan íntimas. Una vez establecida la simbiosis, la planta además controla de manera activa el nivel de proliferación del simbionte dentro de la raíz para prevenir una excesiva colonización, que conllevaría en pérdidas excesivas de compuestos carbonados, poniendo de manifiesto el alto grado de coordinación entre ambos simbiontes.

En el caso de la la colonización por los **hongos formadores de micorrizas arbusculares**, parece producirse en la raíz planta un aumento rápido y transitorio de la hormona SA y una acumulación de compuestos relacionados con la defensa tales como especies reactivas de oxígeno o enzimas hidrolíticas, así como la activación de la ruta de los fenilpropanoides (Pozo et al., 1998; Blilou et al., 1999; Dumas-Gaudot 2000; Fester y Hause, 2005; de Román et al., 2011). Estas reacciones podrían tener un papel importante en el establecimiento o el control de la simbiosis (Dumas-Gaudot et al., 1996; García-Garrido y Ocampo, 2002). De hecho tanto los AMF como *Rhizobium* se muestran sensibles a las respuestas de defensa de la planta que son reguladas por esta hormona. Numerosos estudios han puesto de manifiesto tanto los efectos negativos de SA en la intensidad de colonización de la planta por parte ambos simbiontes

(Martínez-Abarca et al., 1998; Blilou et al., 2000b; Van Spronsen et al., 2003; Stacey et al., 2006; Herrera-Medina et al., 2007; de Román et al., 2011), así como el requerimiento de una inhibición la respuesta de defensa de la planta reguladas por esta hormona para el establecimiento de ambas simbiosis (Dumas-Gaudot, et al 2000; Soto et al., 2009). Aunque actualmente se desconocen los mecanismos exactos mediante los cuales los AMF pueden interferir con la señalización dependiente de SA de la planta, parece ser que de manera similar al programa simbiótico que se activa en la planta tras la percepción de los factores Nod de *Rhizobium*, que interfiere con la respuesta MTI (Maunoury et al., 2010), los AMF podrían modular la respuesta de defensa mediadas por SA a través de la activación del programa simbiótico activado tras a percepción de los factores myc (Zamioudis y Pieterse, 2012).

Además del SA, durante la interacción entre la planta y el AMF se alteran los niveles de otras fitohormonas tales como JA, ABA o ET (Hause et al., 2007) que pueden interaccionar de manera antagonista con la ruta dependiente de SA. Es posible que como ocurriría en diferentes interacciones patogénicas o con *Rhizobium*, durante la colonización de la planta por los AMF el control de la respuesta de SA se llevara a cabo de manera indirecta mediante la interacción de diferentes rutas hormonales (Pieterse et al., 2009; Jung et al., 2012; Zamioudis y Pieterse, 2012;).

Estudios recientes han demostrado además que de manera similar a determinados patógenos biótrofos y oomicetos, los AMF pueden suprimir la respuesta de defensa de la planta a través de la secreción de determinadas proteínas efectoras que interfieren con el sistema inmune de la planta huésped (Paszkowski, 2006). Recientemente se ha demostrado que *Glomus intraradices* secreta el efector *SP7*, con capacidad suprimir las defensas de la planta (Kloppholz et al,. 2011). La expresión de este efector se induce tras el contacto con las raíces de la planta huésped y es translocado al citosol de la planta. El SP7 es posteriormente transportado al núcleo donde interacciona con el factor de transcripción EFR19, relacionado con la respuesta de defensa de la planta dependiente de etileno, bloqueando la transcripción de los genes de defensa regulados por este factor de transcripción, e interfiriendo por lo tanto con la MTI (Clay et al.2009; Boutrot et al. 2010; Mersmann et al. 2010; Millet et al. 2010; Kloppholz et al 2011).

4. Interacciones beneficiosas: Micorrizas.

Dentro de los microorganismos beneficiosos que interaccionan con la planta en la rizosfera, destacan por su gran relevancia ecológica y su amplia distribución los hongos micorrícicos, que colonizan las raíces de las plantas estableciendo una simbiosis mutualista con la planta denominada **micorriza** (del griego *mikos*, hongo, y *rhiza*, raiz). Debido al carácter mutualista de esta asociación ambos simbiontes se ven beneficiados, el hongo proporciona a la planta nutrientes minerales y agua procedentes del suelo y, a cambio, la planta le cede al hongo hidratos de carbono derivados de la fotosíntesis (Harrison, 1999; Govindarajulu et al., 2005; Javot et al., 2007). Aproximadamente el 90% de las especies vegetales pueden formar este tipo de simbiosis y unas 6000 especies de hongos pueden estar implicadas, de aquí la amplia distribución y el carácter universal de esta simbiosis.

A pesar de la gran biodiversidad de los organismos implicados, existen 7 tipos de micorrizas clasificadas en función de criterios estructurales, funcionales y taxonómicos: Ectomicorrizas, Endomicorrizas o Micorrizas Arbusculares, Ectendomicorrizas, Arbutoides, Monotropoides, Ericoides y Orquidioides (Barea, 1998). En cuanto a las estructuras formadas, tipo de colonización y cantidad de especies fúngicas y vegetales implicadas, se puede decir que las **micorrizas arbusculares** (**AM**, del inglés “arbuscular mycorrhizas”) son las más extendidas y las que presentan mayor importancia desde un punto de vita de beneficios para la planta (Smith y Read, 2008).

4.1 Micorrizas Arbusculares

Las micorrizas arbusculares constituyen probablemente la simbiosis vegetal más antigua (evidencias fósiles y moleculares datan su origen en hace más de 400 millones de años) y la más ampliamente distribuida en la naturaleza, ya que aproximadamente el 80% de las especies vegetales conocidas son capaces de establecer esta asociación. Tan solo algunos taxones vegetales no presentan dicha asociación, como son las familias *Cruciferae*, *Fumariaceae*, *Urticaceae*, *Poligonaceae* y *Quenopodiaceae* (Wang y Qiu, 2006). Por el contrario, solo unas 150 especies de hongos son formadores de micorrizas arbusculares (**AMF)** agrupadas actualmente en 17 géneros (Schüβler y Walker, 2010) constituyendo el phylum Glomeromycota (Fig. 9). La clasificación de

estos hongos es bastante controvertida, probablemente por la dificultad de definir el concepto de especie en estos organismos y de integrar criterios morfológicos y moleculares.

Phylum *Glomeromycota*

Clase *Glomeromycetes*

Orden (4)	**Familia (11)**	**Género (17)**
Glomerales	***Glomeraceae***	***Glomus***
		Funneliformis **(*Glomus mosseae* clade)** ***Rhizophagus*** **(*Glomus intraradices* clade)** ***Sclerocystis***
	Claroideoglomeraceae	***Claroideoglomus*** **(*Glomus claroideum* clade)**
Diversisporales	***Gigasporaceae***	***Gigaspora*** ***Scutellospora*** ***Racocetra*** **(including *Racocetra weresubiae*)**
	Acaulosporaceae	***Acaulospora*** **(including the former *Kuklospora*)**
	Entrophosporaceae	***Entrophospora*** **(with unclear phylogenetic affiliation)**
	Pacisporaceae	***Pacispora***
	Diversisporaceae	***Diversispora*** **(including several *Glomus* species)** ***Otospora*** **(unclear phylogenetic affiliation)**
Paraglomerales	***Paraglomeraceae***	***Paraglomus***
Archaeosporales	***Geosiphonaceae***	***Geosiphon***
	Ambisporaceae	***Ambispora***
	Archaeosporaceae	***Archaeospora*** **(including the former *Intraspora*)**

Figura 9. Clasificación taxonómica del Phylum *Glomeromycota* basada en el análisis 18SDNAr, según Schüβler y Walker, (2010).

Los AMF son simbiontes biótrofos obligados que dependen de la planta hospedadora para completar su ciclo de vida. Durante el establecimiento de la simbiosis, el hongo coloniza biotróficamente el córtex de la raíz, llegando a ser parte integrante de dicho tejido. Desarrolla un extenso micelio externo (ERM, del inglés "*extraradical mycelium*") que a modo de sistema radicular complementario y altamente efectivo, ayuda a la planta a adquirir nutrientes minerales y agua del suelo. La colonización sólo tiene lugar

en la epidermis y en el parénquima cortical de las raíces. El AMF no penetra nunca en el cilindro vascular ni en las regiones meristemáticas (Bonfante, 2001). En el interior de las células del córtex de la raíz, el hongo desarrolla unas estructuras arborescentes denominadas arbúsculos (del latín, "*arbusculum"*, que significa arbusto o árbol pequeño), que se encuentran rodeadas por la membrana plasmática de la célula que los alberga (Smith y Gianinazzi-Pearson, 1988). Es a nivel de los arbúsculos en donde transcurre el intercambio bidireccional de nutrientes entre la planta y el hongo (Balestrini y Bonfante, 2005; Ferrol y Pérez-Tienda, 2009). La planta suministra al hongo compuestos carbonados procedentes de la fotosíntesis y nicho. A cambio, el hongo aporta a la planta nutrientes minerales y agua que absorbe del suelo a través de su micelio externo o extrarradical (Smith y Read, 2008). Además de estos arbúsculos los AMF forman otras estructuras dentro de la raíz de la planta como pueden ser órganos de reserva denominados vesículas. Las vesículas son estructuras globosas e irregulares que actúan como reserva lipídica para el hongo. En algunas especies de AMF estas vesículas pueden derivar en la formación de esporas en el interior de la raíz.

Una de los beneficios más importantes para la planta de esta simbiosis es el incremento en la absorción de nutrientes minerales del suelo, que se traduce en un mayor crecimiento y desarrollo de la planta. La expansión del micelio externo del hongo por el suelo rizosférico permite la captación de nutrientes más allá de la zona de agotamiento que se crea alrededor de las raíces, debido a la absorción por la propia planta. Los principales nutrientes que el hongo aporta a la planta son fosfato y nitrógeno. Estudios moleculares describen transportadores de fosfato, tanto de la planta como del hongo, indispensables para la asociación simbiótica (Javot et al., 2007).

Se asume que esta asociación no presenta especificidad, sin embargo sí que existe una compatibilidad funcional (van der Heijden et al., 1998; Klironomos, 2000; Vandenkoornhuyse et al., 2002) que se podría definir como la "preferencia" de algunas especies vegetales de ser colonizadas por determinados AMF de manera que, aunque la mayoría de las plantas pueden ser colonizadas por cualquier AMF, estos lo hacen con distinta intensidad y eficiencia (Jeffries y Barea, 2001). El grado de esta especificidad podría ser consecuencia del control genético por parte de la planta

huésped, el AMF o bien por la interacción entre ambos simbiontes con el medio (Chanway et al., 1991).

4.2 Beneficios de la simbiosis

El establecimiento de la simbiosis entre planta y hongo micorrízico se basa fundamentalmente en un intercambio bidireccional de nutrientes entre ambos simbionte, sobre todo a nivel de los arbúsculos, por tanto gran parte de los cambios estructurales y morfológicos que experimenta la planta en el transcurso de esta simbiosis están esencialmente ligados a aspectos nutricionales. Además de estos beneficios nutricionales, la formación de la simbiosis también protege a la planta frente a estreses de tipo biótico (ataque de organismos patógenos) (Azcón Aguilar y Barea, 1996; Harrier y Watson, 2004; Liu et al., 2007; Pozo y Azcón-Aguilar, 2007; Koricheva et al., 2009) y abiótico (salinidad, sequia, frio y presencia de metales pesados, entre otros) (Gohre y Paszkowski, 2006; Ruiz-Lozano et al., 2006; Aroca et al., 2007; Ferrol et al., 2009). Adicionalmente, la red de hifas del hongo que se desarrolla en el suelo contribuye a mejorar la estructura del mismo al facilitar la formación de agregados estables y por lo tanto la estructura del suelo (Piotrowski et al., 2004; Rillig y Mummey, 2006).

La mejora nutricional de la planta hospedadora por parte del AMF puede explicarse, en parte, por el aumento de superficie efectiva que el micelio extrarradical del AMF le aporta a la raíz. Así, sabemos que la red de micelio externo puede alcanzar una densidad de hasta 100 metros de hifas por centímetro cubico de suelo (Miller et al., 1995). Las hifas del hongo, por su tamaño (con un diámetro medio de 3-4 µm) y distribución, son capaces de explorar un volumen de suelo mayor que las propias raíces, penetrando en poros y cavidades del suelo en los que las raíces, por su tamaño (diámetro ≥ 10 µm), no pueden penetrar (Bolan, 1991; Jakobsen, 1994). Por otro lado, las hifas de los AMF son capaces de absorber y transportar nutrientes desde largas distancias, superando las zonas de agotamiento en agua y nutrientes minerales que rodean a las raíces. Esto es especialmente importante para aquellos nutrientes que difunden lentamente en la solución del suelo, como es el caso del fósforo inorgánico (Pi) (Jakobsen, 1994; Miller et al., 1995). Otros estudios sugieren que las hifas son

capaces de competir más eficientemente que las raíces con otros microorganismos del suelo por la absorción de nutrientes (Linderman, 1992). Debido a su importante papel en la adquisición de nutrientes minerales por la planta, principalmente Pi, frecuentemente se considera a los AMF como una mera extensión del sistema radical de la planta. Esta visión simplista ignora, entre otras cosas, las necesidades nutricionales del hongo, las cuales pueden competir con las de la planta cuando la disponibilidad de determinados nutrientes es baja. Por lo general, los efectos beneficiosos de las AM son más evidentes bajo condiciones limitantes de algunos nutrientes, especialmente Pi, y aunque los mecanismos reguladores no se conocen bien, se observa una disminución en el grado de colonización de la raíz por los AMF cuando aumenta la fertilidad del suelo. Por tanto, el transporte y la transferencia de Pi se considera el proceso fisiológico clave por el cual los AMF mejoran el crecimiento vegetal (Barea et al., 2008; Ferrol y Pérez-Tienda, 2009), pero también hay evidencias que indican que los AMF juegan un importante papel en la nutrición nitrogenada de la planta (Veresoglou et al., 2012), así como en la absorción de otros nutrientes de baja movilidad en el suelo como el Cu o el Zn (Clark y Zeto, 2000). En la solución del suelo, el Pi está presente en forma de ortofosfato, el cual es rápidamente secuestrado por los cationes presentes en suelo, especialmente en condiciones acidas. La movilidad de estas sales de fosfato es muy baja, por lo que la absorción por la planta consume rápidamente el P presente en la zona de influencia de las raíces, creándose zonas de agotamiento o depleción en este nutriente alrededor de las mismas (Barea et al., 2007; Bucher, 2007). El desarrollo del micelio extrarradical en el suelo aumenta considerablemente la superficie de absorción de las plantas micorrizadas, superando las zonas de depleción de nutrientes y accediendo a lugares donde las raíces no pueden acceder (Smith y Read, 2008). Por esta razón, se considera que en plantas micorrizadas coexisten dos vías de absorción de nutrientes: la vía directa llevada a cabo por las células epidérmicas y la vía indirecta o micorrícica mediante las hifas del hongo (Smith y Smith, 2012). Diferentes estudios transcriptómicos, en distintas especies de plantas colonizadas por diferentes hongos, han puesto de manifiesto que los AMF son capaces de alterar la expresión de diferentes transportadores de nutrientes en las raíces de la planta hospedadora (Gómez et al., 2009; Benedito et al., 2010; Casieri et al., 2012; Gaude et al., 2012; Ruzicka et al., 2012). La inducción o la

expresión especifica en raíces micorrizadas de diferentes transportadores evidencia la posible existencia de diferentes vías de absorción micorrícica para diferentes nutrientes minerales, así como la modulación de la vía directa de absorción de los mismos. En diferentes especies vegetales se han identificado transportadores de Pi que se inducen específicamente durante la simbiosis, concretamente en células colonizadas por arbúsculos. Estos transportadores son los responsables de la absorción de Pi por la planta a través de la vía micorrícica (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005; Maeda et al., 2006). A modo de ejemplo, el transportador *LePT4* de *Solanum Lycopersicum* se expresa específicamente en raíces micorrizadas (Ballestrini et al., 2007; Gómez-Ariza et al., 2009). Puesto que el transporte de Pi es el pilar fundamental del funcionamiento de la simbiosis MA; los genes codificantes para estos transportadores específicos de micorrizas son usados como marcadores de la funcionalidad de la simbiosis. Finalmente, la expresión de transportadores de Pi en el propio AMF en células arbusculadas (Ballestrini et al., 2007; Tisserant et al., 2012) sugiere que, en esa interfase simbiótica, la absorción de Pi ocurre probablemente de forma competitiva, pudiendo ejercer el hongo cierto control sobre la cantidad de fosfato que absorbe la planta (Kiers et al., 2011).

La **transferencia de compuestos carbonados desde la planta al hongo** fue por primera vez descrita por Ho y Trappe, 1973. El carácter simbionte estricto de los AMF, junto con la dificultad para aislar sus estructuras intrarradicales, ha complicado en gran medida el estudio de los mecanismos implicados en la transferencia de los compuestos carbonados desde la planta hacia el hongo. El uso de sustratos marcados radiactivamente ha mostrado que los AMF son capaces de absorber carbohidratos a partir de la planta en forma de hexosas, preferentemente glucosa (Shachar-Hill et al., 1995; Solaiman y Saito, 1997). Puesto que la sacarosa es la principal forma en la que se transportan los fotoasimilados, la utilización de la misma como fuente de energía, tanto por las raíces como por los hongos micorrícicos, requiere que esta sea hidrolizada en glucosa y fructosa. Esto ocurre mediante la acción de invertasas y/o sacarosa sintasas, enzimas que están implicadas en el transporte de la sacarosa a larga distancia y en su metabolismo (Sturm y Tang, 1999). Se ha demostrado que la micorrización altera la expresión de algunos genes que codifican estas enzimas en plantas de maíz (Ravnskov et al., 2003) y tomate (Schaarschmidt et al., 2006; Garcia-

Rodriguez et al., 2007) entre otras. Estos datos sugieren que estas enzimas proveen de metabolitos (hexosas) a las células colonizadas para satisfacer la demanda del hongo y mantener la mayor actividad metabólica asociada a la micorrización. En este proceso de redistribución de carbohidratos parecen estar implicadas hormonas como el JA y sus derivados, y el péptido señal Sys (Tejeda-Sartorius et al., 2008) (ver apartado 4.4.2). Estas enzimas estarían indirectamente involucradas en el mantenimiento de un flujo de sacarosa desde los tejidos fuente (generalmente las hojas) hacia la raíz micorrizada que actúa de sumidero. Trabajos posteriores han puesto de manifiesto que el suministro de carbono de la planta al hongo y el normal desarrollo de la micorrización depende de la actividad de las invertasas apoplásticas (Schaarschmidt et al., 2007a, 2007b; Schaarschmidt y Hause, 2008).

Además en numerosos trabajos se describe una **mayor resistencia de las plantas micorrizadas frente a estreses abióticos,** tales como la salinidad, sequía y contaminación por metales pesados (Arriagada et al., 2007; Miransari, 2010; Smith et al., 2010; Porcel et al., 2012; Ruiz-Lozano et al., 2012; Estrada et al. 2013). Las plantas micorrizadas pueden evitar la **sequía** gracias a un aumento en la capacidad de absorción de agua en suelos con bajos niveles de humedad. Son muchos los trabajos que describen una importante influencia de los AMF en el movimiento del agua dentro, a través y fuera de la planta hospedadora, afectando a la hidratación de los tejidos y a la fisiología de las hojas (Nasim, 2010). Algunos fenómenos como alta conductancia estomática y mayor transpiración pueden aparecer en plantas micorrizadas (Augé et al., 2004). Además, durante la AM simbiosis la planta pude fijar más carbono en condiciones de estrés hídrico, lo cual es especialmente importante cuando la planta se encuentra en ambientes áridos (Duan et al., 1996). Además la glomalina, una glicoproteína hidrofóbica que se deposita en la pared celular de los AMF, puede disminuir la ruptura de macro-agregados durante periodos de sequía y humedad facilitando el crecimiento de la planta en estas condiciones (Miller y Jastrow, 2000).

Con respecto al estrés por **salinidad**, el establecimiento de una simbiosis AM es clave para que la planta pueda sobrevivir en un ambiente con este tipo de estrés (Augé et al., 1992; Wang y Liu, 2001; Jahromi et al., 2008, Aroca et al., 2012; Estrada et al., 2013), mediante una mayor captación de nutrientes, que ayuda a la planta a

restablecer el balance iónico descompensado por el estrés salino (Asghari et al., 2005; Giri et al., 2007), protegiendo actividades enzimáticas claves para resistir este estrés (Rabie y Almadini, 2005) y facilitando la captación de agua (Ruiz-Lozano y Azcón, 1995). Además los AMF son capaces de aliviar el **estrés por metales pesados** en la planta hospedadora, contribuyendo a su vez a la fitorremediación de suelos contaminados con diversos metales pesados (Cd, Pb, Zn y Cu, entre otros) (Janouskova et al., 2006). Los mecanismos por los cuales los AMF son capaces de movilizar los metales pesados están ampliamente descritos: **1)** Depósito de estos metales pesados en la pared celular o vacuolas fúngicas. **2)** Secuestración de metales pesados por sideróforos, depositándolos en el apoplasma de la raíz hospedadora o en el suelo. **3)** Metalotioneinas o fitoquelatinas que ayudan a depositar los metales pesados en células de la planta o del hongo. **4)** Reorganización de los metales pesados que se encuentran en el citoplasma, mediante transportadores localizados en el plasmalema o tonoplasto de ambos simbiontes (Galli et al., 1994; Leyval et al., 1997; Schutzendubel y Polle, 2002). Además, hay indicaciones de que los AMF son capaces de inducir la expresión de genes relacionados con los metales pesados en la panta hospedadora (Repetto et al., 2003; Rivera-Becerril et al., 2005), debido a que el contenido de metales pesados en las plantas micorrizadas está altamente alterado, lo que indica que estos genes están siendo regulados a nivel transcripcional y translacional por los AMF (Ouziad et al., 2005).

Por otro lado, los AMF también son capaces de **mejorar la resistencia de la planta hospedadora frente a estreses de tipo biótico**. Las plantas micorrizadas muestran una mayor resistencia frente a un amplio rango de organismos que atacan las raíces de las plantas, como son los hongos del suelo y bacterias patogénicas, nemátodos o insectos capaces de provocar heridas en la raíz (Azcón-Aguilar y Barea, 1997; Whipps, 2004; Jung et al., 2012). Además en la última década se ha demostrado que los AMF también inducen resistencia frente a patógenos de la parte aérea como hongos necrótrofos y frente a insectos herbívoros (Pozo y Azcón-Aguilar, 2007; Koricheva et al., 2009; Campos-Soriano et al., 2012, Jung et al., 2012) (Fig. 10).

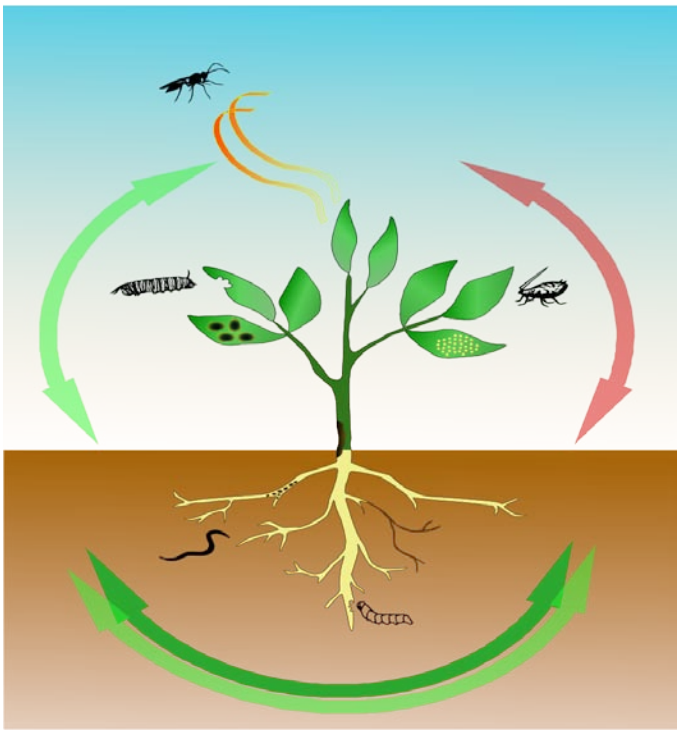

Figura 10. Espectro de efectividad de la resistencia inducida por micorrizas arbusculares. La simbiosis micorrízica arbuscular reduce la incidencia y/o daño que producen los patógenos del suelo a la planta. Esta protección es una combinación de mecanismos tanto locales como sistémicos (abajo). Además esta protección generada en la raíz también es efectiva en la parte aérea de la planta protegiéndola frente a patógenos necrótrofos y herbívoros (izquierda). Además esta simbiosis ayuda indirectamente a la defensa de la planta mediante la emisión de volátiles, los cuales son atrayentes para algunos parasitoides. Por otro lado, los hongos biótrofos, virus e insectos que dañan el floema son más efectivos frente a plantas micorrizadas (derecha). Las flechas verdes indican resistencia, mientras que las rojas indican susceptibilidad en plantas micorrizadas (Pozo y Azcón Aguilar, 2007).

Las plantas micorrizadas presentan un mejor estado de salud, debido en parte a la mejora en la captación de nutrientes y agua de la planta, esto supone un mecanismo indirecto de defensa de la planta, pudiendo aumentar la tolerancia/resistencia de la planta frente a distintos estreses. Sin embargo, experimentos con suplementos nutricionales y otras aproximaciones han demostrado que la resistencia de la planta frente a este estreses no depende solo de la mejora del estado nutricional de la planta (Fritz et al., 2006; Liu et al., 2007), sino que la simbiosis AM provoca cambios significativos en la planta hospedadora y su ambiente. Estos cambios pueden ser originados por los AMF de manera directa compitiendo con hongos patógenos del suelo por los lugares de infección y por el espacio en la raíz (Vigo et al., 2000; Morandi et al., 2002) o de forma indirecta provocando en la raíz de la planta hospedadora un cambio en la producción de exudados lo que conlleva a cambios en la estructura del

suelo, depósito de carbono en el suelo y diversidad microbiana, alterando por completo el entorno de la planta hospedadora a nivel de la rizosfera.

Estos cambios en las comunidades microbianas de la rizosfera pueden influir indirectamente en las distintas interacciones que se pueden dar entre la planta micorrizada y otros organismos tanto beneficiosos como patogénicos (Berta et al., 2002; Barea et al., 2005; Artursson et al., 2006; Lendzemo et al., 2007; Cipollini et al., 2012; Effmert et al., 2012). Además de los cambios en la rizosfera, múltiples modificaciones ocurren dentro de la propia planta hospedadora cuando es colonizada por AMF. En las raíces micorrizadas se pueden observar cambios en la arquitectura, alteración del perfil metabólico y acumulación de ciertos componentes de defensa (Pozo et al., 1996, 1998, 1999, 2002; García-Garrido y Ocampo, 2002; Strack et al., 2003; Hause et al., 2007; Schliemann et al., 2008; Péret et al., 2009). En raíces colonizadas por AMF podemos observar **a)** Acúmulo de apocarotenoides (ciclohexenona y micorradicina), los cuales son el principal componente del pigmento amarillento que se observa en algunas especies de plantas tras ser colonizadas por un AMF, además estos apocarotenoides tienen un importante papel en el control del grado de colonización y funcionalidad de la simbiosis AM (Strack et al., 2003; Strack y Fester, 2006; Floß et al., 2008; Schliemann et al., 2008). **b)** Cambios cualitativos y cuantitativos en el contenido de flavonoides, estos cambios dependen de la planta hospedadora y del estado de desarrollo de la simbiosis (Akiyama et al., 2002; Vierheilig y Piché, 2002). **c)** Cambios en los componentes fenólicos y ROS. **d)** Cambios en el perfil hormonal relacionado con la defensa de la planta, lo cual conlleva a una modificación del sistema inmune de la planta que se encuentra estrechamente relacionado con estas fitohormonas (ver apartado 3). Sin embargo el establecimiento de una simbiosis AM no solo tiene un profundo impacto sobre la raíz de la planta hospedadora, sino que también provoca multitud de cambios en la parte aérea de la planta micorrizada, estando muchos de estos cambios relacionados con la defensa de la planta (Liu et al., 2007; Fiorilli et al., 2009; Kaschuk et al., 2009; Pozo et al., 2009; Aloui et al., 2011; Fester et al. 2011). Además de los cambios directos ocasionados durante la colonización, la modulación del sistema inmune necesaria en la planta hospedadora para permitir la colonización puede resultar en un pre-acondicionando de los tejidos de la planta micorrizada para poder responder de una manera más efectiva y rápida

frente a posteriores ataques de patógenos, fenómeno conocido como "**priming**" (Pozo y Azcón-Aguilar, 2007) (ver apartado 3).

Por tanto debido a las propiedades biofertilizadoras y bioprotectivas de los AMF, la simbiosis AM se postula como una buena alternativa a los fertilizantes y pesticidas químicos en una agricultura sostenible (Harrier y Watson, 2004; Mukerji y Ciancio, 2007; Fester y Sawers, 2011).

4.3 Ciclo de vida

El proceso de micorrización de la raíz consta de varios pasos (Fig. 11):

Germinación de la espora. En ausencia de planta hospedadora, el hongo se mantiene en el suelo en forma de esporas de resistencia. Su ciclo de vida empieza con la germinación de las esporas de resistencia cuando las condiciones ambientales son favorables. La germinación de las esporas no requiere la presencia de la planta hospedadora, aunque puede verse estimulada por la presencia de exudados radicales. También está influenciada por determinados microorganismos del suelo y por las condiciones físico-químicas.

Crecimiento. A partir del tubo de germinación el hongo desarrolla un micelio que se extiende por el suelo de forma errática hasta alcanzar la rizosfera de una planta hospedadora. Si la espora no encuentra una raíz susceptible de ser colonizada para poder continuar su ciclo vital la espora retrae el citoplasma de las hifas producidas y entra de nuevo en un estado de quiescencia debido a su carácter biótrofos obligados (Bonfante y Genre, 2010). Una vez que las hifas alcanzan la rizosfera su crecimiento se ve notablemente estimulado por los exudados radicales existentes en la zona. Antes de que se produzca el contacto físico entre los dos simbiontes, se produce una comunicación molecular entre la raíz de la planta hospedadora y el AMF. Las plantas producen estrigolactonas, presentes en los exudados de la raíz las cuales estimulan el desarrollo y ramificación de las hifas del hongo, promoviendo el crecimiento orientado de estas hifas hacia la raíz, participando activamente en el dialogo entre la planta hospedadora y el AMF (para más detalles ver el apartado 4.4.1) (Akiyama et al., 2005). A su vez el AMF produce moléculas de señalización para la planta, los denominados factores myc (del inglés "*mycorrhization*") por homología a los factores Nod de

Rhizobium, los cuales desencadenan en la planta un pico de Ca^{2+} seguido de una inducción de los genes implicados en el establecimiento de la simbiosis (para más detalles ver el apartado 4.4.1). A continuación se induce una ramificación profusa del micelio y, a partir de ahí, el crecimiento de las hifas ya no es errático, sino que es orientado hacia la raíz. Al entrar en contacto con la superficie de la raíz, la hifa infectiva aumenta de tamaño en su parte apical y forma un hifopodio (Bastmeyer et al., 2002) sobre las células epidérmicas, a partir de la cual se produce una hifa de penetración, que marca el inicio de la colonización de la raíz.

Penetración. La penetración puede ocurrir a través de los pelos radicales jóvenes o bien a través de las raíces secundarias (Tawaraya et al., 2007), pero nunca por las células dañadas o a través de heridas, a diferencia de lo descrito para un gran número de patógenos. La hifa de penetración se introduce en la raíz, bien entre células epidérmicas, o bien directamente a través de la célula epidérmica. Los mecanismos de entrada del hongo en la raíz, en principio parecían ser una suma de procesos mecánicos (debido a la presión ejercida por la hifa de penetración) y enzimáticos (mediante la producción de enzimas pectinolíticos y celulolíticos por parte del hongo) (Smith y Read, 1997; Parniske et al., 2004). Estas enzimas se producen en pequeñas cantidades, por lo que no llegan a producir la degradación total de las paredes celulares de la raíz, sino tan solo la desorganización de sus componentes. Recientes estudios han demostrado la participación activa de la planta hospedadora en el proceso de penetración, con la formación de una estructura en las células corticales que están en contacto con el hongo, está estructura se denomina PPA (del inglés "*PrePenetration Apparatus*"), una especie de columna o túnel por el que pasará el hongo, que implica la reorganización del citoesqueleto y facilita la penetración del hongo en la raíz. Este PPA se forma entre 4 y 5 horas después de la formación del hifopodio. La formación del PPA se inicia cuando el núcleo se aproxima hacia la zona de contacto del hifopodio con la célula vegetal. Desde ahí, el núcleo se desplaza hacia el otro extremo de la célula formando el PPA, una columna citoplasmática compuesta por microtúbulos, microfilamentos y cisternas del retículo endoplasmático. Una vez el núcleo ha atravesado la célula y se ha formado el PPA, la hifa del hongo inicia su crecimiento hacía el interior de la célula a través de dicho túnel citoplasmático (Genre et al, 2008).

Colonización. La hifa de penetración se extiende y avanza entre las células de la epidermis, o a través de ellas, hacia la corteza de la raíz, y con frecuencia forma en el interior de las células unas circunvoluciones no ramificadas llamadas ovillos. Para ello una vez superada la pared celular, la hifa produce la invaginación de la membrana plasmática de la célula hospedadora, quedando completamente envuelta por ella, sin llegar nunca a penetrarla. Posteriormente la hifa alcanza la corteza media a través de los espacios intercelulares. Una vez allí el hongo se desarrolla intercelularmente, actuando la hifa sobre la lamina media, abriéndose paso entre ella, ramificándose y recorriendo longitudinalmente la raíz, por lo que la colonización se extiende rápidamente. Este modelo de colonización descrito corresponde al que se denomina micorriza tipo *Arum*, el formado por la mayoría de las plantas de climas templados. Existe otro modelo de colonización, denominado *Paris*, que lo presentan las plantas tropicales, que no dejan canales intercelulares, y en las que la extensión del hongo dentro de la raíz se produce, mucho más lentamente, mediante el paso célula-célula.

Formación de arbúsculos. En las capas más internas del parénquima cortical, las hifas intercelulares se ramifican lateralmente, penetran en las células y se dividen de forma dicotómica repetidamente, formando unas estructuras con forma de árbol, característica de este tipo de simbiosis, llamadas arbúsculos. En cada célula se forma un solo arbúsculo, que se encuentra rodeado totalmente por la membrana plasmática de la célula hospedadora, con lo que se consigue un gran aumento en la superficie de contacto entre el hongo y la planta. A este nivel la pared celular de hongo queda reducida a su mínima expresión (Bonfante y Perotto, 1995), y experimenta un cambio en su estructura, que pasa a ser amorfa por la desaparición de la estructura fibrilar de la quitina. La interfase que se forma a nivel del arbúsculo es la más especializada de la simbiosis y es aquí en donde tiene lugar preferentemente el intercambio de nutrientes entre la planta y el hongo (Blee y Anderson, 1998; Bonfante, 2001; Balestrini y Bonfante, 2005). La vida media de los arbúsculos es muy corta, entre 3 y 4 días (Alexander et al., 1998) y pasado este tiempo, los arbúsculos degeneran, se retrae el citoplasma de las hifas más finas, se produce el colapso de las mismas y queda en la célula una masa de material fúngico desorganizado que más tarde es reabsorbida y finalmente acaba por desaparecer.

Formación de vesículas. Las hifas pueden formar además unas estructuras globosas, de alto contenido lipídico, conocidas como vesículas, que parecen funcionar como órganos de reserva. La existencia y el número de vesículas depende de la especie fúngica, incluso algunos AMF son incapaces de desarrollarlas.

Desarrollo del micelio extra-radical y formación de esporas. Tras la formación de los primeros arbúsculos, las hifas externas del hongo se fortalecen y ramifican intensamente, constituyendo el micelio externo que se extiende en el suelo y es capaz de explorar un volumen del mismo inaccesible a las raíces. De esta forma aumenta considerablemente la superficie de absorción de la planta y por tanto su capacidad para captar nutrientes y agua más allá de la zona de agotamiento que se crea alrededor de las raíces. Algunas de las hifas extra-radicales darán lugar a nuevas esporas de resistencia, cerrándose así el ciclo de vida del hongo.

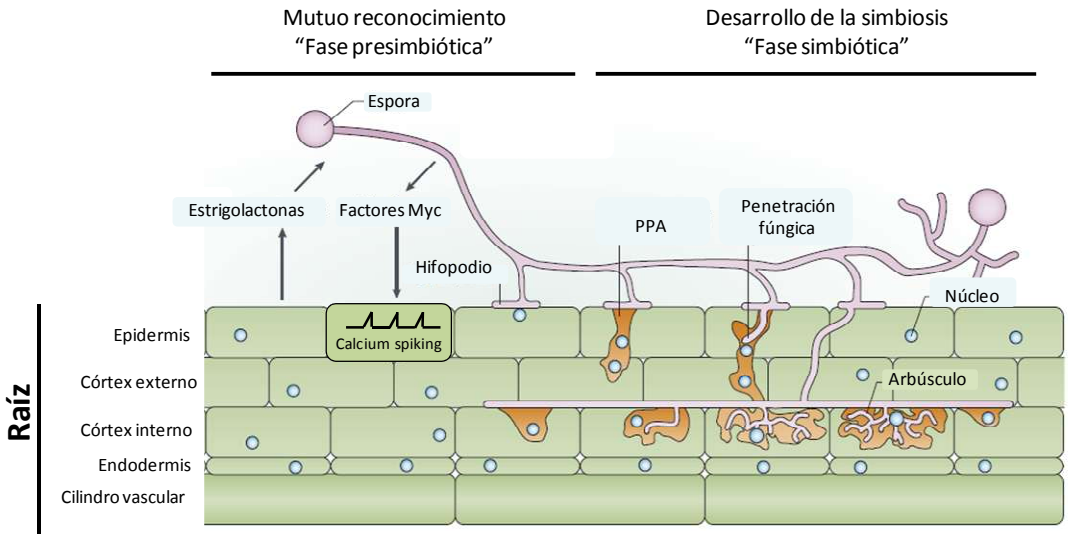

Figura 11. Ciclo de vida de los hongos micorrícicos arbusculares. La espora de resistencia comienza a germinar cuando las condiciones ambientales son favorables. Una vez que las hifas alcanzan la rizosfera su crecimiento se ve notablemente estimulado por los exudados radicales. Antes del contacto físico entre ambos simbiontes, se produce un dialogo molecular entre la raíz de la planta hospedadora y el hongo. Las plantas producen estrigolactonas, las cuales estimulan el desarrollo y ramificación de las hifas del hongo. A su vez el hongo produce factores myc, los cuales desencadenan en la planta un pico de Ca^{2+} seguido de una inducción de los genes implicados en el establecimiento de la simbiosis. A continuación se induce una ramificación profusa del micelio que al entrar en contacto con la superficie de la raíz, la hifa infectiva aumenta de tamaño en su parte apical y forma un hifopodio. La hifa infectiva produce la invaginación de la membrana plasmática de la célula hospedadora, quedando completamente envuelta por ella (PPA), sin llegar nunca a penetrarla. Posteriormente la hifa alcanza la corteza media a través de los espacios intercelulares. Una vez allí el hongo se desarrolla intercelularmente. Las hifas intercelulares se ramifican lateralmente, penetran en las células y se dividen de forma dicotómica repetidamente, formando los arbúsculos, en los cuales tendrá lugar el intercambio de nutrientes entre el hongo y la planta (adaptado de Parniske, 2008).

4.4 Control de la simbiosis

El establecimiento de la simbiosis AM requiere la activación de un complejo programa de desarrollo, tanto en la planta hospedadora como en el hongo, cuyos determinantes genéticos han sido parcialmente descritos en la planta a través de la caracterización de líneas mutantes defectivas en el proceso de micorrización (Reinhardt, 2007; Parniske, 2008; Oldroyd et al., 2009). Al contrario de lo que ocurre en raíces infectadas por

hongos patógenos, las raíces micorrizadas no muestran signos de daño alguno. La colonización de la raíz por los AMF provoca tan solo una activación débil, localizada y transitoria de los mecanismos de defensa de la planta (Smith y Read, 2008). Es decir, la planta permite esa colonización sin mostrar aparentemente demasiada resistencia. Este hecho facilita la interacción estable de ambos simbiontes y es, en cierta medida, la base del biotrofismo de los hongos AM. Durante la interacción con las plantas, muchos microorganismos secretan pequeñas proteínas (**SSPs**, del inglés "*small secreted proteins*"), que entran en las células vegetales modificando el metabolismo y modulando las respuestas de defensa actuando como efectores (Jiang, 2011), como se explica en el apartado 3 de esta Tesis Doctoral. En recientes estudios se ha caracterizado la primera SSP en un hongo AM, denominada **SP7** (Kloppholz et al., 2011). La expresión de gran cantidad de SSPs en la fase intrarradical del hongo micorrícico *Glomus intraradices* y en células arbusculadas (Tisserant et al., 2012) pone de manifiesto la importancia de estas proteínas en la simbiosis.

A grandes rasgos la formación de la simbiosis AM comprende dos fases fundamentales: **Fase presimbiótica**, en la cual se dan procesos de señalización y reconocimiento hongo-planta. **Fase simbiótica**, donde tienen lugar la colonización de la raíz y el desarrollo de estructuras fúngicas intra- y extrarradicales que permiten el establecimiento de una simbiosis funcional.

4.4.1 Fase presimbiótica. Intercambio de señales.

Durante la interacción entre la planta y el AMF se activan en la planta diversas rutas de señalización muy específicas, que preparan a la planta para acoger a su huésped beneficioso. Este proceso comienza con un intercambio de señales moleculares entre ambos simbiontes, que ocurre incluso antes de que haya un contacto físico entre ellos. A esta etapa del proceso de formación de la simbiosis se le conoce como **fase pre-simbiótica**. Algunos de los exudados radiculares de la planta hospedadora inducen cambios en el desarrollo y en el metabolismo del hongo, permitiendo o facilitando que se establezca la simbiosis. Entre estos exudados las **estrigolactonas** cobran una gran importancia en el establecimiento de la simbiosis durante esta etapa presimbiótica, ya que estimulan tanto la germinación de las esporas fúngicas, como el desarrollo

extrarradical del AMF y su posterior ramificación (Giovannetti et al., 1993; Buée et al., 2000; Akiyama et al., 2005).

De forma recíproca el hongo produce los denominados factores **myc**, moléculas difusibles que inducen la expresión de genes en la planta hospedadora, involucrados en el establecimiento de la simbiosis (Kosuta et al., 2003; Weidmann et al., 2004). Además, los factores myc activan procesos de señalización intracelular (Navazio et al., 2007; Kosuta et al., 2008) y estimulan el desarrollo de raíces laterales o secundarias (Oláh et al., 2005). Estas moléculas de señalización han sido recientemente descritas como una mezcla de lipoquitooligosacaridos (**LCO**, del inglés "*lipochitooligosacarides*") sencillos, sulfatados y no-sulfatados (Maillet et al., 2011).

Las estrigolactonas (SLs) son producidas por un amplio rango de plantas. Además, una misma especie puede producir más de un tipo de SLs, lo que sugiere un papel importante en la naturaleza (Xie et al., 2010). Aunque inicialmente las SLs fueron identificadas como metabolitos implicados en la señalización dentro de la rizosfera, recientemente han sido clasificados como fitohormona por su multitud de funciones endógenas en la planta (Kohlen et al., 2012). Entre estas nuevas funciones destaca su papel en la regulación de la arquitectura tanto de la raíz como de la parte aérea de las plantas (Bouwmeester et al., 2007; Gómez-Roldan et al., 2008; Umehara et al., 2008; Kapulnik et al., 2011; Ruyter-Spira et al., 2011).

Las SLs son derivados de los carotenoides y se obtienen a partir de una serie de escisiones oxidativas mediadas por las enzimas **CCD7** y **CCD8** (del inglés "*carotenoid cleavage dioxygenases*"), las cuales están implicadas en la ruta de biosíntesis de los β-carotenos (Matusova et al., 2005; López-Ráez et al., 2008;). Las SLs descritas hasta la fecha muestran una estructura química similar, con un núcleo estructural compuesto por una lactona tricíclica (anillos ABC) unida mediante un enlace éter enólico a un grupo butirolactona (anillo D) (Yoneyama et al., 2009; Zwanenburg et al., 2009) (Fig. 12A). Sin embargo pueden presentar distintos grupos unidos a los anillos A y B, lo que da lugar a los distintos tipos de SLs. La actividad biológica de estas moléculas parece residir en el enlace éter enólico, el cual puede ser roto rápidamente en ambientes acuosos o alcalinos, indicando la corta vida de esta molécula coherente con su papel en señalización (Yoneyama et al., 2009; Zwanenburg et al., 2009; Akiyama et al., 2010;).

Las SLs se describieron por primera vez como moléculas capaces de favorecer la germinación de algunas plantas parásitas pertenecientes a la familia *Orobancheaceae* (Cook et al., 1972; Bouwmeester et al., 2003). Estas plantas parasitas presentan un ciclo de vida similar que comienza con la germinación de sus semillas al detectar la presencia de estrigolactonas en el medio (Bouwmeester et al., 2003) (Fig. 12B).

Posteriormente, se demostró que estas moléculas tienen un papel clave en la fomación de MA (Akiyama et al., 2005; Bouwmeester et al., 2007). Estudios usando líneas mutantes han mostrado que la alteración en la producción de SLs produce una reducción en los niveles de colonización, (Gómez-Roldan et al., 2008; Vogel et al., 2010; Kohlen et al., 2012;). La deficiencia de fosfato o nitrógeno en la planta, inducen la producción de exudados ricos en estrigolactonas, con el fin de atraer a los AMF y mediante el establecimiento de la simbiosis poder suplir estas deficiencias (Yoneyama et al., 2007; López-Ráez et al., 2008) (Fig. 12B). Las SLs presentes en la rizosfera son percibidas por el hongo solo cuando ambos organismos están cerca, a través de un receptor que todavía no está caracterizado, activando el metabolismo del hongo y dando lugar al denominado estado presimbiótico, caracterizado por una intensa ramificación de las hifas en las esporas que están germinando. De esta manera se aumenta la posibilidad de contacto entre el AMF y la raíz hospedadora (Akiyama et al., 2005; Besserer et al., 2006; Bouwmeester et al., 2007) (Fig. 12B). Además, las SLs pueden estimular la germinación de las esporas de ciertos AMF (Besserer et al., 2006) y ejercer un efecto de quimiotaxis, dirigiendo el crecimiento de las hifas hacia la raíz (Sbrana and Giovannetti, 2005). Esta respuesta desencadenada en los AMF parece ser bastante específica, pues no se da frente a otros organismos beneficiosos como *Trichoderma* sp. o *Piriformospora* sp., ni frente a organismos patógenos (Steinkellner et al., 2007).

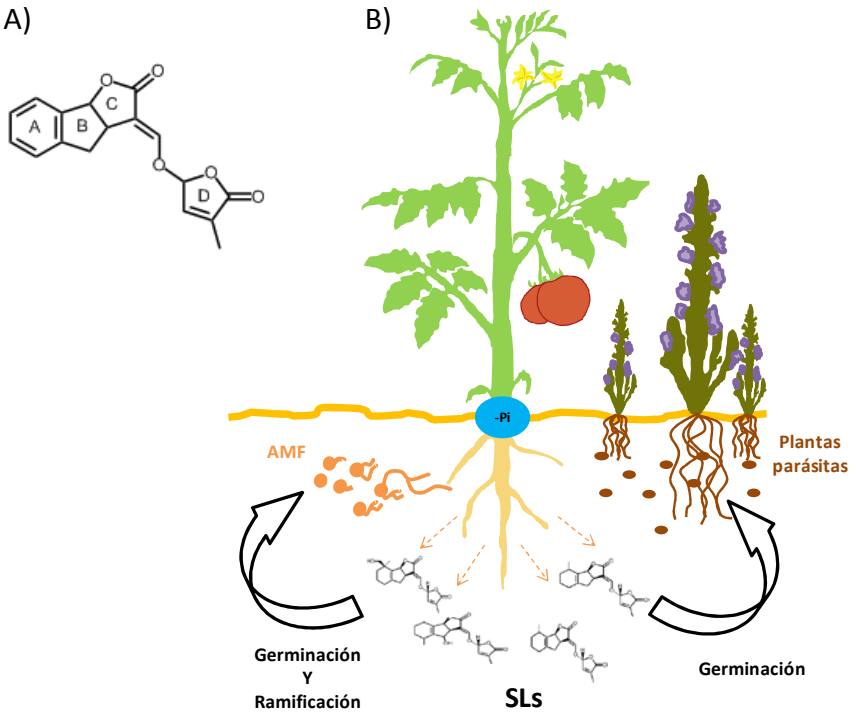

Figura 12. Estructura general (A) y función de las estrigolactonas (B). Ante un déficit de fósforo (-Pi) la planta aumenta la producción de estrigolactonas (SLs), las cuales estimulan la germinación y ramificación de los hongos micorrícicos arbusculares (AMF) promoviendo el establecimiento de la simbiosis micorrícica y así suplir el déficit de Pi. Por otro lado, algunas plantas parasitas también pueden detectar las SLs lo que estimula la germinación de sus semillas que acaban parasitando a estas plantas afectadas en su estado nutricional (adaptado de López-Ráez et al., 2011).

Ruta simbiótica. La percepción temprana de los factores myc (por un receptor todavía no descrito) induce cambios fisiológicos en la célula vegetal que incluyen despolarización de la membrana plasmática como consecuencia de un flujo alterado de iones, seguido por oscilaciones periódicas en los niveles intracelulares de calcio ("*calcium spiking*"), que culminan con la activación de la ruta de señalización **Sym** (del inglés, "*Symbiotic pathway*"). Estas primeras reacciones dan lugar a la activación de un programa genético en la planta huésped que conducirá al desarrollo de la simbiosis (Chabaud et al., 2011; Harrison, 2012).

Esta ruta "*Sym*" está parcialmente caracterizada y tiene componentes que son comunes con la vía de señalización de la simbiosis entre *Rhizobium* y leguminosas, por lo menos durante las primeras etapas del establecimiento de la simbiosis (Oldroyd y Downie, 2004; Parniske, 2008). El clonado de uno de los genes comunes requerido

para el desarrollo de ambas simbiosis, *nork* (del inglés "*nodulation receptor kinase*") / *symrk* (del inglés "*symbiosis receptorlike kinase"*), puso de manifiesto que existen componentes de señalización comunes que son requeridos para el establecimiento de ambas simbiosis (Endre et al., 2002; Stracke et al., 2002). Posteriormente se demostró que los genes *castor* y *pollux* (previamente *sym4* y *sym23*, respectivamente) son indispensables para la admisión de rizobios y micorrizas en la célula de la planta huésped (Imaizumi-Anraku et al., 2005). De esta manera se observó la superposición de programas genéticos requeridos para el establecimiento de ambas simbiosis (Albrecht et al., 1999; Kistner y Parniske, 2002). Otros genes sin embrago son específicos de la nodulación. Por ejemplo *nfr1* y *nfr5* codifican para dos receptores quinasa con dominios LysM esenciales para desencadenar los primeros eventos fisiológicos y morfológicos en respuesta a LCOs. Mutantes de *L. japonicus* en ambos genes son incapaces de formar hilos de infección y desarrollar primordio nodular, a pesar de lo cual presentan un fenotipo normal de micorrización (Madsen et al., 2003; Radutoiu et al., 2003). Sin embrago hasta la fecha no se ha caracterizado ningún componente único y/o específico de micorrización. (Oldroyd y Downie, 2004; Chabaud et al., 2010). No obstante, el establecimiento de la micorrización es un proceso complejo, habiéndose propuesto que pueden existir vías de señalización paralelas que controlen las respuestas tempranas a la micorrización y que todavía están pendientes de caracterización.

4.4.2 Fase simbiótica. Regulación de la simbiosis

Debido al carácter biótrofo de los AMF, la interacción de la planta con AMF muestra algunas similitudes con interacciones con patógenos biótrofos, desencadenando en la planta una respuesta de defensa en cierto modo similar durante los primeros estadios de la interacción (Paszkowski, 2006). En estadios más tardíos, el hongo es capaz de modular activamente la respuesta de defensa de la planta para poder establecer la interacción. Por otro lado, la planta además controla de manera activa el nivel de proliferación del simbionte dentro de la raíz para prevenir una excesiva colonización, que conllevaría pérdidas excesivas de compuestos carbonados.

Las hormonas vegetales parecen tener un papel clave en la modulación de la respuesta de defensa de la planta frente a la colonización por el AMF, así como en el proceso de regulación de la simbiosis por la propia planta. Aunque se trata de un área de investigación poco explorada todavía, trabajos recientes sugieren que el ABA, SA y JAs podrían desempeñar un papel regulador importante en el establecimiento, desarrollo y mantenimiento de la simbiosis (Hause et al., 2007; Herrera-Medina et al., 2007; García-Garrido et al., 2010).

Ácido Abscísico. Hasta el momento el ABA ha sido relacionado principalmente con la protección frente a estreses abióticos (sequía y salinidad), pero su papel durante el proceso de micorrización todavía no está muy definido. Estudios recientes indican que el ABA podría jugar un papel en el establecimiento de la simbiosis. Mutantes de tomate deficientes en la síntesis de ABA, muestran una disminución en el grado de micorrización y sobre todo en la formación de arbúsculos (Herrera-Medina et al., 2007). Por lo tanto, el ABA en raíces es necesario para la colonización de la raíz por AMF, sobre todo bajo condiciones desfavorables para la planta, como puede ser la sequía.

Ácido Salicílico. El papel del SA durante el proceso de micorrización se ha relacionado con la función de esta hormona en la regulación de la respuesta de defensa de la planta (García-Garrido y Ocampo, 2002). Aunque no afecta de manera significativa al grado de micorrización final, la aplicación exógena de SA provoca un retraso transitorio en el desarrollo de la micorrización (Blilou et al., 2000b; Herrera-Medina et al., 2007; de Román et al., 2011), lo que indica un efecto negativo de esta hormona en esta simbiosis.

Por otro lado, durante el proceso de formación del hifopodio y la etapa de penetración de las células epidérmicas por las hifas del AMF se produce en la planta un incremento en los niveles de SA que podría ser consecuencia de una respuesta de defensa de la planta (Blilou et al., 1999; Blilou et al., 2000a; Blilou et al., 2000b). El SA por lo tanto podría desempeñar una función importante en la etapa inicial de contacto entre los dos simbiontes (Gutjahr y Paszkowski, 2009) y en etapas posteriores controlar la colonización por parte del hongo, debido a su papel en el control frente a biótrofos (Fig. 13).

Ácido Jasmónico. Durante la colonización de la raíz por los AMF se produce un incremento en los niveles de JA (Fig. 13) (Hause et al., 2002; Meixner et al., 2005; Stumpe et al., 2005). Este aumento de JA se acompaña de una mayor expresión de genes implicados en la ruta de biosíntesis del JA, concretamente en las células que contienen arbúsculos (Hause et al., 2002). Esto podría indicar que el JA es un regulador del desarrollo de los arbúsculos en la raíz. Se han postulado varios posibles mecanismos para explicar la implicación del JA en la micorrización, como pueden ser la inducción de genes de defensa, la reorganización del citoesqueleto, la alteración del estado sumidero de la raíz, una mejora en el "fitness" de la planta, la redistribución de carbohidratos y más recientemente, un efecto en la expansión de la pared celular de las células vegetales (Hause et al., 2007; Tejeda-Sartorius et al., 2008; Gutjahr y Paszkowski, 2009).

Además del papel del ácido jasmónico, las oxilipinas sintetizadas a través de la rama 9 LOX son también reguladas durante la simbiosis y podrían tener un papel en el control de la extensión de la colonización fúngica en la raíz (León-Morcillo et al., 2012 a, b).

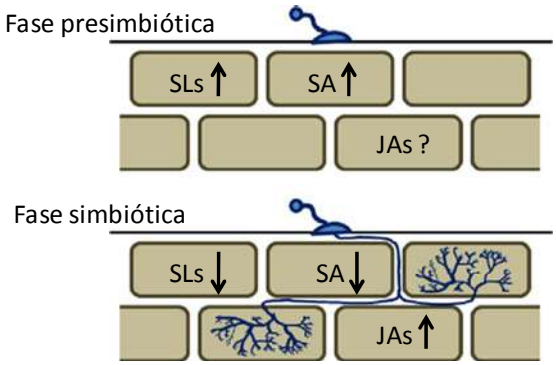

Figura 13. Modelo de los cambios hormonales que ocurren en la raíz asociados a una simbiosis micorrícica arbuscular. En los primeros estadios de la colonización (fase presimbiótica) la producción de estrigolactonas (SLs) es todavía alta. Inicialmente los hongos micorrícicos arbusculares (AMF) son percibidos como organismos invasores y debido a su carácter biótrofo los niveles de ácido salicílico (SA) aumentan en la raíz de la planta hospedadora. Una vez que la simbiosis se ha establecido correctamente (fase simbiótica) la producción de SLs y SA disminuye, mientras que aumenta la biosíntesis de jasmonatos (JAs) (adaptado de Jung et al., 2012).

CAPÍTULO 1: Conserved phytohormone regulation patterns associated to the arbuscular mycorrhizal symbiosis across plant species

Conserved phytohormone regulation patterns associated to the arbuscular mycorrhizal symbiosis across plant species

Fernández I., Merlos M., Ferrol N., López-Ráez J.A., Martínez-Medina A., Flors V., Bonfante P., Pozo M.J.

Resumen.

La simbiosis micorrízica arbuscular (AM) es una asociación mutualista entre los hongos micorrízicos del suelo y la mayoría de las plantas terrestres. Aunque se considera que los hongos AM no tienen especificidad por el hospedador, existe una diversidad funcional entre ambos simbiontes. Por tanto, el resultado final de la interacción depende, en parte, del genotipo tanto de la planta como del hongo. Durante la simbiosis AM tiene lugar una modulación del perfil transcriptómico y de ciertas fitohormonas relacionadas con la defensa en la planta hospedadora. Algunos de estos cambios que se dan en el programa simbiótico de las AM están conservados en plantas muy alejadas filogenéticamente pertenecientes a grupos tan diferentes como pueden ser las monocotiledóneas y dicotiledóneas. En este trabajo hemos realizado un estudio integrativo de la respuesta de diferentes plantas hospedadoras -tomate, soja y maíz- tras la colonización por diferentes hongos micorrízicos -*Glomus mosseae* y *Glomus intraradices*-, combinando múltiples análisis transcriptómicos y hormonales. El análisis de diversas hormonas relacionadas con la defensa de la planta - ácido abscísico, ácido salicílico y compuestos derivados del jasmonato- así como el análisis de genes marcadores de las rutas reguladas por estas hormonas, revelaron cambios significativos en las raíces de plantas micorrizadas con respecto a las plantas no micorrizadas. Estos cambios dependieron en gran medida tanto del genotipo del hongo AM, como de la planta hospedadora involucradas en la simbiosis. Entre estos cambios observados, la regulación de la ruta del los jasmonatos parece estar altamente conservada entre las diferentes especies de plantas hospedadoras, lo cual, parece indicar que los jasmonatos tienen un papel clave durante la simbiosis AM. Además, nuestro estudio mostró que la modulación de la ruta de señalización de los jasmonatos y por tanto de la respuesta de defensa mediada por esta hormona, era altamente dependiente del genotipo del hongo AM, revelando la capacidad de *G.*

intraradices para suprimir la respuesta de defensa de la planta y así lograr una mayor invasión de la raíz.

Adapted from Plant, cell and environment (submitted)

Conserved phytohormone regulation patterns associated to the arbuscular mycorrhizal symbiosis across plant species

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Abstract

Arbuscular mycorrhizal (AM) symbioses are mutualistic associations between soil fungi and most vascular plants. Although AM fungi are considered to lack host specificity, there is a functional diversity among both partners. The final outcome of the interaction seems to be dependent on plant and fungus genotype. Modulation of the transcriptional profile and the level of several defence-related phytohormones in the host plant during AM symbiosis do occur. Some of the components of this AM symbiotic program have been reported to be common in a number of monocotyledons and dicotyledons. An integrative analysis of the response of different host plants -tomato, soybean and maize- to different mycorrhizal fungi –*Glomus mosseae* and *Glomus intraradices*- was performed combining multiple hormone determination and transcriptional analysis. The analysis of the defence-related hormones abscisic acid, salicylic acid and jasmonate derivates compounds and the marker genes of the pathways regulated by these hormones, revealed significant changes in the root of mycorrhizal plants, depending on the AMF and/or the host plant involved. Among them, the regulation of the jasmonates pathway was found to be highly conserved among different plant species, which seems to indicate a key role of jasmonates during the AM symbiosis. Our study further showed a strong control by the AMF genotype on the modulation of the JA-signalling pathway, and hence on the defence related response, indicating that *G. intraradices* have developed the ability to suppress the plant defence response to achieve a higher root invasion.

Key-words: Arbuscular mycorrhiza, phytohormones, maize, soybean, tomato, transcriptional analysis

Introduction

Plants continuously interact with a broad range of organisms present in their environment, giving rise to a large variety of associations. They are frequently challenged by microbial pathogens that threat plant´s fitness, but they also interact with neutral or beneficial microorganisms that positively affect multiple vital parameters such as plant nutrition, growth and help plant to overcome different type of stresses (Barea et al., 2005; Aroca et al., 2009, Raaijmakers et al., 2009). Thus, a microbe free plant may be considered an "exotic exception" (Partida Martinez and Heil, 2012). In nature, severe disease is uncommon since plants count on surveillance systems to detect aggressors and to trigger appropriate defence responses to repeal their attack (Spoel and Dong, 2012). Thus, plants constantly have to fine-tune their defence mechanisms to combat deleterious organisms while allowing associations with beneficials (Pozo and Azcón-Aguilar, 2007; Van wees et al., 2008, Spoel and Dong, 2012; Zamidious and Pieterse, 2012). The response triggered in the plant upon recognition of the microorganism encountered vary depending on the microbe characteristics and lifestyle, and the phytohormones salicylic acid (SA), jasmonic acid (JA) and absicic acid (ABA) play central roles in coordinating such responses (Pieterse et al., 2009; Bari and Jones, 2009; Verhage et al., 2010).

Among plant associations with beneficial microorganisms, arbuscular mycorrhizas (AM) are one of the most widespread interactions on Earth, with a major impact on plant health and ecosystem dynamics (Gianinazzi et al., 2010). About 80% of all land plants, including most agricultural and horticultural crop species, are able to form this kind of mutualistic association with certain soil-borne fungi from the phylum Glomeromycota (Smith and Read, 2008). These fungi, known as arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that obtain carbohydrates from their host, while they improve mineral nutrition and water acquisition by the host plant (Parniske, 2008). Additionally, the symbiosis impacts the plant's ability to overcome biotic and abiotic stresses, commonly improving host resistance to pathogens and tolerance to

unfavourable environmental conditions (Pozo and Azcón-Aguilar, 2007; Singh et al., 2011; Jung et al., 2012; Ruiz-Lozano et al., 2012). Although AMF are considered non specific with respect to host range, there are evidences for "functional diversity". That is, both the plant and fungal genotype determine the benefits of the interaction, being some combinations more efficient than others in terms of nutrition or stress resistance improvement (Cavagnaro et al., 2001; Pozo et al., 2002; Smith et al., 2004; Martinez-Medina et al., 2009). The interaction requires a high degree of coordination between both partners, and bidirectional (plant and fungal) control assure a fair trade of resources between the symbionts (Kiers et al., 2011). Indeed, the plant is able to prevent excessive colonization by the AMF to avoid costs out-weighting the benefits of the interaction (Catford et al., 2003; Breuillin et al., 2010).

Several studies have evidenced alterations in the hormonal and transcriptional profiles of the plant associated to AM development and functioning (Paszkowski 2006; Hause et al., 2007; Requena et al., 2007), many of the changes related to plant defence mechanisms (Liu et al., 2003; Fiorilli et al., 2009; Lopez-Raez et al., 2010; Pozo et al., 2010) likely contributing to the plant control over the symbiosis. The impact of the symbiosis on the plant defence mechanisms may also have consequences on plant interactions with other organisms, commonly priming plant defences against potential attacks (Pozo and Azcón-Aguilar 2007; van Wees et al., 2008, Zamidious and Pieterse, 2012). Moreover, since key defence regulators are common signals in responses to both biotic and abiotic stresses, alterations in phytohormones homeostasis may affect also plant tolerance against abiotic stresses such salinity, drought and heavy metals (Atkinson and Urwin, 2012, Pineda et al., 2012; Ruiz-Lozano et al., 2012). Thus, alterations in phytohormones levels in well established mycorrhizas may underlie at least part of the beneficial effects of the symbiosis on plant health.

Plant-AMF communication starts prior to the physical contact between both symbionts and continues during the entire endosymbiotic phase (Bonfante and Genre, 2010; López-Ráez et al., 2011). Initially, upon mutual recognition the fungus has to deal with the plant immune system to proceed with a successful colonization (Kloppholz et al., 2011). During root colonization, AMF proliferate within the cortex and form specialized structures called arbuscules where the exchange of nutrients between the partners takes place. The growth of the fungal symbiont inside the root implies the alteration of

multiple host cellular processes (Bonfante and Genre, 2010). Since the development of the AM symbiosis is highly dynamic and asynchronous, with new infection events starting continuously, various stages of colonization are present simultaneously in the same roots. Accordingly, gene expression and hormonal profiles should be precisely regulated at the cellular level (Balestrini et al., 2007). Among plant stress-related hormones, ABA, SA and JA are believed to play a key role not only in the establishment, but also in the functioning of the AM symbiosis (Hause et al., 2007; Herrera-Medina et al., 2007; Pozo and Azcon-Aguilar 2007; Lopez-Raez et al., 2010; López-Ráez et al., 2011). Analysis of AM symbiosis in ABA-impaired mutants has shown that ABA is necessary for AM development and functionality (Herrera-Medina et al., 2007; Martin-Rodriguez et al., 2010). In addition, alterations in plant ABA content during AM symbiosis has been reported, although results are controversial ranging from increase (Meixner et al., 2005), no changes (Lopez-Raez et al., 2010; Martinez-Medina et al., 2011; Asensio et al., 2012) to decrease in ABA levels (Aroca et al., 2012).

Similarly, controversial results have also been obtained in relation to the hormones SA and JA. SA is known to have a major role in plant defence against microorganisms with a biotrophic lifestyle (Pieterse et al., 2009). Therefore, AMF as obligate biotrophs are expected to be negatively affected by SA (Pozo and Azcón Aguilar, 2007; Gutjahr and Paszkowski, 2009). Previous studies showed a negative, although transient, effect of SA on fungal root colonization (Herrera-Medina et al., 2007; de Roman et al., 2011). Accordingly, an increase in SA levels and expression of SA-regulated marker genes (*PAL1*, *PR-1a* and *PR2)* has been reported during early stages of symbiosis establishment, coinciding with appresoria formation (Blilou et al., 2000; Gallou et al., 2010). However, contrasting results are reported concerning host SA content in well established associations, since mycorrhizal roots have been reported to have higher, (Khaosaad et al., 2007; Lopez-Raez et al., 2010) unaltered (Campos-Soriano and Segundo, 2011) or lower (Herrera-Medina et al., 2003) SA levels tan non mycorrhizal roots. The results suggest that the changes in SA in mycorrhizal roots are highly dependent on the stage of the symbiosis, the plant and the AMF genotype.

Among all phytohormones analyzed in mycorrhizal plants, particular interest has been devoted to oxilipins, particularly jasmonates (Hause and Schaarschmidt, 2009, León-Morcillo et al., 2012). The oxilipins include biologically active derivatives of JA and

intermediates of JA biosynthesis such as JA-Ile and OPDA (oxo-phytodienoic acid), respectively. These compounds are widely distributed in plants and affect multiple processes involved in development and defence (Creelman and Mullet, 1997; Turner et al., 2002, Pozo et al., 2004; Wasternack and Kombrink, 2010). Increased levels of JA have been reported in mycorrhizal roots of several monocot and dycot species (Hause et al., 2002; Vierheilig and Piché, 2002; Stumpe et al., 2005; Meixner et al., 2005; Lopez-Raez et al., 2010), although there are also reports illustrating unaltered JA levels (Riedel et al., 2008). The regulatory role of the oxilipins and JAs in particular on the symbiosis is far from clear. Studies involving foliar application of JA and wounding resulted in promotion of mycorrhizal root colonization (Regvar et al., 1996; Landgraf et al., 2012), while that the use of JA-impaired mutants resulted in both promotion (Ludwig-Müller et al., 2002; Herrera-Medina et al., 2008) and reduction (Isayenkov et al., 2005) of mycorrhizal colonization. It has been suggested that JA function depends on the degree of mutualism achieved in the plant-AMF interaction (Hause and Schaarschmidt, 2009).

In summary, current data on phytohormone homeostasis in AM roots are often fragmented and highly contradictory, as they were obtained using different plant – AMF systems, frequently in different stages of mycorrhization and determined under different experimental conditions. In the present study, we combine metabolomics and transcriptional approaches to analyze changes in SA, ABA and JA signalling pathways associated to a well established mycorrhizal symbiosis in different host plants, with the aim to determine common features conserved across plant species. We compare plants belonging to distant families such as maize, soybean and tomato interacting with two different AMF species. The results provide original insights into the evolutionary conservation of defence signalling regulation in AM symbiosis among phylogenetically distant plant species and provide hints on the mechanisms underlying functional diversity in AM interactions.

Materials and Methods

Plant growth and AM inoculation

Tomato (*Solanum lycopersicum* L. cv. MoneyMaker), and soybean (*Glycine max* L. Merr. cv.Williams 82) seeds were surface sterilized in 4% sodium hypochlorite , rinsed thoroughly with sterile water and germinated for 3 d in a container with sterile vermiculite at 25ºC in darkness. Subsequently, individual seedlings were transferred to 0.25 l pots with a sterile sand:soil (4:1, v:v) mixture. Plants were randomly distributed and grown in a greenhouse at 24/16ºC with a 16/8 h photoperiod and 70% humidity. Maize (*Zea mays* L.) seeds were surface-sterilized and germinated in humidified filter sterile paper at 27ºC for 3 days. Plantlets were sown in 0.4 l with a sterile soil:peat (9:1, v:v). The mixture of soil:peat was sieved through a 2 mm mesh, sterilized by tyndalization for three consecutive days and dried air. Plants were grown in grown chamber at 25/18ºC with a 16/8 h photoperiod and 60% relative humidity. All plants were watered three times a week, first 3 weeks only water. From the 4th week, Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration was applied. The AM fungi *Glomus intraradices* DAOM 197198 –actually known as *Rhizophagous irregularis* DAOM 197198- and *G. mosseae* BEG12 -actually known as *Funneliformis mosseae* BEG12- were maintained as a soil-sand-based inoculum. The inoculum consisted of thoroughly mixed rhizosphere samples containing spores, hyphae and mycorrhizal root fragments. Pots were inoculated by adding 10% (v:v) *G. mosseae* or *G. intraradices* inoculum. The same amount of soil:sand mix but free from AMF was added to control plants. All plants received an aliquot of a filtrate (20 ml) of both AMF inocula to homogenize the microbial populations. Plants were harvested after 8 weeks of growth, and the fresh weight of shoots and roots was determined. An aliquot of each individual root system was reserved for mycorrhizal quantification. Root of all plants were frozen in liquid nitrogen and stored at -80 °C until use.

Mycorrhizal colonization determination

Roots were stained with trypan blue (Phillips and Hayman, 1970) and examined using a Nikon Eclipse 50i microscope and brightfield conditions. The percentage of total root colonization was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

Hormone quantification

OPDA, JA, JA–Ile, ABA, and SA were analysed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) as described by Flors et al. (2008). A 100 mg aliquot of dry tissue was used per sample. A mixture of internal standards containing 100 ng of [$^2H_6$]ABA, 100 ng of dihydrojasmonic acid, 100 ng of prostaglandin B1, and 100 ng of [$^2H_5$]SA was added to each sample prior to extraction. Individual calibration curves for each tested compound and internal standard were performed before the analysis. The tissue was immediately homogenized in 2.5 ml of ultra pure water and centrifuged at 5000 g for 40 min. Then, the supernatant was acidified and partitioned against diethyl-ether, dried, and resuspended in 1 ml of water/methanol (90:10, v/v). A 20 µl aliquot of this solution was injected into a Waters Acquity UPLC system (Waters). The UPLC was interfaced into a triple quadrupole tandem mass spectrometer (TQD, Waters). LC separation was performed using an Acquity UPLC BEH C_{18} analytical column (Waters) at a flow rate of 300 µl min^{-1}. Quantifications were carried out with MassLynx 4.1 software (Waters) using the internal standards as a reference for extraction recovery and the standard curves as quantifiers.

RNA isolation

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel), and stored at –80ºC until use.

Gene expression analysis by real-time quantitative RT-PCR (qPCR)

Real-time quantitative RT-PCR (qPCR) was performed using the iCycler iQ5 system (Bio-Rad) and gene-specific primers (Table S1). Rutinarely, the first-strand cDNA was synthesized from 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. For the analysis of genes with low expression level, the first-strand cDNA was synthesized with 3 µg of purified total RNA using the BioScript cDNA Synthesis kit (Bioline), according to the manufacturer's instructions. At least three independent biological replicates were analysed per treatment and qPCR reactions were performed in duplicates. Relative quantification of specific mRNA levels was performed using the comparative method of Livak and Schmittgen (2001). Expression values were normalized using the housekeeping genes *SlEF* and *Ubiquitin*, which encodes for the tomato elongation factor-1α and the ubiquitin of tomato respectively; *GmEF1B*, which encodes for the soybean elongation factor-1β; and *ZmEF1*, which encodes for the maize elongation factor-1α. Amplification reactions were run for 35 cycles at 94ºC for 30 s, 58ºC for 30 s, and 72ºC for 40 s. The specificity of each PCR amplification procedure was verified by the melt curve analysis of the PCR product with a heat dissociation protocol (from 58 to 95ºC).

Laser capture microdissection

Roots from 8 weeks-old tomato plants inoculated or not with mycorrhiza were dissected into 5-10mm pieces and fixed in 100% acetone at 4ºC overnight for paraffin embedding. The root pieces were placed in acetone under vacuum for 15 min, and then kept at 4ºC overnight. The next day they were gradually dehydrated in a graded series of acetone: Neoclear (Merck, Darmstadt, Germany) (3:1, 1:1 and 1:3) followed by Neoclear 100% (twice) with each step being carried out on ice for 1 h. The Neoclear was gradually replaced with paraffin (Paraplast Plus; Sigma-Aldrich, St Louis, MO, USA). The embedding step was as described in Balestrini et al. (2007). Sections of 14 µm were cut using a rotary microtome (Microm Hm325) and placed on Leica RNase-free PEN foil slides (Leica Microsystem, Inc., Bensheim, Germany) with diethyl pyrocarbonate-distilled water. The sections were dried at 40ºC in a warming plate, stored at 4ºC, and used within 2 days. Laser microdissection was performed using a

Leica AS laser capture microdissection system (Leica Microsystem, Inc.). The samples were deparaffinized in xylene for 10 min, dipped in 100% ethanol for 2 min, and then air-dried. After collection, an RNA extraction buffer from a Pico Pure kit (Arcturus Engineering, Montain View, CA, USA) was added. The samples were incubated at 42ºC for 30 min, centrifugated at 800 g for 2 min, and stored at 80ºC. RNA was extracted with the Pico Pure kit (Arcturus Engineering), as described by Balestrini et al. (2007), and quantified using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) spectrophotometer. Absence of DNA contaminations was confirmed by PCR assays in samples without retrotranscription. Retrotranscription and PCR amplification were carried out using the One Step RT-PCR kit (Qiagen). The samples were incubated for 30 min at 50ºC, followed by 15 min of incubation at 95ºC. Amplification reactions were run for 40 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 40 s.

Statistical analysis

Data for mycorrhizal root colonization and gene expression levels were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 19 for Windows. When appropriate, DMS's test was applied. Data for hormone content were subjected to two-ways analysis of variance (two-factor ANOVA, fungus and plant). Significance levels were set at 5%.

Results

Root colonization by *G. mosseae* and *G. intraradices* in different plant families

In order to determine phytohormone related changes associated to the mycorrhizal symbiosis in different plant species tomato, soybean and maize were inoculated with the AMF *G. mosseae* and *G. intraradices*. Both fungi were able to colonize the three host plant species tested and to establish the AM symbiosis. Only maize showed a positive growth response to the inoculation with both AMF (Table S1). As previously described (López-Ráez et al. 2010) tomato root colonization by *G. intraradices* was over two-fold higher than the colonization by *G. mosseae* (Fig. 1). A similar colonization pattern was observed in soybean and maize (Fig. 1), suggesting that *G.*

intraradices is a more effective root colonizer than *G. mosseae*. Mycorrhizal colonization levels for tomato and maize were similar while in soybean colonization by both AMF was about two-fold higher than in the other two host plants (Fig.1).

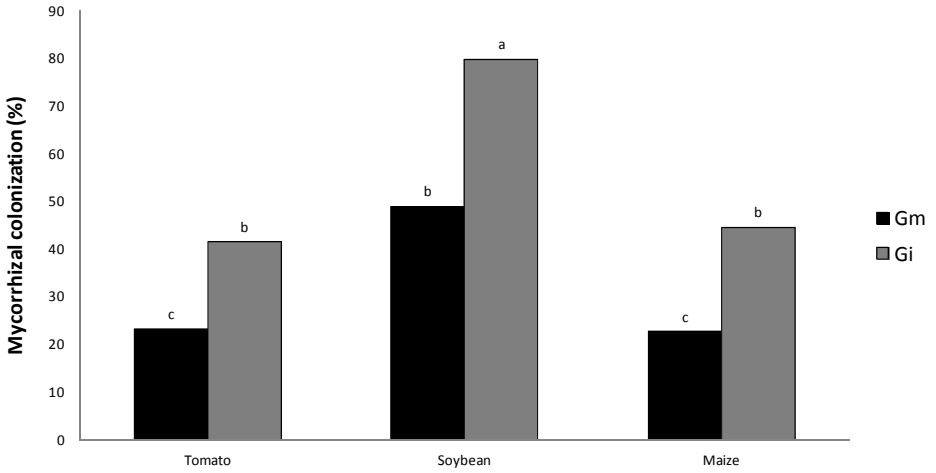

Figure 1. Quantification of mycorrhizal colonization of tomato, soybean and maize roots inoculated with *G. mosseae* (Gm) or *G. intraradices* (Gi). Data represent the means of 4 independent replicates. Data not sharing a letter in common differ significantly ($P \leq 0.05$) according to DMS's test.

Mycorrhiza associated changes related to ABA signalling

To explore the effect of AM symbiosis on ABA levels in the host plant, free ABA was quantified by UPLC-MS/MS in tomato, soybean and maize roots colonized by the AMF *G. mosseae* and *G. intraradices*. ABA content was not significantly altered in mycorrhizal tomato nor did soybean roots compare with non-mycorrhizal control plants, regardless the colonizing fungi (Fig. 2A and B). Conversely, in maize a reduction on ABA was observed in plants colonized by both *G. mosseae* and *G. intraradices* (Fig. 2C).

To further monitor changes related to ABA signalling we compared the expression of ABA biosynthetic and responsive genes in mycorrhizal and non mycorrhizal plants. Nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) catalyze the rate-limiting step in ABA biosynthesis (Taylor et al., 2005). The expression levels of *NCED1* gene were analysed by qPCR using specific primers (Table S2) in the three host plants. No changes were detected in tomato roots (Fig. 2D). In soybean, the expression of *NCED1* was significantly ($p < 0.05$) reduced by both AMF about 3-fold (Fig. 2E). In the case of maize roots, only *G. mosseae* significantly reduced the expression of *NCED1* (Fig. 2F). In

addition to the ABA-biosynthesis gene *NCED1*, the expression of the ABA-responsive genes *Le4*, *Lea* and *ABP9* for tomato, soybean and maize, respectively, were determined by qPCR. These genes encode for dehydrins, proteins known to be associated to drought stress responses (Hanin et al., 2011). As in the case of ABA content and *NCED1* expression, no changes in *Le4* expression levels were observed in tomato roots (Fig. 2G). Similarly, no significant changes were detected for the *ABP9* gene in maize (Fig. 2I). However, the expression of *Lea* in soybean was reduced in *G. mosseae*-colonized plants, while *G. intraradices* did not induce any change in its expression (Fig. 2H).

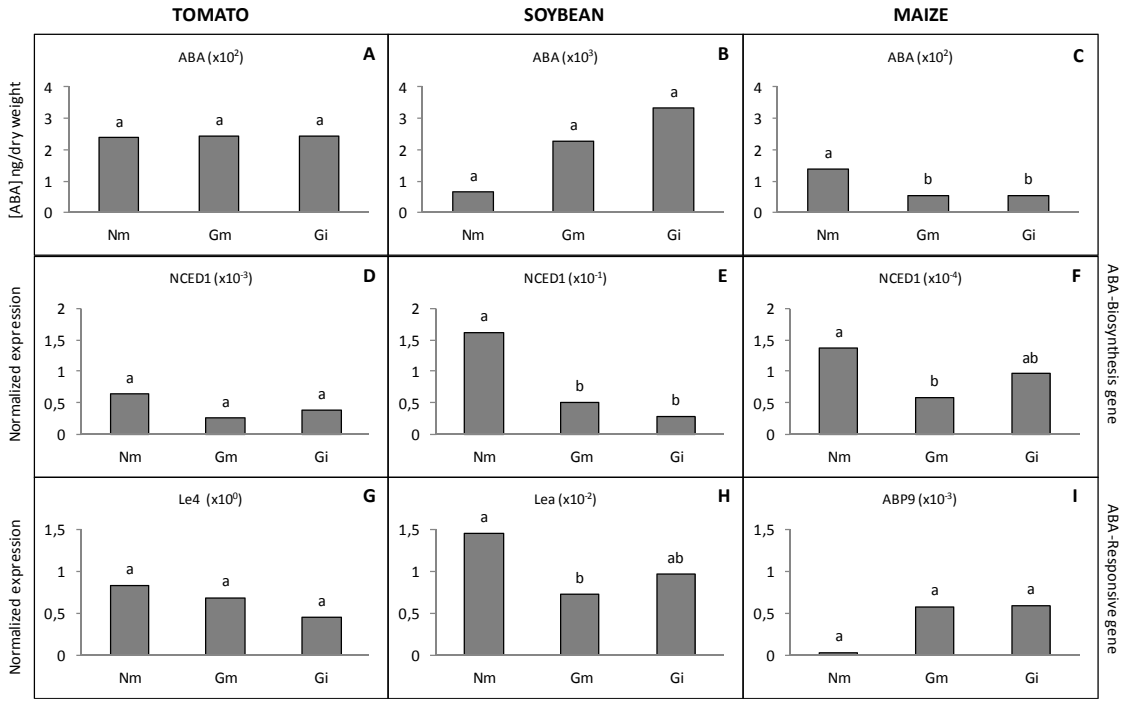

Figure 2. Effect of mycorrhizal colonization on the abscisic acid (ABA) signalling pathway in tomato, soybean and maize roots colonized by either *G. mosseae* (Gm) or *G. intraradices* (Gi). (**A,B,C**), ABA levels in roots of non mycorrhizal plants (Nm) or plants colonized by *G. mosseae* (Gm) or *G. intraradices* (Ri). (**D, E, F**), relative expression of the ABA-biosynthesis gene *NCED1* and (**G, H, I**) relative expression of the ABA-responsive genes *Le4*, *Lea* and *ABP8* in tomato, soybean and maize roots respectively. Gene expression was normalized to the expression of constitutively expressed genes selected as reference for each plant (encoding for elongation factors SlEF1, GmEF1 and ZmEF1). Data points represent the means of five (UPLC-MS/MS) or three (qPCR) independent biological replicates. Data not sharing a letter in common differ significantly ($P \leq 0.05$) according to DMS's test.

Mycorrhiza associated changes related to SA signalling

The levels of the phytohormone SA were also measured by UPLC-MS/MS in *G. mosseae* and *G. intraradices* mycorrhizal roots, in order to determine the conservation of SA-related features in tomato, soybean and maize. It was previously described that

SA concentration increased slightly in roots of tomato plants colonized by *G. mosseae* compared with non-mycorrhizal control plants (Fig. 3A). In soybean, SA levels were not altered in mycorrhizal roots (Fig. 3B). Interestingly, SA content increased in maize roots colonized by *G. intraradices* compared to non-mycorrhizal control plants, while they remained unaltered in *G. mosseae* mycorrhizal plants (Fig. 3C).

The expression of SA-biosynthesis and SA-responsive marker genes was also quantified by qPCR. SA biosynthesis in plants seems to occur through two alternative pathways involving the enzymes PAL (phenyl ammonia lyase) and ICS (isochorismate synthase). Since traditionally PAL has been considered as a marker for SA biosynthesis we used the gene encoding for PAL1 as a molecular marker. Mycorrhizal colonization did not induce changes in *PAL1* expression in any of the host plants analyzed (Fig. 3D, E and F). When analyzing the expression of the SA-responsive gen *PR-1a*, which encodes for the pathogenic related protein PR1a, a significant increase, was observed in *G. mosseae*-colonized tomato roots, with no detectable changes upon colonization with *G. intraradices* (Fig. 3G). The same expression pattern was observed in soybean, with about a 2-fold increase in plants colonized by *G. mosseae* (Fig. 3H). In contrast, only *G. intraradices* induced PR1a expression in maize roots (Fig. 3I), in agreement with the increase in SA content of these plants.

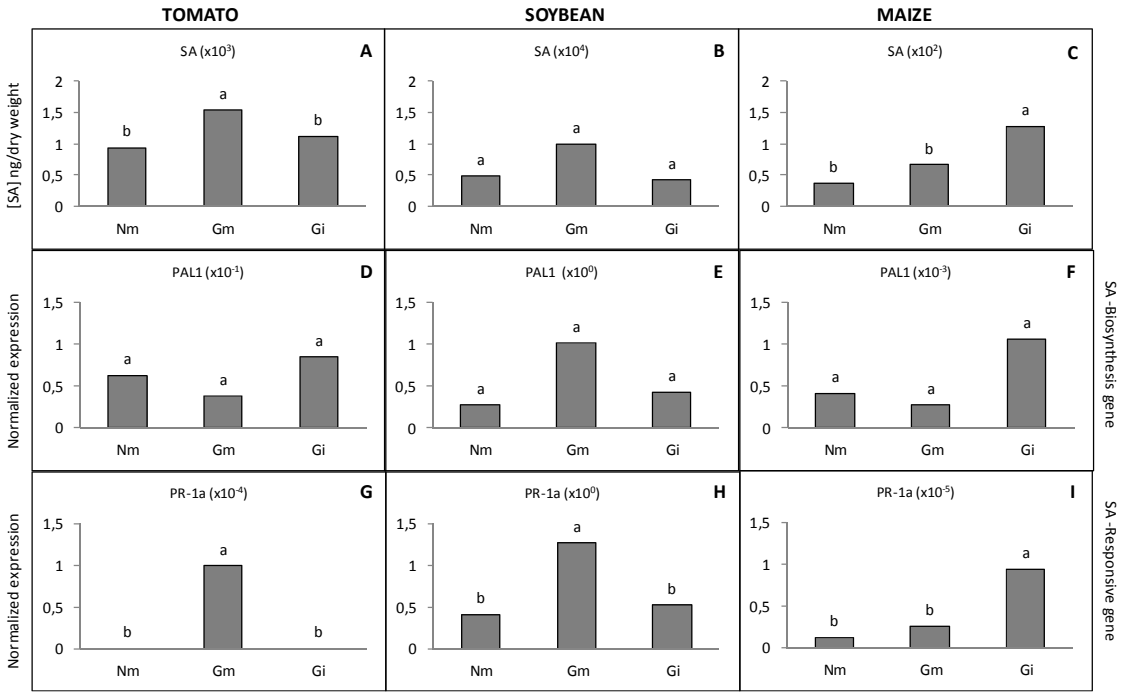

Figure 3. Effect of mycorrhizal colonization on the salicylic acid (SA) signalling pathway in tomato, soybean and maize roots colonized by either *G. mosseae* (Gm) or *G. intraradices* (Gi). **(A,B,C)** SA levels in roots of non mycorrhizal plants (Nm) or plants colonized by G. mosseae (Gm) or *G. intraradices* (Ri). **(D, E, F),** relative expression levels of *PAL1*, related to SA biosintheis and (G, H, I) relative expression of the SA-responsive *PR-1a* genes. Replication, normalization an statistical analysis as described in Figure 2.

Mycorrhiza associated changes related to JA signalling

As aforementioned, among all phytohormones, jasmonates and more generally, oxylipins, have been suggested to play a crucial role as key regulators of AM symbiosis. To study the conservation of JA signalling in mycorrhizas, the levels of different jasmonates were determined in tomato, soybean and maize colonized by either *G. mosseae* or *G. intraradices*. The metabolites analyzed were the JA precursor OPDA, free JA and the bioactive JA-derivative JA-Ile. The levels of OPDA increased significantly ($p < 0.05$) upon *G. mosseae* colonization in the three host plants (Fig. 4A, B and C). However, a different behaviour was observed in plants colonized by *G. intraradices*. In tomato *G. intraradices* induced an increase in OPDA level similar to that induced by *G. mosseae* (Fig. 4A). In contrast, OPDA levels were decreased by *G. intraradices* in soybean and not altered in maize plants (Fig. 4B and C). The content of free JA correlated with those of OPDA in soybean and maize (Fig. 4E and F) but no alteration was observed in JA concentration in mycorrhizal tomato plants (Fig. 4D). When JA-Ile was analyzed, increased levels were detected in tomato and soybean roots colonized

by *G. mosseae*, but no changes in roots colonized by *G. intraradices* were observed (Fig. 4G and H). In maize, no differences in JA-Ile were observed between mycorrhizal and non-mycorrhizal plants (Fig. 4I).

As for the previous hormones, the expression of JA-biosynthetic and JA-responsive genes was also investigated. Here, *LOXA* encoding for the lypoxygenase A was used as an oxylipin biosynthesis marker. Typical JA responsive genes are those encoding for proteinase inhibitors such as PinII (proteinase inhibitor II), R1 (a protease inhibitor) and MC (multicystatin) in tomato, soybean and maize, respectively. In the three plant species a higher expression of *LOXA* was found in roots colonized by *G. mosseae* compared to controls (Fig. 4J, K and L). In contrast, colonization by *G. intraradices* did not significantly alter its expression in any of the host plants (Fig. 4J, K and L). Root colonization by *G. mosseae* but not by *G. intraradices* enhanced the expression level of the JA-responsive marker genes in tomato and maize (Fig. 4M,N,O). Noteworthy, LoxA expression is positively regulated by JA, again supporting that JA responses are activated by *G. mosseae* and not by *G. intraradices* in all plants.

These results illustrated a common pattern of regulation of the jasmonate signalling pathway to mycorrhization that is dependent of the AM fungi involved, and which is partly conserved across the plant species tested.

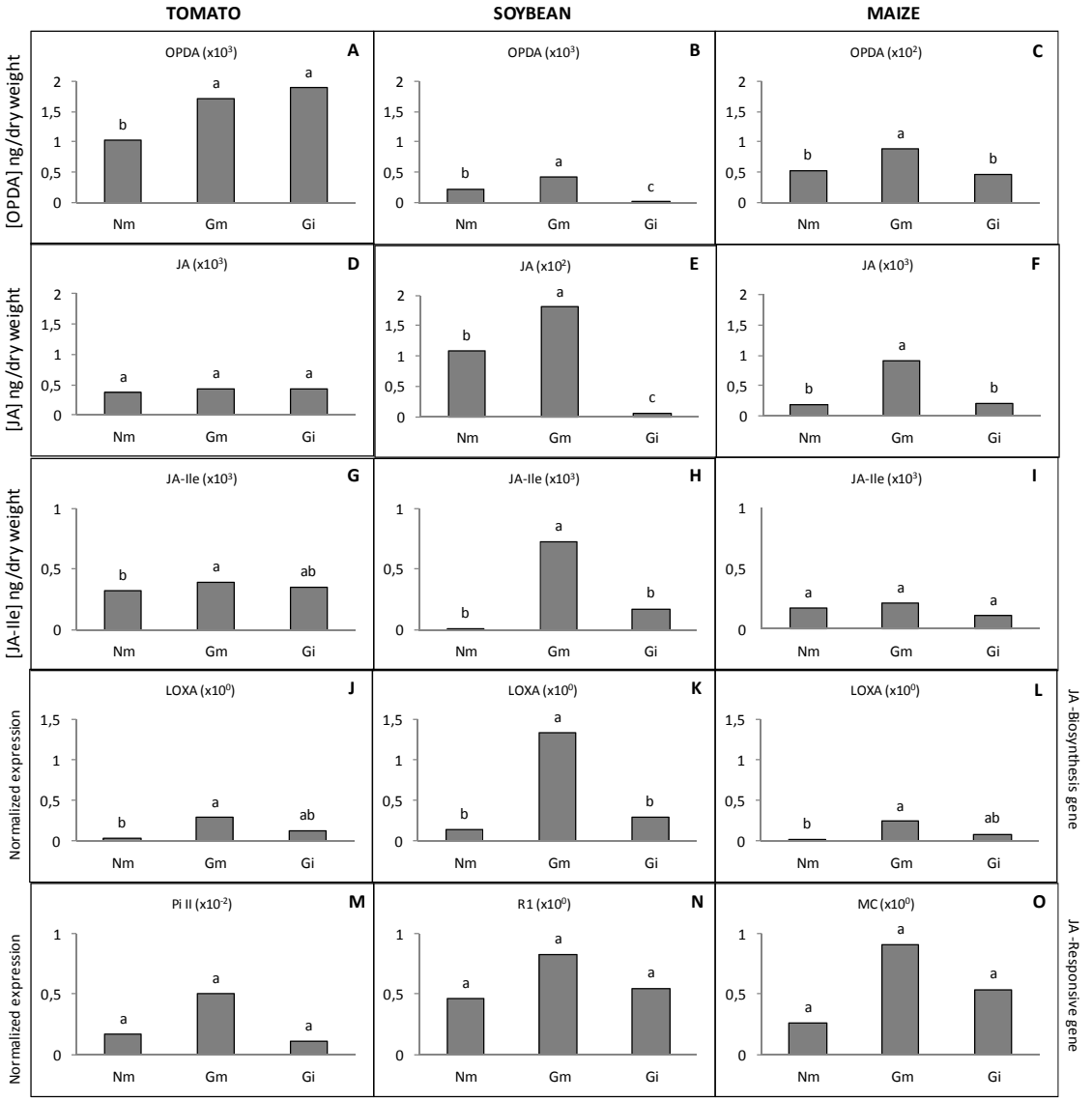

Figure 4. Effect of mycorrhizal colonization on the oxylipin/jasmonate signalling pathway in tomato, soybean and maize roots colonized by either *G. mosseae* (Gm) or *G. intraradices* (Gi). Levels of OPDA (A, B, C), free JA (D, E, F) and JA-Ile (G, H, I) in roots of non mycorrhizal plants (Nm) or plants colonized by *G. mosseae* (Gm) or *G. intraradices* (Gi). (J, K, L) relative gene expression of the oxilipin biosynthesis gene *LOXA* and (M, N, O), relative gene expression for the JA-responsive genes *PinII*, *R1* and *MC*. Replication, normalization an statistical analysis as described in Figure 2.

Global analysis of mycorrhiza associated changes in defence related signalling

The results described above illustrate differences in the impact of the symbiosis on the host hormonal profiles depending of both the host plant and the AMF. Figure 5 summarizes the results regarding the impact of root colonization of tomato, soybean and maize plants by *G. mosseae* or *G. intraradices* on the defence-related hormones ABA, SA and JAs. For each signalling pathway, three different parameters have been analyzed: the hormone content, changes in expression of biosynthesis and responsive

genes. This information is integrated in fig. 5 using a colour code in which (*green*=downregulation and *red*=upregulation) the intensity reflects the number of parameters that are altered, and if the changes in that pathway are positive or negative. The two way ANOVA analysis of the data confirmed that for ABA and JA all parameters (hormone quantification and changes in gene expression) were dependent on the plant and fungal genotypes and their interaction, while for SA the values were mostly affected by the plant (Table S3)

The overall analysis revealed a higher impact on defence related signalling of *G. mosseae* compared to *G. intraradices* in all three tested plants. The data suggest a more exhaustive control of this fungus by the host plant, which may explain the reduced colonization rate detected for *G. mosseae* when compared with *G. intraradices*. In particular, the analysis mainly reveals a negative modulation of the ABA-related pathway upon mycorrhizal colonization, which is more accused in the case of *G. mosseae*. Conversely, a mainly positive regulation of the SA- and JA-related pathways was observed. This conserved enhancement of SA and JA levels in a well established mycorrhization could modulate the plant control over AMF proliferation within the roots.

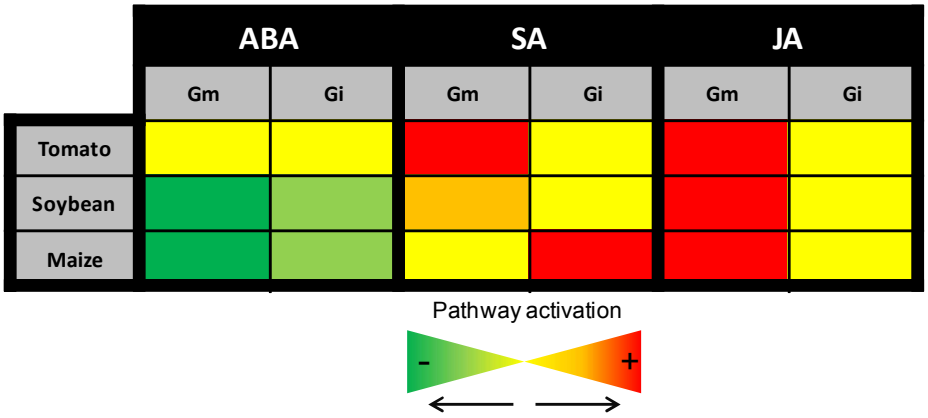

Figure 5. Summary of the changes in the different hormone-related pathways induced in the host plants by the AMF *G. mosseae* and *G. intraradices*. Red indicates up-regulation and green indicates down-regulation of the different hormonal pathways. Changes only in one of the parameters analyzed (hormone content or expression level of marker genes) are indicated in orange when up-regulated and light green when down-regulated. Yellow indicates no changes respect non-mycorrhizal control plants.

Cell specific analysis of gene expression

Regulation of gene expression in arbusculated cells

Mycorrhization is a highly dynamic and asynchronous process within the same root. Laser capture microdissection (LCM) offers an effective way to monitor gene expression in individual cells (Balestrini et al., 2009; Guether et al., 2009; Hogekamp et al., 2011). Therefore, LCM was used to analyze local and systemic changes in gene expression of different hormone-related marker genes in the root cortex of mycorrhizal and non-mycorrhizal tomato plants (Fig. 6). Absence of DNA contamination was confirmed by PCR in RNA samples (data not shown). Equal loading was assessed by analysis of transcript levels of the tomato housekeeping gene *Ubiquitin* (Fig. 6). Expression of the AM fungal specific gene *G. 18S rRNA* was only detected in cells from mycorrhizal roots, and especially in arbusculated cells (Gm+) (Fig. 6). The light band detected in non-arbusculated cells from mycorrhizal roots (Gm-) indicated a limited contamination with fungal DNA, probably due to intercellular hyphae. *SlPT4* encodes a phosphate transporter specifically associated to cells with arbuscules (Gómez-Ariza et al., 2009) used as marker of a functional symbiosis. Accordingly *SlPT4* expression was only detected in Gm+ cells (Fig. 6), indicating a well established mycorrhization and confirming the discrimination between cells with and without arbuscules in our system. The expression of *Ns-Hb1,* encoding for a non-symbiotic haemoglobin and reported to be induced in early mycorrhization stages (Siciliano et al., 2007), showed no differences among the samples, probably because of the well established colonization stage.

The expression of different hormone-related marker genes was also analyzed in these samples. In relation to ABA; NCED1 transcript were not detected in any of the samples, while Le4 transcript were detected in all, with a higher expression level in mycorrhizal roots in both colonized and uncolonized cells. Concerning the SA related *PR-1a*, transcripts were only detected in cells from non-mycorrhizal plants (Nm) in contrast to the data from the whole root system analysis shown in fig. 3. Finally, the analysis of different oxylipin/JA marker genes showed their upregulation in mycorrhizal roots. The JA biosynthesis gene *AOS1* was induced in mycorrhizal roots, both in Gm+ and Gm- cells, compared to non-mycorrhizal cells (Fig. 6). The same pattern was observed for

the gene *AOS3* (Fig. 6), involved in the biosynthesis of oxylipins of the 9 LOX branch (López Raez et al., 2010). In contrast the JA-responsive gene *MC* was only detected in arbuscule containing cells (Gm+) (Fig. 6). *Lin6,* encoding for an invertase with a regulatory role in mycorrhiza, and reported to be JA inducible was induced in mycorrhizal roots in both Gm+ and Gm- cells, probably to a higher extent in arbusculated cells.

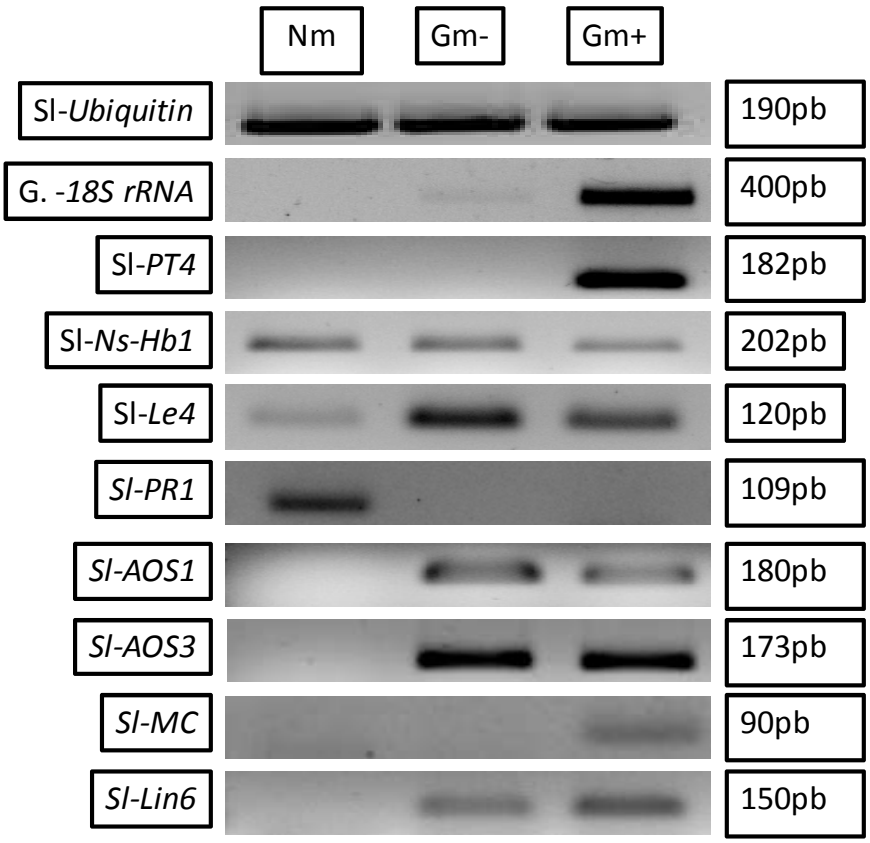

Figure 6. Detection of marker gene transcripts in laser microdissected cell types in non mycorrhizal or *F. mosseae* colonized roots. The analysis was performed by RT-PCR in three different cell types: cortical cells from non mycorrhizal controls (Nm), non colonized cortical cells of mycorrhizal roots *(Fm-)*, and arbuscule containing cortical cells (Fm+). The figure shows gel electrophoresis of the amplification products, and their corresponding size is indicated on the right. *SlUbi,* Ubiquitin, *G.18S,*18S ribosomic RNA*, Sl PT4,*Phosphate transporter4, *SlNsHb1,* non symbiotic hemoglobine*, SlLE4,* dehidrin Le4, *SlPR1,* pathogenesis related 1, *SlAOS1,* allene oxide synthase 1*, SlAOS3,* allene oxydase synthase 3*, SlMC,* multicistatin*, SlLin6* extracellular invertase 6.

Discussion

AM establishment alters the host plant physiology usually leading to benefits that go beyond increased nutrient acquisition. Among them, enhanced resistance to abiotic and biotic stresses is of major importance in natural environments, and may contribute to the evolutionary persistence of AM symbioses (Smith et al., 2010). Some of these

changes may be related to altered hormone levels in the host plant (Pozo and Azcón Aguilar, 2007; Ruiz-Lozano et al., 2012). Moreover, most of the benefits reported for mycorrhizas are dependent on a well established and balanced association (Slezack et al., 2000), and accordingly, control of the symbiosis is of vital importance to keep the interaction on mutualistic terms. Important efforts have been made to unravel the role of plant hormones in the establishment and functioning of AM interactions. Metabolomic and transcriptomic analyses combined with farmacological and genetic approaches have yield abundant information in recent years (Hause et al., 2007). The data are, however, often fragmented and contradictory, likely because the results are dependent on the plant and fungal genotypes involved, stage of the symbiosis, environmental conditions and methods applied. This work aims to analyse the main changes is stress related hormones associated to a well established association in different AMF- host combinations using high resolution techniques (UPLC-MS and qRT-PCR) in order to identify common features among different mycorrhizal systems.

Despite the high promiscuity of AMF species, able to form a successful symbiosis with most plant hosts, a high functional diversity among different combinations have been reported in terms of the morphology, efficiency and gene expression patterns of the symbiosis (Feddermann et al., 2010). The two AMF species used in this study, *G. mosseae* and *G. intraradices,* -formerly *Funneliformis mosseae* and *Rhizofagous irregularis*- are the most common AMF used in functional and biodiversity studies and they are present across drastically different environments (Smith and Read, 1997). A different level of mycorrhizal colonization was observed when comparing both fungi, *G. intraradices* being the most effective colonizer in the three plants. Differences in the colonization by both AMF have been previously shown in several plant species (Pozo et al., 2002; Feddermann et al., 2008; López-Ráez et al., 2010; Nishida et al., 2010; Martínez-Medina et al., 2009). The plants tested also differed in their root colonization levels, soybean reaching higher levels than maize and tomato. As reported in other systems, there was no correlation between colonization level and effects on plant growth, since only maize showed a positive growth response to the colonization.

The quantification of the defence-related hormones ABA, SA and JA, and the analysis of marker genes of the pathways they regulate, revealed significant changes in the

roots of mycorrhizal plants compared to controls, depending on the AMF and/or the plant involved.

Regardless of the AMF involved, the ABA-pathway appeared unaltered (in tomato) or repressed (soybean and maize) upon mycorrhization. Similarly, previous studies have reported no alteration or repression of the ABA-related pathway in mycorrhizal roots (Lopez-Raez et al., 2010; Aroca et al., 2008; Aroca et al. 2013) and leaves (Fiorilli et al., 2009; Asensio et al., 2012). However, increases in root ABA content has also been reported (Danneberg et al., 1992; Meixner et al., 2005). It should be noted that a local increase of ABA could be, at least in part, the result of the synthesis of this hormone by the AM fungus (Esch et al., 1994). Differential transcriptional regulation of genes involved in ABA biosynthesis or responses have also been reported. Interestingly, Meixner *et al*. (2005) using an split-root system showed a local increase on ABA content in AMF colonized *Medicago* roots, but a decrease in the systemic, non colonized parts of the root system. Here, despite of the lack of changes observed in mycorrhizal tomato considering the complete root system, an increase in LE4 expression, an ABA responsive gene, is observed in cortical cells of mycorrhizal roots.

Abscisic acid is known to be a key signal transduction pathway in plant responses to stresses (Hirayama and Shinozaki, 2007) therefore a reduction of ABA in plants may reflect the consequence of mycorrhiza on improved plant fitness (Barker and Tagu, 2000; Ruiz-Lozano, 2003). However, ABA has been shown to play an important role in the development of the arbuscules and their functionality (Herrera-Medina et al. 2007) and may be important in precolonization signalling through the regulation of strigolactone biosynthesis (López-Ráez et al., 2010). Moreover, ABA is able to modulate other defence related pathways through positive and negative interactions. Accordingly, the role of ABA in regulating mycorrhiza development may depend on its crosstalk with the salicylic acid/jasmonate/ethylene pathways (Herrera-Medina et al., 2007; Martín-Rodriguez et al. 2011). The results suggest that mycorrhizal plants have to finely regulate ABA levels to promote colonization and arbuscule development to an optimal level, and at the same time, to efficiently regulate stress responses. In this sense, AM plants have been reported to regulate better and faster their ABA levels than non-AM plants in response to abiotic stresses, allowing a more adequate defence response (Aroca et al. 2008b). Recently it was shown that mycorrhizal lettuce had

lower ABA content in compared to controls in the absence of stress, but this reduction was lost under high salinity conditions where ABA may play a role in the regulation of the stress response (Aroca et al., 2013).

In contrast, the two AMF had different influence on the levels of SA and its related marker genes *PR1* and *PAL*. In one hand, *G. mosseae* induced the SA-related pathway in tomato, while *G. intraradices* had no effect. In contrast, in maize the SA pathway was induced exclusively by *G. intraradices* root colonization. Remarkably no SA related changes were observed in soybean, the plant with higher AM colonization. Salicylic acid is a key phytohormone in the regulation of defence responses against biotrophs (Glazebrook, 2005), and have been assumed to have a negative effect on AM colonization (Herrera Medina et al., 2003; García-Garrido and Ocampo 2002; Gutjahr and Paszkowski, 2009). Indeed, an inverse correlation between SA levels and AM colonization was found in pea and tobacco (Blilou et al., 1999; Herrera-Medina et al., 2003) and enhanced SA levels have been associated to a delay in root colonization, but without affecting the maximal degree of colonization (Herrera-Medina et al., 2003). Thus, enhanced SA levels in the roots could mediate plant control over AMF proliferation, and therefore spatial and temporal regulation of SA within the roots is probably required following the dynamics of the association. Transient increases of SA have been related to the response to hyphopodia formation on the root surface (Blilou et al., 1999, 2000). This weak activation of SA related pathway might be part of the very early plant host immune response to the invading fungus (Zamidious and Pieterse, 2012). The immune response has to be attenuated to enable the accommodation of the fungus within the root tissues, and fungal effectors involved in such suppression have been indentified in *G. intraradices* (Kloppholz et al., 2011). Despite of the presumed decrease in SA signaling in late stages of the association, we report here increases in SA associated to well established symbioses in tomato and maize. Similar increases have been reported in *F. mosseae* colonized clover (Zhag et al., 2013) and barley (Khaosaad et al., 2007). Gallou et al. (2010, 2012) monitored the expression of PR1a in potato roots and showed a very early increase in presymbiotic stages that quickly levelled off as the colonization starts, and then a second induction at later stages of root colonization when arbuscules and vesicles are already present.

Here we show that in tomato, although up regulation of SA was detected in the global analysis of *G. mosseae* colonized roots, PR1 expression was repressed in cortical cells of mycorrhizal roots, suggesting a cell type dependent regulation. As the mycorrhizal development is an asynchronous process, and the activation of some plant defences during AMF colonization seems to occur at the cellular level, probably different regulation patterns may coexist in time in different parts of the root system. Our results suggest that the induction of the SA-related pathway maybe associated with new colonization units in response to appressoria formation and attempts of penetration, and a local repression of the SA-dependent responses in the colonized areas in order to allow a compatible interaction. The results here also support a different dynamic in SA regulation in mycorrhizal roots dependent on the plant and fungus.

The effect of AM symbiosis on JA-signalling pathway showed a conserved pattern among the plant species analyzed, but it was clearly dependent on the colonizing AMF. Root colonization by *G. mosseae* lead to an increase of JA-related compounds and the up-regulation of JA related genes in all, tomato, soybean and maize plants. JA is known to be important in diverse plant processes, as development and plant–microbe interactions (Pozo et al., 2004; Wasternack 2007). Although the role of this phytohormone in AM interactions has not been fully elucidated, the importance of jasmonates as regulators of AM has been evidenced in diverse plant model species (Reviewed in Hause and Schaarschmidt, 2009). An increased level of JA-related compounds was observed in roots of barley (Hause et al., 2002), *Glicine max* (Meixner et al., 2005) or *Medicago truncatula* (Stumpe et al., 2005) among others, upon mycorrhiza establishment. Our results showed an increase of OPDA and in the JA derivative JA–Ile in tomato *G. mosseae* mycorrhizal plants, but not accompanied in free JA (López-Ráez et al. 2010). In contrast, maize plants increase the level of OPDA and free JA upon *G. mosseae* mycorrhization, but no changes were observed in JA–Ile. In addition, soybean plants showed higher levels of the three JA-related compounds. Since the different JA-related compounds may have different functions, this different accumulation pattern may indicate different roles in orchestrating the plant response to mycorrhizal fungi, according to the host plant species.

Interestingly, no major alterations in JA-signalling were observed in *G. intraradices* mycorrhizal plants (with higher colonization levels), compared to non-mycorrhizal plants, independently of the plant species. Similarly, no changes in JA content have been described in *G. intraradices* colonized *Nicotiana* roots (Riedel et al., 2008). These observations demonstrate a strong dependence of the AMF genotype on the modulation of the JA-signalling pathway, and hence on the defence related response. It has been recently speculated that the role of jasmonate in symbiotic interactions depends on the degree of mutualism in the plant–microbe interaction (Hause, 2009). Our results showed however, that the modulation of the JA-regulated signalling pathway depends on the fungal genotype, maybe in relation to its colonization dynamics or its capacity to suppress the plant immune response.

JA biosynthetic genes have been reported to be induced in arbuscule containing cells (Hause et al., 2002). Here, the analysis of the allene oxydase synthase encoding genes *AOS1* and *AOS3* at the cellular level in *G. mosseae* colonized tomato roots revealed an induction in arbuscule containing and non-containing cortical cells of mycorrhizal roots compared to those from non mycorrhizal roots. However, the induction of the JA-responsive marker genes *MC* and *Lin6* was observed only in arbuscule-containing cells, supporting a spatial expression pattern of JA responsive genes. MC codes for a multicistatin involved in plant defence responses, and *Lin6* encodes for an extracellular invertase induced in mycorrhizal roots with an important role in the symbiosis. Both functions, defence and carbon metabolism have been pointed as the major functions of JAs in the symbiosis (Hause et al., 2007). To this regard, JA seems to contribute to the regulation of colonization, positively, promoting accomodation of the fungus and, negatively, restricting colonization (Hause et al., 2007, Gutjahr and Paszkowski 2009), and seem to contribute to increasing the sink strength of mycorrhizal roots for carbohydrates to support fungal growth (Schaarschmidt et al., 2006). Besides that, they have been propose to contribute to the reorganization of the cytoskeleton needed for fungal accommodation (Genre and Bonfante, 2002; Genre et al., 2005). The multifunctionality of these hormones, the existence of different active forms and the spatial and temporal regulation of genes related to JA and other oxilipins (Hause et al., 2002; Gallou et al., 2012; León Morcillo et al., 2012) suggest the need of a precise control of the JA related homeostasis for correct functionality of the symbiosis.

In summary, we found that *G. intraradices* showed higher root colonization rates in all plants tested, but the changes in stress related signalling in the host roots were lower than in *G. mosseae* colonized roots. This finding may suggest a lower control of the plant over *G. intraradices* colonization, maybe related to the fungal ability to suppress the plant defence response to achieve a higher root invasion. Indeed it has been recently reported that *G. intraradices* can deliver the effectors protein SP7, which attenuates the plant immune response to enable the accommodation of the fungus within plant roots (Kloppholz et al., 2011). How conserved and efficient is this mechanism in other AM fungi remains to be determined. It might be that these differences in the modulation of host defence system also contribute to the different capability of different mycorrhizal fungi to induce systemic resistance and higher bioprotection ability observed by *G. mosseae* compared to *G. intraradices* (Pozo et al., 2002).

Hormonal balance is highly important for the coordinated defense response, leading to eventually to AM formation. Our results provide an overview on the changes related to stress related hormones in a well established mycorrhizal symbiosis, confirming that the changes depend on the plant and fungal partners involved. To understand the role of these signaling pathways in the control and regulation of the symbiosis hormone measuring should be performed at different stages of the interaction. Probably the data showed here will be very different in early stages of the association, thus time course studies are required. Finally, the changes revealed here are related to a well established association, and may therefore underlie the impact of the symbiosis on plant response to stresses, since these hormones are central to defense responses to biotic and abiotic stresses (Pineda et al., 2013). The differences in the changes triggered by the different AMF may give hints for the expected effect of each interaction to specific stresses, and this knowledge may pave the way to a more rational selection of the appropriate AMF for specific problems.

SUPPLEMENTARY DATA

Table S1. Root fresh weights in tomato plants, soybean and maize colonized by *G. mosseae* (Gm) or *G. intraradices* (Gi). Nm indicates control non-mycorrhizal plants.

Root fresh weight (g)			
	Tomato	**Soybean**	**Maize**
Nm	**2.07±0.26 a**	**2.08±0.17 a**	**6.24±0.38 b**
Gm	**1.42±0.06 b**	**2.04±0.12 a**	**8.64±0.37 a**
Gi	**2.40±0.21 a**	**1.74±0.13 a**	**6.58±0.16 b**

Numbers represent the average of 5 independent replicates ± SE. Different letters indicate statistically significant differences between means ($P < 0.05$).

Table S2. Primers used for gene expression analysis in this study.

ID	Tomato Gene	Primers (5'-3')
X14449	Elongation factor 1α (Sl-*EF1*)[4]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
Z97215	9-Cis-expoxycarotenoid dioxygenase (Sl-*NCED1*)[2]	ACCCACGAGTCCAGATTTC GGTTCAAAAAGAGGGTTAGC
X51904	Desiccation protective protein (Sl-*Le4*)[2]	ACTCAAGGCATGGGTACTGG CCTTCTTTCTCCTCCCACCT
M83314	Phenylalanine ammonia lyase (Sl-*PAL1*)[1]	CGTTATGCTCTCCGAACATC GAAGTTGCCACCATGTAAGG
M69247	Pathogenesis related protein (Sl-*PR-1a*)[2]	GTGGGATCGGATTGATATCCT CCTAAGCCACGATACCATGAA
U09026	Lipoxygenase A (Sl-*LOXA*)[2]	GGTTACCTCCCAAATCGTCC TGTTTGTAACTGCGCTGTG
K03291	Proteinase inhibitor II (Sl-*Pi-II*)[3]	GAAAATCGTTAATTTATCCCAC ACATACAAACTTTCCATCTTTA
X58253	Ubiquitin (Sl-*Ubiquitin*)[1]	ACCAAGCCAAAGAAGATCAAGC GTGAGCCCACACTTACCACAGT
AY885651	Phosphate transporter (Sl-*PT4*)[5]	GAAGGGGAGCCATTTAATGTGG ATCGCGGCTTGTTTAGCATTTC
HE817883.1	*Glomus intraradices* (G.-*18S rARN*)[6]	TGTTAATAAAAATCGGTGCGTTGC AAAACGCAAATGATCAACCGGAC
AJ271093	Allene oxide synthase (Sl-*AOS1*)[2]	CACCTGTTAAACAAGCGAAAC TCCAGCTCGTTCACCTTCTT
AF454634	Allene oxide synthase (Sl-*AOS3*)[2]	GCGGAGGAGTTCAATCCAG CGCATGAAAAACTCCACAACC
AF083253	Multicystatin (Sl-*MC*)[3]	GAGAATTTCAAGGAAGTTCAA GGCTTTATTTCACACAGAGATA
AF506005.1	Cell-wall Invertase (Sl-*LIN6*)[1]	AGCACATTTATTCGCCTTCAACAA TTTGTGACGTGGCATAATAAGAT

[1]This work; [2]Lopez-Raez et al., 2010; [3]Uppalapati *et al.*, 2005; [4]Rotenberg *et al.*, 2006; [5]Balestrini et al., 2007; [6]Porcel et al., 2005.

ID	Soybean Gene	Primers (5'-3')
Glyma02g44460	Elongation factor 1β (Gm-*EF1*)[3]	AAGGGAGGCTGCTAAAAAGC CAACTGTCAAGCGTTCCTCA
Z97215	9-Cis-expoxycarotenoid dioxygenase (Gm-*NCED1*)[1]	GGATCGATGCTCCTGAATGT TTTCGTCCGAGCTTGTTTCT
NM_001248671	Desiccation protective protein (Gm-*Lea*)[6]	GTCGACGAGATACGGCTAACCC CCAGGGCAAGCTTTCTCCTTT
X52953	Phenylalanine ammonia lyase (Gm-*PAL1*)[1]	CCAAGGAACCCCTATTGGAGCT GTGTTGCTCAGCACTTTGGA
NM_001251239	Pathogenesis related protein (Gm-*PR-1α*)[4]	ATGTGTGTGTTGGGGTTGGT ACTTTGGCACATCCAAGACG
AI443430	Lipoxygenase A (Gm-*LOXA*)[2]	CCGAGAGCATCCAAATACAA GCTCTATTATCGTTTGGACA
TC407960	Proteinase inhibitor (Gm-*R1*)[1]	TTCAGGAATTCAAGCCCATC CCCCACACCGTTTGTAGAGT

[1]This work; [2]Pat Moy et al., 2004; [3]Yamaguchi et al., 2011; [4] de Roman et al., 2011; [5] Porcel et al., 2005.

ID	Soybean Gene	Primers (5'-3')
Nm_001112117	Elongation factor 1α (Zm-*EF1*)[1]	TGTTGCTGTGAAGGATCTGAA ATGATGATGACCTGGGAGG
ZMU95953	9-Cis-expoxycarotenoid dioxygenase (Zm-*NCED1*)[2]	AGTTGTTGTCACCCAGTCCAG CACGCACCGATAGCCACA
GU237073.1	Desiccation protective protein (Zm-*ABP9*)[1]	TCCAGTCCGCTTGCTTAGTT GTTTGTACAACCCAGGCTCA
L77912.1	Phenylalanine ammonia lyase (Zm-*PAL1*)[1]	AGTACACGGACCACCTGACC TCCAGCTCGTTCACCTTCTT
NM_001159109.1	Pathogenesis related protein (Zm-*PR-1a*)[1]	AGAACCTCTTCTGGGGCAGT GGGTCGTAGTTGAGGTGAT
NM_001112510.1	Lipoxygenase A (Zm-*LOXA*)[1]	ATCCTCAGCATGCATTAGTCC AGTCTCAAACGTGCCTCTTGTT
NM_001158213.1	Multicystatin (Zm-*MC*)[1]	AATCTGAACCCGAGCCTTCT GGAAAGCCCATCATTCTCAA

[1]This work; [2]Capelle et al., 2010.

Table S3. The two-way (fungus and plant) ANOVA analysis for all hormones were analysed (*P*-values).

	***P*-value**										
	ABA			SA			JA				
	[ABA]	B-Gene	R-Gene	[SA]	B-Gene	R-Gene	[OPDA]	[JA]	[JA-Ile]	B-Gene	R-Gene
Plant	**0,000**	**0,000**	**0,000**	**0,013**	**0,018**	**0,000**	**0,000**	0,122	0,381	**0,000**	**0,001**
Fungus	**0,046**	**0.002**	0,116	0,294	0,461	0,054	**0,000**	**0,000**	0,093	**0,000**	0,109
Plant x Fungus	**0,011**	**0,000**	0,091	0,426	0,467	**0,029**	**0,000**	**0,000**	0,117	**0,001**	0,566

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CAPÍTULO 2: Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway

Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway

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RESUMEN.

La simbiosis micorrízica arbuscular es una asociación mutualista entre los hongos del suelo y la mayoría de las plantas terrestre. Esta simbiosis afecta significativamente a la fisiología de la planta hospedadora en términos de nutrición y resistencia a diferentes estreses. A pesar de la falta de especificidad de la interacción con respecto al rango del hospedador, existe una diversidad funcional entre las distintas especies de hongos micorrízicos arbusculares. Por tanto la interacción se encuentra finamente regulada dependiendo del caracterer del hongo y de la planta. Determinadas hormonas vegetales parecen orquestar las modificaciones que ocurren en la planta hospedadora durante la simbiosis. Usando plantas de tomate como modelo, se realizó un análisis global de la respuesta de la planta hospedadora tras la colonización por diferentes hongos micorrízicos, combinando múltiples análisis hormonales y transcripcionales. El análisis de algunas de las hormonas vegetales etileno, ácido abscísico, ácido salicílico y compuestos relacionados con el jasmonato, mostró respuestas comunes y divergentes en raíces de tomate frente a los hongos micorrízicos *Glomus mosseae* y *Glomus intraradices*. Estos hongos presentaron diferentes patrones de colonización y por tanto provocaron diferentes respuestas en la planta hospedadora. Los análisis hormonales y transcripcionales revelaron la regulación de la ruta de las oxilipinas durante la simbiosis micorrízica arbuscular, indicando un papel clave de los jasmonatos en la regulación de esta simbiosis. Además, los resultados sugieren que las respuestas específicas de la planta hospedadora frente a diferentes hongos micorrízicos arbusculares, podrían ser responsables de las diferencias en la modulación de la fisiología de la planta tras la colonización.

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Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway

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Abstract

Arbuscular mycorrhizal (AM) symbioses are mutualistic associations between soil fungi and most vascular plants. The symbiosis significantly affects the host physiology in terms of nutrition and stress resistance. Despite the lack of host range specificity of the interaction, functional diversity between AM fungal species exists. The interaction is finely regulated according to plant and fungal characters, and plant hormones are believed to orchestrate the modifications in the host plant. Using tomato as a model, an integrative analysis of the host response to different mycorrhizal fungi was performed combining multiple hormone determination and transcriptional profiling. Analysis of ethylene-, abscisic acid-, salicylic acid-, and jasmonate-related compounds evidenced common and divergent responses of tomato roots to *Glomus mosseae* and *Glomus intraradices*, two fungi differing in their colonization abilities and impact on the host. Both hormonal and transcriptional analyses revealed, among others, regulation of the oxylipin pathway during the AM symbiosis and point to a key regulatory role for jasmonates. In addition, the results suggest that specific responses to particular fungi underlie the differential impact of individual AM fungi on plant physiology, and particularly on its ability to cope with biotic stresses.

Key words: Arbuscular mycorrhiza, hormones, jasmonates, LC-MC/MC, mycroarrays, oxylipins.

Introduction

About 80% of all terrestrial plants, including most agricultural and horticultural crop species, are able to establish mutualistic associations with soil fungi from the phylum Glomeromycota (Smith and Read, 2008). The resulting symbiosis is known as arbuscular mycorrhiza (AM) and is widely distributed throughout the world. This association is considered to be older than 400 million years and a key step in the evolution of terrestrial plants (Smith and Read, 2008). Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that colonize the root cortex of the host plant inter- and intracellularly forming specialized and highly branched structures called arbuscules (Parniske, 2008). The fungus obtains carbohydrates from the host plant and, in return, they assist the plant in the acquisition of mineral nutrients (mainly phosphorus) and water. AM symbiosis is maintained throughout the life of the plant, affecting its physiology significantly (Parniske, 2008). Accordingly, the symbiosis not only influences plant nutrition, but also impacts the plant's ability to overcome biotic and abiotic stresses (Pozo and Azcón-Aguilar, 2007). While AMF are considered non-specific with respect to host range, there are differences in their growth patterns within the roots, functionality, and with regards to their effects on plant nutrition and resistance to stress (Cavagnaro et al., 2001; Pozo et al., 2002; Smith et al., 2004).

AM establishment and functioning require a high degree of coordination between the two partners based on a finely regulated molecular dialogue that orchestrates complex symbiotic programmes (Paszkowski, 2006; Hause et al., 2007; Requena et al., 2007). Upon recognition of the fungal partner, the plant actively accommodates the fungus in the root tissue (Genre et al., 2008) and controls its proliferation, which implies an important transcriptional reprogramming in the plant. Understanding the molecular basis of the AM symbiosis is an ongoing challenge, and, so far, some plant genes specifically associated with the establishment and development of the symbiosis have been identified in different plant species, mostly in legumes (Grunwald et al., 2004; Hohnjec et al., 2005; Liu et al., 2007; Siciliano et al., 2007; Guether et al., 2009).

Some of the changes in the host are related to modifications in the relative abundance of plant hormones, most of which are thought to play a role in the symbiosis (Hause et al., 2007). Among plant hormones, ethylene (ET), salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) are known to be key elements in fine-tuning the plant defence response during interaction with other organisms (Pieterse et al., 2009). In the case of the interaction with AMF, it is accepted that there is an inverse correlation between root colonization and the levels of ET and SA (Blilou et al., 1999; Herrera-Medina et al., 2003, 2007; Riedel et al., 2008). Conversely, a positive correlation for ABA and mycorrhizal establishment has been evidenced (Herrera-Medina et al., 2007). JA and its derivatives, known as jasmonates, have received special attention since they are believed to play a major role in the AM symbiosis. However, experimental data are highly controversial (Gutjahr and Paszkowski, 2009; Hause and Schaarschmidt, 2009). Increased JA levels in mycorrhizal roots compared with non-mycorrhizal controls have been described in *Medicago truncatula* (Hause et al., 2002; Meixner et al., 2005), while they remained unaltered in *Nicotiana attenuata* (Riedel et al., 2008). In addition, studies using reverse genetics approaches with plant mutants affected in JA biosynthesis or signalling have shown positive and negative regulatory roles of the JA pathway in the symbiosis (Isayenkov et al., 2005; Herrera-Medina et al., 2008; Tejeda-Sartorius et al., 2008). Besides the use of different plant and experimental systems, these controversies might be partly due to the overlapping yet distinct signalling activities of its precursor oxo-phytodienoic acid (OPDA) and jasmonate derivatives such as the isoleucine conjugate JA–Ile (Stintzi et al., 2001; Taki et al., 2005; Wang et al., 2008). Moreover, jasmonates belong to a diverse class of lipid metabolites known as oxylipins that include other biologically active molecules (Wasternack, 2007; Mosblech et al., 2009).

In the present study, the agriculturally and economical important crop tomato has been used as a model system to carry out integrative analysis of the transcriptional and metabolic changes that take place during AM symbiosis. The plant response to two related AMF, *Glomus mosseae* and *Glomus intraradices* that showed in previous studies different colonization patterns and functionality was compared in an attempt to provide insights into the common and differential host responses to AMF. The effects of AMF colonization on the content of ABA, SA, ET, and different JA-related

compounds in the host plant were assessed, and correlated with the modifications in their transcriptional profiles. The results provide original insights into our understanding of the AM symbiosis and its impact on plant physiology, and they pave the way for further analyses of the regulatory network controlling this association.

Materials and Methods

Plant growth, AM inoculation, and chemical treatments

The AMF *G. mosseae* (BEG 12) and *G. intraradices* (BEG 121) were maintained as a soil-sand-based inoculum. Tomato seeds (*Solanum lycopersicum* L. cv. MoneyMaker) were surface sterilized in 4% sodium hypochlorite containing 0.02% (v/v) Tween-20, rinsed thoroughly with sterile water and germinated for 3 d in a container with sterile vermiculite at 25 ºC in darkness. Subsequently, individual seedlings were transferred to 0.25 l pots with a sterile sand:soil (4:1) mixture. Pots were inoculated by adding 10% (v:v) *G. mosseae* or *G. intraradices* inoculum. The same amount of soil:sand mix but free from AMF was added to control plants. All plants received an aliquot of a filtrate (<20 µm) of both AM inocula to homogenize the microbial populations. For each treatment, a total of nine plants were used. Plants were randomly distributed and grown in a greenhouse at 24/16 ºC with a 16/8 h photoperiod and 70% humidity, and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration. Plants were harvested after 9 weeks of growth, and the fresh weight of shoots and roots was determined. An aliquot of each individual root system was reserved for mycorrhizal quantification. For microarray and hormone analyses, three pools each consisting of roots from three independent plants were used.

For methyl jasmonate (MeJA) treatment tomato plants were grown hydroponically in 3.0 l plastic containers with Long Ashton nutrient solution containing 25% of the standard phosphorus concentration and with constant aeration. The nutrient solution was replaced once a week. Four-week-old plants were individually transferred to 50 ml plastic tubes filled with nutrient solution with or without 50 µM MeJA (Sigma-Aldrich) and maintained for 24 h. Then, the roots were rinsed with sterilized deionized water and stored at -80 ºC until use.

Mycorrhizal colonization determination

Roots were stained with trypan blue (Phillips and Hayman, 1970) and examined using a Nikon Eclipse 50i microscope and bright-field conditions. The percentage of total root colonization and frequency of intraradical fungal structures, arbuscules, and vesicles was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

Phosphorus content

The total phosphorus content of the leaves was measured at the CEBAS-CSIC (Spain). Shoots were briefly rinsed with deionized water and mature leaves were oven-dried at 60 ºC for 72 h, weighed, and ground to a fine powder. Then, samples were extracted with deionized water. Tissue phosphorus concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Iris Intrepid II, Thermo Electron Corporation) after acid digestion. Three biological replicates each consisting of a pool of leaves from three independent plants were measured for each treatment.

Hormone quantification

OPDA, JA, JA–Ile, ABA, and SA were analysed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) as described by Flors et al. (2008). A 50 mg aliquot of dry tissue was used per sample. A mixture of internal standards containing 100 ng of [$^2H_6$]ABA, 100 ng of dihydrojasmonic acid, 100 ng of prostaglandin B1, and 100 ng of [$^2H_5$]SA was added to each sample prior to extraction. Individual calibration curves for each tested compound and internal standard were performed before the analysis. The tissue was immediately homogenized in 2.5 ml of ultra pure water and centrifuged at 5000 g for 40 min. Then, the supernatant was acidified and partitioned against diethyl-ether, dried, and resuspended in 1 ml of water/methanol (90:10, v/v). A 20 µl aliquot of this solution was injected into a Waters Acquity UPLC system (Waters). The UPLC was interfaced into a triple quadrupole tandem mass spectrometer (TQD, Waters). LC separation was performed using an Acquity UPLC BEH C_{18} analytical column (Waters) at a flow rate of 300 µl min^{-1}.

Quantifications were carried out with MassLynx 4.1 software (Waters) using the internal standards as a reference for extraction recovery and the standard curves as quantifiers.

Ethylene release was determined by gas chromatography–mass spectrometry (GC-MS). Excised roots were placed on wet filter paper in a 90 mm diameter Petri dish, sealed with sticky tape and a rubber stopper on the top, and incubated for 1 h at room temperature. A 1 ml aliquot of headspace gas per plate was sampled with a syringe, and ET was measured in a Hewlett Packard 5890 gas chromatograph fitted with a flame ionization detector (FID). Analyses were carried out at 65 ºC with the injector and FID held at 120 ºC and 105 ºC, respectively. Five independent replicates per treatment were measured.

RNA isolation

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel), and stored at -80 ºC until use.

Microarray hybridization and data analysis

The Affymetrix GeneChip Tomato Genome Array (Affymetrix) was used. A 5 µg aliquot of total RNA was used as starting material. cDNA synthesis, cRNA production, and fragmentation were carried out as described in the Expression Analysis Technical Manual (Affymetrix). The GeneChip Arrays were hybridized, stained, washed, and screened according to the manufacturer's protocol at the Unidad de Genómica of the Universidad Complutense de Madrid (http://www.ucm.es/info/gyp/genomica/) (Madrid, Spain). Three biological replicates, each consisting of pools of three independent plants, were used for microarray analysis of roots colonized or not by *G. mosseae* or *G. intraradices*. For the MeJA experiment, two biological replicates from MeJA- or mock-treated roots were used. Probe signal summarization, normalization, and background subtraction were performed using the multichip analysis RMA algorithm (Irizarry et al., 2003) in the 'affy' package with default parameters. The

statistical test for differentially expressed genes was performed using the software 'Cyber-T' (Baldi and Long, 2001), which allows a better variance estimation by calculating the moderated t-statistic using empirical Bayesian techniques. Genes were considered as differentially regulated if P <0.01 and the ratio compared with the controls was ≥1.7 or ≤0.6. For the MeJA treatment, genes were considered as differentially regulated if P <0.01 and the ratio compared with the controls was ≥2 or ≤0.5. Updated annotation of the differentially regulated genes was obtained by tblastx against the NCBI nr-database. An E-value $<10^{-15}$ was required to take into account the blast result. Microarray data will be deposited in the Tomato Functional Genomics database (http://ted.bti.cornell.edu/cgi-bin/TFGD/array/ home.cgi).

Gene expression analysis by real-time quantitative RT-PCR (qPCR)

Real-time qPCR was performed using the iCycler iQ5 system (Bio-Rad) and gene-specific primers (Supplementary Table S1). The first-strand cDNA was synthesized with 1 μg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Three independent biological replicates were analysed per treatment. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene SlEF, which encodes for the tomato elongation factor-1α.

Statistical analysis

Data for hormone and phosphorus content and mycorrhization levels of tomato roots were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 14.1 for Windows. When appropriate, Fisher's LSD test was applied.

Results

Root colonization by *G. mosseae* and *G. intraradices*, and physiological status of the plant

In order to identify plant responses directly related to the AM symbiosis and not to potential nutritional effects, an experimental system that allowed a robust mycorrhizal

colonization without significant changes in plant growth and nutrition was used. Nine weeks after inoculation the shoot and root mass and phosphorus (Pi) content of the tomato plants colonized by either *G. mosseae* or *G. intraradices* were not significantly different from those in the non-mycorrhizal controls (Supplementary Table S2). The colonization intensities of *G. mosseae* (23%) and *G. intraradices* (41%) differed significantly ($P < 0.01$). Moreover, both AMF showed different colonization patterns. They developed intraradical hyphae, arbuscules, and vesicles, but with different frequencies. Although the relative abundance of arbuscules was similar in both interactions, the proportion of vesicles, fungal reservoir structures, was higher in *G. intraradices*- but scarce in *G. mosseae*-colonized roots (Fig. 1A, C and D). Besides the presence of fungal structures, the functionality of the symbiosis was also checked by molecular methods. The tomato gene *LePT4* encodes a phosphate transporter specific for the AM symbiosis which is expressed in arbusculated cells and considered a marker for a functional symbiosis (Balestrini et al., 2007). A high *LePT4* expression was detected in all mycorrhizal roots, although the levels were almost 2-fold higher in *G. intraradices*- than in *G. mosseae*-colonized roots (Fig. 1B), in agreement with the differences observed in root colonization (absolute arbuscule abundance).

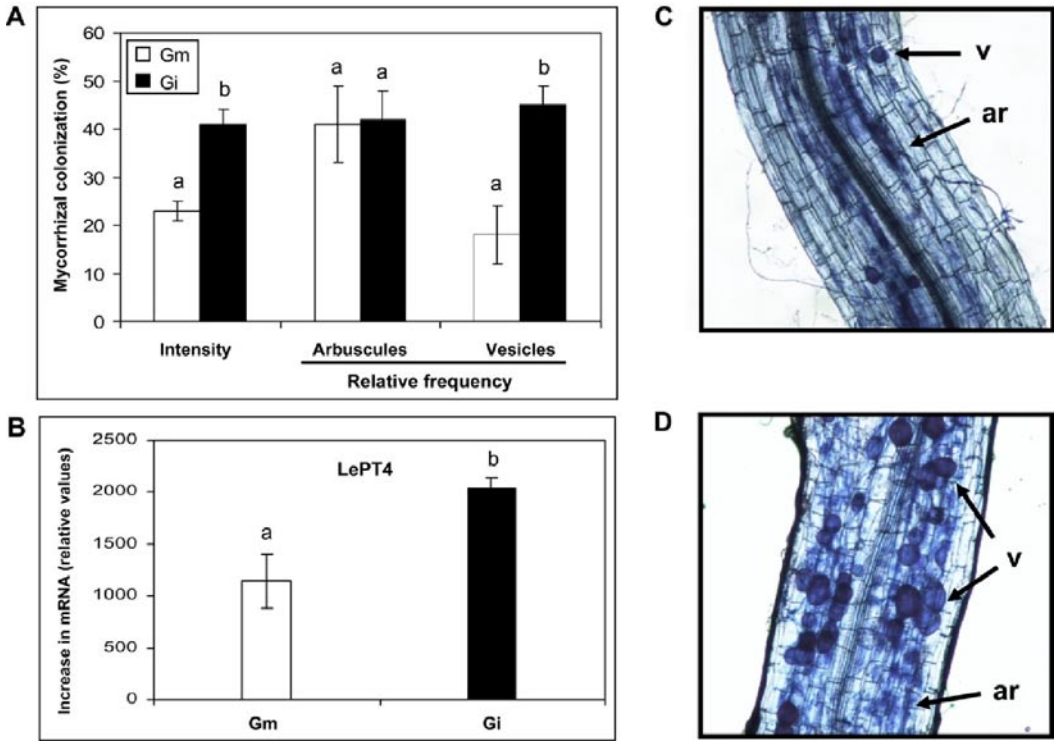

Figure 1. Mycorrhizal colonization and expression analysis of the marker gene *LePT4* of tomato roots inoculated with *G. mosseae* or *G. intraradices*. (A) Intensity of mycorrhizal colonization by *G. mosseae* (Gm) or *G. intraradices* (Gi), and relative frequency of arbuscules and vesicles. (B) Gene expression analysis by real-time qPCR for the mycorrhizal marker gene *LePT4*. Data points represent the means of five (A) or three (B) replicates (±SE). Data not sharing a letter in common differ significantly (P <0.01) according to Fisher's LSD test. The right-hand panels show photographs of root samples after trypan blue staining. (C) *Glomus mosseae* colonizes the root cortex to a lower extent, forming a large number of arbuscules but a limited number of vesicles. (D) *Glomus intraradices* extensively colonize the root cortex forming arbuscules and a large number of vesicles. Arrows indicate arbuscules (ar) and vesicles (v).

Impact of root colonization by *G. mosseae* or *G. intraradices* on defence-related hormones

To investigate the responses of tomato plants to AMF and evaluate the impact of the interaction on hormone homeostasis, the levels of JA, ABA, SA, and ET in tomato roots colonized by the two fungi were determined. Analysis by UPLC-coupled tandem mass spectrometry (LC-MS/MS) allowed the simultaneous quantification of free JA, the JA precursor OPDA, JA–Ile, ABA, and SA from each sample. The levels of OPDA were significantly (P <0.05) higher in roots colonized by both AMF (Fig. 2A). In contrast, JA levels were not altered in mycorrhizal roots (Fig. 2B). Unlike free JA, the levels of its bioactive derivative JA-Ile were higher in *G. mosseae*-colonized roots compared with non-mycorrhizal controls, whereas no significant differences were observed upon *G. intraradices* colonization (Fig. 2C). In spite of this, differences between roots colonized by both fungi were not statistically significant. It was noteworthy that a clear increase

on SA content was only detected in *G. mosseae*-colonized roots (Fig. 2D). Conversely, ABA content did not change upon mycorrhizal colonization by either of the AMF studied (Fig. 2E), but a clear reduction of ET levels in roots colonized by both AMF compared with non-mycorrhizal plants was observed by GC-MS (Fig. 2F).

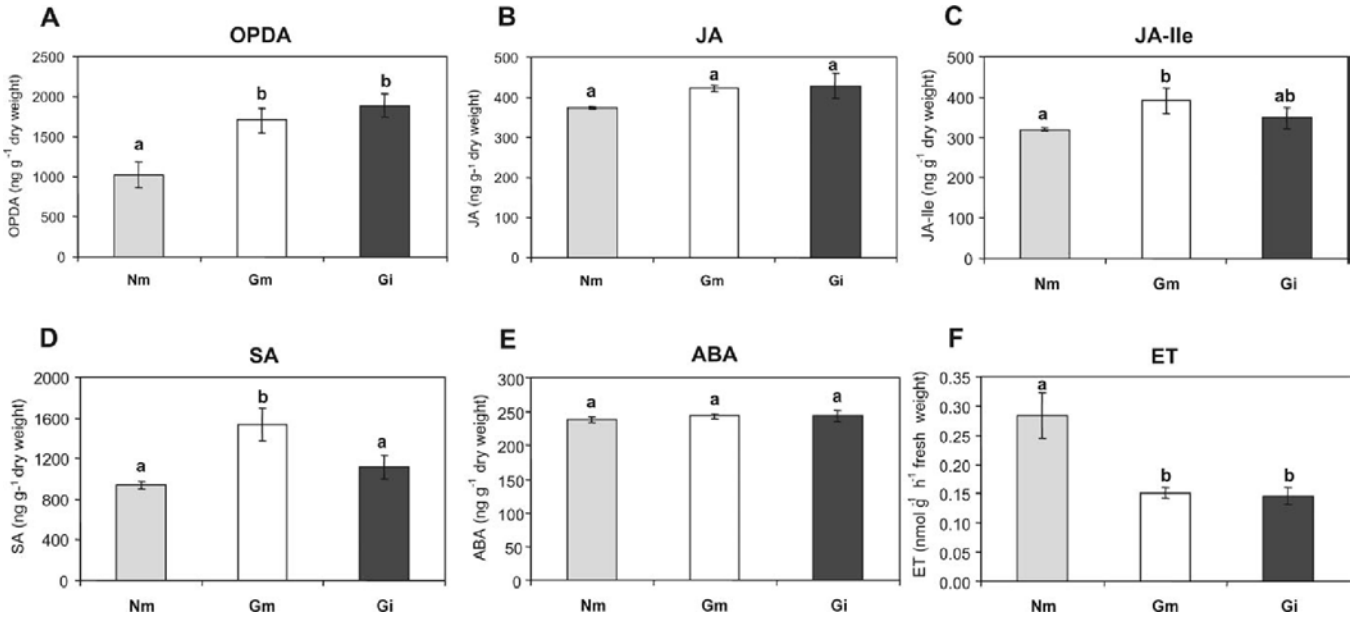

Figure 2. Hormonal content in non-mycorrhizal (Nm) and *G. mosseae*- (Gm) and *G. intraradices*- (Gi) colonized roots. Levels of (A) oxo-phytodienoic acid (OPDA), (B) free jasmonic acid (JA), (C) jasmonic acid isoleucine (JA-Ile), (D) salicylic acid (SA), (E) abscisic acid (ABA), and (F) ethylene (ET). Data points represent the means of five replicates (±SE). Data not sharing a letter in common differ significantly (P <0.05) according to Fisher's LSD test.

Changes in plant gene expression during interaction with *G. mosseae* or *G. intraradices*

To gain further insight into the plant changes related to the symbiosis, a global gene expression profiling of the roots from plants colonized by *G. mosseae* or *G. intraradices* as compared with non-mycorrhizal plants was performed. The Affymetrix Tomato Genome Genechip Array containing >9200 tomato genes was used. In agreement with the similarity in the Pi content of the plants, no differences were observed in the expression of Pi nutrition marker genes present in the array such as the Pi transporter *LePT1*, acid and purple phosphatases and kinases, and other marker genes for Pi starvation such as the iron deficiency-specific-4 (*IDS4*) and the tomato Pi starvation-

induced (*TPSI1*). Thus, the transcriptional changes reported in the present study are not expected to be related to differences in Pi nutrition.

As a first approach, the aim was to determine whether the hormonal changes observed in mycorrhizal plants (Fig. 2) correlated with transcriptional regulation of their metabolic genes. For that, the expression of tomato genes present in the array involved in the metabolism of the hormones analysed was scrutinized (Supplementary Table S3). Consistent with the results from the hormonal analysis, only an induction of the genes encoding enzymes related to the metabolism and regulation of jasmonates, and more generally to oxylipins-LOXA, AOS1, AOS3, JAME, and the jasmonate ZIM domain 2 (JAZ2)-was observed in mycorrhizal plants. However, no changes for the genes related to ABA, SA, and ET were observed, except for the gene encoding a 1-aminocyclopropane-1-carboxylic acid oxidase (ACO1) (Supplementary Table S3).

Comparing the global transcriptional profiles, 162 genes were differentially regulated ($P < 0.01$) in the roots upon *G. mosseae* colonization. Of these, 101 genes (62%) were up-regulated. In *G. intraradices*-colonized roots the expression of 173 genes differed significantly from non-mycorrhizal roots, of which 103 genes (60%) were up-regulated. When comparing the expression profiles in the two mycorrhizal systems, 59 genes were co-regulated during the interaction with both AMF (Table 1). The overlap was considerably higher for up-regulated genes. Among the co-induced genes, previously described markers of the AM symbiosis were found such as those encoding a chitinase, glutathione S-transferase, β-1,3-glucanase, patatin, β-D-xylosidase, a pathogenesis PR10-like protein, and DXS-2, a key enzyme of the mevalonate-independent pathway of carotenoid biosynthesis (Hohnjec et al., 2005; Liu et al., 2007; Siciliano et al., 2007; Walter et al., 2007; Floss et al., 2008), suggesting a role in the AM interaction conserved across plant species. Besides these markers, the microarray analysis revealed the common induction in mycorrhizal roots of key genes in the biosynthesis of oxylipins. In plants there are two main branches of the oxylipin pathway, determined by two different types of lipoxygenases (LOXs), 9-LOX and 13-LOXs (Fig. 3). The 13-LOX pathway leads to the biosynthesis of JA and derivatives, and, as described above, some genes in this pathway were up-regulated during AM interaction (Fig. 3). In addition, the microarray analysis revealed the induction of genes encoding key enzymes of the 9-LOX branch, LOXA and AOS3. LOXA is involved in the production of

lipid 9-hydroperoxides by adding molecular oxygen to either linolenic or linoleic acid at the C-9 position (Ferrie et al., 1994). *AOS3* encodes a root-specific and jasmonate-regulated allene oxide synthase that catalyses the biosynthesis of γ- and α-ketols, and 10-OPDA, an isomer of the JA precursor 12-OPDA (Fig. 3) (Itoh et al., 2002; Grechkin et al., 2008). Furthermore, the gene encoding a divinyl ether synthase (DES), also related to the 9-LOX branch and involved in the formation of the divinyl ether fatty acids colnelenic and colnelic (Itoh and Howe, 2001), was also induced by the two AMF (Table 1 and Fig. 3).

Besides the common genes, there were specific sets of genes regulated only by either *G. mosseae* or *G. intraradices*. Among those significantly ($P < 0.01$) induced exclusively in *G. intraradices*-colonized roots, a group of genes related to the biosynthesis of carotenoids, namely *DXR*, *PSD*, *ZDS*, and *CtrL-b*, stood out (Supplementary Table S4 and Fig. S1). The production of the carotenoid cleavage products mycorradicin and cyclohexenone derivatives is associated with mycorrhization. Indeed, mycorradicin known as the 'yellow pigment'-is responsible for the typical yellow coloration of some mycorrhizal roots and has been correlated with a functional symbiosis (Walter et al., 2007). The first step in this biosynthetic pathway is catalysed by DXS-2, induced transcriptionally during interaction with both fungi but to a higher extent in *G. intraradices*- than in *G. mosseae*-colonized roots (Table 1). Although not significant under the present selection criteria, a moderate increase in the expression of *DXR*, *PSD*, *ZDS*, and *CtrL-b* was also observed in *G. mosseae*-colonized roots. Thus, the induction of the carotenoid pathway is not exclusive to *G. intraradices*-colonized roots; more probably it correlates with the root colonization level.

Table 1. Genes regulated in roots colonized by both *G. mosseae* and *G. intraradices* compared with non-mycorrhizal roots, and their changes in expression after MeJA treatment

ID	Annotation	Ratio Gm	Ratio Gi	MeJA
BT014524	Serine protease/subtilisin-like	28.06	36.26	0.72
X72729	Ripening-related protein ERT1b	25.96	38.42	1.25
M69248	Pathogenesis protein PR1b1	19.71	16.74	2.56[a]
AW622368	Esterase/lipase/thioesterase	17.59	42.64	0.11[a]
AI897365	Putative proteinase inhibitor	17.34	7.42	543.59[a]
AI487223	Anthocyanin acyltransferase	8.63	3.40	112.45[a]
AF454634	Allene oxide synthase 3 (AOS3)	8.49	3.42	236.13[a]
AB015675	Copalyl diphosphate synthase	7.69	10.41	1.25
U09026	Lipoxygenase A (LOXA)	6.59	2.78	13.95[a]
AB010991	3b-hydroxylase (Le3OH-1)	5.13	9.60	0.94
BG631079	β-1,3-Glucanase	5.07	5.35	0.72
BI933750	1-Deoxy-D-xylulose 5-phosphate synthase 2 (DXS-2)	5.00	9.92	2.65[a]
AB041811	β-D-Xylosidase (LEXYL1)	4.72	4.49	1.00
BG125734	Calcium/lipid-binding protein	4.49	15.13	0.91
BT013355	Pathogenesis protein PR-P2	4.45	6.57	9.79[a]
BG626023	Electron carrier (ACD1-like)	4.12	6.88	1.10
BI423134	Germin-like protein	3.92	5.78	1.20
BI423255	Germin-like protein (GLP6)	3.44	4.67	1.18
X94946	Proteinase inhibitor II (Cevi57)	3.42	2.12	4.18[a]
BM412305	EF-hand-containing protein	3.24	4.56	0.81
BF114155	Glutathione S-transferase	3.20	2.76	9.19[a]
AF090115	Heat shock protein HSP17.4	3.20	7.48	1.58
CK720570	Patatin-like protein	3.19	6.04	2.89[a]
AF515615	Lysine-rich protein (TSB)	3.18	3.67	0.47[a]
AI895164	Fatty acid desaturase (FAD)	3.05	3.12	0.46[a]
AJ785041	Cytochrome P450 CYP81C6v2	2.89	2.65	360.27[a]
BT014484	Glucosyltransferase	2.83	3.91	6.26[a]
BG630947	β-Galactosidase (TBG5)	2.79	5.27	0.55
BT014016	Cysteine synthase (cs1)	2.76	5.94	0.99
BI923212	Germin-like protein (GLP9)	2.71	3.04	0.75
CK716273	Miraculin-like protein	2.69	2.48	2.27[a]
AF049898	Gibberellin 20-oxidase-1 (20ox-1)	2.67	3.36	0.61
AW220405	Germin-like protein (ger2a)	2.56	2.86	0.73
AW626187	Unkonwn	2.48	2.83	0.74
AW034398	Subtilisin-like protease (sbt4a)	2.47	3.19	1.70
U30465	Class II chitinase (Chi2;1)	2.41	3.01	6.34[a]
CN384809	1-Aminocyclopropane-1-carboxylic acid oxidase (ACO)	2.35	2.27	12.03[a]
AF317515	Divinyl ether synthase (DES)	2.29	1.96	3.55[a]
X79337	Ribonuclease le	2.24	1.88	2.44[a]
BG628191	Jasmonate ZIM domain 2 (JAZ2)	2.23	2.49	14.78[a]
L77963	Metallothionein II-like protein (MTA)	2.23	2.06	0.31[a]
AY455313	Methylesterase/methyl jasmonate esterase (JAME)	2.16	2.82	1.16
AJ271093	Allene oxide synthase 1 (AOS1)	2.14	2.52	23.83[a]
AA824679	Dihydrolipoamide S-acetyltransferase (LTA2)	2.00	1.96	0.51
Y15846	Pathogenesis protein PR10-like	1.93	2.72	0.52
AI899627	Unknown	1.92	2.47	3.89[a]
BT013881	Cytochrome P450 (CYP721A7v1)	1.86	2.45	1.42
BG625959	Enoyl-[acyl-carrier-protein] reductase	1.81	2.24	0.23[a]
AI780669	Glutathione S-transferase	0.49	0.50	7.29[a]
BM411685	Zinc finger (B-box type)	0.48	0.49	1.80
AW649455	GDSL-motif lipase/hydrolase	0.48	0.54	0.33[a]
AF437878	bHLH transcriptional regulator	0.44	0.32	2.29[a]
AW218614	UDP-glycosyltransferase	0.43	0.36	4.85[a]

AW442015	Cysteine-type endopeptidase	0.40	0.60	0.67
BI207994	Cytochrome P450 CYP72A15	0.36	0.54	4.17[a]
M21775	Metallocarboxypeptidase inhibitor	0.36	0.35	0.82
AI771889	Cytochrome P450 CYP72A57	0.27	0.44	5.12[a]
BI205190	UDP-glycosyltransferase	0.25	0.39	21.66[a]
BM411019	Unknown	0.16	0.39	1.96

Genes significantly up- or down-regulated in roots colonized by *G. mosseae* (Gm) and *G. intraradices* (Gi) compared with non-mycorrhizal roots are sorted according to the fold change in expression in *G. mosseae*-colonized roots. MeJA shows the changes in expression levels of mycorrhiza-regulated genes upon treatment with 50 µM methyl jasmonate compared with mock-treated roots.

[a] Significant (P <0.01) changes in MeJA-treated roots.

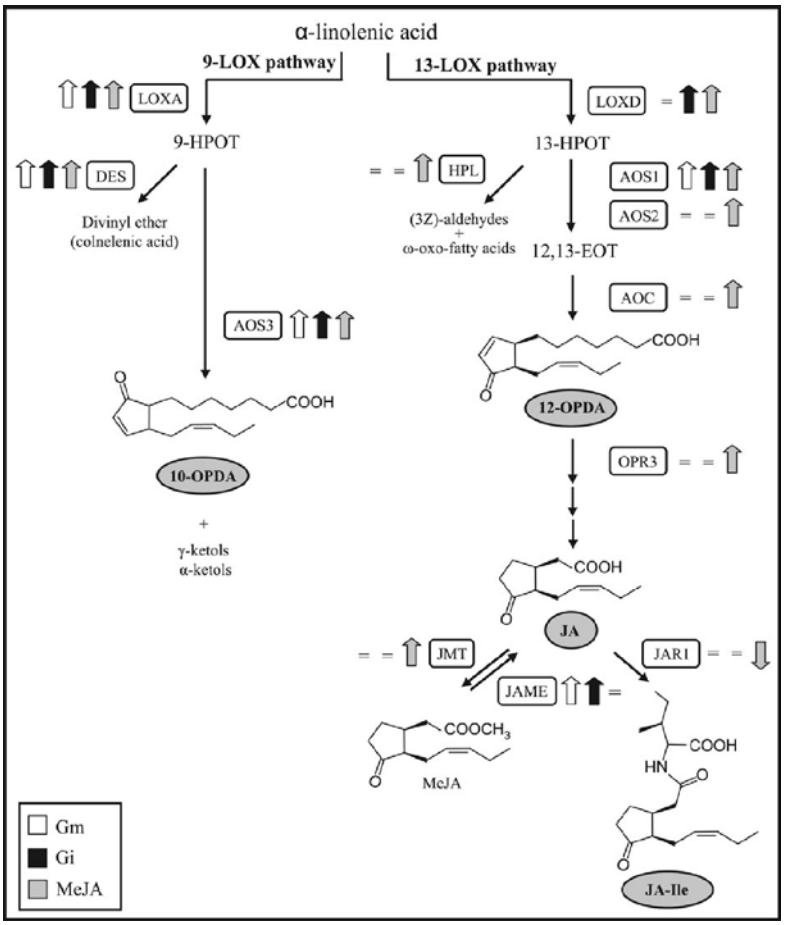

Figure 3. Induction of the oxylipin biosynthetic pathway in arbuscular mycorrhizal symbiosis. Metabolic scheme of the oxylipin pathway including the 9-LOX and 13-LOX branches (modified of Wasternack, 2007). Shaded boxes show the metabolites analysed by LC-MS/ MS in the present study. Thick arrows indicate the direction of the changes in expression levels compared with non-mycorrhizal control roots (up- or down-regulated) of the genes coding for the enzyme cited, as determined by the transcriptomic analysis; = indicates no changes in gene expression. LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, oxo-phytodienoic acid reductase; JMT, jasmonic acid carboxyl methyltransferase; JAME, methyl jasmonate esterase; JAR1, jasmonate-amino synthetase; HPL, hydroperoxide lyase; and DES, divinyl ether synthase.

With regards to *G. mosseae*, a major part of the differentially regulated genes upon mycorrhization were related to defence and wounding responses associated with jasmonates (Supplementary Table S5), including the typical JA marker genes encoding proteinase inhibitors I and II (PinI and II), multicystatin (MC), polyphenol oxidase, and threonine deaminase (Wasternack et al., 2006). Remarkably, despite the strong induction of these genes in *G. mosseae*-mycorrhizal roots, they remained unaltered in *G. intraradices*-colonized roots.

To confirm the regulation of the different signalling pathways, the expression of the hormone biosynthetic genes and several response marker genes was analysed by real-time qPCR. In addition, potential markers of AM symbiosis, orthologues of genes previously described as AM induced in other plant species, such as *DXS-2*, *PR-10*, and the chitinase *Chi2;1*, were also checked (Table 2). There was a very good correlation between the expression data obtained by qPCR and microarray analysis, confirming the global transcript profiling analysis. The analysis highlights the induction of the key biosynthetic genes from the 9-LOX branch (*LOXA*, *AOS3*, and *DES*) in mycorrhizal roots, the induction of *LOXA* and *AOS3* being markedly higher in *G. mosseae*-colonized roots (Table 2), and the striking differential induction in those roots of the typical JA-regulated, wound-related genes such as *PinII* and *MC*.

According to the ABA content, the levels of NCED (encoding a 9-cis-epoxycarotenoid enzyme) involved in ABA biosynthesis, and the ABA-inducible gene *Le4* (Kahn et al., 1993) remained unaltered in the mycorrhizal plants. The qPCR analysis showed unaltered expression of all the ET-related genes tested, not validating the up-regulation of *ACO1* observed in the microarray analysis. In relation to SA, the gene coding for PR1a, a common marker of SA-regulated responses (Uknes et al., 1993), was only detected in *G. mosseae*-colonized roots (Table 2), supporting the increased SA levels detected previously by LC-MS/MS.

The mycorrhiza-related transcriptome is partially mimicked by MeJA treatment

The induction of genes associated with jasmonates in mycorrhizal roots suggested a possible role for these hormones in the plant response to AMF. To assess whether this

is the case, a microarray analysis was carried out to identify JA-regulated genes, and the transcriptional profile was compared with those obtained from mycorrhizal roots. Root treatment with MeJA resulted in 1398 differentially regulated genes (P <0.01), most of them being up-regulated (~60%) (Supplementary Table S6). Even though two different systems were used for growing the plants, 66% of the genes co-regulated upon *G. mosseae* and *G. intraradices* colonization were also regulated by MeJA (Table 1). Among them, the jasmonate biosynthetic and regulatory genes derived from the 13-LOX branch and induced by mycorrhiza (*AOS1* and *JAZ2*) were also significantly (P <0.01) induced by MeJA. Other JA metabolic genes such as *AOC*, *OPR3*, and the jasmonic acid carboxyl methyl transferase (*JMT*), not altered by mycorrhiza, were also up-regulated by MeJA, supporting the reported positive feedback in jasmonate biosynthesis (Fig. 3 and Supplementary Table S6). In addition, the genes involved in defence responses to wounding and herbivory, known to be regulated by JA in aerial parts of the plant (Wasternack et al., 2006), were all up-regulated by MeJA in the roots. Remarkably, as stated before, most of these genes were induced in *G. mosseae* mycorrhizal roots (Supplementary Table S5), but not in those colonized by *G. intraradices*. The genes *LOXA*, *AOS3*, and *DES*, induced by both AMF and acting on the 9-LOX branch, were also induced by MeJA, illustrating a positive regulation of this branch by JA (Table 1 and Supplementary Table S6).

Table 2. Expression analyses of marker genes for AM symbiosis and hormone pathways by quantitative real time RT-PCR (qPCR) in roots colonized by *G. mosseae* (Gm) or *G. intraradices* (Gi)

ID	Annotation	Pathway /Marker	Microarray		qPCR	
			Gm	Gi	Gm	Gi
BI933750	1-Deoxy-D-xylulose 5-phosphate synthase 2 (DXS-2)	AM	5.00[a]	9.92[a]	8.59[a]	20.08[a]
U30465	Class II chitinase (Chi2;1)	AM	2.41[a]	3.01[a]	2.70[a]	2.23[a]
Y15846	Pathogenesis protein PR10-like	AM	1.93[a]	2.72[a]	4.82[a]	5.81[a]
AY885651	Phosphate transporter LePT4	AM	–	–	1141.69[a]	2046.48[a]
U09026	Lipoxygenase A (LOXA)	Oxylipins	6.59[a]	2.78[a]	7.46[a]	3.38[a]
AF454634	Allene oxide synthase 3 (AOS3)	Oxylipins	8.49[a]	3.42[a]	16.60[a]	6.28[a]

AF317515	Divinyl ether synthase (DES)	Oxylipins	2.29[a]	1.96[a]	3.28[a]	2.94[a]
U37840	Lipoxygenase D (LOXD)	JA	1.27	2.17[a]	0.79	2.35[a]
AJ271093	Allene oxide synthase 1 (AOS1)	JA	2.14[a]	2.52[a]	2.51[a]	3.32[a]
AF230371	Allene oxide synthase 2 (AOS2)	JA	0.97	0.89	0.98	0.84
AF384374	Allene oxide cyclase (AOC)	JA	1.34	1.29	1.32	1.41
AJ278332	12-Oxophytodienoate 3 reductase (OPR3)	JA	0.94	1.05	1.09	1.15
AF083253	Multicystatin (MC)	JA	9.27[a]	1.64	8.35[a]	0.76
K03291	Proteinase inhibitor II (PinII)	JA	5.92[a]	0.94	7.39[a]	1.06
CN384809	1-Aminocyclopropane-1-carboxylic acid oxidase (ACO1)	ET	2.35[a]	2.27[a]	0.79	1.76
X58885	Ethylene-forming enzyme (EFE)	ET	0.69	1.13	0.61	1.54
AY394002	CTR1-like protein kinase (CTR4)	ET	1.11	1.13	0.71	0.83
Z97215	9-cis-Epoxycarotenoid (NCED1)	ABA	0.96	0.69	0.68	0.57
X51904	Le4	ABA	–	–	0.82	0.48
M69247	Pathogenesis-related protein PR1a	SA	ND	ND	Ct 30.67	ND

Numbers indicate fold change in expression levels compared with non-mycorrhizal controls.
Ct, the threshold cycle; ND. non-detected;– not present in the tomato array.
[a] Significant (P <0.01) changes.

Discussion

It is widely accepted that AM establishment induces transcriptional changes in the host plant (Grunwald et al., 2004; Hohnjec et al., 2005; Liu et al., 2007; Fiorilli et al., 2009), and the involvement of a number of plant hormones in mycorrhiza formation and functioning has been proposed (Hause et al., 2007; Herrera-Medina et al., 2007; Riedel et al., 2008; Fiorilli et al., 2009; Grunwald et al., 2009). However, the precise mechanisms underlying plant-AMF interactions are still unknown. Here, the plant response to the colonization by the two AMF *G. mosseae* and *G. intraradices* was analysed using tomato as the model plant. A different level and structure of mycorrhizal colonization were observed when comparing both fungi, *G. intraradices* being the most effective colonizer. This higher colonization level intensity was accompanied by a larger induction of host genes coding for symbiosis-related elements such as the mycorrhiza-specific phosphate transporter LePT4 and those involved in the

biosynthesis of mycorrhiza-related carotenoids (Walter et al., 2007). Differences in the colonization by *G. intraradices* and *G. mosseae* isolates have been previously shown in several plant species (Pozo et al., 2002; Feddermann et al., 2008), supporting that at least part of the morphological features in the AM colonization, and probably of the hormonal and transcriptional changes in the host, are related to the AMF genotype.

Simultaneous quantification of several hormones by LC-MS/MS and GC-MS revealed significant changes in the levels of defence-related hormones in mycorrhizal tomato roots, depending on the AMF involved. While ABA and free JA levels remained unaltered in mycorrhizal plants compared with controls, ET and OPDA levels were significantly down- and up- regulated, respectively, during the interaction with both fungi. In contrast, the levels of the JA derivative JA-Ile and SA were elevated exclusively in roots colonized by *G. mosseae*, the AMF with a lower colonization level. The significant reduction in ET production in roots colonized by both AMF is in agreement with studies in other mycorrhizal plants and during plant interaction with other beneficial endophytic fungi (Barazani et al., 2007; Riedel et al., 2008). This reduction, together with data from pharmacological and genetic approaches to analyse the role of ET in the symbiosis, indicates that a precise regulation of ET levels is required for AM establishment (Azcón-Aguilar et al., 1981; Zsogon et al., 2008).

As mentioned above, OPDA levels were elevated in roots colonized by both AMF. Surprisingly, the higher content of OPDA was not accompanied by increased levels of free JA. Free JA increased up to several fold in roots of barley, cucumber, *M. truncatula*, and soybean upon mycorrhization (Hause et al., 2002; Vierheilig and Piche, 2002; Meixner et al., 2005; Stumpe et al., 2005). Accordingly, a conserved role for JA in the establishment and functionality of the AM symbiosis was proposed. However, analysis of the JA content in *N. attenuata* showed unaltered levels in mycorrhizal roots (Riedel et al., 2008). It is also noteworthy that no changes in OPDA content were found in mycorrhizal barley or *Medicago* roots (Hause et al., 2002; Stumpe et al., 2005). Therefore, it is possible that OPDA and other oxylipins, but not free JA, are the main players in orchestrating the plant response to mycorrhizal fungi in the Solanaceae family. In this regard, organ- and plant species-specific patterns of accumulation of different JA-related compounds have been described, tomato being among the plants with higher levels of OPDA relative to the JA concentration before and after wounding

(Miersch et al., 2008) and in response to pathogen attack (Vicedo et al., 2009).

At the transcriptional level, some of the genes related to the biosynthesis and metabolism of jasmonates showed a moderate increase in their expression in mycorrhizal roots. LOXD and AOS1 are involved in the early steps of the pathway, so their higher expression levels might support the increase in the JA precursor 12-OPDA. Induction of the JA biosynthetic genes *AOS* and *AOC* was previously shown in arbusculated cells in barley and *M. truncatula* roots (Hause et al., 2002). However, changes in the expression of *AOC* and later biosynthetic genes were not detected in the tomato array. The picture is more complex because, besides JA, other oxylipins play important roles in biological processes such as plant defence and development (Wasternack, 2007; Mosblech et al., 2009). Interestingly, the microarray analysis revealed a strong induction of the genes coding for LOXA and AOS3, key enzymes in the 9-LOX branch of the oxylipin pathway which give rise to the formation of ketols and 10-OPDA. 10-OPDA is a structural isomer of the JA precursor 12-OPDA (Itoh et al., 2002; Grechkin et al., 2008). Because of the transcriptional activation of the 9- and 13-LOX branches in mycorrhizal roots and the inability of LC-MS/MS to discriminate between the two isomers, it is likely that the increased OPDA levels observed correspond to a mixture of both isomers. In addition to its function as a JA precursor, 12-OPDA per se plays a role in plant defence signalling, regulating the expression of a specific subset of genes (Stintzi et al., 2001; Taki et al., 2005). Despite the lack of information about 10-OPDA in vivo, a similar role for this OPDA isomer in plant defence has been postulated (Itoh et al., 2002). Further supporting the activation of the 9-LOX branch, up-regulation of *DES* in mycorrhizal tomato roots was also found. The encoded enzyme catalyses the biosynthesis of colnelenic and colneleic acids for which a role in defence against plant pathogens has been proposed in tobacco and potato (Mosblech et al., 2009). It is noteworthy that the 9-LOX branch is largely root specific. Indeed, *AOS3* is exclusively expressed in the roots (Itoh et al., 2002), and *LOXA* and *DES* show only very low basal expression levels in shoots (Ferrie et al., 1994; Itoh and Howe, 2001). Recently, the relevance of the 9-LOX pathway in plant interactions with nematodes and pathogens has been demonstrated (Vellosillo et al., 2007; Gao et al., 2008). Thus, it is plausible that the activation of the 9-LOX pathway is part of the strategy of the plant to control AMF development within the roots. Supporting this

hypothesis, increased mycorrhization levels were found in the tomato mutant *jai1* (Herrera-Medina et al., 2008; JAL-R et al., unpublished data), in which *AOS3* expression is undetectable (Itoh et al., 2002). Moreover, although *LOXA* and *AOS3* were significantly induced in roots colonized by both AMF, a higher up-regulation was found in the interaction with the lower colonization rate, tomato-*G. mosseae*. As a side effect, higher levels of 9-LOX-derived products may be responsible for the enhanced resistance to root pathogens in mycorrhizal plants. We previously compared *G. mosseae* and *G. intraradices* in terms of their ability to protect tomato plants against *Phytophthora parasitica* var. *nicotianae*, and only *G. mosseae* efficiently induced resistance (Pozo et al., 2002). More recently, resistance to this pathogen has been demonstrated to depend on the 9-LOX branch in tobacco roots (Fammartino et al., 2007). Accordingly, the stronger activation of this branch of the oxylipin pathway in *G. mosseae*-colonized roots may contribute to the enhanced resistance of these roots against *P. parasitica*. In addition to the higher up-regulation of 9-LOX biosynthetic genes, *G. mosseae* differentially triggered other metabolic changes related to defence in tomato roots. Only roots colonized by this AMF showed a moderate but significant increase in the levels of JA-Ile, one of the most active forms of JA with specific biological roles (Staswick and Tiryaki, 2004; Fonseca et al., 2009). JA-Ile is formed by the action of JAR1, which catalyses the conjugation of JA to isoleucine synthesized from threonine by a threonine deaminase (TD). Remarkably, a gene encoding a TD was up-regulated >6-fold in roots colonized by *G. mosseae*, but not by *G. intraradices*, which is in agreement with the differences in JA-Ile content between roots colonized by the two AMF. In *N. atenuatta* the defensive role of this enzyme is linked to its mediation of JA-Ile signalling, leading to the accumulation of direct defences such as protease inhibitors (Kang et al., 2006). In agreement with the elevated levels of JA-Ile only in *G. mosseae*-colonized roots, all wound inducible marker genes (coding for proteinase inhibitors I and II, polyphenol oxydase, arginase 2, multicystatin, etc.) were up-regulated exclusively in those roots. It is noteworthy that only JA-Ile, and not JA or OPDA, is able to promote interaction of the SCFcoi1 ubiquitin ligase complex and JAZ proteins in *Arabidopsis*. This interaction liberates MYC2, a positive regulator of JA (reviewed in Memelink, 2009) which is essential in rhizobacteria-induced systemic resistance (Pozo et al., 2008). Thus, it is tempting to speculate that elevated JA-Ile

levels and related transcripts in *G. mosseae*-colonized plants may be related to its ability to induce mycorrhiza-induced resistance. As for JA-Ile, SA content and expression of its marker gene *PR1a* were significantly elevated only in *G. mosseae*-colonized roots. SA is a key phytohormone in the regulation of plant defence responses, especially in interactions with biotrophic pathogens. In agreement with the biotrophic character of AMF, a negative regulatory role of SA in the AM symbiosis has been proposed (Gutjahr and Paszkowski, 2009). Indeed, an inverse correlation between SA levels and AM colonization was found in pea and tobacco (Blilou et al., 1999; Herrera-Medina et al., 2003). Accumulation of SA in *G. mosseae*-colonized barley roots has also been described (Khaosaad et al., 2007). Therefore, the enhanced SA levels could modulate the plant control of AMF proliferation within the roots. Additionally, because of the role of SA in regulating systemic defence responses and induced resistance (Vlot et al., 2009), the increase in SA levels might be key in the induction of resistance by mycorrhiza. Taken together, the results show common and divergent responses of the plant to mycorrhizal colonization by different AMF at the hormonal and transcriptional levels. Remarkably, the differences in the response are very significant: only ~35% of the genes regulated during the interaction with *G. mosseae* and *G. intraradices* overlap. A similar overlap (~30%) was found in the response of *M. truncatula* to the same fungi (Hohnjec et al., 2005). The differential responses found in tomato plants interacting with *G. mosseae*, namely stronger induction of the oxylipin 9-LOX branch, increased SA and JA-Ile contents, and the associated induction of *PR1a* and jasmonate-related defence genes points to a more exhaustive control of the fungal partner by the plant that may explain the reduced colonization level of *G. mosseae* when compared with *G. intraradices*. Moreover, it might be that these changes in the host also contribute to the bioprotection ability of this AMF. In conclusion, it is shown here that the maintenance of AM symbiosis implies changes in the content of several phytohormones, which correlate with changes in the expression of genes involved in their biosynthesis and the responses they regulate. A crucial role for oxylipins in this mutualistic symbiosis has been proposed for the first time, and indications of their regulation by jasmonates suggested. Additionally, a different plant response to the colonization by particular AMF is demonstrated, which may underlie the differential impact of individual AMF on plant physiology and,

particularly, on its ability to cope with biotic stresses. Further research is required to elucidate the role of the 9-LOX-derived oxylipins, as well as the metabolic and transcriptional changes needed for the long-term maintenance of the AM symbiosis and its benefits to the host.

SUPPLEMENTARY DATA

Figure S1. Scheme of the biosynthesis of the apocarotenoids cyclohexenone and mycorradicin in mycorrhizal roots (adapted from Walter et al., 2007). Thick arrows indicate significant up-regulation of the corresponding genes as determined by the transcriptomic analysis of mycorrhizal roots (*G. mosseae* or *G. intraradices* colonized) compared with non-mycorrhizal controls; = indicates no changes in gene expression. DXS-2, 1-deoxy-D-xylulose 5-phosphate synthase 2; DXR, 1-deoxy-D-xylulose reductase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zetacarotene desaturase; CrtL-b, lycopene cyclase b; and CCD1, carotenoid cleavage dioxygenase 1.

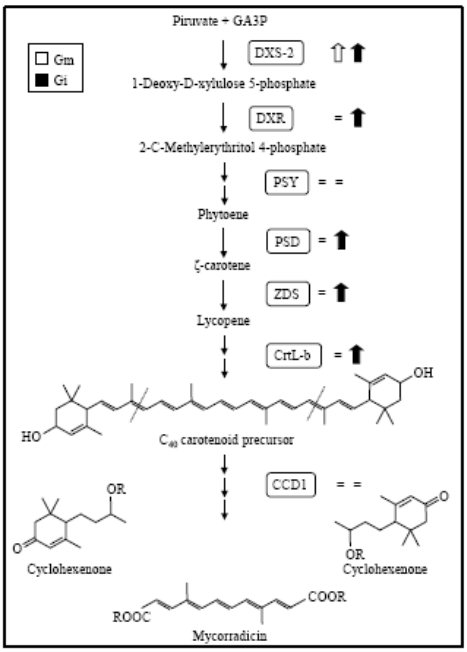

Table S1. Primer sequences used in the real-time qPCR analysis.

ID	Gene	Primers (5'-3')
U09026	Lipoxygenase A (LOXA) [1]	GGTTACCTCCCAAATCGTCC TGTTTGTAACTGCGCTGTG
U37840	Lipoxygenase D (LOXD) [2]	GACTGGTCCAAGTTCACGATCC ATGTGCTGCCAATATAAATGGTTCC
AJ271093	Allene oxide synthase 1 (AOS1) [1]	CACCTGTTAAACAAGCGAAAC GACCTGGTGGCATGTTCGT
AF230371	Allene oxide synthase 2 (AOS2) [2]	AGATTTTCTTCCCGAATATGCTGAA ATACTACTGATTTCATCAACGGCAT
AF454634	Allene oxide synthase 3 (AOS3) [1]	GCGGAGGAGTTCAATCCAG CGCATGAAAAACTCCACAACC
AF384374	Allene oxide cyclase (AOC) [2]	GCACGAAGAAGAGAAGAAAGGAGAT CGGTGACGGCTAGGTAAGTTTC
AJ278332	12-oxophytodienoate 3 reductase (OPR3) [2]	TTGGCTTAGCAGTTGTTGAAAG TACGTATCGTGGCTGTGTTACA
AF317515	Divinyl ether synthase (DES) [1]	CCGGATGAGTTTGTACCTGA ATCTTTGCCTGGACATTGCT
AF083253	Multicystatin [2]	GAGAATTTCAAGGAAGTTCAA GGCTTTATTTCACACAGAGATA
K03291	Proteinase inhibitor II [2]	GAAAATCGTTAATTTATCCCAC ACATACAAACTTTCCATCTTTA
BI933750	1-deoxy-D-xylulose 5-phosphate 2 (DXS-2) [1]	AGACGGTCCAACGCATTGT CCTCTAGGAAATCGGAAACA
U30465	Class II chitinase (Chi2;1) [1]	TGCTGCTTGTGGTGCGAAAG TGCCCATCCACCCGTAG
CN384809	1-aminocyclopropane-1-carboxylic acid oxidase 1 (ACO1) [1]	GGACTCCGCGCTCATACAGA ATAGAGTGGCGCATGGG
X58885	Ethylene forming enzyme (EFE) [1]	CGCAGGAGGCATCATACTTC CTTCCCGTTGGTAATCAC
AY394002	CTR1-like protein kinase (CTR4) [1]	CATCCTCTTTCTTACTGTGAGAAAATTTAGA CATTTCCCTGTATAAAAACGTTCAGTT
Z97215	9-cis-epoxycarotenoid (NCED) [1]	ACCCACGAGTCCAGATTTC GGTTCAAAAAGAGGGTTAGC
AY885651	Phosphate transporter LePT4 [3]	GAAGGGGAGCCATTTAATGTGG ATCGCGGCTTGTTTAGCATTTC
M69247	Pathogenesis related protein PR1a [1]	ATGTGTGTGTTGGGGTTGGT ACTTTGGCACATCCAAGACG
Y15846	Pathogenesis protein PR10-like [1]	CCAAGGCTGTAGAAGCATACC CGTCTCTCATTCGAGCGTTT
X51904	Le4 [1]	ACTCAAGGCATGGGTACTGG CCTTCTTTCTCCTCCCACCT
X14449	Elongation factor 1 (SlEF) [4]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
DQ282611	GintEF [5]	GCTATTTTGATCATTGCCGCC TCATTAAAACGTTCTTCCGACC

Table S3. Expression level of genes involved in hormone metabolism in roots colonized by *G. mosseae* (Gm) or *G. intraradices* (Gi).

ID	Annotation	Roots	
		Gm	Gi
	Jasmonates		
U09026	Lipoxygenase A (LoxA)	6.59	2.78
U09025	Lipoxygenase B (LoxB)	*nd*	*nd*
U37839	Lipoxygenase C (LoxC)	*nd*	*nd*
U37840	Lipoxygenase D (loxD)	1.27	2.17
AJ271093	Allene oxide synthase (AOS1)	2.14	2.52
AF230371	Allene oxide synthase (AOS2)	0.97	0.89
AF454634	Allene oxide synthase (AOS3)	8.49	3.42
AF384374	Allene oxide cyclase (AOC)	1.34	1.29
AJ278332	12-oxophytodienoate 3 reductase (OPR3)	0.94	1.05
BG628687	Jasmonic acid carboxyl methyl transferase (JMT)	1.53	1.62
BT013697	Jasmonate-amino synthetase (JAR1)	1.03	1.10
AY455313	Methyl jasmonate esterase (JAME)	2.16	2.82
BG628191	Jasmonate ZIM domain 2 (JAZ2)	2.23	2.49
	ABA		
Z97215	9-*cis*-epoxycarotenoid (NCED1)	0.96	0.69
AF258808	Aldehyde oxidase 1 (AO1)	0.97	0.93
AF258809	Aldehyde oxidase 2 (AO2)	0.89	0.92
AF258810	Aldehyde oxidase 3 (AO3)	1.13	1.16
AF258811	Aldehyde oxidase 4 (AO4)	*nd*	*nd*
AY074788	Moco sulfurase (FLACCA)	1.29	0.99
AW034203	ABA 8'-hydroxylase	0.62	1.66
Y14809	Beta-carotene hydroxylase (CrtR-b1)	0.63	0.79
BG734834	Zeaxanthin epoxydase	0.78	0.89
AF385366	Violaxanthin epoxydase	*nd*	*nd*
AW219893	ABA-induced protein	0.84	0.99
	ET		
M34289	ACC synthase 2 (ACS2)	*nd*	*nd*
U17972	ACC synthase 3 (ACS3)	*nd*	*nd*
X58885	Ethylene forming enzyme (ACO)	0.69	1.13
CN384809	ACC oxidase 1 (ACO1)	2.35	2.27
AB013101	ACC oxidase 4 (ACO4)	0.64	0.65
AJ715790	ACC oxidase 5 (ACO5)	*nd*	*nd*
AY394002	CTR1-like protein kinase (CTR4)	1.11	1.13
AF110518	CTR1-like protein kinase (ethylene-inducible)	1.12	0.99
	SA		
BG626517	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT)	*nd*	*nd*

Genes involved in the metabolism and signaling of jasmonates, abscisic acid (ABA), ethylene (ET) and salicylic acid (SA) and present in the Affymetrix GeneChip Tomato Genome Array are shown. Data are expressed as fold-change in expression levels compared to the non-mycorrhizal control roots according to the microarray analysis. nd indicates non-detected expression.

Table S4. Genes specifically induced or repressed in tomato roots colonized by *G. intraradices* (Gi). Fold change in their expression level compared with non-mycorrhizal plants or upon methyl jasmonate (MeJA) treatment.

ID	Annotation	Gi	MeJA
AI771939	Class III alcohol dehydrogenase 5	5.04	2.89
AF243040	Receptor-like protein kinase (PRK3)	4.85	0.99
AI899070	Ent-kaurene oxidase (CYP701A)	4.27	0.99
AW030757	Gibberellin 2-oxidase (SlGA2ox3)	4.26	5.49
BI204920	Zinc finger (C3HC4-type RING finger)	4.19	26.42
BI922195	Class III alcohol dehydrogenase 5	3.85	1.44
M80604	Beta-1,3-glucanase	3.31	0.07
BM409727	N-hydroxycinnamoyl/benzoytransferase	3.26	4.83
BT012691	2-oxoglutarate-dependent dioxygenase	3.16	15.39
AF254793	Lycopene beta-cyclase (CrtL-b)	3.02	0.64
CN385777	Zinc finger (C2HC2)	2.87	9.10
AW035980	Amino acid binding (ACR8)	2.81	2.09
BG631850	Phytoene desaturase (PDS)	2.75	0.76
AW625888	1-aminocyclopropane-1-carboxylic acid oxidase (ACO)	2.70	1.63
BG628645	Chitinase	2.65	1.69
BT014289	1-phosphatidylinositol phosphodiesterase-related	2.64	8.45
BG627506	Carbonic anhydrase	2.63	276.72
BT012812	Elongation factor-like	2.60	4.59
BI932691	Unknown	2.57	1.02
AF096246	Ethylene-responsive transcriptional coactivator	2.55	3.31
AW928514	Peroxidase	2.49	0.17
BT013303	4-amino-4-deoxychorismate lyase	2.48	0.30
BI210904	Purine transmembrane transporter (AtPUP3)	2.44	1.77
BG627658	Unknown	2.43	2.42
BE353179	N-acetyltransferase	2.32	4.84
U89256	DNA-binding protein Pti5	2.31	4.70
AW219676	Unknown	2.29	3.53
BT013913	L-lactate dehydrogenase	2.26	1.60
BG643871	Enoyl-[acyl-carrier-protein] reductase	2.24	0.16
CN385420	Calcium binding protein	2.24	5.90
U37840	Lipoxygenase D (LOXD)	2.17	10.79
AI777697	Unknown	2.16	0.91
BT013211	Cystathionine beta-synthase	2.15	0.45
AF195507	Zeta-carotene desaturase (ZDS)	2.13	1.51
BT014379	Histidine decarboxylase	2.11	0.36
AW092785	Unknown	2.11	1.38
BI928506	Annexin	2.11	4.48
BT013586	Heat shock protein 70 kD (Hsc-1)	2.11	3.56
AW223067	Calmodulin	2.09	1.07
AI775872	2-oxoglutarate-dependent dioxygenase	2.07	1.12
BG628954	Unknown	2.02	1.35
BM410464	Sodium symporter-related	2.02	0.78
AI485696	Calmodulin	1.99	0.97
BG628875	Unknown	1.99	0.31
BG627003	S-adenosylmethionine decarboxylase 2	1.97	3.58

BT014384	2-oxoglutarate-dependent dioxygenase	1.96	4.77
AJ784458	Heat shock protein cognate 70	1.95	1.68
AF331705	1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)	1.94	2.42
AY368906	Heat shock protein 90	1.89	1.60
BG630651	Unknown	1.88	1.33
BG735346	Kinesin	1.87	0.67
BG627878	Unknown	1.79	0.41
BT014625	Replication factor C	1.79	0.55
BT013273	Unknown	1.73	1.21
AW092917	Xyloglucan:xyloglucosyl transferase	0.59	0.18
CK715113	Unknown	0.59	0.73
AF218774	Aquaporin PIP1	0.59	0.30
CK720557	Acid phosphatase class B	0.58	0.15
BG628504	Germin-like protein	0.58	1.41
BT014421	Isoflavone reductase	0.58	1.11
AW429117	Unknown	0.58	2.35
CN385091	Peroxidase	0.57	0.56
CK715831	Zinc-binding protein	0.56	0.95
BE451719	Aquaporin PIP2	0.56	0.06
AI898522	2-nitropropane dioxygenase	0.56	3.52
AI490099	Endonuclease (tbn1)	0.56	1.13
CN385508	Proline dehydrogenase	0.55	0.82
BG630440	Unknown	0.55	0.38
CN384666	Germin-like protein	0.55	1.72
AF193439	Glutathione S-transferase/peroxidase	0.54	3.02
BF050423	GDP-mannose 3,5-epimerase	0.54	0.25
CN385367	Glutaredoxin	0.54	4.30
AW737374	MYB transcription factor (AIM1)	0.53	5.77
BT014260	Unknown	0.53	0.16
BI925563	Auxin efflux carrier protein	0.53	5.55
AY324397	Alternative oxidase 1c	0.52	0.92
BG627815	LIM domain protein WLIM1	0.52	0.39
AW625684	Asparagine synthetase (AS1)	0.52	2.64
CN385091	Peroxidase	0.51	0.44
AW217188	Germin-like protein	0.51	1.89
CK715555	Dopamine beta-monooxygenase	0.51	1.40
AY497477	Xyloglucan endotransglucosylase-hydrolase XTH6	0.50	0.39
U18678	Isocitrate lyase	0.50	2.07
BF098450	Pectinesterase inhibitor	0.50	2.25
BI208081	Medium-chain acyl CoA synthetase	0.49	3.72
BG643520	Fasciclin-like arabinogalactan-protein 12	0.49	1.26
BG133955	Cysteine-type peptidase	0.49	0.47
BT013407	Lactoylglutathione lyase	0.49	0.91
CK714985	(1-4)-beta-mannan endohydrolase	0.48	0.87
CN384849	Calmodulin	0.48	1.27
AW033570	Amino acid binding protein	0.48	0.28
AW625370	LOB domain-containing protein	0.46	1.82
AW626006	Unknown	0.46	1.38
AI490099	Putative endonuclease precursor	0.45	1.45
AJ011914	bZIP transcription factor THY5	0.44	0.47
CN385880	Fasciclin-like arabinogalactan protein	0.44	1.06

AW218741	60S ribosomal protein L6	0.43	1.32
CK575023	Nam-like protein	0.43	5.96
BG126009	Unknown	0.43	1.02
BG734719	Unknown	0.43	0.33
AW649367	Carbohydrate transporter	0.43	20.75
AW738551	Carbohydrate transporter	0.43	24.15
BG626715	Apyrase-like protein	0.43	1.26
BT013228	Transcription factor Y	0.42	0.14
BT013477	Glycolate oxidase	0.42	2.37
Z68309	Metallothionein-like protein	0.42	0.93
BM408981	Copper-binding protein	0.41	1.29
BG127578	60S ribosomal protein L6	0.39	1.17
AJ417830	Extensin-like protein	0.36	0.13
AW626016	Cationic peroxidase	0.36	0.09
AY344540	Alpha-dioxygenase DOX-2	0.35	0.33

Table S5. Genes specifically induced or repressed in tomato roots colonized by *G. mosseae* (Gm). Fold change in their expression level compared with non-mycorrhizal plants or upon methyl jasmonate (MeJA) treatment.

ID	Annotation	Gm	MeJA
BG630425	Proteinase inhibitor I	30.86	159.77
AJ133600	Extensin-like protein	14.15	3.49
BG631444	Proteinase inhibitor I	13.86	165.58
BI931127	Putative miraculin	13.09	928.35
AF083253	Multicystatin	9.27	291.27
BG631366	Unknown	9.10	321.97
BG628187	Proteinase inhibitor I	8.27	3.51
BG625870	Polyphenol oxidase (PPO)	6.68	20.19
BG628643	Threonine deaminase	6.43	111.68
K03291	Proteinase inhibitor II	5.92	425.73
BG626001	Serin protease/subtilisin-like	5.71	30.52
BT013249	Inducible plastid-lipid associated protein	4.00	170.19
AF242849	Wound-inducible carboxypeptidase	3.95	9.47
BG629245	Unknown	3.95	0.52
AF146690	Pto-responsive gene 1 protein	3.86	231.96
X77373	Glycine rich protein	3.42	3.94
AJ133601	Gamma-thionin	3.40	1.36
BI203812	Phosphatase (psi14A)	3.39	41.71
Y10149	Subtilisin-like protease	3.16	0.47
CN385118	Unknown	3.07	3.04
BG130169	Extensine-like	2.78	9.48
AJ459816	Phosphatase (psi14B)	2.75	5.49
AJ459817	Phosphatase (psi14A)	2.57	3.11
BI210284	Unknown	2.55	5.94
X71593	Peroxidase CEVI-1	2.53	0.75
AJ635324	Polyphenol oxidase	2.44	1.47
AW648097	Unkonwn	2.35	7.95
AI773917	Arogenate dehydrogenase	2.33	20.60
AW649666	1-aminocyclopropane-1-carboxylate oxidase (ACO)	2.32	0.72
AY026343	Non-symbiotic hemoglobin class 1 (Glb1)	2.27	1.10
BG629220	1-aminocyclopropane-1-carboxylic acid oxidase (ACO6)	2.20	0.43
AW622064	LOB domain 37 (LBD37)	2.15	1.74
BG734632	Unknown	2.13	0.63
BF112635	1-aminocyclopropane-1-carboxylate oxidase (ACO)	2.09	0.60
X94944	Lipid desaturase-like protein (cevi19)	2.08	0.52
CN384955	Unknown	2.08	2.93
AW649659	Gibberellic acid-induced gene (gasa4)	2.07	0.08
AI779060	LOB domain	2.05	1.59
BG127217	Gamma-thionin/defensin	2.04	1.28
BI206094	NIP2	2.02	1.27
L76632	Osmotin-like protein	1.97	1.56
AW625586	Xyloglucan-specific fungal endoglucanase inhibitor protein precursor (Xegip)	1.90	0.32
BG131634	Lipase	1.86	0.91
CN385868	Auxin-responsive protein	1.85	8.45
BG629316	Unknown	1.84	1.54
BE460442	Phosphopyruvate hydratase	1.83	0.16

BG627786	Proline rich protein	1.82	0.90
AF022874	Phosphate transporter 2 (LePT2)	1.78	0.07
AY568721	Wound/stress protein	1.77	0.56
AI782210	Unknown	1.73	0.29
BG629955	Arginine decarboxylase (adc1)	1.73	1.96
CN384480	Peroxidase CEVI-16	1.71	1.25
BF097029	Unknown	0.60	0.37
BI207106	NADPH:quinone oxidoreductase	0.58	4.31
BT013653	Protein serine/threonine kinase	0.58	1.10
AF022020	IAA9	0.58	1.26
AW035650	Serine/threonine phosphatase	0.58	1.13
AI895632	Auxin-inducible SAUR protein	0.57	0.67
BT013320	Nodulin MtN3	0.57	0.72
AI899248	Transcription factor APETALA 2	0.57	0.92
BM535315	Carboxylesterase	0.56	1.29
CK715114	UDP-glucosyltransferase	0.56	2.82
BF112381	Cytochrome c oxidase	0.55	1.86
AF204783	Unknown	0.55	1.23
BE460421	Carboxylesterase	0.55	1.09
AB026983	Small heat shock protein (leer-sHSP)	0.54	1.17
BT014402	Cohesin	0.54	1.48
AI772299	Cytochrome P450 (CYP76C1)	0.54	0.64
AI898501	DNAJ heat shock protein	0.54	1.83
BM412737	Protein binding/transcription regulator (BT1)	0.52	6.52
AF146691	Eli3 protein	0.52	24.52
BI933005	Short-chain dehydrogenase/reductase (SDR)	0.52	6.02
BT014359	Purine transmembrane transporter	0.50	0.75
BG631327	Unknown	0.50	0.34
AY007562	Glutathione S-transferase	0.50	10.69
AW929113	Class I small heat shock protein (HSP15.7-CI)	0.49	1.13
BM410870	Class I small heat shock protein (HSP26.5-P)	0.49	0.92
AJ606077	Spermine synthase (spe4)	0.49	0.48
AI779519	Unknown	0.47	0.66
BI210938	Unknown	0.46	0.38
BT012739	ADP-ribose pyrophosphohydrolase	0.46	0.66
X92855	Mannitol dehydrogenase	0.45	3.44
BM410706	L-ascorbate peroxidase	0.45	1.18
BT013685	Zinc finger	0.44	11.81
BG132890	Dihydroneopterin triphosphate pyrophosphohydrolase (NUDIX1)	0.44	0.83
BM410261	3-ketoacyl-CoA synthase 6	0.42	0.74
AI771544	Homeobox-leucine zipper protein HAT5	0.42	0.92
BT014212	Non-phosphorylating glyceraldehyde dehydrogenase	0.38	1.09
BT014113	Phosphosulfolactate synthase-related protein	0.36	1.17
BM412443	Calcium-binding EF hand family protein	0.36	0.58
BT014414	Flavonoid glucosyltransferase	0.36	20.43
BM535201	Nodulin ENOD18	0.35	2.49
AI773696	Lipid transfer protein (LTP2)	0.35	0.86
AW648171	Urease accessory protein G (ureG)	0.35	0.88
BG126449	Calcium:hydrogen antiporter (CAX3)	0.33	1.10
BM409988	Flavonoid glucosyltransferase	0.31	1.44
BM536272	Oxidoreductase 2OG-Fe(II) oxygenase	0.30	0.77
AF123256	Class II small heat shock protein Le-HSP17.6	0.30	1.13

AW624885	Unknown	0.28	2.09
AW223624	UDP-glycosyltransferase	0.28	1.55
U72396	Class II small heat shock protein Le-HSP17.6	0.11	1.34

Table S6. Genes showing significant differential expression in tomato roots upon treatment with 50 µM MeJA. Available at *JXB* online.

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CAPITULO 3: Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato

Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato

Juan A. López-Ráez, Tatsiana Charnikhova, Ivan Fernández, Harro Bouwmeester, Maria J. Pozo

Resumen.

Las estrigolactonas son una nueva clase de hormonas vegetales que han emergido como importantes moléculas de señalización en el control de la arquitectura de la planta. Además, son elementos clave para la comunicación de la planta con diferentes organismos de la rizosfera. Las estrigolactonas son exudadas por la raíz al suelo, donde actúan en la señalización entre las plantas y los hongos micorrízicos arbusculares (AM), favoreciendo la detección de la planta hospedadora por los hongos AM. Ademas las estrigolactonas actúan también como estimulantes para la germinación de las semillas de las plantas parasíticas de la raíz. Bajo condiciones de limitación de fosfato, las plantas aumentan la secreción de strigolactones a la rizosfera para promover la formación de la simbiosis AM. En el capitulo anterior, mediante el uso de la plantas de tomate como modelo, hemos demostrado que la simbiosis AM induce cambios en los perfiles hormonales y transcripcionales. En el presente estudio utilizando el mismo sistema modelo, hemos demostrado analíticamente que la producción de estrigolactonas se reduce considerablemente durante una simbiosis AM. Teniendo en cuenta el doble papel de la estrigolactonas en la rizosfera como señal para los hongos AM y las plantas parásitas, en ente trabajo se discuten las posibles consecuencias de los cambios en la producción de estrigolactonas sobre la interacción de la planta con ambos organismos.

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Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato

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Abstract

Strigolactones are a new class of plant hormones emerging as important signals in the control of plant architecture. In addition, they are key elements in plant communication with several rhizosphere organisms. Strigolactones are exuded into the soil, where they act as host detection signals for arbuscular mycorrhizal (AM) fungi, but also as germination stimulants for root parasitic plant seeds. Under phosphate limiting conditions, plants up-regulate the secretion of strigolactones into the rhizosphere to promote the formation of AM symbiosis. Using tomato as a model plant, we have recently shown that AM symbiosis induces changes in transcriptional and hormonal profiles. Using the same model system, here we analytically demonstrate, using liquid chromatography-tandem mass spectrometry, that strigolactone production is also significantly reduced upon AM symbiosis. Considering the dual role of the strigolactones in the rhizosphere as signals for AM fungi and parasitic plants, we discuss the potential implications of these changes in the plant interaction with both organisms.

Key words: Arbuscular mycorrhiza, biocontrol, root parasitic plants, strigolactones, tomato.

Introduction

Arbuscular mycorrhizal (AM) symbiosis is a mutualistic association established between certain soil fungi and most terrestrial plants, and it is considered a key step in the evolution of aquatic into terrestrial plants (Parniske, 2008). During the symbiosis, AM fungi obtain carbohydrates from their host plant and, in return, the plants obtain water and mineral nutrients (mainly phosphorous and nitrogen) from their fungal partners. In addition to improving the nutritional status, the symbiosis enables the plant to perform better under stressful conditions (Pozo and Azcón-Aguilar, 2007; Parniske, 2008). AM symbiosis establishment and functioning requires a high degree of coordination between the two partners based on a finely regulated molecular dialogue that orchestrates the complex symbiotic program (Paszkowski, 2006; Hause et al., 2007; Requena et al., 2007). The chemical dialogue starts in the rhizosphere, before there is any contact between the partners. Strigolactones are exuded by the plant roots into the soil and act as host detection signals for the AM fungi, stimulating their metabolism and hyphal branching (Akiyama et al., 2005; Parniske, 2008). In the rhizosphere, the strigolactones are also germination stimulants for root parasitic plant seeds of the family Orobancheaceae, such as *Striga*, *Orobanche* and *Phelipanche* spp. (Bouwmeester et al., 2007). In accordance with their AM fungi attracting role, under low nutrient conditions (mainly phosphorous and nitrogen), plants produce more strigolactones (Yoneyama et al., 2007; López-Ráez et al., 2008a), but this also increases the risk that the plant is abused by the parasitic weeds to establish a parasitic interaction (Bouwmeester et al., 2007). Recently, strigolactones have also been recognized as a novel class of plant hormones because, in addition to their role as signaling molecules in the rhizosphere, they also control plant architecture signaling inhibition of shoot branching (Gómez-Roldán et al., 2008; Umehara et al., 2008). Other phytohormones, such as ethylene, salicylic acid, abscisic acid and jasmonates have been proposed to be important regulators in the mutualistic interaction between plants and AM fungi (Herrera-Medina et al., 2003; Hause et al., 2007; Herrera-Medina et al., 2007; Riedel et al., 2008). We have recently shown that AM symbiosis alters both hormonal and transcriptional profiles in tomato roots, especially the oxylipin pathway (López-Ráez et al., 2010). With respect to strigolactones, there are indications

that regulation of their levels occurs in the host plant once the symbiosis has been established. It was shown that AM fungal inoculation of maize and sorghum led to a reduction of *Striga hermonthica* infection (Lendzemo et al., 2005), and it was proposed that this reduced infection was caused, at least partially, by a reduction in the production of strigolactones in the mycorrhizal plants (Lendzemo et al., 2007; Sun et al., 2008), although no analytical evidence was provided. In the present work, a reduction in strigolactone production during AM symbiosis is also shown in tomato plants, indicating a conserved response in dicotyledonous and monocotyledonous plant species. Moreover, the reduction in strigolactones by AM symbiosis is, for the first time, analytically demonstrated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The potential use of AM fungi as a sustainable alternative in programs for parasitic weed control is discussed.

Materials and methods

Plant growth and AM fungal inoculation

Tomato seedlings (*Solanum lycopersicum* L. cv. BetterBoy) were grown in pots with a sterile sand:soil (4:1) mixture and inoculated or not with a 10% (v:v) soil-sand-based inoculum containing *Glomus mosseae* (BEG12) or *Glomus intraradices* (BEG 121) propagules. Five plants per treatment were used. Plants were randomly distributed and grown in a greenhouse and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorous concentration (López-Ráez et al., 2010). Plants were harvested after eight weeks. For assessment of mycorrhizal colonization, roots were cleared and stained with trypan blue (Phillips and Hayman, 1970). The percentage of total root colonization was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

Germination bioassay and strigolactone analysis using liquid chromatography-tandem mass spectrometry

For strigolactone analysis, root exudates were collected, purified and concentrated as described previously (López-Ráez et al., 2008b). Briefly the substrate in the pots was

rinsed to remove any accumulated strigolactones. After 5 h, 500 ml nutrient solution was applied to the pots, the root exudates were collected and the roots harvested. Then, the crude exudates were concentrated and purified by solid phase extraction on a C_{18} SEPAK cartridge. The exudates solution was loaded onto the pre-equilibrated column and the active fraction eluted with 60% acetone:water. Within each experiment, the exudates were diluted to the same ratio of root fresh weight per ml of root exudates before analysis. For strigolactone analysis in root extracts, 0.5 g of roots were ground in a mortar with liquid nitrogen and then extracted twice with 0.5 ml of acetone in a 2 ml tube. Tubes were vortexed for 2min and centrifuged for 5min at 8000×g in a table top centrifuge. The organic phase was carefully transferred to 2ml glass vials and stored at −20 ºC until use.

Germination bioassays with *Phelipanche ramosa* seeds, as well as strigolactone analysis and quantification by LC-MS/MS, were performed as described in López-Ráez et al. (2008a, b), with the exception that LC-MS/MS analyses were performed using a Xevo tandem quadrupole (TQ) mass spectrometer (Waters) equipped with an ESI source.

Statistical analysis

Data for mycorrhization levels and strigolactone content were subjected to one-way analysis of variance (ANOVA) using SPSS Statistics v. 14.1 for Windows. To analyze the results of germination bioassays, ANOVA after arcsine [square root (X)] transformation was used.

Results and discussion

Germination stimulatory activity of root exudates from AM fungi-colonized tomato plants

The regulation of strigolactone production during AM symbiosis was assayed using tomato plants and two different AM fungi. In the case of *G. intraradices*, the percentage of colonized roots reached 75% after eight weeks of growth, whereas *G. mosseae* colonized only about 5% of the roots in this experiment. Strigolactone levels

in root exudates from mycorrhizal and non-mycorrhizal plants were estimated using a germination bioassay for *P. ramosa* seeds (López-Ráez et al., 2008a). A clear reduction (of about 50%) in the germination stimulatory activity of the exudates from roots colonized by *G. intraradices*, but not *G. mosseae*, was observed (Fig. 1A), revealing an inverse correlation between mycorrhization level and stimulation of germination. A similar reduction in germination of *Striga hermonthica* seeds was observed with root exudates from mycorrhizal sorghum (Lendzemo et al., 2007) and maize plants (Sun et al., 2008). The results show that AM symbiosis can induce a reduction in the germination stimulatory activity of the exudates of tomato plants, as previously shown for monocotyledonous plants (Lendzemo et al., 2007; Sun et al., 2008), suggesting that this is conserved across the plant kingdom. To assess whether the reduction is related to alterations in the secretion process or to biosynthesis, we also analyzed the germination stimulatory activity of root extracts. A similar reduction in germination was found with *G. intraradices* colonized roots, whereas germination with extracts of *G. mosseae*-colonized roots was not significantly different from the non-colonized control (Fig. 1B). These results suggest that the observed decrease in germination stimulant activity is caused by reduced production rather than by a decrease in exudation. Recently, we have shown that some of the changes in the host hormonal and transcriptional profiles associated with the AM symbiosis depend on the fungal species involved in the interaction (López-Ráez et al., 2010). To determine whether the absence of a reduction in *G. mosseae*-colonized plants is related to the AM genotype or to the degree of mycorrhization, we analyzed the germination stimulatory activity in an independent experiment in which the root colonization by *G. mosseae* was about 55%. Here, a significant reduction in the stimulatory activity was observed (Fig. 1C). Thus, the reduction in germination stimulatory activity depends on a fully established mycorrhizal association, and it is not just a consequence of the fungal presence in the rhizosphere or a particular response to specific AM species.

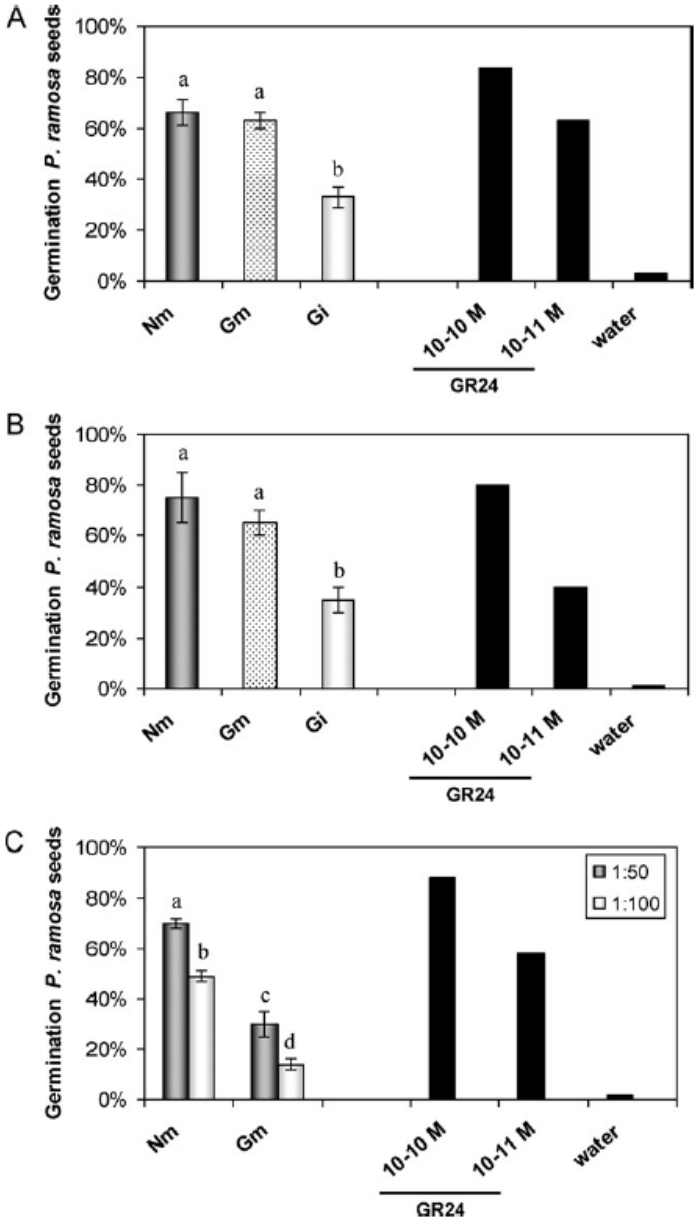

Figure 1. Effect of mycorrhizal colonization of tomato roots (cv. BetterBoy) inoculated with *G. mosseae* or *G. intraradices* on germination stimulatory activity of the root exudates and root extracts. Germination of *P. ramosa* seeds induced by the root exudates (A) or root extracts (B) of tomato plants colonized by *G. mosseae* (Gm) or *G. intraradices* (Gi) (5% and 75% colonization, respectively) or non-colonized (Nm). (C) Germination of *P. ramosa* seeds induced by the root exudates of tomato plants colonized by *G. mosseae* (Gm) (55% colonization) or non-colonized (Nm). Two different dilutions (1:50 and 1:100) of the root exudates were used. GR24 (10^{-10} and 10^{-11} M) and demineralized water (closed bars) were used as positive and negative controls, respectively. Bars represent the mean of five independent replicates ±SE. Different letters indicate statistically ($P < 0.01$) significant differences according to Fisher's LSD test.

LC-MS/MS analysis and quantification of strigolactones

To confirm that the reduction in the germination stimulatory activity observed in *G. intraradices*-colonized plants is the consequence of a reduction in the production of strigolactones by the plant, the root exudates from mycorrhizal and non-mycorrhizal plants were analyzed and the strigolactones quantified by LC-MS/MS. The three major tomato strigolactones detected (solanacol and two didehydro-orobanchol isomers) (López-Ráez et al., 2008b) were significantly ($P < 0.01$) reduced in the exudates of *G. intraradices*-colonized plants compared with the non-mycorrhizal controls (Fig. 2A). The reduction in the levels of solanacol and the didehydro-orobanchol isomers 1 and 2 was 54, 42 and 40%, respectively. This matches very well with the approximately 50% reduction in the germination of *P. ramosa* seeds (Fig. 1A and B). The other strigolactone described thus far in tomato (orobanchol) was also detected, but its concentration was too low for accurate quantification. In agreement with the germination bioassay, no differences in strigolactone levels were observed in root exudates from plants colonized by *G. mosseae* (Fig. 2A). In conclusion, we analytically confirm here, for the first time, that AM symbiosis reduces the production of strigolactones by the host plant, and that this reduction is dependent on the extent of root colonization. It has been previously suggested that a reduction in strigolactones might be involved in the regulation of mycorrhization as a plant strategy to avoid excessive mycorrhizal colonization, a phenomenon known as autoregulation (García-Garrido et al., 2009). The mechanism by which AM symbiosis reduces strigolactone production is unknown. A direct effect of mycorrhization on strigolactone biosynthesis or an indirect effect through the improvement of the plant nutritional status by the symbiosis are possible explanations. Further research is needed to determine the contribution of each of these mechanisms. Since strigolactones are rhizosphere signaling molecules for both AM fungi and root parasitic plants, the fact that AM symbiosis reduces strigolactone production by the host plant opens the possibility of using these beneficial fungi to develop a parasitic weed control strategy. Indeed, the benefits of this mutualistic association on plant fitness are well known. AM symbiosis not only influences plant nutrition (Parniske, 2008), but also helps the plant to overcome both biotic and abiotic stresses (Pozo and Azcón-Aguilar, 2007). One of

these biotic stresses reduced through the use of AM fungi is the infection of cereals by *Striga*, as demonstrated by Lendzemo et al. (2005). Here, we show that this may also hold for *Orobanche*/*Phelipanche* parasites infecting dicotyledonous hosts. In addition, we show that this effect is due to reduced biosynthesis of strigolactones in the roots upon AM infection. As AM fungi colonize roots of most agricultural and horticultural species, this environmentally friendly biocontrol strategy can potentially be used in the majority of important crops worldwide that suffer from these root parasites (López-Ráez et al., 2009).

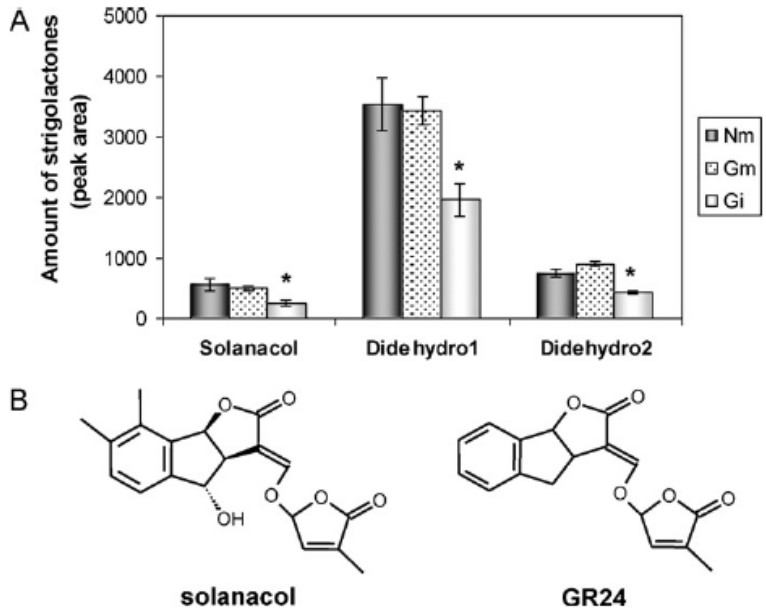

Figure 2. Strigolactone content in tomato root exudates analyzed by LC-MS/MS and strigolactone structures. (A) Strigolactone content in tomato root exudates. Bars correspond to the amount (according to the peak area) of the strigolactone solanacol and the didehydro-orobanchol isomers 1 (Didehydro1) and 2 (Didehydro2) of tomato plants colonized by *G. mosseae* (Gi) or *G. intraradices* (Gi) (5% and 75% colonization, respectively) and non-colonized (Nm). Bars represent the mean of five independent replicates ±SE. Asterisks (*) indicate statistically ($P < 0.01$) significant differences according to Fisher's LSD test. (B) Chemical structure of the tomato strigolactone solanacol and the synthetic strigolactone analogue GR24.

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CAPÍTULO 4: An essential role of systemin in AM symbiosis through the regulation of strigolactone biosynthesis

An essential role of systemin in AM symbiosis through the regulation of strigolactone biosynthesis

Iván Fernández, Juan A. López-Ráez, Juan M. García, Tatsiana Charnikhova, Concepción Azcón-Aguilar, Harro Bouwmeester, María J. Pozo

Resumen.

La hormona péptidica sistemina (Sys), la cual es procesada a partir de su precursor denominado prosistemina (PS), se expresa en la parte aérea de varias especies de plantas del grupo de las solanáceas. La Sys, así como otros péptidos similares, son importantes moléculas de señalización implicadas en los primeros estadíos de la respuesta de defensa de la planta frente al ataque de herbívoros y patógenos. La Sys actúa como molécula señal en la respuesta de defensa de la planta mediante la regulación de la ruta de biosíntesis de los jasmonatos. En el presente estudio mostramos por primera vez que las plantas de tomate son capaces de expresar en la raíz el gen que codifica para la PS. Además, observamos que la expresión de PS en la raíz, independiente de la ruta de los jasmonatos, es esencial para el establecimiento de una simbiosis AM. Por otro lado las estrigolactonas son consideradas una nueva clase de hormonas vegetales. Las estrigolactonas en la rizosfera actúan como estimulantes de la germinación de plantas parásitas de la raíz, actuando además como factores de ramificación de las hifas de los hongos AM. En este estudio hemos observado que líneas de tomate que tienen comprometida la producción de PS, mostraban una disminución en la producción de estrigolactonas, lo que sugiere que la PS puede controlar el establecimiento de la simbiosis AM a través de la regulación de la biosíntesis de estrigolactonas.

In preparation, 2013

An essential role of systemin in mycorrhizal symbioses through the regulation of strigolactone biosynthesis

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Abstract

The peptide hormone systemin, which is processed from a longer precursor prosystemin (PS), is a central regulator of the wound response in the Solanaceae family. Systemin acts in early defence responses against herbivores through the activation of a signalling cascade leading to the biosynthesis of jasmonates. Systemin is believed to be expressed only in aboveground tissues, and only recently it has been related to defence responses to microbial pathogens. We report here unexpected and novel functions of systemin, since we demonstrate for the first time that prosystemin is expressed in roots of tomato plants and it has a critical role in the AM symbiosis establishment and regulates root system architecture. We provide evidences that these functions are independent of the jasmonate signalling pathway. Surprisingly, mutants altered in prosystemin expression (overexpresor and antisense lines) showed strongly altered levels of strigolactones, plant hormones with key signalling functions in the rhizosphere. Strigolactones play a central role in the communication between plants and mycorrhizal fungi in the presymbiotic stages, acting as hyphal branching factors for germinating spores of AM fungi. Our results provide evidences supporting that prosystemin promotes mycorrhizal establishment through the regulation of strigolactone biosynthesis.

Key words: Arbuscular mycorrhiza, jasmonates, prosystemin, roots, strigolactones, systemin.

INTRODUCTION

In nature plants have to face with numerous challenges including abiotic and biotic stresses such as drought stress, UV light, wounding, pathogen attack, etc to survive. A strategy to cope with these environmental factors is the production of chemical responses that are mediated by plant hormones (Wasternack et al., 2006; Bari and Jones, 2009; Pieterse et al., 2009). Among them, ethylene, salicylic acid, abscisic acid and jasmonic acid (JA) are known to be key elements in regulating plant defence responses during interaction with other organisms (Bari and Jones, 2009; Pieterse et al., 2009). Over the last two decades JA and its derivates, known as jasmonates, have received special attention because of their role in the signal transduction pathway activated upon wounding of leaves by insects (Schilmiller and Howe, 2005; Wasternack, 2007) and due to their involvement in the interaction of plants with other microorganisms (Pozo et al., 2004; Hause and Schaarschmidt, 2009). Within this scenario, tomato plant was taken as a model system to study the wounding response and, as a consequence, a number of wound-response tomato mutants related to jasmonates have been isolated and characterized. Based on their response to different stimuli and their capacity to synthesize JA, these mutants can be classified as affected in jasmonate biosynthesis, jasmonate perception and signalling, and systemin function (Schilmiller and Howe, 2005; Wasternack et al., 2006). A key element in plant wound-response is systemin, the first peptide signal discovered in plants (McGurl et al., 1992). Systemin was first identified in tomato leaves and consists in an 18-amino acid peptide hormone, which is processed from the C-terminal of a 200-amino acid precursor, called prosystemin, upon insect attack. It induces and amplifies both the local and systemic production of defence proteins, such as poliphenol oxidases and protease inhibitors, by regulating the biosynthesis of jasmonates and, therefore, protecting plants against insect herbivores (McGurl et al., 1992; Bergey et al., 1996). Systemin or systemin-like peptides (hydroxyproline-rich homologues) have been only described in the Solanaceae family (McGurl et al., 1992; Wasternack, 2007; Pearce, 2011). However,

recently small peptides associated to the plant innate immune response and involved in the wound signalling have also been identified in other plant species as *Arabidopsis* and sweet potato (Huffaker et al., 2006; Chen et al., 2008). So far the gene encoding prosystemin has been described to be expressed in all plant tissues except in the roots (McGurl et al., 1992; Narvaez-Vasquez and Ryan, 2004; Wasternack et al., 2006).

We have recently shown that arbuscular mycorrhizal (AM) symbioses, that are mutualistic associations between soil fungi and most vascular plants (Parniske, 2008), alter both transcriptional and hormonal profiles, specially the oxylipin pathway, pointing to a key regulatory role of jasmonates in the establishment of this type of symbiosis (López-Ráez et al., 2010). Indeed, these phytohormones have received special attention because they are believed to play a major role in the AM symbiosis, although the data are controversial (Hause et al., 2007; Gutjahr and Paszkowski, 2009; Hause and Schaarschmidt, 2009). Interestingly, by microarray analysis a number of defence genes related to the jasmonate-wounding response and associated to systemin, such as proteinase inhibitors, polyphenol oxidases, threonine deaminase and multicystatin, were found to be up-regulated in tomato roots colonized with the AM fungus *Glomus mosseae* (López-Ráez et al., 2010), suggesting that systemin could be involved in the establishment and/or development of AM symbiosis. This symbiotic interaction starts through a mutual recognition between the two partners in the rhizosphere, where the strigolactones play a crucial role as host detection signals. Strigolactones are derived from carotenoids through oxidative cleavage by carotenoid cleavage dioxygenases, being classified as apocarotenoids (Matusova et al., 2005; López-Ráez et al., 2008a; Rani et al., 2008). They are considered a new class of plant hormones (Gomez-Roldan et al., 2008; Umehara et al., 2008), that in the rhizosphere act as germination stimulants of root parasitic plants and as hyphal branching factors for germinating spores of AM fungi (Akiyama et al., 2005; Bouwmeester et al., 2007).

In the present work, using tomato as a model plant, the involvement of jasmonates and jasmonate-related molecules on AM symbiosis was assessed. The study revealed the appearance of a new key player - systemin - in the regulation of the establishment of this symbiotic interaction. Its relationship with other important plant signalling molecules in AM symbiosis, as the strigolactones, was also analyzed.

Materials and Methods

Plant material and chemicals

Tomato (*Solanum lycopersicum* L.) cv. Castlemart and Betterboy were used in this study. Tomato mutants affected in JA biosynthesis or signalling *jai1*, *spr1*, *spr2*, *def1* and corresponding wild-type cv. Castlemart were kindly provided by Dr. Gregg Howe (Michigan State University, US). Tomato mutants constitutively expressing the prosystemin encoding gene in sense and antisense orientation were used. Seeds of the mutant *ps-* and its corresponding wild-type cv. BetterBoy were kindly provided by Dr. Clarence Ryan (Washington State University, Pullman, US). Seeds of the mutant ps^{+} and its corresponding wild-type cv. Castlemart were kindly provided by Dr. Gregg Howe (Michigan State University, US). Seeds of *Phelipanche ramosa* were provided by Dr. Maurizio Vurro (Instituto delle Produzioni Alimentari, Bari, Italy). The synthetic strigolactone analogue GR24 was kindly provided by Binne Zwanenburg (Radboud University, Nijmejen, The Netherlands), and the strigolactone standard solanacol was provided by Dr. Koichi Yoneyama (Utsunomiya University, Japan).

Growth conditions and experiments

Tomato seeds were surfaced-sterilized in 4% sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water and germinated for 3 d on a container with sterile vermiculite at 25°C in darkness. Plants were randomly distributed and grown in a greenhouse at 24/16°C with 16/8 h photoperiod and 70% humidity and watered three times a week with Hewitt nutrient solution (Hewitt, 1966). For each treatment, five independent plants were used. For mycorrhizal experiments the AMF *G*. *mosseae* (BEG 12), *G*. *intraradices* (BEG 121) and *Gigaspora margarita* were maintained as a soil-sand based inoculum, and plants were grown as described previously (López-Ráez et al., 2010). Fungal structures within the roots were detected through histochemical staining with trypan blue (Phillips and Hayman, 1970) and examined using a Nikon Eclipse 50i microscope and bright-field conditions. Parameters of mycorrhizal colonization were determined as described by Trouvelot and co-workers (Trouvelot et al., 1986) and calculated using the program MYCOCALC (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

For MeJA and wounding treatments tomato cv. BetterBoy plants were grown hydroponically in 3 L plastic containers with Hewitt nutrient solution with constant aeration. The nutrient solution was replaced once a week. In the case of MeJA, four week-old plants were individually transferred to 50 mL plastic tubes filled with nutrient solution with or without 50 µM MeJA (Sigma-Aldrich) and maintained for 4 or 24 h. For wounding treatment roots were damaged using forceps and then kept into a 50 mL plastic tubes with nutrient solution for 24h. Upon treatments, the roots were rinsed with sterilized deionised water, frozen in liquid nitrogen and stored at -80ºC until use.

For strigolactone production and analysis, seedlings of *ps*$^{-}$ and *ps+* mutants and corresponding wild-type (cv. BetterBoy) were grown in a greenhouse as described previously (López-Ráez et al., 2008b). Since strigolactone production is promoted under low phosphate conditions (López-Ráez et al., 2008b; Yoneyama et al., 2007), one week before root exudates collection the substrate was rinsed and then the plants were watered with Hewitt nutrient solution without phosphate. The root exudates were collected, purified and concentrated as described in López-Ráez et al. (2008b). Roots from each pot were then collected separately, frozen in liquid nitrogen and stored at at -80ºC until use.

For *in vitro* mycorrhization studies, non-transformed tomato root organ cultures (ROC) from the mutant *ps*$^{-}$ were established basically as described by Bago et al. (2006). Monoxenic AM cultures were initiated by placing a mycorrhizal carrot root segment from an established monoxenic culture used as inoculum, consisting of Ri T-DNA (*Agrobacterium rhizogenes*)-transformed carrot (*Daucus carota* L. clone DC2) roots colonized with the AM fungus *Glomus intraradices* DAOM 197198. Petri plates were incubated in the dark at 24 °C until hyphal development was observed and roots were colonized by the fungus. For the experiment with exogenous application of systemin, the peptide obtained from Genescript (USA) was applied to the medium as an aqueous solution to obtain final concentrations of 1, 10 and 100 pmol.

For the rescue experiment with strigolactones, *ps*- plants inoculated with *G. intraradices* were grown as described before (López-Ráez et al., 2010) and treated with semi-purified tomato strigolactones from the beginning. Once a week, plants were watered with Hewitt nutrient solution (25% of the standard phosphorous concentration) supplemented with 0, 1 or 10 nM of natural strigolactones. Tomato

strigolactones were obtained from root exudates of the wild-type BetterBoy. The exudates from 20 plants were collected, pooled and concentrated as previously described (López-Ráez et al., 2008b). For purification, the C18 SEPAK cartridge was washed with 40% acetone. Then, strigolactones were collected by elution with 50% acetone. The strigolactone concentration was assessed by germination bioassays with *P. ramosa* seeds (López-Ráez et al., 2008b). The germination activity of the tomato exudates was compared with that of known concentrations of the strigolactone analogue GR24. Eight-week old plants were harvested and the root system reserved for mycorrhizal quantification.

Grafting experiments and mycorrhizal colonization

Tomato plants for the mutants *ps-* and *ps+* and corresponding wild-types, cv BetterBoy and Castlemart, respectively were sterilized, inoculated with *G. mosseae* and grown as described above. Three-week old plants were sectioned within the hypocotyls region and the combinations of scion and rootstock were aligned and sealed with a sterile silicone clamp to form a graft union. Seedlings were incubated in a controlled chamber at 21/18ºC with 9/15 h photoperiod and 80% humidity for one week. Following healing of the graft union, seedling were transferred to the greenhouse and grown as described above. Plants were harvested after 8 weeks of growth for mycorrhizal quantification.

RNA isolation gene expression analysis by real time quantitative RT-PCR (qPCR)

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel) and stored at -80ºC until use. Real time quantitative RT-PCR (qPCR) was performed using the iCycler iQ5 system (Bio-Rad) and gene specific primers (Table 1). The first strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Three independent biological replicates were analyzed per treatment. Relative quantification of specific

mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene *SlEF*, which encodes for the tomato elongation factor-1 α.

Table 1. Primers used for gene expression analysis in this study.

ID	Tomato Gene	Primers (5'-3')
X14449	Elongation factor 1α (Sl-*EF1*)[2]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
DQ282611	*G. intraradices* elongation factor 1-α (*GintEF-2*)[4]	GCTATTTTGATCATTGCCGCC TCATTAAAACGTTCTTCCGACC
AY885651	Phosphate transporter (Le-*PT4*)[3]	GAAGGGGAGCCATTTAATGTGG ATCGCGGCTTGTTTAGCATTTC
M84801	Prosystemin (Sl-*PS*)[1]	AATTTGTCTCCCGTTAGA AGCCAAAAGAAAGGAAGCAAT

[1]This work; [2]Rotenberg *et al.*, 2006; [3]Balestrini et al., 2007; [4]Benabdellah et al., 2009.

Prosystemin cDNA cloning from tomato roots

The cloning of the full-length cDNA prosystemin was performed by RT-PCR over RNA from tomato roots treated with MeJA (50 µM) and using specific primers (*PS-root F*: 5'-CTAAGAATTCCATGGGAACTCCTTC-3' and *PS-root R*: 5'-GTTTCTCGAGTTTATTATTGTCTG-3'), designed according to the tomato *prosystemin* sequence isolated from the shoots, with AccuPrime Taq DNA Polymerase High-Fidelity (Invitrogen). PCR program involved: 94ºC for 1 min, 35 cycles of 94ºC for 30 s, 52ºC for 30 s and 68º for 1 min, and a final extension at 68ºC for 10 min. The amplified cDNA fragment of 618 bp was modified using the A-tailing procedure, cloned into pGEM-T Easy vector (Promega) according to the manufacturer instructions and then sequenced by both strains.

Germination bioassay and strigolactone analysis using liquid chromatography-tandem mass spectrometry

For strigolactone analysis on root extracts, 0.5 g of root tissue was ground in a mortar with liquid nitrogen. The samples were extracted with 1 ml of cold acetone in 2 ml eppendorf tube. The tubes were vortexed for 5 min and then centrifuged for 5 min at 8000 *g* in a table centrifuge. Then the organic phase was carefully transferred to a

clean 2 ml tube. The pellets were re-extracted with another 1 ml of acetone. The combined acetone fractions were used for germination bioassays with *P. ramosa* seeds as described previously (López-Ráez et al., 2008b). Strigolactone analysis and quantification on root exudates were performed by LC-MS/MS as described in López-Ráez et al. (2008b).

Statistical analysis

Data for mycorrhization levels in roots, qPCR analysis and strigolactone content in tomato root exudates were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 14.1 for Windows. To analyze the results of germination bioassays, ANOVA after arcsine [squareroot (X)] transformation was used. When appropriate, data were subjected to the Duncan's honestly significant difference test.

RESULTS

Prosystemin mutants are unable to establish the arbuscular mycorrhizal symbiosis

Oxylipins, which include the plant hormones jasmonates, are thought to play a major role in arbuscular mycorrhizal (AM) symbioses in different plant species, supporting the idea of an evolutionary conserved model of root colonization (Hause and Schaarschmidt, 2009; Hause et al., 2007; López-Ráez et al., 2010). To investigate the involvement of jasmonate signalling we performed a study on mycorrhizal colonization by the AM fungus *Glomus mosseae* in several tomato mutants affected either in the jasmonate/prosystemin biosynthesis or signalling pathways (Fig. 1). The JA biosynthesis *def1* and *spr2* mutants showed colonization levels similar to those in the corresponding wild type Castlemart. The JA insensitive mutant *jai1*, deficient in JA signalling showed colonization levels higher than those in the wild type, as previously described (Herrera-Medina et al., 2008). Remarkably, higher differences were found with the prosystemin related mutants. In *ps+*, constitutively expressing prosystemin, a higher colonization level was observed, while almost no colonization was detected in the mutant constitutively expressing prosystemin in the antisense orientation (*ps-*).

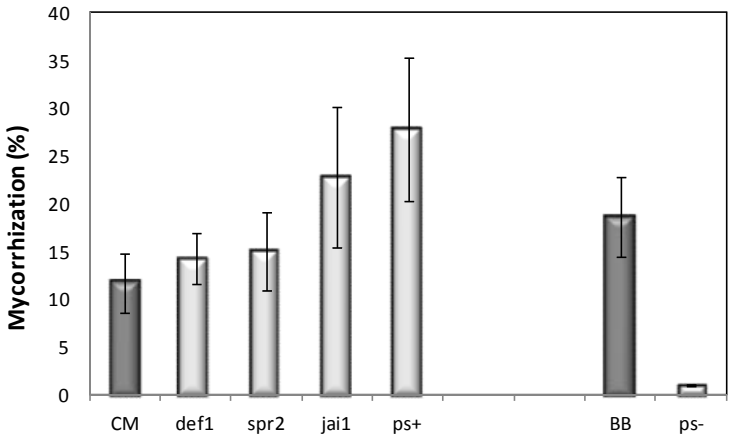

Figure 1. Mycorrhizal colonization of tomato mutants affected either in jasmonate/prosystemin biosynthesis or related signaling pathway, inoculated with *G. mosseae*. Data represent the means of 5 independent replicates (±SE). CM and BB castlemart and Betterboy wildtype cultivars. *jai1*, jasmonate insensitive mutant, *def1* and *spr2* tomato mutants deficient in JA production. *ps+* prosytemin overexpresor line, *ps-*, prosystemin antisense line.

The histochemical analysis revealed that only an antisense mutant for prosystemin (McGurl et al., 1992) (herein referred to as *ps*-) showed an almost complete absence of mycorrhization compared with corresponding wild-type (Fig 2A). In the *ps*- mutant, the typical internal structures formed during AM symbiosis - arbuscules and vesicles - were not observed, being the AM colonization restricted to some dispersed entry points that did not extend within the root (Fig. 2B). To further assess the involvement of prosystemin in this mutualistic association, we examined the mycorrhizal colonization of a tomato transgenic line overexpressing the gene prosystemin (McGurl et al., 1994) (herein referred to as *ps+*) with *G. mosseae*. Conversely to the antisense mutant, *ps+* roots were about 2-fold more colonized than the wild-type (Fig 2A), showing an increased number of arbuscules and vesicles (Fig. 2B). The histochemical analysis was further verified by quantifying the amount of AM fungus within the root tissues using quantitative real time polymerase chain reaction (qPCR) with total RNA from tomato colonized roots. Transcripts of the *Glomus* gene *GintEF-2*, encoding the elongation factor EF-2 (Benabdellah et al., 2009), accumulated over 2.5-fold in *ps+* roots compared with the wild-type, whereas no transcripts were detected in *ps*- (Fig. 2C). Similar results were obtained when using the gene *LePT4* (Fig. 2C), which encodes an AM-specific plant phosphate transporter. This gene is specifically expressed in arbusculated cells and considered as a marker for a functional symbiosis (Balestrini et al., 2007; Nagy, 2005). Therefore, the results demonstrate that prosystemin, or its systemin derivative, is necessary for an appropriate mycorrhizal establishment and functioning.

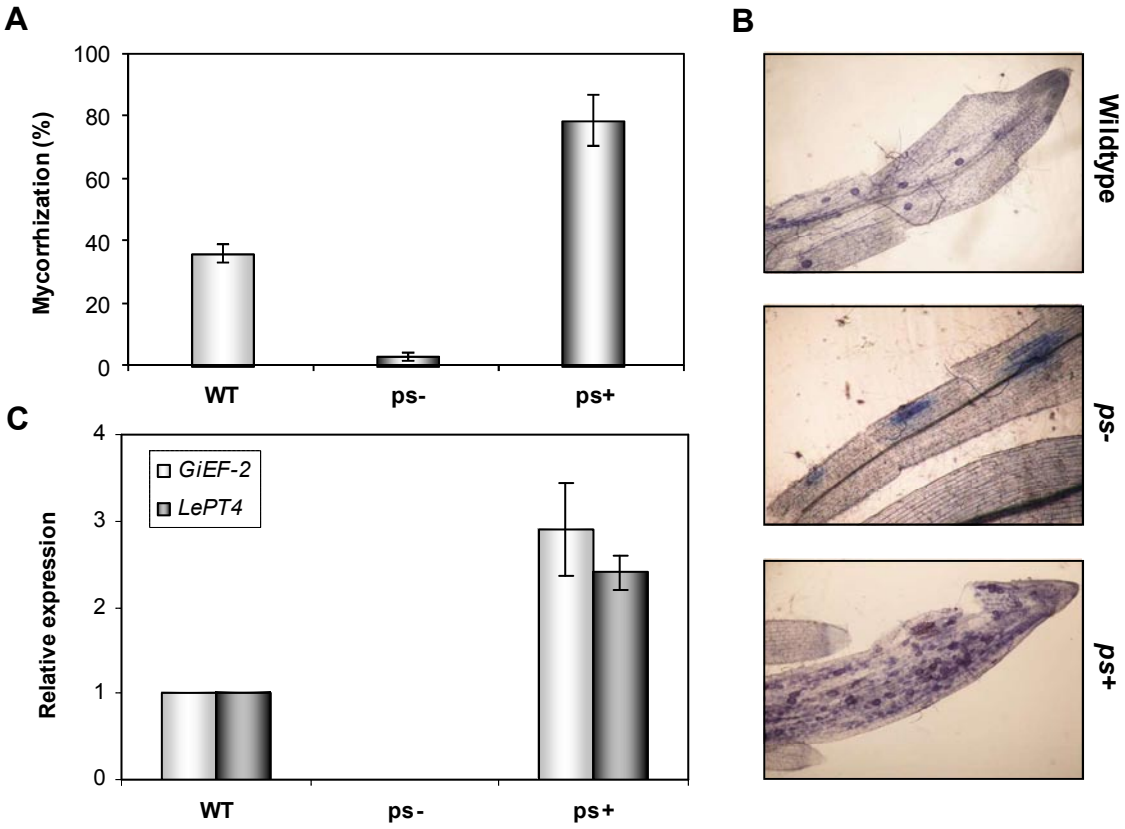

Figure 2. Colonization of roots of tomato plants affected in prosystemin expression, *ps-*, *ps+* and its corresponding wild-type (WT), by the arbuscular mycorrhizal fungus *G. mosseae* (A) Intensity of mycorrhizal colonization determined by histochemical staining (B) Photographs of root samples after trypan blue staining of fungal structures (C) Gene expression analysis by real-time qPCR for the mycorrhizal markers gen *GintEF-2* and *LePT4*. Changes in gene expression were calculated by using the $2^{-\Delta(\Delta Ct)}$ method. Data points represent the means of five (A) or three (C) replicates (±SE).

To address whether such striking reduction in the ability to establish the symbiosis of the mutant was exclusively associated to *G. mosseae*, different AMF (including fungi from different genera) were also checked for their ability to colonize *ps*- plants. As shown in Figure 3, none of the AMF assayed, *G. mosseae*, *G. intraradices* and *Gigaspora margarita*, was able to colonize the *ps*- mutant, indicating that the mutant is dramatically affected in its capacity to establish AM symbioses, and that this effect is common for even very divergent AMFs.

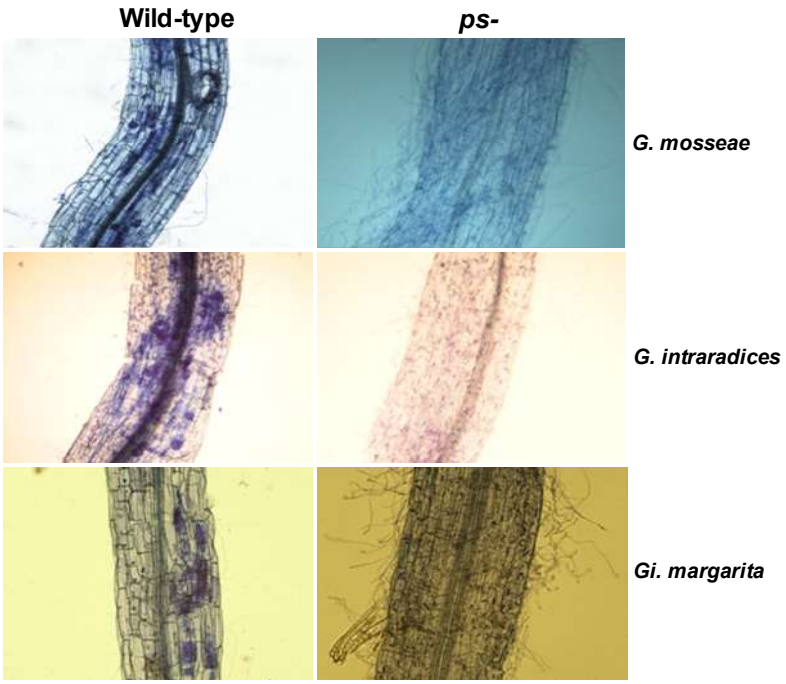

Figure 3. Histochemical staining of fungal structures in tomato roots of wild type or *ps-* mutant inoculated with different arbuscular mycorrhizal fungi: *Glomus mosseae*, *G. intraradices* and *Gigaspora margarita.*

Prosystemin antisense mutants are not deficient in JA accumulation

Although prosystemin signalling is related to jasmonate biosynthesis in the shoots, the absence of defects in mycorrhizal colonization of the JA mutants tested (Fig. 1) suggested that the effect of PS mutants is not related to deficiencies in JA production. In order to rule out any important deficiency in the JA content of the roots in the *ps-* mutants the content of the JA related compounds OPDA, JA and JA-Ile and were determined by UPLC-MS analysis (Fig. 4). Also, the levels of the other phytohormones involved in plant defence response (ABA and SA) were analysed. None of these metabolites were reduced in the root systems of *ps-* plants. Accordingly, PS function in the roots related to mycorrhizal establishment is likely JA, ABA and SA independent.

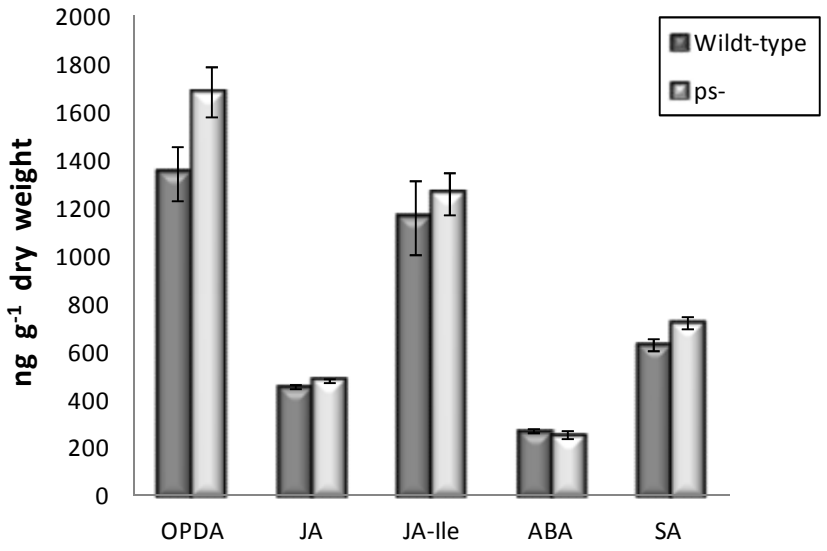

Figure 4. Levels of oxophytodienoic acid (OPDA), free jasmonate (JA), jasmonic acid isoleucine conjugate (JA-Ile), salicylic acid (SA) and abscisic acid (ABA) were determined by UPLC-MS in roots of the mutant *ps-* and corresponding wild-type (BB). Data points represent the means of 4 replicates (±SE).

Prosystemin is required in the roots for mycorrhizal establishment

It was reported that the prosystemin gene is expressed in all plant tissues except the roots (McGurl et al., 1992; Narvaez-Vasquez and Ryan, 2004) where AM colonization takes place. In this scenario, two hypotheses might be envisaged: either systemin is required in the shoots for a correct symbiosis establishment, or it is synthesized in the shoots and then basipetally transported to the roots. To test these hypotheses, we performed a grafting experiment to compare the mycorrhizal colonization in different combinations of rootstocks and scions from the *ps-* and ps*+* mutants and corresponding wild-type. Surprisingly, mycorrhization was drastically reduced only in the grafts with rootstocks from the antisense line (*ps-*), regardless the scion (Fig. 5A), indicating that expression of prosystemin in the roots is required for mycorrhizal establishment. As previously, qPCR analysis of the marker genes *GintEF-2* and *LePT4* confirmed the lack of AM colonization and symbiosis functioning, respectively (Fig. 5B).

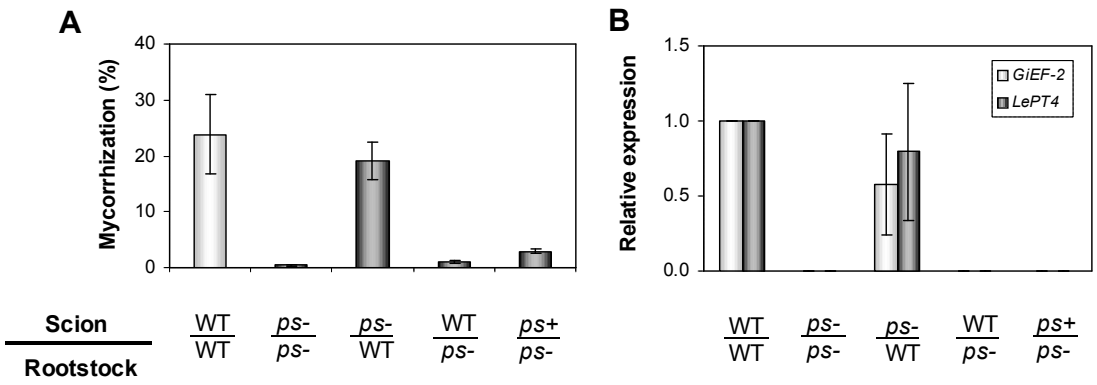

Figure 5. Mycorrhizal colonization by *G. mosseae* (A) and expression analysis of the mycorrhizal markers genes *GintEF-2* and *LePT4* (B) in grafting of tomato plants with different combinations of rootstocks and scions from the *ps-* and *ps+* mutants and their corresponding wild-type (WT). Gene expression analyses were performed by real-time qPCR. Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of five (A) or three (B) replicates (±SE).

Prosystemin is expressed and transcriptionally regulated in tomato roots

According to the grafting experiments, prosystemin expression is required in the roots for AM establishment; however, prosystemin is reported to be not expressed in roots (McGurl et al., 1992). To clarify this point, we aimed to determine whether there is indeed prosystemin gene expression in tomato roots. Interestingly, although at low levels, transcripts encoding prosystemin were detected for the first time in roots (Fig. 6A). The amplified product was cloned and sequenced, and the nucleotide sequence was identical to that of prosystemin from the shoots. We then aimed to determine if the *PS* gene is constitutively expressed or transcriptionally regulated in the roots. We firstly analyzed the possible regulation related to mycorrhizal colonization and we found that prosystemin expression was 7-fold higher in *G. mosseae* colonized roots (Fig. 6A).

Systemin is known to be involved in the regulation of local and systemic wound responses by activating the jasmonate pathway through a positive feedback loop (Wasternack, 2006). Thus, its expression levels in leaves are induced by artificial wounding or herbivory and by exogenous application of JA. We addressed if a similar regulation was taken place in roots, and, consequently, transcripts of prosystemin accumulated after wounding or MeJA treatment of the roots. Indeed, its expression levels in the roots were enhanced about 70-fold after 24 h wounding, and above 300-fold after treatment with 50 µM MeJA (Fig. 6B). Additionally to the transcriptional

analysis, the presence of the prosystemin peptide in mycorrhizal roots was verified by immunobloting analysis using crude protein root extracts and systemin antiserum. The increase at the transcriptional level seems to correlate with the peptide levels (Fig. 6C).

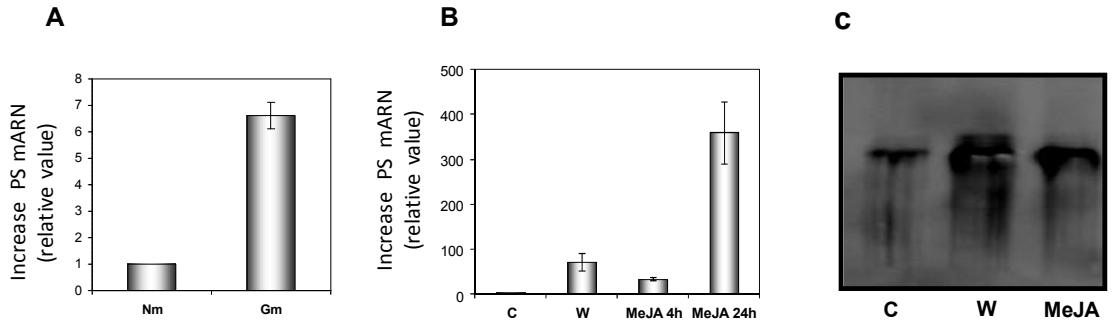

Figure 6. Effect of *G. mosseae* and different elicitors on the level of prosystemin/systemin in roots of tomato plants. (A) Expression of prosystemin gene in roots of tomato plants colonized by *G. mosseae* (Gm) during 8 weeks with respect to the non-mycorrhizal control plants (Nm). (B) Expression of prosytemin in roots of 4-weeks old plants treated for 4h and 24h with Methyl-jasmonate (MeJA), or after 24h of wounding (W) in relation to the non-treated control plants (C). Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of 3 independent biological replicates (±SE). (C) Westernblot analysis of systemin in roots after wounding and MeJA (24h) treatment.

Monoxenic cultures confirmed a deficient AMF development in root cultures of the *ps*- mutant

Respect to the role of prosystemin in AM symbiosis, its increase in mycorrhizal roots is in agreement with increased levels of jasmonates in mycorrhizal tomato (López-Ráez et al., 2010). Therefore, it is logical to speculate with a possible role of prosystemin in regulating jasmonates during AM establishment. However, the fact that the mutants affected in jasmonates biosynthesis and signalling were not impaired in their capacity to establish the symbiosis (Fig. 1), point to a role of prosystemin independent of JA signalling. To further investigate the mechanisms underlying the absence of AM colonization in the prosystemin antisense mutant and decipher the exact role of systemin in the AM symbiosis, we used an *in vitro* system to monitor the mycorrhization process. Root organ cultures (ROC) from *ps*- plants were established and then inoculated with hyphae and spores from monoxenic cultures of the AM fungus *G. intraradices*, as previously described (Bago et al., 2006). As in the *in vivo* experiment, mycorrhizal colonization of *ps*- ROCs was completely inhibited (Fig. 7). External mycelia developed abundantly on ROC from the wild-type roots, and

hundreds of spores were formed (Fig. 7C and E), while no external mycelium nor spores were found in the monoxenic cultures from *ps-* roots (Fig. 7D and F). Some of the roots were stained for the observation of intraradical fungal structures, but accordingly with the development of external mycelium, root colonization was only observed in wild-type roots (Fig. 7G) and it could not be detected in *ps-* (Fig. 7H). Remarkably, this fact did not correlate with aborted infection events, as observed in other mutants where AM hyphae reach the roots, form appresoria but are blocked at this stage. On the contrary, no hyphal development was observed in the plates containing *ps-* roots, while profuse development of external mycelia and spores occurred in the ROCs from the wild-type (Fig. 7).

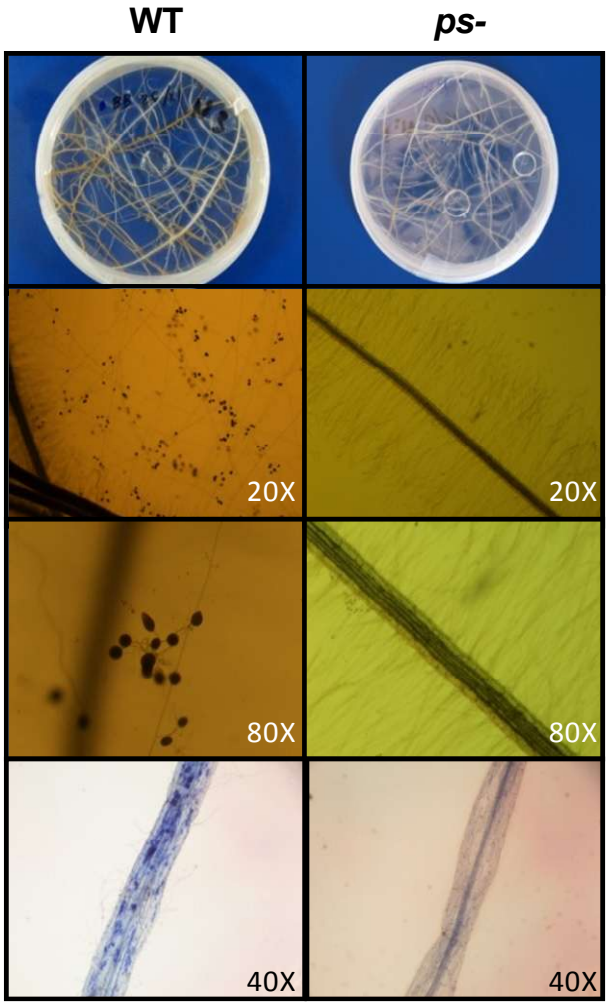

Figure 7. Fungal development and mycorrhizal colonization in monoxenic cultures of *G. intraradices* and non transformed roots of the wild type and *ps-mutant* and its corresponding wild-type (BB) development *in vitro*. General aspect of the cultures (A and B). External mycelia developed and spores of *G. intraradices* in cultures with wild type roots (C and E) but not in those of the *ps*-mutant (D and F). Intraradical colonization in wild type roots (G) and in *ps-* (H).

The results described above suggest a defect in the very early stages of the symbiotic interaction in the mutant, likely at the chemical communication between the two partners. Interestingly, the mycorrhizal phenotype was partly rescued upon exogenous

application of systemin. Different concentrations in the picomolar order were tested and added to the media and different parameters as external mycelium development, spore formation and viability, formation of Branched Absorbing Structures (BAS), root development and pigmentation were evaluated (Table 2). The application of 10 pM of synthetic systemin significantly increased all determined parameters (Table 2 and Fig. 8).

	External mycelium	Spore formation	BAS formation
wt			
Control	++	++	+
1 pmol	+++	+++	+++
10 pmol	++	+	+++
100 pmol	+++	+++	+++
ps-			
Control	-	-	-
1 pmol	-	-	-
10 pmol	+	+	-
100 pmol	-	-	-

Table 2. Effect of systemin application on fungal development in monoxenic cultures of roots from wild type Beterboy (wt) and the prosystemin antisense mutant *ps-*. Synthetic systemin was added to the growing media at the indicated concentrations. Development of external mycelium, spore formation and BAS formation were scored in arbitrary categories ranging from no structures observed (-) to high abundance (+++). BAS: Branching absorbing structures, ramifications formed in the external mycelia of AMF responsible of nutrient absorption

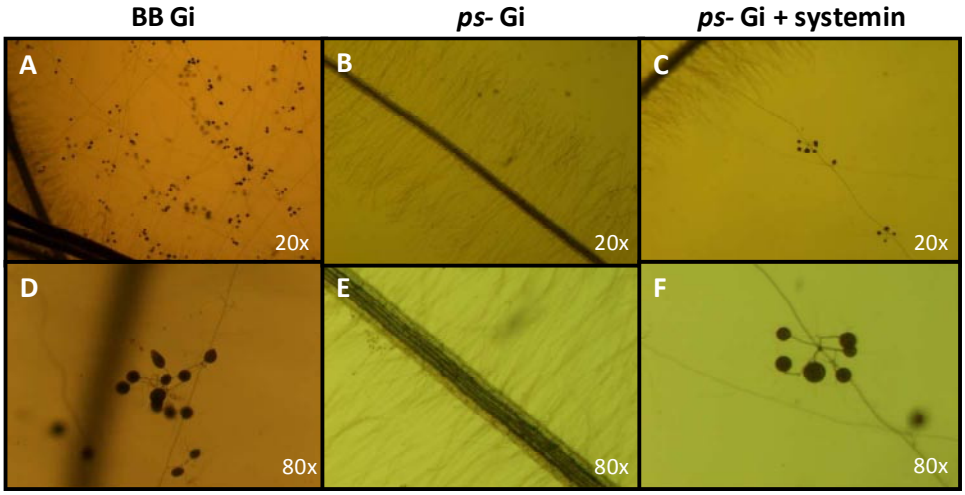

Figure 8. Exogenous application of systemin partially rescues the *ps-* phenotype. Development of external mycelia and spore formation was abundant in the wild type monoxenic cultures, but absent in *ps-* cultures. Supplementation of the media with 10 pm of systemin resulted in several well developed mycelial hyphae and the formation of some apparently healthy and viable fungal spores.

Strigolactones levels are altered in the prosystemin mutants

The previous results (lack of mycorrhizal colonization and absence of hyphal development in ROCs of the *ps-* plants) suggested that *ps-* is affected in presymbiotic signalling. A key role of the plant-derived molecules strigolactones (SLs) have been described in this process. SLs induce hyphal branching in germinating AMF spores, favouring the contact between the two symbionts and, therefore, the initiation of the colonization process. Accordingly the phenotype of *ps-* mutant may indicate a possible connection of systemin signalling with SLs and we decided to analyze the ability to produce strigolactones by this prosystemin antisense mutant. SLs were first described as important signalling molecules in the rhizosphere acting as germination stimulants of root parasitic plant seeds and being active at extremely low concentrations (pico- and nanomolar) (Akiyama et al., 2005; Bowmeester et al., 2007). Bioassays determining the germination of the root parasitic plant *Phelipanche ramosa* constitute a highly sensitive method to relatively quantify SLs (López-Ráez et al., 2008b). Firstly, root exudates from wild-type and mutant plants were collected and a germination bioassay was carried out. A 60% reduction in the germinating stimulatory activity of *P. ramosa* seeds was detected in the root exudates from the mutant compared to the wild-type (Fig. 9A), suggesting a reduced production of SLs. The possible presence of seed germination inhibitors was excluded by spiking the root exudates from the mutant with the synthetic SL analogue GR24 (Fig. 9A). Since SLs are synthesized in the roots and then secreted to the rhizosphere, a reduction in the SLs exuded may be due to a defect in the biosynthesis or the secretion of SLs. To address a possible defect in secretion, root extracts were also compared in another germination bioassay. A similar reduction in the germinating stimulatory activity was observed when root extracts were used (Fig. 9B), indicating a reduction in SL production and not in the exudation. The defect in SL production was further confirmed by analyzing the exudates by liquid chromatography-tandem mass spectrometry (LC-MS/MS). About 60% reduction was observed in all the three major SLs described in tomato, solanacol, and the two didehydro-orobanchol isomers (López-Ráez et al., 2008b) (Fig. 9C), which is in agreement with the decrease in the activity observed in the germination bioassays (Fig. 9A and B). Conversely, the production of the three SLs was significantly increased in the prosystemin overexpressing mutant *ps+* (Fig. 9C). Overall, the results show a

correlation between the levels of prosystemin and SLs, and suggest that systemin signalling is regulating SL production in tomato by the activation of its biosynthetic pathway. Moreover, this correlation would explain the absence and stimulation of mycorrhizal colonization observed in both mutants *ps-* and *ps+* (Fig. 1).

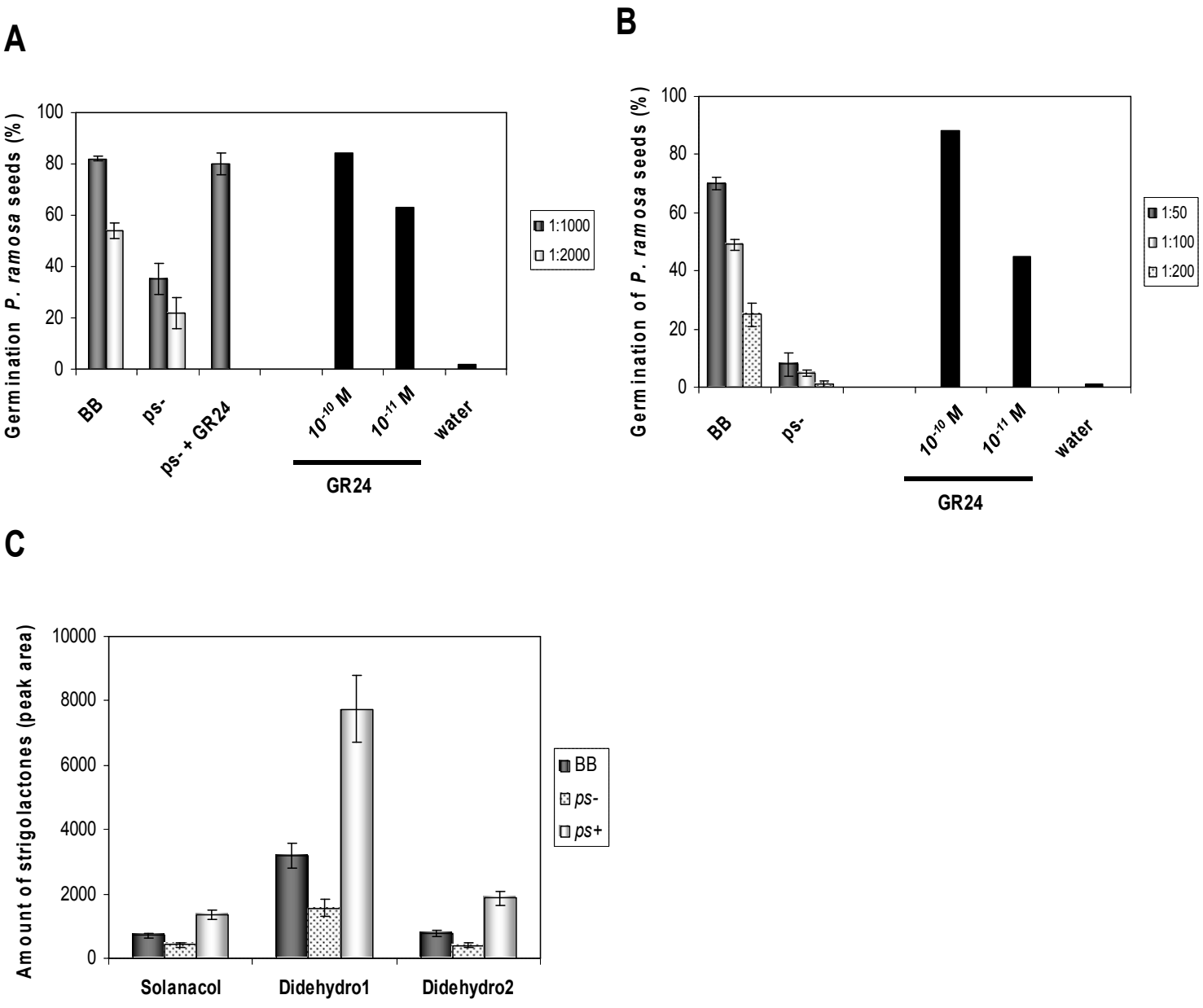

Figure 9. Strigolactones content in tomato roots exudates. Germination of *P. ramosa* seeds induced by exudates (A) and extracts (B) from roots of *ps-* and wild-type (BB) tomato plants. GR24 and demineralized water were used as positive and negative controls, respectively. (C) Amount of strigolactones in root exudates of *ps-, ps+* and wild-type (BB). Different dilutions were assayed to assure linearity between strigolactone content and germination stimulation (1:1000 and 1:2000 for the bioassay with exudates; 1:50, 1:100 and 1:200 for the bioassay with extracts). Bars correspond to the amount (according to the peak area) of the strigolactone solanacol and the didehydro-orobanchol isomers 1 (Didehydro1) and 2 (Didehydro2). Values represent the mean of five independent replicates ±SE.

Exogenous application of natural strigolactones partially revert the mutant *ps-* phenotype

To further confirm that the reduced mycorrhizal colonization observed on the *ps-* mutant was really caused by a reduction in SLs production, a mycorrhizal rescue experiment was performed by applying endogenous tomato SLs. Mutant *ps-* plants were grown in pots, inoculated with *G. intraradices* and watered twice a week with different concentrations (0, 1 or 10 nM) of tomato SLs. These values are within the range of physiological concentrations of SLs described in plants. After 8 weeks, mycorrhizal colonization was 10-fold higher in *ps-* plants treated with an as low concentration as 1 nanomolar, whereas no significant differences were detected upon 10 nM application (Fig. 10 A, B and C). When the host root-AM fungus interaction was analyzed in detail under the microscope, not only a larger colonization rate was observed in 1 nM SL-treated plants but an increased hyphal development was also detected in the proximity of the host root (Fig. 10D). Since 1 nM should be closer to the SLs concentration in the rhizosphere and no effect was observed after 10 nM application, it seems that exist a fine-tuned regulation for the action of the SLs as hyphal branching stimulants.

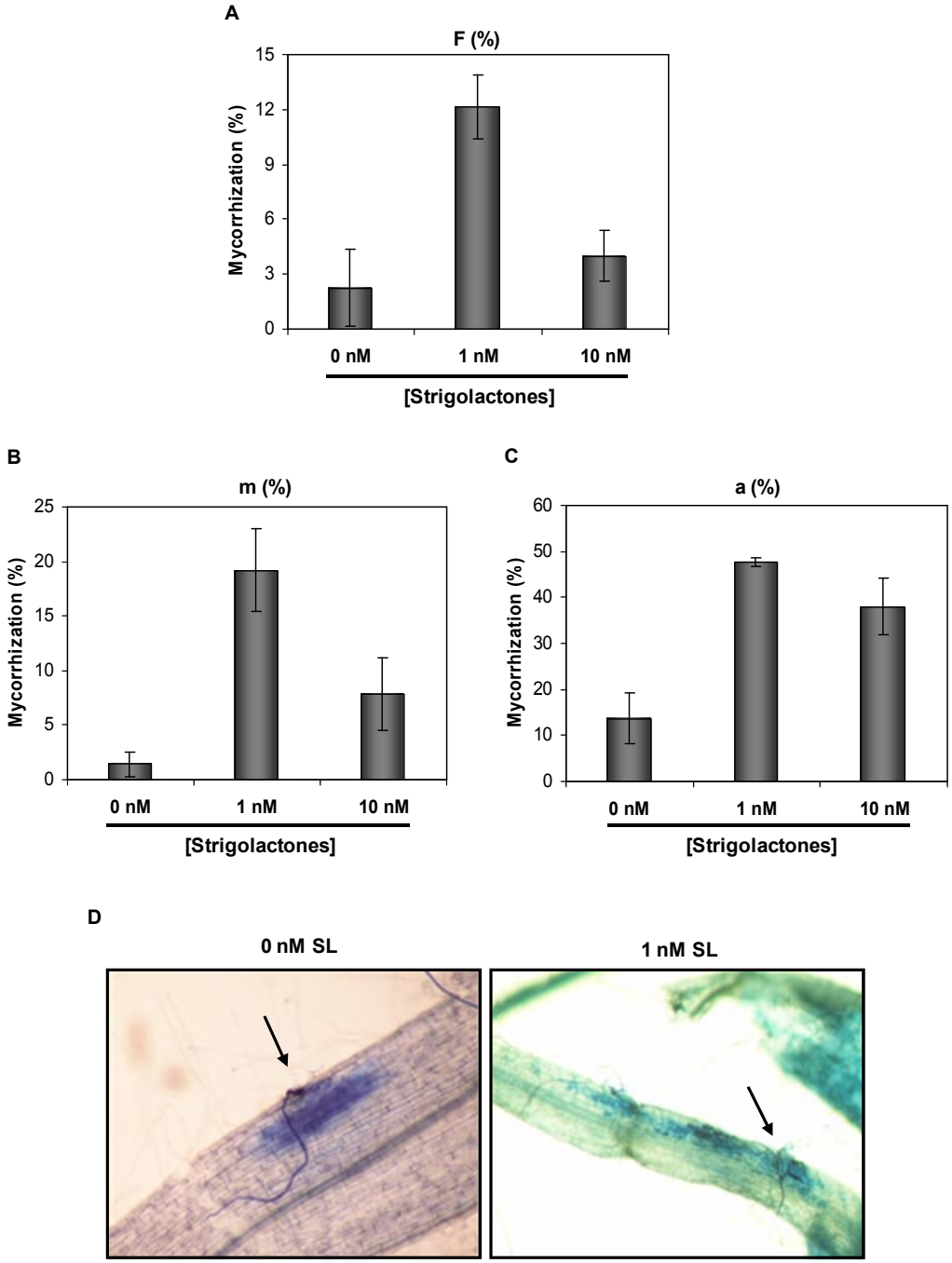

Figure 10. Mycorrhizal colonization by *G. intraradices* of roots of the *ps*- tomato mutants supplied with different concentrations of natural strigolactones, obtained from wild-type tomato. Mycorrhization was quantified according to the Trouvelot et al. (1986) method. Frequency of colonization in the whole root (F%, A), colonization intensity (m%, B) and arbuscular abundance (a%, C) in mycorrhizal zones of the root were determined using the MYCOCALC software. Numbers represent the mean of five independent replicates ±SE. (D) Pictures of colonization units of *G. intraradices* in roots of the *ps*- tomato plants supplied, or not, with 1 nM SLs. Arrows indicate entry points at the root surface.

Prosystemin mutants display altered root architecture

Besides the function of SLs as signalling molecules in the rhizosphere, SLs have also endogenous functions within the plant. They have been recently classified as a new class of plant hormones involved in the regulation of plant architecture through the

inhibition of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008), and affecting also root system architecture (RSA) (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Because of the altered levels in SLs production of the different mutants studied, we decided to investigate whether they also display altered root architecture, a key feature for AM symbioses. Wild-type and *ps-* and *ps+* mutant plants were grown in vitro and their root systems photographed at several time points to follow their development (Fig. 11). Already after 3 days significant differences were found regarding the length of the main root and the number of new lateral roots, showing the prosystemin overexpresor an increase in both parameters. After 14 days both mutants showed higher number of lateral roots of first order, while the number of second order lateral roots was significantly lower in *ps+*. Besides these differences in the number of lateral roots, obvious differences were also observed concerning their length. *ps-* mutant had longer lateral roots, while in *ps+* most lateral roots were very small, probably aborted. Finally, the addition of systemin to the media resulted in a RSA similar to that in *ps+* in relation to the number of lateral roots (3d) and intermediate phenotypes between *ps-* and *ps+* in relation to the length of the lateral roots. Remarkably, these roots also show a “hook” like shape (arrow) that resembles precisely that describe by Koltai and co-workers in tomato plants upon addition of SLs (Koltai et al., 2010).

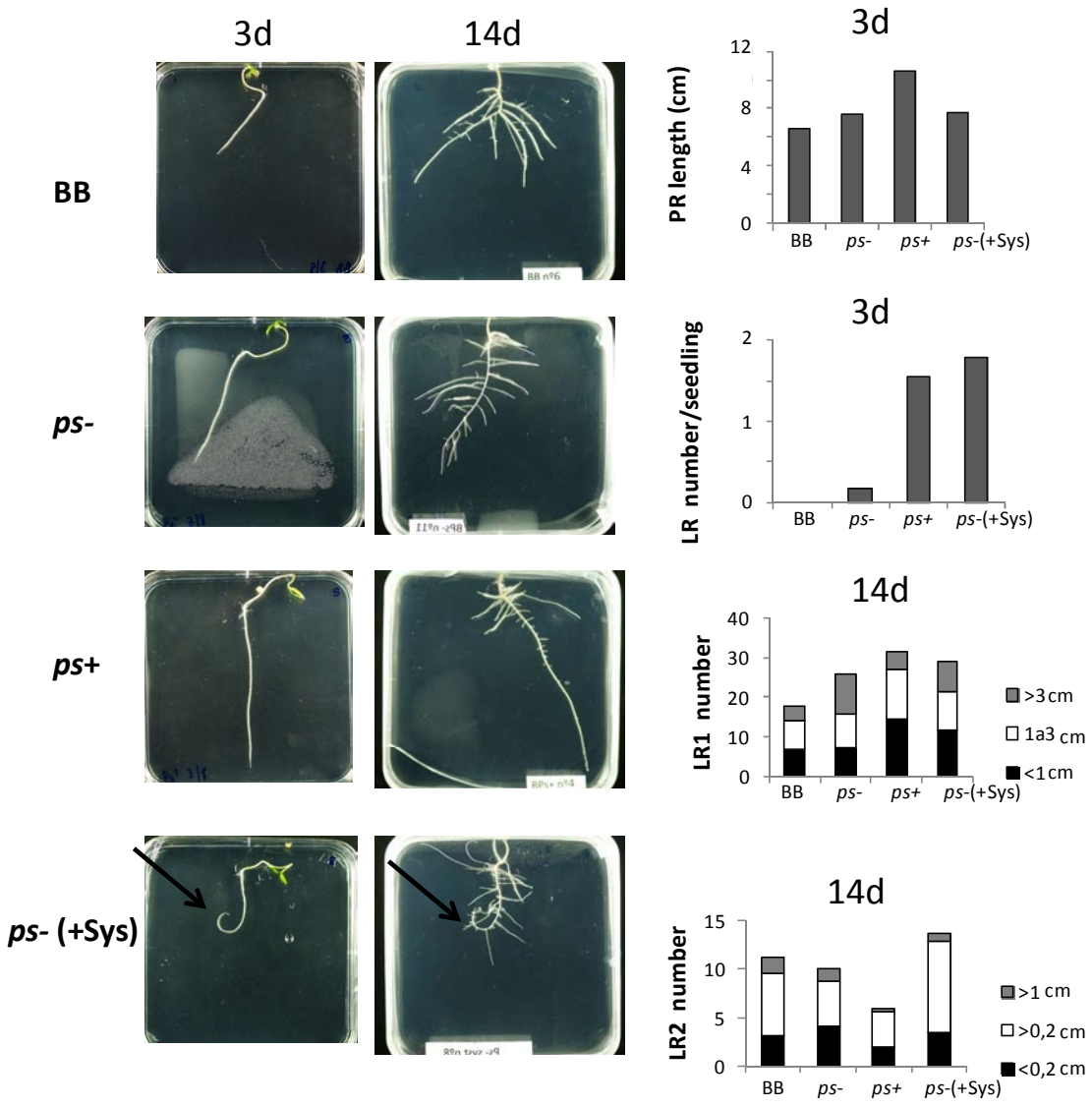

Figure 11. Analysis of the root system architecture in wild-type and prosystemin mutant plants. Plants were grown *in vitro* in MM media and the root systems photographed at different time points. The figure illustrates the images corresponding to 3 and 14 days of development. ps-(+sys) prosystemin antisense plants growing in MM medium supplemented with 10 pM of systemin. After 3 days of growth, the length of the main root was determined (PR length), and the number of lateral roots per seedling (LR number/seedling). After 14 days, the number of lateral roots of first (LR1 number) and second order (LR2 number) were determined and categorized according to their length as indicated in the legends in the right.

Concluding Remarks

Oxylipins, which include the plant hormones jasmonates, are thought to play a major role in arbuscular mycorrhizal (AM) symbioses in different plant species. Prosystemin regulates wound signalling in leaves through the activation of the octadecanoid pathway leading to the biosynthesis of JA, and accordingly, prosystemin mutants are often considered JA biosynthesis mutants. However, the study of mycorrhizal

colonization of several tomato mutants affected either in jasmonates/prosystemin biosynthesis or signalling related pathway revealed that only an antisense mutant for the pro-hormone prosystemin (*ps*-) showed an almost complete absence of mycorrhization compared with corresponding wild-type. JA levels in the roots of *ps*-mutants were not altered, and thus, prosystemin, or its systemin derivative, is necessary for AM symbiosis establishment and function, independently of JA signalling. This critical role of PS in mycorrhizal symbiosis was found to be widespread for even very divergent AMFs, belonging to different families, what further corroborate the essential regulatory role of this signalling molecule in mycorrhizal symbiosis. According to the bibliography, prosystemin gene has been reported to be expressed in all plant tissues except the roots, were AM colonization do occurs. Through grafting experiments aimed to find a shoot derived signal regulating mycorrhization, we showed that, unexpectedly, prosystemin expression was required in the roots of tomato plants for correct mycorrhization, regardless the scion tested. In fact, we were able to show for the first time that prosystemin is indeed expressed in the roots although at very low levels. To further investigate the exact role of this signalling peptide in AM colonization we monitored the mycorrhization process in monoxenic cultures. In contrast to wild-type roots, no external mycelium nor spores were found in the monoxenic cultures from *ps*- roots, suggesting a defect in the very early stages of the symbiotic interaction in the mutant, likely at the chemical communication between the two partners. Strigolactones have been described to play a crucial role as host detection signals. They induce hyphal branching in germinating AMF spores, favouring the contact between the two symbionts and, therefore, the initiation of the colonization process. We addressed weather *ps*- mutants were defective in the production or exudation of strigolactones, and a reduction of SLs production was confirmed in the mutant compared to the wild-type both by activity assays and chemical quantification, confirming a correlation between the levels of prosystemin and SLs. Accordingly, systemin signalling seems to regulate SLs production in tomato by the activation of its biosynthetic pathway. SLs have also endogenous functions within the plant; therefore alteration in SLs production in the antisense line could also affect root architecture, a feature of essential importance for the AM symbioses. Significant differences were found regarding the length of the main root and the

number of lateral roots, systemin apparently promoting the initiation of new lateral roots but not their extension. It is known that secondary roots are more prone to be mycorrhizal. Our study points to new functions for the signalling peptide systemin as a key element in the establishment of mycorrhizal symbiosis, in the modification of root architecture and the production of SLs.

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CAPÍTULO 5: Transcriptional regulation of the prosystemin gene in roots of tomato plants

Transcriptional regulation of the prosystemin gene in roots of tomato plants

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Resumen.

La Sistemina (Sys) es el primer péptido extracelular de señalización descubierto en plantas. Esta hormona peptídica juega un papel clave en la respuesta de defensa de la planta frente al daño por herida y al ataque de insectos herbívoros. La Sys se origina a partir de un precursor denominado prosistemina (PS) mediante un proceso de catálisis. Varios estudios muestran niveles constitutivos del mRNA de PS en la parte aérea de varias plantas pertenecientes al grupo de las solanáceas, pero hasta el momento la expresión del gen que codifica para la PS no se ha detectado en raíz. En el capitulo anterior nuestro grupo demostró que las raíces de tomate son capaces de expresar el gen de la PS. Además la PS en la raíz de tomate es regulada a nivel transcripcional, siendo su expresión clave para el establecimiento de una simbiosis micorrízica arbuscular, de forma independiente a la ruta de los jasmonatos. En este estudio analizamos en profundidad la regulación transcripcional de la de PS en raíces de tomate en respuesta a la interacción con diferentes hongos beneficiosos y patógenos, y tras el tratamiento con diferentes elicitores. La interacción con ciertos hongos, dependiendo de las características de su pared celular, aumentó el nivel de la expresión génica de PS desde etapas muy tempranas. La aplicación de MAMPs y moléculas de señalización relacionadas con la interacción planta-microorganismo como el óxido nítrico también aumentaron la expresión del gen de la PS. Por lo tanto, la PS parece ser un componente importante en la ruta de señalización de la regulación de la respuesta de defensa de la planta durante la interacción de la raíz con diferentes microorganismos del suelo. Mediante el análisis *in silico* de la secuencia del promotor de la PS identificamos elementos cis de respuesta a patógenos y organismos beneficiosos. Debido a que la expresión de la PS está limitada a un pequeño número de plantas del grupo de las solanaceas, en este estudio buscamos posibles homólogos de la PS en otras familias de plantas, identificando en raíces de soja un gen que

codifica para el péptido GmProPep914. Este péptido comparte varias características de las descritas para la PS, aumentando su expresión en respuesta a la simbiosis micorrízica arbuscular. Además encontramos importantes similitudes entre los promotores de ambos genes.

In preparation, 2013.

Transcriptional regulation of the prosystemin gene in roots of tomato plants

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Abstract

Systemin (Sys) was the first extracellular signal peptide discovered in plants. Sys play a key role in defence responses to wounding and insect herbivory. Previous studies have shown low constitutive levels of PS mRNA in the aerial part of several solanaceous plant, but not in roots. Remarkably, we previously showed that PS gene is expressed in the roots, is transcriptionally regulated and is essential of the establishment of AM symbioses, probably independently of the jasmonate pathway. In this study we analyze the transcriptional regulation of PS expression in tomato roots in response to interaction with different beneficial and pathogenic fungi and upon treatment with different defence elicitors. The contact with various fungi increased the level of PS gene expression since very early stages of the interaction, depending on the characteristics of their fungal cell walls. Application of MAMPs and signal molecules related to plant-microbe interactions as nitric oxide also increase the PS expression. Therefore PS seems to be a component in the signalling pathway regulating plant immune responses during root interactions with soil microbes. By *in silico* analysis of the promoter sequence we identified cys regulating elements related to responses to pathogens and symbiosis that may contribute to the precise regulation of the gene. Because systemin is restricted to a subtribe of solanaceae, we looked for potential homologues in other plant families and identified the peptide encoding gene *GmPROPEP914* in roots of soybean. The peptide share several characteristics with those described for prosystemin and it is also upregulated upon mycorrhization. Moreover, important similarities were found in the promoter of both genes.

Key words: MAMPs, Nitric oxide, myc factors, fungi, elicitors, prosystemin, roots, peptídic hormones, signalling, plant-microbe interaction

Introduction

In plants, peptides regulate diverse processes related to both development and defence (Matsubayashi and Sakagami, 2006). Under biotic stress, the expression of genes encoding endogenous peptide precursor proteins is activated in the plant. These peptides are known to act as molecular messengers alerting the plant of a potential attack and inducing defences. Among them, systemin (Sys) was the first extracellular signal peptide discovered in plants (Pearce et al., 1991). Systemin, which is found only in some species of the Solanaceae family, is an 18-amino-acid polypeptide hormone, processed from its larger prosystemin (PS) precursor, 200-amino acid protein. Prosystemin is synthesized in vascular phloem parenchyma cells (Narváez-Vásquez and Ryan, 2004) and has a single-copy gene in the tomato genome (McGurl et al., 1992).

PS play a key role in wounding and insect herbivore signalling defence response. In tomato plants, after wounding or herbivory attack, Sys is proteolytically processed from PS and released into the cell wall apoplast, where Sys interacts with a membrane receptor. This interaction causes a rapid depolarization of the plasma membrane, the alkalinization of the extracellular medium or cell wall apoplast, transient increases in cytoplasmic free Ca^{2+} concentrations, the activation of a mitogen-activated protein kinase (MAPK), and the release of linolenic acid from cell membranes, leading to the biosynthesis of oxo-phytodienoic acid (OPDA) and jasmonates (JA) through the octadecanoid pathway (Ryan, 2000; Wasternack et al., 2006). Therefore Sys plays a central role in the activation of JA-related defences responses (Ryan and Pearce, 2003) through the induction of JA-biosynthesis genes including, lipoxygenase, allene oxide synthase, PS itself and several JA dependent defence related as classes of proteinase inhibitors (*PI-I* and *PI-II*), polyphenol oxidase and leucine aminopeptidase (Constabel et al., 1998; Ryan, 2000). Further, recent findings report a role of PS in the regulation of volatile- mediated plant signalling (Corrado et al., 2007; Degenhardt et al., 2010) and the activation of the phenylpropanoid and polyamine biosynthetic pathways in tomato (Chen et al., 2006). Previous studies have shown low constitutive levels of PS mRNA

within the vascular phloem parenchyma cells of leaves, petioles and stems, but not in roots (Ryan, 1992; Narváez-Vásquez and Ryan, 2004). Remarkably, recent studies carried out in our group have shown that PS gene is expressed in the roots, is transcriptionally regulated and have important functions in plant biotic interactions and root architecture regulation (Fernández et al., unpublished).

The above indicates that the PS gene has a key role in the signalling of the defence responses in a number of closely related solanaceous plants. In early stages of plant biotic interactions, the plant, through its innate immune system is capable of recognizing conserved molecules present in a wide range of microorganisms. These molecules have been termed traditionally elicitors, although more recently the terms of MAMPs ("Microbe-Associated Molecular Patterns") (Nürnberger and Brunner, 2002) have been introduced. In addition to the MAMPs, plants are also able to recognize compounds from the action of microbe on the plant itself; these elicitors have been called MIMPs ("Microbe-Induced Molecular Patterns") (Bent and Mackey, 2007). When the plant recognizes this molecules starts a signaling cascade that finetune the defense response to be triggered depending on the nature of the elicitor. In this early stage of interaction in which the plant recognize the nature of the other partner, the PS seems to have an important role as signaling molecule both local and systemic level (Pearce et al., 1991; Narváez-Vásquez et al., 1994, 1995; Lucas and Lee, 2004).

In this cascade of signaling that occurs in the early stages of recognition among plant and microbe, nitric oxide (NO) seems to be involved in different process depended of the kind of interaction, (Romero-Puertas et al., 2004; Wendehenne et al., 2004; Nagata et al., 2008). In non-compatible pathogenic interactions NO is rapidly produced, triggering hypersensitive cell death and activating the expression of several defense-related genes (Delledone, 2005; Perazzolli et al., 2006), but when this interaction is compatible, NO plays an important role in non-programmed cell death (Asai and Yoshioka, 2009). On the other hand, several studies also have demonstrated production of NO during early (Shimoda et al., 2005; Nagata et al., 2008; del Giudice et al., 2011) and late stages of beneficial interactions (Baudouin et al., 2006; Pii et al., 2007; Horchani et al., 2011). Therefore, NO appears as a central component in early signaling, possibly orchestrating a number of downstream signaling pathways.

In addition, recently the PS has been shown to play in defence response against microbial pathogens (El Oirdi et al., 2011), and its function has been mostly related to its regulatory role in JA signalling. JA has been shown to be a key element in plant-microbe beneficial interactions (Pozo et al., 2004). Among them, it seems to play a regulatory role in arbuscular mycorrhizal (AM) symbiosis (Hause et al., 2007; Tejeda-Sartorius et al., 2008; Gutjahr and Paszkowski, 2009; López-Ráez et al., 2010).

AM are mutualistic associations formed between the roots of 80 % of terrestrial plant species and fungi from the small phylum *Glomeromycota* (reviewed by Schüßler and Walker, 2010). This symbiosis is probably the oldest and most widespread plant symbiosis on Earth (450 million years) (Smith and Read, 2008), which indicates a considerable selective advantage for both partners. Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that require the host plant to complete their life cycle. The fungus colonizes the root cortex and forms intracellular structures called arbuscules, where the exchange of nutrients between the partners takes place. The extracellular hyphal network spreads widely into the surrounding soil, thereby reaching out of the nutrient depletion zone and improving the supply of inorganic nutrients, especially phosphate and nitrate (Smith et al., 2011). In return, the heterotrophic fungal partner receives photosynthates from the host plant (Smith and Smith, 2011). Mutual benefits are the basis of the evolutionary success of the interaction, ensured through a tight bidirectional control of the mutualism (Kiers et al., 2011). From the plant side, this regulation implies important changes in the plant primary and secondary metabolism and regulation of the plant defence mechanisms (Harrison, 1999; Hause and Fester, 2005).

Increased levels of jasmonates in mycorrhizal roots have been reported, supporting the possible role of PS/JA in AM symbiosis (López-Ráez et al., 2010; Fernández et al., unpublished). Further the use of JA-impaired mutants resulted in a reduction of mycorrhizal root colonization (Isayenkov et al., 2005), and PS overexpression in tomato plants resulted in a higher arbuscule abundance in mycorrhizal tomato roots (Tejeda-Sartorius et al., 2008). Interestingly, our studies further have shown that independently of JA root levels, PS root expression have a critical role in arbuscular mycorrhizal symbiosis establishment in tomato plants (Fernández et al., unpublished).

In addition, to better understand the role of the PS in the early stages of the plant-microbe interactions is necessary to study the possible regulation of the PS by several molecules involved in the recognition among plant and fungus as MYC factor. MYC factors are small molecules derived from chitin (3 kDa) (Navazio et al., 2007; Bucher et al., 2009), which consist of a mixture of singles, sulfated and no-sulfated (LCO) lipochitooligosacarides (Maillet et al., 2011). MYC factors are produced by the AMF and recognized by cell membrane receptors in the plant (not characterized so far), before that physical contact with the fungus occurs, which triggers a symbiotic program in the host that involves important cellular changes to accommodate the fungus within the roots and establish the symbiosis (Siciliano et al., 2007; Genre et al., 2013).

Analogous to Sys, the peptide defence signal Hyp-rich glycopeptide systemin (HypSys) was later indentified in tomato (Pearce and Ryan, 2003) and other solanaceous plants such as tobacco (*Nicotiana tabacum*) (Pearce et al., 2001; Pearce, 2011). Although SYS and HypSys do not show sequence homology, they have been reported to share functional similarities (Ryan and Pearce, 2003). Due to the multifaceted role Sys/HypSys in solanaceous plants it would be logical to think that other signalling peptides could share similar functions in other plant families. The search for Sys/HypSys-related peptides in other plant species has led to the discovery of other families of defence-related peptides. In Arabidopsis (*Arabidopsis thaliana*), the AtPeps (a 23-amino-acid polypeptide) were found to protect plants from pathogen attack (Huffaker et al., 2006, 2007). More recently, soybean (*Glycine max*) has been discovered to produce a peptide signal, GmPep914, which activates the transcription of pathogen defence genes (Yamaguchi et al., 2011). As PS root expression has been reported for first time to have a critical role in arbuscular mycorrhizal symbiosis establishment in tomato plants, the response of this signalling peptide to AM symbiosis has not been examined. This may serve to gains information on the function of PS during AM symbiosis. In this study we address these issues by analysing the expression pattern of PS in root tissues during different stages of AM symbiosis and PS response to different elicitors. In addition to gain further insight into the role of

peptidic signalling in AM symbiosis, we examined the expression pattern of the PS homologue, *GmPROPEP914*, in mycorrhizal roots of soybean.

Material and Methods

Biological material

Plants

Tomato (*Solanum lycopersicum* L. cv. MoneyMaker) and transgenic tomato plants with SlPS promoter::GUS fusion constructions were used. The transgenic plants consisted in the fusion of a 2-Kb prosystemin promoter region to GUS gen (U12639), which encodes for the *β-glucuronidase* activity (Jacinto et al., 1997). The transgenic seeds were kindly provided by Prof. Clarence Ryan (Washington State Univ.). All the seeds were surface sterilized in 4% sodium hypochlorite, containing 0.02% (v/v) Tween-20, and germinated in sterile water at 25 ºC in darkness. After 1 week, seedlings were transferred to **hydroponic conditions** in tanks containing Long Ashton nutrient solution and with constant aeration. Plants were grown at day and night temperatures of 24 and 16°C, respectively, 16/8 h photoperiod and 70% humidity. After 2 weeks, the plants were used for elicitation treatments.

Soybean (*Glycine max* L. Merr. cv.Williams 82) seeds were surface sterilized in 4% sodium hypochlorite, rinsed thoroughly with sterile water and germinated for 3 days in a container with sterile vermiculite at 25ºC in darkness. Individual seedlings were transferred to 0.25 l **pots** containing a sterile sand:soil (4:1) mixture. Plants were grown in a greenhouse at 24/16 ºC with a 16/8 hours photoperiod and 70% humidity, and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration.

Fungi

In vitro interactions*.* The AMF *G. intraradices* was grown in monoxenic cultures, using Ri T-DNA (*Agrobacterium rhizogenes*)-transformed carrot (*Daucus carota* L. clone DC2) according to St-Arnaud et al., 1996. Cultures were established in bi-compartmental Petri plates to allow separating the root compartment from the hyphal compartment. Cultures were started by placing a mycorrhizal carrot root segment in the root

compartment containing M medium (Chabot et al., 1992). Petri plates were incubated in the dark at 24ºC until the hyphal compartment, which contained M medium without sucrose (M–C medium), was profusely colonized by the fungus (approximately 20 weeks). The content of the root compartment was then removed, and dishes were used for the experiments. Cultures of the pathogens *Fusarium oxysporum f* sp. lycopersici and *Phytophtora parasitica* were grown for 5 days on PDA at 28ºC.

Ex vitro assays. The AMF *G. intraradices* inoculum was multiplied using *Sorghum vulgare* as trap plant. The inoculums was used as a soil sand based mix containing rhizospheric soil, spores, hyphae and chopped colonized roots. The inoculum density was found to be 35 infective propagules per gram of soil.

Assays

"Early stage" interaction experiment

A small hole (approximately 3mm diameter) was carefully made in the side and the lid of the Petri dishes (120 mm diameter) containing the *G. intraradices, F. oxysporum* and *P. parasitica* cultures. Three-weeks old tomato plants, grown in hydroponic conditions as described above were transferred to the Petri dishes, one plant per dish, extending the roots on the surface of the culture and the shoot extending beyond the hole, in open air conditions, according to Voets et al., 2005. Petri dishes were covered, in order to keep the root system in the dark, and plants were kept in a growth chamber at 24ºC, 16/8 h photoperiod and 70% humidity for 4, 24, 72 and 96 hours.

"Well established" symbioses experiment

Soybean, tomato and the arbuscular mycorrhizal fungus (AMF) *G. intraradices* were used for assays on a well established symbiosis. Pots with a sterile sand:soil (4:1) mixture were inoculated by adding 10% (v:v) *G. intraradices* inoculum. The same amount of soil:sand mix but free from AMF propagules was added to control non-mycorrhizal plants. All plants received an aliquot of a filtrate (20 ml) of both AMF inoculums to homogenize the microbial populations in the rhizosphere. Plants were randomly distributed and grown in a greenhouse at 24/16 ºC with a 16/8 hours photoperiod and 70% humidity, and watered three times a week with Long Ashton

nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration. Also, *G. intraradices* and tomato were used in split-root system assay (for more details to see Catford, 2003). Soybean and tomato plants were harvested after 8 weeks of growth. An aliquot of each individual root system was reserved for mycorrhizal quantification, and the rest frozen in liquid nitrogen for molecular analysis.

Chemical and mechanical treatments

Roots of 2-week-old tomato plants and PS::GUS transgenic-tomato lines from the hydroponic culture, were treated for 24 h with MeJA 50µM (Sigma-Aldrich) and chitosan (Sigma-Aldrich) at 0.15% (w:v) in 10mM acetic acid.

Roots of 2-week-old tomato plants were treated with the NO-releasing compounds sodium nitroprusside (SNP; 200 µM; Sigma-Aldrich), S-nitrosoglutathione (GSNO; 350 µM; Calbiochem) and DETA-NONOate (500 µM; Cayman chemical), for 1, 3 and 6 hours (Bustos-Sanmamed et al., 2011).

For wounding assays Roots of 2-week-old tomato plants of the hydroponic culture and leaves of soybean grown in pots for 8 weeks were wounded three times using sterile tweezers. These samples were harvested 24h after wounding for molecular analysis.

Fungal elicitors

Homogenates of *G. intraradices* cell wall were obtained from *G. intraradices* monoxenic cultures. The mycelium of *G. intraradices* monoxenic culture was carefully removed from the growing medium by addition of 10mM sodium citrate. *Fussarium oxyxporum* was grown on PDA and mycelia was carefully removed from the plate and washed. Cell walls from both *G. intraradices* and *F. oxysporum* mycelia was obtained by filtering and washing with chloroform:methanol (1:1; v/v) and acetone as described by Ren and West, 1992 and then lyophilized and grinded to powder. Fungal exudates containing *myc* factors were obtained from approximately 10^8 germinating spores of *G. intraradices*. Spores were collected from *G. intraradices* monoxenic cultures, by carefully removing the medium with 10mM sodium citrate. Spores were washed and incubated in 30 mL sterile distilled water at 24ºC in dark for several weeks as previously described (Chabaud et al., 2011). The germinating spore suspensions were

then collected and filtrated twice through 0.45 and 0.22 Millipore filters. Roots of 2-week-old tomato plants hydroponically grown were treated with fungal cell walls at 0.1% (w:v) in distilled water for 6 and 24 hours or with the MYC factor suspension from *G. intraradices* for 3, 6 and 24h.

Histochemical analysis

For **mycorrhiza determination**, histochemical staining of fungal structures was performed according to Phillips and Hayman (1970) and examined using a Nikon Eclipse 50i microscope and brightfield conditions. The percentage of total root colonization was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

For determine the **β-Glucuronidase activity**, histochemical assays were performed in extraction buffer (lacking PVP) containing 20% methanol and 1 $mg*ml^{-1}$ of X-GLUC (5-bromo-4-chloro-3-indolyl glucuronide; Research Organics Inc., Cleveland, Ohio, USA) as substrate. Freshly collected tissues were immersed into substrate solution, vacuum-infiltrated, and incubated for 48 h at 37 °C (Jefferson et al., 1987). Samples were rinsed with water and for better visualization of the stained tissue; fruits, flowers and leaves were rinsed at room temperature with increasing concentrations of ethanol (70–100%) in order to remove chlorophyll.

RNA isolation and gene expression analysis

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel), and stored at –80ºC until use. Real-time qPCR was performed using the iCycler iQ5 system (Bio-Rad) and gene-specific primers (Table S1). The first-strand cDNA was synthesized with 5 µg of purified total RNA using the BioScript cDNA Synthesis kit (Bioline) according to the manufacturer's instructions. Quantification of specific mRNA levels was performed using the comparative method of Livak and Schmittgen, 2001. Expression values were normalized using the housekeeping gene *SlEF*, which encodes for the tomato elongation factor-1α (X14449.1) as described in López-Ráez et al., 2010.

Regulatory motif analysis of promoter regions

The Sl prosystemin (M84801) and Gm PROPE914 (Gma.28320) mRNA sequences were found in the online database NCBI (http://www.ncbi.nlm.nih.gov/). Gene structures information and promoters sequences were obtained using the on-line databases SOL Genomics Network (http:// solgenomics.net/) and NCBI. In order to study the presence of potential cis- regulatory elements related to signalling in plant-biotic interactions, a fragment of the Sl prosystemin and Gm PROPEP914 promoters (-2 Kb upstream to the translational starting site) were subject to an *in silico* analysis using the database PLACE (http://www.dna.affrc.go.jp/PLACE/), and the Genomatix software suite (www.genomatix.de).

Statistical analysis

The results were analyzed with the software SPSS Statistics v. 19 for Windows. The Dunnett's and Student's t-test were applied where appropriate. Mean ±SE were calculated. Significance levels were set at 5%.

Results

Prosystemin is transcriptionally regulated in the roots

In the previous chapter we show that, prosystemin (PS) is expressed in tomato roots, although at very low levels. First we compare the basal level of expression in shoot and root systems by quantitative real time RT-PCR. PS expression is much lower in roots than in aerial tissues, about $6x10^4$ fold decrease (data not shown); thus PS expression in the roots is close to the detection limit, difficulting the gene expression analysis. To analyze the regulation of PS by the JA-related pathway in the root system, the roots of 3 weeks old tomato plants were treated with Methyl-Jasmonate (MeJA) for 24 hours, and root PS expression level was analized. We observed a significant increase (by 200 fold) in PS expression level in MeJA treated plants with respect to control plants (Fig.1A). In addition, we analized PS expression in response to wounding. 24 hours after mechanisc wounding in roots, the expression of PS increased by 30 fold,

compared to control plants (Fig.1A). A similar regulation pattern has been widely described in the aerial part.

On the other hand, we studied PS expression in tomato roots during early interactions with different fungi. We observed an increase of PS expression in tomato root after 4 hours of plant interaction with the beneficial *G. intraradices* (Fig. 1B) and after 24 hours with pathogenic fungi *Fusarium oxysporum* (Fig. 1C) compared with control plants. In both interactions the increase of PS expression was sustained up 72 hours and the expression levels were elevated at least until 96 hours after contact. The prosystemin induction was different depending on the fungus. In contrast, the pathogenic oomycete *Phytophtora parasitica* did not induce the PS expression in tomato roots (Fig. 1D). Thus, PS is transcriptionally regulated by wounding and JA as in the shoots, but it is also quickly induced in roots in response to fungi, and the induction seems to depend on the microbe encountered.

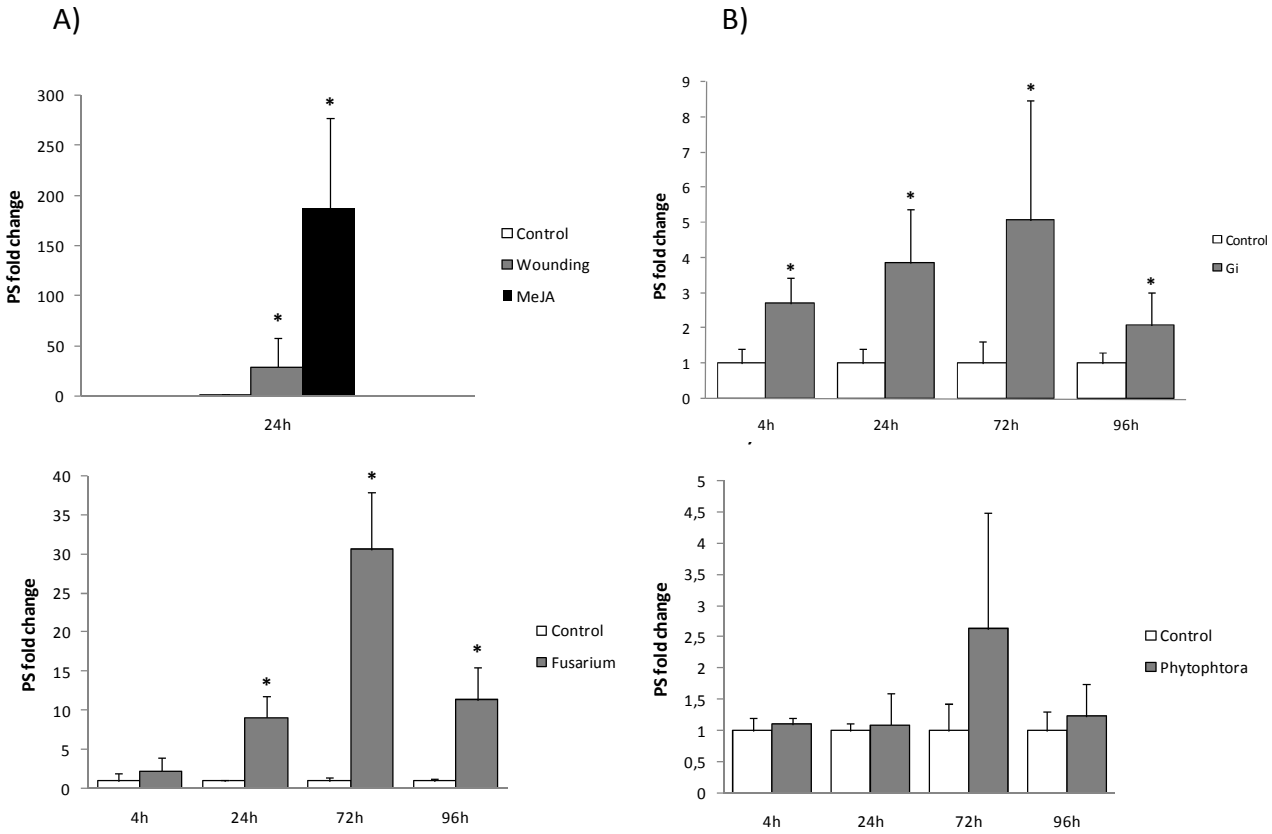

Figure. 1. Prosystemin root expression. A) Roots of 3 weeks-old tomato plants treated with wounding and MeJA for 24h. B) Roots of 3 weeks-old tomato plants in contact with mycorrhizal fungus G*lomus intraradices* and pathogenic fungi *Phytophtora parasitica* and *Fusarium oxysporum* during 4,24, 72 and 96h. Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of three independent biological replicates (±SE). Asterisk indicate statisticaly significant differences compared to control plants (Dunnett's test, $P \leq 0.05$).

Prosystemin expression in root is induced by MAMPs

To gain further insight into the regulation of prosystemin (PS) and its possible role in plant immune response to the recognition of different MAMPs, we analyzed the effect of different potential elicitors. We first tested PS expression in roots of plants treated with *G. Intraradices* (Gi) and *Fusarium oxysporum* cell wall. Additionally, we tested PS expression in roots of plants treated with chitosan, a major component of cell wall of these fungi. 6h posttreatment only the cell wall of *Fusarium oxyxporum* increased PS expression compared with non-treated plants (Fig. 2A). In contrast, 24h posttreatment, chitosan and both fungal cell walls increased PS expression compared with control plants (Fig. 2A), further supporting the role of PS in signalling downstream fungal MAMPs recognition. Intriguingly 6 h after contact with Gi cell walls there was no

induction of PS expression, while in contact with the living fungus PS was induced by that time.

To deepen in this aspect, we also analyzed the effect of the mycorrhizal factor ("MYC factor") of Gi in prosystemin (PS) root regulation. MYC factor are small molecules derived from chitin (Fig. 2C) and produced by the arbuscular mycorrhizal fungi (AMF). These molecules were recognized by the plant cell membrane receptors, in early stages of the interaction, favoring the establishment of symbiosis. After treating the plants with the MYC factor solution, we observed a transient induction (by 2 fold) of PS at 6h posttreatment with respect to mock treated plants However expression levels at 3 and 24h after treatment were similar to that in mock-treated plants (Fig.2B). Accordingly, before physical contact with the AMF, the plant recognises the *myc* factor and among the early responses triggered PS expression in induced.

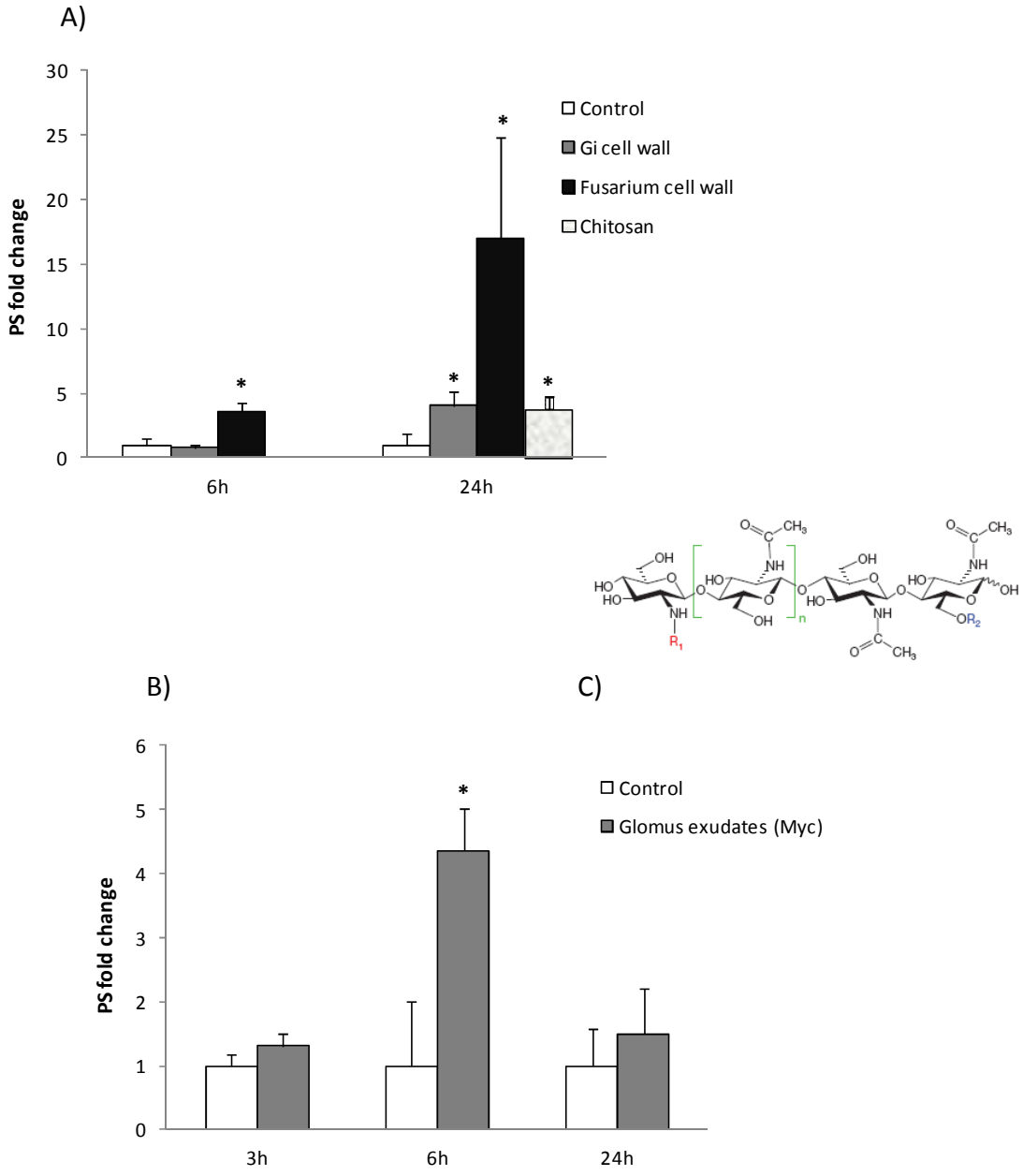

Figure. 2. Effect of different elicitors on prosystemin root expression of tomato plants. A) Expression of prosytemin in roots of 3-weeks old plants treated for 6h and 24h with cell wall of both *Glomus intraradices* and *Fusarium oxysporum*. Also plants were treated for 24h with chitosan. B) Effect of *myc* factor from *Glomus intraradices* on prosystemin root expression of tomato plants. C) *myc* factor structure. Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of four independent biological replicates (±SE). Asterisk indicate statisticaly significant differences compared to control plants (Student's t-test, $P \leq 0.05$).

Prosystemin is regulated by early signalling events in plant-microbe interactions

Several studies have provided compelling evidence that nitric oxide (NO) is an important molecule involved in early signalling processes in plant-microbe interactions. To analyze if NO production may mediate the plant early response to AMF derived signals leading to PS induction, plants were treated with the *G. intraradices* exudates (*myc* factor). *myc* factor triggered an early burst of NO which occurred within 3 hours (Fig. 3A). NO signal declined thereafter to basal levels, showing no differences with non-treated control plants 6 hours after treatment, although an increase in NO signal was observed again 24h after application of *myc* factor (Fig. 3A). The results confirm the occurrence of NO production in the presymbiotic phase of the AMF-plant interaction, and the pick at 3h may be responsible of the PS induction observed 6h after *myc* application.

To confirm if NO may regulate PS expression, we determine the PS expression in roots treated with three different NO donors, in order to emulate in vitro the NO production that occurs during early stages of the plant-AMF interaction. Roots were treated for 1h, 3h and 6h with the NO donors sodium nitroprusside (SNP), DETA-NONOate (DNN) and S-nitrosoglutathione (GSNO). We found a transient induction of PS with an increase of PS expression (by 30 fold) at 3h in all the NO donor treatments (Fig. 3B), supporting the relation between NO and PS-related plant responses. However, in roots treated for 1h and 6h with the NO donors PS expression remained unaltered (Fig. 3B). Thus, our data support the induction of PS expression by Myc factor could be mediated by NO.

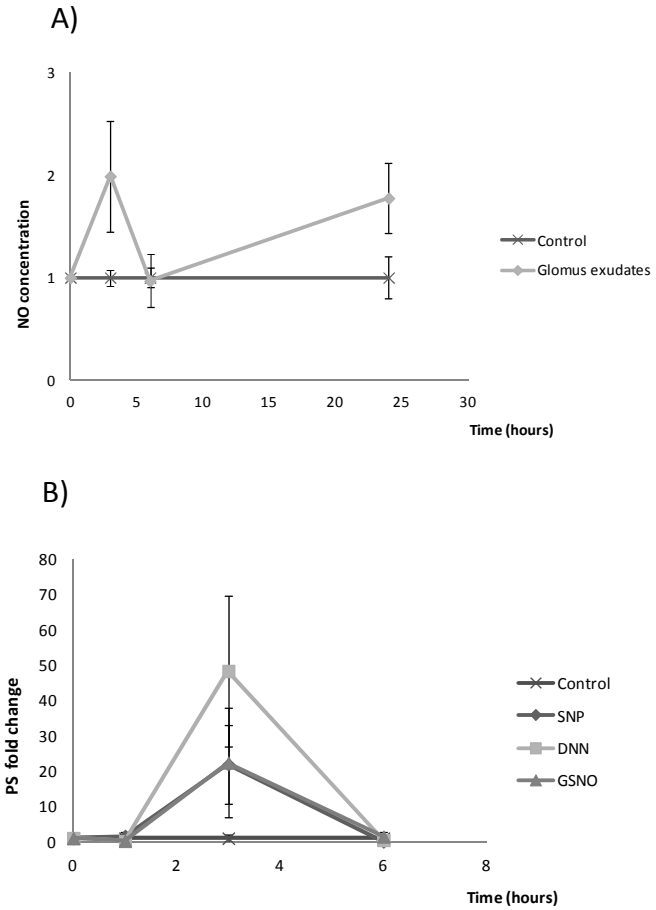

Figure. 3. Effect of nitric oxide on prosystemin root expression of tomato plants. The roots were treated with the nitric oxide donors SNP, DNN and GSNO for 1h,3h and 6h. Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of three independent biological replicates (±SE).

Localization of prosystemin expression in induced roots

Two approaches were followed to determine spatial localization of PS expression within the roots. For well established mycorrhizas laser microdisection was performed in tomato roots colonized by *G. mosseae* as described in chapter 1. No expression was observed in any of the cell types analyzed, so PS is not locally induced in arbusculated cells or if it is, the expression is below the detection limit.

To precisely localize the expression of prosystemin (PS) during early interactions and elicitor treatments, we used a reporter gene approach to monitor activation of PS promoter in tomato roots induced with MeJA, chitosan, the beneficial *G. intraradices* and the pathogens *F. oxysporum* and *P. parasitica*. Transgenic plants carrying a construct of the 2-Kb promoter region of PS fused to the glucuronidase gen were used (Jacinto et al., 1997). Unfortunately, no GUS activity was observed in any of the

treatments. To evaluate the responsiveness of the promoter, we monitored the expression of the GUS gene by quantitative real time RT-PCR, using specific primers for the GUS gen, in roots elicited with MeJA and chitosan. In these treatments, although GUS precipitation was not detected, an increase in GUS expression was observed (Fig. 4), resembling the PS expression pattern. Thus we confirmed the responsiveness and transcriptional regulation of the PS gene to these stimuli, although levels of expression are too low to achieve detectable GUS activity levels.

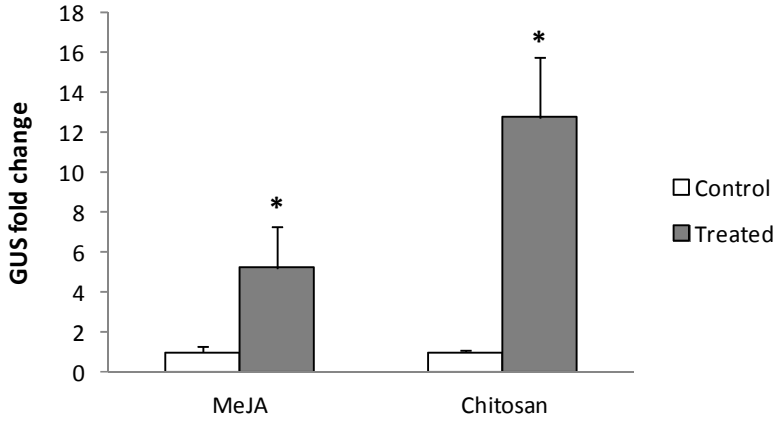

Figure. 4. Prosystemin localization in roots of elicited tomato plants. Expression of GUS in roots of PS::GUS lines elicited with MeJA and chitosan for 24h. Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of three replicates (±SE). Asterisk indicate statisticaly significant differences compared to control plants (Student's t-test, P≤0.05).

In-silico characterization of the prosystemin promoter

To study the presence of potential Cis- elements involved in the transcriptional regulation of PS during plant-microorganism interactions, such as symbiosis, pathogen and hormonal-responsive motifs, the tomato PS promoter was *in-silico* analyzed with the PLACE and the MatInspector databases (Fig. 5). We identify several putative regulatory motifs, including: three symbioses related NODCON1/2 (AAAGAT and CTCTT) motifs that are described as binding sites for mycorrhization related transcription factors (Tf). We also identify two defence related W boxes (TTGA), that coordinate responses to pathogens; two motifs for elicitors response (TGAC); one elements for phosphorous starvation respond (ATAT); and two potential binding sites for Ca^{2+} related response Tfs (GCTT and CGCG). Also were found 5 motifs related hormonal responsive, such as gibberellins (AGTT), auxins (TGTC), acid abscisic (ACGT)

and jasmonates (CACATG). The presence of these motifs is in concordance with the PS expression patterns observed in this study. In addition, all cis-acting elements found are closely related to the symbiosis process, so supporting the regulation of PS in the establishment of the arbuscular mycorrhizal symbiosis.

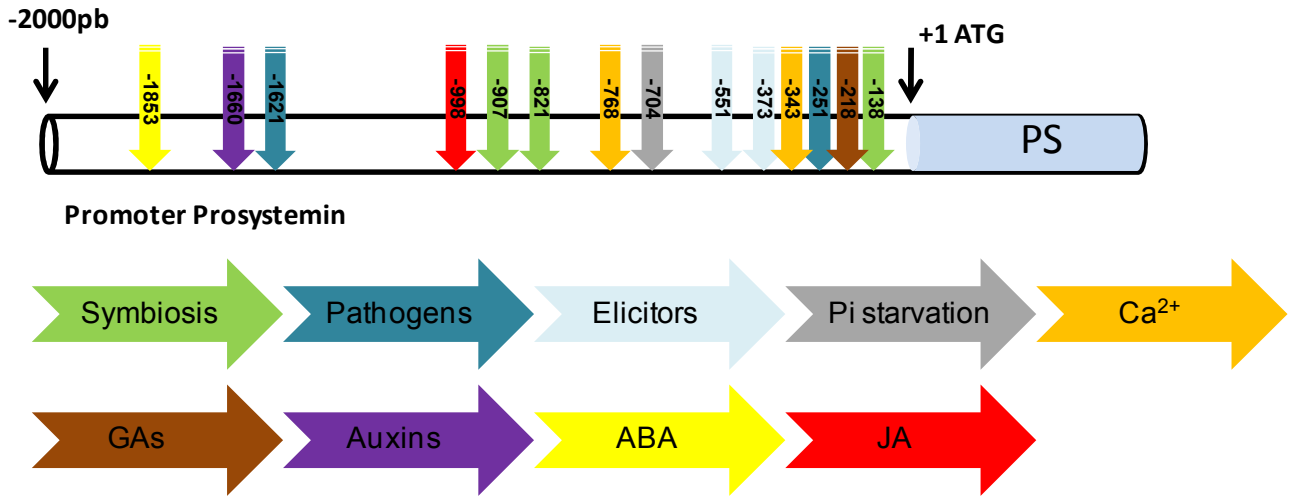

Figure. 5. Structural features of the promoter from the *PS* (SlPS). The localization of several important cis-acting elements is indicated in the SlPS.

Wounding and mycorrhiza induced expression of the possible homologues of prosystemin GmPropep914 and GmPropep890, in soybean plants

As prosystemin (PS) seems to play a key role in several signalling-related plant processes in solanaceus we hypothesized that homologues peptides should be present in other plant families. In soybean, the propeptides GmPropep914 and GmPropep890 have been recently described to share similar molecular and biochemical characteristics with PS (Yamaguchi et al., 2011). Here we studied the response of both propeptides in the JA-related processes wounding and mycorrhization. The expression levels of both genes *PROPEP914* and *PROPEP890* increased in plant shoots 24h after wounding (by 9 fold) indicating a similar regulation pattern (Fig. 6A). In contrast only

PROPEP914 expression also increased in roots mycorrhizal of soybean plants (by 3 fold), but *PROPEP890* expression was not altered with respect to non-mycorrhizal control plants (Fig. 6B). We further analyzed the *PROPEP914* promoter to compare it with the *PS* promoter (Fig. 7). Remarkably, both promoters presented several commune cis-acting elements including those related to symbiosis, response to pathogens, elicitors, Pi starvation, Ca^{2+} and abscisic acid. The result shows a high similarity between both promoters.

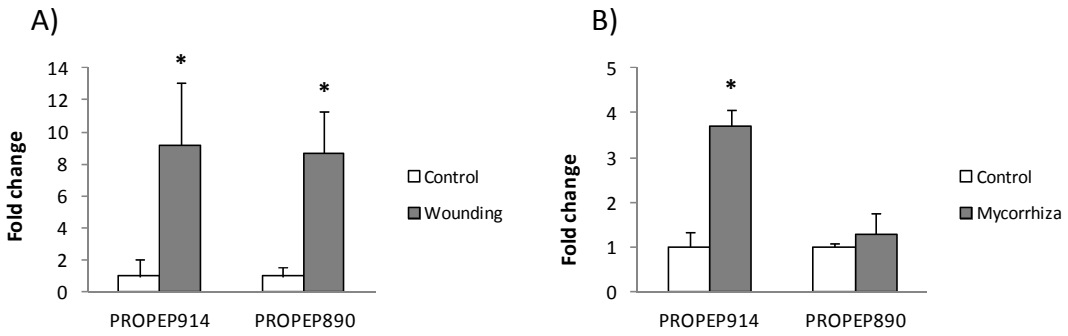

Figure.6. Effect of wounding and mycorrhization in the expression level of the prosystemin homologues GmPropep914 and GmPropep890 in soybean. Shoots of soybean were harvested upon 24h of wounding (A) and roots after 8 weeks of mycorrhization (B). Changes in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method. Data points represent the means of three replicates (±SE). Asterisk indicate statisticaly significant differences compared to control plants (Student's t-test, $P \leq 0.05$).

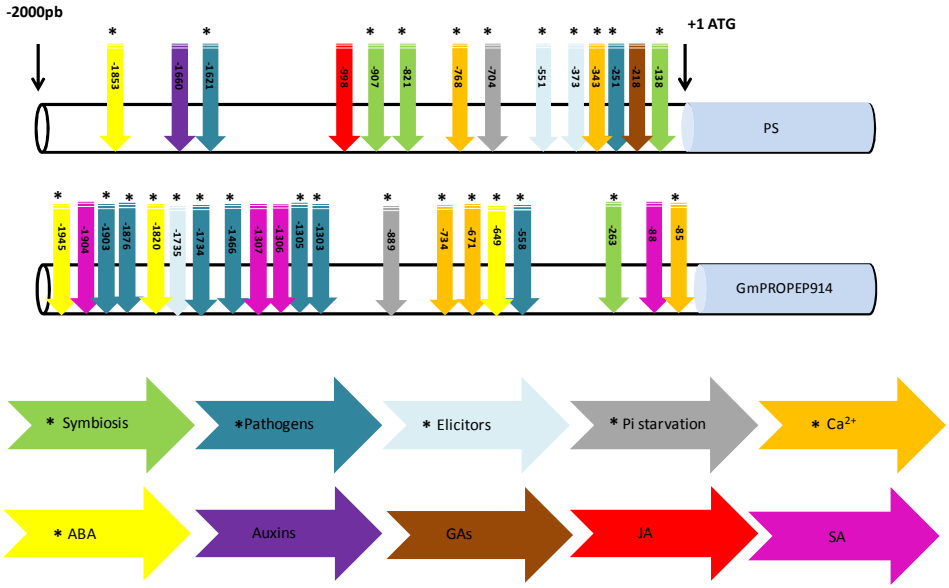

Figure. 7. *In-silico* comparative between *PS* and *PROPEP914* promoters. The localization of several important cis-acting elements is indicated in the *PS* and *PROPEP914* promoters. Asterisk indicate cis-acting elements present in both promoters.

Discussion

Prosystemin (PS) is a peptidic hormone of 200 amino acids, present in some species of solanaceous plants (McGurl et al., 1992). Within the aerial parts of the plant, PS mRNA and protein are known to be synthesized in phloem parenchyma cells of vascular bundles of leaves, stems, and flowers (Avilés-Arnaut and Delano Frier, 2012; Narváez-Vásquez and Ryan, 2004) but was not detected in roots. Recently we showed the expression of the gen coding for the PS in tomato root tissues (Fernández et al., unpublished). In the present study, we found that the expression level of PS gene in root was much lower compare to that observed in shoots. This low expression in the root is probably why PS was not previously reported in root tissues.

In leaves, the role of PS in the regulation of plant defense response against wounding and herbivory is well characterized and it occurs through the regulation of the octadecanoid pathway (McGurl et al., 1992, 1994; Ryan et al., 2003). Upon wounding or methyl-jasmonate (MeJA) exogenous application, systemin (Sys) is processed from PS by proteolisis, although the responsible protease remain unidentified. Within the vascular bundles, Sys initiates a positive amplification loop, in which PS and jasmonic acid (JA) are self-induced as a wave through the plant vasculature for the systemic response (Li et al., 2002; Ryan and Moura, 2002; Lee and Howe, 2003; Stenzel et al., 2003). Similarly, here we show the induction of PS expression in MeJA treated and wounded roots, supporting the connection between roots expressed PS and JA signalling. Thus, PS is related to JA-signalling in the entire plant.

PS is known to regulate diverse processes in the plant as constitutive and inducible defences against insects, volatile emission and only recently its possible role in defence against microbial pathogens have been reported (Corrado et al., 2007, Degenhart et al., 2010; El Oirdi, 2011). Among them, our recent finding demonstrated a critical role of root expressed PS in the establishment of arbuscular mycorrhizal (AM) symbiosis (Fernández et al., unpublished). According with our results, we can conclude that root expressed PS is not only critical for the symbiosis establishment, but also it is modulated during the different stages of colonization. In early stages of symbiosis PS was found to be up regulated in the roots. To further investigate the regulation PS within the root during plant-microbe interaction; we studied the level of root

expressed PS in response to the pathogenic fungi *Fusarium oxysporum* and *Phytophtora parasitica*. Similarly to the beneficial fungus *Glomus intraradices*, root interaction with the pathogen *F. oxysporum* led to an increase in the level of PS within the root. In contrast, *P. parasitica* did not alter PS expression level in the roots. Interestingly, *G. intraradices* and *F. oxysporum* share chitin and its deacetylated form (chitosan) as major component of the cell wall, while glucans are the major component of *P. parasitica* cell wall (Sbrana et al., 1995; Fukamizo et al., 1992). These observations suggest a role of the components of fungal cell wall in the regulation of root PS during plant-fungus interaction. Exposure to chitin oligomers from fungal cell walls are known to elicit in plant defence responses and constitute well characterized MAMPs (Tanabe et al., 2006; Wan et al., 2008, Ramonell et al., 2005). We tested the regulation of root PS expression in the plant in response to purified *G. intraradices* and *F. oxysporum* cell wall. Plant response to MAMPs from both fungi led to an up regulation of PS within the root. In contrast to *F. oxysporum*, the up regulation of *PS* in response to *G. intraradices* MAMPs was delayed compared to that observed during root-AMF interaction. We then explored the possibility that that *PS* expression in the root is regulated during the presymbiotic stages of AM, prior to direct plant-AMF contact. It is known that the molecular dialogue between the fungus and plant in AM symbioses initiates before physical contact of the partners, with the release of fungal diffusible elicitors, among them the so-called *myc* factors (Navazio et al., 2007; Bucher et al., 2009; Maillet et al., 2011). These diffusible factors are modified chitin oligomers that are perceived by the host, and activate early signalling event and the symbiotic program pathways in the host plant (Navazio et al., 2007; Kosuta et al., 2003, Genre et al., 2013). We observed that Myc factors triggered in the plant a very early transient up regulation of PS within the root, suggesting these observations that root PS is a component in the signalling pathway that leads to AM symbiosis. In response to *myc* factor, Calcagno et al., (2011) observed a transient accumulation of nitric oxide (NO) in *Medicago truncatula* roots, suggesting that this signaling molecule may have a role in the early response of the host plants to AM fungal signals. Further, Martinez-Medina et al., (unpublished) demonstrated the occurrence of NO during early steps of the mycorrhizal symbiosis. In the present study, a transiently up regulation of PS in the root was observed in

response to NO, what could indicate a role of NO as secondary messenger in the PS root modulation during the presymbiotic phase of AM symbiosis.

Further, the *in silico* analysis of the PS promoter sequence revealed the presence of several JA, wounding and pathogenesis responsive cis-acting elements, what further corroborate the importance of the octadecanoid pathway in PS regulation. Similarly, Avilés-Arnaut and Délano-Frier, 2012 found similar cis elements in the PS promoter and confirmed their functionality by promoter deletion analysis. Additionally, we identify several cis-elements potentially involved in symbiosis-related plant responses. Among them NODCON1/2 (AAAGAT and CTCTT), described as binding sites for mycorrhization related transcription factors, were present, further supporting reinforce the involvement of PS, as signaling molecule, during AM symbiosis.

The role of PS in plant defense in plant shoot has been widely described (Pearce, 2011). In this study, we demonstrate that PS is also related to signaling process that occurs in roots during early stages of plant interaction with both, beneficial and pathogenic microorganisms. Further we demonstrated the critical role of PS in the establishment of the AM symbiosis (Fernández et al., unpublished). Due to the essential role of this signaling molecule in mycorrhization, established by most plant species, but the restriction of PS to a very limited plant group, we hypothesized that PS homologous in other plant groups should exist To fulfill similar functions. Similarly to PS in tomato, the propeptide GmProPep980, precursor of the small peptide denominate GmPep980 and has been reported to respond to MeJA (Yamaguchi et al., 2011). Further, the exogenous application of GmPep980 induce the expression of several plant defense genes and the expression of *GmPROPEP980* gene, thus being regulated through a positive feedback loop as in the case of PS. We show here that like *PS* in tomato, *GmPROPEP980* was induced by wounding and mycorrhization, supporting the role of *GmPROPEP980* as orthologous of PS in soybean plants. Additionally, the study of the GmPROPEP980 promoter showed a high similarity to *PS* promoter, regarding to cis elements, what reinforce our hypothesis that those molecules could have similar functions in different plant groups.

In summary, we provide evidences of the transcriptional regulation of PS gen in tomato roots associated to early events in the plant immune response to fungi, and in

particular to early communication with AMF essential for mycorrhiza establishment. We also provide results suggesting the existence of peptides with equivalent functions in other plant species.

SUPPLENTARY DATA

Table S1. Primers used for gene expression analysis in this study.

ID	Gene	Primers (5'-3')
X14449	Elongation factor 1α (Sl-*EF1*)[2]	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC
M84801	Prosystemin (Sl-*PS*)[1]	AATTTGTCTCCCGTTAGA AGCCAAAAGAAAGGAAGCAAT
U12639	β-Glucuronidase (*GUS*)[1]	AACGGGGAAACTCAGCAAGC TGTGAGCGTCGCAGAACAT
Gma.28320	Propeptide 914 (Gm-*PROPEP914*)[3]	ATCATCTTGGTAAGATGGTTAA CTGCATCAGCATGTTACAAC

[1]This work; [2]Rotenberg *et al.*, 2006; [3]Yamaguchi et al., 2011.

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DISCUSIÓN GENERAL

Las plantas en su medio interaccionan con multitud de microorganismos tanto beneficiosos como perjudiciales. Dentro de los microorganismos beneficiosos que interaccionan con las plantas en la rizosfera, destacan por su gran relevancia ecológica y su amplia distribución los hongos micorrícicos arbusculares, que colonizan las raíces de las plantas estableciendo una simbiosis mutualista con la planta denominada micorriza (Gianinazzi et al., 2010; Smith y Read, 2008). Esta simbiosis se encuentra finamente regulada ya que el hongo micorrízico penetra en las células del córtex de la raíz dando lugar a multitud de cambios morfológicos y estructurales en la planta hospedadora. Durante esta simbiosis entre la planta y el hongo se produce un intercambio de nutrientes (Parniske, 2008), sobre todo a nivel de los arbúsculos, por tanto gran parte de los cambios estructurales y morfológicos que experimenta la planta en el transcurso de esta simbiosis están esencialmente ligados a aspectos nutricionales. Además de estos beneficios nutricionales, la formación de la simbiosis también protege a la planta frente a estreses de tipo biótico (ataque de organismos patógenos) (Azcón Aguilar y Barea, 1996; Pozo y Azcón-Aguilar, 2007; Koricheva et al., 2009) y abiótico (salinidad, sequia, frio y presencia de metales pesados, entre otros) (Ruiz-Lozano et al., 2006; Aroca et al., 2007; Ferrol et al., 2009). Para que la planta pueda obtener todos estos beneficios, necesita controlar los niveles de colonización de una manera muy precisa (Slezack et al., 2000), ya que de otra forma la simbiosis podría derivar en una interacción perjudicial para la planta (Catford et al., 2003; Breuillin et al., 2010). En los primeros estadíos de la simbiosis, previos a la colonización, se produce un aumento en la expresión de diferentes genes implicados en la regulación de la respuesta de defensa de la planta (Gallou et al., 2012). Esta activación de la respuesta de defensa de la planta parece indicar, que en los primeros estadíos, la planta reconoce al hongo micorrízico como un potencial agresor y necesita un complejo sistema de señalización para poder determinar el carácter beneficioso del hongo (Pozo y Azcón-Aguilar, 2007; van Wees et al., 2008, Spoel y Dong, 2012; Zamidious y Pieterse, 2012). Algunos de estos genes implicados en la respuesta de defensa de la planta se encuentran regulados por la homeostasis de distintas rutas

hormonales (Pozo y Azcón Aguilar, 2007; Ruiz-Lozano et al., 2012). Por tanto la colonización por parte de los hongos micorrízicos implica cambios hormonales relacionados con la defensa de la planta hospedadora. Son muchos los estudios acerca de estos cambios hormonales durante una simbiosis micorrízica arbuscular (AM), pero los resultados obtenidos son altamente controvertidos (revisado en Hause et al., 2007), posiblemente debido a que, si bien la simbiosis AM no muestra una alta especificidad, dependiendo del genotipo de la planta hospedadora y del hongo micorrízico y las condiciones ambientales, el desarrollo de esta simbiosis puede ser diferente (Kiers et al., 2011). A pesar de esta diversidad funcional, hay algunas fitohormonas que presentan un claro papel en esta simbiosis (Hause et al., 2007; Herrera-Medina et al., 2007; Pozo y Azcón-Aguilar 2007). Muchos estudios muestran un posible papel del ácido salicílico (SA) en el control de la colonización del hongo dentro de la planta hospedadora (Pozo y Azcón Aguilar, 2007; Gutjahr y Paszkowski, 2009), debido a su importante papel en la respuesta de defensa de la planta frente a organismos biótrofos (Pieterse et al., 2009). Por tanto debido a que los hongos AM son biótrofos obligados es lógico pensar que el SA pueda ejercer un control sobre la colonización del hongo. Por otro lado el jasmonato (JA) y sus derivados, más conocidos como jasmonatos (JAs), parecen participar activamente en el desarrollo de la simbiosis AM (Hause y Schaarschmidt, 2009, León-Morcillo et al., 2012), debido a que los niveles de JAs se ven incrementados en plantas micorrizadas (Hause et al., 2002; Vierheilig y Piché, 2002; Stumpe et al., 2005; Meixner et al., 2005). Además, muchos estudios muestran que los JAs están relacionados con la respuesta de defensa de la planta frente a organismos necrótrofos, por tanto, este aumento de JAs en las plantas micorrizadas es uno de los beneficios que aportan los hongos AM a la planta hospedadora, reforzando la defensa de la planta frente al ataque de microorganismos necrótrofos (revisado en Jung et al., 2012). Además no solo los niveles de cada una de estas hormonas son importantes para el desarrollo de la simbiosis sino que la interacción entre ambas hormonas puede ser clave para el proceso. En el proceso de señalización entre ambas hormonas tienen un papel importante otras fitohormonas como pueden ser el ácido abscísico (ABA) o el etileno (ET) (Herrera-Medina et al., 2007; Zsogon et al., 2008; Martín-Rodríguez et al., 2010).

En el **primer capítulo** de esta Tesis Doctoral se ha realizado un estudio integrativo de los cambios en la homeostasis de algunas de estas rutas hormonales implicadas en la regulación de la respuesta de defensa de la planta, provocados por la colonización micorrízica. Para esto hemos utilizando los hongos micorrízicos *Glomus mosseae* y *Glomus intraradices*, y las plantas hospedadoras tomate, soja y maíz. En este estudio hemos observado diferentes grados de colonización dependiendo del hongo y la planta implicados, confirmando la "diversidad funcional" observada en otros estudios (Pozo et al., 2002; Feddermann et al., 2008; Kiers et al., 2011). Además los resultados muestran una dependencia del genotipo del hongo y de la planta en la regulación de estas rutas hormonales durante la simbiosis AM. A pesar de esta "especificidad" en la respuesta de la planta, observamos patrones de regulación conservados en algunas de estas rutas hormonas. La ruta del ABA parece encontrarse inhibida o no alterada en la mayoría de las interacciones llevadas a cabo en este estudio, reforzando lo observado en otros estudios (Martínez-Medina et al., 2011; Aroca et al., 2012; Asensio et al., 2012). La modulación de la ruta del SA mostró una gran variabilidad, siendo altamente dependiente del hongo y la planta hospedadora implicada. Estudios anteriores sugieren que el SA controla el avance del hongo dentro de la raíz. Nuestro trabajo sugiere que los niveles de SA dependen de la fase en la que se encuentre el proceso de micorrización en las diferentes intercciones estudiadas (Herrera-Medina et al., 2003; Khaosaad et al., 2007; Campos-Soriano y Segundo, 2011). Uno de los efectos más claros observados en este estudio es un patrón de regulación del JA muy conservado entre las plantas y altamente dependiente del hongo. La colonización por *G. mosseae* produjo un aumento de los niveles de JA, en las diferentes especies de plantas estudiadas, mientras que *G. intraradices* no desencadenó ningún cambio significativo en los niveles de JA. Este patrón de regulación del JA podría explicar la mayor capacidad de bio-protección por parte de *G. mosseae* comparado con *G. intraradices* (Pozo y Azcón-Aguilar, 2007).

Debido a la alta conservación del patrón de regulación de la ruta del JA en las planta micorrizadas, en el **segundo capítulo** de esta Tesis Doctoral, decidimos profundizar en el estudio de este patrón de regulación. En este trabajo se realizó un estudio más exhaustivo de los niveles de los distintos derivados del JA, así como un análisis de los

niveles de expresión de múltiples genes implicados tanto en la biosíntesis como en la percepción de los diferentes derivados del JA. Los resultados muestran un aumento en el precursor del JA ácido (9S, 13S)-12-oxo-fitodienoico (OPDA), que se encuentra aguas arriba en la ruta de biosíntesis del ácido jasmónico. Sin embargo, los niveles de JA libre o su conjugado jasmonato-isoleucina, no se vieron alterados en este estudio. Estos datos sugieren que el OPDA podría estar orquestando el proceso de micorrización en la familia de las solanáceas y no las otras formas de JA (Hause et al., 2002; Stumpe et al., 2005). Además, el estudio de los niveles de expresión de genes implicados en la ruta de biosíntesis de las oxilipinas, sugieren que durante el proceso de micorrización la planta redirige esta ruta hacia la biosíntesis de diferentes formas de OPDA pertenecientes a las dos principales ramas de la ruta de biosíntesis del JA (9-LOX y 13-LOX). Todos estos datos confirman la importancia de la ruta de los JAs durante el proceso de micorrización.

Además de los JAs, se han descrito otras hormonas muy importantes para el proceso de micorrización. Las estrigolactonas son consideradas nuevas hormonas vegetales con un importante papel en la señalización para el establecimiento de una simbiosis AM (Akiyama et al., 2005; Parniske, 2008). Por este motivo en **el tercer capítulo** de esta Tesis Doctoral, analizamos los niveles de estrigolactonas en plantas de tomate micorrizadas. Nuestros estudios mostraron un claro descenso en la producción de los principales tipos de estrigolactonas en las plantas micorrizadas. Estos resultados parecen indicar que una vez establecida la simbiosis AM, la planta hospedadora modula la señalización en la raíz para limitar la interacción con nuevos hongos micorrízicos.

Para estudiar con más detalle el papel de los JAs en la micorrización en el **cuarto capítulo** de esta Tesis Doctoral, realizamos un estudio del proceso de micorrización mediante el uso de diferentes mutantes en la biosíntesis/percepción del JA (Schilmiller and Howe, 2005; Wasternack et al., 2006), asi como en la biosíntesis de prosistemina (PS). La PS es un propéptido a partir del cual se origina la sistemina (Sys), una hormona peptídica con un papel importante en la regulación de la biosíntesis de los JAs, actuando aguas arriba de esta ruta (McGurl et al., 1992; Bergey et al., 1996). Curiosamente solo se observaron cambios significativos en los niveles de colonización

en los mutantes con los niveles de PS alterados. *ps+* (sobreexpresante de PS) mostró un mayor grado de colonización, mientras que *ps-* (mutante con la producción de PS comprometida) fue incapaz de de establecer una simbiosis AM. Mediante un estudio más exhaustivo de los mutantes de PS, pudimos determinar por primera vez que la raíz de tomate es capaz de expresar el gen que codifica para la PS. Hasta el momento la expresión del gen que cofifica para la PS solo se había descrito en la parte aérea de algunas solanáceas (McGurl et al., 1992; Narvaez-Vasquez y Ryan, 2004; Wasternack et al., 2006). Además, nuestro estudio reveló que la expresión de PS en la raíz es clave para el establecimiento de una simbiosis AM. Esta función de la PS en el establecimiento de una simbiosis AM parece ser independiente del JA, ya que los diferentes mutantes de JA usados en este estudio mostraban unos niveles de colonización similares a las plantas silvestres. Nuestros estudios *in vitro* mostraron que la PS actúa en el proceso de señalización durante la colonización AM, ya que mediante el uso de raíces *ps-* pudimos observar un menor desarrollo del micelio extraradical del hongo así como una menor germinación de las esporas, sugiriendo que la PS tiene un papel en el dialogo presimbiótico que tienen lugar entre la planta hospedadora y el hongo AM. Este papel de la PS en el dialogo presimbiótico podría ser través de la regulación de la biosíntesis de las estrigolactonas en la planta hospedadora, ya que los mutantes *ps-* mostraron niveles reducidos de las principales estrigolactonas, mientras que *ps+* mostraba un aumento en los niveles de estas. Debido a estas alteraciones en los niveles de estrigolactonas y al importante papel de estas en la comunicación entre la planta y el hongo, sugerimos que la PS controla el establecimiento de la simbiosis AM mediante la regulación de la biosíntesis de las estrigolactonas.

Además de esta función en el proceso de señalización durante la simbiosis, pudimos observar que la PS también tiene un papel en la regulación de la arquitectura radical, ya que las raíces de los mutantes *ps-* y *ps+* mostraron variaciones en tamaño de la raíz principal, así como en el número de raíces secundarias y su tamaño. Es posible que la PS pueda realizar estos cambios mediante la regulación de la biosíntesis de las estrigolactonas, pues el papel de estas en la arquitectura radical está ampliamente descrito (Kapulnik et al., 2011; Ruyter-Spira et al., 2011).

Por último en el **quinto capítulo** de esta Tesis Doctoral tratamos de profundizar en la regulación del gen de la PS en la raíz de tomate. En nuestro estudio observamos que la PS se regula transcripcionalmente en la raíz de forma similar a la PS expresada en la parte aérea. Los niveles de expresión de PS en raíz incrementaron tras la el tartamiento con herida y la aplicación de metil-jasmonato (MeJA). Además la expresión de PS en raíz fue modulada tras la interacción con diferentes hongos del suelo, en función de los componentes de la pared celular. Este resultado apunta a un papel de la PS en los primeros estadíos de la interacción de la planta con diferentes hongos del suelo. Además la elicitación de la raíz con diferentes moléculas implicadas en los procesos de señalización temprana, factores *myc* y oxido nítrico, dieron como resultado el incremento en los niveles de expresión del gen que codifica para la PS, reforzando la idea de que la PS tiene un papel en los procesos de señalización en los primeros estadios de la interacción de la planta con diferentes microorganismos. Debido a las importantes funciones de la PS en las plantas de tomate decidimos buscar péptidos de señalización homólogos a la PS en especies alejadas filogenéticamente de las solanaceas. En nuestro estudio observamos que GmProPep914, que es un propéptido presente en soja (Yamaguchi et al., 2011), se regulaba de manera similar a la PS, aumentando su nivel de expresión tras la aplicación de MeJA, herida y durante una simbiosis AM.

Con el fin de determinar si el ProPep914 es el homologo en soja a la PS en tomate, en futuros trabajos estudiaremos el proceso de colonización micorrízica en líneas mutantes de soja con la biosíntesis de este propéptido comprometida. Por otro lado, ampliaremos la búsqueda de estos homologos en especies de plantas alejadas filogenéticamente como pueden ser las monocotiledóneas, ya que recientemente se ha sugerido que ZmPep1 podría ser un homologo a la PS en maíz (Huffaker et al., 2011).

Además en futuros trabajos intentaremos profundizar en el estudio de los mecanismos por los cuales la PS regula el establecimiento de la simbiosis AM, así como su papel en la regulación de la biosíntesis de los JAs.

CONCLUSIONES

1. La simbiosis micorríca arbuscular implica cambios en el contenido de varias fitohormonas relacionadas con diferentes estreses en la planta, que se correlacionan con cambios en la expresión de genes implicados en la regulación de la biosíntesis y respuesta de estas fitohormonas. Estos cambios son dependientes del genotipo de la planta y hongo implicados.

2. El genotipo de los hongos micorrízicos arbusculares influye en la modulación de la señalización de la respuesta de defensa de la planta hospedadora, y puede determinar las distintas estrategias de colonización llevadas a cabo por los diferentes hongos micorrízicos arbusculares.

3. La regulación de la ruta de los derivados del jasmonato es altamente conservada en la simbiosis micorrízica arbuscular en las distintas especies de plantas, apoyando un papel clave de los jasmonatos durante la interacción con los hongos micorrízicos arbusculares.

4. La hormona peptídica sistemina es expresada y transcripcionalmente regulada en raíces de tomate. Además, en la raíz la sistemina tiene un papel en la interacción de la planta con microorganismos mediante la ruta de los jasmonatos.

5. Independientemente de la ruta de los jasmonatos, la expresión de prosistemina en las raíces de tomate juega un papel crítico en el establecimiento de la simbiosis micorrízica arbuscular.

6. La prosistemina parece regular la interacción de hongo micorrízico arbuscular con la planta hospedadora mediante la regulación en la producción de estrigolactonas durante la etapa pre-simbiotica y regulando la colonización durante la fase simbiótica.

CONCLUSIONS

1. Arbuscular mycorrhizal symbiosis implies changes in the content of several stress related phytohormones, which correlate with changes in the expression of genes involved in their biosynthesis and the responses they regulate. These changes are dependent on the genotype of the plant and fungal involved.

2. The genotype of the arbuscular mycorrhizal fungi influence the modulation of the host defence signalling, and may give some hints on the colonization strategies of different arbuscular mycorrhizal fungi.

3. The regulation of the jasmonate-related pathway is highly conserved in mycorrhizal symbiosis across plant species, supporting a key role of jasmonates during the arbuscular mycorrhizal interaction.

4. The hormone peptide systemin is expressed and transcriptionally regulated in tomato roots. Systemin is involved in plant interaction with microorganisms trough of the jasmonate-related pathway.

5. Independent of the jasmonate-related pathway, the expression of prosystemin in tomato roots plays a critical role in the establishment of arbuscular mycorrhizal symbiosis.

6. Prosystemin seems to regulate host plant-AM fungus interaction by affecting strigolactone production during the pre-symbiotic stage and regulating the colonization during the symbiotic phase.

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ANEXOS

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Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi.

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SPECIAL FEATURE

PLANT-MEDIATED INTERACTIONS BETWEEN ABOVE- AND BELOW-GROUND COMMUNITIES

Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi

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Summary

1. Plants possess numerous mechanisms to control infections by deleterious organisms. Unspecific resistance mechanisms may, however, also exert ecological costs when they have a negative effect on beneficial plant–microbe interactions. Such negative effects may even cross the border between a plant's aerial parts and its roots and then affect very central functions such as nutrient uptake and root resistance to micro-organisms. Whereas an impaired nodulation indeed appears common after resistance expression in the leaves, contradictory results have been published for the case of arbuscular mycorrhizal (AM) fungi.

2. We analysed the effect of induction of resistance mechanisms in foliar tissues on AM colonization in soybean plants, using acibenzolar-S-methyl (ASM) as chemical elicitor. By determining different physiological and biochemical parameters, we assessed whether the effects are related to the activation of the plant defence mechanisms or rather to the re-allocation of primary metabolites.

3. Colonization with AM fungi transiently decreased after pathogen resistance mechanisms were elicited in the aerial parts of the plant. The induction with ASM led to a significant, yet moderate, defence response in the roots, which was modulated in mycorrhizal plants. No allocation or fitness costs associated with the induction of resistance were detected in this study.

4. *Synthesis*. Our study confirms a transient negative impact of the elicitation of foliar defences on root–AM interactions. The results show that induced resistance to foliar pathogens can (i) move from the above-ground to the below-ground compartment and (ii) affect mutualistic micro-organisms as well as plant pathogens. We also conclude that (iii) the negative effect is likely linked to changes in the defence status of the plant rather than to changes in resource allocation patterns and (iv) the AM association can modulate the activation of the plant defence mechanisms and overcome such effects.

Key-words: allocation costs, arbuscular mycorrhizal fungi, fitness costs, pathogenesis-related proteins, photosynthesis, plant–soil (below-ground) interactions, PR-1 gene expression, β-1,3-glucanase; chitinase, systemic acquired resistance

Introduction

Higher plants largely rely on mutualisms with a diverse set of organisms such as pollinators, natural enemies of herbivores, and micro-organisms such as leaf endopyhtic fungi and soil-borne rhizobia and fungi. Fungi forming arbuscular mycorrhizas (AM) are believed to associate with the roots of over 80% of all higher plants in nature and have significant influences on the nutrition of the plant (Parniske 2008; Smith & Read 2008) as well as on its defensive status (Pozo & Azcón-Aguilar 2007;

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Kempel *et al*. 2010). As a consequence, AM have a significant impact on plant interactions with other organisms and, ultimately, on ecosystem functioning (van der Heijden, Bardgett & van Straalen 2008).

Plant must, however, also cope with pathogenic micro-organisms, and they do so by activating various defence mechanisms (Pieterse *et al*. 2009). Because plants interact simultaneously with multiple other organisms, their responses to specific events may have a significant impact on other interactions, and these effects can even cross the border between the aerial compartments and the roots (van Dam 2009). In fact, the relevance of induced plant defence responses for above-ground and below-ground communities and their interactions is now being recognized (Bezemer & van Dam 2005; Erb *et al*. 2009; van Dam and Heil 2011). Because of the intimate association of plants with mutualistic micro-organisms, several cross-reactions may occur between plant resistance to pathogens and beneficial associations with, for example, nodulating rhizobia, free-living bacteria in the rhizosphere and mycorrhizal fungi (Pozo & Azcón-Aguilar 2007; Soto *et al*. 2009; Yang *et al*. 2011). In this study, we investigate whether the induction of resistance mechanisms to pathogens in foliar tissues can cross the above-ground–below-ground border and have an impact on the symbiosis of roots with AM fungi.

Systemic acquired resistance (SAR) is an induced resistance in plants that is expressed upon local pathogen infection and confers resistance to a broad spectrum of attackers (Ryals *et al*. 1996; Sticher, Mauch-Mani & Métraux 1997). In most plant species, this response requires the synthesis of the endogenous signalling molecule salicylic acid (SA) in the infected tissue and its accumulation in the systemic tissue (Heil & Ton 2008; Xu *et al*. 2009) and it is characterized by the synthesis of pathogenesis-related (PR) proteins (Durrant & Dong 2004). Fourteen families of PR proteins have been described so far, and chitinases and β-1,3-glucanases are among them (van Loon & van Strien 1999). Chitinases and β-1,3-glucanases catalyse the hydrolysis of chitin and β-D-glucans, respectively, so both possess direct antimicrobial activity by degrading common microbial cell wall components (Dumas-Gaudot *et al*. 1996; van Loon 1997). Thus, PR proteins, together with other elements of SAR, contribute to a broad-spectrum resistance against diverse pathogenic bacteria, fungi and viruses. Because of this lack of specificity, they may also have a negative impact on microbial mutualists of the plant, then leading to significant 'ecological costs' of SAR (Heil 2002).

In fact, several studies have reported negative effects of SAR on beneficial plant–microbe interactions: Martínez-Abarca *et al*. (1998), Ramanujam, Jaleel & Kumaravelu (1998), Lian *et al*. (2000), Heil (2001b) and Faessel *et al*. (2010) reported an inhibition of root nodule formation after chemical induction of pathogen resistance in several Fabaceae species. We can conclude that an above-ground elicitation of SA-dependent resistance responses generally impairs the capacity of plant roots to consecutively establish a symbiosis with nodulating bacteria (van Dam & Heil 2011). By contrast, variable results have been reported concerning AM associations, depending on the application method of the chemical elicitor and experimental conditions (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Tosi & Zazzerini 2000; Sonnemann, Finkhaeuser & Wolters 2002; Faessel *et al*. 2010).

How does a resistance against leaf pathogens affect below-ground symbioses with mutualistic micro-organisms? Unfortunately, the physiological mechanisms of the above-ground–below-ground interactions are unclear in most cases, although a re-allocation of primary metabolites or a systemic resistance expression appears to be the most common reason (van Dam & Heil 2011). Besides the induction of active resistance, elicitation of SAR can cause a reduction in plant productivity and fitness, which results from a shift in the allocation pathways from growth to defence (Heil 2001a; Walters & Heil 2007). A common phenomenon associated to this 'shift from housekeeping to resistance expression' is a reduction in photosynthesis (Logemann *et al*. 1995; Scheideler *et al*. 2002; Swarbrick, Schulze-Lefert & Scholes 2006; Schwachtje & Baldwin 2008; Heil & Walters 2009). Thus, negative effects of foliar resistance expression on below-ground mutualisms could result from re-allocation of assimilates, as described from those cases in which plant tolerance responses increased root susceptibility to root herbivores (Kaplan *et al*. 2008). The goal of our present study was, therefore, to elucidate the physiological mechanisms that underlie any putative phenotypic effects of elicitation of resistance in above-ground tissues on the interaction of roots with AM fungi.

Several chemical elicitors of plant resistance are available, which mimic the mechanism by which pathogen infection induces resistance and thereby elicits SAR (Oostendorp *et al*. 2001). The benzothiadiazole derivative benzo-[1,2,3]-thiadiazole-7-carbothioic acid-S-methyl ester (Görlach *et al*. 1996), also known as acibenzolar-S-methyl (ASM), is a functional analogue of SA and effectively induces systemic resistance against pathogens and nematodes in multiple plant species including soybean (Dann *et al*. 1998; Chinnasri, Sipes & Schmitt 2003; Meyer *et al*. 2006; Faessel *et al*. 2008), *Arabidopsis thaliana* (Dietrich, Ploss & Heil 2004, 2005), barley (Sonnemann, Streicher & Wolters 2005), tobacco (Friedrich *et al*. 1996; Ginzberg *et al*. 1998; Shaul *et al*. 1999), tomato (Baysal, Soylu & Soylu 2003; Rossi Cavalcanti *et al*. 2006) and papaya (Zhu *et al*. 2003).

Considering the contradictory studies on the effect of SAR on mycorrhizas cited above, and the lack of knowledge on the underlying mechanisms, detailed research is required to understand the impact of plant resistance regulation on AM. Using a well-characterized chemical elicitor and a mycotrophic model plant such as soybean allows a detailed investigation of such potential effects and the underlying biochemical and molecular mechanisms. We tested the hypothesis that SAR-expressing soybean plants are colonized by the AM fungus *Glomus mosseae* to a lower extent than untreated plants. In order to assess if such effects are related directly to the defence mechanisms or to the alloca-

tion costs that are associated with the expression of the defence, we analysed defence responses and growth-associated parameters. Protein content, the activity of chitinases and β-1,3-glucanases and the expression of the *PR-1a* gene were chosen as disease resistance markers, whereas plant weight, sugar content, photosynthetic activity and seed production were chosen as markers for allocation and fitness costs.

Materials and methods

PLANT AND FUNGAL MATERIAL, GROWTH CONDITIONS AND CHEMICAL INDUCTION OF RESISTANCE

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams 82) were surface sterilized with a commercial bleach solution (10%, v/v) and germinated under sterile conditions on wet filter paper at 28 °C for 3 days. Plants were grown in 500 mL pots containing a sterile mixture of quartz sand and soil (1:1, v/v).

Arbuscular mycorrhizal fungal inoculum consisted of propagules from *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 12) including spores and chopped *Allium porrum* L. roots colonized by the fungus in a sand:sepiolite (1:1, v/v) substrate. Mycorrhizal inoculation was carried out by mixing with the growing substrate 7% of the inoculum. In control plants 7% sand:sepiolite (1:1, v/v) was mixed with the growing substrate. At potting, all plants received a filtrate (< 20 μm) of the AM fungal inoculum in order to provide the microbial populations accompanying the mycorrhizal inocula but free from AM propagules.

Soybean plants were cultivated in a controlled environment room (25/18 °C day/night temperature, 60% relative humidity, 16 h photoperiod, with a photosynthetic photon flux of 400 μmol photons m^{-2} s^{-1}). Plants were watered three times a week, the first 3 weeks only with water, and then twice a week with water and once a week with 50% Long Ashton nutrient solution (Hewitt 1966) at one-quarter phosphorus strength.

Six weeks after planting half of the plants were sprayed with an aqueous 400 mg L^{-1} Bion® solution at 10 mL per plant in order to induce resistance. Bion® is the name under which ASM is marketed by Syngenta (Basel, Switzerland). The rest of the plants were sprayed with the same amount of distilled water. Plant roots had no direct contact with ASM as the application was done by spraying only the shoots. The experimental design consisted, thus, of four different treatments: non-induced non-mycorrhizal plants (Nm−), induced non-mycorrhizal plants (Nm+), non-induced plants inoculated with *G. mosseae* (Gm−), and induced plants inoculated with *G. mosseae* (Gm+).

PLANT HARVEST AND ASSESSMENT OF MYCORRHIZAL COLONIZATION

Plants were harvested 1 and 2 weeks after the application of ASM (7 and 8 weeks after planting). The root system was carefully washed in running tap water and then the fresh weight of shoots and roots was annotated. About one-third of the root system from each plant was kept for determination of mycorrhizal colonization, and the rest was immediately frozen in liquid nitrogen and stored at −80 °C until protein and RNA extraction. Leaf samples were also frozen and stored at −80 °C.

For the estimation of mycorrhizal colonization, samples from the root system were cleared and stained (Phillips & Hayman 1970), and the percentage of total root length colonized by the AM fungi was determined by using the gridline intersect method (Giovannetti & Mosse 1980).

The remaining plants were harvested at the end of their growth period (17 weeks after planting) to record the seed dry weight per plant and the number of seeds per plant.

SUGAR EXTRACTION AND QUANTIFICATION

Sugars were extracted following a modified procedure of the method described by Bligh & Dyer (1959). One gram of fresh leaf material from the plants harvested 1 and 2 weeks after the application of ASM was homogenized in a mortar filled with 7.5 mL methanol for 2 min. Then 7.5 mL chloroform was added and the mixture was homogenized for 1 min. Finally, 3.75 mL of water with 0.88% NaCl were added, the solution was centrifuged at 4500 ***g*** for 10 min at 0 °C, and the upper layer containing the sugars was kept for further analysis.

Quantification of sugars was carried out following the anthrone method (Morse 1947). One hundred microlitre sugar solution, 900 μL water and 3 mL anthrone solution (200 mg anthrone + 100 mL 72% H_2SO_4) were mixed and boiled for 10 min at 100 °C. Samples were then put on ice to stop the staining reaction. The absorbance was measured at 620 nm in a spectrophotometer. The amount of sugars released was determined by comparison with a glucose standard curve with concentrations ranging from 0 to 400 μg mL^{-1}. One millilitre of each glucose concentration was mixed with 3 mL anthrone solution, boiled and the absorbance measured as described above.

PHOTOSYNTHESIS DETERMINATION

Photosynthetic assimilation was measured with a portable photosynthesis system (Li-Cor 6400, Lincoln, NE, USA) 1 week after ASM treatment. The CO_2 concentration was set at 400 p.p.m. and the photosynthetically active radiation at 1000 μmol m^{-2} s^{-1}. All measurements were performed in the morning, between 9:00 and 12:00 AM.

PROTEIN EXTRACTION AND QUANTIFICATION

Frozen plant material (root and leaf samples) from the plants harvested 1 week after the application of ASM was ground at 4 °C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in 100 mM MacIlvaine extracting buffer (citric acid/Na_2HPO_4), pH 6.8 (1:1, w/v). Crude homogenates were centrifuged at 15 000 ***g*** for 30 min at 4 °C and the supernatant fractions were kept frozen at −20 °C. Protein contents were determined by the method of Bradford (1976) using BSA as standard.

CHITINASE AND B-1,3-GLUCANASE ACTIVITY ASSAYS

Chitinase activity was determined with a fluorimetric assay (Ren, Wee & Chang 2000). Five microlitre methylumbelliferyl β-D-*N,N',N'*-triacetylchitotrioside hydrate (0.5 mg mL^{-1}; Sigma, Alcobendas, Madrid, Spain) were added to 95 μL plant extract (root and leaf samples) in a black 96-well microplate. Samples were incubated at 40 °C for 30 min in a shaker. The fluorescence was measured using a Perkin Elmer luminescence spectrometer LS50 (excitation 365 nm, emission 450 nm). All values were reported as fluorescence units.

The activity of β-1,3-glucanase was performed in 96-well microplates using an adaptation of the method described by Somogyi (1952). *Laminaria digitata* laminarin (Sigma) was used as substrate. The total volume of 180 μL reaction preparation contained 50 μL

Table 1. Primers used for the quantitative real-time PCR

Organism	Gene	Accession number	Primer sequence (5′–3′)
Glomus intraradices	*GintEF* (Benabdellah *et al.* 2009)	DQ282611	F-(5′-GCTATTTTGATCATTGCCGCC-3′) R-(5′-TCATTAAAACGTTCTTCCGACC-3′)
Glycine max	*Gm18S* (Porcel *et al.* 2006)	X02623	F-(5′-CCATAAACGATGCCGACCAG-3′) R-(5′-CAGCCTTGCGACCATTACTCC-3′)
Glycine max	*GmPR-1a*	AF136636	F-(5′-ATGTGTGTGTTGGGGTTGGT-3′) R-(5′-ACTTTGGCACATCCAAGACG -3′)

plant extract (root and leaf samples), 10 μL laminarin (20 mg mL^{-1} in 50 mM Na-acetate buffer, pH 5.0), 60 μL copper reactive and 60 μL arsenic reactive. The absorbance was measured at 650 nm in a spectrophotometer. The amount of sugars released was determined by comparison with a glucose standard curve with concentrations ranging from 0 to 200 μg mL^{-1}.

RNA EXTRACTION AND GENE EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from root and leaf samples using Tri Reagent® (Ambion, Foster City, CA, USA) and treated with DNase RQ1 (Promega, Madison, WI, USA). cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) starting with 1 μg of total DNAse-treated RNA.

Gene expression levels were determined by quantitative real-time PCR, using the primers detailed in Table 1. The sets of primers for *Gm18S* and *GintEF* have been previously used as constitutive expression controls for soybean and *Glomus intraradices*, respectively (Porcel *et al.* 2006; Benabdellah *et al.* 2009). The soybean *PR-1a* gene was selected as marker for SA-regulated defence responses, and the corresponding set of primers was designed using the PRIMER 3 software (Rozen & Skaletsky 2000). Prior to real-time PCR analysis, the specificity of the selected primers was checked by conventional RT-PCR. The efficiency of the primer sets was evaluated by performing real-time PCR on several dilutions of genomic DNA. All quantitative real-time PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). The amplification protocol included an initial denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100 °C) after the final cycle of the PCR. The results obtained for the different treatments were standardized to the soybean 18S rRNA levels, which were amplified with the primers *Gm18S* shown in Table 1.

Real-time PCR experiments were carried out at least three times, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the 2-DDCT method (Livak & Schmittgen 2001). Negative controls without cDNA were included in all PCR reactions.

REPLICATION AND STATISTICAL ANALYSIS

Eighteen replicated plants were used per treatment, four of them were harvested at each time point (7 and 14 days after treatment) and used for the physiological and biochemical analyses and the rest (10 replicated plants) were used for the quantification of seed production. STATISTICA 6.1 software (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. A Student's *t*-test was carried out to detect significant effects of induction of resistance on AM colonization. Mann–Whitney *U* tests were conducted to detect significant effects of induction of resistance on fresh weight, sugar content, photosynthetic activity, protein content, chitinase and β-1,3-glucanase activity, and seed production parameters. Mean ± SE were calculated. Significance levels were set at 5%.

Results

Soybean plants inoculated with *G. mosseae* harvested 1 week after the application of ASM (Gm+) showed significantly lower AM colonization than Gm plants sprayed only with water (Gm−) (Fig. 1a; $t = 2.63$; d.f. = 6; $P = 0.038$; *t*-test). A second harvest of Gm+ plants 2 weeks after ASM treatment revealed that the differences in AM colonization between Gm+ and Gm− plants had levelled off ($t = 0.35$; d.f. = 6; $P = 0.735$; *t*-test). Thus, the negative effect of the induction of systemic resistance on the AM colonization of soybean plants appeared transitory. The absence of AM colonization was confirmed in non-inoculated plants (Nm) (data not shown).

Quantifying the expression level of a fungal 'housekeeping gene' is known to be an accurate and sensitive method to estimate the amount of active, living AM fungus within root tissues (Isayenkov, Fester & Hause 2004). The quantitative real-time PCR of transcript levels of *GintEF*, constitutively expressed in AM fungi (Benabdellah *et al.* 2009), confirmed a significant reduction of the AM fungus in Gm+ roots 1 week after treatment (0.4-fold compared to untreated plants; Fig. 1b). The fungal presence increased with time in both treated and untreated plants, and 2 weeks after the chemical treatment the inhibition was completely overridden. The results confirm that the negative effect of ASM application on AM colonization was transitory.

Resistance induction has been associated to a shift in resources allocation from growth to defence, and this shift may potentially limit the resources available for the fungal symbiont, thus limiting AM colonization. To assess if this is the case, we compared plant biomass, sugar content and photosynthetic activity in induced and non-induced plants. Neither the fresh weight, nor the sugar content, nor the photosynthetic activity changed significantly in either Nm or Gm plants after the induction of resistance ($P > 0.05$; Mann–Whitney *U* test; Table 2).

Nm+ plants produced fewer seeds than Nm− ones (Fig. 2a,b), although the difference was insignificant both for

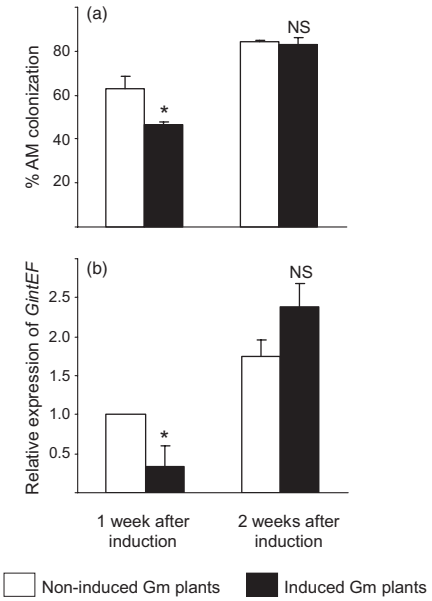

Fig. 1. Determination of arbuscular mycorrhizal (AM) fungal colonization of soybean roots by histochemical and real-time PCR analysis. Effect of the chemical induction of plant resistance to pathogens on AM colonization in soybean plants inoculated with *Glomus mosseae* (Gm) harvested 1 and 2 weeks after the application of acibenzolar-S-methyl (ASM). (a) Percentage of root length colonized by Gm estimated under the dissecting microscope upon staining of fungal structures with trypan blue. Measurements summarize mean percentage of AM colonization + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). The sample size was four plants per treatment. $*P < 0.05$, ns $P > 0.05$ according to *t*-test. (b) Relative transcript levels of the *Glomus* elongation factor gene *GintEF*. Total RNA was extracted from soybean mycorrhizal roots, RNAs were reverse transcribed and gene expression was determined by quantitative real-time RT-PCR using gene-specific primers for *Glomus* elongation factor and normalized to soybean 18S rRNA. The figure represents mean fold change compared to the non-induced plants 1 week after treatment, set at 1. Black bars (■) represent induced plants and white bars (□) non-induced plants. $n = 3$. Changes in gene expression were calculated by using the 2-DDC method. Bars represent SE.

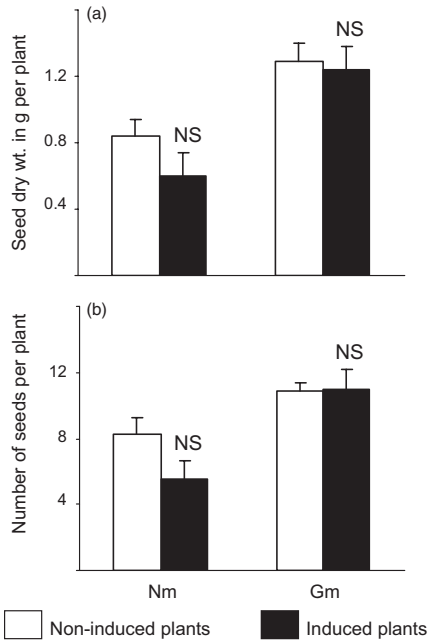

Fig. 2. Seed production. (a) Seed dry weight per plant and (b) number of seeds per plant of soybean. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean parameters + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). The sample size was 8–10 plants per treatment. $*P < 0.05$, ns $P > 0.05$ according to Mann–Whitney *U* test.

seed dry weight per plant ($P = 0.155$; Mann–Whitney *U* test) and numbers of seeds per plant ($P = 0.073$; Mann–Whitney *U* test). No hints towards changes in either the seed dry weight per plant ($P = 0.934$; Mann–Whitney *U* test) or the number of seeds per plant ($P = 0.866$; Mann–Whitney *U* test) were observed between Gm+ and Gm− plants.

We also analysed the impact of ASM treatment on the protein content and defence-related enzyme activities. The chemical induction of resistance led to a significant increase in protein content both in leaves and roots ($P < 0.05$; Mann–Whitney *U* tests) of Gm+ plants harvested 1 week after the application of ASM as compared to Gm− plants (Fig. 3a,b). No significant differences in protein content were observed between Nm+ and Nm− plants ($P > 0.05$; Mann–Whitney *U* test).

Chitinase activity (Fig. 4a,b) in leaves of Gm+ plants ($P < 0.01$; Mann–Whitney *U* test) 1 week after the induction of resistance was significantly higher than in Gm− plants. No

Table 2. Photosynthetic activity, sugar content and fresh weight of soybean plants 1 and 2 weeks after the application of acibenzolar-S-methyl (ASM)

	One week after induction				Two weeks after induction		
Treatment	Photosynthesis (μmol CO_2 m^{-2} s^{-1})	Sugar content (mg g^{-1} fresh wt. leaves)	Fresh wt. shoots (g)	Fresh wt. roots (g)	Sugar content (mg g^{-1} fresh wt.leaves)	Fresh wt. shoots (g)	Fresh wt. roots (g)
Nm−	7.08	3.69	5.19	4.77	10.13	6.61	5.08
Nm+	5.84 ns	4.59 ns	5.47 ns	4.81 ns	9.24 ns	6.82 ns	5.56 ns
Gm−	5.62	3.74	5.70	4.45	8.74	6.99	5.08
Gm+	6.99 ns	3.74 ns	5.40 ns	4.21 ns	8.60 ns	7.59 ns	5.03 ns

Fresh wt., fresh weight; Nm−, non-induced non-mycorrhizal plants; Nm+, induced non-mycorrhizal plants; Gm−, non-induced plants inoculated with *Glomus mosseae*; Gm+, induced plants inoculated with *G. mosseae*. Mean values of Nm− and Nm+ and Gm− and Gm+ in each column were compared using paired Mann–Whitney *U* tests. $n = 4$; ns $P > 0.05$.

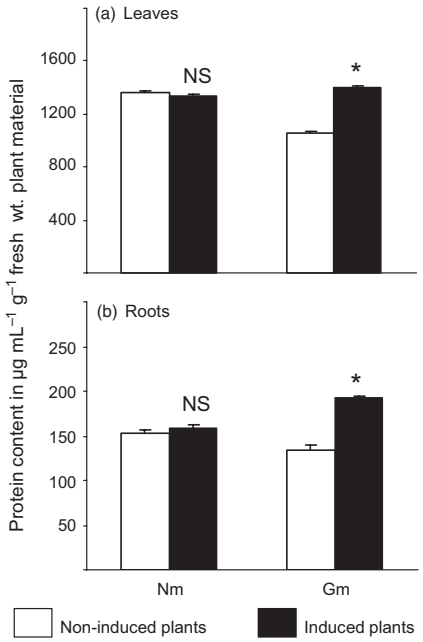

Fig. 3. Protein content. Protein content in µg mL^{-1} g^{-1} fresh weight (fresh wt.) of plant material in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean protein content + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). **P* < 0.05, ns *P* > 0.05 according to Mann–Whitney *U* test.

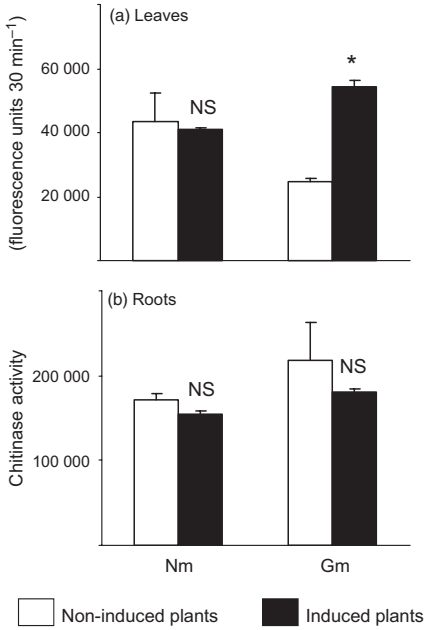

Fig. 4. Chitinase activity. Chitinase activity in fluorescence units min^{-1} in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean chitinase activity + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). **P* < 0.05, ns *P* > 0.05 according to Mann–Whitney *U* test.

significant differences were observed in root chitinase activity between Gm+ and Gm− plants or in either roots or leaves of Nm+ and Nm− plants ($P > 0.05$; Mann–Whitney *U* test).

β-1,3-glucanase activity (Fig. 5a,b) was significantly higher in leaves of induced Nm+ and Gm+ plants compared to non-induced plants, as well as in roots of Nm+ plants when compared with Nm− plants ($P < 0.05$; Mann–Whitney *U* tests). No significant changes in β-1,3-glucanase activity were observed in roots of Gm plants after the induction of resistance ($P > 0.05$; Mann–Whitney *U* test).

Acibenzolar-S-methyl is a functional analogue of SA, and its application induces resistance in plants through the activation of the SA signalling pathway. In order to monitor this pathway we analysed the expression levels of the soybean *PR-1a* gene, coding for an acidic isoform of *PR-1*, a common marker for SA-regulated responses. ASM application resulted in an increase in *PR-1a* transcript levels in shoots in Nm and Gm plants, confirming the activation of the SA signalling pathway (Fig. 6a). In agreement with the reported systemic nature of the elicitor, the expression levels of the gene were also significantly higher in the roots of Nm+ plants. Although the basal level of *PR1a* expression was higher in mycorrhizal plants than in non-mycorrhizal controls, no increase in *PR-1a* expression was observed in roots of Gm plants upon induction by ASM treatment (Fig. 6b). Elevated levels of PR1a expression in roots were still found 2 weeks after induction in Nm+ plants, but again no difference was detected between Gm+ and Gm− plants (Fig. 6c).

Discussion

Plants live in a complex environment and interact with multiple micro-organisms that can behave as pathogens or as mutualists. The level of infection by mutualists such as rhizobia and AM fungi is in part controlled by the same physiological mechanisms that suppress infections by pathogens (Pozo & Azcón-Aguilar 2007; Soto *et al.* 2009). Therefore, plant resistance to pathogens can interfere with mutualistic interactions and *vice versa*, and these effects can systemically move through the plant, from the above-ground to the below-ground compartment and *vice versa* (see references in the Introduction, and van Dam & Heil 2011). Indeed, several studies found inhibitory effects of SAR expression on beneficial plant–microbe interactions, but all clear results have been obtained from experiments with nitrogen-fixing bacteria that form nodules in the roots (Martínez-Abarca *et al.* 1998; Ramanujam, Jaleel & Kumaravelu 1998; Lian *et al.* 2000; Heil 2001b; Faessel *et al.* 2010). In the case of AM fungi, by contrast, three seemingly different responses have been observed: the induction of resistance (i) inhibits AM colonization (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Faessel *et al.* 2010); (ii) leads to a transitory decrease in AM colonization (Tosi & Zazzerini 2000); and (iii) does not have any effect on AM colonization (Sonnemann, Finkhaeuser & Wolters 2002).

Our study agrees most with that of Tosi & Zazzerini (2000), because we also found a transitory decrease in AM coloniza-

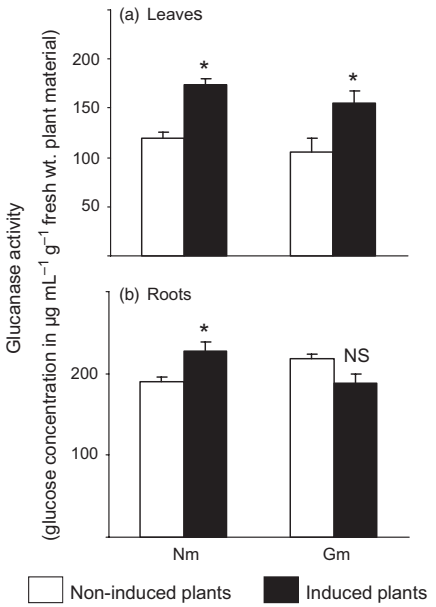

Fig. 5. β-1,3-Glucanase activity. β-1,3-glucanase activity as expressed by amount of glucose released in µg mL^{-1} g^{-1} fresh weight (fresh wt.) of plant material in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean chitinase activity + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). $*P < 0.05$, ns $P > 0.05$ according to Mann–Whitney *U* test.

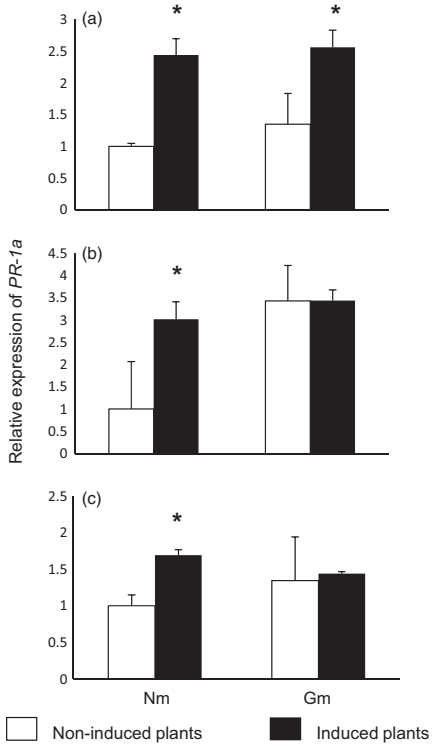

Fig. 6. Fold change in *PR-1a* gene expression upon acibenzolar-S-methyl (ASM) treatment. Total RNA was extracted from soybean (a) shoots and (b, c) roots of non-mycorrhizal (Nm) or mycorrhizal (Gm) plants harvested 1 (a, b) or 2 weeks (c) after resistance induction by treatment with ASM. RNAs were reverse transcribed and gene expression was determined by quantitative real-time RT-PCR using gene-specific primers for soybean *PR-1a* gene and *18S* rRNA. The figure represents mean fold change compared to the non-induced, non-mycorrhizal plants, set at 1. Black bars (■) represent induced plants and white bars (□) non-induced plants. $n = 3$. Changes in gene expression were calculated by using the 2-DDC method. Bars represent SE.

tion. Treating leaves with ASM sufficed to alter root colonization by *G. mosseae*. The long-term inhibitory effect reported by Salazar Costa, Ríos-Ruiz & Rodrigues Lambais (2000) followed the repeated application of SA as soil drench to bean plants, a very intensive treatment compared to standard studies in which chemical elicitors are applied just once (Baysal, Soylu & Soylu 2003; Dietrich, Ploss & Heil 2004, 2005; Sonnemann, Streicher & Wolters 2005; Rossi Cavalcanti *et al.* 2006). It is likely that the constantly low levels of AM colonization were due to this intensive induction of resistance. Furthermore, direct effects of the elicitor on the AM fungus cannot be ruled out at high concentrations of ASM (Faessel *et al.* 2010). In the study by Sonnemann, Finkhaeuser & Wolters (2002), the time lag between the application of ASM as foliar spray and the harvest of barley samples was very long (3 months), so any possible transitory effect of the induction of resistance on AM colonization could have levelled off at the time of data acquisition. In summary, the results of all available studies on negative effects of SAR on AM colonization (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Tosi & Zazzerini 2000; Sonnemann, Finkhaeuser & Wolters2002; Faessel *et al.* 2010; and the present study) are consistent with a transient inhibitory effect of SAR elicitation on mycorrhization. Because, the other way round, AM colonization affects the capacity of a plant to express above-ground resistance to pathogens and herbivores (van Dam & Heil 2011; Vannette & Hunter 2011), our results highlight the relevance of the correct timing of events in studies that aim at understanding the outcome of plant interactions with multiple organisms (Erb *et al.* 2011).

What is the biochemical or genetic mechanism that underlies this phenomenon? Negative effects of SAR induction on the interaction of plants with beneficial microbes can principally result from two different phenomena: (i) the 'metabolic shift' from primary to secondary metabolism that usually occurs during resistance elicitation might lower photosynthesis and, thus, the allocation of assimilates to the root system (as suggested by Russin *et al.* 1990), or (ii) defensive traits such as PR proteins might exert direct negative effects on the beneficial microbes (as suggested by Heil 2001b). In fact, the AM association, although a mutualistic one, is still a form of fungal invasion, and the plant activates its defence mechanisms (Riedel, Groten & Baldwin 2008). Remarkably, local defence responses such as increases of chitinase and β-1,3-glucanase activities are activated during early steps of compatible AM interactions (Ruiz-Lozano *et al.* 1999; Pozo & Azcón-Aguilar 2007), but these enzymatic activities are repressed at a later stage of mycorrhiza formation (Dumas-Gaudot *et al.* 1996; Gianinazzi-Pearson *et al.* 1996; Kapulnik *et al.* 1996). However,

induction of specific hydrolytic enzymes (chitinase, chitosanase and β-1,3-glucanase isoforms) as a response to the AM symbiosis that differ from those induced as the result of general defence mechanisms have been reported in different plants (Dumas-Gaudot *et al*. 1996; Pozo *et al*. 1996, 1998, 1999). The induction of these hydrolytic enzymes and other defence-related compounds in AM symbiosis seems to be involved in the plant control over the fungal symbiont, but it may affect other organisms (Pozo *et al*. 2002; López-Ráez *et al*. 2010). Indeed, the AM symbiosis is usually associated with an enhanced disease resistance or mycorrhiza-induced resistance (Pozo & Azcón-Aguilar 2007). Thus, besides a shift in assimilate allocation patterns, a shift in the delicate balance between host resistance and AM infection (Bennett, Bever & Bowers 2009) provides an alternative explanation of the pattern reported here.

In order to distinguish among these two alternatives we quantified photosynthetic rates and resistance expression in our plants. No changes in photosynthetic activity, sugar content or plant growth were recorded in either mycorrhizal or non-mycorrhizal plants after the induction of resistance. We conclude, therefore, that the negative effects of resistance induction on AM colonization were linked to the biochemical changes associated with the defence response rather than a shift in the allocation pathways from growth to defence. The application of ASM elicited a significant, yet moderate, increase in protein content and PR protein activity. To further analyse the activation of defence responses by ASM, we monitored expression levels of the *PR-1a* gene, commonly used as marker for SA-dependent defence responses (Uknes *et al*. 1993). Gene expression levels confirmed the activation of this signalling pathway not only in shoots, but also in roots of ASM treated non-mycorrhizal plants. SA-dependent responses are effective against biotrophic pathogens (Glazebrook 2005), but they may also impact mycorrhizal fungi because of their biotroph condition (Pozo & Azcón-Aguilar 2007). It is, therefore, likely that the activation of the SA signalling pathway upon ASM treatment results in an inhibition of AM root colonization.

Interestingly, ASM treatment led to a significant change in leaf chitinase activity in Gm plants but not in Nm plants. This pattern was caused by both, a lowered chitinase activity in Gm controls and a higher chitinase activity in Gm-induced plants, compared to the respective groups of Nm plants (Fig. 4a). This pattern is consistent with a successful suppression of chitinase activity by *G. mosseae*, which is transiently lost after ASM treatment. Our results are, therefore, consistent with the hypothesis that a shift in the balance between host resistance and control of AM infection explains why resistance induction transiently inhibits mycorrhization.

It is assumed that the induction of disease resistance incurs allocation and/or fitness costs, but only few studies have demonstrated or quantified them (Heil *et al*. 2000; Cipollini 2002; Heil 2002). In other cases, the results depended on growing conditions (Dietrich, Ploss & Heil 2005). In our study, plant growth was not affected by the induction of resistance. Similar results were reported by Iriti & Faoro (2003), and Zhu *et al*. (2003) and Faessel *et al*. (2008) recorded only transitory negative effects on plant growth. As for the seed production, the induction of resistance was associated with a lower number of seeds per plant and a lower seed dry weight per plant in non-mycorrhizal plants, although the differences were not significant. This trend was not observed in plants inoculated with *G. mosseae*.

In conclusion, our results support the hypothesis that the elicitation of foliar plant defence responses may negatively affect beneficial plant–microbe interactions below-ground, as a decrease in AM colonization of soybean roots was detected after the application of the chemical elicitor ASM to the leaves. The negative effects of the induction of resistance on AM seemed to be directly linked to the biochemical changes associated with the defence response and not to a possible shift in the allocation pathways from growth to defence. Because AM colonization plays an important role in plant nutrition and resistance, these effects likely will affect the future capacity of the plant to survive in the presence of competitors and enemies. Thus, it appears adaptive from the perspective of the plant to minimize these effects as far as possible and indeed, the decrease in AM colonization was only transitory. Because the ability of AM fungi to modulate the plant defence responses likely explains the transitory nature of the effect, we speculate that traits of both the host and its symbiont may be involved in overcoming the negative effects of above-ground resistance expression on the below-ground mutualism. Future studies aiming at an identification of the mechanisms of this co-operative effort would involve studying the interaction under different nutrient conditions and densities of AM colonization (Vannette & Hunter 2011) and monitoring physiological and transcriptomic changes in both plant roots and AM fungi during the various phases of the interaction.

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