DOCTORAL THESIS

Doctoral Programme in Fundamental and Systems Biology

Role of strigolactones in plant defense: hormonal cross-talk and implication in arbuscular mycorrhizal symbiosis.

Rocío Torres Vera

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Consejo Superior de Investigaciones Científicas

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Role of strigolactones in plant defense: hormonal cross-talk and implication in arbuscular mycorrhizal symbiosis.

Memoria presentada por Dña. Rocío Torres Vera, licenciada en Biología, para optar al grado de Doctor en Ciencias Biológicas por la Universidad de Granada con mención internacional.

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(With mention "International Doctor")

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RESUMEN / SUMMERY

RESUMEN

Las fitohormonas son los principales reguladores de las funciones biológicas de las plantas, realizando un papel crucial en el desarrollo, reproducción y defensa de las mismas. Entre las fitohormonas se encuentran principalmente el ácido abscísico (ABA), citoquininas (CKs), auxinas, brasinosteroides (BRs), giberelinas (GAs), ácido salicílico (SA), ácido jasmónico (JA) y etileno (ET) (Robert-Seilaniantz *et al*., 2011; Vanstraelen *et al.*, 2012). En el año 2008, se incluyeron las estrigolactonas (SLs) como una nueva clase de fitohormona, ya que se detectó su papel como inductores de la dominancia apical en el crecimiento de la parte aérea de la planta, inhibiendo el desarrollo de los brotes laterales (Gómez-Roldan *et al*., 2008; Umehara *et al*., 2008). Inicialmente, las SLs fueron descritas como moléculas señal en la rizosfera capaces de inducir la germinación de las semillas de plantas parásitas de raíz (Bouwmeester *et al*., 2003; Cook *et al*., 1972) y más tarde, como inductores de la ramificación de las hifas de los hongos micorrícicos arbusculares (MA), favoreciendo así la simbiosis entre el hongo y la planta hospedadora (Akiyama *et al*., 2005; Bouwmeester *et al*., 2007). Como fitohormonas, más recientemente se ha demostrado que las SLs están también implicadas en otros procesos fisiológicos de la planta como son la senescencia foliar, la arquitectura de la raíz y el desarrollo reproductivo, entre otros (Pandey *et al.*, 2016). Además, se ha detectado una posible implicación en la simbiosis *Rhizobium*-leguminosas (Foo *et al*. 2011; Peláez-Vico *et al*., 2016), ampliándose así su espectro de acción en la rizosfera. Se ha descrito que las fitohormonas regulan los procesos vitales de la planta de manera conjunta, bien presentando una relación sinérgica o antagonista, creando un balance hormonal que determina la respuesta de la planta (Vanstraelen *et al*., 2012). Por ejemplo, se ha identificado cómo la respuesta de

defensa de la planta frente a patógenos y plagas es regulada principalmente por SA, JA y ABA, o cómo la arquitectura del tallo está determinada por la acción conjunta de las auxinas, CKs y SLs (Janssen *et al*., 2014; Robert-Seilaniantz *et al*., 2011; Ton *et al*., 2009). Esta interacción entre hormonas, también llamada *cross-talk*, es una pieza clave del mecanismo regulador hormonal y es el principal tema de esta Tesis.

En la actualidad, uno de los grandes retos en agricultura es cómo hacer frente a las plagas y enfermedades que causan enormes pérdidas en los cultivos (Jung *et al*., 2012; Xie *et al*., 2010). El uso de pesticidas y plaguicidas como control de estos organismos perjudiciales es una solución inviable en el contexto de una agricultura sostenible como requiere la sociedad actual. La mejora de los mecanismos de defensa de la propia planta frente a estos organismos, mediante el uso de microorganismos beneficiosos del suelo, como p.ej. los hongos MA, es una alternativa muy prometedora. Para poder optimizar estos mecanismos se requiere un conocimiento detallado de la regulación de las respuestas de defensa de la planta y de los procesos fundamentales en la biología de la simbiosis y del patógeno o parásito, y en este contexto se enmarca la presente Tesis Doctoral. Por lo tanto, el objetivo general de esta Tesis Doctoral es **el estudio de la regulación de las SLs y su interacción con otras fitohormonas regulando la respuesta de defensa de la planta, así como el establecimiento y desarrollo de la simbiosis MA, haciendo especial énfasis en el papel del ácido jasmónico (JA) y la hormona peptídica sistemina.**

Las plantas parásitas de raíz, comúnmente llamadas jopo para los géneros *Phelipanche* y *Orobanche*, causan graves daños en los cultivos agrícolas. Las estrategias que se usan

actualmente para erradicarlas no son efectivas, ya que su ciclo de vida ocurre mayormente bajo tierra y cuando se detecta la infección, ya es demasiado tarde para eliminarla y ha ocasionado un gran daño a la planta hospedadora. Se están estudiando nuevas estrategias de control frente a estos parásitos focalizadas en eliminar o reducir la infección durante los estadíos iniciales de la interacción (Fernández-Aparicio *et al*., 2016; López-Ráez *et al*., 2009). Los niveles de infección, los problemas que genera y la respuesta de defensa de la planta hospedadora están, en parte, regulados por las fitohormonas. Por ello, en el **capítulo 1** de esta Tesis Doctoral se analizó la regulación transcripcional de distintos marcadores asociados a las diferentes rutas hormonales en raíces de plantas de tomate infectadas por *Phelipanche ramosa* durante los estadíos iniciales de la infección. Los marcadores moleculares indicaron una inducción en la expresión de genes relacionados con JA, SA y ABA, principales hormonas reguladoras de la defensa en la planta (Robert-Seilaniantz *et al*., 2011). **Ésto sugiere una posible inducción de los mecanismos de defensa en el sistema tomate-*P. ramosa* mediante la activación de estas rutas hormonales desde muy temprano en la interacción planta-parásito (Torres-Vera *et al.*, 2016).** Por otro lado, también se analizaron marcadores moleculares para las SLs, observándose una inducción en la expresión de genes de biosíntesis en los estadios iniciales de la infección. **En este capítulo se plantea por primera vez, un posible rol de las SLs en la regulación de la defensa interactuando con las principales fitohormonas en la infección por jopo.** Gracias al papel de las SLs en la rizosfera y su posible implicación en la defensa frente a esta infección, hacen de las SLs una pieza esencial en las futuras estrategias para erradicar/disminuir las infecciones ocasionadas por jopo (Torres-Vera *et al.*, 2016).

En el **capítulo 2** se investiga más detenidamente el papel que podrían desempeñar las SLs en la regulación de la respuesta de defensa en la planta frente a patógenos. Para ello, se analizó el efecto de distintos tipos de patógenos fúngicos, tanto de parte aérea como de raíz, en plantas de tomate deficientes en SLs junto con respecto a sus silvestres. Se observó que las plantas *Slccd7* y *Slccd8* fueron más susceptibles al hongo necrótrofo *Botrytis cinerea* que sus correspondientes plantas silvestres. Asimismo, las plantas *Slccd8* fueron también más sensibles a la infección por *Alternaria alternata*, indicando una posible implicación de las SLs en la infección por patógenos necrótrofos de parte aérea. También se detectó un mayor índice de infección en raíz por el patógeno hemibiótrofo *Fusarium oxysporum f. sp. lycopersici*, lo que podría implicar un amplio espectro de acción de las SLs en defensa. Por otro lado, plantas de Arabidopsis deficientes en SLs *max4-1*, bloqueadas en la enzima CCD8, fueron también más susceptibles a la infección por *B. cinerea*, lo que confirmaría el posible papel de las SLs en defensa e indicaría que esta función es conservada en distintas especies. El análisis hormonal en hojas de plantas *Slccd8* mostraron que las principales hormonas reguladoras de la respuesta de defensa (SA, JA y ABA) se encontraban en concentraciones muy inferiores respecto a las plantas silvestres. Un posterior análisis transcripcional de marcadores moleculares indicó un posible predominio de la vía del JA como la principal vía de regulación de la defensa donde las SLs podrían estar implicadas. **Por tanto, el papel de las SLs en defensa parece ser indirecto actuando de forma sinérgica con las principales vías que regulan los mecanismos de defensa, fundamentalmente con la ruta del JA (Torres-Vera *et al*., 2014).**

Tanto las SLs como el JA presentan un papel en la regulación de la simbiosis MA, aunque aparentemente a diferentes niveles de la interacción ya que las SLs actúan como inductores de la ramificación de las hifas de los hongos MA durante la fase pre-simbiótica, y el JA regulando el establecimiento micorrícico en la raíz en la fase simbiótica (Hause *et al*., 2007; Pozo *et al*., 2015), aunque el papel de este último, no está muy claro aún. Estudios realizados con mutantes afectados en la ruta de señalización del JA, mostraron índices de micorrización alterados respecto a sus correspondientes silvestres (Herrera-Medina *et al*., 2008; Isayenkov *et al*., 2005; Song *et al*., 2013; Tejeda-Sartorius *et al*., 2008). Este comportamiento también está descrito en plantas deficientes en SLs, las cuales presentan unos menores niveles de colonización micorrícica. Estas plantas presentan además una mayor ramificación de la parte aérea y una menor capacidad de estimulación de la germinación de semillas de jopo (Gómez-Roldán *et al*., 2008; Gutjahr *et al*., 2012; Kohlen *et al*., 2012; Koltai *et al*., 2010; Kretzschmar *et al*., 2012). En el **capítulo 3,** se estudió una posible interacción de las SLs y el JA en la regulación de la ramificación del tallo. Para ello, se realizó un análisis de la ramificación aérea total en diferentes plantas de tomate alteradas en JA (*spr1*, *spr2* y *jai1*), detectándose únicamente una mayor ramificación en plantas *spr2* respecto a su silvestre. Curiosamente, estas plantas presentan unos niveles inferiores de micorrización (Song *et al*., 2013; Tejeda-Sartorius *et al*., 2008). Las plantas *spr2* presentaron un patrón de crecimiento similar al de las plantas *Slccd8*, especialmente a la línea L16, plantas con una deficiencia en SLs del 50% respecto al silvestre (Kohlen *et al*., 2012). Estos resultados sugieren una posible deficiencia parcial de SLs en las plantas *spr2.* Tras analizar los niveles de SLs, auxinas y CKs, principales reguladores de la ramificación de la parte aérea (Teichmann y Muhr, 2015), en estas plantas, no fue

posible relacionar directa o indirectamente el fenotipo de *spr2* con una deficiencia en SLs, **no observándose una relación SLs-JA en la regulación de la ramificación aérea.**

Las plantas alteradas en prosistemina (PS) se encuentran alteradas también en sus niveles de JA en condiciones de estrés (Fernández, 2013). La PS es el precursor de una hormona peptídica denominada sistemina presente en Solanáceas (McGurl *et al*., 1992; Ryan y Pearce, 1998). Cuando se produce una herida por herbívora o un ataque por insecto, la PS es inducida y procesada proteolíticamente en el citosol hasta sistemina, induciendo la biosíntesis de JA (Ryan y Pearce, 2003). Fernández y colaboradores observaron que plantas de tomate deficientes en PS (*ps-*) presentaban niveles de micorrización muy bajos, al contrario de las plantas sobreexpresantes (*ps+*), con índices superiores al del silvestre (Fernández, 2013). Además, se detectaron niveles de SLs inferiores y superiores al silvestre en las plantas *ps-* y *ps+*, respectivamente (Fernández, 2013). Esto sugiere que la sistemina podría estar involucrada en la regulación de la simbiosis micorrícica mediante la regulación de los niveles de SLs. En el **capítulo 4,** mediante el tratamiento con sistemina exógena en plantas *ps-* observamos **un efecto directo de la sistemina en la producción de SLs tanto a nivel hormonal como transcripcional en condiciones de alta producción de SLs** (**deficiencia de Pi**, López-Ráez *et al*., 2008; Yoneyama *et al*., 2007). Se ha descrito que el JA está implicado en la regulación de la formación y mantenimiento de los arbúsculos, aunque su papel concreto aún no está claro (Pozo *et al*., 2015). La estrecha relación entre sistemina y JA nos sugirió la posibilidad de que ésta también pudiera estar involucrada en la fase simbiótica de la micorrización. Tras realizar un análisis a nivel microscópico sobre el desarrollo arbuscular en plantas *ps-* y *ps+* micorrizadas con

el hongo MA *Rhizophagus irregularis*, se detectó que **la sistemina está implicada en el desarrollo arbuscular, pero que dicha implicación parece ser independiente de JA y SLs,** ya que ninguno de los mutantes de JA y SLs usados en este estudio tenían comprometido el desarrollo arbuscular de la simbiosis.

La simbiosis MA entre otros beneficios, promueve la resistencia sistémica inducida (ISR), probablemente vía JA, protegiendo a la planta de estreses bióticos (Pieterse *et al*., 1998; Pozo y Azcón Aguilar, 2007; Segarra *et al*., 2009; Van Loon *et al*., 1998). La aplicación de hongos MA como biofertilizantes y agentes de bioprotección en los cultivos agrícolas se presenta como un futuro prometedor y sostenible. Por ello, es necesario conocer los mecanismos que regulan el establecimiento y mantenimiento de la colonización MA, así como la respuesta de defensa de la planta con el fin de optimizar los beneficios de esta simbiosis en cultivos agrícolas. En este trabajo de Tesis Doctoral se ha avanzado en este conocimiento y se han estudiado las interacciones entre diferentes fitohormonas que regulan la respuesta de defensa de las plantas así como la simbiosis MA.

SUMMARY

Phytohormones are the main regulators of the biological functions of plants, playing crucial roles in development, reproduction and stress responses. They include abscisic acid (ABA), cytokinins (CKs), auxins, brassinosteroids (BRs), gibberellins (GAs), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Robert-Seilaniantz *et al*., 2011; Vanstraelen *et al.*, 2012). In 2008, strigolactones (SLs) were included as a new class of phytohormones, due to its role as inductors of apical dominance in shoot architecture, inhibiting the growth of the axillary buds (Gómez-Roldán *et al*., 2008; Umehara *et al*., 2008). Initially, they were described as signalling molecules in the rhizosphere inducing seed germination of root parasitic plants of the *Orobanchaceae* (Bouwmeester *et al.*, 2003; Cook *et al.*, 1972) and later as inducers of hyphal branching of arbuscular mycorrhizal (AM) fungi, thus promoting the symbiosis between the fungus and the host plant (Akiyama *et al*., 2005; Bouwmeester *et al.*, 2007). More recently, it has been proposed a role of SLs in the symbiosis *Rhizobium*-legume (Foo et al., 2011; Pelaez-Vico *et al*., 2016), thus extending its range of action in the rhizosphere. As phytohormones, it has been shown that SLs are also implicated in other physiological processes in the plant such as leaf senescence, root architecture and reproductive development, among others (Pandey *et al*., 2016). It is well known that phytohormones regulate vital processes by interacting with each other (Vanstraelen *et al*., 2012). They can present a synergistic or antagonist relationship, creating a hormonal balance that determines the plant response. For example, it has been shown that the plant defense response against pathogens and pests is regulated mainly by the interaction between SA, JA and ABA, or that shoot branching is mainly determined by the action of auxins, CKs and SLs (Janssen *et al*., 2014; Robert-Seilaniantz *et al*.,

2011; Ton *et al.*, 2009). This interaction between phytohormones, also known as *cross-talk*, is key part in the hormonal regulation, and it is the main subject of this PhD Thesis.

One of the greatest challenges in modern agriculture is how to eradicate the pests and diseases that cause huge crop losses (Jung *et al.*, 2012; Xie *et al*., 2010). The use of pesticides and insecticides as control against harmful organisms is a non-viable solution in the context of sustainable agriculture as required by today's society. In this sense, the improvement of plant defence responses against these organisms using beneficial soil microorganisms, e.g. AM fungi, it is a very promising alternative. In order to implement this strategy, a detailed knowledge of the regulation of the symbiosis, as well as the plant defence responses and the fundamental processes of pathogen/parasite is required, and in this context this Doctoral Thesis is framed. Therefore, the **main objective** of this Doctoral Thesis is **the study of the potential role of SLs in defence and its interaction with other phytohormones regulating plant defence responses during the establishment and development of the AM symbiosis, with particular emphasis on the role of jasmonic acid (JA) and the peptide hormone systemin**.

Root parasitic plants of the genera *Phelipanche* and *Orobanche*, commonly called broomrapes, cause serious damage to agricultural crops. Current control methods used to eradicate these parasitic weeds are not effective. This is due to most of their life cycle occurs underground and therefore, when the infection is detected is too late since an important damage has already been generated to the crop. Thus, new control

strategies are focused on the elimination or reduction of the parasite during the initial stages of the interaction (Fernández-Aparicio *et al*., 2016; Lopez-Ráez *et al.*, 2009). The infection levels and the damages caused by the parasite are partly regulated by the phytohormones of the host plant. For this reason in **Chapter 1**, we analysed the transcriptomic regulation of molecular markers associated to different hormonal pathways in roots of tomato plants infected by *Phelipanche ramosa* during the initial stages of infection. Gene expression analyses showed an induction of markers related to JA, SA and ABA, main regulatory hormones associated to plant defence responses (Robert-Seilaniantz *et al*., 2011). **This suggests a possible induction of the defence mechanisms during the early stages of the tomato-*P. ramosa* interaction, which are associated to these hormonal pathways (Torres-Vera *et al*., 2016).** Molecular markers for the SL signalling pathway were also analysed, showing an induction of these genes during the early stages of infection. **This chapter presents for the first time, a possible role of SLs in the regulation of defence responses, probably through an interaction with the major defence phytohormones.** Due to the role of SLs in the rhizosphere as signalling cues and to their possible involvement in defence responses against broomrapes, SLs could be an key element in future strategies to eradicate this agricultural pest (Torres-Vera *et al.*, 2016).

In **Chapter 2**, the potential role of SLs in plant defence has been explored in more detail. To this end, the resistance/susceptibility of different SL-deficient tomato plants against different fungal – shoot- and root-borne – pathogens was analysed. It was observed that the SL-deficient plants *Slccd8* and *Slccd7* were more susceptible to the necrotrophic pathogen *Botrytis cinerea* than their corresponding wild-types. *Slccd8*

plants were also more susceptible to the infection by the necrotrophic fungus *Alternaria alternata*, suggesting a possible involvement of SLs in the defence response against this type of air-borne pathogens. A higher infection rate was also observed in *Slccd8* roots by the hemibiotrophic pathogen *Fusarium oxysporum f. sp. lycopersici*, suggesting a broad action spectrum of SLs in defence responses. On the other hand, the SL-deficient Arabidopsis plants *max4-1*, blocked at the CCD8 step, were also more susceptible to the infection by *B. cinerea*, indicating that the SL' role in defence seems to be conserved across plant species. Hormonal analyses in leaves of *Slccd8* plants showed that all the main defence phytohormones (SA, JA and ABA) were reduced in the transgenic line compared to wild-type plants. A further transcriptomic analysis showed a possible predominance of the JA signalling pathway as the main defence pathway where SLs may be involved. **Therefore, the role of SLs in defence seems to be an indirect effect, acting synergistically with the major pathways that regulate the defense mechanisms, primarily with the JA pathway (Torres-Vera *et al*., 2014).**

Both SLs and JA play a role in the regulation of AM symbiosis, although apparently to different levels of the plant-AM fungus interaction. SLs act as inducers of hyphal branching of AM fungi during the pre-symbiotic phase, while JA acts regulating hyphal growth and mycorrhizal establishment during the symbiotic phase inside the root (Hause *et al*., 2007; Pozo *et al*., 2015), although its specific role is still unclear. Studies with mutants affected in JA levels and signalling showed altered mycorrhizal rates compared to their corresponding wild-types (Herrera-Medina *et al*., 2008; Isayenkov *et al*., 2005; Song *et al.*, 2013; Tejeda-Sartorius *et al*., 2008). This behaviour is also described for SL-deficient plants, which exhibit lower levels of mycorrhizal

colonization. SL-deficient plants also present a higher shoot branching and a lower ability to stimulate seed germination of broomrape (Gomez-Roldan *et al*., 2008; Gutjahr *et al.*, 2012; Kohlen *et al*., 2012; Koltai *et al*., 2010; Kretzschmar *et al*., 2012). In **Chapter 3**, the possible interaction between SLs and JA in shoot branching regulation was investigated. The total number of branches in different tomato plants altered in JA levels or signalling (*spr1*, *spr2* and *jai1*) was analysed, only detecting a greater branching in *spr2* plants respect to the wild-type. *Spr2* plants showed a similar growth pattern to *Slccd8* plants, especially with the line L16 which has a deficiency in SLs of about 50% (Kohlen *et al*., 2012). This result suggested a possible partial SL deficiency in in *spr2* plants. However, when analysing the SL levels in these plants, no differences between the mutant and the wild-type were observed. In addition, no differences in the levels of auxin and CKs, key regulators of shoot branching (Teichmann and Muhr, 2015), were detected in *spr2* plants. **Thus, we did not observe any JA-SLs relationship in the regulation of shoot branching**.

Plants affected in prosystemin (PS) levels also present altered JA levels under stress conditions (Fernández, 2013). PS is the precursor of systemin, a peptide hormone present in the *Solanaceae*. Systemin promotes JA biosynthesis and signalling, inducing plant defences against biotic stress as pathogen infection and herbivory in the leaves (Ryan and Pearce, 2003). Fernandez and co-workers found out that PS-deficient (*ps-*) tomato plants showed lower levels of mycorrhizal colonization than the corresponding wild-type. Conversely, PS-overexpressing (*ps+*) plants showed higher levels of colonization (Fernandez, 2013). These authors also showed that *ps-* and *ps+* plants presented lower and higher SL levels, respectively (Fernandez, 2013). This fact suggests

that systemin might be involved in the regulation of mycorrhizal symbiosis by regulating SL levels. In **Chapter 4**, after exogenous application of systemin into *ps-* plants grown under low Pi conditions, an increase in SL levels was observed. In addition, the expression of molecular markers for SL biosynthesis was promoted by the treatment with systemin. These results suggest **a direct effect of systemin in SL production under conditions of high SL production (Pi deficiency,** Lopez-Ráez *et al*., 2008; Yoneyama *et al.*, 2007), **and therefore a role during the pre-symbiotic phase of AM symbiosis**.

JA is involved in the regulation of the formation and maintenance of arbuscules, although its role is still unclear (Pozo *et al*., 2015). The close relationship between systemin and JA suggested the possibility that systemin could be also involved in the symbiotic phase of mycorrhizal colonization. After performing a microscopic analysis of the arbuscular development in *ps-* and *ps+* plants colonized by the AM fungus *Rhizophagus irregularis*, **it was observed that indeed systemin is involved in arbuscular development.** However, since none of the JA- and SL- deficient mutants used in this study had committed the arbuscule development, **the systemin role here seems to be independent of JA and SLs**.

Among other benefits, AM symbiosis promotes induced systemic resistance (ISR), probably through the JA pathway, thus protecting the host plant of both biotic and abiotic stresses (Pieterse *et al.*, 1998; Pozo and Azcón Aguilar, 2007; Segarra *et al.*, 2009; Van Loon *et al.*, 1998). The application of AM fungi as biofertilizers and bioprotection agents into agricultural crops is a promising and sustainable strategy for

future agriculture. However, before its application it is necessary to a deeper knowledge about the mechanisms that regulate the establishment and maintenance of AM symbiosis, and the plant defence responses in order to optimize the benefits of this beneficial symbiosis in agriculture. In this Doctoral Thesis, we have advanced in this knowledge and studied the interactions between the different phytohormones that are involved in these processes.

INTRODUCCIÓN GENERAL

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Las fitohormonas son moléculas producidas por las plantas con la finalidad de regular sus procesos fisiológicos, donde se incluyen principalmente los distintos estadíos de desarrollo de la planta y la respuesta de ésta frente a estreses tanto abióticos como bióticos. Son producidas en pequeñas cantidades actuando en el mismo tejido donde se generan o bien actuando a larga distancia, transportándose a través de los vasos del xilema o floema. Las fitohormonas regulan los procesos vegetativos interaccionando unas con otras de una manera antagonista o sinérgica, creando un balance hormonal que provoca principalmente, cambios en la expresión de genes clave en los distintos procesos. Las principales fitohormonas son las auxinas, citoquininas (CKs), giberelinas (GAs), brasinosteroides (BRs), etileno (ET), ácido abscísico (ABA), ácido salicílico (SA), ácido jasmónico (JA) (Robert-Seilaniantz *et al*., 2011; Vanstraelen *et al.*, 2012) y las recientemente incluídas estrigolactonas (SLs) (Gómez-Roldan *et al*., 2008; Umehara *et al*., 2008). Actualmente, el papel que desempeñan las SLs en los procesos fisiológicos de las plantas es el menos conocido, por lo que continuamente surgen estudios que revelan nuevos procesos donde las SLs intervienen. Se ha descrito que las SLs presentan un importante papel endógeno regulando fases del desarrollo tanto en la parte aérea como en la raíz de las plantas (Vanstraelen *et al.*, 2012). Además, en condiciones de estrés nutricional son exudadas a la rizosfera desempeñando un papel como moléculas señal tanto para los hongos micorrícicos arbusculares (MA) como para las plantas parásitas de raíz (Akiyama *et al*., 2005; Bouwmeester *et al*., 2003).

En esta Tesis profundizaremos en el estudio de la regulación de algunos de los principales procesos donde se encuentran implicadas las SLs, así como su interacción con otras fitohormonas en dicha regulación.

1. ESTRIGOLACTONAS (SLs)

1.1. ESTRUCTURA QUÍMICA DE LAS SLs

Las SLs son unos compuestos derivados de los carotenoides (López-Ráez et al., 2008a; Matusova et al., 2005). Hasta la fecha, sólo hay 19 SLs descritas que muestran una estructura química similar, aunque se ha propuesto que pueden existir más de mil (Saint Germain, 2013; Xie et al., 2010). Presentan un núcleo estructural compuesto por una lactona tricíclica (**anillos ABC**) que está unida mediante un enlace **éter enólico** a un grupo butirolactona (**anillo D**) (Yoneyama *et al*., 2009; Zwanenburg *et al*., 2009) (**Fig. 1**). En general, las SLs pueden dividirse en dos grupos de diastereoisómeros en base a la orientación de la unión BC: **tipo estrigol** y **tipo orobancol** (**Fig. 1**) (López-Ráez, 2016). La **parte AB** puede ser modificada mediante metilación, hidroxilación, epoxidación o ketolación, dando lugar a la diversidad de SLs (**Fig. 1**). La actividad biológica de estas moléculas parece residir en el enlace éter enólico, el cual puede ser hidrolizado rápidamente en ambientes acuosos o alcalinos, siendo responsable de la corta vida media de esta molécula, coherente con su papel en señalización (Akiyama *et al*., 2010; Yoneyama *et al*., 2009; Zwanenburg *et al*., 2009).

Fig 1: Estructura general de las SLs naturales.

1.2. BIOSÍNTESIS DE LAS SLs

Los exudados de raíz de plantas tratadas con fluoridona, un inhibidor de la biosíntesis de carotenoides, y de plantas deficientes en la biosíntesis de carotenoides**,** presentaban bajos niveles de SLs, mostrando así que las SLs derivan de los mismos (López-Ráez *et al*., 2008a; Matusova *et al*., 2005).

Una de las principales funciones endógenas de las SLs es la inhibición de la ramificación de la parte aérea de la planta (Gómez-Roldán *et al*., 2008; Umehara *et al*., 2008). De hecho, mutantes de Arabidopsis (*Arabidosis thaliana*), arroz (*Oryza sativa)* y guisante (*Pisum sativum)* alterados en los genes ***CCD7*** y ***CCD8***, codificantes para las **dioxigenasas de rotura de carotenoides 7 y 8,** respectivamente, mostraron un fenotipo altamente ramificado y presentaron niveles de SLs muy reducidos. Este fenotipo podía ser recuperado mediante la aplicación exógena de GR24 (un análogo sintético de SLs), incluyendo así a CCD7 (**MAX3** en Arabidopsis, **D17/HTD1** en arroz***,*** **RMS5** en guisante y **DAD3** en petunia/*Petunia hybrida)* y CCD8 (**MAX4** Arabidopsis, **D10** arroz, **RMS1** guisante y **DAD1** petunia) en la ruta biosíntética de las SLs (Gómez-

Roldán *et al*., 2008; Umehara *et al*., 2008) (**Fig. 2**). Otros estudios similares sugirieron que en Arabidopsis**, un citocromo P450 (CYP711A1, MAX1)**, también es necesario para la biosíntesis de SLs, actuando corriente abajo de CCD7 y CCD8 (Alder *et al*., 2012; Booker *et al*., 2005). Posteriormente, una enzima de función desconocida y con capacidad para fijar hierro **DWARF27** (**D27**) era identificada en arroz como una β-carotenoide isomerasa importante para la biosíntesis de SLs. Esta enzima convierte los metabolitos all-*trans*-β-carotenos en 9-*cis*-β-carotenos, los cuales sirven de sustrato para la enzima CCD7 (Lin *et al*., 2009) (**Fig. 2**). Por el momento, no se han identificado ortólogos de D27 en otras especies de plantas distintas a arroz, pero se han encontrado secuencias nucleotídicas homólogas en la mayoría de las especies de plantas (Lin *et al*., 2009). Recientemente, se ha demostrado que la acción secuencial de CCD7 y CCD8 da lugar a carlactona, un precursor común de la biosíntesis de SLs y compuestos SL-like en Arabidopsis (Brewer *et al*., 2016) (**Fig. 2**). En Arabidopsis, la carlactona es modificada por MAX1, produciendo ácido carlactonoico, la molécula mayoritaria en Arabidopsis (**Fig. 2**). En el resto de plantas, la biosíntesis de SLs es algo diferente. La carlactona es oxidada por la acción de una carlactona oxidasa (MAX1-like, OS900), convirtiéndose en 4-desoxi-orobancol y posteriormente en orobancol por una orobancol sintasa (MAX1-like, OS1400) (Brewer *et al*., 2016) (**Fig. 2**). Sin embargo, estas cuatro enzimas parecen insuficiente para producir las diferentes SLs, lo que sugiere que debe haber más enzimas aún desconocidas asociadas a esta ruta biosintética (Beveridge *et al*., 2010). MAX1 de Arabidopsis contiene un anclaje a la membrana del retículo endoplasmático, lo que sugiere una localización citosólica, en contraste con la localización plastidial de D27, CCD7 y CCD8 (Booker *et al*., 2005).

1.3. SEÑALIZACIÓN/PERCEPCIÓN DE LAS SLs

Las SLs se unen a una proteína receptora llamada **D14** (**DAD2** en petunia), que es una α-β-hidrolasa. Tras esta unión se produce un cambio estructural en el receptor y la hidrólisis de las SLs (**Fig. 3**). Ésto promueve la interacción de la proteína D14 con una proteína **F-box** (**MAX2** en petunia y Arabidopsis, **D3** en arroz y **RMS4** en guisante), provocando la ubiquitinación y degradación a través del proteosoma de ciertas proteínas represoras. La proteína **D53,** descubierta en arroz (Arite *et al*., 2009), es un represor que en presencia de SLs es ubiquitinizado y destruído por el complejo D14-D3, desencadenando así la cascada de señalización de SLs (Zhou *et al*., 2013) (**Fig. 3**). La degradación de D53 activa la transcripción de un **factor de transcripción TCP denominado** BRANCHED 1 (**BRC1**) en Arabidopsis y guisante, FINE CULM1 (**FC1**) en arroz y TEOSINTE BRANCHED1 (**TB1**) en monocotiledóneas, reprimiendo el desarrollo de la yema e inhibiendo la ramificación del tallo (Braun *et al*, 2012; Dun *et al*, 2012) (**Fig. 3**). Este mecanismo de señalización mediante la degradación de represores por acción del proteosoma es similar al descrito para otras hormonas como auxinas, JA y GAs (Shabek y Zheng, 2014).

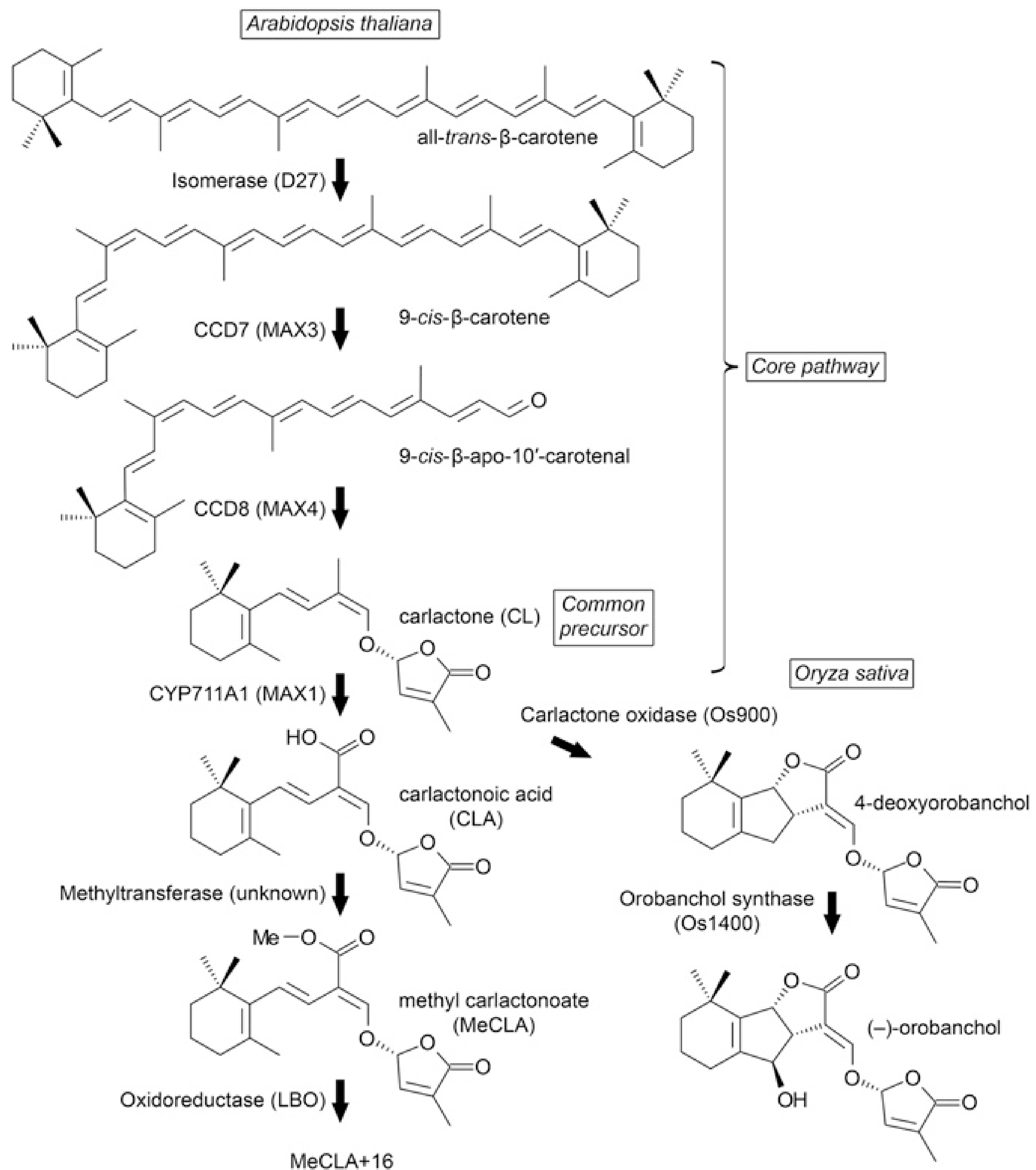

Fig. 2: Ruta biosintética de las SLs. Una isomerasa y dos enzimas CCDs convierten el β-caroteno en carlactona (CL), el común precursor de las distintas SLs y compuestos relacionados (SL-like). En arroz, CL es oxidado por varios citocromos P450 a SLs de tipo orobancol o estrigol. En Arabidopsis, CL es oxidado por el citocromo P450 MAX1 a ácido carlactonoico (CLA), que es convertido a metilcarlactonato (MeCLA), que finalmente es reducida a un producto desconocido SL-like (MeCLA+ 16 Da) (Brewer *et al*., 2016).

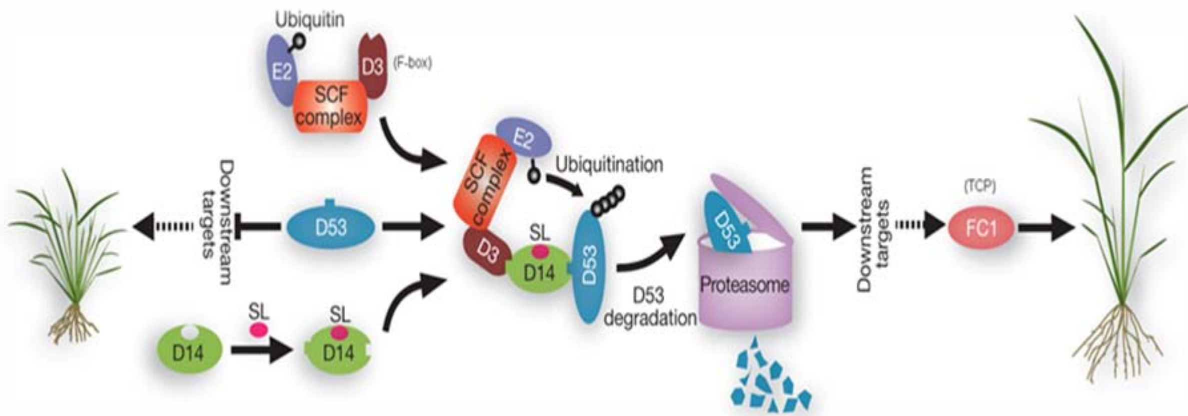

Fig. 3: La SL bioactiva unida a D14 induce un cambio conformacional en D14 permitiendo que SCDD3 y D53 también se unan. Esta unión provoca posteriormente la ubitiquitinización de D53, un regulador negativo, que es degradado por el protosoma y promueve la expresión de genes de respuesta a las SLs, como por ejemplo FC1 en arroz, que inhibe la ramificación de la parte aérea. Adaptado de Zhou *et al*., 2013.

1.4. FUNCIONES DE LAS SLs EN LA PARTE AÉREA DE LA PLANTA

1.4.1. Inducción de la dominancia apical en el crecimiento aéreo.

Según las necesidades de la planta, ésta crece predominantemente de forma apical o bien ramificándose lateralmente. Se ha observado que las **SLs presentan un papel inhibidor en el crecimiento de los brotes laterales, favoreciendo así la dominancia apical** (Gómez-Roldan *et al*., 2008; Umehara *et al*., 2008) (**Fig. 4**). De hecho, diversas plantas deficientes en SLs mostraron un fenotipo altamente ramificado que era revertido tras la aplicación de GR24 (Al-babili *et al*., 2015).

1.4.2. Inhibición de la formación de raíces adventicias.

Las raíces adventicias son aquellas que crecen a partir de otro órgano que no es la raíz primaria como los tallos, hojas o raíces viejas. Cumplen básicamente una función de

sostén y, parcialmente, de absorción de agua y nutrientes. Las **SLs actúan reprimiendo su formación** (Kholen *et al*., 2012; Rasmussen *et al.*, 2012) (**Fig. 4**). En mutantes de Arabidopsis, arroz, guisante y tomate (*Solanum lycopersicum*) deficientes en SLs se observó un alto número de raíces adventicias que fue reducido tras la aplicación exógena de GR24 (Kholen *et al*., 2012; Rasmussen *et al.*, 2012).

1.4.3. Inducción de la senescencia foliar.

La senescencia foliar es promovida en condiciones de deficiencia nutricional en el suelo, provocando que los nutrientes de las hojas senescentes sean transportados a tejidos más jóvenes y a semillas. Las **SLs, que también son inducidas por el estrés nutricional, promueven esta senescencia** (**Fig. 4**). Se observó que mutantes de arroz y Arabidopsis deficientes en SLs, presentaban un menor número de hojas senescentes comparado con las correspondientes plantas silvestres, fenotipo que fue recuperado tras la aplicación exógena de GR24 (Ueda y Kusaba, 2015; Yamada *et al*., 2014).

1.4.4. Inducción del desarrollo de flores y frutos.

En distintos estudios se ha observado que los mutantes deficientes en la biosíntesis de SLs en tomate (*Slccd8*) y petunia (*dad1*) presentaban flores más pequeñas respecto a su correspondiente silvestre (Kohlen *et al*., 2012; Snowden *et al*., 2005). De igual modo, en *Slccd8* también se mostró que los frutos eran de menor tamaño (Kohlen *et al*., 2012). Estos resultados sugieren que las **SLs podrían tener también un papel regulatorio en el desarrollo floral y del fruto** (**Fig. 4**).

1.5. FUNCIONES DE LAS SLs EN LA RAÍZ

1.5.1. Regulación del desarrollo de la raíz primaria y de raíces laterales.

Al igual que en la regulación del desarrollo de los brotes laterales en la parte aérea, las **SLs inducen el crecimiento de la raíz principal inhibiendo a su vez el crecimiento de raíces laterales** (Ruyter-Spira *et al*., 2011; Koren *et al*., 2013) (**Fig. 4**). Se observó que la raíz principal de mutantes deficientes en SLs de Arabidopsis era más corta, además de presentar una mayor cantidad de raíces laterales que en las plantas silvestres, aunque tras la aplicación exógena de GR24, el fenotipo silvestre fue recuperado (Kapulnik *et al*., 2011a; Koltai *et al*., 2009; Ruyter-Spira *et al*., 2011).

1.5.2. Inducción del crecimiento de pelos radicales.

Se ha demostrado que las SLs también promueven la elongación de pelos radicales, probablemente para facilitar la captación de fósforo (P) en condiciones desfavorables (Kapulnik *et al*., 2011a) (**Fig. 4**).

1.6. FUNCIONES DE LAS SLs EN LA RIZOSFERA

1.6.1. Inducción de la ramificación de las hifas de hongos micorrícicos arbusculares (MA).

En condiciones de deficiencia de P o nitrógeno (N) en el suelo, las plantas son capaces de inducir la producción de exudados vegetales ricos en SLs, con el fin de atraer a los hongos MA. Estos hongos, mediante el establecimiento de la simbiosis micorrícica arbuscular (MA) en la raíz, ayudan a suplir estas deficiencias nutricionales (Balzergue *et*

al., 2011; López-Ráez *et al*., 2008; Yoneyama *et al*., 2012). Las **SLs** tras ser exudadas a la rizosfera, **favorecen la colonización micorrícica mediante la inducción del desarrollo y ramificación de las hifas de los hongos MA** (Akiyama *et al*., 2005) (**Fig. 4**). De esta manera, se aumenta la posibilidad de contacto entre el hongos MA y la raíz hospedadora (Akiyama *et al*., 2005; Besserer *et al*., 2006; Bouwmeester *et al*., 2007). Se ha sugerido que las SLs podrían también ejercer un efecto de quimiotaxis, dirigiendo el crecimiento de las hifas hacia la raíz (Sbrana y Giovannetti, 2005), aunque este hecho no ha sido demostrado. Para más información sobre la simbiosis MA, ver el apartado 2.

1.6.2. Inducción de la nodulación en la simbiosis *Rhizobium*-leguminosas.

Existen algunos indicios que involucran a las SLs en la interacción *Rhizobium*-leguminosas. Soto y colaboradores analizaron el establecimiento de la simbiosis *Rhizobium*-alfalfa (*Medicago sativa*) en presencia de diferentes concentraciones de GR24. Mostraron como las **plantas tratadas con GR24 presentaban más nódulos** (estructuras donde se da la fijación de Nitrógeno) que las plantas sin tratar (Soto *et al*., 2010). Además, plantas de guisante deficientes en SLs mostraron bajos niveles de nodulación, que eran parcialmente recuperados tras la aplicación de GR24 (Foo *et al.*, 2011) (**Fig. 4**). Más recientemente, se ha observado que las SLs afectan a la motilidad bacteriana, favoreciendo la movilidad *swarming* (Peláez-Vico *et al*., 2016). Estos resultados indican el papel importante de las SLs como moléculas señal en la rizosfera, facilitando la interacción entre la planta y diferentes microorganismos beneficiosos. Por otro lado, algunos estudios mostraron como la aplicación de GR24 *in vitro* inhibió el crecimiento de distintos patógenos tanto de raíz como de parte aérea, lo que indica un posible efecto de las SLs en microorganismos patógenos además de simbiontes (Dor

et al., 2011, Belmondo *et al*., 2017). Sin embargo en otros trabajos se ha descartado su efecto sobre hongos fitopatógenos a concentraciones fisiológicas (revisado en López-Ráez *et al*., 2017)

1.6.3. Inducción de la germinación de semillas de plantas parásitas de raíz.

Las **SLs** se describieron por primera vez como **estimulantes de la germinación de las semillas de plantas parásitas de raíz** de la familia *Orobanchaceae* (Bouwmeester *et al*., 2003; Cook *et al*., 1972) (**Fig. 4**). Por tanto, las SLs juegan un papel dual en la rizosfera como señal de detección del hospedador tanto para hongos MA y *Rhizobium*, como para estas plantas parásitas (Bouwmeester *et al*., 2003; Xie *et al*., 2010). Las plantas parásitas de raíz son parásitos obligados que adquieren nutrientes y agua a través del hospedador causándole graves daños (Bouwmeester *et al*., 2003; Estabrook y Yoder, 1998). Se cree que originalmente las SLs fueron las moléculas señal para favorecer la micorrización y que más tarde, en la evolución, serían utilizadas por estas plantas parásitas como señal para la localización de posibles hospedadores (López-Ráez *et al*., 2017). Para más información sobre las plantas parásitas de raíz, ver el apartado 3.

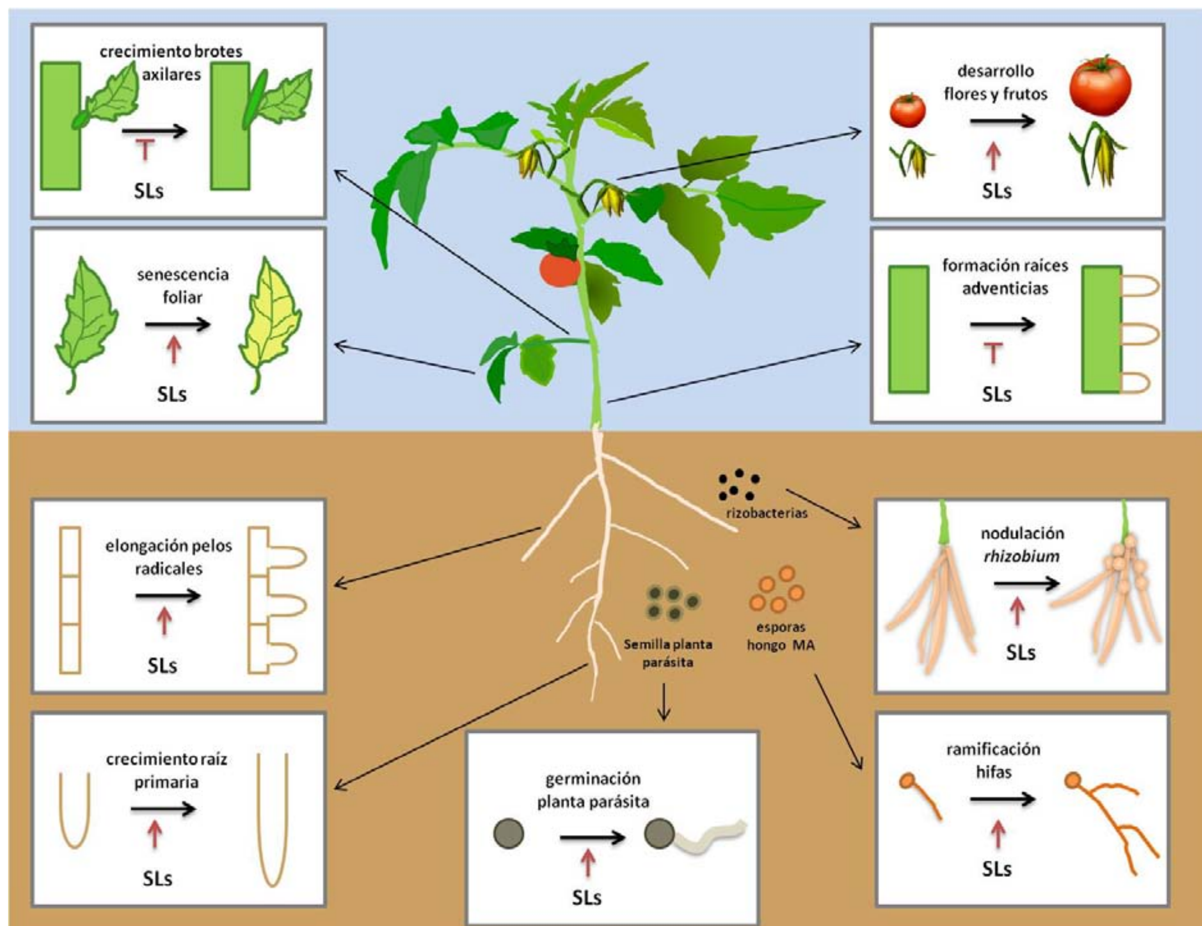

Fig 4: Funciones de las SLs como fitohormona y como molécula señal en la rizosfera.

1.7. REGULADORES DE LA BIOSÍNTESIS DE SLS

1.7.1. Deficiencia de fósforo (P).

En el suelo, el P es muy inaccesible para las plantas ya que está presente como fosfato inorgánico (Pi), formando complejos insolubles que a menudo limitan el crecimiento de las plantas (Peret *et al*., 2011). **Bajo condiciones deficientes de P** (y en menor medida de N), las plantas han desarrollado una serie de estrategias, como **alterar la arquitectura tanto de la parte aérea como radical y establecer simbiosis con hongos MA, procesos que son regulados por las SLs** (Pandey *et al*., 2016). Por tanto, estas condiciones de estrés promueven la biosíntesis y exudación a la rizosfera de éstas

fitohormonas/moléculas señal, regulando positivamente su biosíntesis a nivel transcripcional (Jamil *et al*., 2011; López-Ráez *et al*., 2008; Yoneyama *et al*., 2012).

1.7.2. Sequía / Salinidad.

En plantas de lechuga (*Lactuca sativa*) y tomate se mostró una influencia negativa de la salinidad/sequía sobre la producción de SLs (Aroca *et al*., 2013; Liu *et al*., 2015; Ruiz-Lozano *et al*., 2016). Por el contrario, en presencia de hongos MA en el suelo, que favorecen la tolerancia de la planta a estos estreses, se observó un aumento en la producción de SLs bajo estas condiciones desfavorables (Aroca *et al*., 2013; López-Ráez, 2016; Ruiz-Lozano *et al*., 2016). Una relación entre SLs y este tipo de estreses también se ha detectado en plantas de Arabidopsis deficientes en SLs, ya que mostraban hipersensibilidad al estrés en comparación con las plantas silvestres (Bu *et al*., 2014; Ha *et al*., 2014), pero tras el tratamiento con GR24 aumentó su tolerancia (Ha *et al*., 2014). Estos resultados nos indican que el **estrés osmótico por salinidad/sequía altera la producción de SLs**, aunque deben realizarse más estudios para entender como esta producción se encuentra regulada y cuál es su impacto en los mecanismos de tolerancia de la planta.

1.7.3. Alta intensidad lumínica.

Se ha sugerido que la luz es un regulador de los niveles de SLs, afectando positivamente a sus niveles hormonales y transcripcionales. Ésto se observó en estudios con plantas de tomate y *Lotus japonicus,* aunque parece que la regulación de las SLs por la luz depende tanto de la intensidad, provocando mayores niveles de SLs

un alto ratio luz roja/roja lejana, así como del tiempo de exposición lumínica, siendo mayor el efecto con el tiempo (Koltai et al., 2011; Nagara et al., 2015).

1.7.4. Micorrización.

Las SLs han sido relacionadas con una posible **autorregulación de la micorrización,** ya que en el caso de una colonización fúngica excesiva, la relación, normalmente mutualista, podría tornarse hacia el parasitismo (Vierheilig, 2004). De hecho, se ha evidenciado que la planta hospedadora, una vez bien establecida la simbiosis, deja de favorecer la micorrización mediante la reducción de la producción y exudación de SLs a la rizosfera (Aroca *et al*., 2013; Fernández-Aparicio *et al*., 2010; López-Ráez *et al.,* 2011). De manera similar, se ha visto en la interacción *Rhizobium*-leguminosa que los niveles de SLs son también más reducidos en plantas noduladas que en las no noduladas (Mabrouk *et al*., 2007; Peláez-Vico *et al*., 2016). Estos resultados sugieren que los mecanismos por los cuales se regula la producción de SLs durante las interacciones simbióticas, están conservados en distintos sistemas.

1.8. INTERACCIÓN DE LAS SLs CON OTRAS FITOHORMONAS.

1.8.1 Auxinas/CKs

1.8.1.1 Regulación de la ramificación aérea.

La molécula señal reguladora del crecimiento apical más estudiada son las auxinas, que se producen en las hojas jóvenes y en el ápice del tallo principal, siendo transportadas basipetalmente a través de la planta en las células vasculares del cambium. Se cree que la cantidad de auxinas producidas es proporcional a la actividad del meristemo del

brote y proporciona a los tejidos situados por debajo información sobre el estado del crecimiento de la parte aérea (Li y Bangerth, 1999). Las **SLs que inhiben el crecimiento de los brotes laterales** (Gómez-Roldan *et al*., 2008; Umehara *et al*., 2008) **y las CKs que lo promueven** (Wickson y Thimann, 1958)**, están reguladas a su vez por las auxinas** (Brewer *et al*., 2009; Dun *et al.*, 2012). Se han propuesto dos hipótesis distintas, pero no necesariamente excluyentes entre sí, sobre cómo estas hormonas regulan el crecimiento lateral:

Hipótesis del mensajero secundario: **Las auxinas se mueven basipetalmente en el tallo, inhibiendo los niveles de CKs** (Tanaka *et al*., 2006) **y promoviendo la expresión de genes de biosíntesis de SLs** (Arite *et al*., 2007; Foo *et al*., 2005; Hayward *et al*., 2009; Zou *et al*., 2006). Las SLs y CKs se sintetizan principalmente en la raíz y se mueven acropetalmente hasta las yemas laterales, regulando la ramificación de forma antagonista entre ellas (Dun *et al*., 2012). Ambas hormonas parecen converger en un gen diana específico del brote denominado ***TB1/BRC1/FC1*** que codifica un factor de transcripción TCP que reprime el desarrollo de la yema (Braun *et al*., 2012; Dun *et al*., 2012) (**Fig. 3**), aunque éste responde a SLs y CKs de manera diferente (Müller y Leyser, 2011). En estudios con guisantes, tras la decapitación de la zona apical del tallo las CKs que promueven el desarrollo de las yemas axilares, eran sintetizadas de forma local en el tallo en lugar de en la raíz (Shimizu-Sato *et al*., 2009). En consecuencia, las **SLs se postularon como la señal de larga distancia** para la regulación de la ramificación de la parte aérea.

Hipótesis del transporte/canalización de las auxinas: El flujo de auxinas en el tallo principal actúa inhibiendo el flujo de auxinas de los brotes laterales, impidiendo su

crecimiento (Shinohara *et al*., 2013; Waldie *et al*., 2014). Este **flujo de auxinas es regulado a través de las SLs**, que mantienen el flujo procedente del ápice como flujo dominante en el tallo. Ésto impide que nuevos brotes puedan crear su propio flujo de auxinas hacia el tallo, inhibiendo su crecimiento. Las SLs regulan el flujo de auxinas actuando sobre la **corriente del transporte polar de auxina** (**PATS,** del inglés "*Polar Auxin Transport Stream*")**.** La polarización y la localización de las **proteínas exportadoras de auxinas PIN-FORMED (PIN)** en las membranas celulares, es indispensable para dicho transporte (Wisniewska *et al*., 2006). En estudios con Arabidopsis, se ha observado como las SLs promueven la apolarización de las proteínas PIN, reduciendo la disponibilidad de estas proteínas para el transporte de auxinas, manteniendo el flujo del ápice como dominante en el tallo (Shinohara *et al*, 2013).

1.8.1.2. Regulación de la ramificación de la raíz.

Al igual que en la parte aérea, el factor determinante en el crecimiento de la raíz es el flujo de auxinas que se establece gracias a la **corriente PATS** (Grieneisen *et al*., 2007; Zazimalova *et al*., 2010). Al igual que en la regulación del desarrollo de los brotes laterales en la parte aérea, las **SLs inducen el crecimiento de la raíz principal inhibiendo a su vez el crecimiento de raíces laterales** mediante la regulación de los transportadores de auxinas PIN (Ruyter-Spira *et al*., 2011; Koren *et al*., 2013). De forma similar a las SLs, las **CKs pueden afectar negativamente a la formación de raíces laterales,** ya que también actúan a través de la alteración del transporte de auxinas en el primordio de raíces laterales (Bishopp *et al*., 2011).

1.8.2. Auxinas:

Además de la implicación de las auxinas y las SLs en la ramificación de la parte aérea y de la raíz, ambas interaccionan regulando el desarrollo de **raíces adventicias y de pelos radicales de forma sinérgica**, siendo necesaria la señalización de las auxinas para suprimir la formación de las raíces adventicias (Kapulnik *et al*., 2011b), así como para la inducción del crecimiento de pelos radicales (Rasmussen et al., 2012). Por otro lado, las auxinas son unas de las principales hormonas reguladoras del **desarrollo de flores y frutos**. Se ha observado que estas hormonas presentan un importante papel en el fenotipo relacionado con la deficiencia en SLs (Kohlen *et al*., 2012), mostrando flores y frutos menos desarrollados que las plantas silvestres.

Además, las SLs parecen interactuar con las auxinas en la fase pre-simbiótica de la colonización micorrícica. Hanlon y Coenen mostraron que la presencia de **auxinas es necesaria para que las hifas fúngicas se dirijan hacia la raíz,** siendo este efecto favorecido por la biosíntesis de SLs (Hanlon y Coenen, 2011). Se ha observado como los mutantes de guisante *bsh* alterados en auxinas presentaron bajos niveles de SLs, pudiendo ser responsables de la disminución de la colonización MA en estos mutantes, ya que era parcialmente recuperado el fenotipo micorrícico tras la aplicación de GR24 (Foo, 2013). Estos resultados sugieren que las auxinas podrían regular la fase inicial de la colonización mediante el control de los niveles de SLs.

1.8.3. Etileno (ET):

Poco se sabe de la relación entre las SLs y el etileno en la regulación funcional de la planta. Sin embargo, se sabe que las SLs promueven la senescencia, en parte, afectando a los niveles de ET (Ueda y Kusaba, 2015) y se observó que el efecto de las

SLs en el crecimiento de pelos radicales depende tanto de la biosíntesis como de la señalización de ET (Kapulnik *et al*., 2011b).

1.8.4. Ácido abscísico (ABA):

La implicación del ABA en la simbiosis micorrícica podría estar relacionada con una interacción con las SLs, ya que mutantes de tomate deficientes en ABA, presentaron niveles bajos tanto de colonización micorrícica como de producción de SLs (López-Ráez *et al*., 2010). Por otro lado**,** en la rizosfera, las semillas de las plantas parásitas de raíz son liberadas de su estado de latencia para posteriormente germinar gracias a la reducción de sus niveles de ABA (Chae *et al*., 2004)**.** En estudios mediante la aplicación de GR24, se ha observado como la inhibición de éstos niveles de ABA es mediada por las SLs (Lechat *et al*., 2012). Por tanto, el **ABA parece estar estrechamente ligada al papel que desempeñan las SLs en la rizosfera.** Además, se ha sugerido que el ABA promueve la biosíntesis de SLs en condiciones de salinidad/sequía (López-Ráez, 2016).

2. SIMBIOSIS MICORRÍCICA

2.1. DEFINICIÓN Y CARACTERÍSTICAS GENERALES DE LA SIMBIOSIS MICORRÍCICA.

Los microorganismos presentes en la rizosfera pueden establecer interacciones de forma tanto beneficiosa como perjudicial con las plantas. Entre las relaciones beneficiosas más extendidas destaca la simbiosis mutualista entre los hongos micorrícicos y las plantas, denominada **micorriza** (Frank, 1885). La asociación tiene un claro carácter mutualista, viéndose ambos simbiontes beneficiados ya que el hongo proporciona a la planta nutrientes minerales y agua procedentes del suelo y mejora su

tolerancia a estreses, y a cambio, la planta le cede al hongo hidratos de carbono derivados de la fotosíntesis (Smith *y* Read, 2010). En una clasificación global, encontramos dos principales grupos de micorrizas: **ectomicorrizas y endomicorrizas** (Smith y Read, 2010). Las ectomicorrizas se caracterizan por el desarrollo de sus hifas en el exterior de la raiz, formando un micelio en forma de manto que no penetra en las células corticales de la raíz. Por el contrario, en las endomicorrizas o **micorrizas arbusculares (MA)** parte de las estructuras del hongo se desarrollan intracelularmente en el interior de la raíz. Además de estos dos grandes grupos, se distinguen otros grupos menores de asociaciones micorricicas: Ectendomicorrizas, Arbutoides, Monotropoides, Ericoides y Orquidioides (Smith y Read, 2010).

Las MA son las más extendidas y probablemente las que presentan mayor importancia desde el punto de vista de los beneficios para la planta (Smith y Read, 2010). Más del 80% de las plantas terrestres, incluyendo angiospermas, gimnospermas y plantas inferiores como helechos y briófitos, son capaces de establecer este tipo de simbiosis. Los hongos que participan en esta simbiosis pertenecen a la división **Glomeromycota (**Schüßler *et al*., 2001). La mayoría de las micorrizas arbusculares están caracterizadas por la presencia de **arbúsculos** (formados por la ramificación dicotómica de las hifas dentro de las células vegetales), **hifas intraradicales** (inter e intracelulares) y **micelio extraradical** (donde se forman las esporas).

2.2. CICLO DE VIDA DE LOS HONGOS MICORRÍCICOS ARBUSCULARES

2.2.1. Fase pre-simbiótica:

Germinación de la espora. Las esporas se encuentran en el suelo como estructuras de resistencia en espera de unas condiciones favorables de su entorno, ya que su germinación está influenciada tanto por factores abióticos (Tª, pH, luz, humedad, etc) como bióticos (microorganismos, exudados vegetales, etc). Si la espora una vez germinada no encontrara un posible hospedador, ésta puede retraer hasta 10 veces el citoplasma de sus hifas, entrando en un estado de quiescencia (Bonfante y Genre, 2010).

Crecimiento y desarrollo de las hifas. Tras germinar las esporas, se desarrolla un **tubo germinativo** que dará origen a una **hifa,** que tras su crecimiento y ramificación formará una red de **micelio de hifas** con la finalidad de conectar con una planta hospedadora (Smith y Read, 2010) (**Fig. 5**). Este crecimiento es errático hasta alcanzar la rizosfera de una planta, donde el micelio es guiado para el contacto con la raíz mediante exudados vegetales de la planta (Larose *et al.*, 2002), principalmente SLs (Akiyama *et al*., 2005). De forma recíproca, el hongo produce unas moléculas señal denominadas **factores Myc** (lipoquitooligosacáridos y oligómeros de quitina de cadena corta), moléculas difusibles que permiten el reconocimiento del simbionte por la planta, induciendo la expresión de genes en la planta hospedadora y que están involucradas en el establecimiento de la simbiosis (Bonfante y Genre, 2015). Estos factores Myc estimulan, entre otros procesos, el desarrollo de raíces laterales (Oláh *et al*., 2005), facilitando así el contacto hongo-planta.

Penetración de las hifas en la raíz. El hongo penetra en la planta hospedadora a través de las raíces laterales (Tawaraya *et al*., 2007), formando una estructura de penetración denominada **hifopodio** (Smith y Read, 2010) (**Fig. 5**). La zona de la raíz donde se forma el hifopodio y penetra la hifa, se denomina **punto de entrada.** Poco tiempo después de formarse el hifopodio, la raíz de la planta hospedadora produce una especie de túnel denominado **aparato de pre-penetración (PPA),** del inglés "PrePenetration Apparatus"), que provoca la invaginación de la membrana plasmática, facilitando la entrada progresiva del hongo en la célula epidérmica, sin llegar realmente a penetrarla (Genre *et al*., 2008).

2.2.2. Fase simbiótica:

Crecimiento intracelular de las hifas. Una vez que las hifas del hongo han atravesado la epidermis, se dirigen hacia las células corticales donde desarrollan los principales órganos de intercambio de nutrientes que caracterizan a la simbiosis MA, los **arbúsculos**. Aquí las hifas se ramifican recorriendo longitudinalmente la raíz (**Fig. 5**).

Formación de arbúsculos. En las capas más internas del parénquima cortical, las hifas 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 (**Fig. 5**). En cada célula se forma un solo arbúsculo, que se encuentra rodeado totalmente por una membrana periarbuscular gracias a la invaginación de la membrana plasmática, **creando un espacio o matriz intersticial entre el hongo y la membrana vegetal**. Con ello, se consigue un gran aumento en la superficie de contacto entre el hongo y la planta, aumentando su actividad metabólica y facilitando

el intercambio de nutrientes entre ambos simbiontes (Smith y Read, 2010). Los arbúsculos son unas estructuras muy dinámicas, generándose y degradándose continuamente, con una vida media de 7-10 días (Walter *et al.*, 2010). Una vez degradado el arbúsculo, la célula recupera su morfología, siendo capaz de acoger nuevamente la formación de otro arbúsculo (Walter *et al*., 2010).

Formación de vesículas. En todo el parénquima cortical, las hifas pueden formar tanto intra como extracelularmente, unas estructuras globosas de alto contenido lipídico conocidas como vesículas, que parecen funcionar como órganos de reserva para el hongo (Smith y Read, 2010) (**Fig. 5**). En algunas especies de hongos MA estas vesículas pueden derivar en la formación de esporas en el interior de la raíz, como es el caso de *Rhizophagous irregularis* (Smith y Read, 2010).

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 hasta llegar a formar unas estructuras parecidas a los arbúsculos llamados **BAS** por sus siglas en inglés *"Branched Absorbing Structures"* (Smith y Read, 2010), encargadas de la absorción de nutrientes. El conjunto de hifas y los BAS constituyen 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 depleción que se crea alrededor de las raíces (Smith y Read, 2010). Algunas de las hifas extra-radicales darán lugar a **nuevas esporas de resistencia**, cerrándose así el ciclo de vida del hongo.

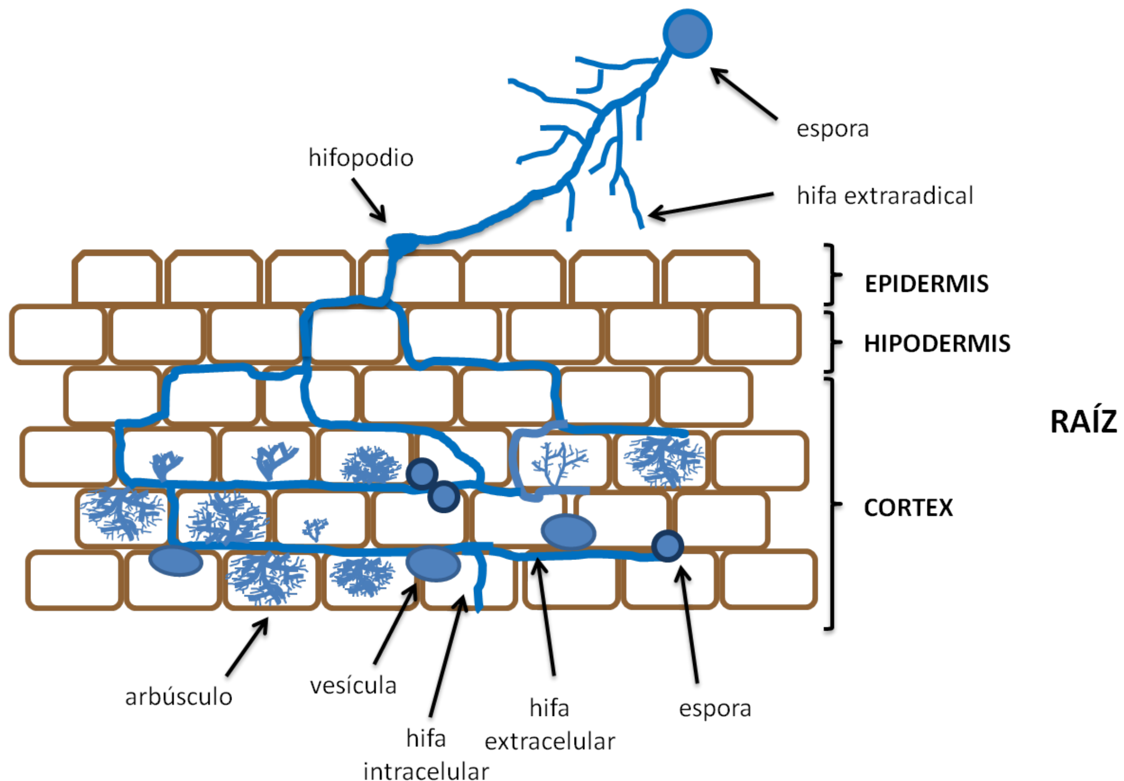

Fig. 5: Características de la simbiosis micorrícica arbuscular. Tras germinar la espora del hongo MA se genera el tubo germinativo e hifas extraradicales que contactan con la raíz formando el hifopodio. Las hifas internas pueden crecer colonizando intra y extracelularmente la raíz, formando arbúsculos (estructura de intercambio nutricional), vesículas (estructuras de reserva) y en algunas especies, esporas (estructuras reproductivas).

2.3. BENEFICIOS DE LA SIMBIOSIS MICORRÍCICA ARBUSCULAR (MA)

Como se ha comentado, la simbiosis MA aporta a ambos socios grandes beneficios, principalmente nutricionales. La simbiosis permite a la planta adquirir mayor cantidad de agua y nutrientes minerales, especialmente en el caso del fósforo. 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**) (Harrier y Watson, 2004; Koricheva *et al*., 2009; Pozo y Azcón-Aguilar, 2007) **y abiótico** (**salinidad, sequia, frio y presencia de metales pesados, entre otros)** (Ferrol *et al*., 2009; Gohre y Paszkowski, 2006; López-Ráez *et al*., 2016). 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 (Piotrowski *et al*., 2004; Rillig y Mummey, 2006).

Las plantas micorrizadas muestran una mayor resistencia frente a organismos patógenos que atacan las raíces de las plantas como son los **hongos del suelo y bacterias patógenas, nemátodos o insectos capaces de provocar heridas en la raíz** (Azcón-Aguilar y Barea, 1996; Jung *et al*., 2012; Whipps, 2004) y también frente a **patógenos de la parte aérea como hongos necrótrofos y frente a insectos herbívoros** (Campos-Soriano *et al*., 2012, Jung *et al*., 2012; Koricheva *et al*., 2009; Pozo y Azcón-Aguilar, 2007). En parte, las plantas micorrizadas son más resistentes a los patógenos del suelo debido a las alteraciones que se producen en la raíz y en el entorno de la planta, a una mayor competencia por el espacio radicular y a alteraciones de la producción de exudados de la planta (Morandi *et al*., 2002; Vigo *et al*., 2000). Sin embargo, además de los cambios directos ocasionados durante la colonización, la modulación del sistema inmune necesaria en la planta hospedadora para permitir la micorrización, 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 tanto del suelo como aéreos. Este pre-acondicionamiento de la planta viene definido por un proceso conocido como **"priming"**, que activa la **resistencia sistémica inducida (ISR)** en la planta (Ver apartado 4, Pozo y Azcón-Aguilar, 2007; Martinez-Medina et al., 2017). Este fenómeno se basa en cambios en los perfiles hormonales relacionados con la defensa de la planta hospedadora. Se ha propuesto que la interacción del hongo MA con la planta hospedadora, provoca un aumento en los niveles de SA, principal hormona reguladora de la defensa frente a patógenos biótrofos. Sin embargo, tras una inicial inducción, los

niveles de SA disminuyen, estableciéndose correctamente la simbiosis MA en la raíz. Tras el establecimiento micorrícico, se produce una regulación de la síntesis de JA que mediará la inducción de resistencia sistémica por micorriza (MIR) del hospedador (Jung *et al*., 2012; Pozo *et al*., 2015).

Por tanto, debido a las propiedades biofertilizadoras y bioprotectoras de los hongos MA, **la inoculación con estos hongos se postula como una buena alternativa a los fertilizantes y pesticidas químicos para una agricultura más sostenible y respetuosa con el medio ambiente** (Fester y Sawers, 2011; Harrier y Watson, 2004; Mukerji y Ciancio, 2007). Concretamente, el **uso de los hongos MA para la eliminación de las infecciones por plantas parásitas de raíz** se ha propuesto en la actualidad como una estrategia prometedora que será comentada en profundidad en el apartado 3.5.

3. PLANTAS PARÁSITAS DE RAÍZ

3.1. DEFINICIÓN Y TIPOS DE PLANTAS PARÁSITAS

En algún momento de la evolución, algunas plantas invadieron los tejidos de otras plantas para obtener de ellas los nutrientes necesarios para llevar a cabo su ciclo de vida. Son las denominadas plantas parásitas, que pueden ser clasificadas en dos grupos (Press y Graves, 1998):

- Plantas **holoparásitas**: Son parásitos obligados ya que no presentan clorofila y tras la germinación, son dependientes totalmente de su hospedador para obtener los requerimientos nutricionales.
- Plantas **hemiparásitas**: Son parásitos facultativos debido a que presentan pequeñas cantidades de clorofila y por lo tanto, pueden satisfacer, aunque de forma parcial, sus

requerimientos fotosintéticos, pudiendo vivir sin el hospedador un periodo más o menos largo de tiempo.

Estos parásitos, según donde se fijen en el hospedador, pueden diferenciarse en parásitos de raíz o de parte aérea.

3.2. TAXONOMÍA

Las plantas parásitas son un grupo diverso taxonómica y biogeográficamente, incluyendo tanto angiospermas como gimnospermas. Hay aproximadamente 3000-4000 especies parásitas de angiospermas en total (Press, 1998). Las plantas parásitas de raíz, económicamente más dañinas, son miembros de la familia **Orobanchaceae**, principalmente especies pertenecientes a los géneros ***Striga*, *Orobanche* y *Phelipanche***. Las especies de *Orobanche* y *Phelipanche*, comúnmente conocidos como **jopos**, se encuentran en las regiones templadas del hemisferio norte incluyendo la cuenca mediterránea, así como en las regiones subtropicales y tropicales, especialmente en las regiones áridas y semiáridas. La región mediterránea, donde grandes superficies muestran una alta infestación (Joel *et al*., 2013; Parker, 2009), parece ser el centro del origen del jopo. Estas plantas parásitas infectan cultivos importantes a nivel agronómico, incluyendo miembros de las familias de las Solanaceae, Fabaceae, Compositae, Cruiciferae, y Umbelliferae, causando grandes pérdidas económicas y llegando incluso a perder hasta el 100% de la producción agrícola (Joel *et al*., 2013; Parker, 2009). El principal problema para el control de estos parásitos es que la mayoría de su ciclo de vida ocurre bajo tierra (**Fig. 6**), lo que dificulta su detección, por lo que cuando son detectadas suele ser demasiado tarde,

cuando ya se han producido un gran daño en la planta hospedadora. Por ello, es necesario el desarrollo de nuevas estrategias de control centradas en los estadíos iniciales de su ciclo de vida (López-Ráez *et al*., 2009; Fernández-Aparicio *et al.*, 2011).

3.3. CICLO DE VIDA DE LAS PLANTAS PARÁSITAS DE RAÍZ

Las principales fases del ciclo de vida son las siguientes:

3.3.1. Acondicionamiento y germinación: Cuando las semillas del parásito se encuentran cerca de las raíces de una posible planta hospedadora , éstas responden gracias a unos inductores químicos específicos emitidos por el hospedador a la rizosfera. Para que los **inductores químicos, principalmente SLs**, sean efectivos, las semillas deben previamente haber pasado por un periodo de acondicionamiento/estratificación en el cual, las semillas activan su metabolismo y se preparan para la germinación. Cuando las **semillas germinan,** se desarrolla una estructura específica denominada **radícula** (Joel *et al*., 2013). En el extremo de la radícula se origina el **haustorio**, cuya función es triple: adhesión a la raíz hospedadora, penetración y absorción de nutrientes (López-Ráez *et al*., 2009; Fernández-Aparicio *et al.*, 2011) (**Fig. 6**).

3.3.2. Adhesión y penetración: El **haustorio** se fija a la raíz de la planta hospedadora y va creciendo y penetrando en ella absorbiendo agua, sales minerales y fotosintatos, actuando como puente de unión entre las dos plantas. Estas plantas parásitas parecen desarrollarse en los espacios intercelulares gracias al efecto de diversas enzimas líticas (endocelulasas, proteasas, ligninas, etc), así como la aplicación por parte del parásito

de fuerzas mecánicas que facilitan la penetración en la epidermis y el córtex de la raíz. De esta manera, el parásito alcanza el cilindro central donde establece conexiones con los haces vasculares de la raíz, a través de los cuales obtiene los nutrientes que necesita para completar su ciclo de vida (Joel *et al*., 2013).

3.3.3. Desarrollo del parásito: Tras conectar con el hospedador, el parásito desarrolla un órgano denominado **tubérculo** que acumula los nutrientes provenientes del hospedador (Joel *et al*., 2013). Posteriormente, este tubérculo desarrolla numerosas protuberancias con aspecto de raíces pero sin capacidad de absorción denominadas **rizomas**, que son capaces de diferenciar nuevos haustorios y volver a infectar al hospedador y que además, sirven de anclaje al patógeno (Joel *et al*., 2013). El ciclo finaliza con la formación de un **tallo** que emerge del suelo y en el que se producen las **flores** que generan un gran número de semillas (una media de 200.000 por planta), incrementando así enormemente el banco de semillas en el suelo (Joel *et al*., 2013) (**Fig. 6**). Estas semillas pueden permanecer latentes en el suelo durante más de 20 años.

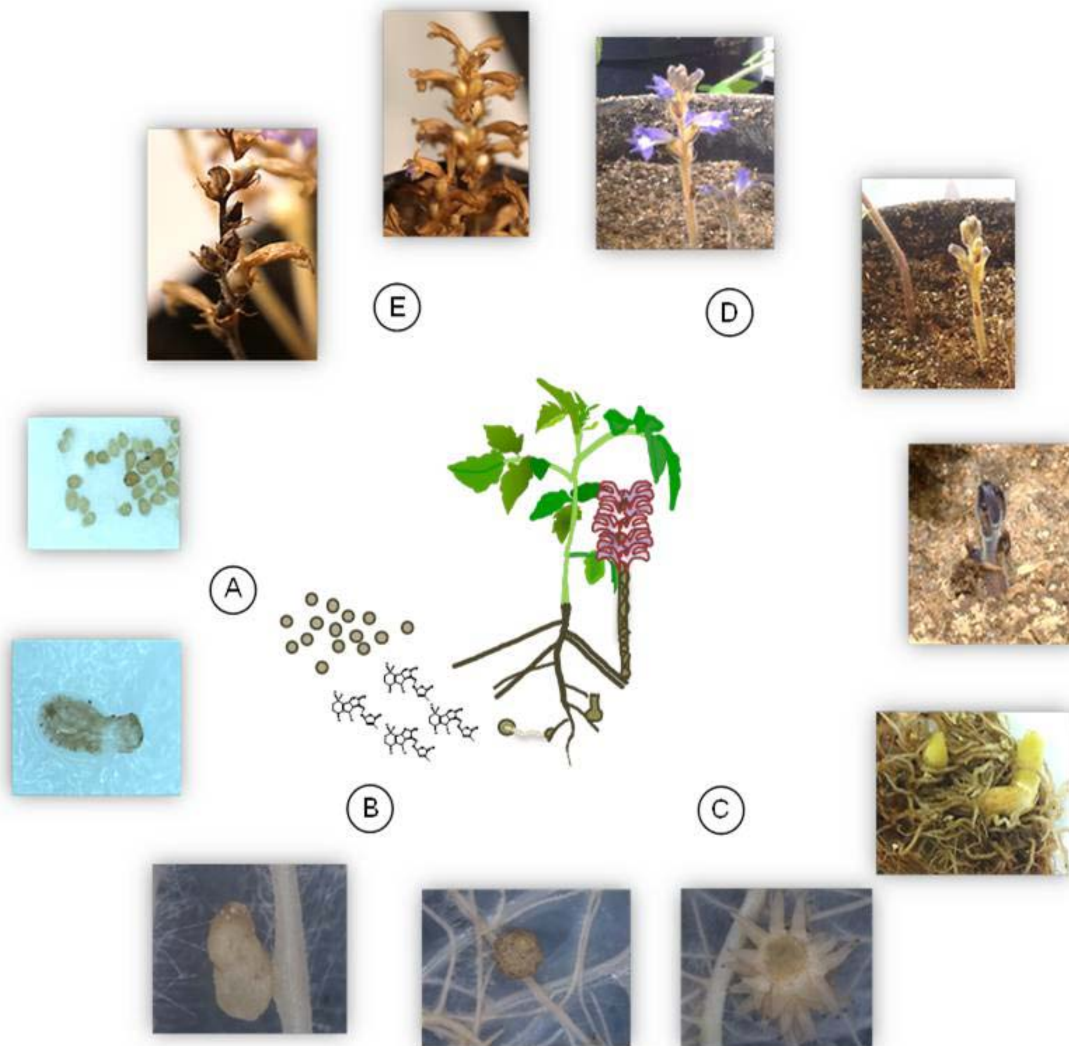

Fig. 6: Ciclo de vida de las plantas parásitas de raíz de la familia Orobanchaceae. **A**, Estimulantes del hospedador, incluyendo SLs, inducen la germinación de semillas. **B**, Semillas germinadas desarrollan una radícula que contacta y penetra en la raíz del hospedador, estableciendo el haustorio. **C**, El parásito crece formando un tubérculo bajo tierra. **D**, Desarrollo de flores. **E**, Tras la fertilización, se producen cápsulas con semillas maduras que son liberadas al suelo incrementando el banco de semillas. Fotos: Rocío Torres.

3.4. REACCIÓN DE LA PLANTA HOSPEDADORA AL ATAQUE POR EL PARÁSITO

Existen plantas con capacidad de resistir o tolerar el ataque de estas plantas parásitas, pueden presentar múltiples componentes de resistencia, incluyendo tanto defensas constitutivas como inducibles. La primera barrera defensiva incluye la ausencia o reducción de la producción de SLs, inhibiendo así la germinación, la

inhibición/reducción de la formación de haustorios y/o la formación de barreras mecánicas frente a la infección tales como engrosamiento de las paredes de las células de la raíz. Si el parásito consigue contactar y adherirse a la raíz, la planta hospedadora sintetiza y libera compuestos citotóxicos como ácidos fenólicos o fitoalexinas, forma barreras físicas como la lignificación y suberización de las paredes celulares o provoca la muerte celular programada (PCD) en forma de una respuesta de hipersensibilidad (HR), evitando así la conexión del parásito con el sistema vascular del hospedador y el progreso de la infección (Ver apartado 4; Cameron *et al*., 2006; Rodenburg *et al*., 2010).

3.5. PROBLEMÁTICA Y ESTRATEGIAS AGRÍCOLAS PARA LA ERRADICACIÓN DEL JOPO

La principal dificultad para controlar las plantas parásitas de raíz en situaciones agrícolas se debe principalmente a las propiedades de sus semillas: **su alto número, su pequeño tamaño, su extrema longevidad y su facilidad para la dispersión** (Joel *et al*., 2013). Estas características incrementan rápidamente el banco de semillas parásitas en el suelo aunque el área infectada inicialmente fuera reducida. El hecho de que la mayor parte del ciclo de vida de estas malas hierbas se produzca bajo el suelo dificulta enormemente el diagnóstico de la infección y por lo general sólo se detecta después de un daño irreversible en la cosecha. Existen estrategias agrícolas que reducen el banco de semillas de plantas parásitas en los cultivos, incluyendo **medidas fitosanitarias, desbroce manual, métodos de siembra alternativos, inundaciones, solarización del suelo, rotación de cultivos o cultivos intercalados y cultivos trampa, entre otros** (Fernández-Aparicio *et al*., 2011; Joel *et al*., 2013; López-Ráez *et al*., 2009)**.** Estas medidas por sí solas no son suficientemente eficaces para evitar o eliminar la

infección por completo del banco de semillas del parásito, pero pueden impedir o reducir la producción y dispersión de semillas. La reducción de la infección se puede lograr mediante la integración de estas medidas con medidas adicionales, tales como el **uso de variedades de cultivo resistentes (mejora genética clásica, mutagénesis e ingeniería genética), fumigación del suelo, principalmente mediante la aplicación de herbicidas, y medidas de biocontrol.** Sin embargo, una vez que las semillas parásitas están en el suelo, es prácticamente imposible erradicarlas por completo (Fernández-Aparicio *et al*., 2011; López-Ráez *et al*., 2009). El verdadero desafío es integrar prácticas que realmente sean eficaces, poco costosas y de fácil manejo para el agricultor y que no afecten negativamente a la producción del cultivo. El método de control más eficiente hasta la fecha, la fumigación, es peligroso para la salud humana y para el medio ambiente. Por tanto, se necesitan nuevos métodos, más respetuosos con el entorno para un control más eficaz contra estos parásitos. Las nuevas estrategias deberían estar centradas en los pasos iniciales de la interacción huésped-parásito, sobre todo en la etapa de germinación inducida por las SLs (Fernández-Aparicio *et al*., 2011; López-Ráez *et al*., 2009).

3.5.1. Nuevas estrategias.

3.5.1.1. Control a través de la modificación de la señal de germinación (SLs): Este control se puede abordar o bien induciendo la germinación suicida de las plantas parásitas mediante la aplicación de SLs sintéticas, o mediante la inhibición/reducción de la síntesis de SLs mediante fertilización del suelo, ingeniería genética o promoviendo la micorrización de las plantas hospedadoras (Fernández-Aparicio *et al*., 2011; López-Ráez *et al*., 2009). Recientemente, se ha propuesto un nuevo mecanismo

descrito como “quenching” mediante la aplicación de compuestos químicos como el bórax y la tiourea. Estos compuestos alcalinizan el suelo, favoreciendo así la degradación de las SLs rápidamente tras su exudación a la rizosfera, impidiendo la germinación de las semillas de plantas párasitas de raíz (Kannan *et al.*, 2015).

3.5.1.2. La simbiosis micorrícica como estrategia de control de plagas de plantas parásitas de raíz: El hecho de que las SLs jueguen un doble papel en la rizosfera como moléculas de señalización tanto para hongos MA como para plantas parásitas de raíz, abre la posibilidad del uso de la simbiosis MA como una estrategia para el control de esta plaga. Como se ha comentado anteriormente, cuando los niveles de micorrización de la raíz son altos, la planta disminuye la producción de SLs posiblemente con el fin de evitar una mayor colonización micorrícica que pudiera ser parasítica en vez de mutualista (López-Ráez *et al*., 2015, Vierheilig, 2004). Esta disminución de las SLs, se ha estudiado como una posible estrategia para disminuir y controlar a las plantas parásitas de raíz. De hecho, se ha demostrado en plantas de maíz (*Zea mays*), sorgo (*Sorghum bicolor*), guisante (*Pisum sativum*) y tomate *(Solanum lycopersicum*) que la micorrización disminuye la capacidad de infección de *Striga hermonthica* y *Phelipanche ramosa*, y que dicha disminución, al menos parcialmente, es debido a la reducción de la producción de SLs (Fernández-Aparicio *et al*., 2010; Lendzemo *et al*., 2005; López-Ráez *et al*., 2015). Mediante esta estrategia, además de una menor infección, la planta hospedadora obtendría los beneficios de la simbiosis micorrícica, siendo por tanto una estrategia totalmente sostenible.

4. DEFENSA DE LA PLANTA FRENTE A PATÓGENOS

Las plantas poseen un sistema inmunológico, que aunque no se basa en células especializadas como en animales, si les permite reconocer posibles agresores y responder activando diversos mecanismos de defensa incluyendo la producción de barreras físicas o sustancias tóxicas (Jones y Dangl, 2006). Para ello, activan diferentes mecanismos dependiendo del tipo de agresor y de las condiciones ambientales de la planta frente a éste. Inicialmente, tras el ataque de un patógeno, la planta puede detectar al patógeno mediante receptores de membrana que reconocen moléculas características del organismo agresor (PAMPS, del inglés, Pathogen Associated Molecular Pathogens; HAMPs, Herbivore associated molecular patterns). Este reconocimiento desencadena una respuesta de defensa inicial y rápida donde se incluyen estrategias como la fortificación de la pared celular, la síntesis de callosa y lignina, la acumulación de compuestos con actividad antimicrobiana como las fitoalexinas y las proteínas PR (del inglés "*Pathogenesis-Related protein*") o la activación de la muerte celular programada, entre otros (Spoel y Dong, 2012).

4.1. RESISTENCIA SISTÉMICA ADQUIRIDA E INDUCIDA

Tras el contacto inicial con el patógeno o con ciertos elicitores y la posterior activación de la defensa inicial de la planta, puede desencadenarse otro mecanismo de defensa que actúa a nivel sistémico protegiendo otras zonas de la planta no infectadas. Este fenómeno se conoce como resistencia inducida. Existen distintos tipos de resistencia inducida dependiendo del estímulo que actúe como inductor y los mecanismos específicos responsables de esta resistencia.

La **resistencia sistémica adquirida** es inducida por un patógeno avirulento o de baja virulencia y se denomina **SAR** (del inglés *"Systemic Acquired Resistance"*). Esta resistencia protege a la planta de posibles futuras infecciones virulentas. Se trata de una resistencia de larga duración que protege frente a un amplio rango de patógenos (Durrant y Dong, 2004; Fu & Dong, 2013, Vlot *et al*., 2009). La inducción de la resistencia SAR puede producirse tras la activación de la defensa inicial y se caracteriza por la activación de un conjunto de **genes PR,** que principalmente codifican proteínas con actividad antimicrobiana (Fu & Dong, 2013, van Loon *et al*., 2006).

Por otro lado, los microorganismos beneficiosos del suelo como los **hongos MA** entre otros, son capaces de **producir una resistencia sistémica inducida en la planta**, denominada **ISR** (del inglés *"Induced Systemic Resistance"*) (Pieterse *et al*., 2014). Al igual que la SAR, es una resistencia duradera con un amplio espectro de acción, protegiendo a la planta hospedadora de bacterias, hongos, virus e incluso insectos. La respuesta de defensa ISR no conlleva, en general, un coste energético añadido para la planta puesto que la respuesta defensiva se desencadena tras el reconocimiento de un posible atacante. Por tanto la ISR va asociada a un estado de precondicionamiento o priming de las defensas en los distintos tejidos que permite a la planta responder de manera más rápida y contundente en caso de un ataque (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017).

4.2. IMPLICACIÓN DE LAS PRINCIPALES FITOHORMONAS EN LA DEFENSA DE LA PLANTA

Determinadas fitohormonas cobran un papel esencial en la modulación del sistema de defensa de la planta, activando la respuesta apropiada. **SA** y **JA** se reconocen como las principales hormonas de defensa en las plantas (Browse, 2009; Pieterse *et al*., 2009;

Vlot *et al*., 2009). A pesar de ello, son múltiples las evidencias que demuestran que otras hormonas como ABA, ET, GAs y BRs, entre otros, así como su homeostasis, tienen también un papel importante en la modulación del sistema inmune (Pieterse *et al*., 2009; Robert et al., 2011). Dependiendo del tipo de agresor, la planta varía la proporción y el tiempo de aparición de las distintas hormonas, generando una combinación casi específica de señales para cada interacción (de Vos et al., 2005) que interactúan entre sí de manera sinérgica o antagónica, lo que permite a la planta priorizar unas vías de señalización sobre otras para refinar su respuesta de defensa activando aquellos mecanismos que sean más eficaces frente al invasor al que se enfrenta (de Vos *et al*., 2005; Pieterse *et al*., 2009).

4.2.1. Ácido salicílico (SA):

Esta fitohormona se encarga de regular principalmente la respuesta de defensa frente a **patógenos biótrofos** (Glazebrook, 2005), debido a que es el principal regulador de la muerte celular programada. La biosíntesis de SA comienza durante la respuesta de defensa inicial, actuando como inductor de la expresión de los **genes PR** (Dong, 2004). Una vez que su ruta hormonal es activada, pueden **inducir la respuesta de defensa SAR.** La proteína reguladora NPR1 (del inglés "*Nonexpressor of Pathogenesis-Related Protein 1*") 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, Vlot et al., 2009).

4.2.2. Ácido jasmónico (JA):

El JA y sus derivados, conocidos como jasmonatos (JAs), son compuestos de carácter lipídico sintetizados a partir de la ruta biosintética de las oxilipinas (Gfeller *et al*., 2010; Wasternack y Hause, 2013). Esta ruta está determinada por dos diferentes tipos de lipooxigenasas (LOXs), las vías 9-LOX y 13-LOX. La vía 13-LOX dirige la biosíntesis de los JAs y la 9-LOX dirige otro tipo de oxilipinas (Wasternack and Hause, 2013) (**Fig. 7**). Los JAs están implicados en multitud de procesos relacionados con el desarrollo de la planta como la formación floral y de tubérculos, promoción de la senescencia o la implicación en el crecimiento de la raíz, entre otros (Wasternack y Hause, 2013). Además de su función en el desarrollo de la planta, los JAs presentan un papel esencial en la defensa de las plantas frente a estreses abióticos y bióticos aunque su papel principal reside en la activación de la defensa frente a **necrótrofos e insectos.** La ruta de biosíntesis de los JAs es activada tras una **herida local o herbivoría,** aumentando los niveles de JA (De Vos *et al*., 2005; Narváez-Vásquez y Ryan, 2004; Stenzel *et al*., 2003). Además, el JA puede actuar como señal regulando las respuestas de defensa a nivel sistémico, y es esencial en **la ISR.** Entre otros genes, el JA activa la expresión de los genes que codifican para inhibidores **de proteasas** (***PIs***, del inglés "*Protease Inhibitors*"), como el gen *PinII* que está involucrado en la defensa de la planta frente a insectos y *Botrytis cinerea* (Wasternack y Hause, 2013; El Oirdi et al., 2011), entre otros compuestos con gran efecto negativo en herbívoros (Halitschke y Baldwin, 2004; Howe, 2004). Por otro lado, el JA regula la producción de compuestos volátiles tras el ataque del patógeno, lo que atrae a enemigos naturales de los herbívoros, señalizando lo que se denomina la defensa indirecta (Waternack y Hause, 2013).

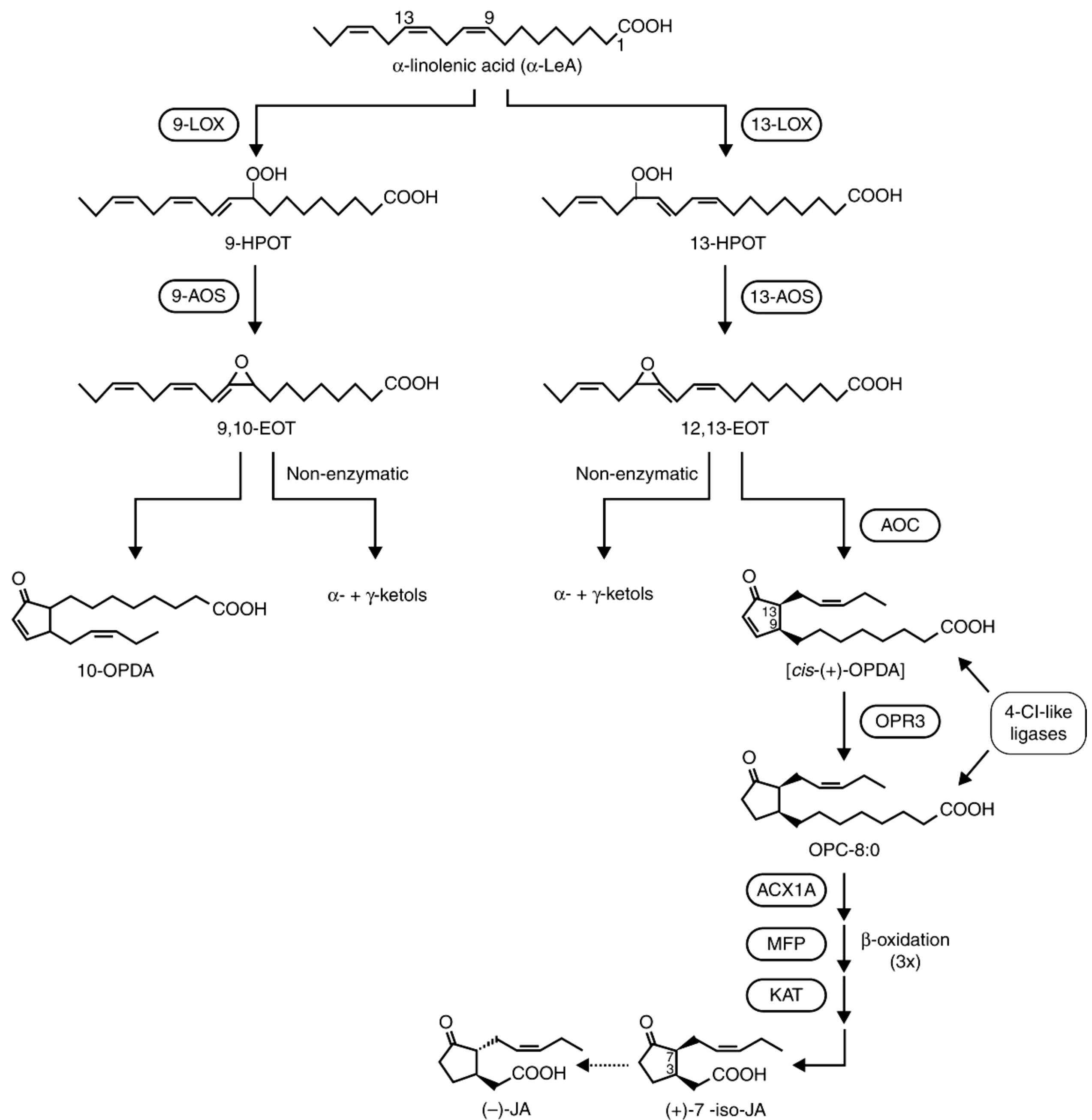

Fig. 7: Esquema metabólico de la ruta biosintética de las oxilipinas incluyendo las ramas de la 9- y 13- lipoxigenasa (9-LOX y 13-LOX). La biosíntesis de jasmonatos tiene lugar a partir de la vía 13-Lox (Wasternack, 2007).

4.2.3. Sistemina:

Es una hormona peptídica presente en la familia de las solanáceas como tomate, patata (*Solanum tuberosum*), boniato (*Ipomoea batatas*) y pimiento (*Capsicum annuum*), entre otros, y está asociada a las respuestas de defensa mediadas frente a herida y herbivoría reguladas por JAs (Constabel *et al*., 1998; Ryan y Pearce, 1998). Es un oligopéptido constituido por 18 aminoácidos, derivado de un pro-péptido de 200 aminoácidos conocido como **prosistemina (PS)** (McGurl *et al*., 1992; Pearce, 2011; Ryan y Pearce, 1998). Cuando se produce una **herida por herbivoría o un ataque por insecto**, la PS en el citosol es procesada proteolíticamente hasta sistemina (Ryan y Pearce, 2003). Tras la unión de la sistemina a su receptor, se produce la **activación de la defensa**, que viene fundamentada por la activación de la ruta de biosíntesis de los **JAs**. De hecho, se inducen los niveles de las **proteínas PIs** y las **cistatinas** entre otros, cuya función es disminuir la digestibilidad de las hojas ingeridas por el herbívoro, siendo ésta la finalidad de los productos de la ruta de biosíntesis del JA (Howe, 2004). Además, pueden inducir la expresión de genes que codifican para proteasas, siendo la más común la **leucina-aminopeptidasa A (LAP-A),** inducida tras un daño provocado por insecto. Esta interacción Sistemina-JAs implica una retroalimentación positiva entre ambas ya que la expresión del gen codificante para la PS es inducido por JA. Al igual que los JAs, la PS induce la defensa indirecta ya que activa la producción de volátiles que atraen a insectos parasitoides de los herbívoros (Conrrado *et al*., 2007). A pesar de esta clara conexión con la ruta dependientes de JA, recientemente se ha puesto de manifiesto a nivel transcriptómico (Coppola *et al.*, 2015) y metabolómico (Pastor *et al*., submitted) que la función de la sistemina va más allá de su interacción con esta ruta, afectando también a otras rutas hormonales.

4.2.4. Etileno (ET):

Es una fitohormona gaseosa que presenta un importante papel en procesos de desarrollo y senescencia en la planta, y también en la regulación de las respuestas de la planta frente a estreses tanto abióticos como bióticos. Concretamente frente a patógenos, el ET puede modular la respuesta de defensa **interaccionando antagónicamente con la ruta hormonal del SA y del ABA y presentando una relación sinérgica con el JA** (Broekgarden et al., 2015). El ET participa conjuntamente con el JA en la señalización de la defensa, induciendo la síntesis de compuestos antimicrobianos como las defensinas, tioninas e isoformas básicas de numerosas proteínas PR, como glucanasas o quitinasas, e interaccionando también en la respuesta **ISR** de la planta **(**Broekgarden et al., 2015; Pieterse et al., 2014). A pesar de su relación sinérgica para la activación de respuestas defensivas frente a necrótrofos, también puede antagonizar ciertas respuestas dependientes de JA destinadas a combatir los daños por insectos (Tianet al., 2014)

4.2.5. Ácido abscísico (ABA):

Posee un papel clave en la respuesta de defensa frente a **estreses abióticos, concretamente frente a sequía y salinidad** (Aroca *et al*., 2012; Ruiz-Lozano *et al*., 2009; Tuteja, 2007; Wasilewska *et al*., 2008). Recientemente, se ha observado también una relación de esta hormona con la regulación de la **defensa frente a estreses bióticos** (Asselbergh *et al*., 2008; Cao *et al*., 2011; Ton *et al*., 2009 Lee y Luan, 2012). Su papel depende en gran medida de su interacción con otras rutas hormonales implicadas en la defensa (Grant y Jones, 2009; Spoel y Dong, 2008). De hecho, se ha

observado un comportamiento antagónico con las rutas hormonales dependientes de SA y JA/ET (de Torres-Zabala *et al*., 2009; Jiang *et al*., 2010; Robert-Seilaniantz *et al*., 2011). Sin embargo, existen otros estudios donde su efecto en la defensa frente a patógenos es sinérgico. En este caso, ABA parece estar implicada en la deposición de callosa en la hoja durante el ataque de patógenos biótrofos, quedando encapsulados y limitando la invasión (Ton *et al*., 2009).

OBJETIVOS / OBJETIVES

OBJETIVOS

En la actualidad, uno de los grandes retos en la agricultura es cómo hacer frente a las plagas y enfermedades que causan enormes pérdidas en los cultivos (Jung *et al*., 2012, Xie *et al*., 2010). El uso de pesticidas y plaguicidas como control de organismos perjudiciales es una solución inviable en el contexto de una agricultura sostenible como requiere la sociedad actual. El desarrollo de nuevas estrategias sostenibles y eficaces para su control requiere de un mayor conocimiento de los procesos de infección por los agresores y de la respuesta de la planta a éstos. De hecho, la estimulación de los mecanismos de defensa de la propia planta frente a estos organismos, por ejemplo, mediante el uso de microorganismos beneficiosos del suelo, como los hongos micorrícicos arbusculares (MA), es una alternativa muy prometedora. Para poder optimizar estos mecanismos, se requiere un estudio profundo de la regulación hormonal de las respuestas de defensa de la planta y de los procesos fundamentales en la biología de la simbiosis y de la infección por el patógeno o parásito, y en este contexto se enmarca la presente Tesis Doctoral.

Las estrigolactonas (SLs) desempeñan un papel clave en el establecimiento de la simbiosis MA y en la germinación de las plantas parásitas (Akiyama *et al*., 2005; Bouwmeester *et al*., 2007). Por su doble papel en la rizosfera, tanto positivo como negativo, se podrían utilizar estrategias basadas en el metabolismo de estas SLs en el control de malas hierbas parásitas (Joel *et al*., 2013). Por un lado, el favorecimiento de la micorrización por parte de las SLs podría potenciar el sistema inmune de las plantas frente a patógenos ya que los hongos MA promueven la resistencia sistémica inducida

(ISR) (Jung *et al*., 2012; Pozo *et al*., 2015). Por otro lado, como fitohormonas las SLs juegan un papel relevante en diferentes procesos fisiológicos de la planta, entre los cuales también podría incluirse la respuesta de defensa a través de su interacción con otras fitohormonas involucradas en defensa y que se encuentran reguladas durante la simbiosis MA (Robert-Seilaniantz *et al*., 2011; Pozo *et al*., 2015).

Por lo tanto, el objetivo general de esta Tesis Doctoral es **el estudio de la regulación y función de las SLs durante el establecimiento de distintas interacciones planta-organismo tanto beneficiosas (micorrizas arbusculares), como perjudiciales (infección por plantas parásitas o hongos fitopatógenos), y su interacción con otras fitohormonas en la modulación de las respuestas de defensa de la planta, haciendo especial énfasis en el papel del ácido jasmónico (JA) y la hormona peptídica sistemina.** Para alcanzar este objetivo general, se han definido los siguientes **objetivos específicos**:

Objetivo 1. Dilucidar cómo se regula la producción de SLs a nivel transcripcional y hormonal.

Objetivo 2. Estudiar los mecanismos de defensa en la interacción temprana planta-planta parásita de raíz y el posible papel de las SLs en dicho proceso.

Objetivo 3. Analizar la posible implicación de las SLs en la respuesta de defensa de las plantas frente a patógenos fúngicos.

Objetivo 4. Dilucidar una posible interacción entre las SLs y el JA en la regulación de los mecanismos de defensa, simbiosis MA y arquitectura de la parte aérea de la planta.

Objetivo 5. Estudiar el papel de las SLs en estadios avanzados de la simbiosis MA y su interacción con la hormona peptídica sistemina.

OBJECTIVES

Currently, one of the greatest challenges in agriculture is how to eradicate pests and diseases that cause huge crop losses (Jung *et al*., 2012; Xie *et al*., 2010). The use of pesticides as control strategy against harmful organisms is a non-viable solution in the context of sustainable agriculture as required by today's society. The improvement of plant defense mechanisms against these organisms, by use of beneficial soil microorganisms, e.g. arbuscular mycorrhizal (AM) fungi, it is a very promising alternative. In order to optimize these mechanisms, a exhaustive knowledge of the regulation of these defense responses, as well as of the fundamental processes of pathogen/parasite infection and beneficial symbiosis is required. In this context, this Doctoral Thesis is framed.

Strigolactones (SLs) play a key role in the establishment of mycorrhizal symbiosis and in the germination of parasitic plants (Akiyama *et al*., 2005; Bouwmeester *et al*., 2007). Due to its dual role in the rhizosphere, both positive and negative, these metabolites may play a crucial role in development of new control strategies against parasitic weeds (Joel *et al.*, 2013). On one hand, the promotion of AM symbiosis by SLs may

enhance the immune system of plants against pathogens by induced systemic resistance (ISR) (Jung *et al.*, 2012; Pozo *et al.*, 2015). On the other hand, SLs, as phytohormones, play a role in several physiological processes in the plant, among which defense responses may be also included. It is well known that multiple plant hormones, including those related with defense, are involved in the regulation of AM symbiosis through interaction with each other (Robert-Seilaniantz *et al.*, 2011).

Therefore, the **main objective** of this Doctoral Thesis is **the study of the regulation of SLs during the establishment of different plant-organism interactions including the beneficial AM symbiosis or deleterious interactions with parasitic weeds and pathogenic fungi, and their interaction with other plant hormones modulating plant defense responses, with particular emphasis in their potential relationship with jasmonic acid (JA) and the peptide hormone systemin**. To achieve this overall objective, we defined the following **specific objectives**:

Objective 1. To elucidate how SL production is regulated at the transcriptional and hormonal level.

Objective 2. To study the plant defense responses against root parasitic plants during the early stages of the interaction, and the potential role of SLs in this process.

Objective 3. To analyze the possible involvement of SLs in plant defense responses against fungal pathogens.

Objective 4. To investigate the possible interaction between SLs and JA in the regulation of defense responses, in AM symbiosis and shoot architecture.

Objective 5. To study the potential role of SLs in further stages of AM symbiosis, as well as its interaction with the peptide hormone systemin.

MATERIAL Y MÉTODOS GENERAL

MATERIAL Y MÉTODOS GENERAL

A continuación se expone de forma breve y resumida parte del material y las principales metodologías que se han usado para esta Tesis Doctoral. Para más información, en cada capítulo se describe más extensamente los Materiales y Métodos específicos.

Material vegetal y organismos usados

En la tabla 1 se enumeran y describen las distintas líneas vegetales usadas en esta tesis doctoral para el estudio de la función de las rutas hormonales reguladas por SLs, JA o PS en los procesos estudiados. Asi mismo, la tabla 2 enumera los organismos usados en este trabajo para estudiar los distintos tipos de interacciones de las plantas incluyendo hongos beneficiosos y patogénicos y plantas parásitas de raíz.

Planta mutante/transgénica	Alteración	Cultivar	Referencia	Capítulo
Slccd7 líneas 6931, 6936 y 7170	deficiente en SLs	Tomate cv. M82	Vogel *et al.*, 2010	3 y 4
Slccd8 lineas L16, L09	deficiente en SLs	Tomate cv. Craigella	Kohlen *et al.*, 2012.	2, 3, y 4
max4-1 *AtCCD8*	deficiente en SLs	Arabidopsis cv. Columbia (Col-0)	Sorefan *et al.*, 2003	2
ps-	deficiente en PS	Tomate cv. Betterboy, (BB)	McGurl *et al.*, 1992	4
ps+	sobreexpresante en PS	Tomate cv. Betterboy (BB)	McGurl *et al.*, 1994	4
spr1 *"suppressor of prosystemin-mediated responses1"*	deficiente en JA	Tomate cv. Castlemart (CM)	Lee y Howe, 2003	3 y 4
spr2 *"suppressor of prosystemin-mediated responses2"*	deficiente en JA	Tomate cv. Castlemart (CM)	Li *et al.*, 2003	3 y 4
jai1 *"jasmonic acid insensitive1"*	insensible a JA	Tomate cv. Castlemart (CM)	Li *et al.*, 2001	3 y 4

Tabla 1: Líneas vegetales usadas en esta Tesis Doctoral.

Especie	Tipo de organismo	Donde infecta	Capítulo
Phelipanche ramosa	planta parásita	raíz	1, 3 y 4
Botrytis cinerea	patógeno necrótrofo	Hoja	2
Alternaria alternata	patógeno necrótrofo	hoja	2
Fusarium oxysporum	patógeno hemibiótrofo	raíz	2
Rhizophagus irregularis	hongo micorrícico arbuscular	raíz	3 y 4
Funneliformis mosseae	hongo micorrícico arbuscular	raíz	3

Tabla 2: Organismos beneficiosos o perjudiciales aplicados a las plantas en los distintos apartados de esta Tesis Doctoral.

Tinción y cuantificación de los niveles de micorrización

Las raíces micorrizadas se incubaron en 10 % de KOH a 4 °C durante 2 días. Se lavaron con agua destilada tres veces para posteriormente ser acidificadas con 2 % de ácido acético. Para la tinción de las estructuras micorrícicas se usó 5 % de tinta china (Sheaffer, CT, USA) en 2 % de ácido acético a 90 °C durante 10 min. El exceso de tinta fue eliminado tras lavar las raíces tres veces con agua destilada (Vierheiling *et al.*, 1998). Las raíces teñidas se mantuvieron en 100 % de glicerol y las estructuras fúngicas dentro de la raíz se observaron por microscopía de campo claro y se cuantificaron mediante el método de intersección en cuadrícula “Gridline intesection method” descrito por Giovannetti y Mosse, (1980).

Extracción de ARN y análisis de la expresión génica mediante qRT-PCR

El ARN total tanto de raíces como de hojas se extrajo usando "Tri-Reagent" (Sigma-Aldrich, St. Louis, MO, USA) de acuerdo con las instrucciones de uso del fabricante. El ARN se trató con "RQ1 DNase" (Promega, Madison, WI, USA) y fue purificado a través de una columna de sílice (silica) usando "NucleoSpin RBA Clean-up Kit" (Macherey-Nagel, Düren, Germany). Antes de almacenar el ARN a – 80 °C, se cuantificó usando un estectrofotómetro Nanodrop 2000C (Thermo Scientific, Wilmington, DE, USA) y se comprobó su integridad mediante electroforesis en gel de agarosa. Se sintetizó ADNc a partir de un 1 µg de ARN total purificado usando el "iScript cDNA Synthesis Kit" (Bio-Rad, Hercules, CA, USA) acorde a las instrucciones de uso del fabricante. La expresión de genes marcadores para las diferentes vías de señalización hormonal se analizó en raíces y hojas de plantas de tomate mediante la reacción en cadena de la polimerasa con transcriptasa inversa en tiempo real (qRT-PCR). Todas las reacciones de qRT-PCR eran realizadas usando "iQ SYBR Green Supermix" (Bio-Rad) y "iCycler iQ5 system" (Bio-Rad), con 1 µl de ADNc monocatenario (dilución 1:10) y cebadores específicos para cada gen. Los genes de las tablas suplementarias correspondientes a cada capítulo de la Tesis se usaron como genes marcadores específicos para cada estudio. El protocolo de amplificación incluye una desnaturalización inicial a 95°C durante 3 min seguido de 35 ciclos a 95°C durante 30s, 58°C durante 30s y 72°C durante 30s. La especificidad de los diferentes amplicones era probada por análisis de curva de melting (de 70 a 100°C) al final del protocolo de amplificación. Entre tres y seis réplicas biológicas independientes fueron analizadas por tratamiento y cada reacción de PCR se realizó por triplicado. Una cuantificación relativa de los niveles específicos de ARNm se

realizaron usando el método comparativo $2^{-\Delta(\Delta Ct)}$ (Livak and Schmittgen, 2001). Se normalizaron los valores de expresión usando el gen constitutivo *SlEF-1α*, que codifica para el factor de elongación de tomate 1α.

Cuantificación hormonal

Las fitohormonas fueron analizadas mediante cromatografía líquida de alta o ultra eficiencia acoplada a un espectrómetro de masas en tándem (HPLC-MS/MS y UPLC-MS/MS, respectivamente) como se describe en López-Ráez *et al*. (2008) para la cuantificación de SLs y en Flors *et al*. (2008) para el resto de fitohormonas. Se usaron cinco réplicas biológicas independientes por genotipo y/o tratamiento y una alícuota de 100 mg de tejido (hojas o raíces) liofilizado por muestra. La cuantificación se llevó a cabo con el software MassLynx 4.1 (Waters) usando estándares internos como referencia para la recuperación de la extracción y curvas estándar como cuantificadores. Los análisis hormonales fueron realizados por el Servicio de Instrumentación Científica de la Estación Experimental del Zaidín-CSIC (España), el Laboratorio de Bioquímica y Biotecnología Vegetal de la Universitat Jaume I (España) y "Laboratory of Plant Physiology of Wageningen University" (Holanda).

Cuantificación de SLs mediante bioensayos con semillas de *Phelipanche ramosa*

- **Obtención de extractos de raíz:** Para analizar los niveles de SLs, 0.5 g de tejido de raíz se molió en un mortero con nitrógeno líquido. Las muestras se extrajeron con 1 ml de acetona pura fría en un tubo eppendorf de 2 ml. El tubo se agitó mediante vórtex durante 5 min y se centrifugó durante 5 min a 8000 g en una centrífuga de mesa. La

fase orgánica se transfirió cuidadosamente a un tubo de 2 ml limpio. El precipitado se re-extrajo añadiendo otra vez 1 ml de acetona. Las fracciones de acetona combinadas se usaron para los bioensayos de germinación con semillas de *P. ramosa*.

- Pre-acondicionamiento y bioensayo de germinación con semillas de *P. ramosa*: El pre-acondicionamiento y los bioensayos de germinación fueron adaptados y modificados de Matusova *et al*. (2004), siendo realizados bajo condiciones estériles. Las semillas de *P. ramosa* se esterilizaron superficialmente en 2% de hipoclorito sódico con 0.02% (v/v) de Tween 20 durante 10 min y enjuagadas con agua desmineralizada estéril. Posteriormente, se secaron durante 60 min en una cabina de flujo de aire laminar. Aproximadamente, entre 50-100 semillas fueron repartidas en discos de papel de fibra de vidrio (GFFP, 9 mm de diámetro) y puestas en placas de Petri estériles (9 cm de diámetro) cubiertas con papel de filtro Whatman humedecido con 3 ml de agua desmineralizada estéril. Las placas de Petri fueron selladas con parafilm e incubadas para el pre-acondicionamiento de las semillas a 21°C en oscuridad durante 10-12 días. Tras el pre-acondicionamiento, alícuotas de los extractos de raíz (50 µl) se añadieron a los discos por duplicado. Se realizaron previamente diluciones seriadas con cada extracto vegetal. En cada bioensayo la estrigolactona sintética GR24 (10^{-7} M) y agua desmineralizada fueron incluidos como control positivo y negativo, respectivamente. Tras 7 días, las semillas germinadas y no germinadas fueron cuantificadas usando un microscopio Nikon Eclipse 50i de campo claro. Las semillas eran consideradas como germinadas cuando la radícula sobresalía a través de la cubierta de la semilla. Se analizaron seis réplicas biológicas por genotipo de planta y tratamiento. Las semillas de *P. ramosa* (recolectadas en cultivo de plantas de tomate) fueron amablemente

proporcionadas por el Dr. Maurizio Vurro y la Dra. Angela Boari (Instituto di Scienci delle Produzioni Alimentari, Bari, Italia).

CAPÍTULO 1

Expresión de marcadores moleculares asociados a vías de señalización de defensa y biosíntesis de SLs durante la interacción temprana tomate-*Phelipanche ramosa*

Torres-Vera R, García JM, Pozo MJ, López-Ráez JA

RESUMEN

Las plantas parásitas de raíz de la familia de las Orobanchaceae causan daños severos en importantes cultivos agrícolas en todo el mundo. Estas malas hierbas parásitas son difíciles de controlar porque su ciclo de vida sucede principalmente bajo el suelo. Esto hace difícil el diagnóstico de la infección que ocurre normalmente cuando el daño en el cultivo es irreversible. Por tanto, las estrategias de control más efectivas contra estas malas hierbas deben focalizarse en los estadíos iniciales de la interacción. Usando como modelo de sistema a tomate-*Phelipanche ramosa*, hemos explorado la respuesta del hospedador durante la fase inicial de la infección por la planta parásita mediante la monitorización de la expresión de genes marcadores de diferentes vías hormonales relacionadas con la defensa. Para ello se seleccionaron y analizaron dos estadíos diferentes de colonización. Los resultados sugieren que las tres principales vías hormonales en la regulación de la defensa - ácido salicílico (SA), ácido jasmónico (JA) y ácido abscísico (ABA) – son inducidos tras la infección, estando probablemente involucradas en la respuesta de defensa contra estos parásitos de raíz. Además, se observó una inducción de los genes de biosíntesis de estrigolactonas (SLs) *SlD27* and *SlCCD8*. Nuestros resultados sugieren una compleja regulación hormonal de las respuestas de defensa de la planta durante los estadíos tempranos de la interacción

planta-parásito, y sugieren un papel adicional de las SLs, estimulantes de la germinación del parásito, en este estadío post-ataque.

Adapted from Physiological and Molecular Plant Pathology (2016)

Expression of molecular markers associated to defense signaling pathways and strigolactone biosynthesis during the early interaction tomato-*Phelipanche ramosa*

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1. ABSTRACT

Root parasitic plants of the family Orobanchaceae cause severe damage to important agricultural crops worldwide. These parasitic weeds are difficult to control since most of their lifecycle occurs belowground. This hinders the diagnosis of infection and normally when irreversible damage has been caused to the crop. Therefore, new and more effective control strategies against these parasitic weeds should be focused on the initial stages of the interaction. Using tomato-*Phelipanche ramosa* as model system, we have explored the host response during the initial phase of parasitic infection by monitoring the expression of marker genes of different defense-related hormonal pathways. Two different colonization stages were selected and analyzed by quantitative real-time PCR. The data suggest that the three principal defense regulating hormonal pathways - salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) - are induced after infection, being therefore, likely involved in the defense response against these root parasites. In addition, induction of the strigolactone (SL) biosynthesis genes *SlD27* and *SlCCD8* was observed. Our results suggest a complex regulation of plant defenses during the early stages of plant-parasite interaction

involving the classical defense hormones, and suggest an additional role of the parasite germination stimulants SLs at this post-attachment stage.

Keywords: Defense responses, molecular markers, phytohormones, root parasitic plants, strigolactones.

2. INTRODUCTION

Over 4000 species of angiosperms are able of directly invade and parasite other plants (Heide-Jorgensen, 2013; Nickrent *et al*., 1998). A small number of these species has become weeds parasitizing major crops with important economic value as vegetables, legumes and cereals. Among them, root parasitic plants of the family Orobanchaceae are the most damaging agricultural pests, causing large crop losses (Parker, 2013). These parasitic weeds are mainly integrated by broomrapes (*Orobanche* and *Phelipanche* spp.) and witchweeds (*Striga* spp.). *Orobanche* and *Phelipanche* spp. form a large group of obligated root-holoparasites, lacking chlorophyll and being completely dependent on their host. They occur mainly in more temperate climates such as the South-East of Europe, Middle East, West Asia, North Africa and in the Americas (Parker, 2013). Most broomrape species have a narrow host range and grow on perennial host plants (Fernández-Aparicio *et al*., 2011; Parker, 2013). However, the species *Phelipanche ramosa* (formerly *Orobanche ramosa* (Joel, 2009)) shows an extremely wide host range. It affects, among others, a great variety of Solanaceae crops, such as eggplants, tobacco, pepper, potato and tomato (**Fig. 1**), causing up to 75% losses on crop production (Joel, 2009; Parker, 2013).

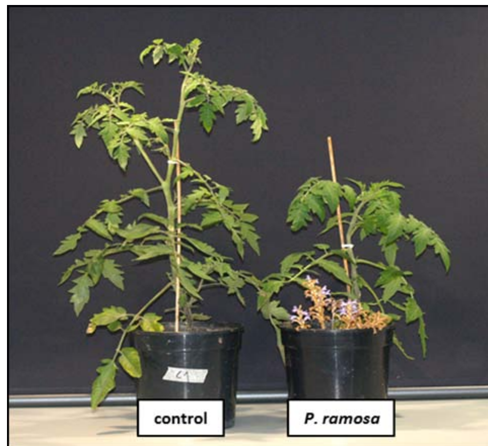

Figure 1. Photograph of a tomato plant infected by the root parasitic plant *Phelipanche ramosa* compared with a non-infected plant.

The parasite life cycle starts when the seeds present in the soil perceive signals from the host plant that induce germination (**Fig. 2**). These signals are mainly strigolactones (SLs), secondary metabolites produced and exuded by the host plant into the rhizosphere under conditions of nutritional deficiency (Bouwmeester *et al*., 2007; Cook *et al.*, 1972; López-Ráez *et al*., 2008; Yoneyama *et al*., 2007). Upon germination, the parasite forms a radicle that grows toward the host root, probably guided by a SL gradient (Fate *et al*., 1990), thus facilitating the contact with the root. Next, the radicle penetrates into the host root establishing a connection with the vascular tissues through the haustorium, which serves as an attachment organ (Joel *et al*., 2013). Through the haustorium, the parasitic plant obtains water, nutrients and other substances from the host needed for its own growth and development (Joel *et al.*, 2013; Pérez-De-Luque *et al*., 2008), and that negatively affects plant fitness and crop yield. Upon the initial vascular connection, the parasite develops a tubercle, a swelling in the young parasite above the host root, being this the first specific step of parasitic plant growth (**Fig. 2**) (Joel *et al*., 2013). After emergence from the soil, the parasite

flowers and produces new mature seeds that are released into the soil, increasing the seed bank (López-Ráez *et al*., 2009; Xie *et al*., 2010). In the absence of a suitable host, the parasite seeds can remain dormant in the soil for many years ensuring the persistence of the parasitic seed bank (Fernández-Aparicio *et al*., 2011). Parasitic weeds are difficult to control because they are intimately associated with the host root and most of their life cycle occurs belowground (**Fig. 2**). This fact hinders the diagnosis of the infection that is generally detected when an irreversible damage has been caused to the crop (Cardoso *et al.*, 2011; Joel *et al*., 2013; López-Ráez *et al*., 2009). Current control strategies against these parasites such as hand weeding, fumigation, solarization, cultural practices, and use of catch and trap crops are being used without the desirable success. Therefore, new control strategies should focus on the pre-attachment stage (seed germination) or on the initial steps during the host-parasite interaction (post-attachment) (Fernández-Aparicio *et al.*, 2011, Joel *et al*., 2013; López-Ráez *et al*., 2009, Pérez-De-Luque *et al*., 2008).

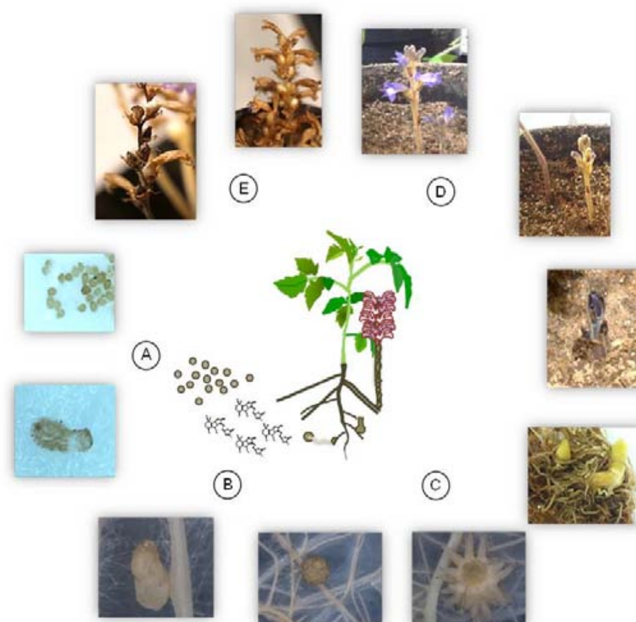

Figure 2. Life cycle of root parasitic plants of the Orobanchaceae. **A**, Seed germination is elicited by host-derived stimulants, including strigolactones. **B**, Germinated seeds develop a radicle that contacts and penetrates the host root, establishing the haustorium. **C**, Parasite grows forming a tubercle belowground. **D**, Emergence of the flowering shoots. **E**, After fertilization, capsules with new mature seeds are produced and released into the soil increasing the seed bank.

Data on plant defense responses and on the hormonal signaling pathways involved during this initial phase of the parasitic infection are scarce. It was shown that the host plant can respond to the attack by inducing callose deposition, suberization, lignification, as well as accumulating and secreting toxic compounds (allelochemicals) into the rhizosphere in order to impede the penetration of the parasite within the roots (Pérez-De-Luque *et al*., 2008). All these defense mechanisms are regulated to some extent by phytohormones; however there is very little information regarding the specific role and the regulation of these hormones during the plant-parasitic plant interaction. The best known phytohormones associated to plant defense responses against biotic stress are salicylic acid (SA) and jasmonic acid (JA). SA-dependent signaling is mainly induced against biotrophic pathogens, while JA-mediated signaling is mainly involved in defense against fungal necrotrophic pathogens and insect herbivores (Pieterse *et al*., 2009; Yoder *et al.*, 2010). Abscisic acid (ABA) is the hormone most studied in the response of plants to abiotic stress, especially water-related stresses (Osakabe *et al.*, 2014). In addition, an important role in the regulation of plant defense responses has been proposed (Christmann *et al*., 2006; Ton *et al.*, 2009). Interestingly, the signaling pathways of these phytohormones can interact, either antagonistic or synergistically depending on diverse factors, to promote different defense responses (Pieterse *et al*., 2009; Robert-Seilaniantz *et al*., 2011). Other phytohormones such as ethylene, brassinosteroids (BRs), gibberellins (GAs), cytokinins (CKs) and auxins are also known to participate in the regulation of defense responses, although their involvement is less explored (Robert-Seilaniantz *et al*., 2011).

As described above, SLs are the main signaling molecules inducing the germination of root parasitic weed seeds. In addition, they also act as host detection cues for symbiotic arbuscular mycorrhizal (AM) fungi, favoring symbiosis establishment (Bouwmeester *et al.*, 2007; Akiyama *et al.*, 2005). Accordingly to their role as molecular cues in the rhizosphere, SLs are mainly produced in the roots and they have been detected in the root extracts and root exudates of monocot and dicot plants (Andreo-Jiménez *et al*., 2015; Xie *et al.*, 2010). Since 2008, SLs are classified as a new class of plant hormones controlling several different processes within the plants (Andreo-Jiménez *et al*., 2015; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Xie *et al.*, 2010). They play a pivotal role as modulators of the coordinated development of roots and shoots in response to nutrient deprivation, especially phosphorus shortage (reviewed in Ruyter-Spira *et al*., 2013). Moreover, a new role for SLs in defense responses against necrotrophic fungi has recently been proposed (Stes *et al.*, 2015; Torres-Vera *et al.*, 2014). They are biosynthetically derived from the carotenoids (López-Ráez *et al.*, 2008; Matusova *et al.*, 2005), through the conversion of all-*trans*-β-carotene to 9-*cis*-β-carotene mediated by a β-carotene isomerase (D27) (Fig. S3) (Alder *et al*., 2012). This compound is transformed into carlactone by sequential oxidative cleavage by two carotenoid cleavage dioxygenases (CCD7 and CCD8) (Alder *et al*., 2012), thus SLs belong to the apocarotenoids, as the phytohormone ABA (**Fig. 3**) (Walter *et al.*, 2011). Carlactone is converted into SLs by the action of a different cytochrome P450 (MAX1 and/or MAX1-like) (Zhang *et al*., 2014). SL perception and signaling require an α/β-hydrolase (D14) and a F-box leucine-rich repeat protein (MAX2) (**Fig. 3**) (Hamiaux *et al.*, 2012). SL binding to this complex induces the

degradation of the repressor D53 (Class I Clp ATPase) via the ubiquitin-proteasome, leading to the expression of SL-responsive genes (Zhou *et al.*, 2013).

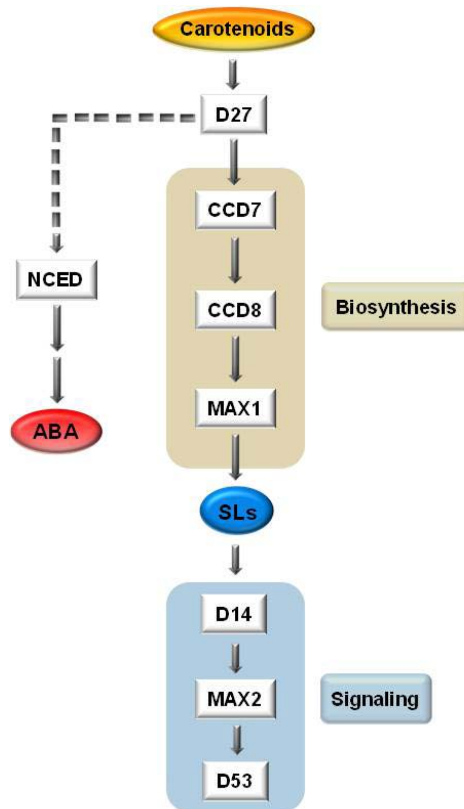

Figure 3. Schematic representation of the biosynthesis and signalling pathway of strigolactones. The dotted arrows indicate possible involvement on the biosynthesis. Enzymes: D27, β-carotene isomerase; CCD7, carotenoid cleavage dioxygenase 7; CCD8, carotenoid cleavage dioxygenase 8; MAX1, cytochrome P450; D14, α/β-hydrolase; MAX2, F-box protein; D53, Class I Clp ATPase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

Improvement of plant defense responses requires an extensive understanding of the regulation of the different hormonal signaling pathways involved, as well as of the interaction between them. In the present study, using tomato-*P. ramosa* as model system, the host response during the initial phase of parasitic infection, after seed germination, has been explored by monitoring the expression of marker genes for the main defense-related hormonal pathways and for the SL signaling pathway. Our results

suggest an involvement of the classical defense phytohormones JA, SA and ABA, and give insight into a new possible role of SLs during the early stages of interaction.

3. MATERIALS AND METHODS

3.1. Plant material

Tomato seeds (*Solanum lycopersicum*, 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 days on moistened filter paper at 25°C in darkness.

P. ramosa seeds (collected from a tomato field) were kindly provided by Maurizio Vurro and Angela Boari (Instituto di Scienze delle Produzioni Alimentari, Bari, Italy). The seeds were sterilized and pre-conditioned as previously described by López-Ráez *et al*., (2008). Upon pre-conditioning, they were treated with 10^{-10} M of the synthetic SL GR24 (Chiralix, Nijmegen, the Netherlands) and incubated for 3 days at 25°C in darkness and a wet environment to induce seed germination.

3.2. Plant growth and parasite infection

To study host plant responses during the initial phase of *P. ramosa*-tomato infection, an *in vitro* plastic-bag assay was carried out basically as described by Amsellem *et al*., (2001), but with some modifications. Briefly, the germinated seeds of tomato plants were placed within plastic-bags (245 x 315mm) containing a sheet of wet filter paper (Whatman International, Kent, UK). After 2 weeks, the shoots were pulled out of the

plastic-bags, maintaining the root system inside the bag (**Fig. 4**). After 3 weeks of growth, ~250 pre-germinated *P. ramosa* seeds per tomato plant were added along the roots randomly. The plastic-bags were sealed with adhesive tape and covered with a dark wrap. The tomato-*P. ramosa* system was disposed vertically in a greenhouse at 24/16°C with 16/8 h photoperiod and 70% humidity. Plants were watered twice a week with Long Ashton nutrient solution (Hewitt *et al.*, 1966), containing 25% of the standard phosphorous concentration.

Figure 4. *In vitro* plastic-bag tomato-*P. ramosa* system used in the present study. Picture courtesy of Dr. Maurizio Vurro (**B**).

Infected roots from tomato plants, containing about 5 tubercles per plant, were harvested at 2 different stages of the initial host-parasite plant interaction: early stage (young tubercles) and late stage (mature tubercles) (**Fig. 5**). Tubercles were carefully removed from collected samples and the roots were immediately frozen in liquid nitrogen and stored at -80°C. Non-infected roots from tomato plants from the same age were used as control. Four independent biological replicates per time point were analyzed and two independent infection experiments carried out.

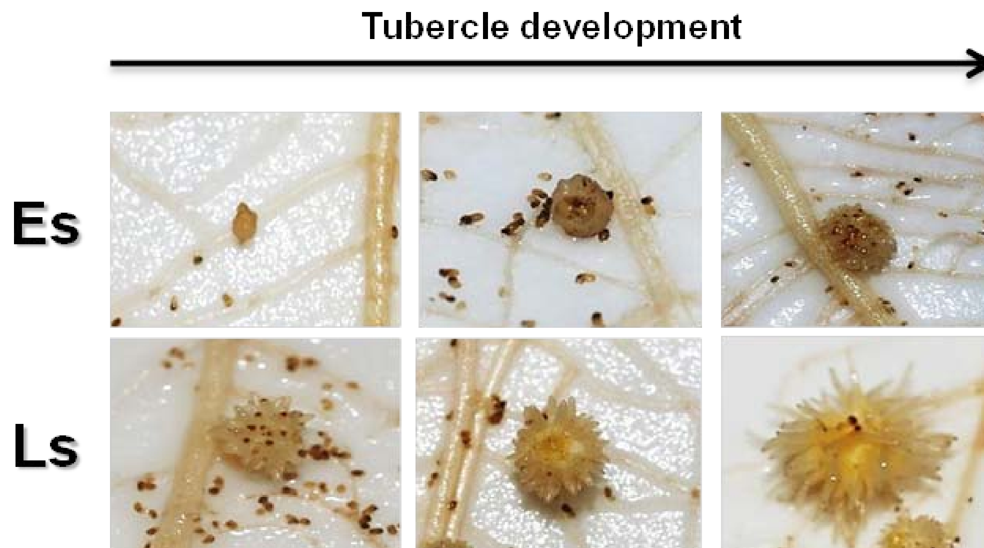

Figure 5. Close-up depicting the different stages of tomato-*P. ramosa* infection. Two different stages of infection are shown: early stage (Es) characterized by the formation of young tubercles (3-7 dai) and late stage (Ls), characterized by the formation of mature tubercles (7-15 dai).

3.3. RNA extraction and gene expression analysis by qRT-PCR

Total RNA from roots was extracted using Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA), purified through a silica column using the NucleoSpin RBA Clean-up Kit (Macherey-Nagel, Düren, Germany). Before storage at -80ºC, RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its integrity checked by gel electrophoresis. The first-strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The expression of marker genes for the different hormonal signaling pathways was analysed in non-infected and infected roots from tomato plants by real-time quantitative polymerase chain reaction (qRT-PCR). All qRT-PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ5 system (Bio-Rad),

using 1 µl of single stranded cDNA (diluted 1:10) and specific primers for each gene. The genes *LoxD*, *PinII*, *LoxA* (JA and oxilipin pathway); *PR1a* (SA); *LeNCED1* and *Le4* (ABA); *IAA11* (Auxin); *CRF5* (CK); *GAST1* (GA); and *SlD27*, *SlCCD7*, *SlCCD8*, *SlD14* (SLs) were used as marker specific genes (**Table S1**). The amplification protocol included an initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s. The specificity of the different amplicons was checked by a melting curve analysis (from 70 to 100°C) at the end of the amplification protocol. Four independent biological replicates were analyzed per treatment and time point, and each PCR reaction was done in triplicate. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak *et al*., 2001). Expression values were normalized using the housekeeping gene *SlEF-1α*, which encodes for the tomato elongation factor-1α.

3.4. Statistical analysis

Data from gene expression were subjected to one-way analysis of variance (ANOVA) using the software Statgraphics Centurion (version XVI) for Windows. Where appropriate, Fisher's LSD test was applied to determine the statistical significance.

4. RESULTS

4.1. Impact of *P. ramosa* infection during early interaction on defense-related phytohormones

In order to elucidate the host response during the initial phase of parasitic plant infection, the expression of marker genes from different defense-related hormonal signaling pathways was analyzed by real time qRT-PCR. Roots from tomato plants infected and non-infected by *P. ramosa in vitro* at two different stages of this initial infection phase were used. For the early stage, roots infected by young tubercles (~3-7 days after inoculation, dai) were harvested. For the late stage, roots infected by mature tubercles (~7-15 dai) were collected (**Fig. 5**). Similar expression patterns were obtained in an independent infection experiment (data not shown).

4.1.1. Effect on oxylipin- and JA-related genes.

The oxylipins are an important class of secondary metabolites generally associated to stress responses and innate immunity in plants (Eckardt, 2008; Wasternack *et al.*, 2013). The best-characterized oxylipin is JA, which is accumulated in response to various stresses, in particular to wounding and pathogen infection (Eckardt, 2008). The oxylipin biosynthetic pathway is determined by two different types of lipoxygenases (LOXs), 9-LOX and 13-LOX (Wasternack *et al.*, 2013). The 13-LOX pathway leads to the biosynthesis of JA and derivatives, known as jasmonates, while the 9-LOX pathway leads to the non-JA oxylipins. An important role of this 9-branch oxylipin pathway in the plant response to pathogens and pests was shown (Vellosillo *et al.,* 2007). The expression of marker genes for these two branches of the oxylipin pathway was

monitored. The expression of the gene *LoxD*, which encodes for a tomato 13-LOX (Heitz *et al.*, 1997), was significantly ($P < 0.05$) increased about 5-fold at the early stage of infection compared to non-infected control plants (**Fig. 6A**). No further induction of *LoxD* was detected at the later stage (**Fig. 6A**). In addition to a JA-biosynthesis gene, the expression of the JA-responsive gene *PinII*, encoding for a proteinase inhibitor highly induced by JA (El-Oirdi *et al.*, 2011; Wasternack *et al.*, 2013), was assessed. About 25-fold induction of *PinII* transcript levels was observed at the early stage (**Fig. 6B**). Increased expression levels of *PinII*, although to a lesser extent, were also detected at the late stage (**Fig. 6B**). Unlike for the 13-LOX pathway, no significant differences in gene expression at any of the stages analyzed were observed for the 9-LOX gene *LoxA* (**Fig. 6C**). Therefore, it seems that the 13-LOX is the mainly branch of the oxylipin pathway involved in the host-parasitic plant interaction, at least during the initial phase of infection.

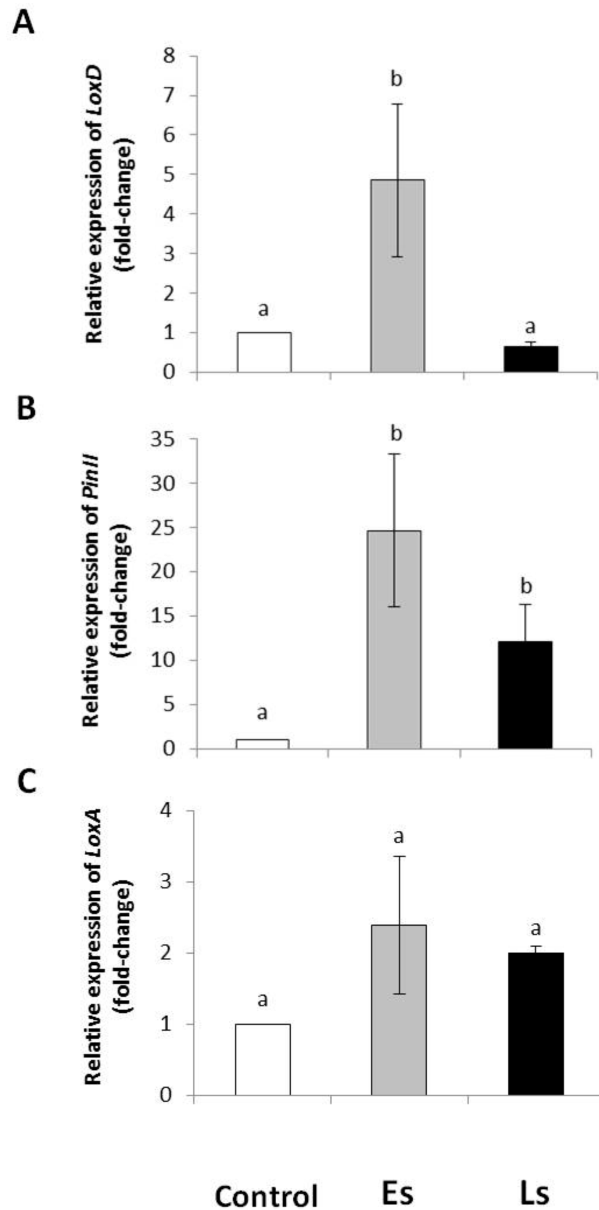

Figure 6. Effect of *P. ramosa* infection during early stages of interaction in marker genes associated to jasmonic acid (JA) and oxylipin signalling pathways. Gene expression analysis by real time qRT-PCR in tomato roots at the different stages of infection: Es (grey bars) and in Ls (closed bars) compared to non-infected plants (control). Expression of the JA biosynthesis gene *LoxD* (**A**), the JA-responsive gene *PinII* (**B**) and the 9-LOX oxylipin biosynthesis gene *LoxA* (**C**). Bars represent the means of four independent replicates (± SE). Bars with different letters are significantly different ($P < 0.05$) according to Fisher's least-significant difference

4.1.2. Expression of SA- and ABA-related genes

As mentioned, SA is the main phytohormone implicated in the defense against biotic stress. The induction of SA upon pathogen attack promotes the presence of the so-

called pathogenesis-related proteins (PR) (Ebrahim *et al*., 2011). The expression of the tomato gene *PR1a*, which encodes an acidic PR protein and is considered as a common marker of SA-regulated responses (Uknes *et al.*, 1993), was increased about 8 times during the early stage compared with non-infected control plants, while no differences were detected in more advance stages of the interaction (**Fig. 7A**).

Another important hormone that has emerged as a regulator of plant defense is ABA (Christmann *et al.*, 2006; Ton *et al.*, 2009). When the expression of the ABA biosynthesis gene *LeNCED1,* coding a 9-*cis*-epoxycarotenoid dioxygenase (**Fig. 3**) (Thompson *et al.*, 2000), was analyzed, about 4-fold increase was detected at the early stage (**Fig. 7B**). Although not significant, a slight induction of about 2-fold was also observed for this gene at the late stage (**Fig. 7B**). In addition to the biosynthesis gene, an increase of the ABA-responsive gene *Le4*, encoding for an ABA-inducible dehydrin (Kahn *et al*., 1993), was detected along the infection, with a maximum of about 10-fold induction at the late stage (**Fig. 7C**). The expression profiles suggest an increase of both SA and ABA during the initial phase of the host-parasite interaction.

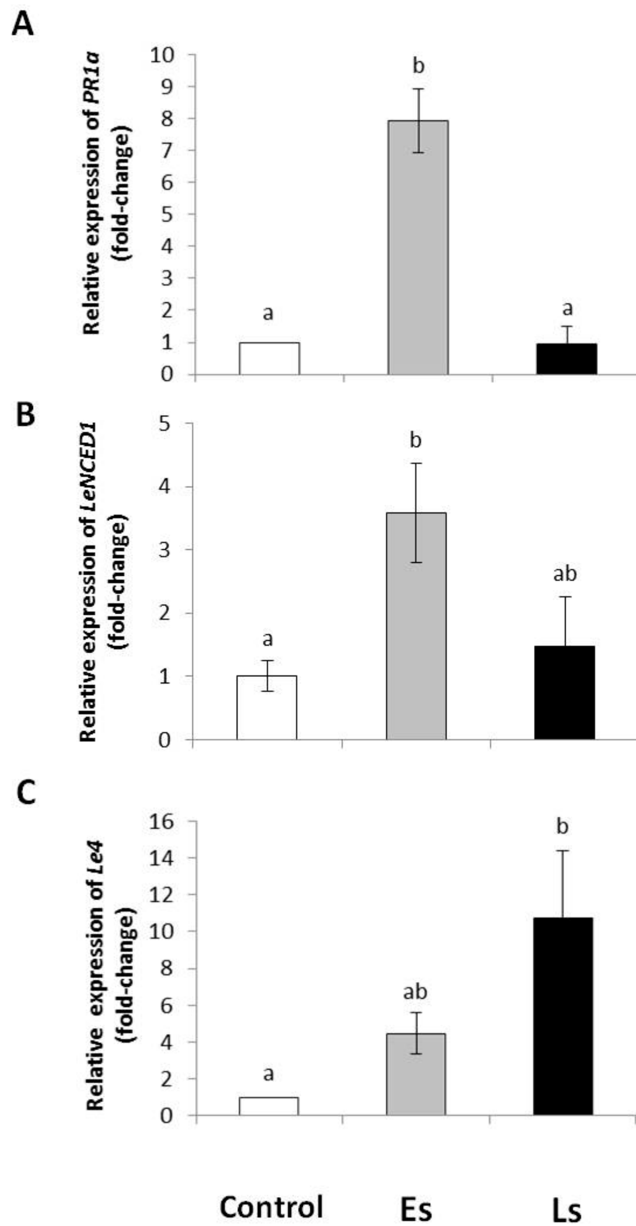

Figure 7. Effect of *P. ramosa* infection on the expression of marker genes associated to the salicylic acid (SA) and abscisic acid (ABA) signalling pathways. Expression levels of the SA-responsive gene *PR1a* (**A**), the ABA biosynthesis gene *LeNCED1* (**B**) and the ABA-responsive gene *Le4* (**C**). See legend of Figure 2.

4.2. Effect of *P. ramosa* infection markers in other hormonal pathways

In addition to the 'classical' defense hormones, other phytohormones traditionally considered as regulators of plant architecture such as auxins, CKs and GAs have been

also recently identified as potential regulators of plant defense responses (Robert-Seilaniantz *et al.*, 2011). To assess whether these hormonal pathways are affected upon infection by *P. ramosa*, the expression of the corresponding molecular markers was analyzed. The auxin-responsive gene *IAA11*, that acts as a transcriptional repressor of the auxin signaling pathway (Gupta *et al*., 2013); the transcription factor *CRF5*, a CK response factor (Shi *et al.*, 2012); and the GA-responsive gene *GAST1*, of unknown function (Serrani *et al.*, 2008), were checked. The expression of these marker genes was not altered upon the interaction tomato-*P. ramosa*, suggesting no involvement of these phytohormones in the process, at least at the early stages of the post-attachment interaction (**Fig. 8**).

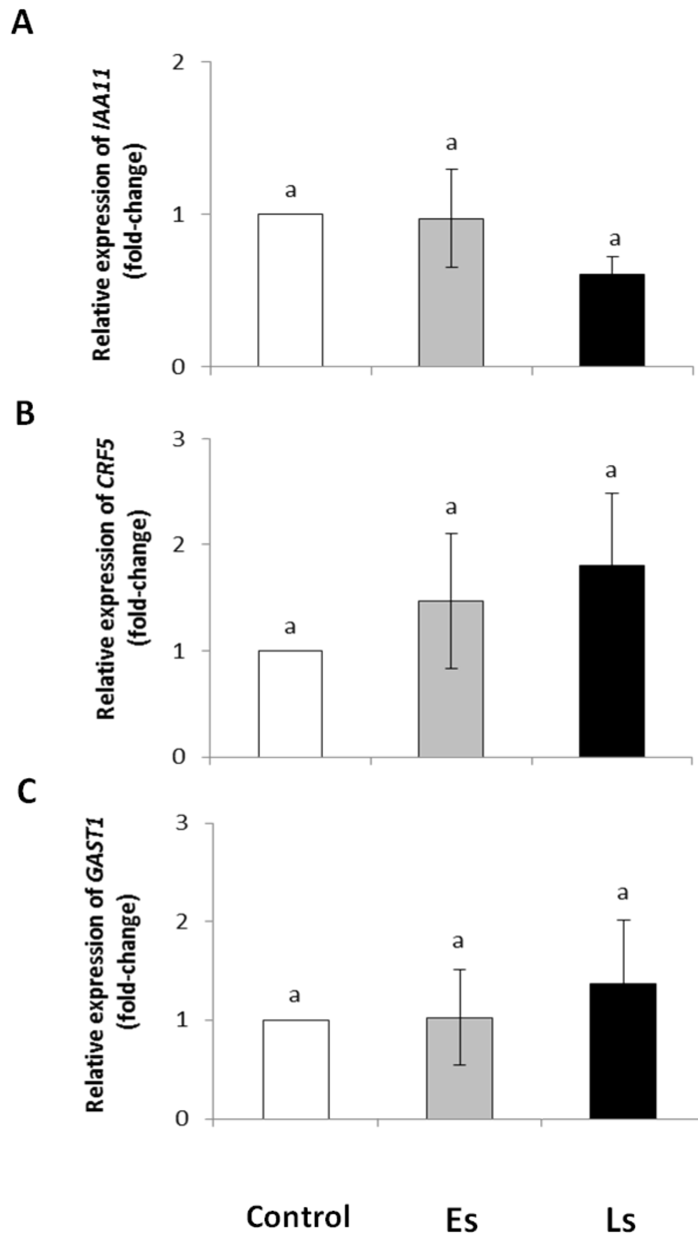

Figure 8. Gene expression analysis of genes related to auxin, cytokinins (CK) and gibberellic acid (GA) pathways. Expression analysis of the auxin-responsive gene *IAA11* (**A**), the CK-responsive gene *CRF5* (**B**) and the GA-responsive gene *GAST1* (**C**). See legend of Figure 2.

4.3. Impact of *P. ramosa* infection on the SL pathway

SLs are crucial cues in the pre-infection stage of host-root parasitic plant interactions by inducing parasite seed germination (Bouwmeester *et al*., 2007; Cook *et al*., 1972).

The potential involvement of SLs during the early stages of the interaction, after germination, was also investigated in our tomato -*P. ramosa* system. The expression of both SL biosynthesis and responsive genes (**Fig. 3**) was analyzed by qRT-PCR. The tomato orthologue to the β-carotene isomerase (D27) gene has not been characterized so far. The putative tomato sequence for *SlD27* was searched using BLAST against the D27 rice sequence. A sequence with an open reading frame (ORF) of 1018 bp (XM_004247285) was found. This ORF encodes a predicted 265 amino acid protein with 80, 76 and 67% homology to D27 proteins from *Medicago*, rice and Arabidopsis, respectively (**Fig. S1**). Specific primers for this sequence were designed (**Table S1**). The expression of *SlD27* was induced more than 6-fold at the early stage of infection compared with non-infected control plants (**Fig. 9A**). Although not significant, a slight induction of *SlD27* of about 3 times was also observed at the later stage (**Fig. 9A**). The transcript levels of the biosynthesis gene *SlCCD7* did not change at any of the stages studied (**Fig. 9B**). However, the expression of the other dioxygenase - *SlCCD8* - was increased over time, with a maximum of about 6-fold at the late stage (**Fig. 9C**). The transcript levels of the tomato SL-receptor D14 orthologue, used as a SL signaling marker, were also analyzed. As for *SlD27*, the corresponding tomato *SlD14* gene has not been characterized. The putative D14 orthologue sequence in tomato was searched by BLAST and specific primers designed. No changes in expression were observed for *SlD14* at the early stage (**Fig. 9D**). Conversely, a significant induction of more than 2 times was detected at the later stage (**Fig. 9D**). Thus, the SL biosynthetic pathway is activated during early interaction with root parasitic plants.

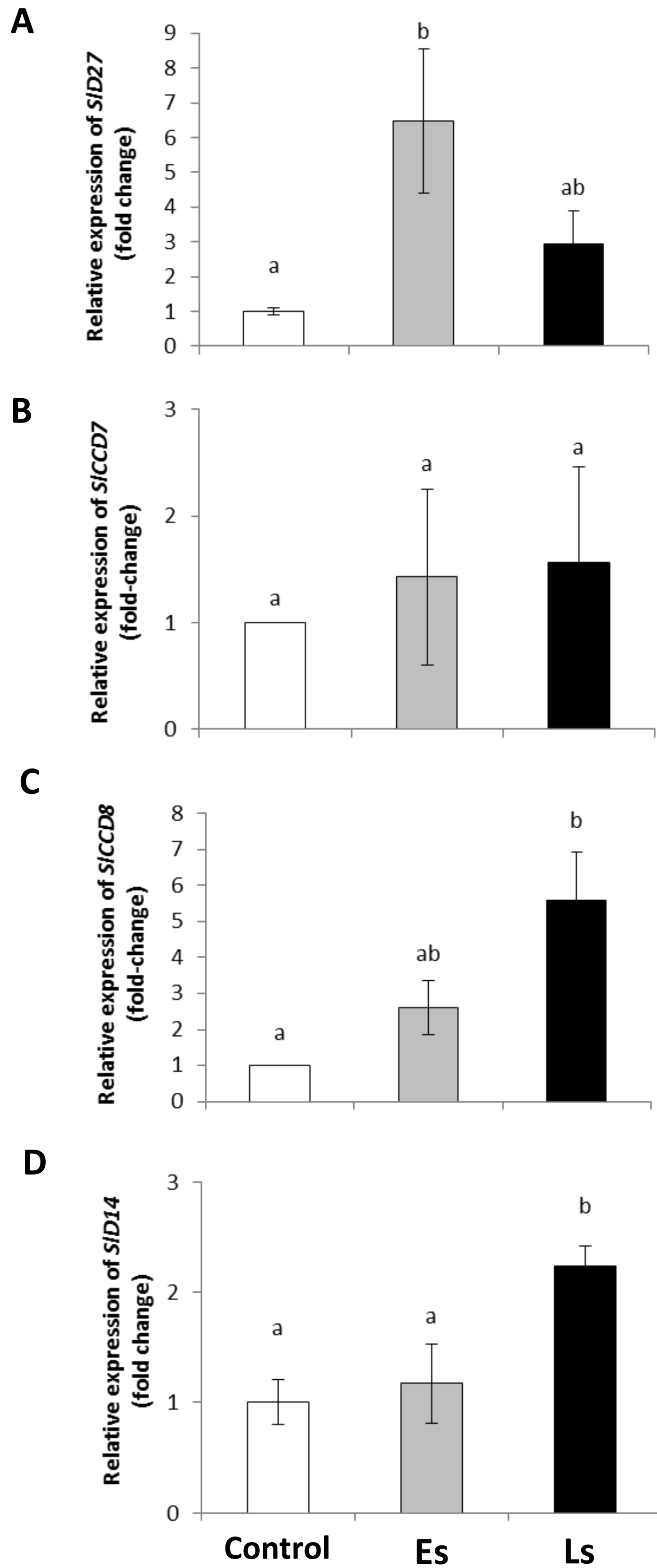

Figure 9. Effect of *P. ramosa* infection during early stages of interaction in marker genes associated to strigolactones (SLs). Expression of the biosynthesis genes *SlD27* (**A**), *SlCCD7* (**B**), *SlCCD8* (**C**) and the SL-receptor *SlD14* (**D**). See legend of Figure 2.

5. DISCUSSION

Root parasitic plants of the family *Orobancheaceae* are difficult to manage. Current strategies used to eradicate these parasitic weeds are not effective enough since most of their life cycle occurs belowground (Cardoso *et al*., 2011; Joel *et al*., 2013; López-Ráez *et al.*, 2009). Therefore, new strategies should be focused on eliminating/reducing the parasite during the early stages of the interaction. Here, we have used an *in vitro* tomato-*P. ramosa* system to study the local host response at the hormonal level during this initial post-attachment phase of parasitic infection by monitoring the expression of specific marker genes. An increase in the expression of both JA-biosynthesis (*LoxD*) and JA-responsive (*PinII*) genes was observed at the early stage of the interaction (3-7 dai). Moreover, in the case of *PinII*, this induction was maintained at the later stage (7-15 dai). Induction of JA-biosynthesis genes, including two LOXs and one allene oxide synthase (AOS), during this initial phase of induction was previously observed in the system Arabidopsis-*P. ramosa* (Vieira Dos Santos *et al.*, 2003). Likewise, using a microarray analysis an induction of *LOX* and JA-responsive genes was reported during the interaction Medicago-*Orobanche crenata* at 35 dai in the partially resistant genotype SA4087 (Dita *et al.*, 2009). In the association sunflower-*Orobanche cumana*, a stronger induction of JA-related marker genes was also shown in the sunflower resistant genotype LR1 compared with the susceptible one (Letousey *et al.*, 2007). An activation of the JA pathway has also been observed in the interaction of rice and sorghum with the hemiparasite *Striga hermonthica* (Hiraoka *et al*., 2008; Mutuku *et al.*, 2015; Swarbrick *et al*., 2008). Moreover, in the case of rice, Mutuku and co-workers have recently shown that the JA-deficient mutant *hebiba* was

more susceptible to *S. hermonthica* than the corresponding wild-type and that the resistant phenotype was recovered by JA application (Mutuku *et al.*, 2015). Altogether, the data indicate an involvement of the JA signaling pathway in the defense response of the host at the initial stages of host-parasitic plant interactions, as it happens in other plant-pathogen interactions such as fungi and bacteria.

A prominent role of the other classical defense hormonal pathway - SA - has been also proposed in the activation of resistance to root parasitic plants. Indeed, an increased expression of SA-related marker genes has been observed in several non-compatible host-parasite interactions and in resistant genotypes (Dita *et al*., 2009; Letousey *et al.*, 2007; Swarbrick *et al*., 2008). Moreover, it was reported in several plant species that the exogenous application of SA induced a reduction in the infection levels (Al-Wakeel *et al.*, 2012; Kusumoto *et al*., 2007; Sillero *et al.,* 2012). Accordingly, we have found an induction of the SA marker gene *PR1a* during the early interaction tomato-*P. ramosa*, confirming the involvement of SA in defense responses at these stages. Interestingly, the expression of *PR1a* decreased at a later stage, returning to the levels of the non-infected plants. Therefore, it seems that just an early peak of SA might be enough for the activation of the host defense responses against root parasitic weeds.

Conversely to JA and SA signaling pathways, even though an involvement of ABA in defense against biotic stresses was proposed (Christmann *et al.*, 2006; Ton *et al*., 2009), its role in host-root parasitic plant interaction has been less explored. Here, an increase in the expression of both ABA biosynthesis (*LeNCED1*) and responsive (*Le4*) genes has been observed from the beginning of the interaction, indicating a possible increase of this phytohormone and suggesting its involvement in early defense

responses in host-root parasite associations at this stage. In agreement with this hypothesis, it was shown that the expression of an ABA response element binding factor was promoted during the compatible interaction between rice and *S. hermonthica* (Swarbrick *et al.*, 2008). Moreover, the induction of the ABA-responsive gene *Hadef1*, encoding for a plant defensin, was reported in a resistant sunflower genotype upon *O. cumana* infection (De Zélicourt et al., 2007; Letousey et al., 2007). Also in pea and *M. truncatula*, proteomic analyses showed a higher proportion of ABA-responsive proteins in an *O. crenata* resistant genotype (Castillejo *et al.*, 2004, 2009). Therefore, it seems clear that ABA is associated with defense responses during the host-root parasite interactions as are JA and SA, although its exact role as well as its interaction with the other defense-related phytohormones need to be further investigated.

SLs are multifunctional molecules classified as a new class of plant hormones having a crucial role in the pre-infection phase of the host-root parasitic weed interaction (Andreo-Jiménez *et al.*, 2015; Bouwmeester *et al.*, 2007; Ruyter-Spira *et al.*, 2013). Surprisingly, very little or nothing is known beyond its function as germination stimulants in this parasitic association. In the present study, we have investigated whether SLs are regulated during the post-attachment stage. Interestingly, the expression of the biosynthesis genes *SlD27* and *SlCCD8* was induced during the early interaction (Fig. 8). Moreover, an increase in the gene encoding for the SL receptor D14 was observed at the later stage (7-15 dai), suggesting a promotion of SLs' biosynthesis and signaling during the more advanced stages of the infection. Recently, a role of SLs in defense responses has been proposed (Torres-Vera *et al.*, 2014). It was shown that the SL-deficient tomato antisense line *Slccd8* was more susceptible to the

foliar necrotrophic fungi *Botrytis cinerea* and *Alternaria alternata* than the corresponding wild-type, which was associated with a reduction in the levels of JA (Torres-Vera *et al.*, 2014). Since the JA pathway is involved in defense against root parasitic weeds, it could be that SLs are related to defense responses against these root parasites through their interaction with JA, although this possibility should be further studied. In addition to JA, an interaction between SLs and ABA has been proposed (Aroca *et al*., 2013; Liu *et al.*, 2015; Ruiz-Lozano *et al.*, 2016). Accordingly, a reduction in ABA levels was also detected in the antisense line *Slccd8* (Torres-Vera *et al.*, 2014). Here, we show that the expression pattern of the ABA-responsive gene *Le4* correlated with that of *SlCCD8*, supporting the ABA-SLs interaction. As ABA signaling seems to be involved in defense against root parasitic plants, this could be mediated by its interaction with SLs.

On the other hand, increased levels of SLs during the early stages of AM symbiosis establishment, where SLs are also important cues, have been also shown (Aroca *et al*., 2013; Liu *et al.*, 2015; Ruiz-Lozano *et al.*, 2016). In this case, it was proposed that the host plant is sensing the presence of the AM fungus, probably through the production of fungal effectors (Kloppholz *et al.*, 2011), and promotes the production of SLs to improve mycorrhizal colonization. A similar strategy has been shown for certain microbial pathogens such as *Pseudomonas syringae* and *B. cinerea* to 'deceive' the host and reduce the defense responses (Laurie-Berry *et al.*, 2006; Weiberg *et al.*, 2013). In a similar way, it might be that the parasitic weeds produced effectors to promote SL production by the host, thus inducing a higher parasitic seed germination and infection. So far, these putative parasite effectors have not been characterized.

6. CONCLUSIONS

Unravelling the study of plant defense mechanisms against root parasitic plants is definitely exciting and promising. The results presented in this study suggest that the three major defense hormonal pathways - JA, SA and ABA - are necessary to regulate the defense response during the initial phase of infection of tomato plants by *P. ramosa*, although further analytical studies are needed to confirm this fact. In addition, a new role of SLs in defense, probably through its interaction with the other defense phytohormones, in the regulatory network controlling root parasitic plants is envisaged, opening up a new area of SL research. However, further investigation is needed to decipher the mechanisms behind this new function and for the development of new biotechnological approaches against these parasitic weeds.

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SUPLEMENTARY INFORMATION

ID	Gene	Pathway	Primers (5'-3')
U37840	Lipoxygenase D (LoxD)[1]	JA	GACTGGTCCAAGTTCACGATCC ATGTGCTGCCAATATAAATGGTTCC
K03291	Proteinase inhibitor II (PinII)[1]	JA	GAAAATCGTTAATTTATCCCAC ACATACAAAACTTTCCATCTTTA
U09026	Lipoxygenase A (LoxA)[2]	Oxylipins	GGTTACCTCCCAAATCGTCC TGTTTGTAACTGCGCTGTG
M69247	Pathogenesis related protein (PR1a)[2]	SA	ATGTGTGTGTTGGGGTTGGT ACTTTGGCACATCCAAGACG
Z97215	9-*cis*-epoxycarotenoid 1 (NCED1)[2]	ABA	ACCCACGAGTCCAGATTTC GGTTCAAAAAGAGGGTTAGC
X51904	Le4[2]	ABA	ACTCAAGGCATGGGTACTGG CCTTCTTTCTCCTCCCACCT
Solyc03g120380	Auxin response factor 9 (IAA11)[3]	Auxin	GCAGCAGCAACAACAACAAC CAAAAACGGTCGTCCATCCA
Solyc01g095500	Cytokinin response factor 5 (CRF5)[4]	Cytokinins	ACGATGACGACGAGAGGAAT CTGACACCGCAAACTTTTT
X63093	GA-responsive gene (GAST1)[5]	Gibberellins	CAACAACAGAGAAAATAACCAAC TTATACGATGTCTTTGAACACC
SGN-U577652	β-carotene isomerase (SlD27)[6]	SLs	TTGGCTAGTTGGACCTTGTG CAAAGTTTGGCACCATATTCA
GQ468556	Carotenoid cleavage dioxygenase 7 (SlCCD7)[7]	SLs	AGCCAAGAATTCGAGATCCC GGAGAAAGCCCACATACTGC
JF831532	Carotenoid cleavage dioxygenase 8 (SlCCD8)[7]	SLs	CCAATTGCCTGTAATAGTTCC GCCTTCAACGACGAGTTCTC
XM_004238045	SL-receptor (SlD14)[6]	SLs	GACATTTGCCACATCTTAGC TTTTGGTTTGGTTGACGC
X14449	Elongation factor 1 (SlEF-1α)[8]	Housekeeping	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC

[1]Uppalapati *et al.*, 2005; [2]López-Ráez *et al.*, 2010; [3]Gupta *et al.*, 2013; [4]Shi *et al.*, 2012; [5]Serrani *et al.*, 2008; [6]This work; [7]Kohlen *et al.*, 2012; [8]Rotenberg *et al.*, 2006

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

```
SlD27 -------------------------MEANLVLSYQSFSKVSMRNIKINTYYKYNKLAYPS
MtD27 -------------------------MDSKMIAHNMSLTPTLAQWKKLRLKPKHTFVVG--
OsD27 METTTLVLLLPHGGAGGVRPAAAATAKRSYVMRRCCSTVRAVMARPQEAPASAPAKKTET
AtD27 -------------------------MNTKLSLSQTKIFTFTTWFNDTRSGLDRRSSISPT
                                . .                  .   .

SlD27 TCCVLTKPPHNNHSSNCH----------VYKDNWFDHLAINHLSQSIQATTGLRN--KKS
MtD27 VLARPTDDISEETLRKTN----------VYKDNWFDKLAINHLSKSVQAATGISN--NKS
OsD27 AAMMSTVQTETAAAPPAT----------VYRDSWFDKLAIGYLSRNLQEASGLKN--EKD
AtD27 LCSKPVYSGKLKAAKETARIETSNTKNASIEDSFFSKIAINYLSKNLQDAAGISSSSKST
           .                        .*.:*.::**.:**:.:* ::*: .  :.

SlD27 GYEGFVEAARVVYVNFNSTHQANLVIESLQRAFPKPILSLVKMLLPESKWAREYCAIFTT
MtD27 GFDSLVEAATVASQKFNTTQQQGIILDALDRAFPKPILSVIRRVMPPSKLAREYFAVFTT
OsD27 GYESLIDAALAISRIFSLDKQSEIVTQALERALPSYILTMIKVMMPPSRFSREYFAAFTT
AtD27 DYDRLVDTATRVSRNFDTKQQHEFVLSSLDRALPTVISSLIKMAFPPSKVSRELFALFTT
      .:: ::::*      *.  :*  :: .:*:**:*. * ::::  :* *: :**  * ***

SlD27 IFFPWLVGPCEVKESEFNGRKENNVVHIKKCRFLEETNCVGMCTNLCKMPSQLFIKDTLG
MtD27 IFFAWLLGPSEVRESEINGRREKNIVHIKKCRFLEETNCVGMCINLCKMPSQLFIKDSFG
OsD27 IFFPWLVGPCEVMESEVEGRKEKNVVYIPKCRFLESTNCVGMCTNLCKIPCQKFIQDSLG
AtD27 ISFAWLVGPSEVRETEVNGRKEKSVVYIEKCRLSVIS-----------------------
      * *.**:**.** *:*.:**:*:.:*:* ***:   :

SlD27 ISVNMVPNFDDMSCEMIFGQDPPPLDKDPAFRQPCYK-LCKLNNKHQADCNSQIKKKDHL
MtD27 MPVNMVPNFDDMSCEMIFGQEPPASTDDPALKQPCYK-LCKAKKNHATQCLS--------
OsD27 MKVYMSPNFEDMSCEMIFGQQPP--EDDPALKQPCFRTKCVAKQNHGVNCSI--------
AtD27 ---LFGPTFDH-------------------------------------------------
          : *.*:.

SlD27 EIS
MtD27 ---
OsD27 ---
```

Figure S2. Alignment of the putative β-carotene isomerase SlD27 amino acid sequence from tomato with those from known D27 proteins: Mt, *Medicago truncatula*; Os, *Oryza sativa*; At, *Arabidopsis thaliana*. Protein alignment was carried out using the program ClustalW. Asterisks indicate amino acids conserved in all four genotypes. Amino acid conserved in three or two plant species are indicated by two or one dot, respectively.

CAPÍTULO 2

¿Contribuyen las estrigolactonas a la regulación de las defensas en planta?

Torres-Vera R, García JM, Pozo MJ, López-Ráez JA

RESUMEN

Las estrigolactonas son moléculas multifuncionales involucradas en diversos procesos tanto fuera como dentro de la planta. Como moléculas señal en la rizosfera, favorecen el establecimiento de la simbiosis micorrícica arbuscular, pero además, también actúan como señal de detección del hospedador para las plantas parásitas de raíz. Como fitohormonas, están involucradas en la regulación de la arquitectura de la planta, desarrollo de raíces adventicias, crecimiento secundario y desarrollo reproductivo, aunque se están descubriendo nuevas funciones continuamente.

En el presente estudio se investigó la posible implicación de las estrigolactonas en la regulación de las respuestas de defensa de la planta. Con este propósito se evaluó la resistencia/susceptibilidad frente a diferentes patógenos fúngicos de diferentes plantas de tomate y Arabidopsis deficientes en la producción de estrigolactonas. Las plantas de tomate *Slccd8* fueron más susceptibles que su silvestre a la infección con los patógenos fúngicos foliares *Botrytis cinerea* y *Alternaria alternata*. El mismo resultado se observó en plantas de tomate *Slccd7*, siendo esta más susceptible que el silvestre a *B. cinerea*. Los mutantes de Arabidopsis *max4-1* eran también menos resistentes a *B. cinerea* que su correspondiente silvestre, confirmando el resultado obtenido en las plantas de tomate. La línea *Slccd8* fue también infectado con el hongo patógeno de suelo *Fusarium oxysporum,* siendo la línea mutante de nuevo más susceptible a este patógeno que su silvestre. El análisis del contenido en fitohormonas mediante HPLC-

MS/MS mostró una reducción en el contenido de las principales hormonas relacionadas con defensa, el ácido jasmónico, ácido salicílico y ácido abscísico en plantas *Slccd8*, sugiriendo que las homeostasis de las hormonas están alteradas en estas plantas. Además, los niveles de expresión del gen marcador de respuestas reguladas por jasmonato *PinII*, involucrado en la resistencia de tomate frente a *B. cinerea*, estaban reducidos en el mutante. De acuerdo a estos resultados, proponemos que las estrigolactonas juegan un papel en la regulación de la defensa de las planta a través de su interacción con otras hormonas relacionadas con la regulación de respuestas defensivas, especialmente con la vía dependiente del ácido jasmónico.

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Do strigolactones contribute to plant defence?

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1. ABSTRACT

Strigolactones are multifunctional molecules involved in several processes outside and within the plant. As signalling molecules in the rhizosphere, they favour the establishment of arbuscular mycorrhizal symbiosis, but they also act as host detection cues for root parasitic plants. As phytohormones, they are involved in the regulation of plant architecture, adventitious rooting, secondary growth and reproductive development, and novel roles are emerging continuously. In the present study, the possible involvement of strigolactones in plant defence responses was investigated. For this purpose, the resistance/susceptibility of different tomato and Arabidopsis strigolactone-deficient plants against different fungal pathogens was assessed. Tomato plants *Slccd8* were infected with the foliar fungal pathogens *Botrytis cinerea* and *Alternaria alternata*, being more susceptible than corresponding wild-type. A similar result was obtained for the tomato line *Slccd7,* being more susceptible to *B. cinerea*. Arabidopsis mutant *max4-1* was also less resistant to *B. cinerea* than wild-type plants, confirming the results from tomato plants. *Slccd8* plants were also infected with the soil fungal pathogen *Fusarium oxysporum,* being again more susceptible to this pathogen than the wild-type. A reduction in the content of the defence-related

hormones jasmonic acid, salicylic acid and abscisic acid was detected by HPLC-MS/MS in the *Slccd8* plants, suggesting that hormone homeostasis is altered. Moreover, the expression level of the jasmonate-dependent gene *PinII*, involved in the resistance of tomato to *B. cinerea*, was lower in *Slccd8* than in the corresponding wild-type. Accordingly, we propose that strigolactones play a role in the regulation of plant defences through their interaction with other defence-related hormones, especially with the jasmonic acid signalling pathway.

Keywords: Arabidopsis, defence responses, fungal pathogens, phytohormones, strigolactones (SLs) and tomato.

2. INTRODUCTION

Strigolactones (SLs) are plant hormones that were initially identified as signalling molecules in the rhizosphere. They are mainly produced in the roots and have been detected in the root exudates of a wide range of monocot and dicot plant species, which suggests their importance in nature (Xie *et al*., 2010). In the rhizosphere, SLs act as host detection cues for symbiotic arbuscular mycorrhizal fungi and root parasitic plants of the Orobanchaceae (reviewed in López-Ráez *et al*., 2011). They are derived from the carotenoids through sequential oxidative cleavage by *c*arotenoid *c*leavage *d*ioxygenases (CCD7 and CCD8) (Gomez-Roldan *et al*., 2008; López-Ráez *et al*., 2008; Matusova *et al*., 2005; Umehara *et al*., 2008), thus belonging to the apocarotenoids class, which also includes the plant hormone abscisic acid (ABA) (López-Ráez *et al*., 2010a).

As phytohormones, it has been suggested that SLs play a pivotal role in plants as modulators of the coordinated development of roots and shoots in response to nutrient deficient conditions (Koltai and Kapulnik, 2011). Accordingly, their biosynthesis and exudation into the rhizosphere is induced under such adverse nutritional conditions (López-Ráez *et al*., 2008; Yoneyama *et al*., 2007). They were shown to regulate above-ground architecture by the inhibition of lateral shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Since then, extensive efforts have been devoted to examine the novel roles and functions of SLs in plant physiology. Thus, it has been shown that they stimulate secondary stem growth, acting as positive regulators of cambial activity (Agusti *et al*., 2011). They also regulate below-ground plant architecture by affecting primary root length, lateral root initiation and root hair development (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011). More recently, it has been shown that they repress adventitious rooting and positively affect reproductive development (Kohlen *et al*., 2012; Rasmussen *et al*., 2012). In all of these functions, a cross-talk between SLs and other signalling pathways regulated by phytohormones such as auxin, ethylene (ET) and ABA, seems to play a prominent role (reviewed in Kohlen *et al*., 2011). Novel functions for SLs are emerging at a fast pace, broadening our understanding of their relevance in plant physiology. However, no relationship with defence responses has been addressed so far.

Plants are exposed to ever-changing and often unfavourable environmental conditions, which cause both abiotic and biotic stresses. Consequently, plants have evolved sophisticated mechanisms to flexibly adapt themselves to overcome such stress conditions. Most of these mechanisms—if not all—are regulated by plant

hormones (Robert-Seilaniantz *et al*., 2011). In recent years, significant progress has been made in the identification of the key components in plant responses to biotic stress. The phytohormones salicylic acid (SA), jasmonic acid (JA) and derivatives—known as jasmonates (JAs)–and ABA are the major players in the signalling networks regulating plant defence responses against pathogens and pests, although others such as ET, brassinosteroids, gibberellins and auxins, are now known to contribute to modulate these responses (Robert-Seilaniantz *et al*., 2011; Ton *et al*., 2009).

In the Chapter 1 of this Thesis, we observed that in tomato plants infected by the parasitic plant *P. ramosa* the defence responses depend mainly on the JA signalling pathway (Torres-Vera *et al*., 2016). In addition, an induction of the expression levels of the SL biosynthesis gene *SlCCD8* during the initial phase of the infection was detected, suggesting a possible contribution of SLs in defence regulation (Torres-Vera *et al.*, 2016). In the present study, the potential involvement of SLs in plant defence is investigated. For that, the resistance/susceptibility of different tomato and Arabidopsis SL-deficient plants was assayed against different foliar and soil fungal pathogens.

3. MATERIALS AND METHODS

3.1. Plant and fungal material, and growth conditions

Tomato (*Solanum lycopersicum* L.) plants of the SL-deficient transgenic lines *Slccd7* (6931, 6936 and 7170), *Slccd8* (L9), and their wild-type (wt) cv. M82, cv. Craigella (LA3247) respectively. *Slccd7* seeds were kindly provided by Dr.Walter (Leibniz Institute of Plant Biochemistry, Ger). Both *Slccd7* and *SLccd8* are antisense CCD7 and

CCD8 RNA interference (RNAi) lines, respectively (Vogel *et al*., 2010; Kohlen *et al*., 2012). Tomato seeds 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 ˚C in darkness. Subsequently, individual seedlings were transferred to 0.25 l pots with a sterile sand:soil:vermiculite (3:1:1) mixture. Plants were randomly distributed and grown in a greenhouse at 24/16 °C 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 4 weeks of growth and used the leaves for infection assays. The rest of leaves were stored at –80 °C until use for hormonal quantification and gene expression analysis.

Seeds of the SL-deficient Arabidopsis mutant *max4-1* (with a mutation in the biosynthetic gene *AtCCD8*) (Sorefan *et al*., 2003) and the corresponding wt cv Columbia (Col-0) were kindly provided by Prof. Leyser (University of York, UK). Arabidopsis seeds were surface sterilized applying the technique described above. Then, individual seedlings were transferred to 4-cm square compartments with a sterile commercial peat moss-based mix soil (Compo, Spain). Plants were randomly distributed and grown in a chamber at 21/16 °C with a 16/8 h photoperiod and 70% humidity. Arabidopsis plants were watered and harvested as described above.

The necrotrophic fungi *Botrytis cinerea* CECT2100 (Spanish Collection of type cultures, Universidad de Valencia) was kindly provided by Dr. Flors (Universidad Jaume I, Spain) and *Alternaria alternata* (isolated from an infected tomato field) were used in a detached leaf assay. *Fusarium oxysporum f. sp. radicis lycopersici* (*Fol*) ZUM 2407 (JPO-

DLO, Wageningen, The Netherlands) was kindly provided by Dr. Jaizme-Vega (Instituto Canario de Investigaciones Agrarias, Spain).

3.2. Infection assays with fungal pathogens

Bioassays with *B. cinerea* and *A. alternata* were performed as described by Vicedo *et al.* (2006). Conidia were collected from 10-15 day-old PDA plates supplemented with 20% of lyophilized tomato leaves. Suspensions of spores were incubated for 2h in the dark in Gamborg's B5 medium (Gamborg *et al*., 1968) supplemented with 10mM sucrose and 10 mM KH_2PO_4. Detached leaves from 4 week-old *Slccd8* plants were treated by applying 5 µl of a suspension containing 10^6 spores ml^{-1}. Leaves from tomato *Slccd7*, *Slccd8* and Arabidopsis *max4-1* and corresponding wild-types were also infected by inoculation with 5-mm-diameter PDA plugs containing actively growing *B. cinerea* hyphae (1 week old). The leaves were maintained at 20 °C and 100% relative humidity. Fungal hyphae grew concentrically, resulting in visible necrosis 48 h after inoculation. Fungal spreading was measured daily. A disease index from 0 (healthy leaf) to 3 (much damaged leaf) was established for infection quantification.

The assays with *Fol* were carried out mainly as described by López-Berges *et al*. (2009). Briefly, the fungus was grown for 7-10 d in PDA plates, and an inoculum containing spores and hyphae was obtained. One week-old tomato seedlings from *Slccd8* and wt plants were inoculated with *Fol* by cutting and immersing the roots in 20 ml of a solution containing the inoculum in sterilized demiwater. For control-treatment the roots were cutted and immersed in sterilized demiwater. Seedlings were replanted in the pots and grown in a greenhouse for 4 weeks. The number of dead plants was

recorded weekly. Ten independent biological replicates per genotype and per pathogen treatment were analysed.

3.3. *In vitro* assay with *B. cinerea* and GR24

Five mm-diameter PDA plugs containing actively growing *B. cinerea* hyphae were applied to the middle of a Petri dish with PDA medium. Six different concentrations of GR24 (Chiralix) were tested, 0, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. Four sterilized 5 mm-diameter fibreglass discs were applied to each plate, and 10 µL of the different GR24 solutions were added to each disc. Plates were incubated at 25 °C and high humidity for 5 d. Ten individual plates were used.

3.4. Hormone quantification

The JA precursor 12-oxo phytodienoic acid (OPDA), JA, ABA and SA were analysed by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) as described by Flors *et al*. 2008. Five independent biological replicates per genotype and a 100 mg aliquot of dry leaves tissue per sample were used. Quantifications were carried out with MassLynx 4.1 software (Waters) using internal standards as a reference for extraction recovery and the standard curves as quantifiers. Hormone analysis was conducted at the Scientific Instrumentation Service at the Estación Experimental del Zaidín-CSIC (Spain).

3.5. RNA extraction and gene expression analysis by qRT-PCR

The expression of marker genes for the different signalling pathways regulated by JA, SA and ABA was analysed in the leaves of *Slccd8* and wt from non-inoculated plants by real-time quantitative polymerase chain reaction (qRT-PCR). The qRT-PCR was conducted using the iCycler iQ5 system (Bio-Rad, Hercules, CA, USA) and specific primers for each gene **(Table S1).** The genes *PinII*, *PR1a* and *Le4* were used as markers for JA, SA and ABA, respectively. Total RNA from leaves was extracted using Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA), purified through a silica column using the NucleoSpin RBA Clean-up Kit (Macherey-Nagel, Düren, Germany) and stored at −80 °C until use. 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. Six independent biological replicates were analysed per genotype. Relative quantification of specific mRNA levels was performed using the comparative 2–Δ(Δ*Ct*) method (Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene *SlEF*, which encodes for the tomato elongation factor-1α.

3.6. Statistical analysis

Data for pathogen infection, hormone and gene expression quantification were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 20 for Windows. When appropriate, Fisher's LSD test was applied to determine the statistical significance.

4. RESULTS

4.1. Susceptibility of the SL-deficient tomato plants *Slccd8* and *Slccd7* to *B. cinerea*

Kohlen and co-workers generated the *Slccd8* knock-down line L09, which exhibited a decrease in SLs production of 95% when compared to the wild type line (Kohlen *et al*., 2012). Leaves from L09 plants and wt were infected with the pathogen *B. cinerea,* a generalist necrotrophic fungus that affect plant leaves from multiple plants. Two different experiments were carried out using L09 plants, one inoculating the pathogen with agar plugs containing active growing hyphae and another with isolated *B. cinerea* spores. After inoculation, the disease symptoms were measured at different time points. *Slccd8* showed more severe symptoms of *B. cinerea* infection than the corresponding wt in both experiments (**Fig. 1**). In the assay with agar plugs, the leaves from *Slccd8* plants presented a ~40% more of infection than its wt 5 days after inoculation (dai). In fact, heavily infested leaves were only observed in *Slccd8* at 5 dai (**Fig. 1A**). This increased susceptibility in *Slccd8* was maintained over time. At 9 dai, 41% of the leaves from *Slccd8* were heavily infested (disease index 3), whereas only 15% of wt leaves reached this lesion diameter (**Fig. 1A**). The same disease pattern against *B. cinerea* was observed when leaves were inoculated with isolates spores (**Fig 1B**): *Slccd8* plants presented more infection than the wt over time. At 8 dai, *Slccd8* plants showed a 25% increase of leaves having level 3 symptoms in relation to the wt (**Fig. 1B**). Therefore, the results clearly showed that the SL-deficient tomato line *Slccd8* is more susceptible to *Botrytis cinerea,* regardless of the inoculum source.

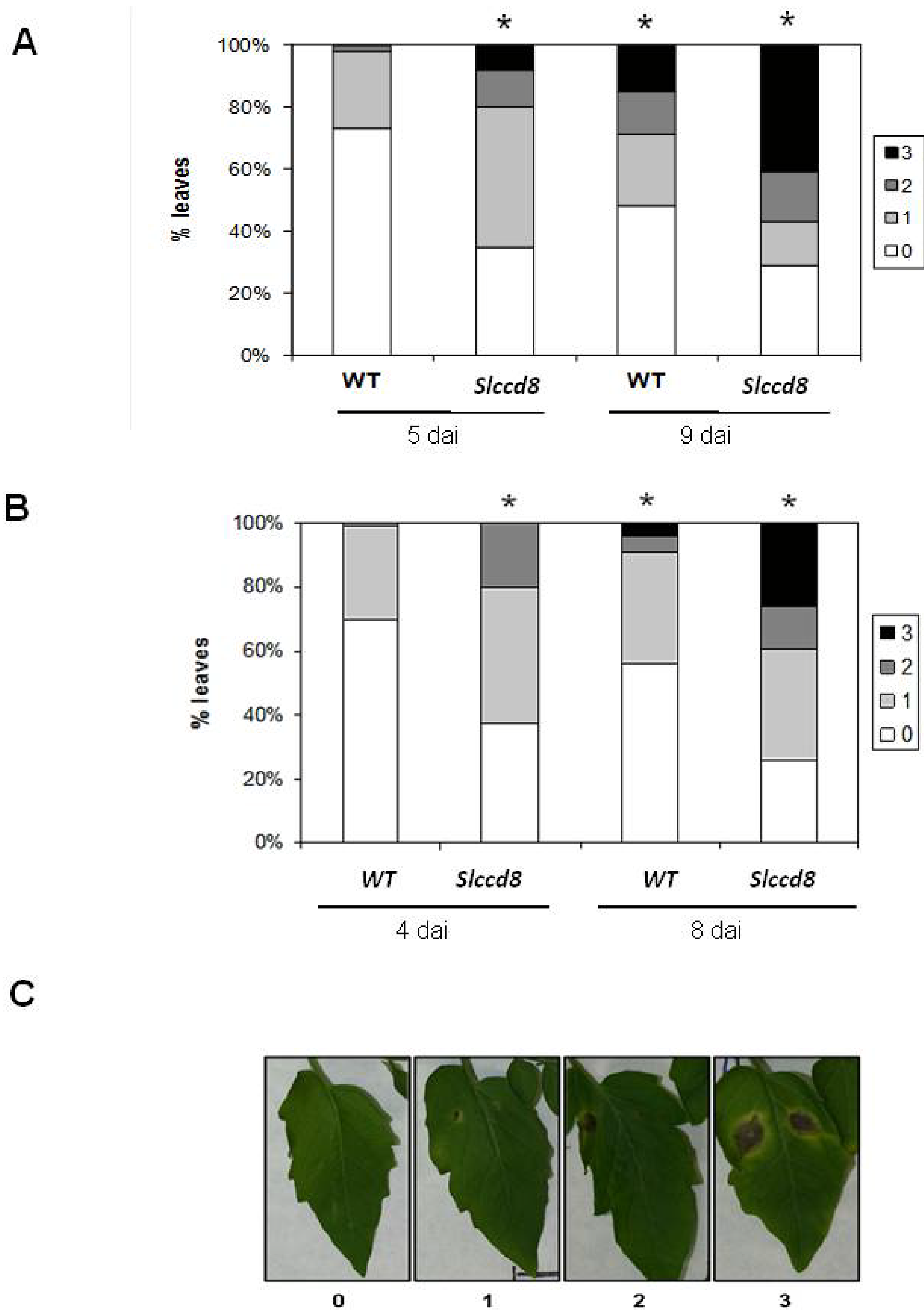


Fig 1. Effect of *Botrytis cinerea* inoculation on the SL-deficient tomato plants *Slccd8*. Evaluation of fungal spreading in leaves of *Slccd8* and the corresponding wild-type (WT) at 5 and 9 days after inoculation (dai) with 5-mm-diameter plugs containing active growing hyphae (**A**), or at 4 and 8 dai by applying 5 µl of a suspension containing 10^6 spores ml^{-1} (**B**). *B. cinerea* disease symptoms in tomato leaves were evaluated following the disease index illustrated in (**C**). Asterisks (*) indicate statistically significant different distributions ($P < 0.01$) of the disease severity classes according to Fisher's least-significant difference (LSD) test. Ten independent biological replicates per genotype were used.

Vogel and co-workers studied different lines altered in the expression levels of and quantified SL levels. Lines 6936 and 7170 presented about 90% reduction in SL levels, whereas an 80% reduction was detected in line 6931 (Vogel *et al*., 2010). We conducted an assay with these 3 different *Slccd7* transgenic lines inoculating *B. cinerea* with PDA plugs containing actively growing hyphae. Leaf damage was measured and the infection rate determined at 2 and 3 dai (**Fig. 2**). At both time points, the SL-deficient lines were more susceptible to *B. cinerea* than the corresponding wt. At 2 dai, the transgenic lines 6931, 6936 and 7170 presented percentages of infected leaves of ~40, 60 and 70% respectively, being these percentages inversely correlated to the SL levels in the lines (**Fig. 2A**). The same pattern was observed at 3 dai, where ~30, 50 and 40%, respectively were heavily infected (disease index 3). At this time point, only 10% of wt leaves reached this level of infection (**Fig. 2A**).

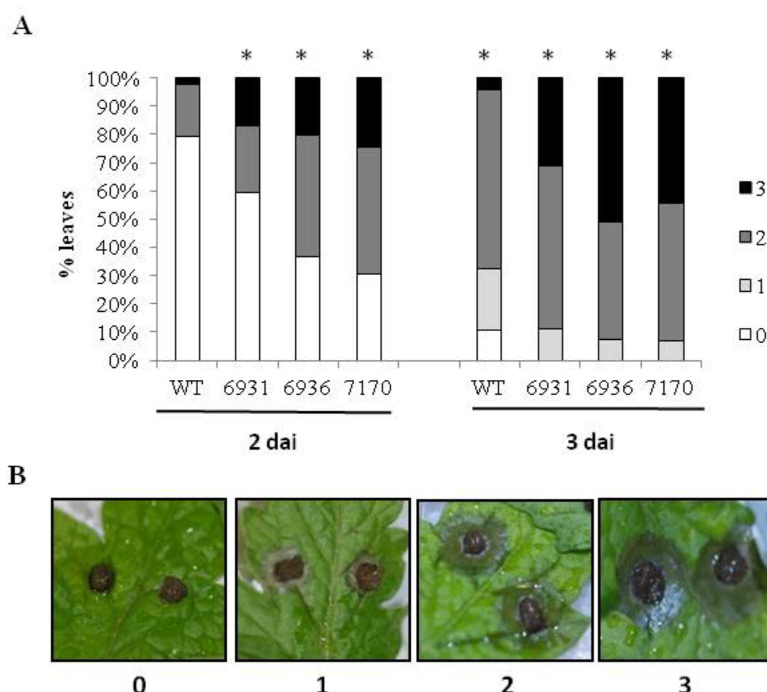


Fig 2. Effect of *Botrytis cinerea* inoculation on the SL-deficient tomato plants *Slccd7* (6931, 6936 and 7170 lines). Evaluation of the spreading lesions in leaves from the *Slccd7 lines* and the corresponding wild-type (WT) at 2 and 3 days after inoculation with 5-mm-diameter plugs containing active growing hyphae (**A**). Disease index of *B. cinerea* symptoms in tomato leaves used (**B**). Asterisks (*) indicate statistically significant different contributions ($P < 0.01$) of the disease severity classes according to Fisher's least-significant difference (LSD) test. Ten independent biological replicates per genotype were used.

4.2. Susceptibility of the SL-deficient tomato plants *Slccd8* to *A. alternata*

In order to ascertain if the increased susceptibility of SL- deficient plants is broad spectrum or restricted to *B. cinerea,* the susceptibility of *Slccd8* plants (line L09) to another foliar fungal pathogen as *A. alternata* was studied using isolated spores as inoculum. The same trend as for *B. cinerea* was observed after inoculation with *A. alternata*. In this case, 40% of the *Slccd8* leaves were heavily infected (index 3) at 5 dai, while only 12% of the wt leaves reached that level of infection (**Fig. 3**).

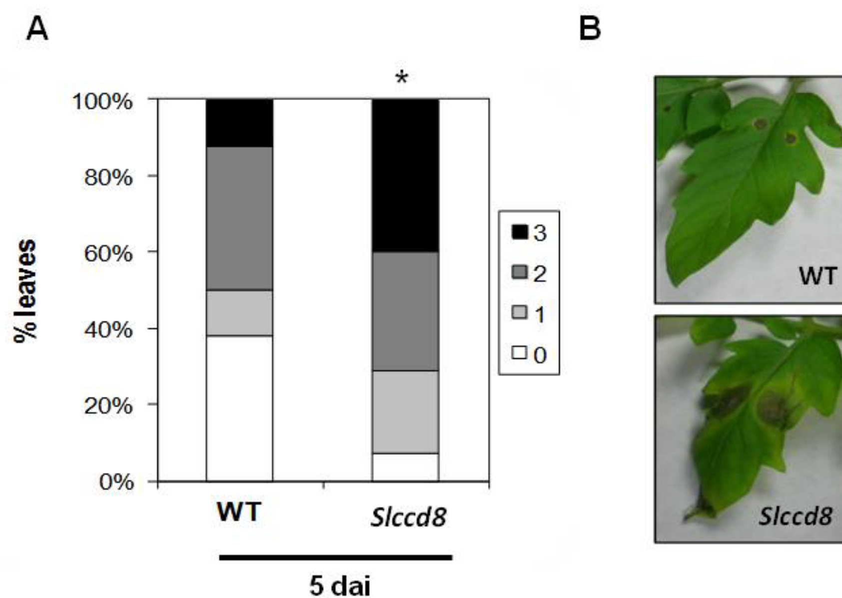


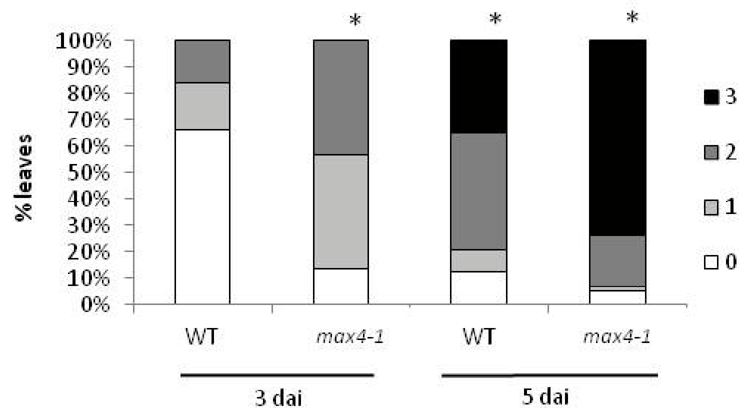
Fig 3. Effect of *Alternaria alternata* inoculation on the SL-deficient tomato plants *Slccd8* (L09 line). Evaluation of fungal spreading lesions in leaves from *Slccd8* and corresponding wild-type (WT) at 5 days after inoculation (dai) by applying 5 µl of a suspension containing 10^6 spores ml^{-1} (**A**). Close-up of *Slccd8* and WT leaves inoculated with *A. alternata* at 5 dai (**B**). Asterisks (*) indicate statistically significant different distributions ($P < 0.01$) of the disease severity classes according to Fisher's least-significant difference (LSD) test. Ten independent biological replicates per genotype were used.

4.3. Susceptibility of the SL-deficient Arabidopsis plants *max4-1* to *B. cinerea*

A possible conservation across plant families of the function of SLs in defence was analysed using Arabidopsis plants. Leaves from the SL-deficient Arabidopsis plants

max4-1 and their corresponding wt were inoculated with *B. cinerea* using PDA plugs containing active growing hyphae, and the spreading lesion diameter was measured at 3 and 5 dai. The *max4-1* plants were more susceptible to *B. cienerea* than the wt and this effect was maintained over time **(Fig. 4).** At 3 dai, 85% of the leaves from the mutant were infected, whereas in the wt only 35% of them showed symptoms (**Fig. 4A**). At 5 dai, ~ 75% of leaves from *max4-1* plants were heavily infected (disease index 3), while only ~ 35% were infected in the wt.

A



B

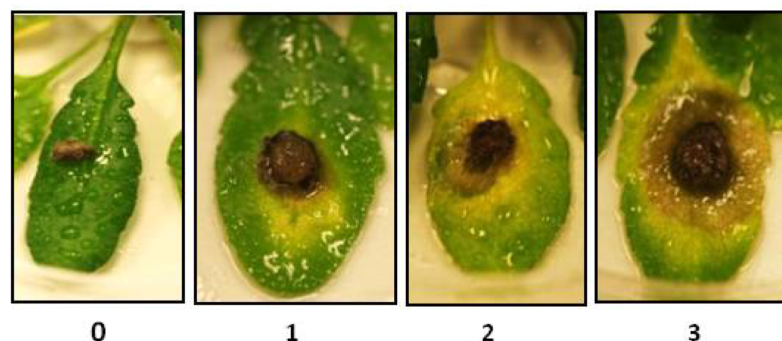


Fig 4. Effect of *Botritys cinerea* inoculation on the SL-deficient Arabidopsis plants *max4-1*. Evaluation of fungal spreading lesions in leaves from *max4-1* and corresponding wild-type (WT) at 3 and 5 days after inoculation (dai) with 5-mm-diameter plugs containing active growing hyphae (**A**). Disease index of *B. cinerea* symptoms in Arabidopsis leaves (**B**). Asterisks (*) indicate statistically significant different distributions ($P < 0.01$) of the disease severity classes according to Fisher's least-significant difference (LSD) test. Ten independent biological replicates per genotype were used.

4.4. Impact of the soil-borne pathogen *F. oxysporum* in the SL-deficient tomato plants *Slccd8*

Because of the increased susceptibility to the the foliar pathogens *B. cinerea* and *A. alternata* observed in the SL deficient lines, we wondered if the deficiency in SLs production also causes susceptibility against soil pathogens. For that, an infection assay was carried out with the soil-borne fungal pathogen *F. oxysporum f. sp. radicis lycopersicy* (*Fol)*. This fungus is a hemi-biotrophic pathogen that infects plants through the roots. *Slccd8* and wt seedlings were inoculated with *Fol*, grown in pots and plant survival monitored weekly. Two independent experiments were performed with similar results. As in the case of *B. cinerea*, plant susceptibility was higher in the *Slccd8* line than in wt plants (**Fig. 5**). The characteristic disease symptoms such as stunting, progressive wilting, defoliation and plant death were observed after 2 weeks inoculation in *Slccd8* plants. At 4 weeks after inoculation, 70% of the *Slccd8* plants died, whereas only a 30% of mortality was observed in wt plants (**Fig. 5A**). The presence of the pathogen within the plant roots was confirmed by surface sterilization and incubation of the fragments in PDA plates.

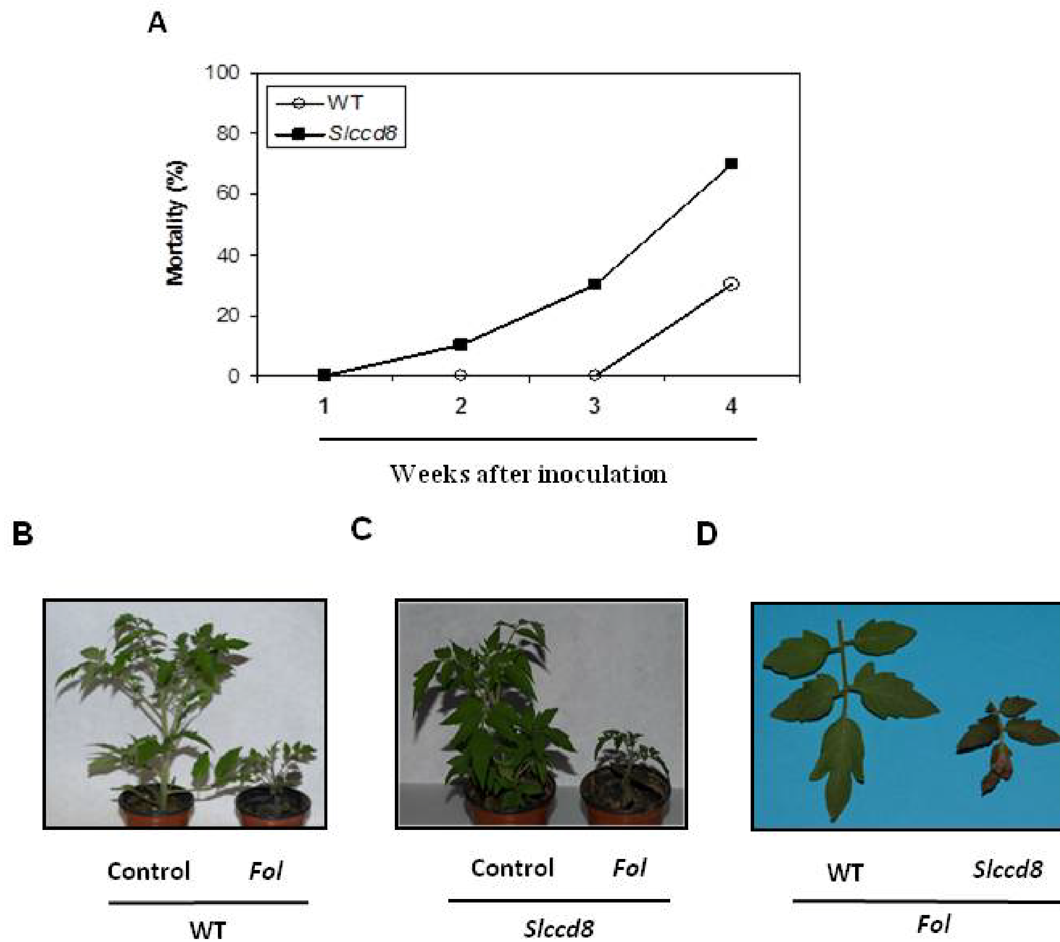
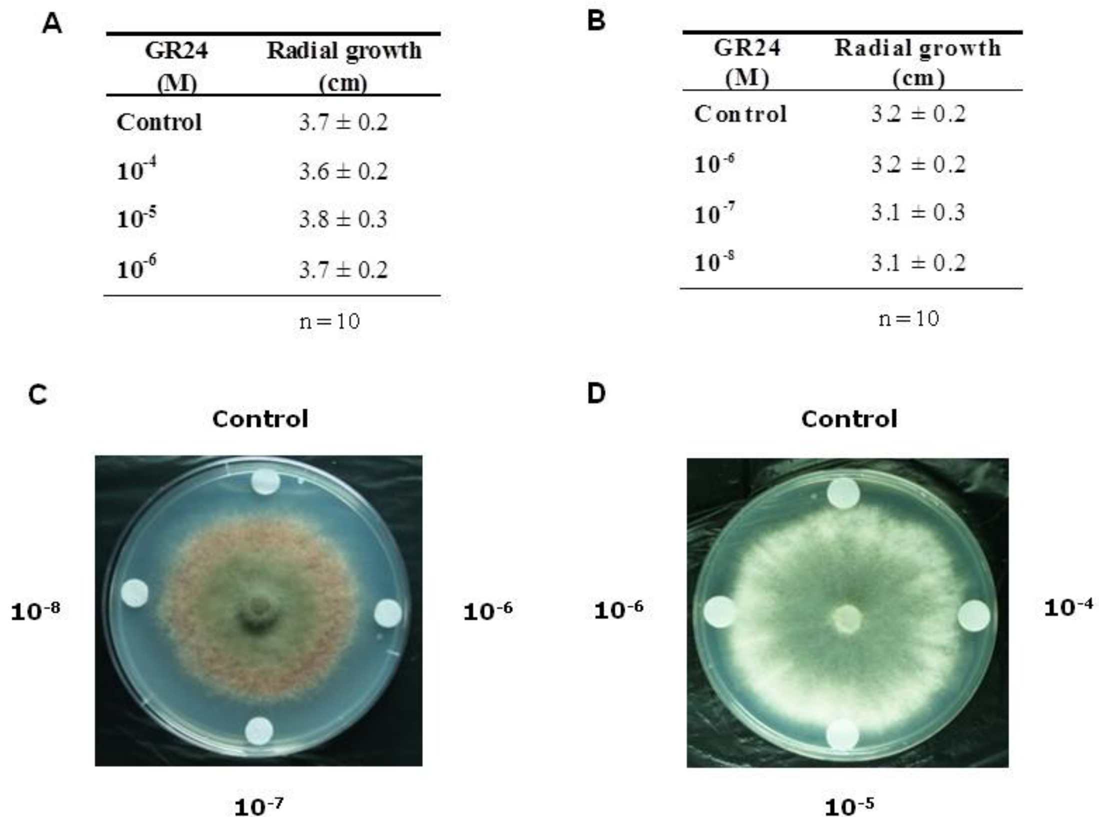


Fig 5. Effect of *Fusarium oxysporum f. sp. radicis lycopersici (Fol)* inoculation on the SL-deficient tomato plants *Slccd8* (L09 line). Evaluation of the mortality of *Slccd8* plants compared with wild-type (WT) plants at different time points after inoculation of the roots (**A**). Close-up of non-infected and infected WT plants **(B)**, Slccd8 plants **(C)** and *Slccd8* and WT leaves from infected plants **(D)** 4 weeks after inoculation. Ten independent biological replicates per genotype were used.

4.5. SLs do not affect fungal development in *B. cinerea*

SLs stimulate hyphal branching in germinating spores of arbuscular mycorrhizal fungi (Akiyama *et al.,* 2005) and have been proposed to affect the development of certain fungal pathogens as well (Dor *et al*., 2011). To check whether SLs have a direct effect on *B. cinerea* development, an *in vitro* assay using different concentrations of the synthetic SL analogue GR24, all within the physiological concentration range (Xie *et al*., 2010). No effect on the *B. cinerea* growth pattern was observed by any of the GR24

concentrations tested **(Fig. 6)**, indicating that there is not a direct effect of SLs on *B. cinerea* development. This observation is in agreement with a previous study in which no alterations in the growth pattern of *B. cinerea* after 10^{-6} M GR24 application were detected (Steinkellner *et al.*, 2007).



A

GR24 (M)	Radial growth (cm)
Control	3.7 ± 0.2
10^{-4}	3.6 ± 0.2
10^{-5}	3.8 ± 0.3
10^{-6}	3.7 ± 0.2
	n = 10

B

GR24 (M)	Radial growth (cm)
Control	3.2 ± 0.2
10^{-6}	3.2 ± 0.2
10^{-7}	3.1 ± 0.3
10^{-8}	3.1 ± 0.2
	n = 10

Fig. 6 Effect of the synthetic SL analogue GR24 on fungal development of *Botrytis cinerea*. Radial growth of *B. cinerea* in vitro determined 5 days after application of 10 µl of different concentration 10^{-6}, 10^{-7} and 10^{-8} **(A)** and 10^{-4}, 10^{-5} , 10^{-6} M **(B)** of the GR24. As a control, 10 µl of sterilized demiwater was used. Data represent the means of 10 independent replicates ± standard error. Representative plates showing no effect of GR24 on *B. cinerea* growth at low **(C)** and high concentrations **(D).**

4.6. Defence-related hormone analysis by HPLC-MS/MS in the SL-deficient tomato plants *Slccd8*

Plant resistance/susceptibility to pathogens is generally associated to changes in the hormone-regulated defence signalling pathways (Robert-Seilaniantz *et al.*, 2011). To

determine whether the increased susceptibility of *Slccd8* plants to *B. cinerea* was related to alterations in the hormonal profiles, the content of the defence-related phytohormones JA, SA, ABA and the JA precursor OPDA was analysed in the leaves from *Slccd8* plants and compared to the levels in wt plants. Analysis by HPLC-MS/MS allowed the simultaneous quantification of all four compounds from each sample. The levels of JA were clearly lower (about 70%) in *Slccd8* than in the wt **(Table 1).** Although a trend to reduction was observed for OPDA, the values were not significantly different. A significant (p < 0.01) reduction of about 50% was also observed in the levels of SA in the mutant leaves **(Table 1).** As in the case of SA and JA, ABA content was also reduced (about 35%) in *Slccd8* compared to the wt **(Table 1).**

	Genotype	
Hormone (ng/g dry weight)	WT	*Slccd8*
OPDA	4495 ± 473	3672 ± 557
JA	157 ± 37	41 ± 16**
SA	2375 ± 165	1251 ± 60*
ABA	2515 ± 79	1624 ± 118*

Table 1. Defence hormonal content in the SL-deficient tomato plants *Slccd8*. Levels of JA precursor 12-oxo phytodienoic acid (OPDA), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) were measured by HPLC-MS/MS in leaves from *Slccd8* plants and corresponding wild-type (WT). Data represent the means of 5 independent replicates ± standard error. . Asterisks (*) indicate statistically significant differences according to Fisher's least-significant difference (LSD) test (*P < 0.05 and **P < 0.01)

4.7. Gene expression of defence-related markers by RT-PCR in the SL-deficient tomato plants *Slccd8*

The expression of defense related marker genes corresponding to the different hormone regulated signalling pathways was analysed by qRT-PCR. The genes *PR1a* and

Le4 were used as markers for SA- and ABA-regulated responses, respectively (López-Ráez *et al*., 2010b). *PR1a* encodes a pathogenesis-related protein which is inducible by SA, and *Le4* encodes for an ABA-inducible dehydrin. Interestingly, the expression levels of *PR1a* and *Le4* were not altered significantly in leaves of *Slccd8 plants* compared to those in the wt. As JA marker gene we used *PinII*, encoding for the proteinase inhibitor II, involved in the plant defence against insects and *B. cinerea* (El Oirdi *et al*., 2011; Wasternack, 2007). The expression of *PinII* was reduced by more than four-fold in the *Slccd8* plants **(Fig. 7)**. The gene expression data suggest that, despite the reduction in the basal level of the three phytohormones (SA, ABA and JA), only JA-dependent responses were affected significantly.

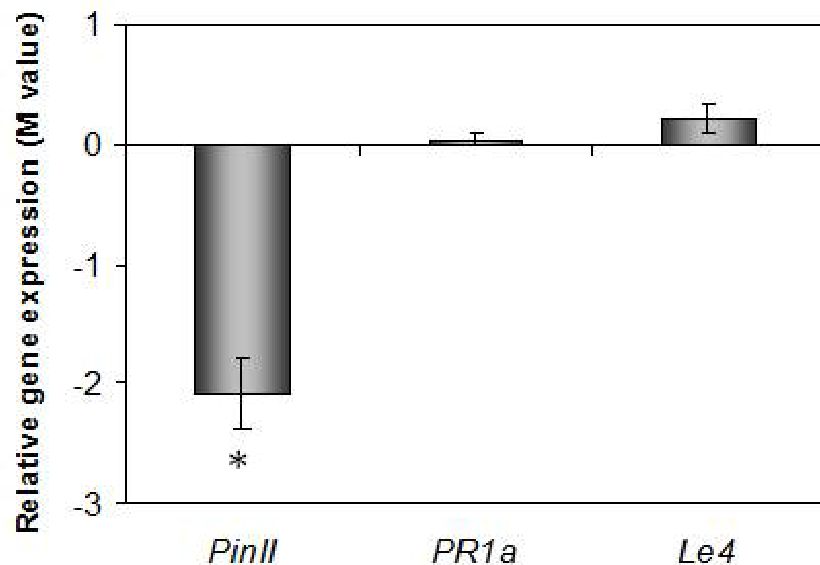


Fig. 7 Expression levels in the SL-deficient tomato plants *Slccd8* compared with the corresponding wild-type (WT). Gene expression analysis by real-time quantitative polymerase chain reaction (qRT-PCR) of molecular markers for different defense signalling pathways. M values (log_2 ratio) are represented. The M value is zero if there is no change, and ´+1´ and ´-1´ indicate a two-fold induction and reduction, respectively. *PinII*, *PR1a* and *Le4* genes were the markers for the jasmonic acid (JA)-, salicylic acid (SA)- and abscisic acid (ABA)-dependent pathways, respectively. Bars represent the means of six independent replicates [±standard error]. Asterisk (*) indicates significantly different (P < 0.01) expression levels according to Fisher's least-significant difference (LSD) test.

5. DISCUSSION

Effective and sustained control of fungal pathogens is one of the most important problems in current agriculture. These microorganisms, foliar and soil fungal pathogens cause a high economic impact in crops. Among them, *B. cinerea* is a non-specific necrotrophic pathogen that attacks a broad range of hosts. It causes grey mould in leaves and fruits from many plants through the production of toxins and causing necrosis in the host to achieve infection. It is one of the most prominent, widespread and destructive diseases in crops (Van Baarlen et al., 2007; Dean et al., 2012). Similarly, *A. alternata* is other foliar and necrotrophic pathogen on a large number of host plants,causing a range of diseases by destruction of host tissues (Thomma, 2003). On the other hand, one of the most destructive hemi-biotrophic pathogens in soils is *F. oxyspoum*, ubiquitous pathogen that causes vascular wilt on a wide range of plants including tomato (Dean et al., 2012).

SLs are multifunctional molecules involved in several processes in plant physiology and plant communication. In this work, we investigated a possible new role of SLs in the regulation of plant defence responses. For that, the resistance/susceptibility of SL-deficient plants from tomato (*Slccd8* and *Slccd7*) and Arabidopsis (*ma4-1*) and the corresponding wild type genotypes to different pathogens was investigated.

The results indicated a higher susceptibility of *Slccd8 and Slccd7* plants against *B. cinerea* than the wt. Interestingly, the Arabidopsis SL- deficient mutant *max4-1* was also more susceptible to *B. cinerea* than its wt. These results suggest a conserved role of SLs in defence across plant species. *Slccd8* plants were also more susceptible to another necrotrophic pathogen as *A. alternata,* reaffirming the SL role in the defence

against necrotrophic pathogens of plant leaves. Moreover, *Slccd8* was more susceptible to the soil fungal pathogen *Fol*, with a hemi-biotrophic life stile. This suggests that the SLs could have a broad spectrum action against different types of pathogens targeting different plant organs.

A direct effect of SLs on fungal pathogens was proposed (Dor *et al*., 2011). Dor and co-workers reported that the application of the synthetic SL GR24 *in vitro* resulted in the growth inhibition of pathogens such *as Fol, A. alternata* or *B. cinerea*, although very high GR24 concentrations were used. In the present work, using concentratios within a physiological range, we have not observed any effect of GR24 on *B. cinerea* germination or growth. The results suggest that there is no a direct effect of SLs on fungal development, and that the possible role of SLs in defence responses might be through an indirect effect. It is well known that plant defence responses depend on phytohormones such as SA, JAs, ET and ABA (Robert-Seilaniantz *et al*., 2011). Here, a decrease in SA, JA and ABA levels was observed in leaves of the *Slccd8* plants compared to the wt (**Table 1**), indicating an altered hormonal balance in the SL-deficient plants. This fact suggests a cross-talk between SLs and the signalling pathways related to defence hormones, and accordingly, an indirect involvement of SLs in plant defence. An interaction between SLs and other phytohormones has been shown for their previously described physiological functions (reviewed in Kohlen *et al*., 2011). For instance, a cross-talk between SLs, auxin and cytokines has been proposed to control shoot branching (Domagalska and Leyser, 2011). Similarly, SLs interactions with auxin and ET seemto regulate root system architecture and root hair formation (Kapulnik *et al*., 2011b; Ruyter-Spira *et al*. 2011). As far as we know, no relationship between SLs and SA and JA has been reported. JA- and SA-dependent signalling

pathways are considered mutually antagonistic (Robert-Seilaniantz *et al*., 2011). However, evidences of synergistic interactions were also reported, suggesting that hormone interactions could be tailored to pathogen by the relative concentration of each hormone (Mur *et a*l., 2006). This seems to be the case in the *Slccd8* plants where both hormones are reduced, given rise to a more susceptible plant.

Unlike SA and JA, a regulatory role for ABA in SL biosynthesis was proposed as tomato ABA-deficient mutants showed a reduced capacity of producing SLs (López-Ráez *et al*., 2010). ABA has emerged as an important regulator of biotic defence responses (Ton *et al*., 2009). The ABA-deficient mutant *sitiens* was shown to be more resistant to *B. cinerea* than wild-type plants (Audenaert *et al*., 2002), suggesting a role of ABA in tomato susceptibility to this pathogen. Conversely, a positive effect of ABA in early defence responses against *B. cinerea* has also been reported (Abuqamar *et al*., 2009). As for the other phytohormones, it seems likely that the impact of ABA in defence depends on their interaction with other hormones. Here, we show that SLs may also alter ABA levels. Therefore, it seems likely that the effect of SLs on ABA content may impact on the plant's ability to cope with stresses.

Among the hormonal changes observed in the *Slccd8* plants, those produced in the levels of JA- were the most pronounced. Moreover, the expression analysis of marker genes showed a strong reduction in the level of the JA-responsive gene *PinII*. Necrotrophic pathogens colonize senescent or dead plant tissues and are generally sensitive to defence responses regulated by JA (Pieterse *et al*., 2009). In this sense, JA is known to be the key signalling pathway regulating defences against *B. cinerea*, and *PinII* has been proven to be important for tomato resistance to *B. cinerea* (El Oirdi *et*

al., 2011). PinII is a proteinase inhibitor which accumulates on wounding and damage caused by herbivores and pathogens or by treatment with exogenous JA (Wasternack, 2007). El Oirdi *et al*. (2011) reported that tomato plants silenced in *PinII* were more susceptible to this pathogenic fungus, showing that this enzyme is required for resistance against *B. cinerea* in tomato. Therefore, the reduction in JA content and *PinII* basal levels could well explain the increased susceptibility of *Slccd8* to the different fungal pathogens.

The SA pathway is often activated in response to biotrophic and hemi-/biotrophic fungal pathogens leading to the expression of suites of PR genes (Glazebrook *et al*., 2005). Recently it has been shown that tomato lines impaired in SA signalling, but not in JA, were affected in the susceptibility to the hemibiotrophic pathogen Fol. Indeed, *NahG* tomato plants, deficient in the accumulation of SA, were much more susceptible to Fol than the corresponding wildtype (Di et al., 2017). Accordingly, it is possible to speculate that the increased susceptibility of *Slccd8* plants to *Fol* could be modulated through the lower accumulation of SA in these plants, although a reduction in the SA dependent defense responses could not be confirmed, since the expression of the SA-marker gene *PR1a* was not altered.. The contribution of JA signalling to the differential response of the *Slccd8* lines to *Fol* should not be ruled out, since some reports showed a complex effect of JA on plant resistance to this an others soil fungal pathogens (Thaler et al., 2004).

We can conclude that the SL-deficient plants are more susceptible to necrotrophic fungal pathogens. The reduced levels of the phytohormones JA, SA and ABA in *Slccd8* suggest a role of SLs in the regulation of plant defence responses through their

interaction with other signalling pathways, especially with the JA-related pathway. The results provide insights into a possible new role for SLs and open up a new area of research related to the involvement of SLs in the regulatory network controlling plant responses to stresses. Unravelling the role of SLs in defence is definitely exciting and promising. However, further research is needed to define the mechanisms that may regulate this new function and to assess whether this effect can be extended to other pathosystems.

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SUPPLEMENTARY INFORMATION

ID	Gene	Pathway	Primers (5´-3')	Reference
K03291	Wound-induced proteinase inhibitor II (PinII)	JA	GAAAATCGTTAATTTATCCCAC ACATACAAACTTTCCATCTTTA	Graham et al., 1985
M69247	Pathogenesis related protein (PR1a)	SA	GTGGGATCGGATTGATATCCT CCTAAGCCACGATACCATGAA	Lopez-Raez et al., 2010b
X51904	Le4	ABA	ACTCAAGGCATGGGTACTGG CCTTCTTTCTCCTCCCACCT	Lopez-Raez et al., 2010b
X14449	Elongation factor 1 (SlEF)	Housekeeping	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC	Rotenberg et al., 2006

ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid

Table S1: Genes analysed, signalling pathways they represent and primer sequences used in the RT-PCR analysis.

CAPÍTULO 3

Estudio de una posible interacción entre los jasmonatos y las estrigolactonas

Torres-Vera R, López-Valverde FJ, Martín L, Pozo MJ, López-Ráez JA.

RESUMEN

Las estrigolactonas (SLs) son hormonas vegetales que regulan la arquitectura de la planta, incluyendo entre otros aspectos, la ramificación de la parte aérea. Además, juegan un papel importante en la rizosfera, por un lado actuando como moléculas señal para la germinación de semillas de jopo, y por otro, induciendo la ramificación de las hifas de hongos micorrícicos arbusculares (MA). De hecho se ha demostrado en distintas especies que plantas con niveles reducidos de SLs presentan una alta ramificación de la parte aérea, baja colonización micorrícica y bajos niveles de germinación de semillas de jopo. Aparte de las SLs, otras hormonas participan en la regulación de la simbiosis MA, como es el caso del ácido jasmómico y sus derivados (jasmonatos, JAs). Estudios previos evidenciaron niveles alterados de colonización micorrícica en algunas plantas alteradas en la ruta del JA. Ésto, junto con los niveles alterados de JA en plantas deficientes en la producción de SLs mostrados en capítulos anteriores, podrían sugerir una posible conexión entre ambas hormonas. Para evaluar esta posible interacción, realizamos un estudio fenotípico en varios mutantes relacionados con la ruta de señalización regulada por JA y se compararon con varias líneas de plantas de tomate silenciadas en el gen *Slccd8* y por tanto deficientes en la producción de SLs. Sólo el mutante deficiente en JA *spr2* mostró mayores niveles de ramificación en parte aérea, observándose una similitud con el fenotipo de la línea *Slccd8* con deficiencia moderada en SLs, sugiriendo una posible interacción JA-SLs, sin

embargo el mutante no presentó diferencias significativas en el contenido de SLs en comparación con el genotipo silvestre. Puesto que otras hormonas como las auxinas y las citoquininas (CKs) regulan el crecimiento de las yemas axilares y están involucradas también en la ramificación de la parte aérea, se realizó un estudio sobre el contenido hormonal de *spr2* para explorar un posible efecto indirecto en la homeostasis hormonal que pueda explicar el fenotipo observado.

Study of a possible cross-talk between jasmonates and strigolactones

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1. ABSTRACT

Strigolactones (SLs) are hormones that regulate plant architecture including shoot branching. In addition, they play an important role in the rhizosphere, on one hand they act as signaling molecules for the broomrape seed germination and on the other hand, they induce hyphal branching in arbuscular mycorrhizal (AM) fungi. In fact, plants with reduced SL biosynthesis/signalling in several species presented a high shoot branching and low levels of broomrape seed germination and mycorrhizal colonization. In addition to SLs, others key hormones participate in the regulation of AM symbiosis as in the case of jasmonic acid and its derivates (jasmonates, JAs). Altered levels of mycorrhizal colonization in some JA-related plants in previous studies were appreciated. Moreover, JA levels were altered in SLs altered plants. These data could suggest a possible connection between these two hormones. To assess a possible interaction between both hormones, we carried out a phenotypic study in several JA-related mutants in comparison with the SL-deficient transgenic tomato plants *Slccd8*. Only the JA-deficient mutant *spr2* displayed a higher shoot branching level, showing a similarity with the phenotype of a *Slccd8* line that presents a moderate SL deficiency, suggesting a possible JA-SL interaction. Other hormones such as auxins and cytokinins (CKs) regulate the axillary bud outgrowth, being also involved

in shoot branching. Therefore, a study about the hormonal content in *spr2* plants was also performed to explore potential direct or indirect effect of SLs.

Keywords: Cross-talk, jasmonates, mycorrhization, shoot branching, *spr2*, strigolactone, tomato.

2. INTRODUCTION

Strigolactones (SLs) are a group of carotenoid-derived plant hormones (López-Ráez *et al*., 2008; Mantusova *et al*., 2005). Mainly, they are biosynthesized in the roots and in some parts of the stem (Foo *et al*., 2001; reviewed by Dun *et al*., 2009), being transported through the xylem, regulating biological functions in different parts of the plant. SLs play an important role in both shoot and root architecture (Arite *et al*., 2012; Cheng *et al*., 2013; Kapulnik *et al*., 2011; Koltai *et al*., 2010; Rasmussen *et al*., 2012; Ruyter-Spira *et al*., 2011). They were recognized as a class of phytohormone after being detected their role as inductors of shoot apical dominance (Gómez-Roldán *et al*., 2008; Umehara *et al*., 2008). Accordingly, high shoot branching has been identified in different SL-biosynthesis/signalling deficient plants from several plant species. Among them there are included *ramosus (rms)* in pea (*Pisum sativum*), *decreased apical dominance (dad)* in petunia (*Petunia hybrida*), *more axillary growth (max)* in Arabidopsis (*Arabidopsis thaliana*), and *dwarf (d)* in rice (*Oryza sativa*) (Reviewed in Janssen *et al*., 2014).

During shoot branching, axillary meristems (axillary buds) are formed in the axil of each leaf with the potential to develop into branches. In addition to SLs, the main hormones that regulate bud dormancy or outgrowth are auxins and cytokinins (CKs). Auxins, produced mostly by the apical bud, were the first plant hormones determined

as involved in shoot branching, controlling the shoot tip apical dominance and consequently, inhibiting axillary bud outgrowth (Janssen *et al*., 2014) (**Fig. 1**). In fact, studies tested how the replacement of the shoot apex with exogenous auxins, maintained the inhibition of axillary buds of several species as Arabidopsis and pea (Cline, 1996). CKs show the opposite physiological role to auxins, since they promote directly axillary bud outgrowth (Shimizu-Sato *et al*., 2009). Several studies demonstrated that either exogenous CK application or overexpression of genes encoding enzymes involved in CK biosynthesis often induce bud outgrowth of Arabidopsis, pea, tobacco (*Nicotiana tabacum*) and lupin (*Lupinus angustifolius)* (King and Van Staden, 1988; Medford *et al*., 1989; Miguel *et al*., 1998) (**Fig. 1**). In addition, some mutants with a great CK level displayed an increased shoot branching (Dun *et al*., 2006).

Nowadays, the mechanisms by which these hormones regulate the shoot branching are unknown. Two different hypotheses were proposed. Initially, “the canalization hypothesis” was proposed, which suggests that auxin transport in the main stem can inhibit bud outgrowth by regulating the ability of axillary buds to establish their own polar auxin transport stream (PATS) to export auxins into the stem (Domagalska and Leyser, 2011). In this hypothesis, shoot apical meristem seems to control the flow of PATS due to SLs capacity to reduce auxins transport through the regulation of the PIN-FORMED (PIN) transporters in xylem parenchyma cells, thus inhibiting the outgrowth of axillary buds (Crawford *et al*., 2010, Lin *et al*., 2009). On the other hand, application of CKs to not-growing buds of pea resulted in the upregulation and polarization of PIN proteins, which suggests that CKs might contribute to the activation of dormant buds through the stimulation of the auxin export from the buds to the stem (Kalousek *et al*.,

2010). The second hypothesis "the second messenger hypothesis" proposes that auxins altered the biosynthesis of a second messenger that regulate bud outgrowth directly. CKs and SLs are candidates to serve as second messengers (Brewer *et al*., 2009; Domagalska and Leyser, 2011; Kolhen *et al*., 2011). CKs can move acropetally into the axillary bud and auxins might restrict CKs availability in the bud through inhibition of CK biosynthesis, as was observed in Arabidopsis and tobacco plants (Eklöf *et al*., 1997, Nordström *et al*., 2004). Moreover, apical decapitation increased CK levels in the axillary meristems in chickpea (*Cicer arietinum*) (Turnbull et al., 1997) and the expression of the CK biosynthesis genes in the pea nodal stem was induced by decapitation or by the application of the auxin transport inhibitor 2, 3, 5-triiodobenzoic acid (TIBA), but reduced by auxins (Tanaka *et al*. 2006). The CK-promoting effects on bud outgrowth are antagonized by SLs. Auxins contribute to the regulation of SL production by stimulating the transcription of SL biosynthesis genes (Teichmann *et al.,* 2015). Accordingly, removal of the apical auxin source leads to a strong decrease in SL biosynthesis gene expression, which therefore reduces the production of SLs in Arabidopsis and pea (Foo *et al*., 2005, Hayward *et al*., 2009, Sorefan *et al*., 2003). Acting as second messengers in buds, the activity of CKs and SLs might converge on the local control over common bud-specific targets. The two models are not mutually exclusive. Both could be active, each being important under different physiological and/or environmental conditions.

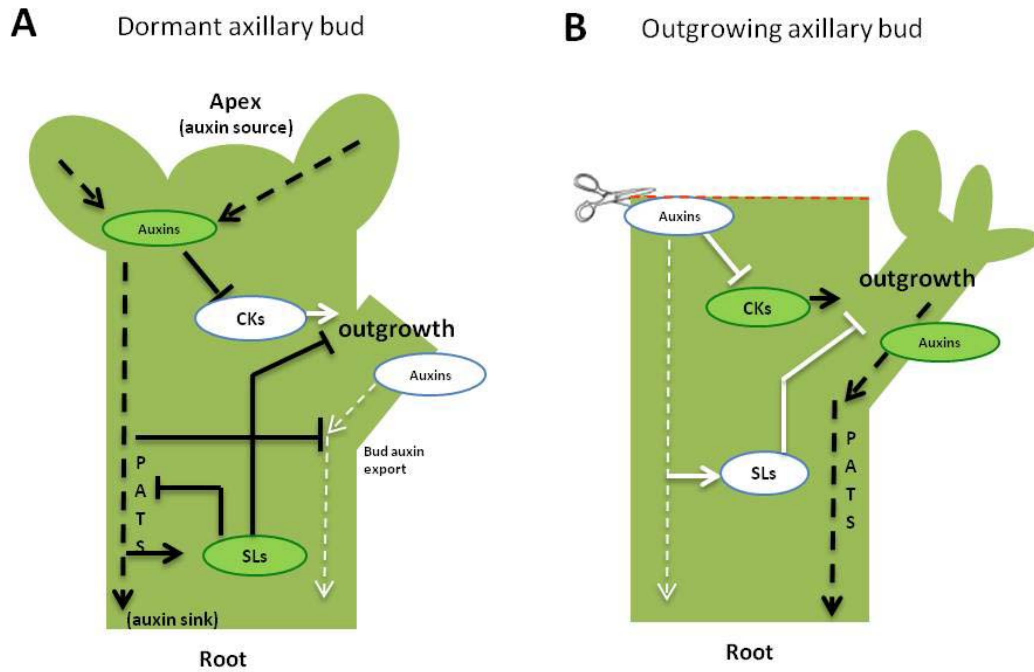


Fig. 1 Schematic summary of the models about hormonal regulation of shoot branching. In a plant without manipulation (**A**), the apex is the dominant source of auxins, which are transported basipetally by the polar auxin transport stream (PATS). According to the second messenger model, auxins induce and decrease the biosynthesis of strigolactones (SLs) and cytokinins (CKs), respectively. Both hormones present opposed effects on bud outgrowth. According to the auxin transport canalization model, the axillary bud needs to establish its own auxin export for bud outgrowth. However, it competes with the shoot apex for the stem as a shared auxin sink. High auxin levels in the stem coming from shoot apex prevent the formation of an initial auxin export flux from the bud, suppressing bud outgrowth. The SLs control the flow of PATS in the main stem, inhibiting the outgrowth of axillary buds. After decapitation (**B**), the apex is no longer the dominant auxin source. Biosynthesis of SLs is not promoted anymore while CK biosynthesis is induced. Therefore, the auxin level in the main stem is reduced, facilitating the establishment of an initial auxin export stream from the axillary bud. Black lines and green circles designate active pathways; white lines and circles indicate suppression or down-regulation of the respective pathway". Illustration modified from Teichmann and Muhr., 2015.

Besides the SL role in plant architecture, they were initially described as signalling molecules in the rhizosphere under nutritional stress, mainly under phosphorous (P) starvation (Kohlen *et al*., 2011; López-Ráez *et al*., 2008; Umehara *et al*., 2008; Yoneyama *et al*., 2007). Firstly, they were described as inducers of seed germination of root parasitic plants of the *Orobanchaceae*, including the genera *Striga, Orobanche* and *Phelipanche* (Bouwmeester *et al*., 2003; Cook *et al*., 1966). Later, it was shown that they act as pre-symbiotic signals, inducing hyphal branching of arbuscular mycorrhizal fungi (AMF), favouring root mycorrhization (Akiyama *et al*., 2005; Besserer

et al., 2006). Arbuscular mycorrhizas (AM) are mutualistic symbioses that promote a nutritional exchange between the plant and AMF through fungal structures denominated arbuscules (Bonfante and Perotto, 1995; van der Heijden *et al.,* 2015). The AMF offers the plants an improved nutrients uptake in the soil. In turn, the AMF obtains of host plant the necessary carbohydrates for its survival (Neumann and George, 2010). Several studies revealed that SL-deficient plants were less colonized (Foo *et al*., 2013; Gómez-Roldán *et al*., 2008; Kohlen *et al*., 2012; Kretzschmar *et al*., 2012; Vogel *et al*., 2010). Interestingly, optimal mycorrhizal colonization causes a decrease in SL production (Aroca *et al*., 2013; Fernández-Aparicio *et al*., 2010; López-Ráez *et al.,* 2011). Likely, this SL reduction is preventing a possible excessive colonization of AMF, a phenomenon that could be associated to a mechanism of autoregulation of the symbiosis (García-Garrido *et al*., 2009; Vierheiling *et al*., 2004).

AM symbiosis establishment and maintenance is finely regulated by hormones (Pozo *et al*., 2015). In addition to SLs, others key hormones, including jasmonic acid (JA) and its derivatives (herein referred as jasmonates, JAs) participate in the regulation of the symbiosis. JA is a phytohormone biosynthesized through the octadecanoid pathway, being the final product of the peroxidation of linolenic acid (Wasternack and Hause, 2013). JAs fulfil multiple functions in plants such as regulation of defense and wounding responses, development processes and AM symbiosis (Avanci *et al*., 2010; Vierheiling, 2004). However, their exact role in AM symbiosis is not clear (Hause *et al*., 2007; Pozo *et al*., 2015). Different studies showed positive and negative effects on mycorrhizal colonization after foliar JA treatment in several plants species (Ludwig-Müller *et al*., 2002; Regvar *et al*., 1996). An increase in JAs levels in mycorrhizal roots has been documented in diverse plants, including tomato (*Solanum lycopersicum*)

(Hause *et al*., 2002; López-Ráez *et al*., 2010; Meixner *et al*., 2005; Vierheilig and Piché, 2002). Accordingly, an increase in expression has been described for several JA biosynthesis and responsive genes (Hause *et al*., 2007; León-Morcillo *et al*., 2012; López-Ráez *et al*., 2010, Fernández *et al.,* 2014). In addition, various studies have tested the effect of JAs on the AM symbiosis by analysing different JA-deficient mutants, yielding opposite results. Low mycorrhization levels were detected in the barrel medic (*Medicago truncatula*) mutant *MtAOC1* (Isayenkov *et al*., 2005). In tomato it was described that *jai1*, a JA insensitive mutant, had higher mycorrhizal colonization levels than the wild-type (Herrera-Medina *et al*., 2008). Another mutant studied was the *suppressor of prosystemin-mediated responses2 (spr2)* mutant that carries a loss-of-function mutation in *LeFAD7* gene that encodes a fatty acid desaturase, being just at the beginning of the oxylipin pathway. It was shown that the *spr2* mutant had a reduced mycorrhization (Song *et al*., 2013; Tejeda-Sartorius *et al*., 2008) ant this phenotype was recovered after the exogenous application of MeJA (Tejeda-Sartorius *et al*., 2008). So far, a possible interaction between SLs and JAs has not been investigated. In the present study, we aim to explore a potential cross-talk between these two hormonal pathways. For that, the shoot branching phenotype of different JA mutants including *spr2* was carefully analysed and compared to those from the different SL-deficient tomato lines *Slccd8* (Kohlen *et al*., 2012). Moreover, the levels of SLs and other phytohormones were analysed in both the roots and the shoots of s*pr2*.

3. MATERIALS AND METHODS

3.1. Plant material, growth conditions and AMF inoculum

Tomato seeds (*Solanum lycopersicum*) from the JA-related mutants *spr1*, *spr2, jai1* and their corresponding wild-type cv Castlemart were kindly provided by Clarence Ryan. Two different SL-deficient lines *Slccd8,* L16 and L09, and their corresponding wild-type cv Craigella, were also used. *Slccd8* lines are antisense CCD8 RNA interference (RNAi) lines (Kohlen *et al*., 2012). All seeds were sterilised and germinated according to López-Ráez *et al*. (2010). The seedlings were transplanted in pots with sterile mix of sand-soil-vermiculite (3:1:1). The plants were randomly distributed and grown in a greenhouse at 24/16 °C with 16/8h photoperiod and 70% humidity and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing the 25% of standard phosphorus concentration. For mycorrhizal treatments, *Rhizophagus irregularis* (BEG 121) and *Funneliformis mosseae* (BEG 12), renamed from *Glomus intraradices* and *Glomus mosseae* respectively (Schüßler and Walker, 2010), were inoculated by adding to the potting mix a 10% (v:v) inoculum consisting of sand-sepiolite (1:1) with fungal propagules. A filtrate of the microbial community, but without AMF propagules, was applied to the control plants according to López-Ráez *et al*. (2010). The plants were harvested after 8 weeks of growth. Before harvesting, the shoot branching phenotype-number of primary, secondary and tertiary order branches- was analysed in each genotype. Root and leaf material was immediately frozen in liquid nitrogen and stored at -80 °C. Between three and six biological replicates per plant genotype and treatment were analysed.

The *jai1* mutant is female sterile and, therefore, cannot be maintained as a homozygous line. Homozygous *jai-1/jai-1* seedlings had to be selected in the F2 populations. The selection of homozygous mutants was based in the inability of the *jai1* mutant to accumulate anthocyanin in the hypocotyl and was confirmed by PCR (Li *et al*., 2004).

3.2. Analysis of AM colonization

Fungal structures inside roots were detected through histochemical staining with dark ink (Vierheilig *et al*., 1998). The samples were examined using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan) under bright-field conditions. The percentage of root length colonised by the AMF was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

3.3. Obtention of root extracts

For SL analysis, 0.5 g of root tissue was ground in a mortar with liquid nitrogen. The samples were extracted with 1 ml of cold pure 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 *Phelipanche ramosa* seeds.

3.4. Preconditioning and germination bioassay with *P. ramosa* seeds

Preconditioning and germination bioassays were adapted and modified from Matusova *et al*. (2004), being performed under sterile conditions. The seeds of *P. ramosa* were surface sterilized in 2% sodium hypochlorite containing 0.02% (v/v) Tween 20 for 10 min, and rinsed thoroughly with sterile demineralized water. Subsequently, the seeds were dried for 60 min in a laminar air flow cabinet. Approximately, between 50-100 seeds were spread on a glass-fibre filter paper (GFFP) disc (9 mm diameter) and put into sterile Petri dishes (9 cm diameter) covered with Whatman filter paper wetted with 3 ml of demineralized water. Petri dishes were sealed with parafilm and incubated for preconditioning at 21°C in darkness during 10-12 days. After preconditioning of seeds, aliquots (50 µl) of root extracts were added to duplicated discs. Serial dilutions from each extract were previously made. The synthetic germination stimulant GR24 (10^{-7} M) and demineralized water were included as positive and negative controls, respectively, in each bioassay. After 7 days, the germinated and ungerminated seeds were counted using a Nikon Eclipse 50i microscope and brightfield conditions. Seeds were considered germinated when the radicle had protruded through the seed coat (Matusova *et al*., 2004). Six biological replicates per plant genotype and treatment were analysed. *P. ramosa* seeds (collected from a tomato field) were kindly provided by Dr. Maurizio Vurro and Angela Boari (Instituto di Scienci delle Produzioni Alimentari, Bari, Italy).

3.5. Hormone analysis by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS)

SLs (solanacol, orobanchol and didehydro-orobanchol isomers) were analysed by UPLC-MS/MS as described in López-Ráez *et al.* (2008), where it was observed that the peak area of didehydro-orobanchol isomer is quite broad suggesting that it is composed of more than one isomer, possibly even up to three isomers (López-Ráez *et al.*, 2008). JA and its isoleucine conjugate (JA-Ile), indole-3-acetic acid (IAA), Kinetin, abscisic acid (ABA) and salicylic acid (SA) were also analysed by UPLC-MS/MS as described by Flors *et al*. (2008).

3.6. Statistical analysis

Data from AMF colonization, seed germination, phenotypical analysis and hormone quantification were subjected to one-way analysis of variance (ANOVA) using the software Startgraphics Centurion (version XVI) for Windows. When appropriate, Fisher's LSD test was applied to determine the statistical significance.

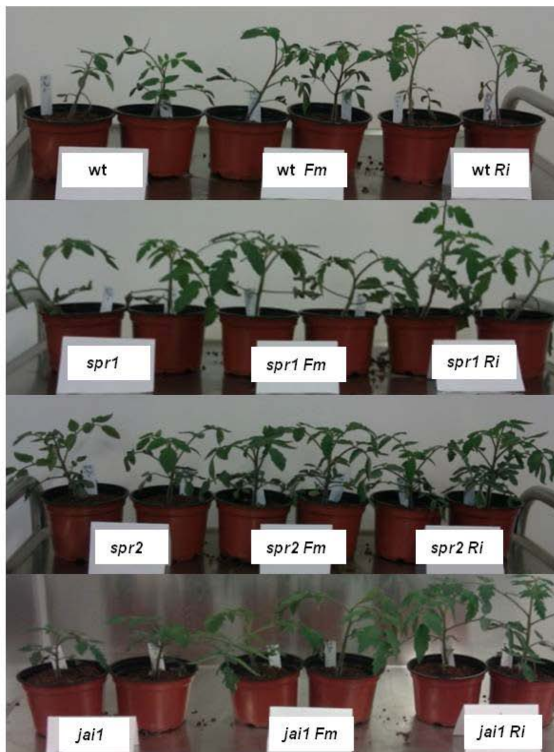
4. RESULTS AND DISCUSSION

4.1. Effect of JA biosynthesis/signalling deficiency and AM symbiosis on shoot architecture of tomato plants

The shoot branching phenotype of different JA-related mutants were analysed by quantifying the total number of branches. Besides the *spr2* mutant (Li *et al.,* 2003), described above, the *suppressor of prosystemin-mediated responses1 (spr1)* and the *jasmonic acid insensitive1 (jai1)* mutants were used. *Spr1* is affected in a signaling step

that couples systemin perception to activation of the octadecanoid biosynthetic pathway, showing an inhibition of the JA biosynthesis (Lee and Howe, 2003). Finally, the *jasmonic acid insensitive1 (jai1)* mutant is a line impaired in jasmonoyl-L-*isoleucine* (JA-Ile) perception due to a deletion mutation in LeCOI1 receptor (Li *et al*., 2001). Since it was shown that AM symbiosis reduces SL production (Aroca *et al*., 2013; Fernández-Aparicio *et al*., 2010; López-Ráez *et al*., 2011) mutant plants and the corresponding wild-type Castlemart were growth with and without AMF (*F. mosseae* and *R. irregularis*) under P-deficient conditions, to explore the effect of AM symbiosis on shoot branching (**Fig. 2A**). Plants were grown for 8 weeks to allow a good plant development and mycorrhization in all plants. Under these conditions, no significant differences in AM colonization were observed (data not shown).

The total number of shoot branches in *spr1, spr2, jai1* and their wild-type was quantified. No changes in the branching pattern of the mutants *spr1* and *jai1*, with and without mycorrhiza were observed when compared to the wild-type (**Fig. 2B**). Similarly, no changes were detected in the phenotype of the wild-type between mycorrhizal and non-mycorrhizal plants (**Fig. 2B**). Conversely, the shoot branching levels were almost 2-fold higher in *spr2* plants than in the wild-type in the absence of AM symbiosis. The differences were even larger in mycorrhizal plants. Here, *spr2* plants colonized by *F. mosseae* and *R. irregularis* were 2.5- and 3.8-fold more branched, respectively, than mycorrhizal wild-type plants (**Fig. 2B**)



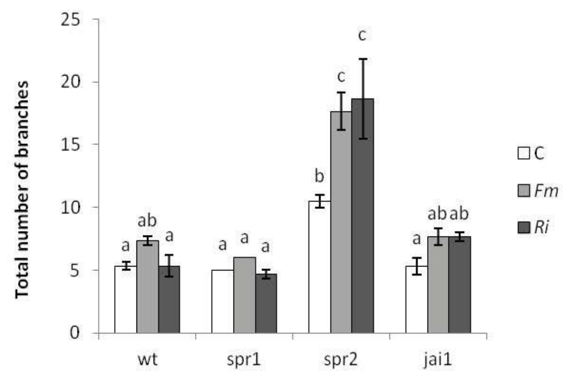


Fig. 2 Analysis of shoot architecture in three JA-related tomato mutants (*spr1*, *spr2* and *jai1*) and their corresponding wild-type (wt) in non-mycorrhizal (C) and mycorrhizal plants colonized by *Funneliformis mosseae* (*Fm*) *and Rhizophagus irregularis* (*Ri*) after 8 weeks of growth. (**A**) Example of plant morphology of the different genotypes with and without mycorrhiza. (**B**) Total number of shoot branches in non-mycorrhizal and mycorrhizal plants from the different genotypes. Data bars correspond to the mean values ± SE of three independent biological replicates. Data not sharing a letter in common differ significantly ($p < 0.05$) according to Fisher's least-significant difference (LSD) test.

From all the JA-related mutants tested, only *spr2* plants presented higher branching respect to wild-type with and without mycorrhiza, and the branching was more pronounced in mycorrhizal *spr2*. Interestingly, the high shoot branching of *spr2* presents similarity with the phenotype described for the SL-deficient tomato plants by Kohlen *et al.* (2012). Therefore, the results might suggest a possible alteration of the SL levels in the *spr2* mutant. *Spr2* mutant is deficient in the oxylipin biosynthesis pathway that includes 2 different branches related to two different types of lipoxygenases (LOXs), 9-LOX and 13-LOX (Wasternack and Hause, 2013). The 13-LOX pathway leads to the biosynthesis of JAs, and the 9-LOX pathway leads to the biosynthesis of other type of oxylipins. Thus, the branched phenotype of *spr2* could be related to an interaction

between the SLs and JAs or other non-JA oxylipins. Therefore, we focused on the phenotypic study of *spr2* for a deeper analysis.

4.2. Comparative analyses of the shoot branching phenotype between *spr2* and *Slccd8* lines

To compare the branching phenotype of *spr2* with that of the SL-deficient plants, *spr2* was grown in parallel with a set of the tomato transgenic lines *Slccd8*, L16 and L09, both altered in the expression of the SL biosynthesis gene *SlCCD8* that encode a carotenoid cleavage dioxygenase (Kohlen *et al.*, 2012). Line L16 is considered an intermediate deficient line, showing a SL reduction of about 50%, while line L09 has a 95% reduction. In these lines, the reduction of SL production was inversely correlated with the levels of lateral shoot branching in the plants (Kohlen *et al*., 2012). In addition, these lines displayed reduced mycorrhizal colonization percentages, decreasing 27% and 65%, respectively compared with the wild-type (Kohlen *et al*., 2012). An exhaustive quantification of different branching orders in *spr2* and lines L16 and L09 compared with corresponding wild-types was performed. For this study, we used both non-inoculated and inoculated plants with the AMF *R. irregularis*. As in the previous experiment, the mycorrhization levels were optimal after 8 weeks of growth, and no significant differences between plants were found at this stage.

Spr2 had a significantly ($p < 0.05$) higher number of branches of all different orders compared to the wild-type. The number of 1st order branches in *spr2* was 1.6-fold higher than in the wild-type (**Fig. 3B**), while branching was 9-fold higher for the 2nd order branches. No branches of the 3rd order were observed in wild-type, whilst *spr2*

presented an average of 3 branches per plant (**Fig. 3B**). Regarding the inoculation with *R. irregularis*, no changes in primary and tertiary order were observed in the mycorrhizal respect to non-mycorrhizal wild-type plants. However, mycorrhizal wild-type plants displayed 5-fold more secondary branching than non-mycorrhizal ones (**Fig. 3B**). In the first experiment, no changes in the branching phenotype were observed between mycorrhizal and non-mycorrhizal wild-type plants (**Fig. 2B**), maybe due to the more rough quantification used, non-classified by orders. As in the case of the wild-type, mycorrhizal *spr2* did not exhibit changes in primary and tertiary branching, but there was a 1.8-fold increase of secondary branching respect to non-mycorrhizal *spr2* (**Fig. 3B**). Therefore, the biggest differences between *spr2* and wild-type are found in the second order of branching. This can be observed in more detail in the supplemental **figure 1SA**. As in the first experiment (**Fig. 2**) the branched phenotype of *spr2* was more prominent upon mycorrhization (**Fig. 3**). In the same way, the SL deficient lines L16 and L09 were also analysed. Primary and tertiary branching were not significantly altered in any of the lines when compared to the wild-type (**Fig. 3D**). However, both lines displayed an increase of secondary branching, being ~ 2.5-fold and ~4-fold more branched, respectively (**Fig. 3D**). Both wild-type and L09 did not present changes in their branching phenotype regarding their mycorrhizal status. In contrast, in L16 the secondary and tertiary branching increased about 2-fold in mycorrhizal plants respect to non-mycorrhizal ones, although it was not statistically significant (**Fig. 3D**). The differences in the second order branching patternis represented in more detail in the supplementary **figure 1SB**. In summary, lines L16 and L09 exhibited more shoot branching than wild-type, being this fact, as expected, more evident in the line L09, corroborating the results described by Kohlen and co-workers (2012). The 2nd order

branches were more abundant in line L09, just as observed in *spr2*. However, after mycorrhizal colonization only line L16 showed a further increase in branching, especially in the secondary branching, as in the case of mycorrhizal *spr2* plants (**Fig. 3**).

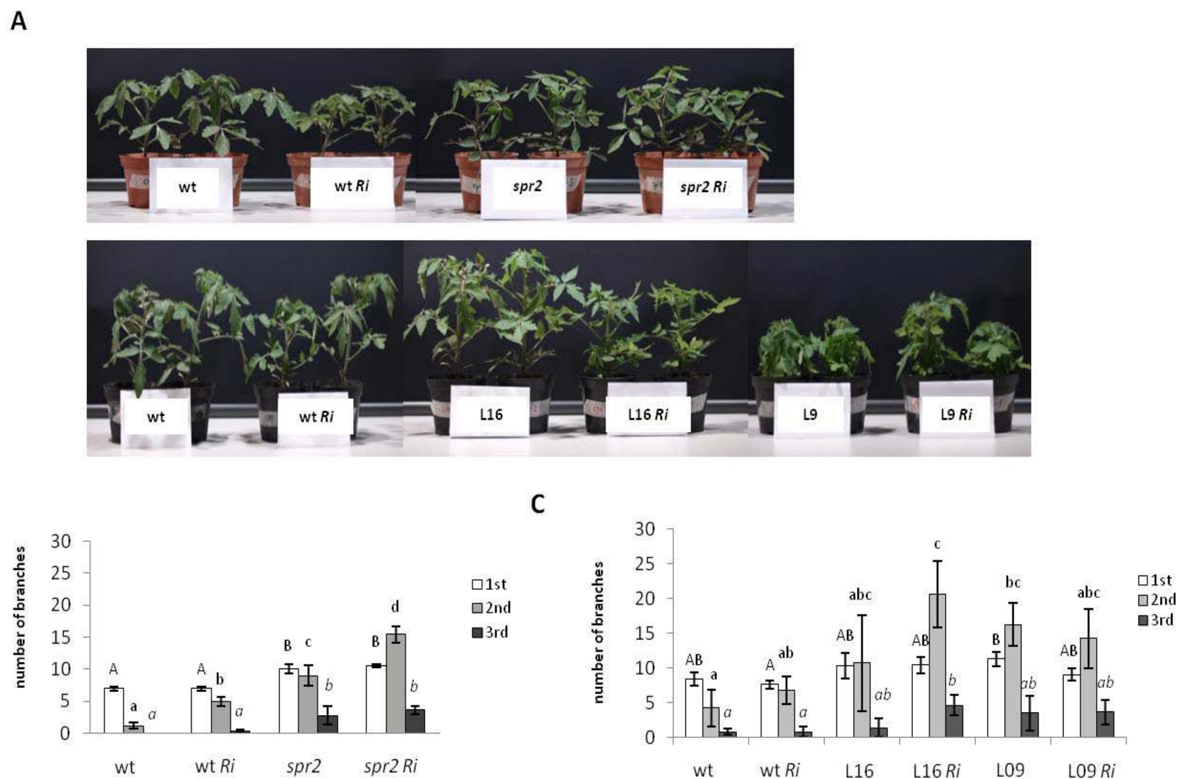


Fig. 3 Shoot architecture analysis of tomato non-mycorrhizal and mycorrhizal plants colonized by *Rhizophagus intraradices* (*Ri*) after 8 weeks of growth. *Spr2* mutants and *Slccd8* transgenic lines (L16 and L09) and their wild-types (wt) were used. An example of plant morphology of the different genotypes with and without mycorrhiza (**A**). Primary (1st), secondary (2nd) and tertiary (3rd) branching in *spr2* (**B**), L16, L09 (**C**) and their corresponding wild-type (wt) with and without mycorrhiza. Data bars represent the mean values ± SE of six independent biological replicates. Data not sharing a letter in common differ significantly ($p < 0.05$) according to Fisher's least-significant difference (LSD) test.

These results suggest a possible SL- threshold level to induce shoot branching, as proposed in the model shown in **Figure 4**. Above and below this level, even if a reduction in SL content occur due to optimal mycorrhization, no additional phenotypic changes in branching would be observed, as observed in the case of wild-type and L09.

The phenotypic analysis suggests that the *spr2* mutant presents a partial reduction in the content of SLs, according to the resemblance of its branched phenotype with Sl*ccd8* silenced line L16. In the proposed model, both *spr2* and L16, with moderately reduced SLs levels, show a similar behaviour presenting additional phenotypic changes after a decrease in SL content upon mycorrhizal colonization (**Fig. 4**). Since *spr2* is a JA-deficient mutant, the results might indicate an interaction between the signaling pathways of SLs and JA. Supporting this hypothesis, lower JA levels in the SL-deficient tomato line L09 were observed (Torres-Vera *et al.*, 2014).

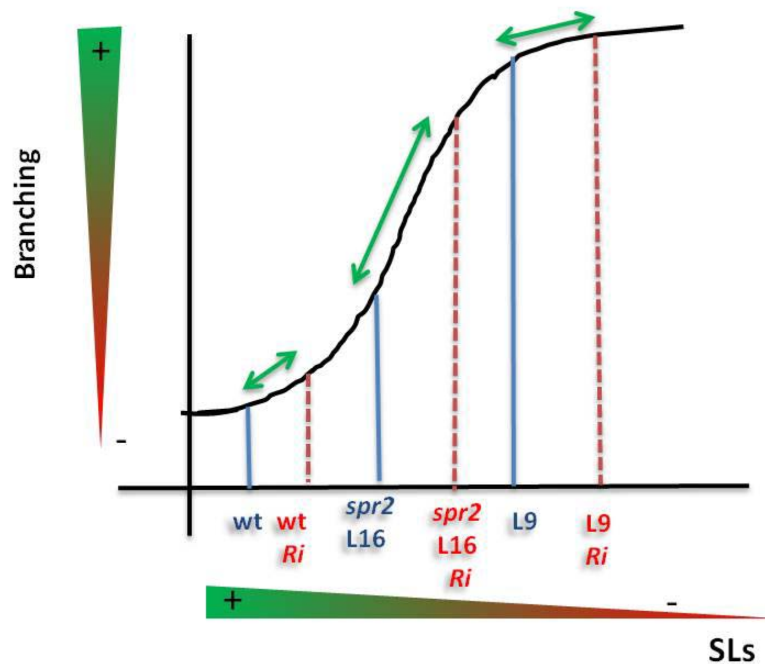


Fig. 4 Proposed model for the regulation of shoot branching by SLs in plants. JA-deficient mutant (*spr2*) and SL-deficient transgenics lines L16 and L09, non-mycorrhizal (blue line) and mycorrhizal plants colonized by *Rhizophagus irregularis* (*Ri*) (red line) were compared.

4.3. Quantification of SL levels in *spr2* mutants by germination bioassay with *P. ramose*

In order to probe our hypothesis about the JA-SLs cross-talk, the SL content in root extracts from the *spr2* mutant and the wild-type were quantified by germination bioassays with *P. ramosa* seeds. For that, samples from non-mycorrhizal and mycorrhizal plants colonized by *R. irregularis* were used. The synthetic germination stimulant GR24 (10^{-7}-10^{-9}M), used as a positive control, always induced germination of *P. ramosa* seeds (up to ~ 70%). Water alone, used as a negative control, did not induce any germination. To avoid saturation of the germination response, a series of root extract dilutions (1:50, 1:100 and 1:200) with demineralised water were used. As expected, the germination percentage inversely correlated with the dilution of the root extracts (**Fig. 5**). *P. ramosa* seed germination induced by root extracts from *spr2* plants did not differ significantly ($p < 0.05$) from that of root extracts from the wild-type, neither in non-mycorrhizal nor mycorrhizal plants (**Fig. 5**). Therefore, the data do not support a partial SL deficiency in *spr2* plants. However, we cannot fully exclude a contribution of SLs to the *spr2* phenotype since the bioassay method used is only an indirect way to quantify the levels of SLs produced by plant roots.

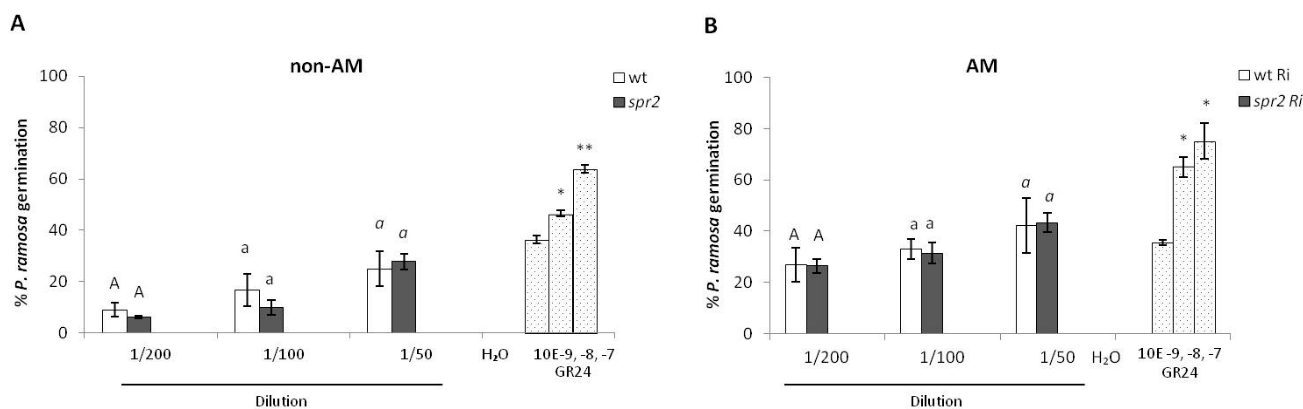


Fig. 5 Germination of *Phelipance ramosa* seeds induced by root extracts from the tomato mutant *spr2* and the corresponding wild-type (wt) in the absence of mycorrhiza (non-AM) (**A**) or in plants colonized by *Rhizophagus intraradices (Ri)* (**B**). GR24 (10^{-7}-10^{-9} M) and demineralized water were used as positive and negative controls, respectively. Within each experiment, different concentrations of the root extracts were tested. Data bars represent the means values ± SE of six independent biological replicates. Data not sharing a letter in common differ significantly ($p < 0.05$) according to Fisher´s least-significant difference (LSD) test.

4.4. Quantification of SL levels in *spr2* mutants by UPLC-MS/MS

To contrast the results obtained in the bioassays, the levels of SLs in the root extracts from *spr2* and wild-type plants were quantified by UPLC–MS/MS, according to peak area. The presence of SLs in a wide variety of plant species has been demonstrated, observing mixtures of several SL compounds (reviewed by Xie *et al*., 2010; Liu *et al.*, 2011; Proust *et al*., 2011). They are synthesized in a few different plant parts, but roots are considered the main site of SL biosynthesis (reviewed by Xie *et al*., 2010). Solanacol, orobanchol and didehydro-orobanchol isomers have been shown to be the major SLs present in tomato plants (López-Ráez *et al.*, 2008; Kohlen *et al*., 2012). Solanacol and the didehydro-orobanchol isomers were not significantly reduced in roots extracts of *spr2* plants compared with wild-type, and orobanchol – was not detected in any of the samples (**Table 1**).

SLs (peak area)	ROOT Genotype	
	Wt	*spr2*
Solanacol	34 ± 14 (a)	56 ± 9 (a)
Orobanchol	nd	nd
didehydro-orobanchol	45 ± 15 (a)	96 ± 39 (a)

Table 1: Levels of SLs in root extracts from the *spr2* tomato mutant and its wild-type (wt). Content of solanacol, orobanchol and didehydro-orobanchol isomers in roots was measured by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS), according to the peak area. Data represent the means of four independent replicates ± standard error (SE), nd indicates non-detected. Data not sharing a letter in common differ significantly ($p < 0.05$) according to Fisher's least-significant difference (LSD) test.

Kohlen and co-workers (2012) observed differences in the localization of the different types of SLs. Orobanchol was abundantly detected in root exudates and extracts but it was also found in the xylem sap of tomato plants. However, neither solanacol nor any of the didehydro-orobanchol isomers were detected in the xylem sap, whereas they were abundant in root exudates and extracts. This differential distribution could suggest that orobanchol might be the SL regulating shoot branching, while solanacol and the didehydro-orobanchol isomers could have a preferential role as signaling molecules in the rhizosphere (Kohlen *et al*., 2012). Low levels of solanacol and DDH were detected in *spr2* and wild-type plants. This could be related to measurement problems in the chromatographic method or during the extraction, since SLs are produced in very low concentrations (pico- and nano-molar concentrations) and an accurate quantification is difficult. Nevertheless, according to the bioassay and the UPLC-MS quantification, *spr2* plants do not seem to display a deficiency in SL levels, although a possible defect in the SL transport from plant roots to shoots should be studied in subsequent experiments.

On the order hand, the high shoot branching of *spr2* mutant could also be related to potential alterations of other phytohormones known as regulators of branching such as CKs and auxins.

4.5. Hormonal profiles in root and shoot extracts from *spr2* plants

To determine whether the increase of shoot branching in the *spr2* mutant was related to alterations in the levels of other architecture-related hormones, the hormonal profiles in leaves and roots of *spr2* and wild-type were compared by UPLC-MS/MS.

As expected, the levels of JA and JA-Ile were reduced in *spr2* compared to the wild-type both in roots and shoots (**Table 2**). JA deficiency in *spr2* was previously described by Li and co-workers (2003). In the leaves, *spr2* presented a significant reduction in JA levels (about 18%), while there were not significant differences in the JA content in roots (**Table 2**). *Spr2* displayed a higher deficiency in JA-Ile, being more than 50% lower than in the wild-type in both leaves and roots (**Table 2**).

SA and ABA are the main defense hormones together with JA, and intensive crosstalk among them has been described (Osakabe *et al.*, 2014; Pieterse *et al.*, 2009), being ABA a carotenoid-derived plant hormone as SLs (López-Ráez *et al.,* 2008; Ohmiya, 2009). ABA has been also related to plant architecture, although the precise role of ABA in the shoot branching is not clear (Rameau *et al*., 2015). No difference in ABA levels were detected between *spr2* and wild-type (**Table 2**), ruling out a possible role of ABA in the branching phenotype of this mutant. SA normally presents an antagonistic cross-talk with JA (Robert-Seilaniantz *et al*., 2011), however, unexpectedly, SA content was not in *spr2* plants (**Table 2**). Finally, as previously mentioned, a cross-talk between

SLs, auxins and CKs has been proposed to control shoot branching (Domagalska and Leyser, 2011). It has been known for decades that auxins inhibit the activation of axillary buds, while CKs have the opposite effect (Müller *et al.,* 2011). Thus, altered levels of auxins and CKs could potentially explain the branched phenotype in *spr2* plants.

The main auxin, IAA, was analysed and higher levels were found in leaves of spr2 plants, although the increase was not statistically significant (**Table 2**). Moreover, no-changes were found in the IAA levels of *spr2* roots compared to the wild-type (**Table 2**). The results, therefore, do not support a role for auxins in the *spr2* phenotype. However, it should be noted that SLs modulate polar auxin transport provided by the asymmetric localization of auxin efflux to control branching, not requiring notable changes in the total auxin levels (Domagalska and Leyser, 2011). Therefore, it might still be that local auxin levels are responsible of the phenotype. In the present study, auxin content was measured in total leaves. It would have been interesting to analyse IAA levels locally at the different tissues along the shoot. On the other hand, that the role of CKs in promoting shoot branching is well stablished (Shimizu-Sato *et al*., 2009). The analysis of kinetin levels (main CK) revealed a decrease, although non-significant, in *spr2* leaves respect to wild-type plants (**Table 2**). Moreover, the level of this hormone decreased significantly 1.7 fold in roots of *spr2* compared to wild-type (**Table 2**). However, since CKs are reported as positive regulators of branching, a deficiency in the CK production would not explain the branched phenotype of *spr2*.

hormone (ng/g dry weight)		LEAF Genotype		ROOT Genotype	
		WT	*spr2*	WT	*spr2*
JA	JA	108 ± 3	88 ± 3 **	126 ± 18	113 ± 14
	JA-Ile	234 ± 46	118 ± 9 *	796 ± 134	322 ± 66 *
ABA	ABA	2206 ± 113	2075 ± 144	95 ± 16	95 ± 15
SA	SA	472 ± 71	476 ± 35	454 ± 44	505 ± 66
auxin	IAA	796 ± 325	1946 ± 581	248 ± 34	219 ± 39
CK	Kinetin	42954 ± 4969	26796 ± 7387	11223 ± 1764	6583 ± 651*

Table 2: Levels of hormones in the *spr2* tomato mutant and its wild-type (wt). Content of jasmonic acid (JA), jasmonic acid–isoleucine conjugate (JA-Ile), indole-3-acetic acid (IAA), Kinetin, abscisic acid (ABA), salicylic acid (SA) in leaves and roots was measured by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS). Data represent the means of four independent replicates ± standard error (SE). Asterisks (*) indicate statistically significant differences according to Fisher's least-significant difference (LSD) test (*p < 0.05 and **p < 0.01).

Nevertheless, the development of shoot branching as a whole is mainly controlled by the auxin/CK balance (Shimizu-Sato *et al.*, 2009; Skoog and Miller, 1957). Thus, the apparently conflicting results found in relation to the IAA and CK content of *spr2* plants could be explained by a hormonal imbalance between auxin/CK in these plants. Accordingly, we compared the IAA/kinetin ratio. A hormonal balance in favor of IAA with respect to kinetin both in leaves and roots of *spr2* plants was observed, being the difference more pronounced in leaves (~ 4-fold) (**Table 3**). Again, the results were unexpected since higher auxin/CK ratio would increase apical dominance instead of branching. However, a possible differentiation between the auxin/CK ratio in the buds and/or main stem could occur, which could explain the observed phenotype (**Fig. 6**). In addition, we cannot exclude that other auxins and/or CKs than the ones analyzed are involved in this process and could be responsible of the *spr2* phenotype. Therefore, further studies with more special resolution locally in the axillary bud outgrowth and

main stem and including other auxins and CKs should be performed in order to clarify any involvement of these hormones in the phenotype observed.

Hormonal balance (ratio)	LEAF Genotype		ROOT Genotype	
	WT	*spr2*	WT	*spr2*
IAA/kinetin	0,019	0,073	0,022	0,033

Table 3: Hormonal balance of auxins/citokinins (CKs) in *spr2* plants respects its corresponding wild-type (wt). Hormonal ratio of indole-3-acetic acid (IAA) / kinetin in shoot and roots was quantified from the average value of hormonal levels (ng/g dry weight of plant) analysed by UPLC-MS/MS.

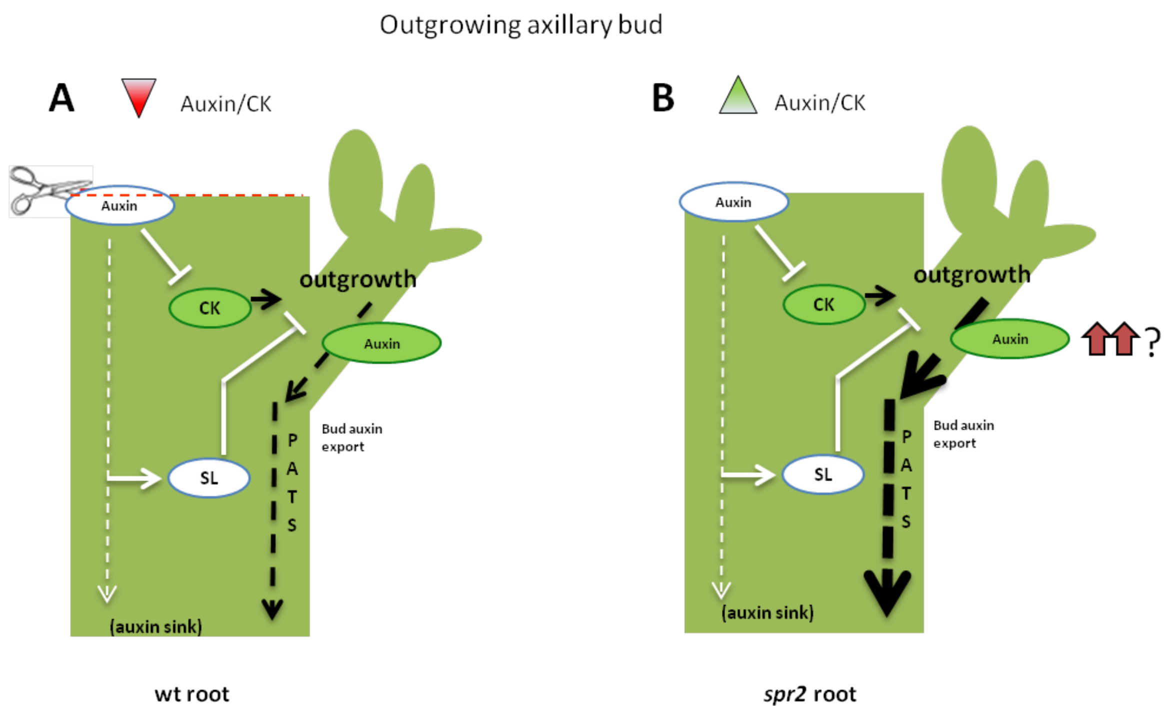


Fig. 6 Schematic illustration of the hypothethical differences between wild-type (wt) and *spr2* plants in the control of bud outgrowth. After decapitation of wt (**A**), the apex as the primary auxin source is removed. The auxin/citoquinin (CK) total balance in the shoot declined. *Spr2* (**B**) presents no-significant changes in the auxins, CKs and strigolactones (SLs) content, being unclear the hormonal regulation. However, *Spr2* presents high level of auxin/CK total balance in the shoot. Black lines and green circles designate active pathways; white lines and circles indicate suppression or down-regulation of the respective pathway. Red arrows designate possible difference in the regulation of *spr2* bud outgrowth respect to wt.

5. CONCLUSIONS

In summary, in this study we explored a possible cross-talk between JA and SL because of the similarity between the branched shoot phenotype of SL- and JA-deficient tomato plants, that suggested a potential deficiency in SL production of *spr2* plants. The levels of SLs and others hormones involved in the regulation of shoot branching were analysed, but a role of these hormones in the branched phenotype of *spr2* plants could not be concluded, and no evidences for a direct or indirect effect of SL in this phenotype was found. Additional and more detailed research will be needed to ascertain the hormone regulation responsible of the branching phenotype of this mutant.

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SUPLEMENTARY INFORMATION

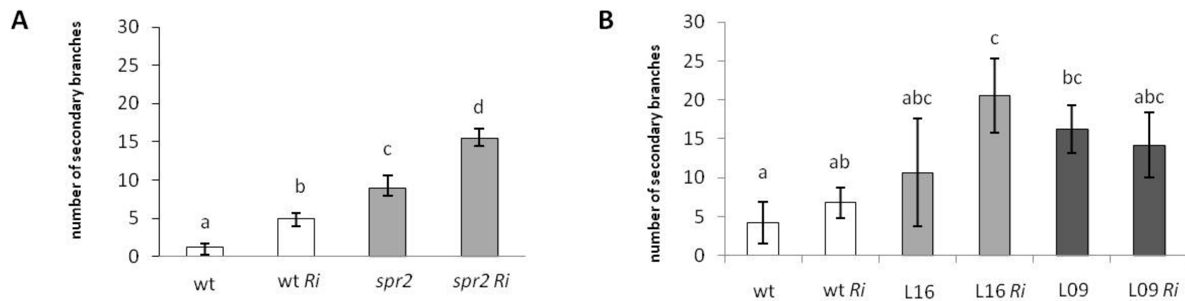

Fig. S1 Secondary (2nd) branching analysis in shoots of tomato plants non-mycorrhizal or colonized by *Rhizophagus intraradices* (*Ri*) after 8 weeks of growth. *Spr2* mutants and *Slccd8* transgenic lines (L16 and L09) and their corresponding wild-types (wt) were analyzed. in *spr2* (**A**), L16, L09 (**B**) and their respective wild-types with and without mycorrhiza. Data bars represent the mean values ± SE of six independent biological replicates. Data not sharing a letter in common differ significantly ($p < 0.05$) according to Fisher's least-significant difference (LSD) test.

CAPÍTULO 4

Regulación de las estrigolactonas por la sistemina. Posible efecto en la micorrización.

Rocío Torres-Vera, Juan García, Estefanía Berrio, Bettina Hause, María J. Pozo, Juan A. López-Ráez

RESUMEN

Las estrigolactonas (SLs) son fitohormonas con diversas funciones tanto exógenas como endógenas en las plantas. Bajo condiciones de estrés nutricional, las plantas exudan SLs a la rizosfera. Por un lado, inducen la germinación de semillas de plantas parásitas de raíz y por otro, inducen la ramificación de las hifas de los hongos micorrícicos arbusculares (HMA). Por consiguiente, las plantas deficientes en SLs muestran bajos niveles de colonización micorrícica y de germinación de semillas de plantas parásitas de raíz. La sistemina es una hormona peptídica, procesada a partir de su precursor más grande denominado prosistemina (PS) que está presente en ciertas Solanáceas. La sistemina induce la biosíntesis del ácido jasmónico (JA) en respuesta al ataque de herbívoros y patógenos en las hojas de las plantas. En estudios previos, observamos que las plantas de tomate deficientes en la producción de PS (*ps-*) muestran bajos niveles de colonización micorrícica y de producción de SLs. Estos resultados mostraron por primera vez la presencia de sistemina en las raíces, su participación en la simbiosis MA, y una posible correlación con las SLs. En el presente estudio, hemos analizado el posible rol de la sistemina como regulador de la producción de SLs mediante análisis transcripcionales y hormonales usando plantas *ps-* y tratamientos exógenos con sistemina. Por otro lado, con el fin de dilucidar si las SLs también están involucradas en otras etapas de la colonización micorrícica, se analizó el

desarrollo arbuscular en plantas deficientes en SLs (*Slccd7* y *Slccd8*) y en plantas alteradas en PS (*ps-* y *ps+*) mediante microscopía confocal. Nuestros resultados sugieren que la sistemina regula la producción de SLs, pero que también presenta un rol independiente a ellas en la regulación de la simbiosis MA.

In preparation, 2017

Strigolactone regulation by systemin. Possible effect in mycorrhization.

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1. ABSTRACT

Strigolactones (SLs) are phytohormones with diverse exogenous and endogenous functions in plants. Under conditions of nutritional stress, the plants exude SLs into the rhizosphere. On one hand, they induce the seed germination of root parasitic plants and on the other, they induce hyphal branching of arbuscular mycorhizal fungi (AMF). Accordingly, SL-deficient plants show low levels of mycorrhizal colonization and of seed germination of root parasitic plants. Systemin is a peptide hormone, processed from a larger precursor known as prosystemin (PS) and present in the Solanaceae. Systemin induces jasmonic acid (JA) biosynthesis in response to herbivore and pathogen attacks in leaves. In previous studies, we showed that PS-deficient (*ps-*) tomato plants displayed low levels of mycorrhizal colonization and SL production. These results showed for the first time the presence of systemin in the roots, its involvement in AM symbiosis, and a possible correlation with SLs. In the present study, we have analyzed the possible role of systemin as regulator of SL production by studing *ps-* plants treated with exogenous systemin by hormonal and transcriptional analyses. Moreover, in order to envisage if SLs are also involved in further stages of mycorrhizal colonization, arbuscule development was analysed by confocal microscopy

in SL-deficient plants (*Slccd7* and *Slccd8*) and in PS-altered plants (*ps*- and *ps+*). Our results suggest that systemin regulates the production of SLs, but that it also presents a SL-independent role in mycorrhizal regulation.

Keywords: Apocarotenoids, AM symbiosis, arbuscule development, phosphorous starvation, root, strigolactone, systemin, tomato.

2. INTRODUCTION

Strigolactones (SLs) are a family of plant hormones derived from the carotenoids (López-Ráez *et al*., 2008a) through the conversion of all-*trans*-β-carotene to 9-*cis*-β-carotene mediated by a β-carotene isomerase (D27) (Alder *et al*., 2012). This compound is then transformed into carlactone by sequential oxidative cleavage by two carotenoid cleavage dioxygenases (CCD7 and CCD8) (Alder *et al*., 2012), thus SLs belong to the apocarotenoids, as the phytohormone abscisic acid (ABA) (Walter, 2013) (**Fig. 1**). Carlactone is then converted into SLs by the action of different cytochrome P450 (MAX1) (Zhang *et al*., 2014). SL perception and signaling require an α/β-hydrolase (D14) and a F-box leucine-rich repeat protein (MAX2) (Hamiaux *et al*., 2012) (**Fig. 1**). SL binding to this complex induces the degradation of the repressor D53 (Class I Clp ATPase) via the ubiquitin-proteasome, leading to the expression of SL-responsive genes (Zhou *et al*., 2013). SLs are biosynthesized mainly in the roots and present diverse functions in the plants. On one hand, SLs are phytohormones that regulate several endogenous functions both in roots and shoots (Cheng *et al*., 2013). On the other hand, they play a dual role as signalling molecules in the rhizosphere, inducing germination of root parasitic plant seeds of the Orobanchaceae (Bouwmeester *et al*.,

2003) and stimulating hyphal branching of arbuscular mycorrhizal fungi (AMF) during the pre-symbiotic phase, favouring the mycorrhizal colonization of plant roots (Akiyama *et al*., 2005). In fact, SL-deficient plants are less infected by root parasitic weeds and less colonized by AMF respect to their corresponding wild-types (Gómez-Roldán *et al*., 2008; Gutjahr *et al*., 2012; Kohlen *et al*., 2012; Koltai *et al*., 2010). So far, no role for SLs in further stages of mycorrhizal colonization or functioning - symbiotic phase - such as hyphal growth within the roots or arbuscule development, has been described. Besides its role in mycorrhizas, a role of SLs in another important mutualistic interaction as the *Rhizobium*-legume symbiosis has been shown (Foo and Davis, 2011; Peláez-Vico et al., 2016; Soto *et al*., 2010).

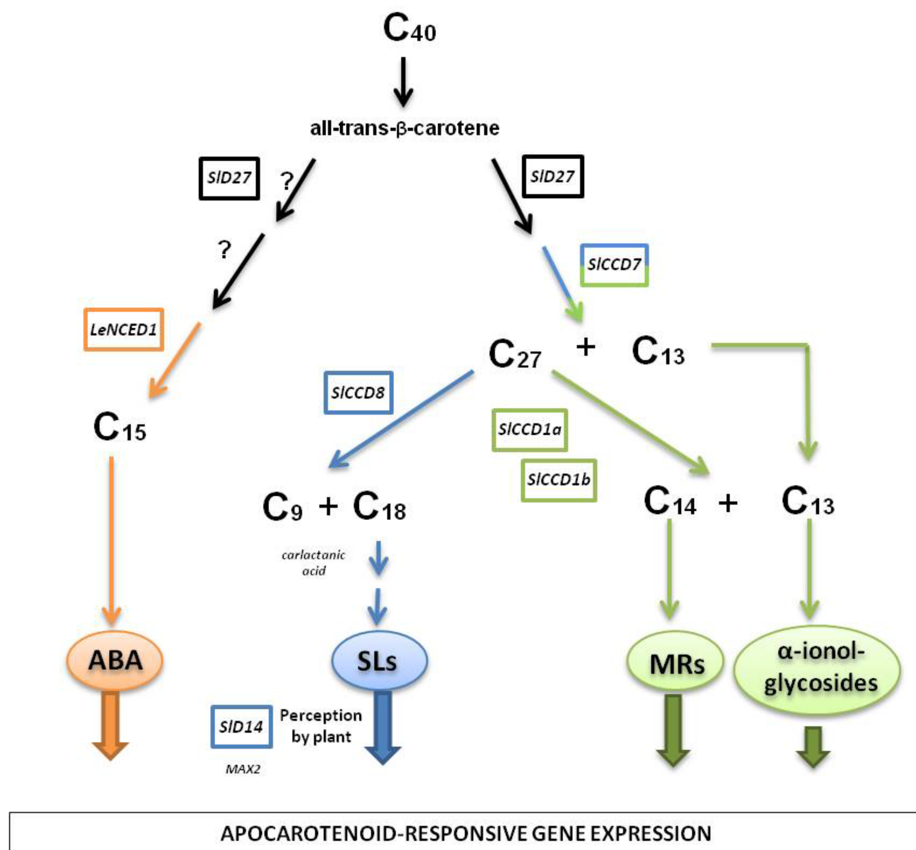

Fig. 1 Schematic summary of apocarotenoid biosynthesis/signalling pathway, starting from the C40 carotenoid precursors. Circles indicate the end-products (SLs, MRs, α-ionol glycosides and ABA) and boxes indicate marker genes analysed by qRT-PCR in this study. The biosynthesis/signaling pathway of each apocarotenoid is indicated with colors, being orange for ABA, blue for SLs and green for MRs/α-ionol glycosides. Question marks indicate steps non-characterized so far.

AMF establish a mutualistic symbiosis with over 80% of plant species (Smith and Read, 2008). The beneficial relationship offers to the plant an improved nutritional uptake, mainly inorganic phosphorus (Pi), especially under nutritional deficient conditions (Smith and Read, 2008). In turn, the fungus obtains from the host plant the necessary carbohydrates for its development and survival (Smith and Read, 2008). The nutritional exchange between both organisms occurs through specialized structures denominated arbuscules, formed in the inner cortex of the host root and characteristics of this symbiosis (**Fig. 2**) (Walter, 2013). The arbuscules present different stages along their life cycle and usually, the mycorrhizal roots possess a heterogeneous population of individual arbuscules (Walter, 2013). The arbuscules are able to develop and mature in a few days (usually 3-4 days), followed by a period of degeneration (forming septa) that finalizes with a period of collapse and death (**Fig. 2**) (Walter, 2013). The whole life cycle of an arbuscule appears to be completed within 7–10 days.

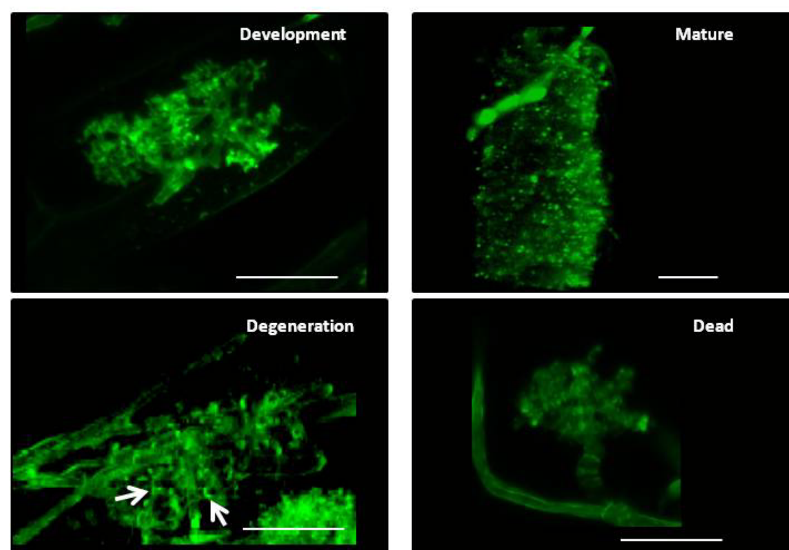

Fig. 2 Confocal images of the different arbuscular stages of the mycorrhizal fungus at the symbiotic phase. Arbuscules were stained with WGA-Alexa Fluor 488 and observed by confocal laser scanning microscopy. The degeneration stage can be recognized by the formation of septa on arbuscule branches (arrow). Scale bars =20 µm.

In addition to SLs, other apocarotenoids as abscisic acid (ABA), mycorradicins (MRs) and ciclohexenones derivatives called α-ionol glycosides are involved in the AM symbiosis. ABA is produced by the oxidative cleavage catalyzed by 9-*cis*-epoxy-carotenoid dioxygenases (NCEDs) (**Fig. 1**) (Walter, 2013) and seems to present an important role in mycorrhizal establishment and arbuscule development (Herrera-Medina *et al.*, 2007; Walter, 2013), although its specific function is not well defined yet. MRs/α-ionol glycosides are known as the yellow pigment, characteristic of mycorrhizal roots (Walter, 2013). They are synthesized by sequential oxidative cleavage by the enzymes CCD7 and CCD1 (**Fig. 1**) (López-Ráez *et al.*, 2015; Vogel *et al.*, 2010) and although their specific function is not clear, they seem to play a role in arbuscule development, regulating its turn-over (Walter *et al.*, 2013). The establishment and functioning of the mycorrhizal symbiosis implies a fine regulation by phytohormones in the host plant where, besides SLs and ABA, other phytohormones as auxin, gibberellins (GAs), ethylene (ET) and jasmonic acid (JA) play a key role in the control of the pre-symbiotic and/or symbiotic phases of mycorrhization (Gutjahr, 2014; Pozo *et al.*, 2015). In particular, JA and its derivates (the jasmonates, JAs) have received special attention, since a positive and negative regulatory role have been proposed for them and experimental data are controversial (Fernández *et al.*, 2014; Gutjahr and Parniske, 2013; Wasternack and Hause, 2013). It has been proposed that JAs affect mycorrhizal establishment by interfering in the host cell wall expansion and arbuscule development (Gutjahr and Paszkowski, 2013). A recent study analyzed the mycorrhizal colonization levels in different tomato lines altered in JA-signalling/biosynthesis, detecting that major changes were only observed in plants with altered levels of prosystemin (PS) (Fernández, 2013). PS is the precursor of

systemin, a 18 amino acids peptide hormone present in the Solanaceae family. Systemin promotes JA biosynthesis and signalling, regulating plant defenses in the leaves against biotic stressors as pathogens and herbivores (Ryan and Pearce, 2003). In PS overexpressing lines (*ps+*) an induction of *Funneliformis mosseae* colonization of about 2-fold, compared to the corresponding wild-type was observed. In contrast, in silenced lines (*ps-*) the colonization levels were negligible (Fernández, 2013).
Fernández and co-workers also analyzed the SLs levels in *ps+* and *ps-* plants, observing a reduction of SL levels in root extracts and exudates from *ps-* plants, while in *ps+* plants a higher production of SLs compared to wild-type was detected (Fernández, 2013). The studies pointed to a key role of systemin in the regulation of the mycorrhizal symbiosis, at least partially through the regulation of SL biosynthesis.

In the present study the regulation of SL biosynthesis by systemin was investigated. To this end, we analysed the expression of marker genes and SL content by seed germination bioassays in PS-altered plants and plants treated with exogenous systemin. In addition, the potential role of SLs and systemin on late stages of mycorrhization, and particularly in arbuscule development was explored. For this, arbuscule development was analyzed in detail by confocal microscopy comparing SL-deficient and PS-altered tomato plants.

3. MATERIAL Y METHODS

3.1. Plant material and growth common conditions

Tomato seeds (*Solanum lycopersicum*) from transgenic plants constitutively expressing the PS encoding gene in sense (*ps+*) (McGurl *et al*., 1994), and antisense orientation

(*ps*-) (McGurl *et al*., 1992) and their respective wild-type (cv Betterboy) were used. Seeds for these lines were kindly provided by Prof. Clarence Ryan (Michigan State University, US). Likewise, SL knock-down transgenic tomato plants *Slccd8* (line L09 in background Craigella) and *Slccd7* (line 7071 in background M82) were used. Seeds for *Slccd7* and its corresponding wild-type were provided by Dr. Michael Walter (Leibniz Institute of Plant Biochemistry, Germany). *Slccd8* and *Slccd7* plants are antisense CCD8 and CCD7, RNA interference (RNAi) lines, respectively (Kohlen *et al*., 2012; Vogel *et al*., 2010). Tomato seeds were sterilised and germinated according to López-Ráez *et al*. (2010b). The seedlings were grown and randomly distributed in a greenhouse at 24/16 °C with 16/8 h photoperiod and 70 % humidity. The plants were harvested after 8 weeks of growth. Root material was immediately frozen in liquid nitrogen and stored at - 80 °C or used directly for several analyses. Six biological replicates per plant genotype and treatment were analysed. Additionally, seeds of the root parasitic plant *Phelipanche ramosa* (collected from a tomato field) were used for the germination bioassays (kindly provided by Dr. Maurizio Vurro and Angela Boari, Instituto delle Produzioni Alimentari, Bari, Italy).

3.2. Systemin effect on SL production

3.2.1. Systemin treatment

The transgenic tomato line *ps*- and its corresponding wild-type were used. One-week-old seedlings were transplanted into 3 L plastic containers and grown hydroponically with Long Ashton nutrient solution (Hewitt, 1966) containing 25 % or 100 % of the standard Pi concentration with constant aeration. The nutrient solution was replaced

once a week. After 4 weeks of growth, half of the plants were treated with 1 nM systemin (GeneScript) during 1 h, using non-treated plants as controls. Then, the plants were incubated in nutrient solution without systemin for 24 h. The roots were then harvested and immediately frozen in liquid nitrogen and stored at -80 ºC until use. Root material was used for molecular analyses and germination bioassays.

3.2.2. Strigolactone analysis using germination bioassays with *Phelipanche ramosa* seeds

Collection of 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.

Preconditioning and germination bioassays with *P. ramosa* seeds: Preconditioning and germination bioassays were adapted from Matusova *et al*. (2004) and performed under sterile conditions. The seeds of *P. ramosa* were surface sterilized in 2 % sodium hypochlorite containing 0.02 % (v/v) Tween 20 for 10 min, and rinsed thoroughly with sterile demineralized water. Subsequently, the seeds were dried for 60 min in a laminar air flow cabinet. Approximately, between 50-100 seeds were spread on a glass-fibre filter paper (GFFP) disc (9 mm diameter) and put into sterile Petri dishes (9 cm diameter) covered with Whatman filter paper wetted with 3 ml of demineralized water. Petri dishes were sealed with parafilm and incubated for preconditioning at 21

°C in darkness during 10-12 days. After preconditioning of the seeds, aliquots (50 µl) of root extracts were added to disc per duplicates. Serial dilutions from each extract were previously made. The synthetic germination stimulant GR24 (10^{-10} and 10^{-11} M) and demineralized water were included as positive and negative controls in each bioassay. After 7 days, the germinated and non-germinated seeds were counted using a Nikon Eclipse 50i microscope of bright field. Seeds were considered germinated when the radicle had protruded through the seed coat (Matusova *et al*., 2004).

3.2.3. RNA extraction and gene expression analysis by qRT-PCR

Total RNA from roots was extracted using Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA), purified through a silica column using the NucleoSpin RBA Clean-up Kit (Macherey-Nagel, Düren, Germany). Before storage at -80°C, RNA was quantified using a Nanodrop 1000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its integrity checked by gel electrophoresis. The first-strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The expression of marker genes for the different apocarotenoid signalling/biosynthesis pathways was analysed in roots from tomato plants by real-time quantitative polymerase chain reaction (qRT-PCR). All qRT-PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ5 system (Bio-Rad), using 1 µl of single stranded cDNA (diluted 1:10) and specific primers for each gene. The following genes were used as molecular markers for the different treatment responses or biosynthetic pathways: *LePT2* (Pi starvation), *SlD27* (apocarotenoids),

SlCCD8 and *SlD14* (SLs), *SlCCD7* (SLs, MRs/α-ionol glycosides), *LeCCD1a* and *LeCCD1b* (MRs/α-ionol glycosides) and *LeNCED1* (ABA) (**Table S1, Fig. 1**).

The amplification protocol included an initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s. The specificity of the different amplicons was checked by a melting curve analysis (from 70 to 100°C) at the end of the amplification protocol. Three independent biological replicates were analyzed per treatment and time point. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method. Expression values were normalized using the housekeeping gene *SlEF-1α*, which encodes for the tomato elongation factor-1α.

3.2.4. Statistical analysis

Data from the germination bioassays and gene expression analyses were subjected to one-way analysis of variance (ANOVA) using the software Statgraphics Centurion (version XVI) for Windows. When appropriate, Fisher's LSD test was applied to determine the statistical significance.

3.3. SL and systemin effect on arbuscule development

3.3.1. Mycorrhizal treatment

For mycorrhizal experiments, the tomato lines *ps-*, *ps+*, *Slccd7*, *Slccd8* and theirs corresponding wild-types were used. Tomato seeds were sterilized as described above. One-week-old the seedlings were transplanted into pots with sterile expanded clay.

For mycorrhizal treatment, the AMF *Rhizophagus irregularis* (BEG 121) and *Funneliformis mosseae* (BEG 12), renamed from *Glomus intraradices* and *Glomus mosseae*, respectively (Schüßler and Walker, 2010), were used adding 10% expanded clay containing fungal propagules. To homogenize the microbial populations in the rhizosphere except the AMF, a filtrate of the mycorrhizal inocula, excluding the AMF propagules, were applied to the control, non mycorrhizal plants. The plants were watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25 % of standard Pi concentration.

3.3.2 Analysis of arbuscule development

Ink staining: Mycorrhizal root segments were incubated in 10 % KOH at 4 °C during 2 days. Then, roots were washed with distilled water three times and acidified with 2% acetic acid. After that, they were stained with 5% dark ink (Sheaffer, CT, USA) in 2 % acetic acid at 90 °C during 10 min. The excess of ink was removed by 3 washes with distilled water (Vierheiling *et al*., 1998). Ink-stained roots were kept in 100 % glycerol and the AM structures were observed and quantified through the Gridline Intersect Method (Giovannetti and Mosse, 1980) by bright field microscopy.

Fluorophore staining: Mycorrhizal root segments were treated during 4 h with 50 % EtOH. After EtOH was removed, 20 % KOH were added and the roots incubated for 10 min at 90 °C and then rinsed with distilled water 2 times. Next, 0.1 m HCl was added and incubated during 2 h and washed twice with distilled water. Finally, they were incubated during 6 h in darkness in PBS buffer with wheat germ agglutinin (WGA) conjugated with the fluorophore AlexaFluor488 (0.2 µg/ml, Molecular Probes, Eugene,

OR, USA), which stains the chitin polymers of fungal cell walls. Images were collected on a confocal laser scanning microscopy LSM710 (CLSM) (Zeiss, Oberkochen, Germany). AlexaFluor488 was excited at 495 nm, and emitted light was collected from 519 nm. Optical sections were acquired at 0.3- to 0.5-µm intervals. Images were processed using the ImageJ software (Wayne Rasband, National Institutes of Health, USA).

4. RESULTS

4.1. Effect of systemin exogenous application on SL production in *ps-* plants

The effect of exogenously applied systemin in the PS-deficient transgenic line *ps-* on SL production was investigated. As described above, *ps-* plants displayed a reduction on SLs respect to the corresponding wild-type (Fernández, 2013) suggesting that systemin might regulate SL biosynthesis. To see whether the potential role of systemin on SL production is a direct effect, *ps-* plants and corresponding wild-type Betterboy, were grown hydroponically under conditions of P deficiency for 4 weeks to increase SL production. Then, part of the *ps-* plants was treated with 1 nM systemin as described in “Materials and Methods”. Subsequently, SL levels in the root extracts were estimated by germination bioassays with seeds of *P. ramosa*. Concentrations of the root extracts from the different samples were previously normalized by diluting the samples to the same volume/fresh root weight. GR24 (10^{-10} and 10^{-11} M) and demineralized water were used as positive and negative controls, respectively. High levels (60 %) of seed germination was observed in extracts from non-treated wild-type plants, while extracts from *ps-* plants showed lower levels of seed germination, about

50 % reduction compared to the wild-type (**Fig. 3**). When *ps-* plants were treated with systemin (*ps-* +S), the stimulatory germination activity in the root extracts increased more than 2-fold respect to *ps-* untreated plants, showing even higher SL levels than in wild-type plants (**Fig. 3**). This result suggests a direct effect of systemin in the regulation of SL production.

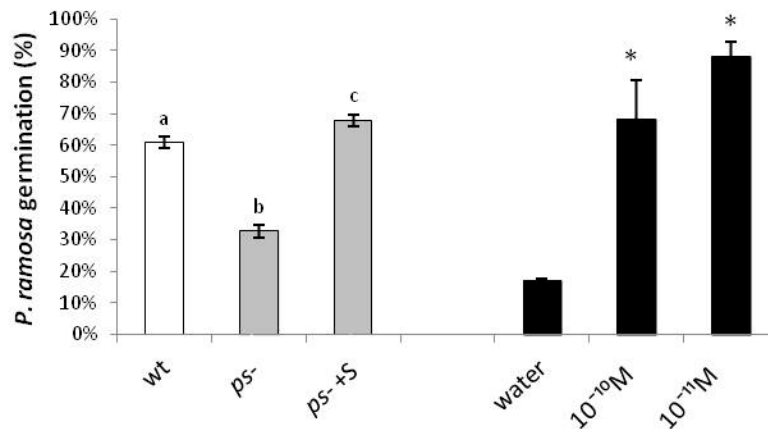

Fig. 3 Germination of *Phelipanche ramosa* seeds induced by root extracts of the prosystemin antisense tomato plants (*ps-*) with exogenous 1 nM systemin treatment (*ps-* +S) and its corresponding wild-type (wt). GR24 (10^{-10} and 10^{-11}M) and demineralized water were used as positive and negative controls, respectively. Germination rate after application on 1:50 dilution of the corresponding root extracts is depicted. Data Bars represent the means values ± SE of six independent biological replicates. Data not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's least-significant difference (LSD) test.

4.2 Transcriptional regulation of SL-related genes by systemin application

Because of the effect systemin application on the germination stimulatory activity of *P. ramosa* seeds, a possible transcriptional regulation of the SL biosynthesis and signalling genes by systemin was studied. In this analysis, *ps-* and wild-type plants grown under optimal nutritional conditions (with 100% Pi) were included and used as controls. Gene expression levels were analysed in root samples by qRT-PCR. The expression of the gene *LePT2*, which encodes for a high affinity Pi transporter (Liu *et al.*, 1998), was used

as a Pi starvation marker. When the wild-type plants were exposed to Pi starvation, the *LePT2* expression was induced 17 fold compared to control plants grown under normal Pi conditions (**Fig. 4**), indicating that plants were indeed sensing the Pi deficiency. When wild-type plants were grown under stress conditions and treated with systemin, the expression of *LePT2* was inhibited about 14 fold respect to the non-treated, Pi stressed wild-type plants, returning *LePT2* to basal expression levels as in control plants (**Fig. 4**). This result suggests a negative regulation by systemin of Pi deficiency responses. On the other hand, the expression levels of *LePT2* in *ps-* plants were not altered under Pi deficient conditions respect to the control plants (**Fig. 4**). However, after addition of systemin, the expression of *LePT2* was significantly ($P < 0.05$) induced (about 20 fold) when compared to non-systemin treated plants (**Fig. 4**). Regarding SL biosynthesis or signalling several marker genes were monitored. The gene *D27* is involved in SL biosynthesis and encodes an enzyme with isomerase activity converting all *trans*-β-carotene into 9-*cis*-β-carotene (**Fig. 1**) (Alder *et al*., 2012; Chapter 1). Wild-type plants grown under Pi deficiency displayed a 200 fold increase in *SlD27* expression when compared to control plants (**Fig. 5A**), while systemin application reduced its expression to basal levels (**Fig. 5A**). A different behaviour was observed in *ps-* plants. No differences in gene expression were observed under Pi deficient conditions, whilst systemin application highly increased *SlD27* expression (about 250 times) compared to non-treated plants (**Fig. 5A**). In addition to *SlD27*, the SL biosynthesis genes *SlCCD7* and *SlCCD8* were analysed, which encode the carotenoid cleavage enzymes 7 and 8, respectively (**Fig. 1**) (Alder *et al*., 2012; Kohlen e*t al*., 2012; Vogel *et al*., 2010). As expected, *SlCCD7* expression increased in wild-type plants grown under stress conditions with respect to those growing without nutritional stress (about 6 times)

(**Fig. 5B**). When systemin was applied to stressed plants, a further increase of about 50% was observed (**Fig. 5B**), suggesting a synergistic effect of low Pi and systemin. In the case of *ps-* plants*,* expression of *SlCCD7* did not change under Pi starvation and, although not significantly, it was a slightly decreased after systemin application (**Fig. 5B**). As for *SlD27* and *SlCCD7*, the expression of *SlCCD8* also increased in wild-type plants grown under Pi stress conditions (about 7 times) (**Fig. 5C**). However, a further decrease of about 5-fold was detected when systemin was applied to Pi-starved plants (**Fig. 5C**). As in the case of *SlD27*, a different behavior for *SlCCD8* was observed in *ps-* plants. Only systemin application to Pi-starved plants significantly ($P < 0.05$) induced its expression levels, while no effect of Pi deficiency was observed (**Fig. 5C**). In addition to the biosynthesis genes, the expression of the gene encoding for the putative SL receptor in tomato, *SlD14,* was studied. The gene *D14* encodes for an α/β-hydrolase enzyme and binding to this receptor activates the SL signaling pathway (**Fig. 1**) (Hamiaux *et al*., 2012). Although no significant, the expression of *SlD14* in wild-type plants showed the same pattern observed for *SlD27* and *SlCCD8*. Its expression increased under low Pi conditions, and this increase was abolished after systemin application (**Fig. 5D**). In the *ps-* line, the expression pattern also resembled those of the SL biosynthesis genes; no changes under Pi deficiency, and induction after systemin application (**Fig. 5D**).

In summary, SL marker genes exhibit a differential regulation between *ps-* and wild-type plants in response to low Pi and systemin treatment, suggesting an involvement of systemin in the transcriptional regulation of both SL biosynthesis and signalling.

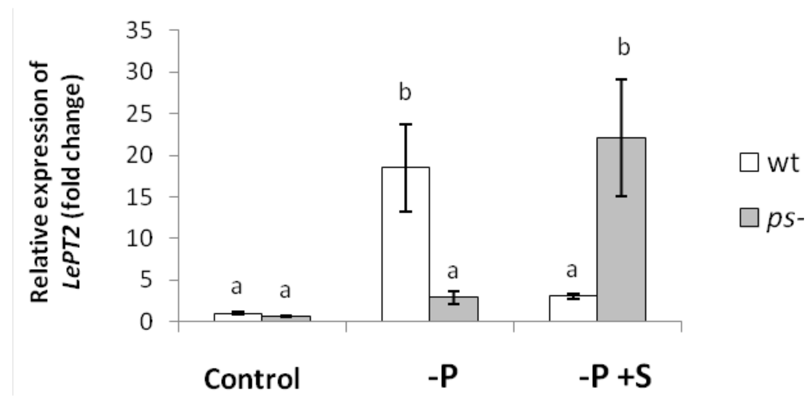

Fig. 4 Gene expression analysis by qRT-PCR for the Pi deficiency marker gene *LePT2* in tomato roots from the PS-deficient plant *ps-* and its corresponding wild-type (wt). The expression levels were analysed in three different growth conditions; Pi optimal conditions (control), Pi deficiency (-P) and Pi deficiency plus exogenous application of 1 nM systemin (-P+S). Data bars represent the mean values ± SE of three independent biological replicates. Data not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's least-significant difference (LSD) test.

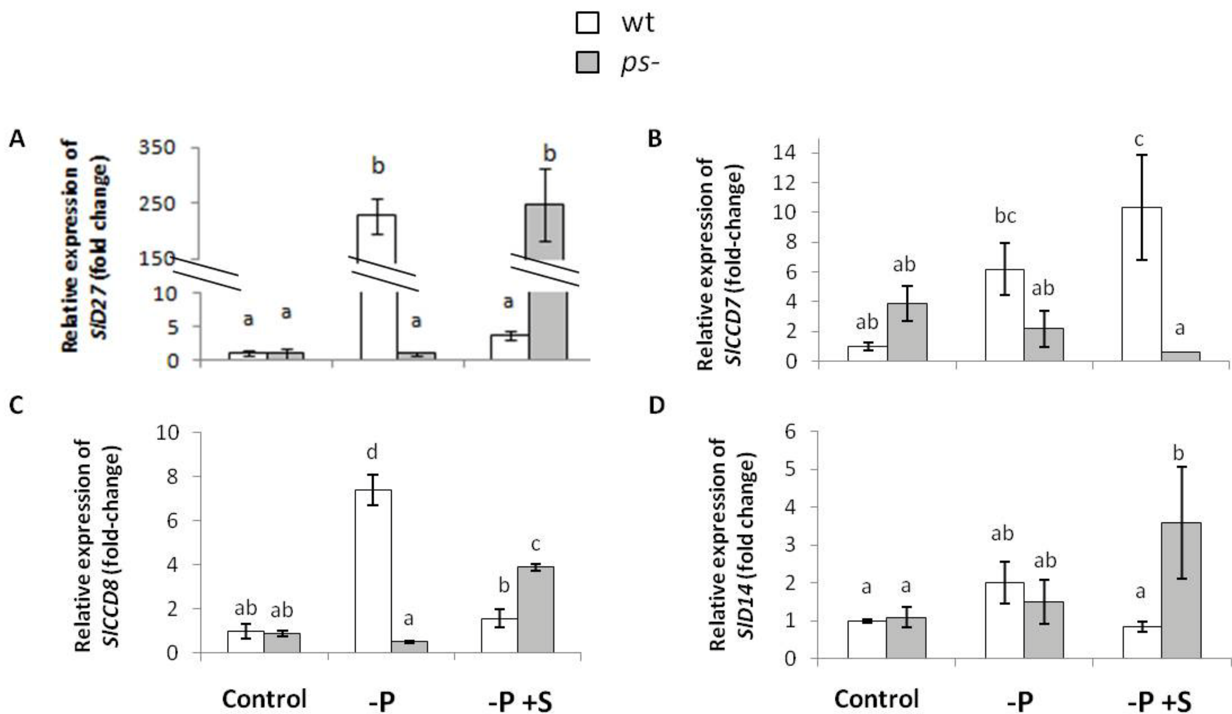

Fig. 5 Gene expression analysis of SL-related genes. The expression levels of the SL biosynthesis genes *SlD27* **(A)** *SlCCD7* **(B)** and *SlCCD8* **(C)**, and the SL perception gene *SlD14* **(C)** were analysed. See legend in Fig. 4.

4.3 Expression of genes related to other apocarotenids

CCD7, in addition to SLs, is involved in the biosynthesis of the apocarotenoids MRs and α-ionol glycosides, which constitute the yellow pigment typical of mycorrhizal roots (**Fig. 1**) (López-Ráez et al., 2015; Vogel et al., 2010; Walter, 2013). Another carotenoid cleaveage enzyme associated to the production of MRs and α-ionol glycosides is CCD1, which cleaves the product from CCD7 (**Fig. 1**) (Walter, 2013). CCD1 is also involved in the biosynthesis of the flavour compound β-ionone (Simkin et al., 2004; Walter, 2013). In tomato, CCD1 is represented by two different isoforms - *SlCCD1a* and *SlCCD1b* (**Fig. 1**) (López-Ráez *et al*., 2015; Simkin *et al*., 2004; Walter *et al*., 2013). When the expression of these two *SlCCD1* isoforms was analyzed, no significant differences were observed among the different treatments or t genotypes (**Fig. 6A, B**).

As mentioned above, ABA also belongs to the apocarotenoids family. It is produced by oxidative cleavage by the action of a 9-*cis*-epoxycarotenoid dioxygenase (NCED1) (**Fig. 1**) (Thompson *et al*., 2000). Thus, this enzyme catalysis a key step in the biosynthesis of ABA and is, therefore, considered a marker for this biosynthetic pathway. Wild-type plants grown under Pi starvation presented a 4 fold increase in the expression of *LeNCED1* respect to control plants. However, under Pi starvation but upon systemin application, *LeNCED1* expression was inhibited returning to the basal levels found in control plants (**Fig. 6C**). No induction of *LeNCED1* was detected in *ps-* plants under Pi deficiency (**Fig. 6C**). However, when systemin was applied under the Pi stress condition, a significant induction (6 fold) of *LeNCED1* expression was observed (**Fig. 6C**).

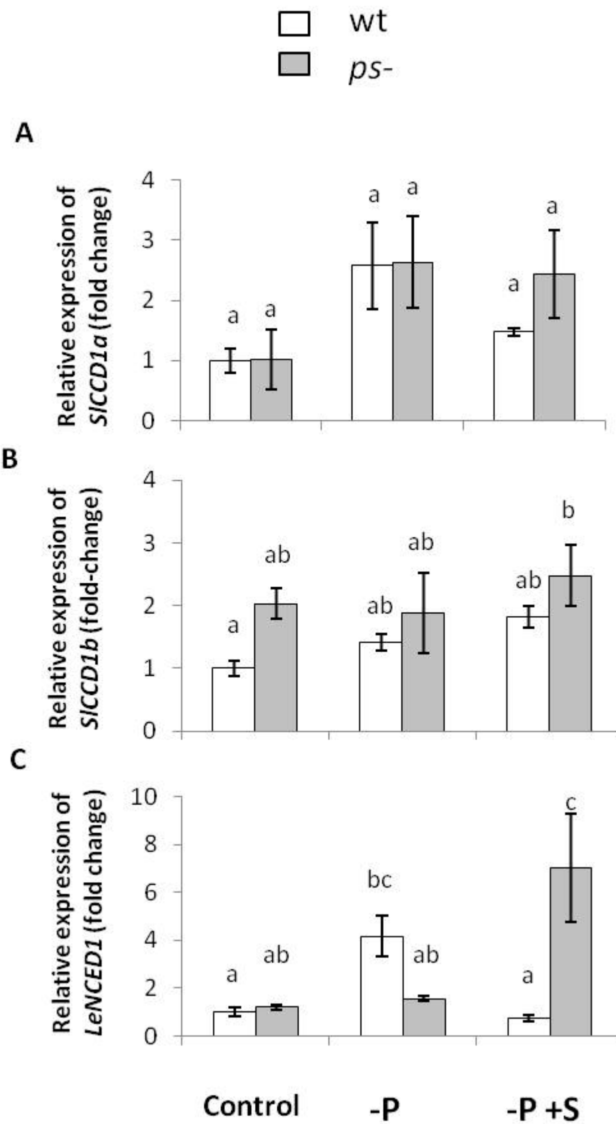

Fig. 6 Gene expression analysis of other apocarotenoid biosynthesis genes not related to SL biosynthesis. The expression levels of the MR/α-ionol glycoside biosynthesis genes *SlCCD1a* **(A)**, *SlCCD1b* **(B)** and the ABA byosinthesis gene *LeNCED1* **(C)** were analysed. See legend in Fig. 4.

4.4. Role of systemin and SLs in arbuscular development

4.4.1. Systemin

Several researches have proposed a role of JAs in the regulation of the mycorrhizal symbiosis, although their specific function is not clear so far (Gutjahr and Parniske,

2013; Hause and Schaarschmidt, 2009; Pozo et al., 2015). Hause and co-workers observed changes in the expression of JA marker genes in arbusculated cells compared to non-colonized cells. Consequently, the authors suggested that JA might affect arbuscule development (Hause et al., 2007). On the other hand, when analysing AM colonization levels in different JA-altered plants, including JA deficient (*spr1*, *spr2, def1*) or JA insensitive (*jai1*) lines, only subtle or not-consistent changes were observed (Fernández, 2013, and supplementary **Fig. S1**), while the most dramatic and consistent changes were observed in PS-altered plants (Fernández and coworkers), and a regulatory role of this hormone was proposed. Besides a role in the presymbiotic phase through the regulation of SL biosynthesis and signalling, a possible role of systemin in later stages, and particularly in arbuscule development was analysed in detail by specific stainings of fungal structures in combination with different microscopy techniques.

Ps+ and *ps-* plants and their corresponding wild-type plants (cv. BetterBoy) were inoculated with the AM fungus *R. irregularis* and the roots harvested at 8 weeks post inoculation, in order to obtain a well-established mycorrhizal symbiosis. Part of the root system from each plant was stained with dark ink and the patterns of mycorrhizal colonization for each genotype were analysed by bright field microscopy. As previously found by Fernández *et al.*, mycorrhizal colonization in *ps-* was practically negligible (3%) (**Fig. 7**). In the case of *ps+*, increased mycorrhizal colonization levels were found with respect to wild-type plants. This result was not evident in the absolute root length colonization percentage (50%), but mycorrhizal colonization was less spread within the root, thus observing differences in the intensity of colonization under the microscope (**Fig. 7**). Besides differences in mycorrhizal colonization rates, *ps+* and *ps-* plants

presented changes in the development of the AM fungus within the roots. Normal arbuscules and fungal development were observed in wild-type roots **(Fig. 7A**). By contrast, *ps-* plants, besides lower mycorrhizal colonization and fungal development within the roots, showed non-developed or dead arbuscules (**Fig. 2** and **7B**), while a higher number and overdeveloped arbuscules were observed in *ps+* roots (**Fig. 2** and **Fig. 7C**). To get a deeper insight on the morphological differences in the arbuscules, the samples were analyzed in more detail using confocal laser microscopy. To this end, roots colonized by *R. irregularis* were stained with the fluorophore WGA-AlexaFluor488. Wild-type plants mostly presented arbuscules in the developing phase (**Fig. 2 and 8A, B**). *Ps-* plants displayed very few arbuscules, poorly developed, being all of them aberrant, undeveloped or even died (**Fig. 2 and 8C, D**). In *ps+,* higher arbuscule development than in wt plants was confirmed, presenting highly branched arbuscules, most of them in the mature phase of their life cycle (**Fig. 2 and 8E, F**). Therefore, the microscopy analyses support a key regulatory role of systemin in arbuscule development within the host root.

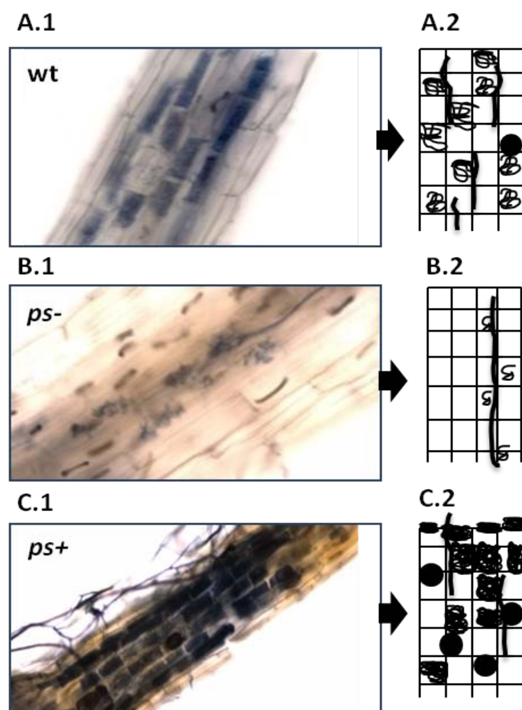

Fig. 7 Arbuscular mycorrhizal colonization by the AM fungus *Rhizophagus irregularis* in tomato roots of wild-type (wt) (**A**), prosystemin antisense *ps-* (**B**) and prosytemin overexpresor *ps+* (**C**) plants at 8 weeks post inoculation. Images of mycorrhizal roots stained with dark ink were observed using a bright field microscope (**A.1, B.1, C.1**). The schemes illustrate the mycorrhizal colonization patterns (**A.2, B.2, C.2**).

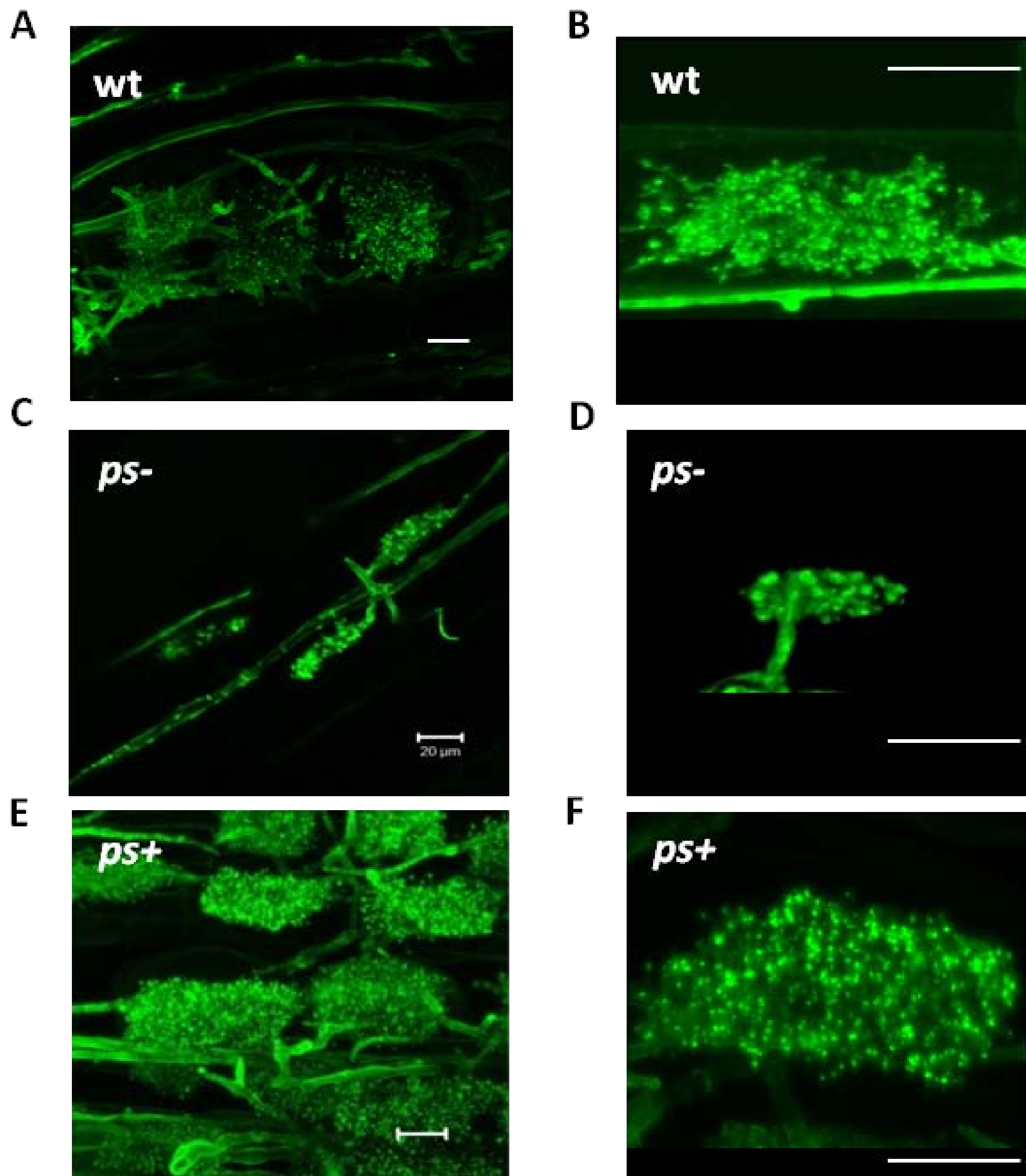

Fig. 8 Arbuscular mycorrhizal colonization by *Rhizophagus irregularis* in tomato roots of wild-type (wt) (**A-B**), prosystemin antisense *ps-* (**C-D**) and prosytemin overexpresor *ps+* (**E-F**) plants at 8 weeks post inoculation. Fluorescent images of arbuscules stained with WGA conjugated with AlexaFluor488 were observed using a confocal laser scanning microscope. Scale bars =20 µm.

4.4.2. Strigolactones

It is very well known that SLs are key molecules regulating the pre-symbiotic phase of AM symbiosis (Akiyama *et al*., 2005; Bouwmeester *et al*., 2007). However, little is

known about their potential role in advanced stages during the symbiotic phase. Considering the conection of systemin and SLs in the regulation of early stages of mycorrhizal colonization, and due to the altered SL levels in the systemin modified lines, it is tempting to speculate that the *ps-* phenotype in arbuscule development may be mediated by SLs. In order to ascertain if SLs are also involved in the regulation of arbuscule development, mycorrhizal colonization was analyzed in detail in different SL-deficient tomato plants and compared with that of the corresponding wild-types by microscopy. The SL-deficient transgenic plants *Slccd7* (line 7071) (Vogel *et al*., 2010) and *Slccd8* (line L09) (Kholen *et al.*, 2012) were inoculated with the AM fungus *R. irregularis*. After 8 weeks of growth mycorrhizal colonization levels were not very high, even in the wild-type plants, where mycorrhization was around 20 % (**Fig. 9**). Nevertheless, as expected, the colonization levels where significantly reduced in both SL-deficient plants compared with the corresponding wild-types (about 50%) (**Fig. 9**). When arbuscule development was analysed in more detail by confocal microscopy and WGA-AlexaFluor488, no differences were found between the arbuscules in *Slccd7* and *Slccd8* plants and those in their respective wild-type plants (**Fig. 9**), in contrast with the clear phenotype observed for PS modified lines (**Fig. 8**), suggesting that SLs are not involved in arbuscule development.

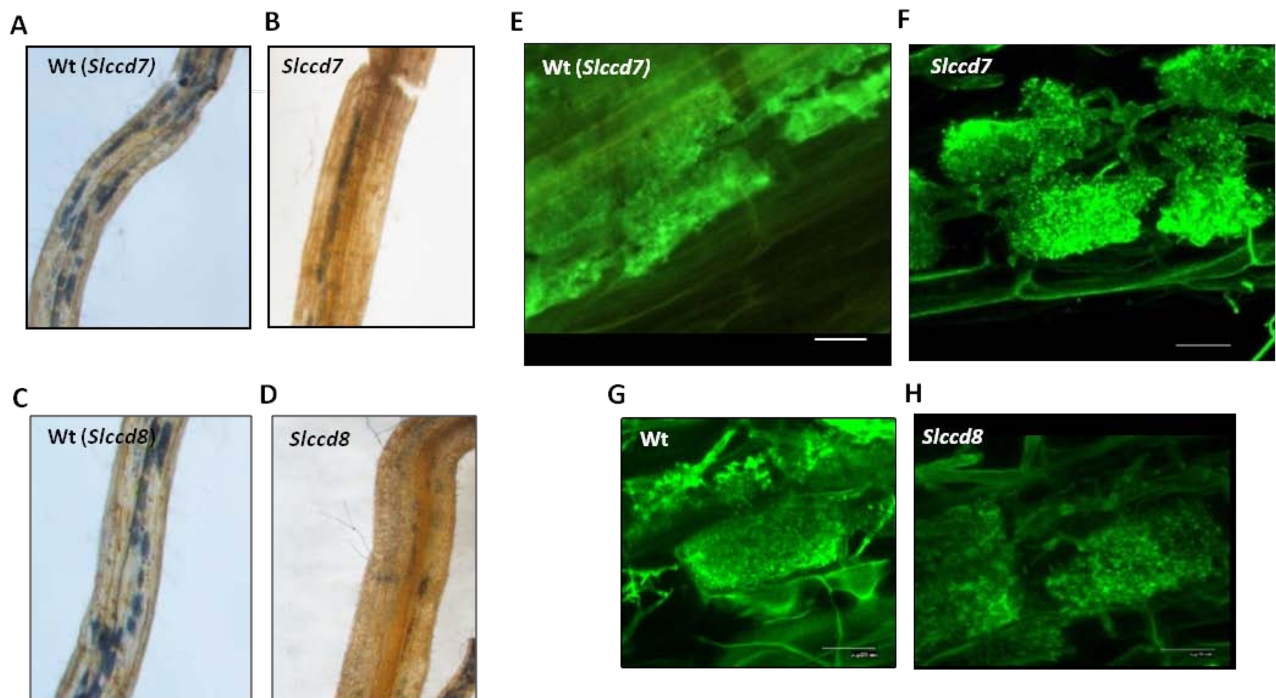

Fig. 9 Arbuscular mycorrhizal colonization by *Rhizophagus irregularis* in the SL-deficient tomato plants *Slccd7* and *Slccd8* and their corresponding wild-types (wt) at 8 weeks of inoculation. Images of mycorrhizal roots stained with dark ink and observed using a bright field microscope (**A–D**). Fluorescent images of arbuscules stained with WGA conjugated with AlexaFluor488 and observed using a confocal laser scanning microscope (**E–H**). Scale bars =20 µm.

5. DISCUSSION

SLs are hormones with several functions in the plants. Among them, they have a role as signalling molecules during the pre-symbiotic phase of AM symbiosis, inducing hyphal branching of the AMF (Akiyama *et al*., 2005). Accordingly, SL-deficient plants show reduced mycorrhizal colonization (Gómez-Roldán et al., 2008; Gutjahr et al., 2012; Koltai et al., 2010). We previously showed that PS-altered plants in tomato presented altered mycorrhization rates, and the differences were proposed to be related to alterations in the SL levels, suggesting that systemin may have a regulatory role in the AM symbiosis probably through the regulation of SL biosynthesis (Fernández, 2013). To study the possible interaction between systemin and SLs, the PS

silenced line *ps-* was treated with exogenous systemin under Pi deficiency conditions, which are well known to promote the SL production (López-Ráez *et al*., 2008a; Yoneyama *et al*., 2007). Here, we have confirmed that *ps-* plants produced less SLs than the corresponding wild-type as described (Fernández, 2013). Interestingly, SL levels in *ps-* plants were restored upon exogenous systemin application, suggesting a direct effect of systemin in the regulation of SL production.

In order to further investigate this systemin-SL cross-talk, we analysed the transcriptional regulation of the SL pathway by systemin. All SL-related genes, both biosynthesis - *SlD27*, *SlCCD7* and *SlCCD8* – and the SL receptor *SlD14*, presented an increased expression under Pi deficiency conditions in wild-type plants, coherent with a positive transcriptional regulation of SL production under low Pi (López-Ráez *et al*., 2008a; Yoneyama *et al*., 2007). Intriguingly, the expression of *SlD27*, *SlCCD8* and *SlD14* returned to the expression levels of control plants when systemin was applied (**Fig. 5**), suggesting a negative regulatory role of systemin of the SL pathway under low Pi conditions at the transcriptional level. Only *SlCCD7* showed a different regulation pattern, being induced after systemin application in wild type plants growing under Pi deficient conditions. In contrast to the regulation in wild type plants, the expression of *SlD27*, *SlCCD8* and *SlD14* was not induced in *ps-* plants exposed to low Pi. These results suggest that plants with low systemin endogenous levels fail to promote SL biosynthesis under Pi deficiency, maybe through a deficient detection of Pi starvation. This reduced sensitivity of *ps-* plants to P deficiency is supported by the fact that the Pi starvation marker *LePT2* was also not induced in *ps-* growing under low P conditions. The expression of *SlD27*, *SlCCD8* and *SlD14* was only induced in *ps-* plants upon the exogenous application of systemin, thus the mutant phenotype being rescued by the

chemical complementation. Therefore, our data indicate that systemin regulates the biosynthesis of SLs under Pi-limiting conditions at the transcriptional level and that this regulation could be positive or negative depending on the systemin content in the plant (exogenous systemin had a negative regulatory effect on wild type, but a positive in the *ps-* mutant). A similar dose-dependent regulation has been previously shown for SLs and auxin in the regulation of the root system archicture (Ruyter-Spira *et al*., 2011), where the effect of SLs in lateral root development depended on the endogenous auxin levels. Therefore, it seems that the regulation of SLs by systemin is very finely regulated, as in the case of auxin. In any case, we demonstrate here that systemin regulates AM symbiosis establishment through the regulation of SL production under Pi deficient conditions.

In addition to a lower colonization rate, we have found that *ps-* plants present aberrant, probably non-functional, arbuscules (**Fig. 8**). Conversely, *ps+* plants displayed very well developed arbuscules, and most of them at the mature stage. These results point to a key role of systemin in AM symbiosis acting in both pre-symbiotic and symbiotic phases of the mycorrhizal symbiosis. We have also observed that mutants altered in the JA biosynthesis/signalling pathway, such *spr1*, *spr2* and *jai1* (Lee and Howe, 2003; Li *et al*., 2003; Li *et al*., 2001), did not show significant changes in SL production, mycorrhizal colonization or arbuscule morphology (**Fig. S1** and Fernández, 2013) suggesting that the systemin function in AM symbiosis is JA-independent.

Despite the important role of SLs in the pre-symbiotic stage of AM symbiosis, little is known about the possible role during the symbiotic phase. Previous studies in petunia showed that the mutant *dad1*, which is blocked at the CCD8 step, did not have any

morphological aberrations in the intracellular mycorrhizal structures (Kretzschmar *et al*., 2012). Similarly, the *d10* and *d17* mutants in rice, affected in the CCD7 and CCD8 genes respectively, did not present differences in arbuscule morphology when compared to wild-type plants (Gutjahr *et al*., 2012). As in petunia and rice, we did not find any significant morphological change in the structure of the arbuscules in the tomato *Slccd7* and *Slccd8* plants. The results support the notion that SLs are not involved in later stages of fungal development in mycorrhizal roots, at least, in arbuscule formation. It might be that they affect hyphal branching within the roots, thus facilitating cell-to-cell colonization, but this fact has not been studied so far.

In contrast to *SlD27* and *SlCCD8*, the expression of *SlCCD7* showed a different regulation pattern, being induced by systemin in wildtype plants. CCD7 plays a pivotal role in both SL and MR/ α-ionol glycoside biosynthesis (López-Ráez *et al*., 2015; Walter, 2013), and this dual function might explain the differential expression pattern of *SlCCD7*. The levels of MRs and α-ionol glycosides that constitute the yellow pigment characteristic of mycorrhizal roots, are highly increased upon mycorrhization, and they are key in the lifespan of the arbuscules (López-Ráez *et al.*, 2015; Walter, 2013). In agreement with this, expression of SlCCD7 is induced in mycorrhizal roots (López-Ráez *et al*., 2015). Since systemin levels are increased in mycorrhizal roots (Fernández I, 2013 DoctoralThesis), the additional increase in *SlCCD7* observed in Pi-starved plants upon systemin application, might be associated to a need of the plant to produce MRs/α-ionol glycosides or another related apocarotenoids, well under mycorrhizal or non-mycorrhizal conditions. Here, we did not detect any change in the expression of the other MRs/α-ionol glycosides biosynthesis genes, *SlCCD1a* and *SlCCD1b,* after

systemin treatment. Therefore, systemin could induce the expression of *SlCCD7* to produce an unknown compound. The fact that this increase does not take place in *ps*-plants suggests that a relatively high endogenous systemin or prosystemin levels are required for the induction.

Finally, ABA is another apocarotenoid regulating AM symbiosis (Pozo *et al*., 2015). It has been observed in different plants species that ABA concentration increased during mycorrhization (Meixner *et al*., 2005; Ludwig-Müller, 2010). In addition, it was shown that the tomato ABA-deficient mutant *sitiens* was less prone to AMF colonization than the corresponding wild-type, a phenotype that was partially restored after exogenous ABA application. Moreover, a reduction in mycorrhizal levels in tomato plants treated with sodium tungstate, a potent inhibitor of the biosynthesis of ABA was reported, confirming a positive role of ABA in mycorrhizal colonization (Herrera-Medina *et al.*, 2007; Martín-Rodríguez *et al*., 2010). These authors proposed that ABA could be involved in arbuscule development as morphological anomalies were detected in the arbuscules of the mutant *sitiens* (Herrera-Medina *et al.*, 2007; Martín-Rodríguez *et al*., 2010). An ABA-SLs interaction has been previously proposed since the ABA-deficient tomato plants *sitiens*, *notabilis* and *flacca* showed lower levels of SLs (López-Ráez *et al*., 2010a). Moreover, SL levels were reduced in wild-type plants grown under Pi starvation and treated with the ABA inhibitor abamineSG (López-Ráez *et al*., 2010a). Similarly, we previously showed that ABA levels are also reduced in *Slccd8* (Torres-Vera *et al*., 2014). Interestingly, it has been recently proposed that the SL biosynthesis gene *SlD27* might be also involved in ABA biosynthesis (**Fig. 1**) (Haider *et al*., 2015). In agreement with this, we have observed here that the expression of the ABA

biosynthesis gene *LeNCED1* presented the same regulation pattern as *SlD27* and *SlCCD8*. Their expression was promoted by Pi starvation and repressed by systemin in wild-type plants, but induced by systemin in *ps-* plants. Therefore, it seems that the ABA-SLs cross-talk could be regulated by the endogenous systemin levels, as shown for SL biosynthesis. Further studies are required to get insights in this three-way interaction and its role in the regulation of the symbiosis.

6. CONCLUSIONS

SLs present an important role in the pre-symbiotic phase of mycorrhization, favouring symbiosis establishment. The peptide hormone systemin is also an important player in mycorrhizal symbiosis. Here, we evidence a positive systemin-SLs interaction during the initial stages of the plant-AM fungus interaction, which seems to be related to the Pi status of the host plant and depends on the root endogenous systemin levels. In addition, the results suggest a possible role of ABA in the regulation of SLs by systemin, pointing to a complex hormone interaction in the regulation of AM establishment (**Fig. 10**). On the other hand, a new role of systemin in arbuscule development, which seems to be SL-independent has been envisaged during later stages of colonization (**Fig. 10**). Therefore, systemin seems to play a pivotal role in the regulation of AM symbioses affecting SL production at the early stages and regulating arbuscule development at later stages of the interaction. Further studies are required to elucidate how these roles are regulated and how the different hormones involved in these processes interact to finetune the mycorrhization process.

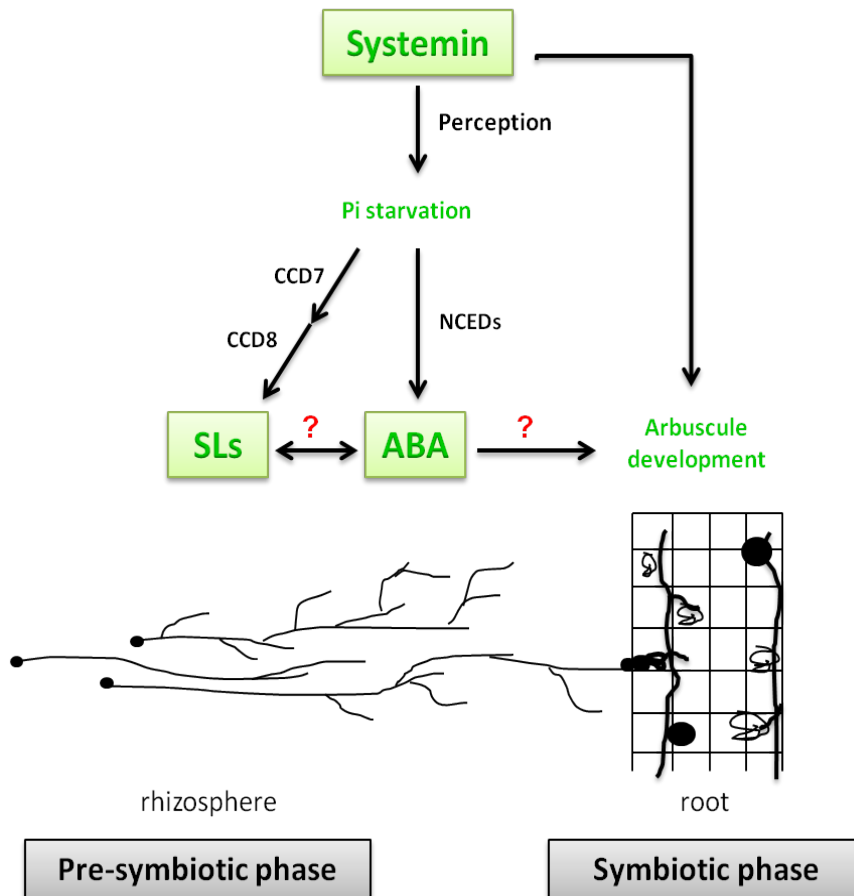

Fig. 10 Schematic model of the systemin effect in AM symbiosis. Systemin, and probably ABA, regulate SL production in roots, probably through the regulation of Pi sensing or Pi deficiency responses in the plant. SLs are induced under low Pi conditions by the biosynthesis genes CCD7 and CCD8, stimulating hyphal branching of arbuscular mycorrhizal fungi (AMF) present in the vecinity of the host root. On the other hand, systemin seems to present an important role in the arbuscule development in a SL-independent manner.

7. ACKNOWLEDGEMENTS

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Institute of Plant Biochemistry for their help conducting the confocal microscopy analyses.

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9. SUPPLEMENTARY INFORMATION

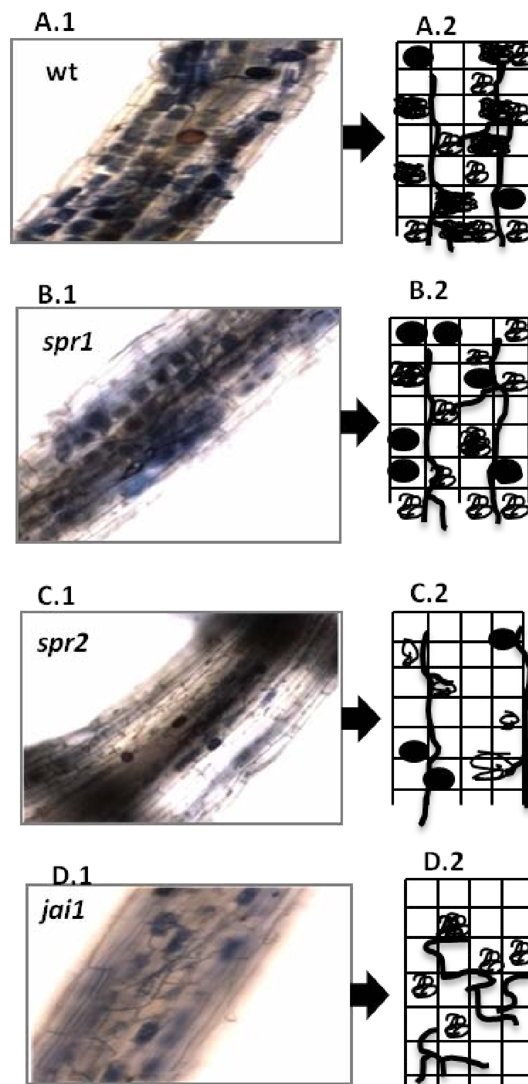

Fig. S1 Arbuscular mycorrhizal colonization by *Rhizophagus irregularis* in tomato roots of wild-type (wt) (**A**), *spr1* (JA signalling mutant) (**B**), *spr2* (JA biosynthesis mutant) (**C**) and *jai1* (JA insensitive mutant) (**D**) at 8 weeks post-inoculation. Images of mycorrhizal roots stained with dark ink were observed using a bright field microscope (**A.1, B.1, C.1**). The schemes illustrate the mycorrhizal colonization patterns observed (**A.2, B.2, C.2**).

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

ID	Gene	Pathway	Primers (5'-3')
AAB82147	Inorganic phospha transporter (*LePT2*)[1]	Pi stress	GGCCAGAGCCAAAAGGAAAATC TGCAACAAACAAGCTTACACAATACA
SGN-U577652	β-carotene isomerase (*SlD27*)[2]	apocarotenoids	TTGGCTAGTTGGACCTTGTG CAAAGTTTGGCACCATATTCA
JF831532	Carotenoid cleavage dioxygenase 8 (*SlCCD8*)[3]	SLs	CCAATTGCCTGTAATAGTTCC GCCTTCAACGACGAGTTCTC
XM_004238045	SL-receptor (*SlD14*)[2]	SLs	GACATTTGCCACATCTTAGC TTTTGGTTTGGTTGACGC
GQ468556	Carotenoid cleavage dioxygenase 7 (*SlCCD7*)[3]	SLs MRs α-ionol glycosides	AGCCAAGAATTCGAGATCCC GGAGAAAGCCCACATACTGC
AY576001	Carotenoid cleavage dioxygenase 1 a (*SlCCD1a*)[4]	MRs α-ionol glycosides	AAGCTTGAGAATTTCTGCA GCCTGTGTAGTTCTCGTTGAT
AY576002	Carotenoid cleavage dioxygenase 1 b (*SlCCD1b*)[4]	MRs α-ionol glycoside	AGAACAGCGTGACGGTTTCACA AGTGTAGTTCTCGTTGATCCGTG
Z97215	Nine-cis-epoxycarotenoid dioxygenase 1 (*LeNCED1*)[6]	ABA	ACCCACGAGTCCAGATTTC GGTTCAAAAAGAGGGTTAGC
X14449	Elongation factor 1 (*SlEF-1α*)[7]	Housekeeping	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC

[1]This work; [2]Chapter 1; [3]Kohlen *et al*., 2012; [4]López-Ráez et al., 2015; [6]López-Ráez *et al.*, 2010; [7]Rotenberg *et al.*, 2006

DISCUSIÓN GENERAL

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Las fitohormonas son reguladores clave en las funciones vitales de las plantas, realizando un papel crucial en su desarrollo, reproducción y en los procesos de defensa de las mismas. Entre las fitohormonas se encuentran principalmente el ácido abscísico (ABA), citoquininas (CKs), auxinas, brasinosteroides (BRs), giberelinas (GAs), ácido salicílico (SA), ácido jasmónico (JA) y etileno (ET) (Robert-Seilaniantz *et al*., 2011; Vanstraelen *et al.*, 2012). En el año 2008, se incluyeron las estrigolactonas (SLs) como una nueva fitohormona, ya que se puso de manifiesto su papel como inductores de la dominancia apical en el crecimiento de la parte aérea de la planta, inhibiendo el crecimiento de los brotes laterales (Gómez-Roldan *et al*., 2008; Umehara *et al*., 2008). Inicialmente, fueron descritas como moléculas señal que inducen la germinación de las semillas de plantas parásitas de raíz (Bouwmeester *et al*., 2003; Cook *et al*., 1972) y más tarde, como inductores de la ramificación de las hifas de los hongos micorrícicos arbusculares (MA), favoreciendo así la simbiosis entre el hongo y la raíz (Akiyama *et al*., 2005; Bouwmeester *et al*., 2007). Además, se ha detectado una posible implicación en la simbiosis *Rhizobium*-leguminosas (Peláez-Vico *et al*., 2016), ampliándose así su espectro de acción en la rizosfera. Por tanto, las SLs además de presentar un papel endógeno como fitohormonas, son moléculas exudadas a la rizosfera con el fin de regular procesos interacciones de la planta con otros organismos (López-Ráez et al., 2017). Como fitohormonas, más recientemente se ha demostrado que las SLs están también implicadas en otros procesos fisiológicos de la planta como son la senescencia foliar, la arquitectura de la raíz y el desarrollo reproductivo, entre otros (Pandey *et al.*, 2016).

En los últimos años numerosos trabajos han mostrado la intrincada interacción de las distintas fitohormonas en la regulación de los procesos vitales de la planta, presentando las distintas vías de señalización que coordinan interacciones sinérgicas o antagonistas, siendo en general el balance hormonal lo que determina la actividad de la planta. Esta interacción entre hormonas, o "*cross-talk*", es fundamental para entender los mecanismos de regulación hormonal en plantas y es el principal tema de esta Tesis. En concreto, en esta Tesis hemos estudiado como las SLs interaccionan con otras hormonas regulando distintos procesos endógenos en la planta y actuando como molécula señal en la rizosfera.

Las plantas parásitas de raíz, comúnmente llamadas jopo para los géneros *Phelipanche* y *Orobanche*, causan graves daños en los cultivos agrícolas. Las estrategias que se usan actualmente para erradicarlas no son efectivas, ya que mayormente el ciclo de vida de estos parásitos ocurre bajo tierra y cuando se detecta la infección, ya es demasiado tarde para eliminarla. Se están estudiando nuevas estrategias focalizadas en eliminar o reducir el parásito durante los estadíos iniciales de la interacción (López-Ráez *et al*., 2009). Los niveles de infección y los problemas que generan están, en parte, regulados por las fitohormonas de la planta hospedadora. Por ello, en el **capítulo 1** se analizó la regulación a nivel transcripcional de distintos marcadores asociados a las diferentes rutas hormonales en raíces de plantas de tomate infectadas por *Phelipanche ramosa* en los estadíos iniciales de la infección. Los marcadores moleculares indicaron una inducción en la expresión de genes relacionados con JA, SA y ABA, principales hormonas reguladoras de la defensa en la planta (Robert-Seilaniantz *et al*., 2011). El análisis de marcadores de otras rutas hormonales, más conocidas por sus funciones en

la regulación en procesos de desarrollo como auxinas, citoquinitas y giberelinas no mostró cambios en estos estadíos iniciales. Ésto sugiere la inducción de mecanismos de defensa regulados por las rutas hormonales relacionadas con defensa en el sistema tomate-*P. ramosa* desde estadíos muy tempranos en la interacción planta-parásito (Torres-Vera *et al.*, 2016). Para confirmar su contribución de estos mecanismos a contener el desarrollo de la planta parasita sería necesario efectuar bioensayos usando mutantes de tomate alterados en estas vías de señalización y comparar la incidencia del parásito con las plantas silvestres. La creación de plantas más resistentes a la infección mediante modificaciones que favorezcan la acumulación o activación de estas hormonas, podría ser clave en futuras estrategias de control a seguir.

Debido al papel que desempeñan las SLs como moléculas señal para la germinación de semillas de plantas parásitas (Bouwmeester *et al*., 2003; Cook *et al*., 1972), también analizamos marcadores moleculares relacionados con la biosintesis de SLs, observándose una inducción en la expresión de estos genes en los estadios iniciales de la infección. Este incremento podría ser explicado por una producción por parte del parásito de moléculas elicitoras que estimulen la producción de SLs que induzcan la germinación. Sin embargo, la activación de los mecanismos defensivos de la planta sugerida por la activación de los marcadores de las tres principales hormonas reguladoras de la defensa, no parece corroborar esta hipótesis. Por tanto, una posible interacción de las SLs, como fitohormonas, con las principales hormonas de defensa en la infección por jopo no es descartable, aunque se deberían realizar estudios en etapas más avanzadas de la infección y hacer un seguimiento en el tiempo de los niveles de las distintas hormonas, incluyendo las SLs. Teniendo en cuenta el papel de las SLs en la rizosfera, y su posible implicación en la defensa frente a esta infección, las SLs podrían

ser una pieza esencial en las futuras estrategias para erradicar las plagas por jopo (Torres-Vera *et al.*, 2016).

Tras vislumbrar en el **capítulo 1** una posible implicación de las SLs en la defensa, en el **capítulo 2** investigamos más detenidamente el papel que podrían desempeñar las SLs en esta función. Para ello, se analizaron distintas infecciones, tanto de parte aérea como de raíz, en distintas plantas de tomate deficientes en SLs junto con sus silvestres. Se observó que las plantas de tomate *Slccd7* y *Slccd8,* fueron más susceptibles a la infección por el patógeno necrótrofo *Botrytis cinerea* que sus correspondientes plantas silvestres. Asimismo, las plantas *Slccd8* fueron más sensibles a la infección por *Alternaria alternata*, reafirmando una posible implicación de las SLs en la infección por patógenos necrótrofos de parte aérea. También se detectó un mayor índice de infección en raíz por el patógeno hemibiótrofo *Fusarium oxysporum*, lo que podría implicar un amplio espectro de acción de las SLs en defensa. Además, plantas de Arabidopsis deficientes en SLs *max4-1*, bloqueadas en el CCD8, fueron también más susceptible a la infección por *B. cinerea*, lo que confirmaría el posible papel de las SLs en defensa y que éste es conservado en distintas especies. El análisis del perfil hormonal en hojas de plantas *Slccd8* mediante HPLC-MS/MS mostró que las principales hormonas reguladoras de la defensa (SA, JA y ABA) se encontraban en concentraciones muy inferiores en comparación con las plantas silvestres. Estos resultados se confirmaron mediante análisis de los niveles transcripcionales de los marcadores de las vías que regulan, observándose un mayor efecto sobre las respuestas reguladas por JA. Por tanto, el papel de las SLs en defensa parece ser indirecto, actuando sinérgicamente con las principales vías que regulan los

mecanismos de defensa, y fundamentalmente con la ruta del JA (Torres-Vera *et al*., 2014). Para confirmar estos resultados habría que realizar estudios de recuperación del fenotipo silvestre mediante aplicación exógena de GR24 (un análogo sintético de SLs), así como estudios mediante injertos con plantas alteradas en los niveles de SLs e infectadas por patógenos de parte aérea, ya que principalmente las SLs se producen en la raíz.

En los **capítulos 1 y 2** se sugiere que las SLs están implicadas en la regulación de la defensa de las plantas, probablemente a través de su interacción con la vía del JA aunque hasta ahora ambas hormonas no habían sido relacionadas. Sin embargo, ambas presentan un papel en la regulación de la simbiosis MA, aunque aparentemente actuando a diferentes niveles de la interacción: las SLs como inductores de la ramificación de las hifas de los HMA en la fase pre-simbiótica, y el JA regulando el establecimiento micorrícico en la raíz en la fase simbiótica (Hause *et al*., 2007; Pozo *et al*., 2015). Estudios realizados con mutantes alterados en los niveles de JA mostraron índices de micorrización alterados respecto a sus correspondientes silvestres (Herrera-Medina *et al*., 2008; Isayenkov *et al*., 2005; Song *et al*., 2013; Tejeda-Sartorius *et al*., 2008). Este comportamiento también está descrito en plantas deficientes en SLs, las cuales presentan unos menores niveles de colonización micorrícica. Estas plantas presentan además una mayor ramificación de la parte aérea (Gómez-Roldán *et al*., 2008; Kohlen *et al*., 2012; Koltai *et al*., 2010; Kretzschmar *et al*., 2012), fenotipo que observamos también en ciertos mutantes de JAs. Estos indicios, junto con los resultados de los **capítulos 1 y 2**, nos hizo considerar la posibilidad de una interacción entre SLs y JA en la regulación de otros procesos en la planta además de en las

respuestas de defensa. En el **capítulo 3** se estudió una posible interacción de las SLs y JA en la regulación de la ramificación de la parte aérea de la planta. Para ello, se realizó un análisis de la estructura de la parte aérea en plantas de tomate alteradas en la ruta regulada por JA (*spr1*, *spr2* y *jai1*), detectándose únicamente una mayor ramificación en plantas *spr2* con respecto al genotipo silvestre. Este resultado fue más acentuado en las plantas *spr2* micorrizadas con los hongos MA *Funneliformes mosseae y Rhizophagus irregularis*, no habiendo cambios en el resto de mutantes inoculados tras la inoculación con hongos MA. Cabe destacar que está descrita la reducción de los niveles de SLs en plantas que presentan una micorrización bien establecida, probablemente para evitar un "exceso" de micorrización que pudiera ser demasiado costoso para la planta (Aroca *et al*., 2013; Fernández-Aparicio *et al*., 2010; López-Ráez *et al*., 2011). Por tanto, estos resultados podrían indicar una posible deficiencia en SLs en las plantas *spr2*, que se vería acentuada en condiciones de micorrización, puesto que reduciría la producción de SLs. Tras comparar plantas *spr2* con plantas deficientes en la producción de SLs (*Slccd8*), se observó que en ambas los mayores índices de ramificación se encontraban en la ramificación secundaria, siendo más acentuadas tras la micorrización por *R. irregularis*. Las plantas *spr2* presentaron un patrón de crecimiento similar al de las plantas *Slccd8*, especialmente a la línea L16, que cuenta con una deficiencia en SLs del 50% respecto al silvestre (Kohlen *et al*., 2012). Ésto sugería una posible deficiencia de SLs intermedia en las plantas *spr2.* Sin embargo, la cuantificación de SLs mediante bioensayos con semillas de *P. ramosa* y por UPLC-MS/MS no corroboró esta hipótesis. Cabe destacar que los análisis se realizaron en raíz, principal productor de SLs, ya que los niveles en hoja están normalmente por debajo de los límites de detección, pero sería interesante analizar posibles diferencias

en la parte aérea de plantas *spr2* respecto a su silvestre para comprobar si el fenotipo observado pudiera corresponder con una distribución anómala de estas en la planta.

Las SLs regulan la ramificación de la parte aérea mediante una interacción sinérgica y antagonista con las auxinas y las CKs, respectivamente (Teichmann y Muhr, 2015). Se analizó un posible efecto indirecto de las SLs en el fenotipo ramificado de *spr2* mediante la cuantificación de los niveles de auxinas y CKs por HPLC/MS-MS, así como el balance auxinas/CKs, tanto en hojas como en raíz. Sin embargo, los resultados obtenidos no permiten explicar el fenotipo de *spr2* en base al contenido de estas hormonas. Por tanto, no se ha podido demostrar un efecto directo o indirecto de las SLs u otras hormonas en el fenotipo ramificado de las plantas *spr2.* Un defecto en el transporte de SLs de la raíz a la parte aérea en plantas *spr2* se podría analizar en futuros estudios.

Además de los mutantes alterados en JA estudiados en el **capítulo 3,** se han analizado en el **capítulo 4** plantas alteradas en prosistemina (PS), que se encuentras alteradas también en sus niveles de JA en condiciones de estrés. La PS es el precursor de una hormona peptídica denominada sistemina presente en Solanáceas (McGurl *et al*., 1992; Ryan y Pearce, 1998). Cuando se produce una herida por herbivoría o un ataque por insecto, la PS es inducida y procesada proteolíticamente en el citosol hasta sistemina, induciendo la biosíntesis de JA (Ryan y Pearce, 2003). Fernández y colaboradores observaron que plantas de tomate deficientes en PS (*ps-*) presentaban niveles de micorrización muy bajos, al contrario de las plantas sobreexpresantes (*ps+*), que mostraron niveles de colonización superiores al silvestre (Fernández, 2013). Además, estas diferencias correlacionaban con niveles de SLs inferiores y superiores al

silvestre en las plantas *ps-* y *ps+*, respectivamente (Fernández, 2013). Éstos resultados sugerían que la sistemina, entre otros mecanismos, podría estar involucrada en la regulación de la simbiosis micorrícica mediante la regulación de los niveles de SLs.

Para investigar si el efecto de la sistemina en la producción de SLs es directo o indirecto, en el **capítulo 4** plantas *ps-* fueron tratadas con sistemina exógena en condiciones de alta producción de SLs (deficiencia de Pi, López-Ráez *et al*., 2008; Yoneyama *et al*., 2007) y se midieron los niveles de SLs mediante bioensayos con semillas de *P. ramosa*. El fenotipo de deficiencia de SLs observado en *ps-* fue revertido tras el tratamiento con sistemina exógena, lo que sugería un efecto directo de la sistemina sobre la producción de SLs. Tras un análisis de la expresión génica de genes marcadores de las SLs, se observó que la sistemina regula los niveles de SLs a través de la activación de su ruta de biosíntesis en condiciones de deficiencia nutricional. Las SLs son derivados de los carotenoides, pertenecientes a la familia de los apocarotenoides, a la que también pertenecen el ABA, micorradicinas (MRs) y α-ionol glucósidos, que comparten con las SLs parte de su ruta biosintética (**Fig.1**, capítulo 4). El análisis de marcadores génicos para estos apocarotenoides sugirió también una posible implicación de la sistemina en la regulación de ABA bajo condiciones de estrés nutricional, aunque más estudios deberían realizarse para corroborar esta posible interacción. La relación entre ABA y SLs ya se había descrito anteriormente, ya que plantas deficientes en ABA o SLs presentaban niveles bajos de ambas hormonas conjuntamente (Torres-Vera *et al*., 2014**;** López-Ráez *et al*., 2010a), y cierto paralelismo en su regulación se ha evidenciado en el **capítulo 1**. Por tanto, los resultados podrían apuntar a una posible interacción entre las tres hormonas, aunque se desconoce aún a qué nivel. El uso combinado de mutantes en las distintas vías sería

necesario para ver cómo se regula esta interacción tripartita y su contribución a la regulación de la simbiosis micorricica y a la defensa de la planta.

Dentro de la fase simbiótica de la micorrización, tanto el JA como ABA, MRs y α-ionol glucósidos, están implicadas en la regulación de la formación y mantenimiento de los arbúsculos, aunque su papel aún no está claro (López-Ráez *et al*., 2015). La estrecha relación entre sistemina y JA, la posible regulación a nivel transcripcional de ABA por sistemina, y la implicación del ABA en la formación de los arbúsculos nos sugirió la posibilidad de que la sistemina también estuviera involucrada en la fase simbiótica de la micorrización. Esta posibilidad fue confirmada tras realizar un estudio microscópico comparativo sobre el desarrollo arbuscular en plantas *ps-* y *ps+* micorrizadas con *R. irregularis*. La implicación de la sistemina en el desarrollo arbuscular parece ser independiente de JA y SLs, ya que ninguno de los mutantes de JA y SLs analizados mostraron alteraciones significativas en los arbúsculos. Diversos estudios habían mostrado que plantas deficientes en ABA y MRs/α-ionol glucósidos mostraron un desarrollo arbuscular anómalo (Herrera-Medina *et al*., 2007; Walter, 2013) por lo que cabría especular con una una posible interacción entre estos apocarotenoides y la sistemina en la regulación de la fase simbiótica de la micorrización. Sería interesante realizar estudios sobre el desarrollo arbuscular en plantas deficientes en estos apocarotenoides y en sistemina para analizar una posible recuperación del fenotipo mediante tratamientos hormonales exógenos.

En conjunto, en este trabajo se han puesto manifiesto la interacción de las SLs con otras hormonas y su posible implicación en diversas funciones en la planta, entre ellas la regulación de respuestas de defensa y de la simbiosis micorricica. Como se comentó

anteriormente, tras una micorrización bien establecida, la planta disminuye los niveles de SLs evitando posiblemente un exceso de colonización por parte del hongo simbionte (López-Ráez *et al*., 2015, Vierheilig, 2004). Este proceso autorregulador se ha propuesto como una posible estrategia para reducir y controlar las plagas de planta parásita de raíz (Fernández-Aparicio *et al*., 2010; Lendzemo *et al*., 2005; López-Ráez *et al*., 2015). Además de este posible efecto protector frente a plantas parásitas, la simbiosis MA puede incrementar los mecanismos de defensa de la planta protegiendo a la planta de estreses tanto abióticos como bióticos (Mycorrhiza Induced Resistance, MIR), probablemente vía JA (Pieterse *et al*., 2014; Pozo y Azcón Aguilar, 2007). Puesta de manifiesto en este trabajo la contribución de las SLs a la regulación de las defensas mediante interacción con otras hormonas, fundamentalmente JA, y la regulación de los niveles endógenos de SLs en la micorrización, una posible función de las SLs en la modulación de las defensas en plantas micorrizadas merece ser explorada en trabajos futuros. EL uso de hongos MA como biofertilizantes y bioprotectores en los cultivos agrícolas se presenta como un futuro prometedor y sostenible, pero para optimizar su aplicación en agricultura es fundamental conocer los mecanismos que regulan su establecimiento y mantenimiento. En este trabajo de Tesis Doctoral se ha avanzado en este conocimiento y se han estudiado las interacciones entre diferentes fitohormonas que regulan dicha asociación, tanto a nivel pre-simbiótico como simbiótico.

CONCLUSIONES

CONCLUSIONES

1. La biosíntesis de las tres principales fitohormonas involucradas en la regulación de las respuestas de defensa, ácido salicílico (SA), ácido jasmónico (JA) y ácido abscísico (ABA) es inducida a nivel transcripcional en los estadíos iniciales de la infección tomate-*Phelipanche ramosa*, lo que sugiere su papel en la defensa de la planta en esta interacción.

2. La ruta de biosíntesis de las estrigolactonas (SLs) es inducida durante la interacción temprana tomate-*P. ramosa*, sugiriéndose un posible papel de estas hormonas en la respuesta defensiva de la planta.

3. En plantas de tomate, las SLs están involucradas en la defensa de las plantas frente a patógenos foliares necrótrofos. Función que parece estar asociada con modificaciones en los niveles de las fitohormonas SA, ABA y principalmente JA.

4. El mutante de tomate *spr2* (deficiente en JA y otras oxilipinas) no muestra alteraciones en los niveles de SLs, aunque sí presenta una menor micorrización y un fenotipo ramificado similar al de las plantas deficientes en SLs.

5. La hormona peptídica sistemina regula el establecimiento de la simbiosis micorrícica arbuscular (MA) a través de la inducción a nivel transcripcional de la biosíntesis de SLs en condiciones de deficiencia de fósforo.

6. La sistemina presenta un papel regulador en el desarrollo arbuscular en la simbiosis MA que es independiente de JA y de SLs.

CONCLUSIONS

1. The biosynthesis of the three main phytohormones involved in the regulation of defense responses, salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) is induced at the transcriptional level at the early stages of the tomato-*Phelipanche ramosa* interaction, suggesting their role in the plant defense mechanisms.

2. The biosynthetic pathway of strigolactones (SLs) is induced during the early interaction tomato-*P. ramosa*, indicating their possible role in defense in this parasitic interaction.

3. In tomato plants, SLs participate in the plant defense against foliar and necrotrophic pathogens in different plants species. Role that seems to be related to the alteration in the SA, ABA and mainly JA signaling pathways.

4. The tomato mutant *spr2* (deficient in JA and other oxylipins) is not deficient in SLs, although it shows lower mycorrhization levels and a branched phenotype similar to that of SL- deficient plants.

5. The peptide hormone systemin regulates the establishment of arbuscular mycorrhizal symbiosis (AM) by inducing the expression of SL biosynthesis genes under phosphate deficient conditions.

6. Systemin plays a regulatory role in the arbuscule development in AM symbiosis, and this function seems to be independent of JA and SLs.

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