Strigolactones cross the kingdoms: plants, fungi, and bacteria in the arbuscular mycorrhizal symbiosis

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Date of submission: August 28, 2017
Number of tables: 2
Number of figures: 4
Fig. 1, 3 and 4 should be in colour online only
Fig. 2 should be in colour in print and online

Word count: 6660

Abstract

Strigolactones firstly evolved as regulators of simple developmental processes in very ancient plant lineages and then assumed new roles to sustain the increasing biological complexity of land plants. Their versatility is also witnessed by the fact that during the evolution they have been exploited, once released in the rhizosphere, as a communication system towards plant-interacting organisms even belonging to different kingdoms. Here we reviewed the impact of SLs on soil microbes giving attention in particular to arbuscular mycorrhizal fungi (AMF). SLs induce several responses in AMF, including spore germination, hyphal branching, mitochondrial metabolism, transcriptional reprogramming and production of chitin oligosaccharides which, in turn, stimulate early symbiotic responses in the host plant. In the specific case study of the AMF *Gigaspora margarita,* SLs are also perceived, directly or indirectly, by the well characterized population of endobacteria with an increase of bacterial divisions and the activation of specific transcriptional responses. SLs dynamic during AM root colonization was also surveyed. Although not essential for the establishment of this mutualistic association, SLs act as positive regulators as they are relevant to achieve a full extent of colonization. This possibly occurs through a complex cross-talk with other hormones such as auxin, abscisic acid and gibberellins.

Key words: arbuscular mycorrhizal fungi, endobacteria, fungi, hormones, mutants, root symbiosis, strigolactones

Abbreviations

ABA: abscisic acid
AMF: arbuscular mycorrhizal fungi
BR: brassinosteroids
CK: cytokinines
CSP: common symbiotic pathway
GA: gibberellin
SLs: strigolactones

Running title: Strigolactones cross the kingdoms

Highlight:

Strigolactones are versatile plant molecules used not only as hormones but also as a communication system to regulate the AM symbiosis through the activation of multiple responses.

Introduction

Among plant-associated microbes, the widespread arbuscular mycorrhizal fungi (AMF) play a key role in nutrient cycling and plant health due to their ability to improve plant mineral nutrition and tolerance to biotic and abiotic stresses. These fungi belong to an ancient monophyletic group, the Glomeromycotina (Spatafora *et al.,* 2016). AMF are obligate biotrophs with coenocytic hyphae and multinucleated asexual spores, although recently hidden sexuality events were proposed to occur (Corradi and Brachmann, 2017). Since AMF establish interactions with more than 80% of land plants, including basal plants like bryophytes and crop plants (Bonfante and Genre, 2010), and may also host endobacteria in their cytoplasm (Bonfante and Desirò, 2017), the AM symbiosis is an excellent model to discuss the exchange of signaling molecules at the inter-kingdom and inter-domain level. Plants have to distinguish among the surrounding microbes the friends or the foes, while AMF have to identify the photosynthetic host which guarantees a flow of reduced carbon. Recent papers have demonstrated that host plants provide lipids to their fungal partners (Bravo *et al.,* 2017; Luginbuehl *et al.,* 2017; Jiang *et al.,* 2017; Keymer *et al*., 2017) and not only sugars as claimed for many years. In turn, AMF transfer to the host plants mineral nutrients. These exchanges are thought to occur primarily in root cortical cells hosting highly branched fungal hyphae, called arbuscules, which are therefore considered key structures of a functional symbiosis (Gutjahr and Parniske, 2013).

While the existence of a conserved signaling transduction pathway, usually defined as the common symbiotic pathway (CSP) since shared by the AM and the rhizobia-legumes symbioses, has been the object of many investigations and summarized in excellent reviews (Oldroyd, 2013; Genre and Russo, 2016; Zipfel and Oldryod 2017), plant and fungal molecules that trigger symbiotic responses in the corresponding AM partner are less well characterized. Bonfante and Genre (2015) have proposed the hypothesis that the molecules involved in inter-kingdom symbiotic signaling, such as strigolactones (SLs), cutin monomers, and chitin-related molecules, also have key roles in development, originally unrelated to symbiosis. Thus, the symbiotic role of these molecules relies on the co-evolved capacity of the AM partners to perceive them as symbiotic signals.

Not only chitin oligosaccharides, but also SLs well fit to this suggestion. SLs derive from carotenoid metabolism (Al Babili and Bouwmeester, 2015); they were first studied as root-exuded molecules that elicit the germination of parasitic plants (Cook *et al.,* 1966). More

recently, SLs were acknowledged as bioactive molecules that stimulate the branching and metabolism of pre-symbiotic hyphae in AMF (Akiyama *et al.,* 2005, Besserer *et al.,* 2006). Finally, SLs emerged as key plant hormones that control several aspects of plant biology and physiology such as the repression of shoot branching (Gomez-Roldàn *et al.,* 2008; Umehara *et al.,* 2008; Waters *et al.,* 2017), the regulation of root system architecture (Koltai *et al.,* 2011; Kapulnik and Koltai, 2014; Sun *et al.,* 2016), the formation of adventitious root and leaf senescence (Waters *et al.,* 2017). SLs production is conserved from Charales to Embryophytes (Delaux *et al.,* 2012). Their function in the rhizosphere seems to be a secondary feature relying on their active release from the roots into the soil (Kretzschmar *et al.,* 2012).

In conclusion, emerging data suggest that SLs function as conserved determinants of plant development that were recruited during the evolution of plant symbiotic and parasitic interactions (Waters *et al.,* 2017).

The aim of the review is to focus on the SLs when released into the rhizosphere: in detail, we will summarize the direct impact of SLs on soil microbes, which proliferate in this specific niche, giving attention to AM and pathogenic fungi. Since these microbes interact with plants, we also review current knowledge on SLs dynamic during plant-microbe interactions, in particular on how the plants regulate SLs synthesis during the colonization. Lastly, we will provide information obtained from the analyses of plant mutants defective in the biosynthesis or in the perception of SLs and highlight how the cross-talk with other hormones could contribute to the control of the extent of plant colonization.

Strigolactones: their impact on arbuscular mycorrhizal fungi

Being released in the rhizosphere, SLs have potential effects on microbes which proliferate in the soil around the roots. Special attention has been given so far to the symbiotic microbes, AMF and rhizobia (Waters *et al.,* 2017), while only a few reports have investigated how saprotrophic or pathogenic fungi respond to SLs.

Akiyama and colleagues (2005; 2010) first described how SLs lead to a specific phenotype during the pre-symbiotic phase of AMF. They based their work also on the use of GR24, a synthetic SLs analog. It is worth to note that several studies on SLs have been carried out using GR24, normally used as a racemic solution of the two enantiomers (±)-GR24, even if in some

cases this detail is not specified. Since stereochemistry was shown to be an important issue for SLs activity (Scaffidi *et al.,* 2014) this could lead to inconsistent results among independent studies.

The molecular mechanisms underlying the AM hyphal branching are still poorly known. SLs treatment boosts fungal metabolism, leading to increased ATP production and mitochondrial division (Besserer *et al.,* 2006; 2008). Our data from RNA sequencing of germinated spores of *G. margarita* after the GR24 treatment confirmed Besserer and colleague's findings, revealing the up-regulation of the expression of mitochondrial genes (Salvioli *et al.,* 2016). The differentially expressed genes involved in fungal respiration after the treatment are listed in Table 1. In addition, other genes resulted GR24-responsive (up- or down-regulated). Among them, the most biologically relevant were: a vacuolar amino acid transporter 1-like, a chitin deacetylase, a chitin synthase, a Mating-type HMG-box protein MAT1-2, a multidrug transporter mdr1 and a cytochrome p450 (Table 1). These data suggests that not only the mitochondrion, but also other cell compartments are sensitive to SLs.

Chitin is a crucial cell wall component of AMF and changes its structural organization along the fungal life cycle (Bonfante, 1988). In addition, chitin oligosaccharides act as signaling molecules eliciting calcium spiking, a key component of a symbiotic pathway involved in the initial stages of root colonization (Genre *et al.,* 2013; Sun *et al.,* 2015). The discovery that GR24 treatment led to an increase in the release of chitin oligomers (Genre *et al.,* 2013) by AMF and, subsequently, to an amplification of the calcium spiking response, offered the first experimental evidence of the interaction between the signaling molecules released by the fungal and plant partners (Bonfante and Genre, 2015). The observation that exposure to chitin oligomers increased the expression of a gene involved in SLs biosynthesis (CCD7) in *Lotus japonicus* together with other genes considered symbiotic markers (Giovannetti *et al.,* 2015), suggests a positive reciprocal feedback in the SL-COs communication system (Fig. 1).

Very little is known about the molecular mechanisms of SLs perception and signal transduction in AMF. So far, homologs of the D14 proteins, the SLs receptors characterized in plants (Waters *et al.,* 2017) have not been found within the only available *Rhizophagus irregularis* genome (Tisserant *et al.,* 2013; Lin *et al.,* 2014). SLs perception may rely on a calcium mediated-process since, by using a transactivator of transcription (TAT) peptide, Moscatiello and colleagues (2014) delivered the bioluminescent calcium reporter aequorin inside *G. margarita* germinating spores

and demonstrated that GR24 evokes a rapid and remarkable elevation in intracellular calcium concentration which is dissipated within 3-4 min. Since oscillations of calcium concentration are often read as a fast cell response to environmental stress (Zhivotovsky and Orrenius, 2011), an alternative hypothesis is that SLs are first perceived by the AMF as foreign molecules (xenobiotics).

To have an overview of fungal responses to SLs we compared transcriptomic data upon GR24 treatment from the two AMF *G. margarita* and *R. irregularis.* We performed GO enrichment analyses starting from public RNA-seq data (NCBI accession numbers: PRJDB3195 for *R. irregularis* and PRJNA267628 for *G. margarita*) (Fig. 2). Many up-regulated genes were related to the nucleus cellular component and DNA-related functions. Interestingly, *R. irregularis* revealed similar patterns with nucleus and organelle as the more enriched cell categories.
Lipid metabolism and/or localization were other enriched categories shared by the two fungal symbionts. Irrespectively of the fact that AMF are auxotrophic for lipids (Bravo *et al.,* 2017; Luginbuehl et *al.,* 2017; Jiang *et al.,* 2017; Keymer *et al*., 2017), lipids are the dominant form of stored carbon in AMF spores (Beilby and Kidby, 1980; Jabaji-Hare, 1988; Gaspar *et al.,* 1994, Bonfante *et al.,* 1994). The mobilization of lipids has possibly a central role during the germination to produce carbohydrates and cellular bioenergetic potential (Lammers *et al.,* 2001; Besserer *et al.,* 2008). In germinating spores, acetyl CoA-derived from lipids breakdown enters the glyoxylate cycle (Lammers *et al.,* 2001) to produce carbohydrates potentially employed in glycogen and chitin synthesis. Taken in the whole, the data suggest that SLs may activate metabolic pathways leading to lipid recycling. This process is probably central not only for hyphal branching, but also for spore germination in both AMF. SLs analogs were indeed shown to stimulate spore germination of *R. irregularis* and *Glomus claroideum* (Besserer *et al.,* 2006). Also our current experiments suggest a significant increase in *G. margarita* germination rate after GR24 treatment (M. Novero, unpublished results).
More recent RNA-seq experiments were performed by Kamel and colleagues (2017) using *R. irregularis* and *Gigaspora rosea* in association with three phylogenetically distant host plants in comparison with non symbiotic germinating spore treated with GR24 or root exudates. They found a core set of secreted proteins (SP) shared by both AMF. Most of these common SPs are small proteins of unknown function that may represent putative host non-specific effector

proteins. The suggestion that SLs may induce the secretion of proteins relevant for the symbiosis already found a confirmation in the findings of Tsuzuki *et al.* (2016). The putative secreted protein 1 (SIS1), highly induced by GR24, was shown to be essential for the correct establishment of the AM symbiosis (Tsuzuki *et al.* 2016).

Taken in the whole, these results suggest that SLs regulate the expression of many fungal secreted proteins whose activity may be operational during both the pre-symbiotic and symbiotic stages, leading to a positive control on host plant colonization.

Strigolactones and prokaryotes: a focus on the endobacteria of AMF

Recent works have discovered an increasing number of cooperative bacterial-fungal associations (Frey-Klett *et al.,* 2011) and revealing an unexpected level of diversity in these interactions (Olsson *et al.,* 2017). Some AMF possess endobacteria inside their cytoplasm, leading to the most intimate interaction so far described between bacteria and fungi. Irrespective of their genetic and functional diversity, fungal-associated bacterial communities constitute a novel type of microbiota, the fungal microbiota (Desirò *et al.,* 2014, Bonfante and Desirò, 2017). The rod shaped endobacterium *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) has a crucial role in the pre-symbiotic life stage of *G. margarita*, enhancing its bioenergetic potential in terms of ATP production (Salvioli *et al.,* 2016). Since it is acknowledged that SLs have an impact on the fungal mitochondrial metabolism (Besserer *et al.,* 2006, 2008), we wondered whether they could be perceived by the endobacterium. It has already been demonstrated that low concentrations of GR24 stimulates nodule formation in the legume-rhizobia interaction (López-Ráez *et al.,* 2017 and references therein). In a recent work McAdam *et al.* (2017) showed that SLs promote infection thread formation probably by influencing the bacterial partner.

When *G. margarita* germinated spores were treated with SLs analogs, *Ca*Gg showed a strong increase of the expression of *fts*Z, a bacterial replication marker (Anca *et al.,* 2009) and an increase in the number of bacteria was observed. The boost of fungal metabolism induced by GR24 may provide energy and nutrients for the bacterium to increase its population.

When compared to a cured line lacking *Ca*Gg (Lumini *et al.,* 2007), the *G. margarita* line containing endobacteria revealed a higher level of transcripts involved in mitochondrial respiration (Table 2), a higher ATP production and a more intense oxygen consume (Salvioli *et al.,* 2016; Vannini *et al.,* 2016). Interestingly, similar effects were observed after GR24 treatment

(Table 2). We speculate that both the endobacterium and SLs have the fungal mitochondrion as the first target, and that the presence of *Ca*Gg could make *G. margarita* more efficient in responding to SLs. This is supported by the observation that a *Ca*Gg peroxiredoxin encoding gene was specifically activated when *G. margarita* spores were treated with GR24 (Salvioli *et al.,* 2016). Interestingly, this bacterial gene, a marker for ROS-scavenger metabolism, was not activated when spores were treated with H_2O_2. The bacterial enzyme could be specifically active against the endogenous ROS produced by the fungal respiration that is boosted by the GR24 treatment.

In summary, current results suggest that SLs are perceived not only by the AMF, but also by their endobacteria. It would be interesting to clarify whether these responses are direct or mediated by the fungal host.

The impact of strigolactones on non AM fungi

Since SLs have a wide distribution throughout the plant kingdom (Delaux *et al.,* 2012; 2014) and are components of root exudates it is likely they could be involved in the communication with other organisms beside AMF and parasitic plants (Garcìa-Garrido *et al.* 2009). Indeed, SLs were shown to have an important role in the control of other biotic interactions (Marzec 2016; López-Ráez *et al.,* 2017). These types of investigations are of high relevance as they could highlight commonalities or specificities in genes and signals, including those exchanged in the rhizosphere, that mediate plant responses to pathogenic and symbiotic microbes (Hayachi and Parniske, 2014).

In plant-microbe interactions, two mode of actions of SLs can be envisaged: a direct effect on the microbial growth or an indirect effect that may arise during the colonization process as a consequence of changes in the host plant metabolism. After the work of Akyiama *et al.* (2005) on AMF, the effects of SLs on the *in vitro* growth of a number of other plant-interacting fungi have been investigated (Steinkellner *et al.,* 2007; Dor *et al.,* 2011; Torres-Vera *et al.,* 2014; Dekker *et al.,* 2017) with sometimes conflicting results possibly related to the different biological systems, experimental conditions, final concentration and type/mixture of SLs stereoisomers.

The application of GR24 into a hole in the medium in front of a colony did not show effect on hyphal branching of *Paxillus involutus, Laccaria bicolor, Amanita muscaria, Cenococcum geophilum* (ectomycorrhizal fungi), *Piriformospora indica* and *Trichoderma* (beneficial fungi), *Rhizoctonia solani, Fusarium oxysporum* and *Verticillium dahliae* (soil-borne pathogens) or

Botrytis cinerea and *Cladosporium sp.* (pathogen of aerial parts) (Steinkellner *et al.,* 2007). With a similar assay (GR24 solutions added to fibreglass discs in front of the fungal colony) Torres-Vera *et al.* (2014) did not observe impact on the growth of *B. cinerea*. Application of eip-GR24 also had no effect on growth of the oomycete *Pythium irregulare* (Blake *et al.,* 2016) or *Fusarium oxysporum* (Foo *et al.,* 2016).

On the other hand, the supply of GR24 embedded in the medium where the fungi were inoculated led to a reduced radial growth of several plant pathogens (*Fusarium oxysporum* f. sp. *melonis*, *Fusarium solani* f. sp. *mango*, *Sclerotinia sclerotiorum* and *Macrophomina phaseolina*, *Alternaria alternata, Colletotrichum acutatum* and *Botrytis cinerea*). In addition, slightly increased hyphal branching was observed for *A. alternata, F. solani* f. sp. *mango* and *B. cinerea* (Dor *et al*., 2011). In a similar assay GR24 reduced the *Sclerotinia sclerotiorum* colony size by 20% (Decker *et al.,* 2017).

The last experimental system was also used by Belmondo *et al.* (2017) who confirmed the sensitivity to GR24 of *B. cinerea*. The reduction in radial growth was indeed exploited in a bioassay for the screening of *B. cinerea* knock-out mutants less sensitive to GR24. Two mutants turned out to be less sensitive to GR24; one is defective of a thioredoxin reductase and the second is lacking a transcription factor belonging to the GATA family. Interestingly, both mutants display an impaired ROS metabolism. In addition, an oxidizing effect was observed in the mitochondrial intermembrane space of a *B. cinerea* strain expressing a redox-sensitive GFP2 upon exposure to GR24. It seems therefore that also in this pathogenic system, in analogy to what has been observed in AMF, ROS and mitochondria are emerging as mediators of SLs actions.

A connection between SLs and ROS was also observed during the early stages of host plant infection by root parasitic plants (Gonzalez-Verdejo *et al.,* 2006).

These results may open new experimental and conceptual perspectives to identify genetic determinants involved in SLs responses in AMF. In an evolutionary perspective it can be hypothesized that SLs may have been first perceived by fungi as a stress/xenobiotic signal and were later co-opted for host detection by AMF (Dor *et al.,* 2011; Belmondo *et al.,* 2017).

SLs biosynthetic mutants were also analysed to study the role of SLs on the outcome of plant-pathogen interactions (Marzec, 2016; Fig. 3). The tomato *slccd8* mutants showed hypersensitivity to *B. cinerea* (Torres-Vera *et al.,* 2014). Very recently, Decker *et al.* (2017) demonstrated that *ccd7* and *ccd8* mutants of the moss *Physcomitrella patens* (which is not an AM host) are more

susceptible to *S. sclerotiorum*, *F. oxysporum* and *Irpex sp*. This effect seems to be mediated by the interaction of SLs with other defence-related hormones rather than a direct effect of SLs on the fungal growth (Torres-Vera *et al.,* 2014; Decker *et al.,* 2017). However, no difference in disease development was observed between SL-deficient and wild-type pea challenged with *Fusarium oxysporum* or the oomycete *Pythium irregulare* (Blake *et al.,* 2016). Thus, so far a general role of SLs on biotic stress cannot be defined.

The AM symbiosis and SLs at a crossroad of root morphogenesis and phosphorus metabolism

While SLs play an important function in the early pre-contact stage of the AM symbiosis, by contrast, their role when the fungus develops in root tissues is not fully clear. Understanding this issue is hampered by the fact both SLs and the AM symbiosis influence several aspects of root biology in particular the root system architecture, including the formation of lateral roots which are the preferential site of AM colonization (Matthys *et al.,* 2016; Oláh *et al.,* 2005; Mukherjee and Ané, 2011; Fusconi 2014). Moreover, the AM symbiosis has a deep impact on mineral nutrient metabolism in particular that of phosphorus (P; Smith *et al.,* 2011), which in turn influences the production of SLs. It is in fact known that SLs biosynthesis and exudation are highly dependent on nutrient availability, with an increase in particular under phosphate (Pi) limiting conditions (López-Ráez *et al.,* 2008) when the AM symbiosis can provide major benefits to the host plant. However, the supply of GR24 to plants with high Pi status did not restore AM colonization (Balzergue *et al.,* 2011; Breullin *et al.,* 2010). Further evidence that SLs are not required for P regulation of AM comes from the observation that SL-deficient mutant can still regulate AM in response to P (Foo *et al.,* 2013a).

These observations indicate that nutrient availability/status is therefore a stronger driver in the control of AM colonization and further support the occurrence of a complex and finely tuned endogenous regulation of the process. In the last decade, several studies, on the basis of pharmacological (treatment with the molecule of interest) and genetic approaches (analysis of mutant lines), highlighted the involvement of other phytohormones (Pozo *et al.,* 2015); in addition, for some of them evidence of cross-talk with SLs metabolism is also emerging. In the following paragraphs we will present data on how SLs metabolism is modified upon mycorrhization, also providing potential explanations of the mycorrhizal phenotype in SLs

mutants.

It is worth to mention that non-host plants produce mainly non-canonical SLs like carlactone and derivatives (albeit this has been analyzed mostly in Arabidopsis, and may not be valid as a general statement for non-host plants; Abe *et al.,* 2014; Seto *et al.,* 2014); these non-canonical SL forms have been reported to be active on AMF (Mori *et al.,* 2016). In addition, SLs treatment does not induce the formation of the symbiosis in non-host roots (Illana *et al.,* 2011). The non AM host status thus does not depend on SLs but is possibly the consequence of the lack of several symbiotic genes (Delaux *et al.,* 2014). In the context of an evo-devo perspective (Bonfante and Genre, 2008), SLs synthesis genes seems to be operational downstream the genes of the CSP (Oldryod *et al.,* 2013). Interestingly, two transcription factors of the CSP, NSP1 and NSP2, were shown to act as regulators of SLs biosynthesis (Liu *et al.,* 2011). Indeed CSP mutants in pea display reduced SLs levels in roots consistent with the hypothesis that CSP positively regulates SLs biosynthesis (McAdam *et al.,* 2017). In addition, very recent data showed that NSP1, which is induced in colonized cortical cells during later stages of AM colonization (Takeda *et al.,* 2013) also contributes to the transcriptional program associated with arbuscule degeneration (Floss *et al.,* 2017). Connection elements are therefore emerging between SLs and the CSP which may contribute to the control of the AM symbiosis not only in the early but also in the late stages of the colonization process.

SLs biosynthesis is regulated during the AM colonization

SLs biosynthesis and exudation into the rhizosphere are induced under nutrient limiting condition and during the early stage of the AM symbiosis (Yoneyama *et al*., 2007; Yoneyama *et al*., 2013; López-Ráez *et al*., 2015). Then, when the AMF profusely colonizes the root (later stages) a decrease of SLs content was observed in tomato, lettuce, pea, cowpea and cotton roots (Lendzemo *et al*., 2009; López-Ráez *et al*., 2011; 2014; Aroca *et al*., 2013; Fernàndez-Aparicio *et al*., 2010). The SLs reduction in mature mycorrhizas has been related to the activation of a control mechanism to limit over-colonization which could be metabolically costly for the host plant (López-Ráez *et al*., 2015). However, the molecular bases of this mechanism are not known. Depending on the plant species, different expression profiles of *CCD7* and *CCD8*, the key genes involved in SLs biosynthesis (Fig. 3; Al Babili and Bouwmeester, 2015) and, so far, the most investigated, were detected during late stages of mycorrhizal colonization.

The spatio-temporal expression pattern of the *CCD7* and *CCD8* genes was investigated in tomato during the AM symbiosis establishment in the whole root system in a time course experiment and, through the laser microdissection technology, in different cell populations (López-Ráez *et al.,* 2015). Interestingly, in mycorrhizal roots, *SlCCD7* was up-regulated compared to non-mycorrhizal roots in all the considered time points and in cortical cells containing arbuscules compared to the cortical cells without arbuscules. By contrast, the expression of *SlCCD8* did not change significantly in any condition. In agreement, no change in *CCD8* expression in the later stage of the symbiosis was also reported in petunia (Breullin *et al.,* 2010). A similar *CCD* expression pattern was observed in the model legume *Medicago truncatula* where only the putative homolog of *CCD7* was up-regulated in mature mycorrhizas (Gomez *et al.,* 2010). However, in the other legume *Lotus japonicus* both *CCD7* and *CCD8* were slightly induced with a comparable expression pattern during the pre-symbiotic (4 days post fungus inoculation - dpi) and late stages (28 dpi) (Guether *et al.,* 2009).

Similarly, high-throughput gene expression analysis in rice mycorrhizal root revealed a strong up-regulation of both *CCD7*/*OsD17* and *CCD8*/*OsD10* during the late stage of the symbiosis (Güimil *et al.,* 2005; Fiorilli *et al.,* 2015). Interestingly, both *CCD* genes and the two rice MAX1 homologs (Cardoso *et al.,* 2014) were also found to be strongly expressed in the host large lateral roots (LLR) compared to the non-host fine lateral roots (FLR) in the presence of AMF, suggesting that the SLs biosynthesis is locally, and not systemically, induced by the presence of the fungus (Fiorilli *et al.,* 2015). Interestingly, the two root types displayed a different Pi content: the non-host FLR have a higher level of Pi compared to the host LLR. These data suggest that in FLR the increase in Pi level may repress the SLs biosynthesis, contributing to make this tissue recalcitrant to AM fungal colonization. It is worth to note that in rice other genes, annotated as CCD8, are up-regulated during AM colonization (Fiorilli *et al.,* 2015). Although they have not been characterized so far, it can be hypothesized that they may be involved in the regulation of SLs metabolism and of the AM symbiosis.

Even if data are fragmentary, there is evidence of a constant *CCD7* gene activation upon mycorrhization. This activation has been related to the involvement of this enzyme also in the production of AM-induced C_{13}/C_{14} apocarotenoids such as α-inol glucoside and mycorradicin (Klingner *et al.,* 1995; Walter *et al.,* 2000; Fester *et al.,* 2002; Vogel *et al.,* 2010). By contrast, the expression of *CCD8*, which is known to specifically catalyze the synthesis of carlactone, a

SLs precursor, is often not regulated by the AM symbiosis.

Remarkably, a SLs reduction was described in mature mycorrhizas (Lendzemo *et al*., 2009; López-Ráez *et al*., 2011; 2014; Aroca *et al*., 2013; Fernàndez-Aparicio *et al*., 2010) but this is not mirrored by a down-regulation of the *CCD7* and/or *CCD8* SLs biosynthetic genes (López-Ráez *et al.,* 2015). It is worth to note that SLs biosynthesis is regulated by a negative feedback mechanism that controls *CCD7* and *CCD8* expression (Simons *et al.,* 2007; Snowden *et al.,* 2005). In addition, an activation of CCD7 in mycorrhizal roots could also mirror the increased production of additional compounds rather than SLs. A recent study could provide a different explanation: among the secreted proteins expressed by *R. irregularis* (Kamel *et al*., 2017) one sequence (RiSP811) has been annotated as a putative α/β hydrolase, the enzymatic activity of SLs receptors described in plants (Hamiaux *et al*., 2012; Nakamura *et al*., 2013; de Saint Germain *et al*., 2016); interestingly, the gene is induced by GR24 exposure and during root colonization. It would be interesting to investigate whether this protein could interact with and hydrolyze SLs and therefore contribute to the degradation of SLs in mycorrhizal roots.

The transport of SLs can be considered a further component of SLs metabolism in roots. The *Petunia hybrida* ABC transporter PLEIOTROPIC DRUG RESISTANCE 1 (PDR1) functions as a cellular SLs exporter (Kretzschmar *et al.,* 2012). *pdr1* mutants have normal level of orobanchol (the most abundant SLs in petunia) in root tissues, but orobanchol exudation is reduced and, as a consequence, the AM colonization is less efficient than in WT plants (Kretzschmar *et al*., 2012; Borghi *et al*. 2016). *PDR1* is up-regulated during the AM colonization and upon Pi starvation. In accordance with this result, PhPDR1 promoter activity was localized in the root tip and in the subepidermal cells of the lateral roots corresponding to hypodermal passage cells which are described, in some plant species, to be the cortical entry points for AMF hyphae and in regions containing or flanking fully developed arbuscules (Sharda and Koide, 2008; Kretzschmar *et al.,* 2012). Sub-cellular localization experiment revealed that the PDR1 protein co-localizes with CCD8/DAD1 in the root tip (Sasse *et al.,* 2015). These data suggest that the regulation of SLs transport might have also a guidance function in the already colonized root, through the induction of intraradical hyphal branching (Kretzschmar *et al*., 2012; Borghi *et al*., 2016).

Up to date the only characterized SLs transporters have been identified in Solanaceae species: the PDR1 from petunia (Kretzschmar *et al*., 2012) and its putative orthologue in *Nicotiana tabacum* PDR6 (Xie *et al*., 2015a). Due to frequent duplication events, the identification of PDR1

homologues in other plant species could be difficult.

The AM colonization of SLs-deficient and insensitive mutants

Pea, rice, petunia and tomato mutants impaired in SLs biosynthesis or export display a reduced level of AM colonization; however, the morphology of intraradical fungal structures is never affected (Gomez-Roldan *et al*., 2008; Breullin *et al*., 2010; Vogel *et al*., 2010; Guthjar *et al*., 2012; Kohlen *et al*., 2012; Kretzschmar *et al*., 2012; Vogel *et al*., 2010; Yoshida *et al*., 2012). Supplementation with GR24 restores the colonization rate of *rms1*/*dad1*/*ccd8* mutant plants (Gomez-Roldan *et al*., 2008, Breullin *et al*., 2010), suggesting that SLs are important but not essential for the AM establishment and that the effect of SLs on AMF is mainly occurring in the rhizosphere, although supplementation with GR24 could also affect root physiology and, indirectly, AM colonization.

Interesting data on the AM symbiosis are coming from the analysis of SLs insensitive plants, that is plants defective in SLs signaling components (Fig. 3). The *d14* rice mutant, lacking the SLs receptor (Fig. 3), shows a slightly higher AM colonization levels compared to wild type, probably due to the higher SLs exudation which results from a feedback mechanism (Yoshida *et al*., 2012). Surprisingly, the AM phenotype in SLs perception mutants defective of downstream signaling components such as the rice *d3* and pea *rms4* (Fig. 3) is rather severe with several aborted infection attempts and a significant reduction of arbuscules and vescicles formation (Yoshida *et al.,* 2012; Foo *et al.,* 2013a) despite they have a normal or an even increased SLs exudation (Yoshida *et al.,* 2012, Gutjahr *et al.,* 2015). It is worth to note that D3/RMS4 F-Box protein is shared by SLs and karrikins signaling pathway. Karrikins are a class of molecules found in aqueous smoke extracts that can promote seed germination of many species (Flematti *et al.,* 2004). Thus, it has been hypothesized that the impaired AM phenotype might be the consequence of the lack of activation of the karrikin signaling (Water *et al.,* 2017). In line with this hypothesis, Gutjahr and colleagues (2015) demonstrated that the rice mutant defective of the karrikin receptor *D14-like* (homolog of the *KAI2* of Arabidopsis) is unable to establish the mycorrhizal symbiosis, a condition mirrored by a complete absence of hyphopodia formation. This is so far one of the most clear-cut mycorrhizal phenotypes so far reported. In line with a potential involvement in early stages of the interaction, the *d14-l* mutant does not show the transcriptional response to germinating spores exudates observed in the wild-type, suggesting the

fascinating hypothesis that the fungal exudates may contain a candidate ligand molecule crucial for the symbiosis. On the other hand, due to the fact that D14-like genes have been found in the genomes of basal land plants, including non AM hosts, and that most plants are not dependent on karrikin for seed germination it has also been suggested that an endogenous, karrikin-like (unknown) compound, plant ligand may exist (Guthjar *et al*., 2015; Waters *et al*., 2017).

SLs / hormones cross-talk during the AM colonization

Several studies indicate possible cross-talks between SLs and other hormones in the regulation of the AM symbiosis, and this makes the understanding of the *in planta* role SLs even more challenging.

Change in auxin level in roots upon AM colonization as well as higher AM colonization rates upon exogenous auxin treatments have been observed in different plants (review in House et al., 2007, Gutjahr 2014). Although the development of fungal structures were not affected, a decrease of the mycorrhization level was observed in pea and tomato mutants affected in indol acetic acid (IAA) biosynthesis, transport or signaling (Foo *et al.,* 2013a; Hanlon *et al.,* 2010). In the pea IAA deficient mutant (*bushy*) the low percentage of mycorrhization was ascribed to a lower SLs biosynthesis and exudation (Foo *et al*., 2005; Foo 2013). Indeed, GR24 treatment could partially restore the AM colonization (Foo 2013). The link between SLs and IAA is strengthened by the recent results obtained by Guillotin and colleagues (2017) who showed a lower AM colonization in the tomato RNAi *Sl-IAA27* line, which has a reduced expression level of an Aux/IAA gene involved in auxin signaling and specifically up-regulated during mycorrhization. Interestingly, the reduced mycorrhization could be elevated with GR24. This study also demonstrated the co-regulation of the NSP1 and the SL biosynthesis gene D27 leading to the hypothesis that Sl-IAA27 positively regulates mycorrhization by controlling SLs biosynthesis.

Likewise, ABA positively regulates AM development and functionality (Herrera Medina *et al*., 2007). ABA biosynthesis knock-out mutants in tomato (*notabilis, sitiens* and *flacca*) display a down-regulation of *LeCCD7* and *LeCCD8* (López-Ráez 2010) which is mirrored by a lower (about 40%) SLs content in root exudates (López-Ráez and Bowmeester 2008; López-Ráez *et al*., 2010). Possibly due to this reduced SLs level, the *sitiens* mutant displayed a reduced number of arbuscules (López-Ráez and Bowmeester 2008; López-Ráez *et al*., 2010), although this has not

been directly tested.

ABA positively interacts with SLs probably at the biosynthetic level (López-Ráez *et al*., 2010). On the other hand, SLs can also influence ABA biosynthesis: ABA content in tomato roots and leaves of the SLs-deficient mutant *SL-ORT1* was significantly lower than that of WT plants (Wu et *al.,* 2017), although the molecular basis of the *ort1* mutation is not known. This data was also confirmed in SLs deficient mutant line *Slccd8* where reduced levels of the defence hormones JA, SA and ABA were found compared with the WT (Torres-Vera *et al.,* 2014). In tomato, Lotus and lettuce plants, a cross-talk between ABA and SLs has been found in mycorrhizal plants under drought and under salinity stress (Aroca *et al.,* 2013; Liu *et al.,* 2015; Ruiz-Lozano *et al.,* 2016; López-Ráez 2016). Since mycorrhizal symbiosis alleviates drought and salinity stresses, SLs-ABA cross-talk may at the basis of the benefit of the AM symbiosis provides to plants under these unfavourable conditions (López-Ráez, 2016).

Gibberellins (GA) have been described as negative regulators of the AM symbiosis. Exogenous application of GA inhibits AM colonization in a dose dependent manner (El Ghachtouli *et al*., 1996; Yu *et al*., 2014; Takeda *et al*., 2015). Accordingly, the GA biosynthesis mutants displayed a higher number of arbuscules and the DELLA proteins, repressors of GA signaling, are essential for their formation (Foo *et al*., 2013b; Floss *et al*., 2013, Yu *et al*., 2014, Martín-Rodriguez *et al*., 2015). A cross-talk between SLs and GA is emerging: a SLs-dependent interaction between the SLs receptor, D14, and the GA signaling repressor, SLR1 was reported (Nakamura *et al*., 2013) and, recently, GA signaling was shown to controls the SLs biosynthesis, through a down-regulation of corresponding genes (Ito *et al*., 2017). Interestingly, in the SLs-deficient mutant (*SL-ORT1*) GA3 content was higher in root than in the WT, while in leaves, the GA level (in particular GA3 e GA9) showed an opposite trend (Wu *et al*., 2017). However SL-deficient mutant in pea has no change in GA content of shoot (de Saint Germain *et al.,* 2013). These observations open the question whether the defect in the AM colonization may arise from a lack of SLs or an increase of GA or from balanced fine tuning of the two hormones.

The role of cytokinins (CK) in the AM symbiosis is less explored (Foo *et al*., 2013b). So far, increase CK level in mycorrhizal plants was reported (Allen *et al*., 1980; Shaul-Keinan *et al*., 2002). Recently, it has been demonstrated that both shoot- and root-specific alterations of CK

474 levels play important roles in the relation between CK homeostasis and the growth effect
475 observed in AM plants (Cosme *et al*., 2016). By contrast, no AM phenotype was detected in
476 the medicago CK-insensitive mutant *cre1* (cytokinin response 1) defective in a cytokinin
477 receptor, suggesting that at least the CRE1-dependent cytokinin signaling is not essential for the
478 AM symbiosis (Foo *et al*., 2013b). So far, little evidence of interaction between CK and SLs
479 metabolism has emerged. CK might inhibit SLs biosynthesis (Bainbridge *et al*., 2005) but
480 contrasting results were obtained for CK content in SLs biosynthesis mutants probably due to the
481 different organs and different species considered. In particular, in pea and Arabidopis SLs-
482 deficient mutants a reduced levels of cytokinin in xylem sap was observed (Beveridge et al.,
483 1994, 1997a,b; Morris et al., 2001; Foo et al., 2007). A decrease content of dihydrozeatin (dhZ)
484 was also detected in leaves of tomato *SL-ORT1* mutant while the root displayed an increase
485 content of CK than WT plants (Wu *et al*., 2017). No differences of CK content were observed in
486 shoot apices of rice *d* mutants (Arite *et al*., 2007) and in shoot tissue of pea SLs-deficient mutant
487 (Foo *et al*., 2007).
488
489 Still little explored is the role of brassinosteroids (BR) in the development of the AM symbiosis.
490 Tomato mutants defective in BR biosynthesis showed decreased mycorrhization (Bitterlich *et al.,*
491 2014). Interestingly, Wang and colleagues (2013) demonstrated that Arabidopsis BES1 (bri1-
492 EMS-suppressor 1), a positive regulator in BR signaling pathway, is a direct target of MAX2, the
493 F-box protein involved in SLs signaling (Fig. 3), and acts as a negative regulator of SLs signaling
494 pathway to promote shoot branching (Wang *et al.,* 2013).
495
496 Overall the deregulation of the AM colonization (lower / higher colonization rate) observed in
497 auxin, ABA and GA mutants indicate that these hormones contribute to control AM
498 establishment. For some of them (auxin, ABA and GA) possible cross-talks with SLs are
499 emerging. While a direct role of SLs on the AMF is evident in the rhizophere, the situation is
500 definitely more complex inside the root tissues. In fact, a mycorrhizal root is a very
501 heterogeneous environment where local and systemic responses occur. In addition, the AM
502 colonization is a very dynamic process with a high arbuscule turnover. Specific spatio-temporal
503 changes in the synthesis, distribution and/or activity of SLs and other hormones are likely to
504 occur and, in the end, mediate the final outcome of the complex network of interactions.

It is also important to underline that there is a distinction between the early stages of the interactions where the fungal metabolism must be activated to favor the contact with the host (active metabolism, release of signaling molecules…) from the late stages where a fine control over fungal proliferation should be set up to guarantee the beneficial mutualistic association. It is tempting to speculate that SLs and the cross-talk with the other phytohormones may contribute to regulate the complex process controlling mycorrhizal formation and arbuscules turn over.

Conclusions

SLs are signal molecules with an ancient origin in the plant kingdom. Their ancestral function of regulators of developmental processes has accompanied the increasing biological complexity of land plants (Waters et al., 2017). Their versatility is also witnessed by the fact that during the evolution they have been exploited, once released in the rhizosphere, as a vocabulary to communicate with soil organisms even belonging to different kingdoms (i.e. AMF and associated bacteria) beside parasitic plants. The range of plant-interacting organisms that may be targets of SLs action could be even wider. SLs biosynthetic mutants often show higher susceptibility to pathogens, possibly due to an altered homeostasis of other defence hormones; however, this is not a universal response since the outcome of some plant-microbe interactions is not influenced by the lack of SLs (López-Ráez *et al*., 2017). To better define the involvement of SLs in plant-pathogen interactions, more detailed studies, possibly extended to different pathosystems, are needed. This information will be instrumental for a safe use of natural or synthetic SLs as innovative tools in the field of agro-biotechnology.

In the specific case of the AM symbiosis studies carried out in the last decade showed that SLs act as positive regulators. Although not essential for the establishment of this mutualistic association, SLs are relevant to achieve a full extent of mycorrhization, primarily by boosting the fungal metabolism and, ultimately, its ability to reach and colonize root tissues. The role of SLs *in planta* is, so far, still ambiguous as the perturbation of SLs biosynthesis and signaling was shown to alter the metabolism of other hormones which also contribute to the correct establishment of the AM symbiosis. In addition, SLs seem to operate in the hub which regulates phosphate metabolism as well as root morphogenesis, two processes that, in host plants, are known to be, to some extent, under the control of the AM symbiosis (Smith *et al.,* 2011; Fusconi, 2014). Understanding the biological relevance of each of the components of this complex

network and how they interact will be the challenging task to be pursued in the future.

Acknowledgements

Research in the authors' lab was supported by the SLEPS and 60% Projects (University of Torino) to LL, and by Mycoceres (CARIPLO) and 60% Projects (University of Torino) to PB. The Authors thank Dr. Marta Vallino for the help in drawing Figure 4 and the three reviewers for their comments and suggestions.

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Table 1. Differentially expressed genes in *G. margarita germinating* spores after 1 week GR24 treatment. A fold change cutoff of +/- 0.5 and an FDR of < 0.05 have been used (Salvioli *et al.,* 2016).

Transcript ID	Log2 Fold Change	Sequence description
Genes involved in fungal respiration		
comp35750_c0	1.3	cytochrome c oxidase subunit 1
comp15252_c0	0.65	ubiquinol-cytochrome c reductase complex core protein 2 precursor
comp15565_c0	0.83	nadh dehydrogenase Fe-S protein 5
comp18263_c0	0.39	nadh dehydrogenase 1 alpha subcomplex 6
comp31224_c0	0.7	ubiquinol-cytochrome c reductase complex 17 kd protein
comp32142_c0	2.25	nadh dehydrogenase subunit 4l
comp34943_c1	1.26	nadh dehydrogenase subunit 52037
comp36626_c0	0.48	cytochrome c oxidase subunit va
comp36884_c0	0.7	cytochrome c oxidase assembly protein cox-16
comp37253_c0	1.17	cytochrome c
comp6965_c0	0.6	ubiquinol-cytochrome c reductase complex 14 kDa protein
comp7520_c0	0.78	nadh dehydrogenase
Genes involved in other pathways		
comp37189_c0	1.18	vacuolar amino acid transporter 1-like
comp37057_c0	1.07	chitin deacetylase
comp5264_c0	-1.65	chitin synthase
comp38121_c0	-0.85	mating type protein mat1-2-1
comp9271_c0	-4.18	ABC multidrug transporter mdr1
comp39141_c0	1.9	cytochrome P450

Table 2. Differentially expressed genes in *G. margarita* germinating spores containing (B+) or not (B-) the endobacteria and after GR24 treatment. A fold change cutoff of +/- 0.5 and an FDR of < 0.05 have been used (Salvioli *et al.,* 2016).

B+ *vs* B-

Transcript ID	Log2 Fold Change	Sequence description
comp35650_c2	0.88	cytochrome c oxidase subunit 1
comp34209_c0	0.54	nadh dehydrogenase subunit1
comp33766_c0	0.25	nadh-ubiquinone oxidoreductase
comp29917_c0	3	nadh dehydrogenase

B+ GR24 *vs* B- GR24

Transcript ID	Log2 Fold Change	Sequence description
comp35750_c0	1.65	apocytochrome b
comp32142_c0	1.44	nadh dehydrogenase subunit 4l
comp34871_c0	1.39	cytochrome c oxidase subunit 3
comp35009_c0	1.36	mitochondrial protein, putative
comp34943_c1	1.28	nadh dehydrogenase subunit 5
comp35650_c2	1.12	cytochrome c oxidase subunit 1

Figure legends

Figure 1. The scheme illustrates the potential interactions between the signaling molecules released by the fungal and plant partners in the AM symbiosis. SLs treatment leads to an increase in the release of chitin oligomers by AMF and, as a consequence, to an amplification of the calcium spiking response in the host plant (Genre *et al.,* 2013); COs induce the expression of CCD7, a SLs biosynthetic gene (Giovannetti *et al.,* 2015), although it has not been proved that this leads to induced SLs production. SLs treatment also stimulates the release of fungal secreted protein, such as SIS1 that positively regulates the AM colonization (Tsuzuki *et al*. 2016).

Figure 2. List of the enriched GO (Gene Ontology) categories in germinating spores of *R. irregularis* (**A**) and *G. margarita* (**B**) after 1 week GR24 treatment. The differential expression analysis was performed as described in Salvioli *et al.* (2016). Briefly, raw reads libraries were trimmed with Trimmomatic V.0.36 (Bolger *et al.,* 2014) and aligned on the reference transcriptomes (Lin *et al.,* 2014; Salvioli *et al.,* 2016) using bowtie2 (Langmead and Salzberg 2012). The DESeq2 1.12.4 Bioconductor package (Love *et al.,* 2014) was used for the identification of differentially expressed genes. Gene Ontology (GO) enrichments were performed with the AgriGO web platform (http://bioinfo.cau.edu.cn/agriGO/) and plotted with ggplot2 R package.

Figure 3. Biosynthesis and signaling pathway of SLs.
CCD: CAROTENOID CLEAVAGE DIOXYGENASE;
D: DWARF (*Oryza sativa* genes);
DAD: DECREASED APICAL DOMINANCE (*Petunia hybrida* genes);
MAX: MORE AUXILLARY GROWTH (*Arabidopsis thaliana* genes);
RMS: RAMOSUS (*Pisum sativum* genes).

Figure 4. Effect of SLs on the host plant, the AM fungus and in its endobacteria during the establishment of AM symbiosis.