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4 **TITLE**

5 **Initiation of arbuscular mycorrhizal symbiosis involves a novel pathway independent**
6 **from hyphal branching**

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ABSTRACT

The arbuscular mycorrhizal symbiosis is a very common association between plant roots and soil fungi, which greatly contributes to plant nutrition. Root-exuded compounds known as strigolactones act as symbiotic signals stimulating the fungus prior to root colonization. Strigolactones also play an endogenous role *in planta* as phytohormones and contribute to the regulation of various developmental traits. Structure-activity relationship studies have revealed both similarities and differences between the structural features required for bioactivity in plants and arbuscular mycorrhizal fungi. In the latter case, bioassays usually measured a stimulation of hyphal branching on isolated fungi of the *Gigaspora* genus, grown *in vitro*. Here, we extended these investigations with a bioassay that evaluates the bioactivity of strigolactone analogs in a symbiotic situation, and the use of the model mycorrhizal fungus *Rhizophagus irregularis*. Some general structural requirements for bioactivity reported previously for *Gigaspora* were confirmed. We also tested additional strigolactone analogs bearing modifications on the conserved methylbutenolide ring, a key element of strigolactone perception by plants. A strigolactone analog with an unmethylated butenolide ring could enhance the ability of *R. irregularis* to colonize host roots. Surprisingly, when applied to the isolated fungus *in vitro*, this compound stimulated germ tube elongation but inhibited hyphal branching. Therefore, this compound was able to act on the fungal and/or plant partner to facilitate initiation of the arbuscular mycorrhizal symbiosis, independently from hyphal branching and possibly from the strigolactone pathway.

KEYWORDS

Arbuscular mycorrhiza; strigolactone; symbiosis; *Rhizophagus*; signalling

ABBREVIATIONS

SL: strigolactone; AM: Arbuscular Mycorrhiza; WT: wild-type; KL: KAI2 ligand

INTRODUCTION

Strigolactones (SLs) are carotenoid-derived compounds produced by plants and exuded by roots into the rhizosphere. They were first characterized as germination stimulants for seeds of the parasitic weeds *Striga* and *Orobanch*e (Cook et al. 1966). It was later discovered that SLs also stimulate Arbuscular Mycorrhizal (AM) fungi (Akiyama et al. 2005; Besserer et al. 2006), which develop a root endosymbiosis with most land plants and play important roles in natural and agricultural ecosystems (Smith and Read 2008; Chen et al. 2018). More recently still, SLs were identified as a novel class of phytohormones, contributing to the control of shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008) and a number of other developmental traits in shoots and roots (Lopez-Obando et al. 2015; Matthys et al. 2016). The bioactivity of SLs on such diverse organisms raises interesting questions about the evolution of their perception.

Since the identification of SLs as phytohormones, genetics has allowed rapid progress in the understanding of their biosynthesis, perception and hormonal effects (Seto and Yamaguchi 2014; Al-Babili and Bouwmeester 2015; Bürger and Chory 2020). The core biosynthetic pathway from β -carotene to SLs involves, after *cis/trans* isomerization by DWARF27, two Carotenoid Cleavage Dioxygenase (CCD) enzymes. CCD7 carries out a first cleavage step yielding 9-*cis*- β -apo-10'-carotenal, which is processed by CCD8 into the common SL precursor carlactone (Alder et al. 2012; Bruno et al. 2017). From that step the pathway diverges between plant species to afford a diversity of SL forms (Jia et al. 2018).

Over 30 different natural SLs have been described to date (Yoneyama et al. 2018; Xie et al. 2019; Mori et al. 2020), and numerous chemically synthesized analogs are available (Takahashi and Asami 2018). Canonical SLs comprise four carbon rings named A to D. This general structure is mimicked in GR24 (Fig. 1a), a synthetic compound used as a universal analog in SL studies (Johnson et al. 1981; Zwanenburg and Pospisil 2013). In known natural SLs, the ABC tricyclic lactone is linked by an enol ether bridge to an invariable methylbutenolide D ring. While the C ring can adopt a β - or an α -orientation, all natural SLs have the same *R*-configuration at the C-2' position, which corresponds to the configuration of their common precursor carlactone (Flematti et al. 2016). Non-canonical SLs comprise an enol-ether-D-ring moiety but lack the A, B or C rings, and include carlactone as their simplest form (Yoneyama et al. 2018).

Structure-Activity Relationship studies have been carried out to link SL structural features with their bioactivity on different organisms. Each study used a different set of SL analogs and measured particular biological responses on a given species. Still, some general conclusions can be drawn from these data. Hormonal activities in pea, rice and *Arabidopsis* have generally been measured on SL-deficient *ccd8* mutants (*rms1*, *d10* and *max4* respectively). In all three plant species, bioactivity largely

depends on the methylbutenolide D ring associated with an enol ether unit. At the other end of this enol ether bridge, the C ring can be replaced by an acyclic moiety without substantial loss of activity (Boyer et al. 2012; Boyer et al. 2014; Umehara et al. 2015). The importance of stereochemistry varies among plant species (Flematti et al. 2016). As for variations on the D ring, the conserved methyl group at C4' is absolutely required to suppress outgrowth of pea buds (Boyer et al. 2012). The importance of the D ring has been further supported by recent studies on plant SL receptors (de Saint-Germain et al. 2016; Yao et al. 2016). These receptors, called D14 in rice and *Arabidopsis*, DAD2 in *Petunia* and RMS3 in pea, belong to the alpha-beta fold hydrolase family of proteins (Arite et al. 2009; Hamiaux et al. 2012; Nakamura et al. 2013; Zhao et al. 2013). In a proposed mode of action of these receptors, a nucleophilic attack on the D ring results in the cleavage of SLs and the release of the ABC moiety. The D ring forms a transient covalent linkage with the receptor and is later released as hydroxymethylbutenolide (De Saint Germain et al. 2016; Yao et al. 2016; Seto et al. 2019). The use of profluorescent SL analogs has revealed that the conserved methyl at position C4' on the D ring strongly influences this hydrolytic process (De Saint Germain et al. 2016; Yao et al. 2018). SL receptors of root parasitic weeds seem to share their general properties, including hydrolytic activity, with SL receptors from other plants (Yao et al. 2017; Xu et al. 2018).

Ancient paralogs of SL receptors are involved in the perception of chemically related signals called karrikins (Bythell-Douglas et al. 2013). Karrikins are produced upon combustion of plant material, and possess like SLs a butenolide moiety. In an ecological context, karrikins stimulate seed germination of some plant species and thereby facilitate vegetation recovery following wildfires. They turned out to be of broader interest when it was discovered that *Arabidopsis* mutants in the karrikin receptor KAI2 exhibit germination and seedling development phenotypes, suggesting that a yet unidentified signal (termed karrikin-like or KAI2 ligand, KL) exists in plants to regulate these developmental steps (Conn and Nelson 2016). Interestingly, in addition to the perception of karrikins and KL, KAI2 is involved in the response to a subset of SL stereoisomers (namely those with a 2'S configuration, Scaffidi et al. 2014). A final and very intriguing observation is that rice mutants in the karrikin receptor KAI2 are unable to develop AM symbiosis (Gutjahr et al. 2015). At this stage it remains unclear whether the cause of this phenotype is the absence of the protein itself, or a defect in the perception of a fungus- or plant-derived signal.

The discovery of a role for SLs in AM symbiotic signalling stemmed from the observation that AM fungi grown *in vitro* exhibit profuse hyphal branching in the vicinity of host roots. This morphological response can be reproduced to some extent in isolated fungi grown *in vitro* and treated with root exudates mixed into the medium or applied locally near hyphal tips (Nagahashi et al. 1996; Nagahashi and Douds 1999; Buée et al. 2003). SLs were later identified as essential components of the so-called “branching factor” responsible for this effect (Akiyama et al. 2005; Besserer et al. 2006). Other fungal

responses to SLs have been reported, such as increased spore germination (Besserer et al. 2006; Kountche et al. 2018) or the production of chito-oligosaccharides (Genre et al. 2013). Nevertheless, hyphal branching remains the classical bioassay to measure SL activity on AM fungi. It is mainly carried out on species of the genus *Gigaspora*, although hyphal branching induction by GR24 has also been documented in the model AM fungus *Rhizophagus irregularis* (Cohen et al. 2013). Structure-activity relationship studies on *Gigaspora margarita* have highlighted that linked ABC and D moieties were necessary for bioactivity, although the enol ether bridge between the C and D rings could be replaced by alkoxy or imino ethers (Akiyama et al. 2010a). Truncation of the A ring markedly reduced hyphal branching activity (Akiyama et al. 2010a). In contrast, the B and C rings appear partially dispensable, as attested by the activity of some carlactone derivatives (Mori et al., 2016) and other non-canonical strigolactones (Xie et al., 2019). Although canonical SLs are generally more active, some non-canonical SL forms can reach high levels of activity on AM fungi. For example carlactonoic acid, which is exuded by a wide range of plant species, has been proposed to be a common inducer of hyphal branching (Yoneyama et al. 2018). It is also noteworthy that active SL analogs can trigger different hyphal branching patterns, stimulating the formation of short or long hyphae of different orders (Akiyama et al. 2010a; Mori et al. 2016; Xie et al. 2019).

In addition to this morphological response of AM fungi to SLs *in vitro*, the importance of SLs in AM symbiosis was first shown through the analysis of SL-deficient pea mutants. AM fungi displayed a reduced ability to colonize these mutants, reaching a level of root colonization of only one third of the level observed in WT plants (Gomez-Roldan et al. 2008). Therefore, in addition to their visible effects on fungal development *in vitro*, SLs are important for the symbiotic interaction to take place.

In this article, we focus on SL bioactivity on the AM fungus *R. irregularis*. We report the development of a novel bioassay to measure the activity of SL analogs in symbiotic conditions. We also extend previous structure-activity relationship studies by testing two new SL analogs bearing modifications on the D ring.

MATERIALS AND METHODS

Biological material

The study was carried out on *Medicago truncatula* Gaertn. transposon insertional mutants of the R108 genotype: *ccd8-1*, *ccd7-1* and *d14-1* (Lauressergues et al. 2015). Backcrossed homozygous *ccd8-1* and *ccd7-1* mutants were compared to their wild-type siblings. Homozygous *d14-1* mutants were compared to wild-type R108. Seeds were sterilized and allowed to germinate for 24 h as described in Lauressergues et al. (2015).

Rhizophagus irregularis spores (strain DAOM197198) were purchased from Agronutrition (France). They were rinsed twice with sterile water before use.

Chemicals

(±)-GR24 was purchased from Chiralix (The Netherlands), and corresponding enantiopure stereoisomers were purchased from StrigoLab (Italy). The two other SL analogs (Fig. 1b) were synthesized as described in Boyer et al. (2012). All SL analogs were dissolved in acetone and stored at -20 °C. Freshly prepared 1000X stock solutions in acetone were added to the nutrient solution. An identical volume of acetone was added to nutrient solution for the mock treatments.

Plant growth and AM inoculation

Seedlings were transferred to 50 mL Falcon tubes pierced at the bottom (three holes in the conical part of the tubes) and containing sterilized substrate (OilDri UK) inoculated with 150 spores of *R. irregularis* per plant: 75 spores mixed with the substrate and 75 spores added halfway up the Falcon tube. Each tube was inserted through the lid, pierced to the diameter of the Falcon tube, of a 150 mL pot containing 85 mL of nutrient solution. This volume was sufficient for the duration of the experiments (three weeks). At the start of experiments, the nutrient solution level in pots reached roughly the lower 20 mL mark of the Falcon tubes, then it decreased gradually due to evaporation and plant growth. Throughout the experiments, the substrate acted as a coarse wick to keep all the tube content wet but aerated. At the time of harvest, the holes at the bottom of the tubes were still under the nutrient solution surface. The nutrient solution was a modified half-strength low-phosphate and low-nitrogen Long Ashton solution (Hewitt 1966), containing 7.5 µM Na₂HPO₄, 750 µM KNO₃, 400 µM Ca(NO₃)₂, 200 mg/L MES buffer, pH 6.5. Treatments were applied by adding the tested compounds to the nutrient solution at the beginning of the experiment. The concentration of the tested compounds was 10⁻⁷ M unless otherwise stated. Plants were kept in a growth chamber under a 16 h photoperiod (light intensity: 300 µmol.m⁻².s⁻¹). The temperature was set to 22 °C day and 20°C night, with 70% humidity.

Staining and observation of mycorrhizal structures

Roots were harvested three weeks post-inoculation and carefully separated from the substrate. They were cleared in 10% KOH (w/v) for three days at room temperature and stained with Schaeffer black ink (Vierheilig et al. 1998) for 8 min at 95 °C. Whole root systems were examined under a Leica RZ75 stereomicroscope, and the total number of AM infection units was counted for each plant. Infection units were defined as areas of the root cortex that host fungal structures (arbuscules, but also vesicles and intraradical hyphae), and derive from a single epidermal penetration event (Kobae 2019).

Analysis of AM fungal development in vitro

Experiments were carried out in plates containing M medium (Bécard and Fortin 1988) without sucrose and solidified with 0.4% (w/v) phytagel. SL analogs (10^{-7} M), or the solvent alone for mock treatments, were added to the medium after autoclaving. Spores of *R. irregularis* were suspended in sterile water and 10 μ L droplets containing 1 to 5 spores were placed on the medium. Plates were incubated horizontally at 30 °C under 2% CO₂ for 12 days. Germ tubes could easily be identified as the longest hypha coming out of each spore. Their length, from spore to tip and excluding any branches, was measured using ImageJ software after scanning the plates. The number of hyphal branches of the 1st order (growing from the germ tube) and 2nd order (growing from 1st-order branches) was determined using a dissecting microscope for each germinated spore. The average length of germ tubes, and average number of branches of each order were calculated for all the germinated spores on a plate (typically 30 to 40 spores). For statistical analysis, each plate was treated as a replication unit and 11 plates were analysed for each treatment.

Statistical analyses

Data were analysed using Statgraphics Centurion software (SigmaPlus). Non-parametric tests were used because normality or homoscedasticity criteria were not met. Datasets were analysed using the Kruskal-Wallis test, followed by pairwise comparisons with Dunn's test.

RESULTS

A novel bioassay to measure the effect of SLs on symbiosis initiation

We aimed to assess the bioactivity of SL analogs in a context of interaction with a host plant. To avoid any interference with endogenous SLs, we used as hosts *M. truncatula* mutants affected in the SL biosynthetic pathway. We have previously described *ccd7-1* and *ccd8-1* mutants of this species, which are affected in genes encoding two key enzymes in SL biosynthesis (Lauressergues et al. 2015). On average, *Mtccd8-1* mutants inoculated with spores of *R. irregularis* exhibited after three weeks of co-culture only 2 root colonization events per plant, vs 21.7 in wild-type plants. Exogenous treatment with the SL analog ($\pm$)-GR24 supplied in the nutrient solution restored normal levels of root colonization (Fig. 2a). A similar defect in root colonization was observed with *Mtccd7-1* mutants (Supp. Fig. 1a). In contrast, mutants carrying a mutation in the SL receptor D14 were normally colonized (Supp. Fig. 1b).

Bioactivity of SL analogs on symbiosis initiation: a structure-activity relationship study

The ability of ($\pm$)-GR24 to favour symbiosis initiation, *i.e.* to enhance root colonization of *Mtccd8-1* mutants by *R. irregularis* (Fig.2a), allowed us to use this experimental set-up as a bioassay to test the activity of other SL analogs. The first tested compounds were the ABC and D moieties of GR24,

which can be generated by the hydrolase activity of plant SL receptors (Hamiaux et al. 2012). We observed that the tricyclic ABC lactone or the hydroxymethylbutenolide D ring supplied individually, or as an equimolar mix, were unable to restore *R. irregularis* ability to colonize roots of *Mtccd8-1* mutants (Supp. Fig. 2).

We next tested the importance of SL stereochemistry. Synthetic SL analogs exist in four stereochemical configurations (Fig. 1b). We compared the biological activities of the four enantiopure stereoisomers of GR24. Three of them were highly active in symbiotic conditions, while (–)-GR24 was not significantly active as compared with the mock treatment (Fig. 2b).

We then evaluated two compounds bearing modifications on the conserved D ring part of SLs. A GR24 analog with a reduced C3'-C4' bond, (±)-H₂-2'-*epi*-GR24 (Fig. 1b; compound **35** in Boyer et al. 2012), was able to restore symbiosis initiation in *Mtccd8-1* mutants inoculated with *R. irregularis* (Fig. 3a). Like (±)-GR24, (±)-H₂-2'-*epi*-GR24 exhibited significant activity at the standard concentration of 10⁻⁷ M (Dunn's test vs mock treatment, P=0.017). However, its activity was not statistically significant at 10⁻⁸ M (P=0.052).

Finally, we evaluated an analog lacking the conserved methyl group at C4': (±)-4'-desmethyl-2'-*epi*-GR24 (Fig. 1b). This compound efficiently promoted the symbiosis between *R. irregularis* and *M. truncatula* (Fig. 3b). To compare the bioactivity of (±)-4'-desmethyl-2'-*epi*-GR24 to that of (±)-GR24, we used a dilution series of the two compounds. (±)-4'-desmethyl-2'-*epi*-GR24 was significantly active at concentrations down to 10⁻⁸ M (Dunn's test, P=0.003). Contrary to (±)-GR24, however, it failed to exhibit significant activity at 10⁻⁹ M (Fig. 3b).

Activity of (±)-4'-desmethyl-2'-epi-GR24 on fungal development in vitro

To investigate the effects of (±)-4'-desmethyl-2'-*epi*-GR24 on *R. irregularis* in terms of presymbiotic growth and hyphal morphology, we recorded fungal development from spores laid on solid medium and exposed to (±)-GR24, (±)-4'-desmethyl-2'-*epi*-GR24 or a mock treatment. The SL analogs were diluted into the medium rather than applied locally, which ensures uniform treatment of all spores and allows to know precisely the SL concentration to which the fungus is exposed. After 12 days of incubation the germ tube was measured, and the number of 1st-order and 2nd-order hyphal branches was determined (Fig. 4). Treatment with (±)-GR24 increased the number of 1st- and 2nd-order branches (Fig. 4a). Germ tubes were shorter in *R. irregularis* treated with (±)-GR24 than in control fungi (Fig. 4b). In contrast, (±)-4'-desmethyl-2'-*epi*-GR24 stimulated the elongation of the germ tube (Fig. 4b), while the number of 1st-order and 2nd-order branches was lower than in the control treatment (Fig. 4a). On average across three repeats of the experiment shown in Fig. 4, (±)-4'-desmethyl-2'-*epi*-GR24 increased germ tube length by 23 %. It triggered 2.1-fold and 6.5 fold reductions of the number of 1st-order and 2nd-order branches, respectively. Together, results presented in Fig. 4a and Fig. 3b indicate

that this compound markedly inhibits hyphal branching, while it can efficiently restore fungal colonization of *Mtccd8* mutant roots.

DISCUSSION

In the present work, we aimed to extend previous structure-activity relationship studies for SL activity on AM fungi. These studies have essentially been based on *in vitro* hyphal branching assays. Although such tests are extremely sensitive to SLs and informative from the point of view of fungal development, the relevance of hyphal branching to the symbiosis has not yet been firmly established. It is therefore of interest to supplement these observations with an assessment of SL bioactivity in a context of interaction with a host plant. An obvious obstacle to this in wild-type plants is the presence of endogenous SLs that could mask the activity of added compounds. We thus took advantage of *M. truncatula* mutants affected in the SL biosynthesis pathway. Consistent with the fact that the CCD7 and CCD8 enzymes act consecutively in this pathway (Alder et al. 2012), *Mtccd7-1* and *Mtccd8-1* mutants display similar defects in mycorrhizal root colonization. *Mtccd8* mutants were preferred for the rest of the study because CCD8 is to the best of our knowledge specifically involved in SL biosynthesis, while CCD7 activity can also lead to the formation of C₁₄ and C₁₃ apocarotenoids which could be involved in the progression of AM intraradical colonization (Vogel et al. 2009; Lopez-Raez et al. 2015; Walter et al. 2007; Stauder et al. 2018). Unlike mutants in the SL biosynthesis pathway, mutants affected in the SL receptor D14 were not hampered in their ability to host mycorrhizal interactions (Supp. Fig. 1). This observation is consistent with the analysis of corresponding mutants in rice (Yoshida et al. 2012), and indicates that hormonal effects of SLs *in planta* are not required for the symbiosis to take place. Therefore, defective mycorrhizal colonization of *Mtccd8-1* and *Mtccd7-1* mutants can be attributed to insufficient stimulation of the fungal partner.

The reduced mycorrhizal capacity we observed in *M. truncatula ccd8-1* mutants is more severe than that reported in corresponding mutants of other species like pea (Gomez-Roldan et al. 2008; Foo et al. 2013), rice (Yoshida et al. 2012; Kobae et al. 2018) or petunia (Kretzschmar et al. 2012). One hypothesis to explain this discrepancy could be the presence of biochemically undetectable residual SLs in some of the latter mutants, or alternatively the production in these species of other compounds able to stimulate AM fungi. In any case, the very strong defect in mycorrhizal colonization observed with *Mtccd8-1* mutants, together with the complete restoration of root colonization upon treatment with exogenous SLs (Fig. 2a), allowed the development of a reliable bioassay to assess the bioactivity of SL analogs in symbiotic conditions. Importantly, since the read-out is the number of root colonization sites, bioactivity encompasses the stimulation of any biological process allowing the fungus to progress from spore germination to successful root colonization.

A structure-activity relationship study was undertaken using this bioassay. All tested compounds were variations of the widely used SL analog GR24. In a first part of this study, we evaluated compounds that have already been tested for hyphal branching activity on *G. margarita* (Akiyama et al. 2010a). The ABC and D moieties, supplied individually or as an equimolar mix, were inactive (Supp. Fig. 2). Thus, SL cleavage products released upon SL perception by the host plant (and possibly by the fungus) do not by themselves stimulate the interaction between *R. irregularis* and *M. truncatula*. Among stereoisomers of GR24, (+)-GR24, (+)-2'-*epi*-GR24 and (-)-2'-*epi*-GR24 exhibited similar levels of bioactivity, while (-)-GR24 was inactive (Fig. 2b). These observations show consistency between hyphal branching tests (Akiyama et al. 2010a; Artuso et al. 2015) and our root colonization-based bioassays. They also indicate that the corresponding structural requirements are shared between *R. irregularis* and *G. margarita*. Preferences for SL stereochemical configurations vary across plant species (Boyer et al. 2012; Scaffidi et al. 2014; Umehara et al. 2015; Flematti et al. 2016). We did not observe such discrepancies among the two distant AM species that have been considered so far, although available data are insufficient to conclude that stereochemical requirements are broadly conserved among AM fungi.

In a second part of the structure-activity relationship study, we included compounds bearing modifications on the methylbutenolide D ring. This invariable component of all known natural SLs is of outmost importance for SL perception by plants, but analogs with variations on this part of the molecule have not yet been tested on AM fungi. We first investigated the importance of the C3'-C4' double bond of the D ring. Analysis of (±)-H₂-2'-*epi*-GR24 revealed that reduction of the double bond resulted in a moderate decrease of bioactivity on *R. irregularis* (Fig. 3a). This effect was comparable to that observed on pea bud elongation (Boyer et al. 2012), while the loss of activity on rice tillering and on *Striga* and *Orobancha* seed germination was more pronounced (Umehara et al. 2015; Yamauchi et al. 2018). It was recently shown that the C3'-C4' double bond could be reduced to a single bond in the presence of root exudates (Yamauchi et al. 2018). Since this yielded compounds with limited activity on shoot branching and parasitic seed germination (Boyer et al. 2012; Umehara et al. 2015; Yamauchi et al. 2018), it was postulated that this modification could represent a biochemical pathway to inactivate SLs. Given that the biological reduction of this double bond is less effective on natural SL forms (Yamauchi et al. 2018), and that the resulting compounds retain some activity on *R. irregularis*, this reduction process seems unlikely to interfere significantly with AM symbiotic signalling.

We then focused on the conserved methyl group at C4' of the butenolide D ring. Based on the current understanding of the SL biosynthetic pathway, this methyl group comes from the SL precursor 9-*cis*-β-carotene (Bruno et al. 2017). Studying the effects of (±)-4'-desmethyl-2'-*epi*-GR24, which lacks this methyl group, led to the most striking observations in our study. Indeed, this compound retains high

activity to promote the initiation of symbiosis, as well as on the isolated fungus. These results contrast with observations made on pea: removal of the methyl group at C4' resulted in a complete loss of ability to suppress shoot branching *in planta* (Boyer et al. 2012), and altered the binding and hydrolytic properties of SL analogs with the pea SL receptor RMS3 (de Saint-Germain et al. 2016).

Our observations of *R. irregularis* development *in vitro* raised interesting questions about the relevance of pre-symbiotic fungal development to the initiation of symbiosis. As expected, (±)-GR24 stimulated 1st- and 2nd-order branching (Fig. 4). This confirmed previous observations (Cohen et al. 2013), and showed that the experimental conditions were adequate to detect positive effects of SL analogs on hyphal branching. Interestingly, germ tubes were shorter in *R. irregularis* treated with (±)-GR24 than in control fungi, a response that to our knowledge has not been reported previously. In contrast, (±)-4'-desmethyl-2'-*epi*-GR24 stimulated elongation of the germ tube and inhibited the formation of new hyphal branches, which to our knowledge is a novel combination of effects. This complete contrast with the effects of (±)-GR24 may reflect a trade-off between hyphal branching and extension growth. Still, although some SL analogs stimulate the formation of long branches of low order rather than the formation of many short high-order branches (Akiyama et al. 2010a; Mori et al. 2016; Xie et al. 2019), an inhibition of branching is quite unusual. Such effects have been reported in root-exuded compounds from non-host species, but were accompanied by a similar inhibition of hyphal growth (Akiyama et al. 2010b). In our case, the branching inhibition cannot be attributed to toxicity, since treatment with the same compound stimulated germ tube elongation and enhanced fungal ability to colonize roots. Positive effects on hyphal elongation are more common: root exudates and a number of compounds therein, like flavonoids, polyamines or jasmonate, trigger this response in *Rhizophagus* or *Gigaspora* species (Elias and Safir 1987; Scervino et al. 2005; Cheng et al. 2012; Nagata et al. 2016). Over the years, such effects have however not attracted as much attention as hyphal branching, which is considered a hallmark of fungal stimulation by its host. It is commonly assumed that hyphal branching in itself is important to increase the chances for the fungus to encounter a host root, although this idea has not been supported by experimental evidence. Alternatively, hyphal branching could be a mere side-effect of important physiological or metabolic changes induced by SLs. Our observations challenge both hypotheses, as they indicate that hyphal branching and infectious capacity can be uncoupled. Another implication is that important bioactive compounds may have escaped identification due to the focus on hyphal branching in previous studies.

The fact that (±)-4'-desmethyl-2'-*epi*-GR24 was active on isolated *R. irregularis* (Fig. 4) indicates the existence of a fungal signalling pathway for this compound. Furthermore, the effects are the opposite of those observed upon treatment with (±)-GR24. This rules out the possibility that the fungal SL receptor is simply permissive to the absence of the methyl group at C4'. Rather, it points towards a perception system that allows discriminating between C4'-methylated and unmethylated forms of

GR24, activating two different pathways to trigger different biological responses. This situation brings to mind an interesting parallel with the activation of homologous, yet distinct pathways by SLs and karrikins in plants. The main active karrikin form in *Arabidopsis*, KAR2, is likely structurally similar to endogenous plant compounds such as the elusive KL. Its unmethylated butenolide moiety is identical to the D ring of (±)-4'-desmethyl-2'-*epi*-GR24, and the absence of a methyl group at C4' is important to activate the KL rather than SL pathway in *Arabidopsis* (Yao et al. 2018). Like plants, AM fungi could possess two pathways to deal with methylated and unmethylated butenolides. If the ability of (±)-4'-desmethyl-2'-*epi*-GR24 to restore the symbiosis in *Mtccd8* mutants is to be explained by effects on the fungus alone, this hypothesis implies that activation of the “unmethylated butenolide” pathway somehow makes the SL pathway dispensable.

An alternative and non-exclusive possibility is that in addition to stimulating the fungus, (±)-4'-desmethyl-2'-*epi*-GR24 acts on the plant host to facilitate fungal entry into roots. This hypothesis is consistent with the importance of the receptor protein KAI2 for AM symbiosis in rice (Gutjahr et al. 2015). Its ortholog in *Arabidopsis* seems strongly involved in the perception of C4'-unmethylated butenolides such as KAR2 (Villaécija-Aguilar et al. 2019). Therefore, it can be envisaged that a KAI2 protein in *M. truncatula* responds to unmethylated butenolides in a way that favours root colonization by AM fungi. Such unmethylated butenolides would comprise (±)-4'-desmethyl-2'-*epi*-GR24 as well as potential plant- or fungus-derived signals. At this stage, we do not have data to either support or rule out this possibility.

Interestingly, untreated *Mtccd8-1/R. irregularis* co-cultures failed to establish a successful interaction, but the exogenous supply of either (±)-GR24 or (±)-4'-desmethyl-2'-*epi*-GR24 was sufficient to restore the symbiosis. This suggests that, in addition to the expected absence of SLs, natural equivalents of (±)-4'-desmethyl-2'-*epi*-GR24 or their receptors are absent or inactive in these co-cultures. Further investigations will be needed to understand how the two fungal pathways, and possibly a plant pathway responding to (±)-4'-desmethyl-2'-*epi*-GR24, interplay to allow the initiation of symbiosis.

In conclusion, our structure-activity relationship analysis on the broad requirements for SL structure and stereochemical configuration was congruent with previous hyphal branching assays. A focus on D ring modifications, however, revealed that hyphal branching is not always a good proxy to estimate symbiosis-relevant bioactivity. This study also highlighted the importance of the methyl group on the D ring, and the ability of methylated and unmethylated variants to trigger differential developmental responses in the fungus. Once again, like in many key studies on SL perception by plants, synthetic analogs were instrumental to unravel new biological events. Ultimately, deciphering the perception of SLs and other butenolides by AM fungi should shed light on how these compounds evolved to become both important regulators of plant development and interkingdom mediators.

FIGURE LEGENDS

Fig. 1 Strigolactone analogs used in this study.

- (a) Structure and stereochemistry of the SL analog GR24. The synthetic SL analog ($\pm$)-GR24 (middle) comprises the four characteristic carbon rings, named A to D, of canonical SLs. Different stereochemical configurations of the C and D rings give rise to four possible stereoisomers (left and right).
- (b) Analogs with modifications on the D ring: reduction of the C3'-C4' double bond (left), or absence of the methyl group at C4' (right)

Fig. 2 Mycorrhizal phenotype of *Mtccd8-1* mutants and its rescue by GR24.

Plants were inoculated with *R. irregularis* and watered with a nutrient solution supplemented with the solvent alone (mock), or 10^{-7} M SL analogs. Root colonization was assessed three weeks post-inoculation.

- (a) Wild-type plants or *Mtccd8-1* mutants treated or not with ($\pm$)-GR24;
(b) *Mtccd8-1* mutants treated with enantiopure stereoisomers of GR24.

Bars represent the mean number of infection units per plant, $\pm$ s.e.m. $n = 9-10$ plants per condition. Means followed by the same letter do not differ significantly by Dunn's test ($P > 0.05$)

Fig. 3 Effect of SL analogs modified on the D ring on symbiosis initiation.

Mtccd8-1 mutants were inoculated with *R. irregularis* and watered with a nutrient solution supplemented with SL analogs: (a) ($\pm$)-H₂-2'-*epi*-GR24; (b) ($\pm$)-4'-desmethyl-2'-*epi*-GR24.

($\pm$)-GR24 was used as positive control. Root colonization was assessed three weeks post-inoculation. Bars represent the mean number of infection units per plant $\pm$ s.e.m. $n = 9-10$ plants per condition. Means followed by the same letter do not differ significantly by Dunn's test ($P > 0.05$)

Fig. 4 Effect of ($\pm$)-4'-desmethyl-2'-*epi*-GR24 on *R. irregularis* development *in vitro*.

R. irregularis was grown from spores for 12 days on medium containing 10^{-7} M ($\pm$)-GR24 or ($\pm$)-4'-desmethyl-2'-*epi*-GR24, or the solvent alone (mock), then developmental parameters were measured:

- (a) number of branches of first order (grey bars) and second order (black bars)
(b) length of the germ tube

Bars represent the mean $\pm$ s.e.m, $n = 11$ plates per condition. Means followed by the same letter do not differ significantly by Dunn's test ($P > 0.05$)

SUPPLEMENTARY DATA

Supp. Fig. 1 Mycorrhizal phenotype of *Medicago truncatula ccd7-1* and *d14-1* mutants.

Wild-type and mutant plants were grown in small containers and inoculated with pre-germinated spores of *R. irregularis* as described in Balzergue et al. (2013). Bars indicate the mean number of infection units per plant, $\pm$ s.e.m. $n = 7$ -8 plants per genotype. Means followed by the same letter do not differ significantly by Dunn's test ($P > 0.05$)

Supp. Fig. 2 Effect of ABC and D moieties on symbiosis initiation.

Mtccd8-1 mutants were inoculated with *R. irregularis* and watered with a nutrient solution supplemented with 10^{-7} M ($\pm$)-ABC=CHOH, ($\pm$)-D-OH, or a mixture of both (10^{-7} M each). ($\pm$)-GR24 was used as positive control. Root colonization was assessed three weeks post-inoculation. Bars represent the mean number of infection units per plant $\pm$ s.e.m. $n = 10$ plants per condition. Means followed by the same letter do not differ significantly by Dunn's test ($P > 0.05$)

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647 **Conflicts of interest/Competing interests**

648 The authors declare that they have no conflict of interest.

649 **Ethics approval**

650 Not applicable

651 **Consent to participate**

652 Not applicable

653 **Consent for publication**

654 Not applicable

655 **Availability of data and material**

656 The datasets generated during and/or analysed during the current study are available from the
657 corresponding author on reasonable request.

658 **Code availability** (software application or custom code)

659 Not applicable

660 **Authors' contributions**

661 SR, QT, DL and FDB contributed to the study conception and design. Experiments and data analysis
662 were performed by QT, DL, KM, MC and SR. Chemicals were synthesized by VS and FDB. The
663 manuscript was written by SR, QT and FDB.

Figure 1

Figure 2

a **b**

Figure 3

Figure 4

Figure S1

(±)-ABC=CHOH

(±)-D-OH

Figure S2