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Highlights

•Exudates or volatiles produced by AM fungi stimulate lateral root formation (LRF).

•Volatiles emitted by AM fungi stimulate LRF in a SYM- and host-independent way.

•Exudates produced by AM fungi stimulate LRF in a SYM- and host-dependent way.

•Strigolactones may participate in the volatile-induced changes.

Abstract

Arbuscular mycorrhizal (AM) fungi influence the root system architecture of their hosts; however, the underlying mechanisms have not been fully elucidated. Ectomycorrhizal fungi influence root architecture via volatiles. To determine whether volatiles also play a role in root system changes in response to AM fungi, spores of the AM fungus Gigaspora margarita were inoculated on the same plate as either wild type (WT) Lotus japonicus, the L. japonicus mutant Ljcastor (which lacks the symbiotic cation channel CASTOR, which is required for inducing nuclear calcium spiking, which is necessary for symbiotic partner recognition), or Arabidopsis thaliana, separated by cellophane membranes (fungal exudates experiment), or on different media but with a shared head space (fungal volatiles experiment). Root development was monitored over time. Both germinating spore exudates (GSEs) and geminated-spore-emitted volatile organic compounds (GVCs) significantly promoted lateral root formation (LRF) in WT L. japonicus. LRF in Ljcastor was significantly enhanced in the presence of GVCs. GVCs stimulated LRF in A. thaliana, whereas GSEs showed an inhibitory effect. The expression profile of the genes involved in mycorrhizal establishment and root development were investigated using quantitative reverse transcription-PCR analysis. Only the expression of the LjCCD7 gene, an important component of the strigolactone synthesis pathway, was differentially expressed following exposure to GVCs. We conclude that volatile organic compounds released by the germinating AM fungal spores may stimulate LRF in a symbiosis signaling pathway (SYM)- and host-independent way, whereas GSEs stimulate LRF in a SYM- and host-dependent way.

Keywords

Arabidopsis thaliana; Germinating spore exudates; Lotus japonicus; Root branch; Volatile

1. Introduction

Arbuscular mycorrhizas (AMs) are some of the most widespread symbioses on earth. More than 80% of plants are known to establish AM associations with fungi from the Glomeromycota, which improves the mineral nutrition of plants, particularly of phosphorus (Smith and Read, 2008). In return, plants provide sugar to the fungal symbionts. The establishment of an AM symbiosis proceeds as a series of genetically controlled steps and commences with a pre-symbiotic molecular crosstalk leading to reciprocal perception (Nadal and Paszkowski, 2013). The fungus makes contact with the root surface via a hyphopodium only if successful recognition occurs. The hyphopodium then penetrates into the root leading to the formation of arbuscules, or sites of nutrient exchange (Bonfante and Requena, 2011 and Nadal and Paszkowski, 2013).

It is known that the AM fungal spores germinate spontaneously, even if the hyphal growth after germination during the pre-symbiotic phase is limited. During this period, the hyphae respond to several root-secreted chemicals, resulting in distinct growth patterns. Flavonoids, polyamines, strigolactones, 2-hydroxy fatty acids and cutin monomers are among the active plant compounds that have been found to have effects on the hyphal elongation or branching (Bécard et al., 1992, Ghachtouli et al., 1995, Akiyama et al., 2005, Nagahashi and Douds, 2011, Wang et al., 2012 and Gutjahr and Parniske, 2013). It has been suggested that AM fungi have perception machinery enabling the differential recognition of chemically diverse plant metabolites and the transduction of the information into compound-specific morphological responses (Nadal and Paszkowski, 2013). These early responses are likely to play crucial roles in the preparation for a successful colonization process.

Plants can also sense and respond to signals released by AM fungi. For example, germinating spore exudates (GSEs) have diverse effects on plant roots at both the cellular and molecular level and on the whole root system, including overall root architecture (Bonfante and Requena, 2011). Lateral root formation (LRF) has been demonstrated as a common response to the presence of GSEs in several plant species, including Medicago truncatula Gaertn., Zea mays L., and rice (Oryza sativa L.) (Oláh et al., 2005 and Mukherjee and Ane, 2011). Recently, bioactive AM molecules, also known as MYC factors, have been identified as lipochitooligosaccharides (LCOs) and as short-chain chitooligosaccharides (COs) (esp. CO4 and CO5) (Maillet et al., 2011 and Genre et al., 2013). Although the short-chain COs are mostly involved in eliciting calcium oscillations (Genre et al., 2013), all the GSEs and fungal LCOs have been shown to have the function of inducing LRF in M. truncatula, resulting in a similar phenotype (Oláh et al., 2005). However, the effects of LCOs depends on the functionality of DOES NOT MAKE INFECTIONS 1 (DMI1), DMI2 and DMI3, components of the so-called common symbiosis (SYM) signaling pathway, whereas the GSE-activated lateral root response only requires functional DMI1 and DMI2 (Oláh et al., 2005 and Maillet et al., 2011). Additional metabolites that are present in GSEs may be interpreted by different plant signaling cascades, boosting lateral root development (Nadal and Paszkowski, 2013). By contrast, the lateral root response of rice to AM fungi is independent of the SYM signaling components (Gutjahr et al., 2009a and Mukherjee and Ane, 2011), thus monocotyledons (and

possibly dicotyledons other than M. truncatula) must employ other signaling cues, which could be activated by a variety of additional and chemically diverse fungal triggers (Nadal and Paszkowski, 2013).

Besides the water soluble and diffusible exudates, microorganisms are capable of producing volatile compounds (Kuske et al., 2005, Meldau et al., 2013 and Wenke et al., 2010). It is believed that many interactions between organisms are based on the emission and perception of volatiles (Wenke et al., 2010). Several microorganisms, including plant-growth promoting rhizobacteria and ectomycorrhizal (ECM) fungi, have been shown to stimulate A. thaliana (L.) Heynh. root development by emitting volatiles (Ryu et al., 2003, Ping and Boland, 2004, Farag et al., 2006, Felten et al., 2009, Felten et al., 2010, Splivallo et al., 2009, Gutiérrez-Luna et al., 2010 and Meldau et al., 2013). Volatile metabolites involved in the establishment of ECMs have been documented previously (Krupa et al., 1973, Splivallo et al., 2007 and Splivallo et al., 2009). There is also evidence that roots emit volatile signals that stimulate the directional growth of the AM fungus toward them (Gemma and Koske, 1988 and Koske, 1982). Furthermore, it has been suggested that AMs could alter the profile of the volatile organic compounds released by a plant, although the mechanisms have still to be elucidated (Leitner et al., 2010, Schausberger et al., 2012 and Babikova et al., 2013). However, to date, the effects of geminated-spore-emitted volatile organic compounds (GVCs) on plant root development have not been investigated.

In this study, using an in vitro system (see Fig. 1), we compared the effects of GVCs and GSEs produced by the AM fungus Gigaspora margarita W.N. Becker & I.R. Hall, on the LRF of Lotus japonicus L., which is a legume model plant, a L. japonicus mutant, Ljcastor (which has a defective CASTOR gene, which is required for inducing nuclear calcium spiking, which is indispensable for AM formation) (Novero et al., 2002), and A. thaliana, which does not form symbiotic associations with AM fungi. The spores and the plants were co-cultured on the same plate, and the root development was monitored over time to enable time series analysis. The transcriptional levels of seven AM establishment and LRF-related genes of L. japonicus were also investigated, to determine the activation of genes involved in strigolactone synthesis.

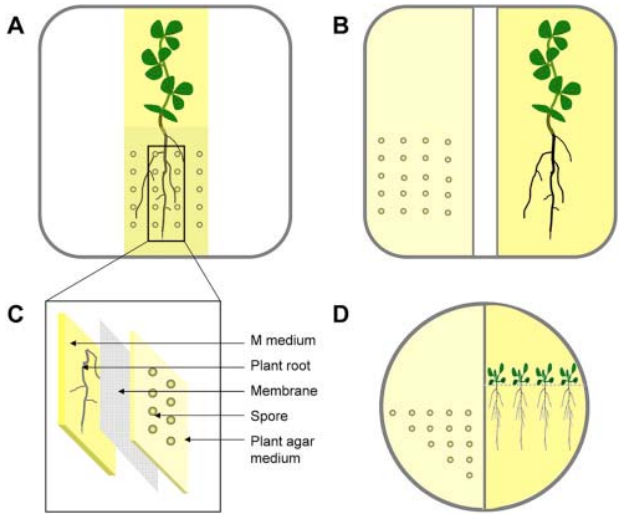

Fig. 1. Schematic diagram to illustrate the experimental procedure. (A) Germinating spore exudate (GSE) treatments: pre-germinated seedlings were transferred to a thin layer of M medium (without sugar) for L. japonicus, or 1/2 MS medium for A. thaliana. Cellophane membranes (4 cm × 7 cm) covered with a thin layer of 0.6% agar medium were placed on top of the plant roots. The plant agar medium was then inoculated with 30 surface-sterilized spores of G. margarita. To diminish the diffusion of the GSEs, the medium beyond the membrane was removed. (C) Magnification of the area indicated in (A) showing the experimental set-up in more detail. (B and D) Experimental set-up to evaluate the impact of germinated-spore-emitted volatile organic compounds (GVCs): Arbuscular mycorrhizal fungal spores and plant seedlings were inoculated in different media on the same square plate (L. japonicus, B) with a 1-cm air gap or on a split-plate (A. thaliana, D).

2. Materials and methods

2.1. Plant materials

Seeds of L. japonicus MG20 and Gifu wild type, and the Ljcator mutant and seeds of A. thaliana Columbia-0 were used in the present study. The Ljcator mutant formerly designated as Ljsym4-2 was generated in a chemical mutagenesis program (mutant line: EMS1749) (Bonfante et al., 2000 and Novero et al., 2002).

L. japonicus seeds were surface sterilized and scarified for 3 min in sulfuric acid, washed three times for 15 min with sterile, distilled water and then placed on 0.6% agar in Petri dishes. Six days after germination the seedlings were transplanted into minimal (M) medium without sucrose (phosphate concentration adjusted to 20 μM) (Bécard and Fortin, 1988).

A. thaliana Columbia-0 seeds were immersed in 70% ethanol solution for 1 min, followed by scarification for 20 min in a commercial bleach (1:5 dilution with distilled water), and finally washed three times by soaking the seeds for 10 min in sterile distilled water. The sterilized seeds were placed on 1/2 Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and incubated overnight at 4 °C before transferring to the climatic chamber.

2.2. AM fungus inoculum

G. margarita (strain deposited in the Bank of European Glomales as BEG 34) spores were collected from pot cultures (3-month-old monoculture of white clover), surface sterilized (Bécard and Fortin, 1988), and stored at 4 °C in sterile water before using. Each of the experimental plates was inoculated with 30 sterilized spores.

2.3. Set-up of GSE and GVC experiments

The system (Fig. 1A) to verify the effects of GSEs was based on the method described by Oláh et al. (2005) with a slight modification (to concentrate GSEs, medium that was not in contact with the cellophane membrane was removed). A commercial cellophane membrane (Model 583 Gel Dryer; Bio-Rad, http://www.bio-rad.com) was used to separate the spores from the plants. Only diffusible compounds can pass through cellophane membrane. Before use, the membranes were washed as described by Volpe et al. (2013). Spores of G. margarita were transferred individually and distributed evenly over the medium.

To evaluate the effect of GVCs on L. japonicus, a separate culture system was established (Fig. 1B). Plants and spores were inoculated onto different media that were separated by an air gap. For this setup, first, a thin layer of 0.6% plant agar medium was poured into the plate, after solidification, the medium was cut in half and one half was then removed. M medium (without sucrose) was poured into the empty half of the plate. At the boundary of the two media, a small strip of each medium was removed to create a gap approximately 1 cm wide between the two media.

To test whether A. thaliana was affected by exposure to GVCs, bipartite Petri dishes were used owing to the relatively small size of A. thaliana (Fig. 1D). The plants and spores were inoculated in separate compartments where seedlings and spores had contact only through a shared headspace.

In all systems, the lower parts of the plates were covered with aluminum foil to reduce the light levels in the area where the spores and roots would grow. For both the GSE and GVC experiments conducted on L. japonicus, there were 30 replicates of each treatment (one plant per plate, 30 L. japonicus in total), whereas for the both the GSE and GVC experiments conducted on A. thaliana, there were 20 replicates of each treatment, with four plants per plate (80 A. thaliana plants in total). Each experiment was conducted separately, and to avoid the diffusion effects of GVCs, the plates used in each treatment in the GVC experiment were laid out in separate climatic chambers. All the plates were placed in a vertical position in climatic chambers with 14 h of light per day at 22 °C and 10 h of dark at 20 °C.

Due to the low germination rate of the seeds and the slow growth rate of the L. japonicus Gifu ecotype, after the effects of GSEs and GVCs on LRF had been compared, only MG20 was used in subsequent tests.

2.4. qRT-PCR

Spores of G. margarita usually need three to five days to germinate (germ tubes emerged from G. margarita spore four days post inoculation; Fig. S1, left panel) and the GSEs or GVCs also need time to accumulate to effective concentrations (an extensive network of hyphae formed 14 days post inoculation; Fig. S1, right panel). Therefore, we collected plant roots for testing gene expression at 4, 7, 10 and 14 days post the start of the experiment.

RNA was extracted using RNeasy Plant Mini Kit (Qiagen) from two root systems per sample for each treatment for use in the quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Three independent biological replicates were performed. Reverse transcription and real-time PCR were performed as described by Guether et al. (2009). CT values of all genes were normalized to the CT values of ubiquitin (TC3806) in each sample. For LjCASTOR and LjPOLLUX amplification, primer sequences were taken from Gutjahr et al. (2009b). For LjMAMI and LjCCD7, primer sequences were taken from Volpe et al. (2013) and Liu et al. (2013) separately. Primers for quantifying expression of LjSCR1 (Scarecrow 1; Lotus Affymetrix ID: Ljwgs_023888.1_at), LjSCR3-1 (Scarecrow 3 like protein A; Lotus Affymetrix ID: Ljwgs_016263.1_at), and LjSCR3-2 (Scarecrow 3 like protein A; Lotus Affymetrix ID: Ljwgs_027761.2_at) were from Guether et al. (2009). The primer sequences of all the genes tested are listed in Table S1.

2.5. Root measurements and biomass assay

All the plates were scanned regularly with a normal scanner (Epson perfection 2450 photo). Total root length, the insertion angle of the lateral root, and the primary root length of both L. japonicus WT and A. thaliana were obtained using the SmartRoot Toolbox (Lobet et al., 2011) based on the platform of ImageJ software (see http://www.uclouvain.be/en-smartroot for the SmartRoot plugin and http://rsb.info.nih.gov/nih-image/ for the ImageJ software). Lateral roots were counted under a dissecting microscope (Leica, MZ12).

At the end of each experiment, the biomass of the plants was determined. Due to the limited amount of biomass that accumulated during the experiments, only the fresh weight was determined. For L. japonicus, the root and shoot biomass of each plant was measured separately. Separating the plant agar from the fine roots of A. thaliana was challenging, therefore only the shoot biomass was determined. For L. japonicus, each plant was weighed separately, and for A. thaliana, the shoot biomass was obtained by weighing the four plants harvested from each plate together.

2.6. Statistics

Data were statistically analyzed, and the means were compared by performing a t-test using the statistical software SPSS 17.0.0 (Statistical Product and Service Solutions, SPSS Inc. Chicago, IL, USA). Data were presented as means and standard errors (SE). GSE experiments were duplicated,

and all GVCs experiments were triplicated. All figures were constructed using Office 2007 (Microsoft Inc. Redmond, WA, USA).

3. Results

3.1. Effects of GVCs versus GSEs on the root development of L. japonicus genotypes

The effects of GSEs and GVCs on LRF were compared in the model legume L. japonicus WT and Ljcastor mutant. The AM fungi were unable to colonize the cortex and form arbuscules in the Ljcastor mutant (Novero et al., 2002).

Cellophane membranes, which only allow diffusible compounds to pass through, were used to separate the spores and the plant root and to investigate the effect of GSEs according to the protocol of Oláh et al. (2005). Spores of G. margarita were transferred individually and dispersed evenly on the medium. GSEs significantly stimulated the LRF of both L. japonicus Gifu and MG20 (from six days and seven days onwards, respectively, $P < 0.05$) (Fig. 2A and C). Although not statistically significant, GSEs appeared to partially inhibit LRF of the Ljcastor mutant compared with the LRF observed on the control plate (Fig. 2E). We conclude that GSEs can enhance LRF of L. japonicus, and the effect is dependent on LjCASTOR gene. Our result confirmed the former findings by Oláh et al. (2005) and Mukherjee and Ane (2011) on M. truncatula.

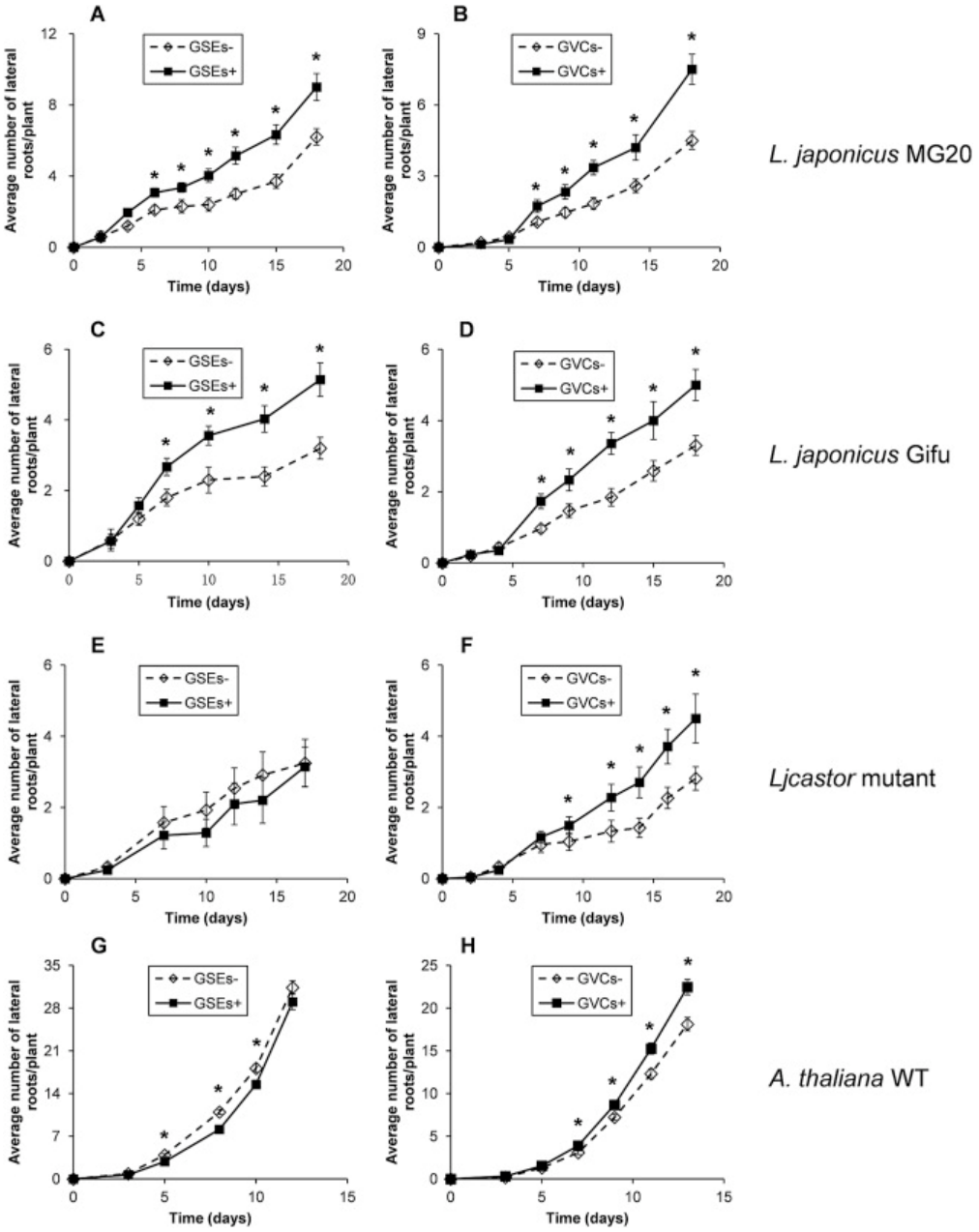

Fig. 2. Effects of GSEs and GVCs on lateral root formation (LRF) in L. japonicus MG20, L. japonicus Gifu, Ljcastor mutant and A. thaliana WT. Curves show the time scale of the effects of GSEs and GVCs released from G. margarita on LRF. In L. japonicus MG20 (A and B) and Gifu (C and D), the stimulation of LRF was statistically significant ($P < 0.05$) within treatments for both the GSE (from day 6 and day 7 onwards, respectively) and for the GVC (from day 7 onwards) treatments. In the Ljcastor mutant, GSEs had no effect on LRF (E); however, GVCs significantly enhanced LRF from day 9 onwards (F) ($P < 0.05$). In A. thaliana plants, GSEs had an inhibitory effect on LRF (G), whereas GVCs stimulated LRF (H). The L. japonicus data are from one representative experiment with 30 plants per treatment, whereas the A. thaliana data was obtained from 80 plants for both the GSE and GVC experiments. GSE and GVC experiments were performed two and three times, respectively. Significant differences are indicated by an asterisk (t-test, $P < 0.05$). Bars show SE.

To test the effect of GVCs on LRF, the two media on which the spores and plants were growing were separated from each other by a 1-cm air gap, but with a shared headspace (Fig. 1B).

Communications between the two organisms were only allowed through the shared headspace. GVCs showed similar stimulatory effects on LRF to those observed for GSEs, and the LRF of both genotypes was significantly enhanced from day seven onwards ($P < 0.05$) (Fig. 2B and D). To test whether the induction of LRF by GVCs was dependent on the common SYM signaling pathway, we also exposed the Ljcastor mutant to GVCs. Surprisingly, not only were the stimulatory effects retained, but LRF was significantly stimulated from day nine onwards ($P < 0.05$) (Fig. 2F).

By contrast, the total root lengths of L. japonicus MG20 were not significantly altered in the presence of GSEs or GVCs (Fig. 3A and B). Similarly, the presence of GSEs or GVCs had no obvious effect on the primary root length (Fig. 4A and B), confirming the results for GSEs obtained by Oláh et al. (2005). However, lateral root density (per centimeter of primary root) was significantly enhanced when L. japonicus MG20 was exposed to GSEs and GVCs ($P < 0.05$) (Fig. S2A and B).

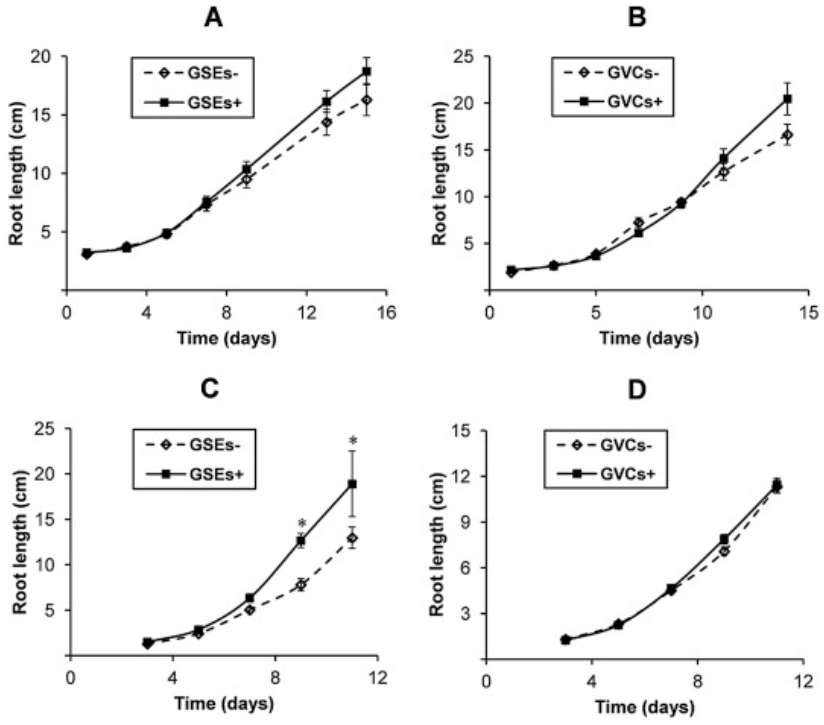

Fig. 3. Total root length in the presence of GSEs or GVCs. (A and B) total root length of L. japonicus MG20; (C and D) total root length of A. thaliana WT. Data were from 30 plants (L. japonicus) or 80 plants (A. thaliana) per treatment. Significant differences are indicated by an asterisk (t-test, $P < 0.05$). Bars show SE.

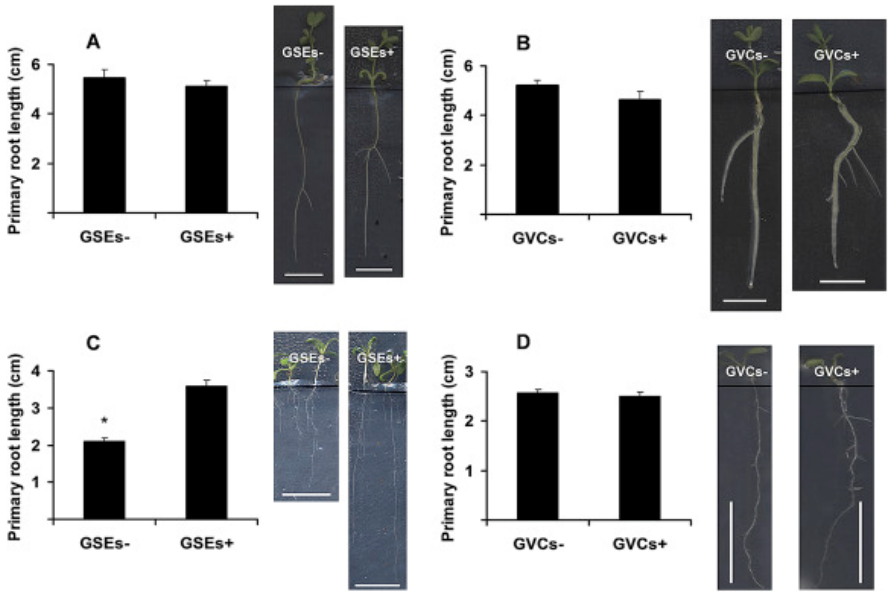

Fig. 4. Primary root length and the growth patterns of plants in the presence of GSEs or GVCs. (A and B) L. japonicus MG20 subjected to GSE or GVC treatment; (C and D) A. thaliana WT subjected to GSE or GVC treatment. Only the primary root of A. thaliana was significantly elongated by the GSE treatment (C). All data and figures were obtained from plants after seven days of treatment. Significant differences are indicated by an asterisk (t-test, $P < 0.05$). Bars show SE. The white scale bars represent 1 cm.

We also recorded the branch angles of lateral roots and found that the branch angles were significantly larger in the presence of GVCs compared with those of the control (Table S2). On the basis of fresh weight measurements, the presence of GSEs or GVCs did not appear to have any effect on the shoot and root biomass of the L. japonicus WT plants (Fig. 5A and B).

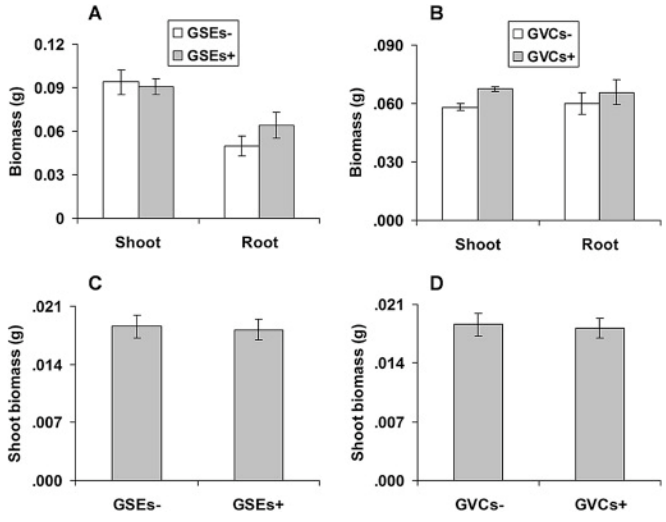

Fig. 5. Biomass of L. japonicus MG20 and A. thaliana WT. (A and B) L. japonicus; (C and D) A. thaliana. All data are based on the fresh weights of plants. For L. japonicus, data from 30 plants per treatment (n = 30); and for A. thaliana, data from 80 plants (n = 20). Bars show SE.

Based on these results, we conclude that GVCs can stimulate LRF in L. japonicus, and differ from the effects of GSEs, since the stimulation seems to be independent of LjCASTOR.

3.2. GVCs stimulate root ramification in A. thaliana

Since GVCs can stimulate LRF in a SYM independent manner, we hypothesized that the stimulation effect would also occur in the AM non-host plant A. thaliana, which lacks many components of the SYM pathway including those required for signal transduction and infection (Venkateshwaran et al., 2013).

GVCs emitted by G. margarita significantly stimulated A. thaliana LRF (Fig. 2H) (from day seven onwards; $P < 0.05$). By contrast, LRF was suppressed by the presence of GSEs on day 5, 8 and 10 ($P < 0.05$) (Fig. 2G). The inhibitory effect diminished over time and after 12 days no significant effect was observed (Fig. 2G).

GVCs had no effect on root elongation, whereas, the total root lengths of A. thaliana WT were significantly enhanced in the presence of GSEs (from day nine onwards; Fig. 3C and D). Elongation of the primary root was also significantly enhanced in the presence of GSEs, whereas exposure to GVCs had no detectable effect on primary root elongation (Fig. 4C and D). Owing to the effect of GSEs and GVCs on LRF and primary root length, lateral root density was also significantly inhibited in the presence of GSEs and significantly stimulated in the presence of GVCs ($P < 0.05$) (Fig. S2C and D). The branch angles of lateral roots were not significantly affected by the presence of either GSEs or GVCs (Table S2). Shoot biomass remained unchanged in the presence of GSEs or GVCs (Fig. 5C and D).

We conclude that GVCs stimulate LRF in a host-independent way.

3.3. Transcriptional response of seven AM-related genes induced in GSE- or GVC-treated L. japonicus WT plants

To verify the possible involvement of GVCs in the AM establishment process, the transcription levels of seven AM establishment- and LRF-related genes were determined by qRT-PCR.

CASTOR and POLLUX are two highly homologous proteins, which are indispensable for AM formation, since they belong to the common SYM pathway (Imaizumi-Anraku et al., 2005 and

Parniske, 2008). LjMAMI, an AM-induced transcription factor, was firstly identified in a GeneChip test by Guether et al. (2009) as one of the most highly expressed genes in arbusculated cells and recently proved to be involved in root branching (Volpe et al., 2013). LjCCD7 is a key enzyme in the biosynthesis pathway for strigolactones (SLs), and a recent study showed that it can trigger root development (Liu et al., 2013). LjSCR1 (Scarecrow 1; Lotus Affymetrix ID: Ljwgs_023888.1_at), LjSCR3-1 (Scarecrow 3 like protein A; Lotus Affymetrix ID: Ljwgs_016263.1_at), and LjSCR3-2 (Scarecrow 3 like protein A; Lotus Affymetrix ID: Ljwgs_027761.2_at) are three mycorrhiza-induced genes belong to the SCARECROW (SCR) family of transcription factors, which were significantly up-regulated in mycorrhized roots (Guether et al., 2009 and Laajanen et al., 2007). It has been shown that SCRs regulate the radial developmental pattern of the root and control the identity of endodermal and cortical cells (Dolan, 2007).

The transcript levels of LjMAMI, LjSCR1 and LjSCR3-2 were not significantly different in plants that had been exposed to GSEs or GVCs compared with those of the controls (Fig. 6). However, after 10 days of exposure to GVCs, the expression of LjCCD7 was significantly induced in plants; four days of exposure to GSEs significantly induced the expression of both LjCASTOR and LjPOLLUX; and 10 days of exposure to GSEs significantly suppressed LjSCR3-1, and the suppression lasted up to 14 days ($P < 0.05$).

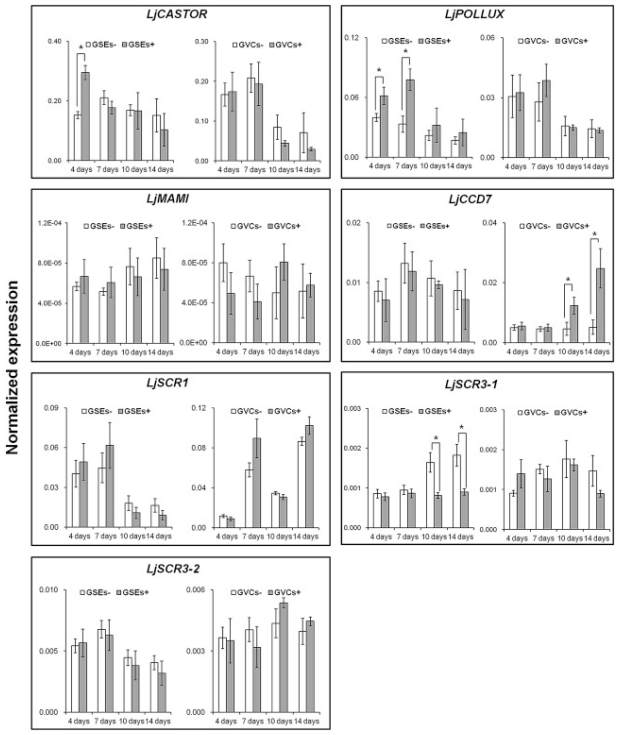

Fig. 6. Transcriptional responses of seven AM-related genes of L. japonicus MG20 after 4, 7, 10 or 14 days exposure to GVCs or GSEs. Significant differences are indicated by an asterisk (t-test, $P < 0.05$). Bars show SE.

In conclusion, LjCCD7 may involve in GVCs induced changes, and LjCASTOR, LjPOLLUX and LjSCR3-1 may be necessary for GSEs induced changes.

4. Discussion

Lateral roots are the preferred target for AM colonization, and perhaps for this reason, their stimulation seems to be a common effect of mycorrhization (Fusconi, 2014 and Harrison, 2005). Although the biological meaning of these effects remains unknown, stimulation of LRF is likely to increase the chances of AM fungal hyphae encountering plant roots during the host-free growth period.

Here, we demonstrated that germinating spores of G. margarita release not only exudates but also volatiles that alter the growth pattern of roots in L. japonicus Gifu and MG20 genotypes, resulting in a similar stimulatory effect on LRF (Fig. 2A–D). This raises the possibility that the stimulatory effect of GSEs on LRF may be a volatile effect. However, when the Ljcastor mutant (which lacks the symbiotic cation channel CASTOR, which is necessary for symbiotic partner recognition) was considered, we found that the GVC-induced effect was independent of the SYM pathway (Fig. 2F); on the contrary, the GSE-induced effect was diminished in the mutant (Fig. 2G), which agrees with earlier findings observed in M. truncatula (Oláh et al., 2005). The transcriptional results of seven AM-establishment and LRF-related genes also confirm the difference between GSE- and GVC-induced effects (Fig. 6). Furthermore, the diminished effect of GSEs on A. thaliana LRF (Fig. 2G) further supports the idea that LRF stimulation induced by GSEs and GVCs employ distinct signaling pathways.

Spores of G. margarita needed 3–5 days to germinate (Fig. S1), and significant changes in LRF always emerged after spore germination (Fig. 2 and Fig. S2), confirming that it is the exudates or volatiles generated by the germinating spores that induced the changes on LRF. The number of lateral roots (Ditengou et al., 2015, Felten et al., 2009 and Maillet et al., 2011) and lateral root density (per centimeter of primary root) (Kapulnik et al., 2011, Ruyter-Spira et al., 2011 and Zamioudis et al., 2013) are all frequently used parameters to represent plant lateral root development. In the present study, the GSEs and GVCs could not only increase the number of lateral roots but also lateral root density (per centimeter of primary root) in L. japonicus (Fig. 1A and B, Fig. S2A and B). Considering the similar length of the primary root (Fig. 4A and B), it is not surprising to find the significant changes also in lateral root density.

LCOs are capable of inducing LRF (Maillet et al., 2011); however, this effect depends on DMI1, DMI2 and DMI3, suggesting that LCOs could be the trigger molecule(s) for GSEs but not for GVCs owing to their non-volatile nature. Thus we hypothesize that there is likely to be a novel effector(s) present in GVCs other than those from GSEs that could induce LRF. It is known that the amount of volatiles released by roots ranges from 57% to 71% of the total exudates (Rovira and

Davey, 1974); however, similar estimations have not been made for fungi. Along the same line, we assume that volatiles (the effective molecules) emitted from germinating spores likely volatilize through the air. Data on the chemical nature of these products is not yet available; however, these volatiles may be water-insoluble and non-polar molecules, suggesting that they cannot pass through the cellophane membrane (polar) and diffuse though the plant agar gel containing considerable moisture to induce LRF.

GVCs and GSEs treatments stimulated LRF but without significant influences on root length and root biomass, raising the question of whether the elongation of lateral root was suppressed by the treatments? Actually, no obviously differences were found among the length of lateral roots in the present study (data not shown). Although not statistically significant, the root biomass of L. japonicus both treated with GVC and GSE were all increased compared with those of control; meanwhile, the root length were also showed increasing trends. These results could potentially eliminate the differences among lateral root length of different treatments.

It is intriguing that the branch angles of lateral roots were significantly altered in the presence of GVCs (L. japonicus MG20; Table S2), which indicates that AM fungi may be able to regulate the root orientation of their potential host via GVCs, which may increase the likelihood of the two symbionts encountering one another. Recent evidence has shown that the branch angle of the lateral root is auxin dependent (Roychoudhry et al., 2013), consequently it is possible that GVCs could activate the auxin signaling pathway of the host plant.

Transcriptional analysis of seven AM colonization-related genes has revealed the possible involvement of LjCCD7 (which is an important component of the SL synthesis pathway) in the GVC-induced effect. As the “branching-factor”, SLs are well known as stimulators of AM fungal hyphal branching (Akiyama et al., 2005 and Besserer et al., 2006). SLs also stimulate the release of short chitooligosaccharides, which facilitate the early plant–fungal communication (Genre et al., 2013). We therefore hypothesize that a regulatory network is involved, whereby the synthesis of SLs is enhanced by the induction of fungal GVCs, therefore increasing the amount of SLs secreted into the rhizosphere, which attracts AM fungal hyphae to localize to the plant root, thus eliciting their molecular dialog. Regarding the relationship between SLs and LRF, although it is claimed that SLs negatively regulated LRF (Kapulnik et al., 2011 and Liu et al., 2013), Ruyter-Spira et al. (2011) found that SLs induced LRF under Pi-limiting conditions (20 μM). In the present study, L. japonicus were also grown under Pi-limiting conditions (20 μM). Consequently, the transcriptional changes of LjCCD7 may partially explain the LRF stimulation effects under the presence of GVCs.

We also found that the expression levels of LjCASTOR and LjPOLLUX were significantly enhanced and that LjSCR3-1 was significantly suppressed by GSEs. There was also a slightly inhibitory effect on LjSCR3-1 expression and a stimulatory effect on LjSCR1 expression in the presence of GVCs. As the SYM pathway component, the transcriptional changes of both

LjCASTOR and LjPOLLUX verified the participation of CASTOR and POLLUX in the induction of LRF by GSEs. This result confirmed the earlier findings by Gutjahr et al. (2009b), who reported that both genes were significantly induced by GSEs using a double-sandwich experiment setup. Scarecrow proteins, members of the GRAS family of transcription factors, have been shown to play central roles in root development (Bolle, 2004). Our results indicate the SCR genes are possibly involved in the GSE- and potentially GVC-induced LRF.

GVCs emitted by the AM fungus G. margarita also stimulated LRF as well as lateral root density in A. thaliana (Fig. 2H and Fig. S2D). LRF is regulated by auxin in conjunction with other phytohormone signaling pathways (Nibau et al., 2008). There is accumulating evidence that ECM and plant growth-promoting rhizobacteria may activate the host auxin-signaling pathway by first, directly producing auxin or ethylene, and second, producing suitable substrates for auxin synthesis (Felten et al., 2009, Ivanchenko et al., 2008, Splivallo et al., 2009 and Zamioudis et al., 2013). However, auxin-producing genes have not been detected in the Rhizophagus irregularis genome (Tisserant et al., 2013). It has recently been shown that sesquiterpenes emitted from an ECM fungus Laccaria bicolor are sufficient to stimulate LRF in A. thaliana (Ditengou et al., 2015), which indicates that there may be pathways other than the auxin-signaling pathway that are as yet undiscovered that are responsible for LRF. It is possible that AM fungi could also produce some of these molecules.

It is intriguing that GSEs significantly stimulated the elongation of the primary root as well as the whole root (Fig. 3 and Fig. 4). Khan et al. (2011) found that LCOs produced by Bradyrhizobium japonicum induced a 35% increase in primary root length in A. thaliana compared with that of the control. Although the structures of the bacterial LCOs are slightly different from AM fungal LCOs, it is likely that LCOs (Maillet et al., 2011) or CO4 and CO5 present in GSEs (Genre et al., 2013) have similar effects on promoting the root elongation of A. thaliana.

We found that the shoot biomass of A. thaliana, which is not a host plant for AM fungi, was not affected by the presence of GSEs or GVCs (Fig. 5C and D). This unexpected result could offer some novel clues to interpret data coming from the field. Veiga et al. (2013) found that the shoot biomass of A. thaliana grown alone (in the absence of an AM host plant) was not influenced by the presence of R. irregularis in the substrate. Taking into account the unchanged biomass of L. japonicus when exposed to GSEs and GVCs, we propose that AM fungi could regulate root development by producing various compounds known as exudates or volatiles at the pre-symbiotic stage, without affecting shoot biomass accumulation.

In conclusion, in this study, we have presented evidence that volatile molecule(s) emitted by germinating G. margarita spores induced LRF in L. japonicus. This response was independent of a genetic component of the SYM pathway, pointing to a consistent difference with LRF induced by GSEs, where SYM dependency has previously been demonstrated for M. truncatula, which

represents the other legume model plant. GVCs emitted by the AM fungus also stimulated LRF in A. thaliana, which is a non-host plant for AM fungi. Out of the seven tested genes, only LjCCD7 was stimulated by the signals of fungal volatiles, raising new questions about the potential interactions among strigolactones, AM fungi and root branching.

Contribution

XS and MT conceived and designed the experiments. XS conducted the experiments, analyzed the data and wrote the manuscript. PB revised the manuscript and advised on the preparation of plant and fungal materials and experimental procedures.

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