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The possible involvement of salicylic acid and hydrogen peroxide in the systemic promotion of phenolic biosynthesis in clover roots colonized by arbuscular mycorrhizal fungus

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ABSTRACT

Arbuscular mycorrhizal fungal (AMF) colonization can induce both the local and the systemic increase in phenolic accumulation in hosts. However, the signaling molecules responsible for the systemic induction is still unclear. In this study, a split-root rhizobox system was designed to explore these molecules, with one half of clover (*Trifolium repense*) roots colonized by AMF, *Funneliformis mosseae* (formerly known as *Glomus mosseae*), and the other not (NM/M). Plants with two halves both (M/M) or neither (NM/NM) inoculated were also established for comparison. The contents of phenols and the accumulation of salicylic acid (SA), hydrogen peroxide (H_2O_2) and nitric oxide (NO) in roots were monitored, the activities of L-phenylalanine ammonia-lyase (PAL) and nitric oxide synthase (NOS) in roots were assayed, and the expressions of *pal* and *chs* (gene encoding chalcone synthase) genes in roots were also quantified using qRT-PCR. Results indicated that when phenolic content in NM/NM plants was lower than that in M/M plants, AMF colonization systemically induced the increase in phenolic content in NM/M plants. Similarly, the accumulations of SA and H_2O_2 were increased by AMF both locally and systemically, while that of NO was only increased locally. Moreover, enzyme assay and qRT-PCR were in accordance with these results. These data suggest that AMF colonization can systemically increase the phenolic biosynthesis, and SA and H_2O_2 are possibly the signaling molecules involved. The role of MeSA, a signaling molecule capable of long distance transport in this process, is also discussed.

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil fungi, classified as the phylum Glomeromycota, and normally establish symbiosis with the roots of most terrestrial plants (Smith and Read, 2008). The benefits to plants following the establishment of symbiosis have been well documented (Smith and Read, 2008), among which the enhanced resistance of plants to pathogens is of great significance (Campos-Soriano et al., 2012; Ismail et al., 2013; Maffei et al., 2014). Several mechanisms have been proposed to be involved in the increase in pathogenic resistance (Pozo et al., 2010; Wehner et al., 2010), e.g. increased pathogenesis-related proteins (PRs), mediated rhizosphere microbial community, improved nutritional status, and promoted antibiotic chemicals. Phenols are important secondary metabolites in plants, contributing greatly to plant resistance to pests (Martin et al., 2008; Wallis et al., 2013). Many experiments indicate that AMF colonization significantly promote the biosynthesis of phenols in plants, which is responsible for the enhanced disease resistance (Zhu and Yao, 2004; López-Ráez et al., 2010; Zhang et al., 2013). In tomato colonized by *Glomus versiforme*, soluble phenolic content in roots was significantly increased by 83.1%, whereas cell wall-bound phenolic content was slightly elevated by about 20% (Zhu and Yao, 2004). The increase in phenolic content in roots resulted in significant decrease in *Ralstonia solanacearum* population in the xylem and surface of tomato roots. It is interesting that AMF affect each phenol species in a different pattern. For example, in tomato roots colonized by *Funneliformis mosseae* (formerly known as *Glomus mosseae*) or

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Rhizophagus intraradices (formerly known as *Glomus intraradices*), the contents of two phenolic acids (caffeic and chlorogenic acid) were significantly decreased, while that of ferulic acid was increased (López-Ráez et al., 2010), and AMF even induced the appearance of novel phenol species or the disappearance of known phenol species (Ponce et al., 2004, 2009). Recently, we demonstrated that signal molecules (e.g. H_2O_2, SA, NO) could be involved in the promoted phenolic biosynthesis induced by AMF (Zhang et al., 2013).

The magnitude of root colonization by AMF varies very much depending on plant species (Saito et al., 2011), fungal species (Ortas et al., 2013) and soil environment (Ndoye et al., 2013). In the case of low colonization, whether AMF induces the increase of phenolic content and pest resistance in uncolonized parts of roots is of practical significance. Surprisingly, this systemic induction has been ever reported in several plant-fungus combinations (Zhu and Yao, 2004; Ceccarelli et al., 2010; Elsharkawy et al., 2012; Maffei et al., 2014), which highlights the utilization of AMF to protect whole plant against pests. Using a split-root system, Zhu and Yao (2004) clearly demonstrated that increased phenols in tomato plants due to systemic induction by *F. mosseae*. significantly suppressed *Ralstonia solani* by 28%, a little lower than that due to local induction (53%). Other plant-fungal combinations showing systemic induction of phenols include artichoke-*F. mosseae* (Ceccarelli et al., 2010), artichoke-*R. intraradices* (Ceccarelli et al., 2010), and etc. This systemic induction has also been clarified by immunocytochemical (Cordier et al., 1998). Cameron et al. (2013) divided the plant disease resistance into two types: systemic acquired resistance (SAR) after pathogen infection and induced systemic resistance (ISR) following root colonization by non-pathogenic rhizobacteria. SAR is a signal molecule-driven process and has been well elucidated in plants (see review by Shah and Zeier, 2013). In contrast, the signaling process involved in the systemic resistance induced by AMF, a kind of ISR, has not yet been characterized so far, although the defense mechanisms associated with ISR by AMF was summarized (Cameron et al., 2013).

Salicylic acid (SA), hydrogen peroxide (H_2O_2) and nitric oxide (NO) are highly motile molecules in plant cells, and actively involved in the signaling processes of SAR (Baxter et al., 2014; Veloso et al., 2014; Wang et al., 2014). For example, SA application up-regulated the expression of pathogenesis-related gene *PR-1* in both shoots and roots of tomato plants, which significantly reduced the number of root-knot nematodes (*Meloidogyne incognita*) that entered the roots and started to develop (Molinari et al., 2014). Lin and Ishii (2009) reported a significant accumulation of H_2O_2 in the xylem fluids of cucumber plants during the acibenzolar-S-methyl induced SAR. Chun et al. (2012) introduced a mammalian NO synthase into tobacco plants and found that its constitutive expression triggered the induction of SAR-associated genes. Meanwhile, they suggested a highly regulatory role for NO and H_2O_2 in SA-, jasmonic acid- (JA-), ethylene-dependant pathways in SAR.

Our previous work demonstrated that AMF colonization induced the phenolic increase in tomato roots in both local and systemic manner (Zhu and Yao, 2004). To further reveal the signaling molecules involved, we monitored the SA, H_2O_2, and NO contents and found that they could be involved in the local induction of phenolic biosynthesis by AMF (Zhang et al., 2013). However, the signaling molecules possibly involved in the systemic induction are still unclear. In this context, we hypothesize that SA, H_2O_2, and NO may also participate in the systemic induction of phenolic biosynthesis. Therefore, this study was designed to explore whether they are involved in the systemic induction of increased phenols in plants colonized by AMF. To achieve this aim, split-root system was employed and the dynamics of contents of signaling molecules were monitored. The expressions of *pal* and *chs* genes (encoding chalcone synthase, CHS) were also analyzed to characterize the response of phenylalanine pathway to AMF colonization.

Material and methods

Plant and fungal material

Clover (*Trifolium repense* L.) and *Funneliformis mosseae* BGC BJ01 (formerly known as *Glomus mosseae*) were selected to establish symbiosis. Clover seeds were commercially obtained and *F. mosseae* were provided by Beijing Ago-Forestry Academy. Fungal inoculants were propagated with clover and sorghum (*Sorghum bicolor*) as hosts for three months in laboratory.

Experimental design

To explore the systemic increase in phenolic content in roots, split-root system was established. Each container consisted of two plastic boxes (8 cm × 8 cm × 6 cm) sticking together (Fig. 1). Growth substrate was the mixture of paddy soils and river sands (1:5 in volume), and the chemical analysis of soils was as following: pH 6.9, organic matter 0.47%, available nitrogen 158 mg kg^{-1}, available phosphorus 27 mg kg^{-1}, and available potassium 465 mg kg^{-1}. River sands were cleaned with tap water to remove any impurities, and then both soils and river sands were sterilized by autoclaving at 121 °C for 2 h.

Substrate of 800 g was filled into each container, with 400 g in each box. Clover seeds were surface disinfected with 0.5% $NaClO_3$ for 10 min and then let germinate on filter paper in a Petri dish. When the radical root extended about 1.5 cm long, root tip of 0.5 cm was excised to encourage the emergence of lateral roots, and then transplanted in sterilized sands, and fertilized with 1/10 strength Hoagland solution. After growth of about 25 days in sands, plants were further transplanted into containers with 12 plants in each one. At transplanting, the root system was carefully separated into two halves of approximately equal size, with one half in each box (Fig. 1).

Three treatments were established, including both boxes of a container inoculated with AMF (M/M), neither box inoculated (NM/NM), one box inoculated while the other not (NM/M). For inoculated box, 40 g inoculants were well mixed with substrate, while for non-inoculated box, 40 g sterilized inoculants were used together with 10 ml filtrate from 40 g unsterilized inoculants to balance the bacterial community. In each treatment, nine containers were prepared, and the experiment totally consisted of 27 containers. Harvest was conducted at 5, 7, and 9 weeks after transplanting (WAT). At each harvest, three containers were harvested as three replicates for each treatment.

Measurement of shoot biomass and root colonization

At each harvest, shoots were carefully cut off and biomass was recorded. In each treatment, roots in different box were separately harvested and cleaned. An aliquot of root samples was cut into 1–2 cm fragments for the measurement of root colonization and another aliquot was stored at −80 °C for enzyme assay and qRT-PCR, while others were used in fresh for the analysis of signal molecules and phenols.

Root fragments were stained with 0.1% trypan blue (Phillips and Hayman, 1970) and mycorrhizal colonization was measured according to Giovannetti and Mosse (1980).

Determination of the contents of phenol, NO, H_2O_2, and SA

The extraction of phenols was modified from Janas et al. (2000). Briefly, 100 mg fresh roots were ground in liquid nitrogen, added