Journal of Plant Physiology 190 (2016) 15–25

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Suppression of allene oxide synthase 3 in potato increases degree of arbuscular mycorrhizal fungal colonization

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ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 29 October 2015
Accepted 11 November 2015
Available online 17 November 2015

Keywords:
Arbuscular mycorrhizal
Oxylipins
9-LOX pathway
Allene oxide synthase

ABSTRACT

Arbuscular mycorrhizal (AM) is a mutually beneficial interaction among higher plants and soil fungi of the phylum *Glomeromycota*. Numerous studies have pointed that jasmonic acid plays an important role in the development of the intraradical fungus. This compound belongs to a group of biologically active compounds known as oxylipins which are derived from the oxidative metabolism of polyunsaturated fatty acids. Studies of the regulatory role played by oxylipins in AM colonization have generally focused on jasmonates, while few studies exist on the 9-LOX pathway of oxylipins during AM formation. Here, the cDNA of Allene oxide synthase 3 (AOS3), a key enzyme in the 9-LOX pathway, was used in the RNA interference (RNAi) system to transform potato plants in order to suppress its expression. Results show increases in *AOS3* gene expression and 9-LOX products in roots of wild type potato mycorrhizal plants. The suppression of *AOS3* gene expression increases the percentage of root with mycorrhizal colonization at early stages of AM formation. *AOS3* RNA interference lead to an induction of *LOXA* and 13-LOX genes, a reduction in AOS3 derived 9-LOX oxylipin compounds and an increase in jasmonic acid content, suggesting compensation between 9 and 13-LOX pathways. The results in a whole support the hypothesis of a regulatory role for the 9-LOX oxylipin pathway during mycorrhization.

1. Introduction

Arbuscular mycorrhizal (AM) symbiosis is a mutually beneficial interaction among higher plants, including the majority of agricultural crop species, as well as soil fungi of the phylum *Glomeromycota* (Smith and Read, 2008). The AM fungus colonizes the root and provides the plant with nutrients and soil water via an external network of hyphae, while the fungus obtains its carbon from the host plant in the form of plant photosynthates (Harrison 1999; Govindarajulu et al., 2005; Javot et al., 2007). This entire process of bidirectional nutrient exchange between plant and fungus is closely linked to and highly dependent upon environmental and biological variables (Koltai et al., 2010). During the establishment of the symbiosis plant cells undergo morphological and functional changes, suggesting that there is a high degree of interaction between both partners at the cellular, molecular and genetic levels. In this regard, a continuous exchange of signals is established between both symbionts in which different classes of plant hormones play a highly important role (Gutjahr, 2014).

Among others functions, plant hormones play an essential role in interactions, such as pathogenic and symbiotic relations of plants with microorganisms (Pieterse et al., 2012; Zamioudis and Pieterse, 2012). Thereby, plant hormones play a role in the process of AM establishment, which involves mutual recognition as well as morphological and physiological adjustments in the root in order to support fungal colonization. Proof of this is mainly based on experiments performed with phytohormone applications on plants colonized by AM fungi and the observation of changes in endogenous levels of these molecules during mycorrhization. Some hormones control the early steps of the interaction mediating pre-symbiotic signaling, while others regulate root morphological adaptations to accommodate the fungus, control the extension of fungal colonization or control symbiosis functionality (Gutjhar, 2014; Foo et al., 2013; Bucher et al., 2014; Pozo et al., 2015). Jasmonic acid (JA) and its derivatives (methyl jasmonate, jasmonate isoleucine), which are believed to play a major role during the establishment of AM symbiosis, have been the subject of particular interest (Hause and Schaarschmidt, 2009).

Jasmonates belong to a group of lipid phytohormones derived mainly from the oxygenation of linoleic and linolenic fatty acids known as oxylipins, which act as signaling molecules in plant responses to stress and are involved in various developmental

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http://dx.doi.org/10.1016/j.jplph.2015.11.003
0176-1617/

processes. The responses regulated by oxylipins include wounding, exposure to ozone, drought as well as participation in plant-microorganism interactions (Howe and Schilmiller, 2002; Wasternack and Hause 2013; Mosblech et al., 2009). Oxylipins are generated by the coordinated activity of lipases, lipoxygenases (LOXs) and a group of cytochrome P450 (CYP74) enzymes adept at metabolizing polyunsaturated fatty acids (Howe and Schilmiller, 2002). The relative specificity of these enzymes for either 9- or 13-hydroperoxides suggests that the oxylipin metabolism is organized into discrete 9-LOX and 13-LOX pathways. In the 13-LOX branch of the oxylipin metabolism, allene oxide synthase (AOS) transforms 13-hydroperoxide linolenic acid (13-HPOT) into an epoxide intermediate (EOT) which is subsequently converted into 12-oxophytodienoic acid (OPDA) by allene oxide cyclase and then into JA after three cycles of β-oxidation (Howe and Schilmiller, 2002; Wasternack and Hause, 2013; Mosblech et al., 2009; Schaller and Stintzi, 2009). Conversely, in the 9-LOX branch, AOS catalyzes the conversion of 9-hydroperoxides of linoleic or linolenic (9-HPOT/D) acids to an unstable intermediate 9,10-epoxy-9,11,15-octadecatri acid (di) enoate (9,10-EOT/D), which is subsequently transformed by non-enzymatic reactions in α- and γ-ketols and 10-oxo 11,15-phytodienoic (10-OPDA) acid or its isomer 10-oxo-11-phytodienoic (10-OPEA) (Hamberg, 2000). In tomato and potato, AOS3 is distinguished from the other two AOS isoforms by its high substrate specificity for 9-hydroperoxides of linoleic and linolenic acid. Potato AOS3 is ten times more active in relation to the 9-HPOT/D substrate than its 13-HPOT/D isomer, and the highest activity was shown with (9S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoic acid (9-HpODE) as a substrate (Itoh et al., 2002; Stumpe et al., 2006).

In general, most research on oxylipins during AM development has focused on JA, although recent studies have shown the involvement of other oxylipins in the process (León-Morcillo et al., 2012a). Experiments involving exogenous applications of JA have shown a dose-response effect on mycorrhization: high and frequent doses produced an inhibitory effect (Ludwig-Müller et al., 2002), while low concentrations of JA boosted root colonization by AM fungus (Regvar et al., 1996). Moreover, plant root colonization by AM fungi has been associated with increases in the endogenous levels of JA (Hause et al., 2002; Meixner et al., 2005). In this regard, the increased accumulation of free and conjugated JA-isoleucine in cells of barley roots colonized by mycorrhizal arbuscules is linked with the induction of genes involved in the biosynthesis of JA and jasmonate-induced protein JIP23 (Hause et al., 2002). In addition, mycorrhization experiments with the JA-deficient tomato plant mutant *spr2*, which lacks the chloroplastic fatty acid desaturase involved in JA biosynthesis (Li et al., 2003), point to a reduction in colonization (Tejeda-Sartorius et al., 2008; León-Morcillo et al., 2012b). On the other hand, studies carried out on the JA-insensitive tomato mutant *jai-1*, defective in terms of the function of the *COI1* tomato homolog in *Arabidopsis* (Feys et al., 1994), have shown an increase in colonization with respect to wild-type tomato plants (Herrera-Medina et al., 2008), suggesting a complex regulatory role for JA in AM symbiosis.

Few studies have been carried out on the changes that occur in the 9-LOX pathway of oxylipins during AM colonization. However, two microarray analyses of mycorrhizal tomato roots colonized with different AM fungi have demonstrated the existence of significant regulation of the 9-LOX gene biosynthetic pathway during the formation of the symbiosis (García-Garrido et al., 2010; López-Ráez et al., 2010). In addition, a fatty acid profile analysis of *Medicago truncatula* plants inoculated with *Rhizophagus irregularis* did not show any significant differences between the 9-LOX and 13-LOX derivatives except in relation to JA (a 13-LOX oxylipin) which reached high levels in mycorrhizal roots (Stumpe et al., 2005). This suggests that the 9-LOX pathway may play a more important role in Solanaceae plants than in other plant families. In this regard, *LOXA* and *AOS3* genes involved in the 9-LOX pathway were induced in tomato roots with a well-established colonization by *R. irregularis,* and their expression appears to be dependent on a certain degree of AM fungal colonization (León-Morcillo et al., 2012b), suggesting that the 9-LOX pathway could be involved in controlling the spread and extent of AM fungi in the roots rather than in the establishment and functionality of the symbiosis.

The aim of this study is to gain a better understanding of the effect of the potential regulatory role played by the 9-LOX oxylipin pathway on the development of the fungus in Solanaceae roots. The cDNA of tomato *AOS3* was therefore used in the RNA interference (RNAi) system for the transformation of potato plants to suppress *AOS3* expression. The suppression of potato *AOS3* gene makes plants significantly more susceptible to colonization by AM fungus *R. irregularis* and lead to an alteration in oxylipin compounds. The results support close relationship between the two pathways of the oxylipin metabolism, and reinforce the hypothesis of a regulatory role for the 9-LOX pathway of oxylipins during mycorrhization.

2. Material and methods

2.1. Plant material and AM inoculation

Solanum tuberosum L. ssp. *andigena* (line 7540) or *Solanum lycopersicum* L. (Mill.) Moneymaker, were grown in a growth chamber (day/night cycle: 16 h, 24 °C/8 h, 19 °C; relative humidity: 50%). Inoculation with *R. irregularis* (DAOM 197198), was carried out in 200 mL pots with a mixture of soil, sand and vermiculite (3:2:1, v:v:v). Each seedling was grown in a separate pot and inoculated with a piece of monoxenic culture containing 50 *R. irregularis* spores and infected carrot roots grown in Gel-Gro (ICN Biochemicals, Aurora, OH, USA). The monoxenic culture (*R. irregularis* and carrot roots) was produced according to previously described method (Chabot et al., 1992). In the non-inoculated treatment, a piece of medium containing only uninfected carrot roots was applied to the plants.

Shoot cultures were established from sprouts and propagated in vitro by monthly subculture of single-node stem explants on a basal MS medium containing mineral salts (Murashige and Skoog, 1962), 2% sucrose and 0.5 g/L 2-(*N*-morpholino) ethane sulfonic acid (MES) solidified with 5.5 g/L agar.

2.2. Estimation of mycorrhizal root colonization

A non-vital trypan blue histochemical staining procedure was used (Phillips and Hayman, 1970). Stained roots were observed with a light microscope, and the intensity of root cortex colonization by AM fungus was determined as described before Trouvelot et al. (1986) and quantified using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured were the percentage of mycorrhizal root length, frequency of mycorrhization (F%), colonization intensity (M%) and arbuscular abundance (a%) in mycorrhizal root fragments. Three microscope slides were analyzed per biological replicate, and each slide contained thirty 1 cm root pieces.

2.3. Isolation and cloning of LeAOS3 cDNA

Amplification of the specific fragment of cDNA coding for *LeAOS3* (deposited in GenBank, accession no. AF454634) was obtained by RT-PCR using RNA isolated from mycorrhizal tomato roots treated with MeJA (50 μM). The RT-PCR program consisted of 5 min incubation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and 5 min at 72 °C