Pre-Print
Leon Morcillo, R.J.; Tamayo Navarrete, M.I.; Ocampo, J.A.; Prat Monguio, S.; Garcia-Garrido, J.M. 2016. Suppression of allene oxide synthase 3 in potato increases degree of arbuscular mycorrhizal fungal colonization. Journal of Plant Physiology. 190: 15-25

Suppression of Allene Oxide Synthase 3 in potato increases degree of arbuscular mycorrhizal fungal colonization

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Summary

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.

Key words: Arbuscular mycorrhizal; Oxylipins; 9-LOX pathway; Allene oxide synthase

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 (Gutjar, 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 (Gutjar, 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 Shaarschmidt, 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 processes. The responses regulated by oxylipins include wounding, exposure to ozone, drought as well as participation in plant-microorganism interactions (Howe and Schilmiller, 2002; Wasternac and House 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; Wasternac and House, 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 *M. 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.

Material and Methods

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 ^{o}C/8 h, 19 ^{o}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.

Phytohormone treatments

After germination, tomato plantlets were grown in 200 mL pots filled for 40 d, and the phytohormone solutions were then applied to soil in a volume of 25 mL per pot. Plants were harvested 6 and 12 h later, and the root system was washed and immediately frozen in liquid nitrogen. The solutions applied contained H_2O (control treatment), 50 µM MeJA (Sigma-Aldrich), 75 µM ABA (Sigma), 70 µM ethephon (Sigma) and 0.5 mM SA (Sigma-Aldrich). All were prepared from stock solutions, and the pH of each solution was adjusted to 7 prior to use.

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.

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 ^{o}C, followed by 30 cycles of 30 s at 95 ^{o}C, 30 s at 60 ^{o}C, 30 s at 72 ^{o}C and 5 min at 72 ^{o}C for extension. Specific primers were designed to amplify a 636 bp fragment of *LeAOS3* according to Gateway® Technology Cloning System (Invitrogen) recommendations: *LeAOS3-F* 5´-CACCGGACCAATGGCTAATACCAAAGACTC-3´ and *LeAOS3RNi-R* 5´-ACCAGGTAATCCCGGTTGTGAACA-3´. The resulting PCR product was purified and ligated into pENTR^{TM}/D-TOPO® (Invitrogen) following the manufacturer's instructions and subsequently sequenced. For the suppression of *StAOS3*, the specific fragment of *LeAOS3* was cloned into a *pBin19RNAi* binary vector (Serra et al., 2009) according to the Gateway® Technology Cloning System manual. The collection of the different mutant lines obtained was screened by RT-PCR using specific oligonucleotides that amplify a fragment comprising the *LeAOS3* gene and part of the upstream region of the T-DNA from pBin19RNAi binary vector that was integrated into the genome of the plant (pBin19RNAi-*F* 5´-CGTCAACATGGTGGAGCACGACA-3´ and *LeAOS3RNAi-R* 5´-ACCAGGTAATCCCGGTTGTGAACA-3´).

Gene structure information and the promoter sequence was obtained using the on-line SOL Genomics Network database (http:// solgenomics.net/) and NCBI. An *in-silico* sequence analysis of 1.5 kb of the *LeAOS3* promoter region was carried out using the PLACE program (Higo et al., 1999) and the Genomatix software suite (www.genomatix.de).

Agrobacterium tumefaciens–mediated transformation

Based on our experience and lab capabilities, we choose potato for genetic transformation. Potato is more amenable to genetic transformation that tomato plants and the high degree of homology between the LeAOS3 tomato protein and its potato homolog StAOS3 enabled the suppression of *StAOS3* in potato plants using *LeAOS3* cDNA. *Agrobacterium tumefaciens* strain *pGV2260* containing the

pBin19RNAi::LeAOS3 vector was used to transform *Solanum tuberosum* ssp. *andigena*. The binary vector was introduced into *pGV2260* by chemical transformation. Bacteria were grown to the late exponential phase (OD_{600} = 1.0) in a YEB liquid medium supplemented with rifampicin (100 µg/mL) and kanamycin (50 µg/mL) and then sub-cultured after 10-fold dilution in a 30 mL fresh medium at 28 °C to an OD_{600} of 0.60. Bacteria were harvested by centrifugation (4000 x g for 10 min) and the resultant pellet was resuspended to an OD_{600} of 0.06 (3 x 10^7 cfu/mL) in the same buffer. *A. tumefaciens*-mediated potato plant transformation was carried out according to the technique described before (Banerjee et al., 2006).

RNA isolation and gene expression analysis by Real-Time Quantitative PCR

For the quantitative reverse transcription PCR (qRT-PCR) experiments, total RNA was isolated from a 0.5 g sample taken from the root, which was a representative part of the root system for each plant and was treated as a biological replicate. Total RNA was isolated from the roots stored at –80 ^{o}C using the RNeasy Plant Mini Kit (Qiagen, MD, USA) following the manufacturer's instructions. cDNAs were obtained from 1 µg of total DNase-treated RNA in a 20 µL reaction volume using the iScript^{TM} cDNA synthesis kit following the supplier's protocol (Bio-Rad, Hercules, CA, USA). qRT-PCR was carried out using an iCycler apparatus (Bio-Rad). Each 20 µL PCR contained 1 µL of diluted cDNA (1:10), 10 µL of 23 SYBR Green Supermix (Bio-Rad) and 200 nM of each primer using a 96-well plate. The PCR program consisted of 3 min incubation at 95 ^{o}C followed by 35 cycles of 30 s at 95 ^{o}C, 30 s at 58–63 ^{o}C and 30 s at 72 ^{o}C. The specificity of the PCR amplification procedure was checked using a melting curve after the final PCR cycle (70 steps of 30 s from 60 ^{o}C to 95 ^{o}C at a heating rate of 0.5 ^{o}C). Experiments were carried out on three biological replicates, and the threshold cycle (C_T) was determined in triplicate. The relative transcription levels were calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The C_T values of all genes were normalized to the C_T value of the *EFα-1* housekeeping gene from tomato and potato. All primer names and corresponding sequences of the genes analyzed are listed in Table S1.

Oxylipin and hormone quantification

Free oxylipin was extracted basically as previously described (Göbel et al., 2002) and analyzed by liquid chromatography coupled to mass spectrometry (HPLC-MS/MS). Individual calibration curves for each tested compound (9(S)-HpODE: 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid; 9(S)-HODE: 9(S)-hydroxy-10(E),12(Z)-octadecadienoic acid; 9-KODA: 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid) and internal standard (13(S)-hydroxy-6(Z),9(Z),11(E))-octadecatrienoic acid) were performed before the analysis. Frozen material (0.5 g) was added to 10 mL of extraction medium [isohexane/2-propanol, 3/2 (v/v) with 0.0025% (w/v) BHT]. After homogenization and vortex, the extract was centrifuged (3,200 × g) at 4 ^{o}C for 15 min. The clear upper phase was collected and the pellet was extracted again with 5 mL of

extraction medium and centrifuged. A 6.7 % (w/v) solution of potassium sulfate was added to the combined organic phase to reach a volume of 32.5 mL. After vigorous shaking, the extract was centrifuged (3,200 × g) at 4 ^{o}C for 10 min. The upper hexane-rich layer containing the oxylipin fatty acid derivatives was collected, dried under nitrogen and resuspended in 0.250 mL of water/methanol (90:10, v/v).

For JA quantification (Flors et al., 2008) a 0.5 g aliquot of frozen root tissue was used per sample. A mixture of internal standard containing 100 ng of dihydrojasmonic acid was added to each sample prior to extraction. Root tissues were homogenized in 2.5 mL of ultra-pure water and centrifuged (5,000 x g) at 4 ^{o}C for 40 min. Then, the supernatant was acidified and partitioned against 2.5 mL of diethyl ether. After centrifugation (5,000 x g) at 4 ^{o}C for 5 min upper organic phase was collected and the aqueous phase was extracted again with 2.5 mL of diethyl ether. The combined organic phase was dried under nitrogen and suspended in 0.250 mL of water/methanol (90:10, v/v).

The samples (20 µL aliquot solution) were analyzed using a HPLC separation module (Alliance 2695, Waters) with a Quattro Micro triple quadrupole mass spectrometer detector (Waters, Milford, MA). Instrument control, data collection, analysis, and management were controlled by MassLynx 4.0 and Quanlynx V4.1 software packages. Separation was performed using an XBridge column C18 3.5 µm 2.1*100 mm connected to a precolumn XBridge 2.1 * 10 mm (Waters).

The mobile phase consisted of methanol and MilliQ water, both added with formic acid at 0.1 %. The gradient started at 40 % of methanol, changed to 90 % in 8 min, and kept during 3 min. Then the gradient changed to the initial condition in 1 min and these conditions were held for 3 min. Retention times of the compounds were: JA (10.17 min), 9-KODA (12.52 min), 9-HpODE (13.48 min) and 9-HODE (13.57 min).

The effluents from the HPLC were introduced into the mass spectrometer using an orthogonal Z-spray electrospray interface (Micromass, Manchester, U.K.). The ionization source temperature was 120 ^{o}C and the desolvatation gas temperature 350 ºC. The cone gas and desolvation gas-flow rates were 600 and 0 L/h, respectively. The capillary voltage was -2.5 kV and the cone voltage was optimized for each compound. Argon gas (2.83 10-3 mbar) was in the collision cell. We optimized the mass spectrometric parameters by continuous infusion of individual solution of each compound at 10 ppm in methanol:water (1:1).The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, using the following transitions: JA 209.5 $\rightarrow$ 58.9, 9-KODA 309 $\rightarrow$ 125, 9-HpODE 311 $\rightarrow$ 185 and 9-HODE 295 $\rightarrow$ 171.

Quantifications were carried out using the internal standards as a reference for extraction recovery and the standard curves as quantifiers. The results were expressed in terms of pmol or nmol x g−1 fresh weight. The analysis was performed on triplicate (technical replicate) on three independent biological samples.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA). The mean values of five biological replication samples for mycorrhization parameters and three replications for qRT-PCR experiments were compared using Duncan's multiple range test (P=0.05).

Results

Cloning and characterization of *LeAOS3*

The complete cDNA coding for *LeAOS3* was obtained by RT-PCR using RNA isolated from mycorrhizal tomato roots. The coding region of cDNA encompasses 1473 bp, corresponding to an allene oxide synthase containing 491 amino acid residues and 55,5 kD belonging to the CYP74 subfamily of P450 cytochromes as has been described previously (Itoh et al., 2002). LeAos3 preserves the major features of the P450 subfamily of CYP74 cytochromes. The features conserved in LeAOS3 comprise some of the major domains of CYP74 enzymes that distinguish them from other P450 monoxygenases, including a small hydrophobic residue of valine (V) (aspartate or isoleucine in other CYP74 enzymes) in the conserved region of helix I, replacing a threonine residue (T) present in all P450 monoxygenases (Chapple, 1998; Paquette et al., 2000). Furthermore, LeAOS3 preserves the consensus sequence NKQC(A/P)(G/A)K(D/N)XV, except for a substitution of isoleucine (I) by valine surrounding the cysteine (C) of the heme group, corresponding to the catalytic site of the enzyme at the C-terminal, characteristic of the CYP74 subfamily (Itoh et al., 2002) (Fig S1).

Comparative analysis of the amino acid sequences of allene oxide synthase protein isoforms involved in the oxylipin metabolism in tomato LeAOS3 present in the 9-LOX pathway, LeAO2 and LeAOS1 involved in the 13-LOX pathway and the StAOS3 protein homologous to LeAOS3 in potato revealed a high degree of similarity (94.7 %) between StAOS3 and LeAOS3 enzymes. However, homology between LeAOS3 sequences and the other two isoenzymes present in tomato (LeAOS1 and LeAOS2) reached only 60 % (Table 1). In this respect, the high degree of homology between the LeAOS3 protein and its homolog StAOS3 in potato and the possibility of easy transforming potato plants enabled the production of transgenic potato plants by genetic silencing using RNA interference from LeAOS3 gene of tomato.

Theoretical analysis of the *LeAOS3* promoter sequence was carried out in order to fully understand the expression pattern and functionality of the *LeAOS3* gene during mycorrhization. This was done by identifying the *Cis* regulatory elements of transcription that may play an important role in its expression during AM symbiosis (Fig S2). The relative positions of the motifs described were indicated with respect to a theoretical transcription start site (TSS), identified according to consensus sequence YYAN(T/A)YY (Yamamoto et al., 2007). The analysis revealed several putative *Cis* regulatory elements that potentially affect symbiotic regulation, several motifs associates with hormone response,

and others regulatory elements specific to roots associated with regulation by light and the circadian cycle were also identified (Fig S2).

To corroborate the function of these *Cis* regulatory elements in the hormone response of the *LeAOS3* gene, tomato plants were treated with phytohormone solutions and RNA was extracted from roots after 6 and 12 h treatments. As expected, the MeJA treatment caused high up-regulation of *LeAOS3* expression. Ethylene and salicylic acid produced a slight increase in gene expression in plant roots after 12 h of treatment. However, ABA did not cause a significant difference in *LeAOS3* gene expression as compared with control roots treated with water, indicating that the putative *Cis* elements in response to ABA do not have the induction capacity of *LeAOS3* under these experimental conditions (Fig. S3).

To complement the characterization of the *LeAOS3* gene in tomato plants, expression patterns were analyzed using qRT-PCR in different organs of the plant: root, leaf, flower and immature fruit. This analysis showed that *LeAOS3* is mainly expressed in the root, while almost no induction was observed in flower and fruit. A small increase in the expression in leaf relative to that observed in flower and fruit was perceived (Fig. S4). These results correlate with one study carried out previously (Itoh et al., 2002), where an accumulation of *LeAOS3* transcripts was detected only in the roots of tomato plants and not in the aerial tissues such as cotyledons, stems, leaves and flowers.

Suppression of *StAOS3* in transformed potato plants

To study the function of the *AOS3* gene and, consequently, the involvement of the 9-LOX oxylipin pathway in AM symbiosis, ssp. *andigena* (line 7540) potato plants were transformed by *A. tumefaciens*, causing function loss in transgenic lines due to gene silencing by RNA interference (RNAi). The high degree of homology between the LeAOS3 tomato protein and its potato homolog StAOS3 (94.7 %) enabled the suppression of *StAOS3* gene expression in potato plants.

Moreover, the expression pattern of *StAOS3* gene was also characterized in the roots of potato mycorrhizal and non-mycorrhizal plants, respectively, at 41 and 62 dai in order to corroborate that *StAOS3* shows an expression pattern associate with AM symbiosis as the previously characterized for *LeAOS3* in tomato (León-Morcillo et al., 2012). We calculated the M value for *StAOS3* potato gene expression in mycorrhizal and non-mycorrhizal plants with respect to gene expression in non-mycorrhizal plants at 41 dai. The expression of the gene was higher in mycorrhizal than in non-mycorrhizal plant roots at 41 and 61 dai although difference in expression between mycorrhizal plants and non-mycorrhizal ones was smaller at 41 dai (Fig. 1A). The oxylipin pattern in roots of potato plants was affected in the same way as for gene expression when comparing inoculated and non-inoculated plants of the same harvest period (41 dai). We measured 9-HODE and 9-HpODE as products from 9-LOX enzyme activity. 9-HpODE is the substrate of potato AOS3 enzyme that catalyses the conversion of 9-HpODE to an unstable intermediate 9,10-epoxy-9,11,15-octadecatrienoic 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). An increase in 9-KODA, 9-HODE and 9-HpODE was noted in mycorrhizal plants with respect to non-mycorrhizal control plants (Fig. 1B) corroborating that the increases in gene expression were parallel to increases in 9-LOX metabolites.

The collection of the different RNAi lines obtained was screened by PCR amplification with specific oligonucleotides of the *LeAOS3* gene and the T-DNA integrated in the plant genome. Due to the low expression level of *StAOS3* in roots of the transgenic plantlets under control growth conditions, the analysis of *StAOS3* expression in RNAi lines was done in response to treatment with MeJA (50 µM), a positive regulator of *AOS3* expression. The treatment enabled us to verify the induction capacity of *StAOS3* in response to MeJA and, thus, to identify the transgenic lines unable to induce gene expression in response to MeJA. Two transgenic lines *AOS3.13* and *AOS3.23,* unable to express the *StAOS3* gene, were therefore identified (Fig. S5).

Effect of *AOS3* suppression on mycorrhization and expression pattern of oxylipin biosynthesis genes in potato plants.

To determine the effect of *AOS3* suppression on the process of AM formation, the pattern of mycorrhization and oxylipin biosynthesis gene expression was studied in wild-type and *AOS3.13* and *AOS3.23* potato RNAi lines inoculated with *R. irregularis*. The degree of fungal colonization was determined using histochemical trypan blue staining. The histochemical analysis parameters showed a higher percentage of root colonization and mycorrhizal frequency in *AOS3.13* and *AOS3.23* RNAi transgenic lines plants as compared with wild-type potato plants. However, no significant changes were observed in the intensity of root colonization and the percentage of arbuscules in colonized roots between the different lines used, transgenic and wild-type plants (Fig. 2).

Fungal colonization and the presence of arbuscules in the whole mycorrhizal root were quantified using qRT-PCR at the molecular level by measuring the accumulation of mRNA for the *GinEF, GinGS* and *StPT4* genes, respectively. *GinEF* gene expression determine the rate of fungal colonization, *GinGS* is a gene marker for fungal functionality and the expression of *StPT4*, a plant gene marker for arbuscules functionality, reflects active arbuscules in potato cells. The M value was calculated for mycorrhizal plants with respect to the expression of each gene in wild-type mycorrhizal plants at 41 dai. The quantitative RT-PCR data showed a significant increase in *GinEF, GinGS* and *StPT4* mRNA accumulation in *AOS3* RNAi lines with respect to wild-type potato plants in the earlier stages of colonization (41 dai). However, no changes were observed in the expression of these genes between RNAi lines and wild-type potato plants at 62 dai, except for the *GinGs* gene of the *AOS3.23* line in which a higher level of expression was detected with respect to wild-type plants (Fig. 3).

The expression pattern of the 9- and 13-LOX metabolism-related genes was also quantified using qRT-PCR. The expression patterns of selected genes were characterized in the roots of mycorrhizal plants at 41 and 62 dai. The M value was calculated for each treatment using as reference the value of the expression of each gene in wild-type mycorrhizal plants at 41 dai. With respect to the genes involved in the 9-LOX pathway, the results show a significant increase in *StLOXA* gene expression in mychorrizal *AOS3* RNAi lines compared with wild-type plants at both mycorrhization stages. In relation to *StAOS3*, similar behavior was detected in its expression in *AOS3.13* and *AOS3.23* lines. As expected, transgenic lines showed low gene expression level at both harvest times. Conversely, a significant induction in mycorrhizal wild-type potato plants at 62 dai was observed. Moreover, no biologically significant differences in the expression of *StDES* (M value in +1/-1 range), a gene encoding a divinyl ether synthase that forms divinyl ethers from fatty acid hydroperoxides, were observed during the experiment between the different lines used (Fig. 4).

With regard to the expression pattern of genes involved in the 13-LOX pathway, the data showed a moderate but significant increase in the expression of *StLOXD* and *StAOS1* in *AOS3* RNAi lines with respect to mycorrhizal wild-type plants mainly at 62 dai. No biologically significant changes were observed (M value in +1/-1 range) in the earlier stages (41 dpi). Nevertheless, in relation to the *StAOS2*, *StAOC* and *StOPR3* genes of the 13-LOX pathway, no biologically significant changes were detected in gene expression during the entire experiment in any of the lines used. However, all of these genes tended to show an increase in gene expression in *AOS3* mycorrhizal RNAi lines in the advanced stages, which is similar to the expression pattern of *StAOS1* and *StLOXD* genes (Fig. 5).

To confirm that *StAOS3* RNA suppression influences 9-LOX metabolic pathway, the amounts of 9-LOX-derived oxylipins were detected by HPLC/MS analysis at 41 dai in roots of mycorrhizal *AOS3.13* and *AOS3.23* RNAi transgenic lines and wild-type potato plants (Fig. 6A). *StAOS3* RNA suppression in transgenic lines lead to a 50 % suppression of 9-KODA content in roots that parallel to an increase (three times) in 9-HpODE, the substrate of AOS3 enzyme. No significant modification was noted from 9-HODE. In order to supplement gene expression measurements of 13-LOX pathway with metabolic data, the jasmonic acid content in roots were analyzed in these plants. The analysis of JA content in roots showed a significant increase in free JA in *AOS3* RNAi lines with respect to mycorrhizal wild-type plants (Fig. 6B).

Discussion

Several studies have shown the involvement of JA, an oxylipin derived from the 13-LOX pathway, in the formation of AM symbiosis. Nevertheless, few studies exist on the changes that occur in the 9-LOX oxylipin pathway during AM colonization. Recent transcriptomic analyses carried out on mycorrhizal tomato roots (García-Garrido et al., 2010; López-Ráez et al., 2010) showed significant upregulation of genes involved in the metabolism of 9-LOX oxylipins. In this regard, *LOXA* and *AOS3* are genes involved in the 9-LOX metabolism whose expression appears to be dependent on a certain degree of AM fungal

colonization and is restricted to the colonized part of the tomato roots (León-Morcillo et al., 2012a; b). As in tomato, we showed here that *StAOS3* gene expression in potato is associated with the mycorrhization process. Further, increases in 9-LOX products were detected for the first time in roots of potato mycorrhizal plants, corroborating that the increases in gene expression were parallel to increases in 9-LOX metabolites. Contrary, in the case of M. truncatula, analysis of the fatty acid profiles of non-mycorrhizal roots and roots colonized by R. irregularis did not show significant differences between the 9-LOX and 13-LOX products of linoleic and α-linolenic acids except in relation to JA (a 13-LOX oxylipin) which reached high levels in mycorrhizal roots (Stumpe et al., 2005). It would therefore be plausible to assume that the 9-LOX pathway plays a more important role in Solanaceae plants than in other plant families. Since 9-LOX pathway is also known to play a defensive role in relation to microbial pathogens in plants (Blée, 2002; Vellosillo et al., 2007; Gao et al., 2007) it is plausible that the activation of the 9-LOX pathway could be a mechanism for controlling AMF development in the roots of both Solanaceae plants.

To obtain additional information concerning the regulation of *AOS3* gene expression patterns and functionality during mycorrhization, a theoretical analysis of the *LeAOS3* promoter sequence was carried out to identify the *Cis* regulatory elements of transcription that could play an important role in its expression during AM symbiosis. The *in silico* analysis of promoter sequence revealed several putative *Cis* regulatory elements that potentially affect symbiotic regulation, such the symbiosis-related OSE1ROOTNODULE and OSE2ROOTNODULE motifs that were identified in genes specifically activated in root cells colonized by AM fungi and rhizobacteria (Stougaard et al, 1990; Vieweg et al., 2004; Fehlberg et al., 2005) whose regulation has been associated with advanced stages of arbuscular development (Fehlberg et al., 2005). It is noticeable the presence of several motifs associated with hormone responses such as two putative G (CACGTG) and T/G (AACGTG) boxes present in other promoters of JA-responsive genes, including biosynthetic genes, genes coding for JAZ proteins and JA-responsive genes such as *PIN-II* (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Fernández-Calvo et al., 2011); two motifs MYC (CAAATG) and MYB (TAACCA) involved in response to ABA, described in the *rd22* and *AtADH1* genes of *Arabidopsis* (Abe et al., 2003); two *Cis* elements LECPLEACS2 (TAAAATAT) related to response to ethylene (Et), described in the *LeAcs2* tomato gene inducible by wounds, floral senescence, ripening or fungal attack (Matarasso et al., 2005); and one W box (TGAC), described in the promoters of gene WRKY1 involved in biotic responses to stress and wound infections developed by pathogens and in early defense responses and salicylic acid (SA) (Eulgem et al., 2000; Xu et al., 2006). The analysis of phytohormone-responsiveness of *LeAOS3* corroborated that this gene showed a strong and early up-regulation by MeJA but ethylene and salicylic acid produced only a slight increase of gene expression in plant roots. However, *LeAOS3* was not responsive to ABA in the experimental conditions tested. These results suggest that *LeAOS3* gene expression could be modulated by typical plant hormones associated with pathogen response (JA, Et, SA). The accumulation of *LeAOS3* transcripts was detected mainly in the plant roots and previous results showed an early increase in the expression of *LeAOS3* after germination, when the root has just emerged from the seed coat, indicating that the

accumulation of gene transcripts is closely linked to soil-exposed tissues (Itoh et al., 2002). In potato, *StAOS3* was shown to be expressed in sprouting eyes, stolons, tubers and roots, but not in leaves (Stumpe et al., 2006). Furthermore, analysis of the *LeAOS3* promoter sequence revealed the presence of potential *Cis* regulatory elements associated with gene expression in roots.

This study aims to gain a greater understanding of the potential effect of this 9-LOX oxylipin pathway control mechanism on AM fungal development in Solanaceae roots. The cDNA of *LeAOS3* was thus used in the RNA interference (RNAi) system to transform potato plants in order to suppress *StAOS3* expression involved in the 9-LOX metabolism. The results show increased susceptibility to AM fungal colonization in the potato RNAi lines of *AOS3* suppression respect to wild-type plants, which mainly affects the frequency of mycorrhization rather than arbuscular formation. These data confirm the regulatory role played by the 9-LOX pathway in controlling AM fungal propagation within the roots. The increased susceptibility to mycorrhizal colonization had already more evident in early development stages of symbiosis, while the higher activation of the 9-LOX pathway was observed in a well-stablished colonization stage (León-Morcillo et al., 2012b). Therefore, it would be plausible to assume that the oxylipin metabolism is involved in regulating plant defense responses during the initial stages of the colonization process which are necessary for establishing AM symbiosis, but the activation of genes from the 9-LOX pathway observed in the well-established stage of colonization reflects the outcome of multiple infection units developed along the colonization period that trigger a high expression rate. In this regard, it cannot be ruled out that increased susceptibility to AM fungal colonization in the RNAi lines of *AOS3* suppression could be a direct consequence of the alteration in the oxylipins pattern in these plants. The transgenic RNAi lines showed a great reduction in 9-KODA concentration in roots, measured here as a marker for AOS3 derived products, and increase their content in the 9-HpODE hydroperoxide, the AOS3 substrate. Thus, the reduction of putative AOS3 derived products (9,10-EOT; 10-OPDA; α–or γ–ketols) and/or accumulation of AOS3 substrate due to *AOS3* suppression might contribute to plant defence modification in response to AMF colonization. Therefore, it is conceivable that the increased susceptibility of *AOS3* suppression RNAi lines is related to a failure in the regulation of plant defense responses in the early stages of colonization and/or to the alteration in the synthesis of secondary metabolites which are important for establishing symbiosis. Functions of oxylipins include direct antimicrobial effect and most oxylipins are able to impair growth of different microbes (Prost et al., 2005). Interestingly, fungal infection of maize by *Cochliobolus heterostrophus* results in the localized production of 9-LOX oxylipins, including 10-OPEA, and a series of related 12- and 14-carbon cyclopente(a)nones which display direct phytoalexin activity against biotic agents, mediate defence gene expression, and can promote cytotoxicity resulting in cell death (Christensen et al., 2015). The authors propose a specialized local role in plant defence for these compounds that suppress the growth of fungi and herbivores including *Aspergillus flavus, Fusarium verticillioides*, and *Helicoverpa zea*. At this point, it would be very interesting for the future research to study the evolution of these compounds in roots during the mycorrhization process and to analyse their possible inhibitory role against AM fungi.

Moreover, the analysis of gene expression patterns involved in the 9- and 13-LOX pathway in *AOS3* RNAi lines showed moderately significant induction of the *LOXA*, *LOXD* and *AOS1* genes with respect to wild-type Mycorrhizal plants. This induction is a direct consequence of *AOS3* suppression and lead to a significant increase in free JA in *AOS3* RNAi lines (scheme general in Fig S6). This suggests the existence of a close relationship between the two pathways of the oxylipin metabolism during AM colonization in Solanaceae. The up-regulation of the 9-LOX oxylipin pathway in mycorrhizal wild-type roots has been shown to be partly dependent on JA pathway activation (León-Morcillo et al., 2012b). Here, we showed that the activation of the 13-LOX pathway could be modulated by alterations in the 9-LOX branch. The differences of free JA content between wild-type and *AOS3* RNAi lines could be also implicated in the differential behavior to mycorrhization in these plants because it has been reported that JA plays an essential role in the colonization of roots by AM fungi. Studies using plant mutants affected in JA biosynthesis or signaling have outstanding the complex regulatory role for JA in AM symbiosis which plays positive and negative roles at different doses and stages of AM development (Tejeda-Sartorius et al., 2008; León-Morcillo et al., 2012b; Herrera-Medina et al., 2008; Isayenkov et al., 2005).

In conclusion the results presented showed that *StAOS3* suppression increase mycorrhizal colonization mainly at early stages. The suppression of *StAOS3* gene expression implies changes in the content of molecular players which participate in the control of AM fungus inside the root during AM colonization, such as reduction in AOS3 products and induction of 13-LOX pathway. Therefore, our results suggest that changes in the homeostasis of oxylipins, including 9-LOX and 13-LOX derived metabolites are responsible for the increased spread of the AM fungus within the root of *AOS3* suppression lines. The results in a whole support the hypothesis of a regulatory role for the 9-LOX oxylipin pathway during mycorrhization.

Acknowlegments

We wish to thank the Tomato Genetics Resource Centre (TGRC) of the University of California for providing tomato seeds. We would also like to thank Michael O'Shea for proof-reading the document. LC-MS/MS analyses were carried out by Dr. Lourdes Sánchez-Moreno at the Scientific Instrumentation Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain. This study was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT) and Fondos Europeos de Desarrollo Regional (FEDER) through the Ministerio de Economía y Competitividad in Spain (AGL2011-25930; AGL2014-52298-P) as well as the Junta de Andalucía (Research Group BIO 260). R. J. León was supported by a research fellowship from the CSIC-JAE program.

Apendix A. Supplementary data

Supplementary data associate with this article can be found in the online version.

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Table 1: Homology between amino acid sequences of LeAOS1 (AJ271093), LeAOS2 (AF230371), LeAOS3 (AF454634) and StAOs3 (AJ868542). Analysis performed with *BioEdit Sequence Aligment Editor*.

Protein	LeAOS1	LeAOS2	LeAOS3	StAOS3
LeAOS1	ID	69%	61%	60%
LeAOs2	69%	ID	49%	49%
LeAOS3	61%	49%	ID	94,7%
StAOS3	60%	49%	94,7%	ID

Legends of Figures

Figure 1. Expression of *StAOS3* and oxylipin content in roots during different developmental stages of arbuscular mycorrhizal formation. (A) qRT-PCR analyses were carried out using cDNA from the roots of non-inoculated and inoculated *andigena* potato plants at 41 and 62 days after colonization (dai) with *R. irregularis*. The value of M is 0 if there is no change and +1 or –1 if there is a 2-fold induction or reduction, respectively, with respect to *StAOS3* gene expression in non-mycorrhizal plants at 41 dai. (B) Oxylipins content in non-inoculated and inoculated *andigena* potato plants at 41 days after colonization. 9-HpODE: 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid; 9-HODE: 9(S)-hydroxy-10(E),12(Z)-octadecadienoic acid; 9-KODA: 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid. Values correspond to the means ± SE of three biological replications. Bars with the same letters are not significantly different (P=0.05) according to Duncan's multiple range test.

Figure 2. Mycorrhizal colonization in *AOS3* RNAi lines and wild-type potato plants. (A) Percentage of mycorrhizal colonization. (B) Mycorrhizal frequency (F%). (C) Intensity of colonization (M%). (D) Arbuscular abundance (a%). Values correspond to the means ± SE of five biological replications. Bars with the same letters are not significantly different (P=0.05) according to Duncan's multiple range test.

Figure 3. Gene expression analysis for AM fungal marker genes *GinEF*, *GinGS* and potato *StPT4*. qRT-PCR analyses were carried out using cDNA from the roots of *AOS3* RNAi lines and wild-type *andigena* potato plants at 41 and 62 days after colonization (dai) with *R. irregularis*. The value of M is 0 if no change is recorded and +1 or –1 if there is a 2-fold induction or reduction, respectively, with respect to the expression of each gene in wild-type mycorrhizal plants at 41 dai that was used as control treatment in which the . Values correspond to the means ± SE of three biological replications. Bars with the same letters are not significantly different (P=0.05) according to Duncan's multiple range test.

Figure 4. Expression analysis of genes involved in the 9-LOX metabolism (*StLOXA, StDES, StAOS3*). qRT-PCR analyses were carried out using cDNA from the roots of *AOS3* RNAi lines and wild-type *andigena* potato plants at 41 and 62 days after colonization (dai) with *R. irregularis*. The value of M is 0 if no change is recorded and +1 or –1 if there is a 2-fold induction or reduction, respectively, with respect to the expression of gene each gene in wild-type mycorrhizal plants at 41 dai. Values correspond to the means ± SE of three biological replications. Bars with the same letters do not differ significantly (P=0.05) according to Duncan's multiple range test.

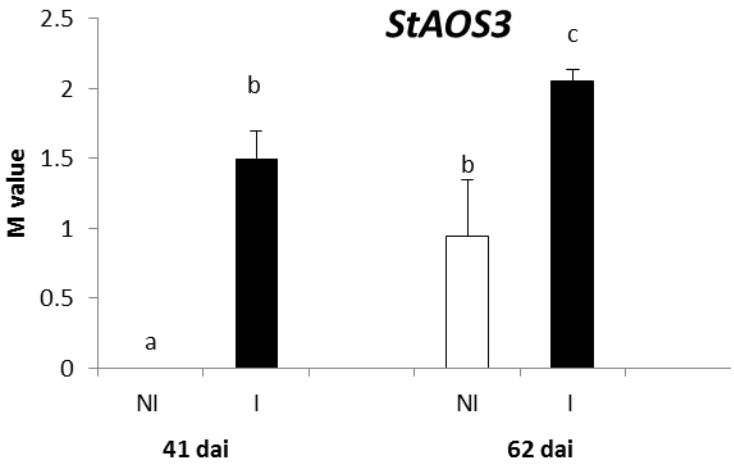
Figure 5. Expression analysis of genes involved in the 13-LOX metabolism (*StLOXD, StAOS1; StAOS2, StAOC, StOPR3*). qRT-PCR analyses were carried out using cDNA from the roots of *AOS3* RNAi lines and wild-type *andigena* potato plants at 41 and 62 days after colonization (dai) with *R. irregularis*. The value of M is 0 if no change is recorded and +1 or –1 if there is a 2-fold induction or reduction, respectively,

with respect to the expression of each gene in wild-type mycorrhizal plants at 41 dai. Values correspond to the means ± SE of three biological replications. Bars with the same letters do not differ significantly (P=0.05) according to Duncan's multiple range test.

Figure 6. Oxylipin and hormone content in mycorrhizal *AOS3* RNAi lines and *andigena* wild-type potato plants at 41 days after colonization (dai) with *R. irregularis*. (A) Oxylipins content in roots. 9-HpODE: 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid; 9-HODE: 9(S)-hydroxy-10(E),12(Z)-octadecadienoic acid; 9-KODA: 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid. (B) Jasmonic acid (JA) content in roots. Values correspond to the means ± SE of three biological replications. Bars with the same letters do not differ significantly (P=0.05) according to Duncan's multiple range test.

Figure 1

A


StAOS3
M value
2.5
2
1.5
1
0.5
0
a
b
b
c
NI
I
NI
I
41 dai
62 dai

B

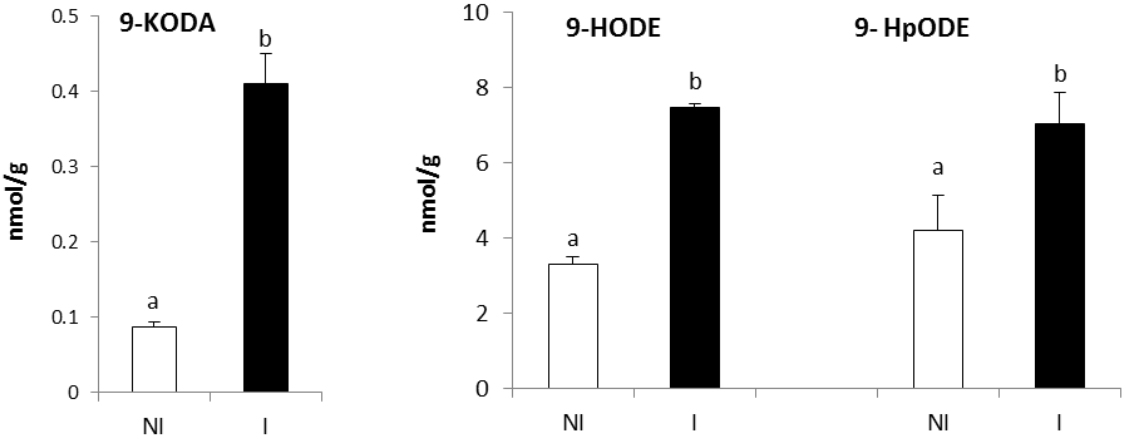

9-KODA
nmol/g
0.5
0.4
0.3
0.2
0.1
0
a
b
NI
I
9-HODE
9- HpODE
nmol/g
10
8
6
4
2
0
a
b
a
b
NI
I
NI
I

Figure 2

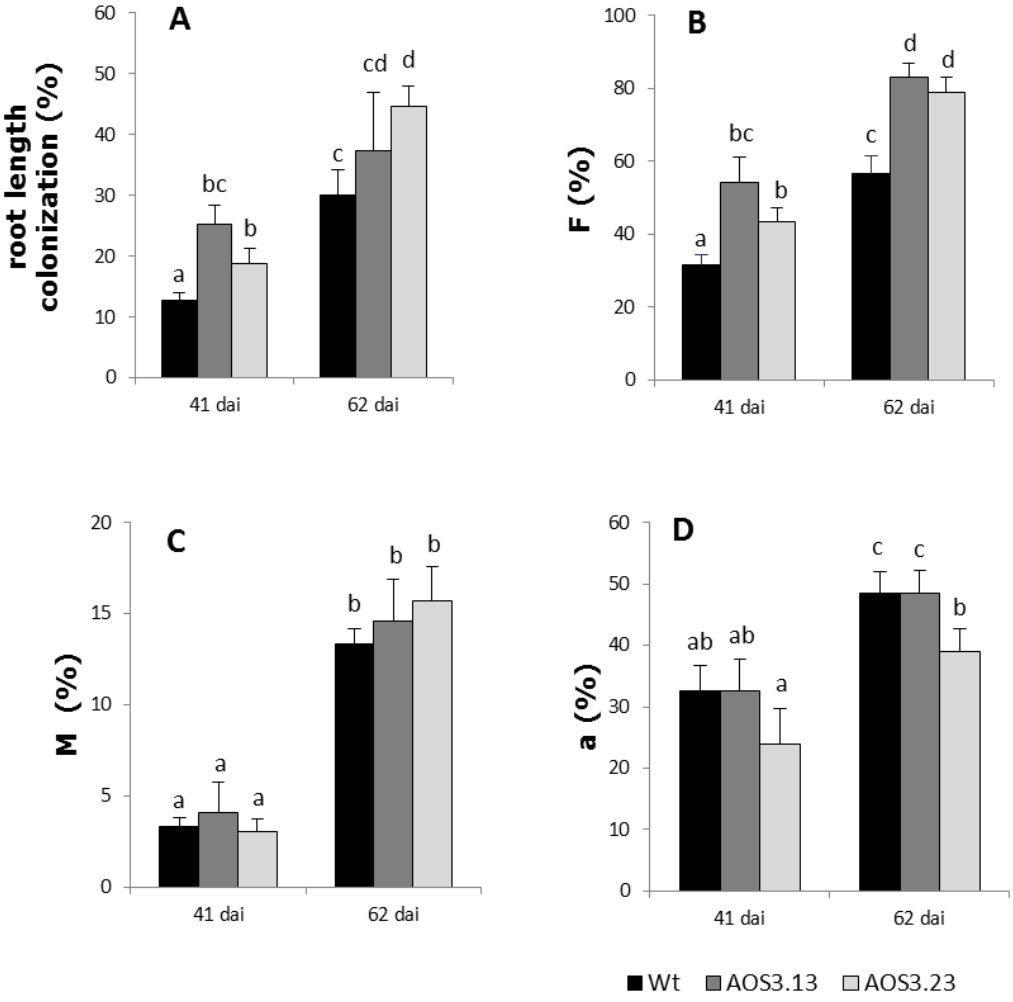

A
root length colonization (%)
60
50
40
30
20
10
0
a
bc
b
c
cd
d
41 dai
62 dai
B
F (%)
100
80
60
40
20
0
a
bc
b
c
d
d
41 dai
62 dai
C
M (%)
20
15
10
5
0
a
a
a
b
b
b
41 dai
62 dai
D
a (%)
60
50
40
30
20
10
0
ab
ab
a
c
c
b
41 dai
62 dai
Wt
AOS3.13
AOS3.23

Figure 3

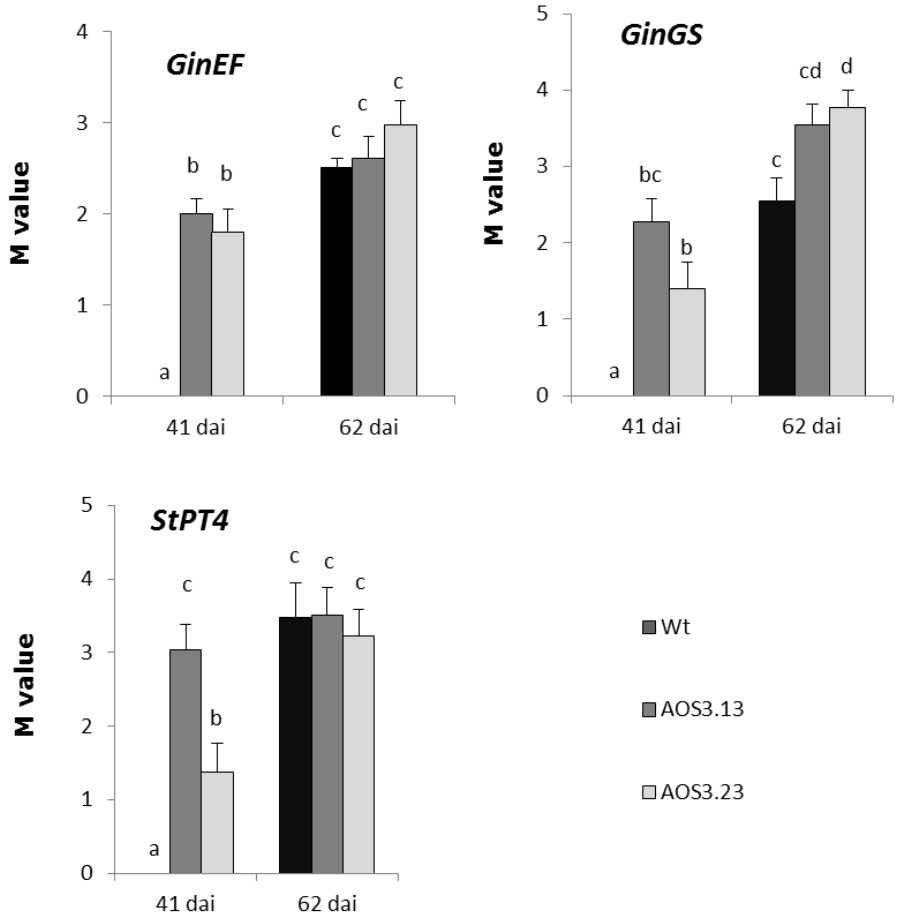

GinEF
M value
4
3
2
1
0
a
b
b
c
c
c
41 dai
62 dai
GinGS
M value
5
4
3
2
1
0
a
bc
b
c
cd
d
41 dai
62 dai
StPT4
M value
5
4
3
2
1
0
a
c
b
c
c
c
41 dai
62 dai
Wt
AOS3.13
AOS3.23

Figure 4

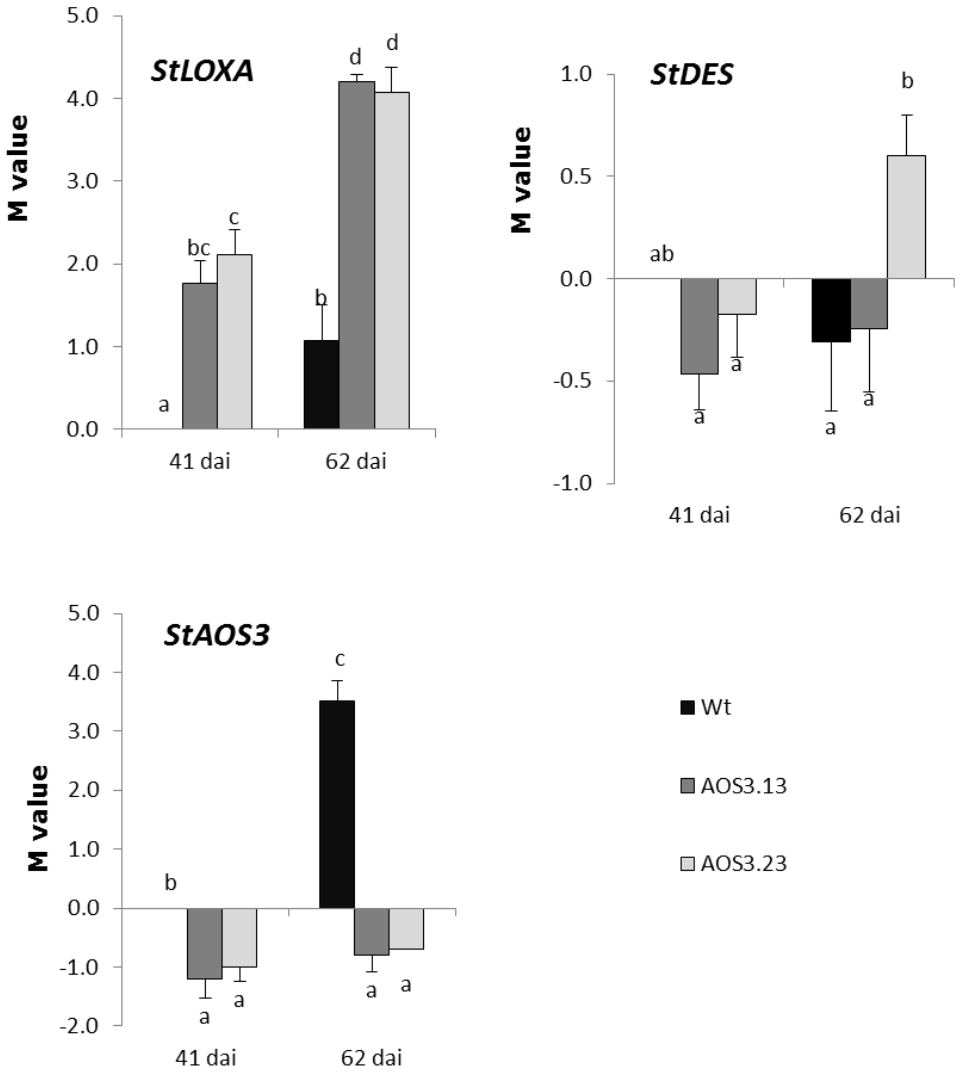

StLOXA
M value
5.0
4.0
3.0
2.0
1.0
0.0
a
bc
c
b
d
d
41 dai
62 dai
StDES
1.0
0.5
0.0
-0.5
-1.0
ab
a
a
a
a
b
StAOS3
b
a
a
c
a
a
-2.0
Wt
AOS3.13
AOS3.23

Figure 5

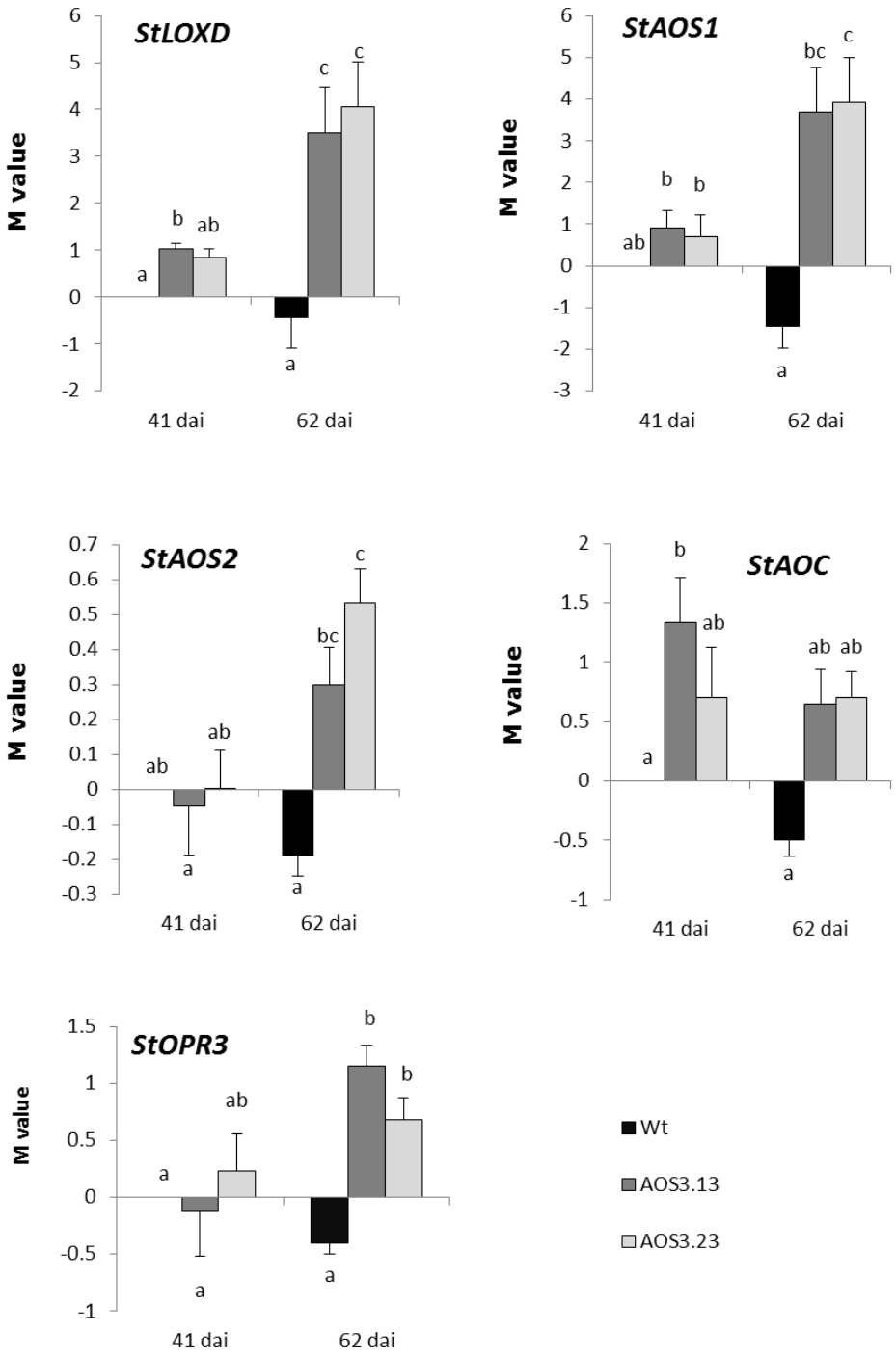
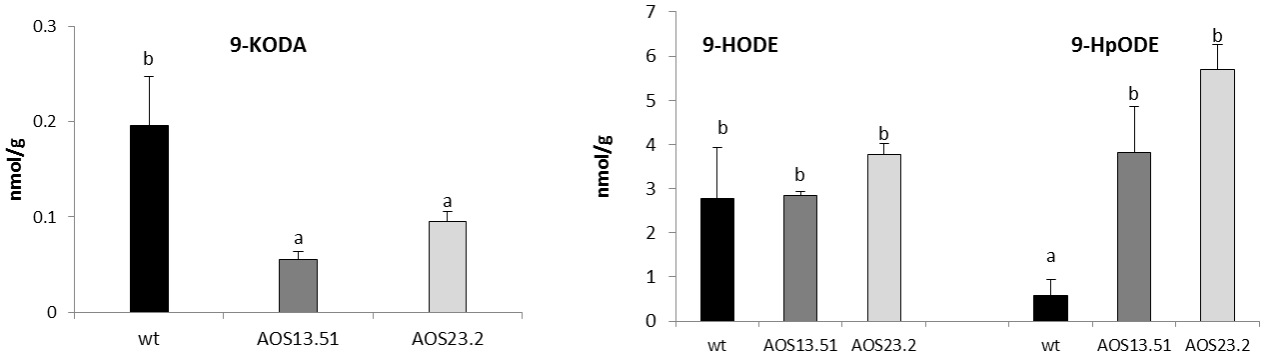
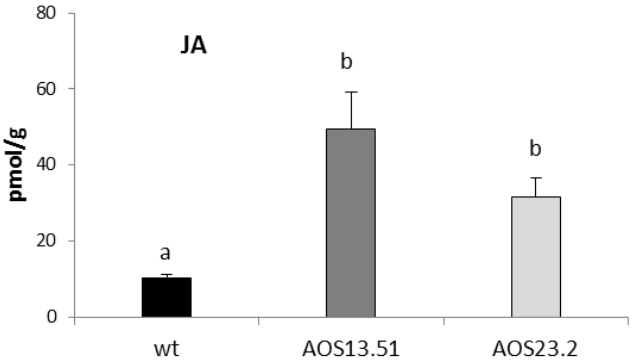

StLOXD
M value
6
5
4
3
2
1
0
-1
-2
a
b
ab
a
c
c
41 dai
62 dai
StAOS1
M value
6
5
4
3
2
1
0
-1
-2
-3
ab
b
b
a
bc
c
41 dai
62 dai
StAOS2
M value
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
ab
a
ab
a
bc
c
41 dai
62 dai
StAOC
M value
2
1.5
1
0.5
0
-0.5
-1
a
b
ab
a
ab
ab
41 dai
62 dai
StOPR3
M value
1.5
1
0.5
0
-0.5
-1
a
a
ab
a
b
b
41 dai
62 dai
Wt
AOS3.13
AOS3.23

Figure 6

A


9-KODA
nmol/g
0.3
0.2
0.1
0
b
a
a
wt
AOS13.51
AOS23.2
9-HODE
9-HpODE
nmol/g
7
6
5
4
3
2
1
0
b
b
b
a
b
b
wt
AOS13.51
AOS23.2
wt
AOS13.51
AOS23.2

B


JA
pmol/g
80
60
40
20
0
a
b
b
wt
AOS13.51
AOS23.2