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# Priming of Anti-Herbivore Defense in Tomato by Arbuscular Mycorrhizal Fungus and Involvement of the Jasmonate Pathway

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**Abstract** Mycorrhizas play a vital role in soil fertility, plant nutrition, and resistance to environmental stresses. However, mycorrhizal effects on plant resistance to herbivorous insects and the related mechanisms are poorly understood. This study evaluated effects of root colonization of tomato (*Solanum lycopersicum* Mill.) by arbuscular mycorrhizal fungi (AMF) *Glomus mosseae* on plant defense responses against a chewing caterpillar *Helicoverpa arimigera.* Mycorrhizal inoculation negatively affected larval performance. Real time RT-PCR analyses showed that mycorrhizal inoculation itself did not induce transcripts of most genes tested. However, insect feeding on AMF pre-inoculated plants resulted in much stronger defense response induction of four defense-related genes *LOXD, AOC, PI-I,* and *PI-II* in the leaves of tomato plants relative to non-inoculated plants. Four tomato genotypes: a wild-type (WT) plant, a jasmonic acid (JA) biosynthesis mutant (*spr2*), a JA-signaling perception mutant (*jai1*), and a JA-overexpressing *35S::PS* plant were used to determine the role of the JA pathway in AMF-primed defense. Insect feeding on mycorrhizal *35S::PS* plants led to higher induction of defense-related genes relative to WT plants. However, insect feeding on mycorrhizal *spr2* and *jai1* mutant plants did not induce transcripts of these genes. Bioassays showed that mycorrhizal inoculation on *spr2* and *jai1* mutants did not change plant resistance against *H. arimigera*. These results indicates that mycorrhizal colonization could prime systemic defense responses in tomato upon herbivore attack, and that the JA pathway is involved in defense priming by AMF.

**Keywords** Defense priming · Arbuscular mycorrhizal fungus · Induced defense · Jasmonate pathway · Tomato · *Glomus mosseae* · *Helicoverpa arimigera*

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## Introduction

Arbuscular mycorrhizal fungi (AMF) are the most important symbionts for the majority of terrestrial plant species (Douds and Millner 1999). Through their widespread extra radical mycelium networks, AMF facilitate plants to acquire nutrients and water in soils that plant roots can not reach. Mycorrhizas not only improve plant nutrition (Evelin *et al.* 2009; Smith *et al.* 2010) and soil stability, but also influence nutrient cycling (Bethlenfalvay and Schüepp 1994; Rillig and Mummey 2006) and a number of important ecosystem processes, including plant diversity, ecosystem variability, and productivity (van der Heijden *et al.* 1998; Vogelsang *et al.* 2006).

Mycorrhizas play a key role in nutrient acquisition for plant growth and reproduction, and thereby have great influences on plant physiology, primary and secondary metabolism, and hormone balance (Smith and Read 2008). The below-ground mycorrhiza may indirectly influence the performance of above-ground herbivorous arthropods through changes in the quality and quantity of plants (Gange 1996; Koricheva *et al.* 2009), and even affect behavior of natural enemies of herbivores (Wooley and Paine 2011) and plant-pollinators (Cahill *et al.* 2008). Therefore, mycorrhizas may influence food chains and webs in ecosystems.

Many studies have showed that arbuscular mycorrhizas (AM) reduce plant diseases (Elsen *et al.* 2008; Zeng 2006). However, effects of mycorrhizal colonization on insect herbivores are much more complicated and unpredictable. Herbivore performance can be affected either positively (Gange and West 1994; Gange *et al.* 1999) or negatively (Gange 2001; Gange *et al.* 1994; Gange and West 1994) by vesicular-arbuscular mycorrhizal (VAM) colonization, depending on both herbivore and fungal species present. This variation may result from specificity between a given plant and AMF. In general mycorrhizal colonization enhances plant resistance to root feeding insects and generalist herbivores, but it may increase plant susceptibility to sucking insects and specialist herbivores (Hartley and Gange 2009).

Defense priming is defined as enhanced readiness of defense responses (Conrath *et al.* 2006; Kim and Felton 2013). Primed plants display faster and/or stronger activation of various cellular defense responses following attack by either pathogens or insects, or in response to other biotic or abiotic stress (Jung *et al.* 2009; Slaughter *et al.* 2012; Ton *et al.* 2006). Some plants previously exposed to herbivore-inducible plant volatiles from neighboring plants under herbivore attack display faster or stronger defense activation and enhanced insect resistance following herbivory (Kessler *et al.* 2006). The primed state in the plant also can be provoked by various natural and synthetic compounds, such as jasmonic acid (JA), salicylic acid (SA), and β-aminobutyric acid (BABA) (Worrall *et al.* 2012). Priming may be initiated in response to an environmental cue that reliably indicates an increased probability of a forthcoming attack. Anti-herbivore defense response often is induced with greater efficiency in plants that have previously experienced insect attack (Karban and Baldwin 1997). Some long-lived trees can maintain a primed state across multiple growing seasons (Zvereva *et al.* 1997). Recent studies demonstrate that the primed state of *Arabidopsis thaliana* plants can be transferred to their progeny, conferring better protection from pathogen attack as compared to the descendants of unprimed plants (Slaughter *et al.* 2012). Tomato plants grown from JA-treated and BABA-treated seeds showed increased resistance against herbivory by spider mites, caterpillars, and aphids, and against fungal pathogens (Worrall *et al.* 2012). The priming responses can last for more than 8 weeks.

Although AM-mediated plant resistance against insect herbivores has been documented in several plant-AMF systems, the underlying mechanisms remain elusive. Besides mechanisms such as improved plant nutrition and quality, increasing evidences show that systematic induction of plant defense responses is involved. Recently defense priming has been suggested as a mechanism of AMF-induced plant resistance (Jung *et al.* 2012).

The objectives of this study were to examine effects of pre-inoculation of tomato with *Glomus mosseae* on resistance to cotton bollworm, as well as on defense responses in tomato leaves, and to determine whether priming is a mechanism of enhanced herbivore resistance of tomato. We also determined involvement of the jasmonate signaling pathway in AMF-induced priming in tomato by using both a defective mutant and over-expression of a transgenic plant in the signaling pathway.

## Methods and Materials

*Plant, Mycorrhizal Fungal and Insect Materials* Tomato seeds (*Solanum lycopersicum* Mill. cv. Jin Bao) were surface-sterilized with 10 % $H_2O_2$ and rinsed five times with sterile distilled water before sowing in autoclaved sand-soil mixture.

The starting inoculum of mycorrhizal fungus *Glomus mosseae* (Gm) (Nicol. and Gerd) Gerdemann and Trappe BEG 167 used, was kindly provided by Prof. Runjin Liu at Qingdao Agricultural University. The mycorrhizal inoculum was produced in pot culture using corn (*Zea mays* L.) plants and autoclaved sand media (Chellappan *et al.* 2002). A mixture of rhizospheric sand from trap cultures (*Z. mays*) containing 35 infective propagules per gram was used for mycorrhizal inoculation.

*Helicoverpa arimigera* (HA, Lepidoptera: Noctuidae) was used to attack tomato plants. These larvae were reared on a semisynthetic diet containing wheat germ (Waldbauer *et al.* 1984) and maintained in an insectary at 23–26 °C, 16 hr/8 hr (day/night) and 65–70 % relative humidity. Homogenously 12-h-molted third instar larvae were used to treat tomato plants.

*Chemicals* TRIzol reagent, AMV reverse transcriptase, Taq polymerase, dNTPs, primer inhibitor, were purchased from TaKaRa (Shuzo Co. Ltd., Shiga, Japan), while 4-morpholine-propanesulfonic acid (MOPS) and diethylpyrocarbonate (DEPC) were purchased from AMRESCO (Solon, OH, USA).

*Experimental Design* *Glomus mosseae* was used for mycorrhizal inoculation in tomato plants, and cotton bollworm *Helicoverpa arimigera* (Lepidoptera: Noctuidae), a generalist chewing caterpillar, was used for insect damage.

Four treatments (Control, HA, Gm, and Gm+HA) were designed to determine effects of defense priming and to exclude possible effects of sole insect attack and AMF inoculation. (1) Control: control plants without insect and mycorrhizal inoculation; (2) HA: plants inoculated with only *H. arimigera*; (3) Gm: plants inoculated with only *G. mosseae*; (4) Gm+HA: plants inoculated with both *G. mosseae* and *H. arimigera*.

*Plant Growth and Mycorrhizal Inoculation* The brown loam soil was collected from the university campus in Guangzhou (China) containing 1.45 % organic matter, 0.725 g/kg total

N, 0.44 g/kg total P, 1.68 g/kg total K, 34.87 mg/kg available N, 1.27 mg/kg available P, and 36.21 mg/kg available K with a pH of 4.72. The soil was autoclaved at 121 °C for 2 hr and mixed with sterilized sand (2:1). Mycorrhizal inoculation of *G. mosseae* in treatments Gm and Gm+HA was obtained by incorporating 120 g of sand containing mycorrhizal inocula into 2.0 kg of the soil/sand mixture before transplanting. The non-mycorrhizal control pots received only the same amount of sterilized sand/soil mixture and 120 g of sand obtained from the growth media of corn plants without mycorrhizal inoculation.

Two pre-germinated tomato seeds were transplanted into each plastic pot with the growth substrate. Ten days later, the seedlings were thinned to one plant per pot. Plants were grown in a growth chamber at 25±1 °C with a 16 hr photoperiod, 150 Md/$m^2$/s PAR, and 60 % relative humidity. Seedlings were watered daily and fertilized every 5 d with 60 ml of Hoagland nutrient solution (Song *et al.* 2010).

*Bioassay* To test whether mycorrhizal infection can enhance host plant resistance against HA, a bioassay was conducted to compare weight gain of HA larvae feeding on the living leaves of mycorrhizal and non-mycorrhizal tomato plants.

Forty days after transplanting, leaves of mycorrhizal and non-mycorrhizal tomato were inoculated with HA third instars. The weight of larva fed on tomato plants was recorded 72 hr after insect inoculation. Before treatment all larvae had been starved for 2 hr and then weighed. Four sets of bioassays were carried out independently, and four pots per treatment were set up for each set of bioassays.

*Transgenic Tomato Plants and Roles of the JA Signaling Pathway* The jasmonic acid (JA) signaling pathway plays an essential role in plant responses to chewing insects (Browse and Howe 2008; Howe and Jander 2008). A JA-overexpressing 35S::prosystemin (*35S::PS*) line, a JA biosynthesis defective mutant *suppressor of prosystemin-mediated responses2* (*spr2*), and a JA-signalling defective mutant *jasmonic acid–insensitive1* (*jai1*) tomato, which were derived from a tomato wild-type (WT, *Solanum lycopersicum* Castlemart) parent (Li *et al.* 2003), were used to determine the role of JA signaling in mycorrhiza-mediated defense priming. Seeds for the 35S::prosystemin (*35S::PS*) transgenic plants were collected from a 35S::prosys/35S::prosys homozygous line (Howe and Ryan 1999) that was backcrossed five times using tomato cv Castlemart as the recurrent parent.

*Real-Time RT-PCR Analysis* Leaves of tomato plants were harvested 0, 6, 12, 24, and 48 hr after caterpillar inoculation for real-time RT-PCR analysis. Transcripts of selected anti-herbivore defense-related genes (*LOXD*, *AOC*, *PI-I,* and *PI-II*) were verified using the RNA samples isolated from tomato leaves obtained from differential treatments. The total RNA was extracted and isolated according to the method described by Kiefer *et al.* (2000) with slight modification (Song *et al.* 2010). Gene-specific forward/reverse primer sets used in these reactions were (5′-ATCTCCCAAGTGAAACACCACA-3′/R: 5′-TCATAAACCCTGTCCCATTCTTC-3′) for *LOXD*; (5′-CTCGGAGATCTTGTCCCCTTT-3′/R: 5′-CTCCTTTCTTCTCTTCTTCGTGCT-3′) for *AOC*; (5′-CGGTTCCTTCACTCTTTAC-3′/R: 5′-GTGCCATGAGAGTTTCAAA-3′) for *PI-I;* (5′-AATTATCCATCATGGCTGTTCAC-3′/R: 5′-CCTTTTTGGATCAGATTCTCCTT-3′) for *PI-II*; and (5′-TCCATCTCGTGCTCCGTCT-3′/R: 5′-GAACCTTTCCAGTGTCATCAACC-3′) for *Ubi3* that was used as a reference. Real-time PCR reactions were carried out with 0.2 μl (0.15 μM) of each specific primers, 1 μl cDNA, 12.5 μl of the SYBR green master mix (Quanti Tech SYBR Green kit, Qiagen, Gmbh Hilden, Germany), and the final volume was made up to 25 μl with RNase-free water. In the negative control, cDNA was replaced by RNase free water. The reactions were performed on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA, USA). The program used for real-time PCR was 3 min initial denaturation at 95 °C, followed by 35 cycles of denaturation for 20 sec at 95 °C, annealing for 20 sec (*LOXD*: 56.9 °C; *AOC*: 56.5 °C; *PI-I*: 47.6 °C; *PI-II*: 55 °C; *Ubi3*: 51.5 °C), and extension for 20 sec at 72 °C. The fluorescence signal was measured immediately after incubation for 2 sec at 75 °C following the extension step, which eliminates possible primer dimer detection. At the end of the cycles, melting temperatures of the PCR products were determined between 65 °C and 95 °C. The specificity of amplicons was verified by melting curve analysis and agarose gel electrophoresis.

Three independent biological replicates for each treatment were used for qRT-PCR analyses. The reference gene was constitutively expressed in all treatments (Song *et al.* 2010, 2011). Relative expression of the target genes to reference gene *Ubi* in the same tissue was calculated by a Double-standard Curves method.

*Statistical Analysis* For each treatment, four replicates were maintained in a completely randomized design. SAS 8.0 (SAS Institute, Cary, NC, USA) package for windows was used for statistical analysis. Data were analyzed with a one-way analysis of variance with the significant differences among means identified by Tukey's multiple range test ($P<0.05$).

## Results

*Effects of Mycorrhizal Colonization on Tomato Resistance Against Helicoverpa arimigera* Mycorrhizal colonization significantly enhanced tomato resistance of both WT and

JA-overexpressing *35S::PS* plants against *HA* (Table 1, Fig. 1). The *HA*-larvae fed mycorrhizal plants of WT and *35S::PS* tomato gained 62.3 and 78.2 % less weight relative to non-inoculated corresponding control, respectively. However, JA biosynthesis mutant *spr2* and JA signaling mutant *jai1* of tomato plants were very susceptible to HA infestation. HA larvae fed on the two mutants gained significantly more weight than those fed on WT and *35S::PS* plants (Fig. 1). Furthermore, mycorrhizal colonization did not affect their resistance against HA. In addition, *spr2* plants had lower mycorrhizal colonization in their roots, but *jai1* plants had similar mycorrhizal colonization with WT and *35S::PS* plants (Table 1).

*Induction of Defense-Related Genes in Mycorrhizal Plants Upon Insect Attack* Quantitative real time RT-PCR was used to detect the transcripts of four defense-related genes: genes that encode lipoxygenase D (*LOXD*) and allene oxide cyclase (*AOC*), which are two key enzymes of the jasmonic acid biosynthesis pathway; two wound-response genes that encode serine protease inhibitors (*PI-I* and *PI-II*), in the leaves of tomato plants. No significant difference in transcript levels of the four genes was found among four treatments (Control, HA, Gm, or Gm+HA) at the beginning time point of insect inoculation (Fig. 2). However, 6 hr after insect inoculation, transcript levels of *LOXD, AOC, PI-I,* and *PI-II* in treatment Gm+HA were significantly higher relative to those in the other three treatments, while insect feeding (treatment) alone induced transcripts of *AOC*. Twelve hours after the insect inoculation and thereafter, pre-inoculation of tomato plants with AMF *G. mosseae* and later inoculation with caterpillar HA (treatment Gm+HA) induced accumulation of *LOXD, AOC, PI-I,* and *PI-II* transcripts over basal levels present in the leaves of non-mycorrhizal and non-insect inoculated control and sole insect inoculation (HA) and *G. mosseae* colonization (Gm) (Fig. 2). In the treatment Gm+HA, *LOXD* transcript levels increased by 49.6-, 62.3- 357.3-, and 7.0-fold (Fig. 2a), and *PI-II* transcript levels increased by 8.5-, 16.7-21.2-, and 10.6-fold (Fig. 2b) at 6, 12, 24, and 48 hr after the insect attack relative to those in the control, respectively. Although insect herbivory induced transcripts of the four tested genes, induction in treatment Gm+HA was stronger than that in the HA treatment. In contrast, mycorrhizal colonization alone only marginally induced *PI-II* transcripts and did not induce the other three genes (Fig. 2a–d).

*Involvement of the JA Signaling Pathway* To determine the role of the JA pathway in AMF-induced insect resistance, four tomato genotypes: JA biosynthesis mutant (*spr2*), JA perception mutant (*jai1*), and JA-overexpressing *35S::PS* line and the corresponding wild-type (WT) were used to study their differential defense responses to insect herbivory and mycorrhizal colonization.

Pre-inoculation of *35S::PS* and WT tomato plants with AMF strongly induced transcripts of defense-related genes *LOXD* and *PI-II* after insect inoculation with HA (treatment Gm+HA) (Figs. 3 and 4). HA herbivory (treatment HA) induced transcript accumulation of *LOXD* and *PI-II* in *35S::PS* and WT tomato plants, but mycorrhizal colonization in the roots of *35S::PS* and WT plants led to stronger induction of *LOXD* and *PI-II* upon insect attack. Insect attack alone increased accumulation of *LOXD* transcripts 4.7-, 11.7-, and 13.3-fold in WT plants, while insect attack on mycorrhizal WT plants induced *LOXD* transcripts 13.2-, 35.9, and 81.1-fold at 12, 24, and 48 hr after the insect inoculation, respectively, relative to untreated control (Fig. 3). The highest transcript levels of *LOXD* and *PI-II* were found in the mycorrhizal pre-inoculated *35S::PS* plants

**Table 1** Percentage of root mycorrhizal colonization in wild-type (WT), JA-overexpressing *35S::PS* line, JA biosynthesis mutant *spr2,* and JA-signaling mutant *jai1* of tomato plants inoculated with AMF *Glomus mosseae* (Gm) and weight gain of *Helicoverpa armigera* (HA) larvae fed on the leaves of these plants

| Tomato line | AMF inoculation | Mycorrhizal colonization (%) | | HA larval weight gain (mg) |
|---|---|---|---|---|
| | | HA-treated | HA-untreated | HA-treated |
| WT | Gm | 53.8±3.2 a | 50.8±1.7 a | 40.6±2.8 c |
| | un-inoculated | – | – | 65.9±7.0 b |
| *35S::PS* | Gm | 50.4±1.5 a | 52.6±2.8 a | 27.1±2.3 c |
| | un-inoculated | – | – | 48.2±1.6 b |
| *spr2* | Gm | 15.8±1.4 b | 16.6±1.6 b | 111.4±4.1 a |
| | un-inoculated | – | – | 115.1±7.5 a |
| *jai1* | Gm | 47.2±3.3 a | 46.2±3.3 a | 119.4±5.1 a |
| | un-inoculated | – | – | 121.9±5.2 a |

Third instar larvae of *H. arimigera* was used to attack tomato plants (WT, *35S::PS*, *spr2, jai1*). Four sets of bioassays were independently carried out and three pots per treatment were set up for each set of bioassays. Values are means ± standard error. Significant differences ($P<0.05$ using Tukey *post-hoc* test) among treatments in the same column are indicated by different letters

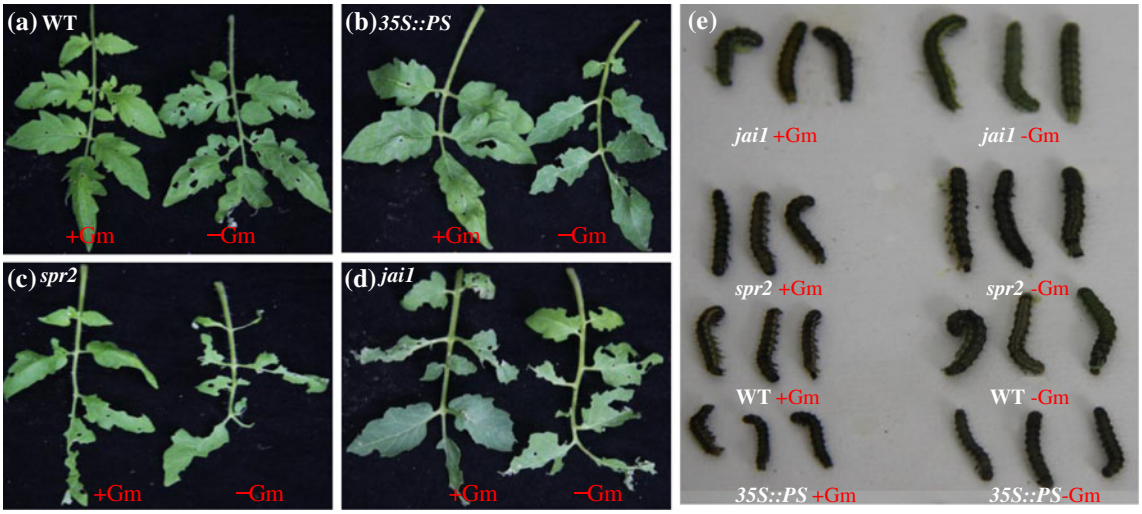


**Fig. 1** Leaf damage (**a**–**d**) of mycorrhizal and non-mycorrhizal tomato plants by *Helicoverpa arimigera* (HA) and HA larval performance fed on different plants (**e**). The photos were taken 72 hr after insect inoculation. *Glomus mosseae* (Gm) was used for mycorrhizal inoculation on tomato plants of four genotypes including wild-type (WT, **a**), JA-overexpressing *35S::PS* line (**b**), JA biosynthesis mutant *spr2* (**c**), and JA-signaling mutant *jai1* (**d**). HA larvae were fed on mycorrhizal (+Gm) and non-mycorrhizal (−Gm) tomato plants of four genotypes, *e.g.* WT, *35S::PS* line, *spr2* and *jai1* mutants (**e**)

after insect inoculation. Sole insect infestation increased accumulation of *LOXD* transcripts 2.5-, 1.7-, 8.6-, and 11.0-fold in *35S::PS* plants, while insect infestation on mycorrhizal pre-inoculated *35S::PS* plants led to 7.6-, 6.4-, 30.4-, and 31.4-fold induction of *LOXD* transcripts at 12, 24, and 48 hr after the insect inoculation, respectively (Fig. 3b–e). Only marginal

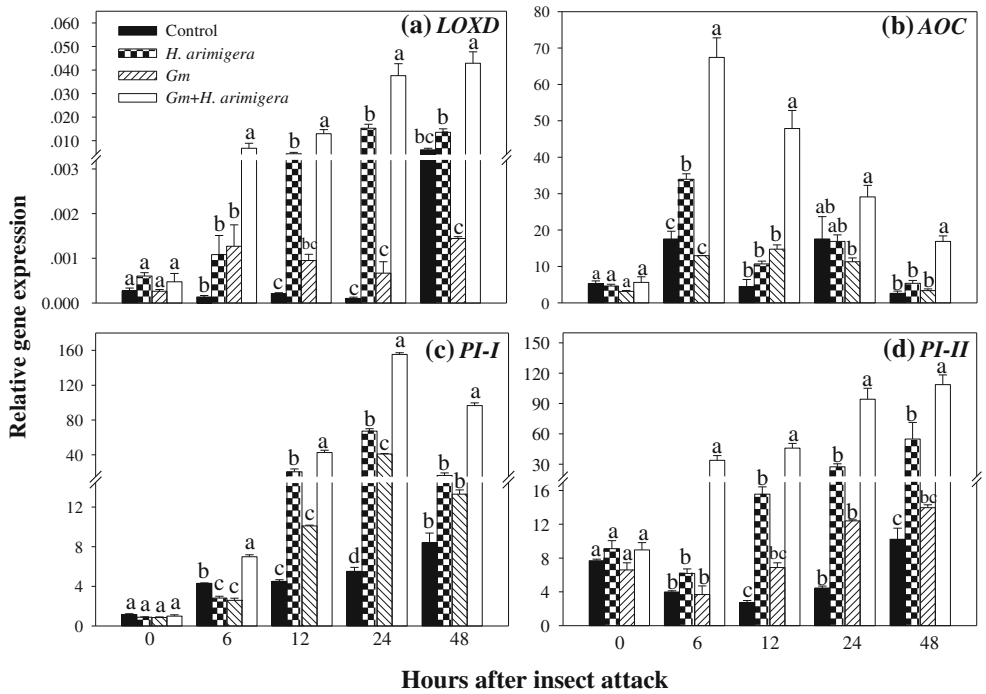


**Fig. 2** Expression of four defense-related genes in leaves of tomato plants in response to mycorrhizal colonization by *Glomus mosseae* (Gm) and herbivore infestation by third instar larvae of *Helicoverpa arimigera* (HA). Four treatments (Control, Gm, HA, Gm+HA) included (1) Control: control plants without insect and mycorrhizal inoculation; (2) HA: plants inoculated with only *H. arimigera*; (3) Gm: plants inoculated with only *G. mosseae*; (4) Gm+HA: plants inoculated with both *G. mosseae* and *H. arimigera*. Quantitative real time RT-PCR was used to detect the transcripts of five defence-related genes encoding (**a**) lipoxygenase D (*LOXD*), (**b**) allene oxide cyclase (*AOC*), (**c**) proteinase inhibitor I (*PI-I*), and (**d**) proteinase inhibitor II (*PI-II*). Values are means + standard error from three sets of independent experiments with three pots per treatment for each set of experiments. For each time point, letters above bars indicate significant difference among treatments ($P<0.05$ according to Tukey's multiple range test)

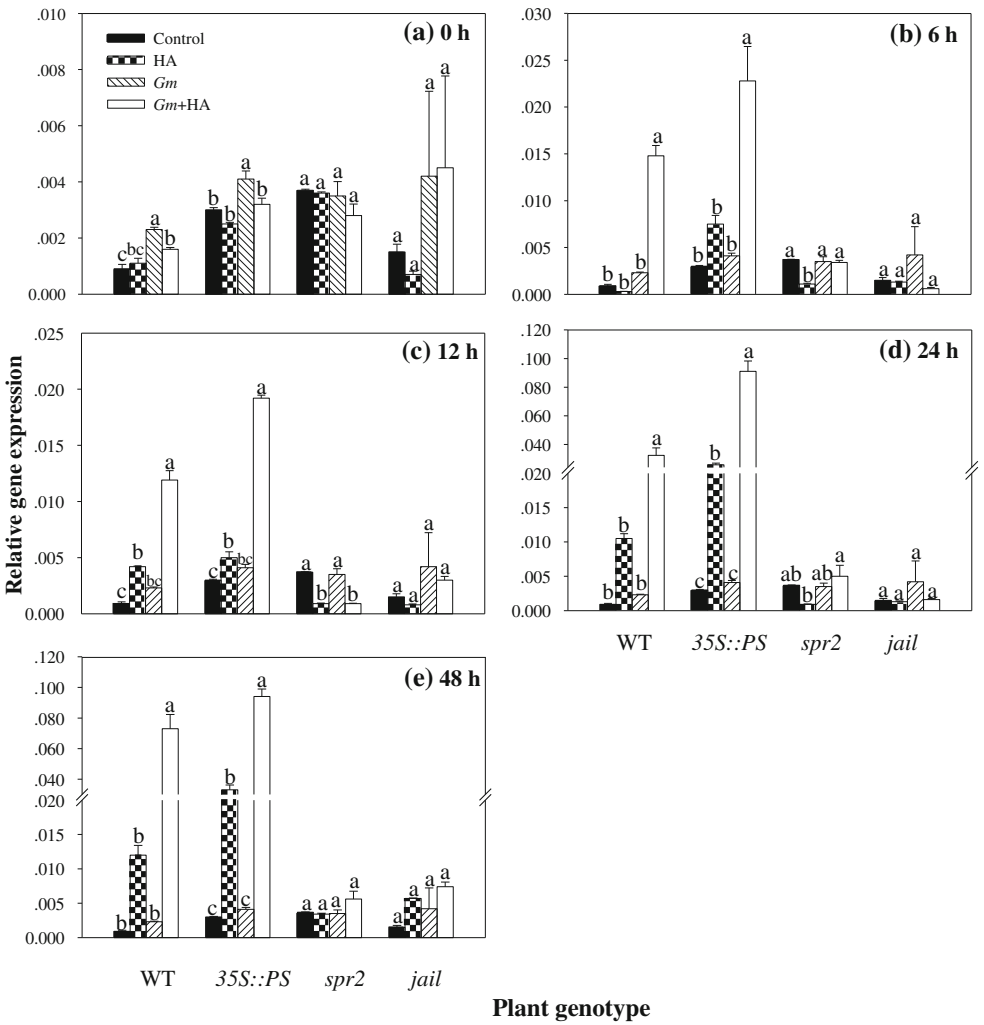


**Fig. 3** Expression of *LOXD* encoding lipoxygenase D in leaves of wild-type (WT), JA-overexpressing *35S::PS* line, JA biosynthesis mutant *spr2,* and JA-signaling mutant *jai1* of tomato plants in response to mycorrhizal colonization by *Glomus mosseae* (Gm) and herbivore infestation by third instar larvae of *Helicoverpa arimigera* (HA). Four treatments (Control, Gm, HA, Gm+HA) were set up as described in Fig. 2. Quantitative real time RT-PCR was used to detect the gene transcripts. Values are means + standard error from three sets of independent experiments with three pots per treatment for each set of experiments. For each genotype within one time point, letters above bars indicate significant difference among treatments ($P<0.05$ according to Tukey's multiple range test)

induction of *LOXD* transcripts was observed in *35S::PS* and WT tomato plants at the beginning of insect treatment (Fig. 3a).

In contrast, AMF pre-inoculation of *spr2* and *jai1* plants did not induce transcripts of *LOXD* and *PI-II* upon HA attack (Figs. 3 and 4). Insect infestation on *spr2* plants slightly induced transcripts of *PI-II*, but mycorrhizal colonization did not have any induced effect. For JA signaling perception mutant *jai1,* no induction of *LOXD* and *PI-II* was observed in either treatment HA or Gm+HA.

## Discussion

Mycorrhizal fungi associate with the majority of plants in all terrestrial ecosystems. There is now increasing recognition of the influence of underground mycorrhizal fungi on plant health and aboveground plant interactions with other organisms (Hartley and Gange 2009; Vogelsang *et al.* 2006). However, less is understood about how soil mycorrhizal fungi influences aboveground plant interactions. This study showed that mycorrhizal colonization enhanced tomato resistance against *H. arimigera*, a chewing generalist herbivore causing severe damage to many important crops. This raises the possibility of use of AMF as a promising alternative for management of some agricultural pests.

The JA signaling pathway plays a crucial role in mediating anti-herbivore defense responses in plants (Howe and Jander 2008). Lipoxygenase (LOX) catalyses the initial reaction in the JA biosynthesis pathway, which inserts molecular oxygen into position 13 of $\alpha$-linolenic acid (Christensen *et al.* 2013). Allene oxide cyclase (AOC) is another important enzyme in JA biosynthesis (Schaller *et al.* 2005). Numerous studies have showed that JA and its derivates play a role in the establishment of functional AM symbiosis (Hause *et al.* 2007; León-Morcillo *et al.* 2012). Hause *et al.* (2002) found that mycorrhizal colonization of barley roots by AMF *Glomus intraradices* led to elevated levels of endogenous jasmonic acid (JA) and increased expression of genes coding for an enzyme of JA biosynthesis (allene oxide synthase). Induction of *LOXD* and *AOC* by AMF indicates that mycorrhizal colonization can also activate the JA pathway, which in turn

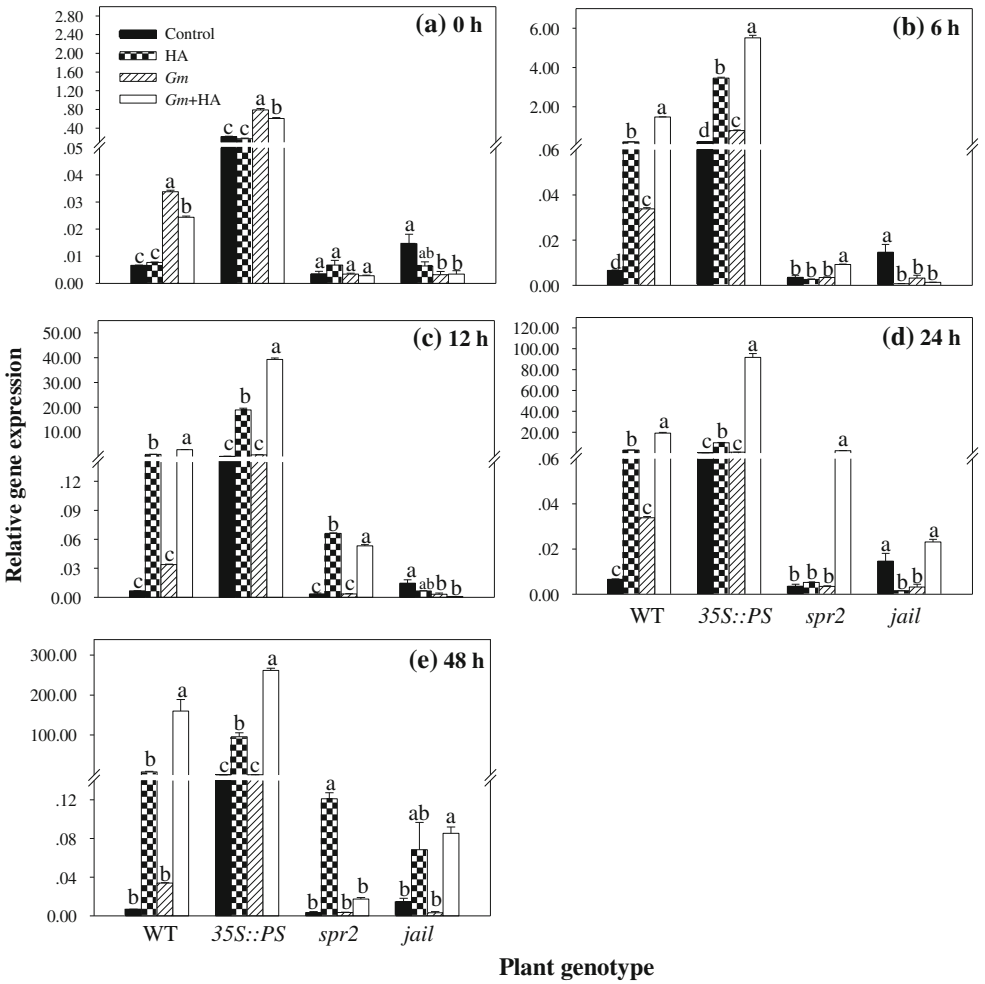


**Fig. 4** Expression of *PI-II* encoding proteinase inhibitor II in leaves of wild-type (WT), JA-overexpressing *35S::PS* line, JA biosynthesis mutant *spr2,* and JA-signaling mutant *jai1* of tomato plants in response to mycorrhizal colonization by *Glomus mosseae* (Gm) and herbivore infestation by third instar larvae of *Helicoverpa arimigera* (HA). Four treatments (Control, Gm, HA, Gm+HA) were set up as described in Fig. 2. Quantitative real time RT-PCR was used to detect the gene transcripts. Values are means + standard error from three sets of independent experiments with three pots per treatment for each set of experiments. For each genotype within one time point, letters above bars indicate significant difference among treatments ($P<0.05$ according to Tukey's multiple range test)

induces broad-spectrum protection against subsequent insect infestation (Christensen *et al.* 2013; De Vos *et al.* 2005).

To determine whether mycorrhizal colonization leads to systemic priming of defense upon insect attack in tomato plants, we compared the different response of mycorrhizal and non-mycorrhizal tomato plants following the inoculation of *H. armigera* larvae in the leaves. Expression levels of defense-related genes in the leaves were examined at 6, 12, 24, and 48 hr after insect inoculation (Fig. 2). Our study showed that mycorrhizal inoculation itself did not affect transcripts of the tested genes (Fig. 2). However, pre-inoculation with *G. mosseae* strongly induced defense responses of all four tested genes in tomato plants upon herbivore attack. Although HA herbivory induced defense responses, mycorrhizal pre-inoculation and late herbivore attack led to much stronger induction of defense-related genes in the leaves, suggesting that root colonization of AMF induces systemic defense and primes defense responses upon herbivore infestation, and consequently, mycorrhizal tomato plants show higher resistance against HA.

Most studies on defense priming focus on priming signals of herbivore-induced volatile compounds (Engelberth *et al.* 2004; Frost *et al.* 2007; Heil and Silvabueno 2007; Ramadan *et al.* 2011; Ton *et al.* 2006). Actually, priming of plant defense can be triggered by certain beneficial microorganisms (Conrath *et al.* 2006; van Hulten *et al.* 2006; Van Wees *et al.* 2008), including AMF (Pozo and Azcon-Aguilar 2007; Pozo *et al.* 2009). The stronger induction of defense-related genes in mycorrhizal tomato plant upon insect herbivory indicates that priming is an important mechanism of mycorrhizal-induced herbivore resistance. Upon AMF colonization, plants modulate their defense responses. As a consequence of this modulation, a mild, but effective activation of the plant immune responses occurs (Hause *et al.* 2002). This activation may lead to a primed state of the plant that allows a quicker and more efficient activation of defense mechanisms in response to subsequent attack by insect herbibores (Jung *et al.* 2012; Pozo and Azcon-Aguilar 2007).

Use of JA biosynthesis (*spr2* and *jai1*) mutants and JA-overexpressing *35S::PS* plants made it possible to identify an essential role the JA signaling pathway in AMF-primed defense in tomato plants. Mycorrhizal *35S::PS* plants showed stronger defense responses to herbivore infestation than mycorrhizal WT plants and non-mycorrhizal *35S::PS*

plants (Figs. 3 and 4). Induction of defense-related genes was significantly higher in mycorrhizal *35S::PS* plants. The mycorrhizal *35S::PS* plants were the most resistant to HA among four tested genotypes (Table 1). However, AMF pre-inoculation and herbivore infestation did not lead to induction of defense-related genes in *spr2* and *jai1* plants. The two mutant plants were the most susceptible to insect infestation, and mycorrhizal colonization did not affect their insect resistance (Table 1 and Fig. 1). These results indicate that the JA pathway is required for AMF-induced systemic priming of defense against HA. Rasmann *et al*. (2012) showed that herbivory in the previous generation primed *Arabidopsis* and tomato for enhanced insect resistance, and *Arabidopsis* mutants that are deficient in jasmonate perception (coronatine insensitive1) did not exhibit inherited resistance, demonstrating that the JA pathway is required for defense priming inheritance.

In summary our results indicate that mycorrhizal-induced herbivore resistance in tomato is associated with priming for an efficient activation of defense responses upon herbivore attack by chewing caterpillar *H. armigera*. The study also indicates that the JA pathway is involved in the AMF-mediated defense priming. Since the majority of land plants establish symbiotic associations with mycorrhizal fungi (Smith and Read 2008), defense priming by common symbiotic organisms may be an important evolutionary strategy for plant defense against herbivores and other biotic stresses.

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