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RESEARCH

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Defense responses and symbiotic functional initiation in trifoliate orange–arbuscular mycorrhizal fungi interaction

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Abstract

Arbuscular mycorrhizal fungi (AMF) trigger beneficial effects on their hosts, but it is unknown how plants modulate their defense responses during root colonization of AMF and the symbiotic benefits are initiated. The purpose of this study was to analyze the root mycorrhizal colonization process of trifoliate orange and the responsive patterns of plant growth, root peroxide hydrogen (H_2O_2), antioxidant enzymes and their encoding gene expression, and sugar, lipid and phosphate transporter protein gene expression at 7—56 days of inoculation (doi) with *Funneliformis mosseae* (*Fm*). *Fm* developed appressoriums on the root surface at 7 doi, followed by abundant arbuscules in root cortical cells at 28 doi, intracellular vesicles at 42 doi, and root mycorrhizal colonization rate of 41.54% at 56 doi. Plant growth improvement by *Fm* started at 28 doi. The immune defense response of roots was initiated at 7 doi, as evidenced by the increase of H_2O_2 levels and superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity, as well as the up-regulation of *PtMn-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT* expression, which lasted until 14 doi. Starting at 28 doi, a sugar transporter gene (*PtSWEET*), a lipid transporter gene (*PtSTR*), and a phosphate transporter gene (*PtPT6*) were initiated to be up-regulated, followed by the up-regulation of *PtSTR2*, *PtPT3*, and *PtPT5* at 42 doi and *PtFe-SOD* at 56 doi. Arbuscule formation and plant growth improvement together at 28 doi suggested that arbuscules trigger improved growth responses of host plants. This study also reveals the initiation of host immune defense response and function in early root AMF colonization.

Keywords Antioxidant defense system, Trifoliate orange, Hydrogen peroxide, Lipids, Mycorrhiza, SWEET sucrose transporter, Symbiosis

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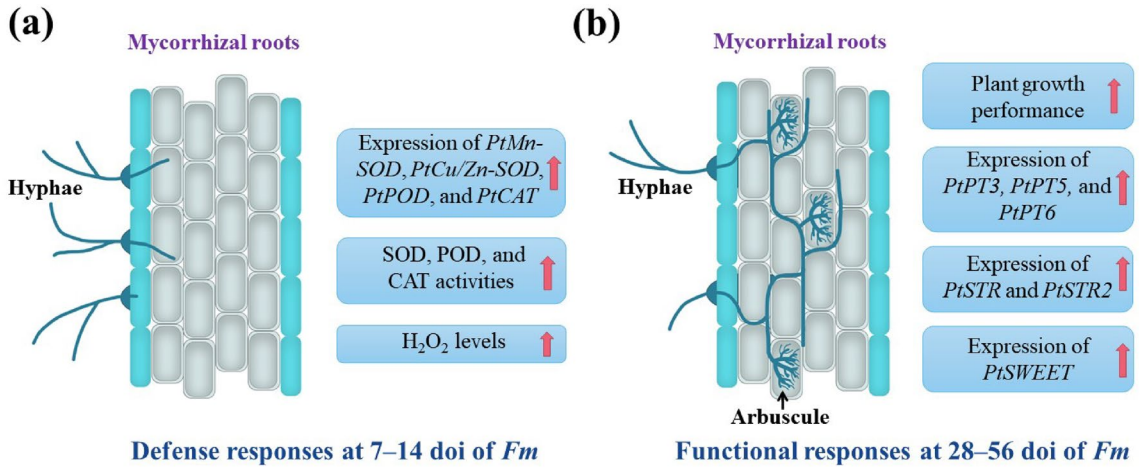
Graphical Abstract



Introduction

Citrus, one of the widely grown fruit trees worldwide, is heavily dependent on arbuscular mycorrhizal fungi (AMF) to facilitate its access to nutrients and water from the soil due to its shallow root systems and few root hairs [1]. AMF roles in citrus plants have garnered a lot of interest [2]. AMF are a widely distributed group of soil fungi that can establish symbiosis with most terrestrial plants, including citrus [3]. AMF can promote growth performance, nutrient acquisition, and stress tolerance of the host plant, as well as crop yield and quality [4–8].

AMF colonization of plant roots starts with the germination of fungal spores in the soil, followed by the continuous growth of hyphae [9]. Under the action of signaling molecules, fungal hyphae contact the surface of root epidermal cells and/or root hairs to form an appressorium, and enter the roots, where fungal hyphae colonize the cortical cells of roots, some of which continuously remifying and fill a cortical cell to form a complex tree-like structure, the arbuscule [9–11]. The tip expansion of intraradical hyphae in AMF of some genera excluding *Gigaspora* and *Scutellospora* forms vesicles within cortical cells [9]. Hydrogen peroxide (H_2O_2) is an important signaling molecule in plants that broadly regulates plant growth and development, responds to stresses, and is involved in plant–microbe interactions [12]. According to Liu et al. [13], H_2O_2 levels in white clover plants increased significantly at the beginning of microbial inoculation and then decreased at the later stages, showing a signaling of H_2O_2 in response to microbial colonization in roots. H_2O_2 burst is necessary and sufficient to induce immune responses [14]. Antioxidant enzymes and their encoding genes, as a natural plant defense system, induce a transient defense response in the early stages of AMF–host plant interaction [15]. Antioxidant enzyme activities are increased significantly at the beginning of root AMF colonization and then decreased in the later stages of AMF growth as the symbiosis develops [16]. Therefore, the response patterns of H_2O_2, antioxidant enzymes, and their encoding genes can reveal the defense response mechanisms of plants to microbial colonization.

Mutual sensing of signals and the exchange of signaling molecules between AMF and the host plant initiate the establishment of symbionts [17]. Arbuscular mycorrhizae are manifested by a bidirectional exchange at the symbiotic interface, where the host plant provides carbon sources (fatty acids and sugars) to the arbuscular mycorrhizal fungus, while the arbuscular mycorrhizal fungus provides the host plant with nutrients, especially phosphorus (P) [18, 19]. Sugar transport and distribution in plants requires the involvement of sugar transporter proteins such as SWEET (sugar will eventually be exported transporters) [20]. Inoculation with AMF in citrus increased the expression of *CsSWEET* gene in fruits [21]. Multiple *SWEET* gene expression was upregulated in AMF–host plants (e.g., *Medicago truncatula* and soybean) [22, 23]. During AMF colonization process, lipids are transported from plant roots to AMF [24] as its main source of carbon [25]. It has been reported that lipid transport proteins (STR/STR2) were involved in mycorrhizal symbiosis in alfalfa [26]. Jiang et al. [27] found that AMF induced lipids synthesis in plants, and lipids entered AMF mainly through STR/STR2, establishing arbuscular mycorrhizae. Yang et al. [28] also

reported that inoculation with AMF in trifoliate orange (*Poncirus trifoliata*; a rootstock used in citrus industry) enhanced host P uptake, mainly through phosphate trasnporter genes (*PtPT3*, *PtPT5*, and *PtPT6*). Thus, sugars, lipids, and *PT* genes play important roles in the symbiosis of AMF and plants, but the underlying mechanisms involved are very complex and need to be studied in depth.

The aim of this study was to further shed light on the mechanism of symbiosis between AMF and trifoliate orange by analyzing the changes in plant growth performance, H_2O_2 levels, antioxidant enzyme activities, and expression of antioxidant enzyme genes and symbiosis-associated genes during the process of root AMF colonization.

Materials and methods

Preparation of AMF inoculum

An arbuscular mycorrhizal fungus *Funneliformis mosseae* (BGC XZ02A) was chosen, because the fungus has been demonstrated to have positive effects on trifoliate orange [29]. This fungus was trapped using white clover as the host plant for about 10 weeks under potted conditions, and the inoculum consisted of fungal colonized root segments, spores (22 spores/g), hyphae, and growth substrates, stored at 4 °C, and used within three months.

Plant culture and experimental design

In March 2022, seeds were removed from trifoliate orange fruits, treated with 10% NaOH to remove pectin from the seed surface, disinfected with 70% alcohol for 10 min, and rinsed three times with distilled water before being placed in pre-autoclaved (121 °C, 0.11 MPa, 2 h) sand (< 2 mm in the diameter) for their germination, where environmental conditions were 30 °C/23 °C (day/night temperature, 16 h / 8 h), with a constant relative air humidity of 75% and a light intensity of 1500 Lux.

After one month, uniformly sized seedlings with four leaves were transplanted into pots (16 × 11 × 15 cm) presupplied with 2.5 kg of autoclaved soil-sand mixture (3: 1, v/v). Meanwhile, 150 g of mycorrhizal fungal inocula was placed around roots of trifoliate orange seedlings as the inoculated treatment, whereas the uninoculated treatment also received an equivalent amount of autoclaved mycorrhizal fungal inocula plus 2 mL of inoculum filtrate through a 20-μm nylon mesh.

Treated seedlings were placed in a controlled greenhouse as described by Cao et al. [30]. This experiment was conducted between May 19 and July 13, 2022. The plants were harvested at 7, 14, 21, 28, 42, and 56 days of inoculation (doi), with 4 pots per treatment harvested each time for a total of 48 pots. The experiment, therefore, consisted of a total of two treatments, each with twenty four replicates.

Determination of plant growth and root mycorrhizal colonization

On the day of each harvest, plant height was measured. After harvest, the taproot length was determined using vernier calipers, and the shoot and root biomass was weighed. Subsequently, root segments with 1–2 cm long were cut and stained with the protocol of Phillips and Hayman [31] with 0.05% trypan blue in lactophenol for 30 s. Root mycorrhizal colonization was observed under a microscope. Root length colonization rate (%) = colonized root length / observed total root length × 100.

Determination of root H_2O_2 concentrations

Root H_2O_2 concentrations were determined as per the protocol outlined by Velikova et al. [32]. The 0.20 g of fresh sample was ground into a homogenate with 5 mL of 0.1% trichloroacetic acid in an ice bath and centrifuged at 10,000 × *g* for 15 min. The 1 mL of supernatant was reacted with 1 mL of 10 mmol/L phosphate buffer (pH 7.0) and 2 mL of 1 mol/L KI, and their absorbance values were recorded at 390 nm.

Determination of root antioxidant enzyme activities

Superoxide dismutase (SOD) activity was determined according to the method described by Wu [33]. The reaction solution consisted of 50 μL of the enzyme extract, 300 μL of 130 mmol/L L-methionine, 300 μL of 750 μmol/L nitroblue tetrazolium, 300 μL of 100 μmol/L EDTA-Na_2, 300 μL of 20 μmol/L riboflavin, and 250 μL of distilled water. CAT activity was measured as per the protocol described by He et al. [34]. A 4-mL reaction solution consisted of 0.2 mL of the enzyme extract, 2.0 mL of 0.1 mmol/L phosphate buffer (pH 7.8), and 1.8 mL of distilled at 25 °C for 3 min, followed by the addition of 0.3 mL of 0.1 mol/L H_2O_2. Peroxidase (POD) activity was assayed using the procedure outlined by Chance and Maehly [35]. A 5-mL mixture contained 2.9 mL phosphate buffers, 1.0 mL of 0.1 mol/L H_2O_2, 1.0 mL of 0.05 mol/L guaiacol, and 0.1 mL of the enzyme extract.

Analysis of relative expression of genes

Root total RNA was extracted by the MiniBEST plant RNA kit (No. 9769; TaKaRa, Dalian, China). The RNA integrity was detected by 1.0% agarose gel electrophoresis, and the concentration of the extracted RNA was calculated by A_{260}/A_{280} ratio. The qualified RNA was reverse transcribed to cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (RR047A; Takara). Five antioxidant enzyme genes (*PtFe-SOD*, *PtMn-SOD*, *PtCu/*

Zn-SOD, *PtPOD*, and *PtCAT*) [34], a sugar transporter protein gene (*PtSWEET*) [36], two fatty acid transporter protein genes (*PtSTR* and *PtSTR2*) [26], and three phosphorus transporter protein genes (*PtPT3*, *PtPT5*, and *PtPT6*) [28] were selected based on previous studies. The genes were identified through the NCBI database (www.ncbi.nlm.nih.gov) and the genome-wide of trifoliate orange (http://citrus.hzau.edu.cn). The Primer Premier 5.0 software was used to design primer sequences of selected genes for qRT-PCR (Additional file 1: Table S1). The cDNA was used as the template. qRT-PCR was performed on an Fast Real-time PCR System (7900HT, ABI, Nanjing, China). The β-actin was employed as an internal reference gene. Each gene had three biological replicates, with three technical replicates per biological replicate. The relative expression of genes was calculated according to the method of Livak and Schmittgen [37], normalized to the gene of the no-*Fm* treatment.

Data analysis

All data analysis was performed in the SAS software (v8.1), where one-way analysis of variance and LSD tests were used at the 0.05 level for significant differences among treatments. The SigmaPlot (v10.0) was used for figure production.

Results

Changes in root length AMF colonization

At 7 doi, *Fm* was found at the root surface, showing a branching pattern and the formation of appressorium (Fig. 1a). At 14 doi, mycorrhizal hyphae had colonized into the roots and formed intraradical hyphae. At 21 doi, well-developed intraradical hyphae were formed in the roots, with obvious branching and visible arbuscules. At 28 doi, intraradical hyphae formed a large number of arbuscules within the cells. At 42 doi, a large number of intercellular vesicles formed by the apical expansion of hyphae could be clearly observed. At 56 doi, a large number of both intraradical hyphae and intercellular vesicles were clearly observed. The mycorrhizal colonization rate of *Fm*-inoculated roots ranged from 2.93% at 7 doi to 41.54% at 56 doi, and the root length colonization rate increased with the increase of *Fm* inoculation time (Fig. 1b).

Changes in plant growth performance

The growth performance of trifoliate orange seedlings changed significantly with the extension of the days of *F. mosseae* inoculation (Fig. 2a). At 7–21 doi, plant height, taproot length, and shoot and root biomass did not differ significantly between *Fm*- and no-*Fm*-inoculated treatments (Fig. 2b–e). Starting from 28 doi, the growth performance in *Fm*-inoculated seedlings was better than that in no-*Fm*-inoculated seedlings. The plant height of *Fm*-inoculated seedlings was significantly higher than that of no-*Fm*-inoculated seedlings by 42.73%, 71.64%, and 84.21% at 28, 42, and 56 doi, respectively. Similarly, shoot biomass was increased by 64.71%, 68.00%, and 96.97% under *Fm*- versus no-*Fm*-inoculated treatment at 28, 42, and 56 doi, respectively, along with 54.55%, 81.25%, and 66.67% significantly higher root biomass in *Fm*-inoculated seedlings than no-*Fm*-inoculated seedlings, respectively. The taproot length of *Fm*-inoculated seedlings showed significant changes from 42 doi, increasing by 31.16% and 24.03% at 42 and 56 doi, respectively, compared with no-*Fm*-inoculated seedlings.

Changes in root H_2O_2 levels

Compared with no-*Fm* inoculation, H_2O_2 levels in roots of *Fm*-inoculated seedlings were significantly increased only at 7 and 14 doi by 63.47% and 34.73%, respectively, along with no significant difference at 21–56 doi (Fig. 3).

Changes in root antioxidant enzyme activities

Compared with no-*Fm* inoculation, *Fm* inoculation significantly increased root SOD activity at 7 and 56 doi by 60.00% and 29.38%, respectively (Fig. 4a). There was no significant difference in SOD activity between the two treatments from 14 doi to 42 doi. Root POD and CAT activities were significantly elevated at 7, 14, and 56 doi after *Fm* inoculation, with 69.46%, 43.26%, and 49.54% increase in POD activity and 199.22%, 93.89%, and 64.46% increase in CAT activity, respectively, plus no significant changes at 21–42 doi (Fig. 4b, c).

Changes in the expression of root antioxidant enzyme genes

Compared with no-*Fm* inoculation, *Fm* inoculation did not significantly affect root *PtFe-SOD* expression at 7–42 doi, but up-regulated root *PtFe-SOD* expression (1.46 folds) at 56 doi (Fig. 5a). *Fm* inoculation also up-regulated root *PtMn-SOD* expression at 7 and 14 doi by 2.43 and 1.78-fold, respectively, compared with no-*Fm* treatment, along with no significant difference at the subsequent 21–56 doi (Fig. 5b). *PtCu/Zn-SOD* expression just got significantly up-regulated (1.60-fold) at 7 doi by *Fm* versus no-*Fm* inoculation, plus no significant change at 14–56 doi (Fig. 5c). Compared with no-*Fm* inoculation, *Fm* inoculation distinctly up-regulated *PtPOD* expression at 7, 14, and 56 doi by 2.43-, 3.36-, and 1.85-fold, respectively, as well as *PtCAT* expression at 7, 14, and 56 doi by 4.06-, 3.46-, and 2.14-fold, respectively, accompanied by no significant difference at 21–42 doi (Fig. 5d, e).

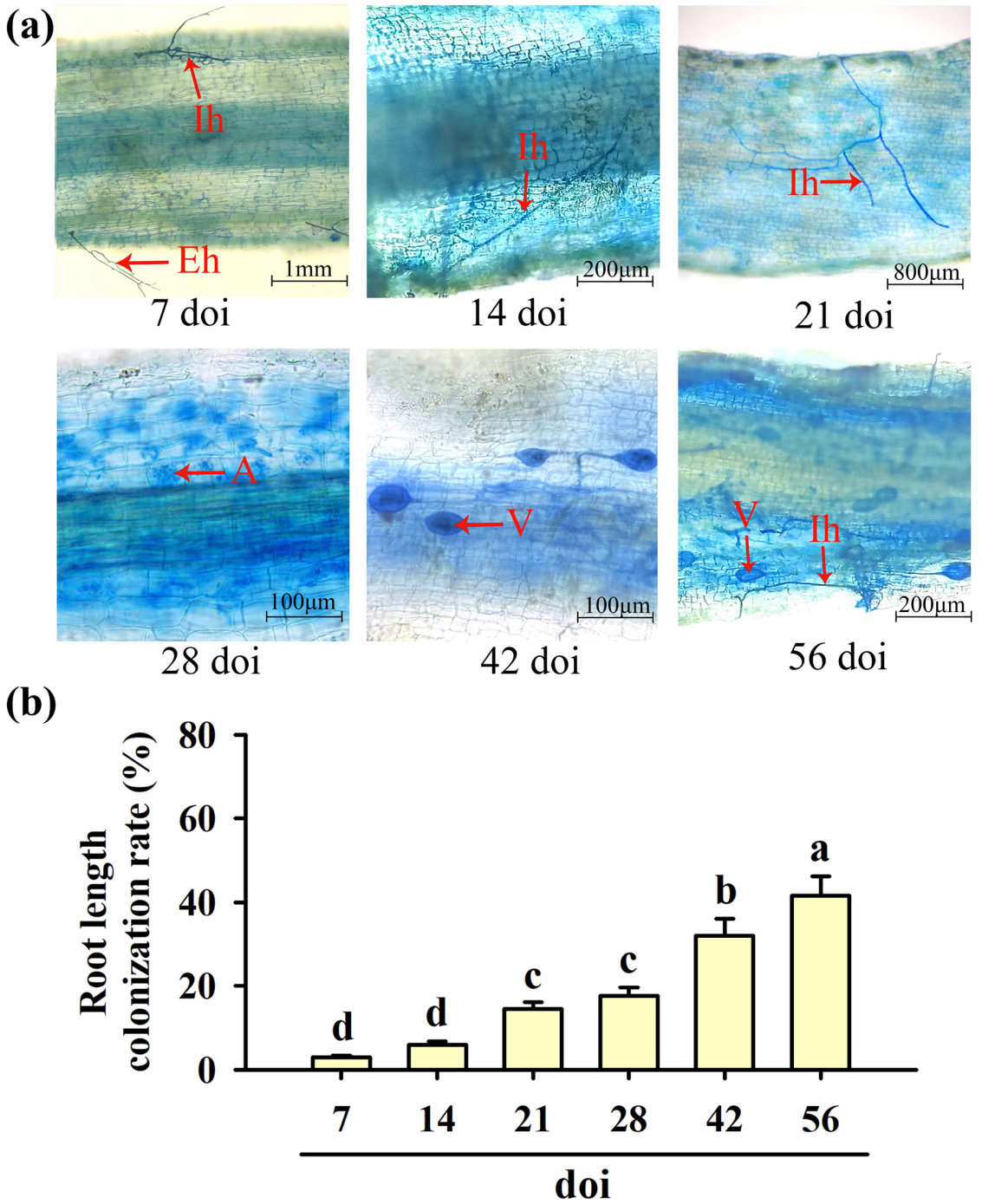


Fig. 1 The colonization process **a** and change in root length colonization rate **b** of *Funneliformis mosseae* in trifoliate orange seedlings. Data (means ± SD, $n = 4$) with different letters on the bar indicate significant ($p \leq 0.05$) differences between treatments. *A* arbuscule, *doi* days of inoculation, *Eh* extraradical hyphae, *Ih* intraradical hyphae, *V* vesicles

Changes in the expression of root *SWEET* and *STR* genes

The expression of root *PtSWEET* was not initiated by *Fm* inoculation at 7–21 doi, whereas the expression of root *PtSWEET* was up-regulated at 28, 42, and 56 doi by 1.50-, 2.00-, and 2.17-fold, respectively, compared with no-*Fm* inoculation (Fig. 6a). Similarly, at 7–21 doi, *Fm* inoculation also did not affect the expression

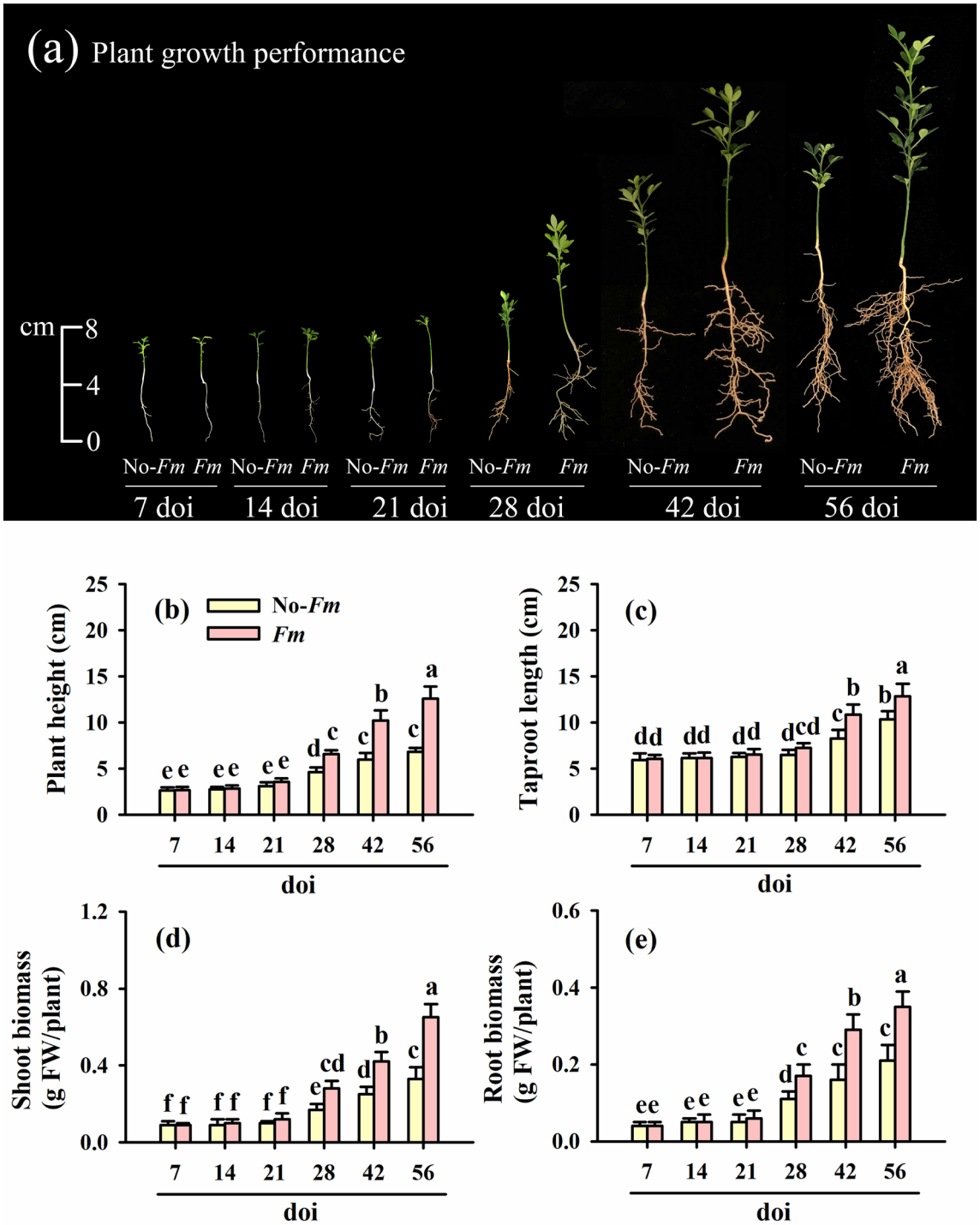


Fig. 2 Changes in plant growth performance (**a**), plant height (**b**), taproot length (**c**), shoot biomass (**d**), and root biomass (**e**) of trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 4$) with different letters on the bar indicate significant ($p \leq 0.05$) differences between treatments

of *PtSTR* and *PtSTR2* in roots (Fig. 6b, c). Starting from 28 doi, *PtSTR* expression was up-regulated by *Fm* inoculation by 1.33 folds at 28 doi, 1.45 folds at 42 doi, and 2.30 folds at 56 doi, respectively, compared with no-*Fm* inoculation. *PtSTR2* expression was up-regulated only at 42 and 56 doi under *Fm*- versus

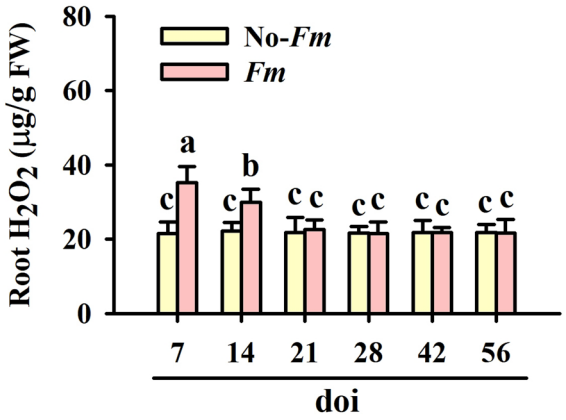


Fig. 3 Changes in H_2O_2 levels in roots of trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 4$) with different letters on the bar indicate significant ($p \le 0.05$) differences between treatments

no-*Fm*-inoculation conditions by 1.49- and 2.12-fold, respectively.

Changes in the expression of root *PT* genes

Fm inoculation did not significantly alter root *PtPT3* and *PtPT5* expression at 7–28 doi and root *PtPT6* expression at 7–21 doi (Fig. 7a–c). Root *PtPT3* and *PtPT5* expression was up-regulated by *Fm* inoculation by 2.46- and 2.30-fold at 42 doi and by 3.22- and 4.76-fold at 56 doi, respectively. Root *PtPT6* expression was up-regulated by *Fm* inoculation at 28, 42, and 56 doi by 1.59-, 1.86-, and 2.13-fold, respectively.

Discussion

The present study showed that *Fm* was able to contact roots of trifoliate orange and form appressorium at 7 doi, and the mycorrhizal colonization rate increased with time, reaching 41.54% at 56 doi. At 7–56 doi, roots of trifoliate orange went through four stages: the formation of appressorium at 7 doi, further expansion of hyphae within mycorrhizal roots at 14–21 doi, formation of arbuscules at 28 doi, and formation of vesicles and numerous intraradical hyphae at 42–56 doi. At 7–21 doi, *Fm* contacted with root surface to form colonization points and appressorium, penetrated epidermal cells into cortical cells to form intraradical hyphae, and then branched to form a developed hyphal network, which was consistent with the results of Sheng et al. [38] in *Pinellia ternata* plants. At 28 doi, the hyphae in roots branched continuously, forming arbuscules and filling the cell. Arbuscules are important sites for nutrient exchange between plant cells and AMF [13, 39, 40], where arbuscules are ensheathed by a host membrane, termed the periarbuscular membrane, which facilitates nutrient exchange [39, 41]. This indicates the functional initiation of arbuscular mycorrhizae in trifoliate orange at 28 doi. In general, the formation of arbuscules precedes the formation of vesicles in some *Glomus* species [42]. Therefore, we found that the apical expansion of hyphae formed intercellular vesicles at 42 doi. Vesicles contain lipid-like droplets that function as nutrient stores, and AMF can use the nutrients stored in the vesicles when mycorrhizal metabolism is reduced [43]. Subsequently there was a large number of intraradical hyphae as well as vesicles in roots at 56 doi, showing the maturation of arbuscular mycorrhizae.

AMF contribute to the growth and development of the host plant after forming a symbiosis in roots [44]. The present study showed that *Fm* inoculation produced a significantly positive effect on plant height ($r = 0.82$, $p < 0.01$), taproot length ($r = 0.70$, $p < 0.01$), shoot ($r = 0.79$, $p < 0.01$) and root biomass ($r = 0.75$, $p < 0.01$) starting from 28 doi (the stage of arbuscule formation), indicating that AMF colonization triggered a positive effect on plant growth of the host, in correlation with the formation of arbuscules in root cortical cells. In white clover, the positive effect of *Paraglomus occultum* on the improvement of shoot and root biomass also occurred at 20 doi,

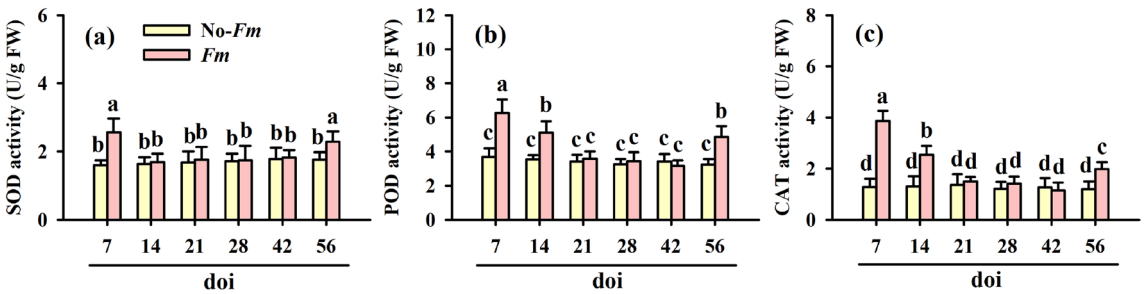


Fig. 4 Changes in superoxide dismutase (SOD) (**a**), peroxidase (POD) (**b**), and catalase (CAT) (**c**) activities in roots of trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 4$) with different letters on the bar indicate significant ($p \le 0.05$) differences between treatments

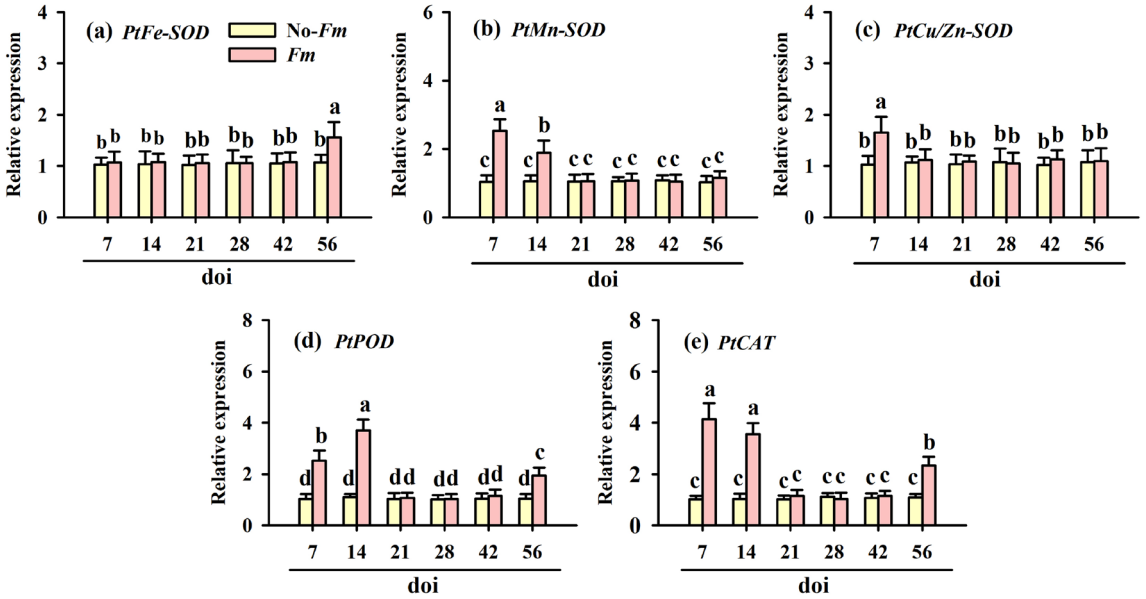


Fig. 5 Changes in relative expression of *PtFe-SOD* (**a**), *PtMn-SOD* (**b**), *PtCu/Zn-SOD* (**c**), *PtPOD* (**d**), and *PtCAT* (**e**) genes in roots of trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 3$) with different letters on the bar indicate significant ($p \leq 0.05$) differences between treatments

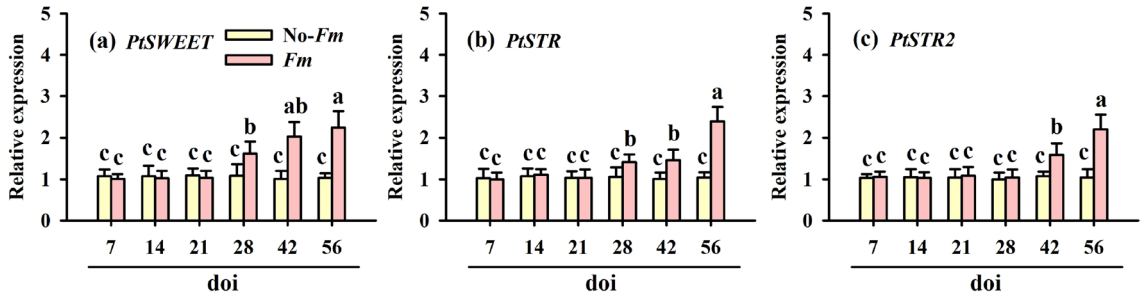


Fig. 6 Changes in relative expression of *PtSWEET* (**a**) and two fatty acid transporter genes (**b**, **c**) in roots of trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 3$) with different letters on the bar indicate significant ($p \leq 0.05$) differences between treatments

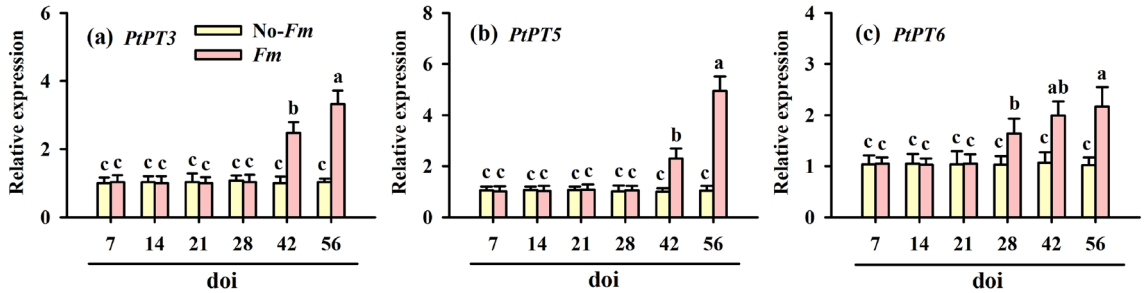


Fig. 7 Changes in relative expression of *PtPT3* (**a**), *PtPT5* (**b**), and *PtPT6* (**c**) genes in roots trifoliate orange seedlings after inoculation with *Funneliformis mosseae*. Data (means ± SD, $n = 3$) with different letters on the bar indicate significant ($p \leq 0.05$) differences between treatments

accompanied by the formation of arbuscules [13]. Similar improvement in host growth by AMF was also reported in sugarcane and drought-stressed trifoliate orange [4, 45]. The mycelium network formed by AMF expands the contact between plant roots and soil, thus promoting plant growth and development [46]. In contrast, before 28 doi, *Fm* inoculation did not significantly improve growth performance of trifoliate orange, because it takes some time for AMF to colonize the host plant and form a symbiotic relationship. It remains to be further determined whether the host supplies more photosynthetic products to root mycorrhizae for their growth in the early stage of mycorrhizal formation [47].

Plant H_2O_2 is dramatically increased after microbial infestation, which is a defense response of the host plant to microbial infestation [48]. The results of the present study showed that root H_2O_2 levels were significantly increased at 7 and 14 doi of *Fm* inoculation, and then no significant change started at 21 doi. This is in agreement with the findings of Song and Song [49] in alfalfa after root colonization of *Glomus intraradices*. Fester and Hause [43] also reported the increase in root H_2O_2 levels after inoculation of *Medicago truncatula* with *G. intraradices*, especially when the mycelium started to penetrate root cortical cells and during arbuscular formation. This suggests that roots of trifoliate orange initiated an immune defense response at 7–14 doi in response to *Fm* colonization through elevated H_2O_2 levels.

Antioxidant enzymes are activated as a defense system during the early stages of AMF colonization of host plants and then inactivated as the symbiosis continues to develop [15, 50, 51]. In this study, activities of root antioxidant enzymes (SOD, POD, and CAT) were significantly increased at the beginning of *Fm* inoculation (7 doi), while root POD and CAT activities continued to be elevated at 14 doi as well, which was consistent with changes in root H_2O_2 levels. Significantly elevated CAT and POD activities were also observed in alfalfa during root early colonization of AMF [49]. Interestingly, at 56 doi, *Fm* inoculation again significantly raised root SOD, POD, and CAT activities. Lokhandwala et al. [52] found in a meta-analysis that AMF inoculation increased antioxidant enzyme activities of host plants by 16%, regardless of stress or not. The increase in antioxidant enzyme activities is a response of plant immune defense in the early stages of AMF colonization [53]. In the later stages of root AMF colonization, AMF enhance the host's antioxidant capacity to resist oxidative damage [54]. In addition, the expression of stress-responsive genes is significantly up-regulated during the early stages of microbial infection of plants [55]. In the present study, the expression of *PtMn-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT* gene was distinctly increased at 7 and 14 doi following *Fm* colonization, further indicating that trifoliate orange recognized root colonization of *Fm* and activated defense responses, triggering the host plant to generate an immune response at the early stage of mycorrhizal fungal colonization [56, 57]. After being recognized as a beneficial fungus, the defense system was removed [49], and thus no change in the antioxidant defense system was found between inoculated versus uninoculated plants. Additionally, *PtFe-SOD*, *PtPOD*, and *PtCAT* expression was again up-regulated at 56 doi of *Fm*. This is in agreement with the findings of Li et al. [58] who reported that AMF inoculation resulted in up-regulation of *CsFe-SOD*, *CsPOD*, and *CsCAT* expression in field citrus, suggesting enhanced antioxidant potential of the host plant at the late stage of AMF colonization. In addition, in the early stage of root colonization of *Fm*, the response of different *PtSOD* genes to mycorrhizal colonization was variable, with *PtMn-SOD* and *PtCu/Zn-SOD* responding first and *PtFe-SOD* responding later. The intrinsic mechanism is not well defined. Van Camp et al. [59] also found that Fe-SOD was closely related to endosymbiont. At 7 doi, SOD activity in roots was significantly increased, along with the up-regulation expression of *PtMn-SOD* and *PtCu/Zn-SOD* and no change in *PtFe-SOD* expression. Similarly, at 56 doi, SOD activity in roots was significantly increased, along with the up-regulation expression of *PtFe-SOD*. This indicated that SOD activity changes under mycorrhizal inoculation conditions are associated with *PtSOD* gene species at different times. The inconsistent results in the enzyme activity and gene expression may be due to differences in transription and translation after gene expression and the distribution and functions of these SOD isoenzymes in plant organelles. Kim et al. [60] also proposed that the down-regulated expression of a *SOD* type can cause changes in the expression of other *SOD* types.

In addition, the establishment of symbiotic associations relies on bidirectional nutrient exchange, such as sugars, lipids, and *PT* gene expression [61]. This study showed that *PtSWEET* gene was significantly up-regulated from 28 doi of *Fm*, which was accompanied by the formation of arbuscules. This suggests that the host plant began providing sugars to the *Fm* at 28 doi. Arbuscules are sites of of nutrient exchange between plants and AMF [62]. Several *SWEET* genes were up-regulated in potato, alfalfa, and soybean with mycorrhizal formation [19, 22, 63], among which the expression of *SWEET1b* in alfalfa was up-regulated in arbuscule-containing cells [19]. The localization of *PtSWEET* protein in mycorrhizal root cells needs further study. AMF induce lipid synthesis in plants, and plants' lipids enter AMF through STR and STR2 proteins located in the periarbuscular membrane as the main carbon source of nutrients [27, 64, 65]. This study

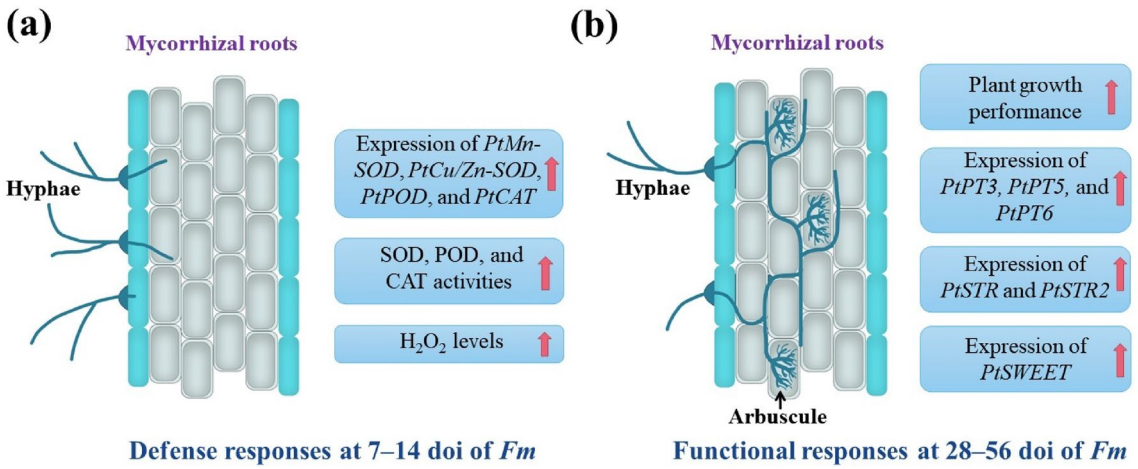


Fig. 8 A model diagram regarding root responses to AMF colonization at 7—56 doi. Here, **a** showed the initiation of root antioxidant defense in response to AMF colonization at 7—14 doi; **b** indicated the initiation of *SWEETs*, *PTs*, and *STRs* at 28—56 doi, after arbuscule formation, possibly accompanied by nutrient exchange between the two partners

showed that *PtSTR* expression was up-regulated from 28 doi, while *PtSTR2* was up-regulated from 42 doi, implying that the host has supplied fungal partners with lipids at 28 doi, of which *PtSTR* was preferentially initiated.

An important function of mycorrhizal mycorrhizae is to up-regulate the expression of host *PT* genes to promote P uptake by the host [66]. For example, *StPT3* in potato, *MtPT4* in alfalfa, and *OsPT11* in rice were identified to import phosphate released by AMF from the symbiotic interface into plant cells to increase plant P levels [67, 68]. In this study, *PtPT6* expression was up-regulated in *Fm*-inoculated plants from 28 doi, while *PtPT3* and *PtPT5* started to be up-regulated only at 42 doi, implying that the mycorrhiza traveled to promote host P uptake at this time, accompanied by the preferential initiation of *PtPT6*. Mycorrhizal extraradical hyphae take up soil inorganic phosphate and transport it within the intraradical hyphae as polyphosphate particles, which are hydrolyzed upon arrival at the fungus-root cell interface (arbuscules) and translocated within the plant as $H_2PO_4^-$ [69]. P exchange occurs during arbuscular formation, so that the response of *PtPTs* expression was initiated only after 28 doi.

Conclusion

During root colonization of *Fm* in trifoliate orange, root defense systems (such as H_2O_2, SOD, POD, and CAT and their corresponding encoding genes) were initiated at 7–14 doi and subsequently maintained unchanged, compared to no-*Fm* (Fig. 8a). At 28 doi, massive formation of arbuscule in the roots was accompanied by growth improvement, up-regulated expression of *PtSWEET* and *PtSTR* and subsequent up-regulated expression of *PtPTs* (Fig. 8b), suggesting the initiation of bidirectional nutrient functions. These results reveal the defense response of the host plant to mycorrhizal fungal colonization and the establishment of a symbiotic association, and also provide a clear understanding of the exchange of nutrients between AMF and the host plant.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00526-0.

Additional file 1: Table S1. Specific primer sequences of genes used for qRT-PCR.

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Author contributions

XQL and ZL conducted the experiment and made data curation. YNZ and QSW designed the experiment. XQL wrote the original manuscript. QSW and MDA revised the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interest regarding the publication of this work.

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