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Arbuscular mycorrhizal fungi enhanced drought resistance in apple by regulating genes in the MAPK pathway

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) can form a symbiotic relationships with most terrestrial plants and play an important role in plant growth and adaptation to various stresses. To study the role of AMF in regulating drought resistance in apple, the effects of drought stress on *Malus hupehensis* inoculated with AMF were investigated. Inoculation of AMF enhanced apple plants growth. Mycorrhizal plants had higher total chlorophyll concentrations but lower relative electrolyte leakage under drought stress. Mycorrhizal plants increased net photosynthetic rate, stomatal conductance, and transpiration rate under drought stress, however, they showed lower inhibition in the quantum yield of PSII photochemistry. Mycorrhizal plants also had higher superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) enzyme activities under drought conditions. Thus, mycorrhizal plants had lower accumulated MDA, H_2O_2, and O_2^- than non-mycorrhizal seedlings. Total sugar and proline concentrations also significantly increased, helping maintain the osmotic balance. Furthermore, mitogen-activated protein kinase (MAPK) cascades, which participate in the regulation of responses of plants and microorganisms to biotic and abiotic stress, were up-regulated in apple plants and AMF during drought. We saw that there were at least two motifs that were identical in MAPK proteins and many elements that responded to hormones and stress from these *MAPK* genes. In summary, our results showed that mycorrhizal colonization enhanced apple drought tolerance by improving gas exchange capacity, increasing chlorophyll fluorescence parameters, creating a greater osmotic adjustment capacity, increasing scavenging of reactive oxygen species (ROS), and using MAPK signals for interactions between AMF and their apple plant hosts.

1. Introduction

Plants are sessile organisms that are regularly exposed to a wide range of environmental stressors such as pathogens, high salinity, drought, and nutrient imbalances, all of which can have adverse effects on plant survival, development, and productivity (Albacete et al., 2014; Ruiz-Lozano et al., 2016). In recent years, of all the environmental stresses, drought has been identified as a key factor that limits plant distribution, growth, and productivity (Ahuja et al., 2010), and as the factor that is most exacerbated by global climate change (Trenberth et al., 2013). Drought can have many effects on plants, such as inhibiting photosynthesis, disrupting water and nutrient relations, and mechanically damaging protoplasts, all of which will lead to decreased growth (Talbi et al., 2015). However, the severity of a drought depends on many different factors, which include the occurrence and

distribution rain, evaporative demands, and water storage in soils (Farooq et al., 2009). Therefore, it is often beneficial to apply targeted agricultural techniques that improve the resistance of plants to drought stress in drought affected geographic areas, and one such technique is the application of arbuscular mycorrhizal fungi (AMF) (Huang et al., 2010).

AMF form beneficial symbiotic relationships with more than 80% of terrestrial plants, including most agricultural and horticultural crop species (Barea et al., 2005; Bucher et al., 2014). AMF are able to pass through the epidermal cells of the root system of a host plant and form branched structures called arbuscules and vesicles. Through this reciprocal symbiotic relationship, the extraradical mycelium of the AM fungal symbionts have the ability to uptake and transfer of water and nutrients to the host plant. Therefore, mycorrhizal plants generally show better nutrient uptake and tolerance against biotic and abiotic

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stressors (Pozo et al., 2015; Hashem et al., 2016). Improving plant drought resistance by inoculating plants with AMF is a well-known agricultural technique (Jayne and Quigley, 2014; Li et al., 2014; Chitarra et al., 2016).

The effect of arbuscular mycorrhizal fungi on the resistance of plants to drought is very complex and includes many metabolites and metabolic pathways (Wu and Xia, 2006). Arbuscular mycorrhizal symbiosis improves plant drought adaptation by up-regulating and down-regulating various physiological and biochemical processes, which have commonly included: the promotion of plant nutrient and water uptake and transport, increased plant osmotic regulation, induced hormone signaling responses, improved gas exchange capacity and water use efficiency, and enhanced antioxidant capacity (Smith et al., 2009; Yang et al., 2014; Huang et al., 2010). In most cases studies, the positive effects of AMF symbiosis on plant tolerance to drought have been described by reporting plant performance, however, the underlying molecular mechanisms generally have yet to be elucidated (Li et al., 2015; Mo et al., 2016; Ruiz-Lozano et al., 2016).

Fortunately, to cope with drought stress, plants have evolved a wide spectrum of adaptive mechanisms (Osakabe et al., 2014). Plants sense and transduce signals that can lead to changes in gene expression, which in turn regulate the corresponding metabolic pathways (Umezawa et al., 2006). A series of rapid perception and active adaptation mechanisms have evolved which can circumvent and/or alleviate damage to plants exposed to stressors. Mitogen-activated protein kinases (MAPK) are serine/threonine protein kinases that are activated by extracellular stimulation through the MAPK cascade reaction (MAPKKK-MAPKK-MAPK) (Colcombet and Hirt, 2008). This pathway plays an important role in the responses of plants to biotic and abiotic stress and in hormonal signal transduction (Pitzschke et al., 2009). Recently, with the sequencing of numerous horticultural crop genomes, numerous members of the MAPK cascade pathway gene family have been identified. At the same time, by using RNA interference (RNAi) technology, virus-induced gene silencing (VIGS) technology, and the insight from gene function-acquired and gene-deficient mutants, the functions of the MAPK cascade in hormone signal transduction and plant stress responses have been determined. Research has shown that in *Arabidopsis*, drought stress rapidly induced the expression of *AtMPK3* and *AtMEKK1* (Huang et al., 2000). Overexpression of *OsMAPK5* positively affected responses to drought, salt stress, and cold stress in rice (*Oryza sativa* L.) (Xiong and Yang, 2003). In *Malus hupehensis* Rehd, the expression of *MhMAPK* was induced when treated with 20% polyethylene glycol and 200 mM NaCl (Duan et al., 2008). However, *MAPK* genes in apple plants undergoing drought stress requires further study.

In recent reports on the transcriptome from the fungus *Rhizophagus irregularis*, the various MAPK signaling components have been identified (Tisserant et al., 2013), which allows us to investigate MAPK in AMF. At the same time, because the signaling pathway in yeast is highly conserved, studies of MAPK in yeast can also help us to understand MAPK in fungi (Liu et al., 2015). In free-living mycelia, fruiting bodies, and ectomycorrhiza, MAPK genes were highly expressed, which indicated that these genes contributed to the symbiotic relationship (Chen and Thorner, 2007; Hamel et al., 2012; Martin et al., 2008).

Apples are one of the most widely cultivated fruits in temperate regions. The land used for apple orchards and production in China is the largest of any country worldwide (Sun et al., 2018; Li et al., 2018). *M. hupehensis* Rehd is a valuable apple rootstock in China, and because of its well-developed roots and apomictic characteristics it is an excellent species for research on roots of fruit trees (Duan et al., 2008). However, there are relatively few studies into how the MAPK genes in AMF and their hosts coordinate to improve stress resistance. Here, using a multidisciplinary approach that focused on photosynthesis, antioxidant systems, osmotic adjustment, expression of stress-response genes, and AMF MAPKs, we characterized plant response to drought stress during mycorrhization process. Overall, the experimental results showed that AMF symbiosis positively affected drought tolerance in this apple species.

2. Materials and methods

2.1. Plant material and experimental treatments

The treatments were conducted in a greenhouse at Northwest A & F University, Yangling, (34°20′N, 108°24′E) Shaanxi Province, China. Seeds of *Malus hupehensis* were stratified in sterilized sand for 50 d at 4 °C. After germination, seedlings were planted in plastic pots (12 cm × 12 cm) filled with a sterilized mixture of one part substrate and two parts sand (sterilized by steaming at 121 °C for 2 h). The amounts of available N, P, and K in the soil mixture were 63.38, 53.63, and 325 mg kg^{-1}, respectively, the pH was 6.0 and the water field capacity was 50.97%. The AMF used in this study was *R. irregularis*, which was provided by Beijing Academy of Agricultural and Forestry Sciences. The inoculant was a mixture of spores, hyphae, colonized root segments, and sand. When the seedlings were planted, half the seedlings were inoculated with AMF, and the other half were inoculated with an equivalent weight of sterilized AMF. Seedlings were then placed in the greenhouse (the relative humidity was 65–95%, and the daytime and nighttime temperatures were 25–30 °C and 16–20 °C, respectively). Before the start of the stress treatment, all *M. hupehensis* seedlings were watered with 100 mL 1/2 strength Hoagland's solution (pH 6.0) once a week. Four treatments were established in our experiments, these were created by crossing two water conditions, well-watered (WW) and drought stress (DS), with two inoculations, AMF (M) inoculated and not inoculated (NM). The four treatments were: (I) well-watered seedlings without AMF inoculation (WW + NM); (II) well-watered seedlings inoculated with the AM fungus *R. irregularis* (WW + M); (III) drought-stressed seedlings without AMF inoculation (DS + NM); and (IV) drought-stressed seedlings with the AM fungus *R. irregularis* inoculation (DS + M). The drought stress treatment began 60 d after seedlings had been planted in pots. At the beginning of the treatment, all the groups were watered thoroughly, the well-watered groups were at 65%–75% field capacity, while water was withheld from the drought-stressed groups for 8 days. Fifty seedlings were assigned to each treatment combination of water amount and AMF inoculation.

2.2. Observation of mycorrhizal colonization and determination of growth indices

The mycorrhizal colonization rate was determined using the methods described by Koske and Gemma (1989) and He et al. (2016) with minor modifications. Root samples of five seedlings were selected at random and washed five times with tap water, then cut into small pieces of about 1 cm, soaked in 10% KOH solution, heated at 90 °C for 60 min, rinsed with tap water three times, and then soaked in 10% H_2O_2 for 10 min to soften the roots, rinsed with tap water three times, soaked in 2% HCL for 30 min, stained with 0.05% trypan blue at 90 °C for 20 min, and finally destained with lactic acid–glycerin (lactic acid/glycerin/water 1:1:1, v/v/v) at room temperature. One hundred root samples were randomly selected for compression and placed under an Olympus microscope to calculate the mycorrhizal colonization rate. AM colonization (%) was calculated as root length infected divided by root length observed × 100 (Wu and Xia, 2006).

At the end of the treatment period, seedling height was measured from the base of the root to the terminal bud and the root length was measured. All seedlings were washed with tap water, 0.1 mol L^{-1} HCL, and finally with distilled water. Seedlings were dried in an oven at 65 °C until they reached a constant weight, once dry, the shoots and roots were weighed. The root/shoot ratio was the ratio of root dry weight over shoot dry weight.

2.3. Determination of photosynthetic and chlorophyll fluorescence parameters

At the conclusion of the experiment, the photosynthetic parameters of seedlings under different treatments were measured using a portable photosynthetic measurement system (Li6400; LICOR, Huntington Beach, CA, USA) from 9:00 to 11:00 a.m. on a cloudless sunny day. From each treatment, 10 fully developed mature leaves from different seedlings were selected to measure the photosynthetic parameters, which included net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and iWUE (instantaneous water use efficiency). We preheated the instruments completely before use and set the parameters as follows: constant airflow rate, 500 $\mu mol\ s^{-1}$; photons, 1000 $\mu mol\ m^{-2}\ s^{-1}$; and cuvette CO_2 concentration, 400 μmol $CO_2\ mol^{-1}$ air.

A PAM-2500 portable chlorophyll fluorimeter (Walz Company, Germany) connected with PamWin-3 software was used to determine the chlorophyll fluorescence-related parameters of the apple seedlings under different treatments. We determined maximum fluorescence yield (Fm) and minimum fluorescence yield (Fo) of leaves ensuring that all leaves from each treatment were in same position and had adapted fully to dark for 30 min prior to measurement. Other chlorophyll fluorescence parameters, such as the maximum quantum yield of PSII (Fv/Fm), photochemical quenching coefficient (qP), PSII actual quantum yield (ΦPSII), non-photochemical quenching coefficient (NPQ), and the ratio of electron transport at PSII (ETR) were calculated automatically by the software.

2.4. Determination of total chlorophyll content and relative electrolyte leakage

After photosynthetic and fluorescence parameters were determined, the total chlorophyll concentration in the leaves of each treatment was determined. Fresh leaf samples (exactly 0.1 g each) were cut into pieces and immediately placed into 8 mL 80% acetone solution and soaked for about 24 h in dark until they were completely decolorized and turned white after shaking 2–3 times. Then, the absorbance of the extraction solution was determined at 663 nm and 645 nm using a UV-1750 ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan). Total chlorophyll content was calculated according to the method described by Arnon (1949). Relative electrolyte leakage (REL) was determined according to the method described by Tan et al. (2017).

2.5. Determination of active substances

Frozen leaf tissue (0.1 g) was ground into a 1 mL extraction solution containing 0.3% TBA thiobarbituric acid (TBA) and 10% trichloroacetic acid (TCA). Malondialdehyde (MDA) concentration was determined using the TBA reaction method, as described previously (Wang et al., 2012). For H_2O_2, frozen leaf tissue (0.1 g) was ground with 1 mL chilled acetone and measured following the method of Patterson et al. (1984). For the rate of O_2^- generation, frozen leaf tissue (0.1 g) was ground with 1 mL 65 mM phosphate buffer solution (PBS) (pH 7.8) and measured according to the method described by Wang et al. (2012). For proline, frozen leaf tissue (0.1 g) was ground with 3% sulfosalicylic acid solution and measured according to the method described by Chołuj et al. (2008). For soluble sugar, frozen leaf tissue (0.1 g) was ground with 1 mL 75% methanol and determined following the method described by Shi et al. (2017).

2.6. Determination of antioxidant enzyme activity

Frozen leaf tissue (0.1 g) was ground with a 1 mL chilled buffer containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM ethylene diamine tetraacetic acid (EDTA), 0.3% Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 12000 g for 20 min at 4 °C and the supernatant was used for the enzyme assays. Activities of CAT, SOD, and POD were measured according to the methods described by Wang et al. (2012).

2.7. Analysis of gene expression

Eight weeks after inoculation (or not) and different water treatments, the root and leaf samples were ground completely in liquid nitrogen. Total RNA was extracted using a Wolact® Plant RNA Isolation kit following the manufacturer's protocol, then 2 μg RNA was used to synthesize the first-strand cDNA. For the qRT-PCR assay, 1 μg of total RNA was used for reverse-transcription, which was followed by PCR-amplification using 1 μL of the product. The reaction system used 20 μL for the qRT-PCR assay included 10 μL of SYBR® Premix Ex Taq™ (TaKaRa) and the use of a LightCycler® 96 instrument (Roche, Switzerland). Primers used in our experiments for qRT-PCR are listed in Supplementary Table 1. The RT-PCR amplification program was as follows: 95 °C for 3 min; 40 cycles at 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 15 s; followed by 72 °C for 3 min and then 81 cycles of 7 s increasing from 55 °C to 95 °C by 0.5 °C each cycle. Three biological replicates were used for all genetic analyses, and MdMDH or GiSR4 were used as the endogenous control to calculate △Ct values (Perini et al., 2014; Tian et al., 2010). Relative quantification values of different genes were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001), and the primer amplification specificity was determined using dissociation curve analysis.

2.8. Sequence analysis of MAPK genes in apple plants and AMF

Phylogenetic trees were constructed with the MEGA6 program (Dong et al., 2018) using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) with Poisson corrections and a bootstrap analysis with 1000 replications. Full-length protein sequences of apple and R. irregularis were obtained from the research described by Zhang et al. (2013) and Liu et al. (2015), respectively. Amino acid sequences of MAPKS were also used to analyze the motif distribution using an online web-site designed for that purpose (http://meme-suite.org/tools/meme) (Supplementary 2).

2.9. Prediction of cis-acting elements in promoters

Upstream regions (1500 bp upstream of the translational start codons) of the selected MdMAPKs were obtained from the apple genome (GDDH13 V1.1) (GDR; http://www.rosaceae.org/) and used to identify putative cis-acting elements (Plant CARE database; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Wang et al., 2017) (Supplementary 3).

2.10. Statistical analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the experimental data, independent-sample T test and one-way analysis of variance (ANOVA) ($P < 0.05$) were used to compare the differences among treatments. A two-way ANOVA was used (i.e., modeled as 'watering', 'inoculation', and 'watering × inoculation') to confirm whether the effects of watering and inoculation, or their interaction, had any significant influence on the results. All data were reported as means ± standard deviation (SD).

3. Results

3.1. AM root colonization and plant growth under different conditions

After 8 weeks, no colonization was detected in the non-inoculated seedlings. In the inoculated plants, AMF and M. hupehensis had established a symbiotic relationship with a colonization rate of > 60%
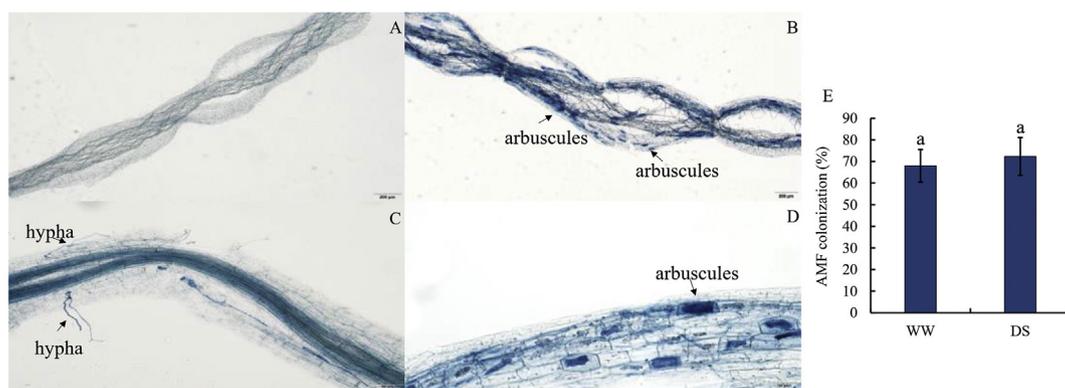
Fig. 1. The development of *R. irregularis* in *M. hupehensis* seedling roots. (A) The root of an uninoculated plant (bars 200 μm). (B) The root of an inoculated plant (bars 200 μm). (C) The hypha in the root of an inoculated plant (bars 50 μm). (D) The arbuscules in the root of an inoculated plant (bars 50 μm). (E) Colonization rate of mycorrhizal *M. hupehensis* seedlings, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on an independent-sample T test. Error bars represent three biological replicates.

Table 1
Plant biomass data of well-watered *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered. Letters indicate significant differences between treatments at $P < 0.05$, based on an independent-sample T test. Data are shown as mean values of five replicates ± SD.

Treatments	Plant height	Root length	Dry weight(g)		Root/shoot ratio
	(cm)	(cm)	Shoot	Root	
WW + NM	7.30 ± 0.34b	5.52 ± 0.36b	0.12 ± 0.01b	0.0064 ± 0.00048b	0.0545 ± 0.01a
WW + M	8.64 ± 0.24a	7.60 ± 0.32a	0.15 ± 0.01a	0.0089 ± 0.00038a	0.0589 ± 0.01a

(Fig. 1). After the AMF inoculation, numerous hyphae could be observed in the epidermal and cortical cells of the mycorrhizal *M. hupehensis* seedling roots, and typical arbuscules could also be found in most cortex cells (Fig. 1). In our experiments, under well-watered conditions, significant differences were observed in plant height, root length, and dry weight (Table 1). However, there was no significant difference in plant growth between the WW seedlings and DS seedlings (data not shown), which was likely due to the relatively short exposure to drought conditions.

3.2. Determination of gas exchange and chlorophyll fluorescence parameters

Under WW conditions, no significant changes in gas exchange or fluorescence were observed (Table 2). Under DS conditions, Pn, Gs, Tr, Fv/Fm, PSII, ETR, and qP of the mycorrhizal seedlings were 51.12%, 66.67%, 87.01%, 5.80%, 28.95%, 18.79%, and 14.29% higher, respectively, compared with non-mycorrhizal seedlings. However, DS conditions led to decreases in iWUE and NPQ (18.92% and 41.41%,

respectively) in the M group compared with the NM group. Under drought stress, only Gs was not significantly different between inoculated and non-inoculated seedlings. A two-way ANOVA revealed that interactions between watering and inoculation had significant interactions for Pn, Gs, Tr, PSII, ETR, qP, and NPQ ($P < 0.05$).
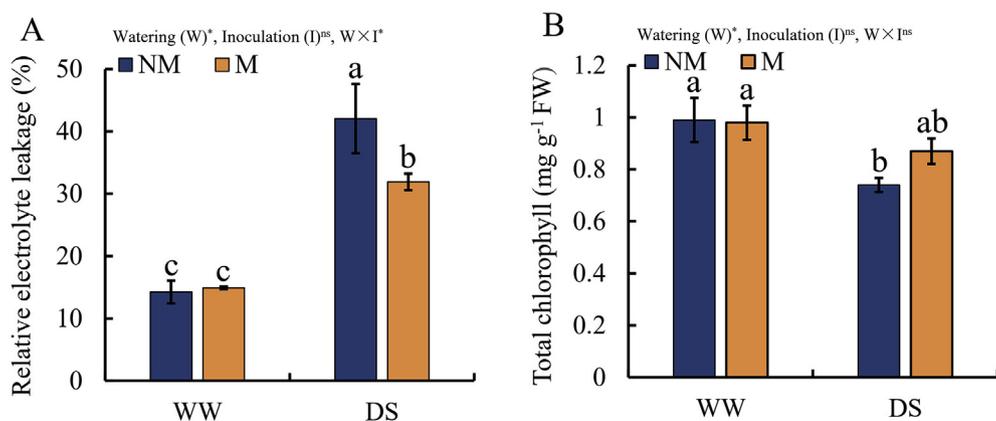
3.3. Determination of REL and total chlorophyll content

Between WW + NM and WW + M conditions, no significant difference in REL was observed, the REL values were 14.25% and 14.92%, respectively (Fig. 2). Drought stress induced an increase in REL in leaves of both inoculated and non-inoculated seedlings. However, of the DS groups, the REL value of the NM seedlings was significantly higher than that of M seedlings. Two-way ANOVA showed that interactions between watering and inoculation had significant interactions for leaf REL. Total chlorophyll concentrations of seedling leaves in the WW treatments were higher than in the DS treatments, regardless of whether they were inoculated or not. Under WW conditions, the total chlorophyll concentration of inoculated and non-inoculated seedlings

Table 2
Leaf gas exchange parameters and leaf chlorophyll fluorescence parameters of *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; *, $P < 0.05$. Data are shown as mean values of four replicates ± SD.

Treatments	Net photosynthesis rate Pn (μmol m^{-2} s^{-1})	Stomatal conductance Gs (mol m^{-2} s^{-1})	Transpiration rate Tr (mmol m^{-2} s^{-1})	iWUE (μmol mmol^{-1})	Maximum fluorescence efficiency Fv/Fm	Actual photosystem II efficiency ΦPSII	Electron transfer rate ETR	Photochemical quenching coefficient qP	Non-photochemical quenching coefficient NPQ
WW + NM	7.51 ± 0.34a	0.12 ± 0.005a	2.55 ± 0.07a	2.95 ± 0.20b	0.74 ± 0.01a	0.59 ± 0.02a	59.5 ± 1.73a	0.91 ± 0.02a	0.3 ± 0.04c
WW + M	7.94 ± 0.09a	0.11 ± 0.018a	2.73 ± 0.35a	2.94 ± 0.35b	0.74 ± 0.02a	0.57 ± 0.04a	58.5 ± 2.38a	0.89 ± 0.04a	0.36 ± 0.03c
DS + NM	3.58 ± 0.85c	0.03 ± 0.009b	0.77 ± 0.13c	4.65 ± 0.54a	0.69 ± 0.01b	0.38 ± 0.02c	41.25 ± 5.56c	0.7 ± 0.04c	0.99 ± 0.06a
DS + M	5.41 ± 0.30b	0.05 ± 0.011b	1.45 ± 0.14b	3.77 ± 0.47b	0.73 ± 0.01a	0.49 ± 0.02b	49 ± 1.83b	0.8 ± 0.03b	0.58 ± 0.02b
Significance									
Watering	*	*	*	*	*	*	*	*	*
Inoculation	*	ns	*	ns	*	*	ns	*	*
W × I	*	*	*	ns	ns	*	*	*	*

Fig. 2. (A) REL and (B) total chlorophyll content of leaves *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; $*$, $P < 0.05$. Error bars represent three biological replicates.

were 0.99 and 0.98 mg g^{-1} FW, respectively, and not significantly different. However, the total chlorophyll concentration of leaves from M plants (0.87 mg g^{-1} FW) was 17.57% higher than that of leaves from NM plants (0.74 mg g^{-1} FW) under drought stress conditions. A two-way ANOVA showed that interactions between watering and inoculation had no significant interactions for total chlorophyll concentration in leaves.

3.4. Determination of active substances

Under well-watered conditions, MDA, $O_2{}^-$, and H_2O_2 concentrations in seedling leaves remained at low levels in both M and NM treatments and were not significantly different (Fig. 3). Under drought conditions, however, the accumulation of ROS and the MDA content in the apple seedlings increased. In the DS treatments, AM inoculation significantly reduced MDA concentrations by 14.30%, but no significant difference was observed in H_2O_2 and $O_2{}^-$. A two-way ANOVA showed that interactions between watering and inoculation had no significant interactions for H_2O_2, $O_2{}^-$, and MDA concentrations in leaves.

3.5. Total soluble sugar and proline concentrations

Concentrations of total sugar and proline showed similar trends to H_2O_2, $O_2{}^-$, and MDA concentrations in leaves under different treatments (Fig. 4). Drought stress led to the accumulation of total sugar and proline in leaves, and the highest levels were found in the DS + M treatment. Total sugar and proline concentrations in leaves under DS + M conditions were 121.68 mg g^{-1} FW and 0.28 μmol g^{-1} FW, respectively, which were 20.57% and 28.27% higher, respectively, than in the DS + NM treatment. Significant differences were found between DS + M and DS + NM in both total sugar and proline concentrations of

leaves. A two-way ANOVA showed that watering and inoculation had a significant interaction with total sugar concentration, but had no significant interaction with proline concentration in leaves.

3.6. Determination of antioxidant enzyme activity

Under WW conditions, CAT, POD, and SOD activity in the mycorrhizal seedlings were enhanced by 36.57%, 87.39%, and 5.85%, respectively, relative to NM conditions (Fig. 5). Under DS conditions, the CAT activity of non-mycorrhizal and AM inoculated seedlings were 416.80 nmol min^{-1}g^{-1} FW and 423.39 nmol min^{-1} g^{-1} FW, respectively, which were not significantly different. However, the POD and SOD activities of the DS + M treatment, 678.34 U g^{-1} FW and 276.78 U g^{-1} FW, respectively, were significantly higher than those of the DS + NM treatment. A two-way ANOVA showed that watering and inoculation had significant interactions with POD and SOD activities, but had no significant interaction with CAT activity in leaves.

3.7. Relative expression of stress-response genes

To better understand the molecular regulatory mechanisms of AMF in seedlings, a series of stress-response genes of apples were analyzed using qRT-PCR, these included genes involved in chlorophyll degradation (*PAO*), genes encoding the key enzyme for the synthesis of proline (*P5CS*), and aquaporin genes (Fig. 6). There were no significant changes in the expression levels between WW + NM or WW + M conditions. However, under drought stress, the expression of *MdPAO* was highly up-regulated, with increases in the DS + NM treatment that were 30.54% higher than in the DS + M treatment. Expressions of *P5CS*, *PIP1-3*, and *PIP1-4* in mycorrhizal plants were increased by 65.15%, 103.67%, and 93.13%, respectively, compared with non-mycorrhizal plants. A two-way ANOVA showed that watering and inoculation had a
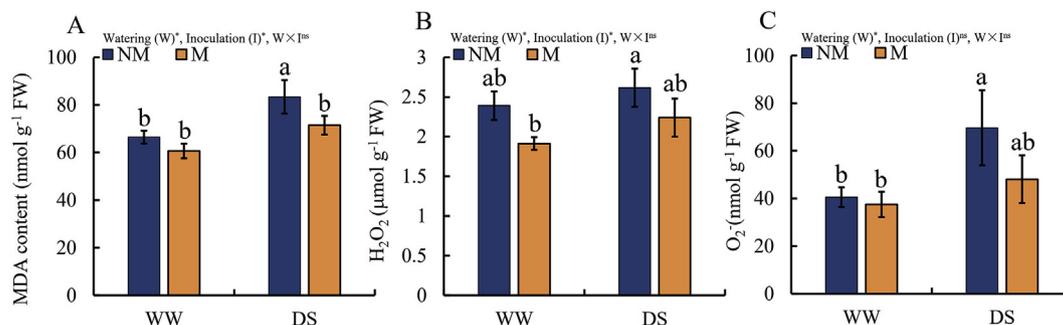
Fig. 3. (A) Malondialdehyde (MDA) concentration, (B) hydrogen peroxide (H_2O_2) concentration, and (C) superoxide anion radical ($O_2{}^-$ concentration) in leaves of *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; $*$, $P < 0.05$. Data are shown as mean ± standard deviation (n = 3). Error bars represent three biological replicates.
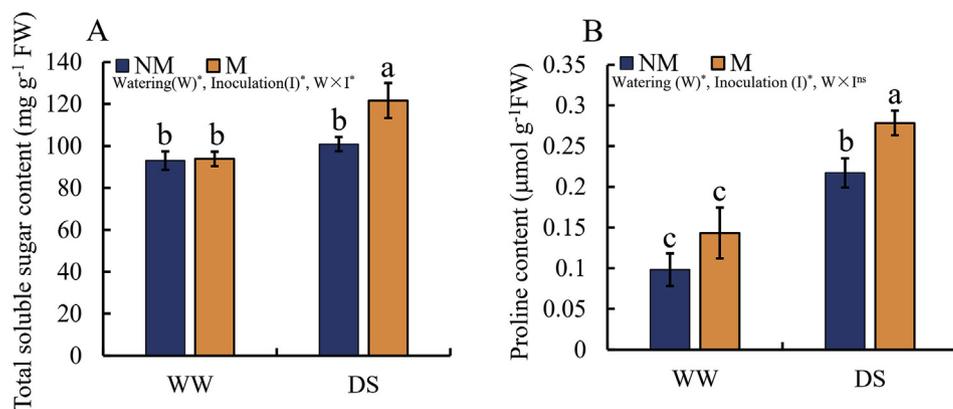
Fig. 4. (A) Total soluble sugar content, and (B) proline content in leaves of *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters that follow the values indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; ∗, $P < 0.05$. Data are shown as mean ± standard deviation (n = 3). Error bars represent three biological replicates.

significant interaction in regards to the expression of *P5CS*, *PIP1-3*, and *PIP1-4*. We also analyzed the expression patterns of *Rir-AQP1* and *Rir-AQP2* in mycorrhizal plants under DS conditions and the expression levels of these two genes were highly up-regulated.

3.8. Phylogenetic relationships and conserved motif analysis

To study the characteristics of *MAPK* genes in apple and AMF, a phylogenetic tree was constructed, which included 19 entries from the apple group and five entries of *R. irregularis* (Fig. 7). MAPK genes were divided into four groups. We identified common motifs within apple and *R. irregularis* MAPK proteins using the Multiple Expectation maximization for Motif Elicitation (MEME) web server (http://meme-suite.org/tools/meme). In the first group, four contained nine motifs, but motif I was not present in the remaining four members. In group II, all contained 8 motifs, but motif IX was not present in these members. In group III, three members contained seven motifs, and only one contained six. In group IV, only *RirMAPK5* contained motif V and VI. There were at least two motifs that were identical in the MAPK genes of apple and *R. irregularis*.

3.9. The expression of MAPK genes during regulation of mycorrhizal symbiosis in response to drought stress

We analyzed the expression patterns of *MAPKs* in apple seedlings and AMF under well-watered and drought-stress conditions. Between them, there were significant changes in the expression levels of eight genes (Fig. 8). For *MdMAPKs*, no significant changes were observed in either WW treatment, however, their expression levels increased in both DS + NM and DS + M treatments. In addition, we observed significant changes in the expression levels of four *MdMAPKs* in mycorrhizal *M. hupehensis* seedlings compared with non-mycorrhizal *M. hupehensis* seedlings. Comparing the two drought treatments, the

expression levels of *MdMAPK16-2*, *MdMAPK17*, and *MdMAPK20-1* in the mycorrhizal plants were enhanced by 36.93%, 58.14%, and 54.14%, respectively. For *RirMAPKs*, expressions of *RirMAPK1* and *RirMAPK3* under drought stress were enhanced by 130.84% and 64.90%, respectively, compared with well-watered conditions. A two-way ANOVA showed that watering and inoculation had significant interactions with the expression of *MdMAPK7-1*, *MdMAPK13-1*, *MdMAPK16-2*, *MdMAPK17*, and *MdMAPK20-1*.

3.10. Analysis of promoter cis-elements of MAPK genes in apple

Next, we analyzed the promoter sequences of these six genes. Promoter cis-regulatory elements are of great significance in regulating gene expression. In this study, putative cis-elements within the *MdMAPK* (< 1.5 kb upstream from the start site of putative translation) were identified using the PlantCARE database (Fig. 9). We found many elements that are responsive to hormones and stress within these genes. Furthermore, most MAPK gene promoters contained ABRE elements, which usually participate in abscisic acid-related responses. Two of the MAPK genes contained a TGA-box, which is an auxin-responsive element. Three of the MAPK genes contained gibberellin-responsive elements. Four of the MAPK genes contained a G-box, CGTCA-motif, and/or TGACG-motif, which participate in MeJA responses. Four of the MAPK genes contained a TCA-element, which is related to salicylic acid responsiveness. Furthermore, a large number of putative hormone-responsive elements suggested that these genes were involved in hormone signal transduction. Moreover, some promotors contained drought-induced elements (MBS), low-temperature response motifs (LTR), and defense and stress responsiveness elements (TC-rich repeats). These results indicated that MAPK genes may be involved in defense against biotic and abiotic stresses in apple.
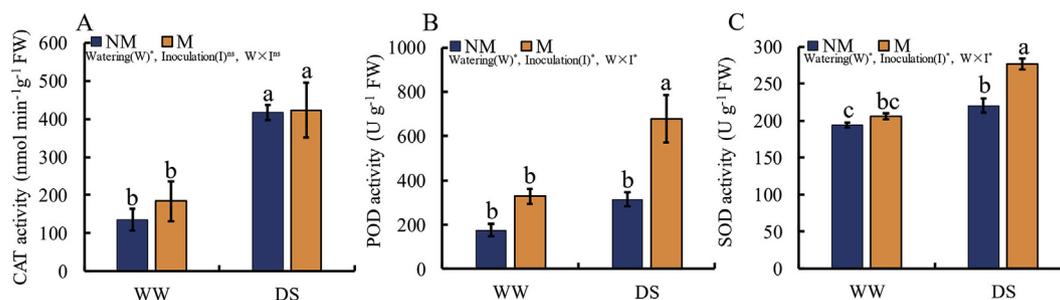
Fig. 5. (A) Catalase (CAT), (B) peroxidase (POD), and (C) superoxide dismutase (SOD) enzyme activity in leaves of *M. hupehensis* seedlings, +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; ∗, $P < 0.05$. Data are shown as mean ± standard deviation (n = 3). Error bars represent three biological replicates.
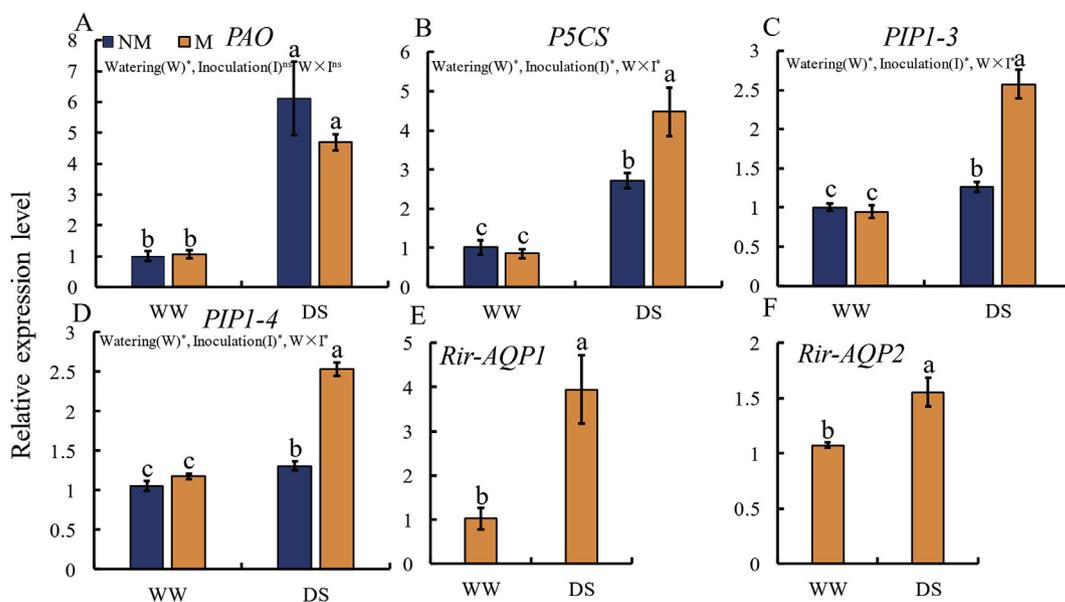
Fig. 6. Changes in expression of stress-response genes (A) *PAO* and (B) *P5CS* in the leaves and (C) *PIP1-3* and (D) *PIP1-4* in the roots of *M. hupehensis* seedlings and (E) *Rir-AQP1* and (F) *Rir-AQP2* in *R. irregularis*. +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; ∗, $P < 0.05$. Data are shown as mean ± standard deviation (n = 3). Error bars represent three biological replicates.

4. Discussion

In our research, we inoculated apple seedlings with *R. irregularis*, which is an AMF that establishes a symbiotic relationship with many plants. We found that the AMF colonization rate in *M. hupehensis* seedlings was not affected by water conditions, which is consistent with the research by Mo et al. (2016). However, we suspected that this was because the duration of the drought stress was not sufficient for its influence to produce significant differences in the AMF colonization rate. The results of this experiment did show that under WW conditions, inoculation of AMF increased plant height, root length, dry weight, and root/shoot ratio. This might be because AMF infects the roots of *M. hupehensis* seedlings and then forms hyphal bridges connecting to the soil. These extra-organic hyphae can extend a few centimeters in the

soil, thereby expanding the absorption area of the roots, extending to areas of the soil that the roots did not reach on their own. Therefore, AMF can help roots to absorb more water and nutrients, which in turn helps plants maintain normal physiological metabolic functions under stressful conditions.

Under stressful conditions, the ability of a plant's antioxidant system to remove reactive oxygen species (ROS) decreases, and a large amount of accumulated ROS can cause damage to plants (Fan and Liu, 2011). Specific antioxidant enzymes, such as CAT, POD, and SOD, are considered important members of the active oxygen scavenging enzyme system. In this research, the results showed that, although drought increased the production of H_2O_2, O_2^-, and MDA in *M. hupehensis* seedlings, their concentrations were lower when inoculated with AM fungi. This trend was supported by the patterns in antioxidant enzyme
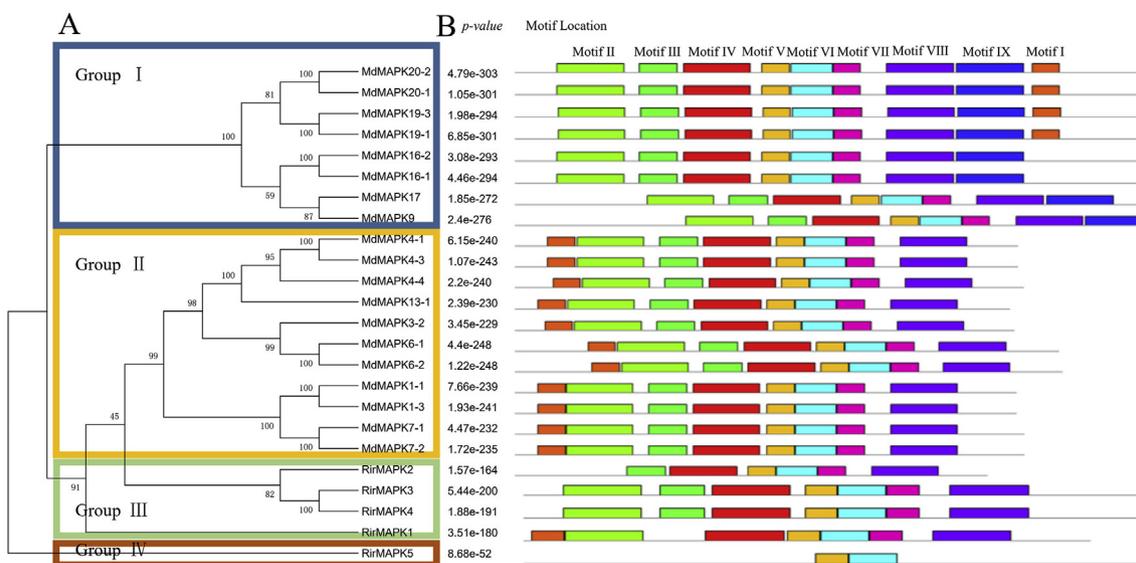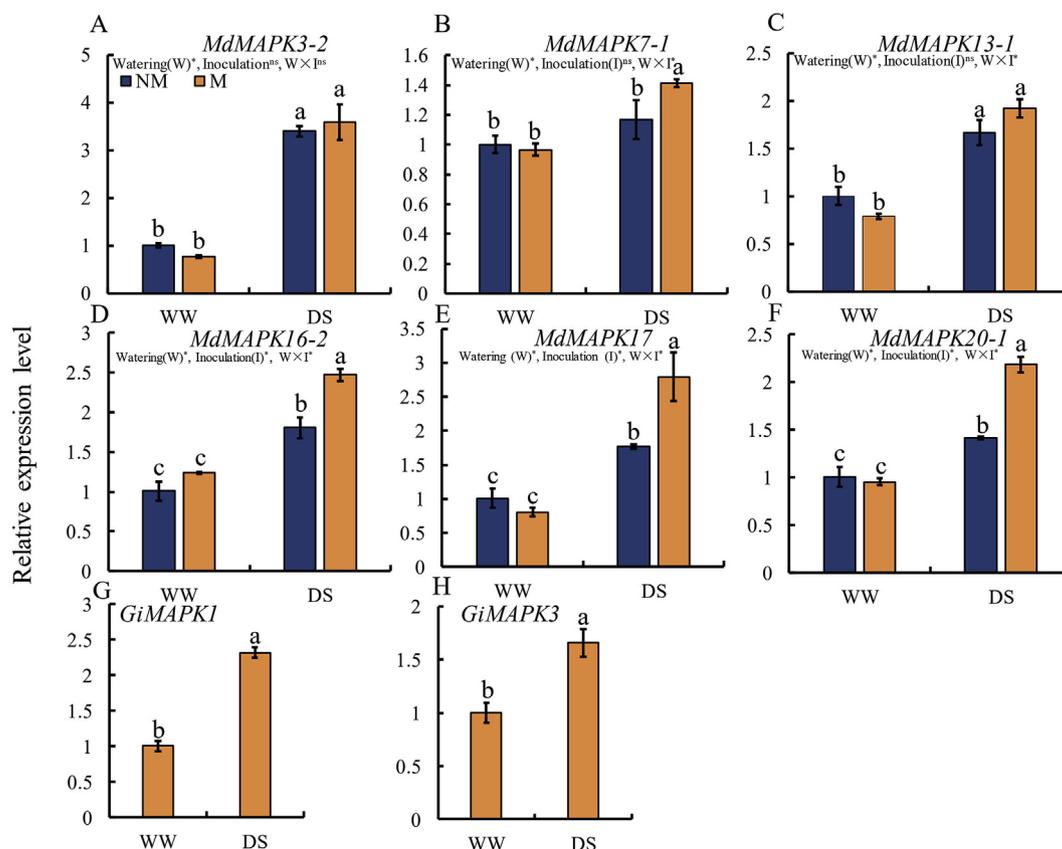

Fig. 7. Phylogenetic relationships and motif distribution of MAPK proteins in apple and *R. irregularis*. (A) The phylogenetic tree for full-length amino acid sequences was constructed with MEGA software and the NJ method. (B) Motifs of the MAPK proteins were analyzed using the MEME (Multiple Expectation Maximization for Motif Elicitation) web server.

Fig. 8. Changes in expression of *MAPK* genes. (A–F) *MAPK* genes in roots *M. hupehensis* seedlings and (G–H) *MAPK* genes in *R. irregularis*. +M = mycorrhizal, +NM = non-mycorrhizal, WW = well-watered, DS = drought-stressed. Letters indicate significant differences between treatments at $P < 0.05$, based on one-way ANOVA and Tukey's tests. Two-way ANOVA output: ns, not significant; *, $P < 0.05$. Data are shown as mean ± standard deviation (n = 3). Error bars represent three biological replicates.

activities under drought stress, where we saw significant increases in SOD and POD activity in plants inoculated with AMF compared to plants that were not (Mo et al., 2016). Therefore, ROS accumulation in *M. hupehensis* seedlings under drought stress was maintained at a relatively low level by enhancing components of the scavenging enzyme system, thereby reducing the degree of cell membrane peroxidation and alleviating serious damage caused by drought stress.

Drought-stressed plants have been shown to accumulate proline and soluble sugars which are known to enhance their ability to tolerate drought conditions (Porcel and Ruiz-Lozano, 2004). Numerous studies have shown that soluble sugars or proline concentrations in mycorrhizal plants were higher than those in non-mycorrhizal plants under drought stress (Huang et al., 2010; Fan and Liu, 2011). Sure enough,

under drought stress, we also observed significant increases in proline and soluble sugar concentrations in mycorrhizal plants compared to non-mycorrhizal plants. At the same time, expression of the key enzyme for the synthesis of proline (*P5CS*) increased, which was consistent with the observed increase in proline concentration. The accumulation of proline and soluble sugars in plant may also provide plants with an osmotic mechanism to improve the water levels in their tissues, thereby resulting in improved growth of AM-associated plants during drought (Porcel and Ruiz-Lozano, 2004).

Photosynthesis is called the most important chemical reaction on earth, and as the basal mechanism in plant metabolism, it is the most important chemical reaction in plants, forming the basis for all plant yields (Yang et al., 2014). It has been well documented that AM
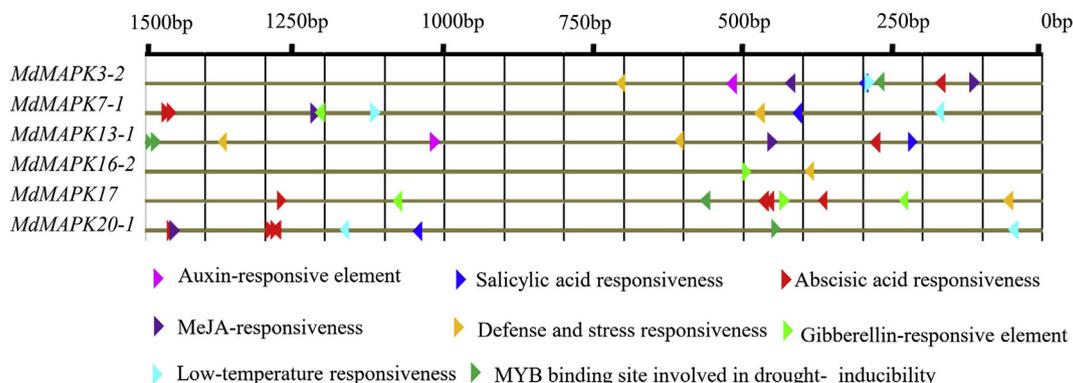
Fig. 9. The main putative cis-elements within 1.5 kb upstream promoter regions of *MdMAPK* genes. The different cis-elements are differentiated using different icons and colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
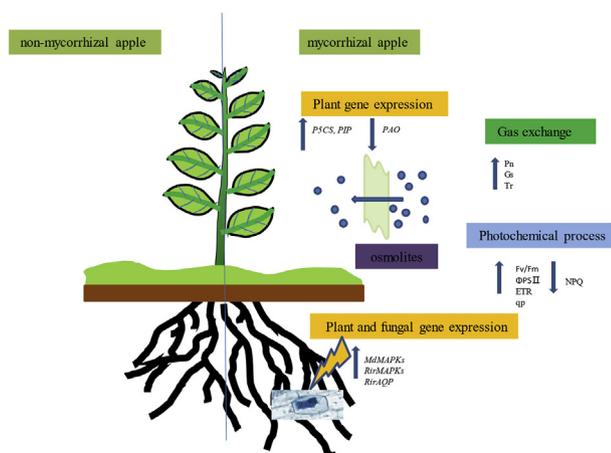
Fig. 10. Biochemical, physiological, molecular, and phenotypic changes induced by AM symbiosis in apple plants under water stress (Chitarra et al., 2016).

symbiosis can alleviate the negative effects of drought stress on net photosynthesis and transpiration rates (Huang et al., 2010; Mo et al., 2016). In this study, the Pn, Gs, and Tr of plants inoculated with AMF were significantly higher than those of the non-inoculated controls. Abiotic stress-generated ROS can damage the photosynthetic apparatus (Gururani et al., 2015). The accumulation of intracellular ROS and its inhibition on photosynthesis can be reduced by increasing the production of ROS-scavenging enzymes. The enhancement of Pn, Gs, and Tr in mycorrhizal apple seedlings under drought stress may have resulted from an improved antioxidant system. In our study, the iWUE of plants increased under drought stress, which was consistent with the pre-existing literature (Chitarra et al., 2016; Mo et al., 2016). However, the iWUE of mycorrhizal *M. hupehensis* seedlings was lower than that of the control group under drought stress, which may have been because AMF helped seedlings adapt to drought stress through mechanisms other than improving their iWUE.

The Fv/Fm value changed very little under normal conditions, but it significantly decreased under stressful conditions (Sheng et al., 2008). In this research, under drought conditions, the Fv/Fm of mycorrhizal *M. hupehensis* seedlings was significantly higher than that of the non-inoculated controls. The same trends also appeared in the ΦPSII, ETR, and qP values, which suggested that inoculation with AM fungi helped *M. hupehensis* seedlings maintain the PSII photochemical efficiency of leaves at a sufficiently high level under drought conditions. NPQ is an indicator that reflects heat dissipation efficiency, which is one of the self-preservation mechanisms used by plants under stress that helps protect photosynthetic mechanisms (Sheng et al., 2008). In our study, under drought stress, NPQ was enhanced by AM symbiosis. The increase in NPQ may mean that, under drought stress, mycorrhizal plants prevent damage to the photosystem reaction center and reduce any interruption to electron transfer in the photosynthetic apparatus (Mo et al., 2016). Chlorophyll is the most effective and important pigment in photosynthesis, and it is the material on which enhanced drought resistance is based. Many studies have indicated that inoculation of AMF can significantly increase the chlorophyll content of plants (Sannazzaro et al., 2006; Sheng et al., 2008). Similarly, this study showed that, under drought stress, inoculation with AMF significantly increased the chlorophyll concentration in *M. hupehensis* leaves. In addition, our data showed that the expression of *PAO*, which is involved in chlorophyll degradation, was increased under drought conditions, but less so in the leaves of mycorrhizal *M. hupehensis* seedlings than in non-mycorrhizal *M. hupehensis* seedlings. Finally, the chlorophyll content of mycorrhizal *M. hupehensis* seedlings decreased less than that of non-mycorrhizal *M. hupehensis* seedlings, which indicated that drought stress had little effect on chlorophyll metabolism of mycorrhizal *M. hupehensis* seedlings,

which were able to maintain chlorophyll metabolism at a sufficiently high level.

Previous studies have shown that apple *PIP* genes were up-regulated under water-stress conditions (Liu et al., 2013). We observed similar results in our research, but we also saw that the up-regulation was greater in mycorrhizal plants, which may further increase drought resistance. The higher expression levels of *Rir-AQP1* and *Rir-AQP2* might lead to a better water uptake capacity as described in the report by Li et al. (2013). When plants are subjected to stress, cell signal transduction is carried out between and within cells to regulate the physiological and biochemical responses of the plants, importantly, the MAPK signal transduction pathway is at the center of the cell signal transduction system. Therefore, the MAPK signal transduction pathway plays a critical role in physiological processes such as plant growth, reproduction, and stress resistance (Hamel et al., 2012). Fungal *MAPKs* promote fungal colonization in plants while plant *MAPKs* stimulate plant immune responses. Previous studies have reported that during the AMF colonization of soybean (*Glycine max*) roots, expression levels of *MAPKs* in roots were linked to the action of *MAPKs* in the AMF (Liu et al., 2015). In our study, under drought stress, inoculation with AMF resulted in significant up-regulation of *MdMAPK7-1*, *MdMAPK16-2*, *MdMAPK17*, and *MdMAPK20-1*. In addition, when experiencing drought stress, *RirMAPK1* and *RirMAPK3* of AMF were also induced. Lastly, we found many elements that responded to hormones and stress from these *MdMAPKs*. Communication between AMF and plants though MAPKs may be vital to the performance of mycorrhizal *M. hupehensis* seedlings under stress. However, there are deeper molecular mechanisms that still require further research.

5. Conclusion

In summary, we suggested that AM symbiosis alleviated the negative effects of drought on apple seedlings by changing the AMF and plant's physiologies and gene expressions resulting in (1) a better ability to balance the distribution of light energy in photochemical and non-photochemical processes, (2) increased antioxidant enzyme activity to scavenge ROS and alleviate oxidative stress injury, (3) more soluble substances to improve osmotic regulation, (4) higher expression of stress-resistant genes, and (5) higher expressions of fungal and apple *MAPK* genes between the two symbiotic parties that cooperated to regulate the mycorrhizal apple seedlings under drought stress (Fig. 10).

Author contributions

Dong Huang and Chao Li collected the public dataset, performed bioinformatics analysis, and drafted the manuscript. Qian Wang, Mengnan Ma, Maoxue Zhang, and Guangquan Jing contributed to the bioinformatics analysis and preparation of all figures and tables. Dong Huang conducted the experiments. Fengwang Ma and Dong Huang conceived this study and reviewed the manuscript. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Cornell University, for help with editing this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.02.020.

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