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Research paper

Overexpression of *PeMIPS1* confers tolerance to salt and copper stresses by scavenging reactive oxygen species in transgenic poplar

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Myo-inositol is a vital compound in plants. As the key rate-limiting enzyme in *myo*-inositol biosynthesis, L-*myo*-inositol-1-phosphate synthase (MIPS) is regarded as a determinant of the *myo*-inositol content in plants. The up-regulation of *MIPS* genes can increase the *myo*-inositol content, thereby enhancing the plant's resistance to a variety of stresses. However, there are few reports on the roles of *myo*-inositol and the identification of *MIPS* in woody trees. In this study, a *MIPS* gene, named as *PeMIPS1*, was characterized from *Populus euphratica* Oliv. The heterologous expression of *PeMIPS1* compensated for inositol production in the yeast inositol auxotrophic mutant *ino1* and the phenotypic lesions of the *atmips1-2* mutant, an *Arabidopsis MIPS1* knock-out mutant. A subcellular location analysis showed that the PeMIPS1-GFP fusion was localized in the nucleus and cytoplasm, but not in the chloroplasts, indicating that PeMIPS1 represented the cytosolic form of MIPS in *P. euphratica*. Interestingly, *PeMIPS1* was not only inducible by drought and high salinity, but also by $CuSO_4$ treatment. The transgenic poplar lines overexpressing *PeMIPS1* had greater plant heights, shoot biomasses and survival rates than the wild type during the salt- or copper-stress treatment, and this was accompanied by an increase in the *myo*-inositol content. The overexpression of *PeMIPS1* resulted in the increased activities of antioxidant enzymes and the accumulation of ascorbate, a key nonenzymatic antioxidant in plant, which partly accounted for the enhanced reactive oxygen species-scavenging capacity and the lowered hydrogen peroxide and malondialdehyde levels in the transgenic poplar. To the best of our knowledge, this study is the first to report the roles of *MIPS* genes in the tolerance to copper stress.

Keywords: antioxidant enzyme activities, ascorbate/glutathione cycle, *myo*-inositol biosynthesis, stress tolerance, transgenic trees.

Introduction

Poplar is widely distributed worldwide and highly regarded for its economic and ecological values. In particular, owing to its fast growth and the high production level, poplar is a predominant 'bioenergy crop' (Polle and Chen 2015). However, as a perennial species, poplar trees are routinely exposed to complex environmental stresses during their long life span, which pose a serious threat to their growth and survival (HL. Wang et al. 2014, L. Wang et al. 2014). To sustain poplar productivity and meet the growing demand for energy and biomaterials, it is necessary to increase our understanding of molecular responses to stress and further improve stress tolerance through genetic engineering.

Populus euphratica, which has the capacity to tolerate extreme environmental stresses, is widely considered an ideal model system for research on abiotic stress resistance in woody plants

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(Ottow et al. 2005). To date, studies of *P. euphratica* have mainly involved single gene functional analyses, transcriptome sequencing and miRNA identification (Ottow et al. 2005, Wang et al. 2014). In *P. euphratica*, a group of core genes that are involved in the complex responses to salinity or drought stress, such as signal transduction, regulation of gene expression, ionic homeostasis, scavenging of reactive oxygen species, accumulation of compatible solutes and growth regulation, have been identified (Ottow et al. 2005, Qiu et al. 2011, Deng et al. 2015, J. Zhang et al. 2016, Chen et al. 2017). However, the stress resistance mechanisms of *P. euphratica* are far from being understood. Identifying more stress-related genes and determining their biological functions will provide valuable information for improving stress tolerance in trees through genetic engineering.

Myo-inositol (MI) is a vital compound in biological kingdoms (Luo et al. 2011). *Myo*-inositol and its derivatives play dual roles in plant stress resistance, being the key metabolites and the second messengers of signal transduction (Donahue et al. 2010, Valluru and Van den Ende 2011). During the de novo synthesis of MI, MIPS is the key rate-limiting enzyme that catalyzes the conversion of D-glucose-6-phosphate to *myo*-inositol-3-phosphate (Loewus and Loewus 1983, Torabinejad et al. 2009). Overexpression of the *MIPS* genes from some herbaceous plants, such as wild halophytic rice (*Porteresia coarctata*), soybean (*Glycine max*), smooth cordgrass (*Spartina alterniflora*), chickpea (*Cicer arietinum*) and sweet potato (*Ipomoea batatas*), conferred tolerance to multiple abiotic stresses, such as salt, dehydration and chilling, which was accompanied by increased MI levels in the transgenic plants (Kaur et al. 2008, 2013, Zhai et al. 2016). Thus, controlling MI synthesis by changing the *MIPS* expression level may provide an effective strategy for manipulating plant tolerance to stress. However, data on *MIPS* genes in perennial woody plants are limited. Although the *MIPS* genes from *Actinidia deliciosus* and *Passiflora edulis* have been isolated and their expression analyzed, the biological functions of these gene are not yet clear (Abreu and Aragão 2007, Cui et al. 2013).

In this study, a *MIPS* gene, namely *PeMIPS1*, was isolated from *P. euphratica* and characterized by functional complementation in the yeast inositol auxotrophic strain *ino1* and the *Arabidopsis atmips1-2* mutant. *PeMIPS1* was induced by various stress treatments, including NaCl, polyethylene glycol (PEG) 6000 and $CuSO_4$. Furthermore, the transgenic poplar 'Shanxin yang' (*Populus davidiana* × *Populus bolleana*) overexpressing *PeMIPS1* exhibited significantly higher tolerance levels to salt and copper stresses than the wild type (WT). To our knowledge, this study is the first to report on the roles of *MIPS* in the resistance to copper stress.

Materials and methods

Plant growth, treatments and collection of tissues

Arabidopsis thaliana used in this study was ecotype Columbia-0. Mutant *atmips1-2* was kindly provided by Prof. Gang Li (Ma et al. 2016). The sterilized *Arabidopsis* seeds were laid on a 0.8% agar plate containing 0.5 × MS medium. The seeds were placed at 22 °C after stratification treatment at 4 °C for 2 days. When the seedlings grew to four-leaf stage, they were transplanted to the soil in the greenhouse at 21–23 °C under 12 h light/12 h dark cycles.

One-year-old rooted cuttings of *P. euphratica* with a height of ~40 cm were used in this study. The uniformly growing *P. euphratica* cuttings were chosen for stress treatments. The pots were dipped in 2 l of solution containing 300 mM NaCl, 30% PEG 6000 or 1 mM $CuSO_4$, respectively. For each treatment, the fourth to sixth leaves from the top of stem were harvested at 0, 12, 24, 48 and 72 h, immediately frozen in liquid nitrogen and stored at −80 °C. The leaves collected from three cuttings were mixed together as a sample, and three separate biological replications were carried out for each sample.

Yeast complementation assay

The *PeMIPS1* cDNA was inserted into the yeast expression vector pAG425GPD-ccdB, and the obtained recombinants were transformed into the yeast *ino1* strain SJY425 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ino1Δ*::HIS3MX6) (Alberti et al. 2007, Luo et al. 2011). The mutant *ino1* transformed with the yeast *INO1* gene was used as a positive control and the empty vector as a negative control. The complete solid synthetic Edinburgh minimal medium without leucine was used for complementation studies. When cultured to 0.5 OD at 600 nm, the transformed yeast cell suspensions were serially diluted at 1:10, and dripped on the screening plate containing 75 mM inositol or not. The yeast cells were observed and photographed after incubation at 30 °C for 2 days.

Subcellular localization studies

The subcellular localization of PeMIPS1 was determined by cloning the open reading frame (ORF) of *PeMIPS1* without the stop codon into the pPZP211-GFP vector. Then, the recombinant vector was further introduced into the mesophyll protoplasts of 'Shanxin yang' by PEG-mediated transformation as described as described in Jeon et al. (2007). After being transformed with plasmid DNA, the protoplasts were incubated at 23 °C for 16 h and then observed using a confocal microscope (Carl Zeiss LSM 880; META, Dresden, Germany). Green fluorescence and chlorophyll autofluorescence were detected at 488 nm and 561 nm, respectively.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted with a PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), and then used for the synthesis of the first-strand cDNA with a PrimeSript™ reagent Kit (Takara, Beijing, China), following the manufacturer's instructions. The qRT-PCR was carried out using SYBR Premix Ex Taq II (Takara), as described previously (Wang et al. 2014). To analyze the expression patterns of *PeMIPS1* in response to various abiotic stresses in

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P. euphratica, a pair of primers was designed based on the 3′ non-coding region of *PeMIPS1*. *PeACTIN7* was selected as the internal control (Deng et al. 2015). For the expression levels of *PeMIPS1* in the different transgenic 'Shanxin yang', a pair of primers specific to the coding region of *PeMIPS1* was used. *PdbEF1β* was employed as the internal control (Yang et al. 2015). The expression levels of *PeMIPS1* were normalized to those of internal control, using the $2^{-\Delta\Delta}$CT method, as described previously. Three biological replicates were performed for calculation of mean expression and standard deviation (SD). The primers used in this study are listed in Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online.

Plasmid construction and plant transformation

The complete ORF of *PeMIPS1* was inserted into a PBI121 vector, and located downstream of cauliflower mosaic virus 35 S promoter. The transgenic 'Shanxin yang' plants were produced by according to Wang et al. (2011). Subsequently, the genomic DNA samples of kanamycin-resistant plants were extracted and used as templates for PCR amplification to further verify the transgenic plants. The forward and reverse primers were designed based on the sequences of CaMV35S promoter and *PeMIPS1*, respectively (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online).

Measurements of MI contents

The measurement of MI content was employed by high-performance liquid chromatography (HPLC), as described in our previous study (Song et al. 2016). Briefly, the rosette leaves of *Arabidopsis* or the fourth to sixth leaves from the top of 'Shanxin yang' seedlings were frozen in liquid nitrogen and ground into a powder. Each sample measuring 1 g was extracted with 2 ml 80% (w/v) ethanol at 60 °C for 30 min. After centrifugation at 4 °C, the supernatant were transferred to a fresh tube and the residue was extracted again. The combined supernatant was evaporated under vacuum, resolved in double-distilled water, and filtered through a 0.45-μm filter. Each injection volume is 10 μl. A SHIMADZU RID-10 A refractive index detector was used and the reference cell was maintained at 30 °C. Standard curve were prepared using gradient dilutions of MI standard (Sigma, Ronkonkoma, NY, USA), and the content of MI was calculated based on the area of the peak.

Assay for stress tolerance

The rooted WT and transgenic plantlets with 9–10 cm in height were transplanted to soil. After 3 months of growth under normal conditions in the illuminating incubator, the uniformly developed WT and transgenic plants were subjected to diverse abiotic stress treatments.

First, we analyzed the tolerance of leaf discs to salt and $CuSO_4$ stress, according to the previous studies (Yang et al. 2015, Ke et al. 2016). Leaf discs with a diameter of 1 cm were punched from fully expanded leaves of the WT and transgenic plants, respectively. The leaf discs from each line were divided into two groups: experimental group and control group. For experimental group, the leaf discs were incubated in 100 mM NaCl or 150 μM $CuSO_4$, while those in water as control. Photographs were taken after treatment for 72 h. Then, the stress tolerance of the WT and transgenic poplar at the whole-plant level was tested. Similarly, the WT and transgenic lines were divided into two groups for each stress treatment. One group were irrigated with 1/4 Hoagland nutrient solution containing 125 mM NaCl or 450 μM $CuSO_4$ every 3 days. Meanwhile, the other group of the WT and transgenic lines were watered with 1/4 Hoagland nutrient solution as control. Photographs were taken after treatment for 3–4 weeks.

Physiological analysis of the transgenic and WT poplar

After the tested plants were treated with 125 mM NaCl or 450 μM $CuSO_4$ for 2 weeks, the fourth to sixth leaves from the top of the tested plants were collected for the following physiological parameters. For histochemical detection of H_2O_2, leaf discs from the fourth to sixth leaves of the WT and transgenic plants were placed in 100 mM NaCl or 150 μM $CuSO_4$ under continuous white light. After treatment for 48 h, leaf discs was stained by 3, 3′-diaminobenzidine (1 mg ml^{-1}, pH 3.8) staining, and then decolorized with 95% ethanol to remove chlorophyll. The visible brown stain indicated the accumulation of H_2O_2 in the samples (Wang et al. 2014). Malondialdehyde (MDA) and H_2O_2 concentrations were determined according to the methods described by Wang et al. (2014) and Jiang and Zhang (2001), respectively.

Measurement of ascorbate (AsA) and glutathione (GSH) was done by using leaves homogenized in 5% meta-phosphoric acid containing 1 mM ethylenediaminete traacetic acid (EDTA) and then centrifuging at 11,500*g* for 12 min at 4 °C. Ascorbate and dehydroascorbate (DHA) content was measured following the methods of Huang et al. (2005) and GSH and glutathione disulfide (GSSG) were examined according to Yu et al. (2003).

Leaves were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, centrifuged at 15,000*g* for 20 min at 4 °C and the supernatant was used for enzyme extract. Ascorbate peroxidase (APX) activity and dehydroascorbate reductase (DHAR) activity were determined according to the method of Nakano and Asada (1981). Monodehydroascorbate reductase (MDHAR) activity was estimated according to the method of Hossain et al. (1984). Glutathione reductase (GR) activity was assayed according to the method of Schaedle and Bassham (1977). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were measured as described in our previous study (Wang et al. 2014).

Statistical analysis

SigmaPlot11.0 and SPSS13.0 software were used for statistical analyses (Wang et al. 2014, Song et al. 2016). Mean values ±

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SD were calculated on the basis of data from three independent replicates. Significant differences were considered to be $P < 0.05$.

Results

Isolation and sequence analysis of PeMIPS1

Two candidate gene sequences encoding MIPS, which shared 96% identity with each other in the ORF, were obtained from *P. euphratica* by a homology-based BLAST algorithm-based search of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nml.gov/) databases. Subsequently, a sequence (1,533 bp) encoding MIPS, named as *PeMIPS1* (*P. euphratica MIPS1*), was isolated from the leaves of *P. euphratica* by reverse transcription-PCR. *PeMIPS1* encoded a 510 amino acid protein, which contained the four highly conserved motifs, GWGGNNG (domain1), VLWTANTERY (domain 2), NGSPQNTFVPGL (domain 3) and SYNHLGNNDG (domain 4), which are found in MIPS proteins from other higher plants (Majumder et al. 2003) (see Supplementary Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Rescue of the yeast inositol auxotrophic mutant ino1 by PeMIPS1

To investigate whether *PeMIPS1* was functional, a complementation experiment was performed in the yeast inositol auxotroph mutant *ino1*, which cannot survive without inositol (Luo et al. 2011). Figure 1a shows that PeMIPS1 rescued the yeast *ino1* mutant and showed no difference from the positive control transformed into the yeast *INO1* gene. However, the negative control introduced into the empty vector could not grow without inositol. Thus, PeMIPS1 could compensate for inositol production in the yeast auxotroph mutant *ino1*.

PeMIPS1 is a functional homolog of MIPS1 in Arabidopsis

We analyzed the ability of PeMIPS1 to rescue the *Arabidopsis atmips1-2* mutant (SALK_023,626) (Ma et al. 2016). As noted previously, *atmips1-2* is smaller overall than the WT. Another phenotype of *atmips1-2* is the presence of lesions in both cotyledons and leaves (Figure 1b) (Donahue et al. 2010, Luo et al. 2011). Figure 1c shows that the transformation of *atmips1-2* with *35S:PeMIPS1* restored the phenotypes in varying degrees to WT and caused a corresponding increase in the MI levels of *atmips1-2* (Figure 1d). Thus, *PeMIPS1* was a functional homolog of its *Arabidopsis* counterpart.

Subcellular localization of PeMIPS1

To determine the subcellular localization of PeMIPS1, we constructed a recombinant vector containing a *PeMIPS1:GFP* gene fusion and transformed it into the mesophyll protoplasts of 'Shanxin yang'. The green fluorescence of PeMIPS1-GFP appeared in the nuclear and cytoplasmic compartments but did not overlap with the red autofluorescence of chloroplasts (Figure 2). Thus, PeMIPS1 appears to be the cytosolic form of MIPS in *P. euphratica*.

Expression profiles of PeMIPS1 in response to different abiotic stresses

We then utilized qRT-PCR to analyze the differential expression patterns of *PeMIPS1* in *P. euphratica* seedlings subjected to independent abiotic stress treatments of 300 mM NaCl, 30% PEG 6000 and 1 mM $CuSO_4$. As shown in Figure 3, the expression level of *PeMIPS1* reached its peak 24 h after the NaCl

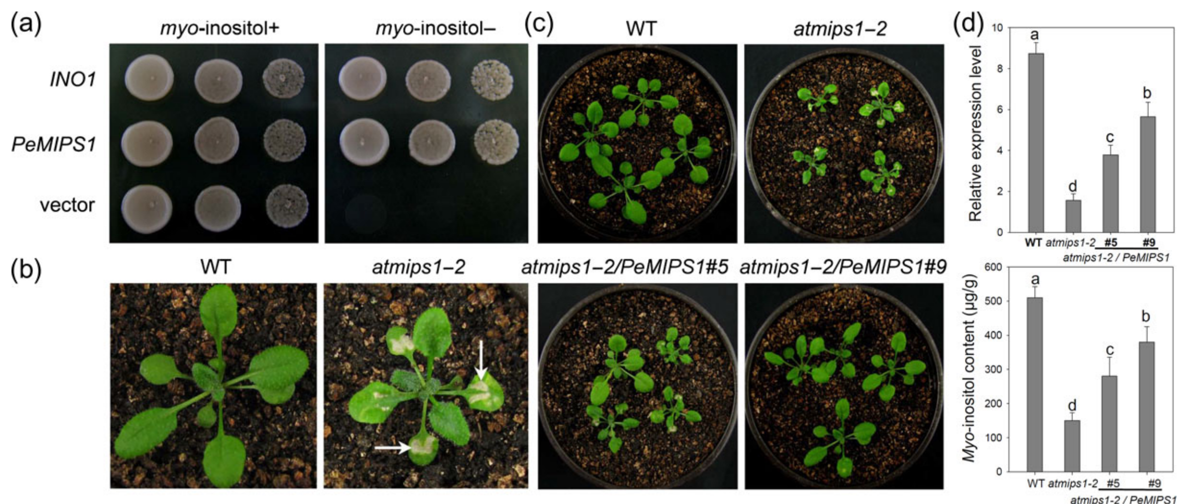

Figure 1. *PeMIPS1* complements the phenotypes of the yeast *ino1* mutant and *Arabidopsis atmips1-2* mutant. (a) Complementation of the yeast *ino1* mutant. The yeast cells transformed with *INO1* or *PeMIPS1* could survive in inositol-free culture, while the negative controls harboring empty vector failed to survive. (b) The WT and *atmips1-2* seedlings grown in soil. The arrows indicated the lesions in the leaves of *atmips1-2*. (c) Morphological phenotypes of the WT, *atmips1-2* and two *atmips1-2/PeMIPS1* lines, respectively. (d) The expression levels of *PeMIPS1* and MI contents in the WT, *atmips1* and two *atmips1-2/PeMIPS1* lines, respectively. *ACTIN2* gene was used as an internal control. Values are means ± SD of three independent biological replicates. The letters indicate *P* value <0.05.

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treatment. For the PEG treatment, the expression level of *PeMIPS1* began to rise at 24 h and increased up to fivefold until 72 h. The accumulation of *PeMIPS1* transcripts increased by 17-fold at 48 h after the $CuSO_4$ treatment. These results indicated that *PeMIPS1* was differentially up-regulated by the three stress treatments.

Production of the transgenic poplar plants overexpressing PeMIPS1

Transgenic 'Shanxin yang' plants overexpressing *PeMIPS1* were generated to determine the possible roles of *PeMIPS1* in abiotic stress responses. We obtained 14 independent transgenic lines by kanamycin screening and PCR verification. Then, the relative expression levels of *PeMIPS1* and the native *PdbMIPS* genes in the WT and transgenic lines were analyzed by qRT-PCR (Figure 4a). The expression levels of the *PdbMIPS* genes remained unchanged among the tested plants, thus eliminating the possibility of interference by native *PdbMIPS* expression (data not shown). Two independent transgenic lines (L4 and L11) with different ectopic expression levels of *PeMIPS1* were chosen for further analyses. Consistent with our expectations, L4 and L11 also had higher MI contents than the WT plants

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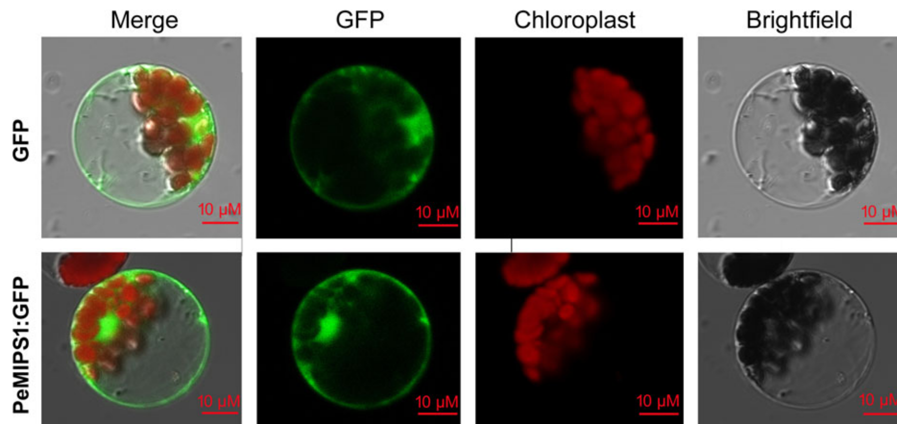

Figure 2. Subcellular localization of PeMIPS1:GFP in the mesophyll protoplasts of Shanxin yang.

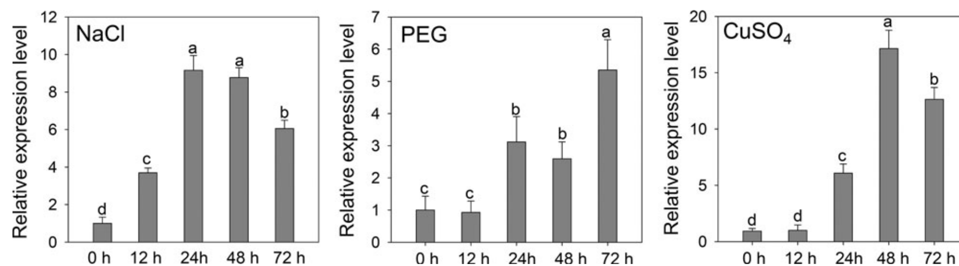

Figure 3. Expression analyses of *PeMIPS1* under diverse stress treatments. The data were normalized to the poplar *EF1β* expression level. Values are means ± SD of three independent biological replicates. The letters indicate *P* value <0.05.

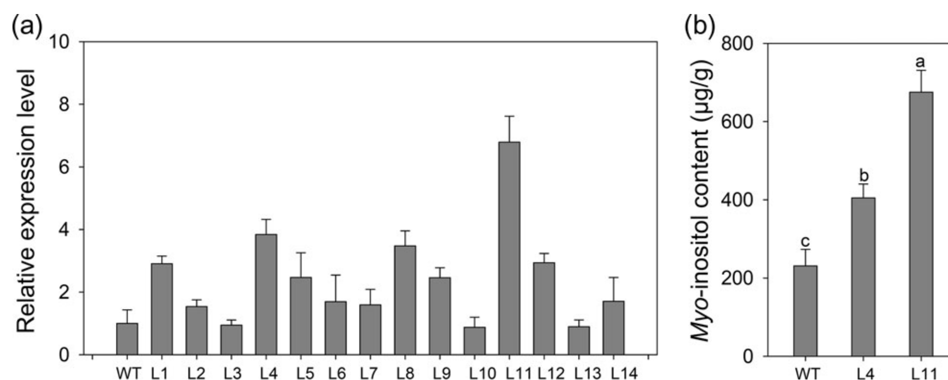

Figure 4. Confirmation of the transgenic poplar plants. (a) Expression levels of *PeMIPS1* in the WT and different transgenic poplar lines (L1–L14); (b) MI contents in the WT and two transgenic lines (L4 and L11). Values are means ± SD of three independent biological replicates. The letters indicate *P* value <0.05.

(Figure 4b). Thus, overexpressing *PeMIPS1* may increase MI synthesis in the transgenic poplar plants.

Enhanced tolerance of transgenic poplar overexpressing PeMIPS1 to salt and Cu stresses

First, the salt tolerance of leaf discs was compared between the selected transgenic lines (L4 and L11) and WT. In the absence of NaCl, there were no significant differences between the WT and two transgenic lines. However, after incubation for 72 h in a medium containing 100 mM NaCl, the leaf discs from the WT showed more severe chlorosis than those from the two transgenic lines (Figure 5a). Then, three salt-tolerance analyses were performed at the whole-plant scale. As shown in Figure 5b, from the bottom to the top, most of the leaves of the WT turned yellow, wilted or even fell off after the salt treatment. By contrast, the leaves on the upper parts of the transgenic plants maintained normal color and growth, although the leaves in the lower part also appeared yellow to varying degrees. Measurements of basic growth indicators showed that the transgenic lines had greater plant heights, shoot biomasses and survival rates than the WT during the salt-stress treatment (Figure 5c–e).

The transgenic plants overexpressing *PeMIPS1* were healthier than the WT under copper-stress conditions in terms of the leaf discs and whole plants (Figure 6). The leaf discs of the two transgenic lines displayed less cellular damage than those of the WT after 72 h of treatment with 150 μM $CuSO_4$ (Figure 6a). On the whole-plant scale, although all of the WT and transgenic lines showed signs of severe copper toxicity, such as growth inhibition and the browning of leaves and stems, the transgenic plants were healthier than the WT (Figure 6b). Furthermore, the survival

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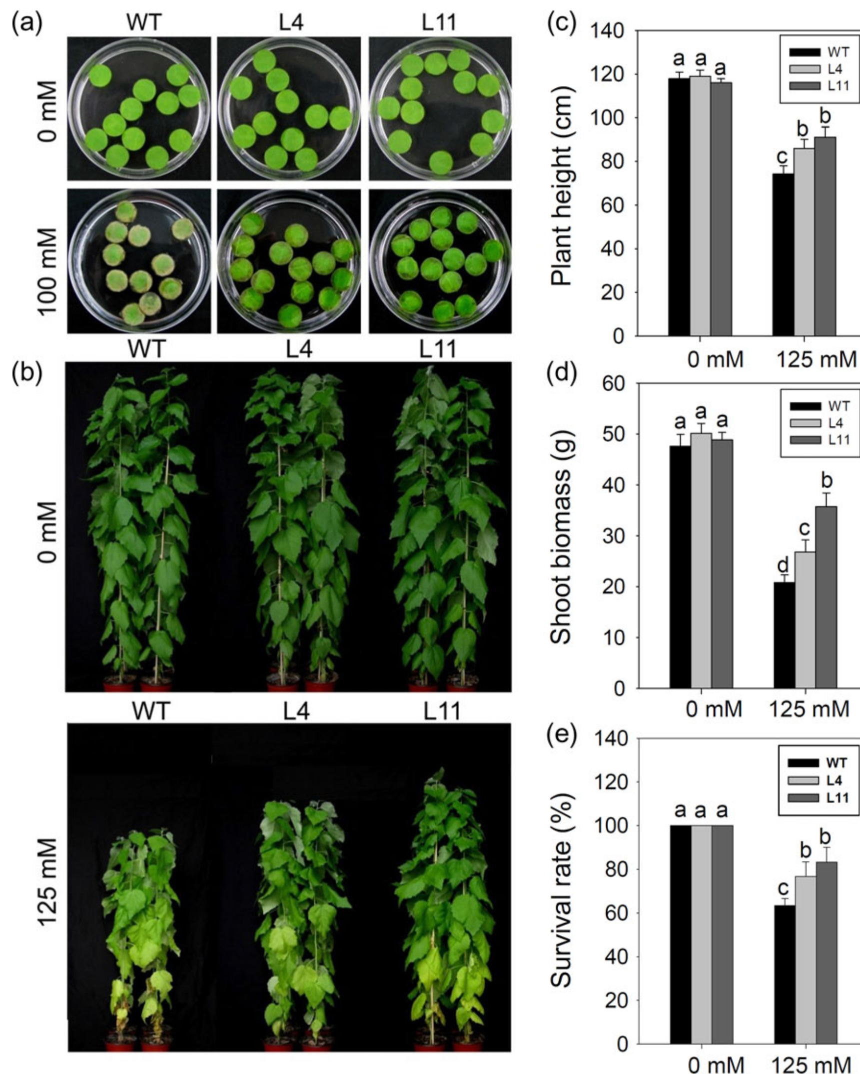

Figure 5. Overexpression of *PeMIPS1* enhanced the tolerance to the salt stress in transgenic plants. (a) Salt-tolerance test of leaf discs. The leaf discs from fully expanded leaves of the WT and each transgenic line were incubated in 100 mM NaCl. (b) Salt-tolerance test of the whole plant. The uniformly developed plants of the WT and each transgenic line were treated with 125 mM NaCl for 3 weeks. (c) Plant height (d) Shoot biomass. (e) Survival rate. Values are means ± SD of three independent biological replicates. The letters indicate *P* value <0.05.

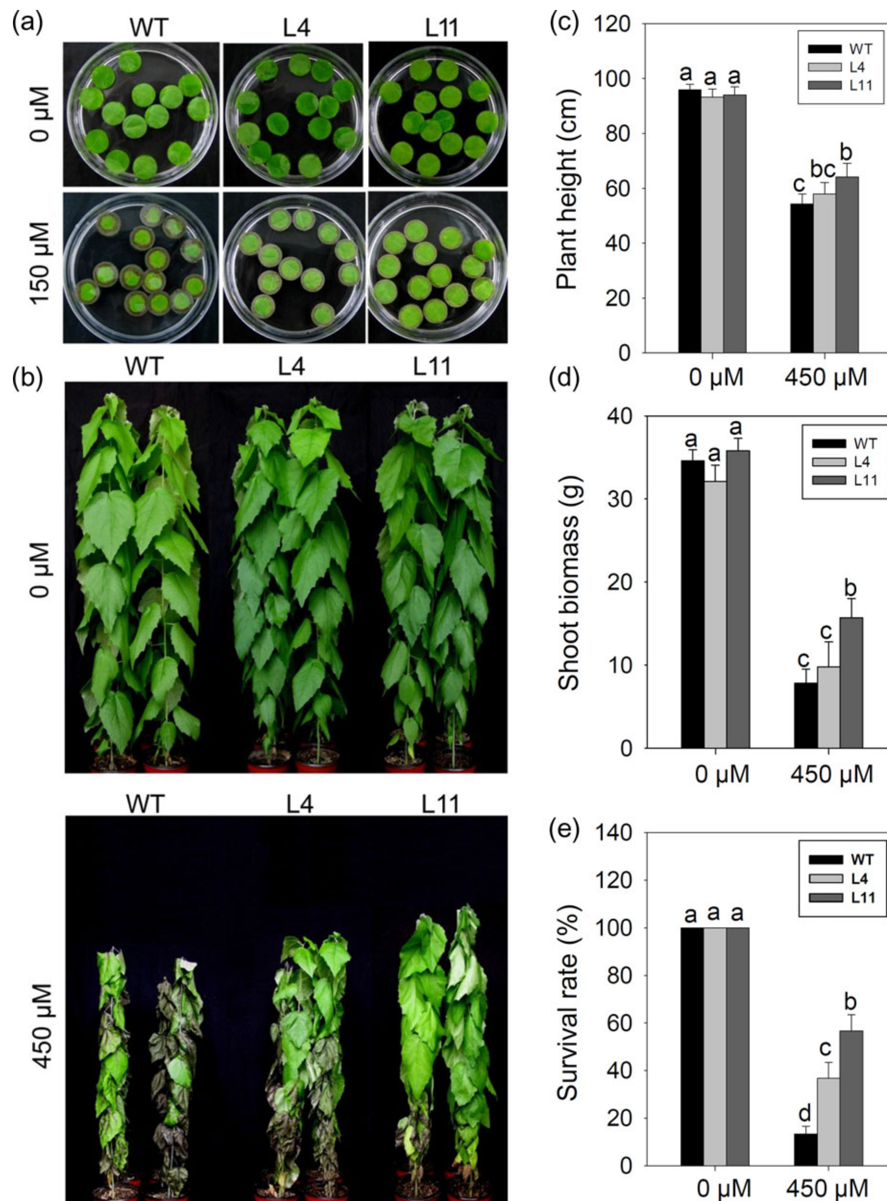

Figure 6. Overexpression of *PeMIPS1* enhanced the tolerance to the copper stress in transgenic plants. (a) Copper tolerance test of leaf discs. The leaf discs from fully expanded leaves of the WT and each transgenic line were incubated in 150 μM $CuSO_4$. (b) Copper tolerance test of the whole plant. The uniformly developed plants of the WT and each transgenic line were treated with 450 μM for 3 weeks. (c) Plant height. (d) Shoot biomass. (e) Survival rate. Values are means ± SD of three independent biological replicates. The letters indicate P value <0.05.

rate of the treated WT was only 13.3%, whereas those of transgenic lines L4 and L11 reached 36.7% and 60%, respectively (Figure 6e). Thus, the overexpression of *PeMIPS1* improved the tolerance levels to salt and copper stresses in transgenic poplar.

Overexpression of PeMIPS1 reduced the level of oxidative damage to the transgenic plants

Both salt and copper stresses can cause oxidative stress by generating reactive oxygen species (ROS) (Schützendübel and Polle 2002, Yasar et al. 2006). We examined the effects of *PeMIPS1* overexpression on the accumulation of ROS in the transgenic poplar. The results showed no significant differences in the H_2O_2 levels between the leaf discs from the two transgenic lines and the WT under normal conditions. However, a lower accumulation of H_2O_2 was observed in the transgenic lines than in the WT upon exposure to either salt or copper stress (Figure 7a). Moreover, the transcript levels of *PeMIPS1* were higher and less H_2O_2 accumulated (Figure 7b). Additionally, the transgenic lines had lower MDA contents than the WT (Figure 7c).

Overexpression of PeMIPS1 enhanced the ROS-scavenging ability of the transgenic plants

Given that the AsA–GSH cycle is a central and efficient antioxidant system for the removal of ROS, we compared the related

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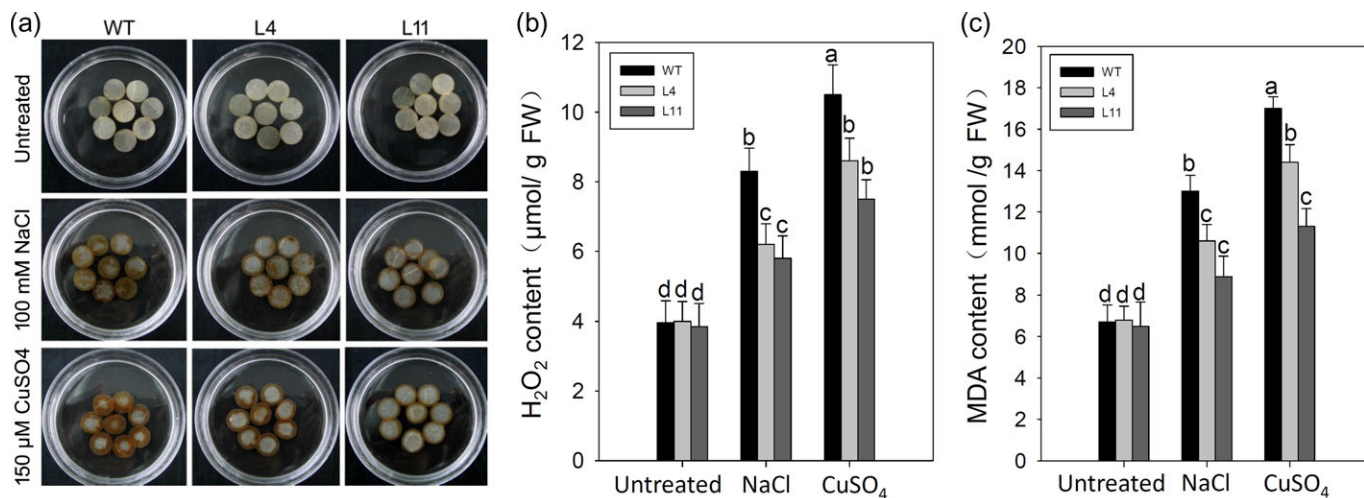

Figure 7. Measurements of H_2O_2 and MDA contents in the WT and transgenic poplar. (a) Histochemical detection of H_2O_2 in leaf discs. (b) H_2O_2 content. (c) MDA content.

nonenzymatic and enzymatic antioxidants between the WT and transgenic plants (Noctor and Foyer 1998). As shown in Figure 8a, the transgenic plants had constitutively higher total AsA (AsA+DHA) contents than the WT. Moreover, the AsA/DHA ratios in the transgenic plants were significantly higher in the WT under salt and copper stresses. There were no differences in the total GSH contents between the WT and transgenic plants under normal and salt conditions. However, compared with the WT, the transgenic plants had an increased GSH content in response to the copper treatment. Additionally, the transgenic plants also had advantageous GSH/GSSH ratios over the WT under normal and salt conditions.

Next, the enzyme activities related to the AsA–GSH cycle, including APX, MDHAR, DHAR and GR, were examined. Ascorbate peroxidase, DHAR and MDHAR are responsible for recycling AsA, in which APX catalyzes the detoxification of H_2O_2 using AsA as a reductant, and DHAR and MDHAR take part in the regeneration of AsA. As shown in Figure 8b, the activities of APX and DHAR were higher in the transgenic plants than in the WT under both salt and copper stresses, while of the MDHAR activity was only increased in the transgenic plants L11 under salt-stress conditions. In addition, the transgenic plants had constitutively higher GR activities than the WT, which reduced GSSG to GSH at the cost of NADPH. The above results indicated that the overexpression of *PeMIPS1* accelerated the rate of the AsA–GSH cycle by increasing the activities of the enzymatic antioxidants in the transgenic poplar lines.

In addition, other ROS scavengers, such as SOD, CAT and POD, were analyzed in the WT and transgenic lines (Figure 8c). There were no differences in the POD activity level between the transgenic plants and the WT under normal or stress conditions. However, compared with the WT, the transgenic plants had higher SOD and CAT activities when treated with NaCl, and higher CAT activities when treated with copper.

Discussion

Planting resistant tree species in soil that is not suitable for growing crops is an effective way of creating both production and environmental benefits. Poplar is an ideal fast-growing tree species that can be propagated by cuttings and genetically engineered (Polle and Chen 2015). Thus, it is of considerable interest to manipulate stress-resistant poplar varieties by genetic engineering to improve its land-use efficiency.

As the key rate-limiting enzyme in MI biosynthesis, MIPS is a determinant of the MI content in plants (Donahue et al. 2010). Studies on herbaceous plants have shown that up-regulating the expression of *MIPS* genes can increase the MI content and ultimately improve the stress tolerance of a plant (Kaur et al. 2008, 2013, Zhai et al. 2016). However, currently, the possible roles of *MIPS* genes in tree species have not been reported. In this study, a gene encoding MIPS, *PeMIPS1*, was characterized from *P. euphratica*. Complementation experiments, which were performed in the yeast inositol auxotroph mutant *ino1* and the *Arabidopsis* mutant *atmip1-2*, clearly demonstrated that *PeMIPS1* encoded a functional MIPS enzyme involved in the biosynthesis of inositol in *P. euphratica*.

To date, two forms of MIPS enzymes, chloroplastic and cytosolic, have been characterized from a wide range of organisms, such as algae, higher plants and animals (Majee et al. 2004). The chloroplastic form is mainly located in the chloroplast, while there are two previously reported subcellular localization patterns for the cytosolic form. Donahue et al. (2010), using stably transformed *Arabidopsis* plants, reported that all three MIPS proteins from *Arabidopsis* were exclusively located in the cytoplasm. By contrast, Latrasse et al. (2013), using transient expression in tobacco BY2 cells, found that MIPS1 localized in the nucleus as well as the cytoplasm. In this study, the PeMIPS1-GFP fusion was localized in the nucleus and cytoplasm, but not in the chloroplasts,

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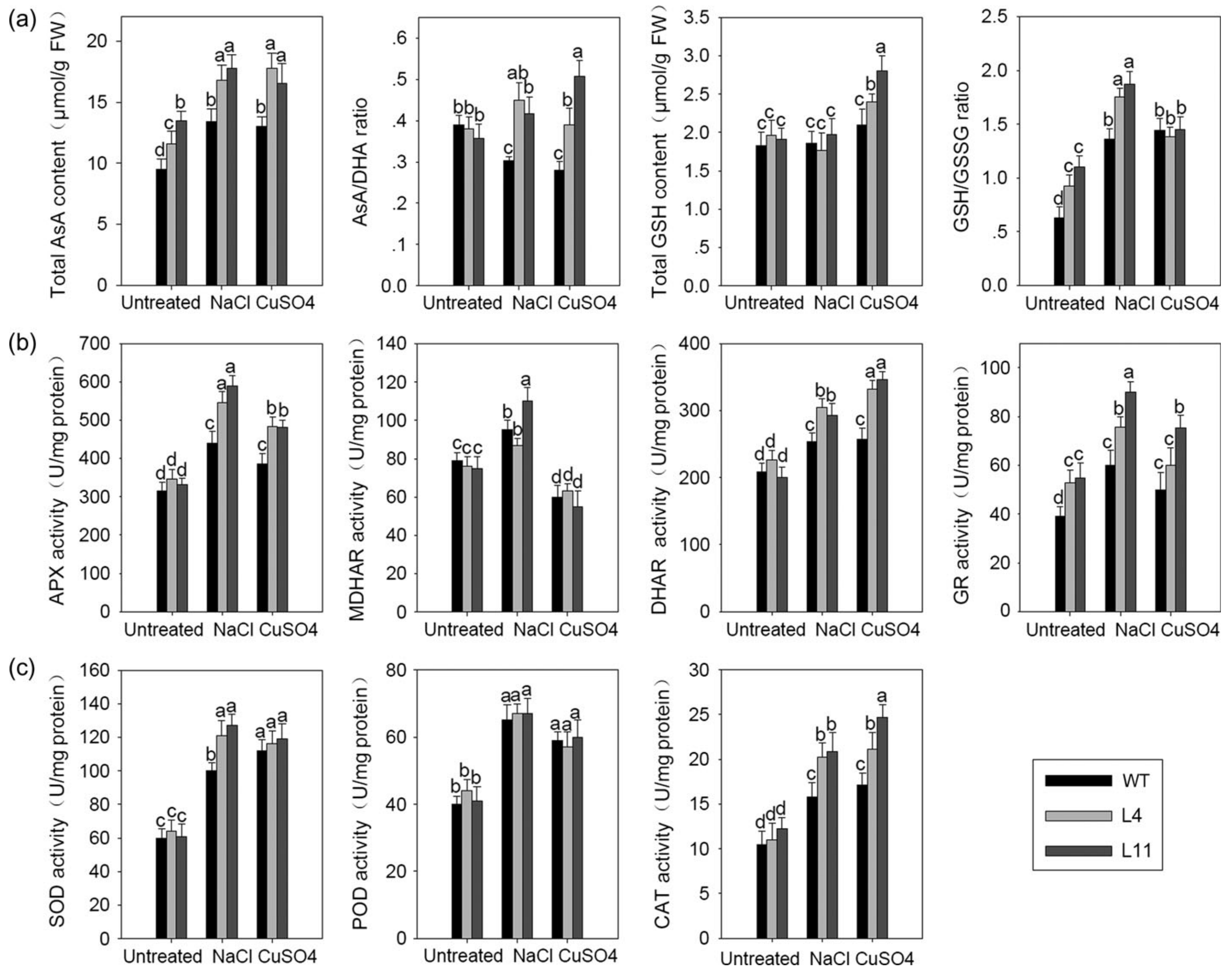

Figure 8. Comparison of the nonenzymatic and enzymatic antioxidants between the WT and the transgenic lines. (a) Measurement of the nonenzymatic antioxidants in the AsA/GSH cycle. (b) Measurement of the enzymatic antioxidants in the AsA/GSH cycle. (c) Measurement of the other enzymatic antioxidants.

which was consistent with the report of Latrasse et al. (2013). Thus, the characterized PeMIPS1 may represent the cytosolic form of *P. euphratica*.

To predict the roles of *PeMIPS1*, we analyzed the expression patterns of *PeMIPS1* in response to a variety of stresses. As shown in Figure 3, *PeMIPS1* was induced by both high salt and drought stresses, which was similar to the expression patterns of the well-characterized *MIPS* homologous genes from herbaceous plants, such as *Arabidopsis*, rice and sweet potato (Zhai et al. 2016). Interestingly, *PeMIPS1* was also observed to be strongly up-regulated by the $CuSO_4$ treatment. To date, little is known about the effects of copper stress on the expression levels of *MIPS* genes in higher plants. Ritter et al. (2014) reported that the *MIPS* gene (Esi0279_0022) was down-regulated in the brown algal model *Ectocarpus siliculosus* during the short-term acclimation to copper stress. This result was inconsistent with the induction of *PeMIPS1* by $CuSO_4$ in this study, implying that the responses of woody plants and algae to copper stress are different. In addition, we found that *PeMIPS1* responded more quickly to salt stress than to PEG or $CuSO_4$ stress. Salt, osmotic and copper stress can cause oxidative stress by generating ROS (Zhang et al. 2016, Yang and Guo 2017). During salt stress, ion toxicity occurs before the production of ROS (Yang and Guo 2017). Therefore, we speculated that *PeMIPS1* can respond more quickly to ion stress than to oxidative stress.

In our study, 'Shanxin yang' was transformed with *PeMIPS1*, and the native PeMIPS1 homolog (PdbMIPS1) shared 100% amino acid identity with PeMIPS1. As expected, the overexpression of *PeMIPS1* enhanced the salt-stress tolerance in the transgenic poplar plants and increased the MI content (Figures 4 and 5). Although *PeMIPS1* was also induced by the PEG, the

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overexpression of *PeMIPS1* produced no changes in the tolerance to drought stress between the WT and transgenic plants. Notably, compared with that of the WT, the transgenic plants' tolerance to copper stress also significantly improved (Figure 6). To the best of our knowledge, this is the first report on the roles of *MIPS* genes in the resistance to copper stress.

We further examined the effects of *PeMIPS1* overexpression on the accumulation of ROS under salt and copper stresses. Reactive oxygen species induced by stress can severely damage cellular structures and macromolecules, such as enzymes, DNA and lipids (Yang and Guo 2017). Therefore, a plant's tolerance to stress is closely related to its capacity to detoxify ROS (Di Baccio et al. 2008). Ascorbate and GSH are the key nonenzymatic antioxidants involved in scavenging ROS through the AsA–GSH cycle in plants, and the ratios of their oxidized to reduced states, AsA/DHA and GSH/GSSG, respectively, are indicators of the cellular redox state (Foyer and Noctor 2011). In our study, the transgenic plants had constitutively higher total AsA contents than the WT, indicating that the overexpression of *PeMIPS1* could result in the accumulation of AsA (Figure 8a). To date, the regulation of the complex AsA biosynthesis network in plants is not fully characterized and it is still unclear whether MI is a precursor of AsA in plants (Lorence et al. 2004, Endres and Tenhaken 2009). However, we hypothesized from the accumulation of AsA and MI in the transgenic plants, that MI may be one of the entry points for the biosynthesis of AsA in poplar. Ascorbate peroxidase and DHAR are responsible for the oxidation and regeneration of AsA, respectively, in the AsA–GSH cycle (Figure 8b) (Foyer and Noctor 2011). After exposure to salt and copper stresses, these two enzymes had higher activities, as well as AsA/DHA ratios, in the transgenic plants than in the WT, indicating that the overexpression of *PeMIPS1* enhanced the recycling of AsA under stress conditions. Interestingly, after exposure to the copper treatment, the transgenic plants showed no increase in MDHAR activity over the WT, indicating that the higher rate of AsA reduction maintained in the transgenic plants was mainly dependent on the enhanced DHAR activity under copper stress (Figure 8b). At the same time, the GR enzyme, which is responsible for reducing GSSG into GSH, had constitutively higher activities in the transgenic plants than in the WT (Figure 8b). This suggested that the overexpression of *PeMIPS1* increased the rate of GSH regeneration in the transgenic plans. In addition to the AsA cycle, the antioxidant enzymes, such as SOD, POD and CAT, play important roles in scavenging ROS (Mittler 2002). Compared with the WT, the transgenic plants increased levels of SOD and CAT, which also partly accounted for the decrease in H_2O_2 and MDA contents in the *PeMIPS1*-overexpressing plants (Figure 8c).

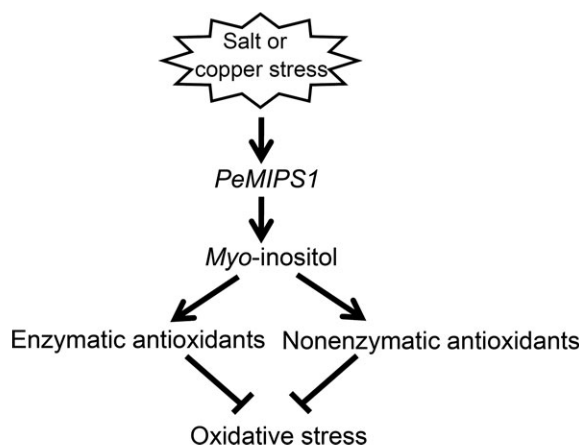

Figure 9. A model of *PeMIPS1* involved in the tolerance to salt or copper stress in poplar. Salt or copper stress promoted the accumulation of MI by inducing the expression of *PeMIPS1*. The increased MI content enhanced the ROS scavenging capacity by increasing the antioxidant enzyme activities as well as the pool size of the nonenzymatic antioxidants.

In addition, the overexpression of the *PeMIPS1* gene may lead to a wide range of metabolic changes in the transgenic poplar. Kusuda et al. (2015) reported that not only various inositol metabolites, but also the basal metabolism, such as glycolysis, the pentose phosphate pathway and the tricarboxylic acidcycle, were induced by the overexpression of the *RINO1*gene, a *MIPS* gene from rice. Thus, further studies are needed to investigate the mechanism of MI that regulates plant tolerance to abiotic stresses.

Thus, salt or copper stress promoted the accumulation of MI by inducing the expression of *PeMIPS1*. The increased MI content enhanced the rate of the AsA–GSH cycle by increasing the enzymatic antioxidant activities, which suppressed the oxidative stress triggered by salt or copper stress. In addition, MI promoted the synthesis of AsA, possibly as a precursor or through another mechanism, thereby enhancing the ROS scavenging capacity by increasing the accumulation of nonenzymatic antioxidants (Figure 9). To the best of our knowledge, this study is the first to report the roles of *MIPS* genes in the tolerance of woody plant to stress. These results provide a promising strategy of constitutively expressing the *PeMIPS1* gene to engineer stress-tolerant poplar trees.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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