

Overexpression of the *Tamarix hispida* *ThMT3* gene increases copper tolerance and adventitious root induction in *Salix matsudana* Koidz.

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Abstract In this work, transgenic *Salix matsudana* expressing the *Tamarix hispida* *ThMT3* gene, which encodes encoding a type 3 metallothionein, showed increased tolerance to copper (Cu) stress. Exposure to 50 μ M Cu completely inhibited rooting of wild-type (WT) plants, but induced numerous adventitious roots in the transgenic plants. The nitric oxide (NO) content in the transgenic plants was higher than that in WT plants. The application of an NO inhibitor, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, decreased superoxide dismutase, catalase and ascorbate peroxidase activities under Cu stress. Auxin application-related genes that are known to improve adventitious roots, such as *Auxin response factor 8*, *auxin resistant 1* and *pinformed*, were highly expressed in transgenic plants under Cu and sodium nitroprusside treatments. These results suggested that the expression of the *ThMT3* gene increased Cu tolerance and NO

production, and the higher NO release contributed to the induction of adventitious roots under Cu stress.

Keywords *Salix matsudana* Koidz. · Phytoremediation · Rooting · Nitric oxide · Copper

Introduction

Phytoremediation of contaminated soils by plants offers an environmentally friendly, cost-effective method to cleanup of toxic pollutants in the environment. There has been an increase in research on improving the ability of plants to remove environmental pollution. For this application, tree species with a large biomass and a tolerance for high levels of toxic metals are needed.

Salix matsudana Koidz. is a large, deciduous, rapidly growing tree that is native to northeastern China, where it experiences a wide range of climatic conditions. The species is one of the most widely distributed and commonly cultivated willow species in China. The species accumulates high level of heavy metals, making it potentially suitable for phytoremediation (Dos Santos Utmazian et al. 2007). However, phytoremediation is limited by the failure of the plants to hyperaccumulate toxins, the unacceptably long time period needed for remediation, and the fact that heavy metal accumulation is restricted mainly to the roots, which complicates their collection for elimination (Dowling and Doty 2009). The efficient genetic engineering of the *Salix* species is highly desirable to address these limitations. *Agrobacterium*-mediated transformation is widely to introduce foreign DNA into plant species. Recently, we developed an *A. tumefaciens*-mediated transformation system for *S. matsudana*, using the embryonic apical region as an explant (Yang et al. 2013).

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Metallothioneins (MTs) are a group of low-molecular-weight Cys-rich metal binding proteins that are present in all eukaryotes, as well as some prokaryotes, and are divided into three classes based on the arrangement of the Cys residues in the N- and C-termini (Cobbett and Goldsbrough 2002). Plant MTs belong to class II and can be further subdivided into four types (MT1, MT2, MT3 and MT4) based on the Cys distribution pattern (Cobbett and Goldsbrough 2002). Numerous studies have observed the expression of plant MTs in response to various heavy metal stresses, such as copper (Cu), zinc and cadmium (Cd). The overexpression of some MTs has led to enhanced heavy metal resistance (Cobbett and Goldsbrough 2002). *Arabidopsis* MTs 2a and 3 enhanced resistance to Cd in *Vicia faba* guard cells (Lee et al. 2004), and the overexpression of *Paxillus involutus*' *PiMT1* in *Hebeloma cyclindrosporum* resulted in higher tolerance to Cu (Bellion et al. 2007). However, the detailed functions of MTs in plants are still poorly understood.

Currently, it is widely accepted that MTs play an important role in Cu homeostasis during plant growth. MT2 from *Brassica juncea* (*BjMT2*) confers a high Cu tolerance to transgenic *Escherichia coli* and *Arabidopsis*. The ectopic expression of *BjMT2* inhibited root growth in the absence of Cu exposure, whereas root growth of the transgenic plant was identical to the wild-type (WT) plants when exposed to high Cu levels, indicating that the overexpression of the MT2 protein interfered with Cu homeostasis, thus affecting root development (An et al. 2006). MT deficiency (*mt1a-2/mt2a-1/mt2b-1/mt3-1*) affects Cu accumulation and distribution in *Arabidopsis* mutants (Benatt et al. 2014). In our previous studies, we found that overexpression of the *Tamarix hispida* *ThMT3* gene, an MT-like gene encoding a type 3 MT, improved the tolerance of transgenic yeast to Zn, Cd and Cu stress (Yang et al. 2011).

Nitric oxide (NO), an important signaling molecule, has roles in the response to Cu. It is a crucial modulator under many stresses, such as pathogen invasion, salt stress, UV-B radiation and drought stress (Shi et al. 2005; Zhang et al. 2007). Recently, NO has been regarded as a molecular signaling response factor in plants against heavy metals, including arsenic (Singh et al. 2009), Cu (Yu et al. 2005), and Cd (Groppa et al. 2008; Laspina et al. 2005). Transgenic antisense-*MT2b* tomato plants showed an increased sensitivity to Cu stress and the Cu resistance can be efficiently rescued by the addition of sodium nitroprusside (SNP) (Wang et al. 2010). Additionally, NO applications induced MT transcription and accumulation in leaves, indicating the possible roles of NO and MTs in response to heavy metals. However, whether the overexpression of MTs improves Cu tolerance, or whether rooting ability is related to NO, has not been demonstrated.

In the present study, the *ThMT3* gene was transformed into *S. matsudana*. The transgenic plants showed increased tolerance to Cu stress. Additionally, adventitious roots were highly induced in transgenic plants under Cu stress, and the improved induction was shown to be related to NO release under Cu stress. Based on this research, we hope to gain a better understanding of the possible protective mechanism of *ThMT3* expression in response to Cu stress in *S. matsudana*.

Materials and methods

Plant materials and transformation

Open-pollinated mature seeds of *S. matsudana* Koidz. var. *matsudana* were collected during late May from 60-year-old trees grown on the campus of Northeast Forestry University, Harbin, China and used for transformation.

A MT-like gene, *ThMT3* (GenBank accession number EH057039), isolated from *T. hispida* was cloned into binary vector PROKII, under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, containing a *neomycin phosphotransferase II* (*npt II*) gene under the control of a nopaline synthase (*nos*) promoter (Chen et al. 2003). The binary vector, PROKII-*ThMT3*, was introduced into *Agrobacterium tumefaciens* strain LBA4404.

Transgenic plants were generated by *Agrobacterium*-mediated transformation of injured *S. matsudana* seeds, according to our previous method, described by Yang et al. (2013). The regenerated, transformed plantlets were cultured on a selective medium successively at 4-week intervals in flasks. Cultures were placed in a culture room at 24 °C with a 16-h photoperiod and a light intensity of 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

PCR and RT-PCR analysis

DNA was extracted from leaves of WT and transgenic plants using modified cetyltrimethylammonium bromide method (Jaakola et al. 2001). The *ThMT3* gene was amplified using primers F: 5'-ATGTCGGGCAAGTGCGGAAACTGCAG-3' and R: 5'-TCAGTGACTGCCACATGTGCAGTTG-3' designed to amplify a 210 bp fragment. The PCR conditions were as follows: 94 °C for 3 min; followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and a final 7 min extension at 72 °C. Total RNA was extracted using a Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesized from 0.5 μg of purified RNA was reverse-transcribed using a Reverse Transcriptase kit (Takara Biotech, Dalian, China). The PCR conditions were identical to those described above.

Heavy metal exposure and growth characteristics

In vitro-grown shoots of about 3 cm height were transferred into 1/2 Murashige and Skoog (MS) (1962) medium supplemented with 0, 10, 30 and 50 μM CuSO_4 , respectively. Growth characteristics, such as plant height, root length and rooting rate (%) were measured after 4 weeks of culture. Five plants were cultured in a flask, with three replicates; three plant lines were tested for each treatment.

Histological observation

Shoots of plants treated with 50 μM CuSO_4 for 4 weeks were collected and sections were made, as described previously (Yang et al. 2013). The sections were de-waxed and observed under a light microscope (Olympus SZ, Japan).

Determination of the copper contents

In vitro-grown shoots of about 6 cm height were cultured on 1/2 MS medium containing 50 and 100 μM CuSO_4 , respectively. After 4 weeks, the roots, stems and leaves of the plants were divided and dried at 80 °C to a constant weight. The samples (0.1–0.2 g) were weighed and dissolved in a mixture of concentrated HNO_3 – HClO_4 (5:1 v/v) and heated at 160 °C for 5 h. After cooling, the beaker was washed with 6 M HCl. Aliquots were analyzed by atomic absorption spectrophotometer (TAS-986), as described previously (Adamis et al. 2003). Absorption was calculated by determining the differences in metal content between non-transgenic and transgenic plant, respectively.

Detection of O_2^- and H_2O_2

The amount of O_2^- and H_2O_2 in the leaves and roots from the WT and three transgenic plant lines (T1, T2, T3) were monitored by incubation with 2 mM nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich) in 20 mM phosphate buffer (pH 6.1) containing 10 mM NaN_3 and in 3,3'-diaminobenzidine (DAB) (D5637, Sigma-Aldrich) (pH 3.8). Chlorophyll was destaining by boiling in alcohol (95 % v/v) for 1 h (Xu et al. 2010).

Transmission electron microscopy (TEM) observation

After treatment with 50 μM CdSO_4 for 4 weeks, leaves were collected. Ultrathin sections were produced according to the procedure in Sridhar et al. (2005). Ultrathin sections were obtained using an ultramicrotome (POWTIME-XL), and observed using an H-7650 TEM.

Confocal laser scanning microscopy (CLSM) observation

For NO and H_2O_2 detection, roots of plants treated with 50 μM CuSO_4 (for 5 h) were monitored using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma) and H_2O_2 -sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA , Sigma, St Louis, MO, USA). Fluorescence was visualized at 488 nm using a confocal laser scanning microscope (LSM 700, Zeiss, Germany).

Effect of NO on rooting

For rooting investigation, 0, 50 and 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide potassium salt (cPTIO, an NO inhibitor) were added into 1/2 MS medium containing 50 μM CuSO_4 . The rooting rate (%) was calculated after 4 weeks of culture. Additionally, the phenotype of the WT and transgenic plants (T1 line) that were cultured on 1/2 MS medium containing 50 μM CuSO_4 , and supplemented with 100 μM cPTIO for 4 weeks was observed.

Measurements of NO and H_2O_2 contents

NO content was determined as described by Zhou et al. (2005). Powdered leaves (0.6 g) were mixed with 3 mL of 50 mM cool acetic acid buffer (pH 3.6, containing 4 % zinc diacetate), and centrifuged at 10,000 $\times g$ for 15 min at 4 °C. Activated charcoal (0.1 g) was added to the supernatant, which was then filtrated. Absorbance was determined at 540 nm. The NO content was calculated by comparison with a standard curve of NaNO_2 .

H_2O_2 contents were measured according to Zhang et al. (2009). Fresh leaves (0.5 g) were excised and ground to a powder in liquid nitrogen and extracted with 5 mL of 5 % trichloroacetic acid (TCA) and 0.15 g activated charcoal. The mixture was centrifuged at 10,000 $\times g$ for 20 min at 4 °C. The supernatant was filtered and used for determination of H_2O_2 . Powders of leaves (0.5 g) were extracted in 3 mL of 50 mM cool acetic acid buffer (pH 3.6, containing 4 % zinc diacetate) with 0.1 g of activated charcoal. The homogenates were centrifuged at 10,000 $\times g$ for 15 min at 4 °C. Absorbance was determined at 540 nm.

Chlorophyll content assay

The fresh leaves (0.5 g) were collected from plants exposed to 50 μM Cu for 4 weeks, and washed with 80 % acetone until the samples turned white. The supernatants were collected and the volume of the samples was fixed to 20 mL using 80 % acetone before being filtered. The

chlorophyll content was measured using a 722 s visible spectrophotometer 722 s (Shanghai, China) at the wavelengths of 663, 645 and 652 nm.

Measurement of SOD, CAT, APX activity and MDA content

Leaf samples (0.5 g) from plants 2 weeks after being treated with 50 μM Cu or Cu + 100 μM cPTIO were ground to a powder with liquid nitrogen respectively. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were measured as described previously (Guo et al. 2006). Measurement of malondialdehyde (MDA) content was conducted as described previously (Lei et al. 2007).

Quantitative real-time RT-PCR

Total RNA was extracted from the WT and transgenic plant shoots under conditions of 50 μM CuSO_4 , 100 μM SNP and 100 μM cPTIO for 5 h, respectively. cDNA was synthesized as described above. PCR was performed with SYBR premix ExTaq (TaKaRa Biotech, Dalian, China), and amplified under the following cycling conditions: 10 s at 95 $^\circ\text{C}$; followed by 40 cycles of 5 s at 95 $^\circ\text{C}$, 30 s at 60 $^\circ\text{C}$; and 1 s at 78 $^\circ\text{C}$ for plate reading. The β -tubulin gene was used for normalization of the qRT-PCR analysis. Primers for β -tubulin (GenBank accession number: KM591694) and functional genes *NR* (KM591683), *GR* (KM591685), *SOD* (KM591686), *CAT* (KM591687), *APX* (KM591688), *ARF8* (KM591689), *AUX1* (KM591690), *PIN* (KM591691), *ABCB* (KM591692) and *PGP* (KM591693) are shown in Table S1.

Statistical analysis

All experiments were repeated independently at least three times. Statistical analyses were carried out using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were compared using Student's *t* test. Differences were considered to be significant if $P < 0.05$.

Results

Overexpression of *ThMT3* increases Cu tolerance

We successfully transformed 13 independent transgenic plant lines, which were confirmed by PCR (Fig. 1a) and RT-PCR analyses (Fig. 1b). Under Cu conditions, plant heights, root growth, and rooting rates of all plants decreased as the Cu concentration increased, however, the plant heights and rooting rates were higher, and the root

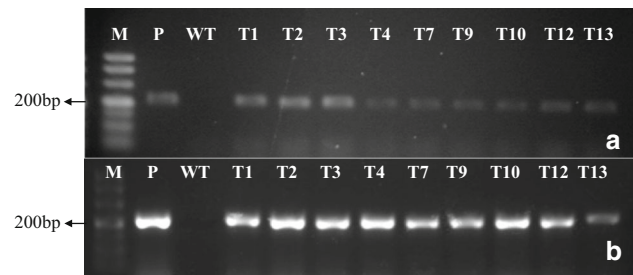


Fig. 1 Molecular analysis of transgenic plants by PCR **a** and RT-PCR **b** analysis. *M* Molecular marker DL500 (*up*). Marker DL2000 (*down*), *P* positive control (PROKII plasmid DNA), *WT* wild type, *T1–T13* Transgenic lines

length was longer, in transgenic plants than in WT plants (Supplementary Fig. S1a–c). In particular, a 50 μM Cu supplement almost completely suppressed the induction of adventitious roots on WT shoots (Fig. 2a, left), while numerous adventitious roots were induced on the transgenic stem within 4 weeks (Fig. 2a right, b). Three plant lines showed the same phenotype. A histological analysis showed that no adventitious roots were induced from the stems of WT plants (Fig. 2c); however, many roots originated from the xylem of the transgenic plant stems and were of multicellular origin (Fig. 2d, e). Thus, we proposed that the *ThMT3* gene improves the rooting of transgenic plants under Cu stress.

The metal contents in the roots, stems and leaves were measured. However, the amounts of Cu in both plants were not significantly different, regardless of lower (Fig. 3a) or higher (Fig. 3b) Cd concentrations.

Oxidative damage caused by Cu

Reactive oxygen species (ROS) are always formed when stress stimulates the photosynthetic electron transport chain (Freeman et al. 2010). The production of ROS in leaves and roots was detected by in situ staining using stains sensitive to O_2^- and hydrogen peroxide (H_2O_2), respectively (Fig. 4). There was no difference between the WT and transgenic plant lines for ROS detection (Fig. 4a, c). The leaves and roots of the WT plant displayed higher O_2^- (Fig. 4b) and H_2O_2 (Fig. 4d) accumulation levels than those measured in three *ThMT3*-transgenic plant lines (T1, T2 and T3) under Cu stress. This suggested that Cu induced a less serious oxidative stress response in transgenic *S. matsudana*.

Overexpression of *ThMT3* protects chloroplasts under Cu stress

Chloroplasts of WT plants treated with 50 μM CuSO_4 for 4 weeks showed visible damage and contained more starch

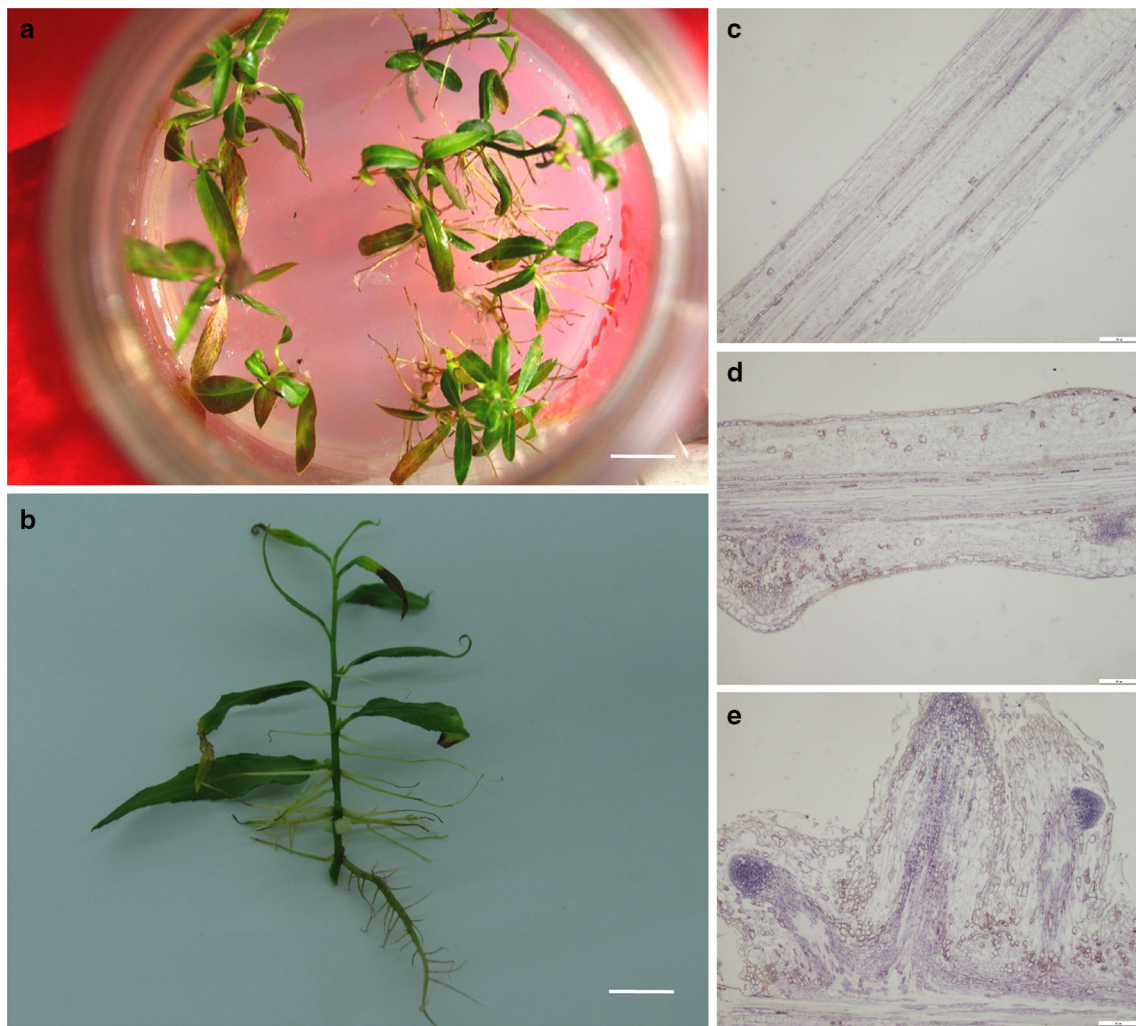


Fig. 2 The overexpression of *ThMT3* increase the rooting efficiency under Cu stress. **a** Wild-type (WT) and transgenic shoots were cultured on 1/2 MS medium containing 50 μM CuSO_4 for 4 weeks. *Left* WT shoots, *Right* transgenic shoots. *Bar* = 10 mm. **b** Transgenic

plant with adventitious roots. *Bar* = 10 mm. Histological observation of transgenic shoots before (**c**), 2 (**d**) and 4 weeks (**e**) after cultured on medium containing 50 μM CuSO_4 . *Bar* = 200 μm

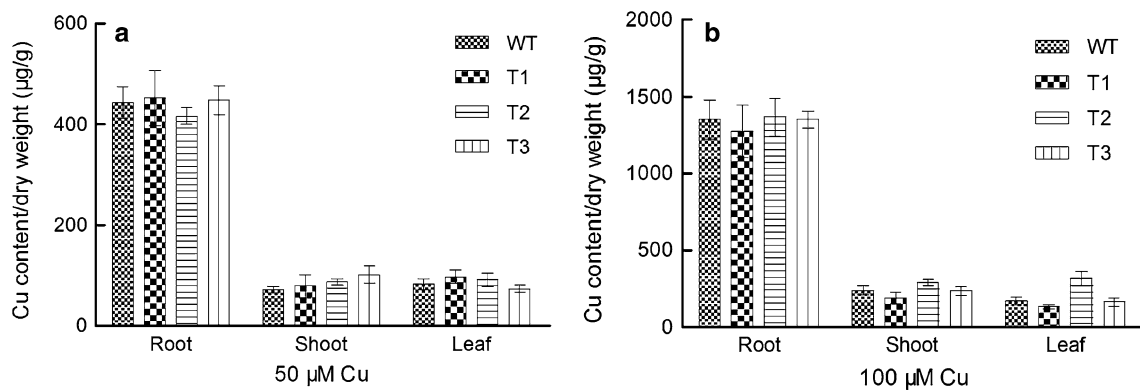


Fig. 3 Cu accumulation in wild-type (WT) and transgenic plants. Both WT and transgenic plants were cultured on 1/2 MS medium containing 50 μM (**a**) and 100 μM (**b**) CuSO_4 for 4 weeks. *T1–T3*

transgenic plant lines. Data are mean \pm SD from three independent experiments. *Significant (*t* test, $P < 0.05$) difference compared with WT plants under each treatment

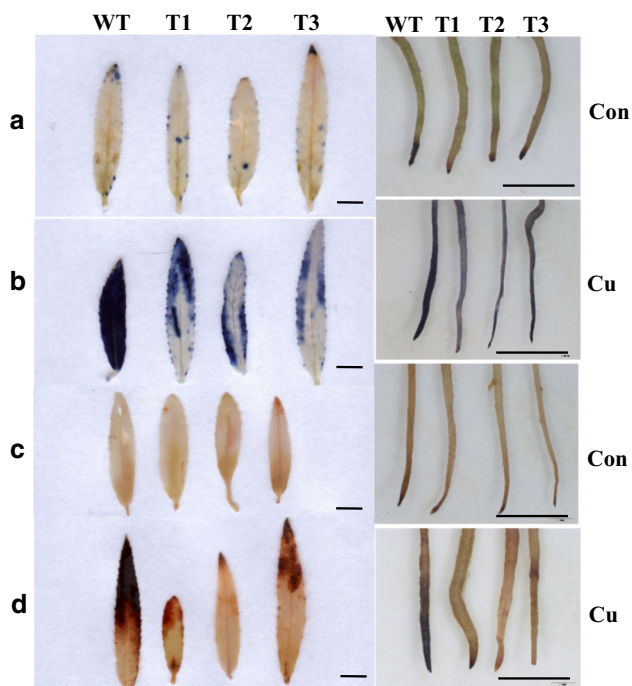


Fig. 4 Overexpression of *ThMT3* decreases ROS levels under Cu stress. Leaves and roots from wild-type (WT) and transgenic plants were pretreated with 50 μM and 100 μM CuSO_4 for 5 h, and were stained with NBT to visualize O_2^- without Cu (a) or with Cu stress (b), or stained with DAB to visualize H_2O_2 without Cu (c) or with Cu stress (d). T1–T3 transgenic plant lines. Bar = 3 mm

granules (Fig. 5a), while the chloroplasts of transgenic plants remained intact (Fig. 5b). When the transgenic plants were cultured in medium containing Cu and cPTIO, starch granules also appeared (Fig. 5c). After receiving Cu treatment for 4 weeks, the leaves yellowed and there was a dramatic decrease in the chlorophyll content of WT plants compared with the transgenic plants (Supplementary Fig. S2).

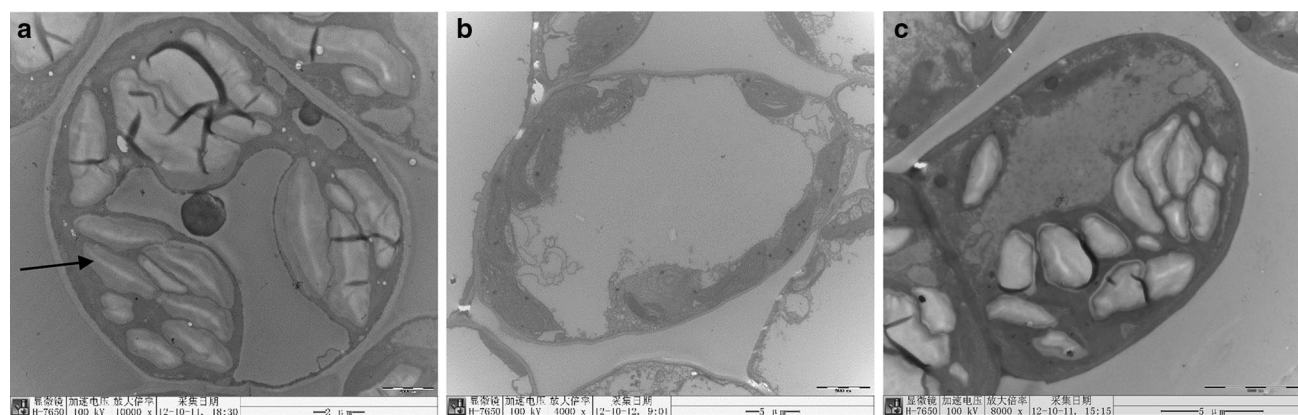


Fig. 5 Transmission electron microscopy observation of mesophyll cells. a mesophyll cells of WT plant cultured on 1/2 MS medium containing 50 μM CuSO_4 for 4 weeks. Bar = 2 μm . b mesophyll cells of transgenic plants cultured on 1/2 MS medium containing

Overexpression of *ThMT3* increases the NO content and rootings under Cu stress

For rooting investigation, 50 and 100 μM cPTIO were added into 1/2 MS medium containing 50 μM CuSO_4 . The results showed that, as the level of the NO inhibitor cPTIO increased, the rooting rate dramatically decreased in transgenic plants under 50 μM Cu stress, suggesting that NO was critically important for rooting in the *ThMT3*-transgenic plants (Supplementary Fig. S3a). No roots showed in the stems of transgenic plants with supplementary of cPTIO in the culture medium containing Cu (Supplementary Fig. S3c), representing the same phenotype as the WT (Supplementary Fig. S3b). Thus, we investigated the potential role of NO in transgenic plants. Only a small amount of NO fluorescence was visible in the roots of the WT (Fig. 6a) and transgenic plants without Cu treatment (Fig. 6b). After Cu treatment, the NO fluorescence increased in the transgenic plant roots compared with that in the WT plant roots (Fig. 6c, d). Without Cu treatment, there was no difference in H_2O_2 between non- (Fig. 6e) and transgenic plant cells (Fig. 6f), with both having low concentrations. After Cu treatment, higher H_2O_2 fluorescence levels appeared in non- (Fig. 6g) and transgenic plants (Fig. 6h). However, the H_2O_2 fluorescence in WT plants (Fig. 6g) was higher than that in transgenic plants (Fig. 6h). Additionally, the NO contents in the roots of non- and transgenic plants were not significantly different (Fig. 7a). However, after Cu treatment, the NO level in WT plants still remained unchanged, while the NO level in transgenic plants was extremely high (Fig. 7a), which was consistent with the NO fluorescence results. We presumed that the increased generation of NO in root cells of the transgenic plants is necessary to protect plants against Cu stress.

50 μM CuSO_4 for 4 weeks. Bar = 2 μm . c mesophyll cells of transgenic plants cultured on 1/2 MS medium containing 50 μM CuSO_4 and supplemented with 100 μM cPTIO for 4 weeks. Bar = 5 μm

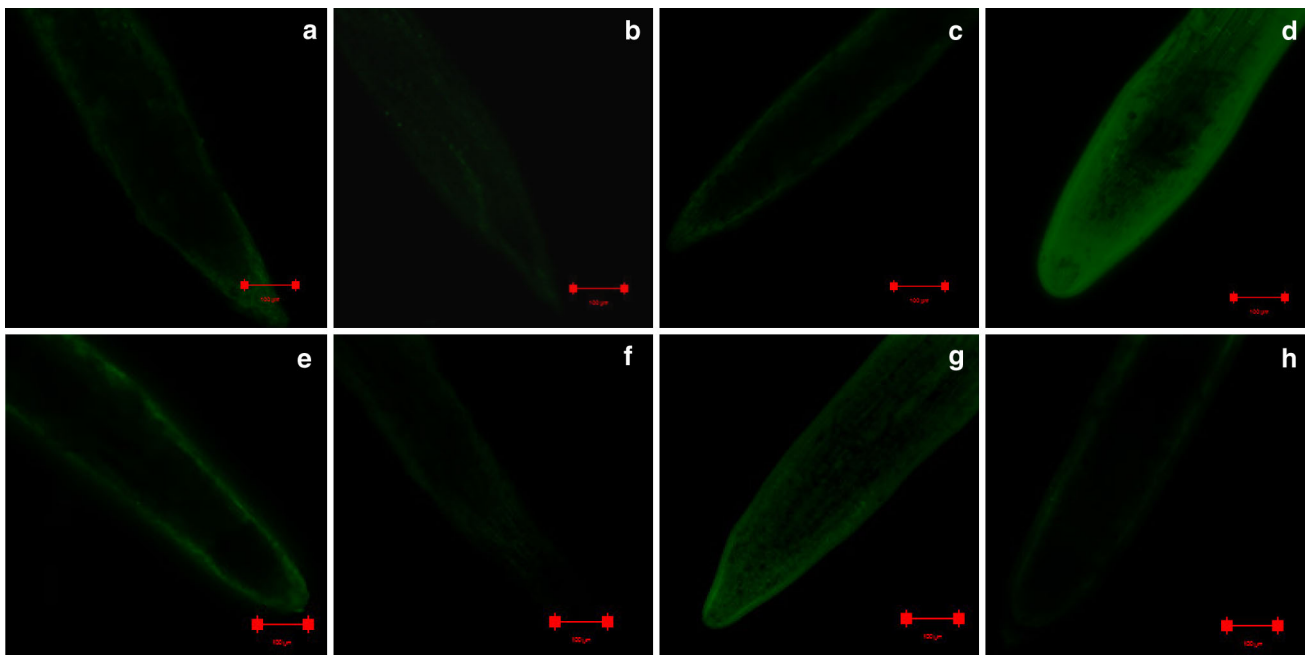


Fig. 6 Nitric oxide (NO) and hydrogen peroxide (H_2O_2) fluorescence observation under Cu stress. Confocal laser scanning microscopy (CLSM) observation of NO fluorescence in wild-type (WT) (a) and transgenic plant (b). NO fluorescence in WT (c) and transgenic plants

(d) treated with $50 \mu\text{M}$ CuSO_4 for 5 h. H_2O_2 fluorescence in WT (e) and transgenic plant (f) H_2O_2 fluorescence in WT (g), and transgenic plants (h) treated with $50 \mu\text{M}$ CuSO_4 for 5 h. Bar = $100 \mu\text{m}$

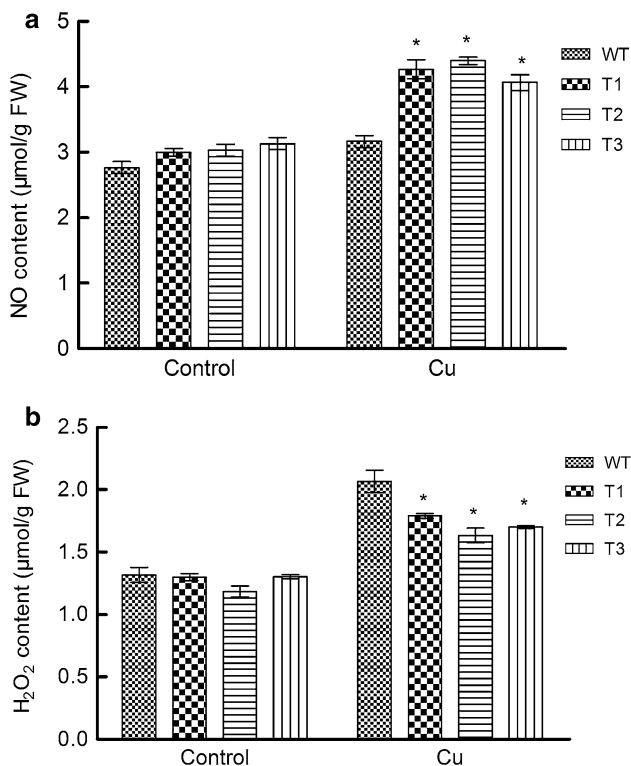


Fig. 7 Measurement of nitric oxide (NO) (a) and hydrogen peroxide (H_2O_2) (b) content in the roots under Cu stress. The roots from wild-type (WT) and transgenic plants were pretreated with $50 \mu\text{M}$ CuSO_4 for 5 h. Data are mean \pm SD from three independent experiments. *Significant (t test, $P < 0.05$) difference compared with WT plants

Overexpression of *ThMT3* decreased H_2O_2 by activating anti-oxidative enzymes under Cu stress

The H_2O_2 content in the roots of non- and transgenic plants were not significantly different (Fig. 7b). However, the H_2O_2 content in plant roots under Cu treatment was lower in transgenic plants than in WT plants, although both showed an increased H_2O_2 content in the roots (Fig. 7b), indicating the H_2O_2 was scavenged in the transgenic plants.

The *ThMT3*-transgenic plants showed significantly different SOD, CAT and APX activity levels, as demonstrated by three transgenic and WT plants under different conditions (Fig. 8a–c), indicating that there is a relationship between *ThMT3* expression and the anti-oxidant enzyme system. The activity levels in transgenic plant increased under Cu stress (Fig. 8a–c), but under the Cu + cPTIO conditions, the SOD, CAT and APX activities were not significantly different between non- and transgenic plants (Fig. 8 a–c). Cu stress also increased the MDA content in non- and transgenic plants (Fig. 8d), which reflected the degree of membrane lipid oxidation. However, the increased level in the transgenic plants was not significantly higher than that in the WT plants (Fig. 8d), indicating membrane lipid oxidation in WT plant similar to that in transgenic plants. Based on our results, we hypothesized that NO plays a central role in adventitious rooting in transgenic plants under Cu stress.

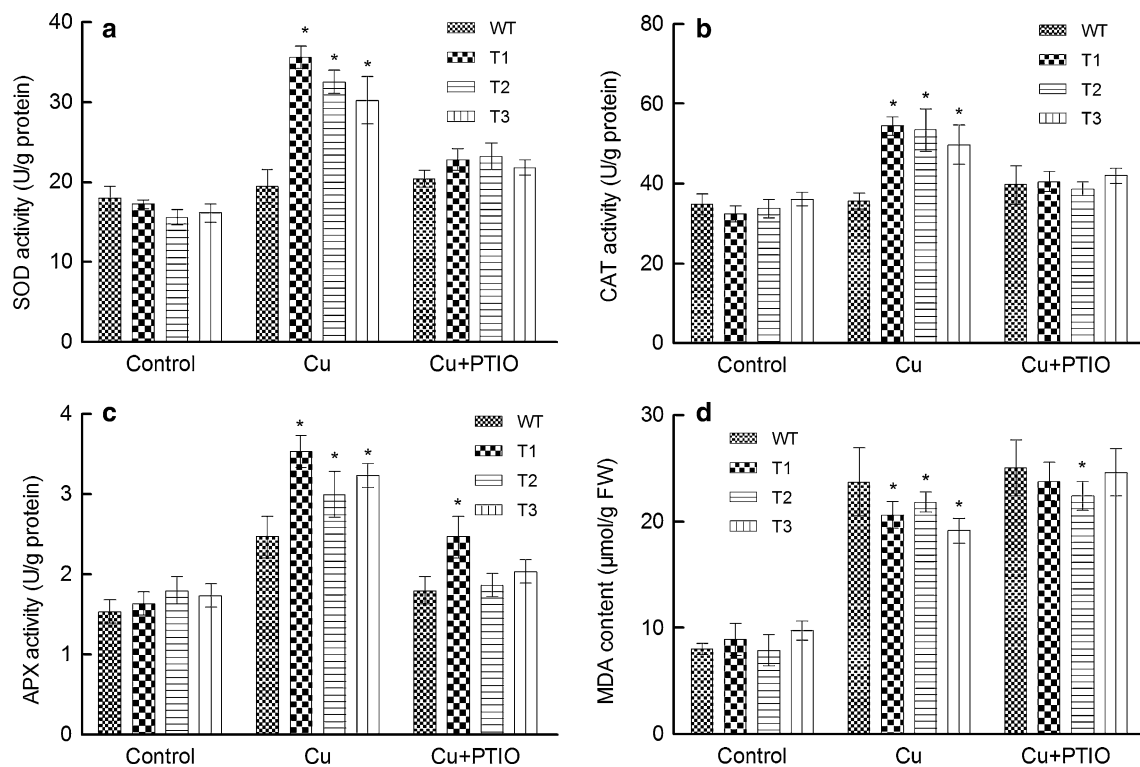


Fig. 8 Measurement of anti-oxidative activity in the roots under Cu stress. Both wild type (WT) and transgenic plants were cultured on 1/2 MS medium containing 50 μM CuSO_4 , 100 μM sodium nitroprusside (SNP), 50 μM Cu + 100 μM SNP, and 50 μM Cu + 100 μM cPTIO for 2 weeks. Activities of Superoxide

dismutase (SOD) **a** catalase (CAT) **b** ascorbate peroxidase (APX) **c** and malondialdehyde (MDA). Data are mean \pm SD from three independent experiments. *Significant (t test, $P < 0.05$) difference compared with WT plants under each treatment

Transcript levels of genes involved in NO biosynthesis, anti-oxidation and adventitious root formation under Cu stress

The expression of genes encoding the NO synthesis enzymes nitrate reductase (NR) and Nitric synthase (NOS) were induced in transgenic plants under Cu treatment, while the levels of these enzymes remained unchanged in WT plants after treatment (Fig. 9a). This suggested that NO was indeed induced in *ThMT3*-transgenic plants by the Cu treatment. Meanwhile, Cu significantly induced genes encoding anti-oxidative enzyme genes, including glutathione reductase (*GR*), *SOD*, *CAT* and *APX* in *ThMT3*-transgenic plants.

Auxin application effectively enhances adventitious root formation (Sukumar et al. 2013), and auxin response factors (ARFs) are transcription factors involved in auxin signaling. There are influx proteins, including auxin resistant 1 (*AUX1*), which mediate auxin movement (Swarup et al. 2008), and proteins, such as pinformed (*PIN1*), ATP-binding cassette type B (*ABCB*), P-glycoprotein (*PGP*) and multidrug resistant (*MDR*), which participate in auxin efflux (Zazimalová et al. 2010). Analysis of the expressions of the genes encoding these proteins showed that, *Auxin*

response factor 8 (*ARF8*), *AUX1* and *PIN1* were highly expressed in transgenic plants under Cu treatment (Fig. 9b) and SNP (Fig. 9c) treatment compared with WT plants, while the expression levels of *ABCB* and *PGP* did not change significantly (data not shown). After adding cPTIO, the expression levels of the genes were similar in transgenic and WT plants (Fig. 9d).

Discussion

In this study, we introduced a *ThMT3* gene from *T. hispida* into *S. matsudana* seeds following our previous reported transformation protocol (Yang et al. 2013) to improve the reported heavy metal tolerance of this important tree species. To the best of our knowledge, this is the first report of the recovery of transgenic *Salix* plants expressing a functional gene. Previous studies have reported transgenic MT-containing plants that displayed an enhanced tolerance to heavy metals (Bellion et al. 2007; Lee et al. 2004). In the current study, the transgenic plants showed better growth than WT plants when under Cu stress. Moreover, 50 μM Cu treatment damaged the chloroplasts of WT plants and increased the number of starch granules instead of thylakoids in the

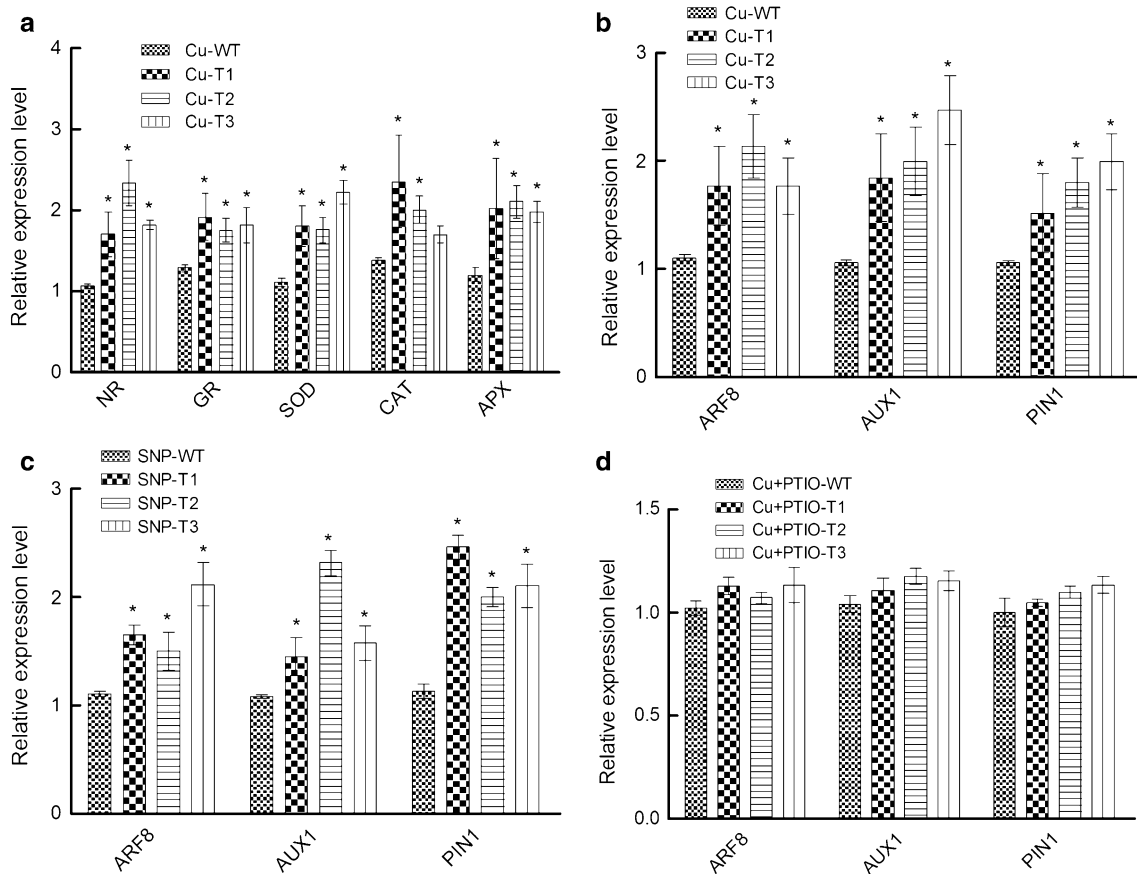


Fig. 9 Expression of NO synthesis, antioxidant and adventitious root formation related genes. **(a)** The transcript levels of *NR* (GeneBank accession number: KM591683), *GR* (KM591685), *SOD* (KM591686), *CAT* (KM591687), *APX* (KM591688) with treatment of 50 μ M CuSO_4 for 5 h, respectively. **(b, c, d)** The transcript levels of *ARF8* (KM591689), *AUX1* (KM591690), *PIN1* (KM591691) under

conditions of 50 μ M Cu **(b)** 100 μ M SNP **(c)** and 50 μ M Cu + 100 μ M cPTIO **(d)** for 5 h, respectively. The gene expression levels were normalized against the β -tubulin (KM591694) gene expression level. Data are mean \pm SD from three independent experiments. *Significant (*t* test, $P < 0.05$) difference compared with WT plants under each treatment

chloroplasts (Fig. 5a), whereas the chloroplasts of transgenic plants remained intact (Fig. 5b). These results suggested that the transgenic plants are Cu-tolerant. The results were consistent with our previous study that demonstrated that transgenic *Saccharomyces cerevisiae* over-expressing *ThMT3* had increased tolerance to Cu (Yang et al. 2011).

In this study, exposure to 50 μ M Cu induced more adventitious roots in transgenic plant stems than in WT plants. Treatment with the NO inhibitor cPTIO decreased the rooting rate of transgenic plants (Supplementary Fig. S3a), suggesting that NO release is related to Cu exposure in *ThMT3*-transgenic plants. However, NO may be an important element to improve the rooting rate. It was reported that Cu treatments caused NO release in the roots and leaves of tomato and NO can efficiently alleviate the Cu toxicity effect (Wang et al. 2010). NO is a radical molecule that participates in multiple plant physiological processes, such as seed germination, plant growth and

response to drought. Exogenous NO protects rice leaves from oxidative stress by increasing the activities of antioxidant enzymes (Hung et al. 2002). Generally, heavy metals cause the rapid release of ROS (Groppa et al. 2008). In the current study, the Cu stress induced a higher antioxidant enzyme activity. However, SOD, CAT and APX activities were similar between transgenic and WT plants when exposed to cPTIO, demonstrating that Cu can induce NO, and that NO may play a special role in Cu tolerance. MDA is one of the main products of lipid peroxidation and is highly toxic. An increased MDA level is an indicator of membrane damage, which is closely associated with the accumulation of ROS caused by stress (Cai et al. 2011). The decreased MDA levels in transgenic plants under Cd and Cu stress (Fig. 8d) were probably connected with the decreased H_2O_2 level that mediated lipid peroxidation. Meanwhile, MTs are thought to function as antioxidants against ROS (Yang et al. 2011). NOS and NR are two major NO synthetic enzymes, and we confirmed that their

expression levels increased in transgenic plants under lower Cu conditions, as confirmed by qRT-PCR results (Fig. 9a). This demonstrated that Cu indeed improved NO synthesis, and that NO probably plays a key role in Cu tolerance in transgenic *S. matsudana*. Additionally, Cu induced *GR*, *SOD*, *CAT* and *APX* gene expression levels (Fig. 9a), which further indicated that the anti-oxidative enzyme system is induced by Cu to increase tolerance.

Cu promoted adventitious roots growth on the stems of transgenic plants (Fig. 2a, b). The same result was reported by An et al. (2006), low concentrations of Cu in the culture solution enhanced root elongation and increased the lateral root number in *Pinus pinaster*. The development of the root system is an extremely complex physiological process, which is affected by many internal and environmental factors (Malamy 2005). One factor, the regulation of auxin transport, is essential for normal shoot and root growth. Polar auxin transport (PAT) is the main transport pattern in plants, and interfering with PAT results in the redistribution of endogenous auxins and inhibits gravity responses, which then affect adventitious root development (Geldner et al. 2001; Muday and Murphy 2002). In the current study, NO may have accumulated in transgenic *S. matsudana*, interfering with PAT to the hypocotyls, and causing accumulation of auxin in stems by an as yet unknown mechanism. Moreover, NO has been reported to regulate adventitious root initiation under the regulation of PAT (Xiong et al. 2009). *ARF8*, *AUX1* and *PIN1* in transgenic plants were highly expressed under Cu (Fig. 9b) or SNP (Fig. 9c) treatment, but did not respond to Cu and cPTIO condition (Fig. 9d), indicating that NO is related to the expression of these genes. In *Arabidopsis*, *ARF8* acts as a positive regulator in adventitious root formation (Gutierrez et al. 2009; Wilmoth et al. 2005). Other studies reported that defects in *AUX1*, *PIN1*, *ABCB19* and *PGP1* reduce initiation frequencies and/or the elongation of lateral roots (Lewis et al. 2011; Marchant et al. 2002; Swarup et al. 2008). However, in the current study, *ABCB28* and *PGP* expression levels in transgenic plants were not significantly different from non-treated plants, probably indicating that *ARF*, *AUX* and *PIN* are involved in adventitious root formation in *ThMT3*-transgenic *S. matsudana*, but not *ABCB* and *PGP*.

In conclusion, overexpression of the *T. hispida ThMT3* gene in *S. matsudana* effectively increased the Cu tolerances and rooting efficiency of the transgenic plants. The NO level in transgenic plants were significantly higher than in WT plants under Cu stress. The application of a NO inhibitor, cPTIO, decreased SOD, CAT and APX activity under Cu stress. Auxin response factors *ARF8* and auxin transport proteins, such as *AUX1* and *PIN1* which are known to improve adventitious roots, were highly expressed in transgenic plants under Cu and SNP treatments. These results suggested that overexpression of the *ThMT3*

gene increased Cu tolerance and NO production in *S. matsudana*, and the higher NO release contributed to the induction of adventitious roots under Cu stress.

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Conflict of Interest The authors declare no competing financial interests.

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