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# Involvements of $H_2O_2$ and metallothionein in NO-mediated tomato tolerance to copper toxicity

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ABSTRACT

Both $H_2O_2$ and NO are involved in multiple physiological responses in plants. Metallothionein (MT) can bind heavy metal ions and reduce metal toxicity. Copper toxicity has become a major problem with increasing agricultural and environmental pollution. Here, we investigated the possible roles of ROS, NO and metallothionein in tomato plant responses to copper toxicity. We found that $Cu^{2+}$ stress caused the rapid release of $H_2O_2$ and chlorotic leaves, and it stunted root growth and development. Cu treatment also caused an increase in NOS enzyme activity and NO release in roots and leaves. Application of the NO donor SNP efficiently alleviated the copper toxicity effect, as shown by increases in chlorophyll content and the biomass of fresh/dry leaves. SNP treatment also induced the transcription and increased activities of anti-oxidant enzymes, including catalase, peroxidase, superoxide dismutase and ascorbate peroxidase, and led to reduced $H_2O_2$ accumulation in the leaves. Special inhibitors or scavengers of NO synthesis diminished the ameliorating effect of NO on copper toxicity. NO application induced MT transcription and accumulation in leaves. Furthermore, the antisense-*MT* transgenic tomato was more sensitive to copper stress, and this effect could not be efficiently reversed by NO treatment. From these data, we propose that NO induces tomato tolerance to copper toxicity through antioxidant enzyme activity and metallothionein accumulation, and that metallothionein acts downstream of NO signaling.


## Introduction

Copper is an essential plant micronutrient that is a component of several transport enzymes and is involved in catalyzing the redox reaction in mitochondria and chloroplasts (Lewis et al., 2001). However, copper also induces toxicity at concentrations even slightly above the optimal level (Guo et al., 2009). Excess leaf copper can induce changes in photosynthetic and respiratory processes, enzyme activities, and DNA and membrane integrity (Hazen et al., 1988; Vinit-Dunand et al., 2002; Alaoui-Sossé et al., 2004; Lombardi and Sebastiani, 2005). Another important feature of copper toxicity is the induction of oxidative stress, as over-accumulation of copper can catalyze the generation of harmful reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide and hydroxyl radicals, all of which can damage biological molecules by lipid peroxidation (Demirevska-Kepova et al., 2004; Contreras et al., 2009).

Plants have evolved protective mechanisms to scavenge ROS and alleviate their deleterious effects. Several antioxidative enzymes, including catalase (CAT EC 1.11.1.6), peroxidase (POD EC 1.11.1.7), superoxide dismutase (SOD EC 1.15.1.1) and ascorbate peroxidase (APX EC 1.11.1.11), are reported to be involved in minimizing ROS damage or oxidative bursts during biotic or abiotic stress responses (Teisseire and Guy, 2000; Drążkiewicz et al., 2007; Posmyk et al., 2009). In addition to antioxidant enzymes, some small chemicals, such as melatonin, are also powerful antioxidants. Unlike other antioxidants, melatonin does not undergo redox cycling and cannot undergo repeated reduction and oxidation (Reiter, 1998; Mayo et al., 2003), and it is referred to as a terminal or suicidal antioxidant. Nitric oxide (NO) is a radical

*Abbreviations:* APX, ascorbate peroxidase; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide potassium salt; $F_0$, minimum chl fluorescence level; Fm, maximum chl fluorescence level; Fv, variable chl fluorescence; Fv/Fm, maximum quantum yield of PSII; SF, sodium ferricyanide; SNP, sodium nitroprusside; L-NAME, N-nitro-L-arginine methyl ester; MT, metallothionein; NO, nitric oxide; NOS, nitric oxide synthase; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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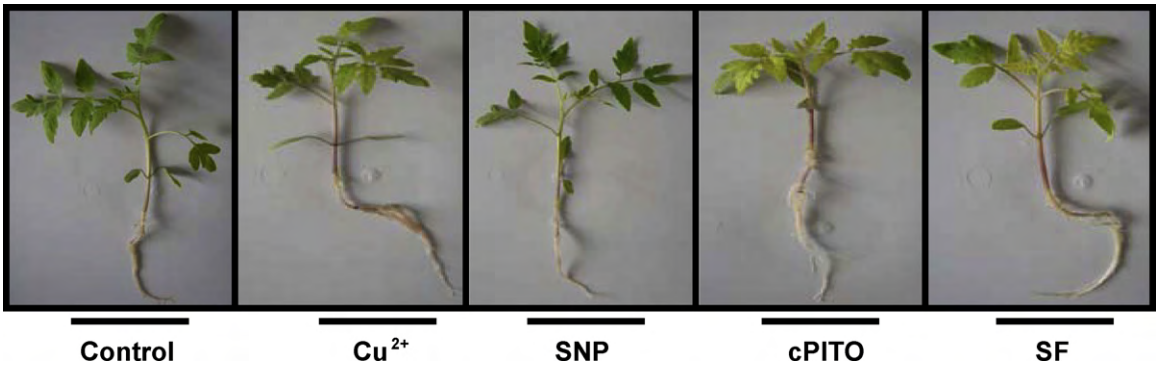


**Fig. 1.** Effects of $Cu^{2+}$, SNP, cPITO and SF on tomato growth and development. The tomato seedlings grew to three-leaf status in soil and were then moved into liquid medium with 1 μM $CuSO_4$ with or without 100 μM SNP, 100 μM cPITO, or 1 μM SF. Photos were taken after 1 week of treatment.

molecule that participates in multiple plant physiological processes (Seregélyes et al., 2003; Wendehenne et al., 2004; Arasimowicz and Floryszak-Wieczorek, 2007).

NO is also an important ROS-interacting signaling molecule (Delledonne et al., 2002). For example, exogenous NO has been shown to protect rice leaves from paraquat-induced oxidative stress by increasing the activities of antioxidant enzymes (Hung et al., 2002), and NO significantly improved the antioxidative capacity of wheat seeds germinated under osmotic stress by increasing the activities of CAT and APX (Zheng et al., 2009).

Metallothioneins (MT) are members of the family of cysteines, low molecular weight proteins. They have the ability to bind both physiological (such as zinc, copper, and selenium) and xenobiotic (such as cadmium, mercury, silver, and arsenic) heavy metals through their cysteine residue thiol groups, which account for nearly 30% of the amino acid content. Expression of metallothionein genes has been reported in a variety of senescing plant tissues, such as leaves and stems, ripening fruits, and wounded tissues, and has been proposed to function in both metal chaperoning and scavenging of ROS. However, direct evidence linking metallothionein metabolism to NO signaling in plant tolerance to heavy metal stress has yet to be demonstrated.

In this study, we investigated the relationships of NO, ROS and antioxidants in copper toxicity in the tomato plant. Our results indicate that NO treatment can significantly alleviate copper toxicity and that both $H_2O_2$ and metallothionein play essential roles during this process. Furthermore, transgenic antisense-*MT* tomato plants show increased sensitivity to copper stress and, unlike in control plants, their stress resistance cannot be efficiently rescued by the addition of sodium nitroprusside (SNP). Taken together, these data indicate a possible mechanism whereby NO signaling is linked to $H_2O_2$ and metallothionein during tomato plants' response to copper stress.

## Materials and methods

### *Chemicals*

Sodium nitroprusside(SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide potassium salt (cPTIO), N-nitro-L-arginine methyl ester (L-NAME), Tungstate were purchased from Sigma (St. Louis, MO, USA). SNP stock was made up as stock solution in distilled water at 100 μM; all the inhibitor was dissolved in water to dilute the indicated concentration when using. NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2DA) was obtained from Invitrogen Inc. (San Diego, CA 92121, USA). Most other chemicals were from Amresco Inc. (Cochran Solon, OH, USA).

### *Tomato and copper stress treatments*

Tomato (*Lycopersicon esculentum* Mill. cv. No. 4 Zhongshu) seeds were germinated on moisture filter paper in an incubator at 28 °C

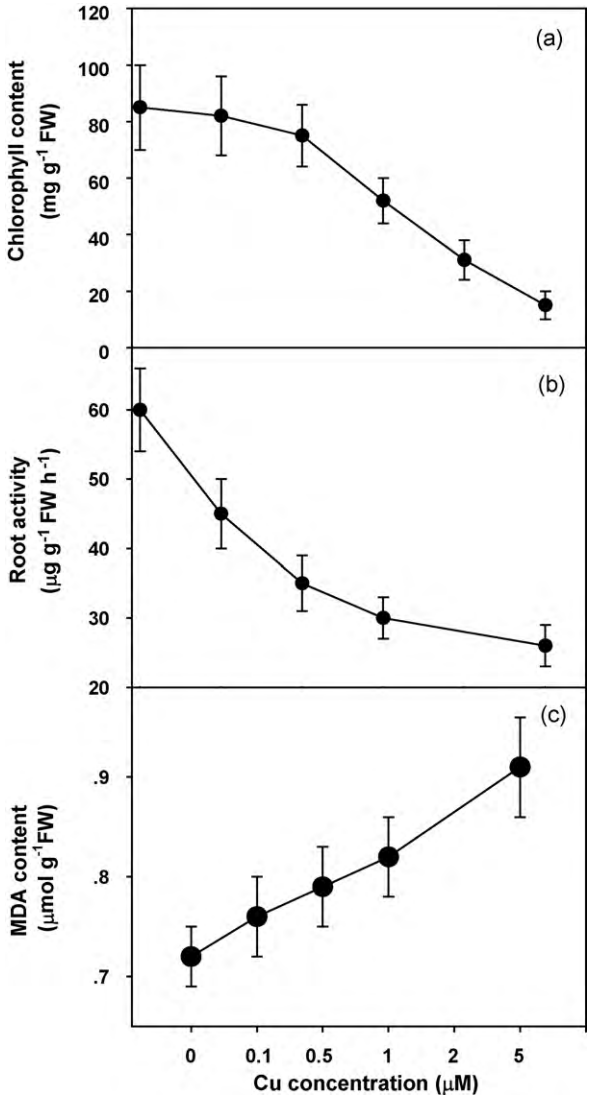


**Fig. 2.** Effects of different concentrations of $CuSO_4$ on tomato chlorophyll content (a), root activity (b) and MDA content (c). Three-week old tomato plants were grown with different concentrations of $CuSO_4$ for 6 days before analysis. Values are the mean ± SE for three independent experiments.

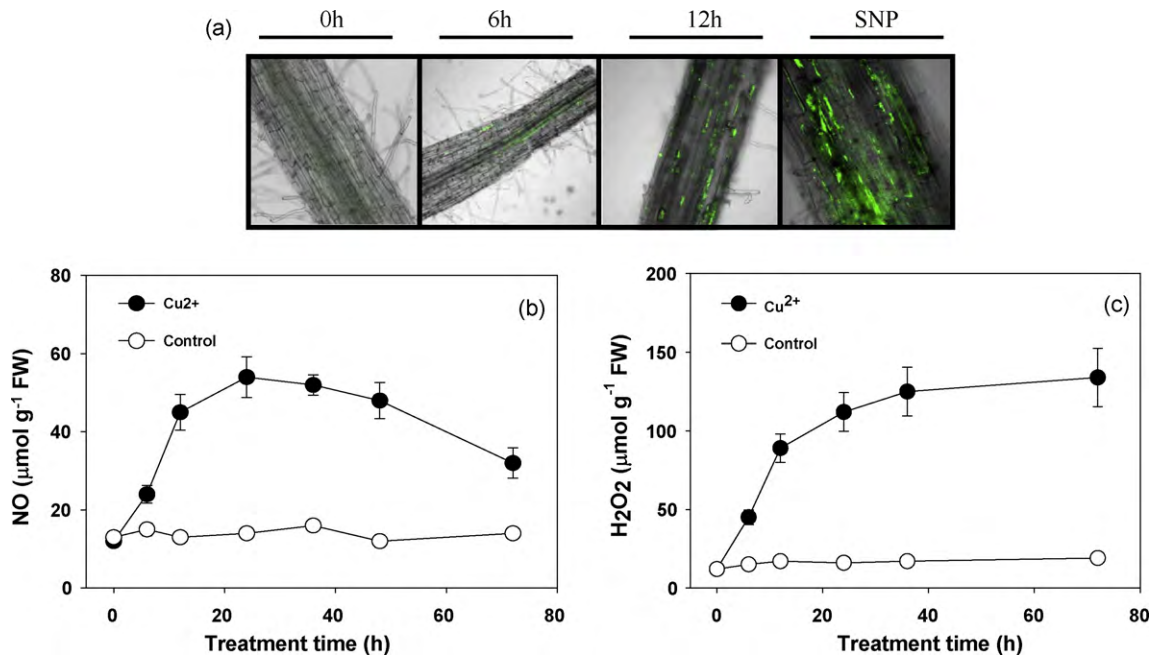


**Fig. 3.** Effects of $Cu^{2+}$ on $H_2O_2$ and NO accumulation in tomato seedlings. Three-week old tomatoes were grown with 1 μM $CuSO_4$. After 6 h or 12 h treatment, roots were stained with DAF and imaged using a confocal microscope. NO accumulation (a) and $H_2O_2$ accumulation (b) in the treated seedlings were measured at indicated time points. Values are the mean ± SE for five independent experiments

for 2 days, and sown in sands in the greenhouse. After 10 days, batches of 10 seedlings were transferred to a plastic tank with 10 L nutrient solution with pH 6.0–6.5 containing aerated full nutrient solution: $Ca(NO_3)_2$ 3.85 mM, $KNO_3$ 2.35 mM, $KH_2PO_4$ 1.36 mM, $MgSO_4$ 2 mM, $H_3BO_3$ 46.3 μM, $MnSO_4$ 9.55 μM, Fe–EDTA 50 μM, $ZnSO_4$ 0.76 μM, $H_2MoO_4$ 0.02 μM, $CuSO_4$ 0.32 μM. The experiment was carried out under natural conditions with an air temperature of 25–30 °C during the day and 18–25 °C during the night. The relative humidity in light and dark was 65–75%. When the tomato seedlings had 6–7 true leaves for about 3 weeks later, different concentrations of $CuSO_4$ were added into the nutrient solution for stress experiments, and at the indicated time, the samples of tomato seedlings were taken and immediately frozen in liquid nitrogen and stored at −80 °C until use. For inhibitory experiments, different concentrations of inhibitors/scavengers were added to the nutrient solution for 2 days before $CuSO_4$ treatment.

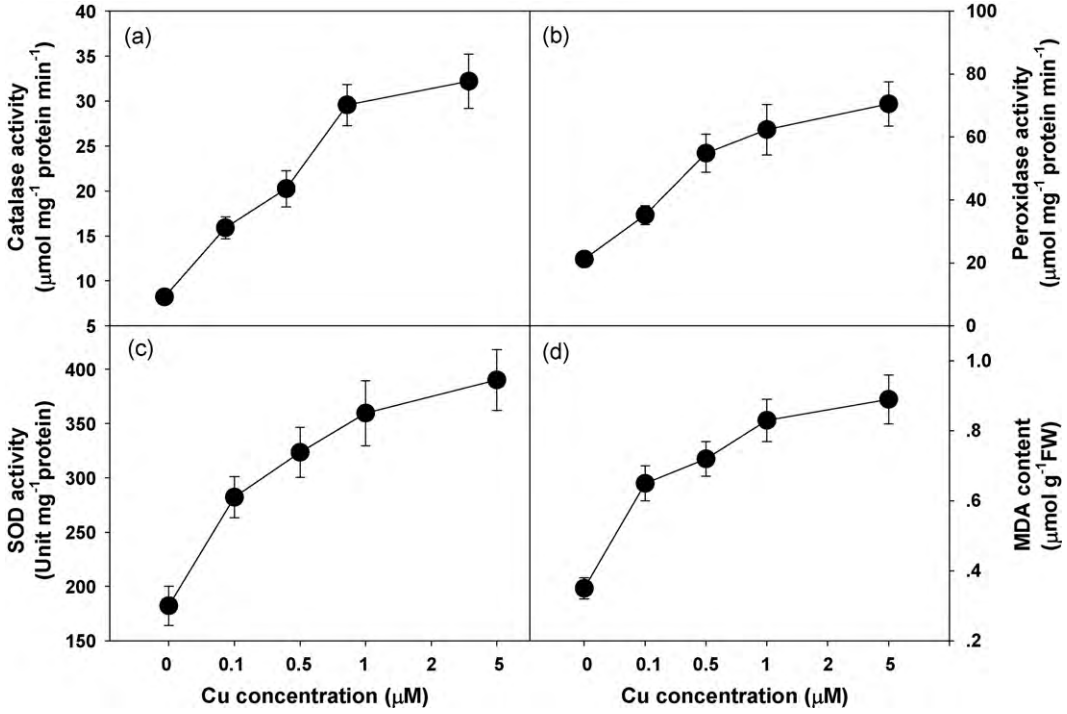


**Fig. 4.** Effects of different concentrations of $Cu^{2+}$ on antioxidant enzyme activities and MDA content. Three-week old tomatoes were grown with different concentrations of $CuSO_4$ for 6 days. Tomato leaves were assayed for activity of catalase (a), peroxidase (b) and SOD (c) enzyme activities and for MDA content (d). Values are the mean ± SE for three independent experiments.

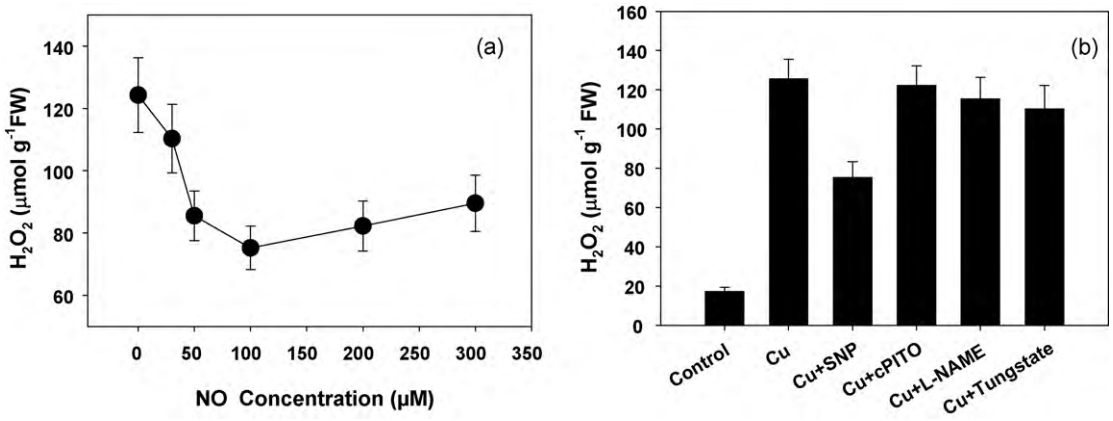


**Fig. 5.** Effects of NO on Cu-induced $H_2O_2$ accumulation in tomato seedlings. (a) Effects of different concentrations of NO on $H_2O_2$ generation in the tomato root. Three-week old tomato plants were grown with 300 M $CuSO_4$ with or without different concentrations of SNP, for 24 h and $H_2O_2$ content in the tomato leaves was measured. Values are the mean ± SE for three independent experiments. (b) Effects of different NO metabolism inhibitors on Cu-induced $H_2O_2$ generation in the tomato roots. Three-week old tomato plants were grown with 1 μM $CuSO_4$ with or without 100 μM SNP, 100 μM cPITO, 100 μM L-NAME, 50 μM tungstate for 24 h and the $H_2O_2$ content in the tomato leaves was measured. Values are the mean ± SE for three independent experiments.

*Chlorophyll content assays*

The fresh leaves (0.5 g) were put into the mortar, adding a spot of quartz sand. The samples were then skived as far as starchiness and skived sequentially by adding 80% of 5 mL acetone up until the samples turned white. The samples were transferred into a 25 mL container, at the same time washing the mortar and the draff repeatedly. Finally, the volume of the samples was fixed using 80% of 25 mL acetone and filtrated. Under the wavelengths of 663 nm, 645 nm, 652 nm, respectively, the content of chlorophyll was measured using a UV–vis Shimadzu 2450 spectrophotometer.

*Root activity assays*

Measurements of root activity were performed according to the triphenyltetrazolium chloride (TTC) method (Li, 2000). The surface liquid of white young roots was blotted with tissue paper and their fresh weights measured. Roots with weights 0.5 g were placed in tubes, filled with 5 mL of 0.4% triphenyltetrazolium chloride (TTC), 5 mL phosphate buffer (0.06 mol $L^{-1}$, pH 7.0). Control experiments (blank runs) were always carried out using the same procedure, but adding 2 mL of 1 mol $L^{-1}$ sulfuric acid first. The tubes were incubated at 37 °C for up to 4 h. The chemical reaction was stopped by adding 2 mL of 1 mol $L^{-1}$ sulfuric acid in the tubes. This step was followed by extraction with 10 mL of 95% ethanol for 24 h, which consisted of taking the root in a new tube. The OD values were recorded with a UV–vis Shimadzu 2450 spectrophotometer at 485 nm.

*MDA content and chlorophyll fluorescence assays*

Measurement of MDA content was performed according to the method of Health and Packer (1981). For chlorophyll measurement, dark-adapted and light-adapted measurements of chlorophyll fluorescence were conducted on the fourth or fifth fully expanded leaf of each plant using a portable chlorophyll fluorometer FMS 2 (Hansatech Instruments, Kings Lynn, UK). The minimal fluorescence (F0) emissions were assessed in leaves after 30 min of dark adaptation. The parameter Fm with all PSII reaction centers closed was determined by a 0.8 s saturating pulse at 6000 μmol $m^{-2}$ $s^{-1}$ in dark-adapted leaves. Then, the leaves were continuously illuminated with a white actinic light (400 μmol $m^{-2}$ $s^{-1}$), which was equivalent to the actual growth light of tomato plants, in order to measure the steady-state value of fluorescence (Fs) and a second saturating pulse at 6000 μmol $m^{-2}$ $s^{-1}$ was imposed to determine the maximal fluorescence level in the light-adapted state (Fm′). The minimal fluorescence level in the light-adapted state (Fo′) was determined by brief application (5 s) of a low-intensity (6 μmol $m^{-2}$ $s^{-1}$) far-red light (730 nm). Using these parameters, the following ratios were calculated: maximum PSII photochemical efficiency, Fv/Fm = (Fm − F0)/Fm, potential PSII photochemical efficiency, Fv/F0 = (Fm − F0)/F0, effective quantum yield of photochemical energy conversion in PSII, $\oint$PSII = (Fm′ − Fs)/Fm′. At least four repetitions were performed for each measurement.

*NO and $H_2O_2$ concentration measures*

A batch of 10 seedlings were ground to a fine powder after rapid freezing by liquid nitrogen and homogenized in extraction buffer (10 mM Tris–Cl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgSO_4$, 5 mM KCl, 5 mM NaCl, 10 μM oxyhemoglobin and 10 units/mL of catalase) in a ratio of 100 mg seeds/1 mL buffer. The extracts were centrifuged at 15,000 × g for 10 min at 4 °C and the supernatant collected and used for NO determination following the hemoglobin assay (Delledonne et al., 1998). Fluorescent probes were used to detect NO. The protocol included seeds subjected to different periods of desiccation or inhibitor/scavenger treatments and dipped in 10 μM 4-amino-5-methylamino-2,7-diaminofluorescein diacetate (DAF-FM), a specific cell permeable probe for NO, which accelerates seed absorption to the NO probe. Following a 30 min treatment, each seed embryo was removed to image NO accumulation with a Zeiss confocal laser-scanning microscope (LSM 510 META, Zeiss, Germany). $H_2O_2$ content was measured as previously reported (Hu et al., 2003).

*Antioxidant enzyme activity measures*

All operations were done at 4 °C. Frozen cell samples were ground to a fine powder with liquid nitrogen and extracted with ice-cold 50 mM phosphate buffer, pH 7.8, containing 0.1% polyvinylpyrrolidone(PVP). Cells (0.5 g) were homogenized in a mortar with 3 mL of phosphate buffer, and homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C. The resulting supernatant was stored at −80 °C. SOD activity was measured at 560 nm according to the Beauchamp and Fridovich method (1971), based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture contained 33 mM NBT, 10 mM methio-

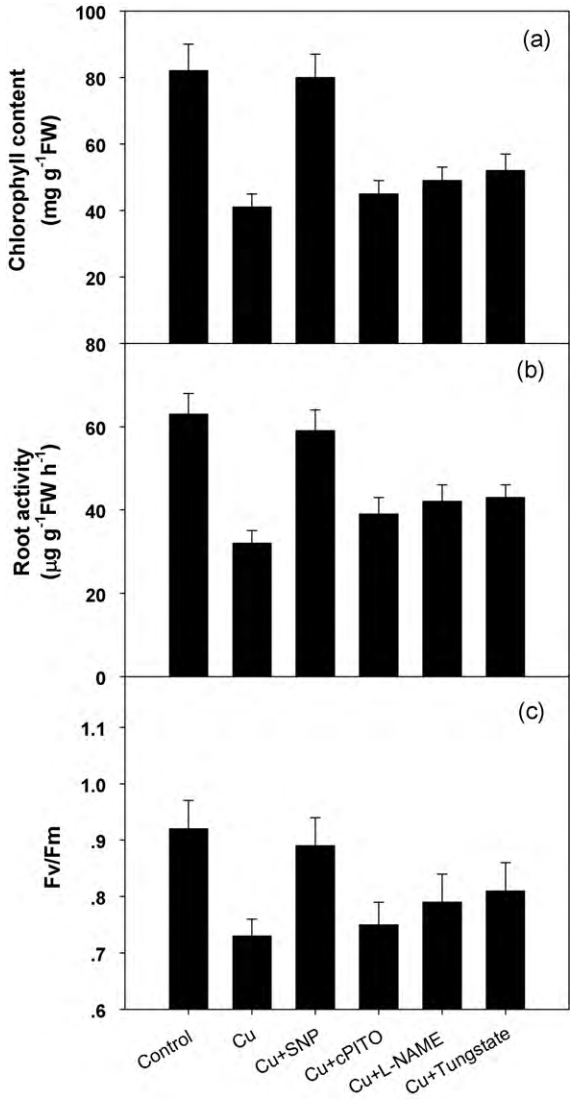


**Fig. 6.** Effects of SNP and NO scavengers and inhibitors on Cu-induced chlorophyll content, root activities and NO fluorescence. Three-week old tomato plants were grown with 1 μM $CuSO_4$ additional 100 μM SNP, 100 μM cPITO, 100 μM L-NAME, or 50 μM tungstate. After 6 days of treatment, tomato leaves or roots assayed for Cu-induced chlorophyll content (a), root activity (b) and NO fluorescence (c). Values are the mean ± SE for three independent experiments.

nine 0.66 mM EDTA and 3.3 mM riboflavin in 50 mM phosphate buffer, pH 7.8. Reactions were carried out at 25 °C for 30 min, at a light intensity of about 74 μmol $m^{-2}$ $s^{-1}$. One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of NBT reduction. Control experiments (blank runs) were always carried out using the same procedure, but in the absence of superoxide dismutase.

Catalase activity was measured using Aebi's (1984) method, in which the disappearance of hydrogen peroxide was monitored spectrophotometrically at 240 nm. The activity was defined as the amount of enzyme which oxidized $H_2O_2$ Amol/min/g fresh weight. Control experiments (blank runs) were always carried out using the same procedure, but in the absence of catalase.

The POD activity was based on the change of absorbance at 470 nm due to the formation of tetraguaicol, the product of the guaiacol oxidation (Doerge et al., 1997). Peroxidase assay medium contained 2.8 mL of phosphate buffer 100 mM (pH 6.0), 0.02 mL of enzyme preparation, 0.1 mL of the guaiacol solution 100 mM, and 0.1 mL of 2.0 mM $H_2O_2$ solution, at 25 °C. One unit of enzyme (U) was defined as the quantity of enzyme sufficient to produce tetraguaicol Amol/min/g fresh weight at 25 °C. Control experiments (blank runs) were always carried out using the same procedure, but in the absence of peroxidase.

For APX analysis, the extraction buffer contained 5 mM ascorbate. The homogenate was centrifuged at 10,000 rpm for 20 min, at 4 °C, and the supernatant was used for enzyme activity. APX activity was determined by measuring the decrease in absorbance of the oxidized ascorbate at 290 nm, according to Nakano and Asada (1980). The reaction mixture contained 0.5 mM ascorbate, 0.1 mM EDTA and 1.2 mM $H_2O_2$ in 50 mM phosphate buffer, pH 7.0. One unit of enzyme was expressed as Amol/min/g fresh weight of oxidized ascorbate, calculated using the extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$ for ascorbate. Control experiments (blank runs) were always carried out using the same procedure, but in the absence of ascorbate peroxidase.

*Transgenic antisense-MT tomato*

A 217 bp fragment from the tomato MT gene (GenBank: L77966) was amplified to use for the antisense construct (see the MT primes as below). The amplified fragment was fused in the reverse orientation to an enhanced CaMV35S promoter and nopaline synthase 3′-termination sequence; this construct was then cloned into the BamHI site at the right border of the pCGN1547 binary vector. The binary vector was transformed into *Agrobacterium tumefaciens* (LBA4404) by the freeze–thaw method. Transgenic tomato plants were generated by the method of McCormick et al. (1986). Transformed shoots were selected on Kanamycin (100 mg/mL). After rooting of shoots, the plantlets were transferred to sterile potting soil and gradually acclimated before transfer to the greenhouse.

*RNA gel blot analysis*

Total RNA was extracted and subjected to RNA gel analysis as described in the method of Maniatis et al. (1982). Total RNA (10 μg) extracted from seedlings was fractionated by electrophoresis on 14% agarose gels with formaldehyde and blotted onto nitrocellulose membrane. Pre-hybridization and hybridization were carried out as recommended in the Dig DNA labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany). To prepare the probe fragments, the primers below were used: (APX1-F:cgtgccattcgtttaacctt; APX1-R:tgggaaactgctccctaatg; CAT1-F:gcattgaaaccaaatccaa; CAT1-R:caacaccaatcgaccaactg; SOD1-F:ctggacttcacgggtttcat; SOD1-R:ttggagtcaaaccaaccaca; POD-F:cgtagttcgtgctggtgcta; POD-R:acatcttgcccttccaaatg; MT-F:gtggaggaaactgtggctgt; MT-R: ttgcacttgcagtcagatcc).

*Protein gel blot analysis*

Differential protein expression analysis was performed using the purified protein method for 2-DE as described above. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel (Hu et al., 2003). Gel proteins were transferred to a PVDF membrane using Bio-Rad mini transblot electrophoretic transfer cells. Filters were then probed with the primary antibodies and signals were detected using an ECL kit (GE company, USA). The primary polyclonal antibodies against plant MT are the products of Agrisera Inc. (Vännäs, Sweden).

## Results

*Copper treatment induced the rapid generation of NO and $H_2O_2$ and the increase in anti-oxidant enzyme activities*

To investigate the potential role of NO and $H_2O_2$ in the tomato response to copper stress, we first measured the effect of Cu ion

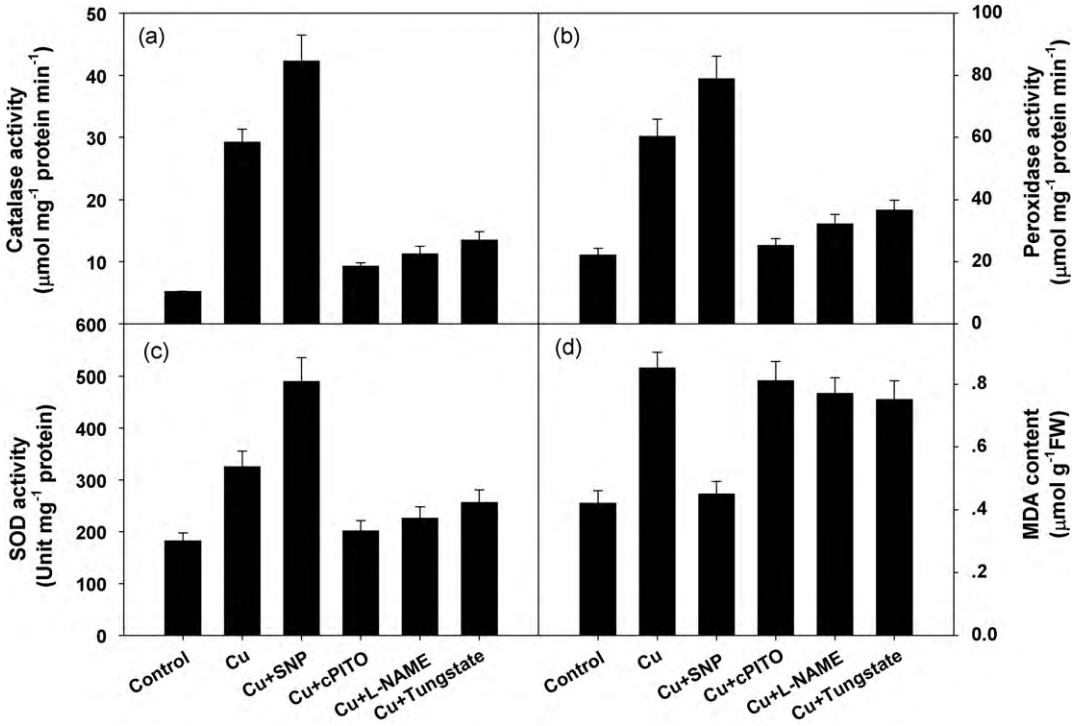


**Fig. 7.** Effects of SNP and NO scavengers and inhibitors on Cu-induced anti-oxidant enzyme activities. Three-week old tomato plants were grown with 11 μM $CuSO_4$ with or without 100 μM SNP, 100 μM cPITO, 100 μM L-NAME, or 50 μM tungstate. After 6 days of treatment, tomato leaves were assayed for catalase (a), peroxidase (b) and SOD (c) activities and MDA content (d). Values are the mean ± SE for three independent experiments.

stress on tomato growth and development, as shown in Fig. 1. One micromolar $CuSO_4$ treatment for 3 days caused yellowing of the leaves, accompanied by a dramatic decrease in chlorophyll content (Fig. 2a) and root activity (Fig. 2b). Cu stress also increased the MDA content (Fig. 2c), which reflects the degree of membrane lipid oxidation.

Stress also induced the rapid accumulation of $H_2O_2$ within the first 6 h and the concentration of $H_2O_2$ reached its maximum value after 48 h of treatment, and then decreased gradually over time (Fig. 3c). Consistent with the decrease in $H_2O_2$, Cu treatment also significantly activated the antioxidant enzymes activities including POD, SOD, APX and CAT, as shown in Fig. 4. Antioxidant enzymes increased with Cu ion concentration in a dose-dependent manner. Cu concentrations of 3 μM caused yellow blight in leaves' rapid plant death (picture not shown).

*NO treatment enhances tomato tolerance to copper stress*

To further confirm the function of NO during the tomato response to Cu stress, we applied different concentrations of the NO donor SNP solution to the copper-treated tomato plants. Treatment with 100 μM SNP efficiently reduced the toxic symptoms of Cu stress (Fig. 1), while the SNP analogue SF did not. NO treatment also efficiently reduced copper-induced $H_2O_2$ accumulation, as shown in Fig. 5a. While SNP at 100 μM showed the maximum effect on alleviating copper-induced $H_2O_2$ accumulation, concentrations of 30 μM and 50 μM also reduced copper-induced $H_2O_2$ content by 21 ± 3% and 48 ± 5%, respectively. SNP treatment also reduced copper-induced decreases in chlorophyll content and root activity, and repressed the copper-induced increase in MAD (Fig. 6). Finally, NO treatment dramatically increased copper-induced antioxidant

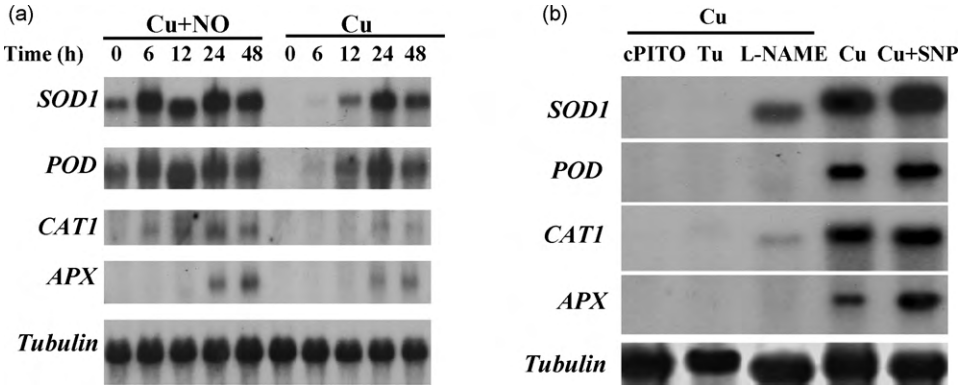


**Fig. 8.** Effects of NO and NO scavengers and inhibitors on Cu-induced anti-oxidant enzyme gene transcription. (a) Three-week old tomato plants were grown with 1 μM $CuSO_4$ with or without 100 μM SNP. *SOD1*, *POD*, *CAT1* and *APX1* mRNA levels were measured at the indicated times. (b) Three-week old tomato plants were grown with 1 μM $CuSO_4$ with or without 100 μM SNP, 100 μM cPITO, 100 μM L-NAME, or 50 μM tungstate. After 12 h of treatment, *SOD1*, *POD*, *CAT1* and *APX1* mRNA levels were measured.

enzyme activities (Fig. 7). We then investigated whether NO affects the anti-oxidant enzymes at the transcriptional level. As shown in Fig. 8a, Cu treatment induced the increased expression of *APX*, *POD*, *CAT1*, and *SOD1*, and addition of the NO donor SNP further improved increased transcription of these genes. These results are in agreement with the finding that NO treatment enhanced copper-induced antioxidant enzyme activities.

*The protective effect of NO against Cu stress is reversed by NO scavengers and inhibitors*

We next examined the role of NO in enhancing tomato tolerance to Cu stress. Pretreatment with the NO scavenger cPTIO strongly blocked the beneficial effect of SNP on copper-induced damage to tomato leaves (Fig. 1), repressed the SNP-induced increase of chlorophyll content and root activities (Fig. 2), and reversed the SNP-induced reduction of copper-induced $H_2O_2$ in tomato leaves (Fig. 5b). Similar results were observed in copper-treated tomato leaves treated with the NOS inhibitor L-NAME or the nitrate reductase (NR) inhibitor tungstate (Figs. 5–7). These results indicated that Cu-induced NO accumulation may be mediated by proteins similar to mammalian NOS enzymes and nitrate reductase. We also found that cPTIO, L-NAME and tungstate pretreatment suppressed the Cu-induced the transcription of *APX*, *POD*, *CAT1*, and *SOD1*. As shown in Fig. 8b, while Cu treatment increased the transcriptional levels of these genes, this effect could be efficiently suppressed by all three inhibitors, consistent with the previous findings.

*NO induces the expression of metallothionein to efficiently reduce the copper toxicity in tomato plants*

Because metallothionein proteins may provide protection against metal toxicity through binding physiological and xenobiotic heavy metals, we next examined the expression of MT. Cu treatment rapidly induced the transcription of MT (Fig. 9a), and additional NO treatment also further increased its expression, while cPITO, L-NAME and tungstate pretreatment prevented MT transcription (Fig. 9b). These data indicate that NO is an important signal for the activation of MT transcription. To further understand the possible relationship of MT and Cu tolerance in tomato, we generated a transgenic RNAi tomato line expressing an antisense metallothionein gene, as shown in Fig. 9c. The RNAi transgenic line showed less accumulation of MT protein after Cu treatment. To determine the relationship between $H_2O_2$, NO and MT during the tomato response to Cu stress, we measured NO fluorescence accumulation in the wild type control tomato line and the MT-antisense transgenic lines, and found that Cu treatment induced similar NO fluorescence intensity in the control line and in the MT-antisense transgenic lines (Fig. 10a). Furthermore, unlike the wild type plants, SNP treatment did not rescue chlorophyll content in Cu-treated transgenic lines (Fig. 10b). In addition, NO accumulation did not show a significant difference between the transgenic line and the wild type control (Fig. 10c), but $H_2O_2$ accumulation was higher in the MT-antisense transgenic lines than in the control plants (Fig. 10c).

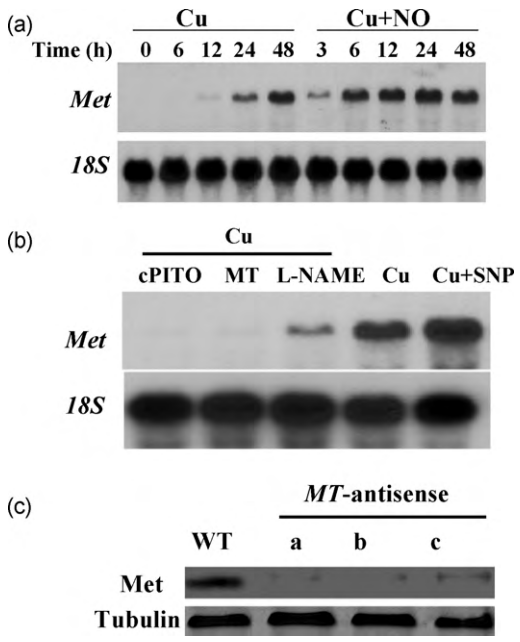


**Fig. 9.** Effects of SNP and NO scavengers and inhibitors on Cu-induced MT transcription. (a) Three-week old tomato plants were grown with 1 μM $CuSO_4$ with or without 100 μM SNP. *MT* mRNA levels were measured at the indicated times. (b) Three-week old tomato plants were grown with 1 μM $CuSO_4$ with or without 100 μM SNP, 100 μM cPITO, 100 μM L-NAME, or 50 μM tungstate. After 12 h of treatment, *MT* mRNA levels were measured. (c) MT protein accumulation in transgenic antisense-*MT* tomato lines. WT indicates untransformed control tomato plant; a, b, and c indicate different individual transgenic lines.

## Discussion

The data reported in this paper demonstrated the new signaling role for NO in mediating the response of tomato to copper stress. Reducing the ROS damage by NO through antioxidant enzyme activities and metallothionein protein both are crucial for the tomato tolerance to copper stress. Our conclusion is based on the following evidence: (i) The production of NO is an early and sustained response to copper stress and is primarily induced in root tissue. (ii) Removal of NO decreases, while addition of NO enhances tomato tolerance to copper stress. (iii) NO induced the activities of antioxidant enzymes and reduced ROS accumulation. (iv) The transgenic tomato with metallothionein antisense line, which is unable to generate metallothionein, is insensitive to NO and more sensitive to copper stress.

Production of NO is an early response to heavy metal stress such as cadmium stress (Ramirez and Gimenez, 2000; Kopyra and Gwóźdź, 2003). Here, we found that copper stress also induced the rapid accumulation of NO. The distribution of NO production was not restricted to root tissue; NO was also detected in the leaf tissue, which indicated a function of NO in addition to resistance to copper intake in the root, which coincided with the result that NO induced the activity of antioxidant enzymes in the leaves. The NO scavenger can efficiently degrade copper-induced NO accumulation, which indicated that copper-induced NO accumulation is special. With respect to NO generation, there are the enzyme independent pathway and enzyme-dependent pathway. For the enzyme-dependent pathway, nitrate reductase and NOS-like enzyme were also reported to be responsible for NO accumulation. Here, we found that the nitrate reductase inhibitor and NOS-like inhibitor can efficiently suppress copper-induced NO accumulation, which indicated that both nitrate reductase and NOS-like may be required for NO accumulation.

$H_2O_2$ is also an early signal in the plant stress response, but too high levels of accumulation without control also caused the by-product toxin effect in plants (Camp and Montagu, 1998; Lu et al., 2009). Here we found that copper stress induced the quickly and sustained generation in the root, and the $H_2O_2$ was always found at high levels with prolonged copper treatment. Many groups have reported that $H_2O_2$ induced NO accumulation in the *Arabidopsis* (Zhao et al., 2007). Previous studies have found that other heavy metals such as cadmium impair the growth of *Arabidopsis* seedlings (Cho and Seo, 2005). Here, we also found that copper suppressed the growth of tomato, and this effect was significantly reverted when

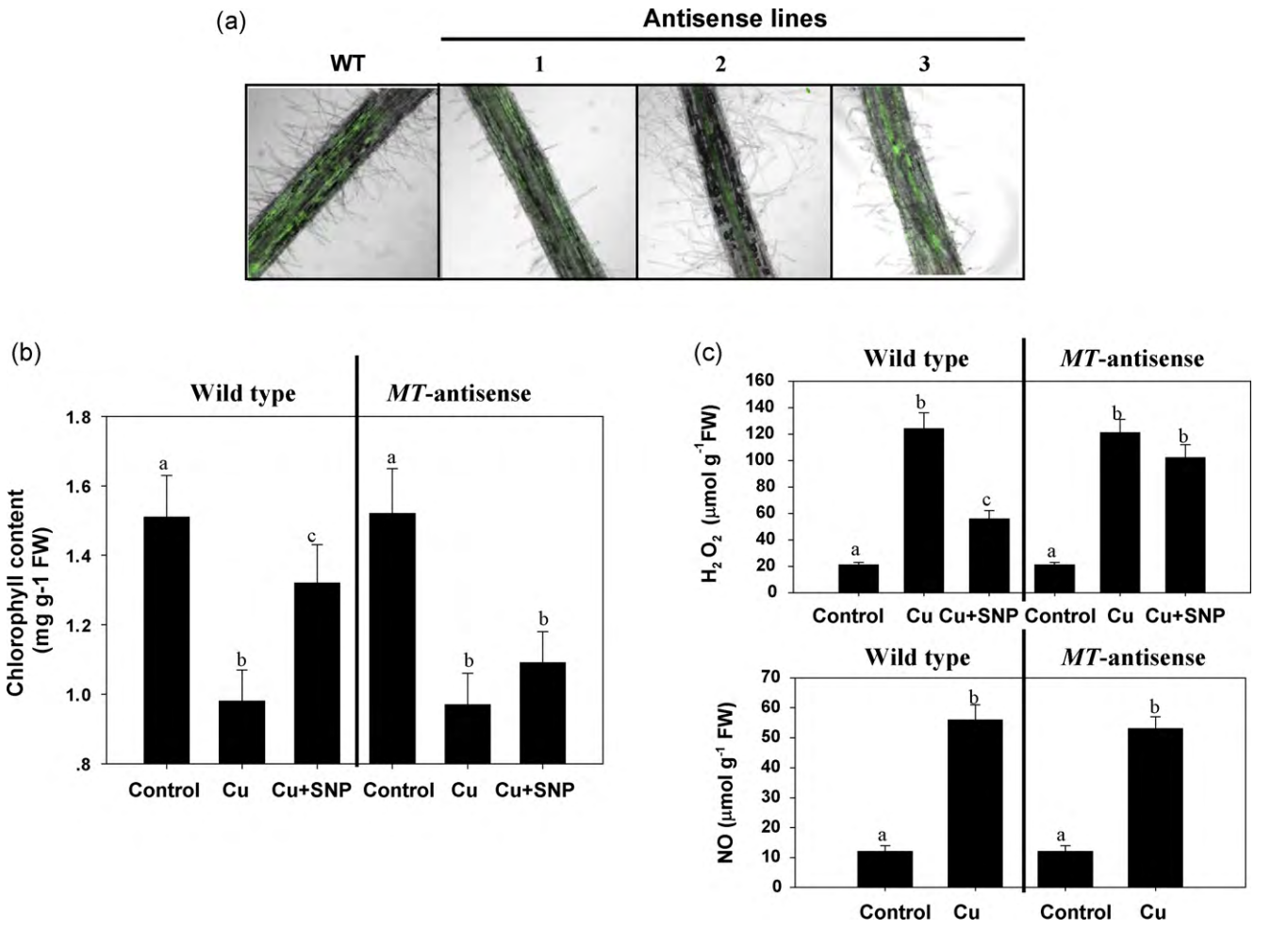


**Fig. 10.** Effects of MT on Cu-induced NO and ROS accumulation in transgenic antisense-*MT* lines. (a) Wild type control tomato and transgenic antisense-*MT* plants were treated with 1 μM $CuSO_4$ for 12 h, and NO fluorescence staining was observed by fluorescence. (b) Chlorophyll content in the leaves of three-week old tomato plants grown with 1 μM $CuSO_4$ with or without 100 μM SNP for 6 days. (c) $H_2O_2$ (upper) and NO (bottom) levels in three-week old tomato plants grown with 300 M $CuSO_4$ with or without 100 μM SNP for 12 h. Values are the mean ± SE for three independent experiments.

the copper-treated seedlings were cultured on the liquid medium supplemented with the NO scavenger cPTIO and L-NAME. A similar observation was recently reported in wheat roots (Groppa et al., 2008; Ling et al., 2009) and *Arabidopsis* seedlings (Martin et al., 2009). Although at least two mechanisms were proposed for the generation in the plant, here, we found that only L-NAME can reduce the effect.

The metabolism of ROS depends on antioxidant enzymes such as SOD, CAT and POD. In the present study, we found that NO reduced early increases in $H_2O_2$ content and lipid peroxidation in tomato leaves caused by excess Cu. These results are in agreement with previous work, in which we demonstrated that NO counteracted copper-increased lipid peroxidation in tomato leaves. We also found that NO treatment can significantly enhance the activities of antioxidants including POD, SOD and APX. Previous reports have also demonstrated that NO can enhance these activities during heavy metal stress (Yu et al., 2005; Shi et al., 2007; Madejón et al., 2009). Because lipid peroxidation is a consequence of AOS production, NO may act as an AOS scavenger in $CuSO_4$-treated tomato leaves.

Metallothioneins (MTs) are small, cysteine-rich, metal-binding proteins that are involved in metal homeostasis and detoxification in both plants and animals (Paris-Palacios et al., 2000; Zhang et al., 2005). Here, we also found that copper stress induced the transcription and expression of MTs, and moreover, that exogenous NO also induces significant accumulation of MTs. We also found that application of an NO scavenger or NOS inhibitor reduced copper-induced MT accumulation, indicating that NO mediates copper-induced MT generation. To further understand the possible function of MT during tomato response to copper treatment, we generated the MT-antisense transgenic line, which shows less MT protein accumulation after copper treatment compared with that in control wild type lines. We also found that SNP treatment cannot alleviate copper stress toxins to these antisense transgenic lines because the low chlorophyll content still remains after additional SNP treatment, but not in wild type lines. Further study showed that $H_2O_2$ accumulation cannot be scavenged after SNP treatment, though copper-induced NO accumulation did not show a difference. These results suggest that metallothioneins play the critical role for assuaging NO-mediated copper stress damage to tomato and metallothioneins should be functional upstream of $H_2O_2$ and downstream of the NO signal. Based on above results, we proposed one model (Fig. 11) to explain the possible role of NO during tomato tolerance to copper stress. In this model, we propose that copper-induced rapid $H_2O_2$ production as the early signal to induce defense response against copper damage. However, extremely high copper stress caused the over-accumulation of $H_2O_2$ and subsequently irreversible damage to tomato. Therefore, tomato evolved a new mechanism to scavenge the $H_2O_2$ over-accumulation through anti-oxidant enzymes or chemicals

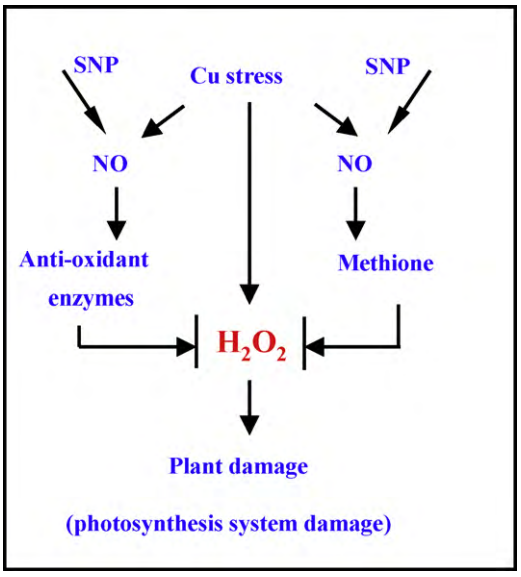


**Fig. 11.** Model for NO alleviation of copper toxicity via regulation of antioxidant enzymes and MT accumulation.

(such as metallothioneins), or secondary signals such as NO to induce accumulation of these anti-oxidant enzymes or chemicals. In our experiments, copper stress induced the $H_2O_2$ accumulation accompanying NO generation, and NO then further induced the antioxidant enzyme activities and metallothionein accumulation to antagonize $H_2O_2$ over-accumulation, and finally enhanced the tomato tolerance to copper stress. It is clear that the SNP cannot assuage copper stress to tomato when there is a shortage of metallothioneins in these transgenic lines to efficiently scavenge ROS over-accumulation.

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