Nitric oxide reduces Cu toxicity and Cu-induced NH_4^+ accumulation in rice leaves

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Summary

Nitric oxide (NO) is a highly reactive, membrane-permeable free radical, which has recently emerged as an important antioxidant. Here we investigated the protective effect of NO against the toxicity and NH_4^+ accumulation in rice leaves caused by excess CuSO_4 (10 mmol L^{-1}). It was found that free radical scavengers (sodium benzoate, thiourea, and reduced glutathione) reduced the toxicity and NH_4^+ accumulation in rice leaves caused by excess CuSO_4 . NO donor sodium nitroprusside (SNP) was also effective in reducing CuSO_4 -induced toxicity and NH_4^+ accumulation in rice leaves. The protective effect of SNP on the toxicity and NH_4^+ accumulation can be reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a NO scavenger, suggesting that the protective effect of SNP is attributable to NO released. Results obtained in the present study suggest that reduction of CuSO_4 -induced toxicity and NH_4^+ accumulation by SNP is most likely mediated through its ability to scavenge active oxygen species.

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Introduction

Copper (Cu) is an essential element in plants involved in various biochemical processes, but excess Cu (above 0.01 mmol L^{-1} in an incubation medium; Wainwright and Woolhouse, 1977) is

harmful to most plants (Fernandes and Henriques, 1991). Due to its widespread industrial and agricultural use, Cu pollution is a major environmental problem. Excess Cu can generate active oxygen species (AOS), such as H_2O_2 and $\text{O}_2^{\cdot -}$ (Girotti, 1985; Fernandes and Henriques, 1991; Chen et al., 2000)

Abbreviations: AOS, Active oxygen species; APX, Ascorbate peroxidase; CAT, Catalase; c-PTIO, 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide; FW, Fresh weight; GR, Glutathione reductase; GS, Glutamine synthetase; GSH, Reduced glutathione; MDA, Malondialdehyde; NO, Nitric oxide; PAL, Phenylalanine ammonia-lyase; PBN, N-Tert-butyl- α -phenylnitrone; POX, Peroxidase; SB, Sodium benzoate; SIN-1, 3-morpholinosydnonimine; SNP, Sodium nitroprusside; SOD, Superoxide dismutase; TU, Thiourea

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and is able to stimulate the production of $\bullet\text{HO}$ in a Fenton-type reaction (Sandmann and Böger, 1980). Cu-increased lipid peroxidation has been demonstrated in *Avena sativa* (Luna et al., 1994), *Brassica napus* (Baryla et al., 2000), *Helianthus annuus* (Gallego et al., 1996), *Holcus lanatus* (Hartley-Whitaker et al., 2001), *Lycopersicon esculentum* (Mazhoudi et al., 1997), *Nicotiana plumbaginifolia* (Savouré et al., 1999), *Oryza sativa* (Lindon and Henriques, 1993; Chen and Kao, 1999), *Phaseolus vulgaris* (Weckx and Clijsters, 1996; Cuypers et al., 2000), and *Silene cucubalus* (De Vos et al., 1993). Thus, excess Cu leads to oxidative stress in plant cells.

NH_4^+ is a central intermediate of nitrogen metabolism (Mifflin and Lea, 1976). Glutamine synthetase (GS) is the key enzyme in NH_4^+ assimilation and catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine (Mifflin and Lea, 1976). Previously, we demonstrated that NH_4^+ accumulation in rice leaves subjected to excess Cu is attributable to a decrease in GS activity (Chen and Kao, 1998). GS in plants has been reported to be particularly prone to degradation under oxidative stress conditions (Ortega et al., 1999; Palatnik et al., 1999; Chien et al., 2002; Ishida et al., 2002). In oat chloroplasts, it has been shown that specific activity of a thylakoid-bound endopeptidase increased under photooxidative environmental conditions or treatment with $\bullet\text{OH}$ or H_2O_2 generating systems (Casano and Trippi, 1992; Casano et al., 1990, 1994). Phenylalanine ammonia-lyase (PAL) catalyzes the elimination of NH_4^+ from phenylalanine and produces *trans*-cinnamate (Hahlbrock and Grisebach, 1979). NH_4^+ released in the PAL-catalyzed reaction is known to be trapped in the glutamine molecule by the action of GS (Razel et al., 1996). Sakurai et al. (2001) provided evidence showing that GS is partly coupled to the reaction of PAL in developing leaves. It has been shown that CdCl_2 treatment results in an increase in NH_4^+ accumulation, an increase in the specific activity of PAL and a decrease in the activity of GS in rice leaves (Hsu and Kao, 2004). Kumar and Knowles (2003) demonstrated that the specific activity of PAL induced by wounding is related to the ability to produce superoxide radicals. Previously, we have shown that NH_4^+ accumulation in rice leaves subjected to excess Cu is attributable to a decrease in GS activity and suggested, but did not prove, that NH_4^+ accumulation is part of an overall expression of oxidative damage caused by an excess of Cu (Chen and Kao, 1998).

Nitric oxide (NO) is a free radical involved in numerous and diverse physiological processes in mammals (Torreilles, 2001) and plants (Lamattina

et al., 2003). Evidence has been obtained for the involvement of NO in plant growth and development, as well as in defense responses (Durner and Klessig, 1999; Wojtaszek, 2000; Beligni and Lamattina, 2001; Lamattina et al., 2003). NO seems to exert a protective function also during abiotic stress. It is reported that NO increases drought tolerance of some species (Garcia-Mata and Lamattina, 2001; Cheng et al., 2002; Neill et al., 2002), salt stress in rice (Uchida et al., 2002), *Lupinus luteus* and reed (Zhao et al., 2004), heat stress in rice (Uchida et al., 2002), and Cd stress in rice (Hsu and Kao, 2004) and *Lupinus luteus* (Kopyra and Gwóźdz, 2003).

Several recent publications demonstrate that NO counteracts the toxicity of AOS generated by diquat or paraquat in potato and rice (Beligni and Lamattina, 1999; Hung et al., 2002) and oxidative stress induced by abscisic acid (Hung and Kao, 2003), methyl jasmonate (Hung and Kao, 2004), and excess Cd (Hsu and Kao, 2004). Orozco-Cárdenas and Ryan (2002) demonstrated that NO blocks H_2O_2 production induced by jasmonic acid in tomato leaves. In the present investigation, we examined the effect of NO on Cu toxicity and NH_4^+ accumulation caused by excess copper in rice leaves.

Material and methods

Plant material and chemicals

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. Seeds were then germinated in Petri dishes with wetted filter paper at 37 °C under dark conditions. After 48 h, uniformly germinated seeds were selected and cultivated in a 500 mL beaker containing half-strength Kimura B solution as described previously (Chu and Lee, 1989). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron with natural sunlight at 30 °C day/25 °C night and 90% relative humidity. The apical 3 cm of the third leaf were used in all experiments. A group of ten segments were floated in a Petri dish containing 10 mL of test solution and application was carried out at 27 °C in the light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Test solutions included CuSO_4 , NO donors, and the NO scavenger 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO). N-Tert-butyl- α -phenylnitrone (PBN), 3-morpholinonylamine (SIN-1), sodium nitroprusside (SNP) and a

solution containing ascorbic acid (Asc) and NaNO_2 were used as NO donors. All chemicals were purchased from Sigma Co (St. Louis, MO, USA).

Determination of protein, H_2O_2 , lipid peroxidation, NH_4^+ , and Cu

The toxicity of detached rice leaves exposed to CuSO_4 (10 mmol L^{-1}) was followed by monitoring decrease of protein content. For protein extraction, leaf segments were homogenized in a 50 mmol L^{-1} sodium phosphate buffer (pH 6.8). The extracts were centrifuged at $17,600g_n$ for 20 min and supernatants used for protein measurements after Bradford (1976) and for the determination of the activities of antioxidant enzymes. The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mmol L^{-1} , pH 6.5) containing 1 mmol L^{-1} hydroxylamine. The homogenate was centrifuged at $6000g_n$ for 25 min and the supernatant mixed with 0.1% titanium chloride in 20% (v/v) H_2SO_4 . The mixture was then centrifuged at $6000g_n$ for 25 min and absorbance measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient $0.28\text{ }\mu\text{mol}^{-1}\text{ cm}^{-1}$. Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). NH_4^+ was determined as described previously (Chien and Kao, 2000). For the determination of Cu, leaves were dried at 65°C for 48 h and ashed at 550°C for 48 h. The ash was incubated with 31% HNO_3 and 17.5% H_2O_2 at 70°C for 12 h and dissolved in 1 mol L^{-1} HNO_3 . Cu content was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto, Japan).

Enzyme assays

The assays of antioxidant enzymes have been described previously (Hurng and Kao, 1994). Peroxidase (POX) activity was measured using a modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient [$26.6\text{ (mmol L}^{-1})^{-1}\text{ cm}^{-1}$ at 470 nm] for tetraguaiacol. Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu, 1987). The activity was calculated using the extinction coefficient [$40\text{ (mmol L}^{-1})^{-1}\text{ cm}^{-1}$ at 240 nm] for H_2O_2 (Kato and Shimizu, 1987). Superoxide dismutase (SOD) and ascorbate peroxidase (APX) were determined ac-

cording to Paoletti et al. (1986) and Nakano and Asada (1981), respectively. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of activity for CAT, POX, SOD, APX, and GR was defined as the amount of enzyme which degraded $1\text{ }\mu\text{mol H}_2\text{O}_2$ per min, caused the formation of $1\text{ }\mu\text{mol tetraguaiacol}$ per min, inhibited 50% the rate of NADH oxidation observed in control, degraded $1\text{ }\mu\text{mol of ascorbic acid}$ per min, and decreased $1A_{340}$ per min, respectively.

For extraction of glutamine synthetase (GS), leaf samples were homogenized with 10 mmol L^{-1} Tris-HCl buffer (pH 7.6, containing 1 mmol L^{-1} MgCl_2 , 1 mmol L^{-1} EDTA and 1 mmol L^{-1} 2-mercaptoethanol) using a chilled pestle and mortar. The homogenate was centrifuged at $15,000g_n$ for 30 min and supernatants used for determination of GS activity after Oaks et al. (1980). One unit of GS activity is defined as $1\text{ }\mu\text{mol L-glutamate } \gamma\text{-monohydroxamate}$ formed per min. Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). One unit of PAL activity was defined as the amount of enzyme, which caused the formation of $1\text{ nmol trans-cinnamic acid}$ per h. For protease extraction, leaf samples were homogenized in a pre-chilled pestle and mortar with 10 mmol L^{-1} Tris-HCl buffer (pH 7.4) containing 10 mmol L^{-1} 2-mercaptoethanol at 4°C . The homogenate was centrifuged at $15,000g_n$ for 30 min and supernatants used for protease assay after Sheoran and Garg (1978). One unit of protease activity was defined as the amount of enzyme which increased A_{280} by 0.01 per h.

Statistical analysis

Statistical differences between measurements ($n = 4$) of different treatments were analyzed following Duncan's multiple range test or Student's *t*-test.

Results

In the present study, the toxicity in detached rice leaves caused by excess CuSO_4 was assessed by monitoring the decrease in protein content. In our previous work, we observed that CuSO_4 and CuCl_2 were equally effective in causing the loss of chlorophyll and protein in detached rice leaves in the light (Chen and Kao, 1999). In Fig. 1 the time courses of protein and MDA contents in detached rice leaves treated with either water or 10 mmol L^{-1} CuSO_4 in the light are shown. The

Figure 1. Changes in the contents of protein (A), MDA (B), H_2O_2 (C), and Cu (D) in rice leaves treated with CuSO_4 . Detached rice leaves were treated with either water or 10 mmol L^{-1} CuSO_4 in the light. Values are means $\pm$ SE ($n = 4$). Asterisks denote values that are significant at $P < 0.05$.

Figure 2. Effect of SB, TU, and GSH pre-treatments on the contents of protein (A), MDA (B), and H_2O_2 (C, D) in rice leaves treated with CuSO_4 . Detached rice leaves were pre-treated with water, 1 mmol L^{-1} SB, 5 mmol L^{-1} TU or 5 mmol L^{-1} GSH for 6 h and then treated with water or 10 mmol L^{-1} CuSO_4 for 18 h (A, B, D) or 2 h (C). Values are means $\pm$ SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

promotion of protein loss by CuSO_4 was evident 4 h after treatment (Fig. 1A). The MDA content in CuSO_4 -treated leaves was observed to be greater than that in water-treated controls throughout the entire duration of incubation (Fig. 1B), indicating that Cu toxicity in detached rice leaves was linked to lipid peroxidation. Lipid peroxidation is caused by AOS (Thompson et al., 1987). The H_2O_2 content remained unchanged for 4 h, and subsequently increased in control leaves (Fig. 1C) but rapidly increased at 2 h and then declined after CuSO_4 -treatment (Fig. 1C). This decline is most likely due to a Cu-catalyzed Fenton reaction (Sandmann and Böger, 1980). It appears that short-term treatment (2 h) with CuSO_4 increased the H_2O_2 content whereas long-term treatment (24 h) caused a decrease (Fig. 1C). To determine if the observed toxicity, lipid peroxidation and change in H_2O_2 was related to an increase of Cu in leaves, the internal Cu concentration was measured. The Cu content of control leaves remained unchanged during 24 h of

incubation in the light (Fig. 1D). However, Cu content in CuSO_4 -treated detached rice leaves increased with increasing duration of incubation (Fig. 1D). The increase in Cu content in CuSO_4 -treated leaves was evident 2 h after treatment (Fig. 1D).

When rice leaves were pre-treated with free radical scavengers such as SB, TU, and GSH, it was found that they inhibited the reduction in protein content (Fig. 2A) and the increase in MDA content (Fig. 2B) caused by CuSO_4 . They were also effective in preventing early increase (Fig. 2C) and late decrease (Fig. 2D) in H_2O_2 content caused by CuSO_4 . These results support the involvement of AOS as the chemical species inducing toxicity of rice leaves by excess Cu.

Plant cells are equipped with several AOS detoxifying enzymes to protect them against oxidative damage. These include SOD, APX, GR, CAT, and POX (Foyer et al., 1997). The striking increase in lipid peroxidation seen in rice leaves

Figure 3. Changes in the specific activities of SOD (A), APX (B), GR (C), CAT (D), and POX (E) in rice leaves treated with CuSO_4 . Detached rice leaves were treated with either water or 10 mmol L^{-1} CuSO_4 in the light. Values are means $\pm$ SE ($n = 4$). Asterisks denote values that are significant at $P < 0.05$.

Figure 4. Effect of SB, TU, and GSH pre-treatments on the specific activities of SOD (A), GR (B), and POX (C) in rice leaves treated with CuSO₄. Detached rice leaves were pre-treated with water, 1 mmol L⁻¹ SB, 5 mmol L⁻¹ TU or 5 mmol L⁻¹ GSH and then treated with either water or 10 mmol L⁻¹ CuSO₄ for 18 h. Values are means ± SE (*n* = 4). Values with the same letter are not significantly different at *P* < 0.05, according to Duncan's multiple range test.

treated with excess Cu may be a reflection of the changes of the specific activities of antioxidant enzymes. As shown in Figs. 3A and E, CuSO₄-treated rice leaves had higher specific activities of SOD and POX than the controls at 2 and 8 h, respectively, after treatment. Low GR specific activity in CuSO₄-treated rice leaves was observed at 2 h after incubation (Fig. 3C). However, higher APX specific activity in CuSO₄-treated rice leaves was observed

only at 24 h after treatment (Fig. 3B). Figure 3D shows that CuSO₄-treated rice leaves had higher CAT specific activity at 12 h and lower CAT specific activity at 24 h than the controls. Pre-treatment of free radical scavengers (SB, TU, and GSH) inhibited the increase in SOD and POX specific activities (Figs. 4A and C) and the decrease in GR specific activity (Fig. 4B) caused by excess Cu. These results further suggest that excess Cu causes strong oxidative stress in rice leaves.

There are reports that NO counteracts oxidative stress in plants (Beligni and Lamattina, 1999; Beligni et al., 2002; Cheng et al., 2002; Hung et al., 2002; Hung and Kao, 2003, 2004; Hsu and Kao, 2004). When rice leaves were pre-treated with NO donor SNP for 6 h, it was observed that the reduction in protein content (Fig. 5A) as well as the increase in MDA content (Fig. 5B) were inhibited. Similar results were obtained by using other NO donors such as PBN, SIN-1, and a mixture of Asc and NaNO₂ (data not shown). SNP pre-treatment was also observed to be effective in preventing early increase (Fig. 5C) and late decrease (Fig. 5D) in H₂O₂. Furthermore, SNP pre-treatment inhibited the increase in SOD and POX specific activities (Figs. 6A and C) and the decrease in GR specific activity (Fig. 6B) caused by excess Cu.

In the present investigation, NO donors rather than NO per se were used. In order to determine whether the protective effect caused by SNP pre-treatment was the result of a release of NO, 100 μmol L⁻¹ c-PTIO, a NO-specific scavenger, was applied together with 100 μmol L⁻¹ SNP. It was found that the protective effect of SNP on CuSO₄-induced toxicity, increase in MDA content, changes in H₂O₂ content and antioxidant enzyme specific activities could be reversed by c-PTIO (Figs. 5 and 6). Clearly, the effect of NO donor SNP is attributable to free NO.

Figure 7A shows that the increase in NH₄⁺ content by CuSO₄ was evident 8 h after treatment. NH₄⁺ accumulation caused by CuSO₄ was observed to be preceded by a decrease in the activity of GS and an increase in the specific activity of PAL and protease (Fig. 7B–D). When rice leaves were pretreated with free radical scavengers (SB, TU, and GSH), it was found that they inhibited the increase in the content of NH₄⁺ (Fig. 8A), the decrease in the activity of GS (Fig. 8B), and the increase in the specific activities of PAL and protease (Figs. 8C and D), suggesting that oxidative stress is involved in NH₄⁺ accumulation in CuSO₄-treated rice leaves. If this assumption is correct, then SNP pre-treatment can be expected to counteract the CuSO₄-induced increase in NH₄⁺ content, decrease in the activity of GS, and increase in the specific activities of PAL and

Figure 5. Effect of SNP pre-treatment on the contents of protein (A), MDA (B), and H_2O_2 (C, D) in rice leaves treated with CuSO_4 . Detached rice leaves were pre-treated with water, $100 \mu\text{mol L}^{-1}$ SNP or $100 \mu\text{mol L}^{-1}$ SNP + $100 \mu\text{mol L}^{-1}$ c-PTIO for 6 h and then treated with water or 10 mmol L^{-1} CuSO_4 for 18 h (A, B, D) or 2 h (C). Values are means $\pm$ SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

protease, and c-PTIO can be expected to reverse the SNP effect. As shown in Fig. 8, this is indeed the case.

Discussion

Copper-mediated AOS formation has been demonstrated in isolated chloroplasts of *Spinacia oleracea* (Sandmann and Böger, 1980), in intact roots of *Silene cucubalus* (De Vos et al., 1993) and *Oryza sativa* (Chen et al., 2000), in detached leaves of *Avena sativa* (Luna et al., 1994), and in intact leaves of *Phaseolus vulgaris* (Weckx and Clijsters, 1996). It has also been shown that excess Cu induced lipid peroxidation (De Vos et al., 1993; Luna et al., 1994; Gallego et al., 1996; Weckx and Clijsters, 1996; Mazhoudi et al., 1997; Chen and Kao, 1999; Savouré et al., 1999; Cuypers et al., 2000; Hartley-Whitaker et al., 2001). These findings suggest that excess Cu causes oxidative stress in plants. Our results presented here not only show that CuSO_4 rapidly increases the content of H_2O_2

(Fig. 1C), but also demonstrate that it causes an increase in the specific activities of SOD and POX and a decrease in the specific activity of GR (Figs. 3A, C, and E). Furthermore, protein loss and lipid peroxidation were observed in CuSO_4 -treated rice leaves (Figs. 1A and B). All these results suggest that excess Cu causes oxidative stress and that Cu-induced toxicity is mediated through oxidative stress. This suggestion is further supported by observations that free radical scavengers (SB, TU, and GSH) inhibit CuSO_4 -induced toxicity (Fig. 2A), lipid peroxidation (Fig. 2B), early increase in H_2O_2 content (Fig. 2C), increase in SOD and POX specific activities (Figs. 4A and C) and decrease in GR specific activities (Fig. 4B).

There is only limited information about the mechanism of Cu-induced H_2O_2 production. SOD is known to catalyze the dismutation of superoxide into H_2O_2 and O_2 . CuSO_4 treatment rapidly induced a significant increase in SOD specific activity in rice leaves (Fig. 3A). It appears that Cu-induced early accumulation of H_2O_2 in rice leaves may be, at least in part, due to Cu-enhanced SOD specific activity. In several plant systems, the accumulation of H_2O_2

Figure 6. Effect of SNP and SNP+c-PTIO pre-treatments on the specific activities of SOD (A), GR (B) and POX (C) in rice leaves treated with CuSO₄. Detached rice leaves were pre-treated with water, 100 $\mu\text{mol L}^{-1}$ SNP or 100 $\mu\text{mol L}^{-1}$ SNP+ 100 $\mu\text{mol L}^{-1}$ c-PTIO for 6 h and then treated with water or 10 mmol L^{-1} CuSO₄ for 18 h. Values are means $\pm$ SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

appears to be mediated by the activation of a plasma-membrane NADPH oxidase complex (Orozco-Cárdenas and Ryan, 1999; Pei et al., 2000; Zhang et al., 2001; Jiang and Zhang, 2002). In the present study, we have not investigated whether the early rapid H₂O₂ production is augmented by the stimulation of plasma-membrane NADPH oxidase. Further research is necessary to clarify this point.

In the present study, we found that NO reduced early increase in H₂O₂ content and lipid peroxidation in rice leaves caused by excess Cu (Figs. 5B and C). These results are in agreement with our previous work, in which we demonstrated that NO counteracted paraquat-increased lipid peroxidation in rice leaves (Hung et al., 2002). Because lipid peroxidation is a consequence of AOS production (Thompson et al., 1987), NO may act as an AOS scavenger in CuSO₄-treated rice leaves.

Ferrer and Barcelo (1999) demonstrated that the NO donor SNP (5 mmol L^{-1}) and NO (55 $\mu\text{mol L}^{-1} \text{ s}^{-1}$) itself were able to inhibit POX activity in the xylem of *Zinnia elegans*. However, our results show that SNP treatment alone did not affect the specific activity of POX in rice leaves (data not shown). Thus, the reduction of CuSO₄-induced increase in the specific activity of POX by SNP is unlikely due to a direct NO-mediated inhibition of POX.

Of particular interest in the present study is the finding that CuSO₄ treatment resulted in a decrease in the activity of GS and an increase in the specific activities of PAL and protease (Figs. 7B–D), which preceded the occurrence of NH₄⁺ accumulation (Fig. 8A). It appears that CuSO₄-induced NH₄⁺ accumulation is mediated through the increase in the specific activities of PAL and protease and the decrease in the activity of GS in rice leaves. NH₄⁺ released from the PAL reaction is known to be trapped in glutamine by the activity of GS (Sakurai et al., 2001). It has been shown that GS in plants is particularly prone to proteolysis under oxidative stress conditions (Ortega et al., 1999; Palatnik et al., 1999; Chien et al., 2002; Ishida et al., 2002), the specific activity of protease increases under photooxidative environmental conditions and treatment with $\cdot\text{OH}$ generating system or H₂O₂ (Casano and Trippi, 1992; Casano et al., 1990, 1994), and the specific activity of PAL induced by wounding is related to the ability to produce superoxide radicals (Kumar and Knowles, 2003). We have previously shown that paraquat, an AOS generating chemical, increased lipid peroxidation, decreased GS activity, and increased NH₄⁺ in rice leaves in the light (Chien et al., 2002). Here, we demonstrated that free radical scavengers (SB, TU, and GSH) and NO donor SNP were able to counteract the CuSO₄-induced decrease in the activity of GS (Fig. 8B), increase in the specific activities of PAL and protease, and increase in NH₄⁺ content (Fig. 8). It appears that changes in the content of NH₄⁺, the activity of GS, and the specific activities of PAL and protease in rice leaves caused by excess Cu are the consequence of oxidative damage.

In the present study, we clearly show that exogenous application of NO donor SNP counteracts

Figure 7. Changes in the content of NH_4^+ (A), the activity of GS (B), and the specific activities of PAL (C) and protease (D) in rice leaves treated with CuSO_4 . Detached rice leaves were treated with either water or 10 mmol L^{-1} CuSO_4 in the light. Values are means $\pm$ SE ($n = 4$). Asterisks denote values that are significant at $P < 0.05$.

Figure 8. Effect of SB, TU, GSH, SNP, and SNP+c-PTIO pre-treatments on the content of NH_4^+ (A), the activity of GS (B), and the specific activities of PAL (C) and protease (D) in rice leaves treated with CuSO_4 . Detached rice leaves were pre-treated with water, 1 mmol L^{-1} SB, 5 mmol L^{-1} TU, 5 mmol L^{-1} GSH, $100 \mu\text{mol L}^{-1}$ SNP or $100 \mu\text{mol L}^{-1}$ SNP+ $100 \mu\text{mol L}^{-1}$ c-PTIO for 6 h and then treated with water or 10 mmol L^{-1} CuSO_4 for 18 h. Values are means $\pm$ SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Cu-induced toxicity and NH_4^+ accumulation in rice leaves. It is not known if endogenous NO plays a role in regulating Cu-induced toxicity and NH_4^+ accumulation in rice leaves. To determine a possible involvement of endogenous NO in meliorating the effects caused by CuSO_4 , c-PTIO was used to deplete endogenous NO. This removal of NO did not increase toxicity symptoms and NH_4^+ accumulation (data not shown), indicating that endogenous NO plays no role in meliorating Cu-induced effects in leaves. Possibly, the amount of endogenous NO in rice leaves is too low to exert a protective role.

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References

- Baryl A, Laborde C, Montillet J-L, Triantaphylidés C, Chagvardieff P. Evaluation of lipid peroxidation as toxicity bioassay for plants exposed to copper. *Environ Pollut* 2000;109:131–5.
- Beligni MV, Lamattina L. Nitric oxide protects against cellular damage produced by methyl viologen herbicides in potato plants. *Nitric Oxide Biol Chem* 1999;3:199–208.
- Beligni MV, Lamattina L. Nitric oxide in plants: the history is just beginning. *Plant Cell Environ* 2001;24:267–78.
- Beligni MV, Fath A, Bethake PC, Lamattina L, Jones RL. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol* 2002;129:1642–50.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- Casano LM, Trippi VS. The effect of oxygen radicals on proteolysis in isolated chloroplasts. *Plant Cell Physiol* 1992;33:329–32.
- Casano LM, Gómez LD, Trippi VS. Oxygen- and light-induced proteolysis in isolated oat chloroplasts. *Plant Cell Physiol* 1990;31:377–82.
- Casano LM, Lascano HR, Trippi VS. Hydroxyl radicals and a thylakoid-bound endopeptidase are involved in light- and oxygen-induced proteolysis in oat chloroplasts. *Plant Cell Physiol* 1994;35:145–52.
- Chen L-M, Kao CH. Effect of excess copper on rice leaves: evidence for involvement of lipid peroxidation. *Bot Bull Acad Sin* 1999;40:283–7.
- Chen L-M, Lin CC, Kao CH. Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H_2O_2 level, and cell wall peroxidase activity in roots. *Bot Bull Acad Sin* 2000;41:99–103.
- Cheng F-Y, Hsu S-Y, Kao CH. Nitric oxide counteracts the senescence of detached rice leaves induced by dehydration and polyethylene glycol but not by sorbitol. *Plant Growth Regul* 2002;38:265–72.
- Chen LM, Kao CH. Relationship between ammonium accumulation and senescence of detached rice leaves caused by excess copper. *Plant Soil* 1998;200:169–73.
- Chien HF, Kao CH. Accumulation of ammonium in rice leaves is response to excess cadmium. *Plant Sci* 2000;156:111–5.
- Chien H-F, Lin CC, Wang J-W, Chen CT, Kao CH. Changes in ammonium ion content and glutamine synthetase activity in rice leaves caused by excess cadmium are a consequence of oxidative damage. *Plant Growth Regul* 2002;36:41–7.
- Chu C, Lee TM. The relationship between ethylene biosynthesis and chilling tolerance in seedlings of rice (*Oryza sativa* L.). *Bot Bull Acad Sin* 1989;30:263–73.
- Cuypers A, Yangonsveld J, Clijsters H. Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of *Phaseolus vulgaris* seedlings during the early stages of metal assimilation. *Physiol Plant* 2000;110:512–7.
- De Vos CHR, Ten Brookum WM, Vooijs R, Schat H, De Kok LJ. Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. *Plant Physiol Biochem* 1993;31:151–8.
- Durner J, Klessig DF. Nitric oxide as a signal in plants. *Curr Opin Plant Biol* 1999;2:369–74.
- Fernandes JC, Henriques FS. Biochemical, physiological, and structural effects of excess copper in plants. *Bot Rev* 1991;57:246–73.
- Ferrer MA, Barcelo AR. Differential effects of nitric oxide on peroxidase and H_2O_2 production by the xylem of *Zinnia elegans*. *Plant Cell Environ* 1999;22:891–7.
- Foster JS, Hess JL. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. *Plant Physiol* 1980;66:482–7.
- Foyer CH, Lopez-Delgado H, Dat JF, Scott IM. Hydrogen peroxide- and glutathione-associated mechanism of acclimatory stress tolerance and signaling. *Physiol Plant* 1997;100:241–54.
- Garcia-Mata C, Lamattina L. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiol* 2001;126:1196–204.
- Gallego SM, Benavides MP, Tomaro ML. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Sci* 1996;121:151–9.
- Girotti AW. Mechanism of lipid peroxidation. *J Free Rad Biol Med* 1985;1:87–95.
- Hahlbrock R, Grisebach H. Enzymic controls in the biosynthesis of lignin and flavonoids. *Annu Rev Plant Physiol* 1979;30:105–30.
- Hartley-Whitaker J, Ainsworth G, Meharg AA. Copper- and arsenate-induced oxidative stress in *Holcus*

- lanatus* L. clones with different sensitivity. *Plant Cell Environ* 2001;24:713–22.
- Heath RL, Packer L. Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 1968;125:189–98.
- Hsu YT, Kao CH. Cadmium toxicity is reduced by nitric oxide in rice leaves. *Plant Growth Regul* 2004;42:227–38.
- Hung KT, Kao CH. Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. *J Plant Physiol* 2003;160:871–9.
- Hung KT, Kao CH. Nitric oxide acts as an antioxidant and delays methyl jasmonate-induced senescence of rice leaves. *J Plant Physiol* 2004;161:43–52.
- Hung KT, Chang CJ, Kao CH. Paraquat toxicity is reduced by nitric oxide in rice leaves. *J Plant Physiol* 2002;159:159–66.
- Hung WP, Kao CH. Effect of flooding on the activities of some enzymes of activated oxygen metabolism, the levels of antioxidants, and lipid peroxidation in senescing tobacco leaves. *Plant Growth Regul* 1994;14:37–44.
- Hyodo H, Fujinami H. The effects of 2,5-norbornadiene on the induction of the activity of 1-aminocyclopropane-1-carboxylate synthase and of phenylalanine ammonia-lyase in wounded mesocarp tissue of *Cucurbita maxima*. *Plant Cell Physiol* 1989;30:857–60.
- Ishida H, Anzawa D, Kokubun N, Makino A, Mae T. Direct evidence for non-enzymatic fragmentation of chloroplastic glutamine synthetase by a reactive oxygen species. *Plant Cell Environ* 2002;25:625–31.
- Jana S, Choudhuri M. Glycolate Metabolism of three submerged aquatic angiosperms during aging. *Aquat Bot* 1981;12:345–54.
- Jiang M, Zhang J. Involvement of plasma-membrane NADPH oxidase in abscisic acid and water stress-induced antioxidant defense in leaves of maize seedlings. *Planta* 2002;215:1022–30.
- Kato M, Shimizu S. Chlorophyll metabolism in higher plant. VII. Chlorophyll degradation in senescencing tobacco leaves: phenolic-dependent peroxidative degradation. *Can J Bot* 1987;65:729–35.
- Kopyra M, Gwózdź FA. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiol Biochem* 2003;41:1011–7.
- Kumar GNM, Knowles NR. Wound-induced superoxide production and PAL activity decline with potato tuber age and wound healing ability. *Physiol Plant* 2003;117:108–17.
- Lamattina L, García-Mata C, Graziano M, Pagnussat G. Nitric oxide: The versatility of an extensive signal molecule. *Annu Rev Plant Biol* 2003;54:109–36.
- Lindon FC, Henriques FS. Copper-mediated oxygen toxicity in rice chloroplasts. *Photosynthetica* 1993;29:385–400.
- Luna CM, González CA, Trippi VS. Oxidative damage caused by an excess of copper in oat leaves. *Plant Cell Physiol* 1994;35:11–5.
- MacAdam JW, Nelson CJ, Sharp RE. Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiol* 1992;99:872–8.
- Mazhoudi S, Chaoui A, Ghorbal MH, El Ferjani E. Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum*, Mill). *Plant Sci* 1997;127:129–37.
- Mifflin BJ, Lea PJ. The pathway of nitrogen assimilation in plants. *Phytochemistry* 1976;15:873–85.
- Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 1981;22:867–80.
- Neill SJ, Desikan R, Clarke A, Hancock JT. Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. *Plant Physiol* 2002;128:13–6.
- Oaks A, Stulen I, Jones K, Winspear MJ, Boesel IL. Enzyme of nitrogen assimilation in maize roots. *Planta* 1980;148:477–84.
- Orozco-Cárdenas M, Ryan CA. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid. *Proc Natl Acad Sci USA* 1999;96:6553–7.
- Orozco-Cárdenas M, Ryan CA. Nitric oxide negatively modulates wound signaling in tomato plants. *Plant Physiol* 2002;130:487–93.
- Ortega JL, Roche D, Sengupta-Gopalan C. Oxidative turnover of soybean root glutamine synthetase. *In vitro* and *in vivo* studies. *Plant Physiol* 1999;119:1483–95.
- Palatnik JF, Carrillo N, Valle EM. The role of photosynthetic electron transport in the oxidative degradation of chloroplastic glutamine synthetase. *Plant Physiol* 1999;121:471–8.
- Paoletti F, Aldinucci D, Mocali A, Capparini A. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal Biochem* 1986;54:536–41.
- Pei Z, Murata Y, Benning G, Thomine S, Klusener B, Allen G, Grill E, Schroeder J. Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* 2000;406:731–4.
- Razel RA, Ellis S, Singh S, Lewis NG, Towers GH. Nitrogen recyclins in phenylpropanoid metabolism. *Phytochemistry* 1996;41:31–5.
- Sakurai N, Katayama Y, Yamaya T. Overlapping expression of cytosolic glutamine synthetase and phenylalanine ammonia-lyase in immature leaf blades of rice. *Physiol Plant* 2001;113:400–8.
- Sandmann G, Böger P. Copper-mediated lipid peroxidation processes in photosynthetic membranes. *Plant Physiol* 1980;66:797–800.
- Savouré A, Thorin D, Davey M, Hua X-J, Mauro S, Van Montagu M, Inzé D, Verruggen N. NaCl and CuSO₄ treatments trigger distinct oxidative defence mechanisms in *Nicotiana plumbaginifolia* L. *Plant Cell Environ* 1999;22:387–96.
- Sheoran IS, Garg OP. Effect of salinity on the activities of RNase, DNase and protease during germination and early seedling growth of mung bean. *Physiol Plant* 1978;44:171–4.

- Thompson JE, Legge RL, Barber RF. The role of free radical in senescence and wounding. *New Phytol* 1987;105:317–44.
- Torreilles J. Nitric oxide: one of the more conserved and widespread signaling molecules. *Front Biosci* 2001;6: 1161–72.
- Uchida A, Jagendorf AT, Hibino T, Takabe T. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Sci* 2002;163: 515–23.
- Wainwright SJ, Woolhouse HW. Some physiological aspects of copper and zinc tolerance in *Agrostis tenuis* Sibth: cell elongation and membrane damage. *J Exp Bot* 1977;28:1029–36.
- Weckx JEJ, Clijsters HMM. Oxidative damage and defense mechanism in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. *Physiol Plant* 1996;96:506–12.
- Wojtaszek P. Nitric oxide in plants: to NO or not to NO. *Phytochemistry* 2000;54:1–4.
- Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song C- P. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol* 2001;126: 1438–48.
- Zhao L, Zhang F, Guo J, Yang Y, Li B, Zhang L. Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. *Plant Physiol* 2004;134: 848–57.