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Endogenous nitric oxide mediates alleviation of cadmium toxicity induced by calcium in rice seedlings

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Abstract

The effect of calcium chloride ($CaCl_2$) on rice seedling growth under cadmium chloride ($CdCl_2$) stress, as well as the possible role of endogenous nitric oxide (NO) in this process, was studied. The growth of rice seedlings was seriously inhibited by $CdCl_2$, and the inhibition was significantly mitigated by $CaCl_2$. However, hemoglobin (Hb) and 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (cPTIO) weakened the promotion effect of $CaCl_2$. The results of NO fluorescence localization suggest that growth accelerated by $CaCl_2$ might be associated with elevated NO levels. The content of Cd, protein thiols (PBT), and nonprotein thiols (NPT) in cell walls, cell organelles, and soluble fractions, respectively, of rice seedlings decreased considerably in the presence of $CaCl_2$, whereas the content of pectin, hemicellulose 1 (HC1), and hemicellulose 2 (HC2) increased significantly. Elimination of endogenous NO in Cd+Ca treatment could promote the transportation of Cd^{2+} to cell organelles and soluble fractions and increase the content of NPT and PBT in leaves. In addition, transportation of Cd^{2+} to cell organelles and soluble fractions was retarded in roots, the content of NPT increased, and the content of PBT decreased. With elimination of endogenous NO in Cd+Ca treatment, the content of pectin, HC1, and HC2 decreased significantly. Thus, Ca may alleviate Cd toxicity via endogenous NO with variation in the levels of NPT, PBT, and matrix polysaccharides.

Key words: nitric oxide; cadmium; calcium; nonprotein thiols; protein thiols; matrix polysaccharides
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Introduction

Calcium ion (Ca^{2+}) and cadmium ion (Cd^{2+}) are divalent cations with radii of 9.9 and 9.7 nm, respectively. Thus, Ca^{2+} and Cd^{2+} are likely transported in the same manner into plant cells and organs. Ca^{2+} is not only a very important mineral nutrient in plants, but also a ubiquitous secondary messenger that mediates many aspects of cell and plant development, as well as stress resistance response (White and Broadley, 2003). For example, intracellular Ca^{2+} levels in plants are modulated in response to various signals, including hormones, light, and abiotic and biotic stresses (Evans et al., 2001; Rudd and Franklin-Tong, 2001; Arasimowicz and Floryszak-Wieczorek, 2007). A universal mechanism for Ca^{2+} signaling is the release of Ca^{2+} from the cell wall and intracellular compartment. How response specificity is regulated during Ca^{2+}-mediated signal transduction is an important biological issue, while the presence of Cd^{2+} in the plant environment disturbs the balance between calcium nutrition and signaling in plants (Wang and Song, 2009), diminishes the absorption of water and nutrients (Kahle, 1993), and induces the development of secondary oxidative stress (Zhang et al., 2005). As a result, plants show characteristic Cd stress responses.

Even though discovered recently, nitric oxide (NO) appears to be a universal messenger molecule, involved in many key physiological processes in plants (Pagnussat et al., 2002; Liu et al., 2009; Moreau et al., 2010). In addition, NO participates in environmental abiotic and biotic stimuli (Lamotte et al., 2004; Lozano-Juste and León, 2010). NO is able to modulate Ca^{2+} channels in the cell wall and implement oscillations in Ca^{2+} concentration (Besson-Bard et al., 2008). For instance, NO accelerates the release of Ca^{2+} from cell organelles to the cytoplasm in guard cells, which is accomplished through the activation of cyclic adenosine diphosphate ribose (cADPR) dependent Ca^{2+} channels (Garcia-Mata et al., 2003). The intracellular concentration of Ca^{2+} is also regulated by NO-activated Ca^{2+} channels in the plasma membrane (Lamotte et al., 2006). In leaves of pea plants, Cd^{2+} decreases the generation of NO, while the quenched NO is alleviated with supplementation of Ca^{2+} (Rodríguez-Serrano et al., 2009), indicating that NO and Ca^{2+} exhibit complex responses to each other. There are two pathways that produce the

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generation of endogenous NO in plants, that is, enzymatic and non-enzymatic pathways (Neill et al., 2003), thus in order to investigate the effect of endogenous NO in plants, the endogenous NO scavengers hemoglobin (Hb) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (cPTIO) were used. cPTIO, able to penetrate cell membranes and diffuse freely into the plant transporting system, serves as an endogenous NO scavenger, while Hb has also been employed as an absorption reagent for endogenous NO (Zhang et al., 2009). Additionally, lanthanum chloride ($LaCl_3$) was used as a Ca^{2+}-channel inhibiter (Perfus-Barbeoch et al., 2002) in order to evaluate the effect of Cd on the Ca^{2+}-channel in the present study.

Many studies have found that the toxicity of Cd^{2+} can be alleviated by application of Ca^{2+} (Fuhrer, 1983; El-Enany, 1995; He et al., 2005; Suzuki, 2005; Österås and Greger, 2006), but the mechanism is not yet understood. Recent studies suggest that NO participates in the flux of Ca^{2+} (Wang et al., 2009) and Cd^{2+} (Besson-Bard et al., 2009). The present investigation was conducted to determine whether Ca^{2+} accelerates the growth of rice against Cd stress and how NO signaling is involved in the alleviation process.

1 Materials and methods

1.1 Plant material, growth conditions and treatments

Seeds of rice (*Oryza sativa* cv. Xiushui 11) were surface-sterilized with 10% sodium hypochlorite for 15 min, rinsed thoroughly with distilled water, and then germinated in sterilized, moist filter papers at 37°C in darkness. After emergence of shoots, uniformly germinated seeds were selected and cultivated in quarter-strength Yoshida's rice nutrient solution (Yoshida et al., 1976). Plants were grown under a 14-hr photoperiod, a 30°C/24°C (day/night) thermoperiod, and 60% relative humidity. Uniform 1-week-old seedlings with 2 leaves were used in the experiments.

The following four treatments were applied: in treatment 1, seedlings treated with 100 μmol/L $CdCl_2$ were supplemented with 1, 5, and 10 mmol/L $CaCl_2$, respectively. Seedlings without any chemical treatments were used as control for this and the following treatments. After 1 week treatment, the length of crown root and shoot was recorded. In treatment 2, seedlings were treated with 100 μmol/L $CdCl_2$ (Cd), 5 mmol/L $CaCl_2$ (Ca), 1 mmol/L $LaCl_3$ (La), 100 μmol/L $CdCl_2$ and 1 mmol/L $LaCl_3$ (Cd+La), 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$ (Cd+Ca), 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO (Cd+Ca+cPTIO), 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 10 μmol/L Hb (Cd+Ca+Hb), respectively. After 1 week treatment, the length of crown root and shoot were recorded. In treatment 3, seedlings were treated with 100 μmol/L $CdCl_2$ (Cd), 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$(Cd+Ca), 100 μmol/L $CdCl_2$ and 1 mmol/L $LaCl_3$ (Cd+La), respectively. After 1 week treatment, the fluorescence of endogenous NO was measured in the roots. In treatment 4, seedlings were treated with 100 μmol/L $CdCl_2$ (Cd), 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$ (Cd+Ca), 100 μmol/L $CdCl_2$, and 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO (Cd+Ca+cPTIO), respectively. After 1 week treatment, the fluorescence of Cd, and the concentrations of Cd, non-protein thiols, protein thiols and cell wall components were measured in root and leaves, respectively.

1.2 Growth analysis

The seedlings were treated for 7 days in quarter-strength Yoshida's rice nutrient solution (control) supplemented with $CaCl_2$, $CdCl_2$, $LaCl_3$, Hb, and cPTIO. After treatment, the length of the shoots and primary roots, and the sum length of the crown roots of nine seedlings were measured.

1.3 Determination of Cd content

After treatment with $CaCl_2$, $CdCl_2$, and cPTIO, roots of seedlings were washed thoroughly with tap water and then three times with deionized water. Leaves and roots were collected, and cell walls, cell organelles, and soluble fractions were isolated according to published procedures (Xiong et al., 2009a). Cd content was assayed by digestion of cell fractions in $HNO_3/HClO_4$ (3/1, *V*/*V*) and characterized by atomic absorption spectrophotometry (Perkin Elmer 2380, USA). Each treatment was repeated three times and the mean was used.

1.4 Fluorescence localization of NO

NO was determined by a fluorescence assay through binding to the specific fluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). After 7 days of treatment, root tips (2 cm in length) were cut, immersed in 20 mmol/L DAF-FM DA prepared in 50 mmol/L Tris-HCl, pH 7.5, for 2 hr, and washed three times with distilled water. Fluorescence measurements were carried out with a laser confocal scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) with an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

1.5 Fluorescence localization of Cd

For Cd localization, the roots of all rice seedlings treated 7 days were immersed in 20 mmol/L disodium ethylenediamine tetraacetic acid (Na_2-EDTA) for 15 min and then rinsed three times with deionized water. Roots and leaves from the same parts of each treated plantlet were hand-sectioned. The sections were then stained with the assay reagent from the LEADMIUM kit (Invitrogen) for 30 min in the dark and then washed three times for 5 min with assay buffer from the kit. Sections were observed on the laser confocal scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) with excitation and emission wavelengths of 488 and 515 nm, respectively.

1.6 Determination of nonprotein and protein thiol content

Plant materials were homogenized manually with mortar and pestle in 0.02 mol/L EDTA under a cold ice bath after 7 days of treatment. The homogenate was centrifuged

at 12,000 ×*g* for 10 min. The supernatant was used for the detection of thiol content, which was measured using Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), DTNB (ε = 13.6 mmol/(L·cm) as described by Sedlak and Lindsay (1968) and Aravind and Prasad (2005). For detection of total thiols (TP), a reaction mixture containing 100 μL sample, 300 μL 0.2 mol/L Tris/HCl, pH 8.2, 20 μL of 0.01 mol/L DTNB, and 1580 μL methanol was incubated for 5 min at room temperature. The absorbance was determined at 412 nm. In order to detect nonprotein thiols (NPT), 500 μL of 100% trichloroacetic acid (TCA) was added to 4500 μL of the centrifuged homogenates with the TCA final concentration of 10%. The mixture was thoroughly mixed and centrifuged at 12,000 ×*g* for 10 min. One milliliter of the supernatant was mixed with 2 mL of 0.4 mol/L Tris buffer (pH 8.9) and 50 μL of DTNB, and absorbance was read within 5 min at 412 nm against a reagent blank. The protein thiols (PBT) were calculated by subtracting the non-protein thiols from total thiols. Each treatment was repeated three times and the mean was used.

1.7 Determination of cell wall component content

Cell wall component content was determined by the method of Xiong et al. (2009a). The crude cell walls were prepared and fractionated into four fractions: pectin, hemicellulose 1 (HC1), hemicellulose 2 (HC2), and cellulose. Cellulose content was assayed according to the methods of Correa-Aragunde (2008). Pectin, HC1, and HC2 contents were assayed according to the level of uronic acid.

1.8 Statistics analysis

All data were presented as means ± one standard deviation (SD). Statistical analyses were performed by analysis of variance (ANOVA) using SAS8.0 software (SAS Institute, Cary, NC). Differences between treatments were separated by Duncan's new multiple range test, taking $P < 0.05$ as significant.

2 Results

2.1 Effect of $CaCl_2$ on the growth of rice seedlings under $CdCl_2$ stress

The growth inhibition of rice seedlings exposed to 100 μmol/L $CdCl_2$ was partially alleviated with 1, 5, and 10 mmol/L $CaCl_2$ (Fig. 1). Five and 10 mmol/L $CaCl_2$ exerted the highest beneficial effect on the growth of shoots, and 5 mmol/L $CaCl_2$ exerted the most beneficial effect on the growth of roots. In addition, 10 mmol/L $CaCl_2$ exerted higher ionic and osmotic stresses than 5 mmol/L. Thus, further experiments were performed using 5 mmol/L $CaCl_2$.

2.2 Effects of $LaCl_3$, cPTIO, and Hb on growth of rice seedlings under $CdCl_2$ stress

The effects of $CaCl_2$, $LaCl_3$, Hb, and cPTIO on the growth of rice seedlings treated with $CdCl_2$ were observed and statistical analysis was performed (Fig. 2). $CaCl_2$ and $LaCl_3$ promoted the growth of rice seedlings retarded by the presence of $CdCl_2$, and $CaCl_2$ exerted a more beneficial effect than that of $LaCl_3$ (Fig. 2). The shoot or root lengths of seedlings treated with Cd+La were longer than for those treated with Cd alone, but shorter than for those treated with Cd+Ca.

As shown in Fig. 2, treatment with Cd+Ca+cPTIO and Cd+Ca+Hb inhibited the growth of seedlings compared to Cd+Ca treatment. Additionally, according to previous studies, cPTIO alone has no effect on the growth of seedlings (Xiong et al., 2009a). cPTIO and Hb weakened the positive effects of $CaCl_2$ on Cd stress, suggesting that NO might mediate calcium-induced alleviation of Cd toxicity.

2.3 Effects of $LaCl_3$, $CaCl_2$ on NO fluorescence of roots under $CdCl_2$ stress

The NO-derived DAF-FM DA green fluorescence was found in root tips of rice with the control treatment (Fig. 3A); however, Cd treatment triggered a significant reduction in NO-dependent fluorescence (Fig. 3B, E). Fluorescence was evoked and reversed significantly when $CaCl_2$ or $LaCl_3$ was added to the Cd treatment (Fig. 3C, D). The fluorescence intensity of NO is shown in Fig. 3E, indicating that growth accelerated by $CaCl_2$ and $LaCl_3$ in the presence of Cd stress might be associated with elevated NO levels.

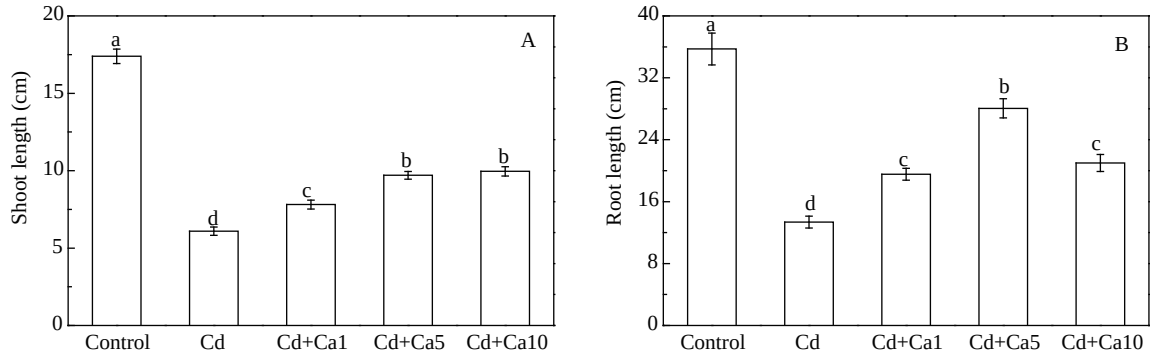

Fig. 1 Effects of different concentrations of $CaCl_2$ on growth of rice seedlings under $CdCl_2$ stress for 7 days. Control: without any chemical treatments; Cd: 100 μmol/L $CdCl_2$; Cd+Ca1: 100 μmol/L $CdCl_2$ and 1 mmol/L $CaCl_2$; Cd+Ca5: 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; Cd+Ca10: 100 μmol/L $CdCl_2$ and 10 mmol/L $CaCl_2$. A: the length of shoots with different treatments; B: the length of roots with different treatments. Each value is the mean ± SD of nine plants (n = 9). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels.

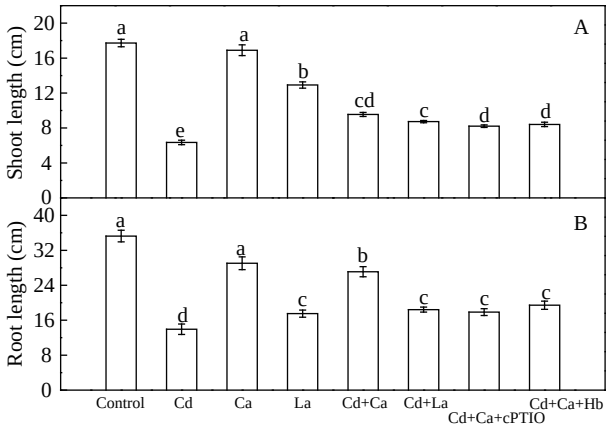

Fig. 2 Effects of $CaCl_2$, $LaCl_3$, cPTIO and Hb on growth of rice seedlings under $CdCl_2$ stress. Control: without any chemical treatments; Cd: 100 μmol/L $CdCl_2$; Ca: 5 mmol/L $CaCl_2$; La: 1 mmol/L $LaCl_3$; Cd+La: 100 μmol/L $CdCl_2$ and 1 mmol/L $LaCl_3$; Cd+Ca: 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; Cd+Ca+cPTIO: 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO; Cd+Ca+Hb: 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 10 μmol/L Hb. (A) the length of shoots with different treatments; (B) the length of roots with different treatments; Each value is the mean ± SD of nine plants (n = 9). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels.

2.4 Distribution of Cd^{2+} in cells with treatment of $CdCl_2$, $CaCl_2$, and cPTIO

Concentrations of Cd^{2+} in cell walls, cell organelles, and soluble fractions of leaf and root cells decreased in response to Cd+Ca treatment compared with Cd treatment (Fig. 4), indicating that $CaCl_2$ restrained the absorption of Cd^{2+} into plant tissue. Cd+Ca treatment supplemented with cPTIO decreased the concentration of Cd^{2+} in cell walls, cell organelles, and soluble fractions of roots, compared with Cd+Ca treatment. Cd+Ca treatment supplemented with cPTIO increased the concentration of Cd^{2+} in cell organelles and soluble fractions of leaves, while decreasing it in cell walls of leaves, compared with Cd+Ca treatment. Finally, the distribution of Cd in roots and leaves

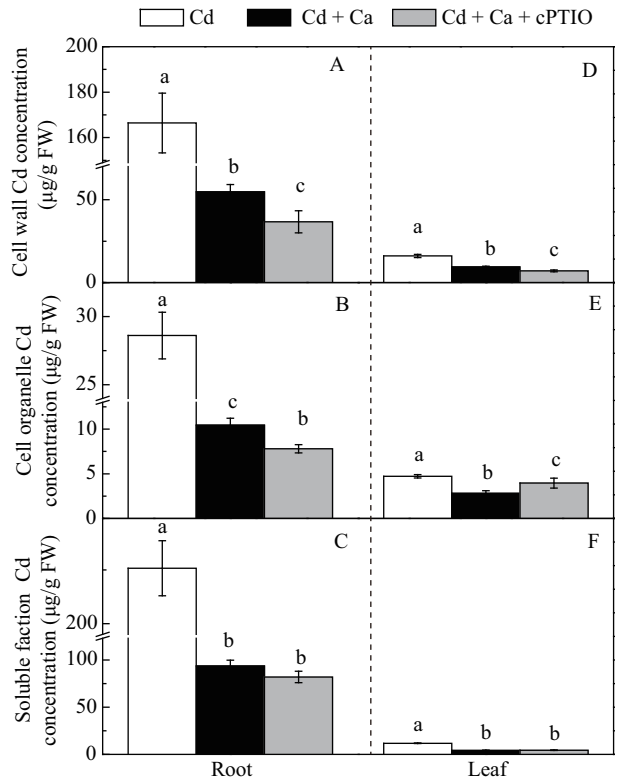

Fig. 4 Cd concentration in cell walls, cell organelles and soluble fraction of roots and shoots in rice seedlings with different treatments (determined by $HNO_3/HClO_4$ digestion and atomic absorption spectrophotometric analysis). Cd: 100 μmol/L $CdCl_2$; Cd+Ca: 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; Cd+Ca+cPTIO: 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO. (A) Cd concentration in cell wall of root; (B) Cd concentration in cell organelle of root; (C) Cd concentration in soluble fraction of root; (D) Cd concentration in cell wall of leaf; (E) Cd concentration in cell organelle of leaf; (F) Cd concentration in cell soluble fraction of leaf. Each value is the mean ± SD of triplicates (n = 3). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels.

varied.

The same phenomena were observed with the visu-

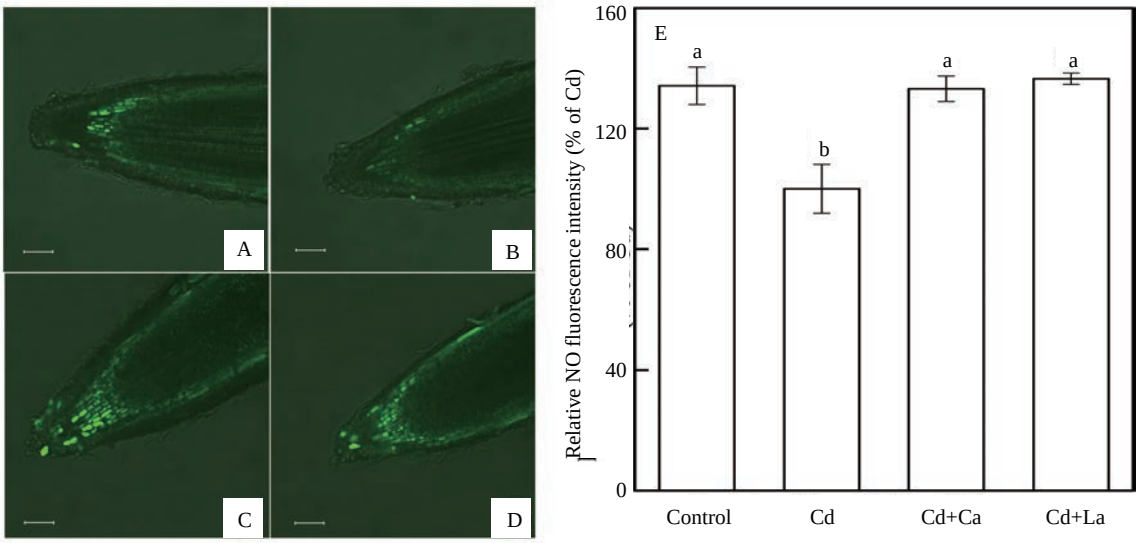

Fig. 3 Effects of $CdCl_2$, $CaCl_2$ and $LaCl_3$ on endogenous NO content in roots of rice seedlings (determined by DAF-FM DA fluorescence assay). (A) without any chemical treatments (Control); (B) 100 μmol/L $CdCl_2$ (Cd treatment); (C) 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$(Cd+Ca treatment); (D) 100 μmol/L $CdCl_2$ and 1 mmol/L $LaCl_3$ (Cd+La treatment); (E) relative NO fluorescence intensity under different treatments. Each value is the mean ± SD of triplicates (n = 3). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels. Bar = 50 μm.

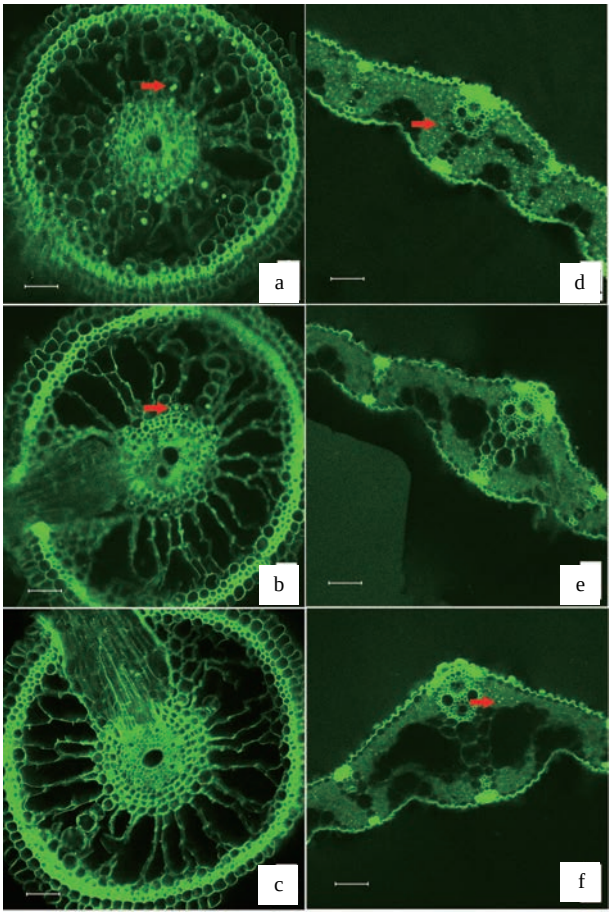

Fig. 5 Fluorescence images of Cd in roots and leaves with different treatments.; (a) root, 100 μmol/L $CdCl_2$; (b) root, 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; (c) root, 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO; (d) leaf, 100 μmol/L $CdCl_2$; (e) leaf, 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; (f) leaf, 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO. Bar = 50 μm; red arrow indicates the Cd fluorescence in cell organelle.

alization of Cd^{2+} in green fluorescence. The detected fluorescence was specifically associated with Cd^{2+}. In plants treated with Cd, Cd^{2+} fluorescence was found in cell walls and cell organelles of roots and leaves (indicated by the arrows in Fig. 5a, d). $CaCl_2$ strongly inhibited the fluorescence of Cd^{2+} in cell organelles of roots and leaves (indicated by the arrows in Fig. 5b, e). Compared to Cd+Ca treatment, the deposition of Cd^{2+} in organelles of root cells was inhibited in the $CaCl_2$+Cd+cPTIO treatment, but deposition of Cd^{2+} to organelles of leaf cells was accelerated (indicated by the arrows in Fig. 5c, f). Therefore, it is possible that endogenous NO-mediated alleviation of cadmium toxicity by calcium on rice seedlings is the consequence of redistribution of Cd^{2+} in cells.

2.5 Changes in thiols and cell wall components in rice seedlings with treatment of $CdCl_2$, $CaCl_2$, and cPTIO

The levels of nonprotein thiols (NPT) and protein thiols (PBT) in roots and leaves decreased significantly after treatment with Cd+Ca compared with Cd treatment alone (Fig. 6). cPTIO increased the level of NPT in roots and leaves of rice seedlings (Fig. 6A). However, PBT had the opposite response in roots and leaves. In rice leaves, the level of PBT increased after supplementation of cPTIO to the Cd+Ca treatment, while in roots, the level of PBT decreased (Fig. 6B). It can be concluded that Ca-induced endogenous NO, along with balance in the production of NPT in rice roots, might "set up" the rice plant to respond to Cd more effectively.

Because cell wall polysaccharides are crucial sites for Cd retention in plants (Xiong et al., 2009a), this study investigated whether changes in cell wall components in rice roots and leaves did vary with different treatments, in order to further investigate the mechanism of detoxification (Fig. 7). There were no significant differences found in the cellulose content of the cell walls of rice leaves and roots between the three treatments. Pectin, HC1, and HC2, all of which contain the uronic acid group, showed the same response to treatments, whether in roots or leaves. Therefore, the addition of Ca to the Cd treatment resulted in an observable increase in the content of pectin, HC1, and HC2. Supplementation of cPTIO in the Cd+Ca treatment decreased the content of pectin, HC1, and HC2. Thus, it was confirmed that NO mediates the detoxification of cadmium by calcium.

3 Discussion

Cadmium is a non-essential element for plant growth, but is taken up by roots and transporters and accumulates in the upper part of plants, thereby affecting crop yield and threatening food safety all over the world. The convergence of Cd^{2+} in plant organs is toxic to many species and causes disturbances in metabolic processes. In the past years, many studies have been conducted on alleviation of Cd toxicity by Ca and possible mechanisms of the protective function of Ca on Cd toxicity (El-Enany, 1995; Suzuki, 2005; Zhang et al., 2008), but there have been no definitive results. In the present study, between concentrations of 1 to 10 mmol/L, 5 mmol/L $CaCl_2$ was optimal for alleviating Cd stress (Fig. 1). $CdCl_2$ significantly inhibited the growth of roots and shoots of rice seedlings. $CaCl_2$ supplemented at a concentration of 5 mmol/L significantly counteracted the growth inhibition caused by $CdCl_2$ (Fig. 1). Furthermore, $LaCl_3$ has been used as Ca^{2+}-channel inhibitor in Arabidopsis and fava bean guard cells (Hamilton et al., 2000; Pei et al., 2000; Perfus-Barbeoch et al., 2002). In the present study, $LaCl_3$ was also used as inhibitor of Ca^{2+} channels. $LaCl_3$ significantly promoted the growth of roots and shoots in rice seedlings retarded by the presence of Cd^{2+} (Fig. 2). It was concluded that blockage of Ca^{2+} channels by La^{3+} protected the rice seedlings against Cd stress. Therefore, it is possible that Ca^{2+} competed with Cd^{2+} in the roots of rice seedlings, thus decreasing the absorption of Cd in the Cd+Ca treatment.

To validate whether endogenous NO participated in Ca^{2+} and Cd^{2+} absorption, two NO-trapping agents, cPTIO and Hb, were used to remove NO from biological tissues and solution by supplementation in the Cd+Ca treatment. With the addition of cPTIO or Hb, the growth of roots and shoots decreased significantly in the Cd+Ca treatment (Fig. 2). Hence, endogenous NO might accelerate the

growth of roots and shoots in Cd+Ca treatment. Over the past years, several reports have postulated that the exogenous NO donor sodium nitroprusside alleviates the inhibition of plants under Cd stress (Kopyra and Gwódź 2003; Rodríguez-Serrano et al., 2009; Xiong et al., 2009a). Hsu and Kao (2004) have reported that reactive oxygen species induce cell damage and inhibition of plant growth; and the reduction of $CdCl_2$-induced toxicity by NO in rice leaves was most likely mediated through its ability to scavenge reactive oxygen species (ROS). Based on these reports, it appears that plants possess several different strategies to cope with Cd toxicity.

In the present study, further examination was made to determine whether NO was involved in the absorption of Cd^{2+}. The NO-specific fluorescent probe DAF-FM DA (Foresi et al., 2007) was used to detect endogenous NO. Cd^{2+} induced a strong decrease in DAF-FM DA fluorescence in roots and leaves of rice seedlings (Fig. 3b), which was in accordance with studies in the roots of pea (*Pisum sativum* L.) and rice, and leaves of pea (Rodríguez-Serrano et al., 2006, 2009; Xiong et al., 2009a). On the contrary, in Arabidopsis plantlets, tobacco BY-2 cells, and Arabidopsis suspension cultures, NO level increased with Cd treatment (Besson-Bard et al., 2009; de Michele et al., 2009; Ma et al., 2010). Plant material, the age of plants, treatment time, and treatment pattern could explain the contradictory effects of endogenous NO. For instance, long-term treatment of Cd does depress the generation of endogenous NO (Rodríguez-Serrano et al., 2006, 2009; Xiong et al., 2009b). To investigate if the Cd^{2+}-dependent reduction of NO observed was due to a Ca deficiency, $CaCl_2$ was introduced in $CdCl_2$ treatment. After supplementation of $CaCl_2$ the reduction of NO-dependent fluorescence by Cd was reversed (Fig. 3c). Further, when the Ca^{2+}-channel inhibitor La^{3+} was added to the $CdCl_2$ treatment, the reduction of NO production was reversed (Fig. 3d). Therefore, it could be concluded that Ca alleviated the Cd toxicity via endogenous NO.

The subcellular levels of Cd^{2+} and the distribution of Cd^{2+} by Cd^{2+}-specific fluorescence were visualized in the cells (Fig. 5). The exogenous supply of Ca reduced the accumulation of Cd in the cell wall; the content and fluorescence of Cd^{2+} were determined in cell organelles and soluble fractions of root and leaf cells (Figs. 4 and 5b, e). Other authors have also found that Ca alleviates Cd toxicity in radish, rice, and Arabidopsis seedlings by reducing Cd^{2+} uptake (Rivetta et al., 1997; Suzuki, 2005). It can be concluded that competition between both elements for the same transporters may be the main reason for the decreased Cd^{2+} accumulation in any part of the cells. In the present work, with scavenging of endogenous NO in addition to Cd+Ca treatment, the Cd content in cell walls, cell organelles, and soluble fractions of root were decreased (Fig. 4), accompanied with decreased fluorescence (Fig. 5c), while in cell organelles and cytoplasm of leaf cells, the Cd content increased and Cd-specific fluorescence recurred (Fig. 5f). Based on these findings, it was assumed that endogenous NO-mediated alleviation of cadmium toxicity by calcium might be a consequence of Cd redistribution in cells.

Plants have developed mechanisms for metal detoxification to counteract Cd toxicity; one of these is binding of Cd^{2+} with intracellular thiol-containing compounds (Song et al., 2004; Vázquez et al., 2006; Kuramata et al., 2009). In the present study, the levels of NPT and PBT were reduced with Ca supplementation, whether in leaves or roots (Fig. 6), which is in accordance with the variation in Cd content (Fig. 4). With supplementation of cPTIO in Cd+Ca treatment, the PBT content increased in leaves, in order to chelate the increased Cd^{2+} in the cytoplasm and cell organelles of leaves. The NPT content increased with the application of cPTIO in Cd+Ca treatment (Fig. 6), indicating that endogenous NO decreased the level of NPT under Cd+Ca treatment. It is possible that the decrease in NPT served to synthesize PBT, which can chelate Cd^{2+} more tightly. In addition, the direct relationship between endogenous and generated PBT would be worth investigating.

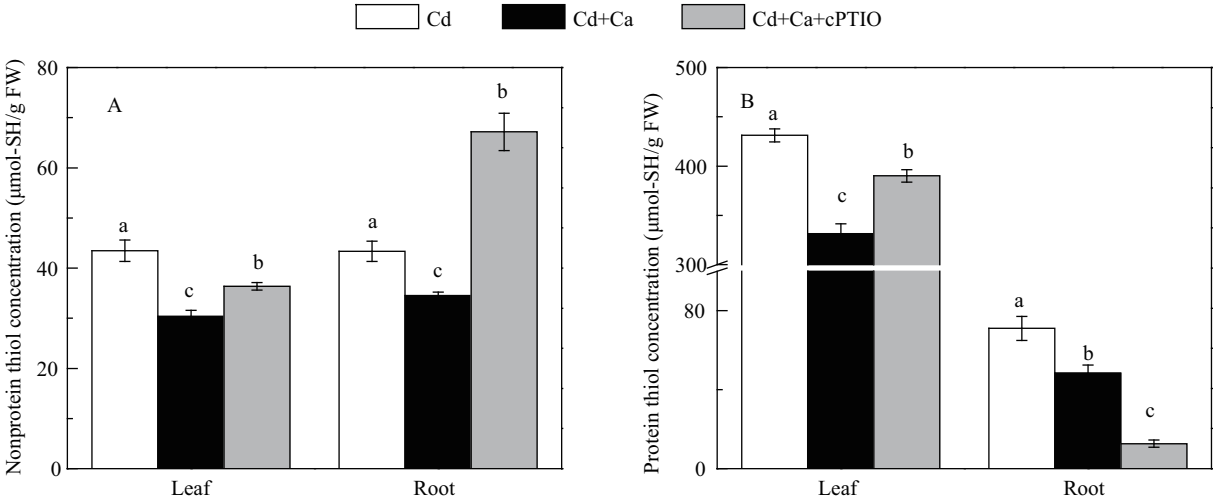

Fig. 6 Effects of $CaCl_2$ and cPTIO on thiol content in rice roots and leaves under $CdCl_2$ stress. Cd: 100 μmol/L $CdCl_2$; Cd+Ca: 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; Cd+Ca+cPTIO: 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO. (A) nonprotein thiol content in roots and leaves of rice seedlings; (B) the protein thiol content in roots and leaves of rice seedlings. Each value is the mean ± SD of triplicates (n = 3). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels.

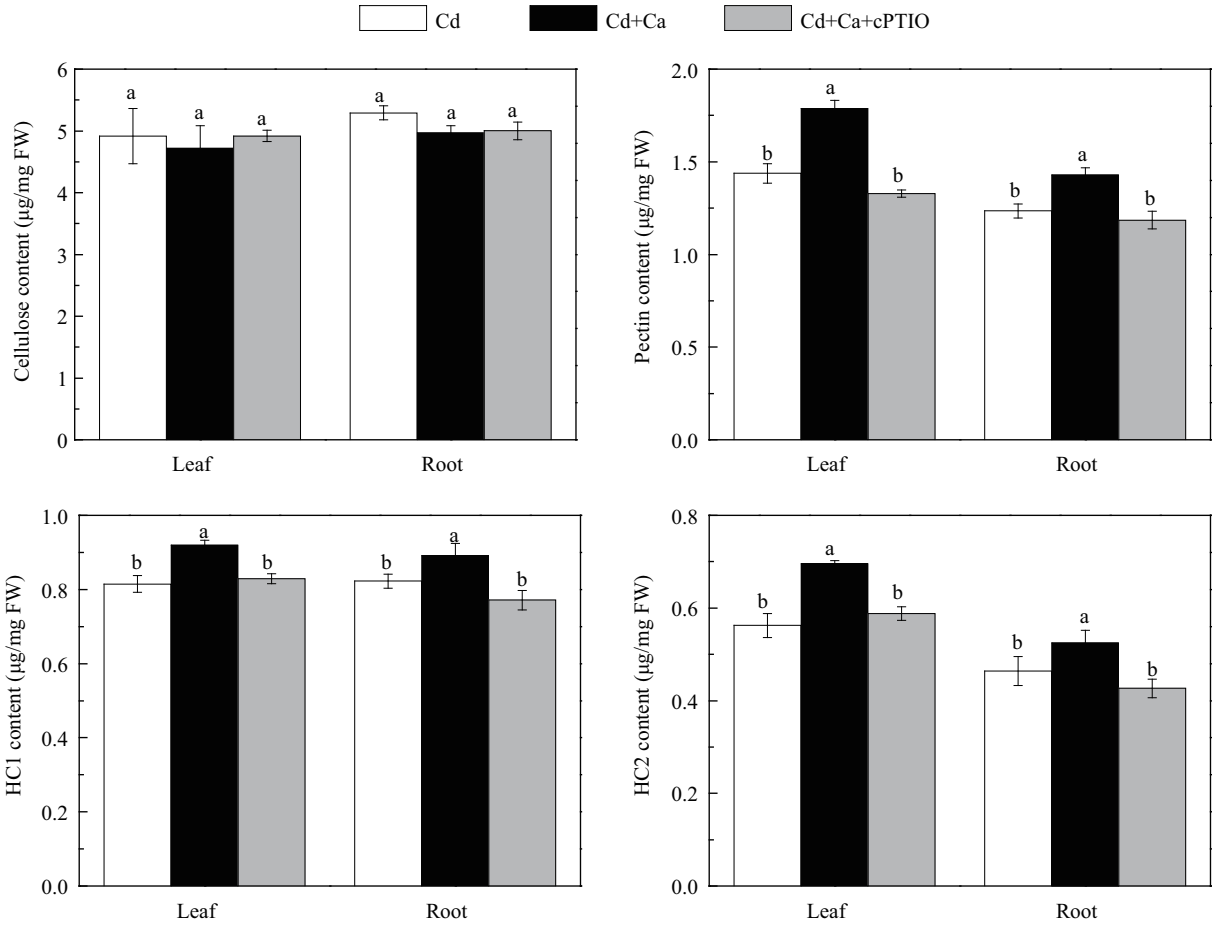

Fig. 7 Effects of $CaCl_2$ and cPTIO on cell wall component (cellulose, pectin, hemicellulose 1 (HC1) and hemicellulose 2 (HC2)) content in rice roots and leaves under $CdCl_2$ stress. Cd: 100 μmol/L $CdCl_2$; Cd+Ca: 100 μmol/L $CdCl_2$ and 5 mmol/L $CaCl_2$; Cd+Ca+cPTIO: 100 μmol/L $CdCl_2$, 5 mmol/L $CaCl_2$ and 200 μmol/L cPTIO. Each value is the mean ± SD of triplicates (n = 3). Data passed Duncan analysis, and different letters show significant differences at 0.05 levels.

The cell wall is the main Ca^{2+} store in plant cells. Many plant processes are mediated by the entry of Ca^{2+} into cytoplasm (White and Broadley, 2003). When plants are incubated for 2 weeks in 200 μmol/L $CdCl_2$, root cell walls exhibit irregular thickening, such as areas of enlarged or unusually formed cells (Suzuki, 2005). Cell wall structure is impacted with Cd stress, in particular for compounds containing uronic acid groups (Barceló et al., 1988; Maruthi et al., 2005; Paynel et al., 2009; Xiong et al., 2009a; Douchiche et al., 2010). As a result, the homeostasis of Ca^{2+} is disturbed, and the plants exhibit toxic symptoms. Xiong et al. (2009a) have discovered that exogenous NO enhances Cd tolerance by increasing pectin and hemicellulose in the root cell walls of rice. It was this evidence on which the present study was based. Cellulose content did not vary with the application of Ca or scavenging of endogenous NO in the cell walls of rice leaf and root. Ca increased the content of matrix polysaccharides (pectin and hemicellulose), both in roots and leaves under Cd stress. Interestingly, the matrix polysaccharide content decreased with scavenging of endogenous NO under Cd+Ca treatment (Fig. 7).

In conclusion, the results demonstrated that Ca accelerated the growth of rice seedlings under Cd stress. On the other hand, by application of Ca^{2+} and La^{3+}, respectively, endogenous NO levels were increased, and the growth of rice seedlings was also recovered under Cd stress. Ca increased the matrix polysaccharides in the cell walls of roots and leaves and decreased the NPT and PBT in the cytoplasm of roots and leaves. With the elimination of endogenous NO in Cd+Ca treatment, matrix polysaccharides in cell walls of roots and leaves decreased, and PBT decreased in roots but increased in leaves. Endogenous NO distributed Cd^{2+} throughout the cell in rice seedlings and influenced the distribution of matrix polysaccharides and NPT and PBT in cell walls and cytoplasm under Cd+Ca treatment. Therefore, it can be concluded that Ca alleviated the Cd toxicity in rice seedlings via endogenous NO signaling.

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References

Arasimowicz M, Floryszak-Wieczorek J, 2007. Nitric oxide as a bioactive signalling molecule in plant stress responses. *Plant Science*, 172(5): 876–887.

Aravind P, Prasad M N V, 2005. Modulation of cadmium-induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate-glutathione cycle and glutathione metabolism. *Plant Physiology and Biochemistry*, 43(2): 107–116.

Barceló J, Vázquez M D, Poschenrieder C, 1988. Cadmium induced structural and ultrastructural changes in the vascular system of bush bean stems. *Botanica Acta*, 101: 254–261.

Besson-Bard A, Courtois C, Gauthier A, Dahan J, Dobrowolska G, Jeandroz S et al., 2008. Nitric oxide in plants: production and cross-talk with Ca^{2+} signaling. *Molecular Plant*, 1(2): 218–228.

Besson-Bard A, Gravot A, Richaud P, Auroy P, Duc C, Gaymard F et al., 2009. Nitric oxide contributes to cadmium toxicity in Arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. *Plant Physiology*, 149(3): 1302–1315.

Correa-Aragunde N, Lombardo C, Lamattina L, 2008. Nitric oxide: an active nitrogen molecule that modulates cellulose synthesis in tomato roots. *New Phytologist*, 179(2): 386–396.

de Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentin M et al., 2009. Nitric oxide is involved in cadmium-induced programmed cell death in Arabidopsis suspension cultures. *Plant Physiology*, 150(1): 217–228.

Douchiche O, Driouich A, Morvan C, 2010. Spatial regulation of cell-wall structure in response to heavy metal stress: cadmium-induced alteration of the methyl-esterification pattern of homogalacturonans. *Annals of Botany*, 105(3): 481–491.

El-Enany A E, 1995. Alleviation of cadmium toxicity on maize seedlings by calcium. *Biologia Plantarum*, 37(1): 93–99.

Evans N H, McAinsh M R, Hetherington A M, 2001. Calcium oscillations in higher plants. *Current Opinion in Plant Biology*, 4(5): 415–420.

Foresi N P, Laxalt A M, Tonón C V, Casalongué C A, Lamattina L, 2007. Extracellular ATP induces nitric oxide production in tomato cell suspensions. *Plant Physiology*, 145(3): 589–592.

Fuhrer J, 1983. Phytotoxic effects of cadmium in leaf segments of *Avena sativa* L., and the protective role of calcium. *Experientia*, 39(5): 525–526.

Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt M R, 2003. Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proceedings of the National Academy of Sciences USA*, 100(19): 11116–11121.

Hamilton D W A, Hills A, Köhler B, Blatt M R, 2000. Ca^{2+} channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proceedings of the National Academy of Sciences USA*, 97(9): 4967–4972.

He Z Y, Li J C, Zhang H Y, Ma M, 2005. Different effects of calcium and lanthanum on the expression of phytochelatin synthase gene and cadmium absorption in *Lactuca sativa*. *Plant Science*, 168(2): 309–318.

Hsu Y T, Kao C H, 2004. Cadmium toxicity is reduced by nitric oxide in rice leaves. *Journal of Plant Growth Regulation*, 42(3): 227–238.

Kahle H, 1993. Response of roots of trees to heavy metals. *Environmental and Experimental Botany*, 33(1): 99–119.

Kopyra M, Gwód E A, 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiology and Biochemistry*, 41(11-12): 1011–1017.

Kuramata M, Masuya S, Takahashi Y, Kitagawa E, Inoue C, Ishikawa S et al., 2009. Novel cysteine-rich peptides from *Digitaria ciliaris* and *Oryza sativa* enhance tolerance to cadmium by limiting its cellular accumulation. *Plant and Cell Physiology*, 50(1): 106–117.

Lamotte O, Courtois C, Dobrowolska G, Besson A, Pugin A, Wendehenne D, 2006. Mechanisms of nitric-oxide-induced increase of free cytosolic Ca^{2+} concentration in *Nicotiana plumbaginifolia* cells. *Free Radical Biology and Medicine*, 40(8): 1369–1376.

Lamotte O, Gould K, Lecourieux D, Sequeira-Legrand A, Lebrun-Garcia A, Durner J et al., 2004. Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. *Plant Physiology*, 135(1): 516–529.

Liu Y G, Shi L, Ye N H, Liu R, Jia W S, Zhang J H, 2009. Nitric oxide-induced rapid decrease of abscisic acid concentration is required in breaking seed dormancy in Arabidopsis. *New Phytologist*, 183(4): 1030–1042.

Lozano-Juste J, León J, 2010. Enhanced abscisic acid-mediated responses in *nia1nia2noa1-2* triple mutant impaired in NIA/NR- and AtNOA1-dependent nitric oxide biosynthesis in Arabidopsis. *Plant Physiology*, 152(2): 891–903.

Ma W W, Xu W Z, Xu H, Chen Y S, He Z Y, Ma M, 2010. Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. *Planta*, 232(2): 325–335.

Maruthi S B B, Diehl S V, Han F X, Monts D L, Su Y, 2005. Anatomical changes due to uptake and accumulation of Zn and Cd in Indian mustard (*Brassica juncea*). *Environmental and Experimental Botany*, 54(2): 131–141.

Moreau M, Lindermayr C, Durner J, Klessig D F, 2010. NO synthesis and signaling in plants – where do we stand? *Physiologia Plantarum*, 138(4): 372–383.

Neill S J, Desikan R, Hancock J T, 2003. Nitric oxide signalling in plants. *New Phytologist*, 159(1): 11–35.

Österås A, Greger M, 2006. Interactions between calcium and copper or cadmium in Norway spruce. *Biologia Plantarum*, 50(4): 647–625.

Pagnussat G C, Simontacchi M, Puntarulo S, Lamattina L, 2002. Nitric oxide is required for root organogenesis. *Plant Physiology*, 129(3): 954–956.

Paynel F, Schaumann A, Arkoun M, Douchiche O, Morvan C, 2009. Temporal regulation of cell-wall pectin methylesterase and peroxidase isoforms in cadmium-treated flax hypocotyl. *Annals of Botany*, 104(7): 1363–1372.

Pei Z M, Murata Y, Benning G, Thomine S, Klüsener B, Allen G J et al., 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, 406(6797): 731–734.

Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C, 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *The Plant Journal*, 32(4): 539–548.

Rivetta A, Negrini N, Cocucci M, 1997. Involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination. *Plant, Cell & Environment*, 20(5): 600–608.

Rodríguez-Serrano M, Romero-Puertas M C, Pazmiño D M, Testillano P S, Risueño M C, Del Río L A et al., 2009. Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and

calcium. *Plant Physiology*, 150(1): 229–243.

Rodríguez-Serrano M, Romero-Puertas M C, Zabalza A, Corpas F J, Gómez M, Del Río L A et al., 2006. Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. *Plant, Cell & Environment*, 29(8): 1532–1544.

Rudd J J, Franklin-Tong V E, 2001. Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. *New Phytologist*, 151(1): 7–33.

Sedlak J, Lindsay R H, 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry*, 25: 192–205.

Song W Y, Martinoia E, Lee J, Kim D, Kim D Y, Vogt E et al., 2004. A novel family of cys-rich membrane proteins mediates cadmium resistance in Arabidopsis. *Plant Physiology*, 135(2): 1027–1039.

Suzuki N, 2005. Alleviation by calcium of cadmium-induced root growth inhibition in *Arabidopsis* seedlings. *Plant Biotechnology*, 22(1): 19–25.

Vázquez S, Goldsbrough P, Carpena R O, 2006. Assessing the relative contributions of phytochelatins and the cell wall to cadmium. *Physiologia Plantarum*, 128(3): 487–495.

Wang C Q, Song H, 2009. Calcium protects *Trifolium repens* L. seedlings against cadmium stress. *Plant Cell Reports*, 28(9): 1341–1349.

Wang Y H, Chen T, Zhang C Y, Hao H Q, Liu P, Zheng M Z et al., 2009. Nitric oxide modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes. *New Phytologist*, 182(4): 851–862.

White P J, Broadley M R, 2003. Calcium in plants. *Annals of Botany*, 92(4): 487–511.

Xiong J, An L Y, Lu H, Zhu C, 2009a. Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. *Planta*, 230(4): 755–765.

Xiong J, Lu H, Lu K X, Duan Y X, An L Y, Zhu C, 2009b. Cadmium decreases crown root number by decreasing endogenous nitric oxide, which is indispensable for crown root primordia initiation in rice seedlings. *Planta*, 230(4): 599–610.

Yoshida S, Forno D A, Cock J H, Gomez K A, 1976. Laboratory Manual for Physiological Studies of Rice (3rd ed.). The International Rice Research Institute, Manila.

Zhang H Y, Jiang Y N, He Z Y, Ma M, 2005. Cadmium accumulation and oxidative burst in garlic (*Allium sativum*). *Journal of Plant Physiology*, 162(9): 977–984.

Zhang X R, Yang H Q, Sui J, Qiao H T, Jiang Q Q, Ran K et al., 2008. Calcium protects grape leaves against cadmium stress by root treatment. *Acta Horticulturae Sinica*, 35(10): 1405–1410.

Zhang Y K, Han X J, Chen X L, Jin H, Cui X M, 2009. Exogenous nitric oxide on antioxidative system and ATPase activities from tomato seedlings under copper stress. *Scientia Horticulturae*, 123(2): 217–223.

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