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ORIGINAL ARTICLE

Cadmium decreases crown root number by decreasing endogenous nitric oxide, which is indispensable for crown root primordia initiation in rice seedlings

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Abstract Cadmium (Cd) is toxic to crown roots (CR), which are essential for maintaining normal growth and development in rice seedlings. Nitric oxide (NO) is an important signaling molecule that plays a pivotal role in plant root organogenesis. Here, the effects of Cd on endogenous NO content and root growth conditions were studied in rice seedlings. Results showed that similar to the NO scavenger, cPTIO, Cd significantly decreased endogenous NO content and CR number in rice seedlings, and these decreases were recoverable with the application of sodium nitroprusside (SNP, a NO donor). Microscopic analysis of root collars revealed that treatment with Cd and cPTIO inhibited CR primordia initiation. In contrast, although SNP partially recovered Cd-caused inhibition of CR elongation, treatment with cPTIO had no effect on CR elongation. L-NMMA, a widely used nitric oxide synthase (NOS) inhibitor, decreased endogenous NO content and CR number significantly, while tungstate, a nitrate reductase (NR) inhibitor, had no effect on endogenous NO content and CR number. Moreover, enzyme activity assays indicated that treatment with SNP inhibited NOS activity significantly, but had no effect on NR activity. All these results support the conclusions that a critical endogenous NO concentration is indispensable for rice CR primordia initiation rather than elongation, NOS is the main source for endogenous NO generation, and Cd decreases CR number by inhibiting NOS activity and thus decreasing endogenous NO content in rice seedlings.

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Keywords Adventitious roots · Cadmium · Crown root · Nitric oxide · Nitric oxide synthase · *Oryza*

Abbreviations

AR	Adventitious roots
CR	Crown roots
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
LR	Lateral roots
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
PR	Primary root
SNP	Sodium nitroprusside

Introduction

Inhibition of root growth is one of the most significant symptoms of cadmium (Cd) and other heavy metals in plants (Metwally et al. 2003; Rodríguez-Serrano et al. 2006; Kim et al. 2007; Groppa et al. 2008). Inhibition of root growth is directly related to reduction of apex length, mitotic activity and percentage of DNA-synthetizing cells, which are caused by distorting microtubules and microtubule-associated proteins (Fusconi et al. 2006, 2007; Tamás et al. 2007). It has also been suggested that growth inhibition of roots by Cd is due to a direct effect of Cd on the nucleus or to interactions with hormones (Laspina et al. 2005). Rice (*Oryza sativa*) is not only a worldwide staple crop, but also an important monocot model plant (Yang et al. 2008). In contrast to the primary root (PR) system in

dicots, rice produces numerous crown roots (CR), which are essential for maintaining normal growth and development (Xu et al. 2005). In rice, while the PR develops during embryogenesis, CR and lateral roots (LR) develop from differentiated cells postembryonically (Inukai et al. 2005). The toxic effects of Cd on rice roots have been reported by several authors (Hsu and Kao 2005; Guo et al. 2007; Yeh et al. 2007). However, in all of these researches, Cd toxicity to rice roots was evaluated by inhibition of root weight or/and elongation, and little attention had been paid to the reduction of CR number. Until now, only a few papers have reported Cd-induced decrease of LR in dicots (Rodríguez-Serrano et al. 2006), yet the effect of Cd on CR emergence in rice seedlings has not been studied and the mechanisms of Cd toxicity to rice CR are still far from clear.

Due to the simplicity of root structure and the availability of root-specific mutants, organization and cell differentiation processes in LR development have been well characterized in a model dicot, *Arabidopsis* (Malamy and Benfey 1997). In contrast, only a few studies have explored root development in monocot cereals (Inukai et al. 2005; Liu et al. 2005; Xu et al. 2005). In rice, the primordia of CR are initiated from several cells of the pericycle layer adjacent to the peripheral vascular cylinder in the stem (Kaufman 1959). The development of CR was divided into 12 successive stages several years ago (Kawata and Harada 1975). However, based on the results of genetics and molecular biology, the process of rice CR development is now divided into seven stages (Itoh et al. 2005): primordia initiation and primordia emergence are divided into different stages, which are controlled by different genes and signaling molecules (Xu et al. 2005).

It is well established that nitric oxide (NO) functions as a crucial signaling molecule in plant signaling and in plant defense responses (Neill et al. 2003). Increasing instances prove that NO plays a significant role in mediating root growth and development (Gouvêa et al. 1997; Pagnussat et al. 2002, 2003; Correa-Aragunde et al. 2004; Lombardo et al. 2006). NO is necessary for maintaining root elongation in *Hibiscus moscheutos* and maize (Tian et al. 2007; Zhao et al. 2007), as well as determining LR development in tomato (Correa-Aragunde et al. 2004). In addition, NO acts as a messenger and mediates the indole-acetic-induced AR developing process in cucumber (Pagnussat et al. 2002, 2004). In contrast, Kopyra and Gwóźdź (2003) have reported that root length of *Lupinus* is reduced by sodium nitroprusside (SNP) concentrations higher than 400 μM. Perrine-Walker et al. (2007) found that the induced growth inhibition of rice roots colonized by *Rhizobium leguminosarum* is due to a toxic accumulation of NO. Groppa et al. (2008) showed that Cd-induced accumulation of NO in wheat roots is involved in root growth inhibition.

In recent decades, the effects of exogenous NO on alleviating heavy metal toxicity of root in plants have been reported. For instance, pretreatment with SNP (a NO donor) alleviated Al-induced inhibition of root elongation and decreased Al accumulation in root apices, as compared with plants without SNP pretreatment (Wang and Yang 2005; Tian et al. 2007). A similar situation was described in *Lupinus* roots under Cd and Pb stresses (Kopyra and Gwóźdź 2003). Cd-induced oxidative stress in wheat roots was alleviated with supplementation of exogenous NO (Singh et al. 2008). All these results indicate that NO could have been involved in the deleterious effect of Cd in plant roots. However, the precise mechanism of Cd-induced inhibition of root growth has not been clearly elucidated; the role of NO in this process remains undetermined.

To further explore the effect of Cd toxicity to CR in monocot cereals and to elucidate the role of NO in this course, we studied the inhibition of CR growth and monitored the change of NO content in Cd-treated rice seedlings. In addition, the protective effects of exogenous NO against Cd toxicity were investigated in rice roots. We also studied the enzymes in relation to endogenous NO production and investigated the effects of Cd and NO on rice CR primordium initiation. The results of this work may help to further understand the mechanisms of Cd-caused inhibition on root growth in monocot cereals.

Materials and methods

Seedling cultivation and treatment

Dehulled seeds of rice (*O. sativa* L., cv. Zhonghua 11, from Institute of Crop Breeding and Cultivation, Chinese Academy of Agricultural Sciences, Beijing, China) were sterilized with 5% sodium hypochlorite for 15 min and rinsed three times with sterilized water. The sterilized seeds were then soaked for 2 days at 37°C in the dark. Six seeds were placed in a glass tube, filled with 30 ml sterilized 1% agar and then cultivated in a controlled cabinet under conditions of a 14/10 h-light, 30/24°C-temperature cycle for 7 days. In the microscopic analysis of CR primordia, the seedlings were cultivated for 7 and 14 days, separately. All chemicals [100 μM $CdCl_2$, 200 μM SNP, 200 μM KNO_2, 200 μM $K_4Fe[CN]_6$, 100 μM tungstate, 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma, St Louis, MO, USA) and 200 μM N^G monomethyl-L-arginine (L-NMMA, Sigma)] were added into the sterilized agar in the form of solutions. At least five independent replicates were used for each treatment.

Measurement of root growth

Seven days after cultivation, rice seedlings were pulled out and washed with sterilized water to remove the agar on the surface of the roots. Lengths of the PR and CR were measured with a ruler; in addition, the number of roots also was calculated. Values were given as mean ± SE of eight independent measurements.

Determination of NO content

Nitric oxide content was determined using the NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA; Invitrogen, Carlsbad, CA, USA) (Zeidler et al. 2004; Foresi et al. 2007). Seven-day-old seedlings were incubated in sterilized water (pH 5.5) containing various compounds (as indicated in the figure legends) for 1 day, then the root apices of CR (about 2 cm from the root apices) were excised and incubated with 2.5 μM DAF-FM DA in HEPES–NaOH buffer (pH 7.5) for 30 min. Thereafter, roots were washed with HEPES–NaOH buffer three times. Fluorescence image was taken with a laser confocal scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) with excitation and emission wavelengths of 488 and 515 nm, respectively. The three-dimensional reconstructed image of the individual root apex was used to calculate the relative fluorescence intensity, and the fluorescence intensity of the individual root apex was expressed in color level on a scale ranging from 0 to 255.

Determination of NOS activity

The activity of NOS was determined with an NOS assay kit (Beyotime, Haimen, China). Briefly, 0.1 g roots were frozen in liquid N_2 and ground to a fine powder; then the powder was homogenized in 1 ml extraction buffer containing 100 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl (PMSF), 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4 and 1% polyvinylpyrrolidone (PVP). The contents were centrifuged at 13,000*g* for 20 min at 4°C; 0.2 ml clear supernatant was added to 0.1 ml assay mixture (containing NADPH, L-Arginine and NOS assay buffer, DAF-FM DA) and reacted at 37°C in the dark for 1 h. The NO content was detected with a laser confocal scanning microscope (LSM 510; Zeiss) with excitation and emission wavelengths of 488 and 515 nm, respectively. The fluorescence intensity was expressed in color level on a scale ranging from 0 to 255, and the fluorescence intensity was named as relative fluorescence unit 1 (RFU_1). In the blank control, 0.2 ml extract buffer, instead of the enzyme extract, was added into 0.1 ml assay mixture and reacted at 37°C in the dark for 1 h. The fluorescence intensity was named as RFU_2. The final relative fluorescence intensity (RFU) was calculated using the formula, $RFU = RFU_1 - RFU_2$. The RFU of control (seedlings without SNP or Cd treatment) was defined as 100%, and the relative NOS activity was expressed with RFU compared to control value.

Determination of NR activity

The activity of NR was determined as described by Tian et al. (2007) with some modifications. Briefly, 0.1 g roots were frozen in liquid N_2 and ground into a fine powder. Then, the powder was homogenized in 1 ml extraction buffer containing 100 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1% Triton X-100, 0.5 mM PMSF, 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4 and 1% PVP. The contents were centrifuged at 13,000*g* for 20 min at 4°C. A total of 200 μl of clear supernatant was added into 0.4 ml of prewarmed assay buffer containing 100 mM HEPES–KOH (pH 7.5), 5 mM KNO_3 and 0.25 mM NADH. The mixed solution reacted at 30°C for 60 min, and then the reaction was stopped by adding Zn-acetate. The nitrite produced was measured colormetrically at 540 nm by adding 1 ml of 1% sulfanilamide in 3 M HCl plus 1 ml of 0.2% *N*-(1-naphthyl) ethylenediamine.

Determination of protein content

The protein content was measured following the method of Bradford (1976), with BSA as a standard.

Microscopic analysis of CR primordia

Crown root primordium microscopic analysis was performed as described (Liu et al. 2005). About 3 mm root collar (the junction between root and stem) was fixed with formalin acetic acid and alcohol (FAA, ethanol:water:formalin:acetic acid, 10:7:2:1, by volume) fixation solution at 4°C overnight, dehydrated and embedded in paraffin. Cross sections (8 μm thickness) were cut with a microtome (Microm HM325; Walldorf, Germany) and stained with safranin and fast green (Sigma). Serial cross sections were observed with a bright-field microscope and photographed with a Zeiss Axiocam HRC digital color camera (Zeiss).

Statistical analysis

The data were analyzed by analysis of variance (ANOVA), and difference (LSD) test was employed to determine the differences among the treatments at $P = 0.05$ level. Statistical analyses were conducted with SPSS software for Windows (Version 11.0) and the graphs were drawn with OriginPro software (Version 7.5G).

Results

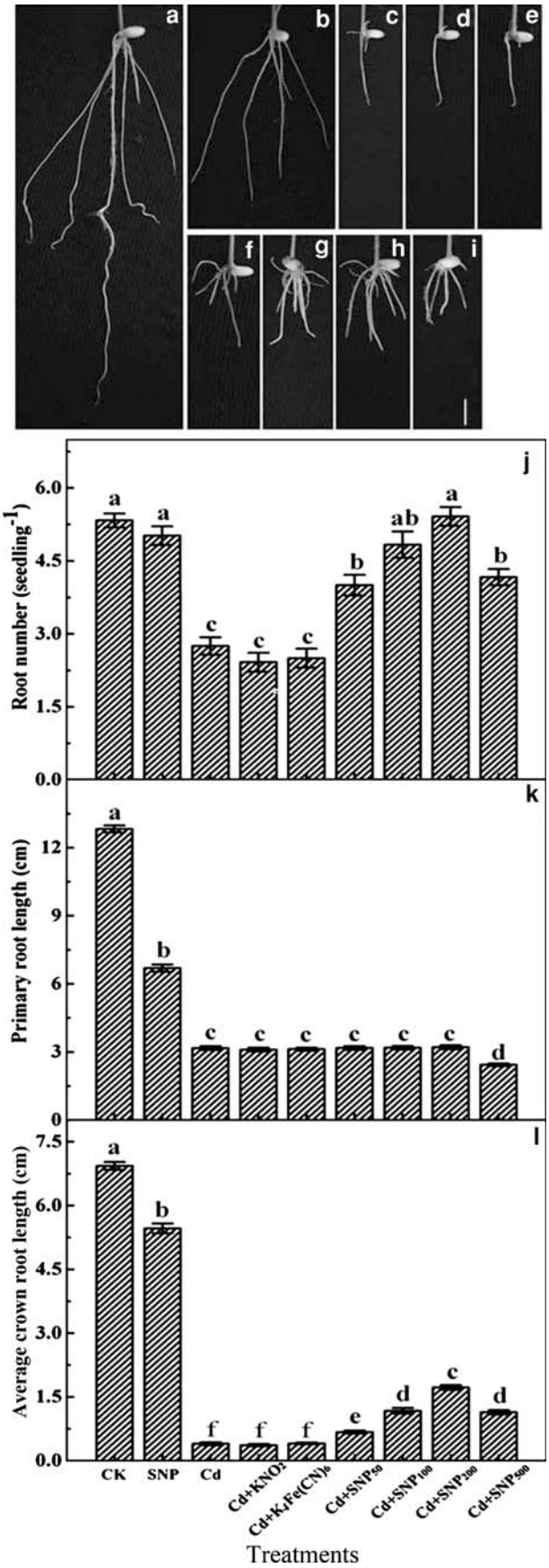
Exogenous NO alleviated Cd-induced inhibition on CR formation

A reduction in CR and PR elongation and a decrease in CR number were observed in rice seedlings treated with 100 μM Cd (Fig. 1). To elucidate the effect of exogenous NO on Cd-treated rice root growth, Cd-treated rice seedlings were treated with different concentrations of SNP simultaneously. The number and elongation of rice CR increased as SNP concentration increased from 50 to 200 μM, and treatment with 200 μM SNP recovered the decreases in CR number and elongation most significantly. In contrast, a reduction in CR number and elongation was observed at SNP concentration of 500 μM. Interestingly, application of low concentrations of SNP (≤200 μM) had no amelioration effect on Cd-induced decrease of PR elongation. A total of 500 μM SNP even deteriorated Cd-caused inhibition on PR elongation. Compared with control and treatment with Cd, treatment with 200 μM SNP had no effect on CR number, but both CR and PR elongations were inhibited significantly (Fig. 1). All these results indicate that low concentrations of SNP (≤200 μM) are useful for alleviating Cd-caused inhibition of CR number and elongation. Treatment with 200 μM SNP had the most significant alleviation effect on Cd-caused root growth inhibitions, so it was applied in the following experiments.

In water solution, SNP decomposes into NO, NO_2^- and $Fe(CN)_6^{4-}$ spontaneously. In order to prove that SNP-released NO, rather than other compositions from SNP decomposition, was responsible for the SNP-induced alleviation effect on Cd-caused root growth inhibition, Cd-treated rice seedlings were treated with 200 μM KNO_2 and 200 μM $K_4Fe(CN)_6$ separately. The results indicated that treatment with neither KNO_2 nor $K_4Fe(CN)_6$ had an amelioration effect on Cd-induced inhibition of rice roots (Fig. 1). This evidence proves the hypothesis that SNP-released NO, rather than other compositions from SNP decomposition, accounts for the alleviation effects on Cd toxicity.

NO was crucial for rice CR number rather than root elongation

Based on the results that low concentration of SNP (≤200 μM) alleviates Cd-induced inhibition of rice CR number and elongation, SNP-released NO rather than other compositions from SNP decomposition accounts for this alleviation, we hypothesize that Cd decreases endogenous NO content in rice seedlings. In order to prove the hypothesis, the effect of NO scavenger (cPTIO) on rice root growth was investigated. Treatment with 200 μM cPTIO reduced the CR number, but affected neither PR nor



◀ **Fig. 1** Effects of varying concentrations of sodium nitroprusside (*SNP*) and compositions from SNP on rice roots grown under 100 μM cadmium (Cd) stress. **a** Control culture without a chemical treatment (*CK*). **b** Cultured with 200 μM SNP alone. **c** Cultured with 100 μM Cd alone. **d** Cultured simultaneously with 100 μM Cd and 200 μM KNO_2. **e** Cultured simultaneously with 100 μM Cd and 200 μM $K_4Fe(CN)_6$. **f–i** Cultured simultaneously with 100 μM Cd and 50, 100, 200 and 500 μM SNP. **j–l** Root number, primary root length and average crown root length of rice seedlings under varying treatments. All seedlings were treated for 7 days in a controlled cabinet. The values are mean ± SE of more than 12 seedlings. *Bar* 1 cm

CR elongation. Interestingly, 200 μM SNP totally recovered cPTIO-caused reduction of CR number, but had no effect on CR elongation. In contrast, PR elongation was inhibited (Fig. 2). All of these phenomena indicate that endogenous NO content is crucial for rice CR number rather than elongation, and endogenous NO content is dispensable for maintaining PR elongation. In contrast, high concentration of exogenous NO even suppresses PR and CR elongation.

NOS, rather than NR, was responsible for endogenous NO generation

In order to ascertain the key enzymes responsible for endogenous NO origination in rice roots, the effects of L-NMMA (NOS inhibitor) and tungstate (NR inhibitor) on rice root growth were investigated. Treatment with L-NMMA decreased CR number significantly and application of 200 μM SNP totally recovered L-NMMA-induced decrease of CR number (Fig. 3). In contrast, tungstate significantly inhibited both PR and CR elongation, but failed to decrease CR number. Treatment with SNP also had no amelioration on tungstate-induced inhibition of CR elongation. The results of enzyme activity assays showed that treatment with Cd inhibited NOS and NR activities significantly, and the Cd-induced inhibition on NOS activity seemed more severe than on NR. Compared with controls, a single treatment with 200 μM SNP decreased NOS activity significantly, but did not affect NR activity. Simultaneous treatment with SNP had no effect on recovering Cd-caused decrease of NOS and NR activities (Fig. 4). Based on these findings, we hypothesize that there is a feedback inhibition relationship between NO content and NOS activity; NOS, rather than NR, plays an important role in endogenous NO generation in rice roots.

Effects of Cd, SNP, cPTIO, L-NMMA and tungstate on NO content

In order to accurately monitor the effects of various treatments on endogenous NO contents in rice roots and confirm whether Cd-induced decrease of CR number and elongation is related to endogenous NO content, the NO content was analyzed by the specific fluorescence intensity

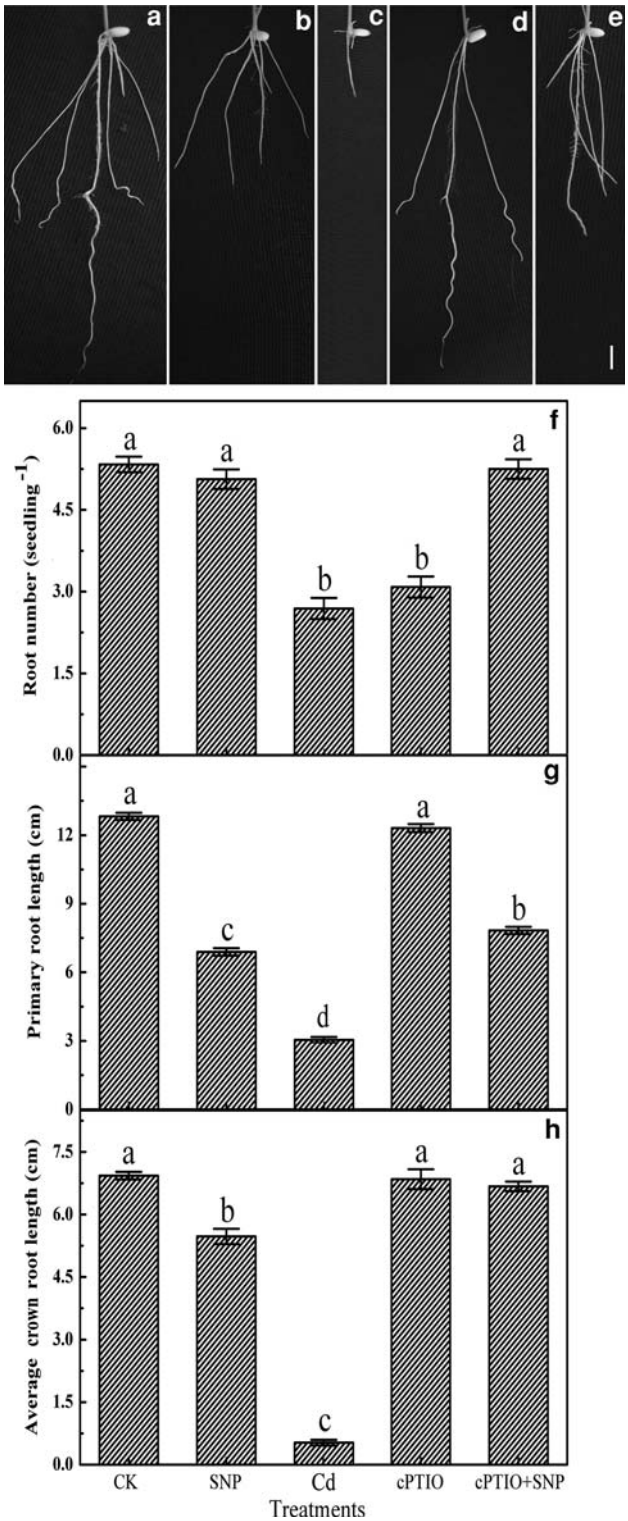
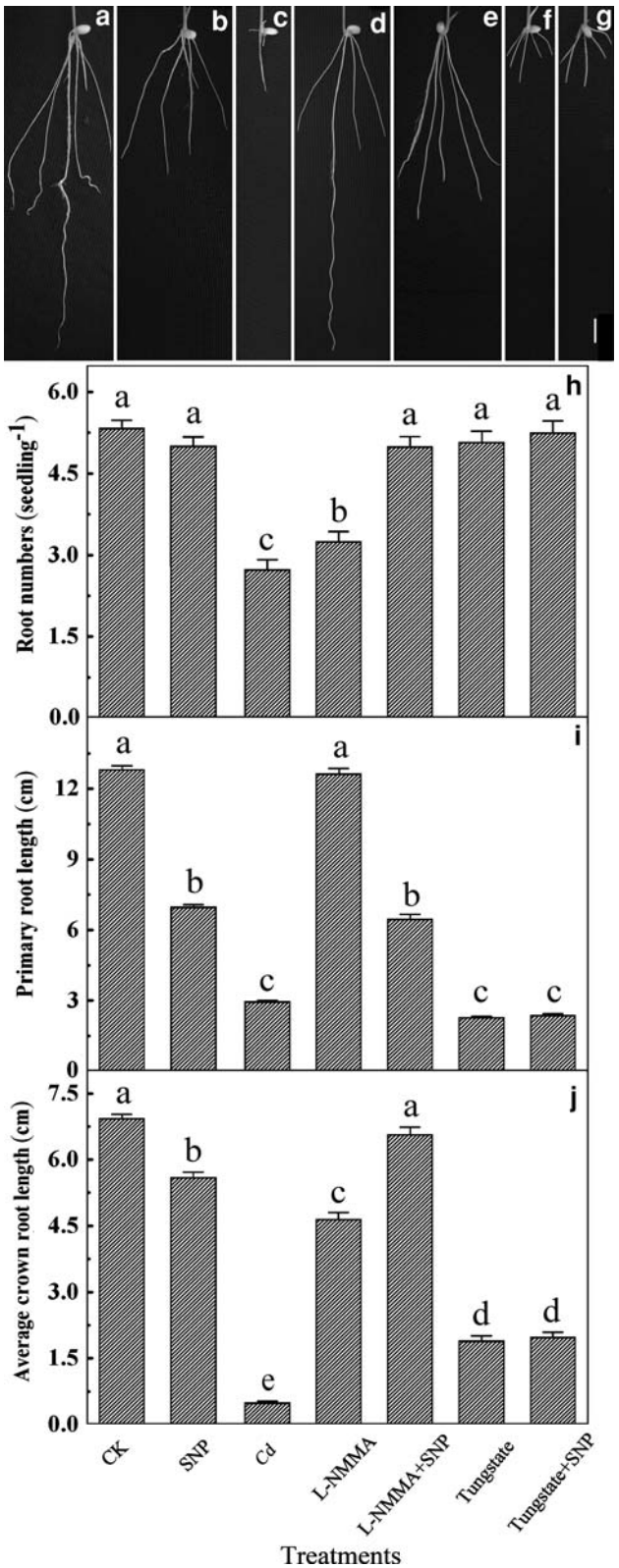


Fig. 2 Effects of NO scavenger (*cPTIO*) on rice root growth. **a** Control culture without chemical treatment (*CK*). **b** Cultured with 200 μM SNP alone. **c** Cultured with 100 μM Cd alone. **d** Cultured with 200 μM cPTIO alone. **e** Cultured simultaneously with 100 μM Cd and 200 μM cPTIO. **f**, **g**, **h** Root number, primary root length and average crown root length of rice seedlings under varying treatments. All seedlings were treated for 7 days in a controlled cabinet. The values are mean ± SE of more than 12 seedlings. *Bar* 1 cm



◀ **Fig. 3** Effects of nitric oxide synthase (NOS) and nitrate reductase (NR) inhibitors (L-NMMA and tungstate) on rice root growth. **a** Control culture without chemical treatment (*CK*). **b** Cultured with 200 μM Cd alone. **c** Cultured with 100 μM Cd alone. **d** Cultured with 200 μM L-NMMA alone. **e** Cultured simultaneously with 200 μM SNP and 200 μM L-NMMA. **f** Cultured with 100 μM tungstate alone. **g** Cultured simultaneously with 200 μM SNP and 100 μM tungstate. **h–j** Root number, primary root length and average crown root length of rice seedlings under varying treatments. All seedlings were treated for 7 days in a controlled cabinet. The values are mean ± SE of more than 12 seedlings. *Bar* 1 cm

of DAF-FM DA in rice roots. No fluorescence was observed when the sample was not loaded with the probe (data not shown), and spontaneous fluorescence was negligible. Compared with controls, treatment with Cd markedly reduced the fluorescence intensity by about 80%. The NO scavenger, cPTIO, reduced the fluorescence intensity almost by 50%. L-NMMA, as an NOS inhibitor, had a similar inhibition on fluorescence intensity as cPTIO

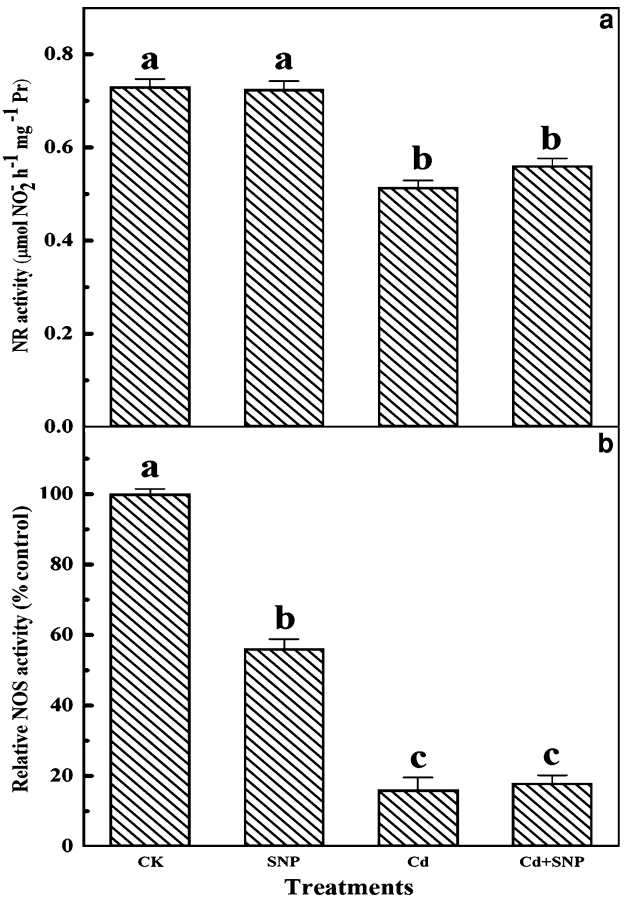


Fig. 4 Effects of cadmium (*Cd*) and sodium nitroprusside (*SNP*) on nitrate reductase (*NR*) activity (**a**) and nitric oxide synthase (*NOS*) activity (**b**) in rice roots. Seven-day-old seedlings were incubated in sterilized water containing chemicals for 1 day, and then the roots were collected for assaying enzyme activities, and the amount of protein used in the analyses of enzyme activity was 51.31 ± 3.22 μg. The values are mean ± SE of more than five seedlings

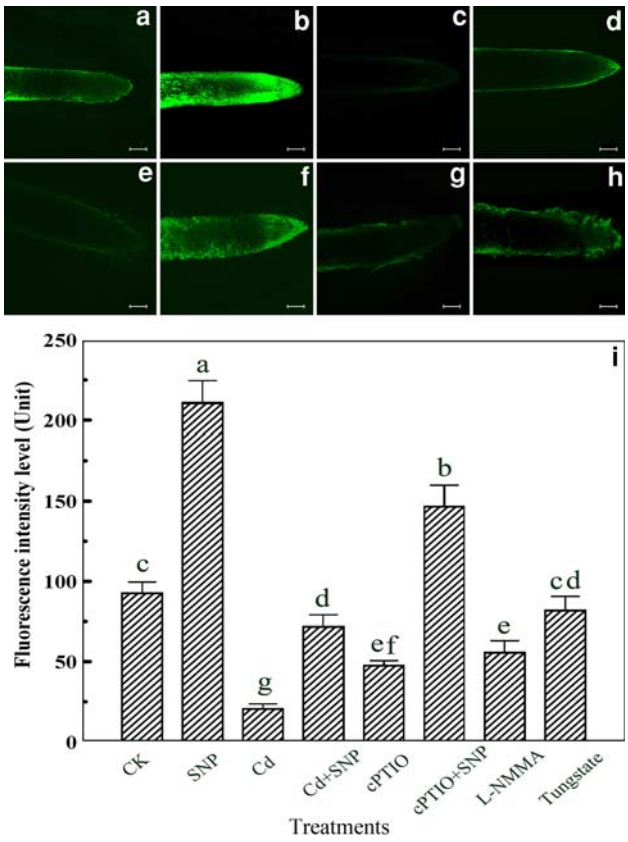


Fig. 5 Effects of cadmium (*Cd*), sodium nitroprusside (*SNP*), NO scavenger (*cPTIO*), nitric oxide synthase (*NOS*) inhibitor (*L-NMMA*) and nitrate reductase (*NR*) inhibitor (*tungstate*) on endogenous NO content in rice roots. Seven-day-old seedlings were incubated in sterilized water containing chemicals for 1 day, and then the root apices were excised and loaded with 2.5 μM NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) for 30 min. Fluorescence density was detected with confocal laser scanning microscopy and expressed in color level on a scale ranging from 0 to 255. Representative fluorescence images of crown root in rice seedlings. **a** Incubated without chemical (control, *CK*). **b** Incubated in 200 μM SNP alone. **c** Incubated in 100 μM Cd alone. **d** Incubated simultaneously in 100 μM Cd and 200 μM SNP. **e** Incubated in 200 μM cPTIO alone. **f** Incubated simultaneously in 200 μM cPTIO and 200 μM SNP. **g** Incubated in 200 μM L-NMMA alone. **h** Incubated in 100 μM tungstate alone. **i** Relative fluorescence density in rice CR apices under varying treatments. All values are mean ± SE of more than five seedlings. *Bar* 200 μm

(Fig. 5). These results suggested that endogenous NO decreased significantly after these treatments, and treatment with Cd caused the most significant decrease in NO content. As L-NMMA decreased the NO content significantly, NOS played a crucial role in endogenous NO generation. In contrast, as a prevalent NR inhibitor, tungstate obviously did not affect the fluorescence intensity in rice roots, indicating that NR is not responsible for endogenous NO generation in 7-day-old rice seedlings. Treatment with 200 μM SNP not only increased root fluorescence intensity significantly, but also recovered the reductions of fluorescence intensity resulting from treatments with Cd and cPTIO (Fig. 5). The results also proved the hypothesis that Cd inhibits NOS activity, which is responsible for endogenous NO generation in rice roots.

NO played a crucial role in CR primordium initiation

To verify the process of Cd-induced inhibition on CR number and to elucidate the role of endogenous NO in maintaining CR emergence, the effects of Cd and cPTIO on rice CR primordium initiation were investigated. Compared with controls, 7-day treatment with Cd decreased the CR number. In the Cd-treated 14-day-old seedlings, no increase in CR number or elongation was observed. A 7-day treatment with cPTIO also decreased the CR number, and 14-day-old seedlings treated with cPTIO showed no increase in CR number (Fig. 6). This result proves that NO is indispensable for CR emergence.

Serial transverse sections of the root collars were analyzed to determine whether decrease in CR was due to arrest of CR primordia initiation or primordia emergence. In the 7-day-old control seedlings, CR, mature primordium and unmature primordium were clearly seen. In the 7-day-old seedlings treated with cPTIO, CR rather than primordium was observed. In the 7-day-old seedlings treated with Cd, the beginning of the primordium was also observable. In the 14-day-old seedlings of control, new CR and primordium had developed. In the 14-day-old seedling under Cd stress, the primordium stayed at the same size as those in the 7-day-old seedlings and no new primordium had initiated. In the section of seedlings treated with cPTIO for 14 days, no new primordium, but a mature CR, was found (Fig. 7). All these results indicate that Cd not only arrests primordia developing into CR, but also inhibits CR primordium initiation. NO is crucial and necessary for CR primordium initiation.

Discussion

Most plants are sensitive to Cd toxicity, which inhibits root and shoot growth, as a consequence of alterations in the photosynthesis rate, and uptake and distribution of macronutrients and micronutrients (Lozano-Rodríguez et al. 1997; Sandalio et al. 2001). Inhibition of root elongation is one of the earliest and distinct symptoms of Cd toxicity; however, Cd-induced decrease in CR number in monocot cereals was neglected by earlier studies. Thus, the precise mechanisms underlying Cd-induced decrease of CR number and elongation remains largely unknown in monocot cereals (Groppa et al. 2008). In the present study, we observed that treatment with 100 μM Cd induced an obvious inhibition of PR and CR elongation in rice

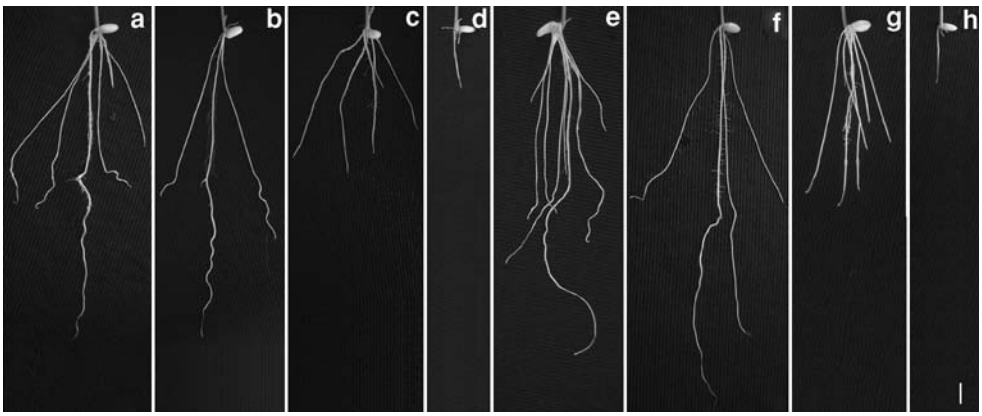


Fig. 6 Effects of NO scavenger (cPTIO), NO donor (SNP) and Cd on rice roots growth. **a** Control culture without chemical treatment (CK) for 7 days. **b** Cultured with 200 μM cPTIO alone for 7 days. **c** Cultured with 100 μM SNP alone for 7 days. **d** Cultured with 100 μM Cd alone for 7 days. **e** Cultured without chemical (CK) for 14 days. **f** Cultured with 200 μM cPTIO alone for 14 days. **g** Cultured with 100 μM Cd alone for 14 days. **h** Cultured with 100 μM Cd alone for 14 days. All seedlings were cultured in a controlled cabinet. The values are mean ± SE of more than 12 seedlings. *Bar* 1 cm

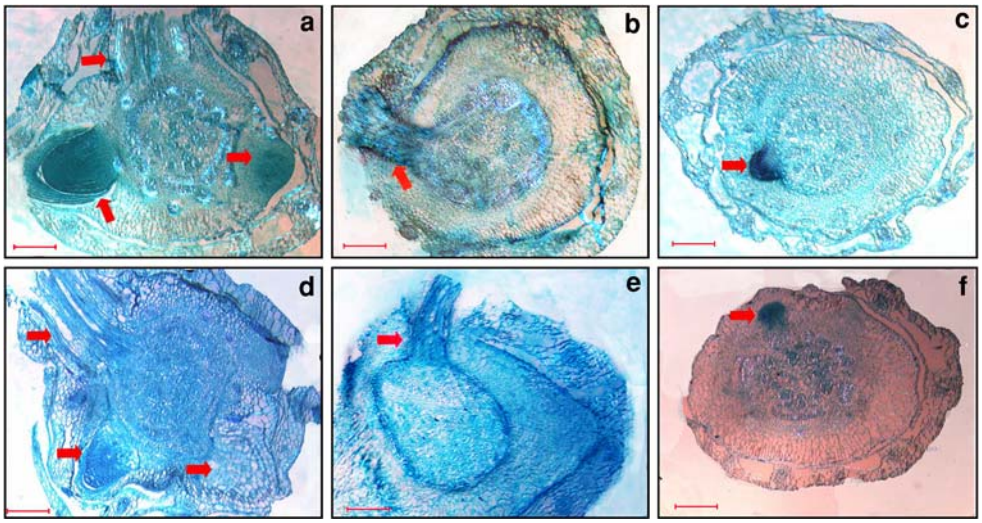


Fig. 7 Transverse sections of root collar (the junction between stem and root) in rice seedlings. Representative images of transverse sections of root collar in rice seedlings. **a** Control culture without chemical treatment for 7 days (CK). **b** Cultured with 200 μM cPTIO alone for 7 days. **c** Cultured with 100 μM Cd alone for 7 days. **d** Cultured without chemical (CK) for 14 days. **e** Cultured with 200 μM cPTIO alone for 14 days. **f** Cultured with 100 μM Cd alone for 14 days. *Red arrows* point to the crown root primordium or mature CR. *Bar* 500 μm

seedlings, in concurrence with previous reports for this (Hsu and Kao 2005; Guo et al. 2007; Yeh et al. 2007) and for other monocot cereals (Metwally et al. 2003; Groppa et al. 2008). Besides, in the present study, it was observed that treatment with 100 μM Cd decreased CR number in rice seedlings significantly.

Nitric oxide is a bioactive molecule that regulates biological processes in phytogenetically distant species (Beligni and Lamattina 2002). The effects of exogenous NO in alleviating heavy metal toxicity in roots in both monocots and dicots have been reported (Kopyra and Gwóźdź 2003; Wang and Yang 2005; Tian et al. 2007; Singh et al. 2008). The present work demonstrates for the first time that application of SNP alleviated Cd-induced reduction of rice CR number (Fig. 1). As SNP has been widely used as a compound to release NO (Furchgott 1995), these results indicate that Cd may inhibit NO homeostasis, leading to the endogenous NO content being lower than required for CR emergence. Moreover, treatment with NO scavenger (cPTIO) had a similar effect on the reduction of CR emergence, and SNP also was able to recover the cPTIO-induced decrease in CR number (Fig. 2). Because cPTIO functions to scavenge endogenous NO, the reduction of CR number by cPTIO indicates that CR emergence is closely related to endogenous NO concentration. Treatments with Cd and cPTIO reduced endogenous NO contents in the root

apices and these effects were reversed by applications of SNP (Fig. 5). These results are consistent with the propositions that endogenous NO content is crucial for rice CR emergence. Cd decreases endogenous NO content and thus suppresses CR formation.

In order to investigate whether the Cd-induced decrease of CR number resulted from the arrest of CR initiation or delay of CR initiation, rice seedlings were treated with Cd and cPTIO for 14 days. In the 2nd week, no new CR was initiated in the cPTIO-treated or Cd-treated rice seedlings (Fig. 6; although in a few seedlings, a new CR was observed). These results indicate that Cd and cPTIO arrest CR initiation in rice seedlings. Serial transverse sections of the root collars indicate that both cPTIO and Cd inhibit CR primordial initiation, and Cd also inhibits the development of immature primordia (Fig. 7). It is possible that CR pimordia initiation and development are two different stages for CR formation, and these two stages are controlled by different genes and signaling molecules (Xu et al. 2005). All these results indicate that endogenous NO is indispensable and important for promoting CR primordia initiation, and Cd decreases CR number by decreasing endogenous NO content in rice seedlings.

In cucumber, it was reported that NO donors were able to mimic the effect of the auxin IAA in inducing the de novo root organogenesis, and the induced roots presented similar anatomic structure (Pagnussat et al. 2002). Results of treatments performed with different SNP concentrations confirmed that the effect was dose dependent, with a maximal biological response at 10 μM SNP, and cPTIO prevented NO donor-induced AR organogenesis (Pagnussat et al. 2002). In rice, the endogenous phytohormone auxin is essential for CR development, and a small number of crown rootless mutants have been found related with auxin signaling (Inukai et al. 2001, 2005; Liu et al. 2005; Xu et al. 2005). Gouvêa et al. (1997) provided the first evidence for the participation of NO in an auxin-induced process in monocot roots. Based on these facts and the results of our experiments, we propose that NO is an importance messenger molecule operating downstream of auxin through a linear signaling pathway during CR organogenesis in rice seedlings. Of course, in order to prove this hypothesis, further research is required.

Although low concentrations of SNP ($\leq$200 μM) recovered Cd-induced decrease of rice CR number, treatment with 200 μM SNP had no effect on CR number in rice seedlings (Fig. 1). Rice seedlings were treated with different concentrations of SNP (1, 10, 50, 100, 200 and 500 μM), but there was no effect on the number of CR (unpublished data). Based on this result, we propose that although a critical content of endogenous NO is indispensable for promoting CR initiation, exogenous NO is useless for inducing CR formation in rice seedlings. In contrast to our results, exogenous NO has been shown to play a role in inducing AR organogenesis in cucumber explants (Pagnussat et al. 2002) and in determining LR development in tomato (Correa-Aragunde et al. 2004) in a dose-dependent manner. The controversial results are possibly due to the fact that different plant species and organs were used. In monocot cereals, CR are genetically determined roots and belong to the normal developmental program of cereals, and endogenous NO is indispensable and adequate for maintaining CR initiation. However, in dicots, AR are formed under unusual circumstances, such as wounding or hormone application at uncharacteristic sites, and belong to the abnormal developmental program (Hochholdinger et al. 2004).

Tian et al. (2007) have shown that cPTIO decreased the fluorescence intensity by 10% and inhibited root elongation by more than 20% in *Hibiscus moscheutos*. On the other hand, SNP markedly recovered cPTIO-induced decrease of root elongation. Based on these results, the authors proposed that maintaining a critical concentration of endogenous NO is essential for root elongation (Tian et al. 2007). In contrast, the results of our study indicated that the NO scavenger, cPTIO, reduced the fluorescence intensity by almost 50% (Fig. 5), but had no effect on CR elongation (Fig. 2). Treatment with cPTIO plus SNP also had no effect on CR elongation in rice seedlings (Fig. 2). These results, however, indicate that endogenous NO was not necessary for maintaining CR elongation in rice seedlings. The controversial findings are possibly due to the difference in the plant roots used in the experiments. It is possible that endogenous NO plays a role in dicot PR elongation rather than in monocot CR. Interestingly, treatments with 100 μM Cd reduced endogenous NO content (Fig. 5) and inhibited CR elongation (Fig. 1) in the rice seedlings, and SNP partially recovered the decrease of CR elongation (Fig. 1). In plant roots, the progressive reduction in the growth rate, which depends on both Cd concentration and exposure time, mainly results from the reduction or inhibition of mitotic activity, along with the appearance of chromosome aberrations in the apical meristems and from inhibition of cell elongation in the extension regions (Fusconi et al. 2007). It has been demonstrated that Cd-caused oxidative stress partially accounts for the inhibition of cell mitotic activity and cell elongation in root apices (Fusconi et al. 2006; Rodríguez-Serrano et al. 2006), and exogenous NO plays an important role in protecting the plant against oxidative stress (Wang and Yang 2005).

Although recent developments lead us back to the fact that no plant NOS gene has yet been identified (Neill et al. 2008), several studies support the presence of NOS activity in plants (Corpas et al. 2006; Valderrama et al. 2007; Chaki et al. 2009). NR and NOS are still supposed to be the two potential enzymatic sources of NO in plants (Neill et al.

2008). To investigate the contribution of the generation mechanism of endogenous NO and unravel the process of Cd-induced decrease in endogenous NO content, pharmacological approaches using both NR and NOS inhibitors have been employed. Similar to treatment with cPTIO and the NOS inhibitor, L-NMMA decreased endogenous NO content (Fig. 5) and CR number (Fig. 3) significantly. Treatment with SNP recovered L-NMMA-induced decrease of endogenous NO content (Fig. 5) and CR number (Fig. 3) obviously. Moreover, enzyme activity assays indicated that treatment with SNP inhibited NOS activity significantly (Fig. 4). These results indicated that NOS-generated endogenous NO was important for CR initiation, and there was a feedback inhibition mechanism between NOS and endogenous NO content. By contrast, as a potent inhibitor of NR, tungstate had no obvious effect on endogenous NO content (Fig. 5). Treatment with tungstate also had no obvious effect on CR number in rice seedlings (Fig. 3). Moreover, enzyme activity assays indicated that treatment with SNP had no observable effect on NR activity (Fig. 4). There was no nitrate supply in the sterilized agar in our study, so NR could not account for NO generation in our experimental system. All of these results highlight that NOS, rather than NR, was responsible for the endogenous NO source in root morphological responses. This is inconsistent with the results of previous studies on *Hibiscus moscheutos* and *Zea mays* (Tian et al. 2007; Zhao et al. 2007). Interestingly, treatment with L-NMMA decreased endogenous NO content and CR elongation significantly, and these decreases were recovered by the application of SNP (Fig. 3). This phenomenon is not in line with our hypothesis that endogenous NO is unnecessary for maintaining CR elongation in rice seedlings. There is a possibility that L-NMMA may inhibit CR elongation by disturbing other physiological processes accounting for CR elongation, and this disturbance may be coincidentally recoverable by the application of SNP. We cannot exclude the possibility that the chemical agents used in the present study may not specifically target NO (Tian et al. 2007). Treatment with tungstate obviously inhibited both PR and CR elongation (Fig. 3), and was consistent with the result of previous reports in which the authors proposed that reduction of the endogenous NO concentration required extended periods of exposure to tungstate (Tian et al. 2007). Our result is inconsistent with this hypothesis: NO concentrations were determined after 1 day, rather than 20 min, exposure to tungstate in our experiment, but the endogenous NO content was still insensitive to tungstate. Therefore, we suppose that tungstate is toxic to plant roots and that tungstate-induced inhibition on root elongation has no relationship with endogenous NO content.

In the present study, based on the observations that 100 μM Cd inhibited NOS activity (Fig. 4) and decreased the endogenous NO content (Fig. 5), similar to cPTIO, Cd-induced decrease of CR number was recoverable with application of exogenous NO. We therefore propose that Cd decreased endogenous NO content by inhibiting NOS activity and thus inhibited CR primordium initiation. Besides, we also observed that treatment with 100 μM Cd inhibited PR and CR elongation in rice seedlings (Fig. 1). This result is consistent with those of Bartha et al. (2005), Barroso et al. (2006) and Rodríguez-Serrano et al. (2006), who found a decrease of NO content and root elongation in 50 μM Cd-treated pea roots. However, Bartha et al. (2005) reported that NO content increased in roots of *Brassica juncea* and *Pisum sativum* exposed to 100 μM Cd, Cu or Zn, whereas Groppa et al. (2008) reported that 100 μM Cd increased endogenous NO accumulation and then inhibited root growth in wheat seedlings. Yet, the authors suggest that these opposite findings on Cd toxicity, endogenous NO content and root elongation could be explained by the different Cd concentrations used, the age of the plants and the time of treatment (Groppa et al. 2008). We agree with these suggestions and infer that differences in plant root species may be another reason that accounts for these opposite findings. It is well known that PR develops during embryogenesis and CR develops from differentiated cells postembryonically (Inukai et al. 2005). In contrast with the PR system in dicot, rice produces numerous CR that are essential for maintaining normal development. It is possible that in the study of Groppa et al. (2008), PR rather than CR was used in the measurement of endogenous NO content and root length, because PR usually was the longest root in young wheat seedlings. In our experiment, exogenous NO had no amelioration effect on Cd-induced inhibition on PR elongation (Fig. 1), and treatment of cPTIO had no effect on PR elongation too (Fig. 3). We hypothesize that PR elongation was independent of endogenous NO content. In previous reports, the results were based on experiments performed on dicots, or the authors did not distinguish PR from CR in monocots. Therefore, these results indicate that rice PR and CR have different signaling pathways during elongation.

The importance of exogenous NO in protecting against the deleterious effects of toxic heavy metals in plant roots and leaves has been shown by many reports (Kopyra and Gwóźdź 2003; Laspina et al. 2005; Wang and Yang 2005; Tian et al. 2007; Singh et al. 2008). Most of the previous studies have emphasized the antioxidative effect of exogenous NO against heavy metal stresses. For instance, Kopyra and Gwóźdź (2003) conclude that the protective effect of exogenous NO in heavy metal-stressed lupin roots may be at least partly due to the stimulation of SOD activity and/or direct scavenging of the superoxide anion. Wang and Yang (2005) demonstrate that exogenous NO reduces

aluminum toxicity by preventing oxidative stress in the root of *Cassia tora*. Tewari et al. (2008) report that exogenous NO decreases copper-induced oxidative damage in the AR of *Panax ginseng*. In the present study, we discovered that exogenous NO alleviated Cd-induced decrease of CR number and elongation (Fig. 1), and exogenous NO also decreased Cd-caused stunting of rice shoots (data not shown). We propose another mechanism for the amelioration of exogenous NO on Cd toxicity; it is possible that exogenous NO recovers Cd-caused decrease of CR number, increases Cd accumulation in CR cell wall and then decreased Cd toxicity in rice seedlings. We have found that exogenous NO increased Cd tolerance in rice seedlings by increasing polysaccharides content and Cd accumulation in the cell wall of rice roots (unpublished data).

In conclusion, our results demonstrate that Cd inhibits NOS activity and decreases endogenous NO content, and then the reduction of endogenous NO content blocks CR primordium initiation and decreases the CR number. While Cd-caused decrease in CR primordium initiation is correlated with decrease in endogenous NO content, Cd-caused inhibition on PR and CR elongation was not directly related with decrease of endogenous NO content in rice seedlings. This finding may provide a new insight to understand the process of CR development in monocot cereals, and the amelioration of exogenous NO on Cd-caused inhibition on rice root may also provide information for protecting crops against heavy metal toxicity.

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