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REGULAR ARTICLE

Involvement of auxin and nitric oxide in plant Cd-stress responses

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Abstract Cadmium (Cd) toxicity inhibited the seedling growth while inducing the occurrences of lateral roots (LR) and adventitious roots (AR). Further study indicated that auxin and nitric oxide (NO) are involved in the processes. In this study, we chose model plant *Arabidopsis thaliana* and Cd-hyperaccumulator *Solanum nigrum* as material to examine the involvement of Cd-induced auxin redistribution in NO accumulation in plants and the effect of NO on Cd accumulation. For this aim, the histochemical staining, NO fluorescence probe (DAF-2DA) detections combined with the pharmacological study were used in this study. By using DR5:GUS staining analysis combined with NO fluorescence probe (DAF-2DA) detection, we found that Cd-induced NO accumulation is at least partly due to auxin redistribution in plants exposure to Cd. Supplementation with SNP donor S-nitrosoglutathione (GSNO) increased the number of LR and AR. In contrast, NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (cPTIO) reversed the effects of NO on modulating root system architecture and Cd accumulation. These results suggest that manipulation of the NO level is an effective approach to improve Cd tolerance in plants by modulating the development of LR and AR, and provide insights into novel strategies for phytoremediation.

Keywords Cadmium · Nitric oxide · Root system architecture · Auxin redistribution

Abbreviations

ABA	Abscisic acid
AR	Adventitious root
CAT	Catalase
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl- 3-oxide
DAB	3-diaminobenzidine
DAF-2 DA	4,5-diaminofluorescein diacetate
DCFH-DA	2,7-dichlorfluorescein-diacetate
GSNO	Sodium nitroprusside
GUS	3-glucuronidase

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IAA	Indole acetic acid
ICP-MS	Inductively coupled plasma-mass spectroscopy
LR	Lateral root
NBT	Nitroblue tetrazolium
NPA	N-1- naphthylphthalamic acid
NO	Nitric oxide
PR	Primary root
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SOD	Super- oxide dismutase
TIBA	2,3,5-triiodobenzoic acid
X-Gluc	5-bromo-4-chloro- 3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium

Introduction

Cadmium (Cd) contamination in soil (higher than 300 nM) is becoming a major global environmental problem. As one of the most toxic nonessential elements, Cd negatively affects plant growth and development (Wagner 1993; Berkelaar and Hale 2000; Macek et al. 2002). When present at high concentrations (such as 5–200 μM or higher), it can cause oxidative damage by generating free radicals and active oxygen species and affecting the contents of micronutrients by competing for protein and transporter binding sites (Zhang et al. 2009). Cd toxicity inhibits transpiration, photosynthesis and synthesis of RNA in plants, resulting in growth retardation and low biomass accumulation (Hsu and Kao 2004). While much research on Cd-induced growth inhibition and oxidative damages has been reported, the effects of Cd on root system development are not fully understood.

In recent years, much attention has been focused on nitric oxide (NO) in plants. As a signaling molecule, NO serves an important role in numerous physiological processes in plants, including developmental, hormonal and environmental responses (Neill et al. 2002; Xu et al. 2010). NO stimulates plant growth and development, such as promoting seed germination, seedling growth and delaying senescence (Libourel et al. 2006; Kolberz et al. 2008). Many studies indicate that NO improves plant tolerance to both abiotic and biotic stresses (Hsu and Kao 2004; Yang et al. 2006; Sun et al. 2007; Zhang et al. 2008; Vital et al. 2008; Xu et al. 2009a). It has been hypothesized that NO may act as an antioxidant in limiting cellular damage by scavenging the reactive oxygen species (ROS) (Laspina et al. 2005; Kopyra and Gwózdz 2003). There is also evidence suggesting that NO may lead to changes in gene expression as a signaling compound in the molecular cascade (Delledonne et al. 2001; Lamattina et al. 2003). NO has a great impact on the root growth of plants. Evidence has been obtained for the involvement of NO in auxin-, ABA- and ethylene-signaling pathways (Pagnussat et al. 2002; Lozano-Juste and León 2010; Wang et al. 2009). NO accumulation in roots was found to induce lateral root formation (Correa-Aragunde et al. 2004) and adventitious root occurrence (Tewari et al. 2008).

There are conflicting results regarding the impact of Cd on NO accumulation in plants. Barroso et al. (2006) and Rodríguez-Serrano et al. (2009) reported a reduction in the NO content in plants treated with 50 μM $CdCl_2$. However, Bartha et al. (2005) and Besson-Bard et al. (2009) found that Cd induced NO accumulation in pea (100 μM Cd), India mustard (100 μM Cd) and Arabidopsis (200 μM $CdSO_4$) roots. These differences are probably due to the fact that Cd effects greatly depend on the plant used, timing of the treatment and Cd concentration. In this study, we chose the model plant *Arabidopsis thaliana* and the Cd-hyperaccumulator *Solanum nigrum* to examine: (1) NO accumulation in roots under Cd stress (50–100 μM $CdSO_4$), (2) the role of NO in the physiological responses of plant to Cd stress, (3) the involvement of Cd-induced auxin redistribution in NO accumulation in plants and (4) the effect of NO on Cd accumulation.

Materials and methods

Plant material and culture conditions

Seeds of *Arabidopsis thaliana* (L.) *DR5:GUS* line in a Col-0 background were used in this study (Ulmasov et al. 1997). Seeds were sown under sterile conditions in Petri dishes containing half-strength MS medium (Sigma, UK) (Murashige and Skoog, 1962), 1% (w/v) agar and 3% (w/v) sucrose.

Five-day-old *A. thaliana* seedlings were transferred into fresh half-strength MS medium containing various concentrations of $CdCl_2$ (0, 5, 15, 30, 50, 100 and 200 μM) with or without indole acetic acid (IAA, 10 nM), auxin transport inhibitors N-1-naphthylphthalamic acid (NPA, 10 μM) or 2,3,5-triiodobenzoic acid (TIBA, 10 μM) (pH 5.7). The plants were then grown under the same culture conditions for 2 d. At least 20 seedlings were used in each experiment.

Seeds of *Solanum nigrum* were kindly provided by the National Southwest Germplasm Resources Lab, CHINA (IP: SCSB-C-003073) in October 2007 and stored at −20°C until use. Seeds were sterilized and plated on half strength hormone-free MS medium. Cultures were maintained at 22–25°C under a 16 h photoperiod. Seven-day-old *S. nigrum* seedlings were transferred to half-strength Hoagland's solution (pH 5.7) (Hoagland and Arnon, 1950). The seedlings were grown for 7 day in half-strength Hoagland's solution and were subsequently transferred to fresh half-strength Hoagland's solution that contained 100 μM $CdCl_2$ with or without NO donor S-nitrosoglutathione (GSNO, 100 μM) or NO scavenger 2-(4-carboxyphenyl)- 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 200 μM) treatments for 7 d. In this solution, 45.3% of Cd was in the free ionic form, and other species predominant was in the $CdCl^+$ form (calculated using the Geochem-PC program) (Parker et al. 1995). For inducing the adventitious root occurrence, the 2 cm of stems above the junction sites between shoots and roots of seedlings were immersed in culture solution. The culture solution was replaced every 2 days.

Detection of NO accumulation in roots

NO was monitored by incubating roots with 15 μM of the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) in 20 mM HEPES–NaOH (pH 7.5) as described by Kolberz et al. (2008). Thereafter, the roots were washed twice for 5 min with fresh buffer and viewed under a Leica laser scanning confocal microscope (excitation 490 nm; emission 515 nm). The fluorescence intensity was quantified and normalized against the levels in untreated controls that are given a value of 100%.

Determination of proline content in roots

Root samples (0.5 g) were ground in 5 ml of 3% (w/v) sulfosalicylic acid, incubated at 100°C for 10 min and then centrifuged for 15 min at 3000 rpm. The supernatant (2 ml) was mixed with 2 ml of distilled water and 6 ml of a mixture of glacial acetic acid, orthophosphoric acid (3:2, v/v) and 25 mg ninhydrin as described by Errabii et al. (2007). After 1 h of incubation at 100°C, the tubes were cooled and 4 ml of toluene was added. The reaction mixture was measured at 520 nm.

Determination of antioxidative enzyme activity in roots

Fresh roots (0.5 g) were ground in liquid nitrogen. The obtained powder was suspended in 3 ml of extraction buffer containing 50 mM sodium phosphate buffer (pH 7.5), 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1 mM EDTA. After centrifugation (30 min, 12,000 g), the supernatant was used to measure enzyme activity. SOD activity was determined by recording the inhibition of the formazan formation rate by the enzyme (Dhindsa et al. 1981). One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50%. CAT activity was determined by measuring the decrease in H_2O_2 absorbance at 240 nm. The reaction mixture contained phosphate buffer 50 mM (pH 7.0), 12.5 mM H_2O_2, and 50 μl enzyme in a volume of 3 ml (Xu et al. 2010).

Detection of H_2O_2 accumulation and plasma membrane integrity in roots

For localizing H_2O_2 produced by *S. nigrum* roots, treated roots were immersed in 1 mg/ml of 3-diaminobenzidine (DAB)-HCl (pH 3.8) for 5 h and cleared by boiling in alcohol (95%, v/v) for 5 min (Ramel et al. 2009). Photos were taken using a Carl Zeiss Imaging System. Ten roots were analyzed in each set of experiments.

The loss of plasma membrane integrity as an estimation of sensitivity to oxidative stress was monitored by Evans blue staining (Baker and Mock 1994). *S. nigrum* roots were stained in 0.25% (v/v) of Evans blue solution for 15 min at room temperature

and subsequently rinsed three times with distilled water for 10 min. Roots (1 cm) were excised and soaked in N,N- dimethylformamide for 1 h at room temperature. The optical density was measured spectrophotometrically at 600 nm.

DR5:GUS staining analysis

For the detection of the expression of GUS, we incubated the *DR5:GUS* seedlings in GUS buffer with the substrate 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium, Sigma) at 37°C for 12 h in the dark (Ulmasov et al. 1997). The seedlings were washed and placed in 75% (w/v) ethanol before examination under the microscope (Zeiss Axioskop). At least 20 seedlings were analyzed for each treatment.

Determination of Cd accumulation

Plantlets were oven-dried at 65–70°C for 1 week. Fifty milligrams of dried plant tissues were ground up and digested in 1 ml of concentrated nitric acid for 2–3 days at room temperature. Samples were then boiled for 1–2 h until completely digested. After adding 4 ml of Millipore-filtered deionized water and briefly centrifuging (6000 rpm, 1 min), the Cd content of each sample was determined by inductively coupled plasma-mass spectroscopy (ICP-MS). Each experiment was repeated at least three times with six plantlets.

For visualization of Cd distribution in intact roots of *S. nigrum*, seedlings were treated with 100 μM $CdCl_2$ with or without NO scavenger cPTIO treatments. A stock solution of Cd specific probe Leadmium™ Green AM dye (Molecular Probes, Invitrogen, Calsbad, CA, USA) was made by adding 50 μl of DMSO to one vial of the dye. This stock solution was then diluted with 1:10 of 0.85% NaCl. Roots were immersed in this solution for 2 h in the dark (Lu et al. 2008). Cd fluorescence was visualized under a Leica laser scanning confocal microscope (S484/15 for excitation and S517/30 for emission).

Statistics

At least 15 roots were analyzed for each treatment, and all the experiment were repeated at least three times. For the statistical analysis, we used Duncan's test ($P<0.05$).

Results

Nitric oxide acts downstream of auxin on modulating lateral root (LR) occurrence in *Arabidopsis* seedling exposed to Cd

As shown in Fig. 1, Cd treatment markedly induced LR occurrence up to 50 μM in *Arabidopsis* (Fig. 1a). However, higher doses suppressed seedling growth, including LR formation. Inhibition of auxin transport by NPA or TIBA completely suppressed LR formation (Fig. 1b), suggested that Cd treatment affected LR occurrence by modulating the auxin pathway. Involvement of NO in LR development and the correlation between auxin distribution and LR has been reported (Potters et al. 2007). Cd-induced NO production in *Arabidopsis* roots has also been reported (Besson-Bard et al. 2009) and has been confirmed in the study (Fig. 2). The effect of auxin redistribution induced by Cd and its effects on NO accumulation in roots, however, remains unclear. To address this issue, we treated *DR5:GUS* seedlings of *A. thaliana* with $CdCl_2$. The DR5 is a synthetic auxin-response element. The *DR5:GUS* seedling is the transgenic *Arabidopsis* expressing the auxin-responsive reporter DR5::uidA (Ulmasov et al. 1997). As shown in Fig. 2, exposure to 50 μM $CdCl_2$ leads to a redistribution of *DR5:GUS* expression, reflecting the alteration in auxin redistribution. With Cd treatment, the auxin level decreased in the root tip but increased in the middle part of roots. In contrast, inhibition of auxin transport by NPA or TIBA increased the auxin build-up in root tips and decreased the auxin level in the root middle part. Accordingly, Cd treatment increased NO accumulation in the middle part of roots, and supplementation with NPA or TIBA reduced the NO level in the zones. This was found to coincide with Cd-induced LR development and NPA- or TIBA-suppressed LR formation (Fig. 1b). We also found that treatment with NPA or TIBA led to auxin accumulation in root tips, as indicated by GUS staining, and to NO accumulation in the root tips. These results indicate that Cd-mediated auxin redistribution affects NO accumulation in roots. However, we found that although Cd treatment reduced auxin distribution in root tips, it increased NO accumulation in the zone (Fig. 2).

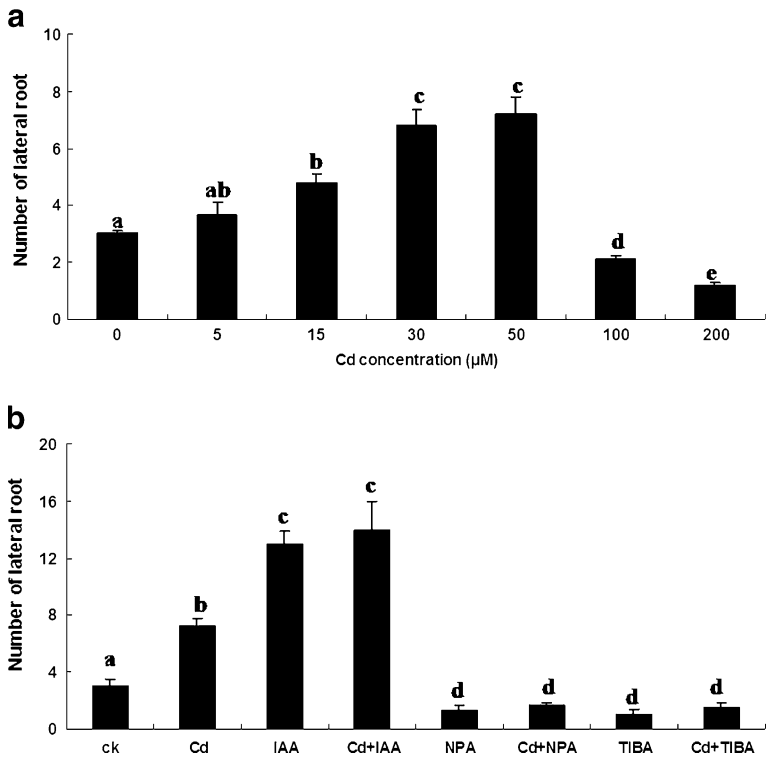

Fig. 1 **a** Effect of Cd treatment on the lateral root occurrence in *Arabidopsis* seedlings exposed to different concentrations of $CdCl_2$ for 7 d. **b** Effect of IAA (10 nM), NPA (10 μM) or TIBA (10 μM) on the lateral root occurrence in *A. thaliana* seedlings exposed to 50 μM $CdCl_2$ for 7 d. ck, untreated control

NO contributes to Cd-induced occurrence of LR and adventitious root (AR) in *S. nigrum*

Cd-induced LR formation has also been observed in the Cd-hyperaccumulator *Solanum nigrum* (Table 1), indicating that Cd-modulated plastic development of the root system architecture is a common phenomenon in plants. Cd toxicity markedly inhibits plantlet growth (Table 1). After 7 days of exposure to 100 μM Cd, the plantlet height decreased by 38%, the fresh weight of the root and shoot was reduced by 51% and 32% respectively, when compared with untreated control seedlings, while the number of LR increased by 29% (Table 1). Cd treatment also stimulated the AR formation in hydroponic seedlings (Table 1, Fig. 3).

In order to determine the accumulation of NO in *S. nigrum* roots under Cd stress, a DAF-2DA fluorescence probe was added to detect the NO level in vivo. 100 μM of $CdCl_2$ triggered an increase of NO production in *S. nigrum* roots (Fig. 4). Compared to untreated control plants, the Cd-induced NO production in roots was almost completely prevented by the NO scavenger cPTIO (Fig. 4), suggesting that the DAF-2DA-dependent fluorescence was related to the amount of endogenous NO. In contrast, supplementation with the NO donor GSNO further increased NO fluorescence in roots (Fig. 4).

To investigate the role of Cd-induced NO in *S. nigrum* seedlings response to Cd stress, we applied the NO scavenger cPTIO to examine plant growth under Cd stress. As shown in Table 1, supplementation with the NO scavenger cPTIO reduced the plantlet height by 14% and the fresh weight of the root by 33.3% and of the shoot by 28.1% versus Cd treatment alone. Cd treatment induced the formation of LR and AR in hydroponic seedlings. The supply of NO scavenger cPTIO decreased the number of LR by 51.6% and AR by 61.5% as compared with Cd treatment alone. In contrast, supplementation with the NO donor GSNO markedly increased the plantlet height and fresh weight and further promoted the formation of LR and AR (Table 1). These data indicate that Cd led to significant inhibition of growth as measured by plantlet height and fresh weight, which were alleviated by treating with the NO donor GSNO.

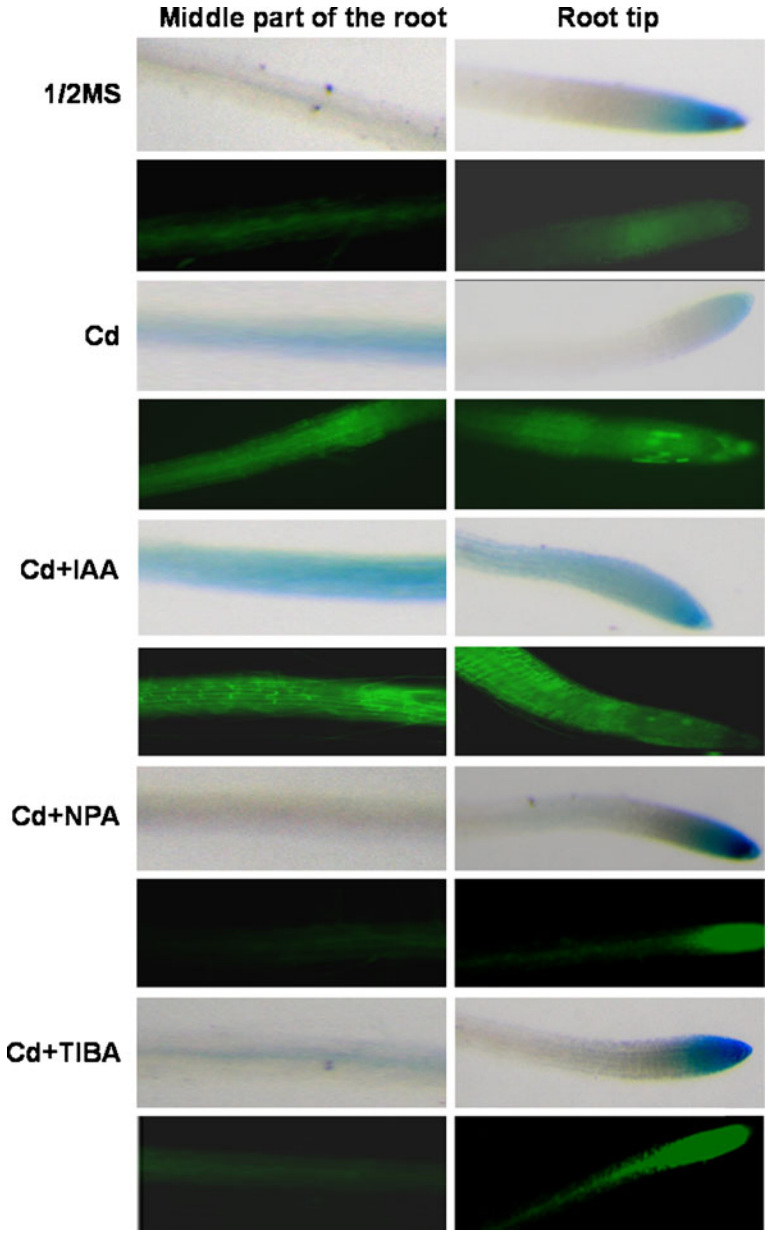

Fig. 2 Effect of Cd toxicity (50 μM) on the auxin level and NO production in roots of *Arabidopsis thaliana*. Five-day-old *A. thaliana* DR5: GUS seedlings were treated with indole acetic acid (IAA, 10 nM), N-1-naphthylphthalamic acid (NPA, 10 μM) or 2,3,5-triiodobenzoic acid (TIBA, 10 μM) for 2 d and then stained with GUS dye. For NO fluorescence probe detection, roots of *A. thaliana* seedlings were loaded with 4,5-diaminofluorescence (DAF-2DA) and then detected by confocal laser scanning microscopy. In each treatment, upper plate represents *DR5:GUS* staining and lower plate represents NO production in *A. thaliana* roots

NO reduced the oxidative damage, promoted proline accumulation and maintained the antioxidative enzyme activity

Cd induced oxidative damage and cell death in plants. To monitor cell viability of *S. nigrum* roots exposed to Cd stress, treated roots were stained with Evans blue dye. Evans blue is a molecule retained only in dead cells, and quantification of this molecule retained by roots can estimate cell death and sensitivity to oxidative stress (Xu et al. 2010). As shown in Table 1 and Fig.5a, Cd treatment induced cell death significantly. Supplementation with the NO scavenger cPTIO further increased cell death and H_2O_2 content (Fig.5b), whereas GSNO reduced cell death and H_2O_2 content, suggesting that Cd-induced NO in roots may protect against oxidative damage and help survival.

The results above indicate that NO is involved in Cd-induced H_2O_2 accumulation in roots. To test if NO-modulated H_2O_2 accumulation is due to an induction of antioxidant system, we investigated the content of proline and the activities of SOD and CAT

Table 1 Physical and chemical measurements of *S. nigrum* seedlings grown in half-strength Hoagland's solution containing 100 μM $CdCl_2$ with or without cPTIO (200 μM) or GSNO (100 μM). Means ± SE, n=6

Indices	Treatment					
	ck	Cd	Cd+cPTIO	cPTIO	Cd+GSNO	GSNO
Plantlet height (cm)	6.43±0.5a	4±0.35 d	3.44±0.21e	6.5±0.4a	4.7±0.35c	5.98±0.43b
Fresh weight in root (g)	0.61±0.13a	0.3±0.06 d	0.2±0.01e	0.62±0.12a	0.39±0.09c	0.53±0.04b
Fresh weight in shoot (g)	2.9±0.36ab	1.97±0.06c	1.42±0.09c	3.1±0.41a	2.4±0.04b	3.4±0.3a
Number of lateral roots	8.8±0.84 d	12.4±0.5b	6±1f	7.5±0.7e	15.9±1.3a	9.5±0.78c
Number of adventitious roots	0	14.8±0.9b	5.7±1 d	0	18.3±1.2a	6.8±1.1c
Proline content (μg g^{-1} FW)	25±3.8de	93.4±6b	59.7±5.6c	23.4±3.1e	125.2±11a	26.2±3.3 d
Evans blue uptake (OD_{600})	0.16± 0.02 d	0.28±0.03b	0.36±0.03a	0.12±0.01e	0.22±0.03c	0.18± 0.02 cd
SOD activity (U mg^{-1} protein)	16.1±1.6e	18.7±0.9b	17.7±1.1c	17±2 d	20.5±1.3a	16.8±1.6 d
CAT activity (μmol H_2O_2 min^{-1} mg^{-1} protein)	12.3±1.9 d	23.3±2.4b	19.1±1.7c	13.4±1.1 d	25.6±2.4a	11±0.9e
H_2O_2 production (μmol g^{-1} FW)	341±27 d	423±47b	531±58a	331±26 d	387±36c	333±25 d
Cd content in seedlings (μg g-1 DW)	0.8±0.12 d	27.5±1.2b	21±2.3c	0.3±0.01e	36±4.1a	0.56±0.02 d

in roots. The accumulation of proline is an important mechanism in the response of plants to cadmium stress (Siripornadulsil et al. 2002). Cd toxicity increased the proline content in roots of *S. nigrum*. Supplementation with the NO scavenger cPTIO reduced the proline content to 64%, whereas GSNO increased the proline content to 134% compared with Cd treatment alone (Table 1), indicating that NO is involved in Cd-induced proline accumulation. Cd toxicity increased SOD and CAT activity (Table 1). Supplementation with the NO donor SNP further increased SOD and CAT activity, whereas, application of the NO scavenger cPTIO decreased SOD and CAT activity.

NO role in Cd accumulation

To investigate the role of NO in Cd accumulation, the NO scavenger cPTIO was applied to determine Cd accumulation under Cd stress. ICP-MS analysis showed that inhibition of NO accumulation by cPTIO reduced the accumulation of Cd by 23.6%, whereas supplementation with GSNO increased Cd accumulation by 25% compared with Cd treatment alone (Table 1).

Cd-specific probe Leadmium™ Green AM dye has been successfully used to detect Cd accumulation in plants (Lu et al. 2008) and was used here to further investigate Cd localization in roots of *S. nigrum* seedlings. A very low level of fluorescence was observed in the roots of seedlings grown in normal half-strength Hoagland's solution with or without GSNO and cPTIO treatment (data not shown). In contrast, an intense fluorescence was observed in Cd-treated roots of *S. nigrum* seedlings. Supplementation with the NO donor GSNO further increased the green fluorescence, especially in vascular tissues, whereas cPTIO reduced the green fluorescence (Fig. 6). These results further supported the assumption that Cd-induced NO accumulation is advantageous for Cd accumulation in plants.

Discussion

NO accumulation alleviates Cd toxicity in *S. nigrum* seedlings

Solanum nigrum, the identified Cd hyperaccumulator, has received extensive attention in recent years. In

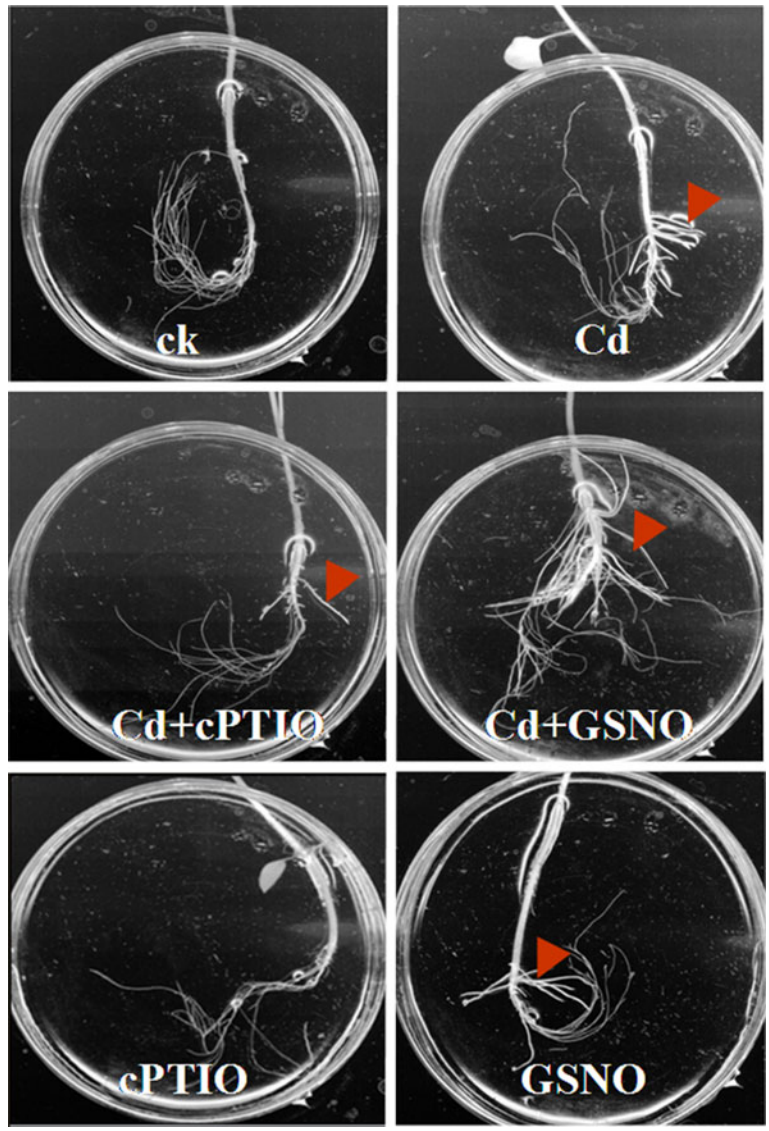

Fig. 3 Effect of NO on adventitious root formation in 100 μM Cd-treated *Solanum nigrum* seedlings with or without cPTIO (200 μM) or GSNO (100 μM) for 7 d. Red arrows indicate the occurrence of adventitious roots. ck, untreated control

this study, we found that NO levels rapidly increased in roots under Cd stress. NO is a bioactive messenger molecule, acts as an antioxidant and quenches Cd-induced ROS (Singh et al. 2008). The protective role of NO in Cd toxicity has been reported in rice shoots (Hsu and Kao 2004), sunflower shoots (Laspina et al. 2005), wheat roots (Singh et al. 2008) and *Medicago truncatula* roots (Xu et al. 2010). In the present study, we found that Cd led to significant inhibition of growth as measured by plantlet height and fresh weight, which were aggravated by treating with the NO scavenger cPTIO. In contrast, supplementation with the NO donor GSNO alleviated Cd toxicity. Cd treatment affected the plasma membrane permeability and thereby inhibited water and nutrition metabolism. Supplementation with GSNO decreased H_2O_2 production and improved plasma membrane integrity as indicated by Evans blue staining, whereas, inhibition of NO accumulation by cPTIO reversed the effects. These results suggest that Cd-induced NO accumulation is advantageous for protecting against Cd stress in plants.

Exogenous NO enhanced the accumulation of proline and glutathione in *Medicago truncatula* roots (Xu et al. 2010). Siripornadulsil et al. (2002) reported that proline counteracts free radical damage in cells. Cd-induced proline accumulation in *S. nigrum* seedlings can reduce free radical damage and maintain a

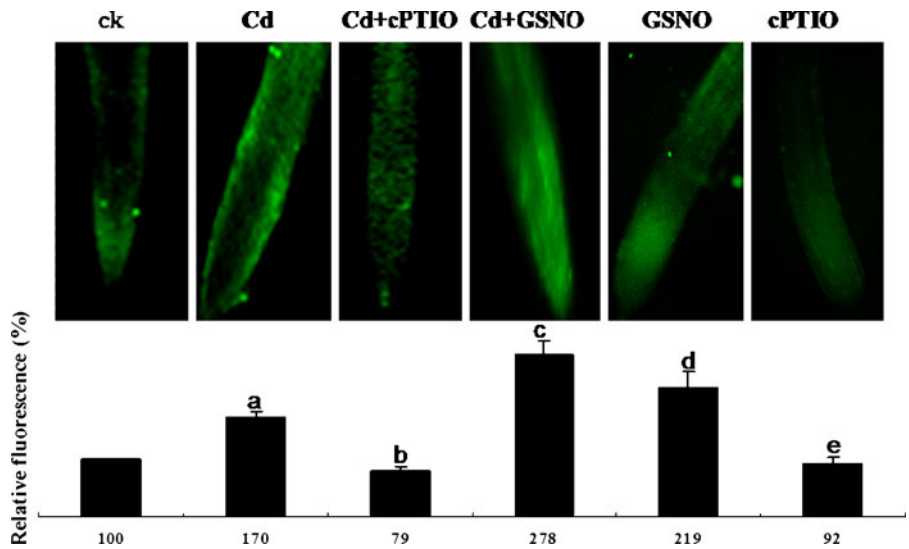

Fig. 4 Effects of cPTIO (200 μM) or GSNO (100 μM) on NO production in *Solanum nigrum* roots exposed to 100 μM $CdCl_2$ for 7 d. Representative images show NO production in roots. ck, untreated control

more reducing environment in *S. nigrum* seedlings, reducing Cd toxicity in plants (Xu et al. 2009b). We found that inhibition of NO accumulation by cPTIO further reduced the proline content and increased the H_2O_2 level and oxidative damage in roots of *S. nigrum*, whereas application of GSNO increased proline content and decreased the H_2O_2 level and oxidative damage, suggesting that Cd-induced NO repressed the ROS burst at least partially by promoting proline accumulation.

Cd-modulated antioxidative enzyme activity and NO roles have been reported, although there are

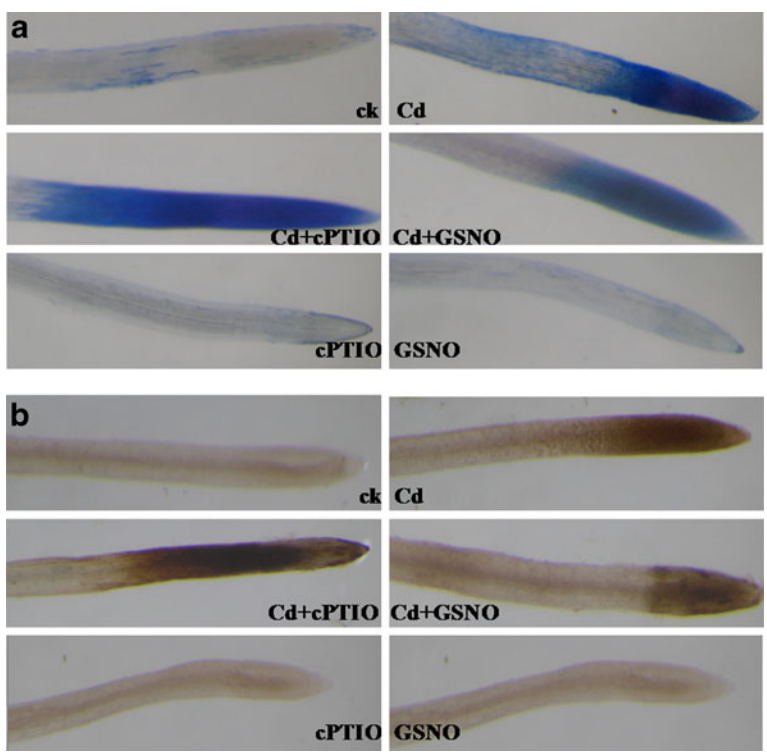

Fig. 5 **a** Evans blue staining showing the effects of NO on the plasma membrane integrity in the roots of *Solanum nigrum* seedlings exposed to 100 μM $CdCl_2$ with or without cPTIO (200 μM) or GSNO (100 μM) for 7 d. **b** DAB staining showing the effect of cPTIO (200 μM) and GSNO (100 μM) on Cd-induced H_2O_2 accumulation in *S. nigrum* roots. ck, untreated control

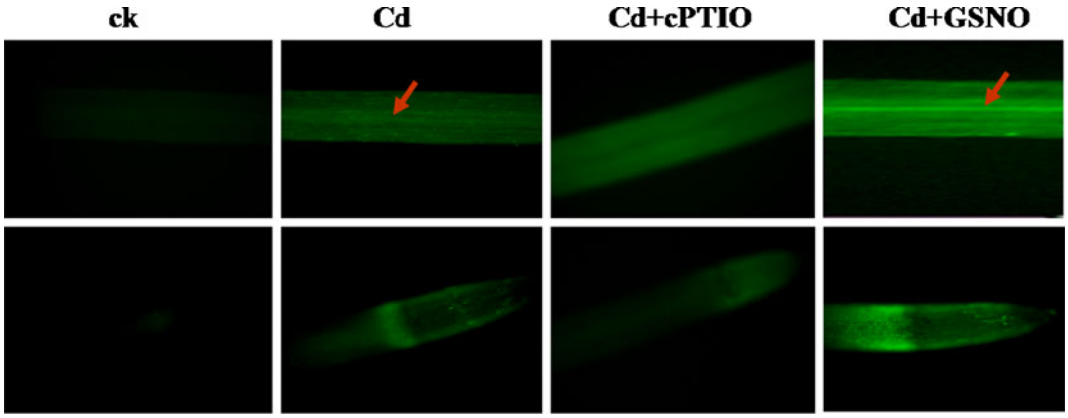

Fig. 6 Leadmium™ Green AM fluorescence dyeing showing the effect of NO on the Cd accumulation in *Solanum nigrum* seedlings exposed to 100 μM Cd with or without cPTIO (200 μM) or GSNO (100 μM) treatments for 7 d. ck, untreated control

conflicting results in these studies (Hsu and Kao 2004; De Michele et al. 2009). Vital et al. (2008) have found that NO inhibits SOD activity in cotton callus. De Michele et al. (2009) have reported that Cd-induced NO production negatively affects CAT activity in Cd-treated *Arabidopsis* suspension cells. The discrepancies may be attributed to differences in plant species and Cd concentrations used and in time points detected in these studies. In this study, we tested the activity of SOD and CAT in the roots of *S. nigrum* seedlings exposed to Cd stress. SOD, as a key enzyme in protecting cells against oxidative stress, catalyses the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. CAT catalyzes the dismutation of H_2O_2 to H_2O and O_2. Both the activity of SOD and CAT were elevated in Cd-treated *S. nigrum* roots, indicating a high H_2O_2 scavenging capacity in *S. nigrum* roots. The findings may partially explain the fact that *S. nigrum*, as a hyperaccumulator, has a strong Cd stress tolerance. We also found that NO increased SOD and CAT activity in Cd-treated *S. nigrum* roots. These studies indicate that Cd-induced NO can both maintain intracellular antioxidative capacity and reduce oxidative damage.

NO is involved in Cd-induced LR/AR formation and auxin redistribution affects NO accumulation in Cd-treated roots

Cd toxicity inhibits primary root (PR) growth, whereas it promotes LR formation. Similar morphological changes in the root system have also been noted in NO-treated plants. The application of exogenous NO increases the number of tomato LR, whereas depletion of intracellular NO arrests LR initiation (Correa-Aragunde et al. 2004). Besson-Bard et al. (Besson-Bard et al. 2009) reported that NO is responsible for Cd-induced growth inhibition of PR in *Arabidopsis*. However, the underlying physiological and molecular mechanisms have not been completely elucidated. In this study, scavenging NO by cPTIO reduced LR formation in both *Arabidopsis* and *S. nigrum* seedlings. These results indicated that though Cd-induced NO production inhibited PR growth, it promoted LR formation. Root traits are closely related to stress tolerance of plants. Several studies indicated that the morphological alterations that result in an increased surface area, such as the formation of root hairs and LR, provide the basis for physiological reactions to abiotic stresses (Schmidt et al. 2000). Our study is in agreement with previous reports showing that NO enhances the tolerance to Cd (Laspina et al. 2005) and Fe deficiency (Martin et al. 2009) in plants. However, these data are contradictory to the results of Besson-Bard et al. (2009) and De Michele et al. (2009) showing that NO contributes to Cd sensitivity of seedling growth and programmed cell death in *Arabidopsis* suspension cultures. This discrepancy might be attributed to differences in plant species used in these studies. *Arabidopsis* is a Cd-sensitive plant species. However, *S. nigrum*, as a trace metal hyperaccumulator, exhibits a higher Cd tolerance than *Arabidopsis*; therefore, it may respond to Cd stress more rapidly and, subsequently, change its root system architecture. After adaptation to Cd stress

conditions by modulating LR and AR formation, plant growth was gradually recovered. In contrast, inhibiting NO production disturbed the formation of LR and AR under Cd toxicity and was therefore disadvantageous for the plant's adaptation to Cd toxicity. Similar results are also observed in Zn-treated *S. nigrum* seedlings (unpublished data), suggesting that trace metal-induced NO production might be an adaptive mechanism for *S. nigrum* response to trace metal stress.

Maintaining the balance of auxin metabolism and distribution in roots is required for root system development under abiotic stresses. The transportation of auxin from root tips into the elongation zone is required to regulate cell elongation and LR development. Both the reduced and excessive levels of auxin in the root tip repress the PR growth and LR formation. Cd treatment inhibited PR growth and promoted LR formation. *DR5:GUS* activity analysis indicated that Cd treatment reduced auxin content in the apex, while it increased the auxin level in the middle part of roots, suggesting that the modulated auxin redistribution by Cd affects the root system development.

Previous reports have shown that NO accumulation in roots has an effect on auxin-modulated growth of LR and AR (Correa-Aragunde et al. 2004; Tewari et al. 2008). Correa-Aragunde et al. (Correa-Aragunde et al. 2004) found that NO is involved in auxin-modulated LR development in tomato. In this study, we found that Cd stress induced the occurrence of LR and AR. Supplementation with the NO scavenger cPTIO reversed the effects of Cd on LR and AR formation, indicating that Cd-induced NO is involved in this process. Correlation between auxin distribution and LR formation has also been reported (Potters et al. 2007). Therefore, we hoped to determine whether Cd-induced NO accumulation is involved in auxin-mediated occurrences of LR and AR. We found that Cd treatment increased the auxin level in the middle part of roots and enhanced NO accumulation in the zone. Supplementation with the auxin transport inhibitors, NPA and TIBA, reversed the effects of Cd on the auxin distribution and, subsequently, NO accumulation in the middle root zones. Build-up of auxin in root tips by NPA or TIBA also produced intense NO fluorescence by DAF-2DA staining in the zone. These results implied that auxin distribution affects NO accumulation in roots and that NO acts downstream to auxin action during LR induction. Although Cd treatment reduced the auxin level in root tips, we found that it increased NO accumulation in the zone. Therefore, we believe that Cd-induced NO accumulation in root occurs through auxin dependent and independent pathways. NO accumulation in root tip regulates the cell-cycle genes, such as *CYCD3;1*, triggering cell cycle activation and LR formation (Correa-Aragunde et al. 2004). The plasticity to modulate the root system architecture is one way to overcome the inability of plants to move towards water and nutrients stores (Correa-Aragunde et al. 2004). Cd-induced NO accumulation promotes the LR and AR formation and, hence, improves the water and nutrient uptake capability and Cd tolerance as indicated by the fresh weight and height of seedlings.

NO contributes to root Cd accumulation

Inhibition of NO accumulation by cPTIO reduced Cd accumulation, suggesting that the endogenous NO induced by Cd has a relation to the Cd accumulation. Several studies have provided evidence showing that NO promotes Cd accumulation in plants. Besson-Bard et al. (2009) found that NO-induced up-regulation of *IRT1*, *FRO2* and *FIT* may be responsible for Cd absorption in *Arabidopsis*. In this study, we found that NO is involved with Cd-induced proline accumulation in *S. nigrum* seedlings. Proline can reduce the free metal ion concentration in cells by chelating metal ions. Therefore, NO-induced proline accumulation promotes the build-up of Cd accumulation in plants. Further study will elucidate the detailed molecular mechanisms involved in Cd-induced NO production in plants.

In summary, the result of the present study revealed that nitric oxide acts downstream of auxin on modulating lateral root occurrence in *Arabidopsis* seedling exposed to Cd and treatment with NO was effective in inducing Cd tolerance in plants. Our study provides an explanation for the role of Cd-induced NO in the LR and AR occurrences and the Cd accumulation in plants. The results presented here should benefit future work to reveal the molecular mechanisms of NO in modulating plant Cd tolerance. Our results suggest that the manipulation of the NO level is an effective approach to improve Cd tolerance in plants.

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