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# Nitrate reductase-mediated NO production enhances Cd accumulation in *Panax notoginseng* roots by affecting root cell wall properties

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ABSTRACT

*Panax notoginseng* (Burk) F. H. Chen is a traditional medicinal herb in China. However, the high capacity of its roots to accumulate cadmium (Cd) poses a potential risk to human health. Although there is some evidence for the involvement of nitric oxide (NO) in mediating Cd toxicity, the origin of Cd-induced NO and its function in plant responses to Cd remain unknown. In this study, we examined NO synthesis and its role in Cd accumulation in *P. notoginseng* roots. Cd-induced NO production was significantly decreased by application of the nitrate reductase inhibitor tungstate but not the nitric oxide synthase inhibitor L-NAME ($N^G$-methyl-L-arginine acetate), indicating that nitrate reductase is the major contributor to Cd-induced NO production in *P. notoginseng* roots. Under conditions of Cd stress, sodium nitroprusside (SNP, an NO donor) increased Cd accumulation in root cell walls but decreased Cd translocation to the shoot. In contrast, the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and tungstate both significantly decreased NO-increased Cd retention in root cell walls. The amounts of hemicellulose 1 and pectin, together with pectin methylesterase activity, were increased with the addition of SNP but were decreased by cPTIO and tungstate. Furthermore, increases or decreases in hemicellulose 1 and pectin contents as well as pectin methylesterase activity fit well with the increased or decreased retention of Cd in the cell walls of *P. notoginseng* roots. The results suggest that nitrate reductase-mediated NO production enhances Cd retention in *P. notoginseng* roots by modulating the properties of the cell wall.



## 1. Introduction

The heavy metal cadmium (Cd) is a widespread environmental pollutant with toxic effects on plant growth and development (Wagner, 1993). For example, Cd reduces plant growth by inhibiting photosynthesis and respiration, which disturbs nutrient assimilation (Chugh and Sawhney, 1999; Choppala et al., 2014). Due to its high solubility, Cd can easily and rapidly be taken up by plant roots and translocated to above-ground parts. Therefore, Cd contamination of crops poses a potential risk to human health and compromises the commercial value of the products, especially for plants with edible roots.

Plants have evolved various mechanisms to resist Cd stress, such as antioxidative enzyme upregulation and metal chelation by phytochelatins (PCs), compartmentalization in vacuoles and metal binding to the cell wall (Di Toppi and Gabbrielli 1999; Wang et al., 2008; Choppala et al., 2014). Among these strategies, Cd deposition in the cell wall is considered to be an effective detoxification mechanism due to the decreased Cd translocation to above-ground parts. In *Athyrium yokoscense*, approximately 70–90% of cellular heavy metals (Cd, Cu and Zn) was found to be located in the cell wall, preventing the metals from entering the cytoplasm (Nishizono et al., 1987). In bush bean roots and leaves, Cd is primarily bound by pectic sites and histidyl groups present in the cell wall (Leita et al., 1996).

Nitric oxide (NO), a crucial gaseous signaling molecule, plays significant roles in modulating several physiological and biochemical functions in plants (Wang et al., 2010). For instance, NO is involved in regulating root growth and development, leaf expansion, cell senescence, stomatal closure and programmed cell death

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(Neill et al., 2003). Furthermore, NO has been shown to affect several biotic and abiotic stresses, such as drought, salinity and heavy metal toxicity (Qiao and Fan, 2008). External application of sodium nitroprusside (SNP, an NO donor) protects tomato plants against salinity-induced oxidative stress by stimulating the activity of antioxidative enzymes. Similarly, exogenous NO application alleviates the toxicity caused by Cd in *Medicago truncatula* roots (Xu et al., 2010). In rice and *Arabidopsis*, exogenous NO enhances Cd deposition in roots and decreases its translocation to shoots (Besson-Bard et al., 2009; Xiong et al., 2009).

The NO biosynthesis in plants is mediated by both non-enzymatic and enzymatic processes. The non-enzymatic pathway involves nitrite dismutation into nitrate and NO (Gotte et al., 2002; Neill et al., 2002). In addition to non-enzymatic production, nitric oxide synthase (NOS, EC1.14.23) and nitrate reductase (NR, EC 1.7.1.1) have been regarded as two key enzymes involved in NO production in plants. In animals, NO is primarily synthesized by the NOS-mediated conversion of L-arginine to L-citrulline and NO (Furchgott, 1995). NOS activity has also been detected in plants because the NOS inhibitor (L-NAME) was reported to block NO synthesis (Crawford, 2006). Nitrate reductase is associated with nitrogen assimilation, which can also generate NO with NAD(P)H as an electron donor (Kaiser et al., 2002). Furthermore, nitrate reductase-mediated NO synthesis is involved in salt stress (Liu et al., 2007), freezing tolerance (Zhao et al., 2009) and Al resistance (Wang et al., 2010). Despite evidence suggesting that exogenous NO plays an important role in the plant response to Cd stress, the origin of Cd-induced NO and its function in this response remain unknown.

*Panax notoginseng* (Burk.) F.H. Chen is one of the precious traditional herbs in China. The roots are used for the treatment of cardiovascular disease, inflammation, trauma and hemorrhage (Yan et al., 2012), and due to its high medicinal value, this plant is widely cultivated in Wenshan autonomous prefecture, Yunnan Province, China. However, Cd levels in soils of *P. notoginseng* cultivation areas and in its roots were recently found to exceed the national standard by 75% and 61%, respectively (Zhu et al., 2012a). Cd contamination of *P. notoginseng* roots is directly correlated with its medicinal safety, making this a primary obstacle for its international trade (Zhu et al., 2012a). Thereby, minimizing or preventing Cd contamination of *P. notoginseng* roots is an urgent problem that remains to be solved. The aim of this study was to investigate whether NO synthesis and activity are involved in Cd accumulation in *P. notoginseng* roots. We provide evidence that Cd-induced NO production occurs through the activation of nitrate reductase. Furthermore, nitrate reductase-dependent NO production enhanced Cd accumulation in *P. notoginseng* roots by modulating root cell wall properties. Our findings will not only be helpful for a better understanding of the physiological mechanisms of Cd-induced NO production in plants, but will also provide a new insight into preventing Cd contamination of *P. notoginseng* through biotechnological approaches.

## 2. Materials and methods

### 2.1. Plant cultivation and treatments

One-year-old *P. notoginseng* plants (approximately 3 g fresh weight/plant) were collected from Wenshan autonomous prefecture, Yunnan Province, China (23°18′ N 104°20′ E). The plants were dug from the soil, rinsed with deionized water, transferred to a glass pot containing 200 mL ½-strength Hoagland nutrient solution, and grown in a growth chamber under 12 h light (100 μmol $m^{-2}$ $s^{-1}$) and temperature of 25 °C; the relative humidity was 60%. The nutrient solution was renewed every other day.

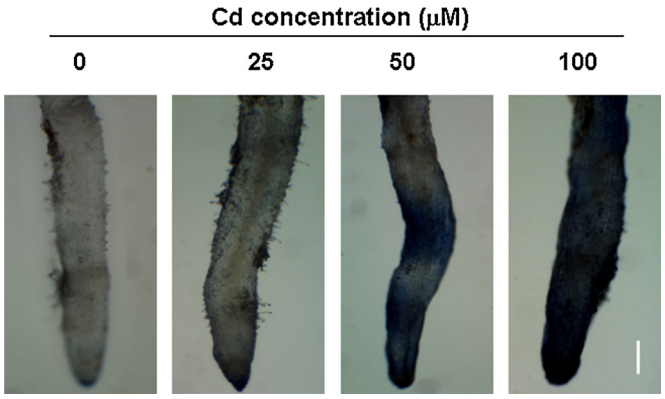


**Fig. 1.** Effects of Cd on *P. notoginseng* root (0–2 cm) membrane integrity, as assessed by Evans blue staining. The experiments were conducted three times, and one set of representative results is shown. The white bar represents 2 mm.

After one week of growth, the plants were transferred to the following treatment solutions: (1) ½-strength Hoagland nutrient solution containing 0 (CK), 25, 50 or 100 μM $CdCl_2$; (2) ½-strength Hoagland nutrient solution containing 50 μM $CdCl_2$ plus 0 or 100 μM SNP (sodium nitroprusside, an NO donor); (3) ½-strength Hoagland nutrient solution containing 50 μM $CdCl_2$ plus 100 μM SNP with or without 100 μM L-NAME ($N^G$ nitro-L-Arg-methyl ester, an NOS inhibitor) or 100 μM tungstate ($Na_2WO_4$, a nitrate reductase inhibitor) or 100 μM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, an NO scavenger). After 24 h, the plants were harvested for analysis.

### 2.2. Detection of plasma membrane integrity

Evans blue staining indicates cell death, and the degree of staining in the root provides a semi-quantitative measurement of membrane permeability (Hamer et al., 2002). Accordingly, the loss of plasma membrane integrity in *P. notoginseng* roots was evaluated by Evans blue staining (Baker and Mock, 1994). After exposure to 0, 25, 50 or 100 μM Cd for 24 h, the roots were thoroughly rinsed with deionized water, gently blotted and weighed. *P. notoginseng* root tips were stained with 0.5% (v/v) Evans blue solution for 10 min at room temperature, rinsed three times with deionized water for a total of 10 min and photographed.

### 2.3. Determination of NO content

The NO content was determined using the NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM), as previously described (Foresi et al., 2007). After treatment, the roots were thoroughly rinsed with deionized water and gently blotted. The root tips were incubated with 15 μM DAF-FM for 20 min at root temperature. The samples were washed three times with phosphate-buffered saline (PBS, 10 mM, pH 7.4), mounted on glass slides and examined with a Leica (DMI 6000) fluorescence microscope (excitation 488 nm; emission 525 nm). Very weak fluorescence in the roots treated with Cd + tungstate was observed. Thus, to observe these roots more clearly, the exposure time for the experiment shown in Fig. 3A was longer than that for the data shown in Fig. 2A.

For quantitative analysis of the NO content, roots were thoroughly rinsed with deionized water, gently blotted and weighed after treatment. These samples were then homogenized with 1 mL of 50 mM acetic acid solution (pH 3.6) containing 4% (w/v) zinc acetate. The extracts were centrifuged at 9500 × *g* at 4 °C for 15 min, and the NO content was measured using an NO detection kit

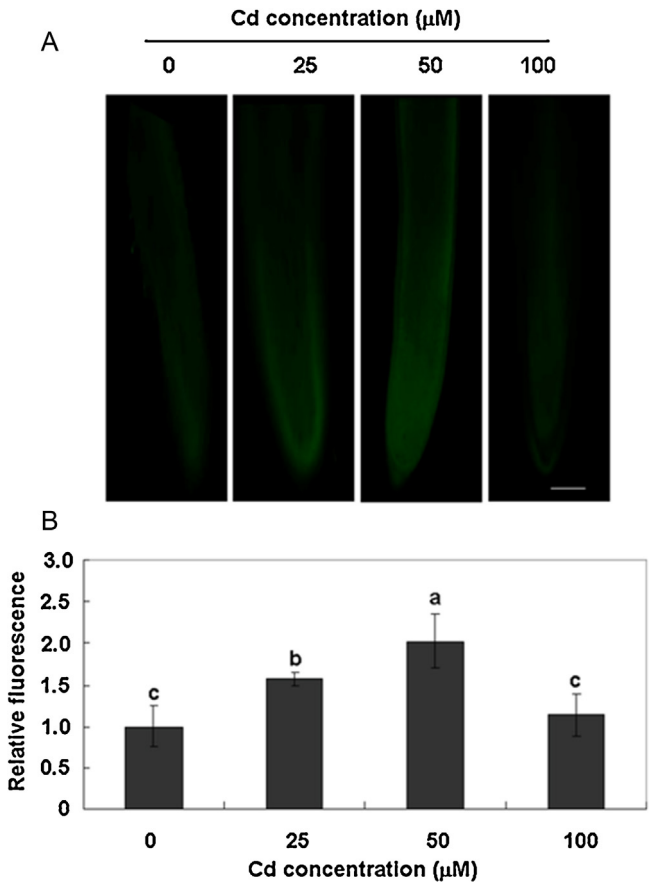


**Fig. 2.** Changes in endogenous NO levels (A) and relative fluorescence intensities (B) in *P. notoginseng* roots (0–2 cm) in response to different concentrations of Cd. *P. notoginseng* roots were treated with 0, 25, 50 or 100 μM Cd for 24 h; the NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) was used for detection. For (B), quantification of the fluorescence intensity was determined using ImageJ. The white bar represents 2 mm. Values represent the means ± SE ($n = 6$).

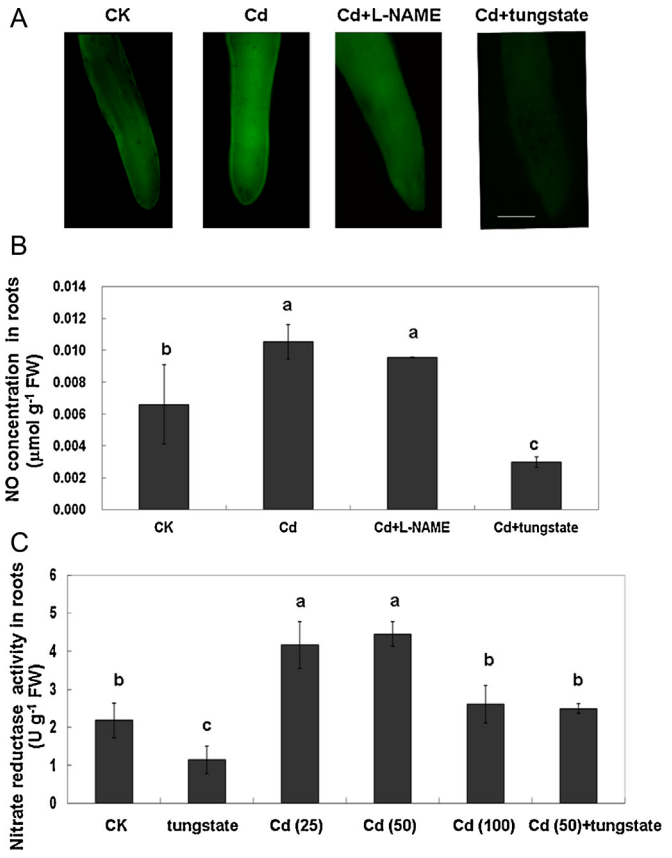


**Fig. 3.** Response of the endogenous NO level (A and B) and nitrate reductase activity (C) to Cd stress in *P. notoginseng* roots. (A) the endogenous NO concentration in *P. notoginseng* roots (0–2 cm) was monitored by the NO-specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM). The experiments were conducted three times, and one set of representative results is shown. The white bar represents 2 mm. (B) Quantitative analysis of endogenous NO production in *P. notoginseng* roots. For A and B, 100 μM tungstate or 100 μM L-NAME ($N^G$-methyl-L-arginine acetate) was added to the 50 μM Cd treatment solution. (C) *P. notoginseng* plants treated with 0, 25, 50 or 100 μM Cd, or 50 μM Cd plus 100 μM tungstate for 24 h. Values are the means ± SE ($n = 6$).

according to the manufacturer's instructions (Beyotime Institute of Biotechnology, China).

### 2.4. Determination of nitrate reductase activity

The activity of nitrate reductase was measured according to the method described by Sun et al. (2014). After treatment, the roots were homogenized with extraction solution containing 50 mM Hepes-KOH (pH 7.5), 5% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μM flavin adenine dinucleotide (FAD). The homogenates were centrifuged at 13,000 × *g* at 4 °C for 20 min. The activity of nitrate reductase was detected by mixing 200 μL supernatant with 500 μL reaction buffer containing 50 mM Mops-KOH buffer (pH 7.5), 10 mM $KNO_3$ and 0.2 mM NADH to initiate the reaction in a 2-mL centrifuge tube. After incubation for 30 min at 25 °C, the reaction was stopped by adding 50 μL of 0.5 M zinc acetate; 1 mL of 1% (w/v) sulfanilamide and 0.02% (w/v) α-naphthylamine were added to the solution, and after 15 min, the absorbance of the mixture was measured at 540 nm using a spectrophotometer.

### 2.5. Determination of Cd content

For measurement of Cd concentrations in tissues, *P. notoginseng* plants were divided into roots, stems and leaves. The plant materials were thoroughly washed with deionized water and weighed. These samples were then transferred to borosilicate tubes and ashed at 550 °C for 12 h. The ash inside the tubes was dissolved overnight in 1 mL of concentrated $HNO_3$ followed by dilution to 10 mL with deionized water. Cadmium was analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, model PS-1000, Leeman Labs., Lowell, MA, USA).

Tissue fractionation was performed as described previously (Su et al., 2014). After treatment, roots were thoroughly washed with deionized water and homogenized with subcellular extraction solution containing 50 mM Tris–HCl (pH 7.5), 1.0 mM DTT and 0.25 mM sucrose. The homogenate was centrifuged at 500 × *g* for 15 min, and the pellet (mainly composed of cell walls and cell wall debris) was designated as the cell wall fraction. After the supernatant was centrifuged at 20,000 × *g* for 20 min, the resulting pellet and supernatant solution were collected and referred to as the organelle fraction and soluble fraction, respectively. All steps were performed at 4 °C. The subcellular fractions were digested with concentrated nitric acid overnight, and the content of Cd was quantified by ICP-AES.

### 2.6. Cell wall fractionation and measurement

Cell wall extraction and fractionation were conducted as described by Zhong and Läuchli (1993), with several modifications. Roots were ground in liquid nitrogen using a mortar and pestle, and

the powder was immersed in 1.5 mL of 75% (v/v) ethanol for 20 min on ice. The homogenates were centrifuged at 6520 × *g* for 10 min. The pellets were homogenized and washed with acetone, methanol and chloroform (1/1, v/v) and methanol, respectively, each supernatant removed after centrifugation between the washes. The final pellets after washed with methanol were considered the cell wall fraction and were freeze-dried for further analysis.

The pectin fraction was extracted three times from cell walls (2 mg) by incubation with 1 mL of water at 100 °C for 1 h each. After centrifugation at 16,500 × *g* for 10 min, the supernatants were combined in a 5-mL Eppendorf tube. The remaining pellets were extracted twice with 1 mL of 4% (w/v) KOH containing 0.1% (w/v) $NaBH_4$ for 12 h and centrifuged at 13,500 × *g* for 10 min to obtain the hemicellulose 1 fraction. The uronic acid content of pectin was determined according to Blumenkrantz and Asboe-Hansen (1973). The total soluble polysaccharide content of hemicellulose 1 was assayed via the phenol sulfuric acid method.

### 2.7. Pectin methylesterase activity assay

Pectin methylesterase (PME, EC 3.1.1.11) activity was assayed according to the method described by Zhou et al. (2012) using MeOH as a standard (Wojciechowski and Fall, 1996). After various treatments, roots were gently blotted, weighed, ground in liquid nitrogen and homogenized with 1 mL 1 M NaCl. The mixture was centrifuged at 2,3000 × *g* at 4 °C for 15 min. Subsequently, 50 μL aliquots of the supernatants were added to the reaction mixtures containing 100 μL of 0.64 mg/mL pectin (Sigma) in 200 mM PBS (pH 7.4) and 10 μL 0.01 U/μL alcohol oxidase (Sigma). The tubes were incubated at 25 °C for 10 min with occasional shaking; 200 μL of 0.5 M NaOH solution containing 5 mg/mL purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) was added, and the mixture was incubated at 25 °C for 30 min. After 640 μL of deionized water was added to the tubes, $A_{550}$ was measured with a spectrophotometer. PME activity was defined as MeOH production per minute at 25 °C.

### 2.8. Statistical analysis

Three to six replicates were included in each experiment, and the data were expressed as the means and SE. All data were analyzed by analysis of variance (ANOVA) using SPSS 13.0 (SPSS Inc., USA), and the least significant difference test was employed to determine differences among the treatments at the $p \leq 0.05$ level.

## 3. Results

### 3.1. Membrane integrity in P. notoginseng roots under Cd stress

To explore the toxic effects of Cd on *P. notoginseng* roots, Evans blue staining was performed after plants were treated with $CdCl_2$ at increasing concentrations (0, 25, 50 or 100 μM) for 24 h. The degree of staining in *P. notoginseng* root tips was gradually enhanced with increasing Cd concentrations (Fig. 1). Weak staining appeared in the root tips after treatment with 25 μM Cd, and visible staining was observed at 50 μM Cd. When the concentration was increased to 100 μM, the root tips of *P. notoginseng* were fully stained and exhibited severe damage. The results indicated that the membrane damage caused by Cd is correlated with increasing Cd concentration.

### 3.2. Cd induced NO production in P. notoginseng roots

To determine whether endogenous NO is affected by Cd stress, NO levels in *P. notoginseng* roots treated with different Cd concentrations were detected using the NO-specific fluorescence probe DAF-2 DA (Fig. 2A and B). The fluorescence intensity was significantly increased to 1.6- and 2.0-fold after roots were treated with 25 and 50 μM Cd for 24 h (Fig. 2A and B). In contrast, the endogenous NO concentration decreased to the control level (CK) when 100 μM Cd was added to the solution, which may be due to heavy injury of the root tips. The strongest Cd-induced NO production was observed at 50 μM Cd; therefore, this concentration was used in the subsequent analyses.

### 3.3. Cd induced NO synthesis by activating nitrate reductase

To investigate the source of Cd-induced NO production in *P. notoginseng* roots, endogenous NO contents were examined after plants were treated with Cd, Cd + L-NAME or Cd + tungstate. Compared with Cd-treated plants, the NO fluorescence intensity was significantly decreased by tungstate; however, L-NAME had little effect on Cd-induced NO production (Fig. 3A). Quantitative analysis of the NO content showed similar results (Fig. 3B); 50 μM Cd increased endogenous NO synthesis by 60% compared with the control, whereas L-NAME and tungstate reduced Cd-induced NO production by 4% and 72%, respectively.

To further validate whether nitrate reductase is involved in Cd-induced NO production in *P. notoginseng* roots, the activity of this enzyme was determined after various treatments (Fig. 3C). Compared with control plants (CK), application of tungstate inhibited nitrate reductase activity by 47%. After exposure to 25 or 50 μM Cd for 24 h, nitrate reductase activity was enhanced by approximately 2.1- and 2.2-fold, respectively, compared with control plants (CK), whereas no difference was found between the control condition and 100 μM Cd. At 50 μM Cd, the addition of tungstate significantly reduced NR activity by 49% in comparison with Cd-treatment only. These results further confirmed that Cd-induced NO production is dependent on nitrate reductase.

### 3.4. NO enhanced Cd retention in roots but decreased its translocation to above-ground parts

To determine the effect of endogenous NO on Cd accumulation and localization in *P. notoginseng* plants, the Cd content of roots, stems and leaves was analyzed. After the 50 μM Cd treatment for 24 h, the root, stem and leaf Cd contents were 139, 1.84 and 0.69 μg/g, respectively (Fig. 4). Compared with Cd alone, exogenous addition of 100 μM SNP to the Cd treatment solution increased the Cd content in roots by 3.7-fold (Fig. 4A), but decreased it by approximately 62% in stems (Fig. 4B) and 55% in leaves (Fig. 4C). In contrast, addition of the NO scavenger cPTIO or nitrate reductase inhibitor tungstate to the Cd + SNP solution significantly decreased the Cd content in *P. notoginseng* roots by 65% (cPTIO) and 46% (tungstate), but increased it by approximately 1.3- (cPTIO) or 3.5-fold (tungstate) in stems and 1.3- (cPTIO) or 2.2-fold (tungstate) in leaves (Fig. 4). These results suggested that NO contributes to Cd retention in roots and thus decreases Cd translocation to above-ground parts.

### 3.5. NO increased Cd retention in root cell walls

Plant root cell walls are the first barrier to preventing Cd from entering cells. Therefore, Cd distribution in the cell wall, organelle and soluble fractions was determined. Compared with 50 μM Cd treatment, exogenous addition of 100 μM SNP significantly increased Cd deposition in *P. notoginseng* root cell walls by 2.9-fold (Fig. 5A). Furthermore, SNP increased Cd accumulation 2.7-fold in each the organelle and soluble fractions of root cells in comparison with the Cd-only treatment (Fig. 5B and C). Compared with the Cd + SNP treatment, exogenous addition of 100 μM tungstate or cPTIO significantly decreased cell wall Cd

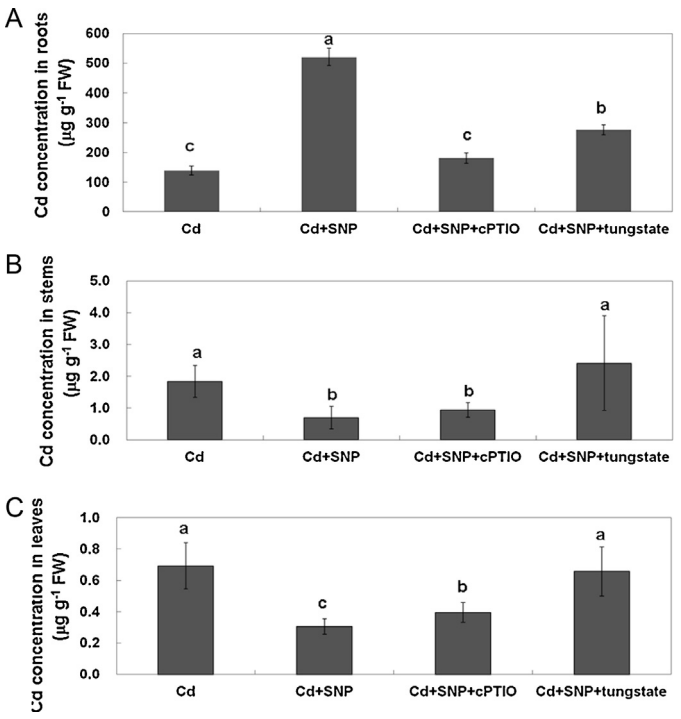


**Fig. 4.** Effects of NO on the Cd distribution in roots (A), stems (B) and leaves (C) of *P. notoginseng*. Plants were treated with 50 μM Cd, 50 μM Cd + 100 μM sodium nitroprusside (SNP), 50 μM Cd + 100 μM SNP + 100 μM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or 50 μM Cd + 100 μM SNP + 100 μM tungstate for 24 h. Values represent the means ± SE ($n = 6$).

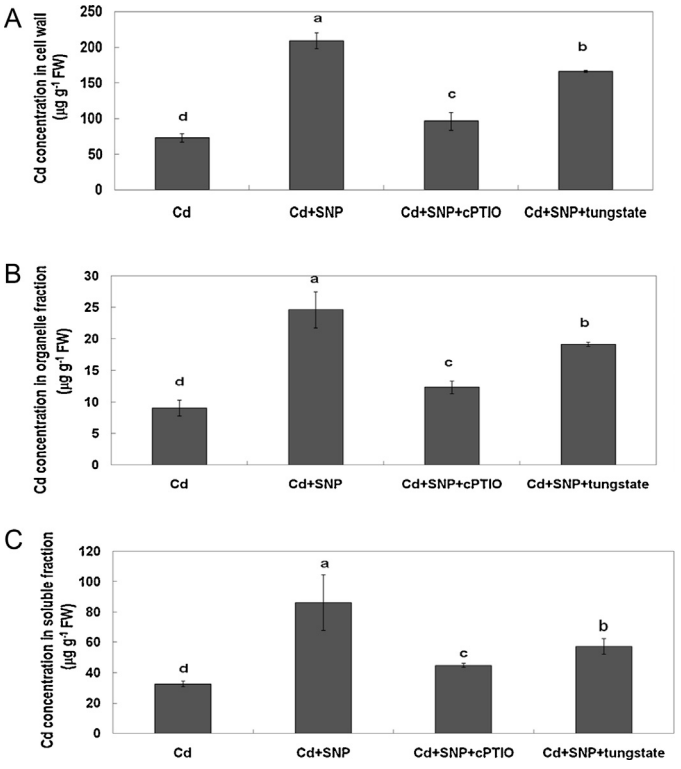


**Fig. 5.** Effects of NO on the Cd distribution in cell wall (A), organelle (B) and soluble (C) fractions of *P. notoginseng* root cells. Plants were treated with 50 μM Cd, 50 μM Cd + 100 μM sodium nitroprusside (SNP), 50 μM Cd + 100 μM SNP + 100 μM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or 50 μM Cd + 100 μM SNP + 100 μM tungstate for 24 h. Values are the means ± SE ($n = 6$).

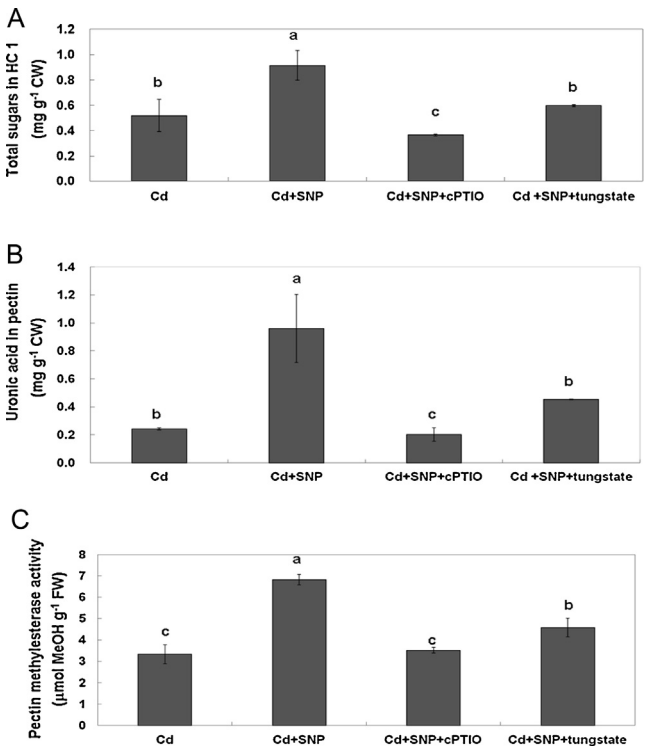


**Fig. 6.** Effects of NO on the concentration of uronic acid as a measure of pectin (A), total sugars as a measure of hemicellulose 1 (B), and activity of pectin methylesterase (PME) (C) in *P. notoginseng* root cell walls. Plants were treated with 50 μM Cd, 50 μM Cd + 100 μM sodium nitroprusside (SNP), 50 μM Cd + 100 μM SNP + 100 μM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or 50 μM Cd + 100 μM SNP + 100 μM tungstate for 24 h. Values represent the means ± SE ($n = 6$).

accumulation by 54% (cPTIO) or 21% (tungstate) (Fig. 5A). Furthermore, exogenous addition of cPTIO or tungstate to the Cd + SNP treatment solution decreased Cd accumulation by 50% (cPTIO) or 22% (tungstate) in the organelle fraction and 48% (cPTIO) or 34% (tungstate) in the soluble fraction of root cells.

### 3.6. NO increased hemicellulose 1 and pectin synthesis as well as pectin methylesterase activity

Compared with the Cd-only treatment, external SNP application significantly increased the concentration of total sugars as a measure of hemicellulose 1 (1.8-fold) and uronic acid as a measure of pectin (4.0-fold) (Fig. 6A and B). However, exogenous addition of cPTIO or tungstate to the Cd + SNP treatment solution decreased the total soluble sugars in hemicellulose 1 by 60% (cPTIO) or 35% (tungstate) (Fig. 6A) and the uronic acid content by approximately 79% (cPTIO) or 53% (tungstate) (Fig. 6B).

The activity of pectin methylesterase (PME, EC 3.1.1.11), the enzyme responsible for catalyzing the demethylation of pectin in plant cell walls, is positively related to the binding of Cd and Al in plants. Based on the amount of methanol released from the pectin extracted from the cell walls of *P. notoginseng* roots, the treatment with Cd + SNP resulted in a significant increase in pectin methylesterase activity in comparison with the Cd-only treatment (Fig. 6C). However, exogenous addition of cPTIO or tungstate to the Cd + SNP solution decreased pectin methylesterase activity by 48% (cPTIO) or 33% (tungstate).

## 4. Discussion

Cadmium is among the most toxic heavy metal pollutants and causes many physiological and biochemical disorders in plants (Rengel et al., 2015). For example, wheat seedlings exposed to Cd exhibit a substantial decline in growth, pigment content and antioxidant enzyme activity (Agami and Mohamed, 2013). Moreover, the 200 μM Cd treatment decreased chlorophyll content and increased the malondialdehyde concentration in *Kandelia obovata* seedlings (He et al., 2014). In the present study, we found that loss of plasma membrane integrity was exacerbated in a Cd concentration-dependent manner (Fig. 1). Furthermore, root hairs were not visible after roots were treated with 50 and 100 μM Cd (Fig. 1). These observations indicated that both plasma membrane integrity and root hair growth were strongly affected by Cd.

Nitric oxide (NO), an important signaling molecule in plants, modulates not only growth and development but also has a critical function in stress resistance. There are several potential sources of NO production in plants, with the pathway generally depending on the species, cells/tissues and growth conditions. For instance, chilling-induced NO production in *Chorispora bungeana* suspension culture cells is dependent on nitric oxide synthase (NOS) activity (Liu et al., 2010), whereas cold acclimation-induced NO production results from enhanced nitrate reductase activity and the up-regulation of *NIA1* gene expression in *Arabidopsis* (Zhao et al., 2009). In red kidney bean roots, external application of tungstate, but not L-NAA ($N^G$-amino-L-arginine, an NOS inhibitor), significantly inhibits Al-induced NO production (Wang et al., 2010). Consistently, the results presented here demonstrate that Cd elicited a marked increase in endogenous NO production in *P. notoginseng* roots (Fig. 2). More specifically, pharmacological results using a nitrate reductase or NOS inhibitor revealed nitrate reductase-dependent NO synthesis to be positively correlated with Cd-induced NO production (Fig. 3). Furthermore, nitrate reductase activity showed trends similar to that of NO concentration under Cd stress conditions. (Fig. 2 and Fig. 3). These results indicate that Cd induced NO production in *P. notoginseng* roots primarily through the nitrate reductase pathway.

The activity of the nitrate reductase can be modulated at both transcriptional and post-translational levels. For example, drought and $NO_3^-$ stresses affect the mRNA abundance of nitrate reductase in wheat and cucumber (Foyer et al., 1998; Li et al., 2012). Furthermore, overexpression of nitrate reductase in tobacco delays both drought-induced reduction in nitrate reductase activity and mRNA abundance (Ferrario-Mery et al., 1998). Phosphorylation of the nitrate reductase is a common example of post-translational modification altering the activity of this enzyme (Kaiser and Huber 2001). The association of 14-3-3 proteins with the phosphorylated nitrate reductase maintains the phosphorylation state and results in its inactivation. It has been shown that divalent cations (such as $Mg^{2+}$) and polyamines can interact with the 14-3-3 protein, enhancing an interaction with its phosphorylated target proteins, such as nitrate reductase and the plasma membrane $H^+$-ATPase (Athwal et al., 1998; Athwal and Huber 2002; Chen et al., 2015). Therefore, these physical and metabolic effectors can be used in biotechnological applications for regulating the activity of nitrate reductase and the related NO production under Cd stress.

Various roles have been proposed for NO in regulating the plant response to toxic metals. For example, Kopyra and Gwózídz (2003) reported that NO counteracts the inhibitory effect of heavy metals and salinity on *Lupinus luteus* root growth (Kopyra and Gwózídz, 2003). External SNP application was found to protect plants against Cd-induced oxidative stress by stimulating the activity of antioxidant enzymes in sunflower and *Medicago truncatula* (Laspina et al., 2005; Xu et al., 2010). In addition to protecting plants against oxidative damage triggered by Cd, NO affects Cd accumulation in plant roots. In *Arabidopsis*, Cd-induced NO production contributes to Cd toxicity by promoting Cd accumulation in roots (Besson-Bard et al., 2009). In rice, Xiong et al. (2009) showed that exogenous NO enhances Cd accumulation in roots by increasing the synthesis of pectin and hemicellulose 1 in the cell wall (Xiong et al., 2009). In support of these findings, we found a significantly increased Cd content in roots, but decreases in stems and leaves, after SNP was added to the Cd treatment solution. However, the addition of tungstate and cPTIO to the Cd + SNP solution significantly decreased SNP-promoted Cd accumulation in roots, but increased the Cd contents of stems and leaves (Fig. 4).

It has been reported that Ca and Fe transporters are partly responsible for Cd uptake into plant cells. For instance, Ca-channel inhibitors can suppress Cd influx into guard cells (Perfus-Barbeoch et al., 2002). In *Arabidopsis*, the Fe transporter IRT1 is involved in Cd uptake into root cells, and NO promotes Cd accumulation in roots via up-regulation of the *IRT1* gene (Besson-Bard et al., 2009). In the present study, we found that SNP not only promoted Cd accumulation in the cell wall, but also increased Cd concentrations in the organelle and soluble fractions of root cells. It is speculated that upregulation of Fe transporter gene expression may contribute to NO-promoted Cd uptake into *P. notoginseng* root cells. The results from subcellular Cd distribution analysis in *P. notoginseng* roots revealed the root cell walls as the major site of Cd binding (Fig. 5). After the plants were treated with Cd, Cd + SNP, Cd + SNP + cPTIO or Cd + SNP + tungstate, Cd in the cell walls accounted for approximately 65% of the total Cd in the *P. notoginseng* root cells (Fig. 5A).

The cell wall is the first barrier for metal entry into the cytoplasm. Primary plant cell walls are mainly composed of cellulose microfibrils and an interwoven matrix of hemicellulose and pectin. Cellulose consists of linear chains of (1,4)-linked β-D-glucose residues and is generally regarded as chemically inactive (Zhu et al., 2013); in contrast, hemicellulose 1 and pectin showed a positive relationship with the retention of Cd and Al in *Arabidopsis* and rice (Shi et al., 2014; Yang et al., 2008; Zhu et al., 2013). Furthermore, pectin methylesterase (PME) catalyzes pectin demethylation, which increases the capacity of pectin to bind metal ions. Implicated in Al and Cd toxicity, negatively charged pectin comprised 35% of the primary cell wall dry mass (Wehr et al., 2003). For example, higher pectin methylesterase activity in rice roots correlated with a greater Al content in Al-sensitive compared with Al-resistant cultivars (Yang et al., 2008). In rice bean (*Vigna umbellata*), the SNP treatment increased pectin methylesterase activity, making cells more sensitive to Al toxicity due to an increased Al content (Zhou et al., 2012). *OsPME14* overexpression was found to result in increased Al retention in the cell wall and increased Al sensitivity in transgenic rice (Yang et al., 2013). Moreover, P deficiency decreased pectin methylesterase activity and reduced Cd retention in pectin, thus alleviating Cd toxicity in *Arabidopsis* (Zhu et al., 2012b). In the present study, we found that increased hemicellulose 1 and pectin contents as well as pectin methylesterase activity correlated with the increased retention of Cd in the cell wall of *P. notoginseng* roots under Cd stress in the presence of SNP (NO donor), whereas cPTIO (NO scavenger) or tungstate (nitrate reductase inhibitor) decreased Cd content in the cell walls. These results indicated that nitrate reductase-mediated NO production promoted Cd accumulation by stimulating hemicellulose 1 and pectin synthesis and by modulating pectin methylesterase activity.

Taken together, the work presented here shows that Cd-induced NO production primarily occurs through activation of the nitrate reductase pathway. Furthermore, nitrate reductase-mediated NO synthesis promotes Cd accumulation in *P. notoginseng* roots by affecting cell wall properties. Therefore, it appears that a decrease in NO production by regulating nitrate reductase activity may be an effective and feasible strategy for controlling Cd contamination in *P. notoginseng* roots.

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