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Genotype-Dependent Effect of Exogenous Nitric Oxide on Cd-induced Changes in Antioxidative Metabolism, Ultrastructure, and Photosynthetic Performance in Barley Seedlings (*Hordeum vulgare*)

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Abstract A greenhouse hydroponic experiment was performed using Cd-sensitive (cv. Dong 17) and Cd-tolerant (Weisuobuzhi) barley seedlings to evaluate how different genotypes responded to cadmium (Cd) toxicity in the presence of sodium nitroprusside (SNP), a nitric oxide (NO) donor. Results showed that 5 μM Cd increased the accumulation of $O_2^{\bullet -}$, H_2O_2, and malondialdehyde (MDA) but reduced plant height, chlorophyll content, net photosynthetic rate (P_n), and biomass, with a much more severe response in the Cd-sensitive genotype. Antioxidant enzyme activities increased significantly under Cd stress in the roots of the tolerant genotype, whereas in leaves of the sensitive genotype, superoxide dismutase (SOD) and ascorbate peroxide (APX), especially cytosol ascorbate peroxidase (cAPX), decreased after 5–15 days Cd exposure. Moreover, Cd induces NO synthesis by stimulating nitrate reductase and nitric oxide synthetase-like enzymes in roots/leaves. A Cd-induced NO transient increase in roots of the Cd-tolerant genotype might partly contribute to its Cd tolerance. Exogenous NO dramatically alleviated Cd toxicity, markedly diminished Cd-induced reactive oxygen species (ROS) and MDA accumulation, ameliorated Cd-induced damage to leaf/root ultrastructure, and increased chlorophyll content and P_n. External NO counteracted the pattern of alterations in certain antioxidant enzymes induced by Cd; for example, it significantly elevated the depressed SOD, APX, and catalase (CAT) activities in the Cd-sensitive genotype after 10- and 15-day treatments. Furthermore, NO significantly increased stromal APX and Mn-SOD activities in both genotypes and upregulated Cd-induced decrease in cAPX activity and gene expression of root/leaf *cAPX* and leaf *CAT1* in the Cd-sensitive genotype. These data suggest that under Cd stress, NO, as a potent antioxidant, protects barley seedlings against oxidative damage by directly and indirectly scavenging ROS and helps to maintain stability and integrity of the subcellular structure.

Keywords Barley (*Hordeum vulgare* L.) · Cadmium (Cd) · Nitric oxide (NO) · Nitric oxide synthase (NOS) · ROS metabolism · Ultrastructure

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Introduction

Cadmium (Cd) is a heavy metal widely recognized as a very dangerous environmental pollutant due to its high toxicity to both plants and animals. Although Cd has a wide variety of toxic effects on plants, the photosynthetic apparatus appears to be particularly susceptible to this

heavy-metal pollutant (di Cagno and others 2001). However, little information is available on the effects of Cd on chloroplast ultrastructure such as the thylakoid system, and this knowledge is important in understanding the relationship between physiological alterations induced by metal stress and photosynthesis. In addition, studies on different plant species have revealed that Cd produces concentration-dependent imbalances in the antioxidant defenses of plants and induces oxidative stress by inducing the generation of reactive oxygen species (ROS) (Sandalio and others 2001; Schützendübel and others 2001; Wu and others 2003). The excess formation of ROS, such as the superoxide radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2), can oxidize various cellular components (for example, protein, lipids, and nucleic acids) and result in lipid peroxidation, membrane leakage, and enzyme inactivation, which can finally lead to oxidative injury and alteration in cell structure (Romero-Puertas and others 2007). Although plants cope with oxidative stress in a complex system, it is well known that the increased ROS levels could be scavenged by antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), and peroxidase (POD, EC 1.11.1.7) (Noctor and Foyer 1998). However, different effects of Cd toxicity on the activity of antioxidant enzymes scavenging ROS were reported (Sandalio and others 2001; Schützendübel and others 2001; Wu and others 2003; Romero-Puertas and others 2004). Moreover, SOD, a key enzyme in superoxide radicals dismutated to H_2O_2, had at least three different isoforms: CuZn-SOD, Mn-SOD, and Fe-SOD (del Río and others 2003); and APX has at least four distinct compartments: chloroplastic stroma (sAPX), thylakoid membrane (tAPX), microbody (mAPX), and cytosol (cAPX) (Song and others 2005). It is meaningful to examine individual SOD and APX isoforms because they have different subcellular locations and different metabolic roles (Ishikawa and others 1998). Yet, little information is available concerning the possible function of different isoforms of these antioxidative enzymes in Cd tolerance, and most of the existing studies did not investigate the alteration in transcription level of antioxidative enzymes in response to Cd stress. Therefore, more research is needed at the subcellular and molecular levels to elucidate genotypic differences in the antioxidative enzyme capacity and the relationship between the corresponding gene expression under Cd stress to gain a deeper insight into the mechanisms of Cd tolerance.

Nitric oxide (NO) is a bioactive gaseous free radical first described in mammals (Schmidt and Walter 1994). Plant scientists started to pay attention to NO in the late 1990s. The explosive growth in the area of research related to NO over the past few years has been rapidly extending to almost all aspects of plant physiology. Undoubtedly, it is an era of great enthusiasm for NO as a plant signaling molecule, and today it is known to play a crucial role in regulating many key physiological processes in plants, such as development and senescence and adaptation to biotic and abiotic stresses (Gould and others 2003; Besson-Bard and others 2008). Gould and others (2003) reported that heat and osmotic and salinity stresses induced a rapid increase in NO production in tobacco cells. Besson-Bard and others (2009) demonstrated that Cd induces NO synthesis in *Arabidopsis thaliana* seedlings. Furthermore, application of exogenous NO in the form of sodium nitroprusside (SNP), as a NO donor, alleviates the toxic effects caused by heat stress in weed calluses by decreasing H_2O_2 and malondialdehyde (MDA) contents and increasing the activities of SOD, CAT, APX, and POD (Song and others 2006). The positive effect of NO on oxidative stresses was found after plant exposure to salt (Zhao and others 2007), heat (Song and others 2006), drought (Mata and Lamattina 2001), and ion deficiency (Graziano and others 2002). It has been reported that exogenously applied NO can enhance germination or break seed dormancy (Beligni and Lamattina 2000), and when no dormancy breakage is required, greater germination rates have been observed by supplementation with an NO donor (Kopyra and Gwóźdź 2003). Once NO is endogenously generated or gets inside the cell from an exogenous source, it reacts with a wide range of targets, including protein and nonprotein thiols and the superoxide anion O^{2-}.

Nitrate reductase (NR, EC 1.6.1.6) and NOS-like enzymes are the two main enzymes involved in NO synthesis in plant cells. NR can catalyze the reduction of nitrate to nitrite and further reduce nitrite to NO; this was observed in ABA-induced stomatal closure (Garcia-Mata and others 2003). In animal cells, NO is catalyzed from L-Arg by the heme-containing enzyme nitric oxide synthase (NOS). Recently, there have been more reports providing evidence for L-Arg-dependent NO synthesis by a putative NOS-like enzyme in plants, and NO production was inhibited by mammalian NOS inhibitors (del Río and others 2003; Guo and others 2003). Therefore, the question arises whether NO participates in Cd tolerance and whether NR and NOS may be important in NO synthesis in barley plants. It is also of great interest to know whether external NO could act as a regulator of antioxidant intervention strategy in preventing oxidative stress in response to Cd stress, and to get a better understanding of how plants adjust to an adverse environment.

The present study reports the genotypic differences in Cd-induced changes in antioxidative metabolism, ultrastructure, and photosynthetic performance, and the role of NO in Cd tolerance using two barley genotypes varying in Cd tolerance. We provided evidence that Cd induced a

significant increase in NO synthesis at the early stage of Cd exposure in barley seedlings, but the Cd-tolerant and Cd-sensitive genotypes showed different responses. Our results supported the hypothesis that NO alleviates Cd-induced oxidative damage in plants via stimulating antioxidative enzyme activities, and it helps to maintain the stability and integrity of the subcellular structure under Cd stress.

Materials and Methods

Plant Material and Growth Condition

The hydroponic experiment was performed using two barley genotypes differing in Cd tolerance, Weisuobuzhi and Dong 17, relatively Cd-tolerant and Cd-sensitive genotypes, respectively (Chen and others 2008). Seeds of individual genotypes were surface sterilized in 1% H_2O_2 for 30 min, rinsed seven times with distilled water, and then germinated in sterilized moist sand in an incubator at 20 ± 1°C. When seedlings reached the two-leaf stage (10 days old), uniform healthy plants were selected and transplanted to 5-l containers filled with 4.5 l basal nutrient solution. The container was covered with a polystyrol plate with seven evenly spaced holes (2 plants per hole) and placed in a greenhouse. Seven days after transplanting, Cd (as $CdCl_2$) and SNP were added to the corresponding containers to form three treatments: basal nutrient solution (control), 5 μM Cd (Cd), and 5 μM Cd + 0.25 mM SNP (Cd + NO). The experiment was laid out as a split-plot design, with treatment as the main plot and genotype as the subplot, and there were six replicates for each treatment. The composition of the basic nutrient solution was (mg l^{-1}): $(NH_4)_2SO_4$, 48.2; $MgSO_4$, 65.9; K_2SO_4, 15.9; KNO_3, 18.5; $Ca(NO_3)_2$, 59.9; KH_2PO_4, 24.8; Fe-citrate, 5; $MnCl_2{\cdot}4H_2O$, 0.9; $ZnSO_4{\cdot}7H_2O$, 0.11; $CuSO_4{\cdot}5H_2O$, 0.04; HBO_3, 2.9; H_2MoO_4, 0.01. The solution pH was adjusted to 5.8 ± 0.1 with NaOH or HCl, as required. The nutrient solution was continuously aerated with pumps and renewed every 5 days.

Plant samples to examine enzyme activities, antioxidants, and chlorophyll were collected after 1, 5, 10, 15, and 25 days of treatment. Fresh leaf fragments were used to measure chlorophyll content and NR activity. For the determination of antioxidative enzymes, NOS activities, and NO content, samples were kept at −70°C. Meanwhile, SOD and APX isoenzyme activities and photosynthesis parameters were measured after 15 days of treatment, and cytochemistry staining of H_2O_2 and $O_2^{\bullet-}$ and leaf/root ultrastructure was also performed. The first fully expanded leaves were used in determining all indices.

Growth Measurement and Metal Analysis

At 25 days after treatment, plants were harvested and separated into roots and tops (shoots and leaves). The roots were soaked with 20 mM Na_2EDTA for 3 h to eliminate the ions on the surface and then washed thoroughly with deionized water. Plant height and root length were measured simultaneously, dried at 80°C, and weighed. Dried shoots and roots were powdered and weighed, then ashed at 550°C for 12 h. The ash was digested with 5 ml of 30% HNO_3 and then diluted using deionized water. The Cd concentration was quantified using flame atomic absorption spectrometry (Shimadzu AA-6300; Chen and others 2007).

Determination of NO Levels and NOS and NR Activity

NO levels were determined according to the method described by Murphy and Noack (1994). Fresh samples were cut into approximately 5-mm segments and immediately incubated with 100 U CAT and 100 U SOD for 5 min to remove endogenous ROS before addition of oxyhemoglobin (5 mM). After 3 min of incubation, NO was quantified by spectrophotometric measurement of the conversion of oxyhemoglobin to methemoglobin. The total NOS activity in roots and leaves of barley seedlings was determined by the L-citrulline assay method described by Ribeiro and others (1999), and the NR activity was assayed based on the method of Zhang and Qu (2003).

Measurement of Chlorophyll Content and Photosynthesis Parameters

Chlorophyll content was determined according to the method of Beligini and Lamattina (1999). A LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE) was used to measure net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r), and intracellular CO_2 concentration (C_i) of the first fully expanded leaves.

Examination of Leaf and Root Ultrastructure

Fresh leaves and root tips were sectioned and fixed with 2.5% glutaraldehyde (v/v) in 100 mM phosphate buffer (PBS, pH 7.0) for 6–8 h. Samples were washed three times with the same PBS and post-fixed in 1% osmium tetroxide (OsO_4) for 1 h. After fixing, samples were dehydrated with ethanol series, infiltrated, and embedded in Spurr's resin overnight. Then the specimen sections were stained with uranyl acetate and alkaline lead citrate, respectively, and ultrathin sections (80 nm) were prepared. Examination of sections was carried out using a transmission electron microscope (JEOL JEM-1230 EX, Japan).

Histochemical Staining of H_2O_2 and $O_2^{\bullet-}$ and Determination of MDA Content

Cytochemistry staining of H_2O_2 and $O_2^{\bullet-}$ was performed as described by Romero-Puertas and others (2004) with minor modifications. In the case of H_2O_2, leaves were vacuum-infiltrated with 10 mM potassium phosphate buffer (PBK, pH 6.5) containing 1% diaminobenzidine (DAB) for 5 min and then incubated at 25°C for 12 h in the dark. Leaves were illuminated until the appearance of brown spots, characteristic of the reaction of DAB with H_2O_2. In the case of $O_2^{\bullet-}$, leaves were infiltrated with 0.1% nitroblue tetrazolium (NBT) in 50 mM PBK (pH 6.4) containing 10 mM Na-azide and illuminated until the appearance of dark spots. Leaves were all bleached in boiling ethanol to visualize the spots after incubation. The level of lipid peroxidation was measured as the amount of malondialdehyde (MDA) which was determined by the thiobarbituric acid (TBA) reaction (Wu and others 2003).

Assay of Antioxidant Enzymes

Fresh roots and leaves (0.3 g) were homogenized in 8 ml of 50 mM PBS (pH 7.8) using a prechilled mortar and pestle. Then the homogenates were centrifuged at 10,000*g* for 15 min and the supernatants were used for enzyme activity assaying. APX, CAT, and POD activities were determined according to the method of Song and others (2005). The activities of SOD and its isoenzymes were assayed by the inhibition of the photochemical reduction of NBT, as described by Yu and Rengel (1999). KCN and H_2O_2 were used to identify the different forms of SOD. KCN inhibits Cu/Zn-SOD but does not affect Mn-SOD or Fe-SOD, whereas H_2O_2 inactivates Cu/Zn-SOD and Fe-SOD without affecting Mn-SOD. Activities of APX isoenzymes in the leaf extracts were determined according to the method of Amako and Asada (1994). Fresh leaves (0.5 g) were homogenized in 10 mM PBK (pH 7.0) containing 1 mM AsA, 20% (w/v) sorbitol, 1 mM EDTA, and 0.1% (w/v) phenylmethanesulfonyl fluoride (PMSF), and the homogenates were centrifuged at 12,000*g* for 10 min. The supernatant was used to determine the activities of sAPX and cAPX. Fifty microliters of supernatant was added to 2.0 ml of 50 mM PBK (pH 7.0) containing 10 μM H_2O_2. One, two, three, and five minutes after the start of the incubation, the incubated mixture (1.98 ml) was sampled and mixed with 10 μl of 100 mM AsA and then 10 μl of 20 mM H_2O_2. The oxidation of AsA was followed by a decrease in the A290. The membrane fraction was washed and suspended in 10 mM PBK (pH 7.0) containing 1 mM AsA. The activities of tAPX and mAPX were assayed separately using the same method that was used to measure the activities of sAPX and cAPX. cAPX and mAPX activities were calculated from the inactivation curve of each isoenzyme.

qRT-PCR Analysis

Total RNA was isolated from roots and leaves with the TRIzol reagent following the manufacturer's recommendation (Invitrogen, Carlsbad, CA, USA). One microgram of each total RNA sample was subsequently used for cDNA synthesis using 0.5 μg of oligo(dT) 12–18 (Invitrogen) and 200 U of Superscript II (Invitrogen) according to the supplier's recommendation. cDNA samples were assayed by quantitative real-time PCR (qRT-PCR) in the iCycler iQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s.

The following primers were used: *POD* (X58396), forward (fw)-5′-TGCTTTGGTCTTATCGCT-3′ and reverse (rv)-5′-GTTTGCCTCATTCTCGTT-3′; *CAT1* (U20777), fw-5′-TGAAGTGCCTCTTGGAT-3′ and rv-5′-AGACG GTGCCTTTGGGT-3′; *cAPX* (AJ006358), fw-5′-AGGG AGGGAGGACAAGC-3′ and rv-5′-ACCAGAGAGGGCA ACAA-3′. Barley *actin* gene was used as control (AY145451): fw-5′-ATGTTTTTTTCCAGACG-3′ and rv-5′-ATCAAGCCAACCCAAGT-3′.

Statistical Analysis

Data presented are the averages of at least three independent replicates. Statistical analyses were performed with the Data Processing System (DPS) statistical software package using ANOVA followed by the Duncan's multiple range test (SSR) to evaluate significant treatment effects at a significance level of $P \leq 0.05$.

Results

Cd Toxicity Symptoms and Cd Concentration

Symptoms of Cd toxicity (5 μM Cd-alone treatment) in barley leaves included brown spots and yellow necrotic patches. Cd toxicity markedly hindered shoot and root elongation and biomass accumulation (Supplementary Fig. S1). Furthermore, time of appearance and severity of Cd toxicity symptoms differed significantly between genotypes ($P < 0.01$). The tolerant genotype Weisuobuzhi was less affected in terms of the above-mentioned growth traits and yellow necrotic patches, whereas the sensitive Dong 17 was more affected and Cd toxicity symptoms also appeared

rapidly and severely. To investigate the protective role of NO, 0.25 mM SNP was added to the 5 μM Cd medium (Cd + NO). After 25 days of treatment, plant height, root length, and biomass increased significantly in both genotypes compared with Cd-alone treatment (Supplementary Fig. S1), and NO effectively inhibited the appearance of Cd toxicity such as chlorosis or necrosis in the leaves.

To prove that the SNP-released NO, rather than other substances associated with SNP decomposition (such as cyanide and Fe), was responsible for the SNP-induced alleviation effect on Cd toxicity, Cd-treated leaflets of Dong 17 were treated with 200 μM $K_4Fe(CN)_6$ and 200 μM SNP + 200 μM cPTIO separately. The results indicated that treatment with neither 200 μM SNP + 200 μM cPTIO nor $K_4Fe(CN)_6$ had an ameliorating effect on Cd-induced inhibition of brown precipitate (Supplementary Fig. S2). This evidence proves the hypothesis that SNP-released NO, rather than other substances from SNP decomposition, accounts for the alleviation effect on Cd toxicity.

Shoot and root Cd concentrations of 123.5–140.6 mg kg^{-1} DW and 1116.1–1147.9 mg kg^{-1} DW were detected after 25 days of 5 μM Cd treatment. The tolerant genotype Weisuobuzhi showed relatively higher Cd concentrations in shoots than the sensitive Dong17, whereas no significant genotype difference was observed in the roots. The addition of NO significantly reduced Cd concentrations in both genotypes compared with Cd-alone treatment, and the sensitive genotype Dong 17 showed much more reduction than Weisuobuzhi [7.7 and 11.5% more reduction than Weisuobuzhi in shoots and roots, respectively (Supplementary Table 1)].

Chlorophyll Content and Photosynthetic Parameters

There was no significant effect on chlorophyll content of the tolerant genotype Weisuobuzhi plants treated with 5 μM Cd except on day 5 when the chlorophyll content was 20.2% lower than in the control. However, a substantial degradation of chlorophyll was detected in the sensitive genotype Dong 17, with the largest reduction of 58.2% after 25 days of Cd exposure. When treated with Cd in the presence of SNP, the chlorophyll content was significantly increased in Weisuobuzhi after 1–10 days and in Dong 17 after 5–10 days compared with Cd-alone treatment (Fig. 1). Thereafter (15–25 days), no differences in chlorophyll content were observed in the Cd + SNP treatments. The sensitive Dong 17 showed much more drastic alteration in chlorophyll content when SNP was added to the Cd treatments. Although the chlorophyll content of the Cd-alone-treated plants remained significantly below that of control all the way through the experiment, after 10 days the plants treated with Cd + NO

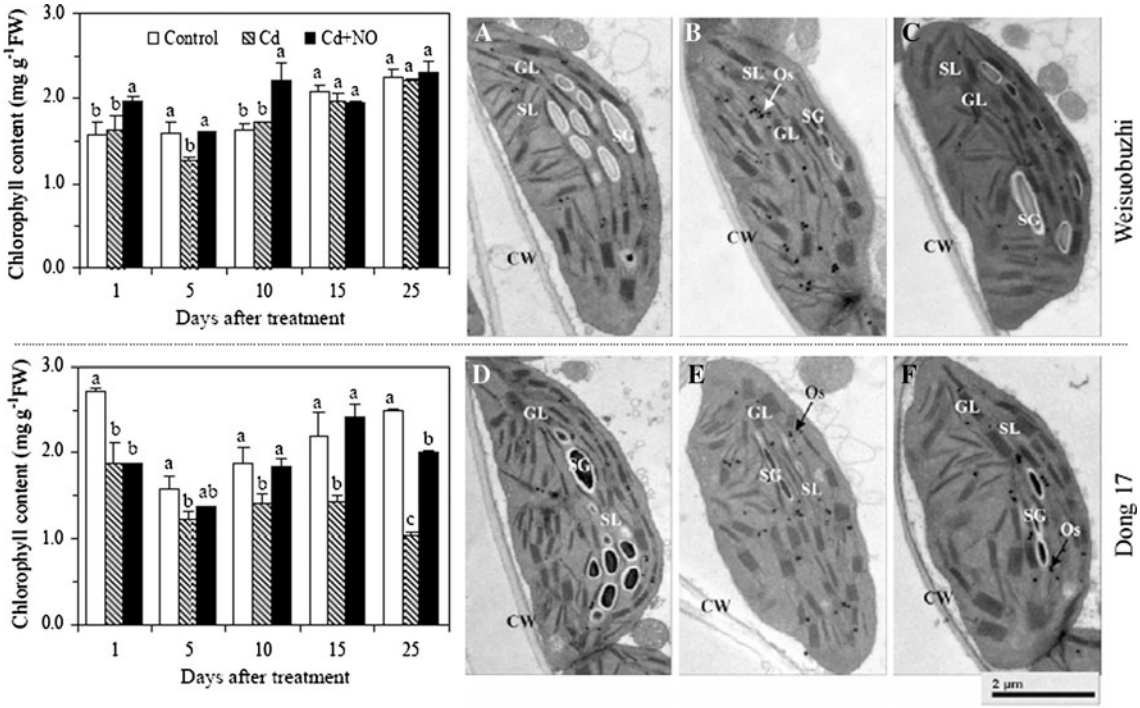

Fig. 1 Chlorophyll contents and transmission electron micrograph of chloroplasts of Weisuobuzhi (*upper*) and Dong 17 (*below*) cultured in basic nutrition (BNS, **A**, **D**), BNS + 5 μM Cd (**B**, **E**), and BNS + 5 μM Cd + 0.25 mM SNP (**C**, **F**). Scale bar = 2 μM. *CW* cell wall, *GL* granum lamellae, *Os* osmiophilic plastolobuli, *SG* starch grain, *SL* stroma lamellae. Figure is representative from three different experiments

showed significant recovery and the chlorophyll contents were very close to the control levels (Fig. 1).

Exposure of barley plants to 5 μM Cd for 15 days reduced the net photosynthetic rate (P_n), stomatal conductance (G_s), and transpiration rate (T_r) by 40.4, 54.5, and 25.7% in Weisuobuzhi and 68.9, 66.0, and 44.9% in Dong 17, respectively, compared to the control, whereas intracellular CO_2 concentration (C_i) increased in both genotypes (Supplementary Table 1). When barley plants were treated with SNP in the presence of Cd, Cd-induced carbohydrate metabolism decay was evidently reduced, especially in Dong 17; P_n, G_s, and T_r increased by 94.6, 23.5, and 21.7% in Dong 17 compared to Cd-alone treatment. In Weisuobuzhi, the alleviation effect of NO was detected only with respect to P_n. Reduced C_i levels were observed in Cd + NO-treated plants of both genotypes compared with Cd-alone treatment (Supplementary Table 1).

Ultrastructure of Chloroplasts and Root Cells

Under normal conditions (control), chloroplasts of barley plants contained dense thylakoids arranged in grana and in membranes interconnecting grana, and their stroma lamellae also had localized starch grains (Fig. 1A, D). Exposure to 5 μM Cd for 15 days resulted in swollen grana/stroma lamellae and loose thylakoid membranes, and the number and size of grana stacking per chloroplast and starch grain decreased significantly compared with the control. Furthermore, increased osmiophilic plastoglobuli and cracked chloroplast membranes were observed. The chloroplast ultrastructural deterioration due to Cd was more prevalent in Dong 17 than in Weisuobuzhi (Fig. 1B, E). Cd + NO treatment markedly increased starch grains and reduced osmiophilic plastoglobuli in both genotypes (Fig. 1C, F).

Electron micrographs of meristem cells from 5-μM-Cd-stressed barley seedlings revealed obvious ultrastructural changes, characterized by cracked karyotheca and reduced vacuolar numbers (Fig. 2B, E). In contrast, root meristem cells appeared completely developed when exogenous NO was added to the Cd experiment (Fig. 2C, F). NO mediated the number of plastids and mitochondria cristae increased in Dong 17, even returning to the normal level of controls (Fig. 2D–F).

NO Eliminated Cd-induced Overaccumulation of $O_2^{\bullet-}$, H_2O_2, and MDA in Leaves

Overaccumulation of ROS in plant cells is the main effect of Cd toxicity. As shown in Supplementary Fig. S3, 5 μM Cd treatment induced a dramatic increase in $O_2^{\bullet-}$ and H_2O_2 production in the leaves of barley seedlings compared with controls, with a much more severe reaction in the sensitive Dong 17 than in the tolerant Weisuobuzhi. Addition of NO significantly diminished Cd-induced ROS accumulation in leaves of both genotypes, particularly of $O_2^{\bullet-}$, and returned accumulation to control levels (Supplementary Fig. S3).

MDA content was measured as an index of lipid peroxidation. During the 25-day experiment period, Cd alone induced an increase in MDA content in both genotypes but the increase was greater in the sensitive genotype Dong 17. MDA content from 5 μM Cd treatment increased by 51.2%

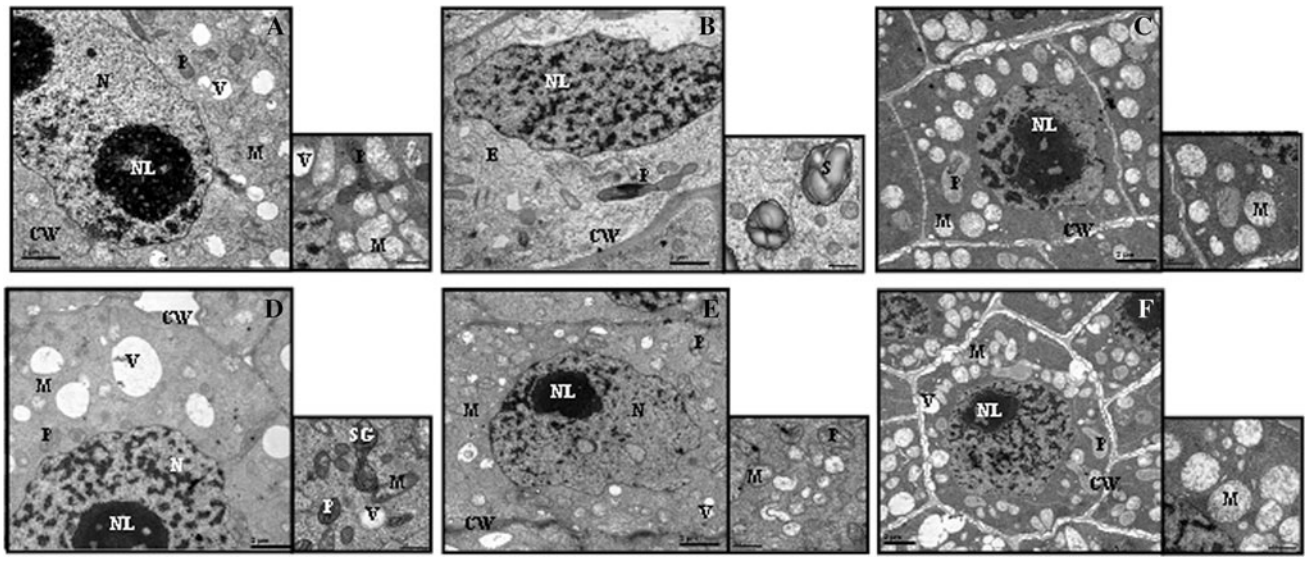

Fig. 2 Transmission electron micrograph of root cells of Weisuobuzhi (*upper*) and Dong 17 (*below*) cultured 15 days in basic nutrition (BNS, **A**, **D**), BNS + 5 μM Cd (**B**, **E**), and BNS + 5 μM Cd + 0.25 mM SNP (**C**, **F**). Scale bars = 2 μM and 1 μM. *CW* cell wall, *E* endoplasmic reticulum, *M* mitochondrion, *N* nucleolus, *P* plastid, *SG* starch grain, *V* vacuole. Figure is representative from three different experiments

in Weisuobuzhi and 65.1% in Dong 17 compared to controls. Exogenous NO markedly reduced Cd-induced MDA accumulation; on day 25 MDA content decreased by 21.1% in Weisuobuzhi and 54.7% in Dong 17 compared with Cd treatment (Supplementary Fig. S3).

Effects of NO Donor on Antioxidant Enzymes

SOD activity showed genotype- and time-dependent variations in response to Cd and Cd + NO treatment (Fig. 3). The expression patterns of the two genotypes were strikingly different. After 1 day, SOD activities in the leaves of the tolerant Weisuobuzhi dropped during Cd stress. It increased dramatically after 5 days and continuously decreased until the end of the experiment (25 days) (Fig. 3A). The SOD activity of the sensitive genotype Dong 17 rapidly increased after 1 day of Cd treatment, continuously decreased until 15 days, and then increased at 25 days (Fig. 3B). The SNP supplement to the Cd treatment had no effect on SOD activity in Weisuobuzhi except for an increase at 15 days (Fig. 3A), whereas in the sensitive Dong 17, the Cd-induced decline in SOD activity was not affected by Cd + NO treatment at the beginning (one day) and the end of the experiment (25 days) but SOD activities were elevated between 5 and 15 days (Fig. 3B). SOD activity in the roots of the tolerant Weisuobuzhi increased significantly after 1 day, remained significantly higher than the control until 5 days, and dropped back to control levels until it increased again at 25 days (Fig. 3C). In Dong 17, slight inhibition or no effect was detected through 15 days, followed by a significant increase at 25 days (Fig. 3D). The addition of SNP induced increased SOD activity in the tolerant Weisuobuzhi to a level even higher than Cd-only treatment between 5 and 15 days, after which SOD activity returned to near control levels (Fig. 3C). In Dong 17 NO-supplemented plants showed significantly higher SOD activity than the control or Cd-treated plants after 10 days of exposure to the end of the experiment (Fig. 3D).

Further study of SOD isoenzymes found that Cd stress reduced Mn-SOD activity in the leaves of both genotypes, especially in Dong 17, whereas it increased root Mn-SOD compared with controls after 15 days of exposure (Fig. 3E). SNP supplementation induced a significant

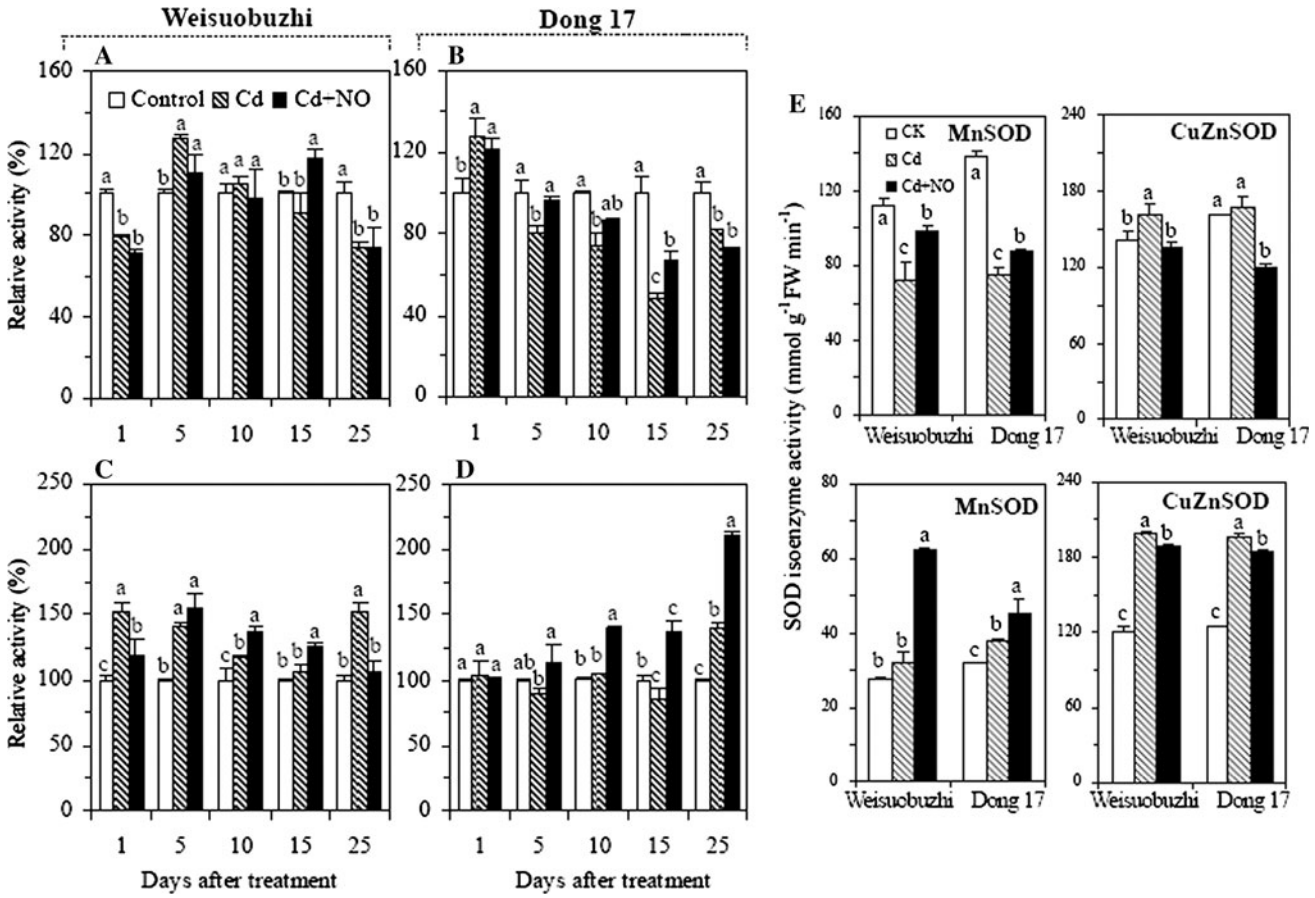

Fig. 3 SOD activity in leaves (*upper*) and roots (*below*) of Weisuobuzhi (**A**, **C**) and Dong 17 (**B**, **D**) exposed to Cd for different days, and SOD isoenzyme activities (**E**). *Error bars* represent SD values ($n = 3$). Control, Cd, and Cd + NO correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively

increase in Mn-SOD activity in the leaves and roots of both genotypes: by 35.9% (leaves) and 17.7% (roots) in Weisuobuzhi and 109.4% and 21.6% in Dong 17 compared to the Cd-alone treatment. Cu/Zn-SOD activity under Cd stress was significantly higher than controls in both genotypes, except in the leaves of Dong 17 (slightly but not statistically significantly higher than that of control). The addition of NO decreased Cu/Zn-SOD activity in both leaves and roots; in the leaves of Weisuobuzhi, it recovered to control levels, whereas in Dong 17, it was significantly lower than controls and Cd-treated plants.

Cd-alone treatment induced an increase in POD activity in both genotypes (Fig. 4A–D). During the experiment, the increase ranged from 70.4 to 189.7% in the leaves of Weisuobuzhi, with the highest increase after day 1, whereas in Dong 17, POD activity increased continuously from 17.5% (day 1) to 77.4% (day 25) compared to controls (Fig. 4A, B). Addition of NO decreased the

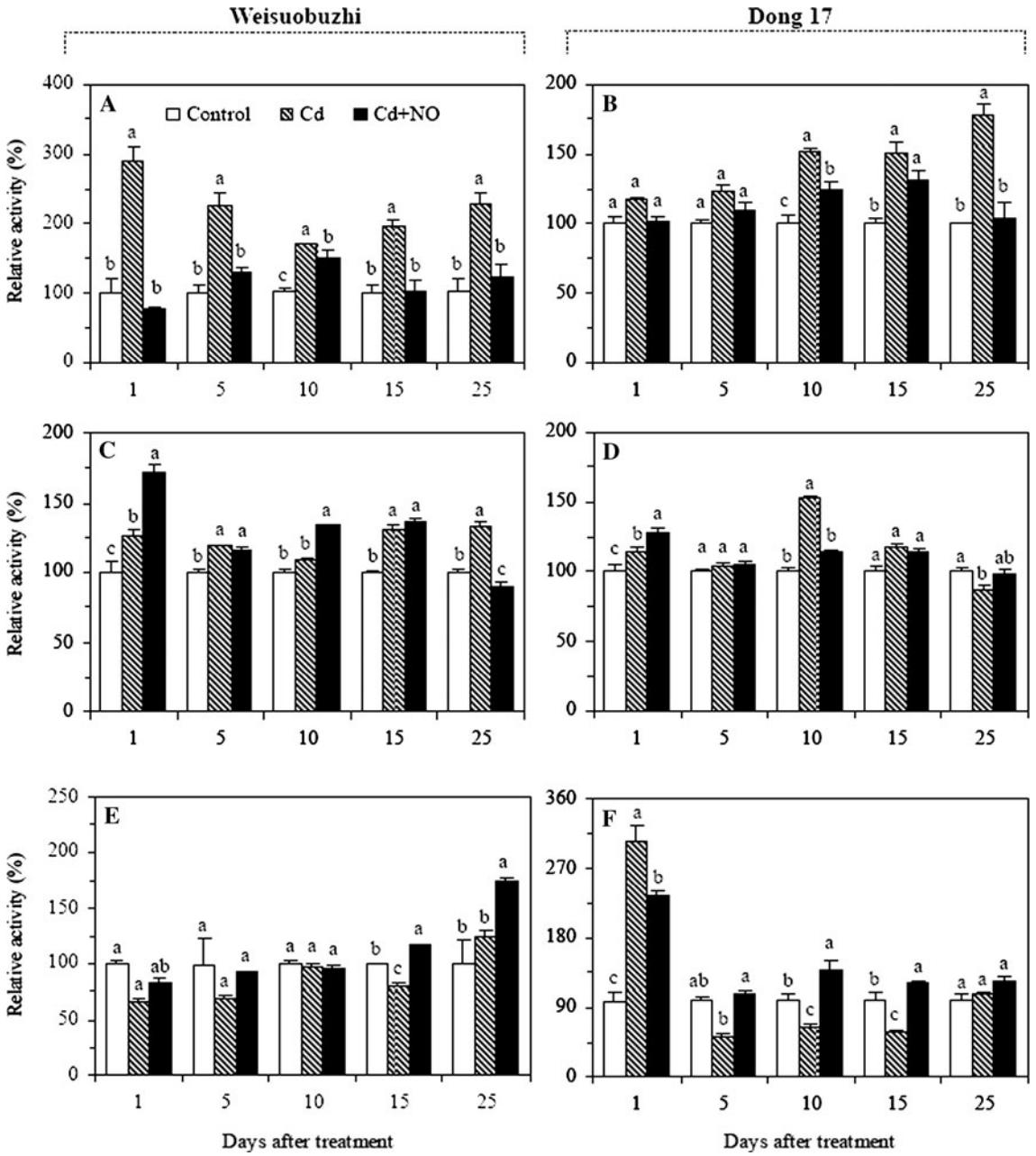

Fig. 4 Effect of NO on POD activity in leaves (**A**, **B**) and roots (**C**, **D**) and CAT activity in leaves (**E**, **F**) of two barley genotypes (*left*, Weisuobuzhi; *right*, Dong 17) exposed to Cd for different days. *Error bars* represent SD values ($n = 3$). Control, Cd, and Cd + NO correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively. Means with a same letter are not significantly different at $P = 0.05$

Cd-mediated increase in POD activity in the leaves of both genotypes (Fig. 4A, B). However, the effect of NO on the response to Cd with respect to POD activity in the roots differed between the genotypes. In Weisuobuzhi, POD activity increased to a higher level than that of Cd-treated plants after 1, 10, and 15 days, whereas in Dong 17, there was no significant difference compared with Cd-treated plants, except for a decrease after 10 days (Fig. 4C, D).

Leaf CAT activity in the tolerant and sensitive genotypes differed significantly in response to the treatments. At the beginning of Cd exposure, CAT activity decreased significantly in tolerant Weisuobuzhi, reaching control levels by the end of the experiment (25 days) (Fig. 4E), whereas in the sensitive Dong 17, CAT activity increased more than threefold after 1 day, decreased sharply to below control levels between 5 and 15 days, and recovered to control levels on day 25 (Fig. 4F). Addition of NO significantly alleviated Cd-induced inhibition of CAT activity in Weisuobuzhi, except on day 10. Similarly, in Dong 17, Cd + NO markedly increased CAT activity after 5–15 days of treatment.

There were significant differences between the genotypes with respect to the effects of Cd on APX activity (Fig. 5). In the tolerant genotype Weisuobuzhi, leaf APX activity in the Cd-alone treatment increased significantly at the beginning of the experiment (day 1) and at the end of the study period (25 days), but decreased slightly on day 5 and 15 (Fig. 5A). Root APX activity was significantly higher than that of controls during the experiment (Fig. 5C). In Dong 17, leaf APX activity increased after 1–5 days, declined significantly after 10–15 days, and recovered to control levels at the end of the experiment (Fig. 5B). Root APX activity was affected from day 5 to 15, with the largest decrease of 25.2% after 10 days of Cd exposure (Fig. 5D). SNP supplementation increased APX activity in the leaves of both Weisuobuzhi and Dong 17 all the way through the experiment except for day 1 (Fig. 5A, B). Similarly, exogenous NO increased APX activity in the roots of both genotypes throughout the 25 days of exposure except for a reduction at day 25 in Weisuobuzhi (Fig. 5C, D).

The sAPX of leaves of the two genotypes had similar responses to Cd (decrease) and to SNP supplementation

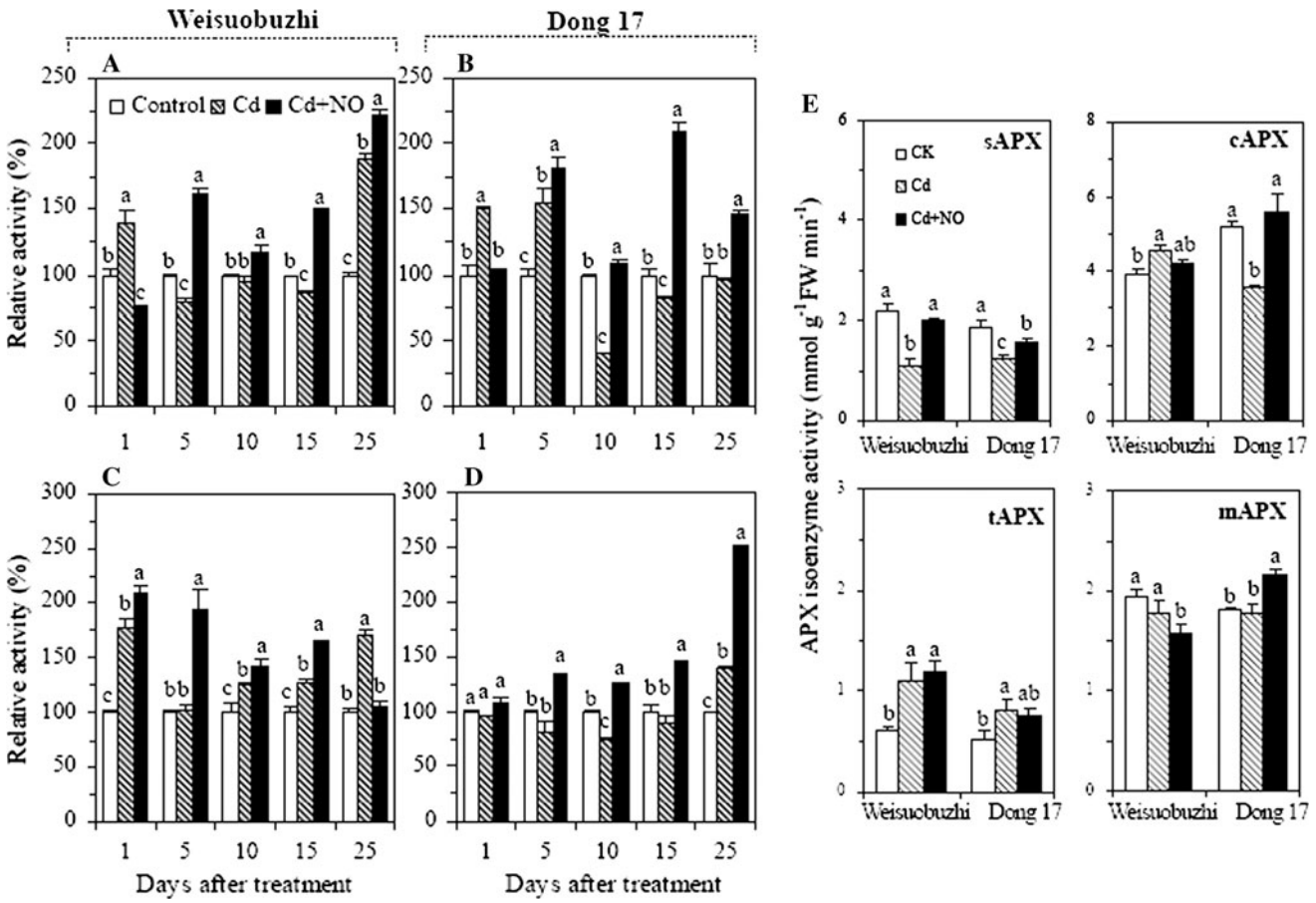

Fig. 5 Effect of NO on APX activity in leaves (*upper panel*) and roots (*bottom panel*) of Weisuobuzhi (**A**, **C**) and Dong 17 (**B**, **D**) exposed to Cd for different days, and APX isoenzyme activities (**E**) in leaves. *Error bars* represent SD values ($n = 3$). Control, Cd, and Cd + NO correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively

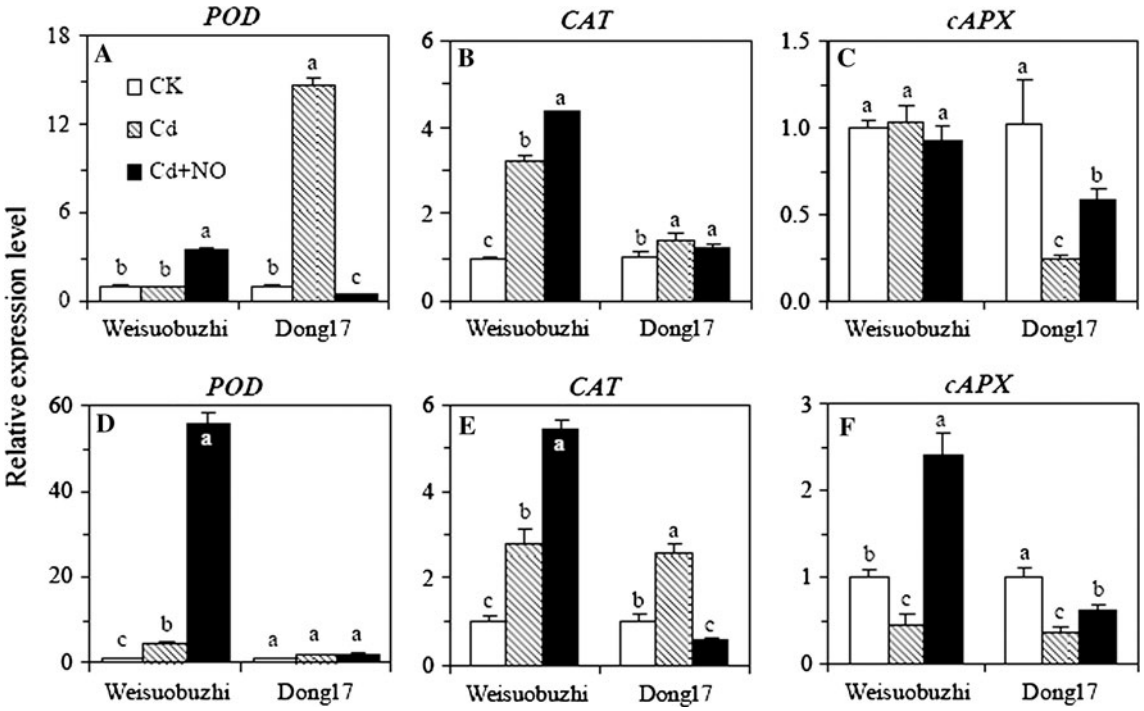

Fig. 6 Effect of NO on the transcript levels of gene expression encoding antioxidant enzymes in leaves (**A**, **B**, **C**) and roots (**D**, **E**, **F**) of two barley genotypes exposed to Cd for different days. *Error bars* represent SD values ($n = 3$). Control, Cd, and Cd + NO correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively

(increase). The tAPX of the two genotypes had a similar response to Cd (increase) and no significant change compared to the Cd treatment when the NO donor was added (Fig. 5E). The response of cAPX and mAPX to Cd and Cd + NO varied between the genotypes. Compared with controls, cAPX activity in Weisuobuzhi increased 16.5% under Cd stress, whereas a 30.8% decrease was detected in Dong 17. Addition of NO (Cd + NO) reduced cAPX activity in Weisuobuzhi to control levels, whereas in Dong 17 cAPX activity increased over that of Cd-treated plants and even over that of controls. mAPX activity in Weisuobuzhi decreased with Cd and Cd + NO treatments; in Dong 17 it showed no obvious change under Cd treatment but increased significantly with the addition of NO (Fig. 5).

Effect of NO Donor on Transcript Levels of Certain Antioxidative Enzymes

Figure 6 shows the quantitative real-time PCR (RT-PCR) results of the relative transcript levels of *POD*, *CAT*, and *cAPX* for the two genotypes. The 5 μM Cd stress upregulated *CAT1* in both roots and leaves of the two genotypes and *POD* in the leaves of Dong 17 and in the roots of Weisuobuzhi by more than tenfold over the controls. However, a significant decrease in *cAPX* transcripts was observed except in the leaves of Weisuobuzhi, where it remained unchanged from that of the controls. In the presence of exogenous SNP, transcript levels of root and leaf *POD* and *CAT1* and root *cAPX* in Weisuobuzhi increased markedly compared with Cd treatment, and transcript levels of leaf *cAPX* showed no significant difference with Cd treatment and controls. However, in Dong 17, only the *cAPX* level increased; *CAT1* and leaf *POD* were downregulated (Fig. 6).

Cd Induces NO Production in Roots and Leaves of Barley Seedlings

NO concentration in the leaves of both genotypes increased dramatically after 1 day of Cd treatment relative to controls, and it decreased significantly after 10–25 days of Cd exposure, especially in the sensitive genotype Dong 17 (average of the three sampling dates decreased by 15.0%) (Fig. 7A, B). Root NO concentration showed significant differences between genotypes (Fig. 7C, D). In Weisuobuzhi, it increased markedly after 1 day of Cd treatment, became nearly similar with the controls during 5-10 days of Cd exposure, and then increased again after 15–25 days (Fig. 7C). Root NO concentration in Dong 17 was similar to that of controls during 1–5 days of Cd exposure, increased markedly on days 10–15, and after that decreased sharply (Fig. 7D). Exogenous SNP supplements increased NO concentration in leaves and roots of the tolerant genotype Weisuobuzhi, except for day 15, compared

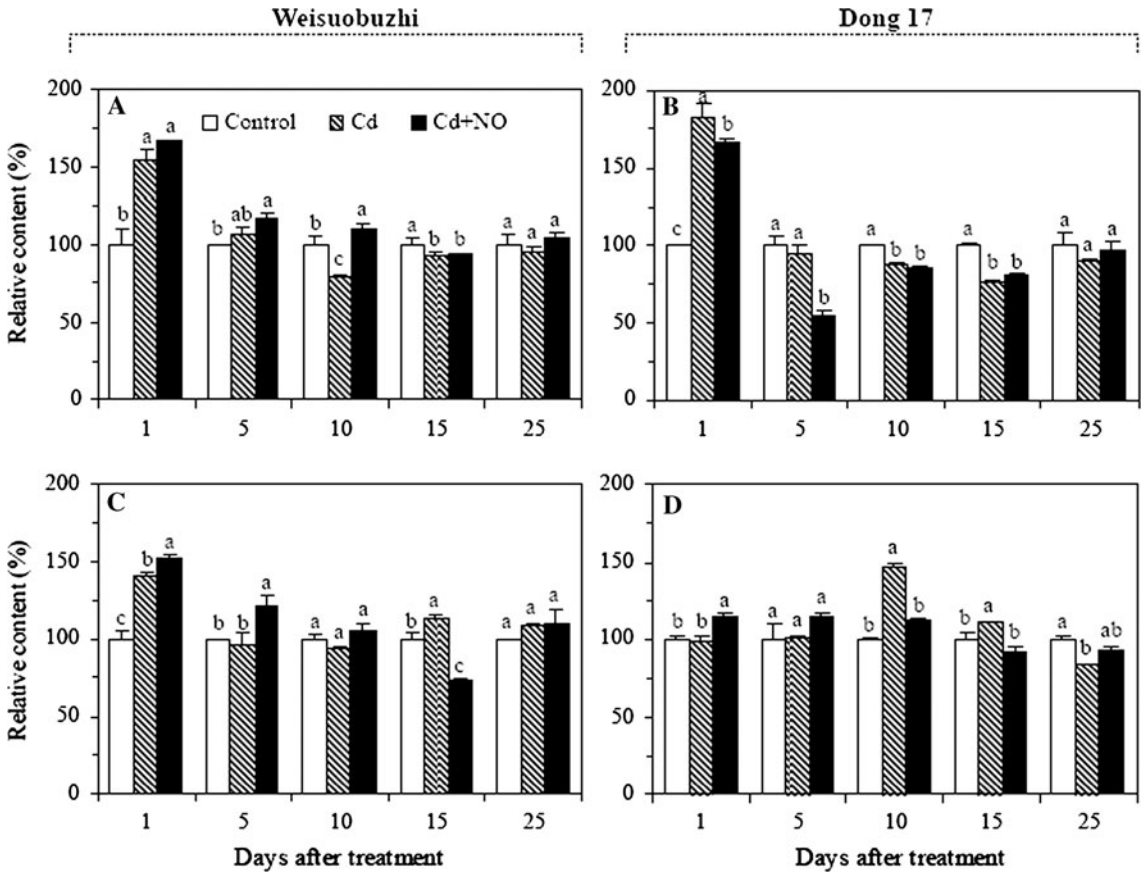

Fig. 7 NO content in leaves (**A**, **B**) and roots (**C**, **D**) of two barley genotypes (*left*, Weisuobuzhi; *right*, Dong 17) exposed to Cd for different days and as affected by SNP. *Error bars* represent SD values ($n = 3$). Control, Cd, and Cd + NO correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively

with controls and Cd treatment (Fig. 7A, C). However, in Dong 17, the NO content in the leaves of Cd + NO-treated plants was lower than in the Cd-alone-treated plants during day 1 to day 5 period (but was still significantly higher than in controls after 1 day), and then remained the same as the Cd-alone-treated plants (Fig. 7B). The NO content of the roots of Cd + NO-treated plants increased on days 1, 5, and 25 and decreased significantly on days 10 and 15 compared with Cd-treated plants (Fig. 7D).

Leaf NOS activity in both genotypes increased sharply the first day of Cd treatment and then decreased significantly (Fig. 8). At day 1 of Cd exposure, NOS activity in Weisuobuzhi and Dong 17 was 65.7 and 45.7%, respectively, higher than in controls, but 30.4 and 47.8% lower on average on the later four sampling dates (Fig. 8A, B). SNP addition effectively alleviated Cd-induced inhibition of NOS activity in leaves of both genotypes, with a pronounced effect in Weisuobuzhi of returning to control levels. NOS activity was induced by Cd in the roots of both genotypes, especially after 10–15 days. Root NOS activity in the Cd + NO treatment increased more than twofold in Weisuobuzhi compared with Cd-alone-treated plants and controls on days 1 and 5, and after that it was downregulated to lower than the level of the Cd-alone treatment (Fig. 8C). In Dong 17, Cd + NO elevated NOS levels above those of controls and Cd-alone treatment except at 15 days (Fig. 8D).

As shown in Fig. 8E and F, leaf NR activity in the two genotypes was similar during exposure to Cd and Cd + NO (5–15 days). Differences were observed at the beginning and the end of the experiment (Fig. 8E, F). NR activity in Weisuobuzhi increased onefold after 1 day of Cd exposure compared to the controls, while in Dong 17, 5 μM Cd induced a marked decline on day 1. After day 1 the NR activity of both genotypes tended to decrease to below control levels. Day 25 showed differences again in the NR activity of the two genotypes, with Weisuobuzhi showing lower and Dong 17 higher enzyme activities than the controls. Exogenous SNP mediated Cd-induced inhibition of leaf NR activity in Weisuobuzhi except on day 1, especially on days 5, 10, and 25. For example, NR activity on day 25 after Cd + NO treatment was 68.2 and 37.6% higher than that after Cd alone and control, respectively. In the sensitive genotype Dong 17, external NO increased NR

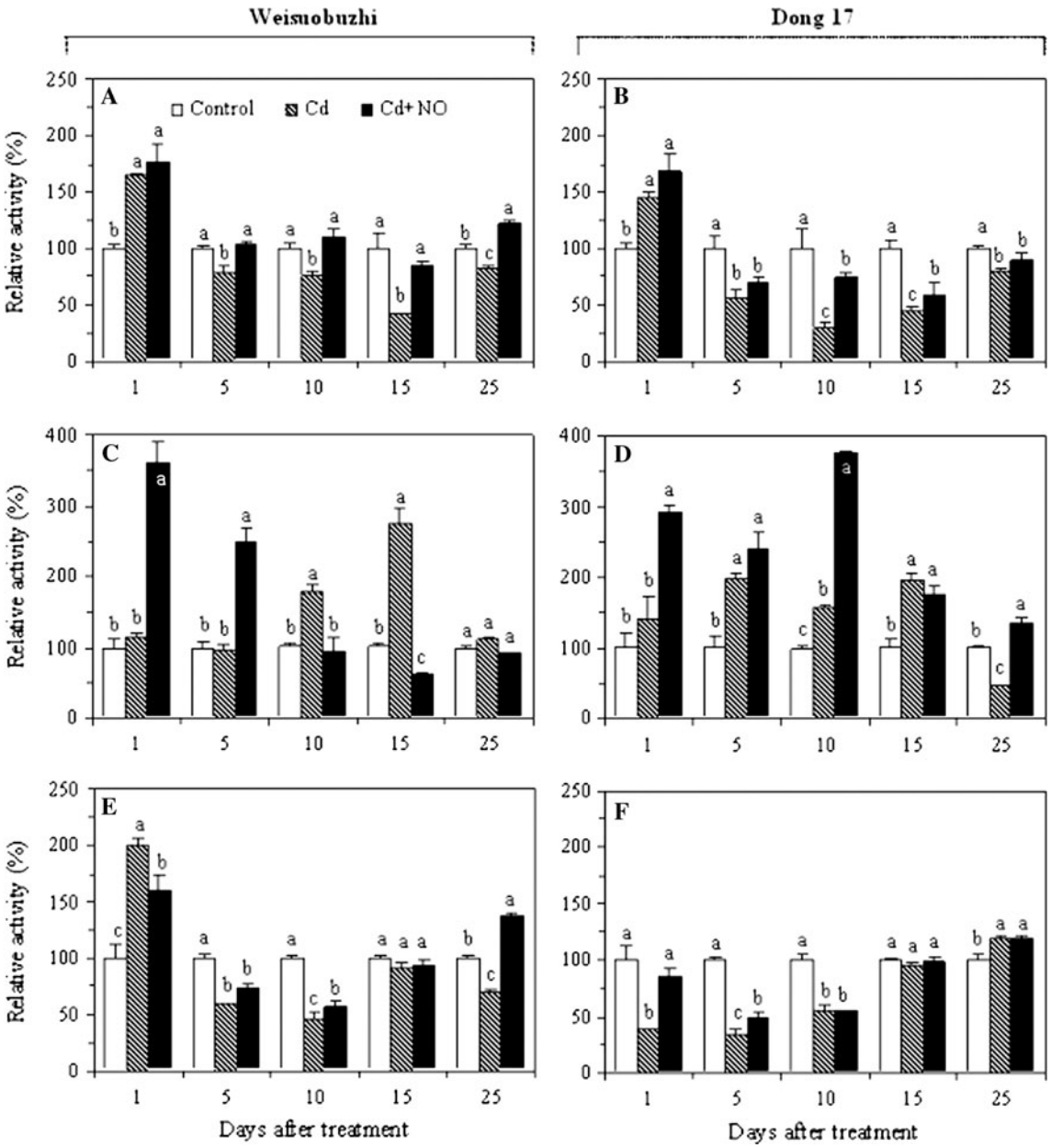

Fig. 8 NOS activity in leaves (**A**, **B**) and roots (**C**, **D**) and NR activity in leaves (**E**, **F**) of two barley genotypes (*left*, Weisuobuzhi; *right*, Dong 17) exposed to Cd for different days and as affected by SNP. NOS activity in the enzyme extraction supernatant solution was assayed by the conversion of L-arginine to L-citrulline in samples with and without the NOS inhibitors of L-NAME (N^G-nitro-L-arginine methyl ester) and L-AG (L-aminoguanidine). *Error bars* represent SD values ($n = 3$). Means with the same letter are not significantly different at $P = 0.05$. Control, Cd, and Cd + N O correspond to basic nutrition solution (BNS), BNS + 5 μM Cd, and BNS + 5 μM Cd + 0.25 mM SNP, respectively

activity after 1–5 days of Cd exposure, after which it remained unchanged as that of the Cd-alone treatment (Fig. 8E, F).

Discussion

Nitric oxide (NO) as a ubiquitous signal molecule participates in major physiological processes in plant growth and development, and in response to biotic and abiotic stresses (Besson-Bard and others 2008, 2009). In this work we analyzed the possible role of exogenous NO in the modulation of the antioxidant defense system and photosynthetic performance against Cd stress in two barley genotypes differing in Cd tolerance. Addition of 0.25 mM SNP to 5 μM Cd medium (NO + Cd) effectively alleviated Cd-induced growth inhibition and toxicity in barley seedlings, described as its ability to prevent inhibition of shoot/root

dry weight, plant height, root length, and chlorophyll content, achieving a similar growth state as that found in barley seedlings growing under control conditions, especially for the Cd-sensitive genotype Dong 17 (Fig. 1, Supplementary Fig. S1, Supplementary Table S1). Our previous study suggested that decreasing leaf chlorophyll content was one of the most general toxicity effects of Cd on plants (Wu and Zhang 2002; Chen and others 2008), which was confirmed again in the present study. A notable reduction of chlorophyll content was detected in the Cd-sensitive genotype Dong 17 exposed to 5 μM Cd, whereas there was no alteration in that of the tolerant Weisuobuzhi. Cd-induced chlorophyll synthesis inhibition was significantly reversed when barley seedlings were treated with exogenous NO (Cd + NO) (Fig. 1), accompanied by the recovery of the rate of photosynthesis, which was severely affected by 5 μM Cd, especially in the sensitive Dong 17 (Supplementary Table 1). This indicated that NO-mediated improvement in photosynthesis, partly due to increasing chlorophyll synthesis, is important for improving plant Cd tolerance. In addition, 5 μM Cd obviously damaged the ultrastructure of chloroplasts, characterized by swollen thylakoid membranes, disturbed the shape of chloroplasts, and induced accumulation of osmiophilic plastoglobuli (Fig. 1B, E), with Dong 17 exhibiting much more severe effects. Interestingly, compared with controls, Cd-alone and Cd + NO treatments both led to less starch accumulation in chloroplast stroma in the two genotypes, indicating that plants fighting against Cd stress might be consuming energy. The addition of NO ameliorated Cd-induced damage to leaf and root ultrastructures. Chloroplasts of Cd + NO-treated plants recovered, becoming similar to those of the control plants (Fig. 1C, F). Root meristem cell ultrastructural observations also revealed a protective effect of NO against Cd-induced swollen mitochondria and plastids with fewer cristae (Fig. 2). These results indicate that NO-mediated stability and integrity of the subcellular structure of barley seedlings under Cd stress contribute to its effective role in preventing Cd-induced leaf chlorosis and inhibition of photosynthesis and growth in barley seedlings.

Reactive oxygen and nitrogen species such as $O_2^{\bullet -}$, H_2O_2, and NO are often produced in large quantities by plants during various stress responses. We investigated the involvement of these molecules in our experiment. Results of histochemical staining and MDA content showed that Cd-caused overaccumulation of $O_2^{\bullet -}$, H_2O_2, and MDA in leaves of barley seedlings was markedly eliminated by the addition of NO (Supplementary Fig. S3), especially in Dong 17. Moreover, the pattern of alteration in certain antioxidant enzymes induced by Cd stress was counteracted in the presence of SNP (Cd + NO) (Laspina and others 2005; Song and others 2006). We obtained similar results in our experiments; for example, addition of 0.25 mM SNP significantly elevated the depressed SOD, APX, and CAT activities in Dong 17 after 10 and 15 days of treatment and suppressed the dramatic increase of POD activity toward control levels in both genotypes (Figs. 3, 4, 5). Thus, it might be deduced that NO indirectly scavenges ROS accumulation by elevating Cd-decreased SOD, APX, and CAT activities, which may account in part for its alleviating effect on Cd-induced oxidative damage in barley seedlings. In addition, according to different subcellular locations and metabolic functions, certain oxidative enzymes always can be distributed in several individual forms (Bowler and others 1992). Thus, individual forms of SOD and APX responding to Cd stress and affected by exogenous NO were detected in this study. SOD isoenzyme analysis showed that Mn-SOD activity decreased in the leaves and Cu/Zn-SOD activity simultaneously increased in the leaves and roots of barley seedlings under Cd stress. Thus, the decrease in total SOD activity in leaves under Cd stress is conceivably due to the decrease of Mn-SOD activity. Moreover, Cd-induced Mn-SOD reduction was significantly elevated by the addition of NO (Cd + NO), indicating that NO protection of Mn-SOD activity from Cd-induced inactivation is important for its role in scavenging ROS under Cd stress (Fig. 3). In addition, different patterns of activity of four individual forms of APX were observed in barley leaves under Cd stress. More interestingly, cAPX activity exhibited a genotypic difference in both how it responds to Cd stress and the addition of NO (Fig. 5E). NO upregulated the Cd-induced decrease in cAPX activity in Dong 17 and downregulated the increased level in Weisuobuzhi, indicating that maintaining a feasibly high cAPX level is beneficial for plant Cd tolerance, and cAPX might participate in NO-mediated Cd detoxification (Fig. 5E).

Lamattina and others (2003) reported that NO might regulate the expression of antioxidative genes to stimulate the relative enzyme activities. NO-regulated antioxidant enzyme (for example, APX, CAT) gene activation was observed in *Arabidopsis* suspension cells (Huang and others 2002). However, the mechanism of NO-mediated alteration of the antioxidative enzyme activities is still not clear. In the present study, the alteration of the transcript levels of some antioxidant enzymes induced by Cd and NO was detected by qRT-PCR analysis (Fig. 6). Results showed that the addition of NO upregulated the transcript levels of leaf/root *POD*, *CAT1*, and root *cAPX* in Weisuobuzhi compared with Cd-alone treatment, whereas in Dong 17, only *cAPX* transcript levels increased and transcription of *CAT1* and leaf *POD* was downregulated. The obvious discrepancy between expression and activity of POD and CAT might be due to the presence of multiple allo- or isoenzymes or enhanced post-translational

modification (Smeets and others 2008). The results of the analysis of *cAPX* transcript levels and enzyme activity showed good agreement between the gene expression data and the measured enzyme activity in the leaves of the two barley genotypes during Cd stress and NO addition (Figs. 5 and 6). Such results suggested that the protective effect of NO in Cd toxicity is at least partly related to its role in upregulating the expression of genes encoding antioxidative enzymes under Cd stress and subsequently eliminating Cd-induced ROS accumulation.

Recently, Cd-mediated NO production has been found in some plant species (Groppa and others 2008; Besson-Bard and others 2009). We also investigated the importance of NO production in Cd tolerance. In barley seedlings exposed to 5 μM Cd, the NO content dramatically increased in the roots and leaves of Cd-tolerant Weisuobuzhi and in the leaves of Cd-sensitive Dong 17 after 1 day of treatment compared with controls, whereas no difference was found in the roots of Dong 17 (Fig. 7). Furthermore, two main enzymes involved in NO production of NR and NOS-like enzymes showed a consistent increased pattern of NO synthesis response to Cd with the activity of NOS-like enzymes in the leaves of both genotypes after 1 day of treatment (Fig. 8A–D). However, Cd induced a genotype-dependent change in NR activity in the leaves of barley seedlings after 1 day of Cd exposure (Fig. 8E, F), that is, an increase in the tolerant genotype but a decrease in the sensitive genotype. These results demonstrated the role of NO synthesis in the Cd tolerance of barley seedlings and the important role of NR and NOS-like enzymes in NO synthesis (Garcia-Mata and others 2003). The Cd-induced increase in NR activity might be related to the Cd tolerance of Weisuobuzhi. Delledonne and others (2001) reported that the cytoprotective role of NO is based mainly on its ability to maintain the cellular redox homeostasis and regulate the level and toxicity of ROS. Meanwhile, an antioxidant function of NO in Cd-stressed plants may be carried out by a direct scavenging of $O_2^{\bullet-}$ (Singh and others 2004), resulting in a reduced amount of $O_2^{\bullet-}$, as shown in Supplementary Fig. S3. However, this ability of NO to exert a protective function against Cd-caused oxidative stress might also be due to the following pathway: reaction with lipid radicals and then blocking the propagation of lipid oxidation, and activation of antioxidant enzymes such as SOD (especially Mn-SOD for sensitive genotype Dong 17), APX (especially cAPX in Dong 17), and CAT.

In summary, exogenous NO dramatically depressed ROS and MDA accumulation, compared with 5 μM Cd treatment, ameliorated Cd-induced damage to leaf and root ultrastructure, and increased chlorophyll content, P_n, G_s, and T_r, thus improving photosynthesis efficiency. Meanwhile, exogenous NO counteracted Cd-induced time- and genotype-dependent responses of antioxidant enzymes via suppressing a Cd-induced dramatic increase in POD activity in the shoots of both genotypes and recovery to near control values, and by elevating Cd-depressed APX and CAT activities in Dong 17 after 10 and 15 days of exposure. Examination of APX and SOD isoenzymes in leaves revealed the NO significantly increased sAPX and Mn-SOD activities in both genotypes, strongly stimulated the Cd-induced decrease in cAPX in the sensitive genotype, and downregulated the increased level in Weisuobuzhi. The results of RT-PCR showed that *POD*, *CAT1*, and *cAPX* responded to Cd stress at the transcript level. External NO upregulated root and leaf *cAPX* and leaf *CAT1* expression in Dong 17 to achieve stimulation. In addition, Cd induced NO synthesis by stimulating NR and NOS-like enzymes in roots and leaves of barley seedlings. A Cd-induced NO burst in the roots of Weisuobuzhi (Cd-tolerant genotype), but not in that of Dong 17 (Cd-sensitive genotype), partly contributed to its Cd tolerance. Our results demonstrated that improved photosynthesis efficiency and the membrane-stabilizing/integrity effect could be the principal protective mechanism for exogenous NO in cytoprotection against Cd toxicity. The results also suggested the potential for NO as a potent antioxidant in plants and that its action may, at least in part, be explained by its ability to directly and indirectly scavenge ROS.

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