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REGULAR ARTICLE

Heme oxygenase-1 is involved in ascorbic acid-induced alleviation of cadmium toxicity in root tissues of Medicago sativa

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Abstract
Aims This study explored molecular mechanism of ascorbic acid (AsA)-mediated enhancement of plant tolerance against cadmium (Cd) stress.
Methods Complex pharmacological, histochemical and molecular approaches were applied to analyse the effect of AsA on the alleviation of Cd stress and corresponding signalling pathway.
Results Cd stress brought about severe oxidative damage and remarkable decrease in AsA content in alfalfa (Medicago sativa) seedling roots. Exogenous AsA not only increased AsA content in vivo, and strengthened the up-regulation of alfalfa heme oxygenase-1 (HO-1) transcript and HO activity triggered by Cd, but also significantly decreased Cd accumulation and oxidative damage, which was confirmed by the histochemical analysis. The responses of AsA were further impaired by the potent inhibitor of HO-1, zinc protoporphyrin IX (ZnPP), which were blocked further when 50 % saturation of carbon monoxide (CO) aqueous solution (in particular) or bilirubin (BR), two catalytic by-products of HO-1, was added, respectively. Molecular evidence illustrated that AsA-triggered the up-regulation of antioxidant enzyme genes, especially *Mn-SOD* and *POD*, were sensitive to ZnPP and reversed by CO.
Conclusions In short, above results suggested that cytoprotective roles triggered by AsA might be, at least partially, through HO-1-dependent fashion by the induction of antioxidant system and lowering Cd accumulation.

Keywords Ascorbic acid · Cadmium toxicity · Heme oxygenase-1 · Medicago sativa · Oxidative damage · Root tissues

Abbreviations

APX	Ascorbate peroxidase
AsA	Ascorbic acid
BR	Bilirubin
BV	Biliverdin IXα
CO	Carbon monoxide
EF-2	Elongation factor 2
HO	Heme oxygenase
HO-1	Heme oxygenase-1
Mn-SOD	Manganese superoxide dismutase
POD	Guaiacol peroxidase
TBARS	Thiobarbituric acid reactive substances
ZnPP	Zinc protoporphyrin IX

Introduction

Cadmium (Cd) is a highly toxic and persistent environmental poison for plants and animals (Sanità and Gabbrielli 1999). Besides the severe seedling growth inhibition and Cd accumulation, one of the indirectly primary responses of plants to Cd exposure is oxidative

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damage, which led to lipid peroxidation of the plasma membrane (Xiang and Oliver 1998; Rodríguez-Serrano et al. 2006; De Michele et al. 2009). It was well established that the excess formation of reactive oxygen species (ROS) in plant cells, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) is one of the principle response to Cd exposure (Romero-Puertas et al. 2004; Besson-Bard et al. 2009; Chao and Kao 2010). It has been further proposed that modulation observed in the bioactivity of antioxidant enzymes/proteins superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), glutathione reductase (GR), ascorbate peroxidase (APX) and peroxiredoxins (PRXes) in response to heavy metal exposure may be partially caused due to the altered metabolic activities of cells that led to changes in ROS level, thus resulting in the maintenance of cellular redox steady state (Gallego et al. 1996; Milone et al. 2003; Finkemeier et al. 2005). Subsequent genetic studies suggested that the up-regulation of some antioxidant enzyme gene expression is beneficial for plant acclimation against heavy metal stress. For instance, overexpression of *GR* in the plastids of Indian mustard could lead to the increased Cd tolerance at the chloroplastic level and decreased Cd accumulation in the shoot tissues (Pilon-Smits et al. 2000).

Besides antioxidant enzymes/proteins, the decline in ascorbic acid (AsA, also named as vitamin C), a low molecular weight antioxidant, could account for the toxicity of Cd in plants (Chao et al. 2010a). In fact, AsA is a multifunctional and high abundance metabolite in plants with key roles in stress tolerance (Noctor and Foyer 1998). A plant-specific pathway of AsA biosynthesis has been described and appears to be controlled by both developmental triggers and environmental cues (Smirnoff and Wheeler 2000). Control of AsA synthesis by respiration in Arabidopsis was also implicated for the possible role in stress response (Millar et al. 2003). Meanwhile, exogenous application of AsA could enhance the tolerance of plant to Cd exposure, chilling, drought, and salt stresses (Shalata and Neumann 2001; Guo et al. 2005; Chao and Kao 2010). The importance role of AsA in protection against abiotic stresses is further strengthened by using Arabidopsis AsA-deficient mutants (Filkowski et al. 2004).

When much attention has focused on the antioxidant role of AsA, some reports indicated that this vitamin also plays a regulatory role in the plant growth via phytohormones (Noctor and Foyer 1998; Smirnoff and Wheeler 2000; Pastori et al. 2003). Moreover, in human beings, a study demonstrated that gastric mucosal protection exerted by vitamin C may due to the induction of heme oxygenase-1 (HO-1) (Becker et al. 2003), and HO-1 was able to promote ulcer healing in a rat model as well (Guo et al. 2003). In fact, HO-1, an inducible isoform of heme oxygenase (HO, EC 1.14.99.3), has emerged in recent years as an important antioxidant enzyme (Ryter et al. 2002). In plant kingdoms, accumulating evidence suggests that HO, which cleaves heme to biliverdin IXα (BV), with the concomitant release of carbon monoxide (CO) and the production of free iron (Fe^{2+}), is an important signalling system involved in the plant response to abiotic stresses and development process (Yannarelli et al. 2006; Zilli et al. 2008; Shekhawat and Verma 2010; Cui et al. 2011; Fu et al. 2011a and b). BV is subsequently reduced by cytosolic biliverdin reductase to form the potent antioxidant bilirubin (BR) (Camara and Soares 2005; Bauer et al. 2008; Guan et al. 2009). Furthermore, HO-1 overexpression and knock-out studies in Arabidopsis clearly showed that HO-1 plays a central role in salt acclimation signalling (Xie et al. 2011). Exogenous application of CO, a by-product of HO, could be advantageous against Cd toxicity in alfalfa (Medicago sativa) seedlings. Interestingly, we also noticed that alfalfa plants pretreated with CO led to significant increases in SOD and POD activities upon thereafter Cd stress (Han et al. 2008).

Although a large number of studies have been conducted on the protection roles of AsA and HO-1 in plant stressful conditions, little information was known about their molecular mechanisms and possible interactions. In this work, by pharmacological, histochemical and molecular approaches,we provided evidence showing that HO-1 up-regulation, at least partially, is involved in AsA-induced Cd tolerance in alfalfa plants. Therefore, this work may further increase our understanding of the mechanisms of AsA amelioration of Cd toxicity in plant kingdoms.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. Ascorbic

acid (AsA) was purchased from Shanghai Medical Instrument, Co., Ltd., China National Medicine (Group), Shanghai, China. Hemin, purchased from Fluka, was used at 10 μM as the HO-1 inducer (Lamar et al. 1996; Xie et al. 2011). Zinc protoporphyrin IX (ZnPP), a specific inhibitor of HO-1 (Fu et al. 2011b; Xie et al. 2011; Bai et al. 2012), was used at 3 μM. The preparation of 50 % saturation of CO aqueous solution was carried out according to the method described in our previous report (Han et al. 2008). The concentrations of above chemicals used in this study were determined in pilot experiments from which the effective responses were obtained.

Plant materials, growth condition and treatments

Commercially available alfalfa (Medicago sativa L. cv. Victoria) seeds were surface-sterilized with 5 % NaClO for 10 min, rinsed extensively in distilled water and germinated for 1 d at 25 °C in the darkness. Uniform seedlings were then chosen and transferred to the plastic chambers and cultured in nutrient medium (quarter-strength Hoagland's solution). Alfalfa seedlings were grown in an illuminating incubator at 25 °C, with a light intensity of 200 $\mu mol m^{-2} s^{-1}$ and 14 h photoperiod. After growing for 5 d, the seedlings were then incubated in water solution containing varied concentrations of $CdCl_2$ and AsA, 3 μM ZnPP, 50 % saturation of CO aqueous solution, 10 μM bilirubin (BR), 10 μM Fe (II) citrate (Fe^{2+}) and 10 μM hemin alone, or the combinations for the indicated times, and/or followed by the indicated treatments as described in the figure legends. Seedlings without chemicals were used as the control (Con). After various treatments, the seedlings were sampled, then used immediately or frozen in liquid nitrogen, and stored at –80 °C until further analysis.

Determination of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was estimated by measuring the amount of TBARS as previously described (Liu et al. 2007). About 200 mg root tissues was ground in 0.25 % 2-thiobarbituric acid (TBA) in 10 % trichloroacetic acid (TCA) using a mortar and pestle. After heating at 95 °C for 30 min, the mixture was quickly cooled in an ice bath and centrifuged at 10,000g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25 % TBA in 10 % TCA. The concentration of lipid peroxides together with oxidatively modified proteins of plants were thus quantified in terms of TBARS amount using an extinction coefficient of 155 $mM^{-1} cm^{-1}$ and expressed as $nmol g^{-1}$ fresh weight (FW).

Determination of reduced ascorbic acid content

Reduced ascorbic acid (AsA) was measured according to the previous method (Law et al. 1983). After various treatments, fresh root tissues were frozen in liquid nitrogen then homogenized in cold 6 % TCA immediately. The homogenate was centrifuged at 12,000g for 20 min at 4 °C, and the supernatant was collected for analysis of AsA. Color was developed in reaction mixtures after the addition of the following reagents: 0.4 ml of 10 % TCA, 0.4 ml of 44 % ortho-phosphoric acid, 0.4 ml 4 % a,a'-dipyridyl in 70 % ethanol, and 0.2 ml 0.3 % (w/v) $FeCl_3$. After vortex mixing, the mixture was incubated at 37 °C for 60 min and the A_{525} was recorded.

Histochemical staining

Histochemical detection of lipid peroxidation was performed with Schiff's reagent as described by Pompella et al. (1987). Histochemical detection of loss of plasma membrane integrity in root apexes was performed with Evans blue described by Yamamoto et al. (2001). All the roots stained with Schiff's reagent or Evans blue were washed extensively, then observed under a light microscope (model Stemi 2000-C; Carl Zeiss, Germany) and photographed on color film (Powershot A620, Canon Photo Film, Japan).

Determination of Cd content

Plant samples were harvested and digested with HNO_3 using a Microwave Digestion System (Milestone Ethos T). Cd contents were determined in root tissues by an Inductively Coupled Plasma Optical Emission Spectrometer (Perkin Elmer Optima 2100DV).

HO activity assay

Heme oxygenase (HO; EC 1.14.99.3) activity was analysed following the method described by Han et

al. (2008). For the HO activity test, the concentration of biliverdin IX (BV) was estimated using a molar absorption coefficient at 650 nm of 6.25 $mM^{-1}cm^{-1}$ in 0.1 M HEPES-NaOH buffer (pH 7.2). One unit of activity (U) was calculated by taking the quantity of the enzyme to produce 1 nmol BV per 30 min. Protein concentration was determined according to the method of Bradford (1976), with bovine serum albumin as the standard.

Transcript quantification

Alfalfa roots were homogenized with mortar and pestle in liquid nitrogen. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the instructions supplied by the manufacturer. Total RNA was reverse-transcribed using an oligo(dT) primer and SuperScript™ Reverse Transcriptase (Invitrogen, Carlesbad, CA, USA). Real-time quantitative RT-PCR reactions were performed using a Mastercycler® ep *realplex* real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® *Premix Ex Taq*™ (TaKaRa Bio Inc., China) according to the manufacturer's instructions. The cDNA was amplified using the following primers: for *MsHO1* (accession number HM212768; Fu et al. 2011a), forward TCTCATTCTCCTCGTTTAGC and reverse TTCGCCTGGTCCTTTGTAT; for *Mn-SOD* (accession number AY145894.1), forward TGTC ATCAGCGGCGTAATCAT and reverse GGGCT TCCTTTGGTGGTTCA; for *POD* (accession number X90695), forward TTTGTCATTGGCAGGTGAT and everse TGAAACTTGGCTGAGGGA; for *APX1* (accession number DQ122791), forward TCCTCT TATGCTCCGTTTG and reverse GTTCCACC CAGTAATCCCA; and for *EF-2* (accession number DQ122789), forward AACGAAATCAAGGACT and reverse AACAACATCACAAACC. Relative expression levels were presented as values relative to that of the corresponding control samples at the indicated times, after normalization to *EF-2* transcript levels.

Statistical analysis

Data are means ± SE from three independent experiments. For statistical analysis, either the *t*-test ($P<0.05$) or Tukey's test ($P<0.05$), was selected where appropriate.

Results

Contrasting responses in TBARS and AsA contents upon Cd exposure

Figure 1 illustrated dose-dependent effects of Cd on TBARS and AsA contents in alfalfa seedling roots. In comparison with the control sample, after 12 h treatment with $CdCl_2$ ranging from 100 to 500 μM (De Michele et al. 2009), AsA content approximately exhibited a dose-dependent decrease. Whereas, TBARS overproduction enhanced with the increasing concentrations of Cd. The abovementioned changes of TBARS and AsA contents were also shown to be time dependent. During the 24 h of treatment, for example, seedlings-treated with Cd exhibited progressive increase in TBARS content, compared with chemical-free control samples (Fig. 2a). Meanwhile, increasing depletion of AsA contents were also observed (Fig. 2b). Taken together, these results clearly indicated a possible interrelationship between AsA and TBARS in Cd-stressed plants.

Cd-induced oxidative damage is sensitive to added AsA or hemin

To assess whether the depletion of AsA was responsible for Cd-induced oxidative damage, exogenous AsA ranged from 200 to 800 μM and the HO-1

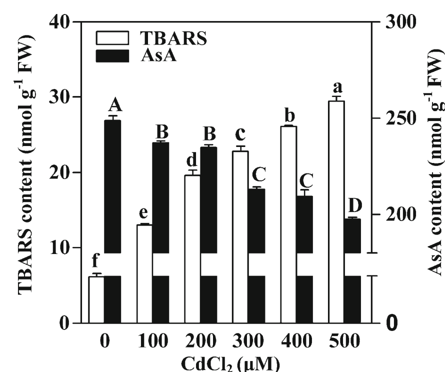


Fig. 1 Dose-dependent effects of Cd on TBARS and AsA contents in alfalfa seedling root tissues. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing indicated concentrations of $CdCl_2$ for another 12 h. Then, TBARS and AsA contents were determined. Values are means ± SE of three independent experiments with at least three replicates. Within each set of experiments, bars with different letters are significantly different at $P<0.05$ according to Tukey's test

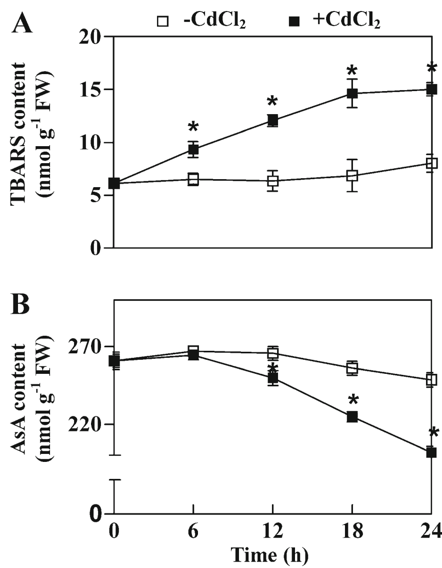


Fig. 2 Time-course of TBARS and AsA contents in the roots of alfalfa seedlings upon Cd exposure. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing 100 μM $CdCl_2$ for the indicated times. Then, TBARS (**a**) and AsA (**b**) contents were determined. Values are means ± SE of three independent experiments with at least three replicates. Asterisks indicate that mean values are significantly different at $P<0.05$ according to *t*-test

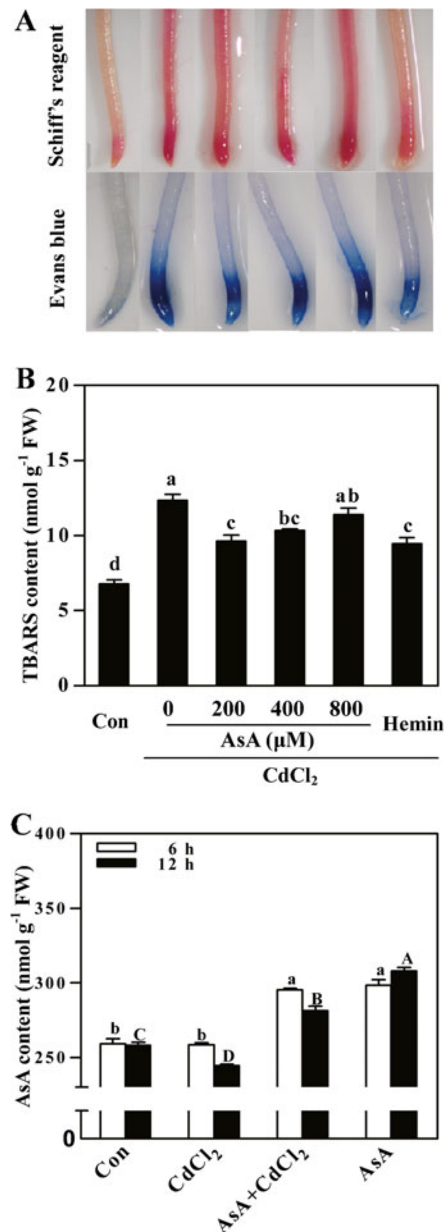


Fig. 3 Effects of AsA on Cd-induced oxidative damage and changes of endogenous AsA content in alfalfa seedling root tissues. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing indicated concentrations of AsA (**a** and **b**) or 200 μM AsA (**c**), 100 μM $CdCl_2$, 10 μM hemin alone, or the combination treatment for another 12 h. Seedlings without chemicals were used as the control (Con). Afterwards, the seedling roots were stained with Schiff's reagent and Evans blue, and immediately photographed under a light microscope (**a**). Bars, 2 mm. TBARS content was also quantified (**b**). Endogenous AsA contents were determined after 6 h or 12 h of treatments (**c**). Values are means ± SE of three independent experiments with at least three replicates. Within each set of experiments, bars with different letters are significantly different at $P<0.05$ according to Tukey's test

inducer hemin (also regarded as the positive control) were added. Meanwhile, assessments of lipid peroxidation and the loss of plasma membrane integrity in roots under various treatments were evaluated by histochemical staining with Schiff's reagent and Evans blue. The roots of alfalfa seedlings treated with Cd alone were stained extensively (Fig. 3a), whereas those treated with the increasing concentrations of AsA and 10 μM hemin exhibited differential light staining, with a maximal responses at 200 μM AsA and 10 μM hemin (in particular). Above results were consistent with the changes in TBARS formation (Fig. 3b). It was also observed that treatment of seedlings with exogenous AsA (200 μM) significantly blocked the decrease of AsA content in vivo triggered by Cd stress for 12 h (Fig. 3c).

Further results showed that similar decreasing effects of AsA and hemin on the Cd-induced TBARS overproduction were observed regardless of whether AsA and Cd were present together or separately (Fig. 4). Therefore, these discoveries ruled out the possibility that the alleviation of Cd-induced oxidative damage by AsA could be resulted from chelation of Cd by AsA.

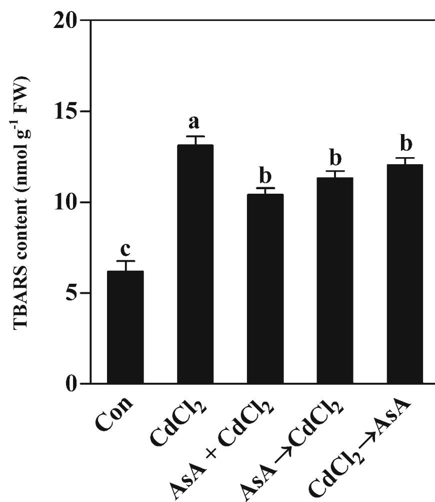


Fig. 4 The effect of AsA on Cd-induced oxidative damage in the roots tissues of 5-day-old alfalfa seedlings by incubating the roots in water solutions supplemented with 100 μM $CdCl_2$, 200 μM AsA and 100 μM $CdCl_2$ together for 12 h, or incubating the roots in 200 μM AsA for 12 h followed by another 12 h incubation in 100 μM $CdCl_2$, or incubating the roots in 100 μM $CdCl_2$ for 12 h followed by another 12 h incubation in 200 μM AsA. Seedlings without chemicals were used as the control (Con). Afterwards, TBARS content was quantified. Values are means ± SE of three independent experiments with at least three replicates. Bars with different letters are significantly different at $P<0.05$ according to Tukey's test

Together, above results proved that the application of exogenous AsA and hemin exhibited protective effects against Cd-induced oxidative damage in alfalfa seedling roots.

Up-regulation of *HO-1* in response to AsA

Previous results showed that pretreatment with AsA could prevent the UV-B-induced up-regulation of HO-1 in soybean plants (Yannarelli et al. 2006). To get better understanding of the association of HO-1 with the alleviation of Cd-induced oxidative damage triggered by AsA, a detailed study was carried out to investigate the changes of alfalfa HO-1 transcripts (*MsHO1*) and HO activities. The results in Fig. 5a demonstrated that Cd-induced *MsHO1* up-regulation was strengthened by the addition of AsA at 6 h of treatments. Meanwhile, AsA at 200 μM was able to significantly induce *MsHO1* gene expression. By contrast, in comparison with the control sample, no significant difference was observed in *MsHO1* gene expression after 3 h of various treatments regardless of whether Cd and AsA were present together or

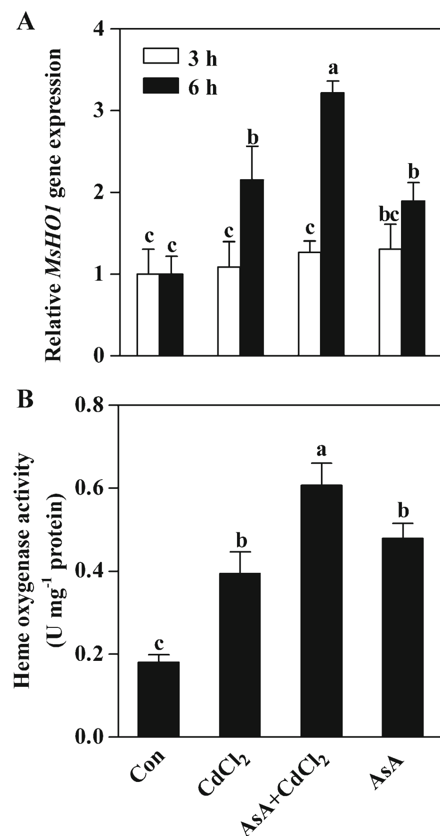


Fig. 5 Changes of *MsHO1* transcript and HO activity in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing 100 μM $CdCl_2$, 200 μM AsA alone, or the combination treatment for the indicated times (**a**) or 6 h (**b**). Seedlings without chemicals were used as the control (Con). Then, the gene expression was analyzed by real-time RT-PCR (**a**). The expression levels of the gene were presented as values relative to the corresponding control samples. HO activity (**b**) was determined after 6 h of various treatments. Values are means ± SE of three independent experiments with at least three replicates. Bars with different letters are significantly different at $P<0.05$ according to Tukey's test

separately. Interestingly, a close correlation was found between *MsHO1* transcript levels and corresponding HO activities after 6 h of various treatments (Fig. 5b).

AsA-triggered responses were sensitive to the specific inhibitor of HO-1 ZnPP, but reversed differentially by CO and BR

In animals, some AsA responses are similar to or mediated by HO-1 (Becker et al. 2003). Endogenous or exogenous CO has been confirmed to induce alfalfa tolerance against Cd toxicity (Han et al. 2008). In the

following tests, as expected, after 12 h of treatments, the Cd content in the AsA- and hemin-treated root tissues exhibited a significant decrease in comparison with Cd-stressed alone samples (Fig. 6). By contrast, a significant reversal was observed when ZnPP, the potent inhibitor of HO-1, was applied together with AsA, which was blocked by the addition of CO. Comparatively, the addition of Fe^{2+} and BR, the other two catalytic by-products of HO-1 resulted in negative responses. In addition, the combination of Cd together with ZnPP brought about a slight increase in Cd content, respect to the Cd stressed alone sample. These results suggested that AsA response on the alleviation of Cd accumulation is HO-1-dependent.

To confirm above deduction, histochemical analysis of lipid peroxidation and the loss of plasma membrane integrity as well as TBARS content were also investigated. As expected, results of Fig. 7 illustrated that when CO or BR (in particular) was applied together with AsA plus ZnPP, the heavy staining of

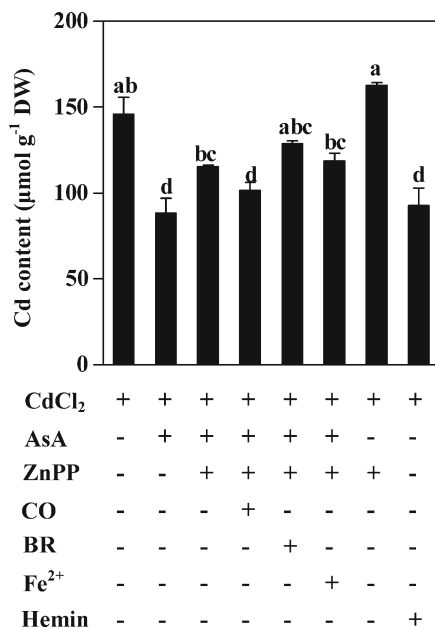
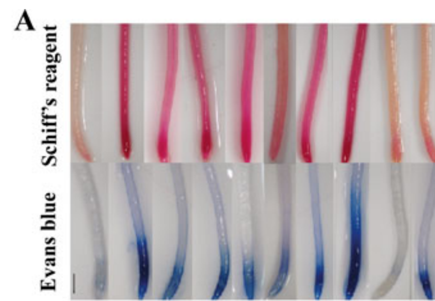


Fig. 6 Effects of $CdCl_2$, AsA, ZnPP, CO, BR, Fe^{2+} and hemin on Cd contents in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing 100 μM $CdCl_2$, 200 μM AsA, 3 μM ZnPP, 50 % saturation of CO aqueous solution, 10 μM BR, 10 μM Fe^{2+}, 10 μM hemin alone, or the combinations for 12 h. Values are means ± SE of three independent experiments with at least three replicates. Bars with different letters are significantly different at $P<0.05$ according to Tukey's test



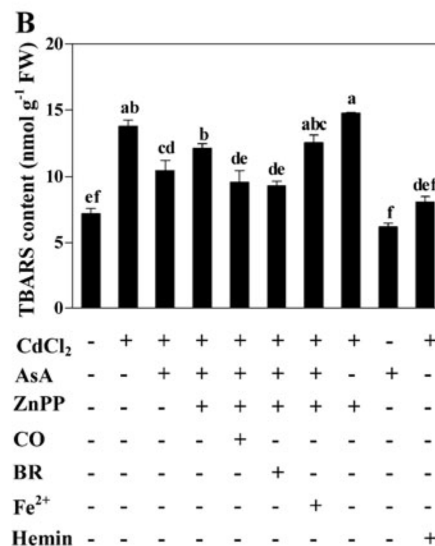
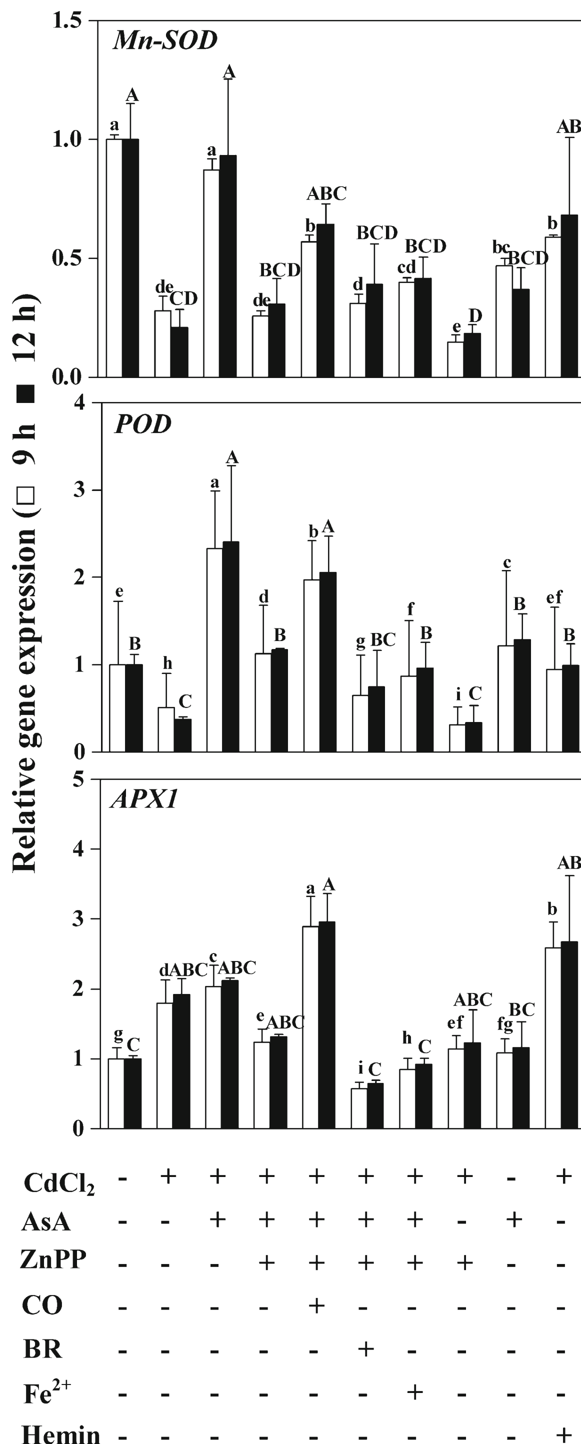


Fig. 7 Effects of $CdCl_2$, AsA, ZnPP, CO, BR, Fe^{2+} and hemin on Cd-induced oxidative damage in alfalfa seedling root tissues. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing 100 μM $CdCl_2$, 200 μM AsA, 3 μM ZnPP, 50 % saturation of CO aqueous solution, 10 μM BR, 10 μM Fe^{2+}, 10 μM hemin alone, or the combinations for 12 h. Seedlings without chemicals were used as the control (Con). Afterwards, the seedling roots were stained with Schiff's reagent and Evans blue, and immediately photographed under a light microscope (**a**). Bars, 2 mm. TBARS content was also quantified (**b**). Values are means ± SE of three independent experiments with at least three replicates. Bars with different letters are significantly different at $P<0.05$ according to Tukey's test

lipid peroxidation and the loss of plasma membrane integrity in the Cd-stressed roots of alfalfa seedlings were relieved. Comparatively, the addition of Fe^{2+} displayed less effective, and the combination of Cd and ZnPP brought about the maximal heavy staining. Additionally, the addition of CO, BR, and Fe^{2+} alone, did not change the staining pattern, in comparison with the chemical-free control samples (data not shown). Changes of TBARS content exhibited the similar tendencies.

Transcript profiles of antioxidant enzyme genes

Cd stress for 9 h and 12 h led to significant changes in the transcripts of *Mn-SOD*, *POD* and *APX1* in alfalfa seedling root tissues (Fig. 8). For example, treatment with 100 μM $CdCl_2$ for 12 h down-regulated the transcripts of *Mn-SOD* and *POD* by 79.0 and 62.5 %, respectively, and the transcript of *APX1* was increased by 91.9 %, compared with the control values. Meanwhile, besides the slight increase of *APX1* transcript, treatment together with AsA mimicked the response of hemin in the obvious reversal of above down-regulation tendencies. Similar results were observed after Cd treatment for 9 h. However, above AsA responses were weaken by the together addition of the potent inhibitor of HO-1, ZnPP. After the combination with CO, there were comparatively increases in the transcripts of *Mn-SOD*, *POD* and *APX1*. The addition of BR and Fe^{2+}, however, resulted in weaker or negative responses. Additionally, in comparison with the Cd stressed alone sample, Cd plus ZnPP differentially decreased above three antioxidant enzyme genes, especially in *POD* and *APX1* transcripts after 9 h of treatments.



Discussion

It has been well documented that Cd stress can indirectly cause the increased generation of ROS and thereafter oxidative damage in plant tissues (Xiang and Oliver 1998; Ortega-Villasante et al. 2005; De Michele et al. 2009). In response to Cd exposure, gene expression of antioxidant genes and antioxidant contents were also altered (Sharma and Dietz 2009). A previous study demonstrated that the decline in AsA content is associated with Cd toxicity of rice seedlings (Chao et al. 2010a). In fact, AsA is the most abundant antioxidant in plants and plays a role in cytoprotective responding against oxidative stress (Noctor and Foyer 1998). Exogenous AsA has been applied to tissues, culture medium or cultivating soil to increase the AsA content in plants for the purpose of improving Cd tolerance (El-Naggar and El-Sheekh 1998; Noctor and Foyer 1998; Erdogan et al. 2005). As expected (Chao et al. 2010a), we observed that the addition of AsA individual or simultaneously to alfalfa seedlings could significantly increase endogenous AsA content under normal growth conditions, or in particular, block Cd-induced decrease of AsA content in vivo (12 h; Fig. 3c).

◀ **Fig. 8** Effects of $CdCl_2$, AsA, ZnPP, CO, BR, Fe^{2+} and hemin on the expression of antioxidant defence genes in alfalfa seedling root tissues. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to water solution containing 100 μM $CdCl_2$, 200 μM AsA, 3 μM ZnPP, 50 % saturation of CO aqueous solution, 10 μM BR, 10 μM Fe^{2+}, 10 μM hemin alone, or the combinations for 9 h and 12 h. Seedlings without chemicals were used as the control (Con). Afterwards, transcript levels of *Mn-SOD*, *POD* and *APX1* were analyzed by real-time PCR. The expression levels of each gene were presented as values relative to the corresponding control samples. Values are means ± SE of three independent experiments with at least three replicates. Within each set of experiments, bars with different letters are significantly different at $P < 0.05$ according to Tukey's test

Our previous observations showed that β-cyclodextrin-hemin complex (CDH)-mediated induction of alfalfa HO-1, a novel antioxidant enzyme confirmed recently in plants as well as previously in animals (Ryter et al. 2002; Shekhawat and Verma 2010), provides critical protection against Cd-induced oxidative damage and toxicity in alfalfa seedlings (Fu et al. 2011b). In this study, by the application of hemin, an HO-1 inducer as the positive control, we further present evidence of the fact that the beneficial effects of AsA on the alleviation of Cd-induced oxidative damage and Cd accumulation in alfalfa seedling roots, both of which are in line with the observations that AsA alleviates Cd toxicity in rice (Chao and Kao 2010), barley (Wu and Zhang 2002), Chlorella vulgaris (El-Naggar and El-Sheekh 1998), freshwater catfish (Kumar et al. 2009), mice (Gupta et al. 2004; Acharya et al. 2008; Donpunha et al. 2011), and broilers (Erdogan et al. 2005). Interestingly, above AsA effect, at least partially, was in a HO-1-depednet manner. This study further supports the conclusion that HO-1 might be a component of the AsA-induced cytoprotective role against Cd toxicity, remarkably similar to those found in animals (Becker et al. 2003).

The following results derived from physiological, histochemical and molecular approaches support above conclusion. First, our results demonstrated that Cd stress elicited a marked decrease in endogenous AsA content approximately in a dose- and time-dependent fashion; meanwhile, a contrasting response in TBARS overproduction was observed (Figs. 1 and 2). Further results indicated that alfalfa plants treated with 200 μM AsA, which blocks Cd-induced decrease of AsA content in vivo (Fig. 3c), mimicked the cytoprotective effects of hemin in the alleviation of Cd-induced oxidative damage and Cd accumulation in alfalfa seedling roots, as compared with the stressed alone sample (Figs. 3 and 6). Some previous results have established a close link between the degree of plant tolerance to Cd stress and the level of AsA (Wu et al. 2004; Chao et al. 2010a and b). These results collectively point to the fact that a reduction of endogenous AsA concentration in root tissues of alfalfa seedlings could be one of the important events in triggering Cd toxicity in plants. The exogenous AsA-induced alleviation of Cd toxicity is unlikely to result from chelation of toxic Cd by AsA, since pretreatments of roots with AsA and Cd individually had an identical effect on TBARS content compared to the treatments with Cd and AsA simultaneously (no significant difference was observed; Fig. 4).

However, the detailed mechanism and signal transduction of above AsA action remain to be determined. Subsequently, we observed that both AsA and hemin exhibited the decreased Cd accumulation (Fig. 6), and the mitigation of Cd-induced oxidative damage (Fig. 3) by inducing some representative antioxidant genes (*Mn-SOD* and *POD*, especially; Fig. 8). The latter of which were confirmed by the histochemical staining for the detection of lipid peroxidation and injury of membrane integrity in root apexes (Fig. 3). Interestingly, we also noticed that there was a strong correlation among the responses of AsA and hemin, the up-regulation of *MsHO1* transcript and induction of HO activity (Fig. 5), and ample evidence has confirmed that the activation of HO-1 plays an important role in plant response to multiple stresses, including heavy metal-induced oxidative stress (Noriega et al. 2004), drought (Liu et al. 2010), and salinity stress (Xie et al. 2011). These results were remarkably similar to those found in animals, showing that HO-1 mRNA expression in gastric epithelial cells is enhanced by acetylic-salicylic acid as well as AsA, and an increase in HO-1 protein level seems to occur only in the presence of AsA as a "non-stressful" stimulus of HO-1 expression (Becker et al. 2003). Meanwhile, AsA was able to strengthen the induction of *HO-1* mRNA by As^{3+} in the absence or presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in Hepa 1c1c7 cells (Elbekai et al. 2007). However, previous results obtained by Noriega et al. (2007), showed that

AsA could decrease the transcripts of soybean *HO-1* upon ultraviolet-B exposure. This discrepancy is likely due to different AsA concentrations used. They applied 10 mM AsA, a concentration is 50 times higher than the concentration (200 μM) used in our study.

To assess whether a HO-1 pathway is involved in AsA-enhanced adaptive plant response against Cd stress or not, we subsequently investigated the effects of specific inhibitor of HO-1, ZnPP. As demonstrated in Figs. 6 and 7, corresponding cytoprotective effects conferred by AsA are blocked by ZnPP which could be differentially reversed when CO (in particular) or BR, two catalytic by-products of HO-1, was added together. Changes of two antioxidant enzyme genes, including *Mn-SOD* and *POD*, approximately exhibited the similar tendencies (Fig. 8). These results, partly in accordance with the findings of several previous studies (Noriega et al. 2004; Fu et al. 2011b), obviously suggested that the up-regulation of HO-1 was associated with the alleviation of Cd toxicity by the induction of antioxidant genes and lowering Cd accumulation. In animal cells, it was also reported that HO-1/CO could induce *Mn-SOD* transcript, or enhance SOD and CAT activities (Garnier et al. 2001; Turkseven et al. 2005).

There are many studies on the responses of APX activity and/or its transcripts in plants upon Cd stress, but different results have been obtained from various plant species upon different conditions. In plants, it was well known that APX in different cellular compartments utilizes two molecules of AsA as its specific electron donor, to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA) (Nakano and Asada 1987). In our test, we noticed that the Cd-induced increases in the *APX1* transcript levels (Fig. 8) were in line with previous results in alfalfa seedlings (Han et al. 2008; Fu et al. 2011b; Li et al. 2012), although the opposite or no significant response was observed in pea seedlings upon higher doses of Cd (Romero-Puertas et al. 2007; Hana et al. 2008). Subsequently, no significant difference was observed in *APX1* transcripts between seedling roots upon Cd stress with or without the addition of AsA for 12 h (Fig. 8). This result was consistent with that in rice plants, showing the gene expression of *OsAPX2* but not *OsAPX1* was induced by exogenous AsA (Chao et al. 2010a). Microarray analysis also confirmed that numerous stress-related genes but not *OsAPX* were induced by exogenous AsA (Tokunaga and Esaka 2007). Therefore, we deduced that different responses of *APX1*, *Mn-SOD* and *POD* upon various treatments (Fig. 8) might be dependent on the concentration of heavy metals, and even different plant species. Additionally, the possibility of the different sensitivities of multiple gene families of *APX* to AsA could not be easily ruled out.

In summary, the results of the present study support the theory that the up-regulation of HO-1 is, at least partially, associated with the AsA-induced cytoprotective role against Cd stress. Afterwards, the product of the HO reaction, CO, might trigger the signal transduction events that lead to the gene expression of antioxidant genes, etc. (Liu et al. 2007; Han et al. 2008; Xie et al. 2008; Bai et al. 2012). Further loss- and gain-of-function mutants to manipulate gene expression of HO isoforms and endogenous AsA content may help to elucidate their physiological importance in the plant responses against heavy metal exposure.

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