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Cadmium-induced heme oxygenase-1 gene expression is associated with the depletion of glutathione in the roots of *Medicago sativa*

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Abstract Following previous findings that cadmium (Cd) induces heme oxygenase-1 (HO1) gene expression in alfalfa seedling roots, we now show that the decreased glutathione (GSH) and ascorbic acid (AsA) contents, induction of *HO-1* gene expression and its protein level by Cd was mimicked by a GSH depletor diethylmaleate (DEM). Meanwhile, above Cd- or DEM-induced decreased GSH content followed by HO-1 up-regulation could be strengthened or reversed differentially by the application of a selective inhibitor of GSH biosynthesis L-buthionine-sulfoximine (BSO), or exogenous GSH and AsA, respectively. The antioxidative behavior of HO-1 induction was further confirmed by histochemical staining for the detection of loss of membrane integrity in a short period of treatment time. Additionally, the induction of *HO-1* transcript was inhibited by the transcriptional inhibitor actinomycin D (ActD) or protein synthesis inhibitor cycloheximide (CX, especially). In contrast, the level of *HO-2* transcript did not change upon various treatments. Together, above results suggested that Cd-induced up-regulation of *HO-1* gene expression is associated with GSH depletion, which is at least existing transcriptional regulation level, thus leading to enhanced antioxidative capability transiently.

Keywords Alfalfa seedling roots · Ascorbic acid · Cd-induced oxidative stress · Glutathione depletion · *HO-1* gene expression

Introduction

In animals, three isoforms of heme oxygenase (HO, EC 1.14.99.3), the rate-limiting enzyme of heme degradation with the concomitant release of carbon monoxide (CO), the production of biliverdin IXα (BV) and free iron (Fe^{2+}), HO-1, HO-2 and HO-3 have been identified, which are products of distinct genes (Maines 1997). HO-2 and HO-3 are constitutively expressed whereas HO-1 is highly inducible. Furthermore, ample evidence has been accumulated showing that HO-1 is a stress responsive protein, and it responds to many kinds of chemically or physiologically produced oxidative stress in various cells and tissues. Among these, glutathione (GSH) depletion caused by many chemicals and other factors, such as cytokine, prostaglandin, endotoxin, ultraviolet A, produces the induction of HO activity by de novo enzyme synthesis associated with an increase in HO-1 mRNA level (Ewing and Maines 1993; Oguro et al. 1996; Horikawa et al. 2002).

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However, the role of HOs in plants was originally focused on its association with the pathway leading to phytochrome chromophores metabolism and functioning in light signalling (Davis et al. 2001; Emborg et al. 2006; Shekhawat and Verma 2010). Previous result also postulated that HO-1 in alfalfa mature nodules is involved in leghemoglobin metabolism (Baudouin et al. 2004), and the expression of HO-1 is usually triggered by diverse stress-inducing stimuli including salinity stress (Xie et al. 2008; Zilli et al. 2008; Ling et al. 2009), cadmium (Cd) exposure (Noriega et al. 2004; Han et al. 2008), UV radiation (Yannarelli et al. 2006), reactive oxygen species (ROS) such as H_2O_2 (Yannarelli et al. 2006; Chen et al. 2009), and nitric oxide (NO) (Noriega et al. 2007; Xuan et al. 2008; Santa-Cruz et al. 2010). Thus, the up-regulation of HO-1 could act as antioxidant barrier against various stresses-induced oxidative damage (Shekhawat and Verma 2010).

It was well established that GSH plays an important role in protecting plants from various environmental stresses, including oxidative stress resulting from Cd, which is a well-known pro-oxidant, and a highly toxic trace element entering the environment mainly from industrial processes and phosphate fertilizers (Sharma and Dietz 2009). Report from our laboratory (Han et al. 2008) has demonstrated that in alfalfa seedling roots, exogenous Cd applied was able to cause the depletion of GSH, increase of CO production, which is consistent with the changes of HO activity and *HO-1* transcripts. Furthermore, CO was regarded as a gaseous signal for the alleviation of Cd-induced oxidative damage by modulating GSH and ascorbic acid (AsA) homeostasis. This observation led to the suggestion that HO-1 may present an alternative mean for production of antioxidants, particularly in those conditions of plant tissues and cells with relatively low levels of GSH and AsA or with compromised defence mechanisms caused by GSH depletion.

In the present investigation, we confirmed this hypothesis and report on the induction of HO-1 expression (transcript, protein, and enzyme activity) in alfalfa seedling roots upon specific chemical agents, the depletor of GSH, mimicking the responses in Cd stressed plants, which has been reported by Tomaro's research group. Related inducible mechanism of HO-1 expression and its relationship with GSH depletion and AsA were also illustrated. Therefore, we suggested the increase in HO-1 expression in response to both GSH and AsA depletion, and Cd exposure in alfalfa plants, consistent with the idea that HO-1 and BV may serve as a potential source of antioxidant in the event of GSH deficiency (Ewing and Maines 1993; Shekhawat and Verma 2010).

Materials and methods

Chemicals

Reduced ascorbic acid (AsA), glutathione reduced form (GSH), a GSH depletor diethyl maleate (DEM), and a selective inhibitor of GSH biosynthesis L-buthionine-sulfoximine (BSO) were from Sigma/Aldrich. Actinomycin D (ActD) and cycloheximide (CX), which used as the transcriptional inhibitor and the protein synthesis inhibitor, were also obtained from Sigma.

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 2 days 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 the illuminating incubator at 25 ± 1°C, with a light intensity of 200 μmol m^{-2} s^{-1} and 14 h photoperiod. After growing for 5 days, seedlings were incubated in quarter strength Hoagland's solution containing either 200 μM or the indicated concentrations of $CdCl_2$, 1 mM DEM, 1 mM BSO, 1 mM AsA, 5 μg/ml ActD and CX alone, or the combination for the indicated time, or then exposed to 1 mM DEM, 2 mM GSH, or 200 μM $CdCl_2$ alone, or the combination treatment as indicated for another indicated time. The pH for both nutrient medium and treatment solutions was adjusted to 6.0 by using NaOH or HCl. After various treatments, the seedlings root tissues were sampled, then immediately used or frozen in liquid nitrogen, and stored at −80°C until further analysis.

Determination of reduced GSH and reduced ascorbic acid contents

Reduced GSH was measured according to the method previously reported (Han et al. 2008). Frozen root tissues were homogenized in cold 5% 5-sulfosalicylic acid. The homogenate was centrifuged at 20,000×*g* in a rotor (model Avanti J-25, Beckman) for 15 min at 4°C and the supernatant was collected for analyses of GSH. Total glutathione (GSH + GSSG) was determined in the homogenates spectrophotometrically at 412 nm, using glutathione reductase, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and reduced GSH content was calculated from the difference between total glutathione and GSSG.

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 12,000×*g* for 20 min at 4°C, and the supernatant was collected for analyse 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.

Determination of thiobarbituric acid reactive substances content

Lipid peroxidation was estimated by measuring the amount of thiobarbituric acid reactive substances (TBARS) as previously described (Han et al. 2008). About 500 mg fresh tissues was ground in 0.25% 2-thiobarbituric acid (TBA) in 10% 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 10,000×*g* for 10 min. The absorbance of the supernatant was recorded 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).

Histochemical analyses

Histochemical detection of loss of plasma membrane integrity in root apexes was performed with Evans blue (Yamamoto et al. 2001). All the roots stained with 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).

HO activity assays

HO activity was analyzed by the method according to our former report (Han et al. 2008). In HO activity test, the concentration of biliverdin IX 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 was determined by using bovine serum albumin (BSA) as a standard (Bradford 1976).

Western-blot analysis for HO-1

Recently, we cloned alfalfa *HO-1* gene from total RNA prepared from seedling plants and named as *MsHO1*, which was deposited in GenBank recently (accession number HM212768). The *MsHO1* gene encodes a single open reading frame of 283 amino acids with a predicated molecular mass of 32.8 kDa. The expression analysis showed that *MsHO1* gene was expressed in leaves, seeds, stems, and root tissues. Further, rabbit polyclonal antibody was made against the mature MsHO1.

Homogenates obtained for HO activity assays were also analyzed by western blotting. Fifty micrograms of protein from homogenates were subjected to SDS–PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad) according to the method described in our previous report (Xuan et al. 2008). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding of antibodies was blocked with 5% non-fat dried milk in phosphate buffered saline (PBS, pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies diluted 1:2,500 in phosphate-buffered saline + 1% non-fat milk. Immune complexes were detected

using horseradish peroxidase-conjugated goat anti-rabbit IgG. The color was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride as the horseradish peroxidase substrate. Additionally, the films were scanned (Uniscan B700^{+}, Tsinghua Unigroup Ltd., Beijing, China) and analysed using Quantity One v4.4.0 software (Bio-Rad, USA).

Transcript quantification

Root tissue was 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. Approximately 5 μg of total RNA was reverse-transcribed using an oligo(dT) primer and SuperScript^{TM} Reverse Transcriptase (Invitrogen, Carlesbad, CA, USA). cDNA was amplified by PCR using the following primers: *HO-1* (accession number HM212768), forward TACATACAAAGGA CCAGGCTAAAG and reverse GTCCCTCACATT CTGCAACAACTG (amplifying a 476 bp fragment); *HO-2* (the AW696919 and BE205128 sequences were assembled to construct the HO-2 cluster sequence), forward CGTTTCATATGCGTACTTG and reverse TTATTTTTCTCATCCCGAC (amplifying a 363 bp fragment); and *EF-2* (accession number DQ122789), forward AATGGCTGATGAGAACC TGC and reverse TTGTCCTCGAACTCGGAGAG (amplifying a 498 bp fragment). To standardize the results, the relative abundance of *EF-2* was determined and used as the internal standard.

The cycle numbers of the PCR reactions were adjusted for each gene to obtain visible bands in agarose gels. Aliquots of the PCR reactions were loaded on 1.2% agarose gels with the use of ethidium bromide. Specific amplification products of the expected size were observed and their identities were confirmed by sequencing. Ethidium bromide stained gels were scanned and analyzed using TotalLab v1.10 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). The ratio of *HO-1* mRNA to *EF-2* was quantified.

Statistical analysis

Values are means ± SD of three different experiments with at least three replicated measurements. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Duncan's multiple test.

Results

Effects of Cd and DEM on reduced GSH and AsA contents, and *HO-1* expression

Dose-dependent effects of Cd on reduced GSH and AsA contents, and *HO-1* expression in alfalfa seedling root tissues were confirmed firstly in Fig. 1. As expected, after 6 h treatment with $CdCl_2$ ranging from 1 to 500 μM, GSH content decreased in a dose-dependent fashion (Fig. 1a). However, in compared with the control sample, a depletion of reduced AsA was only observed in root tissues of plants treated with the higher Cd concentrations (100, 200, and 500 μM $CdCl_2$), whereas no significant changes occurred when 1 and 10 μM $CdCl_2$ were used. Meanwhile, changes of HO-1 expression exhibited the dose-dependent fashion, with a maximal inducible response at 200 μM $CdCl_2$. For example, *HO-1* transcripts increased by 5.2, 28.4, 50.9, and 57.8% at 1, 10, 100, and 200 μM $CdCl_2$ treatment, respectively, in comparison with the values of $CdCl_2$-free control treatment (Fig. 1b). The HO-1 protein level exhibited the similar tendency. We further noticed that respect to 200 μM $CdCl_2$ treatment, the addition of 500 μM $CdCl_2$ produced the weaker effects in the up-regulation of both *HO-1* transcript and its protein level. Thus, 200 μM $CdCl_2$ treatment was used for the following studies.

Reduced GSH content and HO-1 protein expression in alfalfa seedling root tissues at various intervals following $CdCl_2$ and DEM treatments were also compared in Fig. 2. In our test, the addition of 200 μM $CdCl_2$ and 1 mM DEM resulted in a rapid and prominent reduction (49.4 and 40.0% approximately) in root GSH level at 6 h of treatment, respectively (Fig. 2a, c). The GSH level was depressed maximally for 6 or 12 h posttreatment and thereafter rose to 88.6 and 85.4% (24 h posttreatment) of the values at 0 h, respectively, which was till, however, significantly lower than the values at 0 h. In contrast, western blotting data obtained through densitometric analysis revealed that HO-1 protein is induced by at 6 h posttreatment (95.5 and 92.8%, respectively; Fig. 2b, d) in a manner

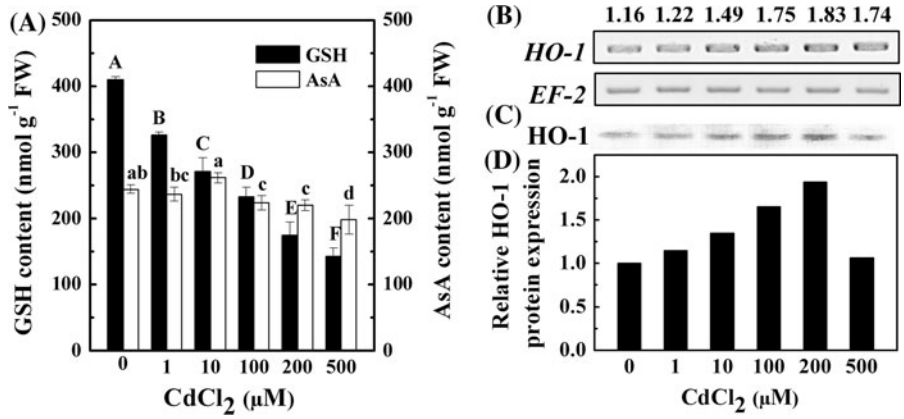

Fig. 1 Dose-dependent effects of Cd on GSH and AsA content (**a**), HO-1 transcripts (**b**) and its protein level (**c**, **d**) in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing indicated concentrations of $CdCl_2$ for another 6 h. Then, GSH and AsA contents were determined. The transcript levels of *HO-1* was also analysed by semi-quantitative RT-PCR (**b**). The *number above the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*. HO-1 protein expression was analysed by western blotting (**c**). Relative HO-1 protein expression taking Cd-free control sample as 1 U (**d**). Values are means ± SD of three different experiments with at least three replicated measurements. Within each set of experiments, *bars denoted by different letters* were significantly different at $P < 0.05$ according to Duncan's multiple test

reciprocal approximately to the effects observed on GSH content (Fig. 2a, c). Together, these results clearly suggest a possible interrelationship between GSH content and HO-1 protein expression in plants, which has been proven in animals previously. We also noticed that the abrupt decreases of GSH level driven by Cd or GSH depletion precede the up-regulation of HO-1 protein expression.

Cd-induced loss of plasma membrane integrity, lipid peroxidation, HO-1 expression is sensitive to GSH depletion and added GSH

To examine whether the depletion of GSH was solely responsible for loss of plasma membrane integrity, lipid peroxidation, and the induction of HO-1 expression, effects of inhibitor of GSH synthesis by BSO, a specific transition-state inactivator of γ-glutamyl cysteine synthetase, the rate-limiting enzyme in GSH biosynthesis, and exogenous GSH were examined. Figure 3 demonstrated that the addition of BSO before Cd stressed conditions for 6 h brought about obvious depletion of GSH in alfalfa seedling roots as well as the increases in the level of HO-1 mRNA, its protein expression and HO activity, all of which could be blocked significantly by the application of exogenous GSH. Similar reversing effects of GSH were observed in Cd stressed alone sample. HO-1 up-regulation driven by GSH depletion was consistent with the histochemical analysis of the loss of plasma membrane integrity after 12 h of treatment. For example, it exhibited the light staining in the root tips upon BSO + Cd treatment in comparison with Cd stressed alone sample, suggesting the antioxidative behavior of HO induction. However, upon Cd stress for 24 h, BSO pretreatment brought about extensive staining in the root tips, which was consistent with the changes of TBARS. Additionally, the expression profile of *HO-2*, another member of the HO gene family, showed the equivalent responses under different treatments approximately, further indicating that Cd-driven HO activation is HO-1-dependent. We also noticed that in GSH-treated samples, two bands with a molecular mass of about 30 kDa appeared apparently, both of which were recognized by the rabbit polyclonal antibody against the MsHO1 protein.

GSH depletion induces HO-1 expression

Similarly, effect of GSH depletion on alfalfa root *HO-1* gene expression was examined by semi-quantitative RT-PCR and western blotting (Fig. 4). BSO pretreatment alone, which acts as a selective inhibitor of GSH biosynthesis, brought about a slight but no significant induction of *HO-1* transcript, its protein

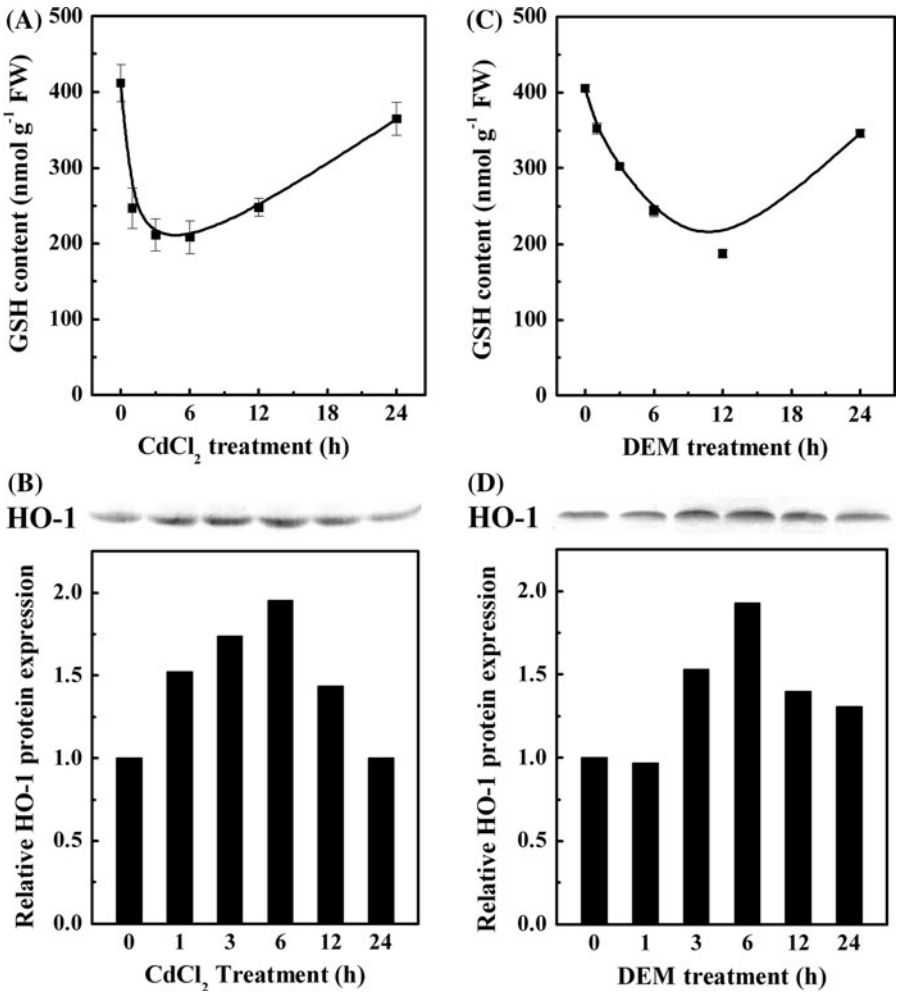

Fig. 2 Changes in endogenous GSH content (**a**, **c**) and HO-1 protein expression level (**b**, **d**) in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing 200 μM $CdCl_2$ (**a**, **b**) or 1 mM DEM (**c**, **d**) for another 24 h. GSH content was determined at the indicated treatment time. Meanwhile, HO-1 protein expression was also analysed by western blotting. Relative HO-1 protein expression taking treated sample at time zero as 1 U. Values are means ± SD of three different experiments with at least three replicated measurements

expression, and HO activity as well as the significant decreased GSH content, in comparison with those of control samples. Comparably, treatment of DEM, a GSH depletor, decreased GSH content obviously, also produced the significant responses in the induction of *HO-1* gene expression, which could be strengthened or blocked obviously by the simultaneous addition of BSO or GSH. However, a slight but no significant enhancement of endogenous GSH content or decrease of HO activity was observed when exogenous GSH was applied. No significant difference was observed by the application of GSH with or without BSO pretreatment. Consistent with the former results shown in Fig. 3c, identical *HO-2* transcript level was observed upon various treatments.

HO-1 induction driven by GSH depletion is regulated at transcriptional level

To determine whether transcripts or protein synthesis in the HO-1 gene expression, the transcriptional inhibitor actinomycin D (ActD) and the protein synthesis inhibitor cycloheximide (CX) was pretreated, separately. The results of Fig. 5 showed that both ActD and CX (especially) blocked the Cd- or DEM-mediated increases in HO-1 mRNA. These results indicated that HO-1 mRNA induction after GSH depletion seems to require the biosynthesis of some transcriptional factor, which is similar to the result reported previously in rats (Oguro et al. 1996).

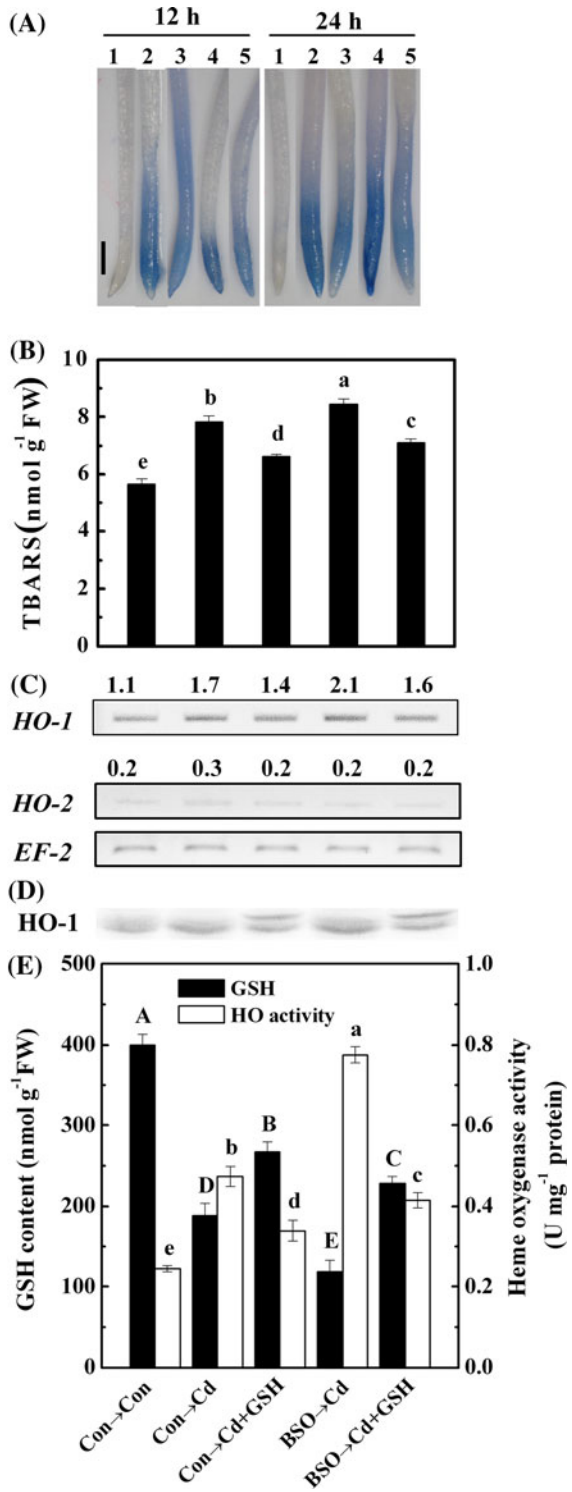

Fig. 3 Effects of BSO and GSH on loss of plasma membrane integrity, lipid peroxidation, *HO-1/2* transcript level, HO-1 protein expression, GSH content, and HO activity in Cd stressed alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing with or without 1 mM BSO for 3 h, and then exposed to 200 μM $CdCl_2$ in the presence or absence of 2 mM GSH for another 6, 12, or 24 h. Sample without chemicals was the control (Con). The roots were stained with Evans blue at the indicated time (**a**), and immediately photographed under a light microscope. *Bar*, 0.5 mm. *Lines 1–5* represent different treatments as follows: *1*, Con → Con; *2*, Con → Cd; *3*, Con → Cd + GSH; *4*, BSO → Cd; *5*, BSO → Cd + GSH. Meanwhile, TBARS content was also determined (**b**, 24 h). The transcript levels of *HO-1* and *HO-2* analysed by semi-quantitative RT-PCR (**c**, 6 h). The *number above the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*. HO-1 protein expression was analysed by western blotting (**d**, 6 h). Meanwhile, GSH content and HO activity (**e**) was also determined. Values are means ± SD of three different experiments with at least three replicated measurements. Within each set of experiments, *bars denoted by different letters* were significantly different at $P < 0.05$ according to Duncan's multiple test

Decrease in GSH content and induction of HO-1 by Cd and GSH depletion treatment are sensitive to the addition of AsA

Figure 6a illustrated that significant decreased GSH and AsA contents appeared in alfalfa seedling roots when Cd and DEM were present separately. By contrast, the combination treatment with AsA attenuated above responses except no significant changes of GSH content was observed in Cd-treated samples regardless of whether AsA was present together or not. Meanwhile, AsA exogenously applied alone brought about the obvious enhancement of GSH and AsA contents ($P < 0.05$). In parallel experiments, AsA differentially blocked the induction of HO-1 expression at both transcriptional and translational levels driven by Cd (ineffectively or slightly) or DEM (slightly or especially) compared with plants not treated with AsA. On the contrary, alfalfa plants being stimulated with AsA alone showed an even higher HO-1 protein expression compared to the similar stimulation in transcriptional level with one of the substances alone (Cd or DEM).

Discussion

The findings presented in this paper demonstrate the induction of *HO-1* gene expression following reduced GSH depletion driven by Cd exposure (Figs. 1, 2). Similar phenomenon has been discovered previously in different animal tissues (Ewing and Maines 1993;

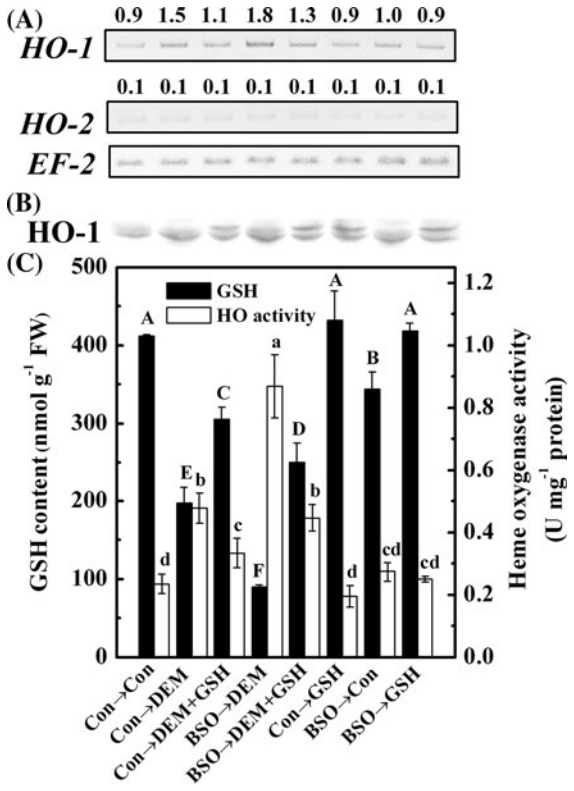

Fig. 4 Effects of DEM, BSO and GSH on *HO-1/2* transcript level, HO-1 protein expression, HO activity, and GSH content in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing with or without 1 mM BSO for 3 h, and then exposed to 1 mM DEM, 2 mM GSH alone, or the combination as indicated, for another 6 h. Sample without chemicals was the control (Con). The transcript levels of *HO-1* and *HO-2* analysed by semi-quantitative RT-PCR (**a**). The *number above the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*. HO-1 protein expression was analysed by western blotting (**b**). Meanwhile, GSH content and HO activity (**c**) was also determined. Values are means ± SD of three different experiments with at least three replicated measurements. Within each set of experiments, *bars denoted by different letters* were significantly different at $P < 0.05$ according to Duncan's multiple test

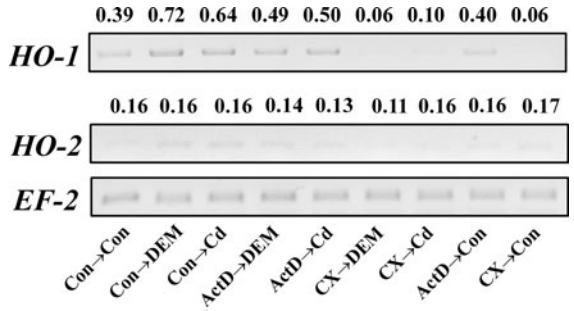

Fig. 5 Effects of ActD and CX on Cd- and DEM-induced HO-1 gene expression in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing with or without 5 μg/ml ActD or CX for 3 h, and then exposed to 1 mM DEM, 200 μM $CdCl_2$ alone, or the combination as indicated, for another 6 h. Sample without chemicals was the control (Con). The transcript levels of *HO-1* and *HO-2* analysed by semi-quantitative RT-PCR. The *number above the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*

Oguro et al. 1996; Horikawa et al. 2002). Our results also define GSH-depleting chemical DEM (Fig. 2) or BSO (Fig. 3) promoting similar responses. Thus, the findings demonstrated that GSH depletion, either by Cd stress or by usage of GSH depletor, or the specific inhibitor of GSH synthesis, causes an increase in transcript and protein level for the inducible form of HO in animals and plants, HO-1.

In animals, ample evidence illustrated that HO-1 is highly induced by a variety of agents or stimuli causing oxidative stress, such as H_2O_2, glutathione depletors, ultraviolet irradiation and hyperoxia (Maines 1997). HO-1 knockout mice exhibit reduced stress defences when exposed to oxidative challenge (Poss and Tonegawa 1997). Similarly, metal irons are other powerful HO-1-inducing agents and Cd was the first metal ion identified in plants with an inducing property reported initially by Tomaro's research group (Noriega et al. 2004; Balestrasse et al. 2005, 2008; Shekhawat and Verma 2010). It was well established that all above factors induced HO-1 expression in animals by a general mechanism: enhancement of HO activity by de novo enzyme synthesis associated with an increase in HO-1 mRNA level. In agreement with these results, we discovered that both Cd and GSH depletor or the specific inhibitor of GSH synthesis could result in the induction of *HO-1* transcript, corresponding HO-1 protein, and HO activity as well as the decreased GSH content in alfalfa seedling roots, all of which could be blocked by GSH exogenously applied (Figs. 3, 4). The application of the transcriptional and protein synthesis inhibitor suggested that HO-1 induction driven by GSH depletion is regulated at transcriptional level (Fig. 5), which also requires the involvement of some transcriptional factors (Oguro et al. 1996). Furthermore, we assumed that HO-1 induction would be beneficial by providing an improved antioxidant defence which was confirmed

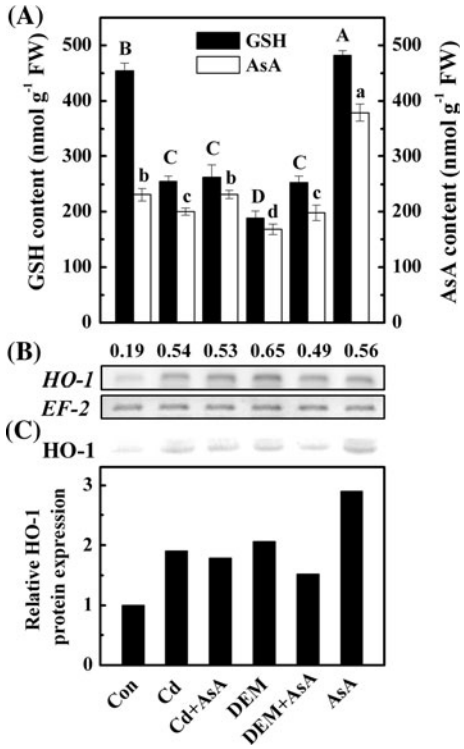

Fig. 6 Changes in endogenous GSH and AsA contents (**a**), HO-1 transcripts (**b**), and its protein level (**c**) in alfalfa seedling roots. Seedlings were incubated in quarter-strength Hoagland's solution for 5 days then transferred to the same solution containing 200 μM $CdCl_2$, 1 mM DEM, 1 mM AsA alone, or the combination as indicated, for another 6 h. Sample without chemicals was the control (Con). Then, GSH and AsA contents were determined (**a**). The transcript levels of *HO-1* was also analysed by semi-quantitative RT-PCR (**b**). The *number above the band* indicates relative abundance of corresponding gene with respect to the loading control *EF-2*. HO-1 protein expression was analysed by western blotting (**c**). Relative HO-1 protein expression taking control sample (Con) as 1 U (**c**). Values are means ± SD of three different experiments with at least three replicated measurements. Within each set of experiments, *bars denoted by different letters* were significantly different at $P < 0.05$ according to Duncan's multiple test

in plants (Han et al. 2008; Yannarelli et al. 2006; Noriega et al. 2007; Santa-Cruz et al. 2010) and therefore might protect plants against Cd-induced oxidative damage.

GSH functions as a heavy metal–ligand and an antioxidant. For example, the GSH level was inversely linked to Cd sensitivity when comparing ten pea genotypes showing differing Cd sensitivity (Metwally et al. 2005). Upon Cd and copper (Cu) exposure, normally, GSH concentration drops as consequence of initiated phytochelatin (PC) biosynthesis. This causes oxidative stress and in turn short-term toxicity (De Vos et al. 1992; Schützendübel et al. 2001). For example, cell imaging by using fluorescent probes of Cd- and mercury (Hg)-treated alfalfa confirmed the rapid accumulation of peroxides and depletion of GSH and homoglutathione (hGSH) causing redox imbalance (Ortega-Villasante et al. 2005). Further, if the ability of high rate PC synthesis is insufficient to cope with the metal load due to the hampered antioxidant system, the up-regulation of HO-1 mediated by the depletion of GSH could act as the second antioxidant barrier against oxidative damage, because BV is an efficient scavenger of ROS and its increase due to HO induction was a response to the oxidative stress generated by Cd in soybean leaves (Noriega et al. 2004). More recently, our work also illustrated that bilirubin (BR), the other end-products of HO activity, could perform similar cytoprotective effect (Wu et al. 2010). Furthermore, the performances of histochemical staining of loss of plasma membrane integrity (12 h, Fig. 3a) support this deduction. Similar deduction has been proposed or confirmed in animal tissues (Ewing and Maines 1993; Oguro et al. 1996; Horikawa et al. 2002). An additional role for the HO induction in Cd-stressed or GSH-depletion conditions can be envisioned relating to the production of CO, which was able to enhance GSH synthesis later (Fig. 2) (Han et al. 2008). Similarly, using the HO-1 knockout mice and inhibitor test, Matsumoto et al. (2006) have confirmed that CO and BV produced from HO-1 suppresses ROS production generated from NADPH oxidase. However, if enhanced GSH content can not compensate the loss of GSH previously and satisfy the requirement for Cd tolerance, the performance of Cd toxicity on loss of plasma membrane integrity and lipid peroxidation were strengthened later (24 h, Fig. 3a, b).

Additionally, the possibility of Cd-induced AsA depletion contributing to the up-regulation of HO-1 could not be easily ruled out. It was well established that GSH and AsA are the main antioxidant compounds in plants under normal growth conditions and heavy metal exposure (Foyer and Noctor 2009; Sharma and Dietz 2009). Subsequently, in view of the fact that and the GSH and AsA depletion, as indicators of oxidative stress, as well as the induction of HO-1 expression appeared in alfalfa seedling roots subjected to 200 μM Cd exposure (Fig. 1a), we

further testify if the change of AsA exhibits the similar behavior as GSH depletion in the induction of HO-1. Results of Fig. 6 showed that the addition of AsA was able to differentially attenuate both elevation of HO-1 expression and levels of reduced GSH content induced by Cd and GSH depletion treatment, separately, suggesting that AsA affords more protection against the oxidative insult elicited by Cd, thus resulting in diminished endogenous antioxidant response. These findings are consistent with those reported by Yannarelli et al. (2006), in which they found that AsA pretreatment prevented the UV-B-induced up-regulation of HO-1 mRNA in soybean plants. Similar effects of AsA on HO-1 gene expression were also confirmed in animals subjected to oxidized low density lipoprotein (LDL), Cd, chromium (Cr) treatments (Harris et al. 2006; Elbekai et al. 2007). For example, pretreatment of smooth muscle cells (SMC) with the antioxidant vitamin C attenuated the induction of HO-1 by moderately oxidized LDL (moxLDL) or highly oxidized LDL (oxLDL) compared with cells not pretreated with vitamin C (Anwar et al. 2005). In fact, AsA can enhance the regeneration of GSSG and spare intracellular reduced GSH by being preferentially oxidized such as Cd exposure, or by GSH depletion treatment (Fig. 6). Therefore, it seems likely that the AsA depletion caused by Cd exposure could result in the induction of HO-1 expression via GSH depletion. By contrast, the significant induction of HO-1 expression was observed when AsA was applied alone. A previous report also showed that HO-1 mRNA was significantly elevated by AsA alone in gastric epithelial cells (Becker et al. 2003). Thus, we deduced that the specific induction of HO-1 by 'non-stressful' stimuli, such as AsA, was not associated with oxidative stress (Immenschuh and Ramadori 2000).

As stated above, it was demonstrated that in alfalfa seedling roots, HO-1 expression can be enhanced by GSH depletion at least at transcriptional level, which mimicked the responses in Cd stressed conditions. Meanwhile, Cd-induced up-regulation of HO-1 gene expression is associated with GSH depletion. Therefore, the present study illustrated the involvement of HO-1 in the antioxidant defense system in GSH-depletion condition transiently, which has been shown to occur in animal kingdoms and plants recently (Shekhawat and Verma 2010).

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