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RESEARCH PAPER

Haem oxygenase-1 is involved in salicylic acid-induced alleviation of oxidative stress due to cadmium stress in *Medicago sativa*

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Abstract

This work examines the involvement of haem oxygenase-1 (HO-1) in salicylic acid (SA)-induced alleviation of oxidative stress as a result of cadmium (Cd) stress in alfalfa (*Medicago sativa* L.) seedling roots. $CdCl_2$ exposure caused severe growth inhibition and Cd accumulation, which were potentiated by pre-treatment with zinc protoporphyrin (ZnPPIX), a potent HO-1 inhibitor. Pre-treatment of plants with the HO-1 inducer haemin or SA, both of which could induce *MsHO1* gene expression, significantly reduced the inhibition of growth and Cd accumulation. The alleviation effects were also evidenced by a decreased content of thiobarbituric acid-reactive substances (TBARS). The antioxidant behaviour was confirmed by histochemical staining for the detection of lipid peroxidation and the loss of plasma membrane integrity. Furthermore, haemin and SA pre-treatment modulated the activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), and guaiacol peroxidase (POD), or their corresponding transcripts. Significant enhancement of the ratios of reduced/oxidized homoglutathione (hGSH), ascorbic acid (ASA)/dehydroascorbate (DHA), and NAD(P)H/$NAD(P)^+$, and expression of their metabolism genes was observed, consistent with a decreased reactive oxygen species (ROS) distribution in the root tips. These effects are specific for HO-1, since ZnPPIX blocked the above actions, and the aggravated effects triggered by SA plus ZnPPIX were differentially reversed when carbon monoxide (CO) or bilirubin (BR), two catalytic by-products of HO-1, was added. Together, the results suggest that HO-1 is involved in the SA-induced alleviation of Cd-triggered oxidative stress by re-establishing redox homeostasis.

Key words: Cadmium, haem oxygenase-1, *Medicago sativa*, oxidative stress, redox state homeostasis, salicylic acid

Introduction

A plant's survival often depends on its ability to acclimate rapidly to various abiotic and biotic environmental stresses by adjusting its cellular homeostasis and balancing multiple pathways in different cellular compartments. It has been well established that the maintenance of homeostatic redox levels in cells is essential for plant adaptive responses to biotic and abiotic stresses, and that failure to establish redox homeostasis usually leads to the phenomenon known as oxidative stress (Dutilleul *et al.*, 2003; Noctor *et al.*, 2007). Salicylic acid (SA) is an endogenous regulator or signal of various physiological processes such as thermogenesis and defence against harmful microorganisms in plants (Raskin, 1992; Chen *et al.*, 1993; Delaney *et al.*, 1994; Durner *et al.*, 1997; Shen *et al.*, 1999; Xie and Chen, 1999). It is also involved in the alleviation of oxidative stress caused by ageing as well as by biotic and abiotic stresses (Yang *et al.*, 2004). Increased SA levels in response to heavy metal stress (Metwally *et al.*, 2003) demonstrate a link between the degree of plant tolerance to heavy metals mediated by the SA signal and redox homeostasis (Sharma and Dietz, 2009). Meanwhile, signalling substances, such as calcium (Ca^{2+}), nitric oxide (NO), hydrogen

peroxide (H_2O_2), and their interactions, have been identified as being potentially involved in the cellular responses of plants to heavy metal toxicity (Rodríguez-Serrano *et al.*, 2009). However, the molecular events involved in SA signalling responsible for the alleviation of heavy metal-induced oxidative stress are still poorly understood (Metwally *et al.*, 2003).

Haem oxygenase (HO; EC 1.14.99.3), the rate-limiting enzyme in the breakdown of haem into carbon monoxide (CO), iron, and biliverdin (BV), has attracted much recent research interest (Ryter *et al.*, 2002; Otterbein *et al.*, 2003; Cao *et al.*, 2011). To date, three HO isozymes have been identified in animals. One of these, HO-1, is a stress response protein induced by various oxidative agents, while the HO-2 and HO-3 genes are constitutively expressed (Ryter *et al.*, 2002). In *Arabidopsis thaliana*, a family of four genes (*HY1*, *HO2*, *HO3*, and *HO4*) encodes HO, and they play a major role in phytochrome chromophore biosynthesis (Muramoto *et al.*, 2002; Shekhawat and Verma, 2010). Furthermore, the antioxidant behaviour of the HO-1/CO system has been demonstrated in *Arabidopsis* (Xie *et al*., 2011, 2012), soybean (Noriega *et al*., 2004; Yannarelli *et al*., 2006), alfalfa (Han *et al*., 2008), and wheat (Huang *et al*., 2006; Xie *et al*., 2008; Wu *et al.*, 2011), and these responses might exhibit interactions with reactive oxygen species (ROS) metabolism or signalling.

Cadmium (Cd) is a heavy metal with a long biological half-life, and is present as a pollutant in agricultural soils due mainly to anthropogenic activities. Normally, Cd induces genetic and biochemical changes in plant metabolism that are related to general and Cd-specific stress responses (Ortega-Villasante *et al.*, 2005; Krantev *et al.*, 2008; Sharma and Dietz, 2009; Brunetti *et al*., 2011). For example, Cd causes oxidative stress in plants resulting in lipid peroxidation of the plasma membrane in plant tissues (Ortega-Villasante *et al.*, 2007). Interestingly, the more Cd-sensitive pea genotypes showed decreased concentrations of glutathione (GSH; γ-glutamyl-cysteinyl-glycine) in their roots, whereas the more tolerant genotypes had increased GSH levels (Metwally *et al.*, 2005). However, homoglutathione (hGSH; γ-glutamyl-cysteinyl-β-alanine) is more abundant than GSH in soybean and alfalfa plants (Matamoros *et al.*, 1999; Baldacci-Cresp *et al.*, 2012). The hGSH is synthesized by homoglutathione synthetase (hGS) and catalyses the conjugation of β-alanine to γ-glutamylcysteine; the latter is synthesized from L-glutamate and L-cysteine by γ-glutamylcysteine synthetase (ECS), the first enzyme in glutathione synthesis. The second enzyme involved in glutathione synthesis is glutathione synthetase (GS, also named GSHS). It was further known that the balance between GSH and oxidized GSH (GSSG) and/or their homologues reduced/oxidized homoglutathione (hGSH/hGSSGh), as well as reduced ascorbic acid (ASA) and its oxidized forms [monodehydroascorbate and dehydroascorbate (DHA)], is crucial for the efficiency of plant antioxidant systems (Noctor *et al.*, 2002). Recently, it has also been found that exposure to Cd at low doses induces HO-1 production which plays a cytoprotective role both *in vitro*, and especially *in vivo* (Noriega *et al*., 2004). The CO released by HO-1 catalysis might act as a signal element for the alleviation of Cd-induced oxidative stress by modulating GSH homeostasis (Han *et al*., 2008).

In animals, previous studies have shown that aspirin, the acetylated derivative of SA, targets HO-1, presumably via the NO-dependent pathway. Induction of HO-1 expression and activity may be a novel mechanism by which aspirin prevents cellular injury under inflammatory conditions and in cardiovascular disease (Grosser *et al.*, 2003). However, little information is known about the specific role of HO-1 in SA-induced antioxidant behaviour in plants. To investigate the hypothesis that a tight link between HO-1-mediated and SA-dependent signalling exists in the alleviation of Cd toxicity, SA-mediated HO-1 up-regulation is first investigated and then its relationship to SA-induced antioxidative behaviour in the root tissues of alfalfa plants is elucidated. Plants were pre-treated with SA, the HO-1 inducer haemin, and a potent HO-1 inhibitor, zinc protoporphyrin (ZnPPIX), alone or in various combinations, and then exposed to Cd. Various redox homeostasis parameters were determined, such as: antioxidative enzyme expression and activities; lipid peroxidation; ROS distribution; and the hGSH/hGSSGh, ASA/DHA, and NAD(P)H/NAD(P)$^+$ ratios, etc. The possible mechanisms driving these parameters, and their significance, are discussed.

Materials and methods

Chemicals

SA was purchased from Shanghai Medical Instrument, Co., Ltd., China National Medicine (Group), Shanghai, China. Haemin, purchased from Fluka, was used as an HO-1 inducer. ZnPPIX, a potent inhibitor of HO-1 (Noriega *et al.*, 2004; Lang *et al.*, 2005; Wu *et al.*, 2011), was obtained from Sigma. Cysteine, γ-glutamylcysteine (γ-EC, the precursors of GSH), and GSH were purchased from Sigma-Aldrich. hGSH was obtained from Shanghai RD BIOSCIENCES, Co., Ltd. The preparation of CO aqueous solution was carried out according to the method described in a previous report (Han *et al.*, 2008).

Plant materials, growth conditions, and treatments

Commercially available alfalfa (*Medicago sativa* L. cv Zhongmu No.1) seeds were surface-sterilized with 5% NaClO for 10 min, and rinsed extensively in distilled water before being germinated for 1 d at 25 °C in darkness. Uniform seedlings were then selected and transferred to plastic chambers and cultured in nutrient medium (quarter-strength Hoagland's solution). Alfalfa seedlings were grown in an 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 d, the seedlings were then incubated in quarter-strength Hoagland's solution with or without 10 μM or the indicated concentrations of SA, 100 μM ZnPPIX, 20 μM haemin, 20 μM Fe (II) citrate (Fe), 100 μM $ZnSO_4$ alone, or the combination treatments for 12 h, and/or exposed to 0 or 50 μM $CdCl_2$, 50% saturated CO aqueous solution, 20 μM bilirubin (BR), 20 μM Fe alone, or the indicated combination treatments for another 24 h or the indicated times. Sample without chemical treatments was used as the control. The pH for both nutrient medium and treatment solutions was adjusted to 6.0 by using NaOH or HCl. After various treatments, the seedlings were sampled, then used immediately or frozen in liquid nitrogen, and stored at –80 °C for further analysis.

Determination of thiobarbituric acid-reactive substances (TBARS), and ASA, DHA, and chlorophyll contents

Lipid peroxidation was estimated by measuring the amount of TBARS as previously described (Han *et al*., 2008). The contents of ASA and DHA, and chlorophyll *a*/*b* were measured according to previous methods (Law *et al*., 1983; Xie *et al*., 2012).

Thiol analysis by HPLC

Low molecular weight thiols and their corresponding disulphide contents were measured according to the methods previously reported, with minor modification (Herschbach *et al*., 2002; Meyer *et al*., 2007; Queval and Noctor, 2007). Frozen root tissues were ground to a fine powder under liquid nitrogen and then extracted into 1 ml of 0.2 M HCl. The combined extracts were centrifuged at 13 000 *g* for 15 min at 4 °C. For determination of thiols plus disulphides (cysteine+cysteine disulphide, γ-EC+γ-EC disulphide, GSH+GSSG, and hGSH+hGSSGh), following neutralization, a 0.2 ml aliquot of the extract supernatant was mixed with 100 µl of 500 mM 2-(*N*-cyclohexylamino)ethane-sulphonic acid (CHES) buffer (pH 8.5), then 20 µl of 10 mM dithiothreitol (DTT) was added and incubated for 30 min, followed by the addition of 20 µl of 30 mM monobromobimane (mBBr) to derivatize thiols. For determination of disulphides (cysteine disulphide, γ-EC disulphide, GSSG, and hGSSGh), following neutralization, 0.2 ml extracts were blocked with *N*-ethylmaleimide (NEM). After removal of NEM, 100 µl of 500 mM CHES was added, followed by reduction of disulphides to thiols with DTT and labelling of thiol groups with mBBr as above. After 15 min under dim light, conjugation of thiols with mBBr was completed and 660 µl of 10% (v/v) acetic acid was added to stabilize the mBBr derivatives. After centrifugation at 10 000 *g* for 10 min, the supernatant was filtered through a 0.22 µm filter, and 50 µl of the mixture was subjected to HPLC analysis (Agilent Technologies, 1200 series Quaternary, Foster City, CA, USA). Thiol derivatives were separated on an Agilent Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm) at a flow rate of 0.8 ml min^{-1}. The linear gradient was from 0% solution B to 10% (v/v) solution B (90% methanol, 0.25% acetic acid, pH 4.3) within 25 min. This composition was maintained for 2 min; thereafter the column was washed with 100% solution B for 10 min and re-equilibrated with 100% solution A (10% methanol, 0.25% acetic acid, pH 4.3) for 5 min. Thiol derivatives were quantified by fluorescence detection (excitation at 380 nm, emission at 480 nm). A standard solution of 0.1 mM cysteine, γ-EC, GSH, and hGSH was used for quantification. Corresponding retention times were 10, 12, 15, and 22 min, respectively.

Pyridine nucleotide analyses

NAD and NADP pool sizes and reduction state were measured in acid and alkaline extracts using the protocol described in Wang and Pichersky (2007). The assays involve the phenazine methosulphate-catalysed reduction of thiazolyl blue tetrazolium bromide (MTT) in the presence of ethanol and alcohol dehydrogenase (for NAD and NADH) or glucose-6-phosphate and glucose-6-phosphate dehydrogenase (for NADP and NADPH). Reduced and oxidized forms are distinguished by preferential destruction in acid or base.

Histochemical analyses

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 as described by Yamamoto *et al.* (2001).

Determination of Cd content in plant tissues

Cd in root tissues was extracted and measured by graphite furnace atomic absorption spectrophotometry (180-80 Hitachi, Tokyo, Japan) as described by Brune and Dietz (1995).

Enzymatic activities assays

HO activity was analysed using the method described in our previous report (Xuan *et al.*, 2008). Frozen alfalfa seedling roots (~200 mg) were homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) for superoxide dismutase (SOD) and guaiacol peroxidase (POD) assay, or their combination, with the addition of 1 mM ASC in the case of ascorbate peroxidase (APX) assay. SOD and guaiacol POD activities were analysed by the methods described in previous reports (Huang *et al.*, 2006; Liu *et al.*, 2007). APX activity was measured as described by Nakano and Asada (1981). Protein was determined by the method of Bradford (1976).

Native gradient PAGE, and SOD and POD activity staining

Native gradient PAGE (5–20%) was performed for 15 h at 4 °C at a constant voltage of 150 V in Tris-glycine buffer, pH 8.3. SOD and POD isozymatic activities on the gel were visualized (Beauchamp and Fridovich, 1971; Janda *et al.*, 1999). For the determination of the relative activity of different isozymes, gels were scanned in the transmission black-and-white mode and the intensity of bands was calculated by using the Quantity One v4.4.0 software (Bio-Rad, Hercules, CA, USA). Then the band intensities of the individual isozymes were expressed as a percentage of the control (C) value.

Western blot analysis for MsHO1

Rabbit polyclonal antibody was prepared against the mature MsHO1 (Fu *et al.*, 2011). A 50 µg aliquot of protein from homogenates was subjected to SDS–PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad). Finally, the developed films were scanned (Uniscan B700^{+}, Tsinghua Unigroup Ltd, Beijing, China) and analysed by using Quantity One v4.4.0 software (Bio-Rad, USA).

Real-time RT-PCR analysis

Total RNA of root tissues was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Real-time quantitative reverse transcription-PCRs (RT-PCRs) were performed using a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® Pre-mix Ex Taq™ (TaKaRa Bio Inc., China) according to the manufacturer's instructions (Xie *et al.*, 2012). Using specific primers (Supplementary Table S1 available at *JXB* online), the expression levels of the genes are presented as values relative to the corresponding control samples under the indicated conditions, with normalization of data to the geometric average of two internal control genes *MSC27* and *Actin2* (Vandesompele *et al.*, 2002).

Confocal analysis of ROS production

Production of ROS was assayed by confocal microscopy with 20 µM 2',7'-dichlorofluorescin diacetate (H_2DCFDA; Calbiochem, La Jolla, CA, USA). Alfalfa seedling roots were loaded with H_2DCFDA for 30 min before being washed in 20 mM HEPES buffer (pH 7.8) three times for 15 min (Mazel *et al*., 2004; Leshem *et al*., 2007). All images were obtained by a TCS-SP2 confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany; with excitation at 488 nm, and emission at 500–530 nm). All manipulations were performed at 25 ± 1 ºC. Production of ROS in root tips was quantified with the Leica software package.

Statistical analysis

Values are shown as the means ±SE of three independent experiments with at least three replicates for each. Differences among treatments were analysed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test, taking $P < 0.05$ as the thresholds.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HM212768, DQ122791, AY054988, AM407888, AM411123, AM411122, AM407889, AM407890, JN979555, X63872, and JQ028730.

Results

Lipid peroxidation

Figure 1A shows that the content of TBARS decreased by 9.0% and 18.1% in seedlings pre-treated with 1 µM and 10 µM SA,

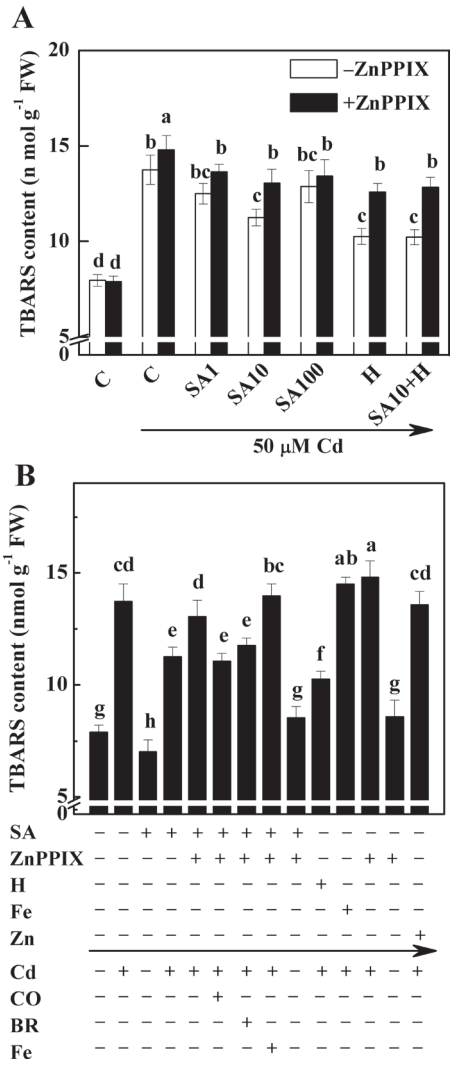

Fig. 1. Effects of salicylic acid (SA), ZnPPIX, haemin, Fe (II) citrate, and $ZnSO_4$ pre-treatment on the concentration of thiobarbituric acid-reactive substance (TBARS) in alfalfa seedling roots subjected to 50 μM $CdCl_2$. Five-day-old seedlings were pre-treated or not with 1, 10, or 100 μM SA (SA1, SA10, and SA100; A), 10 μM SA (SA; B), 20 μM haemin (H), 20 μM Fe (II) citrate (Fe), 100 μM $ZnSO_4$ (Zn) alone, or the combination of treatments, in the presence (+) or absence (–) of 100 μM ZnPPIX for 12 h, and then exposed to 50 μM $CdCl_2$ with or without 50% saturated aqueous CO solution (CO), 20 μM bilirubin (BR), or 20 μM Fe (II) citrate (Fe) for another 24 h. The sample without chemicals was the control (C). Values are means ±SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

respectively, before exposure to 50 μM $CdCl_2$, compared with samples treated with $CdCl_2$ alone. However, treatments with SA concentrations up to 100 μM resulted in a slight decrease but no significant effect on lipid peroxidation. Therefore, 10 μM SA was used in the following experiment.

Previous results suggested that ZnPPIX, a potent inhibitor of HO-1, might be another substrate for methylation by *S*-adenosyl-L-methionine:magnesium protoporphyrin IX methyltransferase (MgPMT) in the synthesis of chlorophyll (Gibson *et al.*, 1963). However, the pilot experiment showed that at least under the research conditions used here, pre-treatment with 100 μM ZnPPIX failed to influence the chlorophyll content in alfalfa seedlings significantly regardless of whether Cd was added or not (Supplementary Fig. S1 at *JXB* online). Similarly (Noriega *et al.*, 2004), further results in this study confirmed that ZnPPIX is a potent inhibitor of MsHO1 (Supplementary Fig. S2). To investigate the physiological role of HO-1 in plant responses to Cd exposure, the changes in content of TBARS in seedlings simultaneously pre-treated with SA and ZnPPIX were investigated (Fig. 1A). As expected, treatment with ZnPPIX significantly increased the content of TBARS (9.2, 16.0, and 4.2%) compared with the values produced by treatment with SA at concentrations of 1, 10, and 100 μM. Meanwhile, seedlings pre-treated with haemin, an HO-1 inducer (Xuan *et al.*, 2008), also showed progressive reductions in the content of TBARS (data not shown), with the maximal response at 20 μM haemin. This response was reversed by the addition of ZnPPIX (22.7%; Fig. 1A). Interestingly, these values, conferred by SA or haemin plus ZnPPIX, were equivalent to the control sample followed by Cd treatment alone (without ZnPPIX pre-treatment), indicating the possible protective role of HO-1. However, no significant additive effects of haemin (20 μM) plus SA (10μM) were observed in the presence or absence of ZnPPIX.

Histochemical staining

Histochemical staining showed that, compared with the Cd-free control sample, root tips of alfalfa plants treated with Cd alone stained extensively with Schiff's reagent and Evans blue (Supplementary Fig. S3 at *JXB* online), and stained more strongly following pre-treatment with ZnPPIX. In contrast, root tips pre-treated with SA or haemin regardless of whether they were treated with Cd later (in particular) or not showed only light staining, which was differentially reversed when ZnPPIX was added together with SA or haemin. Combined with the results on the contents of TBARS (Fig. 1), these results further support the hypothesis that the SA- and haemin-induced cytoprotective effects were HO-1 specific.

SA-induced cytoprotective response is HO-1-specific

If HO-1 was really involved in the SA-mediated cytoprotective response, feeding plants with exogenous CO or BR (two catalytic by-products of HO-1) might, at least partially, block the increase of TBARS caused by SA plus ZnPPIX followed by the addition of Cd. As expected, the increase in the content of TBARS was considerably reduced in an aqueous 50% saturated solution of CO or BR (Fig. 1B). Under similar conditions, however, there was an increase in the content of TBARS when Fe (as a control for haemin decomposition) was added. It was also found that adding Fe or $ZnSO_4$ (as a control for ZnPPIX decomposition) followed by Cd stress could not alleviate the TBARS overproduction caused by Cd stress alone.

Similarly, when CO was applied together with Cd, the heavy staining of lipid peroxidation and the loss of plasma membrane

integrity in the roots of alfalfa seedlings pre-treated with SA plus ZnPPIX were relieved (Supplementary Fig. S3 at *JXB* online). Simultaneously added BR produced a modest alleviation response, in comparison with the addition of Fe. The addition of CO, BR, or Fe alone, however, did not change the staining pattern with respect to the chemical-free control samples. The above results further confirmed that the effect of SA on the alleviation of Cd-induced oxidative stress is mediated specifically by HO-1.

Cd toxicity

In the following experiment, it was discovered that plants treated with Cd at an external concentration of 50 μM exhibited a time-dependent tendency to a decrease in the fresh weight of 100 seedling roots compared with Cd-free control samples (Fig. 2A). The improved root growth with SA or haemin pre-treatment was also shown to be time dependent. However, it was noticed that during the whole treatment period, the reduction effects on root growth of the combination of SA and ZnPPIX were stronger than those of the SA treatment alone. Meanwhile, an obvious decrease in seedling root growth appeared in the sample pre-treated with ZnPPIX followed by Cd exposure, as compared with the Cd-stressed alone sample. When a 50% saturated aqueous solution of CO (in particular) or BR (modestly) was separately applied together with Cd, the inhibition of seedling root growth caused by the combination of SA and ZnPPIX pre-treatment was differentially alleviated. However, no alleviation role of Fe was observed. It was also noticed that the application of CO (in particular) or BR alone brought about the stimulation of seedling growth.

The Cd content in plant seedling roots was simultaneously investigated (Fig. 2B). The uptake of Cd in the SA- and haemin-pre-treated roots exhibited a significant tendency to decrease in comparison with samples subjected to a Cd stress alone. In contrast, the combination of the HO-1 potent inhibitor ZnPPIX blocked the above responses. It was also noticed that the addition of CO (in particular) or BR together with Cd could obviously reverse the above ZnPPIX response, although no significant difference was observed in an Fe-treated sample. Interestingly, a significant increase of Cd accumulation was observed in the sample pre-treated with ZnPPIX followed by the addition of Cd, in comparison with the sample subjected to Cd stress alone. Taken together, the above results strongly indicated the involvement of HO-1 in the SA-mediated modulation of Cd toxicity to root growth.

SA- or haemin-induced MsHO1 expression is sensitive to ZnPPIX

To confirm further whether alfalfa HO-1 (*MsHO1*; Fu *et al.*, 2011) is associated with the above SA responses, a detailed study of the SA-induced expression of this enzyme was undertaken. Further results revealed that when treated with 1, 10, and 100 μM SA, induction of the *MsHO1* gene and its corresponding protein levels peaked at 10 μM SA (Fig. 3A–C). Enzyme activity analysis (Fig. 3D) revealed a similar tendency. Furthermore, the induction of *MsHO1* expression, protein level, and HO activity by 10 μM SA or 20 μM haemin was clearly inhibited by ZnPPIX. Additionally, an obvious decrease in HO activity was observed when ZnPPIX was added alone.

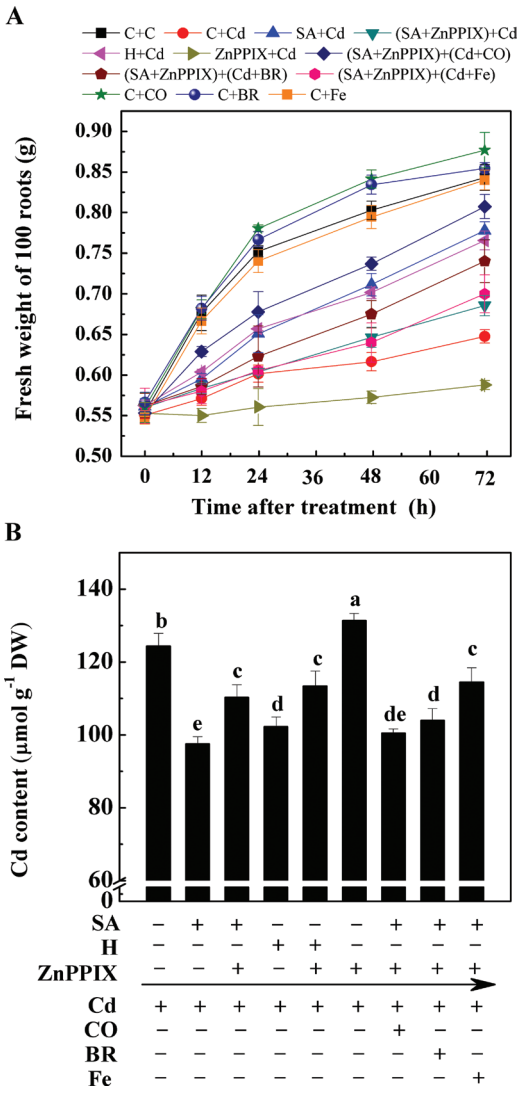

Fig. 2. Effects of salicylic acid (SA), ZnPPIX, and haemin (H) pre-treatment on the $CdCl_2$-induced inhibition of 100 seedling roots (A) and Cd contents (B) in root tissues of alfalfa plants. Five-day-old seedlings were pre-treated or not with 10 μM SA, 20 μM haemin, 100 μM ZnPPIX alone, or the combination treatments for 12 h, and then exposed to 50 μM $CdCl_2$, 20 μM Fe (II) citrate (Fe), 50% saturated aqueous CO solution (CO), 20 μM bilirubin (BR) alone, or the combination treatments for another 72 h. The sample without chemicals was the control (C). Values are means ±SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Antioxidant enzyme activities and their transcripts

In order to determine whether there is a link between the HO-1 and SA-induced alleviation of oxidative stress, the changes in several antioxidant enzyme activities and corresponding transcripts in alfalfa seedling roots were investigated. Using real-time RT-PCR, it was shown that pre-treatment with 100 μM ZnPPIX differentially blocked the SA-induced enhancement of the transcription levels of the *APX1/2* genes, and the total activity of APX in Cd-stressed plants (Fig. 4A, 4E). A similar inhibition

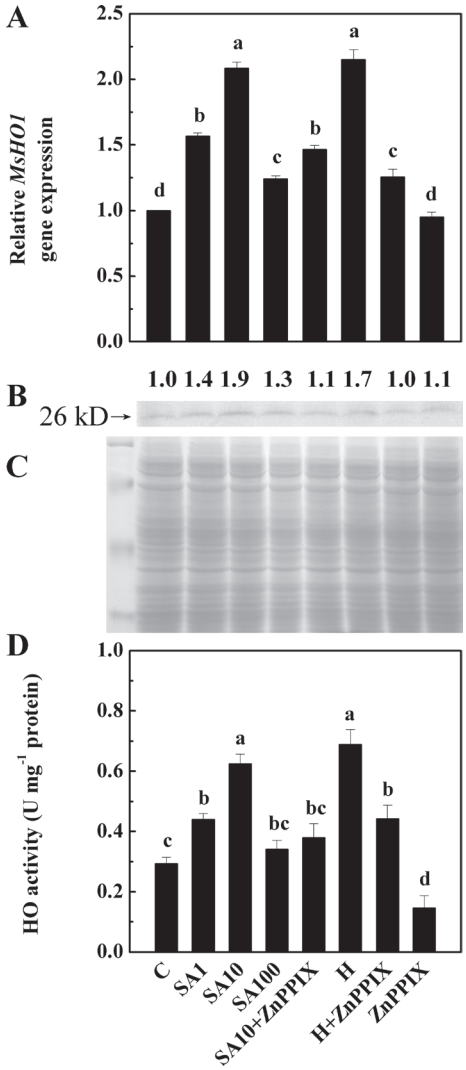

Fig. 3. Comparisons of *MsHO1* transcripts (A), HO-1 protein levels (B and C), and HO activity (D) in alfalfa seedling roots. Five-day-old seedlings were treated or not with 1, 10, and 100 μM SA (SA1, SA10, and SA100), 20 μM haemin (H), 100 μM ZnPPIX alone, or the combination treatments for 12 h. The sample without chemicals was the control (C). The transcript quantification test was carried out after 12 h of treatment, normalized against expression of two internal reference genes in each sample (A). HO-1 protein expression was analysed by western blotting (B). The number above the band indicates the relative abundance of the corresponding MsHO1 protein compared with that of the control sample. Coomassie Brilliant Blue-stained gels were present to show that equal amounts of proteins were loaded (C). HO activity was also determined (D). Three independent experiments were performed, and the results showed similar trends.

was observed in samples pre-treated with ZnPPIX, in comparison with samples subjected to Cd stress alone. Similarly, compared with the samples treated with Cd alone, haemin caused increases in the transcription levels (Fig. 4A) and the total activity of APX (Fig. 4E), and these increases were substantially reduced by pre-treatment with ZnPPIX. These results clearly indicated that HO-1 itself could induce the up-regulation of APX in plants.

Analysis of another two antioxidant enzymes revealed that after 24 h of exposure to Cd, the activity levels of SOD and POD in alfalfa seedling roots fell to 25.5% and 24.5% lower than the control sample, respectively (Fig. 4B, 4F). In contrast, SA and haemin pre-treatment resulted in significantly increased SOD and POD activities, being 15.4% and 13.1% (SOD), 18.1% and 11.9% (POD) higher, respectively, than the sample treated with Cd alone. Furthermore, when the HO-1 inhibitor ZnPPIX was also added, the above observed effects were significantly reversed ($P < 0.05$). Four clear SOD isozymes were detected in the root tissues (Fig. 4C, 4D). Among these, only the SOD-I isozyme was Mn-SOD (as confirmed by the inhibitor test, data not shown; located in the mitochondria and peroxisome), and the rest of the isozymes belonged to the Cu/Zn-SODs (located in the cytosol). Treatment with Cd generally resulted in a decrease in band size. However, the decrease in the amount of SOD isozymes could be partially reversed by pre-treatment with SA and haemin. Surprisingly, slight decreases of SOD isozyme activities were observed when ZnPPIX was added together with SA or haemin (except for the SOD-I isozyme in the sample treated with haemin). Testing another H_2O_2-scavening enzyme, POD, four bands of isozymes could be detected, and the POD-III isozyme contributed the most activity (Fig. 4G, 4H). Among these, the increase in POD-II isozyme activity due to the SA and haemin pre-treatment was most clearly observed in the gel. Pre-treatment of seedlings with ZnPPIX inhibited the accumulation of this isozyme.

Re-establishing redox homeostasis

It was well known that alfalfa plants contained more hGSH than GSH (Cruz de Carvalho *et al.*, 2010). An experiment using HPLC with fluorescence detection showed that under normal growth conditions, the major glutathione pool obtained from alfalfa seedling roots was hGSH (Table 1); the concentration of hGSH is ~8-fold that of GSH, similar to findings of a previous report (Ortega-Villasante *et al.*, 2007). By using the HPLC assay, it was observed that treatment with Cd for 24 h significantly reduced the content of hGSH and increased hGSSGh and GSH contents (GSSG was not detected) in alfalfa seedling roots. However, pre-treatment with SA or haemin reduced or significantly eliminated the effects of Cd treatment alone on the changes of hGSH and hGSSGh. When SA or haemin was added together with the potent HO-1 inhibitor, ZnPPIX, before treatment with Cd, the changes in the content of hGSH and hGSSGh, induced by SA or haemin, were totally prevented. Similarly, a high ratio of hGSH/hGSSGh was also observed in the sample pre-treated with SA or haemin, compared with the sample treated with Cd alone. Furthermore, treatment with ZnPPIX before Cd exposure obviously enhanced the hGSSGh content and decreased the GSH and hGSH contents, leading to a decreased hGSH/hGSSGh ratio. Additionally, the application of SA alone increased the GSH and hGSH contents and the hGSH/hGSSGh ratio. Similar tendencies were also observed in the responses of the ascorbic acid pool (ASA and DHA). These results were consistent with changes in Cd toxicity (Fig. 2) and lipid peroxidation (Fig 1; Supplementary

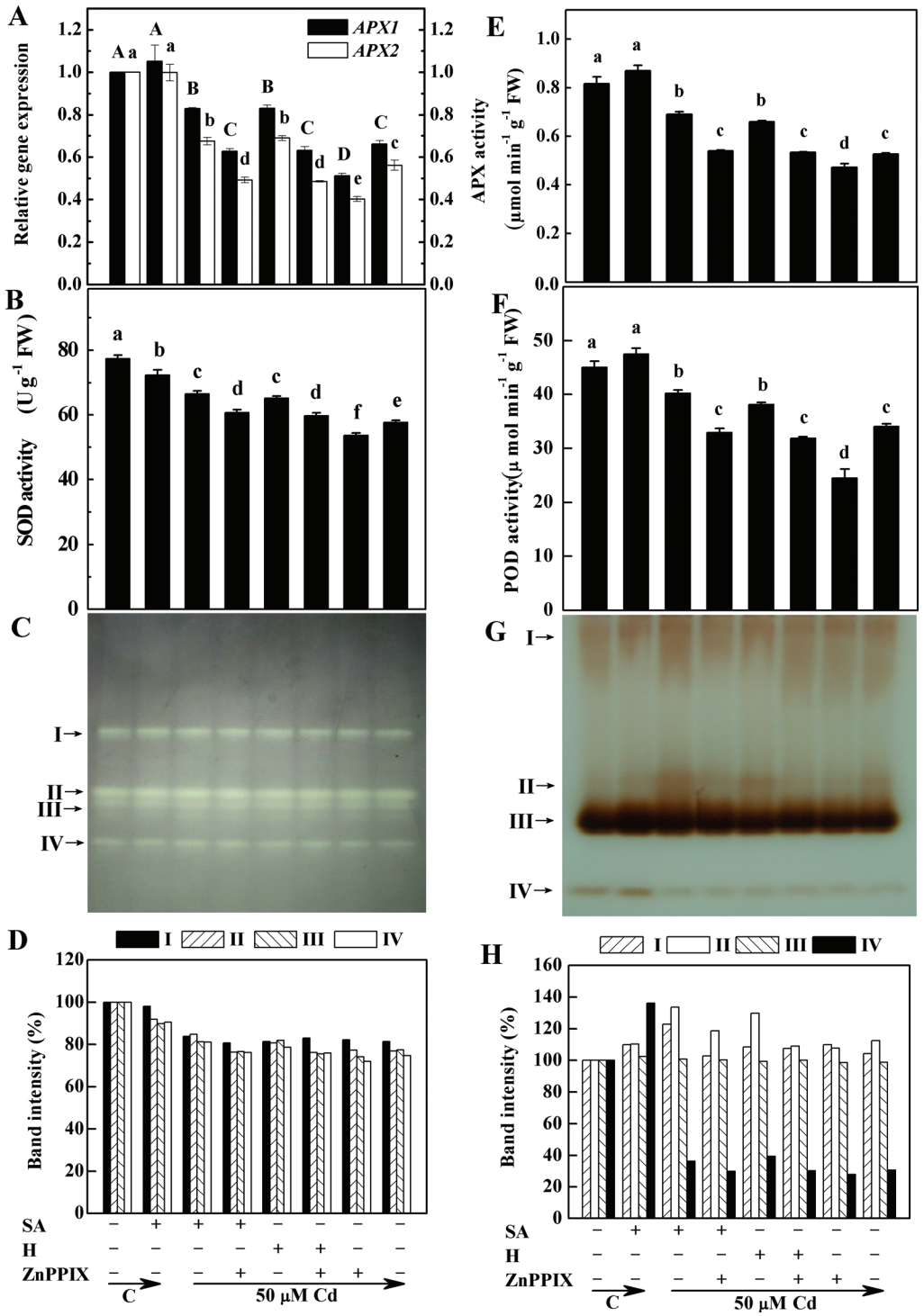

Fig. 4. Effects of salicylic acid (SA), ZnPPIX, and haemin (H) pre-treatment on the expression and activities of ascorbate peroxidase (APX), and total and isozyme activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) in the root tissues of alfalfa upon Cd stress. Five-day-old seedlings were pre-treated or not with 10 μM SA, 20 μM haemin (H), 100 μM ZnPPIX alone, or the combination treatments for 12 h, and then exposed to 50 μM $CdCl_2$ for another 24 h. The sample without chemicals was the control (C). Then, the *APX1/2* transcript quantification test was carried out, normalized against expression of two internal reference genes in each sample (A). SOD (B), APX (E), and POD (F) activity was also determined. Values are means ±SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test. For the determination of the in-gel activity of SOD (C) and POD isozymes (G), extracts of root apices containing 100 μg of protein were loaded onto native gradient PAGE (5–20%) and, following electrophoresis, the gels were stained. Relative activities of different SOD and POD isozymes are also shown in (D) and (H), respectively. Band intensities of the individual isozymes are expressed as a percentage of the control values. The arrows indicate the bands corresponding to various isozymes.

Table 1. Cysteine (cysteine and cysteine disulphide), γ-EC (γ-EC and γ-EC disulphide), glutathione (GSH and GSSG), homoglutathione (hGSH and hGSSGh), and ascorbic acid (ASA and DHA), and the hGSH/hGSSGh and ASA/DHA ratios in alfalfa seedling roots. Five-day-old seedlings were treated with 0 or 50 μM $CdCl_2$ for 24 h with or without 12 h pre-treatment with 10 μM SA, 20 μM haemin (H), 100 μM ZnPPIX alone, or their combination treatments. The sample without chemicals was the control (C).

Values are means ±SE of three independent experiments. Different letters within columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.
[a]GSSG was not detected.

Treatment	Cysteine (nmol g^{-1} FW)	Cysteine disulphide (nmol g^{-1} FW)	γ-EC (nmol g^{-1} FW)	γ-EC disulphide (nmol g^{-1} FW)	GSH[a] (nmol g^{-1} FW)	hGSH (nmol g^{-1} FW)	hGSSGh (nmol g^{-1} FW)	hGSH/ hGSSGh	ASA (nmol g^{-1} FW)	DHA (nmol g^{-1} FW)	ASA/ DHA
C→C	52±1 c	2±1 b,c	15±2 d	1±0 a,b	35±4 d	282±6 a	60±4 d	4.69	319±7 c	95±8 d	3.36
SA→C	55±3 b,c	3±0 a,b	14±2 d	0±0 b	42±1 c,d	310±12 a	57±7 d	5.44	587±7 a	68±7 e	8.63
C→Cd	63±3 b	1±0 c	39±6 b	2±1 a,b	60±10 b	96±7 c	112±11 b	0.86	294±4 c,d	222±6 b	1.32
SA→Cd	77±1 a	2±0 b,c	49±1 a	2±1 ab	79±7 a	142±24 b	76±3 c	1.88	452±6 b	177±9 c	2.55
SA+ZnPPIX→Cd	65±3 b	4±0 a	35±3 b,c	3±2 a,b	59±3 b	86±29 c	111±5 b	0.77	283±4 d	291±8 a	0.97
H→Cd	76±1 a	1±0 c	49±3 a	2±1 a,b	79±8 a	155±17 b	83±13 c	1.87	426±9 b	208±10 b	2.05
H+ZnPPIX→Cd	63±5 b	3±0 a,b	34±3 b,c	2±1 a,b	58±5 b	96±14 c	106±10 b	0.91	291±6 c,d	294±8 a	0.99
ZnPPIX→Cd	58±4 b,c	2±0 b,c	29±2 c	3±1 a	52±7 b,c	49±23 d	128±8 a	0.39	230±4 e	270±14 a,b	0.85

Fig. S3 at *JXB* online). Comparatively, similar to the responses of GSH, Cd-induced cysteine and γ-EC contents were strengthened by the addition of SA or haemin, which was reversed by ZnPPIX, respectively.

A wealth of evidence has accumulated that NAD (including NAD^+ and NADH) and NADP (including $NADP^+$ and NADPH) may be among the fundamental common mediators of various biological processes, including energy metabolism, antioxidation, and the generation of oxidative stress (Ying, 2008). The present results showed that, consistent with the responses of hGSH/hGSSGh and ASA/DHA, Cd caused a consumption of reduced NADPH, but a significant increase in $NADP^+$, as evidenced by the observed decrease in NADPH/$NADP^+$ (Supplementary Table S2 at *JXB* online). Meanwhile, the NADH/NAD^+ ratio was also decreased, which was caused mainly by the increases in the NAD^+ content, consistent with previous findings (Iturbe-Ormaetxe *et al.*, 1998). In contrast, pre-treatment with either SA or haemin could block these tendencies, both of which were significantly reversed when ZnPPIX was added as well. It was also observed that pre-treatment with ZnPPIX alone led to a significantly decreased NAD(P)H/$NAD(P)^+$ ratio.

Since it was possible that the observed re-establishment of redox homeostasis was caused by the modulation of ROS, the distribution of ROS in the root apexes was investigated. Supplementary Fig. S4 at *JXB* online shows that the ROS level increased 2.76-fold following treatment with 50 μM Cd. Conversely, pre-treatment with SA or haemin inhibited ROS formation, which was blocked by the combination with ZnPPIX pre-treatment. It was also observed that SA applied alone produced an increase in ROS distribution compared with the control sample.

Gene expression analysis of GSH/hGSH and ASA pools

As there were changes in the pools of GSH/hGSH and ASA after Cd exposure, the expression of genes involved in their synthesis and/or metabolism, namely *ECS*, *GS*, *hGS*, *GR1*, *GR2*, and *MDHAR*, was analysed. Cd produced a clear induction of *ECS* and *GS* transcripts, which were strengthened by the pre-treatment with SA or haemin, and further blocked by simultaneously added ZnPPIX (Fig. 5A, 5B). Changes in *GR1*, *GR2*, and *MDHAR* exhibited similar tendencies (Fig. 5D–F). Upon Cd exposure, however, the expression of *hGS* declined significantly, compared with Cd-free control samples. Moreover, in the presence of SA or haemin, *hGS* expression was elevated, and this was clearly blocked by the addition of ZnPPIX (Fig. 5C). Further results confirmed different restoration effects of CO (in particular) and BR on the ZnPPIX-induced inhibition of the expression of above genes, which were consistent with the partial reversal of oxidative damage and Cd toxicity (Figs 1, 2; Supplementary Fig. S3 at *JXB* online). However, the combination of Fe, as well as the CO, BR, or Fe alone treatments brought about weaker responses. Significant effects in the down-regulation of *hGS* and *MDHAR* transcripts were also observed when ZnPPIX was applied alone.

Discussion

The beneficial effect of HO-1 on SA-induced alleviation of oxidative stress and Cd toxicity caused by Cd stress

This study confirms that SA arrested Cd-induced oxidative stress and toxicity in alfalfa seedling roots (Figs 1, 2; Supplementary Fig. S3 at *JXB* online), which is in agreement with the observations that SA alleviates Cd toxicity in barley (Metwally *et al.*, 2003), cucumber (Shi and Zhu, 2008), pea (Popova *et al.*, 2009), and rice seedlings (Mishra and Choudhuri, 1999; Guo *et al.*, 2007), but contrasts with the negative role of SA observed in *Arabidopsis* (Zawoznik *et al.*, 2007).

Further data support a linear signal transduction cascade involving up-regulation of MsHO1 downstream of the SA responses. First, exogenous application of haemin, an HO-1 inducer, confers a similar cytoprotective role to SA in the alleviation of oxidative stress and Cd toxicity (Figs 1, 2;

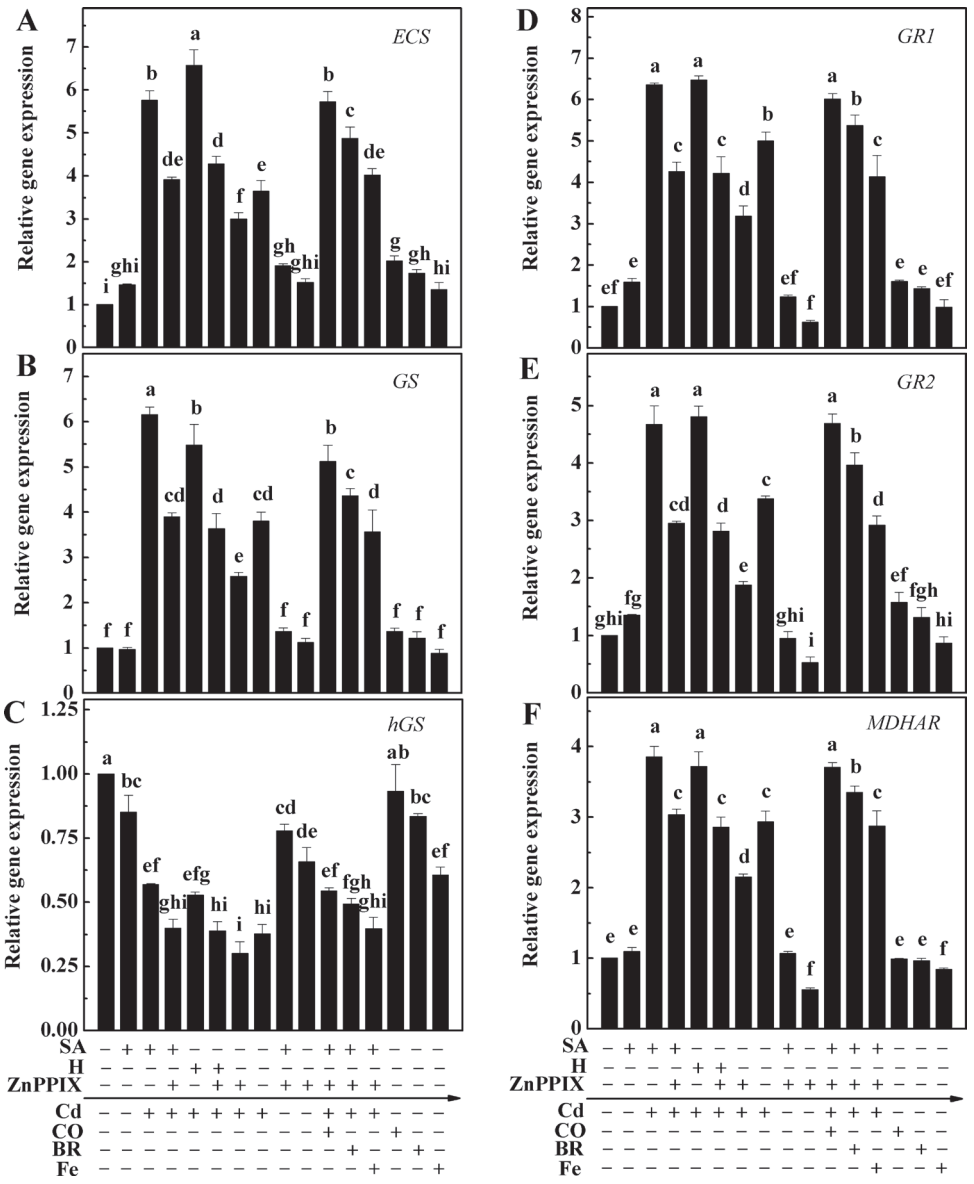

Fig. 5. Effects of salicylic acid (SA), ZnPPIX, haemin (H), CO, bilirubin (BR), and Fe (II) citrate (Fe) on gene expression in the root tissues of alfalfa seedling upon Cd stress. Five-day-old seedlings were pre-treated or not with 10 μM SA, 20 μM haemin (H), 100 μM ZnPPIX alone, or the combination treatments for 12 h, and then exposed to 50 μM $CdCl_2$, 50% saturated aqueous CO solution (CO), 20 μM bilirubin (BR), 20 μM Fe (II) citrate (Fe), or the combination treatments for another 24 h. Then, the transcript levels of *ECS* (A), *GS* (B), *hGS* (C), *GR1* (D), *GR2* (E), and *MDHAR* (F) were analysed by real-time RT-PCR. Expression levels of genes are presented relative to the control samples, normalized against expression of two internal reference genes in each sample. Date are the means ±SE of at least three independent experiments. Within each set of experiments, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Supplementary Fig. S3 at *JXB* online). Treatment with SA and haemin trigger *MsHO1* gene expression, at the translational, transcriptional, and enzymatic levels (Fig. 3). In animals, ample evidence has illustrated that HO-1 is highly induced by a variety of agents or stimuli causing oxidative stress, such as H_2O_2, GSH sdepletors, UV irradiation, and hyperoxia (Ryter *et al.*, 2002). Following these stimuli, the induction of HO activity by *de novo* enzyme synthesis is normally associated with an increase in HO-1 mRNA and corresponding protein levels. The maximal induction of *MsHO1* expression conferred by 10 μM SA (Fig. 3) also matches its cytoprotective performance in the alleviation of Cd-induced overproduction of TBARS (Fig. 1). The present findings are consistent with those reported by Grosser *et al.* (2003) and Oberle *et al.* (2002), that HO-1 amplifies the therapeutic effects of certain stimuli in animals, such as aspirin and pentaerythrityl trinitrate (PETN), a long-acting NO donor.

Subsequent experiments showed that the potent HO-1 inhibitor ZnPPIX (Supplementary Fig. S2 at *JXB* online) could block responses of SA and haemin in the induction of *MSHO1* gene expression (Fig. 3), alleviating overproduction of TBARS (Fig. 1) and oxidative stress (Supplementary Fig. S3 at *JXB* online), as well as lowering the Cd toxicity (Fig. 2). These

observations confirmed that the SA- and haemin-induced cytoprotective effects were MsHO1 specific.

In fact, the above cytoprotective activities of HO-1 might be due to the catalytic products of its enzymatic reactions (Noriega *et al.*, 2004; Shekhawat and Verma, 2010). For example, previous work (Han *et al.*, 2008) showed that exposure to Cd induced the production of endogenous CO in alfalfa seedling roots, consistent with the changes in HO-1 gene expression, and that CO pre-treatment decreased the Cd-dependent oxidative stress, mainly via the modulation of enzymes associated with GSH metabolism. In addition, BV exhibits antioxidant and cytoprotective effects that may enhance the HO-1 responses in animals and plants (Piantadosi 2002; Dulak and Józkowicz, 2003; Noriega *et al.*, 2004; Matsumoto *et al.*, 2006). Interestingly, in the experimental conditions used here, it was also observed that the increase in the content of TBARS conferred by ZnPPIX plus SA or haemin followed by exposure to Cd could be differentially reversed when CO or BR was added (Fig. 1). Similar cytoprotective responses were observed in the histochemical staining for the detection of peroxidation of lipids and injury of plasma membrane integrity in root apexes (Supplementary Fig. S3 at *JXB* online). The addition of CO (in particular) and BR could differentially reverse the seedling root growth inhibition and Cd accumulation in SA plus ZnPPIX-pre-treated alfalfa plants (Fig. 2). However, treatment with Fe^{2+} failed to alleviate the inhibition of seedling root growth. These various pieces of pharmacological evidence therefore support the idea that SA and up-regulation of *MsHO1* might be on a linear signalling pathway in the process of the alleviation of oxidative stress and Cd toxicity.

Redox state homeostasis is involved in HO-1-mediated responses

In plants, HO-1/CO is associated with antioxidant processes when subjected to various abiotic stresses, including salinity stress (Xie *et al.*, 2011), UV-B radiation (Yannarelli *et al.*, 2006), and Cd toxicity (Noriega *et al.*, 2004; Han *et al.*, 2008). Exposure to Cd induced a reduction in the amounts of GSH and ASC, as well as the activities of catalase (CAT), GSH reductase (GR), and POD (Rodríguez-Serrano *et al.,* 2006). In this report, it was further shown that the up-regulation of *MsHO1* driven by SA pre-treatment is an early event in the stimulation of antioxidative enzyme expression, which simultaneously alleviated Cd-induced lipid peroxidation and toxicity. Subsequent data support the establishment of redox homeostasis downstream of MsHO1-mediated responses. The growth of alfalfa plants pre-treated with SA and haemin clearly increased when compared with samples treated with Cd alone (Fig. 2A), and they suffered considerably less Cd-induced oxidative injury (Fig. 1; Supplementary Fig. S3 at *JXB* online). Further experiments confirmed that this was due to induced activation of the antioxidant detoxifying enzymes APX, SOD, and POD, including total or isozymic activities, or the corresponding transcripts (Fig. 4). These increased enzymatic activities resulted in partial prevention of oxidative injury to membranes (Fig. 1) in root tissues. These effects were confirmed by the histochemical staining for the detection of peroxidation of lipids and injury of plasma membrane integrity (Supplementary Fig. S3) as well as the ROS distribution (Supplementary Fig. S4) in root apexes. Moreover, the protective roles of SA and haemin in the activation of antioxidant detoxifying enzymes and the distribution of ROS were suppressed differentially by the potent HO-1 inhibitor ZnPPIX. These data further support the hypothesis that MsHO1 up-regulation may mediate SA-induced antioxidant behaviours as well as the alleviation of Cd toxicity (Fig. 2).

In higher plants, it is well known that Cd toxicity is mediated by oxidative stress, and that Cd not only inhibits plant growth, but also affects GSH and ASA metabolism. Redox buffering in the apoplasts protects the plasmalemma from oxidation (Foyer *et al.*, 2001). It is known that reduced GSH levels play a central role in protecting plants from environmental stresses, including oxidative stress or toxicity from exposure to certain heavy metals (Xiang and Oliver, 1998; Sharma and Dietz, 2009). For example, in a comparison of 10 pea genotypes showing differing Cd sensitivity, the GSH level and the GSH/GSSG ratio were inversely linked to Cd sensitivity (Metwally *et al.*, 2005). In alfalfa and soybean plants, a GSH homologue, hGSH, is also abundantly present instead of, or in addition to, GSH (Matamoros *et al.*, 1999). As expected, the quantification of GSH and hGSH pools by HPLC methods (Table 1) showed that hGSH was significantly more abundant than GSH in alfalfa seedling roots under normal growth conditions. It was also suggested that GSH and hGSH play a major role in plant development and plant adaptation to biotic and abiotic stresses (Baldacci-Cresp *et al.*, 2012). Most importantly, many of the roles ascribed to GSH are also performed by hGSH, particularly the control of the cellular redox status and ROS scavenging (Dalton *et al.*, 1986). In this study, a relationship between hGSSGh accumulation and the amount of oxidative stress has been demonstrated, as the serious oxidation of hGSH (Table 1) was coincident with the accumulation of H_2O_2 (Supplementary Fig. S4 at *JXB* online) as well as the decreased *APX1/2* transcripts and corresponding activity (Fig. 4A, 4E) in Cd-treated alfalfa seedling roots. Previously, it was shown that the enhancement of reduced GSH concentrations and high GSH/GSSG ratios might provide some explanation for the cytoprotective role of CO in mediating Cd-induced oxidative stress in alfalfa root tissues (Han *et al.*, 2008). Similar responses of hGSH/hGSSGh and ASA homeostasis to SA and haemin were observed (Table 1). In contrast, both SA- and haemin-induced restoration of hGSH/hGSSGh and ASA/DHA was obviously blocked by the addition of ZnPPIX (Table 1), in agreement with the reversed effects on the inhibition of lipid peroxidation (Fig 1; Supplementary Fig. S3), the enhancement of antioxidant enzyme expression (Fig. 4), and the decreased ROS distribution (Supplementary Fig. S4). Changes in the expression of the genes involved in GSH, hGSH, and ASA synthesis and/or metabolism, *ECS*, *GS*, *hGS*, *GR1*, *GR2*, and *MDHAR* (Fig. 5), were correlated with the parameters of GSH/hGSH and ASA pools (Table 1). These results may provide an explanation for the cytoprotective role of MsHO1 in mediating SA-induced alleviation of Cd toxicity and corresponding oxidative stress in plant tissues.

Although a significant increase in reduced GSH content was observed in Cd-treated samples, it was also noticed that the total

level of GSH, hGSH, and hGSSGh was lower in Cd-treated seedlings in comparison with the control sample (Table 1). The observed phenomenon of enhanced reduced GSH content could be partially explained by the induction of GSH synthesis genes (*ECS* and *GS*) in Cd-stressed seedlings (Fig. 5A, 5B). In view of the fact that the major glutathione pool obtained from alfalfa seedling roots was hGSH, the obvious down-regulation of *hGS* transcripts (Fig. 5C), the important synthetic gene responsible for the synthesis of the hGSH pool, could be used to explain the decrease in total levels of GSH, hGSH, and hGSSh, which might be the result of the increase in synthesis of phytochelatins (PCs) in Cd-stressed plants (Zhu *et al.*, 1999).

hGSH/hGSSGh and ASA/DHA are also regarded as important redox couples through their interaction with NAD(P)H/$NAD(P)^+$, providing the conditions to support mitochondrial oxidative phosphorylation, generation of ATP, and hence key anabolic activities (May *et al.*, 1998). It has also been suggested that tolerance to metal toxicity is more dependent on the availability of reduced cell metabolites, such as NAD(P)H, than on the antioxidant enzyme capacity of plant tissues (León *et al.*, 2002). It is further suggested that the higher NAD(P)H/$NAD(P)^+$ ratios conferred by SA and haemin pre-treatment (Supplementary Table S2 at *JXB* online) could favour the functionality of the ascorbate–glutathione cycle, and this is confirmed by the inducible responses of *APX1/2*, *GR1/2*, and *MDHAR* expression and APX activities (Figs 4, 5). The reversal response of the NAD(P)H/$NAD(P)^+$ ratio triggered by the HO-1 inhibitor ZnPPIX further confirms that the SA- and haemin-induced cytoprotective effects are HO-1 specific.

In conclusion, the data for the first time showed the up-regulation of MsHO1 involving the SA-induced amelioration of Cd-induced toxicity and oxidative stress in the root tissues of alfalfa seedlings, and provide additional information on important aspects of SA signalling functions in plants. The significant alteration of antioxidant enzyme expression, the hGSH/hGSSGh, ASA/DHA, and NAD(P)H/$NAD(P)^+$ ratios observed here, and their previously demonstrated induction by SA (Mishra and Choudhuri, 1999; Metwally *et al.*, 2003; Shi and Zhu, 2008; Clemente *et al.*, 2012), confirm the involvment of MsHO1 in SA-induced cytoprotection against Cd toxicity and its part in these interrelated events.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Effects of SA and ZnPPIX on the chlorophyll content of alfalfa seedling leaves upon Cd stress.

Figure S2. Effects of ZnPPIX on the activity of purified MsHO1 protein.

Figure S3. Effects of salicylic acid (SA), ZnPPIX, and haemin (H) pre-treatment on $CdCl_2$-induced lipid peroxidation (A) and the loss of plasma membrane integrity (B) in the root tips of alfalfa (*Medicago sativa*).

Figure S4. Confocal images of ROS production in root tips of alfalfa *(Medicago sativa*).

Table S1. The sequences of primers for real-time RT-PCR.

Table S2. Reduced and oxidized nicotinamide (NADH and NAD^+, NADPH and $NADP^+$), and the ratio of NADH/NAD^+ and NADPH/$NADP^+$ in alfalfa seedling roots.

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