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Heme Oxygenase is Involved in the Protection Exerted by Jasmonic Acid Against Cadmium Stress in Soybean Roots

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Abstract The present study was undertaken to test the influence of exogenously applied jasmonic acid (JA) upon the oxidative stress exerted by Cd in soybean plants. It was found that depending on its concentration, JA can improve plant antioxidant responses against Cd. Pretreatment with 20 μM JA effectively ameliorated Cd-induced oxidative stress as indicated by the decrease in thiobarbituric reactive substance (TBARS) levels, enhancement of glutathione (GSH) content, and diminution of H_2O_2 and O_2^- formation. On one hand, the activities of classic antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) were also augmented by JA treatment. This behavior was not observed in plants treated with Cd alone. On the other hand, 20 μM JA caused the enhancement of heme oxygenase (HO) activity (71% with respect to controls) and the amount of protein (60% with respect to controls). However, no gene induction was observed. Pretreatment with 20 μM JA before the addition of Cd provoked the highest values of HO activity and protein expression (138 and 122%, respectively). Once again, these enhancements were not correlated with transcript levels. Plants pretreated with Zn-protoporphyrin IX (ZnPPIX), a well-known irreversible HO-1 inhibitor, could not cope with the oxidative damage caused by Cd. This indicates that HO-1 is involved in the protection exerted by JA against the oxidative stress due to Cd treatment.

Keywords Soybean plants · Jasmonic acid · Cadmium · Antioxidant enzymes · Heme oxygenase-1

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Introduction

Jasmonates are ubiquitously occurring, lipid-derived compounds with signal functions in plant responses to abiotic and biotic stresses, as well as in plant growth and development. Jasmonic acid (JA) and its various metabolites are members of the oxylipin family. Many members alter transcript levels positively or negatively in a regulatory network with synergistic and antagonistic effects in relation to other plant hormones such as salicylate, auxin, ethylene, and abscisic acid (Wasternack 2007).

Different physiological activities of JA have been described. Jasmonates have been proposed as signaling molecules during biotic and abiotic stresses such as pathogen attack, wounding, or osmotic stress (Farmer and Ryan 1992; Gundlach and others 1992; Sembdner and Parthier 1993; Blechert and others 1995; León and others 2001). When jasmonates are exogenously applied to plant tissues, they exert either inhibitory or promotive effects in growth and development (Sembdner and Parthier 1993).

Recent investigations indicated that Cd induces accumulation of jasmonic acid in *A. thaliana* and runner bean plants (Maksymiec and others 2007). This signaling factor can inhibit growth of various plant tissues (Maciejewska

and Kopcewicz 2003) through depression of elongation or cell cycle processes (Świątek and others 2002; Merkouropoulos and Shirsat 2003). This indicates that Cd can influence plant growth indirectly through induction of the jasmonate signaling pathway. This assumption was supported by accumulation of jasmonate-like inducible proteins after metal exposure during initial plantlet elongation (Gianazza and others 2007) and by the alleviating effect of salicylic acid (an antagonist of jasmonates) pretreatment on Cd-induced inhibition of rice root growth and oxidative damage (Guo and others 1993).

JA modifies the resistance of *W. arrhiza* to Pb and increases the stress or/and enhances the plant resistance depending on its concentration. JA at higher doses acts as a stressor and stimulates Pb biosorption as well as lipid peroxide formation, leading to inhibition of plant growth and metabolite degradation. On the other hand, JA at lower concentrations, especially at 0.1 μM, displays beneficial effects which may help plants avoid cumulative damage upon exposure to Pb (Piotrowska and others 2009).

Heme oxygenase (HO, EC 1.14.99.3) catalyzes the oxidative degradation of heme and has well-known antioxidant properties in mammals by means of its products biliverdin IXα and carbon monoxide (Kikuchi and others 2005). Plant HOs are represented by a small gene family with four members in total. This family can be categorized into two distinct classes on the basis of amino acid sequence alignments in HO proteins. One subfamily includes *HO1*-like genes (including *HO3* and *HO4* of *Arabidopsis*), all of which have the canonical HO active site, and the other includes *HO2* genes (Davis and others 2001; Emborg and others 2006), which also can be distinguished by the absence of a positionally conserved histidine considered to be an important ligand for heme binding (Davis and others 2001). All four members of the HO family in *Arabidopsis* are transcriptionally active with substantially overlapping patterns of expression (Emborg and others 2006). In the recent results from Matsumoto and others (2004) it has been found that *HO1* is clearly the one most highly expressed, followed by *HO2*, with both *HO3* and *HO4* expressed at low levels.

We have previously shown that HO is induced in plant tissues as a result of Cd treatment and confers protection against oxidative stress (Noriega and others 2004; Balestrasse and others 2005). Recently, it has been demonstrated that carbon monoxide mitigates salt-induced inhibition of root growth and suppresses programmed cell death in wheat primary roots by inhibiting superoxide anion overproduction (Ling and others 2009). Moreover, it has been reported that reactive oxygen species (ROS) are involved in *HO-1* upregulation in soybean leaves subjected to Cd treatment (Balestrasse and others 2008). We hypothesized that JA may also participate in this process because it regulates the oxidative status and mediates other responses.

The aim of the present study was to investigate whether JA could protect soybean roots against Cd-induced oxidative stress through the modulation of HO activity. Soybean plants were subjected to Cd after pretreatments with different concentrations of JA. Overall, our results indicate that JA is involved in the signaling pathway leading to *HO-1* upregulation under Cd and that a balance between JA and ROS is important to trigger the antioxidant response against oxidative stress.

Materials and Methods

Plant Material and Growth Conditions

Determination of Growth

Seeds were germinated in pots with distilled water (20 seeds in 10 ml for each treatment) in the presence of different JA concentrations (2–200 μM) for 5 days at 50 rpm in an Incubator Shaker model G 25 (New Brunswick Scientific) at 24 ± 2°C and protected from light. Controls were run with distilled water. Afterward, the plants were treated hydroponically with Hoagland solution (Hoagland 1957) (6 plants in 300 ml for each treatment) containing Cd (50–500 μM) for 48 h. The hydroponic medium was continuously aerated in a controlled climate room at 24 ± 2°C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 μmol m^{-2} s^{-1}. Plants were then harvested. When the effect of Zn-protoporphyrin IX (ZnPPIX), a well-known HO irreversible inhibitor, was investigated, roots were pretreated with 22 μM ZnPPIX for 4 h before Cd addition. Controls were run with Hoagland solution. For fresh weight determination, plants were filtered, washed three times with distilled water, kept on filter paper for a few minutes to remove excess liquid, and weighed. Three different experiments were performed, with three replicate measurements for each parameter assayed.

Thiobarbituric Acid Reactive Substances (TBARS) Determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated roots (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3,500×*g* for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 1 ml 4% butylated hydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000×*g* for 15 min and the absorbance

was measured at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Glutathione Determination

Nonprotein thiols were extracted by homogenizing 0.3 g of roots in 3.0 ml of 0.1 N HCl (pH 2.0), and 1 g PVP. After centrifugation at 10,000×*g* for 30 min at 4°C, the supernatants (0.1 ml) were used for analysis. Total glutathione content (GSH plus GSSG) was determined in the homogenates by spectrophotometry at 412 nm using yeast-GR, DTNB, and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine, and GSH content was calculated from the difference between the total glutathione content and GSSG content (Anderson 1985).

Ascorbate and Dehydroascorbate Determination

Ascorbate (AS) and dehydroascorbate (DAS) were determined as described by Law and others (1983). The tissue was homogenized in 10% (w/v) TCA and the supernatant (0.2 ml) was used for the assay. For the concentration quoted, half of the preparation sample was assayed for total ascorbate content and the other half was assayed for As only. DAS concentration was then deduced from the difference. Sodium hydroxide (10 µl, 5 M) was added to 400 µl of extract and mixed; then the mixture was centrifuged for 2 min at 3,500×*g*. To a 200-µl sample of the supernatant were added 200 µl of 150 mM NaH_2PO_4 buffer (pH 7.4) and 200 µl of water. To another 200 µl of supernatant, 200 µl of buffer and 100 µl of 10 mM dithiothreitol were added, and after thorough mixing and being left at room temperature for 15 min, 100 µl of 0.5% (w/v) *N*-ethylmaleimide was added. Both samples were vortex-mixed and incubated at room temperature for 30 s. To each the following was added: 400 µl of 10% (w/v) TCA, 400 µl of 44% (v/v) H_3PO_4, 400 µl of 4% (w/v) bipyridyl in 70% (v/v) ethanol, and 200 µl of 3% (w/v) $FeCl_3$. After vortex mixing, samples were incubated at 37°C for 60 min and the absorbance at 525 nm was recorded. A standard curve of AS or DAS was used for calibration.

Classic Antioxidant Enzymes

Extracts for determination of SOD, CAT, and APX activities were prepared from 0.3 g of roots homogenized under ice-cold conditions in 3 ml of extraction buffer containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4°C. The homogenates were centrifuged at 10,000×*g* for 20 min and the supernatant fraction (0.1 ml) was used for the assays.

CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first-order reaction constant ($k' = k$[CAT]) of the decrease in H_2O_2 absorption was determined, and the catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1}.

Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana and others (1986). The reaction mixture consisted of 50–150 µl of enzyme extract and 3.5 ml O_2^- generating solution which contained 14.3 mM methionine, 82.5 µM NBT, and 2.2 µM riboflavin. Extracts were brought to a final volume of 0.3 ml with 50 mM K-phosphate (pH 7.8) and 0.1 mM Na_2EDTA. Test tubes were shaken and placed 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the lights off. The reduction in NBT was followed by reading absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under assay conditions.

APX activity was measured immediately in fresh extracts and was assayed as described by Nakano and Asada (1981) using a reaction mixture (1 ml) containing 50 mM K-phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM Na-ascorbate, and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm ($\varepsilon = 2.8$ mM^{-1} cm^{-1}). One unit of APX forms 1 µmol of ascorbate oxidized per minute under the assay conditions.

Histochemical Analysis

To analyze H_2O_2 generation, roots were excised and immersed in a 1% solution of 3,3′-diaminobenzidine (DAB) in Tris–HCl buffer (pH 6.5), vacuum-infiltrated for 5 min, and then incubated at room temperature for 16 h in the absence of light. Roots were illuminated until the appearance of a brown color, characteristic of the reaction of DAB with H_2O_2.

In the same way to show O_2^- production, roots were excised and immersed in a 0.1% solution of NBT in K-phosphate buffer (pH 6.4) containing 10 mM Na-azide, vacuum-infiltrated for 5 min, and illuminated until the appearance of dark spots, characteristic of blue formazan precipitate.

Heme Oxygenase Preparation and Assay

Roots (0.3 g) were homogenized in a Potter-Elvehejm homogenizer using 4 vol of ice-cold 0.25 M sucrose

solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.2 mM EDTA, and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20,000×*g* for 20 min and supernatant fractions (0.1 ml) were used for activity determination. Heme oxygenase activity was determined as previously described with minor modifications (Muramoto and others 2002). The standard incubation mixture in a final volume of 500 ml contained 10 mmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 250 ml HO (0.5 mg protein), and 200 nmol hemin. Incubations were carried out at 37°C for 60 min. Activity was determined by measuring biliverdin formation, which was calculated using the absorbance change at 650 nm using a ε value of 6.25 mM^{-1} cm^{-1} (vis_{max} = 650 nm).

Western Blot Analysis for HO Protein

Proteins from roots were subjected to denaturing SDS-PAGE in a Mini-PROTEAN 3 System (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE was performed in 12% gels for HO (4% stacking gels) and run according to Laemmli (1970). The separated polypeptides were transferred to a nitrocellulose membrane at 25 V/300 mA for 2 h in a Mini-Trans-Blot Electrophoretic System (Bio-Rad Laboratories) according to the manufacturer's instructions. The membranes were washed in 25 mM Tris–HCl (pH 8.5), 192 mM glycine, and 20% (v/v) methanol. The blots were blocked by incubation for 2 h in 2% (w/v) powdered nonfat dry milk dissolved in T-TBS [20 mM Tris–HCl (pH 7.6), 137 mM NaCl, and 0.1% (v/v) Tween 20] before reaction with an appropriate antibody. The membrane was incubated with rabbit antibody *A. thaliana* HO-1 (dilution 1:2000) (Muramoto and others 1999). The antibody for HO-1 was diluted in T-TBS with 2% (w/v) powdered nonfat dry milk and washed twice for 10 min both times with T-TBS. Immunoblot with anti-β-tubulin (Sigma, St. Louis, MO) was used as a loading control. Goat anti-rabbit horseradish peroxidase conjugate was used as a secondary antibody and incubated for 1 h. The blots were washed twice for 10 min before color development (ECL immunodetection system, ECL Western Blotting Protocols, Dako). The intensity of bands was analyzed with Gel-Pro Analyzer 3.1 software (Media Cybernetics, Bethesda, MD). The films were scanned (Fotodyne Incorporated, Hartland, WI) and analyzed using Gel-Pro Analyzer 3.1 software.

Isolation of RNA and RT-PCR Analysis

Total RNA was extracted from soybean roots by using the Trizol reagent (Gibco BRL). Four micrograms of total RNA was treated with RNase-free DNase I (Promega, Madison, WI) and then 1.0 μg was reverse-transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen, Carlsbad, CA). PCR reactions were carried out using *Glycine max* HO-1 and 18S-specific primers, as previously described (Yannarelli and others 2006). The PCR profile was set at 94°C for 1 min and then 29 cycles at 94°C for 0.5 min, 54°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. Each primer set was amplified using an optimized number of PCR cycles to ensure the linearity requirement for semiquantitative RT-PCR analysis. The amplified transcripts were visualized on 1.5% agarose gels with the use of ethidium bromide. Gels were then scanned (Fotodyne Incorporated) and analyzed using Gel-Pro Analyzer 3.1 software.

Protein Determination

Protein concentration was evaluated using the method of Bradford (1976), with bovine serum albumin as a standard.

Statistics

All values reported in this work represent the means of three independent experiments. The mean values ± standard deviation (SD) are given in the Tables and Figures. A one-way analysis of variance (ANOVA) test was used to confirm the significance of the data. Comparison with control and treatments was performed using Tukey's test.

Results

Growth of Soybean

Jasmonic acid influenced soybean root growth in a dose-dependent manner (Fig. 1a). Stimulation was observed after application of 2 and 20 μM JA leading to a significant increase in the roots' fresh weight (40 and 88%, respectively). In contrast, JA at the highest concentration range of 100–200 μM considerably suppressed biomass production. For example, a significant diminution (25%) was obtained in plants treated with 200 μM JA. These results may not necessarily contradict a senescence-promoting role of JA, as described by He and others (2002). One possible explanation is that application of JA at low concentrations could promote root growth by protecting the plant tissue against the oxidative damage. The same results were already found in experiments carried out with low concentrations of nitric oxide or 5-aminolevulinic acid, both of which can act as a herbicide when used at higher doses (Noriega and others 2007; Balestrasse and others 2010).

Preliminary experiments showed that the growth of soybean was inhibited by the presence of Cd in the nutrient solution. Plants exposed to Cd showed obvious symptoms of toxicity such as brown roots and growth inhibition.

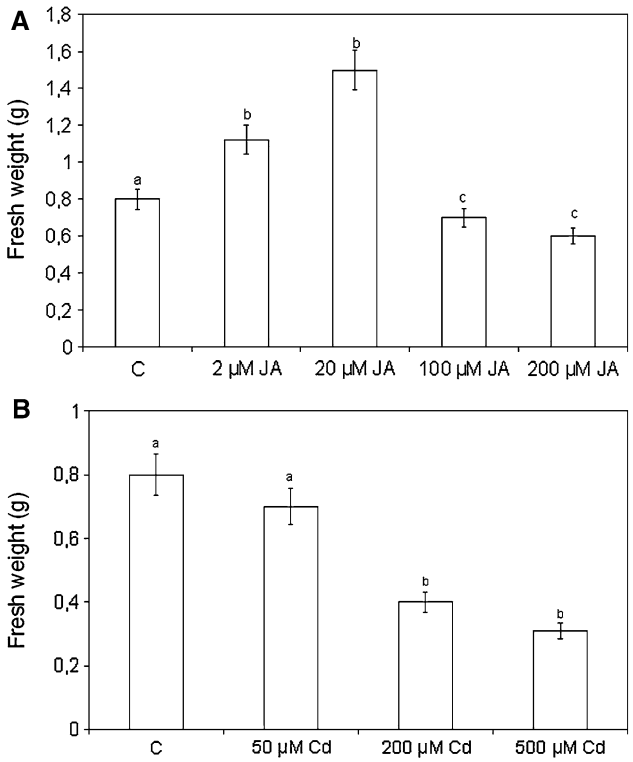

Fig. 1 Effect of Cd and JA pretreatment on fresh weight of soybean roots. Experiments were performed as described in "Materials and Methods" Section. Values are the mean of three independent experiments and *bars* indicate SE. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

Fresh weight of roots decreased proportionally with increasing Cd concentrations and the most phytotoxic effect was observed at 500 µM (Fig. 1b). At lower doses, Cd brought about a significant decrease in biomass production. Therefore, Cd at 200 µM combined with JA at the concentration range of 2–200 µM was used in the following experiments.

Lipid Peroxidation

The increment in TBARS is a good reflection of the oxidative damage to membrane lipids and other vital molecules such as proteins, DNA, and RNA. Figure 2 shows that TBARS levels increased 1.6-fold with respect to controls under Cd treatment, which is in agreement with results of other studies (Deng and others 2010). To complete this analysis, the effect of different JA concentrations ranging from 2 to 200 µM were evaluated. Figure 2 indicates that membrane damage in roots was observed in roots grown in the presence of 200 µM JA, whereas under other concentrations no differences were observed with respect to controls.

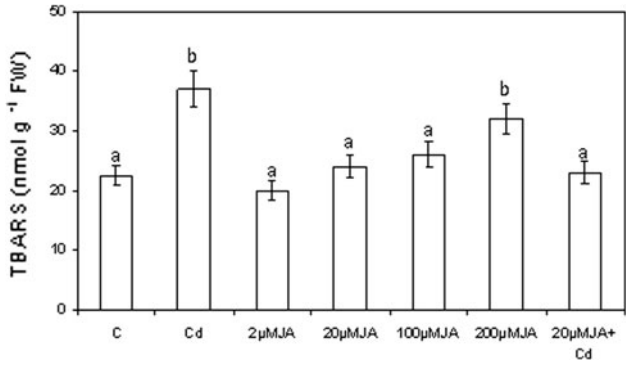

Fig. 2 Effect of Cd and JA pretreatment on TBARS formation in soybean roots. Experiments were performed as described in "Materials and Methods" Section. Values are the mean of three independent experiments and *bars* indicate SE. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

Pretreatment with 20 µM JA totally prevented the enhancement of TBARS levels by Cd (Fig. 2). Taking into account this fact, 20 µM JA was employed to perform the following experiments. This result is supported by Anjum and others (2011), in which exogenous application of 50 µM JA alleviated the adversities of drought stress in soybean leaves.

Glutathione Content

GSH is a leading substrate for enzymatic antioxidant functions and it is also a known radical scavenger. Previous reports from our laboratory demonstrated that Cd induces the formation of oxidant species and therefore affects GSH content in soybean plants (Balestrasse and others 2001; Noriega and others 2004). Data in Fig. 3 show that GSH concentrations in soybean roots treated with Cd decreased 68% with respect to controls. JA did not affect this parameter but pretreatment with JA before the addition of Cd totally prevented the decrease previously observed in GSH content.

Ascorbate and Dehydroascorbate Behavior

Ascorbate levels were markedly decreased in plants treated with Cd (62%). Significant increases were found in DAS levels (32%) and therefore a corresponding decrease in the AS/DAS ratio was observed. No differences were observed in the presence of JA. Pretreatment with JA before the addition of Cd showed a protective effect compared to the results obtained with Cd alone (Table 1).

H_2O_2 and O_2^- Localization in situ

To obtain clues about the mechanism of JA in its defense against Cd, H_2O_2 and O_2 localization in situ was

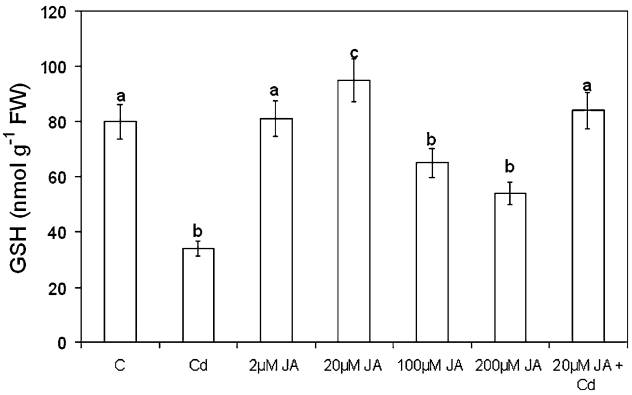

Fig. 3 Effect of Cd and JA pretreatment on GSH content of soybean roots. Experiments were performed as described in "Materials and Methods" Section. Values are the mean of three independent experiments and *bars* indicate SE. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

Table 1 Ascorbic acid and dehydroascorbic acid determinations in soybean roots

	AS (μmol g^{-1} FW)	DAS (μmol g^{-1} FW)	AS/DAS
C	0.21 ± 0.01^a	0.46 ± 0.02^a	0.46^a
Cd	0.08 ± 0.01^b	0.61 ± 0.03^b	0.13^b
20 μM JA	0.23 ± 0.02^a	0.44 ± 0.02^a	0.52^c
20 μM JA + Cd	0.16 ± 0.01^b	0.38 ± 0.02^b	0.42^a

AS and DAS content were assayed as described in "Materials and Methods" Section. Values are the mean ± SE of three independent experiments. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

performed. Histochemical methods were applied as shown in Fig. 4. Cd produced a 30% H_2O_2 spot area versus total root area, whereas pretreatment with 20 μM JA prevented this effect and the spot area was similar to that of controls. Pretreatment with 20 μM JA avoided partially H_2O_2 accumulation. Data in Fig. 4 show that roots of Cd-treated soybean plants produced a 65% O_2^- spot area versus total root area. Pretreatment with 20 μM JA partially prevented the O_2^- production induced by Cd. These data indicate that ROS accumulation can be induced by Cd, which may result in injury to the plant, and that 20 μM JA is effective in enhancing Cd tolerance in soybean plants.

Antioxidant Enzyme Activities

To further investigate the protective action of 20 μM JA on oxidative stress in soybean plants, antioxidant enzyme activities were determined. Analyses were performed on CAT, SOD, and APX in plants subjected to Cd with or without JA pretreatment. Interestingly, Table 2 shows that

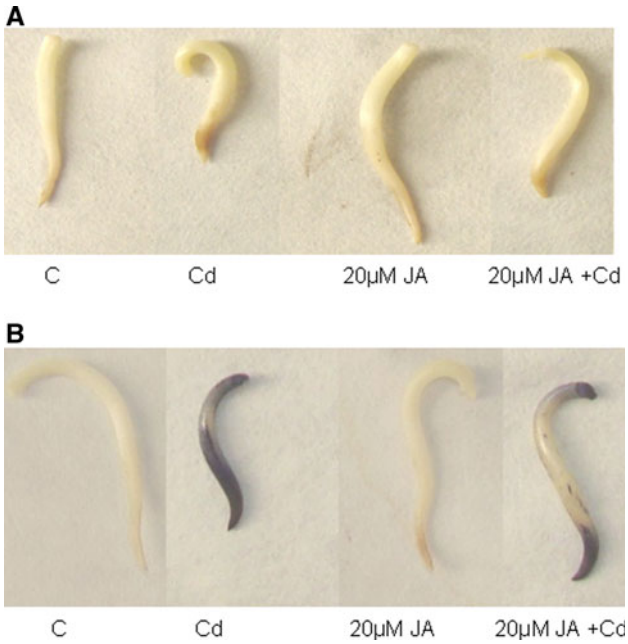

Fig. 4 H_2O_2 and O_2^- localization in situ. Experiments were performed as described in "Materials and Methods" Section

in our experiment, the activities of CAT and SOD were slightly enhanced after Cd stress (15% with respect to controls), whereas APX remained at control levels. Higher SOD activity can efficiently remove O_2^-, which leads to the production of H_2O_2, and H_2O_2 can be scavenged by CAT and glutathione reductase in the Halliwell–Asada pathway. Nevertheless, this augmentation seems not to be high enough to cope with the oxidative damage. When plants were pretreated with 20 μM JA, CAT and SOD activities were enhanced (51 and 29% with respect to controls), and once again no significant changes in APX activities were observed.

Experiments carried out in plants treated with both compounds revealed that 20 μM JA protects soybean plants against oxidative damage by enhancing CAT and SOD activities (57 and 25% with respect to controls).

Table 2 Antioxidant enzyme activities in soybean roots subjected to 200 μM Cd and 20 μM JA pretreatment

Treatment	CAT (pmol/mg protein)	SOD (U/mg protein)	APX (U/mg protein)
Control	120 ± 12^a	14.3 ± 0.5^a	0.004 ± 0.001^a
Cd	138 ± 9^b	16.4 ± 0.7^b	0.006 ± 0.002^a
20 μM JA	181 ± 12^c	18.5 ± 0.6^c	0.004 ± 0.001^a
20 μM JA + Cd	188 ± 14^c	17.9 ± 0.5^c	0.005 ± 0.001^a

Enzymatic activities were assayed as described in "Materials and Methods" Section. Values are the mean ± SE of three independent experiments. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

Effect of JA on Heme Oxygenase-1 Activity, and Protein and Transcript Levels

On one hand, previous findings from our group demonstrated the protective role that HO-1 plays against oxidative stress in Cd-treated plants (Noriega and others 2004; Balestrasse and others 2005), and on the other hand, the present data indicate that pretreatment with 20 μM JA seems to neutralize the effects brought about by Cd. These results prompted us to investigate the behavior of HO-1 as a consequence of JA treatment. Figures 5 and 6 show that application of 20 μM JA caused the enhancement of HO-1 activity (71% with respect to controls) and protein expression (60% with respect to control) but not of transcript levels. Plants subjected to Cd showed an increase of HO-1 activity (66%) with respect to controls. Pretreatment with 20 μM JA before the addition of Cd provoked a major enhancement of HO-1 activity and protein expression (138 and 122% with respect to controls) but once again not in transcript levels. HO-1 has been described as a feature of plant response to stress conditions (Xie and others 2008). To asses whether HO-1 is involved in the protection against Cd exerted by 20 μM JA, experiments were carried out in plants treated with ZnPPIX, a well-known irreversible HO-1 inhibitor. Plants with inhibited HO-1 activity cannot cope with the oxidative damage caused by Cd (Table 3). We can assume that protection exerted by 20 μM JA may also be due to the augmentation of this antioxidant enzyme activity. When *Arabidopsis HY1* (*HO1*) expression was analyzed, it was found that it was significantly suppressed by MeJA in a time-dependent manner (Zhai and others 2007). Nevertheless, our data show that soybean plants treated with both JA and Cd revealed a major enhancement in HO-1 activity that did not correlate with transcript levels. This fact led us to suppose that increased transcript levels of HO-1 could result in transcript accumulation and/or protein accumulation, but it was not detectable 48 h after treatment. The enhancement found in the amount of proteins reinforces this hypothesis. Further studies are necessary to clarify this behavior; in this sense, not only the plant species but also stress and treatment must be considered.

Discussion

In previous reports we have demonstrated the protective role that HO-1 plays against oxidative stress caused by Cd in soybean plants (Noriega and others 2004; Balestrasse and others 2005). Taking into account the fact that JA induces the expression of a variety of genes involved in the activation of defense mechanisms (Koiwa and others 1997; Harms and others 1998), in the present study we evaluated whether this phytohormone could enhance HO activity, conferring major protection against Cd.

Our data demonstrated that depending on its concentration, JA can improve the plant antioxidant response against Cd. This model was appropriate to determine the beneficial effect of exogenously added JA. Although 200 μM showed a pro-oxidant effect, 20 μM JA reduced oxidative damage. Pretreatment with 20 μM JA effectively ameliorated Cd-induced oxidative stress, as indicated by the decrease in H_2O_2 and O_2^- formation and the decrease in TBARS and the increase in GSH and AS root contents with respect to plants treated with Cd alone.

Plants have an efficient system for decomposing ROS, using the enzymes SOD and guaiacol peroxidase in chloroplasts (Asada 1999). In general, the responses of the antioxidant system, and especially those of antioxidant enzymes, to oxidative stress are variable and depend on plant species, cultivar, and age and also on the duration of stressor action (Hodges and others 1996).

The activities of classic antioxidant enzymes such as CAT and SOD were enhanced by Cd with respect to controls and a more important increase was found in the presence of JA (Table 2). These data are in agreement with reports that show a protective effect of JA in plants subjected to Cd and other stresses (Maksymiec and Krupa 2002; Poschenrieder and others 2006). Moreover, increased SOD activity correlated with increased protection from damage associated with oxidative stress (Asada 1999), and results from our study showed that the SOD activity in soybean roots increased significantly. This implies that enhancement of SOD activity scavenges O_2^- radicals to protect the cell from oxidative damage. The control of the steady-state O_2^- levels by SOD is an important protective mechanism against cellular oxidative damage because O_2^- acts as a precursor of more cytotoxic or highly reactive oxygen derivatives such as peroxynitrite or HO (Halliwell and Gutteridge 1989). Therefore, SOD is usually considered the first line of defense against oxidative stress.

Data here reported also showed an enhancement of CAT activity in soybean roots. This augmentation is necessary to scavenge H_2O_2 in peroxisomes and cytosol, where it might have diffused from chloroplasts as a result of SOD activity. Different cell compartments may activate different defensive systems to reduce excessive ROS. The increased peroxidation of lipids and peroxidase activity that we have observed in our study of soybean roots could reflect a similar process of oxidative stress with the implication of peroxidase activity as part of the antioxidant response against H_2O_2. However, Franck and others (2004) argued for a stress response of soybean and suggest alternative defense mechanisms involving homeostatic regulation and controlled degradation processes to maintain integrity and vital functions of the cell.

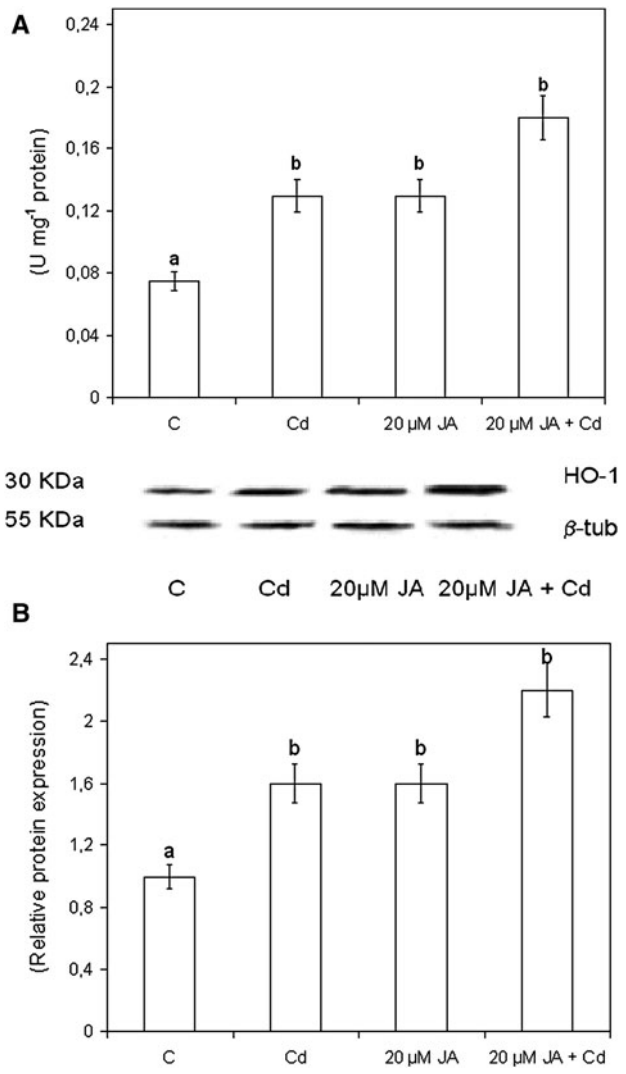

Fig. 5 Effect of Cd and JA pretreatment on HO activity (**a**) and amount of proteins (**b**) in soybean roots. HO-1 protein expression was analyzed by Western blotting as described in "Materials and Methods" Section. β-tubulin (β-tub) immunoblotting was performed as an internal control of protein loading. Relative HO-1 protein expression taking control as 1 U. Experiments were performed as described in "Materials and Methods" Section. Values are the mean of three independent experiments and *bars* indicate SE. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

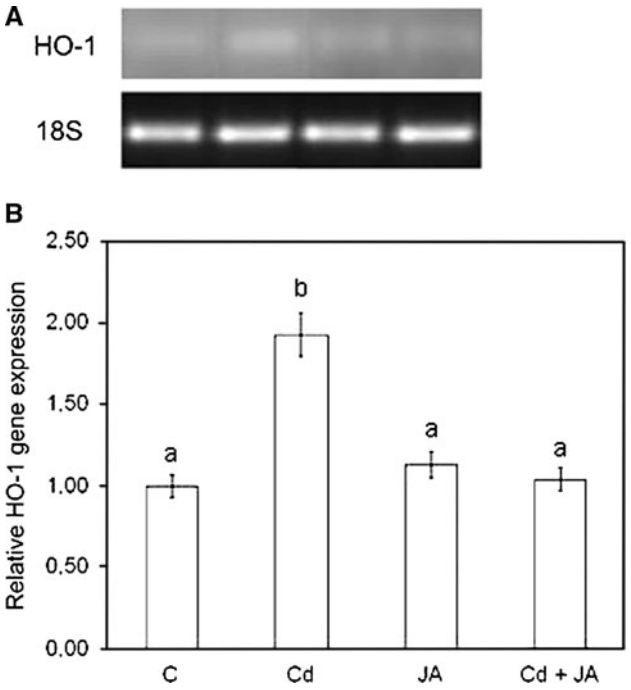

Fig. 6 Effect of Cd and JA pretreatment on soybean root transcript levels. **a** HO-1 mRNA expression was analyzed by semiquantitative RT-PCR as described in "Isolation of RNA and RT-PCR Analysis" subsection. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. **b** Relative *HO-1* transcript expression taking control as 1 U. Data are means of three independent experiments and *bars* indicate SE. Different letters within *bars* indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

Nevertheless, the molecular mechanism that mediates JA enhancement of antioxidant enzyme activities is not completely understood. Interestingly, we found that HO and CAT activities were similar with respect to JA pretreatment under Cd (Fig. 5). A recent study showed that the time course induction of these enzymes in soybean nodules subjected to Cd are similar (Balestrasse and others 2008). These results suggest a close relationship between the signal transduction pathways involved in the response of HO and CAT after oxidative stress generation and support the antioxidant role of HO. In addition, there was no correlation between *HO-1* transcript levels and enzyme activity (Fig. 5). Previous reports have also demonstrated that the enhancement of HO activity is associated with an increase in *HO-1* transcript levels and protein content (Yannarelli and others 2006; Noriega and others 2007; Han and others 2008). Although this mechanism can account for the changes observed in HO activity, the incidence of post-translational modifications or different HO isoforms under stress conditions needs to be addressed.

Experiments carried out in plants treated with JA in the absence of Cd showed that JA itself cannot upregulate *HO-1* mRNA expression (Fig. 6). This observation indicates that a certain balance between JA and ROS is required to trigger the full response. Indeed, the specific inhibition of HO by Zn-protoporphyrin IX increased the oxidative stress parameters after treatment with low dose of Cd (Table 3). Moreover, we have previously shown similar results in leaves and nodules of Cd-treated plants (Noriega and others 2004; Balestrasse and others 2005). Biliverdin, one of the products of HO, is an efficient scavenger of ROS and it can account for the antioxidant properties of this enzyme in animals and plants (Noriega and others 2004).

Table 3 Effect of ZnPPIX on HO-1 activity, TBARS levels, and GSH content in soybean roots

Treatment	HO-1 (U/mg protein)	TBARS (nmol/g FW)	GSH (nmol/g FW)
Control	0.070 ± 0.010^{a}	22.5 ± 0.2^{a}	80.5 ± 0.5^{a}
Cd	0.120 ± 0.010^{b}	37.1 ± 0.4^{b}	38.0 ± 0.2^{b}
ZnPPIX	0.020 ± 0.006^{c}	21.3 ± 0.2^{a}	82.1 ± 0.4^{a}
ZnPPIX + Cd	0.020 ± 0.004^{c}	62.1 ± 0.3^{c}	15.4 ± 0.3^{c}

Enzymatic activity as well as TBARS levels and GSH content were assayed as described in "Materials and Methods" Section. Values are the mean ± SE of three independent experiments. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple-range test

More recently, it has been shown that CO released by HO is an important signal molecule for the tolerance mechanisms against cadmium and salt stress (Han and others 2008; Xie and others 2008). It would be interesting to determine whether CO also has a role in Cd acclimation. These data led us to assume that a slight enhancement in HO transcript levels could have occurred which contributed to an increase in enzyme activity. 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). Taking into account that, on one hand, GSH and ASA levels were decreased and, on the other hand, HO activity, protein amount, and transcript levels were increased in soybean roots subjected to Cd exposure (Figs. 5, 6), we further suggest that this enhancement in enzyme activity and upregulation clearly indicates that HO is directly involved in the antioxidant defense system of soybean plants through its antioxidant byproducts.

Based on this study and supporting literature, we speculate that JA may be involved in the Cd-specific signaling pathway that mediates the HO response under Cd treatment. The present study, together with previous results (Noriega and others 2004), supports the model explaining the mechanisms involved in HO response in Cd-treated soybean plants. In this model JA is implicated in the HO signaling pathway and, together with ROS, could modulate the activity of this enzyme under Cd treatment. Moreover, JA can enhance the antioxidant system allowing improved plant defense against the subsequent oxidative damage. Interestingly, although JA could directly potentiate Cd-induced *HO-1* transcription, the pretreatment may protect and enhance by inducing free radical scavenging enzymes that suppress the inhibitory effects of elevated ROS accumulation at this higher dose, resulting in an appropriate balance of ROS–JA to trigger the full HO response. HO plays an important role during stress conditions. Moreover, AS/DAS ratios remained unaltered in plants pretreated with 20 μM JA before the addition of Cd, indicating that in this case the oxidative–redox state is maintained by the heme catabolism products. Interestingly, Maksymiec (2011) demonstrated that the inhibitors of the JA pathway greatly weakened the inhibitory effect of Cd in *P. coccineus* roots, whereas no significant effect was observed in *A. cepa.*

In conclusion, JA applied at lower concentrations, especially at 20 μM, displays a protective function against Cd stress, leading to restoration of fresh weight. Similar data have been also reported by Maksymiec and Krupa (2002), indicating that exogenous MeJA at 20 μM did not enhance the inhibitory action of Cu and Cd on *Arabidopsis thaliana* growth. This protection exerted by 20 μM JA against Cd toxicity in soybean plants may be explained by the observed enhancement of HO-1 activity. Our hypothesis is also supported by other reports that demonstrated the effect of MeJA on the accumulation of phytochelatins in *A. thaliana* in response to Cu and Cd, leading to enhanced plant tolerance to stress (Maksymiec and others 2007). Moreover, it has been shown that low JA concentrations protect proteins in *W. arrhiza* exposed to Pb (Piotrowska and others 2009).

The explanation for the JA-induced increase in protein level despite the presence of toxic concentrations of a heavy metal may be stimulation of the expression of genes resulting in the synthesis of proteins with protective functions (Piotrowska and others 2009). Our results revealed that JA at 20 μM may be involved in inhibition of lipid peroxide formation through the stimulation of nonenzymatic (ascorbate, glutathione) antioxidant machinery as well as the enhancement of an antioxidant enzyme such as HO-1, which may be responsible for tight regulation of ROS homeostasis during heavy-metal stress.

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