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Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress

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Abstract Heme oxygenase (HO, EC 1.14.99.3) catalyses the oxidative conversion of heme to biliverdin IXα (BV) with the concomitant released of carbon monoxide and iron. Recently, plant HOs have been involved in the defence mechanism against oxidative stress. The goal of this study was to evaluate the time-course of HO-1 and catalase (CAT, EC 1.11.1.6) gene expressions in nodules and roots of soybean plants subjected to Cd treatment. No significant changes were observed up to 24 h. After 48 h of 200 μM Cd exposure, an up-regulation of HO-1 mRNA (110%) occurred in nodules. On the other hand, a down-regulation was found in roots (39%). While there was an augmentation in CAT transcript levels (30%) in nodules, an important diminution (52%) was evidenced in roots. Changes observed in gene expression were also found in protein levels and activities. These data suggest that an induction of CAT and HO-1 occurred in nodules as a response of cell protection against oxidative damage. However, after 72 h treatment, a down-regulation of HO-1 mRNA was found either in nodules or in roots (78% and 94%, respectively), while a similar response was evidenced for CAT (40% and 83%, respectively). These results are consistent with our previous findings suggesting that oxidative stress produced by Cd were more pronounced in roots than in nodules of soybean plants. Moreover, this behaviour could explain the major viability observed in nodules respect to roots, and provide a new insight into the processes involved in the antioxidant defence system in plant tissues.

Keywords Cadmium stress · Catalase · Heme oxygenase · Soybean

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Abbreviations

BV	Biliverdin
CAT	Catalase
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
HO-1	Heme oxygenase-1
HO	Heme oxygenase
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species

Introduction

Cells have the ability to protect themselves against oxidative stress through the up-regulation of a wide range of genes. Among them, heme oxygenase-1

(HO-1) has attracted particular interest in mammalian tissues because it is up-regulated by stress conditions and generates products that might have important biological activities (Maines 1997). HO's are widely distributed enzymes and their main function is associated with the degradation of heme to iron, carbon monoxide and biliverdin (BV), the latter being converted to bilirubin by the cytosolic enzyme biliverdin reductase. Both BV and bilirubin possess antioxidant properties (Stocker et al. 1987). Furthermore, CO could inhibit excessive NO generation and suppress the catalytic activity of cytochrome P450 monooxygenases, enzyme systems that account for endogenous generation of reactive oxygen species (ROS). The increase in free iron induces the up-regulation of ferritin which in turn causes overall reduction of free iron and thereby attenuates oxidative susceptibility (Vile et al. 1994). Genes encoding HO's have been isolated from a wide variety of organisms including mammals, red algae, cryptophytas, cyano- and pathological bacteria (Ortiz de Montellano and Wilks 2001; Terry et al. 2002; Kikuchi et al. 2005; Maines and Gibbs 2005). In cyanobacteria and some algae, the function of HO is to provide cofactors to the photosynthetic apparatus. In plants, the only role attributed to HOs is their participation in the biosynthetic pathway leading to phytochrome chromophore formation, functioning in light signaling (Muramoto et al. 1999; Davis et al. 2001). We have recently reported that HO is involved in the antioxidant defense system in soybean leaves and nodules as was previously shown to be the case in animal tissues (Noriega et al. 2004; Balestrasse et al. 2005a). But it is still unknown if this increase in HO activity under cadmium (Cd) stress is regulated at the transcript levels.

In plants, catalase (CAT) represents a primary enzymatic defense against oxidative stress induced by senescence, chilling, dehydration, osmotic stress, wounding, paraquat, ozone and heavy metals (Gallego et al. 1996; Gadjev et al. 2006; Park et al. 2006), because it catalyses the breakdown of H_2O_2 (Scandalios et al. 2000). Catalases occur as multiple isozymes encoded by a small family, and it belongs to a group of antioxidant enzymes which protective role is modulated at the transcriptional level (Jithesh et al. 2006).

These findings prompted us to further examine: (1) whether soybean HO could also be involved in the antioxidant defense in soybean roots, as it occurs in nodules and leaves, (2) whether the changes in HO activity and protein content are associated to a regulation of its gene expression in roots and nodules and (3) whether HO behaviour could be compared to that of a classical antioxidant heme enzyme such as CAT. To this end, we have investigated the time course of HO-1 and CAT gene expressions in soybean nodules and roots subjected to 50 and 200 μM Cd treatments. In addition, we have evaluated HO and CAT protein expression and activity.

Material and methods

Plant material and growing conditions

Seeds of soybean (*Glycine max* L., A6445RG) were surface sterilized with 5% v/v sodium hypochlorite for 10 min and then washed with distilled water four times. The seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and were planted in vermiculite for 5 days. After germination, plants were removed from pots; roots were gently washed and transferred to separated containers for hydroponics. Five liter pots were used, containing 15 plants each. Plants were germinated and grown 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}. The hydroponic medium consisted of nitrogen-free Hoagland nutrient solution (Hoagland and Arnon 1950). The medium was continuously aerated (100 ml min^{-1}) and replaced every three days. After 4 weeks growth, plants were treated with nutrient solution devoid of Cd (control) or containing 50 and 200 μM Cl_2Cd during different times ranging from 24 to 72 h. After treatment, roots (approximately 0.9 ± 0.3 g each) and nodules (0.09 ± 0.02 g each) were separated and used for determinations. Determinations were performed by triplicate in three different experiments employing 15 plants for each treatment.

Heme oxygenase preparation and assay

Tissues (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 chloroplasts were used for activity determination. Heme oxygenase activity was assayed as previously described with minor modifications (Muramoto et al. 2002). The assays (1 ml final volume unless otherwise indicated) contained, 250 µl HO (0.5 mg protein), 10 µM hemin, 0.15 mg ml^{-1} bovine serum albumin, 50 µg ml^{-1} (4.2 µM) spinach (Spinacia oleracea) ferredoxin (Sigma Chemical Co.), 0.025 units ml^{-1} spinach ferredoxin-$NADP^+$ reductase (Sigma Chemical Co.). The reaction was started by adding NADPH to a final concentration of 100 µM, samples were incubated at 37°C during 60 min, BV formation was calculated using the absorbance change at 650 nm. The concentration of BV was estimated using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES-NaOH buffer (pH 7.2).

Catalase preparation and assay

Extracts for determination of CAT activity were prepared from 0.3 g of nodules or roots, homogenized under ice-cold conditions in 3 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4°C. The homogenates were then centrifuged at 10,000*g* for 20 min and the supernatant fraction was used for the assays. Catalase 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 ($k' = k \times [CAT]$) of the decrease in H_2O_2 absorption was determined and the CAT content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} (Chance et al. 1979).

Western blot analysis for heme oxygenase-1 and catalase

Homogenates obtained for HO-1 or CAT activity assays were also analyzed by Western immunoblot technique. About 40 µg of protein from homogenates were subjected to sodium dodecyl sulfate (SDS)-poliacrylamide gel electrophoresis using a 12% acrylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK), according to Laemmli (1970). Separated proteins were then transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies raised against *Arabidopsis thaliana* HO-1 (Muramoto et al. 1999) diluted 1:2000 in Tris–NaCl buffer plus 1% non-fat milk or human anti-catalase rabbit immunoglobulin (Calbiochem) in the same conditions. Goat anti-rabbit horseradish peroxidase conjugate was used as secondary antibody. Inmunoblot with anti-tubulin (Sigma, St Louis, MO) was used as a loading control. Immune complexes were detected using the enhanced chemiluminescence western blotting procedure (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden). The films were scanned (Photodyne Incorporated, WI, USA) and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA).

Isolation of RNA and RT-PCR analysis

Total RNA was isolated using Trizol reagent (Gibco BRL), treated with RNase-free DNase I (Promega), and reverse transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Gibco BRL). PCR reactions were carried out using *Glycine max.* HO-1 and 18S specific primers, as previously described (Yannarelli et al. 2006). In addition, 2 µl of the reverse-transcribed material was amplified by use of a primer pair specific to *Glycine max.* CAT cDNA (sense primer, 5′-CTG CTGGAAACTATCCTGAGTG-3′; antisense primer, 5′-ATTGACCTCTTCATCCCTGTG-3′). 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 umber of PCR cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. Ethidium bromide stained gels were scanned (Photodyne Incorporated, WI, USA) and analyzed using Gel-Pro Analyser 3.1 software (Media Cybernetics, MD, USA). The ratios of HO-1 mRNA to 18S mRNA and CAT mRNA to 18S mRNA were quantified.

Protein determination

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

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Effect of Cd treatment on HO-1 gene expression in soybean nodules and roots

We have previously reported that Cd treatment results in an increase of HO activity in soybean roots nodules (Balestrasse et al. 2005a). To further characterize this induction, here we analyze HO-1 transcript levels in nodules in response to 50 and 200 μM Cd at different times ranging from 24 to 72 h. Semi-quantitative RT-PCR analysis showed that up to 48 h 200 μM Cd treatment resulted in an induction of HO-1 gene expression (Fig. 1a). Densitometric analysis revealed that after a 48 h exposure the steady-state level of HO-1 mRNA increased by 110% (Fig. 1b). When gene expression in roots was analyzed, a 39% decrease in HO-1 transcript levels was found (Fig. 2a, b). These results demonstrate that in soybean plants exposed to 200 μM Cd for 48 h, HO-1 gene expression is induced in nodules, but down-regulated in roots. However, after exposure to either 50 or 200 μM Cd for 72 h, transcript levels in both nodules and roots were decreased (Fig. 1, 2).

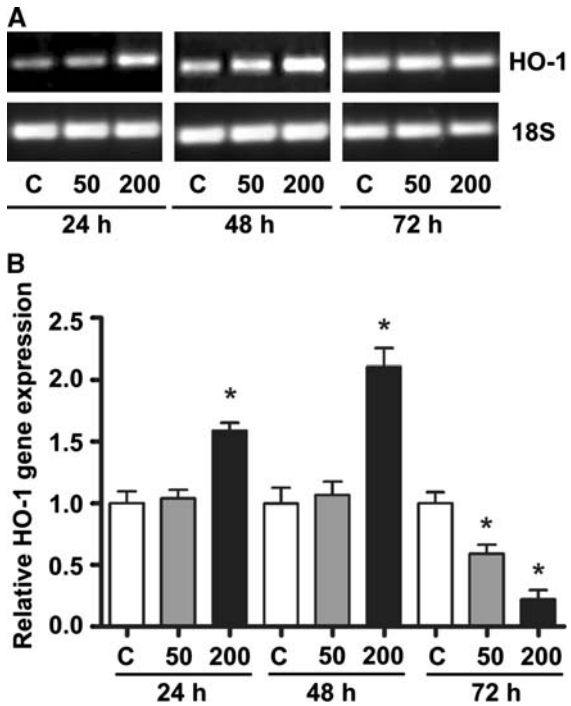

Fig. 1 HO-1 gene expression in soybean nodules exposed to different cadmium concentrations (50 or 200 μM) during 24, 48 or 78 h. (**a**) HO-1 mRNA expression was analyzed by semiquantitative RT-PCR as described in Materials and methods. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (**b**) Relative HO-1 transcript expression taking control as 1 unit. Values are the mean of three independent experiments ($n = 9$) and bars indicate S.E. $^*P < 0.001$ respect to control according to Tukey's multiple range test

Effect of Cd treatment on CAT gene expression

To compare HO-1 gene expression with that of a classical antioxidant enzyme, CAT gene expression was analyzed in nodules (Fig. 3) and roots (Fig. 4) following treatment with 50 or 200 μM Cd. Exposure of plants to 200 μM Cd for 48 h led to an increase in CAT mRNA in nodules (30%) (Fig. 3b), but to a decrease in roots (52%) (Fig. 4b). Exposure to 50 μM Cd for 72 h resulted in a 63% decrease in CAT transcript level in roots (Fig. 4b) but no difference was observed in nodules (Fig. 3b). On the other hand, CAT mRNA levels decreased in nodules (40%) and roots (83%) following exposure to 200 μM Cd (Figs. 3, 4).

Heme oxygenase and CAT protein accumulation and activities

To assess whether transcriptional regulation correlated with HO-1 and CAT protein contents, immunoblot analysis were performed. Because the greatest increase in transcript levels was observed in plants treated with 200 μM Cd for 48 h, protein

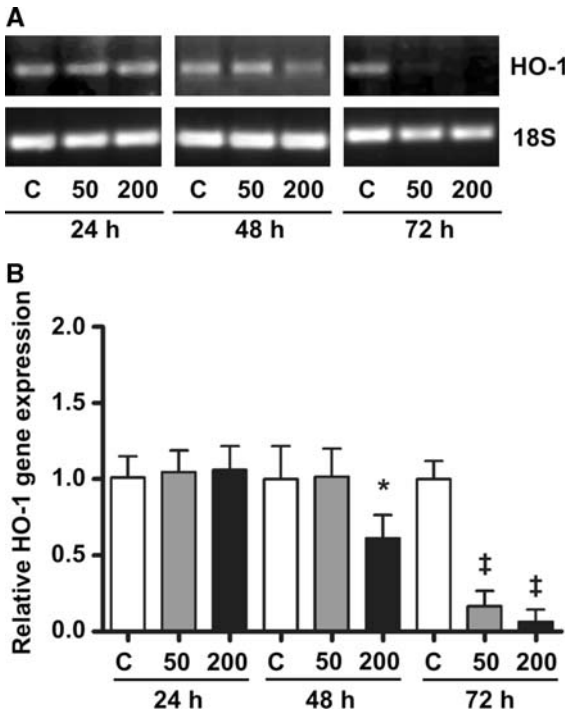

Fig. 2 HO-1 gene expression in soybean roots exposed to different cadmium concentrations (50 or 200 μM) during 24, 48 or 78 h. (**a**) HO-1 mRNA expression was analyzed by semiquantitative RT-PCR as described in Materials and methods. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (**b**) Relative HO-1 transcript expression taking control as 1 unit. Values are the mean of three independent experiments ($n = 9$) and bars indicate S.E. $*P < 0.05$, $‡P < 0.001$ respect to control according to Tukey's multiple range test

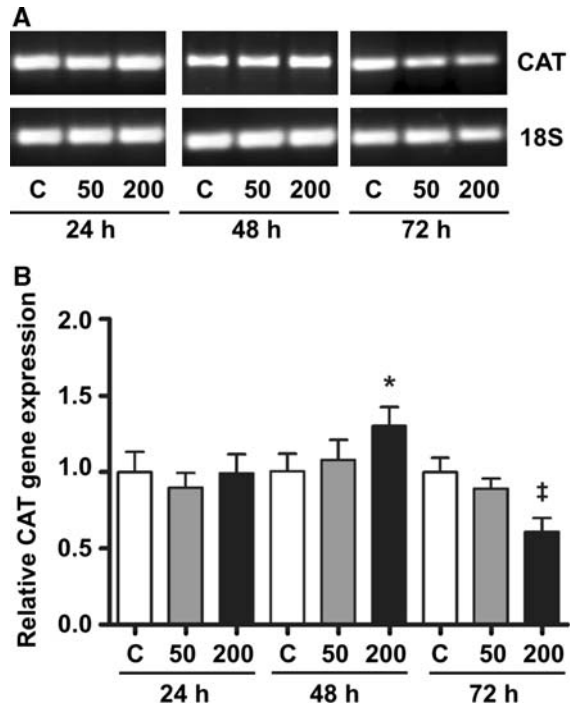

Fig. 3 Catalase gene expression in soybean nodules exposed to different cadmium concentrations (50 or 200 μM) during 24, 48 or 78 h. (**a**) CAT mRNA expression was analyzed by semiquantitative RT-PCR as described in Materials and methods. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (**b**) Relative CAT transcript expression taking control as 1 unit. Values are the mean of three independent experiments ($n = 9$) and bars indicate S.E. $*P < 0.05$, $‡P < 0.001$ respect to control according to Tukey's multiple range test

analyses focused on these conditions. In root extracts, the antibody raised against *Arabidopsis thaliana* HO-1 recognized a single band of approximately 30 kDa, a mass consistent to that previously report for HO of soybean nodule and other species (Baudoin et al. 2004) (Fig. 5). Densitometric analysis indicated that only HO-1 mRNA levels in nodules correlated with protein accumulation (Fig. 5b) and enzyme activity (Table 1). No correlation was found between transcript levels and protein amount (Fig. 5b) or activity (Table 1) in roots. CAT transcript levels did correlate with the amount of CAT protein. As shown in Fig. 5b, CAT protein increased 3.5-fold increased in nodules and decreased by 65% in roots following 200 μM Cd treatment. As shown in Table 2, in all cases CAT transcript and protein levels correlated with enzyme activity.

Discussion

Heme oxygenase has been recently described as a molecule involved in the defense against Cd induced oxidative stress in soybean leaves and nodules (Noriega et al. 2004; Balestrasse et al. 2005a). In this study, we have demonstrated that augmented HO activity in the nodules was associated to enhanced HO-1 mRNA levels and protein amount. In addition, we showed for the first time a different behavior in roots, implicating that differential response occurred in both tissues under Cd treatment.

Several authors have demonstrated that oxidative stress caused by Cd in plants leads to increased expression and activities of antioxidant enzymes, such as CAT (Chaoui et al. 1997; Metwally et al. 2003, 2005; Skorzynska-Polit et al. 2003). Moreover, Skorzynska-Polit et al. (2003) showed that antioxidant

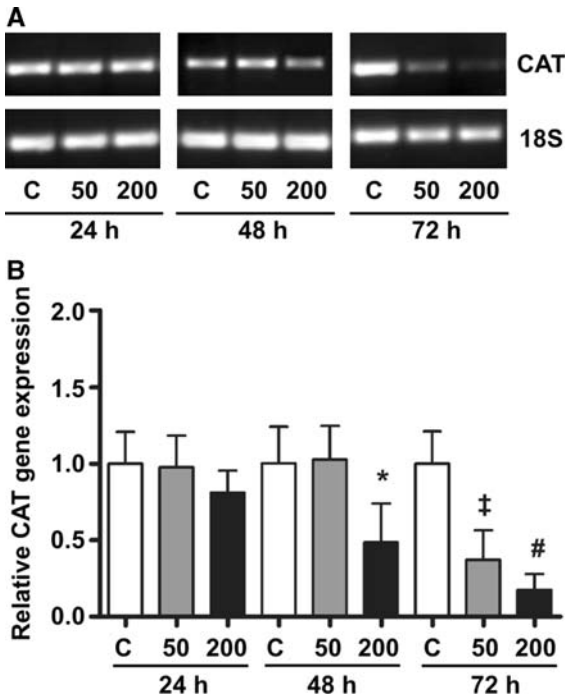

Fig. 4 Catalase gene expression in soybean roots exposed to different cadmium concentrations (50 or 200 μM) during 24, 48 or 78 h. (**a**) CAT mRNA expression was analyzed by semiquantitative RT-PCR as described in Materials and methods. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (**b**) Relative CAT transcript expression taking control as 1 unit. Values are the mean of three independent experiments ($n = 9$) and bars indicate S.E. $*P < 0.05$, $‡P < 0.01$, $\#P < 0.001$ respect to control according to Tukey's multiple range test

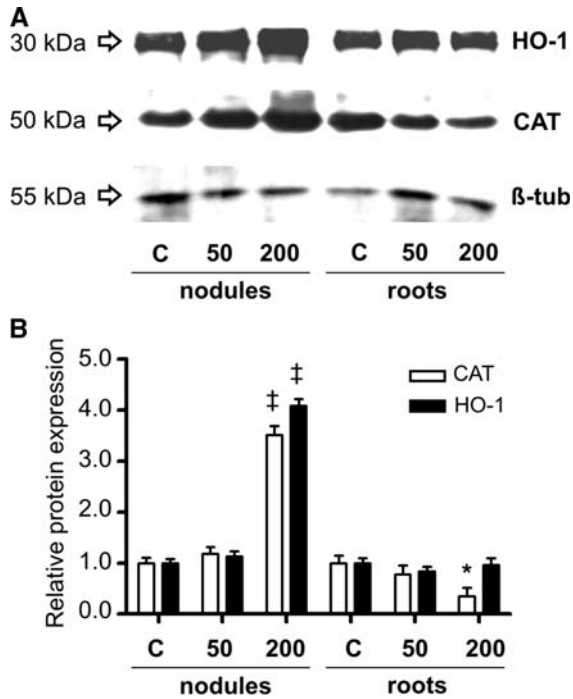

Fig. 5 HO-1 and CAT protein expression in soybean nodules and roots exposed to different cadmium doses (50 or 200 μM) after 48 h. (**a**) HO-1 and CAT protein expression was analyzed by Western-blot as described in Materials and methods. β-tubulin (β-tub) inmunoblotting was performed as an internal control of protein loading. (**b**) Relative HO-1 and CAT protein expression taking control as 1 unit. Values are the mean of three independent experiments ($n = 9$) and bars indicate S.E. $*P < 0.05$, $‡P < 0.001$ respect to control according to Tukey's multiple range test

activities change depending on enzyme and external Cd concentration. There is also evidence that a decrease in CAT activity may occur as a result of oxidative stress in different plant species (Shim et al. 2003). In the present study, CAT activity was enhanced in nodules whereas a significant decrease was observed in roots after 48 h of 200 μM Cd treatment. These changes may be explained, at least in part, through mechanisms that involve regulation of CAT gene and protein expression. These data are consistent with a previous report (Balestrasse et al. 2001) that showed evident signals of oxidative stress generation in soybean plants treated with 200 μM Cd, such as enhanced TBARS content and unaltered GSH levels in nodules and increased TBARS content and decreased GSH levels in roots. In view of these results, we established that soybean roots are more affected than nodules by Cd exposure.

Table 1 Effect of Cd treatment on HO-1 activity in nodules and roots

Treatment	HO-1 (U/mg protein)	
	Nodules	Roots
Control	4.21 ± 0.41a	0.30 ± 0.02a
50 μM	3.90 ± 0.22a	0.35 ± 0.03a
200 μM	42.15 ± 4.21b	0.31 ± 0.02a

Enzyme activity was assayed as described in Materials and methods section. Data are mean values of three independent experiments ± S.E. Each value represents three replicates. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of the enzyme forms 1 nmol of biliverdin/30 min under the assay conditions

Recent reports demonstrated that following an acute Cd treatment an enhancement of HO activity occurs in nodules as a response to this insult

Table 2 Effect of Cd on catalase activity in nodules and roots

Treatment	CAT (pmol/mg protein)	
	Nodules	Roots
Control	1.1 ± 0.1a	0.71 ± 0.05a
50 μM	1.2 ± 0.1a	0.70 ± 0.05a
200 μM	2.5 ± 0.2b	0.31 ± 0.02b

Data are mean values of three independent experiments ± S.E. Each value represents three replicates. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

(Balestrasse et al. 2005a). Nevertheless, until now there is no information about the mechanism underlying this behavior. Up to 48 h, an enhancement of HO-1 mRNA in nodules was observed as a result of 200 μM Cd treatment. When protein content and activity were studied, a positive correlation was found, among these parameters. This fact leads to an increase in the synthesis of BV, which is an efficient scavenger of ROS (Otterbein et al. 2003). These results are in agreement with that obtained in soybean leaves employing a physical stressor, such as UV-B radiation (Yannarelli et al. 2006). These authors have demonstrated that HO is up regulated in a dose-dependent manner as a mechanism of cell protection against oxidative damage, and that such response occurred as a consequence of HO-1 mRNA enhancement involving ROS formation.

A different behavior was found in roots. After 48 h treatment, the diminution of HO-1 mRNA in this tissue was not paralleled with protein content and activity, which remained unchanged respect to controls. At this time point, CAT activity was also diminished implying that the antioxidant defenses could be overwhelmed. In this context, Balestrasse et al. (2005b) demonstrated that there were alterations in roots and nodules viability from 48 up to 144 h treatment, with roots being more affected than nodules. Moreover, it was demonstrated the occurrence of marked nitrogen metabolism perturbations and the appearance of senescence indicators in both tissues (Balestrasse et al. 2004). These results could now be explained through the data obtained at 72 h Cd treatment, when HO-1 and CAT transcripts were significantly decreased either in nodules or in roots treated with 200 μM Cd.

Taking together, the early and the present data let us suppose that in nodules the induction of HO occurred as a hallmark of cell protection against oxidative damage. Catalase is also up-regulated to safeguard normal cellular functions and survival, but the enhancement in oxidative stress parameters (Balestrasse et al. 2001) demonstrated that the observed induction of HO and CAT could not fully cope with the oxidative stress elicited by Cd. However, in roots, CAT activity is decreased by a down-regulation in transcript and protein contents. These results, together with those found for HO-1, may explain the major root sensitive to Cd-induced oxidative stress. It is worth to mention that Cd concentration is higher in roots than in other tissues (Lozano-Rodríguez et al. 1997) because of the presence of specific metal-binding peptides, mainly chelatins (Gratão et al. 2005). It emerges from our results that the oxidative insult was far more important in roots than in nodules. Endogenous production of ROS induces modifications of proteins, such as fragmentation, increased susceptibility to proteolysis and cross-linking reactions (Berlett and Stadtman 1997). In this regard, Romero-Puertas et al. (2002) showed that in leaves of Cd-treated pea plants, CAT was oxidized and subjected to increased proteolytic degradation caused by Cd toxicity. Therefore, the oxidative stress generated by Cd could be responsible for the diminution of HO and CAT expressions in roots. It is worth to mention that, the heme pool is much more abundant in nodules than in roots due to the symbiosis with *Bradyrhizobium japonicum* (Santana et al. 1998) and that HO-1 is involved in leghemoglobin metabolism in mature alfalfa nodules (Baudouin et al. 2004). In animals, the efficiency of heme degradation is enhanced by the ability of heme itself to activate HO-1 transcription (Poss and Tonegawa 1997). Up to day, the ability of hemin to induce plant genes has not been demonstrated. Because of the high availability of free heme pool, the induction of HO can occur in nodules together with heme-proteins synthesis.

In conclusion, our data demonstrated that as a consequence of the high stress elicited by Cd, a down-regulation of HO-1 and CAT gene expression occurred in soybean roots, whereas an up-regulation was found in the nodules, at least up to 48 h Cd exposure. This behavior could explain the major viability observed in nodules respect to roots and also demonstrate that HO-1 shows a similar response to a classical heme protein involved in the antioxidant

defense system in plant tissues. Findings here reported showed that an inducible HO plays a key role in the enzymatic antioxidant defense system in higher plants.

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