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Heme oxygenase up-regulation under salt stress protects nitrogen metabolism in nodules of soybean plants

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ABSTRACT

The behaviour of enzymes involved in nitrogen metabolism, as well as oxidative stress generation and heme oxygenase gene and protein expression and activity, were analysed in soybean (*Glycine max* L.) nodules exposed to 50, 100 and 200 mM NaCl concentrations. A significant increase in lipid peroxidation was found with 100 and 200 mM salt treatments. Moreover, superoxide dismutase, catalase and peroxidase activities were decreased under 100 and 200 mM salt. Nitrogenase activity and leghemeoglobin content were diminished and ammonium content increased only under 200 mM NaCl. At 100 mM NaCl, glutamine synthetase (GS) and NADH-glutamate dehydrogenase (GDH) activities were similar to controls, whereas a significant increase (64%) in NADH-glutamate synthase (GOGAT) activity was observed. GS activity did not change at 200 mM salt treatment, but GOGAT and GDH significantly decreased (40 and 50%, respectively). When gene and protein expression of GS and GOGAT were analysed, it was found that they were positively correlated with enzyme activities. In addition, heme oxygenase (HO) activity, protein synthesis and gene expression were significantly increased under 100 mM salt treatment. Our data demonstrated that the up-regulation of HO, as part of antioxidant defence system, could be protecting the soybean nodule nitrogen fixation and assimilation under saline stress conditions.

1. Introduction

Nitrogen is an essential plant macronutrient and its availability has a major influence on both yield and product quality. Leguminous plants are able to take up significant amounts of nitrogen through dinitrogen fixation by forming nodules in their roots in symbiosis with rhizobia. In bacteroids, dinitrogen is reduced to NH_4^+ by the enzyme nitrogenase and it is then exported into the surrounding nodule plant cell cytoplasm and assimilated into organic compounds (Simon-Rosin et al., 2003). Ammonium is toxic for the cell and it needs to be rapidly assimilated. This process is carried out by the concerted action of two highly regulated pathways (Masclaux-Daubresse et al., 2006): one through the action of NADH-glutamate dehydrogenase (GDH), which synthesizes glutamate from 2-oxoglutarate and ammonium and, alternatively, the couple glutamine synthetase (GS)/NADH-glutamate synthase (GOGAT), where NH_4^+ is incorporated into glutamine by GS, which is then converted with 2-oxoglutarate to glutamate by GOGAT (Azevedo et al., 2006). Glutamine (Gln) and glutamate (Glu) are donors for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Coruzzi and Last, 2000). GDH plays a minor role in glutamate biosynthesis, and principally participates in its oxidation (Khadri et al., 2001). This enzyme is present in large amounts in nodule cytosol and may also play an important detoxification role (Chopra et al., 2004).

Cultivation of agricultural crops in soil is limited by salt stress, which arises from the excessive uptake of salt by plants and it is an unavoidable consequence of high ion concentrations. Excessive amounts of salt in the soil, most commonly NaCl, has detrimental effects on plant growth and productivity (Soussi et al., 1998; Reynolds et al., 2005). In this regard, it is known that salinity could affect the activities of enzymes involved in nitrogen metabolism (Mansour et al., 2002; Santos et al., 2002), but the mechanism underlying this phenomenon still remained unclear. It is known that the establishment and activity of the legume–*Rhizobium* symbiosis is susceptible to salinity (Rao et al., 2002). The responses of plant genotypes to salinity differ widely, depending on the form and dosage of salt and the stage of plant growth (Rogers et al., 1998). In addition, under hyperosmotic conditions the balance between synthesis and deactivation of reactive oxygen species (ROS) is disturbed, enhancing intracellular oxida-

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tive stress as a secondary effect (for a review, see Parida and Das, 2005).

Heme oxygenase (HO) catalyses the stereo-specific cleavage of heme to biliverdin with the release of free iron and carbon monoxide (Tenhunen et al., 1968). It has been widely studied in animal tissues, particularly in the liver (Tomaro and Batlle, 2002 and references herein). Heme oxygenase is involved in heme degradation and participates in the antioxidant machinery of the cells by means of its product biliverdin, which in turn is subsequently converted to bilirubin through the action of biliverdin reductase. It has been demonstrated that one of the three known mammalian isoforms, HO-1, is induced in animal tissues by many factors including its own substrate heme, several heme-proteins, heavy metals, UV-A radiation, hypoxia, hyperoxia, among others (Tomaro et al., 1991). Reports from our laboratory have shown for the first time the presence, in soybean leaves and nodules, of one HO closely related to the HO-1 of mammalian cells, on the basis of its induction by prooxidants and its antioxidant behaviour under Cd and UV-B stresses, indicating that HO plays a protective role against oxidative cell damage in soybean plants (Noriega et al., 2004; Balestrasse et al., 2005, 2006; Yannarelli et al., 2006). In view of these considerations, it is of interest to examine: (1) whether salt-generated oxidative stress could provoke HO induction and (2) whether this induction affects the enzymes involved in nitrogen metabolism in soybean nodules. To this end, the effects of NaCl on HO behaviour and oxidative stress parameters were analysed. Besides, mRNA and protein expression of the main assimilation enzymes, such as GS and GOGAT were determined. The activities of nitrogen fixation and assimilation enzymes were also evaluated. Findings here reported first provided evidence about the protective effect of HO-1 on soybean nodule nitrogen fixation and assimilation under saline stress conditions.

2. Material and methods

2.1. Chemicals

ATP, NADH, NADPH, 2-oxoglutarate, γ-glutamyl hydroxamate and NBT were from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Plant material and treatments

Surface sterilised soybean seeds (*Glycine max* L.) (A6445RG) were germinated directly in plastic pots containing moistened vermiculite in controlled environmental chambers, with a photoperiod of 16 h, photon flux density of 175 μmol m^{-2} s^{-1}, and a day/night regime of 25/20 °C and were simultaneously inoculated with *Bradyrhizobium japonicum* strain E 109 (INTA, Castelar). After this, they were watered daily with a nutrient solution (Hoagland and Arnon, 1950) during the first 5 days and then with a N-free nutrient solution. After 4 weeks, plants were treated with nutrient solution devoid of salt (Control) or containing 50, 100 or 200 mM of NaCl. After 10 days of treatment, nodules were isolated and used for the determinations.

2.3. 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 leaves (0.3 g) were homogenised in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 × *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 100 μl 4% butylated hydroxytoluene (BHT) in ethanol was 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. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.4. Leghemoglobin determination

Nodules (0.3 g) were homogenised in 3 ml of extraction medium containing 0.02% (w/v) potassium ferricyanide and 0.1% sodium bicarbonate. Leghemoglobin was estimated in the red supernatant (nodule cytosol) obtained after centrifugation of homogenates, using a fluorometric method as described by La Rue and Child (1979). Bovine hemoglobin was used as standard.

2.5. Ammonium determination

Plant material (0.3 g of nodule) was homogenised in 3 ml of 0.3 mM H_2SO_4 and centrifuged at 15,000 × *g* for 15 min. Ammonium content was measured in the supernatant by the phenol-hypochlorite method (Weatherburn, 1967). A calibration curve with NH_4Cl was used as the standard.

2.6. Nitrogen fixation assay

Nitrogen fixation was measured as acetylene reduction activity (Hardy et al., 1968). Nodules were enclosed in 100 ml bottles sealed with rubber stoppers containing C_2H_2 (10%, v/v) in air. Gas samples (0.5 ml) were taken 60 min later and analysed for ethylene in a Konik 3000 HRGC chromatograph equipped with a hydrogen flame ionisation detector (Hewlett Packard fused silica capillary HP-Plot Al_2O_3 column; oven temperature: 120 °C; carrier gas: N_2 at a rate of 30 ml min^{-1}).

2.7. Superoxide dismutase, peroxidase and catalase preparations and assays

Extracts for determination of SOD, CAT and POD activities were prepared from 0.3 g of nodules homogenised 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 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} (Chance et al., 1979). Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana et al. (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 light bank consisting of six 15 W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light 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 amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions (Giannopolitis and Ries, 1977). POD activity was determined in the homogenates by measuring the increase

in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction contained extract, 50 mM buffer K-phosphate 50 mM, pH 7, 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2. One unit of POD forms 1 μmol of guaiacol oxidised per min under the assay conditions.

2.8. Heme oxygenase preparation and assay

Nodules (0.3 g) were homogenised in a Potter-Elvehejm homogenizer using 1.2 ml 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) contained 250 μl of extract (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 30 min and 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). One unit of the enzyme forms 1 nmol of biliverdin in 30 min under assay conditions.

2.9. Preparations and assays of nitrogen assimilation enzymes

Extracts for the determination of GDH and GOGAT activities and expression were prepared from 0.3 g of nodules homogenised in 3.0 ml of extraction buffer containing 100 mM MES NaOH buffer (pH 6.8), 100 M sucrose, 2% (v/v) 2-mercaptoethanol and 15% (v/v) ethylene glycol at 4 °C. Homogenates were centrifuged at 10,000 × *g* for 20 min, and the supernatant was used for the assays. GDH and GOGAT were determined in the homogenates by measuring the decrease in absorption at 340 nm due to NADH oxidation in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.6), 0.1% (v/v) 2-mercaptoethanol, 100 μM NADH, 2.5 mM 2-oxoglutarate and 200 mM $(NH_4)_2SO_4$ or 100 mM glutamine for GDH and GOGAT, respectively (Groat and Vance, 1981). One unit of GDH and GOGAT oxidizes 1 μmol of NADH per min under the assay conditions. Plant material from GS determination was extracted in 50 mM Tris–HCl buffer (pH 7.5) containing 1.0 mM 2-mercaptoethanol and 2 mM EDTA, and homogenised at 4 °C. Following centrifugation (15,000 × *g* for 30 min), GS activity was measured in an assay buffer consisting of 50 mM Tris–HCl (pH 7.5), 4.0 mM ATP, 80 mM Na-glutamate, 30 mM $MgSO_4$, 10 mM NH_2 OH and 30 mM cysteine (Kanamori and Matsumoto, 1972). A standard curve was prepared using γ-glutamyl hydroxamate. One unit of GS forms 1 μmol of γ-glutamyl hydroxamate per min under the assay conditions.

2.10. 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 *G. max* HO-1 and 18S specific primers, as previously described (Yannarelli et al., 2006). In addition, the reverse-transcribed material was amplified by use of a primer pair specific to *G. max* GS cDNA (sense primer, 5′-ATGAGGAGCAAAGCAAGGAC-3′; antisense primer, 5′-CCATACCAGGGTTCTTCAGC-3′) and GOGAT cDNA (sense primer, 5′-GCGGCTATTCTGAAGGACTG-3′; antisense primer, 5′-TACGGGGTTCCTCATACTGC-3′). The PCR profile for GS was set at 94 °C for 1 min and then 31 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. GOGAT amplification was carried out using 29 cycles under the same conditions. Each primer set was amplified using an optimised number 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 analysed using Gel-Pro Analyser 3.1 software (Media Cybernetics, MD, USA). The ratios of HO-1, GS and GOGAT mRNA to 18S mRNA were quantified.

2.11. Western-blots analysis for HO, GOGAT and GS proteins

Proteins from nodules were subjected to denaturing SDS-PAGE in a Mini-PROTEAN 3 System (Bio-Rad Laboratories, CA, USA). SDS-PAGE was performed in 7.5% for GOGAT and 12% gels for GS and HO (4% stacking gels), respectively 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 non-fat dry milk dissolved in TTBS (20 mM Tris–HCl at 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 antibodies against barley leaf GOGAT for GOGAT (dilution 1:1000) or purified GS from *Phaseolus vulgaris* root nodules (Cullimore and Miflin, 1984) for GS (dilution 1:1000) or *Arabidopsis thaliana* HO-1 (dilution 1:2000) (Muramoto et al., 1999). The antibodies for GOGAT, GS and HO-1 were diluted in TTBS with 2% (w/v) powdered non-fat dry milk. The blots were washed twice for 10 min each with TTBS. 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 inmunodetection system, ECL Western Blotting protocols, Dako). The intensity of bands was analysed with Gel-Pro Analyzer 3.1 software (Media Cybernetics). The films were scanned (Fotodyne Incorporated, WI, USA) and analysed using Gel-Pro Analyzer 3.1 software (Media Cybernetics).

2.12. Protein determination

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

2.13. Statistics

All treatments were repeated four times, with newly grown plants. Data in the text and tables indicate mean values ± S.E. Differences among treatments were analysed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

3. Results

3.1. Oxidative stress generation

In order to evaluate the oxidative stress generated by saline conditions, TBARS formation was determined in soybean plants subjected to 50, 100 or 200 mM NaCl during 10 days. TBARS formation remained unaltered in nodules treated with 50 mM NaCl while a 29 and 64% increase was found with 100 and 200 mM salt treatments, respectively (Fig. 1).

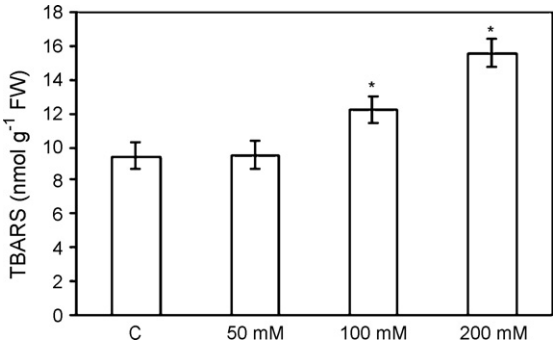

Fig. 1. Effect of different salt concentrations on nodules TBARS content. Values are the mean of four independent experiments and bars indicate S.E. $^{*}P < 0.05$ compared with control according to Tukey's multiple range test.

Taking into account the fact that oxidative stress could be produced by a decrease in antioxidant defences, the activities of the main antioxidant enzymes, such as SOD, CAT and POD were also analysed. As shown in Table 1, SOD, CAT and POD activities decreased with 100 mM NaCl (40, 30 and 27%, respectively, with respect to controls). These decrease were higher under 200 mM salt treatment (78, 48 and 60%, respectively), whereas 50 mM NaCl did not affect these enzyme activities, compared to control values (Table 1).

Finally, the effect of different salt concentrations on HO activity, which is considered a new antioxidant enzyme in plant tissues, was assessed. As it can be seen in Table 1, HO activity remained unchanged after 50 mM salt treatment but an impressive increase of 3.2-fold was observed at 100 mM NaCl respect to controls. Interestingly, 200 mM salt condition produced a drastic decrease (80%) in HO activity.

3.2. Leghemoglobin content and nitrogen fixation

Leghemoglobin (Lb) content and nitrogenase activity were measured as indicators of nodules effectiveness. Both parameters showed a similar behaviour at the three salt concentrations with a decrease of 67 and 60% in Lb and nitrogenase activity at 200 mM salt, respectively, and no changes were observed neither in 50 mM nor in 100 mM NaCl, with respect to control values (Table 2).

3.3. Ammonium content

The ammonium levels were enhanced in soybean nodules only at the highest salt concentration. A significant increase (100%) was observed with 200 mM NaCl, respect to the controls (Fig. 2). Under 50 and 100 mM salt treatment, the ammonium content remained similar to control values.

3.4. Activities of enzymes involved in nitrogen assimilation

A significant increment by about 50% was found in GS and GOGAT activities at 50 mM NaCl, while GDH activity remained unaffected (Table 3). At 100 mM NaCl, GS and GDH activities were similar to controls, whereas a significant increase (64%) in GOGAT activity was observed. Despite that GS activity did not change at 200 mM salt treatment, GOGAT and GDH significantly decreased (40 and 50%, respectively), compared to controls (Table 3).

Table 2
Effect of different salt concentrations on nodules leghemeoglobin content and nitrogenase activity

Treatment	Leghemeoglobin (mg mg^{-1} protein)	Nitrogenase activity (μmol ethylene h^{-1} mg^{-1} protein)
Control	0.21 ± 0.01^{a}	0.45 ± 0.02^{a}
50 mM NaCl	0.20 ± 0.02^{a}	0.43 ± 0.01^{a}
100 mM NaCl	0.18 ± 0.01^{a}	0.41 ± 0.02^{a}
200 mM NaCl	0.07 ± 0.01^{b}	0.18 ± 0.02^{b}

Data are mean values of four independent experiments ± S.E. Different letters within columns (a and b) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

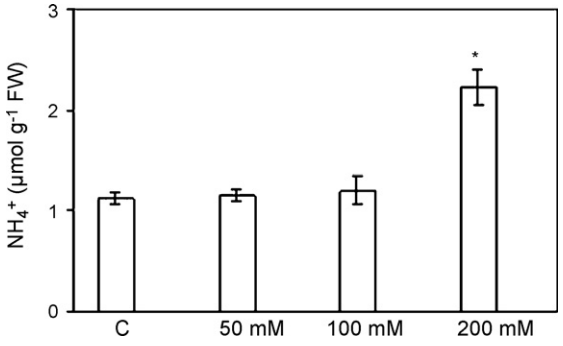

Fig. 2. Effect of different salt concentrations on nodules ammonium content. Values are the mean of four independent experiments and bars indicate S.E. $^{*}P < 0.05$ compared with control according to Tukey's multiple range test.

3.5. Gene and protein expression of enzymes involved in nitrogen assimilation

Gene expression of GS and GOGAT were subsequently analysed (Fig. 3). Both enzymes showed increased transcript levels at 50 mM salt treatment (31 and 21%, respectively). Under higher NaCl concentration, GS mRNA remained unchanged (100 mM) and decreased by about 28% at 200 mM salt, respect to controls (Fig. 3b). On the other hand, a 51% increase was found in GOGAT gene expression at 100 mM NaCl, but a significant decrease (42%) was observed under 200 mM salt concentration (Fig. 3b). As it can be seen in Fig. 4, GS and GOGAT protein levels showed a similar behaviour to that found for gene expression. It is noteworthy that mRNA and protein levels were also positively correlated with enzyme activities.

Table 1
Effect of different salt concentrations on nodules antioxidant enzyme activities

Treatment	SOD (U mg^{-1} protein)	CAT (pmol mg^{-1} protein)	POD (U mg^{-1} protein)	HO-1 (U mg^{-1} protein)
Control	11.5 ± 0.1^{a}	1.20 ± 0.10^{a}	0.30 ± 0.03^{a}	1.04 ± 0.01^{a}
50 mM NaCl	11.2 ± 0.1^{a}	1.10 ± 0.10^{a}	0.32 ± 0.02^{a}	1.06 ± 0.01^{a}
100 mM NaCl	6.9 ± 0.1^{b}	0.85 ± 0.02^{b}	0.22 ± 0.02^{b}	3.20 ± 0.30^{b}
200 mM NaCl	2.5 ± 0.2^{c}	0.62 ± 0.02^{c}	0.12 ± 0.01^{c}	0.20 ± 0.02^{c}

Data are mean values of four independent experiments ± S.E. Enzymatic activities were assayed as described in Section 2. Different letters within columns (a–c) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table 3
Effect of different salt concentrations on nitrogen assimilation enzymes

Treatment	GS (U mg^{-1} protein)	GOGAT (U mg^{-1} protein)	GDH (U mg^{-1} protein)
Control	0.36 ± 0.02^{a}	0.33 ± 0.04^{a}	3.35 ± 0.29^{a}
50 mM NaCl	0.54 ± 0.02^{b}	0.50 ± 0.01^{b}	3.86 ± 0.44^{a}
100 mM NaCl	0.35 ± 0.03^{a}	0.54 ± 0.05^{b}	3.84 ± 0.41^{a}
200 mM NaCl	0.41 ± 0.04^{a}	0.20 ± 0.02^{c}	1.65 ± 0.78^{b}

Data are mean values of four independent experiments ± S.E. Enzymatic activities were assayed as described in Section 2. Different letters within columns (a–c) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

3.6. Effect of salt stress on HO-1 gene expression

We analysed the expression of HO-1 mRNA in soybean nodules in response to 50, 100 and 200 mM NaCl treatments (Fig. 3). Semi-quantitative RT-PCR revealed that 100 mM NaCl enhanced HO-1 gene expression, whereas the level of 18S was unaffected throughout all the experiments (Fig. 3a). Densitometric analysis showed that HO-1 mRNA increased by about 79% after 100 mM NaCl treatment respect to controls, while at 50 and 200 mM salt exposure no significant changes were observed (Fig. 3b). These results demonstrated that 100 mM NaCl-treated plants overexpressed the HO-1 gene transcript.

3.7. Heme oxygenase protein expression

To assess whether the aforementioned different mRNA levels reflected changes in HO protein expression, inmumoblot analysis were performed. The HO-1 antibody raised against *A. thaliana* recognised a single band of approximately 30 kDa in nodules of soybean plants, as demonstrated in a previous report (Fig. 4a) (Balestrasse et al., 2005). As shown in Fig. 4b, HO-1 protein level increased two-fold at 100 mM NaCl, respect to controls. The lowest (50 mM) and the highest (200 mM) salt treatment did not affect HO-1 protein expression, which remained similar to control values. These results indicated that the enhanced mRNA levels determined at 100 mM NaCl were associated with augmented HO-1 protein expression.

4. Discussion

There is abundant literature about the effects of salinity on ROS generation (Tejera et al., 2004; López-Gómez et al., 2007) and on the activities of nitrogen fixation and assimilation enzymes (Cordovilla et al., 1999; Khadri et al., 2001; Dluzniewska et al., 2007), but the mechanisms implicated remains unclear. In addition, no information is still available regarding HO behaviour under salt stress. In this report, we demonstrated the effect of salt stress on gene expres-

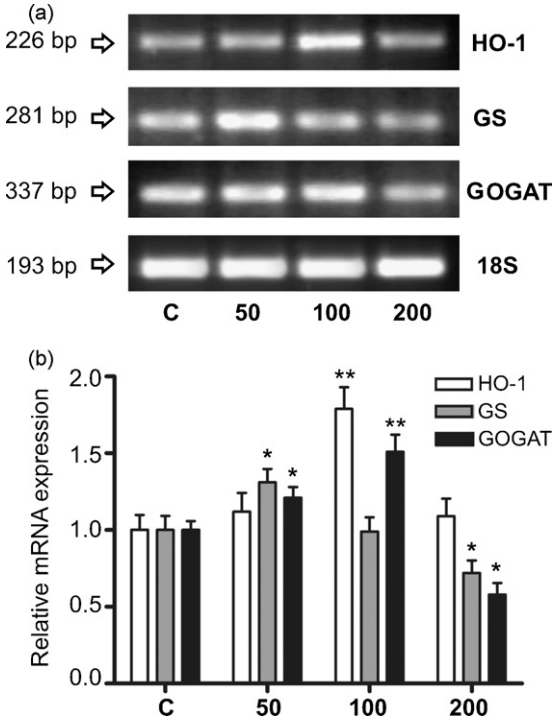

Fig. 3. HO-1, GS and GOGAT transcript levels in nodules of control (C) and salt-treated (50, 100 and 200 mM) soybean plants. (a) HO-1, GS and GOGAT mRNA expressions were analysed by semi-quantitative RT-PCR as described in Section 2.10. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (b) Relative HO-1, GS and GOGAT transcript levels expressed as the ratio of HO-1, GS and GOGAT mRNAs to 18S mRNA. Values are the mean of four independent experiments and bars indicate S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control according to Tukey's multiple range test.

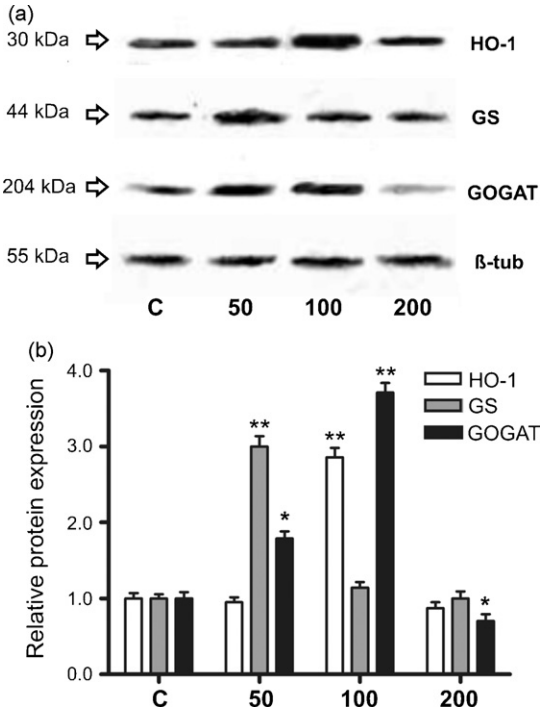

Fig. 4. HO-1, GS and GOGAT protein expressions in nodules of control (C) and salt-treated (50, 100 and 200 mM) soybean plants. (a) HO-1, GS and GOGAT protein expressions were analysed by Western blotting as described in Section 2.11. β-Tubulin (β-tub) inmunoblotting was performed as an internal control of protein loading. (b) Relative HO-1, GS and GOGAT protein expressions expressed as the ratio of HO-1, GS and GOGAT to β-tub. Values are the mean of four independent experiments and bars indicate S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control according to Tukey's multiple range test.

sion, protein amount and activity of two key enzymes involved in nitrogen assimilation such as GS and GOGAT together with HO.

Oxidative stress may be defined as an increment of oxidant species and/or a depletion of antioxidant defences. Our data shown that, under 50 mM NaCl, TBARS levels and antioxidant enzyme activities remained unchanged respect to controls, indicating that this salt concentration did not produce oxidative damage (Fig. 1 and Table 1). An increase in TBARS content and a significant decrease in the classical antioxidant enzymes (SOD, CAT and POD) activities were observed when plants were treated with 100 mM NaCl (Fig. 1 and Table 1). The present study make evident that under this salt concentration an impressive increase in HO gene expression, protein amount and activity occurred (Figs. 3 and 4 and Table 1). Previous reports have shown that an enhancement of HO activity contributes to counteract oxidative stress generation (Noriega et al., 2004; Balestrasse et al., 2005; Yannarelli et al., 2006). This antioxidant response may prevent Lb breakdown and nitrogenase activity diminution (Table 2), as it is widely accepted that this two parameters vary in parallel under different situations of stress conditions (Balestrasse et al., 2003). It is interesting to note that under 100 mM NaCl SOD, CAT and POD activities are inhibited, while HO is markedly increased. The antioxidant protection afforded by the latter may allow the correct nitrogen fixation. On the other hand, under 200 mM NaCl, TBARS content increased and the antioxidant enzymes (SOD, CAT, POD and HO) activities were diminished (Table 1), indicating that an oxidative burst occurred. It is noteworthy, that HO-1 gene expression and protein amount were unaffected under 200 mM NaCl, but its activity was drastically diminished. Taking into account the fact that heme compounds up-regulate HO activity (Tenhunen et al., 1968; Tomaro et al., 1991), we can consider that the significant diminution of Lb content could be responsible for this inhibition (Table 2). It is well known that Lb transports and regulates O_2 concentration in the nodules (Denison and Okano, 2003). In this way, the inhibition of nitrogenase could result from the effect of high salt on bacteroid O_2 uptake (Fernández-Pascual et al., 1996; Bolaños et al., 2006).

Previous reports have described that external salinization affects different steps of nitrogen metabolism. Moreover, enzymes involved in primary nitrogen assimilation also exhibit salt-dependent regulation. In order to know the effect of salt treatment on enzymes involved in nitrogen assimilation, the activities of GS, GOGAT and GDH were evaluated. GS increased at the lowest salt concentration and remained similar to control values at 100 and 200 mM NaCl (Table 3). GOGAT activity increased at 50 and 100 mM salt concentrations and significantly decrease (40%) at the highest salt treatment. These results are in agreement with those found by Cordovilla et al. (1999) which demonstrated that GS appeared to be more tolerant to salinity than GOGAT. Besides, it has been reported that GOGAT reduction in nodules resulted in plant having impaired nitrogen assimilation and altered carbon/nitrogen metabolic flux (Cordoba et al., 2003). GDH did not show any change at 50 and 100 mM NaCl, but decreased by about 50% when plants were treated with 200 mM NaCl (Table 3). The significant reduction in GOGAT and GDH activity could explain the enhancement of ammonium content found at 200 mM salt treatment (Fig. 2). Interestingly, these data also demonstrated that enzyme responses (induction or repression) often differ among species, cultivars and analysed tissues (Popova et al., 2002; Gu et al., 2004; Zhou et al., 2004).

In order to get hints on GS and GOGAT regulation under salt stress, their respective mRNA levels were determined in nodules by semi-quantitative RT-PCR. Data obtained herein indicated that 50 and 100 mM NaCl enhanced GOGAT mRNA levels, whereas GS mRNA only increased under 50 mM salt treatment (Fig. 3). Both enzymes showed a down-regulation in plants treated with 200 mM NaCl. Accordingly, a similar behaviour was found when the specific protein contents were assessed by Western blotting (Fig. 4). These results are positively correlated with enzymes activities and indicate that GS and GOGAT are regulated at transcriptional level in nodules under salt-induced oxidative stress.

5. Conclusion

Summing up, 100 mM NaCl treatment elicited the induction of HO-1 mRNA, protein amount and activity. The involvement of ROS in this process let us assume that the observed HO-1 mRNA enhancement could occur as a mechanism of cell protection against oxidative damage. However, no induction was found in plants subjected to 200 mM salt when ROS production overwhelmed the antioxidant capacity of the cell. Our data demonstrate for the first time the response of HO-1 to the oxidative stress generated by salinity in plants. In addition, our results strongly indicate that salt-induced up-regulation of HO-1 mRNA is involved in the effective nitrogen fixation and assimilation in nodules of soybean plants.

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References

Azevedo, R.A., Lancien, M., Lea, P.J., 2006. The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino Acids 30, 143–162.
Balestrasse, K.B., Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2003. Effect of cadmium stress on nitrogen metabolism in nodules and roots of soybean plants. Funct. Plant Biol. 30, 57–64.
Balestrasse, K.B., Noriega, G.O., Batlle, A.M.C., Tomaro, M.L., 2005. Involvement of heme oxygenase as antioxidant defence in soybean nodules. Free Radic. Res. 39, 145–151.
Balestrasse, K.B., Noriega, G.O., Batlle, A.M.C., Tomaro, M.L., 2006. Heme oxygenase activity and oxidative stress signaling in soybean leaves. Plant Sci. 170, 339–346.
Becana, M., Aparicio-Tejo, P., Irigoyen, J.J., Sánchez-Díaz, M., 1986. Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. Plant Physiol. 82, 1169–1171.
Bolaños, L., Martín, M., El-Hamdaoui, A., Rivilla, R., Bonilla, I., 2006. Nitrogenase inhibition in nodules from pea plants grown under salt stress occurs at the physiological level and can be alleviated by B and Ca. Plant Soil 280, 135–142.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.
Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527–605.
Chopra, J., Kaur, N., Gupta, A.K., 2004. The role of ammonium assimilating enzymes in lentil roots and nodules. Biol. Plantarum 47, 105–109.
Cordoba, E., Shishkova, S., Vance, C.P., Hernández, G., 2003. Antisense inhibition of NADH glutamate synthase impairs carbon/nitrogen assimilation in nodules of alfalfa (*Medicago sativa* L.). Plant J. 33, 1037–1049.
Cordovilla, M.P., Ligero, F., Lluch, C., 1999. Effect of salinity on growth, nodulation and nitrogen assimilation in nodules of faba bean (*Vicia faba* L.). Appl. Soil Ecol. 11, 1–7.
Coruzzi, G., Last, R., 2000. Amino acid. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), Biochemistry and Molecular Biology of Plants, pp. 358–410.
Cullimore, J.V., Miflin, B.J., 1984. Immunological studies on glutamine synthetase using antisera raised to the two plants forms of the enzyme from *P. vulgaris* root nodules. J. Exp. Bot. 35, 581–587.
Denison, F.R., Okano, Y., 2003. Leghaemeoglobin oxygenation gradients in alfalfa and yellow sweetclover nodules. J. Exp. Bot. 54, 1085–1091.
Dluzniewska, P., Gessler, A., Dietrich, H., Schnitzler, J., Teuber, M., Rennenberg, H., 2007. Nitrogen uptake and metabolism in *Populus* × *canescens* as affected by salinity. New Phytol. 173, 279–293.
Fernández-Pascual, M., de Lorenzo, C., de Felipe, M.R., Rajalakshmi, S., Gordon, A.J., Thomas, B.J., Minchin, F.R., 1996. Possible reasons for relative salt stress tolerance in nodules of white lupin cv. Multolupa. J. Exp. Bot. 47, 1709–1716.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol. 59, 309–314.
Groat, R.G., Vance, C.P., 1981. Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Plant Physiol. 67, 1198–1203.
Gu, R., Fonseca, S., Puskás, L.G., Hackler Jr., L., Zvara, Á., Dudits, D., Pais, M.S., 2004. Transcript identification and profiling during salt stress and recovery of *Populus euphratica*. Tree Physiol. 24, 265–276.
Hardy, R.W., Holstein, R.D., Jackson, E.K., Burns, R.C., 1968. The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43, 1185–1207.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.
Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Station Circular 347, 1–32.
Kanamori, T., Matsumoto, H., 1972. Glutamine synthetase from rice plant roots. Arch. Biochem. Biophys. 125, 400–412.
Khadri, M., Pliego, L., Soussi, M., Lluch, C., Ocaña, A., 2001. Ammonium assimilation and ureide metabolism in common bean (*Phaseolus vulgaris*) nodules under salt stress. Agronomie 21, 635–643.
La Rue, T.A., Child, J.J., 1979. Sensitive fluorometric assay for leghemoglobin. Anal. Biochem. 92, 11–15.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
López-Gómez, E., San Juana, M.A., Diaz-Vivancosa, P., Mataix Beneytob, J., García-Legaz, M.F., Hernández, J.A., 2007. Effect of rootstocks grafting and boron on the antioxidant systems and salinity tolerance of loquat plants (*Eriobotrya japonica* Lindl.). Env. Exp. Bot. 60, 151–158.
Mansour, M.M.F., Salama, K.H.A., Al-Mutawa, M.M., Abou Hadid, A.F., 2002. Effect of NaCl and polyamines on plasma membrane lipids of wheat roots. Biol. Plantarum 45 (2), 235–239.
Masclaux-Daubresse, C., Reisdorf-Cren, M., Pageau, K., Lelandais, M., Grandjean, O., Kronenberger, J., Valadier, M.H., Feraud, M., Jouglet, T., Suzuki, A., 2006. Glutamine synthetase–glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink–source nitrogen cycle in tobacco. Plant Physiol. 140, 444–456.
Muramoto, T., Kohchi, T., Yokota, A., Hwang, I., Goodman, H.M., 1999. The *Arabidopsis* photomorphogenic mutant HY1 is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid haeme oxygenase. Plant Cell 11, 335–348.
Muramoto, T., Tsurui, N., Terry, M.J., Yokota, A., Kohchi, T., 2002. Expression and biochemical properties of a ferredoxin-dependent haeme oxygenase required for phytochrome chromophore synthesis. Plant Physiol. 130, 1958–1966.
Noriega, G.O., Balestrasse, K.B., Batlle, A.M.C., Tomaro, M.L., 2004. Heme oxygenase exerts a protective role against oxidative stress in soybean leaves. Biochem. Biophys. Res. Commun. 323, 1003–1008.
Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotox. Environ. Safe. 60, 324–349.
Popova, O., Ismailov, S., Popova, T., Dietz, K.J., Golldack, D., 2002. Salt-induced expression of NADP-dependent isocitrate dehydrogenase and ferredoxin-dependent glutamate synthase in *Mesembryanthemum crystallinum*. Planta 215, 906–913.
Rao, D.L.N., Giller, K.E., Yeo, A.R., Flowers, T.J., 2002. The effect of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum*). Ann. Bot. 89, 563–570.
Reynolds, M.P., Mujeeb-Kazi, A., Sawkins, M., 2005. Prospects for utilising plant-adaptive mechanisms to improve wheat and other crops in drought- and salinity-prone environments. Ann. Appl. Biol. 146, 239–259.
Rogers, M.E., Grieve, C.M., Shannon, M., 1998. The response of lucerne (*Medicago sativa* L.) to sodium sulphate and chloride salinity. Plant Soil 202, 271–280.
Santos, C.V., Falcão, I.P., Pinto, G.C., Oliveira, H., Loureiro, J., 2002. Nutrient responses and glutamate and proline metabolism in sunflower plants and calli under Na_2SO_4 stress. J. Plant Nutr. Soil Sci. 165 (3), 366–372.
Simon-Rosin, U., Wood, C., Udvardi, M.K., 2003. Molecular and cellular characterisation of LjAMT2;1, an ammonium transporter from the model legume *Lotus japonicus*. Plant Mol. Biol. 51, 99–108.
Soussi, M., Ocaña, A., Lluch, C., 1998. Effects of salt stress on growth, photosynthesis and nitrogen fixation in chickpea (*Cicer arietinum* L.). J. Exp. Bot. 49, 1329–1337.
Tejera, N.A., Campos, R., Sanjuán, J., Lluch, C., 2004. Nitrogenase and antioxidant enzyme activities in *Phaseolus vulgaris* nodules formed by *Rhizobium tropici* isogenic strains with varying tolerance to salt stress. J. Plant Physiol. 161 (3), 329–338.
Tenhunen, R., Marver, H.S., Schmid, R., 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. PNAS USA 61, 748–755.
Tomaro, M.L., Batlle, A., 2002. Bilirubin: its role in cytoprotection against oxidative stress. Int. J. Biochem. Cell Biol. 34, 216–220.
Tomaro, M.L., Frydman, J., Frydman, R.B., 1991. Heme oxygenase induction by $CoCl_2$, Co-protoporphyrin IX, phenylhydrazine, and diamide: evidence for oxidative stress involvement. Arch. Biochem. Biophys. 226, 610–617.
Weatherburn, M.W., 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971–974.
Yannarelli, G.G., Noriega, G.O., Batlle, A., Tomaro, M.L., 2006. Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species. Planta 224, 1164–1172.
Zhou, W., Sun, Q.J., Zhang, C.F., Yuan, Y.Z., Zhang, J., Lu, B.B., 2004. Effect of salt stress on ammonium assimilation enzymes of the roots of rice (*Oryza sativa*) cultivars differing in salinity resistance. Acta Bot. Sin. 46, 921–927.