Effects of salt stress on the expression of antioxidant genes and proteins in the model legume *Lotus japonicus*

Maria C. Rubio*, Pilar Bustos-Sanmamed*, Maria R. Clemente and Manuel Becana

Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Apartado 13034, 50080 Zaragoza, Spain

* These authors contributed equally to this work.

Author for correspondence: *Manuel Becana*

Tel: +34 976716055

Fax: +34 976716145

becana@eead.csic.es

Abstract:

Salt stress negatively affects many physiological processes in plants. Some of these effects may involve the oxidative damage of cellular components, which can be promoted by reactive oxygen species and prevented by antioxidants.

The protective role of antioxidants was investigated in *Lotus japonicus* exposed to two salinization protocols: S1 (150 mM NaCl for 7 d) and S2 (50, 100 and 150 mM NaCl, each concentration for 6 d). Several markers of salt stress were measured and the expression of antioxidant genes was analyzed using quantitative reverse transcription–polymerase chain reaction and, in some cases, immunoblots and enzyme activity assays.

Leaves of S1 plants suffered from mild osmotic stress, accumulated proline but no Na⁺, and showed induction of many *superoxide dismutase* and *glutathione peroxidase* genes. Leaves of S2 plants showed increases in Na⁺ and Ca²⁺, decreases in K⁺, and accumulation of proline and malondialdehyde. In leaves and roots of S1 and S2 plants, the mRNA, protein and activity levels of the ascorbate-glutathione enzymes remained constant, with a few exceptions. Notably, there was consistent up-regulation of the gene encoding cytosolic dehydroascorbate reductase, and this was possibly related to its role in ascorbate recycling in the apoplast.

The overall results indicate that *L. japonicus* is more tolerant to salt stress than other legumes, which can be attributed to the capacity of the plant to prevent Na⁺ reaching the shoot and to activate antioxidant defenses.

Keywords:

antioxidants • dehydroascorbate reductase • gene expression • glutathione peroxidases • model legumes • salt stress • superoxide dismutases

Introduction:

Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop production. Currently, *c.* 20% of the world's cultivated land is affected by salinity, which results in the loss of *c.* 50% of agricultural yield (for reviews see Zhu, 2001; Bartels & Sunkar, 2005). Salt stress (NaCl) has both osmotic (cell dehydration) and toxic (ion accumulation) effects on plants, impairing growth, ion homeostasis, photosynthesis and nitrogen fixation, among other key physiological processes (Zhu, 2001; Munns, 2002; Tejera *et al.*, 2004; Bartels & Sunkar, 2005). The relative contributions of these two effects to the decrease in the overall performance of the plant vary with the intensity and duration of salt stress (Munns, 2002), as well as with the plant species, cultivar and tissue (Hernández *et al.*, 2000; Jungklang *et al.*, 2004).

Several studies have shown that reactive oxygen species (ROS) and oxidative stress may be mediating at least some of the toxic effects of NaCl on legumes (Hernández *et al.*, 1999, 2000; Jungklang *et al.*, 2004) and other vascular plants (Lee *et al.*, 2001; Mittova *et al.*, 2004; Attia *et al.*, 2008). Thus, in plants under optimal growth conditions, antioxidant defenses are able to cope with ROS, whereas, in plants exposed to salinity or other types of stressful conditions, the antioxidant capacity may be overwhelmed by ROS production. Tight control of the steady-state concentrations of ROS by antioxidants is critical to prevent oxidative damage in plant cells while simultaneously allowing ROS to perform useful functions as signal molecules (Mittler *et al.*, 2004). The antioxidant defenses of plants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and the four enzymes of the ascorbate-glutathione cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MR), dehydroascorbate reductase (DR) and glutathione reductase (GR). This cycle is fueled by ascorbate and glutathione, which are major redox compounds involved also in other important processes, such as photosynthesis, nitrogen fixation and organ development (Nakano & Asada, 1981; Dalton *et al.*, 1986; Pignocchi *et al.*, 2003). In some legume species and tissues, glutathione is partially or completely replaced by homoglutathione, another thiol tripeptide with presumably analogous functions (Evans *et al.*, 1999; Loscos *et al.*, 2008).

To our knowledge, all previous studies on antioxidants and salt stress, with two exceptions (Hernández *et al.*, 2000; Attia *et al.*, 2008), have examined the effects of NaCl on enzyme activities and metabolites but not on mRNA levels. Even in those cases, the expression of only a few genes has been analyzed, in pea (*Pisum sativum*) and *Arabidopsis thaliana*. This paucity of molecular data can be attributed in part to the unsuitability of many crops for extensive gene expression analysis, because they have large genomes with a high number of gene copies and because most antioxidant proteins are encoded by multigene families sharing high sequence identities. Also, northern blots are not amenable for quantification of mRNAs that are low in abundance or encode closely related proteins. These problems can be circumvented by using model plants that have been (or are being) fully sequenced, in combination with quantitative reverse transcription–polymerase chain reaction (qRT-PCR) for the gene-specific and highly sensitive determination of mRNA levels. To gain a deeper insight into the effects of salt stress on plants, and in particular into the protective role of antioxidants, we used the model legume *Lotus japonicus*. This species grows in Japan and other East Asian countries but virtually nothing is known about its tolerance to salt stress in its natural habitat. Plants of *L. japonicus* were exposed to two salinization protocols, one causing only osmotic effects and the other also having toxic effects as a result of Na⁺ accumulation; then, several physiological and biochemical markers related to salt stress were measured and the expression of more than 20 genes encoding antioxidant enzymes was analyzed in detail. Osmotic stress induced the expression of specific *SOD* and *GPX* genes in leaves but not in roots, whereas cytosolic DR (DRc) was clearly up-regulated in leaves and roots in both salt stress treatments.

These and other results are discussed in terms of the relatively high tolerance of *L. japonicus* to salt stress observed in this work.

Materials and Methods:

Biological material and plant treatments

Seeds of *Lotus japonicus* (Regel) Larsen cv. MG20 were scarified, surface-disinfected and germinated in agar plates. Seedlings were transferred to pots containing vermiculite that had been washed with B&D nutrient solution (Broughton & Dilworth, 1971). Plants were grown for 33 d under controlled environment conditions (23°C : 18°C (day:night), 70% relative humidity, 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 16-h photoperiod) and were watered twice a week with B&D solution supplemented with 5 mM NH_4NO_3 .

Two salinization protocols were used. In S1, plants (26 d old) were treated with 150 mM NaCl for 7 d. In S2, plants (15 d old) were treated successively with 50, 100 and 150 mM NaCl, each concentration for 6 d. Pots were watered twice with the different NaCl concentrations, which were prepared in nutrient solution. Plant material to be used for biochemical and molecular analyses was harvested directly in liquid nitrogen and stored at -80°C .

Physiological parameters and biochemical markers

The dry weight (DW) of the shoot was obtained after drying at 60°C for 78 h. The leaf area per plant was determined using the image analysis application CARNOY v2.1 (Schols *et al.*, 2002). The leaf water potential (Ψ_w) was measured in the leaves in the upper third of the plant, 2 h after the beginning of the photoperiod, with a pressure chamber (SKPM 1400 model; Skye Instruments, Powys, UK). The leaf contents of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), sulfur (S), iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) were measured by inductively coupled plasma/atomic emission spectrometry. Photosynthetic pigments were quantified spectrophotometrically in acetone extracts (Lichtenthaler, 1987). Proline was measured by the acid ninhydrin method (Bates *et al.*, 1973). Soluble protein was measured using the Bradford dye-binding microassay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

Oxidative damage of proteins was measured on western blots using the OxyBlot protein oxidation kit (Chemicon, Temecula, CA, USA), based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine to form the corresponding hydrazones (Levine *et al.*, 1990). Oxidative damage of lipids was quantified as the content of malondialdehyde, a cytotoxic aldehyde arising from lipid peroxidation. Briefly, the method involved extraction of malondialdehyde with 5% (weight/volume (w/v)) metaphosphoric acid containing 0.04% (w/v) butylhydroxytoluene and subsequent reaction with thiobarbituric acid at low pH and 95°C to form (thiobarbituric acid)₂-malondialdehyde adducts. These were extracted with 1-butanol and quantified by high-performance liquid chromatography with a photodiode array detector (Iturbe-Ormaetxe *et al.*, 1998). The identity of the malondialdehyde adduct was verified by scanning of the peak and co-elution with standards of 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St Louis, MO, USA).

Gene expression

Total RNA was extracted with the RNAqueous isolation kit (Ambion, Austin, TX, USA) and treated with DNaseI (Roche, Penzberg, Germany) at 37°C for 30 min. cDNA was synthesized from DNase-

treated RNA with (dT)₁₇ and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR analysis was performed with an iCycler iQ instrument using iQ SYBR-Green Supermix reagents (Bio-Rad) and gene-specific primers (Supporting Information Table S1). The PCR programme consisted of an initial denaturation and *Taq* polymerase activation step of 5 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of primers and the absence of contaminating genomic DNA were verified, respectively, using amplicon dissociation curves and by PCR analysis of RNA samples before reverse transcription. The amplification efficiency of primers, calculated using serial dilutions of root and leaf cDNAs, was > 75%, except for the primers of peroxisomal APX (APXpx), cytosolic GR (GRc) and plastidic GR (GRp), whose efficiencies were > 65%. Expression levels were normalized using *ubiquitin* as a reference gene and were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Threshold cycle (C_T) values were in the range of 17–19 cycles for *ubiquitin* and 22–29 cycles for the genes of interest.

Immunoblot analysis

Proteins were extracted from roots and leaves at 0°C with 50 mM potassium phosphate buffer (pH 7.8), 0.1% (v/v) Triton X-100 and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Proteins were separated in 12.5% sodium dodecyl sulfate gels (Bio-Rad), transferred onto polyvinylidene fluoride membranes and challenged with optimal concentrations of the primary antibodies (Table S2). The secondary antibodies were anti-guinea pig (DRc) and anti-rabbit (other proteins) immunoglobulin G conjugated to horseradish peroxidase (Sigma-Aldrich) and were used at dilutions of 1 : 10 000 and 1 : 20 000, respectively. Immunoreactive proteins were visualized using the SuperSignal West Pico (Pierce, Rockford, IL, USA) chemiluminescent reagent for peroxidase detection.

Antioxidant enzymes

All enzymes were routinely extracted from 0.1 g of leaves or roots with a mortar and pestle at 0–4°C and assayed at 25°C within the linear range. General blanks were prepared by replacing the extracts by the buffers of the extraction media. Specific blanks for each sample were prepared as described in the references given for each assay and used to correct for nonenzymatic rates.

SOD was extracted in 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1% Triton X-100 and 1% (w/v) soluble polyvinylpyrrolidone (PVP-10), and its activity was determined by the ferric cytochrome *c* method using a xanthine/xanthine oxidase system to generate superoxide radicals. SOD isoforms were individualized and identified on 15% acrylamide native gels using the nitroblue tetrazolium method (Beauchamp & Fridovich, 1971) and the inhibitors KCN (3 mM) and H₂O₂ (5 mM). The relative abundance of SOD isoforms was determined by densitometry using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

CAT was extracted in 50 mM potassium phosphate buffer (pH 7.0) and 0.5% PVP-10, and its activity was measured by following the decomposition of H₂O₂ at 240 nm ($\epsilon = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) for 1 min (Aebi, 1984). APX was extracted with the same medium as for CAT but adding 5 mM ascorbate immediately before extraction, and its activity was measured by following ascorbate oxidation at 290 nm ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) for 2 min (Asada, 1984). GR was extracted with 50 mM potassium phosphate buffer (pH 7.8), 1% PVP-10, 0.2 mM EDTA and 0.1% Triton X-100, and its activity was measured by following NADPH oxidation at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) for 3 min (Dalton *et al.*, 1986). MR and DR were extracted with the same medium as for GR but without Triton and with 10 mM β -mercaptoethanol. MR activity was determined by following NADH oxidation at 340 nm

($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) for 90 s (Dalton *et al.*, 1993) and DR activity by following ascorbate formation at 265 nm ($\epsilon = 14.1 \text{ mM}^{-1} \text{ cm}^{-1}$) for 3 min (Nakano & Asada, 1981).

Antioxidant metabolites

Ascorbate and dehydroascorbate were extracted from roots and leaves with 1 M perchloric acid and quantified using the ascorbate oxidase method (Bartoli *et al.*, 2000) with some modifications (Loscos *et al.*, 2008). Reduced and oxidized (homo)glutathione were extracted with 5% (w/v) sulfosalicylic acid and quantified using an enzymatic recycling procedure (Law *et al.*, 1983).

Results:

Physiological state of the plants and oxidative damage

Plants of *L. japonicus* at the vegetative growth stage were exposed to two types of salt stress (S1 and S2) as described in the Materials and Methods. This plant material was first characterized by measuring several important physiological parameters. The leaf Ψ_w values (mean $\pm$ SE; $n = 12\text{--}15$) of S1 (-1.26 ± 0.04 MPa) and S2 (-1.38 ± 0.04 MPa) plants were slightly lower than those of control plants (-0.81 ± 0.03 MPa), indicating that salinity was causing mild water stress. Plant growth was assessed by measuring the shoot DW and total leaf area (Fig. 1a). The shoot DW increased and decreased slightly (*c.* 16%) in S1 and S2 plants, respectively, but the leaf area per plant declined with the two salt treatments, especially (37%) in S2 plants. The leaf contents of Na^+ , K^+ and Ca^{2+} remained unaffected in S1 plants, whereas Na^+ increased 2.6-fold, Ca^{2+} increased by 27% and K^+ decreased by 28% in S2 plants (Fig. 1a). There were no changes in the leaf contents of other elements measured in this work (Mg, P, S, Fe, Cu and Mn), except for an increase (34%) in Zn in both S1 and S2 plants.

Several markers of stress were measured to assess the physiological status of the plants. These included proline, soluble protein, photosynthetic pigments and metabolic products of oxidative damage. Proline is an osmoprotectant (Khedr *et al.*, 2003, and references therein) and is used as a marker of drought (Bates *et al.*, 1973) and salt (Díaz *et al.*, 2005) stress. Proline was found to be such a marker here, as its concentration increased 6- and 14-fold in the leaves and 4- and 6-fold in the roots of S1 and S2 plants, respectively (Fig. 1b). By contrast, the content of soluble protein did not vary in the leaves during either salt treatment and increased by 59% in the roots of S2 plants (Fig. 1b). The concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids also remained unchanged in S1 and S2 plants. Finally, we measured the levels of protein oxidation (total carbonyl groups) and lipid peroxidation (malondialdehyde) as markers of oxidative stress in leaves and roots. We could not detect enhanced levels of protein oxidation in leaves or roots of S1 or S2 plants (data not shown). The content of malondialdehyde in the two organs did not change in S1 plants, but increased by 2-fold in roots and by 1.7-fold in leaves of S2 plants (Fig. 2).

Expression of SOD isoforms

The main objective of this work was to determine the effects of salt stress on the expression of the antioxidant enzymes of *L. japonicus*. The first group of enzymes studied was the SODs, which are metalloenzymes that catalyze the dismutation of superoxide radicals into O_2 and H_2O_2 . There are three

types of SODs in plants which differ in the metal cofactor at the active site and in subcellular localization. All of them were found in *L. japonicus* leaves and roots, including cytosolic CuZnSOD (CuZnSODc), plastidic CuZnSOD (CuZnSODp), cytosolic FeSOD (FeSODc), plastidic FeSOD (FeSODp) and mitochondrial MnSOD. The steady-state mRNA levels of all these SODs remained constant in roots during both NaCl treatments (data not shown). In sharp contrast, the transcript levels of all *SOD* genes increased in leaves of S1 plants, with the exception of *FeSODc*, which was not affected in S1 plants but decreased in S2 plants (Fig. 3a).

The relative abundance of SOD proteins during both types of salt treatment was estimated by immunoblot analysis using polyclonal antibodies raised against CuZnSODp, MnSOD and FeSODc. However, the FeSODc antibody also recognizes the FeSODp isoform (Rubio *et al.*, 2007), and FeSODc and FeSODp could not be resolved in gels; therefore, the protein levels of these two isoforms were quantified together. In leaves, the protein level of CuZnSODp increased in S2 plants and that of FeSOD (FeSODc + FeSODp) decreased during both salt treatments (Fig. S1). In roots, there was also an increase in the protein level of MnSOD in S1 and S2 plants (Fig. S1), whereas no significant changes were observed in the protein levels of CuZnSODp or FeSOD in S1 or S2 plants (data not shown).

The total SOD activity (means $\pm$ SE; $n = 6-13$) in leaves and roots of control plants was 11.02 ± 0.34 and 26.36 ± 1.08 units mg^{-1} of protein, respectively, and remained unchanged during both salt treatments. The isoform composition of SODs in the two plant organs was determined on native gels stained for SOD activity. In root extracts, most SOD activity (values in parentheses are percentages of total SOD activity) corresponded to MnSOD (48%) and CuZnSODc (43%), whereas CuZnSODp (6%) and FeSOD (3%) were much less abundant. By contrast, in leaf extracts we found an intense activity band of MnSOD (62%) and less intense activity bands of CuZnSODp (two bands summing to 25%), CuZnSODc (10%) and FeSOD (2%) (Fig. 3b). As occurred for the corresponding protein levels, the activities of FeSODc and FeSODp could not be resolved on native gels. The specific activity of CuZnSODc remained constant in the leaves of S1 plants but increased by 80% in those of S2 plants (Fig. 3b). However, the specific activities of the other SOD isoforms did not change in the leaves or roots of S1 or S2 plants.

Expression of GPX genes

The GPX isoforms are encoded by a small multigenic family and catalyze the reduction of H_2O_2 and lipid hydroperoxides using thioredoxin preferentially as the electron donor. The isoforms are located in different cellular compartments, where they, among other possible functions, protect membranes against lipid peroxidation and act as sensors in signaling cascades (Herbette *et al.*, 2007). Very recently, we identified six *GPX* genes in *L. japonicus* (Ramos *et al.*, 2009) and have now used this molecular information to quantify their mRNA levels in S1 and S2 plants (Fig. 4). Interestingly, the *GPX1* and *GPX6* genes, which putatively encode chloroplastic isoforms, were up-regulated in the leaves of plants exposed to either of the two salt treatments. In leaves, the *GPX4* gene, encoding presumably a cytosolic isoform, was up-regulated in S1 plants but down-regulated in S2 plants (Fig. 4). By contrast, we could not detect any change in the transcript levels in the roots of S1 or S2 plants (data not shown).

Expression of CAT and ascorbate-glutathione cycle enzymes

The expression of critical enzymes involved in the scavenging of H_2O_2 was also studied under salt stress. The peroxisomal enzyme CAT decomposes H_2O_2 to water and O_2 , whereas the enzymes of the

ascorbate-glutathione cycle, which are localized in the cytosol, chloroplasts, mitochondria and peroxisomes, reduce H_2O_2 to water using ascorbate and glutathione. The steady-state mRNA levels of these enzymes were determined by qRT-PCR, which allowed the separate expression analysis of four *APX*, two *MR*, two *DR* and two *GR* genes (Fig. 5). In the leaves of S1 plants, the only relevant findings were a modest up-regulation of the genes encoding thylakoidal APX (*APX_t*) and *DR_c*, as well as the down-regulation of the gene encoding stromal APX (*APX_s*). By contrast, in the leaves of S2 plants only the mRNA level of *DR_c* was increased. However, in the roots, *CAT* was up-regulated in S1 and down-regulated in S2 plants, whereas *GR_c* was down-regulated in S1 and *DR_c* was up-regulated in both salt treatments (Fig. 5).

The relative protein levels of CAT and some of the ascorbate-glutathione cycle enzymes were also determined in roots and leaves. In both plant organs, the CAT and APX antibodies recognized single immunoreactive protein bands with apparent molecular masses of *c.* 50 and 30 kDa, respectively, as predicted from the amino acid sequences. The levels of CAT and APX proteins remained essentially constant during both salt treatments (data not shown). Also, as expected from the DR protein sequences of *A. thaliana* and *L. japonicus* (see accession numbers in Table S2), the antibodies raised against *DR_c* and *DR_p* of *A. thaliana* recognized immunoreactive bands of 28 and 30 kDa, respectively (Fig. S2). Each of the two antibodies cross-reacted with the other isoform, although with much lower affinity. *DR_p* was not detectable in root extracts and *DR_c* was much less abundant in leaf than in root extracts. In both salt treatments, the protein level of *DR_c* increased in roots and leaves, whereas that of *DR_p* remained constant in leaves (Fig. S2).

The salt stress treatments had no significant effects on the specific activities of the enzymes involved in H_2O_2 scavenging, with the notable exception of DR. Total DR (*DR_c* + *DR_p*) activity increased by 2.2- and 1.4-fold in the roots and leaves of S2 plants, respectively (Fig. 6). We thus conclude that the expression of *DR_c* is enhanced in response to salt stress at the levels of mRNA, protein and (probably) enzyme activity.

Ascorbate and homoglutathione

The concentrations of total ascorbate (reduced ascorbate + dehydroascorbate) and total homoglutathione (reduced + oxidized homoglutathione) were also determined in plants exposed to salt stress. The total ascorbate content of leaves and roots was 3 and 0.5 $\mu\text{mol g}^{-1}$ of fresh weight (FW), respectively, and remained unaffected by the salt treatments. Similarly, the ascorbate redox state (proportion of reduced to total ascorbate) remained unchanged in the range of 91–94% in leaves and 78–79% in roots. The concentration of total homoglutathione and its redox state (proportion of reduced to total homoglutathione) also remained unchanged in either salt treatment. Thus, the contents of total homoglutathione in leaves and roots were, respectively, *c.* 1.0 and 0.3 $\mu\text{mol g}^{-1}$ of FW, and the corresponding redox states were *c.* 97–98% and 94–95%.

Discussion:

In this work, we have investigated the effects of two salinization protocols on the expression of > 20 genes encoding antioxidant enzymes in leaves and roots of *L. japonicus*. Expression was analyzed at the mRNA level and, for many of the genes, also at the protein and enzyme activity levels. As an essential

first step, the effects of each type of salt treatment on important physiological and biochemical markers of plants were examined.

The first conclusions of this study stem from the comparison between the S1 and S2 treatments. Plants subjected to the S1 treatment showed a decrease in leaf Ψ_w but no detectable accumulation of Na^+ or any change in K^+ and Ca^{2+} in the leaves. There was also a decrease in the total leaf area per plant and a slight increase in the shoot DW. Consequently, salinity, at the S1 stage, was causing exclusively an osmotic effect, which was also evident from the increase in the proline concentration in leaves and roots. Proline is an osmoprotectant rapidly synthesized in response to cell dehydration, but may play additional roles in ROS scavenging and in regulation of stress-responsive genes (Zhu, 2001; Khedr *et al.*, 2003; Díaz *et al.*, 2005). Dehydration of leaf tissue in S1 plants may decrease cell expansion and hence the area of newly formed leaves without any detrimental effect on the shoot DW. In comparison to S1 plants, the prolonged salinization experienced by S2 plants did not cause a further decline of leaf Ψ_w , but led to decreases in shoot DW, leaf area and K^+ , and to increases in Na^+ , Ca^{2+} , proline and malondialdehyde. These results indicate that S2 plants were suffering from the toxic effects of NaCl in addition to the osmotic effects seen in S1 plants. The toxic effects observed in S2 plants, namely, moderate growth retardation, disturbance of K^+ and Ca^{2+} homeostasis and lipid peroxidation, were previously reported in plants exposed to long-term salt stress (Cavalcanti *et al.*, 2004; Sánchez *et al.*, 2008). However, in contrast to other authors (Hernández *et al.*, 1999; Sánchez *et al.*, 2008), we did not observe any effect of salt on the leaf contents of Mg, P, S, Fe and Mn, and found an increase, rather than a decrease, in Zn content. These data indicate that plant growth was not limited by nutrients under our experimental conditions. Based on the effects of salinity on plant growth, we also conclude that *L. japonicus* is more tolerant than some important crop legumes. Thus, the shoot FW of *L. japonicus* did not vary with the S1 treatment and was reduced by 28% with S2, but declined by 40% in pea plants (Hernández *et al.*, 1999) and by 84% in common bean (*Phaseolus vulgaris*) plants (Jungklang *et al.*, 2004) treated with 100 mM NaCl for 12–14 d. A likely explanation for the greater tolerance of *L. japonicus* plants is their capacity to prevent Na^+ reaching the shoot at the S1 stage. Even at the S2 stage, Na^+ had only increased 2.6-fold, considerably lower than the 47-fold increase of Na^+ in the shoot of pea plants (Hernández *et al.*, 1999).

The differences between the S1 and S2 treatments also permitted interpretation of the changes in gene expression. The first important observation was the induction of many *SOD* and *GPX* genes in the leaves, but not in the roots, of S1 plants. This finding reveals that the S1 plants already perceived the salt stress, albeit only the osmotic component because there was no detectable ion accumulation or oxidative damage at this stage. Therefore, we conclude that the up-regulation of *SOD* genes is a plant's response to cell dehydration and not to salinity itself, which is fully consistent with the observation that superoxide radicals, which induce *SOD* genes, are overproduced in the chloroplasts during dessication (Menconi *et al.*, 1995). The mechanism for the induction of the *CuZnSODc* and *CuZnSODp* genes with salt stress may not necessarily be transcriptional, in the light of the recent discovery that, in *A. thaliana* plants exposed to oxidative stress, the mRNA levels of these two *SOD* genes are regulated through the interaction with a specific microRNA (Sunkar *et al.*, 2006). In the case of *GPX*s, the activation of the genes that presumably code for the chloroplastic isoforms persisted in the leaves of S2 plants. The induction of the *GPX1* and *GPX6* genes suggests that chloroplast membranes are protected during salt stress, a suggestion also supported by the maintenance of levels of photosynthetic pigments. The induction of *GPX* genes is probably mediated by ROS, in agreement with the finding that the *GPX1* promoter of *Citrus sinensis* is induced by salt through H_2O_2 signaling (Avsian-Kretchmer *et al.*, 2004). Taking our results together, we propose that S1 plants avoid oxidative damage by a combination of Na^+ exclusion from the leaves and activation of *SOD* and *GPX* genes. This activation would be brought about by an enhanced ROS production triggered by cell dehydration. By contrast, in the S2 treatment, Na^+ accumulated in the leaves with detrimental effects on plant growth and metabolism, as indicated above.

The expression of the enzymes involved in H_2O_2 scavenging was studied in leaves and roots during salt stress. The mRNA level of CAT in roots increased in S1 but decreased in S2 plants, although these changes were not reflected in protein or activity levels. There were also changes in the transcript levels for some enzymes of the ascorbate-glutathione cycle. Thus, the mRNA levels of APXt and APXs showed opposite patterns in the leaves of S1 plants, whereas the GRc mRNA level decreased significantly in the roots. Again, we failed to detect changes in the enzyme activities or in the concentrations of ascorbate and homogluthathione, suggesting that the ascorbate-glutathione cycle is still functional in S1 or S2 plants. However, the most striking observation was a consistent up-regulation of DRc in leaves and roots. The mRNA and protein levels increased in S1 and S2, whereas the enzyme activity was enhanced only in S2. Because these increases were not accompanied by changes in other activities or in the metabolites of the ascorbate-glutathione cycle, it is likely that DR performs additional roles. These may be related to the need to keep ascorbate reduced in the apoplast, and maybe other compartments, as ascorbate is a cofactor of several enzymes involved in the formation and stabilization of cell walls and in the synthesis of phytohormones (Arrigoni & De Tullio, 2002). Although DR is apparently absent from the apoplast, this contains up to 10% of the total ascorbate of leaves (Pignocchi & Foyer, 2003). In the apoplast, ascorbate is rapidly oxidized and dehydroascorbate needs to be returned to the cell for reduction by DRc (Pignocchi & Foyer, 2003). Thus, the requirement for a high DR activity in the cytosol in connection with ascorbate regeneration in the apoplast would explain, at least in part, why the *DRc*, but not the *DRp*, gene is up-regulated in salt-stressed *L. japonicus* plants.

Our data also raised some intriguing questions. First, why was the increase in *SOD* mRNA levels in S1 plants not reflected in changes in protein or enzyme activity levels? These discrepancies have previously been reported in plants and may be attributed, among other factors, to post-transcriptional regulation of the enzyme (Attia *et al.*, 2008, and references therein). However, the increase of CuZnSODc activity in S2 plants could also reflect a delay between gene activation at the S1 stage and the synthesis of functional enzyme. Secondly, why did the increase in *SOD* and *GPX* mRNA levels occur in leaves but not in roots? This finding may be related to the need to protect the photosynthetic machinery of the chloroplasts, which are a major site of ROS production in plants (Asada, 1984). This explanation is consistent with the induction in leaves of some genes encoding important antioxidant enzymes of chloroplasts: CuZnSODp, FeSODp, APXs, GPX1 and GPX6. Thirdly, why did oxidative damage occur in S2 plants despite the maintenance of antioxidant levels? Two possible explanations are that malondialdehyde is generated at cellular membranes that are particularly sensitive to ROS attack, and that oxidative damage is the result of an excess of ROS production rather than of insufficient antioxidant protection, as suggested for other plant systems such as soybean (*Glycine max*) nodule senescence (Evans *et al.*, 1999). Further work on these and other far-ranging issues will certainly help to elucidate the roles of ROS and antioxidants in the salt stress perception, response and tolerance of plants.

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Fig. 1

Effect of salt stress on some physiological and biochemical markers of *Lotus japonicus*. Plants were separated at random into three groups as follows: control plants (white bars) were left untreated and harvested when 33 d old; S1 plants (gray bars) were treated when 26 d old with 150 mM NaCl for 7 d; S2 plants (black bars) were treated when 15 d old with 50, 100 and 150 mM NaCl, each concentration for 6 d. (a) Shoot dry weight (DW), leaf area and leaf metal content. (b) Proline and soluble protein contents in leaves and roots. Data are means $\pm$ SE of 50–90 (shoot DW), 8–13 (leaf area and metal contents) or 3–9 (proline and protein) replicates from at least three series of plants grown independently. Asterisks denote significant differences from control plants based on Student's *t*-test ($P < 0.05$). FW, fresh

Fig. 2

Effect of salt stress on the malondialdehyde content of leaves and roots of *Lotus japonicus*. Treatments: control plants (white bars); S1 plants (gray bars); S2 plants (black bars). Data are means $\pm$ SE of 5–10 replicates from at least four series of plants grown independently. Asterisks denote significant differences from control plants based on Student's *t*-test ($P < 0.05$).

Fig. 3

Effect of salt stress on the expression of superoxide dismutase (SOD) isoforms in leaves of *Lotus japonicus*. Treatments: control plants (white bars); S1 plants (gray bars); S2 plants (black bars). (a) Steady-state mRNA levels were quantified by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). The mRNA levels were normalized with respect to *ubiquitin* and are expressed relative to those of control plants, which were arbitrarily given a value of 1. Data are means $\pm$ SE of five to six biological replicates from at least two series of plants grown independently. Asterisks denote up-regulation (> 2.0 -fold) or down-regulation (< 0.5 -fold) of genes with respect to control values. (b) Specific activities of SOD isoforms. Data are means $\pm$ SE of 5–13 replicates from at least five series of plants grown independently. Asterisks denote significant differences from control plants based on Student's *t*-test ($P < 0.05$).

Fig. 4

Effect of salt stress on the steady-state mRNA levels of glutathione peroxidase (GPX) isoforms in leaves of *Lotus japonicus*. Treatments: S1 plants (gray bars); S2 plants (black bars). The mRNA levels were normalized with respect to *ubiquitin* and are expressed relative to those of control plants, which were arbitrarily given a value of 1. Data are means $\pm$ SE of four biological replicates from two series of plants grown independently. Asterisks denote up-regulation (> 2.0 -fold) or down-regulation (< 0.5 -fold) of genes with respect to control values.

Fig. 5

Effect of salt stress on the steady-state mRNA levels of the genes encoding catalase (CAT) and the ascorbate-glutathione pathway enzymes in leaves and roots of *Lotus japonicus*. Treatments: S1 plants (gray bars); S2 plants (black bars). The mRNA levels were normalized with respect to *ubiquitin* and are expressed relative to those of control plants, which were arbitrarily given a value of 1. Data are means $\pm$ SE of five to six biological replicates from at least two series of plants grown independently. Asterisks denote up-regulation (> 2.0 -fold) or down-regulation (< 0.5 -fold) of genes with respect to control values. APX, ascorbate peroxidase; DR, dehydroascorbate reductase; GR, glutathione reductase; MR, monodehydroascorbate reductase; c, cytosolic; px, peroxisomal; p, plastidic; s, stromal; t, thylakoidal.

Fig. 6

Effect of salt stress on dehydroascorbate reductase (DR) activity in leaves and roots of *Lotus japonicus*. Treatments: control plants (white bars); S1 plants (gray bars); S2 plants (black bars). Data are means $\pm$ SE of three to eight replicates from at least three series of plants grown independently. Asterisks denote significant differences from control plants based on Student's *t*-test ($P < 0.05$).