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Antioxidant gene–enzyme responses in *Medicago truncatula* genotypes with different degree of sensitivity to salinity

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Antioxidant responses and nodule function of *Medicago truncatula* genotypes differing in salt tolerance were studied. Salinity effects on nodules were analysed on key nitrogen fixation proteins such as nitrogenase and leghaemoglobin as well as estimating lipid peroxidation levels, and were found more dramatic in the salt-sensitive genotype. Antioxidant enzyme assays for catalase (CAT, EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11) and guaiacol peroxidase (EC 1.11.1.7) were analysed in nodules, roots and leaves treated with increasing concentrations of NaCl for 24 and 48 h. Symbiosis tolerance level, depending essentially on plant genotype, was closely correlated with differences of enzyme activities, which increased in response to salt stress in nodules (except CAT) and roots, whereas a complex pattern was observed in leaves. Gene expression responses were generally correlated with enzymatic activities in 24-h treated roots in all genotypes. This correlation was lost after 48 h of treatment for the sensitive and the reference genotypes, but it remained positively significant for the tolerant one that manifested a high induction for all tested genes after 48 h of treatment. Indeed, tolerance behaviour could be related to the induction of antioxidant genes in plant roots, leading to more efficient enzyme stimulation and protection. High induction of CAT gene was also distinct in roots of the tolerant genotype and merits further consideration. Thus, part of the salinity tolerance in *M. truncatula* is related to induction and sustained expression of highly regulated antioxidant mechanisms.

Introduction

Salinity represents, today, the major cause of land degradation and crop productivity limitation around the world. Soil salinity could be because of soil-type characteristic, an excessive water evaporation and use of low-quality water for crop irrigation. This constraint affects most of the agricultural lands in the arid and semi-arid regions. High salinity adversely affects plants causing hyperosmotic stress and ion disequilibrium, and generating

Abbreviations – APx, ascorbate peroxidase; ARA, acetylene reduction activity; CAT, catalase; DW, dry weight; Lg, leghaemoglobin; MDA, malonyldialdehyde; NDPK, nucleoside diphosphate kinase; NFW, nodule fresh weight; POX, guaiacol peroxidase; ROS, reactive oxygen species; RT, real-time; SOD, superoxide dismutase.

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secondary effects such as accumulation of reactive oxygen species (ROS) and growth retardation (Hasegawa et al. 2000, Zhu 2001). Plant responses to salt stress involve complex and diverse mechanisms and include the mobilization of numerous regulatory pathways and genes (Gruber et al. 2009, Merchan et al. 2007), which makes the research complicated in order to provide solutions to salinity constraint. Salt tolerance can be accomplished with defence against the primary salt effects mainly by ionic homeostasis and osmotic adjustment, and enhanced defence on the secondary effects by ROS scavenging and growth resume (Zhu 2001). Not all these mechanisms are operating or are equally significant in every salt-tolerant plant species or varieties. Information on the relative importance of each mechanism in adaptation of a tolerant plant to salinity is fundamental for breeding crops that withstand increased concentrations of salt maintaining their productivity. Research programmes interested in improving crop production under salinity constraint are focussed on selection of natural tolerant varieties by traditional selection practices (Drevon et al. 2002). Also biotechnological tools could be used to obtain transgenic tolerant plants overexpressing some genes related to salt stress tolerance (Lee et al. 2007, Mansour and Salama 2004, Sairam and Tyagi 2004, Tseng et al. 2007). Antioxidant enzymes are widely reported to play a crucial role in the protection of plant tissues under stressful conditions, mainly salinity and drought (França et al. 2007, Lima et al. 2002, Polidoros et al. 2003, Fotopoulos et al. 2008, Fotopoulos et al. 2010). It has been reported that certain species can develop increased salt tolerance because of an increase of antioxidant enzymes and other compounds (Lopez et al. 1996, Shalata et al. 2001). Various studies also indicate that genetically engineered plants overexpressing antioxidant genes, thus having higher levels of the relative ROS scavenging enzymes, may display improved tolerance towards abiotic stresses, including salt stress (Badawi et al. 2004, M'Hamdi et al. 2009, Tseng et al. 2007, Zhao and Zhang 2006). Thus, study of antioxidant genes, enzymes or compounds that provide a certain degree of tolerance to salt stress can be useful in understanding salt tolerance and breeding salt-tolerant cultivars.

Regarding symbiosis of legume plants with their nitrogen-fixing symbiotic bacteria, the mechanism of stress response is furthermore complex because of the interaction of plant with the microsymbiont. Nodules of legume–rhizobia symbiotic association are key organs of symbiosis that are extremely sensitive to oxidative stress injured by salinity (Mhadhbi et al. 2004, Nandwal et al. 2007). Indeed, nodules are protein-rich organs, requiring high energy levels and oxygen uptake for their functioning. This could generate ROS within nodules as metabolic co-products, even without stress (Matamoros et al. 2003). Under salt stress, ROS production is amplified, and high ROS concentration could deteriorate nodule's structure if not controlled. Oxidative stress control in nodules and other plant tissues is assured by some morphological (as changes in nodule cortex leading to the limitation of oxygen flux through the oxygen diffusion barrier) and physiological changes (Matamoros et al. 1999), and mainly the mobilization of antioxidant enzymes as superoxide dismutase (SOD) and peroxidases (França et al. 2007, Mhadhbi et al. 2008, 2009, Türkun et al. 2005, Fotopoulos et al. 2006).

Medicago truncatula is the model plant for studying legume–rhizobia symbiosis, and numerous molecular, genetic, proteomic and physiological studies have been focussed on its symbiosis with *Sinorhizobium meliloti* (reviewed by Rose 2008). This legume plant represents one of the major evolution success stories of plant adaptation to its environment. In previous studies, we identified *M. truncatula–S. meliloti* symbioses presenting contrasted behaviours in some traits such as nodule number and form, efficiency, drought and salt stress tolerance (Mhadhbi and Aouani, 2008, Mhadhbi et al. 2005, 2009). The aim of this work was to assess the importance of antioxidative defence in manifestation of salt tolerance in contrasting, tolerant/sensitive, *M. truncatula* genotypes. Towards this aim, we examined several antioxidant systems in relation to the ability of nodules to function properly under salt stress, the induction of antioxidant responses under short-term salt treatments in roots and leaves and the establishment of antioxidant defence after prolonged exposure.

Materials and methods

Plant material and NaCl treatment

This study was conducted on three *M. truncatula* genotypes: Jemalong (A17), a reference line with moderate sensitivity to salt and two Tunisian lines; TN6.18, a salt sensitive and TN1.11, a salt tolerant (Lazrek et al. 2009). After scarification, seeds were placed at 4°C for 4 days in Petri dishes containing 0.9% agar medium for germination. Seedlings were transferred in sterile perlite:sand (3:1) pots and inoculated with *S. meliloti* strain TII7 (10^9 colony forming unit (cfu)) and watered twice weekly with nitrogen-free nutritive solution [macro elements (m*M*): KH_2PO_4 (0.25), K_2SO_4(0.7), $MgSO_4{\cdot}7H_2O$ (1) and $CaCl_2$ (1.65); microelements (μ*M*): H_3BO_3 (4), $MnSO_4{\cdot}H_2O$ (6.6), $ZnSO_4{\cdot}7H_2O$ (1.55), $CuSO_4{\cdot}5H_2O$ (1.56), $Na_2MoO_4{\cdot}7H_2O$ (0.12) and $CoSO_4{\cdot}7H_2O$ (0.12)]. Iron is added as Fe-K-EDTA (45 μ*M*). Non-inoculated plants were watered

with a nitrogen-enriched nutritive solution (with 2 m*M* of urea). Plants were grown in a growth chamber at 22/16°C day/night temperatures, at 60–70% relative humidity, with a photosynthetic photon flux density of 100 µmol m^2 s^{-1} and a 16/8 h photoperiod.

For salt treatment, inoculated plants were treated progressively with 25, 50 and 75 m*M* NaCl to avoid osmotic shock. At the stage of flowering, plants were harvested and nodule samples were immediately processed for enzyme activity assays. Plant parts were oven-dried at 60°C for 3 days, and dry weight (DW) was determined.

Non-inoculated 40-day-old plants were treated with 75, 150, 300 and 600 m*M* NaCl solution for 24 and 48 h. Root and leaf samples were harvested and half of these samples were used for enzyme activity assays. The remaining samples were frozen in liquid nitrogen and stored at −80°C for subsequent expression analyses.

Choice of NaCl concentrations used for experiments was based on preliminary experiments, together with previous bibliographic reports.

Acetylene reduction assay

Nitrogenase (EC 1.7.9.92) was assayed by acetylene reduction activity (ARA) based on the method of Hardy et al. (1968) using gas chromatograph (Hewlett PACKARD, HP 4890A, USA) with a Porapak-T column. Nodule-bearing roots were incubated in 10% C_2H_2 atmosphere. After 60 min, three replicates of 0.5 ml samples were withdrawn from the root atmosphere of each plant and ethylene production was measured. Pure acetylene and ethylene were used as internal standards. Specific ARA is calculated as micromoles of reduced acetylene per gram of nodule fresh weight (NFW).

Leghaemoglobin content

The content of leghaemoglobin (Lg) in nodules was determined by the extraction of the pigment in Drabkin's solution using the method described by Shiffman and Lobel (1970) with few modifications. Fresh nodules (100 mg) were homogenized in 3 ml Drabkin's solution. The homogenate was centrifuged at 5000 *g* for 15 min. The supernatant was added to 10 ml of Drabkin's solution, homogenized and centrifuged for 30 min at 15 000 *g*. The supernatant was then collected and absorbance was determined at 540 nm. Lg content was determined using bovine hemoglobin (Sigma, St. Louis, MO) as standard.

Lipid peroxidation assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid reactive substances (TBARS) method modified according to Singh et al. (2007). Nodules (300 mg) were homogenized in 3 ml of 0.1% TCA solution. The homogenate was centrifuged at 10 000 *g* for 20 min and 0.5 ml of the supernatant was added to 1 ml of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The samples were then centrifuged at 10 000 *g* for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of malonyldialdehyde (MDA) is calculated using the extinction coefficient $\varepsilon = 155$ mM^{-1} cm^{-1}.

Enzyme activity assays and protein determination

All operations were performed at 4°C to maintain enzyme activity. Extracts were prepared by homogenizing 200 mg of nodules, non-inoculated roots or leaves in a mortar with 10% (w/w) polyvinyl–polypyrrolidone and 1 ml of 50 m*M* phosphate buffer (pH 7.8) containing 0.1 m*M* ethylene diamine tetra acetic acid (EDTA), 0.1% (v/v) triton X-100, 1 m*M* phenylmethylsulphonyl fluoride as an inhibitor of proteases, added with 5 m*M* ascorbate for ascorbate peroxidase (APx) activity. Extracts were centrifuged at 13 000 *g* for 20 min, and the supernatant was used to determine enzyme activities. SOD (EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), APx (EC 1.11.1.11) and guaiacol peroxidase (POX, EC 1.11.1.7) activities were determined as previously described (Mhadhbi et al. 2005). Units used for graphical representation are expressed in micromoles of specific substrate oxidized per milligram of total protein. Enzyme activities tested represent the total activity of a multienzymatic system. These were measured in three independent experiments and each measurement was repeated twice. Protein content of each sample was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

RNA isolation, cDNA synthesis and real-time PCR assay

Total RNA was prepared from roots and leaves with the Qiagen RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) followed by DNase digestion (RNase-free DNase Set; Qiagen). RNA integrity was analysed spectrophotometrically and by gel electrophoresis. For real-time (RT)-PCR analyses, 1 µg of total RNA was converted into cDNA using Primescript 1st Strand Synthesis Kit according to the manufacturer's protocol (Takara Bio, Shiga, Japan). Subsequently, RT-PCR was performed with Opticon2 DNA Engine (MJ Research, Waltham,

Table 1. Characteristics of primers used to amplify antioxidant genes. T_m = melting temperature.

Gene	Primer	Nucleotide sequence	Length of amplified fragment (bp)	T_m (°C)
CAT	Cat-middle5-F:	5′CCAACCCCAAGTCCCACATT	405	54
	Cat-middle5-R:	5′CAGGCCAAGTCTTAGTTACATCAA		
ACT 11	Actin11-F:	5′ATGCCATCCTTCGTCTTGA	182	53
	Actin11-R:	5′GCTGGTCCTGGCTGTCTC		
CytAPx	Apx-cyt-F:	5′GGTCGCTTGCCTGATGC	206	55
	Apx-cyt-R:	5′CCACCCAACAACTCCGTAAA		
Cu SOD	SodA9-F:	5′CGGCACGAGGGAACATAG	284	54
	SodA9-R:	5′GTCGCTCATCATCGGTAGGA		
Fe SOD	SodB3-F:	5′TCTTGCAACTGAGGAGGAC	145	52
	SodB3-R:	5′AGGACGCCGATTCTGATA		

MA). The reaction mix contained 2 µl cDNA in RT buffer, 0.75 µ*M* of each primer (Table 1) and 1× master mix (SYBR Green Super Mix, Invitrogen, Carlsbad, CA). Reactions were carried out in quadruplicate and the thermocycler conditions were 50°C for 2 min, 95°C for 2 min, then 35 cycles of 95°C for 30 s, melting temperature for 45 s, 72°C for 45 s, 80°C for 2 s, plate read at 78°C, followed by 72°C for 10 min. The melting temperature of the products was calculated in the Opticon2 DNA Engine and ranged between 52 and 55°C (Table 1). Relative quantification of gene expression and statistical analysis were performed using the REST software (Pfaffl et al. 2002). Actin 11 gene was used as structural unchanged reference gene. It should be noted that among the gene members of multigenic family, only a single gene of CAT and APx have been tested. Indeed, a single CAT expressed sequence tag is available in the *M. truncatula* database, while for APx the cytosolic isoform chosen has been reported as the major component of antioxidant defence (Davletova et al. 2005).

Statistical analyses

Data variance between treatments was assessed by STATISTICA software (ANOVA/MANOVA) and comparison of means by higher significant difference (HSD) Tukey test ($P \leq 0.05$). For the calculation of the plant DW and nitrogen fixation, 9–10 replicates were used per treatment. Six replicates were used for enzyme activity and protein content. Correlations among growth parameters, nitrogen-fixing capacity and antioxidant enzyme activities were performed for each *M. truncatula* line.

Results

Effects of NaCl stress on nodule growth, function and antioxidant enzyme activities

Plant growth was assessed with total biomass (DW), and nodule activity estimated by nitrogen-fixing capacity (total and specific ARA), Lg content and structural stability (lipid peroxidation – MDA). These indicators were sensitive to salt stress in all lines examined (Table 2).

Table 2. Changes in the plant biomass (DW g plant^{-1}), the NFW (mg plant^{-1}), the nitrogen-fixing capacity (total ARA, µmol h^{-1} plant^{-1} and specific ARA µmol h^{-1} g^{-1} NFW), the nodule protein content (protein mg g^{-1} NFW), the Lg content (mg g^{-1} NFW), the lipid peroxidation rate (MDA, µmol g^{-1} NFW) and the antioxidant activities (SOD as unit mg^{-1} protein and CAT, POX, APx as µmol mg^{-1} protein) in nodules of *Medicago truncatula* lines TN6.18, Jemalong A17 and TN1.11 under salt stress (75 m*M* NaCl) compared with those grown under non-stressful conditions (control). In each line, data denoted with different letters are statistically different according to HSD Tukey test ($P \leq 0.05$).

		TN6.18		A17		TN1.11	
		Control	NaCl	Control	NaCl	Control	NaCl
Nodule functioning indices	DW	1.68b	1.1c	2.14a	1.87b	2.05a	1.83b
	Total ARA	11.45c	6.2e	13.98a	8.71d	12.86b	8.22d
	NFW	154.34c	139.53d	193.10a	170.54b	171.20b	151.46c
	Specific ARA	74.18a	44.43c	72.39a	51.07b	75.20a	54.27b
	Protein	17.65a	13.6cd	18.08a	16.61b	14.79c	12.65d
	MDA	14.2c	24.3a	14.86c	18.67b	11.62d	14.79c
	Lg	3.75ab	2.62c	4.36a	3.49b	4.15a	3.36b
Antioxidant enzymes	SOD	20.45bc	26.03a	18.33c	19.94c	21.35b	22.85b
	CAT	78.98a	58.6c	87.48a	64.43b	82.47a	67.74b
	POX	43.57b	62.45a	39.25b	57.22a	33.88c	61.87a
	APx	25.25b	27.77ab	20.97c	32.85a	16.75d	25.66b

Under normal conditions, TN6.18 exhibited lower production of whole plant biomass and lower total ARA compared with A17 and TN1.11. However, specific ARA assessed as activity per gram of NFW was similar in three analysed symbioses. Under NaCl stress, TN6.18 line exhibited the higher sensitivity in almost all nodule parameters assessed. Accordingly, DW, total and specific ARA, MDA and Lg were significantly different in nodules of TN6.18 compared with A17 and TN1.11 under NaCl stress conditions. Protein content in TN6.18 was significantly lower only from A17. The nitrogen-fixing capacity (total and specific ARA) decline under salt stress was relatively more important in symbiosis involving TN6.18 line (41/40%). Such breakdown of ARA activity was parallel to analogue declines of biomass production. It was also correlated with the decline of Lg content ($r = 0.92$, $P \leq 0.001$). A17 and TN1.11 also exhibited decrease in plant biomass, ARA, protein content and Lg in the presence of NaCl; however, the observed decreases were not significantly different between the two lines (Table 2).

On the other hand, lipid peroxidation assessed as MDA content of TN6.18 and A17 was similar under physiological conditions; however, under NaCl stress TN6.18 exhibited the highest MDA content compared with A17 and TN1.11 (Table 2). Noteworthy that TN1.11 exhibited the lowest MDA content under physiological and NaCl stress conditions compared with reference line A17. Specifically, A17 exhibited a 56.61% increase of nodular MDA content compared with 36.83% of TN1.11 under NaCl stress.

Nodule antioxidant enzyme activities of CAT, SOD, APx and POX were determined in nodules of all lines under control and NaCl conditions (Table 2). CAT activity was not significantly modified among different lines under control conditions. However, under NaCl conditions, CAT activity significantly decreased in all lines, with TN6.18 exhibiting significantly lower activity compared with A17 and TN1.11. The opposite effect of salinity was observed for SOD, APx and POX activities. Specifically, SOD activity increased in nodules of TN6.18, A17 and TN1.11, with TN6.18 exhibiting significantly higher increase compared with A17 and TN1.11. Similarly, POX activity increased significantly in response to salinity in all lines, with TN1.11 exhibiting the highest increase compared with A17 followed by TN6.18. Salinity induced an increase of APx activity in nodule of all lines that was significant in A17 and TN1.11.

Analysis of relationships between parameters used to estimate nodule efficiency and the antioxidant activities in nodules was performed under salt stress comparatively to unstressed symbioses to assess the modulation of this relationship under salt stress. The results showed that for non-stressed symbioses, the two essential indices of symbiosis effectiveness, DW and ARA, were positively correlated ($r = 0.83$, $P \leq 0.001$). A positive correlation was also recorded between CAT and DW ($r = 0.77$, $P \leq 0.001$), CAT and ARA ($r = 0.71$, $P \leq 0.004$), APx and DW ($r = 0.53$, $P \leq 0.02$) and APx and ARA ($r = 0.79$, $P \leq 0.001$). Moreover, CAT and APx activities were positively correlated ($r = 0.64$, $P \leq 0.004$). No significant correlation was found for SOD and POX. Under salt stress, some of these relationships were affected. Specifically, correlation levels were decreased by salt stress for all analysed parameters as APx with effectiveness indices, where it was faintly correlated with DW ($r = 0.47$, $P \leq 0.05$) and none significantly correlated with ARA. Moreover, correlation between CAT and APx activity was non-significant under stressful conditions.

Antioxidant enzyme responses to NaCl in roots and leaves

The activities of antioxidant enzymes SOD, CAT, APx and POX were investigated in roots and leaves of control and salt-stressed plants under non-symbiotic conditions. Salt stress was applied at 0, 75, 150, 300 and 600 m*M* NaCl for 24 and 48 h. Each line exhibited a specific pattern of response and no generalizations could be suggested. There was not a trend of increasing enzymatic activity with increasing concentration of NaCl or at longer exposure, although such observations were made in certain cases.

Salinity-induced antioxidant enzyme responses in roots of all lines were examined, with the more pronounced induction observed after 48 h of exposure (Fig. 1). After 24 h of treatment, SOD was increased under 150, 300 and 600 m*M* for A17 and TN1.11 lines. However, for TN6.18 SOD increase was only reported under 600 m*M*. After 48 h, the increase was revealed under all NaCl concentrations, and it was more pronounced for the reference line A17 compared with the two local lines mainly when submitted to higher NaCl concentrations (300 and 600 m*M*). The same trend of kinetic activity was revealed on Cu, Zn and Fe SODs' specific isoforms (data not shown). POX stimulation seemed to be more important within A17 roots until 300 m*M*, after what these drop near the control values. TN6.18 POX increased parallel to salt concentration. However, for the highly tolerant TN1.11, POX was increased (52%) at 75 m*M* NaCl but not thereafter (Fig. 1). APx showed a similar behaviour for the two local lines after 48 h with an increase until 300 m*M*, then a decrease of the activity. However, the increase of A17 root APx was evident even for plants cultivated with

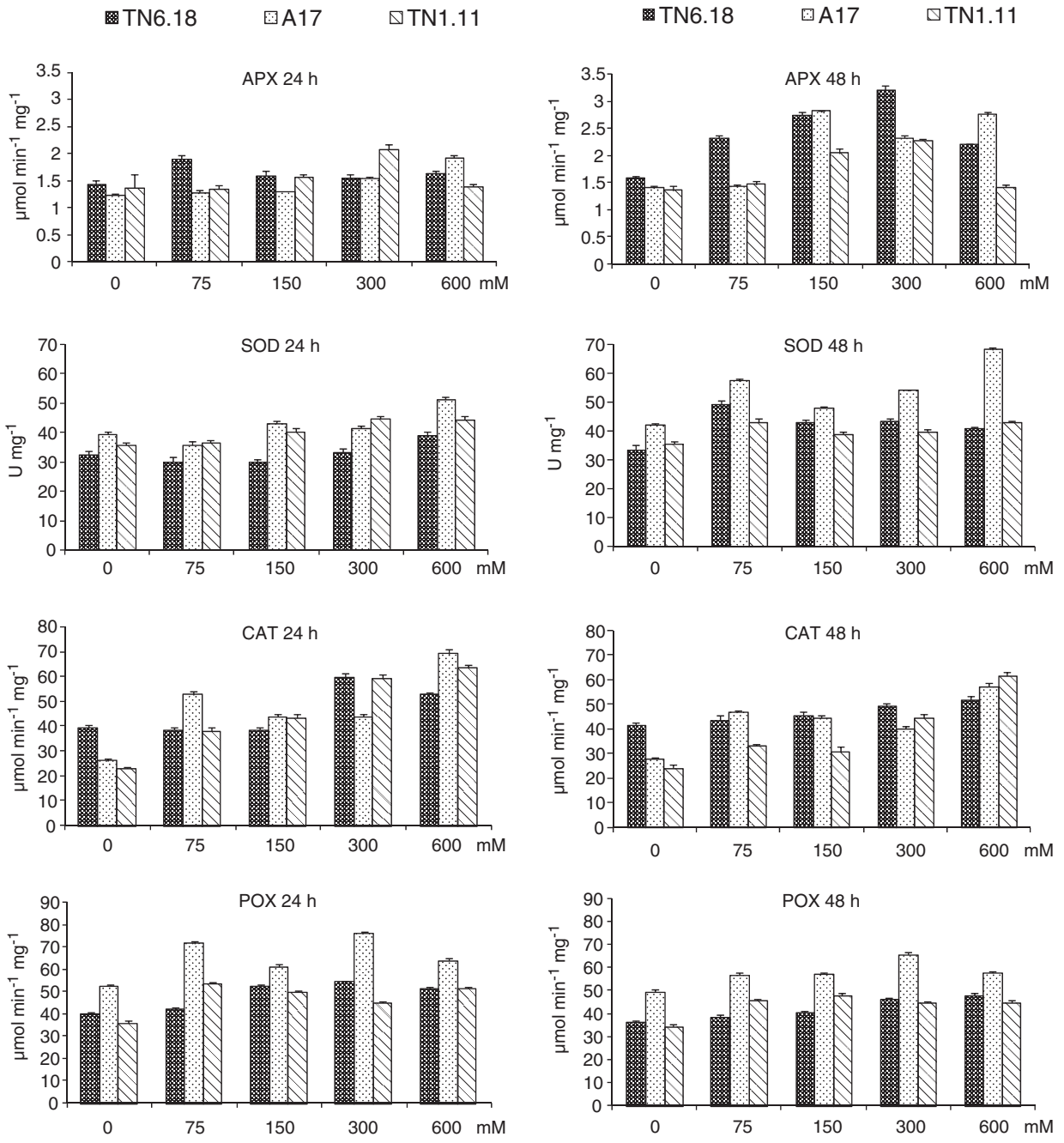

Fig. 1. Variation of total antioxidant enzyme activities; SOD (unit mg^{-1} protein), CAT (µmol mg^{-1} protein), POX (µmol mg^{-1} protein) and APx (µmol mg^{-1} protein) in roots of TN1.11, Jemalong A17 and TN6.18 *Medicago truncatula* lines depending on NaCl concentration (0, 75, 150, 300 and 600 m*M*) and period of stress application (24/48 h). Data are means ± SE at $P \leq 0.05$.

600 m*M*, both after 24 and 48 h of salt treatment. CAT activity increased at 24 and 48 h in TN1.11 and A17 lines. For TN1.11 the increase was gradual, whereas for A17 there was one increase at 75 m*M* and a second at 600 m*M*. TN6.18 displayed an increase at 300 m*M* that remained constant or slightly reduced at 600 m*M*.

In leaves, the total SOD and specific isoforms increased mainly for plants cultivated at 300 m*M* NaCl, then decreased at 600 m*M* for TN6.18 and A17, but not for TN1.11 (Fig. 2). TN6.18 line POX activity was highly induced at 75 and 150 m*M* (2.5 fold) in TN6.18, but the stimulation rate dropped at 300 and 600 m*M* (1.5

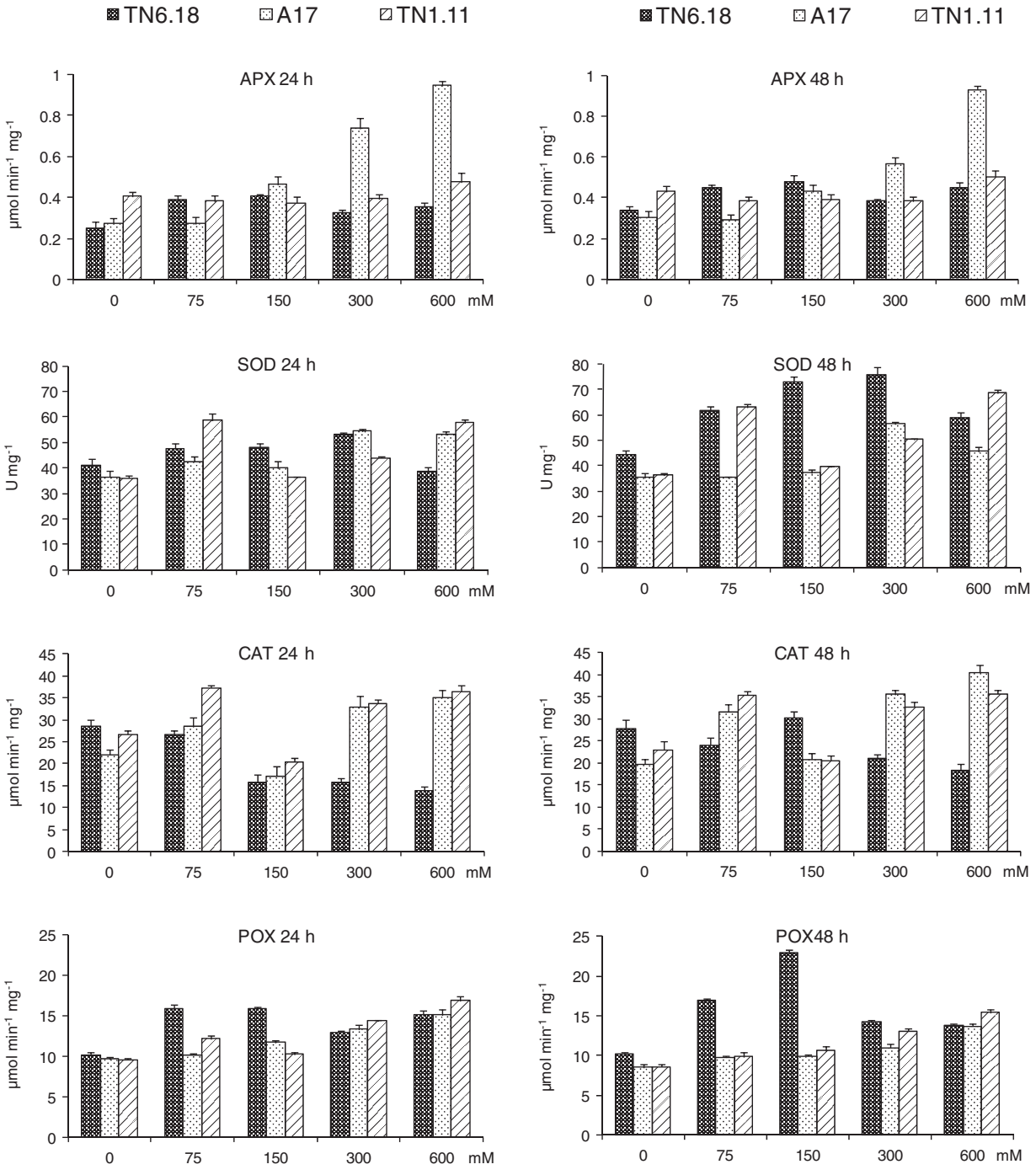

Fig. 2. Variation of total antioxidant enzyme activities; SOD (unit mg^{-1} protein), CAT (µmol mg^{-1} protein), POX (µmol mg^{-1} protein) and APx (µmol mg^{-1} protein) in leaves of TN1.11, Jemalong A17 and TN6.18 *Medicago truncatula* lines depending on NaCl concentration (0, 75, 150, 300 and 600 m*M*) and period of stress application (24/48 h). Data are means ± SE at $P \leq 0.05$.

fold). In TN1.11 and A17 leaves, we noted a progressive increase of POX activity parallel to NaCl concentration. A progressive increase was also revealed for APx activity; but with higher average (3-fold) for A17 leaves than for those of the local lines (Fig. 2). A contrasting trend was revealed for CAT activity, mainly under severe stress (300 and 600 m*M*), where the tolerant TN1.11 and A17 leaves displayed increased activity contrary to the decreased activity in the salt-sensitive TN6.18 leaves.

Gene expression responses to NaCl in roots and leaves

Gene expression analysis was performed on plants grown under 150 and 300 m*M* NaCl. POX was omitted from molecular analyses, because of lack of suitable primers amplifying single alleles. The statistically significant changes in gene expression were the following (Fig. 3) after 24 h of treatment, *APx* was induced in roots of A17 at 150 m*M*. *CAT* was highly induced in TN1.11 and A17 roots, both at 150 and 300 m*M* NaCl. *FeSOD* was suppressed at 150 m*M* NaCl in TN6.18 roots and 300 m*M* in A17. After 48 h of treatment, all the four analysed genes, encoding *CAT*, *cytAPx*, *CuSOD* and *FeSOD*, were positively and significantly induced in the roots of the tolerant TN1.11 line at 150 m*M* and even more at 300 m*M* NaCl (Fig. 3). In A17 roots, there was an induction in *CAT* at 150 m*M* and reduction in *APx* at 300 m*M* NaCl. TN6.18 displayed a suppression of *CAT* gene at 150 m*M* and high induction at 300 m*M* NaCl.

Antioxidant gene expression in leaves followed a complex pattern (Fig. 3). The significant changes at 24-h treatment were observed in TN1.11 *APx* and *CAT* genes that were suppressed, while in TN6.18 *APx* was induced and *CAT* was suppressed. In A17 all genes responded with induction that was significant for *APx*, *CAT* and *CuSOD*. In the prolonged 48-h treatment, the significant changes were in A17 (*APx* suppression and *CuSOD* induction) and TN6.18 (*CuSOD* suppression).

The relation between gene expression and enzyme activities was examined, and no correlation was observed for the leaves. However, a positive correlation between the biochemical and the molecular levels of enzyme–gene expression was recorded in roots (Table 3). In 24-h treated roots, a parallel induction of genes and enzymatic activities was observed as response to salt application in the tolerant TN1.11 and A17 lines, but not in the sensitive one TN6.18. After 48 h of salt application, the positive correlation remained significant and even more obvious only in roots of the highly tolerant TN1.11 line (Table 3).

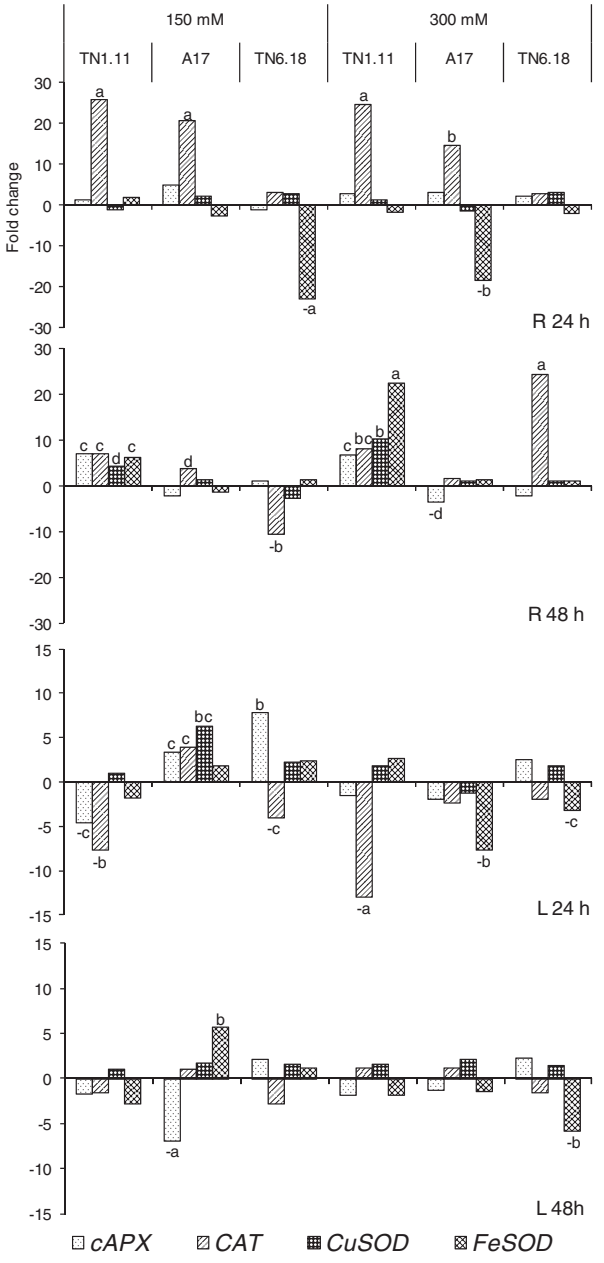

Fig. 3. Effect of short-term salt stress (150 and 300 m*M* NaCl, during 24 and 48 h) on the relative expression of antioxidant genes; SOD isozymes (Cu/Zn SOD and Fe SOD), CAT and APx in roots (R) and leaves (L) of TN1.11, Jemalong A17 and TN6.18 *Medicago truncatula* lines. Data denoted with different small letters are significantly changed (− suppressed, or + induced) compared with control data according to SE at $P \leq 0.05$. Data not denoted are not significantly different from control values.

Discussion

Effects of salinity on nodule function and antioxidant enzymes

Salinity, as expected, exerted an adverse effect on nodule function of all the examined genotypes. Nevertheless, nodules of the salt-sensitive TN6.18 genotype displayed a higher metabolic slowdown, as determined by nodule protein and Lg accumulation, decrease of nitrogen-fixing capacity and accumulation of membrane peroxides. A similar result was reported for sensitive chickpea (Mhadhbi et al. 2008) and alfalfa (Naya et al. 2007) genotypes. Cell membrane stability estimated by MDA level is another indicator of salt stress tolerance (Farooq et al. 2009, Mansour and Salama 2004). MDA

Table 3. Relationship between the activities and the gene expressions of antioxidant enzymes; SOD (total and specific isozymes), CAT and APx in roots of TN6.18, Jemalong A17 and TN1.11 *Medicago truncatula* lines, submitted to salt stress (150/300 m*M* NaCl) for 24 and 48 h. +, induced; −, suppressed; ns, not significantly changed.

	TN6.18				A17				TN1.11			
	Enzyme activity		Gene expression		Enzyme activity		Gene expression		Enzyme activity		Gene expression	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
APx	ns/ns	+/+	ns/ns	ns/ns	ns/+	+/+	+/+	−/−	ns/+	+/+	ns/+	+/+
CAT	ns/+	+/+	ns/ns	−/+	+/+	+/+	+/+	+/ns	+/+	+/+	+/+	+/+
Cu/ZnSOD	ns/ns	ns/ns	ns/+	ns/ns	+/+	ns/+	+/ns	ns/ns	+/+	+/+	ns/ns	+/+
FeSOD	ns/ns	+/+	−/ns	ns/ns	+/+	ns/ns	−/−	ns/ns	+/+	+/+	+/ns	+/+
Total SOD	ns/ns	+/+			+/+	ns/+			+/+	+/+		

accumulation was greater in the sensitive *M. truncatula* TN6.18 line, a result that is similar with reports on *M. sativa*-sensitive cultivars (Wang and Han 2009).

The antioxidant enzyme activities varied differentially under stress conditions. Decline of CAT activity in legume nodules under salt stress has been previously reported (Hernandez-Jimenez et al. 2002, Mhadhbi et al. 2004, Rubio et al. 2002), and similar results were obtained in this study. It has been suggested that CAT might not have the primary role in nodule antioxidant defence under water and salt stresses (Matamoros et al. 2003, Mhadhbi et al. 2008, Tejera et al. 2004), although it is significant in the nodulation process and the nodule functioning (Jamet et al. 2003, Sigaud et al. 1999). However, it should be noted that decrease of CAT activity in the nodules of TN1.11 was at the range of 17% of the control level, which is much lower than that of TN6.18 and A17 that was at the range of 26% of the control level.

SOD has been reported to play a crucial role in symbiosis establishment and functioning of ROS homeostasis regulation (Santos et al. 2000). SOD levels increased about 27.3% by salt application only in TN6.18, the sensitive genotype, indicating a putative protective role to avoid nitrogenase inhibition in highly damaged nodules (Mhadhbi et al. 2004, 2008, 2009, Puppo and Rigaud 1986, Tejera et al. 2004). On the contrary, in the salt-tolerant lines, SOD displayed a slight increase (7–9%) by salt stress which is in agreement with several reports mentioning unchanged SOD levels under moderate stresses (Mhadhbi et al. 2004, Rubio et al. 2002). Unlike SOD, APx activity was increased under salinity treatments. The increase was significant at the range of 53–56% the control levels only in tolerant genotypes, while a slight 10% increase was observed in the sensitive genotype. This enzyme was also reported to play critical role in maintaining the nodule metabolism (Dalton et al. 1998). However, no enhancement of APx activity was reported in nodules under stress conditions (Rubio et al. 2002, Tejera et al. 2004).

POX activity was dramatically increased in stressed nodules of all genotypes. However, POX increase in the tolerant TN1.11 was almost double of the increase of the two other genotypes (82.6% compared with 43.3 and 45%, respectively). This enzyme might be critical for the antioxidative defence in nodules of *M. truncatula* under salt stress, and it was suggested as a potent biochemical marker for drought and salt stress tolerance (Mhadhbi et al. 2004, 2008, 2009). Moreover, the stimulation of POX activity under salt and water stresses and its protective role under these constraints have been widely reported in legume–rhizobia nodules (Matamoros et al. 2003, Mhadhbi et al. 2004, 2008, 2009, Jebara et al. 2010, Nandwal et al. 2007), as well as in other plant tissues (Cavalcanti et al. 2007, Raza et al. 2007).

Overall, our results of the comparison of nodule function and antioxidant mechanisms of the three genotypes can be summarized as follows: (1) nodules of the sensitive TN6.18 genotype displayed worst values for each parameter of nodule function examined in control as well as in stressed plants (with exception of the higher protein content, which, however, displayed more significant decrease in TN6.18 under stress), (2) antioxidant enzymes displayed different responses that were similar in all genotypes; CAT activity decreased while SOD, APx and POX increased, (3) nodules of the tolerant genotype displayed lower CAT decrease and higher APx and POX increase expressed as percentage of the control levels, compared with the sensitive one. On the contrary, SOD increase was higher in the sensitive genotype and (4) the most important differences between the moderately tolerant A17 and the tolerant TN1.11 were the level of decrease in CAT activity (26.3% for A17 compared with 17.86% of TN1.11) and the increase of POX activity (45% for A17 compared with 82.6% of TN1.11).

Assessment of nodule functioning under salt stress was performed under 75 m*M* NaCl adopted after preliminary experiments where diverse concentrations were assessed to obtain the concentration allowing the examination of differences between symbioses but also allowing a

relatively good growth for the most sensitive symbiosis to obtain functional nodules for physiological and biochemical analyses. Then, in the experiments for the induction of antioxidant defence in roots and leaves of non-inoculated plants, a 'pharmacological' approach was selected. Induction was assessed after a short-term treatment (24 h) and the establishment of steady-state antioxidant defense was assessed after prolonged exposure (48 h) using a range of salt concentrations from low (75 m*M*) to toxic (600 m*M*). Lower concentrations (75 m*M*) were used to establish the limit of induction while higher (150 m*M*) was used to assess the magnitude of the response in more severe stress that plant could be submitted in natural conditions. Indeed, 150 m*M* is the salinity level of the original soil where the tolerant line (TN1.11) naturally grows (Lazrek et al. 2009). Salt concentration of 300 m*M* was used as the higher concentration to assess the response of plant to a severe stress from which the plant can recover, while salt concentration of 600 m*M* was used as an extreme concentration at the limit of tolerance of *S. meliloti*, the microsymbiont of *M. truncatula* (Jebara et al. 2001, Zahran, 1999) but toxic to the plant itself (visible beginning of necrosis at the border of the leaves at the 24-h treatment).

Biochemical and molecular responses of antioxidant mechanisms to salinity in roots and leaves

The well-established involvement of ROS on the physiological effects of salinity in plants prompted the study of the role of antioxidant mechanisms in salinity tolerance in various species. It is now recognized that although there is considerable variation in the responses, these mechanisms are part of salinity tolerance (Ashraf 2009). This was evident in that study, strengthening the view that antioxidant gene–enzyme systems are important in stress tolerance in plants. However, it is not easy to make generalizations on the role of each particular antioxidant as there are no systematic similarities in the mode of the response among different species.

One striking observation is that the tolerant TN1.11 line had in most of the measurements the lower control levels of antioxidant enzyme activities. Increases of TN1.11 enzymatic activities were rather gradual with increasing concentrations of NaCl, while the other two lines exhibited more complex patterns. The same has also been observed in a recent study involving wild and cultivated tomato species. Under control conditions, the salt-sensitive cultivated tomato *Solanum lycopersicum* had higher levels of all antioxidants (except SOD) than *Solanum pennellii*, a salt-tolerant wild relative. However, under salt stress, the wild species showed greater induction of all antioxidants except peroxidase (Frary et al. 2010). This gradual response may be advantageous for the tolerant genotypes considering the possible signalling role of accumulated ROS during the initial phase of salinity stress (Miller et al. 2009). If the ROS level cannot reach a threshold needed for signalling because of higher basal antioxidant capacity of the sensitive genotype, this could adversely affect the timely deployment of the required response.

Nevertheless, the response of all the genotypes examined in this study was an increase in enzymatic activity for all the studied antioxidants above control levels after salt application in almost any concentration (with the exception of CAT in the sensitive TN6.18, which displayed a decrease in leaves). This concord with other reports (Farooq et al. 2009, França et al. 2007, Lima et al. 2002). Enzyme stimulation in roots was stronger for the tolerant TN1.11 and A17 lines than the sensitive one (TN6.18). This supports the notion that tolerance to salt stress is at least in part associated with enhancement of the antioxidant machinery. Behaviours of the antioxidant enzymes in the contrasting *M. truncatula* lines concord with reports on many tolerant/sensitive contrasting cultivars of various plant species (Abogadallah et al. 2010, Ben Amor et al. 2006, França et al. 2007, Frary et al. 2010, Mittova et al. 2002, Raza et al. 2007, Sairam and Srivastava 2002, Sekmen et al. 2007, Sreenivasulu et al. 2000, Vaidyanathan et al. 2003). POX and CAT responses in leaves were similar to those of roots treated with analogous NaCl concentrations; however, SOD and APx were differentially modulated. This variation of antioxidant enzymes between leaves and roots could depend on differences of the mechanisms responsible for stress manifestation in each organ (Cavalcanti et al. 2007). POX activation in roots was more intense for tolerant lines. POX is among the most important protective enzyme under stress (Mhadhbi et al. 2004, 2008, 2009). It is essentially involved in the lignification and the cell wall enforcing. So, in the roots (directly submitted to salt effect) of the tolerant lines, POX stimulation confers a best protection of tissue integrity which is confirmed by the low rate of MDA accumulation. However, in leaves the moderate stimulation of POX allows a relatively higher rate of shoot growth (Table 2). POX is essentially involved in the protection of nodules and other plant tissues against salt-mediated oxidative stress. The localization of this enzyme in tissues (*in situ* hybridization) as well as the purification and the characterization of polymorphic isoforms between contrasting symbioses (Mhadhbi et al. 2005) combined with molecular analysis of gene expression could provide further insight into the understanding

of the role of POX in salt-tolerance mechanisms of symbiosis.

The increased enzymatic activity of antioxidant genes was not concomitant with a similar increase in gene expression. The most spectacular effect on antioxidant gene expression was a dramatic increase of CAT gene expression in roots of the tolerant TN1.11 and the moderately tolerant A17, 24 h after salt treatment in both concentrations tested. On the contrary, CAT expression was repressed in leaves. In prolonged 48-h treatment, the response of *CAT* was moderate in the two tolerant lines, while it became strong in the sensitive line only at the high concentration of 300 m*M* salt. This remarkable CAT response may be significant for manifestation of salt tolerance because CAT overexpressing transgenic plants display enhanced salt tolerance in many species (M'Hamdi et al. 2009, Tseng et al. 2007, Zhao and Zhang 2006). Moreover, CAT may play a regulatory role as it has recently been implicated in the protection of the cellular ROS signalling transduction pathway as well as in the deployment of salt tolerance responses. It has been known for a while that CATs can interact with other proteins or factors. In spinach leaf peroxisomes, CAT is member of an enzymatic complex that enables a rapid metabolic channelling (Heupel and Heldt 1994). *Arabidopsis* and tobacco CATs are able to bind calmodulin that is increasing their activity (Yang and Poovaiah 2002). Then, *Arabidopsis* CATs were shown to interact with the nucleoside diphosphate kinase (NDPK), $NDPK_1$, and this interaction was regarded as part of a cellular ROS signalling system (Fukamatsu et al. 2003). More recently it was found that *Arabidopsis* CATs CAT2 and CAT3 were able to interact with salt overly sensitive (SOS_2), an important mediator of salt stress response, which also interacted with NDPK 2 ($NDPK_2$). $NDPK_2$ is involved in several signalling pathways including phytochrome, auxin and H_2O_2 signalling, while the interaction of SOS_2 with CAT and $NDPK_2$ suggests a connection between salt and H_2O_2 signalling in *Arabidopsis* (Verslues et al. 2007). These authors speculate that CAT could prevent $NDPK_2$ inactivation by H_2O_2, preventing its accumulation in a zone around the SOS_2–$NDPK_2$–CAT complex. They also raised the possibility of a similar role of CAT in protection of ABI_1 and ABI_2 which also interact with SOS_2 and are H_2O_2 sensitive. Taken together, these reports provide support for a putative role of CAT in salt stress tolerance aside from the classical housekeeping H_2O_2 scavenging by targeted protection of significant signalling components which are both H_2O_2-sensitive and H_2O_2-dependent. Our finding that *CAT* expression and CAT enzymatic activity are strongly enhanced in the tolerant genotypes under salt stress conditions fits the above model involving CAT in the protection of signalling components responsible for salt stress defence. Another important characteristic of gene expression in the tolerant genotype was that it uniquely exhibited induction of expression of all the tested genes in prolonged (48 h) NaCl treatment. The other genotypes displayed rather weak responses with a notable exception of *CAT* expression in the sensitive TN8.16 where the gene significantly decreased at 150 m*M* and significantly increased at 300 m*M* NaCl. It was reported that the resistance to stresses in tolerant plant species is associated with the upregulation of antioxidant genes (Clement et al. 2008, França et al. 2007, Sreenivasulu et al. 2000). Our results regarding the tolerant genotype are well coherent to these reports. Thus, the high salt tolerance of TN1.11 line could be related to a continuous induction of antioxidant genes, in plant roots, leading to a longer and stronger enzyme stimulation and consequently, protective response.

In summary, antioxidant gene–enzyme responses are contributing to defence of *M. truncatula* genotypes to salinity stress. The tolerant genotype has characteristic differences compared with a sensitive one. These differences encompass: (1) lower control levels and gradual increase of antioxidant enzyme activities with increasing concentrations of NaCl in the roots of the tolerant genotype that might enable ROS levels to reach a signalling threshold earlier and more efficiently than the sensitive one while the response is analogous to the insult, (2) differential POX responses in roots and leaves of the tolerant genotype enabling better protection in the roots that are facing the high NaCl level and allowing sustained growth in shoot that is dealing with secondary effects, (3) a remarkable *CAT* expression response in the roots of the tolerant genotype that can be associated in addition to the 'housekeeping' H_2O_2 scavenging, with a protective CAT role in stress signalling components and (4) induction of gene expression of all the tested genes in the tolerant genotype in a prolonged NaCl treatment that was analogous to salt concentration, securing continuous support of transcripts for the antioxidative cellular machinery. These differences can be an essential part of the salt stress tolerance conferred by antioxidant mechanisms and may help us both better understand and breeding salt-tolerant crops. Indeed; these data show that salt tolerance is related to the induction of antioxidant genes and efficient stimulation of antioxidant enzymes that are tissue and genotype specific and may help to identify genetic markers relative to the salt tolerance response.

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