

Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases

Maria C. Rubio^a, Esther M. González^b, Frank R. Minchin^c, K. Judith Webb^c, Cesar Arrese-Igor^b, Javier Ramos^a and Manuel Becana^a

^a*Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Apdo 202, E-50080 Zaragoza, Spain*

^b*Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, E-31006 Pamplona, Spain*

^c*Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, United Kingdom*

**Corresponding author, e-mail: becana@eead.csic.es*

The antioxidant composition and relative water stress tolerance of nodulated alfalfa plants (*Medicago sativa* L. x *Sinorhizobium meliloti* 102F78) of the elite genotype N4 and three derived transgenic lines have been studied in detail. These transgenic lines overproduced, respectively, Mn-containing superoxide dismutase (SOD) in the mitochondria of leaves and nodules, MnSOD in the chloroplasts, and FeSOD in the chloroplasts. In general for all lines, water stress caused moderate decreases in MnSOD and FeSOD activities in both leaves and nodules, but had distinct tissue-dependent effects on the activities of the peroxide-scavenging enzymes. During water stress, with a few exceptions, ascorbate peroxidase and catalase activities increased moderately in leaves but decreased in nodules. At mild water stress, transgenic lines showed, on average, 20% higher photosynthetic activity than the parental line, which suggests a superior tolerance of transgenic plants under these conditions. However, the untransformed and the transgenic plants performed similarly during moderate and severe water stress and recovery with respect to important markers of metabolic activity and of oxidative stress in leaves and nodules. We conclude that the base genotype used for transformation and the background SOD isozymic composition may have a profound effect on the relative tolerance of the transgenic lines to abiotic stress.

Abbreviations - APX, ascorbate peroxidase; C_i , internal CO₂ concentration; GPX, guaiacol peroxidase; MnSOD, FeSOD, CuZnSOD, superoxide dismutases containing Mn, Fe, or Cu plus Zn as metal cofactors; Ψ_w , water potential.

Introduction

Water stress has profound effects on crop production. Even plants with an optimum water supply experience transient water shortage periods, where water absorption cannot compensate for water loss by transpiration (Kramer and Boyer 1997). In addition, many other environmental stresses, such as cold, salinity and high temperature, have a water stress component. At the molecular and cellular levels, drought and other adverse conditions induce oxidative stress in plant tissues (Thompson et al. 1987, Smirnoff 1993), which can be diagnosed by the accumulation of lipid peroxides, oxidized proteins, or modified DNA bases (Moran et al. 1994, Halliwell and Gutteridge 1999).

Superoxide dismutases (SODs) are ubiquitous metalloenzymes that catalyze the dismutation of superoxide radical to H_2O_2 and O_2 . The superoxide radical is a potential precursor of the highly oxidizing hydroxyl radical and, therefore, SODs are a critical defense of plants, other aerobic organisms, and some anaerobes against oxidative stress (Halliwell and Gutteridge 1999).

Three classes of SODs, differing in their metal cofactor, are known in plants. All three SODs are nuclear-encoded but localized in different subcellular compartments. Typically, CuZnSODs are in the cytosol and chloroplasts, MnSODs in the mitochondria and peroxisomes, and FeSODs in the chloroplasts (Bowler et al. 1994, del Río et al. 1998). Transgenic plants overexpressing SODs in the chloroplasts, mitochondria, and cytosol have been generated (Bowler et al. 1991, Van Camp et al. 1996). In some cases, transgenic plants showed superior tolerance to oxidative stress induced by incubation of leaf disks with methylviologen or by exposure of plants to ozone (Bowler et al. 1991, Sen Gupta et al. 1993). In other cases, but using the same stress inducers, no beneficial effects were found (Tepperman and Dunsmuir 1990, Pitcher et al. 1991). These contradictory results were ascribed to differences in the SOD constructs, in the methodology used to analyze the transformants, and in the growth conditions of the plants (Slooten et al. 1995, Allen et al. 1997).

The above-mentioned studies were performed mainly with tobacco, but important crop legumes are now amenable for transformation (Christou 1994). In these plants, SOD and ascorbate peroxidase (APX) play critical protective roles in nodule activity (Puppo and Rigaud 1986, Dalton et al. 1998). Conceivably then, overexpression of antioxidant enzymes in legumes

could provide additional protection to the process of N₂ fixation, especially during senescence and under stress conditions. In a previous study, we have analyzed the SOD composition of several transgenic lines of alfalfa and characterized three of them at the molecular level (Rubio et al. 2001). Using the three selected lines (1-10, 4-6 and 10-7) along with the parental line (N4), we have carried out the present study with two objectives. First, to determine the effects of water stress on important physiological and biochemical parameters of leaves and nodules. Second, to find out whether transgenic plants overexpressing SOD isozymes in different subcellular compartments outperform untransformed plants during water stress. To fulfil these objectives, we have studied in detail the antioxidant composition and relative water stress tolerance of nodulated alfalfa plants of the elite genotype N4 and three derived transgenic lines.

Materials and methods

Plant material and propagation

Alfalfa (*Medicago sativa* L.) lines used in this study were provided by B. McKersie (Research Triangle Park, NC, USA). Line N4 (WT) is the nontransgenic parental line. Lines 1-10 (MnSODmit) and 4-6 (MnSODchl) were transformed to overexpress *Nicotiana plumbaginifolia* MnSOD in the mitochondria or in the chloroplasts, respectively. Line 10-7 (FeSODchl) was transformed to overexpress *Arabidopsis thaliana* FeSOD in the chloroplasts. All three constructs included the 35S promoter. Details of gene constructs, transformation protocols, screening of transformants, propagation of clones, and inoculation of plants with *Sinorhizobium meliloti* strain 102F78 were described earlier (Rubio et al. 2001). Plants were grown on pots containing a 2/1 (v/v) mixture of perlite and vermiculite in growth cabinets set at the following conditions: 16-h photoperiod, PPFD of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 25/20°C (day/night), and 70/80% RH (day/night). Plants were irrigated three days a week alternatively with distilled water and nutrient solution supplemented with 0.5 mM NH₄NO₃ (Gogorcena et al. 1997).

Application of water stress

Approximately 40 days after transferring plants to pots, they were separated at random into five groups (see Figure legends for details of plant numbers). These were labeled as control (unstress

ed), mild water stress (S1), moderate water stress (S2), severe water stress (S3), and recovery (R). Control plants were kept under optimal water conditions throughout the experiment, whereas the other four groups were subjected to water stress by withholding irrigation. Water potential (Ψ_w) was measured with a pressure bomb (Soil Moisture Equipment, Santa Barbara, CA, USA) using representative leaves, situated in the middle of the plant, to monitor the progression of water deficit. Measurements were made on two to four leaves from different plants within each treatment.

Plants from the S1, S2, and S3 stress treatments were harvested when leaf Ψ_w values (\pm SE of 12-20 replicates) reached -1.29 ± 0.03 MPa, -1.80 ± 0.02 MPa, and -2.83 ± 0.06 MPa, respectively. The S3 stage was usually reached after 7 days of withholding water. For the recovery treatment, plants were allowed to attain the S3 stress stage and were then rewatered for 3 days. Control plants were harvested in between S3 and R plants. Average Ψ_w values (\pm SE of 8-19 replicates) for control and R plants were -0.93 ± 0.08 MPa and -1.15 ± 0.05 MPa, respectively. Samples of leaves (0.25 g) and nodules (25 mg) to be used for biochemical analyses were flash-frozen in liquid N₂ and stored at -80°C.

Physiological parameters

Photosynthesis was measured in the same type of leaves using a LI-6200 portable photosynthesis system equipped with a LI-6250 CO₂ analyzer (Li-COR, Lincoln, NE, USA). Gas-exchange parameters of leaves, including stomatal conductance, transpiration and internal CO₂ concentration (C_i), were measured simultaneously with photosynthesis using the same equipment. Leaf areas were determined with a portable area meter LI-3000A. All measurements were made on three to five leaves of different plants from each treatment.

In vivo nitrogenase activity and root respiration were determined as H₂ and CO₂ evolution, respectively, from sealed roots in a 79% Ar, 21% O₂ gas stream generated using an open flow-through gas system (Witty and Minchin 1998) with electrochemical H₂ sensors (City Technology, Portsmouth, UK) and an IR gas analyzer (ADC, Hoddesdon, UK). Measurements were made on intact, undisturbed plants housed in a controlled environment cabinet (Gogorcena et al. 1997).

Assay of total SOD activity

All enzymes were extracted at 0-4°C and activities were measured spectrophotometrically at 25°C within the linear region for both time and enzyme concentration.

Leaves (0.1 g) were thoroughly ground in a plastic centrifuge tube with 2 ml of a medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% (w/v) PVP-10, and 0.1% (v/v) Triton X-100. The extract was centrifuged at 13000g for 20 min. Nodules (25 mg) were homogenized in an Eppendorf tube with 200 µl of the same extraction medium. The extracts were centrifuged at 13000g for 5 min and the supernatants used to assay SOD activity. Dialysis of the extracts was not necessary when using the method described in detail below.

Total SOD activity was assayed in a medium consisting of 1 ml of superoxide-generating solution, 20 µl of 0.5 mM KCN, and 25 µl (leaves) or 5 µl (nodules) of enzyme extract. The superoxide-generating solution contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1 mM xanthine, and 1 mM ferric cytochrome *c*. The reaction was initiated by addition of sufficient xanthine oxidase (approximately 16 µl of diluted enzyme) to produce an increase in the absorbance at 550 nm of 0.050 per min. Diluted xanthine oxidase was prepared by adding 20 µl of stock solution (Sigma) to 800 µl of 50 mM potassium phosphate (pH 7.8). This preparation was kept in ice and discarded every day. A low concentration of KCN (10 mM) was included in the assay medium of total SOD to inhibit mitochondrial cytochrome *c* oxidase without affecting CuZnSOD activity. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of ferric cytochrome *c* by 50% (McCord and Fridovich 1969). Boiled extracts showed < 4% residual SOD activity.

Analysis of SOD isozyme composition

SOD isozymes were individualized and identified on 15% polyacrylamide native gels by incubation with specific inhibitors (3 mM KCN or 5 mM H₂O₂ for 1 h) and subsequent staining for SOD activity (Rubio et al. 2001). Activity bands resistant to KCN but inhibitable by H₂O₂ were assigned to FeSOD isozymes and those resistant to both inhibitors to MnSOD isozymes. Representative gels were also stained for peroxidase activity to rule out the possibility of artifactual SOD activity for any of the bands. The same leaf and nodule extracts as those loaded

on gels were assayed for KCN-insensitive SOD activity (MnSOD+FeSOD) using the ferric cytochrome *c* method and including 3 mM KCN in the assay medium. The individual FeSOD and MnSOD activities were then calculated by applying the relative proportions calculated by gel densitometry to the KCN-insensitive SOD activity.

Assay of other antioxidant enzymes

APX, catalase (EC 1.11.1.6), and guaiacol peroxidase (GPX, EC 1.11.1.7) were extracted from 0.1 g leaves or 25 mg nodules with 2 ml or 200 μ l, respectively, of optimized media. The extraction medium for leaf APX contained 50 mM potassium phosphate buffer (pH 7.8), 1% PVP-10, 5 mM ascorbate, and 0.1% Triton X-100. The extraction media for nodule APX and all the other enzymes from leaves and nodules had identical composition to the medium for leaf APX except that 0.1 mM EDTA was included.

APX activity was assayed by following the disappearance of ascorbate at 290 nm (Asada 1984) for 3 min (40-s lag period) using 30 μ l (leaves) or 5 μ l (nodules) of extract. Additional controls were run to correct for APX-independent ascorbate oxidation. These controls included boiling of enzyme extracts, incubation with the inhibitors KCN (1 mM) and *p*-chloromercuriphenylsulfonic acid (0.5 mM; *p*CMPSA), and omission of extract (nonenzymatic activity) or H₂O₂ (ascorbate oxidase activity). Nonenzymatic activity was virtually zero, but oxidase activity was especially high in nodules and APX activities in leaves and nodules were corrected accordingly. As expected, APX activities were lost either by boiling the extracts or in the presence of the above inhibitors. *p*CMPSA inactivates cytosolic and chloroplastic APX but has no effect on GPX (Amako et al. 1994). Therefore, the inhibition of APX activity by *p*CMPSA indicated that ascorbate oxidation by the GPX present in the extracts was negligible and that genuine APX activity was being measured.

Catalase activity was assayed by following the decomposition of H₂O₂ at 240 nm (Aebi 1984) for 2 min with 30 μ l extract (leaves) or for 1 min with 5 μ l extract (nodules). As expected, boiling of extracts and addition of 1 mM KCN or 5 mM aminotriazole in the assay medium inhibited catalase activity.

GPX activity was measured by following the oxidation of pyrogallol at 430 nm (Amako et al. 1994) for 3 min with 10 μ l extract (leaves) or for 1 min with 2.5 μ l extract (nodules).

Extracts were preincubated for 5 min with 0.5 mM *p*CMPSA in 50 mM potassium phosphate buffer (pH 7.0). Then, 20 mM pyrogallol and 0.1 mM H₂O₂ were added to initiate the reaction. Because APX may also catalyze pyrogallol oxidation to some extent, *p*CMPSA was included in the assay medium to inactivate APX and thus ensure the accurate measurement of GPX activity. Additional controls included boiling of extracts and incubation with inhibitors. The small residual activity (<15%) found after boiling nodule extracts was used to correct GPX activities of nodules. GPX activity was completely inhibited by 1 mM KCN.

Oxidative damage of lipids and proteins

The content of lipid peroxides in leaves was measured in terms of 2-thiobarbituric acid reactive substances (TBARS) exactly as previously described (Iturbe-Ormaetxe et al. 1998), except that the absorbance of the chromogen in the butanol phase at 532 nm was corrected by the nonspecific absorption at 600 nm (Dhindsa et al. 1981).

Oxidized proteins were quantified by derivatization of carbonyl groups with 2,4-dinitrophenyl hydrazine to form the corresponding dinitrophenyl-hydrazones, according to the procedure of Levine et al. (1990), which was adapted for plant tissue as described by Matamoros et al. (1999).

Statistical analyses

Six series of plants grown independently under identical environment conditions and subjected to the same water stress treatments were required to obtain sufficient leaf and nodule material. Thus, each series was considered to be a repetition of the whole experiment. Physiological parameters of control and stressed plants were measured from at least four repeat series and values did not significantly differ among series based on analysis of variance. The same occurred with biochemical parameters, although in this case at least two repeat series of plants were used. To study the effect of water stress for each line, means were subjected to analysis of variance and compared with the Duncan's multiple range test. The number of independent samples (replicates) used for the calculation of the means is stated in each table or figure.

Results

Effects of water stress on physiological parameters

Three stress levels were applied to plants by withholding water, giving an average leaf Ψ_w of -1.29 MPa (S1), -1.80 MPa (S2), and -2.83 MPa (S3). Plants at the S1 and S2 stages were visually indistinguishable from control (unstressed) plants (leaf Ψ_w of -0.93 MPa), whereas those at the S3 stage exhibited some wilting. Rewatering of these plants for three days (R) caused complete rehydration of plant tissue (leaf Ψ_w of -1.15 MPa). Measurements of photosynthesis and gas-exchange parameters during stress and recovery indicated that the three stress levels were physiologically relevant and that their effects were reversible to different extents.

Photosynthesis was similar in control plants of all four lines (Fig. 1), as were the dry weights of leaves, stems, roots, and nodules (data not shown). Mild water stress had no effect on photosynthesis of transgenic lines but inhibited that of WT by 20%. Moderate and severe water stress inhibited photosynthesis of all lines by 25% and 50%, respectively. After rewatering of plants, photosynthetic activities recovered >86% of the control values (Fig. 1). Simultaneous measurements of gas-exchange parameters indicated that there was significant stomatal closure already at mild stress (83% decrease in stomatal conductance for WT and 45-70% for transgenic lines) and that this was further aggravated at moderate stress (72-85% decrease for all lines) and at severe stress (82-90% decrease for all lines). However, the photosynthesis/ C_i ratios are clearly more relevant than the values of stomatal conductance per se to assess the relative contribution of stomatal vs nonstomatal factors to the stress-induced decline of photosynthesis (Long and Hallgren 1985). For all lines, the photosynthesis/ C_i ratios (in percent) of plants at mild or moderate stress were similar or slightly superior to those of control plants (Fig. 1), indicating that the inhibition of photosynthesis at mild and moderate stress can be entirely accounted for by stomatal closure (Long and Hallgren 1985). In contrast, the photosynthesis/ C_i ratio substantially decreased in severely-stressed plants of all lines except perhaps in FeSODchl (Fig. 1). This indicates that the additional decrease in stomatal conductance at severe stress

relative to moderate stress (<10%) cannot explain the additional decrease (20-36%) of photosynthesis and hence that nonstomatal factors are also limiting photosynthetic activity.

Rates of N₂ fixation were measured on intact WT and MnSODmit plants, since the latter was the only line expressing the transgene in the nodules. Control (unstressed) plants showed comparable values for nitrogenase activity (14.2 ± 0.4 and 15.9 ± 1.2 mmol H₂ min⁻¹ g⁻¹ dry weight (mean \pm SE of 3 replicates), for WT and MnSODmit, respectively. These values decreased by 81% and 78%, respectively, at mild water stress and became too low to measure at moderate and severe water stress. In WT and MnSODmit plants, water stress produced a decrease in nitrogenase-linked respiration and an increase in the O₂ diffusion resistance of nodules and in the carbon cost of nitrogenase activity. However, there were no significant differences between the lines in relation to the magnitude of these effects.

Effects of water stress on antioxidant activities and oxidative damage

The total SOD activity of leaves remained relatively unchanged during water stress, except for a 25% decrease in the activity of the MnSODchl and FeSODchl plants at severe stress (Fig. 2). Rewatering of plants produced a complete recovery of total SOD activity, except for FeSODchl (80% of control). Separate determinations of MnSOD (KCN/H₂O₂-insensitive) and FeSOD (KCN-insensitive) using SOD activity gels in the presence of inhibitors revealed significant differences. The MnSOD activity of WT decreased by approximately 30% during stress, whereas that of MnSODchl was unaffected. Upon rewatering, the MnSOD activity of WT recovered to 75% of control and that of transgenic lines recovered completely. The MnSOD activity of FeSODchl declined by 28% at mild and moderate stress but then increased to nearly control values at severe stress and recovery; in contrast, its FeSOD activity increased slightly at mild stress and decreased to approximately 35% of control at severe stress and following rewatering. The FeSOD activity of MnSODchl, unlike its MnSOD activity, increased slightly at mild stress but then decreased at severe stress by 30% relative to the control (Fig. 2).

Water stress had distinct effects on the enzyme activities involved in the removal of H₂O₂ in leaves. GPX activity did not change, whereas total APX activity (cytosolic plus chloroplastic) varied moderately and catalase activity was markedly affected (Fig. 3). Total APX activity of WT remained constant at mild and moderate water stress, whereas the activity of MnSODmit

and MnSODchl increased between 50 and 80% in the same conditions and that of FeSODchl increased between 20 and 40% at moderate and severe stress. Comparisons of APX activities extracted in the absence and presence of ascorbate enabled us to estimate cytosolic APX activity (Amako et al. 1994). This activity accounted for 70% of total APX in WT and FeSODchl and 50% in MnSODmit and MnSODchl. Likewise, the increase of total APX activity in the latter two lines during water stress was found to be mainly due to an increase in cytosolic APX activity (data not shown). The catalase activity of leaves increased by 2-fold in WT at moderate stress and by 71% in MnSODmit at mild stress, but in both lines the activity declined below control levels during intensification of stress and after rewatering of plants (Fig. 3). Catalase activity of MnSODchl showed a 4-fold increase at mild water stress and then progressively returned to control values. In contrast, the activity of FeSODchl was unaffected by mild water stress, increased approximately 2-fold at moderate and severe stress, and declined below control values after recovery of plants. The protein content in leaves of MnSODchl and FeSODchl showed decreases of only 16 to 24% at severe stress and upon recovery (Fig. 3).

Preliminary quantification of oxidized lipids and proteins in leaves showed that they accumulated significantly at severe water stress but not at mild and moderate stress. Likewise, no detailed measurements of oxidative damage were made in nodules because initial data showed no differences in the accumulation of lipid peroxides between WT and MnSODmit (the only line expressing the transgene in nodules). We therefore measured oxidative damage only in leaves of control (unstressed) plants and in those subjected to severe stress and recovery (Fig. 4). Severe water stress caused a general, although modest (23 to 36%), increase in TBARS in the leaves of all four lines. It also led to a moderate accumulation of oxidized proteins (carbonyl groups) in leaves of MnSODmit (44%) and MnSODchl (27%), but had no effect on the other two lines. Upon rewatering of plants, TBARS and total carbonyls returned to control levels in all four lines (Fig. 4).

Antioxidant enzyme activities were measured in nodules of the same plants. In all four lines, the total SOD activity of nodules was not affected by mild stress, decreased by 20% at

moderate stress and by 30% at severe stress, then remained at this level after recovery of plants (Fig. 5). There was a progressive decline of MnSOD activity of all lines, with decreases in the range of <25% at mild stress, 20 to 40% at moderate stress, and 40 to 60% at severe stress. Recoveries were to >70% of the control values. However, there were only minor changes in the FeSOD activity of FeSODchl and moderate decreases in that of WT, whereas the decline in MnSODmit and MnSODchl was in the range of 40 to 60%. Upon rewatering of plants, the FeSOD activity of MnSODmit and MnSODchl returned to the mild stress levels, but that of WT and FeSODchl remained 20% lower (Fig. 5).

Water stress also had a differential effect on the activities involved in H₂O₂ scavenging in nodules, although the responses were rather similar for all four lines (Fig. 6). The APX activity of FeSODchl was only slightly (<20% decrease) affected by any stress treatment, whereas that of the other lines declined by 40% at mild stress and recovered partially or completely to the control values with progression of water stress and rewatering of plants. The GPX activity of nodules was slightly affected by mild or moderate stress, and declined by 35 to 45% at severe stress for all lines. Catalase activity, unlike its leaf counterpart, was inhibited by moderate and severe water stress, albeit at levels always <30%. This activity remained at values 20% lower than the controls during rewatering. The soluble protein content of nodules decreased by only 20 to 30% at severe stress and remained at this level in the transgenic lines during recovery, but returned to control values in WT (Fig. 6).

Discussion

In plants of all four lines subjected to severe water stress (leaf Ψ_w of -2.8 MPa), photosynthesis was inhibited by 50% (Fig. 1) and the leaf content of oxidized lipids and proteins increased by 23 to 44% (Fig. 4) relative to control (unstressed) plants. This reflects a general superior tolerance of alfalfa to water stress with respect to other legumes such as pea, where severe water stress (reached with a leaf Ψ_w of -1.9 MPa) caused the virtual suppression of photosynthesis and an increase of 45 to 67% in the oxidative damage of the leaves (Iturbe-Ormaetxe et al. 1998).

At mild stress, transgenic lines showed, on average, 20% higher photosynthetic activity

than the WT (Fig. 1). This relatively worse photosynthetic performance of WT plants should be mainly ascribed to a larger decrease in stomatal conductance (83% in WT vs 45-70% in transgenics), which suggests variations in stomatal density or size. The photosynthesis/ C_i ratios (Fig. 1) indicate that in most, if not all, lines the inhibition of photosynthesis at mild and moderate stress is due to stomatal closure, whereas at severe stress both stomatal and nonstomatal factors are involved. One such nonstomatal factor is probably damage of the photosynthetic machinery, as there is accumulation of oxidized lipids and proteins in the leaves at severe stress (Fig. 4).

The response of SODs and associated antioxidant enzymes to water stress was compared in WT and transgenic alfalfa. This was deemed of interest because previous work had showed that in water-stressed pea leaves there is a correlative increase between cytosolic CuZnSOD and APX activities (and transcripts), suggesting the coordinated expression of both enzymes (Mittler and Zilinskas 1994). In alfalfa leaves, however, the cytosolic CuZnSOD activity was low and in general there was no correlation between H_2O_2 -producing (MnSOD or FeSOD) and H_2O_2 -scavenging (APX, catalase, GPX) enzyme activities. The possible exception would be the MnSO Dchl plants, where MnSOD and APX activities clearly exceeded the control values during water stress (compare Figs. 2 and 3). The maintenance of higher MnSOD and APX activities during stress in MnSODchl plants may be related to the fact that a fraction of both enzymes is located to the chloroplasts, because it did not occur in MnSODmit plants, which bear the same construct but targeted to the mitochondria.

An important finding of this work was the contrasting behavior of APX and catalase activity in leaves with respect to nodules during water stress (compare Figs. 3 and 6). In general, both enzyme activities (especially catalase) increased in leaves but declined in nodules relative to control values. The increase in APX and catalase activities in leaves is probably a response to the enhanced production of H_2O_2 under water stress. This H_2O_2 excess would be generated by photorespiration in the peroxisomes rather than by overexpression of SODs because water stress is known to enhance photorespiratory rates, the increase in catalase (peroxisomal) was considerably greater than in APX (extra-peroxisomal), and there was no obvious correlation

between overexpressed SODs (especially FeSOD) and APX or catalase. Reports of the effects of water stress on catalase activity have been so far contradictory. While several authors found only minor changes (reviewed by Smirnoff 1993), others observed similar increases in catalase activity to those reported here in water-stressed leaves of young pea plants (Mittler and Zilinskas 1994). However, we found a decline of activity in older plants from different pea cultivars (Moran et al. 1994, Iturbe-Ormaetxe et al. 1998), suggesting that the response of catalase activity to water stress is age- and cultivar-dependent. In alfalfa nodules, contrary to the leaves, water stress caused a decrease in the enzyme activities that produce or scavenge H_2O_2 (Figs. 5 and 6). The decline in APX, and especially in catalase, may be the result of a general but reversible slowing down of nodule metabolism, as similar decreasing trends were observed for SODs, GPX, and soluble protein.

We conclude that in general there were no major differences between WT and transgenic alfalfa for most parameters used in this study as markers of water stress tolerance. Dry weight production, photosynthetic activity, leaf soluble protein content, and MnSOD, FeSOD and GPX activities were similar for all four lines at moderate and severe water stress and following rewatering of plants. Furthermore, transgenic plants suffered from oxidative stress (judged by the accumulation of damaged lipids and proteins) and recovered from it similarly to the WT (Fig. 4). These conclusions are important because previous work on transgenic tobacco overexpressing MnSOD (Slooten et al. 1995) or FeSOD (Van Camp et al. 1996) in the chloroplasts has shown enhanced tolerance to oxidative stress induced by methyl viologen in leaf discs. While convenient for many purposes, this assay is unlikely to reflect tolerance of plants to oxidative stress generated in natural conditions. In fact, plants overproducing FeSOD were no more tolerant to salt stress (Van Camp et al. 1996).

The oxidative damage to cell components under water stress conditions is mediated by superoxide radicals (Smirnoff 1993) and probably hydroxyl radicals because there was oxidation of amino acid residues of proteins to carbonyl groups (Moran et al. 1994; Fig. 4). Transgenic alfalfa plants of the RA3 genotype overexpressing MnSOD in the chloroplasts showed lower membrane injury than the WT as judged by electrolyte leakage from leaf discs (McKersie et al. 1996). Our data show similar membrane damage, estimated as peroxidation of membrane lipids, and similar overall cellular damage, estimated as oxidation of membrane and soluble proteins.

Assuming equal reliability of the markers used to assess stress tolerance, several reasons may explain the different results. (a) *The use of different base genotypes.* Thus, McKersie et al. (1996) used the RA3 genotype (poor agronomic interest) for water stress experiments, whereas we have used the N4 genotype (selected for its field performance), and it is well-known that important variations exist in the tolerance to abiotic stress among cultivars of most crops, including legumes (Moran et al. 1994, Iturbe-Ormaetxe et al. 1998). (b) *The different nitrogen source of plants.* The plants used by McKersie et al. (1996, 1999) were grown under nonnodulating conditions and may differ in stress tolerance from the N₂-fixing plants used in this study. This was shown in pea and alfalfa, which are more tolerant to water stress in symbiosis than when using combined nitrogen (Antolín et al. 1995, Frechilla et al. 2000). (c) *The different background SOD isozyme composition of the leaves.* McKersie et al. (1999) found high CuZnSOD activities and no FeSOD activity, whereas we observed low CuZnSOD activity and high MnSOD and FeSOD activities. We conclude that all these factors, along with the use of tissue-specific promoters, may have a major impact on the performance of the derived transgenic lines and hence should be considered in any attempt to improve crop tolerance to abiotic stress by genetic engineering.

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Legends to Figures

Fig. 1. Effect of water stress on photosynthesis and on the photosynthesis/ C_i ratio of alfalfa WT and the derived transgenic lines MnSODmit, MnSODchl, and FeSODchl. For each line, bars represent control (well-watered plants, o), mild water stress (S1), moderate water stress (S2), severe water stress (S3), and recovery (R). Data are means \pm SE of 10-21 replicates.

Fig. 2. Effect of water stress on SOD activities of leaves from alfalfa WT and the derived transgenic lines MnSODmit, MnSODchl, and FeSODchl. Designation of water stress and recovery treatments is as described in the legend to Fig. 1. Data are means \pm SE of 4-5 replicates.

Fig. 3. Effect of water stress on H_2O_2 -scavenging enzyme activities and protein content of leaves from alfalfa WT and the derived transgenic lines MnSODmit, MnSODchl, and FeSODchl. Designation of water stress and recovery treatments is as described in the legend to Fig. 1. Data are means \pm SE of 4-5 replicates.

Fig. 4. Effect of water stress on the oxidative damage of lipids and proteins of leaves of alfalfa WT and the derived transgenic lines MnSODmit, MnSODchl, and FeSODchl. Designation of water stress and recovery treatments is as described in the legend to Fig. 1. Data are means \pm SE of 3-4 replicates.

Fig. 5. Effect of water stress on SOD activities in nodules of alfalfa WT and the derived transgenic lines MnSODmit, MnSODchl, and FeSODchl. Designation of water stress and recovery treatments is as described in the legend to Fig. 1. Data are means \pm SE of 4-5 replicates.

Fig. 6. Effect of water stress on H₂O₂-scavenging enzyme activities and protein content in nodules of alfalfa WT and the derived transgenic lines Designation of water stress and recovery treatments is as described in the legend to Fig. 1. MnSODmit, MnSODchl, and FeSODchl. Data are means \pm SE of 4-5.

Fig. 1

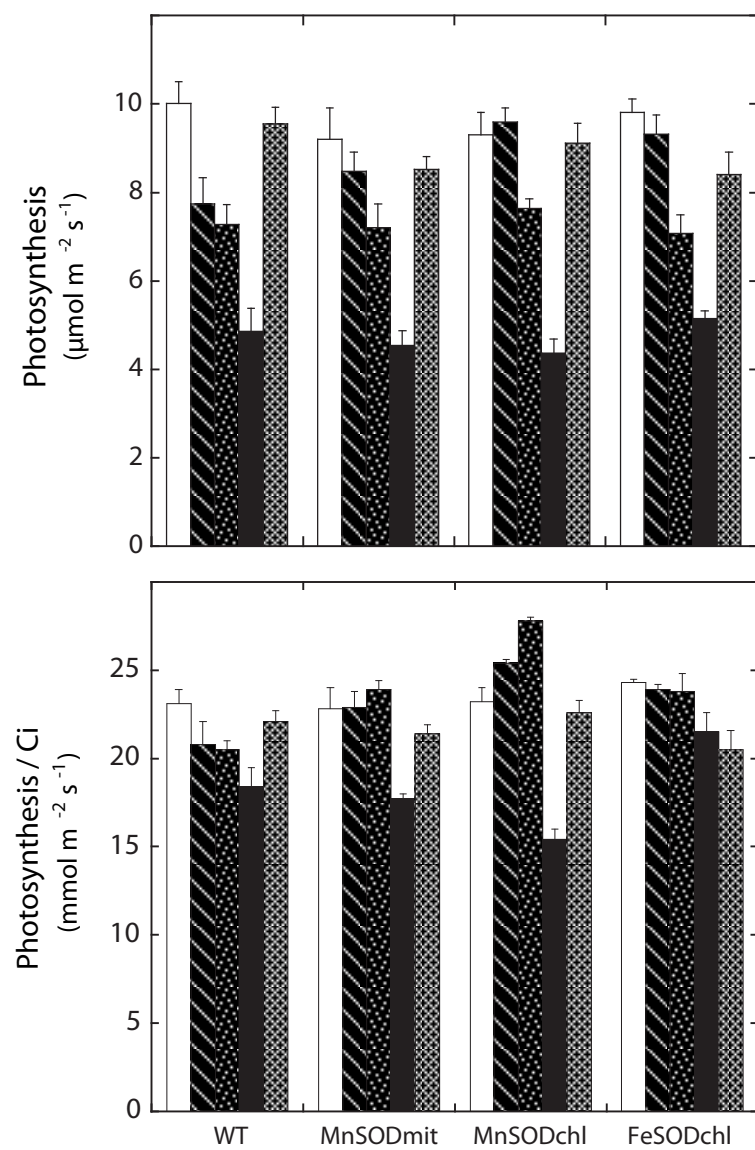


Fig. 2

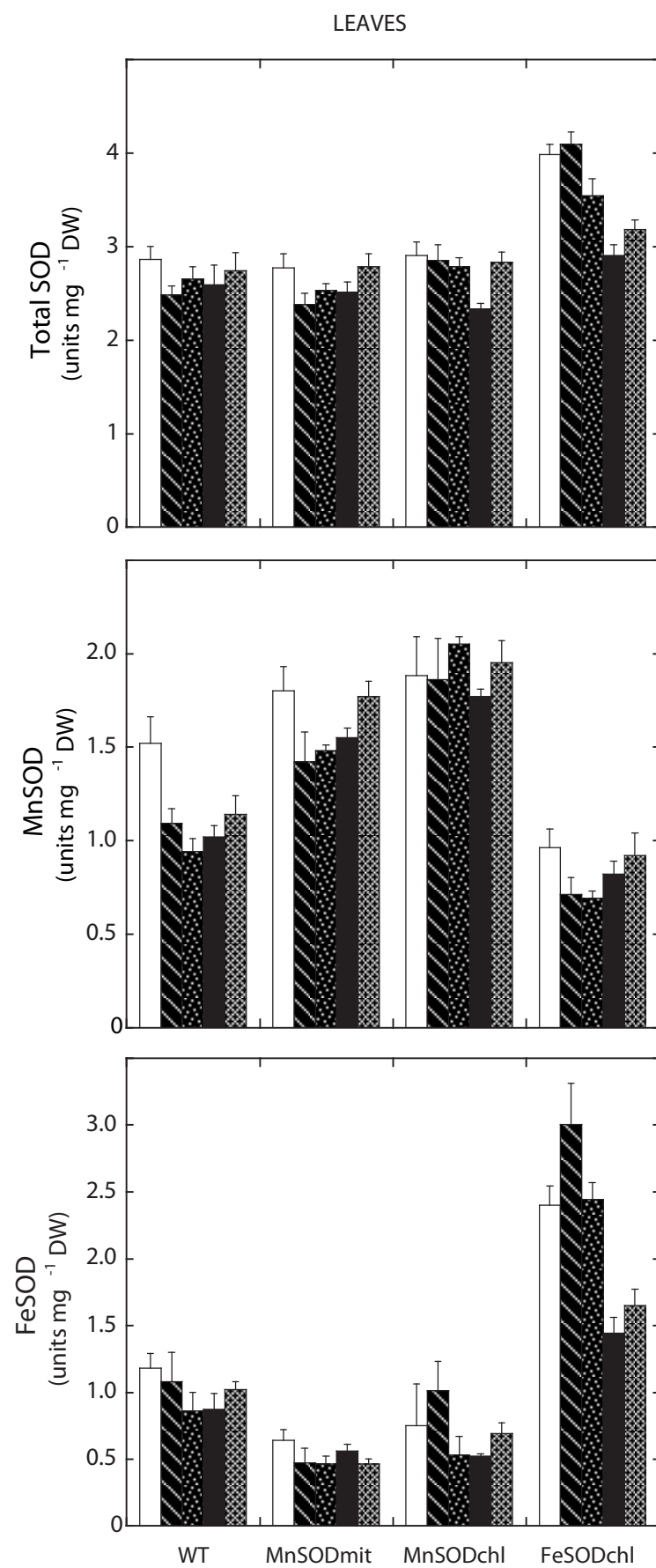


Fig. 3

LEAVES

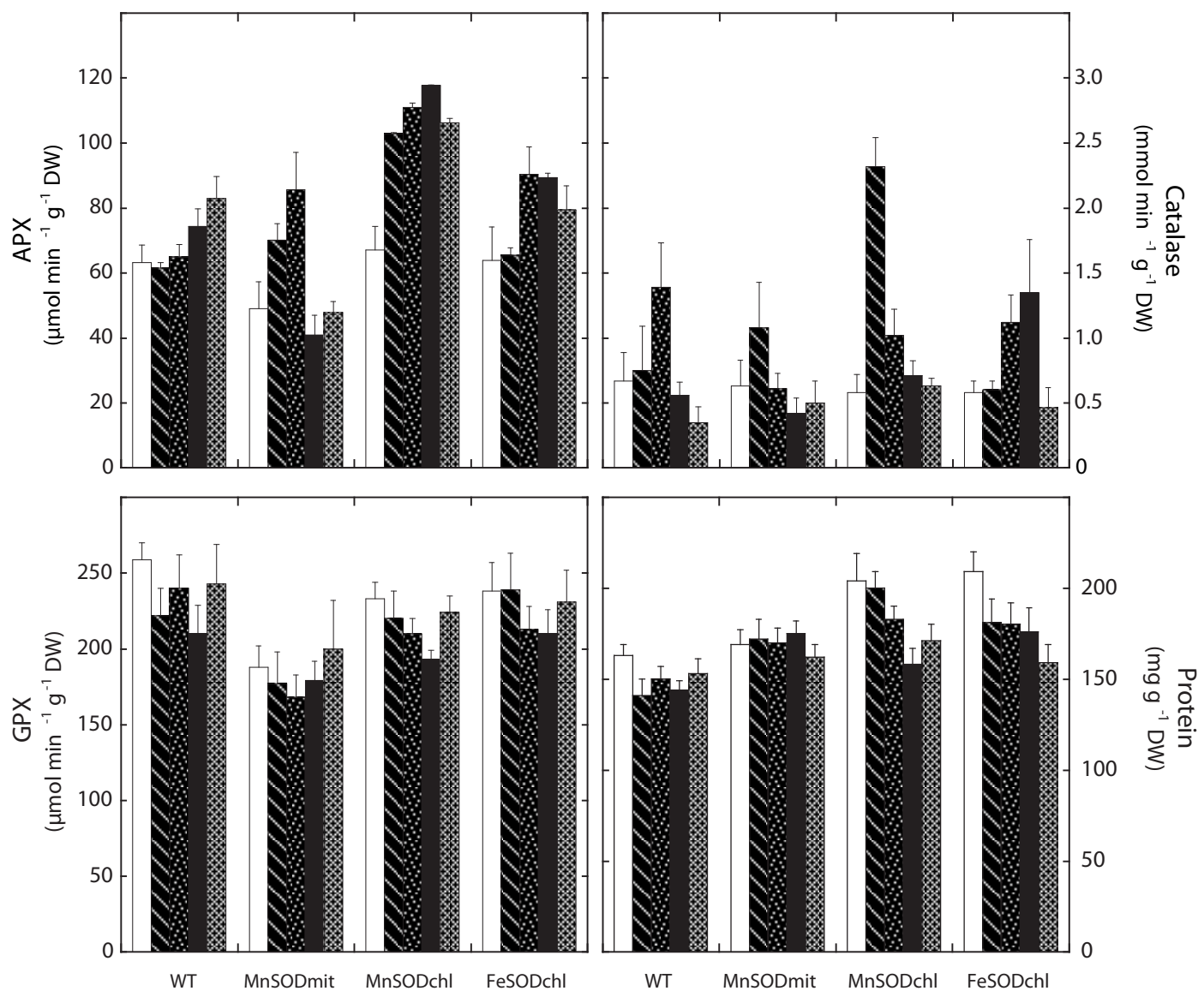


Fig. 4

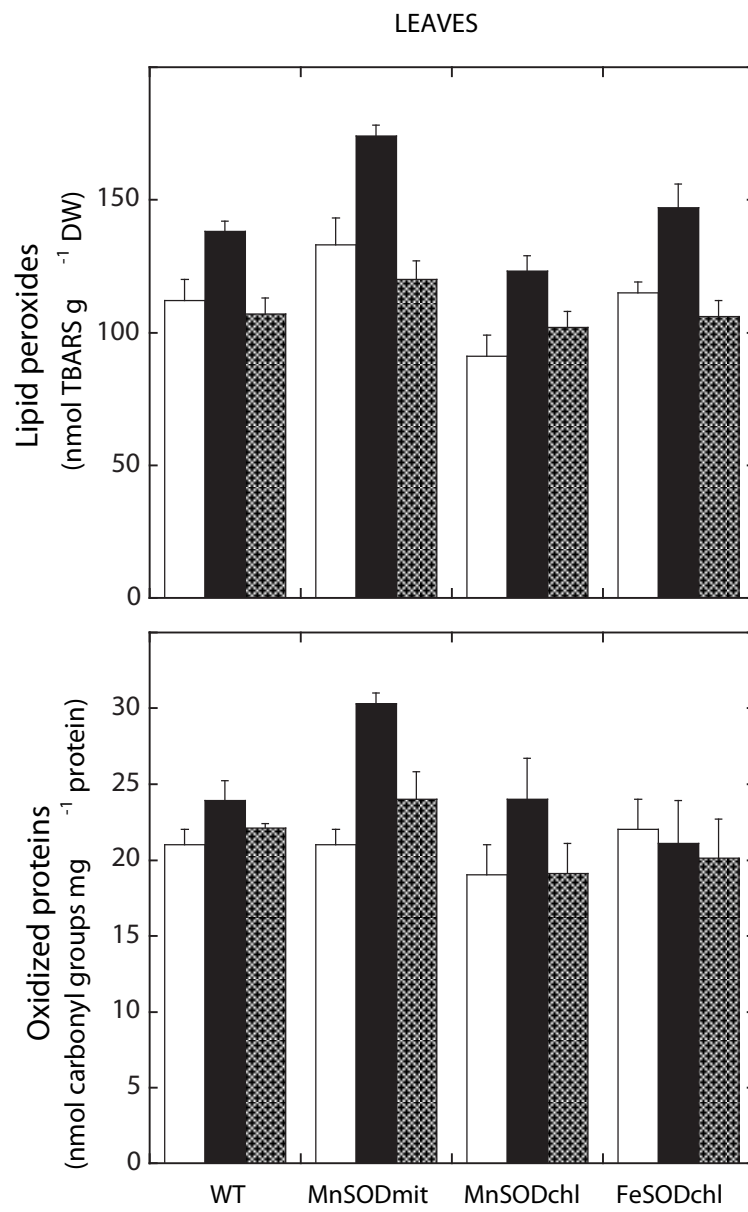


Fig. 5

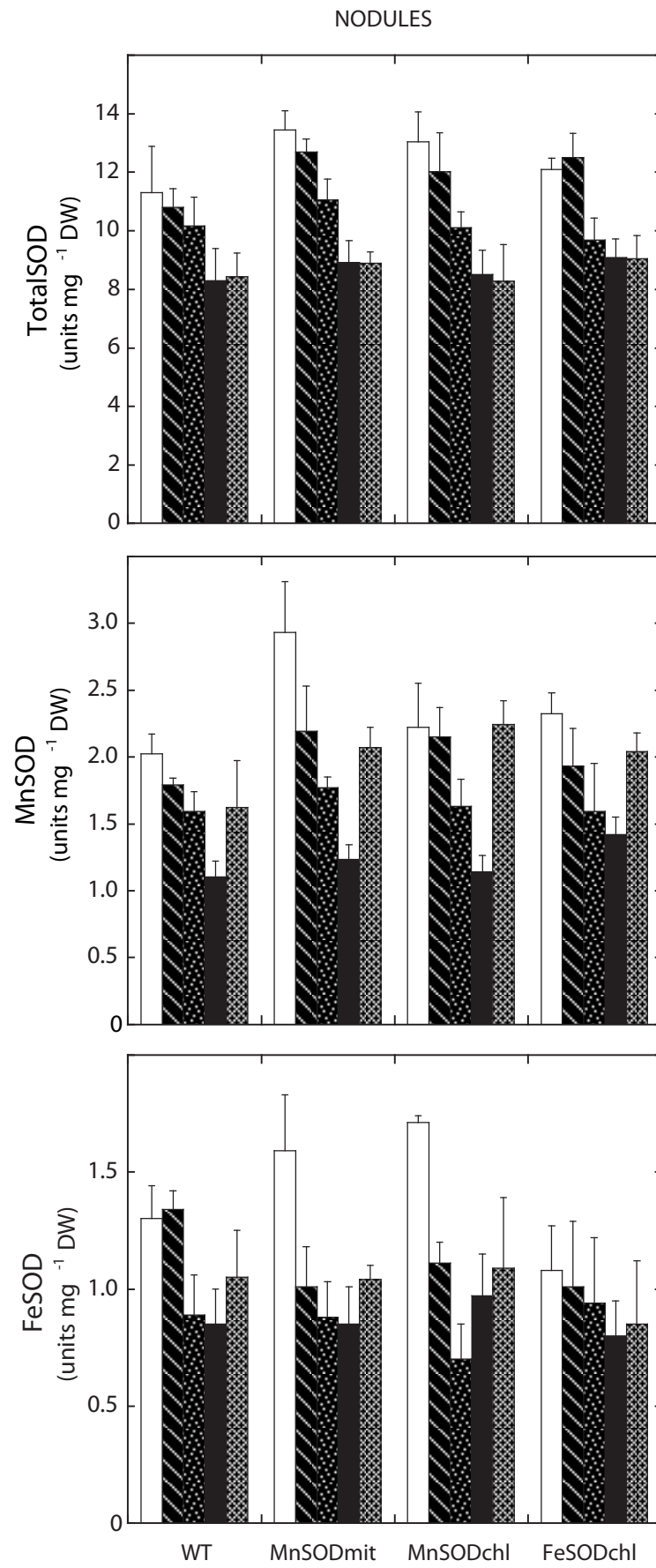


Fig. 6

NODULES

