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Expression Studies of Superoxide Dismutases in Nodules and Leaves of Transgenic Alfalfa Reveal Abundance of Iron-Containing Isozymes, Post-translational Regulation, and Compensation of Isozyme Activities

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Nucleotide sequences were deposited in GenBank as accession nos. AF377344 (*Medicago sativa* nodule FeSOD cDNA) and AF077224 (*Vigna unguiculata* nodule FeSOD cDNA).

The composition of antioxidant enzymes, especially superoxide dismutase (SOD), was studied in one nontransgenic and three transgenic lines of nodulated alfalfa plants. Transgenic lines overproduced MnSOD in the mitochondria of nodules and leaves (line 1-10), MnSOD in the chloroplasts (line 4-6), and FeSOD in the chloroplasts (line 10-7). In nodules of line 10-7, the absence of transgene-encoded FeSOD activity was due to lack of mRNA, whereas in nodules of line 4-6 the absence of transgene-encoded MnSOD activity was due to enzyme inactivation or degradation. Transgenic alfalfa showed a novel compensatory effect in the activities of MnSOD (mitochondrial) and FeSOD (plastidic) in the leaves, which was not caused by changes in the mRNA levels. These findings imply that SOD activity in plant tissues and organelles is regulated at least partially at the post-translational level. All four lines had low CuZnSOD activities and an abundant FeSOD isozyme, especially in nodules, indicating that FeSOD performs important antioxidant functions other than the scavenging of superoxide radicals generated in photosynthesis. This was confirmed by the detection of FeSOD cDNAs and proteins in nodules of other legumes such as cowpea, pea, and soybean. The full-length cDNA encoding alfalfa nodule FeSOD was characterized and the deduced protein found to contain a plastid transit peptide. Comparison of sequences and other properties reveals that there are two types of FeSODs in nodules.

The superoxide dismutase (SOD) family of enzymes represents a primary line of defense against the superoxide radical and derived reactive oxygen species in all organisms. Three types of SOD, differing in the metal at the active site, may coexist in plants. The CuZnSOD isozymes are localized in the cytosol, chloroplast stroma, glyoxysomal matrix, apoplast, nucleus, and mitochondrial intermembrane space; the MnSOD isozymes are localized in the mitochondrial and peroxisomal matrices and in the glyoxysomal membrane; and the FeSOD isozymes are primarily localized in the chloroplast stroma (del R□o et al. 1992; Bowler et al. 1994; Ogawa et al. 1996; Schinkel et al. 1998). Complete cDNA clones encoding some enzymes of each type have been isolated and used to construct transgenic plants overexpressing SODs in the chloroplasts, mitochondria, and cytosol (Bowler et al. 1991; Scandalios 1993; Van Camp et al. 1996). These studies were performed using model plants for transformation, such as tobacco, but important crop legumes can now be transformed and regenerated with relative ease (Christou 1994). Because legumes are unique amongst crop plants in their ability to fix atmospheric N_2 in symbiosis with rhizobia, transformation of these plants with antioxidant genes could provide additional advantages related to its protective role in nodule activity (Puppo et al. 1982; Dalton et al. 1998; Matamoros et al. 1999).

In this work, we have analyzed the antioxidant composition of nodulated plants of the elite genotype N4 and three derived transgenic lines overproducing, respectively, MnSOD in the mitochondria, MnSOD in the chloroplasts, and FeSOD in the chloroplasts. Transgenic alfalfa plants showed a compensatory effect in expression of MnSOD and FeSOD in the leaves. All four lines displayed low CuZnSOD activities and abundant FeSOD and MnSOD activities in nodules and leaves. The full-length cDNA sequence encoding alfalfa nodule FeSOD was characterized and nodules of different legumes were screened for the presence of FeSOD cDNAs and proteins. Results show that the FeSODs of nodules belong to two types of enzymes that can be distinguished biochemically and immunologically and that are probably located in different subcellular compartments.

RESULTS

Composition of SOD isozymes in alfalfa nodules and leaves.

SOD activity staining of nondenaturing gels preincubated with potassium phosphate buffer alone or supplemented with either 3 mM KCN or 5 mM H_2O_2 revealed that alfalfa nodules (Fig. 1) and leaves (Fig. 2) contain the three types of SOD that can be found in plants. These corresponded, in order of increasing mobility, to the MnSOD (KCN-insensitive, H_2O_2-insensitive), FeSOD (KCN-insensitive, H_2O_2-sensitive), and CuZnSOD (KCN-sensitive, H_2O_2-sensitive) isozymes. Nodule extracts of all lines produced a single band of mitochondrial MnSOD activity (Fig.1). However, in nodules and leaves of lines transformed to overproduce *Nicotiana plumbaginifolia* MnSOD in mitochondria (1-1, 1-6, 1-9, and 1-10), the MnSOD activity band was considerably more intense than in the nontransgenic line (Figs. 1 and 2), which probably reflects the inability of these gels to separate alfalfa and *Nicotiana* MnSODs. In contrast, in leaves of lines producing MnSOD in the chloroplasts (4-1, 4-6, and 4-7), two MnSODs were resolved (Fig. 2).

Nodules of all lines examined contained a distinct FeSOD isozyme, which was also present, albeit at lower levels, in the leaves (compare Figs. 1B and 2B). The presence of abundant FeSOD activity in alfalfa nodules and leaves is in clear contrast with previous reports showing no FeSOD in leaves (McKersie et al. 1999; 2000). Extracts of leaves, but not of nodules, of lines transformed to produce *Arabidopsis thaliana* FeSOD in the chloroplasts (10-4 and 10-7) showed three extra bands of FeSOD activity (Fig. 2). In contrast, in nodules there were four CuZnSOD isozymes, the first two of them (according to relative mobility) at higher levels than in the leaves (Fig. 1). Nodules also had a major band showing SOD activity, which was labeled as X. This band accounted for 70% of the total activity (calculated from data in Table 1) and was assigned to the MnSOD type (KCN-insensitive, H_2O_2-insensitive). However, its electrophoretic mobility (similar to that of plant FeSODs)

and apparent molecular mass (45 kDa) are clearly different from those of typical plant MnSODs (Sevilla et al. 1982).

It is possible that at least part of the activity of band X is due to bacteroid MnSOD. Thus, activity stain gels of extracts from highly purified bacteroids, broken by sonication, revealed a single MnSOD isozyme which had identical mobility to band X (Fig. 1).

Transcripts of SOD isozymes in alfalfa nodules and leaves.

Out of the nine lines initially screened, we selected line N4 and three transgenic lines (1-10, 4-6, and 10-7) for further study. These lines were representative of each different construct. Expression of *MnSOD* and *FeSOD* genes in nodules and leaves of the four selected lines was analyzed by reverse transcription (RT)-PCR and northern blots. Gene-specific primers were used to distinguish the mRNAs encoded by the endogenous genes (alfalfa *MnSOD* and *FeSOD)* and the transgenes (*Nicotiana MnSOD* and *Arabidopsis FeSOD)*.

Consistent with activity data (Fig. 1), RT-PCR analysis revealed that the absence of *Arabidopsis* FeSOD activity in nodules of line 10-7 was due to lack of message, whereas the absence of *Nicotiana* MnSOD activity in nodules of line 4-6 was probably due to inactivation or degradation of the enzyme because the corresponding mRNA was detectable at levels similar to those found in line 1-10 (Fig. 3). The analysis also showed that the leaves of all four lines express endogenous *MnSOD* and *FeSOD*, that the leaves of lines 1-10 and 4-6 express *Nicotiana MnSOD*, and that the leaves of line 10-7 express *Arabidopsis FeSOD* (Fig. 3).

Northern blot analysis of the steady-state mRNA levels confirmed the presence of abundant message for endogenous MnSOD and FeSOD in nodules and leaves of all four lines, as well as the absence of *Arabidopsis* FeSOD mRNA in nodules of line 10-7 and its presence in the corresponding leaves (Fig. 4). Most interesting, northern

blots showed abundant mRNA for *Nicotiana* MnSOD in the leaves of line 4-6 and less abundant mRNA in the leaves of line 1-10. The two mRNAs were also present in the corresponding nodules (Fig. 4). The differences in mRNA size between the *Nicotiana* MnSODs targeted to chloroplasts (line 4-6) and to mitochondria (line 1-10) reflect the differences in the original constructs, with the chloroplast transit peptide being significantly larger than the mitochondrial peptide (Bowler et al. 1991) .

Antioxidant enzymes of alfalfa nodules and leaves.

The activities of SOD and other antioxidant enzymes were measured in the four selected lines. The antioxidant activities of nodules from control plants were measured with optimized methods including specific controls to ensure measurement of genuine activities in the presence of leghemoglobin, which may interfere with determination of guaiacol peroxidase (GPX) or SOD activity under certain conditions (Puppo et al. 1982). There were no significant differences in the total SOD activity or in the activities of the H_2O_2-scavenging enzymes in nodules for any of the four lines. However, when MnSOD and FeSOD activities were individualized by densitometry, MnSOD activity in line 1-10 was found to be 45% higher than in line N4 and FeSOD activity of line 4-6 was 32% higher (Table 1).

Total SOD, APX, GPX, and catalase activities were similar in leaves of all lines, except in line 10-7, which exhibited a total SOD activity 39% higher than in line N4 (Table 2). Separate quantification of SOD isozymes showed that the leaves of lines 1-10 and 4-6 had approximately 20% more MnSOD activity and 40% less FeSOD activity. Conversely, the leaves of line 10-7 had 37% less MnSOD activity and twice as much FeSOD activity. These results confirmed that the leaves of the transformed plants expressed the MnSOD or FeSOD encoded by the transgenes.

Characterization of the FeSOD cDNA and deduced protein of alfalfa nodules.

The finding of abundant FeSOD activity in alfalfa nodules and the absence of

sequences for any FeSOD from nonphotosynthetic tissue prompted us to isolate and characterize the corresponding cDNA. Degenerate primers were used to screen an alfalfa nodule cDNA library. As expected, the positive clones contained the 5' and 3' regions of an FeSOD cDNA, judging by the high homology (>70% identity) with other cDNAs encoding FeSODs from higher plants. The complete cDNA sequence of alfalfa nodules (Fig. 5) contains 10 nucleotides within the 5'-untranslated region (UTR), 942 nucleotides within the open reading frame (ORF), and 205 nucleotides within the 3'-UTR that precedes a poly(A+) tail. The 3' end also contains a consensus signal for polyadenylation.

The ORF encodes a protein of 313 amino acids (Fig. 5), with a calculated molecular mass of 35.4 kDa and isoelectric point of 5.79. The enzyme contains the residues thought to be essential for FeSOD activity (Tyr-91, Trp-133, and Asn-218) and for metal binding (His-83, His-135, Asp-235, and His-239), as well as the residues (Ala-130, Gln-131, Trp-133, and Ala-219) proposed as primary candidates to distinguish FeSODs from MnSODs (Van Camp et al. 1990; Bowler et al. 1994). The deduced protein of alfalfa nodules also contains a signal peptide that, based on the N-terminal sequences of other FeSODs (Van Camp et al. 1990), is 57 amino acid residues long (Fig. 5). The signal peptide is very rich in Ser (19%) and Thr (11%) compared to Arg (5%), and is devoid of Glu, Asp, and Tyr. All these characteristics are typical of plastid transit peptides (von Heijne et al. 1989). The mature protein has a calculated molecular mass of 29.4 kDa and isoelectric point of 5.09, which is significantly different from the values predicted (molecular mass of 23.0 to 25.3 kDa; isoelectric point of 5.42 to 6.07) for the FeSODs of other plants. The deduced amino acid sequence of alfalfa nodule FeSOD has high homology (>70 %) with the FeSODs from other higher plants and lower homology (<70%) with the enzymes from cyanobacteria and green algae. However, there are two exceptions: the nodule enzyme showed only 58% identity with *Arabidopsis* FeSOD isozyme-1 and 50% identity with rice FeSOD.

Because FeSODs are detected only ocassionally in plants, we screened

extracts of other legume nodules for the presence of FeSOD. This provided information on the distribution and abundance of the enzyme and allowed a comparison with the alfalfa nodule FeSOD. Using SOD activity gels, we found FeSOD in nodules of cowpea, mungbean, pea, bean, and soybean, but not in those of lupine and broad bean (data not shown). The same primers used for alfalfa served to isolate clones and obtain the complete cDNA sequence of cowpea nodule FeSOD as well as partial sequences for pea and soybean nodule FeSODs. All derived protein sequences were used to construct a phylogenetic tree of known FeSODs (Fig. 6). It is interesting to note that two isozymes have been described for *Arabidopsis* and that the partial sequence we found for soybean nodules differs from that reported for leaves, suggesting that FeSOD may be present as a multigenic family at least in some plants. Phylogenetic analysis revealed three large clusters, which, as expected, are fully consistent with homology data: a cluster including the FeSODs of cyanobacteria, green algae, rice, and *Arabidopsis* isozyme-1; a cluster including the FeSOD of *Raphanus* and *Arabidopsis* isozyme-2; and a cluster including legume FeSODs. Interestingly, in this third cluster two groups can be recognized (Fig. 6) and this appears to be correlated with subcellular localization. Prediction programs reveal that the FeSODs of alfalfa and pea nodules contain a plastid signal peptide, whereas the FeSODs of cowpea nodules and soybean leaves and nodules lack any recognizable plastid peptide. The presence of two types of FeS ODs in nodules is confirmed by the absence of cross-reactivity between an antibody raised to cowpea nodule FeSOD and alfalfa or pea nodule FeSODs (data not shown) .

DISCUSSION

In this work we report that all three types of SODs are present in nodules and leaves of alfalfa (Figs. 1 and 2). The isozymic composition of SOD in our extracts

(low CuZnSOD activity, high FeSOD activity) clearly contrasts with that reported by other authors (McKersie et al. 2000) for alfalfa leaves (high CuZnSOD activity, virtually no FeSOD activity). These differences may be due to variations in plant development or to nutritional factors, such as the nitrogen source (N_2 versus combined nitrogen) and the micronutrient supply (which is known to differentially affect expression of SOD isozymes; eg. Kurepa et al. 1997). Whatever the reason, our results indicate that important variations in the SOD isozyme pattern do not affect the health and growth of plants. This may well reflect compensation in the activities of the SOD isozymes, which would require their expression to be tightly regulated. Thus, overproduction of MnSOD in the leaves of lines 1-10 and 4-6 was matched by lower FeSOD activities, resulting in similar total SOD activities in the leaves of the two lines and in the nontransgenic line (Table 2). Conversely, overproduction of FeSOD in line 10-7 was paralleled by a lowering in MnSOD activity, although in this case the 2-fold excess of the former was not compensated for completely and the total SOD activity of leaves in line 10-7 remained 40% greater than in line N4. Northern analysis indicated that the compensation of activities is not due to changes in transcript abundance. Thus, the mRNA level of endogenous MnSOD in leaves of line 10-7 (which expresses *Arabidopsis* FeSOD) is even higher than in line N4, and the mRNA level of endogenous FeSOD in lines 1-10 and 4-6 (which express *Nicotiana* MnSOD) is similar to or higher than in line N4 (Fig. 4). Therefore, the compensation between MnSOD and FeSOD activities occurs, at least in part, at the post-translational level.

In nodules, only line 1-10 produced the transgene-encoded SOD (Fig. 1). However, the *Nicotiana* MnSOD mRNAs were found in both lines 1-10 and 4-6, albeit at low levels (Figs. 3 and 4). The absence of *Nicotiana* MnSOD activity in nodules of line 4-6, despite expression of the gene, can be attributed to degradation of the enzyme, perhaps as a result of the inability of nodule plastids to process the protein bearing a chloroplastic prepeptide. Another surprising observation is the absence of transcript for *Arabidopsis* FeSOD in nodules of line 10-7, despite its abundance in

the corresponding leaves. This may be ascribed at least in part to a weak activity of the 35S promoter in alfalfa nodules. However, this would still leave unexplained why constructs with identical promotor and chloroplastic targeting sequences are express ed (MnSOD in line 4-6) or not expressed (FeSOD in line 10-7) in nodules. To verify the differences in expression among the three transgenic lines, nodules of exactly the same plant were used to extract RNA (for northern analysis) and protein (for activity gel analysis). This experiment confirmed that nodules of line 1-10 expressed transcript and activity, nodules of line 4-6 expressed transcript but not activity, and nodules of line 10-7 did not express the transcript.

The abundance of FeSOD in alfalfa nodules deserved special attention, as this type of SOD, in contrast to CuZnSOD and MnSOD, is not ubiquitous in plants. Furthermore, there are contradictory reports about the presence of FeSOD in leaves of some species, such as common bean and cowpea. Early studies reported that FeSODs were confined to a few families of higher plants (Bridges and Salin 1981). However, it seems now that the gene is more widely distributed than previously thought but remains silent due to lack of appropriate inducing conditions (Bowler et al. 1994). Our finding of FeSOD in nodules of healthy plants of alfalfa and other legumes is consistent with that hypothesis. The FeSODs of alfalfa and pea nodules are closely related and are synthesized as precursor plastidic proteins, which suggests that these enzymes play a role in plastid metabolism other than the scavenging of superoxide radicals associated with photosynthesis. The occurrence of abundant ferritin in the plastids may ensure an adequate Fe supply for the synthesis of FeSOD, especially under conditions in which this isozyme is up-regulated (reviewed by Becana et al. 1998). The presence of at least two types of FeSODs further underscores the multifaceted nature of antioxidant protection in nodules and suggests that the various SOD isozymes may have evolved to perform specific defensive or regulatory roles that are essential for optimal nodule functioning. Clearly, further work is necessary to characterize these roles.

MATERIALS AND METHODS

Plant material and propagation.

Alfalfa (*Medicago sativa* L.) clones used in this study were generously provided by Dr. Bryan McKersie. Clone N4 is the nontransgenic parental line. Clones 1-1, 1-6, 1-9, and 1-10 were transformed with pSOD1, which was generated as a transcriptional fusion of the MnSOD preprotein from *Nicotiana plumbaginifolia* to the cauliflower mosaic virus 35S promoter (Bowler et al. 1991). Clones 4-1, 4-6, and 4-7 were transformed with pSOD4, which harbors the mature MnSOD-encoding sequence of *N. plumbaginifolia* fused to a chloroplast transit peptide sequence (small subunit of Rubisco from pea) under the control of the 35S promoter (Bowler et al. 1991). Clones 10-4 and 10-7 were transformed with pSOD10, which encodes FeSOD from *Arabidopsis thaliana* and is coupled to the same chloroplast targeting sequence under the control of the 35S promoter (Van Camp et al. 1996). All three binary vectors (pSOD1, pSOD4, and pSOD10) contained the *nptII* gene as a selectable marker, under the control of the nopaline synthase *(nos)* promoter (Herrera-Estrella et al. 1983).

Briefly, the transformation procedure was as follows. Alfalfa petiole explants from the parental line were co-cultivated with *Agrobacterium tumefaciens* C58C1 pMP90 for 3 d in the dark on SH induction medium (Van Camp et al. 1996; McKersie et al. 2000). The explants were plated on SH medium containing kanamycin (50 mg l^{-1}) and somatic embryos were matured on BOi2Y development medium containing sucrose but no growth regulators or antibiotics (McKersie et al. 2000). Putative transformants were screened for the presence of T-DNA by PCR. Positive plants were transplanted to growth medium in a greenhouse and were screened by determining SOD activity in leaf extracts and isozyme composition on nondenaturing gels (McKersie et al. 2000). Clones were vegetatively propagated by cuttings taken from plants before flowering, briefly dipped into commercial hormone rooting powder,

and placed in beakers containing nutrient solution supplemented with 1 mM KNO_3. Cuttings were left to root in the growth cabinet under low light conditions for approximately 25 d. At that time, plants were transferred to pots containing a perlite/vermiculite mixture and watered once with nutrient solution supplemented with 2 mM KNO_3. After 3 d and 7 d, plants were inoculated twice with *Sinorhizobium meliloti* 102F78. Growth conditions were as previously described (Gogorcena et al. 1997). No obvious phenotypic differences between the transgenic and the parental line were observed.

Isolation of FeSOD cDNA of alfalfa nodules.

An alfalfa nodule cDNA λZAP library (kind gift of Dr. Carroll P. Vance) was PCR-screened with degenerate oligonucleotide primers (forward: 5'-GC[A/T]TTCAAC AA[T/C]GC[T/A]GC[T/A]CAGG-3'; reverse: 5'-TC[A/C]AG[G/A]TAGTAAGCATGCTC CCA-3') based on conserved sequences of FeSODs (GenBank accession numbers in parentheses) from *Chlamydomonas reinhardtii* (U22416), *Nicotiana plumbaginifolia* (M55909), *Arabidopsis thaliana* isozyme-2 (M55910), and soybean leaves (M64267). The 5'- and 3'-end regions were amplified using the above primers in combination with T3 (5'-GCAATTAACCCTCACTAAAGGG-3') and T7 (5'-GCGTAA TACGACTCACTATAGGGC-3') primers. The PCR mix contained 0.2 μM of primers, 240 μM dNTPs, 1.5 mM $MgCl_2$, 0.05% W-1 detergent (Life Technologies, Paisley, U.K.), and 1.25 units of *Taq* polymerase (Life Technologies), in a final volume of 25 μl of the PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl). The PCR cycling protocol consisted of an initial denaturation step at 95C for 3 min, 40 cycles (62C for 45 s, 72C for 60 s, 95C for 45 s), and a final elongation step at 72C for 10 min.

The resulting PCR products were size-fractionated on 1% agarose gels, purified (Concert; Life Technologies), and subcloned into the linearized vector pGEM-T Easy (Promega, Madison, WI). The DNA inserts from several clones were sequenced on both strands with an ABI Prism 377 sequencer (Applied Biosystems, Foster City,

CA). Homology searches were done with the BLAST algorithm (Altschul et al. 1997). Sequence alignments and analyses were performed using the Gap and PileUp programs of the Genetics Computer Group package (Madison). Predictions of subcellular localization and signal peptide analyses were performed using the programs MitoProtII (Claros 1995), PSORT (Nakai and Kanehisa 1992), ChloroP and TransitP (Center for Biological Sequence Analysis, Department of Biotechnology, Technical University of Denmark, Denmark), along with criteria stated by von Heijne et al. (1989).

RT-PCR and northern analyses.

Total RNA (approximately 100 μg) was extracted from 200 to 400 mg of leaves or nodules by a phenol-LiCl procedure (Verwoerd et al. 1989), using RNaseOut (Life Technologies). RNA concentration and purity was determined by spectrophotometry and visualized by electrophoresis in agarose-formaldehyde gels.

For RT-PCR, the RNA (5 μg) was primed with oligo$(dT)_{20}$ and reverse-transcribed in a total volume of 25 μl using Moloney murine leukemia virus reverse transcriptase (Promega). PCR was carried out using gene-specific primers based on internal sequences of *Medicago truncatula MnSOD* (AW688895), alfalfa *FeSOD* (AF 377344), tobacco *MnSOD* (X14482), and *Arabidopsis FeSOD-2* (M55910). For alfalfa *MnSOD*, the forward primer was 5'-TGTCATCAGCGGCGAAATC-3', and the reverse primer 5'-TTCTGCATTAACCTTTTGTATC-3'. For alfalfa *FeSOD*, forward: 5'-AAGGAAAGCAGACTTGATG-3', reverse: 5'-TCTCCTCCTCTTTCTCC-3'. For *Nicotiana MnSOD,* forward: 5'-GCGGCTTGCAGACCTTTT-3', reverse: 5'-GATAGCG CTATGCAATTTGG-3'. For *Arabidopsis FeSOD*, forward: 5'-AATGAAAAACTCAAAG TAGTG-3', reverse: 5'-ACTCACTGTCACTGAAGTC-3'. PCR reactions (25 μl) contained 0.75 to 2 μl of first-strand cDNA (taken from a resuspension of the cDNA in 100 μl of water), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM of primers, and 2.5 units of *Taq* polymerase (Life Technologies) in a total volume of 25 μl. Reactions were

initiated by a denaturation step at 94C for 4 min, followed by 25 cycles (94C for 30 s, 54C for 45 s, and 72C for 45 s), and a final extension step at 72C for 10 min. After amplification, the reaction products were resolved by electrophoresis on a 1% (w/v) agarose gel and stained with ethidium bromide. In all cases, preliminary runs were used to verify that the number of amplification cycles was below signal saturation. Images were captured using a Gel-Doc 2000 DNA Gel Analysis and Documentation System (Bio-Rad, Hercules, CA) using Quantity One 4.1.1 software.

For northern hybridization, RNA (10 μg) was separated on 1.2% agarose denaturing (formaldehyde) gels and capillary transferred overnight in 20 x SSC to Hybond-N^+ nylon filters (Amersham Pharmacia Biotech, Uppsala, Sweden). The RNA was fixed to the filter by exposure to UV light for 5 min and then at 80C for 2 h. Probes were obtained directly by gel purifying the products of the RT-PCR experiment. Random priming was used to ^{32}P-label the probes (Megaprime, Amersham Pharmacia Biotech). Filters were prehybridized at 42C for 2 h with 50% formamide, 125 mM sodium phosphate (pH 7.2), 250 mM NaCl, 1 mM EDTA, and 7% SDS. Hybridization was performed overnight at the same conditions as for prehybridization with addition of the ^{32}P-labeled probes. The filters were washed succesively with 2 x SSC for 10 min, 0.5 x SSC for 10 min, and 0.1x SSC for 3 min all SSC media contained 0.1% SDS). Signals were detected using a storage phosphorscreen (Imaging Screen-K; Eastman Kodak, Rochester, NY) and quantified with a Molecular Imager FX (Bio-Rad) using Quantity One 4.1.1 software.

RNA extractions and RT-PCR and northern experiments were repeated twice using nodules and leaves from two series of plants grown independently. Similar results were obtained and representative data are shown.

Assay of antioxidant enzymes.

Samples of nodules or leaves to be used for biochemical analyses were flash-frozen in liquid N_2 and stored at -80C. All enzymes were extracted at 0-4C and

activities were measured spectrophotometrically at 25C within the linear region for both time and enzyme concentration. Total SOD activity was assayed by the inhibition of the reduction of ferric cytochrome *c* by the superoxide radicals generated with a xanthine/ xanthine oxidase system. 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). A low concentration of KCN (10 μM) was inc luded in the assay medium of total SOD to inhibit mitochondrial cytochrome *c* oxidase withoutaffecting CuZnSOD activity. To assay KCN-insensitive SOD activity, the final KCN concentration was increased to 3 mM. Boiled extracts showed < 4% residual SOD activity.

The isozymic pattern of SODs in nodule and leaf extracts was analyzed by activity staining following electrophoresis on nondenaturing polyacrylamide gels (0.75-mm thick, 15% resolving gel, 4% stacking gel). The activity stain was based on the inhibition by SOD of the reduction of nitroblue tetrazolium by superoxide radicals generated photochemically (Beauchamp and Fridovich 1971). Identification of isozymes was based on the differential inhibition of SOD activity on gels preincubated with 3 mM KCN or 5 mM H_2O_2 for 1 h. Isozymes were quantitated by densitometry using National Institutes of Health software.

APX, GPX, and catalase were extracted with optimized media essentially as described by Gogorcena et al. (1995). APX and catalase activities were assayed by following the disappearance of ascorbate at 290 nm (Asada 1984) and of H_2O_2 at 240 nm (Aebi 1984), respectively. GPX activity was measured by following the oxidation of pyrogallol at 430 nm with preincubation for 5 min with 0.5 mM *p*-chloromercuriphenyl-sulfonic acid (Amako et al. 1994). Because APX may also catalyze pyrogallol oxidation to some extent, *p*-chloromercuriphenyl-sulfonic acid was included in the assay medium to inactivate APX and thus ensure the accurate measurement of GPX activity.

Statistical analyses.

Six series of plants grown independently under identical environment conditions were required to obtain sufficient nodule and leaf material. Each series comprised plants of all four lines. All measurements were made on plants belonging to at least two series and were pooled for statistical analysis. All data were subjected to analysis of variance and, when this was significant, means were compared with the Duncan's multiple range test. The number of samples used for the calculation of the means is stated in each table. Experiments to analyze gene expression by RT-PCR and northern blot were performed in duplicate, each replicate corresponding to extracts of nodules and leaves from two series of plants.

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Legends for Figures

Fig. 1. Isozyme SOD composition of nodules from alfalfa line N4 and derived transgenic lines. Prior to activity staining, the gels were incubated with (A) potassium phosphate buffer, (B) buffer plus 3 mM KCN, or (C) buffer plus 5 mM H_2O_2. Lanes were loaded with 40 μg protein.

Fig. 2. Isozyme SOD composition of leaves from alfalfa line N4 and derived transgenic lines. Panel description is identical to that of Figure 1. Lanes were loaded with 40 μg protein.

Fig. 3. RT-PCR analysis of SOD isozymes of alfalfa nodules and leaves. Gene-specific primers were used to distinguish mRNAs of alfalfa (*Msat MnSOD* and *Msat FeSOD)* and mRNAs encoded by the transgenes (*Nplu MnSOD* and *Atha FeSOD)*. Abbreviations are as follows: *Msat, Medicago sativa; Nplu, Nicotiana plumbaginifolia; Atha, Arabidopsis thaliana.* Size of PCR products is indicated in bp. To ensure uniform amounts of template, PCR reactions were performed simultaneously using ubiquitin primers (Horvath et al. 1993).

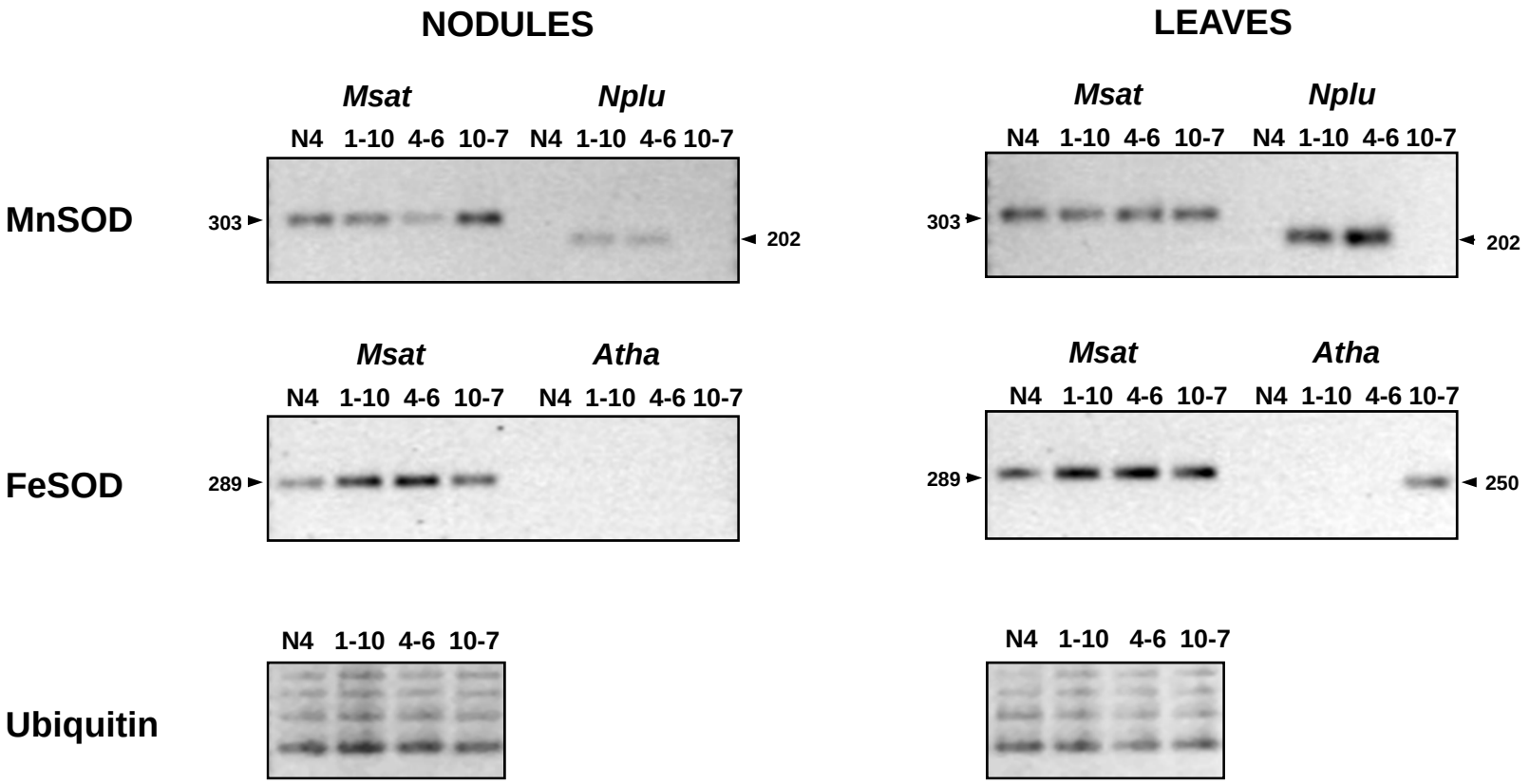
Fig. 4. Northern blot analysis of SOD isozymes of alfalfa nodules and leaves. Probes were generated from the primers used for RT-PCR. All the bands had the expected size of 1100 to 1200 bp according to RNA molecular weight markers. Uniform loading and integrity of RNA was verified by spectrophotometry and visualization of RNA with ethidium bromide (EtBr).

Fig. 5. Nucleotide and deduced amino acid sequences of FeSOD from alfalfa nodules. The arrow indicates the putative cleavage site of the signal peptide. Residues that distinguish FeSOD from MnSOD are indicated with a black circle. Residues essential for catalytic activity and metal binding are indicated with a plus

mark and a white circle, respectively. A putative polyadenylation sequence at the 3' end is underlined.

Fig. 6. Unrooted phylogenetic tree of FeSOD proteins from cyanobacteria, green algae, and higher plants. The tree was calculated using the neighbor-joining method of the CLUSTAL W suite of programs. The numbers correspond to percentages of 1000 'bootstraps'. The bar represents 0.01 substitutions per site. Abbreviations and GenBank accession numbers are as follows: *Anabaena, Anabaena* strain PCC7120 (AF173990); *Atha*-1, *Arabidopsis thaliana* isozyme-1 (AF061852); *Atha*-2, *Arabidopsis thaliana* isozyme-2 (P21276); *Crei, Chlamydomonas reinhardtii* (JC4611); *Gmax*-leaf, *Glycine max*, leaf isozyme (M64267); *Gmax*-nod, *Glycine max* nodule isozyme (partial sequence); *Msat*-nod, *Medicago sativa* nodules (AF377344); *Nplu, Nicotiana plumbaginifolia* (A39267); *Osat, Oryza sativa* (AB014056); *Pbor, Plectonema boryanum* (P50061); *Psat*-nod, *Pisum sativum* (partial sequence); *Rsat, Raphanus sativus* (AF061583); *Synechocystis, Synechocystis* strain PCC6803 (P77968); *Vung*-nod, *Vigna unguiculata* nodules (AF077224); *Zaet, Zantedeschia aethiopica* (AF094831).

Fig. 3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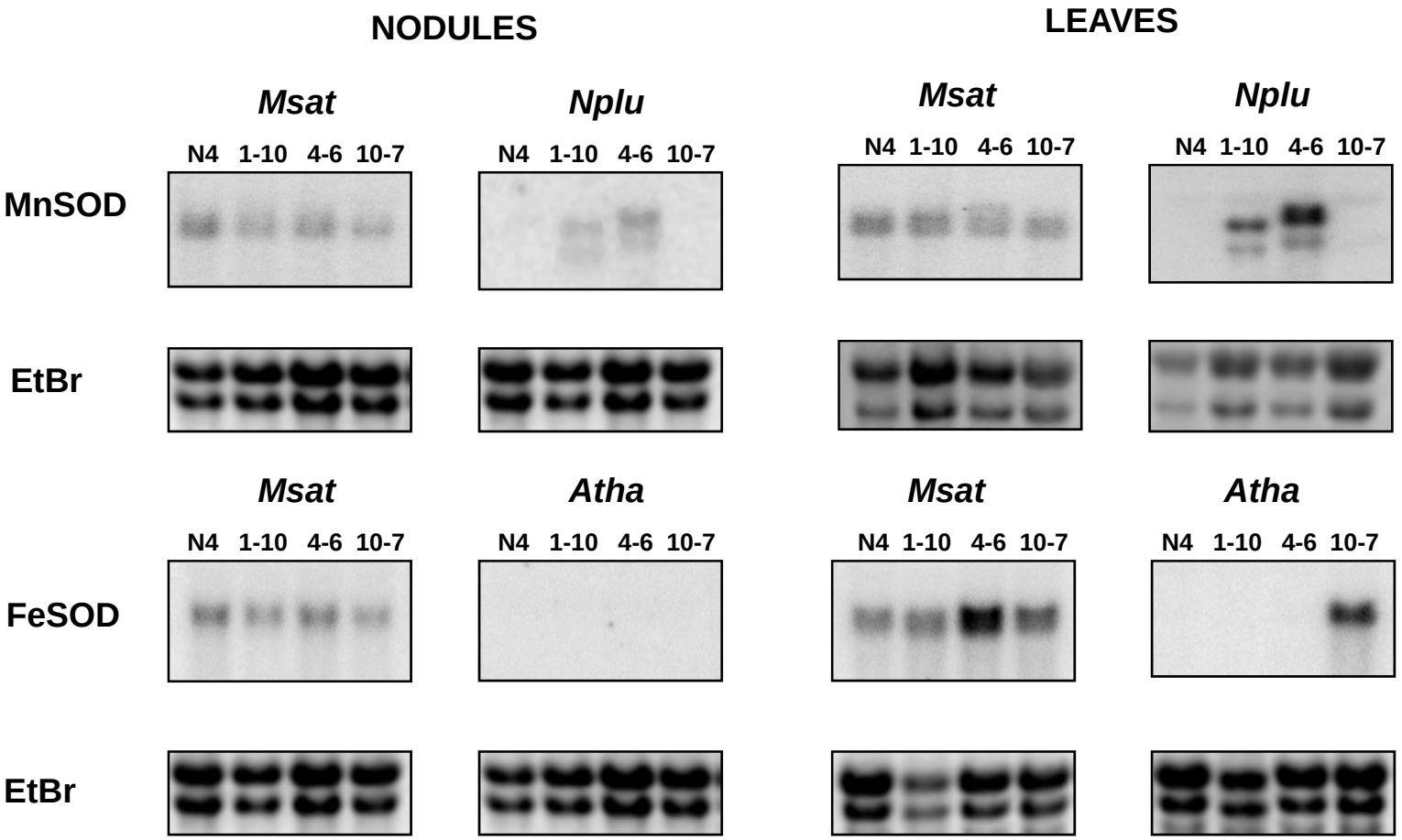
LEAVES
Msat
Nplu
Atha
N4
1-10
4-6
10-7
MnSOD
FeSOD
Ubiquitin
303
202
289
250

Fig. 4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LEAVES
Msat
Nplu
Msat
Nplu
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N4 1-10 4-6 10-7
MnSOD
EtBr
Msat
Atha
Msat
Atha
N4 1-10 4-6 10-7
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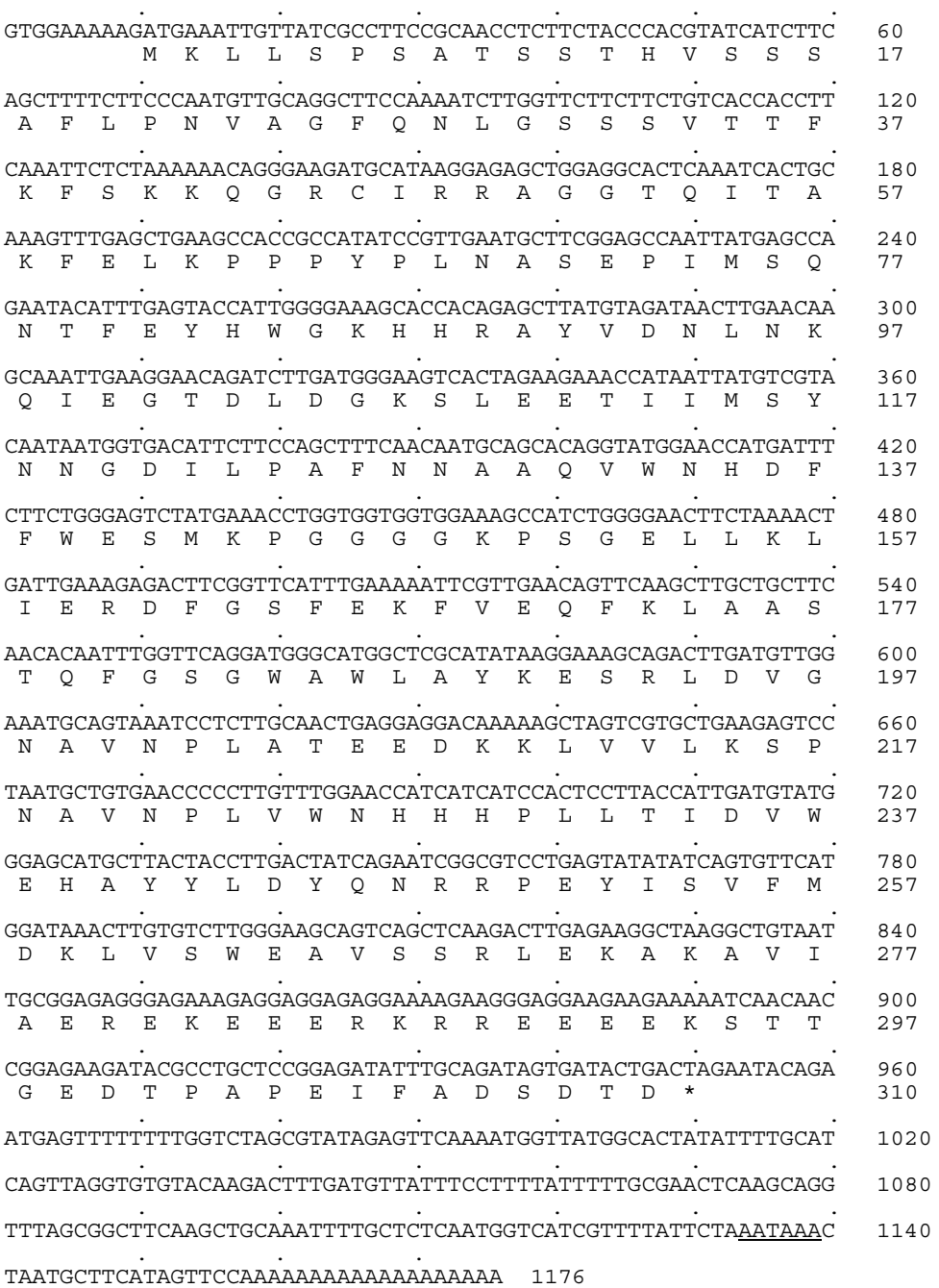
N4 1-10 4-6 10-7
N4 1-10 4-6 10-7
FeSOD
EtBr

Fig. 5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N T F E Y H W G K H H R A Y V D N L N K 97
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I E R D F G S F E K F V E Q F K L A A S 177
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E H A Y Y L D Y Q N R R P E Y I S V F M 257
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D K L V S W E A V S S R L E K A K A V I 277
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A E R E K E E E R K R R E E E E K S T T 297
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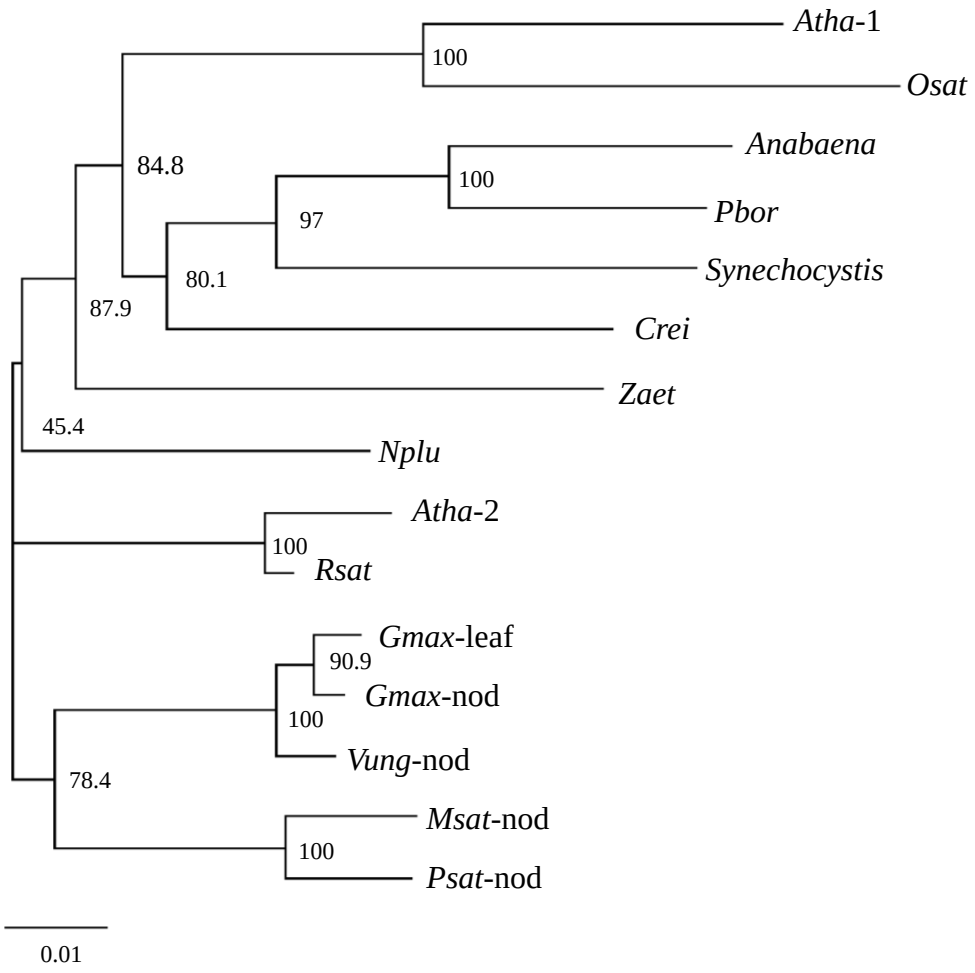
Fig. 6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Atha-2
100
Rsat
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100
Vung-nod
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Msat-nod
100
Psat-nod
0.01

Table 1. Antioxidant enzyme activities in nodules of line N4 and three derived transgenic lines of alfalfa[z]

Enzyme	N4	1-10	4-6	10-7
Total SOD	11.29 ± 1.59 a	13.43 ± 0.66 a	13.03 ± 1.03 a	12.09 ± 0.38 a
MnSOD	2.02 ± 0.15 a	2.93 ± 0.38 b	2.22 ± 0.33 ab	2.32 ± 0.16 ab
FeSOD	1.30 ± 0.14 ab	1.59 ± 0.24 ab	1.71 ± 0.03 b	1.08 ± 0.19 a
APX	92.7 ± 6.8 a	99.1 ± 11.2 a	104.3 ± 12.5 a	87.8 ± 11.8 a
GPX	683 ± 45 a	825 ± 111 a	820 ± 101 a	805 ± 69 a
Catalase	8.9 ± 0.8 a	9.1 ± 1.1 a	8.4 ± 1.5 a	7.1 ± 1.0 a
Protein	182 ± 6 a	198 ± 16 ab	212 ± 10 ab	220 ± 18 b

[z] Units are as follows: SOD (units mg^{-1} dry weight), APX and GPX (μmol min^{-1} g^{-1} dry weight), catalase (mmol min^{-1} g^{-1} dry weight), and protein (mg g^{-1} dry weight). Means ± SEM (n=4-7) were compared using one-way analysis of variance and the Duncan's multiple range test. For each parameter, means followed by the same letter do not differ significantly at $P<0.05$.

Table 2. Antioxidant enzyme activities in leaves of line N4 and three derived transgenic lines of alfalfa[z]

Enzyme	N4	1-10	4-6	10-7
Total SOD	2.86 ± 0.14 a	2.77 ± 0.15 a	2.96 ± 0.15 a	3.98 ± 0.11b
MnSOD	1.52 ± 0.14 a	1.80 ± 0.13 b	1.88 ± 0.21 b	0.96 ± 0.10 c
FeSOD	1.18 ± 0.11 a	0.64 ± 0.08 b	0.75 ± 0.31 b	2.40 ± 0.14 c
APX	63.2 ± 5.4 a	49.1 ± 8.3 a	67.1 ± 7.2 a	63.9 ± 10.2 a
GPX	259 ± 11 a	188 ± 14 b	233 ± 11 a	238 ± 19 a
Catalase	0.67 ± 0.22 a	0.63 ± 0.20 a	0.58 ± 0.14 a	0.58 ± 0.09 a
Protein	163 ± 6 a	169 ± 8 a	204 ± 15 b	209 ± 11 b

[z] Enzyme units and statistical analysis of means ± SEM (n=4-5) are as in Table 1. For each parameter, means followed by the same letter do not differ significantly at $P<0.05$.