

Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (*Hevea brasiliensis*)

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Abstract

A genomic clone encoding manganese-containing superoxide dismutase (SOD; EC 1.15.1.1) was isolated from a *Hevea brasiliensis* genomic library made in λ phage EMBL3 by using a heterologous cDNA probe of MnSOD from *Nicotiana plumbaginifolia*. The nucleotide sequence of 4968 bp from the genomic clone was determined. Based on the putative translation initiation codon and stop codon, PCR primers were designed and utilized for cloning the full-length cDNA from total mRNA. Of the two distinct cDNAs of MnSOD isolated, MnSOD-A has a perfect match with exons of the nuclear gene, while MnSOD-B has a 90.2% homology and is 6 nucleotides longer than MnSOD-A in the putative transit peptide region. The nuclear gene comprises 6 exons and 5 introns, giving a total length of 3211 bp. The sequences of 1400 bp upstream of the initiation codon and 320 bp downstream of the stop codon were also determined. Southern analysis of genomic DNA from *Hevea* probed with a genomic fragment indicated there are at least two genes of MnSOD in *Hevea*. Northern blot analysis showed that MnSOD transcripts were present in all tissues examined (leaf, petiole, root, latex, callus) with young leaves showing the highest levels in intact plants. The transcript level in embryogenic callus was nearly 50-fold higher than in mature leaves. In addition, transcripts of MnSOD could be induced 3- to 5-fold in response to sucrose, ethephon and Murashige-Skoog salts.

Introduction

Superoxide dismutase (SOD; superoxide: superoxide oxidoreductase, EC 1.15.1.1) functions as a defense mechanism against highly reactive oxygen species by catalyzing the conversion of superoxide anion (O_2^-) to oxygen and hydrogen

peroxide (H_2O_2) [14]. SODs are categorized into two major families on the basis of metal cofactors at the active site and their evolutionary homology: copper and zinc-containing SOD (Cu/ZnSOD), and manganese or iron-containing SOD (Mn or FeSOD) [3]. In higher plants two forms of Cu/ZnSOD have been found, one in the cytosol and

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L11707.

another in chloroplasts [1, 13]. FeSOD has also been found in the chloroplasts of some plant families [3, 40]. In plants, MnSOD is found primarily in mitochondria and peroxisomes [4, 6, 12].

The MnSOD gene has been clones and characterized from *Escherichia coli* [39], *Bacillus stearothermophilus* [7], *Mycobacterium leprae* [38], *Halobacterium* [27] and yeast [26]. In addition, cDNAs of MnSOD have been isolated from man [5], rat [19], maize [41], and *Nicotiana plumbaginifolia* [6]. The striking homology of MnSOD at the amino acid level from phylogenetically distant organisms suggests that it is a highly conserved enzyme in the biological kingdom [16]. In addition, MnSOD was found highly inducible by oxidative stress in *E. coli* [17, 30], and by a number of environmental stresses in plants [6].

Low levels of SOD have been found to be associated with dry bark syndrome of the commercial rubber tree, *Hevea brasiliensis*. This is a physiological disorder characterized by a severe reduction or cessation of latex flow resulting from premature senescence of latex-producing cells (laticifers) located just beneath the bark [10]. Interestingly, affected trees also contain high levels of superoxide and hydrogen peroxide suggesting that the accumulation of oxyradicals, and the inadequate protection provided by lower levels of SOD and catalase in *Hevea* latex, might be responsible for development of this syndrome.

We report here on the isolation and characterization of genomic and cDNA clones encoding MnSOD in rubber. This is the first report of a genomic sequence of MnSOD isolated from a higher eukaryote. We also present data on tissue-specific expression and accumulation of MnSOD mRNA in response to sucrose, ethephon and MS salts.

Materials and methods

Plant materials, culture and treatments

Hevea brasiliensis (Muell. Arg.) cv. RRIM C600 plants (3 years old) were grown from freshly harvested seeds, a generous gift of the Rubber Re-

search Station of IRCA (Institut de Recherches sur le Caoutchouc Agronomique), Abidjan, Ivory Coast, and maintained in a greenhouse with a mean day-time temperature of 32 °C and a mean night-time temperature of 22 °C. During the months of September to April, daylight was supplemented with an additional 4 h of artificial illumination (high-pressure sodium, 400 W, GE Lucalux). Fully expanded mature leaves (one to two months old) were subjected to immediate RNA extraction or cut into several sections to fit the dimensions of a 90 mm Petri plate for *in vitro* treatments. Each Petri plate contained 30 ml of sterile liquid containing various treatments as indicated. Ethephon (2-chloroethyl phosphonic acid; Sigma) was made fresh in sterile water just prior to use. Murashige-Skoog (MS) salts were prepared based on the conventional formula [29]. All the treated plant materials were incubated in a growth chamber at 25 °C with a light/dark cycle of 14/10 for 48 h. For the *in vivo* treatment with ethephon, leaves (attached to 3-year-old trees) were sprayed with an atomizer, then sealed in a plastic bag. Control leaves (on the same tree but opposite to treated leaves) were also sealed in a plastic bag. Embryogenic callus cultures of rubber (RRIM C600) was maintained on solid MS medium supplemented with 2 µg/ml of 2,4-D and 1 µg/ml of kinetin at 25 °C with a photoperiod of 12 h/12 h (light/dark in a growth chamber).

Construction and screening of a genomic library

A genomic library was constructed in EMBL3 [15] from genomic inserts of 12–20 kb in size generated by partial *Sau3A* digestion and size fractionation through sucrose gradient centrifugation of high-molecular-weight *Hevea* genomic DNA isolated as described by Shure *et al.* [34]. The host strain *Escherichia coli* LE392 was infected with 10⁶ pfu of independent recombinant phage, and plated at a density of 50 000 pfus per 150 mm (diameter) NZY plates. Plaques were transferred to Nylon Nytran filters (Schleicher & Schuell), lysed and baked as described by the manufacturer's instructions.

The filters were prehybridized for 2 h at 60 °C in 5 × SSPE, 5 × Denhardt's solution, 0.1% (w/v) SDS and 100 µg/ml denatured salmon testes DNA, after which the heterologous MnSOD cDNA probe from *Nicotiana plumbaginifolia* [6], which was labeled with random hexamers according to the manufacturer's instructions (Promega), was added at 6 × 10⁵ cpm/mL (specific activity > 1 × 10⁹ cpm/µg). The hybridization proceeded at the same temperature for 15 h. The filters were washed at 65 °C in 1 × SSC/0.1% (w/v) SDS for 20 min. The washed filters were briefly dried at room temperature and exposed to X-Omat AR film (Kodak) for 12 h. All positive clones were plated in duplicate and rescreened by the same procedure.

Sequence analysis

On the basis of the restriction maps of the genomic clones, a 5.0 kb *Eco* RI-*Sal* I fragment from phage clone λHSM3 and a 7.2 kb fragment from λHSM2 were subcloned into the plasmid vector pUC13 [28]. A series of nested deletions derived from the 5.0 kb *Eco* RI-*Sal* I fragment in pUC13 and a portion of 7.2 kb *Eco* RI fragment in pUC13 were obtained by the exonuclease III method of Henikoff [18]. DNA sequencing was performed by the Sanger dideoxy-method [32] using double-stranded supercoiled plasmid as described by Craft *et al.* [23]. All the reagents for sequencing reactions came from U.S. Biochemicals (Sequenase Version 2.0 Sequencing Kit).

cDNA cloning by PCR

Total RNA was isolated from leaves, roots, and petioles of 3-year-old rubber trees (RRIM C600) growing in the greenhouse, and embryogenic callus (RRIM C600) by using the phenol extraction method as described by Ausubel *et al.* [2]. Based on the sequencing data derived from the genomic clone, two oligonucleotides were synthesized. HSP5 (5'-GACGAATTCATGGCTCTGCGA-TCT-3') is an upstream primer which overlaps

the ATG initiation codon (underlined) and includes 12 nucleotides of the coding region. P4657 (5'-CATCTAGAGATGGAAAGCACT-CTAAG-3'), designed as a downstream primer, is composed of the last 5 nucleotides of the sixth exon (stop anti-codon is underlined) and 21 nucleotides of the 3'-untranslated region. Either *Eco* RI (in HSP5) or *Xba* I (in P4657) sites were engineered into the primers by changing 2 or 3 nucleotides, respectively *Eco* RI and *Xba* I sites are in bold type) near the 5' end of the primers for the convenience of subcloning of the amplified products. Total RNA (6.2 µg) isolated from young leaves was reverse-transcribed into cDNA by M-MLV reverse transcriptase (BRL) and the downstream primer. The first-strand cDNA was amplified by *Taq* DNA polymerase (Perkin-Elmer Cetus) in a Perkin-Elmer thermal cycler as described by Kawasaki [22]. Thirty cycles of amplification were performed (1 min at 94 °C for denaturation; 1 min at 55 °C for annealing; and 2.5 min at 72 °C for extension) and 5% of the product was analyzed on a 1.2% (w/v) agarose gel. The remaining product was then fractionated on a 1.2% (w/v) agarose gel and the fragment with the expected size was isolated from the gel by electroelution as described by Sambrook *et al.* [31], digested with *Eco* RI and *Xba* I, and ligated to pUC13. The resulting cDNA clones were sequenced as described above.

Primer extension

An antisense 31-nucleotide primer, HSME, corresponding to nucleotides +1492 to +1524 of the genomic sequence (Fig. 1) was synthesized and used to direct the synthesis of cDNA from total RNA of young leaves, as described by Dean *et al.* [11]. The extended product was analyzed on a 6% (w/v) denaturing polyacrylamide gel.

Southern hybridization of Hevea genomic DNA

Nuclear DNA isolated from rubber leaves was digested with *Eco* RI, *Eco* RI + *Bam* HI, *Hind* III,

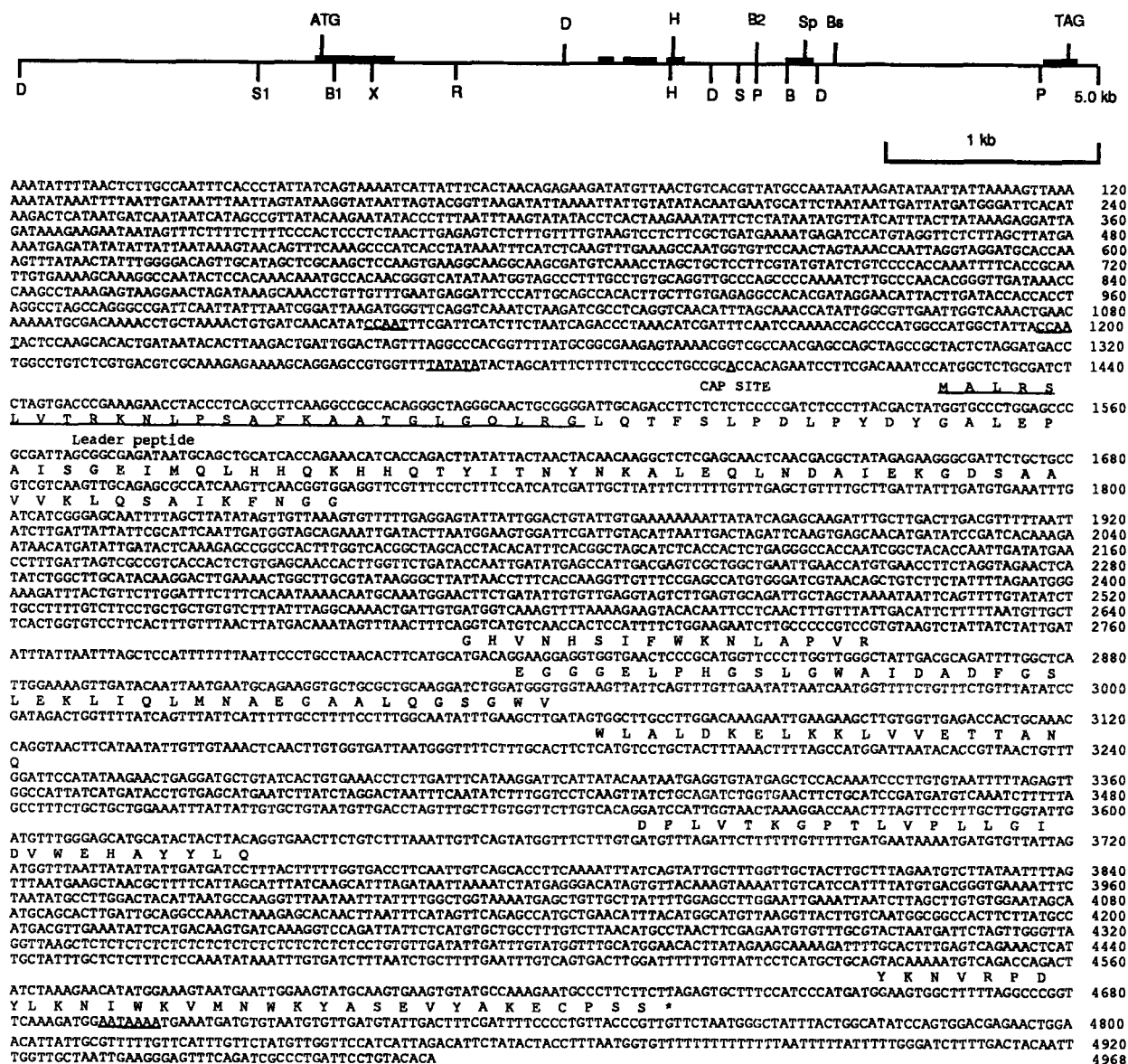


Fig. 1. Top: restriction map of the 5 kb fragment derived from λHSM3. Bars represent exons; ATG indicates the site of the initiator codon (in exon I) and TAG represents the stop codon (in exon VI). Symbols used for restriction enzymes: B, *Bam* HI; B1, *Bgl* I; B2, *Bgl* II; Bs, *Bst* EII; D, *Dra* I; H, *Hind* III; P, *Pst* I; R, *Eco* RV; S, *Sac* I; Sp, *Sph* I; S1, *Stu* I. Bottom: nucleotide sequence of the *Hevea* MnSOD gene and flanking 5' and 3' regions. The deduced amino acid sequence encoded by the six exons of the MnSOD gene is shown below the DNA sequence. The CAAT box, the TATA box, the mRNA transcription start site (CAP site), polyadenylation signal (AATAAA) and putative transit peptide are underlined. Numbers on the right represent the nucleotide position relative to the *Dra* I site in the upstream region.

Bst EII, and *Bst* EII + *Stu* I, and electrophoresed on 0.8% (w/v) agarose gels, blotted and hybridized to ³²P-labeled *Bst* EII-*Stu* I fragment corresponding to the first 5 exons and introns of the

rubber MnSOD genomic clone. The conditions were identical to those used for the library screening with the exception that Southern hybridizations were performed at 65 °C. After hybridiza-

tion the blots were washed at 65 °C for 1 h in 0.1 × SSC and 0.1% SDS as described by Sambrook *et al.* [31].

Northern blot analysis

Total RNA was isolated from fresh tissues as described [2] and quantified spectrophotometrically. Twenty µg of total RNA was electrophoresed in 1.2% (w/v) agarose gels, blotted and hybridized to ³²P-labeled probe of the rubber MnSOD cDNA as described [31]. The bands of northern blot analyses were quantified by one-dimensional scanning using an LKB Enhanced Laser Densitometer (LKB Ultrascan XL).

Results

Isolation of the MnSOD gene and nucleotide sequence

Using *Nicotiana plumbaginifolia* MnSOD cDNA as a probe [6], four genomic clones were isolated from the rubber genomic library, three of which were characterized and found to be overlapping fragments containing the same nuclear gene. Nucleotide sequence of the genomic clones was determined and compared with the MnSOD cDNA sequences from *N. plumbaginifolia* [6] such that approximate positions of the putative exons were determined. On the basis of the genomic sequence, PCR primers were designed and utilized for cloning the full-length cDNA corresponding to the isolated genomic clone. The cDNA clone isolated by PCR was sequenced and was found to contain a single open reading frame of 702 nucleotides which encodes a polypeptide of 233 amino acids. By homologous alignment between the cDNA and the genomic clone, positions of introns and exons were determined (Fig. 1). The biological activity of the isolated genomic clone was confirmed by the identical match between the exons and the cDNA. The nucleotide sequence of the genomic clone comprises 6 exons and 5 introns which make a total length of 3217 nucleotides (Fig. 1). The first, and largest, exon

contains 297 nucleotides and the second, smallest exon, has only 47 nucleotides. The first intron and the last intron are similar in size with 972 and 986 nucleotides, respectively. Exon-intron junctions essentially observe the consensus sequence of 5'-AG-GT-3' with some exceptions. Two transcription initiation sites, as determined by primer extension, were located at positions -24 and -26 upstream of the ATG initiation codon (Fig. 2). The sequence context of the -24 site is in agreement with the consensus sequence commonly found in other higher plant genes [21]. Thirty nucleotides upstream of the transcription start site is a putative TATA box (ATATAT) [8]. There are two 'CAAT' box-like sequences: CCAATAC at position -204 and CCAATTT at -297 upstream of the transcription start site. Interestingly, there are two tandem 'TGACG'-like

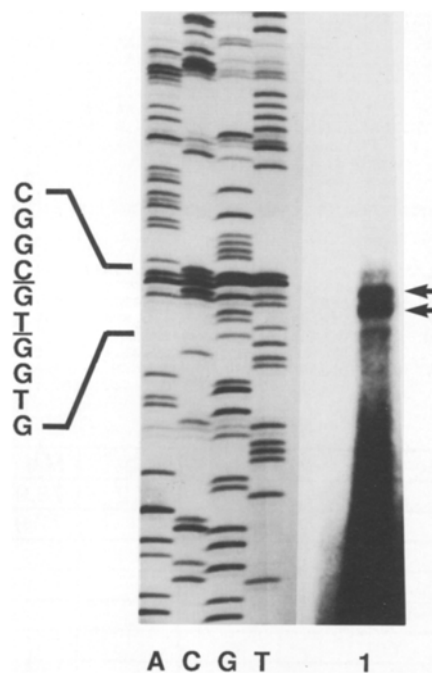


Fig. 2. Determination of transcription start site by primer extension. An antisense nucleotide primer, HSME (corresponding to nucleotides +1492 to +1524 of the genomic sequence in Fig. 1) was annealed to total RNA from ethephon-treated young leaves at 50 °C. The primer-extended products were subjected to electrophoresis on a 6% acrylamide-urea gel. The major products of the primer extension are indicated by arrows on the right; the sequence of the region in the marker lanes corresponding to these two bands are shown on the left.

boxes, TGACCTGGCCTGTCTCGTGACG, from position -87 to -66 upstream of the transcription start site. This motif was found to be responsible for the binding of transcription factor ASF-1, which has been demonstrated to confer gene expression in roots [25]. At the 3' end, a hexanucleotide consensus sequence of AATAAA (polyadenylation signal) was found 53 nucleotides downstream of the stop codon.

Deduced amino acid sequence of Hevea MnSOD has a striking homology with other MnSODs

Comparison of amino acid sequences of MnSOD from *Hevea* and other eukaryotic species reveals

a striking homology, with the strongest homology between *Hevea* and other higher plants (80–90%; Fig. 3). In addition, comparison between *Hevea* and phylogenetically distant prokaryotes shows strong conservation in several regions. As expected, the putative metal (Mn) binding ligands as determined in prokaryotes [35] are well conserved at His-28, His-76, Asp-165 and His-169 of the deduced amino acid sequence in *Hevea* (Fig. 5). In addition, amino acids adjacent to these sites have been changed much more slowly than more distant amino acids. The most highly conserved region is the one surrounding the last two ligands, as these two sites are separated by only 4 amino acids.



Fig. 3. Top: pairwise comparison of the primary amino acid sequence of mature MnSOD-A (H-A) and MnSOD-B (H-B) from *H. brasiliensis* with those of *N. plumbaginifolia* (Tob [6]), maize (Mai [41]), man (Hum [5]), rat [19], yeast (Yst [26]), *E. coli* (Eco [36]), *Mycobacterium leprae* (Myc [38]), and *Halobacterium cutirubrum* (Hal [27]). The gaps introduced for optimal alignment are shown by dashed bars; a perfect match is indicated by a dot. Junctions of exons are shown by double underlining. The putative Mn(III) ligand-binding sites (His-28, His-76, Asp-165 and His-169 relative to MnSOD cDNA of *Hevea*) are shown by arrows. Bottom: amino acid homology comparison of MnSOD from the organisms listed in upper panel. Homology comparisons were made by optimal pairwise alignment of amino acid sequences of the various species.

MnSOD in Hevea is encoded by a small multigene family

The strong hybridization bands generated by the double digestions of *Eco* RI-*Bam* HI and *Stu* I-*Bst* EII, respectively, on a filter of *Hevea* genomic DNA probed with the *Stu* I-*Bst* EII fragment (Fig. 4) correspond in size to the bands in similar restriction digestions of the phage λ HSM3 identified by hybridization to the MnSOD cDNA probe from *N. plumbaginifolia*. The size of these bands is in accordance with the predicted size from the genomic sequence. Based on the ge-

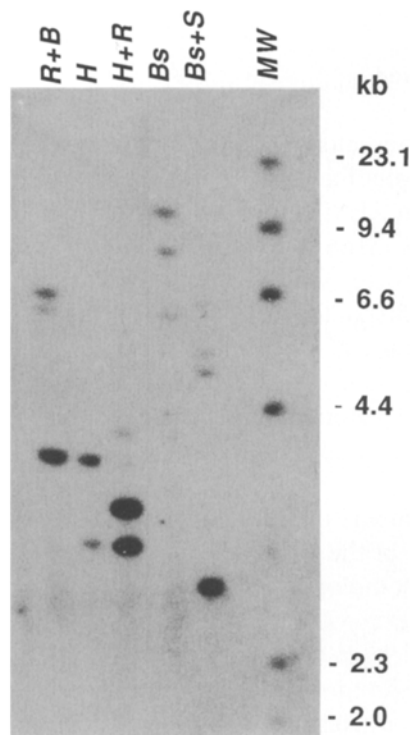


Fig. 4. Southern hybridization analysis of rubber genomic DNA. Genomic DNA was digested to completion with the enzymes as indicated, electrophoresed in a 0.8% (w/v) agarose gel and blotted to a nylon membrane. The blot was hybridized to the 2.8 kb *Stu* I-*Bst* EII genomic fragment corresponding to the first 5 exons and introns of the rubber MnSOD nuclear gene from λ HSM3. The restriction endonucleases used are *Eco* RI (R), *Bam* HI (B), *Hind* III (H), *Bst* EII (Bs), and *Stu* I (S). The size (in kb) of the hybridizing fragments, based on the migration of *Hind* III-digested λ DNA, is indicated in the right-hand lane.

nomonic sequence, as well as restriction maps of λ HSM3 and λ HSM2, the occurrence of hybridizable bands other than a 3.8 kb fragment in *Eco* RI-*Bam* HI double digestion and a 2.8 kb in *Stu* I-*Bst* EII double digestion would indicate that there is more than one gene of MnSOD in *Hevea*. The detection of additional bands by both double digestions, as well as by other single digestions, clearly indicates that there are at least two copies of MnSOD in *Hevea*. In support of this is the isolation of the second MnSOD cDNA (MnSOD-B) from *Hevea* (Fig. 3). Of the 11 PCR-generated cDNA clones we sequenced, 8 identical clones are grouped as MnSOD-A, whose sequence corresponds exactly to that of exons in the isolated genomic clone; the other three identical clones show significant nucleotide sequence difference from that of MnSOD-A, and therefore is designated as MnSOD-B. This indicates that the difference between the two types of cDNA clones are not caused by PCR errors but of reality. Comparison of nucleotide sequences between MnSOD-A and MnSOD-B shows that they are two distinct forms of MnSOD, with a 90.2% homology at the nucleotide level in the coding region. The sequence divergence between these two genes is greater than that between MnSOD-A from *Hevea* and the reported MnSOD from *N. plumbaginifolia*. In addition, MnSOD-B encodes two more amino acids (arginine and leucine) than MnSOD-A in the putative transit peptide, making this region of MnSOD-B more basic.

Organ-specific expression of MnSOD mRNA

To examine the expression of MnSOD genes in various tissues in *Hevea*, total RNA isolated from young leaves, mature leaves, young petiole, roots and embryogenic callus as well as latex poly(A)⁺ RNA, were analyzed by northern hybridization (Fig. 5A). The results demonstrated that there was considerable variation in the levels of expression of MnSOD in different tissues of *Hevea*. The highest level of MnSOD transcript was found in embryogenic callus, which was 30- to 50-fold

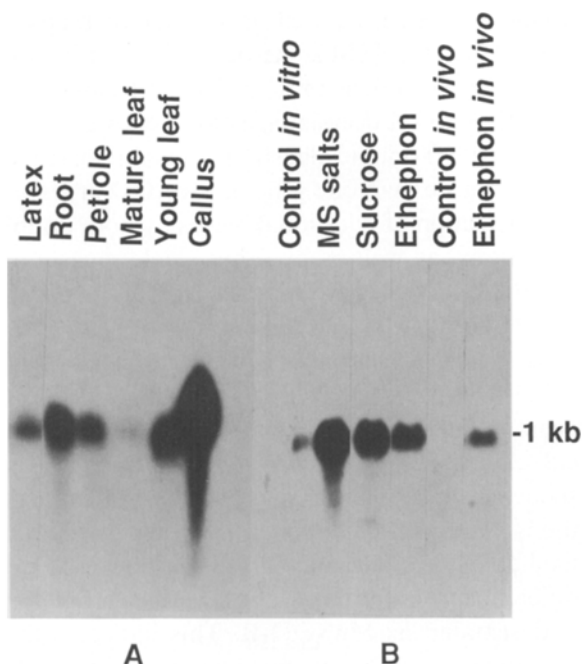


Fig. 5. A. Northern blot analysis of MnSOD mRNA in various tissues of rubber. Total RNA (20 μ g) from root, petiole, mature leaf, young leaf, callus and poly(A)⁺ mRNA from latex (1 μ g) were electrophoresed in a 1.2% (w/v) denaturing agarose gel and blotted to a nylon membrane. The blot was hybridized to a radiolabeled full-length cDNA of rubber MnSOD (pSM25). B. Northern blot analysis of MnSOD mRNA in response to various stresses. Mature leaves of *Hevea* were excised and treated for 48 h as described in Materials and methods. Total RNA (20 μ g) from each treatment was separated electrophoretically, transferred to nylon membrane, and probed as described above. Controls for both *in vivo* (attached to tree) and *in vitro* (excised leaf) treatments were included. Molecular size was estimated from the comigration of RNA samples of known molecular weights (data not shown).

higher than that of mature leaves. In intact plants, young leaves (7 days old) revealed the highest level (4–6-fold higher than mature leaves), followed by roots, petiole and mature leaves which had the lowest level. A strong signal was also detected for latex RNA, but this level was not comparable with other tissues due to the use of poly(A)⁺ mRNA. However, these experiments clearly demonstrated that MnSOD was expressed in all the tissues examined.

Accumulation of MnSOD mRNA in response to ethephon, sucrose and MS salts

To investigate the extent to which MnSOD mRNA is induced by various environmental stresses, leaves were excised from trees, sliced into sections and incubated with 7% (w/v) sucrose, 0.02% (w/v) ethephon (an ethylene-generating compound) or 1 $\times$ MS salts for 48 h. Total RNA was isolated and analyzed by northern hybridization. The results (Fig. 5B) showed that all three *in vitro* treatments resulted in a 3- to 5-fold induction of MnSOD mRNA over control levels. A similar magnitude of induction by ethephon under *in vivo* conditions was also observed.

Discussion

A genomic clone of MnSOD has been isolated from a genomic library of *Hevea brasiliensis* produced in λ EMBL3 using a heterologous cDNA probe of MnSOD from *N. plumbaginifolia*. The cDNA corresponding to the genomic clone and a second distinct form of cDNA have been isolated from total RNA of rubber leaves using the polymerase chain reaction. Both cDNA cloning results and genomic Southern analysis indicate that there is a small multigene family of MnSOD in *Hevea*.

A comparison of deduced transit peptide sequences of the two cDNAs from *Hevea* revealed a 90% homology with an introduced gap (Fig. 6). This putative transit peptide is characterized by being rich in both uncharged and positively charged amino acids, but devoid of acidic residues, characteristic of transit peptides for mitochondria-targeted proteins [20, 33]. In addition, the presence of an invariable arginine at the penultimate position of the transit peptide has been detected in both MnSOD from *Hevea* and all other eukaryotic MnSODs examined (Fig. 6). We believe that the unique feature of the transit peptide present in both MnSOD-A and -B might be related to their subcellular location.

MnSOD transcripts have been demonstrated to be present in young leaves, mature leaves, peti-

	+	+	++	+		+
H-A	MALRSLVTRKNLPSAFKAATGL				GQLRQ--LQTFSLPDL	PYDYGALEP
H-B	MALRSLVARKTISAFKAATGL				RLGQLRG--IQTYSLPDL	PYDYGALEP
TOB	MALRTLVSRRTLATGLGF				RQQLRG--LQTFSLPDL	PYDYGALEP
ZEA	MALRTLASKKVLSPFPFGGAGRPLAAAASARG--VTTVTLPDL					SYDFGALEP
HUM	MLSRVCGTSRQLAPAL				GVLGSRQ--	KHSLPDL
RAT	MLLRAACSAGRRLLGP				AASTAGSBH--	KHSLPDL
YST	MFAKTAANLTKKGGLS				LLSTTARBT--	KVTLPLDKWDFGALEP

Fig. 6. Comparisons of transit peptide sequences of MnSOD. The sources of the MnSODs marked on the left are H-A (*Hevea* MnSOD-A), H-B (*Hevea* MnSOD-B), TOB (*Nicotiana plumbaginifolia*; [6]), ZEA (*Zea mays* [41]), HUM (man [5]), RAT [19], and YST (yeast [26]). Gaps are introduced to facilitate optimal alignment of sequences. A dash (–) indicates the putative junction between transit peptide and mature protein; a ‘+’ denotes the positions of positively charged amino acids.

ole, latex, roots and callus of *Hevea* by use of a probe derived from MnSOD cDNA of *Hevea* (Fig. 5A). The amount of MnSOD mRNA varies considerably between tissues, with the highest level detected in callus, and in the young leaves of intact plants. A remarkably high level of MnSOD transcript was also reported in cell suspensions in *N. plumbaginifolia* [6]. Cultured cells are believed to be under heavy oxidative stress and, therefore, defense mechanisms which protect the cells from oxyradicals have been induced [37].

Bowler *et al.* [6] found that the key factor causing the induction of MnSOD in suspension cells of *N. plumbaginifolia* was sugar. In rubber, sucrose was also found to cause a dramatic induction of MnSOD mRNA, whereas the same concentration of mannitol, a non-metabolizable osmoticum used in plant tissue culture, did not (data not shown). These results support the conclusion obtained by Bowler *et al.* [6] that the induction by sucrose was due to metabolic activities of cells related to the carbon source and not to its osmotic effects. However, the induction of MnSOD mRNA in *Hevea* by MS salts is in dramatic contrast with the observation in *N. plumbaginifolia* [6], where MS salts were found to have no inductive effect. This might be attributed to different experimental conditions or to an intrinsic difference in the regulation of MnSOD in these two plants. If the latter is true, it would be intriguing to investigate the regulation of the *Hevea* MnSOD genomic clone after it is introduced into *N. plumbaginifolia*.

Ethephon was also found to increase expression of MnSOD (Fig. 5B). Ethephon was re-

ported to induce the expression of hevein, an abundant soluble protein [9], chitinase, an anti-fungal enzyme (J.J. Gaynor and V. Pujade-Renaud, unpublished data), as well as a number of other defense-related products found in rubber latex [24]. This is particularly interesting in rubber as ethephon is routinely applied to the bark of rubber trees to increase latex yield. Latex is produced by highly specialized cells called laticifers which are derived from the cambium layer and are found as a continuous syncytium of cells just beneath the bark. When the bark is cut, latex, containing rubber particles (*cis*-1, 4-polyisoprene) and a variety of organelles, flows freely. Latex flow generally ceases after a few hours when sufficient rubber has coagulated at the wound site. Rupture of specialized organelles known as lutoids (derived from the vacuole) has been reported to accelerate this coagulation. Ethephon treatment increases latex flow presumably by preventing or delaying the rupture of lutoid membranes and, thus, preventing premature coagulation of latex.

It is interesting to note that a physiological disorder common to low-yielding varieties of *Hevea* known as dry bark syndrome is correlated with low levels of both SOD and catalase activities. These very low levels of SOD and virtual disappearance of catalase exhibited in these trees is believed to result in the accumulation of superoxide radicals and hydrogen peroxide which can damage lutoid membranes [10]. As MnSOD functions as a superoxide scavenger, high levels of MnSOD induced by ethephon might aid in preventing lutoid disruption by superoxide radicals. With the availability of the cloned MnSOD

gene, we are currently examining both the quantitative and qualitative regulation of MnSOD in healthy and diseased trees as influenced by stress and other environmental factors. In addition, we are presently dissecting the putative *cis*-acting elements upstream of the MnSOD coding sequence responsible for this unique spatial and temporal pattern of expression.

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References

- Asada K, Urano M, Takahashi M: Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. *Eur J Biochem* 36: 257–266 (1973).
- Ausubel FM, Brent R, Kingston RE, Moore DD, Steidman JG, Smith JA, Struhl K: Current Protocols in Molecular Biology. John Wiley, New York (1990).
- Bannister JY, Bannister WH, Rotillo G: Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit Rev Biochem* 2: 111–180 (1987).
- Baum JA, Scandalios JG: Isolation and characterization of the cytosolic and mitochondrial superoxide dismutases of maize. *Arch Biochem Biophys* 206: 249–264 (1981).
- Beck Y, Oren R, Amit B, Levanon A, Gorecki N, Hartman JR: Human Mn superoxide dismutase cDNA sequence. *Nucl Acid Res* 15: 9076–9076 (1987).
- Bowler C, Alliotte T, De Loose M, Van Montagu M, Inze D: The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. *EMBO J* 8: 31–38 (1989).
- Bowler C, Van Kaer L, Van Camp W, Van Montagu M, Inze D: Characterization of the *Bacillus stearothermophilus* manganese superoxide dismutase gene and its ability to complement copper/zinc superoxide dismutase deficiency in *Saccharomyces cerevisiae*. *J Bact* 172: 1538–1546 (1990).
- Breathnach R, Chambon P: Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50: 349–384 (1981).
- Broekaert W, Lee H, Kush A, Chua N-H, Raikhe N: Wound-induced accumulation of mRNA containing a bevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc Natl Acad Sci USA* 87: 7633–7637 (1990).
- Chrestin H: Biochemical aspects of bark dryness induced by overstimulation of rubber trees with ethrel. In d'Auzac J, Jacob J-L, Chrestin H (eds), *Physiology of Rubber Tree Latex*, pp. 431–442. CRC Press, Florida (1989).
- Dean FM, Dunsmuir P, Bedbrook J: Confirmation of the relative expression levels of the petunia (Mitchell) *rbcS* gene. *Nucl Acid Res* 15: 4655–4668 (1987).
- Droillard MJ, Paulin A: Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. *Plant Physiol* 94: 1187–1192 (1990).
- Duke MV, Salin M: Isozymes of cuprozinic superoxide dismutase from *Pisum sativum*. *Phytochemistry* 22: 2369–2373 (1983).
- Fridovich I: Superoxide dismutases. *Adv Enzymol* 58: 61–97 (1986).
- Frschauf A-M, Kehrach H, Poustka A, Murray N: Lambda replacement vectors carrying polylinker sequences. *J Mol Biol* 170: 827–842 (1983).
- Grace SC: Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria. *Life Sci* 47: 1875–1886 (1990).
- Hassan HM, Fridovich I: Intracellular production of superoxide radical and hydrogen peroxide by redox active compounds. *J Biol Chem* 196: 385–395 (1979).
- Henikoff S: Unidirectional digestion with exonuclease III in DNA sequence analysis. *Meth Enzymol* 155: 156–163 (1987).
- Ho YS, Crapo JD: Nucleotide sequences of cDNAs coding for rat manganese-containing superoxide dismutase. *Nucl Acids Res* 15: 10070–10070 (1987).
- Hurt EC, van Loon APGM: How proteins find mitochondria and intramitochondrial compartments. *Trends Biochem Sci* 11: 204–207 (1986).
- Joshi CP: An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucl Acids Res* 15: 6643–6653 (1987).
- Kawasaki ES: Amplification of RNA. In: Innis MA, Gel-fand DH, Sninsky JJ, White TJ (eds), *PCR Protocols*, pp. 21–27. Academic Press, London (1990).
- Kraft R, Tardiff J, Frauter KS, Leinwand LA: Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase. *Biotechniques* 6: 544–547 (1988).

24. Kush A, Goyvaerts E, Chye M-L, Chua N-H: Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *Proc Natl Acad Sci USA* 87: 1787–1790 (1990).
25. Lam E, Benfey PN, Gilmartin M, Fang R-X, Chua N-H: Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. *Proc Natl Acad Sci USA* 86: 7890–7894 (1989).
26. Marres CM, van Loon APGMY, Oudshoorn P, Steeg HV, Grivell LA, Slater EC: Nucleotide sequence analysis of the nuclear gene coding for manganese superoxide dismutase of yeast mitochondria, a gene previously assumed to code for the Rieske iron-sulphur protein. *J Biochem* 147: 153–161 (1985).
27. May BP, Dennis PP: Evolution and regulation of the gene encoding superoxide dismutase from the archaebacterium *Halobacterium cutirubrum*. *J Biol Chem* 264: 12253–12258 (1989).
28. Messing J: New M13 vectors for cloning. *Meth Enzymol* 101: 20–78 (1983).
29. Murashige T, Skoog F: A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol Plant* 15: 473–479 (1962).
30. Privalle CT, Beyer WFJ, Fridovich I: Anaerobic induction of ProMn-superoxide dismutase in *Escherichia coli*. *J Biol Biochem* 264: 2758–2763 (1989).
31. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
32. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-termination inhibitors. *Proc Natl Acad Sci USA* 74: 5463–5467 (1977).
33. Schatz G: Signals guiding proteins to their correct locations in mitochondria. *Eur J Biochem* 165: 1–6 (1987).
34. Shure MSW, Fedoroff N: Molecular identification and isolation of the *waxy* locus in maize. *Cell* 35: 225–233 (1983).
35. Stallings WC, Patridge KA, Strong RK, Ludwig ML: The structure of manganese superoxide dismutase from *Thermus thermophilus* HB8 at 2.4 Å resolution. *J Biol Chem* 260: 16424–16432 (1985).
36. Steinman HM: The amino acid sequence of manganese superoxide dismutase from *Escherichia coli* L. *J. Biol Chem.* 253: 8708–8720 (1978).
37. Tanaka K, Masuda R, Sugimoto T, Kawamura Y, Kuboi T: An H₂O₂-decomposing system in cultured tobacco cells. *Agric Biol Chem* 54: 2003–2008 (1990).
38. Thangaraj HS, Lamb FI, Colston MJ: Nucleotide and deduced amino acid sequence of *Mycobacterium leprea* manganese superoxide dismutase. *Nucl Acids Res* 17: 8378–8378 (1989).
39. Touati D: Molecular genetics of superoxide dismutases. *Free Radicals Biol Med* 5: 393–402 (1988).
40. Van Camp W, Bowler C, Villarroel R, Tsang EWT, Van Montagu M, Inze D: Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*. *Proc Natl Acad Sci USA* 87: 9903–9907 (1990).
41. White JA, Scandalios JG: Isolation and characterization of a cDNA for mitochondrial manganese superoxide dismutase (SOD-3) of maize and its relation to other manganese superoxide dismutases. *Biochim Biophys Acta* 951: 61–70 (1988).