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The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*

Chris Bowler, Thierry Alliotte[1], Marc De Loose, Marc Van Montagu and Dirk Inzé

Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium
[1]Present address: L'Air Liquide S.A., 75, quai d'Orsay, F-75321 Cedex 07, France

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Superoxide dismutases (SODs) are metalloproteins that catalyse the dismutation of superoxide radicals to oxygen and hydrogen peroxide. The enzyme has been found in all aerobic organisms examined, where it plays a major role in the defence against toxic reduced oxygen species which are generated in many biological oxidations. Here we report the complete primary structure of a plant manganese superoxide dismutase (MnSOD), deduced from a cDNA clone of *Nicotiana plumbaginifolia*. The plant protein is highly homologous to MnSODs from other organisms and also contains an N-terminal leader sequence resembling a transit peptide for mitochondrial targeting. The location of the mature protein within the mitochondria has been demonstrated by subcellular fractionation experiments. We have analysed the expression profile of this MnSOD and found that it is dramatically induced during stress conditions, most notably in tissue culture as a result of sugar metabolism and also as part of the pathogenesis response of the plant, being induced by ethylene, salicylic acid, and *Pseudomonas syringae* infection. This induction is always accompanied by an increase in cytochrome oxidase activity, which suggests a specific protective role for MnSOD during conditions of increased mitochondrial respiration.

Key words: ethylene/mitochondrial respiration/*Nicotiana plumbaginifolia*/pathogen infection/salicylic acid/superoxide dismutase

Introduction

Superoxide radicals ($O_2^{-}\cdot$) are ubiquitously generated in many biological oxidations within all compartments of the cell (for review, see Fridovich, 1978). The toxicity of superoxide radicals has been attributed to their interaction with other cellular constituents, in particular with hydrogen peroxide (Halliwell, 1984). In the presence of trace amounts of iron salts, the combination of superoxide radicals and hydrogen peroxide leads rapidly to the formation of highly reactive hydroxyl radicals ($OH\cdot$) as follows:

$$H_2O_2 + O_2^{-}\cdot \xrightarrow[Fe^{3+}]{Fe^{2+}} OH^- + O_2 + OH\cdot$$

Hydroxyl radicals are thought to be in large part responsible for oxygen toxicity *in vivo*. Being one of the most reactive species known to chemistry, they are able to react with DNA, proteins, lipids and almost any other organic constituent of living cells.

Organisms have evolved a battery of mechanisms which prevent the formation of such deleterious oxygen species, one example being the superoxide dismutases (SOD; E.C. 1.15.1.1), a class of metalloproteins which catalyse the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Their importance has been demonstrated in SOD-deficient mutants of *Escherichia coli* (Carlioz and Touati, 1986) and yeast (Biliński *et al.*, 1985; van Loon *et al.*, 1986) which are hypersensitive to oxygen.

Three classes of SOD can be distinguished according to their metal cofactor: copper/zinc, manganese and iron forms (for review, see Bannister *et al.*, 1987). The iron enzyme (FeSOD) is present in prokaryotes and in some plants. The manganese SOD (MnSOD) is widely distributed among prokaryotic and eukaryotic organisms; in eukaryotes it is most often found in the mitochondrial matrix. The iron and manganese SODs are very similar in their primary, secondary, and tertiary structure (see for example, Parker and Blake, 1988). The copper/zinc enzyme (Cu/ZnSOD) represents a distinct class, found almost exclusively in eukaryotic species, where it is often present as several isoforms. One of these is always present in the cytosol, and a chloroplastic isoform is frequently found in plant cells (Halliwell, 1984).

The isolation of cDNAs and genes encoding SODs from various species has progressed rapidly (for review, see Touati, 1988). In plants, however, despite some physiological data (Bannister *et al.*, 1987), very little is known at the molecular level. A cDNA clone encoding a cytosolic Cu/ZnSOD has been isolated from maize (Cannon *et al.*, 1987), but until now no sequence data has been available from a plant MnSOD.

The possibility of obtaining amino acid sequence data directly from proteins separated on two-dimensional gels (Bauw *et al.*, 1987) greatly facilitates the screening of cDNA libraries for the isolation of important genes. We have used this approach to isolate a cDNA clone from *Nicotiana plumbaginifolia* encoding a manganese superoxide dismutase. The plant protein shows clear homology with known MnSODs, and contains a leader sequence for mitochondrial targeting. The mitochondrial localization was confirmed by subcellular fractionation. We have demonstrated that the expression of this MnSOD is dramatically increased in plant cells during conditions of metabolic stress in tissue culture and during the pathogenesis response. This induction always accompanies an enhanced cytochrome oxidase activity, which, in turn, is likely to reflect an increase in mitochondrial respiration as caused by the stressing agent.

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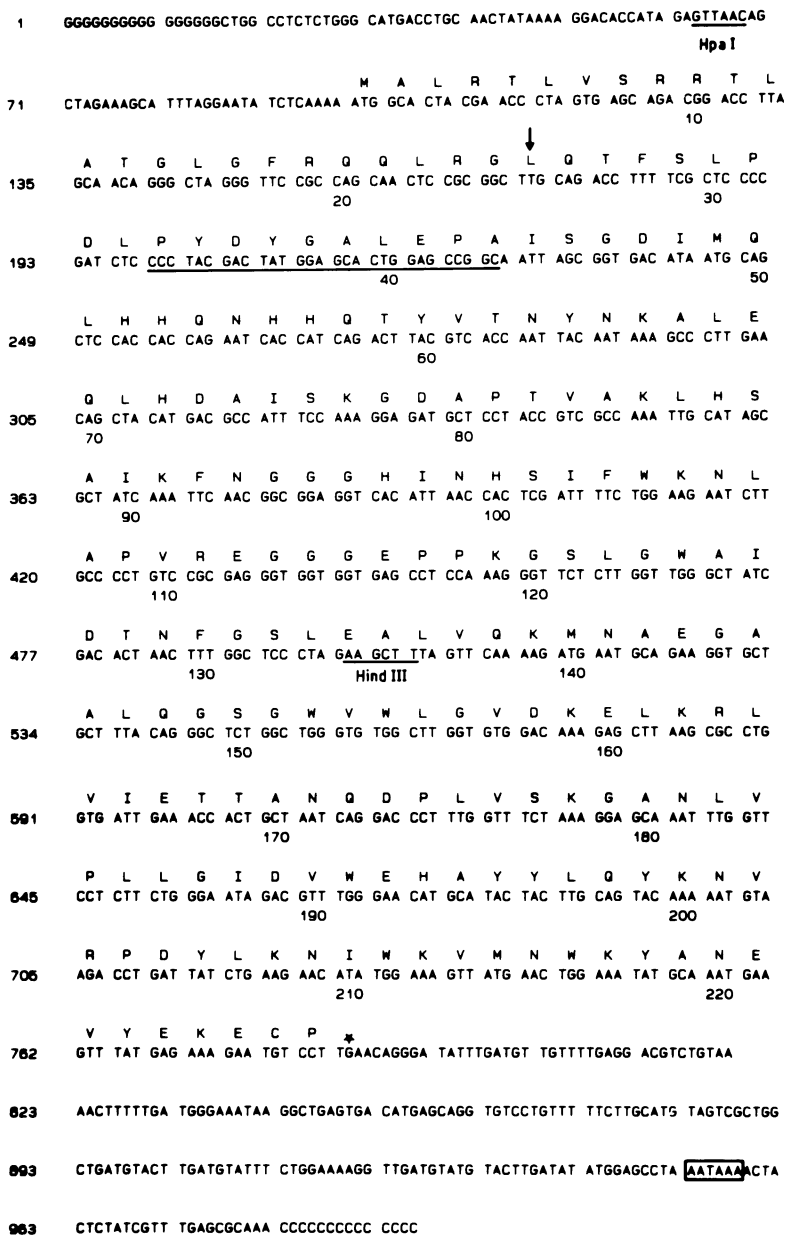


Fig. 1. Sequence of a manganese superoxide dismutase (MnSOD) of *N.plumbaginifolia*. Complete nucleotide sequence of the pSOD1 cDNA insert of 996 bp with its flanking G/C homopolymer tails added during the cloning procedure. The sequence homologous to the oligonucleotide probe is underlined. The potential polyadenylation site AATAAA (position 953) is indicated by boxing. The predicted coding sequence starting at position 99 (start codon ATG) to position 783 (stop codon TGA marked with an asterisk) has been translated into the corresponding amino acid sequence, written above the nucleotide sequence in the one-letter code. The arrow on amino acid 25 points to the N-terminus of the mature protein (Bauw *et al.*, 1987). The position of the *Hpa*I and *Hin*dIII sites of the cDNA insert are indicated.

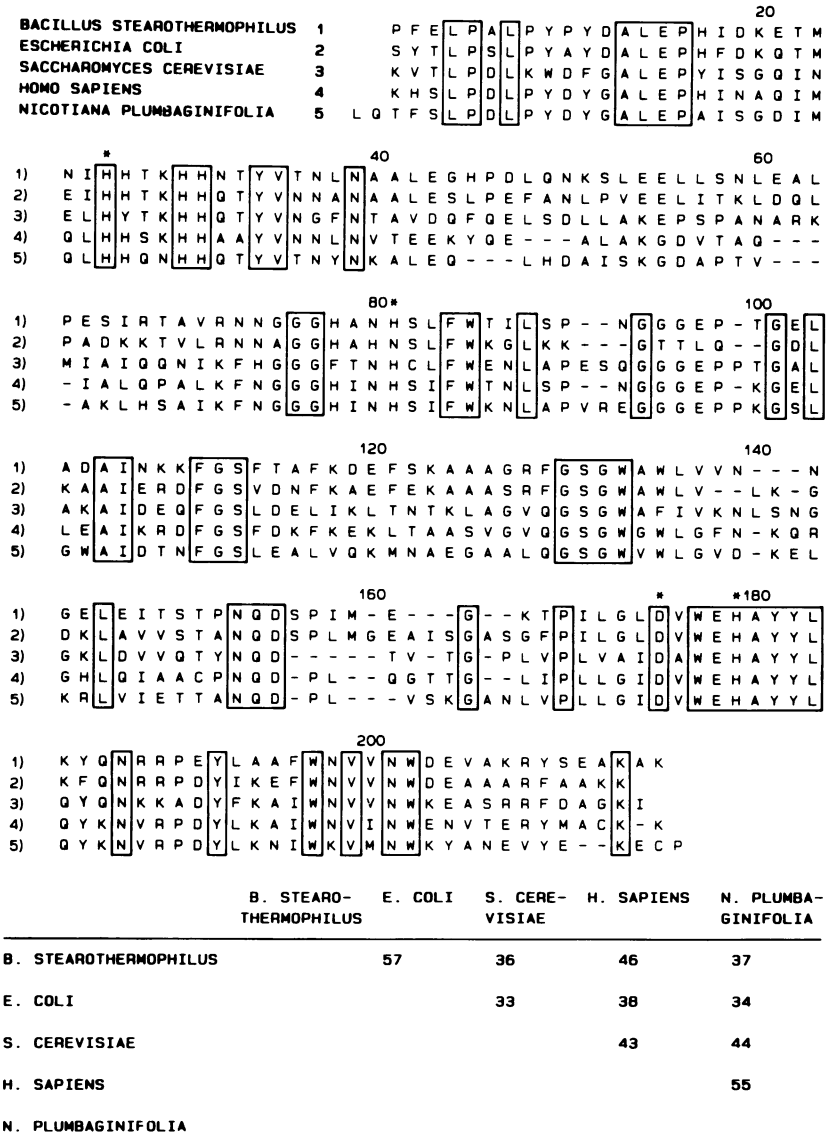
We propose that this correlation is due to the specific need for MnSOD to protect against the oxidative stress generated in mitochondria during conditions of increased mitochondrial respiration. The availability of a MnSOD cDNA thus provides a sensitive marker for studying the response of plants to oxidative challenge and to stress situations in general.

Results

Isolation and sequence analysis of a cDNA clone encoding a MnSOD from N.plumbaginifolia

We have previously determined the N-terminal amino acid sequence of a MnSOD from *N.plumbaginifolia* (Bauw *et al.*, 1987). From this an oligonucleotide probe was designed containing deoxyinosines at ambiguous codon positions (see Materials and methods), and used to screen a cDNA library derived from a *N.plumbaginifolia* cell suspension culture. A clone, designated as pSOD1, was selected for further analysis.

The entire sequence of the 996-bp cDNA insert is shown in Figure 1. It contains one continuous open-reading frame, corresponding to 228 amino acids. The presumed initiation codon (ATG) at nucleotide position 99 is preceded by two in-frame stop codons. The sequence starting from amino acid 25 is identical to the previously determined N-terminal amino acid sequence of the mature protein. The molecular weight of the mature protein, calculated from the cloned sequence



	B. STEARO-THERMOPHILUS	E. COLI	S. CERE-VISIAE	H. SAPIENS	N. PLUMBA-GINIFOLIA
B. STEAROTHERMOPHILUS		57	36	46	37
E. COLI			33	38	34
S. CEREVISIAE				43	44
H. SAPIENS					55
N. PLUMBAGINIFOLIA					

Fig. 2. Comparison between the MnSOD sequence of *N.plumbaginifolia*, *Bacillus stearothermophilus* (Brock and Walker, 1980), *E.coli* (Steinman, 1978), *Saccharomyces cerevisiae* (Marres *et al.*, 1985), and *Homo sapiens* (Beck *et al.*, 1987). (**a**) Optimal alignment between the five species. Only the mature protein is shown. The positioning of the N-terminus of human MnSOD is according to Barra *et al.* (1984). Gaps introduced to obtain maximal homology are indicated by horizontal lines. Residues conserved in all five species are enclosed in boxes. Asterisks indicate the ligands to the metal (see text). (**b**) Pairwise quantitative homologies between the five species. Numbers shown in the matrix represent the percentage of homology corrected for the percentage of deletions, in optimal pairwise alignments. This analysis was performed using Genalign programme (Intelligenetics, Inc.) with standard parameters, version 5.1 for UNIX, based on the 'Region method' algorithm described by Sobel and Martinez (1986).

is 22.8 kd, which compares well with our previous estimate of 24 kd (Bauw *et al.*, 1987).

There is considerable homology between the *N.plumbaginifolia* mature protein and the MnSOD of bacteria, yeast and human (Figure 2a). In addition, the ligands to the Mn(III) ion: His-26, His-81 (His-80 for *E.coli*), Asp-175, and His-179, are all conserved in the plant sequence (Stallings *et al.*, 1985; Bannister *et al.*, 1987). Pairwise homologies show that the plant enzyme is more closely related to human and yeast than to bacterial MnSOD (Figure 2b).

Profile of SOD isoenzymes and their subcellular location in N.plumbaginifolia

SOD assays performed directly on non-denaturing gels revealed two major bands of SOD activity in protein samples obtained from leaves of *N.plumbaginifolia* (Figure 3a). Inhibition studies with H_2O_2 and KCN, routinely used to distinguish between Cu/Zn-, Mn-, and FeSOD (for example, Bridges and Salin, 1981) demonstrated that the upper band was a MnSOD (by its resistance to H_2O_2 and KCN) and that the lower band was a Cu/ZnSOD (as judged by its sensitivity to both inhibitors). No FeSOD activity (resistant to KCN and sensitive to H_2O_2) was detected.

The sequence of 24 amino acids upstream from the mature protein of the MnSOD (Figure 1) has features typical of a leader sequence for translocation to the mitochondrial matrix (Schatz, 1987). It contains five arginines interspersed with uncharged amino acids, and is devoid of acidic residues but rich in the hydroxylated amino acids serine and threonine.

In order to confirm the subcellular location of MnSOD we separated extracts from leaves into chloroplast, mito-

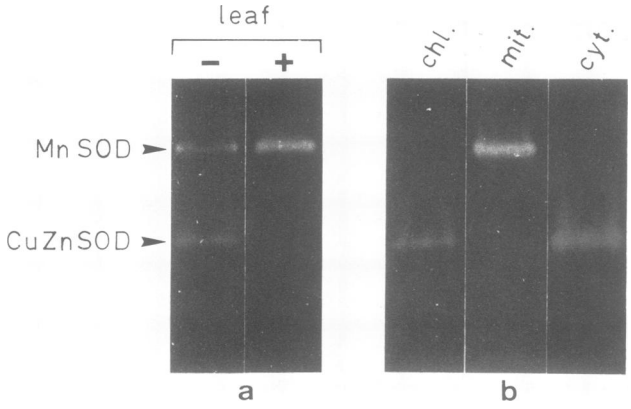


Fig. 3. Profile of SOD isozymes from *N.plumbaginifolia*. Protein samples were separated on non-denaturing 10% acrylamide gels run overnight at 120 V constant voltage and stained for SOD activity. **(a)** SOD activity in total extracts of leaves from *N.plumbaginifolia*. Both lanes contain 200 μg protein samples, one treated with the inhibitors H_2O_2 and KCN (+) and the other untreated (−). **(b)** Subcellular location of MnSOD and Cu/ZnSOD. Extracts from leaves were separated into chloroplast, mitochondrial and cytosolic fractions. Samples containing 50 μg protein (200 μg for cytosolic fraction) were separated on gels and stained for SOD activity. Abbreviations: chl., chloroplasts; cyt., cytosol; mit., mitochondria.

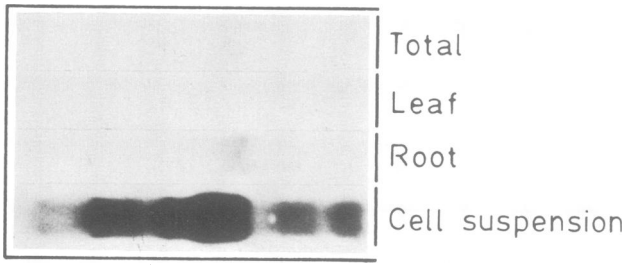


Fig. 4. Northern analysis of MnSOD mRNA in *N.plumbaginifolia*. Hybridization of a pSOD1-derived probe to total RNA from whole plants, leaves, roots and dark-grown cell suspension cultures. The size of the hybridizing RNA is 1.25 kb.

chondrial, and cytosolic fractions and assayed the SOD activity present in each sample (Figure 3b). The results clearly demonstrate that the MnSOD activity is present exclusively in the mitochondrial fraction. The Cu/ZnSOD appeared to be primarily associated with the soluble cytosolic fraction although some of the activity copurified with the chloroplast-containing fraction.

MnSOD mRNA levels in different tissues

MnSOD has previously been shown to be a highly abundant protein in *N.plumbaginifolia* cell suspension cultures (Bauw *et al.*, 1987). To characterize the expression at the mRNA level, Northern analysis on total RNA with a probe synthesized from the pSOD1 cDNA insert was carried out. This revealed considerable variations in the expression of MnSOD in plant cells (Figure 4). Expression is very weak in leaves from intact plants, 2- to 3-fold higher in roots, and ~50 times higher in dark-grown cell suspension cultures.

The high level expression of MnSOD in cell suspensions is caused by sugars

The dramatic difference between the expression of MnSOD in leaves and cell suspensions could be due to the differences

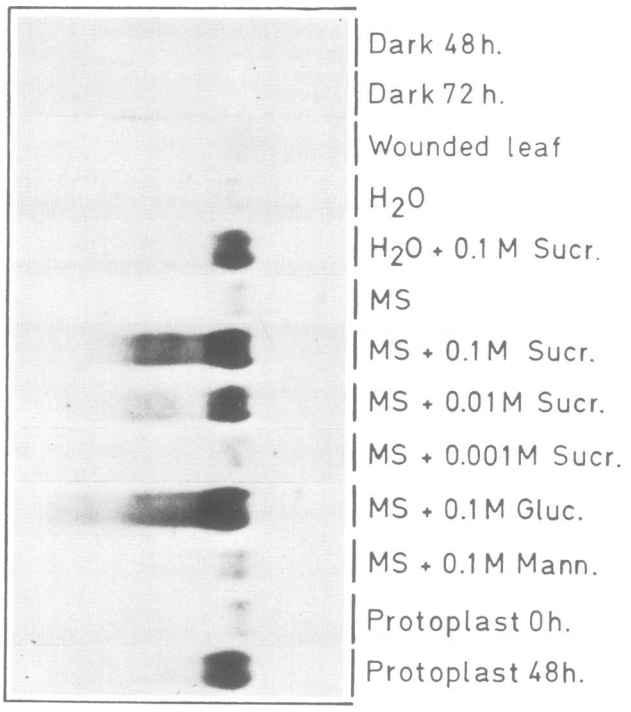


Fig. 5. Effect of sugars on the accumulation of MnSOD mRNA. Lanes 1 and 2 represent RNA extracted from whole plants incubated in the dark for 48 and 72 h respectively. The control for wounding (lane 3) involved making several cuts on leaves of an intact plant and extracting RNA 24 h later. Two samples of protoplasts are shown (lanes 12 and 13): immediately after isolation (time 0) and after a further 48 h incubation at low-light intensity in K3 medium (Nagy and Maliga, 1976), supplemented with 0.4 M glucose, 0.1 mg/l naphthalene acetic acid (NAA) and 0.2 mg/l 6-benzylaminopurine (BAP). All other samples were extracted from leaf discs incubated for 48 h under dark conditions in the indicated liquid media. Abbreviations. gluc., glucose; mann., mannitol; sucr., sucrose.

in the cell types or to differences in culture conditions. To resolve this question, *N.plumbaginifolia* leaf discs were incubated for 48 h in the dark in Murashige and Skoog liquid medium and assayed for MnSOD mRNA. Figure 5 shows that leaf tissue maintained in growth medium contained levels of MnSOD mRNA comparable to those seen in cell suspension cultures. This response was not due to a switch-off of photosynthesis, as a 72-h exposure of whole plants to the dark did not result in increased expression of MnSOD. Also, incubation of leaf discs in the dark in pure water caused only a weak (2- to 3-fold) induction, similar to the effect of wounding whole plants. Thus, the massive induction of MnSOD in tissue culture appears to be an intrinsic effect of the medium itself. This induction was also seen when freshly isolated protoplasts were incubated in regenerating medium (Figure 5).

To analyse the effect of the medium, sucrose and salts were tested separately on leaf discs. We found sucrose to be the crucial factor for induction and its effect was greatest in the presence of salts. A linear dose−response could be observed at different concentrations of sucrose (0.001−0.1 M), with the highest level of expression being reached after a 48-h incubation. Since the response was amplified by salts, we tested combinations of iron, manganese, copper and zinc ions in the presence of sucrose and we concluded that the enhancement in induction was not due to these salts

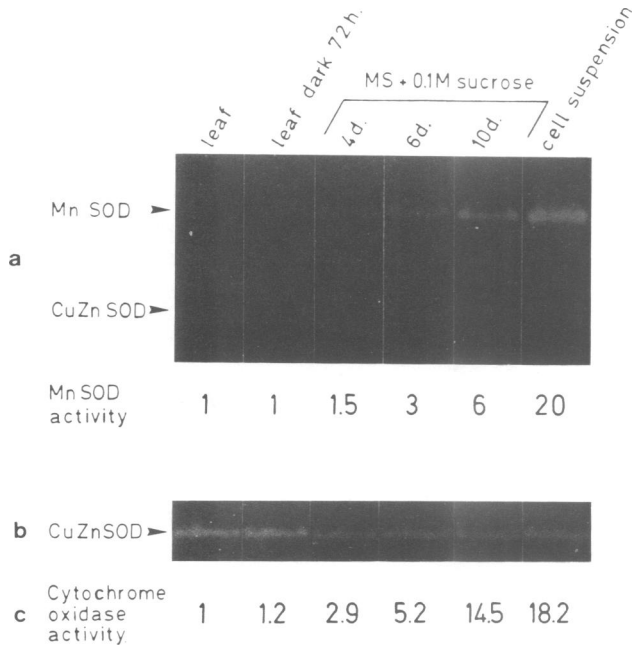


Fig. 6. Analysis of SOD and cytochrome oxidase activities. (**a**) Induction of MnSOD activity in leaf pieces incubated in MS medium with sucrose and in cell suspensions, as revealed by SOD staining on non-denaturing gels. Changes in MnSOD activity were best visualized in samples containing 50 μg protein. The Cu/ZnSOD activity could only be weakly detected at these concentrations. Values given for MnSOD activity are shown as fold increases in relation to the activity in an untreated leaf. (**b**) Visualization of Cu/ZnSOD activity in samples containing 200 μg protein. (**c**) Cytochrome oxidase activity measurements shown as fold increases over the activity in an untreated leaf. Experimental conditions have been described in Figures 4 and 5. Leaf discs were incubated for the periods indicated in MS + 0.1 M sucrose supplemented with 0.5 mg/l NAA and 0.1 mg/l BAP.

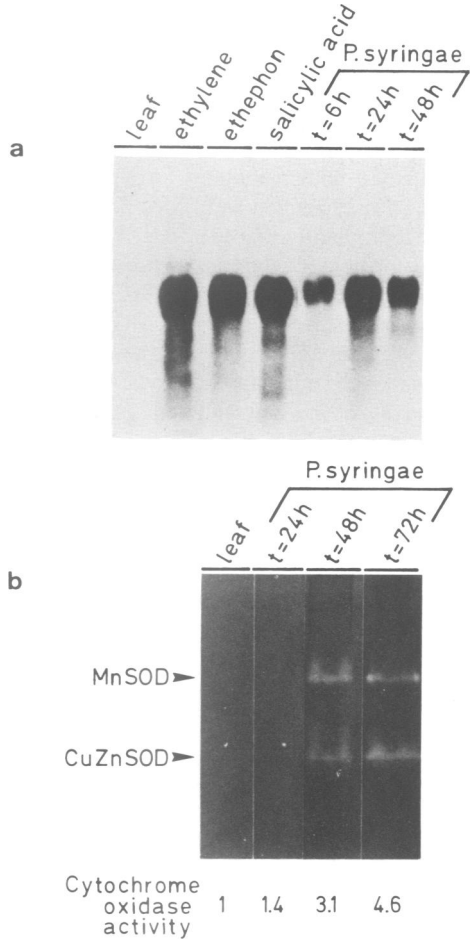


Fig. 7. Induction of MnSOD during the pathogenesis response. Whole plants were incubated under an atmosphere of ethylene (10 p.p.m. in purified air), or sprayed with ethephon (1 mg/ml) or salicylic acid (10 mM) and left for 48 h. *P.syringae* infections were performed as described in Materials and methods, and samples taken at the times indicated. (**a**) Northern blot analysis showing changes in MnSOD mRNA caused by the different treatments. (**b**) SOD activity gels and cytochrome oxidase activities measured in protein samples obtained from the same plants from which RNA was extracted. 75 μg total protein from each sample was loaded on the gel. Cytochrome *c* oxidase values are expressed as in Figure 6.

in particular (data not shown). The massive increase in MnSOD expression was also produced by glucose, but not by mannitol. Since mannitol is used as a non-catabolizable osmoticum in plant tissue culture (Street, 1959), our observation suggested that the induction of MnSOD by sugars was due to a trophic rather than an osmotic effect.

Analysis of SOD and cytochrome oxidase activities

From SOD assays performed on non-denaturing protein gels we estimated the abundance of the MnSOD protein in cell suspension cultures to be ~20 times higher than that found in leaves of intact plants (Figure 6a). When leaf discs were incubated with sucrose, the MnSOD protein was shown to follow a similar, albeit delayed, induction profile in relation to its mRNA. These experiments confirmed that sucrose caused an accumulation of MnSOD in leaf discs similar to that seen in cell suspension culture.

In contrast to the MnSOD, the Cu/ZnSOD showed no significant alteration in expression (Figure 6b). The lack of response from the latter enzyme, however, may reflect the specific role this enzyme plays in controlling free-radical concentrations in chloroplasts. If plants are treated with paraquat, a herbicide that induces superoxide production in chloroplasts (Halliwell, 1984), chloroplastic and cytosolic Cu/ZnSOD is induced in maize (Matters and Scandalios, 1986). We have found a similar induction in *N.plumbaginifolia* while MnSOD expression was virtually unchanged (data not shown). A possible interpretation of these observations could be that superoxide radicals produce a specific triggering molecule in each subcellular compartment, which is capable of acting as a signal to induce the nuclear gene(s) coding for the particular superoxide dismutase(s) associated with that compartment.

As the MnSOD proved to be the mitochondrial isozyme, its induction could be considered as a response against superoxide radicals generated in mitochondria by enhanced respiratory oxidation of sugars. It has been established that superoxide radicals are produced from the mitochondrial respiratory chain of eukaryotes (Loschen *et al.*, 1974), so we attempted to measure the activity of this pathway by measuring cytochrome *c* oxidase activity. This is the terminal enzyme, catalysing the reduction of oxygen to water. In Figure 6c, we show a clear correlation, under the conditions tested, between the activities of MnSOD and cytochrome oxidase.

The involvement of SOD in the pathogenesis response

To extend our information concerning MnSOD expression, we looked for an additional system to study. Previous reports have demonstrated that changes in SOD activity occur when a plant is infected with pathogens (Arrigoni *et al.*, 1980; Zacheo *et al.*, 1982; Zacheo and Bleve-Zacheo, 1988), and evidence is accumulating that the generation of superoxide radicals is responsible for the occurrence of necrotic lesions upon infection (Sekizawa *et al.*, 1987; Doke and Ohashi, 1988). To study the involvement of SOD in the defence response of *N.plumbaginifolia*, we chose to examine the effect of ethylene (and ethephon), salicylic acid, and infection with *Pseudomonas syringae*. Ethylene is known to induce several proteins involved in plant defence responses (Ecker and Davis, 1987) and endogenous levels of ethylene control many processes within a plant (Yang and Hoffman, 1984). Ethephon, which is hydrolyzed to ethylene in the plant, can mediate similar responses (van Loon, 1985). Several benzoic acid derivatives, such as salicylic acid can also induce stress responses; for example, it has been shown to induce several of the pathogenesis-related (PR) proteins (Hooft van Huijsduijnen *et al.*, 1986). We used a strain of *Pseudomonas syringae* that is non-pathogenic to *N.plumbaginifolia*, but which does elicit the hypersensitive response. Infection is characterized by the rapid development (within 6–12 hours) of localized necrosis around the infected area which prevents further spread of bacteria to other parts of the leaf.

Figure 7a shows the MnSOD mRNA to increase considerably following treatment for 48 h with ethylene, ethephon and salicylic acid. MnSOD induction in response to *P.syringae* appeared most apparent after 24 h and was followed by a slow decay. Analysis of SOD activity in infected material revealed that both the MnSOD and the Cu/ZnSOD increased (Figure 7b), which distinguishes the response from that mediated by sucrose, in which only the MnSOD was appreciably induced. Similar to earlier experiments, however, infection increased cytochrome *c* oxidase activity (Figure 7b).

Discussion

We have isolated a cDNA clone encoding a MnSOD from *N.plumbaginifolia*. The protein was identified from its N-terminal amino acid sequence (Bauw *et al.*, 1987), and this sequence was used to design an oligonucleotide probe for the screening of a cDNA library from *N.plumbaginifolia* cell suspension cultures. The complete amino acid sequence, obtained from the cDNA sequence, confirmed the preliminary identification of the MnSOD and revealed a high degree of homology with MnSODs from other organisms. As expected, the plant protein shows a higher homology with other eukaryotic MnSODs (yeast and human) as compared to bacterial MnSODs.

In animals and yeast the MnSOD protein appears to be located exclusively in the mitochondrial matrix (Geller and Winge, 1984; Marres *et al.*, 1985; Bannister *et al.*, 1987). However, the subcellular location in plants was uncertain. In the present case, the *N.plumbaginifolia* cDNA has a leader sequence of 24 amino acids with the characteristic features of a transit peptide for translocation to the mitochondrial matrix (Schatz, 1987). Although the import mechanism of nuclear-encoded mitochondrial proteins is being well characterized in organisms such as yeast and *Neurospora crassa*, few studies have been performed in plants. Thus, we considered it prudent to check the location with subcellular fractionation which demonstrated that the MnSOD activity copurified exclusively with the mitochondrial fraction. Further evidence has been obtained by expressing the plant preprotein in yeast, where it has been found to be efficiently targeted into yeast mitochondria, with concomitant cleavage of the leader sequence (Bowler *et al.*, in preparation).

The MnSOD was initially identified as a highly abundant protein in *N.plumbaginifolia* cell suspension cultures (Bauw *et al.*, 1987), which was confirmed at the mRNA level. In contrast, the expression in leaves was found to be very low (~50-fold less at the mRNA level and 20-fold less at the protein level). However, this basal expression could be dramatically induced at the mRNA level within 48 h by incubation of leaf discs or protoplasts in tissue culture medium with sucrose. A corresponding increase at the protein level was found to be somewhat delayed (Figure 6), perhaps indicating an additional post-transcriptional control mechanism. The critical factor for messenger induction was found to be sucrose (or glucose) and the level of induction was dose-dependent. This response appears to be specific for the MnSOD isozyme, as the Cu/ZnSOD remained unaffected.

Sugars do not induce the MnSOD through an osmotic effect, since mannitol did not increase gene expression in leaf discs. The effect of sugars may thus reflect their role as a carbon source: cytochrome *c* oxidase activity necessary for respiration was clearly stimulated by sucrose. The production of superoxide radicals from the mitochondrial respiratory chain is known to occur from NADH dehydrogenase and from ubiquinol-cytochrome *c* reductase (Loschen *et al.*, 1974; Ksenzenko *et al.*, 1983). As such, one would expect a concomitant need for SOD activity within the mitochondria in times of high respiratory activity. This requirement has been verified by studies showing that a yeast mutant lacking a mitochondrial MnSOD is unable to grow on a non-fermentable carbon source such as ethanol (van Loon *et al.*, 1986). Although a cytosolic SOD remains functional in this mutant, compartmentalization evidently precludes it from substituting for the mitochondrial SOD. Correspondingly, the specific and dramatic induction of MnSOD and not Cu/ZnSOD in *N.plumbaginifolia* could indicate that only the former can protect against the severe oxidative stress created in mitochondria by tissue culture.

The availability of our cDNA clone allowed further analysis of the role of MnSOD under more natural physiological conditions. To determine whether it forms a part of the plant response to pathogens, we analysed SOD protein and mRNA profiles in leaves after treatment with ethylene, salicylic acid and *P.syringae*. All these treatments enhance plant respiration (Laties, 1982; Raskin *et al.*, 1987) and all increased the abundance of MnSOD transcript. Following *P.syringae* infection the Cu/ZnSOD was found to be induced in concert with the MnSOD, in contrast to the more restricted response to sucrose. However, the correlation of MnSOD activity with cytochrome *c* oxidase activity is clearly conserved in response to both treatments. Our results thus reinforce the hypothesis that MnSOD must be induced whenever mitochondrial activity increases, regardless of the cause.

Taken collectively, these results reveal the connection between cytochrome oxidase and MnSOD expression. An obvious hypothesis is that this close correlation reflects a common regulatory mechanism which allows a stoichiometric 'switching on' of all the genes involved in mitochondrial function. In yeast, it has been shown that the expression of MnSOD and cytochrome oxidase are coupled, and a gene which controls oxygen-regulated functions has been identified (Lowry and Zitomer, 1984). It is possible that further characterization of the response in plants may reveal a similar mechanism, although the presence of two respiratory pathways (cyanide-sensitive, the other cyanide-resistant) would presumably make the situation somewhat more complex. However, the function of the enzyme might suggest that its induction would somehow be mediated by superoxide radicals. If superoxide radicals were the triggering factor, it should be possible to block the induction by the addition of free radical scavengers. Using ascorbate, commonly used as a free radical scavenger, we attempted to suppress induction of MnSOD in leaf discs incubated with sucrose. All attempts, however, were unsuccessful (data not shown). The lack of an effect makes it difficult to draw any conclusions, since it may simply reflect ascorbate sequestration rather than effectiveness.

As such, the mechanism of MnSOD induction in *N.plumbaginifolia* whether mediated as a general response or via superoxide radicals, remains unclear. By isolating the gene and constructing transgenic plants containing MnSOD promoter−reporter gene fusions, a more sensitive characterization will be possible which should allow further elucidation of the regulatory mechanism.

Materials and methods

Plant material

N.plumbaginifolia var. P2 was used as plant source. All plant material was sterile-grown at 25°C on Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) unless stated otherwise. In particular, whole plants, from which leaf and root samples were taken, were grown with a 16-h light/8-h dark cycle on solid MS medium containing 0.1 M sucrose. Cell suspension cultures were shaken at 110 r.p.m. (Queue Radial Shaker) under dark conditions in liquid medium with 0.1 M sucrose and supplemented with 0.5 mg/l naphthalene acetic acid (NAA) and 0.1 mg/l 6-benzylaminopurine (BAP). They were subcultured every 3 days. To maintain leaf discs in MS medium for periods longer than 48 hours, 0.5 mg/l NAA and 0.1 mg/l BAP were added. Protoplasts were prepared as described by Zambryski *et al.* (1984).

Construction and screening of cDNA library

The cDNA library from a *N.plumbaginifolia* cell suspension culture was constructed in plasmid pUC18 (Yanisch-Perron *et al.*, 1985) using a procedure adapted from Gubler and Hoffman (1983). A detailed description of the method used has been published elsewhere (De Loose *et al.*, 1988). The oligonucleotide sequence, 5'-CCITAIGAITAIGGIGCICTIGAICCIGC -3', was designed with deoxyinosines at ambiguous codon positions (Ohtsuka *et al.*, 1985) to match the previously determined MnSOD N-terminal amino acid sequence (Bauw *et al.*, 1987). The screening of the cDNA library was essentially performed according to standard procedures (Maniatis *et al.*, 1982).

The probe was end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP to a sp. act. of 5×10^7 c.p.m. per μg DNA. A filter containing 5×10^4 bacterial clones from the cDNA library was hybridized overnight with the probe in 6 × SSC, 5 × Denhardt's, and 0.05% sodium pyrophosphate at 40°C. Subsequently, the filter was washed four times for 30 min in 6 × SSC, 0.5% SDS at 40°C, and autoradiographed on Kodak XAR-5 film with intensifying screens. Twelve clones showed a positive signal. The clone with the largest cDNA insert, pSOD1, was selected for further analysis.

Subcellular fractionation

Leaf material (5 g) was homogenized and fractionated into chloroplast, mitochondrial and cytosolic fractions essentially as described by Boutry *et al.* (1987). Chloroplasts were obtained from a two-step Percoll gradient consisting of 80% Percoll and 40% Percoll. For the isolation of mitochondria, the gradient was made from 45% Percoll and 21% Percoll. All Percoll solutions contained 0.1% BSA and 0.25 M sucrose.

Organellar fractions were removed from the interfaces and were diluted 10-fold in suspension medium (0.4 M mannitol, 10 mM KH_2PO_4, 0.1% BSA, pH 7.2). A pellet was obtained by centrifugation at 13 000 r.p.m. for 10 min which was then resuspended in suspension medium. Organelles were lysed with 0.05% Triton X-100 and insoluble material removed by a further centrifugation step. The supernatant thus obtained was used for SOD assays. The presence/absence of mitochondria within each sample was checked with cytochrome oxidase assays performed spectrophotometrically as described (Darley-Usmar *et al.*, 1987).

RNA preparation and analysis

Total RNA was prepared from fresh tissue as described by Jones *et al.* (1985) and quantified spectrophotometrically. For Northern analysis, 12 μg was denatured in formaldehyde, electrophoresed and transferred to nylon membranes according to Maniatis *et al.* (1982). To obtain highly specific probes, the internal *Hpa*I−*Hin*dIII fragment from pSOD1 was recloned in the *Sma*I−*Hin*dIII sites of pGem2, and ^{32}P-labelled single-stranded riboprobes were synthesized using T7 RNA polymerase (Promega). Northern hybridizations were performed overnight at 68°C in 50% formamide, 5 × SSPE, 0.25% non-fat milk powder, 0.5% SDS, 10% dextran sulfate, and 20 μg/ml denatured herring sperm DNA. The filters were washed twice with 3 × SSC, 1% SDS, and twice with 1 × SSC, 1% SDS, at 68°C, and were autoradiographed on Kodak XAR-5 film with intensifying screens for 5−10 h at −70°C. To quantify MnSOD mRNA levels in each sample, hybridizing bands were cut out from the filter and the amount of bound radioactivity was measured by scintillation counting.

Superoxide dismutase assays

Plant material was homogenized in an equal volume of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.8, 0.1% BSA, 0.1% ascorbate, 0.05% β-mercaptoethanol, 0.2% Triton X-100), and clarified by centrifugation at 13 000 r.p.m. for 12 min. Total protein was measured by the protein-dye binding assay (Bradford, 1976) with a kit supplied by BioRad Laboratories. Samples were separated on non-denaturing 10% polyacrylamide gels and the SOD activity was localized on these gels using the *in situ* staining technique of Beauchamp and Fridovich (1971). Inhibitor studies were performed directly on gels in order to distinguish between Cu/Zn-, Min- and FeSOD isoforms. These were carried out in the manner described by Sandalio *et al.* (1987). Relative levels of MnSOD activity were estimated by comparing staining reactions on gels containing different protein concentrations (10−200 μg).

Bacterial infections

P.syringae infections were performed on non-flowering plants grown in soil under greenhouse conditions. An end-log culture grown in LPG medium (0.3% yeast extract, 0.5% bactopeptone, 0.5% glucose) was diluted to 1×10^7 cells/ml and injected into the leaves with a hypodermic syringe.

Miscellaneous

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982). The DNA sequences were determined on both strands by the procedure of Maxam and Gilbert (1980).

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