



Role of superoxide dismutases (SODs) in controlling oxidative stress in plants

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Abstract

Reactive O₂ species (ROS) are produced in both unstressed and stressed cells. Plants have well-developed defence systems against ROS, involving both limiting the formation of ROS as well as instituting its removal. Under unstressed conditions, the formation and removal of O₂ are in balance. However, the defence system, when presented with increased ROS formation under stress conditions, can be overwhelmed. Within a cell, the superoxide dismutases (SODs) constitute the first line of defence against ROS. Specialization of function among the SODs may be due to a combination of the influence of subcellular location of the enzyme and upstream sequences in the genomic sequence. The commonality of elements in the upstream sequences of Fe, Mn and Cu/Zn SODs suggests a relatively recent origin for those regulatory regions. The differences in the upstream regions of the three FeSOD genes suggest differing regulatory control which is borne out in the research literature. The finding that the upstream sequences of Mn and peroxisomal Cu/Zn SODs have three common elements suggests a common regulatory pathway. The tools are available to dissect further the molecular basis for antioxidant defence responses in plant cells. SODs are clearly among the most important of those defences, when coupled with the necessary downstream events for full detoxification of ROS.

Key words: Defence systems, detoxification, ROS, SOD, superoxide dismutase.

Introduction

Reactive O₂ species (ROS) are produced in both unstressed and stressed cells. Plants have well-developed defence systems against ROS, involving both limiting the formation of ROS as well as instituting its removal. Under unstressed conditions, the formation and removal of O₂ are in balance. However, the defence system, when presented with increased ROS formation under stress conditions, can be overwhelmed. Plants respond to a rise in ROS that the defence system is unable to remove with increased enzymatic or non-enzymatic antioxidant processes (Alscher and Hess, 1993), but the mechanisms underlying these processes is not well understood.

Within a cell, the superoxide dismutases (SODs) constitute the first line of defence against ROS. O₂⁻ is produced at any location where an electron transport chain is present, and hence O₂ activation may occur in different compartments of the cell (Elstner, 1991), including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. This being the case, it is not surprising to find that SODs are present in all these subcellular locations (Fig. 1). While all compartments of the cell are possible sites for O₂⁻ formation, chloroplasts, mitochondria and peroxisomes are thought to be the most important generators of ROS (Fridovich, 1986).

It has been shown that phospholipid membranes are impermeable to charged O₂⁻ molecules (Takahashi and Asada, 1983). Therefore, it is crucial that SODs are present for the removal of O₂⁻ in the compartments where O₂⁻ radicals are formed (Takahashi and Asada, 1983). Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD),

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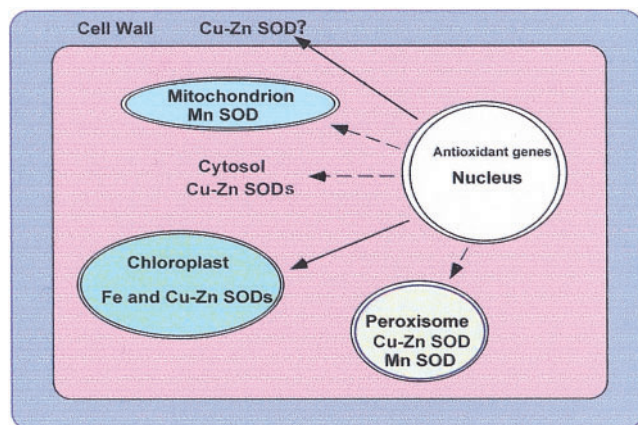


Fig. 1. Locations of SODs throughout the plant cell.

manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD), and these SODs are located in different compartments of the cell. Fe SODs are located in the chloroplast, Mn SODs in the mitochondrion and the peroxisome, and Cu-Zn SODs in the chloroplast, the cytosol, and possibly the extracellular space (Fig. 1). Comparison of deduced amino acid sequences from these three different types of SODs suggest that Mn and Fe SODs are more ancient types of SODs, and these enzymes most probably have arisen from the same ancestral enzyme, whereas Cu-Zn SODs have no sequence similarity to Mn and Fe SODs and probably have evolved separately in eukaryotes (Kanematsu and Asada, 1990; Smith and Doolittle, 1992) (see the later discussion of sequence analysis). The evolutionary reason for the separation of SODs with different metal requirements is probably related to the different availability of soluble transition metal compounds in the biosphere in relation to the O_2 content of the atmosphere in different geological eras (Bannister *et al.*, 1991). Each SOD group will be studied in turn.

Iron SODs

The group of Fe SODs probably constitute the most ancient SOD group. It has been suggested that iron was probably the first metal used as a metal cofactor at the active site of the first SOD because of an abundance of iron in soluble Fe (II) form at the time (Bannister *et al.*, 1991). As the levels of O_2 in the environment increased, the mineral components of the environment were oxidized. The decrease in available Fe (II) in the environment caused a shift to the use of a more available metal, Mn (III).

Fe SOD is found both in prokaryotes and in eukaryotes. In eukaryotes it has been isolated from *Euglena gracilis* (Kanematsu and Asada, 1979) and higher plants. Fe SOD is inactivated by H_2O_2 and is

resistant to KCN inhibition. In all plant species examined to date, it is inferred that it is located in the chloroplast. Polyclonal antibodies were raised against water lily (*Nuphar luteum*) Fe SOD (Salin and Bridges, 1981). When these antibodies were incubated with protoplasts from water lilies, it was shown that the antibodies predominantly associated with the chloroplasts (Salin, 1988). Fe SOD has also been localized in the chloroplast in *N. luteum*, and a potential chloroplastic targeting sequence was found in soybean Fe SOD. Three Fe SODs were reported in *Arabidopsis thaliana* (Kliebenstein *et al.*, 1998). The absence of Fe SOD in animals has given rise to the proposal that the Fe SOD gene originated in the plastid and moved to the nuclear genome during evolution. Support for this theory comes from the existence of several conserved regions that are present in plant and cyanobacterial Fe SOD sequences, but absent in non-photosynthetic bacteria (Bowler *et al.*, 1994). Previously, it was thought that Fe SOD was not present in all plants. The presence of Fe SOD activity in Ginkgoaceae, Nymphaeaceae, and Cruciferae was concluded to be a random occurrence of the enzyme in the plant kingdom (Salin and Bridges, 1981). More recently, Fe SOD genes were isolated in three plant species lacking phylogenetic relationships to one another: *N. plumbaginifolia*, *A. thaliana* (Van Camp *et al.*, 1990), and *Glycine max* (Crowell and Amasino, 1991). All three Fe SOD plant sequences encode a unique tripeptide (SRL for *N. plumbaginifolia* and *G. max* and ARL for *A. thaliana*) close to the carboxyl terminus of the enzyme. Although this sequence has been shown to direct the proteins to peroxisomes in other proteins, it has yet to be determined whether this is a functional sequence or not. The conserved SRL/ARL sequence is not present in the prokaryotic Fe SOD proteins showing that it is not obligatory for the enzyme function (Van Camp *et al.*, 1994). SOD populations in rice (*Oryza sativa*) and maize have been extensively studied, but there have been no reports of the presence of Fe SOD in either rice or maize.

There are two distinct groups of Fe SOD. The first group is a homodimer formed from two identical 20 kDa subunit proteins, with 1–2 gram atom of iron in the active centre. This type of Fe SOD has been isolated from *Escherichia coli* (Yost and Fridovich, 1973); *Photobacterium sepoa* and *P. leiognathi* (Puget and Michelson, 1974); the facultative anaerobe, *Thiobacillus denitrificans* (Baldensperger, 1978); the purple sulphur bacterium, *Chromatium vinosum* (Kanematsu and Asada, 1978); and the plant species, *Ginkgo biloba*, *Brassica calpestris*, and *Nuphar luteum* (Salin and Bridges, 1980). The second Fe SOD group, found in most higher plants, is a tetramer of four equal subunits with a molecular weight of 80–90 kDa. Members of this group contain 2–4 gram atoms of iron in the active centre. Proteins in this group have been isolated from three prokaryotes,

Mycobacterium tuberculosis (Kusunose *et al.*, 1976), *Thermoplasma acidophilum* (Searcy and Searcy, 1981), and *Methanobacterium bryantii* (Kirby *et al.*, 1981), in addition to one eukaryote, *Tetrahymena pyriformis* (Barro *et al.*, 1990).

Manganese SODs

As mentioned previously, as the levels of O₂ in the environment increased, the amount of available Fe (II) in the environment decreased, causing a shift to the more available metal, Mn (III). As a consequence, Mn SODs are likely to be second only to Fe SODs in antiquity and certainly evolved from the ancestral Fe SODs, perhaps by way of the cambialistic SODs (discussed later). Mn SODs occur in mitochondria and peroxisomes. Mn SODs carry only one metal atom per subunit. These enzymes cannot function without the Mn atom present at the active site. Even though Mn and Fe SODs have a high similarity in their primary, secondary, and tertiary structure, these enzymes have diverged sufficiently that Fe (II) could not restore the activity of Mn SOD and vice versa (Fridovich, 1986). Catalysis by Mn SODs is through the attraction of negatively charged O₂⁻ molecules to a site formed from positively charged amino acids present at the active site of the enzyme. The metal present in the active site then donates an electron directly to the O₂⁻, reducing one O₂⁻ molecule, which in turn forms H₂O₂ by reacting with a proton (Asada, 1994; Bowler *et al.*, 1994).

Mn SOD is either a homodimeric or a homotetrameric enzyme with one Mn (III) atom per subunit. The enzyme is not inhibited by potassium cyanide (KCN) or inactivated by H₂O₂ and is present in both eukaryotes and prokaryotes. Plant Mn SODs have approximately 65% sequence similarity to one another, and these enzymes also have high similarities to bacterial Mn SODs (Bowler *et al.*, 1994).

Although Mn SOD is known as the mitochondrial enzyme of eukaryotes, a Mn-containing SOD has also been located in the peroxisomes. The presence of one peroxisomal and one mitochondrial Mn SOD was shown by using immunolocalization assays in watermelon (del Río *et al.*, 1992). Four genes that encode Mn SOD were reported in maize (*Zea mays*) (Zhu and Scandalios, 1993). Deduced amino acid sequences from these four isoenzymes have a mitochondrial targeting sequence, indicating that all are located in the mitochondria. In *Nicotiana glauca*, two nuclear-encoded Mn SOD genes were isolated and the tissue-specific expression Mn SOD was shown by analysing promoter fusion with β-glucuronidase (GUS) in transgenic plants (Van Camp *et al.*, 1996). Multiple transcripts for Mn SOD have been reported in human (*Homo sapiens*) tissues. These different transcripts have been found to be the product of the same gene, but result from alternative polyadenylation or

alternative splicing and polyadenylation (Zhu and Scandalios, 1993). In addition to maize, Mn SOD has been found in the mitochondria of tobacco (*Nicotiana tabacum*; Bowler *et al.*, 1994), mung beans (*Vigna mungo*; Reddy and Venkaiah, 1982), watermelon (Sandalo and del Río, 1987), carnations (*Dianthus caryophyllus*; Droillard and Paulin, 1990), peas (*Pisum sativum*; Foster and Edwards, 1980), spinach (*Spinacia oleracea*; Jackson *et al.*, 1978), and some other plants. A peroxisomal Mn SOD was reported in pea for the removal of O₂ formed as a result of xanthine oxidase action (del Río *et al.*, 1983, 1998; Sandalo and del Río, 1987), but none of the known Mn SOD sequences appear to have a transit peptide sequence for peroxisomal targeting (Bowler *et al.*, 1994).

In green algae and cyanobacteria, Mn SOD is found in the thylakoid membrane (Kanematsu and Asada, 1979; Okada *et al.*, 1979). Although a thylakoid bound form of Mn SOD was reported in spinach plants (Hayakawa *et al.*, 1985), after further investigation it was suggested that this Mn SOD activity was the result of a non-specific or mimic Mn SOD activity (Palma *et al.*, 1986) and that Mn SOD is present only in the mitochondria and peroxisomes of the plant cells.

Copper-zinc SODs

When the atmosphere was completely replenished with oxygen, Fe (II) was almost completely unavailable and insoluble Cu (I) was converted into soluble Cu (II). At this stage, Cu (II) began to be used as the metal cofactor at the active sites of SODs. Since Fe SODs and Mn have similar electrical properties, the transition from the use of iron to the use of manganese required little change in SOD protein structure. Thus, Mn and Fe SODs are structurally very similar. However, the electrical properties of Cu-Zn⁴ SODs differ greatly from those of Fe and Mn SODs. Therefore, a major change in the structure of the protein occurred after Cu become a metal cofactor (Bannister *et al.*, 1991). Fe and Mn SODs are present both in prokaryotic and in eukaryotic organisms whereas Cu-Zn SODs have been found mostly in eukaryotes. However, Cu-Zn SODs have been demonstrated in some bacteria, including *Photobacterium leiognathi*, *Caulobacter crescentus*, and pseudomonads. Three hypotheses that might explain the presence of Cu-Zn SOD in prokaryotes are these:

- (1) Cu-Zn SODs evolved independently in prokaryotes and eukaryotes.
- (2) Cu-Zn SOD originated in the eukaryotes, and the eukaryotic gene was transferred into the prokaryotes. This hypothesis was first proposed by Martin and

⁴ Every molecule of the Cu-Zn SOD enzyme contains both an atom of copper and an atom of zinc, as denoted by the hyphen.

Fridovich (Martin and Fridovich, 1981) and then found support from Bannister and Parker (Bannister and Parker, 1985) because of the 30% similarity between the amino acid sequences of the Cu-Zn SODs in ponyfish and in its symbiont *Photobacterium leiognathi*. After taking point mutations into consideration this similarity increased to 44%, bringing yet more support to the hypothesis (Leunissen and de Jong, 1986). However, the presence of Cu-Zn SOD in *Caulobacter crescentus* (Steinman, 1982) and in pseudomonads that are not symbionts (Steinman, 1985) suggests the hypothesis requires further refinement.

- (3) Cu-Zn SOD first originated in prokaryotes and then was transferred to eukaryotes. However, this hypothesis entails the unlikely requirement that prokaryotic and eukaryotic enzymes have a common ancestor that had Cu-Zn SODs before the time that prokaryotes and eukaryotes separated, which, in turn, was before the availability of Cu (II) in the atmosphere.

Cu-Zn SODs are found throughout the plant cell. There are two different groups of Cu-Zn SODs. The first group consists of cytoplasmic and periplasmic forms, which are homodimeric. The second group comprises the chloroplastic and extracellular Cu-Zn SODs, which are homotetrameric (Bordo *et al.*, 1994). The active sites of each subunit function independently. When these subunits are separated and then coupled with an inactive subunit, newly formed enzymes show full activity, providing evidence that the functional interactions between the subunits are not essential for full catalytic activity (Fridovich, 1986).

Tetrameric extracellular SOD (EC-SOD) has been found exclusively in mammals to date and has a unique structure. The enzyme is a glycoprotein and is primarily located in the interstitial matrix of tissues and the glycocalyx of cell surfaces, anchored to heparin sulphate proteoglycans (Carlsson *et al.*, 1995). Only a small fraction of EC-SODs are found in extracellular fluids such as plasma, lymph, synovial fluid, and cerebrospinal fluid (Carlsson *et al.*, 1995). The expression of EC-SOD was disrupted by homologous recombination in embryonic stem (ES) cells (Carlsson *et al.*, 1995). They observed that these mice showed no abnormality and no induction of other SODs or other antioxidant enzymes up to 14 months of age. However, when they subjected these mice to oxidative stress by exposure to high O₂ concentrations, the null mutant mice displayed a remarkably reduced tolerance. This shows that under unstressed conditions, there is enough protection provided in extracellular space against O₂ (Carlsson *et al.*, 1995). Evidence has been provided for the existence of an extracellular

Cu-Zn SOD in plants (Streller *et al.*, 1994; Streller and Wingsle, 1994; Ogawa *et al.*, 1996).

Cu-Zn SOD exists in both chloroplastic and cytosolic forms. Deduced amino acid sequences of these two isoforms show approximately 68% similarity, whereas there is approximately 90% similarity among the chloroplastic Cu-Zn SODs (Cu-Zn SOD_{chl}) and 80–90% similarity among the cytosolic Cu-Zn SODs (Cu-Zn SOD_{cyt}). One Cu-Zn SOD_{chl} isoform (Ogawa *et al.*, 1996) and two proposed 'cytoplasmic' isoforms (Kanematsu and Asada, 1990) have been identified in spinach (*Spinacia oleracea*). Cu-Zn SOD_{chl} is a soluble enzyme and is localized in the stroma (Asada and Kiso, 1973; Asada *et al.*, 1973). Localization studies performed with an immunogold-labelled antibody raised against Cu-Zn SOD_{chl} from spinach leaves showed that this soluble enzyme is not uniformly distributed in the chloroplast but rather is localized mainly on the stromal face of thylakoid membranes (Ogawa *et al.*, 1995) where photosystem I (PSI) is located. The two other Cu-Zn SODs are considered 'cytoplasmic' because they have not been detected in intact chloroplasts (Ogawa *et al.*, 1996). However, when immunogold-labelled antibodies raised against 'cytosolic' Cu-Zn SOD were used in localization experiments, it was shown that these enzymes were located in the nucleus and apoplast (Ogawa *et al.*, 1996). More than 40% of the immunogold particles were found in the apoplast and approximately 25% was found in the nucleus (Ogawa *et al.*, 1996). Researchers proposed that Cu-Zn SOD in the apoplast functions in lignification and that in the nucleus it protects the cell against fatal mutations caused by O₂⁻ molecules (Ogawa *et al.*, 1996, 1997). The occurrence of a peroxisomal Cu-Zn SOD from watermelon, which represented about 18% of the total SOD activity in the cell, was also previously reported (Sandalio and del Río, 1987). Presence of such a peroxisomal Cu-Zn SOD also was shown in rat liver cells (Dhaunsi *et al.*, 1992).

Cambialistic SODs

A cambialistic Fe/Mn SOD is an enzymatically active protein that can accommodate either metal ligand. Some bacterial species can use a common SOD for both Fe and Mn and utilize either of these two metals as the active metal cofactor, according to availability of the metal. A great similarity in primary, secondary, and tertiary structures of Fe and Mn SODs have been observed. The metal ligands and other structurally and functionally important residues are highly conserved between these enzymes. In addition, the similar electrical properties of Fe and Mn allow the enzyme to function with either metal without a conformational change in its protein structure. Similarly, a hybrid SOD produced by using *E. coli* Fe and Mn subunits has been shown to be partially inhibited

with H₂O₂ treatment in proportion to its content of Fe (Clare *et al.*, 1984). Three-dimensional structures of Mn and Fe SODs also show high similarity, further demonstrating the homology present between these two enzymes (Stallings *et al.*, 1984).

SOD expression and activity under oxidative stress

Bipyridinium herbicides can bind to the thylakoid membrane of the chloroplasts and transfer the electrons to O₂ in a chain reaction causing continuous formation of O₂⁻. As a result NADP⁺ cannot be reduced and carbon fixation ceases. Methyl viologen (1,1'-dimethyl-4,4'-bipyridylium dichloride) is a bipyridylium herbicide widely used in plants to study O₂-mediated damage in the chloroplast (Dodge, 1994). Methyl viologen also affects electron-transducing reactions in the endoplasmic reticulum and in mitochondria. Methyl viologen treatment affects the level of chloroplastic SOD as well as mitochondrial and cytosolic SODs (Dodge, 1994; Van Camp *et al.*, 1994).

Effects of oxidative stress on SOD activities in *Arabidopsis*

SOD enzyme activities and protein levels were determined in 16-d-old whole *A. thaliana*. Two control groups, untreated and 0.05% surfactant-treated groups, and two methyl viologen-treated groups (1 × 10⁻⁶ M and 5 × 10⁻⁵ M) were used as the experimental groups. Treatments were repeated three times with different set of plants and replicate samples were taken from each treatment group. One set of these replicas was used for the activity experiments on native gels, and the other set was used for immunoblot experiments using antibodies raised against purified *A. thaliana* Fe and Mn SODs. Each experiment was repeated three times and one representative picture of these experiments is presented in Fig. 2. Both concentrations of methyl viologen caused an increase in the amount of Fe SOD enzyme (Fig. 2). After the methyl viologen treatment, the maximum increase in Fe SOD activity was observed 5 h after the treatments for both methyl viologen-treated groups and the level of activity in both groups was similar. Another Fe SOD response to stress is the appearance of novel Fe SOD activity bands (Fig. 2). These bands were observed both in DCMU- and in methyl viologen-treated plants. Densitometric scanning showed that the novel Fe SOD enzyme activity was present 5 h after the treatment, and it showed a consistent increase over the 41 h time-course. These bands were not observed in the control plants. Denaturing gel electrophoresis was performed using the collected samples in order to determine the effects of stress treatment at the protein level. Proteins

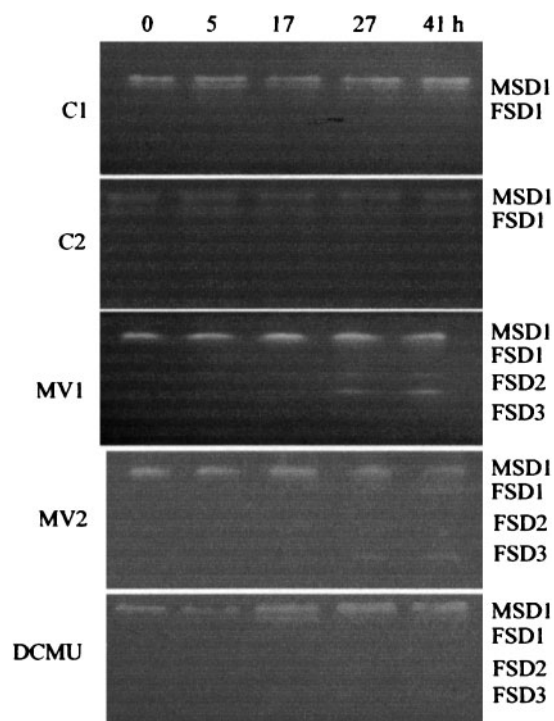


Fig. 2. Appearance of two novel Fe SOD activity bands after treatment of *Arabidopsis thaliana* with methyl viologen and DCMU. Gel C1: untreated plants. Gel C2: surfactant-treated plants. Gel MV1: 5 × 10⁻⁵ M methyl viologen-treated plants. Gel MV2: 1 × 10⁻⁶ M methyl viologen-treated plants. Gel DCMU: DCMU-treated plants.

from denaturing gel electrophoresis were transferred to the nitrocellulose membranes. After these membranes were incubated with the Fe SOD antibody, one band at 25 kDa was observed.

Although second and third Fe SOD activity bands were observed in methyl viologen and DCMU-treated plants, no secondary bands were observed in immunoblots in any of the treatment groups, at any time point. Although an increase in the protein level was observed in both methyl viologen-treated groups, protein amounts increased in both control groups as well (Fig. 2), suggesting that this increase was not a result of the oxidative stress treatments.

Arabidopsis was subjected to a series of oxidative stresses and changes were observed in the seven SODs—three Fe SODs denoted FSD1, FSD2, and FSD3; three Cu-Zn SODs denoted CSD1, CSD2, and CSD3; and one Mn SOD denoted MSD1—that are present in that plant, both at the mRNA and the protein level (Kliebenstein *et al.*, 1998). They reported increases in FSD2 in response to UV irradiation, and to high light at the mRNA level, but no response of FSD2 mRNA to ozone exposure. They found that FSD1 is under the control of a circadian clock at the mRNA level. Since enzyme activities were not reported (Kliebenstein *et al.*, 1998), it was not possible to make direct comparisons between their data and those of this study. However, it

is of interest to note that they report FSD2 responding to specific stresses, as has been shown here for methyl viologen stress. Thus, it appears that members of the Fe SOD family are specialized for specific responses to stress. The seven known *A. thaliana* SODs seem to be sufficiently distinct in sequence so as not to cross-hybridize. A Southern hybridization in which FSD1, MSD1, CSD1 (present in the cytosol), and CSD2 (present in the chloroplast) were used to probe *A. thaliana* genomic DNA showed no evidence of cross-hybridization (Fig. 3).

Responses of antioxidants to methyl viologen were also detected in pea leaves (Donahue *et al.*, 1997). It was observed that there is a positive correlation between leaf age and susceptibility to methyl viologen. The younger leaves had higher SOD, APX, and GR activity in methyl viologen-treated and control plants. These authors reported the presence of Fe SOD in pea plants for the first time. It was also reported that the changes they observed in the enzyme activity level as a result of stress treatment did not always correlate with the changes in the mRNA levels.

Exposure to severe salt stress results in increases in SOD activities in pea plants, and in Fe SOD in particular (Gomez *et al.*, 1999; Hernández *et al.*, 1993, 1995), with corresponding decreases in Cu-Zn SOD II, suggesting the participation of the SODs in an intracellular signalling pathway (see below). Exposure to less severe salt stress resulted in increases in Cu-Zn SOD activities.

Effects on Mn SOD

Treatment of *A. thaliana* with methyl viologen resulted in an increase in the amount of mitochondrial Mn SOD enzyme activity. Increase in the activity was between 50–75% for both 5×10^{-5} M and for 1×10^{-6} M methyl viologen-treated plants. No change in the activity was

observed in the control groups or the DCMU-treated group over the 41 h time-course (Fig. 2). Activity changes caused by methyl viologen were similar in both Mn and Fe SOD enzymes. In contrast to the changes observed in Fe SOD activity, DCMU did not cause any changes in Mn SOD enzyme activity. Kliebenstein *et al.* also reported that there was no effect of their series of oxidative stress treatments on Mn SOD in *A. thaliana* (Kliebenstein *et al.*, 1998).

By contrast, Mn SOD was reported to respond positively to salt stress (Gomez *et al.*, 1999; Hernández *et al.*, 1993, 1995), manganese toxicity (Gonzalez *et al.*, 1998), chilling stress (Lee *et al.*, 1999; Lee and Lee, 2000), and drought (Wu *et al.*, 1999). An explanation for the differences in responses between the Mn and Fe SODs in plants can be correlated with their disparate sub-cellular locations and the sites of action of the various oxidative stresses that were used. The stresses that did not affect Mn SOD may all have their site of action in the chloroplast.

The effects of methyl viologen and water deprivation on SOD enzyme activities were investigated in the late vegetative growth stage of pea leaves (Iturbe-Ormaetxe *et al.*, 1998). Plants that were moderately deprived of water (D1) and sprayed with 100 μ M methyl viologen (PQ) showed an increase in total Cu-Zn activity, but a decrease in the amount of Fe SOD activity was observed after treating the plants with methyl viologen and water deprivation. Mn SOD activity was unchanged under both conditions. It was concluded that the Fe SOD activity was being inhibited by methyl viologen, but the Cu-Zn and the Mn SOD activities were not being affected (Iturbe-Ormaetxe *et al.*, 1998).

Overexpression of SODs can lead to protection against specific stresses. Mn SOD can protect when targeted to the chloroplast. Plants overexpressing SODs and other

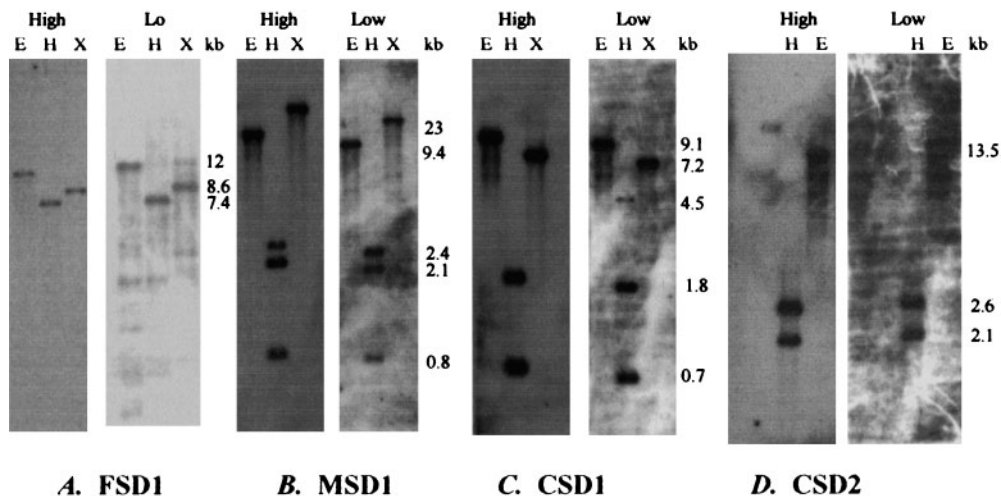


Fig. 3. Southern hybridization of *Arabidopsis* genomic DNA with SOD probes.

scavenging enzymes have been engineered with the goal of increasing stress tolerance. However, the scavenging pathway is quite complex. Therefore, both successful and unsuccessful results have been obtained from attempts to create resistant plants (Perl *et al.*, 1993; Slooten *et al.*, 1995; Tepperman and Dunsmuir, 1990; Van Camp *et al.*, 1996).

The site of action for ozone has been suggested to be in the apoplast, but when Mn SOD was overexpressed in the chloroplasts of tobacco plants, less damage was observed in the leaves. When the over-production of the enzyme was in the mitochondria, less protection was observed. Also, it was shown that high levels of chloroplastic Mn SOD activity protected the plant from visible injury caused by ozone, suggesting that ozone may cause oxidative stress in the chloroplast, as well as the apoplast (Van Camp *et al.*, 1994).

When an *A. thaliana* Fe SOD gene was targeted into *N. tabacum* cv. Petit Havana SR1 chloroplasts, an increased protection against O₂ generated both in the plasmalemma and photosystem II was observed (Van Camp *et al.*, 1996). An *A. thaliana* Fe SOD was overexpressed in poplar and a protective effect on PSII was observed under limiting carbon dioxide conditions (Arisi *et al.*, 1998). However, in the study of Van Camp *et al.*, induction of Cu-Zn SODs was suppressed, lending more credence to the hypothesis that a cross-family signalling pathway for SOD induction exists in the plant cell (Van Camp *et al.*, 1994). By contrast, when mitochondrial Mn SOD was targeted to the chloroplasts of tobacco plants, protection was only observed against stress generated in the plasmalemma. An increase in the activity of APX, DHAR, and MDAR, other scavenging pathway enzymes, was also observed. It was concluded that the protection provided by overproduction of Mn SOD was dependent on whether the other enzymes, DHAR and MDAR, were or were not present in elevated levels (Slooten *et al.*, 1995). Localization of imposed stress as well as its type seems to play an important role in enhancing tolerance against oxidative stress.

Two alfalfa genotypes were transformed with *Nicotiana* Fe SOD (McKersie *et al.*, 1999, 2000) and the effect of the transformation on winter survival was observed. Although increased winter survival was observed, it could not be correlated with increased protection of the photosynthetic apparatus nor with increased ability to withstand primary freezing injury. It was proposed that Fe SOD is acting to protect oxidative stress-sensitive processes in the root, allowing superior winter survival (McKersie *et al.*, 1999, 2000; Samis *et al.*, 2002).

When tomato chloroplastic and cytosolic Cu-Zn SOD genes were transferred into potato plants, elevated levels of tolerance against the stress caused by methyl viologen was observed. A higher tolerance was observed in plants that carry the cytosolic Cu-Zn SOD gene compared to

plants expressing the chloroplastic Cu-Zn SOD gene (Perl *et al.*, 1993). By contrast, tobacco plants transformed with the Cu-Zn SOD gene exhibited an approximately 50-fold increase in Cu-Zn SOD expression with no increased tolerance (Tepperman and Dunsmuir, 1990). The authors suggest that this is due to increased formation of H₂O₂. When the overexpression results in a moderate increase in SOD activity, then it is more likely there will be tolerance against oxidative stress, since the equilibrium between O₂⁻ radicals and H₂O₂ is maintained (Perl *et al.*, 1993).

The effect of high light stress is inhibition of photosystem II through D1 protein, which is associated with PSII electron transduction reactions (Foyer and Mullineaux, 1994). Photoinhibition of PSII is not well understood. However, it has been shown that plants with elevated levels of SOD are tolerant to high light stress photoinhibition of PSII (Van Camp *et al.*, 1994).

When plants were treated with methyl viologen, it was observed that the plants that had Mn SOD expressed in their chloroplast had remarkable protection, accompanied by an increased SOD activity against methyl viologen stress compared to control plants in light (Bowler *et al.*, 1994). This protective effect was less observable in plants that were kept in the dark, since the H₂O₂ scavenging system is not activated without light (Foyer and Halliwell, 1976; Nakano and Asada, 1980). Even though Mn SOD is not inactivated by H₂O₂, the balance between generated O₂⁻ and H₂O₂ is disrupted in these transgenic plants, which may increase the formation of OH. Although increased protection was observed in plants that had foreign Mn SOD targeted into the mitochondria, this effect was not as remarkable compared to plants that had Mn SOD targeted into the chloroplasts. This may be due to the majority of the O₂ being generated in the chloroplast in plants exposed to methyl viologen in light (Bowler *et al.*, 1991). The effects of methyl viologen treatment in pea protoplasts were also investigated. The levels of total SOD activity remained unchanged for 9 h after methyl viologen treatment, but after 12 h a significant increase was observed. This increase was mainly in Mn SOD enzyme activity, whereas chloroplastic Cu-Zn SOD showed a decrease, and cytosolic Cu-Zn SOD activity remained constant. It was suggested that the decrease in chloroplastic SOD is caused by increased H₂O₂ formation as a result of increased SOD activity (Doullis *et al.*, 1998).

The effects of methyl viologen were investigated in *N. plumbaginifolia* (Tsang *et al.*, 1991). In these studies a significant increase was observed in mRNA levels for Mn, Fe and cytosolic Cu-Zn SODs in methyl viologen-treated plants that were kept in light. In the plants that were kept in the dark following the treatment, Mn and Fe SOD transcript levels did not change, but an increase in the amount of cytosolic Cu-Zn SOD mRNA was observed (Tsang *et al.*, 1991). The effect of methyl

viologen treatment was not investigated at the enzyme or protein level, however.

The effects of SO₂ on Cu-Zn SOD enzyme activity were investigated in two pea cultivars, cvs Progress and Nugget insensitive and sensitive, respectively, to SO₂ (Madamanchi *et al.*, 1994). After the treatment of these plants with SO₂, activities of both cytosolic and chloroplastic SODs increased in cv. Progress whereas the activities of both enzymes decreased in cv. Nugget. Chloroplastic Cu-Zn SOD mRNA levels decreased in cv. Nugget after the treatments, whereas in cv. Progress there was a recovery in the amount of transcripts present after an initial decrease (Madamanchi *et al.*, 1994).

Taken together, these results suggest that regulation of expression of plant Fe and Mn SODs may differ. The subcellular location of the protein also appears to play a role. In some instances, both chloroplast and cytosolic Cu-Zn SODs afford protection against the same stresses as Fe SOD. There appears, also to be specialization within the Fe SOD family. The upstream sequences of all *A. thaliana* SOD genes have been compared in order to determine similarities or differences among their respective regulatory regions.

Comparing the information in sod genomic sequences

With the complete sequencing of the *A. thaliana* genome (The *Arabidopsis* Genome Initiative, 2000), it is a

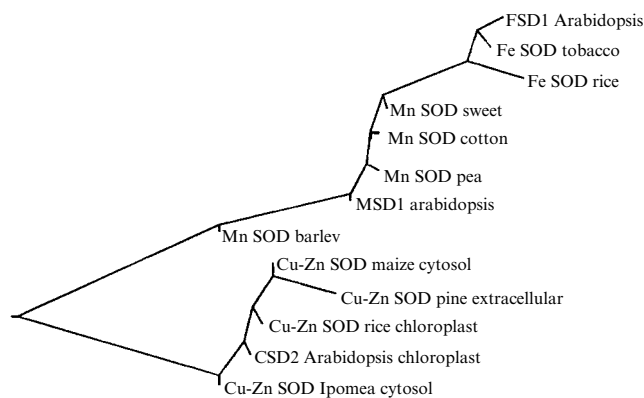


Fig. 4. Relatedness of SOD protein sequences in some plant cells.

propitious time to ask what the appropriate application of computation to that genome might reveal about the evolution and regulation of SODs in plants. The protein sequences of all seven *A. thaliana* SODs, plus the protein sequences of SODs from a variety of other plant species, were extracted from GenBank and aligned using the Megalign program in LaserGene. A subset of that alignment was put into a cladogram (a phylogenetic tree with branch lengths proportional to the computed quantity of sequence divergence) within Megalign to obtain Fig. 4. This cladogram clearly supports the hypotheses that the Fe SODs are of greatest antiquity and that the Cu-Zn SODs evolved independently of the Fe and Mn SODs. The evidence that the antiquity of the Mn SODs exceeds that of the Cu-Zn SODs is less convincing; however, a more refined computational study of plant and prokaryotic SODs, which are being planned under the authors bioinformatics research umbrella, should shed more light on this and related questions.

The upstream regions of the seven *A. thaliana* SOD open reading frames were extracted with the intention of mining them for promoter sequences related to the regulation of gene expression under different stresses. Bioinformatics tools were developed for searching an upstream region for particular promoter sequences that bind to known transcription factors. (The area of computational tools for analysing promoter sequences is in its infancy (Prestridge, 2000), and existing tools are limited in their abilities to address questions and to analyse an entire genome.) The ABA responsive element (ABRE) appears to be associated with genes responding to osmotic stress (high osmoticum, salt, desiccation, and cold) and binds to several similar sequences of eight nucleotides (Choi *et al.*, 2000; Guan and Scandalios, 1998); the consensus sequence YACGTGGC was used. NF-κB is a transcription factor that activates immunoglobulin-κ genes; the consensus sequence GGRNNYYCC was used (Smith *et al.*, 2000). The heat shock protein gene promoter consensus sequence is the palindromic sequence TTCNNGAA (Santos *et al.*, 1996). Finally, the Y-box motif has consensus sequence GATTGG and mediates redox-dependent transcription activation (Guan and Scandalios, 1998). These tools were used to search the seven promoter regions for these four consensus sequences. In Table 1, diamonds (◆) summarize the exact

Table 1. A comparison of the upstream regions of the seven SODs using the identification of sequences known to bind to four transcription factors

Transcription factors	CSD1	CSD2	CSD3	FSD1	FSD2	FSD3	MSD1
ABRE	◆					◆	
NF-κB	◆	◆				◆	◆
Heat shock element			◆		◆		◆
Y-box			◆				◆

or close matches found at plausible upstream locations (within 1000 nucleotides of the ATG where transcription begins). The different patterns of consensus sequences found suggest that the phenomenon of differential expression of the SOD genes under different stresses can be explained, at least in part, through promoter sequence analysis. It is planned to extract the upstream sequences for all known or putative *A. thaliana* genes and to compare these sequences on a genome-wide basis with new computational tools that are being developed. From these comparisons, it is hoped to identify the versions of each promoter sequence that are actually present in the *Arabidopsis* genome. Employing information about what genes are co-regulated, it is also hoped to identify what promoter sequence combinations correspond to what kind of regulation.

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