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Differential Regulation of Superoxide Dismutases in Plants Exposed to Environmental Stress

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Superoxide dismutases (SODs) are metalloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The enzyme is ubiquitous in aerobic organisms where it plays a major role in defense against oxygen radical-mediated toxicity. In plants, environmental adversity often leads to the increased generation of reduced oxygen species and, consequently, SOD has been proposed to be important in plant stress tolerance. Here we describe the isolation of a cDNA clone encoding a cytosolic copper/zinc SOD from *Nicotiana plumbaginifolia*. Using this, together with previously isolated cDNAs encoding the mitochondrial manganese SOD and the chloroplastic iron SOD as probes in RNA gel blot analyses, we have studied SOD transcript abundance during different stress conditions: in response to light, during photoinhibitory conditions (light combined with high or low temperatures), and in response to a xenobiotic stress imposed by the herbicide paraquat. Evidence is presented that iron SOD mRNA abundance increases whenever there is a chloroplast-localized oxidative stress, similar to the previous finding that manganese SOD responds to mitochondria-localized events. The diverse effects of the different stress conditions on SOD mRNA abundance thus might provide an insight into the way that each treatment affects the different subcellular compartments.

INTRODUCTION

Superoxide radicals ($O_2^{\cdot -}$) are toxic by-products of oxidative metabolism (Fridovich, 1986). Their toxicity has been attributed to their interaction with hydrogen peroxide to form highly reactive hydroxyl radicals (OH·) through the reaction:

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

These hydroxyl radicals are thought to be largely responsible for mediating oxygen toxicity in vivo: they can react indiscriminately with DNA, proteins, lipids, and almost any constituent of cells (for reviews, see Halliwell, 1987; Cadenas, 1989; Fridovich, 1989). The electron transport chains present within the different subcellular compartments are well-documented sources of reactive oxygen species (reviewed in Asada and Takahashi, 1987; Cadenas, 1989). For example, the oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, thus forming superoxide radicals (Asada and Takahashi, 1987). In addition to this, when absorbed light energy exceeds the capacity of the photosystems to direct it through photosynthetic electron transport, photosynthesis is inhibited (photoinhibition) and pigments and membranes become severely damaged (photooxidation) (for review, see Powles, 1984). This can arise during conditions of high light intensities and also when a temperature stress (chilling or heat) accompanies illumination. The damage that occurs during such conditions is thought to be mediated, at least in part, by oxygen radicals (Barényi and Krause, 1985; Wise and Naylor, 1987).

Both enzymic and nonenzymic mechanisms have evolved to overcome oxygen toxicity. These include antioxidants such as glutathione, ascorbate, and α-tocopherol; catalases and peroxidases, which remove H_2O_2; and superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide. Three classes of SOD have been identified, based on the metals present at the active site: copper/zinc (Cu/ZnSOD), iron (FeSOD), and manganese (MnSOD). The iron enzyme is present in prokaryotes and within the plastids of some plants. The MnSOD is widely distributed among prokaryotic and eukaryotic organisms, and in eukaryotes it is most often found in the mitochondrial matrix. These two enzymes are likely to have arisen from the same ancestral enzyme, whereas the Cu/ZnSOD has no sequence similarity to them and appears to have

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evolved separately in eukaryotes. It is normally present in the cytosol, and some plants contain also a chloroplastic isoform (for review, see Bannister et al., 1987).

Our previous studies on SOD have been performed using *Nicotiana plumbaginifolia*. We have been able to demonstrate the presence of all three types of SOD in this plant, and subcellular fractionations showed that MnSOD was present in the mitochondrial matrix (Bowler et al., 1989b) and that FeSOD was in the chloroplast stroma (Van Camp et al., 1990). Cu/ZnSOD activity appeared only to be present in the cytosol. cDNAs encoding the nuclear-encoded FeSOD and MnSOD have been isolated recently (Bowler et al., 1989a, 1989b; Van Camp et al., 1990), and we report here the isolation of a cDNA encoding the cytosolic Cu/ZnSOD. Using these three cDNAs as probes in RNA gel blots, we have examined the abundance of SOD transcripts in *N. plumbaginifolia* during different stress conditions. The results presented form a basis for elucidating the regulatory mechanisms controlling SOD abundance within the different subcellular compartments.

RESULTS

Isolation and Sequence Analysis of a cDNA Clone Encoding a Cytosolic Cu/ZnSOD from *N. plumbaginifolia*

To isolate cDNAs encoding Cu/ZnSOD, we designed an oligonucleotide probe based on a conserved region of known plant Cu/ZnSOD sequences (see Methods). This was used to screen a cDNA library derived from a *N. plumbaginifolia* cell suspension culture. A hybridizing clone that contained the largest insert was designated pSOD3 and was selected for further analysis.

The entire sequence of the 807-bp cDNA is shown in Figure 1. It contains an open reading frame of 456 nucleotides, corresponding to 152 amino acids. Although there is no in-frame stop codon upstream of the methionine codon at position 89, it is the first possible initiation codon within the primary gene transcript (D. Hérouart, unpublished results).

The deduced amino acid sequence has considerable homology to Cu/ZnSODs from other plants, as given in Figure 2 and Table 1. The figure shows that although chloroplastic and cytosolic Cu/ZnSOD sequences have much in common, they do have amino acid residues that distinguish them from each other. In this regard, the *N. plumbaginifolia* sequence clearly resembles cytosolic Cu/ZnSODs more than chloroplastic sequences (see Table 1), in agreement with our previous fractionation data (Van Camp et al., 1990). Nonetheless, the possibility remains that a chloroplastic Cu/ZnSOD isoform also exists in *N. plumbaginifolia* because its presence has been demonstrated in tomato, a close relative (Kwiatowski and Kaniuga, 1986).

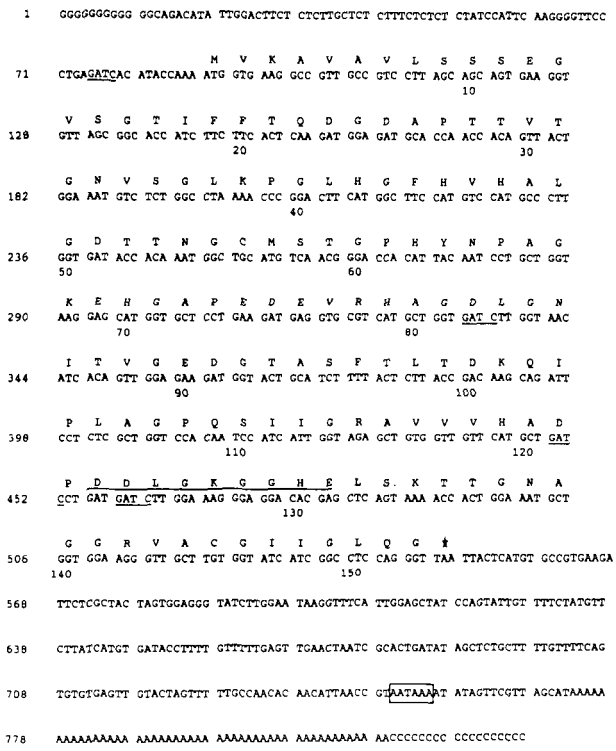

Figure 1. Sequence of a Cytosolic Cu/ZnSOD from *N. plumbaginifolia*.

The complete nucleotide sequence of the pSOD3 cDNA insert is shown, together with the G/C homopolymer tails added during the cloning procedure. The sequence homologous to the oligonucleotide mixture used as a probe is denoted by a line above the nucleotide sequence. The potential polyadenylation site AATAAA (position 750 to 755) is indicated by the box. The predicted coding sequence starting at position 89 has been translated into the corresponding amino acid sequence, written above the nucleotide sequence in the one-letter code. The positions of the Sau3A sites are underlined. The sequence has been transferred to the EMBL Data library as Accession No. X55974.

Light Effects on SOD mRNA Abundance in Green Leaves and in Etiolated Seedlings

To determine the effects of high photosynthetic activities on the levels of mRNAs encoding chloroplastic ($FeSOD_{chl}$), cytosolic ($Cu/ZnSOD_{cyt}$), and mitochondrial SOD ($MnSOD_{mit}$), we examined the effect of light on the abundance of transcripts present in green leaves of dark-adapted mature *N. plumbaginifolia* plants and in etiolated seedlings.

When mature plants grown in a 16-hr photoperiod are placed in the dark for 3 days, there is a significant reduction of SOD transcript levels when measured by RNA gel blot analysis: $FeSOD_{chl}$ is reduced approximately 30-fold,

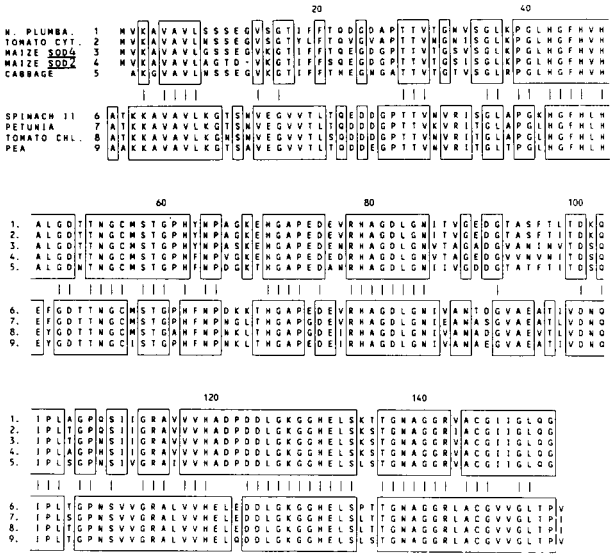

Figure 2. Comparison of the Cu/ZnSOD Sequence from *N. plumbaginifolia*.

The Cu/ZnSOD sequence from *N. plumbaginifolia* (row 1) is compared with the cytosolic Cu/ZnSODs from tomato (Perl-Treves et al., 1988) (row 2), maize *SOD4* (Cannon and Scandalios, 1989) (row 3), maize *SOD2* (Cannon et al., 1987) (row 4), and cabbage (Steffens et al., 1986) (row 5), and with the chloroplastic Cu/ZnSODs of spinach (Kitagawa et al., 1986) (row 6), petunia (Tepperman et al., 1988) (row 7), tomato (Perl-Treves et al., 1988) (row 8), and pea (Reed Scioli and Zilinskas, 1988) (row 9). For the chloroplastic Cu/ZnSODs, only the mature protein is shown. Two errors made in the original publication of the pea sequence have been corrected here (B. Zilinskas, personal communication). Residues conserved in each of the two types of Cu/ZnSODs are enclosed in boxes, and residues conserved in all nine sequences are indicated by vertical lines between the cytosolic and chloroplastic sequences.

$MnSOD_{mit}$ 12-fold, and $Cu/ZnSOD_{cyt}$ by twofold to threefold, as indicated in Figure 3A, lanes L and 0. However, a subsequent exposure to white light for 17 hr is sufficient to restore SOD mRNA to levels equivalent to or higher than those measured before the dark treatment. $FeSOD_{chl}$ mRNA levels increased rapidly upon exposure to light, and the maximal increase was approximately 30-fold. Increases in $MnSOD_{mit}$ mRNA lagged slightly behind and reached levels approximately 20-fold higher than were originally seen in dark-adapted material. $Cu/ZnSOD_{cyt}$ mRNA increased approximately 10-fold (Figure 3A). For comparative analysis the abundance of mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*), which is known to be strongly affected by light, is also shown (Figure 3A). As expected, *rbcS* transcript abundance also decreased during the dark period and was subsequently induced (approximately 50-fold) by light.

The observed increases in abundance of all these transcript families may reflect a general increase in transcription mediated by light. To test this, we analyzed the light responsiveness of a β-1,3-glucanase (De Loose et al., 1988). This enzyme has been shown to be highly regulated during development and is induced in response to pathogens (Castresana et al., 1990). The results in Figure 3A show that β-1,3-glucanase mRNA abundance is not strongly affected by light, demonstrating that the observed increases in SOD mRNA have at least some degree of specificity.

The small variations observed in β-1,3-glucanase mRNA in the different samples are consistent with that normally seen (Castresana et al., 1990) and are a reflection of mRNA abundance in different material (caused, for example, by slight differences in the age of the leaves) rather than loading artifacts.

Effects resulting from the exposure of etiolated seedlings to white light are shown in Figure 3B. Again, the $FeSOD_{chl}$ and *rbcS* mRNAs increase in response to the light stimulus, although $FeSOD_{chl}$ induction lagged behind that of *rbcS*: whereas increases in the levels of steady-state *rbcS* mRNA were detectable after 1 hr of light, $FeSOD_{chl}$ mRNA levels were only slightly higher after 5 hr. Nonetheless, after 24 hr, the increase in both mRNAs was comparable and was approximately the same as was found in dark-adapted green leaves exposed to light (i.e., 30-fold for $FeSOD_{chl}$ and 50-fold for *rbcS*). In spite of these effects, neither $MnSOD_{mit}$ nor $Cu/ZnSOD_{cyt}$ mRNA abundance was affected by light in etiolated seedlings (Figure 3B).

This contrasts with the observations made in dark-adapted mature leaves, particularly for the $MnSOD_{mit}$ mRNA, which increased approximately 20-fold in response to light in that material. The lack of any effect may be a consequence of the higher levels of $MnSOD_{mit}$ and $Cu/ZnSOD_{cyt}$ transcripts, which are already present in etiolated seedlings; the mRNAs of both are approximately 20-fold more abundant in etiolated material than they are in dark-adapted leaves.

$MnSOD_{mit}$ activity was previously found to parallel closely the activity of the respiratory electron transport chain of mitochondria, the chief site of superoxide formation within this organelle (Bowler et al., 1989a). The high levels of $MnSOD_{mit}$ mRNA in etiolated seedlings is presumably a reflection of the fact that such seedlings depend upon sugar breakdown as their source of energy and reducing power. Measurements of cytochrome oxidase activity confirmed that mitochondrial respiration was indeed higher in etiolated seedlings than in dark-adapted green leaves and the same trend was also found in $MnSOD_{mit}$ enzyme activity (data not shown). Hence, in etiolated seedlings, perhaps $MnSOD_{mit}$ activity may already be sufficiently high to obviate the need for more upon light exposure, whereas in green leaves the basal level of activity may not be sufficient to cope with the increased

Table 1. Pairwise Homologies (Expressed as Percent) between Different Cu/ZnSOD Sequences[a]

	Cytosolic				Chloroplastic			
Plant	Tomato	Maize *SOD4*	Maize *SOD2*	Cabbage	Spinach II	Petunia	Tomato	Pea
N. plumbaginifolia	93	87	83	81	65	65	63	61
Tomato cyt.		85	81	80	66	63	62	64
Maize *SOD4*			89	82	66	64	64	64
Maize *SOD2*				78	67	64	64	64
Cabbage					65	63	61	63
Spinach II						90	90	90
Petunia							93	90
Tomato chl.								90
Pea								

[a] Sequences are given in Figure 2.

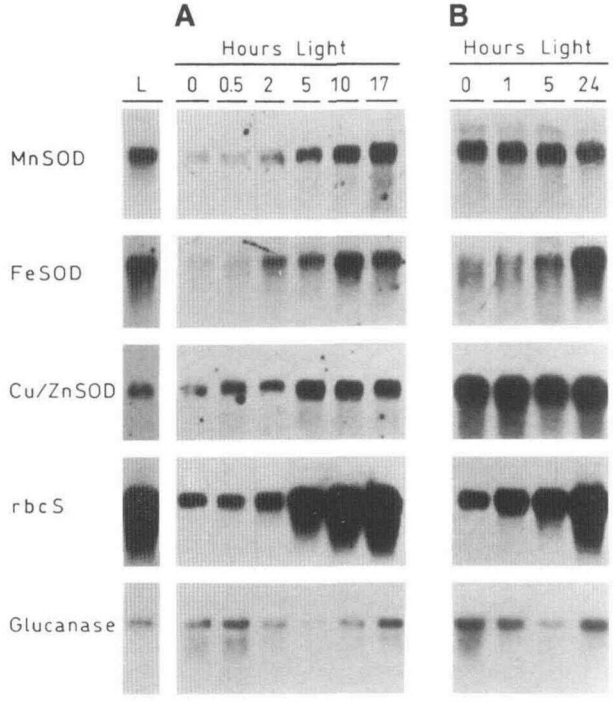

Figure 3. RNA Gel Blot Analysis Showing the Effect of White Light (Approximately 5000 Lux) on SOD, *rbcS*, and β-1,3-Glucanase mRNA Abundance in Leaves and Seedlings of *N. plumbaginifolia*.

(A) Three-day dark-adapted mature green leaves of *N. plumbaginifolia*.
(B) Seven-day-old etiolated seedlings of *N. plumbaginifolia*.
The times at which RNA samples were taken are indicated above each lane. Lane L shows the levels of the different transcripts in mature leaves derived from plants grown in a 16-hr photoperiod (the RNA sample was taken during the light period). The $FeSOD_{chl}$ mRNA isolated from etiolated seedlings consistently appears (see also Figure 4) as a diffuse band.

mitochondrial respiration arising from the breakdown of sugars created by enhanced photosynthetic activity.

Induction Mechanism of $FeSOD_{chl}$ mRNA Accumulation by Light

The effects of white light on SOD transcript abundance in both green leaves and etiolated seedlings reveal that $FeSOD_{chl}$ is the most strongly affected by this stimulus. Because this mRNA encodes the chloroplastic SOD, this is perhaps not surprising. Light effects in plants are often mediated by photoreceptors, particularly the red-/far red-absorbing phytochrome (for review, see Tobin and Silverthorne, 1985). In a classic phytochrome response, irradiation of plant material for a short time with red light can mimic the induction of gene expression by white light. To determine whether the effects of light on $FeSOD_{chl}$ mRNA abundance in etiolated seedlings were mediated by phytochrome, plant material was irradiated with red light and samples were taken at various times afterward for RNA analysis. The results shown in Figure 4A reveal that $FeSOD_{chl}$ mRNA levels are not affected by red light, contrasting with *rbcS* mRNA, which responds, albeit weakly, to red light (Figure 4A), as previously reported (Kaufman et al., 1984). From this we conclude that phytochrome is not a major component of the light-regulated accumulation of $FeSOD_{chl}$ mRNA.

The changes in photosynthetic activity that occur during a normal diurnal cycle also did not affect abundance of $FeSOD_{chl}$ (nor $MnSOD_{mit}$ nor $Cu/ZnSOD_{cyt}$) transcripts (data not shown). These observations, thus, appear to suggest that the changes in $FeSOD_{chl}$ mRNA abundance caused by light are not simply due to a light stimulus but rather may be a response to the increased stress placed upon the photosystems of dark-adapted cells by light irradiation. Because oxygen radicals are generated primarily by the leakage of electrons from photosystem I and

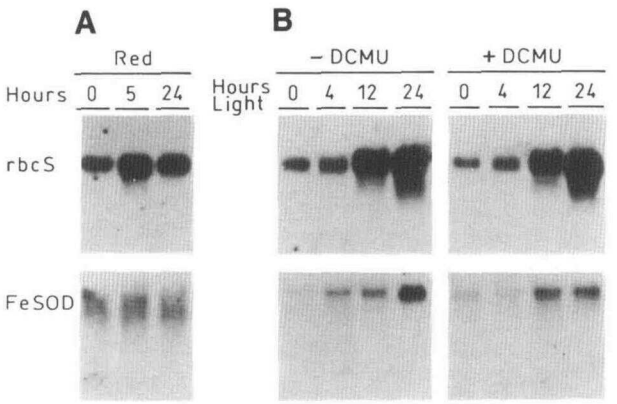

Figure 4. Induction Mechanism of $FeSOD_{chl}$ mRNA Accumulation by Light.

(A) Response of *rbcS* and $FeSOD_{chl}$ transcripts to red light in etiolated seedlings. Seven-day-old seedlings were irradiated with red light (20.13 μmol m^{-2} sec^{-1}) for 15 min. Samples were taken for RNA analysis at time 0, and 5 and 24 hr after irradiation.
(B) The effect of DCMU on the light-mediated changes in *rbcS* and $FeSOD_{chl}$ transcript abundance. Leaf discs that had been maintained in the dark for 3 days in the presence or absence of DCMU were exposed to white light, and RNA samples were taken at the times indicated. For further details, see Methods.

from ferredoxin (Asada and Takahashi, 1987), any block in photosynthetic electron transport in photosystem II would be expected to reduce oxyradical formation from photosynthesis. If oxidative stress is the mediator of $FeSOD_{chl}$ mRNA accumulation by light, then one would predict that such a blockage would reduce significantly the accumulation of $FeSOD_{chl}$ mRNA. We attempted to impose such a block using 3-(3,4-dichlorophenyl)-1,1′-dimethylurea (DCMU), which prevents the reoxidation of Q_A (the primary electron-accepting quinone of photosystem II) in the photosystem II reaction center and which has been shown to lower the amount of superoxide formed from photosynthetic electron transport (Asada et al., 1974). The results are shown in Figure 4B. Whereas *rbcS* mRNA levels in 3-day dark-adapted leaf discs increased in response to light to the same levels regardless of the presence or absence of DCMU (approximately 50-fold), the augmentation of $FeSOD_{chl}$ mRNA levels was reproducibly both retarded and reduced in the presence of DCMU. Hence, these results suggest that it is not light per se but rather oxidative stress arising from photosynthetic electron transport that modulates $FeSOD_{chl}$ gene expression. This might also explain the observed increase in $FeSOD_{chl}$ mRNA in 3-day dark-adapted plants when they are exposed to light (Figure 3B). In this case, the prolonged period of chloroplast inactivity in the dark probably results in a reduction in photosynthetic efficiency upon subsequent illumination because of the inadequate maintenance of the photosynthetic apparatus in the dark. Thus, electrons will be more likely to leak to oxygen and to generate superoxide, thus necessitating increased chloroplastic SOD activity.

Differential Regulation of SOD mRNA Abundance during Temperature Stress

To examine the effect of photoinhibitory conditions on the levels of SOD mRNAs, we subjected mature *N. plumbaginifolia* plants previously grown with a 16-hr photoperiod to high and low temperatures in combination with light. For comparison, we also studied the effect of temperature stress alone by maintaining the plants in the dark. These plants had been dark adapted for 3 days before the temperature stress. Thus, in these plants the levels of SOD mRNA were severalfold lower at the outset of the experiment than in the plants used for the light experiments (as shown in Figure 3A). These differences are not always apparent but these and subsequent results should be viewed with reference to Figure 3A (lanes L and 0).

Phenotypically, plants subjected to temperature stress in combination with illumination become wilted (after approximately 5 hr of heat shock and after 15 hr of chilling), whereas dark-maintained plants appear to remain healthy. However, these wilted plants regain normal appearance upon return to ambient temperature after 2 to 3 hr, and for this reason we also studied SOD mRNA abundance during this recovery period. A combination of chilling with light only increased $FeSOD_{chl}$ mRNA abundance (approximately fourfold), whereas $MnSOD_{mit}$ and $Cu/ZnSOD_{cyt}$ were unaffected, as shown in Figure 5A. However, during the subsequent recovery period, both these transcripts became more abundant: $Cu/ZnSOD_{cyt}$ mRNA increased by approximately 10-fold and $MnSOD_{mit}$ mRNA by approximately fivefold, whereas $FeSOD_{chl}$ mRNA abundance was less affected. In dark-maintained plants, only $Cu/ZnSOD_{cyt}$ was affected by chilling (Figure 5B): mRNA abundance increased by approximately 10-fold after 24 hr of chilling and remained at similar levels during the examined period of recovery.

The effects of heat shock combined with light and dark are shown in Figures 5C and 5D. Steady-state levels of $FeSOD_{chl}$ mRNA were not consistently affected during heat shock in combination with light, which contrasts with the effects of chilling (Figure 5A). This may suggest that the mechanism of photoinhibition caused by light in combination with either low-temperature or high-temperature stress is not the same, i.e., that oxyradicals generated in the chloroplasts may be responsible in part for the former but not the latter. Unfortunately, our knowledge of the processes involved is not yet sufficient to explain this observation. $MnSOD_{mit}$ mRNA also did not show any reproducible changes in these experiments, although $Cu/ZnSOD_{cyt}$ mRNA increased rapidly by up to 10-fold

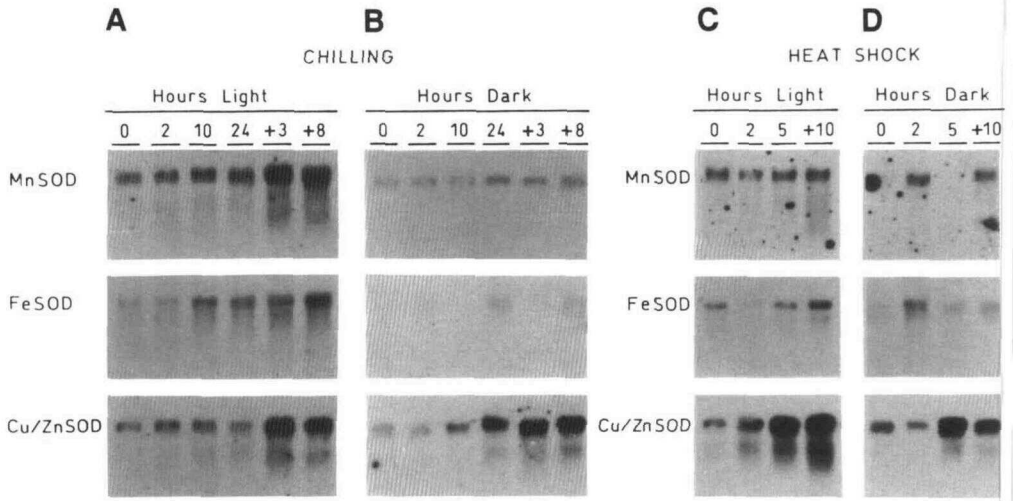

Figure 5. Changes in SOD mRNA Abundance during Temperature Stress in the Light and Dark.

(A) Effect of chilling in the light. Plants maintained in a normal 16-hr photoperiod were exposed to a temperature of 4°C in combination with continuous white light of approximately 5000 lux. The stress was initiated at the beginning of the normal light period and was maintained for 24 hr. Leaf samples were taken for RNA extraction at time 0, 2, 10, and 24 hr. After chilling treatment, plants were returned to normal temperatures with light, and additional samples taken after 3 and 8 hr.
(B) Effect of chilling in the dark. All experimental conditions were as described in **(A)**, except that 3-day dark-adapted plants were used and were maintained in darkness throughout the experiment.
(C) Heat shock effects in combination with light.
(D) Effect of heat shock in the dark. The source of plant material used in **(C)** and **(D)** was identical to that described in **(A)** and **(B)**, respectively. Plants were either maintained in continuous light (approximately 5000 lux) **(C)** or continuous darkness **(D)** and kept at 37°C for 5 hr. Samples were taken at time 0, 2, and 5 hr, and after 10 hr of recovery at normal temperatures.

during heat shock and remained high for at least 10 hr afterward, essentially independent of the light regime.

From the experiments involving a temperature stress, $Cu/ZnSOD_{cyt}$ appears to be the most responsive of the SOD mRNAs. It is the only one to be strongly induced by heat shock and during the recovery period after chilling stress. In both cases, the response is essentially independent of light. This implies that the cytosol may be the chief site of oxyradical formation under these conditions. In *Salmonella typhimurium*, many of the proteins induced by oxidative stress are also induced by heat shock (Morgan et al., 1986), leading to the proposal that heat shock may itself be a form of oxidative stress. This has been further supported by the finding that SOD was induced by heat shock in *Escherichia coli* (Privalle and Fridovich, 1987). Conversely, hydrogen peroxide has been found to induce the heat shock response in *Drosophila* (Compton and McCarthy, 1978; Becker et al., 1990) and to induce thermotolerance in *Neurospora crassa* (Kapoor et al., 1990). It is possible that in addition to eliminating excess superoxide, $Cu/ZnSOD_{cyt}$ raises cytoplasmic levels of H_2O_2 during heat shock, which can then activate the defense mechanism of the cell.

Induction of SOD by Paraquat

Bipyridyl herbicides such as paraquat are redox-active compounds that become reduced within the cell and subsequently transfer their electrons to oxygen, forming the superoxide anion (reviewed in Asada and Takahashi, 1987; Halliwell and Gutteridge, 1989). During illumination, it is known that the electrons are donated from photosystem I, ensuring that superoxide is formed primarily in the chloroplasts. Paraquat can also mediate toxic effects in the dark, albeit to a lesser extent; in this case, the major electron donor may be a microsomal NADPH-cytochrome P-450 reductase, as it is in animal cells (Halliwell and Gutteridge, 1989).

Plants sprayed with 5×10^{-5} M paraquat were maintained in the presence and absence of light, and leaf samples were taken at various time intervals. As in the chilling and heat shock experiments, the source material for light experiments was plants grown in a 16-hr photoperiod, whereas the plants used for the dark experiments had been adapted to the dark for 3 days before paraquat treatment. The results in Figure 6 reveal the changes in steady-state SOD mRNAs caused by light-dependent and

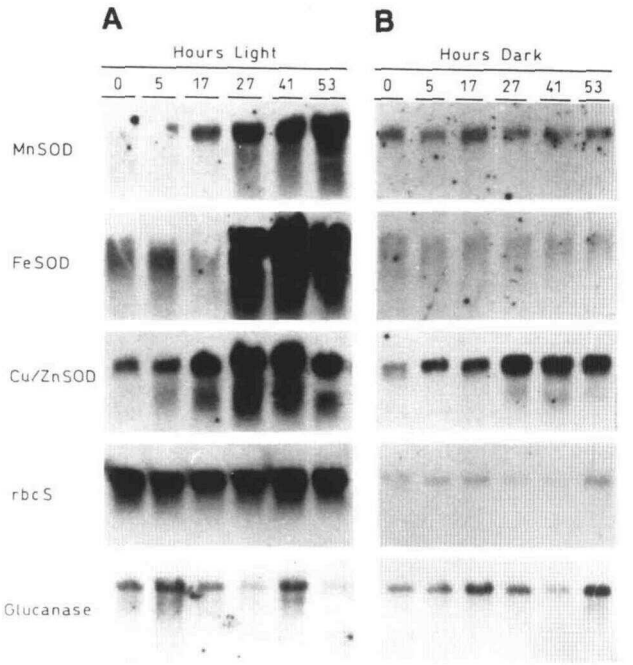

Figure 6. Effect of Paraquat on SOD, *rbcS*, and β-1,3-Glucanase mRNA Abundance.

(A) Light-mediated paraquat effects. Plants grown in a 16-hr photoperiod were sprayed with 5×10^{-5} M commercial paraquat (PROTEX, Deurne, Belgium) and illuminated constantly with white light (approximately 5000 lux). Samples were taken for RNA analysis at the times indicated. As seen earlier for RNA isolated from etiolated seedlings, $FeSOD_{chl}$ mRNA appears as a smear in contrast to $MnSOD_{mit}$, $Cu/ZnSOD_{cyt}$, *rbcS*, and β-1,3-glucanase mRNAs.

(B) Dark-mediated paraquat effects. Experimental conditions were as described in **(A)**, except that 3-day dark-adapted plants were used and were maintained in complete darkness during the course of the experiment.

dark-dependent paraquat toxicity. Clearly, $FeSOD_{chl}$ transcript abundance is increased greatly by paraquat in the light (approximately 40-fold), as are $MnSOD_{mit}$ and $Cu/ZnSOD_{cyt}$ mRNA (by 30-fold and 15-fold, respectively). The effects of paraquat in the dark are much less dramatic, presumably reflecting the unavailability of electrons from photosynthetic electron transport. In the absence of light, $Cu/ZnSOD_{cyt}$ mRNA increased approximately 12-fold, whereas $MnSOD_{mit}$ and $FeSOD_{chl}$ transcripts were unaffected.

Because all three transcript levels were increased in response to paraquat during illumination, we tested the specificity of this response by again hybridizing with *rbcS* and β-1,3-glucanase probes. In both the light and the dark, it is clear that their expression was unaffected by paraquat (Figure 6), suggesting that the SOD mRNA accumulated specifically during these conditions.

DISCUSSION

In this paper, we report the changes in SOD mRNA levels that occur under a variety of stress treatments. Although SOD enzyme activity was not measured, the diverse responses of the SOD mRNAs suggest that oxidative stress is an influencing component of environmental stress. Because the SOD mRNAs encode enzymes that are present in different compartments, the results also can be used to help understand how each stress may affect the different subcellular compartments. So what do these results tell us about the possible mechanism of SOD gene induction and to what extent are oxygen radicals directly involved in signaling these responses? The experiments with DCMU clearly implicate oxidative stress within the chloroplasts as a component in $FeSOD_{chl}$ gene induction, and this is further supported by the effects of paraquat which, in the presence of light, causes the formation of superoxide radicals preferentially within the chloroplasts. Hence, whereas the inhibition of superoxide formation by DCMU reduced the light-mediated $FeSOD_{chl}$ mRNA increases, the generation of superoxide by paraquat enhanced $FeSOD_{chl}$ mRNA levels. *rbcS* mRNA was unaffected by these treatments, revealing that the responses of $FeSOD_{chl}$ were not a general reflection of other photosynthetic components. This suggests that $FeSOD_{chl}$ gene expression is controlled by the oxidative stress itself and not as part of a global response for the induction of genes involved in photosynthesis.

Although the combined action of paraquat and light imposes an oxidative stress mainly upon the chloroplasts, it was found that both $MnSOD_{mit}$ and $Cu/ZnSOD_{cyt}$ mRNA abundance increased in addition to $FeSOD_{chl}$ (Figure 6). It is likely, therefore, that although superoxide is generated within a specific compartment, the ensuing damage can affect other compartments in the cell. This is not particularly surprising when one considers the reactivity of hydroxyl radicals and related oxygen species. What is of more interest, however, is that the formation of oxidative stress within the different compartments can by itself mediate changes in steady-state SOD mRNA levels. Previously we showed increases in $MnSOD_{mit}$ mRNA and enzyme activity when mitochondrial activity is stimulated (Bowler et al., 1989a), and although we inferred from this that the increased $MnSOD_{mit}$ activity was a response to the increased oxidative stress that subsequently occurs, we did not dissect the phenomenon of mitochondrial electron transport from mitochondrial oxidative stress. Thus, it was possible that $MnSOD_{mit}$ gene expression was simply coregulated with other genes involved in mitochondrial

respiration (as may be the case in yeast; Lowry and Zitomer, 1984) and that reactive oxygen species were not involved in any way in the induction mechanism. The observed increases in $MnSOD_{mit}$ mRNA abundance by the combination of paraquat and light reveal that this is not the case in plants because paraquat does not stimulate mitochondrial activity (data not shown). Therefore, although in many cases $FeSOD_{chl}$ and $MnSOD_{mit}$ mRNA accumulation closely correlates with the metabolic activity within their respective organelles, they do appear to be responsive to the ensuing oxidative stress itself and not to the general metabolic phenomena. In light of these precedents, we believe that $Cu/ZnSOD_{cyt}$ gene expression is likely to be controlled in a similar way.

Our results indicate that these three SODs can be used as molecular probes to study the oxidative stress that occurs specifically in plant cytoplasm, mitochondria, and chloroplasts as a result of environmental adversity. The diverse responses of the SOD mRNAs reported in this paper reveal that each condition produces its own unique effects, and further study of these should help to clarify the overall importance of oxidative stress phenomena in plants. In addition, these results can be used as a basis for elucidating the mechanisms by which such compartment-specific regulation is controlled. We are currently attempting to identify the signal molecules that mediate such fine-tuned specificity.

METHODS

Construction and Screening of the cDNA Library

The cDNA library from a *Nicotiana plumbaginifolia* cell suspension culture in plasmid pUC18 has been described elsewhere (De Loose et al., 1988). The oligonucleotide mixture 5′-GA$^{T}_{C}$GA$^{T}_{C}$CTIGGIAA$^{A}_{G}$GGIGGICA$^{T}_{C}$GA-3′ was designed with deoxyinosines at four of the eight ambiguous codon positions (Ohtsuka et al., 1985) to match a highly conserved sequence at the 3′ end of known plant $Cu/ZnSOD_{cyt}$ sequences (see Figures 1 and 2). The screening of the cDNA library was performed as previously described by Bowler et al. (1989a). Twelve clones from 5×10^4 originally screened showed positive signals, and the clone with the largest insert, designated pSOD3, was selected for further analysis.

Plant Material

Soil-grown *N. plumbaginifolia* var P2 plants (approximately 45 cm in height) were used to examine the effect of different stress treatments on SOD mRNA abundance in whole plants. These plants were maintained at 24°C in a 16-hr light/8-hr dark cycle. To evaluate the effects of white light, plants were first preadapted in the dark for 3 days before being exposed to light of approximately 5000 lux. The effects of heat shock, chilling, and paraquat treatment in combination with darkness were tested on 3-day dark-adapted plants, and for light experiments, the plants used had been maintained in a normal 16-hr photoperiod. In all cases, the stress was initiated at the beginning of the normal light period. For further information on experimental design, see text and individual legends to figures. All treatments were done at least in duplicate; samples were immediately frozen in liquid nitrogen and stored at −70°C until used. Samples from dark-maintained plants were taken using a dim green safety light.

For experiments with etiolated seedlings, *N. plumbaginifolia* seeds were germinated in the dark on Murashige and Skoog medium (Murashige and Skoog, 1962) for 7 days. Approximately 250 seeds were plated per 90-mm Petri dish, and three to four dishes were used for each RNA preparation. After irradiation with white or red light, the seedlings were uprooted with care and frozen in liquid nitrogen. Where appropriate, the seedlings were collected with the aid of a green safety light.

The effects of DCMU on light-regulated expression were tested using leaf discs from mature *N. plumbaginifolia* leaves. These were cut to an equal size (0.7 × 2.2 cm) and floated top-side-up in Petri dishes containing water. Before these were illuminated, they were dark adapted for 3 days. Approximately 12 hr before light irradiation, DCMU was added under a green safety light to a final concentration of 10 μM (from a 20 mM stock dissolved in ethanol). To the control material the equivalent amount of ethanol was added. During subsequent illumination, samples were taken at various times for RNA analysis. Approximately 15 leaf discs were used for each RNA extraction.

RNA Preparation and Analysis

Total RNA was prepared as described by Jones et al. (1985) and quantified spectrophotometrically. For RNA gel blot analysis, 12 μg was denatured in formaldehyde, electrophoresed, and transferred to nylon membranes according to Maniatis et al. (1982). To obtain highly specific single-stranded RNA probes, internal fragments from the cDNAs of pSOD1 ($MnSOD_{mit}$), pSOD2 ($FeSOD_{chl}$), and pSOD3 ($Cu/ZnSOD_{cyt}$) were recloned into selected sites of pGem2 (Promega). The 5′ HpaI-HindIII fragment from pSOD1 (Bowler et al., 1989a) was cloned into the SmaI-HindIII sites of pGem2, from pSOD2 a 5′ PstI-HindIII fragment (Van Camp et al., 1990) was cloned into the corresponding sites within the pGem2 polylinker, and from pSOD3 the 5′ Sau3A fragment (see Figure 1) was recloned into the dephosphorylated BamHI site of pGem2. Single-stranded, ^{32}P-labeled riboprobes were synthesized using T7 RNA polymerase (for $MnSOD_{mit}$ and $FeSOD_{chl}$) or SP6 RNA polymerase (for $Cu/ZnSOD_{cyt}$). Riboprobes specific for β-1,3-glucanase and the small subunit of *rbcS* were synthesized from pGem2-based plasmids, which were kind gifts of Fernanda de Carvalho (Castresana et al., 1990) and Chok-Fun Chui (Mazur and Chui, 1985). Each SOD probe specifically recognized a single band on RNA gel blots. However, it is currently not known whether this represents more than one transcript for a particular SOD. In some experiments, $FeSOD_{chl}$ mRNA appears as a smear, which indicates that $FeSOD_{chl}$ mRNA is rather unstable as compared with $MnSOD_{mit}$,$Cu/ZnSOD_{cyt}$, *rbcS*, and β-1,3-glucanase mRNAs. The three SOD probes did not cross-hybridize with any of the other SOD mRNAs, and any other cross-hybridizing bands occasionally seen on blots were not reproducible.

RNA gel blot hybridizations were performed overnight at 68°C in 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM

sodium phosphate, 1 mM EDTA, pH 7.4), 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 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, and twice with 1 × SSC, 0.5% SDS, at 68°C, and were autoradiographed on Kodak XAR-5 film with intensifying screens for 2 to 72 hr at −70°C. The relative amounts of mRNA in different samples were determined by densitometric scanning of the autoradiographs using an Ultrascan laser densitometer (LKB model 2202). These results were then compared with the values obtained by cutting hybridizing bands from the filters and determining the amount of bound radioactivity by scintillation counting.

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