Overexpression of Superoxide Dismutase Protects Plants from Oxidative Stress

Induction of Ascorbate Peroxidase in Superoxide Dismutase-Overexpressing Plants

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Photosynthesis of leaf discs from transgenic tobacco plants (Nicotiana tabacum) that express a chimeric gene that encodes chloroplast-localized Cu/Zn superoxide dismutase (SOD+) was protected from oxidative stress caused by exposure to high light intensity and low temperature. Under the same conditions, leaf discs of plants that did not express the pea SOD isoform (SOD−) had substantially lower photosynthetic rates. Young plants of both genotypes were more sensitive to oxidative stress than mature plants, but SOD+ plants retained higher photosynthetic rates than SOD− plants at all developmental stages tested. Not surprisingly, SOD+ plants had approximately 3-fold higher SOD specific activity than SOD− plants. However, SOD+ plants also exhibited a 3- to 4-fold increase in ascorbate peroxidase (APX) specific activity and had a corresponding increase in levels of APX mRNA. Dehydroascorbate reductase and glutathione reductase specific activities were the same in both SOD+ and SOD− plants. These results indicate that transgenic tobacco plants that overexpress pea Cu/Zn SOD II can compensate for the increased levels of SOD with increased expression of the H2O2-scavenging enzyme APX. Therefore, the enhancement of the active oxygen-scavenging system that leads to increased oxidative stress protection in SOD+ plants could result not only from increased SOD levels but from the combined increases in SOD and APX activity.

All aerobic organisms must possess the means to protect themselves from the toxic effects of reduced oxygen species. Oxidative damage occurs when the capacity of cellular antioxidant systems is overwhelmed by oxygen-centered radicals and other oxidants generated within the cell. In plants, environmental conditions such as extreme temperatures and/or water stress, especially in combination with high light intensities, ambient ozone, or sulfur dioxide, and some pathogens can cause oxidative stress damage by overproduction of toxic oxygen species (Bowler et al., 1992).

SOD (EC 1.15.1.1), the first enzyme in the detoxifying process, converts O2− radicals to H2O2. In plants, Cu/Zn SOD isoforms are found primarily in chloroplasts and in the cytosol, and Mn SODs are located primarily in mitochondria (Rabinowitch and Fridovich, 1983). In addition, peroxisomal localization of Mn SOD has been reported in pea (Sandalio et al., 1987). Tobacco (Nicotiana tabacum) plants also contain chloroplast-localized Fe SOD (Van Camp et al., 1990). In chloroplasts, H2O2 is reduced by APX (EC 1.11.1.11) using ascorbate as an electron donor. Oxidized ascorbate is then reduced by reactions that are catalyzed by monodehydroascorbate reductase, DHAR (EC 1.8.5.1), and GR (EC 1.6.4.2) in a series of reactions known as the Halliwell-Asada pathway (Bowler et al., 1992).

Analysis of transgenic plants that overexpress these putative protective enzymes should provide interesting insights into their relative contributions to oxidative stress tolerance. We have reported that leaf disc from transgenic tobacco plants that overexpressed chloroplast-localized pea Cu/Zn SOD had greater resistance to photooxidative damage and to methyl viologen-mediated oxidative stress than did control plants (Sen Gupta et al., 1993). Bowler et al. (1991) have demonstrated increased resistance to methyl viologen in transgenic tobacco plants that overexpressed mitochondrial Mn SOD and a modified, chloroplast-targeted Mn SOD, and Perl et al. (1993) have shown that transgenic potato plants that expressed tomato Cu/Zn SODs also have enhanced protection from methyl viologen toxicity. Alternatively, transgenic tobacco plants that overexpressed high levels of chloroplastic Cu/Zn SOD from Petunia hybrida did not have detectable increases in resistance to methyl viologen (Tepperman and Dunsuir, 1990) or ozone (Pitcher et al., 1991). These discrepancies in published reports seem to indicate that factors other than SOD overexpression may be involved in the establishment of increased oxidative stress resistance in transgenic plants.

Results reported here indicate that overexpression of chloroplast-localized Cu/Zn SOD in transgenic tobacco can lead to alterations in the expression of another protective enzyme, namely APX. We suggest that the combined increase in SOD

Abbreviations: APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; SOD, superoxide dismutase.

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and APX may be necessary for the increased stress protection observed in transgenic SOD plants.

**MATERIALS AND METHODS**

**Development of Transgenic Plants**

The development of transgenic tobacco plants that overexpress pea Cu/Zn SOD II was described by Sen Gupta et al. (1993). Briefly, a chimeric gene construct that contained a cDNA encoding the chloroplastic Cu/Zn SOD II subunit from pea (Ishii et al., 1990) was ligated into the NcoI and XbaI sites of the expression vector pRTL2, which places it between a cauliflower mosaic virus 35S promoter with a duplicated enhancer region and a cauliflower mosaic virus 35S termination and polyadenylation signal. The completed chimeric SOD gene cassette was transferred into the HindIII site of the binary shuttle vector pBIN 19 and mobilized to Agrobacterium tumefaciens (Nicotiana tabacum cv Xanthi) leaf discs were inoculated and approximately 20 kanamycin-resistant plants were regenerated essentially as described by Horsch et al. (1985). These plants were assayed for alterations in SOD isozyme profile, and two independently derived transgenic plants that expressed a SOD isoform that corresponded with pea Cu/Zn SOD II were selected for detailed analysis.

**Plant Materials**

The two selected primary transgenic plants that overexpressed pea Cu/Zn SOD were self-pollinated. Progeny (T2) were grown from seed in a growth room at 25°C and a photoperiod of 12 h at 150 μmol quanta m⁻² s⁻¹. Three- to 4-week-old seedlings were transplanted into pots and grown in the greenhouse (15-h days, 30°C day and 22°C night) with daily watering. These plants were screened for expression of the pea Cu/Zn SOD II isoform by the non-denaturing polyacrylamide gel assay method (Beauchamp and Fridovich, 1971). This analysis gave a 3:1 segregation ratio ($P = 0.06$ by $\chi^2$ analysis) of plants that overexpressed pea Cu/Zn SOD II (SOD⁺) to those that did not (SOD⁻) for both T2 populations, indicating the presence of single, functional transgene insertions in both lines. Leaf discs of SOD⁺ and SOD⁻ plants were collected from the first fully expanded leaves at the rosette stage (approximately 10 cm tall) and the bolting stage (30-40 cm tall) and from the fifth leaf of mature plants (approximately 125 cm tall) after the emergence of the first flower. Five SOD⁺ and five SOD⁻ plants were removed to determine Chl content (Amon, 1949). After centrifugation in a microcentrifuge, aliquots of the supernatant were removed to determine enzyme activity and protein concentration (Bradford, 1976). Lysed chloroplast extracts of known Chl concentration were also centrifuged, and the supernatant was used to determine enzyme activity and protein content. All extracts were prepared at 0 to 4°C, and enzyme assays were run at 25°C.

**Measurement of Photosynthesis**

Net photosynthesis was measured by $O_2$ evolution from leaf discs (3.6 cm diameter) with a Hansatech gas-phase $O_2$ electrode system (Hansatech Instruments Ltd., Pentney, King's Lynn, UK) under saturating $CO_2$ as described previously (Sen Gupta et al., 1993). Steady-state rates of photosynthesis for leaf discs of five SOD⁺ and five SOD⁻ plants were measured as the rate of $O_2$ evolution at 25°C and 975 μmol quanta m⁻² s⁻¹ before and after the stress treatments described above. Photosynthetic rates of nonstressed leaf discs of both genotypes did not change during the stress period.

**Enzyme Assays**

Two leaf discs from each designated leaf were used for all enzyme assays. Leaf tissue (0.3–0.5 g) was ground in liquid $N_2$, suspended in the appropriate homogenization solution, and rapidly homogenized in a glass tissue grinder. Aliquots were removed to determine Chl content (Arnon, 1949). After centrifugation in a microcentrifuge, aliquots of the supernatant were removed to determine enzyme activity and protein concentration. Enzyme assays were run at 25°C. The homogenization solution for SOD contained 50 mm KPO₄ (pH 7), 0.1 mm EDTA, and 1% (w/v) polyvinylpyrrolidone as described by Dhindsa et al. (1981). The SOD activity was measured spectrophotometrically as described by Beyer and Fridovich (1987). In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of nitroblue tetrazolium by 50%. The specific activity of SOD was expressed as units mg⁻¹ protein or units mg⁻¹ Chl. The homogenization solution for APX contained 50 mm Hepes (pH 7.0), 1 mm ascorbate, 1 mm EDTA, and 1% (v/v) Triton X-100. Enzyme activity was determined by measuring the oxidation of ascorbate at 290 nm in 1 mL of solution that contained 50 mm Hepes (pH 7.0), 1 mm EDTA, 1 mm $H_2O_2$, and 25 μL of enzyme extract. The specific activity of APX was expressed as μmol ascorbate oxidized h⁻¹ mg⁻¹ protein or μmol ascorbate oxidized h⁻¹ mg⁻¹ Chl. The extraction solution for GR was the same as that used for SOD. Enzyme
activity was determined by monitoring the oxidation of NADPH at 340 nm in 1 mL of solution that contained 0.1 M Tris-HCl, pH 7.8, 2 mM EDTA, 50 μM NADPH, 0.5 mM GSSG, and 25 μL of enzyme extract. The specific activity of GR was expressed as μmol NADPH oxidized h⁻¹ mg⁻¹ protein. DHAR was extracted by a method described by Jahnke et al. (1991) and assayed by following an increase in protein. DHAR was extracted by a method described by Nakano and Asada (1981). The specific activity of DHAR was expressed as μmol dehydroascorbate reduced h⁻¹ mg⁻¹ protein.

**Chloroplast Isolation**

Chloroplasts were isolated from leaves of SOD⁺ and SOD⁻ plants on a 40% Percoll gradient according to a method described by Berkowitz and Gibbs (1982). Ascorbate (1 mM) was added to the isolation medium and the chloroplast resuspension medium to ensure retention of APX activity. For enzyme analyses, isolated chloroplasts were suspended in the appropriate enzyme extraction solution and lysed by repeated freezing and thawing.

**Northern Blot Analysis**

Total RNA was prepared from leaves of SOD⁺ and SOD⁻ plants as described by Chomczynski and Sacchi (1987). Twenty-microgram samples were run on 1.2% agarose-18% formaldehyde gels and transferred to nitrocellulose membranes (Sambrook et al., 1989). Membranes were probed with a 32P-labeled APX cDNA from tobacco (R.P. Webb and R.D. Allen, unpublished data) This tobacco APX cDNA is homologous to an APX cDNA from pea reported by Mittler and Zilinskas (1991). Hybridizations were carried out in 50% formamide, 5X SET (1X SET is 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM EDTA), 10% dextran sulfate, and 0.5% SDS overnight at 42°C. After hybridization, filters were washed three times for 30 min each in 2X SET and 0.5% SDS at 60°C. Hybridization signals were detected by exposure to x-ray film (Hyperfilm, Amersham, Arlington Heights, IL) for 24 h at −80°C with an intensifying screen, and signal intensities were estimated from autoradiograms using a Molecular Dynamics scanning laser densitometer (Texas Tech University Institute for Biotechnology Core Facility).

**RESULTS**

**Increased Oxidative Stress Resistance in SOD-Overexpressing Plants**

Exposure of leaf discs from the first fully expanded leaf of bolting SOD⁻ tobacco plants to low temperature and high light intensity (1500 μmol quanta m⁻² s⁻¹, 3°C) caused a time-dependent reduction of steady-state photosynthesis subsequently measured at 25°C (Fig. 1). After 6 h of exposure, SOD⁻ leaf discs were unable to recover any of their initial photosynthetic capacity. Under the same conditions, leaf discs of bolting SOD⁺ plants retained the capacity to recover nearly full photosynthetic activity (94% of initial rate), even after a stress treatment of 6 h.

The extent of oxidative stress damage in leaf discs of both SOD⁺ and SOD⁻ plants was dependent on their develop-

![Figure 1. Steady-state photosynthetic rates (μmol O₂ m⁻² s⁻¹) at 25°C and 975 μmol quanta m⁻² s⁻¹ for leaf discs from bolting-stage SOD⁺ plants (■) and SOD⁻ plants (●) after exposure to 1500 μmol quanta m⁻² s⁻¹ and 3°C for the times indicated. Values are means ± sd; n = 5 for SOD⁺ and n = 10 for SOD⁻.](http://www.plantphysiol.org)
Table 1. Comparison of SOD and APX activities in SOD+ and SOD− plants

<table>
<thead>
<tr>
<th>SOD Specific Activity</th>
<th>APX Specific Activity</th>
<th>SOD/APX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosette</td>
<td>Rosette</td>
<td>Rosette</td>
</tr>
<tr>
<td>SOD+</td>
<td>85.6 ± 4.1</td>
<td>45.3 ± 3.3</td>
</tr>
<tr>
<td>SOD−</td>
<td>25.7 ± 2.3</td>
<td>17.0 ± 5.3</td>
</tr>
<tr>
<td>SOD*/SOD−</td>
<td>3.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table I. Comparison of SOD and APX activities in SOD+ and SOD− plants

Specific activities of SOD (units mg−1 protein) and APX (µmol ascorbate oxidized h−1 mg−1 protein) in whole-leaf extracts from tobacco plants that express pea Cu/Zn SOD (SOD+) and sibling plants that do not express pea Cu/Zn SOD (SOD−) at three developmental stages. Since stress treatments did not affect the specific activities of SOD or APX, only values for nonstressed samples are given. Values are means ± SD; n = 5 for SOD− and n = 10 for SOD+.
DHAR specific activity decreased by 14% in leaves of rosette-stage plants after stress treatment and by nearly 18% in flowering plants. Levels of GR specific activity in leaves increased by between 21 and 30% in response to stress treatment in all cases except for SOD+ plants at the flowering stage.

**DISCUSSION**

Progeny of transgenic tobacco plants that overexpress chloroplast-localized Cu/Zn SOD from pea segregated 3:1 for SOD overexpression to give SOD+ and SOD− populations. Levels of SOD specific activity were at least 3 times higher in SOD+ plants than in SOD− plants. Resistance to oxidative stress caused by exposure to high light and low temperature co-segregated with SOD overexpression. This tight linkage strongly indicates that the stress resistance is caused by SOD overexpression and not by random somaclonal variations in the transgenic plants or by selection during regeneration.

The increased levels of chloroplastic Cu/Zn SOD in our SOD+ plants might be expected to lead to higher production of H2O2, which, unless quickly removed, could react with remaining O$_2^-$ to form the highly toxic OH radical (Beyer et al., 1991). However, since overexpression of pea Cu/Zn SOD II in these plants clearly leads to increased protection from oxidative stress (Figs. 1 and 2; Sen Gupta et al., 1993), it is apparent that their H2O2-scavenging systems are sufficient. It seems likely that the proportional increase in APX activity that occurred in SOD+ plants plays an important role in facilitating the increased stress protection provided by SOD overexpression. Elevated levels of SOD and APX have been correlated with increased levels of oxidative stress resistance in several cases (Jansen et al., 1989; Jahnke et al., 1991), and pretreatment of plants with ethylene, which led to increased levels of APX, has been shown to provide increased protection from subsequent exposure to ozone or methyl viologen (Mehlhorn, 1990). These results support the hypothesis that SOD and APX play integral roles in the protection of plants from oxidative stress.

Since levels of APX or other enzymes were not given in previous reports on transgenic SOD-overexpressing plants (Tepperman and Dunsmuir, 1990; Bowler et al., 1991; Perl et al., 1993), it is impossible to determine at this time whether increased APX activity is associated with SOD overexpression and increased stress protection in all cases. Tepperman and Dunsmuir (1990) reported that levels of SOD overexpression in tobacco plants that contained a chimeric petunia Cu/Zn SOD gene that were at least 10 times higher (30- to 50-fold increase) than the 2- to 3-fold increase reported by other groups (Bowler et al., 1991; Perl et al., 1993; Sen Gupta et al., 1993). Since these plants failed to develop increased stress protection, it would be particularly interesting to examine whether the extremely high SOD activity in these plants outstripped the capacity of the native H2O2-scavenging systems to respond.

The complementary relationship between SOD and H2O2-scavenging enzymes is also important in other organisms (cf. Elroy-Stein et al., 1986; Mao et al., 1993). Kelner and Bagnell (1990) reported that human cell lines transfected with a Cu/Zn SOD overexpression construct had elevated levels of GSH.

### Table II. Activities of SOD and APX in chloroplasts of SOD+ and SOD− plants

<table>
<thead>
<tr>
<th>SOD Specific Activity</th>
<th>APX Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>SOD+</td>
<td>3258.3 ± 51</td>
</tr>
<tr>
<td>SOD−</td>
<td>976.8 ± 54</td>
</tr>
<tr>
<td>SOD+/SOD−</td>
<td>3:3</td>
</tr>
</tbody>
</table>

| SOD+ | NS | 12.4 ± 1.2 | 13.1 ± 0.8 | 13.3 ± 1.2 |
| SOD− | 5 | 10.8 ± 1.1a | 11.6 ± 0.9a | 10.9 ± 1.4a |
| SOD+ | NS | 12.5 ± 1.9 | 13.3 ± 0.5 | 14.3 ± 1.3 |
| SOD− | 5 | 10.8 ± 0.8a | 11.1 ± 1.0a | 11.5 ± 1.7a |

<table>
<thead>
<tr>
<th>GR Activity</th>
<th>Rosette</th>
<th>Bolting</th>
<th>Flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD+</td>
<td>6.8 ± 1.4</td>
<td>5.2 ± 1.3</td>
<td>6.6 ± 0.85</td>
</tr>
<tr>
<td>SOD−</td>
<td>8.2 ± 1.4a</td>
<td>7 ± 0.7a</td>
<td>6.5 ± 1.2</td>
</tr>
<tr>
<td>SOD+</td>
<td>6.8 ± 0.9</td>
<td>6.2 ± 0.5</td>
<td>6.2 ± 1.1</td>
</tr>
<tr>
<td>SOD−</td>
<td>8.2 ± 1.7a</td>
<td>7.5 ± 0.9a</td>
<td>7.8 ± 0.9a</td>
</tr>
</tbody>
</table>

*a Significant changes in mean specific activity due to stress treatment determined by t test (P < 0.005).
peroxidase as well as SOD. Cell lines with the greatest resistance to oxidative stress (methyl viologen) maintained SOD:GSH peroxidase ratios that were similar to nontransfected cells.

Although our SOD* plants maintained a ratio of SOD:APX specific activity that was essentially identical to that in SOD* plants, this ratio changed from 0.45 in rosette plants to approximately 0.75 in bolting plants (Table I). Since rosette plants of both genotypes were the most stress sensitive, this could indicate that a high SOD:APX ratio, combined with increased activity of these enzymes, is necessary for optimum stress resistance. However, attempts to assess the relative contributions of SOD and APX to stress protection are complicated by the age-associated changes in stress resistance. The increase in oxidative stress resistance that occurred during maturation of both SOD* and SOD* plants could be related to the 25 to 30% increase in SOD activity that occurred between rosette and bolting stages (Fig. 2). However, since stress resistance also increased in SOD* plants between the bolting and flowering stages while SOD activity remained stable, other maturation-related factors are likely to be involved. We believe that the dramatic developmental increase in stress protection in SOD* plants may reflect the synergistic effects of SOD overexpression with the maturation of other quenching systems that may include antenna pigments and pools of antioxidant compounds such as ascorbate, GSH, and tocopherols. Furthermore, since SOD overexpression can affect the expression of APX, it is possible that it could affect the development of these other protective mechanisms as well.

Increased levels of SOD and APX can clearly lead to enhanced oxidative stress protection in plants. Our results also indicate that, under the stress conditions used, levels of DHAR and GR activity are not limiting. These enzymes are certainly necessary to maintain the intact oxyradical-scavenging system, but since DHAR and GR levels were the same in SOD* and SOD* plants, it is apparent that increased levels of these enzymes are not necessary for the increased oxidative stress protection observed. Rao and Alschier (1991) have shown that SO2-tolerant pea cultivars have higher levels of GR than SO2-sensitive peas. However, transgenic tobacco plants that overexpressed bacterial GR were not more resistant to methyl viologen (Foyer et al., 1991) or ozone exposure (Aono et al., 1991), although, in the latter case, a qualitative improvement in methyl viologen resistance was noted. Interestingly, GR was the only enzyme in our analyses that responded positively to the oxidative stress treatment (Table II). This response occurred in all samples except SOD* plants at the flowering stage. We interpret these results to indicate that, under the conditions used, these highly resistant flowering plants did not experience sufficient stress to induce GR activity.

We do not know the mechanism by which APX activity and mRNAs are induced in transgenic SOD-overexpressing plants. We speculate that APX gene expression could be upregulated in these plants as a direct or indirect response to a constitutive increase in H2O2 putatively associated with SOD overexpression, but this relationship has not been demonstrated. We are hopeful that further analysis of these plants will provide insights into the regulatory relationships between SOD and APX gene expression and the role of these enzymes in the establishment of oxidative stress tolerance.

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Overexpression of Superoxide Dismutase


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