Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1629–1633, February 1993
Plant Biology

Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase

(tobacco/photoinhibition/methyl viologen)

ASHIMA SEN GUPTA*, JEANIE L. HEINEN*, A. SCOTT HOLADAY*, JOHN J. BURKE†, AND RANDY D. ALLEN*‡§

Departments of *Biological Sciences and Agronomy, ‡Horticulture and Entomology, Texas Tech University, Lubbock, TX 79409; and †Department of Agriculture–Agricultural Research Service Cropping Systems Research Laboratory, Route 3, Box 215, Lubbock, TX 79401

Communicated by Joseph E. Varner, November 25, 1992

ABSTRACT **Transgenic tobacco plants that express a chimeric gene that encodes chloroplast-localized Cu/Zn superoxide dismutase (SOD) from pea have been developed. To investigate whether increased expression of chloroplast-targeted SOD could alter the resistance of photosynthesis to environmental stress, these plants were subjected to chilling temperatures and moderate (500 μmol of quanta per m^2 per s) or high (1500 μmol of quanta per m^2 per s) light intensity. During exposure to moderate stress, transgenic SOD plants retained rates of photosynthesis ≈20% higher than untransformed tobacco plants, implicating active oxygen species in the reduction of photosynthesis during chilling. Unlike untransformed plants, transgenic SOD plants were capable of maintaining nearly 90% of their photosynthetic capacity (determined by their photosynthetic rates at 25°C) following exposure to chilling at high light intensity for 4 hr. These plants also showed reduced levels of light-mediated cellular damage from the superoxide-generating herbicide methyl viologen. These results demonstrate that SOD is a critical component of the active-oxygen-scavenging system of plant chloroplasts and indicate that modification of SOD expression in transgenic plants can improve plant stress tolerance.**

Oxygen is essential for the existence of aerobic life, but toxic oxygen species, which include the oxygen-centered superoxide (O_2^-), and hydroxyl (·OH), free radicals, as well as hydrogen peroxide (H_2O_2), are generated in all aerobic cells. Injury caused by these oxygen derivatives is known as oxidative stress (see refs. 1–3 for reviews). Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) constitutes the first line of cellular defense against oxidative stress.

Oxidative stress is a major damaging factor in plants exposed to environmental stress, and there is strong evidence for the protective role of SOD in plants (see refs. 4 and 5 for reviews). Most plants contain a number of SOD isozymes that are located in various cellular compartments. In pea, Cu/Zn-containing SOD isoforms are found in chloroplasts and in the cytosol, whereas a Mn-containing enzyme is located in mitochondria (6). Tobacco has a more complex complement of SODs that includes at least five distinct isozymes (7, 8). Most notably, tobacco includes a distinct Fe-containing SOD in chloroplasts, as well as chloroplastic and cytosolic Cu/Zn SODs and mitochondrial Mn SOD.

Superoxide radicals are produced continuously in plant chloroplasts as O_2 is reduced to O_2^- by electrons from the photosystems (the Mehler reaction) (4, 5). SOD converts O_2^- to H_2O_2 that is then scavenged in chloroplasts by a series of oxidation/reduction reactions, known as the Halliwell–Asada pathway, that use ascorbate, reduced glutathione, and NADPH as electron donors (4).

The inhibition of photosynthesis that can occur when excess excitation energy reaches the reaction center is commonly referred to as photoinhibition. High light intensity, especially at extreme temperatures or water deficit, can cause increased electron flow to O_2, resulting in greater production of O_2^- and H_2O_2. Although oxygen radicals appear to be involved in photoinhibition (9–11), the role of SOD in limiting the oxidative damage associated with photoinhibition has not been directly demonstrated (12, 13).

To investigate the possible protective functions of SOD in plant chloroplasts, we have developed transgenic tobacco plants that overexpress chloroplast-localized Cu/Zn SOD. These plants were analyzed for photosynthetic rate when exposed to light and temperature conditions that inhibit photosynthesis and for their ability to recover photosynthetic capacity after stress. Our results indicate that these transgenic plants have improved photosynthetic function at chilling temperatures and moderate light intensity, and they recover more effectively from severe stress than control plants. These changes correlate with increased resistance to oxidative damage caused by the herbicide methyl viologen (MV).

MATERIALS AND METHODS

Plant Transformation. Chimeric gene constructs were developed to overexpress chloroplastic SOD subunit in plant cells (Fig. 1). Chloroplastic Cu/Zn SOD cDNA from pea (14, 15) was amplified by polymerase chain reaction using mutagenic primers that introduced an *Nco* I site at the translation start codon (ACATGG to CCATGG) and an *Xba* I site within the 3′ untranslated sequence. After digestion with *Nco* I and *Xba* I, this fragment was ligated into the *Nco* I and *Xba* I sites of the expression vector pRTL2 (a gift from J. R. Carrington, Department of Biology, Texas A&M University). This vector includes a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer and a CaMV 35S terminator sequence. The cDNA insert was fused at the translation initiation codon within the 5′ untranslated sequence of the tobacco etch virus (TEV) that provides highly efficient translational initiation. The completed chimeric gene cassette was excised with *Hin*dIII and ligated into the *Hin*dIII site of the binary shuttle vector pBIN 19 (16) and mobilized to *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating. Transformation of tobacco leaf disks was performed according to Horsch *et al.* (17), and >20 putative transgenic plants were regenerated.

SOD Isozyme Analyses. Transgenic plants that expressed pea chloroplastic Cu/Zn SOD were identified by analysis of SOD isozymes in leaves, using a method described by Beauchamp and Fridovich (18) as modified by Bowler *et al.* (8). Leaf extracts from transgenic tobacco plants, untrans-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SOD, superoxide dismutase; MV, methyl viologen.
§To whom reprint requests should be addressed.

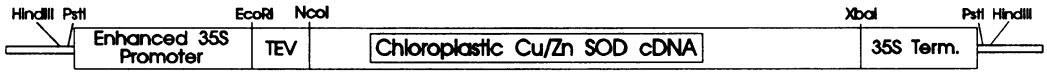

FIG. 1. Chimeric gene construct developed to overexpress pea chloroplastic SOD subunit in plant cells. This gene cassette was inserted into the binary plant transformation vector pBIN 19 and introduced into plant cells by *Agrobacterium*-based transformation. See text for further description.

formed tobacco plants (Xanthi), and pea plants were electrophoresed in nondenaturing polyacrylamide gels and negatively stained for SOD activity. Intensities of pea Cu/Zn SOD bands and native tobacco SOD bands on activity gels were estimated with a Molecular Dynamics scanning laser densitometer system (provided by the Texas Tech University Biotechnology Institute). Analysis of SOD isozymes extracted from crude chloroplast preparations of leaves of transgenic tobacco plants was also performed to determine whether the transgene product was correctly targeted to tobacco chloroplasts. Briefly, 1 g of leaf tissue was ground gently in 10-ml aliquots of 20 mM Hepes, pH 7/0.4 M sorbitol/2 mM $MgCl_2$ and the slurry was filtered through a 20-μm mesh to remove unbroken cells. Chloroplasts were pelleted by centrifugation at 200 × *g* and washed in the same buffer. Extracts were analyzed by SOD activity gel as described above. Two independently transformed plants that expressed the introduced gene at highest levels were self-pollinated, and the offspring of these plants (hereafter referred to as transgenic SOD plants) were grown in a greenhouse and used for our analyses.

Analysis of Photoinhibition. Net photosynthetic rates were measured for transgenic SOD plants and untransformed plants (Xanthi) by measuring O_2 evolution from 10-cm^2 leaf disks with a gas-phase, O_2 electrode system (Hansatech Instruments, Pentney, King's Lynn, U.K.) under saturating CO_2. Leaf disks were subjected independently to either moderate or severe stress regimes. Control leaf disks of each genotype were maintained at 25°C and the light intensity of the stress treatments for the duration of the experiments. To ensure that measurement of O_2 evolution accurately represented photosynthesis, rates of $^{14}CO_2$ fixation were also determined. Since both measurements correlated closely in all cases, only the O_2 evolution data are presented.

Moderate stress treatment. Leaf disks from transgenic SOD plants and Xanthi plants were equilibrated at 500 μmol of quanta per m^2 per s and 25°C for 1 hr, after which photosynthetic rates were determined at 470 μmol of quanta per m^2 per s and 25°C. Disks were then chilled to 10°C at 500 μmol of quanta per m^2 per s on a cooling block. Leaf disks were removed at designated intervals and photosynthetic rates were measured at 470 μmol of quanta per m^2 per s and 10°C.

Severe stress treatment. Leaf disks were equilibrated at 1500 μmol of quanta per m^2 per s and 25°C for 1 hr. Photosynthetic rates were determined at 975 μmol of quanta per m^2 per s and 25°C. The disks were then rapidly cooled to 3°C at 1500 μmol of quanta per m^2 per s and kept under these conditions for 4 hr. After this treatment, photosynthetic rates measured at 975 μmol of quanta per m^2 per s and 3°C were <1 μmol of O_2 per m^2 per s for all leaf disks. Leaf disks were then quickly warmed to 25°C, and O_2 evolution was monitored at 975 μmol of quanta per m^2 per s continuously until steady-state photosynthetic rates were reached (within 30 min for both transgenic SOD and Xanthi leaf disks).

MV Treatment. MV damage was analyzed as described by Bowler *et al.* (8) with modifications. Leaf disks (1.5 cm^2) collected from transgenic SOD plants and untransformed Xanthi plants were transferred to 3.5-cm Petri dishes containing 3 ml of MV solutions at various concentrations. Samples were vacuum infiltrated for 5 min and incubated at 21°C for 16 hr in darkness. Leaf disks were then illuminated (500 μmol of quanta per m^2 per s) for 2 hr, then incubated in darkness at 30°C for an additional 16 hr.

Cell leakage analysis. The conductivity of the decanted MV solution was measured with an Orion model 120 conductivity meter. The MV solutions were recovered from the conductivity meter cell and autoclaved, with the damaged leaf disks, for 15 min to release all solutes. The conductivity of the MV solution was again determined and the percentage of electrolyte leakage attributable to MV treatment was determined by dividing the conductivity value of the test sample by the conductivity of the sample after autoclaving (100% electrolyte leakage).

Pheophytin measurements. Pheophytin is a breakdown product of chlorophyll that results from the loss of the Mg moiety. After MV treatment, pigments were extracted from leaf disks in 80% acetone and the percentage of chlorophyll converted to pheophytin was determined by an increase in absorbance at 553 nm relative to absorbance at 665 nm (8, 19).

RESULTS

Analysis of SOD isoforms from leaves of two transgenic SOD plants that accumulated significant amounts of pea chloroplastic Cu/Zn SOD is shown in Fig. 2. Densitometric analysis of SOD activity gels indicated that transgenic SOD plants accumulated the pea SOD isoform to levels ≈2-fold higher than that of endogenous chloroplastic Fe SOD or Cu/Zn SODs in untransformed tobacco plants. Notably, native Cu/Zn SOD isoforms were not detectable in any transgenic SOD plants that expressed detectable amounts of pea chloroplastic Cu/Zn SOD, indicating that expression of the introduced Cu/Zn SOD gene somehow interfered with the activity of native tobacco Cu/Zn SOD isoforms. Pea Cu/Zn SOD was detected in chloroplasts isolated from transgenic plants (Fig. 2), indicating that this enzyme was incorporated into tobacco chloroplasts.

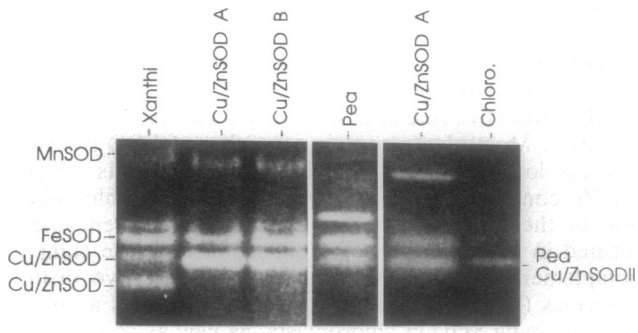

FIG. 2. Samples (25 μg) of total protein from leaf extracts from Cu/Zn SOD transgenic tobacco plants, untransformed tobacco plants (Xanthi), and pea plants were electrophoresed in nondenaturing polyacrylamide SOD activity gels. Native tobacco isoforms are indicated at left. Mn SOD is located in mitochondria. Fe SOD is found in chloroplasts, as is the high-mobility Cu/Zn SOD isoform (bottom band). The other (slower) Cu/Zn SOD isoform is found in the cytosol. Pea isoforms include (from top to bottom) mitochondrial Mn SOD, cytosolic Cu/Zn SOD I, and chloroplastic Cu/Zn SOD II. Note that the native tobacco chloroplastic Cu/Zn SOD isoform was not detectable in the extracts of transgenic leaves. Chloroplasts isolated from transgenic SOD plants (Chloro.) contain bands that correlate with native Fe SOD and pea Cu/Zn SOD II.

Photosynthetic rates determined by O_2 evolution from leaf disks of Xanthi plants and transgenic SOD plants before photoinhibitory treatments were virtually identical, averaging 35 μmol of O_2 per m^2 per s (Fig. 3). When leaf disks of Xanthi plants were exposed to moderate stress (10°C, 500 μmol of quanta per m^2 per s), their photosynthetic rates decreased by ≈95% to a mean of 1.75 μmol of O_2 per m^2 per s. However, under identical conditions, photosynthetic rates of transgenic SOD leaf disks were reduced by only 75%, to a mean of 8.75 μmol of O_2 per m^2 per s. Although reduced photosynthesis was observed in leaf disks of both genotypes during moderate stress conditions, leaf disks of transgenic SOD plants exhibited higher photosynthetic capacity than Xanthi leaf disks. It should be noted that net photosynthetic rates of both Xanthi and transgenic SOD leaf disks rapidly recovered after moderate stress exposure when they were warmed to 25°C and maintained at 50 μmol of quanta per m^2 per s for 30 min (Fig. 3). This indicates that, under these moderate stress conditions, little if any long-term oxidative damage had occurred in leaf disks of either genotype.

Since complete recovery of photosynthesis was observed in leaf disks of both Xanthi and transgenic SOD plants after moderate stress, increased stress levels were necessary to determine whether differences existed in their capacity to cope with and to recover from more severe stress. After exposure to 1500 μmol of quanta per m^2 per s and 3°C for 4 hr, photosynthetic rates for both Xanthi and transgenic SOD leaf disks were <1 μmol of O_2 per m^2 per s. When warmed to 25°C and measured at 975 μmol of quanta per m^2 per s, photosynthesis in leaf disks from Xanthi plants recovered, within 30 min, to a mean steady-state rate of 12 μmol of O_2 per m^2 per s (Fig. 4). Thus, after severe stress, the photosynthetic capacity of Xanthi leaf disks was only 36% of that measured before stress treatment, indicating that substantial oxidative damage that could not be rapidly reversed had occurred. Under the same conditions, photosynthetic rates of leaf disks from transgenic SOD plants recovered to a mean steady-state rate of 31 μmol of O_2 per m^2 per s. Hence, transgenic SOD leaf disks retained photosynthetic capacity after severe stress that was nearly 90% of that before stress exposure, indicating that little oxidative damage had occurred. Although leaf disks of both Xanthi and transgenic SOD plants exhibited complete inhibition of photosynthesis during exposure to severe stress conditions, most of this inhibition was rapidly reversed in transgenic SOD leaf disks,

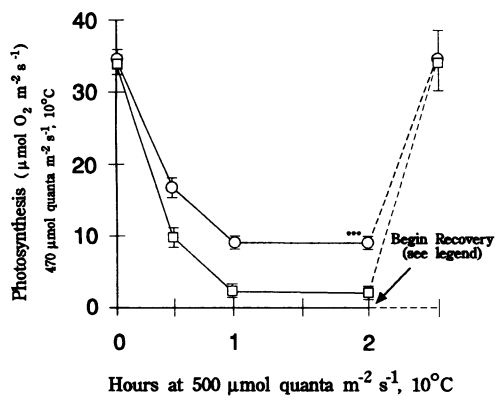

FIG. 3. Net photosynthetic rates were measured for transgenic SOD plants (○) and untransformed Xanthi plants (□) during moderate photoinhibitory stress (500 μmol of quanta per m^2 per s and 10°C). After stress treatment, leaf disks were allowed to recover for 30 min at 25°C and 50 μmol of quanta per m^2 per s. Photosynthetic rates for control leaf disks of each genotype maintained at 25°C and 500 μmol of quanta per m^2 per s did not change for the duration of the experiment. Values are means ± SD (n = 10–12 plants of each genotype). ***, P = 0.001.

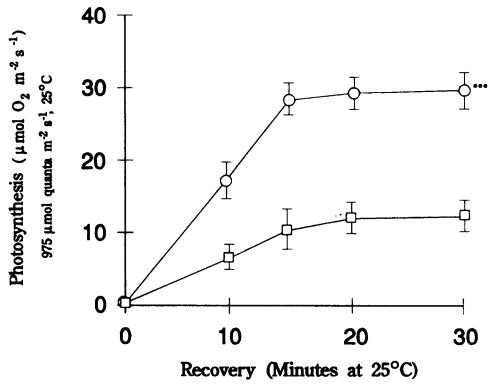

FIG. 4. Net photosynthetic rates were measured for transgenic SOD plants (○) and untransformed Xanthi plants (□) at 25°C after exposure to severe photoinhibitory stress (1500 μmol of quanta per m^2 per s, 3°C for 4 hr). Photosynthetic rates for control leaf disks of each genotype maintained at 25°C and 1500 μmol of quanta per m^2 per s did not change for the duration of the experiment. Values are means ± SD (n = 10–12 plants of each genotype). ***, P = 0.001.

indicating that they had suffered substantially less damage than Xanthi leaf disks. These results demonstrate that significant reduction in the levels of photoinhibitory stress can be achieved in leaves of transgenic tobacco plants that overexpress pea chloroplast Cu/Zn SOD.

To directly correlate the enhanced photosynthetic performance of transgenic SOD leaves under chilling and high light intensity with increased resistance to oxidative stress, leaf disks from transgenic SOD and Xanthi plants were treated with MV, a contact herbicide that causes massive, light-mediated accumulation of O_2^- in photosynthetic tissues (20) (Fig. 5). The extent of cellular damage was quantified by solute leakage, which is a measure of membrane disruption (8). Leaf disks of Xanthi plants showed a dose-dependent increase in membrane damage, reaching nearly complete disruption (≈90% of maximum solute leakage) at 2.4 μM MV. Tissues of transgenic SOD plants showed significantly less damage at 0.6 and 1.2 μM MV than Xanthi tissues. However, at 2.4 μM MV the extent of damage in transgenic leaf disks was not significantly different from that of control samples. These observations were extended by analysis of pheophytin production in MV-treated leaf disks as a measure of chlorophyll damage (Fig. 6). Chlorophyll damage in transgenic SOD leaf disks at 1.2 μM MV was reduced by an average of 40% compared with Xanthi leaf disks, but at 2.4 μM MV, much less difference was seen. These results confirm that transgenic SOD plants exhibit increased resistance to MV-

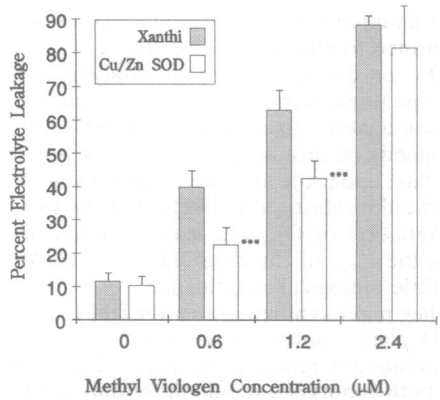

FIG. 5. Analysis of cellular damage in MV-treated leaf disks of transgenic SOD and untransformed Xanthi plants by measurement of percent solute leakage in treated tissues compared with autoclaved tissues. Values are means ± SD (n = 9 plants of each genotype). ***, P = 0.001.

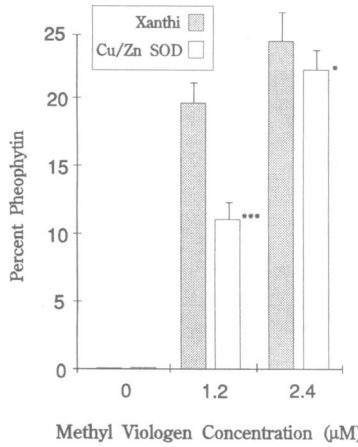

FIG. 6. Analysis of oxidative damage in MV-treated leaf disks from transgenic SOD plants and Xanthi plants by measurement of the conversion of chlorophyll to pheophytin. Values are means ± SD (n = 5 plants of each genotype). ***, P = 0.001; *, P = 0.05.

mediated oxidative damage, although significant differences in MV tolerance between transgenic SOD plants and Xanthi plants could be detected only over a relatively narrow range of MV concentrations.

DISCUSSION

The results presented clearly indicate that overexpression of pea chloroplast Cu/Zn SOD in tobacco leaves can improve their photosynthetic performance under moderate stress and maintain photosynthetic capacity after severe oxidative stress. We interpret these results to indicate that, under stressful conditions, transgenic SOD tissues have lower levels of O_2^- than control tissues, leading to higher net rates of photosynthesis and reduced oxidative damage. Since it is unlikely that pea Cu/Zn SOD is inherently superior to tobacco chloroplast SOD isoforms, we believe that the difference in stress tolerance in transgenic SOD plants is directly related to increased levels of active SOD in their chloroplasts. These results do not necessarily indicate that O_2^- causes chloroplast damage directly. Rather, rapid dismutation of O_2^- in transgenic SOD plants could prevent its reaction with H_2O_2 to form highly reactive ·OH (8, 21). Increased levels of SOD and other oxidative stress response enzymes have been correlated with reduced photoinhibition in MV-resistant varieties of *Conyza bonariensis* (9) and the addition of exogenous SOD or catalase to thylakoids has also been shown to reduce photoinhibition (22). Our results show that direct manipulation of SOD gene expression alone can effect tolerance to photoinhibitory stress.

Since O_2^- can dismutate without catalysis at a relatively high rate, one might question the need for increased SOD in plants. Spontaneous dismutation is highly pH-dependent, since it depends on protonation of O_2^- to $HO_2\cdot$ with a pK_a of 4.8 (23). Thus, under the alkaline conditions of the stroma of illuminated chloroplasts, it is likely that the rate of uncatalyzed dismutation of O_2^- is at least 4 orders of magnitude lower than the V_{max} of Cu/Zn SOD ($\approx 2 \times 10^9$ $M^{-1}\cdot s^{-1}$) (24) and is of little biological significance.

The higher photosynthetic rates for leaf disks from transgenic SOD plants compared with those for Xanthi plants under moderate low temperature stress (Fig. 3) are interesting, since both genotypes should differ only in their capacity to scavenge toxic oxygen species. Other genetically controlled factors that could cause differences in photosynthetic performance at low temperature are presumably the same in both genotypes. Therefore, the results presented here implicate the direct involvement of oxidative stress in the rapidly reversible reduction of photosynthesis at low temperature in tobacco.

Transgenic SOD plants also exhibited increased resistance to oxidative damage caused by exposure to low concentrations of MV, but at higher concentrations, this protective effect disappeared. Tepperman and Dunsmuir (25) were unable to detect any significant differences in resistance to MV between tobacco plants that expressed high levels (30- to 50-fold above normal SOD levels) of petunia chloroplast Cu/Zn SOD and control plants. Although the discrepancies between our results and those of this previous study can be largely explained by differences in assay methods, it is also possible that moderate increases in Cu/Zn SOD activity (as in our plants) can provide more effective protection from MV damage than very high SOD levels. In fact, Elroy-Stein *et al.* (26) have reported that moderate increases of Cu/Zn SOD provide MV resistance in human and mouse cells but large increases do not. Since Cu/Zn SODs are sensitive to end-product (H_2O_2) inhibition, it is possible that, when cells that express high levels of Cu/Zn SOD are treated with MV, they produce a burst of H_2O_2 that simply deactivates the enzyme.

The breakdown in stress resistance seen in our transgenic plants at 2.4 μM MV could indicate that the introduced Cu/Zn SOD is deactivated under these conditions. Bowler *et al.* (8) have reported significant protection from MV-induced oxidative damage in transgenic tobacco plants that overexpressed H_2O_2-insensitive, chloroplast-localized Mn SOD. Preliminary analysis in our laboratory indicates that tobacco plants that contain an analogous Mn SOD construct are substantially more resistant to high concentrations of MV than the transgenic Cu/Zn SOD plants described here (A.S.G., A.S.H., and R.D.A., unpublished results).

Further analyses of transgenic plants that overexpress chloroplast SODs will undoubtedly yield fundamental information about the effects of oxidative stress on chloroplasts, cells, and whole plants. Additional modifications of other components of the active-oxygen-scavenging system of plants will help to elucidate the interactions between these protective mechanisms. We remain hopeful that investigations of this type will eventually provide significant improvements in the tolerance of cultivated plants to environmental stress.

This research was supported by Texas Advanced Technology Program Grant 03644083 (to R.D.A. and A.S.H.) and United States Department of Agriculture Cooperative Agreement 58-7MN1-9-952 (to R.D.A. and J.J.B.). A.S.G. was supported by a fellowship from the National Research Council.

1. Cadenas, E. (1989) *Annu. Rev. Biochem.* **58,** 79–110.
2. Bannister, J. V., Bannister, W. H. & Rotilio, G. (1987) *CRC Crit. Rev. Biochem.* **22,** 111–180.
3. Halliwell, B. (1987) *Chem. Phys. Lipids* **44,** 327–340.
4. Bowler, C., Van Montagu, M. & Inzé, D. (1992) *Annu. Rev. Plant Physiol.* **43,** 83–116.
5. Rabinowitch, H. D. & Fridovich, I. (1983) *Phytochem. Photobiol.* **37,** 679–690.
6. Duke, M. V. & Salin, M. L. (1983) *Phytochemistry* **22,** 2369–2373.
7. Van Camp, W., Bowler, C., Villarroel, R. E., Tsang, W. T., Van Montagu, M. & Inzé, D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9903–9907.
8. Bowler, C., Slooten, L., Vandenbranden, S., De Ryke, R., Botterman, J., Sybesma, C., Van Montagu, M. & Inzé, D. (1991) *EMBO J.* **10,** 1723–1732.
9. Jansen, M. A. K., Shaaltiel, Y., Kazzes, D., Canaani, O., Malkin, S. & Gressel, J. (1989) *Plant Physiol.* **91,** 1174–1178.
10. Richter, M., Rühle, W. & Wild, A. (1990) *Photosynth. Res.* **24,** 237–243.
11. Sopory, S. K., Greenberg, B. M., Mehta, R. A., Eldman, M. A. & Mattoo, K. (1990) *Z. Naturforsch.* **45c,** 412–417.
12. Hodgson, R. A. J. & Raison, J. K. (1991) *Planta* **183,** 222–228.

13. Hodgson, R. A. J. & Raison, J. K. (1991) *Planta* **185,** 215–219.
14. Scioli, J. R. & Zilinskas, B. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7661–7665.
15. Isin, S. H., Burke, J. J. & Allen, R. D. (1990) *Plant Mol. Biol.* **15,** 789–791.
16. Bevan, M. W. (1984) *Nucleic Acids Res.* **12,** 8711–8721.
17. Horsch, R. B., Fry, J. E., Hoffmann, N. J., Eicholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227,** 119–123.
18. Beauchamp, C. O. & Fridovich, I. (1971) *Anal. Biochem.* **44,** 276–287.
19. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917.
20. Babbs, C. H., Pham, J. A. & Coolbaugh, R. C. (1989) *Plant Physiol.* **90,** 1267–1270.
21. Halliwell, B. (1982) in *Superoxide Dismutase*, ed. Oberley, L. W. (CRC, Boca Raton, FL), Vol. 1, pp. 89–124.
22. Wild, A., Richter, M. & Rühle, W. (1990) *Curr. Res. Photosynth.* **2,** 377–380.
23. Halliwell, B. & Gutteridge, J. M. C. (1985) *Free Radicals in Biology and Medicine* (Clarendon Press, Oxford).
24. Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezoli, M. S., Banci, L. & Hallewell, R. A. (1992) *Nature (London)* **358,** 347–351.
25. Tepperman, J. M. & Dunsmuir, P. (1990) *Plant Mol. Biol.* **14,** 501–511.
26. Elroy-Stein, O., Bernstein, Y. & Groner, Y. (1986) *EMBO J.* **5,** 615–622.