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Overexpression of Copper/Zinc Superoxide Dismutase in the Cytosol of Transgenic Tobacco Confers Partial Resistance to Ozone-Induced Foliar Necrosis[1]

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Ozone damage to plants has been attributed to the action of oxygen free-radicals and other ozone degradation products against which cellular antioxidant systems have been considered to be a front-line defense. The activity of superoxide dismutase (SOD), one such antioxidant, has been shown to increase in ozonated plants. Past work with pea (*Pisum sativum* L.) in our laboratory showed that the cytosolic Cu/Zn-SOD isoform and its transcript were most responsive to ozone, compared to chloroplastic Cu/Zn-SOD. In the current work we tested the hypothesis that plants that constitutively overexpress cytosolic SOD are more tolerant of ozone. Pea cytosolic Cu/Zn-SOD was overproduced in the cytosol of two cultivars of transformed tobacco (*Nicotiana tabacum*), Bel W3 and Wisconsin 38. Young and recently expanded leaves of transgenic plants of both cultivars showed less foliar necrosis than nontransformed controls when exposed to acute doses of ozone. We suggest that this may demonstrate the importance of Cu/Zn-SOD in the cytosol as a protector of the integrity of the plasma membrane and possibly other cellular constituents.

Oxygen is essential for the metabolism of aerobic organisms, including plants; however, its participation in cellular events results in ever-present toxic oxygen products. Therefore, enzyme systems have evolved to either remove or limit the production of toxic oxygen species. We have been investigating SOD and APX. SOD removes superoxide radicals by dismutation to oxygen and H_2O_2, which is subsequently removed by APX. Thus, the SODs and APXs work in concert to keep superoxide and H_2O_2 concentrations low, which in turn limits the generation of hydroxyl radicals, the most dangerous of all the oxyradicals. Likewise, contributing to cellular defenses against reactive oxygen are nonenzymatic antioxidants and other enzymes, including catalase, which also scavenges H_2O_2, and enzymes that regenerate oxidized ascorbate (Inze and Van Montagu, 1995, and refs. cited therein).

In addition to those reactive oxygen species generated as an inevitable consequence of aerobic metabolism, the air pollutant ozone presents additional oxidative stress. Despite increasing environmental awareness and regulations designed to limit industrial and automotive emissions, ozone remains an important summer air pollutant and thus a threat to commercial crops (Heagle, 1989), causing more crop damage than all other air pollutants combined (Bowler et al., 1992). It directly damages leafy crops and causes reduced yield from seed and root crops.

We have used pea, bean, and tobacco (*Nicotiana tabacum*) as model systems in which to investigate responses to ozone stress. Previously, we found that the cytosolic isoform of Cu/Zn-SOD from pea was more responsive to ozone than was its chloroplastic counterpart (Pitcher et al., 1991b, 1992); its expression was induced simultaneously with the appearance of necrotic injury. Furthermore, we found no protection from ozone imparted to tobacco that overexpressed chloroplastic Cu/Zn-SOD at 15-fold the level of the endogenous plastid enzyme (Pitcher et al., 1991a). Thus, we were interested in continuing the investigation of the relationship between cytosolic Cu/Zn-SOD and its possible role in ozone protection, especially if present at elevated levels prior to ozone exposure. Previously in this laboratory, pea cytosolic Cu/Zn-SOD cDNA was cloned and characterized (White and Zilinskas, 1991). Currently, we report the use of this cDNA to construct transgenic tobacco plants that constitutively overproduce Cu/Zn-SOD in the cytosol. To our knowledge, this is the first report that demonstrates that constitutive overproduction of Cu/Zn-SOD in the cytosol of tobacco is associated with reduced foliar necrosis on young and newly expanded leaves in response to acute ozone fumigations.

MATERIALS AND METHODS

Plant Material and Growth

The full-length cDNA encoding pea cytosolic Cu/Zn-SOD (White and Zilinskas, 1991) was cloned into the unique *Xba*I and *Sac*I sites of the plant transformation vector pBI121, downstream of the constitutive cauliflower mosaic virus 35S promoter and upstream of the *nos* termination sequence, in place of the *uid*A (GUS) reporter gene. This vector, which carries an *nptII* gene to allow for selection by kanamycin resistance, was then mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating.

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Abbreviations: APX, ascorbate peroxidase; ppb, parts per billion; SOD, superoxide dismutase; W38, *N. tabacum* cv Wisconsin 38.

Leaf pieces of *Nicotiana tabacum* cv Bel W3 or *Nicotiana tabacum* cv Wisconsin 38 (W38) were transformed as described by Horsch et al. (1985) and regenerated on Murashige-Skoog basal medium (Sigma) solidified with 0.8% agar and containing 3% Suc, 200 μg/mL kanamycin, 10 μM BA (Sigma), and 0.1 μM naphthalene acetic acid (Sigma) or 1 μM naphthalene acetic acid for Bel W3 or W38 transformants, respectively. For nontransformed controls, leaf pieces were inoculated with *Agrobacterium*-free medium but were otherwise carried through identical steps of regeneration and propagation with the exception that they were not exposed to kanamycin. Regenerated plants were grown to maturity and then self-pollinated; the resulting seeds were germinated on kanamycin-containing medium both for determination of segregation ratios and for selection of plants for experimentation. Transgenic progeny of the self-pollinated primary transformants (T_0) of Bel W3 are termed CZ followed by the number of the primary transformant; in W38, the terms S1 and S3 indicate progeny of two primary transformants.

Plants were grown in a computer-controlled greenhouse with supplemental lighting in the winter to provide a minimal light period of 14 h; temperature was maintained between 21 and 25°C. Plants were grown in Promix BX (Premier Brands, Red Hill, PA) supplemented three times weekly with Peters 20–20–20 (N-P-K) (Grace Sierra Horticultural Products, Milpitas, CA) in the early stages of this work and later with Peters Excel Cal-Mag, 15–5–15 (N-P-K); in each case Peters S.T.E.M. (soluble trace element mixture) was provided biweekly.

Preparation of Extracts

Leaves or roots were ground to a powder with mortar and pestle under liquid N_2 and were then homogenized at a ratio of 1 g of leaf powder/5 mL of ice-cold buffer (100 mM sodium phosphate, pH 7.8, 1 mM EDTA, 5 mM ascorbate) and 6% insoluble PVP. After the sample was filtered through Miracloth (Calbiochem), the filtrate was centrifuged at 39,000*g* for 15 min at 4°C. Aliquots of the supernatant were frozen in liquid N_2 and stored at −70°C. Protein concentration was determined by the Bradford (1976) method with BSA as a standard.

SOD Activity and Immunoblot Assays

SOD isozymes in total soluble tobacco leaf extracts were separated by PAGE using the Laemmli (1970) system and a 15% polyacrylamide resolving gel with or without SDS in the gel and running buffer for denaturing and native gels, respectively.

After SDS-PAGE, polypeptides within the gel were electroblotted in transfer buffer (25 mM Tris-Cl, 192 mM Gly, 0.1% SDS, 20% methanol) to 0.1 μm of nitrocellulose. After the blot was blocked in Tris-buffered saline containing 3% BSA, it was incubated overnight with antibodies raised in rabbits against SDS-denatured pea cytosolic Cu/Zn-SOD, which cross-reacts with both chloroplastic and cytosolic isozymes in pea but not with Mn-SOD (Scioli and Zilinskas, 1988). The blot was subsequently incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

To quantitate SOD isozyme activity, native gels were stained as described by Beauchamp and Fridovich (1971) so that, where present, SOD inhibited the background-staining reaction in proportion to its concentration. Quantitation was by scanning with an Ultrascan XL laser densitometer (LKB, Bromma, Sweden), in conjunction with Gelscan software (Pharmacia). For the identification of the individual SOD isozymes, i.e. Cu/Zn-, Mn-, and Fe-SOD, gels were soaked in 2 mM KCN or 5 mM H_2O_2 prior to activity staining. KCN inactivates Cu/Zn-SOD; H_2O_2 inactivates Cu/Zn- and Fe-SOD (Fridovich, 1982).

Ozone Stress Tests

For ozone fumigations, a loaf design chamber, 6 × 12 × 4 feet high along the center beam, approximately 260 cubic feet, was constructed on a benchtop in a greenhouse and covered with 0.008-in polyvinylchloride glazing. Plants were placed within this tent on two turntables, each 5 feet in diameter, which rotated two times/min. Ozone was generated by passing high-grade oxygen through an OREC model 0341–0 ozone generator (Ozone Research and Equipment Corp., Phoenix, AZ) and then feeding it into a charcoal-filtered air stream pulled into the tent by slight negative pressure of 0.0017208 atm below normal atmospheric pressure. Air turnover was about once every 3 min. Ozone concentration was monitored continuously with a model 1003 ozone monitor (Dasibi Environmental Corp., Glendale, CA). Vegetative plants were ozonated in the early-to-mid-bolting stages of development, at which time they had 10 to 14 leaves. Leaves were numbered from the top of the plant to the bottom, counting as leaf No. 1 the first leaf from the top that was more than 10 cm in length. Plants at this stage were maintained in 6-inch-diameter standard plastic pots. Nontransformed control and transgenic plants were evenly distributed on each turntable. After ozonation, each plant was tagged so that its identity was unknown to the raters, and 48 h after the end of ozone fumigation, visible foliar damage was determined, usually by two or more independent raters, using a method based on a system described earlier (Pitcher et al., 1991a). This visible injury-rating system integrated scores for intensity and extent of injury. The highest possible injury index was 100%. Seven categories of severity of injury were assigned the following values: 0, no injury; 0.05, slight silvering; 0.10, severe silvering; 0.25, small adaxial necrotic flecks; 0.50, larger adaxial flecks; 0.75, large bifacial necrotic areas; and 1.0, very large bifacial necrotic areas. The percentage of area per leaf exhibiting each range of severity was multiplied by the assigned value, and then the sum per leaf was reported as the percentage of visible injury. Photographs of typical injury types were used as an aid in rating.

Experimental Design

Two transgenic W38 lines (S1 and S3) were tested against nontransformed W38 controls; four transgenic Bel W3 lines

(CZ5, CZ6, CZ11, and CZ12) were tested against their nontransformed Bel W3 controls. Equal numbers (six to eight) of one transgenic plant type and its nontransformed control were ozonated in each experiment, with the exception that in three experiments both S1 and S3 plants were ozonated with their W38 controls. Ten experiments were conducted with W38 plants; eight were done with Bel W3 plants.

RESULTS

Overproduction of Cytosolic Cu/Zn-SOD

For each transgenic line the number of apparent insertion loci was determined by kanamycin selection. Of the Bel W3-based transformants used in this work, CZ11 is a T_2 generation homozygous line that has one insertion locus, CZ12 and CZ5 are T_1 lines with one insertion locus, and CZ6 is a T_1 line with two insertion loci. Of the W38-based transformants, both S1 and S3 are T_2 generation plants derived from selfing of hemizygous T_1 plants with two and one insertion loci, respectively. Plants from all lines (except CZ11) were selected on kanamycin before transplanting to soil.

SOD overproduction in each transgenic line was analyzed in representative nonozonated plants from the same group of plants used for ozonation. Typically, leaf No. 5, the most recently fully expanded leaf, was sampled. Presence of pea cytosolic Cu/Zn-SOD in transgenic tobacco was verified by immunodetection (Fig. 1). Its activity was compared with that of the endogenous tobacco cytosolic Cu/Zn-SOD activity of the appropriate nontransformed controls using activity gel analysis. As determined from several gels and shown in a representative gel (Fig. 2), CZ12 and CZ11 showed 1.5- to 2-fold, and CZ5 and CZ6 showed 3-fold the activity of the cytosolic Cu/Zn-SOD of the nontransformed Bel W3 control. S1 showed 3- to 6-fold, and S3 showed 1.5- to 3-fold the activity of the cytosolic Cu/Zn-SOD of the W38 nontransformed control.

Protection from Ozone Stress

Both W38 and Bel W3 tobacco plants overexpressing pea cytosolic Cu/Zn-SOD in the cytosol showed moderate to substantial protection from ozone when subjected to acute fumigations (Figs. 3 and 4). As is typical of ozone injury, in all cases, younger leaves were less injured than older leaves. Leaf No. 5 typically is the most recent fully expanded leaf. Leaves younger than No. 3 were not injured. Leaves that were visibly senescent prior to the ozonation were not included in the statistical analysis.

Injury to leaves of W38-nontransformed controls ranged from an average of 7% on leaf No. 3 to a high of 38% on leaf No. 7, whereas injury to Bel W3-nontransformed controls ranged from an average of 9% on leaf No. 3 to a high of 26% on leaf No. 7.

T_2 progeny of two primary transformants (T_0) of W38 were used in the ozonations. There was no significant difference between the two independent transgenic lines. Statistically significant protection was seen in leaves 5 through 8, which were injured about 50% less than the nontransformed W38 controls.

T_1 progeny of three primary transformants of Bel W3 and T_2 progeny (CZ11) of a fourth primary transformant were ozonated. Statistically significant protection was seen in leaves 3 and 4 in all four transgenic lines, in leaves 5 and 6 in three of the four transgenic lines, and in leaves 7 and 8 in one transgenic line. Protection ranged from about 30 to 50% less injury than the nontransformed controls in leaves 3 through 6. However, the degree of protection and consistency among the different transgenic lines observed in the younger leaves were not seen in leaves 7 and 8, in which two transgenic lines were more injured than the controls. The physiological significance of this is not currently understood and will be investigated further.

DISCUSSION

It is well known that SOD activity increases with oxidative stress, including that resulting from ozone exposure (Chanway and Runeckles, 1984; refs. cited in Bowler et al., 1992). We previously reported a preferential increase of cytosolic (as opposed to chloroplastic) Cu/Zn-SOD transcript, protein, and activity in pea and activity in bean in response to ozone. The increased enzyme activity coincided with the development of injury rather than with the initial ozone insult (Pitcher et al., 1991b, 1992).

Recent work by others has indicated increases in cytosolic Cu/Zn-SOD transcript level and/or activity in connection with ozone stress treatments. Willekens et al. (1994) reported a 4- to 5-fold increase in the cytosolic Cu/Zn-SOD transcript that appeared simultaneously with the appearance of visible injury in ozone-sensitive *N. tabacum* cv PBD6; in contrast, no change was observed in transcript levels for any of the SOD isozymes in ozone-resistant *Nicotiana plumbaginifolia*, which did not show visible injury in response to the same ozone dosages (exposure for 7 con-

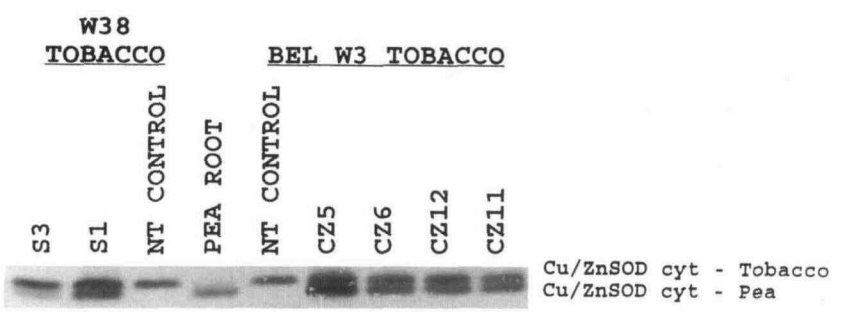

Figure 1. Immunodetection of pea cytosolic Cu/Zn-SOD in total soluble extracts of transgenic tobacco leaves and pea root. Seventy micrograms of protein/lane were subjected to SDS-PAGE as described in "Materials and Methods," transferred to a nitrocellulose membrane, and developed with antibodies to pea Cu/Zn-SOD. NT, Nontransformed; cyt, cytosolic.

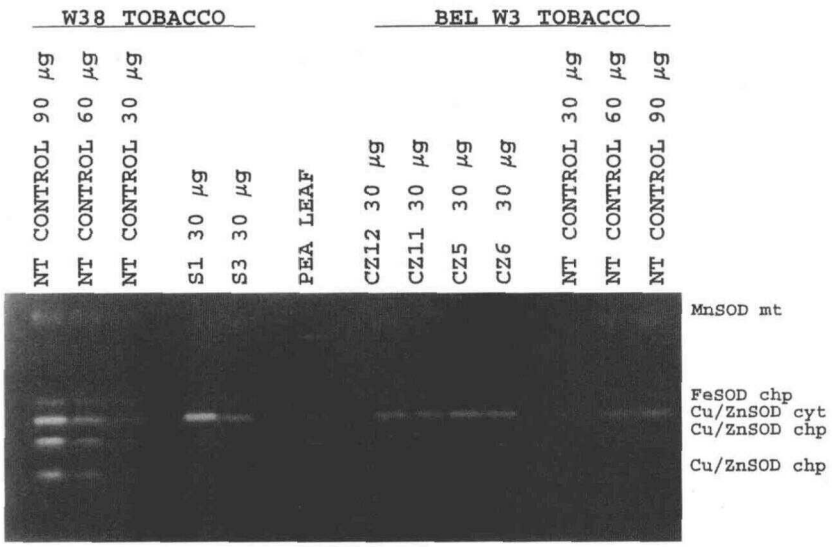

Figure 2. *Overproduction of transgenic Cu/Zn-SOD in tobacco leaves quantified by activity gel analysis using soluble extracts of leaf No. 5 prepared as described in "Materials and Methods." The transgenic cytosolic Cu/Zn-SOD comigrates with endogenous tobacco cytosolic Cu/Zn-SOD. NT, Nontransformed; mt, mitochondrial; chp, chloroplastic; cyt, cytosolic.*

secutive d at 120 ppb for 14 h/d). Sharma and Davis (1994) reported a 2- to 3-fold increase in the cytosolic Cu/Zn-SOD transcript in *Arabidopsis thaliana* 10 h after the start of a 6-h exposure to 300 ppb ozone. Herouart et al. (1993) showed that with very high ozone doses (500 ppb for 5 h) the cytosolic Cu/Zn-SOD transcript in tobacco SR1 increased 12-fold during fumigation and continued to increase 2 h postfumigation; however, the Mn-SOD transcript increased only after the end of fumigation and the transcript encoding the plastid FeSOD decreased. Small increases in activity of endogenous Cu/Zn-SODs in ozone-sensitive tobacco cv PDB6 were reported at the time of visible injury after 7 consecutive d of ozone fumigation at 120 ppb for 14 h/d (Van Camp et al., 1994). Conklin and Last (1995) report that ozone treatment of *A. thaliana* caused a preferential accumulation of transcripts encoding cytosolic antioxidants including glutathione *S*-transferase, APX, and Cu/Zn-SOD, whereas there was a decline in the transcript levels for the

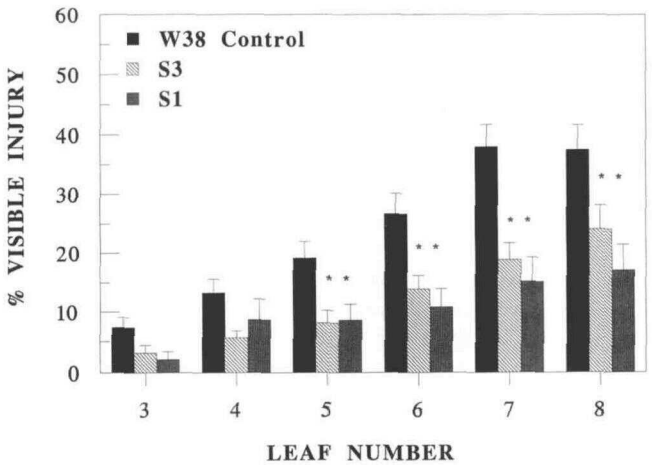

Figure 3. Effect of single acute ozone fumigations (200 or 300 ppb for 4.5 or 6 h) on visible injury in two transgenic lines of W38 tobacco constructed to overproduce pea cytosolic Cu/Zn-SOD in the cytosol. Results from 10 experiments were compiled. An equal number of transformed plants and nontransformed controls were ozonated in each experiment: typically six to eight control and six to eight transgenic plants. T_2 progeny of two independent transformants, named S1 and S3, were used in these experiments. Both S1 and S3 plants were used in three experiments; S1 plants only were used in four experiments; S3 plants only were used in four experiments. Means for controls were compiled from all experiments. Means marked by asterisks (*) are significantly less than the respective control at the 0.05 level as determined by an LSD test within analysis of variance. Bars indicate ± SE.

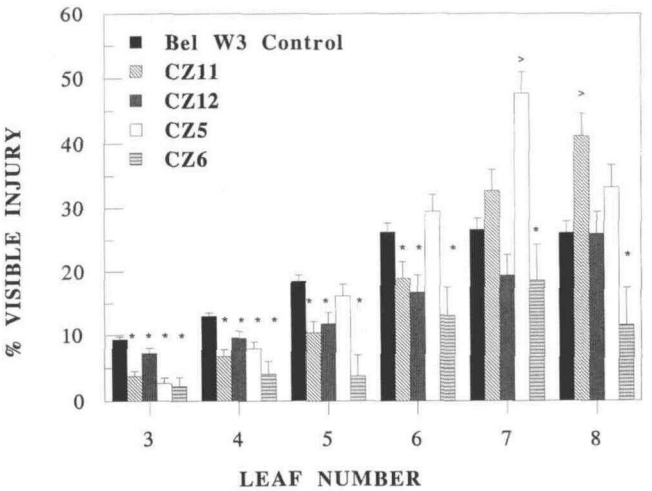

Figure 4. Effect of single acute ozone fumigations (300 ppb for 4.5 h) on visible injury in four transgenic lines of Bel W3 tobacco constructed to overproduce pea cytosolic Cu/Zn-SOD in the cytosol. Results from eight experiments were compiled. In each experiment, eight nontransformed controls were ozonated with eight transformed plants from one line; thus, each transgenic line was used in two experiments. T_1 kanamycin selected progeny of CZ12, CZ5, and CZ6 and homozygous T_2 progeny of the primary transformant CZ11 were used in these experiments. Means for controls were compiled from all eight experiments. Means marked by asterisks (*) are significantly less than the control at the 0.05 level as determined by an LSD test within analysis of variance. Means marked by > are significantly greater than the respective control. Bars indicate ± SE.

two chloroplastic antioxidative enzymes Fe-SOD and glutathione reductase. Bahl et al. (1993) reported large increases in mRNAs encoding Mn-SOD and cytosolic Cu/Zn-SOD in *N. tabacum* cv SR1 when treated with automobile exhaust and ozone, with very little response of the chloroplastic Fe-SOD. They offered the interpretation that the increased cytosolic Cu/Zn-SOD is a consequence of and protective reaction against the buildup of oxidative stress within the cell as a result of damage to the cell membrane. It is then reasonable to suppose that, if extra SOD were constitutively present in the cytosol, the effects of ozone damage might be ameliorated. This work addresses that question.

Ozone damage is considered to occur from radicals formed from the reaction of ozone with the aqueous solution bathing the cell; these oxyradicals can then attack lipid and protein components of the membrane itself, inducing chain reactions from which the products can further damage the membrane. Thus, the initial cellular site of ozone attack is considered to be the plasma membrane (Heath, 1987). H_2O_2 and hydroxyl radicals produced in the apoplastic fluids as a result of the ozone insult can directly penetrate an intact membrane prior to its total damage. Once inside the cell they can continue to react with cellular components, producing as a consequence superoxide radicals that can be attacked by SOD. Furthermore, the lipid peroxidation and damage of the plasma membrane cause a buildup of oxidative stress within the cell (Bahl et al., 1993). Because the development of necrotic symptoms indicates that the plasma membranes of large groups of cells have been lysed, a decline in that symptoms would indicate that, among other things, the integrity of a larger number of cell membranes has been maintained. The present results show that a constitutive overexpression of Cu/Zn-SOD in the cytosol, in the range of 1.5- to 6-fold, reduces ozone-induced necrosis in the ozone-sensitive tobacco cv Bel W3 as well as cv W38. Since the only known protective function of SOD is to remove superoxide radicals, in the case of these plants engineered to produce higher levels of cytosolic Cu/Zn-SOD, the superoxide radicals generated as a byproduct of ozone fumigation must be present at a position where they are both accessible to the cytosolic SOD and inherently dangerous to the cellular constituents, including the plasma membrane. The reduced necrosis is consistent with the explanation that the elevated cytosolic Cu/Zn-SOD activity provides direct and indirect protection to the integrity of the plasma membrane.

This is not to argue against the possibility that SOD overproduced in other cellular compartments can likewise be effective in preventing ozone injury. Although the cytosol is the first cellular compartment to be reached after the plasma membrane, the oxyradicals will move to other compartments if not effectively scavenged within the cytosol. However, we did not see protection from acute ozone exposures of W38 tobacco engineered to overproduce petunia chloroplastic Cu/Zn-SOD by 15-fold in the chloroplast (Pitcher et al., 1991a). In contrast to that work, Van Camp et al. (1994) reported a 3- to 4-fold reduction in visible necrotic damage to ozone-sensitive tobacco (cv PBD6) engineered to produce Mn-SOD in the chloroplast at 2- to 4-fold the levels of the endogenous SOD; plants were subjected to 7 consecutive days of ozone at 120 ppb for 14 h/d. They noted a lesser degree of protection when Mn-SOD was overproduced in the mitochondria. Whether the observed difference in the response to ozone exposure of the transgenic W38 and PBD6 tobacco can be accounted for by cultivar, level of overexpression of the introduced transgene, the SOD isozyme overproduced, the single versus chronic exposure, or other factors is yet unresolved.

In conclusion, our work shows that overproduction of Cu/Zn-SOD in the cytosol provides some protection to tobacco exposed to a single acute ozone dosage. We offer the hypothesis that this protection occurs largely as a result of enhanced preservation of the integrity of the plasma membrane, although the exact mechanism is not known. Furthermore, it remains to be seen what importance this extra cytosolic SOD will have for the overall physiological status of these plants when they are subjected to other oxidative stresses and are tested at other stages of growth and under field conditions.

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