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Communication

Overproduction of Petunia Chloroplastic Copper/Zinc Superoxide Dismutase Does Not Confer Ozone Tolerance in Transgenic Tobacco[1]

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ABSTRACT

Transgenic tobacco (*Nicotiana tabacum* cultivar W38) plants that overproduce petunia chloroplastic Cu/Zn superoxide dismutase were exposed to ozone dosages that injure control tobacco plants. Based on foliar injury ratings, there was no consistent protection provided to the transgenic plants. These data indicate that an increase in the chloroplastic Cu/Zn superoxide dismutase alone is not sufficient to reduce ozone toxicity.

Ozone damage is thought to result from the action of free radicals and other oxidants produced by the interaction of O_3 and its degradation products with plant tissue constituents (9). Cellular antioxidant systems are a front-line defense against oxygen free radicals. The activity of SOD,[2] one such enzymatic antioxidant, has been shown to increase in response to O_3 (7, 8, 14). Whether this increase is part of a protective, defense response or is a secondary consequence of injury is subject to debate. Lee and Bennett (12) reported a substantial increase in SOD activity in bean plants treated with EDU, a compound capable of conferring tolerance on O_3-susceptible plants (4). They also attributed the "natural" tolerance of young *versus* old leaves in the O_3-susceptible BBL290 bean cultivar to a higher endogenous SOD content in young leaves. They suggested that SOD plays a major role in detoxifying O_3-induced superoxide radicals which are at least in part responsible for ozone injury.

We have been investigating the role of SOD in several O_3-stressed model systems including *Pisum sativum, Phaseolus vulgaris,* and *Nicotiana tabacum.* This report concerns tobacco. Tepperman and Dunsmuir (19) transformed "Wisconsin 38" tobacco with DNA encoding petunia chloroplastic Cu/Zn-SOD, which was expressed in the chloroplast such that the transgenic plants overproduced the chloroplastic SOD. Inasmuch as certain histological (20) and physiological evidence (22) identifies the chloroplast as a primary site of O_3-induced injury, we proposed to use the transformed tobacco to test the hypothesis that tobacco plants with elevated levels of chloroplastic SOD are rendered tolerant to O_3 at dosages that normally injure tobacco.

MATERIALS AND METHODS

Nicotiana tabacum (tobacco) cv Wisconsin 38 plants were grown from seeds produced by selfing original transformants overproducing petunia chloroplastic Cu/Zn-SOD (19). Plants for assay were selected by rooting on kanamycin (200 μg/mL). Three plant types were tested: the control W38.11, which had been transformed with the kanamycin resistance gene only, and independent transformants SOD 35/1.4 and SOD 13.22 (hereafter called S1 and S2, respectively), which overproduced the petunia chloroplastic Cu/Zn-SOD (19). Vegetative plants aged 3 to 4 months with 9 to 11 nonsenescent leaves were used, uniform plants being chosen within each experiment. The newest leaf over 2 cm long was designated as leaf 1. Phenotypically there were no distinguishing features between the control and the two SOD transformants.

Ozone fumigation tests were conducted with five plants of each genotype in a dynamic flow system contained within a greenhouse (13); an equal number were placed in a similar chamber minus O_3. Ozone was produced by a generator (OREC model 0341-0) supplied with high grade oxygen. Ozone concentration in the chamber was measured by the neutral potassium iodide method (10). A dosage of 0.30 ppm O_3 for 6 h was used because it was capable of eliciting a differential response from indicator tobacco cultivars Bel W3 and BelB, sensitive and tolerant, respectively. Forty-eight hours after ozonation, leaves 1 through 8 were rated for visible injury. The experiment was conducted three times. Injury data for leaves 5 through 8 were subjected to analysis of

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[2] Abbreviations: SOD, superoxide dismutase; EDU, ethylenediurea.

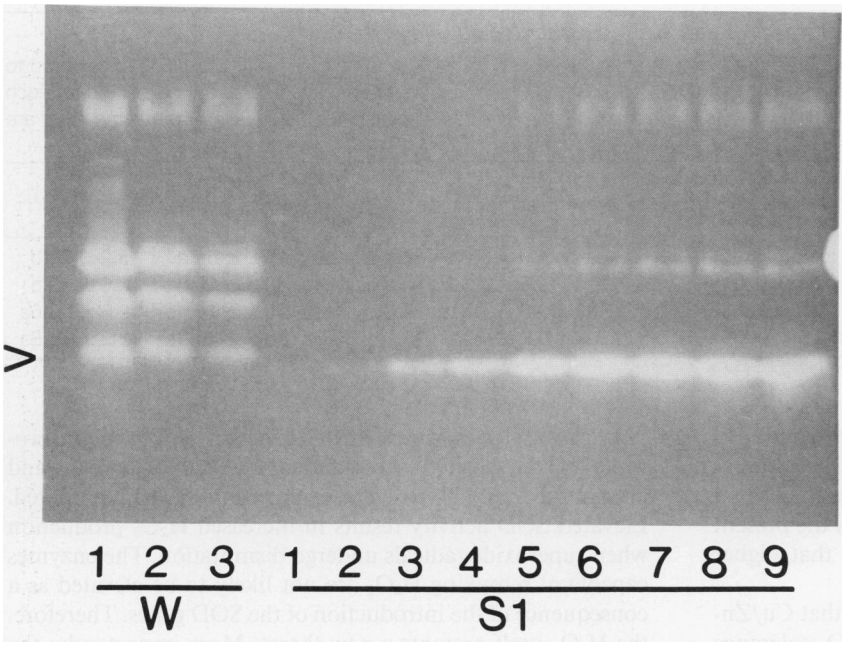

Figure 1. Quantitation of the chloroplastic Cu/Zn-SOD overexpression in transgenic tobacco on activity gels according to Beauchamp and Fridovich (1). SOD activity of W38.11 control tobacco (W) was compared with that of transgenic plants 35/1.4 (S1). Lanes were loaded with crude soluble leaf protein extract as follows: W, 1 to 3: 500, 250, and 100 μg; S1, 1 to 9: 0.5, 1, 5, 10, 20, 30, 40, 50, and 60 μg.

variance; data from young leaves 1 through 4 were excluded from analysis inasmuch as these typically showed no injury.

The visible injury rating system integrated scores for intensity and extent of injury, the highest possible injury index being 100%. Seven categories of injury intensity 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/leaf exhibiting each range of severity was multiplied by the assigned value, and then the total sum per leaf was reported as injury index.

Immediately following ozonation in experiment 1, leaf 6 from nonozonated and ozonated plants was harvested, frozen in liquid N_2, and stored at −70°C until prepared for analysis. Leaves were ground to a powder in liquid N_2, then homogenized in ice-cold buffer (100 mM sodium phosphate, pH 7.8, 0.1 mM EDTA and 10 mM β-mercaptoethanol) at a ratio of 2 g leaf powder/10 mL buffer. After filtration through Miracloth (Calbiochem), the filtrate was centrifuged at 39,000g 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 method (2). To assess the degree to which the SOD was overexpressed in the transformants, immunoblot and SOD activity gel analyses were employed as described earlier (1, 16). Immunoblots and activity gels were analyzed on an Ultrascan XL Laser Densitometer (LKB, Sweden), using GELSCAN software (Pharmacia).

RESULTS AND DISCUSSION

In transgenic tobacco, the overproduction of Cu/Zn-SOD was shown by immunoblot and gel activity analysis to be approximately 15 times that of the control genotype, with both transformed types showing similar levels of overexpression of the SOD, as measured by densitometric analysis of the immunoblot and activity gels. There were no differences in SOD activity between nonexposed plants and O_3-exposed plants sampled immediately after ozonation. Figure 1 shows SOD activity gel containing a dilution series of leaf extracts from transformant 35/1.4 (S1) as compared with the control tobacco transformant W38.11 (W). The fastest migrating band corresponds to the endogenous chloroplastic Cu/Zn-SOD of tobacco in the control samples (W) and the introduced petunia chloroplastic Cu/Zn-SOD together with the endogenous activity in the transgenic (S1) extracts as demonstrated earlier by Tepperman and Dunsmuir (19).

All genotypes exhibited visible foliar injury as a result of O_3 fumigation (Table I, Fig. 2). Leaves 1 through 4 of all three genotypes were nonsymptomatic, thus exhibiting O_3 resistance typical of young, developing leaves. In all genotypes, leaf 5 (which was nearly fully expanded) was intermediate in sensitivity, while maximum damage was seen in leaves 6 through 8 (which were fully expanded). Statistical analysis of injury data by leaf age (Table I) indicated a significant decrease in injury in only 4 of 24 possible comparisons between transgenic plants overexpressing SOD and control plants. Had SOD played a protective role, one would have expected, if not complete protection, at least a consistent decrease in injury in each comparison. The data demonstrate that a large amount of additional Cu/Zn-SOD in the chloroplast does not protect the ozonated plants.

We have also tested whether the acknowledged tolerance of Bel B tobacco relative to very sensitive Bel W3 involved elevated levels of SOD. Immunoblots and SOD activity gels showed the same level of SOD for the two cultivars, thus eliminating dissimilarities in SOD levels as a possible reason for their differing sensitivities (data not shown).

EDU is an O_3 protectant which, in our hands, completely protects a large number of plant species, including tobacco, pea, and beans from visible injury at the ozone dosage used in the present study. Lee and Bennett (12) proposed that the

Table I. *Foliar Injury Index in Response to Ozone in Control and Transgenic Tobacco Overproducing SOD*

Visible foliar injury index as a function of leaf age was measured in control (W38.11) and transgenic tobacco lines (S1 and S2) engineered to overproduce petunia chloroplastic Cu/Zn-SOD, 48 h subsequent to a 6 h exposure to 0.3 ppm ozone. The means and SE are presented for each of three experiments, with $n = 5$ for each individual genotype. Means in a horizontal row (within one experiment) followed by the same letter are not significantly different at the 0.05% level using an LSD test.

Leaf Number	Experiment 1			Experiment 2			Experiment 3		
	Control	S1	S2	Control	S1	S2	Control	S1	S2
5	11.6 ± 4.0a	26.2 ± 9.0a	24.0 ± 5.4a	7.8 ± 6.1a	5.6 ± 3.3a	1.0 ± 1.0a	27.6 ± 10a	18.2 ± 5.7a	5.2 ± 2.4b
6	31.2 ± 8.7a	48.4 ± 8.2a	42.8 ± 10.6a	29.0 ± 10b	6.2 ± 1.2a	9.0 ± 4.1a	47.0 ± 14.4a	33.8 ± 10.8a	10.6 ± 4.7b
7	26.2 ± 8.8a	44.2 ± 15.0a	61.2 ± 7.2b	13.8 ± 7.5a	32.6 ± 12.2a	29.8 ± 13.1a	44.0 ± 14.8a	55.5 ± 14.4b	32.8 ± 12.6a
8	6.6 ± 6.6b	43.0 ± 14.0a	44.4 ± 19.0a	5.6 ± 5.6a	19.0 ± 6.0a	9.2 ± 6.5a	40.7 ± 13.3a	56.5 ± 18.3a	42.2 ± 15.6a

protective action of EDU is the result of an increase in SOD activity. This idea has been refuted in two published studies (6, 7) and in our own work (L.H. Pitcher, E. Brennan, and B.A. Zilinskas, unpublished results). Neither does the present work with transgenic tobacco support the idea that higher SOD levels protect plants from O_3 damage.

The conclusion drawn from the present study that Cu/Zn-SOD overexpression does not *per se* guarantee O_3 tolerance extends a principle developed in other laboratories in which stress from paraquat-generated superoxide radicals was not ameliorated by increased SOD. Tepperman and Dunsmuir (19) showed that elevated chloroplastic Cu/Zn-SOD in tobacco was not able to reduce paraquat's toxicity. Studies with "engineered" prokaryotic and eukaryotic systems have provided examples in which higher levels of SOD activity promote greater sensitivity to paraquat (see refs. 17 and 18 and citations therein). Two related explanations have been offered. Elevated SOD activity results in increased H_2O_2 production when superoxide radicals undergo dismutation. The enzymes capable of removing H_2O_2 are not likely to be elevated as a consequence of the introduction of the SOD genes. Therefore, the H_2O_2 itself presents a new threat. More importantly, the likelihood of production of very toxic hydroxyl radicals is greatly increased via the interaction of the superoxide radicals and H_2O_2 in the Haber-Weiss reaction. In a complementary vein, Yim *et al.* (23) recently reported that Cu/Zn-SOD itself is able to catalyze hydroxyl radical production directly from H_2O_2. In our study, we report some cases (Table I) in which SOD-overproducing plants had more O_3-induced injury than the control plants, but we cannot provide anything but speculation at this time as to why this occurred.

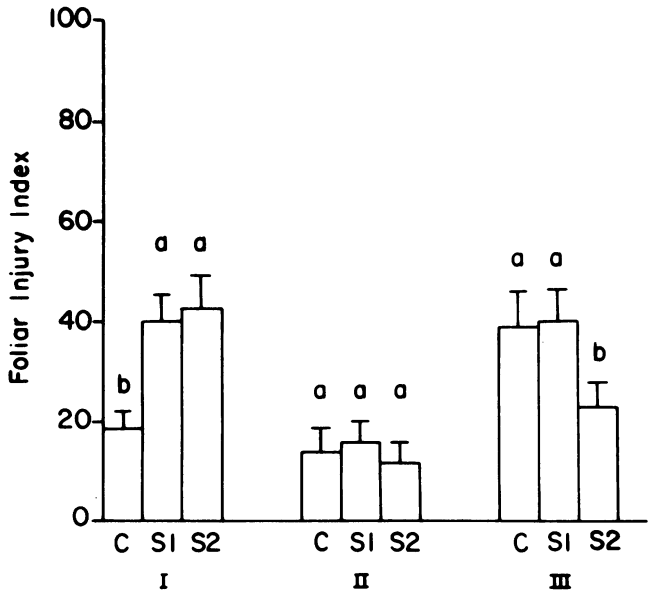

Figure 2. Visible foliar injury index in response to ozone in control (C, W38.11) tobacco and two independent transgenic lines (S1 and S2) that overproduce the petunia chloroplastic Cu/Zn-SOD. The ratings for three independent experiments are presented. Leaves 5 through 8 on five plants of each genotype were rated as described in "Materials and Methods." The indices presented are means ± SE of these values ($n = 20$). Means within one experiment, designated by the same letter, are not significantly different at the 0.05 level, using an LSD test. Details of the experimental protocol are described in the text.

While overexpression of SOD may not necessarily be beneficial, numerous studies with bacteria, protists, animals, and plants indicate that SOD activity is required for life in oxygen-containing atmospheres. Thus, a double mutant of *Escherichia coli* lacking all SOD had reduced viability and a high mutation rate and was very sensitive to paraquat; these characteristics could be counteracted by a plasmid that overproduced SOD (3). Likewise, a *Drosophila* SOD null mutant had increased sensitivity to O_2, reduced fertility, and shortened lifespan (15).

Finally, there are a few reports of protection provided against paraquat toxicity by organisms engineered to produce higher levels of SOD (for example, see refs. 5, 11, 21). Interestingly, at least in some cases where such has been noted, the levels of H_2O_2 detoxifying enzymes appear to increase in an adaptive response to the higher H_2O_2 levels generated via increased SOD activity (5, 11).

In summary, in this work we find no consistent protection provided to transgenic plants engineered to overproduce the chloroplastic Cu/Zn-SOD. It is clear from the examples listed above that there are many factors that enter into the response of organisms to oxidative stress, and the variation we see within individual experiments and among leaves of different ages from the same plant genotype is probably due to the complexity of the overall antioxidant system.

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