Plant Physiol. (1993) 103: 1155-1163

Superoxide Dismutase Enhances Tolerance of Freezing Stress in Transgenic Alfalfa (*Medicago sativa* L.)[1]

Bryan D. McKersie*, Yurong Chen, Mitchel de Beus[2], Stephen R. Bowley, Chris Bowler[3], Dirk Inzé, Kathleen D'Halluin, and Johan Botterman

Department of Crop Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (B.D.M., Y.C., M.d.B., S.R.B.); Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium (C.B., D.I.); and Plant Genetic Systems N.V., Plateaustraat 22, B-9000 Gent, Belgium (K.D'H., J.B.)

Activated oxygen or oxygen free radicals have been implicated in a number of physiological disorders in plants including freezing injury. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into O_2 and H_2O_2 and thereby reduces the titer of activated oxygen molecules in the cell. To further examine the relationship between oxidative and freezing stresses, the expression of SOD was modified in transgenic alfalfa (*Medicago sativa* L.). The Mn-SOD cDNA from *Nicotiana plumbaginifolia* under the control of the cauliflower mosaic virus 35S promoter was introduced into alfalfa using *Agrobacterium tumefaciens*-mediated transformation. Two plasmid vectors, pMitSOD and pChlSOD, contained a chimeric Mn-SOD construct with a transit peptide for targeting to the mitochondria or one for targeting to the chloroplast, respectively. The putatively transgenic plants were selected for resistance to kanamycin and screened for neomycin phosphotransferase activity and the presence of an additional Mn-SOD isozyme. Detailed analysis of a set of four selected transformants indicated that some had enhanced SOD activity, increased tolerance to the diphenyl ether herbicide, acifluorfen, and increased regrowth after freezing stress. The F_1 progeny of one line, RA3-ChlSOD-30, were analyzed by SOD isozyme activity, by polymerase chain reaction for the Mn-SOD gene, and by polymerase chain reaction for the *neo* gene. RA3-ChlSOD-30 had three sites of insertion of pChlSOD, but only one gave a functional Mn-SOD isozyme; the other two were apparently partial insertions. The progeny with a functional Mn-SOD transgene had more rapid regrowth following freezing stress than those progeny lacking the functional Mn-SOD transgene, suggesting that Mn-SOD serves a protective role by minimizing oxygen free radical production after freezing stress.

Activated oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical are associated with a number of physiological disorders in plants. Although activated oxygen is produced as a by-product of normal cell metabolism, its levels are enhanced by exposure to chemical and environmental stress. Increased production of activated oxygen species has been associated with the development of injury symptoms resulting from diverse environmental stresses, including chilling (Wise, and Naylor, 1987; Senaratna et al., 1988), drought (Price and Hendry, 1991), desiccation (Senaratna et al., 1985; Leprince et al., 1990), flooding (Hunter et al., 1983; Van Toai and Bolles, 1991), freezing (Kendall and McKersie, 1989), and ice encasement (McKersie et al., 1988). Tolerance to these environmental stresses correlates with an increased capacity to scavenge or detoxify activated oxygen species (McKersie et al., 1988; Kendall and McKersie, 1989; Malan et al., 1990). A number of enzymes are involved in the selective detoxification of activated oxygen species. One enzyme, SOD (EC 1.15.1.1), catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen (Bowler et al., 1992). The hydrogen peroxide is removed by a number of different peroxidase enzymes such as those in the ascorbate-GSH cycle present in the chloroplast (Foyer et al., 1991; Salin, 1991).

SODs are metalloproteins classified into three distinct types according to their metal cofactor: Cu/Zn (Cu/Zn-SOD), Mn (Mn-SOD), and Fe (Fe-SOD). The Cu/Zn-SOD is found almost exclusively in eukaryotes in the cytosol and/or chloroplast. The Mn-SOD group is widely distributed in prokaryotes and in the mitochondrial matrix of eukaryotes. Fe-SOD has been found in prokaryotes and recently within the chloroplast of some plant families (Bannister et al., 1987; Bowler et al., 1992).

Several SOD cDNAs have been cloned from plants (Bowler et al., 1992; Perl et al., 1993), and transgenic plants have been produced that exhibit enhanced SOD activity. In some cases, these transgenic plants exhibited enhanced tolerance of oxidative stress (Bowler et al., 1991; Perl et al., 1993; Sen Gupta et al., 1993), whereas in other cases, they did not (Tepperman and Dunsmuir, 1990; Pitcher et al., 1991). Tobacco (*Nicotiana tabacum*) transformed with the *Nicotiana plumbaginifolia* Mn-SOD cDNA showed reduced cellular damage when treated with paraquat, an oxygen free radical-generating herbicide (Bowler et al., 1991). Similarly, transgenic potato (*Solanum tuberosum*) plants expressing a tomato

[1] Financial assistance was provided by the Natural Sciences and Engineering Research Council of Canada (strategic grant) and the Ontario Ministry of Agriculture and Food.

[2] Present address: Department of Plant Science, University of Alberta, Edmonton, Alberta, T6G 2P5 Canada.

[3] Present address: Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021.

* Corresponding author; fax 1-519-763-8933.

Abbreviations: F_o, initial fluorescence; F_{max}, maximal fluorescence; LD_{50}, lethal dose causing 50% death; npt, neomycin phosphotransferase; PCR, polymerase chain reaction; SOD, superoxide dismutase.

Cu/Zn-SOD were also more resistant to paraquat (Perl et al., 1993). On the other hand, tobacco plants that were transformed using a petunia (*Petunia hybrida*) chloroplastic Cu/Zn-SOD cDNA had a 30- to 50-fold increase in chloroplastic Cu/Zn-SOD activity, but the plants did not have enhanced tolerance to oxidative stress imposed by the herbicide paraquat (Tepperman and Dunsmuir, 1990) nor were they more tolerant to ozone (Pitcher et al., 1991). Tomato (*Lycopersicon esculentum*) transformed with the same construct did not exhibit increased tolerance to photoinhibition at low temperatures (4°C) and high light (Tepperman and Dunsmuir, 1990). More recently, Sen Gupta et al. (1993) demonstrated increased resistance to photoinhibition in tobacco plants that expressed a cDNA for pea (*Pisum sativum*) Cu/Zn-SOD. These transgenic tobacco plants retained photosynthetic rates that were approximately 20% higher than nontransformed tobacco plants when subjected to chilling temperatures (3°C) under moderate light intensities. These differences among the experiments on transgenic SOD plants are possibly due to differences in the level of expression, with extreme overexpression being detrimental. In this respect, the balance between the activities of SOD and the subsequent hydrogen peroxide scavenging enzymes, such as ascorbate peroxidase and GSH reductase, is probably critical.

We have introduced the *N. plumbaginifolia* Mn-SOD cDNA (Bowler et al., 1989) into alfalfa (*Medicago sativa* L.) to evaluate its effect on the persistence of this perennial. These plants were developed to test our previous hypothesis that there is an involvement of oxidative stress in mediating injury after freezing stress (Kendall and McKersie, 1989) and specifically to assess the role of SOD in freezing tolerance. In this report we describe our evaluation of the primary transformants and the inheritance of the SOD transgene in one transformant that exhibited enhanced freezing tolerance.

MATERIALS AND METHODS

Plant Material

An alfalfa (*Medicago sativa* L.) plant RA3 from Regen S (Walker et al., 1978) was utilized in the transformation experiments and was vegetatively propagated by cuttings. *Agrobacterium*-mediated transformation was conducted using the method described by D'Halluin et al. (1990). The T-DNA vectors, pMitSOD and pChlSOD, were described previously by Bowler et al. (1991). Both contain a cDNA encoding Mn-SOD isolated from *Nicotiana plumbaginifolia* (Bowler et al., 1989) under the control of the cauliflower mosaic virus 35S promoter (Odell et al., 1985) and the *neo* gene, encoding npt-II and conferring kanamycin resistance, under the control of the nopaline synthase (*nos*) promoter as a selectable marker (Herrera-Estrella et al., 1983). In pMitSOD, the Mn-SOD contained its own transit peptide sequence for mitochondrial targeting (Bowler et al., 1991). The pChlSOD vector contained a transit peptide sequence that targets the Mn-SOD cDNA product to the chloroplast (Bowler et al., 1991).

Regenerated plants were selected on 50 mg L^{-1} of kanamycin and tested for expression of the *neo* gene before transplanting to the growth room (D'Halluin et al., 1990). All plants were grown in a growth room with a 16-h photoperiod (300 μmol m^{-2} s^{-1} PPFD), 21/18°C day/night temperature, watered daily, and fertilized every 3rd d with a 0.1% (w/v) 20:20:20 fertilizer. The primary transformants were propagated by cuttings to increase the number of plants available for biochemical, physiological, and genetic analysis.

Native PAGE Determination of SOD Isozyme Banding Pattern

Six 7.0-mm leaf discs from the first fully expanded leaves were placed in 120 μL of homogenizing buffer consisting of 0.1 M Tris-HCl (pH 7.2), 0.5 M Suc, 6 mM Cys-HCl, 6 mM ascorbic acid, 1% (v/v) Tween 80, 4% PVP, 0.02 M diethyldithiocarbonic acid, 30 mM sodium metabisulfate, 0.4 mM NAD, 0.1% BSA, 1 mM DTT, 0.5 mM EDTA, 10% DMSO, 1% PEG, and 1% β-mercaptoethanol (Pitel and Cheliak, 1984). Alfalfa contains secondary products that inactivate enzymes in homogenates; this complex buffer was used to prevent this inactivation (C. Duxbury and B.D. McKersie, unpublished data). The samples were homogenized using a power-driven pestle for 15 to 20 s and then centrifuged at 13,600*g* for 15 min at 4°C, and the supernatant was removed for electrophoresis and protein determination.

Total protein was determined spectrophotometrically at 595 nm using Coomassie protein reagent (Bradford, 1976). A 0.75-mm, 13% separating gel with a 4% stacking gel was loaded with 70 μg of protein. The staining solution consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.03 mM riboflavin, 0.326% (v/v) *N-N-N'-N'*-tetraethylene diamide, and 1.25 mM nitroblue tetrazolium (Beauchamp and Fridovich, 1971). Gels were floated in the staining solution in the dark for 30 min, removed from the stain, and illuminated on a light box for 20 min.

SOD Activity

SOD activity in leaf extracts of alfalfa was based on the indirect spectrophotometric method described by Spychalla and Desborough (1990) except that diethylene triamine pentaacetic acid was used instead of EDTA in the assay mixture. Briefly, 200 mg of leaves were ground in liquid nitrogen and extracted with 4 mL of homogenizing buffer (Pitel and Cheliak, 1984). The extract was filtered through filter paper and loaded onto a Pharmacia PD-10 column of Sephadex G-250. The eluent in 0.5- to 3.5-mL fractions was collected and assayed immediately for SOD activity and protein content (Bradford, 1976). SOD activity was calculated in varying volumes of extract according to the method of Giannopolitis and Ries (1977).

PCR Analysis

DNA was extracted from leaf discs as described by Edwards et al. (1991). PCR was performed with specific primers either for *neo* gene (P_1 and P_3) or for the Mn-SOD transgene in the pChlSOD vector (CP and SP_2), on a 5-μL DNA solution in a GTC-2 Genetic Thermal Cycler (Precision Scientific, Inc., Chicago, IL) using 250 pmol of each primer, 100 μM of each deoxyribonucleotide triphosphate, 3 units of Taq DNA polymerase (Promega), and 5 μL of 10× Taq DNA polymerase

buffer (Promega) in a 50-μL reaction mixture. The reactions were overlaid with 50 μL of light mineral oil. The amplification was started with one 5-min cycle at 94°C followed by 30 cycles of 1.5 min at 94°C, 2 min at 65°C (for npt-II primers) or at 55°C (for Mn-SOD primers), and 3 min at 72°C, and this was followed by one 5-min cycle at 72°C. Each PCR sample (25 μL) was electrophoresed on a 1.2% agarose gel and visualized by staining with ethidium bromide.

The PCR primers used for analysis of the *neo* gene were computer selected, and the primer sequences were P_1, 5'-AGCTGTGCTCGACGTTGTCAC-3', and P_3, 5'-GGTGGGCGAAGAACTCCAGCA-3'. A 732-bp fragment was expected if the template DNA contained this portion of the *neo*-coding sequence. The sequences of the PCR primers used for analysis of the introduced Mn-SOD cDNA were CP, 5'-ATGACTGGATTCCCAGTG-3', and SP_2, 5'-AGAAACCAAAGGGTCCTG-3', which were complementary to the region encoding the Rubisco small subunit transit peptide and an internal sequence of the Mn-SOD cDNA, respectively. A 545-bp product was expected. The CP primer was used to reduce complementarity with the alfalfa Mn-SOD genes.

Cytological Examination

Young roots from newly rooted cuttings were pretreated in ice water at 0°C for 16 to 18 h, fixed in a 3:1 (v/v) mixture of ethanol and glacial acetic acid for 24 h, and stained in 1% acetocarmine for 2 h. Squashes were made in 45% acetic acid, and chromosome number was counted from the root-tip cells in metaphase.

Herbicide Treatments

Trifoliolate leaves of RA3 and primary transformants were weighed and floated on 3.0 mL of acifluorfen (Rohm and Haas, Spring House, PA) solution in a Petri plate for 16 h at room temperature in the dark, then illuminated for 2 h (400 μmol m^{-2} sec^{-1} PPFD), and returned to the dark for a further 16 h at 30°C. Three trifoliolate leaves were used for each herbicide treatment, and the experiment was replicated three times. The herbicide solution was removed from the Petri plate and adjusted to 3.0 mL with deionized water to compensate for evaporation. The conductance was expressed as $\mu\Omega^{-1}$ cm^{-2} 20 mg^{-1} fresh weight. All conductances were corrected by subtracting the conductance value per 20 mg of trifoliolate leaves floating on water (zero herbicide control).

Using a Hansatech Modulated Fluorescence Measurement System (Hansatech Ltd., Norfolk, England), the parameters F_o and F_{max} of each trifoliolate were measured. This allowed for the calculation of photosynthetic quantum yield [$\Phi_{II} = (F_{max} - F_o)/F_{max}$] as an indication of the fitness of the photosynthetic pathway after stress (Gentry et al., 1989). Operating conditions were 1 s of saturating light pulse, 8000 μmol m^{-2} s^{-1} PPFD at the end of the probe, and 2000 μmol m^{-2} s^{-1} PPFD at the tissue.

The parameters, conductivity and quantum yield, were analyzed statistically as a split-plot arrangement. Herbicide treatments were assigned to the whole plot, and plants were assigned to the split plots. Comparisons among plants were made based on a protected LSD at the 5% level of probability.

Freezing Tolerance

Twenty-four cuttings of each plant were prepared and propagated for evaluation under six different environmental stress treatments, no stress, and freezing to −8, −10, −12, −14, and −16°C. Cuttings were grown in 2.2-cm^2 root trainer trays, with four plants per experimental unit, for 7 weeks to establish a good root system, and then they were clipped to 2 to 3 cm above the soil surface and allowed to grow back for 1 week. The plants were then acclimated for 4 weeks in a growth cabinet with a 12-h photoperiod (200 μmol m^{-2} s^{-2} PPFD) at 2°C. The plants were watered every 2nd d and fertilized every third watering with 0.1% (w/v) 20:20:20 fertilizer. After acclimation the plants were defoliated to 2 to 3 cm above soil. The nonstressed cuttings of each plant were put directly back into the growth room with a 16-h photoperiod (400 μmol m^{-2} s^{-1} PPFD), 21/18°C day/night temperature. For freezing, 20 cuttings of each plant (five groups of four) were put in a programmable freezer in the dark and held at −2°C for 48 h to freeze the soil medium. The temperature was then decreased at a constant rate of 2°C h^{-1}. Plants were subsampled at −8, −10, −12, −14, and −16°C, packed in Styrofoam chips at 2°C for 48 h to reduce the rate of thawing, and then transferred to the growth room. Shoot regrowth was cut from the surviving plants in two consecutive harvests at 28 and 56 d and dried for 48 h at 80°C. The values for the regrowth data were expressed as average regrowth of the two harvests per four plants. Plants that did not survive were assigned a regrowth value of zero and were included in the calculation. This experiment was replicated three times and was statistically analyzed as a split-plot design with intensity of stress as the whole plot and lines as split plots.

Inheritance of SOD Transgene and Freezing Tolerance

For the inheritance study, the primary transformant RA3-ChlSOD-30 was crossed to the agronomically desirable breeding line C2–3 obtained from the University of Guelph breeding program. C2–3 rather than RA3 was used for this test cross because RA3 exhibits in-breeding depression when self-pollinated. Reciprocal crosses were made without emasculation. F_1 seeds were germinated on moist filter paper in a Petri dish at room temperature for 3 to 5 d, transplanted to pots, and grown in a growth room as described above.

Protein samples were collected from fully opened young leaves for Mn-SOD analysis using PAGE as above. Plants were classified as MnSOD$^+$ (band present) or MnSOD$^-$ (band absent). PCR analysis was conducted on the progeny both for the *neo* gene and for the Mn-SOD transgene as described above. To measure freezing tolerances, we divided the progeny into two populations, MnSOD$^-$ and MnSOD$^+$. The plants were grown, acclimated, and subjected to freezing stress as described previously for the primary transformants except that the plants were grown in individual 8-cm-diameter pots, which allowed more shoot growth. At least four plants were randomly sampled from each population at each freezing temperature. Regrowth of the plants after freezing stress was determined as total herbage yield after 28 and 56 d.

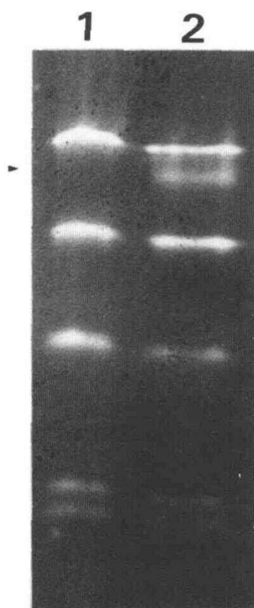

Figure 1. Native PAGE of SOD isozymes from the leaves of alfalfa (*M. sativa* L.) clone RA3 (lane 1) and the transgenic plants RA3-ChlSOD-30 (lane 2). The arrowhead indicates the presence of the additional Mn-SOD isozyme.

RESULTS

Analysis of Primary Transformants

The putative transformants were regenerated and selected first for expression of the *neo* gene (data not shown). For pMitSOD and pChlSOD, 40 and 33 plants, respectively, were selected, propagated by cuttings, and screened using native PAGE gels to determine the SOD isozyme banding pattern. RA3 had five SOD isozymes, and 20 of the putatively transgenic plants showed the presence of an extra high mol wt isozyme (Fig. 1). In RA3, the highest mol wt isozyme was resistant to both hydrogen peroxide and sodium cyanide and, therefore, was characterized as Mn-SOD; the other four isozymes were inhibited by both hydrogen peroxide and cyanide and, therefore, were characterized as Cu/Zn-SOD. No Fe-SOD isozymes were detected. The additional isozyme of the putatively transgenic plants was resistant to both inhibitors, confirming that it was Mn-SOD. The intensity of this extra SOD band varied quite dramatically among the transformants. Based on visual assessment of intensity, two plants with the highest expression were selected from each of the constructs for further detailed evaluation; these four were subsequently called RA3-ChlSOD-30, RA3-ChlSOD-64, RA3-MitSOD-5, and RA3-MitSOD-38. These names designate the original genotype that was transformed, the vector used, and the plant number of the primary transgenic.

Only four of the primary transformants were selected for more detailed evaluation because it was necessary to propagate these primary transformants with cuttings, and large numbers of plants were required to accurately evaluate stress tolerances. The primary transformants were examined because alfalfa is an autotetraploid species that exhibits inbreeding depression. Therefore, selfing the primary transformant would not have given genetically identical progeny but, instead, a segregating population of progeny with an anticipated lower level of stress tolerance due to in-breeding depression. Therefore, propagation with cuttings was the only method available to maintain the original genotype for evaluation of stress tolerance.

Chromosome counts were made from root-tip cells in metaphase on two of the transformants, RA3-ChlSOD-30 and RA3-ChlSOD-64; both had normal karyotype (2n = 32). The presence of the Mn-SOD transgene in RA3-ChlSOD-30 and RA3-ChlSOD-64 was confirmed using PCR amplification with synthetic primers specific for the Mn-SOD cDNA (SP_2) and the chloroplast transit peptide (CP) sequences (Fig. 2). The expected 545-bp fragment was obtained from both primary transformants tested. These two primary transformants also had the expected 732-bp fragment following PCR amplification with the primers specific for the *neo* gene (data not shown).

Total SOD activity was measured in leaf extracts from the transgenic plants. In the preliminary analysis a nonenzymic inhibition of Cyt *c* reduction by the leaf extract was suspected because the inhibition was not related predictably to the concentration of SOD added (volume of extract). Therefore, the extract was passed through a desalting Sephadex G-250 column before the enzyme assay to remove the interfering compounds, and, subsequently, this extract was used to determine the amount of protein required to give 50% inhibition of the xanthine oxidase reduction of Cyt *c*. Plotting the data as the rate of Cyt *c* reduction without SOD (V) relative to the rate with SOD (v) according to the method of Giannopolitis and Ries (1977) gave a linear relationship to both precolumn (Fig. 3) and postcolumn protein (data not shown). The slope of the regression line is related to the Cyt *c* concentration and the affinity of SOD and Cyt *c* for superoxide.

Apparently the presence of the additional Mn-SOD iso-

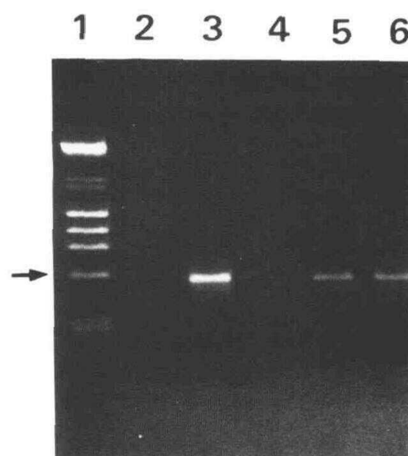

Figure 2. PCR amplification of the Mn-SOD transgene in transgenic alfalfa (*M. sativa* L.) RA3-ChlSOD-30 and RA3-ChlSOD-64 using primers specific for the chloroplast transit peptide sequence and an internal sequence of the Mn-SOD cDNA. Lane 1, λ DNA *Hin*dIII/ϕX174 DNA *Hae*III digest; lane 2, RA3; lane 3, pChlSOD plasmid; lane 4, water; lane 5, RA3-ChlSOD-30; lane 6, RA3-ChlSOD-64. The arrow indicates the expected 545-bp fragment.

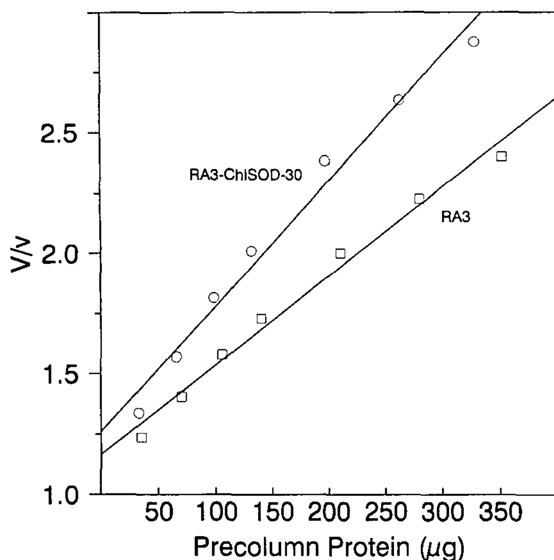

Figure 3. SOD activity (percentage inhibition) in leaf extracts from RA3 and RA3-ChlSOD-30 alfalfa (*M. sativa* L.) after desalting through a Sephadex G-250 column. Activities are expressed as rate of Cyt c reduction without SOD (*V*) relative to rate with SOD (*v*). The lines represent the linear regression equations: for RA3, $y = 1.16 + 0.0037x$, $r^2 = 0.987$; for RA3-ChlSOD-30, $y = 1.26 + 0.0052x$, $r^2 = 9.82$. $n = 3$.

zyme enhanced the affinity of the crude extract for superoxide in addition to increasing total activity. Comparison of the leaf extracts from the four transgenic plants and the RA3 control indicated that the transgenic plants had approximately twice the amount of total SOD activity (Table I). Although the relative activity of SOD varied in leaf age and growth conditions among the plants, the transgenics consistently had a 30 to 100% increase in total SOD activity. The inclusion of 100 μM cyanide$^-$ in the assay mixture inhibits Cu/Zn-SOD activity (Bridges and Salin, 1981) and, therefore, estimates Mn-SOD activity. In the presence of cyanide$^-$ three of the transgenic plants had approximately 45 to 95% increase in Mn-SOD activity relative to RA3 (Table I), but the stimulation of total SOD in the transgenic plants and the amount of Mn-SOD activity did not mathematically correspond. The Mn-SOD transgene may have stimulated the activity of Cu/Zn-SOD in the transgenic plants by an as-yet unknown mechanism, but this discrepancy may also be due to interference in the Mn-SOD assay.

The herbicide acifluorfen was used to generate oxidative stress in a detached leaf system and to quantify the oxidative stress tolerance of the transgenic plants. Acifluorfen is a photobleaching, *p*-nitrodiphenylether herbicide that promotes accumulation of the Chl precursor protoporphyrin; in the light, this generates singlet oxygen that causes peroxidation in the tonoplast, plasmalemma, and chloroplast envelope (Jacobs et al., 1991). Leaves treated with 333 μM acifluorfen varied in the amount of electrolyte leakage, a measure of free radical damage to cellular membranes (Table II). Both ChlSOD transformants and one of the MitSOD transformants showed a significant reduction in electrolyte leakage. Although there was a dramatic reduction with the herbicide treatment in quantum yield, as calculated from Chl fluorescence, there was no statistically significant difference among the plants in this experiment. It was postulated that the injury in this experiment was too severe to detect the small changes in tolerance to acifluorfen. Therefore, two transgenic plants and the RA3 nontransgenic control plant were subjected to a broad range of herbicide concentrations to more accurately determine their tolerance of this herbicide by comparing their LD_{50} values (Fig. 4). Conductivity measurements for RA3-MitSOD-38 and RA3-ChlSOD-64 were significantly lower than RA3 at concentrations greater than 200 μM. According to the conductivity test these transgenic plants had LD_{50} values of 283 and 345 μM, respectively, which were slightly higher than the value of 255 μM for RA3. Chl fluorescence showed that acifluorfen had an LD_{50} of 462 μM for RA3-ChlSOD-64 compared to 330 μM for RA3-MitSOD-38 and the RA3.

Four of the transgenic plants and RA3 were subjected to a freezing stress. The plants were propagated by cuttings, grown in the growth room to establish the root and crown system, acclimated at low temperature and short photoperiod, and then subjected to the freezing treatments. The relative

Table I. *Total and Mn-SOD activity of young, fully expanded leaves of the primary transformants of alfalfa (M. sativa L.) expressing the Mn-SOD transgene*

Values represent units of activity μg^{-1} of postcolumn protein, where one unit inhibits by 50% the reduction of Cyt c by superoxide.

Plant	Total SOD	Mn-SOD
RA3	13.0	2.34
RA3-MitSOD-5	24.3[a]	4.57[a]
RA3-MitSOD-38	24.7[a]	3.89[a]
RA3-ChlSOD-30	25.3[a]	3.43[a]
RA3-ChlSOD-64	24.7[a]	2.88 NS

[a] Significant difference between the transgenic plant and the nontransgenic RA3 control at the 5% level of probability according to a protected *t* test. $n = 3$.

Table II. *Effect of the Mn-SOD transgene on electrolyte leakage (conductance) and photosynthetic quantum yield of transgenic alfalfa (M. sativa L.) after treatment with 333 μM acifluorfen*

	Conductance[a]	Quantum Yield
	$\mu\Omega^{-1}$ cm^{-2} 20 mg^{-1} fresh weight	$\Phi_{II} = (F_{max} - F_o)/F_{max}$
Nonstressed	0	0.79
RA3	91	0.16
RA3-MitSOD-5	96	0.23
RA3-MitSOD-38	72[b]	0.13
RA3-ChlSOD-30	73[b]	0.15
RA3-ChlSOD-64	61[b]	0.18

[a] Conductance values represent the difference between nonstressed and stressed samples. [b] Statistically significant difference between the stressed transgenic and RA3 at the 5% level of probability according to analysis of variance and a protected LSD test.

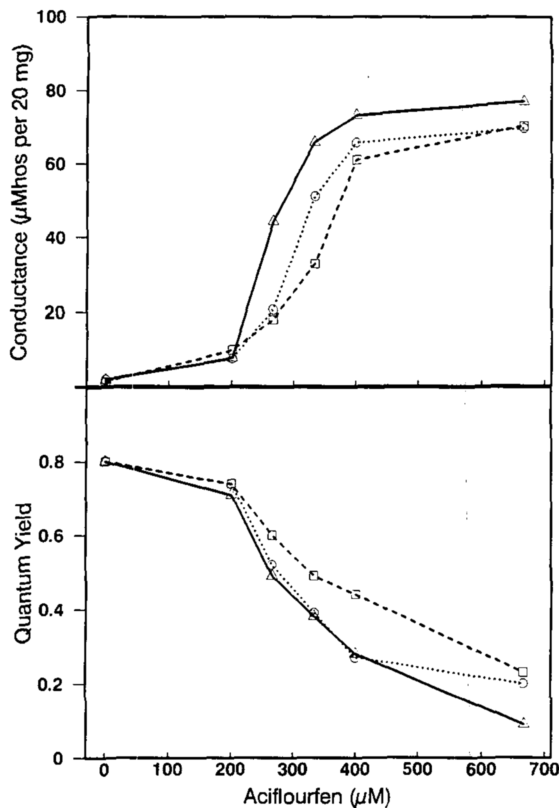

Figure 4. Effect of acifluorfen concentration on electrolyte leakage (conductance) and photosynthetic quantum yield of leaves from alfalfa (*M. sativa* L.) plants RA3 (△), RA3-MitSOD-38 (○), and RA3-ChlSOD-64 (□). The LSD at the 5% level of probability is 7.7 for conductance and 0.06 for quantum yield.

stress tolerance of the plants was best quantified by shoot dry matter production (regrowth) after the stress treatments, which integrated lethal and sublethal injuries at the cellular level to the crown and root tissues.

The regrowth of RA3 did not differ significantly from any of the transgenic plants when not subjected to stress. Analysis of variance of the regrowth data after freezing stress indicated that there were no statistically significant two- or three-way interactions among the freezing temperature, plant, or harvest treatments; therefore, it was possible to examine the main effects directly. Increasing the intensity of the freezing stress from −8 to −16°C progressively reduced regrowth with an average lethal temperature causing 50% death of −12.5°C. The plants had significantly different amounts of regrowth ($P = 0.0016$) after the freezing stress. One chloroplast-targeted transformant, RA3-ChlSOD-30, produced more than twice the amount of regrowth averaged across all intensities of freezing stress compared to RA3 (Table III). Regrowth of this transgenic plant was greater than RA3 at every freezing temperature from −8 to −16°C (data not shown). Two of the three other transformants, RA3-MitSOD-5 and RA3-ChlSOD-64, had greater regrowth after freezing than RA3, but this difference was not statistically significant at the 5% level of probability.

Table III. *Stress tolerances of the primary transformants of alfalfa (M. sativa L.) expressing the Mn-SOD transgene*

Values for freezing represent the average shoot regrowth (mg dry matter per plant) after freezing to temperatures of −8 to −16°C, averaged over two subsequent harvests.

Plant	No Stress	Freezing
RA3	229	46
RA3-MitSOD-5	202	73
RA3-MitSOD-38	181	54
RA3-ChlSOD-30	220	108[a]
RA3-ChlSOD-64	182	75

[a] Statistically significant difference between the transgenic plant and RA3 at the 5% level of probability according to analysis of variance and a protected LSD test. $n = 3$.

Analysis of the F_1 Progeny

To determine whether the increased tolerance of freezing was due to the presence of the Mn-SOD transgene or whether it was due to tissue culture-induced or epigenetic variation, inheritance of these traits in the F_1 progeny was examined. The primary transformant, RA3-ChlSOD-30, which was more tolerant of freezing, was selected. To determine the number of functional genetic loci that had been integrated into the genome of RA3-ChlSOD-30, native PAGE analysis of Mn-SOD isozymes in the F_1 progeny was conducted (Table IV). Segregation ratios in the F_1 progeny from RA3-ChlSOD-30 × C2-3 were 29 Mn-SOD$^+$ to 39 Mn-SOD$^-$, which followed the expected 1:1 ratio for a one-gene insertion. A two-gene insertion into an autotetraploid genome, such as alfalfa, would be expected to give a 5:1 ratio of Mn-SOD$^+$ to Mn-SOD$^-$. Reciprocal crosses did not differ. Therefore, the isozyme data indicated that RA3-ChlSOD-30 had a functional Mn-SOD transgene that was transmitted to the progeny in accordance with a one-locus insertion model.

PCR amplification of F_1 plants from the cross RA3-ChlSOD-30 × C2-3 with primers specific for the Mn-SOD transgene showed that in the Mn-SOD$^+$ plants 97% (28 of 29) had the expected 545-bp fragment (Fig. 5, top; Table IV).

Table IV. *Inheritance of the Mn-SOD transgene in the F_1 progeny of the cross RA3-ChlSOD-30 × C2-3 according to native PAGE analysis of Mn-SOD isozymes and PCR analysis of the Mn-SOD and npt-II sequences*

Values represent the number of plants in the tested progeny that had (+) or did not have (−) the transgene according to each test.

Mn-SOD		npt-II	No. of Plants
Isozyme	PCR	PCR	
+	+	+	24
+	+	−	4
+	−	+	1
+	−	−	0
−	+	+	12
−	+	−	3
−	−	+	18
−	−	−	6

PCR amplification of DNA from these same plants with primers for the *neo* gene showed that 86% (25 of 29) had the expected 732-bp fragment (Fig. 5, bottom; Table IV). These results confirmed the expected co-segregation of the additional Mn-SOD isozyme with the PCR fragments of the Mn-SOD and *neo* transgenes.

PCR amplification of DNA from 39 F_1 plants from the cross RA3-ChlSOD-30 × C2-3, which were classed as Mn-SOD$^-$ by native PAGE, showed that 15 plants contained the Mn-SOD transgene fragment and 24 plants did not. This suggested that there was another independent insertion of a nonfunctional Mn-SOD transgene in this alfalfa genome.

PCR amplification of DNA from the Mn-SOD$^-$ plants with primers specific for the *neo* gene indicated that 30 of 39 plants that did not have MnSOD expression had the expected amplified 732-bp fragment from the *neo* gene. This segrrgation in the MnSOD$^-$ plants is best explained by the presence of two additional loci. Thus, there were apparently three loci of the *neo* gene and two loci (one functional and one nonfunctional) of the Mn-SOD gene inserted in the genome of RA3-ChlSOD-30. This suggested that there were three insertion events: one with the full-length fragment from the left to right border and two with partial segments, one containing part of the Mn-SOD and the other containing only the *neo* gene.

The F_1 progeny from the cross RA3-ChlSOD-30 × C2–3 were grouped into Mn-SOD$^+$ and Mn-SOD$^-$ populations according to Mn-SOD isozyme analysis by native PAGE and

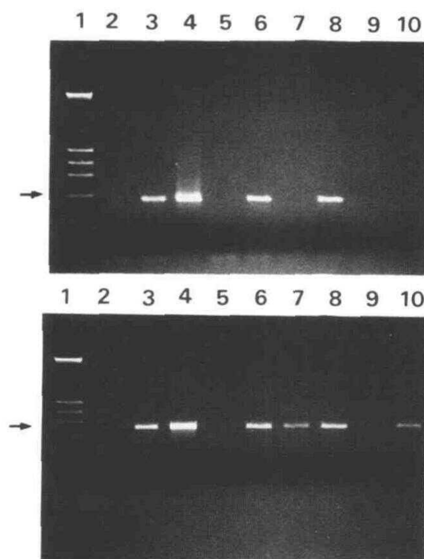

Figure 5. PCR amplification of DNA from the progeny of the cross RA3-ChlSOD-30 × C2-3 using primers specific for the chloroplast transit peptide sequence (CP) and an internal sequence of the Mn-SOD cDNA (top) and primers specific for the npt-II gene (bottom). Lanes 1, λ DNA *Hin*dIII/ϕX174 DNA *Hae*III digest; lanes 2, C2-3; lanes 3, RA3-ChlSOD-30; lanes 4, pChlSOD plasmid; lanes 5, water; lanes 6, Mn-SOD$^+$ plant 1; lanes 7, Mn-SOD$^+$ plant 2; lanes 8, Mn-SOD$^-$ plant 1; lanes 9, Mn-SOD$^-$ plant 2; lanes 10, Mn-SOD$^-$ plant 3. The arrows indicate the expected fragment.

Table V. *Regrowth of two alfalfa populations differing in expression of the Mn-SOD transgene after freezing*

The progeny of the cross RA3-ChlSOD-30 × C2-3 were separated into Mn-SOD$^+$ and Mn SOD$^-$ populations. After acclimation at 2°C for 4 weeks, the plants were frozen to temperatures from −8 to −16°C and allowed to regrow for two 28-d cycles of regrowth. Regrowth of the two populations not subjected to freezing was not significantly different and averaged 2.58 g/plant at each cycle of regrowth. Values are the dry weight (g) of shoot regrowth averaged across the five freezing temperatures shown in Figure 6. LSD at 5% level of probability is 0.36.

Cycle of Regrowth	Mn-SOD$^+$	Mn-SOD$^-$
1	1.06	0.98
2	1.87	1.26

subjected to freezing stress. The regrowth of plants from the two populations did not differ significantly if they were not subjected to stress. It should be noted that the regrowth in this freezing test (Table V) is greater than in the previous experiment (Table III) because the plants in this experiment were grown from seed in large pots, whereas previously small root trainers were used to propagate the cuttings. As the intensity of stress increased, regrowth declined. Differences in regrowth between the populations were apparent at −10, −12, and −14°C but not at −8 or −16°C (Fig. 6). When averaged across all freezing temperatures from −8 to −16°C, the Mn-SOD$^+$ population had an average regrowth that was significantly ($P = 0.015$) greater than the Mn-SOD$^-$ population. Therefore, the increase in freezing tolerance was co-segregating with the Mn-SOD transgene in the F_1 progeny of RA3-ChlSOD-30. Increased tolerance was more dramatic when measured on the second cycle of regrowth (Table V).

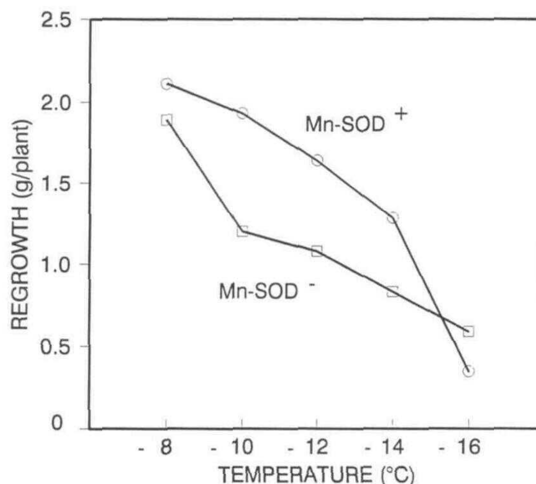

Figure 6. Effect of freezing temperature on the regrowth of two alfalfa (*M. sativa* L.) populations from the cross RA3-ChlSOD-30 × C2-3 differing in expression of the Mn-SOD transgene. Same experiment as described in Table V. Values are averaged over two cycles of regrowth. The LSD at 5% level of probability is 0.56.

DISCUSSION

The expression of the cDNA encoding Mn-SOD from *N. plumbaginifolia* in alfalfa resulted in the presence of a novel SOD isozyme in leaf extracts. This allowed the rapid screening of a large number of primary transformants using native PAGE to identify plants with high expression of the Mn-SOD transgene in the presence of native Mn-SOD expression. We anticipated substantial variability in expression among the putatively independent transformants because this is common in transformation experiments (Dean et al., 1988), and in fact only 20 of the 73 plants exhibiting npt-II activity also exhibited Mn-SOD transgene expression. This disproportionate selection may have been due to our inability to detect low levels of Mn-SOD transgene expression.

One of the transgenic alfalfa plants, RA3-ChlSOD-30, was particularly interesting because it had a greater total SOD activity in leaves, greater tolerance of the herbicide acifluorfen, and greater regrowth after freezing stress than did RA3. The observation that tolerance of these chemical and environmental stresses was altered by the presence of the Mn-SOD transgene indicates a common involvement of activated oxygen in the injury response. The increased tolerance of freezing was heritable and co-segregated with the active Mn-SOD transgene, suggesting that enhanced stress tolerance was the result of the Mn-SOD transgene and not an artifact of the tissue culture process. Transgenic tobacco plants transformed with these same vectors, pMitSOD and pChlSOD, exhibited increased tolerance of paraquat, a superoxide-generating herbicide (Bowler et al., 1991).

The presence of the Mn-SOD transgene gave an increase in the rate of regrowth from alfalfa crowns after being subjected to a freezing stress. Regrowth of shoots from the defoliated crowns of alfalfa is due largely to the mobilization of carbohydrate and protein reserves from the root and crown. Therefore, regrowth integrates a number of processes related to the general health of the plant. It requires active apical meristems or shoot buds, an active vascular transport system, and viable root cells to hydrolyze the stored reserves. Disruption of any of these processes would be expected to reduce regrowth, which may explain why it is a much better indicator of stress tolerance than simply survival (Bowley and McKersie, 1990). The enhanced regrowth after freezing stress is not due to a general enhancement of vigor because there was no difference among the primary transformants or the progeny if they were not subjected to stress.

A relationship between freezing and oxidative stresses was originally postulated based on studies detailing the injury symptoms in the microsomal membranes isolated from winter wheat (*Triticum aestivum*) crowns after freezing. These biochemical and biophysical changes were simulated in vitro by oxygen free radical treatment (Kendall and McKersie, 1989). Both in situ freezing and in vitro treatment with superoxide increased the gel-to-liquid crystalline phase transition temperature, promoted deesterification of membrane phospholipids, and led to the accumulation of free fatty acids in the membrane bilayer. Also, tolerance of the free radical-generating herbicides paraquat (Kendall and McKersie, 1989) and acifluorfen (G. Bridger, D.E. Falk, and B.D. McKersie, unpublished data) increased during the acclimation of winter wheat seedlings to freezing stress. Although speculative, Mn-SOD is probably not changing the primary site that perceives the freezing stress. It is more likely that Mn-SOD functions to minimize the deleterious accumulation of activated oxygen that occurs as a consequence of the primary freezing injury. Enhanced Mn-SOD activity may enable the injury to be contained within a few cells, thereby preventing development of secondary injury and allowing repair of the primary lesion. This assumption is consistent with the observation that differences among the plants were greatest at the second cycle of regrowth, not the first.

The Mn-SOD transgene in the vectors pChlSOD and pMitSOD was under the control of the cauliflower mosaic virus 35S promoter. This is generally considered to be a constitutive promoter, but its response to low temperature during either acclimation or freezing is not known. Although this study of the transgenic Mn-SOD alfalfa suggests that progress might be made in the development of plants with enhanced environmental stress tolerance through the manipulation of SOD and other scavengers of activated oxygen, more precise regulation of these genes during acclimation and freezing is probably required to achieve significant advances. We also need more information concerning the effects of acclimation on the activity of SOD and other free radical-scavenging systems in the various tissues of the alfalfa plant. Finally, the effects of the SOD transgene on the activity of other free radical-scavenging systems must be evaluated. The results presented here indicate that this detailed evaluation is necessary to understand the relationship between oxidative and freezing stress.

ACKNOWLEDGMENTS

The skilled technical assistance of Danuta Gidzinski and Lori Wright with the growth and analysis of the transgenic plants and critical review of the manuscript by Olivier Leprince are gratefully acknowledged.

Received March 26, 1993; accepted July 29, 1993.
Copyright Clearance Center: 0032-0889/93/103/1155/09.

LITERATURE CITED

Bannister JV, Bannister WH, Rotilio G (1987) Aspects of the structure, function and applications of superoxide dismutase. CRC Crit Rev Biochem **22:** 111–180

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:** 276–287

Bowler C, Alliotte T, De Loose M, Van Montagu M, Inzé D (1989) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. EMBO J **8:** 31–38

Bowler C, Slooten L, Vandenbranden S, De Rycke R, Botterman J, Sybesma C, Van Montagu M, Inzé D (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J **10:** 1723–1732

Bowler C, Van Montague M, Inzé D (1992) Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol **43:** 83–116

Bowley SR, McKersie BD (1990) Relationships among freezing, low temperature flooding, and ice encasement tolerance in alfalfa. Can J Plant Sci **70:** 227–235

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bridges SM, Salin ML (1981) Distribution of iron-containing superoxide dismutase in vascular plants. Plant Physiol **68:** 275–278

Dean C, Jones J, Favreau M, Dunsmuir P, Bedbrook J (1988) Influence of flanking sequences on the variability in expression levels of an introduced gene in transgenic tobacco plants. Nucleic Acids Res **16:** 9267–9283

D'Halluin K, Botterman J, De Greef W (1990) Engineering of herbicide-resistant alfalfa and evaluation under field conditions. Crop Sci **30:** 866–871

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19:** 1349

Foyer CH, Lelandais M, Edwards EA, Mullineaux PM (1991) The role of ascorbate in plants, interactions with photosynthesis and regulatory significance. *In* EJ Pell, KL Steffen, eds, Active Oxygen/Oxidative Stress and Plant Metabolism. American Society of Plant Physiologists, Rockville, MD

Gentry B, Briantais JM, Baker NR (1989) The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta **990:** 87–92

Giannopolitis CN, Ries SK (1977) Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol **59:** 309–314

Herrera-Estrella L, De Picker A, Van Montagu M, Schell J (1983) Expression of chimeric genes transferred into plant cell using Ti plasmid-derived vector. Nature **310:** 115–120

Hunter MIS, Hetherington AM, Crawford RMM (1983) Lipid peroxidation—a factor in anoxia intolerance in Iris species? Phytochemistry **22:** 1145–1147

Jacobs JM, Jacobs NJ, Sherman TD, Duke SO (1991) Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporhyrin in organellar and plasma membrane enriched fractions of barley. Plant Physiol **97:** 197–203

Kendall EJ, McKersie BD (1989) Free radical and freezing injury to cell membranes of winter wheat. Physiol Plant **76:** 86–94

Leprince O, Deltour R, Thorpe PC, Atherton NM and Hendry GAF (1990) The role of free radicals and radical processing systems in loss of desiccation tolerance in germinating maize (*Zea mays* L.). New Phytol **116:** 573–580

Malan C, Greyling MM, Gressel J (1990) Correlation between Cu/Zn superoxide dismutase and glutathione reductase, and environmental and xenobiotic stress tolerance in maize inbreeds. Plant Sci **69:** 157–166

McKersie BD, Senaratna T, Walker MA, Kendall EJ, Hetherington PR (1988) Deterioration of membranes during aging in plants: evidence for free radical mediation. *In* L Nooden, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, New York, pp 441–463

Odell J, Nagy F, Chua N (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810–812

Perl A, Perl-Treves R, Galili G, Aviv D, Shalgi E, Malkin S, Galun E (1993) Enhanced oxidative stress defense in transgenic potato expressing tomato Cu, Zn superoxide dismutases. Theor Appl Genet **85:** 568–576

Pitcher LH, Brennan E, Hurley A, Dunsmuir P, Tepperman JM, Zilinskas BA (1991) Overproduction of petunia chloroplastic copper zinc superoxide dismutase does not confer ozone tolerance in transgenic tobacco. Plant Physiol **97:** 452–455

Pitel JA, Cheliak WM (1984) Effect of extraction buffers on characterization of isozymes from vegetative tissues of 5 conifer species. A User's Manual. Petawawa National Forestry Institute, Canada Forestry Service

Price AH, Hendry GAF (1991) Iron-catylized oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. Plant Cell Environ **14:** 477–484

Salin ML (1991) Chloroplast and mitochondrial mechanisms for protection against oxygen toxicity. Free Radical Res Commun **12:** 851–858

Sen Gupta A, Heinen JL, Holoday AS, Burke JJ, Allen RD (1993) Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc Natl Acad Sci USA **90:** 1629–1633

Senaratna T, Mackay CE, McKersie BD, Fletcher RA (1988) Uniconazole-induced chilling tolerance in tomato and its relationship to antioxidant content. J Plant Physiol **133:** 56–61

Senaratna T, McKersie BD, Stinson RH (1985) Simulation of dehydration injury to membranes from soybean axes by free radicals. Plant Physiol **77:** 472–474

Spychalla JD, Desborough SL (1990) Superoxide dismutase, catalase, and α-tocopherol content of stored potato tubers. Plant Physiol **94:** 1214–1218

Tepperman JM, Dunsmuir P (1990) Transformed plants with elevated level of chloroplastic SOD are not more resistant to superoxide toxicity. Plant Mol Biol **14:** 501–511

Van Toai T, Bolles CS (1991) Postanoxia injury in soybean (*Glycine max*) seedling. Plant Physiol **97:** 588–592

Walker K, Yu P, Sato S, Jaworski E (1978) Hormonal control of organ formation in callus of *Medicago sativa* L. cultured *in vitro.* Am J Bot **65:** 654–659

Wise RR, Naylor AW (1987) Chilling-enhanced photooxidation. Evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. Plant Physiol **83:** 278–282