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Water-Deficit Tolerance and Field Performance of Transgenic Alfalfa Overexpressing Superoxide Dismutase[1]

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Transgenic alfalfa (*Medicago sativa*) expressing Mn-superoxide dismutase cDNA tended to have reduced injury from water-deficit stress as determined by chlorophyll fluorescence, electrolyte leakage, and regrowth from crowns. A 3-year field trial indicated that yield and survival of transgenic plants were significantly improved, supporting the hypothesis that tolerance of oxidative stress is important in adaptation to field environments.

Many of the degenerative reactions associated with several biotic, abiotic, and xenobiotic stresses are mediated by toxic, reactive oxygen intermediates formed from superoxide, such as the hydroxyl radical (Scandalios, 1993; Allen, 1995). These stresses include the herbicide paraquat (Bowler et al., 1991; Herouart et al., 1993), ozone (Van Camp et al., 1994), anoxia (Monk et al., 1989), pathogens (Mehdy, 1994), desiccation (Senaratna et al., 1985a, 1985b), and freezing (Kendall and McKersie, 1989; McKersie et al., 1993). The mechanisms to detoxify oxygen radicals are varied, and the complex interactions among the antioxidants in different subcellular compartments, cells, and tissues are only now being elucidated. SOD is an essential component of these defense mechanisms because it dismutates two superoxide radicals to produce hydrogen peroxide and oxygen (Scandalios, 1993; Allen, 1995). The observation that water deficiency caused the chloroplasts of wheat (*Triticum aestivum* L.) to reduce oxygen to superoxide because of a drought-impaired electron transport system (Price et al., 1989) prompted us to hypothesize that plants overexpressing SOD might have improved tolerance of water deficit. Previously, an Mn-SOD cDNA from *Nicotiana plumbaginifolia* was introduced into alfalfa; the primary transformants and their F_1 transgenic progeny showed increased survival and vigor after exposure to sublethal freezing stress (McKersie et al., 1993). We now report the results of two further experiments with these transgenic plants, indicating that manipulation of genes associated with oxidative stress tolerance can also improve survival and vigor after exposure to water deficit in controlled environments and over three winters in natural field environments. These preliminary studies indicate that a rigorous analysis of the oxidative stress response will aid in the genetic improvement of environmental stress tolerance in crop plants.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L.) clone RA3 was previously transformed by *Agrobacterium tumefaciens* with binary vectors that contained different constructs developed to overexpress the Mn-SOD cDNA from *Nicotiana plumbaginifolia* (Bowler et al., 1991). Expression of the Mn-SOD cDNA was controlled by the cauliflower mosaic virus 35S promoter, and a transit peptide sequence was included to target the protein to either the MitSOD or ChlSOD. Segregation analysis of F_1 progeny from the primary transformants for the presence of the transgenes by PCR and for the gene product (SOD activity) by native PAGE indicated that each transformant had a one-locus insertion of the T-DNA (Chen, 1993; McKersie et al., 1993).

Because alfalfa is a heterozygous, cross-pollinating, autotetraploid plant, seed from these plants would be segregating for not only the transgenes but other genes associated with stress tolerance, growth, and vigor. Therefore, seed propagation was avoided intentionally because it was presumed that cuttings of the primary transgenic plants would give the most uniform genetic material to compare the relative effects of the transgenes. Rooted cuttings were grown in plastic pots in Turface (Applied Industrial Material, Deerfield, IL), with a 16-h photoperiod (400 μmol m^{-2} s^{-1} PPFD at the top of the leaf canopy), 21/18°C day/night temperatures, and 65% RH. The plants were fertilized five times per week with a dilute solution containing 20–20–20 N, P, K fertilizer (Plant Products, Brampton, Ontario, Canada).

Total SOD activity was measured in leaves, crowns, and roots of 10 to 16 plants, established from cuttings of each genotype (control or transgenic), using the Cyt *c* reduction method (Spychalla and Desborough, 1990). Protein was determined using the method of Bradford (1976). Units of activity were calculated as described by Giannopolitis and Ries (1977).

Four water-deficit experiments were conducted in a controlled environment at different times. For each experiment a

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Abbreviations: ChlSOD, Mn-SOD targeted to the chloroplast; F_m, maximal fluorescence; F_v, variable fluorescence; MitSOD, Mn-SOD targeted to the mitochondria; SOD, superoxide dismutase.

minimum of three replicates was used; a single plant propagated from a cutting was considered as the experimental unit for statistical analysis. Alfalfa is a perennial forage legume; therefore, to establish a crown and root system, cuttings were grown through at least one cycle of defoliation before use in the water-deficit experiments and reselected for morphological similarity to ensure that a similar degree of water deficit was experienced by each plant. Sixteen days after defoliation, when the stem length was approximately 30 cm (stage 1; Kalu and Fick, 1983), water-deficit stress was induced by withholding water for up to 9 d.

In the first experiment, leaf water potential at midday was determined using an Arimad-2 pressure chamber (ARI Kfar Charuv-Water Supply Accessories, Tel Aviv, Israel). Data were statistically analyzed using a completely randomized model.

In the second experiment, chlorophyll fluorescence was measured to estimate injury to PSII. Plants were dark adapted for 30 min, and then young, fully expanded leaves were detached and chlorophyll fluorescence was measured using a Hansatech modulated fluorescence measurement system (Hansatech, Norfolk, UK). The initial fluorescence was measured with modulated light (yellow light-emitting photodiode). F_m was measured with a 1-s pulse of 2000 μmol m^{-2} s^{-1}. F_v/F_m was calculated as a measure of the quantum efficiency of PSII (Krause and Weis, 1991). Data were statistically analyzed as a randomized complete block (replications) model.

In the third experiment, membrane injury was measured by electrolyte leakage. Three leaf discs (0.5 cm in diameter) from the third or fourth leaves were incubated in 10 mL of H_2O. Conductivity readings were expressed as the percentages of conductivity after 60 min relative to conductivity after boiling and statistically analyzed as a completely randomized model.

In the fourth experiment, plants were deprived of water and then defoliated 5 cm from the crowns and irrigated daily. Shoot regrowth was removed in two consecutive harvests at 16 and 32 d. Data were statistically analyzed using a completely randomized model.

The field trial was conducted at the Elora Research Station (Elora, Ontario, Canada) following protocols authorized by Agriculture Canada (test no. T92-UOG-ALF-01, approved February 14, 1992). The plots were established in spring 1992 by transplanting rooted cuttings of each of the transgenic and control genotypes. Soil at this location is a clayed brunisolic gray-brown luvisol-London. Fertilizer (P and K) was applied following the last harvest of each year according to soil test analysis. The test was arranged in a randomized complete block design with four replications (blocks), and the plot size was 1 × 1.5 m. Plants were harvested twice in the year of transplanting, 1992 (middle July and late August) and three times in 1993 and 1994 (early June, middle July, and late August) prior to the onset of flowering. Total herbage yield was calculated as the sum of these harvests. Stand counts were taken at the beginning of the experiment and in the fall and spring of each year and expressed as a percentage relative to the initial counts.

Statistical analysis was performed using the Statistical Analysis System, version 6.03, Proc GLM (SAS Institute Inc., Cary, NC). Significant differences were determined according to the analysis of variance at the 5% level of probability, and mean comparisons were made using a protected LSD test.

RESULTS

Leaves and roots of the two transgenic plants, RA3-ChlSOD-30 and RA3-MitSOD-5, had higher total SOD activity than the RA3 control, but activity in the crowns was slightly lower (Table I). Variability in SOD activity among propagules was similar for the RA3 controls and for the transgenic plants, suggesting that the transgene was neither lost nor silenced using this propagation method, but molecular analysis was not conducted in this set of experiments to confirm this. Also, the RA3 control used in this and subsequent experiments was not exposed to cell culture conditions, whereas the transgenic plants had been during transformation. Somaclonal variations may have existed, but the transgenic plants were fertile (Chen, 1993) and displayed no visual differences from RA3.

Under our treatment conditions to induce water deficit, leaf water potential changed coincidently in control and transgenic plants. Until 3 d, leaf water potential remained relatively constant, but a dramatic reduction occurred in all plants 5 d after withholding water (Table II). These treatment conditions were repeated in the following experiments.

The chlorophyll fluorescence parameter, F_v/F_m, was used to determine injury to PSII during water deficit (Fig. 1). In nonstressed plants, the F_v/F_m was approximately 0.8 and remained unchanged after withholding water for 3 d. At 5 d, F_v/F_m decreased drastically in RA3 but was unchanged for both SOD transformants, suggesting that this degree of water deficit caused less injury in the transgenic plants.

Disruption of membrane integrity was estimated by leakage of cytoplasmic solutes from leaf discs. After 1 or 3 d of water deficit, there were no statistically significant differences in electrolyte leakage among the nontransgenic and SOD-transgenic plants (Fig. 2). After 5 d of water deficit, electrolyte leakage from leaves increased but significantly less in the SOD-transgenic plants, again suggesting less injury in the transgenic plants.

Regrowth of shoots from defoliated crowns and roots, used to indicate the viability of these tissues, their level of stored nutrients, and their ability to mobilize and translocate these nutrients to the developing shoot, was not dif-

Table I. *Total SOD activities in leaves, crowns, and roots of RA3 and transgenic alfalfa (M. sativa L.) expressing an Mn-SOD cDNA with ChlSOD or MitSOD transit peptides*

Plants were replicated by cuttings and sampled under normal growing conditions. Values are the mean ± SE of 10 to 16 replicates.

Sample	RA3	RA3-ChlSOD-30	RA3-MitSOD-5
		units mg^{-1} protein	
Leaves	6.9 ± 1.1	10.5 ± 0.4	17.3 ± 3.0
Crowns	46.1 ± 3.3	41.3 ± 3.6	41.7 ± 3.9
Roots	19.0 ± 1.0	21.8 ± 2.3	30.2 ± 5.9

Table II. *Effect of withholding water in a controlled environment on the leaf water potential of RA3 and transgenic alfalfa (M. sativa L.) expressing an Mn-SOD cDNA with ChlSOD or MitSOD transit peptides*

RA3, RA3-ChlSOD-30, and RA3-MitSOD-5 were individual plants that were replicated from cuttings and grown in separate pots for 16 d after defoliation prior to imposition of the water deficit. The experiment was analyzed statistically using a completely randomized design with n = 1–5 for each plant × day of treatment; LSD for comparison between means of each plant × day of treatment at the 5% level of probability = 0.16; LSD for comparison between means for each day at the 5% level of probability = 0.10.

Days without Water	Leaf Water Potential			
	RA3	RA3-ChlSOD-30	RA3-MitSOD-5	Mean
		MPa		
0	−0.65	−0.58	−0.54	−0.59
1	−0.74	−0.78	−0.65	−0.71
3	−0.55	−0.71	−0.88	−0.72
5	−1.49	−1.66	−1.65	−1.61
7	−1.59	−1.64	−1.59	−1.61
9	−1.95	−1.99	−1.86	−1.93

ferent among treatments or genotypes when the water deficit was imposed for 5 d or less (Fig. 3). After 7 and 9 d, the relative regrowth of RA3 and RA3-ChlSOD-30 decreased but not RA3-MitSOD-5, which maintained a relatively high vigor equivalent to almost 80% of its nonstressed value at 9 d.

The field performance of these transgenic plants expressing Mn-SOD was compared using two independent transformants of ChlSOD, two of MitSOD, the RA3 control, and two plants randomly selected from commercial cultivars,

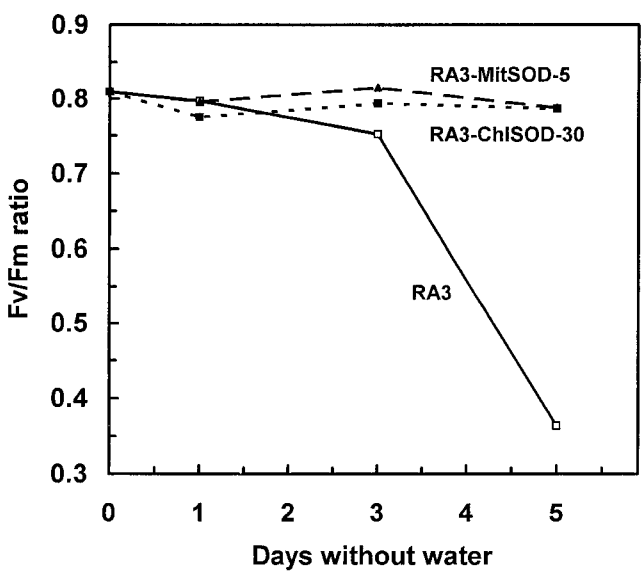

Figure 1. Photosynthetic efficiency of young leaves of alfalfa (*M. sativa*) during water-deficit stress as determined by the chlorophyll fluorescence parameter F_v/F_m. Values for F_v/F_m were not statistically different among days or plants at d 0, 1, and 3, but were significantly different between RA3 and each of the transgenic plants at d 5 according to analysis of variance. LSD at the 5% level of probability (n = 3) for comparison between means was 0.12.

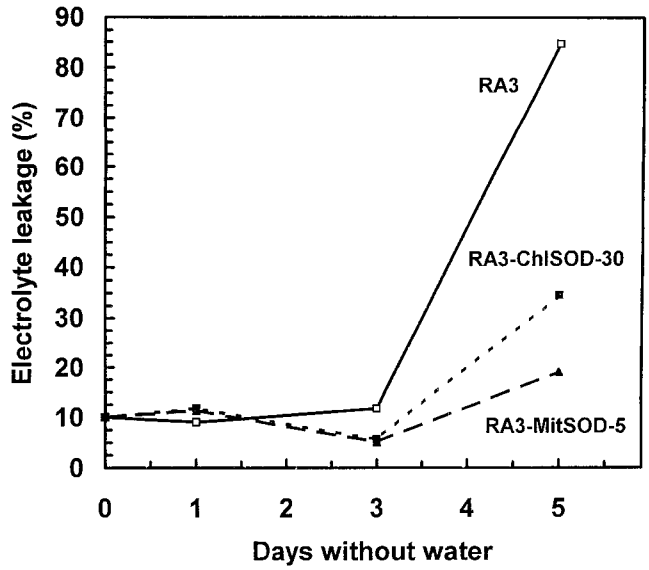

Figure 2. Electrolyte leakage from leaves of alfalfa (*M. sativa*) during water-deficit stress. Values are expressed as the amounts of electrolyte leakage after 60 min relative to total leakage after boiling expressed as a percentage. Values were not significantly different among plants at d 0, 1, and 3, but were significantly different at d 5 according to analysis of variance. LSD at the 5% level of probability (n = 4–12) for comparison between means was 11%.

designated A7 and A9. RA3 is not an agronomic genotype of alfalfa and is not adapted to the Ontario climate because of a lack of winterhardiness, disease tolerance, and other unidentified attributes. In a previous transgenic field trial that we conducted at this Elora site (test no. P658916, authorized by Agriculture Canada on September 27, 1989), RA3 showed only 34% survival following the first winter (S.R. Bowley and B.D. McKersie, unpublished data). RA3 was used in the transformation experiments, because at the time (1989) it was one of the few genotypes of alfalfa that would form somatic embryos in culture and therefore could be transformed. The majority of plants adapted to Ontario's field environment at that time were nonembryogenic. This has subsequently changed because of an intensive plant-breeding effort (Bowley et al., 1993).

In the field trial, the 1992 season was cooler with higher precipitation than normal, and the 1994 season was warmer with lower precipitation than normal. Precipitation between April 1 and September 30 was 750, 588, and 456 mm in 1992, 1993, and 1994, respectively; the crop heat unit accumulation (Brown and Bootsma, 1993) from May 10 to September 30 was 2504, 2576, and 2710, respectively. The yield of RA3 alfalfa was dramatically lower than the two commercial type genotypes, A7 and A9 (Table III). The transgenic plants had almost twice the yield of RA3 in 1992, the year of transplanting, before they experienced any winterkill. This may be an expression of other types of abiotic stress tolerance, perhaps tolerance of transplanting (water deficit), but changes in other attributes, even disease tolerance, cannot be discounted at this time. In 1993, after one winter, yields of the transgenic plants were again approximately double those of RA3, and in 1994, after two win-

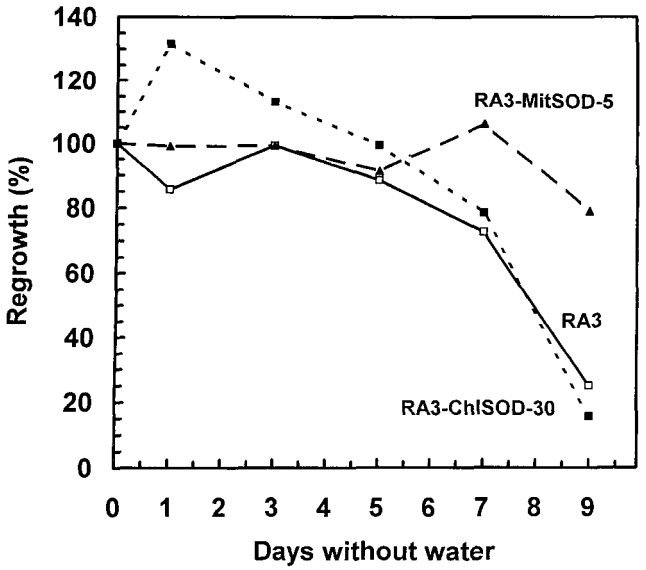

Figure 3. Regrowth of shoot dry matter from defoliated crowns and roots of alfalfa (*M. sativa*) after water-deficit stress. Regrowth was determined as total shoot dry matter from two consecutive harvests 16 and 32 d after water-deficit stress, defoliation, and rewatering. Values are expressed relative to nonstressed plants. Nonstressed values were: RA3, 2.78 ± 0.07 (SE) g dry matter/plant; RA3-ChlSOD-30, 3.28 ± 0.16; RA3-ChlSOD-5, 2.60 ± 0.24 (n = 2–4). LSD for comparison between means of each plant × day of treatment at the 5% level of probability = 0.63 g/plant or 22%.

ters, they were 3- to 5-fold higher. This was in part due to increased survival (Table IV). After experiencing two winters, between 26 and 66% of the transgenic plants had survived compared to only 17% survival for RA3. An analysis of SOD activity and other antioxidants in different subcellular compartments, cell types, tissues, and organs of the alfalfa plants in the field was not conducted, but the relative level of SOD expression from the native genes and transgenes might differ between the controlled environment (Table I) and the field. This analysis now seems necessary to fully understand these effects of SOD transgene expression.

DISCUSSION

Antioxidant levels and the activities of oxygen free radical-scavenging enzymes have been correlated with tolerance to several different environmental stresses. As plants acclimate or experience sublethal levels of stress, their potential to scavenge free radicals often increases (Senaratna et al., 1985b; Kendall and McKersie, 1989; Bridger et al., 1994). The biophysical and biochemical changes in cell membranes that occur following the exposure of plants to a lethal stress (freezing or desiccation) can be mimicked when isolated membranes are treated with free radicals in vitro (Senaratna et al., 1985a, 1987; Kendall and McKersie, 1989). Also, transgenic plants that overexpress one or more components of the Halliwell-Asada pathway, such as SOD or glutathione reductase, often have increased tolerance to free radical-generating herbicides (Bowler et al., 1991; Herouart et al., 1993) and/or environmental stresses, such as

Table III. *Herbage yield of transgenic alfalfa (M. sativa L.) expressing an Mn-SOD cDNA in field trials*

The test was conducted at Elora (Ontario) and established by transplanting rooted cuttings of each plant in 1- × 1.5-m plots. Plots were harvested twice in the year of transplanting (1992) and three times in 1993 and 1994. RA3 is the nontransgenic control, and A7 and A9 are plants randomly selected from a commercial cultivar. Values are the sum of all harvests in one year. LSD (0.05) is between means in a column at the 5% level of probability (n = 4).

Plant	1992	1993	1994
		g dry matter m^{-2}	
RA3	151	265	37
RA3-ChlSOD-30	291	465	140
RA3-ChlSOD-64	286	652	198
RA3-MitSOD-5	189	353	135
RA3-MitSOD-38	266	510	193
A7	342	956	860
A9	321	920	848
LSD (0.05)	130	150	104

chilling (Gupta et al., 1993a, 1993b), ozone (Van Camp et al., 1994), and freezing (McKersie et al., 1993), although this relationship is not always observed (Pitcher et al., 1991).

Our analysis of the Mn-SOD transgenic alfalfa plants indicates that the transformation produced a new Mn-SOD isozyme, increased SOD activity, enhanced vigor after freezing (McKersie et al., 1993) and water deficit in controlled environments, and enhanced herbage yield and survival in the field. Collectively, our data support the hypothesis that increased tolerance to several different environmental stresses can be achieved through increased tolerance of oxidative stress. Yet, it is somewhat surprising that this positive response occurs as the consequence of the overexpression of a single transgene, especially when both genetic and physiological studies have clearly shown that tolerance to freezing (Stone et al., 1993) and to drought (Estill et al., 1993) are quantitative genetic traits that are not easily manipulated by plant breeding (Fowler et al., 1983). The observed effects of the Mn-SOD transgene on alfalfa's tolerance to freezing and water deficit and its field performance might simply be due to the increased efficiency by

Table IV. *Survival of alfalfa (M. sativa L.) expressing an Mn-SOD transgene in field trials over 3 years*

Data were taken from the same field plots as herbage yield (Table III). LSD (0.05) is between means in a column at the 5% level of probability (n = 4), and NS indicates that the values were not significantly different. Values represent counts of surviving plants as percentages of spring 1992 stand counts.

Plant	Fall 1992	Spring 1993	Fall 1993	Fall 1994
		% survival		
RA3	97	72	42	17
RA3-ChlSOD-30	88	71	27	26
RA3-ChlSOD-64	98	87	67	57
RA3-MitSOD-5	88	77	57	42
RA3-MitSOD-38	94	86	66	66
A7	100	92	89	88
A9	94	87	83	77
LSD (0.05)	NS	15	22	15

which superoxide is removed from the cells of the transgenic plants during periods of stress. This explanation is most likely too simplistic, because it is also possible that increased SOD activity has altered the expression of other genes associated with stress tolerance, and therefore the effect of the SOD is indirect. The product of SOD is hydrogen peroxide, which has been implicated as an elicitor of several different genes related to both biotic and abiotic stress tolerances (Chen et al., 1993; Levine et al., 1994; Prasad et al., 1994). Further support is given to this assumption by the observation that increased expression of Cu/Zn-SOD in transgenic tobacco plants increased the activity of another free radical-scavenging enzyme, ascorbate peroxidase (Gupta et al., 1993b). Currently, we hypothesize that the improved field performance of these plants is due to an enhanced overall defense system induced by a higher cellular titer of hydrogen peroxide. Our observations indicate that the mechanisms of oxidative stress tolerance need to be better understood to assist in the further development of crop plants with improved environmental stress tolerance.

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