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Manipulating freezing tolerance in transgenic plants

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Abstract

Winterhardiness is a composite of tolerances to freezing, desiccation, ice-encasement, flooding and diseases. From one point of view, winterhardiness may not be easily manipulated by genetic engineering technology because many different genes are involved in the tolerance of these diverse stresses. However, these various stresses have similarities. They promote formation of activated forms of oxygen, promote membrane lipid and protein degradation, cause similar biophysical changes in membrane structure, and culminate with increased leakage of cytoplasmic solutes and loss of cellular membrane functions. These similarities led to the hypothesis that winter injury might be reduced in crop plants if their tolerance of oxidative stress was increased.

Towards that objective we created transgenic alfalfa (*Medicago sativa* L.) plants that overexpress either Mn-SOD or Fe-SOD cDNA (provided by Dirk Inzé, Universiteit Gent). Petiole explants were transformed using *Agrobacterium tumefaciens* and plants were regenerated by somatic embryogenesis. The primary transgenic plants were screened using PCR (polymerase chain reaction), Southern hybridization and native PAGE for SOD activity. Greenhouse and laboratory studies showed a minimal difference in stress tolerance between the primary transgenic and non-transgenic plants. In the first field trial, four primary transgenic plants expressing two forms of the Mn-SOD cDNA had greater survival after two winters than the non-transgenic RA3. Similar results were obtained in a second field trial, comparing 18 independent transformants with Mn-SOD targeted to the mitochondria, 11 independent transformants with Mn-SOD targeted to the chloroplast and 39 independent transformants with Fe-SOD targeted to the chloroplast, expressed in three different non-transgenic plants. The transgenic plants averaged over 25% higher survival than the non-transgenic controls after one winter. There was no effect of subcellular targeting or SOD type on field survival, but there was variation among independent transformants containing the same SOD construct. Activated oxygen therefore appears to be one of the possible causes of winter injury, and it should be possible to reduce winter injury in transgenic plants by constitutive overexpression of SOD.

Introduction

Winterhardiness is a complex trait involving tolerances to freezing, water deprivation, ice-encasement (severe anoxia), flooding (milder anoxia) and disease. The combination and severity of these stresses that crops must tolerate varies from environment to environment and from year to year. Dif-

ferent crops, even in the same environment, experience different stresses because of their growth habit. For example, a winter annual crop like wheat (*Triticum aestivum* L.) grows close to the ground and is covered by snow, whereas a woody fruit crop grows above the snow and is not insulated against cold air temperatures. In northern climates, management of our major crops is based on the avoidance of winter injury. For example, a summer annual is grown in these areas instead of a winter annual, *i.e.* spring wheat is grown instead of winter wheat. Also, production practices for perennial forage crops, such as alfalfa (*Medicago sativa* L.), include planting before critical seeding dates, harvesting before critical fall harvest dates, leaving shoot growth to hold snow that will insulate the plants over-winter, and grading to improve surface drainage.

Crop production practices have limits in their ability to ensure winter survival and a genetic solution to this problem is desired. Although genetic variability for winterhardiness exists within plant species and in species closely related to many of our crop plants, exploitation of the variability has been limited using conventional methods of hybridization and plant breeding to transfer winterhardiness from wild species into high yielding, economically desirable cultivars. Even application of techniques involving inter-species hydrizations by embryo rescue or protoplast fusion have not led to great advances in stress tolerance. One of the reasons for the limited progress in improving winterhardiness is the difficulty in combining the many genes associated with yield, quality, disease and stress tolerances in one plant. Another reason is the difficulty in quantifying the degree of winterhardiness in individual plants from segregating populations.

These difficulties prompted us to explore the feasibility of using a genetic engineering approach to improve winterhardiness. Alfalfa was chosen as our model because it is a perennial crop whose productivity in our environment in Ontario is limited by its ability to survive winter, and because the tissue culture system for somatic embryogenesis has been well defined (McKersie and Bowley 1993) allowing it to be easily transformed by *Agrobacterium tumefaciens* (D' Halluin *et al.* 1990). The major difficulty was the choice of the genes to be introduced or modified by genetic engineering. As other chapters in this book and elsewhere illustrate, there is no shortage of candidates. Freezing tolerance, which is just one aspect of winterhardiness, is a quantitative genetic trait encoded by genes at multiple loci (Fowler *et al.* 1983, Grant 1983).

We decided to focus our efforts not on individual aspects of freezing, anoxia or desiccation tolerance but on the collective stresses that cause winter injury. In other words, even though freezing, anoxia and desiccation are recognized as being distinctly different types of environmental stresses, they have common physiological elements. One of those common elements is that each of these stresses promotes oxidative stress. There is also evidence to suggest that many symptoms of disease reactions in plants occur because of oxidative stress. Our hypothesis was that if we could enhance the plant's tolerance of oxidative stress, then we would improve its ability to survive the combination of the multiple stresses associated with winter.

Physiological evidence

The physiological and biochemical evidence that links oxidative stress with freezing, anoxia and desiccation stress is only correlative, but corresponds with three major types of experimental observations. The injury symptoms that are observed in cellular membranes isolated from plants exposed to freezing temperatures, ice-encasement, desiccation, or the free radical generating herbicide paraquat are essentially indistinguishable. Secondly, the membranes from acclimated plants are more tolerant of *in vitro* free radical treatment than those from

non-acclimated plants. Thirdly, as plants acclimate at low temperatures, they acquire coincidentally increased tolerance to freezing stress, ice-encasement stress and free-radical generating herbicides. Finally, when plants are subjected to a freezing stress, their antioxidant levels are depleted coincident with the loss of viability. Each of these points will be discussed in more detail.

Table 1. Degradation of microsomal membranes following freezing injury to winter wheat crowns. Microsomal membranes were isolated from the crowns of 7-day-old (non-acclimated) winter wheat (*Triticum aestivum* L. *cv.* Fredrick) seedlings after a lethal freezing treatment

	Protein (μg/g)	Phospholipid (nmol/g)	Microviscosity (poise)	Protein thiols (DA fluorescence)
Control	1031	527	2.1	59
Frozen	445	132	3.8	33

Data from (Borochov *et al.* 1987)

The first evidence from our laboratory linking these stresses was the observation that microsomal membranes isolated from winter wheat crowns after a lethal freezing (Borochov *et al.* 1987, Kendall and McKersie, 1989, Kendall *et al.* 1985, Pukacki *et al.* 1991) or ice-encasement stress (Hetherington *et al.* 1987, 1988), membranes isolated from soybean seeds after lethal desiccation (Senaratna *et al.* 1984), and membranes isolated from bean leaves after treatment with paraquat (Chia *et al.* 1982) or after natural senescence (McKersie and Thompson 1978), exhibited very unique physical properties. All of these different stresses result in a characteristic degradation of cellular membranes with symptoms that included degradation of membrane proteins, reduction in protein thiols, degradation of membrane phospholipid and increased membrane lipid microviscosity (decreased fluidity) (Table 1). Wide-angle x-ray diffraction of these membranes contained gel phase domains at physiological temperatures, and had dramatically elevated gel to liquid-crystalline phase transition temperatures. The degradation of membrane phospholipids occurred in an apparently random manner (Borochov *et al.* 1987, Kendall *et al.* 1985, Kendall and McKersie 1989, Pukacki *et al.* 1991), and resulted in the accumulation of degradation products, particularly free fatty acids, in the membrane bilayers. This accumulation of free fatty acids likely caused the physical changes in phase properties and fluidity of the microsomal membranes (Senaratna *et al.* 1987).

Exactly the same physical and chemical changes as caused by freezing *in planta* were found in isolated wheat microsomal membranes treated with an aqueous source of superoxide radicals *in vitro* (Table 2). Furthermore, membranes from plants acclimated to tolerate lower freezing temperatures withstood a longer duration of treatment with superoxide before being degraded (Figure 1). Perhaps the microsomal membranes from acclimated plants contained different levels of antioxidants, or perhaps the membranes acquired tolerance of free radical treatment by changes in their lipid composition. The changes in lipid composition that occur during acclimation have been well documented in numerous studies (*e.g.* Lynch and Steponkus 1987, Uemura and Steponkus 1994).

These *in vitro* experiments prompted us to examine the relationship between antioxidant levels and freezing tolerance at the whole plant level (Figure 2). Three wheat cultivars that differed in their potential to acclimate to freezing stress were grown in acclimating conditions for varying periods of time up to 5 weeks. Plants were then either frozen to de-

termine LT_{50} or the leaves were treated with a 5 µl drop of a 0.1 mM paraquat solution to define tolerance of oxidative stress. LT_{50} was measured as survival and paraquat injury was measured as the length of burn on the leaf. Across stages of acclimation, the LT_{50} for each cultivar was correlated with paraquat tolerance. In other words, as the wheat seedlings acclimated, they coincidentally developed greater tolerance to both freezing and paraquat (Bridger *et al.* 1995). Others have shown that

Table 2. Degradation of microsomal membranes following freezing injury *in planta* is similar to degradation induced by superoxide *in vitro*.
Microsomal membranes were isolated from the crowns of 7-day-old (non-acclimated) winter wheat (*Triticum aestivum* L. cv. Fredrick) seedlings before (control) or after a lethal freezing treatment (frozen). Control membranes were treated *in vitro* with superoxide generated by xanthine oxidase for 30 min and re-isolated (superoxide). The lipid phase transition temperature (Phase Tt) was measured by wide angle x-ray diffraction as the highest temperature at which gel phase was detected. Phospholipid and free fatty acid content are given relative to the control amounts as a percentage.

	Phase Tt (°C)	Phospholipid (%)	Free Fatty Acid (%)
Control	0	100	100
Frozen	40	46	315
Superoxide	40	21	405

Data from (Kendall and McKersie 1989)

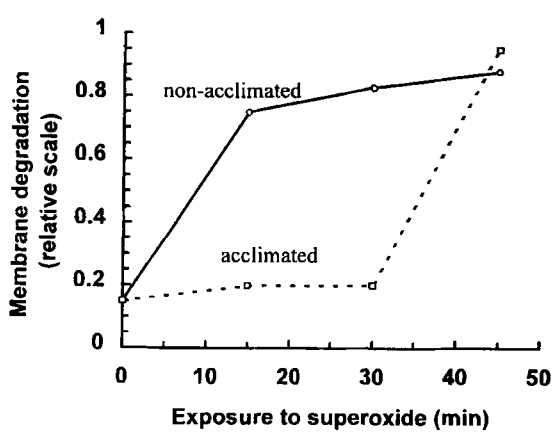

Fig. 1. Changes in free radical tolerance of wheat microsomal membranes during acclimation. Microsomal membranes were isolated from the crowns of winter wheat (*Triticum aestivum* L. *cv.* Fredrick) that were 7-days-old (non-acclimated) or were 7-days-old and then acclimated for 28 days at 2 °C (acclimated). The membranes were exposed *in vitro* to superoxide generated from the enzymatic reaction of xanthine oxidase for varying periods of time, were re-isolated and analyzed for the presence of free fatty acids. Values indicate the relative amount of phospholipid degradation detected after exposure to varying doses of superoxide. Data from (Kendall McKersie 1989).

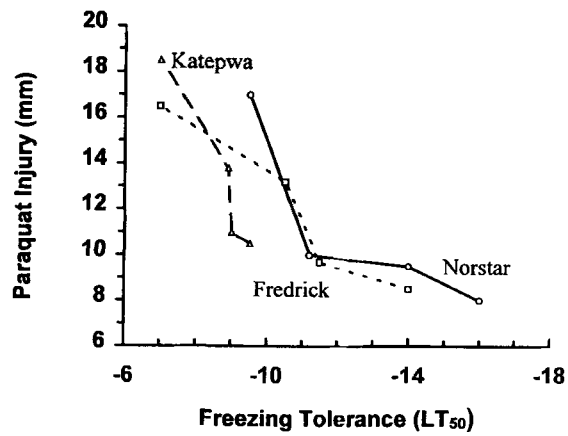

Fig. 2. Correlation between the development of freezing and oxidative stress tolerances in three cultivars of winter wheat. Winter wheat (*Triticum aestivum* L) seedlings of *cv.* Katepwa, Fredrick and Norstar were acclimated for 0, 1, 3 or 5 weeks at 2°C. Some seedlings were frozen to determine LT_{50} (°C) as survival based on regrowth. Others were treated with a 5 µl drop of 0.1 mM paraquat on the leaf and the length of burning (mm) determined after 31 h. LSD at the 5 % level of probability (n=3) for comparison between means is 2.1 °C for LT_{50} and 3.6 mm for paraquat injury. Data from Bridger *et al.* (1995).

wheat seedlings also acquire tolerance of ice-encasement as they acclimate in these growth conditions (*e.g.* Andrews and Pomeroy 1989).

The final line of evidence linking oxidative and freezing stresses is that freezing injury depletes antioxidant levels (Figure 3). In this experiment, alfalfa plants were either acclimated at 2 °C for 4 weeks or not acclimated, and then frozen at a range of temperatures as low as -14 °. Alfalfa leaves do not cold acclimate to the same degree as roots and crowns (McKenzie *et al.* 1988). The reduced glutathione (GSH) levels of leaves were depleted at the highest temperature, -2 °C. GSH levels of roots and crowns were depleted at a lower freezing temperatures than the leaves and at lower temperatures in the acclimated plants than in the non-acclimated ones (Schubert 1994). In leaves, crowns and roots, the glutathione pool became increasingly reduced as the plants were injured by freezing and this correlated with freezing injury in these different tissues.

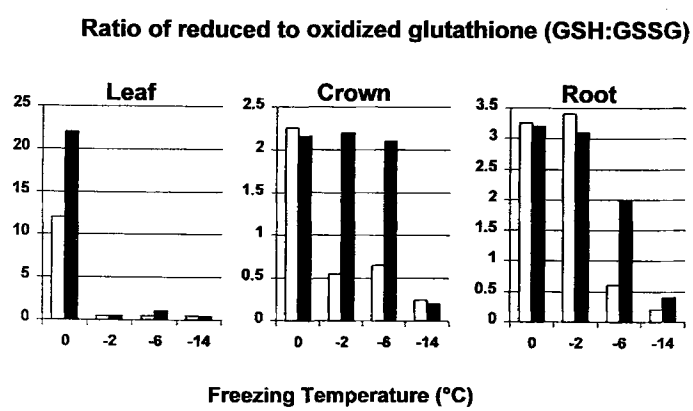

Fig. 3. The reduced:oxidized glutathione (GSH:GSSG) ratio after freezing.
Alfalfa (*Medicago sativa* L. *cv.* Excalibur) plants were acclimated at 2 °C for 4 weeks (closed bars) or not-acclimated (open bars). Plants were then frozen to varying temperatures at 2 °C h^{-1} and leaf, crown and root samples were analyzed. LSD at the 5% level of probability (n=3) for comparison between means are 3.4 for leaf, 1.0 for crown and 1.3 for root samples. Data from Schubert (1994).

Superoxide dismutase

These observations prompted us to propose our hypothesis as stated earlier. To test it, we have collaborated with Chris Bowler and Dirk Inzé at the Laboratorium voor Genetica, Rijksuniversiteit, Gent and Kathleen D'Halluin and Johan Botterman at Plant Genetic Systems, Gent. Superoxide dismutase (SOD) catalyzes the dismutation of two superoxide molecules to hydrogen peroxide and oxygen (Bannister *et al.* 1987, Bowler *et al.* 1992, 1994, Foyer *et al.* 1994, Scandalios 1990, 1993). A Mn-SOD cDNA was isolated from *Nicotiana plumbiginofolia* (Bowler *et al.* 1989). Binary vectors were constructed controlling the expression of the MnSOD cDNA with the CaMV35S promoter and targeting the Mn-SOD protein to either the mitochondria or chloroplasts (Bowler *et al.* 1991). When the MnSOD was expressed in *N. tabacum* after transformation using *Agrobacterium tumefaciens*, its expression increased the plant's tolerance to paraquat (Bowler *et al.* 1991).

These vectors were used to transform an alfalfa plant called RA3 using *Agrobacterium tumefaciens* (McKersie *et al.* 1993). This RA3 plant was used because when we did these transformations in 1988, this was one of the few alfalfa genotypes that would regenerate by somatic embryogenesis and which therefore could be transformed. Other regenerating lines have subsequently been developed (Bowley *et al.* 1993) because RA3 is not a good agronomic type of alfalfa and is not adapted to the field environment in Ontario.

The plants regenerated from these experiments were screened for the presence of the transgene using PCR, the presence of a new SOD isozyme on

native PAGE gels, the presence of unique T-DNA insertion using Southern hybridization and inheritance of the transgenes to F1 and F2 progeny (De Beus 1991, Chen 1993, McKersie *et al.* 1993). Total SOD activity in these plants was only modestly increased (Table 3), in part because increased Mn-SOD activity seems to be accompanied by lower Cu/Zn-SOD activity in most transgenic plants (unpublished observation). The transgenic plants did not have increased tolerance of the herbicide paraquat in an excised leaf assay, but they were slightly more tolerant of the diphenyl ether herbicide, acifluorfen, in a similar assay (McKersie *et al.* 1993). They also tended to have higher shoot regrowth from crown and root tissues after freezing stress (Figure 4). In this experiment, the primary

Table 3. Total superoxide dismutase activities in leaves, crowns and roots of RA3 and two transgenic alfalfa (*Medicago sativa* L.) plants expressing a Mn-superoxide dismutase cDNA with chloroplast (ChlSOD) or mitochondrial (MitSOD) transit peptides. Plants were replicated by cuttings and sampled under normal growing conditions. Values are the mean of 10-16 replicates ± std error.

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±1.0	21.8±2.3	30.2±5.9

Data from McKersie *et al.* (1996).

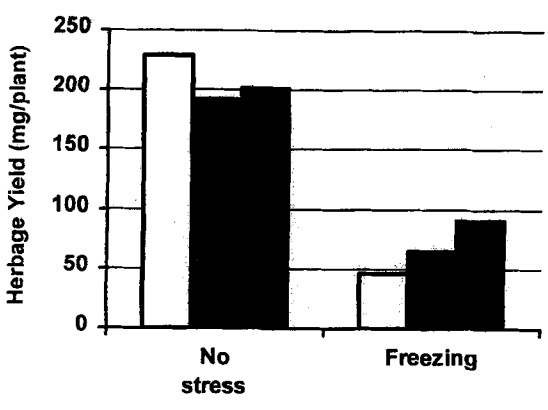

Fig. 4. Regrowth following freezing from crowns of of transgenic alfalfa (*Medicago sativa* L.) expressing a Mn-superoxide dismutase cDNA.
The non-transgenic control (RA3) and the primary transgenic plants of alfalfa (*Medicago sativa* L.) containing either the pChlSOD or pMnSOD T-DNA were propagated by cuttings to establish replicate plants. Plants were cold acclimated at 2 °C for 4 weeks, defoliated and frozen to temperatures between -8 and -16 °C at 2 °C h^{-1} and sampled at 2 °C intervals. The no-stress treatment was not frozen after acclimation, but defoliated and immediately transferred to the growth cabinet. The frozen plants were thawed at 2 °C, then regrown in the same growth cabinet and shoot dry matter was removed in two successive harvests at 28 and 56 days. Values for freezing are the mean of all freezing temperatures. Data from McKersie *et al.* (1993).

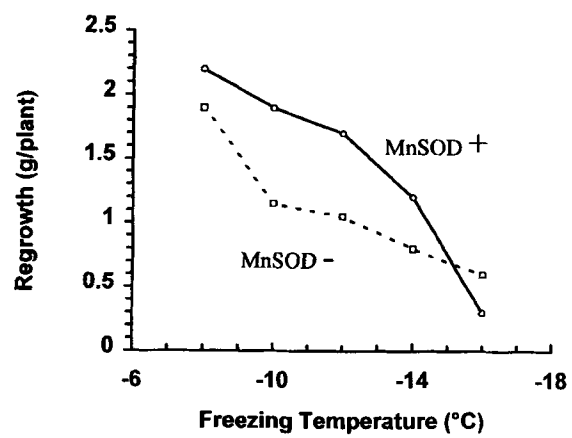

Fig. 5. Freezing tolerance of F1 progeny segregating for the MnSOD transgene.
The F1 progeny of one transgenic plant (designated as RA3-ChlSOD-30) were produced by cross pollination with another plant designated as C2-3. Seeds were germinated and the plants separated into two populations based on the presence or absence of the transgenic MnSOD isozyme in native PAGE analysis. The plants were acclimated at 2 °C for 4 weeks and frozen at 2°C h^{-1} to the designated temperatures. Regrowth of herbage was determined after 28 and 56 days as a measure of plant vigor and freezing tolerance. LSD at the 5% level of probability (n=3) for comparison between means is 0.56. Data from McKersie *et al.* (1993).

transgenic alfalfa plants were propagated by cuttings to produce sufficient replicates, acclimated at 2 °C and then frozen to temperatures ranging from -8 to -16 °C. As the temperature dropped, alfalfa gradually lost vigor but it was not killed until the temperatures fell below -14 °C. This gradual loss of vigor resulted from less shoot regrowth because either an increasing number of crown buds were killed or an increasing proportion of the root was damaged preventing translocation of nutrients to the buds. However, following freezing to temperatures between -8 and -16 °C , the transgenic plants expressing the MnSOD had higher shoot dry matter over two successive harvests compared to the non-transgenic control (Figure 4). The tolerance of freezing was heritable to the F1 progeny (Figure 5). Those plants that had inherited the MnSOD transgene also had less freezing injury compared to those progeny lacking the transgene over the temperature range between -8 and -14°C.

Our hypothesis that enhanced tolerance of oxidative stress provides increased tolerance to multiple environmental stresses was supported further by observations that these same plants have increased tolerance of water deprivation (McKersie et al., 1996). When deprived of water in growth cabinets for 5 days, plants expressing the MnSOD cDNA tended to have smaller changes in the chlorophyll fluorescence ratio, Fv/Fm (Figure 6), and less electrolyte leakage (Figure 7).

Transgenic field trials

The most definitive test of our hypothesis has come from the evaluation of these plants in field trial of these plants (McKersie et al., 1996). In 1992, a replicated test was established using transplanted cuttings of four of these primary transgenic plants, two with mitochondrial targeting (pMitSOD) and two with chloroplast targeting (pChlSOD). As stated earlier the RA3 plant that we had originally transformed was not adapted to our Ontario climate and

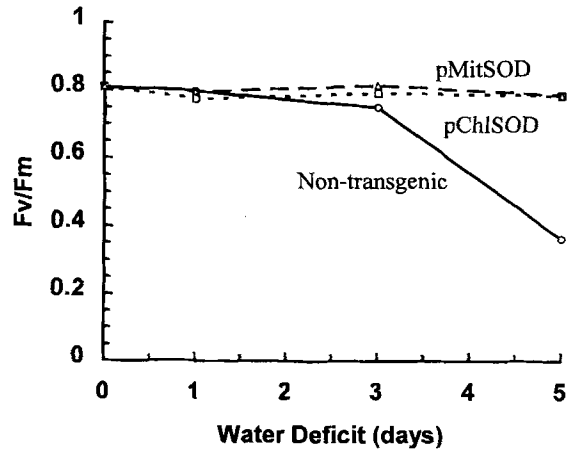

Fig. 6. Photosynthetic efficiency during water deficit stress of leaves of transgenic alfalfa (*Medicago sativa* L.) expressing a Mn-superoxide dismutase cDNA.
The non-transgenic control (RA3) and the primary transgenic plants of alfalfa (*Medicago sativa* L.) containing either the pChlSOD or pMnSOD T-DNA were propagated by cuttings to establish replicate plants. The plants were deprived of water for varying periods of time. Young leaves were sampled and measured by chlorophyll fluorescence to determine Fo and Fm values. LSD at the 5 % level of probability (n=3) for comparison between means is 0.12. Data from McKersie *et al.* (1996).

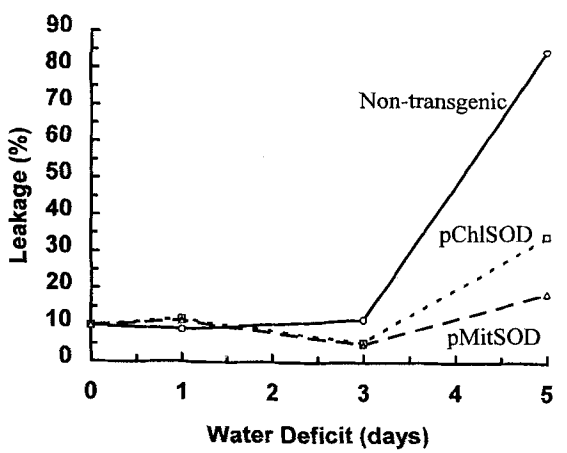

Fig. 7. Electrolyte leakage during water deficit stress from leaves of transgenic alfalfa (*Medicago sativa* L.) expressing a Mn-superoxide dismutase cDNA .
Transgenic and control alfalfa plants were propagated and treated as in figure 6. Leaves were excised and incubated in 10 ml of water for 60 min. Total electrolytes were determined by conductivity before and after boiling expressed as a percentage. LSD at the 5 % level of probability (n=4-12) for comparison between means is 11 %. Data from McKersie *et al.* (1996).

its persistence in field plots was relatively low compared to commercial alfalfa varieties. Therefore, after two years, only 17 % of the original plants survived (Figure 8). Perhaps this was fortuitous because it provided an excellent test case; all SOD transgenic plants had much higher survival than the non-transgenic RA3. This increased survival contributed to higher forage yields after both one and two winters (Figure 9). The forage yields in the year of seeding (1992) are also interesting because those plants with the MnSOD transgene had numerically higher yields before the plants had experienced any winter stress. The reason for this apparent increased vigor is unknown, but since this effect was not observed in controlled environment experiments (*e.g.* Figure 4), it possibly reflects tolerance of transplanting or water deprivation.

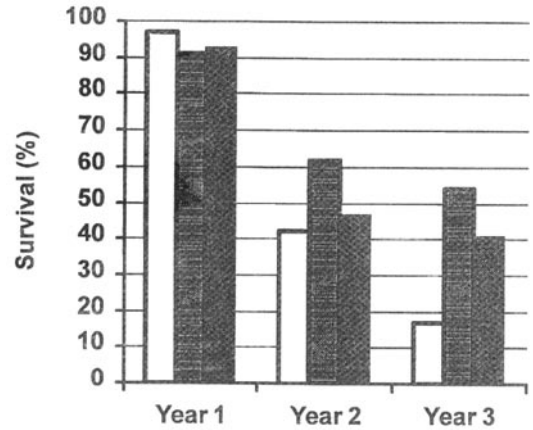

Fig. 8. Survival of alfalfa (*Medicago sativa* L.) expressing a Mn-superoxide dismutase transgene in field trails over 3 years. The test was conducted at Elora, Ontario and established by transplanting rooted cuttings of each plant in 1 x 1.5 m plots. Plants were counted in the autumn of the year of seeding (1992) and in each subsequent year, and expressed as a percentage of the original number of plants. RA3 is the non-transgenic control (open bars). The mean of two independent transgenic plants containing the pMitSOD are shown by hatched bars and two independent transgenic plants containing the pChlSOD are shown by solid bars. LSD values at the 5 % level of probability (n=4) are 19 for year 2 and 13 for year 3. Data from McKersie *et al.* (1996).

Mechanism of superoxide dismutase protection

Our current hypothesis is that the SOD does not prevent primary freezing injury in alfalfa, but instead prevents or reduces secondary injury that spreads from this primary lesion via activated oxygen. In other words, we propose that a primary freezing lesion occurs and that this causes the increased production of activated forms of oxygen; oxidative injury spreads to adjacent cells and tissues and the resulting damage leads to the death of the plant. In alfalfa, the crowns, crown buds, and roots are the critical tissues for plant survival; leaves can be lost with no adverse effect on plant survival. Localized freezing injury probably occurs at the same temperature in transgenic and control plants. The increased antioxidant potential in the transgenic plants, however, prevents this injury from spreading to other buds or meristems, and the plant is able to recover, admittedly with lower vigor than if it had not experienced this freezing injury. The effect of the SOD transgene is therefore believed to be in reducing the degree of freezing in-

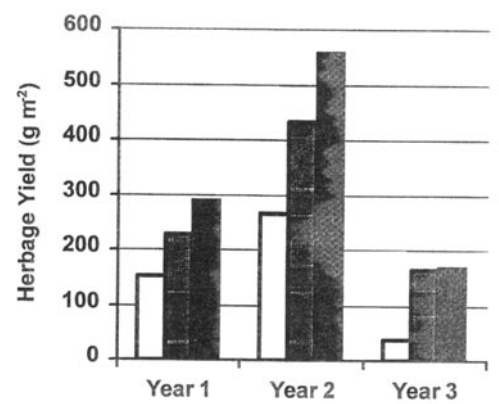

Figure 9. Herbage yield of transgenic alfalfa (*Medicago sativa* L.) expressing a Mn-superoxide dismutase cDNA in field trials.
Data were taken from the same field plots as figure 8. Plots were harvested twice in the year of transplanting (1992) and three times in 1993 and 1994. Values represent the sum of all harvests in one year for total dry matter and are expressed as g m^{-2}. RA3 is the non-transgenic control (open bars). The mean of two independent transgenic plants containing the pMitSOD are shown by hatched bars and two independent transgenic plants containing the pChlSOD are shown by solid bars. LSD values at the 5 % level of probability (n=4) are 113 for year 1, 130 for year 2 and 90 for year 3. Data from McKersie *et al.* (1996).

jury that the transgenic alfalfa plants suffered, not in whether the injury occurred.

The source of oxygen free radicals produced after freezing injury has not been defined but there are several potential candidates. We have previously shown that microsomal membranes from wheat seedlings isolated from frozen tissue have a greater tendency to form superoxide than those from control plants (Kendall and McKersie 1989). Realize also that as water freezes in the plant tissue, any dissolved oxygen is excluded from the ice and accumulates to saturating, or perhaps supersaturating, levels in the liquid. Therefore, freezing seems to promote both the tendency of the tissue to donate electrons to oxygen and to increase the availability of oxygen as a substrate for the reaction. The actual donor of the electron to oxygen is possibly a dysfunctional electron transport chain, such as that of the chloroplast or mitochondrion (Lawrence *et al.* 1995, Morre *et al.* 1995, Morre, 1986). Other possiblilities include the NADH oxidase system on the plasmalemma (Otter and Polle 1994), cell wall peroxidases (Bhaumik *et al.* 1995), or soluble enzymes including acetaldehyde oxidase or xanthine oxidase (Bhaumik *et al.* 1995). Numerous reviews discuss the involvement of oxygen free radicals and their implication in various metabolic disorders in plants and humans (Elstner 1991, Gutteridge and Halliwell 1990, Halliwell 1987a,b, Halliwell and Gutteridge 1984, Low and Merida 1996, McKersie and Leshem 1994).

Conclusions and future studies

These results support our hypothesis that we might genetically engineer crop plants with increased winterhardiness by improving tolerance of oxidative stress. However, there are many questions that remain to be addressed. We are not sure why the introduced SOD has led to the improved stress tolerance exhibited by the transgenic plants. It may function to simply lower the titer of free radicals in the frozen tissue or stop the spread of oxidative stress after freezing, as originally proposed from the observations in our physiological studies. Alternatively, higher SOD activity may increase the steady-state levels of hydrogen peroxide. Hydrogen peroxide has recently been shown to be an elicitor in several stress and disease responses (Baker and Orlandi 1995, Levine *et al.* 1994). Therefore, the expression of the SOD transgene might be activating or enhancing a whole defense system not just a single enzyme. Understanding how this SOD transgene has increased stress tolerance is critical to our future efforts to develop more winterhardy crops. For example, we do not know if this is the best candidate gene to be manipulating. The metabolic pathways that detoxify activated oxygen are complex, and SOD is only one of many enzymes in this pathway (Bowler *et al.* 1994, 1992, Foyer *et al.* 1994, Scandalios 1990, 1993). Perhaps other enzymes such as ascorbate peroxidase or glutathione reductase are better candidates for genetic manipulation (Foyer *et al.* 1994). We also do not know what the negative side effects of increased SOD activity might have in plant metabolism, if any. A promoter other than CaMV35S for the SOD transgene may be required, such as one regulated by cold (*e.g.* Jiang *et al.* 1996) or ABA (*e.g.* Marcotte *et al.* 1989), so that high SOD levels do not accumulate in the summer months. Alternatively, its expression should perhaps be targeted to specific tissues at specific stages of development, for example roots and crowns after an acclimation period. Even though we have not detected effects due to subcellular targeting of SOD to mitochodrial and chloroplasts in the experiments to date, it remains probable that subcellar targeting of the protein would also have an effect on the efficacy of the transgene in protection form different types of stress.

In conclusion, we are now fairly confident that engineering increased tolerance of oxidative stress will lead to improvements in the winterhardiness of alfalfa and other perennial crops, but there is con-

siderable research and development that needs to be done to turn that optimism into an economic reality.

Acknowledgements

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