

Down-Regulating α -Galactosidase Enhances Freezing Tolerance in Transgenic Petunia¹

Joyce C. Pennycooke², Michelle L. Jones, and Cecil Stushnoff*

Department of Horticulture and Landscape Architecture, Colorado State University, Fort Collins, Colorado 80523 (J.C.P., C.S.); and Department of Horticulture and Crop Science, The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, Ohio 44691 (M.L.J.)

α -Galactosidase (α -Gal; EC 3.2.1.22) is involved in many aspects of plant metabolism, including hydrolysis of the α -1,6 linkage of raffinose oligosaccharides during deacclimation. To examine the relationship between endogenous sugars and freezing stress, the expression of α -Gal was modified in transgenic petunia (*Petunia* \times *hybrida* cv Mitchell). The tomato (*Lycopersicon esculentum*) *Lea-Gal* gene under the control of the Figwort Mosaic Virus promoter was introduced into petunia in the sense and antisense orientations using *Agrobacterium tumefaciens*-mediated transformation. RNA gel blots confirmed that α -Gal transcripts were reduced in antisense lines compared with wild type, whereas sense plants had increased accumulation of α -Gal mRNAs. α -Gal activity followed a similar trend, with reduced activity in antisense lines and increased activity in all sense lines evaluated. Raffinose content of nonacclimated antisense plants increased 12- to 22-fold compared with wild type, and 22- to 53-fold after cold acclimation. Based upon electrolyte leakage tests, freezing tolerance of the antisense lines increased from -4°C for cold-acclimated wild-type plants to -8°C for the most tolerant antisense line. Down-regulating α -Gal in petunia results in an increase in freezing tolerance at the whole-plant level in nonacclimated and cold-acclimated plants, whereas overexpression of the α -Gal gene caused a decrease in endogenous raffinose and impaired freezing tolerance. These results suggest that engineering raffinose metabolism by transformation with α -Gal provides an additional method for improving the freezing tolerance of plants.

Many plants increase in low temperature and/or freezing tolerance in response to low nonfreezing temperatures, a phenomenon known as cold acclimation. For decades, the study of low temperature stress has had a primary goal of cataloging and understanding the biochemical and physiological changes occurring during cold acclimation (Levitt, 1980; Guy, 1990; Thomashow, 2001). It is known that the disruption of cellular membranes, particularly the plasma membrane, is the primary site of injury during a freeze-thaw cycle and that this injury usually results from dehydration associated with freezing (Steponkus, 1984). Other consequences of freeze-induced cellular dehydration include the generation of reactive oxygen species that are damaging to other cellular components (McKersie, 1991). As such, plants have developed mechanisms to deal with these multiple stresses.

It has long been established that changes in gene expression occur upon exposure to cold acclimation

(Guy et al., 1985). In the last decade, extensive research to identify and characterize cold-responsive (COR) genes has been undertaken. Hajela et al. (1990) isolated the COR genes from *Arabidopsis* that encode polypeptides thought to have protective roles against dehydration. Expression profile experiments in *Arabidopsis* demonstrated that extensive changes in gene expression occur during cold acclimation and that a substantial number of the genes that are up-regulated by the cold response are involved in metabolism (Fowler and Thomashow, 2002; Seki et al., 2002). Hence, equally important in understanding the mechanisms of low temperature stress in plants are the products of these genes. In addition to COR gene expression, numerous biochemical and physiological changes occur in plants during cold acclimation and are likely to have roles in freezing tolerance. Notable changes include alterations in membrane composition (Steponkus, 1984) and accumulation of compatible solutes such as soluble sugars, Pro, and Glycyl betaine (Guy, 1990). Sugars have been shown to be effective cryoprotectants in vitro (Carpenter and Crowe, 1988; Pennycooke and Towill, 2000), and there is evidence indicating a role in freezing tolerance in cold-acclimated membranes (Sanitarius, 1973), cells (Sanitarius and Milde, 1977), and plants (Olien and Clark, 1993; Taji et al., 2002; Strand et al., 2003).

Raffinose family oligosaccharides (RFOs), particularly raffinose, play a role in the acquisition of cold tolerance in many plant species, including herbaceous (Bachmann et al., 1994; Taji et al., 2002) and

¹ This work was supported in part by The Colorado Institute of Research in Biotechnology, by the Ohio Floriculture Foundation, and by funds from the Colorado Agricultural Experiment Station (project nos. 690 and 738).

* Present address: Department of Horticulture and Crop Science, The Ohio State University/Ohio Agricultural Research and Development Center, 1680 Madison Ave, Wooster, OH 44691.

* Corresponding author; e-mail stushnof@lamar.colostate.edu; fax 970-491-7745.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.024554.

woody plant species (Stushnoff et al., 1993). Concentration of RFOs in *Ajuga reptans* has been shown to be the lowest in summer and highest in fall and winter (Bachmann et al., 1994). Raffinose accumulation in pansies (*Viola wittrockiana*) has been demonstrated during low temperature-induced cold acclimation (Stushnoff et al., 1998). Furthermore, Arabidopsis plants with altered rates of raffinose biosynthesis increased accumulation of raffinose and galactinol upon cold acclimation (Taji et al., 2002). However, studies of deficient mutants and/or transgenics involving overexpression and down-regulation of the enzymes responsible for RFO metabolism are limited. Raffinose biosynthesis is regulated by the action of two enzymes, galactinol synthase (GolS; catalyzes the first committed step) and raffinose synthase. Raffinose degradation proceeds by the action of α -galactosidase (α -Gal; EC 3.2.1.22), which catalyzes the hydrolytic cleavage of the terminal-linked moiety from Gal-containing oligosaccharides.

Synthesis, degradation, and transport of soluble sugars are thought to cooperatively control their endogenous concentration in higher plants in response to environmental conditions (Bachmann et al., 1994; Strand et al., 2003). Understanding the control of the enzymatic mechanism responsible for driving RFO accumulation and degradation presents a unique opportunity to develop applications for producing harder crop varieties. Fowler and Thomashow (2002) reported that at least three putative GolS genes in Arabidopsis are members of the CBF regulon, whereas Taji et al. (2002) showed that one of these GolS (AtGolS3) was induced in response to low temperature and overexpression of CBF3/DREB1a.

We reasoned that if raffinose accumulates during cold acclimation to induce freezing tolerance, then down-regulating α -Gal might result in raffinose accumulation and subsequent freezing tolerance in the absence of cold acclimation. To investigate the function of α -Gal in RFO metabolism and to further elucidate the role of raffinose in freezing stress tolerance in plants, we generated transgenic plants with altered raffinose content and compared them with wild-type

plants after exposure to low temperature. The results presented confirm α -Gal inhibition as the mode of RFO accumulation and subsequent freezing tolerance. Molecular and physiological evaluations of transgenic petunia (*Petunia \times hybrida* cv Mitchell) with the α -Gal antisense and sense gene constructs are discussed.

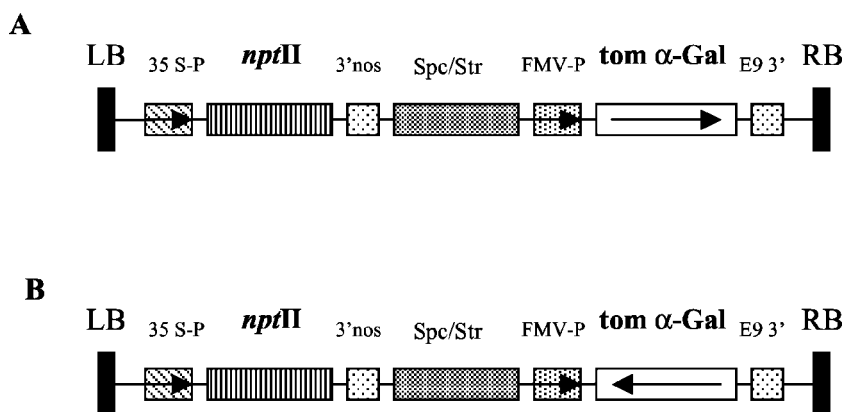
RESULTS

Creation of Transgenic Lines That Overexpress and Suppress α -Gal

The respective sense and antisense constructs (Fig. 1) were used to produce transgenic petunia plants. Nineteen antisense and six sense independent transgenic lines were identified as T₁ seedlings that segregated 3:1 for kanamycin resistance. Of the kanamycin-resistant rooted plantlets that were identified, the presence of the transgene and NPT II were confirmed by PCR and NPT II enzyme-linked immunoabsorbant assay (data not shown). Two independent transgenic sense lines (S3 and S7) and six independent antisense lines (150, 149, 147, 146, 105, and 91) were selected for further analyses. Northern-blot analysis indicated that α -Gal transcripts were reduced in antisense lines compared with wild type, whereas sense plants had an increased abundance of α -Gal transcripts (Fig. 2A). α -Gal activity assays confirmed that α -Gal activity had been down-regulated in antisense lines and that overexpression of α -Gal in sense lines resulted in increased activity (Fig. 2C). Activity in the sense lines was three times higher than in wild-type plants.

At 8 weeks of age, the transgenics were visually indistinguishable from the wild-type plants. However, a striking observation from this study is that seed germination was affected by the presence of the transgenes indicated by a 20% to 40% decrease in antisense seed germination and a 10% decrease in sense seed germination after 16 d compared with wild-type plants (data not shown).

Figure 1. Composition of sense (A) and antisense (B) constructs used in transformation of petunia. The tomato *Lea-Gal* gene (1,540 bp) was placed in the antisense orientation relative to the Figwort Mosaic virus promoter (FMV-P), upstream of the E9 3'-terminator sequence (B). LB and RB, Left and right borders; *nptII*, *Spc/Str*: resistance genes encoding kanamycin and spectinomycin/streptomycin; 3'nos, E9 3', terminator sequences.



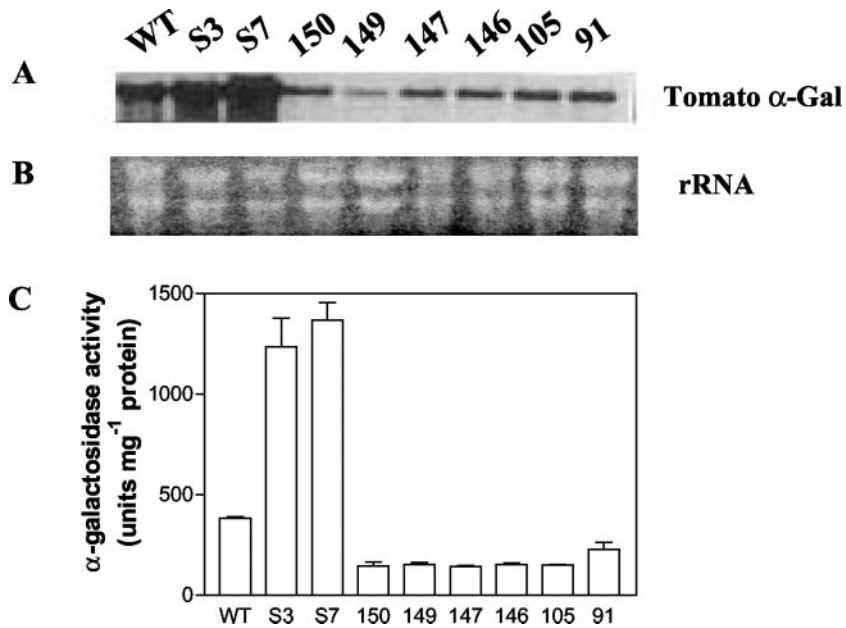


Figure 2. Northern-blot analysis of total RNA isolated from wild-type, sense (S3 and S7), and antisense (150–91) lines (A). Ten micrograms of total RNA was separated by electrophoresis through agarose and was hybridized with an α - ^{32}P -labeled tomato *Lea-Gal* probe. Ribosomal RNA stained with ethidium bromide was used as a loading control (B). α -Gal activity in wild-type, sense, and antisense lines (C).

Changes in Carbohydrate Metabolism in Wild-Type and Transgenic Lines

Several soluble sugars were detected in wild-type and transgenic lines, including Suc, raffinose, stachyose, Glc, Fru, and Gal. Changes in individual sugar contents, total sugar content, and starch at nonacclimating or cold-acclimating temperatures are shown in Figure 3. Suc was the main soluble sugar in wild-type petunia plants. Raffinose was barely detectable in nonacclimated control wild-type and sense plants, but the inhibition of α -Gal brought about increased raffinose content in antisense plants. Stachyose, another member of the RFO family, was also increased in antisense lines but to a lesser degree. Gal content was relatively low in all plants, probably due to immediate phosphorylation by a galactokinase. These data suggest that the increase in RFO content in antisense lines is due to α -Gal inhibition. When the plants were cold acclimated, we noticed even further increases in raffinose content in all lines with a 6-fold increase in the wild-type and a 2- to 3-fold increase in antisense lines. With the exception of wild-type plants, there were no appreciable changes in stachyose content upon cold acclimation. Suc content also increased in cold-acclimated wild-type and sense lines but to a lesser degree in only three antisense lines.

Total soluble sugars in wild-type and transgenic plants were determined under nonacclimating and cold-acclimating conditions. The content of total soluble sugars in nonacclimated antisense lines was approximately three to five times higher than those of nonacclimated wild-type and sense lines. When plants were cold acclimated, total soluble sugars increased 6-fold in wild-type, 2- to 3-fold in antisense, but only 1-fold in sense transgenic lines. More than

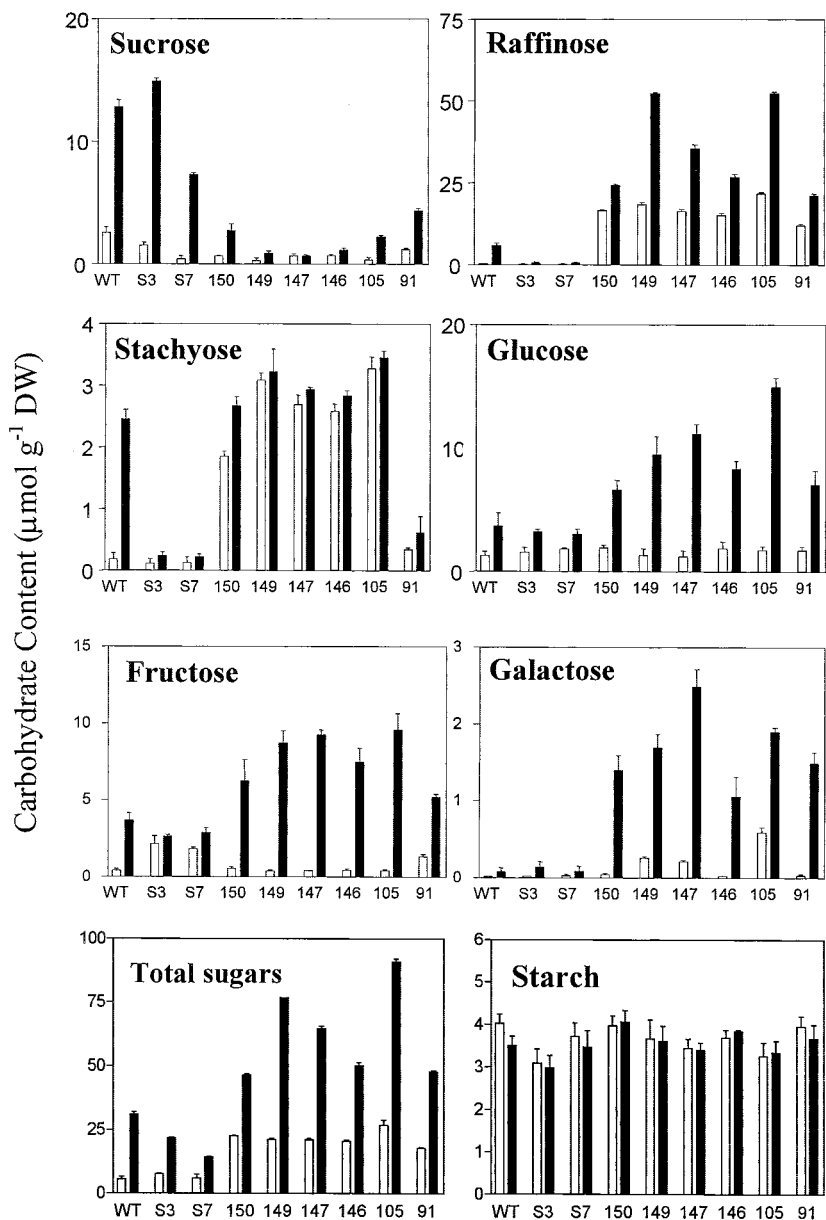
50% of the increase in total soluble sugars was due to raffinose accumulation in cold-acclimated antisense lines and Suc accumulation in cold-acclimated wild-type and sense lines. Petunia leaves contained a relatively small pool of starch 2 h into the photoperiod and remained largely unchanged for nonacclimated or cold-acclimated transgenic lines (Fig. 3).

Freezing Tolerance and Raffinose Content

Freezing tolerance was determined by electrolyte leakage, visual observation of lesions, and by assessing the ability to regenerate new growth. The levels of freezing tolerance varied among antisense lines, with 149 and 105 being the most tolerant and 91 being the least tolerant (Fig. 4A). With the exception of 91, all antisense lines were significantly more freezing tolerant than wild type ($P < 0.05$). In contrast, sense lines showed impaired increase in freezing tolerance. When plants were grown under cold-acclimating conditions, there were increases in tolerance in antisense lines and to a lower extent in wild-type but not in sense lines (Fig. 4B). Tolerance levels averaged -5°C in cold-acclimated wild-type and -3°C in cold-acclimated sense lines, whereas they ranged from -6°C to -8°C in cold-acclimated antisense lines. Thus, antisense lines tolerated temperatures that were 3°C to 5°C lower than those tolerated by wild-type and sense lines.

The pattern of freezing injury and ability to recover from damage were monitored on whole plants for 1 week after removal from the freezing stress. As shown in Figure 5A, the top portion of the shoots clearly sustained more injury than the bottom one-half of the shoots. Lesions on individual leaves began from the tip of the leaf and progressed toward the

Figure 3. Carbohydrate content in wild-type, sense (S3 and S7), and antisense (150–91) lines 2 h into the photoperiod. Sugar content was determined by HPLC-pulsed amperometric detection. Data are from two replicated experiments with three replicates for each genotype. Bars represent the mean \pm SEM; $n = 6$; nonacclimated (white bars); cold-acclimated (black bars): 15°C for 7 d, 10°C for 7 d, 5°C for 7 d, and 3°C for 3 d. Starch was determined enzymatically and is estimated as micromoles of Glu per gram of dry weight.



petiole. Only antisense lines produced new auxiliary buds after damage to the shoot tip. Wild-type and sense lines never recovered and subsequently died. On a rating of 0 to 10 (0, no injury; 10, dead), nonacclimated antisense lines fell in the range of 1 to 6, whereas nonacclimated wild-type and sense lines were rated at 9 and 10, respectively. When plants were cold acclimated, the gradation of freezing injury from top to bottom was less evident in all antisense lines except 91, but remained unchanged in wild-type and sense lines (Fig. 5B).

At 2 h into the photoperiod, sink tissues of nonacclimated wild-type and T_2 plants contained significantly less raffinose than source tissues of the same plants ($P < 0.05$; Table I). When plants were cold acclimated, there was a significant shift in raffinose

content from larger leaves to younger leaves in all antisense lines except 91, but not in wild-type and sense lines ($P < 0.05$).

To examine the relationship of raffinose to freezing tolerance, we plotted the changes in electrolyte leakage against the raffinose content present in sink leaves at the time the plants were subjected to the freezing test (Table II). There was a significant correlation between the temperature at which 50% of electrolytes were released from the plant tissue (TEL_{50}) estimated from electrolyte leakage curves and raffinose content in nonacclimated antisense lines ($P < 0.05$), but little or no correlation was observed in nonacclimated wild-type ($r = 0.2397$) or sense lines ($r = 0.5190$ and 0.6142 , respectively). Differences in maximum level of freezing tolerance between anti-

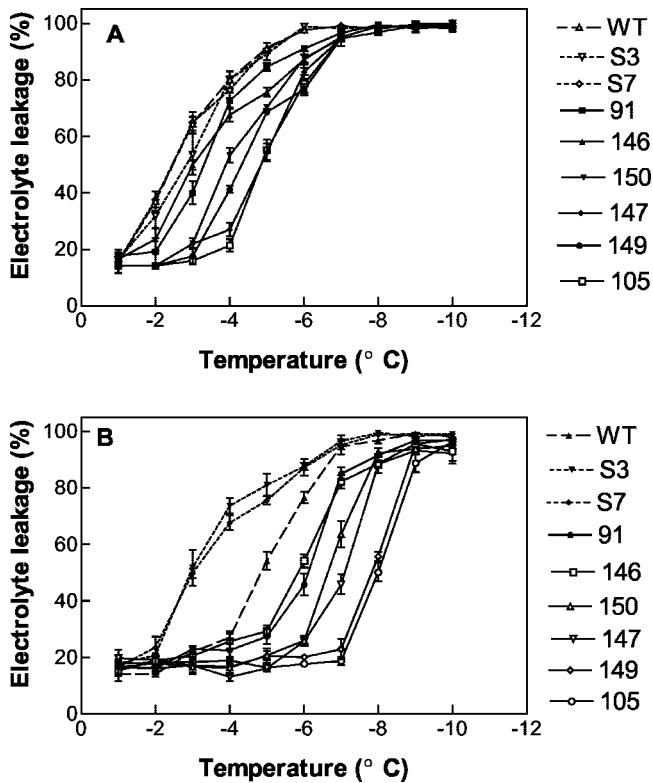


Figure 4. Effect of α -Gal expression and suppression on whole-plant freezing tolerance as indicated by TEL_{50} values in nonacclimated (A) and cold-acclimated plants (B). Sense lines (S3 and S7); antisense lines (150–91). Cold acclimation consisted of 15°C for 7 d, 10°C for 7 d, 5°C for 7 d, and 3°C for 3 d. Plants were frozen at a rate of 1°C h⁻¹ to -8°C. Three leaves were drawn from the top portions of two separate plants for each genotype at the temperatures indicated above and were tested for electrolyte leakage as described in “Materials and Methods.”

sense and sense lines were more closely related to the capacity of plants to accumulate raffinose than their capacity to accumulate Suc (data not shown).

DISCUSSION

Compelling evidence in the literature supports the hypothesis that soluble sugars function in part to enhance stress tolerance in cold-hardy plants (Bachmann et al., 1994; Strand et al., 2003). We have focused on raffinose, which is one member of the RFO family, and have studied its potential role in freezing tolerance by taking a reverse genetics approach in a relatively freezing-sensitive plant. In recent years, researchers have isolated and manipulated key genes involved in the biosynthetic pathway of sugars mostly raffinose and Suc (Taji et al., 2002; Strand et al., 2003). Others have used mutants to study freezing sensitivity resulting from sugar deficiencies (Uemura et al., 2003). Cold-inducible raffinose accumulation is caused by the activation of raffinose biosynthesis and the inactivation of raffinose hydrolysis (Bachmann et al., 1994; Castonguay and

Nadeau, 1998). We know that the α -Gal gene is constitutively expressed in petunia, with 88% nucleotide homology to that of the tomato (*Lycopersicon esculentum*) α -Gal (Pennycooke et al., 2003). In view of this observation, we tested the hypothesis that raffinose is protective against freezing stress by generating transgenic petunia plants with a tomato *Lea-Gal* gene and compared these with wild-type plants after exposure to low temperature. Our results demonstrated that altering rates of raffinose synthesis can imitate (antisense transgenics) or attenuate (sense transgenics) the cold acclimation responses and strongly influence the development of freezing tolerance.

Under nonacclimating conditions, the raffinose content in antisense lines was about 12 to 22 times higher than that of control wild-type plants. Upon cold acclimation, the raffinose content in antisense lines increased further (about 2- to 3-fold). In such a highly concentrated RFO environment, these oligosaccharides may interact with membrane phospholipids and proteins to stabilize their structures as water is removed during freezing. Although also contributing to the stabilization of membranes and proteins, RFO are also thought to enhance the ability of Suc to promote glass formation at low moisture contents and extremely low temperatures by preventing Suc from forming ice as water is removed. The extremely high viscosity of the glassy state prevents membrane fusion and retards the rates of chemical reactions that can lead to tissue deterioration (Pennycooke and Towill, 2001).

The concentration of Suc compared with that of raffinose or stachyose in nonacclimated and cold-acclimated wild-type plants suggest that Suc is the dominant transport sugar in petunia. The total soluble sugar content increased significantly when the



Figure 5. Phenotype of wild-type, sense (S3 and S7), and antisense (150–91) plants 1 week after freezing stress. A, Nonacclimated; B, cold-acclimated at 15°C for 7 d, 10°C for 7 d, 5°C for 7 d, and 3°C for 3 d. Two plants per genotype were frozen at a rate of 1°C h⁻¹ to -8°C (only one was photographed). After the freezing stress, three leaves were drawn from the top portions of the two separate plants for each genotype at specific temperatures and were tested for electrolyte leakage. Plants were incubated at 4°C overnight and were then placed at 22°C for 1 week. The tips of all plants fell off 2 d after freezing. Full damage is not apparent in B: 149, 105, 147, and 150 due to sampling for electrolyte leakage and abscission due to injury.

Table I. Raffinose content in nonacclimated and cold-acclimated sink and source leaves of wild-type and transgenic petunia lines

Treatment	Genotype	Raffinose Content	
		Sink leaves	Source leaves
$\mu\text{mol g}^{-1}$ dry weight			
Nonacclimated	WT	0.23 \pm 0.18	0.53 \pm 0.11
	S3	0.22 \pm 0.17	0.46 \pm 0.13
	S7	0.23 \pm 0.18	0.50 \pm 0.11
	150	16.69 \pm 0.29	19.87 \pm 0.36
	149	18.47 \pm 0.62	24.98 \pm 0.42
	147	16.39 \pm 0.64	22.54 \pm 0.34
	146	15.22 \pm 0.68	18.98 \pm 0.22
	105	21.78 \pm 0.43	26.67 \pm 0.32
	91	12.23 \pm 0.46	15.46 \pm 0.23
Cold acclimated	WT	5.91 \pm 0.82	5.00 \pm 0.88
	S3	0.71 \pm 0.10	0.66 \pm 0.21
	S7	0.70 \pm 0.12	0.77 \pm 0.67
	150	24.30 \pm 0.26	22.97 \pm 0.55
	149	52.34 \pm 0.23	50.46 \pm 0.13
	147	35.60 \pm 1.15	33.77 \pm 0.82
	146	26.84 \pm 1.02	23.41 \pm 0.57
	105	52.25 \pm 0.62	49.89 \pm 0.66
	91	21.28 \pm 0.52	20.54 \pm 0.35

Sense lines (S3 and S7); antisense lines (150, 149, 147, 146, 105, and 91).

Petunia plants were nonacclimated (22°C, 16/8-h photoperiod) or gradually cold acclimated from 15°C to 3°C, 12/12-h photoperiod for 24 d. Sink leaves (-2 cm^2) from the top one-half of the plants and source leaves (-4.5 cm^2) from the bottom one-half of the plants were sampled 2 h into the photoperiod. Raffinose content was determined by HPLC-PAD. Data are from two replicated experiments with three replicates for each genotype.

Values are mean \pm SEM; $n = 6$.

plants were in the acclimated state. Similarly, in *Arabidopsis* there is a strong up-regulation of transcripts and enzyme activity for sugar biosynthesis at low temperatures (Strand et al., 2003). Such an accumulation may serve a dual role in storing carbon and providing cryoprotectants. On the other hand, starch accumulation remained unchanged in wild-type and transgenic plants during cold acclimation. Starch synthesis is relatively sensitive to cool temperature (Pollock et al., 1983), and as a result, alternative forms of carbohydrate storage are required for plants that continue to grow during cooler months. In the antisense lines, 50% or more of the increase in total soluble sugar content was due to the increase in raffinose compared with wild type, in which case Suc contributed more than 50%. Stachyose was detected at relatively low levels in wild-type plants but increased significantly when the plants were cold acclimated although less than raffinose. Similarly, in cold-treated *AtGolS2* (encoding galactinol synthase) overexpressing transgenic *Arabidopsis*, large amounts of raffinose but not stachyose were detected (Taji et al., 2002). However, in the frost hardy *A. reptans*, stachyose was by far the most dominant form of RFO (Bachmann et al., 1994). It is possible that stachyose could also play a role in developing some

freezing tolerance in petunia considering that it was blocked in the freezing-sensitive sense plants. The possibility must be considered that the lack of sugar accumulation might be responsible for the freezing tolerance deficiency of the sense plants. Uemura et al. (2003) showed that the sugar deficiency of *sfr4* cells during cold acclimation was largely responsible for the impaired increase in freezing tolerance but supplementation of *sfr4* plants with Suc at 2°C restored the freezing tolerance of the *sfr4* mutant to a level shown by wild type after conventional cold acclimation.

Freezing stress tolerance was predicted based on the relationship between raffinose accumulation and decreased electrolyte leakage. Although Suc made up more than 50% (w/w) of the increase in total soluble sugars in cold-acclimated wild type, there was no significant correlation between Suc accumulation and TEL₅₀ values in wild-type plants (data not shown). This suggests that raffinose and/or a combination of raffinose and Suc was partly responsible for inducing freezing tolerance in petunia. It is important to note that the raffinose content in nonacclimated antisense lines was about 3-fold higher than that in cold-acclimated wild-type plants, yet the freezing tolerances were similar. Also, the freezing tolerance of cold-acclimated antisense line 150 was greater than that of the nonacclimated plant despite similar raffinose levels. Collectively, these results suggest that there may be some interaction with raffinose and other mechanisms that occur upon cold acclimation, or that a threshold level is sufficient to induce a response. In addition to a cryoprotective role, raffinose is probably also important for providing energy for other critical mechanisms of freezing tolerance such as the synthesis of specific stress tolerance proteins and lipid changes. As would be expected from a quantitative trait such as freezing tol-

Table II. Pearson's correlation coefficient between raffinose content and electrolyte leakage in wild-type and transgenic petunia lines

Genotype	Correlation Coefficient ^a	
	Nonacclimated	Cold acclimated
	p^b	
Wild type	0.2397 (0.64)	0.8712 (0.002)
S3	0.5190 (0.29)	0.6167 (0.26)
S7	0.6142 (0.11)	0.6190 (0.25)
150	0.8848 (0.02)	0.8987 (0.02)
149	0.7541 (0.01)	(0.9234) (0.003)
147	0.7102 (0.02)	0.8245 (0.04)
146	0.851 (0.03)	0.8657 (0.03)
105	0.9083 (0.01)	0.9245 (0.002)
91	0.703 (0.09)	0.7761 (0.05)

Sense lines (S3 and S7); antisense lines (150, 149, 147, 146, 105, and 91).

^a Correlation coefficient was calculated from six reps of sugar content and electrolyte leakage data using SAS software (Cary, NC).

^b Values < 0.05 are significant at $P = 0.05$.

erance, different factors may come into play at different times during the development and maintenance of freezing tolerance.

The data presented here suggest that modifying the endogenous raffinose levels by gene manipulation had a significant effect on freezing tolerance in comparison with a cold-acclimation treatment alone. Consistent with the findings of Yelenosky and Guy (1989), wild-type petunia plants increased in freezing tolerance upon cold acclimation (by 1°C). In our study, cold-acclimated antisense transgenic lines ranged from a 3°C to 6°C increase in freezing tolerance. Similarly, Taji et al. (2002) demonstrated that overexpression of AtGolS2 in transgenic Arabidopsis caused an increase in endogenous galactinol and raffinose and improved cold and drought tolerance. The rationale behind using transgenic studies rather than cold acclimation treatments alone to evaluate freezing tolerance is that an inherent genetic factor is more stable than an environmental factor that could introduce variables in the experiment and might invalidate evaluation of true cold hardiness.

Wild-type and transgenic lines were able to cold acclimate when subjected to a gradual low temperature exposure from 15°C to 3°C. When petunia plants were exposed directly to 5°C, chilling injury was observed (data not shown). This suggests that the 5°C cold acclimation regime may have initially caused injury that impeded acclimation at the onset. Although Yelenosky and Guy (1989) reported that petunia cold acclimated but lacks the capacity to further increase in freezing tolerance after cold acclimation at 5°C for 7 d, we found that the development of cold tolerance in petunia progressed slowly, requiring 18 d of progressive acclimation to attain a steady level of cold tolerance. The cellular changes that enable the plant to endure low temperature stress are likely to develop very gradually. In contrast, cold acclimation in Arabidopsis (Gilmour et al., 1988) and spinach (*Spinacia oleracea*; Yelenosky and Guy, 1989) progresses rapidly.

It is essential to have a reliable screening method to measure the development of freezing tolerance in plants. In addition to electrolyte leakage data, we looked at the pattern of freezing injury and the ability of the plants to repair damage and resume normal growth. The observation that injury in the nonacclimated plants was progressively worse from the top of the shoot toward the base was interesting and may reflect a freezing injury gradient related to tissue maturity. Carbohydrate profiles in leaves vary during development. Our data revealed that nonacclimated young leaves (sinks) already contained raffinose and concentrations increased with increasing leaf age (source). This observation may explain the uncharacteristic freezing sensitivity in nonacclimated sink tissues relative to source tissues. In contrast, a change in the partitioning of raffinose was observed when the plants were cold acclimated whereby anti-

sense sink leaves accumulated more raffinose than source leaves. Collectively, these results indicated that cold acclimation influenced younger leaves more than older ones, shifting the transition in cold-acclimated leaves toward younger tissue compared with nonacclimated leaves. Although our test conditions did not permit monitoring temperature differences within plants, others have noted that substantial differences in temperature occur within a given plant under freezing conditions in the field (Renquist, 1985). The shoot is a highly heterogeneous structure, therefore, different freezing processes can occur in areas less than a few millimeters apart (Levitt, 1980). Whether this was related to the temperature at which freezing occurred and subsequent rates of ice propagation or due to inherent levels of cold hardiness was not determined.

In this study, the combination of molecular and physiological approaches demonstrated the role of raffinose in low temperature stress. Through antisense technology, α -Gal was shown to be an essential component of the cold hardiness pathway by providing a direct route to modify raffinose accumulation in target tissues needed for freezing stress tolerance. It was demonstrated that down-regulation of α -Gal in petunia is sufficient to decrease electrolyte leakage and subsequently confer tolerance to freezing stress. The lack of sugar accumulation in the sense lines is closely related to increased electrolyte leakage and impairment of freezing tolerance. Freezing temperatures often cause severe losses in agricultural productivity. Although traditional breeding approaches have met with limited success in improving the freezing tolerance of economic crops, biotechnology may offer new strategies. Late spring freezes can cause substantial damage to plants like petunias because they are less cold tolerant and lack rapid acclimation response mechanisms compared with other species that can tolerate ice in their tissues such as the Brassicas and spinach. Although fall conditions with shorter days and cooler temperatures may trigger some acclimation, there is substantial room for improvement to extend the season. This information has potential practical implications by possibly extending the growing season of petunia, permitting earlier spring planting and later fall production when frost injury is most likely. The α -Gal gene, which was targeted here, is not limited to petunia and thus may provide a means of improving the freezing tolerance of other economic crops.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sterile seeds of petunia (*Petunia × hybrida* cv Mitchell) were germinated on solid modified Murashige and Skoog basal medium (one-half strength of ammonium nitrate and potassium nitrate, termed one-half Murashige and Skoog medium) containing 3% (w/v) Suc and 0.8% (w/v) agar at pH 5.8. Seeds were germinated in Magenta boxes and were incubated at 22°C day, 20°C night, with a 16/8-h photoperiod under cool-white fluorescent light at

60 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Two-week-old agar-grown seedlings were transferred to potting medium (4P mix; Fafard, Agawam, MA) and were gradually acclimated before being transferred to 12-cm pots maintained in a greenhouse under controlled-environment conditions: day/night temperature regime of 22°C/20°C; cool-white fluorescent light at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$; and a 16-h photoperiod. Supplemental lighting was provided during the winter months by 430 W Agro Sun Lamps (Denver).

Constructs and Plant Transformation

The tomato *Lea-Gal* (*Lycopersicon esculentum*) cDNA (GenBank accession no. AF191823; Feurtado et al., 2001) in the phagemid vector pBK-CMV was obtained from Prof. J. Derek Bewley (University of Guelph). For the sense construct, the 1,540-bp *BamH I/XhoI* fragment from the *Lea-Gal* clone was inserted into the corresponding sites of pBluescript. The *XbaI/KpnI* fragment from the resultant construct was inserted into the *XbaI* and *SmaI* sites of the transformation vector pMON981 obtained from (Monsanto, St. Louis). For the antisense construct, the 5' *BamH I/XbaI* 3' 1,540-bp fragment was ligated in the inverted orientation into the 5' *XbaI/BamH I* 3' sites of pMON981. The respective sense and antisense constructs (Fig. 1), which contained the *Lea-Gal* coding sequence under the control of the FMV-P, were introduced into *Agrobacterium tumefaciens* LBA4404 and used to produce transgenic petunia plants using a modified leaf disc cocultivation protocol of Jorgensen et al. (1996). Transformed seedlings were selected on the basis of kanamycin resistance, and the rooted plantlets were further screened by PCR of the α -Gal gene and enzyme-linked immunoabsorbant assay (Patho-Screen kit for NPT II; Agdia Inc., Elkhart, IN). T₂ plants were used for subsequent analyses.

RNA Extraction and Northern-Blot Analysis

Total RNA was extracted from leaves of 8-week-old wild-type and transgenic plants using the TRIZOL reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA was separated by electrophoresis on a 1% (w/v) agarose gel containing 2.2 M formaldehyde and blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH). The membrane was probed with 10⁶ cpm mL⁻¹ ³²P-labeled *Lea-Gal* cDNA. Hybridizations and washes were performed as described by Jones et al. (1995).

α -Gal Extraction and Activity Assay

The extraction of α -Gal was performed as described by Smart and Pharr (1980). The resulting α -Gal was assayed using *p*-nitrophenyl- α -D-galactopyranoside (PNPG). Assay mixtures contained 100 μL of enzyme in 400 μL of McIlvaine buffer (pH 6.0; McIlvaine, 1921) at 30°C. The reaction was started by adding 100 μL of 30 mM PNPG and was terminated after 20 min by adding 2.4 mL of 5% (w/v) Na₂CO₃. Blanks were prepared by adding enzyme after Na₂CO₃. Absorbance was read at 400 nm and for quantifying enzyme activity; the amount of *p*-nitrophenol released was calculated using a molar extinction coefficient of 1.83 $\times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of enzyme activity was defined as one micromole of PNPG hydrolyzed per minute. Total protein was estimated by the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951).

Carbohydrate Analyses

For total soluble sugars and starch analyses, leaf tissues were sampled 2 h into the photoperiod from all stages of leaf development from 8-week-old plants. Samples from each plant were pooled together and were immediately drenched in liquid nitrogen. Total soluble sugars were extracted from lyophilized leaf tissue (5 mg) in 5 mL of 80% (v/v) ethanol at 90°C for 20 min. Samples were centrifuged at 1,000 rpm for 2 min and the supernatant was collected. A total of three rounds of the hot ethanol and centrifugation steps were performed. The supernatants of these samples were pooled and evaporated to dryness under nitrogen gas overnight at room temperature. The samples were resuspended in 5 mL of deionized water and were filtered through a 0.22- μm filter membrane. Cell extracts were analyzed by HPLC (Dionex, Sunnyvale, CA) equipped with a CarboPac PA 10 column and

pulsed amperometric detection system. Two separate experiments were conducted and each treatment was replicated three times.

Starch was measured in the residue as described by Haissig and Dickson (zharv1979) with slight modifications. The starch was gelatinized in 100 mM sodium acetate buffer (pH 4.5) at 95°C for 1 h followed by hydrolysis with amyloglucosidase enzyme (Sigma A7420; Sigma, St. Louis) at 45°C for 16 h. The reaction was terminated by incubating at 95°C for 20 min. Starch was determined spectrophotometrically by reference to a Glc standard curve.

To test the correlation of raffinose content and freezing tolerance, samples were collected 2 h into the photoperiod from sink leaves (approximately 2 cm²) located at the top one-half of the plants and source leaves (approximately 4.5 cm²) located at the bottom one-half of the plants. The transition from sink to source is defined by a shift from 25% expansion (sink) to 40% to 50% expansion (source; Taiz and Zeiger, 2002). Samples were prepared in the same manner and analyzed for raffinose content as described for soluble sugar content.

Freezing Tolerance Studies

Eight-week-old wild-type and T₂ transgenic plants were used to evaluate freezing tolerance. Plants of each type were divided into two groups. Plants in one group were used to measure freezing tolerance without cold acclimation and were maintained at 22°C with a 16/8-h photoperiod. Plants in the other group were transferred to a cold-acclimation chamber with a gradual exposure at 15°C for 7 d, 10°C for 7 d, 5°C for 7 d, and 3°C for 3 d with a 12-h photoperiod. Whole plants were used to evaluate freezing tolerance and were placed in a programmable freezer (Tenney Jr.; Lunaire, Williamsport, PA). It has long been suggested that roots are more susceptible to freezing injury than shoots (Chen et al., 1983). As such, Styrofoam chips were added to the pots to protect the root crowns. To initiate extracellular ice nucleation, the plants were misted with 0°C tap water and were kept at -1°C for an additional 3 h before lowering the temperature at a rate of 1°C h⁻¹ to various temperatures (lowest temperature, -9°C) and each temperature was held for 30 min. Sink leaves (approximately 2 cm²) were sampled from the top portion of the plants at predetermined temperatures and were held at 4°C overnight for electrolyte leakage tests. Whole plants were monitored for 1 week at 22°C, and freezing injury lesions and the ability to recover were evaluated.

Freezing injury was determined by electrolyte leakage, which provides a good estimate of cell damage. This was expressed as TEL₅₀ (Webb et al., 1994). To determine electrolyte leakage, leaf discs (5 mm in diameter) were immersed in individual cells of a leakage tray of a 100-well ASAC seed analyzer (Neogen, East Lansing, MI) containing 2 mL of deionized water, previously tested to read 0 conductivity. After the conductivity of the exosmosed solution was measured (T₁), the leaf discs were frozen at -80°C overnight and were thawed at room temperature (22°C) to destroy compartmentalization so that total potential leakage (T₂) could be determined. Relative percentage of injury represents the mean leakage as a percentage of the mean total leakage from frozen-killed samples (T₁/T₂ \times 100). Sampling for this freezing assay included three leaves from two separate plants for each genotype.

ACKNOWLEDGMENTS

We thank John Ray, John Waddell, and Ann McSay (Colorado State University, Fort Collins) for technical assistance. We also thank Prof. J. Derek Bewley (University of Guelph, Guelph, Ontario, Canada) for providing the tomato *Lea-Gal* cDNA and Monsanto Corp. (St. Louis) for the pMON981 transformation vector. Also, special thanks to Dr. Larry V. Gusta (University of Saskatchewan, Saskatchewan, Canada) and Prof. J. Derek Bewley (University of Guelph, Guelph, Ontario, Canada) for reviewing the manuscript.

Received March 31, 2003; returned for revision May 8, 2003; accepted July 1, 2003.

LITERATURE CITED

Bachmann M, Matile P, Keller F (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans*: cold acclimation, transloca-

- tion and sink to source transition: discovery of chain elongation enzyme. *Plant Physiol* **105**: 1335–1345
- Carpenter JF, Crowe JH** (1988) The mechanism of cryoprotection of proteins by solutes. *Cryobiology* **25**: 244–255
- Castonguay Y, Nadeau P** (1998) Crop physiology and metabolism: enzymatic control of soluble carbohydrate accumulation in cold acclimated crowns of alfalfa. *Crop Sci* **38**: 1183–1189
- Chen TH, Gusta LV, Fowler DB** (1983) Freezing injury and root development in winter cereals. *Plant Physiol* **73**: 773–777
- Feurtado JA, Banik M, Bewley JD** (2001) The cloning and characterization of α -galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seed. *J Exp Bot* **52**: 1239–1249
- Fowler S, Thomashow M** (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**: 1675–1690
- Gilmour SJ, Lin C, Thomashow MF** (1988) Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol* **87**: 745–750
- Guy CL** (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu Rev Plant Physiol Plant Mol Biol* **41**: 187–223
- Guy CL, Niemi KJ, Brambi R** (1985) Altered gene expression during cold acclimation of spinach. *Proc Natl Acad Sci USA* **81**: 3673–3677
- Hajela RK, Norvath DP, Gilmour SJ, Thomashow MF** (1990) Molecular cloning and expression of cor (cold-regulated) genes in *Arabidopsis thaliana*. *Plant Physiol* **93**: 1246–1252
- Haissig BE, Dickson RE** (1979) Starch measurement in plant tissue using enzymatic hydrolysis. *Physiol Plant* **47**: 151–157
- Jones ML, Larsen PB, Woodson WR** (1995) Ethylene-regulated expression of a carnation cysteine proteinase during flower petal senescence. *Plant Mol Biol* **28**: 505–512
- Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA** (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Mol Biol* **31**: 957–973
- Levitt J** (1980) Responses of Plants to Environmental Stresses: Vol 1. Chilling, Freezing and High Temperature Stresses, Ed 2. Academic Press, New York
- Lowry H, Rosebrough N, Fan A, Randall R** (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275
- McIlvaine TC** (1921) A buffer solution for colorimetric comparison. *J Biol Chem* **49**: 183–186
- McKersie BD** (1991) The role of oxygen free radicals in mediating freezing and desiccation stress in plants. In E. Pell and K. Steffen, eds., *Active Oxygen/Oxidative Stress and Plant Metabolism*. American Society of Plant Physiologists, Rockville, MD, pp 107–118
- Olien CR, Clark JL** (1993) Changes in soluble carbohydrate composition of barley, wheat, and rye during winter. *Agronomy J* **85**: 21–29
- Pennycooke JC, Jones ML, Vepachedu R, Stushnoff C** (2003) PCR-based cloning of an α -galactosidase gene from deacclimated petunia (*Petunia X hybrida*). *Acta Hort* (in press)
- Pennycooke JC, Towill LE** (2000) Cryopreservation of shoot tips from *in vitro* plants of sweet potato (*Ipomoea batatas* Lam.) by vitrification. *Plant Cell Rep* **19**: 733–739
- Pennycooke JC, Towill LE** (2001) Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. *CryoLetters* **22**: 381–389
- Pollock CJ, Lloyd EJ, Stoddard JL, Thomas H** (1983) Growth, photosynthesis and assimilate partitioning in *Lolium temulentum* exposed to chilling temperatures. *Physiol Plant* **59**: 257–262
- Renquist AR** (1985) The extent of fruit bud radiant cooling in relation to freeze protection with fans. *Agric Meteorol* **36**: 1–6
- Sanitarius KA** (1973) The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. *Planta* **113**: 105–114
- Sanitarius KA, Milde H** (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. *Planta* **136**: 163–166
- Seki M, Narusaka M, Ishida J, Nanjo T, Frujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T et al.** (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high salinity stresses using a full-length cDNA microarray. *Plant J* **31**: 279–292
- Smart EL, Pharr DM** (1980) Characterization of α -galactosidase from cucumber leaves. *Plant Physiol* **66**: 731–734
- Strand A, Foyer CH, Gustafsson P, Gardestrom P, Hurry V** (2003) Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. *Plant Cell Environ* **26**: 523–535
- Steponkus PL** (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Annu Rev Plant Physiol* **35**: 543–584
- Stushnoff C, Remmele RL, Essensee V, McNeil M** (1993) Low temperature induced biochemical mechanisms: implications for cold acclimation and de-acclimation. *NATO ASI Ser I* **16**: 647–657
- Stushnoff C, Seufferheld MJ, Creegan T** (1998) Oligosaccharides as endogenous cryoprotectants in woody plants. In P.H. Li and T.H.H. Chen, eds., *Plant Cold Hardiness: Molecular Biology, Biochemistry and Physiology*. Plenum Press, New York, pp 301–309
- Taiz L, Zeiger E** (2002) Translocation in plants. In L. Taiz and E. Zeiger, eds., *Plant Physiology*, Ed 3. Sinauer Associates, Sunderland, MA, pp 690
- Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K** (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J* **29**: 417–426
- Thomashow MF** (2001) So what's new in the field of cold acclimation? Lots! *Plant Physiol* **125**: 89–93
- Uemura M, Warren G, Steponkus** (2003) Freezing sensitivity in the *sfr4* mutant of Arabidopsis is due to low sugar content and is manifested by loss of osmotic responsiveness. *Plant Physiol* **131**: 1800–1807
- Webb MS, Uemura M, Steponkus PL** (1994) A comparison of freezing injury in oat and rye: two cereals at the extremes of freezing tolerance. *Plant Physiol* **104**: 467–478
- Yelenosky G, Guy CL** (1989) Freezing tolerance of citrus, spinach, and petunia leaf tissue: osmotic adjustment and sensitivity to freeze induced cellular dehydration. *Plant Physiol* **89**: 444–451