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DISSERTATION

MOLECULAR AND PHYSIOLOGICAL ANALYSES OF A TEMPERATURE DEPENDENT α-GALACTOSIDASE IN *PETUNIA X HYBRIDA* 'MITCHELL'

Submitted by

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In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Fall 2002

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOYCE C. PENNYCOOKE ENTITLED MOLECULAR AND PHYSIOLOGICAL ANALYSES OF A TEMPERATURE DEPENDENT α-GALACTOSIDASE IN *PETUNIA X HYBRIDA* 'MITCHELL' BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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Advisor

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ABSTRACT OF DISSERTATION

MOLECULAR AND PHYSIOLOGICAL ANALYSES OF A TEMPERATURE DEPENDENT α-GALACTOSIDASE IN *PETUNIA X HYBRIDA* 'MITCHELL'

Raffinose family oligosaccharides (RFO) have been implicated in the acquisition of tolerance to low temperature stresses. Studies on the biochemical basis of plant tolerance to low temperatures have focused primarily on the cold acclimation response, while retention and loss of the cold acclimated state have been largely neglected. Alpha-galactosidase (α–Gal) is a key catabolic enzyme of RFO involved in the cold hardiness pathway, cleaving the terminal-linked moiety from galactose-containing oligosaccharides. Petunia was chosen as a model for this research because preliminary results in our laboratory demonstrated petunia's capability to cold acclimate. This research was based on the primary hypothesis that as cold acclimation occurs, specific soluble sugars increase and as tissues deacclimate, the sugar levels decrease. Based on this, a second hypothesis was formulated; that down regulation of the α -Gal gene may be an important element in the accumulation or maintenance of RFO levels that are required to enhance freezing tolerance. The objectives were to determine the role of α -Gal in deacclimation and in freezing tolerance. This was accomplished by examining α -Gal activity and transcript accumulation during raffinose catabolism and overexpression and downregulation of the α -Gal gene in petunia.

A cDNA clone *petgal*, was isolated from *Petunia x hybrida* cv Mitchell RNA by RT-PCR using degenerate oligosaccharide primers designed to amplify the α-Gal cDNA.

The putative α-Gal cDNA sequence has high nucleotide sequence homology (>80%) to other known α-Gals. Southern blot analysis suggests that α-Gal represents a single gene family. This study showed a comprehensive analysis of *petgal* expression including non tissue-specific expression, no developmental regulation and expression in response to increased temperature. Increases in α-Gal transcript one hour after deacclimation correspond with increases in α-Gal activity suggesting that warm temperature may regulate RFO catabolism by increasing the transcription of the α-Gal gene.

To examine the relationship between endogenous sugars and freezing stress, the expression of α-Gal was modified in transgenic petunia. The α-Gal cDNA from tomato seed under the control of the Figwort Mosaic Virus promoter was introduced into petunia using the *Agrobacterium tumefaciens*-mediated transformation.

Besides lower germination percentages in transgenic plants, there were no phenotypical differences between wild type and transgenic plants. RNA gel blot analysis demonstrated an increase in α-Gal transcript accumulation in sense plants and a decrease in antisense plants. Antisense inhibition of the α-Gal gene resulted in an accumulation of raffinose and enhanced freezing tolerance of petunia. Among the antisense lines examined, several different levels of freezing tolerances were observed with A105 being the most tolerant line and A91 being the least tolerant line. Freezing stress tolerance was predicted based on the relationship between raffinose accumulation and decreased electrolyte leakage. Overexpression of the α-Gal gene inhibited low temperature tolerance when compared to antisense petunia lines suggesting that α-Gal plays a major role in low temperature tolerance. 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.

As an adjunct to this study, phenolic extracts from plants subjected to two different cold acclimation regimes to induce chilling tolerance were analyzed for specific phenolic acids and assessed for their antioxidant capacity. Gentisic acid was induced in significant quantities upon cold acclimation ($p < 0.05$). The data suggest that phenolic metabolism may be a consequence of cold stress and probably not related to protective functions.

Knowledge from this research has important implications whereby petunia may be grown later into fall when frost injury is most likely. But most importantly, the target gene (α -Gal), is not limited to petunia and thus may provide a means of improving the freezing tolerance of other economic crops.

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Chapter 1

Literature Review

1.1 Origin and introduction of petunia

Petunia was first recorded as a species in the late 1700s when it was thought to be a member of the *Nicotiana* genus (Pelletier and Ferault, 1976). The genus *Petunia* originated in South America and wild relatives are abundant in Argentina, Bolivia, Brazil and Uruguay (Sink, 1984). Petunia is a member of the *Solanaceae* family (Corr, 1998). As a result of the breeding work of the French botanist Petun, early cultivars were found in European private gardens by 1850 (Kessler, 1999).

Today, *Petunia x hybrida* is one of the major bedding plants in the world because of its versatility and variety. It is grown for its large colorful flowers. Commercial petunia originated as a result of chance crossing between *Petunia axillaries* and *Petunia violacea* (Weddle, 1976). Currently, there are a number of petunia cultivars on the market: grandifloras (single and double), multifloras (single and double) and floribunda (Corr, 1998).

Petunia x hybrida is a popular model system for scientific research for many reasons. The flowers have large sexual organs, enabling easy manipulation for pollination. The fruit is a capsule that consists of hundreds of seeds which makes propagation easy. Seeds may remain dormant for 3-4 months but this can be overcome by treatment with GA_3 at 100 ppm for 24 h before germination. *P. hybrida* has a relatively short life span, with only two months for flowering and one month for fruit ripening. It is

also self-compatible, thus continuation of inbred lines is possible. In addition, *P. hybrida* is a diploid with only seven pairs of chromosomes, as such working with genetics is relatively simple.

The use of petunias for transgenic studies to improve certain traits such as plant vigor, flower longevity and salt tolerance is common (Joseph et al., 1995; Clark et al., 1999; Chaing 2000) because transformation protocols are already established and are relatively uncomplicated (Jorgensen et al., 1996).

1.2 Chilling tolerance of petunia

Petunia is a cool season crop that is responsive to day-night temperature differential. Optimum day temperatures are 16 °C to 18 °C and night temperatures are 13 °C to 16 °C (Corr, 1998). In the southern United States, plants do well in full sun and are hardy in areas with mild or no winters. A hard frost will kill petunias. Not much is known about petunia's chilling tolerance but Yelonosky and Guy (1989), presented evidence that cold acclimated petunia increased in freezing tolerance by about -1 °C as a result of low temperature induced osmotic adjustment.

1.3 Biochemical and physiological mechanisms of low temperature tolerance/injury

Most tropical and subtropical and some temperate species exhibit limited tolerance to low nonfreezing temperatures (Levitt, 1980). The critical temperatures for chilling injury in tropical species are in the range of 0 °C to 12 °C and temperatures below 5 °C for temperate species. Chilling injury refers to a metabolic and physiological dysfunction caused by low nonfreezing temperature on various physiological processes in

plants (Levitt, 1980). Chilling injury is affected by the duration of low temperature exposure, light intensity and humidity. Tolerance varies among plant species, tissues, and developmental stages. Visual observations of chilling injury become apparent days or weeks after chilled stressed plants are transferred to non-chilling temperatures. These include wilting, water soaked lesions, chlorosis and eventually necrosis leading to death (Lyons, 1973). The most widely used method to determine chilling injury is electrolyte leakage of cellular components (Levitt, 1980). Since the plasma membrane is the primary site of damage due to chilling stress injury, electrolyte leakage measured by conductivity is rather quick and simple. Ultrastructural studies using electron microscopy may also show damage (such as general disorganization of organelles and membrane discontinuities) to cell components even before whole plant symptoms are apparent (Ikler et al., 1976). Triphenyl tetrazolium chloride (TTC) test measures the capability of a plant tissue to carry out respiration. Presumably, inhibition of TTC reduction is an indication of enzyme inactivation and a reliable indicator of chilling stress (Stergios and Howell, 1973). The degree of chilling injury may also be determined based on changes in ethylene production and enzyme activities (Wang, 1982). The classical visual symptoms such as wilting, chlorosis and water soaked lesions are not always quantitative but a scoring index may be used such as in the tissue browning assay, which quantifies damage (Sufferfeld et al., 1999). The most decisive indication of low temperature stress tolerance is evaluation of recovery based upon regrowth when whole plants are tested. This assay is especially applicable in herbaceous perennials (Zurawicz and Stushnoff, 1977).

Chilling injury results in a loss of cell homeostasis in which a number of biochemical and physiological mechanisms are affected. These include loss of

protoplasmic streaming, changes in membrane structure and function, loss of mitochondrial activity, alterations in respiratory rates, increased proteolysis, loss of enzyme activity and metabolic inbalances (Morris, 1982).

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). The term cold acclimation is usually used to describe the outcome of a range of biochemical and physiological processes associated with the increase in cold tolerance. This phenomenon is the process by which certain plants increase in cold tolerance upon exposure to low non-freezing temperature (Guy, 1990). Some of the more common biochemical and physiological processes include changes in endogenous metabolites, lipid composition, and reactive oxygen species.

Several lines of evidence suggest that endogenous metabolic changes accompany the transition to the cold hardy state. These endogenous metabolites include soluble sugars such as raffinose family oligosaccharides (RFOs), polyols such as mannitol and sorbitol, and amino acids such as proline and glycine betaine. RFOs are the most abundant soluble carbohydrates in the plant kingdom (French, 1954). The biosynthesis of RFOs depends on galactose donation from galactinol, originally to sucrose creating the trisaccharide raffinose. Subsequent galactose donation to the existing RFOs forms higher degree polymerizations of the family. Galactinol is formed from UDP-galactose and myo-inositol by the action of galactinol synthase, which is the rate limiting, committed enzyme step in the RFO-biosynthetic pathway. Raffinose degradation occurs through sequential exo-hydrolysis by α-galactosidase (Dey, 1985) (Fig. 1.0). In many plants

RFOs serve as storage (Keller and Matile, 1985) and transport (Hendrix, 1968) metabolites. Less attention has been given to the equally important function of conferring cold tolerance. In several species, sucrose, raffinose and other members of the raffinose family oligosaccharides have been implicated in the acquisition of tolerance to low temperature stresses (Stushnoff et al., 1993; Imanishi et al., 1999; Habb and Keller, 2002). Crawford and Huxter (1977) reported that maize and peas, both chilling sensitive species were capable of growing at chilling temperatures if an adequate supply of sugar was maintained in their roots. Raffinose accumulation in pansies has been demonstrated during low temperature induced cold acclimation (Stushnoff et al., 1998). Similarly in *Citrus sinensis* L. cv Valencia (Yelonosky and Guy, 1977) and in young grapefruit (Purvis and Yelonosky, 1983) sucrose levels increased at cool temperatures, but the authors suggested that this was a consequence of low temperature rather than a prerequisite of cold hardening.

These endogenous metabolites confer cold tolerance by one of two mechanisms or both; osmoprotection or osmoregulation (Crowe et al., 1987; Crowe et al., 1988; Yelonosky and Guy, 1989). During osmoprotection, the water replacement theory described by Crowe et al. (1987), proposes that sucrose interacts with membrane phospholipids and proteins to stabilize their structures as water is removed. It is universally accepted that the increase in compatible solutes results in increased cellular osmolarity. As temperatures drop below freezing, ice formation is generally initiated in the extracellular spaces of plant cells and, because the chemical potential of ice is less than that of water, water moves out from inside the cell to the extracellular spaces where it freezes (prevents lethality caused by intracellular ice formation). As such, the

accumulated compatible solutes act as osmolytes, which facilitate the retention of water in the cytoplasm thus preventing dehydration resulting from water movement to the appoplast as extracellular ice forms (osmoregulation) (Yelenosky and Guy, 1989; Thomas and James, 1993). Yelenosky and Guy (1989) provided evidence that petunia, citrus and spinach had minor increases in cold tolerance resulting from low temperature induced osmotic adjustment. Other suggested functions of endogenous metabolites include carbon source and storage, signaling and free radical scavenging. Because starch synthesis is relatively sensitive to cool temperature, RFO accumulation offers an alternative form of carbohydrate storage and provides a ready supply of reduced nitrogen and carbon upon relief from stress. The signaling capabilities of certain osmolytes have been implicated in mediating responses to abiotic stresses. Nelson et al. (1999) suggested that myo-inositol serves as a leaf-to-root signal to facilitate sodium uptake in ice plants. Many of the endogenous metabolites that are produced in response to the biochemical and physiological adaptations to cold stress do so by various mechanisms. Proline for example, has been shown to induce chilling tolerance by acting as an osmoprotectant (Christian, 1955), a hydroxyl scavenger (Smirnoff and Cumbes, 1989) and serving as a sink for energy to regulate redox potential (Saradhi and Saradhi, 1991).

The changes in lipid composition that occur during cold acclimation have been well documented in several studies (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). Several hypotheses have been put forward to explain the various mechanisms of low temperature tolerance. The membrane phase change hypothesis proposed by Lyons and Raison (1970) was among the first to explain the fundamental basis for chilling sensitivity by causing membrane-associated enzymes to undergo conformational changes

that alter catalysis or render them inactive. There are two distinctly different types of membrane phase separations. First, there are those lipid phase separations caused directly by low temperature and these transitions are reversible upon warming. Secondly, there are those caused by changes in the lipid composition during the stress treatments that are environmentally irreversible.

Membrane lipid composition was suggested to differ between chilling insensitive and chilling sensitive species. Several studies showed that chilling insensitive species have a higher degree of unsaturated fatty acids in membranes, which explains why they would not exhibit a phase change during exposure to chilling temperatures (Miller et al., 1974; Terzaghi et al., 1989; Li et al., 1990). Lipid bilayers exist in one of two states, a gel phase or a liquid-crystalline state. In the gel phase, van der Waals interactions between the fatty acid chains restrict lateral and rotational movement of the molecules. The more mobile liquid-crystalline phase forms when extreme temperatures disrupt the van der Waals interactions. The number and position of double bonds, fatty acid chain length and the lipid head group all affect the transition temperature between the two phases. At physiological temperatures, saturated phospholipids are usually in the gel phase but addition of one or two double bonds per pair of acyl chains reduces the transition temperature to below 0 °C (Lyons and Raison, 1970).

In recent years mutant analysis provided very compelling evidence in support of the membrane phase change hypothesis. The *fad* chilling sensitive mutants for acyl chain desaturases substantially decrease levels of lipid unsaturation and are defective in regenerating chloroplast membranes at low temperature. The membrane phase change hypothesis appears to have explained the discontinuities in Arrhenius plots of respiration

rates for isolated mitochondria from chilling sensitive crops compared to chilling tolerant crops (Lyons and Raison, 1970; Downton and Hawker, 1975; Terzaghi et al., 1989).

Since the membrane phase change hypothesis has been put forward, many studies have shown its importance with regard to chilling responses in plants (Wang and Baker, 1979; Sommerville and Browse, 1991; Vijayan and Browse, 2002). However, others have contradictory evidence from studies with purified enzymes such as PEP carboxylase (Uedan and Sugiyama, 1976) and Phosphofructokinase (PFK) (Bredemeijer et al., 1991) which suggest that membrane association is not a requirement to produce low temperature discontinuity in Arrhenius plots, but rather a consequence of protein rearrangement independent of lipid fluidity.

Another hypothesis associated with chilling sensitivity in plants proposed that injury results from low temperature impairment of protein structure and function. Guy et al. (1998), proposed that the direct effects of low temperature may inhibit protein biogenesis at any stage (transcription, translation, membrane translocation, posttranslational processing or folding), prevent folded proteins from remaining in the native conformation and finally, cause changes in the cellular "milieu" in response to low temperature which could indirectly disrupt the native conformation of a protein.

Privalov (1990) presented a thermodynamic explanation and evidence that cold denaturation is caused by temperature-dependent interactions of protein nonpolar groups with water. Other forces such as H-bonds and van der Waals forces that participate in protein structure may be altered at low temperature (Chen and Schellmaan, 1989). As the temperature is lowered, bulk water becomes more hydrogen-bonded and more highly ordered, thus the entropic advantage of folding nonpolar groups is lessened as

temperature decreases. Regardless, it is believed that the hydrophobic effect seems to be dominant over the other forces associated with low temperature protein conformational stability. Reduction of hydrophobic forces could contribute to dissociation of many "cold labile" enzymes (Privalov, 1990). Phosphofructokinase, chloroplast superoxide dismutase, PEP carboxylase and Rubisco are among a few enzymes exhibiting *in vitro* cold inactivation and thus characterized as "cold labile" enzymes (Privalov, 1990).

There is increasing physiological and biochemical evidence to support the view that activated oxygen plays a role in low temperature stress. Powles (1984) presented a hypothesis suggesting that during chilling-induced injury in the light, there is a transient disruption of the photosystems that makes them "leaky" and results in the transfer of energy to molecular oxygen, thus forming activated oxygen species. The most common reactive oxygen species (ROS) encountered during chilling and membrane peroxidation include the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$) (McKersie, 1991). Isolated wheat microsomal membranes treated with an aqueous source of superoxide radicals, *in vitro*, exhibited very unique physical membrane changes such as degradation of membrane phospholipid and increased membrane lipid microviscosity (McKersie and Bowley, 1998). Futhermore, cold acclimated wheat tolerated longer exposure to superoxide radicals before degradation. The authors suggested that perhaps the microsomal membranes isolated from cold acclimated plants acquired tolerance by changes in lipid composition or contained different levels of antioxidants.

Besides lipids, ROS also damage nucleic acids and proteins (Fridovich, 1991). While the superoxide radical does not directly attack DNA, the hydroxyl radical does.

When DNA is exposed to the superoxide radical, this results in damage due to the Haber-Weiss reaction. This reaction involves catalytic levels of Fe (III), which mediate the production of the hydroxyl radical by a series of reactions (Brawn and Fridovich, 1981). The hydroxyl radical is extremely reactive and can react within a few molecular diameters of its site of generation within a cell. The source of ROS produced after cold stress injury has not been well defined but there are several potential candidates mentioned in the literature. For example, when water freezes in plant tissues, any dissolved oxygen is excluded from the ice and accumulates to saturating and sometimes supersaturating levels within the tissues. As a result, these tissues donate electrons to oxygen and increase the availability of oxygen as a substrate for reactivity. Likely candidates for electron donors to oxygen are the dysfunctional electron transport chain of the chloroplast or mitochondrion (Morre, et al., 1995; Lawrence et al., 1995). Other biological sources include the NADH oxidase system (Otter and Polle, 1994), acetaldehyde oxidase and cell wall peroxidases (Bhaumik et al., 1995).

It is known that these ROS are generated in living systems, that they constitute a threat to such systems, and that defenses such as antioxidants, superoxide dismutases (SODs), catalases, peroxidases, and repair systems are necessary adaptations to living with environmental stresses (Beyer et al., 1991; Bielski and Cabell, 1991; Kerdinaimongkol and Woodson, 1999). The photosynthetic electron system and Calvin cycle enzymes are the most vulnerable targets of damage by ROS (Foyer et al., 1991). It makes sense therefore that 20% - 40% of the ascorbate (a potent antioxidant) found in plants is localized in the chloroplast. The remaining ascorbate is found in the cytosol and vacuole but is accessible to the chloroplast via chloroplast membrane translocators. The

ascorbate-glutathione cycle was demonstrated in the chloroplast by Asada and Takahashi (1987), which functions to scavenge hydrogen peroxide by ascorbate peroxidase and maintains the ascorbate pool in the reduced form.

Quantitative measurements of ROS in biological systems are technically difficult but in recent years several methods have been developed. Kendall and McKersie (1989) used the spin trap Tiron method to show a two- to five-fold increase in superoxide activity in wheat microsomes isolated from lethally frozen crown tissue. The 2,2' azinobis (3-ethylbenzothiazoline-6-sulfonc acid) diammonium salt (ABTS) assay measures the ability of antioxidants in the sample to scavenge the $ABTS^{\bullet}$ radical. Trolox is a water-soluble vitamin E analog that is used as the standard and the free radical scavenging ability is determined by the trolox equivalent antioxidant capacity (Miller and Evans, 1997). The fox assay measures the ability to scavenge hydroperoxide radicals and is suitable for measuring initial rather than advanced fatty acid oxidation. For this reason this assay is ideal for detecting early membrane-associated stress events in plant tissues (DeLong et al., 2002).

1.4 Alpha-galactosidase in plants

The distribution of α-galactosidase in plants has been studied extensively to determine its role in hydrolysis of transport sugars of the RFO series (French, 1954; Thomas and Webb, 1978; Gao and Schaffer, 1999). Alpha-galactosidases catalyze the hydrolysis of various storage substances like galacto-oligosaccharides, -lipids and -proteins. Studies of plant α-galactosidases have mostly focused on seed tissues since seeds contain large amounts of such storage compounds, which are utilized as an energy

source during germination (Bewley and Black, 1994; Feurtado et al., 2001). Alpha-galactosidase activites however have been studied in photosynthetic tissues (Thomas and Webb, 1978; Smart and Pharr, 1980; Chrost and Schmitz, 2000). Multiple forms of the α-galactosidase enzyme have been described (Keller and Pharr, 1996). These can be divided into two groups, acid and alkaline, based on their activity response to pH. Most studies have dealt with the acid forms of the enzyme, which play important roles in seed germination (Keller and Pharr, 1996). In the Cucurbits, Gaudreault and Webb (1986) described an alkaline α-galactosidase from young leaves of *Cucurbita pepo*, in addition to multiple acid forms of the enzyme. The alkaline forms were unique in that they showed high affinity for stachyose and little activity toward raffinose compared with the acid forms, for which raffinose was found to be the preferred substrate (Gaudreault and Webb, 1996; Pharr and Hubbard, 1994). For plants synthesizing and translocating RFOs, appreciable levels of α-galactosidase activity were found in leaves and petioles at different stages of development, roots, flowers and dry and germinating seeds (Thomas and Webb, 1978). The occurrence of α-galactosidase activity indicates an effective intracellular compartmentation between its location and sites of RFO biosynthesis, accumulation and movement in the tissue. RFOs, substrates for α-galactosidase, are synthesized and stored generally in the cytoplasm (Obendorf, 1997). The presence of a signal peptide in the tomato α-galactosidase (Feurtado et al., 2001) may destine it for one of two locations: the cell wall region, where galactomannan is located and or protein bodies, where the enzyme has been found in soybean (Herman and Shannon, 1985). During germination, RFOs are transported from the cytosol to the protein bodies (Obendorf, 1997). The presence of a signal peptide on the tomato α-galactosidase does

allow it to be transported into the ER, and thence to a protein body or extracellular site (Feurtado et al., 2001).

In plants not synthesizing RFOs, α-galactosidases seem to be involved in galactolipid metabolism of plastid membranes (Chrost and Krupinska, 2000). Galactolipids are the major lipid components of thylakoid membranes. The degradation of these plastid galactolipids during senescence requires hydrolytic enzymes such as α-galactosidases. Chrost and Krupinska (2000) demonstrated that among the genes associated with senescence in barley leaves, two are coding for putative α-galactosidases. It is not surprising then, that the location of α-galactosidase in these species is concentrated in the photosynthetic lamellae of chloroplasts (Dey and Pridham, 1972).

RFOs, mainly raffinose and stachyose are the major factors responsible for flatulence following ingestion in soybean-derived products (Guimaraes et al., 2001). Removal of RFO from seeds or soymilk by the hydrolysis of α-galactosidases would have a positive impact on the acceptance of soy-based foods. Studies are in progress characterizing α-galactosidases from germinating soybean seeds and on the possibility of using these enzymes for hydrolysis of the RFO content in soymilk (Guimares et al., 2001).

Alpha-galactosidase is widely distributed in plants and cDNAs have been cloned from several sources including guar (Overbeeke et al., 1989), coffee bean (Zhu and Goldstein, 1994), soybean (Davis et al., 1996), pinto bean (Davis et al., 1997) and tomato seed (Feurtado et al., 2001) to name a few. Alpha-galactosidases have broad substrate specificities hydrolyzing many types of bonds thus enabling many different roles in plant and animal metabolism. The precise specificity of the enzyme is somewhat dependent on

the nature of the glycoconjugate (Zhu and Goldstien, 1994). The isolation of α-galactosidase from the above mentioned sources showed the role of the enzyme in seed germination and assimilate partitioning (Feurtado et al., 2001) and seroconversion of blood group B to O (Davis et al., 1996; Davis et al., 1997).

1.5 Molecular genetics of low temperature tolerance

Many aspects of plant responses to low temperature have been reviewed (Weiser, 1970; Steponkus, 1984; Guy, 1990). In the most recent review on the subject of cold stress, Thomashow (2001), noted that the "cold acclimation research landscape has dramatically changed". This is due to the significant insights on novel approaches of low temperature signaling, regulatory pathways, molecular genetics and mutational studies.

Cold acclimation is a complex phenomenon and its signal transduction pathway consists of: i) perception of cold signals, ii) transduction of these signals into biochemical processes by releasing second messengers (eg. calcium), iii) cytosolic events involving protein kinases and phosphatases, iv) transfer of cold-specific signals to the nucleus, v) activation of cold acclimation-specific genes, and vi) development of cold tolerance. In alfalfa (Dhindsa and Monroy, 1994) and Arabidopsis (Knight et al., 1996), low temperature triggers the process of cold acclimation by opening the calcium channels on the plasma membrane allowing the cell wall calcium to enter the cytosol. Dhindsa and Monroy (1994) suggested that the roles of cold and calcium are separate and demonstrated that calcium alone can induce cold acclimation-specific genes at room temperature but cold is essential to stabilize the expression of these genes. Although the mechanisms involving the steps between calcium influx and gene expression are not well

understood, it is believed that certain calcium-dependent protein kinases and phosphatases are involved. Alfalfa plants treated with the protein phosphatase inhibitor okadaic acid, increased transcript levels at normal growth temperatures and did not accumulate transcripts upon low temperature exposure in plants treated with the protein kinase inhibitor staurosporine (Monroy et al., 1998).

Knowledge of the biosynthetic pathways of metabolites involved in cold hardiness has the potential for enhancing stress tolerance. To study gene expression patterns the revolutionary Affymetrix GeneChip microarray technology or metabolic engineering may be used. Using Affymetrix Genechip arrays, Fowler and Thomashow (2002), demonstrated transcription of approximately 8000 Arabidopsis genes in cold acclimated and non acclimated plants. Approximately 306 genes were identified as being cold-responsive at various time points. Of these cold responsive genes, transcript levels for 218 genes increased by 3-fold or greater while those for 88 genes decreased. As such, the results of their study indicated the existence of a multiple low temperature regulatory pathway in addition to significant down regulation of certain genes during cold acclimation.

Metabolic engineering refers to strategies for manipulating plant metabolism, which involves targeting specific enzymes or regulatory pathways. Manipulating an existing pathway may be accomplished by overproduction of a rate-limiting enzyme (inserting extra copies of the gene of interest using a sense construct) or by down regulation of gene expression (shutting off genes). Down regulation of gene expression may be accomplished by antisense technology or partial sense or cosuppression mechanisms (see below). A few attempts to understand chilling tolerance mechanisms

have been successful using these approaches (Kerdinaimongkol and Woodson; 1999, Chiang, 2000). Chilling injury and other abiotic stresses have been associated with increased production of reactive oxygen species (ROS). Catalase assumes the role of a primary hydrogen peroxide scavenger. Kerdinaimongkol and Woodson (1999) showed that tomato plants transformed to suppress catalase activity increased in oxidative and chilling stress sensitivity. While Chiang 2000, demonstrated that petunia plants overexpressing the mannitol-1-phosphate dehydrogenase enzyme (MTL D) resulted in two transgenic lines with increased mannitol levels and enhanced chilling tolerance compared to wild types and low mannitol expressing transgenic lines.

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 genes has been undertaken. Hajela et al. (1990) isolated the COR (cold responsive) genes from *Arabidopsis thaliana*. They found that the transcript levels for at least one member of the COR6.6, COR15, COR47 and COR78 gene families began to accumulate within the first 4 h exposure to low temperature (5 °C) and remained elevated for as long as the plants were kept at low temperature. Inspection of the COR15a and COR78 promoter sequences revealed sequence repeats, which they referred to as "CRT" (C-repeats) (Baker et al., 1994). Further analysis led to the identification of transcriptional activators that bind to the CRT, designated CBF1, CBF2 and CBF3 (Gilmour et al., 1998). Interestingly, constitutive expression of the CBF1/DREB1b gene in transgenic Arabidopsis induced the expression of multiple cold-responsive CRT/DRE-containing genes in the absence of a low temperature stimulus (Jaglo-Ottosen et al., 1998). Thus the "CBF regulon" (CRT/DRE-containing genes that

are induced by the CBF/DREB1 transcription factors) results in the discovery of a novel cold acclimation pathway containing genes that do not require a low temperature stimulus for activation (Thomashow, 2001).

Chemical mutagenesis has been used to isolate several cold acclimation mutants (Sommerville and Browse, 1991; McKown et al., 1996; Xin and Browse, 1998). *Arabidopsis thaliana* is a chilling tolerant species, but mutations in its lipid metabolism result in chilling sensitivity (Somerville and Browse, 1991). Miquel et al. (1993) showed that the *fad2* mutant for oleate desaturase grew normally at 22 °C but could not survive extended low temperature exposure. Warren et al. (1998) identified the *sfr* (sensitivity to freezing) mutants from Arabidopsis. These mutants decrease the level of tolerance that would normally be attained upon cold acclimation. The *sfr4* mutant fails to accumulate sucrose during cold acclimation, which results in its increased sensitivity to cold temperatures. Like the *fad* mutants, the *sfr4* and *sfr7* mutations caused perturbations of fatty acids pools in leaf tissue following cold acclimation. In contrast, the *eskimo1* (*esk1*) mutant results in increased cold tolerance in both nonacclimated and cold acclimated Arabidopsis plants (Xin and Browse, 1998). In nonacclimated *esk1* mutant plants there was a 30-fold and 2-fold accumulation of proline and soluble sugars, respectively, compared to nonacclimated wild type plants. It is believed that increases in proline and soluble sugars contributed to enhancing cold tolerance of the *esk1* mutants. The *hos1* gene has been recently identified and shown to encode a protein with RING-finger motif (Lee et al., 2000). Zhu and colleagues have proposed that HOS1 and HOS2 appear to encode negative regulators of low-temperature signal transduction and function upstream of the CBF/DREB1 transcription. Mutations in these genes result in induction of

COR78/RD29a genes in response to low temperature (Ishitani et al., 1998; Lee et al., 1999).

1.6 Cloning plant genes

Clones may be characterized through their sequence data. The DNA sequence data in itself is not valuable until it is analyzed and, if possible, also applied. The sequence of nucleotides within a DNA molecule can yield a wealth of information such as amino acid sequence and composition, gene structure contained within sequences, post translational processing and cellular location. Knowing the nucleotide sequence of a clone is helpful to determine the biological function of the deduced protein.

Cloning a gene is an intense process requiring not only molecular biology techniques, but also microbiology, genetics and protein biochemistry approaches. A range of techniques is available for cloning of plant genes and knowledge of one or more of the following is necessary: i) the sequence of the gene or gene products: screening of libraries with a probe (cDNA or synthetic oligonucleotide) allows the isolation of clones with homologous sequences; ii) the location of the gene in the genome: map-based cloning involves "walking" from a known linked marker to the gene of interest; iii) the pattern of gene expression: differential screening of cDNA libraries may be used or subtraction of libraries may allow the isolation of cDNA clones unique to particular tissues or stages of development and iv) the function of the gene: genes may be cloned by analysis of their ability to add a function or a trait to a line that is lacking the gene such as transposon insertion (Sawahel and Kiichi, 1995).

A typical scheme for cloning involves choosing the source of DNA for cloning and producing a collection of the DNA fragments that can be inserted into a vector. This construct is introduced into a population of bacteria and then screened for the desired sequence. A good choice of DNA for cloning would be complementary DNA (cDNA), a DNA copy of an mRNA. Synthesis of cDNA requires the isolation of total RNA from specific tissue or under certain environmental conditions. Reverse transcriptase is used to produce a 1st strand DNA copy of the mRNA of interest using two gene specific primers. This is then converted to double-stranded DNA in a second strand synthesis reaction involving DNA-polymerase. This technique is known as RT-PCR. The polymerase chain reaction (PCR) is a technical revolution in molecular biology. Kary Mullis first described it in 1990. This is a process by which DNA is amplified exponentially *in vitro*. PCR makes use of the instability of the double helix at high temperature, the specific hybridization of oligonucleotides and action of heat tolerant DNA polymerases such as Taq polymerase (from *Thermus aquaticus*). PCR can be divided into three steps: denaturation, annealing and extension. The key to PCR lies in the fact that two oligonucleotides (primers) are designed to anneal to opposite strands of DNA so that their 3' hydroxyl groups point towards each other. The extension (polymerization) of the primers replicates the homologous binding sequence for the other primer. PCR with DNA Taq polymerase, results in products with 3'-A overhang because of the enzymes' capacity to add a single A to a double-stranded DNA. Vectors with a complementary T can be used to clone these products with high efficiency. This approach is known as TA cloning (Zhou et al., 1995).

Transformation of the clone into *Escherichia coli (E. coli)* may be achieved via electroporation or heat shock. *E. coli* cells must first be prepared in such a manner that they become permeable or competent. Competent cells are those that are capable of assimilating exogenous DNA. The idea of electroporation is to create holes in the cell membranes, accomplished by discharging a brief electric pulse across electrodes of an electroporater. The initial screening of cells begins with differentiation between those that are transformed and those that are not. It is relatively easy since the vector contains a selectable maker or antibiotic resistance gene. Transformed cells cultured on medium with the selectable marker will grow while nontransformed cells will not. Another method of identifying a recombinant vector is by gene inactivation. Vectors with a polycloning site within the coding region of a part of a β-galactosidase gene allow blue-white screening. The gene LacZ codes for the β-galactosidase enzyme. Though *E. coli* normally uses β-galactosidase to cleave lactose (hence LacZ), the enzyme hydrolyzes any β-galactoside including the chromogen (color yielding substrate) 5-bromo 4-chloro 3-indoyl β-D galactoside (X-Gal). Appropriate *E. coli* hosts harboring plastids with wild type lacZ confer the β-galactosidase phenotype. Insertion of a DNA fragment in the poycloning site disrupts the production of an effective peptide, preventing complementation to form an active β-galactosidase. When cells synthesizing β-galactosidase are grown on media containing X-Gal and the lacZ inducer isopropyl-β-D thiogalactose pyranoside (IPTG), the enzyme releases the indole derivative from galactose (a blue precipitate). Transformed cells with the vector and the insert should be white and those transformed only with the vector should be blue. Restriction digests or

PCR of the plasmid DNA may be done to confirm the presence of the insert (Sambrook et al., 1989).

1.7 *Agrobacterium*-mediated transformation

The introduction of foreign DNA into plants may be achieved in several ways; *Agrobacterium*-mediated transformation (Mozo and Hooykaas, 1992), microparticle bombardment (Birch and Bower, 1994) and micro-injection laser-mediated transformation (Guo et al., 1995) are just a few. The most effective approach in those plants amendable to the technique is probably *Agrobacterium*-mediated transformation (using *A. tumefaciens* or *A. rhizogenes*). *A. tumefaciens* may be viewed as a natural genetic engineer. As a soil pathogen, this bacterium causes crown gall tumors in wounded dicotyledonous plants (Zupan and Zambryski, 1995). Gall inducing strains contain a single copy of a plasmid (the transfer or Ti plasmid) that includes a segment (the transfer or T-DNA) that is stably incorporated into the plant genome. Mobilization of the T-DNA to plant cells is mediated by gene products located in another region of the Ti plasmid, the virulence (*vir*) region and in chromosomal (*chv*) genes (Zambryski, 1988). The T-DNA is flanked by 24-bp direct repeats (T-DNA border repeats). In plant vectors, therefore, the genes that are to be transferred to plant cells are cloned between the border repeats along with a plant selectable marker. The exact mechanisms involved in the integration of the T-DNA into the plant chromosome are not well understood. However, Zupan and Zambryski (1995) suggested that T-DNA could integrate into plant cells by illegitimate recombination.

Binary vectors are often used in plant tranformation systems. These vectors are capable of autonomous replication to the Ti plasmid. As the name suggests, binary vectors are constructed from two plasmids, one with the T-DNA border sequences, a selectable marker expressible in plants, and a wide host range replicon capable of replicating in *E. coli* and *A. tumefaciens* (Bevan, 1984). The other plasmid, known as the helper plasmid, contains the *vir* regions without the T-DNA (Gynheung, 1987). Using *E. coli* as a host, the triparental mating system may be used to transfer the recombinant molecule to *A. tumefaciens* carrying a helper Ti plasmid (Ditta et al., 1980; Gynheung, 1987).

The key to plant transformation is in designing the vector construct. Regulation of transgene expression is important. A promoter (short DNA sequence) is used to initiate transcription and it affects when, where, and how a gene product is produced. Promoters in transgenic plants are derived from i) pathogens e.g. the cauliflower mosaic virus 35S (CaMV35S) is a constitutive promoter, that is expressed all the time and in all plant parts (Bourgue, 1995) or ii) plant genes e.g. the *cab* promoter from chlorophyll a/b binding protein is light inducible (Millar and Kay, 1991). Selectable markers, usually antibiotic or herbicide resistance genes are used to distinguish between transformed plants and wild type (non-transformed) plants. The *npt II* (Bevan et al., 1983) or *Bar* (Elzen et al., 1985) genes for example are placed downstream of the host promoter and can be expressed in foreign hosts to yield active enzymes which inactivates kanamycin (antibiotic) and bialaphos (herbicide) respectively. Reporter genes are also selectable markers used when the timing or location of a transgene is important. The *E. coli* gene for β-glucuronidase (GUS) is especially useful as a reporter gene in plant transformation systems (Lambe et

al., 1985). When plant cells expressing β-glucuronidase are cultured in X-glucuronide (X-gluc), a blue color is produced that can be detected histochemically or measured quantitatively with a fluorometer. However, the disadvantage of using this marker is that destructive sampling is necessary for histochemical analysis. The luciferase (LUC) and green florescent protein (GFP) reporter genes may be used for expression in living cells.

The orientation of the gene in the construct determines whether that gene will be over-expressed or down regulated. The key of over-expressing any gene lies in the strength of the promoter. A promoter that is upstream of the gene of interest in a forward orientation (5' – 3') will initiate transcription in a sense direction. The coding region of the gene requires a translation initiation and termination codon and care is required to maintain the codons in frame. As a further refinement, the codon usage may need to be adjusted to maximize plant expression. Introns may also be used to enhance expression.

Downregulation of plant transgenes may result from homology-based gene silencing. One particularly important phenomenum in petunia requires transcript homology, is post-transcriptional and is referred to as cosuppression. Additions of transgenes encoding chalcone synthase into petunia frequently result in lack of anthrocyanin pigment in the floral petals. This is due to post-transcriptional degradation of chalcone synthase RNA from both the transgenes and the endogenous chalcone synthase genes that have coding sequence identity with each other (Jorgensen et al., 1996).

A new approach for shutting off an endogenous cellular gene, targets the gene's mRNA rather than the gene itself. This can be accomplished with a vector that carries a portion of the target gene downstream of a strong promoter in backward orientation

(antisense 3' – 5'), so that the RNA transcribed from the vector is complementary in sequence to the mRNA transcribed from the corresponding cellular gene. The hypothesis is that when the antisense RNA is present in excess, it base-pairs to all the mRNA to form a double-stranded RNA that cannot be translated into protein and is rapidly destroyed. In several cases, this strategy has led to reduced expression of the corresponding cellular gene. Particularly dramatic results were achieved with antisense constructs for chalcone synthase to inhibit flower pigmentation (van de Krol et al., 1988), ACC synthase to decrease galactosidase activity, reduce ethylene production and delay fruit ripening in tomato fruit (Sozzi et al., 1998) and catalase to enhance sensitivity to oxidative and chilling stress (Kerdinaimongkol and Woodson, 1999). To signal the end of transcription, a nopaline (nos) 3' polyadenylation sequence is designed in the construct.

Antibiotic resistance and the enzyme-linked immunosorbent assay (ELISA) are merely initial screening techniques for the presence of transgenes. These tests confirm the presence of the antibiotic genes in the construct but do not directly determine the presence of the target gene. Northern blots or RT-PCR of RNA isolated from transgenics demonstrates the presence of mRNA transcripts and are better estimations of the presence of the transgene. To quantify the gene products, western blots can be done using polyclonal antisera raised against the enzyme of interest.

1.8 Limitations in plant transformation

Generally foreign genes introduced into a plant genome can retain expression but the level of expression varies widely among transformants. Although a growing list of plant species have been successfully transformed, many technical problems associated

with plant transformation remain to be resolved. Many of the direct transfer methods (microparticle bombardment, microinjection) are highly genotype dependent because of the need for tissue culture. Improvement of genotype-independent cell culture and plant regeneration techniques are required to allow more general application of plant transformation techniques.

Gene expression may not be stable in some systems because of gene silencing, co-suppression, T-DNA deletion during recombination and position effect (Matzke and Matzke, 1995). Chalcone synthase (CHS) is an enzyme in the biosynthetic pathway for anthocyanin, a purple pigment found in flowers and maize grains. It was expected that over expression of the cloned petunia CHS in petunia might result in flowers with a deeper purple color. Surprisingly, the regenerated transgenics produced flowers with either uncolored sectors or flowers lacking color entirely. Somehow the presence of the additional petunia gene had led to its inactivation as well as the endogenous gene (Jorgensen et al., 1996). This phenomenon is known as cosuppression and is still not understood. Loss of expression of some genes may be due to methylation (up to 30% of cytosines in plants may be methylated as 5-methylation) and differential methylation may explain differences in the expression of transgenes (Lambe et al., 1995). When trangenes insert into endogenous genes, this causes the genes to become inactivated, a phenomenon known as position effect. Transgenes can insert downstream of a strong promoter or tissue specific promoter that causes change in the expected expression patterns.

1.9 Importance of this research

In response to cold stress tolerance, the role of RFO catabolic enzymes (mainly α-galactosidase) is largely unexplored. Previous studies have shown that the activity of RFO biosynthetic enzymes can change dramatically in response to stress (Bachmann and keller, 1995), plant developmental stage (Pharr et al., 1985) and leaf age (Holthaus and Schmitz, 1991). Data from this research provides information on the role and characterization of α-galactosidase during deacclimation.

The combination of molecular and physiological approaches contributes to the understanding of RFOs role in freezing tolerance. Since RFOs have been shown to be an essential component of cold stress tolerance, these studies provide a direct route to modify RFO accumulation in target tissues and subsequently enhance freezing tolerance. This has implications for late fall production of petunia when frost is most likely. Also the target gene (α-galactosidase) is not limited to petunia hence this approach may open the way to the development of strategies for the production of transgenic plants with enhanaced tolerance to cold stress.

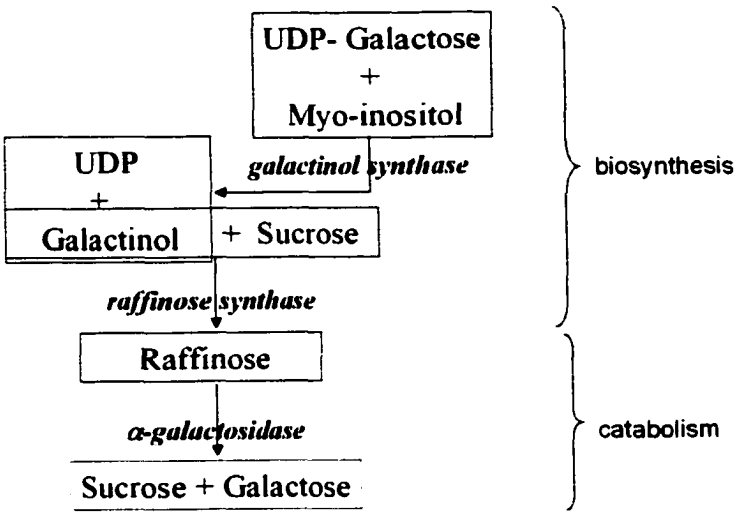


Fig. 1.0 RFO metabolic pathway

Chapter 2

Characterization of an α-galactosidase from petunia in response to cold acclimation and deacclimation

2.1 Abstract

Raffinose family oligosaccharides (RFO) have been implicated in the acquisition of tolerance to low temperature stresses. Studies on the biochemical basis of plant tolerance to low temperatures have focused primarily on the cold acclimation response, while retention and loss of the cold acclimated state have been largely neglected. This study examined α -galactosidase activity and transcript accumulation during raffinose catabolism. The raffinose metabolite has been strongly associated with cold hardiness in many species. Variations in soluble carbohydrate content and enzyme activity in *Petunia x hybrida* cv Mitchell were examined under controlled environmental conditions. Cold acclimation of plants that were previously grown at warm conditions (25 °C) was accompanied by a ten-fold accumulation of raffinose. Membrane leakage of cold acclimated chilled plants was significantly lower than in nonacclimated chilled plants. Petunia deacclimated after only one hour of exposure to warm temperatures (25 °C) as indicated by electron micrographs and at the same time α-galactosidase activity increased two-fold, corresponding to a significant decrease in raffinose levels. Northern blot analysis indicated that the level of α -galactosidase transcript increased in plants that were

deacclimated for one hour compared to cold acclimated plants. The enzyme was characterized with optimum activities at pH 6.0 and 45 °C. The increase in α-galactosidase activity and transcript accumulation during deacclimation, suggests a temperature-dependent regulatory role of α- galactosidase in raffinose catabolism.

2.2 **Introduction**

Raffinose family oligosaccharides (RFO) are the most widely occurring oligosaccharides in the plant kingdom (French, 1954). Most biochemical and physiological studies concerning RFO metabolism have been performed on seeds of legumes and leaves of cucurbits. Besides storage in seeds and carbon transport in leaves, RFO are equally important in the cold hardiness pathway (Kandler and Hopf, 1980). In plants, there is ample evidence to suggest that RFO are involved in the acquisition of tolerance to low temperature stresses during cold acclimation (Stushnoff et al., 1993; Bachmann et al., 1994; Castonguay and Nadeau, 1998). Raffinose levels in *Ajuga*, *Forsythia* and *Medicago* have been shown to be the lowest in summer and highest in fall and winter (Bachmann et al., 1994; Flinn and Ashworth, 1995; Castonguay et al., 1995). Raffinose and stachyose are known to accumulate in the needles of gymnosperms (Kandler and Hopf, 1982; Hinesley et al., 1992), and dormant buds and cortical stem tissues of woody plants (Stushnoff et al., 1993) during the winter season. Studies on the biochemical basis of plant tolerance to low temperatures have focused primarily on the cold acclimation response, the process by which plants increase their tolerance to freezing in response to low non-freezing temperatures (Guy, 1990), while studies on the deacclimation process have been largely neglected.

Given the extent of the accumulation of RFO in cold acclimated plants, the activity of key enzymes associated with carbohydrate metabolism could play determining roles in low temperature adaptation. Raffinose biosynthesis is regulated by the action of two enzymes, galactinol synthase and raffinose synthase. RFO catabolism proceeds by sequential exo-action of α-galactosidase which catalyses the hydrolytic cleavage of the terminal-linked moiety from galactose-containing oligosaccharides (Dey, 1985). Our primary hypothesis is that as cold acclimation occurs, specific soluble sugars increase and as tissues deacclimate, the sugar levels decrease. The goal of this study was to examine the role of α-galactosidase in this process by investigating α-galactosidase activity and transcript accumulation during cold acclimation and deacclimation of *Petunia x hybrida* cv Mitchell in leaves.

2.3 **Materials and methods**

2.3.1 Plant growth and treatments

Petunia x hybrida cv Mitchell plants were grown from seed and maintained in a greenhouse at 25 °C with a 16 h light / 8 h dark (16/8 h) photoperiod. Supplemental lighting was provided during the winter months by 430 watt Agro Sun lamps. Eight-week-old plants were transferred to a growth chamber to induce cold acclimation by incubating at 15 °C for 7 d, 10 °C for 7 d, 5 °C for 7 d and subsequently at 3 °C for 3 d with a 12 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. Plants were deacclimated by incubating previously cold acclimated plants at 25 °C with a 16/8 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1} for varying periods of time. Acclimation to low temperature and deacclimation in response to 25 °C were

monitored by evaluating susceptibility to chilling injury (CI). CI stress tests were conducted on nonacclimated, cold acclimated and deacclimated plants by incubating at 2 °C for 14 days with a 12 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. CI was determined by visual observation, ultrastrutural studies of leaf membrane integrity and by measuring electrolyte leakage with a 100 well ASAC 1000 seed analyzer (Neogen, East Lansing, MI) one day following chilling treatment. To determine electrolyte leakage, leaf discs (5 mm in diameter) were immersed in individual cells of a leakage tray containing 2 ml of deionized water, previously tested to read 0 conductivity. After the conductivity of the exosmosed solution was measured (T_1), the leaf discs were frozen at –80 °C overnight, and thawed at room temperature (22 °C) to destroy compartmentalization so total potential leakage (T_2) could be determined. Relative % injury represents the mean leakage as a percentage of the mean total leakage from frozen-killed samples (T_1/T_2 x 100). Electron microscopy was performed at the Electron Microscopy Center at Colorado State University. Leaf samples were fixed for 3 h in 3% glutaraldehyde in 0.05 M phosphate buffer at pH 7.0. The samples were then washed 3 times within the next hour in 0.05 M phosphate buffer and postfixed for 2 h in 1% aqueous osmium tetroxide. They were dehydrated in a graded ethanol series and embedded in Spurs's plastic for electron microscopy.

2.3.2 Soluble carbohydrate extraction and analysis

Whole leaf tissues were sampled from all stages of development from the entire plant. Samples from each plant were pooled together and immediately drenched in liquid nitrogen. Samples were freeze dried and ground into a fine powder. Carbohydrate

extraction was done using the hot 80 % ethanol extraction protocol as described by Wang et al., 1999 with some modifications. Soluble carbohydrate analyses were determined by HPLC (Dionex, Sunnyvale, CA) equipped with a CarboPac PA 10 column and a pulse amperometric detection (PAD) system. Samples were eluted isocratically with 500 mM NaOH. The soluble carbohydrates were quantified from peak area calculations and related to regression curves of a 14-sugar standard using the external standard method.

2.3.3 α-galactosidase extraction and assay

The enzyme assay was adapted from Gaudreault and Webb (1983) with slight modifications. Leaf tissue was collected from 8-week-old plants and extraction was carried out at 4 °C. Approximately 20 g fresh weight of whole leaf tissue were homogenized in a Virtis 45 homogenizer in 100 ml chilled extraction buffer consisting of 0.5M sucrose, 0.1M KCl, 50mM Tris-Cl, 10mM $Na_2S_2O_5$, 10mM phenylmethyl sulfonyl fluoride (PMSF), 1.5% PVPP at pH 6.3. The crude extract was used for α-Gal activity with p-nitrophenyl-α-D-galactopyranoside (PNPG) as the substrate.

Assay mixtures contained 100 µl of enzyme preparation 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 terminated after 20 min by adding 2.4 ml of 5 % Na_2CO_3. Blanks were prepared by adding enzyme after Na_2CO_3. 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 X 10^4 M^{-1} cm^{-1}. One unit of enzyme activity was defined as one µmol of PNPG hydrolyzed min^{-1}.

2.3.4 RNA extraction and gel blot analysis

To determine if α-galactosidase is regulated by deacclimation at the level of transcription, RNA gel blot analysis was conducted using a tomato α-galactosidase cDNA obtained from Dr. D. Bewley (University of Guelph) as a probe (Genbank accession # AF191823). Primary foliage leaves of cold acclimated and deacclimated petunia plants were frozen in liquid nitrogen and stored at – 80 °C until used for RNA extraction. Total RNA was extracted using TRIZOL reagent following the manufacturers recommendations (Gibco, BRL). RNA was quantified spectrophotometrically and 10 μg of total RNA was separated by electrophoresis through a 1 % (w/v) agarose gel containing 2.2 M formaldehyde. Equal loading of RNA samples was confirmed by visual observation of the RNA gel following staining with ethidium bromide. The separated RNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and cross-linked with a controlled UV light source (Stratalinker, Stratagene, La Jolla, CA). Membranes were prehybridized for 2 h at 42 °C and hybridized for 20 h at 42 °C with 5 X 10^5 cpm ml^{-1} ^{32}P- labeled cDNA as described by Jones et al. (1995). Membranes were washed at 55 °C in 2X SSC and 0.1 % SDS for 45 min and 0.2X SSC and 0.1 % SDS for 30 min. Transcript accumulation was visualized using a phosphoimager (Storm 840, Molecular Dynamics, Sunnyvale, CA.).

The statistical significance between treatments was tested using analysis of variance (ANOVA). All experiments were repeated at least once.

2.4 **Results**

2.4.1 α-galactosidase properties

Alpha-galactosidase from nonacclimated petunia leaves exhibited activity over a broad pH range, from 4.0 to 8.5, with maximal activity at pH 6.0 (Fig.2.0). At higher or lower pH values, activity decreased sharply.

The effect of temperature on α-galactosidase activity was evaluated by varying the incubation temperature of the assay. For all three treatments, enzyme activity was highest at an assay temperature of 45 °C after which activity dropped rapidly (Fig. 2.1). The Q_{10} was 1.8 for a temperature range of 4 °C to 100 °C. Enzyme activity at 30, 40 and 45 °C was significantly higher in deacclimated than nonacclimated or acclimated plants ($p \leq 0.05$).

2.4.2 Raffinose accumulates during cold acclimation

Five soluble sugars; glucose, sucrose, fructose, raffinose and stachyose were compared in nonacclimated and cold acclimated plants (Fig 2.2). Cold acclimation of plants that were previously grown at warm temperature (25 °C) induced a significant increase in raffinose from 0.01 μmoles g^{-1} dw to 0.082 μmoles g^{-1} dw ($p \leq 0.05$) while the levels of stachyose dropped. Raffinose was scarcely detectable in nonacclimated plants but was the only sugar that accumulated during cold acclimation. Levels of all other sugars decreased. The total soluble sugar content diminished by 18.8 % following cold acclimation from 3.19 μmoles g^{-1} dw to 0.6 μmoles g^{-1} dw.

2.4.3 Activity of α-galactosidase and the relationship to raffinose content

Analyses of the crude enzyme extracts revealed a significant increase in enzyme activity during deacclimation compared to nonacclimation and cold acclimation ($p \leq 0.05$) (Fig. 2.3). Raffinose content increased during low temperature induced cold acclimation and decreased with deacclimation as α-galactosidase activity increased (Fig. 2.4). Petunia deacclimated after 1 h of exposure to warm temperature. This was demonstrated at the ultrastructural level with pronounced chilling injury to various cell membranes: discontinuity of plasma and chloroplast membranes and ill-defined tonoplast (Fig. 2.5). Raffinose content remained constant for up to 24 h following deacclimation.

2.4.4 Northern blot analysis

Alpha-galactosidase mRNA was most abundant following 1 h of deacclimation. After 3 h of deacclimation, mRNA levels had decreased to control levels (Fig. 2.6).

2.4.5 Chilling injury of leaves

Nonacclimated plants had severe symptoms of CI when removed from chilling temperatures. Chilled plants were left at 25 °C for one day and sampled to determine electrolyte leakage as a measure of membrane damage. Significantly lower electrolyte leakage was observed in cold acclimated plants compared to nonacclimated plants (Fig. 2.7). CI evaluated by electrolyte leakage, was lower in cold acclimated plants and equal to non chilled stressed plants, suggesting no membrane injury. Non acclimated plants died after one week at 25 °C (Fig. 2.8).

2.5 **Discussion**

Under controlled environmental stress conditions, petunia provides a useful model to increase understanding of the biochemical and physiological mechanism of cold acclimation and deacclimation. Petunia plants cold acclimated when exposed to low nonfreezing temperatures and were not injured when subjected to chilling stress at 2 °C for 2 weeks. Leaf discs excised from nonacclimated chilled plants had increased electrolyte leakage and damaged membranes while leaf discs from cold acclimated chilled plants were intact and similar to non chilled control plants. While Guy et al. (1988) and Yelenosky and Guy (1989) reported that petunia lacked the capacity to further increase in freezing tolerance following cold acclimation at 5 °C for 7 d, we found that the development of chilling tolerance in petunia progresses slowly, requiring 18 days of progressive acclimation to attain a steady level of chilling tolerance. The cellular changes that enable the plant to endure low temperature stress are likely to develop very gradually. In contrast, cold acclimation in both *Arabidopsis* (Gilmour et al., 1988) and spinach (Yelenosky and Guy, 1989), progresses rapidly.

Evidence for CI in deacclimated plants comes from visual symptoms, leakage and ultrastructural studies, which indicate that disorganized plasma membranes are within cells of chilled stressed petunia leaves. Deacclimation of petunia following the first hour of exposure to 25 °C, is associated with increased α-galactosidase activity and the catabolism of raffinose. This rapid loss of hardiness in petunia suggests it could be useful as a model system to study the metabolic changes that occur during cold acclimation and deacclimation.

The raffinose content of petunia leaves increased ten-fold when growing conditions were changed from long to short days and from warm to cold temperatures. Similarly, the raffinose content of spruce roots increased when the plants were subjected simultaneously to a change from long to short days and from warm to cold conditions (Wiemken and Ineichen, 1993). While soluble raffinose accumulated during cold acclimation, the total sugar content decreased by 18.8 % on a dry weight basis. In some species, the type of carbohydrate accumulated is a more critical factor than the total sugar content (Sauter and Cleve, 1991; Stushnoff et al., 1993). These findings and other reports suggest that RFO are strongly associated with greater cold hardiness, and may act as cryoprotectants in cold acclimated plant cells (Imanishi et al., 1999). Certain soluble sugars can serve as good cryoprotectants because of their capacity to lower the freezing point temperatures and RFO are no exceptions (Stushnoff et al., 1998).

Multiple isoforms of α-galactosidase with different pH optima have been reported from various species (Smart and Pharr, 1980; Chrost and Schmitz, 2000). The acid form of the enzyme has been reported to play important roles in seed germination and assimilate partitioning in the phloem (Smart and Pharr, 1980; Keller and Phar, 1996; Gao and Schaffer, 1999). It has been reported that two other enzymes (raffinose synthase and galactinol synthase) associated with the accumulation of RFO during cold acclimation are cold stable (Castillo et al., 1990), therefore we were interested in determining the temperature dependence of α-galactosidase.

The α-Gal from petunia was relatively thermophilic, with maximum activity at 45 °C for leaf extracts from all three treatments. Enzyme activity was relatively low in nonacclimated and cold acclimated plants but a sharp rise in α-galactosidase was observed

during deacclimation at the time when raffinose levels decreased rapidly. The evidence presented here suggests that warm temperature induced and/or increased α-galactosidase activity in the leaves. High temperature inductibility of α-galactosidase was demonstrated in plants after only one hour of deacclimation. Increases in α-galactosidase transcript at one hour after deacclimation correlate with increases in α-galactosidase activity suggesting that temperature may regulate RFO catabolism by increasing the transcription of α-galactosidase gene(s).

Petunia retains the capacity to cold acclimate at a relatively late developmental stage (8 weeks), thus by increasing tolerance to chilling temperatures, the growing season of bedding plants like petunia could be extended into the fall when temperatures are cooler and chilling injury is most likely. Furthermore, petunia deacclimates after only 1 hour of exposure to warm temperature and while this has obvious implication during the inclement fall weather, altering this response could be valuable for the improvement of floricultural products. Alpha-galactosidase responded to warm temperature (deacclimation) with increased activity, suggesting a regulatory role for α-galactosidase in raffinose catabolism. This increased activity may be due to gene activation and as such the α-galactosidase gene may provide a genetic target for manipulating cold hardiness in plants.

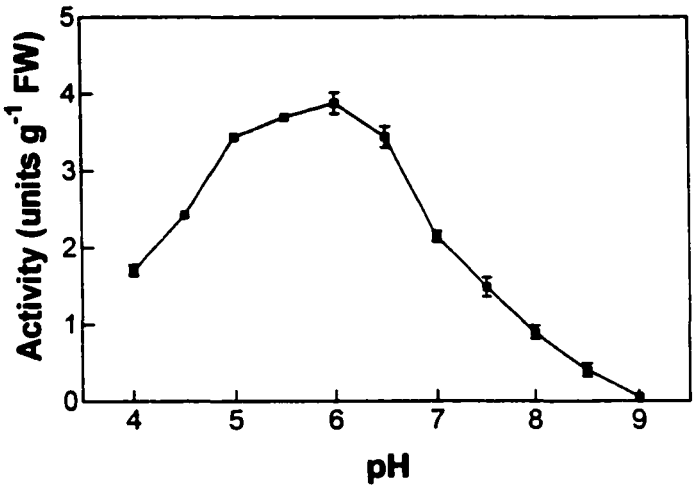


Fig. 2.0 Effect of pH on α-galactosidase activity in nonacclimated petunia leaves with PNPG substrate. The buffers used were McIlvaine (pH 4 to 6.5), Hepes-NaOH (pH 7 to 7.5) and Tris-Cl (pH 8 to 9). Assay temperature was 30 °C. Bars indicate SEM; n= 10 (two separate exp, 5 reps each).

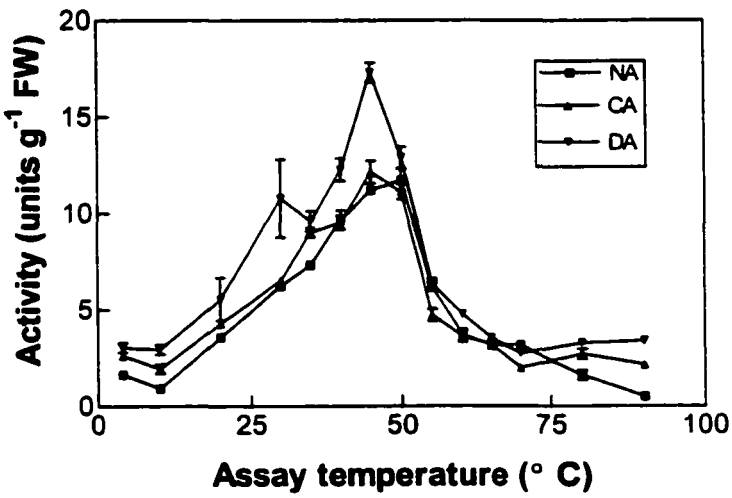


Fig. 2.1 Effect of reaction temperature on α-gal activity of nonaclimated (NA), cold acclimated (CA) and deacclimated (DA) petunia using PNPG as the substrate. Assay pH was 6.3. Bars indicate SEM; n = 10 (two separate exp, 5 reps each)

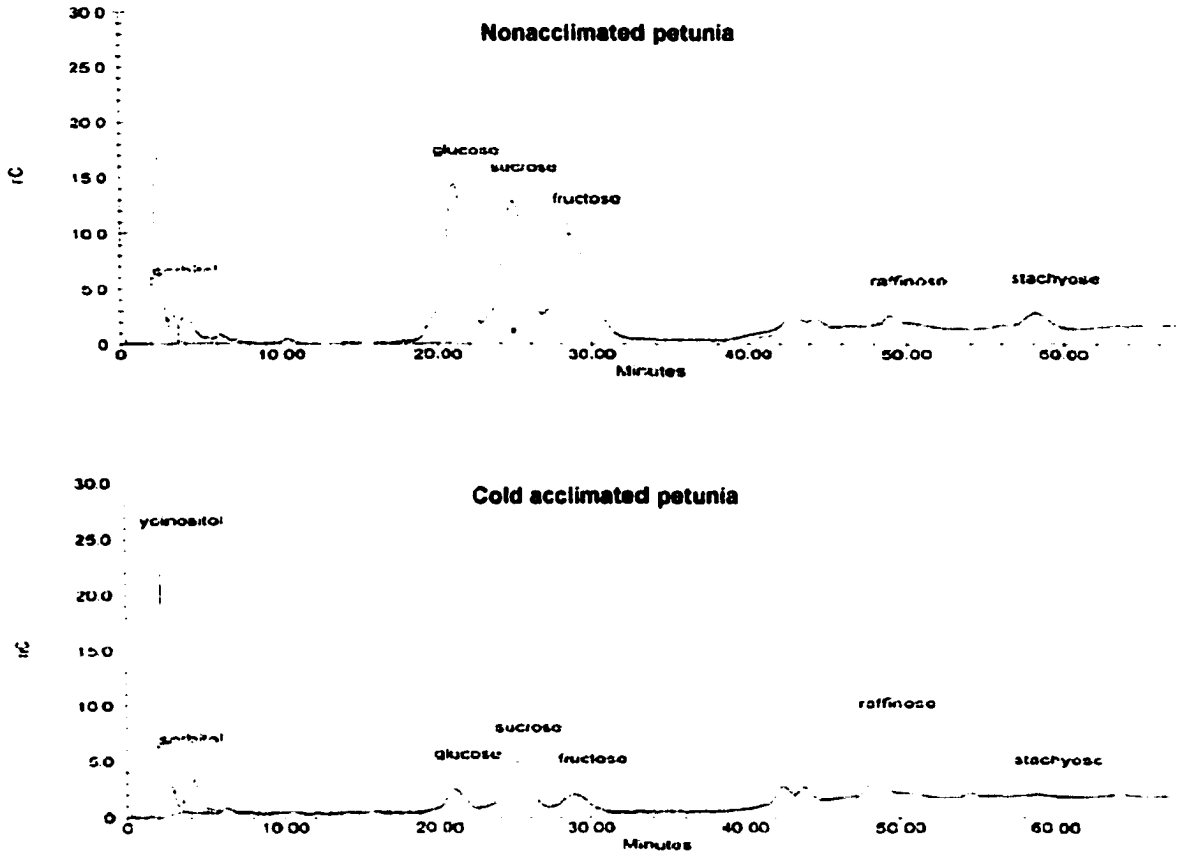


Fig. 2.2 HPLC chromatograms showing the changes in soluble carbohydrate content in petunia leaves after exposure to low temperature; nonacclimated: 25 ° C for 24 d at 16 h photoperiod; cold acclimated: 15 °C for 7 d, 10 °C for 7 d, 5 °C for 7 d and subsequently 3 °C for 3 d with a 12 h photoperiod.

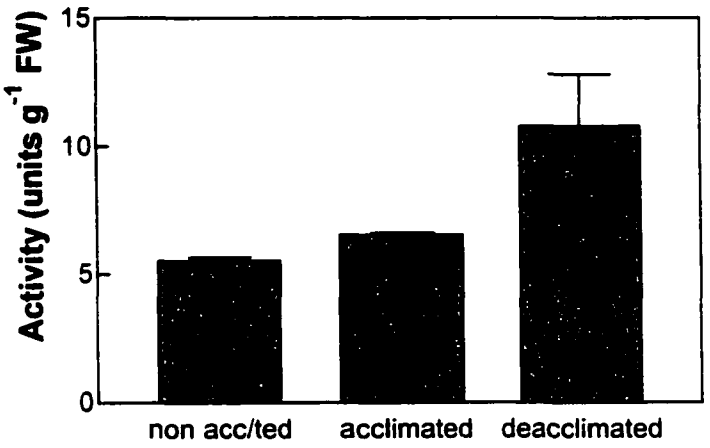


Fig. 2.3 Alpha-galactosidase activity in petunia leaves. Non acclimated: 25 °C for 24 d at 16 h photoperiod, cold acclimated: 15 °C for 7 d, 10 °C for 7 d, 5 °C for 7 d and subsequently 3 °C for 3 d with a 12 h photoperiod, deacclimated: previously cold acclimated plants were incubated at 25 °C for 1 h at 16 h photoperiod. Assay conditions as described in materials and methods. Error bars indicate SEM; n = 10 (two separate exp, 5 reps each).

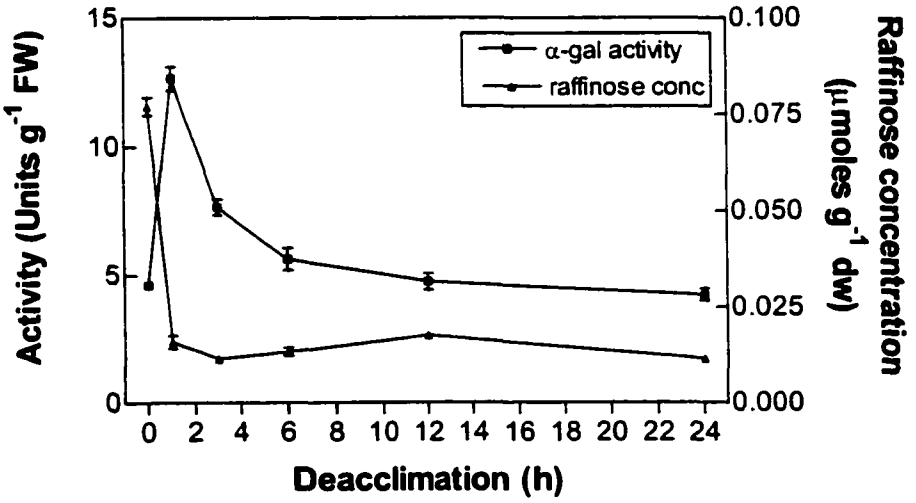


Fig. 2.4 Changes in alpha-galactosidase activity and raffinose content in response to deacclimation. Cold acclimated plants (0 h deacclimation) were deacclimated by incubating at 25 °C and sampled at various times for alpha-gal activity using PNPG as the substrate and for raffinose content using HPLC.

Fig. 2.5 Ultrastrutural changes observed in A. Cold acclimated, chilled petunia leaves following 1 h of deacclimation X 25K; and B. non chilled control leaves X 15K; In A., the cell membrane is discontinuous and retreats from the slightly uneven cell wall (W) (double arrows), the tonoplast is ill-defined and in the insert, a discontinuity (arrow) of the chloroplast (C) membrane is apparent. In B, the mitochondria (M) and chloroplast are normal and the tonoplast appears to be intact. Vacuole (V)

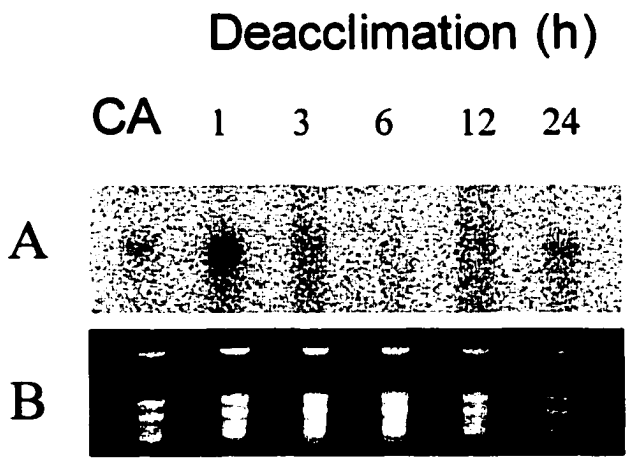


Fig. 2.6 Gel blot analysis of RNA from cold acclimated (CA) and deacclimated petunia. The blot (A) was hybridized with a tomato alpha-galactosidase cDNA probe. Equal loading of total RNA (10 μg) in each lane of an agarose gel is shown by ethidium bromide staining (B).

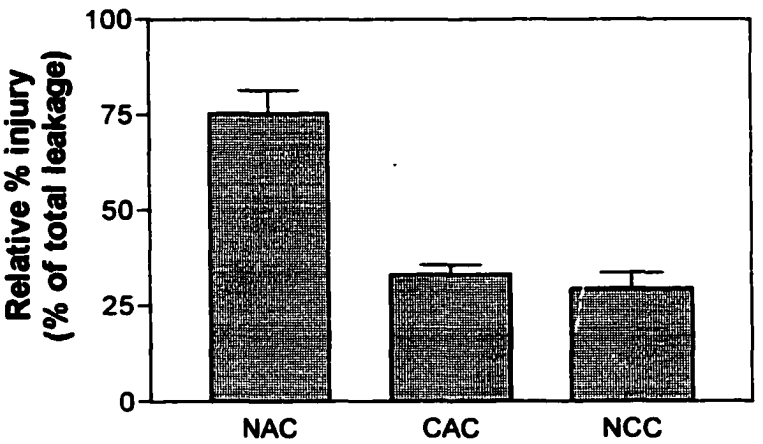


Fig. 2.7 Chilling tolerance of leaves from non acclimated and cold acclimated plants as evaluated by electrolyte leakage 1 day at 25 °C following chilling treatment. NAC: non acclimated chilled; CAC: cold acclimated chilled; NCC: non chilled control. Non acclimated: 25 °C for 24 h at 16 h photoperiod; cold acclimated: 15 °C for 7 d, 10 °C for 7 d, 5 °C for 7 d and subsequently 3 °C for 3 d with a 12 h photoperiod; chilling treatment: 2 °C for 14 d with a 12 h photoperiod. Error bars indicate SEM; n = 16 (two separate exp, 8 reps each).

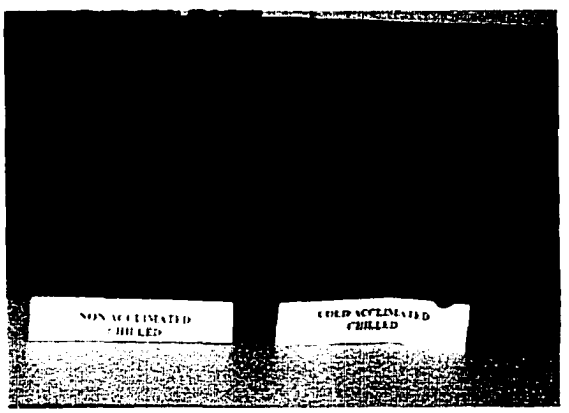


Fig. 2.8 Chilling injury of nonacclimated and cold acclimated petunia as evaluated by visual observations one week at 25 °C following chilling.

Chapter 3

PCR-based cloning of an α-galactosidase gene from deacclimated petunia (*Petunia* x *hybrida* 'Mitchell')

3.1 Abstract

Alpha-galactosidase is a key catabolic enzyme of the raffinose family oligosaccharides (RFO) involved in the cold hardiness pathway, cleaving the terminal-linked moiety from galactose-containing oligosaccharides. A cDNA clone *petgal*, was isolated from *Petunia x hybrida* cv Mitchell leaf RNA by RT-PCR using degenerate oligosaccharide primers designed to amplify the α-galactosidase cDNA. The putative α-galactosidase cDNA sequence has high nucleotide sequence homology (>80%) to other known α-galactosidases. Southern blot analysis suggests that α-galactosidase represents a single gene family. This study showed a comprehensive analysis of *petgal* expression including non tissue-specific expression, no developmental regulation and expression in response to increased temperature. Increases in α-galactosidase transcript one hour after deacclimation corresponded with increases in α-galactosidase activity suggesting that warm temperature may regulate RFO catabolism by increasing the transcription of the α-galactosidase gene. The evidence presented here suggests that warm temperatures induced and/or increased α-galactosidase activity in leaves. This rapid loss of hardiness in petunia makes it a useful model system to study rapid changes in metabolism that occur during deacclimation.

3.2 **Introduction**

Plants capable of cold acclimation require a growth period or incubation at low non-freezing temperature to trigger the appropriate genes needed for subsequent cold tolerance. During this period of cold acclimation, several biochemical and physiological processes and gene expression are altered in plants (Guy, 1990). Previous biochemical and physiological studies have demonstrated the accumulation of specific metabolites such as the raffinose familiy oligosaccharides (RFO) upon low temperature exposure (Bachmann et al., 1994; Imanishi et al., 1998). When *Chlorella vulgaris* was subjected to a cold shock for 30 h, the sucrose and raffinose concentration increased by 30-fold (Salerno and Pontis, 1989). One of the suggested functions of these metabolites is membrane protection upon low temperature stress. In spite of ample evidence demonstrating a significant shift in carbohydrate status of plants at low temperature, very few studies of the enzymes involved in carbohydrate biosynthesis and even fewer on RFO catabolism have been undertaken (Guy et al., 1992; Castonguay and Nadeau, 1998).

Consistent with the proposed role of RFO in cold acclimation, the galactinol synthase and raffinose synthase activities in developing seeds and leaves of soybean and kidney bean increased 3- to 4- fold upon exposure of whole plants to 4 °C (Castillo et al., 1990). Castonguay and Nadeau (1998) showed that α-galactosidase, the key enzyme involved in the breakdown of RFOs, increased activity during spring dehardening at the time when RFO levels decreased rapidly.

Alpha-galactosidase is widely distributed in microorganisms, humans and plants and cDNAs have been cloned from several sources including yeast (Liljestrom, 1985), human (Bishop et al., 1986), *E. coli* (Liljestrom and Liljestrom, 1987), guar (Overbeeke

experiment, plants were deacclimated for 1 h at various temperatures (25 °C – 40 °C). Five mm leaf disc samples were collected for electrolyte leakage tests as described in chapter 2 to determine the approximate threshold temperature for heat-induced damage of petunia. Samples for Southern blots were collected from leaves and for RNA gel blots, from various tissues and at different developmental stages. For developmental regulation of α-Gal, randomly selected seedlings were used and the first eight true leaves were numbered according to their appearance order. Leaf area measurements of the expanding leaves were recorded weekly, close to a fixed reference time of day. The leaf relative expansion rate was determined as a function of developmental stage.

3.3.2 Isolation, cloning and sequencing of α-Gal

A putative alpha-galactosidase cDNA was isolated from petunia total RNA using the Access RT-PCR System according to the manufacturers recommendations (Promega, Madison, WI). The reverse transcriptase polymerase chain reaction (RT-PCR) kit included AMV reverse transcriptase (AMV RT) from Avian Myeloblastosis Virus for the synthesis of first strand DNA and the thermostable T*fl* DNA polymerase from *Thermus flavus* (Kaledin et al., 1981) for second strand cDNA synthesis and DNA amplification.

Total RNA was obtained from leaves of 8-week old deacclimated petunia plants using the TRIZOL reagent according to the manufacturers recommendations (Gibco, BRL, Rockville, MD).

Degenerate primers Gal5'(5'ATGGGRTGGARYAGCTGGAAYCA 3') and Gal3' (5' CTDARWGGHCCDGCCCAWACCTC 3') were designed based on other plant α-Gal sequences to obtain the mature α-Gal from petunia. RT- PCR reactions were

carried out in a volume of 50 µl containing (final concentration) 1X AMV T*fl* Reaction Buffer, 0.2mM dNTP mix, 5µM Gal5' primer, 5µM Gal3' primer, 1mM $MgSO_4$, 0.1u/µl AMV RT, 0.1 u/µl T*fl* DNA polymerase, and 1µg total RNA sample. Cycling was performed in a Mastercycler (Eppendorf, Westbury, NY) under the following conditions: reverse transcription at 48 °C for 45 min, AMV RT inactivation and RNA/cDNA/primer denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1.5 min and extension at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. A unique PCR product of the expected size (855bp) was observed on agarose gel electrophoresis, purified by phenol/ chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation and then ligated into the pCR 2.1-TOPO expression vector, according to the manufactures recommendations (Invitrogen, Carlsbad, CA). The TOPO system relies on the TA cloning method. Clones were screened (blue/white screening) on LB plus kanamycin (50mg/ml) plates supplemented with X-gal (20mg/ml) and IPTG (200mg/ml). Putative transformants (white colonies) were further analyzed by doing plasmid minipreps according to the boiling lysis method (Sambrook et al., 1989) and digested with *EcoR* I restriction enzyme. The putative clone designated *petgal* was sequenced using a Perkin Elmer 377 (ABI Prism, v.3.2) DNA sequencer by the DNA sequencing service provided by the Department of Biochemistry and Molecular Biology at Colorado State University.

3.3.3 Sequence analyses and amino acid alignment

Sequence analyses were carried out using World Wide Web resources such as BLAST searches of the National Center for Biotechnology Information (NCBI) database

(Altschul et al., 1997) and Vector NTI Suite computer software (Informax, Bethesda, MD) for detecting restriction enzyme sites within the sequence. The deduced *petgal* protein sequence was aligned with other known α-Gal sequences in the database using the Vector NTI Suite software (InforMax, Bethesda, MD).

3.3.4 α-galactosidase extraction and activity assay

The enzyme assay was adapted from Gaudreault and Webb (1983) with slight modifications. Twenty grams fresh weight of leaf tissue were homogenized in a Virtis 45 homogenizer in 100 ml chilled extraction buffer consisting of 0.5 M sucrose, 0.1 M KCl, 50 mM Tris-HCl, 10 mM $Na_2S_2O_5$, 10 mM PMSF, 1.5 % PVPP at pH 6.3. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10, 000 x g for 10 min. The crude enzyme extract was assayed using the synthetic substrate *p*-nitrophenyl-α-D-galactopyranoside (PNPG). Assay mixtures contained 100 µl of enzyme preparation in 400 µl of McIlvaine buffer (pH 6.0) at 30 °C. McIlvaine buffer pH 6.0 was prepared by using approximately 35 ml of 100 mM citric acid and 60 ml of 200 mM Na_2HPO_4 (McIlvaine, 1921). The reaction was started by adding 100 µl of 30 mM PNPG and terminated after 20 min by adding 2.4 ml of 5 % Na_2CO_3. Blanks were prepared by adding enzyme after Na_2CO_3. 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 $1.83 \times 10^4\ M^{-1}\ cm^{-1}$. One unit of enzyme activity was defined as one µmol of PNPG hydrolyzed min^{-1}.

3.3.5 DNA extraction and Southern blot analysis

Genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Briefly, 0.8 g of leaf sample from 8-week old petunia was ground to a fine powder in liquid nitrogen (LN) and added to 750 µl prewarmed (65 °C) extraction buffer (4% (w/v) CTAB, 100 mM Tris-HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA and 0.1% β-mercaptoethanol) and incubated in a 65 °C water bath for 30 min with occasional vortexing. Approximately 560 µl of chloroform was added and mixed gently for 15 min at RT. Samples were spun at 8000 rpm for 5 min and the aqueous phase was removed and placed in new tubes. An equal volume of ice-cold isopropanol was added and tubes were laid in an icebox and mixed gently on a rocker (Boekel) until the DNA precipitated out of the solution (about 10 min). The DNA precipitate was recovered by spinning at 8000 rpm for 10 min at 4°C. The DNA pellet was collected with a sterile toothpick and dissolved in 50 µl TE at 65 °C for 15 min before RNase treatment (10 µg/µl) at RT for 30 min. To precipitate the DNA, $1/10^{th}$ of the volume of 3 M Na-acetate (pH 6.0) and twice the volume of 95% ethanol were added, tubes were inverted twice and stored at –20 °C for 30 min. The DNA was pelleted by spinning at 8000 rpm for 10 min at 4 °C and washed with cold 70% ethanol (8000 rpm for 2 min). The resultant DNA was vacuum dried (DNA Speed Vac, Savant) on low for 10 min and the dried pellet was resuspended in 50µl TE. The DNA was quantified at 260nm.

Petunia genomic DNA samples (10µg) were digested with *Xba* I, *EcoR* I or *Nco* I. The digested DNA was separated on a 1% agarose gel at 80v for 3 h. The gel was soaked in depurinization solution (0.25M HCl) for 10 min with gentle shaking, in denaturation

buffer (0.5 M NaOH, 1.5 M NaCl) for 45 min and in neutralization buffer (1 M Tris pH 8.0, 1.5 M NaCl) for 30 min. The separated DNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and cross-linked with a controlled UV light source (Stratalinker, Stratagene, La Jolla, CA). After air-drying at room temperature, the membrane was prehybridized in 20 ml pre-warmed solution (43 °C) (10 ml formamide, 5 ml 20X SSPE, 2 ml 50X Denhardt's (1966) solution, 2 ml 10% SDS, 200 µl denatured salmon sperm DNA (10 mg ml^{-1}) and 0.8 ml H_2O) for 2.5 h at 43 °C and hybridized for 20 h at 43 °C with 10^6 cpm ml^{-1} ^{32}P-labeled cDNA. Probes were synthesized using a random primed DNA labeling kit (DECAprime™, Ambion, Austin, TX) with DNA fragments derived from PCR-amplified *petgal* as follows. Eight µl of purified *petgal* cDNA insert, 1µl H_2O, and 2.5 µl 10X decaprimer buffer were mixed. After a quick spin at 12000 rpm the mixture was heated at 100 °C for 5 min. The tube containing the mixture was snap frozen in LN and immediately put on ice. After thawing, 5 µl 5X reaction buffer (-dATP), 1.5 µl H_2O, 5 µl [α-^{32}P] dATP (0.25 mCi/ml) and finally 1 µl of Klenow enzyme were added to the mixture. The reaction was incubated in a 37 °C water bath for 15 min. Adding 1 µl EDTA solution to the mixture stopped the reaction. The labeled DNA was passed through a Micro Bio-spin column (Biorad, Hercules, CA) to separate the probe from unincorporated nucleotides. Two µl of the reaction mixture was added to 175 µl TE and the total radioactivity was measured using a liquid scintilation counter (Beckman LS6000IC, Somerset, NJ).

Following hybridization, the membrane was washed at 50 °C in 2X SSC and 0.1 % SDS for 30 min and 0.2X SSC and 0.1 % SDS for 15 min. The membrane was

wrapped in plastic wrap and placed on a phospho screen at RT for 3 d. Fragments were visualized using a phosphoimager (Storm 840, Molecular Dynamics, Sunnyvale, CA).

3.3.6 RNA extraction and northern blot analysis

Samples were collected from leaves of 8-week old acclimated and deacclimated plants at various temperatures and time points and from flowers, leaves, stems and roots of 8-week old non acclimated plants. To determine if α-Gal is developmentally regulated, samples were collected from the first eight true leaves of 8-week-old plants. Leaves were sorted into four groups; 1^{st} - 2^{nd}, 3^{rd} - 4^{th}, 5^{th} - 6^{th} and 7^{th} - 8^{th} according to their appearance order (Fig. 3.6a). Leaf relative expansion rate as determined by weekly measurements was used as a function of developmental stage. Samples were frozen in LN and stored at –80 °C until used for RNA extraction. Total RNA was extracted using the TRIZOL reagent according to the manufacturers recommendations (Gibco, BRL, Rockville, MD). RNA was quantified spectrophotometrically and 10 μg of total RNA was separated by electrophoresis through a 1 % (w/v) agarose gel containing 2.2M formaldehyde. Equal loading of RNA samples was confirmed by visual observation of the RNA gel following staining with ethidium bromide. The separated RNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and cross-linked with a controlled UV light source (Stratalinker, Stratagene, LaJolla, CA). After air-drying at room temperature, membranes were prehybridized in 20 ml pre-warmed solution (43 °C) (10 ml formamide, 5 ml 20X SSPE, 2 ml 50X Denhardt's (1966) solution, 2 ml 10% SDS, 200 μl denatured salmon sperm DNA (10 mg ml^{-1}) and 0.8 ml H_2O) for 2.5 h at 43 °C and hybridized for 20 h at 43 °C with 10^6 cpm ml^{-1} ^{32}P-labeled cDNA. Probes were

synthesized using a random primed DNA labeling kit (DECAprime™, Ambion, Austin, TX) with DNA fragments derived from PCR-amplified *petgal* as described for Southern blots.

Following hybridization, the membranes were washed at 50 °C in 2X SSC and 0.1 % SDS for 30 min and 0.2X SSC and 0.1 % SDS for 15 min. The membranes were wrapped in plastic wrap and placed on phospho screens at RT for 3 d. Transcripts were visualized using a phosphoimager (Storm 840, Molecular Dynamics, Sunnyvale, CA).

3.4 **Results**

3.4.1 Amplification and sequencing of a partial cDNA encoding petunia α-Gal

The partial cDNA amplification was performed by the RT-PCR method. The partial cDNA encoding α-Gal was amplified as a single PCR product of the putative 855 bp fragment length (Fig. 3.0). The product amplified by RT-PCR was cloned into the pCR 2.1 TOPO vector (designated *petgal*). Eight white colonies were screened and two colonies revealed the inserts. The double stranded plasmid cDNA of these two putative clones were sequenced.

3.4.2 Sequence analysis and comparison of deduced amino acid sequences

The nucleotide sequence of the respective cDNA clone (designated *petgal*) was sequenced and compared to known α-Gal sequences compilied in the NCBI GenBank. The nucleotide sequence of the cloned *petgal* contains an ORF of 285 amino acids (Fig. 3.1). The calculated molar mass of the polypeptide is 31.3 KD with an isoelectric point of 4.86. The predicted protein has a compositional bias for Gly (10.88 %), Ser (9.47 %) and

Asp (8.42 %). A comparison of the cDNA sequence to others in GenBank revealed striking homologies (between 42 % to 88 %) with human, microbial and plant α-Gal genes.

Based on the alignment of the petunia α-Gal with other known α-galactosidases, it appears that the protein is conserved among species (Fig. 3.2). Table 3.0 shows the nucleotide and amino acid identities between petunia α-Gal and those from other species.

3.4.3 Southern blot analysis

Southern hybridization analysis of petunia genomic DNA digested with three restriction enzymes indicated that there is a single gene present for petunia α-Gal. No more than two fragments of the genomic DNA digested with the restriction enzymes were detectable (Fig.3.3). In the case of *Xba* I and *Eco*R I only a single fragment was detectable. In the *Nco* I lane, two fragments gave positive signals. These results suggest that the gene represented by the *petgal* clone sequence is a single copy gene with an intrinsic *Nco* I site. The restriction enzyme map of *petgal* shows the *Nco* I site (Fig.3.4).

3.4.4 Northern blot analyses of α-Gal

The cDNA clone described above was used to probe northern blots prepared with RNA from a number of tissues, i.e. flowers, leaves, stems and roots and leaves at four stages of development and from leaves of cold acclimated and deacclimated plants. Alpha-galactosidase transcripts were detected at the same levels in leaves, stems and flowers and to a lesser degree in roots (Fig. 3.5a). Petunia α-Gal activity was the same in all tissues tested (ranging from 4.7 ± 1.2 to 5.1 ± 0.5 units g^{-1} FW) ($p<0.05$) (Fig. 3.5b).

Petunia plants grown for 4 weeks had eight or more leaves (Fig. 3.6a). At this time there was a clear growth gradient, with younger leaves growing faster than older leaves (Fig. 3.6b). The activity of α-Gal showed no relationship with growth rate (Fig. 3.6c) and neither did the mRNA transcript acccumulation (Fig. 3.6d). RNA from leaves at varying developmental stages revealed a constitutive expression of the α-Gal gene.

To analyze the regulation of gene expression by temperature, mRNA transcripts were monitored at cold acclimation and at 25 °C deacclimation time points from 30 min and up to 180 min (Fig. 3.7a). As shown in Figure 3.7a, accumulation of α-Gal mRNA began at 1 h following deacclimation. Alpha-galactosidase mRNA transcript levels remained constant for up to 90 min but by 120 min mRNA accumulation had decreased to control levels. Increased gene expression could not, however, be detected before the first hour of deacclimation. A similar trend was observed for α-Gal activity (3.7b). To learn how deacclimating temperatures other than 25 °C affect cloned gene expression, a temperature dose-response experiment was performed. Total RNA was isolated from petunia plants deacclimated for 1 h at 25 °C to 40 °C. Although all treatments induced *petgal* transcripts, deacclimation at 30 °C resulted in the greatest mRNA accumulation (Fig. 3.8a). At 35 °C deacclimation, transcript levels decreased but at 40 °C transcripts were barely detectable. Alpha-galactosidase activity followed a similar trend (Fig. 3.8b). As indicated by electrolyte leakage tests plants were heat stressed upon exposure to temperatures at 35 °C and above (Fig 3.9).

3.5 **Discussion**

In an attempt to understand the role of α-Gal in deacclimation, a cDNA clone representing a gene encoding an α-Gal protein was isolated and its expression at the RNA level was also characterized.

The *petgal* sequence shown above encodes a 31 KD protein. Comparison of the nucleotide sequence as well as the translation in 6 reading frames showed high homology to a number of α-Gal genes in the database. From these results it was concluded that the *petgal* cDNA encodes a petunia α-Gal.

The relatively high Asp content in the petgal sequence probably assumes a role in the active site, as in guar (Overbeeke et al., 1989). Asp-262 in petunia is conserved in the plant α-Gals shown. Likewise, tyrosine-35 in petunia is conserved in other plant α-Gals and is also believed to be an important residue for α-Gal enzyme activity (Zhu et al., 1995).

Southern hybridization using *petgal* as a probe suggests that the α-Gal represents a single gene in petunia. Similarly, only one gene is present for tomato seed α-Gal (Feurtado et al., 2001) while two different α-Gal genes are present in barley (Chrost and Krupinska, 2000). The number of genes in other plant species has not been investigated.

The presence of the enzyme in different plant tissues suggests that α-Gal is not only involved in raffinose degradation but plays a broader role in galactomannan remobilization during seed germination (Feurtado et al., 2001) and in plants not synthesizing these oligosaccharides, α-Gal seems to be involved in galactolipid metabolism of plastid membranes (Chrost and Krupinska, 2000). Alpha-galactosidase

activity and transcript accumulation was detected in all of the tissues tested suggesting that *petgal* is not tissue specific but rather is involved in non-specific degradation of raffinose. In tomato for example, EST clones were isolated from seed (Genbank accession number AF191823), ovary (Genbank accession number AI898528) and root (Genbank accession number BE451601). Although *petgal* is constitutively expressed, transcript levels in roots were slightly lower than that of the other tissues. Young rapidly expanding leaves, flowers and stems are not only very active in photosynthetic carbon partitioning, but they are also metabolic sinks thus the translocated oligosaccharides found in these regions are expected to be rapidly hydrolyzed (Webb and Gorham, 1965). It can be speculated that the temperature stimulus to evoke gene expression would be more easily perceived in the above ground tissues than in roots.

Petgal is not developmentally regulated, as its pattern of mRNA transcripts is present virtually unchanged throughout plant development. Since α-Gal activity is fairly constant during the various stages of development, presumably the transcripts are being used to replace enzyme that is being turned over. Consistent with our findings is the observation that α-Gal activity in leaves remained constant at all stages of development (Thomas and Webb, 1978). On the other hand, the activity of stachyose synthase, an RFO biosynthetic enzyme donating a second galactosyl moiety to raffinose, can change dramatically in response to plant developmental stage and leaf age (Holthaus and Schmitz 1991). The cucurbits translocate RFOs predominantly; hence the biosynthetic enzymes (galactinol synthase, raffinose synthase, stachyose synthase) are expected to be upregulated in mature leaves (source tissues). Alpha-galactosidase is therefore expected to be upregulated in young leaves (sink tissues) to degrade RFO into sucrose and

galactose. Since petunia translocates sucrose primarily, large quantities of α-Gal are not necessary for RFO degradation. Constant levels of α-Gal activity in petunia were observed throughout leaf development.

Another interesting finding in this study is the rather rapid activation of petgal with the onset of deacclimation. These changes in mRNA levels are likely to be involved in the metabolic changes that occur during loss of hardiness. This information provides a rather clear picture for the temperature dependence of *petgal*.

Induction of α-Gal mRNA corresponding to *petgal* was detected in 25 °C deacclimated plants but elevated temperatures enhanced increased accumulation of transcripts. The data suggest that higher temperatures are required to induce significant levels of *petgal* gene expression in deacclimating petunia. Furthermore transcript levels rapidly decreased when the temperature was increased from 35 °C to 40 °C, approximately the threshold temperature for heat-induced damage of petunia. While the temperature activity curve presented in chapter two showed that α-Gal activity is highest at 45 °C, *in vivo* activity is curtailed above 35 °C. This suggests that further increases in transcript accumulation were inhibited by heat stress on the plant. The knowledge of heat stress on plant productivity and metabolism is well documented (Boyer 1982, Burke 1990). Chen et al., 1982 used the 2, 3, 5 –triphenyl tetrazolium chloride (TTC) reduction test to show that enzyme inactivation by high temperature is a good indicator of heat stress.

To better define the physiological role of α-Gal in plant deaclimation, an α-Gal gene was isolated from petunia (*petgal*). This study showed a comprehensive analysis of the expression of *petgal* including non tissue-specific expression, no developmental

regulation and expression in response to increased temperatures. The data revealed that the single gene family shows distinct expression patterns upon temperature treatments, allowing predictions of when function is expected to become physiologically important. The data presented in this study are also important for future research in devising experimental strategies to assess phenotypes of loss of function mutants and transgenic plants that over- and or under-express α-Gal.

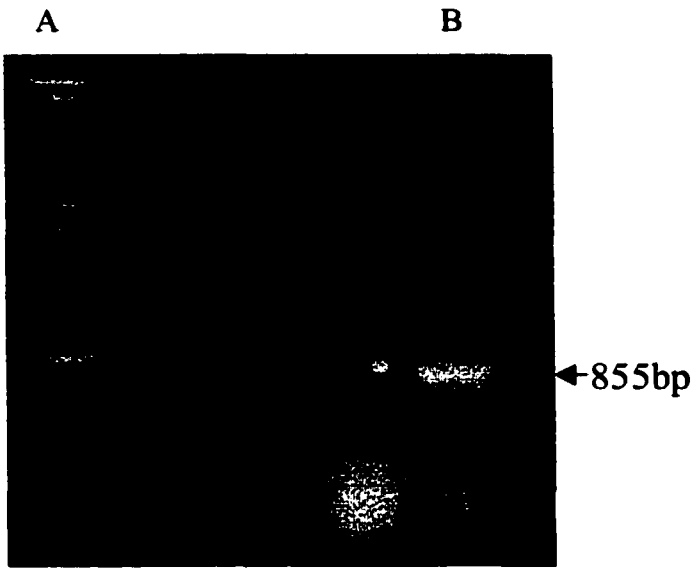


Fig. 3.0 The cDNA amplification of petunia α-galactosidase by RT-PCR. Lanes: A. molecular weight marker, B. *petgal* PCR fragment.

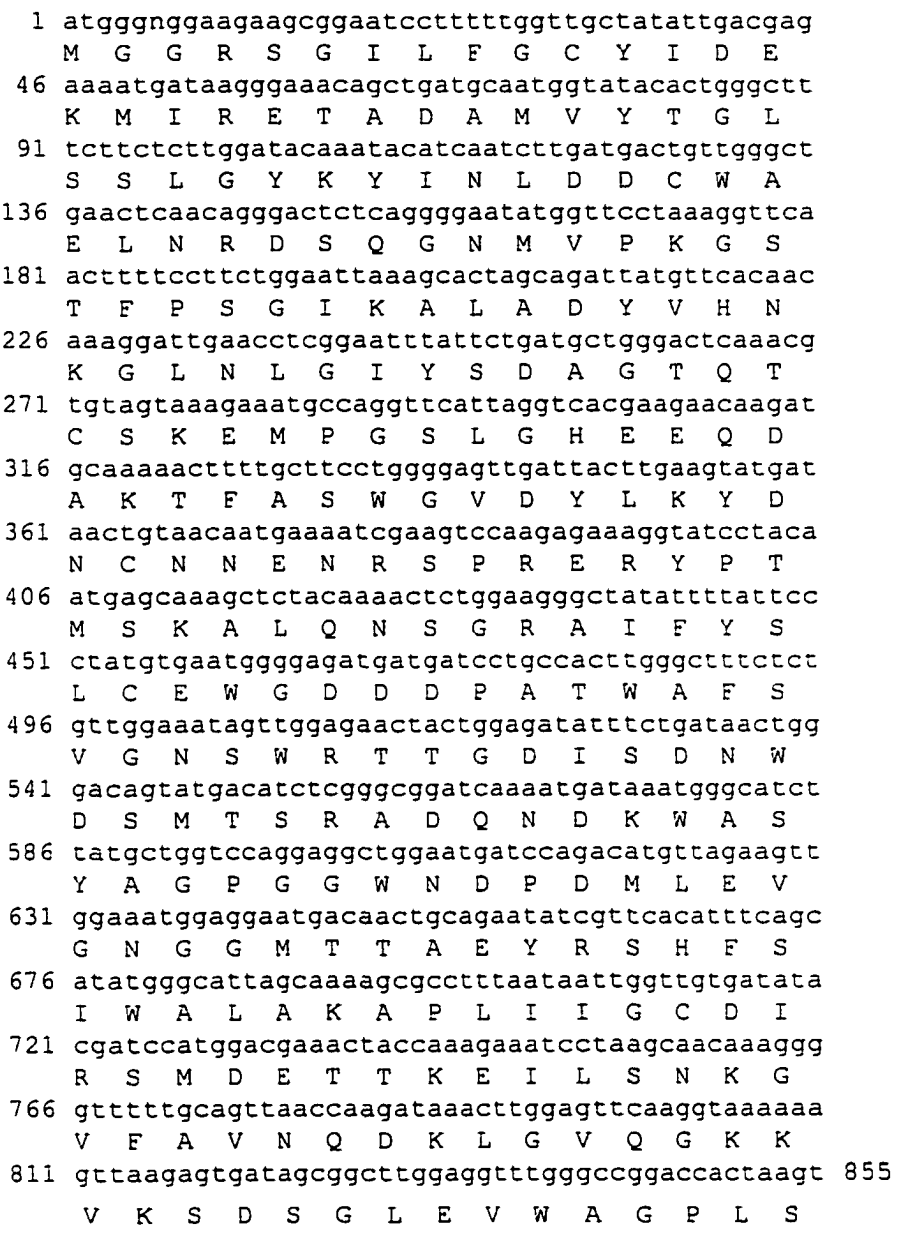


Fig. 3.1 Nucleotide sequence and amino acid translation of the petunia α-galactosidase cDNA clone

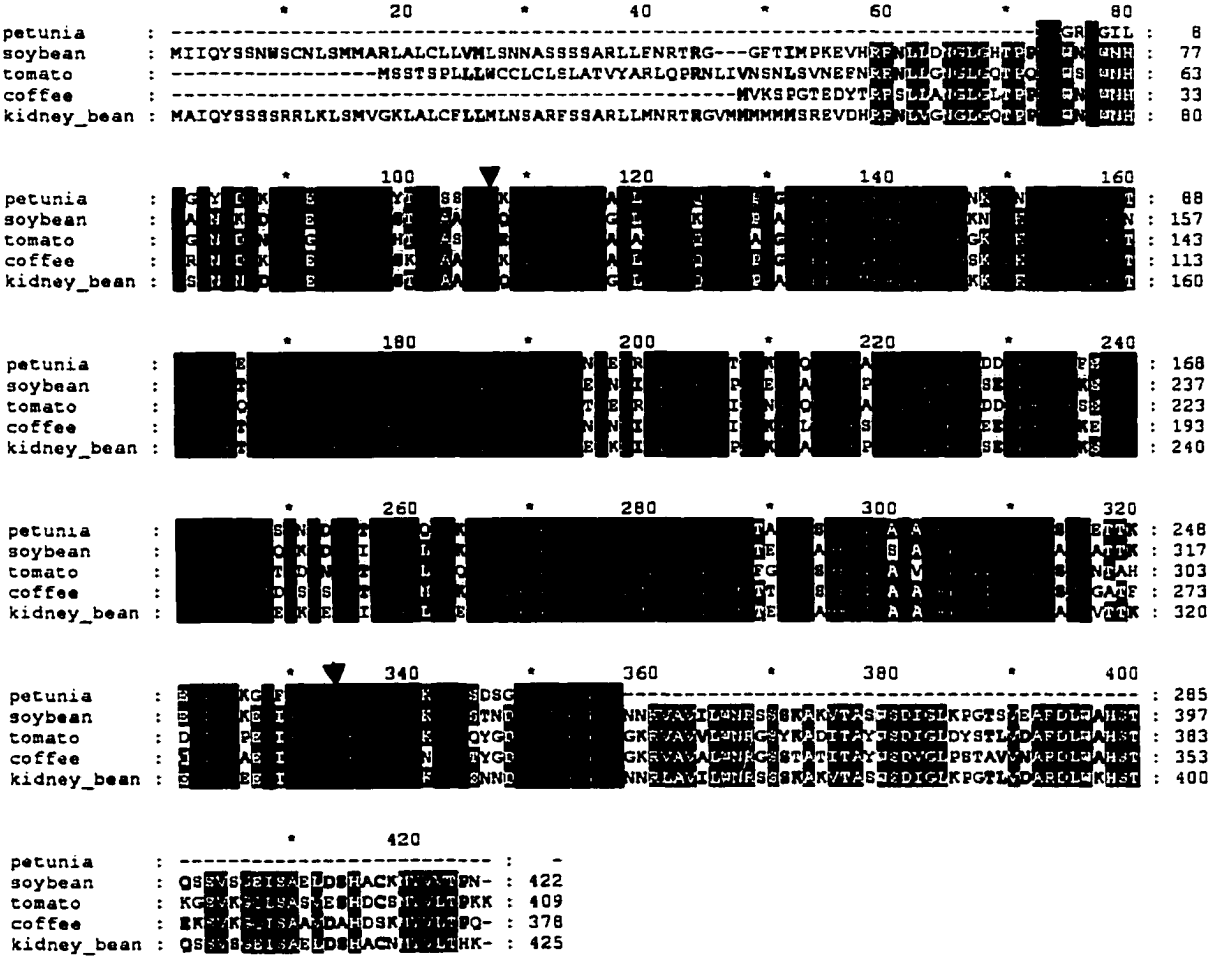


Fig. 3.2 Amino acid sequence alignment of petunia (*Petunia* x *hybrida*) with soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), coffee (*Coffee arabica*) and kidney bean (*Phaseolus vulgaris*). The alignment was performed using the vector NTI Suite software (Informax). Identical amino acids are shaded in black and conserved substitutions are shaded in grey. Amino acids in the α-galactosidase catalytic site are indicated with an arrow.

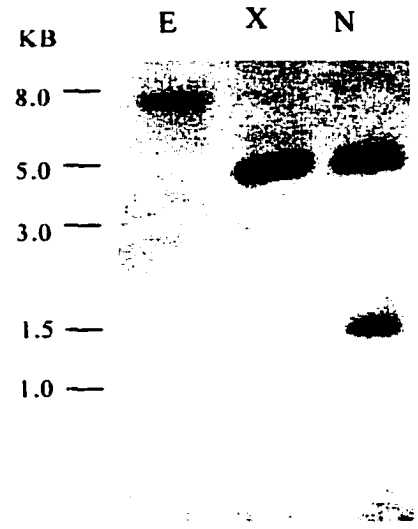


Fig 3.3 Southern blot analysis using *petgal* cDNA as a probe. Ten micrograms of total genomic DNA were digested with *EcoR* I (E), *Xba* I (X) or *Nco* I (N). The fragments were separated by electrophoresis in a 1 % gel and transferred onto Nytran membrane which was hybridized with *petgal* as a probe. Molecular size standards in kilobases are shown to the left.

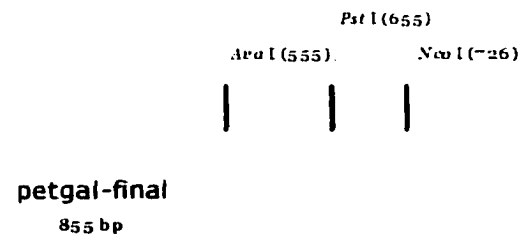


Fig 3.4 The restriction enzyme map of *petgal* cDNA showing selected restriction enzyme sites. The map was constructed using the Vector NTI Suite software (Informax).

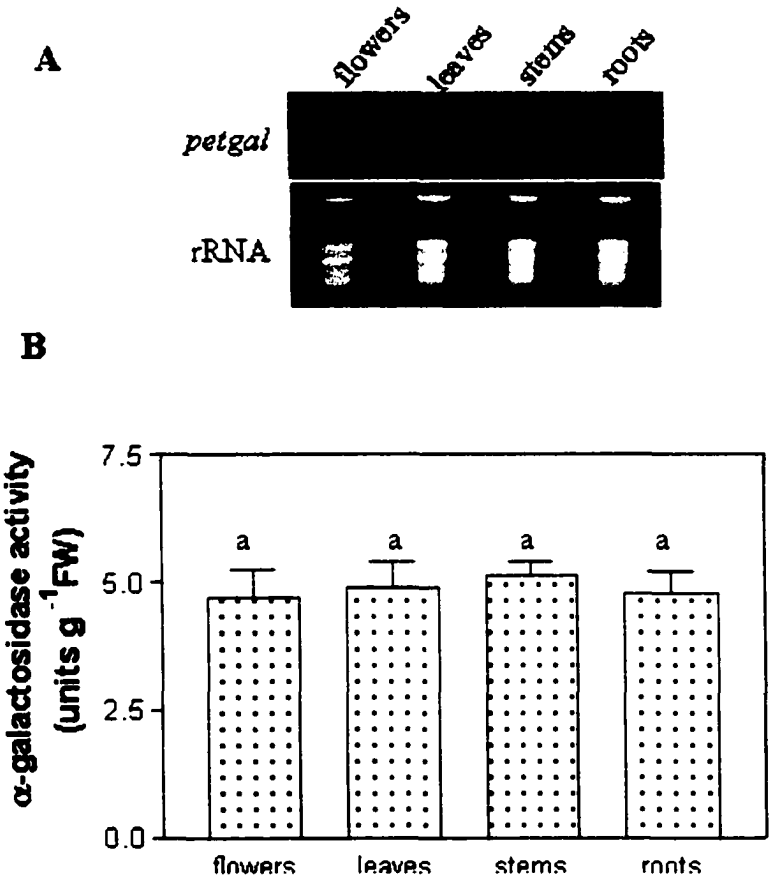
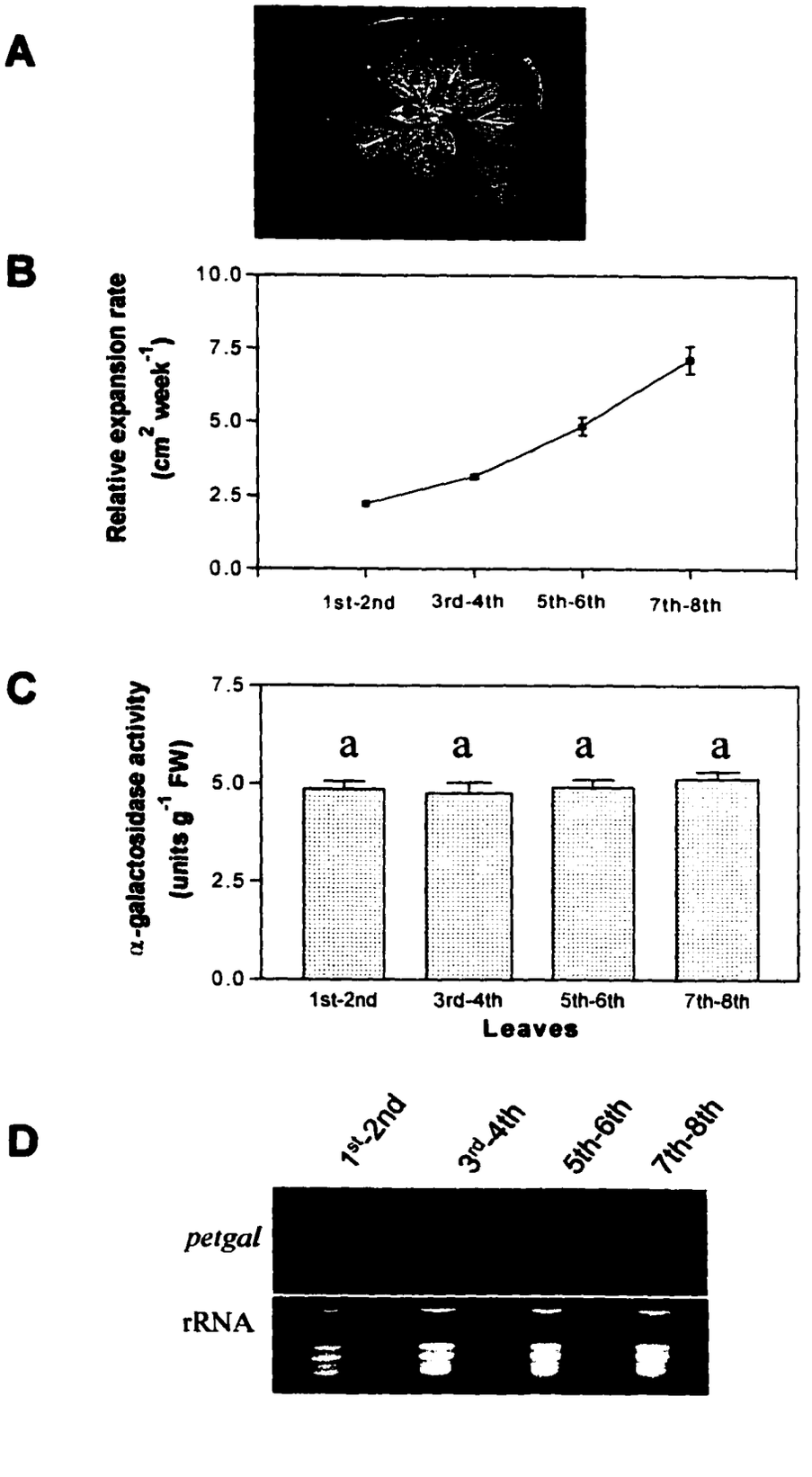


Fig. 3.5 Constitutive expression of *petgal*. A. Northern blot analysis of total RNA isolated from various tissues of plants growing under normal conditions. Ten micrograms of total RNA were separated by electrophoresis through agarose and hybridized with α-^{32}P-labeled *petgal*. Ribosomal RNA stained with ethidium bromide was used as a control. B. α-galactosidase activity in various tissues. Error bars are standard deviation of 5 replicates each from two separate experiments. [a] Treatments with the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS).

Fig.3.6 Non-developmental regulation of *petgal*. A. Petunia plant showing the first eight true leaves numbered according to their appearance order. B. Leaf relative expansion rate as a function of developmental stage. Leaves were classified in four groups. C. α-Gal activity in the four leaf groups. D. Northern blot analysis of the four leaf groups. Ribosomal RNA stained with ethidium bromide was used as a control. [a] Treatments with the same letter are not significantly different at $p<0.05$ by Tukey's Studentized Comparison Test.



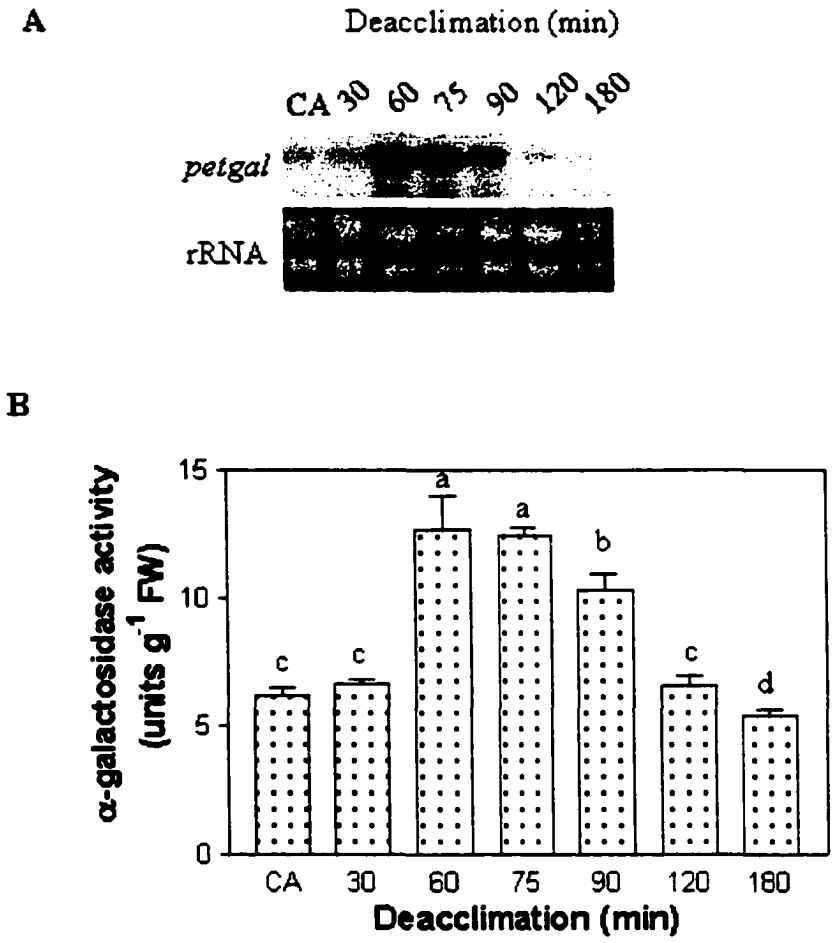


Fig 3.7 Deacclimation-induced expression of *petgal* in petunia. A. Northern blot analysis of petunia plants deacclimated at 25 °C for the indicated lengths of time. See materials and methods for cold acclimation (CA) regime. Each lane was loaded with 10 μg total RNA, separated by electrophoresis through agarose and hybridized with α-^{32}P-labeled *petgal*. Ribosomal RNA stained with ethidium bromide was used as a control. B. α-galactosidase activity in petunia plants deacclimated at 25 °C for varying periods of time. Error bars are standard deviation of 5 replicates each for two separate experiments. [a] Treatments with the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS).

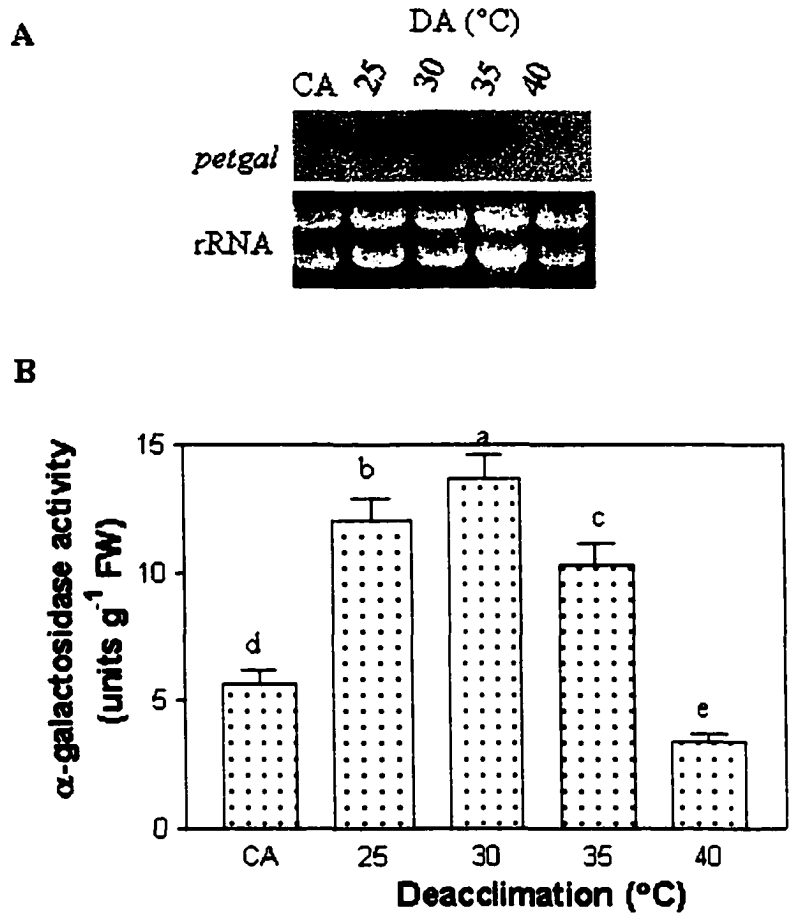


Fig. 3.8 Temperature dose response of *petgal*. A. Northern blot analysis of plants deacclimated (DA) for 1 h at the indicated temperatures. See materials and methods for cold acclimation (CA) regime. Each lane was loaded with 10 μg total RNA, separated by electrophoresis through agarose and hybridized with α-^{32}P-labeled *petgal*. Ribosomal RNA stained with ethidium bromide was used as a control. B. α-galactosidase activity of petunia plants following deacclimation for 1 h at various temperatures. Error bars are standard deviation of 5 replicates each for two separate experiments. [a] Treatments with the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS).

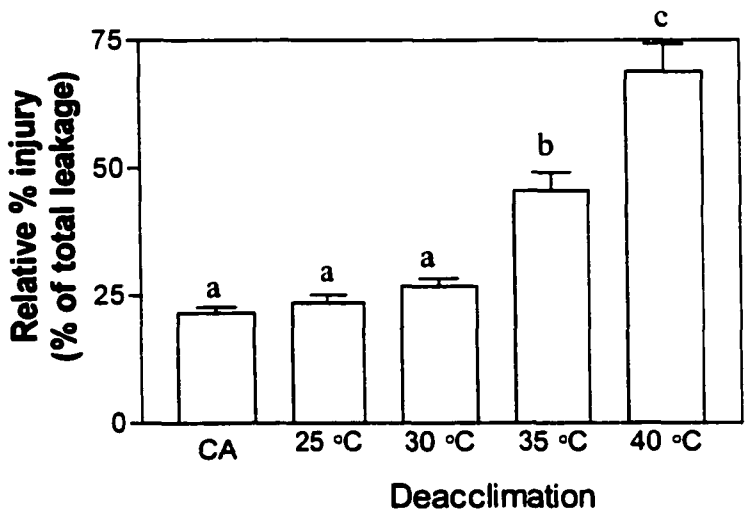


Fig 3.9 Electrolyte leakage as a measure of heat stress on deacclimated petunia plants. Plants were deacclimated by incubating previously cold acclimated (CA) plants (see materials and methods for CA regime) for 1 h at the deacclimating temperatures indicated. Five mm leaf discs were sampled to determine electrolyte leakage. Relative % injury represents the mean leakage as a percentage of the mean total leakage from frozen- killed samples. Error bars are the standard deviation of 5 replicates. [a] Treatments with the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS).

Chapter 4

Transformation of petunia with the α-galactosidase gene in the sense and antisense orientations

4.1 Abstract

Alpha-galactosidase is involved in many aspects of plant metabolism, including hydrolysis of the α-1,6 linkage of raffinose oligosacharides during deacclimation. The evidence to date for the importance of raffinose oligosaccharides and cold stress is only correlative. To further examine the relationship between endogenous sugars and freezing stress, the expression of α-galactosidase was modified in transgenic petunia. The α-galactosidase cDNA from tomato seed under the control of the Figwort Mosaic Virus promoter was introduced into petunia using the *Agrobacterium tumefaciens*-mediated transformation. The putatively transgenic plants were selected for resistance to kanamycin and screened for neomycin phosphotransferase activity (ELISA test). Putative transgenics were also screened by PCR for the α-galactosidase gene. RNA gel blot analysis demonstrated an increase in α-galactosidase transcript accumulation in sense plants and a decrease in antisense plants. Antisense inhibition of the α-galactosidase gene resulted in an accumulation of raffinose and enhanced freezing tolerance of petunia. Freezing stress tolerance was predicted based on the relationship between raffinose accumulation and decreased electrolyte leakage. Overexpression of the α-galactosidase gene inhibited low temperature tolerance when compared to antisense petunia lines suggesting that α-galactosidase plays a major role in low temperature tolerance.

4.2 **Introduction**

Antisense technology has been used very successfully to reduce the expression of various genes in several plant species. Down regulation of chalcone synthase in petunia to inhibit flower pigmentation (van de Krol, 1988), ACC synthase to decrease galactosidase activity in tomato fruit (Sozzi et al., 1998) and catalase to study oxidative and chilling stresses in tomato (Kerdinaimongkol and Woodson, 1999) are a few examples. The key to the success of antisense technology is the promoter used to drive the antisense transcripts. The Figwort Mosaic Virus (FMV) and Cauliflower Mosaic Virus (CaMV) 35S promoters are two very powerful promoters and while the CaMV35S is the most common promoter used, both are active in a wide range of plant tissues and lead to a non-specific reduction in gene expression. The rationale behind the application of antisense genes in plants is the *in vivo* synthesis of complementary RNA, which subsequently hybridizes to its target RNA and prevents expression. Applications of antisense strategies in plant systems are expanding as knowledge of this technology broadens.

Alpha-galactosidase (α-Gal) has been detected in various plant tissues with great diversity of biochemical characteristics in a number of developmental and physiological processes. Considerable attention has been given to its role in seed germination (Feurtado et al., 2001), fruit ripening (Sozzi et al., 1998), leaf senescence (Chrost and Krupinska, 2000), bean product quality (Guimaraes et al., 2001), assimilate partitioning (Thomas and Webb, 1978) and to a lesser extent plant cold hardiness (Castonguay and Nadeau, 1998).

Several lines of evidence suggest that raffinose family oligosaccharides (RFOs), particularly raffinose, play a role in the acquisition of cold tolerance in plants (Stushnoff et al., 1993; Bachmann et al., 1994). However, the evidence for the importance of RFOs and cold stress tolerance is correlative. What are lacking are studies of deficient mutants and/or transgenics involving overexpression and downregulation of the enzymes responsible for RFO metabolism. Alpha-galactosidase is one of the RFO catabolic enzymes hydrolyzing the α-1,6 linkage of raffinose to produce galactose and sucrose. Understanding the control of the enzymatic mechanism responsible for driving RFO degradation presents a unique opportunity to develop applications for producing hardier crop varieties. Petunia was chosen as a model for this research because transformation protocols for petunia were previously successful in our laboratory, but most importantly preliminary results demonstrated petunia's capability to cold acclimate. This research was based on the hypothesis that down regulation of α-Gal may be an important element in the accumulation or maintenance of RFO levels that are required to enhance cold tolerance. The results presented confirm α-Gal inhibition as the mode of RFO accumulation and subsequent freezing tolerance. Molecular and physiological evaluations of transgenic petunia with the α-Gal antisense and sense gene constructs are discussed.

4.3 **Materials and methods**

4.3.1 Plant material and culture conditions

Petunia x hybrida cv Mitchell used in this research were obtained from Dr. M.L. Jones, Colorado State University. Seeds were surfaced sterilized in 70 % ethanol for 2 min followed by 5 % Clorox bleach (0.26 % sodium hypochorite) with 0.1 % Tween 20

for 8 min and then rinsed in three washes of sterile distilled water. Surface sterile seeds were routinely cultured on solid modified MS basal medium (half strength of ammonium nitrate and potassium nitrate, termed ½ MS medium) containing 3 % (w/v) sucrose and 0.8 % (w/v) agar at pH 5.8. Seeds were germinated in Magenta boxes and incubated at 25 °C day, 22 °C night, with a 16/8 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. Nodal segments were cultured on full strength MS and maintained as described above.

4.3.2 Sense and antisense vector construction

The tomato α-Gal cDNA (GenBank accession AF191823) in the phagemid vector pBK-CMV was obtained from Dr. D. Bewley, University of Guelph. The tomato cDNA was electroporated into *Escherichia coli* (*E. coli*) cells as follows: 2 μl (2 ng/μl) of tomato α-Gal cDNA was carefully mixed with 40 μl of thawed competent *E. coli* DH5α cells in a chilled 1 mm cuvette. The cuvette was placed on ice for 1 min before electroporating at 1.43 V (BTX^R electroporator, Genetronic Inc., San Diego, CA). Immediately, the cuvette was removed and 960 μl liquid LB (10 g Bacto tryptone, 5 g Bacto yeast, 5 g NaCl pH 7.0 / L) was added and mixed gently by pipetting up and down. The mixture was then pipetted into a sterile microfuge tube and incubated at 37 °C for 1 h with continuous shaking (200 rpm). The culture was plated on solid LB plates supplemented with kanamycin (50 mg/ml), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (20 mg/ml in N,N'-dimethylformamide) and isopropyl-beta-D-thiogalactopyranoside (IPTG) (200 mg/ml). The plates were incubated at 37 °C overnight. Sixteen white colonies were selected for plasmid DNA isolations according to

the boiling lysis method described by Sambrook et al., 1989. Digestion with *Bam*H I and *Xho* I resulted in a 1540 bp fragment which was confirmed as the tomato α-Gal gene (GenBank accession AF191823) by the DNA sequencing service provided by the Department of Biochemistry and Molecular Biology, Colorado State University (CSU).

Figure 4.1 illustrates the designs leading to the final sense and antisense constructs in pMON981. The plant transformation vector pMON981 was obtained from Monsanto. It consists of two strong constitutive promoters, the 35S Cauliflower Mosaic Virus (CaMV 35S) and the Figwort Mosaic virus (FMV), kanamycin and spectomycin resistance genes and the E9 and Nos 3' termination signals. For the sense construct, the 1540 bp tomato α-Gal fragment digested from phagemid pBR-CMV with *BamH* I and *Xho* I was inserted into the corresponding sites of pBluescript (sense construct 1) as follows: pBluescipt was digested with *BamH* I and *Xho* I at a final concentration of 1 U/μl each followed by a phenol chloroform extraction and ethanol precipitate according to the procedure by Sambrook et al., 1989. It was necessary to dephosphorylate the vector arms to prevent self ligation of pBluescript. This was accomplished with calf intestinal alkaline phosphatase (CIAP) according to the method by Sambrook et al., 1989. The dephosphorylated pBluescript was purified by phenol chloroform extraction and concentrated by an ethanol precipitate. The ligation reaction was carried out in a 10 μl volume containing (final concentration) 1 μg tomato α-Gal cDNA insert, 1 μg dephosphorylated pBluescript vector, 1X T_4 ligase buffer, 0.05 U/μl T_4 DNA ligase and 4 μl H_2O in a 1:1 (vector : insert) ratio. The reaction was incubated at 4 °C overnight. The presence of the insert was confirmed by restriction digestion and sequencing of the plasmid DNA at the Department of Biochemistry and Molecular Biology, CSU. The *Xba*

I- *Kpn* I fragment of the sense construct 1 containing the α-Gal cDNA was ligated into the *Xba* I and *Sma* I sites of the dephosphorylated plant transformation vector pMON981 in a 1:1 (vector : insert) ratio to yield the sense construct 2. The *Sma* I site on the pMON981 vector gives a blunt end so the *Kpn* I end of the linearized *Xba* I - *Kpn* I fragment was made blunt with Klenow fragment (Promega protocols and application guide, Madison, WI) before ligation. All enzymes were purchased from Promega (Madison, WI). The presence of the insert was confirmed by sequencing at the Department of Biochemistry and Molecular Biology, CSU.

For the antisense construct, the 5' *Bam*H I - *Xba* I 3' 1540 bp fragment was ligated in the inverted orientation into the 5' *Xba* I - *Bam*H I 3' sites of the dephosphorylated pMON981 plant transformation vector in a 1:1 (vector : insert) ratio. The α-Gal insert was verified by sequencing at the Department of Biochemistry and Molecular Biology, CSU.

4.3.3 Plant transformation and plant culture

The sense and antisense constructs were introduced into competent *Agrobacterium tumefaciens* LBA4404 cells as follows: LBA4404 cells were grown at 28 °C overnight in modified LB (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5 / L) supplemented with streptomycin (50 mg/ml). The culture was diluted at 1:100 into 10 ml LB and incubated at 28 °C for 8 h. Approximately 2 ml was chilled on ice for 5 min and then pelleted by centrifuging at 5000 rpm for 3 min at 4 °C. The pellet was gently resuspended in 1 ml cold 10 mM Tris pH 7.5 and was pelleted as above. The pellet was resuspended in 200 μl of cold LB. Care was taken to ensure that cells were well

resuspended and not clumped at this stage. About 2 μg of sense or antisense plasmid DNA was added to the cells and frozen in liquid nitrogen (LN) for 5 min. The cells were immediately transferred to a 37 °C water bath and incubated for 5 min. Fresh LB (1 ml) was added and the cells were recovered at 28 °C for 3 h. Between 100 and 300 μl of cells were plated on LB plus spectomycin (50 mg/ml) selection plates at 28 °C for 3 days. Doing plasmid DNA isolations and checking for the inserts through restriction digests confirmed the appropriate transformation.

These cells were used to produce transgenic petunia plants using a modified leaf disc cocultivation protocol of Jorgensen et al. (1996). Using a sterile 5 mm cork borer, leaf discs were cut from aseptically grown plants and dipped in *Agrobacterium* cells adjusted to an OD @600 nm of 0.15. Leaf discs were blotted dry on sterile filter paper and transferred to cocultivation medium (CCM) [containing MS basal salts, MS vitamins, 3 % (w/v) sucrose, 0.05 % (w/v) MES, thidiazuron (TDZ) (0.001 mg/ml), 0.8 % (w/v) agar, pH 5.8] for 2 days in darkness at 25 °C. For callus production, leaf discs were transferred to petri dishes containing selection/regeneration medium (SSM) (containing the components of the CCM supplemented with carbenicillin (0.5 mg/ml) and kanamycin (0.15 mg/ml)) and incubated in darkness for 2-3 weeks at 25 °C. For shoot proliferation, explant calli were transferred to Magenta boxes containing SSM without TDZ and incubated for 2-3 weeks at 25 °C with a 16/8 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. One shoot per callus was excised and transferred to selection and rooting medium (SRM) containing the components of the CCM supplemented with carbenicillin (0.5 mg/ml), kanamycin (0.2 mg/ml) and IBA (4×10^{-6} mg/ml) and

incubated as above. Rooted plantlets were numbered and maintained on antibiotic free MS medium as described in section 4.3.1.

4.3.4 Screening putative transgenics

4.3.4.1 α-Gal activity assay

The enzyme assay was adapted from Gaudreault and Webb (1993) with slight modifications. Leaf tissue was collected from 8 week-old plants and extraction was carried out at 4 °C. Approximately 20 g fresh weight of whole leaf tissue were homogenized in a Virtis 45 homogenizer in 100 ml chilled extraction buffer consisting of 0.5 M sucrose, 0.1 M KCl, 50 mM Tris-Cl, 10 mM $Na_2S_2O_5$, 10 mM PMSF, 1.5 % PVPP at pH 6.3. The crude extract was used for α-Gal activity using p-nitrophenyl-α-D-galactopyranoside (PNPG).

Assay mixtures contained 100 µl of enzyme preparation 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 terminated after 20 min by adding 2.4 ml of 5 % Na_2CO_3. Blanks were prepared by adding enzyme after Na_2CO_3. 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 X 10^4 M^{-1} cm^{-1}. One unit of enzyme activity was defined as one µmol of PNPG hydrolyzed min^{-1}. Total protein was estimated by the Lowry method using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

4.3.4.2 Polymerase chain reaction (PCR)

The presence of the α-Gal transgene was demonstrated in the plants by PCR analysis. Genomic DNA was isolated (as described in 3.3.5) from leaves of aseptically grown nontransformed (wild type control) and transformed plantlets that rooted on kanamycin. Two specific oligonucleotide primers were designed from the tomato α-Gal sequence. The primers were 5'-CAC TCT AGA GGG GCT CTC TTG AAC ATT-3' and 5'-GTA GGA TCC CTC CTC CAG CAT TGT GGT-3'; yielding a fragment of approximately 14.9 KB. PCR was carried out in a 50 µl reaction volume containing 1X buffer including $MgCl_2$, 5X Master Taq enhancer, 10 mM dNTPs, 5 µM of each primer, 2 µg template DNA and 5 U/µl *Tag* polymerase. PCR was performed in a thermal cycler, (Mastercycler, Ependorf, Westbury, NY) with an initial hotstart at 94 °C followed by 30 cycles, with each cycle consisting of 94 °C for 1 min of denaturation, 65 °C for 1.5 min annealing and 72 °C for 2 min of elongation. This was followed by a final extension at 72 °C for 7 min.

4.3.4.3 NPT II ELISA test

The *nptII* gene that encodes neomycin phosphotransferase (NPT II) confers resistance to a wide range of aminoglycosides including kanamycin. Therefore, this gene was used as a selectable marker in the transformation constructs. T_1 transgenic lines previously screened by PCR were subjected to the ELISA test according to the manufacturers recommendations (PathoScreen kit for NPT II, Agdia Inc.).

4.3.4.4 RNA extraction and nothern blot analysis

Eight-week old wild type and T_2 plants growing under normal conditions (as described in 4.3.5) were used for RNA extraction and northern blot analysis as described in 3.3.6.

4.3.5 Production and selection of transgenic lines

Aseptically grown wild type plants and transgenic lines were transferred to potting medium (4P mix, Fafard, Agawam, MA) and gradually acclimated before transferring to 12 cm pots. Plants were maintained in a greenhouse under standard conditions used for greenhouse production of petunia (Kessler, 1999). Supplemental lighting was provided during the winter months by 430 W Agro Sun lamps, (Denver, CO). Plants were allowed to flower and emasculations followed by pollinations (5 flowers per plant) were performed on each line over the course of 2 months. All flowers pollinated were at the same developmental stage i.e. one day after anthesis. Non pollinated flowers were removed on a daily basis to prevent cross pollination. Fruits produced from tagged flowers were checked daily from 25 days after pollination until the fruit was uniformly brown and not dehisced. Petunia seeds have a 3-4 month dormancy hence three-week old seeds were treated with 100 ppm GA_3 for 24 h before germinating on ½ MS medium supplemented with 0.2 mg/ml kanamycin or without kanamycin. T_1 transgenic lines segregating at a 3 : 1 ratio were used to produce T_2 plants.

4.3.6 Plant growth parameters and trait analyses

If the transgenic petunia plants are to become commercially important they must be able to germinate and produce vigorous plants. To determine if the α-Gal transgenes affected the plants ability to produce seeds that will germinate normally, 4-month old T_0 seeds were used for the germination assay. Seeds from 10 capsules from 2 different plants of the same line that were pollinated on the same day and harvested on the same day were grouped together as a seed lot. The seeds were germinated as described in 4.3.1. Germination tests consisted of 100 seeds per line with 5 plates and 20 seeds per plate. Germination (defined as the emergence of the radicle with fully opened cotyledons) data were taken on days 4, 6, 8, 10, 12, 14 and 16. The germination assay was replicated once using seeds from the same seed lots. The same assay was repeated as above except that seeds were treated with 100 ppm GA_3 for 24 h before sowing.

Eight- and twelve-week old wild type and T_2 plants respectively were grown as described in 4.3.5. Data were collected for flower number, plant height, width, shoot dry mass and root dry mass. Plant height (cm) was measured as the distance from the soil level to the primary shoot apical meristem. To calculate plant width, two diameter measurements were taken; first as the diameter of the widest portion of the plant and then the diameter measured two nodes down of the first measurement [diameter 1 + diameter 2) /2]. Plant height and width were used to calculate plant size as (height + width)/2). Plants were then cut at the soil level and the shoot dry weight (dw) determined. Roots were washed to remove the soil before the dry weight was determined. Dry weights were determined after drying in an oven (70 °C) for 5 days. Data were collected from four plants per line for each treatment and analyzed for statistical significance using ANOVA

and for mean differences by Tukey's Studentized Comparison Tests (SAS computer software).

4.3.7 Soluble sugar analyses

Samples were collected from leaves at all stages of development from 8-week old wild type and T_2 plants and prepared in the same manner and analyzed for soluble carbohydrate content as described in 2.3.2. Two separate experiments were conducted and each treatment was replicated 3 times.

Correlation coefficients were calculated for the relationships between raffinose content and electrolyte leakage for WT and transgenic lines.

4.3.8 Freezing tolerance studies

A technique to screen seedlings for freezing tolerance using petri dishes was developed. This technique allowed a high throughput initial screen for several seedlings of different lines. Wild type and T_2 seeds were cultured on solid ½ MS medium in 5.5 cm petri dishes at 25 °C with a 16/8 h photoperiod for 2 weeks. Seedlings of each type were divided into two groups. Seedlings in one group were used to measure freezing tolerance without cold acclimation and were maintained at the above conditions for an additional 24 days. Seedlings in the other group were transferred to a cold acclimation chamber with a gradual exposure at 15 °C for 7 days, 10 °C for 7 days, 5 °C for 7 days and 3 °C for 3 days with a 12 h photoperiod. Cotyledons from cold acclimated seedlings were wrapped in moistened tissue paper and were placed in a programmable freezer (Tenney Jr., Lunaire Ltd., Williamsport, PA). The samples were equilibrated at 0 °C for 30 min. Ice

seeding (nucleation) was initiated by the presence of the moistened tissue paper and confirmed visually following equilibrium. The temperature was lowered at a rate of 1 °C h^{-1} to various temperatures (lowest temperature, - 9 °C) and each temperature was held for 30 min. Samples were removed at predetermined temperatures and were placed in a refrigerator (4 °C) to allow contents to thaw overnight. Nonacclimated wild type and T_2 cotyledons were subjected to an identical freezing regime as described above. Freezing tolerance was expressed as the temperature at which 50 % of electrolytes were released from the plant tissue (LT_{50}). Electrolyte leakage tests were conducted as described in 2.3.1.

In a separate freezing experiment, 5 mm discs from mature leaves of 8-week old plants grown in the greenhouse (as described in 4.3.5) were used to evaluate freezing tolerance. These plants were subjected to the same cold acclimation and freezing regime described for the seedlings. Soluble carbohydrate content was determined for the selected lines that were subjected to the freezing stress regime to examine the relationship between sugar content and freezing tolerance indicated by electrolyte leakage tests.

4.4 **Results**

4.4.1 Plant transformation

Petunia was transformed via *Agrobacterium* with the tomato α-Gal gene, either in the sense or antisense orientation, under the control of the strong constitutive promoter, FMV (Fig. 4.2). T_0 primary transformants were selected in tissue culture based on kanamycin resistance, and the rooted plantlets were further screened by PCR (Fig. 4.3) and NPT II ELISA (Fig. 4.4). No amplification or positive NPT II were observed for wild

type plants. Several independent transgenic lines were established, transferred to soil potting media (4P mix, Fafard) and grown in the containment greenhouse to generate seeds. Two independent transgenic sense lines (S3, S7) and 6 independent antisense lines (A150, A149, A147, A146, A105 and A91) were selected for further analyses. Approximately 75 % of their T_1 generation seedlings were resistant to kanamycin (Table 4.1). For further confirmation of insertion of the transferred genes, northern blot analyses were carried out on T_2 plants using a tomato α-Gal cDNA as a probe. The α-Gal transcript detected in leaves of antisense lines were lower than that of wild type but much lower than those of sense lines (Fig. 4.5a). Accumulation of transcripts from sense lines was greater than both WT and antisense lines. Alpha-galactosidase activity followed a similar trend whereby activity from antisense lines was significantly lower than that of WT and sense lines ($p< 0.05$) (Fig. 4.5c).

4.4.2 Effect of transgene inheritance on progeny phenotype

Overall, antisense transgenic lines had significantly lower germination rates compared to wild type and sense lines ($p<0.05$) (Fig. 4.6). Among the antisense lines, A146 and A91 had germination percentages closest (69.3 ± 1.7 & 86.3 ± 1.16 respectively) to that of wild type and sense lines. Treatment with GA_3 did not improve the germination rates (Fig. 4.6b).

At eight and twelve weeks old, there were no discernable differences in the phenotypic appearances of transgenic plants compared to wild type plants (Tables 4.2, 4.3 & 4.4). The size of all plants ranged from 15.4 ± 1.18 cm to 17.73 ± 1.40 cm (Table 4.2). Similarly, shoot, root and total dry mass were not significantly different between

wild type and transgenic lines ($p<0.05$) (Table 4.3). Also, under normal growth conditions, the number of flowers did not differ among wild type and transgenic lines (Table 4.4).

4.4.3 Soluble sugar content in wild type and transgenic lines

Six soluble sugars; galactose, glucose, fructose, sucrose, raffinose and stachyose were detected in non acclimated and cold acclimated WT and transgenic petunia plants. Table 4.5 summarizes the changes in the soluble sugar content of antisense lines compared to sense lines and WT under non acclimated and cold acclimated conditions. In non acclimated WT plants, raffinose was scarcely detectable (0.04 ± 0.01 μmol g^{-1} dw) and not significantly different from that of sense lines (0.04 μmol g^{-1} dw and 0.02 ± 0.01 μmol g^{-1} dw) but increased significantly in all antisense lines (12.69 ± 1.69 μmol g^{-1} dw to 22.2 ± 2.81 μmol g^{-1} dw) ($p<0.05$). With the exception of A91, sucrose levels were generally lower in non acclimated antisense lines compared to that of WT. The total soluble sugar content in the non acclimated antisense lines ranged from 19.02 ± 11.6 μmol g^{-1} dw to 27.67 ± 21.4 μmol g^{-1} dw and were significantly higher than that of WT (1.65 ± 1.8 μmol g^{-1} dw) and sense lines (4.64 ± 2.1 μmol g^{-1} dw and 3.15 ± 1.9 μmol g^{-1} dw) ($p<0.05$).

Cold acclimation of plants that were previously grown at warm temperature (25 °C) resulted in a significant increase in raffinose concentration in all lines but especially so in the antisense lines. Following cold acclimation, the raffinose concentration in WT increased 150-fold (from 0.04 ± 0.01 μmol g^{-1} dw to 6.34 ± 0.98 μmol g^{-1} dw) but only increased 2.5-fold (from 22.2 ± 2.81 μmol g^{-1} dw to 53.52 ± 2.03 μmol g^{-1} dw) in the best

antisense line (A105). Total soluble sugars increased significantly following cold acclimation ($p<0.05$).

4.4.4 Freezing tolerance of wild type and transgenic lines

When wild type and transformed lines that had been cold acclimated were frozen to –9 °C, only two antisense lines had LT_{50} values of –8 °C (Table 4.6). The levels of freezing tolerance varied among antisense lines with A105 and A149 being the most tolerant and A91 being the least tolerant. The freezing tolerances of non acclimated wild type and sense lines were similar at LT_{50} values of –2.5 °C in mature leaves and –3.0 °C in cotyledons. There were no significant differences in LT_{50} values of cotyledons and mature leaves of wild type and transgenic lines ($p<0.05$). With the exception of A91, all antisense lines were significantly more freezing tolerant than wild type and sense lines ($p<0.05$).

As shown in Table 4.6, freezing tolerance, expressed as the temperature at which 50 % of electrolytes were released from leaf tissue (LT_{50}), averaged -5 °C in cold acclimated wild type and -3 °C in cold acclimated sense lines whereas it 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 freezing tolerance as indicated by total electrolyte leakage was significantly correlated to the levels of raffinose accumulation in antisense transgenic lines ($p<0.05$) (Table 4.7).

4.5 **Discussion**

Since RFO have been shown to be associated with several abiotic stress tolerances, including freezing tolerance, analyses of transgenic lines carrying the antisense gene of α-Gal were compared to wild type and sense transgenic lines. Alpha-galactosidase is known to play a key role in the plant cold hardiness pathway specifically in RFO catabolism (Castillo et al., 1990; Castonguay and Nadeau, 1998). Consequently, it can be expected that a comparative study using transgenic lines can contribute to the understanding of plant cold tolerance.

An efficient transformation regeneration procedure for petunia was used, resulting in about 35 % transformation efficiency. The use of green healthy leaf explants from *in vitro* grown plantlets was likely responsible for the high transformation rate. Previously, leaf explants from non-vigorous *in vitro* plantlets resulted in yellowing and subsequent death of the leaf discs 14 days after cocultivation. It is believed that the healthy leaf explants are self-sustaining until calli formation, which in turn enhance the transformation efficiency. Segregation analysis suggested that the T_0 transgenic lines selected for further analyses appeared to have an integrated T-DNA locus on a single chromosome since 75 % of their T_1 segregating seedlings were resistant to kanamycin.

Morphologically, transgenic lines were not different from wild type plants. However a striking observation from this study is that seed germination was affected by the presence of the transgene as indicated by a decrease in germination rate of the antisense lines. Bewley and Black (1978) suggested that α-Gal present in embryos during and following germination is involved in the degradation of RFO as an early source of carbohydrate prior to the mobilization of the major hemicellulose or starch reserves.

Alpha-galactosidase activity unlike that of endo-β-mannanase (works co-operatively with α-Gal to degrade cell wall galactomannan during seed germination) appears to be present early in seed germination (Feurtado et al., 2001). Furthermore, early seedling growth was not affected by the antisense transgene suggesting that mobilization of carbohydrate reserves is not specific to α-Gal. To further establish the effect of the antisense transgene on germination, seeds were imbibed with 100 ppm GA_3 for 24 h before sowing. This had no consistent effect on the germination rate, indicating that germination inhibition was not due to embryo dormancy (ratio of endogenous GA to ABA) that has been shown in numerous studies to be overcome by GA application (Bewley and Black, 1994; Gallardo et al., 2002).

In this study the hypothesis that down regulation of α-Gal coincides with raffinose accumulation and subsequent stress tolerance was tested. Compelling evidence in the literature supports the hypothesis that RFOs function in part to enhance stress tolerance in many plants. The reduction of RFO hydrolysis by insertion of α-Gal in the antisense orientation was expected to result in an accumulation of RFO. 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. While also contributing to the stabilization of membranes and proteins (Santarius, 1973), RFO are also thought to enhance the ability of sucrose to promote glass formation at low moisture contents by preventing sucrose from forming ice as water is removed. In fact, raffinose is so competent at disrupting sucrose crystallization that much effort is dedicated to eliminating it from expressed juice during sugar beet processing (Kerr et al., 1997). 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 levels of sucrose compared to that of either raffinose or stachyose in both non acclimated and cold acclimated WT plants suggest that sucrose is the dominant transport sugar in petunia. The total soluble sugar content increased significantly when the plants were in the acclimated state. In the antisense lines, fifty percent or more of the increase in total soluble sugar content was due to the increase in raffinose compared to WT in which case sucrose contributed more than fifty percent. Freezing stress tolerance was predicted based on the relationship between raffinose accumulation and decreased electrolyte leakage.

Downregulation of α-Gal in petunia resulted in an increase in freezing stress tolerance while overexpression of α-Gal resulted in freezing tolerance comparable to that of wild type plants. The data presented here suggest that manipulation of endogenous raffinose content by gene regulation has a significant effect on freezing tolerance in comparison to a cold acclimation treatment alone. Consistent with the findings of Yelonosky and Guy (1989), cold acclimated wild type petunia increased in freezing tolerance by about 1 °C. Cold acclimated antisense transgenic lines ranged from a 3 °C to 6 °C increase in freezing tolerance. The rationale behind using transgenic studies rather than cold acclimation treatments only to evaluate freezing tolerance is that an inherent genetic factor is more stable than an environmental factor which could introduce variables in the experiment and might invalidate evaluation of true cold hardiness.

Earlier (chapter 3) it was demonstrated that the α-Gal gene is constitutively expressed. In view of this observation, evaluation of freezing tolerance of wild type and

transgenic lines at the seedling and mature plant stages were conducted. Also, because environmental adversities can have a serious impact on growth at any stage of development, this experiment was performed to determine to what extent acclimation could induce tolerance in the plants. Both wild type and transgenic lines at both stages of development were able to cold acclimate when subjected to a gradual low temperature exposure but to a lesser extent in wild type and sense lines. Mature plants acclimated to the same levels of freezing tolerance as the plants having only cotyledonous leaves. The cotyledons function as photosynthetic organs until true leaves can develop and become functional. Thus, it is not surprising that cotyledons and true leaves would have similar levels of freezing tolerance despite their substantial structural and biochemical differences. These results contradict the findings of Prasad (1996) that acclimation induced freezing tolerance is developmentally regulated in developing maize seedlings but are comparable to those observed by others using leaf tissue from spinach (Guy et al., 1984; Fennel and Li, 1985). Among the antisense lines examined, several different levels of freezing tolerances were observed, probably due to whether the plants were homozygous or heterozygous for the α-Gal gene. Evaluation of subsequent generations (T_3 and beyond) could determine whether a line might be homozygous.

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 demonstated that downregulation of α-Gal in petunia is

sufficient to decrease electrolyte leakage and subsequently confer tolerance to freezing stress.

Freezing temperatures often cause severe losses in agricultural productivity. While traditional breeding approaches have met with limited success in improving the freezing tolerance of economic crops, biotechnology may offer new strategies. This study has demonstrated the use of genetic modification of α-Gal in petunia to enhance the plant's capability to tolerate freezing temperatures. This information has obvious implications during production whereby petunia may be planted earlier in the spring and grown later into fall when frost injury is most likely. But most importantly, 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.

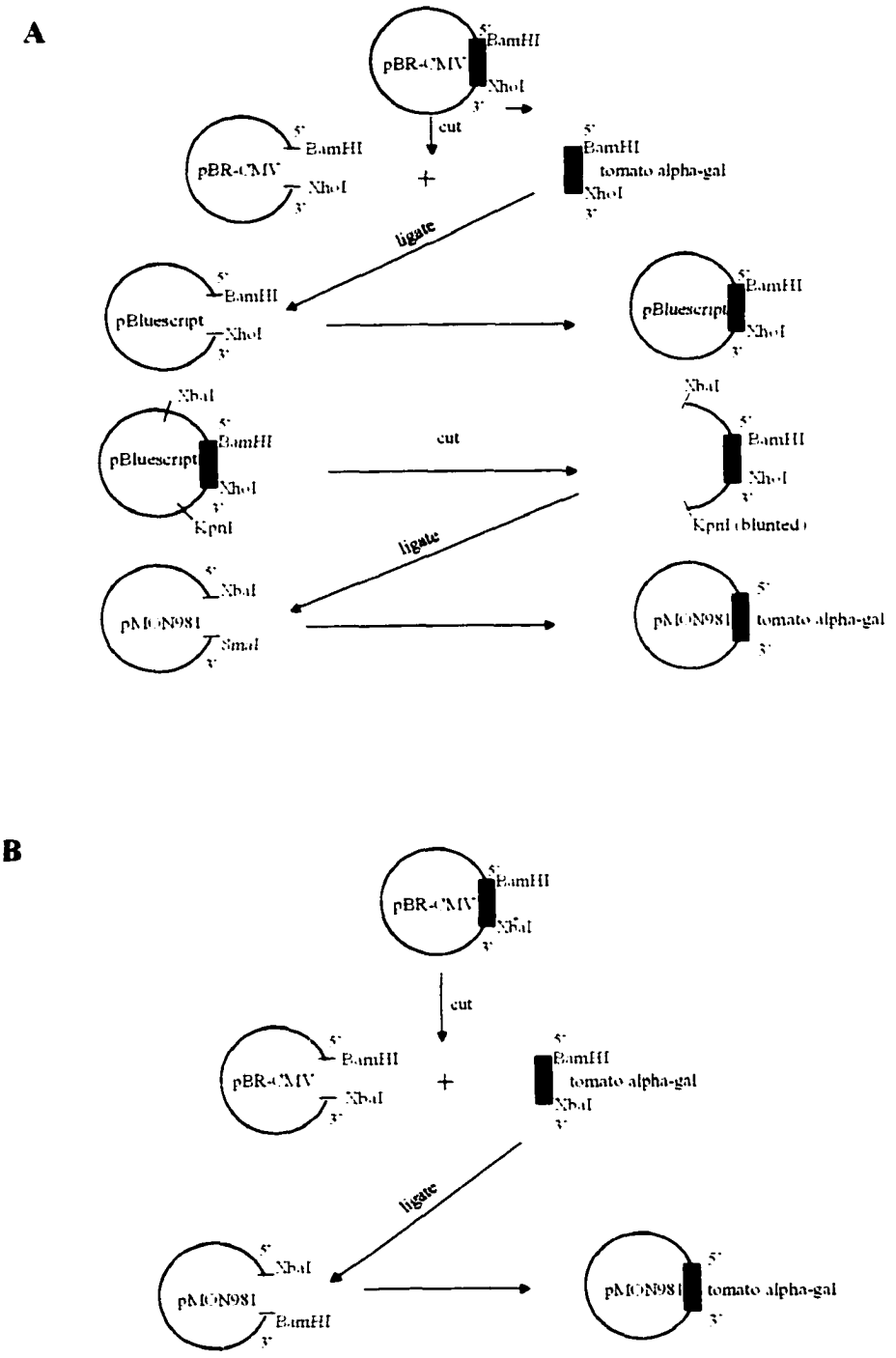


Fig. 4.1 Step by step designs leading to the sense (A) and antisense (B) contructs. pBR-CMV: phagemid vector with tomato α-Gal cDNA; pBluescript: plasmid vector; pMON981: plant transformation vector.

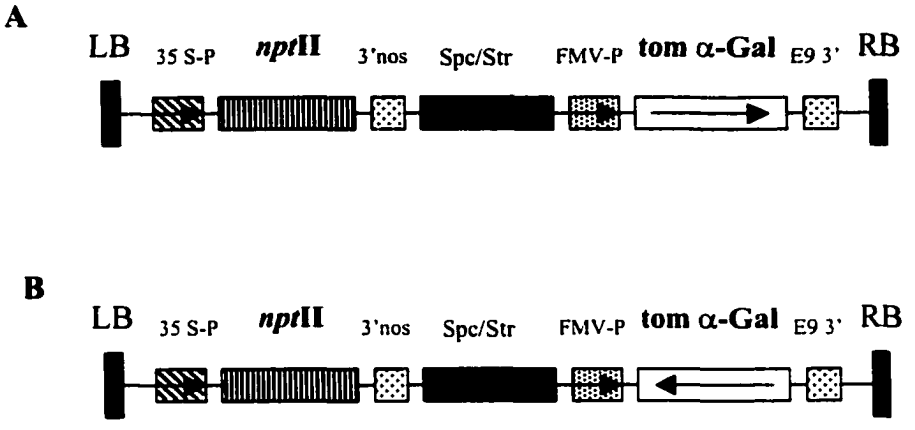


Fig. 4.2 Composition of sense (A) and antisense (B) constructs used in transformation of petunia. The tomato α-Gal gene (1540bp) was placed in the antisense orientation relative to the FMV-P, upstream of the E9 3' terminator sequence (B). LB, RB: left and right borders; 35S-P, FMV-P: Cauliflower and Figwort mosaic virus promoters; *npt*II, Spc/Str: resistance genes encoding kanamycin and spectomycin/streptomycin; 3'nos, E9 3': terminator sequences.

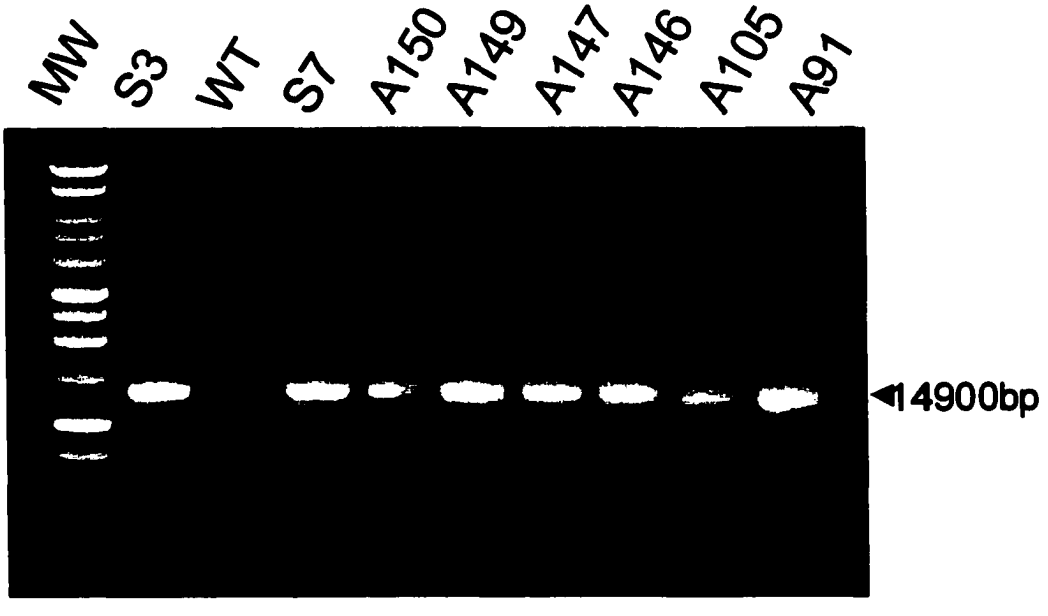


Fig. 4.3 Gel electrophoresis of PCR products amplified from putative transgenic sense (S3, S7) and antisense (A150 – A91) lines. A 14900 bp fragment was amplified with primers designed from the tomato α-Gal sequence. MW: 1 Kb molecular weight marker; WT: wild type negative control

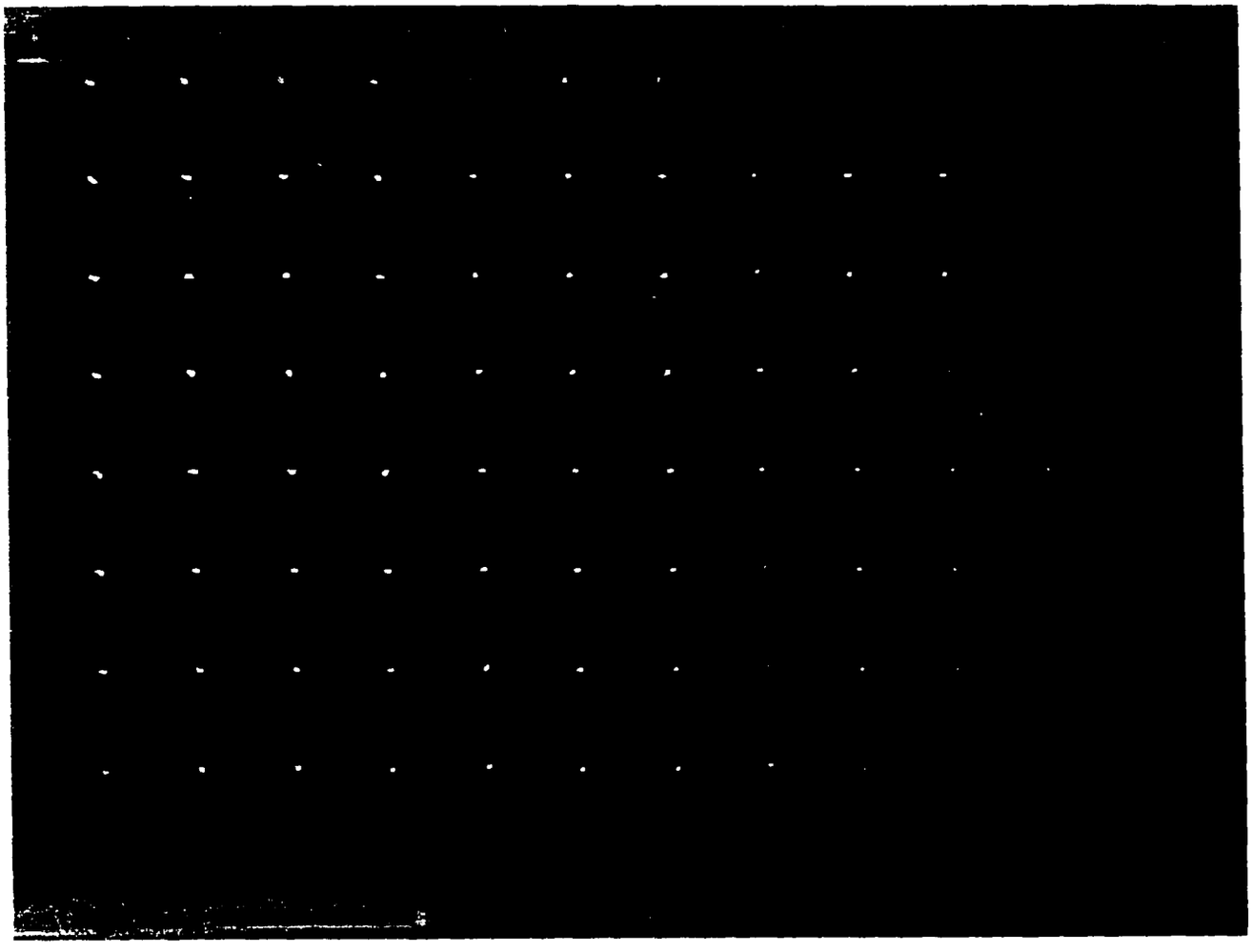


Fig. 4.4 NPT II (neomycin-phosphotransferase II) enzyme assay (ELISA). Lanes 1 & 2: standards (ng/ml) as positive controls. Lanes 3 – 12: leaves from WT (negative control) and T_0 putative transformants. Positive sense (S3, S7) and antisense (A150 – A91) lines were used for further evaluations.

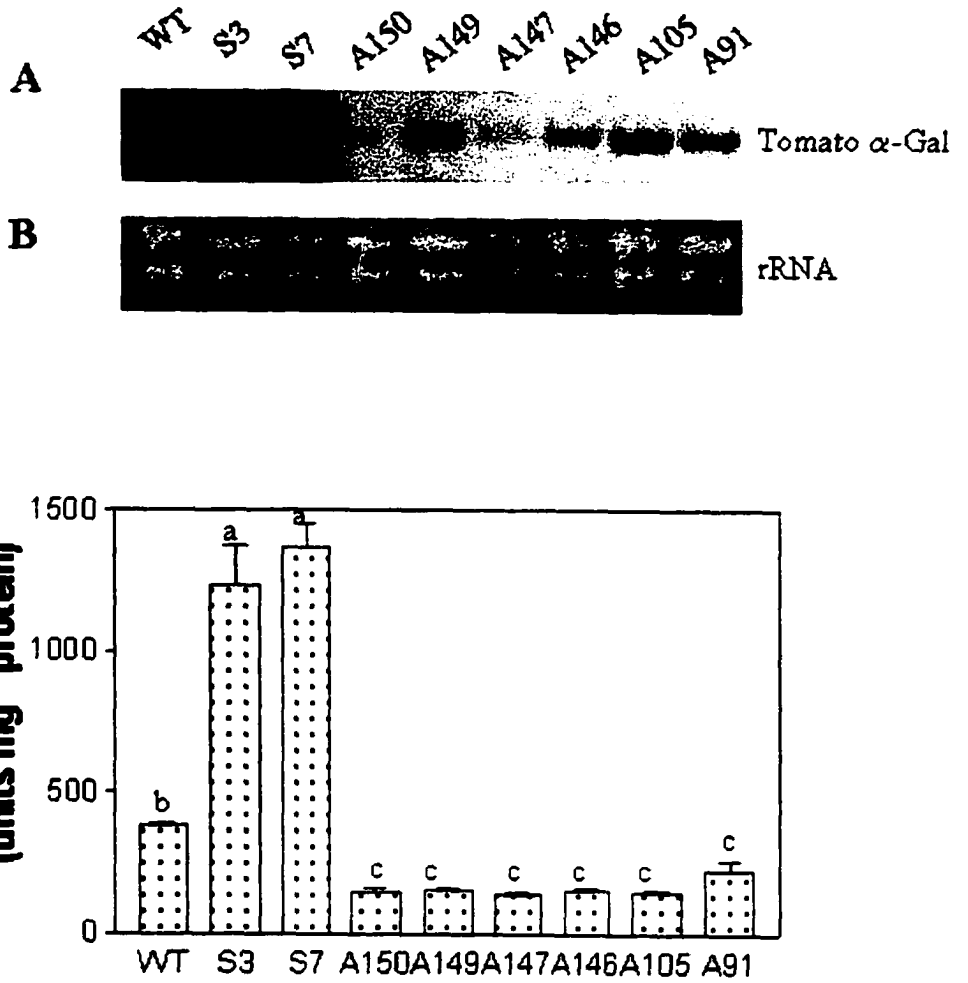


Fig. 4.5 Northern blot analysis of total RNA isolated from WT, sense (S3, S7) and antisense (A150 – A91) lines (A). Ten μg of total RNA were separated by electrophoresis through agarose and hybridized with a α-^{32}P-labeled tomato α-Gal. Ribosomal RNA stained with ethidium bromide was used as a loading control (B). Alpha-galactosidase activity in Wt, sense and antisense lines (C).

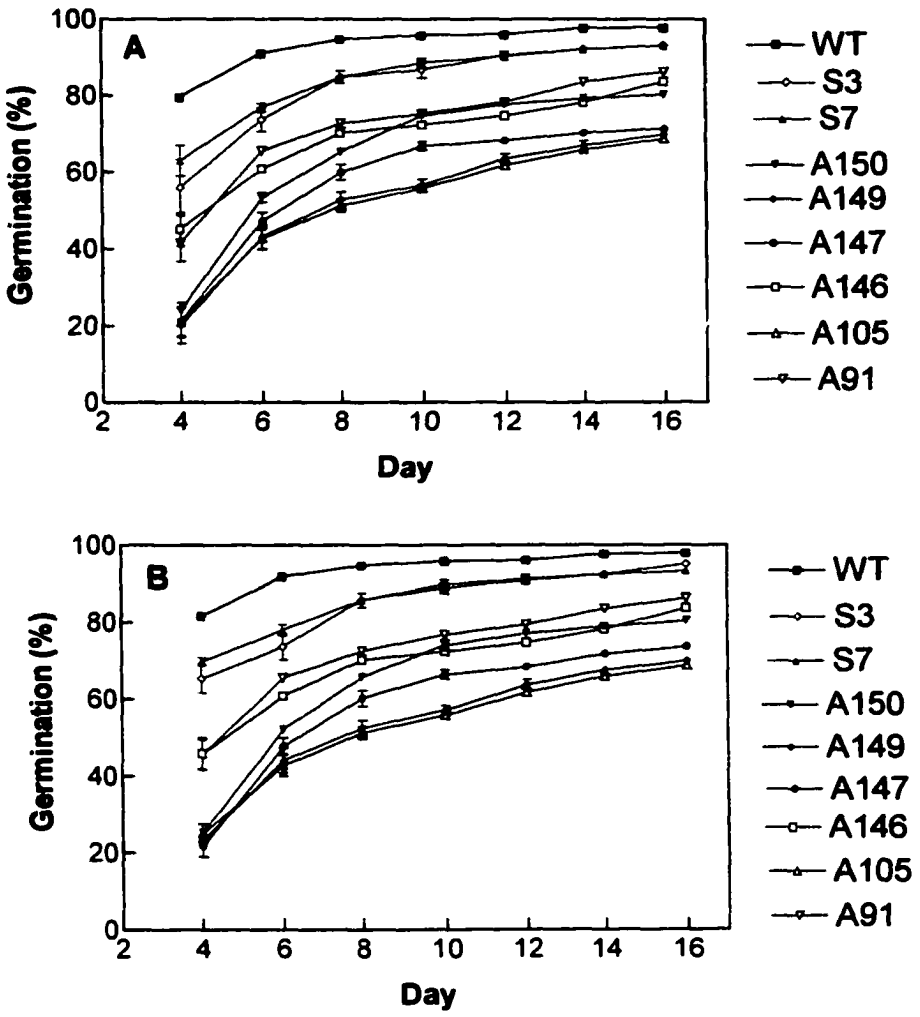


Fig. 4.6 Germination of WT and 4-month-old T_0 seeds of sense (S3, S7) and antisense (A150 – A91) lines on solid ½ MS media (A). Germination of seeds from the same seed lots as in A but seeds were treated with 100 ppm GA_3 for 24 h before sowing (B). Error bars are standard deviation of 5 reps each from two separate experiments. n= 200 seeds / line

Table 4.1 Analysis of kanamycin resistance in T_1 transgenic plants

Genotype	Segregation ratio	χ^2
S3	190 : 60	0.13 *
S7	175 : 65	0.55 *
A150	135 : 55	1.58 *
A149	80 : 20	1.30 *
A147	115 : 35	0.22 *
A146	160 : 40	2.60 *
A105	82 : 18	2.61 *
A91	188 : 62	0.005 *

Goodness of fit to 3:1 ratio tested by Chi-square analysis. Data calculated with df=1.

* significant at 0.05; $\chi^{2\,(0.05)} = 3.84$

Table 4.2 Size of eight-week-old wild type and transgenic plants

Genotype	Height (cm)	Width[γ] (cm)	Size[ϕ] (cm)
WT	13.050 ± 2.12 a	22.08 ± 1.02 a	15.73 ± 0.85 a
S3	12.63 ± 1.54 a	21.90 ± 0.98 a	15.54 ± 0.89 a
S7	12.96 ± 2.20 a	20.53 ± 1.90 a	15.40 ± 1.18 a
A150	13.80 ± 0.96 a	19.88 ± 1.18 a	15.92 ± 1.42 a
A149	13.85 ± 2.54 a	21.08 ± 2.15 a	16.48 ± 0.99 a
A147	13.73 ± 1.32 a	21.73 ± 1.85 a	15.85 ± 0.88 a
A146	13.15 ± 0.72 a	20.93 ± 2.34 a	15.78 ± 1.40 a
A105	15.08 ± 0.59 a	22.08 ± 1.71 a	17.73 ± 1.152 a
A91	13.95 ± 1.06 a	21.38 ± 2.05 a	16.18 ± 1.27 a

Data are from two replicated experiments. Values are mean ± sd.
[a] Numbers within columns followed by the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS, Cary, NC).

Table 4. 3 Dry mass of 12-week-old wild type and transgenic plants

Genotype	Shoot Dry Mass (g)	Root Dry Mass (g)	Total Dry Mass (g)
WT	20.62 ± 4.67 a	5.49 ± 1.23 a	26.11 ± 4.99 a
S3	19.97 ± 3.72 a	5.25 ± 1.19 a	25.25 ± 2.98 a
S7	20.45 ± 2.51 a	5.50 ± 0.45 a	25.95 ± 2.52 a
A150	21.45 ± 2.92 a	6.13 ± 0.15 a	27.58 ± 3.02 a
A149	22.44 ± 4.66 a	5.28 ± 0.38 a	27.72 ± 4.35 a
A147	20.33 ± 1.56 a	6.13 ± 0.15 a	26.45 ± 1.49 a
A146	21.00 ± 2.92 a	5.80 ± 0.22 a	26.80 ± 2.87 a
A105	21.70 ± 3.73 a	5.88 ± 0.25 a	27.57 ± 3.58 a
A91	20.43 ± 3.25 a	5.63 ± 0.85 a	26.05 ± 2.61 a

Data are from two replicated experiments. Values are mean ± sd.
[a] Numbers within columns followed by the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS, Cary, NC).

Table 4.4 Number of flowers on 12-week old plants

Genotype	Number of flowers
WT	17.5 ± 1.00 a
S3	18.25 ± 0.96 a
S7	17.5 ± 1.00 a
A150	17.25 ± 0.50 a
A149	17.75 ± 1.50 a
A147	18.00 ± 1.41 a
A146	18.50 ± 1.29 a
A105	17.25 ± 0.50 a
A91	17.50 ± 0.58 a

Data are from two replicated experiments. Values are mean ± sd.
[a] Numbers within columns followed by the same letter are not significantly different at $p < 0.05$ by Tukey's Studentized Comparison

Table 4.5 Changes in soluble sugar content (μmol g^{-1} dw) of non acclimated and cold acclimated WT and transgenic petunia

Cold ACC	Genotype	Galactose	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Total Sugars
NO	WT	0.009±0.01	0.67±0.15	0.38±0.08	0.55±0.03	0.04±0.01	0.005	1.65 ± 1.8
	S3	0.021±0.03	1.32±0.82	1.2±0.68	1.98±0.19	0.042	0.035	4.64 ± 2.1
	S7	0.002	1.64±0.62	1.45±1.02	0.04	0.02±0.01	0.005	3.15 ± 1.9
	A150	0.004	1.35±0.83	0.44±0.30	0.675±0.05	17.55±1.00	2.86±0.97	22.88 ± 16.7
	A149	0.24±0.04	0.66±0.73	0.39±0.22	0.07	17.27±1.18	3.51±0.82	22.13 ± 16.6
	A147	0.24±0.04	0.30±0.30	0.37±0.13	0.07	17.36±1.34	2.8±1.02	21.15 ± 16.8
	A146	0.018±0.03	1.40±0.23	0.38±0.13	0.88±0.04	15.711±0.09	2.7±0.40	21.10 ± 14.8
	A105	0.70±0.02	1.27±0.14	0.047	0.06	22.2±2.811	3.32±0.49	27.67 ± 21.4
	A91	0.003	1.81±0.61	1.75±0.35	2.38±0.36	12.69±1.69	0.39±0.85	19.02 ± 11.6
YES	WT	0.01	5.31±1.18	4.81±1.12	13.77±4.05	6.34±0.98	2.54±1.6	32.79 ± 11.4
	S3	0.33±0.11	3.11±1.44	2.50±0.77	15.03±4.22	0.56±0.13	0.44±0.2	22.00 ± 13.9
	S7	0.003	2.42±0.99	3.36±0.8	7.63±2.1	0.60±0.1	0.40±0.33	14.44 ± 7.0
	A150	1.71±0.31	8.57±3.06	7.02±1.66	3.73±1.39	24.00±5.79	1.87±1.14	46.92 ± 20.6
	A149	1.31±0.46	13.46±4.29	10.45±1.71	0.66±0.18	52.27±3.05	4.09±1.14	82.26 ± 47.9
	A147	2.55±0.44	13.72±1.45	9.96±0.87	0.62±0.14	36.38±8.24	2.49±0.99	65.73 ± 32.9
	A146	0.97±0.58	9.46±3.19	7.91±3.64	0.54±0.15	28.50±3.95	2.94±0.88	50.35 ± 25.7
	A105	1.77±0.15	16.30±1.93	13.43±4.49	2.40±1.93	53.52±2.03	3.37±0.6	90.82 ± 48.5
	A91	2.69±0.42	7.50±1.39	5.97±2.21	8.58±2.9	22.23±4.24	1.66±1.04	48.66 ± 18.2

Sugar analysis by HPLC-PAD. Data are from two replicated experiments with 3 replicates for each genotype. Values are mean ± SEM; n = 6.0; Cold ACC: cold acclimated at 15 °C for 7 days, 10 °C for 7days, 5 °C for 7 days and subsequently 3 °C for 3 days.

Table 4.6 Comparison of freezing tolerance as indicated by LT_{50} values during nonacclimation and cold acclimation of cotyledons and mature leaves of wild type and transgenic plants

Treatment	Genotype	LT_{50} values of developmental stages	
		cotyledons	mature leaves
		° C	
Nonacclimated	WT	-2.5 a	-2.0 a
	S3	-2.5 a	-2.0 a
	S7	-2.5 a	-2.0 a
	A91	-3.0 a	-3.0 a
	A105	-5.0 b	-4.5 b
	A146	-5.0 b	-4.5 b
	A147	-5.0 b	-5.0 b
	A149	-4.5 b	-4.5 b
	A150	-4.0 b	-4.0 b
Cold acclimated	WT	-4.5 b	-5.0 b
	S3	-3.0 a	-3.0 a
	S7	-3.0 a	-3.0 a
	A91	-5.5 b	-6.0 bc
	A105	-8.0 d	-8.0 d
	A146	-6.0 bc	-6.0 bc
	A147	-6.5 c	-7.0 cd
	A149	-8.0 d	-8.0 d
	A150	-6.5 c	-6.5 c

LT_{50} values were determined from electrolyte leakage tests.
Data are from two replicated experiments. Values within columns followed by different letters are significantly different at $p < 0.05$ by Tukey's Studentized Comparison Test (SAS, Cary NC).

Table 4.7 Pearson's correlation coefficient between raffinose content and electrolyte leakage in WT and transgenic petunia lines

Genotype	Correlation coefficient[a] (p-value)[b]	
	Non acclimated	Cold acclimated
WT	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)
A150	0.8848 (0.02)	0.8987 (0.02)
A149	0.7541 (0.01)	(0.9234) (0.003)
A147	0.7102 (0.02)	0.8245 (0.04)
A146	0.851 (0.03)	0.8657 (0.03)
A105	0.9083 (0.01)	0.9245 (0.002)
A91	0.703 (0.09)	0.7761 (0.05)

[a] correlation coefficient was calculated from 6 reps of sugar content and electrolyte leakage data using SAS computer software (Cary, NC).
[b] values < 0.05 are significant at P = 0.05.

5.2 **Introduction**

Phenolic compounds are widely distributed in plants (Sellapan et al., 2002). They are particularly important in fruits and vegetables, to which they contribute color and flavor (Macheix et al., 1991). Moreover, phenolic compounds of fruits and vegetables contribute to antioxidant intake and are presumed to have a health protective action in humans (Kroon and Williamson, 1999). However, the role of phenolic compounds in plant abiotic stresses has received much less attention. The structural chemistry of polyphenols predicts their potential role as free radical scavengers (antioxidants) and this has been well documented (Rice-Evans et al., 1997). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Rice-Evans et al., 1997). Antioxidant activity of a compound may be determined by inhibition of the radical formation, or by elucidating scavenging ability in a system generating the radical. The 2,2' azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical (ABTS*) decolorisation assay discussed below was based on the latter approach (Miller and Rice-Evans, 1997). In the presence of MnO_2, ABTS is oxidized to the activated ABTS radical cation (ABTS*), a blue colored radical with absorption at 734 nm. The presence of antioxidants in the plant extract suppresses the formation of the colored radical cation in comparison with the antioxidant potency of standard amounts of Trolox (6-hydroxy-2,5,7,8-tetramethychroman 2-carboxylic acid), a water soluble vitamin E analogue. Total

antioxidant capacity (TAC) is directly related to the extent of the suppression. TAC is measured as the Trolox Equivalent Antioxidant Capacity (TEAC).

Plant cells contain antioxidant metabolites such as β-carotene, α-tocopherol, ascorbate and glutathione that maintain reactive oxygen species (ROS) at low steady-state levels (Asada, 1992). Several studies have demonstrated that antioxidant enzymes such as superoxide dismutase (SOD) (McKersie et al., 1993), glutathione reductase and catalase (Prasad, 1996) confer protection against ROS in plants. Anderson et al. (1995) showed that in nonacclimated seedlings, chilling injury is partly due to the build up of ROS while chilling tolerance in cold acclimated seedlings is due in part to an increased antioxidant system that protects against the accumulation of ROS. It has also been demonstrated that certain abiotic stresses induce the production of phenolic compounds in plants (Christie et al., 1994). Several authors (Christie et al., 1994; Prasad 1996; Rice-Evans et al., 1997) have reported the synthesis, oxidation and the content of certain phenolic compounds in response to the induction of some type of stress, but few have examined increases in phenolic acids relative to low temperature stress. The objective of this study was to determine the total phenolics content and relate this to ABTS antioxidant activity in response to two cold acclimation regimes tested in petunia.

5.3 **Materials and methods**

5.3.1 Plant materials and treatment

Petunia x hybrida cv Mitchell plants were grown from seed and maintained in a greenhouse at 25 °C with a 16/8 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. Eight-week old plants were transferred to a growth chamber to induce cold

acclimation by incubating at 15 °C for 1 week, 10 °C for 1 week, 5 °C for 1 week and 3 °C for 1 week or at 5 °C for 4 weeks with a 12 h photoperiod under cool white fluorescent light at 60 μmole m^{-2} s^{-1}. Non acclimated plants were maintained at 25 °C for 4 weeks with a 16/8 h photoperiod. Plants were subjected to chilling stress by incubating at 2 °C for 2 weeks with a 12 h photoperiod. Leaf samples were collected at one-week intervals and processed accordingly for total phenolics, antioxidant capacity, and electrolyte leakage. Sampling for identification of phenolic acids was done at the end of the fourth week of acclimation.

5.3.2 Extraction and measurement of total phenolics

The total phenolic content was determined using a modified Folin-Ciocalteu colorometric method (Spanos and Wrolstad, 1990). For this, 600 mg leaf tissue was macerated in 50 ml 80 % (v/v) acetone in a mortar and pestle. The sample was placed into 50 ml tightly covered plastic tubes and incubated in darkness at 4 °C overnight. The sample was then filtered through a Buchner funnel under vacuum with filter paper. Sample concentration was adjusted by vacuum spinning 1 ml of the acetone extract for about 2.5 h on medium heat (45 °C). The pellet was resuspended in 200 μl 80 % (v/v) acetone just before use. Precisely, 450 μl H_2O, 2.5 ml 1/10 dilution Folin-Ciocalteu reagent and 2.0 ml 7.5 % (w/v) Na_2CO_3 were added to 50 μl of phenolic extract. After vortexing for 10 sec, the mixture was incubated at 45 °C in a shaking water bath for 15 min. Sample tubes were allowed to cool to room temperature before reading the absorbance at 765 nm. Blanks were prepared as above but the sample was replaced with 50 μl 80 % (v/v) acetone. A gallic acid standard curve was prepared from a freshly made

1 mg ml^{-1} gallic acid (in 80 % (v/v) acetone) stock solution. Total phenolics was calculated from the gallic acid standard curve and expressed as milligram gallic acid equivalents per gram fresh weight (mg GAE g^{-1} FW). Assays were carried out in triplicate extracts per sample. The experiment was repeated once. The analysis of variance (ANOVA) statistical test was applied to the data using SAS computer software.

5.3.3 Identification of phenolic acids

Leaf samples were collected at the end of the fourth week of the non acclimated and cold acclimation treatments. Dry, pulverized samples (500 mg) were suspended in 2 ml absolute methanol and incubated overnight at 4 °C in darkness. Samples were evaporated to dryness under reduced pressure, resuspended in 200 µl absolute methanol and filtered using a 0.22 µm GV Durapore centrifugal filter (Millipore, Bedford, MA). Phenolic acids were determined by HPLC (Dionex, Sunnyvale, CA) equipped with a P580 pump, connected to an ASI-100 automated sample injector and attached to a reverse phase C_{18} column (5- µm particle size, 25 cm x 4.6 mm). The system consisted of a PDA-100 photodiode array variable UV/VIS detector. Phenolic acids were analyzed with a mobile phase of 0.1 % trifluroacetic acid (TFA) in water (solution A) and absolute acetonitrile (solution B), detection at 280 nm and a flow rate of 1 ml min^{-1}. A multi step gradient with an initial injection volume of 15 µl was used as follows: 0 – 20 min: 20 – 70 % B, 20 – 40 min: 70 % B; 40 – 45 min: 70 – 20 % B. The phenolic acids were quantified from peak area calculations and retention times using the external standard method. All phenolic compounds used as standards were of analytical and HPLC grade.

5.3.4 ABTS* decolorisation assay for antioxidant capacity

The ABTS* assay was done according to Miller and Rice-Evans (1997). A 5 mM stock solution of ABTS was prepared by adding 54.8 mg to 20 ml H_2O. Approximately 1 g of the oxidizing agent, MnO_2 was added to the ABTS stock solution to generate the ABTS*. The mixture was stirred occasionally for 20 min at room temperature and excess MnO_2 was removed by first filtering through a Buchner funnel under vacuum, then with a 0.2 μM syringe-end filter. A 5 mM phosphate buffer saline (PBS) was prepared from a 25 mM stock solution, pH 7.4. The ABTS* solution incubated in a 30 °C water bath was diluted using 5 mM PBS to an absorbance of 0.7 (± 0.02) at 734 nm. Trolox standards of final concentration (0 - 20 μM) were prepared from a 0.5 mM stock solution. Precisely 1 ml of ABTS* solution was added to 100 μl of Trolox standards or samples, vortexed for 10 s and absorbance was read in a temperature controlled $SPECTRA_{max}$ $Plus^{384}$ spectrophotometer (Molecular Dynamics, Sunnyvale, CA) at 30 °C exactly 1 min after the start of the reaction. A PBS blank was run for each assay. Measurements were taken from three different dilutions per sample within the range of the dose-response curve. Each dilution was run three times. The activity of antioxidants was estimated as the mean value as the Trolox Equivalent Antioxidant Capacity (TEAC). The experiment was repeated once and ANOVA statistical test was applied to the data using SAS computer software.

5.3.5 Electrolyte leakage

Chilling injury was determined by measuring electrolyte leakage with a 100 well ASAC 1000 seed analyzer (Neogen, East Lansing, MI) one day at 25 °C following

chilling treatment. Leaf discs (5 mm in diameter) were immersed in individual cells of a leakage tray containing 2 ml of deionized water, previously tested to read 0 conductivity. After the conductivity of the exosmosed solution was measured (T_1), the leaf discs were frozen at –80 °C overnight and thawed to destroy compartmentalization so total potential leakage (T_2) could be determined. Relative % injury represents the mean leakage as a percentage of the mean total leakage from frozen-killed samples (T_1/T_2 x 100).

5.4 **Results**

5.4.1 Temporal changes in total phenolics during cold acclimation

Plants that were subjected to a constant 5 °C cold acclimation regime showed a significant increase ($p< 0.05$) in total phenolics by the third week of incubation while non acclimated plants and those that were gradually cold acclimated from 15 °C to 3 °C, showed very little variation in phenolics content over the 4 week period (Fig 5.1). Overall the highest concentration of total phenolics was observed after 3 weeks in 5 °C cold acclimated plants at 105 ± 8.4 mg GAE g^{-1} FW x 10^4. There were no significant differences in total phenolics between non acclimated plants and those that were gradually cold acclimated from 15 °C to 3 °C over the entire 4 week acclimation period ($p> 0.05$).

5.4.2 Identification of specific phenolic acids

The following phenolic acids were identified in petunia plants: Rosmarinic acid, ρ-coumaric acid, litospermic acid, o-coumaric acid and gentisic acid (Fig. 5.2). The concentration levels of these compounds ranged from 6.7 μg g^{-1} DW rosmarinic acid in

non acclimated plants to 1443.9 μg g^{-1} DW gentisic acid in 5 °C cold acclimated plants. All five phenolic acids were observed in plants that were subjected to the cold acclimation regimes but to a lesser extent in plants that were acclimated gradually from 15 °C – 3 °C. Gentisic acid was the only phenolic acid not detected in non acclimated plants.

5.4.3 Total antioxidant activity

Among the three treatments tested, plants cold acclimated at a constant 5 °C had the highest TEAC value at 3.8 μM g^{-1} FW (Fig. 5.3). By the third week of incubation, plants that were gradually cold acclimated from 15 °C - 3 °C had very little antioxidant capacity; significantly lower than that of non acclimated plants ($p < 0.05$).

5.4.4 Chilling tolerance

Chilling tolerance was estimated based on electrolyte leakage data. A low percentage of total leakage translates to chilling tolerance. Generally, electrolyte leakage in chilled plants was least in plants that were gradually cold acclimated from 15 °C - 3 °C and similar to that of non chilled control plants (Fig. 5.4). By the second week of incubation, plants cold acclimated at 5 °C showed similar electrolyte leakage to non acclimated chilled plants. In the following weeks, these plants induced some chilling tolerance as indicated by electrolyte leakage.

5.4.5 Relationship between total phenolics, total antioxidant capacity and chilling tolerance.

There was a positive linear ($R^2 = 0.90$) relationship between total phenolic content and the total antioxidant capacity of 5 °C cold acclimated plants ($p < 0.05$) (Fig. 5.5). The higher phenolic content resulted in a greater antioxidant capacity. However, there was no linear relationship between total phenolic content and total antioxidant capacity in plants that were gradually cold acclimated from 15 °C - 3 °C. The relationships of antioxidant capacity and chilling tolerance of plants were also determined (Fig. 5.6). The correlation coefficient, R^2, was 0.63 for plants that were cold acclimated at a constant 5 °C and 0.02 for plants that were gradually cold acclimated from 15 °C - 3 °C. These values indicate that the antioxidant capacity is moderately related to chilling tolerance in 5 °C cold acclimated plants but not related to chilling tolerance in plants that were gradually cold acclimated from 15 °C to 3 °C.

5.5 **Discussion**

As only a few of the entire spectra of compounds could be identified and quantified by HPLC, the spectrophotometric determination of total phenolics estimates the total amount of phenolics present in samples. Attention should be focused on gentisic acid, not because it was the most predominant phenolic acid but because it was induced upon cold acclimation. The levels of gentisic acid, (661 $\mu g\ g^{-1}$ DW detected in plants that were gradually cold acclimated from 15 °C to 3 °C and 1443.9 $\mu g\ g^{-1}$ DW detected in

plants that were cold acclimated at a constant 5 °C), are comparable to the levels detected in various medicinal species of the *Lamiaceae* family (Zgorka and Glowniak, 2001). As gentisic acid was induced upon cold acclimation, it may be possible, by manipulating its biosynthetic pathway, to trigger acclimation mechanisms in plants under cold stress.

The results showed that the antioxidant capacity was related to the content of phenolic compounds in these samples. Samples with higher content of total phenolics had higher TEAC values, while those with lower total phenolic levels had reduced TEAC values. Petunia plants that were cold acclimated at 5 °C for 4 weeks accumulated the most phenolics overall and had the highest levels of antioxidant capacity. However, unlike plants that were gradually cold acclimated, these plants did not exhibit signs of cold tolerance until the third week of incubation. After the first week at 5 °C, these plants began to show symptoms of chilling stress. Increased electrolyte leakage, parallel to that of non acclimated chilled plants often correlates with chilling injury. It is known that ROS induced by chilling stress, may trigger a series of deleterious processes, such as lipid peroxidation and degradation of proteins and nucleic acids in the cell (Fridovich, 1978) and that several secondary metabolites including phenolics may accumulate to mediate these stresses (Christie et al., 1994). Therefore the rapidity with which these plants were able to recover from the chilling stress indicates some kind of antioxidant protection. The relationship between antioxidant capacity and chilling tolerance in these plants supports the idea of some protection by radical scavenging. In normal circumstances, ROS concentrations are likely to remain low because of the activity of protective enzymes such as catalase, glutathione reductase and guaiacol peroxidsae (Prasad, 1996).

The induction of chilling tolerance in plants that were gradually cold acclimated in the absence of accumulated phenolics seems to support the idea of other endogenous metabolites assuming the role of a protective mechanism other than radical scavenging. In chapters 2 and 4, it was demonstrated that an increase in endogenous soluble carbohydrates in cold acclimated plants was associated with inducing cold tolerance. Apart from their role as hydroxyl scavengers, these compounds stabilize membranes by acting as osmoregulators (Yelonosky and Guy, 1989). As indicated by electrolyte leakage data, the plants that were gradually cold acclimated were not damaged, thus cold tolerance appeared to be a prophylactic rather than a repair mechanism. This indicates that petunia requires a gradual cold acclimation regime to induce chilling tolerance.

These results collectively provide correlative evidence to suggest that pre-exposure of the plants to a mild chilling stress induced antioxidant accumulation and eventually chilling tolerance. Among various phenolic acids, gentisic acid seems to be induced upon cold acclimation. Also in cold acclimated plants, chilling tolerance was not only due to an induced antioxidant defense system but other biochemical and physiological mechanisms should be contributing to tolerance in this system. These data also suggest that the 5 °C cold acclimation regime may have initially caused injury that impeded acclimation at the onset or that phenolic metabolism is probably a consequence of cold stress. Improvement in chilling tolerance upon accumulation of specific phenolic compounds suggests that chilling injury is reversible and can be ameliorated by an antioxidant defense system.

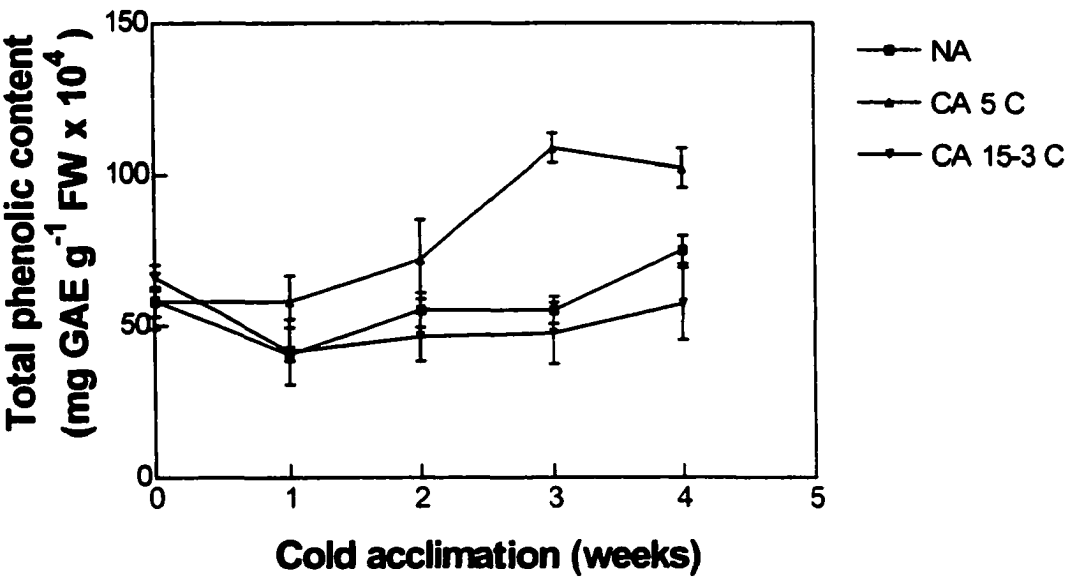


Fig. 5.1 Total phenolic content in petunia during cold acclimation. Samples were collected weekly from leaves at all stages of development from the entire plant under various conditions. NA: non acclimated; CA 5 C: a constant 5 °C cold acclimation regime; CA 15-3 C: gradual cold acclimation at 15 °C for 1 wk, 10 °C for 1 wk, 5 °C for 1 wk and 3 °C for 1 wk. Data represent the mean ± SD of 6 replicates and are expressed as milligrams of Gallic Acid Equivalent (GAE) g-1 FW x 104.

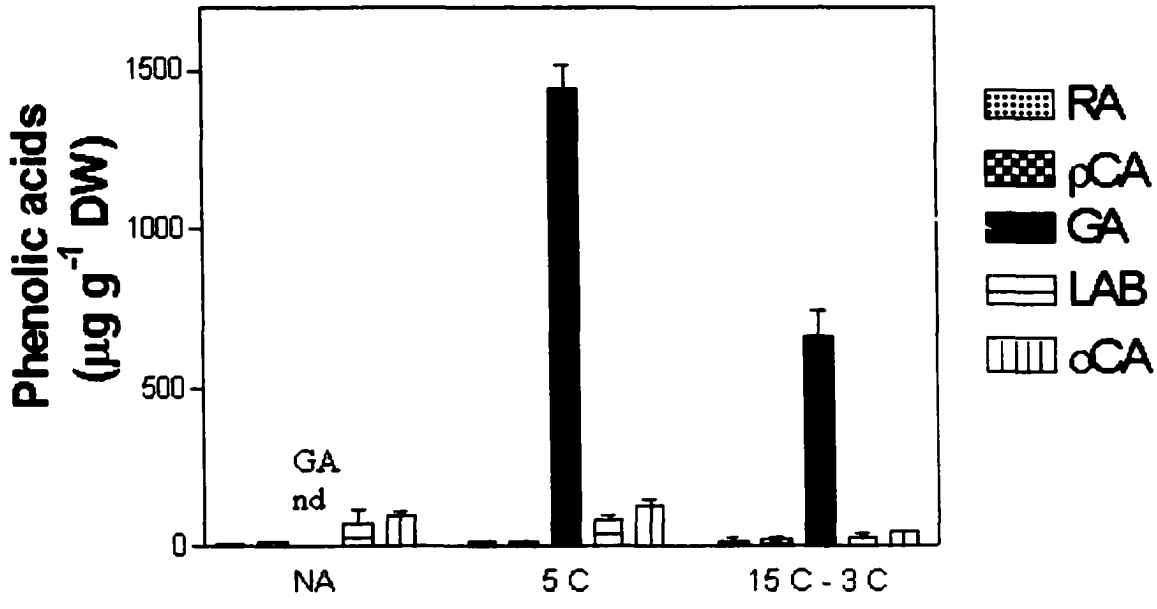


Fig. 5.2 Specific phenolic acids in petunia during cold acclimation. Samples were collected from leaves at all stages of development from the entire plantat the end of the 4 week cold acclimation regime. NA: non acclimated; 5 C: cold acclimated at 5 °C for 4 weeks; 15C - 3 C: gradual cold acclimation at 15 °C for 1 wk, 10 °C for 1 wk, 5 °C for 1 wk and 3 °C for 1 wk; nd: not detected. Ra: Rosmarinic acid, *p*CA: *p*-coumaric acid, GA: Gentisic acid, LAB: Lithospermic acid B, *o*CA: *o*-coumaric acid. Data represent the mean ± SD of 6 replicates.

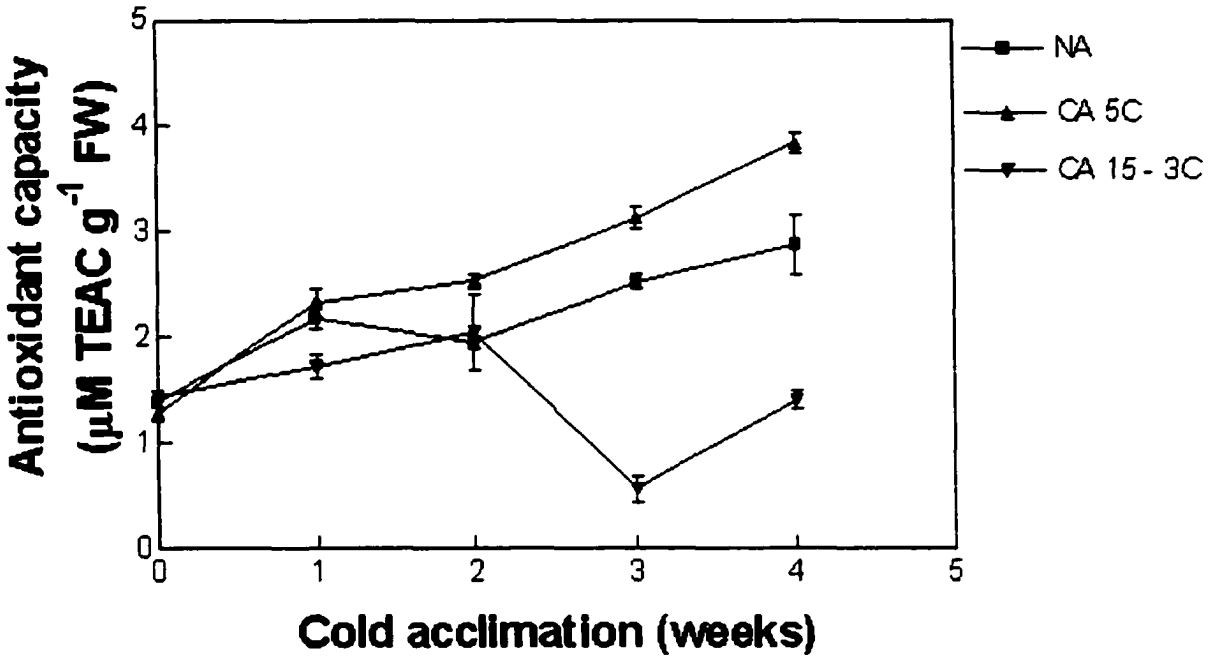


Fig. 5.3 Antioxidant capacity in petunia during cold acclimation. Leaf samples were collected weekly at all stages of development from the entire plant under various conditions. NA: non acclimated; CA 5 C: a constant 5 °C cold acclimation regime; CA 15-3 C: gradual cold acclimation at 15 °C for 1 wk, 10 °C for 1 wk, 5 °C for 1 wk and 3 °C for 1 wk. Data represent the mean ± SD of 12 replicates and are expressed as μM Trolox Equivalent Antioxidant Capacity (TEAC) g^{-1} FW.

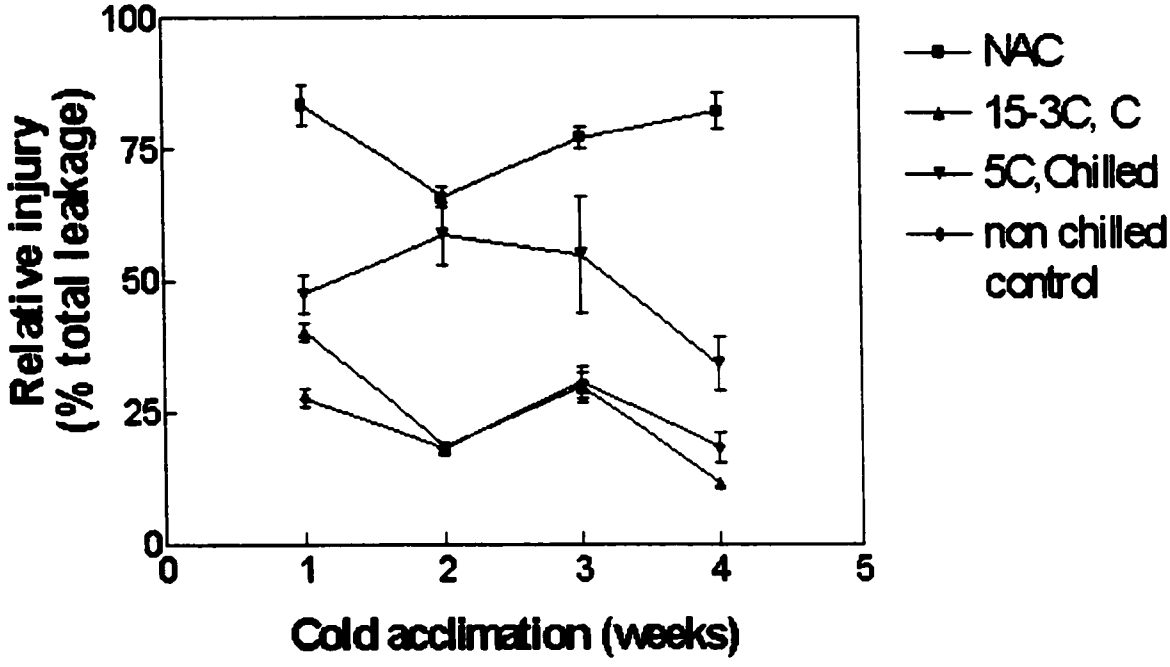


Fig. 5.4 Relative chilling injury as indicated by electrolyte leakage of petunia leaves following cold acclimation and chilling. Plants growing under various conditions were sampled weekly and exposed to the chilling treatment (2 °C for 2 wks) (except non chilled control). NAC: non acclimated plants chilled at 2 °C for 2 wks; 15-3C, C: gradual cold acclimation at 15 °C for 1 wk, 10 °C for 1 wk, 5 °C for 1 wk and 3 °C for 1 wk followed by chilling at 2 °C for 2 wks; 5C, chilled: a constant 5 °C cold acclimation regime followed by chilling at 2 °C for 2 wks; non chilled control: non acclimated plants not chilled. Samples for electrolyte leakage tests were incubated at 25 °C for 1 day following the chilling treatment. Data represent the mean ± SD of 6 replicates and are expressed as a % of the total leakage.

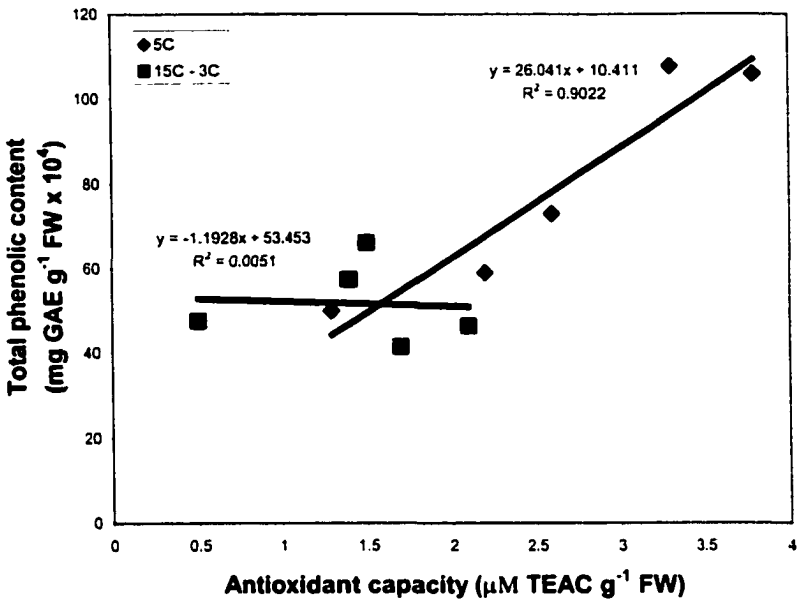


Fig. 5.5 Relationship between total phenolic content and antioxidant capacity in petunia plants subjected to two cold acclimation regimes. 5C: plants cold acclimated at a constant 5 °C for 4 weeks; 15 C – 3 C: gradual cold acclimation at 15 °C for 1 week, 10 °C for 1 week, 5 °C for 1 week and 3 °C for 1 week.

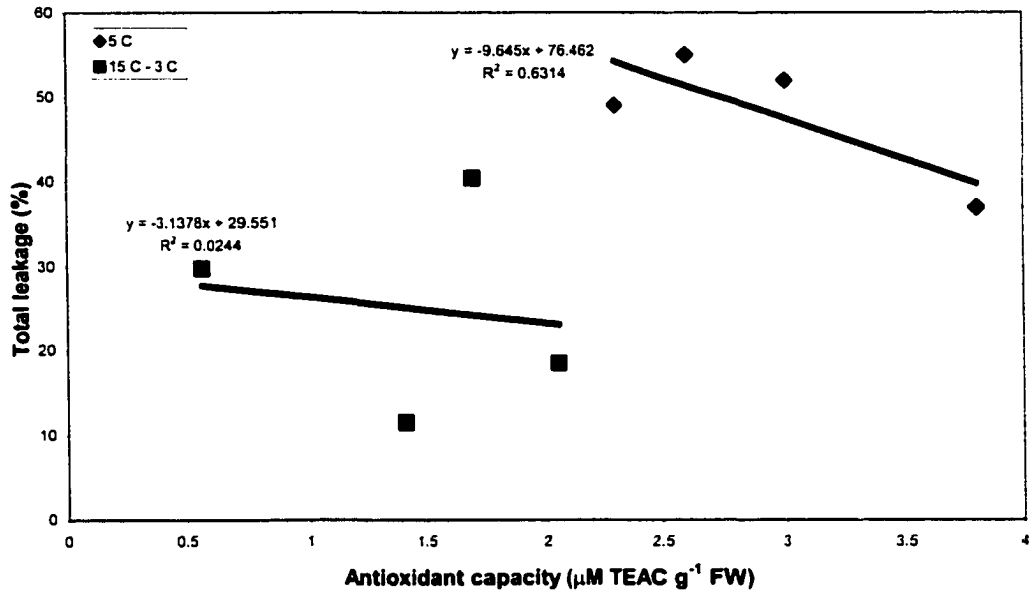


Fig. 5.6 Relationship between antioxidant capacity and chilling tolerance as indicated by total electrolyte leakage in petunia plants subjected to two cold acclimation regimes. 5C: plants cold acclimated at a constant 5 °C for 4 weeks; 15 C – 3 C: gradual cold acclimation at 15 °C for 1 week, 10 °C for 1 week, 5 °C for 1 week and 3 °C for 1 week.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389 - 3402

Anderson MD, Prasad TK, Stewart CR (1995). Changes in isozyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol* 109: 1247 -1257

Asada R (1992). Ascorbate peroxidase – a hydrogen peroxide scavenging enzyme in plants. *Plant Physiol* 85: 235 - 241

Asada K, Takahashi M (1987). Production and scavenging of active oxygen in photsynthsis. In: Photoinhibition. D. K. kyle, C. B. Osmond, C. J. Arntzen eds., Elsevier Science Publishhers, Armsterdam pp 227 - 284

Bachmann M, Matile P, Keller F (1994). Metabolism of raffinose oligosaccharides in leaves of *Ajuga reptans* L. *Plant Physiol* 105: 1335 - 1345

Bachmann M, Keller F (1995). Metabolism of the raffinose family oligosccaharides in Leaves of *Ajuga reptans* L. –inter and intracellular compartmentation. *Plant Physiol* 109: 991 - 998

Baker SS, Wilhelm KS, Thomashow MF (1994). The 5' region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought-, and ABA-regulated gene expression. *Plant Mol Biol* 24: 701 - 713

Beyer W, Imlay J, Fridovich I (1991). Superoxide dismutases. *Prog Nucl Acids Res* 40: 221 - 253

Bevan MW (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucl Acids Res*. 12: 8711 - 8721

Bevan MW, Flavel RB, Chilton MD (1983). A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 304: 184 -187

Bewley JD, Black M (1978). Physiology and biochemistry of seeds in relation to their germination, Vol. 1. Development germination and growth. Berlin, Springer-Verlag.

Bewley JD, Black M (1994). Seed. Physiology of development and germination. 2nd Edition, Plenum Press, New York, Pgs 126 - 128

Bhaumik G, Srivastava KK, Selvamurthy W, Purkayastha SS (1995). The role of free radicals in cold injuries. *Int J Biometeorol* 38: 171- 175

Bielski BHJ, Cabelli DE (1991). Highlights of current research involving superoxide and perhydroxyl radicals in aqueous solutions. *Int J Radiat Biol* 59: 291 - 319

Birch RG, Bower R (1994). Principles of gene transfer using particle bombardment. In: Particle Bombardment for Gene Transfer. N. S. Yang and P. Christou eds., Oxford University Press, NY, pp 3 - 37

Bishop DF, Calhoun DH, Bernstein HS, Hantzopoulos P, Quinn M, Desnick RJ (1986). Human α-galactosidase A: nucleotide sequence of a cDNA clone encoding the mature enzyme. *Proc Natl Acad Sci USA* 83: 4859 - 4863

Bishop DF, Kornreich R, Desnick RJ (1988). Structural organization of the human alpha-galactosidase A-gene- further evidence for the absence of a 3' unstranslated region. *Proc Natl Acad Sci USA* 85: 3903 - 3907

Bourque JE (1995). Antisense strategies for genetic manipulations in plants. *Plant Sci.* 105: 125 - 149

Boyer JS (1982). Plant productivity and environment. *Science* 218: 443 - 448

Brawn K, Fridovich I (1981). DNA strand scission by enzymatically-generated oxygen radicals. *Arch Biochem Biophys* 206: 414 - 419

Bredemeijer GMM, Burg HCJ, Claassen PAM, Stiekema WJ (1991). Phosphofructokinase in relation to sugar accumulation in cold-stored potato tubers. *J Plant Physiol* 138 (2): 129 - 135

Burke JJ (1990). High temperature stress and adaptations in crops. Wiley Publishing Inc., New York, NY

Castillo EM, de Lumen BO, Reyes PS, de Lumen HZ (1990). Raffinose synthase and galactinol synthase in developing seeds and leaves of legumes. *J Agric Food Chem* 38: 351 - 355

Castonguay Y, Nadeau P, Leschasseur P, Chouinard L (1995). Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winter hardiness. *Crop Science* 35: 509 - 516

Castonguay Y, Nadeau P (1998). Enzymatic control of soluble carbohydrate accumulation in cold-acclimated crowns of alfalfa. *Crop Sci* 38 (5): 1183 - 1189

Chen H, Shen, Z, Li, PH (1982). Adaptability of crop plants to high temperature stress. *Crop Sci* 22: 719 - 725

Chen B, Schellman JA (1989). Low-temperature unfolding of a mutant of phage T4 lysozyme. 1. Equilibrium studies. *Biochem* 28: 685 - 689

Chiang Y (2000). Overexpression of mannitol-1-phosphate dehydrogenase increases mannitol production and confers salt and chilling tolerance in transgenic petunia cv. Mitchell. PhD thesis, CSU

Christian JH (1955). The influence of nutrition on the water relations of *Salmonella oranienburg*. *Aust J Biol Sci* 8: 75 - 82

Christie PJ, Alfenito MR, Walbot V (1994). Impact of low temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* 194: 541 - 549

Chrost B, Krupinska K (2000). Genes with homologies to known α-galactosidases are expressed during senescence of barley leaves. *Physiologia Plant* 110: 111 - 119

Chrost B, Schmitz K (2000). Purification and characterization of multiple forms of α-galactosidase in *Cucumis melo* plants. *J Plant Physiol* 156: 483 - 491

Clark DG, Gubrium EK, Barrett JE, Nell TA, and Klee HL (1999). Root formation in ethylene-insensitive plants. *Plant Physiology* 121: 53 - 59

Corr, B (1998). Petunia. In: Ball Redbook. V. Ball, ed. Batavia, IL, USA pp 678 - 686

Crawford RMM, Huxter TJ (1977). Root growth and carbohydrate metabolism at low temperatures. *J Exp Bot* 28: 917 - 925.

Crowe JH, Crowe JF, Carpenter LM, Wistrom CA, (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem J* 242: 1- 10

Crowe JH, Crowe JF, Carpenter LM, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ (1988). Interaction of sugars with membranes. *Bioche et Biophy Acta* 947: 367 - 384

Davis MO, Hata DJ, Johnson SA, Walker JC, Smith DS (1996). Cloning, epression and characterization of a blood group B active recombinant α-galactosidase from soybean *(Glycine max)*. *Biochem Mol Biol International* 39: 471

Davis MO, Hata DJ, Johnson SA, Jones DE, Harmata MA, Evans ML, Walker JC, Smith DS (1997). Cloning, sequence and epression of a blood group B active recombinant α-galactosidase from pinto bean *(Phaseolus vulgaris)*. *Biochem Mol Biol International* 42 (3): 453 - 467

Denhardt DT (1966). A membrane-filter technique for the detection of complementary DNA. *Biochem Biophy Res Communications* 23: 641 - 646

den Herder IF, Rosell AM, Van Zuilen CM, Punt PJ, Van den Hondel CA (1992). Cloning and expression of a number of the *Aspergillus niger* gene family encoding α-galactosidase. *Mol Gen Genet* 233: 404 - 410

DeLong JM, Prange RK, Hodges DM, Forney CF, Bishop MC, Quilliam M (2002). Using a modified ferrous oxidation-xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. *J Agri Food Chem.* 50 (2): 248 - 354

Dey PM (1985). D-galactose-containing oligosaccharides. In: Biochemistry of Storage Carbohydrates in Green Plants. R. A Dixon ed. Academic Press Inc. Fl

Dey PM, Pridham JB (1972). Biochemistry of α-galactosidases. *Adv Enzymol* 36: 91 - 130

Dhindsa RS, Monroy AF (1994). Low temperature signal transduction, gene expression, and cold acclimation: multiple roles of low temperature. In: Molecular and Cellular Mechanisms of Stress Tolerance in Plants. J. H. Cherry ed., Springer-Verlag, Berlin, Germany

Ditta G, Stanfield S, Corbin D, Helinski D (1980). Broad-host range DNA cloning system for gram-negative bacteria: construction of a gene bank for *Rhizobium meliloti*. *Proc Natl Acad Sci USA*. 77: 7347 - 7351

Downton WJS, Hawker JS (1975). Evidence of lipid-enzyme interaction in starch synthesis in chilling-sensitive plants. *Phytochem* 14: 1259 - 1263

Elzen PJM, Tounsend J, Lee KY, Bedbrook Jr (1985). A chimeric hydromycin resistant gene as a selectable marker in plant cells. *Plant Mol Biol.* 5: 299 - 302

Fennel A, Li PH (1985). Rapid cold acclimation and deacclimation in winter spinach. *Acta Horti* 168: 179 - 183

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: (359) 1239 - 1249

Flinn CL, Ashworth EN (1995). The relationship between the carbohydrates and flower bud hardiness among three Forsythia taxa. *J Amer Soc of Hort Sci* 120: 607 - 613

Fowler S, Thomashow M (2002). Regulons and regulatory networks involved in plant freezing tolerance. (abstract no. 50). 39th meeting of the Society for Cryobiology, Breckenridge, CO

Foyer CH, Lelandais M, Edwards EA, Mullineaux PM (1991). The role of ascorbate in plants, interactions with photosynthesis, and regulatory significance. In: Active Oxygen/Oidative Stress and Plant Metabolism. E. Pell and K. Steffen eds., ASPP pp 131 - 144

French D (1954). The role of raffinose family of oligosaccharides. *Adv Carbohydrate Chem* 9: 149 - 184

Fridovich I (1978). The biology of oxygen radical. *Science* 201: 875 - 880

Fridovich I (1991). Molecular oxygen : Friend and foe. In: Active Oxygen/Oidative Stress and Plant Metabolism. E. Pell and K. Steffen eds., ASPP pp 1 - 5

Gallardo K, Job C, Groot SPC, Puype M, Demol H, Vanderkerckhove J, Job D (2002). Proteomics of Arabidopsis seed germination. A comparative study of wild-type and gibberellin-defecient seeds. *Plant Physiol* 129: 823 - 837

Gao Z, Schaffer AA (1999) A novel alkaline alpha-galactosidase from melon fruit with a substrate preference for raffinose. *Plant Physiol* 119: 979 - 987

Gaudreault PR, Webb JA (1983). Partial purification and properties of an alkaline α-galactosidase from mature leaves of *Cucurbita pepo*. *Plant Physiol* 71: 662 - 668

Gaudreault PR, Webb JA (1986). Alkaline α-galactosidase activity and galactose metabolism in the family cucurbitaceae. *Plant Sci* 45: 71 - 75

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol* 87: 745 - 750

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998). Low temperature regulation of *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Proc Natl Acad Sci USA* 82: 3673 - 3677

Guiseppin ML, Almkerk JW, Heistek JC, Verrips CT (1993) Comparative study on the production of guar α-galactosidase by *Saccharomyces cerevisiae* SU50B and *Hansenula polymorpha* 8/2 in continuos cultures. *Appl. Environ. Micrbiol.* 59: 52 - 59

Guimaraes VM, Tavares de Rezende S, Moreira MA, Goncalves de Barros E, Felix CR (2001). Characterization of α-galactosidases from germinating soybean seed and their use for hydrolysis of oligosaccharides. *Phytochemistry* 58: 67 - 73

Guo Y, Liang H, Berns MW (1995). Laser-mediated gene transfer in rice. *Physiol Plant.* 93: 19 - 24

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

Guy CL, Haskell D, Jierwiriyapant U, Lowery L (1988). Freezing tolerance in the genus *Petunia. HortScience* 23 (3): 66

Guy CL, Huber JLA, Huber SC (1992). Sucrose phosphate synthase and sucrose accumulation at low temperature. *Plant Physiol* 100: 502 - 508

Guy CL, Anderson JV, Haskell DW, Li Q-B (1994). Caps, cors, dehydrins and molecular chaperons: their relationship with low temperature responses in spinach. In Biochemical and Cellular Mechanisms of Stress Tolerance in Plants. Ed. JH Cherry, Springer-Verlag, Berlin. pp 479 - 499

Guy C, Haskell D, Li Q, Zhang C (1998). Molecular chaperones: Do they have a role in cold stress responses of plants. In: Plant Cold Hardiness: molecular biology, biochemistry and physiology. P.H. Li and T.H.H. Chen eds. Plenum Press, NY pp 109 - 129

Gynheung A (1987). Binary Ti vectors for plant transformation and promoter analysis. In: Methods in Eenzymology Vol 153. Academic Press, Inc., NY, pp 292 - 305

Habb CI, Keller F (2002). Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galatosyltransferase (GGT), from *Ajuga reptans* leaves. *Physiologia Plant* 114 (3): 361 - 371

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

Hendrix JE (1968). Labeling pattern of translocated stachyose in squash. *Plant Physiol* 43: 1631 - 1636

Herman EM, Shannon LM (1985). Accumulation and subcellular localization of α-galactisidase-hemagglutinin in developing soybean cotyledons. *Plant Physiol* 77: 886 - 890

Hinsley LE, Pharr DM, Snelling LK, Funderburk SR (1992). Foliar raffinose and sucrose in four conifer species: relationship to seasonal temperature. *J Amer Soc of Hort Sci* 17: 852 - 855

Holthaus U, Schmitz K (1991). Distribution and immunolocalization of stachyose synthase in *Cucumis melo* L. *Planta* 185: 479 - 486

Ilker R, Waring AJ, Lyons JM, Breidenbach RW (1976). The cytological responses of tomato-seedling cotyledons to chilling and the influence of membrane modifications upon these responses. *Protoplasma* 90: 229 - 252

Imanishi HT, Suzuki T, Masuda K, Harada T (1998). Accumulation of raffinose and stachyose in shoot apices of Lonicera caerulea L. during cold acclimation. *Scientia Horticulturae* 72: 255 - 263

Imanishi H, Kawaguchi T, Suzuki T, Masuda K, Harada T (1999). Accumulation of raffinose during cold acclimation in *Lonicera caerlea* L. plantlets cultured *in vitro*. *Cryoletters* 20: 235 - 242

Ishitani M, Xiong L, Lee H, Stevenson B, Zhu J-K (1998). HOSI, a genetic locus involved in cold responsive gene expression in Arabidopsis. *Plant Cell* 10: 1151 - 1161

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Scabenberger O, Thomashow MF (1998). *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* 280: 104 - 106

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 cosuppresion phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Mol Biol* 31: 957 - 973

Joseph NM, Timothy M, Holton A, Koes RE (1995). Floriculture: genetic engineering of commercial traits. *TIBECH* 13: 350 - 355

Kaledin AS, Siliusarenko AG, Gorodetskii SI (1981). Isolation and properties of DNA

polymerase from the extreme thermophilic bacterium *Thermus rubber*. *Biokhimiya* 46: 1576

Kandler O, Hopf H (1980). In *Biochemistry of plants*: A comprehensive treatise. Academic Press, New York

Kandler O, Hopf H (1982). Oligosaccharidres based on sucrose (sucrosyl oligosaccharides). In: *Encyclopedia of Plant Physiology* (new series), vol 13A, Eds A. Person and M. Zimmerman, Springer-Verlag, New York, pp 348 - 383

Keller F, Matile P (1985). The role of the vacuole in storage and mobilization of stachyose in tubers of *Stachys sieboldii*. *J Plant Physiol* 119: 369 - 380

Keller F, Pharr DM (1996). Metabolism of carbohydrates in sink and sources: galactosyl-sucrose oligosaccharides. In: E. Zamski, *Crops*. Marcel Dekker, New York, pp 157 - 183

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

Kerdinaimongkol K, Woodson WR (1999). Inhibition of catalase by antisense RNA increases susceptibility to oxidative stress and chilling injury in transgenic tomato plants. *J Am Soc Hortic Sci*. 124 (4): 300 - 336

Kerr PS, Pearlstein RW, Schweiger BJ, Becker-Manley MF, Pierce JW (1997). Nucleotide sequences of galactinol synthase from zucchini and soybean. United States Patent 5 648 210

Kessler JR (1999). Greenhouse production of petunia. *Southern Floriculture*, Auburn University pp 15 - 19.

Knight H, Trewavas AJ, Knight MR (1996). Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* 8: 489 - 503

Kroon PA, Williamson G (1999). Hydroxycinnamates in plants and food: current and future perspectives, review. *J Sci Food Agric* 79: 355 - 361

Lambe P, Dinant M, Matagne RF (1995). Differential long-term expression and methylation of the hydromycin phosphotransferase (hph) and β-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum*) callus. *Plant Sci.* 108: 51 - 62

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680 - 685

Lawrence K, Bhalla P, Misra PC (1995). NADH-dependent redox activities on the

external face of plasma membrane vesicles of chickpea roots. *J Plant Physiol* 146: 763 - 765

Lee H, Xiong L, Ishitani M, Stevenson B, Zhu J-K (1999). Cold regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. *Plant Journal* 17: 301 - 308

Lee H, Xiong L, Ishitani M, Stevenson B, Zhu J-K (2000). (abstract no. 107). 11th International Conference on Arabidospis Research, p.54

Levitt J (1980). Responses of Plants to Environmental Stresses: Vol 1. Chilling, Freezing and High Temperature Stresses. New York: Academic 2nd ed.

Li G, Knowles PF, Murphy DG, Marsh D (1990). Lipid-protein interactions in thylakoid membranes of chilling-resistant and –sensitive plants studied by spin label electron spin resonance spectroscopy. *J Biol Chem* 265: 16867 - 16872

Liljestrom PL (1985). The nucleotide sequence of the yeast *MEL*1 gene. *Nuclei Acids Res* 13: 7257 - 7268

Liljestrom PL, Liljestrom P (1987). Nucleotide sequence of the *melA* gene, coding for α-galactosidase in *Escherichia coli* K-12. *Nucleic Acids Res* 15: 2213 - 2220

Lowry H, Rosebrough N, Fan A, Randall R (1951). Protein measurement with the Folin phenol reagent. *J. Biol Chem* 193: 265 - 275.

Lynch DV, Steponkus PL (1987). Plasma membrane alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). *Plant Physiol* 83: 761 - 767

Lyons JM (1973). Chilling injury in plants. *Ann Rev Plant Physiol* 24: 445 - 466

Lyons JM, Raison JK (1970). Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. *Plant Physiol* 45: 386 - 389

Macheix JJ, Sapis JC, Fleuriet A (1991). Phenolic compounds and polyphenoloxidase in relation to browning in grapes and wines. *Critical Reviews in Food Science and Nutrition.* 30 (4): 441 - 486

Matzke MA, Matzke AJM (1995). How and why do plants inactivate homologous (trans) genes? *Plant Physiol* 107: 679 - 685

McKersie B, Chen YR, de Beus M, Bowley SR, Bowler C, Inze D, D'Halluin K, Botterman J (1993) Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medigo sativa* L). *Plant Physiol* 103 (4): 1155 - 1163

McKersie BD (1991). The role of oxygen free radicals in mediating freezing and

desiccation stress in plants. In: Active Oxygen/Oxidative Stress and Plant Metabolism. E. Pell and K. Steffen eds., ASPP pp 107 - 118

McKersie BD, Bowley SR (1998). Active oxygen and freezing tolerance in transgenic plants. In: Plant Cold Hardiness: molecular biology, biochemistry and physiology. P.H. Li and T.H.H. Chen eds. Plenum press, NY pp 203 - 214

McKown R, Kuroki G, Warren G (1996). Cold responses of *Arabidopsis* mutants impaired in freezing tolerance. *J Exp Bot* 47: 1919 - 1925

McIlvaine TC (1921). A buffer solution for colorimetric comparison. *J Biol Chem* 49: 183 - 186

Miller RW, Roche IDL, Pomery MK (1974). Structural and functional responses of wheat mitochondria membranes to growth at low temperatures. *Plant Physiol* 53: 426 - 433

Miller AJ, Kay SA (1991). Circadian control of CAB gene-transcription and messenger-RNA accumulation in Arabidopsis. *Plant Cell.* 3 (5): 541 - 550

Miller NJ, Rice-Evans CA (1997). Factors influencing the antioxidant activity determined by the ABTS* radical cation assay. *Free Radical Research* 26: 195 - 199

Miquel M, James D, Dooner H, Browse J (1993). *Arabidopsis* requires polyunsaturated lipids for low temperature survival. *Proc Natl Acad Sci* 90: 6208 - 6212

Monroy AF, Sangwan V, Dhindsa RS (1998). Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for cold-inactivation. *Plant J.* 13: 653 - 660

Moore DH, Brightman AO, Hidalgo A, Nava P (1995). Selective inhibition of auxin-stimulated NADH oxidase activity and elongation growth of soybean hypocotyls by thiol reagents. *Plant Physiol* 107: 1285 - 1291

Morris LL (1982). Chilling injury of horticultural crops: An overview. *Hortscience* 17 (2): 161- 172

Mozo T, Hooykaas JJ (1992). Design of a novel system for the construction of vectors for Agrobacterium-mediated plant transformation. *Mol Gen Genet* 236: 1 - 7

Mullis KB (1990). The unusual origin of polymerase chain reaction. *Scientific American* 36 - 43

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8: 4321 - 4325

Nelson DE, Koukoumanos M, Bohnert HJ (1999). Myo-inositol-dependent sodium uptake in ice plant. *Plant Physiol* 119: 165 - 172

Obendorf RL (1997). Oligosaccharides and galatosyl cyclitols in seed desiccation tolerance. *Seed Science Research* 7: 63 - 74

Otter T, Polle A (1994). The influence of apoplastic ascorbate on the activities of cell-associated peroxidase and NADH oxidase in needles of Norway spruce (*Picea abies* L.). *Plant Cell Physiol* 35: 1231 - 1238

Overbeeke N, Fellinger AJ, Toonen MY, van Wassenaar D, Verrips CT (1989). Cloning and nucleotide sequence of the α-galactosidase cDNA from *Cyamopsis tetragonoloba* (guar). *Plant Mol Biol* 13: 541 - 550

Pelletier G, Ferault M (1976). Relationship between androgenesis and somatic embryogenesis *in vitro* in *Nicotania* and *Petunia* genus. *CR Acad Sci DNAT* 285 (15): 1633 - 1976

Pennycooke JC, Towill LE (2001). Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* {L} Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. *CryoLetters* 22: 381 - 389

Pharr DM, Huber SC, Sox HN (1985). Leaf carbohydrate status and enzymes of translocate synthesis in fruiting and vegetable plants of *Cucumis sativus* L. *Plant Physiol.* 7: 104 - 108

Pharr DM, Hubbard NL (1994). Melons: Biochemical and physiological control of sugar accumulation. In Encyclopedia of Agricultural Science, Vol 3. Academic Press, New York, pp 25 - 37

Powles SB (1984). Photoinhibition of photosynthesis induced by visible light. *Ann Rev Plant Physiol.* 35: 15 - 44

Prasad TK (1996). Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids and protease activities. *The Plant Journal* 10 (6): 1017 - 1026

Pravalov PL (1990). Cold denaturation of protein. *Crit Rev Biochem Mol Biol* 25: 281 - 305

Purvis AC, Yelonosky G (1983). Translocation of carbohydrates and proline in young grapefruit trees at low temperatures. *Plant Physiol.* 73: 877 - 880

Rice-Evans CA, Miller NJ, Paganga G (1997). Antioxidant properties of phenolic compounds. *Trends Plant Sci* 2 (4): 152 -159

Salerno GL, Pontis HG (1989). Raffinose synthesis in *Chlorella vulgaris* cultures after a

cold shock. *Plant Physiol* 89: 648 - 651

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning – a laboratory manual, 2nd ed, Cold Spring Harbor Laboratory Press, New York

Santarius 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.

Saradhi A, Saradhi PP (1991). Proline accumulation under heavy metal stress. *J Plant Physiol* 138: 554 - 558

Sauter JJ, van Cleve B (1991) Poplar storage of protein in xylem sap. *J Plant Physiol* 139: 19 - 26

Sawahel W, kiichi F (1995). Gene cloning in plants: innovative approaches. *Bio Tech* 19: 106 - 114

Sellapan S, Akoh CC, Krewer G (2002). Phenolic compounds and antioxidant capacity of Georgia grown blueberries and blackberries. *J Agric Food Chem* 50: 2432 - 2438

Seufferheld MJ, Stushnoff C, Forsline PL, Terraas Gonzalez GH (1999). Cryopreservation of cold-tender apple, *Malus domestica* Borkh, germplasm. *J. Amer. Soc Hort. Sci* 124: 612 - 618

Sink, KC (1984). In: Petunia. Springer-Verlag, Berlin, p 256.

Smirnoff N, Cumbes QJ (1989). Hydroxyl radical scavenging activity of compatible solutes. *Phytochem* 28: 1057 - 1060

Smart EL, Pharr DM (1980) Characterization of alpha-galactosidase from cucumber leaves. *Plant Physiol* 66: 731 - 734

Sommerville C, Browse J (1991). Plant lipids: metabolism mutants and membranes. *Science* 252: 80 - 87

Sozzi GO, Camperi SA, Cascone O, Fraschina AA (1998). Galactosidases in tomato fruit ontogeny: decreased galactosidase activities in ACC synthase fruit during ripening and reversal with endogenous ethylene. *Aust J Plant Physiol.* 25: 237 - 244

Spanos GA, Wrolstad RE (1990). Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. *J of Agric and Food Chem* 38: 1565 - 1571

Steponkus PL (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Annu Rev Plant Physiol.* 35: 543 - 584

Stergios BG, Howell GS (1973). Evaluation of viability test for cold stressed plants. *Hortsci* 98: 325 - 330.

Stushnoff C, Remmele RL, Essensee V, McNiel 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: Plant Cold Hardiness: molecular biology, biochemistry and physiology. P.H. Li and T.H.H. Chen eds. Plenum press, NY pp301 - 309

Terzaghi WB, Fork DC, Berry JA, Field CB (1989). Low and high temperature limits to PSII. A survey using trans-parinaric acid, delayed light emission, and Fo chlorophyll fluorescence. *Plant Physiol* 91: 1494 - 1500.

Thomas H, James AR (1993). Freezing tolerance and solute changes in contrasting genotypes of *Lolium perenne* L. acclimated to cold and drought. *Annals of Botany* 72: 249 - 254

Thomas B, Webb JA (1978). Distribution of α-galactosidase in *Cucurbita pepo*. *Plant Physiol* 62: 713 - 717

Thomashow MF (2001). So what's new in the field of cold acclimation? Lots! *Plant Physiol* 125: 89 - 93

Uedan K, Sugiyama T (1976) Purification and characterization of a phosphenolpyruvate carboxylase from maize leaves. *Plant Physiol* 57: 906 – 910

Uemura M, Steponkus PL (1994). A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. *Plant Physiol* 104: 479 - 496

van der Krol AR, Lenting PE, Veenstra J, van der Meer IM, Koes RE, Gerats AGM, Mol JNM, Stuitje AR (1988). Antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature* 333: 866 - 869

Vijayan P, Browse J (2002). Photoinhibition in mutants of Arabidopsis deficient in thylakoid unsaturation. *Plant Physiol* 129 (2): 876 - 885

Velioglu YS, Mazza G, Gao L, Oomah BD (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J Agric Food Chem* 46: 4113 - 4117

Wang CY (1982). Physiological and biochemical responses of plants to chilling stress. *Hortsci* 17 (2): 173 – 186.

Wang CY, Baker JE (1979). Effects of two free radical scavengers and intermittent warming on chilling injury and polar lipid composition of cucumber and sweet pepper fruits. *Plant and Cell Physiol* 20: 243 - 251

Wang HL, Lee PD, Liu LF, Su JC (1999). Effect of sorbitol induced osmotic stress on the changes of carbohydrate and free amino acid pools in sweet potato cell suspension cultures. *Botanical Bulletin of Academia Sinica* 40: 219 - 225

Warren G, McKown R, Teutonico R, Kuroki G, Veale E, Sagen K (1998). *Arabidopsis* mutants impaired in freezing tolerance after cold acclimation. In: Plant Cold Hardiness: molecular biology, biochemistry and physiology. P.H. Li and T.H.H. Chen eds. Plenum Press, NY, pp 45 - 56

Webb JA, Gorham PR (1965). Radical movement of ^{14}C translocates from squash phloem. *Can J Bot* 43: 97 - 103

Weddle, LC (1976). Petunia. In: Bedding Plants. JW Mastalerz, ed. Pennsylvania Flower Growers pp 252 - 271.

Weiser CJ (1970). Cold resistance and injury in woody plants. *Science* 169: 1269 - 1278

Wiemken V, Ineichen K (1993) Effect of temperature and photoperiod on the raffinose content of spruce roots. *Planta* 190 (3): 387 - 392

Xin Z, Browse JA, (1998). Constitutive freezing tolerant mutants in Arabidoposis: A genetic approach to signaling transduction in cold acclimation. In: Plant Cold Hardiness: molecular biology, biochemistry and physiology. P.H. Li and T.H.H. Chen eds. Plenum Press, NY pp 35 - 44

Yelenosky G, Guy CL (1977). Carbohydrate accumulation in leaves and stems of 'valencia' orange at progressively colder temperatures. *Bot Gaz* 138: 13- 17

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

Zambryski P (1988). Basic processes underlying *Agrobacterium* mediated DNA transfer to plant cells. *Annu Rev Genet* 22: 1 - 30

Zgorka G, Glowniak K (2001). Variation of free phenolic acids in medicinal plants belonging to the *Lamiaceae* family. *J Pharmaceutical and Biomedical Anaysis* 26: 79 87

Zhou M-Y, Clark SE, Gomez-Sanchez CE (1995). Universal cloning method by TA strategy. *Bio Tech* 19: 34 - 35

Zhu A, Goldstein J (1994). Cloning and functional expression of DNA encoding coffee bean α-galactosidase. *Gene* 140: 227 – 231

Zhu A, Wang ZK, Goldstein J (1995). Indentification of Tyrosine-108 in coffee bean alpha-galactosidase as an essential residue for the enzyme activity. *Biochim Biophys Acta* 1247: 260 - 264

Zupan JR, Zambryski P (1995). Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol* 107: 1041 – 1047

Zurawicz E, Stushnoff C (1977). Influence of nutrition on cold tolerance of 'Redcoat' strawberries. *J Amer Soc Hort Sci* 102 (3): 342 - 346