DISSERTATION

OVEREXPRESSION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE INCREASES MANNITOL PRODUCTION AND CONFERS SALT AND CHILLING TOLERANCE IN TRANSGENIC PETUNIA *CV. MITCHELL*

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERFISION BY YU-JEN CHIANG ENTITLED OVEREXPRESSION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE INCREASES MANNITOL PRODUCTION AND CONFERS SALT AND CHILLING TOLERANCE IN TRANSGENIC PETUNIA *CV. MITCHELL*

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ABSTRACT OF DISSERTATION

OVEREXPRESSION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE INCREASES MANNITOL PRODUCTION AND CONFERS SALT AND CHILLING TOLERANCE IN TRANSGENIC PETUNIA *CV. MITCHELL*

Diploid petunia plants (*Petunia hybrid, cv. Mitchell*) transformed with a bacterial gene (*mtl D*) encoding mannitol-1-phosphate dehydrogenase enzyme (MTL D), resulting in high mannitol expression, were developed and studied in this research. Transgenic lines and wild type control plants were exposed to salinity and chilling stress with the express goal of delineating the impact of manniol on these abiotic stresses. Phenotypically, there was no difference in growth between in vivo wild type and transgenic lines under non-stress conditions. However, transgenic lines expressing high mannitol levels were found to exhibit a greater capacity to tolerate salinity and chilling stresses compared to wild type and transgenic lines expressing low mannitol levels. Enhanced salinity tolerance was observed in seed germination (T2 generation), and in vegetative growth and floral development of transgenic lines expressing high mannitol levels. Also, based upon foliage symptoms and membrane leakage, transgenic lines expressing high mannitol levels were more tolerant of chilling stress compared to with wild type and transgenic lines expressing low mannitol levels. Carbohydrate analysis of wild type and transgenic plants showed that mannitol was the single carbohydrate most affected by plant lines. Two transgenic lines (M3 and M8) always had higher mannitol

expression levels when compared to wild type and the other two transgenic lines (M2 and M9) under non-stress and stress conditions. Therefore, the function of high mannitol expression should be considered in developing improved tolerance of petunia plants to salinity and chilling stresses. However, assuming 90 % water content in leaf tissue, leaf osmotic potential of high mannitol expressing lines contributed by mannitol accounted for only 0.006 %-0.01 % of increased osmoregulation caused by salinity stress, and 0.04 %-0.06 % of the increase in osmotic potential caused by chilling stress, respectively. Quantitatively, mannitol appears not to play a role as an osmoregulator in osmotic adjustment in response to both salinity and chilling stresses. Rather, the data from this research suggest that mannitol may function as an important osmoprotectant in enhancing salt and chilling tolerance of those transgenic petunia lines expressing high mannitol levels.

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Literature Review

1.1 Introduction of petunia

Petunia is the one of the leading annual plants in American gardens. The first recorded petunia was *P. nyctaginiflora*, a white, sweet-scented, night-blooming flower, which was discovered in Brazil in 1823 (Coats, 1968). Taxonomically, petunia belongs to the genus *Petunia* which is a member of the family *Solanaceae* (Karunanandaa et al., 1994).

Modern cultivated petunia is presumed to have originated from a result of chance hybridization between two variable species *Petunia axillaris* and *Petunia violacea* (Weddle, 1976). In the 1920's and 1930's , breeders discovered the efficacy of inbreeding and individual selection, and separate color-type varieties began to appear, first in the small flower (hybrida) and later in the grandiflora (diploid) type (Weddle, 1976). For the modern cultivated petunia, there are five main type : (1) grandiflora; (2) multiflora singles (small flowers); (3) grandiflora double; (4) multiflora double; and (5) Superbissima or Califonia Giant (Weddle, 1976). Cytoplasmic male sterility has been found in *Petunia hybrida* (Perl et al., 1992). Gametophytic self-incompatibility also was found in *Petunia inflata* (Kao et al., 1994).

The petunia is a half-hardy perennial (Weddle, 1976). It likes full sun and warm

day temperatures (Rosen, 1992). It can thrive under hot weather and dry conditions (Weddle, 1976). In contrast, extremely humid and poorly drained soil can cause petunia to suffer several diseases (Rosen, 1992). Most appropriate growth conditions for petunia include full sunlight, 75 ^{0}F, humidity 75-85 % and soil pH 6.5 (Carpenter and Carlson, 1974). In addition, petunias can not initiate and develop flower buds below 56 ^{0}F (Weddle, 1976).

Commercially, routine transformation procedures are available for the petunias, and selecting desirable qualities such as flower color, plant habit, vigor and stand ability have been improved by introducing foreign genes and cross-fertilization of different transgenic lines (Joseph et al., 1995).

1.2 *Agrobacterium*-mediated gene transfer and expression of introduced genes

Agrobacterium tumefaciens is a gram negative soil-borne bacterium that causes crown gall diseases in dicotyledonous plants (Zupan and Zambryski., 1995). The first study showing pathogenic infection of *Agrobacterium tumefaciens* to dicotyledonous plants was published by Smith and Townsend (1907) early in the 20th century. Since then many scientists throughout this world focused their research on this organism to learn how it can cause tumor formation in the plants. However, the finding of the virulent strains of *Agrobacterium tumefaciens* having a large extrachromosomal element involved in crown gall induction was not discovered until 1977 by Chilton et al.

There are many different strains of *Agrobacterium tumefaciens* that can cause crown galls on plant tissues. A common feature is the Ti plasmid (tumor inducing

plasmid). Ti plasmids are large, containing 100-200 kb of DNA as a circular, extrachromosomal genetic element (Zaenen et al.,1994). The sequences of different Ti plasmids are not homologous (Hooykaas and Schilperoort,1994). However, there are two regions that are similar in sequence of the different Ti plasmids. One is known as *vir* (virulent) region and another one is T-DNA (transfer DNA) region (Gelvin,1993). The T-DNA is delimited by 25-bp direct repeats that flank the T-DNA (Zupan and Zambryski, 1995). Any DNA segment between these borders will be transferred to plant cells. A set of genes in the T-DNA segment involving phytohormones (auxin and cytokinin) and opine biosynthesis now is known as a main cause for inducing tumor formation and abnormal development in the transformed plant cells (Anzai et al., 1989; Bakkeren et al., 1989). However, T-DNA can not encode the products that mediate or induce its own transfer. The processing and transfer of T-DNA are mediated by the products encoded by the *vir* genes (resides on the Ti plasmid) and *chv* (chromosomal) genes (Hooykeas and Schilperoort, 1992; Stache and Nester, 1986). By introducing T-DNA into plant cells, the *chv*A and *chv*B genes are needed for the attachment of Agrobacterium to plant cell walls (Hooykeas and Schilperoot, 1992). While the known *chv* genes are constitutively expressed, the *vir* genes are silent until they are induced by certain phenolic compounds released from wounding plant tissues (Stachel and Nester, 1986). Control of *vir* gene expression is mediated by *Vir*A and *Vir*G proteins, a two component regulatory system (Winans, 1992). The product of *vir* A gene can detect the phenolic compounds released from wounded plant tissues resulting in autophosphorylation (Zupan and Zambryski, 1995). Then, the phosphorylated vir A protein can transfer its phosphate to the *Vir* G

protein (Jiu et al., 1990). It is believed that phosphorylated *Vir* G protein can bind the promoter region of virulence genes and activate the *vir* gene transcription (Tamamoto et al., 1990).

Following *vir* gene activation, the production of a transfer intermediate begins with the generation of the T-strand, as ss copy of the T-DNA (Stachel et al., 1986). The T-DNA generation and transfer process is mediated by a number of genes in the *vir* region (Stachel et al., 1986). T-DNA of Ti plasmid can enter into *E. coli* via conjugation and integration via homologous recombination in *E. coli* (Hooykaas and Schilperoort, 1992). However, how the T-DNA enter into plant cells and integrate into plant chromosome are still unknown. It is suggested that T-DNA can enter into plant cells by conjugal transfer and integrate into plant genome by illegitimate recombination (Gheysen et al., 1991; Zupan and Zambryski, 1995).

Agrobacterium plasmids have been exploited as a vector to deliver foreign DNA into plants; this is the most widespread transformation system in use now (Glick and Thompsom., 1993). Two types of transformation vectors have been developed for *Agrobacterium*-mediated gene transfer. The vectors that integrate into Ti plasmid for maintenance in *Agrobacteria* are known as cointegrating vectors whereas the vectors that are capable of autonomous replication in trans to the Ti plasmid are known as "binary vectors" (Nehra., 1991).

The cointegrating vectors are based on disarmed Ti plasmid containing no phytohormone genes but retaining intact border sequences and *a vir* region (Nehra, 1991). In addition, the construction of cointegrating vectors also needs a homologous sequence

between the intermediate vector plasmid and the disarmed Ti plasmid to allow the occurrence of recombination (Nehra, 1991).

Binary vectors are constructed from two plasmids, one plasmid contains the T-DNA border sequences, a selectable marker expressible in plants, and a wide host range replicon that replicates in both *A.tumefaciens* and *E. coli* (Ditta et al., 1980; Bevan, 1984). The second plasmid containing the vir region but having no T-DNA is known as a helper plasmid (Gynheung, 1987). Foreign DNA can be inserted into a binary system using *E. coli* as a host and the recombinant molecule can be transferred to A. tumefaciens carrying a helper Ti plasmid by triparental matings(Gynheung, 1987).

The selection marker gene is an important component of the constructed vectors. The expression of selection markers is applied to distinguish the transformed plant cells and non-transformed ones. In general, selection markers are based on the sensitivity of plant cells to antibiotics and herbicides because expression of (bacteria) genes coding for enzymes which detoxify such compounds in plant cells can prevent transformed plant cells from death (Hooykaas and Schilperoort, 1992).

The most extensively used selection marker genes for plant transformation is neomycin phosphotransferase (NPT II) gene derived from the bacterial transposon Tn5 (Bevan et al., 1983). This NPT II enzyme is known to inactivate kanamycin antibiotics. In addition, some other selection markers such as hygromycin phosphotransferas (HPT) gene from *E. coli*, dihydrofolate reductase (DHFR) from mouse and bar gene streptomyces hygroscope which are known respectively to resist hygromycin, methtrexate and bialaphos herbicide also have been applied in some plant transformation events (Elzen

et al., 1985; Eichholtzz et al., 1987). Furthermore, another group of marker genes known as reporter genes is used as selection markers in common. The most widely used reporter gene in plant transformation is the B-glucuronidase (GUS) gene (Jefferson, 1987). The expression of GUS gene can be measured by fluorometric and histochemical assays in transformed plant tissues (Jefferson et al., 1987; Hooykaas and Schilperoort, 1992).

The *Agrobacterium*-mediated transformation method has now become a routine method for transformation of dicot plant species. Many plant tissues such as leaves, meristems, embryos, stem pieces, root explants and cotyledons can be the starting sources for transformation (Glick and Thompson, 1993). Even callus or protoplasts also can be the starting sources. However, the protoplast cocultivation method is limited in its application because it is time-consuming for plant regeneration and availability of protoplast regeneration system has been developed in only few plant species (Nehra, 1991). In 1985, Horsch et al. developed a leaf disk transformation system which combines foreign gene transfer, selection of transformed cells, and plant regeneration in a single process. Recently, this transformation system has become a routine method for transformation of dicotyledonous plant species with slight modifications in tissue culture of different explant tissues and selection schemes (Gaser and Fraley, 1989).

In general, wounded tissues of certain species, notably amongst the Solanaceae, are able to produce sufficient quantities of signal molecules to induce effective transformation (Holford et al., 1992). With less amenable species, it is necessary to consider both the bacteria strain and the cocultivation conditions for inducing the formation of *Agrobacterium*-competent cells (Holford et al., 1992). *Agrobacterium*-

competent cells, infers that there are cells within explants that possess particular metabolic states which enable them to respond to certain physiological signals and to become highly susceptible to transformation by *Agrobacterium tumefaciens* (Sangwan et al., 1992). Without phytohormone or preculture treatment, the wound site of tissues can become attached to *Agrobacteria* but appear not to be transformed because phytohormone can induce wounded cells to become dedifferentiating cells (Sangwan et al., 1992). The dedifferentiating may allow some unknown sort of wound-induced reaction to occur because non-dedifferentiated infected cotyledon epidermal layer cells of some species such as flax and soybean generate non-transformed shoots (Basiram et al., 1987; Hinchee et al., 1988; Sangwan et al., 1992).

It is known that the *vir* genes of *Agrobacterium* can be induced by some phenolic compounds. In fact, the addition of phenolic compounds such as acetosyringone to the culture medium has been proven to induce the activity of *vir* genes and enhance the transformation percentage in several plant species (Holford et al., 1992). Several studies also have shown that the optimal induction of vir genes is obtained when the pH is lower than 5.8-6.0(Alt-Moerbe et al., 1988; Holford et al., 1992). Thus, both the low appropriate pH and the presence of phenolic compounds can enhance the frequency of transformation. In addition, bacteria cell density, antibiotics, bacteria strains and starting sources need to be considered for producing successful transformation.

The incidence of chimeric regenerated plants via *Agrobacterium*-mediated transformation also has been reported (Dong and McHughen, 1993). For this problem, Mathew et al. (1995) demonstrated that gradually increasing antibiotic (kanamycin)

concentrations can decrease the chance to get chimeric strawberry regenerants.

The understanding of how transgenic plants incorporate foreign genes and how the foreign gene is expressed in transformant progeny is important for genetic engineering of crop plant species. In general, foreign genes introduced into a plant genome can retain their expression (Fraley et al., 1986). However, the level of expression varies widely among transformants (Fraley et al., 1986 ; Deroles and Gardner, 1988ab).

Several possible explanations for the variability of foreign gene expression such as rearrangement of T-DNA in the genome, co-suppression, position effect, chimeric transformants and T-DNA deletion during recombination have been suggested (Fraley et al., 1986; Matzke et al., 1989; Dong and McHughen, 1993; Schmulling and Schell, 1993). For the evaluation of transgenic plants, the rooting of selected regenerated shoots in the presence of an antibiotic used in transformant cell selection is the first criteria for selecting possible transgenic plants (Horsch et al., 1985). Recallusing assay of leaf explants taken from selected putative transgenic plants is also an useful method to test the expression of a foreign gene introduced into plants (McHughen and Jordan, 1989). Southern and/or Northern blot hybridization and inheritance of progeny can be applied as a final means to verify the stable expression of introduced foreign gene in the transgenic plants (Deroles and Gardner, 1988a; Deroles and Gardner, 1988b). In addition, the enzyme - linked immunosorbent assay (ELISA) and Western blot can also be applied as a measurement to quantify the expression levels of enzymes encoded by novel introduced genes in the transgenic plant.

1.3 Salt tolerance in plants

Salinity is a serious and costly problem for global agriculture (Carvajal et al., 1998). On a global scale, it is estimated that there are between 400 and 950 x 10^6 hectars of salt affected land (Epstein et al., 1980). Primarily, excessive soil salinity is a major challenge in arid to semi-arid regions (Greenway and Munns, 1980). Saline irrigation under poor drainage conditions is a common cause, inhibiting the growth and yields of crop plants (Chaudhory et al., 1997).

In general, the effects of salinity on plants have been recognized not only in terms of survival, but also on its effect on plant growth (Flowers and Yeo, 1989). Since growth is concerned, plants are categorized on the basis of their performance in response to salinity from the most salinity-sensitive glycophytes to the most salinity-tolerant halophytes (Greenway and Munns, 1980).

Plants exposed to salinity encounter two basic problems : (1) osmotic stress caused by external salt; (2) toxic effect of excessive salt accumulation within the plants (Munns, 1993). Under saline conditions, ions such as sodium and chloride can be continually transported to the leaf cells and accumulate in the vacuole of leaf cells until a saturation is reached (Flowers and Yeo, 1989). Subsequently, salt overflows and accumulates in the apoplastic cell walls, and then accumulated apoplastic salt results in cell dehydration (Flowers and Yeo, 1986). The result of cell dehydration may cause cell death, firstly of mature leaves and eventually of young leaves (Boyer, 1965; Neumann, 1997). Furthermore, when necrosis or abscission increase, the amount of photosynthetic products available for growth is decreased. Thus, growth is suppressed. In addition, excessive

sodium and chloride ions, the predominant ions in saline soil, create high ionic imbalances that may impair the membrane selectivity (Bohra and Dorffling, 1993). It has been reported that high levels of sodium ions can displace calcium ion from membranes and result in damaging their integrity (Cramer et al., 1990). High levels of sodium also can affect the selectivity for potassium uptake which is a major nutrient in metabolic process (Lazef and Cheeseman, 1988). These long-term toxic effects of salt accumulation can undoubtedly influence both vegetative and reproductive development (Khatun and Flowers, 1995). It was demonstrated that plant growth is initially inhibited by cellular responses to the osmotic effects of external salt, and then following further inhibition is caused by the toxic effects of excessive salt accumulation within plant cells (Munns, 1993). All of these negative effects result in decreased photosynthesis, respiration, transpiration and other metabolic events (Pier and Berkowitz, 1987; Flowers and Yeo, 1989).

In general, halophytic plants exhibit a greater capacity to tolerate salinity stress in comparison with glycophytic plants. *Mangrove* can grow in a saline environment by the exclusion mechanism which is accomplished by active pumping of salt back into the sea water (Ball, 1988). In addition, it was found that ice plants can pump excessive salt out by the epidermal bladder cells which locate on the surface of leaves or shoots (Bohnert et al., 1995). In fact, all plants are salt excluders (Flowers and Yeo, 1989). However, halophytes can sustain this exclusion for longer and at higher salinity than glycophytes (Flowers and Yeo, 1989). Interestingly, the ice plants can even use sodium as an osmoticum against osmotic stress caused by salinity or low temperature stress (Adams et

al., 1992). This is in contrast to glycopohytic plants which attempt to limit sodium uptake or partion sodium into the older tissues (Cheeseman, 1988).

Salt tolerance in not controlled by a single trait but is the consequence of complex gene interaction (quantitative trait). It has been suggested that glycophytes and halophytes have similar genetic make up which is needed for the salt tolerance and that the main difference between these two groups is in their capacity, which may dependent on a relatively small number of regulatory genes to exhibit the potential when required (Hasegawe et al., 1990; Amzallag et al., 1990; Moshe, 1994). Thus, the salt tolerance of halophytes over that of glycophytes may be simply from more efficient performance of a few basic tolerance mechanisms (Bohnert et al., 1995). Basically, possible mechanisms of plants to tolerate salinity can be considered from (1) osmotic adjustment; (2) osmoprotection; (3) ion homeostasis; (4) water-use efficiency (Nelson et al., 1998).

Upon exposure to the salinity stress, many plants (both halophytes and glycophytes) accumulate many organic solutes such as saccharides, polyols and nitrogen-containing compounds in their cells (Hare et al., 1998). It is generally accepted that the increase in compatible solutes can result in increasing cellular osmolarity (Cushman et al., 1990b). Thus, water can keep moving into the cytoplast by cellular osmotic adjustment (Morgan, 1984; Loescher, 1987). In this class, these accumulated compatible solutes are proposed to act as osmolytes which facilitate the retention of water in the cytoplasm and allow sodium sequestration to the vacuole or apoplast (Bhonert et al., 1995; Thomas et al., 1995). The function for osmolytes in osmotic adjustment is an accepted concept, but this is probably not their only purpose (Sheveleva et al., 1997). Other suggested functions

include osmoprotection, carbon or nitrogen source, free radical scavenging and signaling (Bohnert et al., 1995; Hare et al., 1999). In osmoprotection, it is suggested that these organic compatible solutes can stabilize membranes, proteins and enzymes by their water-like hydration property (Holmberg and Bulow, 1988). In addition, soluble sugars such as sucrose, polyols such as mannitol and sorbitol and amino acids such as glycine betaine have been known as a hydroxyl radical scavenger (Smirnoff, 1993). They may support plant natural antioxidative system to alleviate oxidative stress (Shen et al., 1997). Furthermore, in view of their highly reduced nature, it seems that these organic compatible solutes might be valuable storage compounds for reducing power and source of carbon/nitrogen upon relief from stress (Hare et al., 1999). Recently, myo-inositol was found to act as a facilitator of sodium uptake and long-distance transport in ice plants (Nelson et al., 1999). The authors of this report suggested that myo-inositol may serve not only as a source for the production of compatible solutes but also as a leaf- to- root signal to promote sodium uptake. The signaling capacities of sucrose and hexose also have been suggested in mediating responses to environmental stress (Graham et al., 1994).

It has been known that some halophytic plant species have evolved specialized complex mechanisms such as salt glands which allow them to adapt to saline environments (Niu et al., 1995). In general, glycophytic plants do not have these specialized structures such as salt glands and they just limit sodium uptake and partition sodium into older tissues which are later sacrificed (Bohnert et al., 1995). To glycophytes, salt exclusion in the root and salt compartamentation in the leaf cell vacuoles appear to be critical for salt tolerance (Rausch et al., 1996). Under salinity stress, kinetic steady state of ion transport

may be disturbed by high salt levels (Binzel et al., 1988). Continuously, high levels of Na^+ and Cl^- may result in dehydration, ionic imbalance and toxicity (Bohra and Dorffing, 1993). Therefore, it is essential for plants to re-establish cellular ion homeostasis to maintain metabolism in response to salinity stress (Niu et al., 1995).

Recently, the proteins (involved in the transport of Na^+ across plasma membrane and tonoplast) such as plasma membrane H^+-ATPases, vaculolar H^+-ATPases and vaculolar H^+-PPiases have been cloned to study their structural functions and expression (Sze et al., 1992; Leigh et al., 1994; Sussman, 1994). The Na^+/H^+ antiporters are still not cloned in both plant plasma membrane and tonoplast (it has been cloned in bacteria and animal tissues), whereas physiological and biochemical analysis support the existance of Na^+/H^+ antiporters which involve mediating Na^+ fluxes (Dupont, 1992). All these proteins are suggested to be involved in the regulation of sodium ion uptake, compartmentation and exclusion (Rausch et al., 1996). It has been reported that mRNA expressing levels of plasma membrane H^+-ATPases of roots and leaves are increased by salinity stress in certain halophytic plant species when compared with their controls (Braun et al., 1986; Niu et al., 1993b). Furthermore, it was suggested that salt exclusion at the plasma membrane via a Na^+/H^+ antiporter driven by the plasma membrane H^+-ATPase is likely to be the primary basis for cellular ion homeostasis under salinity stress (Binzel et al., 1988; Watad et al., 1991). Vacuolar compartmentation of Na^+ is also an important mechanism for plants to tolerate salinity stress. Since high vacuoler H^+-ATPase and vacuolar H^+-PPiase activities are found in certain plant species in response to salinity stress, it has been suggested that sodium ion can be transported into the vacuole via

Na^+/H^+ antiporters driven by the vacuolar H^+-ATPases and the vacuolar H^+-PPiases (Binzel et al., 1988; Colombo and Cerama, 1993; Rausch et al., 1996).

It has been reported that cytosolic Ca^{2+}-homeostasis can be disturbed by influx of Na^+ (Schumaker and Sze, 1990). Thus, it is obvious that Na^+ which crosses plasma membranes and tonoplasts may influence cellular signaling transduction by affecting the cellular distribution of Ca^{2+} which is thought to be a second messenger in signaling transduction (Rausch et al., 1996). In addition, it was suggested that the characteristics of potassium channels (high affinity and low affinity) are important determinants of plants capacity to tolerate salinity stress (Benlloch et al., 1994). Inability to perform a high discrimination between K^+ and Na^+ or a high maintained K^+ level in the cytoplasm could result in increased salt sensitivity of plants (Nelson et al., 1998). Increased calcium in the growth medium is known to help plants to tolerate salinity (Lahaye and Epstein, 1971). Especially, calcium is known to sustain potassium transport and potassium/sodium selectivity in salt-stressed plants (Epstein, 1998). A protein, phosphatase calcineurin was shown to be essential for salt tolerance in yeast, and this salt tolerance in yeast was found to be dependent on signaling transduction involving Ca^{2+} and protein phosphorylation/dephosphorylation (Mendoza et al., 1994). Recently, a product of SOS3 gene, as a calcium-binding protein (contain 3 calcium binding sites), was discovered in Arabidopsis thaliana plants (Liu and Zhu, 1998). The authors of this report also suggested that SOS3 protein responds to the calcium messengers by activating a protein phosphatase or inhibiting a protein kinase to regulate potassium and sodium discrimination/transport systems. These two research reports provide the molecular evidence that calcium is the

essential part for potassium/sodium discrimination.

Regulation of water permeability should also be considered as a mechanism for plants to tolerate salinity. It was reported that transcripts of Mip (major intrinsic protein) genes encoding aquaporin-like protein are increased in ice plants under salinity stress (Bohnert et al., 1995; Yamada et al., 1995). In addition, tonoplast and plasma membrane aquaporin transcripts also were found to increase in pea and *Arabidopsis* in response to osmotic stress (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992). The exact mechanism of water-specific transporter is still unclear. However, it has been suggested that induction of water-specific transporters/or aquaporin-like proteins at the initial stages of salinity stress may supply the mechanisms leading to osmoregulation and/or increased vacuolar osmolyte levels, at least, to a limited degree (Maurel et al., 1993; Bartels and Nelson, 1994; Bohnert et al., 1995).

Among various mechanisms of plants to tolerate salinity stress, the synthesis and accumulation of organic compatible solutes is the most recognizable feature in plants exposed to salinity stress. Several genes involved in production of proline, glycine betaine, mannitol and pinitol have been engineered into certain plants such as tobacco and Arabidopsis respectively (Tarcynski et al., 1992; Kavi Kishor et al., 1995; Hayash et al., 1997; Shevelena et al., 1997). Furthermore, transgenic plants expressing any one of these compounds are found to exhibit a greater capacity to tolerate salinity in comparison with the controls. Since the accumulation levels of these compounds are relatively low, it has been suggested that they may act as osmoprotectants and/or free radical scavengers, rather than osmolytes (Karakas et al., 1997; Hare et al., 1998). These successful transgenic

examples can be used as guidance for the engineering of salt-sensitive crop plants.

1.4 Chilling tolerance in plants

Temperature is an important environmental factor that limits plant growth and development. Numerous plants such as cotton, soybean, maize, rice, banana, tomato, sugarcane and pineapple that originated from tropical and subtropical regions show stress symptoms caused by low, nonfreezing temperatures (Tokuhisa and Browse, 1999). In general, the growth and development of plants originating from tropical and subtropical regions are limited by temperatures below 10-12 ^{0}C (Paull, 1990). Plants originating from temperate regions can survive and grow at temperatures limiting growth of tropical or subtropical plants. However, many temperate-origin plants are still sensitive to temperatures below 3-5 ^{0}C if these plants are exposed for long-term periods (Bramlage and Meir, 1990).

Chilling injury is known as a physiological dysfunction by exposure of plants to low but nonfreezing temperatures (Lyons, 1973). Generally, the chilling injury symptoms develop rapidly after chilled plants are transferred back to nonchilling temperatures. Chilling injury depends on plant species, tissues and developmental stages (Lyons, 1973). In addition, environmental factors such as duration of exposure to low temperature, humidity and light intensity also influence severity of chilling injury (Wilson, 1987). Visual symptoms of chilling injury include wilting, chlorosis, necrosis and bleaching (Wang, 1982; Paull, 1990). Retarded seedling growth, damaged reproductive organs, accelerated senescence and inhibited seed germination also have been reported (Wilson, 1976; Van

Hasselt and Strikwenda, 1976; Wilson, 1987).

Physiological and biochemical responses of plant sensitivity to chilling temperatures are often the subject of controversy in published reports. The hypotheses explaining chilling responses include : changes in membrane structure and function; cessation of protoplasmic streaming; alterations in respiration rates; changes in ethylene synthesis; and many biochemical and compositional changes (Morris, 1982). Among these hypotheses, changes in membrane function and structure are generally accepted as the primary chilling response (Wang, 1982). This hypothesis was first developed by Lyons and Raison (1970). According to this hypothesis, physical phase transition of membranes from a flexible liquid-crystalline to a solid-gel phase is thought as the primary response of chilling-sensitive plants to chilling temperatures. Following this primary chilling response the membrane alterations may cause secondary chilling responses such as stimulation of ethylene, loss of membrane integrity, loss of compart mentation, increase in the activation energy of membrane-associated enzymes, cessation of cytoplasmic streaming, reduction in energy supply, decrease in photosynthetic rate, imbalance of metabolism and accumulation of toxic substances depending on the length of exposure and the susceptibility of plant species and tissues (Wang, 1982).

Although physical changes in membranes are generally agreed on as the primary chilling response of plants to chilling stress, the concept that the composition of membrane lipids may be related to the capacity of plants to tolerate chilling stress are discussed in many studies (Lyons and Asmundson, 1965; Miller et al., 1974; Wang and Baker, 1979). Numerous studies have shown that the phase change temperatures of membranes is

determined by the ratio of saturated to unsaturated fatty acids (Miller et al., 1974; Kane et al., 1978; Wang and Baker, 1979). These studies suggest that high unsaturated fatty acids in membranes correlate with chilling tolerance of plants.

However, some conflicting reports demonstrate that the degree of unsaturation of fatty acids may not be necessarily correlated with the physical state of the membrane and the sensitivity of plants to chilling (Patterson et al., 1978; Priestley and Leopold, 1979; Sharom et al., 1994). All these studies suggest that phase changes of membrane lipids is a symptom rather than a cause of chilling injury. Some studies also suggest that sterols and cholesterol or the lipid-protein complex also may be involved in regulating the membrane fluidity (Lyons, 1973; McElhaney, 1974).

Recently, chilling-induced oxidative stress has received more notice than ever. Considerable evidence shows that oxidative stress is a significant factor in relation to chilling injury in plants (Burdon et al., 1994; Prasad et al., 1994). Chilling injury of plants is thought to be mediated by the formation of reactive oxygen species under chilling stress associated illumination (Wise and Naylor, 1987). Many studies have shown that reactive oxygen species such as superoxide, H_2O_2 and hydroxyl radicals are generated by chilling and membrane peroxidation in chilling-sensitive plants (Gilmour et al., 1988; Burdon et al., 1994). Reactive oxygen species not only damage membrane lipids but also damage nucleic acids, proteins and other cellular components (Asada, 1994; Tokuhisa and Browse, 1999). It has been documented that reactive oxygen species cause membrane peroxidiation by the increased formation of malondialdehyde (Smirnoff, 1993). In addition, it also has been known that reactive oxygen species can form carbonyl

derivatives by attacking amino acid residues in proteins and cause protein to be degraded by forming intra-and inter molecular cross-links (Stadtman, 1992). Thus, the targets most susceptible to damage by reactive oxygen species in chloroplasts could be both photosynthetic electron transport system and Calvin cycle enzymes (Shen et al., 1997b).

Plants naturally can detoxify reactive oxygen species by using a combination of antioxidants and antioxidative enzymes such as glutathione, B-carotene and ascorbate, and a regeneration cycle involving superoxide dismutase, ascorbate peroxidase and glutathione reductase (Foyer et al., 1994; Bruggemann et al., 1999). Several studies have reported that chilling tolerant species have more efficient antioxicative systems than sensitive ones (Jahnke et al., 1991; Walker and Mckersie, 19933). It has been suggested that the induction of certain antioxidative enzymes in maize is signal by H_2O_2 (Prasad et al., 1994). Recently, overaccumulation of glycine betaine in transgenic *Arabidopsis thaliana* also was found to enhance chilling tolerance of plants (Hayashi et al., 1997). In this study, glycine betain is suggested as a radical scavenger rather than an osmolyte.

Few studies have shown a positive correlation between sugar levels/composition and tolerance to chilling stress. Maintenance of adequate sugar supply in roots has been associated with the capacity of maize and peas to grow at chilling temperatures (Crawford and Huxter, 1977). High levels of reducing sugars in grapefruit peel were found to correlate positively with chilling tolerance (Purvis and Rice, 1983). King et al., (1988) also reported that carbohydrate depletion caused by dark treatment may relate to increased chilling injury. However, the accumulation of sugars is suggested to be a response to low temperature, rather than a direct factor to determine chilling tolerance (Wang, 1982).

Assays of chilling sensitivity are needed, and information derived from chilling assays are required to enhance the understanding of chilling-induced physiological, biochemical and even molecular responses. Furthermore, information obtained from chilling assays is also useful to the improvement of plants to tolerate chilling stress. Visual symptoms of chilling injury in whole plants or tissues usually are observed after chilling plants are transferred back to nonchilling temperatures. However, it is known that chilling injury can occur before occurrence of visual symptoms.

The most widely used method of measuring chilling sensitivity has been the leakage of cellular contents by conductivity (Wilson, 1987). However, a serious problem with measurements of electrolyte leakage is that visual symptoms of chilling injury can occur before a significant increase in electrolyte leakage (Wilson, 1987). A simple assay called TTC test is also usually used in assay of chilling injury. In a TTC test, the reduction of triphenyl tetrazolium chloride is used as an indicator showing the respiring capacity of chilling tissues (Stergios and Howell, 1973). The degree of the reduction of triphenyl tetrazolium chloride can be measured at 400 mM wave length with a spectrophotometer. Since chilling-induced damage can occur in a photosynthetic system, chlorophyll fluorescence also is very useful for assaying the degree of chilling injury (Bruggeman et al., 1999). Moreover, changes in respiration rate, stimulation ethylene, and changes in concentrations of certain compounds and enzymes also are suggested to provide a quantitative assay of the degree of chilling injury (Wang, 1982).

Molecular techniques are frequently used as tools for studying and improving tolerance of plants to chilling stress (Prasad et al., 1994; Bruggemann et al., 1999;

Tokuhisa and Browse, 1999). Furthermore, plants engineered with genes encoding antioxidative enzymes, desaturases and enzymes producing glycine betaine were found to be positively correlated with their chilling tolerance (Gupta et al., 1993; Kodama et al., 1994; Hayashi et al., 1997).

Since chilling tolerance is a quantitative trait, improvement of chilling tolerance may be an inherently incremental process, requiring the expression of many genes (Tokuhisa and Browse, 1999). Instead of classical genetic and breeding methods, the modern molecular techniques provide the chances to clone genes associated with chilling tolerance and determine the function of each cloned gene in chilling tolerance. Moreover, these genes can be introduced into chilling-sensitive horticultural plant species by genetic engineering. Thus, modern molecular techniques can be useful measures to alleviate the disadvantages of classical genetic and breeding methods that depend upon long term programs and limited genetic sources.

Chapter 2

Overexpression of Mannitol-1-Phosphate Dehydrogenase Increases Mannitol Production and Confers Salt Tolerance in Transgenic Petunia Plants

2.1 Abstract

Petunia plants (*cv. Mitchell*) were transformed with a gene encoding mannitol-1-phosphate dehydrogenase (MTLD) resulting in high mannitol levels. This study was conducted to investigate the effect of mannitol expression levels on seed germination, and vegetative growth and floral development of wild type and transgenic lines under salinity stress. Four T1 progeny lines that exhibited 3:1 Mendelian kanamycin resistance were established from nine individual putative T0 transgenic shoots. Apparently, the incidence of escapes occurred at a high frequency. Furthermore, only two of these four T1 transgenic lines had the capacity for high mannitol expression. Phenotypically, there was no difference in growth between *in vivo* wild type and transgenic lines under non-stress conditions. However, transgenic lines expressing high mannitol levels were found to exhibit a greater capacity to tolerate salinity stress compared to wild type and transgenic lines expressing low mannitol levels. Enhanced salinity tolerance was observed in seed germination (T2 generation), and in vegetative growth and floral development of transgenic lines expressing high mannitol levels. Carbohydrate analysis of wild type and T1 transgenic plants, showed that mannitol was the single carbohydrate most affected by

plant line. Thus, the mechanistic function of mannitol should be considered in tolerance of petunia plants to salinity stress. In addition, assuming 90 % water content in leaf, leaf osmotic potentials of high mannitol expressing lines contributed by mannitol accounted for only 0.006 %-0.01 % of osmotic potential generated from all solutes under salinity stress. Mannitol should thus be considered an osmoprotectant rather than an osmolyte. Enhanced myo-inositol, sucrose and raffinose levels in both T1 high mannitol expressing and low mannitol expressing lines were detected after 28 days in salt treatments. These soluble carbohydrates in an enhanced level (78 % of total soluble carbohydrates) also should not be neglected in the tolerance of plants to salinity stress. It is suggested that mannitol may function as an important stress-protective component in enhancing salt tolerance of transgenic lines expressing high mannitol levels in this study

2.2 Introduction

Many plants can accumulate biocompatible solutes such as amino acids, soluble sugars and polyols in response to salinity stress (Cushman et al., 1990). Properties of these biocompatible solutes are commonly considered as osmolytes and osmoprotectants (Bohnert et al., 1995). As osmolytes, these biocompatible solutes can contribute to osmotic adjustment in plants in response to osmotic stress caused by salinity (Morgan, 1984; Loescher, 1987). As osmoprotectants, they can act at low concentrations to stabilize membranes, proteins and other macromolecular structures by their water-like hydration property (Holmberg and Bulow, 1998). In addition, many of these biocompatible solutes also are suggested to serve as carbon or nitrogen sources and free

radical scavengers (Nelson et al., 1998).

Among such biocompatible solutes, sugar alcohols are widely distributed in bacteria, fungi, algae, lichens, animals and many higher plants (Bieleski, 1982). In fungi and algae, sugar alcohols are found to accumulate in response to various environmental stresses (Jennings, 1984; Kirst, 1989). These accumulated sugar alcohols are suggested to have a positive correlation with stress tolerance of these nonvascular plants (Kirst, 1989; Smirnoff and Cumbes, 1989). Mannitol, a six carbon noncyclic sugar alcohol, is a major photosynthetic metabolite in celery plants and is also found to accumulate in response to salinity stress (Bieleski, 1982; Pharr et al., 1995). Similarly, studies have shown that pinitol accumulates in ice plants exposed to salt stress, and becomes a major carbohydrate exceeding 700 mM in the cytosol and in chloroplasts (Paul and Cockburn, 1989; Adams et al., 1992). In addition, sorbitol accumulation was also found to correlate with adaptation to salt stress in Plantage (Briens and Larher, 1983). The primary functions of accumulated sugar alcohols in naturally salt-tolerant plants have been suggested to be osmolytes and osmoprotectants (Loescher, 1987; Cushman et al., 1990a; Bartel and Nelson, 1994; Pharr et al., 1995). However, other possible functions such as free radical scavengers and storage of reduced carbon source still should be considered due to their biocompatible characteristics (Nelson et al., 1998).

By using cloning techniques and genetic engineering, many genes encoding stress-tolerance reactive enzymes have been isolated from different sources and introduced into target plants to examine their possible functions (Bartels and Nelson, 1994). Furthermore, many studies have shown that stress tolerance can be improved in plants transformed with

genes encoding enzymes related to the synthesis of biocompatible solutes such as mannitol, proline, glycine betaine, fructan and ononitol (Tarczynski et al., 1993; Kavi Kishor et al., 1995; Pilon-Smits et al., 1995; Sheveleve et al., 1997; Hayashi et al., 1997). Since low concentrations of these biocompatible solutes are found to accumulate in the transgenic plants in response to stresses, they may not play an absolute role in osmotic adjustment (Hare et al., 1998). They may act as osmoprotectants rather than osmolytes. Smirnoff and Cumbes (1989) reported that *in vitro* proline, mannitol and sorbitol at low concentrations can act as hydroxyl radical scavengers to inhibit hydroxylation of salicylate and protect enzymes from inactivation. Thus, it should be considered that accumulation of biocompatible solutes may protect stressed plants by their possible free radical scavenging properties.

Several studies have shown that transformation of tobacco and *Arobidopsis thaliana* with a *E. Coli mtl D* gene encoding mannitol 1-phosphate dehydrogenase resulted in mannitol synthesis, and increased stress tolerance (Tarczynski et al., 1993; Thomas et al., 1995; Karakas et al., 1997). However, Karakas et al.(1997) reported that tobacco plants engineered with *mtl D* genes tolerated salinity stress in comparison to wild-type plants; their transgenic tobacco plants grew slower and were 20-25 % (dry weight basis) smaller than wild-type ones under non-stress conditions. This research group suggested that improved salinity tolerance in transgenic tobacco plants may have resulted from the slower growth which allows plants to decrease ion uptake and prevent sodium and/or chloride ions to build up in cytoplasm. Thus, mannitol may have not acted as a primary effector to result in increased salinity tolerance in transgenic-tobacco plants

(Karakas et al., 1997; Pharr et al., 1999). However, the stress-protective function of mannitol rather than an osmolyte in transgenic tobacco plants still can not be completely neglected.

A recent study by Shen et al.(1997a) showed that the presence of mannitol in chloroplasts of transgenic tobacco can result in increase resistance to oxidative stress. In a further study conducted by the same group, it was suggested that mannitol can act as a hydroxyl radical scavenger to protect SH-enzymes and other SH-regulating chloroplast components such as ferredoxin, thioredoxin and glutathione from inactivation during oxidative stress (Shen et al., 1997b). Reactive oxygen species are commonly generated in plants exposed to various environmental stresses and subsequently they may result in damaging nucleic acids, membranes and proteins (Asada, 1994; Burden et al., 1994). To prevent the damage caused by reactive oxygen species, plants naturally can produce antioxidants and antioxidative enzymes. However, under stress conditions, the normal antioxidative systems may not be adequate to lessen the damage caused by excessive reactive oxygen species generated in stressed plants (Smirnoff, 1993). Thus, based on the results published by Shen et al.(1997b) and Smirnoff (1993), mannitol may act as a hydroxyl radical scavenger to supply normal antioxidative systems in stressed plants, and then alleviate oxidative damage caused by various environmental stresses.

The study reported here was conducted to investigate : (1) the effect of mannitol expressing levels on seed germination, and on vegetative growth, and floral development of petunia *cv.Mitchell* plants engineered with *mtl D* genes under salinity stress and (2) soluble carbohydrate levels of these transgenic plants exposed to high salinity.

2.3 Materials and Methods

2.3.1 Plant material and culture conditions

Petunia *C.V. Mitchell < P.axillaris* x (*P. Axillaris* x *P.hybrida 'Rose du Ceil* ') > was used throughout all following experiments. Seeds obtained from Purdue University were surface-sterilized in 20% Clorox bleach (1.2 % sodium hypochlorite) with 3 drops of Tween 80 for 10 min, and then were treated with four rinses in sterile distilled water. After surface-sterilized seeds were blotted dry on the filter papers, they were placed individually on medium containing ½ MS salt., 30 gL^{-1} sucrose and 0.8 % (w/v) agar. Approximately 100 seeds were cultured in two baby food jars and incubated in a growth chamber (day 25 ^{0}C-night 20 ^{0}C, 16 hr photo period). Seeds began to germinate after 4 days in culture. Aseptic plant seedlings from these germinated seeds were used in the following experiments.

2.3.2. Gene constructs, plant transformation and regeneration

Agrobacterium tumefaciens (strain LBA 4404) with the pCaMVMTLDS containing a 35S cauliflower mosaic virus promoter, the *E.Coli mtl D* (Mannitol-1-phosphate dehydrogenase) gene and a nopaline synthase termination signal subcloned into the binary vector p Bin 19 (Tarczynski et al. 1992) was gifted from Dr. Hans Bohnert the University of Arizona.

Transgenic plants were produced using a leaf disc cocultivation protocol following the method published by Horsch et al.(1985) with modification. Leaf disks were cut

from the leaves of aspetically grown plants cultured in the baby food jars , and then immersed in a culture of *A. tumefaciens* grown overnight in LB with 50 mg/L kanamycin at 28 ^{0}C. After gentle shaking about 10 min to ensure that all edges were infected, the 50 infected leaf disks were blotted dry and cultured upside-down on the co-cultivation medium containing basal MS salts (Marashige and Skoog, 1962), B-5 Vitamins (Gamborg et al., 1968), 30 gL^{-1} sucrose, 0.8 % agar, 0.25 μM IAA and 2.5 μM BAP at 25 ^{0}C in the dark for 48 hours to increase the chance of co-cultivation. Following completion of co-cultivation, these putative co-cultivated leaf disks were transferred to the shoot regeneration/selection medium containing all components of co-cultivation medium, 500 μg/ml carbenicillin and 300 μg/ml kanamycin in the 25 ^{0}C and 16 hr photoperiod. About 40 non-co-cultivated leaf disks also were culture on the shoot regeneration/selection medium as a control. After co-cultivated leaf disks were cultured three weeks in the shoot regeneration/selection medium, regenerated adventitious shoots were excised and transferred to the rooting medium containing all components of basal MS medium and 100 μg/ml kanamycin. After roots appeared, the rooted plantlets were transferred to the antibiotic-free rooting medium for further growth.

2.3.3. Rooting and callusing assay

Aseptically grown rooted plantlets of each putative transgenic line were excised into 4-5 segments and cultured in the rooting medium containing 100 μg/ml kanamycin for the re-rooting assay. Only all shoot segments in the same line that showed positive root formation were chosen and transferred to the antibiotic-free rooting medium. At the same

time, leaf explants of these positive rooting lines (two leaf disks of each putative transgenic plantlets in the same line were chosen) were plated on shoot regeneration/selection medium to test if callus or shoot formation occurred. Only the lines showing callus formation were chosen for further experiments.

2.3.4. Recallusing assay and multiplication of T0 plants

Aseptically grown plantlets of each transgenic line were excised into 3 segments and cultured for multiplication in a rooting medium containing 100 μg/ml kanamycin. After roots appeared, three rooted plantlets of each transgenic line were transferred to soil for acclimation. Two weeks later, a recallusing assay was carried out to test *in vivo* plants of each transgenic line and wild type to ensure that the transformed plants were stable.

2.3.5. Production and selection of T1 transgenic plants

Wild type plants and T0 plants of each transgenic line were allowed to flower and set seeds in a containment greenhouse. Cross-pollination was prevented by placing paper bags on flowers before opening and one day after self-pollination. The investigation of inheritance of kanamycin resistance for T1 seeds followed a report published by Deroles and Gardner. (1988a). Seeds were collected from each transgenic line and two capsules of seeds were separately germinated on the MS medium with 0 μg/ml and 200μg/ml kanamycin in the same culture conditions as wild-type seed germination (2.3.1). Numbers of green and white seedlings were counted after three weeks. Only the seed germinating from transgenic lines showing about a 3:1 segregation ratio were chosen for

further use. About 30 seedlings of each transgenic line with a 3:1 segregation ratio were cultured in the medium and used as stock plant materials for further physiological experiments. At the same time, about 20 kanamycin-resistant seedlings of transgenic lines with a 3:1 segregation ratio were acclimated in the soil and grown in the growth chamber to flower and set seeds (self-cross). T1 plants from which all seeds that germinated in the1/2 MS medium containing 200 μg/ml kanamycin, were then chosen and allowed to set more seeds. These homozygous seeds of each transgenic line were then used as stock source for the further stress-imposing T2 seed germination experiments.

2.3.6. NPT II ELISA test of T1 transgenic lines

A PathoScreen Kit for neomycin phosphotranferase II (product of Agdia Inc; catalog No. PSP 73000) was used throughout this experiment. Sample preparation (10 T1 plants of each transgenic line with a 3:1 segregation ratio) were subjected to an ELISA test according to the instructions provided in the kit.

2.3.7. Salinity treatments

For the T0 generation, eight rooted plants (four plants for the control) of the wild type and of each transgenic line (M2, M3, M4, M5, M8, M9) were transferred from the rooting medium containing 100 μg/ml kanamycin (2 week old) to the potting soil medium for 2 weeks acclimation (4 week old). Then, roots of these acclimated plants were cleaned by tap water and transplanted to the pots (4 inches) containing sand medium for four more weeks (8 week old). These 8-week old plants then were stressed by adding 50

mM NaCl for the first three days, increasing 25 mM every three days up to 200 mM for 30 days in a greenhouse (August and October, 1997) . The NaCl was mixed with1/2 strength Hollagand's solution (pH = 6.0) and added as 100 ml of mixed solution to each pot every day. This experiment was repeated twice.

For the T1 generation, twenty four 8 week old plants of wild type and each transgenic line with a 3:1 segregation ratio (M2, M3, M8 and M9) and a positive reaction in NPT II ELISA test were stressed and grown in a growth chamber (day 25 ^{0}C/night 20 ^{0}C cycle, 75 % relative humidity and 16 hr photoperiod). Twenty four plants of each T1 line were separated into the control group and stressed groups. Three replications were applied in this experiment.

All excised shoots (2 cm in length) of T0 and T1 plants grew slower in the rooting medium containing 100 μg/ml kanamycin than wild-type shoots grown in the kanamycin-free rooting medium. To compensate for this situation, excised shoots of both T0 and T1 generation were placed on the rooting medium containing 100 μg/ml kanamycin 4 days earlier than excised shoots of wild type. Thus, both wild type and transgenic lines were of comparable size before imposing salinity.

2.3.8. Growth measurements

In the salt experiments, four wild type plants and four of each T0 transgenic lines were collected after 30 days. Plants (including 4 plants of the control) were divided into shoots and roots, and oven-dried at 70 ^{0}C for five days. Shoot and root dry weight were measured at this time.

For the T1 generation, the same number of wild type plants and of each transgenic line were collected after 28 days. Plants (including the control plants) were first measured for fresh weight of both shoots and roots. The divided shoot and root parts were oven-dried at same conditions as the T0 generation. Dry weight of both shoots and roots were measured, and water content was calculated on a fresh weight basis.

2.3.9. Mannitol and carbohydrate extraction

At the end of the salt stress treatments, fresh upper leaves (~ 4-5 cm^2) were harvested from wild type and T0 transgenic plants (including the control plants). Fresh upper leaf tissues were first ground with liquid nitrogen in a ceramic mortar, and then 0.1 M NaOH was added to extract carbohydrates from these frozen leaf tissues. The extract was stored at 4 ^{0}C about half an hour. Later, the extract was centrifuged at 1000 xg for 5 minutes, and the supernatant was collected for HPLC analysis.

Leaf harvest and carbohydrate extraction of salt-stressed T1 plants (including the control plants) was the same as for the T0 generation. Instead of fresh leaf tissues, freeze dried samples were used and the extract was stored at 4 ^{0}C about half an hour to rehydrate the freeze dried tissue and solubilize carbohydrates uniformly. Carbohydrate extraction of T2 seeds was conducted the same as T1 plants.

2.3.10. HPLC analysis

Leaf tissue of T0 plants was analyzed by a Dionex HPLC system coupled with a pulsed electrochemical detector and a CarboPac-MA1 analytic column maintained at 25

^{0}C. Soluble carbohydrates of T1 plants and mannitol of T2 seeds were also analyzed by Dionex HPLC. Instead of a CarboPac-MA1 column, a CarboPac-100 analytic column maintained at 35 ^{0}C was used. External standards were used to calculate concentration of all sugars.

2.3.11. Measurements of osmotic potential

Osmotic potentials of upper leaves were measured by a vapor pressure osmometer (model 5520, Wescor,Inc) at the end of salt treatments. Whole upper leaves (one leaf for each plant) containing no petiole were collected from each plant of wild type and T1 transgenic lines (3 plants for each line). Leaves were then treated with liquid nitrogen. After frozen leaves were thawed, they were crushed and 20μl leaf juice samples were measured. Three replications were applied for measurements of osmotic potential.

2.3.12. Conductivity measurements

Upper leaves (one leaf for each plant) of wild-type and T1 transgenic lines (including the control plants) were removed from each plant at the end of salt treatments. Leaf tips of each plant were then punched into leaf disks (0.5 mm in diameter) with a cork borer. Five leaf disks from leaf tips of each salt-stressed and control lines were first treated with liquid nitrogen and then placed in each measuring cell. Freeze-killed leaf disks of each line were measured for maximum leakage for each line. Five other leaf disks of each line were then placed in the measuring cells, and then 2 ml distilled water was

added into each cell.

The leakage of electrolytes was measured by a DAC-1000 conductance meter. Total leakage measuring time was 240 minutes, and the sampling interval was 20 minutes. Relative conductivity represents the mean amount of leachate from the salt-stressed or the control samples at each sampling point as a percentage of the mean maximum amount of leachate from frozen-killed salt-stressed or control samples at the end of measuring time. This experiment was replicated three times.

2.3.13. Growth parameters and seed germination studies

Seed-surface sterilization procedures were as described in 2.3.1. Instead of four rinses in sterile distilled water, seeds for salt treatments were rinsed with a sterile solution of 200 mM NaCl. Surface-sterilized seeds were then placed on the seed germination medium the same as described in 2.3.1 with 0 mM or 200 mM NaCl respectively. Approximately 100 seeds were placed on each petri dish and incubated in the same culture conditions as described in 2.3.1. Over 400 seeds of each wild type and T2 transgenic line were sown onto petri dishes and germination was scored for the salt-stressed and control seeds. Seeds were scored as germinated when the radicle was at least 5 mm long and the cotyledons began to appear.

2.4 Results

2.4.1 Events of *Agrobacterium*-mediated transformation of Petunia hybrida *cv. Mitchell* with a *E. Coli mtl D* gene

Three weeks after cocultivated leaf disks were cultured in the shoot regeneration/kanamycin selection medium, calli formed on the wounded surfaces of most cocultivated leaf disks, and even shoots regenerated from some of them. In contrast, neither callus formation nor shoot regeneration were observed in the non-cocultivated leaf disks which were cultured on the shoot regeneration/kanamycin selection medium. Nine well-developed regenerated shoots were excised from 9 individual cocultivated leaf disks respectively (Table 2.1) and immediately transferred to the rooting medium containing 100 ug/ml Kanamycin for the primary rooting test. Three weeks later, roots appeared in all nine regenerated shoots, but two of them had slower root development in comparison with the others. All 9 rooted shoots were then transferred to the kanamycin-free rooting medium for further growth.

In the following re-rooting test, all aspetically grown rooted plantlets of each putative transgenic line were excised into 4-5 segments and cultured on the rooting medium containing 100 μg/ml kanamycin. Seven of these 9 putative transgenic lines showed positive root formation in all excised segments (Table 2.1). These seven lines were used for an additional callusing test. Later, six of these 7 positive rooting lines formed callus in all tested leaf disks cultured on the shoot regeneration/selection medium containing 300 μg/ml kanmycin (Table 2.1). The six transgenic lines passing re-rooting and callusing assays were multiplied in the rooting medium containing 100 μg/ml kanamycin and then transferred to a greenhouse. All six T0 transgenic lines grown in a greenhouse also passed the recallusing assay (Table 2.1). These six transgenic lines were multiplied, and transferred to the containment greenhouse and used to produce the self-

fertilized T1 generation. In addition, these six T0 transgenic lines were used as a source for mannitol analysis and in the preliminary salt treatments. Interestingly, it was observed that in vitro transgenic T0 plantlets showed slower growth in the kanamycin-containing medium in comparison with those ones which grow in the kanamycin-free medium. Kanamycin in the medium appears to result in slower growth which needs to be considered when conducting trials with transgenic plants.

2.4.2 Inheritance of kanamycin resistance in T1 transgenic lines

Inheritance of kanamycin resistance was monitored by sowing T1 seeds on the medium containing 200 μg/ml kanamycin, and scoring the number of green resistant seedlings and white sensitive seedlings to determine a segregation ratio for kanamycin resistance. Seeds obtained from self-crossed wild-type plants showed essentially no kanamycin-resistant seedlings (data not shown). In a subsequent observation, the growth of pale germinated wild-type seedlings was still inhibited even after they were transferred from medium containing 200 μg/ml kanamycin to medium containing 100 μg/ml kanamycin (Fig. 2.2). Progeny segregation ratios of transgenic lines M2, M3, M8 and M9 are approximate to 75 % green and 25 % white respectively (Table 2.2), and are similar to the inheritance pattern of a single dominant gene (3 : 1). These four transgenic lines were considered to have the integrated *mtl D* gene on a single chromosome. The ratios of transgenic lines M4 and M5 were approximate to 47 % : 53 % and 59 % : 41 % ,respectively (Table 2.2). The kanamycin resistant trait of these two transgenic lines can

not be considered due to a single dominant gene according to traditional Mendelian inheritance. While no studies were conducted to determine the mode of inheritance the nearly 1:1 segregation pattern, suggests either a hypomorphic allele (leaky gene that function improperly) or a multiple copy insertion. Therefore, these two transgenic lines M4 and M5 were not used in any physiological investigations or carbohydrate analysis of T1 generation. A NPT II (neomycin phosphotrasferase II) ELISA test was used to further verify transformation of T1 transgenic lines M2, M3, M8 and M9. Compared with the standards, all four selected T1 transgenic lines showed positive comparable concentrations in the NPT II ELISA test (Fig. 2.1). In addition, continuous growth of T1 seedlings surviving in medium containing 200 μg/ml kanamycin (line M2, M3, M8 and M9) also was observed after they were transferred to medium containing 100 μg/ml kanamycin (Fig. 2.2). Thus, only these 4 lines, M2, M3, M8 and M9, were maintained for further physiological investigations.

2.4.3 Growth and mannitol expression levels of T0 transgenic plants in the presence of NaCl

Thirty days after greenhouse grown plants of wild-type and T0 transgenic lines were cultured in the absence of added NaCl, dry weight of roots and shoots did not significantly differ between wild type and all six T0 transgenic lines (Table 2.3). However, expression of mannitol levels in wild type and T0 transgenic lines were quite different (Table 2.3). The lowest mannitol levels were detected in wild type and

transgenic lines M4 and M9. The second lowest mannitol level was detected in line M2, and the highest mannitol levels were obtained from lines M3, M5 and M8. Thus, it appears that plant development of T0 transgenic lines was not influenced by introduced *mtl D* genes in comparison with wild type under non-salinity stress.

Thirty days after plants of wild type and T0 transgenic lines were stressed by stepwise increased salinity, mannitol levels of salt stressed wild type and all salt stressed transgenic lines were similar to non-stressed plants (Table 2.3 and 2.4). Mannitol expression levels in lines M3, M5 and M8 were still higher than those in wild type and transgenic lines M2, M4 and M9 (Table 2.4). Similarly, growth (dry weight of roots and shoots) of transgenic lines expressing high mannitol levels also was better than that of the low expression transgenic lines under salinity stress (Table 2.4). Furthermore, some high expression lines maintained flowers after 30 days in the presence of added NaCl, whereas wild type and transgenic lines expressing low mannitol levels did not maintain flowers after salinity treatments were completed (Fig. 2.3 and 2.4). Mannitol expression levels seemed to act as a decisive factor to influence the capacity of petunia plants to tolerate salinity stress.

Root growth (dry weight basis) of wild type and all transgenic lines was reduced by salinity in comparison with non-stressed ones (Table 2.3 and 2.4). In contrast, shoot growth (dry weight basis) of stressed plants was not significantly reduced by salinity stress (Table 2.3 and 2.4). The growth patterns observed in this study were not similar to some previous studies reporting improved osmotic-stress tolerance associated with increased energy allocation to the roots for increasing water uptake (Pelleschi et al., 1997; Blum and

Sullivan, 1997).

2.4.4 Non-structural carbohydrate analysis of T1 transgenic plants in the presence of added NaCl

Instead of greenhouse conditions, all T1 transgenic plants were cultured and stressed with salinity in a growth chamber (day 25 ^{0}C/night 20 ^{0}C cycle and 16 hr photoperiod) to have more uniform growth conditions and obtain more accurate measurements and analysis.

Under non-salinity stress, all soluble carbohydrate levels, but mannitol did not differ among wild type and all T1 transgenic lines (Table 2.5). Mannitol was the only compound affected by plant line, being higher in transgenic lines M3 and M8, and lower in wild type and transgenic lines M2 and M9 (Table 2.5). Expression of mannitol levels of T1 transgenic plants seemed not to affect other carbohydrates synthesis in comparison with wild type plants in the absence of salt stress.

Twenty eight days after salinity stress, inositol, glucose, fructose, sucrose, raffinose, and stachyose levels were not significantly different among wild type and all transgenic lines (Table 2.6). Mannitol expression levels of lines M3 and M8 were 2.5~3.6 times than those of wild type and transgenic lines M2 and M9. Mannitol levels of all lines were reduced by the salinity stress treatments.

To understand the effect of salinity on soluble carbohydrate metabolism of wild type and all T1 transgenic lines, both wild type and transgenic lines were classified into high mannitol expressing (M3 and M8) and low mannitol expressing (wild type, M2 and

M9) groups. Levels of inositol, sucrose and raffinose of both low mannitol and high mannitol expressing groups were significantly enhanced by salinity stress (Table 2.7). In contrast, reduced mannitol and fructose levels occurred in both mannitol expressing groups under salinity stress, but mannitol levels of the high-mannitol expressing group were still approximately equal to the non-stressed low-mannitol expressing group (Table 2.7). In addition, both glucose and stachyose levels of high mannitol expressing and low mannitol expressing groups were not significantly different whether they were stressed by salinity or not (Table 2.7). Thus, it could be concluded that salinity did cause a shift of carbohydrate metabolism in petunia plants. Furthermore, total levels of soluble carbohydrates analyzed in wild type and in each transgenic line were increased about 2 times by salinity in comparison with the non-stressed ones.

2.4.5 Growth of T1 transgenic plants in the presence of NaCl

To compensate for slow growth of T1 transgenic plantlets in the presence of 100 μg/ml kanamycin, wild - type shoots were planted in the kanamycin-free medium 4 days later than the T1 transgenic shoots cultured on the rooting medium containing 100 μg/ml kanamycin. Experimental wild type and transgenic lines of comparable size were then obtained after they were transferred from *in vitro* conditions to acclimation conditions. Further observation showed that growth of T1 transgenic plants was the same as wild type plants under *in vivo* conditions. This observation conflicted with Karakas et al.(1997) who reported slow growth of tobacco plants engineered with *mtl D* genes even under non-

salinity conditions.

Twenty eight days after plants of wild type, and T1 transgenic lines M2, M3, M8 and M9 were grown in the absence of added NaCl, both shoot and root growth (dry weight basis) did not differ among wild type and all transgenic lines (Table 2.8, Fig. 2.5 and 2.7). Water content of both shoots and roots also was similar in all plants whether high mannitol expressing or not (Table 2.8).

Phenotypically, plant growth in wild type and T1 transgenic lines did not show any significant symptoms of salt injury at the beginning of salt treatments (data not shown). However, twenty eight days after salt treatments were completed, leaf wilting symptoms of leaves were observed in the middle and lower parts of plants of wild type and transgenic lines expressing low mannitol levels (line M2 and M9), whereas comparable plants of transgenic lines expressing high mannitol levels (M3 and M8) did not wilt (Fig. 2.6). In addition, it was observed that root development of transgenic lines M3 and M8 was much better in comparison with wild type, and transgenic lines M2 and M9 under salinity stress (Fig. 2.7). Furthermore, these phenotypic observations of stressed plants also were found to be consistent with their dry weight measurements. Under salinity stress, dry weight and water content (shoots and roots) of wild type, and transgenic lines M2 and M9 were significantly lower than those of transgenic lines M3 and M8 (Table 2.9).

Apparently, shoot and root mass (dry weight) of both high mannitol expressing and low mannitol expressing groups were significantly reduced by salinity stress (Table 2.10). However, the high mannitol expressing group still accumulated much more dry weight (both shoots and roots) compared with the low mannitol expressing group under

saline conditions (Table 2.10). Similarly, decreased water content, induced by salinity stress was observed in both high mannitol expressing and low mannitol expressing groups, but a higher water content was maintained by the high mannitol expressing group (Table 2.10). Thus, it seems that salt tolerance of T1 transgenic lines expressing high mannitol levels (M3 and M8) is better than the wild type and the other two transgenic lines expressing low mannitol levels (M2 and M9). Since mannitol was the single compound affected by altered gene expression plant line in this study, we can conclude that mannitol plays an important role in transgenic lines M3 and M8 to improve tolerance to salinity stress.

2.4.6 Flower buds and opened flowers in the presence of NaCl

Under non-saline conditions, the number of flower buds did not differ among wild type and transgenic lines (Fig. 2.8). In fact, there was also no difference in flower bud development among all plant lines at the beginning and mid phases of salinity stress. However, a stronger flower-bud development potential was detected in transgenic lines M3 and M8 compared to wild type and transgenic lines M2 and M9 after the mid-point of imposed salinity stress (Fig. 2.9). Furthermore, twenty eight days after plants were stressed by salinity, the number of flower buds on transgenic lines M3 and M8 was significantly higher than that of wild type and the other two transgenic lines expressing low mannitol levels (M2 and M9).

Similarly, the number of opened flowers did not differ among all plant lines in non-

stressed conditions, and at the mid-point of salinity stress (Fig. 2.10 and 2.11). However, transgenic lines M3 and M8 maintained many more flowers than wild type and the other two transgenic lines (M2 and M9) at the end of salinity stress (Fig. 2.11).

These data suggest that reproductive development of transgenic lines M3 and M8 can be maintained at a higher rate in comparison with wild type and, transgenic lines M2 and M9 under saline conditions.

2.4.7 Osmotic potential of salt-stressed plants

All leaf samples (upper leaves) used in the measurements of osmotic potential were harvested 28 days after plants were cultured in the absence or presence of added NaCl. Osmotic potentials were not significantly different among wild type and all transgenic lines under non-stressed conditions (Fig. 2.12). Osmotic potentials also did not differ among wild type and all transgenic lines grown under salinity stress (Fig. 2.13). However, the osmotic potentials of salt-stressed plants were significantly lower about -2.2~ -2.5 Mpa in comparison with those of non-stressed ones (Fig. 2.12 and 2.13). Apparently, osmotic adjustment occurred in the plants in response to salinity stress.

2.4.8 Relative conductivity and leakage rate of salt-stressed plants

Relative conductivity of non-salt stressed wild type and transgenic lines was not significantly different (Fig. 2.14). In all lines relative conductivity ranged between 5 %

and 12.5 % after 240 minutes of measurement.

Relative conductivity of all lines was significantly increased by salinity. However, transgenic lines M3 and M8 did have lower relative conductivity in compared to wild type and the other transgenic lines (M2 and M9) (Fig. 2.15).

Calculated leakage rates (% x min^{-1}) of wild type and all transgenic lines were similar under non-salt treatments (Fig. 2.16), and among all salinity-stressed plant lines when Turkey's Multiple Comparison Test ($P<0.05$) was used as a statistically comparative method. A large amount of apoplastically accumulated salt which was released into the measuring cells may interfere with the reading of electrolyte leakage throughout the whole measuring process.

2.4.9 T2 seed germination in the presence of NaCl

Significantly, higher mannitol accumulation (0.12-0.17 μmole/g dry weight) were found in the T2 seeds of transgenic lines M3 and M8 in comparison with wild type, and the low expression transgenic lines M2 and M9 (0.03-0.07 μmole/g dry weight). These results are consistent with the analysis of their parental T1 plant. Five days after T2 seeds were cultured on the NaCl-free medium, germination percentages of wild type and transgenic line M2 were higher than transgenic lines M3, M8 and M9 (Fig. 2.18). Around 86 % seed germination was observed in wild type and transgenic line M2, whereas germination percentages of transgenic lines M3, M8 and M9 were about 70-78 %. Apparently, the germination rate of transgenic lines M3, M8 and M9 were slower than

those of wild type and transgenic M2. The slow germinating capacity of transgenic lines M3, M8 and M9 can be avelliated by a prolonged-culture period. Germination percentages of wild type and all transgenic lines did attain ~ 90 % ten days after seeds were cultured in the NaCl-free medium (Fig. 2.18). Furthermore, no significant increase in germination was observed in all plant lines after 10 days in culture (Fig. 2.18 and 2.20).

In general, seed germination was significantly decreased by salinity in this study. Fifteen days after T2 seeds of all plant lines were cultured on the medium containing 200 mM NaCl, no seed germination was observed in any of the plant lines, except M3, which had about 1 % germination (Fig. 2.19). Germination increased in line M3 and M8 (30 % and 23 % respectively) after 25 days in salt treatments. At this point, germination percentages of wild type, line M2 and M9 were only 6-12 %. Germination percentages of all plant lines continue to increase after 45 day in salt treatments, abtaining 54 and 56 % respectively in M3 and M8 (Fig. 2.19 and 2.21). Germination percentages of wild type, M2 and M9 were 25 to 31 %, significantly lower than those of M3 and M8. In addition, fresh weight of plantlets from transgenic lines M3 and M8 was higher than those of wild type and, transgenic lines M2 and M9 after 45 days in salt treatments (Fig. 2.22).

2.5 Discussion

In *E. Coli*, the *MTL D* enzyme is known to convert mannitol-1-phosphate derived from phosphorylation of mannitol to fructose-6-phosphate which is used in glycolysis

(Tarczynski et al., 1992). In transgenic *mtl D* tobacco and *Arabidopsis thaliana*, however, the *mtl D* product (mannitol-1-phosphate dehydrogenase) converts fructose-6-phosphate and NADH to mannitol-1-phosphate and NAD^+ (Thomas et al., 1995). It is suggested that mannitol-1-phosphate is dephosphorylated by a non-specific phosphatase to form mannitol in transgenic tobacco plants (Tarczynski et al., 1992). Furthermore, transgenic tobacco and *Arabidopsis thaliana* plants containing *mtl D* genes were found to have increased capacity to tolerate salinity stress in comparison with the comparable control plants (Tarczynski et al., 1992; Tarczynski et al., 1993; Thomas et al., 1995; Karakas et al., 1997).

Petunia (*cv. Mitchell)* plants engineered with *mtl D* genes were obtained via *Agrobacterium*-mediated transformation. Six T0 individual transgenic lines were established from 9 well-developed regenerated shoots through a series of kanamycin selection screening tests (Table 2.1). Apparently, even though the selection period was short and only well-developed regenerated shoots were selected, some escapes still occurred. Furthermore, when segregation patterns of kanamycin-resistance was determined for the progeny of these six T0 transgenic lines, a single dominant gene was found in transgenic lines M2, M3, M8 and M9. However, non-Medelian segregation ratios were observed in transgenic lines M4 and M5 (Table 2.2). Our observation is similar to Deroles and Gardner (1988a) who reported a high proportion of plants whose gene was transmitted to progeny at lower than Mendelian ratios. The reasons why escapes occurred in kanamycin-resistant positive selected T0 plants and why low ratios of the transmitted kanamycin-resistant trait occurred in the progeny of transgenic lines M4

and M5 are unknown. Possible explanations include chimeric transformants, the rearrangement of T-DNA in the genome, deletion of T-DNA during recombination, position effects and integration of T-DNA in the cytoplasmic genome such as chloroplastic or mitochondrial (Horsch et al., 1985; Budar et al., 1986; Dong and McHughen, 1993).

It was found that slow growth occurred in both T0 and T1 transgenic plants when they were cultured in the medium containing 100 μg/ml kanamycin. In contrast, the growth of transgenic plants whether T0 or T1 showed no growth differences when compared with wild-type plants in the kanamycin-free medium. Since kanamycin was the only component that differed in the medium, its presence must influence the growth of transgenic plants. To compensate for slow growth of transgenic plants in the presence of kanamycin, wild-type shoots were planted in the kanamycin-free medium 4 days later than the transgenic plants cultured in the medium containing 100 μg/ml kanamycin. Wild-type and transgenic plants of similar size were then obtained under *in vitro* conditions. Furthermore, in a subsequent observation, *in vivo* wild type and transgenic plants did not show any phenotypical differences in both vegetative and reproductive development. The results observed in this study do not agree with Karakas et al. (1997) who reported slow growth of tobacco plants engineered with *mtl D* genes under non-salinity stress. In their case, slow growth of transgenic plants was not caused by the introduced *mtl D* gene nor the NPT II gene themselves. Furthermore, observed slow growth in their transgenic tobacco plants may have been caused by the inserted positions of introduced genes. Although total carbohydrate pools are similar between wild type and transgenic type, the positions of the introduced gene may still affect the synthesis of other substances which

are important to growth.

Results from soluble carbohydrate analysis and growth measurements of T0 and T1 transgenic lines grown in a greenhouse or in a growth chamber showed that transgenic lines expressing higher mannitol levels grew better than those lines expressing lower mannitol levels in response to salinity stress. It did show that there was a positive relationship between mannitol expressing levels and the extent of salt tolerance in plants.

Based upon soluble-carbohydrate analysis of T1 transgenic plants, mannitol was the only compound that differed among plant lines with or without salinity stress (Table 2.5 and 2.6). Apparently, higher mannitol expressing levels in T1 transgenic lines M3 and M8 did not influence synthesis of other carbohydrate. This result was similar to the report by Karakas et al. (1997).

Salinity stress did cause metabolic shifts in wild type and all T1 transgenic lines (Table 2.7). Enhanced inositol, sucrose and raffinose levels were detected in all plant lines after 28 days in salt treatments. These three soluble carbohydrates represented ~ 78 % of total amount of all soluble carbohydrates analyzed in all salt-treated plants. Sugar accumulation and metabolic shifts in carbohydrate patterns have long been known as important factors in plants response to osmotic stress caused by environmental stresses (Ingram and Bartels, 1996; Hare et al., 1998). Sugars that accumulate in large quantity are often thought to act as osmolytes, osmoprotectants or carbon sources in response to stresses. However, signaling by sucrose and myo-inositol has also been suggested in several studies (Graham et al., 1994; Nelson et al., 1999). Increased raffinose levels were also found associated with enhanced inositol and sucrose levels under salinity stress in this

study. In RFO-exporting plants, inositol is commonly found to combine with galactose to form galacitinol which can provide glactosyl residues to incorporate in the formation of RFO (Dey, 1985; Bohnert et al., 1995). Enhanced raffinose levels are commonly known to be induced by cold acclimation in cold-hardy plants (Stushnoff et al., 1993). However, since increased raffinose levels by salinity were observed in this study, functions of raffinose may not be restricted just to cold tolerance.

Mannitol is naturally produced in petunia *cv. Mitchell*, but at a relatively minor level in comparison to other soluble carbohydrates (Table 2.7). However, the function of mannitol in petunia plants in response to salinity stress should not be underestimated because only 3 times the mannitol concentration, even as low as 3 μM. g^{-1} dw enhanced transgenic petunia plant tolerance to salinity stress in comparison to wild-type petunia plants. Evolutionarily, less mannitol accumulation in petunia may be caused by long-term artificial breeding and a better culture environment which does not need specific stress-protective functions. Furthermore, it has been reported that transgenic tobacco plants which produced just two times as much proline as control plants had improved tolerance in response to imposed drought and salinity stress (Kavi Kishor et al., 1995). Thus, even relatively low levels of a protectant compound should not be neglected in plants under environmental stress.

Increased compatible solute production in response to osmotic dehydration caused by environmental stresses has been reported (Bartels and Nelson, 1994). These compatible solutes are primarily thought to act as osmolytes in osmotic adjustment in response to stresses (Zimmermann, 1978; Greenway and Munns, 1980; O'Neil, 1983).

However, several recent reports have shown that increased levels of mannitol, fructans trehalose, proline and glycine betaine in transgenic plants such as tobacco and *Arabidopsis thaliana* may not be adequate to account for osmotic adjustment as an explanation for the enhanced stress tolerance observed (Kave Kishor et al., 1995; Pilon-Smits et al., 1995; Thomas et al., 1995; Romero et al., 1997; Karakas et al., 1997; Hare et al., 1998). These accumulated compatible solutes have been suggested to act as osmoprotectants rather than osmolytes. In our study, it was observed that osmotic potentials were adjusted about 2.5 Mpa in plants in response to salinity stress. However, using the Van't Hoff equation, osmotic potential = - CiRT{C = concentration of solute(mol kg^{-1}), i = a constant that accounts for ionization of the solute from perfect solution ,R = 0.00831 kg · Mpa mol^{-1} K^{-1} , and T = temperature (^{o}K)} ,osmotic potentials of mannitol produced in transgenic lines M3 and M8 treated with salinity are about 0.0002-0.0003 Mpa respectively (assuming 90 % water content in leaf and T=298 ^{0}K). The osmotic potentials generated from additional mannitol found in stressed transgenic lines M3 and M8 are roughly 0.008 % and 0.012 % of the total osmotic difference (2.5 Mpa) between non-salt treated plants and salt-treated plants, and account for only 0.006 %-0.01 % of osmotic potentials caused by salinity stress. Apparently, mannitol cannot be thought of as an osmolyte for osmotic adjustment in this study, and should rather be considered as an osmoprotectant. It was observed that mannitol levels in all plant lines were significantly reduced by salinity. However, why mannitol levels were decreased by salinity stress is unknown. One possibility is that mannitol may be catabolized by the cytosolic mannitol degradative enzymes which are enhanced by salinity. Enzymes which degrade mannitol have been found in the cytosol of

celery plant cells (Stoop et al., 1996). The regulatory mechanism of cytosolic mannitol is still unknown in petunia plants. However, since wild-type petunia (*cv. Mitchell*) did produce mannitol naturally, mannitol expression in the petunia plant cells may also be regulated by a series of mannitol degradative and synthetic enzymes which depend on environmental signals. Another possibility is that mannitol may act as a free radical scavenger to detoxify reactive oxygen species caused by salinity stress and then result in decreased mannitol levels detected in petunia plants.

Mannitol levels in all plant lines used in this study were similar to the levels of antioxidants such as ascorbate and glutathione found in tomato plants and red seaweed *Mastocarpus stellatus*, and were higher than the levels of antioxidants found in plants of *Nicotiana plumbaginifolia* L (Bruggemann et al., 1997; Collen and Davison, 1999; Savoure et al., 1999). Thus, levels of mannitol found in petunia plants in this study are consistent with reported physiological levels of other antioxidants.

Excessive reactive oxygen species are commonly generated in plants exposed to drought and salinity. However, it has been reported that normal antioxidative systems may not be sufficient to alleviate damage of oxidative stress caused by drought and salinity (Smirnoff, 1993). Shen et al. (1997a) reported that the presence of mannitol in chloroplasts of transgenic tobacco plants can result in increased resistance to oxidative stress. The authors of this report suggested that mannitol may act as a hydroxyl radical scavenger to protect SH-enzymes and other SH-regulating chloroplast components from inactivation during oxidative stress (Shen et al., 1997b). Unfortunately, they did not show if the presence of mannitol in chloroplasts of transgenic tobacco plants can result in

improving plants' tolerance to salinity or other environmental stresses. However, combined with other reports which show improved salinity tolerance in transgenic plants expressing mannitol as osmoprotectant levels, the possibility that mannitol acts as a hydroxyl radical scavenger to lessen damage caused by hydroxyl radicals is quite likely. The fate and location of mannitol in the transgenic petunia plants were unknown in this study. However, improved salt tolerance found in transgenic lines M3 and M8 can be attributed to their higher mannitol expressing characteristic when compared with M2 and M9, the other transgenic lines.

Relative conductivity as a % of total potential conductivity is commonly used to measure the degree of membrane damage. In this study, relative conductivity was lower in transgenic lines M3 and M8 than in wild type and transgenic lines M2 and M9 (Fig. 2.15). Thus, it appears that less membrane damage occurred in transgenic lines expressing higher mannitol levels. Leakage rates, however, were not significantly different between wild type and all transgenic lines, which does not agree with the results observed in the calculated relative conductivity. In fact, high relative conductivity (about 60 %) also was found in transgenic lines M3 and M8 even though the relative conductivity of these two lines was still lower in comparison with wild type and the other transgenic lines (about 80 %). Plant cells leaking at such high level normally do not survive. It is likely that high leakage was due to accumulation of NaCl in the apoplast from the high salt stress treatments.

Molecules such as sucrose and polyols may function to bind water to their high affinity OH groups to maintain membranes, proteins and other macromolecules in

hydrated status (Bartels and Nelson, 1994; Pharr et al., 1995). Since mannitol expressing levels detected in the high mannitol expressing group were only about 0.6 % of total soluble carbohydrates induced by salinity, it is unlikely that mannitol itself is adequate to protect macromolecules by its OH groups. Compounds such as sucrose and raffinose enhanced by salinity seemed to be more likely candidates to provide hydration rather than mannitol. Although the suggested hydration function of mannitol can not be completely excluded from this study, the possibility of mannitol to act as a hydroxyl radical scavenger seems more likely than its hydration function in a quantitative sense. Mannitol may alleviate membrane lipid peroxidation and denaturation of proteins by its hydroxyl radical scavenging function.

It is commonly known that reproductive development such as flowering and fruit set in plants can be severely affected by salinity stress. Therefore, one purpose of this study was to investigate the effect of mannitol expressing levels on the flowering capacity of wild type and transgenic petunia plants under saline conditions. It was observed that the number of opened-flowers and flower buds decreased in all plant lines after 28 days in salt treatments in comparison with non-stressed ones. Apparently, flowering capacity of all plant lines was affected by salinity stress. Furthermore, flowering capacity of wild type and all transgenic lines did not show any significant difference at the beginning and mid-way phases (50 mM-175 mM). However, flowering capacity of transgenic lines expressing high mannitol levels was maintained at a higher rate in comparison with lines expressing low mannitol levels after 28 days in salt treatments (Fig. 2.9 and 2.11). Thus, it seems that high mannitol expression in transgenic plants can also sustain floral growth at

a higher rate in comparison with low-mannitol expressing levels in wild type and the other transgenic lines. This result observed in this study was similar to Kavi Kishor et al. (1995) who reported higher capacity of flowering and seed setting found in salinity-stressed transgenic tobacco expressing high proline levels in comparison with the control ones.

Boron is an essential element for plant reproductive development and is generally considered as immobile in most species (Dell and Huang, 1997; Bellaloui et al., 1999). It was found that boron can be transported in the phloem of celery and peach plants by forms of sorbitol-boron-sorbitol and mannitol-boron-mannitol complexes, respectively (Hu et al., 1997). Two recent papers published by Brown et al. (1999) and Bellaloui et al. (1999) showed that transgenic tobacco plants expressing sorbitol (not found in wild-type tobacco) exhibit an apparent increase in phloem boron transport and result in increased tolerance of transgenic tobacco plants to boron deficiency. Since the sorbitol amount (0.37-0.87 μmole/gfw) detected in transgenic tobacco plants is similar to the mannitol amount (~0.3 μmole/gfw) detected in non-stressed transgenic lines M3 and M8, it may be that mannitol plays a similar function to sorbitol, to facilitate boron uptake in petunia plants. Furthermore, higher mannitol expressing levels in transgenic lines may form and transport much more mannitol-boron-mannitol complex to support floral development in comparison with lower mannitol expressing lines under saline conditions. Unfortunately, whether mannitol can be transported out of cells in petunia plants is still unknown.

Higher T2 seed germination of transgenic lines (M3 and M8) compared to wild type and transgenic lines (M2 and M9) in the presence of NaCl was observed in this study (Fig. 2.19). Since mannitol levels detected in the seeds of transgenic lines M3 and M8

were not adequate to provide osmoregulation in response to NaCl osmoticum, it is more likely that mannitol acts as a stabilizing agent to prevent aggregation of proteins and membrane disruption, or as a detoxifying agent to prevent oxidative stress during imbibition. However, at present there is no evidence to explain the exact mechanism of the effect of mannitol on the tolerance to salinity stress observed in seeds of both low mannitol expressing and high mannitol expressing lines. Overall, results obtained from this T2 seed germination experiment are similar to those of Thomas et al. (1995), who reported that seed germinating capacity of transgenic *Arabidopsis athaliana* plants containing mannitol is maintained at a higher rate in comparison with the controls under high salinity.

In summary, this study was designed to investigate the effect of mannitol expressing levels on the growth of wild-type and transgenic petunia (*cv. Mitchell*) plants under saline conditions. Our results showed that transgenic petunia plants expressing high mannitol levels had an increased capacity to germinate seed, and better vegetative and floral development under salinity stress. Since mannitol levels in high mannitol expressing lines were not adequate to contribute meaningfully to osmotic adjustment caused by salinity stress, it is suggested that mannitol may act as an osmoprotectant or hydroxy radical scavengers, rather than an osmolyte. Results presented here agree with several previous reports that showed a positive correlation between mannitol expression and transgenic plants such as tobacco and *Arabidopsis thalinia* under salinity stress (Tarcznski et al., 1993; Thomas et al., 1995; Karakas et al., 1997). Further studies are necessary to investigate if the location of mannitol production (in cytosol, chloroplasts or

mitochondria) results in different mannitol expressing levels in transgenic lines and if mannitol in the presence of chloroplasts or mitochondria can result in increased capacity of transgenic plants to tolerate oxidative stress caused by salinity.

Table 2.1 ***Agrobacterium*-mediated transformation events of *petunia hybrida. Mitchell* (*mtl D* gene)**

	Shoot selection medium (300 mg/L Kanamycin)	Re-rooting medium (100mg/L Kanamycin)	Callus assay (300 mg/L Kanamycin)	Recallus assay (300 mg/L Kanamycin)
Cocultivation (+)	9	7	6	6
Cocultivation (-)	0	0	0	0

* Fifty leaf disks were used for *Agrobacterium*-mediated transformation.
* Shoots for re-rooting assay were from in vitro plantlets.
* Leaf disks for callus assay were from in vitro plantlets.
* Leaf disks for recallus assay were from in vivo plants.

Table 2.2 Analysis of kanamycin resistant trait in T1 transgenic plants (self-fertilization of T0 plant)

Plant line	No. of tested capsule	Segregation ratio (green : white)
M2	1	152 : 49
M3	1	198 : 56
M4	2	57 : 65
M5	1	161 : 112
M8	2	108 : 38
M9	1	171 : 52

Table 2.3 Mannitol expression levels (μmole/gfw) and dry weight per plant of petunia T0 plants in absence of added NaCl (30 days)

Plant type	Mannitol (μmole/g) [x]	Root (g) [y]	Shoot (g) [y]
Wild	0.04a[z]	1.11a	2.81a
M2	0.06b	1.31a	2.91a
M3	0.12c	1.04a	3.11a
M4	0.05a	1.06a	3.12a
M5	0.11c	1.11a	3.16a
M8	0.10c	1.05a	3.18a
M9	0.05a	0.93a	2.85a

Two trials for all experiments except mannitol concentration which was determined only once.
Four plants were used for each replication.
[y] Root and shoot weight (per plant) were measured on a dry weight basis.
[x] Mannitol concentration was calculated on a fresh weight basis.
[z] Means followed by the same letter within each column are not significantly different at P<0.05 by Turkey's Studentized Comparison Test.

Table 2.4 Mannitol expression levels (μmole/gfw) and dry weight per plant petunia T0 plants in the presence of 200 mM NaCl (30 days)

Plant type	Mannitol (μmole/g) [x]	Root (g) [y]	Shoot (g) [y]
Wild	0.05a[z]	0.13a	2.04a
M2	0.06a	0.15a	2.37a
M3	0.13b	0.25b	3.05b
M4	0.06a	0.16a	2.45a
M5	0.11b	0.23b	3.11b
M8	0.11b	0.25b	3.04b
M9	0.05a	0.14a	2.27a

Two trials for all experiments except mannitol concentration which was determined only once.
Four plants were used for each replication.
[x] Mannitol concentration was calculated on a fresh weight basis.
[y] Root and shoot weight were measured on a dry weight basis.
[z] means followed by the same letter within each column are not significantly different at

Table. 2.5 Soluble carbohydrate and mannitol concentrations (μmol.g^{-1} dry weight basis) in upper leaves of wild type and T1 transgenic lines after 28 days for the non-salt stress treatment.

Plant line	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
WT	3.29±0.25 a[z]	0.92±0.05 a	17.70±0.51 a	48.29±2.04 a	16.05±1.28 a	2.59±0.55 a	0.47±0.01 a
M2	4.97±0.62 a	1.42±0.14 a	17.12±2.19 a	47.57±3.56 a	21.19±0.88 a	4.01±0.92 a	0.48±0.16 a
M3	4.14±0.41 a	3.29±0.22 b	17.27±2.11 a	56.68±6.03 a	18.02±2.11 a	3.40±0.34 a	0.57±0.14 a
M8	4.33±0.20 a	3.19±0.25 b	15.60±1.78 a	50.87±5.45 a	19.61±1.57 a	4.82±0.41 a	0.58±0.13 a
M9	3.37±0.21 a	1.20±0.03 a	14.66±0.82 a	49.80±6.39 a	15.63±0.84 a	3.55±0.19 a	0.73±0.20 a

Data are from three replicated experiments, in μmol/g dw for each sugar and polyol.
Values are mean ± SEM.
[z] Means followed by the same letter within each column are not significantly different at P < 0.05 by Tukey's Studentized Comparison Test.

Table. 2.6 Soluble carbohydrate and mannitol concentrations (μmol.g^{-1} dry weight basis) in upper leaves of wild type and T1 transgenic lines 28 days after salt treatments were completed (NaCl concentration were added from 50 mM and increased 25 mM every 3 days until 200 mM NaCl was reached)

Plant line	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
WT	19.53±0.54 a[z]	0.28±0.07 a	15.28±2.15 a	24.78±2.25 a	103.00±14.44 a	8.32±1.07 a	0.85±0.14 a
M2	24.31±1.66 a	0.40±0.10 a	15.33±0.91 a	22.96±3.14 a	99.34±5.41 a	10.12±0.96 a	0.81±0.05 a
M3	20.50±3.00 a	0.91±0.07 b	16.21±2.45 a	28.41±4.22 a	104.20±16.91 a	10.83±0.34 a	0.83±0.15 a
M8	25.41±2.60 a	1.02±0.15 b	17.96±0.65 a	18.70±1.00 a	108.10±20.69 a	11.39±2.03 a	0.77±0.13 a
M9	19.18±1.65 a	0.32±0.06 a	14.82±1.46 a	19.71±2.35 a	105.91±5.10 a	10.13±1.81 a	0.80±0.05 a

Data are from three replicated experiments, in μmol/g dw for each sugar and polyol. Values are mean ± SEM.

[z] Means followed by the same letter within each column are not significantly different at $P < 0.05$ by Tukey's Studentized Comparison Test.

Table. 2.7 Carbohydrate analysis ($\mu m.g^{-1}$ dw) of high-mannitol expressing and low-mannitol expressing groups under non-salinity and salinity stress

Salt treatment	Group	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
No	Low-mannitol expressing	3.88±0.55 a[1]	1.18±0.14 a	16.49±0.93 a	48.55±0.66 a	17.62±1.79 a	3.38±0.42 a	0.56±0.09 a
	High-mannitol expressing	4.23±0.09 a	3.24±0.05 b	16.62±0.65 a	53.78±2.91 a	18.82±0.80 a	4.11±0.71 a	0.57±0.01 ab
Yes	Low-mannitol expressing	21.01±1.65 b	0.33±0.03 c	15.14±0.16 a	22.48±1.49 b	102.7±1.90 b	9.52±0.60 b	0.82±0.02 bc
	High-mannitol expressing	22.95±2.45 b	0.96±0.06 a	17.09±0.88 a	23.56±4.86 b	106.2±8.95 b	11.11±0.28 b	0.80±0.03abc

Wild type , T1 transgenic lines M2 and M9 were collected as low-mannitol expressing group. T1 transgenic lines, M3 and M8 were collected as high-mannitol expressing group.

Data are from three replications, presented in $\mu m.g^{-1}$ dw for each sugar and polyol. Values are mean ± SEM.

[1] Means followed by the same letter within each column are not significantly different at $P < 0.05$ by Tukey's Studentized Comparison Test.

Table 2.8 Dry weight and water content of wild type and transgenic plants after 4 weeks in the absence of NaCl

Plant line	Shoot (DW)	Shoot(H_2O %)	Root (DW)	Root (H_2O %)	Root / Shoot ratio
WT	4.044 ±0.201 a	89.14±0.30 a	0.995±0.058 a	89.37±0.22 a	0.285 ±0.017 a
M2	3.571 ±0.287 a	89.48±0.29 a	1.033±0.086 a	89.66±0.27 a	0.312 ±0.023 a
M3	3.608 ±0.181 a	89.47±0.30 a	1.004±0.091 a	89.21±0.11 a	0.307 ±0.012 a
M8	3.826 ±0.031 a	89.50±0.19 a	1.020±0.115 a	89.31±0.32 a	0.284±0.014 a
M9	3.379 ±0.236 a	90.20±0.32 a	0.989±0.076 a	89.17±0.24 a	0.303±0.019 a

Data are from three replicated experiments. Values are mean ±SEM.
[z] means followed by the same letter within each column are not significantly different at P<0.05 by Turkey's Studentised Comparsion Test.

Table 2.9 Dry weight (g.Plant $^{-1}$) and water content (% FW) of wild type and transgenic petunia plants (T1 generation) after 4 weeks in the presence of 200 mM NaCl

Plant line	Shoot (DW)	Shoot (H_2O %)	Root (DW)	Root (H_2O %)	Root/Shoot ratio
WT	1.94±0.07 a[z]	82.44±1.01 a	0.37±0.01 a	83.24±0.48 a	0.19±0.01 a
M2	2.01±0.09 a	82.89±0.43 a	0.36±0.03 a	83.53±0.50 a	0.18±0.02 a
M3	3.10±0.16 b	86.15±0.37 b	0.87±0.06 b	85.78±0.40 b	0.30±0.03 b
M8	2.85±0.21 b	86.45±0.45 b	0.77±0.03 b	86.16±0.16 b	0.27±0.02 b
M9	1.94±0.07 a	82.19±0.37 a	0.35±0.04 a	81.33±0.58 a	0.18±0.02 a

Data are from three replicated experiments, presented in μmol/g dw for each sugar and polyol.
Values are mean ±SEM.
[z] means followed by the same letter within each column are not significantly different at P<0.05 by Turkey's Studentised Comparison Test.

Table 2.10 Dry weight (g. plant^{-1}) and water content (% FW) of high-mannitol expressing and low-mannitol expressing groups 4 weeks in the presence and absence of NaCl

Salt treatment	Group	Shoot (DW)	Shoot (H_2O %)	Root (DW)	Root (H_2O %)	Root/Shoot ratio
No	Low- mannitol expressing	3.67±0.20 a[z]	89.61±0.31 a	1.01±0.01 a	89.40±0.14 a	0.30±0.01 a
	High-mannitol expressing	3.72±0.11 a	89.49±0.01 a	1.01±0.02 a	89.26±0.05 a	0.29±0.01 a
Yes	Low-mannitol expressing	1.97±0.03 c	82.51±0.21 c	0.36±0.01 c	82.70±0.69 c	0.18±0.02 b
	High-mannitol expressing	2.98±0.13 b	86.33±0.13 b	0.82±0.05 b	85.97±0.19 b	0.28±0.01 a

Wild type , T1 transgenic lines M2 and M9 were collected as low-mannitol expressing group. T1 transgenic lines, M3 and M8 were collected as high-mannitol expressing group. Data are from three replications. Values are mean ± SEM.

[z] Means followed by the same letter within each column are not significantly different at $P < 0.05$ by Tukey's Studentized Comparison Test.

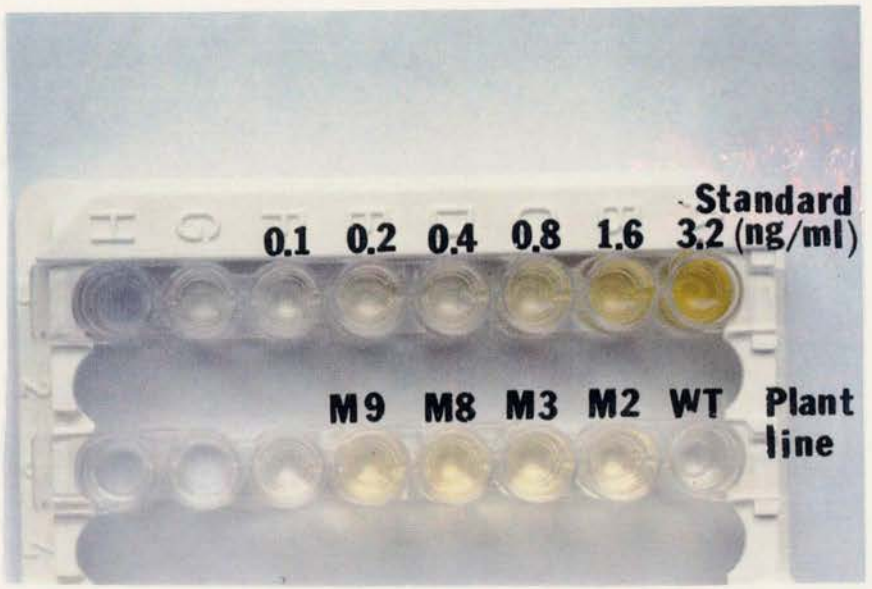

Fig. 2.1 Identification of kanamycin-resistant trait of T1 transgenic lines (M2, M3, M8 and M9) by a NPT II (neomycin-phosphotransferase II) ELISA test.

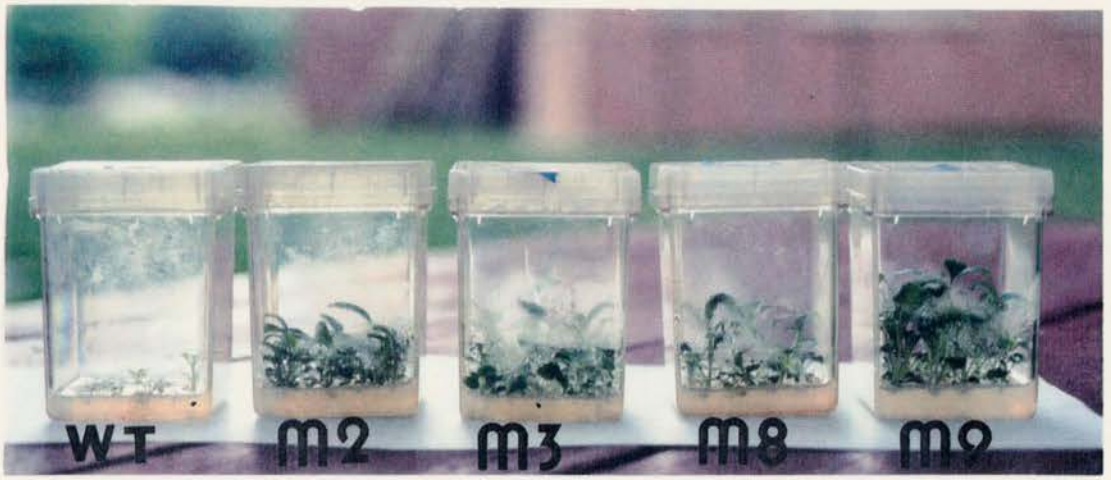

Fig. 2.2 Phenotype of wild type and T1 transgenic seed lines in the presence of 100 μg/ml kanamycin about 3 weeks after they were transferred from seed germination medium containing 100 μg/ml kanamycin.

Fig. 2.3 Phenotype of wild type, and T0 transgenic lines (M2, M4 and M9) 30 days after salt treatments were completed

Fig. 2.4 Phenotype of wild type and T0 transgenic lines (M3, M5, and M8) 30 days after salt treatments were completed.

Fig. 2.5 Phenotype of wild type and T1 transgenic lines (M2, M3, M8, and M9) 28 days after non-salt treatments were completed.

Fig. 2.6 Phenotype of wild type and T1 transgenic lines (M2, M3, M8 and M9) 28 days after salt treatments was completed (NaCl were added from 50 mM and increased 25 mM every day until 200 mM NaCl was reached)

Fig. 2.7 Phenotype of root growth of wild type and T1 transgenic lines (M2, M3, M8 and M9) after 28 days in non-salt or salt treatments (salt treatments as described in Fig. 2.6)

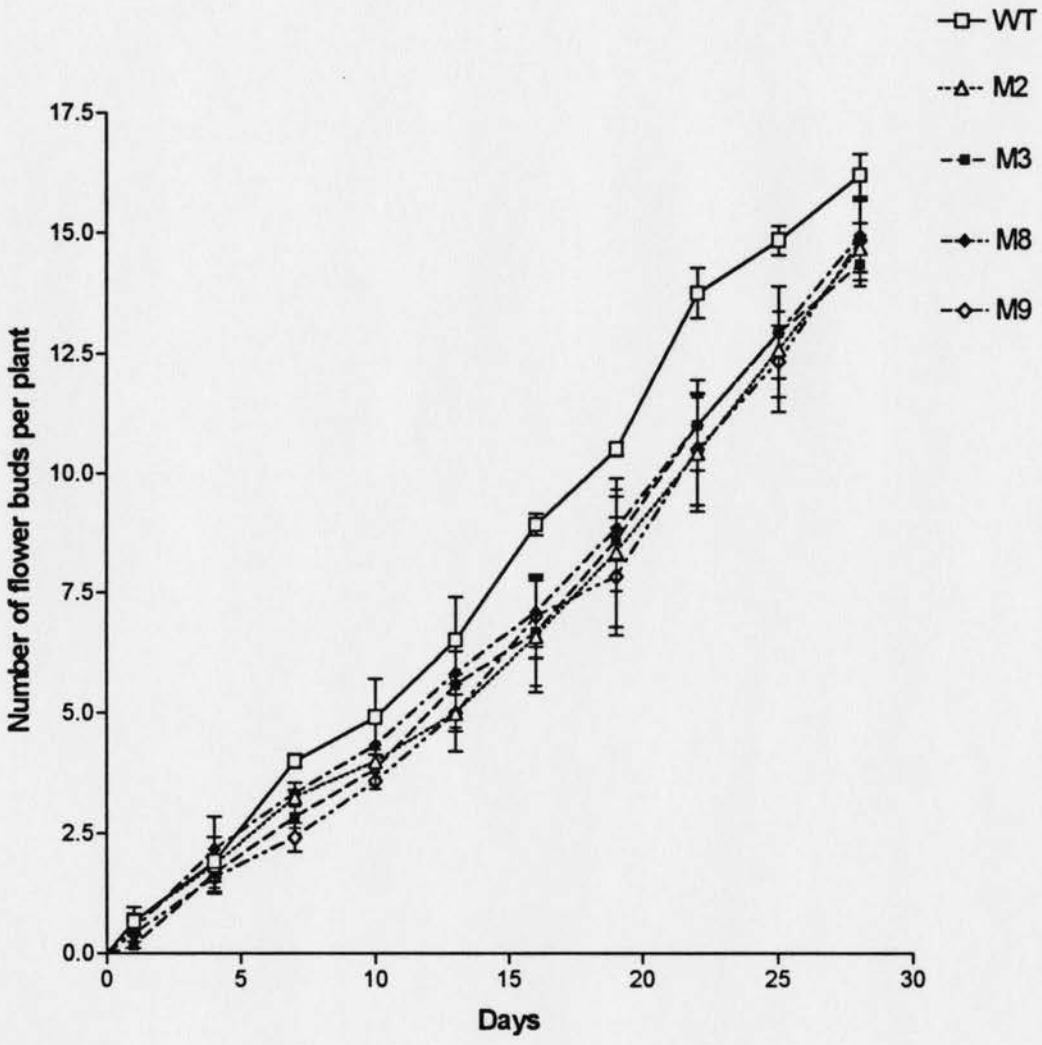

Fig. 2.8 Number of flower buds (per plant) of wild type and T1 transgenic lines after 28 days growth with no salt stress.

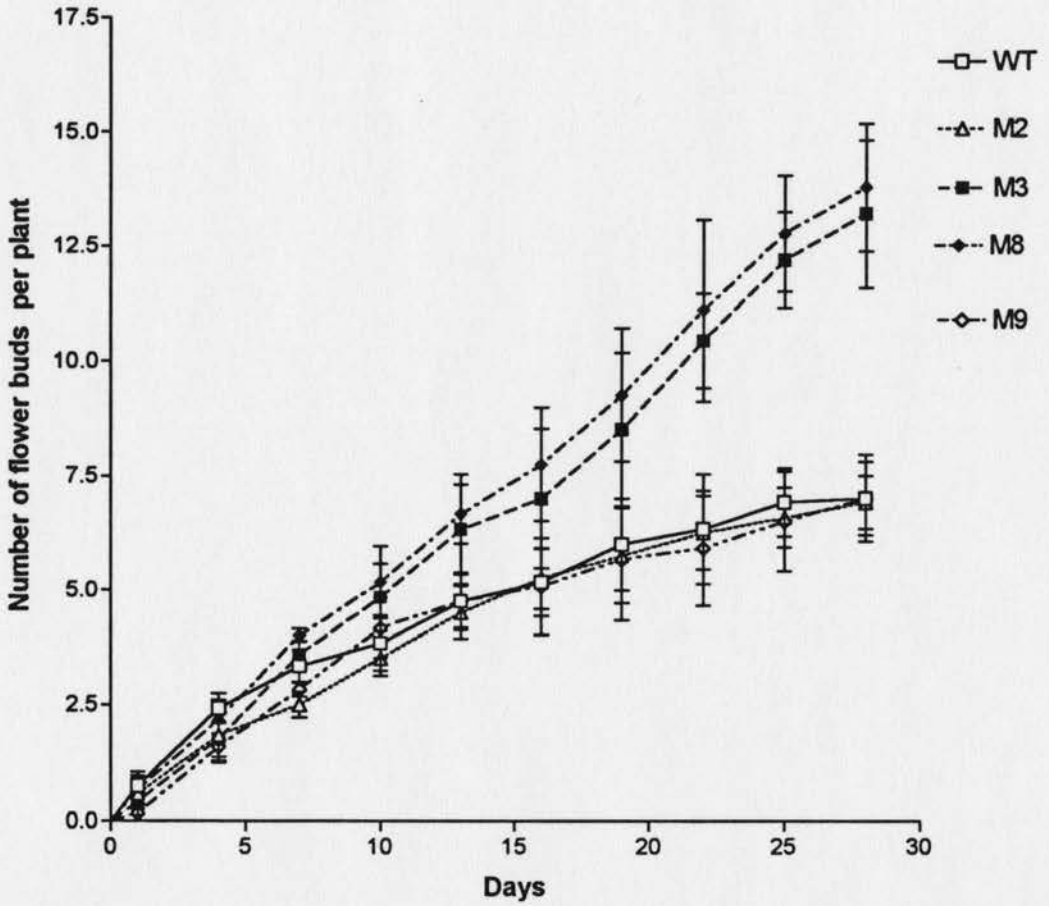

Fig. 2.9 Number of flower buds (per plant) of wild type and T1 transgenic lines after 28 days of salt treatment (salt treatments as described in Fig.2.6)

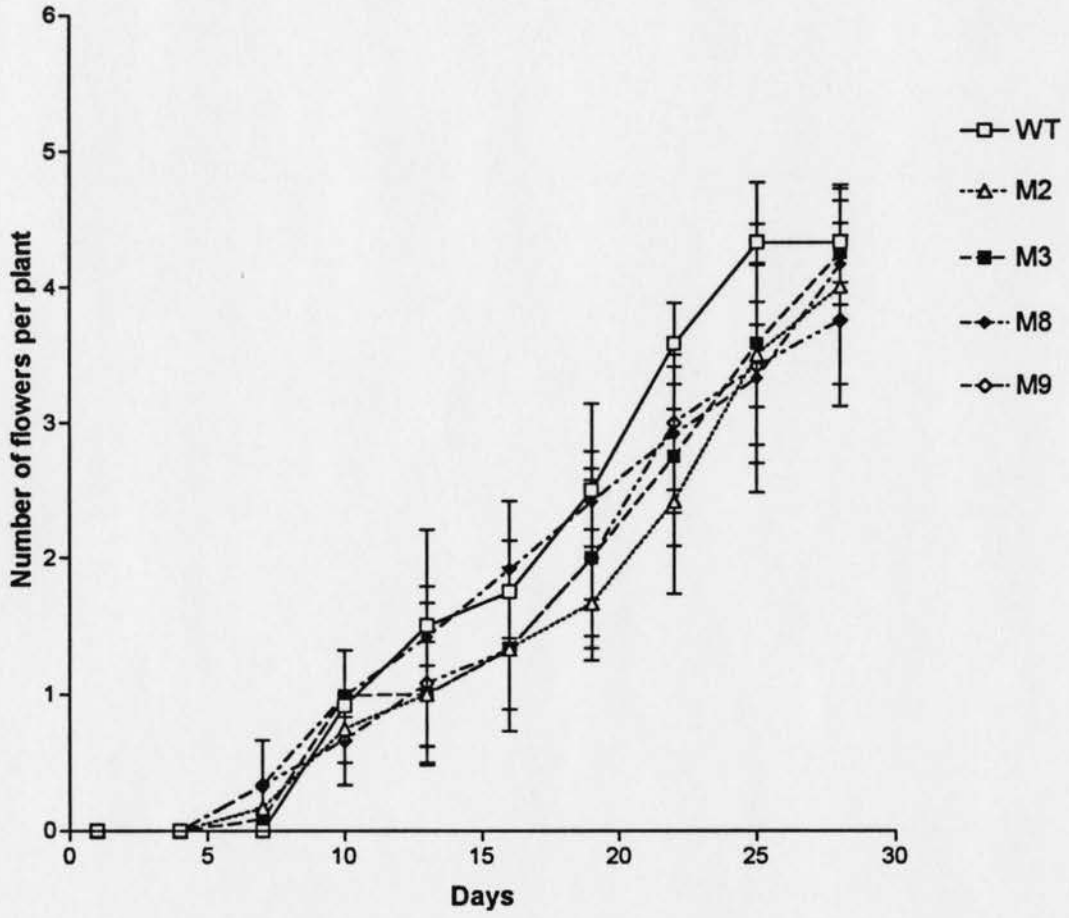

Fig. 2.10 Number of flowers (per plant) of wild type and T1 transgenic lines after 28 days of growth with no salt stress.

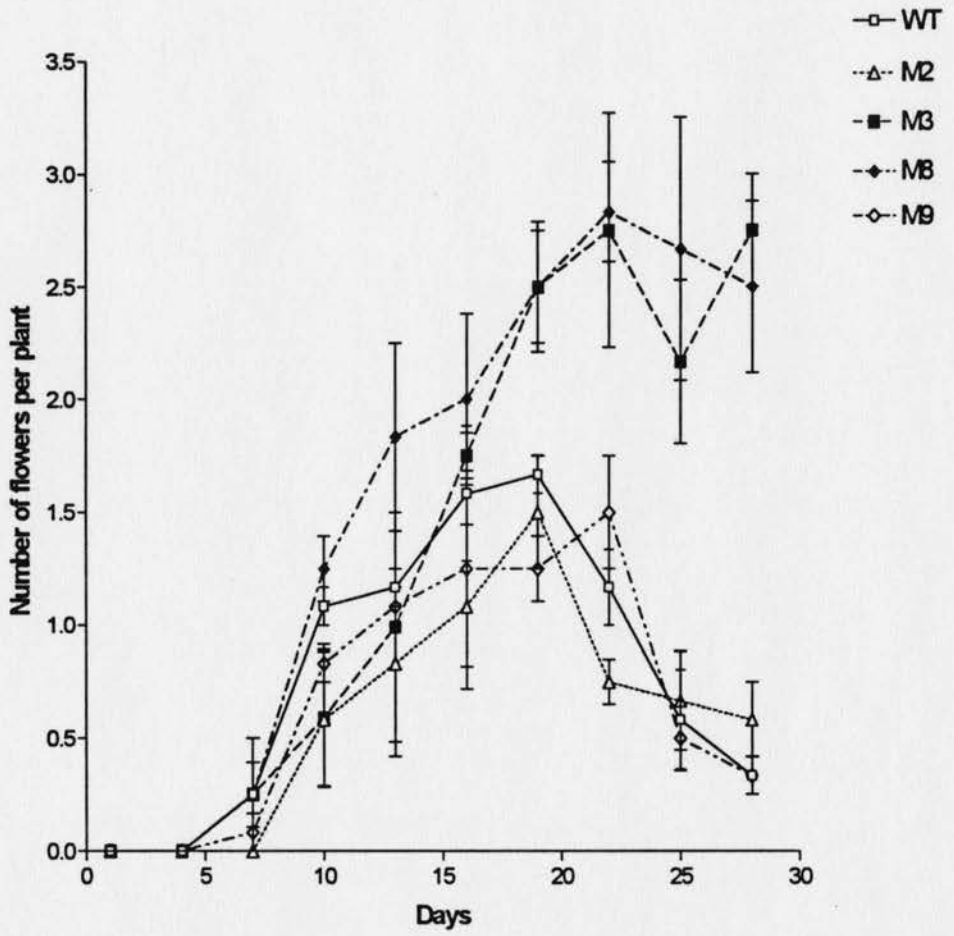

Fig. 2.11 Number of flowers (per plant) of wild type and T1 transgenic lines after 28 days growth with salt treatments (salt treatments as described in Fig. 2.6)

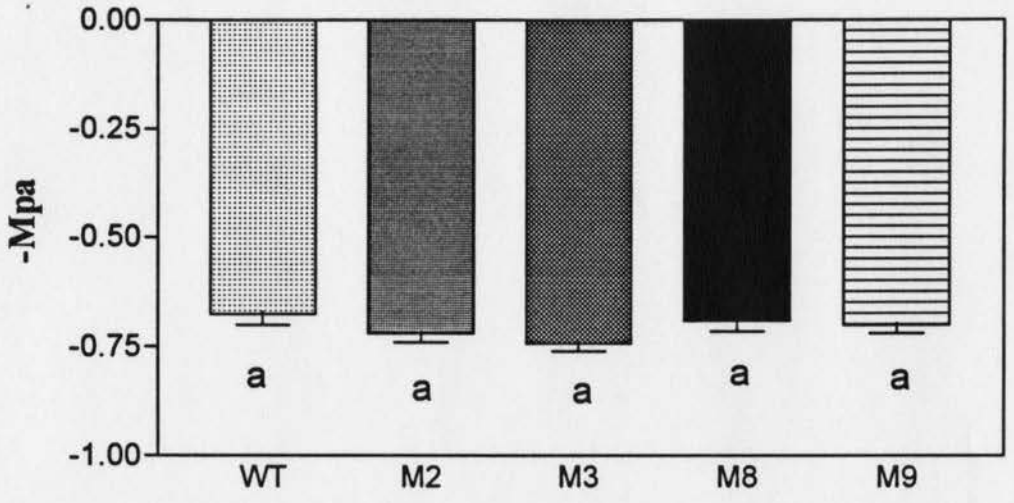

Fig. 2.12 Osmotic potential of wild type and T1 transgenic lines after 28 days growth with no salt stress.

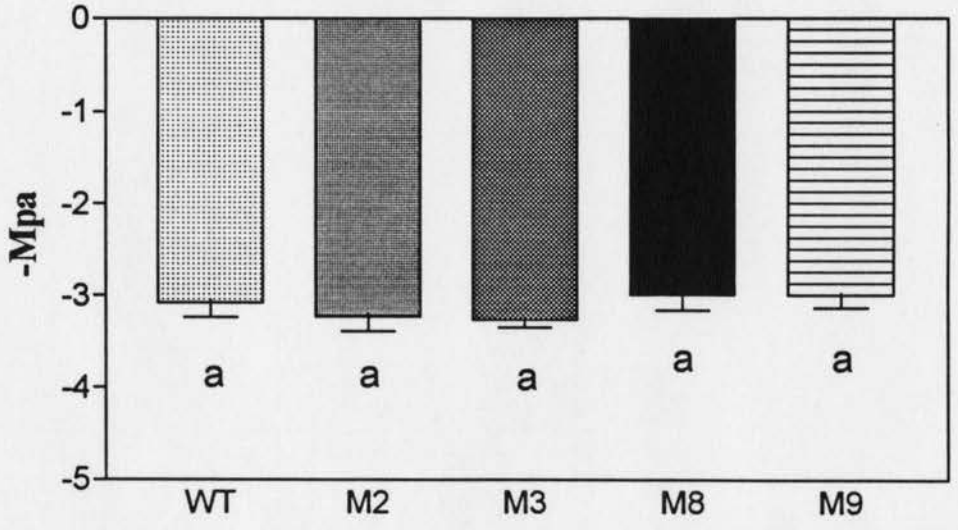

Fig. 2.13 Osmotic potential of wild type and T1 transgenic lines 28 days after salt treatments were completed

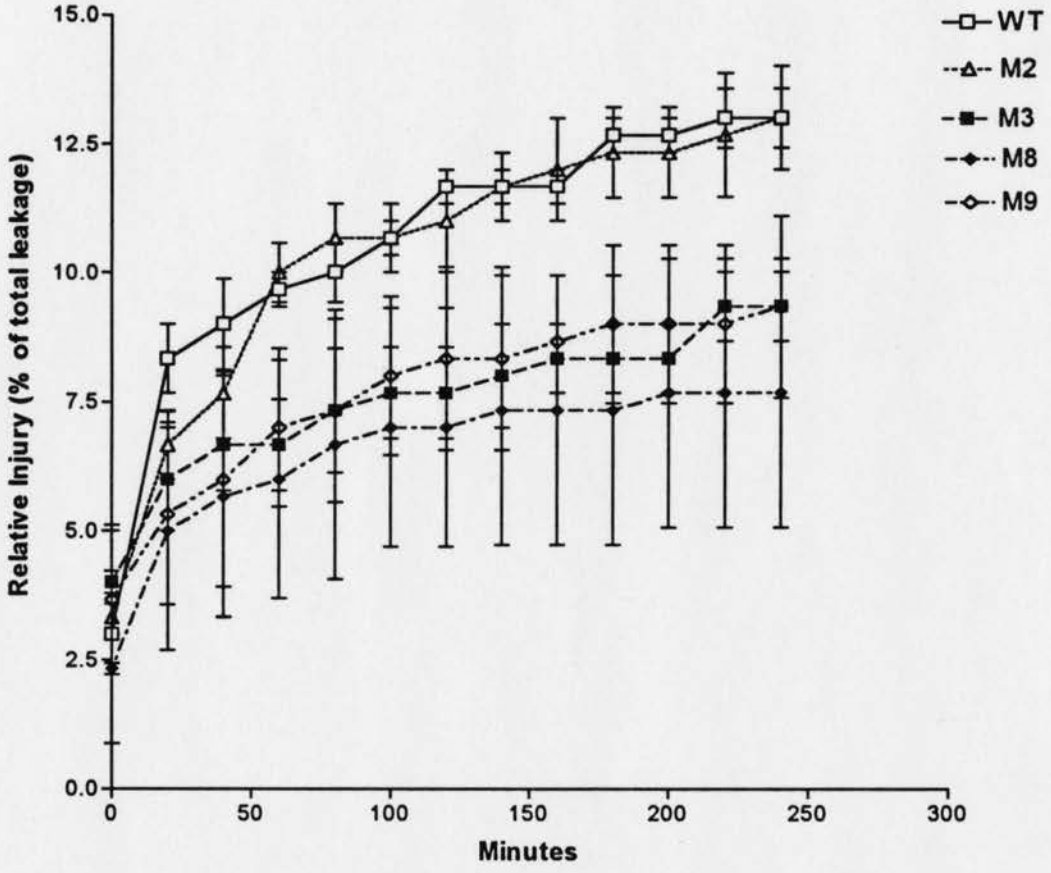

Fig. 2.14 Electrolyte leakage of upper leaves of wild type and T1 transgenic lines after 28 days growth with no salt stress.

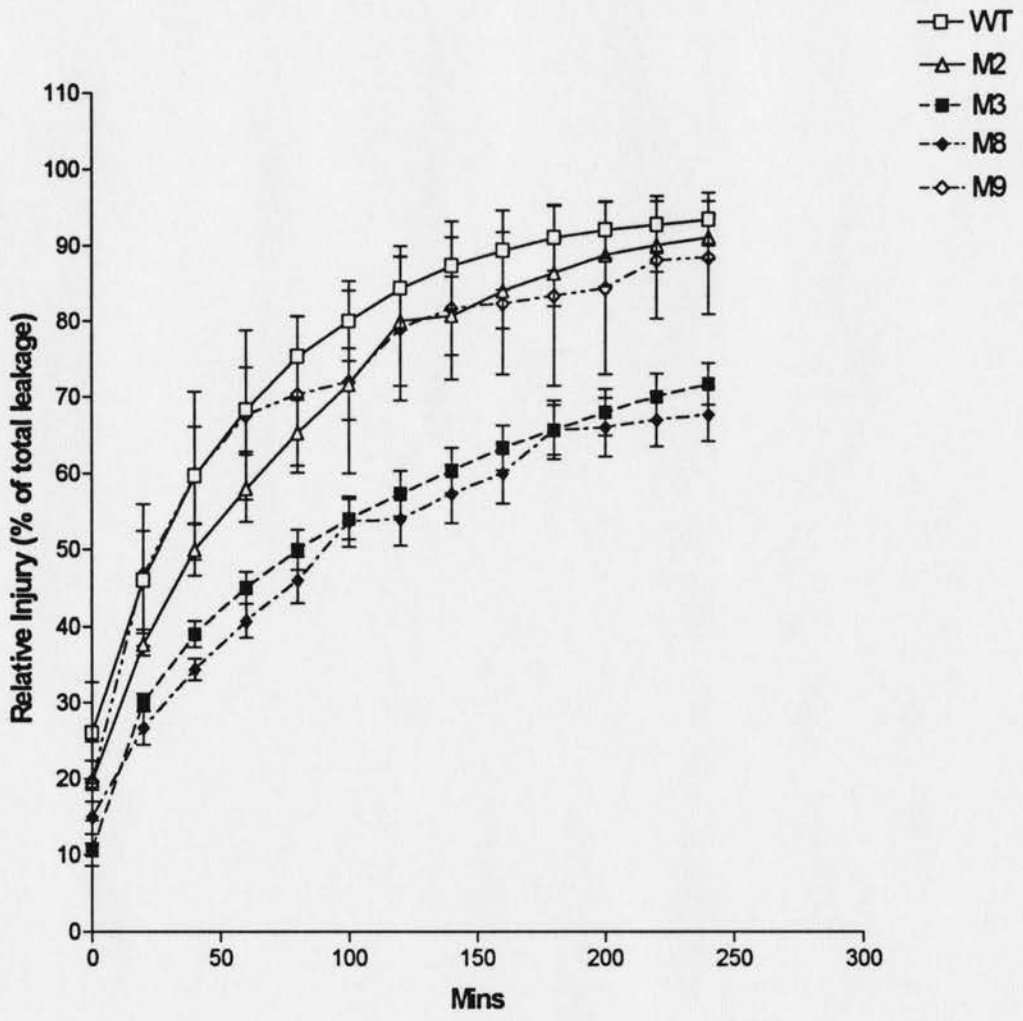

Fig. 2.15 Electrolyte leakage of upper leaves of wild type and T1 transgenic lines after 28 days in the salt treatments (salt treatments as described in Fig. 2.6)

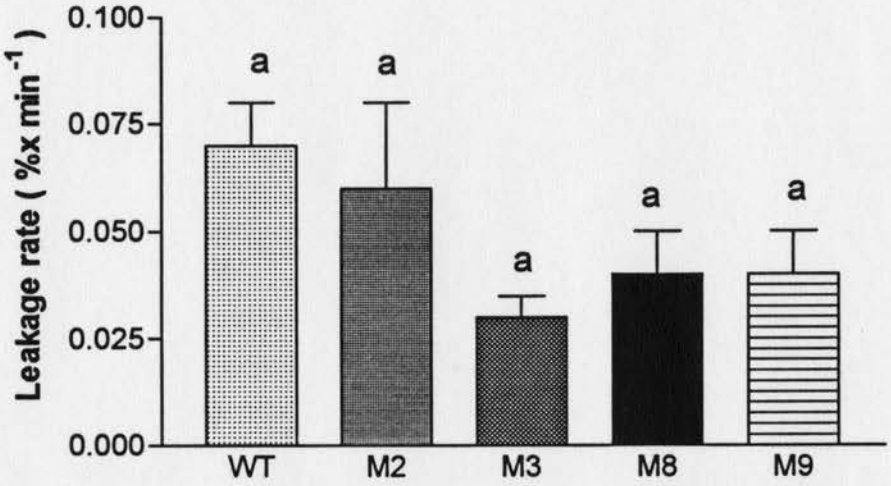

Fig. 2.16 Leakage rate of wild type and T1 transgenic lines 28 days after non-salt treatments were completed

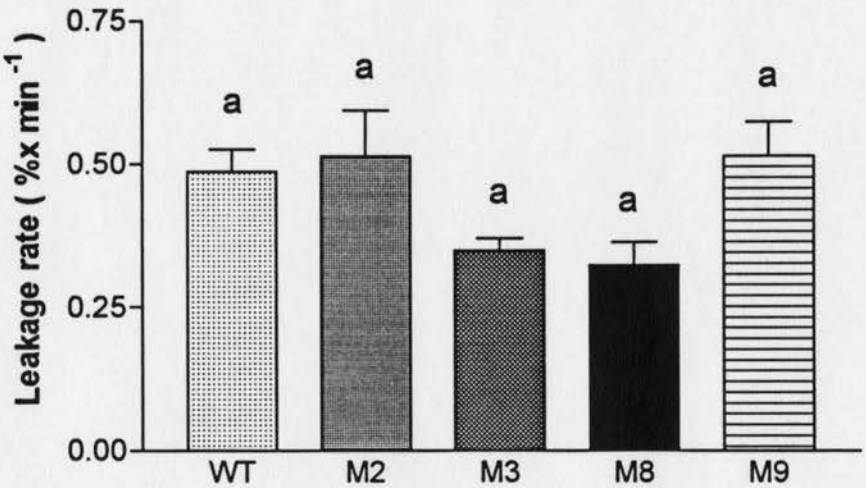

Fig. 2.17 Leakage rate of wild type and T1 transgenic lines 28 days after salt treatments were completed

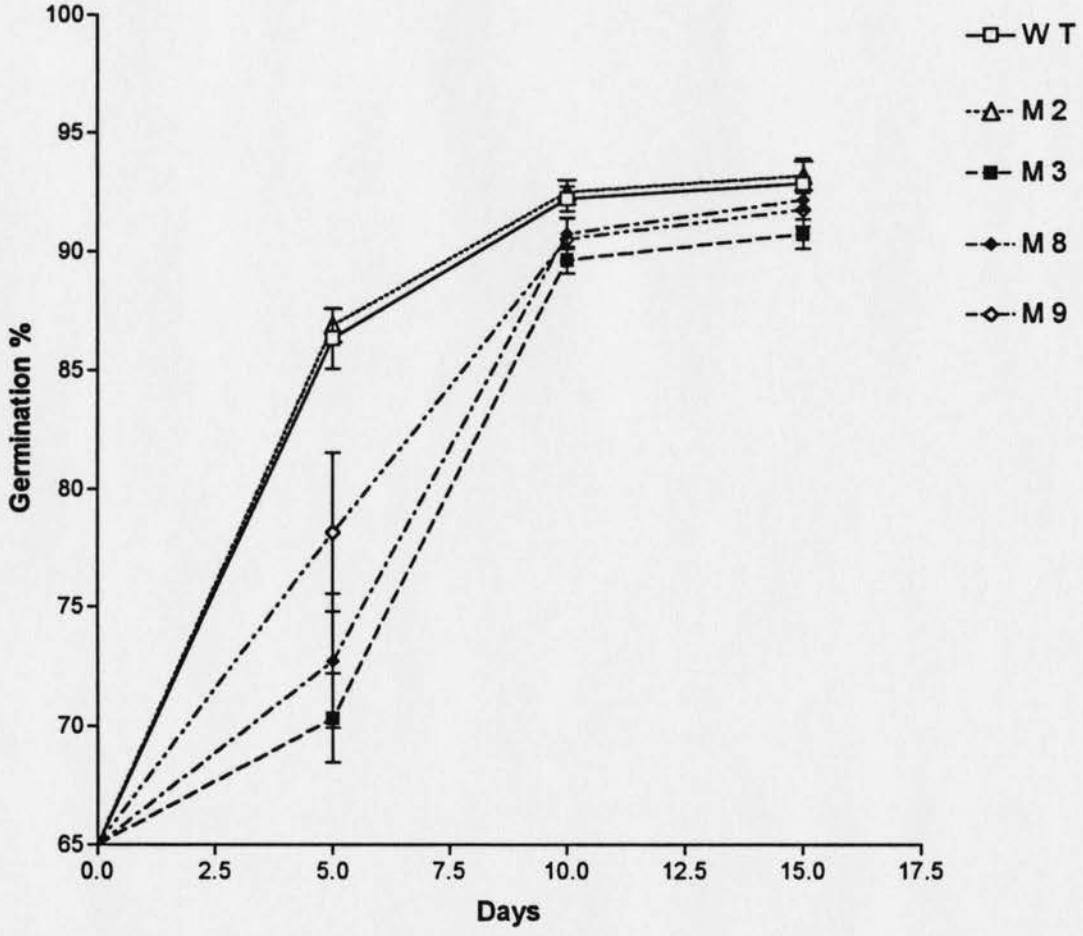

Fig. 2.18 Seed germination of wild type and T2 transgenic lines after 15days in the NaCl - free medium.

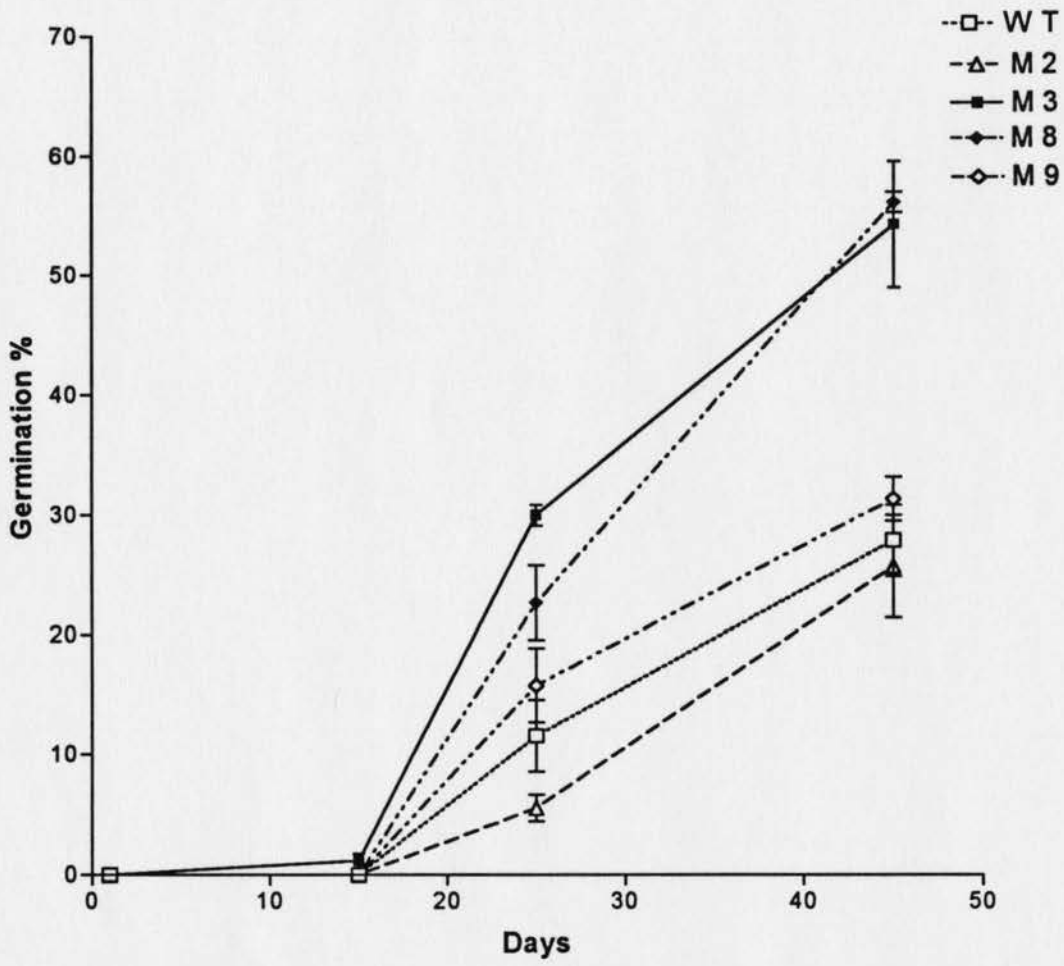

Fig. 2.19 Seed germination of wild type and T2 transgenic lines after 45 days in the medium containing 200 mM NaCl.

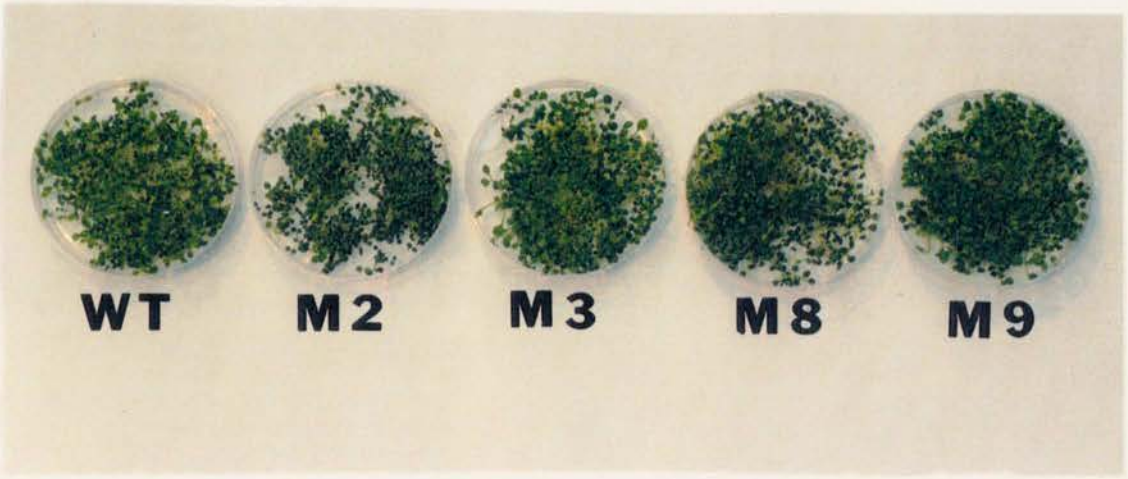

Fig. 2.20 Phenotype of T2 seed germination in wild type and transgenic lines (M2, M3, M8 and M9) after 15 days in the absence of NaCl

Fig. 2.21 Phenotype of T2 seed germination in wild type and transgenic lines (M2, M3, M8 and M9) after 45 days in the presence of 200 mM NaCl

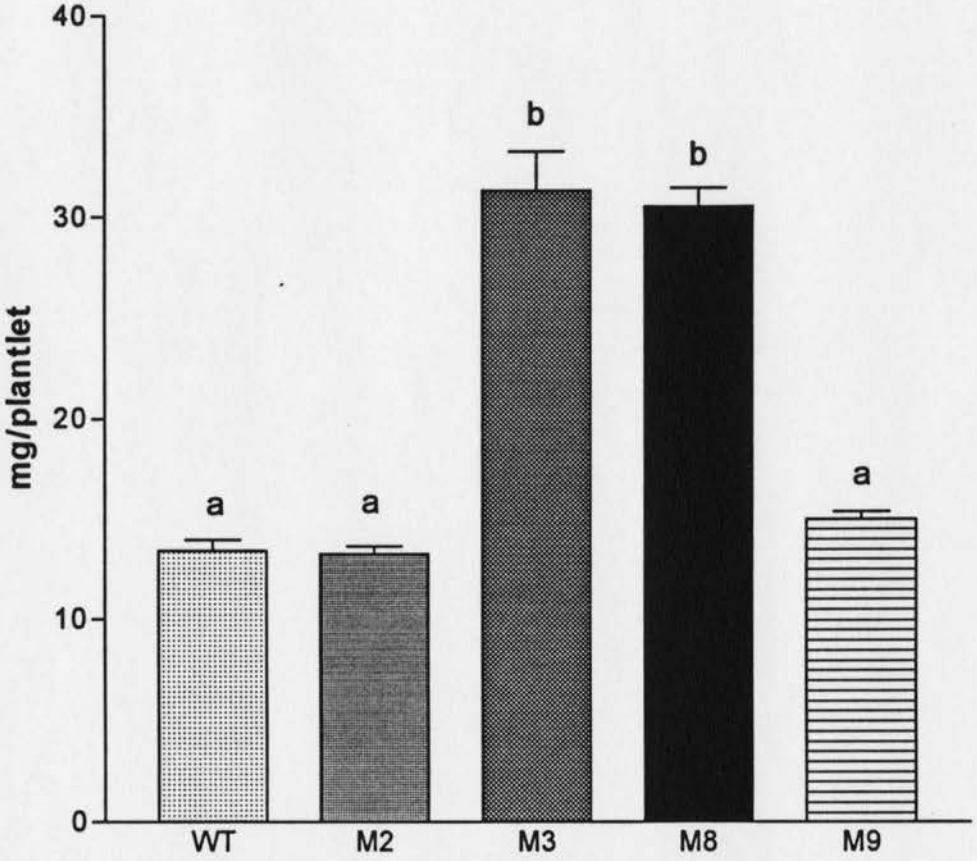

Fig. 2.22 Fresh weight of germinated wild-type and T2 transgenic plantlets after 45 days in the medium containing 200 mM NaCl

Chapter 3

Effect of Mannitol-Expressing Levels to Wild-Type and T1 Transgenic Petunia *cv.Mitchell* growing under Chilling Treatments

3.1 Abstract

"Mitchell diploid petunia, transformed with an *E.Coli mtl D* gene were tested for response to chilling stress. After a 3 week chilling treatment, transgenic lines expressing high mannitol levels were more tolerant of chilling stress in comparison with wild type and transgenic lines expressing low mannitol levels based upon foliage and membrane leakage. Carbohydrate analysis showed that mannitol was the carbohydrate that differed among transgenic lines under normal conditions. Thus, the role of mannitol could be considered in tolerance of plants to chilling stress. Furthermore, leaf osmotic potentials of high-mannitol expressing lines generated from mannitol were only 0.04-0.06 % of osmotic potentials generated from all solutes. Mannitol may act as an osmoprotectant, rather than an osmolyte. Shifts of carbohydrate metabolism were found in both low-mannitol expressing and high-mannitol expressing lines when subjected to chilling stress. Wild type and transgenic lines were classified into high- mannitol expressing and low-mannitol expressing groups based on their mannitol accumulation. The high-mannitol expressing group maintained higher mannitol, fructose and sucrose levels when compared to the low-mannitol expressing group. These sugar levels may also play a role in enhancing the high-

mannitol expressing group to tolerate chilling stress. Floral development was inhibited by chilling stress in both wild type and transgenic lines. High mannitol levels did not help floral development in plants under chilling treatments. However, flowering capacity was resumed in some of transgenic plants expressing high-mannitol levels shortly after transfer to a greenhouse. The data provided in this report suggest that mannitol may act as an osmoprotectant, with the high mannitol expressing transgenic lines imparting increased tolerance to chilling stress in *Mitchell* diploid petunia.

3.2. Introduction

Chilling injury is known as a physiological dysfunction caused by exposure of plants to low but nonfreezing temperatures (Lyons, 1973). The symptoms of chilling injury vary with plant species/cultivars, tissues and growth stages. Chilling injuries generally develop rapidly after transfer of plants or tissues to non- chilling temperatures. Environmental factors such as light intensity, relative humidity and growth conditions prior to chilling also affect the development of chilling injury (Wilson, 1987). Symptoms related to chilling injury include reduced or retarded germination and seedling development, wilting, tissue chlorosis and necrosis, increased electrolyte leakage caused by altered membranes, imbalances in metabolism and accelerated senescence (Saltveit and Morris, 1990).

Plant sensitivity to low temperatures has been recorded for many decades. However, the mechanism of chilling injury is still unclear, especially the possible primary causes of chilling injury. Numerous theories have been proposed as explanations. One of

the earlier theories developed by Lyons and Raison (1970), proposed that chilling can cause physical phase transitions in membrane lipids from a liquid crystalline to a solid gel phase. The phase change temperature of a membrane is determined by the ratio of saturated to unsaturated fatty acids. According to this theory, phase transitions are the primary cause to initiate chilling injury. Then, primary chilling injury (phase transition) induces time-dependent secondary responses such as an increase in permeability, changes in activation energy of membrane-bound enzymes, stimulation of ethylene production, reduction in photosynthesis and interference in energy production (Lyons and Raison, 1970; Raison et al., 1971; Wang, 1982). Other theories to explain the cause of primary chilling response are :(1) increase in the concentration of cytosolic Ca^{2+} (Minorsky, 1985), (2) conformation change in some key regulatory enzymes (Graham et al., 1979), (3) decrease in the rate of cytoplasmic streaming and (4) alteration of cytoskeleton (Woods et al., 1984).

Occurrence of photooxidative stress in plant tissues chilled for prolonged periods has been known for many years. Many reports have shown that reactive oxygen species are generated by chilling and cause membrane peroxidation in chilling sensitive plants (Wise and Naylor, 1987; Burden et al., 1994; Prasad et al., 1994). Reactive oxygen species not only damage membrane lipids, but also damage nucleic acid , proteins and other cellular components (Asada, 1994; Tokuhisa and Browse, 1999). In fact, reactive oxygen species are also generated in plant cells in the normal growth conditions. To prevent damage, plants naturally produce numerous efficient antioxidants such as glutathione, *B*-carotene and ascorbate and antioxidative enzymes such as superoxide

dismutase, ascorbate peroxidase and glutathione reductase (Asada and Takahashi, 1987). Sen et al. (1993) reported that transgenic tobacco plants expressing a pea chloroplastic Cu/Zn superoxide dismutase exhibited a greater recovery from photoinhibition after a chilling treatment 13^{0}C for 4 hours than that of non-transgenic plants. However, under chilling temperatures, the enzymatic protection is less effective due to reduced enzymatic reaction. Thus, endogenous antioxidants may play a more effective role (Hasselt, 1990). Recently, Shen et al. (1997ab) showed that mannitol, a possible hydroxyl radical scavenger, may protect plants against photooxidative stress. Thus, functions of polyols should be considered in chilling induced photooxidation.

In contrast to numerous research reports focusing on the development of chilling injury, the role and composition of soluble sugars on chilling tolerance have been studied much less. It has been shown that there is a positive relationship between invertase activity and accumulation of reducing sugars at low temperature (5^{0}C) in detached grapefruit flavedo tissues (Purvis and Rice, 1983). King et al. (1988) reported that dark treatment of tomato seedlings prior to chilling stress results in increasing the severity of chilling injury, and that light exposure of seedlings prior to chilling results in increasing the chilling tolerance. Similar results also were obtained in cotton and maize seedlings (Rikin and Guier, 1979; Prasad et al., 1994). These evidences all suggest that carbohydrate depletion caused by dark treatment may relate to increased chilling injury (King et al., 1988).

Soluble sugars and sugar alcohols have been known to play important roles in protecting plants from stresses such as salinity, drought and frost. They also may have

either direct or indirect roles in chilling tolerance of plants. It has been suggested that soluble sugars and sugar alcohols can enhance chilling tolerance by osmotic adjustment, stabilization of membranes and proteins, and by serving as energy sources for plants (Purvis, 1990).

The study reported here was designed to investigate chilling tolerance using transgenic petunia plants expressing high mannitol levels and altered soluble carbohydrate levels under chilling treatments.

3.3. Material and methods

3.3.1. Plant material

Wild type and T1 transgenic lines (M2, M3, M8, and M9) used in this study were the same as described previously in 2.3.7. The shoot rooting and plantlet acclimation processes also were the same as described in 2.3.7. However, instead of sand medium, plants were grown in the potting soil medium for four more weeks under the same conditions as described previously in 2.3.7.

3.3.2. Chilling treatments

No reference to chilling temperatures, nor to duration required to cause injury (CI) of petunia *cv.Mitchell* has been published . Therefore, prior to conducting chilling experiments, with transgenic lines, two chilling cycles, day 5 ^{0}C/night 3 ^{0}C and day 3 ^{0}C/ night 0 ^{0}C at 12hr photoperiod were first tested.

No significant symptoms of chilling injury were observed in the wild-type plants using the first chilling cycle (day 5 ^{0}C/ night 3 ^{0}C) after 10 days. In contrast to the results

of the first chilling cycle, the second chilling cycle (day 3 ^{0}C/night 0 ^{0}C) did cause significant necrotic spots in the upper leaves of wild-type plants after 10 days in the chilling treatments and following 2 days in a growth chamber (day 25 ^{0}C and night 20 ^{0}C at 16 hr photoperiod). . Thus, the second chilling cycle (day 3 ^{0}C/night 0 ^{0}C at 12 hr photo period) was chosen. Further tests, using a 3 -week chilling duration with this cycle produced severe chilling injuries and wilting of wild-type plants.

Eight-week-old plants of each line were separated into two groups. The control group (4 plants for each line) remained in the growth chamber 25 ^{0}C day/20 ^{0}C night at 16 hr photoperiod. Another group was immediately transferred to a cold room to induce chilling stress (day 3 ^{0}C/night 0 ^{0}C, 12 hr photoperiod and 75 % relative humidity) for 3 more weeks. The control and chilling-stressed plants were watered every two days and fertilized with a complete soluble fertilization once every two weeks.

3.3.3. Leaf tissue harvesting

At the end of chilling treatment, upper leaves of wild-type and transgenic lines were collected directly from the cold room (the chilling group) or growth chamber (the control group). The collected leaf samples were freeze dried and used to measure soluble carbohydrates and osmotic potential.

3.3.4. Evaluation of chilling injury

After chilling treatments were completed, plants of both wild-type and transgenic lines were held at room temperature and visual injury/relative conductivity were measured after one day.

3.3.5. Measurements of carbohydrates, osmotic potential and relative conductivity

Carbohydrate analysis, and measurements of osmotic potential and relative conductivity of the chilling and control plants were as described previously in 2.3.9, 2.3.10, 2.3.11 and 2.3.12.

3.4. Results

3.4.1. Visual chilling symptoms of plants

Exposure of 8-week-old wild-type and T1 transgenic plants (lines of M2, M3, M8, and M9) after 3 weeks in a (day 3 ^{0}C/night 0 ^{0}C) cycle, produced visible leaf or whole plant wilting in wild type plants and transgenic lines M2 and M9 (lines expressing low mannitol levels) after transfer to room temperature for one day (Fig. 3.1). However, plants of transgenic line M3 and M8 (lines expressing high mannitol levels) regained turgor or remained turgid after the same treatments and only a few leaves showed necrosis (Fig. 3.2). Similar visual results were observed in 6-week old plants after the same treatments (Fig. 3.3). In fact, in a pre-experiment of this study, exposure of 8-week old wild type and transgenic lines after 10 days in a day 3 ^{0}C/night 0 ^{0}C cycle and a following two- day light treatment (12 hr photo period) at day 25 ^{0}C/night 20 ^{0}C, necrotic or chlorotic spots occurred in the leaves of wild type, and transgenic line 2 and 9 but not in transgenic line 3 and 8 (Fig. 3.4). Although no wilting symptoms were observed in this pre-experiment, results of this pre-experiment still indicated that leaves of wild type and transgenic lines expressing low mannitol levels actually can be damaged at an earlier stage of the chilling treatments.

The eight-week-old plants were chosen for replicated experiments because this is the beginning stage of flower-bud development. In addition to the effect of chilling on the vegetative growth, we also wanted to examine the effect of chilling on the reproductive growth of plants. Unfortunately, there were no visual differences of reproductive development among wild type and all transgenic lines whether high-mannitol expression or not during chilling period (Fig. 3.1 and 3.2).

3.4.2. Osmotic potential of chilled plants

All leaf samples (upper leaves)used in the measurements of osmotic potential were collected at the end of both chilling and non-chilling treatments. Osmotic potentials were not significantly different among wild type, nor among the transgenic lines under non-chilling treatments (Fig. 3.5). Furthermore, osmotic potentials also did not differ among wild type and all transgenic lines under chilling treatments (Fig. 3.6). However, the osmotic potentials of non-chilled plants were significantly higher, about 0.4 MPa when compared with those of chilled plants (Fig. 3.5 and 3.6). Thus, it appears that osmotic adjustment occurred in the leaves of plants exposed to chilling treatments.

3.4.3. Relative conductivity and leakage rate of chilled plants

Upper leaf samples used in this experiment were collected one day after transfer of chilled plants to room temperature, and leaf samples of non-chilled plants were collected

directly from the 25 ^{0}C growth chamber. Relative conductivity of non-chilled wild type and transgenic lines did not differ at any point of the measurement period (Fig. 3.7). However, relative conductivity of chilled plants did differ among wild type and transgenic lines (Fig. 3.8). Relative conductivity of transgenic lines M3 and M8 was significantly lower than other lines.

Calculated leakage rates of wild type and transgenic lines were similar under non-chilling treatments (Fig. 3.9). Lower leakage rates were detected in transgenic lines M3 and M8 in chilled plants compared to other lines (Fig. 3.10). Interestingly, leakage rates of chilled wild type and transgenic lines M2 and M9 were higher than those of non-chilled ones, but no difference of leakage rates were observed in transgenic line M3 and M8 whether chilled or not. These data indicate that less chilling damage occurred in transgenic lines M3 and M8.

3.4.4. Analysis of non-structual carbohydrates of chilling

Under non-chilling treatments, there were no differences in content of inositol, glucose, fructose, sucrose, raffinose and stachyose between wild type and transgenic lines (Table 3.1). Mannitol was the only soluble sugar affected by plant line, being higher in transgenic lines M3 and M8 and lower in wild type and transgenic lines M2 and M9 (Table 3.1). Mannitol concentrations of transgenic lines M3 and M8 was about 3 times that of wild type and transgenic lines M2 and M9. Mean mannitol levels of transgenic lines M2 and M9 was higher than of the wild type, but the differences were not significant.

Furthermore, expression of higher mannitol levels in transgenic lines did not affect synthesis of other carbohydrate because there was no significant concentration difference among sugars of wild type and transgenic lines.

In general, leaf soluble carbohydrates of both wild type and transgenic lines were decreased by chilling except for raffinose and stachyose (Table 3.2). These results indicated that metabolic processes were changed by chilling in petunia plants. Interestingly, mannitol levels of transgenic line M3 and M8 were still higher than wild type and the other two transgenic lines, even though mannitol levels of wild type and all transgenic lines declined following chilling treatments (Table 3.2). Mannitol levels of transgenic line M3 and M8 were still about 4 times higher than those of wild type and transgenic line M2 and M9. In addition, sucrose levels of transgenic line M8 were significantly higher than wild type and the other transgenic lines but not M3 under chilling treatments.

In a subsequent carbohydrate analysis, wild type and transgenic lines were catagorized into high-mannitol expressing (M3 and M8) and low-mannitol expressing (WT, M2 and M9) groups based on their response to chilling treatments and mannitol-expression levels (transgenic line M3 and M8 showed less relative conductivity and higher mannitol levels than those of wild type and transgenic line M2 and M9). Levels of all soluble carbohydrates, but mannitol did not differ between low-mannitol expressing and high-mannitol expressing groups under non-chilling conditions (Table 3.3). However, under chilling conditions, levels of mannitol, fructose, sucrose and raffinose from the high-mannitol expressing group were always higher than those of low-mannitol expressing

group, but levels of inositol, glucose and stachyose were not different between these two groups (Table 3.3). Furthermore, in this study, the effect of chilling did cause a shift of carbohydrate metabolism. Levels of inositol and mannitol were decreased by chilling treatments, and levels of raffinose were significantly enhanced by chilling treatments (Table 3.3). Interestingly, similar levels of glucose, fructose and sucrose were found in the high-mannitol group treated with chilling and in both non-chilled groups (Table 3.3).

3.5. Discussion

Visually, recovery of chilled transgenic lines M3 and M8 expressing high mannitol levels was greater than in wild type and the other transgenic M2 and M9 lines after 3 weeks in the chilling treatments (Fig. 3.1 and 3.2). Only a few wilting or necrotic leaves occurred in the chilling lines M3 and M8 in comparison with other lines. In a subsequent experiment, death or stunted growth was observed in the chilled wild type, and transgenic lines M2 and M9, but transgenic lines M3 and M8 did show continuous growth and some of them even flowered about 10 days after transfer to the greenhouse (data not shown). The most widely used method of measuring chilling injury has been the leakage of cellular contents by conductivity, which is thought to reflect membrane damage (Wilson, 1987). In this study, less relative conductivity and lower leakage rate were observed in the transgenic lines expressing high mannitol levels. Mannitol seems to play a role in protecting plants from chilling injury.

Photooxidation of photosynthesis in various plant species at low temperatures has been reported (Bloom et al., 1998; O 'Kane et al., 1996 and Prasad et al., 1994). Under chilling conditions, excess light energy can not be converted to CO_2 fixation and then may cause damage to the photosynthetic system by the formation of reactive oxygen species (Foyer et al., 1994). Main targets of reactive oxygen species include proteins, membrane lipids, leaf pigment and other cellular components (Hasselt, 1990; Asada, 1994). Studies have shown that reactive oxygen species can cause membrane lipid peroxidation by increased formation of malondialdehyde and protein degradation by promotion of intra- and inter molecular cross-linking such as protein fragmentation and -s-s bonding (Stadtman, 1992, and Shen et al., 1997b). It has been suggested that these reactive oxygen species accumulate and cause damage due at low temperatures to reduction of scavenging enzymes and antioxidants (Tokuhisa and Browse, 1999). Recent studies have shown that transgenic tobacco expressing glutathione reductase from *E.Coli* and pea can tolerate more serious oxidative stress in comparison to the wild type (Sen et al., 1993). However, Bruggemann et al. (1999) reported that transgenic tomato expressing glutathione reductase is identical on chilling sensitivity of the photosynthetic apparatus to the wild type. Thus, the antioxidative system may be questioned as a sole mechanisom to protect plants from chilling stress.

Many osmoprotectants such as glycine betaine, proline, reducing sugars and sugar alcohols increase in response to various stresses. One of their suggested functions is to scavenge free radicals (Kavi Kishor et al., 1995; Shen et al., 1997b, and Hayashi et al., 1997). Purvis (1981) reported that the greatest tolerance of a grapefruit peel to chilling

injury coincided with high proline levels. Overexpressing glycine betaine in transgenic *Arabidopsis thaliana* also was found to enhance tolerance to chilling stress (Hayashi et al., 1997). Among the reactive oxygen species, hydroxyl radicals cause most of the oxidative damage during drought and chilling stress (Shen et al., 1997a). It has been suggested that mannitol can react with hydroxyl radicals to form mannitol radicals, which are then converted to mannose in the presence of oxygen (Franzini et al., 1994, and Shen et al., 1997a). Furthermore, mannitol is suggested to protect thiol-regulated enzymes such as phosphoribulokinase, thioredoxin, ferredoxin and glutathione from damage caused by hydroxyl radicles (Shen et al., 1997b). In our study, although the fate and location of mannitol were unclear, the greatest recovery in plants from chilling stress also coincided with high mannitol levels. Furthermore, using the Van 't Hoff equation, osmotic potential = -ciRT, osmotic potential of mannitol produced in transgenic line M3 and M8 treated with chilling is about 0.0005, and 0.0006 MPa respectively (assuming 90 % water content in leaf and T= 279 ^{0}K). The osmotic potentials generated from accumulated mannitol of transgenic line M3 and M8 are roughly 0.04 % and 0.06 % of osmotic potentials generated from all solutes. Thus, mannitol is not likely an effective osmolyte for osmotic adjustment in this study, and it should rather be considered as an osmoprotectant.

The mechanism of plants to tolerate chilling stress is unclear. However, there is increasing but uncertain evidence that carbohydrate level and composition can affect chilling tolerance (Crawford and Huxter, 1972, and Purvis, 1990). In carbohydrate analysis in this study, chilling caused a shift of carbohydrate metabolism in wild type and transgenic plants (Table 4.3). However, a positive correlation between soluble sugar

levels and chilling was not observed. It is known that there are strong correlations between the soluble carbohydrate levels/composition of plants and frost hardiness (Fischer and Holl, 1991 ; Stushnoff et al., 1993 and Imanihi et al., 1998). Since chilling stress is quite different from freezing stress, the positive correlations between soluble carbohydrate level/composition and chilling tolerance should not be expected in this study. Only a few studies have shown a positive correlation between soluble carbohydrate level/composition and chilling tolerance (Taylor et al., 1972; Purvis and Rice, 1983).

Maintenance of adequate sugar supplies in tomato was found to be associated with better growth at chilling temperatures (King et al., 1988). In this study, maintenance of higher soluble sugar levels in the high-mannitol expressing group treated with chilling stress also were found to be associated with chilling tolerance. Thus, these soluble carbohydrate levels also should be considered to play a role in petunia plants to tolerate chilling stress.

The diversion of carbon to polyol biosynthesis under abiotic stress has been reported by Bieleski, 1982 and Bohnert et al., 1995. It has been suggested that inositol can serve as a source of raffinose family oligosacchanides (RFO) such as raffinose, stachyose and verbascose (Bohnert et al., 1995) . However, a positive correlation between inositol and the raffinose group levels was not found in this study.

It is hard to describe how the increased raffinose and unchanged stachyose levels occurred in both chilling mannitol expressing groups from the data provided in this study. However, since raffinose and stachyose are important products as osmolytes or osmoprotectants in cold-acclimated plants, they may provide similar functions in chilling-

treated plants or they may increase in response to cold acclimation conditions present in the chilled plants.

A series of enzymes to degrade mannitol have been located in the cytosol of celery sink tissues (Stoop et al., 1996). In celery tissues, mannitol is converted to fructose-6-P through a series of enzymatic activities. Decreased mannitol levels found in both chilled high-mannitol expressing and low-mannitol expressing groups could be that mannitol degradative enzymes in the cytosol were strongly induced in response to chilling stress.

Similar levels of glucose, fructose and sucrose were found in the chilled high-mannitol expressing and other non-chilled mannitol-expressing groups. In contrast, levels of glucose, fructose and sucrose, of the chilled low-mannitol expressing group, were lower in comparison with those of non-chilled ones. Since fructose, glucose and sucrose are important substrates in plant metabolism, maintenance of these soluble sugars may also involve enhancing tolerance of the high-mannitol expressing group to chilling stress. Why soluble sugar levels can be maintained in the chilled high-mannitol expressing group is a good question. In this study, mannitol production is the only single carbohydrate affected by plant lines. Thus, the possible role of mannitol as a free radical scavenger to prevent photooxidative stress from plants under chilling treatments associated with light can not be neglected. We suggest here that mannitol may shield chilling-treated plants from the attack of reactive oxygen species to membranes, nucleic acids, proteins and other cellular components. Thus, the rates of photosynthesis and respiration can be sustained in the high-mannitol expressing group at chilling temperatures.

No further reproductive development of both wild type and transgenic lines under

a chilling environment was observed. However, the flowering capacity was resumed in the high mannitol-expressing lines. These observations were confirmed with several previous studies (Caulfield and Bunce, 1988; Wang and Baker, 1979). It has been suggested that the delays in reproductive development enable plants to partition more biomass into vegetative development (Wang and Baker, 1979).

In summary, our results showed that transgenic petunia plants expressing high mannitol levels did possess a greater capacity to tolerate long-term chilling stress than that of wild type and transgenic lines expressing low mannitol levels. Mannitol levels in high-mannitol expressing lines were not adequate to protect by osmotic adjustment caused by chilling stress. Therefore, the functions of mannitol should be considered as free radical scavengers or osmoprotectants, rather than osmolytes.

The fate and location of mannitol in the transgenic lines was not studied. High mannitol expressing transgenic lines from introduction of the *mtl D* gene may be due to incorporation into chloroplasts, thus preventing mannitol from degradation caused by the cytosolic mannitol-degradative enzymes which may be strongly induced by chilling. Thus, plant metabolism can be continued in the high-mannitol expressing lines under chilling stress associated with light. Or mannitol itself may very effectively quench reactive oxygen species directly preventing damage to photosystem II in the chloroplast. Future studies are necessary to investigate if isolated chloropasts of high-mannitol expressing lines do contain higher mannitol levels than those of low-mannitol expressing lines. Moreover, the rates of photosynthesis in both low-mannitol expressing and high-mannitol expressing lines exposed to the short-term or long-term chilling stress also should be

investigated to distinguish if higher mannitol levels can decrease the degree of photooxidiation caused by chilling associated with light.

Table. 3.1 Carbohydrate and mannitol concentrations (μmol.g^{-1} dry weight basis) in upper leaves of nonchilled wild type and T1 transgenic lines

Plant line	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
WT	9.39±0.94 a[z]	0.86±0.01 a	23.54±1.30 a	42.95±8.56 a	23.75±3.09 a	1.85±0.51 a	0.83±0.28 a
M2	11.39±0.92 a	1.15±0.05 a	21.02±2.00 a	52.57±2.79 a	24.84±4.65 a	2.55±0.82 a	0.56±0.07 a
M3	11.07±0.76 a	3.32±0.31 b	20.25±3.37 a	50.56±7.63 a	25.84±2.63 a	2.76±0.36 a	0.63±0.18 a
M8	10.18±0.56 a	3.45±0.07 b	19.01±1.24 a	49.08±6.66 a	25.79±1.14 a	2.54±0.52 a	0.64±0.17 a
M9	8.18±1.58 a	1.13±0.12 a	25.25±1.91 a	43.55±2.08 a	20.93±1.54 a	2.60±0.34 a	0.74±0.20 a

Data are from three replicated experiments, in μmol/g dw for each sugar and polyol.
Values are mean ± SEM.

[z] Means followed by the same letter within each column are not significantly different at $P < 0.05$ by Tukey's Studentized Comparison Test.

Table.3.2 Carbohydrate and mannitol concentrations (μmol.g^{-1}dry weight basis) in upper leaves of wild type and T1 transgenic lines 3 weeks after chilling treatments were completed

Plant line	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
WT	6.46±1.01 a[z]	0.23±0.05 a	17.54±3.75 a	36.43±6.15 a	11.25±2.25 a	5.08±1.16 a	062±0.11 a
M2	4.94±0.63 a	0.36±0.03 a	17.22±2.19 a	35.13±6.00 a	13.11±1.08 a	4.81±0.34 a	0.46±0.09 a
M3	5.04±0.66 a	2.09±0.49 b	20.31±6.30 a	48.57±8.40 a	18.10±1.54 ab	6.39±0.14 a	0.63±0.10 a
M8	4.11±0.14 a	2.28±0.41 b	19.85±1.73 a	51.87±5.42 a	20.56±2.43 b	5.90±0.72 a	0.37±0.06 a
M9	3.75±0.64 a	0.21±0.03 a	19.45±3.51 a	35.59±2.59 a	11.54±2.74 a	5.28±0.73 a	0.27±0.04 a

Data are from three replicated experiments, in μmol/g dw for each sugar and polyol.
Values are mean ± SEM.

[z] Means followed by the same letter within each column are not significantly different at P < 0.05 by Tukey's Studentized Comparison Test.

Table.3.3 Carbohydrate analysis of high-mannitol expressing and low-mannitol expressing groups under non-chilling and chilling treatments

Chilling treatment	Group	Inositol	Mannitol	Glucose	Fructose	Sucrose	Raffinose	Stachyose
No	Low-mannitol expressing	9.99±0.71 a[z]	1.05±0.01 a	23.21±1.22 a	46.36±3.11 a	23.17±1.17 a	2.33±0.24 a	0.60±0.02 a
	High-mannitol expressing	10.63±0.45 a	3.39±0.07 b	20.64±0.39 ab	49.83±0.75 a	25.82±0.03 a	2.65±0.11 a	0.64±0.01 a
Yes	Low-mannitol expressing	5.05±0.79 b	0.26±0.08 c	18.07±1.21 bc	35.72±0.39 b	11.51±0.86 b	5.06±0.14 b	0.45±0.10 a
	High-mannitol expressing	4.58±0.47 b	2.18±0.01 d	20.08±0.23 ac	50.22±1.65 a	19.33±1.17 a	6.15±0.25 c	0.50±0.13 a

Wild type , T1 transgenic lines M2 and M9 were collected as low-mannitol expressing group. T1 transgenic lines, M3 and M8 were collected a high-mannitol expressing group.
Data are from three replications, presented in $\mu m.g^{-1}$ dw for each sugar and polyol. Values are mean ± SEM.
[z] Means followed by the same letter within each column are not significantly different at $P < 0.05$ by Tukey's Studentized Comparison Test.

Fig. 3.1 Phenotype of wild type and T1 transgenic lines M2 and M9 after one day at 25 °C, following a 3-week chilling treatment (3 °C day, 0 °C night, 12hr photoperiod).

Fig. 3.2 Phenotype of wild type and T1 transgenic lines M3 and M8 after one day at 25 °C, following a 3-week chilling treatment (3 °C day, 0 °C night, 12 hr photoperiod).

Fig. 3.3 Phenotype of 6 week-old wild type and T1 transgenic lines after one day at 25 ^{0}C following a chilling treatment for 3 weeks at 3 ^{0}C day, 0 ^{0}C night, 12 hr photoperiod.

Fig.3.4 Phenotype of wild type and T1 transgenic lines two days after 10-day chilling treatments were completed

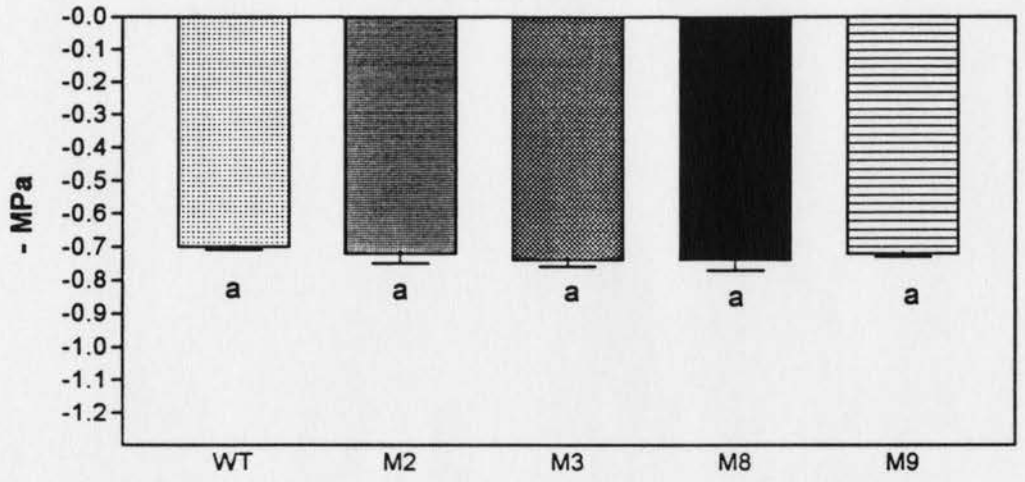

Fig.3.5 Osmotic potential of upper leaves of wild type and T1 transgenic lines 3 weeks after non-chilling treatments were completed. Values are mean ±SEM.

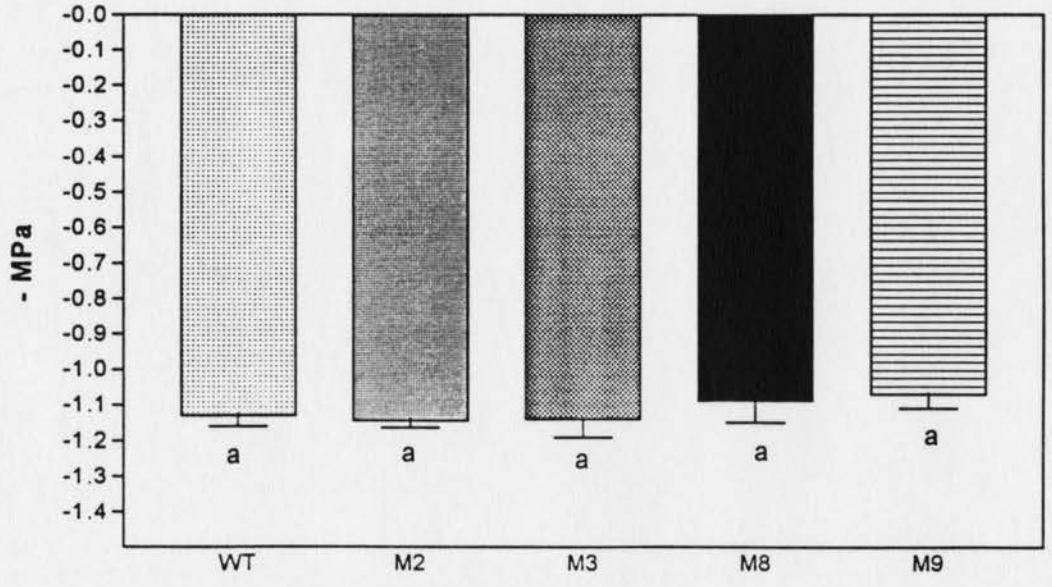

Fig.3.6 Osmotic potential of upper leaves of wild typd and T1 transgenic lines 3 weeks after chilling treatments were completed. Values are mean ±SEM.

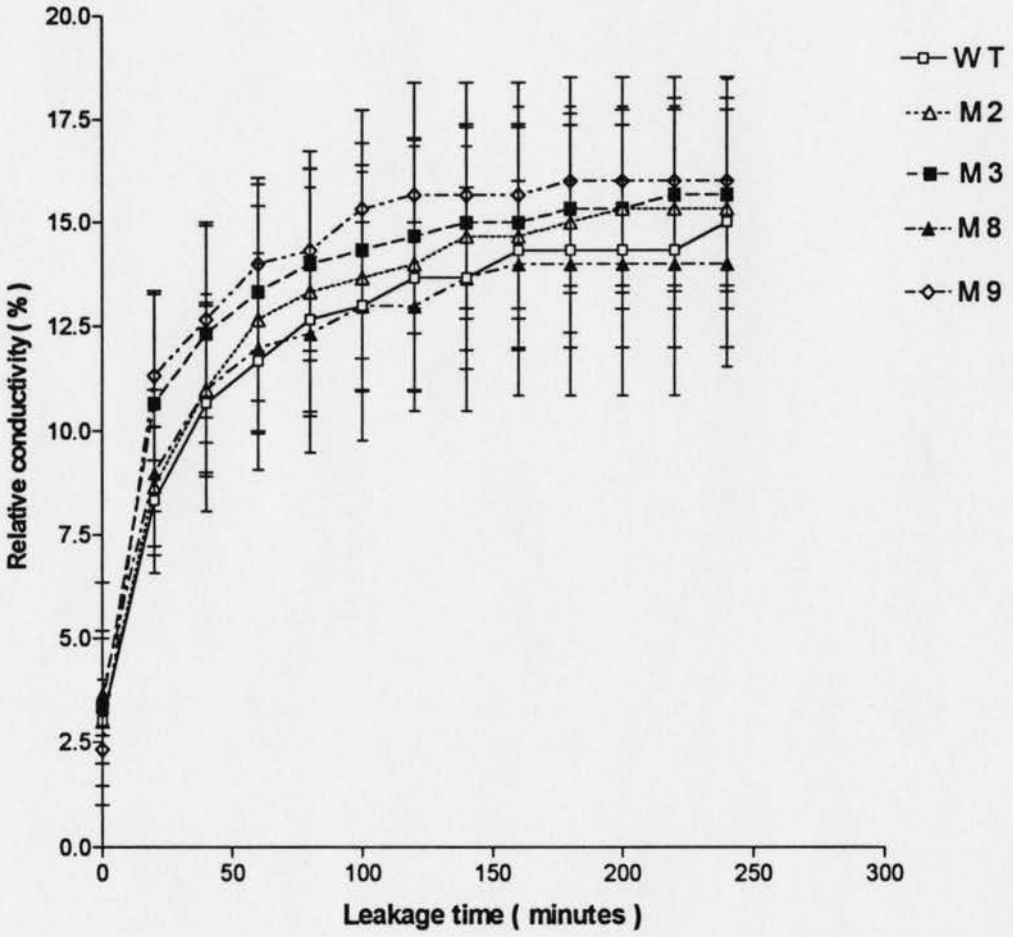

Fig. 3.7 Relative conductivity of upper leaves of wild type and T1 transgenic lines 3 weeks after non-chilling treatments were completed. Values are mean ±SEM. (25 ^{0}C day, 20 ^{0}C night).

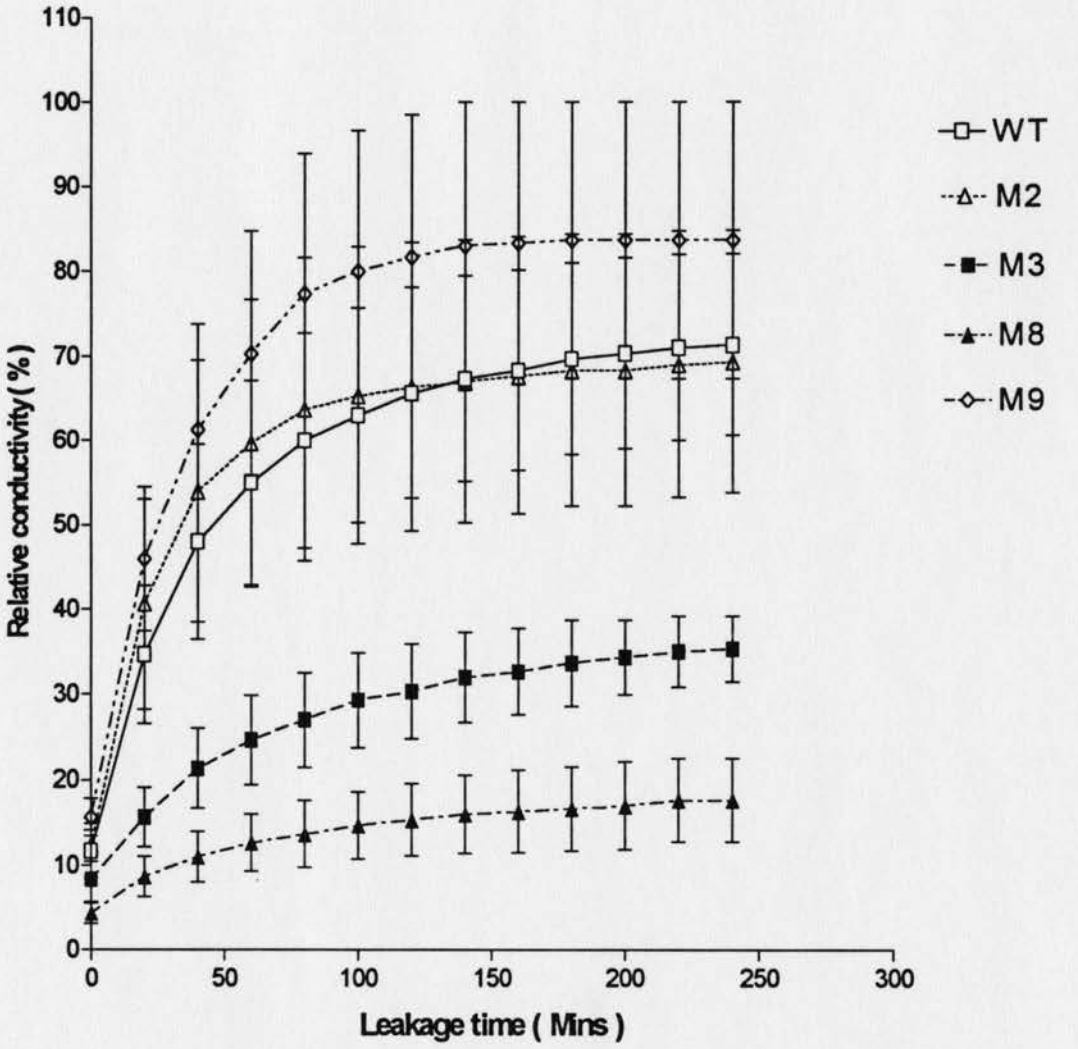

Fig. 3.8 Relative conductivity of upper leaves of wild type and T1 transgenic lines one day after 3-week chilling treatments (3 ^{0}C day, 12 ^{0}C night) were completed. Values are mean ±SEM.

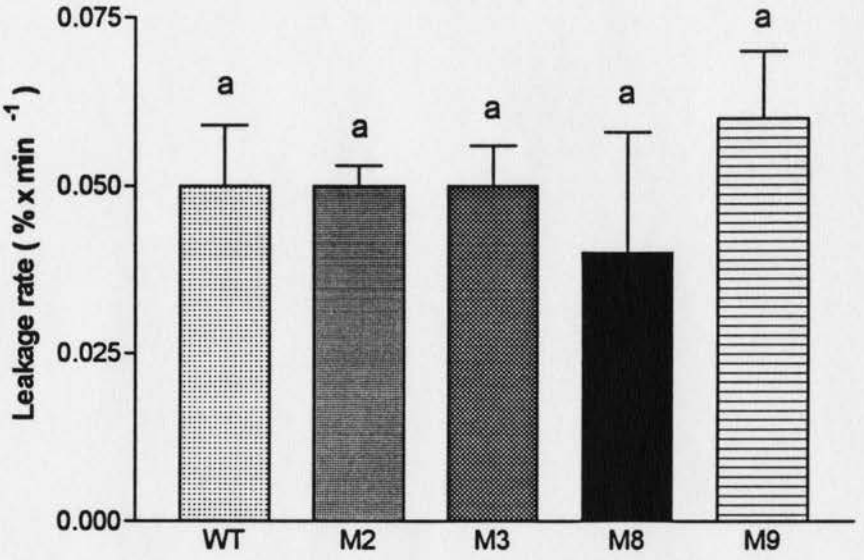

Fig. 3.9 Leakage rate of upper leaves of wild type and T1 transgenic lines 3 weeks after a non-chilling treatment was applied. Values are mean ±SEM.

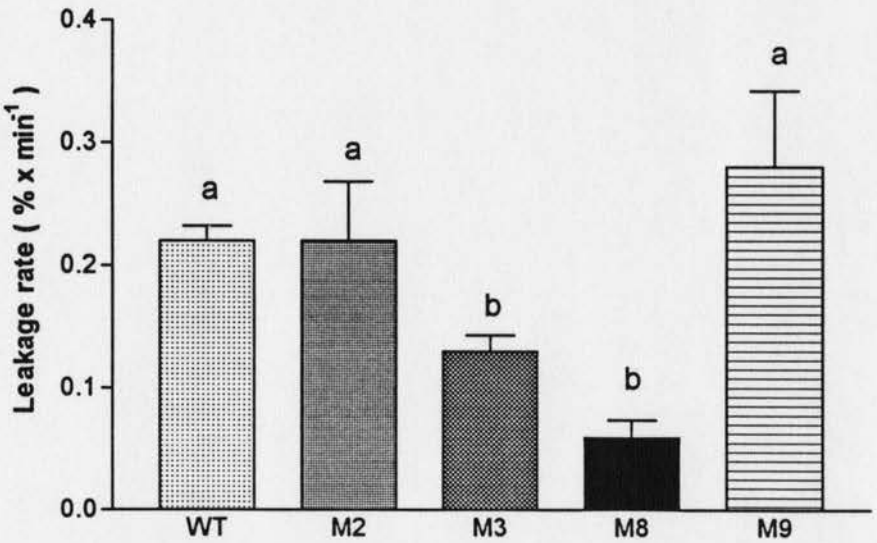

Fig. 3.10 Leakage rate of upper leaves of wild type and T1 transgenic lines one day after a 3-week chilling treatment was applied. Values are mean ±SEM.

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