AN ABSTRACT OF THE DISSERTATION OF

Eung-Jun Park for the degree of Doctor of Philosophy in Horticulture presented on June 15, 2005. Title: Enhancement of Cold Tolerance in Tomato Plants by Genetic Engineering of Glycinebetaine Biosynthesis.

Abstract approved:

Tony H. H. Chen

Tomato [*Lycopersicon esculentum* Mill.] plants are chilling sensitive and do not naturally accumulate glycinebetaine (GB), a metabolite that functions as a stress protectant in plants. While GB can increase tolerance of tomato plants to salt and drought stresses, its effect on chilling tolerance has not been examined. To evaluate whether GB improves chilling tolerance, tomato (cv. Moneymaker) plants were treated with foliar-applied GB. GB-treated plants exhibited enhanced chilling tolerance. During chilling treatment, GB-treated plants maintained lower $H_20_2$ levels but a higher degree of catalase activity compared with the controls. The protective effect of GB on enhanced chilling tolerance disappeared within a week after the application. These results suggested genetic engineering of a biosynthetic GB pathway into tomato plants may provide constitutively enhanced chilling tolerance.

Tomato plants (cv. Moneymaker) were transformed with a chloroplast-targeted *codA* gene of *Arthrobacter globiformis,* which encodes choline oxidase (COD) that catalyzes the conversion of choline to GB. Transgenic plants accumulated GB and their chloroplasts contain up to 86 % of total leaf GB. Over various developmental phases, transgenic plants were more tolerant of chilling stress than their wild-type (WT) counterparts. Transgenic plants also maintained lower $H_20_2$ levels but a higher degree of catalase activity under chilling stress compared with the controls. Finally, GB accumulation in the chloroplasts of transgenic plants was positively correlated

with their level of chilling tolerance, although GB levels in transgenic plants were very low compared to those in natural GB-accumulator plants.

To increase the amount of GB in transgenic tomato plants, three *codA* expression cassettes were constructed to target the chloroplasts (Chl-codA), cytosol (Cyt-codA), or both locations (ChlCyt-codA). Targeting COD to the cytosol or to both locations resulted in higher GB accumulations in the Cyt-codA and ChlCyt-codA lines compared to the Chl-codA lines. Regardless of targeted location and the different amounts of GB, all three types exhibited similar degrees of enhanced tolerance to various abiotic stresses compared with the WT plants.

The *codA* transgenic plants produced significantly enlarged flowers and fruits, which are a consequence of increased cell number and size. Fruit enlargement was caused by a pleiotropic effect of the *codA* gene transfer. Expression analysis revealed altered expression of genes that are involved in cell division. These results suggest that enlarged flowers and fruits are the pleiotropic effects of *codA* transgene expression, which may be useful for further improvement of these traits.

All together, these results demonstated that exogenous GB application enhanced chilling tolerance of non-transgenic tomato plants, and moreover introduction of a GB biosynthetic pathway into tomato confers enhanced tolerance to abiotic stresses, as well as increase in sizes of flowers and fruits.

# Enhancement of Cold Tolerance in Tomato Plants by Genetic Engineering of Glycinebetaine Biosynthesis

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Eung-Jun Park

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Eung-Jun Park, Author

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## CONTRIBUTION OF AUTHORS

Dr. Norio Murata contributed to the ideas and suggestions to the manuscripts. Dr. Atsushi Sakamoto also provided suggestions and assisted with western-blot analysis. Jeanine DeNoma assisted in producing the chloroplast-targeting *codA* transgenic plants. Raweewan Yuwansiri assisted with plasmid construction. Finally, Zoran Jeknić assisted with many aspects of experimental procedures throughout the entire period of my works.

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**CHAPTER 1**

## Improvement of cold tolerance in horticultural crops by genetic engineering

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**Abstract**

Chilling and freezing temperatures often adversely affect the productivity and quality of horticultural plants. Attempts to enhance cold tolerance through traditional breeding have achieved limited success, mainly due to the complexity of the genetics associated with a plant's response to low temperatures. Recently, the improvement of cold tolerance by genetic engineering has been achieved in many species. Nevertheless, transgenic plants with greater tolerance have been reported in only a few horticultural examples. Their production has led to a new era in improving performance. In this review, we first present recent advances in understanding cold acclimation. We then describe a few cases in which improved cold tolerance has been achieved with transgenic plants.

## Introduction

Low temperature (LT) is a major limiting factor in the geographical distribution of horticultural plants, often adversely affecting crop development, growth, and productivity. This results in huge losses to economic yield around the world. Furthermore, many important species are often cultivated in areas where temperatures fall below the optimum required for their normal growth and development (McKersie and Leshem, 1994). For example, the growing season of annual plants is generally established by the length of the frost-free period, so that economic losses often occur in tropical and sub-tropical species due to unseasonable episodes of LT stress. The performance of perennial species can also be affected by cold conditions (Wisniewski and Bassett, 2003). These may include extremely low midwinter temperatures, frost that occurs early in the fall before plants have become fully acclimated to the cold, or frost in the spring when deacclimation, i.e., the loss of cold hardiness, has occurred. The result is damage to over-wintering buds, developing blossoms, or even vegetative tissues.

In addition to the direct effects of LT on plant survival and productivity, susceptibility to injury limits the areas in which woody ornamentals and perennial fruit crops can be planted. Therefore, LT-stress tolerance has been one of the primary considerations in deciding which horticultural species can be established in a particular geographical region. Recent advances in plant molecular biology have not only allowed a better understanding of the adaptive responses to LT but also have led to the development of new approaches for improving cold tolerance.

In this review, we first present the current understanding of both LT-induced injury and how some species adapt to cold temperatures. We then focus on various attempts to protect higher plants against LT stress through genetic engineering, with special emphasis being placed on a few cases in which transgenic horticultural crops with enhanced cold tolerance have been developed.

## Low-temperature stress in horticultural crops

Chilling and freezing are the two major types of LT stresses in plants. Chilling stress occurs at temperatures either above 0 °C or below 0 °C in the absence of ice formation. In contrast, freezing stress is associated with temperatures below 0 °C and always involves the formation of ice crystals in the tissues.

### A. Chilling injury

Chilling injury occurs in many tropical and sub-tropical horticultural plants, such as banana, beans, cucumber, melon, tomato, and citrus. In temperate regions, these crops are commonly exposed to low, but non-freezing temperatures (0 ~ 15 °C) during the growing season (Paull, 1990). The extent of chilling injury depends on the species, variety, part of the plant affected, and its developmental stage, as well as the severity and duration of the cold period (Saltveit and Morris, 1990). In chilling-sensitive plants, injury can occur at all stages of development, except for seeds that are dormant and dry (Saltveit and Morris, 1990). Seeds of some sensitive plants, such as soybeans, sweet corn, and cotton, are particularly vulnerable during the initial stages of imbibition, when low temperatures can result in seed death, decreased germination rates, increased decay, and the production of low-vigor or abnormal seedlings (Herner, 1990). If exposure to chilling occurs after a sensitive plant has become established, it may exhibit wilting and/or low leaf water potentials (Pardossi et al., 1992). These symptoms are caused primarily by the uncontrolled opening of the leaf stomata while the permeability of the roots to water is simultaneously reduced (McWilliam et al., 1982).

The reproductive stage, which includes the development of floral organs, flowering, fruiting, and seed formation, is most sensitive to LT (McKersie and Leshem, 1994). Although storage at low temperatures is the most effective means for maintaining fruit viability, the refrigeration of chilling-sensitive commodities, e.g.,

tomato, cucumber, or banana, often results in a loss of quality, as manifested by pitting, discoloration, abnormal ripening, and weakening of the tissues, thereby rendering the product susceptible to pathogenic decay (Wang, 1990).

Many different mechanisms have been explored to describe the process of chilling injury; the phase transition of membrane lipids has been suggested as the primary cause. Lyons (1973) has proposed that LT is first perceived by the acyl tails of the phospholipid molecules in cell membranes. This results in a membrane phase transition, i.e., the lateral phase separation of membrane lipids to form distinct domains of gel and liquid-crystalline phases. These changes are purportedly dependent on lipid composition, with a greater level of unsaturated lipids being correlated with a lower number of phase transition events (Lyons and Raison, 1970). For example, an analysis of the molecular species of individual phospholipid classes has shown a negative relationship between chilling tolerance and the sum of the relative contents of the 16:0/16:0 and 16:0/16:1$^{t}$ fatty acids in the phosphatidylglycerol of the thylakoid membranes (Murata, 1983). Many efforts to alter the degree of unsaturation have resulted in modified chilling sensitivity (Murata et al. 1992; Kodama et al., 1994; Moon et al., 1995). Ultimately, changes in the membrane lipid phase transition prevent proper functioning of integral membrane proteins. This results in increased leakiness of the membranes, which then leads to a loss of compartmentation, decreased rates of mitochondrial oxidative activity, reduced energy supplies and utilization, lowered photosynthetic rates, disrupted metabolism, the accumulation of toxic substances, cell autolysis, and death (Wang, 1982).

The inhibition of photosynthesis is the first of the secondary events following membrane phase transition. This failure to maintain photosynthesis is associated with steps involved in regulating stomatal aperture (McKersie and Leshem, 1994). In chilling-tolerant species, the size of the stomatal opening is generally reduced at LT, in part because water conductivity through the root plasma membrane is decreased (McWilliam et al., 1982). For example, some tolerant plants can maintain steady water potentials by closing their stomata and preventing excessive transpirational water loss,

whereas chilling-sensitive species often exhibit wide-open stomata at LT (Emaus et al., 1983). Cold-induced stomatal closure might also occur through the direct effects of temperature on the guard cells themselves (Honour et al., 1995). In addition, LT may affect the enzymes and ion channels responsible for the active maintenance of guard-cell osmotic potential (Ilan et al., 1995). Stomatal aperture is also influenced by plant growth regulators, such as abscisic acid (ABA). ABA binds to receptors on the outside of the stomatal guard-cell plasma membrane, and induces a signal transduction cascade involving increases in cytoplasmic calcium (Assmann and Shimazaki, 1999). This action eventually reduces guard-cell osmotic potential via loss of $K^+$ and $Cl^-$, causing the stomata to close.

When chilling-sensitive plants are exposed to LT under strong illumination, electron transport through Photosystem II (PS II) is inhibited. McKersie and Leshem (1994) have presented several hypotheses to explain the causes of such 'photoinhibition'. First, LTs promote excessive excitation of the photosystems by reducing the demand for chemical energy in processes such as $CO_2$ fixation. Consequently, over-excitation favors the transfer of energy from light to oxygen molecules, causing photo-oxidative damage to the reaction center D1 protein. Second, the activity of enzymes that scavenge reactive oxygen species (ROS) decreases at LT (Richter et al., 1990); the accumulated ROS subsequently "escape" to other sites in the chloroplast or cytosol where they initiate degradation reactions. Third, turnover of the D1 protein in the reaction center is slow at LTs (Gong and Nilsen, 1989) and, therefore, its assembly into new PS II complexes is blocked. Finally, LTs inhibit the formation of zeaxanthin that quenches excitation energy and dissipates it as heat, further reducing the cells' ability to rid themselves of excess energy (Demming-Adams, 1990).

Another major contributing factor to chilling injury is oxidative stress. In addition to this chilling-induced photoinhibition in sensitive plants, membrane phase transition and dysfunction of other metabolic and repair processes, including a potential decrease in scavenger system capacities, can all contribute to the

proliferation of ROS. For example, catalase activity can decrease in response to LTs in various crop plants (Fadzillah et al., 1996; Hsieh et al., 2002). This decline is likely caused by the failure of repair synthesis and suppressed translation (Feierabend et al., 1992), as well as reduced expression of the catalase gene (Hsieh et al., 2002).

Transgenic tomato plants overexpressing the antisense catalase gene show a 2- to 8-fold reduction in total catalase activity, and a 2-fold increase in levels of $H_2O_2$ in leaf extracts (Kerdnaimongkol and Woodson, 1999). These plants are more sensitive to exogenous applications of $H_2O_2$ and also become more susceptible to chilling stress. Suppression of catalase activity in these plants likely leads to a failure to scavenge the ROS that accumulate, resulting in increased sensitivity to oxidative stress. In contrast, transgenic tobacco, genetically engineered with chloroplastic *CuZn-SOD* from pea, retains higher rates of photosynthesis under intense light and LTs, compared with wild-type (WT) plants (Gupta et al., 1993). Moreover, overexpression of a mitochondrial manganese superoxide dismutase (*MnSOD*) and superoxide dismutase (*FeSOD*) in the chloroplasts of transformed maize enhances foliar tolerance to chilling and oxidative stress when leaf discs are incubated in the pro-oxidant herbicide methyl viologen (van Breusegem et al., 1999a, 1999b).

**B. Freezing injury**

Freezing injury occurs when the external temperature drops below the freezing point of water (0 °C). Some plants susceptible to chilling injury can be killed by the first touch of frost, while many that are native to cold climates can survive extremely low temperatures without damage (Levitt, 1978). When plant tissues freeze, ice forms in their intercellular spaces, reducing water potential and leading to a loss of water from the cells. At –10 °C, more than 90 % of the osmotically active water will generally move out of the cells into those spaces (Thomashow, 1998). Therefore, freeze-induced dehydration has a number of damaging effects on cells, e.g., multiple forms of membrane lesions and the denaturation of proteins (Thomashow, 1998).

Upon thawing, freezing-damaged protoplasts lose turgor and are unable to prevent cellular water and osmolites from leaking into their surroundings.

At the whole-plant level, symptoms of freezing injury may include desiccation or burning of foliage; water-soaked areas that progress to necrotic spots on leaves, stems, or fruit; and death of portions or the entire plant (Ingram et al., 2001). The various effects of such stress include freezing of citrus fruits; midwinter damage to deciduous crops; and early-frost injury to flowers, vegetables, and developing fruit; as well as limiting the potential number of perennial species that can be planted within a particular cold hardiness zone (Ashworth, 1986). Spring frost damage in reproductive organs usually causes internal and external morphological abnormalities that affect normal fruit development or even cause abscission. In several of the early-blooming members of *Rosaceae* (e.g., strawberry, cherry, peach, almond, and apricot), floral damage is the main form of freezing injury (Rodrigo, 2000). Varying levels of freezing tolerance are associated with different plant organs: in cabbage (Brassica *oleracea* L. var. *capitata*), the relative tolerance is petiole < upper pith (stem) < middle pith < lamina < lower pith (Manley and Hummel, 1996). It has been suggested that the cold hardiness mechanism in the foliar portions of cauliflower (*Brassica oleracea* L. var. *botrytis*) is not expressed in the curd because freezing always proves fatal, as evidenced by florets having a flaccid and water-soaked appearance upon thawing (Fuller et al., 1989).

Plants have evolved two major means for surviving freezing stress. The first, supercooling of the cellular water, is an avoidance mechanism by which protoplasmic water can remain unfrozen to its homogeneous ice nucleation point of approximately –40 °C, when the source of the nucleation agent is absent (Thomashow, 1998). Deep supercooling has been observed in the dormant flower buds and ray parenchyma cells of a number of deciduous fruit crops, including grape, blueberry, and several *Prunus* species (Ashworth, 1989). In some woody plants, anatomical and biochemical adaptations to their cell walls and vascular systems are believed to limit the spread of ice-frozen tissues into supercooled areas (Fujikawa et al., 1997). For example, the

degree of esterification of pectins is modified in tissues that experience deep supercooling. Such modifications may change the pore size of the cell walls and inhibit ice penetration into supercooled compartments (Wisniewski and Davis, 1995). Nevertheless, supercooled plant tissues can suffer irreversible damage once ice nucleation occurs.

The second, and most common, mechanism for survival is the development of tolerance to freezing temperatures. Cold acclimation is the process by which plants acquire tolerance through their exposure to low but non-freezing temperatures (Thomashow, 1998). Our current understanding of cold acclimation will be explained in more detail below.

**C. Approaches and their limitations in overcoming LT problems**

To overcome the problems associated with low-temperature stress and to improve production efficiency, more stress-tolerant crops must be developed. Traditional breeding strategies that have attempted to utilize the natural genetic variation within a species, interspecific or intergeneric hybridization, or induced mutation using tissue culture techniques, have met with only limited success (Flowers and Yeo, 1995). Those approaches are confounded by the complexity of stress-tolerance traits, low genetic variance in yield components under stress conditions, and a lack of efficient selection techniques (Cushman and Bohnert, 2000). Furthermore, quantitative trait loci (QTL) that are linked to tolerance at one stage of development can differ from those associated with tolerance at other stages (Foolad and Lin, 2000). Once identified, these useful QTLs not only require extensive breeding to restore the desirable traits but also prove time-consuming when removing the chromosomal segments that interfere with the recurrent parent genome. That is, when conventional breeding programs try to introduce such a single cold-tolerance trait into a high-yielding variety, many undesirable attributes (which often decrease crop values) are also transferred to the offspring. Therefore, an expensive and time-consuming process

of backcrossing is required to develop a high-quality variety with one additional feature. Moreover, crossing is limited only to individuals of the same or closely related species.

In contrast to traditional breeding, genetic engineering with a small number of stress-tolerance genes appears to be a more attractive and rapid approach. First, it allows the introduction of isolated genes, i.e., single traits, into a crop without affecting other desirable attributes. Second, genetic engineering empowers breeders to transfer genetic material between unrelated plant species or even genes from phylogenetically distant species, such as a virus, bacteria, or animals. Thus breeders can more precisely produce a plant variety with a single new trait.

Present engineering strategies rely on the transfer of one or a couple of genes that encode either biochemical pathways or endpoints of signaling pathways (Nelson et al., 1998). These gene products can protect, directly or indirectly, against LT stresses. Such strategies invariably are based on the existence of correlations between a specific stress-protective function and a consequence of that stress. Some of the approaches reported in the literature (Tables 1.1 and 1.2) include the overexpression of biosynthetic enzymes for protein kinases, transcription factors, and cold-regulated, oxidative stress-related, lipid-modifying, or compatible solute-synthesis genes.

## Molecular biology of cold acclimation

### A. Cold acclimation

To survive winter, most species living in temperate regions undergo adaptive changes in the fall. Some plants acquire tolerance to freezing temperatures via prior exposure to low, nonfreezing temperatures, a process called cold acclimation (Hughes and Dunn, 1996). For example, non-acclimated rye is killed at about –5 °C, but after being cold-acclimated, can survive freezing to about –30 °C (Thomashow, 1999). This acclimation process is correlated with a number of cellular and metabolic changes.

Recent studies have started to define the molecular basis of these changes, which has led to the characterization of a large number of genes upregulated by LTs (reviewed by Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Browse and Xin, 2001; Zhu, 2001; Chinnusamy and Zhu, 2002; Shinozaki et al., 2003). Although the regulatory means for controlling LT responses are not fully understood, a better knowledge of cold-acclimation mechanisms would undoubtedly provide new strategies for improving freezing tolerance in horticultural plants.

## B. Morphological, anatomical, and physiological/biochemical changes during cold acclimation

The process of cold acclimation is very complex, involving a number of morphological, physiological, and biochemical alterations. The most obvious changes are morphological, involving the partial or total loss of aerial organs and the formation of specialized organs, such as buds and tubers (Ferullo and Griffith, 2001). For example, the prostrate or rosette growth form is assumed to be a morphological consequence of development at LT, and can serve as a selection criterion for cold hardiness (Roberts, 1984). Anatomical analyses have also been used to find morphological "markers" for an effective breeding program. In the case of the potato, hardier species, such as *Solanum acaule* and *S. commersonii*, have smaller cells, a thicker palisade, and two to three times greater stomatal indices than do non-hardy species, such as *S. tuberosum* (Li and Palta, 1978).

The effect of cold temperatures on the ultrastructure of plant cells has been long studied (see review by Kratsch and Wise, 2000). The extent of the alterations in cell components is apparently related to the degree of chilling and the length of exposure. Studies have suggested that cold temperature-related changes involve a wide range of components. Garber and Steponkus (1976) have reported the formation of a paracrystalline array of proteins in the thylakoid membranes of cold-acclimated spinach plants. In a comparison with particles in the membranes of non-acclimated

thylakoids, they have also noted a decreased number of particles, representing the PS II complex, on the inner fracture face of acclimated thylakoid membranes and a homogenization of two sizes of particles.

Cold-hardening temperatures cause both univacuolated and multivacuolate mesophyll cells to form in acclimated plants, whereas only univacuolate cells exist in non-acclimated plants (Huner et al., 1983). The chloroplast ultrastructure in the former exhibits an increase in smaller granal stacks while the size of the photosynthetic unit remains the same. O'Neill et al. (1981) have found that the vesiculated smooth endoplasmic reticulum enlarges in cold-hardened leaf cells, suggesting that freezing tolerance is enhanced because more substrate is available for vesicle formation and subsequent extension of the plasma membrane. Consequently, this allows the plant to adapt to the reduction in membrane surface area that occurs during freeze-induced dehydration and later rehydration during thawing.

Cellular and metabolic changes during cold acclimation include a rise in the levels of sugars, soluble proteins, proline, and organic acids, as well as the appearance of new isoforms of proteins and an altered lipid membrane composition (Hughes and Dunn, 1996). In early fall, all perennial plants accumulate carbohydrate reserves in the form of starch or fructan, which is converted into soluble sugars when the cold period begins (Levitt, 1978). Their principal forms are oligosaccharides, such as sucrose, raffinose, and stachyose (Bachmann et al., 1994; Olien and Clark, 1995; Hill et al., 1996). The storage of soluble sugars is concomitant with an increase in the activities of several enzymes associated with carbohydrate metabolism, i.e., amylase, sucrose phosphate synthase, sucrose synthase, Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (Rubisco), and others (Hurry et al., 1994; Nielsen et al., 1997; Reimholz et al., 1997). In fact, this accumulation of free sugars seems to involve the enhancement of all photosynthetic pathways (Hurry et al., 1994). In addition, increased amylase and sucrose synthase activities are related to the appearance of new, cold-specific isozymes (Nielsen et al., 1997; Reimholz et al., 1997). A rise in the steady-state levels of mRNAs that encode sucrose phosphate synthase has also been

observed in potato (Reimholz et al., 1997). This indicates that changes in carbohydrate metabolism, with respect to cold acclimation, are under transcriptional and/or post-transcriptional control.

Other compatible solutes, such as proline (Pro) and glycinebetaine (GB), are also accumulated in response to LTs. An increase in the content of those solutes under stress conditions has been found in a number of species; their levels have, in some cases, been positively correlated with freezing tolerance (Bohnert et al., 1995; Rhodes and Hanson, 1993). A causal relationship between Pro accumulation and tolerance to freezing stress has been demonstrated in *Arabidopsis thaliana* (Nanjo et al., 1999). Transgenic *Arabidopsis* plants with an antisense construct of proline dehydrogenase (*AtProDH*), which catalyzes Pro degradation, accumulate higher levels of Pro and show enhanced tolerance to freezing stress (Nanjo et al., 1999). In contrast, Xin and Browse (1998) have found that, under normal growing conditions, a freezing-tolerant *Arabidopsis* mutant, *eskimo1*, can accumulate a 30-fold higher level of Pro due to greater expression of the Δ1-pyroline-5-carboxylate synthetase (*P5Cs*) gene, which catalyzes Pro biosynthesis, compared with WT plants.

Glycine betaine accumulates naturally in distantly related plant species in response to various stresses (Rhodes and Hanson, 1993). In strawberry leaves, levels of GB can rise nearly two-fold after four weeks of cold-acclimation treatment, during which time their cold tolerance increases from –5.8 to –17 °C (Rajashekar et al., 1999). Naidu et al. (1991) have reported that the concentration of GB can more than double (from 7.9 to 17.9 μmol/g dry weight) in cold-acclimated wheat seedlings in response to 5 d of cold stress (4 °C). Likewise, GB in the winter type of barley accumulates to five times the basal level over three weeks at 5 °C, but is only doubled in some spring types (Kishitani et al., 1994). In that barley near-isogenic lines of the same cultivar, accumulated levels of GB in leaves at LTs were well correlated with the percentage of green leaves that survive freezing injury (–5 °C). Furthermore, the exogenous application of GB improves freezing tolerance in plants that are not natural accumulators (Harinasut et al., 1996; Chen et al., 2000; Makela et al., 2000; Sakamoto

et al., 2000; Park et al., 2003). Studies *in vitro* have shown that GB is effective in stabilizing the structures of enzymes and proteins, as well as in protecting membrane properties (Papageorgiou and Murata, 1995).

Temperature and water availability affect both physical and biological properties of cell membranes. Therefore, it is assumed that plasma membranes undergo chemical alterations during cold acclimation so as to adjust to stress. During freezing, the formation and growth of extracellular ice crystals can cause lesions in the plasma membrane, resulting in the loss of osmotic responsiveness during subsequent thawing (Steponkus, 1984). Cold-acclimated plants manifest not only a rise in the degree of lipid unsaturation, but also changes in lipid composition and the ratio of lipid to protein in membranes (Palta et al., 1993). Steponkus et al. (1988) have shown that increased levels of unsaturated species of phosphatidylcholine mimic the behavior of protoplasts from acclimated plants, and suffer less expansion-induced lysis during thawing. In *Arabidopsis thaliana*, the lipid composition of the plasma membrane changes significantly as plants acclimate (Uemura et al., 1995). The proportion of phospholipids increases from 46.8 to 57.1 mol% of the total lipids, with little change in the proportions of the phospholipid classes. Although the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine rises, mono-unsaturated species are still the predominant species. The proportion of cerebrosides decreases from 7.3 to 4.3 mol%, with only small changes in the proportions of the various molecular species. Finally, the proportion of free sterols decreases from 37.7 to 31.2 mol%, but with only small changes in the proportions of sterylglucosides and acylated sterylglucosides (Uemura et al., 1995).

### C. Cold-regulated (*COR*) genes

Biochemical and physiological changes that occur during cold acclimation are regulated by LTs through modifications in gene expression. Cold-regulated (*COR*) gene expression is critical to plants for imparting tolerance to both chilling (Gong et

al., 2002; Hsieh et al., 2002) and freezing (Thomashow, 1999). Moreover, the expression of specific genes up-regulated by LT is highly correlated with the development of freezing tolerance (Thomashow, 1999).

Because tolerance is inducible, it has been commonly assumed that this induction involves the synthesis of novel peptides, which, by means of their enzymatic activity or structural properties, confer tolerance to the tissue. A number of *COR* genes have been characterized from different plant species (Hong et al., 1988; Houde et al., 1992; Lin and Thomashow, 1992; Monroy et al., 1993). For example, Lin and Thomashow (1992) have isolated four *COR* genes from *Arabidopsis thaliana*. Their Northern hybridization has indicated that the level of mRNA from each of these genes increases dramatically during the first 4 h of treatment at 5 °C. The level then remains high for the duration of the acclimation period, and declines after the plants are transferred to warm temperatures. All four *COR* genes are ABA-responsive and accumulate if plants are sprayed at room temperature with that growth regulator. The peptides from these genes are boiling-stable, in contrast to most peptides that are denatured and form a precipitate if an aqueous solution is boiled (Guy, 1990; Thomashow, 1999).

**D. CRT/DRE binding factors (*CBF*s)**

Many *COR* genes have in their promoter regions one or several copies of the *CRT* (C-repeat)/*DRE* (Dehydration Responsive Element) *cis*-element, which has the core sequence CCGAC that is responsive to LT as well as dehydration (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). Stockinger et al. (1997) have isolated *CBF1* (*CRT*-binding factor 1), a cDNA clone for *CRT/DRE*-binding protein. It contains a DNA binding motif (AP2 domain) that is found in the *Arabidopsis APETALA2* (*AP2*) protein, where it functions in floral morphogenesis, as well as in the tobacco *EREBP1* family, where it is involved in ethylene-responsive gene expression (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). More recently, Liu et al. (1998)

have used a yeast one-hybrid program to isolate five *CRT/DRE*, which they have named *DREB*s (*DRE*-binding proteins) and have classified into two groups - *DREB1* and *DREB2*. Although each group contains similar AP2 domains, it has low sequence similarity outside that domain. Three *DREB1* proteins are encoded by genes tandemly repeated in the order *DREB1B* (=*CBF1*), *DREB1A* (=*CBF3*), and *DREB1C* (=*CBF2*). Two *DREB2* proteins, *DREB2A* and *DREB2B*, also exist (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). Expression of *DREB1A* and its homologues, *DREB1B* and *DREB1C*, is induced by LT stress, whereas expression of *DREB2A* and *DREB2B* is induced by dehydration and salt stresses (Liu et al., 1998). Therefore, two independent *DREB* proteins, *DREB1* and *DREB2*, function as transcription factors in LT and dehydration signal transduction pathways, respectively, to activate *CRT/DRE* *cis*-elements.

Zarka et al. (2003) have recently found the transcription factors involved in *CBF2* expression, and have identified a 125-bp promoter segment containing two regions, designated *ICEr1* and *ICEr2* (induction of *CBF* expression region 1 or 2). By themselves, the regions are only weakly responsive to low temperatures, but in combination, impart a robust cold response.

Further analysis of the cold-sensing mechanism involved in *CBF* regulation has revealed that a cold shock is not required for bringing about the accumulation of *CBF* transcripts, but instead, the thermosensing circuitry is likely to monitor absolute temperature and act like a rheostat increasing output (i.e. the levels of *CBF* transcripts), with greater degrees of input (i.e., lower temperatures) (Zarka et al., 2003). Even though this cold-sensing mechanism becomes desensitized within a few hours of exposure to a given LT, e.g., 4 °C, this desensitization does not preclude a robust response to a further decrease in temperature. Plants that have been adapted to 4 °C for 14 d and that have low levels of CBF transcripts produce more transcript when transferred to 0 °C or –5 °C. In contrast, resensitizing to that temperature requires between 8 and 24 h of exposure to 22 °C.

## E. Inducer of *CBF* expression (*ICE*)

The *CBF/DREB1* genes are themselves induced by LTs. This induction is transient, preceding that of downstream genes with the *DRE/CRT cis*-element (Thomashow, 1999). Gilmour et al. (1998) have proposed that a transcriptional factor, present at warm temperatures, recognizes the *CBF* promoters and is activated in response to LTs by a signal transduction pathway that also exists at warm temperatures. Recently, Chinnusamy et al. (2003) also have identified a transcriptional activator, *ICE* (Inducer of *CBF* Expression), that can recognize the promoters of the *CBF* genes and induce their expression.

*ICE1* encodes a MYC-like helix-loop-helix (bHLH) transcriptional activator (Chinnusamy et al., 2003). A dominant-negative *ice1* mutation blocks expression of *CBF3* and decreases the expression of many genes downstream of *CBF*s, such as *RD29A, COR15A* and *COR47*. This leads to a significant reduction in plant chilling and freezing tolerance. Interestingly, transgenic *Arabidopsis* lines that constitutively overexpress *ICE1* do not show *CBF3* expression at warm temperatures, but have a higher level of *CBF3* transcript at low temperatures, suggesting that cold-induced modification of the *ICE1* protein or a transcriptional cofactor is necessary for *ICE1* to activate the expression of *CBF*s (Chinnusamy et al., 2003).

Finally, in contrast to *CBF3* expression, the *ice1* mutation produces somewhat greater levels of cold-induced *CBF2* transcripts, indicating that the mechanisms for expression differ within the *CBF/DREB1* gene family (Chinnusamy et al., 2003).

## F. Mutational analysis of freezing tolerance in *Arabidopsis thaliana*

Many *Arabidopsis* mutants with increased or decreased freezing tolerance have been isolated and have proven very useful in identifying the genes and proteins involved in cold acclimation. A classical mutagenesis approach has enabled the isolation of *sfr* (sensitivity to freezing) mutants that fail to gain tolerance after cold

acclimation (Warren et al., 1996). Based on an analysis of these mutants for cold-induced gene expression, the *sfr6* mutants are deficient in the genes *kin1, cor15a, and cor78/rd29A*, all of which contain the *CRT/DRE* motif in their promoters (Knight et al., 1999). Further study with the *sfr6* mutant has found that the expression of cold-induced genes is activated post-transcriptionally by the interaction of *CBF1/DREB1* and *DREB2* with the *CRT/DRE* promoter element (Boyce et al., 2003). In contrast, the freezing sensitivity of cold-acclimated *sfr4* has shown the greatest deficit among the *sfr* mutants (Warren et al., 1996) is due to its continued susceptibility to membrane lesions that are caused by lyotropic formation of the hexagonal II phase, and which are also associated with the low sugar content in this mutant's cells (Uemura et al., 2003).

A constitutively freezing-tolerant mutant, *eskimo1*, exhibits greater tolerance than WT plants in the absence of cold acclimation (Xin and Browse, 1998). These mutant plants accumulate a 30-fold greater level of Pro due to higher expression of the *P5Cs* gene compared with WT plants under normal growing conditions. However, the expression of several cold-regulated genes involved in freezing tolerance is not increased. This suggests that *ESKIMO1* may activate a different signal transduction pathway from the *CRT/DRE*-related pathway (Xin and Browse, 1998).

Ishitani et al. (1997) have isolated a large number of mutants with deregulated cold-responsive gene expression, including *cos* (constitutive expression of osmotically responsive genes), *los* (low expression of osmotically responsive genes), and *hos* (high expression of osmotically responsive genes). Xiong et al. (1999) have characterized an *Arabidopsis* mutant, *hos5*, which exhibits increased expression of the osmotic stress-responsive *rd29A* gene under osmotic stress but not when under cold stress. Moreover, this osmotic-stress hypersensitivity found in *hos5* does not affect the *aba* or *abi* mutants, suggesting that its sensitivity only in the *hos5* mutant is ABA-independent. The *hos1* and *hos2* mutants show enhanced induction of both *CBF*s and their downstream *cor* genes only under cold stress (Ishitani et al., 1998; Lee et al., 2001). Non-acclimated *hos1* and *hos2* mutants are less cold-tolerant than WT plants, whereas the expression of *CBF*s is maintained at a higher level for up to 24 h during this stress.

*HOS1* and *HOS2* are, therefore, assumed to be negative regulators of cold signaling pathways by modulating the expression level of the *CRT/DRE* binding factors. *HOS1* encodes a RING finger protein as a possible E3 ubiquitin ligase that is present in the cytoplasm at normal growth temperatures, but accumulates in the nucleus upon LT treatment. Therefore, Lee et al. (2001) have proposed that *HOS1* displays cold-regulated nucleo-cytoplasmic partitioning, which may play an important role in communicating cold-generated signals in the cytoplasm to the nucleus by targeting certain positive regulators of *CBF*s for ubiquitination and degradation.

The *los1* mutant, in which the *COR* genes are no longer induced by LT, enhances the expression of *CBF/DREB1*s but fails to develop freezing tolerance. This mutant, defective in the translation elongation factor 2 genes, blocks new protein synthesis specifically at low temperatures, indicating that cold-induced transcription of *CBF/DREB1*s is feedback-inhibited by either their gene products or the products of their downstream target gene (Guo et al., 2002). Another mutant, *los2*, is also involved in the accumulation of cold-responsive gene transcripts. The *LOS2* gene encodes a bi-functional enolase involved in the glycolytic pathway. *LOS2* protein can bind to the promoter of *STZ/ZAT10*, a zinc finger transcriptional repressor. Induction of *STZ/ZAT10* is strongly increased and sustained in the *los2* mutant whereas its expression is rapid and transient in WT plants (Lee et al., 2002). The *los4* mutant plants are very sensitive to chilling stress, particularly in the dark; their chilling sensitivity is reversed by constitutive expression of the *CBF3* gene (Gong et al., 2002). The *LOS4* gene, considered the first positive regulator in the expression of *CBF*s, encodes a DEAD-box RNA helicase, suggesting that RNA metabolism is involved in cold acclimation.

Stockinger et al. (2001) have proposed that transcriptional activation of a *COR* gene by *Arabidopsis CBF1* might be mediated by homologs of the yeast histone actyltransferase (*HAT*) *GCN5* and the transcriptional adaptor proteins *Ada2*. In *Arabidopsis*, the *AtADA2* proteins interact with the *AtGCN5* protein. Moreover, both those proteins are found to interact with *CBF1*. Recently, Vlachonasios et al. (2003)

have reported that isolating the *Arabidopsis* mutants, which are disrupted by T-DNA insertion of *ADA2* and *GCN5*, leads to induced expression of *CBF*s, as is seen in WT plants. However, subsequent transcription of the *COR* genes is reduced in both mutants. Non-acclimated *ada2b-1* mutant plants are more freezing-tolerant than the non-acclimated wild-types, suggesting that *ADA2b* may directly or indirectly repress a tolerance mechanism that does not require the expression of *CBF* or *COR* genes.

Another series of freezing-sensitive mutant plants, *frs1* (freezing sensitive 1), show a wilty phenotype and excessive water loss in both cold-acclimated and non-acclimated plants, but they recover after treatment with exogenous ABA (Llorente et al., 2000). Complementation analysis has revealed that the *frs1* mutation is a new allele of the *ABA3* locus, and that gene expression in those mutants is altered in response to dehydration. Therefore, the freezing tolerance triggered by ABA-regulated proteins probably protects plants mainly from freezing-induced cellular dehydration.

The involvement of $IP_3$ (Inositol 1,4,5-trisphosphate) has been demonstrated in the stress signal pathway (Xiong et al. (2001). A mutation, *fiery1* (*fry1*), shows enhanced expression of ABA- and stress-responsive genes when treated with ABA, cold, drought, or salt. *FRY1* encodes an enzyme with inositol polyphosphate 1-phosphatase that is involved in the catabolism of $IP_3$. In response to ABA and osmotic stress, there is a transient increase of inositol 1,4,5-trisphosphate ($IP_3$) in WT plants (Lee et al., 1996), whereas the *fry1* mutant accumulates a significantly higher level of $IP_3$. In contrast to the greater induction of cold-responsive genes, the *fry1* plants are defective in their cold acclimation and germination. Hence, *FRY1* is a negative regulator of cold-responsive gene expression through the modulation of $IP_3$ levels (Xiong et al., 2001).

**Genetic engineering for cold tolerance in plants**

Genetic engineering, with either one or a small number of genes being introduced into a crop species, has achieved considerable progress toward improving

tolerance to LT stresses, including chilling and freezing. Among the LT-induced genes already isolated, several major groups have been adopted for enhancing tolerance. These include protein kinases, transcription factors, cold-regulated genes, oxidative stress-related genes, lipid-modifying genes, and compatible solute-synthesis genes (Tables 1.1 and 1.2).

**A. Protein Kinase**

In early cold signaling, low temperatures are sensed via alterations in membrane fluidity or through cytoskeletal reorganization that affects the calcium channels (Knight and Knight, 2001). Under cold stress, these transient increases in cytosolic $Ca^{2+}$ are mainly perceived by $Ca^{2+}$ binding proteins, e.g., calmodulin and $Ca^{2+}$ -dependent protein kinases (*CDPKs*) (Zielinski, 1998). Overexpression of a rice calcium-dependent protein kinase (*OsCDPK7*) results in increased chilling- and osmotic-stress tolerances in rice (Saijo et al., 2000). Expression of *OsCDPK7* also induces some stress-responsive genes in response to high-salt or drought conditions, but not the cold, suggesting that the downstream pathways leading to such cold or salt/drought tolerance differ (Saijo et al., 2000).

In plants, various stresses, including the cold, stimulate the accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (Hasegawa et al., 2000). These ROS serve as a signal that induces scavengers and other protective mechanisms, as well as the damaging agents that contribute to stress injury in plants (Prasad et al., 1994). Mitogen-activated protein kinase (*MAPK*)-signaling pathways are actively involved in transducing oxidative signaling (Ligterink and Hirt, 2001). Kovtun et al. (2000) have shown that the *Arabidopsis* mitogen-activated protein kinase kinase kinase (ANP1) can be induced specifically by $H_2O_2$. Furthermore, transgenic tobacco plants that express a constitutively active tobacco *ANP1* (*NPK1*) display elevated tolerance to multiple environmental stress conditions and repressed expression in auxin-inducible promoters (GH3). NDP kinase (*NDPK*) is

believed to be a housekeeping enzyme that maintains the intracellular levels of all dNTPs except ATP (Moon et al., 2003). This enzyme is also associated with $H_2O_2$-mediated *MAPK* signaling in plants. Proteins from transgenic *Arabidopsis* plants that overexpress *Arabidopsis NDPK2* (*AtNDPK2*) show high levels of autophosphorylation and *NDPK* activity, and have fewer ROS than the wild-type. In contrast, mutants that lack *AtNDPK2* have higher levels of ROS than do WT plants. Constitutive expression of *AtNDPK2* in *Arabidopsis* plants confers enhanced tolerance to multiple environmental stresses that elicit ROS accumulation *in situ* (Moon et al., 2003).

**B. Transcription factors**

Transgenic expression of the *CBF* genes leads to improved cold tolerance in many species (Tables 1.1 and 1.2). Constitutive expression of the *CBF1* or *CBF3* genes in transgenic *Arabidopsis* plants not only induces multiple *COR* genes without prior cold treatment, but also renders those plants more freezing-tolerant than the controls (Liu et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Qin et al. 2004; Kitashiba et al. 2004). However, use of the strong constitutive 35S cauliflower mosaic virus (*CaMV*) promoter to drive expression of *CBF3* results in severe growth retardation under normal conditions. In comparison, the overexpression of *CBF3*, as driven by the stress-inducible promoter such as *RD29A* or ABRC1, gives rise to only minimal effects on plant growth, with greater tolerance to stress (Kasuga et al., 1999 and 2004; Lee et al. 2003). Jaglo-Ottosen et al. (2001) have reported that overexpression of the *Arabidopsis CBF1* genes in canola increases tolerance in both acclimatized and non-acclimatized plants. Furthermore, they have found that transcripts encoding *CBF*-like proteins also accumulate in response to LT in wheat and rye (which cold-acclimate), as well as in tomato, a freezing-sensitive species that does not cold-acclimate. Hence, components of the *CBF* cold-responsive pathway are believed to be highly conserved in flowering plants, and are not limited only to those that cold-acclimate. Furthermore, transgenic expression of *CBF1* in tomato plants

induces a higher level of the *CATALASE1* (*CAT1*) gene, resulting in improved chilling tolerance with less $H_2O_2$ under either normal or LT conditions (Hsieh et al., 2002). Those research results have suggested that heterologous *CBF1* expression in transgenic tomato may induce several oxidative stress-responsive genes to protect plants from chilling stress. Finally, *Arabidopsis* plants that overexpress *CBF3* not only have elevated levels of *COR* proteins, but also higher Pro and total sugar contents (Gilmour et al., 2000). Increased levels of the latter two occur with cold acclimation in a wide variety of plants, and are thought to contribute to the enhancement of freezing tolerance, in part, by stabilizing membranes.

In addition to *CBF*s, analysis of the expression patterns of genes induced by both dehydration and LT have demonstrated broad variations in the timing of their induction and in their responsiveness to ABA (reviewed by Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). The transient increase in ABA during cold stress as well as the enhancement of freezing tolerance by its exogenous application indicates that this growth regulator must play a critical role in cold acclimation (Thomashow, 1999). Analysis of the promoter regions of *cor15a*, *rd29a*, and *cor6.6* has revealed the presence of *ABRE*s (ABA-responsive elements), or PyACGTGGC (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al., 1995). Gene expression through these *ABREs* is regulated in plants by the transacting protein that comprises the basic domain/leucine zipper (bZIP) structure (Aguan et al., 1993; Kusano et al., 1995; Lu et al., 1996; Choi et al., 2000). Kim et al. (2001) have cloned a novel cold-inducible zinc finger protein from soybean, *SCOF1*, containing two $C_2H_2$-type zinc fingers and a putative nuclear localization signal, KRKRSKR. *SCOF1* is weakly induced by ABA as well as LT. Its level of transcription is increased up to 3 d after cold stress; in contrast, expression of *DREB1* decreases to a minimum level within 1 d (Liu et al., 1998). This temporal sequence in the expression pattern of *DREB1* and *SCOF1* indicates that the initial induction of *COR* gene expression by *DREB1* is synergistically increased by *SCOF1* during cold stress (Kim et al., 2001). Constitutive expression of *SCOF1* in *Arabidopsis* results in the expression of multiple *COR* genes

(*cor15a, cor47*, and *rd29b*) and freezing tolerance. Although it does not directly bind to *ABRE* or *CRT/DRE* motifs, it does enhance the DNA binding activity of *SGBF1*, a bZIP transcription factor. Thus, *SCOF1* interacts with *SGBF1* to regulate *COR* gene expression through activation of *ABRE* in the ABA-dependent pathway of cold-stress signal transduction (Kim et al., 2001).

Microarray analysis has been used to monitor the complex changes in response to LT in the *Arabidopsis* transcriptome. Seki et al. (2001) identified 40 transcriptional factors that belong to AP2/EREB, zinc-finger, ERF, WRKY, bZIP and MYB families and Fowler and Thomashow (2002) have provided a direct evidence for the activity of at least 15 cold-regulated transcription factors not participating in the CBF cold-response pathway. *Arabidopsis* plants expressing the pepper ERF (ethylene-responsive factor) /AP2 affected expression of genes that contain either a GCC or a CRT/DRE box in their promoter region and displayed tolerance against freezing and pathogen stresses (Yi et al. 2004). In addition, the expression of *Osmyb4* or zinc-finger protein from rice in *Arabidopsis* transgenic plants also increases LT tolerance, suggesting that the existence of cross-talk between the LT stress and other stresses (Vannini et al. 2004; Mukhopadhyay et al. 2004).

**C. *COR* genes**

LT induces the expression of many *COR* genes (reviewed by Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Browse and Xin, 2001; Zhu, 2001; Chinnusamy and Zhu, 2002, Shinozaki et al., 2003). In *Arabidopsis*, these include the *lti* (low temperature-induced), *kin* (cold-induced), *rd* (responsive to desiccation), and *erd* (early dehydration-inducible) genes. Their products help plants adapt to subsequent LT stress.

Overexpression of *cor15a,* which encodes a polypeptide, modestly increases the freezing tolerance of chloroplasts in non-acclimated *Arabidopsis* plants (Artus et al., 1996; Steponkus et al., 1998). This effect appears to result from the mature

*cor15a*-encoded polypeptide, *cor15am*, decreasing the propensity of membranes to form deleterious hexagonal II phase lipids upon freeze-induced dehydration (Artus et al., 1996; Steponkus et al., 1998). Furthermore, Hara et al. (2003) have suggested that a citrus *cor15a*, dehydrin (a Group-2 LEA protein), improves tolerance in transgenic tobacco plants by scavenging radicals to protect membrane systems. Transgenic *Arabidopsis* plants constitutively expressing multiple dehydrin genes including *RAB18* and *COR47* or *LTI29* and *LTI30* exhibited lower LT50 values and improved survival under freeing stress compared to the control plants (Puhakainen et al. 2004). They also found that the acidic dehydrin LTI29 was translocated from cytosol to the vicinity of the membranes during cold acclimation in transgenic plants. However, the overexpression of individual cold-induced genes has provided little improvement in tolerance at the whole-plant level (Artus et al., 1996; Kaye et al., 1998; Steponkus et al., 1998). Therefore, those individual components of freezing tolerance perhaps can work only within the context of a broader cold-acclimation response (Browse and Xin, 2001). Ndong et al. (2002) have reported that the wheat *wcs19* is a stromal protein belonging to a new class of organelle-targeted Group-3 *LEA* proteins. Its constitutive expression in *Arabidopsis* can protect only cold-acclimated leaves from freezing-induced damage.

**D. Compatible solute-synthesis genes**

Compatible solutes, or osmoprotectants, are highly soluble compounds that are nontoxic at high concentrations and which are accumulated at elevated levels by many plant species in response to abiotic stresses (Rhodes and Hanson, 1993). These solutes include polyols and sugars, e.g., mannitol, sorbitol, and trehalose; amino acids, such as proline; and betaines and related compounds (Rhodes and Hanson, 1993; McNeil et al., 1999). With advances in plant molecular genetics, the roles of compatible solutes have been strengthened by the performance of transgenic plants that overexpress or express

genes related to their biosynthesis under various stress conditions (reviewed by Chen and Murata, 2002)

**1. Sugars**

In the plant kingdom, most species do not seem to accumulate detectable amounts of trehalose, a non-reducing disaccharide of glucose, with the notable exception of the highly desiccation-tolerant "resurrection plants" (Wingler, 2002). Genetic transformation for enhanced accumulation of trehalose in dicot species has resulted in undesirable pleiotropic effects, including stunted growth and altered metabolism under normal conditions (Goddijn et al., 1997; Romero et al., 1997). On the other hand, overexpression of E. *coli* trehalose-6-phosphate synthase/phosphatase (*ots A/B*) in rice is driven by either a constitutive promoter (Jang et al., 2003) or a stress-inducible or tissue-specific promoter (Garg et al., 2002). As a result, trehalose is accumulated in amounts of up to approximately 1 mg per gram fresh weight (FW), which contrasts with the negligible levels seen in non-transgenic controls. Transgenic lines produced by both groups (Garg et al., 2002; Jang et al., 2003) exhibit enhanced tolerance to various stresses without growth retardations or morphological alterations. In addition, increased trehalose levels are correlated with higher soluble carbohydrate contents and an elevated capacity for photosynthesis under stress and non-stress conditions. These observations are consistent with a suggested role in modulating sugar sensing and carbohydrate metabolism, rather than in osmoregulation (Garg et al., 2002).

In addition, *Arabidopsis* plants with increased expression of sucrose phosphate synthase (*SPS*) mitigate the inhibitory effect of cold stress on photosynthesis, and maintain the mobilization of carbohydrates from source leaves to sinks, leading to improved freezing tolerance (Strand et al., 2003). Antisense expression of a tomato *Lea-Gal* gene, which encodes *α-galactosidase* to catalyze the hydrolysis of raffinose, results in an increase in endogenous raffinose and enhanced tolerance by both non-

acclimated and cold-acclimated plants (Pennycooke et al., 2003). Transgenic expression of genes, which are involved in fructan synthesis such as bacterial fructan polymerase (*SacB*) or wheat fructosyltransferase (*wft1 and wft2*) increased freezing tolerance in tobacco and perennial ryegrass, respectively (Konstantinova et al. 2002; Hisano et al. 2004).

**2. Proline (Pro)**

In many plants, the stress-inducible accumulation of free proline (Pro) is caused by both the activation of Pro biosynthesis and the inactivation of Pro degradation (Kiyosue et al., 1996). Transgenic *Arabidopsis* plants with an antisense proline dehydrogenase (*AtProDH*), which catalyzes Pro degradation, accumulate higher levels of Pro (~ 0.6mg/g FW) and show enhanced freezing tolerance, with a 33 % survival rate and 59 % ion leakage, versus a 100 % mortality rate in WT plants (Nanjo et al., 1999). In addition, Konstantinova et al. (2002) have introduced Δ1-pyroline-5-carboxylate synthetase, as derived from *Arabidopsis thaliana* and *Vigna aconitifolia* (Lines AtP5Cs and VacP5Cs), into tobacco plants. In that research, Pro contents have increased up to 8- and 15-fold, respectively, under cold stress. After the transgenic tobacco is exposed to freezing temperatures under field conditions, they show higher survival rates (50 to 90 %), compared with the WT plants (0 % survival).

**3. Glycinebetaine (GB)**

Glycine betaine (GB) is among the common betaines most widely distributed in higher plants. However, several taxonomically distant species, e.g., *Arabidopsis*, rice, and tobacco, are considered non-accumulators (Rhodes and Hanson, 1993), such that exogenous GB applications to these species actually improve their growth under various stresses (Harinasut et al., 1996; Chen et al., 2000; Makela et al., 2000;

Sakamoto et al., 2000). Genes that encode the enzymes involved in GB biosynthesis have been cloned from higher plants, *E. coli*, and microorganisms (Rozwadowski et al., 1991; Deshnium et al., 1995; Rathinasabapathi et al., 1997; Nyyssola et al., 2000). Furthermore, genetic engineering of the GB biosynthetic pathway into non-accumulators has proven effective in enhancing stress tolerance (see review by McNeil et al., 1999; Sakamoto and Murata, 2000, 2002; Chen and Murata, 2002).

Rice plants have been transformed with the *codA* gene, which encodes choline oxidase (*COD*) that converts choline to GB, with or without the target sequence for chloroplasts (Sakamoto et al., 1998). When *COD* is designed to remain in the cytosol, GB levels are five times greater (~5 μmol/g FW) than when it is targeted to the chloroplasts. Under stress conditions, the content of GB in transgenic plants appears to be much lower (up to 5 μmol/g FW) than in GB accumulators (up to 40 μmol/g FW), regardless of the host plant species. For example, transgenic tobacco plants, engineered to produce GB, accumulate that compound in quantities of no more than 0.1 μmol/g FW (Holmstrom et al., 2000; Huang et al., 2000). On the other hand, transgenic utilization of two bacterial N-methyltransferase enzymes, such as glycine sarcosine methyltrasferase (*ApGSMT*) and dimethylglycine methyltransferase (*ApDMT*), in *Arabidopsis* plants accumulated much higher levels of GB than that of choline monooxigenase (*CMO*) transgenic *Arabidopsis* plants (Waditee et al. 2005).

In contrast, exogenous application of choline as a substrate to several species of transgenic plants can greatly increase GB levels (Nuccio et al., 1998; Huang et al., 2000). Nevertheless, modeling of the kinetics for labeled choline metabolites, after [14-C]-choline has been applied to transgenic tobacco that expresses a spinach gene for *CMO*, has demonstrated that choline import into the chloroplasts is a major constraint on the synthesis of GB in these organelles (McNeil et al., 2000). Hence, two major limiting factors have been hypothesized for the accumulation of GB in transgenic plants – substrate availability and the transport of choline across chloroplast membranes (Nuccio et al., 1998, 2000; Huang et al., 2000; McNeil et al., 2000). In particular, the availability of substrate can be increased by the engineering of

phosphoethanolamine N-methyltransferase (*PEAMT*), a key enzyme in the choline-biosynthetic pathway (McNeil et al., 2001). When *PEAMT* is introduced into transgenic tobacco plants already engineered to produce GB, its overexpression enables plants to accumulate up to 50 times more free choline and 30 times more GB than those transformed with vector alone (McNeil et al., 2001).

Recently, transgenic plants expressing the *codA* gene show reduced oxidative damage under LT stress (Park et al. 2004). Despite having higher levels of $H_2O_2$ the transgenic lines were phenotypically normal and recorded higher activities of $H_2O_2$ scavenging enzymes under both normal and stress conditions (Park et al. 2004). It has suggested that $H_2O_2$ produced by *COD* activity in transgenic lines stimulates the expression of antioxidant enzymes to maintain safe levels of $H_2O_2$, resulting in enhanced tolerance to oxidative stress induced by LT stress.

**D. Lipid-modifying genes**

Because membranes are critical sites of LT-induced injury, the engineering of more stress-tolerant cell membranes has attracted much attention. Increased levels of unsaturated membrane lipids lead to higher fluidity, improved cold tolerance, and greater photosynthetic ability in transgenic plants (Tables 1.1 and 1.2). For example, tobacco plants have been transformed with acyl-ACP:glycerol-3-phosphate acyltransferase (*GPAT*), from both a chilling-sensitive squash plant and chilling-tolerant *Arabidopsis* (Murata et al., 1992). Levels of saturated phosphatidyl glycerol decline in the transgenic tobacco containing the *Arabidopsis* enzyme, in contrast to plants with the squash enzyme. Correspondingly, transformants with the *Arabidopsis* enzyme are more chilling-tolerant than those with the squash enzyme. The cDNAs for either *Arabidopsis GPAT* or spinach *GPAT* have also been introduced into rice (Yokoi et al., 1998; Ariizumi et al., 2002). Under LT conditions, both the level of unsaturated fatty acids and the rate of net photosynthesis in the leaves of transgenic plants are much higher than in the controls.

A chloroplast ω-3 fatty acid desaturase has been shown to increase the amount of dienoic and trienoic fatty acids and, consequently, enhance resistance (Kodama et al., 1994). A broad-specificity Δ9-desaturase gene (*Des9*) from the cyanobacterium *Anacystis nidulans* or *Synechococcus vulcanus* has also been inserted into tobacco plants, resulting in enhanced chilling tolerance (Ishizaki-Nishizawa et al., 1996; Orlova et al. 2003). The enzyme introduces a *cis*-double bond in specific saturated fatty acids of various membrane lipids. Because lipid biosynthetic activities in higher plants are localized to the plastid and endoplasmic reticulum, the enzyme is fused to a transit peptide of the pea *Rubisco* small subunit in order to achieve correct targeting. As a result, the amount of affected *Δ9*-monosaturated fatty acids in the transgenics is increased up to 17-fold (Ishizaki-Nishizawa et al., 1996). Furthermore, when tobacco plants are exposed to 1 °C for 11 d, those expressing *Des9* show no signs of chlorosis compared with the WT plants.

**E. Oxidative stress-related genes**

Reactive oxygen species (ROS), including the superoxide ($O_2^-$), hydroxyl radicals ($OH^-$) and hydrogen peroxide ($H_2O_2$), cause oxidative damage to cells during several important metabolic processes, including the mitochondrial electron transport system and the chloroplast photosystems (Foyer et al., 1994). The amount of ROS increases when plants are exposed to various environmental stresses (reviewed by Foyer et al., 1994; Mittler, 2002). Under such stresses, PS I uses oxygen as an alternative electron acceptor. This oxygen reduction results in the production of superoxide and its dismutation product $H_2O_2$. Hydrogen peroxide and superoxide cooperate in the formation of highly toxic oxygen species, such as hydroxyl radicals (Wise, 1995). Furthermore, peroxidation of the membrane lipids or photoinhibition of PS I can be observed in chilling-sensitive plants (Gupta et al., 1993). These symptoms typically occur in plants suffering from oxidative stress. Therefore, this enhanced protection is also advantageous under LT conditions

To scavenge ROS, plants have developed enzyme systems that include superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), catalase (*CAT*), monodehydroascorbate reductase (*MDAR*), dehydroascorbate reductase (*DHAR*), and glutathione reductase (*GR*). Recent studies have demonstrated that plants produce ROS as signaling molecules to control their biological processes in response to abiotic stress (Neill et al., 2002; Vranová et al., 2002). In addition, researchers have attempted to develop transgenic plants with modified ROS-scavenging mechanisms as a means of increasing the functional capacity of such enzymes against LT-induced stress (Tables 1.1 and 1.2).

Manipulation of the expression of enzymes involved in ROS-scavenging systems has focused on *SOD, APX,* and *GR*, all isoenzymes that are mainly targeted into the cytosol, mitochondria, or plastids (Gupta et al., 1993; McKersie et al., 1993; Kornyeyev et al. 2003; Foyer et al., 1995; Yoshimura et al. 2004). For example, transgenic tobacco, which is genetically engineered with chloroplastic *CuZn-SOD*, shows improved resistance to intense light and LTs, even though the elevated activity of the native *APX* associated with higher *SOD* activity in these plants may be most critical to their recovery of photosynthesis (Gupta et al., 1993). Overexpression of *Mn-SOD* from tobacco also confers protection against freezing damage in alfalfa (McKersie et al., 1993) as well as chilling damage in cotton (Allen, 1995) and maize (van Breusegem et al., 1999a, 1999b). During field trials, transgenic alfalfa that potently expresses *Mn-SOD* and *Fe-SOD* in the chloroplasts has higher winter survival rates and herbage yield than do the control plants, with no detectable difference in the pattern of primary freezing injury, as shown by vital staining (McKersie et al., 1999, 2000). Transgenic overproduction of *APX* in cotton increases four-fold higher activity of *APX* than that of WT during exposure to 10 °C and 500 μmol photons/$m^2/s^1$ (Kornyeyev et al. 2003). These plants did not exhibit as large of an increase in cellular $H_2O_2$ that was evident in WT shortly after the imposition of the chilling treatment, together with showing less PS I and PS II photoinhibition. Transgenic tobacco that expresses both Glutathione S-transferase (*GST*) and glutathione peroxidase (*GPX*)

genes in the cytoplasm also shows improved tolerance to chilling temperatures and salt stress during germination and seedling development (Roxas et al., 1997, 2000). This improvement in growth appears to depend on increased oxidation of the glutathione pool, which is necessary for the effective scavenging of toxic $H_2O_2$ by *GPX* and for the maintenance of other antioxidants, such as ascorbate and tocopherols. Furthermore, transgenic tobacco plants overexpressing *Chlamydomonas* GPX in chloroplast seems to be more tolerant to several stresses, including chilling, salt and oxidative stress, than the cytosol-targeted lines (Yoshimura et al. 2004). This can be explained by the fact that the major sites of ROS production in the plant cell are the organelles with highly oxidizing metabolic activities or with sustained electron flows; chloroplasts, mitochondria, and microbodies. In addition, transgenic tobacco plants overproducing alfalfa NADPH-dependent aldose/aldehyde reductase also show higher tolerance to LT photoinhibition and cadmium stress (Hegedüs et al. 2004).

**Genetic engineering for cold tolerance in horticultural crops**

Even though many species have been transformed for enhanced tolerance to chilling (Table 1.1) and freezing (Table 1.2), the number of transgenic horticultural crops with such traits is relatively small.

**A. The *CBF1* transgenic tomato**

The *CRT/DRE* binding factor 1(*CBF1*) induces expression of the *COR* genes, resulting in enhanced tolerance to chilling (Table 1.1) or freezing (Table 1.2) without cold acclimation. *CBF1* homologues have been isolated from various chilling-tolerant and -sensitive species, indicating that cold-regulated *CBF*-like genes are not limited only to plants that can be cold-acclimated (Jaglo-Ottosen et al., 2001). Constitutive expression of the *Arabidopsis CBF1* gene in tomato increases tolerance to chilling, but not to freezing stress (Hsieh et al., 2002). Moreover, using known *Arabidopsis COR*

cDNAs, such as *COR47, COR15a*, and *KIN1*, as probes with *CBF1* transgenic tomatoes does not hybridize to any tomato transcripts, implying that alternative tomato protein(s) may be functioning as stress protectants (Hsieh et al., 2002).

Hsieh et al. (2002) have also found that the *CBF1* gene induces higher levels of the *CATALASE1* (*CAT1*) gene and catalase activity, resulting in improved oxidative tolerance with lower $H_2O_2$ content under either normal or LT conditions. In chilling-sensitive maize, *CAT3* gene expression and its enzymatic activities are increased during cold acclimation; the improvement of chilling tolerance conferred by acclimation is correlated with up-regulation of this gene (Prasad, 1997). Transgenic tomato plants that overexpress antisense *CAT1* are also more sensitive to oxidative stress and chilling injury (Kerdnaimongkol and Woodson, 1999). Hsieh et al. (2002) have proposed that a putative *CRT/DRE* binding site may exist on the maize *CAT3* and rice *CAT-A* promoter, based on DNA sequence analysis. Recently, Seki et al. (2001) have reported that the *Arabidopsis CAT3* gene is induced by cold, drought, and the overexpression of *DREB1a* (=*CBF3*). Therefore, based on all these studies, Hsieh et al. (2002) have concluded that the enhancement of chilling tolerance in *CBF1* transgenic tomato may be due to the induction of the *CAT1* gene.

*CBF1* transgenic tomato plants exhibit apparent dwarfism along with reductions in fruit set and number of seeds per fruit. Similar growth retardation has been observed in *Arabidopsis* transformed with the *DREB1a* construct (Kasuga et al., 1999). However, the application of $GA_3$ to transgenic tomato plants can reverse this phenomenon while not affecting their chilling tolerance. This suggests that overexpression of *CBF1* may interfere with GA biosynthesis in transgenics. Kasuga et al. (1999) have achieved enhanced tolerance without harming growth by replacing the *CAMV* 35S promoter with an inducible promoter in transgenic *Arabidopsis*. Their results demonstrate the potential benefit of using a stress-inducible promoter, such as *RD29A*, as a way to overcome the problem of dwarfism in *CBF1* transgenic plants.

**B. The *CBF1* transgenic strawberry**

Floral damage is a serious consequence of exposure to freezing temperatures, particularly in early-blooming species, such as strawberry, cherry, peach, almond, and apricot (Rodrigo, 2000). In strawberry plants, their early bloom and extreme mid-winter temperatures may increase the occurrence of injuries to flower and crown tissues, respectively. Therefore, Owens et al. (2002) have cloned orthologs of the *Arabidopsis CBF1* gene from strawberry (*Fragaria* X *ananassa* Duchesne) and sour cherry (*Prunus cerasus* L.), and have studied the expression of *CBF1* in both species. They have also used *Agrobacterium*-mediated transformation in strawberry, with the *Arabidopsis CBF1* gene driven by a constitutive CaMV 35S promoter. This has led to enhanced freezing tolerance in transgenic leaf tissues, but not in the receptacle tissues, although the level of *CBF1* transcript is similar in both (Owens et al., 2002).

In *Arabidopsis* transformed with reporter genes driven by the *cor15a* and *cor78* promoters, Horvath et al. (1993) have noted that the reporter is not expressed in the ovaries following cold acclimation, despite reporter gene expression being detected in many vegetative and reproductive tissues. Moreover, the *Arabidopsis sfr6* mutant, which is deficient in freezing tolerance, shows no expression of the *COR* genes in response to LTs, even though their expression of *CBF3* following cold exposure is normal (Knight et al., 1999). Altogether, these observations raise the possibility that either 1) different signal transduction pathways lead to the induction of *CBF* in strawberry floral tissues, or 2) cold acclimation can be induced by an alternative pathway or by a *CBF* paralog that has yet to be detected in such experiments (Owens et al., 2002).

### C. The *α-Gal* transgenic petunia

Transcriptome-profiling experiments in *Arabidopsis* indicate that extensive changes in gene expression occur during cold acclimation, and that a substantial number of the genes that are up-regulated during that time are involved in metabolism

under cold conditions (Fowler and Thomashow, 2002). In addition to *COR* gene expression, one notable biochemical/physiological alteration during cold acclimation is the accumulation of compatible solutes, such as soluble sugars (Guy, 1990). In *Arabidopsis*, transcripts and enzyme activity are strongly up-regulated for sugar biosynthesis at LTs (Strand et al., 2003), which suggests that such an accumulation may be related to carbon storage and a role as a cryoprotectant. Uemura et al. (2003) have studied the sugar-deficient mutant (*sfr4*), and have shown that this defect in its cells during cold acclimation is largely responsible for the impaired increase in freezing tolerance. However, supplementing those mutant plants with sucrose restores their tolerance to a level similar to that found with the wild-types after cold acclimation. Moreover, Castonguay and Nadeau (1998) and Taji et al. (2002) have reported that variations in freezing tolerance are more closely related to a plant's capacity to accumulate raffinose family oligosaccharides (RFOs) than to its ability to store sucrose.

In response to environmental conditions, the endogenous concentration of soluble sugars in higher plants is controlled through synthesis, degradation, and transport (Strand et al., 2003). RFOs, particularly raffinose, accumulate as a reaction to cold stress, with altered rates of raffinose biosynthesis (Taji et al., 2002). This biosynthesis is regulated by two enzymes, galactinol synthase (*GolS*) and raffinose synthase (*RafS*) (Pennycooke et al., 2003). Raffinose degradation proceeds by the action of α-galactosidase (*α-Gal*), which hydrolyzes Gal-containing oligosaccharides (Pennycooke et al., 2003). In addition, at least three putative *GolS* genes in *Arabidopsis* are members of the *CBF* regulon (Fowler and Thomashow, 2002), while one of them is induced in response to LTs and the overexpression of *CBF3/DREB1a* (Taji et al., 2002).

The tomato *Lea-Gal* gene (α-galactosidase) has been introduced into petunia in the sense and antisense orientations (Pennycooke et al., 2003). The content of total soluble sugars in non-acclimated antisense lines is approximately three to five times higher than that in the non-acclimated WT and sense lines. More than 50 % of the

increase in total soluble sugars is due to the accumulations of raffinose in cold-acclimated antisense lines and sucrose in cold-acclimated WT and sense lines (Pennycooke et al., 2003).

The raffinose content of non-acclimated antisense plants is up to 22-fold higher than in the wild-types, and up to 53-fold greater after cold acclimation. Under non-acclimated conditions, all except one antisense line are significantly more freezing tolerance (up to a 2 °C increase) than the WT plants. Freezing tolerance is about –5 °C for cold-acclimated WT plants, whereas the cold-acclimated antisense lines range from –6 to –8 °C. Thus, down-regulating α-Gal in petunia results in an elevated level of raffinose and increased tolerance at the whole-plant level for both non-acclimated and cold-acclimated plants. In contrast, overexpression of the α-Gal gene causes endogenous raffinose to decrease, along with the impairment of freezing tolerance. Moreover, differences in the maximum level of tolerance between the antisense and sense lines are more closely related to the capacity of plants to accumulate raffinose rather than sucrose (Pennycooke et al., 2003). These results, therefore, suggest that engineering for raffinose metabolism via transformation with the antisense construct of α-Gal provides an additional method for improving freezing tolerance.

**D. The *codA* transgenic tomato**

Tomato plants, which normally do not accumulate glycinebetaine (GB), are susceptible to chilling stress. We have transformed tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) with the *codA* gene, which encodes choline oxidase (*COD*), using the transit peptide sequence of a small subunit of the tobacco Rubisco gene to target the *COD* into chloroplasts (Park et al., 2003 and 2004). These transgenic plants can accumulate up to 0.23 μmol of GB per gram FW in their leaves. After exposure to stress conditions, GB levels in their leaves are lower than those reported in *Arabidopsis* (0.8 ~ 1.2 μmol/g FW; Hayashi et al., 1997), *Brassica juncea* (0.64 ~ 0.82 μmol/g FW; Prasad et al., 2000), or rice (1.1 ~ 5.3 μmol/g FW; Sakamoto et al., 1998), and are much lower than those in natural GB-accumulators such as spinach (30 to 40

μmol/g FW) (Rhodes and Hanson, 1993). Therefore, it may be necessary to generate plants with the capacity to synthesize higher levels of GB by enhancing the availability of choline, the GB precursor (McNeil et al., 2001).

In further physiological studies, seeds of homozygous transgenic lines have been found to germinate earlier, with and without cold treatment. When exposed to low temperatures, the dispersion rates of germination events increase in the wild-type, but not in the transgenic lines, compared with those under non-stress conditions. Within one week after the end of the chilling treatment, seeds of two transgenic lines show higher germination rates (93 % and 83 %) than those of WT seeds (53 %). *In-vitro* growth of transgenic seedlings, under either a 16-h photoperiod or 24-h darkness, also have enhanced tolerance to LT stress. Although neither the WT nor the L1 and L2 transgenic plants grow at all in the light during chilling, upon transferring all the plant types to warm conditions, the transgenic seedlings (with a 100 % survival rate) resume normal growth while the WT seedlings suffer extensive chilling injury, as manifested by necrotic lesions and moribund shoot tips. When chilled in darkness, transgenic seedlings continue to grow even at LT conditions, but the development of WT plants is completely inhibited. Upon their return to the greenhouse after 4 d of LT stress, five-week-old greenhouse-grown transgenic plants recover more quickly, with significantly less extensive chilling injury ($P<0.01$). Furthermore, only about 20 to 25 % of the total leaf area on the transgenic plants is damaged, compared with about 55 % injury on the WT plants.

When WT and transgenic plants that contain 2 ~ 3 open flowers in the first inflorescence are exposed to LT, the former retain only about 82 % of their flowers, but all flowers remain on the latter. Although this stress reduces the number of fruit in both types of plants, fruit set is higher ($P\leq0.052$) in the transgenics. Consequently, they yield, on average, 30 % more fruit after an episode of chilling stress. Therefore, these results indicate that the endogenous production of GB in transgenic tomato plants, as a consequence of *codA* expression, increases cold tolerance not only at the

vegetative but also the reproductive stage, both of which are known to be most sensitive to environmental stresses.

**Conclusions and perspectives**

With the advances made in the molecular biology of plant cold acclimation, our understanding of injuries and LT tolerances has improved significantly in the past ten years. This has contributed to the impressive results gained in research to improve plant cold tolerance by genetic engineering. In addition to studies of "model plant systems", various genes related to the cold-defensive mechanism have been shown to confer tolerance in crop species, such as rice, maize, and canola, as well as a few horticultural plants, i.e., tomato, strawberry, and petunia. We expect that genetic engineering will continue to be used for generating additional horticultural species with enhanced tolerance.

To date, most of the successful examples of engineered tolerance have been achieved by transferring a single cold-tolerance gene, although the particular conferring mechanisms have greatly differed. Constructs with the sense orientation for overexpression or the antisense orientation to down-regulate gene expression have been successfully used. In the case of transformation with transcription factors, such as the *CBF* gene, this single transgene has been capable of inducing the expression of a battery of downstream *COR* genes. This approach appears to be the most effective way to confer a high level of tolerance. In addition, these cold-hardy transgenic plants very often show improved tolerance to salinity, oxidative stress, and drought as well. Nevertheless, when driven by a constitutive promoter, high expression for this type of gene often leads to undesirable side effects, such as lower transformation and regeneration frequencies, difficulty in obtaining transgenic plants with a high level of *CBF* transgene expression, severe growth retardation, and reduced seed and fruit formation. Fortunately, a stress-inducible promoter can be used to alleviate such problems (Kasuga et al., 1999). Alternatively, if one can identify and clone a small

number of key *COR* genes involved in cold acclimation, a plant can be transformed with those genes, either singularly or in combination with a few such genes, to produce transgenic plants with improved tolerance but minimal detrimental effects.

Further improvement of cold tolerance via genetic engineering is still possible. First, a better understanding of the stress protection pathways in plants may allow us to selectively activate a minimum number of necessary genes at the right time and at the right place, as nature does. Second, better transformation technologies in horticultural species will allow us to improve tolerance in additional crops. Moreover, introducing different genes, which are individually effective in enhancing tolerance by various modes of action, into a single plant may result in the production of extremely stress-tolerant plants. However, it is also will be necessary to ensure the genetic manipulation that is achieved does not perturb growth or yield. Finally, the performance of transgenic plants should be verified in the lab as well as in the field; collaboration among different plant-science disciplines will lead to better evaluation of their practical utility.

Table 1.1 Summary of engineered chilling tolerance in plants

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| **Protein kinase** | | | |
| *OsCDPK7* *(Ca-dependent protein kinase)* | Rice | Rice | Saijo et al. (2000) |
| *OsMAPK5* *(Mitogen-activated protein kinase)* | Rice | Rice | Xiong and Yang (2003) |
| *AtDBF2* *(protein kinase)* | *Arabidopsis* | Tobacco | Lee et al. (1999) |
| **Transcription factors** | | | |
| *ICE1* *(Inducer of CBF expression1)* | *Arabidopsis* | *Arabidopsis* | Chinnusamy et al. (2003) |
| *CBF1* *(CRT/CRE binding factor)* | *Arabidopsis* | Tomato | Hsieh et al. (2002) |
| | " | " | Lee et al. (2003) |
| *SCOF-1* *(soybean cold-inducible factor1)* | Soybean | Tobacco | Kim et al. (2001) |
| *DREB1A* *(dehydration-responsive element binding protein)* | *Arabidopsis* | Tobacco | Kasuga et al. (2004) |
| *Osmyb4* *(myeloblastosis binding factor)* | Rice | *Arabidopsis* | Vannini et al. (2004) |
| *OSISAP1* *(zinc-finger protein)* | Rice | *Arabidopsis* | Mukhopadhyay et al. (2004) |

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| *CuCOR19* | Citrus | Tobacco | Hara et al. (2003) |
| *RCI3* | *Arabidopsis* | *Arabidopsis* | Llorente et al. (2002) |
| *(Rare cold-inducible gene)* | | | |
| ***Compatible solute synthesis genes*** | | | |
| *COD/codA* | *Arthrobacter* | *Arabidopsis* | Alia et al. (1998); |
| *(choline oxidase)* | *globiformis* | | Hayashi et al. (1997) |
| | " | Rice | Sakamoto et al. (1998) |
| | " | Tomato | Park et al. (2003 and 2004) |
| *BADH* | Barley | Rice | Kishitani et al. (2000) |
| *(betaine aldehyde dehydrogenase)* | | | |
| *bet a and bet b* | E. *coli* | Tobacco | Holmstrom et al. (2000) |
| *(choline dehydrogenase and betaine aldehyde dehydrogenase)* | | | |
| *ApGSMT andApDMT* | *Aphanothece* | *Arabidopsis* | Waditee et al. (2005) |
| *(glycine sarcosine methyltransferase and dimethyltransferase)* | *halophytica* | | |
| *TPSP* | E. *coli* | Rice | Garg et al. (2002); |
| *(trehalose-6-phosphate synthase/ phosphatase)* | | | Jang et al. (2003) |

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| **Lipid-modifying genes** | | | |
| *GPAT* | *Arabidopsis* | Tobacco | Murata et al. (1992) |
| *(glycerol-3-phosphate* | " | Rice | Yokoi et al. (1998) |
| *acyltransferase)* | Arabidopsis and Spinach | " | Ariizumi et al. (2002) |
| | Squash | Tobacco | Sakamoto et al. (2003) |
| *fad7* | *Arabidopsis* | Tobacco | Kodama et al. (1994) |
| *(chloroplast ω-3 fatty acid desaturase)* | | | |
| *des9* | *Anacystis* | Tobacco | Ishizaki-Nishizawa et |
| *(acyl-lipid Δ9 desaturase)* | *nidulans* | | al. (1996) |
| | *Synechococcus vulcanus* | " | Orlova et al. (2003) |
| ***Oxidative stress-related genes*** | | | |
| *Cu/Zn-SOD* | Pea | Tobacco | Gupta et al. (1993) |
| *(Cu/Zn superoxide dismutase)* | | | |
| *Mn-SOD* | Tobacco | Maize | van Breusegem et al. |
| *(Mn-superoxide dismutase)* | | | (1999a) |
| *Fe-SOD* | Tobacco | Alfalfa | McKersie et al. (2000) |
| *(Fe-superoxide dismutase)* | *Arabidopsis* | Maize | van Breusegem et al. (1999b) |
| *GST/GPX* | Tobacco | Tobacco | Roxas et al. (2000) |
| *(Glutathione S-transferase/peroxidase)* | | | |
| *GPX* | *Chlamydomonas* | Tobacco | Yoshimura et al. |
| *(glutathione peroxidase)* | | | (2004) |

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| *Nt107* *(Glutathione S-transferase)* | Tobacco | Tobacco | Roxas et al. (1997) |
| *APX* *(Ascorbate peroxidase)* | Tobacco and Spinach | Tobacco | Yabuta et al. (2002) |
| | Pea | Cotton | Kornyeyev (2003) |
| *ALR* *(aldose/aldehyde reductase)* | Alfalfa | Tobacco | Hegedüs et al. (2004) |

Table 1.2 Summary of engineered freezing tolerance in plants

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| **Protein kinases** | | | |
| *AtNDPKs* *(NDP kinase)* | *Arabidopsis* | *Arabidopsis* | Moon et al. (2003) |
| *ANP1* *(mitogen-activated protein kinase kinase kinase)* | *Arabidopsis* | Tobacco | Kovtun et al. (2000) |
| *NPK1* *(mitogen-activated protein kinase kinase kinase)* | *Tobacco* | Maize | Shou et al. (2004) |
| **Transcription factors** | | | |
| *ICE1* *(Inducer of CBF expression1)* | *Arabidopsis* | *Arabidopsis* | Chinnusamy et al. (2003) |
| *CBF1* *(CRT/DRE binding factor)* | *Arabidopsis* | *Arabidopsis* | Jaglo-Ottosen et al. (1998); Kasuga et al. (1999); Gilmour et al. (2000) |
| | - | Canola | Jaglo-Ottosen et al. (2001) |
| | - | Strawberry | Owens et al. (2002) |
| | Canola | Canola | Gusta et al. (2002) |
| | - | Flax | - |
| | Sweet cherry | *Arabidopsis* | Kitashiba et al. (2004) |
| *SCOF-1* *(soybean cold-inducible factor1)* | Soybean | Tobacco | Kim et al. (2001) |

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| *OsDREB1A* *(dehydration-responsive element binding protein)* | Rice | *Arabidopsis* | Dubouzet et al. (2003) |
| *ZmDREB1A* | Maize | *Arabidopsis* | Qin et al. (2004) |
| *Osmyb4* *(myeloblastosis binding factor)* | Rice | *Arabidopsis* | Vannini et al. (2004) |
| *CBF4* | *Arabidopsis* | *Arabidopsis* | Haake et al. (2002) |
| *CaPF1* *(ERF/AP2 transcription factor)* | Hot pepper | *Arabidopsis* | Yi et al. (2004) |
| **Cold-regulated (*COR*) genes** | | | |
| *COR15a* *(cold-regulated gene)* | *Arabidopsis* | *Arabidopsis* | Artus et al. (1996); Steponkus et al. (1998) |
| *CuCOR19* *(dehydrin gene)* | Citrus | Tobacco | Hara et al. (2003) |
| *Wcs19* *(late embryogenesis abundant protein)* | Wheat | *Arabidopsis* | Ndong et al. (2002) |
| *RAB 18 and COR47* | *Arabidopsis* | *Arabidopsis* | Puhakainen et al. (2004) |
| *LTI29 and LTI30* | - | - | - |
| **Compatible solute-synthesis genes** | | | |
| *COD/codA* *(choline oxidase)* | *Arthrobacter globiformis* | *Arabidopsis* | Sakamoto et al. (2000) |
| | - | Tobacco | Konstantinova et al. (2002) |
| *COD/cox* | *Arthrobacter pascens* | *Arabidopsis* | Huang et al. (2000) |

| Gene | Origin | Transgenic species | References |
|---|---|---|---|
| *AtProDH* *(Antisense proline dehydrogenase)* | *Arabidopsis* | *Arabidopsis* | Nanjo et al. (1999) |
| *Lea-Gal* *(Antisense α-galactosidase)* | Tomato | Petunia | Pennycooke et al. (2003) |
| *SPS* *(Sucrose phosphate synthase)* | *Arabidopsis* | *Arabidopsis* | Strand et al. (2003) |
| *wft1/wft2* *(fructosyltransferase)* | Wheat | Ryegrass | Hisano et al. (2004) |
| *SacB* *(levan sucrase)* | *Bacillus subtillis* | Tobacco | Konstantinova et al. (2002) |
| **Oxidative stress-related genes** | | | |
| *Mn-SOD* *(Mn-superoxide dismutase)* | Tobacco | Alfalfa | McKersie et al. (1999) |
| | Wheat | Canola | Gusta et al. (2002) |
| *Fe-SOD* *(Fe-superoxide dismutase)* | Tobacco | Alfalfa | McKersie et al. (2000) |

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# CHAPTER 2

## Exogenous Application of Glycinebetaine Increases Chilling Tolerance in Tomato Plants

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**Abstract**

Tomato plants do not naturally accumulate glycinebetaine (GB), a metabolite that functions as a stress protectant in plants. While GB can increase tolerance to salt and drought stresses, its effect on chilling tolerance has not been examined. Therefore, we evaluated whether exogenously applied GB could improve the tolerance of plants of *Lycopersicon esculentum* Mill. cv. Moneymaker, which were first sprayed with different concentrations of GB (0, 0.1, 1.0, or 10.0 mM), then exposed to chilling stress at 3 °C. GB-treated plants exhibited enhanced chilling-stress tolerance, as defined by both their Photosystem II (PSII) activity during the treatment period and by the recovery of their growth at 25 °C following chilling stress. Based on the measurements of these parameters, we determined the optimal GB concentration to be 1.0 mM. Such a level improved PSII activity and reduced ion leakage in plants of various ages (i.e., 4, 6, and 8 weeks old). This beneficial, protective effect on PSII activity continued for up to 3 d, but then disappeared within a week after the application. Endogenous GB levels in treated plants were 0.01 to 1.07 μmol $g^{-1}$ chlorophyll; only ~21.6% of the total GB content in their leaves was localized to the chloroplasts. In addition, the highest levels of GB were found in meristematic tissues, including the shoot apices and flower buds. Furthermore, when plants were treated with 1.0 mM GB, their levels of $H_2O_2$ and catalase activity, as well as expression of the catalase gene (*CAT*), were higher under non-stress conditions (Day 0) than those of the control plants. One day after exposure to chilling stress, GB-treated plants had significantly enhanced catalase activity and *CAT* expression compared with the control plants, although $H_2O_2$ levels in those treated plants remained unchanged. During chilling treatment, plants that had received GB maintained lower $H_2O_2$ levels but a higher degree of catalase activity compared with the controls. Therefore, these results suggest that exogenously applied GB improves chilling tolerance, first by increasing endogenous $H_2O_2$ content, then, subsequently, by enhancing the levels of catalase expression and catalase activity.

## Introduction

In nature, low temperatures are a major factor limiting the geographical distribution and productivity of many plant species, including important agricultural and horticultural crops. Over time, plants have developed various protective mechanisms to cope with such abiotic stresses as cold, salt, and drought. One mechanism is the accumulation of compatible solutes (Bohnert *et al.*, 1995), a variety of small organic metabolites that are very soluble in water and non-toxic at high concentrations. These metabolites allow cells to retain water without disturbing their normal functions (Yancey *et al.*, 1982). Representative compatible solutes, which differ among plant species, include certain polyols, sugars, amino acids, betaines, and related compounds (Rhodes and Hanson, 1993).

The best-known betaine in plants is glycinebetaine (GB) which is dipolar but electrically neutral at physiological pH (Rhodes and Hanson, 1993). GB is synthesized at elevated rates in response to abiotic stresses (Allard *et al.*, 1998; Nomura *et al.*, 1995). Levels of GB accumulation are correlated with the extent of increased tolerance by plants (Rhodes and Hanson, 1993). Exogenous applications can improve the growth and survival of numerous species under stress (Alia *et al.*, 1998; Allard *et al.*, 1998; Chen *et al.*, 2000; Jokinen *et al.*, 1999; Mäkelä *et al.*, 1998, 1999; Zhao *et al.*, 1992). GB also effectively stabilizes the quaternary structures of enzymes and complex proteins, and maintains a highly ordered state of membranes when *in-vitro* temperatures or salt concentrations are extreme (Papageorgiou and Murata, 1995). Finally, introducing the GB biosynthetic pathway into non-accumulator plant species also increases their tolerance to various abiotic stresses (For reviews, see Chen and Murata, 2002; Sakamoto and Murata, 2000).

Tomato plants (*Lycopersicon esculentum* Mill.) are of tropical origin and do not naturally accumulate GB (Wyn Jones and Storey, 1981). Most cultivated genotypes suffer chilling injury when grown at less than 10 °C (Graham and Patterson, 1982; Patterson *et al.*, 1987). Extended exposure below 6 °C can kill plants; below 13 °C,

fruit-set may be inhibited (Atherton and Rudich, 1986). Although they are not natural accumulators (Wyn Jones and Storey, 1981), exogenous GB application to tomato plants increases their tolerance to salt and drought stresses (Makela *et al.*, 1998, 1999).

Because molecular and cellular responses to salt, drought, and cold are often interlinked, and because various kinds of proteins and smaller molecules are produced simultaneously in response to these stresses, we have now evaluated whether exogenously applied GB can also result in improved chilling tolerance in tomato. Here, such tolerance was assessed by measuring various growth parameters to determine by which mechanism the accumulation of GB might protect plants exposed to low temperatures.

## Results

### Exogenous GB application enhances chilling tolerance in tomato plants

Following exogenous GB applications, we examined PSII activity while the plants were chilled at 3 °C for 3 d, then allowed to recover for 2 d at 25 °C. We also examined the resumption of growth at 2 and 4 weeks after the chilling treatment.

One day after GB application (Day 0), PSII activity in GB-treated plants did not differ significantly from the water-treated (control) plants (Figure 2.1a). During the chilling period, however, activity declined in both types (Figure 2.1a), although the rate of decline was much slower for the GB-treated plants. After 2 d at 25 °C, PSII activity in the control plants declined to 41% of the original value (at Day 0); GB-treated plants maintained a level of activity that was 49 to 55% of normal.

We also investigated the long-term effects of chilling stress on subsequent recovery of their height development (cm) when plants were grown in a greenhouse for up to 4 weeks. Immediately after the stress period, growth did not differ significantly among plants treated with various GB concentrations (0.1 to 10.0 mM) during the post-chilling recovery at 25 °C (Figure 2.1b). Nevertheless, the endogenous level of total GB in leaves of plants treated with 10.0 mM GB was >100-fold higher than that of the 0.1 mM GB-treated plants (Table 2.1). However, all treated plants grew much faster than did the controls at 4 weeks after chilling stress (Figure 2.1b, c). Based on these preliminary results, we selected 1.0 mM GB as the optimal concentration for further evaluation of the responses of GB-treated plants to chilling stress, as defined in terms of both its enhancement of PSII activity during the chilling treatment as well as improved recovery after this stress period.

### GB application enhances chilling tolerance at various stages of plant growth

To evaluate whether this GB effect was limited to a particular developmental stage, we applied 1.0 mM during three distinct growth periods: 1) 4-week-old plants (non-flowering) that possessed six to seven compound leaves without flower buds; 2) 6-

**Table 2.1.** Total glycinebetaine (GB) levels in leaves, and percentage of GB in chloroplasts isolated from leaves of GB-treated tomato plants[1]

| GB treatment (mM) | GB | | | Intact chloroplasts (%) | GB in chloroplasts (%) |
|---|---|---|---|---|---|
| | Leaves | | Isolated chloroplast | | |
| | μmol $g^{-1}$ FW | nmol $mg^{-1}$ chlorophyll | nmol $mg^{-1}$ chlorophyll | | |
| 0.1 | 0.09±0.01 | 7.78±0.98 | 0.98±0.11 | 58.9±6.4 | 21.55±3.20 |
| 1.0 | 1.09±0.13 | 89.21±1.69 | 1.75±0.94 | 59.7±5.8 | 3.28±0.41 |
| 10.0 | 10.88±1.38 | 900.49±102.48 | 3.20±3.98 | 61.2±5.5 | 0.58±0.07 |

[1]Mean values ± S.D. from three experiments. GB content in chloroplasts was corrected for percentage of broken chloroplasts present. Percentage of GB found in chloroplasts was calculated by comparing leaf and chloroplast contents, expressed on a chlorophyll basis.

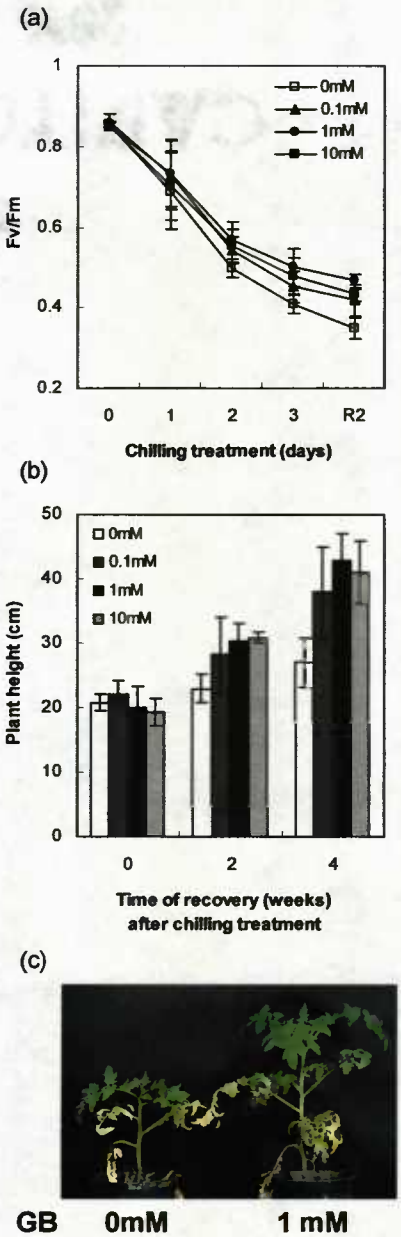


**Figure 2.1.** Enhanced chilling tolerance in glycinebetaine (GB)-treated tomato plants. (a) Five-week-old plants were sprayed with either water (control) or solutions containing various concentrations of GB (0.1, 1.0, or 10.0 mM). One day after foliar application, plants were chilled at 3 °C for 3 d, then placed in greenhouse at 25 °C for 2 d (R2). Photosystem II (PSII) activity (Fv/Fm) was measured at each time point indicated. (b) Following chilling treatment described in (a), GB- and water-treated plants were transferred to greenhouse at 25 °C; heights (cm) were measured 0, 2, and 4 weeks later. (c) Plants treated with either 0 or 1 mM GB for 1 d, chilled for 3 d, then grown at 25 °C for 4 weeks.

week-old plants with flower buds pre-anthesis; and 3) 8-week-old plants with open flowers. At 3 d after these chilling treatments, the GB-treated plants had maintained 61 to 71% of their relative PSII activity (compared with pre-stress values) while the control plants retained only as much as 51% of the original value (Figure 2.2a). Exogenous GB was more effective (10% higher rate) in protecting PSII activity at 4 weeks than when applied to 8-week-old plants.

Much greater membrane injury was observed among our control plants, regardless of their age (Figure 2.2b). For example, the control plants exhibited up to 75% ion leakage compared with less than 40% of pre-stress values for the GB-treated plants.

**Beneficial effect of GB on enhanced chilling tolerance is diminished after one week**

To evaluate how long the beneficial effect of glycine betaine endured, we applied 1 mM GB to tomato plants at 1, 3, or 7 d before beginning the chilling treatment. At 3 d post-chilling, we found that GB pre-treatment at both 1 and 3 d provided similar levels of protection to PSII activity, whereas PSII activity in plants pre-treated with GB at 7d was not significantly increased compared to that in the control plants (Figure 2.3), i.e. 46.7% (GB-treated) and 50.1% (control).

**Localization of GB in treated plants**

To determine the translocation of exogenously applied GB to different tissues, we applied 20 mM of solution to 7-week-old plants, of which their first inflorescences included unopened flower buds (<0.5 cm), and observed GB movement from the leaves to the shoot apices and flower buds (Figure 2.4). High levels were found mainly in the shoot apices (20.4 μmol $g^{-1}$ FW) and flower buds (18.7 μmol $g^{-1}$ FW) at 3 d after application, while levels in the stems and roots were not significantly changed. However, GB content in older leaves was rapidly reduced by Day 5, down to 19% of that measured at Day 1. These data clearly demonstrate that exogenously applied GB was readily taken up by the leaves and translocated to various other organs.

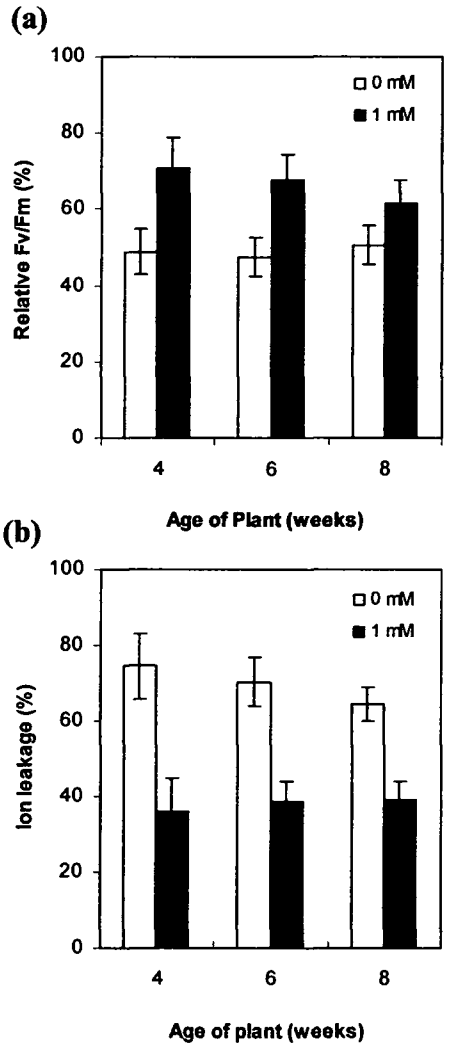


**Figure 2.2.** Improved chilling tolerance in glycinebetaine (GB)-treated tomato plants at various developmental stages. Either water or 1 mM GB solution was foliar-applied to 4-, 6-, or 8-week-old plants. One day after GB treatment, chilling stress was given. PSII activity (a) and ion leakage (b) were measured at end of treatment. Results are mean ± SE from three independent experiments (three to five plants per experiment).

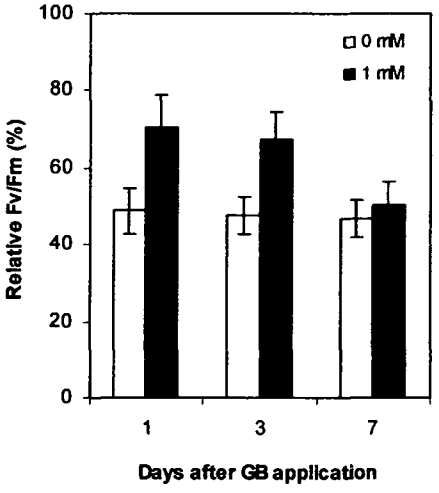


**Figure 2.3.** Duration of glycinebetaine (GB)-enhanced chilling tolerance. Five-week-old plants were sprayed with either water or 1 mM GB. At 1, 3, or 7 d after GB pre-treatment, GB-treated plants were exposed to chilling stress for 3 d. PSII activity was measured at end of chilling period. Results are mean ± SE from three independent experiments (three to five plants per experiment).

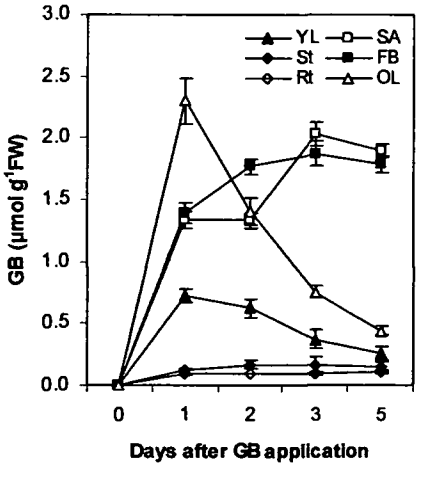


**Figure 2.4.** Glycinebetaine (GB) levels in various organs of GB-treated tomato plants. Means of three experiments; bars indicate standard errors. YL, young leaves (<1 cm); SA, shoot apices; St, stems; FB, flower buds; Rt, roots; OL, old leaves directly exposed to 20 mM GB solution.

We also analyzed GB contents in the chloroplasts of treated plants, reasoning that some of the glycinebetaine had to reach the chloroplasts in order to exert any protective effect. Various levels of GB accumulated at 1 d after our foliar application, depending on the concentration used in the spray solution (0.1, 1.0, or 10.0 mM; Table 2.1). Although total GB contents in the leaves ranged from 0.01 to 1.07 μmol $mg^{-1}$ chlorophyll, only a limited amount of GB (1.00 to 3.65 nmol $mg^{-1}$ chlorophyll) was detected in the chloroplasts, regardless of the concentration of GB originally applied. Moreover, only 0.6 to 21.6% of the total leaf GB was localized in the chloroplasts of GB-treated plants.

**Exogenous application of GB enhances levels of catalase expression and catalase activity with only marginal increases in $H_2O_2$ production**

Because chilling is a form of oxidative stress, we analyzed the effects of GB application using several parameters related to tolerance: $H_2O_2$ levels, catalase activity, and expression of the catalase gene.

Under non-stress conditions, $H_2O_2$ levels were 11% higher in the GB-treated plants than in the controls (Figure 2.5a). At Day 1 of chilling stress, levels in the latter rose to about 50% of their pre-stress amounts (i.e., at Day 0), whereas $H_2O_2$ content in the GB-treated plants remained unchanged. However, levels increased in both types as the stress period continued. This rate was much greater in the control plants than in the GB-treated plants during the first day of chilling, but then $H_2O_2$ content continued to increase at a similar rate for both types until Day 3 (Figure 2.5a). During 3 d of incubation at 3 °C, contents in the control plants were 2.4-fold higher than their original value (Day 0), whereas the GB-treated plants accumulated up to 2.0-fold more $H_2O_2$.

Under non-stress conditions, catalase activity in GB-treated plants was 12% higher compared with the controls (Figure 2.5b). It then increased up to 57% for the former versus 18% for the latter during Day 1 of chilling (Figure 2.5b). Thereafter, activity decreased very rapidly for both plant types. At the end of the treatment period (Day 3),

catalase activity in the control and GB-treated plants was reduced to 36% and 38%, respectively, of their initial values (Day 0). Nevertheless, catalase activity in GB-treated plants was much higher than that in the control plants (Figure 2.5b).

The expression pattern for the catalase gene (*CAT1*) was correlated with its enzyme activity (Figure 2.5c), with GB application temporarily increasing expression under non-stress conditions. Expression at Day 1 after chilling increased much more in the GB-treated plants than in the controls. However, no significant difference was observed in the level of catalase expression at Day 3 of the stress treatment.

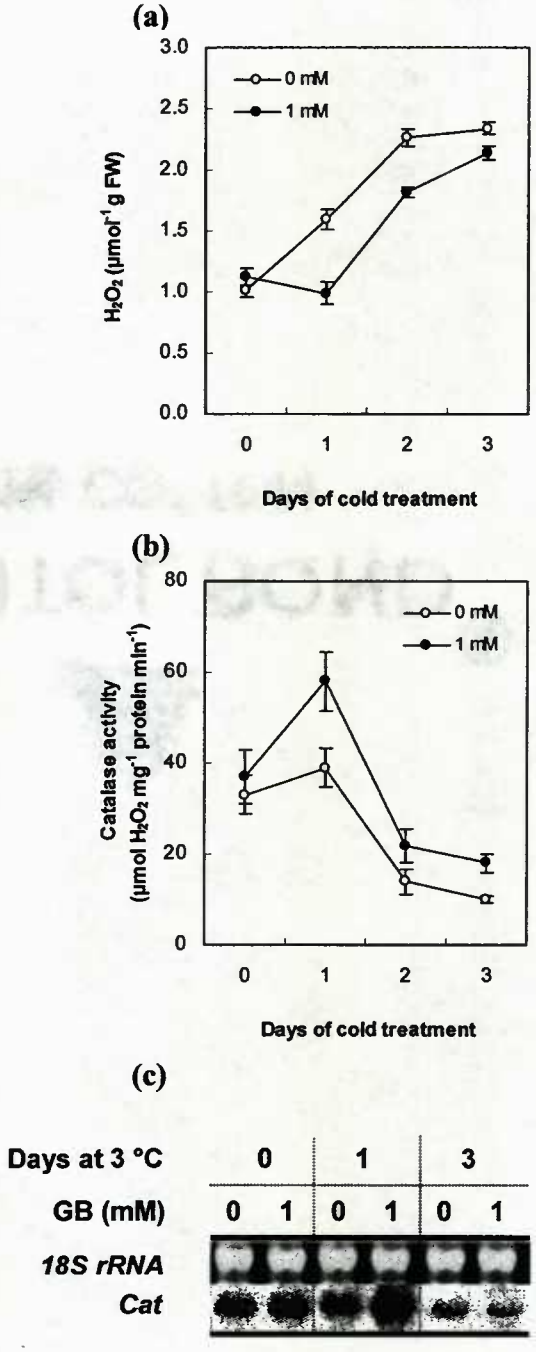


**Figure 2.5.** Effects of glycinebetaine (GB) on levels of hydrogen peroxide (a), catalase activity (b), and catalase expression (c) as determined by northern blot analysis. Either water or 1 mM GB solution was foliar-applied to 5-week-old plants. One day after GB treatment, plants were chilled at 3 °C for 3 d. Twenty micrograms of total RNA was loaded for each lane. Hybridization probe (*CAT1*) was $^{32}$P-labeled.

**Discussion**

Although tomato plants do not naturally accumulate GB (Wyn Jones and Storey, 1981), GB was taken up readily when foliar-applied (Table 2.1). These plants accumulated various levels in their leaves (0.09 to 10.88 μmol $g^{-1}$ FW) depending on the concentration applied (0.1 to 10.0 mM; Table 2.1), and also exhibited enhanced tolerance at different stages of development (Figures 2.1 and 2.2). In natural GB-accumulator plants, absorption is induced under stress conditions, with the level of GB being correlated with the degree of enhanced tolerance to stress (Rhodes and Hanson, 1993). However, we found that higher concentrations of applied GB did not further increase chilling tolerance in GB-treated plants (Figure 2.1), even though those sprayed with 10 mM GB accumulated 100-fold higher levels of endogenous GB than those treated with 0.1 mM GB (Table 2.1). This result agrees with that from our earlier work, which focused on GB-accumulating transgenic tomato plants (Park *et al.*, 2004). Previously, those chloroplast-targeted *codA* plants that accumulated GB in their leaves at levels of 0.09 to 0.30 μmol $g^{-1}$ FW exhibited enhanced chilling tolerance at various ages. Based on all these data, we can now suggest that a threshold level of endogenous GB (>0.09 μmol $g^{-1}$ FW) provides sufficient protection against low temperatures.

The effects of GB accumulation in the chloroplasts have been extensively examined in genetically engineered plants. Transgenic production of GB in those cellular components protects plants from stress more efficiently than when GB is accumulated in the cytosol (Sakamoto *et al.*, 1998). Moreover, chloroplast GB levels are correlated with the degree of stress tolerance (Park *et al.*, 2004). In transgenic rice plants transformed with the *codA* gene for chloroplast-targeted choline oxidase, the photosynthetic machinery is more efficiently protected against salt and cold stresses than in those that express a *codA* gene whose product is not targeted to chloroplasts, even though the latter accumulates five times more GB (Sakamoto *et al.*, 1998).

We also have reported previously that chloroplast-targeting *codA* transgenic tomato plants accumulate up to 86% of the GB in their leaf chloroplasts, and that the

highest correlation between GB contents and chilling tolerance levels is manifested in the protection of PSII (Park *et al.*, 2004). However, in the current study, the proportion of GB in the chloroplasts of GB-treated plants accounted for up to 22% of the total leaf GB when 0.1 mM glycinebetaine was applied. Likewise, the amount of GB in the chloroplasts did not vary, regardless of the concentration used (Table 2.1). In GB-accumulating transgenic plants, the targeting of a GB-catalyzing enzyme to different subcellular compartments, e.g., the peroxisome, mitochondria, and cytosol, also enhances tolerance by the photosynthetic machinery to salt and chilling (Holmstrom *et al.*, 2000; Kishitani *et al.*, 1994; Takabe *et al.*, 1998). Our result demonstrates that GB was mostly localized in the cytosol and that only a limited amount was translocated into the chloroplasts. Therefore, it is possible that GB synthesized in the cytosol can be transported into those aforementioned compartments, and that even a small amount may be sufficient to confer protection in them against chilling stress.

When GB was foliar-applied to our tomato plants, large amounts of GB were translocated into the meristematic tissues, including the flower buds and shoot apices (Figure 2.4). GB-treated plants also showed improved growth rates during their post-chilling recovery period (Figure 2.1b). Furthermore, a pre-treatment with GB at 7 d prior did not later enhance PSII activity under chilling stress, whereas plants sprayed with GB at 1 or 3 d before chilling was begun maintained higher PSII activities than did the control plants (Figure 2.3). Partial translocation of GB has been reported with assimilates, especially to actively growing and expanding plant portions, thus indicating that its long-distance transport is phloem-mobile (Mäkelä *et al.*, 1996). Furthermore, exogenous application of GB results in higher yields in the greenhouse and field, mainly due to improved net photosynthesis (Jokinen *et al.*, 1999; Mäkelä *et al.*, 1998). Greater absorption in the reproductive organs of GB-accumulating transgenic plants also is associated with enhanced tolerance to salt and chilling stresses, again resulting in high crop yields (Park *et al.*, 2004; Sulpice *et al.*, 2003). Therefore, more accumulation of GB in the meristematic tissues might further increase tolerance of those organs in GB-treated plants, thereby improving their growth rates

after exposure to low temperatures. In addition, such translocation of GB likely causes a decrease in its level in leaves of GB-treated plants, which consequently diminishes the beneficial effect of protecting PSII activity from chilling stress. Therefore, our result provides a motive for introducing a biosynthetic GB pathway into tomato plants in order to enhance chilling tolerance without requiring frequent exogenous applications of glycinebetaine.

It is interesting to note that GB application increased levels of $H_2O_2$ in GB-treated plants over those in the control plants under non-stress conditions (Figure 2.5a). Sulpice *et al.* (2002) have reported that applying GB to both canola and *Arabidopsis* leaf discs induces the accumulation of both glutamine and glycine. Accumulation of the latter in canola, however, is restricted when GB is supplied along with glycolate-pathway inhibitors, suggesting a possible interaction between GB accumulation and photorespiration at the mitochondria. Glycolate from the chloroplasts diffuses to the peroxisome, where it is oxidized to glyoxylate by a glycolate oxidase (GO)-mediated reaction that yields $H_2O_2$. Therefore, it is very likely that the GB absorbed by our tomato plants also increased GO activity, resulting in a higher accumulation of $H_2O_2$ in GB-treated plants than in the controls.

Following chilling, GB-treated plants maintained lower levels of $H_2O_2$ than those in water-treated plants, even though glycinebetaine is known to be ineffective in scavenging reactive oxygen species (Smirnoff and Cumbes, 1989). Thus, the increased tolerance to oxidative stress should be an indirect effect of GB, such as the induction of catalase. $H_2O_2$ is a secondary messenger in plants (Neil *et al.*, 2002). Although toxic levels lead to programmed cell death, a relatively non-toxic amount modifies gene expression and enhances plant stress responses (Inzé and van Montagu, 1995). At low levels, $H_2O_2$ can stimulate protection against such oxidative stress by inducing expression of antioxidant enzymes, e.g., CAT3 (catalase3), resulting in enhanced tolerance to chilling (Prasad *et al.*, 1994). Tomato plants pre-treated with $H_2O_2$ also improve their chilling tolerance because of increased catalase activity (Kerdnaimongkol and Woodson, 1997). We also observed greater levels of both

catalase expression and activity under non-stress conditions (Figure 2.5b, c). Furthermore, catalase activity in GB-treated plants increased up to 57% compared with only 18% in the control plants during Day 1 of chilling (Figure 2.5b). Demiral and Türkan (2004) have reported that exogenous application of GB increases catalase activity in a salt-sensitive rice cultivar under high salt stress. They suggest that such enhanced stimulation activity might result from an induction of synthesis of the enzyme via the production of $H_2O_2$. This phenomenon has been observed in a number of transgenic plants transformed with a gene for choline oxidase that renders $H_2O_2$ as a by-product of the reaction (Alia *et al.*, 1999; Park *et al.*, 2004; Prasad and Pardha Saradhi, 2004). All of those plants produce higher levels of $H_2O_2$ and catalase activity, suggesting that maintaining $H_2O_2$ at a particular non-toxic threshold level in *codA* transgenics might induce the expression of genes responsible for enzymatic detoxification of $H_2O_2$.

Likewise, treatments involving $H_2O_2$ increase the levels of both *cat3* transcripts and CAT3 activity, resulting in higher survival and growth rates upon exposure to chilling stress (Prasad *et al.*, 1994). We also observed that GB applications elevated the expression of catalase correspondent to its enzyme activity (Figure 2.5). Therefore, it is likely that the induced tolerance conferred by exogenously applied GB might have been the result of a $H_2O_2$-mediated antioxidant mechanism that included enhanced catalase expression and catalase activity. However, it still remains to be revealed whether expression of other genes is affected by GB treatment. Currently, we are conducting tomato microarray analysis, which will help us to understand the mode of GB action in conferring chilling tolerance. Preliminary results have shown that a number of genes involved in the electron transport pathways in both chloroplasts and mitochondria are over-expressed in response to GB application (data not shown).

In conclusion, exogenous application of glycinebetaine caused tomato plants to accumulate GB. A threshold level (>0.09 μmol $g^{-1}$ FW) in GB-treated plants provided sufficient protection against low temperatures. The majority of the GB was localized in the cytosol and only a small proportion was translocated into the chloroplasts,

indicating that GB can be translocated from the cytosol to protect various subcellular compartments. High levels of GB were found in meristematic tissues, including the shoot apices and flower buds, and the growth of GB-treated plants was increased following chilling. These results suggest that a higher accumulation of GB in those meristematic tissues may further improve their tolerance of low temperatures, resulting in enhanced recovery-growth rates during post-chilling periods under non-stress conditions. Our application of GB also increased levels of $H_2O_2$, catalase expression, and catalase activity in the absence of stress. Following chilling, the GB-treated plants maintained lower levels of $H_2O_2$ and higher levels of catalase activity throughout the treatment period, compared with the performance of those parameters in the control plants. In addition to a possible direct, protective effect of GB on macromolecules, such as membranes and proteins, it is likely that the induced chilling tolerance conferred by exogenous application may have resulted from the induction of $H_2O_2$-mediated antioxidant mechanisms, e.g., enhanced catalase expression and catalase activity.

## Experimental Procedures

### Growth conditions and GB treatments

Seeds of the tomato (*Lycopersicon esculentum* cv. 'Moneymaker') were surface-sterilized with 70% ethanol and 25% commercial Clorox, and thoroughly rinsed with distilled water. They were then held for 3 d in the dark at 25 °C on two layers of sterile filter paper in plastic Petri dishes. Uniformly germinated seedlings were grown in the greenhouse (25±3 °C, 16-h photoperiod, 400 to 500 μmol $m^{-2}$ $s^{-1}$).

Three to five plants per treatment were sprayed with either a water control or a solution of GB (0.1, 1.0, or 10.0 mM). Tween-20 (0.005 % v/v) was included as a wetting agent. Following the foliar applications, GB-treated plants were kept in the dark for 24 h, then transferred to a cold growth chamber (3 ± 0.5 °C, 16-h photoperiod, 400 to 500 μmol $m^{-2}$ $s^{-1}$) for 3 d. Afterward, these chilled plants were allowed to recover in a greenhouse at 25 °C.

The duration of any beneficial effect of GB on tolerance was measured by foliar-applying a 1.0 mM GB solution to 5-week-old plants at 1, 3, or 7 d before the chilling treatment began. For GB translocation analysis, 20 mM of GB solution was sprayed on 7-week-old plants of which the first inflorescences contained flower buds at the pre–anthesis stage. These solutions were applied only to leaves that were the second or third from the bottom. Other portions of the plant were covered with plastic wrap to avoid having GB absorbed through their roots or other leaves.

### GB determination

GB contents were determined via HPLC (Waters Corp., Milford, MA) using a refractive index detector (RI 2414) and a Sugar-Pak I HPLC column (6.5 mm i.d. x 300 mm; Waters Corp.), as described by Park *et al.* (2004).

**Chloroplast isolation**

After GB-treated plants were held in the dark for 24 h, their intact chloroplasts were obtained with an isolation kit (Sigma, St, Louis, MO) according to the manufacturer's protocol. The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. Total chlorophyll concentration was determined in 80% (v/v) acetone via the manufacturer's instructions.

**Protein extraction, catalase assay, and $H_2O_2$ quantification**

Compound leaves, the third to fifth from the top of each plant, were collected after each treatment (water or GB), then washed three times with distilled water, and stored at –80 °C. Proteins were extracted and catalase activity was calculated via methods described by Alia *et al.* (1999). Levels of $H_2O_2$ were determined as described by Park *et al.* (2004).

**Measurement of chlorophyll fluorescence and ion leakage**

Chlorophyll fluorescence was measured at the end of the dark treatment period. The ratio of variable to maximum fluorescence (Fv/Fm) was recorded at RT using a pulse-modulated Fluorescence Monitoring System (FMS1; Hansatech, UK). Ion leakage was determined with a conductivity meter (Model 35; Yellow Springs Instrument) from three to five leaf discs per sample, according to the procedure described by Park *et al.* (2004).

**Isolation of RNA and Northern blotting analysis**

Leaves were harvested at the end of the dark period, and total RNA was isolated using the Plant RNeasy kit (Quiagen, Valencia, CA). Gel electrophoresis and blotting of RNA to a Nytran nylon membrane (Schleicher and Schuell, Keene, NH) was performed as described by Skinner and Timko (1998). RNA blots were probed and washed using Ultrahyb Solution, following the manufacturer's instructions (Ambion,

Austin, TX). For northern analysis, a cDNA probe fragment for tomato catalase (*CAT1*; accession no. M93719) was amplified by RT-PCR with the following set of primers: Tcat1-F, 5'- CAGGAGAACTGGAGGATACTTGAT-3'; and Tcat1-R, 5'-ATACGCGAATATCCTAGTCTGGAG-3'). Labeled probes were generated using a High Prime Labeling Kit according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN).

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## CHAPTER 3

# Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage

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# ABSTRACTS

Tomato (*Lycopersicon esculentum* Mill.) plants, which normally do not accumulate glycinebetaine (GB), are susceptible to chilling stress. Exposure to temperatures below 10 °C causes various injuries and greatly decreases fruit set in most cultivars. We have transformed tomato (cv. Moneymaker) with a chloroplast-targeted *codA* gene of *Arthrobacter globiformis,* which encodes choline oxidase to catalyze the conversion of choline to GB. These transgenic plants express *cod*A and synthesize choline oxidase, while accumulating GB in their leaves and reproductive organs up to 0.3 and 1.2 $\mu mol \cdot g^{-1}$ fresh weight (FW), respectively. Their chloroplasts contain up to 86 % of total leaf GB. Over various developmental phases, from seed germination to fruit production, these GB-accumulating plants are more tolerant of chilling stress than their wild-type counterparts. During reproduction, they yield, on average, 10-30 % more fruit following chilling stress. Endogenous GB contents as low as 0.1 $\mu mol \cdot g^{-1}$ FW are apparently sufficient to confer high levels of tolerance in tomato plants, as achieved via transformation with the *codA* gene. Exogenous application of either GB or $H_2O_2$ improves both chilling and oxidative tolerance concomitant with enhanced catalase activity. These moderately increased levels of $H_2O_2$ in *codA* transgenic plants, as a byproduct of choline oxidase catalyzed GB synthesis, may activate the $H_2O_2$-inducible protective mechanism, resulting in improved both chilling and oxidative tolerances in GB-accumulating *codA* transgenic plants. Thus, introducing the biosynthetic pathway of GB into tomato through metabolic engineering is an effective strategy for improving chilling tolerance.

## INTRODUCTION

Chilling injury, i.e., physical and physiological changes induced by exposure to low temperatures, is a primary factor in limiting crop production worldwide. This stress causes significant economic losses to producers in scattered geographic regions. Many species that originated in tropical and subtropical regions are susceptible when temperatures fall below about 15 °C (McKersie and Leshem, 1994; Paull, 1990). Such stress can delay growth and development, reduce productivity, and even cause mortality (McKersie and Leshem, 1994). The most vulnerable stage in susceptible plants is the reproductive phase, which includes the formation of reproductive organs, flowering, fruiting, and seed development (McKersie and Leshem, 1994). Although sensitive crops can be grown in greenhouses in northern climates, the energy required to maintain minimal temperatures increases production costs. A second critical impact of chilling is on post-harvest storage of perishable fruits and vegetables. Refrigeration is often used to preserve their quality, but some commodities cannot be stored at low temperatures because this may inhibit normal ripening or hasten spoilage (McKersie and Leshem, 1994; Paull, 1990).

Developing germplasm with increased chilling tolerance would provide a long-term solution to this problem. Chilling tolerance is controlled by many genes (McKersie and Leshem, 1994), and success in improving it by using traditional breeding approaches has been limited. Economic incentives for introducing such crop plants have encouraged researchers to explore genetic engineering methods for enhancing cold tolerance. Various genes have been utilized to produce transgenic plants (Chen and Murata, 2002; Yuwansiri *et al.*, 2002). Recently, an *Arabidopsis* CBF1 gene was used to generate tomato plants with increased chilling tolerance (Hsieh *et al.*, 2002). However, most previous engineering studies have focused only on seed germination and seedling growth; to our knowledge, the production of fruit and seeds in these species has not been examined. Because many of the world's major food crops are grown specifically for their fruits or seeds, engineered chilling-stress

tolerance in reproductive organs will significantly impact production under cold regimes.

Plants have evolved various mechanisms for adapting to harsh environments. For example, they may accumulate glycinebetaine (GB), a so-called "compatible solute," in response to salt and cold stresses (Allard *et al.*, 1998; Kishitani *et al.*, 1994; Nomura *et al.*, 1995; Wyn Jones and Storey, 1981). While several taxonomically distant species, e.g., spinach and wheat, are natural accumulators of GB, others, such as *Arabidopsis*, potato, rice, and tomato, are considered non-accumulators (Wyn Jones and Storey, 1981). Research has indicated that GB might play an important role in enhancing tolerance. First, its accumulation is induced under stress conditions, with levels being correlated with the extent of increased tolerance (Rhodes and Hanson, 1993). Second, exogenous application of GB improves the growth and survival of numerous species under stress (Allard *et al.*, 1998; Alia *et al.*, 1998; Chen *et al.*, 2000; Jokinen *et al.*, 1999; Mäkelä *et al.*, 1998, 1999; Sakamoto and Murata, 2000; Zhao *et al.*, 1992). Third, GB effectively stabilizes the quaternary structures of enzymes and complex proteins, and maintains a highly ordered state of membranes when *in-vitro* temperatures or salt concentrations are extreme (Papageorgiou and Murata, 1995). Furthermore, introducing the GB biosynthetic pathway into non-accumulators increases tolerance to various abiotic stresses (Chen and Murata, 2002).

The tomato (*Lycopersicon esculentum* Mill.) is a very popular vegetable worldwide. Because this species originated in the Tropics, most cultivated plants suffer chilling injury when exposed to temperatures below 10 °C (Graham and Patterson, 1982; Patterson *et al.*, 1987). Extended exposure below 6 °C can kill plants; below 13 °C, fruit-set may be inhibited (Atherton and Rudich, 1986). Cold stress delays the onset and reduces the rate of seed germination, resulting in poor performance (Atherton and Rudich, 1986; Foolad and Lin, 2000). At later stages, growth and development decline. Chilling may also interfere with flowering reducing fruit yield substantially (Foolad and Lin, 2000). These latter negative effects are due mainly to reduced pollen viability and the failure of anthers to release pollen grains

when nighttime temperatures are low (Picken, 1984). Moreover, when fruits are stored at low temperatures post-harvest, ripening may be slow and abnormal, with chilling-injured tomatoes becoming blotchy and exhibiting increased disease susceptibility (Paull, 1990).

Although tomato plants do not normally accumulate glycinebetaine (Wyn Jones and Storey, 1981), exogenous GB is taken up readily when applied to their leaves. Rapid translocation to other parts of the plant (Mäkelä *et al.*, 1996) results in increased tolerance to both salt and drought (Mäkelä *et al.*, 1999) and higher yields in the greenhouse and field (Jokinen *et al.*, 1999; Mäkelä *et al.*, 1998). Therefore, introducing a biosynthetic GB pathway into plants might enhance their tolerance to chilling without requiring exogenous GB applications.

Here, we report research on engineering GB biosynthesis in tomato plants. In this study, tolerance to chilling was assessed at various developmental stages to determine whether the accumulation of GB *in vivo* protects reproductive organs against cold stress during two critical phases: flowering and fruiting.

## RESULTS

### Expression of *codA* gene in transgenic tomato

We used *Agrobacterium*-mediated transformation (Fray and Earle, 1996) to transform tomato (cv. 'Moneymaker') with a *codA*-expression cassette (Figure 3.1a). PCR analysis demonstrated that all 45 independently generated primary transgenic plants possessed a stably integrated gene for hygromycin resistance (*hpt*). Staining for ß-glucuronidase (GUS) activity showed that 28 of the 45 plants were GUS-positive. Western blotting analysis revealed that all 28 accumulated a 64-kDa protein corresponding to choline oxidase, while no such protein was detected in the WT (Figure 3.1b). Those 28 transgenic plants were then self-pollinated to produce 19 homozygous lines.

The GB levels in the leaves of nine transgenic lines ranged from 0.1 to 0.3 $\mu mol \cdot g^{-1}$ FW (Figure 3.1c). No GB was detected in WT plants. GB contents were also measured in various organs of the L1 and L3 transgenic plants (Figure 3.1d). The highest levels were observed in shoot apices and reproductive organs. For example, in L1 plants, GB levels were 2.8 to 3.8 times greater in petals, anthers, and pistils than in leaves. GB levels in seeds and stems were 70 % and 52 % of those in leaves, respectively. A similar pattern of GB accumulation was observed in various organs of L3 plants (Figure 3.1d).

### Localization of GB in chloroplasts of transgenic plants

Because choline oxidase is specifically targeted to the chloroplasts, we analyzed GB content in the isolated fraction of chloroplasts from leaves of five transgenic lines. The percentage of GB found in the chloroplast was calculated by comparing leaf and chloroplast contents, expressed on a chlorophyll basis and corrected for the percentage

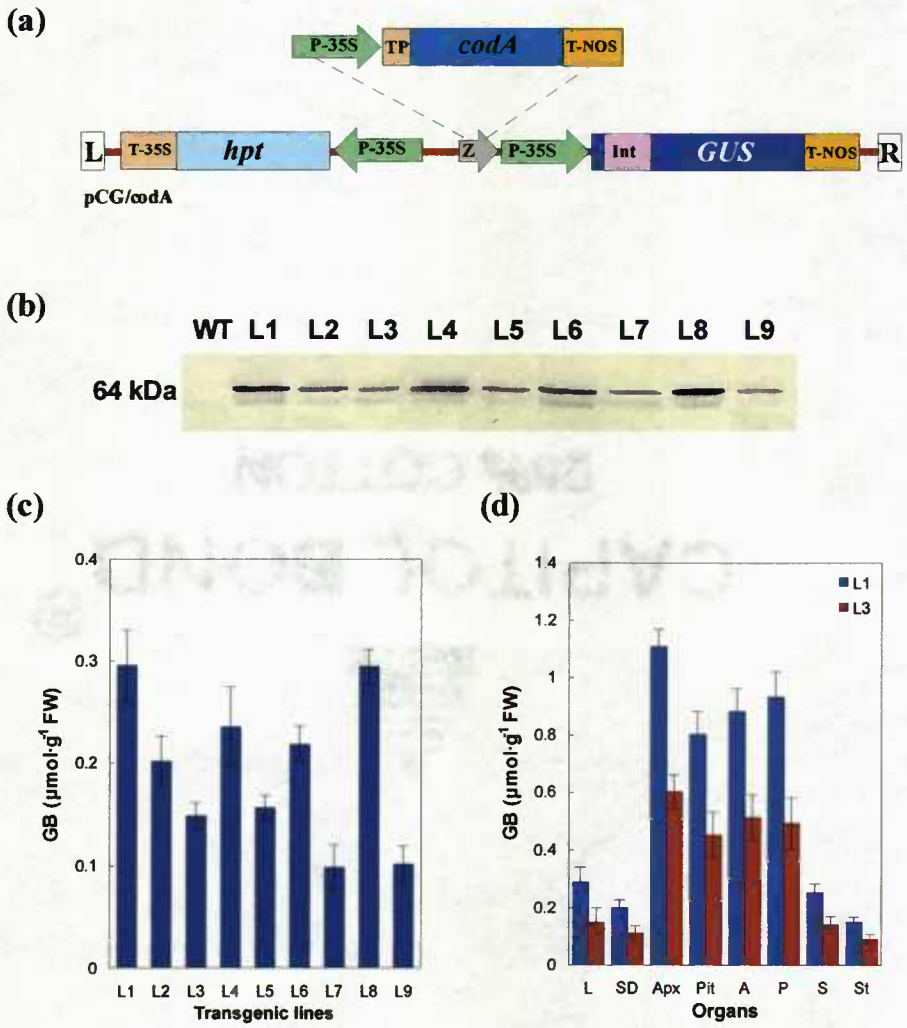


**Figure 3.1.** Characterization of the *codA* transgenic plants **(a)** T-DNA region of binary vector pCG/codA: P35S, CaMV 35S promoter; *codA*, gene for choline oxidase from *Arthrobacter globiformis*; TP, transit peptide sequences of small subunit of Rubisco; T-Nos, terminator sequence of gene for nopaline synthase; GUS, β-glucuronidase gene; Int, intron of gene for catalase; *hpt*, gene for hygromycin phosphotransferase; T35S, CaMV 35S terminator sequence; LB, left T-DNA border; RB, right T-DNA border. **(b)** Western blotting analysis: WT, wild-type plant; L1 through L9, independent transgenic lines (T0 generation). **(c)** Glycinebetaine (GB) levels in leaves of 5-week-old seedlings from WT and nine independent transgenic (T3) lines. Means of three experiments; bars indicate standard errors. **(d)** GB levels in various organs of 8-week-old L1 and L3 plants. Means of three experiments; bars indicate standard errors. L, leaves; SD, seeds; Apx, shoot apices; Pit, pistil; A, anther; P, petal; S, sepal; St, stem.

**Table 3.1.** Glycinebetaine (GB) levels in chloroplasts isolated from leaves of 5 transgenic lines [1].

| Transgenic line | GB in leaves ($nmol \cdot mg^{-1}$ chlorophyll) | GB in isolated chloroplasts ($nmol \cdot mg^{-1}$ chlorophyll) | % Intact chloroplasts | % GB in chloroplasts |
|---|---|---|---|---|
| L1 | 23.5±2.8 | 9.32±1.8 | 57.9±5.1 | 68.6±6.3 |
| L3 | 15.3±1.9 | 6.89±1.2 | 58.7±5.8 | 76.7±7.7 |
| L5 | 14.7±2.1 | 7.09±1.1 | 55.9±4.8 | 86.3±6.9 |
| L7 | 11.3±1.8 | 4.28±0.6 | 63.2±5.7 | 60.0±5.6 |
| L9 | 10.8±1.5 | 3.58±0.5 | 55.2±6.7 | 60.0±7.5 |

[1]Mean values ± S.D. from three experiments. GB content in chloroplasts was corrected for the percentage of broken chloroplasts present. The percentage of GB found in the chloroplast was calculated by comparing leaf and chloroplast contents, expressed on a chlorophyll basis.

of broken chloroplasts present. Consequently, we estimated that 60-86 % of total leaf GB was localized in the chloroplasts (Table 3.1). These levels were later used to establish correlations between GB content and other parameters associated with chilling injury, including Fv/Fm, $H_2O_2$ level, catalase activity, and % ion leakage.

**Tomato plants accumulating low GB levels show high degree of chilling tolerance**

*In vitro*-grown WT seedlings accumulated different amounts of glycinebetaine when grown on media containing various GB concentrations (0.01 to 10000 μM). Their endogenous levels ranged from 0.07 to 8.94 $\mu mol \cdot g^{-1}$ FW (Figure 3.2a). Following chilling stress, growth of seedlings fed with 1 to 1000 μM GB was much better than that of seedling grown on medium containing no GB. Seedlings that accumulated 0.14 to 0.42 $\mu mol \cdot g^{-1}$ FW, which corresponds to 1 to 100 μM of GB in the medium, showed no significant difference in growth. Higher GB levels (>1000 μM ) resulted in smaller plants, indicative of phytotoxicity of supernormal GB. This demonstrates that even a low level of endogenous GB (0.14 $\mu mol \cdot g^{-1}$ FW) can confer a high degree of protection against chilling.

We observed similar results in greenhouse-grown WT plants sprayed with various levels of GB (0.1 to 10.0 mM). Endogenous accumulation of GB ranged from 0.09 to 1.66 $\mu mol \cdot g^{-1}$ FW (Figure 3.2b), and only ~20 % GB was found in the chloroplasts of treated plants regardless of endogenous GB levels (data not shown). These plants exhibited greater tolerance to chilling than the untreated WT control (Figure 3.2b). Nevertheless, their growth during post-chilled period in a greenhouse (25 °C) was not significantly different even though the endogenous levels of GB in 10.0 mM GB-treated plants were over 100-fold higher than in 0.1 mM GB-treated plants (Figure 3.2b). This demonstrates that 0.09 $\mu mol \cdot g^{-1}$ FW GB induces almost full protection against chilling (Figure 3.2b), as defined by the expression of *codA* in transgenic plants.

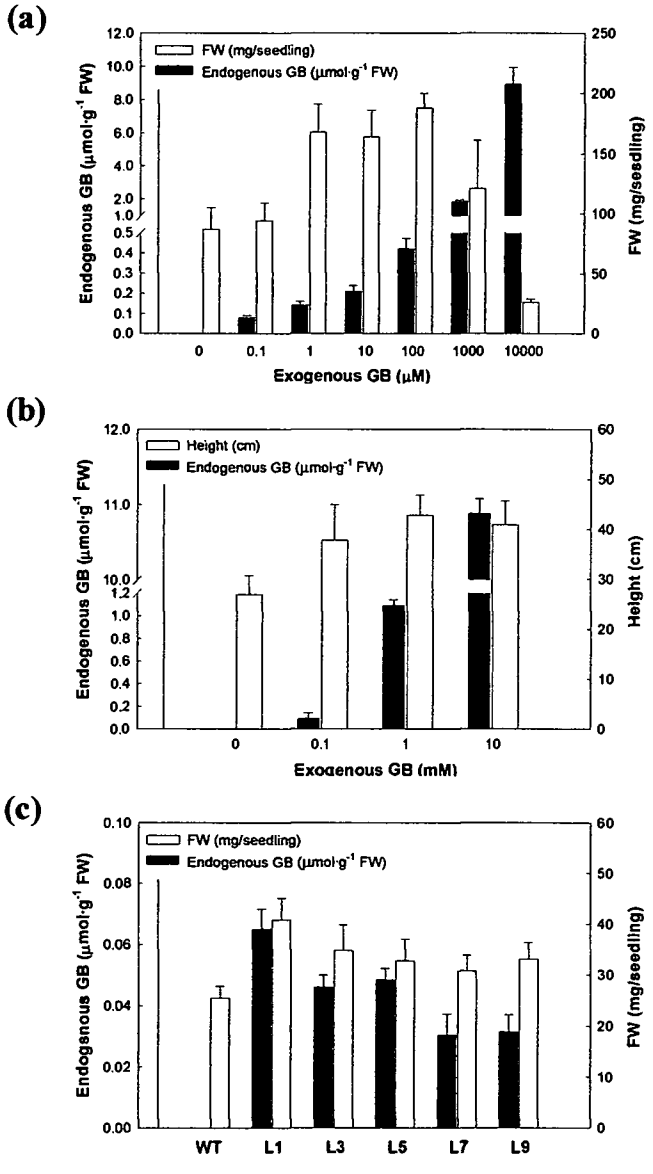


**Figure 3.2.** Protection against chilling in tomatoes accumulating various levels of glycinebetaine (GB). **(a)** Seeds from WT plants germinated in 0.1 to 10,000 μM GB for 5 d, then were transferred to MSP medium containing same amount of GB. After reaching approximately 2 cm tall, seedlings were chilled at 3 °C for 7 d under 16-h photoperiod, and then transferred to warm growth chamber (25 °C). Endogenous GB (μmol·g$^{-1}$ FW) and fresh weight (FW, mg per seedling) were measured 7 d later. **(b)** Five-week-old greenhouse-grown WT plants were sprayed with either a water control or various concentrations of GB in solution (0.1, 1.0, or 10.0 mM). One day after GB

foliar application, plants were moved to 2 °C for 5 d. Plants were then returned to greenhouse and heights (cm) were measured 4 weeks later. **(c)** Uniform 10-d-old seedlings of WT and five independent homozygous transgenic lines accumulating different levels of GB were excised just above root system (approximately 2.0 cm long) and transplanted into MSP medium. After 3 d, they were chilled at 3 °C for 7 d, and then transferred to 25 °C. Fresh weight (mg per seedling) was measured after 7 d of recovery at 25 °C. All values are means ± SE of results from three experiments.

**Transformation with *codA* gene protects seeds against chilling**

Seeds were incubated in a growth chamber for 14 d at 17/7 °C (day/night) and under a 16-h photoperiod before being moved to room temperature (25 °C). By the end of the chilling treatment, ~1.4 % of the WT seeds had germinated, whereas that rate was up to 30.5 % for seeds from the five transgenic lines (Figure 3.3a). This improvement was significantly correlated with the amount of GB accumulated in the transgenic seeds (Figure 3.3c; $P < 0.01$). Later exposure to 25 °C resulted in 60 to 93 % of the transgenic seeds germinating within 1 d compared with only 16 % of the WT seeds. Furthermore, the sprouted and rooted seedlings of five transgenic lines were larger and grew more vigorously than those of WT (Figure 3.3b). This demonstrates that transformation with the *codA* gene significantly increases chilling tolerance at the germination stage and promotes more vigorous seedling growth.

**GB accumulation enhances chilling tolerance in young seedlings**

*In-vitro* seedlings were chilled in a cold room (3 °C) for 7 d. No growth was observed in either WT or transgenic seedlings during the cold treatment (data not shown). Upon transfer to a warm growth chamber (25 °C), transgenic seedlings were healthy and grew much better than WT. On the contrary, WT seedlings showed extensive chilling injury including wilting, bleaching of the chlorophyll, and necrosis (data not shown). After 7 d at 25 °C, hypocotyl growth in the chilling-treated WT seedlings was only 38 % of that measured with the WT (non-chilled) control (Figure 3.4a). In contrast, tolerance was enhanced for the chilling-stressed seedlings of all transgenic lines, as shown by their relative hypocotyl growth (59.1 ~ 62.3 % of non-chilled control; Figure 3.4a). In addition, their root systems were better developed than those of the WT (Figure 3.4b).

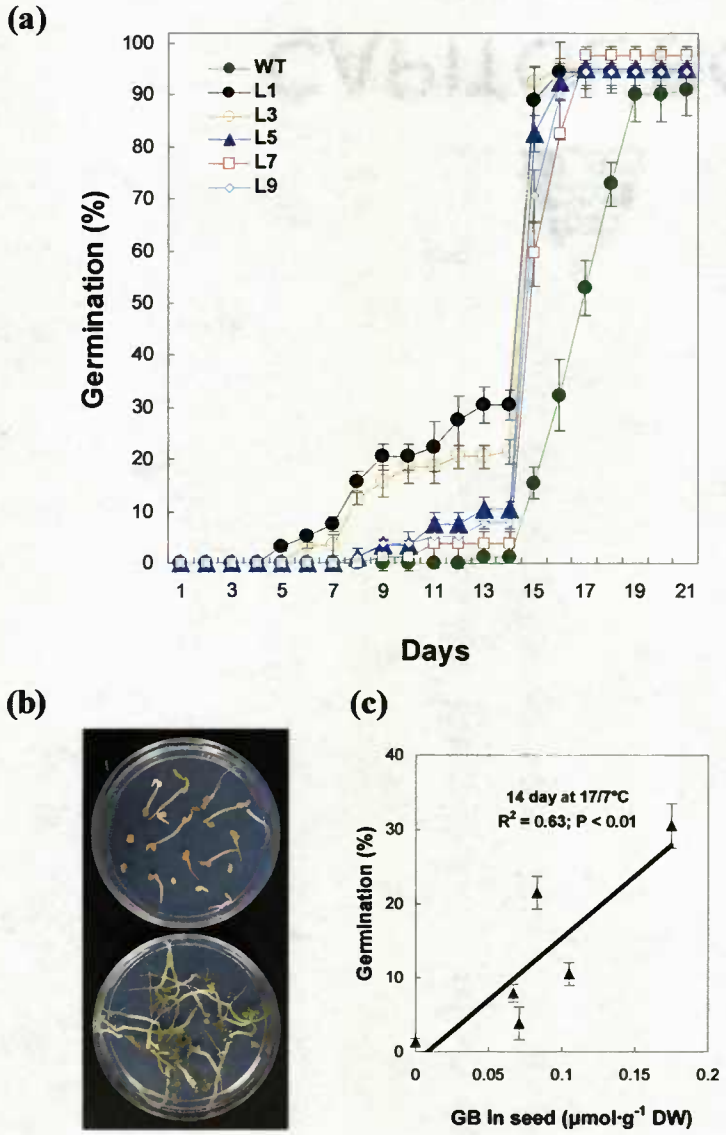


**Figure 3.3.** Effects of low temperature on seed germination. Seeds of WT and 5 independent homozygous transgenic lines (L1, L3, L5, L7, and L9) were incubated for 14 d at 17/7 °C (light/dark) and 16-h photoperiod. **(a)** Germination was recorded daily for 21 d; values represent mean ± SE of three experiments. **(b)** Correlation between % germination and glycinebetaine contents in seeds of transgenic lines. **(c)** Photo taken 4 d after seedlings returned to 25 °C after treatment for 14 d at 17/7 °C.

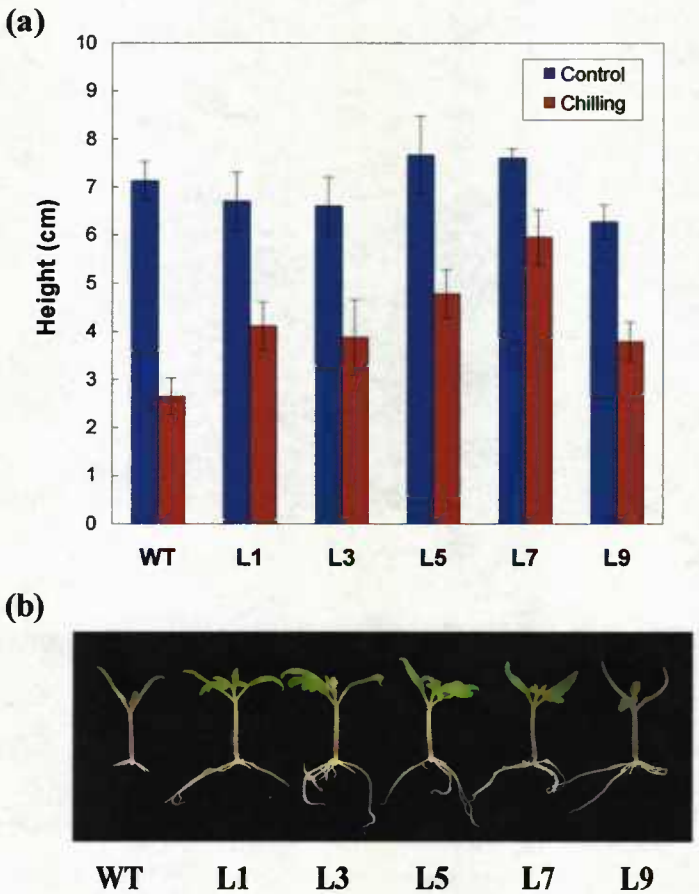


**Figure 3.4.** Effects of low temperature (3 °C) on *in-vitro* growth. **(a)** Uniform 10-d-old seedlings of WT and five independent homozygous transgenic lines that accumulated different levels of glycinebetaine were excised just above root system (approximately 2.0 cm long) and transplanted into MSP medium. After 3 d of growth, seedlings were chilled at 3 °C for 7 d, then transferred to 25 °C. Response to cold was examined by measuring hypocotyl length (cm) 7 d after this transfer. **(b)** Photo taken 5 d after seedlings returned to 25 °C following 7 d of chilling at 3 °C under 16-h photoperiod.

**GB accumulation enhances chilling tolerance in greenhouse-grown non-flowering transgenic tomato**

Five-week-old greenhouse-grown seedlings were chilled in a cold room (3 °C) for 5 d. To examine the responses of both WT and transgenic plants to chilling stress, we measured four parameters: chlorophyll fluorescence (Fv/Fm), $H_2O_2$ level, catalase activity, and % ion leakage. Correlations were graphed by comparing these values (obtained at Day 3 in the greenhouse) with the level of GB accumulated in the chloroplasts of the transgenic lines (Figure 3.5).

Under normal greenhouse conditions, growth did not differ between the WT and transgenic lines. However, during the chilling treatment, Photosystem II activity declined in both WT and transgenic plants (Figure 3.5a), though at a much slower rate of decline for the latter. After 3 d at 25 °C, PS II activity in the WT plants declined to 40 % of the original value (Day 0); transgenic plants maintained 45 to 63 % of their original activity. Moreover, Fv/Fm was significantly correlated with the amount of GB accumulated in the chloroplasts of each transgenic line (Figure 3.5b; $R^2$=0.65, $P < 0.01$).

Under non-stress conditions, $H_2O_2$ levels in the transgenic lines were 9 to 16 % higher than those in the WT plants. The levels of $H_2O_2$ increased in both WT and transgenic plants during the cold-treatment. The rate of increase was much higher in WT during the first day of cold-treatment and then continued at the similar rate in both WT and transgenic plants until Day 3 (Figure 3.5c). After 3 d of incubation at 25 °C, $H_2O_2$ contents in the WT plants were 2.3-fold higher than their original value (Day 0), whereas the transgenics accumulated 1.7 to 2.1-fold more $H_2O_2$. GB content showed a moderately negative correlation with $H_2O_2$ level in the chloroplasts (Figure 3.5d; $R^2$=0.54, $P < 0.05$).

Under non-stress conditions, catalase activity in the transgenic lines was 10 to 20 % higher than those in WT plants. Catalase activity increased up to 40 % (L1) versus 20 % (WT) during Day 1 of chilling (Figure 3.5e). Thereafter, the activity of catalase

decreased at rates similar for all genotypes. At the end of the treatment (GH3), the catalase activity in WT plants was reduced to 44 %, whereas the transgenics maintained up to 67 % of their initial activity (Day 0). Nevertheless, catalase activity for the five transgenic lines was not significantly correlated with their chloroplastic GB levels (Figure 3.5f; $R^2$=0.30, P > 0.05).

Chilling stress caused severe ion leakage in WT plants, whereas the transgenics were much less affected (Figure 3.5g). The correlation between chloroplastic GB content and % ion leakage, however, was low (Figure 3.5h; $R^2$=0.39, P > 0.1).

**Fruit-set is improved in transgenic tomato plants after chilling**

When two to three flowers of the first inflorescence had reached anthesis, WT and transgenic (L1and L3) plants were chilled at 3 °C for 7 d, and then returned to the greenhouse. Thereafter, 20 % of the WT plants died within 3 or 4 d. In contrast, survival rates were 100 % for L1 and 96 % for L3. During the first week back in the greenhouse, the WT leaves became severely necrotic and only about 82 % of WT flowers were retained (Figure 3.6a, c; Table 3.2). Their fruit-set was greatly delayed compared with L1 plants (Figure 3.6f). Some WT clusters retained all of their flowers but none had fruit three weeks after chilling (Figure 3.6e). In contrast, leaves of L1 plants showed only mild evidence of injury (Figure 3.6b). These plants also retained all of their flowers (Figure 3.6d). Although chilling reduced the number of fruits on both WT and L1 plants, fruit set was higher ($P \leq 0.052$) for the latter.

**Tolerance to methyl viologen (MV)-induced oxidative stress in GB-accumulating transgenic plants as well as in GB- and $H_2O_2$-treated WT plants**

MV-mediated damage to the photosynthetic apparatus was defined by the reduction in PSII activity after leaf discs were incubated in water or 20 μM MV for 1 d at 25 °C.

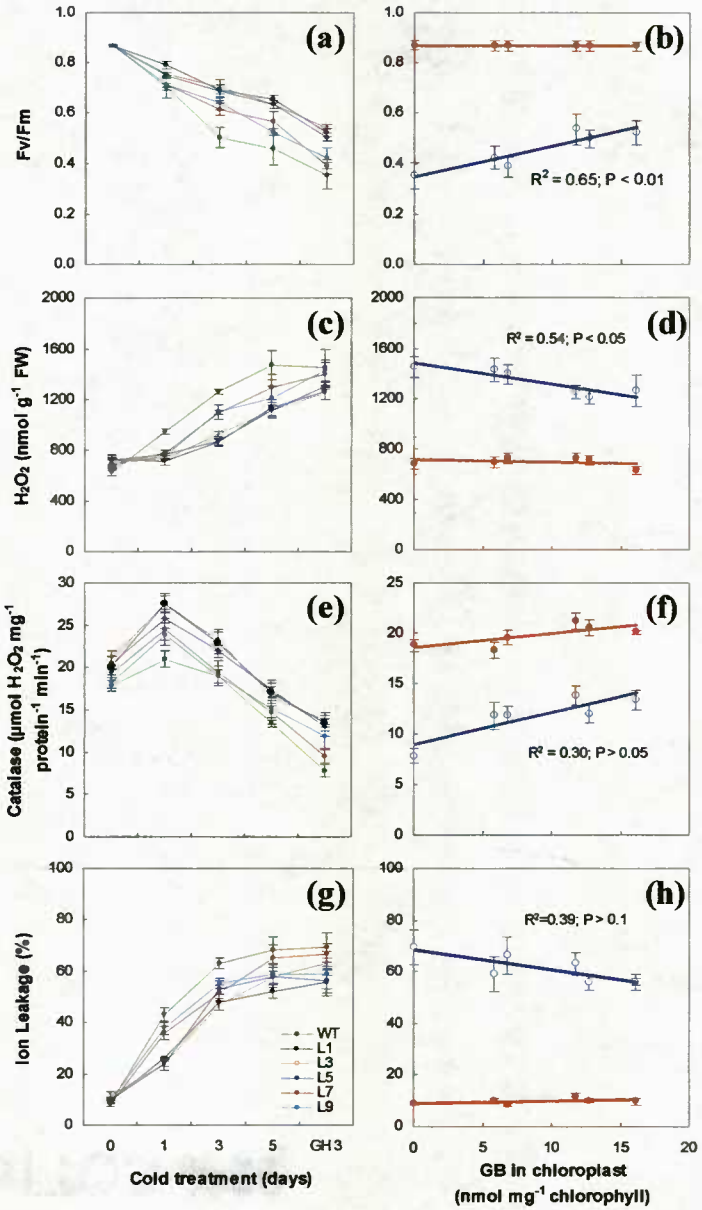


**Figure 3.5.** Effects of chilling on various growth parameters. Five-week-old greenhouse-grown WT and independent homozygous transgenic lines (L1, L3, L5, L7, and L9) were chilled (3 °C) for 5 d, then returned to greenhouse. **(a, b)** Chlorophyll fluorescence (Fv/Fm), **(c, d)** $H_2O_2$ levels, **(e, f)** catalase activity, and **(g, h)** % ion leakage at 0, 1, 3, and 5 d after chilling, and at R3 (i.e., after 3 d of recovery in warm greenhouse). Different GB levels in chloroplasts of 5 transgenic lines were used to establish correlations with values obtained at R3. Results are mean ± SE from three independent experiments: (○), non-stressed controls in warm greenhouse (25 °C); (●), chilling-stressed plants in cold room (3 °C).

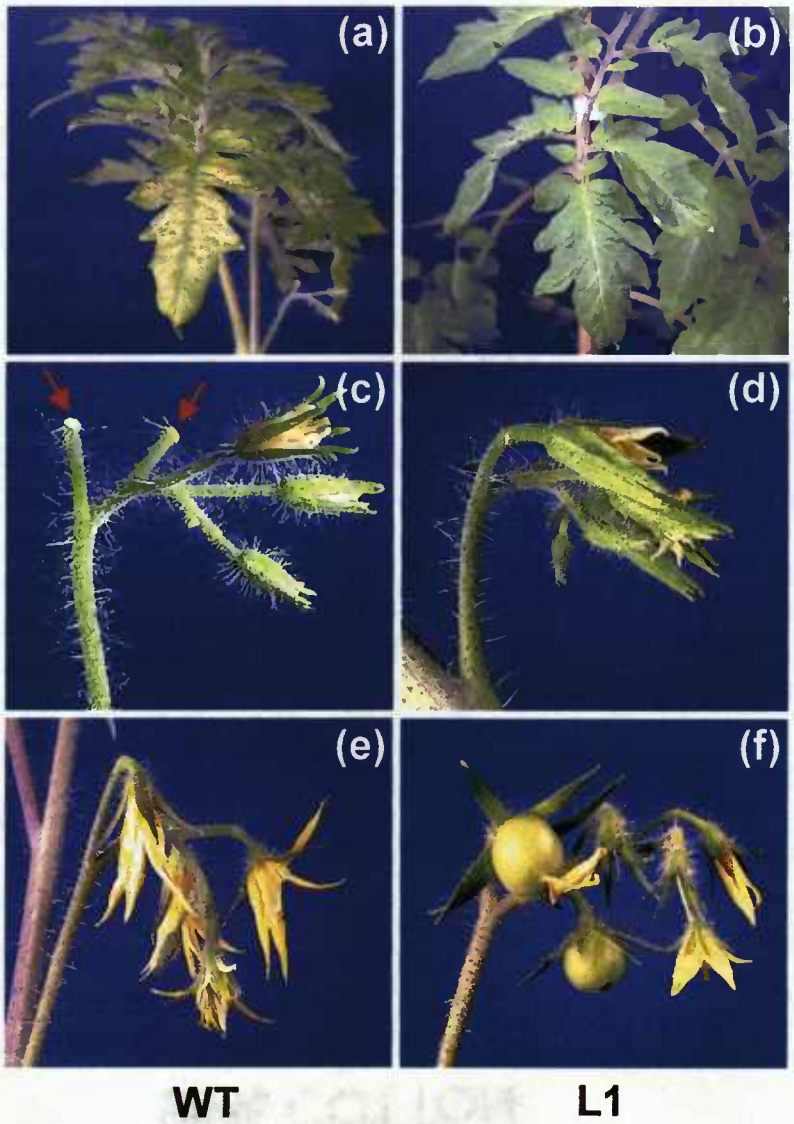


**Figure 3.6.** Levels of chilling tolerance at various reproductive phases. Nine-week-old WT **(a**, **c**, and **e)** and L1 transgenic plants **(b**, **d**, and **f)**, at stage when 2 or 3 flowers of first inflorescence had reached anthesis, were incubated at 3 °C (16-h photoperiod) for 7 d, then transferred to greenhouse. Upon exposure to warm temperature, WT plants immediately developed bleached areas on leaves **(a)** compared with those of L1 transgenic plants (**b**). During subsequent 7-d recovery period at 25 °C, WT plants dropped their flowers (**c**) while transgenics did not **(d)**. Three weeks after cold treatment, flowers remaining on WT plants failed to set fruit (**e**), whereas those on L1 transgenic plants did **(e)**. Flower retention is quantified in **(c** and **d)**; fruit set, in **(e** and **f)**.

discs also maintained PS II activity of up to 76 % of that measured from the water control (Figure 3.7b). In contrast, Fv/Fm in non GB-treated leaf discs was reduced by about 62 %; similar results in the response to MV-induced oxidative stress were obtained with $H_2O_2$-treated plants. Moreover, WT leaf discs treated with $H_2O_2$ had about 2-fold higher Fv/Fm values compared with those calculated for the water-treated WT (Figure 3.7c).

**Exogenous application of either $H_2O_2$ or GB improves chilling tolerance in WT plants**

Five-week-old greenhouse-grown plants were sprayed with various levels of either $H_2O_2$ (10.0 to 1000.0 mM) or GB (0.1 to 10.0 mM). Within 2 h of $H_2O_2$ application in the dark, endogenous $H_2O_2$ levels in those treated plants had become similar to those measured in the water-treated controls (data not shown). Following chilling stress at 3 °C in the dark for 3 d, $H_2O_2$ contents in the water-treated plants had increased significantly, up to 2.4-fold higher than those treated with 10-1000 mM $H_2O_2$ (Figure 3.8a). Under non-chilled conditions, catalase activity in the $H_2O_2$–treated plants had increased up to 14 % of that in the water-treated plants (Figure 3.8c). While catalase activity was reduced in both water- and $H_2O_2$-treated plants following chilling treatment, in the latter it was maintained at a level 67 % higher than in the former. Similar trends were observed in our GB-treated plants. One day after GB application, levels of both $H_2O_2$ and catalase activity did not differ significantly from the control (water-treated) plants (Figure 3.8b, d). However, as the chilling stress progressed, GB-treated plants maintained lower levels of $H_2O_2$ (Figure 3.8b). After 1 d of a given chilling stress, catalase activity in the GB-treated plants increased up to as much as 65 % of their pre-stress values, compared to only 16 % increase in control plants (Figure 3.8d). Although the catalase activity was considerably reduced in both GB-treated and control plants after 3 d of chilling, those treated with GB still maintained higher levels of catalase activity than the control (Figure 3.8d). In addition, plants

treated with either $H_20_2$ or GB maintained much higher values of Fv/Fm than those calculated for the water-treated plants (Figure 3.8e, f).

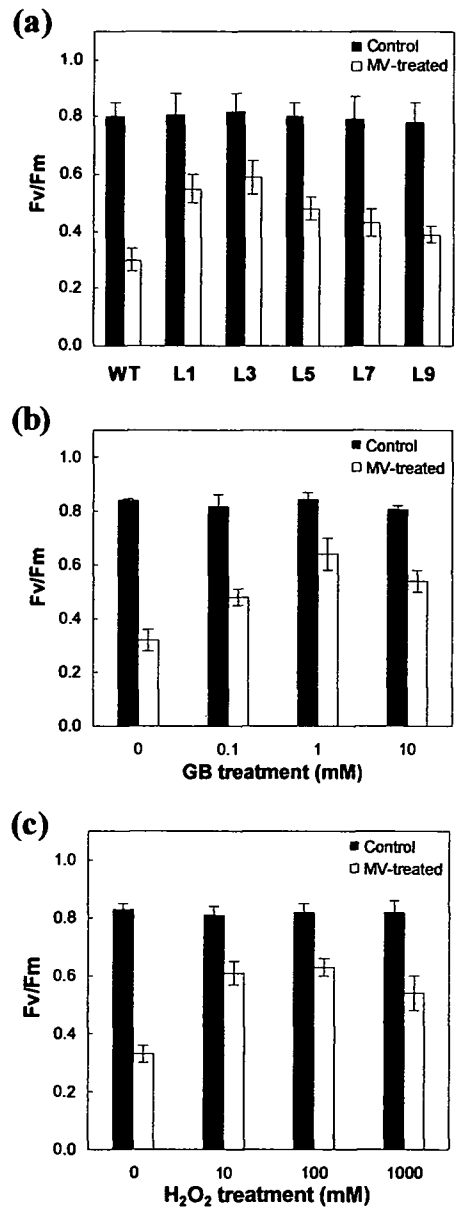


**Figure 3.7.** Improved tolerance to methyl viologen-induced oxidative stress in transgenic plants **(a)**, and WT plants treated with various concentrations of glycinebetaine (0.1, 1.0, and 10.0 mM) **(b)**, or $H_2O_2$ (10, 100, and 1000 mM) **(c)**. Leaf discs (1.0 $cm^2$) collected from those plants were incubated in water (control) or 20 μM methyl viologen following vacuum infiltration for 30 min. Reduction in Fv/Fm was measured after 1 d of treatment. Results are mean ± SE from three independent experiments (n = 27).

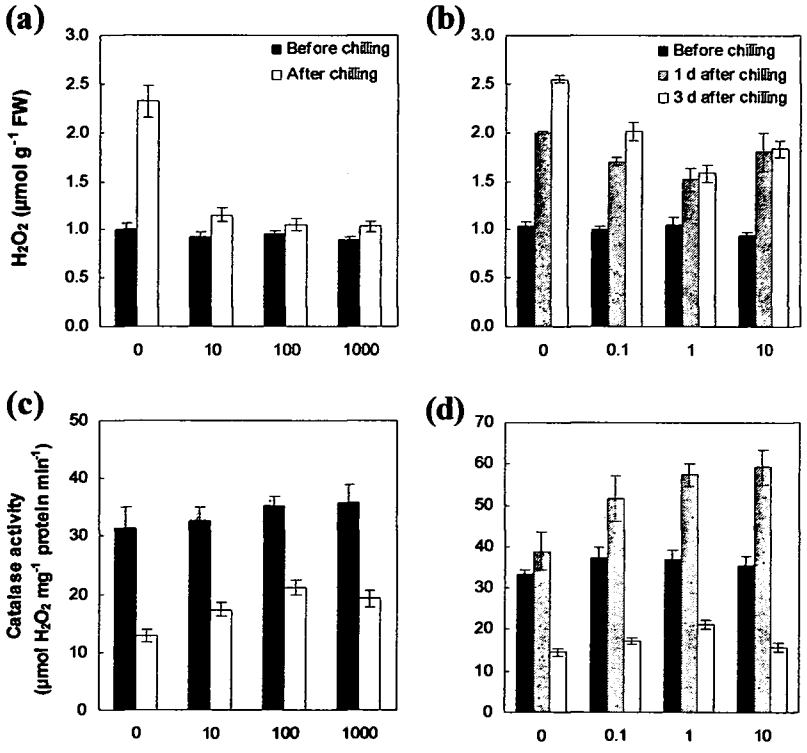


**Figure 3.8.** Effects of exogenous application of glycinebetaine (GB) and $H_2O_2$ on endogenous $H_2O_2$ level, catalase activity and Fv/Fm in response to chilling stress. Five-week-old greenhouse-grown WT tomato plants were sprayed with either water (control), or solutions containing various concentrations of GB or $H_2O_2$. Endogenous levels of $H_2O_2$ (**a** and **b**), catalase activity (**b** and **d**) and Fv/Fm (**e** and **f**) in $H_2O_2$-treated and GB-treated plants, respectively, were measured before chilling as well as 1d after chilling for $H_2O_2$-treated plants and 1d and 3d after chilling for GB-treated plants.

## DISCUSSION

We have successfully expressed a bacterial gene for choline oxidase in transgenic tomatoes. These plants accumulate GB and exhibit enhanced chilling tolerance at several developmental stages. However, their amounts of accumulation in leaves (0.09 to 0.30 $\mu mol \cdot g^{-1}$ FW) are much lower than those reported in other species that express *codA* constructs (Chen and Murata, 2002). Our levels are also much lower than those measured in natural GB-accumulators, such as spinach (30 to 40 $\mu mol \cdot g^{-1}$ FW) (Rhodes and Hanson, 1993). Accumulated GB contents in transgenic tomato plants are likely not high enough to be osmotically significant.

In this study, GB accumulated to different levels in various organs, with amounts measuring up to 3.8 times greater in shoot apices and reproductive organs (Figure 3.1d). Similar results have been observed in *codA* transgenic *Arabidopsis* plants (Sulpice et al., 2003). Because the *codA* gene is driven by a constitutive CaMV 35S promoter, it should be expressed uniformly throughout the plant. However, partial GB translocation has been reported with assimilates, especially to actively growing and expanding plant portions, thus indicating that long-distance transport of GB is phloem-mobile (Mäkelä *et al.*, 1996). In addition, GB import across the plasma membrane can occur via a transporter of GB/proline/γ-amino butyric acid (*LePro T1*), as occurs in tomato pollen (Schwacke *et al.*, 1999), and/or by means of a proline/GB transporter (*AmT1*), such as that found in GB-accumulating mangrove (Waditee *et al.*, 2002).

Considering the low amount of GB that accumulated in our *codA* transgenic plants, we must ask whether such levels are sufficient to confer chilling tolerance. Here, we have addressed this question in two ways. First, we showed that exogenous application of various GB concentrations caused WT plants to accumulate different levels of glycinebetaine. Second, we tested tolerance in transgenic plants that accumulated various GB contents. After these plants were subjected to chilling, their degree of sensitivity was evaluated in terms of their growth. In the first experiment, *in-vitro* WT seedlings grown in media containing various concentrations of GB accumulated to

different levels (Figure 3.2a). Following chilling, their growth was much better than for the non-accumulating control. This suggests that a threshold level of endogenous GB (>0.1 $\mu mol \cdot g^{-1}$ FW) can provide full protection against low temperatures. Similar results were found in greenhouse-grown WT plants sprayed with various GB concentrations. All these results indicate that levels as low as 0.09 $\mu mol \cdot g^{-1}$ FW can confer a high level of chilling tolerance in tomato plants, as achieved through their expression of *codA*.

In transgenic *Arabidopsis* plants that express this gene, choline oxidase is appropriately transported to the targeted chloroplasts (Hayashi *et al.*, 1997), and almost all the accumulated GB is found in that compartment (Sakamoto *et al.*, 2000). Likewise, in our chloroplast-targeted transgenic tomato leaves, 60 to 86 % of the total GB was localized there (Table 3.1). Among the parameters tested, GB levels in the chloroplasts were most highly correlated with PSII activity during chilling (Figure 3.5).

In transgenic rice plants transformed with the *codA* gene for chloroplast-targeted choline oxidase, the photosynthetic machinery is more efficiently protected against salt and cold stresses than in those that express a *codA* gene whose product is not targeted to chloroplasts, even though the latter accumulates five times more GB (Sakamoto *et al.*, 1998). Therefore, localization of GB in the chloroplasts might be responsible for the effective protection seen in our transgenic tomatoes. However, targeting the enzyme that catalyzes GB synthesis to other compartments should be considered because utilizing mitochondria and the cytosol can also enhance tolerance of the photosynthetic machinery to salt and chilling (Holmstrom *et al.*, 2000; Takabe *et al.*, 1998).

Despite the relatively low GB concentration in our transgenic tomato, plants that expressed the *codA* gene exhibited significantly increased chilling tolerance at various developmental stages, from seed germination to fruit formation. This is of agricultural importance because, under field conditions, the incidence of stress is unpredictable and plants can be exposed to low temperatures any time during their life cycle. Such

enhanced tolerance should greatly reduce the negative impact of chilling and stabilize tomato production in vulnerable regions.

We reported previously that exogenous application of GB could shield tomato plants against chilling injury (Park *et al.*, 2003). This is in addition to its known protective role against salt and drought stresses (Jokinen *et al.*, 1999; Mäkelä *et al.*, 1998, 1999). GB accumulation results from *in-vivo* expression of the *codA* gene as well as through exogenous applications. The mechanism for inducing such tolerance might be explained several ways. First, GB stabilizes the oxygen-evolving PSII complex by stimulating its repair when plants are exposed to various stresses (Alia *et al.*, 1999; Hayashi *et al.*, 1997; Holmstrom *et al.*, 2000; Papageorgiou *et al.*, 1991; Sakamoto and Murata, 2000). Our transgenic plants also exhibited efficient protection of PS II in a GB dose-dependent manner. In contrast, our WT plants grown under light presumably suffered from photo-damaging effects, perhaps as a result of competition between photochemical inactivation of the PS II complex and the counteracting recovery of that complex from such damage (Figure 3.5a).

As a second possibility, the Rubisco enzyme in the presence of GB is protected from inactivation during salt stress by the stabilization of its conformation (Nomura *et al.*, 1998). For example, Bourot *et al.* (2000) have shown that GB acts as a molecular chaperone in *E. coli*, assisting in enzymatic refolding.

Third, GB can stabilize membrane integrity against extreme temperatures (Gorham, 1995), reduce membrane lipid peroxidation (Chen *et al.*, 2000), and protect complex II electron transport in the mitochondria (Hamilton and Heckathorn, 2001). GB might also destabilize double-helixed DNA and lower the melting role in promoting transcription and replication under high-salt conditions (Rajendrakumar *et al.*, 1997).

Finally, chilling may induce cellular membrane dysfunction or protein denaturation, causing a disturbance in the electron transport system embedded on mitochondrial or chloroplastic membranes. This, in turn, may interrupt electron transport, leading to the production of reactive oxygen species (ROS). Increased

intracellular concentrations of ROS can directly damage cellular components or hinder the repair of PS II by inhibiting *de novo* protein synthesis (Nishiyama *et al.*, 2001). Tomato plants exposed to chilling temperatures show higher concentrations of cellular hydrogen peroxide and decreased catalase activity (Hsieh *et al.*, 2002; Kerdnaimongkol and Woodson, 1997). Such sensitivity varies diurnally, being highest when chilling occurs at the end of a dark period (King *et al.*, 1982; Kerdnaimongkol and Woodson, 1997). Those seedlings then become more sensitive to chilling injury when exposed to the light because photoinhibition induces a reduction in catalase activity (Kerdnaimongkol and Woodson, 1997). However, exogenous application of $H_2O_2$ increases tolerance, indicating that oxidative stress might function as a secondary messenger to induce a rise in antioxidant defenses (Hsieh *et al.*, 2002; Kerdnaimongkol and Woodson, 1997).

Hydrogen peroxide appears to have dual effects on the chilling response. At low levels, it can stimulate protection against such oxidative stress by inducing expression of antioxidant enzymes, e.g., CAT3 (catalase3) (Prasad *et al.*, 1994). However, $H_2O_2$ can also accumulate to damaging levels in those tissues due to low antioxidant-enzyme activities (Prasad *et al.*, 1994). Under non-stress conditions, our transgenics contained up to 16 % more $H_2O_2$ than did the WT plants. As chilling continued, the transgenics maintained their original $H_2O_2$ level (Figure 3.5c) while the WT plants accumulated 1.5-fold of their original amount. After this initial period, the transgenic plants accumulated lower levels than the WT (Figure 3.5d).

Catalase activity in both WT and transgenics during the first day of chilling increased by 20 and 40 %, respectively (Figure 3.5e). Thereafter, the rate of decrease in activity was similar for both genotypes, such that the transgenics continuously maintained higher levels of activity. Such an increase in $H_2O_2$ and catalase activity in transgenic plants expressing the *codA* gene is not unique to tomato. For example, in *codA Arabidopsis* plants, transformation with this gene increases the amount of $H_2O_2$ by about 50 % under non-stress conditions (Alia *et al.*, 1999). When these transformed plants are exposed to 5 °C for 24 h, activities of both catalase and ascorbate

peroxidase rise significantly. Likewise, in transgenic tomatoes that over-express an *Arabidopsis* CBF1 gene, expression of the *CATALASE1* (*CAT1*) gene is highly induced (Hsieh *et al.*, 2002). This results in greater tolerance to both chilling and oxidative stress as well as lower hydrogen peroxide concentrations and higher catalase activity compared with WT plants (Hsieh *et al.*, 2002).

When we exogenously applied $H_2O_2$ or GB to the WT, those plants also exhibited improved chilling tolerance associated with enhanced catalase activity compared to water-treated controls (Figure 3.8). Although endogenous levels of hydrogen peroxide in the $H_2O_2$-treated plants became similar to those in the water-treated plants within 2 h after exogenous application (data not shown), as the chilling stress was prolonged, both $H_2O_2$- and GB-treated plants always maintained lower amounts of $H_20_2$ and higher catalase activity than those treated with water. Similar results have been observed in maize seedlings treated with hydrogen peroxide. There, applications of $H_2O_2$ increase the levels of both *cat3* transcripts and CAT3 activity, resulting in higher survival and growth rates upon expose to chilling stress (Prasad *et al.*, 1994). Therefore, it is likely that the induced chilling tolerance conferred by our exogenous applications of $H_2O_2$ and GB could have been the result of a $H_2O_2$-mediated antioxidant mechanism that included enhanced catalase activity.

Methyl viologen, which is known to generate ROS, has been intensively investigated for its oxidative action in plants. During light exposure, the major site of electron donation to MV has been shown to be PSI (Fujii *et al.*, 1990). The ROS produced by reduced MV might also propagate to the reaction centers of PSII, resulting in the loss of photosynthetic efficiency (Lannelli *et al.*, 1999). Moreover, MV tolerance in plants has been correlated with its scavenging capacities for ROS (Bowler *et al.*, 1991); the combined action of MV and light imposes enhanced oxidative stress mainly on the chloroplasts (Fujii *et al.*, 1990). In WT plants treated with either GB or $H_2O_2$, or GB-accumulating transgenic plants, we observed greater tolerances to chilling and MV-induced oxidative stresses (Figure 3.7). All those plants exhibited higher catalase activity under chilling conditions. Therefore, we might

speculate that, in codA transgenic plants, GB may prevent chilling-suppressed catalase activity and, subsequently, prohibit the detrimental accumulation of $H_2O_2$. Furthermore, moderately increased levels of $H_2O_2$ in *codA* transgenic plants may activate the $H_2O_2$-inducible protective mechanism, including catalase, resulting in improved chilling tolerance in GB-accumulating codA transgenic plants. Thus, both products of the choline oxidase-catalyzed reaction, i.e., GB and $H_2O_2$, may contribute to enhancing the chilling tolerance of transgenic plants by maintaining their catalase activities under chilling conditions.

Fruit-set under stressful conditions, including low temperatures, is a complex phenomenon involving many traits closely associated with reproductive structures and events. These include pollen and ovule viability, effective pollination, and early fruit development (Picken, 1984). The effects of chilling are reduced by transformation with the *codA* gene. Our results suggest that enhanced tolerance in transgenic plants resulted from a high accumulation of GB in those organs. In fact, GB levels in the petal, pistil, and anther were 2.8 to 3.8 times higher than in the leaves. This finding supports those seen with *codA* transgenic *Arabidopsis*. Sulpice *et al.* (2003) found that GB contents in flowers, siliques, and inflorescence apices are about five times greater than in leaves, and that expression of the *codA* gene significantly relieves the detrimental effects of salt stress on reproductive organs, particularly the development of anthers, pistils, and petals. When we grew tomatoes in a greenhouse without manual pollination, the frequency of fruit-set was about 50 % in both WT and transgenic plants. When flowering plants were chilled at 3 °C for 7 d, approximately 20 % of the WT died within one week whereas almost all the transgenic plants survived. Frequency of flower drop by chilled plants was approximately 18 % in the WT, but only 0 and 12 % in the L1 and L3 plants, respectively, resulting in higher fruit set rates for the latter transgenics. Even though some WT flowers eventually set fruit, their development was delayed by at least three weeks. Therefore, GB-accumulating transgenic plants produced an average of 10 to 30 % more fruit after chilling.

Although the engineering of tolerance to abiotic stress appears promising (Chen and Murata, 2002; Yuwansiri *et al.*, 2002), no reports are available on the enhancement of cold tolerance in reproductive tissues. Our study demonstrates the importance of transformation with the *codA* gene during flowering and fruiting because many food crops are grown for their fruits. Furthermore, the reproductive phase is generally the time in which a plant is most sensitive to environmental stress. Therefore, our results may benefit efforts to improve crop yields in cold regions.

## EXPERIMENTAL PROCEDURES

### *Agrobacterium* strain and binary vector

Our expression cassette contained 1) the *codA* gene from *Arthrobacter globiformis* under the control of the 35S promoter of cauliflower mosaic virus (CaMV35S), 2) the transit peptide of the small subunit of Rubisco, and 3) a NOS terminator. This cassette was inserted into binary vector pCAMBIA 1201 (CAMBIA, Canberra, Australia) at the *EcoRI* and *HindIII* sites (Figure 4.1a). The resulting plasmid, pCG/codA, was introduced into *Agrobacterium tumefaciens* EHA101 (Hood *et al.*, 1993) by the freeze-thaw method (Walkerpeach and Velten, 1994).

### Transformation of tomato plants

Tomato (*Lycopersicon esculentum* cv. 'Moneymaker') was transformed with *A. tumefaciens* EHA101 (pCG/codA) by an improved version of the protocol described by Frary and Earle (1996). Here, a suspension culture of tobacco cells was omitted as a feeder layer. Antibiotic-resistant shoots were selected and stained for GUS activity (Jefferson, 1987). GUS-positive shoots were rooted *in vitro*, then transferred to soil and grown in a greenhouse. Integration of the *hpt* gene into the tomato genome of T0 plants was confirmed by PCR (Abedinia *et al.*, 1997). Expression of the *codA* gene in those plants was verified by western blotting analysis, using antibodies raised in rabbit against choline oxidase (Hayashi *et al.*, 1997). For repeated self-pollination and production of homozygous lines, we retained only those transgenic lines that expressed choline oxidase at high levels and exhibited a 3:1 resistant:susceptible segregation ratio for hygromycin resistance in the T1 generation. This indicated the insertion of a single transgene. Seed germination for the homozygous transgenic lines was 100 % on an MSG medium (half-strength MS basal medium containing 10 g $L^{-1}$

sucrose and 7 g $L^{-1}$ agar; pH 5.7) (Frary and Earle, 1996) supplemented with 20 mg $L^{-1}$ hygromycin.

**Quantifying glycinebetaine**

Young, fully expanded leaves (50 mg) were powdered with a steel pestle in a 2-ml microfuge tube in liquid nitrogen. The powder was suspended in 0.5 ml of ice-cold methanol:chloroform:water (MCW; 60:25:15) and vortexed thoroughly. The grinding pestle was washed with 0.5 ml distilled water; its contents were then combined with MCW-plant homogenate in the same tube. The resulting homogenate was shaken on a gyrator at 150 rpm for 5 min, then centrifuged at 2500 rpm for 10 min at room temperature (RT) to separate phases. The upper methanol-water (MW) phase was transferred to a clean tube and the volume was measured. The upper aqueous phase was treated with strong anion exchange resin AG 1-X8 (Bio-Rad), as described by Bessieres *et al.* (1999), with slight modifications. The resin was first washed three times with deionized distilled water (dd-water), then suspended in three volumes of dd-water. Micro Bio-spin Chromatography columns (Bio-Rad) were packed with AG 1-X8 resin by adding 1 ml of the resin slurry. Centrifuging followed at 500 rpm for 3 min to eliminate excess water and air bubbles and to form a tight and uniform resin bed. Afterward, 0.5 ml of each sample (MW-phase) was gently loaded onto an AG 1-X8 packed column, which was placed in a 2-ml microfuge tube and centrifuged at 500 rpm for 3 min. The resin was washed once with 0.5 ml dd-water and the two flow-through fractions were combined.

GB was analyzed by HPLC as described by Naidu (1998), except that refractive indices were used for estimations. The HPLC system (Waters Corp., Milford, MA, USA) comprised an Alliance separation module (2695 XL), a photodiode array detector (PDA 2996), and a refractive index detector (RI 2414). A Sugar-Pak I HPLC column (6.5 mm i.d. x 300 mm; Waters Corp.) was maintained at 90 °C in a retrofitted column oven. Samples were held at 4 °C in the refrigerated compartment during the

entire analysis. The mobile phase was dd-water containing 5 mg $L^{-1}$ Ca-EDTA; the flow rate was 0.5 ml $min^{-1}$. Data were analyzed with Millennium software (Waters Corp.) and the amount of GB in each sample was estimated from the refractive index by referring to standard GB solutions. PDA spectral data (190 to 350 nm) were used for authentication.

**Chloroplast isolation**

We placed five- to six-week-old transgenic plants in the dark for 3 d, then isolated intact chloroplasts with an isolation kit (Sigma, USA) according to manufacturer's protocol. The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. Total chlorophyll concentration was determined in 80 % (v/v) acetone via the manufacturer's instructions.

**Protein extraction, western-blot analysis, and catalase assay**

Compound leaves that were third to fifth from the top of the plant were gathered. Proteins
were extracted and catalase activity was determined via methods described by Alia *et al.* (1999). Western-blot analysis was performed as previously reported by Sakamoto *et al.* (1998).

**$H_2O_2$ quantification**

Levels of $H_2O_2$ in the third to fifth compound leaves were determined as described by Alia *et al.* (1999), with modifications. Leaves (100 mg FW) were frozen in liquid nitrogen and homogenized in 1 ml of 5 % ice-cold trichloroacetic acid (TCA) with 50 mg of activated charcoal. The homogenate was centrifuged at 13,000 rpm for 20 min

at 4 °C. After the pH was adjusted to 8.4 by adding 7 M ammonium solution, the supernatant was filtered through a Millex-HA filter (0.4 μm; Millipore, Bedford, MA). The filtrate (0.2 ml) was mixed with 0.4 ml of colorimetric reagent (0.9 mM 4-(2-pyridylazo) resorcinol and 0.9 mM potassium titanium oxalate). Following incubation at 45 °C for 1 h, absorbance was recorded at 508 nm.

**Measurement of chlorophyll fluorescence and ion leakage**

The induction of chlorophyll fluorescence was recorded at RT using a pulse-modulated Fluorescence Monitoring System (FMS1; Hansatech, UK). After adaptation in the dark for 20 min, the ratio of variable to maximum fluorescence (Fv/Fm) was calculated. Chlorophyll fluorescence was measured with five of the third to fifth compound leaves.

Three leaf discs (1-cm diam.) per sample were excised and immersed in vials containing deionized water, then shaken at 150 rpm for 1 h. Ion leakage was determined with a conductivity meter (Model 35; Yellow Springs Instrument). After the samples were autoclaved to release all ions, conductivity was re-measured. The percentage of ion leakage was calculated, using 100 % to represent values obtained after autoclaving.

**Effects of exogenous GB application on chilling tolerance of WT tomato plants**

To produce *in-vitro* seedlings, seeds from WT plants were germinated for 5 d on filter papers soaked with various concentrations of GB solution (0.1 to 10,000.0 μM). They were then transferred to MSP media (full-strength MS basal medium with 30 g $L^{-1}$ sucrose and 7 g $L^{-1}$ agar; pH 5.7) that contained the same GB amounts. When seedlings were approximately 2 cm tall, they were chilled at 3 °C for 7 d (16-h photoperiod), then transferred to a warm growth chamber (25 °C). After 7 d, FW (mg

per seedling) and endogenous GB level in the entire seedling ($\mu mol \cdot g^{-1}$ FW) were measured.

In another experiment, five-week-old greenhouse-grown WT plants were sprayed with either a water control or one of three concentrations of GB solution (0.1, 1.0, or 10.0 mM). Tween-20 (0.005 % v/v) was included as a wetting agent. One day after the foliar application, endogenous GB levels were measured in leaves ($\mu mol \cdot g\ FW^{-1}$). Afterward, the plants were transferred to a cold room (3 °C) for 5 d. They were then returned to the greenhouse where heights (cm) were measured at 0 and 4 weeks.

**Chilling stress at various developmental stages**

WT plants and those from homozygous *codA*-transgenic lines (L1, L3, L5, L7, and L9) were examined for the effect of chilling at the stages of seed germination, *in-vitro* seedling development, non-flowering phase in the greenhouse, and flowering (L1 and L3 only). General growth and treatment conditions included: greenhouse (25±3 °C, 16-h photoperiod, 400-500 $\mu mol\ m^{-2}\ s^{-1}$); growth chamber (3, 7, or 25±0.5 °C, 16-h photoperiod, 200 $\mu mol\ m^{-2}\ s^{-1}$); and cold room (3±1 °C, 16-h photoperiod, 50 $\mu mol\ m^{-2}\ s^{-1}$).

**Seed germination**

Twenty sterilized seeds were placed in a Petri dish filled with 25 ml of an MSG medium (three dishes/genotype/treatment). They were then chilled in a growth chamber [17/7 °C (light/dark)]. After 14 d, the dishes were incubated at 25 °C for another 7 d. Germinated seeds, i.e., those with 2- to 3-mm-long radicles, were counted daily, and results were expressed as percent germination over time. This experiment was replicated three times.

**_In-vitro_ seedling development**

Seeds were germinated in Petri dishes as described above. Uniform 10-d-old seedlings were excised just above their root systems (approximately 2.0 cm long) and transplanted into Magenta GA-7 vessels containing 50 ml of an MSP medium. After 3 d, they were chilled at 3 °C for 7 d in a cold room, and then transferred to a warm growth chamber (25 °C). Seedling responses to cold were quantified by FW (mg) and hypocotyl length (cm). This experiment was repeated three times with 15 replicates each (45 seedlings/genotype/treatment).

**Growth of non-flowering plants in greenhouse**

Five-week-old greenhouse-grown transgenic and WT plants (five of each) were chilled for 5 d (3 °C) in a growth chamber. Afterward, they were returned to the greenhouse. We determined the extent of injury by measuring chlorophyll fluorescence (Fv/Fm), $H_2O_2$ levels, catalase activity, and % ion leakage at 0, 1, 3, and 5 d after the chilling treatment as well as at R3 (i.e., after 3 d of recovery in a warm greenhouse). This experiment was repeated three times (five plants/treatment).

**Effect of chilling at the reproductive phase**

Eight-week-old plants, in which two to three flowers of the first inflorescence had reached anthesis, were chilled (3 °C) in a cold room for 7 d, and then returned to the greenhouse. One week later, they were assessed for injury (defined as % flower retention). At the end of Week 3, the plants were examined for fruit set, as a percentage of flower number. This experiment was repeated three times with five plants (six to nine flowers/plant/treatment).

**Exogenous application of GB or $H_2O_2$ to WT plants**

Five-week-old greenhouse-grown WT plants were sprayed with a water control, GB solutions (0.1, 1.0, or 10.0 mM), or $H_2O_2$ solutions (10, 100, or 1000 mM) at the end of the dark period. Tween-20 (0.005 % v/v) was included as a wetting agent. After the foliar applications, the GB-and $H_2O_2$-treated plants were kept in the dark for 24 h and 4 h, respectively, then transferred to a cold room (3 °C) for 3 d in the dark. Leaves collected from the third to fifth compound leaves of either the GB-treated or $H_2O_2$–treated plants were washed three times with distilled water and stored at -80 °C.

**Methyl viologen treatment**

Leaf discs (1.0 $cm^2$) were collected from the third or fourth compound leaves of five-week-old greenhouse-grown WT and transgenic plants. For the second set of MV treatments, leaf discs collected from five-week-old greenhouse-grown WT plants were incubated in GB solutions (0.1, 1.0, or 10.0 mM) for 24 h in the dark following vacuum infiltration for 30 min. For the last experimental set, five-week-old greenhouse-grown WT plants were sprayed with $H_2O_2$ solutions (10, 100, or 1000 mM) at the end of the dark –period, then further incubated in the dark for 4 h. All these prepared leaf discs were transferred to 6.0-cm Petri dishes containing 5 ml of either water or 20 μM methyl viologen (MV) solutions. Following vacuum infiltration for 30 min, the leaf discs were illuminated (100 μmol $m^{-2}$ $s^{-1}$) at 25 °C for 24 h.

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# CHAPTER 4

## Accumulation of glycinebetaine in various subcellular compartments confers similar levels of enhanced tolerance to abiotic stresses

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## Abstract

We transformed tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) plants with a gene for choline oxidase (*codA*) from *Arthrobacter globiformis*. The gene product was targeted to the chloroplasts (Chl-codA), cytosol (Cyt-codA), or both compartments simultaneously (ChlCyt-codA). The three types of transgenic plants that resulted accumulated different levels and proportions of glycinebetaine (GB) in their chloroplasts and cytosol. Nevertheless, all three types exhibited similar degrees of enhanced tolerance to various abiotic stresses, including chilling, high salt, and oxidation, despite the different amounts of GB accumulated in their targeting sites. We conclude that (1) the accumulation of GB above a threshold level is effective in protecting cells against various abiotic stresses and (2) the location of that accumulation appears not to be a critical factor in conferring stress tolerance. It is possible that small amounts of GB, synthesized in one location, may be translocated to various subcellular compartments, thereby protecting those compartments against such stresses.

## Introduction

In natural environments, productivity and geographical distribution of plants are limited by adverse conditions, such as cold, salinity, and drought. Plants have developed various protective mechanisms to ensure their own survival. One common mechanism is the accumulation of compatible solutes; e.g., organic metabolites that are of low molecular weight, highly soluble in water, and non-toxic at high concentrations (Bohnert et al. 1995). Compatible solutes, which differ among plant species, include betaines, polyols and sugars, and amino acids (Rhodes and Hanson 1993).

Glycinebetaine (GB) is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals, and microorganisms (Rhodes and Hanson, 1993). In both higher plants and *E. coli*, two enzymes are required for the production of GB via two-step oxidation of choline (Hanson et al. 1985, Landfald and Strøm 1986). By contrast, GB synthesis in two soil bacteria, *Arthrobacter globiformis* and A. *pascens*, requires only a single enzyme - choline oxidase (COD) - to catalyze the direct conversion of choline to GB (Ikuta et al. 1977). GB is accumulated in many plant species at elevated levels in response to various abiotic stresses, resulting in improved stress tolerance (Rhodes and Hanson 1993). Possible roles for GB under those conditions include the stabilization of complex proteins and membranes, protection of the transcriptional and translational machinery, and intervention as a molecular chaperone in the refolding of enzymes (For reviews, see Chen and Murata 2002, Sakamoto and Murata 2000).

Genetic engineering to introduce GB biosynthetic pathways to non-accumulator plants appears to be a promising approach in efforts to increase tolerance against environmental stresses (For reviews, see Chen and Murata, 2002, Sakamoto and Murata, 2000). However, levels of GB, on a fresh-weight basis, differ considerably between transgenic plants (0.05 – 5.00 μmol $g^{-1}$ FW) and natural accumulators under stress conditions (4 – 40 μmol $g^{-1}$ FW; Rhodes and Hanson 1993). One possible

protective mechanism proposed for transgenic plants is that GB might be compartmentalized at certain sites within cells to confer substantial protection against stresses even when the overall level of accumulation is low. For example, in rice plants transformed with a *codA* gene, targeting the enzyme to the chloroplasts elicits a higher level of protection than that shown by non-targeting transgenic plants against photoinhibition induced by salt and cold stresses, although levels of GB are up to 5 times higher in the non-target transgenics (Sakamoto et al. 1998). These results indicate that chloroplast compartmentalization of GB biosynthesis efficiently protects the photosynthetic machinery under those stress conditions.

Tomato plants do not normally accumulate GB. Under drought or saline conditions, for example, foliar application of GB to non-accumulator plants enhances stomatal conductance without affecting ABA metabolism, while also increasing protein and chlorophyll contents, and decreasing photorespiration (Mäkelä et al., 1998, 1999). Furthermore, GB accumulation in the chloroplasts of transgenic tomatoes is positively correlated with their level of tolerance to chilling stress (Park et al., 2004). Our objective in the current study was to further increase the amount of GB accumulated in transgenic tomato plants. To do so, we constructed three *codA* expression cassettes that targeted the chloroplasts, cytosol, or both locations, and investigated whether subcellular compartmentalization of that accumulation was a critical determinant for the enhancement of tolerance to various sources of stress.

## Results

### *Localization of GB in codA transgenic plants*

Because COD was targeted to different cellular compartments, i.e., the chloroplasts and cytosol, we analyzed GB levels in the isolated fraction of chloroplasts as well as total content in the leaves from five transgenic lines per construct (Table 4.1). Transgenic plants accumulated various amounts of GB in their leaves, while no GB was detected in the WT (Fig. 4.2a). Moreover, the Cyt-and ChlCyt-codA lines accumulated up to 8.6- and 11.1-fold more GB, respectively, in their leaves than did the Chl-codA lines. In contrast to total leaf contents, chloroplast GB levels were much higher in the Chl-codA lines (3.6 – 9.3 nmol $mg^{-1}$ chlorophyll) than in the Cyt-codA lines (1.3 – 1.9 nmol $mg^{-1}$ chlorophyll). The ChlCyt-codA lines also accumulated GB in their chloroplasts, but to a lesser extent compared with the Chl-codA lines (3.7 – 6.7 nmol $mg^{-1}$chlorophyll). This result demonstrates that 60 to 86% of total leaf GB was localized in the chloroplasts from the Chl-codA transgenic lines, compared with 3 to 9% for Cyt-codA and 10 to 16% for ChlCyt-codA (Table 4.1, Fig. 4.2a). These levels of GB in the chloroplasts were later used to establish correlations between GB content and tolerance to several abiotic stresses.

From each construct, we selected the one line that accumulated the highest level of GB in its leaves, and measured GB content in various plant organs. These three construct lines also were used later to evaluate their responses to salt and chilling stresses. The highest levels of GB were observed in the reproductive organs. In particular, GB accumulations in anther tissues were 2.8- to 3.8-fold greater than in the leaves (Fig. 4.2b). Levels were lower in the seeds and stems than in the leaves. These patterns of GB accumulation in the organs were very similar among all three types of transgenic lines.

**Table 4.1.** Total glycinebetaine (GB) levels in leaves, and percentage of GB in chloroplasts isolated from leaves of five transgenic tomato lines per construct[1]

| Construct | Transgenic line | Total GB in leaves ($\mu mol\ g^{-1}$ FW) | % GB in isolated chloroplasts |
|---|---|---|---|
| Chl-codA | L1 | 0.30±0.04 | 68.6±6.3 |
| | L2 | 0.16±0.01 | 76.7±7.7 |
| | L3 | 0.15±0.01 | 86.3±6.9 |
| | L4 | 0.10±0.02 | 60.0±5.6 |
| | L5 | 0.10±0.02 | 60.0±7.5 |
| Cyt-codA | T1 | 1.43±0.20 | 3.3±0.4 |
| | T2 | 0.89±0.12 | 5.6±0.7 |
| | T3 | 0.63±0.09 | 6.2±0.8 |
| | T4 | 0.54±0.07 | 6.3±0.8 |
| | T5 | 0.38±0.06 | 8.7±1.1 |
| ChlCyt-codA | LT1 | 1.81±0.25 | 9.6±1.2 |
| | LT2 | 1.07±0.15 | 11.4±1.5 |
| | LT3 | 1.02±0.14 | 10.0±1.3 |
| | LT4 | 0.87±0.12 | 11.7±1.5 |
| | LT5 | 0.67±0.09 | 13.2±1.7 |

[1]Mean values ± S.D. from three experiments. GB content in chloroplasts was corrected for percentage of broken chloroplasts present. Percentage of GB found in chloroplasts was calculated by comparing leaf and chloroplast contents, expressed on a chlorophyll basis.

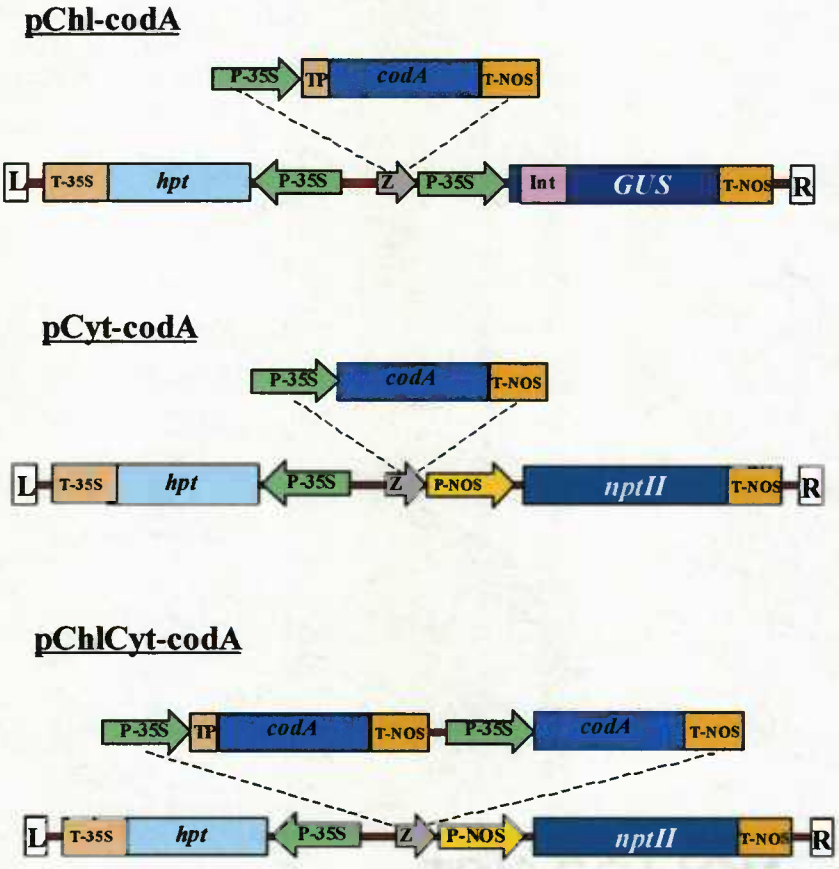


**Fig. 4.1**. Schematic representation of T-DNA regions for pChl-codA, pCyt-codA, and pChlCyt-codA expression cassettes. *codA*, gene encoding choline oxidase; P-35S, CaMV 35S promoter; TP, transit peptide sequences of small subunit of Rubisco; T-*nos*, terminator sequence of gene for nopaline synthase; P-Nos, promoter sequence of gene for nopaline synthase; GUS, β-glucuronidase gene; Int, intron of gene for catalase; *nptII*, gene conferring resistance to kanamycin; *hpt*, gene conferring resistance to hygromycin; L, left T-DNA border; R, right T-DNA border.

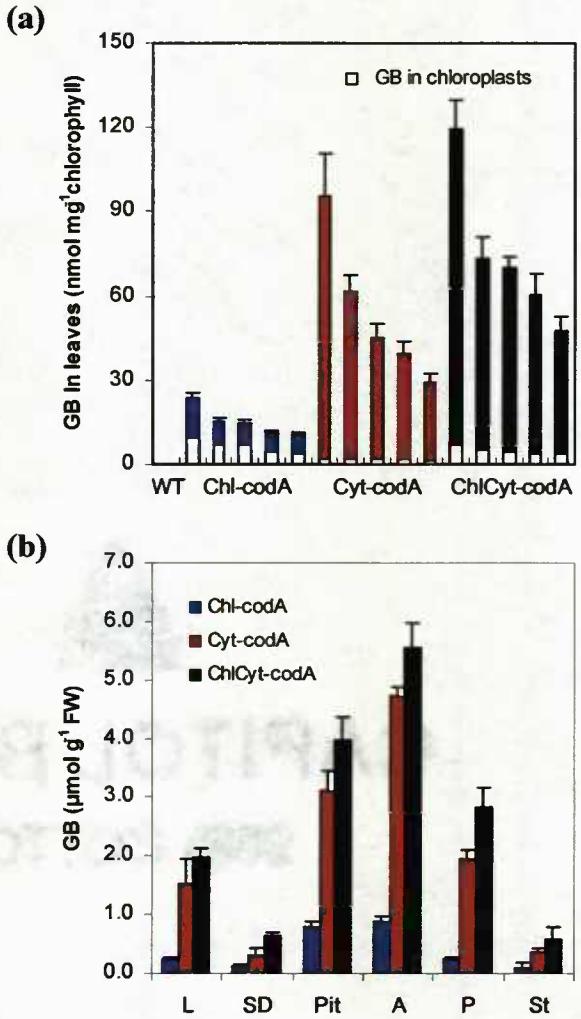


**Fig. 4.2.** GB levels in leaves of 5-week-old tomato seedlings from wild-type (WT) and transgenic lines. (a) GB in chloroplasts (□) and cytosol (■). Means of three experiments; bars indicate standard errors. (b) GB levels in various organs of one transgenic line per construct. Means of three experiments; bars indicate standard errors. L, leaves; SD, seeds; Pit, pistils; A, anthers; P, petals; St, stems.

***GB accumulation protects transgenic tomato seeds from chilling and salt stress***

To evaluate whether higher levels of GB accumulation in seeds further increased the degree of stress tolerance, seeds of the WT and three types of transgenic lines, all of which accumulated different amounts of GB (Fig. 4.2b), were exposed to low temperature (3 °C) or high salt (125 mM NaCl). By the end of the 2-week chilling treatment, 1.4% of the WT seeds had germinated, whereas that rate was increased up to 23% in the transgenic lines (Fig. 4.3a). Upon exposure to 25 °C for 1 d, germination was comparable among all three types of transgenics, with rates calculated at 82.5% (ChlCyt-codA), 89.0% (Chl-codA), and 92.8% (Cyt-codA), compared with only 16.0% for the WT (Fig. 4.3a). Moreover, those WT seeds took 5 d to reach 90% germination after their transfer to 25 °C.

To induce salt stress, seeds of WT and transgenic lines were exposed to 125 mM NaCl , following one day of imbibition with water. After two weeks of treatment, germination was calculated at 25% for the WT versus at least 91% for any of the transgenics (Fig. 4.3b). Furthermore, already by Day 5 of their exposure, all transgenic lines had achieved 50% germination, compared with less than 5% germination for the WT. These results indicate that GB accumulation in tomato seeds enhanced their tolerance to both chilling and salt stress, even though no significant differences were observed among those three types of transgenic lines in this germination phase.

***GB accumulation in both cytosol and chloroplasts protects tomato plants from chilling stress***

Under normal greenhouse conditions, growth did not differ between the WT and transgenic lines. Chilling stress caused severe ion leakage in the former, while the latter were much less affected (Fig. 4.4a). After plants were chilled at 3 °C for 3 d, ion leakage in the Chl-, Cyt-, and ChlCyt-codA transgenic lines was 28.7%, 38.5%, and 32.2%, respectively, whereas that of WT plants was about 50%. After 3 d of recovery at 25 °C, leakage from WT cells was further increased to 65% but was much less in the transgenics (Fig. 4.4a). After 3 d at 3 °C, PS II activity in the WT plants declined

to 40% of the original value (Day 0); transgenic plants maintained 49 to 52% of their initial activity (Fig. 4.4b). Although this PSII damage was further magnified in both WT and transgenic plants after 3 d of recovery at 25 °C, the transgenics appeared to be far less harmed than the WT.

Under non-stress conditions, $H_2O_2$ levels were 17 to 21% higher in the transgenic lines than in the WT plants (Fig. 4.4c). However, those levels increased in both WT and transgenics during cold treatment. After 1 d of incubation at 3 °C, $H_2O_2$ contents in WT plants were 3.7-fold higher than their original value (Day 0), whereas transgenic accumulations were 2.2- to 2.4-fold greater (Fig. 4.4c). Under non-stress conditions, catalase activities in the transgenic lines were 8 to 13% higher than in the WT, but, on Day 1 of the stress period, had increased to 34% (Chl-codA), 27% (Cyt-codA), and 33% (ChlCyt-codA) versus 12% for the WT (Fig. 4.4d). At the end of the treatment (Day 3 at 25 °C), catalase levels in both the WT and the transgenic plants were severely reduced compared with their initial activity (Day 0), but this decline was much less in the transgenics.

### *GB accumulation in chloroplasts of transgenic plants is related to enhanced stress tolerance in a dose-dependent manner*

No changes in heights (from the original value of 2 cm) were observed in either the WT or the transgenic *in vitro* seedlings during cold treatment at 3 °C for 7 d (data not shown). However, after recovering for 7 d in a warm growth chamber (25 °C), the growth of transgenic seedlings was significantly greater than that of the WT (Fig. 4.5a; $P < 0.01$). After 7 d at 25 °C, WT hypocotyls grew only 35% over their original heights. In contrast, the growth of transgenic seedlings following chilling stress was enhanced [46 ~ 290% of their original height at Day 0; Fig. 4.5a]. The Chl-codA lines appeared to be more tolerant of low temperature than any other transgenic lines. Moreover, among those three engineered groups, hypocotyl growth in the Chl-codA seedlings was most positively correlated with GB chloroplast levels (Fig. 4.5a; $R^2=0.67$). The Cyt-codA

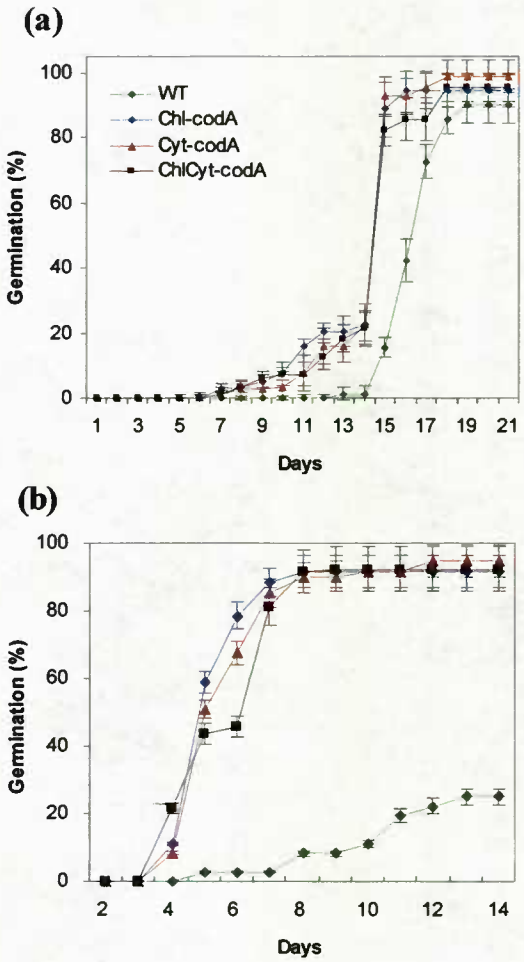


**Fig. 4.3**. Effects of chilling and salt stress on germination of three types of *codA* transgenic tomato lines. (a) Seeds of WT and one transgenic line per construct were incubated for 14 d at 17/7 °C (light/dark) and 16-h photoperiod, then returned to warm growth chamber at 25 °C for 7 d. (b) Seeds of WT and one transgenic line per construct were allowed to imbibe water for 1 d, then placed for 14 d on media containing 125 mM NaCl. Germination rates for both experiments were recorded daily; values represent mean ± SE of three experiments.

The following excerpt (Figure 27) reveals more about how students use their data as evidence to support their claims and how they report the results through their writing during the phase of *Analyzing and Interpreting Results*. The letters A through F indicate how Mr. Field scaffolded students to develop argumentation in the discourse included in Figure 27. Mr. Field's instruction directed toward argumentation is highlighted in bold.

As outlined in Figure 27, Mr. Field emphasized the importance of using evidence that students collected themselves through experimentation in order to support their claims and hypotheses (A). Mr. Field also encouraged students to understand in what ways that evidence (data) supported their claims or did not, thereby developing students' argumentation (B). This step of CLEA is called *Analyzing and Interpreting Results* and is considered to be the most important stage in which students develop their reasoning skills by sharing ideas with peers in groups or with the teacher as a whole class. Mr. Field expected his students to understand what evidence they could use to support their own claims, how they could explain what happened using evidence, and how students could connect those results or conclusions to their own hypotheses or questions that were derived from their claims (C). Furthermore, Mr. Field provided opportunities for students to extend their thinking skills by describing the patterns of data to *generally explain* the mechanism of what happened (D) as well as reporting their exact evidence to be used to *justify* what happened (E). During this process, Mr. Field encouraged students to think of any factor which limited their experiment in terms of getting evidence (data) refuting their claims (F).

lines showed the lowest correlation ($R^2$=0.28) while the ChlCyt-codA lines were intermediate ($R^2$=0.44).

GB accumulations in our transgenic lines also improved their tolerance to salt stress (125 mM NaCl), as seen by their *in vitro* growth (Fig. 4.5b). Two weeks after salt treatment, the transgenic seedlings were up to 72% heavier than the WT. Although growth did not differ significantly among the three transgenic groups, the Chl-codA lines again exhibited the highest correlation between the level of salt tolerance and GB content in their chloroplasts (Fig. 4.5b; $R^2$=0.69). $R^2$ values for the ChlCyt-codA and the Cyt-codA were 0.44 and 0.16, respectively.

We used the reduction in PSII activity to illustrate the extent of MV-mediated damage to the photosynthetic apparatus. Under non-stress conditions, Fv/Fm values were not significantly different between the WT and transgenic plants (data not shown). Treatment with MV caused a severe decline in photosynthetic activity for leaf discs from all genotypes (Fig. 4.5c). However, activity in the transgenic lines remained 11 to 96% higher than in the WT plants. Again, the highest correlation between tolerance to MV-induced oxidative stress and GB levels in their chloroplasts was observed in the Chl-codA plants ($R^2$=0.72).

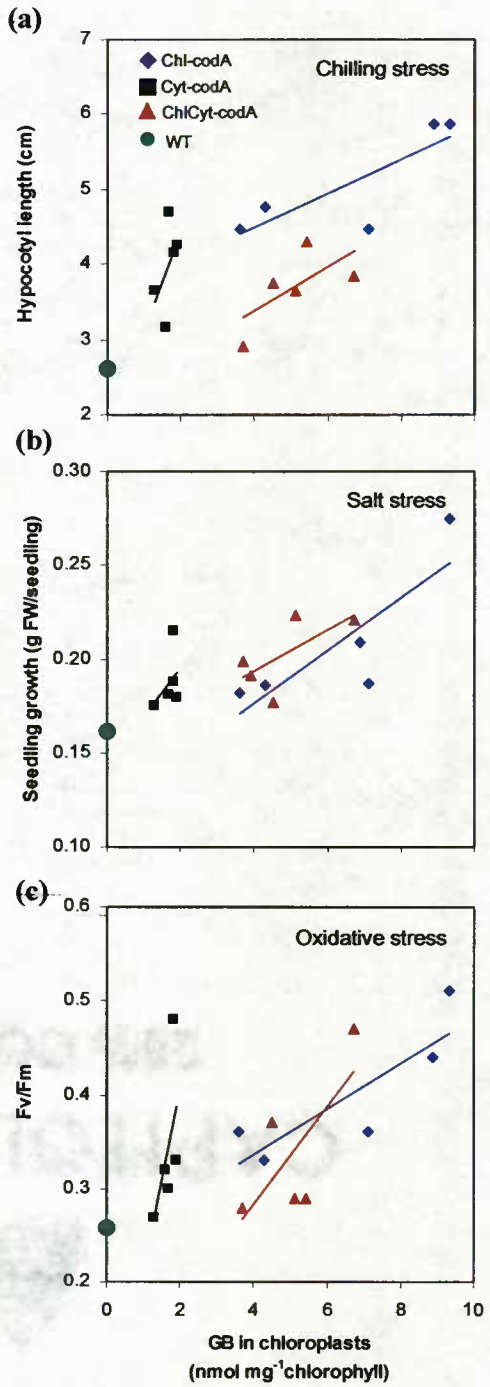


**Fig. 4.5**. Correlations between glycinebetaine (GB) levels in chloroplasts and tolerance to three sources of stress. (a) Chilling: uniform 10-d-old seedlings of WT and five independent transgenic lines per construct that accumulated different levels of GB in their chloroplasts were excised just above root systems (approximately 2.0 cm

long) and transplanted into growth media. After 3 d, seedlings were chilled at 3 °C for 7 d, then transferred to 25 °C. Response to cold was examined by measuring hypocotyl length (cm) 7 d after this transfer. (b) Salt: same procedure described for (a) was applied before salt stress. Seedlings were then transplanted to MSP media containing 125 mM NaCl. Response was examined by measuring fresh weight (g) per seedling after 2 weeks of treatment. (c) Oxidation: leaf discs (0.8 $cm^2$) collected from 5-week-old WT and five independent transgenic lines per construct were incubated in water containing 5 μM methyl viologen following vacuum infiltration for 30 min. Reduction in Fv/Fm was measured after 1 d of treatment. Results are mean ± SE from three independent experiments (n = 27 per genotype).

**Discussion**

We have previously transformed tomato plants with a bacterial gene for COD, which results in the accumulation of GB by *codA*-transgenic tomato plants (Park et al. 2004). These plants exhibit enhanced chilling tolerance during their entire life cycle, from seed germination to the reproductive stage. However, targeting this transgene to the chloroplasts elicits only a very low level of accumulation in the leaves (0.09 – 0.30 μmol $g^{-1}$ FW; Park et al. 2004). This amount of GB is far less than that detected under stress conditions in natural GB-accumulators, such as spinach (30 – 40 μmol $g^{-1}$ FW) (Rhodes and Hanson 1993). A positive correlation has been reported between betaine accumulation and the acquisition of tolerance to salt and cold in maize (*Zea mays*) and barley (*Hordeum vulgaris*), respectively (Rhodes *et al.* 1989, Kishitani *et al.* 1994). Moreover, exogenous applications of betaine to the leaves or roots enhance tolerance to various stresses in several species, both natural accumulators and non-accumulators (Harinasut *et al.* 1996, Mäkelä *et al.* 1996, Allard *et al.* 1998, Hayashi *et al.* 1998). These studies have suggested that it might be possible to further improve stress tolerance by genetic manipulation to increase GB levels in non-accumulators (McCue and Hanson 1990).

Nonetheless, genetic engineering of GB biosynthesis with the *codA* gene has been mostly targeted to the chloroplasts in a number of higher plants (reviewed by Sakamoto and Murata 2000). Such transgenic plants accumulate GB exclusively in their chloroplasts, and exhibit tolerance to various abiotic stresses at a wide range of developmental stages (Sakamoto et al. 2000, Park et al. 2004). Furthermore, Sakamoto et al. (1998) have reported that, in rice plants transformed with the *codA* gene for chloroplast-targeted COD, their photosynthetic machinery is more efficiently protected against salt and cold stresses than in those that express a *codA* gene whose product is not targeted to chloroplasts, even though the latter type of plants accumulate five times more GB. Therefore, it is important to know whether it is the localization of GB synthesis in various cellular compartments and/or the particular GB concentration that affects tolerance to abiotic stresses in transgenic tomato plants.

Two major factors limit the accumulation of GB in chloroplasts of transgenic plants: the availability of endogenous choline itself (Huang et al. 2000) and the transport of choline across the chloroplast envelope (McNeil et al. 2000). Huang et al. (2000) have introduced the metabolic steps for oxidation of choline to GB into three diverse species -- *Arabidopsis thaliana*, *Brassica napus*, and tobacco (*Nicotiana tabacum*). In all three, the use of exogenous choline significantly increases GB accumulation, suggesting that this supplement is required for the enhancement of GB levels in transgenic plants. Furthermore, the establishment of a model for the labeling kinetics of choline metabolites has revealed that the import of choline into chloroplasts limits GB synthesis in those compartments (McNeil et al. 2000). In plants, the biosynthesis of choline occurs exclusively in the cytosol (McNeil et al. 2000). Therefore, McNeil et al. (2001) have attempted to increase the endogenous choline supply through a transgenic approach, using phosphoethanolamine N-methyltransferase (PEAMT), a key enzyme in the plant choline-biosynthetic pathway. Their research has provided the first example of engineered biosynthesis of choline, and has demonstrated that GB accumulation can be significantly increased in transgenic plants that normally accumulate only to very limited levels (McNeil et al. 2001). Those PEAMT transgenic plants contain up to 50-fold more free choline and accumulate 30-fold more GB than plants transformed with the vector alone. However, those previous experiments did not evaluate the effects on abiotic stress tolerance by transgenic lines with elevated GB contents.

In the current study, we transformed tomato plants with the *codA* gene that was targeted to the cytosol (Cyt-codA) or to both chloroplasts and cytosol (ChlCyt-codA) in order to increase GB levels. Following transformation, we further evaluated whether increases in GB synthesis in the cytosol of transgenic tomato plants could confer higher abiotic tolerance compared with chloroplast-targeting transgenic plants that had low GB levels. Targeting *codA* genes to the cytosol or to both locations allowed much higher GB accumulations in the leaves of the Cyt-codA (0.38 – 1.43 $\mu$mol $g^{-1}$ FW) and ChlCyt-codA transgenic lines (0.67 – 1.81 $\mu$mol $g^{-1}$ FW) than in the

Chl-codA lines (0.10 – 0.30 μmol $g^{-1}$ FW) (Table 4.1). We first chose one line per construct that produced the highest amount of GB in their leaves -- Chl-codA (0.3 μmol $g^{-1}$ FW), Cyt-codA (1.43 μmol $g^{-1}$ FW), and ChlCyt-codA (1.81 μmol $g^{-1}$ FW). Higher amounts of GB were also found in seeds of the ChlCyt-codA (1.1 μmol $g^{-1}$ FW) and Cyt-codA (0.31 μmol $g^{-1}$ FW) lines compared with those of the Chl-codA (0.1 μmol $g^{-1}$ FW) lines.

In the first experiment, seeds of the WT and three transgenic lines were exposed to chilling or salt stress (Fig. 4.3). Afterward, germination in all three transgenic lines was much better than in the WT (Fig. 4.3a). However, those rates did not differ significantly among the transgenic lines, although their GB contents did.

In the second experiment, 5-week-old WT and transgenic seedlings were exposed to chilling stress. All three transgenic lines showed improved tolerance over the wild type, as defined by their % ion leakage, chlorophyll fluorescence (Fv/Fm), $H_2O_2$ level, and catalase activity (Fig. 4.4). Nevertheless, their degree of tolerance did not differ among the three, regardless of their leaf GB levels (Fig. 4.4). Therefore, these results suggest that GB accumulation in the chloroplasts or the cytosol confers similar levels of enhanced tolerance to abiotic stresses.

Among our transgenic tomato lines, chloroplasts of the Cyt-codA lines accumulated 3.3 to 8.7% of the total GB in their leaves, compared with the Chl-codA line (60.0 – 86.3%) (Table 4.1; Fig. 4.2a). Although chloroplast GB levels in the ChlCyt-codA lines accounted for only 9.6 to 13.2% of the total leaf GB, this concentration was much higher than that in the Cyt-codA lines but was similar to that in the Chl-codA lines (Fig. 4.2a). Targeting the GB-catalyzing enzyme to different subcellular compartments, including the peroxisome, mitochondria, and cytosol, has also been shown to enhance tolerance by the photosynthetic machinery to salt and chilling (Kishitani et al. 2000, Takabe *et al.* 1998, Holmstrom *et al.* 2000). Moreover, exogenous application of GB to tomato plants enhances their tolerance to salt, drought, and chilling (Mäkelä *et al.* 1999, Park et al. 2004). In our study, the absorbed GB remained mostly in the cytosol but a very limited amount was translocated into the

chloroplasts (Table 4.1, Fig.4.2). Therefore, it is possible that GB synthesized in the cytosol can be transported into other subcellular compartments, such as the chloroplasts, peroxisome, and mitochondria. Likewise, although that amount of translocated GB may be very limited, it is sufficient to confer stress tolerance to those compartments.

Previously, we showed that *codA*-transgenic tomato plants exhibit efficient protection of their PSII in a GB dose-dependent manner (Park et al. 2004). In this study, we also observed that the Chl-codA lines had the highest linear relationship between levels of GB in their chloroplasts and degrees of tolerance to chilling, salt, and oxidative stress, with $R^2$ values of 0.67, 0.69, and 0.72, respectively (Fig. 4.5). The Cyt-codA lines had the lowest correlation ($R^2 = 0.16 - 0.31$) while the ChlCyt-codA lines were intermediate ($R^2 = 0.44 - 0.51$). These results suggest that GB accumulation in the chloroplasts of transgenic tomato plants increases stress protection in a dose-dependent manner, such that any further rise of GB levels in their chloroplast could improve even more their stress tolerance. In addition, we speculate that one possible cause for the low $R^2$-value from the Cyt-codA lines is that translocation of GB from the cytosol to different subcellular compartments was very limited, making it difficult to see any GB dose-dependent relationship in the chloroplasts. Furthermore, levels of abiotic tolerance in the ChlCyt-codA lines were not further improved compared with the performance of the other two types of transgenic lines, even though they might have dual protective effects on both the chloroplasts and other subcellular compartments. Therefore, future work is needed to elucidate whether (1) GB accumulation in the cytosol is actually transported to other subcellular compartments, (2) those compartments are protected by such accumulations if indeed GB is translocated, and (3) increasing choline availability in the chloroplasts further enhances stress tolerance.

In conclusion, tolerance to several abiotic stresses was enhanced in transgenic tomato plants in which the *codA* gene was targeted to the chloroplasts, cytosol, or both compartments. Although each transgenic line accumulated different amounts of GB in

either the chloroplasts or the cytosol, their levels of tolerance were similar. This likely resulted because GB synthesized from the cytosol was translocated to different subcellular compartments, thereby conferring stress protection. Furthermore, the amounts of GB translocated into other compartments, albeit very limited, was sufficient to reach the protective levels obtained by the chloroplast-targeting transgenic plants.

## Materials and Methods

### *Construction of plasmids*

The binary vector pCG/codA for chloroplast-targeted expression of the *codA* gene (P35S::TP-codA::TNos) was constructed as described by Park et al. (2004). From this point forward, we will clearly distinguish between the binary transformation vectors and localization of the *codA* gene product by referring to pCG/codA as pChl-codA.

For non-targeted, i.e., cytosolic, expression of the *codA* gene, we used the binary vector pGAH/codANT (Hayashi et al., 1997). In this vector, P35S::codA::TNos lacks TP (transit peptide of the small subunit of Rubisco from tobacco). Therefore, its product is mainly localized in the cytosol. Again, pGAH/codANT will be hereafter referred to as pCyt-codA.

To obtain simultaneous expression of *codA* in both the chloroplasts and the cytosol, we constructed a third binary vector as follows: the pUC/codANT cloning vector (Hayashi et. al., 1997) carries a P35S::codA::TNos expression cassette between the *HindIII* and *EcoRI* sites. pUC/codANT was cut with *EcoRI* and treated with Mung Bean Nuclease (New England BioLabs, Beverly, MA) to remove the 3'- and 5'-single-stranded extensions. This plasmid was mixed with a HindIII linker (New England BioLabs) and ligated by T4 DNA ligase (Promega, Madison, WI). The P35S::codA::TNos expression cassette was then excised with *HindIII* and ligated upstream of P35S::TP-codA::TNos into the *HindIII*-digested and APS-dephosphorylated (APS-Shrimp Alkaline Phosphatase; Roche Applied Science, Indianapolis, IN) pGAH/codA binary vector (Hayashi et al., 1997) to create pCytChl-codA.

### *Transformation*

The general transformation procedure for tomato (*Lycopersicon esculentum* cv. 'Moneymaker') followed that described by Park et al. (2004), with a few

modifications. Antibiotic-resistant shoots were chosen from selection media containing hygromycin (5 mg $L^{-1}$) or kanamycin (50 mg $L^{-1}$). Integration of the *hpt* or *npt* gene into the tomato plants was confirmed by PCR. For repeated self-pollination and production of homozygous lines (T3), we germinated putative transgenic seeds on an MSG medium (Park et al., 2004) supplemented with 20 mg $L^{-1}$ hygromycin or 150 mg $L^{-1}$ kanamycin. Five transgenic lines per construct were chosen for all three types (i.e., Chl-codA, Cyt-codA, and ChlCyt-codA). These five lines, showing various levels of GB, were used for further characterizations.

***Biochemical analyses***

General biochemical analyses, including GB and $H_2O_2$ quantifications and a catalase assay, were conducted with compound leaves that were the third to the fifth from the top of each plant, based on procedures described by Park et al. (2004).

***Chloroplast isolation***

We placed 6-week-old transgenic plants, at the pre-flowering stage, in the dark for 2 d, then extracted intact chloroplasts with an isolation kit (Sigma, St, Louis, MO) according to the manufacturer's protocol. The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. Total chlorophyll concentrations were determined in 80% (v/v) acetone, via the manufacturer's instructions.

***Measurement of chlorophyll fluorescence and ion leakage***

The induction of chlorophyll fluorescence was recorded at RT using a pulse-modulated Fluorescence Monitoring System (FMS1; Hansatech, UK). After adaptation in the dark for 30 min, the ratio of variable to maximum fluorescence (Fv/Fm) was measured.

Five leaf discs (0.8-cm diam) per sample were used to determine ion leakage, as described previously (Park et al., 2004). Both measurements were conducted with the

third to fifth compound leaves.

***General plant growth and abiotic stress treatments***

WT plants and those from five transgenic lines per construct were examined at the stages of seed germination, *in-vitro* seedling development, or the non-flowering phase. General growth and treatment conditions included: greenhouse (25±3 °C, 16-h photoperiod, 400-500 μmol $m^{-2} s^{-1}$); growth chamber (3, 7, or 25±0.5 °C, 16-h or 24-h photoperiod, 500 μmol $m^{-2} s^{-1}$); and cold room (3±1 °C, 16-h photoperiod, 100 μmol $m^{-2} s^{-1}$).

***1) Chilling stress treatment***

Uniform 10-d-old *in-vitro* seedlings were excised just above their root systems (approximately 2.0 cm long) and transplanted into Magenta GA-7 vessels containing 50 ml of an MSP medium (full-strength MS basal medium with 30 g $L^{-1}$ sucrose and 7 g $L^{-1}$ agar; pH 5.7). After 3 d, they were chilled at 3 °C for 7 d in a cold room under continuous light, and then transferred to a warm growth chamber (25 °C; 16-h photoperiod). Seedling responses to cold were quantified by hypocotyl length (cm). This experiment was repeated three times with 15 replicates each (45 seedlings/genotype/treatment).

***2) Salt stress treatment***

Uniform 10-d-old *in-vitro* seedlings were excised just above their root systems (approximately 2.0 cm long) and transplanted into Magenta GA-7 vessels containing 50 ml of an MSP medium. Those seedlings were placed in a growth chamber (25 °C) under continuous light for two weeks, after which the fresh weight (FW) per seedling was measured.

### *3) Oxidative stress*

Leaf discs (0.8 $cm^2$) were collected from the third or fourth compound leaves of 5-week-old greenhouse-grown WT and transgenic plants, and were subjected to vacuum infiltration for 30 min in the dark. The discs were then transferred to 6.0-cm Petri dishes containing 5 ml of either water or 5 μM methyl viologen (MV) solutions, and were placed under lights (100 μmol $m^{-2}$ $s^{-1}$) at 25 °C for 16 h.

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# CHAPTER 5

## Engineered enlargement of tomato flowers and fruits using a transgene for choline oxidase

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**Abstract**

The domestication and genetic improvement of crop plants have been achieved through dramatic increases in the sizes of flowers and fruits. However, the molecular mechanisms that regulate the size of reproductive organs are poorly understood. Tomato plants expressing a transgene for choline oxidase (*codA*) accumulated glycinebetaine and exhibited alterations in expression of genes that are involved in cell division. Moreover, increases in cell number and cell size resulted in enlarged flowers and fruits, which reflected the pleiotropic effects of expression of the *codA* transgene and suggest a novel approach to the improvement of crop plants.

**Introduction**

In nature, the intrinsic sizes of plant organs exhibit not only remarkable uniformity within species but also conspicuous differences among related species. Increases in organ size have long been a primary target in the domestication and genetic improvement of crop plants but studies of the developmental mechanisms that regulate the inherent sizes of plant organs have only recently been initiated. Moreover, most characterized genes that control organ size in plants are associated with the development of leaves, roots, and shoot apical meristems. Less attention has been paid to the developmental and molecular mechanisms that regulate the sizes of reproductive organs, such as flowers and fruits.

Certain aspects of flower development, such as the establishment of floral organ identity, have been well characterized (1). By contrast, very little is known about the ways in which the patterns and sizes of flowers are controlled. In *Arabidopsis*, the *AINTEGUMENTA* (*ANT*) gene is involved in the control of organ growth during flower development. Ectopic expression of *ANT* results in the development of enlarged floral and shoots in *Arabidopsis* (2). The increased size of sepals in such transgenic plants is a result of increased cell division, whereas the increased sizes of petals, stamens, and carpels are primarily attributable to increased cell expansion (2).

In *Lycopersicon*, the genus to which tomatoes belong, the weights of fruits exhibit extreme variability, ranging from a few grams to a kilogram (3). A number of major quantitative trait loci (QTLs) that control the size and shape of fruits have been extensively exploited. Several QTLs, including *fw, locule-number, fascinated, ovate, sun* and *fs8.1*, control the size or the shape of tomato fruits (4). To date, only one gene with a major effect on fruit size, *fw2.2*, has been cloned and molecular studies have revealed that natural allelic variations in *fw2.2* alone can alter fruit size by as much as 30% (5).

We showed previously that transformation of tomato plants with the *codA* gene for choline oxidase from the soil bacterium (*Arthrobacter globiformis*) allows them to

synthesize glycinebetaine (GB), which is not normally accumulated in tomato. The synthesis and accumulation of GB resulted in enhanced tolerance to chilling stress at various stage of plant development, including reproductive stages (6). We report here that our *codA* transgenic plants produced significantly enlarged flowers and fruits as a result of the pleiotropic effects of the *codA* gene. Our observations suggest a novel approach to the improvement of the flower and fruit sizes of crop plants.

## Results and Discussion

### Transformation with the *codA* gene resulted in enlarged flowers and fruits

We introduced a *codA*-expression cassette into a market-type tomato cultivar (*Lycopersicon esculentum* cv. Moneymaker) and determined levels of GB (6). A transgenic line used in this study exhibited a 3:1 resistant:susceptible segregation ratio for hygromycin resistance in the T1 generation, indicating a single insertion of the transgene. In plants that were homozygous for the transgene, elevated levels of GB were found in flower buds, shoot apices, and the locular cavities of red fruits, in which levels of GB were at least double those in leaves (7). When wild-type (WT) and transgenic tomato plants were grown hydroponically in a greenhouse (8), a number of phenotypic differences ($p \leq 0.001$; Student's t-test) were observed between WT and *codA* transgenic plants (Table 5.1). In particular, individual flowers from transgenic plants were approximately twice as heavy as WT flowers (Table 5.1) and the lengths of petals were approximately 1.7-fold larger than the lengths of WT counterparts (Fig. 5.1A and Table 5.1). The difference in flower size became apparent well before anthesis, when the ovaries were also unusually large (Fig. 5.1A). The number of petal segments did not differ significantly between WT and transgenic plants ($6.24 \pm 0.43$ versus $6.30 \pm 0.51$; means $\pm$ SEM; $P = 0.24$; $n = 50$ flowers from WT and transgenic plants, respectively), whereas elevated numbers of both carpels and stigma-lobes were observed in the transgenic flowers (9). However, the *codA* transgenic plants produced approximately 30% fewer flowers per inflorescence than did the WT plants. Without hand-pollination, the rate (42%) of fruit-set per inflorescence of transgenic plants was lower than that of WT plants (68%; Table 5.1).

Ripe transgenic tomatoes were also significantly larger (Fig. 5.1B) and 54 % heavier on average (Table 5.1) than ripe WT tomatoes. Figure 5.1C compares the normal distribution of weights of WT tomatoes with that of transgenic tomatoes. The median weight of transgenic tomatoes was more than 60 % greater than that of WT

tomatoes, and the weights of 25% of individual transgenic tomatoes represented by the upper whisker were approximately twice the median weight of WT fruit (Fig. 5.1C).

After fertilization, each carpel develops into a locule in the fruit. However, some varieties produce fruits with unusually large numbers of locules, often producing larger fruit with a greater diameter (3). Multilocular-type carpels were observed from very early stages in the transgenic flower buds and the transgenic fruits contained six or seven locules, as compared to the two or three locules in the case of WT fruits (Table 5.1; Figs. 5.2C and 5.2D). In transgenic plants, the number of seeds per fruit was significantly smaller than that in WT plants (Table 5.1). Fertilization and the subsequent development of seeds are essential for the full development of ovaries into fruit. Poor seed-set has been shown to be associated with underdeveloped and small tomato fruit (10). However, it is possible that this correlation might be due to linkage of separate genes that control fruit weight and the number of seeds per fruit. In fact, in a study of relationships between many traits and fruit size, the number of seeds per fruit was the trait that was most weakly correlated with fruit weight (3).

We produced more than thirty individual GB-accumulating transgenic lines. All of them exhibited the enlarged sizes of flowers and fruits, indicating that *codA* transgene is responsible for the observed effects on flower and fruit sizes. In addition, we found that the increase in fruit size was caused primarily by pleiotropic effects of the introduction of the *codA* transgene, rather than by secondary effects of morphological alterations, such as smaller numbers of flowers per inflorescence and lower rates of fruit-set (11)

**Enlarged transgenic petals and carpel tissues were due to increases in both cell size and cell number**

The sizes of plant organs are generally determined by both cell number and cell size. To investigate whether the increased growth of transgenic plants was related to enhanced cell division and/or to the enhanced post-mitotic expansion of cells during

**Table 5.1**. Comparison of the characteristics of reproductive organs of *codA* transgenic and wild-type (WT) tomato plants.

| | WT | *codA* |
|---|---|---|
| No. of flowers per inflorescence | 8.7 ± 0.7 | 6.1 ± 0.6 |
| Fresh weight of flower (mg) | 96 ± 15 | 194 ± 22 |
| Length of petal (mm) | 10.1 ± 1.5 | 17.3 ± 1.8 |
| Fruit-set (%) | 68 ± 9 | 42 ± 7 |
| Fresh weight per red fruit (g) | 93 ± 11 | 143 ± 14 |
| No. of locules per fruit | 2.7 ± 0.3 | 6.5 ± 0.9 |
| No. of seed per fruit | 120 ± 18 | 45 ± 8 |

Five pairs of WT and *codA* transgenic plants were grown hydroponically. Ten flowers per plant were weighed three to five days after anthesis. Ten of the first fruits on each plant were harvested at random when fully ripe (red). Results are means ± SEM of results from two independent experiments.

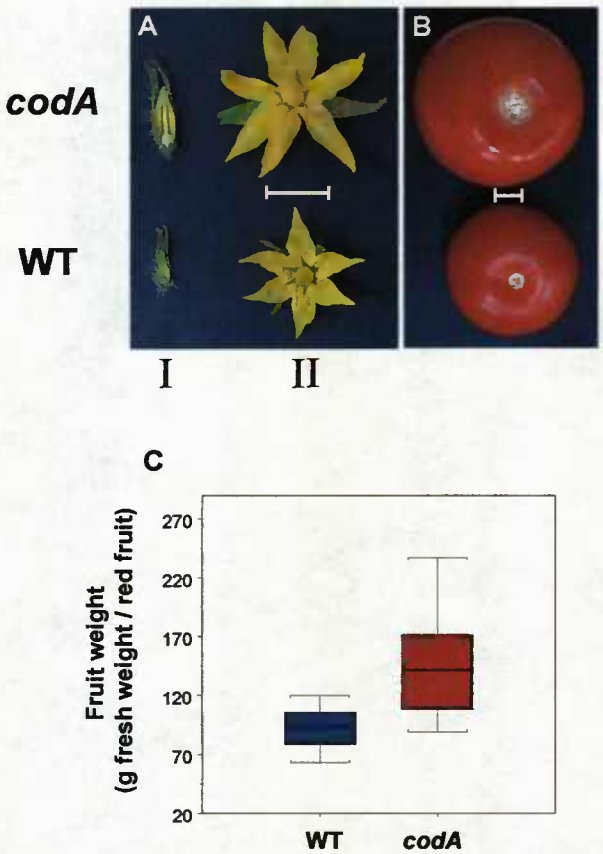


**Fig. 5.1**. Comparison of the size of flowers (A) and fruits (B) of *codA* transgenic and wild-type tomato plants. WT and *codA* represent wild-type and *codA* transgenic plants, respectively. I, Two days before anthesis; and II, Seven days after anthesis. Sepal and petal tissues were removed manually from flower buds at stage I. The histogram in (C) shows the pattern of distribution of fruit weights from WT and transgenic plants (n = 100 for WT, n = 97 for transgenic plants). Each bar in panel (A) and (B) corresponds to 1 cm, respectively.

fruit development, we performed histological analysis using cross sections of carpel and petal organs harvested two or three days before anthesis (Fig. 5.2). In petal tissues of transgenic plants, cells were strikingly enlarged (Figs. 5.2A and 5.2B), while cell numbers per unit area were significantly reduced ($P \leq 0.01$). The proportion of enlarged cells (> 25 μm in length) in transgenic petals was much higher (13.4%) than that in WT petals (8.4%), whereas the proportions of medium and small (12 – 25 μm and < 12 μm in length, respectively) cells were not significantly different (P = 0.22 and P= 0.44 for medium and small cells, respectively) between the WT and transgenic petals.

Two or three days before anthesis, transgenic carpels were already noticeably bigger than their WT counterparts (Fig. 5.1A), and they were composed of many locules, which were often arranged in double tiers (Figs. 5.2C and 5.2D). The outer wall of the pericarp of transgenic carpels was also much thicker, in most cases, than that in the WT (Figs. 5.2C and 5.2D). The transgenic carpels contained fewer cells per unit area of the outer wall of the pericarp ($P < 0.01$) than did the WT (Figs. 5.2E and 5.2F), whereas there was no significant difference ($P = 0.25$) in the inner wall of pericarp. However, the outer wall of the pericarp of transgenic carpels had significantly more cell layers (P < 0.01) than the outer wall of the WT pericarp (Figs. 5.2E and 5.2F). Therefore, enlargement of transgenic reproductive organs, including petal and carpel tissues, was caused by increases not only in cell size but also in cell number.

**Levels of transcripts of genes involved in the regulation of cell division were altered in transgenic plants**

To determine how insertion of the *codA* transgene might induce the enlargement of flowers and fruits via changes in gene expression, we used a tomato cDNA microarray (Tom1; this microarray covers approximately 12,000 tomato genes) to compare gene expression in flower buds (two or three days before anthesis) of WT and transgenic

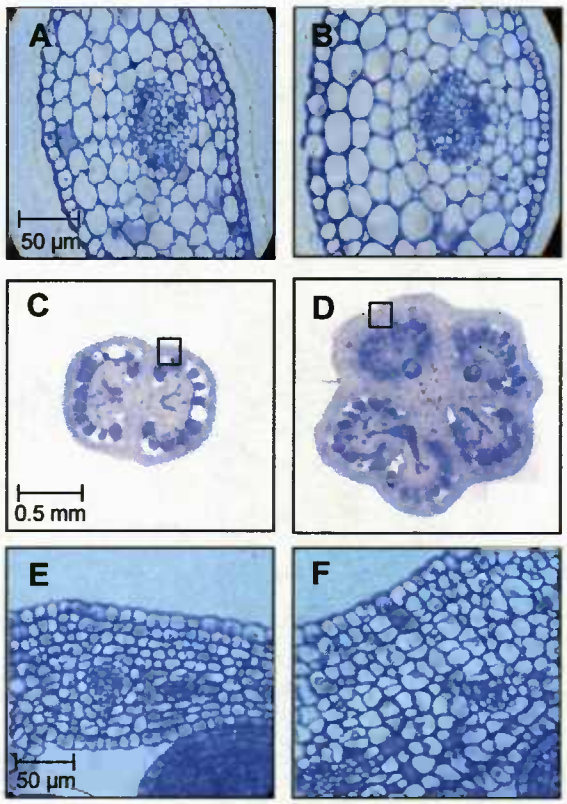


**Fig. 5.2.** Histological analysis of longitudinal sections of petals and transverse sections of carpels from WT and *codA* transgenic plants two to three days before anthesis. Upper photos (A and B) show petal tissues. Middle photos (C and D) show entire carpels. Lower photos (E and F) show the outer wall of the pericarp. Sections on the left (A, C and E) are from WT plants; sections on the right (B, D and F) are from *codA* transgenic plants. The boxes in C and D indicate areas that are shown at higher magnification in E and F. Carpel and petal tissues were fixed in 2.0 % FAA, dehydrated through an ethanol-water series and embedded in plastic resin.

plants (12). The expression of thirty genes was enhanced in the transgenic flower buds (13), and we confirmed the enhanced expression of genes for cyclin D (*CycD3-1*), cyclin-dependent protein kinase p34/cdc2 (*CDKA1*,), and RUB1- conjugating enzyme (*RCE1*), by semi-quantitative RT-PCR (14). The expression of these three genes exhibited very similar patterns; the levels of the transcripts of each gene in the flower buds of transgenic plants were much higher than those in WT plants. After anthesis, the levels of these transcripts fell to levels lower than those in WT plants (Fig. 5.3). The levels of expression of these genes did not differ significantly between the leaves of WT and transgenic plants.

Genetic analysis of the control of the sizes of plant organs is just beginning to reveal the coordination between cell growth and the cell cycle. It has been suggested that one of the plant D-type cyclins is a regulator of organ size, as are the D-type cyclins in animal cells (15). D-type cyclins appear to regulate the G1- to S-transition in the cell cycle and to act as important integrators of various signals. Three genes for D3 cyclins have been cloned from young tomato fruit and have been shown to play different respective roles during fruit development (16). In the G1 phase, Cdc2, a homolog of cyclin-dependent kinase A, is produced and has been shown to interact with CycD3 (17). To date, two CDKAs, designated Lyces;CycA1;1 and Lyces;CycA2;1, have been isolated from tomato fruits, and they have similar patterns of expression throughout the development of tomato fruits (18). The CDKA/CycD3-1 complex of tobacco, formed in insect cells, can phosphorylate and inactivate the tobacco retinoblastoma-related (RB-related) protein, which is a product of a tumor-suppressor gene. In mammals, the phosphorylation of RB protein activates E2F-controlled genes, which are required for S progression through the S phase of the cell cycle (19). The ubiquitin-protein conjugation pathway consists of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) (20). In *Arabidopsis*, a mutation in the RCE1 protein, which functions analogously to E2, results in various organs of reduced length, such as leaves, petioles, and siliques (21). Moreover, *rce1* mutants are deficient in their responses to anxin. Thus, it seems

likely that the RUB-conjugating pathway is required for auxin-dependent pattern formation in the developing embryo.

We examined levels of the transcripts of the *fw2.2* gene, which is a key controller of fruit size that acts as a negative regulator of cell division. This gene was not included in the Tom1 cDNA microarray. As shown in Figure 5.3, levels of the transcripts were very similar in the leaves and flower buds of WT and transgenic plants. After anthesis, however, the level of the transcript in transgenic flowers was significantly reduced relative to that in WT flowers. The *fw2.2* gene encodes a negative repressor of cell division, whose activity is largely confined to the cell-division phase of fruit development (5). A noteworthy feature of the *fw2.2* gene is that changes in its promoter are associated with changes in mitotic activity at the early stages of fruit development, as well as a shift in the timing of gene expression (22).

Our present working hypothesis is that the regulation of genes that are involved in cell division, such as *fw2.2*, might somehow be responsible for increases in the sizes of the *codA* transgenic tomato plants. We are now examining the mechanism by which the *codA* transgene affects cell division in tomato plants. We plan also to examine whether the *codA* transgene can be used to increase the sizes of reproductive organs in other plant species.

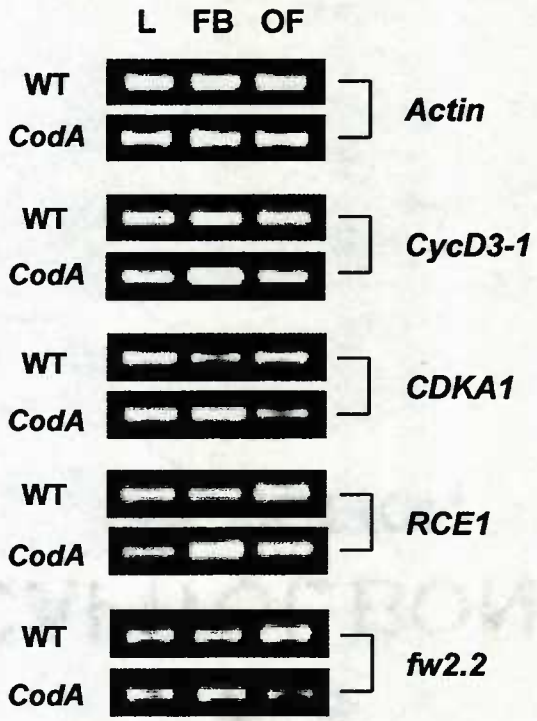


**Fig. 5.3**. Results of semi-quantitative analysis by RT-PCR of levels of various transcripts in WT and *codA* transgenic plants. We examined levels of transcripts of genes for cyclin D (*CycD3-1*), cyclin-dependent kinase A (*CDKA1*), ubiquitin-conjugating enzyme (*RCE1*), and *fw2.2*, using the gene for actin as the control. L, young leaves; Fb, flower buds two to three days before anthesis; and OF, opened flowers seven days after anthesis.

**References and Notes**


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8. Plant growth conditions are available as supporting material on Science Online
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12. The microarray and experimental procedures are available as supporting material on Science Online
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**Supporting Online Materials**

7. **Table 5.S1**. Levels of glycinebetaine (GB) in various organs of *codA* transgenic tomato plants.

| Organ | GB (μmol/g FW) |
|---|---|
| Leaves | 0.28 ± 0.05 |
| Shoot apices[a] | 0.59 ± 0.06 |
| Flower buds[b] | 0.65 ± 0.08 |
| Stems | 0.09 ± 0.02 |
| Roots | 0.03 ± 0.01 |
| Seeds | 0.20 ± 0.03 |
| Red fruit: | |
| Epidermis | 0.07 ± 0.01 |
| Pericarp | 0.23 ± 0.03 |
| Locular cavity | 0.58 ± 0.05 |

Levels of GB were determined in *codA* transgenic tomato plants that had been grown in a hydroponic system. Data are means ± SEM of results from three independent experiments with three plants per experiment.
[a]Shoot apices (< 10 mm).
[b]Flower buds before anthesis

11. **Flower-removal experiment**

Table S2. Effects of the *codA* transgene on the size and morphology of fruits

| | WT | | | *codA* | | |
|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 1st | 2nd | 3rd |
| Red fruit (g) | 85.8 ± 11.8 | 78.7 ± 10.3 | 72.2 ± 8.5 | 185.7 ± 32.5 | 101.6 ± 12.9 | 86.7 ± 10.7 |
| Width of fruit (cm) | 5.1 ± 0.6 | 4.9 ± 0.4 | 4.5 ± 0.2 | 6.9 ± 0.5 | 5.8 ± 0.2 | 5.1 ± 0.3 |
| Height of fruit (cm) | 4.2 ± 0.2 | 4.1 ± 0.2 | 4.0 ± 0.1 | 5.6 ± 0.4 | 4.8 ± 0.3 | 4.2 ± 0.2 |

Five pairs of WT and *codA* transgenic plants were grown hydroponically in a greenhouse. On the first five inflorescences of each plant, only three flowers per inflorescence were allowed to remain after manual pollination. The other inflorescences were removed soon after their appearance. The parameters shown above were measured at harvest when fruits were fully ripe.

8. **Plant growth conditions.** Five pairs of WT and the *codA* transgenic plants were grown hydroponically in a greenhouse at 25 °C with a 16-h photoperiod (400-500 μmol $m^{-2}$ $s^{-1}$). Hydroponic nutrients (Flora Series; General Hydroponics, Sebastopol, CA) were freshly applied every other week and the pH was maintained between 5.7 to 5.9. All flowers were tagged at anthesis and fresh weights of flowers were measured three to five days after anthesis. Ten of the first fruits from each plant were harvested at random when fully ripe and all characteristics were recorded.

12. **Analysis of transcripts with the tomato cDNA microarray**. Total RNA was extracted from pools of flower tissues derived from five pairs of WT and *codA* transgenic plants, respectively. Both the quantity and the quality of RNA samples were checked with an Agilent Bioanalyzer 2100 (Agilent Biotechnology, Town, MA, USA). The basic experimental procedure for tomato microarray analysis followed the protocol described by Zhang et al. with a few modifications (35). In brief, 10 μg of total RNA were used to prepare labeled cDNA with a 3DNA Array Detection Cy3/Cy5 kit (Genisphere, Montvale, NJ, USA). Both cy3 and cy5 primers, which consisted of poly(dT) fused to a capture sequence, were used to synthesize the first-strand cDNA. Before hybridization, a tomato microarray slide (Tom1), obtained from the Center for Gene Expression Profiling at Cornell University (http://bti.cornell.edu/CGEP/ CGEP.html), was pre-hybridized in 5x SSC, 0.1% SDS, 1% BSA for 45 min at 45 °C. Each preparation, labeled with either the Cy3 or the Cy5 capture sequence, was allowed to hybridize overnight at 60 °C to probes on the Tom1 array in a total volume of 68 μl of hybridization buffer, as described by the manufacturer of the kit. The arrays were then washed in 2x SSC, 0.2% SDS at 60 °C for 10 min, in 2x SSC at room temperature for 10 min, and in 0.2x SSC at room temperature for 10 min. Then they were rinsed in 95% ethanol and centrifuged dry at 1,500 rpm for 3 min in a clinical centrifuge (HN-SII; International Equipment Co., Needham Heights, MA). Arrays were hybridized a second time for 3 h at 60 °C with 68 μl of hybridization buffer that contained Cy3 and Cy5 capture reagents, as

described by the manufacturer of the kit. After the second hybridization, arrays were washed and dried as described above but in darkness. Hybridized arrays were scanned with a ScanArray 4000 system (GSI Lumonics, Watertown, MA, USA). Competitive hybridization was repeated by hybridizing two chips with the dye-labeling reversed. The intensities of spots were measured with Quantarray (GSI Lumonics). The intensity of fluorescence from each spot on both the Cy3 and the Cy5 images was quantified and local background-fluorescence levels were subtracted. Cy3 and Cy5 images were normalized by adjusting the median signal intensities of the two images ("global normalization") with Microsoft Excel macro. The array data were compared and queried with Microsoft Access software (Microsoft, Redmond, WA, USA).

13. **Table S3.** Changes in the expression of genes in the flower buds of *codA* transgenic tomato plants.

| Unigene ID | Regulation | | Annotation |
|---|---|---|---|
| | Up (-fold) | Down (-fold) | |
| | | | **Signal transduction** |
| U143532 | 3.1 | | GTP-binding nuclear protein RAN2 |
| U144463 | 4.3 | | GDP-dissociation inhibitor - common tobacco |
| U145565 | 2.2 | | **Cyclin-dependent protein kinase p34cdc2 [*Lycopersicon esculentum*]*** |
| U152464 | 2.4 | | **cyclin D [*Helianthus tuberosus*]*** |
| U159909 | 4.3 | | Calcium/calmodulin-dependent protein kinase CaMK3 [*Nicotiana tabacum*] |
| U144099 | | 3.0 | ARF GTPase-activating domain-containing protein [*Arabidopsis thaliana*] |
| U146230 | | 2.6 | GTP-binding protein ypt2 |
| U146565 | | 2.2 | GTP-binding protein - garden pea |
| U147336 | | 2.5 | cyclin family [*Arabidopsis thaliana*] |
| | | | **Transcription factor** |
| U149142 | 2.3 | | Zinc finger protein–like [*Arabidopsis thaliana*] |
| U154664 | | 2.3 | Zinc finger protein [*Arabidopsis thaliana*] |
| | | | **Defense/ stress response** |
| U143160 | 2.7 | | Acyltransferase 2 [*Capsicum chinense*] |
| U143414 | 2.4 | | NP24 protein precursor (pathogenesis-related protein PR P23; salt-induced protein) |
| U143849 | 2.8 | | Peroxidase [*Nicotiana tabacum*] |
| U144671 | 2.3 | | Putative peroxidase [*Solanum tuberosum*] |
| U143518 | | 3.1 | TSI-1 protein |
| U144354 | | 3.5 | Thylakoid-bound ascorbate peroxidase [*Nicotiana* |

| | | | |
|---|---|---|---|
| | | | *tabacum*] |
| U145080 | | 2.3 | Elicitor inducible protein [*Nicotiana tabacum*] |
| U146833 | | 2.3 | Avr9/Cf-9 rapidly elicited protein 146 [*Nicotiana tabacum*] |
| | | | **Metabolism** |
| U143454 | 3.3 | | Putative chloroplast thiazole-biosynthetic protein [*Nicotiana tabacum*] |
| U143745 | 2.9 | | Precursor to malate dehydrogenase (oxaloacetate-decarboxylating) (NADP; EC 1.1.1.40) |
| U146553 | 2.2 | | Precursor to delta-9 stearoyl-acyl carrier protein desaturase |
| U148184 | 4.2 | | Omega-6 fatty acid desaturase [*Sesamum indicum*] |
| U151189 | 2.4 | | Calcineurin-like phosphoesterase family [*Arabidopsis thaliana*] |
| U152658 | 2.7 | | Indole-3-acetate beta-glucosyltransferase -related [*Arabidopsis thaliana*] |
| U145461 | | 3.4 | Pin1-type peptidyl-prolyl cis/trans isomerase [Malus x domestica] |
| U146087 | | 2.5 | NAD-dependent epimerase/dehydratase family [*Arabidopsis thaliana*] |
| U155547 | | 2.2 | Ribulose bisphosphate carboxylase small chain 2A, chloroplast precursor (RuBisCO small subunit 2A) |
| | | | **Protein biosynthesis/ modification** |
| U143296 | 2.1 | | **RUB1 conjugating enzyme [*Olea europaea*]*** |
| U143395 | 2.2 | | Elongation factor 1-alpha (EF-1-α) |
| U143905 | 2.1 | | Precursor to proteinase inhibitor type II TR8 |
| U145296 | 2.5 | | Xaa-Pro aminopeptidase 2 [*Lycopersicon esculentum*] |
| U147915 | 3.0 | | Arginine/serine-rich splicing factor RSp41 |

| | | | |
|---|---|---|---|
| | | | [*Arabidopsis thaliana*] |
| U148415 | 3.8 | | PQ loop repeat-containing protein [*Arabidopsis thaliana*] |
| U143349 | | 3.9 | Subtilisin-like proteinase (EC 3.4.21.) |
| U144085 | | 3.7 | 40S Ribosomal protein S27 (RPS27B) [*Arabidopsis thaliana*] |
| U150273 | | 2.8 | Putative DEAH-box RNA helicase [*Oryza sativa* (japonica)] |
| U146693 | | 2.6 | Eukaryotic initiation factor 4 (eIF4), putative [*Arabidopsis thaliana*] |
| U147049 | | 2.7 | Putative RING protein [*Populus* x *canescens*] |
| U147454 | | 2.3 | Protein similar to von Hippel-Lindau binding protein [*Arabidopsis thaliana*] |
| U148554 | | 2.5 | Trigger factor-related protein [*Arabidopsis thaliana*; involved in protein folding] |
| U149951 | | 2.7 | Hydroxycinnamoyl/benzoyltransferase-related protein [*Arabidopsis thaliana*] |
| | | | **Electron transport** |
| U144467 | 2.1 | | Cytochrome P450, putative [*Arabidopsis thaliana*] |
| U146545 | 3.4 | | NAD(P)H:quinone oxidoreductase [*Solanum tuberosum*] |
| U147194 | 3.0 | | Cytochrome P450 [*Petunia* x *hybrida*] |
| U167387 | 2.2 | | Cytochrome c oxidase, subunit VIIIb; COX VIII-H [*Mus musculus*] |
| U143344 | | 3.0 | Ferredoxin I [*Solanum tuberosum*] |
| | | | **Transport** |
| U146431 | 5.3 | | Transporter-related [*Arabidopsis thaliana*] |
| U147496 | 2.3 | | Probable potassium channel protein SKT3 [Solanum tuberosum] |

| | | | |
|---|---|---|---|
| U149487 | | 3.7 | ABC transporter family protein [*Arabidopsis thaliana*] |
| | | | **Hormone/ pigment biosynthesis** |
| U143397 | | 2.9 | Phytoene synthase 1, chloroplast precursor (Fruit ripening-specific protein pTOM5) |
| U144054 | | 2.5 | 1-Aminocyclopropane-1-carboxylate synthase [*Pelargonium x hortorum*] |
| U149412 | | 2.2 | Gibberellin 20-oxidase-3; 20ox-3 [*Lycopersicon esculentum*] |
| | | | **Cell division/ growth** |
| U145211 | 2.5 | | Pectin esterase (pectin methylesterase) family [*Arabidopsis thaliana]* |
| U147117 | | 2.9 | Syntaxin SNAP33 [*Arabidopsis thaliana*] |
| | | | **DNA binding/repair** |
| U154274 | 2.2 | | N-Acetylglucosamine-phosphate mutase [*Arabidopsis thaliana*] |
| U145406 | | 2.8 | Remorin 1 [*Lycopersicon esculentum*] |
| U145438 | | 2.3 | S-Adenosylmethionine:2-demethylmenaquinone methyltransferase-like protein [Oryza sativa (japonica)] |
| U155986 | | 2.5 | Probable RAD51B-like DNA-repair protein [imported; *Arabidopsis thaliana*] |

Numbers in columns 'up' and 'down' indicate relative changes (-fold) at the transcript level in transgenic flower buds two to three days before anthesis, as compared to WT flower buds. This table lists genes associated with more than two-fold changes. An asterisk plus bold face indicates genes whose elevated expression was confirmed by semi-quantitative RT-PCR

14. **Semi-quantitative RT-PCR.** Total RNA (2 $\mu$g) was treated with DNase I (Promega, Medison, WI, USA) and reverse-transcribed with 0.1 $\mu$M oligo-dT as primer in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). The cDNAs of interest were specifically amplified with the following sets of primers: CycD3-1-F, 5'-ATGAGTTTCATTTGGGTTTTCAAT-3', and CycD3-1-R, 5'-CAGATTCA -TTTTCCACTTAAGAGT-3'; CDKA1-F, 5'-AAGGTTGGAGCAGGAAGACGAGGG-3', and CDKA1-R, 5'-TATGAAGAACTCTATGAGAATGAC-3'; RCE1-F, 5'-CCCAATGAAAGATGACCTCATG-3', and RCE1-R, 5'-ACATTGGATTCAAACATC -TTCGGG-3'; and fw2.2-F, 5'-AATCCAGGTCAATGAAACAACTT-3', and fw2.2-R, 5'-CCATATCAAAGCCACGGTTC-3'. As a control for RT-PCR, a 525-bp cDNA fragment encoding tomato actin was amplified with primers: Act-F, 5'-CCAAAAGCCAATCGAGAGAA-3', and Act-R, 5'-GGTACCACCACTGAGGACGA-3'. PCR was performed with1 $\mu$l of the RT reaction mixture after reverse transcription. After an initial cycle (3 min at 94 °C, 3 min at 57 °C, and 3 min at 72 °C), reaction cycle was as follows: 45 s at 95°C, 45 s at 57°C, and 45 s at 72°C. A final elongation step was allowed to proceed for 5 min at 72°C. The amount of first-strand cDNA used and the number of cycles were chosen for amplification within linear range. The PCR products were verified by sequencing and then separated on an agarose gel (1.2 %), stained with ethidium bromide.

23. **Supporting references and notes**

X. Zhang *et al.*, *Plant J.* **39**, 905 (2004).

## CONCLUSIONS

Exogenous application of glycinebetaine (GB) caused tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker) to accumulate GB (0.09 – 10.88 μmol $g^{-1}$ FW). GB-treated plants exhibited similar levels of enhanced chilling tolerance regardless of endogenous levels of GB. The majority of the GB was localized to the cytosol with only a small proportion translocated to the chloroplasts, indicating that cytosolic GB can be translocated to protect various subcellular compartments. The growth of GB-treated plants was increased following chilling, and GB accumulated to high levels in meristematic tissues, including shoot apices and flower buds. These results suggest increased GB accumulation in meristematic tissues improves the tolerance of these tissues to chilling, which results in enhanced recovery-growth rates during post-chilling periods under non-stress conditions. The application of GB also increased levels of $H_2O_2$, catalase expression, and catalase activity in the absence of stress. Following chilling, the GB-treated plants maintained lower levels of $H_2O_2$ and higher levels of catalase activity throughout the treatment period, compared with the performance of those parameters in the control plants. In addition to a possible direct protective effect of GB on macromolecules, such as membranes and proteins, it is likely that the induced chilling tolerance conferred by exogenous application may have resulted from the protective induction of $H_2O_2$-mediated antioxidant mechanisms, e.g., enhanced catalase expression and catalase activity. Furthermore, the protective effect of GB on the activity of PSII continued for up to 3 d, but then disappeared within a week after the application, which provide a motive for introducing a biosynthetic GB pathway into tomato plants in order to enhance chilling tolerance without requiring frequent exogenous applications of glycinebetaine.

To evaluate the hypothesis described above, transgenic tomato plants (cv. Moneymaker) were generated with a chloroplast-targeted *codA* gene of *Arthrobacter globiformis,* which encodes choline oxidase that catalyzes the conversion of choline to GB. While these transgenic plants accumulated the majority of GB (~86%) in the

chloroplasts, their total leaf-GB concentration was very low (0.1 – 0.3 $\mu mol \cdot g^{-1}$ FW) compared to natural GB-accumulators, such as spinach (~ 40 $\mu mol \cdot g^{-1}$ FW). Transgenic plants were more tolerant of chilling stress than their wild-type (WT) counterparts during their entire life cycle from seed germination to reproductive stages. This result showed that endogenous GB concentrations as low as 0.1 $\mu mol \cdot g^{-1}$ FW are sufficient to confer high levels of tolerance in transgenic tomato plants, in agreement with the GB foliar-application. Exogenous application of either GB or $H_2O_2$ improves both chilling and oxidative tolerance concomitant with enhanced catalase activity. The *codA* transgenic plants also accumulated moderately increased levels of $H_2O_2$, which is likely produced as a by-product of choline oxidase-mediated reaction. Therefore, this marginal increase of $H_2O_2$ in our transgenic plants may activate the $H_2O_2$-inducible protective mechanism, resulting in improved chilling and oxidative tolerances in GB-accumulating *codA* transgenic plants. Thus, introducing the GB biosynthetic pathway into tomato through metabolic engineering is an effective strategy for improving chilling tolerance. The level of accumulated GB in the chloroplast-targeting *codA* transgenic plants was very low compared to natural accumulators, where GB accumulation is known to induce under stress conditions and levels correlate with the extent of increased tolerance. Furthermore, we found that chloroplast GB levels are correlated with the degree of chilling-stress tolerance.

To determine if increased amount of GB in transgenic tomato plants would result in further increase in chilling-stress tolerance, tomato (cv. Moneymaker) plants were transformed with a *codA* gene whose product was targeted to either the chloroplasts (Chl-codA), the cytosol (Cyt-codA), or both compartments simultaneously (ChlCyt-codA). Targeting the activity of *codA* gene to the cytosol or to both locations resulted in higher GB accumulations in the leaves of the Cyt-codA and ChlCyt-codA transgenic lines than in the Chl-codA lines. The chloroplasts of the Cyt-codA lines contained 3.3 to 8.7% of the total GB, indicating that GB synthesized in the cytosol can be translocated into other subcellular compartments. While three types of transgenic lines accumulated different amounts of GB in either the chloroplasts or the

cytosol, their levels of chilling tolerance were similar, likely as a result that cytosolic GB is translocated to subcellular compartments and consequently protected those compartments. Furthermore, the amount of GB translocated into other compartments, while limited, was sufficient to confer the protection, suggesting the location of GB accumulation is not a critical factor in enhancing stress tolerance in tomato plants.

It is interesting to note that the *codA* transgenic plants produced significantly enlarged flowers and fruits, which are caused by increases in both cell number and cell size. Fruit size of transgenic tomatoes remained significantly larger than that of the WT plants in a flower removal experiment, which was conducted to compensate for potential effects of differences in fruit load between WT and transgenic plants, indicating that fruit enlargement resulted from a pleiotropic effect of the *codA* gene transfer. Transgenic plants also exhibited alterations in expression of genes involved in cell division, including cyclin D, cyclin-dependent protein kinase A, ubiqutin-conjugating enzyme, and *fw2.2* mRNA, which is a key gene determining fruit size. These results suggest that the transformation with the *codA* gene might be a novel approach to improve yield traits in agricultural and horticultural crop plants.

Although GB accumulation was shown to be effective in enhancing chilling tolerance of tomato plants, several mechanisms still remains to be revealed whether (1) marginal increase of $H_2O_2$-in GB-accumulating tomato plants affects the expression of other genes that might be responsible to enhance their chilling tolerance, (2) cytosolic GB is transported to other subcellular compartments other than the chloroplasts, e.g., the mitochondria, peroxisome, etc., (3) those compartments are protected by such low levels of GB accumulations if indeed GB is translocated, (3) increasing choline availability in the chloroplasts further enhances stress tolerance, (4) genetic engineering of the *codA* gene into different plant species other than tomatoes also enlarge the sizes of reproductive organs.

Based on all results shown in this study, I conclude that; (1) GB accumulation in both the WT and the *codA* transgenic plants induces $H_2O_2$-mediated antioxidant mechanisms, that include enhanced catalase expression and catalase activity, (2) GB

levels in the chloroplasts of the *codA* transgenic plants are positively correlated with the degrees of their chilling tolerance, (3) GB accumulated in the cytosol can be translocated into different subcellular compartments, including the chloroplasts, and consequently confer sufficient chilling tolerance to those organelles, (4) transformation of tomato plants with a *codA* gene increases the sizes of flowers and fruits, as a consequence of increased cell numbers and sizes.