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Exogenous Application of Glycinebetaine Increases Chilling Tolerance in Tomato Plants

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Tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) plants are chilling sensitive, and do not naturally accumulate glycinebetaine (GB), a metabolite that functions as a stress protectant. We reported previously that exogenous GB application enhanced chilling tolerance in tomato. To understand its protective role better, we have further evaluated various parameters associated with improved tolerance. Although its effect was most pronounced in younger plants, this benefit was diminished 1 week after GB application. When administered by foliar spray, GB was readily taken up and translocated to various organs, with the highest levels being measured in meristematic tissues, including the shoot apices and flower buds. In leaves, the majority of endogenous GB was found in the cytosol; only 0.6–22.0% of the total leaf GB was localized in chloroplasts. Immediately after GB application, levels of H_2O_2, catalase activity and expression of the catalase gene (*CAT1*) were all higher in GB-treated than in control plants. One day after exposure to chilling stress, the treated plants had significantly greater catalase activity and *CAT1* expression, although their H_2O_2 levels remained unchanged. During the following 2 d of this chilling treatment, GB-treated plants maintained lower H_2O_2 levels but had higher catalase activity than the controls. These results suggest that, in addition to protecting macromolecules and membranes directly, GB-enhanced chilling tolerance may involve the induction of H_2O_2-mediated antioxidant mechanisms, e.g. enhanced catalase expression and catalase activity.

Keywords: Catalase — Chilling tolerance — Glycinebetaine — Tomato.

Abbreviations: GB, glycinebetaine; ROS, reactive oxygen species.

Introduction

In nature, low temperature is 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 means for coping 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 and avoid disturbing their normal functions when exposed to abiotic stresses (Yancey et al. 1982). Representative compatible solutes, which differ among 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 (Zhao et al. 1992, Alia et al. 1998, Allard et al. 1998, Mäkelä et al. 1998, Jokinen et al. 1999, Mäkelä et al. 1999, Chen et al. 2000). 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 Sakamoto and Murata 2000, Chen and Murata 2002).

Tomato plants (*Lycopersicon esculentum* Mill.) do not naturally accumulate GB (Wyn Jones and Storey 1981). Being of tropical origin, most cultivated genotypes suffer chilling injury when grown at <10°C (Graham and Patterson 1982, Patterson et al. 1987). Exposure to temperatures below 13°C may inhibit fruit-set (Atherton and Rudich 1986), while extended exposure to temperatures below 6°C can kill plants. Although they are not natural accumulators (Wyn Jones and Storey 1981), exogenously applied GB increases their tolerance to stress from salt and drought (Mäkelä et al. 1998, Mäkelä et al. 1999) and chilling (Park et al. 2004). Nevertheless, the mechanisms by which GB increases this stress tolerance are poorly understood. As the first step towards better comprehension of GB-enhanced tolerance in plants, we report here the characterization of various parameters associated with chilling tolerance in tomato plants.

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Results

Exogenous GB application enhances chilling tolerance in tomato plants

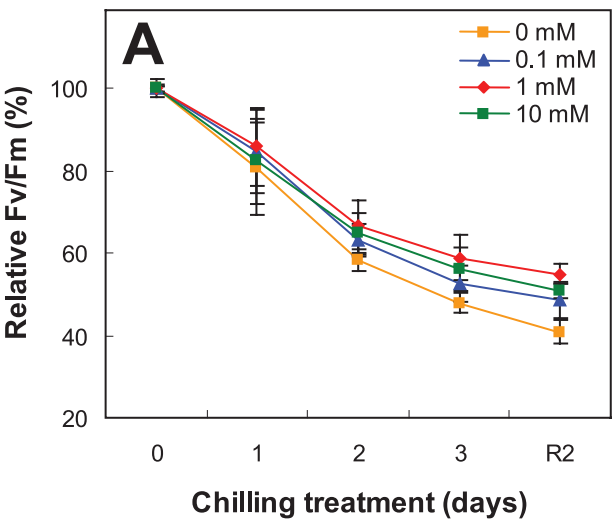
Following exogenous GB applications, PSII activity was measured daily during 3 d of chilling at 3°C, then for another 2 d at 25°C. One day after GB application, but before chilling had begun (i.e. day 0), PSII activity in GB-treated plants did not differ significantly from that of the control plants (Fig. 1A). During the following 3 d chilling period, however, activity declined in both treated and non-treated plants, although the rate of decline was slower for the former (Fig. 1A). After 2 d at 25°C, PSII activity declined further, to about 40% of the original value (day 0) in the controls and to 50–55% in the treated plants.

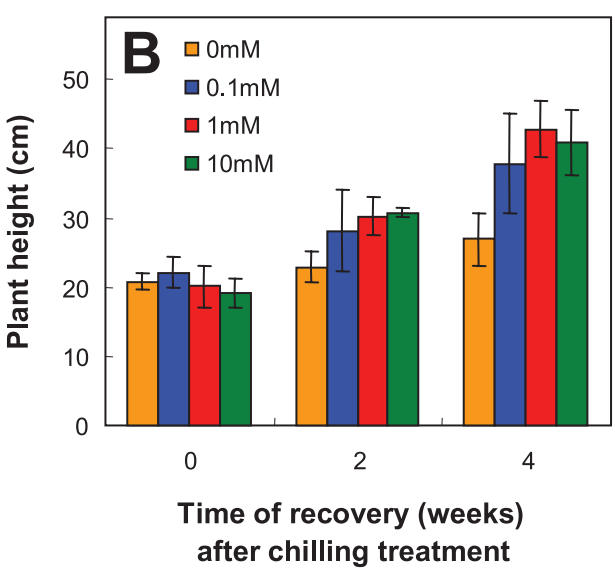
The long-term effect of chilling stress was assessed during the recovery period in a greenhouse maintained at 25°C. Growth in terms of height was measured for up to 4 weeks. Immediately after the chilling treatment, growth did not differ significantly among plants treated with various GB concentrations (0.1–10.0 mM) and the control plants (Fig. 1B). Nevertheless, the endogenous level of GB in leaves of plants treated with 10.0 mM GB was >100-fold higher than in those treated with only 0.1 mM GB (Table 1). All GB-treated plants recovered and grew faster than the controls in the 4 weeks following our stress treatment (Fig. 1B, C). Based on these results, we selected a concentration of 1.0 mM GB for further evaluation of this chilling response.

GB application enhances chilling tolerance at various stages of plant growth

To determine whether this GB benefit was limited to a particular developmental stage, we applied GB (1.0 mM) during three distinct growth periods: (i) 4-week-old non-flowering plants with 6–7 compound leaves; (ii) 6-week-old plants with flower buds at pre-anthesis; and (iii) 8-week-old plants with open flowers. After 3 d of chilling, the GB-treated plants retained 60–70% of their relative pre-stress PSII activity while the control plants retained only 50% (Fig. 2A). Based on their relative PSII activities, exogenous application of GB was more effective in protecting younger than older plants against chilling stress. For example, 4-week-old plants had about 10% higher PSII activity than did 8-week-old plants.

We also used ion leakage measurements to assess the extent of membrane damage caused by chilling stress. Regardless of age, exogenous GB applications reduced the severity of damage in all treated plants (Fig. 2B). Again, the protective





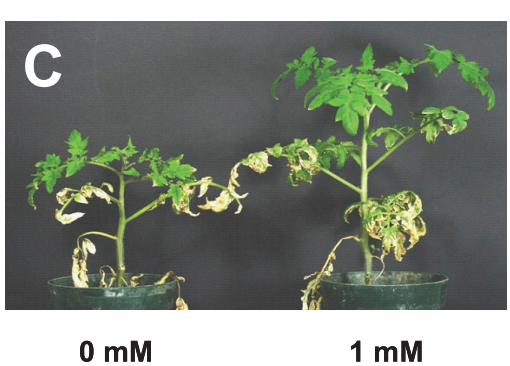
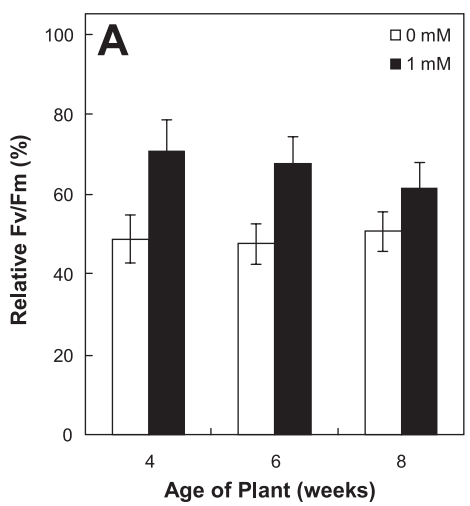


Fig. 1 Enhanced chilling tolerance in glycinebetaine (GB)-treated tomato plants. (A) Five-week-old plants were sprayed with either water (control) or solutions containing various concentrations of GB (0.1, 1.0 or 10.0 mM). One day after foliar application, plants were chilled at 3°C for 3 d, then placed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

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

GB treatment (mM)	GB			Intact chloroplasts (%)	GB in chloroplasts (%)
	Leaves		Isolated chloroplast		
	μmol g^{-1} FW	nmol mg^{-1} Chl	mol mg^{-1} Chl		
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

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



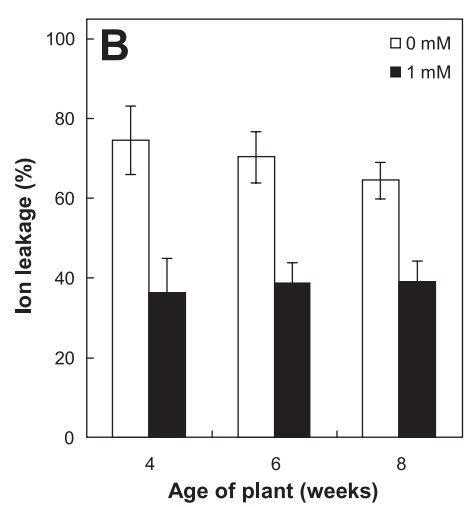


Fig. 2 Improved chilling tolerance in glycinebetaine (GB)-treated tomato plants at various developmental stages. Either water or 1 mM GB solution was foliar applied to 4-, 6- or 8-week-old plants. One day after GB treatment, chilling stress was given. PSII activity (A) and ion leakage (B) were measured at the end of treatment. Results are the mean ± SE from three independent experiments (3–5 plants per experiment).

effect of GB was most pronounced in younger plants, as evidenced by a nearly 50% decline in ion leakage compared with the control plants.

Beneficial effect of GB-enhanced chilling tolerance is diminished after 1 week

To evaluate how long the beneficial effect of GB could persist, we applied 1 mM GB to 5-week-old tomato plants at 1, 3 or 7 d before the beginning of the chilling treatment. At 3 d post-chilling, pre-treatments at both 1 and 3 d provided similar levels of protection to PSII activity (approximately 70%). In contrast, both the control and the plants treated at 7 d retained only about 50% of their original activity (Fig. 3).

Translocation of GB

To determine if the benefit of exogenously applied GB was limited to only the tissues actually treated, or whether it could be translocated to other tissues, we sprayed leaves of 7-

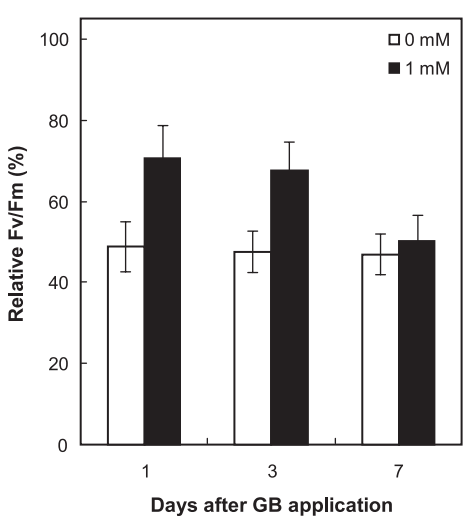


Fig. 3 Duration of glycinebetaine (GB)-enhanced chilling tolerance. Five-week-old plants were sprayed with either water or 1 mM GB. At 1, 3 or 7 d after GB pre-treatment, GB-treated plants were exposed to chilling stress for 3 d. PSII activity was measured at the end of the chilling period. Results are means ± SE from three independent experiments (3–5 plants per experiment).

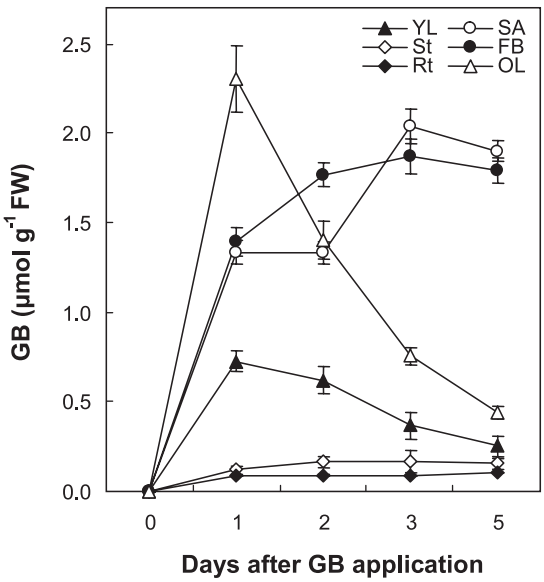


Fig. 4 Glycinebetaine (GB) levels in various organs of 7-week-old GB-treated plants. Means of three experiments; bars indicate standard errors. YL, young leaves (<1 cm in length); SA, shoot apices; St, stems; FB, flower buds; Rt, roots; OL, old leaves directly exposed to 20 mM GB solution.

week-old plants (with the first inflorescence containing unopened flower buds <0.5 cm in length) with 20 mM GB solution (Fig. 4). GB content in the treated leaves peaked at day 1 (2.2 µmol g^{-1} FW) but was rapidly reduced thereafter. By day 5, leaf content was reduced to only about 20% of the amount at day 1. Concomitant with this decrease, GB contents increased in other organs, thereby indicating that the GB was absorbed by the directly treated leaves and readily transported throughout the plant. The highest GB levels were found in the shoot apices (2.0 µmol g^{-1} FW) and flower buds (1.9 µmol g^{-1} FW) at day 3 after GB application (Fig. 4), and showed little change in those organs from day 3 to day 5. In young leaves, the GB level reached 0.7 µmol g^{-1} FW at day 1, then declined gradually from day 2 to day 5. The lowest amounts of GB were found in the stem and roots, and remained unchanged throughout the experimental period.

Localization of GB inside the cell

The photosynthetic apparatus is one of the most susceptible to cold stress; the state of the PSII system after exposure to low temperatures is a very good indicator of plant chilling tolerance. We reasoned that some of the GB had to reach the chloroplasts in order to exert any protective effect on that system. Therefore, we quantified GB contents in isolated chloroplasts of treated plants. Various levels of GB accumulated at 1 d after foliar application, depending on the concentration used in the spray solution (Table 1). Although total GB contents in the leaves ranged from 7.8 to 900.5 nmol mg^{-1} Chl, only a limited amount was detected in the chloroplasts (0.98–3.20 nmol mg^{-1} Chl). Therefore, only about 0.6–22.0% of the total GB found in leaves was localized in chloroplasts.

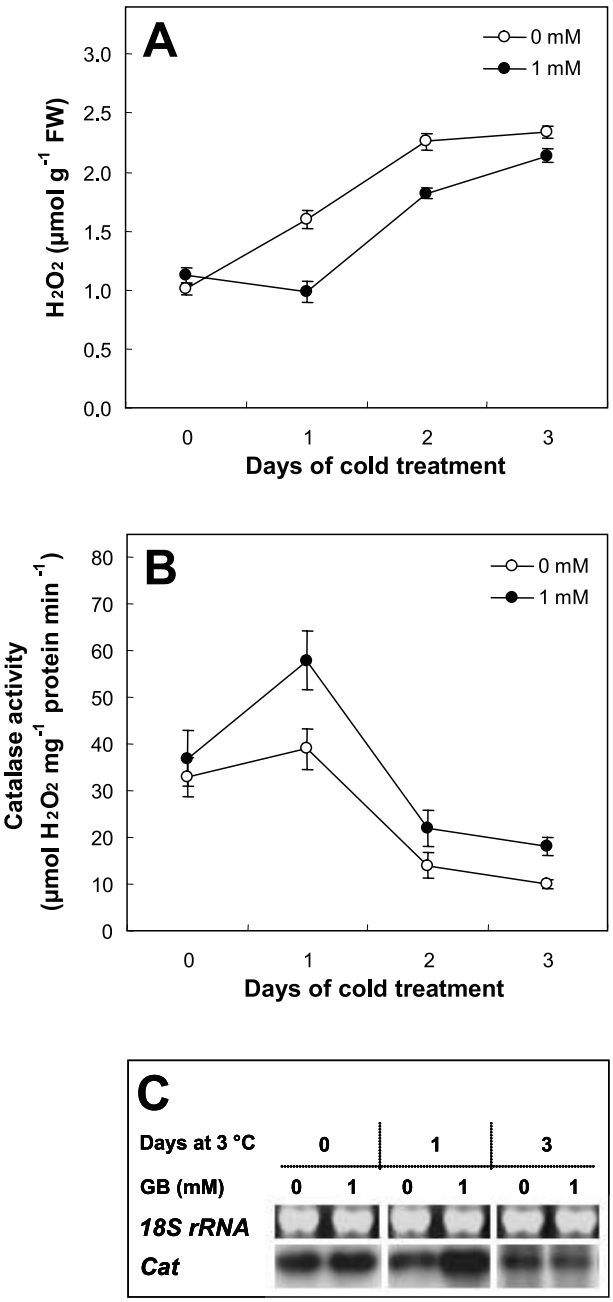


Fig. 5 Effects of glycinebetaine (GB) on levels of hydrogen peroxide (A), catalase activity (B) and catalase gene (*CAT1*) expression (C), as determined by Northern blot analysis. Either water or 1 mM GB solution was foliar applied to 5-week-old plants. One day after GB treatment, plants were chilled at 3°C for 3 d. A 20 µg aliquot of total RNA was loaded for each lane. The hybridization probe (*CAT1*) was ^{32}P labeled.

Exogenous application of GB increases catalase activity, catalase gene expression and levels of H_2O_2

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

Under non-stress conditions, H_2O_2 levels were about 10% higher in the GB-treated than in the control plants (day 0; Fig. 5A). On day 1 of the chilling stress, levels of H_2O_2 in the controls rose by about 50%, whereas those in the treated plants remained unchanged. A steady increase in H_2O_2 levels was observed in both the GB-treated and the control plants until day 3 (Fig. 5A). After 3 d of chilling stress, H_2O_2 contents in the latter were 2.4-fold higher than their original values (day 0), whereas the former accumulated up to 2.0-fold more H_2O_2.

Catalase activity, under non-stress conditions (day 0), was 12% higher in GB-treated than in control plants (Fig. 5B). During day 1 of chilling stress, it increased by up to 57 and 18% in the treated and control plants, respectively (Fig. 5B). Thereafter, catalase activity decreased very rapidly for both plant types. At the end of the treatment period (day 3), activity in the GB-treated and control plants was reduced to 38 and 36%, respectively, of their initial values (day 0). Nevertheless, activity in GB-treated plants remained higher compared with the control plants (Fig. 5B).

The expression pattern for the catalase gene (*CAT1*) was well correlated with its enzyme activity (Fig. 5C). Transcript levels increased in the GB-treated plants under non-stress conditions (day 0), reaching their highest point at day 1 of chilling stress, then falling below the values observed at day 0.

Discussion

Although tomato plants do not naturally accumulate GB (Wyn Jones and Storey 1981), we have demonstrated here that GB is taken up readily when foliar applied (Table 1 and Fig. 4). These plants accumulated various levels in their leaves (0.09–10.88 μmol g^{-1} FW) depending on the concentration applied (0.1–10.0 mM; Table 1), and also exhibited enhanced tolerance to chilling stress at different stages of development (Fig. 1 and 2). In natural GB-accumulator plants, accumulation is induced under stress conditions, 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 those plants (Fig. 1), even though plants sprayed with 10 mM GB accumulated 100-fold higher levels of endogenous GB than those treated with 0.1 mM GB (Table 1). This result agrees with that from our earlier work, which focused on GB-accumulating transgenic tomato plants (Park et al. 2004). There, transgenic chloroplast-targeted *codA* plants accumulated GB in their leaves at levels from 0.09 to 0.30 μmol g^{-1} FW, and also 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. Plants are more efficiently protected from abiotic stress when the chloroplast-targeted *codA* gene is used and GB accumulates inside the chloroplasts rather than within the cytosol (Sakamoto et al. 1998). Moreover, chloroplast GB levels are correlated with the degree of stress tolerance conferred (Park et al. 2004). In transgenic rice, the photosynthetic machinery is also better protected against salt and cold stresses when plants are transformed with the chloroplast-targeted *codA* gene than with the non-targeted *codA* gene, the product of which is mainly localized in the cytosol, even though the latter genotype can accumulate up to five times more GB (Sakamoto et al. 1998).

We also have reported previously that chloroplast-targeted *codA* transgenic tomato plants accumulate up to 86% of the GB in their leaf chloroplasts, and that the highest correlation between GB content and level of chilling tolerance is manifested in the protection of PSII (Park et al. 2004). However, in the current study, the proportion of GB in the chloroplasts of treated plants accounted for only up to 22% of the total leaf content when 0.1 mM GB was applied. The amount of GB in chloroplasts, however, did not vary as much as the total GB content found in leaves, regardless of the concentration of GB used (Table 1). In salt-stressed spinach plants, GB originating from other subcellular compartments does not enter the chloroplasts during the isolation procedure (Robinson and Jones 1986). Thus, the GB content measured in isolated chloroplasts does not result from contamination by extra-chloroplastic GB. However, GB may leak out of the chloroplast during the isolation procedure (Robinson and Jones 1986). Therefore, GB content in isolated chloroplasts should be considered as a minimal estimate of its concentration in vivo. The actual in vivo GB concentration in chloroplasts could be up to 1.5 times higher than that determined in isolated chloroplasts (Robinson and Jones 1986).

The concentration of GB in the chloroplasts isolated from GB-treated tomato plants ranged from 0.98 to 3.2 nmol mg^{-1} Chl depending on the concentration of exogenous GB applied (Table 1). These concentrations are much lower, even when corrected by a factor of 1.5 to account for loss during chloroplast isolation, than those found in natural GB-accumulators. In spinach, for example, the concentration of GB in the chloroplasts isolated from control and salt-stressed plants was 0.67 and 6.65 μmol mg^{-1} Chl, respectively (Robinson and Jones 1986). Such a large concentration gradient across the chloroplast envelope suggests the existence of a specific transport mechanism. Although little is known about the transport of compatible solutes in plants, Schwacke et al. (1999) demonstrated that the product of the tomato gene *LeProT1*, a homolog of an *Arabidopsis* proline transporter, transported proline and γ-aminobutyric acid with low affinity and GB with high affinity

when expressed in yeast. Apparently, in GB-treated tomato plants, the transport of GB from cytosol to chloroplasts is very inefficient (Table 1). Nevertheless, this small amount of GB in chloroplasts might be sufficient to confer full protection against chilling stress.

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 of the photosynthetic machinery to salt and chilling stresses (Kishitani et al. 1994, Takabe et al. 1998, Holmstrom et al. 2000). Our result demonstrates that exogenously applied GB is mostly localized in the cytosol, and that only a limited amount is translocated to the chloroplasts. Therefore, it is possible that both foliar-applied GB and GB that is synthesized in the cytosol can be transported into those aforementioned cell compartments. Thus, even a small amount of GB may be sufficient to confer protection against chilling stress.

When GB was foliar applied to our tomato plants, large amounts of GB were translocated into meristem-containing tissues, including the flower buds and shoot apices (Fig. 4). Differing levels of GB in various plant organs indicated active and possibly regulated translocation and accumulation from the original site of application. 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). Our GB-treated plants also showed improved growth rates during their post-chilling recovery period (Fig. 1B), indicating that a higher concentration of GB in the shoot apices may either reduce the level of injury during chilling or enhance the recovery following an episode of chilling stress.

Exogenous application of GB also results in higher yields in the greenhouse and field, mainly due to improved net photosynthesis (Mäkelä et al. 1998, Jokinen et al. 1999). GB stabilizes the oxygen-evolving PSII complex by stimulating its repair when plants are exposed to various stresses (Papageorgiou and Murata 1995, Hayashi et al. 1997, Alia et al. 1999, Holmstrom et al. 2000, Sakamoto and Murata 2000). 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 PSII repair by inhibiting de novo protein synthesis (Nishiyama et al. 2001). In this study, GB-treated plants exhibited increased levels of PSII activity compared with control plants (Fig. 1A, 2A). In addition, GB stabilizes membrane integrity against extreme temperatures (Zhao et al. 1992, Gorham 1995), reduces membrane lipid peroxidation (Chen et al. 2000) and protects complex II electron transport in the mitochondria (Hamilton and Heckathorn 2001). We used ion leakage measurements to assess the extent of membrane damage caused by chilling stress and found that exogenous GB application significantly reduced the severity of damage in GB-treated plants compared with controls (Fig. 2B).

Greater accumulation in the reproductive organs of GB-accumulating transgenic plants is also associated with enhanced tolerance to salt and chilling stresses, again prompting high crop yields (Sulpice et al. 2003, Park et al. 2004). Therefore, greater accumulation of GB in the meristematic tissues might further increase tolerance of those organs in treated plants, thereby improving their growth rates after exposure to low temperatures. However, such translocation of GB probably causes a decrease in its level in the leaves of treated plants, which consequently diminishes the beneficial effect. For our plants, a pre-treatment with GB at 7 d before the start of the chilling treatment did not enhance PSII activity compared with the control, whereas those sprayed with GB at 1 or 3 d before chilling had higher PSII activities than the control plants (Fig. 3). Therefore, our result provides a motive for introducing a biosynthetic GB pathway into tomato plants by genetic engineering in order to enhance chilling tolerance without requiring frequent and timely exogenous applications of GB.

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 (Fig. 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 in mitochondria. Glycolate from the chloroplasts diffuses to the peroxisome where it is oxidized to glyoxylate by a glycolate oxidase-mediated reaction that yields H_2O_2. Therefore, it is very likely that the GB absorbed by our tomato plants might also have increased glycolate oxidase activity, resulting in a higher accumulation of H_2O_2 in GB-treated than in control plants.

Following chilling, treated plants maintain lower levels of H_2O_2 than do control plants, even though GB is known to be ineffective in scavenging ROS (Smirnoff and Cumbes 1989). Thus, the increased tolerance to oxidative stress is probably an indirect effect of GB, such as through the induction of catalase. H_2O_2 is a secondary messenger in plants (Neill 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 the expression of antioxidant enzymes, e.g. catalase 3, 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, Park et al. 2004). We also observed greater levels of both catalase gene expression and catalase activity under non-stress conditions (Fig. 5B, C). Furthermore, catalase activity in GB-treated plants increased by 57% compared with a rise of only 18% in

our control plants during day 1 of chilling (Fig. 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 have suggested that such enhanced activity might result from the increased synthesis of catalase protein induced by elevated H_2O_2 production during salt stress. This phenomenon has been observed in a number of transgenic plants transformed with the *codA* gene for choline oxidase, which gives H_2O_2 as a by-product of that reaction (Alia et al. 1999, Park et al. 2004, Prasad and Saradhi 2004). All of those plants have higher levels of H_2O_2 and catalase activity, suggesting that maintaining H_2O_2 at a particular non-toxic threshold level in *codA* transgenic plants, as well as the increased H_2O_2 content in GB-treated plants, might induce the expression of genes responsible for enzymatic detoxification of H_2O_2.

Likewise, treatments that cause H_2O_2 production also increase the levels of both *cat3* transcripts and catalase 3 activity, resulting in higher survival and growth rates upon exposure to chilling stress (Prasad et al. 1994). Previously, it has been reported that overexpression of the *Arabidopsis CBF1* (CRT/DRE-binding factor1) gene in tomato enhances chilling, oxidative and water stress tolerance. This was associated with increased levels of catalase activity and *CAT1* expression, suggesting that enhancement of tolerance to various stresses in these transgenic tomatoes may be partially, if not solely, due to the induction of the *CAT1* gene (Hsieh et al. 2002a, Hsieh et al. 2002b). We also observed that GB applications elevated expression of the catalase gene correspondent to its enzyme activity (Fig. 5). Therefore, it is likely that the induced tolerance conferred by exogenously applied GB results from H_2O_2-mediated induction of antioxidant mechanisms that include enhanced catalase gene expression and increased 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 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 overexpressed in response to GB application (data not shown).

In conclusion, exogenously applied GB is effectively taken up through the tomato leaf surface and then translocated into different organs. A relatively low endogenous level of GB (>0.09 μmol g^{-1} FW) in treated plants can provide sufficient protection against chilling temperatures. The majority of the GB is localized to the cytosol, with only a small amount being translocated into the chloroplasts and probably other subcellular compartments. This action effectively protects the photosynthetic apparatus and enhances chilling tolerance in treated plants. Interestingly, large amounts of GB can be found in meristematic tissues, including the shoot apices and flower buds of treated plants. We believe that the high levels in these tissues may be critical for plant survival and enhanced recovery of growth after release from chilling temperatures. Finally, exogenous, foliar application of GB increases levels of H_2O_2, catalase gene expression and catalase activity. In addition to a possible direct protective effect on macromolecules such as membranes and proteins, it is likely that the induced chilling tolerance conferred by exogenously applied GB may result from the induction of H_2O_2-mediated antioxidant mechanisms, e.g. enhanced catalase expression and catalase activity.

Materials and Methods

Plant material, GB application and chilling treatment

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

Exogenous, foliar application of GB was carried out by spraying 3–5 plants per treatment with a solution of GB (0.1, 1.0 or 10.0 mM). Control plants were sprayed with water only. Tween-20 [0.005% (v/v)] was included as a wetting agent in all treatments. Following the foliar applications, both control and GB-treated plants were kept in the dark for 24 h (day 0), then transferred to a cold growth chamber (3 ± 0.5°C, 16 h photoperiod, 50 μmol m^{-2} s^{-1}) for 3 d. Afterwards, all chilled plants were allowed to recover in a greenhouse at 25°C.

GB treatment at different developmental stages

To evaluate the effectiveness of GB at different developmental stages, tomato plants were sprayed with a 1.0 mM solution of GB at three distinct growth periods: (i) 4-week-old non-flowering plants with 6–7 compound leaves; (ii) 6-week-old plants with flower buds at pre-anthesis; and (iii) 8-week-old plants with open flowers. After the chilling treatment, PSII activity and ion leakage were used to determine the degree of chilling tolerance.

Duration of the beneficial effect of GB

To evaluate the duration of the protective effect of GB against low temperatures, 5-week-old plants were sprayed with a 1.0 mM GB solution at 1, 3 or 7 d before the start of the chilling period. After the chilling treatment, PSII activity was used to evaluate the degree of tolerance.

Translocation of GB

For GB translocation analysis, 20 mM GB solution was applied by spraying only the second or third leaf from the bottom of 7-week-old plants that had flower buds in the first inflorescence pre-anthesis. Other plant parts were covered with plastic wrap to prevent any accidental or cross-contamination with GB. Treated plants were kept in the dark for 24 h before being transferred to the greenhouse (25 ± 3°C, 16 h photoperiod, 400–500 μmol m^{-2} s^{-1}). The concentration of GB in different organs—young leaves (<1 cm in length), shoot apices, stems, flower buds, roots, and the second or third leaf from the bottom—was determined daily via HPLC over a 5 d period. In this experiment, a higher concentration of GB (20 mM) was used to ensure that the concentrations of translocated GB in different organs tested were above the detection limit (0.1 μmol g^{-1} FW) of our HPLC-based detection system.

GB determination

Extraction and quantification of GB by HPLC was carried out as described by Park et al. (2004). To prevent any carry-over contamina-

tion, samples were first thoroughly washed with distilled water to remove any remaining GB from their surfaces.

Chloroplast isolation

After the GB-treated plants were held in the dark for 24 h, their intact chloroplasts were isolated with a Chloroplast Isolation Kit (Sigma, St Louis, MO, USA). The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. The total Chl concentration was determined in 80% (v/v) acetone according to the manufacturer's instructions. GB content was obtained by HPLC as described by Park et al. (2004). The percentage of GB in the chloroplasts was calculated by dividing the amount of GB in isolated chloroplasts by the total GB in the leaves, then further dividing this value by the percentage of intact chloroplasts.

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, washed three times with distilled water and then stored at –80°C. Protein extraction and catalase activity determination were carried out as described by Alia et al. (1999); H_2O_2 levels were determined as described by Park et al. (2004).

Measurement of chlorophyll fluorescence

Chl fluorescence was measured at the end of the dark treatment period as described by Park et al. (2004). Its induction was recorded at room temperature using a pulse-modulated Fluorescence Monitoring System (FMS1; Hansatech, Norfolk, UK). After adaptation in the dark for 20 min, the ratio of variable to maximum fluorescence (F_v/F_m) was calculated. Chl fluorescence was measured with five of the third to fifth compound leaves. The percentage of F_v/F_m was calculated based on 100% of the values obtained under non-stressful conditions.

Ion leakage assay

Three leaf discs (1 cm diameter) 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 Spring Instrument, Yellow Spring, OH, USA). 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.

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, USA). Gel electrophoresis and blotting of RNA to a Nytran nylon membrane (Schleicher and Schuell, Keene, NH, USA) were performed as described by Skinner and Timko (1998). RNA blots were probed and washed using an Ultrahyb Solution, following the manufacturer's instructions (Ambion, Austin, TX, USA). For Northern analysis, a cDNA probe fragment for tomato catalase (*CAT1*; accession no. M93719) was amplified by reverse transcription–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, USA).

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