Glycine betaine improves thylakoid membrane function of tobacco leaves under low-temperature stress

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Abstract

Glycine betaine (GB) is an effective compatible solute that improves the tolerance in plants to various stresses. We investigated the effects of 2 mM GB applied to the roots of a tobacco (*Nicotiana tabacum* L.) cultivar on enhancing photosynthesis under low-temperature (LT) stress (5/5 °C, 12/12 h, 300 μmol m^{-2} s^{-1}) and in the subsequent recovery (25/18 °C) from the stress. The net photosynthetic rate, intrinsic efficiency measured as the ratio of variable to maximum fluorescence, and actual efficiency of the photochemistry of photosystem 2 as well as the ATPase activity in the thylakoid membrane decreased, and a distinct K step in the fluorescence transient O-J-I-P appeared under cold stress. Exogenous GB alleviated the decrease in all these parameters. The LT-stress induced the accumulation of 33–66 kDa polypeptides and decreased the proportion of unsaturated fatty acids in the thylakoid membrane. In plants subjected to LT-stress, GB protected these polypeptides from damage and enhanced the proportion of unsaturated fatty acids. An increase in non-radiative energy dissipation (NPQ) may be involved in the improvement of the function of the thylakoid membrane by GB since exogenous GB protected violaxanthin de-epoxidase and enhanced NPQ.

Additional key words: ATPase; chlorophyll fluorescence induction; fatty acids; net photosynthetic rate; peptides; *Nicotiana*; violaxanthin de-epoxidase; xanthophyll cycle.

Introduction

Photoinhibition—the interruption of photosystem 2 (PS2) activity under intense irradiance—is caused by an imbalance between the rate of PS2 photo-damage and repair, especially under stress (Nishiyama *et al*. 2006). To avoid or minimize photoinhibition, photosynthetic organisms have evolved several strategies (Niyogi *et al*. 2001) that can be distinguished as short- and long-term responses; these strategies are aimed at diminishing over-excitation of the reaction centres to facilitate the dissipation of excess absorbed photon energy. In long-term responses, a long-term exposure to high irradiance induces modulation of gene expression, leading to a reduction in the amount of chlorophyll (Chl) and the number of light-harvesting complex (LHC) proteins in the photosynthetic apparatus. This in turn results in the assembly of smaller-size functional light-harvesting Chl antenna in the chloroplast thylakoids and effectively diminishes the over-excitation of the PSs (Jin *et al*. 2003). In the short-term response, within few minutes after plant exposure to excessive irradiance, an irradiance-dependent xanthophyll cycle is activated. This activation involves the reversible de-epoxidation of violaxanthin (V) and the formation of zeaxanthin (Z) *via* antheraxanthin (A); these reactions are catalyzed by violaxanthin de-epoxidase (VDE) in the thylakoid membrane. In this process, VDE is the key enzyme of the xanthophyll cycle and plays an important role in protecting the photosynthetic apparatus from the damaging effects of excessive radiant energy. Therefore, enhancing plant resistance by improving the xanthophyll cycle under stress is one of the focuses of stress physiology research (Maren *et al*. 2007).

Received 4 January 2008, *accepted* 9 May 2008.
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Abbreviations: A – antheraxanthin; Chl – chlorophyll; DGDG – digalactosyl diacylglycerol; DTT – 1,4-dithiothreitol; F_v/F_m – intrinsic efficiency of PS2 photochemistry; GB – glycinebetaine; IUFA – index of unsaturated fatty acids; LHC – light-harvesting complex; LT – low temperature; MGDG – monogalactosyl diacylglycerol; NPQ – non-radiative energy dissipation; OEC – oxygen evolving complex; P_N – net photosynthetic rate; PG – phosphatidylglycerol; PS – photosystem; SQDG – sulfoquinovosyldiglyceride; V – violaxanthin; VDE – violaxanthin de-epoxidase; Z – zeaxanthin; Φ_{PS2} – actual efficiency of PS2 photochemistry.
Acknowledgments: This work was supported by National Natural Science Foundation of China (No.30671259) and by the Tobacco Supervising Organization of Shandong province in China (KN77).

Glycine betaine (GB) is a naturally occurring compatible solute, which is widely distributed in plants, animals, and bacteria (Prasad and Saradhi 2004). Demiral and Türkan (2004) reported that GB protects higher plants against stresses not only through osmotic adjustments but also by stabilizing many functional units such as the oxygen evolving complex (OEC) of PS2, cell membranes, quaternary structures of complex proteins, and enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase. Barley (Nomura *et al*. 1995) and wheat (Ma *et al*. 2006a) are natural accumulators of GB; they improve plant resistance by GB over-accumulation under stress. The resistance of some GB non-accumulators such as tobacco (Quan *et al*. 2004) and tomato (Park *et al*. 2007) could be improved by transferring the GB synthesis gene or by applying exogenous GB. However, most studies have focused on the physiological role of GB in the anti-oxidative system (Raza *et al*. 2007), PS2 function (Allakhverdiev *et al*. 2003), and biosynthesis pathways (Chen and Murata 2002). The systemic and comprehensive effects of GB on thylakoid membrane function under low-temperature (LT) stress have not been adequately analyzed yet. Therefore we used tobacco plants, which are a cold-sensitive and non-GB accumulating species, to study the mechanisms underlying the involvement of root-applied GB in the protection of photosynthetic apparatus of thylakoid membranes.

Materials and methods

Plant growth: Tobacco (*Nicotiana tabacum* L.) seeds were germinated in a plastic tray containing a mixture of organic fertilizer and soil. After the expansion of the 4th leaf, the seedlings were transplanted into earthen pots (height 20 cm; diameter 15 cm) containing a 1.5-kg mixture of organic fertilizer and soil. The plants were grown normally for 60 d in a greenhouse (25/18 °C, 300 μmol m^{-2} s^{-1}) until the 7th leaf expanded.

GB application: Before exposing the plants to cold stress, 2 mM aqueous GB mixed with Hoagland solution was applied to the plant roots for 3 d (C+GB); control (C) received a mixture of de-ionized water and Hoagland solution.

LT-stress treatment: After GB application, some of the GB-treated and non-GB-treated plants were exposed to LT-stress by growing them in an artificial chamber (5/5 °C, 12/12 h, 300 μmol m^{-2} s^{-1}) for 5 d; the plants were subsequently recovered (25/18 °C, 12/12 h, 300 μmol m^{-2} s^{-1}) for 3 d. The plants were divided into 4 groups: (*1*) C = moderate temperature and no GB pre-treatment; (*2*) LT = LT-stress and no GB pre-treatment; (*3*) C+GB = moderate temperature and GB pre-treatment; and (*4*) LT+GB = LT-stress and GB pre-treatment.

GB determination: Leaves at the same position in each plant were used in all experiments. The GB content in these leaves was determined by high-performance liquid chromatography (HPLC) (*Shimadzu-LC-6A; Shimadzu*, Japan) using the procedure described by Ma *et al*. (2007).

Photosynthetic rate: A portable infrared gas analyzer (*CIRAS-2; PP Systems*, UK) was used to estimate the net photosynthetic rate (P_N). The external air was scrubbed of CO_2 and mixed with pure CO_2 to achieve a reference concentration of 360 μm^3 m^{-3}, and the irradiance was set at 800 μmol m^{-2} s^{-1} (greater than the saturating irradiance of the tobacco leaves, *i.e*. 600 μmol m^{-2} s^{-1}). The temperature inside the leaf chamber was maintained at 25 °C.

Chl *a* fluorescence: The intrinsic efficiency (F_v/F_m), actual efficiency (Φ_{PS2}), and non-radiative energy dissipation (NPQ) of PS2 of the same tobacco leaves, which were used for GB and P_N determination, were measured with a portable pulse-modulated fluorometer *FMS-2* (*Hansatech Instruments*, UK). These measurements were performed after the leaves had been irradiance-adapted (800 μmol m^{-2} s^{-1}) for 30 min in a leaf-clip connected to the fluorometer (Jiang *et al*. 2003).

Thylakoid membrane Ca^{2+}-ATPase and Mg^{2+}-ATPase activities: Chloroplasts were isolated according to Zhao *et al.* (2007). Fresh leaves (2 g) were homogenized in an ice-cold isolation buffer containing 0.4 M sucrose, 15 mM *Tricine* (pH 7.8), and 5 mM $MgCl_2$ (buffer *A*) in a tissue homogenizer. The homogenate was filtered through 4 layers of gauze and centrifuged at 3 000×*g* at 4 °C for 5 min. The supernatants and most of the loose pellets were discarded. The remaining chloroplast deposit was suspended in buffer *A* for further use.

The activity of coupling factor (ATPase) in the thylakoid membrane is generally determined according to the activity of ATP hydrolase that hydrolyzes ATP after being activated by ions; these two ATPase activities are classified into 2 types according to the type of ions used, *i.e*. Mg^{2+}- and Ca^{2+}-dependent ATPase, respectively (Ma *et al*. 2006b).

Ca^{2+}-ATPase activity was determined following the activation of the coupling factor by trypsinase according to Zhao *et al*. (2007). The chloroplast suspension (1 cm^3) was added to 1 cm^3 of a medium containing 250 mM *Tricine* (pH 8.0), 20 mM ethylene diamine tetraacetic acid (EDTA), 10 mM ATP, and 2 mg cm^{-3} trypsinase, and incubated at 20 °C for 10 min. Then, 0.1 cm^3 bovine serum albumin (10 mg cm^{-3}) was added to the mixture to terminate the reaction. Next, 0.5 cm^3 of the incubated chloroplast suspension was added to 0.5 cm^3 of the reaction mixture containing 500 mM *Tricine* (pH 8.0), 10 mM ATP, and 50 mM $CaCl_2$. The mixture was incubated at 37 °C for 10 min and centrifuged at 3 000×*g*

for 1 min; the resulting supernatant was used to determine the content of inorganic phosphorus.

For the measurement of Mg^{2+}-ATPase activity, 1 cm^3 of the chloroplast suspension was added to 1 cm^3 of a medium containing 250 mM *Tricine* (pH 7.0), 500 mM NaCl, 50 mM $MgCl_2$, and 50 mM 1,4-dithiothreitol (DTT), and incubated at high irradiance at 25 °C for 5 min; 0.1 cm^3 bovine serum albumin (10 mg cm^{-3}) was added to the mixture to terminate the incubation. Incubated chloroplast suspension (0.5 cm^3) was then added to 0.5 cm^3 reaction mixture containing 500 mM *Tricine* (pH 8.0), 50 mM ATP, and 50 mM $MgCl_2$. The reaction mixture was incubated at 37 °C for 10 min and centrifuged at 3 000×*g* for 1 min; the supernatant was used to determine the content of inorganic phosphorus (Zhao *et al.* 2007).

Analysis of O-J-I-P Chl *a* fluorescence induction transients, JIP-test: Chl *a* fluorescence was measured at 25 °C using a plant efficiency analyzer (*PEA; Hansatech*, UK) using an excitation irradiance of 3 000 μmol m^{-2} s^{-1}. Fluorescence transients were recorded during a 60-s pulse generated by an array of 6 light-emitting diodes. Fluorescence was measured using a PIN-photodiode after passing the emitted fluorescence through a long-pass filter. All fluorescence transients were recorded in a time span ranging from 10 μs to 1 s with a data acquisition rate of 10 μs for the first 2 ms and a resolution of 12 bits. After 2 ms, the instrument automatically switches to a slow digitization rate. The JIP-test was employed to analyze the fluorescence transient for each Chl *a*. The following data from the original measurements were used: maximum fluorescence intensity (F_m); fluorescence intensity at 50 μs considered as the minimum intensity (F_0); and fluorescence intensity at 300 μs (K-step), at 2 ms (J-step), and at 30 ms (I-step). The relative variable fluorescence (V_t) at 300 μs (K-step), 2 ms (J-step), and 30 ms (I-step) were calculated using the formula $V_t = (F_t - F_{50\mu s})/(F_m - F_{50\mu s})$ according to Jiang *et al*. (2003).

Analysis of thylakoid polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: Chloroplasts were prepared according to Zhao *et al*. (2007). The thylakoid membranes were prepared according to the modified procedure of Nakatani and Barber (1977). The chloroplast suspension was centrifuged at 1 200×*g* at 4 °C for 10 min. The supernatants and most of the loose pellets were discarded; the deposit was washed in buffer containing 10 mM tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$ (buffer *B*) and centrifuged at 4 800×*g* and 4 °C for 10 min. The deposit containing the thylakoid membranes was suspended in a buffer containing 0.1 M saccharose, 10 mM *Tricine* (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$ (buffer *C*) and the final Chl concentration was adjusted to 1.7 kg m^{-3}. The thylakoid membranes were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Parida *et al*. (2003).

Analysis of index of unsaturated fatty acids in thylakoid membrane: Polar lipids [monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), and phosphatidyl glycerol (PG)] were extracted from the thylakoid membrane pellet using a chloroform/methanol mixture (2 : 1, v/v) and then separated by two-dimensional thin-layer chromatography (TLC) according to the modified method of Droppa *et al*. (1987). One-dimensional TLC was performed using acetone/benzene/water (91 : 30 : 8, v/v/v) as the solvent; two-dimensional TLC was then performed using chloroform/methanol/ammonia (130 : 70 : 10, v/v/v). After the second extraction using benzene/petroleum ether (1 : 1, v/v), the combined extracts were esterified with 0.4 M NaOH. The concentration of fatty acid methyl esters was determined by a gas chromatograph analyzer (*GC-9A; Shimadzu*, Japan) by using a methyl esterified arachidic acid as an internal standard. The conditions for GC were as follows: glass column, 2 m×3 mm; *Chromosorb W. AW. DWCS,* 80–100 mesh; solid phase, 15 % diethylene glycol succinate (DEGS); column temperature, 190 °C; detector temperature, 290 °C; and pneumatophore pure N_2 with flow velocity of 100 cm^3 min^{-1}. Quantification was performed after normalization by using the processing software of the apparatus. The index of unsaturated fatty acids (IUFA) in MGDG, DGDG, SQDG, and PG was calculated using the following formula: IUFA [mol%] = $(C_{16:1} + C_{16:1t} + C_{18:1} + 2\,C_{18:2} + 3\,C_{18:3}) \times 100$ (Zhao *et al*. 2007).

DTT treatment *in vivo*: Leaves along with their stalks at the same position in each group were immersed in 6 mM DTT at low irradiance (10 μmol m^{-2} s^{-1}) for 6 h. According to Darkó *et al.* (2000), the concentration of *in vivo* absorbed DTT by transpiration was 2 mM.

(A+Z)/(V+A+Z) assay: The proportion of components of the xanthophyll cycle, *i.e*. V, A, and Z, was determined by HPLC (*LC-6A; Shimadzu*, Japan) performed according to the procedure of Thayer and Björkman (1990). A *Spherisorb C185 u* 10-μm column (4.6×250.0 mm) was used for gradient elution. The mobile phase A was a mixture of acetonitrile, methanol, and tricine (72 : 8 : 3, v/v/v) and mobile phase B was a mixture of methanol and hexane (5 : 1, v/v). The xanthophyll components were detected by a UV detector at 440 nm and quantified by comparing their relative peak surface areas.

VDE activity assay: The extraction of VDE was performed according to the method of Bugos *et al*. (1999) with some modifications. Chloroplasts were suspended in an STN buffer containing 20 mM HEPES (pH 7.5) and 6 mM $MgCl_2$ at 4 °C for 30 min. The suspension was centrifuged at 20 000×*g* at 4 °C for 10 min; the deposit (thylakoid membranes) suspended in STN buffer was

freeze-thawed 5 times. The suspension was then centrifuged at 12 000×*g* at 4 °C for 10 min; the resulting supernatant was a crude extract of VDE. The enzyme activity was determined using a UV-spectrophotometer (*UV-1601; Shimadzu,* Japan) at 410 nm. The enzyme extract (10 cm^3) was added to a mixture containing 100 μM V, 270 μM MGDG, 0.2 mM sodium citrate (pH 5.1), and sodium ascorbate (1 cm^3). One unit of enzyme activity was defined as the amount of enzyme that digested 1 μmol of V per time unit (Latowski *et al*. 2000).

Statistical analysis: All experiments were repeated at least 3 times. All data obtained were subjected to analysis of variance, and the differences between the mean values were compared by a least significant difference (LSD) test at an α-level of 0.05. Statistical analyses of the data were performed with *SAS* version *9.0*.

Results

Effects of root-applied GB on the GB content of tobacco seedling leaves: Under both moderate temperature and cold stress, almost no GB accumulation was observed, but the application of 2 mM GB to the roots significantly increased the GB content of the leaves [mmol kg^{-1}(dry mass)]: C 1.33±4.16b, C+GB 953.33±50.33a, LT 35.67±5.13b, LT+GB 983.43±35.12a (p<0.05). These results were consistent with those of Ma *et al*. (2007), who reported the role of exogenous GB in the improvement of drought tolerance in tobacco plants.

Effects of root-applied GB on the photosynthetic apparatus of tobacco leaves: Under moderate temperature, root-applied GB had small effects on P_N of the leaves (Fig. 1). The P_N was significantly reduced (p<0.05) by cold stress; it then increased to the level typical for moderate temperature (C) after 3 d of recovery. The P_N in the LT+GB group was higher than that in the LT group, suggesting that GB pre-treatment had a positive effect in tobacco leaves (p<0.05).

GB did not have any effect on the F_v/F_m and Φ_{PS2} under moderate temperature (Fig. 2*A,B*); however, F_v/F_m and Φ_{PS2} decreased markedly at LT (p<0.05) and then increased to the level typical for moderate temperature conditions (C) after 3 d of recovery. From days 3 to 5, the F_v/F_m and Φ_{PS2} of the LT+GB group were obviously higher than those of the LT group both during stress and after recovery for 1 d.

Under moderate temperature, GB did not have any effect on the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the thylakoid membrane (Fig. 2*C,D*). The activities of both ATPases decreased with the maintenance of LT-stress but increased to more than the level typical for moderate temperature (C) after 3 d of recovery. The activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the LT+GB group were higher than those in the LT group during stress treatment (p<0.05) and recovery.

As shown in Fig. 3, scarcely any difference in V_t was observed between the C and CK+GB groups at moderate temperature. In contrast, two important observations were made regarding the effect of GB on the O-J-I-P fluorescence transients in the LT and LT+GB groups after a 5-d exposure to LT: (*1*) The shapes of the O-J-I-P fluorescence transients recorded in the LT and LT+GB groups differed from those recorded in the C and C+GB groups, and (*2*) a conspicuous difference was observed between the absent and present stress conditions with regard to the extent to which the shape of the transients was altered by LT, especially the appearance of the K step (*ca*. 300 μs) and the increase in V_J (*ca*. 2 ms). LT-stress resulted in a considerably large increase in the fluorescence intensity at the K and J steps in the LT group than that in the LT+GB group. Hence under LT, electron transport in PS2 was inhibited to a greater extent in the LT group than in the LT+GB group, and the O-J-I-P fluorescence transient was sensitive enough to reveal these differences in the response to LT-stress (Strauss *et al*. 2006).

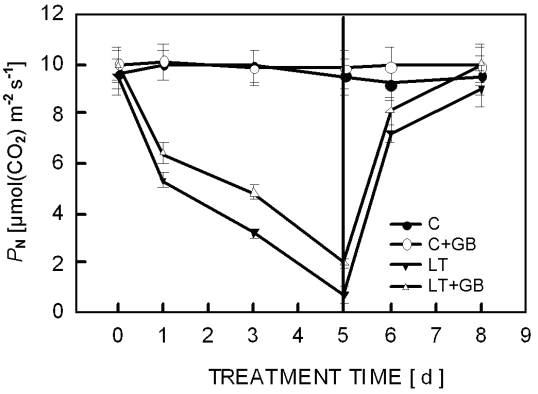

Fig. 1. Effects of glycine betaine (GB) on the net photosynthetic rate (P_N) of tobacco leaves. External air was scrubbed of CO_2 and mixed with a supply of pure CO_2 to prepare a reference concentration of 360 μm^3 m^{-3}, and the irradiance was set at 800 μmol m^{-2} s^{-1}. The temperature inside the leaf chamber was maintained at 25 °C. Means of 10 replicates ±SE.

Effects of root-applied GB on the components in the thylakoid membrane of tobacco leaves: Under cold stress, almost no new polypeptides were formed in the thylakoid membrane (Fig. 4); however, the accumulation of some polypeptides increased, especially that of the 50 kDa polypeptides. The accumulation of these peptides increased in a time-dependant manner under cold stress but decreased after recovery from the stress. Under LT, the accumulation of 50 kDa polypeptides in the LT+GB group was similar to that in the LT group, but it slowly decreased after recovery. The accumulation of the 33 kDa

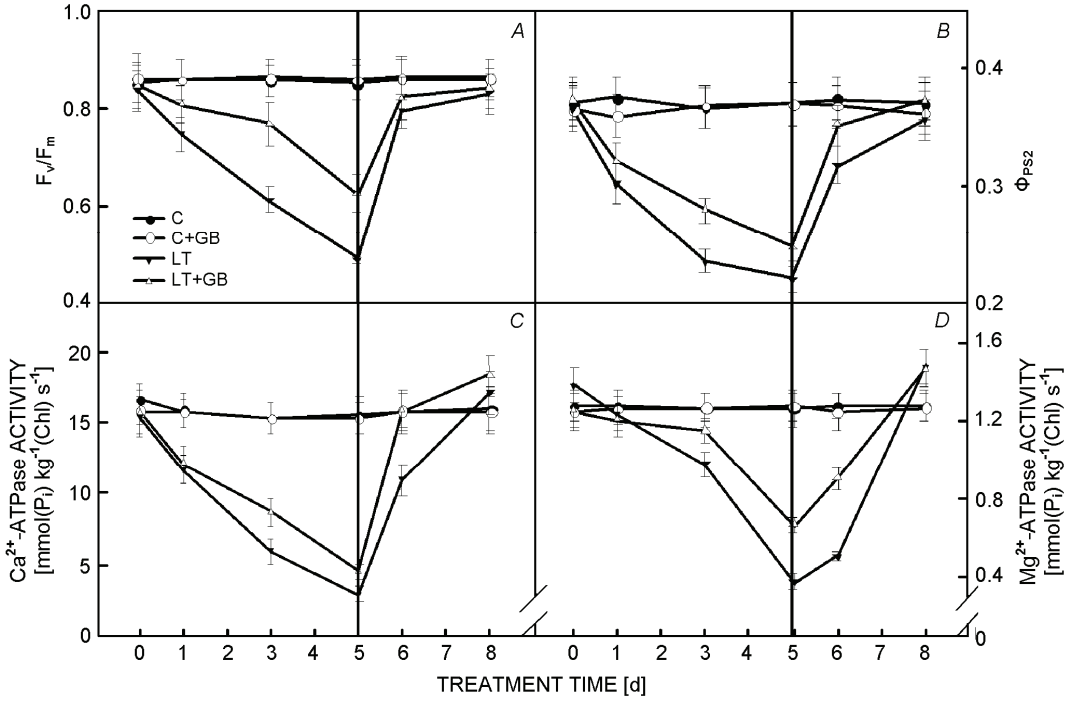

Fig. 2. Effects of glycine betaine (GB) on intrinsic (*A*) and actual efficiency (*B*) of the photochemistry of photosystem (PS) 2 and on the activities of Ca^{2+}-ATPase (*C*) and Mg^{2+}-ATPase (*D*) in the thylakoid membrane of tobacco leaves. Means of 20 (*A, B*) or 3 (*C, D*) replicates ±SE.

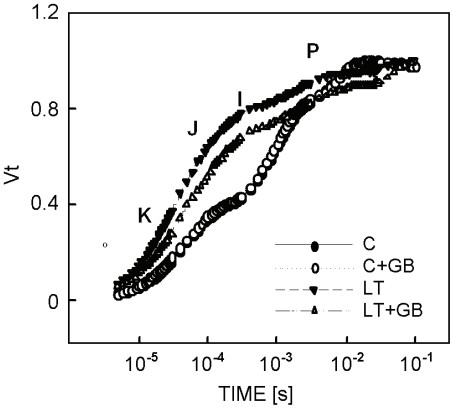

Fig. 3. Effects of glycine betaine (GB) on the fluorescence transient kinetics O-J-I-P plotted on a logarithmic time scale from 50 μs to 1 s of dark-adapted tobacco leaves after 5 d of moderate (C and C+GB groups) and low (LT and LT+GB groups) temperature treatments. The leaves were excited with 3 000 μmol m^{-2} s^{-1} of red radiation for 1 s. Original data with normalization of the variable fluorescence from 0 to 1, which corresponded to the relative variable fluorescence, *i.e.* $V_t = (F_t - F_{50\mu s})/(F_m - F_{50\mu s})$ are presented.

polypeptides also slightly increased under LT-stress, but no evident difference in the accumulation of these peptides was observed between the LT and LT+GB groups.

Under moderate temperature, GB exerted few effects on the IUFA of the thylakoid membrane lipids (Fig. 5). The IUFA of MGDG, DGDG, SQDG, and PG decreased under cold stress and increased with recovery from the stress. At almost all time points of the cold-treatment period, the IUFA in the LT+GB group was higher than that in the LT group ($p<0.05$). This indicated that the unsaturated fatty acids in the thylakoid membrane lipids were decreased by LT and GB which might help maintain high IUFA in the thylakoid membrane lipids.

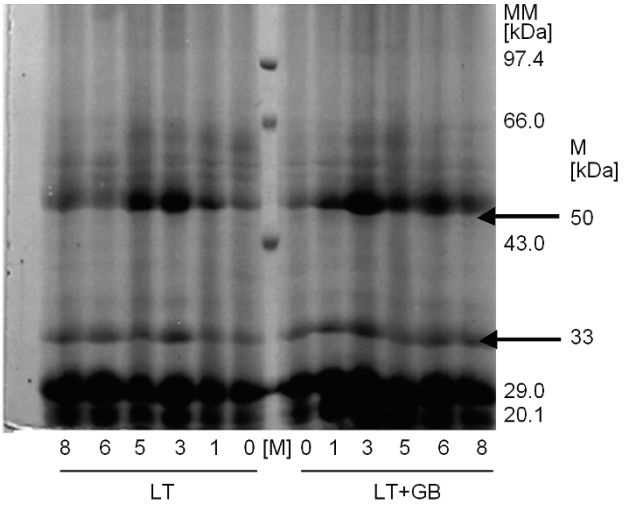

Fig. 4. Effects of glycine betaine (GB) on thylakoid polypeptides of tobacco leaves. The numbers 0, 1, 3, 5, 6, and 8 represent the treatment and recovery days; MM is molecular mass, M represents molecular mass markers.

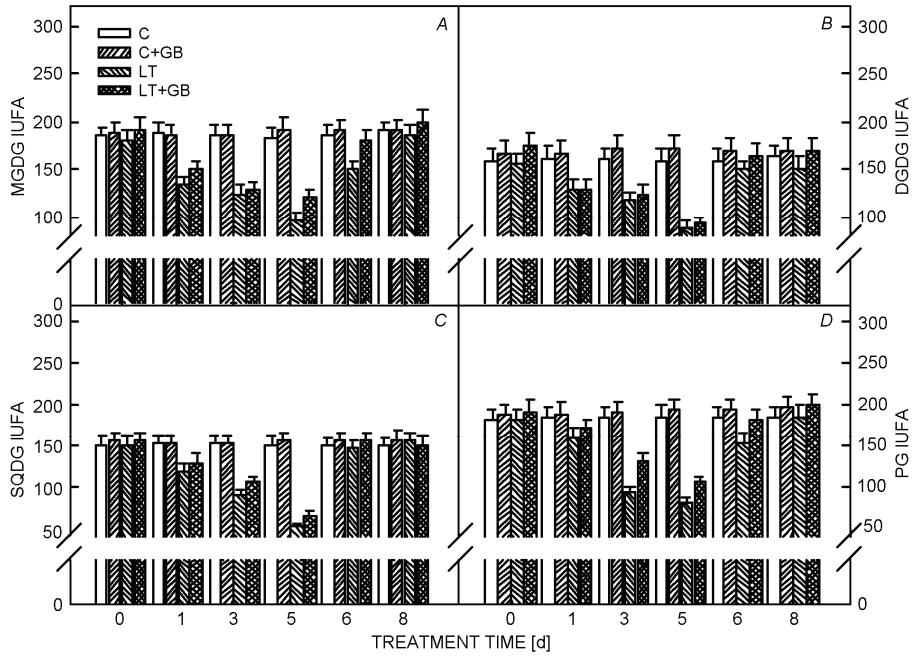

Fig. 5. Effects of glycine betaine (GB) on the index of unsaturated fatty acids (IUFA) in monogalactosyl diacylglycerol (MGDG) (*A*), digalactosyl diacylglycerol (DGDG) (*B*), sulfoquinovosyl diacylglycerol (SQDG) (*C*), and phosphatidyl glycerol (PG) (*D*) in the thylakoid membrane of tobacco leaves. The IUFA in MGDG, DGDG, SQDG, and PG was calculated as IUFA [mol%] = ($C_{16:1}$ + $C_{16:1t}$ + $C_{18:1}$ + 2$C_{18:2}$ + 3$C_{18:3}$)×100.

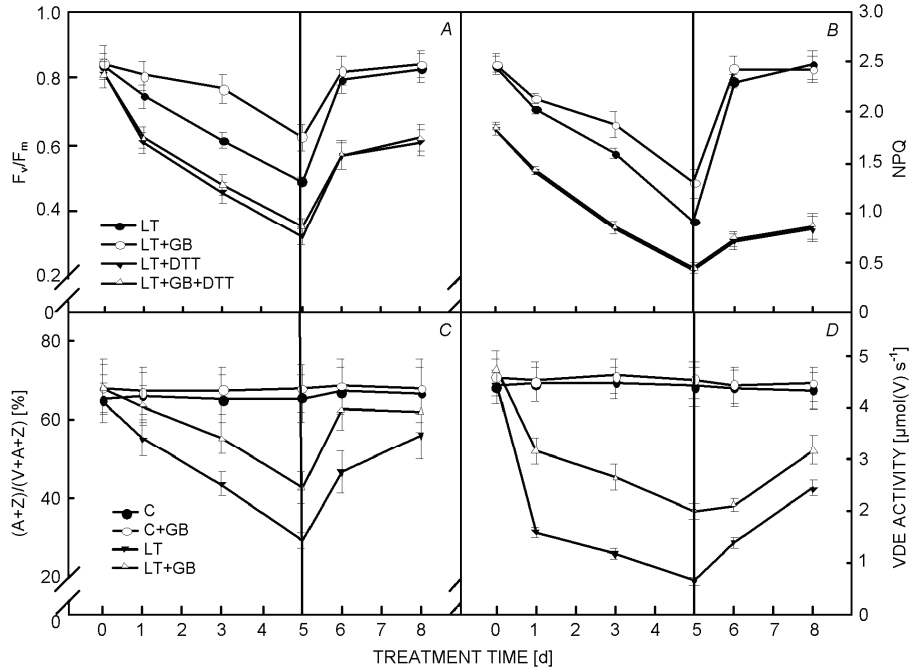

Fig. 6. Effects of glycine betaine (GB) and 1,4-dithiothreitol (DTT) on the intrinsic efficiency of the photochemistry of PS2 (*A*), non-radiative energy dissipation (NRD) (*B*), (A+Z)/(V+A+Z) (*C*), and violaxanthin de-epoxidase (VDE) activity (*D*) of tobacco leaves. DTT (6 mM) was applied to the plants for 6 h. Means of 10 (*A, B*) or 3 (*C, D*) replicates ±SE.

Involvement of xanthophyll cycle in the improvement of thylakoid membrane function of tobacco leaves by GB: Based on the data presented in Fig. 2*A,B*, we suggest that GB could markedly improve the efficiency of photochemistry of PS2 and alleviate photoinhibition under stress; it is to be clarified whether xanthophyll cycle-dependent thermal dissipation is involved in this GB-induced improvement. In this experiment, F_v/F_m and non-

photochemical quenching (NPQ) were determined after inhibiting the xanthophyll cycle by DTT. Considering that GB had hardly any effect on thylakoid membrane function under moderate temperature (Figs. 1–3), the C and C+GB groups were excluded from this experiment.

Fig. 6*A* shows that photoinhibition was more severe after DTT treatment at LT. The F_v/F_m of the LT+DTT and LT+GB+DTT groups were only approximately 0.33; this is markedly lower than the values obtained under the same cold-stress without DTT ($p<0.05$). The F_v/F_m did not return to that value under moderate temperature until 3 d of recovery. Thus photoinhibition was more severe when the xanthophyll cycle was inhibited by DTT, and exogenous GB was not able to alleviate the inhibition of DTT. After DTT treatment, the changes in NPQ were similar to those in F_v/F_m (Fig. 6*B*). However, in the absence of DTT, GB could significantly increase F_v/F_m and NPQ under stress ($p<0.05$).

Fig. 6*C* shows that the changes in (A+Z)/(V+A+Z) with temperature were similar to those observed in Fig. 6*D*. Under moderate temperature, GB scarcely had any effect on (A+Z)/(V+A+Z) and the VDE activity of the xanthophyll cycle, but (A+Z)/(V+A+Z) decreased evidently under cold stress ($p<0.05$). On the 5th day under stress, (A+Z)/(A+Z+V) in the LT+GB group only decreased to 60.99 % of that in the CK group; but it decreased to 48.12 % of that in the LT group. However, the level of (A+Z)/(A+Z+V) in both groups returned to the C level after 3 d of moderate temperature recovery (Fig. 6*C*). Under stress, the changes in the VDE activity were similar to those in (A+Z)/(V+A+Z), but this activity did not return to the C level until 3 d of recovery (Fig. 6*D*).

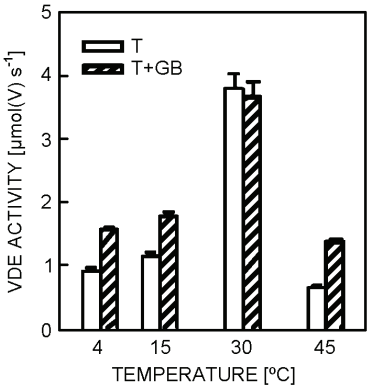

Fig. 7. Effects of glycine betaine (GB) on the activity of violaxanthin de-epoxidase (VDE) *in vitro* at different temperatures. The solutions containing VDE were incubated at various temperatures for 30 min. T, temperature treatment; T+GB, 50 mM GB pre-treatment and then temperature treatment. Means of 3 replicates ±SE.

We also determined the effects of GB on VDE activity *in vitro* at different temperatures (Fig. 7): VDE activity increased with an increase in temperature from 4 to 30 °C but decreased at 45 °C. In this experiment, maximal VDE activity was observed at 30 °C. GB pre-treatment significantly increased VDE activity under low- and high-temperature stresses ($p<0.05$); however, almost no effect was observed at 30 °C, which may be the optimal temperature for maximal VDE activity ($p>0.05$). Thus GB protects VDE from damage under low- or high-temperature stress, but does not have any effect on VDE activity at optimal temperature.

Discussion

We demonstrated that root-applied GB was absorbed by tobacco plants and improved the photosynthetic capacity of tobacco leaves under cold stress (Fig. 1). This implies that the applied GB might accumulate in the cytosol or/and in the chloroplast (Park *et al*. 2004). Moreover, according to the position of the GB peak in the chromatogram map, the molecular structure of GB was not altered during the process of GB absorption from the soil. Therefore, it was feasible to increase GB content in the leaves of the plant by root-applied GB for improving cold resistance.

Protective effects of root-applied GB on the photosynthetic apparatus of tobacco leaves under LT-stress: The photosynthetic capacity of cold-susceptible plants decreased when exposed to LT; this decline was associated with a decrease in the quantum efficiency of PS2 as well as in the activities of PS1, ATP synthase, and the stroma enzymes of the C_3 carbon reduction cycle (Allen and Ort 2001). Sakamoto and Murata (2002) suggested that transgenic plants with the GB synthesis gene could protect the photosynthetic apparatus under stress. In our experiment, P_N of tobacco leaves decreased at LT (Fig. 1), which was accompanied with a decrease in F_v/F_m and Φ_{PS2}. However, the levels of all these parameters returned to the C level after 3-d recovery from stress (Fig. 2*A,B*). Hence under LT-stress, moderate irradiance (300 μmol m^{-2} s^{-1} in this experiment) was strong enough to cause photoinhibition in tobacco leaves. GB treatment could help maintain higher P_N, F_v/F_m, and Φ_{PS2} under stress and recovery from stress compared with no-GB treatment, suggesting that GB can improve photosynthesis and alleviate photoinhibition.

Photoinhibition is associated with the thylakoid membrane on which the electron transfer chain and other protein complexes, including ATPase, associated with photosynthesis are embedded. Fig. 2*C,D* indicates that ATPase activity decreased at LT; this decrease in ATPase activity would disturb the ΔpH balance and decrease the ATP supply for CO_2 assimilation, which may be involved in the reduction of P_N under cold stress (Fig. 1). GB pre-treatment alleviated the decrease of ATPase activity and

the feedback inhibition of photosynthesis, which could increase the P_N under cold stress.

The polyphasic increase in the fluorescence transients provides some information on PS2 photochemistry. In Fig. 3, the typical O-J-I-P curve changed and a distinct K step appeared. The appearance of K, which is used as a specific indicator of OEC damage, was caused by an inhibition of an electron donor to the secondary electron donor of PS2, namely Y_Z (Guissé *et al*. 1995); this negative effect was alleviated by GB pre-treatment. This was consistent with the effect of GB on ATPase (Fig. 2*C,D*) and polypeptides (Fig. 4) in the thylakoid membrane.

Components of thylakoid membrane involved in the protective effects of GB on photosynthetic apparatus: The function of the thylakoid membrane is based on its fluidity and integrity, which are affected by its components, such as lipids, membrane proteins, pigments, and ions. Gillet *et al.* (1998) reported that drought induced an increase in the concentration of 34 kDa thylakoid proteins. Similar studies by Huseynova *et al.* (2007) showed that the concentration of the 40.5 kDa thylakoid membrane protein increased under water stress. We found (Fig. 4) that the accumulation of polypeptides with a molecular mass of approximately 33–66 kDa, *e.g*. 33 and 50 kDa polypeptides, was affected by cold stress. Based on the molecular mass and the time of appearance of such polypeptides, we suggest that the increase in the accumulation of these polypeptides might be induced by LT and not by protein disaggregation. These findings imply that GB can not only protect some intrinsic protein complexes in the thylakoid membrane from damage by cold but also those induced by LT from damage by cold stress.

The specialized structure of the thylakoid membrane lipids is crucial to photosynthesis (Vijayan and Browse 2002). The functions of membrane proteins are influenced by the lipid matrix in which they were embedded (Huseynova *et al*. 2007), especially PG lipid molecules relative to the PS2 structure (Kanervo *et al*. 1997). Fig. 5 shows that the IUFA of MGDG, DGDG, SQDG, and PG decreased at LT. GB pre-treatment could increase the IUFA of the fatty acids of membrane lipids; it particularly increased the IUFA of PG (Fig. 5). This increase was propitious for the PS2 function and the maintenance of other protein complexes in the thylakoid membrane. Therefore, an increase in the components of the thylakoid membrane may be involved in the mechanisms underlying the alleviation of photoinhibition under LT-stress by GB.

Improvement in photoinhibition by GB application may be relative to xanthophyll cycle-dependent NPQ: The photosynthetic apparatus can protect itself by increasing xanthophyll cycle-dependent energy dissipation around PS1 and PS2 to dissipate excessive energy under cold stress (Havaux *et al*. 2000). The xanthophyll cycle plays a major role in photoprotection and Z was a key factor in NPQ (Gisselsson *et al*. 2004). In our study, F_v/F_m and Φ_{PS2} clearly decreased under LT- stress; similar to NPQ; the levels of these two parameters returned to the C level after temperature recovery from LT (Figs. 2 and 6*B*). Further, under the cold stress, changes in (A+Z)/(V+A+Z), NPQ, and VDE activity occurred simultaneously. When DTT was used to inhibit the xanthophyll cycle *in vivo* (Darkó *et al*. 2000), F_v/F_m and NPQ were decreased to a greater extent than (A+Z)/(V+A+Z) and VDE activity (Fig. 6*A,B*). These results implied that xanthophyll cycle-dependent NPQ is important for photoprotection under cold stress. However, the VDE activity did not return to the C level until 3 d after recovery from the stress (Fig. 6*D*), indicating that VDE was affected more than NPQ by cold stress. It also implied that the VDE-mediated decreases in NPQ and (A+Z)/(V+A+Z) under cold stress could be compensated by other mechanisms such as the aggregation of light-harvesting complexes (LHCs) (Tang *et al*. 2007). These mechanisms might enable NPQ (Fig. 6*B*) and (A+Z)/(V+A+Z) (Fig. 6*C*) to return to the C level 3 d after recovery from the stress when the VDE activity was not restored to its normal level (Fig. 6*D*).

However, DTT is not a specific inhibitor of VDE; it can also inhibit some other proteins with a structure similar to that of VDE. But the VDE activity may still be involved in the GB-induced increase in NPQ and decrease in photoinhibition because of the close relationship between NPQ and the xanthophyll cycle (Jiang *et al*. 2001).

What is the mechanism underlying the GB-induced protection of VDE under stress? The functions of membrane proteins are influenced by the lipid matrix in which they are embedded (Huseynova *et al*. 2007). VDE is localized in the thylakoid membrane (Havaux *et al*. 2000); except for VDE as a protein protected by GB, the state of thylakoid membrane must be involved in the VDE activity, and its improvement might be beneficial to VDE. In this experiment, GB pre-treatment increased the IUFA of the fatty acids in the membrane lipids (Fig. 5). This was propitious to the maintenance of protein complexes in the thylakoid membrane, including VDE.

We demonstrated that GB could alleviate photoinhibition by not only maintaining a high IUFA of the thylakoid membrane lipids but also by protecting protein complexes such as ATPase and VDE. Protecting the xanthophyll cycle by improving VDE activity by applying GB to plant roots might be an important approach for alleviating photoinhibition under cold stress.

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