Overaccumulation of glycine betaine enhances tolerance of the photosynthetic apparatus to drought and heat stress in wheat

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Abstract

To investigate the role of glycine betaine in photosynthesis under stress, a transgenic wheat (*Triticum aestivum* L.) line T6 overaccumulating glycine betaine and its wild type Shi4185 were used. Seedlings were exposed to conditions of drought (30%, PEG-6000), heat (40°C) and their combination. The results revealed ultrastructural damage to the chloroplast and thylakoid lamellae with the withered phenotype by both drought and heat stress, and the damage was exacerbated by the combination of drought and heat. The appearance of a K step in the typical O-J-I-P curve and the decrease of Hill activity indicated a reduction of oxygen evolving complex function caused by stress. The greater damage was found in wild type than T6. Overaccumulation of glycine betaine in T6 could protect lipids in the thylakoid membrane from damage and stabilize the index of unsaturated fatty acids under stress. A lower ratio of monogalactosyl diacylglycerol/digalactosyl diacylglycerol and higher phosphatidylglycerol content in the thylakoid membrane of T6 were also observed under stress. These effects can promote stability of the thylakoid membrane. Otherwise, glycine betaine overaccumulation decreased photoinhibition of PSII under stress. The results also suggest that xanthophyll cycle-dependent non-radiative energy dissipation may be involved in the GB-mediated effects on PSII function under stress conditions.

Additional key words: chloroplast ultrastructure; fatty acids; Hill activity; lipids; nonradiative energy dissipation; oxygen-evolving complex; thylakoid membrane.

Introduction

Wheat is often affected by environmental stresses, particularly the combination of drought and heat in the field during late stages of development. The study of abiotic stress in wheat has advanced considerably in recent years. However, stress conditions are traditionally studied by applying a single source of stress such as drought, salt, or heat, and fewer studies have considered the effects of combined sources of stress. Several studies have revealed that plants respond differently to individual sources of stress compared to combinations of stress (Rizhsky *et al*. 2002, Mittler 2006, Shulaev *et al*. 2008). These reports highlight the need for further studies of how plants respond to combinations of stress.

The photosynthetic activity of the chloroplast is one of the most stress-sensitive physiological processes. Stress damages the thylakoid membrane, disturbs its functions, and ultimately decreases photosynthesis and crop yield (Shah and Paulsen 2003, Huseynova *et al*. 2007, Zhao *et al*. 2007). Preservation of the photosynthetic apparatus is an important strategy for enhancing crop yield under stress. Glycine betaine (GB) is regarded as one of the most effective osmoprotectants due to its many advantages, in addition to being a compatible solute (Demiral and Türkan 2004, Yang *et al*. 2007). Wheat can

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Abbreviations: BADH – betaine aldehyde dehydrogenase; Chl –chlorophyll; DS – drought stress; DGDG – digalactosyl diacylglycerol; DTT – 1,4-dithiothreitol; F_v/F_m – maximal quantum yield of PSII photochemistry; GB – glycine betaine; HS – heat stress; IUFA – index of unsaturated fatty acid; MGDG – monogalactosyl diacylglycerol; NPQ – nonphotochemical quenching of chlorophyll fluorescence; OEC – oxygen evolving complex; PG – phosphatidylglycerol; PS – photosystem; PSI – photosystem I; PSII – photosystem II; q_E – high energy state quenching; q_I –photoinhibitory quenching; SQDG – sulfoquinovosyldiglyceride; VDE – violaxanthin de-epoxidase; V_t – variable fluorescence; WT – wild type.
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naturally accumulate GB, but the levels accumulated *in vivo* are too low for adequate protection of the photosynthetic apparatus under stress. Transgenic wheat that overexpresses betaine aldehyde dehydrogenase (BADH) cDNA can overcome this GB deficiency (Ding *et al*. 2003). Here we show an improved protection of the thylakoid membrane structure, composition and function in *BADH* transgenic wheat overaccumulating GB during stress, and compare the different damages between drought, heat and the combination of both factors.

Materials and methods

Plant materials and stress treatments: Two wheat lines were used in this experiment. One is a transgenic wheat line overexpressing a gene encoding betaine aldehyde dehydrogenase (BADH), 99T6 (T6). T6 was generated by introducing a pABH9 plasmid encoding the *BADH* gene under the control of a maize ubiquitin promoter and a bar gene by microprojectile bombardment. The *BADH* gene was cloned from *Atriplex hortensis* L. (Guo *et al.* 2000, Ding *et al.* 2003). The other line is the wild type, Shi4185 (WT). Seeds of both WT and T6 were germinated on filter papers moistened with water for 24 h after being sterilized with 0.2% sodium hypochlorite. Then the germinating seeds were placed into plastic pots (height 8 cm; diameter 10 cm) containing quartzite with the appropriate density. The wheat plants were grown in an artificial chamber at 25 ± 1°C with a photon flux density of 300 μmol m^{-2} s^{-1} of the photosynthetically active radiation, a relative humidity of 75–80%, and a photoperiod of 14/10 h (light/dark). Hoagland nutrient solution was sprayed on the leaves every day. All treatments were performed in parallel when the third leaf was fully expanded. Drought was imposed by 30% (w/v) PEG-6000 (osmotic potential was about –1.88 MPa) until plants reached a relative water content (RWC) of 83–86% (typically 3 d). A combination of drought and heat stress was applied by subjecting drought-stressed plants to a high temperature of 40°C for 3 h in an artificial chamber. The heat stress alone was conducted by exposure to a high temperature as described above, but the plants were not previously stressed by water deficiency. All of the treatments included single water stress (DS), single heat stress (HS), the combination (DS+HS) and well-watered plants (CK). All of the experiments were performed in triplicate and all of the measurements were repeated at least three times.

GB content was determined using high-performance liquid chromatography (HPLC) (*LC-6A, Shimadzu Corp*., Kyoto, Japan) according to the procedure described by Ma *et al*. (2007).

Chlorophyll (Chl) *a* fluorescence analysis: The maximum quantum yield of PSII (F_v/F_m) and nonphotochemical quenching of Chl fluorescence (NPQ) were measured with a portable pulse-modulated fluorometer *FMS-2* (*Hansatech Instruments Ltd*., King′s Lynn, UK). For quenching analyses, the leaves were illuminated with actinic light intensities of 800 μmol(photon) m^{-2} s^{-1} for 16 min (which was found to be sufficient for the induction of steady state light conditions) and subsequently kept for a further 10 min in the dark. For q_{NSV} (NPQ), q_{ESV} (q_E) and q_{ISV} (q_I), the Stern–Volmer type parameters were calculated according to Krause and Jahns (2003) as $q_{NSV} = (F_m - F'_m)/F'_m = F_m/F'_m - 1$; $q_{ESV} \approx (F_{mr}/F'_m - 1)F_m/F_{mr} = F_m/F'_m - F_m/F_{mr}$; $q_{ISV} \approx (F_m - F_{mr})/F_{mr} = F_m/F_{mr} - 1$; with F_{mr} representing the maximum fluorescence measured after 10 min in the darkness.

Analysis of O-J-I-P Chl *a* fluorescence induction transient (JIP-test): In the preliminary, the leaves of wheat seedling were dark-adapted for different times (2, 4, 6, 8, 10, 15, 20, 25, and 30 min) under 3,000 μmol m^{-2} s^{-1} firstly, and we found that the F_v/F_m ratio had no obvious change after 10 min. Then, the leaves of wheat seedling were dark-adapted for 15 min under different excitation light intensity (500; 1,000; 1,500; 2,000; 2,500; and 3,000 μmol m^{-2} s^{-1}), and we found that the F_v/F_m ratio had no obvious change when the light intensity was above 2,000 μmol m^{-2} s^{-1}. The F_p is the highest level of F_m. Therefore, F_p is equal to F_m in our experiment.

The polyphasic Chl *a* fluorescence transients (OJIP) were measured at 25°C using a *Plant Efficiency Analyzer* (*PEA, Hansatech Ltd*, King′s Lynn, UK) with excitation light intensity of 3,000 μmol m^{-2} s^{-1}. Leaves were dark-adapted for 15 min before they were measured. Fluorescence transients were recorded during a 60-s light pulse of red radiation (about 3,000 μmol m^{-2} s^{-1}) provided by an array of six light emitting diodes (peak 650 nm). The fluorescence signals were recorded within a time span ranging from 10 μs to 1 s with a data acquisition speed of 10^5 points per s for the first 2 ms and of 1,000 points per s after 2 ms. The JIP-test was employed to analyze each Chl *a* fluorescence transients. The following data from the original measurements were used: Maximum fluorescence intensity (F_m), the fluorescence intensity at 50 μs was considered the minimum intensity (F_o); the fluorescence intensity at 300 μs (K step), the fluorescence intensity at 2 ms (J step) and the fluorescence intensity 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) as $V_t = (F_t - F_{50\mu s})/(F_m - F_{50\mu s})$ were all calculated according to Jiang *et al*. (2003).

The amplitude of the K step (W_K) was expressed as the ratio of $V_K/V_J = W_K$ (Wen *et al.* 2005).

Hill activity of the chloroplast: Chloroplast isolation and Hill activity assays were determined following the procedures modified from Ye and Qian (1985). 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 four 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 suspended in buffer *A* was used to examine the Hill activity of the chloroplast.

Chl content of the chloroplast suspension was measured according to Arnon (1949).

The Hill activity of isolated chloroplasts was determined by the photoreduction of potassium ferricyanide ($K_3[Fe(CN)_6]$). A medium containing 0.5 M Tris-HCl (pH 7.6), 0.05 M $MgCl_2$, 0.1 M NaCl, 0.01 M $K_3Fe(CN)_6$, and 0.1 cm^3 chloroplast suspension was diluted with distilled water [0.15 mg(Chl) ml^{-1}]. This solution was exposed to 800 μmol m^{-2} s^{-1} irradiance for 1 min at 20°C, and the reaction was terminated by adding 10% trichloroacetic acid (TCA). Then, the mixture was centrifuged at 1,000 × *g* for 2 min, and the supernatant was extracted for the Fe $(CN)_6^{4-}$ assay. Hill activity was expressed as μmol(O_2) mg^{-1}(Chl) h^{-1} (Zhao *et al.* 2007).

Analysis by electron microscopy: Healthy fresh leaves were cut from the plants. Small pieces (1 mm^2) were washed by distilled water and taken for electron microscopy. Leaf fragments were fixed in 2% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) for 12 h at 4°C. After being washed, the segments were post-fixed in 1% osmium tetroxide for 4 h in the same buffer, dehydrated in a graded series of ethanol and embedded in *Epon-812*. Ultrathin sections cut on a *Reichert Ultratome* (*OM-U3*, *Reichert-Jung*, Vienna, Austria) were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (*JEOL-1200EX*, Japan).

Determining the contents of polar lipids and fatty acids in thylakoid membrane: The polar lipids (MGDG, DGDG, SQDG, PC, and PG) were extracted from the pellet of thylakoid membrane by a mixture of chloroform/methanol (2:1, v/v) and then separated by two-dimensional thin-layer chromatography (TLC) according to a modified method reported by Droppa *et al*. (1987). One-dimensional TLC was conducted using acetone/benzene/water (91:30:8, v/v/v) and then the two-dimensional TLC was conducted with 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 fatty acid methyl ester was determined by a gas chromatogram analyzer (*GC-9A, Shimadzu*, Japan) using a methyl-esterified arachidic acid as an internal standard. The conditions were: 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, pneumatophore pure N_2 with flowing velocity 100 cm^3 min^{-1}. Quantification was calculated by normalization, which was done with the processing software of the apparatus. The index of unsaturated fatty acids (IUFA) in the polar lipids was calculated as IUFA= ($C_{16:1}$ mol % + $C_{16:1t}$ mol % + $C_{18:1}$ mol % + $2C_{18:2}$ mol % + $3C_{18:3}$ mol %) × 100, respectively (Deng and Wang 1982).

DTT treatment *in vivo*: DTT treatment was performed according to Tang *et al.* (2007) with some modifications. Leaves with stalks were taken from the same position on plants from each wheat line and submerged in 10 mM DTT for 3 h at 50 μmol m^{-2} s^{-1}.

Statistical analysis was conducted using the procedures of DPS (Zhejiang University, China). All pairwise comparisons were analyzed using *Duncan*'s test. Differences between the means among wheat lines or treatments were compared using *Duncan*'s multiple range tests at 0.05 probability levels.

Results

Association between GB content and T6 and WT phenotype: Owing to the constitutive expression of the transgenic *BADH* gene, the GB content in T6 was significantly higher than that in WT in the absence of stress treatment (Fig. 1) (Guo *et al.* 2000, Ding *et al.* 2003). Stress-induced GB levels increased significantly in the leaves of the 2 lines, especially in T6, showing that the accumulation of GB sensitized the wheat plants to these stresses. Meanwhile, although exposure to all stressors produced the withered phenotypes in both the wheat lines, the withering was more serious in WT than in T6, suggesting the positive effect of overaccumulated GB on the stress tolerance of wheat (Fig. 2).

Effects of GB and stress on chloroplast and thylakoid ultrastructure: To test the improvement of GB in protecting the photosynthetic apparatus from stress, chloroplast and thylakoid ultrastructure were observed as shown in Fig. 3. Under normal condition, no significant differences were found between WT and T6. The chloroplasts were typically long and oval, arranged regularly along the cell wall, with a ratio of length to width of about three (Fig. 3*A,E*). A typical granal stack was found to have lamellae that were closely arranged and assembled to form the grana, which was lens-shaped with a typical arrangement of grana and stroma thylakoid (Fig. 3*A′,E′*). After drought treatment, some damage of the chloroplast envelope was found, and the shape of the chloroplast was spherical, especially in WT (Fig. 3*B′,F′*). The chloroplasts from WT plants deviated from the cell wall to the center of the cell. Meanwhile, drought stress resulted in swollen and loosely scattered thylakoid

lamellae in WT, but this was not obvious in T6 (Fig. 3*B*, *F*). Similar changes were also found under heat stress (Figs. 3*C,G*, 1*C′,G′*). The most severe damage was found under the condition of combined stress (Fig. 3*D,H,D′,H′*). It was evident that the stress-induced damage to chloroplasts and thylakoid was more severe in WT than T6, suggesting that overaccumulation of GB resulted in protection of the photosynthetic apparatus in wheat leaves.

Effects of GB and stress on the lipid and fatty acid components in thylakoid membrane: To testify the involvement of lipid composition in GB improvement in the ultrastructures of chloroplast and thylakoid, we detected the lipid and fatty acid compositions in the thylakoid membrane as shown in Table 1 and Fig. 4.

In the thylakoid membranes from the two wheat lines, the main fatty acid (Table 1) of the MGDG and DGDG was 18:3. The main fatty acids of PC were 16:0, 18:2 and 18:3; while 16:1(3t) was only present in PG (Table 1). Stress decreased the relative content of the 18:3 fatty acid in the MGDG and DGDG. Overaccumulation of GB had little effect on the fatty acid composition under normal condition. However, the relative contents of the 18:3 fatty acid in the MGDG and DGDG were significantly (p<0.05) higher in T6 than in WT under different stress conditions. Similar results were observed in the fatty acid compositions in SQDG, PG and PC. Overaccumulation of GB had little significant effect on the relative content of the 18:3 fatty acid in the SQDG. PG is particularly important for maintaining the structure and function of the thylakoid membrane. Drought, heat and the combination of both resulted in a significant decrease of the relative content of 16:1(3t) in the PG profile. This decrease was greater in WT than in T6.

The results presented in Fig. 4 show that almost all of the stress conditions decreased the contents of all five lipids, especially combined stress. However, the content of PG was hardly affected by heat stress. Overaccumulation of GB had little effect on MGDG, but did have evident effects on DGDG, PG and SGDG. The content of the PC was much lower than other lipids, but overaccumulation of GB resulted in a significant increase in PC lipids under both normal and stress conditions. Heat and combined stress obviously increased the MGDG/DGDG, overaccumulation of GB in T6 reduced this increase significantly. Under both normal and stress conditions, significantly smaller MGDG/DGDG ratios were observed in T6 than in WT due to the increase of DGDG.

Fig. 5 shows that almost all stresses decreased the IUFA of SQDG and PG, especially under the stress combination; the small effect of GB on the IUFA of MGDG and SQDG, but obvious effect of GB on the IUPA of PG under stress; heat stress decreased the IUFA of DGDG in WT, but not in T6.

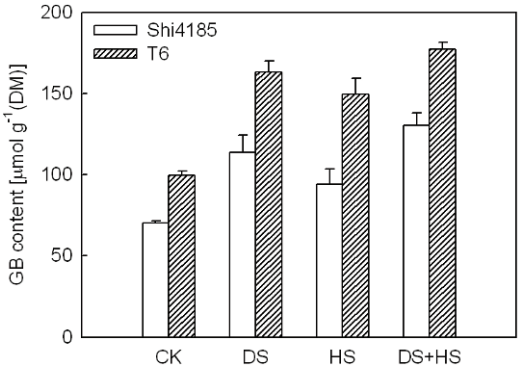

Fig. 1. Glycine betaine (GB) contents in leaves of wheat plants subjected to drought, heat, and their combination. CK – well-watered control; DS – drought-stressed; HS – heat-stressed; DS+HS – combination of drought- and heat-stressed. Each bar represents the mean ± SD of three replications.

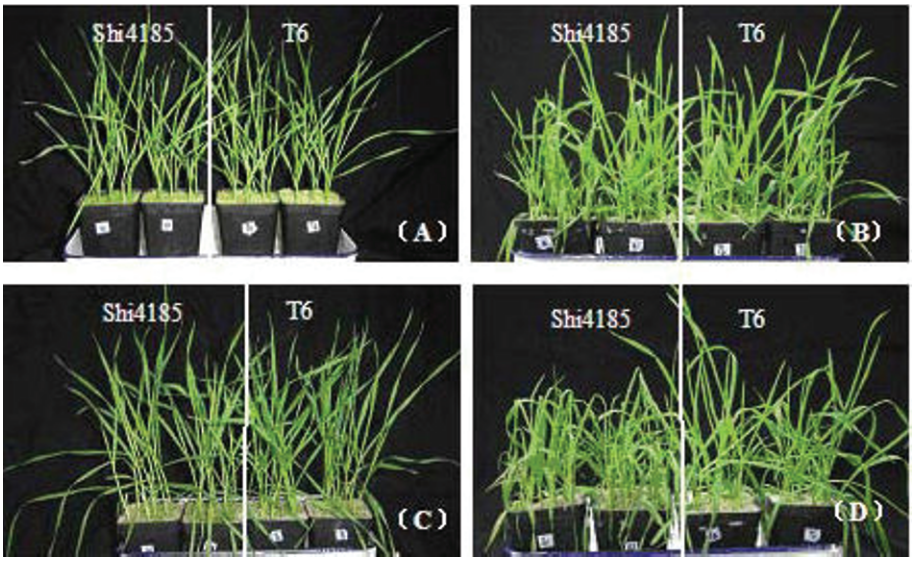

Fig. 2. The morphological difference between transgenic wheat and wild plants subjected to drought, heat, and their combination. *A*: well-watered control (CK). *B*: drought-stressed (DS). *C*: heat-stressed (HS). *D*: A combination of drought- and heat-stressed (DS+HS).

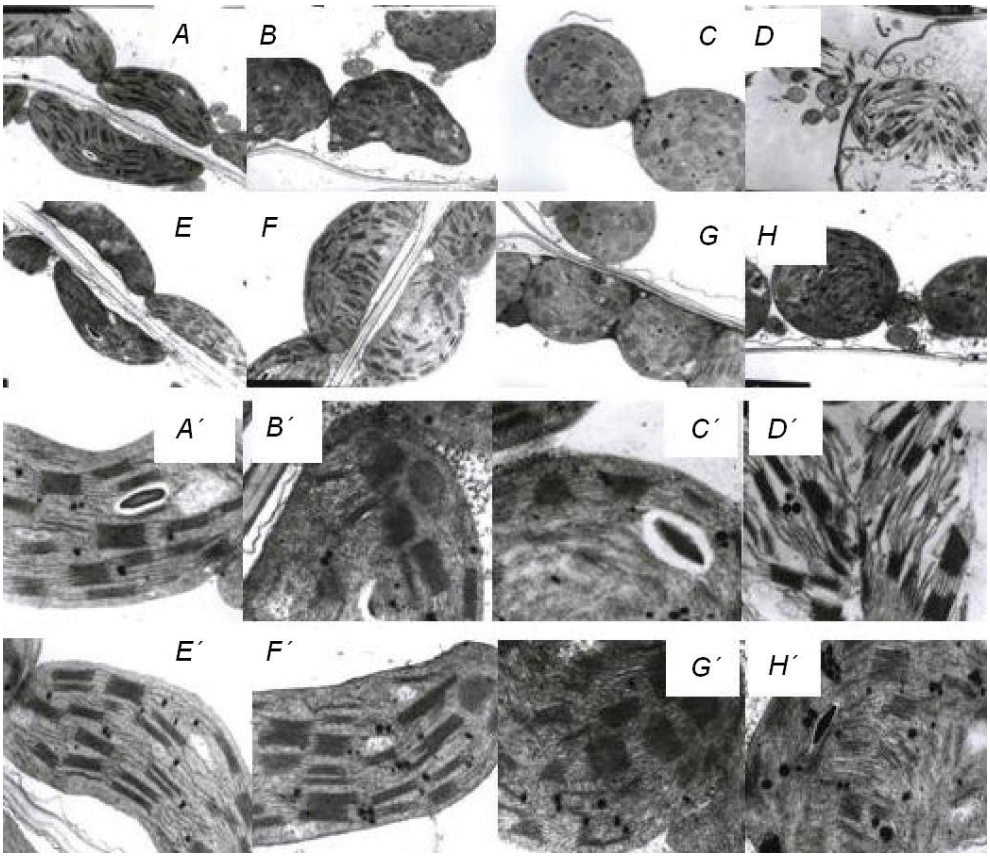

Fig. 3. Chloroplast ultrastructure (× 10,000) of wheat leaves in wild type Shi4185 (*A–D*) and transgenic line T6 (*E–H*) subjected to drought, heat, and their combination. *A*,*E*: typical chloroplast under normal condition; *B*,*F*: drought-stressed chloroplast; *C*,*G*: heat-stressed chloroplast; *D*,*H*: chloroplast treated with stress combination of drought and heat. Thylakoid ultrastructure (× 25,000) in wild type Shi4185 (*A′–D′*) and transgenic line T6 (*E′–H′*) leaves subjected to drought, heat, and their combination. *A′*,*E′*: typical thylakoid under normal condition; *B′*,*F′*: drought-stressed thylakoid; *C′*,*G′*: heat-stressed thylakoid; *D′*,*H′*: thylakoid treated with stress combination of drought and heat.

Different compositions of fatty acids were found between drought and heat stresses and the greatest fatty acid changes were observed when these stresses were combined (Table 2). The effects of stress on the IUFA in T6 leaves were less than in WT, but the responses of different fatty acids to stresses were not identical in the different wheat lines. For example, 16:0 and 18:3 were the major species of fatty acid in the thylakoid membrane and stress increased the level of 16:0 greatly in WT, but only slightly in T6. In contrast, stress decreased the level of 18:3 markedly in WT and only slightly in T6.

Effects of GB and stress on Hill activity and the O-J-I-P fluorescence transient: The polyphasic fast-phase fluorescence induction curve provides valuable information on photosystem II (PSII) function (Strasser and Govindjee 1992). We performed the JIP-test under different stress conditions as shown in Figs. 6*A,B*. Drought stress had little effect on the fluorescence induction curves in either wheat line. However, under heat- and combined stress, the JIP phase disappeared, but the K step (at about 0.3 ms) was emerged, and the I level approached the maximum fluorescence level. These changes were more obvious in WT than in T6. These results may be related to a transient oxidation of the PQ pool (and Q_A^-) by PSI (Schansker *et al.* 2005).

Furthermore, original data of the fluorescence transients with normalization of the variable fluorescence from 0 to 1, which corresponded to the relative variable fluorescence, *i.e.* $V_t = (F_t - F_{50\mu s})/(F_m - F_{50\mu s})$ were presented, which is shown in Figs. 6*C,D*. Similar changes were observed under different stress condition, but K step appeared significantly increased, and the largest K increase was found under the combined stress condition, especially in WT.

In order to compare the changes in the amplitude of K step, the portion between F_o and F_J on the fluorescence induction curve was normalized, *i.e.*, $V = (F–F_o)/(F_J–F_o)$. The amplitude of K step (W_K) was expressed as the ratio of $V_K/V_J = W_K$, which was shown in Fig. 7*B*. A higher W_K was observed in stressed plants than in nonstressed plants, and the largest K increase was found under the condition of combined stress. Moreover, the increase of W_K was greater in WT than in T6.

Table 1. Effects of overaccumulated glycine betaine on the fatty acid composition [mol%] of the thylakoid membrane in wheat leaves under different stress conditions. The abbreviation for fatty acid is denoted by ratios, indicating the number of carbons: the number of unsaturated bonds. The 3t of 16:1(3t) denotes a trans-double-bond at the third carbon. Means ± SD, n = 3. Means in a column followed by the same letter are not different at the level of P = 0.05. tr – trace (< 0.5 %). FA – fatty acid composition; CK – well-watered plants; DS – drought-stressed plants; HS – heat stressed plants; and DS+HS – plants stressed by the combination of drought and heat; Shi4185 – the wild type line; T6 – the transgenic wheat line with overaccumulation of BADH.

Lipid	Fatty acid	Shi4185				T6			
		CK	DS	HS	DS+HS	CK	DS	HS	DS+HS
MGDG	16:0	3.6±0.2^{f}	6.4±0.4^{b}	6.0±0.2^{bc}	10.2±1.03^{a}	2.1±0.02^{g}	4.1±0.4^{e}	5.6±0.2^{c}	5.4±0.3^{cd}
	16:1(3t)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	18:0	0.6±0.05^{d}	0.0^{f}	0.3±0.04^{e}	1.0±0.19^{c}	0.8±0.1^{cd}	1.7±0.14^{b}	0.0^{f}	3.0±0.2^{a}
	18:1	0.9±0.3^{f}	1.4±0.1^{e}	3.1±0.3^{a}	0.7±0.02^{g}	1.4±0.01^{e}	2.4±0.05^{c}	2.0±0.3^{d}	3.0±0.18^{ab}
	18:2	3.5±0.1^{c}	3.1±0.3^{cd}	3.1±0.1^{cd}	4.6±0.1^{a}	3.5±0.1^{c}	2.8±0.01^{de}	4.0±0.3^{b}	2.0±0.1^{e}
	18:3	91.4±0.7^{a}	89.2±1.4^{b}	87.5±1.7^{bc}	83.5±0.8^{d}	92.2±0.7^{a}	89.0±0.87^{b}	88.3±1.4^{bc}	86.7±1.1^{c}
DGDG	16:0	13.0±0.4^{cd}	13.8±0.6^{c}	25.6±1.3^{a}	23.4±0.4^{b}	12.8±0.4^{cd}	7.4±0.1^{e}	12.8±0.3^{cd}	12.8±0.1^{cd}
	16:1(3t)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	18:0	2.4±0.6^{c}	3.4±0.4^{b}	0.0^{e}	0.0^{e}	5.7±0.3^{a}	1.4±0.1^{d}	0.0^{e}	5.7±0.1^{a}
	18:1	3.1±0.1^{c}	2.9±0.2^{d}	3.8±0.5^{c}	9.0±0.2^{a}	2.8±0.3^{d}	1.1±0.1^{e}	8.0±0.3^{b}	0.0^{f}
	18:2	1.8±0.1^{bc}	1.7±0.02^{bc}	1.5±0.04^{cd}	2.1±0.3^{ab}	0.1±0.3^{f}	0.5±0.02^{e}	2.0±0.3^{ab}	2.8±0.1^{a}
	18:3	79.7±1.7^{b}	78.2±0.4^{bc}	69.2±0.3^{e}	65.4±1.4^{f}	78.7±0.3^{bc}	89.6±1.3^{a}	77.2±0.3^{cd}	78.8±0.9^{bc}
SQDG	16:0	30.0±1.2^{d}	31.7±1.3^{d}	46.2±0.7^{c}	62.8±1.6^{b}	28.1±0.3^{de}	12.9±0.5^{f}	46.2±0.2^{c}	68.3±0.7^{a}
	16:1(3t)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	18:0	7.9±0.4^{c}	15.8±0.5^{b}	0.0^{f}	4.3±0.2^{d}	3.2±0.3^{de}	39.7±1.4^{a}	0.0^{f}	3.7±0.2^{d}
	18:1	4.4±0.3^{e}	1.7±0.2^{f}	22.8±0.8^{b}	14.6±0.4^{c}	9.4±0.3^{d}	0.2±0.0^{g}	22.8±0.6^{a}	9.6±0.3^{d}
	18:2	8.5±0.2^{b}	7.4±0.4^{c}	4.3±0.2^{ef}	4.8±0.2^{e}	10.0±0.3^{a}	6.5±0.3^{d}	4.3±0.1^{ef}	4.2±0.4^{ef}
	18:3	49.3±1.4^{a}	43.3±0.4^{b}	26.7±1.1^{d}	13.5±0.7^{ef}	49.3±0.3^{a}	40.7±0.4^{c}	26.7±0.5^{d}	14.2±1.2^{e}
PG	16:0	17.1±0.5^{e}	31.0±0.1^{c}	33.3±1.3^{b}	57.0±0.5^{a}	17.6±0.3^{e}	23.6±0.6^{d}	23.1±1.3^{d}	29.2±1.5^{c}
	16:1(3t)	38.9±1.3^{a}	35.1±0.7^{b}	22.8±0.7^{c}	18.9±0.3^{d}	38.1±0.3^{a}	34.3±0.8^{b}	32.8±1.4^{bc}	24.4±0.5^{c}
	18:0	0.0^{d}	0.0^{d}	0.0^{d}	7.6±0.2^{a}	0.8±0.3^{c}	0.0^{d}	0.0^{d}	11.1±0.2^{b}
	18:1	14.4±0.2^{b}	8.1±0.9^{ef}	16.4±0.3^{a}	10.8±0.1^{d}	9.0±0.3^{e}	2.9±0.02^{g}	9.3±0.1^{e}	13.2±1.3^{bc}
	18:2	14.2±0.3^{ab}	11.6±0.2^{bc}	14.2±0.2^{ab}	0.2±0.0^{e}	11.8±0.3^{bc}	16.2±0.1^{a}	15.8±0.3^{a}	5.4±0.4^{d}
	18:3	15.4±0.5^{cd}	14.2±0.7^{e}	13.4±0.5^{e}	11.0±0.1^{d}	22.7±0.3^{a}	23.0±0.3^{a}	18.9±0.9^{b}	16.7±0.3^{c}
PC	16:0	36.1±1.2^{c}	44.1±0.5^{b}	48.4±1.2^{a}	47.1±1.5^{a}	35.2±0.3^{c}	43.4±0.3^{b}	47±1.7^{a}	47.1±1.6^{a}
	16:1(3t)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	18:0	13.3±0.3^{c}	15.9±0.7^{b}	19.5±0.3^{a}	16.1±0.8	9.3±0.3^{d}	6.0±0.3^{e}	12.5±0.2^{c}	16.1±0.6^{b}
	18:1	13.5±1.1^{bc}	13.1±0.4^{bc}	22.0±0.6^{a}	16.5±0.1^{b}	15.2±0.3^{b}	10.2±0^{d}	15.8±1.1^{b}	16.5±0.8^{b}
	18:2	19.9±0.3^{a}	13.3±0.3^{cd}	0.4±0.1^{e}	13.8±0.5^{cd}	18.5±0.3^{a}	15.3±0.3^{b}	14.7±1.3^{bc}	13.8±0.5^{cd}
	18:3	20.2±1.2^{ab}	13.7±0.6^{d}	9.7±0.2^{e}	6.5±0.3^{f}	21.8±1.1^{a}	18.1±0.5^{c}	10±0.7^{e}	6.5±0.1^{f}

Meanwhile, Hill activity in the chloroplast was detected as shown in Fig. 7*A*. Contrary to the change of phase K in the fluorescence transients, all stress conditions lead to a decrease of Hill activity in the chloroplast. The most significant decrease was found under the condition of combined stress, especially in WT (p<0.01). Both the results in Fig. 7*A* and *B* suggested that all stress resulted in OEC damage and the GB-mediated effects on PSII function under stress conditions.

Effects of GB and stress on NPQ: NPQ was closely related to the characteristic of thylakoid membrane. To study the improvement of overaccumulated GB in energy dissipation, we tested NPQ and its components. Fig. 8*A* demonstrates that drought, heat stress, and the combination of both resulted in significantly increased NPQ in both WT and T6, and the greatest NPQ was found under conditions of combined stress. NPQ encompasses both high energy state quenching (q_E) and photoinhibitory quenching (q_I) (Johnson *et al.* 1993). The changes of q_E and q_I were consistent with the observed NPQ under different conditions of stress (Fig. 8*B,C*). Fig. 8 also shows that NPQ, q_E and q_E/NPQ were all higher in T6 than in WT (Fig. 8*A,B,C*).

We further examined F_v/F_m and NPQ after using DTT to inhibit the xanthophyll cycle, which is the major contributor to NPQ. The results (Fig. 9) showed that photoinhibition was more severe after DTT treatment in plants exposed to stress. For example, under the combined stress, the F_v/F_m in WT was approximately 0.42, and this is markedly lower than that under the same conditions without DTT (p<0.05). In T6, the F_v/F_m was almost the same as in WT, and was much lower with DTT than without. The results indicate that greater photoinhibition occurred when the xanthophyll cycle was inhibited by DTT, and the ability of GB to improve F_v/F_m

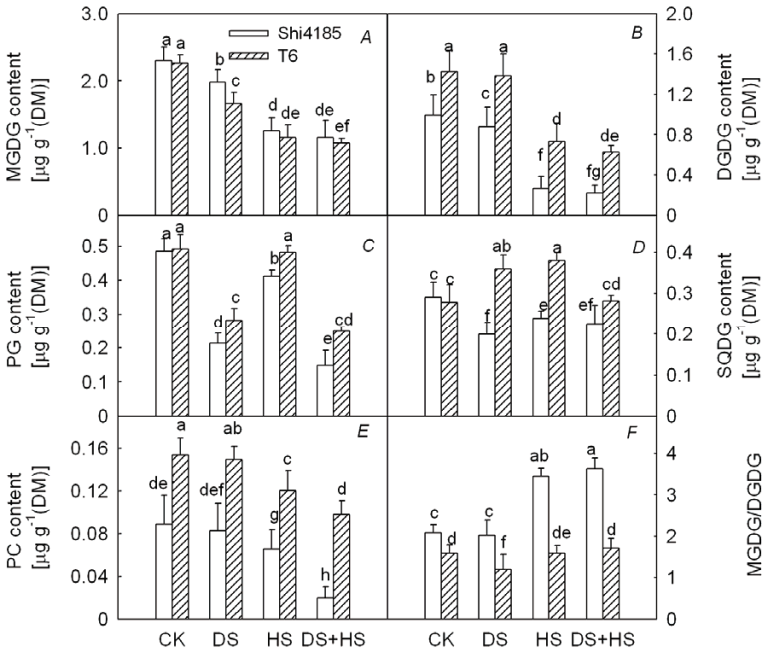

Fig. 4. Changes of lipid contents in the thylakoid membrane of wheat leaves subjected to drought (DS), heat (HS) and their combination (HS+DS). MGDG – monogalactosyl diacylglycerol; DGDG – digalactosyl diacylglycerol; PG – phosphatidyl-glycerol; SQDG – sulfoquinovosyl diacylglycerol. PC – phosphatidylcholin; CK – well-watered plants. Each bar is the mean ± SD of three replications. Bars with the same letter were not significantly different at $p<0.05$.

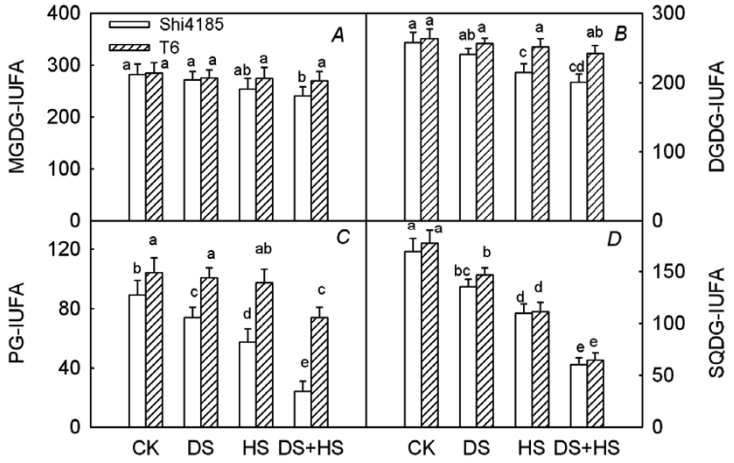

Fig. 5. Effects of different stresses and overaccumulated glycine betaine on the index of unsaturated fatty acid (IUFA) of thylakoid membrane lipids of wheat leaves subjected to drought (DS), heat (HS) and their combination (HS+DS). IUFA = 18:1 % + (18:2×2) % + (18:3×3) %. Each bar is the mean ± SD of three replications. Bars with the same letter were not significantly different at $p<0.05$. CK – well-watered plants.

Table 2. Effects of different stress and glycine betaine on the total fatty acid composition [mol%] of the thylakoid membrane in the leaves of transgenic wheat line (T6) and its wild type (Shi4185). The abbreviation for fatty acid is denoted by ratios, indicating the number of carbons: the number of unsaturated bonds. The 3t of 16:1(3t) denotes a trans-double-bond at the third carbon. IUFA (index of unsaturated fatty acid) = 18:1 % + (18:2×2) % + (18:3×3) %. Means ± SD, $n = 3$. Means in a row followed by the same letter are not different at the level of $P = 0.05$. FA – fatty acid composition, CK – well-watered wheat plants, DS – drought-stressed plants, HS – heat stressed plants, DS+HS – a combination of drought and heat stress. Statistical analysis as in Table 1.

Fatty acid	Shi4185				T6			
	CK	DS	HS	DS+HS	CK	DS	HS	DS+HS
16:0	11.2±0.8^{g}	19.9±0.4^{c}	26.2±1.4^{b}	39.8±1.5^{a}	15.1±1.1^{f}	15.5±0.3^{f}	17.3±0.5^{de}	18.2±0.4^{d}
16:1(3t)	1.7±0.3^{g}	4.3±0.02^{e}	3.8±0.4^{f}	5.5±0.4^{c}	5.3±0.3^{cd}	6.8±0.2^{b}	5.6±0.4^{c}	10.4±0.2^{a}
18:0	2.4±0.04^{e}	7.1±0.8^{b}	6.1±0.05^{c}	8.0±0.1^{a}	2.0±0.04^{ef}	2.2±0.5^{ef}	4.0±0.2^{d}	7.1±0.02^{a}
18:1	1.8±0.1^{f}	1.9±0.04^{f}	2.8±0.6^{d}	4.8±0.3^{b}	6.2±0.6^{a}	2.3±0.03^{e}	3.6±0.7^{c}	2.0±0.1^{f}
18:2	3.0±0.2^{g}	5.3±0.01^{e}	4.6±0.1^{f}	19.2±0.2^{a}	4.5±0.1^{f}	6.3±0.4^{d}	8.0±0.3^{c}	9.2±0.4^{b}
18:3	79.8±1.3^{a}	61.4±1.1^{de}	56.5±0.4^{ef}	22.6±1.4^{h}	65.8±1.2^{bc}	66.8±1.6^{b}	62.3±0.4^{cd}	53.0±1.4^{fg}
IUFA	247.2±1.7^{a}	196.7±1.3^{d}	181.5±1.1^{e}	111.2±1.5^{g}	210.6±1.5^{bc}	215.3±0.3^{b}	206.5±1.9^{bc}	173.4±1.8^{ef}

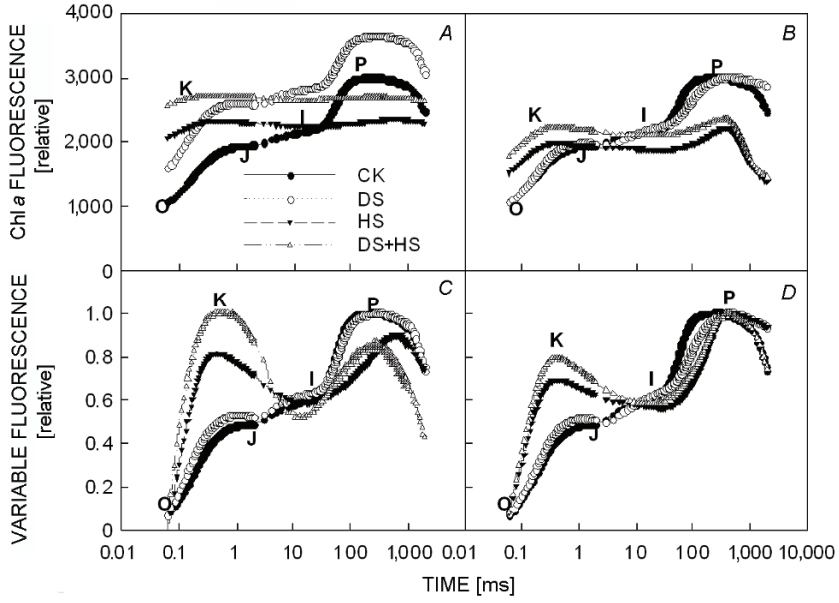

Fig. 6. The Chl *a* fluorescence induction curves of wild type leaves (*A*) and transgenic plants leaves (*B*). The fluorescence transients were double normalized between the F_o and F_m levels plotted on a logarithmic time scale in wild type (*C*) and transgenic plants (*D*) subjected to drought (DS), heat (HS) and their combination (HS+DS), the relative variable fluorescence at any time t is defined as: $V_t = (F_t–F_o)/(F_m–F_o)$. Values are means ±SD from four replicates. CK – well-watered plants.

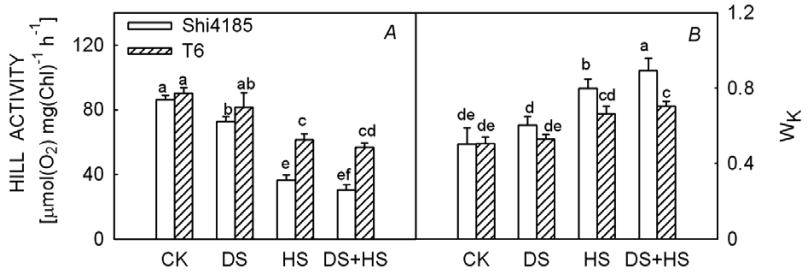

Fig. 7. Hill activity of chloroplast (*A*) and the changes in the amplitude (W_K) of the K-step of O-J-I-P fluorescence transients, which is expressed as the ratio $W_K = V_K/V_J$ (*B*) in wheat leaves subjected to drought (DS), heat (HS) and their combination (HS+DS). The relative variable fluorescence at any time t is defined as: $V_t = (F_t–F_o)/(F_m–F_o)$. Each bar is the mean ± SD of four replications. Bars with the same letter were not significant different at $p<0.05$.

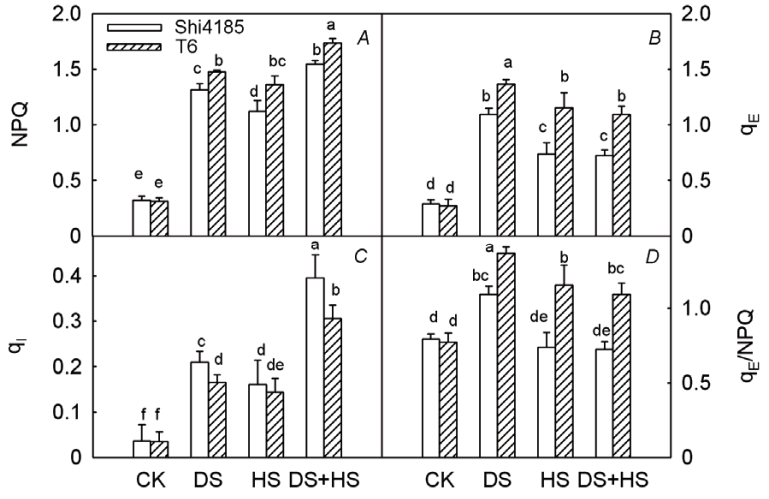

Fig. 8. Changes in nonphotochemical quenching (NPQ) (*A*), q_E- (*B*) and q_I-component (*C*) of NPQ, and ratio q_E/NPQ (*D*) of wheat leaves subjected to drought (DS), heat (HS) and their combination (HS+DS). Each bar is the mean ± SD of four replications. Bars with the same letter were not significantly different at $p<0.05$. CK – well-watered plants.

and NPQ were both inhibited (Fig. 9*B*). These results suggest that xanthophyll cycle-dependent thermal dissipation might be one of the most important mechanisms mediating GB-based improvement of PSII functions. This is consistent with the findings of Wang *et al.* (2008) in tobacco.

Discussion

The more severe damage to the structure and function of the photosynthetic apparatus by the combined stress of drought and heat than by either alone and the improvement of GB: As the apparatus of photosynthesis, the chloroplast is often subjected to stress that results in membrane damage, including expansion and rupture of the thylakoid membrane (Ristic and Cass 1992, Han *et al*. 1997). Our results presented in Fig. 3 confirmed this and we observed that the damage induced by heat and drought together was more severe than by either alone. This structural damage may be one of the most important factors involved in the withering phenotype of the wheat plants (Fig. 2) under stress.

The electron transfer chain and protein complexes, including PSII, are embedded in the thylakoid membrane. A polyphasic rise of fluorescence transients can provide information on the photochemistry of PSII. Under normal condition, WT and T6 plants exhibited a typical polyphasic rise of Chl fluorescence transients, including the O-J-I-P phases. Exposure to stress induced a rapid rise in the polyphasic fluorescence transients. The level at around 0.3 ms was labeled as K, and the O-K transient was the fastest phase observed in the OJIP transient, which ultimately became the OKJIP transient (Fig. 6). In the polyphasic fluorescence transients, phase K is often considered as an indicator of OEC damage caused by the inhibition of electron transfer from the donor to the secondary electron donor of PSII, namely Y_Z (Guissé *et al.* 1995). Exposure to stress resulted in the marked appearance of a K step (about 0.3 ms), especially under conditions of combined stress (Figs. 6, 7*B*). These findings suggest damage to OEC was caused by the stress.

Consistent with previous studies, we demonstrated that overaccumulation of GB by transformation of a GB synthesis gene into plants can protect the photosynthetic apparatus from stress-induced damage (Sakamoto and Murata 2002) and enhance the stress tolerance of plants (Figs. 2, 3). In Fig. 3 we show that stress caused ultrastructural damage to the chloroplast and thylakoid, but the damage was less severe in T6 than in WT. These results were also consistent with the observations of the polyphasic fluorescence transients and OEC activity (Figs. 6, 7*A*).

Overaccumulated GB can improve the structure and function of the photosynthetic apparatus under stress probably by steadying the lipid in thylakoid membrane: The changes caused to the structure and composition of the thylakoid membrane by stress may be involved in the functional impairment of PSII and OEC. In general, the function of the thylakoid membrane is based on fluidity and integrality, which are determined by its composition of lipids, membrane proteins, pigments, ions *etc*. Garab *et al.* (2000) reported that the membrane protein can inhibit the formation of nonbilayer structures in the thylakoid lamellae, without this inhibition, excess lipids are secreted. On the other hand, the functions of protein complexes were influenced by the lipid matrix (Vijayan and Browse 2002, Huseynova *et al*. 2007).

Stress can cause changes in the fluidity and composition of membrane (Larkindale and Huang 2004), followed by the formation of nonbilayer lipid structures (Gounaris *et al*. 1984). DGDG is considered a bilayer-prone lipid. In contrast, MGDG has characteristics that allow it to form nonbilayer lipid structures (Webb and Green 1991, Bruce 1998), and it does not contribute to the stability of the membrane. Therefore, the low ratio of MGDG/DGDG suggests high stability of the thylakoid membrane. In this study, exposure to stress decreased both MGDG and DGDG contents (Figs. 4*A,B*); however, the MGDG/DGDG (Fig. 4*F*) ratio was high in WT because the DGDG level decreased to a greater extent than the MGDG one. This effect did not contribute to the stability of the thylakoid membrane. Further, this effect was smaller in T6 than in WT because of the relative stability of MGDG/DGDG in T6 (Fig. 4*F*). This result is consistent with the findings of the previous reports (Williams *et al*. 1992, Williams 1998).

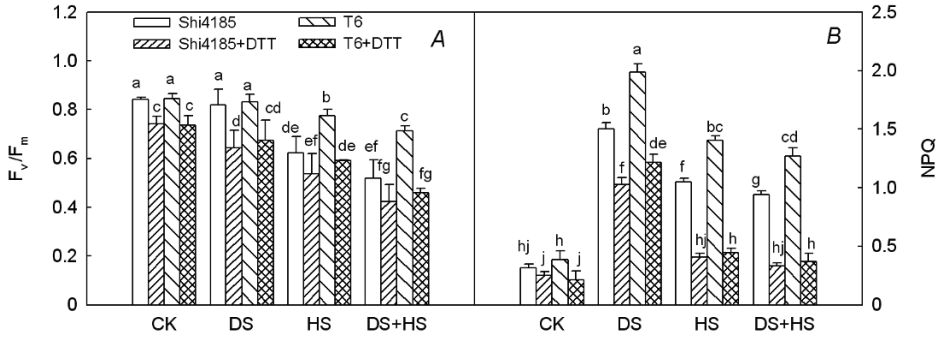

Fig. 9. Effects of different stresses and overaccumulated glycine betaine on F_v/F_m (*A*) and NPQ (*B*) of wheat leaves in the absence or presence of 10 mM 1,4-dithiothreitol (DTT, treated for 3 h), then wheat leaves along with their stalks were immersed into water (CK), 30% PEG-6000 (DS), 40°C (HS) or 30% PEG-6000+40°C (DS+HS) for 3 h. Each bar is the mean ± SD of four replications. Bars with the same letter were not significantly different at $p< 0.05$.

Phosphatidylglycerol (PG), an acidic/anionic thylakoid phospholipid, is limiting for chloroplast structure and function (Yu and Benning 2003). PG was closely related to both the formation and function of the photosynthetic apparatus (Hagio *et al.* 2002, Xu *et al.* 2002, Domonkos *et al.* 2004), and especially the PSII structure (Kanervo *et al*. 1997). The higher relative content of 16:1(3t) in the PG is important for maintaining the structure and function of thylakoid membrane (Pick *et al.* 1985, Sigrist *et al.* 1988, Tremolieres *et al.* 1989). The majority of PG is localized to the site of oxygenic electron transport (Wada and Murata 1998). Exposure to stress decreased the content of PG (Fig. 4*C*) as well as the relative content of 16:1(3t) in PG (Table 1), especially under combined stress conditions. However, the content of PG was higher in T6 than in WT under different stress conditions (Fig. 4*C*). The high relative content of 16:1(3t) in PG in T6 (Tables 1 and 2) facilitated the maintenance of the structure and function of the thylakoid membrane.

Moreover, our results suggested that PC was present in wheat leaf thylakoids (Table 1), which was consistent with the findings of Siegenthaler *et al.* (1989); further, the significant effect of overaccumulated GB on lipid contents was also observed in the case of PC under both normal and stress conditions (Fig. 4*E*). However, Dorne *et al.* (1990) reported that the occurrences of PC in thylakoid preparations probably reflect the contamination of the thylakoids by the envelope membranes. Therefore, both the presence of PC and the mechanism underlying the effect of GB on PC need further research.

Damage of thylakoid membranes also results from the changes of IUFA. Gounaris *et al.* (1983) reported that the increase of saturated fatty acids in MGDG and DGDG can decrease the tendency of nonbilayer lipid structure formation. Thomas (1986) and Karim *et al.* (1999) reported that an increase in saturated fatty acids in mature leaves elevated the melting temperature of plasma membranes, thereby increasing the heat tolerance of the plant. These results suggest that low IUFA may be favorable for tolerating heat stress. However, as shown in Fig. 5 and Table 2, under all stress conditions, the IUFA of lipids in the thylakoid membrane was significantly lower than that under normal condition, especially PG-IUFA and SQDG-IUFA. We suggest that a significant decrease in IUFA under stress conditions is also disadvantageous to the stability of the thylakoid membrane because it tends to reduce the fluidity of the thylakoid membrane. However, a smaller decrease of IUFA in T6 as compared to that in WT can contribute to the stability of the thylakoid membrane.

Xanthophyll cycle-dependent nonradiative energy dissipation may be involved in the improvement of GB: The photosynthetic apparatus could protect itself under stress by increasing the xanthophyll cycle around PSI and PSII to dissipate excessive energy (Havaux *et al*. 2000). The xanthophyll cycle plays a major role in photoprotection and the resulting zeaxanthin plays a key role in NPQ (Gisselsson *et al*. 2004). Based on the results above (Figs. 6, 7) and our previous studies (Wang *et al*. 2008), GB could improve the efficiency of PSII photochemistry and alleviate photoinhibition under stress conditions. Our data in Fig. 9 suggest that exposure to stress resulted in an obvious decrease of F_v/F_m, in parallel with an increase in NPQ (Figs. 8*A*, 9). NPQ is primarily composed of high energy state quenching (q_E) and photoinhibitory quenching (q_I) (Johnson *et al.* 1993). In general, q_E is a major contributor to NPQ and is xanthophyll cycle-dependent (Demmig-Adams 1990). Furthermore, NPQ is essential for protecting the leaf from irradiation-induced damage (Johnson *et al*. 1993, Horton *et al*. 1996). Fig. 8 also shows that the stress-induced increase of NPQ was accompanied by q_E and q_E/NPQ increases, in agreement with previously reported results.

How q_E could increase while the membrane is damaged (and thus no ΔpH can be maintained) by stress? According to Demmig and Winter (1988), upon darkening of leaves, the relaxation of fluorescence quenching showed biphasic kinetics. One portion of total nonphotochemical quenching, designated $q_{E\text{-}fast}$, was linearly related to the acidity of the thylakoid lumen. The remaining component, designated $q_{E\text{-}slow}$, relaxed much more slowly and was previously shown to have the characteristics of a heat dissipation process. This second type of fluorescence quenching was suggested to be related to the operation of the xanthophyll cycle in thylakoid membranes with zeaxanthin acting as a fluorescence quencher and mediating radiationless dissipation. However, the ΔpH requirement for q_E had little effect on the maximum q_E capacity (Noctor *et al.* 1991). This is consistent with our results presented in Fig. 8.

Overaccumulation of GB in T6 resulted in increased NPQ and q_E, but not q_I (Fig. 8). These observations suggest that the xanthophyll cycle may be involved in the improvement of protective mechanisms against photoinhibition by GB overaccumulation. To test this hypothesis, DTT was used to inhibit the xanthophyll cycle by inhibiting violaxanthin de-epoxidase (VDE) *in vivo*. The results in Fig. 9 show that F_v/F_m and NPQ were significantly inhibited by DTT treatment, especially under stress conditions. The decrease of NPQ was more significant than the decrease of F_v/F_m. Furthermore, as shown in Fig. 8, the increase of NPQ caused by overaccumulation of GB was lost. These results imply that xanthophyll cycle-dependent nonradiative energy dissipation plays an important role in photoprotection and in GB-mediated alleviation of photoinhibition under stress.

The increase of NPQ is dependent on the increase of pH gradient built up across the thylakoid membrane (ΔpH) and the formation of zeaxanthin in xanthophyll cycle (Demmig *et al*. 1990). The accepted hypothesis is that when the thylakoid membrane lumen pH dropped, the violaxanthin (Vx) is rapidly convertible to zeaxanthin (Zx), then Zx and ΔpH induced conformational changes

in LHCII, resulting in forming the center of energy dissipation (Krieger *et al*. 1992, Gilmore 1997). GB can improve NPQ by protecting VDE from photodamage directly, since VDE is a key enzyme in the xanthophyll cycle and is localized in the thylakoid membrane (Havaux *et al*. 2000). Also, GB may improve NPQ indirectly by protecting lipids (Figs. 4, 5, Tables1, 2) in thylakoid membrane, for ΔpH is closely related to the fluidity and integrality, which are determined by its composition. However, GB cannot protect VDE from damage caused by DTT (Wang *et al.* 2008). But it is important to consider that DTT is not a specific inhibitor of VDE and may also interfere with other proteins.

In conclusion, our results demonstrate that chloroplast and thylakoid structure and function were damaged by different types of stress and that the damage was more severe when sources of stress were combined. Overaccumulation of GB alleviated the effects of this damage. GB may stabilize the lipid composition of the thylakoid membrane, and GB appears to enhance xanthophyll cycle-dependent nonradiative energy dissipation, these may be involved in the protection of thylakoid structure and function.

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