Overaccumulation of glycine betaine enhances tolerance to drought and heat stress in wheat leaves in the protection of photosynthesis

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Abstract

We investigated the different responses of wheat (*Triticum aestivum* L.) plants to drought- (DS) and heat stress (HS), and analyzed the physiological mechanisms of glycine betaine (GB) involved in the improvement of wheat tolerance to the combination of these stresses. The transgenic wheat T6 line was generated by introducing a gene encoding betaine aldehyde dehydrogenase (BADH) into the wild-type (WT) Shi4185 line. The gene was cloned from the Garden Orache plant (*Atriplex hortensis* L.). Wheat seedlings were subjected to drought stress (30%, PEG-6000), heat stress (40°C), and their combination. Photosynthetic gas exchange, water status and lipid peroxidation of wheat leaves were examined under different stresses. When subjected to a combination of drought and heat, the inhibition of photosynthesis was significantly increased compared to that under DS or HS alone. The increased inhibition of photosynthesis by the combined stresses was not simply the additive stress effect of separate heat- and drought treatments; different responses in plant physiology to DS and HS were also found. HS decreased the chlorophyll (Chl) content, net photosynthetic rate (P_N), carboxylation efficiency (CE) and apparent quantum yield (AQY) more than DS but DS decreased the transpiration rate (E), stomata conductance (g_s) and intercellular CO_2 concentration (C_i) more than HS. GB over-accumulation led to increased photosynthesis not only under individual DS or HS but also under their combination. The enhancement of antioxidant activity and the improvement of water status may be the mechanisms underlying the improvement of photosynthesis by GB in wheat plants.

Additional key words: lipid peroxidation; photosynthetic gas exchange; stress combination; transgenic wheat; water status.

Introduction

The effects of stress conditions on plants are traditionally evaluated by applying a single stress such as drought or heat. However, in the field, the combination of some stresses such as drought and heat often occur simultaneously. Therefore, the effects of individual stresses applied in experimental conditions may not correlate well with those in natural conditions (Mittler 2006). For instance, the transcriptome analysis of tobacco plants subjected to a combination of DS and HS revealed a new pattern of defense response different from those found with drought or heat stress applied separately (Rizhsky *et al*. 2002). Different stresses may induce conflicting or antagonistic responses. During heat stress, for example, plants open their stomata to cool their leaves by transpiration; however, if heat stress is combined with drought, the plants are unable to open their stomata so that the leaf temperature remains high (Rizhsky *et al*. 2002). Some plant physiological studies have shown that

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Abbreviations: APX – ascorbate peroxidase; AQY – apparent quantum yield; AsA – ascorbic acid; BADH – betaine aldehyde dehydrogenase; C_i – intercellular CO_2 concentration; Car – carotenoid; CAT – catalase; CE – carboxylation efficiency; Chl – chlorophyll; CK – well-watered control; DM – dry mass; DS – drought stress; DS+HS – combination of drought- and heat stress; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; GSH – reduced glutathione; GSSG – oxidized form of glutathione; HS – heat stress; MDA – malondialdehyde; OA – osmotic adjustment; POD – peroxidase; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; ROS – reactive oxygen species; RWC – relative water content; SFM – water-saturated fresh mass; SOD – superoxide dismutase; Φ_{PSII} – actual efficiency of PSII photochemistry, ψ_s – osmotic potential.
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water stress can enhance the tolerance of PSII to high temperatures (Lu and Zhang 1999), but others reported that drought greatly exacerbated the effects of high temperatures on plant growth and photosynthesis (Xu and Zhou 2005). These data highlight the need for further research on plant responses to stress combinations. Wheat is one of the most important food crops planted worldwide. The combination of drought- and heat stress is the major stress condition that restricts wheat growth and production but relatively little is known about how their combination impacts wheat plants. GB is a quaternary ammonium compound synthesized naturally in a wide variety of organisms. Many studies indicate that GB might play an important role in enhancing plant tolerance to individual DS and HS (Khan *et al.* 2009). However, until now, there have been no reports on whether GB can protect wheat plants against the combination of DS and HS.

In our previous study, we found that both DS and HS induced ultrastructural damage to the chloroplast and thylakoid lamellae with the withered phenotype by both DS and HS, and the damage was exacerbated by the combination of those stresses. Overexpression of GB could protect the chloroplast and thylakoid lamellae from damage not only with DS or HS separately, but also when they were applied in combination (Wang *et al*. 2010). In this study, we performed further analysis on the different effects of DS, HS, and their combination on photosynthesis, and analyzed the mechanisms underlying the improvement of photosynthesis by GB by utilizing the same GB transgenic and wild-type wheat lines as in our previous study. The results suggested that a combination of drought- and heat stress increased the inhibition of photosynthesis compared to heat- or drought stress alone. Overexpressed GB increased the tolerance of the plants to all stresses in terms of maintaining photosynthesis rates. The improvement of water balance and antioxidant metabolism may be involved in GB increasing photosynthesis under stress conditions. These results may provide useful information for designing strategies to improve the tolerance of wheat to stress combinations in the field.

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°C ± 1°C with a photon flux density of 300 μmol m^{-2} s^{-1} of the photosynthetically active radiation, a relative humidity of 75% to 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% to 86% (typically 3 d). A combination of DS and HS 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, the high humidity (about 75%) in the chamber was maintained to avoid possible drought stress due to water evaporation caused by high temperature. All of the treatments included single DS, single 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.

Determination of GB: The 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).

Photosynthetic gas-exchange parameters measurements: The measurements of photosynthetic gas-exchange parameters including net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), apparent quantum yield (AQY) and carboxylation efficiency of photosynthesis (CE) were carried out with fully expanded attached leaves using a portable infrared gas analyzer (*CIRAS-2*, *PP Systems*, Norfolk, UK). The light-saturating net photosynthetic rate was recorded at a CO_2 concentration of 360 μl l^{-1} and saturating light of 800 μmol m^{-2} s^{-1}. The relative air humidity in the chamber was about 75%. The measurements on these photosynthetic parameters lasted approximately 10 min, during which no significant recovery was observed.

To determine apparent quantum yield (AQY), we first measured the P_N-PPFD (photosynthetic photon flux density) response curve. P_N-PPFD was also measured by using a portable infrared gas analyzer *CIRAS-2*. CO_2 and air temperature in the leaf chamber were maintained at 360 μmol mol^{-1} and 25ºC, respectively. PPFD started at 1400 μmol m^{-2} s^{-1} and decreased stepwise to 50 μmol m^{-2} s^{-1}. Apparent quantum yield (AQY) was calculated from the initial slopes by linear regression using PPFD values below 200 μmol m^{-2} s^{-1}.

The photosynthetic response to C_i was measured at saturating PPFD by step changes of CO_2 concentrations measured in a cuvette. Different CO_2 concentrations were generated inside the leaf chamber by using a CO_2 regulator (*CIRAS-2*, *PP Systems*, Norfolk, UK). By plotting P_N *versus* C_i we calculated the CO_2-response curve. The *in vivo* carboxylation efficiency (CE) was estimated as the slope of the linear portion of the CO_2-response curve.

Chl *a* fluorescence analysis: PSII chlorophyll fluorescence measurements were performed using an *FMS-2* portable pulse modulated fluorometer (*Hansatech*, King´s Lynn, UK). The minimal fluorescence level (F_o) with all PSII reaction centers open was measured by the measuring modulated light sufficiently low (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence, and the maximal fluorescence level (F_m) with all PSII reaction centers closed were determined by a 0.8-s saturating pulse at 8,000 μmol m^{-2} s^{-1} in dark-adapted for 20 min leaves. Next, the actinic radiation (300 μmol m^{-2} s^{-1} PPFD) was turned on after the fluorescence signal leveled off, the steady-state fluorescence (F_s) and the light-adapted maximum fluorescence (F_m') were determined, then the actinic radiation was removed and the minimal fluorescence in the light-adapted state (F_o') was determined by irradiating the leaf segment for 2 s with infrared radiation. Maximum quantum yield of PSII (F_v/F_m) and the actual PSII efficiency under irradiance (Φ_{PSII}) were calculated according to Genty *et al*. (1989), $F_v/F_m = (F_m - F_o)/F_m$, $\Phi_{PSII} = (F_m' - F_s)/F_m'$.

Assays of thylakoid membrane Ca^{2+}-ATPase and Mg^{2+}-ATPase activities: Chloroplasts were isolated according to the method by Zhao *et al*. (2007). Briefly, 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 debris were discarded. The remaining chloroplast pellet 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 activation by ions; these two ATPase activities are classified into 2 types according to the type of ions used, Mg^{2+}- and Ca^{2+}-dependent ATPases (Ma *et al*. 2006).

Ca^{2+}-ATPase activity was determined following the activation of the coupling factor by trypsin 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 ethylenediamine tetraacetic acid (EDTA), 10 mM ATP, and 2 mg cm^{-3} trypsin, 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 in 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 reaction. The incubated chloroplast suspension (0.5 cm^3) was then added to the 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, and the supernatant was used to determine the content of inorganic phosphorus.

Water status measurements: Leaf relative water content (RWC) was calculated from (FM – DM)/(SFM – DM) ×100%, where FM is the fresh mass, SFM is the water-saturated fresh mass, and DM is the dry mass after oven-drying samples at 80°C for 24 h (Ma *et al*. 2007). Osmotic adjustment (OA) was determined using a vapour pressure osmometer (*5520, WESCOR*, Logan, UT, USA). For the measurement of osmotic potential at full turgor (ψ_s^{100}), tissues were rehydrated with deionized water for 6–8 h at 4°C in the dark. OA was calculated as the difference of ψ_s^{100} at full turgor (RWC = 100%) between unstressed (ψ_{sc}^{100}) and stressed (ψ_{ss}^{100}) treatment: OA (MPa) = $\psi_{sc}^{100} - \psi_{ss}^{100}$

Determinations of Chl, free proline and soluble sugar contents: The Chl and carotenoid (Car) contents were determined spectrophotometrically according to Arnon (1949). The proline content was determined spectrophotometrically by the ninhydrin method described by Bates *et al*. (1973). The soluble sugar content was determined colorimetrically using a phenolsulphuric acid technique (Tissue and Wright 1995).

Measurements of ion leakage and MDA levels: The ion leakage from the cellular membrane was determined according to Fan *et al*. (1997). Malondialdehyde (MDA) level was assayed according to Prochazkova *et al*. (2001).

Determinations of superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) productions: The rate of $O_2^{\cdot -}$ generation was determined by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to a previously described method (Beauchamp and Fridovich 1971, Cakmak and Marschner 1992). H_2O_2 content was determined

according to Sairam and Srivastava (2002). The concentration of H_2O_2 was estimated by measuring the spectrum absorbance of the titanium-hydroperoxide complex and using a standard curve plotted with known concentration of H_2O_2.

Assays of antioxidant enzyme activities: Total superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities were determined as described by Prochazkova *et al*. (2001). Samples (1 g FM) were excised and ground with a pestle in an ice-cold mortar with 8 ml of 0.05 M Na_2HPO_4/NaH_2PO_4 (pH 7.8) buffer containing 1 mM EDTA-Na_2. The homogenates were filtered through four layers of gauze and then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were collected and used to assay antioxidative enzymatic activities.

Assays of nonenzyme antioxidants: Ascorbic acid (AsA) was determined according to Arakawa *et al*. (1981). Wheat leaves were homogenized in 5 ml of 5% trichloroacetic acid (TCA), and centrifuged at 4,000 × *g* for 10 min. 1 ml of clear supernatant was added to 1 ml of 5% TCA and 1 ml alcohol, and then to 0.5 ml 0.4% phosphoric acid (H_3PO_4)-alcohol, 1 ml of 0.5% 4,7-diphenyl-1,10-phenanthroline (BP)-alcohol and 0.5 ml 0.03% ferric trichloride ($FeCl_3$)-alcohol. The reaction mixture was incubated at 30°C in a water bath for 90 min. The reaction was terminated at the room temperature, after which the absorbance spectrum of the supernatant at 534 nm was determined with a spectrophotometer (*UV1601, Shimadzu Corp*., Kyoto, Japan).

Total glutathione (GSH + GSSG) was determined by an enzymatic cycling assay method described by Noctor and Foyer (1998b). GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. GSH was determined by subtraction of GSSG from the total glutathione content.

Assays of GB scavenging $O_2^{\cdot -}$ and H_2O_2: The ability of GB to scavenge superoxide anions ($O_2^{\cdot -}$) and H_2O_2 was determined according to the procedure by Luo *et al*. (2008) with some modifications. Each reaction mixture contained 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.8) buffer, 65 mM methionine (Met), 500 μM nitroblue tetrazolium (NBT), 100 μM Na_2EDTA (pH 7.6), 66 μM riboflavin, and an appropriate aliquot of enzyme extract and/or GB solution at the final concentrations indicated in Fig. 6 in which the amount of enzyme extract was equal to that of the GB solution. The ability of GB to scavenge $O_2^{\cdot -}$ was expressed as the quotient of the absorbance spectrum at 560 nm in the presence of GB subtracted from that in the absence of GB, divided by the latter absorbance value. For assays of GB scavenging H_2O_2, the absorbance spectrum was measured at 520 nm.

Statistical analysis was conducted using the procedures of *DPS* (*Data Processing System*, 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 and discussion

GB content in wheat leaves: To correlate the presence of GB with protection of the photosynthesis process from stress, we measured the GB content in leaves of wheat plants subjected to drought, heat and their combination. As shown in Fig. 1, the GB content of the transgenic wheat line T6 was significantly higher than that of the wild-type Shi4185 line (WT) when no stress treatment was given due to the constitutive expression of the transgenic *BADH* gene. When subjected to DS, HS and DS+HS, GB levels in the leaves of both lines increased significantly higher than that of CK, especially T6, indicating that the wheat plants were sensitive to these stresses and responded by accumulating GB in the leaves.

It has been reported that the accumulation of GB in plants is induced by stress conditions (Gorham 1995). Our study confirms that wheat synthesizes and accumulates GB *in vivo* naturally, and drought, heat as well as a combination of DS and HS all induced GB accumulation in wheat leaves (Fig. 1). Furthermore, DS+HS induced a greater level of GB than individual DS or HS. Fig. 1 also shows that the stress-induced GB was significantly higher in the T6 than the WT line. This was unexpected considering the constitutive expression of the *BADH* transgene under a constitutive maize ubiquitin promoter (Ubi1). We hypothesize that the overaccumulation of GB in T6 can protect the BADH protein itself

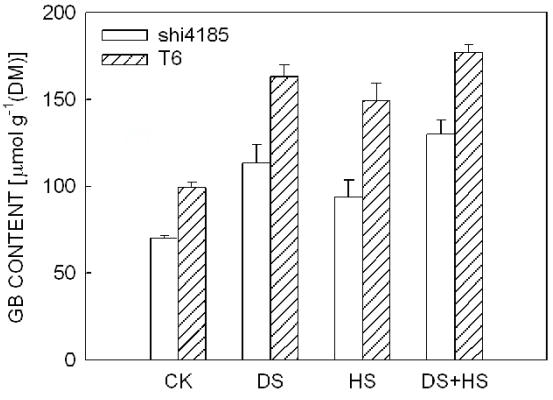

Fig. 1. Glycine betaine (GB) contents in leaves of wheat plants subjected to drought, heat and their combination. CK: well-watered control. DS – drought stress, HS – heat stress, DS+HS – combination of drought- and heat stress. Each bar represents the mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

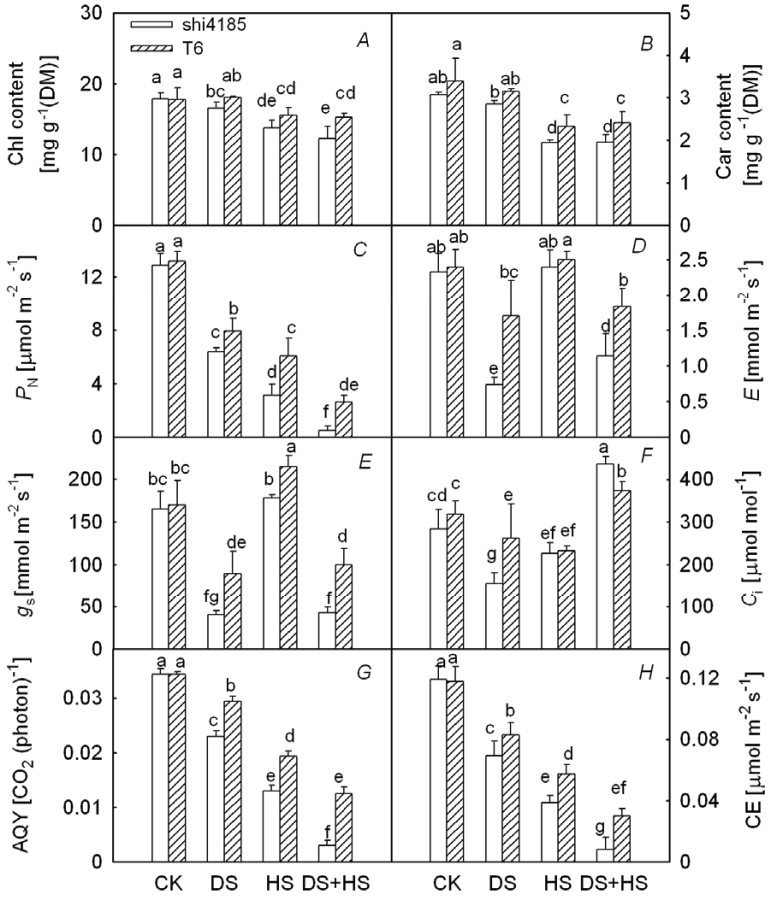

Fig. 2. Chlorophyll (Chl) (*A*) and total carotenoid (Car) (*B*) contents, net photosynthetic rate (P_N) (*C*), transpiration rate (*E*), (*D*), stomatal conductance (g_s), (*E*), intercellular CO_2 concentration (C_i), (*F*), the apparent quantum yield (AQY), (*G*) and the carboxylation efficiency of photosynthesis (CE), (*H*) of wheat plants subjected to drought (DS), heat (HS) and their combination (DS+HS). CK – well-watered control. Each bar is the mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

from stress-induced damage, which probably leads to accumulation of more GB in T6 than WT under stress conditions. However, the underlying mechanisms need to be validated in the future.

Effects of overaccumulation of GB and environmental stresses on CO_2 assimilation and the relevant gas exchange parameters: As the foundation of crop growth and yield, photosynthesis is one of the most important metabolic processes. Therefore, the maintenance of high photosynthesis is a key factor for crop yield under stress. In our previous study, we found that HS or DS could damage the ultrastructure of chloroplast and thylakoid lamellae, but the injury was exacerbated when both stresses were combined. Overaccumulation of GB alleviated this damage. In this study, we investigated the effects of overaccumulated GB and environmental stress on various physiological parameters of CO_2 assimilation in wheat leaves.

As shown in Fig. 2*A,B*, under normal conditions, both the Chl and carotenoid (Car) contents were nearly the same in T6 as in WT. HS decreased Chl and Car contents significantly in both wheat lines ($P<0.05$), but the decrease was smaller in T6 than in WT. DS did not decrease Chl and Car levels significantly, and there was just a slight decrease of Chl in the WT line. When DS and HS were imposed simulaneously, however, a drastic decline of Chl and Car levels were observed in both wheat lines, indicating that the combined stress reduced the pigments compared with the individual stress. However, the Chl and Car contents were maintained at a relatively higher level in T6 than in the WT plants under all stress conditions, suggesting that increased GB provided protection from the damage by environmental stresses.

Drought, heat, or the two stresses together significantly affected CO_2 assimilation and other gas exchange parameters. Fig. 2*C* shows that net photosynthetic rates (P_N) of both WT and T6 were significantly inhibited by HS and DS and were dramatically decreased upon exposure to DS+HS. Similar results were observed in the apparent quantum yield (AQY) and the carboxylation efficiency of photosynthesis (CE) as shown in Fig. 2*G,H*, respectively. However, the effects of different stresses on E and g_s were different from those of P_N, CE and AQY. For instance, DS resulted in a decrease in g_s and E, but HS resulted in opening stomata and increasing E. When HS was accompanied by DS, g_s and E were suppressed by the combination stress treatment. These results are consistent with the reports from Rizhsky *et al*. (2002). Additionally, drought or heat stress alone decreased C_i in the plants. When the plants were subjected to both DS and HS, however, C_i was increased significantly. Compared to the changes of C_i to g_s and P_N under the

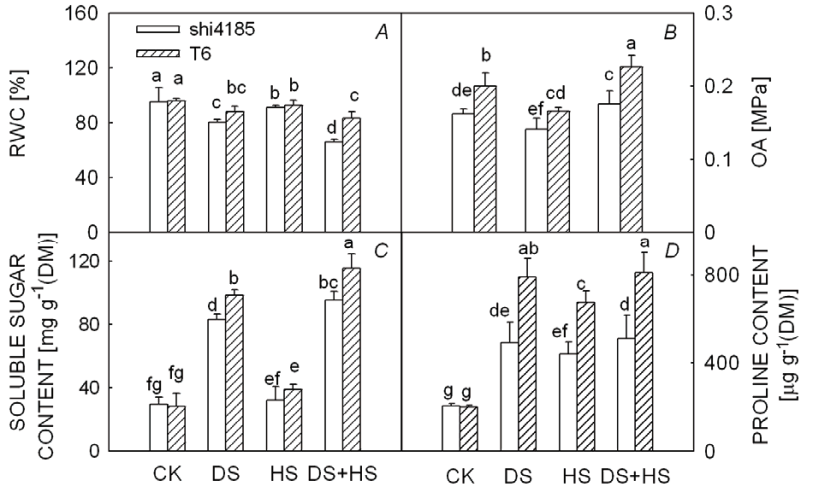

Fig. 3. Leaf relative water content (RWC) (*A*), osmotic adjustment (OA) (*B*), total soluble sugars (*C*) and proline (*D*) contents of wheat plants subjected to drought (DS), heat (HS) and their combination (DS+HS). CK – well-watered control. The values are mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

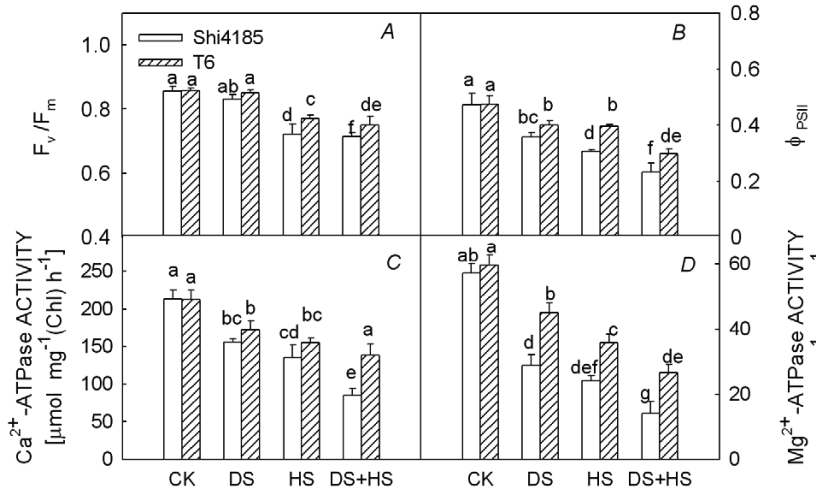

Fig. 4. Maximal efficiency of PSII photochemistry (F_v/F_m) (*A*), actual efficiency of PSII (Φ_{PSII}) (*B*), the activities of Ca^{2+}-ATPase (*C*) and Mg^{2+}-ATPase (*D*) in the thylakoid membrane of wheat leaves subjected to drought (DS), heat stress (HS) and their combination (DS+HS). Each bar is the mean ± SD of four replications. Bars with the same letter were not significantly different at $P<0.05$.

identical stress conditions, we suggest that the decrease of C_i under drought stress may be related to the g_s decrease, but the increase of C_i under the combination of stresses may involve the decreased CO_2 assimilation. These results suggest that the aggravated damage of photosynthesis by stress combination is not an additive effect, but rather may be a synergic effect. The data in Fig. 2 also revealed that overaccumulation of GB in T6 improved P_N and various gas exchange parameters under all stresses. In particular, the most evident effect of GB is on g_s, then on AQY, and then finally on CE.

The maintenance of cell water balance may be involved in the improvement of GB in g_s: What are the mechanisms of GB improving P_N as well as gas exchange parameters under stress conditions, especially under DS+HS? It is known that the improvement of GB on P_N (Fig. 2*C*) results mostly from its effects on gas exchange parameters. Among these parameters, E and C_i are involved in g_s, and furthermore, g_s is related to the water status of the cell. Otherwise, ATP supply and energy translation are involved in AQY and CE. Then, in the following experiments, we measured the changes of the water status, ATPase activity and PSII photochemistry of wheat leaves under these stresses.

Fig. 3*A* shows that HS induced no significant effects on RWC of leaves in both WT and T6, which may be due to the short duration of the HS treatment (3 h). In contrast, drought stress resulted in a significant decrease in RWC, and DS+HS deteriorated the RWC further. Overaccumulation of GB in T6 alleviated the decrease in RWC compared to WT.

To investigate the mechanism underlying the improvement of overaccumulated GB in water status in the transgenic wheat line, we further detected the Ψ_s^{100} of the wheat leaves subjected to DS, HS and DS+HS, and estimated the osmotic adjustments (OA) of the two wheat lines. As shown in Fig. 3*B*, the two wheat lines responded to all three stresses with a significant decrease in osmotic potential which resulted in the appearance of OA. The response of OA to DS was greater than that to HS, and the greatest OA was observed under DS+HS. The OA of T6 was greater than that in the WT plants, suggesting that stress-induced GB accumulation in the two wheat lines as well as the overaccumulation of GB in T6 may all contribute to the maintenance of OA and RWC in wheat leaves.

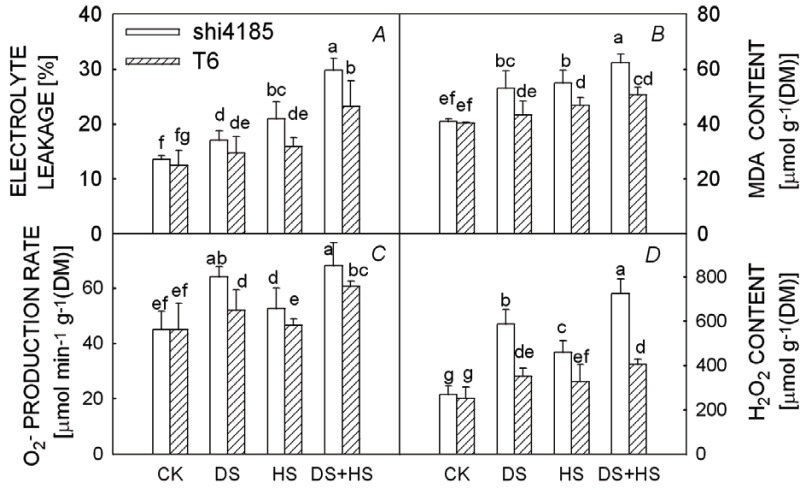

Fig. 5. Ion leakage (*A*), MDA content (*B*), the production rate of $O_2^{\cdot -}$ (*C*) and H_2O_2 content (*D*) of the wheat leaves subjected to drought (DS), heat stress (HS) and their combination (DS+HS). Each bar is the mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

Considering the increased GB level in T6 was not sufficient to match the OA, other metabolites probably also contributed to the observed OA. We also detected the total soluble sugars and proline levels in the wheat leaves (Fig. 3*C,D*), and the results indicated that total soluble sugars and proline levels increased under DS, HS and DS+HS, and those were dramatically higher in T6 than in WT. This was consistent with OA levels in Fig. 3*B*. These findings suggest that the overaccumulated GB in T6 is related to the accumulations of other metabolites, which are in turn related to changes in OA and improvements in RWC. These results are consistent with other previous reports (Ma *et al.* 2007).

From the results above, we conclude that the increase of GB itself (Fig. 1) and other metabolites (*e.g.* soluble sugars and proline, Fig. 3*C,D*) induced by GB in T6 can decrease the osmotic potential of the plant cell (Fig. 3*B*), which can enhance the absorbance of water into the plant cells, resulting in opening stomata (Fig. 2*E*) and ultimately improving water status (Fig. 3*A*) of wheat plant cells.

The improvement of GB in AQY and CE may be related to the enhanced activity of the protein complex in the thylakoid membrane: GB can improve the photosynthetic capacity not only by increasing stomatal conductance but also by maintaining protein activity in the chloroplast (Nomura *et al.* 1998) under stress conditions. Considering that levels of AQY and CE are related to the excitation energy capture and translation as well as ATP anabolism in photosynthesis, the characteristics of PSII photochemistry and ATPase activity in the thylakoid membrane were detected in this study (Fig. 4).

The maximal efficiency (F_v/F_m) and actual efficiency (Φ_{PSII}) of PSII photochemistry are usually used as a sensitive indicator of plant photosynthetic performance responding to stress conditions (Maxwell and Johnson 2000), and both F_v/F_m and Φ_{PSII} represent a measure of the functional status of the PSII. Fig. 4 shows that, under nonstress conditions, no significant differences in F_v/F_m and Φ_{PSII} of PSII photochemistry were observed between T6 and WT. HS and DS+HS decreased F_v/F_m significantly (Fig. 4*A*), but individual DS had little effect on it and all three stress conditions decreased Φ_{PSII} (Fig. 4*B*). The combination of drought- and heat stress resulted in a more drastic decline of F_v/F_m and Φ_{PSII} than each stress alone. Compared to WT, overaccumulation of GB in T6 alleviated the decrease of both F_v/F_m and Φ_{PSII} under stress conditions, indicating that PSII complex in T6 could better withstand photoinduced inactivation than WT under stress conditions.

As shown in Fig. 4*C,D*, DS, HS and DS+HS significantly ($P<0.05$) decreased the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the thylakoid membrane, but the extent of the decrease was different among the three groups of stress treatments. The combined stress treatment had a more drastic decline of these enzyme activities than with application of each stress type alone. Fig. 4 also revealed that the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in T6 were always higher than those of WT during stress treatments, suggesting the protection of overaccumulated GB from damage in ATPase under stress conditions. All these results were consistent with our previous study (Wang *et al*. 2010).

Effects of environmental stress and GB on membrane integrity: DS and HS can damage the cell membranes (Chaidee and Pfeiffer 2006), including the chloroplast membrane and thylakoid lamellae (Wang *et al.* 2010) and disturb the cell metabolism and functions (Terence *et al.* 2003). Lipid peroxidation resulting from the accumulation of reactive oxygen species (ROS) is involved in this process. To investigate the improvement of GB in protection of the cell membrane, we measured the lipid peroxidation in both wheat lines.

Cell membrane stability estimated by ion leakage has been widely used to differentiate the tolerance and susceptibility of plants to stresses (Rehman *et al.* 2004). Fig. 5*A* shows that both DS and HS increased ion leakage significantly ($P<0.05$), with the increase being slightly greater under HS than DS, and the greatest increase was

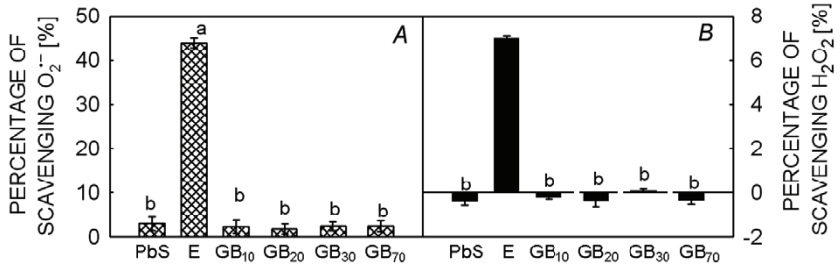

Fig. 6. Glycine betaine (GB) scavenges $O_2^{\cdot -}$ (*A*) and H_2O_2 (*B*). Pbs – phosphate buffer solution. E – enzyme solution extracted from wheat leaves. GB concentration: 10 mM (GB_{10}), 20 mM (GB_{20}), 30 mM (GB_{30}), and 70 mM (GB_{70}), respectively. Each bar is the mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

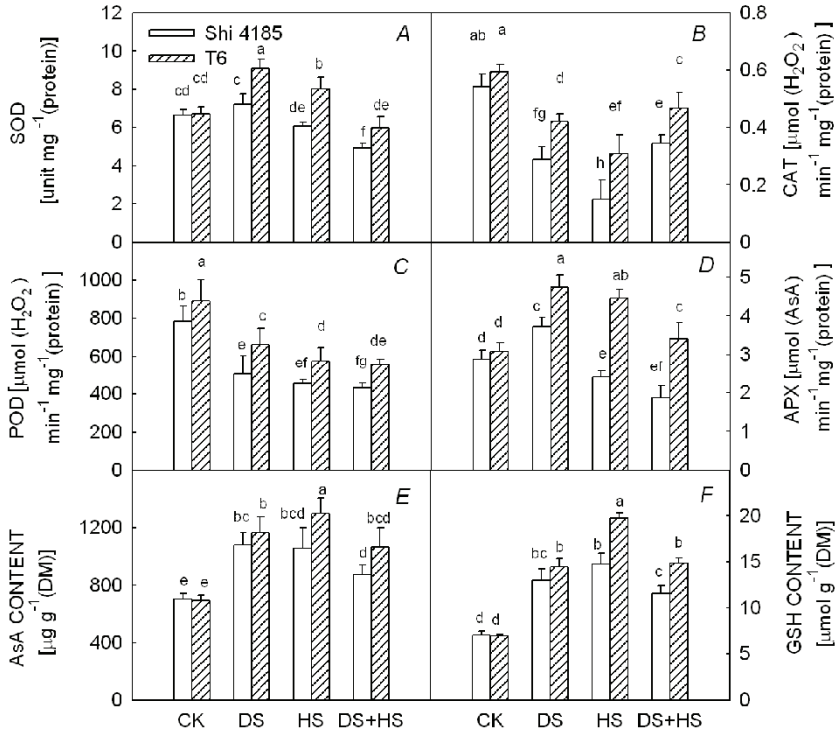

Fig. 7. The activities of superoxide dismutase (SOD) (*A*), catalase (CAT) (*B*), peroxidase (POD) (*C*), and ascorbate peroxidase (APX) (*D*) as well as the accumulation of ascorbic acid (AsA) (*E*) and glutathione (GSH) (*F*) in wheat leaves subjected to drought (DS), heat (HS) and their combination (DS+HS). The values are mean ± SD of three replications. Bars with the same letter were not significantly different at $P<0.05$.

observed under DS+HS ($P<0.01$). Compared with g_s (Fig. 2*E*) and RWC (Fig. 3*A*), the effect of heat stress on cell membrance integrity (Fig. 5*A*) was greater than that on water balance; on the other hand, the effect of drought stress on water balance was greater than that on cell membrane integrity. Perhaps the heat stress fluidifies the lipids of the membrane in this process in addition to damage from ROS. Overaccumulation of GB in T6 could protect the cell membrane from damage under different stress conditions, which resulted in less ion leakage in T6 than in WT, especially under the stress combination.

Cell membrane stability is affected by lipid peroxidation caused by ROS accumulation under stress conditions (Terence *et al.* 2003), which results in the production of malondialdehyde (MDA). As shown in Fig. 5*B*, both DS and HS increased MDA levels in both wheat lines, and much more MDA was generated under DS+HS. Similar results were observed in $O_2^{\cdot -}$ and H_2O_2 production. However, all these levels, including $O_2^{\cdot -}$ and H_2O_2 as well as MDA, were lower in T6 than WT, suggesting that the overaccumulated GB can help to eliminate ROS (Fig. 5*C,D*) and alleviate lipid peroxidation (Fig. 5*B*), thus maintaining the cell membrane stability (Fig. 5*A*). These results are consistent with previous studies *in vitro* (Saneoka *et al.* 2004).

GB can help to scavenge $O_2^{\cdot -}$ and H_2O_2 indirectly: Under normal conditions, the production and elimination of ROS is balanced, and there is no excess of ROS in plant cells. However, ROS accumulated when this balance was disturbed by the stress conditions. From the results in Fig. 5, overaccumulation of GB in T6 can help to decrease ROS levels. However, we needed to know whether GB eliminates ROS directly or indirectly. Thus, the ability of GB to eliminate ROS in a concentration-dependent manner was examined. Extracts of wheat cells which included antioxidant enzymes and phosphate buffer solution (PBS) were used as two controls (Fig. 6). As expected, PBS treatment cannot eliminate $O_2^{\cdot -}$ and H_2O_2. The same results were observed in different concentrations of GB. These results suggest that GB is ineffective in scavenging $O_2^{\cdot -}$ and H_2O_2 directly. By contrast, the enzyme solution extracted from wheat cells showed high levels of $O_2^{\cdot -}$ and H_2O_2 scavenging activity.

Next, we evaluated the antioxidative defense system in both wheat lines *in vivo* (Fig. 7). To do so, we first investigated the activities of several key antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) under different stress conditions. Different responses among the four antioxidant enzymes to different stresses were observed in T6 and WT. In the WT line, the responses of SOD (Fig. 7*A*) and APX (Fig. 7*D*) to DSs were observed as increased activity, but that of CAT (Fig. 7*B*) and POD (Fig. 7*C*) were detected as decreased activity. When they were subjected to HS, however, almost all the antioxidant enzyme activities were decreased except for SOD. These results indicate that there are specific responses of different antioxidant enzymes to different stresses. A stress combination of drought and heat inhibited SOD and APX activities further, but pretreatment of DS increased the tolerance of CAT to HS. A better stabilization in SOD activity than other antioxidant enzymes under stress conditions was also observed. The T6 line with abundantly expressed GB had increased activities of all four antioxidant enzymes, especially APX, followed by CAT, both of which can eliminate H_2O_2; this was consistent with the greater decrease of H_2O_2 accumulation in T6 as shown in Fig. 5*D*.

In contrast to the antioxidant enzymes, the accumulations of two nonenzyme antioxidants, namely ascorbic acid (AsA) and glutathione (GSH), were significantly increased by all conditions (Fig. 7*E,F*). In particular, under HS, the contents of both AsA and GSH were increased significantly, but almost all activities of the four enzymes were decreased by HS (Fig. 7*A–D*), suggesting the enzymes had a weaker tolerance than the nonenzyme antioxidants to HS. The increase of nonenzyme antioxidant levels may be one of the specific responses when wheat plants are exposed to HS. Comparing the increase in the levels of the two nonenzyme antioxidants with separate DS or HS treatment, DS+HS had decreased levels, but they were still higher than in the nonstressed control. Overaccumulation of GB increased AsA and GSH to higher levels in T6 than WT under all stress conditions, but the increase, especially in GSH, was more significant under HS than DS.

These results demonstrate that overaccumulation of GB *in vivo* can induce an enhanced activation of the antioxidative defense system including antioxidative enzymes and antioxidants, which results in the reduction of ROS in the transgenic T6 plants (Fig. 6*C,D*) as compared with the WT; therefore, lipid peroxidation and the damage of cell membrane is alleviated (Fig. 6*A,B*).

However, GB does not have the capability of eliminating ROS directly, but how the accumulation of GB *in vivo* can enhance the antioxidant metabolism is not clear. It has been documented that, *in vitro*, GB stabilizes the structures and activity of enzymes against the damaging effects of excessive salt, cold and heat (Gorham 1995). For instance, GB protects the Rubisco enzyme from inactivation under salt stress by the stabilization of its conformation (Nomura *et al.* 1998). The accumulation of GB in the chloroplast in transgenic plants showed a more significant improvement in the tolerance to abiotic stress than that in the cytosol (Park *et al.* 2007). In this study, some antioxidant enzymes examined were primarily localized in the chloroplast (Noctor and Foyer 1998a); thus, we hypothesize that the accumulation of GB in the chloroplast could help to stabilize the structures of these enzymes under stress conditions. The increased nonenzymatic antioxidants such as AsA and GSH in T6 may be the result of GB activating the corresponding synthesis pathways. However, further research is needed to confirm the mechanism.

In conclusion, the photosynthesis and gas exchange parameters of wheat leaves respond to DS, HS and DS+HS with different ways, especially with g_s (Fig. 2*E*). DS alone results in stomatal closure, but under HS alone, the stomatal openings are the same as with the nonstressed control. However, when applied with DS, HS cannot lead to stomatal opening, consistent with the report by Rizhsky *et al*. (2002). DS+HS aggravates the inhibition of photosynthesis (Fig. 2*C*), which may be related to the decrease of AQY (Fig. 2*G*) and CE (Fig. 2*H*) as well as g_s (Fig. 2*E*). However, the aggravated damage to the photosynthesis process may not be the result of an additive effect, but rather of a synergic effect by the stress combination. The results above are consistent with our previous study demonstrating damage to the ultrastructure of the chloroplast and thylakoid lamellae by the same stress conditions (Wang *et al.* 2010). We also found in this present study that the balance of water metabolism may play a major role in the different behaviors of g_s; meanwhile, the antioxidant metabolism may be more important in the changes of AQY and CE. In general, we found that HS inactivated the activities of antioxidant enzymes to a greater extent than DS, but the increases of nonenzyme antioxidants (AsA and GSH) were observed under all stress conditions. Thus, the improvement of water balance and antioxidant metabolism may be involved in the protection of the photosynthesis process by GB under stress conditions, most likely by indirect elimination of ROS.

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