Plant Biotechnol Rep (2010) 4:213–222
DOI 10.1007/s11816-010-0139-y

ORIGINAL ARTICLE

Improvement of heat and drought photosynthetic tolerance in wheat by overaccumulation of glycinebetaine

Gui-Ping Wang · Zhen Hui · Feng Li · Mei-Rong Zhao · Jin Zhang · Wei Wang

Received: 23 February 2010 / Accepted: 28 April 2010 / Published online: 26 May 2010

Abstract Within their natural habitat, crops are often subjected to drought and heat stress, which suppress crop growth and decrease crop production. Causing overaccumulation of glycinebetaine (GB) has been used to enhance the crop yield under stress. Here, we investigated the response of wheat (*Triticum aestivum* L.) photosynthesis to drought, heat stress and their combination with a transgenic wheat line (T6) overaccumulating GB and its wild-type (WT) Shi4185. Drought stress (DS) was imposed by controlling irrigation until the relative water content (RWC) of the flag leaves decreased to between 78 and 82%. Heat stress (HS) was applied by exposing wheat plants to 40°C for 4 h. A combination of drought and heat stress was applied by subjecting the drought-stressed plants to a heat stress as above. The results indicated that all stresses decreased photosynthesis, but the combination of drought and heat stress exacerbated the negative effects on photosynthesis more than exposure to drought or heat stress alone. Drought stress decreased the transpiration rate (Tr), stomatal conductance (Gs) and intercellular CO_2 concentration (Ci), while heat stress increased all of these; the deprivation of water was greater under drought stress than heat stress, but heat stress decreased the antioxidant enzyme activity to a greater extent. Overaccumulated GB could alleviate the decrease of photosynthesis caused by all stresses tested. These suggest that GB induces an increase of osmotic adjustments for drought tolerance, while its improvement of the antioxidative defense system including antioxidative enzymes and antioxidants may be more important for heat tolerance.

Keywords Glycinebetaine · Transgenic wheat · Drought stress · Heat stress · Photosynthesis

G.-P. Wang · Z. Hui · F. Li · M.-R. Zhao · J. Zhang · W. Wang (✉)
State Key Laboratory of Crop Science/College of Life Science, Shandong Agricultural University, Daizong Street 61, Tai'an 271018, Shandong, People's Republic of China
e-mail: wangw@sdau.edu.cn

G.-P. Wang
Shandong Institute of Pomology, Tai'an 271000, Shandong, People's Republic of China

Introduction

Wheat is one of the most important food crops planted worldwide. Drought and heat stress are the major stress conditions that restrict wheat growth and production. Although drought and heat stress have been extensively studied in wheat (Bahieldina et al. 2005; Frances et al. 2006; Luo et al. 2008), relatively little is known about the differences between mechanisms of survival responses to drought, heat and the combination of these stresses. The response of plants to combined stress is different from that of exposure to a single source of stress. In particular, different stresses might require conflicting or antagonistic responses (Mittler 2006). Therefore, analyzing the differences in tolerance of wheat to drought, heat and the combination of these stresses would greatly benefit the development of urgently needed new wheat cultivars with enhanced stress tolerance.

Glycinebetaine (GB) plays an important role in enhancing plant tolerance to drought or to heat stress (Sakamoto and Murata 2002; Yang et al. 2007; Khan et al. 2009), but it is not known if it can protect wheat plants against the combination of both drought and heat stress. In this study, we interrogated this issue by performing a physiological analysis of a transgenic wheat line with

overaccumulated GB (Guo et al. 2000) and its wild-type Shi4185 line. Our study suggests that there are different physiological responses of wheat photosynthesis to different stresses, and that the combination of drought and heat stress decreased the photosynthesis process more drastically than drought or heat stress alone. Overaccumulation of GB not only improves the tolerance of wheat plants to drought or heat but also to the combination of these stresses. We further analyzed the mechanisms underlying the role of GB in improvement of photosynthesis under the different stress conditions.

Materials and methods

Plant materials and stress treatments

The experiments were carried out with two wheat lines provided by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China. One is a transgenic wheat line overexpressing a *BADH* gene encoding betaine aldehyde dehydrogenase (BADH), designated as 99T6 (T6), and the other was its wild-type (WT) Shi4185 line. T6 was generated by introducing a pABH9 plasmid encoding the *BADH* gene cloned from *Atriplex hortensis* L. (Guo et al. 2000) under the control of a maize ubiquitin promoter and a *bar* gene by microprojectile bombardment.

Seeds of both the T6 and WT plants were sown in earthenware pots (8 L, 24 cm diameter) filled with 3 kg of soil composed of loam and organic fertilizer at the ratio 7:3 and 250 g of complete fertilizer (NH_4NO_3:K_3PO_4:KNO_3 = 20:10:20). About 10 seeds were initially sown in each pot, and four plants per pot were left after germination. All plants in the pots grew in a greenhouse with conventional cultivation conditions (25–30°C/15–20°C (day/night), 300–400 μmol m^{-2} s^{-1}). At the flowering stage, the wheat plants with identical growth status were subjected to various stress treatments in a controllable growth chamber. The various treatment groups included drought-stressed plants (DS), well-watered plants subjected to heat stress (HS), drought- and heat-stressed plants (DS + HS), and control well-watered plants (CK). Drought stress was imposed by controlling irrigation until the relative water content (RWC) of the flag leaves decreased to between 78 and 82% (typically for 6–7 days). Heat stress was applied by transferring wheat plants to a growth chamber pre-conditioned at a temperature of 40°C, and the plants were exposed to this heat for 4 h. During heat stress, high humidity in the growth chamber was maintained to avoid possible drought stress due to water evaporation caused by the high temperature. A combination of drought and heat stress was applied by subjecting the DS plants to a heat stress treatment as above (40°C for 4 h). At the end of treatments, all the plants were returned to the greenhouse, and the detection and sampling were executed immediately. All experiments were repeated at least three times with at least three plants for each wheat line.

Determination of GB content

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

Measurements of photosynthetic gas exchange parameters

The measurements of photosynthetic gas exchange parameters including net photosynthesis rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were carried out with flag leaves using a portable infrared gas analyzer (Ciras-2; PP Systems, Norfolk, UK). The light-saturating photosynthetic rate was recorded at a CO_2 concentration of 360 μL L^{-1} and temperature of 25°C with relative humidity of 80% and saturating light (800 μmol m^{-2} s^{-1}). The measurements on these photosynthetic parameters lasted approximately 10 min, during which no significant recovery was observed.

Water status measurements

Leaf RWC of wheat flag leaves was determined according to Ma et al. (2006). Leaf water potential (ψ_w) was measured with a HR-33 T dew point micro voltmeter (Wescor, Logan, UT, USA) after equilibration in the chamber for 2.5 h. Leaf osmotic potential (ψ_s) and osmotic potential at full turgor (ψ_s^{100}) were determined using a vapor pressure osmometer (5520; Wescor). Osmotic adjustment (OA) was determined according to Lv et al. (2007). For the measurement of osmotic potential at full turgor (ψ_s^{100}, RWC = 100%), the 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} between unstressed (ψ_{sc}^{100}) and stressed (ψ_{ss}^{100}) treatments:

$$\mathrm{OA(MPa)} = \psi_{sc}^{100} - \psi_{ss}^{100}.$$

Determinations of photosynthetic pigments, free proline, soluble protein, free amino acid and soluble sugar contents

Photosynthetic pigments were determined in 80% (v/v) acetone according to Porra et al. (1989). Soluble protein content was determined according to the Bradford method (1976). Proline content was determined spectrophotometrically by

the ninhydrin method described by Bates et al. (1973). For determining the content of total free amino acids, about 50 mg of sample was ground with 5 ml of 10% acetic acid and centrifuged at 5,000*g* at 4°C for 10 min. Then, 50 ml of the supernatants were used to determine free amino acid contents by ninhydrin reaction (Troll and Cannan 1953). The soluble sugar content was determined colorimetrically using a phenolsulphuric acid technique (Tissue and Wright 1995).

Measurements of nitrate reductase (NR) and glutamine synthetase (GS) activities

Nitrate reductase activity was determined as described by Baki et al. (2000) with some modifications. Briefly, the reaction medium (total volume 9 ml) consisted of 100 mM KNO_3, isopropanol (1%, v/v), and 100 mM kalium phosphate, pH 7.5. The reaction was terminated after 30 min at 30°C in the dark by adding 1 ml trichloroacetic acid (TCA) (30%, w/v). The nitrite formed was measured colorimetrically by adding 4 ml of 1% (W/V) sulfanilamide in 3 M HCl and 4 ml of 0.2% (w/v) alpha-Naphthylamine, and absorption was determined at 540 nm.

Glutamine synthetase activity was determined by the mean value of the synthetase reaction (Hadži-Tašković Šukalivić 1986). The volume of the reaction mixture was 2.2 ml, including 0.5 ml of enzyme extract. Hydroxamate formation was measured in an assay mixture after 15 min at 30°C, and the absorbance was measured at 540 nm.

Measurements of ion leakage and malonaldehyde (MDA) levels

The ion leakage from the cellular membrane was determined via conductivity measurement according to Fan et al. (1997). The MDA level was assayed according to Quan et al. (2004).

Determinations of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) productions

The assay for O_2^- was performed as described by Wang and Luo (1990). The concentration of H_2O_2, determined according to a method described by Sairam and Srivastava (2002), was estimated by measuring the spectrum absorbance of the titanium–hydroperoxide complex and using a standard curve plotted with a known concentration of H_2O_2.

Assays of antioxidant enzyme activities and non-enzyme antioxidants

Total superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities were determined as described by Prochazkova et al. (2001). Ascorbic acid (AsA) was determined according to Arakawa et al. (1981) with a spectrometer (UV1601; Shimadzu, Kyoto, Japan). Total glutathione (GSH) was determined with an enzymatic cycling assay method by Noctor and Foyer (1998b).

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was conducted using the DPS (Data Processing System) procedures (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

The GB content of wheat flag leaves

The GB content of transgenic line T6 was significantly higher than that of the WT when no stress treatment was given (Table 1) due to the constitutive overexpression of the transgenic *BADH* gene (Guo et al. 2000). When subjected to drought, heat or a combination of drought and heat stress, the GB contents in the leaves of the two lines increased significantly ($P < 0.05$), especially in the T6 line, over that of the well-watered controls (CK), indicating that the wheat plants sensitively responded to these stresses.

Overaccumulation of GB enhanced photosynthesis of wheat flag leaves under drought and heat stresses

Both the chlorophyll and carotenoid contents in T6 were almost the same as in the WT under normal conditions (Fig. 1a, b). Drought and heat stress decreased chlorophyll and carotenoid contents significantly in both wheat lines ($P < 0.05$), but the decrease was smaller in T6 than in WT. Heat stress decreased them to a slightly greater extent than drought stress in this experiment. When a combination of drought and heat stress was imposed, a drastic decline of chlorophyll and carotenoid contents was observed in both wheat lines, showing that the stress combination aggravated the damage of photosynthetic pigments. The chlorophyll and carotenoid contents remained higher in T6 than in WT under all three stresses, suggesting the protection of overaccumulated GB from the damage induced by environmental stress.

Figure 1c shows that Pn of both WT and T6 were significantly inhibited by all stresses; heat stress inhibited it to a greater extent than drought stress, and it was greatly

Table 1 GB contents (μmol/g DW) in flag leaves of wheat plants subjected to drought, heat or the combination of these stresses

Treatment	CK	DS	HS	DS + HS
Shi4185	89.14 ± 2.89 f	123.57 ± 12.34 cd	107.59 ± 10.76 cde	141.16 ± 11.37 c
T6	121.71 ± 6.42 cd	170.68 ± 8.04 b	140.22 ± 10.46 c	207.23 ± 6.19 a

Values are the mean ± SE of three replications. Means in a column followed by the same letter were not significantly different at $P < 0.05$

CK Well-watered control, *DS* drought-stressed, *HS* heat-stressed, *DS + HS* combination of drought- and heat-stressed

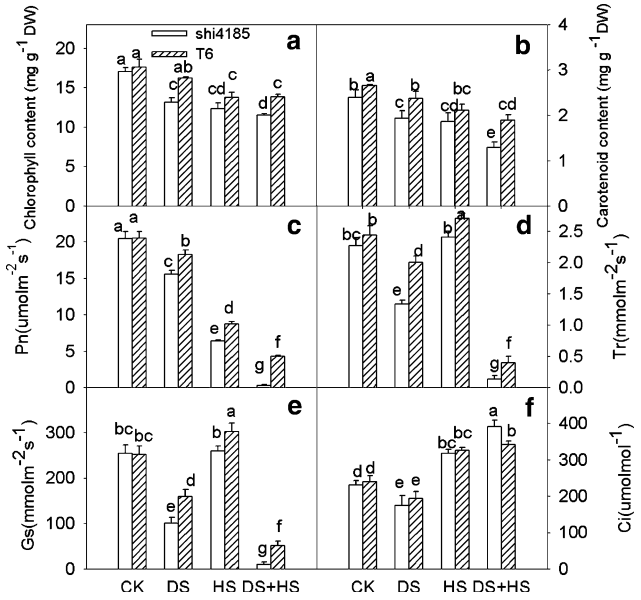

Fig. 1 Chlorophyll (**a**) and total carotenoid (**b**) contents, net photosynthesis rate (*Pn*, **c**), transpiration rate (*Tr*, **d**), stomatal conductance (*Gs*, **e**) and intercellular CO_2 concentration (*Ci*, **f**) of wheat flag leaves subjected to drought, heat or the combined stresses. Each *bar* is the mean ± SE of three replicates. *Bars* with the *same letter* were not significantly different at $P < 0.05$

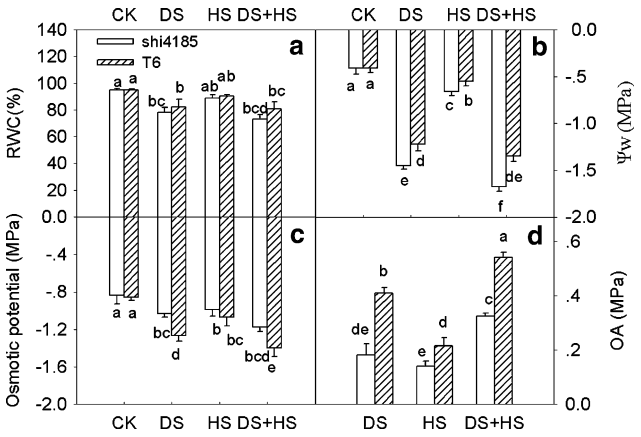

Fig. 2 Changes of leaf *RWC* (**a**), water potential (ψ_w, **b**), osmotic potential (ψ_s^{100}, **c**) and osmotic adjustment (*OA*, **d**) induced by drought, heat and their combination in wheat flag leaves. The values are mean ± SE of three replicates. *Bars* with the *same letter* were not significantly different at $P < 0.05$

aggravated by the heat and drought stress combination. However, the different stresses did not have the same effects on Tr (Fig. 1d) and Gs (Fig. 1e) as on Pn. For instance, drought stress decreased Gs and Tr, while heat stress increased these parameters. When drought stress was accompanied by heat stress, Gs and Tr were suppressed further. Figure 1f shows that drought stress decreased Ci, but it was increased by heat stress. When the plants were subjected to a combination of drought and heat stresses, however, the Ci was increased significantly although Gs decreased. GB overaccumulation in T6 improved Pn and gas exchange parameters under all three stresses.

Effects of overaccumulated GB on the water status of wheat flag leaves under drought and heat stresses

As shown in Fig. 2a, b, heat stress had hardly any effect on RWC and resulted in only a slight decrease in the ψ_w of both WT and T6, which may be attributed to the short duration of heat treatment (4 h). By contrast, drought resulted in a significant decrease of RWC and ψ_w, and a combination of drought and heat stress deteriorated them further. The decreases of RWC and ψ_w were greater in WT than in T6 under drought stress, suggesting the improvement of overaccumulated GB in the water balance of the transgenic line.

There were no obvious differences in ψ_s^{100} between WT and T6 under normal conditions (Fig. 2c). While all stress conditions decreased ψ_s^{100} in both wheat lines, the combination of drought and heat stresses decreased it to a greater extent than application of each stress factor alone. However, under all stress conditions, ψ_s^{100} was significant lower in T6 than in WT.

The decrease of saturated osmotic potential resulted in the appearance of OA under the stress treatments (Fig. 2d). The response of OA to drought stress was greater than that to heat stress, and the greatest OA was observed with exposure to the stress combination. The OA of T6 was greater than that of WT due to GB overaccumulation, consistent with the different responses of ψ_s^{100} between the two wheat lines.

We also detected the contents of osmotically active compounds, including the total soluble sugar, free proline, total free amino acids, and soluble proteins in the flag

leaves. The results (Fig. 3) indicated that drought and combined stress induced the accumulations of almost all osmotically active compounds in both wheat lines, but these increases were greater in T6 than in WT. Compared to drought and combined stress, heat stress increased them to a lesser extent. While heat and combined stress decreased the soluble protein content in both plants, it was slightly higher in T6 than in WT (Fig. 3c), consistent with previous reports (Demiral and Türkan, 2006; Ma et al. 2007; Raza et al. 2007).

Effect of overaccumulated GB on the activities of NR and GS under drought and heat stresses

Drought increased the activities of NR and GS (Fig. 3e, f), but heat and combined stresses significantly decreased them ($P < 0.05$), indicating the sensitivity of these two enzymes to heat. These effects were not consistent with the induced accumulation of free proline (Fig. 3b) and free amino acids (Fig. 3d) by drought and heat stress combination, suggesting that both NR and GS were not the major contributors to these increases. However, the activities of NR and GS were always higher in T6 than in WT under different stresses, suggesting that these enzymes may be specifically involved in the higher proline and free amino acid levels in T6 under stress conditions.

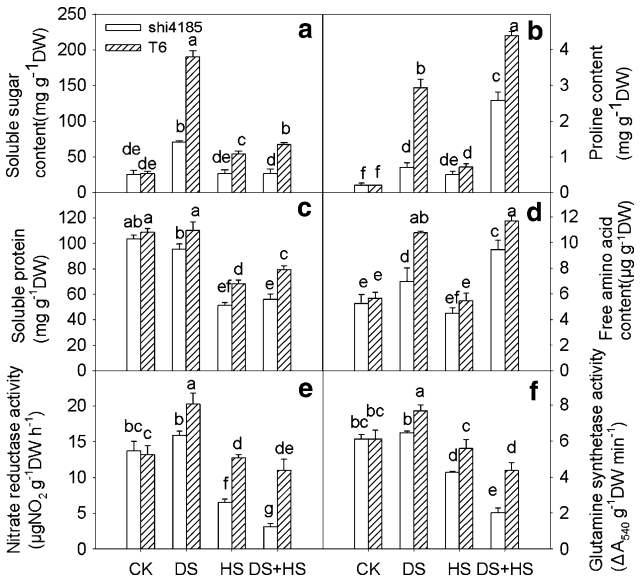

Fig. 3 Total contents of soluble sugars (**a**), proline (**b**), soluble protein (**c**), and free amino acids (**d**). The activities of nitrate reductase (*NR,* **e**) and glutamine synthetase (*GS,* **f**) induced by drought, heat and their combination in wheat flag leaves. The values are mean ± SE of three replicates. *Bars* with the *same letter* were not significantly different at $P < 0.05$

Effects of overaccumulated GB on membrane leakage and MDA levels in wheat leaves under drought and heat stresses

Figure 4a shows that while both drought and heat stress increased ion leakage significantly ($P < 0.05$), the increase was greater under heat than drought stress, and the greatest increase was observed under their combination ($P < 0.01$). Overaccumulation of GB resulted in less ion leakage in T6 than in WT, especially under the stress combination. Similar results were observed with MDA levels (Fig. 4b). Figure 4c shows that all stresses increased O_2^- and H_2O_2 productions. Compared to drought stress, heat stress increased them to a lesser extent, and the greatest increase was observed under stress combination. However, all these levels of O_2^-, H_2O_2, ion leakage and MDA were lower in T6 than in WT, indicating that overaccumulation of GB can help to eliminate reactive oxygen species (ROS) and alleviate membrane damage.

Effects of overaccumulated GB on antioxidant enzyme activities

We investigated the activities of several key antioxidant enzymes, namely SOD, CAT, POD and APX, under different stress conditions. Different responses of the four antioxidant enzymes to different stresses were observed in T6 and WT. All stresses increased the activity of SOD (Fig. 5a) with the greatest increase observed under stress combination, but that pattern was reversed with CAT (Fig. 5b) and APX (Fig. 5c) activities. However, different responses of POD activity (Fig. 5d) to drought and heat stress were observed, with drought stress increasing POD activity but heat stress decreasing it. GB overaccumulation

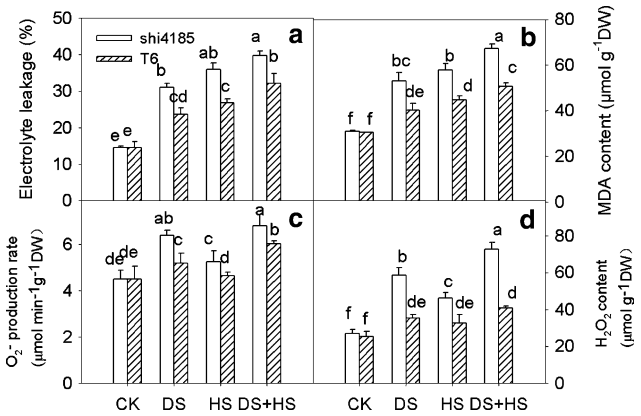

Fig. 4 Ion leakage (**a**), MDA content (**b**), O_2^- production rate (**c**) and H_2O_2 content (**d**) of the flag leaves in the wild-type and transgenic line after exposure to drought, heat stress and their combination. Each *bar* is the mean ± SE of three replicates. *Bars* with the *same letter* were not significantly different at $P < 0.05$

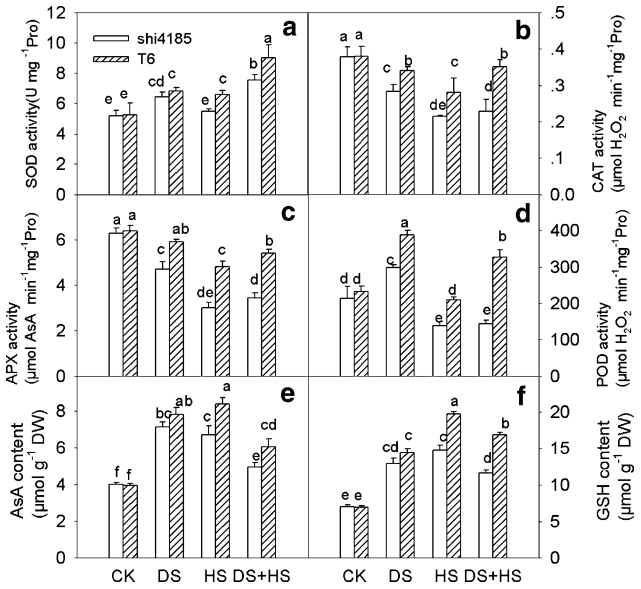

Fig. 5 The activities of SOD (**a**), CAT (**b**), APX (**c**) and POD (**d**) as well as the accumulation of AsA (**e**) and GSH (**f**) in the wild-type and transgenic lines after exposure to drought, heat and their combination. The values are mean ± SE of three replicates. *Bars* with the *same letter* were not significantly different at $P < 0.05$

in T6 increased activity of all the four antioxidant enzymes, especially APX and CAT, all which can eliminate H_2O_2, and this was consistent with the greater decrease of H_2O_2 accumulation in T6 (Fig. 4d).

Different from the antioxidant enzymes, the accumulations of two non-enzyme antioxidants, AsA and GSH, were significantly increased by all stress conditions (Fig. 5e, f), especially under heat stress condition. Compared to drought or heat stress alone, the stress combination had lower non-enzyme antioxidant accumulation, but their overall levels were still increased by these stress treatments. GB overaccumulation in T6 increased accumulations of both AsA and GSH under stress conditions, and the increase was more significant, especially of GSH, under heat stress than drought stress.

Discussion

The different physiological responses of wheat leaves to drought, heat stress and their combination

The response of plants to different stress stimulation can vary, and, particularly when they occur together, different stresses might require conflicting or antagonistic responses (Mittler 2006). Flag leaves are the major contributor to wheat yield that is the result of photosynthesis, but photosynthesis is often decreased seriously by drought, heat stress or both at late stages of growth within the natural habitat. In the present study, we observed the different responses and interrogated the underlying mechanisms of Pn to drought, heat, and the combination of both stresses in wheat flag leaves. First, our results suggested that the stress combination resulted in a greater decrease in Pn of wheat flag leaves than drought or heat stress alone, and heat stress reduced Pn more than drought stress could (Fig. 1c) in this experimental condition. The negative effect of drought stress on Pn may result from the decreased Gs (Fig. 1e) that resulted in the decreased Ci (Fig. 1f); on the other hand, heat stress increased Gs (Fig. 1e) and Ci (Fig. 1f), suggesting that there are different mechanisms underlying the decrease in Pn caused by drought and heat stress. We propose that the decrease of Pn may result from the stomatal factors under drought stress, but that it may be due to non-stomatal factors under heat stress and stress combination. Second, heat stress increased Tr, but drought stress decreased it (Fig. 1d). This effect may be another cause for the severe decrease in Pn by the stress combination because, under heat stress, the leaf temperatures will be increased, and Tr is an important way for decreasing leaf temperature. However, under the stress combination of drought and heat, Tr was decreased as a result of the stomata closing, which can increase the leaf temperature further, resulting in the inactivation of some enzymes with heat sensitive characteristic such as NR and GS (Fig. 3e, f). These observations were consistent with those of Rizhsky et al. (2002). Third, drought stress limited the water content more seriously than heat stress (Fig. 2), but the heat stress decreased the activity of some enzymes (Figs. 3e, f, and 5d) and the cell membrane integrity (Fig. 4a) more effectively than did the drought stress.

However, we must give attention to the inconsistent results in this paper compared with others. In the present study, as well as in two other papers of ours (Wang et al. 2010a, b), we demonstrated that drought can decrease the tolerance of the wheat flag leaves to heat stress. However, in another experiment, our results demonstrated that drought can induce higher tolerance of PSII to heat stress (data will be published in another journal), which is consistent with the results by Lu and Zhang (1999) who reported that water stress increased the thermostability of PSII in wheat plants. But those experiments were conducted in the field, and the drought condition as well as the growth stage of the wheat plants was also different from that in this paper. So, these conflicting results may be due to the different growth stage of the plant and the experimental conditions tested; the mode of drought as well as the duration of heat stress treatment may also be involved in.

Overaccumulation of GB improves the photosynthesis of wheat flag leaves under stress

Our results in Fig. 1 and some other reports suggested that GB can improve the photosynthetic capacity not only by

increasing stomatal conductance but also by maintaining protein activity in the chloroplast (Mäkelä et al. 2000; Yang et al. 2005) under stress conditions. But what is its mechanism? Previous reports suggested that, under heat stress, GB can attenuate the effects of heat-induced inhibition of the PS II and will alleviate the inhibitory effect of heat stress on the repair of photosystem II during photoinhibition and protect it against heat-induced injuries (Allakhverdiev and Murata 2004; Allakhverdiev et al. 1996, 2007). What is more, the improvement of GB in photosynthesis may be related to the protection of GB on proteins in the thylakoid membrane (Allakhverdiev et al. 1996, 2003, 2007; Kreslavski et al. 2007). Our previous data also suggested that overaccumulation of GB can enhance the tolerance of PSII and ATPase activities to drought and heat stress and protect the structures of chloroplast and thylakoid from the damage by stress (Wang et al. 2010a, b); this was consistent with other reports (Mamedov et al. 1993). Furthermore, our results also suggested that the improvement of GB on the water status and the antioxidative defense system, including antioxidative enzymes and antioxidants, were involved in its mechanisms.

GB-induced increase of osmotic adjustment may be more important than that of the antioxidative defense system in the improvement of drought tolerance

Our study confirms that wheat synthesizes and accumulates GB in vivo naturally under normal conditions; however, heat, drought stress and their combination induced GB to higher levels (Table 1). The accumulation of GB in vivo increased significantly by introducing the *BADH* gene into wheat, which resulted in the enhanced tolerance of wheat to stress and increased Pn (Fig. 1c) in transgenic plants, although the content of GB was much lower than that of wheat with foliar-applied GB (Ma et al. 2006). These results were consistent with previous reports (Saneoka et al. 1995).

Of course, we wanted to understand the mechanisms of GB improving Pn under different stress conditions. In general, the Pn results from gas exchange parameters. GB may maintain the photosynthetic capacity not only through increasing stomatal conductance but also by maintaining Rubisco activity (Yang et al. 2005; Sage and Kubien 2007) under stress. Stomata movement is related to the water status, and OA is an important mechanism underlying plant responses to osmotic stress (Zhang et al. 1999; Chimenti et al. 2002). From Fig. 2a–c, the decreases of RWC, water potential (ψ_w) and osmotic potential were greater when caused by drought stress than by heat stress. Correspondingly, overaccumulation of GB in T6 also improved those parameters to a greater extent under drought stress than under heat stress and may be related to the greater OA in T6 (Fig. 2d) in drought conditions. The greater OA in T6 under drought stress than heat stress resulted from the accumulation of solutes (Fig. 3a–d).

Furthermore, the synthesis of proline (Fig. 3b) and free amino acids (Fig. 3d) were related to NR and GS, two key enzymes in nitrogen metabolism. NR activity is positively associated with photosynthesis, and GS plays a positive role in the link between carbon and nitrogen metabolism (Lam et al. 1996). From Fig. 3e, f, we observed that the activities of NR and GS were more sensitive to heat stress than drought stress, and especially to the stress combination. However, we found that overaccumulation of GB in T6 increased the activities of NR and GS significantly, which may be involved in the higher proline and free amino acid contents in T6 (Fig. 3) under stress conditions. Furthermore, GB-induced accumulation of solute compounds in T6 can decrease the osmotic potential of the plant cell (Fig. 2c), which can enhance absorption of water into the plant cells and result in the improvement of cell water status (Fig. 2a) and stomatal opening (Fig. 1e).

We must also give some attention to the increase of proline content induced by single heat stress and the combined drought and heat stresses, which is contrary to the results by Rizhsky et al. (2004) who reported that the proline content in *Arabidopsis* plants was decreased by exposure to heat stress (38°C) for 6 h. These conflicting results may be due to differences in the plant species tested as well as the duration of heat stress treatment, and further research is needed to determine if the phenomenon is plant specific.

GB-induced improvement of the antioxidative defense system may be more important than that of OA for heat tolerance

Cell membrane stability estimated by ion leakage has been widely used to differentiate the tolerance and susceptibility of plants to stresses (Blum and Ebercon 1981; Rehman et al. 2004). Figure 4a shows that both drought and heat stress can increase ion leakage significantly ($P < 0.05$), but heat stress could increase it more than drought stress in this experiment, and the greatest increase was observed under the combined stresses ($P < 0.01$). Cell membrane stability is often affected by lipid peroxidation caused by ROS under stress conditions (Sudhakar et al. 2001), which results in the production of MDA as shown in Fig. 4b. Overaccumulation of GB can help to alleviate lipid peroxidation (Fig. 4b) and maintain the cell membrane stability (Fig. 4a), consistent with previous in vitro studies (Saneoka et al. 2004; Lv et al. 2007). However, GB cannot eliminate ROS directly (Smirnoff and Cumbes, 1989; Wang et al. 2010b); therefore, the

enhanced activities of the antioxidative enzymes and antioxidant contents (Fig. 5) may be the major factors involved in the GB-mediated decrease of ROS (Fig. 4c, d), consistent with the previous reports (Sakamoto and Murata 2001; Yang et al. 2007).

How the overaccumulation of GB in vivo enhanced 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 stress tolerance than that in the cytosol (Park et al. 2007). In this study, the antioxidant enzymes examined are primarily localized in the chloroplast (Noctor and Foyer 1998a), thus it is hypothesized that the overaccumulated GB in vivo may be partly distributed in the chloroplast in transgenic plant T6, and stabilized the structures of these enzymes under stress conditions. The increased non-enzyme antioxidants such as AsA and GSH in T6 may be the involvement of the corresponding enzymes synthesizing them by overaccumulated GB probably. The further research is needed.

Additionally, the results in Fig. 4c, d show that the effects of GB on H_2O_2 elimination were greater than that on O_2^-, consistent with the greater protection of overaccumulated GB to CAT, APX and POD (Fig. 5b–d) than SOD (Fig. 5a). The results in Fig. 5e, f also show the stress-induced increase of non-enzyme antioxidants including AsA and GSH; while heat stress inhibited the antioxidative enzyme activity, it increased and stabilized the non-enzyme antioxidant levels.

In conclusion, photosynthesis in wheat flag leaves is affected by drought, heat, and the combination of these stresses in different ways. The combination of drought and heat further decreased the photosynthesis process over the inhibition with either stress alone. Overaccumulation of GB in T6 helped to increase the tolerance of the plants, via the photosynthesis process, not only to individual drought or heat but also to the combined stresses. This effect of GB on the photosynthesis and tolerance of the plants may be the result of its improvement of water balance and antioxidant metabolism. Furthermore, the tolerance to drought may be affected more by the GB-induced increase of OA, while the GB-induced improvement of the antioxidative defense system may be more important for the heat tolerance in the conditions tested. The results in this paper would greatly benefit the development of urgently needed new wheat cultivars with enhanced stress tolerance in the field.

Acknowledgments We are grateful to Prof. S. Y. Chen (Institute of Genetics, Chinese Academy of Sciences, China) for her kindly presentation of the transgenic wheat lines to us. This study is supported by National Natural Science Foundation of China (No. 30671259) and by National Key technology R&D program (No. 2006BAD01A02-15).

References

Allakhverdiev SI, Murata N (2004) Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage-repair cycle of Photosystem II in *Synechocystis* sp. PCC6803. BBA-Bioenergetics 1657:23–32. doi:10.1016/j.bbabio.2004.03.003

Allakhverdiev SI, Feyziev YM, Ahmed A, Hayashi H, Aliev JA, Klimov VV, Murata N, Carpentier R (1996) Stabilization of oxygen evolution and primary electron transport reactions in photosystem II against heat stress with glycinbetaine and sucrose. J Photochem Photobiol B 34:149–1578. doi:10.1016/1011-1344(95)07276-4

Allakhverdiev SI, Hayashi H, Nishiyama Y, Ivanov AG, Aliev JA, Klimov VV, Murata N, Carpentier R (2003) Glycinebetaine protects the D1/D2/Cytb559 complex of photosystem II against photo-induced and heat-induced inactivation. J Plant Physiol 160:41–49. doi:10.1078/0176-1617-00845

Allakhverdiev SI, Los DA, Mohanty P, Nishiyama Y, Murata N (2007) Glycinebetaine alleviates the inhibitory effect of moderate heat stress on the repair of photosystem II during photoinhibition. Biochim Biophys Acta 1767:1363–1371. doi:10.1016/j.bbabio.2007.10.005

Arakawa N, Tsutsumi K, Sanceda NG, Kurata T, Inagaki C (1981) A rapid and sensitive method for the determination of ascorbic acid using 4, 7-diphenyl-1, 10-phenanthroline. Agric Biol Chem 45:1289–1290

Bahieldina A, Mahfouz HT, Eissa HF, Saleh OM, Ramadan AM, Ahmed IA, Dyer WE, ElItriby HA, Madkour MA (2005) Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. Physiol Plant 123:421–427. doi:10.1111/j.1399-3054.2005.00470.x

Baki GKAE, Siefritz F, Man HM, Weiner H, Kaldenhoff R, Kaiser WM (2000) Nitrate reductase in *Zea mays* L. under salinity. Plant Cell Environ 23:515–521. doi:10.1046/j.1365-3040.2000.00568.x

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207. doi:10.1007/BF00018060

Blum A, Ebercon A (1981) Cell membrane stability as measure of drought and heat tolerance in wheat. Crop Sci 21:43–47

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein–dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Chimenti CA, Pearson J, Hall AJ (2002) Osmotic adjustment and yield mentanence under drought in sunflower. Field Crop Res 75:235–246. doi:10.1016/S0378-4290(02)00029-1

Demiral T, Türkan I (2006) Exogenous glycinebetaine affects growth and praline accumulation and retards senescence in two rice cultivars under NaCl stress. Environ Exp Bot 56:72–79. doi:10.1016/j.envexpbot.2005.01.005

Fan L, Zheng S, Wang X (1997) Antisense suppression of phospholipase Dα retards abscisic acid and ethylene promoted senescence of postharvest Arabidopsis leaves. Plant Cell 9:2183–2196. doi:10.1105/tpc.9.12.2183

Frances MD, William JH, William HV, Charlene T, Kerry MK, Okkyung KC, Susan BA (2006) Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature. Eur J Agron 25:96–107. doi:10.1016/j.eja.2006.04.003

Gorham J (1995) Betaines in higher plants: biosynthesis and role in stress metabolism. In: Wallsgrove (ed) Amino acids and their derivatives in higher plants. Cambridge University Press, Cambridge, pp 171–203

Guo BH, Zhang YM, Li HJ, Du LQ, Li YX, Zhang JS, Chen SY, Zhu ZQ (2000) Transformation of wheat with a gene encoding for the betaine aldehyde dehydrogenase (BADH). Acta Botanica Sinica 42:279–283. doi:cnki:ISSN:11-1896.0.2000-03-011 (in Chinase, with English abstract)

Hadži-Tašković Šukalović V (1986) Activity and distribution of nitrogen-metabolism enzymes in the developing maze kernel. Physiol Plant 67:247–252. doi:10.1111/j.1399-3054.1986.tb02451.x

Khan MS, Yu x, Kikuchi A, Asahina M (2009) Genetic engineering of glycine betaine biosynthesis to enhance abiotic stress tolerance in plants. Plant Biotechnol 26:125–134

Kreslavski V, Carpentier , Klimov VV, Murata N, Allakhverdiev SI (2007) Molecular mechanisms of stress resistance of the photosynthetic apparatus. Biol Membr 24:195–217

Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:569–593. doi:10.1146/annurev.arplant.47.1.569

Lu CM, Zhang JH (1999) Effects of water stress on photosystemII photochemistry and itst hermostability in wheat plants. J Exp Bot 50:1199–1206

Luo Y, Li WM, Wang W (2008) Trehalose: protector of antioxidant enzymes or reactive oxygen species scavenger under heat stress? Environ Exp Bot 63:378–384. doi:10.1016/j.envexpbot.2007.11.016

Lv SL, Yang AF, Zhang KW, Wang L, Zhang JR (2007) Increase of glycinebetaine synthesis improves drought tolerance in cotton. Mol Breed 20:233–248. doi:10.1007/s11032-007-9086-x

Ma QQ, Wang W, Li YH, Li DQ, Zou Q (2006) Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum* L) by foliar-applied glycinebetaine. J Plant Physiol 163:165–175. doi:10.1016/j.jplph.2005.04.023

Ma XL, Wang YJ, Xie SL, Wang C, Wang W (2007) Glycinebetaine application ameliorates negative effects of drought stress in tobacco. Russ J Plant Physiol 54:472–479. doi:10.1134/S1021443707040061

Mäkelä P, Kärkkäinen J, Somersalo S (2000) Effect of glycinebetaine on chloroplast ultrastructure, chlorophyll and protein content, and RuBPCO activities in tomato grown under drought or salinity. Biol Plant 43:471–475. doi:10.1023/A:1026712426180

Mamedov M, Hayashi H, Murata N (1993) Effects of glycinebetaine and unsaturation of membrane lipids on heat stability of photosynthetic electron-transport and phosphorylation reactions in Synechocystis PCC6803. Biochim Biophys Acta 1142:1–5. doi:10.1016/0005-2728(93)90077-S

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11:15–19. doi:10.1016/j.tplants.2005.11.002

Noctor G, Foyer CH (1998a) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol 49:249–279. doi:10.1146/annurev.arplant.49.1.249

Noctor G, Foyer CH (1998b) Simultaneous measurement of foliar glutathione, γ-glutamylcysteine and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione. Anal Biochem 264:98–110. doi:10.1006/abio.1998.2794

Nomura M, Hibino T, Takabe T, Sugyama T, Yokota A, Miyake H, Takabe T (1998) Transgenically produced glycinebetaine protects ribulose 1, 5-bisphosphate carboxylase/oxygenase from inactivation in *Synechococcus* sp. PCC7942 under salt stress. Plant Cell Physiol 39:425–432

Park EJ, Jeknić Z, Pino MT, Murata N, Chen THH (2007) Glycinebetaine accumulation in chloroplasts is more effective than that in cytosol in protecting transgenic tomato plants against abiotic stress. Plant Cell Environ 30:994–1005. doi:10.1111/j.1365-3040.2007.01694.x

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975:384–394. doi:10.1016/S0005-2728(89)80347-0

Prochazkova D, Sairam RK, Srivastava GC, Singh DV (2001) Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. Plant Sci 161:765–771. doi:10.1016/S0168-9452(01)00462-9

Quan RD, Shang M, Zhang H, Zhao YX, Zhang JR (2004) Improved chilling tolerance by transformation with *betA* gene for the enhancement of glycinebetaine synthesis in maize. Plant Sci 166:141–149. doi:10.1016/j.plantsci.2003.08.018

Raza SH, Athar HR, Ashraf M, Hameed A (2007) Glycinebetaine-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. Environ Exp Bot 60:368–376. doi:10.1016/j.envexpbot.2006.12.009

Rehman H, Malik SA, Saleem M (2004) Heat tolerance of upland cotton during fruiting stage evaluated using cellular membrane thermostability. Field Crop Res 85:149–158. doi:10.1016/S0378-4290(03)00159-X

Rizhsky L, Liang HJ, Mittler R (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol 130:1143–1151. doi:10.1104/pp.006858

Rizhsky L, Liang HJ, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of *Arabidopsis* to a combination of drought and high temperature. Plant Physiol 134:1683–1696. doi:10.1104/pp.103.033431

Sage RF, Kubien DS (2007) The temperature response of C3 and C4 photosynthesis. Plant Cell Environ 30:1086–1106. doi:10.1111/j.1365-3040.2007.01682.x

Sairam PK, Srivastava GC (2002) Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. Plant Sci 162:897–904. doi:10.1016/S0168-9452(02)00037-7

Sakamoto A, Murata N (2001) The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress resistant transgenic plants. Plant Physiol 125:180–188

Sakamoto A, Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ 25:163–171. doi:10.1046/j.0016-8025.2001.00790.x

Saneoka H, Nagasaka C, Hahn DT, Yang WJ, Premachandra GS, Joly RJ, Rhodes D (1995) Salt tolerance of glycinebetaine-deficient and containing maize lines. Plant Physiol 107:631–638

Saneoka H, Moghaieb REA, Premachandra GS, Fujita K (2004) Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in *Agrostis palustris* Huds. Environ Exp Bot 52:131–138. doi:10.1016/j.envexpbot.2004.01.011

Smirnoff N, Cumbes Q (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28:1057–1060. doi:10.1016/0031-9422(89)80182-7

Sudhakar C, Lakshmi A, Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding genotypes of

mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci 161:613–619. doi:10.1016/S0168-9452(01)00450-2

Tissue DT, Wright SJ (1995) Effect of seasonal water availability on phenology and the annual shoot carbohydrate cycle of tropical forest shrubs. Funct Ecol 9:518–527

Troll W, Cannan RK (1953) A modified photometric ninhydrin method for the analysis of amino and imino acids. J Biol Chem 200:803–811

Wang AG, Luo GH (1990) Quantitative relation between the reaction of hydroxylamine and superoxide anion radicals in plants. Plant Physiol Commun 6:55–57. doi:cnki:ISSN:0412-0922.0.1990-06-032 (in Chinese with English abstract)

Wang GP, Li F, Zhang J, Zhao MR, Hui Z, Wang W (2010a) Overaccumulation of glycine betaine enhances tolerance of the photosynthetic apparatus to drought and heat stress in wheat. Photosynthetica 48:30–41

Wang GP, Zhang XY, Li F, Luo Y, Wang W (2010b) Overaccumulation of glycine betaine enhances tolerance to drought and heat stress in wheat leaves in the protection of photosynthesis. Photosynthetica 48(1):117–126

Yang XH, Liang Z, Lu C (2005) Genetic engineering of the biosynthesis of glycinebetaine enhances photosynthesis against high temperature stress in transgenic tobacco plants. Plant Physiol 138:2299–2309. doi:10.1104/pp.105.063164

Yang XH, Wen XG, Gong HM, Lu QT, Yang ZP, Tang YL, Liang Z, Lu CM (2007) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225:719–733. doi:10.1007/s00425-006-0380-3

Zhang J, Nguyen HT, Blum A (1999) Genetic analysis of osmotic adjustment in crop plants. J Exp Bot 50:291–302