The protection of wheat plasma membrane under cold stress by glycine betaine overproduction

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Abstract

We aimed to study the protection of wheat plasma membrane (PM) under cold stress (0 - 2 °C) by the overaccumulation of glycine betaine (GB). For this, we used wild-type winter wheat (*Triticum aestivum* L.) cv. Shi 4185 (WT) and 3 transgenic lines (T1, T4, and T6) expressing the *BADH* gene isolated from *Atriplex hortensis* L. Under cold stress, the transgenic lines with higher GB content maintained better membrane integrity and higher plasma membrane H^+-ATPase activity than WT. In these transgenic lines, ROS production and membrane lipid peroxidation were lower, while antioxidative enzyme activities and compatible solute contents were higher in comparison with WT. This may be attributable to their enhanced cold-stress tolerance mediated by GB overproduction.

Additional key words: antioxidative enzymes, *BADH* gene, compatible solutes, H^+-ATPase, lipid peroxidation, ROS production, *Triticum aestivum*.

Introduction

The enhancement of the cold-stress tolerance of winter wheat is an important strategy to increase wheat production. In higher plants, glycine betaine (GB) could improve tolerance to stresses such as drought (Zhao *et al*. 2007) and cold (Kishitani *et al*. 1994, Chen *et al*. 2000). Increasing GB biosynthesis through genetic engineering could enhance the cold stress resistance of transgenic plants (Allard *et al*. 1998, Sakamoto and Murata 2002), however, the mechanism through which GB biosynthesis enhances cold-stress resistance remains unclear. Plasma membrane is the primary site of injury when plant cells are subjected to low temperatures (Uemura *et al*. 2006), an undamaged plasma membrane is crucial to the survival of the whole cell (Kosová *et al*. 2007). Therefore, it is necessary to develop a strategy to reduce the damage to the wheat plasma membrane by increasing the expression levels of GB under cold stress.

Guo *et al*. (2000) transformed wheat with the *BADH* gene for betaine aldehyde dehydrogenase (BADH) isolated from *Atriplex hortensis* and 3 independent transgenic lines – T1, T4, and T6 were obtained. The overexpression of *BADH* induced GB overaccumulation in these transgenic wheat lines and they demonstrated increased salt-stress tolerance. In this study, we studied strategies for improving the cold stress tolerance of these transgenic lines, and attempted to elucidate the mechanisms through which GB protects the plasma membrane.

Materials and methods

We used wild-type winter wheat (*Triticum aestivum* L.) cultivar Shi 4185, and three wheat lines transformed with the *BADH* gene isolated from *Atriplex hortensis* L. (T1, T4 and T6). The transgenic wheat lines were produced by the Institute of Genetics, Chinese Academy of Sciences, Beijing, China. The *BADH* cDNA was cloned into the plasmid pABH9, which contained the ubiquitin promoter and the *bar* gene of maize; this plasmid was transferred into wheat cells by microprojectile bombardment (Guo *et al*. 2000), and several transgenic lines with different GB contents were obtained. After being surface-sterilized with 0.1 % carbendazim solution for 10 min, wheat seeds

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Abbreviations: APX - ascorbate peroxidase; BADH - betaine aldehyde dehydrogenase; CAT - catalase; GB - glycinebetaine; IUFA - index of unsaturated fatty acid; MDA - malondialdehyde; PM - plasma membrane; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; WT - wild type.

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were germinated on filter papers moistened with water for 24 h at 25 °C. Thereafter, the germinating seeds were placed on nylon gauze and cultured in Hoagland's solution in trays (25 × 18 × 5 cm). The wheat seedlings grew in the growth chamber under a 14-h photoperiod with a photosynthetic photon flux density (PPFD) of 300 μmol m^{-2} s^{-1} and at day/night temperature of 25/15 °C. When the third leaf was fully expanded, the seedlings were subjected to low temperature (2/0 °C) for 2 d. After stress treatment, the wheat plants were allowed to recover at 25/15 °C for 2 d.

The GB content in wheat leaves was analyzed by high-pressure liquid chromatography (HPLC) (*LC 6-A*, *Shimadzu,* Kyoto, Japan) as described by Ma *et al*. (2007). The malondialdehyde (MDA) content and the extent of electrolyte leakage in wheat leaves were determined by the procedure of Alia *et al*. (1997).

The activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) were estimated using a UV-visible spectrophotometer (*UV-1601*, *Shimadzu*, Kyoto, Japan). CAT and APX activities were done according to Alia *et al*. (1999). CAT activity was assayed based on the decrease in absorbance at 240 nm due to the degradation of H_2O_2, one unit CAT activity is defined as an absorbance change of 0.1 units per min. The assay of APX activity depended on the decrease in absorbance at 290 nm as ascorbate was oxidized, one enzyme unit was defined as the decrease of 0.1 units in absorbance per min. The activity of SOD was determined by measuring the ability to inhibit the photoreduction of NBT following the method of Bartoli *et al*. (1999), one unit of enzyme activity was defined as the quantity of SOD required to produce a 50 % inhibition of reduction of NBT. POD activity was based on the method as described by Chance and Maehly (1955), one unit it was defined as an absorbance change of 0.1 units per min at 470 nm. The specific activity for all enzymes was expressed as in U mg^{-1}(protein). The protein content was assayed according to Bradford (1976) using BSA as standard.

The rate of production of the superoxide anion (O_2^-) was estimated according to the method described by Wang and Luo (1990), with some modifications. The reaction mixture was composed of 0.5 cm^3 extract, 0.5 cm^3 phosphate-buffered saline (PBS) (50 mM, pH 7.8), and 1 cm^3 hydroxylamine (1 mM). After the mixture was warmed in a water bath at 25 °C for 1 h, 1 cm^3 of sulfanilic acid (17 mM) and α-naphthylamine were added; subsequently, the mixture was warmed at 25 °C for 25 min and the absorbance of the mixture at 530 nm was determined using a spectrophotometer. The O_2^- content was calculated as described by Elstner *et al.* (1976), with nitrite as a standard. H_2O_2 was measured by the method of Ferguson *et al*. (1983).

The plasma membranes (PMs) of leaf cells were isolated and purified using the procedure described by Larsson *et al*. (1994). Leaves (20 g f.m.) were homogenized in a blender in a reaction buffer composed of 250 mM sucrose, 1 mM ethylene diamine tetraacetic acid (EDTA), 50 mM Tris-HCl (pH 7.8), 0.6 % polyvinyl pyrrolidone (PVP) (m/v), 1 mM phenylmethylsufonyl-fluoride (PMSF), 1 mM dithiothreitol (DTT), and 0.1 % bovine serum albumin (BSA). The leaf extract was filtered through 2 layers of nylon cloth. This filtrate was centrifugated, first at 10 000 *g* for 15 min and at 80 000 *g* for 30 min after discarding the pellet. The second pellet was resuspended in the buffer as described above. The PMs were purified by passing the resuspended pellet through 3 sets of an aqueous polymer 2-phase system composed of 6.4 % (m/m) polyethylene glycol 3350 and 6.4 % (m/m) dextran T-500. The final membrane pellets were obtained by diluting the processed pellets from the third upper phase with the resuspension buffer in the ratio of 2:1 and centrifuging at 80 000 *g* for 40 min. The pellet was suspended in the resuspension buffer and stored at -80 °C until use. All isolation steps were carried out at 4 °C. The efficiency of purification was estimated using 1 mM Na_3VO_4 (an inhibitor of PM H^+-ATPase activity). Na_3VO_4 inhibited approximately 85 % of the H^+-ATPase activity of the purified PM protein vesicles; this indicates that the purified PM protein could be used for the measurement of PM H^+-ATPase activity. ATP hydrolytic activity was monitored according to Wang *et al*. (2001).

Fatty acid methyl esters of PMs were prepared as described by Xue *et al*. (1997). The samples were analyzed using a gas chromatograph analyzer (*GC-2010 Shimadzu* Kyoto, Japan) equipped with a flame ionization detector. The analytical conditions were as follows: a DB-23 quartz capillary column (30 × 0.25 × 0.25 mm ID), a column temperature of 190 °C, and a detector temperature of 250 °C. The flow velocities of H_2 and air were 40 and 400 cm^3 min^{-1}, respectively. The pure N_2 had a flow velocity of 8.4 cm^3 min^{-1} and a pressure of 187.4 kPa. The quantification was performed by normalization, which was done using the processing software of the analyzer.

Data are presented as the mean ± SD of 3 independent experiments for each treatment. Significant differences were detected using the two-way analysis of variance (*ANOVA*). The means were compared using the least significant difference test at $P < 0.05$.

Results

Before cold stress, the GB content in the leaves of the transgenic lines T1, T4, and T6 was higher than that in the WT by 19, 69 and 71 %, respectively. After 2 d of cold treatment, the GB content in all plants increased significantly ($P < 0.05$); this increase was higher in the transgenic lines than in the WT. The increased GB content remained also after recovery from the cold stress; however, GB content was lower during recovery compared to that under cold stress (Fig. 1*A*).

Before cold stress, the H^+-ATPase activity of WT and T6 did not differ significantly. With continued stress, PM H^+-ATPase activity decreased in both lines, however, the

decline in the H^+-ATPase activity in WT was more significant than that in T6. During recovery, the PM H^+-ATPase activity of T6 recovered to 98 % of its initial level; in contrast, the PM H^+-ATPase activity of WT recovered to only 80 % (Fig. 1*B*).

Under cold stress, the increase in the electrolyte leakage was greater in WT than in the transgenic lines. The electrolyte leakage continued to be lower in the transgenic lines than in the WT even during recovery (Fig. 1*C*).

Prior to cold treatment, there was no obvious difference between the MDA content in the transgenic lines and WT. Low temperatures induced greater increase in the MDA content in WT leaves than in the leaves of the transgenic lines. After 2 d of recovery, the MDA content in all the transgenic lines decreased to initial level; however, in WT remained bout 10 % higher (Fig. 1*D*).

The proline content also increased under cold stress in both WT and the transgenic lines; moreover, this increase was higher after 2 d of stress than after 1 d (Fig. 1*E*). Similar results were observed in the case of the soluble sugar content (Fig. 1*F*). Compared to WT, the transgenic lines showed higher increases in both proline and soluble sugar contents. After 2 d of recovery, the contents of both these compatible solutes decreased to their initial levels.

After exposure to a low temperature, the O_2^- content significantly increased in both WT and the transgenic lines. During recovery, the O_2^- content declined; however, during both stress and recovery, the O_2^- content was significantly lower in the transgenic lines than in WT ($P < 0.05$) (Fig. 2*A*). Similar results were observed for the H_2O_2 content (Fig. 2*B*).

In both transgenic lines and WT, the response of CAT activity to cold stress and recovery was not steady, but it was always higher in the transgenic lines than in WT (Fig. 2*C*). POD activity was increased by cold stress and decreased during recovery ($P < 0.05$) (Fig. 2*D*), but the

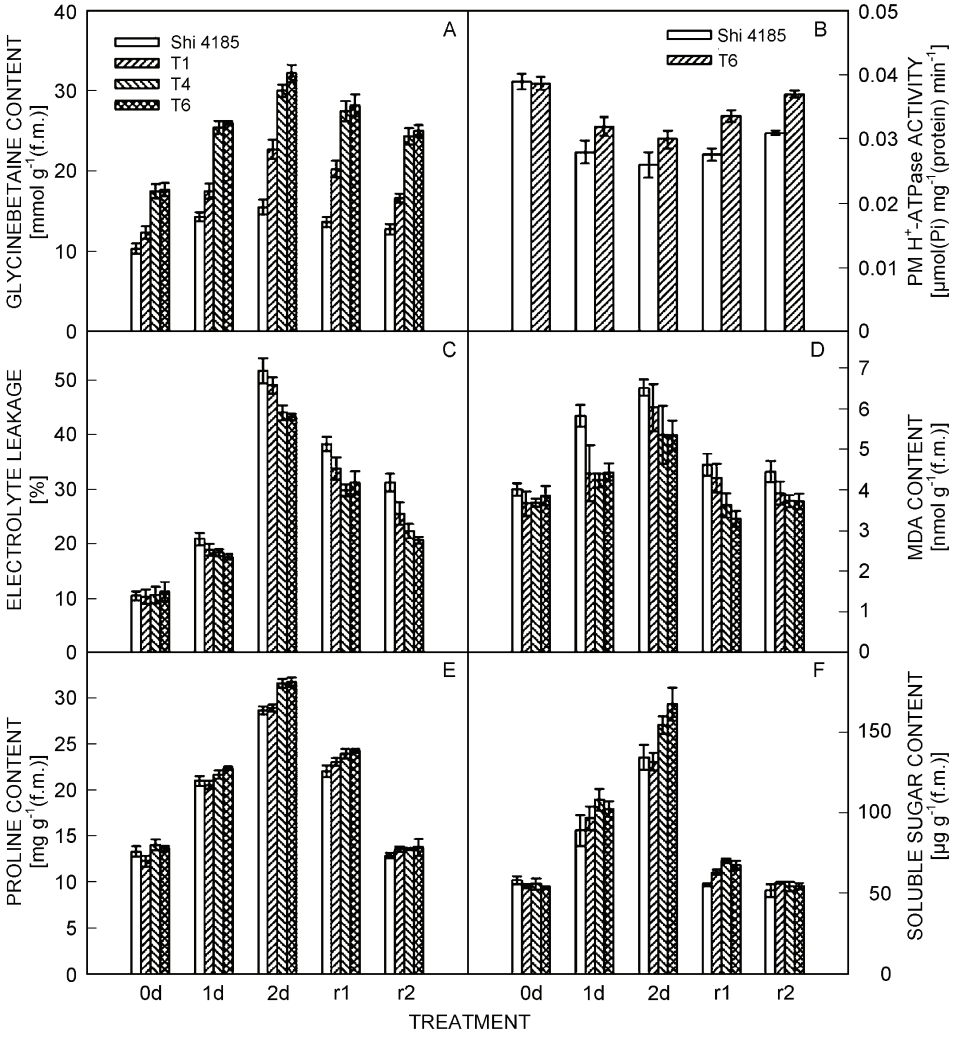

Fig. 1. GB content (*A*), PM H^+-ATPase activity (*B*), electrolyte leakage (*C*), MDA content (*D*), proline content (*E*), and soluble sugar content (*F*) in leaves of WT and transgenic wheat lines before cold stress (0 d), under cold stress 2.0 °C (1 d, 2 d) and during recovery (r 1, r 2). Means ± SD, $n = 3$.

improvement of it by GB overproduction was not steady. Similar results to POD were observed for APX activity (Fig. 2*F*). SOD activity was not sensitive to low temperature (Fig. 2*E*).

During cold stress, neither the compositions of the unsaturated fatty acids nor the index of unsaturated fatty acid (IUFA) were significantly altered in the transgenic line T6; however, certain alterations were observed in the contents of myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0) in WT. Compared to WT, the T6 line showed lower proportion of stearic acid (18:0) and oleic acid (18:1); however, T6 showed higher content of linoleic acid (18:2) and linolenic acid (18:3); therefore, the IUFA in T6 was higher than that in WT ($P < 0.05$) (Table 1).

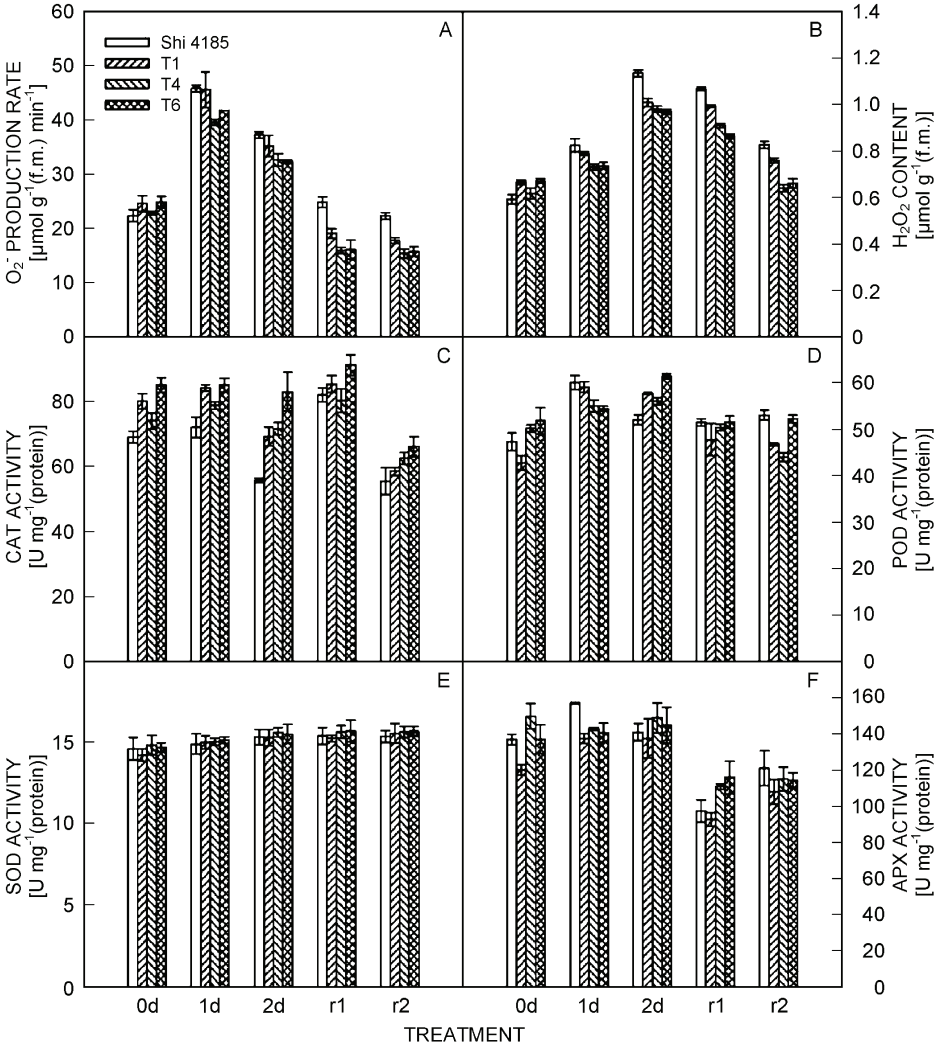

Fig. 2. Superoxide anion (O_2^-) production rate (*A*) , H_2O_2 content (*B*) and the activities of CAT (*C*), POD (*D*), SOD (*E*), and APX (*F*) in leaves of WT and transgenic wheat lines before cold stress (0 d), under cold stress 2.0 °C (1 d, 2 d) and during recovery (r 1, r 2). Means ± SD, $n = 3$.

Discussion

Before imposition of stress, the GB levels in transgenic plants were higher than those in WT (Fig. 1*A*) due to the overexpression of the *BADH* gene (Guo *et al*. 2000, Ding *et al*. 2003). Further, the up-regulation of GB content by cold stress was higher in transgenic lines than in WT. It is interesting to note that although the ubiquitin promoter is considered to be constitutive, the transgenic lines accumulated more GB under stress conditions.

Electrolyte leakage can indicate PM injury induced by cold stress. In this study, although the duration of cold stress was only 2 d, it induced an electrolyte leakage of approximately 50 % (Fig. 1*C*); this indicates great damage to the plasma membrane. This short-term cold stress often occurs during early spring; this can hamper the spike differentiation in wheat and result in a great reduction in yield and quality. Therefore, enhancing the tolerance of

Table 1. Fatty acid composition of the plasma membrane in the leaves of the WT and transgenic T6 line before cold stress (0d), under cold stress 2.0 °C (1d, 2d) and during recovery (r1, r2).under conditions of cold stress and recovery. The values represent the percentage of all lipids. Values within the rank followed by a different letter are significantly different at $P = 0.01$. Means ± SD, $n = 3$. IUFA = (18:1) + (18:2) × 2 + (18:3) × 3.

Lines	Fatty acids	0d	1d	2d	r1	r2
WT	14:0	5.21 ± 0.13d	5.86 ± 0.22c	6.71 ± 0.30a	6.34 ± 0.09b	6.40 ± 0.25b
	16:0	13.62 ± 0.51d	14.29 ± 0.31c	15.98 ± 0.14a	15.15 ± 0.17b	13.77 ± 0.22d
	18:0	3.47 ± 0.21e	4.16 ± 0.34c	4.85 ± 0.011a	4.09 ± 0.11d	4.32 ± 0.17b
	18:1	4.73 ± 0.35c	5.02 ± 0.17bc	4.87 ± 0.20bc	5.37 ± 0.20a	5.06 ± 0.23b
	18:2	9.87 ± 0.33a	9.16 ± 0.28b	8.53 ± 0.26c	8.61 ± 0.16c	9.51 ± 0.31ab
	18:3	63.10 ± 0.82a	61.51 ± 0.77b	59.06 ± 0.54d	60.71 ± 0.42c	60.94 ± 0.48c
	IUFA	213.77a	207.87b	199.65e	204.72d	206.9c
T6	14:0	5.88 ± 0.12ab	5.32 ± 0.20c	5.44 ± 0.16c	6.04 ± 0.11a	5.67 ± 0.21b
	16:0	13.45 ± 0.23b	13.04 ± 0.17bc	13.96 ± 0.21a	12.76 ± 0.30c	13.49 ± 0.41b
	18:0	3.46 ± 0.30b	3.50 ± 0.13ab	3.41 ± 0.14c	2.92 ± 0.07d	3.52 ± 0.13a
	18:1	2.36 ± 0.11a	2.21 ± 0.09ab	1.98 ± 0.08b	2.18 ± 0.10ab	2.30 ± 0.10a
	18:2	10.76 ± 0.34b	11.34 ± 0.24a	11.07 ± 0.28ab	11.32 ± 0.15a	11.14 ± 0.37ab
	18:3	64.09 ± 0.52b	64.59 ± 0.32a	64.14 ± 0.22b	64.78 ± 0.45a	63.88 ± 0.67b
	IUFA	216.15c	218.66b	216.54c	219.16a	216.22c

wheat to low temperatures is very important. Our results indicated that, under cold stress, the PMs of the transgenic lines were injured to a lesser extent than those of WT; this indicates that over-accumulation of GB can protect the PM from cold-induced damage. Cold stress was also observed to induce an increase in the MDA content (Fig. 1*D*); this suggests that lipid peroxidation occurred during stress. MDA content was even more sensitive to cold stress than electrolyte leakage.

It is necessary to understand how GB alleviates membrane damage. Studies have documented that H^+-ATPases in the PM are more sensitive to cold stress than other ATPases (Arora and Palta 1991). The results of this study (Fig. 1*B*) showed that PM H^+-ATPase activities in both WT and transgenic plants decreased under cold stress; this decrease was attributable to the cold-induced deactivation of membrane proteins. Further, our results suggested that the accumulation of GB in transgenic wheat plants could protect the PM H^+-ATPases. Kosová *et al.* (2007) suggested that irreversible dysfunction of the plasma membrane as a consequence of freeze-induced dehydration is the primary cause of freezing injury. One of the most effective mechanisms in plants to mitigate this injury is to accumulate compatible solutes (Uemura *et al*. 2006), which can maintain the integrity and function of PM against cold stress. Studies have also suggested that sugars, GB, and proline may function as cryoprotectants by helping to maintain the ordered vicinal water layer around proteins, thereby effectively stabilizing them against stress-induced inactivation and degradation (Hincha 2006). The results of this study demonstrate that overexpression of GB in transgenic plants can increase the accumulation of soluble sugars and proline (Fig. 1*E*,*F*). We speculated that the increased accumulation of soluble sugars and proline may also be involved in the protection provided by GB to PM H^+-ATPase under cold stress as also suggested by Quan *et al.* (2004).

Cold stress induced the increased generation of ROS, however, the generation of ROS was considerably less in transgenic lines than in WT (Fig. 2*A*,*B*). The structure and activity of antioxidant enzymes may possibly be protected by GB under cold stress (Sakamoto and Murata 2002). Higher CAT activity and lower H_2O_2 content were observed in transgenic lines than in WT under cold stress. Similar results have been reported in transgenic plants expressing the *codA* gene (Alia *et al*. 1999). However, SOD is not very sensitive to cold (Fig. 2*E*). The over-accumulation of proline and soluble sugars in transgenic plants may also be responsible for the decrease in the O_2^- content (Smirnoff and Cumbes 1989, Shen *et al*. 1997).

The fluidity of the PM is predominantly affected by the contents of unsaturated fatty acids and membrane-bound proteins. In this study, we observed that, among all the unsaturated fatty acids, linolenic acid, which is a major member influencing the IUFA, occurred in the highest percentage (Table 1). The topic of utmost interest was the difference in the proportion of oleic acid, linoleic acid, and linolenic acid between WT and T6 ($P < 0.05$). In general, the proportion of linolenic and linoleic acids is based on the proportion of their synthesis and desaturation from oleic acid 18:1 (Browse and Slack 1983). The higher linolenic acid and linoleic acid contents, and the lower stearic acid and oleic acid contents in T6 may be due to the enhanced fatty acid metabolism caused by GB.

In conclusion, cold stress injures the PM to a great extent. GB overexpression can improve the cold resistance in the *BADH*-transgenic wheat lines. The protective activity of GB may involve improvement in the efficiency of ROS elimination and in the fluidity of the PM.

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