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**Characterization of Two Winter Wheat Varieties' Responses to Freezing in a
Frigid Region of China**

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Running title: Glycine Betaine in two cold-resistant winter wheat varieties

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Abstract

Cold stress is one of the main abiotic stresses faced by winter wheat, which results in significant yield loss, especially in the harsh winter of the Heilongjiang province. Glycine betaine (GB), an important osmolyte in higher plants, helps in stabilization of the plasma membrane and its protection from cold stress. In the present study, two winter wheat varieties, Dongnongdongmai1 (DM1) and Jimai22 (J22), which differ in their cold-resistance were planted under natural conditions and used for analyzing the relative electrical conductivity, malondialdehyde (MDA) content, betaine aldehyde dehydrogenase (BADH) activity, expression of BADH, and the content of GB. The cold-resistant variety, DM1, showed a greater increase in BADH activity and GB content and decreased MDA content than J22 under freezing conditions. GB was observed to have an obvious role in inhibiting the MDA content. This was reflected by the expression of BADH and enhanced tolerance to cold stress upon GB accumulation, which helped in membrane stabilization. The results of the present study confirmed the role of GB in conferring cold-resistance in the DM1 winter cultivar and could benefit other studies aimed at improving the tolerance of other wheat cultivars as well as other crops.

Keywords: Winter wheat, Cold resistance, BADH, Glycine betaine

INTRODUCTION

Abiotic stresses cause severe injury and yield loss in crops. Two of the most common abiotic stresses are drought and low temperature, which induce osmotic stress. At temperatures below 0°C, plants are subjected to freezing injury, caused by the destruction of the protoplasm and could be fatal for the plant (Yan et al. 2011). Many physiological and biochemical mechanisms enable plants to adapt to freezing stress; one of them being the accumulation of compatible solutes, such as polyols, proline, and betaine.

Glycine betaine (GB) is a solute that accumulates in different cellular compartments to maintain the osmotic balance of the cells (Hanson and Scott 1980). It acts as molecular chaperone in the stabilization of the protein tertiary structure (Yancey et al. 1982) under water deficit or salt stress (Hanson and Scott 1980, Weretilnyk and Hanson 1990). In higher plants, glycine betaine is synthesized from choline via a two-step reaction, where betaine aldehyde act as an intermediate compound. The first reaction is catalyzed by choline monooxygenase (CMO; Brouquisse et al. 1989; Yang et al. 2015), whereas the second one is catalyzed by betaine aldehyde dehydrogenase (BADH; Weigel et al. 1986), which convert betaine aldehyde to glycine betaine. To date, *BADH* has been cloned from several plants, such as *Atriplex hortensis*, *Spinacia oleracea*, *Suaeda liaotungensis*, *Solanum tuberosum* and *Sesuvium portulacastrum* etc. and has been successfully transferred into many plants, including *Triticum aestivum*, *Solanum tuberosum*, and *Medicago sativa* (Ahmad et al., 2014; Di et al. 2015). Transgenic plants carrying the *BADH* gene show enhanced tolerance to various abiotic stresses. However, the evaluation for stress tolerance in most of these studies was conducted in glass houses under artificial environment. Furthermore, for simulation of cold stress, the plants were subjected to

only a few days of cold treatment, with temperatures reaching only a few degrees below zero. In contrast, under field conditions, for example in Heilongjiang Province, winters last for more than 5 months with minimum temperature reaching -30°C . In addition, a considerable difference exists between the day- and night-time temperatures of this region. Thus, it is anticipated that the response of plants to stress would differ under laboratory and natural conditions.

Dongnongdongmai 1 (DM1) is a winter wheat cultivar that can survive the harsh winters of Heilongjiang province with an overwintering survival rate exceeding 70% (Wang et al. 2009, Liu et al. 2013). But the overwintering survival rate of Jimai22 (J22) was no more than 20%. In the present study, we examined the metabolic profiles of GB biosynthesis pathway in order to analyze the cold tolerance of winter wheat. The results of this study not only find one of the reason of cold resistance but also utilization of this cultivar in breeding programme for cold resistance and also help in contribution of osmolyte accumulation to freezing tolerance of this species.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two winter wheat varieties (*Triticum aestivum* L., $2n=6x=42$, AABBDD), with significantly different overwintering survival rate, were used in the present study. One variety was Dongnongdongmai 1 (DM1), developed at the Northeast Agricultural University located in Harbin ($44^{\circ}04'\text{N}$ – $46^{\circ}40'\text{N}$, $125^{\circ}42'\text{E}$ – $130^{\circ}10'\text{E}$), Heilongjiang Province, China and validated by the Heilongjiang Province Crop Variety Certification Committee in 2007. This was the first winter wheat variety that could be grown in the cold areas of China with an overwintering survival rate exceeding 70%, reaching even 90% in some years (Xie et al. 2015). The other variety used was Jimai

22 (J22), which was developed by Shandong Province Academy of Agricultural Sciences (34°87' N – 37°32' N, 116°45' E – 117°57' E). J22 is the main winter wheat variety cultivated in the Huanghuai wheat growing area and it is a cold-resistance variety in that area. But in Heilongjiang Province, the overwintering survival rates of J22 for six consecutive years (2007–2012) were 0% (Xie et al. 2015). The field trial was carried out in a completely randomized design. The two winter wheat varieties were planted in the Northeast Agricultural University campus on September 3, 2013 and September 7, 2014, respectively. Each variety was hand-sown in a 30m² plot with 20 rows that were 5 m long with an inter-row distance of 30 cm. Close spacing of individual plants is 2cm. Fertilizers were applied at the rate of 17.5 g N m⁻² as urea and 9.0 g P m⁻² as single superphosphate to soil before sowing. Field preparations consisted of deep ploughing and harrowing.

Temperature Changes during the Test Period

As cold acclimation allows hardy plants to develop efficient tolerance mechanisms needed for winter survival (Allard et al. 1998), the wheat varieties used in the present study were acclimated by daytime temperatures below 10°C, night temperatures above 0°C, and the average daily temperature below 10°C (Wang et al. 2013). In the present study, the acclimation was performed from October 12 to November 14 in 2013 and from October 10 to November 10 in 2014. During the freezing stage, the average temperature was below 0°C; this stage started from November 15, 2013 and November 11, 2014, in the present study. The temperature (maximum, minimum, and average) details are provided in Fig. 1.

The details of the sampling dates in both the years are shown in Table 1. The sampling days spanned through the initiation of tiller stage to almost the coldest

period of the winter. The seedlings reached the two or three tiller-stage by November 1 in 2013 and 2014. We investigated the number of seedlings at this stage in order to know the cross-winter survival rate of the winter wheat varieties. The field was covered with snow after November 15 in 2013 and November 21 in 2014. At each sampling date, 30 seedlings were harvested from three accessions of each cultivar. Crowns were cut from each seedling sample. Few crowns from each sample were used to measure the relative electrical conductivity, malondialdehyde (MDA) content, and BADH activity. Other crowns were snap-frozen in liquid nitrogen and stored at -80°C for further estimation of GB content and the expression of *BADH* gene. We investigated the number of survival seedlings at April 3 in 2014 and April 6 in 2015 and calculated the cross-winter survival rate.

Detection of Physiological and Biochemical Parameters Related to low Temperature Stress

Plant fresh weight, MDA, relative electrical conductivity (REC), GB content BADH activity, and were done from the crowns (2-3cm shoots part above the roots containing the tillering nodes) of acclimatized DM1 and J22 plants and is repeated using triplicates for both the years.

Plant fresh weight

The seedling fresh weight was scored immediately after removal from the field.

MDA contents

MDA contents were determined according to the published methods of Bates et al. (1973) with minor modifications. Crown tissue (0.5g) was homogenized in 3 mL of 10%

trichloroacetic acid (TCA) using a pestle and mortar. The homogenates were then centrifuged at 8 000g for 10 min. One-mL aliquot of the supernatant was incubated with 1 mL of 0.5% thiobarbituric acid (TBA) in 10% TCA in boiling water for 15 min. After centrifugation at 8000g for 10 min, the absorbance of the supernatant was read at 450, 532, and 600 nm.

Relative electrical conductivity

Relative electrical conductivity was detected using a conductivity meter (DDS-11A, Shanghai, China; Li, 2000). $REC = R_{\text{fresh}} / R_{\text{kill}}$. Where R_{fresh} = electrical conductivity of the water in which the crown was immersed for 1h, and R_{kill} = electrical conductivity of the water after boiling the crown tissue for 15min in immersed water.

BADH enzyme activity

The BADH enzyme activity was measured according to the previously published procedures (Liu et al. 1997). Crown tissue (0.5g) was homogenized in ice-cold 50 mM potassium phosphate buffer (2ml; pH 7.0, 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ D-isoascorbic acid, 2% (w/v) PVP and 0.05% (w/v) Triton X-100). The homogenates were centrifuged at 12,000 g for 15 min at 4 °C, and the supernatants were collected and used in the enzyme assays. BADH activity reaction mixture contained 100 mM K-phosphate buffer (pH8.0), 5mM DTT, 0.5 mM NAD⁺ and 0.5 mM betaine aldehyde. Measurements of fluorescence were carried out with a Hitachi fluorescence spectrometer (U3900, Japan; excitation 340 nm, emission 460 nm). Enzyme activity was expressed as initial rates for the reduction of NAD⁺ per mg total protein.

GB content

GB content was estimated according to methods of Moghaieb et al. (2004). Crown samples used for betaine determination were transferred to vials subjected to methanol extraction, and stored in the dark at 4 °C. The methanol extracts were passed through Dowex-1-H- and Dowex-50-H+ columns. Betaine was eluted with 6M NH₄OH and then analyzed by HPLC (Agilent 1260 Infinity, Germany) with a SB-C8 Analytical HPLC column. The standard material of betaine (> 95%) was purchased from ChromaDex (CA, USA).

Total RNA Isolation and cDNA Synthesis

All the samples were ground to a fine powder in liquid nitrogen with a pestle and mortar. Total RNA was extracted using a Plant Total RNA Kit (Tiangen, Beijing, China) according to manufacturer's instructions. The quality and concentration of the total extracted RNA was determined by spectrophotometric analysis. For quantitative real-time polymerase chain reaction (qRT-PCR), 1 µg total RNA was used for reverse transcription using Bioneer RocketScript RT PreMix (Seoul, Korea). The resulting cDNA was diluted 5-fold in a final volume of 20 µL and used as a template for subsequent PCR analyses.

qRT-PCR Analysis

BADH transcript levels were analyzed using qRT-PCR. Reactions were performed in triplicate, in a 96-well plate, with an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using SYBR Green detection chemistry. Reactions were carried out in a total volume of 20 µL that contained 10.4 µL 2X TransStartTop Green qPCR SuperMix with 0.4 µL Passive Reference Dye II (50X; Transgene, Beijing, China), 2 µL cDNA, and 0.4 µL (300 nM) of each gene-specific

primer. The following standard thermal profile was used for all the PCRs: polymerase activation (94°C for 30 s), amplification, and quantification cycles repeated 40 times (94°C for 5 s, 60°C for 34 s, and 72°C for 10 s). Finally, a dissociation analysis of the PCR products was performed by running a gradient from 60 to 95°C to confirm the presence of a single PCR product and the specificity of qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. A pair of primers for *BADH* was designed using Primer Premier 5.0 software. The relative expression levels were calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT, \text{target} - CT, \text{actin})_{\text{time x}} - (CT, \text{target} - CT, \text{actin})_{\text{time 0}}$ (Livak and Schmittgen, 2001). The primer sequences used in the reactions are shown in Table 2.

Statistical Analysis

Statistical analyses of data were performed using SPSS 20.0. Analysis of variance (ANOVA) was used to evaluate the effects on physiological and biochemical parameters in response to low temperature stress.

The relative difference rate (RDR) in various parameters was defined as the ratio of the difference of observed values for DM1 and J22 and the observed value for J22 at each sampling date. The equation used was as follows:

$$RDR \% = \frac{\text{DataDM1} - \text{DataJM22}}{\text{DataJM22}} \times 100\%$$

RESULTS

Comparative Analysis of Cross-winter Survival Rate of the Two Wheat Varieties

From the coldest period to the next April in 2013 and 2014, the average temperature differences were approximately 30°C and the lowest temperature was below -25°C.

Fig. 2 shows that the cross-winter survival rate of DM1 were much higher than in J22,

which were above 90% for both the years. The survival rate in J22 was not more than 25% in two years. It was verified in a previous experiment that DM1 was a more cold-resistant variety than J22.

Comparative Analysis of Plasma Membrane Stability in Crowns of the Two Wheat Varieties At Low Temperature

There were no obvious differences in the REC between the two wheat varieties at the first sampling date for both the years. However, the REC was significantly lower ($P \leq 0.05$) in DM1 than in J22 in the samples collected on the next seven dates (Fig. 3A). The REC rapidly increased in DM1 in the samples collected on December and November 15. The minimum and the maximum value of REC in the two varieties were obtained in the samples collected on October 1 and January 15. The REC of DM1 ranged from 0.15 to 0.73 and 0.19 to 0.71 in the years 2013 and 2014, respectively, whereas it ranged from 0.19 to 0.84 and 0.21 to 0.82 in J22 for these years.

With the temperature falling below 0°C, there was a significant increase in the levels of MDA content in both the varieties. The MDA content in DM1 was lower than that in J22 after November 1. From December 1 to January 15, the MDA content decreased in DM1 (Fig. 3B)

Cold Stress Induces Accumulation of GB and Improves the Activity of BADH

GB contents did not differ in both cultivars until November (Fig. 3C). However, the contents increased rapidly on November 15, and were 2- to 2.25-fold higher than the content in the October 1 samples. Although the difference in GB content was inconspicuous at basal levels in the two varieties, the total accumulation of GB was 21% higher in DM1, the more tolerant winter wheat cultivar, than in J22 ($P \leq 0.05$).

BADH enzyme activity demonstrated a trend similar to that of GB accumulation. The activity in DM1 was 1.3-fold higher than in J22 on December 1; the highest activity was observed from October 1 to January 15. After December 1, as the temperature continued to fall, the activity of BADH was inhibited in both the varieties (Fig. 3D).

Up-Regulation of BADH Gene Expression in Winter Wheat under Cold Stress

To determine whether BADH expression was associated with cold stress, RNA extracted from the crown of wheat varieties was used for qRT-PCR analysis. The expression of BADH in both the varieties showed notable up-regulation with the decrease in temperature from October 1 to December 1 (Fig. 3E). The relative expression reflected the level of the transcript. BADH exhibited similar expression patterns in the two wheat varieties. The maximum transcript level was detected in DM1 on December 1, which was around 6-fold higher than the expression in the October 1 samples. However, in comparison with the BADH expression levels in DM1, the expression level of J22 was lower during the same period. The up-regulated pattern of BADH in the two varieties waned from December 15 to January 15.

GB Protects the Plasma Membrane Stability

As shown in Fig. 3A–Fig. 3E, for all the sampling dates, the GB content and expression of BADH, as well as its activity, was higher in DM1 than in JM22. In contrast, the REC and MDA content was lower in DM1. These differences increased with the decrease in temperature and revealed the same trend for both the years. To depict the changes in the above-mentioned parameters clearly, relative difference rates were calculated to compare the changing trends of the different parameters during the

cold treatment period (Fig. 4). The difference rates of BADH expression reached the maximum value on October 1 in both the years, thereafter decreasing in an undulating pattern to 16.12% and 18.68% in 2013 and 2014, respectively. Along with the changing *BADH* expression, the difference rate of BADH activity increased from October 1 to January 15. The difference rates for the GB content in DM1 and J22 were much higher, which changed approximately from 0% to 40% in both the years. However, during this period, the difference rates for REC decreased rapidly from -10% to -14% and MDA content also decreased from -3% to -22%. REC in DM1 (samples collected on December 1) was around 30% lower than in J22 in both the years. Moreover, damage to the plasma membrane was less severe in DM1 compared to J22.

DISCUSSION

Short-term cold stress can hamper spike differentiation in wheat, and result in a great yield reduction. It also affects protein and carbohydrate quality of wheat grain. Therefore, enhancing the tolerance of wheat to low temperatures is very important. In previous research using DM1 and J22, the plant materials were planted in the growth cabinet which cannot reflect the natural environment accurately. But the present study was performed in the field, during winter and, plants response to the stresses was more reliable than in growth cabinet. Cold stress not only affects the lipid composition of plasma membrane but also membrane permeability, which can be assessed by relative electrical conductivity (Zhang et al. 2010). In the present study, the decline in temperature induced an increase in REC by approximately 0.6 in DM1 and 0.7 in J22 (Fig. 3A). The increased REC indicated damage of plasma membrane. However, the extent of injury was lower in DM1 than in J22. Cold stress was also observed to induce an increase in the MDA content (Fig. 3B). The increasing MDA

content suggested that lipid peroxidation occurred during the stress. The level of lipid peroxidation in cold-resistant variety DM1 was lower than the level in cold-sensitive variety J22.

GB is an osmoprotectant found in many organisms, including higher plants such as maize (Quan et al. 2004, Chen and Murata 2008), *Arabidopsis* (Xing and Rajashekar 2001), tomato (Park et al. 2006), and wheat (Ma et al. 2006). It maintains the water balance between plant cells and the environment and stabilizes macromolecules, enzyme activities, and membranes under stress conditions (Joseph et al. 2013). Alteration of the lipid composition of the cell membrane is known to be associated with low temperature tolerance since cell membranes are the first sites of injury (Liu et al. 2008). Chilling stress affects membranes due to the impairment of membrane permeability by the transition of lipids from liquid crystalline phase to gel phase. GB acts a membrane stabilizer contributing to the stabilization and integrity of cellular membranes when plants are subjected to low temperature conditions (Park et al. 2006). As evident from the relative difference rate analysis during the sampling period, the GB content and the activity of BADH in DM1 were much higher than in J22. Concomitantly, the increase in MDA content and REC was lower in DM1 than in J22. All these attributes have been confirmed to be involved in the defense response of plants by protection of the plasma membrane and inhibition of MDA production (Chen and Murata 2008; Zhang et al. 2010). In the present study, GB content increased slowly during the acclimation period. However, there was a significant increase in the GB content in the November 15 samples for both the years when the average temperature was below 0°C. This finding indicates that GB accumulation at the end of acclimation period plays an important role in response to the freezing temperatures. In addition, the cold-resistant variety DM1 synthesized and

accumulated more GB than did the cold-sensitive variety J22 during the cold acclimation and freezing stage, as shown in Fig. 3D. It also had 1.3-fold higher BADH activity than the J22 variety under cold stress.

In conclusion, our results indicate that the expression of BADH and the accumulation of GB plays key role in membrane stability which contribute in the cold stress response in the cold-resistant winter wheat variety DM1.

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Figure Captions

Fig. 1. Details of temperature in the year 2013 and 2014.

Fig. 2. The cross-winter survival rate of two winter wheat varieties in two years. Values shown are mean $\pm$ SE of three replicates per plant.

Fig. 3. Effect of cold stress on REC, MDA, GB, BADH activity, and BADH expression in the wheat cultivars DM1 and J22 in the year 2013 and 2014. The two winter wheat varieties were planted on the field and sampled 8 times per year. (A) REC, (B) MDA content, (C) GB content, (D) BADH activity, (E) BADH mRNA expression. Significance of difference was tested at $P = 0.05$ using ANOVA post hoc LSD. Values shown are mean $\pm$ SE of three replicates per plant.

Fig. 4. Relative difference rate of various parameters between DM1 and J22 cultivars at each sampling date. The RDR was defined as the difference in the observed values in DM1 and J22 divided by the value in J22 at each sampling date.

Table 1 Sampling Dates

Year	Dates (month/date)							
2013	10/1	10/15	11/1	11/15	12/1	12/15	12/30	1/15
2014	10/1	10/15	11/1	11/15	12/1	12/15	12/30	1/15

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Table 2 Primers used for real-time polymerase chain reaction

Gene name	Amplicon size (base pairs)	GenBank accession number	Forward and reverse primer 5'→3'
BADH	104	AY050316.1	F: GTCTGGCTGGTGCCGTGATTTCTG R: GAAGCAAGGCTGCGAGCAGTTCAC
GAPDH	148	EF592180.1	F: TAAGGGTGGTGCCAAGAAGGT R: AGCAAGAGGAGCAAGGCAGTT

For Review Only

Fig. 1. Details of temperature in the year 2013 and 2014.

207x229mm (300 x 300 DPI)

Fig. 2. The cross-winter survival rate of two winter wheat varieties in two years. Values shown are mean $\pm$ SE of three replicates per plant.

125x75mm (100 x 100 DPI)

Fig. 3. Effect of cold stress on REC, MDA, GB, BADH activity, and BADH expression in the wheat cultivars DM1 and J22 in the year 2013 and 2014. The two winter wheat varieties were planted on the field and sampled 8 times per year. (A) REC, (B) MDA content, (C) GB content, (D) BADH activity, (E) BADH mRNA expression. Significance of difference was tested at $P = 0.05$ using ANOVA post hoc LSD. Values shown are mean $\pm$ SE of three replicates per plant.

209x253mm (300 x 300 DPI)

Fig. 4. Relative difference rate of various parameters between DM1 and J22 cultivars at each sampling date. The RDR was defined as the difference in the observed values in DM1 and J22 divided by the value in J22 at each sampling date.

71x74mm (300 x 300 DPI)