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BRIEF COMMUNICATION

Effect of glycinebetaine on function of thylakoid membranes in wheat flag leaves under drought stress

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Abstract

Two wheat cultivars, HF9703（drought tolerant）and SN215953 (drought sensitive) were used to examine the effects of glycinebetaine (GB, 100 mM) on lipid composition and function of thylakoid membranes under drought stress. GB application mitigated negative effect of drought on Ca^{2+}-ATPase and Hill reaction activities, chlorophyll content, gas exchange and photosynthesis. These positive effects of GB application maybe, in part, correlated with improving the lipid composition of the thylakoid membranes.

Additional key words: Ca^{2+}-ATPase, chlorophyll content, gas exchange, Hill reaction activity, lipids, *Triticum aestivum*.

Approximately 70 ~ 90 % of wheat grain yields depend on successful photosynthesis of the flag leaves (Bidinger *et al*. 1977). High photosynthesis of flag leaves in a hot and dry summer is critical since the basal leaves of the plants have begun to senesce during this stage.

The thylakoid membrane is the site of the photosynthetic light reactions. These membranes maintain a lipid skeleton and pigment-protein complexes. The protein complexes, especially those of photosystem 2 (PS 2) are highly vulnerable to environmental stresses. For example, it was reported that salt stress caused inactivation of PS 2 reaction center (Allakhverdiev *et al*. 2001) and led to peripheral protein divorced from PS 2 complex (Miyao and Murata 1989). Giardi *et al*. (1996) reported that drought stress resulted in membrane fatty acid desaturation.

It is well known that plants often accumulate compatible solutes under unfavourable conditions including glycinebetaine (GB), which can protect membrane functionality and induce osmotic adjustment. Both external application of GB and plant transformation with the aim of manipulation in GB biosynthesis pathway have been shown to increase the tolerance of the plants to adverse environment (for review see, Cherian *et al*. 2006). Some data suggest that GB also protects the photosynthetic apparatus from damage under stress condition (Xing *et al*. 1999, Zhao *et al*. 2001, Allakhverdiev *et al*. 2003). However, whether and how GB protects the photosynthetic apparatus and function by changing lipid composition in thylakoid membranes under stress conditions remains still unclear and warrants investigation. In the present experiments, we used two wheat cultivars with different drought tolerance and determined their responses to GB application under drought stress. The parameters including GB content, gas exchange, Hill reaction activity, chloroplast Ca^{2+}-ATPase activity, chlorophyll content and lipids in wheat flag leaves were investigated. Our data can help to better understanding of the mechanism of GB ability to improve the drought resistance of wheat plants.

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Abbreviations: c_i - intercellular CO_2 concentration; DGDG - digalactosyl diacylglycerol; g_s - stomatal conductance; GB - glycinebetaine; IUFA - index of unsaturated fatty acid; MGDG - monogalactosyl diacylglycerol; OEC - oxygen-evolving complex; PS 2 - photosystem 2; P_N - net photosynthesis rate; PG - phosphatidylglycerol; PC - phosphatidylcholin; SQDG - sulfoquinovosyl diacylglycerol.

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Seeds of two winter wheat (*Triticum aestivum* L.) cultivars, HF9703 (drought-tolerant) and SN215953 (drought-sensitive), were sown in pot filled with soil. Plants were well-watered until the heading stage, then, aqueous 100 mM GB solution containing 0.1 % *Tween 20* was sprayed on the leaves until runoff twice a day for three days, as described by Ma *et al.* (2006). Control plants were sprayed with water containing 0.1 % *Tween 20*. Drought stress (DS) was induced by withholding irrigation after GB application. The soil water content at 20 ~ 40 cm depth was 73.2 % of water saturated soil in controls and 45.9 % under drought treatment when the leaves were used for analyses.

Flag leaves were excised and cleaned with tissue paper. Samples (0.5 g) for each replicate were stored in liquid nitrogen. GB was analyzed using high performance liquid chromatography (HPLC; *Shimadzu-LC-6A*, Kyoto, Japan) followed the procedures modified by Chen *et al.* (2000). Briefly, samples were extracted in methanol at 4 °C for 24 h. and then in methanol:chloroform:water (ultra pure; 10:5:6). GB was concentrated in the upper aqueous phase with a rotor-evaporator and the pellet was dissolved in ultra pure water. The resuspended samples were passed through columns of *Amberlite CG250* cation and *Dowex1-X2* anion exchange resins. GB was eluted with 4 M NH_4OH and the resuspension was concentrated to dryness. GB pellets were dissolved in methanol and the mixture was filtered through a 0.25 μm membrane before applying it to the HPLC system. The HPLC was equipped with *Spherisorb 10SCX* (4.6 × 250 mm) column and guard column. The mobile phase was $NH_4H_2PO_4$. The detection wavelength was set at 195 nm. Peak areas was determined and quantified using the samples against GB standards.

For determination of leaf chlorophyll contents (Chl *a* and *b*), frozen leaf discs were ground under dim light in a mortar containing liquid N_2 and pigments were extracted by ice-cold methanol (100 %, v/v). After centrifugation, Chl content was analyzed using a spectrophotometer (*Shimadzu, UV-160A*, Kyoto, Japan) at 665.2 and 652.4 nm and the amounts of chlorophyll were calculated as reported by Lichtenthaler (1987).

Chloroplasts were prepared following the modified procedure from Ye and Qian (1985). Flag leaves were ground and the homogenates were filtered through four layers of cheesecloth. The filtered homogenates was then centrifuged at 1 °C for 1 min at 300 *g*. The supernatants was collected and centrifuged for 10 min at 3 000 *g*. The pellet was stored at low temperature.

The polar lipids were extracted from the thylakoid membranes following the method of Su (1980) and then separated by two-dimensional thin-layer chromatography (TLC; *Qingdao Haiyang Chemical Plant*, China). After a second extraction using benzene:petroleum ether (1:1, v/v), the combined extracts were esterified with 0.4 M NaOH. The fatty acid methyl esters were determined using a gas chromatogram analyzer (*Shimadzu GC-9A*), using a methyl esterified arachidic acid as an internal standard. The conditions were: glass column 2 m × 3 mm, *Chromosorb W. AW.DWCS* 80～100 mesh, solid phase 15 % diethylene glycol succinate (DEGS), column temperature 190 °C, detector temperature 290 °C, pneumatophore pure N_2 with flowing velocity 100 $cm^3\ min^{-1}$. Quantification was calculated by normalization, which is done with the processing software of the apparatus.

The Ca^{2+}-ATPase activity was determined following coupling factor activation by trypsinase according to Huang (1985). 1 cm^3 chloroplasts suspension was added to 1 cm^3 medium containing 50 mM Tris-HCl with pH 8.0, 4 μM EDTA, 2 μM ATP, 2 mg cm^{-3} trypsin, following 10 min incubation at 20 °C, 0.1 cm^3 bovine serum albumin (10 mg cm^3) was added to terminate the incubation. Then, 0.8 cm^3 incubated chloroplasts suspension was added to 1 cm^3 reaction mixture containing 150 mM Tris-HCl with pH 8.0, 50 mM ATP and 5 mM $CaCl_2$. After 10 min at 37 °C, centrifuged at 300 *g* 1 min, the supernatant was used to determine the content of inorganic phosphorus.

Hill reaction activity was determined following the procedures modified from Ye and Qian (1985). The medium containing 0.5 M Tris-HCl with pH 7.6, 0.05 M $MgCl_2$, 0.1 M NaCl, 0.01 M $K_3Fe(CN)_6$ and 0.1 cm^3 chloroplasts suspension was diluted with distilled water. The mixture was exposed to irradiance of 800 μmol $m^{-2}\ s^{-1}$ for 1 min at 20 °C, the reaction was terminated by 10 % TCA. Then the mixture was centrifuged at 1 000 *g* for 2 min, and the supernatant was extracted for the assay of $Fe(CN)_6^{4-}$.

The net photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular CO_2 concentrations (c_i) were measured with an open system with infrared gas analyzer (*CIRAS-I, PP Systems*, Hertfordshire, UK) between 10:00 and 12:00, at CO_2 concentration 360 μg g^{-1}, and O_2 concentration 21 %, irradiance of about 1 200 μmol $m^{-2}\ s^{-1}$, relative humidity of air 80 % and the air temperature between 24 and 30 °C.

Each treatment was repeated three times and all measurements were made at least three times within each treatment. Data are represented with mean ± standard errors (SE). All data were subjected to analysis of variance (*ANOVA*) and the means were compared by Fischer's LSD test.

Both wheat cultivars accumulated GB under all treatments (CK, DS and DS+GB) although at different degrees. Drought stress caused increases in GB contents in both cultivars. GB pre-treatment increased GB content in both wheat cultivars compared to DS-treated plants. These results indicate that foliar-applied GB was absorbed and stored by wheat flag leaves (Table 1).

Net photosynthetic rate (P_N) of both wheat cultivars was significantly inhibited by drought stress, and the inhibition in SN215953 was greater than in HF9703. Foliar-applied GB increased net photosynthetic rate under drought stress condition. P_N in GB pretreated HF9703 and SN215953 was 16.2 and 26.5 % higher than that in non-GB treated plants under stress condition, respectively.

Drought stress decreased stomatal conductance (g_s)

Table 1. Effects of foliar-application of glycinebetaine on the endogenous GB content [μg g^{-1}(d.m.)], net photosynthetic rate, P_N, [μmol(CO_2) m^{-2} s^{-1}], stomatal conductance, g_s, [mmol(H_2O) m^{-2} s^{-1}], internal CO_2 concentration, c_i, [μmol mol^{-1}], Hill reaction activity, HRA, [μmol(O_2) mg^{-1}(Chl) min^{-1}], chloroplast Ca^{2+}-ATPase activity, CAA, [μmol(Pi) mg^{-1}(Chl) min^{-1}] and chlorophyll content, Chl, [g m^{-2}] in wheat flag leaves of cvs. HF9703 and SN215953 under drought stress. Means ± SE, n = 3. Means in a column followed by the same letter are not different at the level of P = 0.05. CK - well-watered wheat plants, DS - drought-stressed plants, DS+GB - drought-stressed plants pre-treated with 100 mM GB. Means compared by Fischer's LSD test: A (a) - statistically significant compared to CK within cultivar, B (b) - statistically significant DS+GB compared to DS within cultivar, C (c) - statistically significant of the same treatment between cultivars; a, b, c $P < 0.05$; A, B, C, $P < 0.01$).

Parameters	HF9703			SN215953		
	CK	DS	DS+GB	CK	DS	DS+GB
GB	12.7 ± 0.32	32.2 ± 2.51A	56.8 ± 2.45AB	7.5 ± 0.41C	28.2 ± 1.82Ac	49.2 ± 3.21ABC
P_N	15.8 ± 1.21	12.5 ± 1.11A	14.5 ± 1.22b	15.7 ± 1.35	9.2 ± 1.35Ac	12.5 ± 0.85aBc
g_s	358.0 ± 8.96	235.0 ± 10.5A	265.0 ± 14.5Ab	390.0 ± 15.2c	279.0 ± 12.2AC	285.0 ± 15.9Abc
c_i	203.0 ± 5.89	235.0 ± 8.25A	205.0 ± 9.25B	192.0 ± 5.89c	246.0 ± 8.25Ac	208.0 ± 8.52aB
HRA	526.0 ± 25.2	386.0 ± 12.5A	508.0 ± 18.2B	528.0 ± 28.6	337.0 ± 5.88AC	412.0 ± 15.2ABc
CAA	42.2 ± 12.5	35.2 ± 2.53A	38.5 ± 13.2b	45.8 ± 13.2	25.2 ± 8.25AC	37.5 ± 12.2aB
Chl	0.3 ± 0.05	0.2 ± 0.02a	0.3 ± 0.05b	0.4 ± 0.04c	0.3 ± 0.01AC	0.3 ± 0.03ac

but increased intercellular CO_2 concentration (c_i) significantly in both wheat cultivars, suggesting that both stomatal and non-stomatal factors were involved in the reduction of P_N (Xue *et al*. 1992). Pre-treatment with GB led to little change in g_s in both wheat cultivars under drought stress, but did result in significant decrease in c_i, suggesting that GB improved CO_2 assimilation inside the flag leaves (Table 1).

Under drought stress, Hill reaction activity was reduced by 26.6 and 36.1 % in leaves of HF9703 and SN215953 plants, respectively, suggesting that an injured PS 2 function was involved in the reduction of photosynthesis, and that the drought-resistant wheat cultivar HF9703 could retain better PS 2 function than the drought-sensitive cultivar SN215953. Foliar-applied GB enhanced the Hill reaction activities of both wheat cultivars significantly, indicating that GB could protect PS 2. Similar responses of Ca^{2+}-ATPase activities and chlorophyll contents to drought stress and GB application were observed (Table 1). Similarly, Liu *et al*. (2006) reported that osmotic dehydration decreased the content of Chl *a+b* and inhibited PS 2 activity more in the drought-sensitive than the drought-resistant wheat cultivar. They also found that the contents of major PS 2 proteins including the D1 and D2 proteins in the PS 2 reaction centre and the light-harvesting Chl *a/b*-protein complex in periphery, all of them embed in the thylakoid membrane, declined with increasing water stress.

Five lipid classes including monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), phosphatidylglycerol (PG) and phosphatidylcholin (PC), and and six fatty acids including palmic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid ((18:2) and linolenic acid (18:3) in the thylakoid membranes in the flag leaves of both cultivars were detected in the present experiment, to explore whether the effects of GB on function of the thylakoid membranes are associated with lipid and fatty acid composition of the thylakoid membranes (Table 2). The results suggested that the main fatty acid of MGDG and DGDG was 18:3, and the main fatty acids of SQDG, PG and PC were 16:0 and 18:2. The index of unsaturated fatty acid (IUFA) of MGDG was increased in SN215953 but decreased in HF9703 in response to DS. GB application had no effect on the fatty acid composition in both cultivars. In HF9703, the DS treatment increased SQDG saturated fatty acid profile and the relative content of fatty acid 16:1(3t) in the PG. However, reverse changes were observed in SN215953. GB pre-treatment decreased the saturated fatty acid content in SQDG, and increased the relative content of 16:1(3t) in the PG profile significantly in SN215953, but not in HF9703 (Table 2).

Drought stress resulted in altered lipid and fatty acid components, which may affected the thylakoid membrane structure and function (McConn *et al*. 1998). The GB pre-treatment partially restored the normal lipid profile under drought stress conditions, thus GB may protect the thylakoid membrane function from damage and improve the photosynthesis capacity of wheat flag leaves (Table 2).

It is well known that drought stress usually results in the accumulation of reactive oxidative species (ROS) and following membrane disorganization may be a part of the general damage (De Longo *et al*. 1993, Reddy *et al*. 2004). It is likely that GB could protect membrane against oxidative stress by stimulation of anti-oxidative enzymes (Ma *et al*. 2004, 2006), or GB could eliminate ROS directly (Smirnoff *et al*. 1989, Shen *et al*. 1997). Additionally, there is some evidence for the involvement of GB in the protection of the transcription and translation machinery under stress conditions (Rajendrakumar *et al*. 1997, Allard *et al*. 1998, for review see Cherian *et al*. 2006).

In conclusion, under drought stress, GB improved

photosynthesis. The positive effects of GB application led to stabilization of the function of the thylakoid membranes, suppression of chlorophyll degradation and enhancement of Ca^{2+}-ATPase and Hill reaction activities, possibly by affecting the membrane lipid and fatty acid composition.

Table 2. Effects of foliar-application of GB on the fatty acid composition [mol%] of the thylakoid membrane in the flag leaves of cvs. HF9703 and SN215953 under drought stress. The abbreviation for fatty acid is denoted by ratios, indicating the number of carbons: the number of unsaturated bonds. The 3t of 16:1(3t) denotes a trans-double-bond at the third carbon. IUFA = 18:1 % + (18:2×2) % + (18:3×3) %. Means ± SE, *n* = 3. Means in a column followed by the same letter are not different at the level of *P* = 0.05. tr - trace (< 0.5 %). FA - fatty acid composition, CK - well-watered wheat plants,DS - drought-stressed plants, DS+GB - drought-stressed plants pre-treated with 100 mM GB. Statistical analysis as in Table 1.

Lipid	FA	HF9703			SN215953		
		CK	DS	DS+GB	CK	DS	DS+GB
MGDG	16:0	0.8 ± 0.3	1.8 ± 0.4A	1.0 ± 0.3AB	1.8 ± 0.4C	1.8 ± 0.6	1.5 ± 0.7abc
	16:1(3t)	-	-	-	-	-	-
	18:0	-	1.0 ± 0.6A	-	-	-	0.6 ± 0.3AB
	18:1	1.9 ± 0.2	2.9 ± 0.3A	2.0 ± 0.6b	2.4 ± 0.2C	1.2 ± 0.3Ac	2.0 ± 0.2aBc
	18:2	5.7 ± 0.6	7.8 ± 1.2a	5.8 ± 0.9B	5.3 ± 0.5	3.1 ± 0.3Ac	4.8 ± 0.4bc
	18:3	91.6 ± 1.4	86.6 ± 2.7a	91.2 ± 1.6	90.6 ± 1.0	93.9 ± 1.2	91.2 ± 0.8
	IUFA	288.1	278.3a	288.4	284.8	289.1c	285.2
DGDG	16:0	8.2 ± 1.2	9.0 ± 1.3a	9.5 ± 1.2ab	6.0 ± 2.1C	5.4 ± 0.6aC	6.2 ± 1.2bc
	16:1(3t)	-	-	-	-	-	-
	18:0	-	tr	-	-	1.1 ± 0.7AC	tr
	18:1	4.5 ± 0.5	3.1 ± 0.6a	2.4 ± 0.5ab	1.1 ± 0.4C	0.5 ± 0.1aC	0.5 ± 0.3aC
	18:2	4.7 ± 0.6	4.4 ± 0.7a	3.8 ± 0.4Ab	3.4 ± 0.5c	2.9 ± 0.8AC	2.1 ± 0.6Abc
	18:3	82.6 ± 1.0	83.5 ± 1.3	84.4 ± 2.0	89.5 ± 2.1c	90.2 ± 1.3c	90.8 ± 2.0
	IUFA	261.7	262.4	263.2	276.4c	276.9c	277.1
SQDG	16:0	28.2 ± 2.1	28.1 ± 1.9	30.9 ± 2.3ab	28.1 ± 1.4	30.2 ± 1.1a	32.3 ± 1.0a
	16:1(3t)	-	-	-	-	-	-
	18:0	-	2.8 ± 0.5A	1.0 ± 0.3Ab	-	-	1.1 ± 0.6AB
	18:1	-	-	1.2 ± 0.4AB	2.8 ± 0.7AC	-	-
	18:2	8.1 ± 0.9	10.0 ± 1.3a	6.4 ± 1.6Ab	10.0 ± 1.3c	3.1 ± 0.4AC	5.0 ± 1.8ABc
	18:3	63.7 ± 0.9	59.1 ± 1.6	60.7 ± 1.4	59.1 ± 1.1c	66.8 ± 1.2c	61.6 ± 1.4bc
	IUFA	207.3	207.3	196.1a	200.1	206.6a	194.8ab
PG	16:0	19.1 ± 2.4	17.7 ± 1.6A	20.2 ± 1.8b	26.7 ± 1.1c	28.9 ± 0.0aC	28.4 ± 0.7ac
	16:1(3t)	20.4 ± 0.8	23.2 ± 0.4a	24.8 ± 1.2A	19.2 ± 1.0	16.7 ± 1.0ac	20.9 ± 2.1Bc
	18:0	-	-	-	-	2.3 ± 2.1AC	-
	18:1	5.3 ± 0.3	4.1 ± 0.8a	-	4.1 ± 0.4c	2.0 ± 0.2Ac	-
	18:2	19.2 ± 0.8	18.8 ± 0.6	18.0 ± 1.2a	14.8 ± 1.0C	13.0 ± 2.0c	12.0 ± 1.2ac
	18:3	36.0 ± 0.7	36.4 ± 0.6	37.2 ± 1.4	35.2 ± 0.8	36.8 ± 1.8	38.7 ± 1.3
	IUFA	151.7	150.9	151.2	139.3c	138.4c	140.1c
PC	16:0	25.0 ± 1.0	25.4 ± 1.2	26.2 ± 0.9	25.4 ± 0.8	26.4 ± 1.2	26.2 ± 1.6
	16:1(3t)	-	-	-	-	-	-
	18:0	-	-	1.7 ± 1.1B	-	-	-
	18:1	8.9 ± 1.0	6.9 ± 0.8a	4.4 ± 1.2ABB	3.9 ± 0.2C	2.3 ± 0.1aC	2.0 ± 0.2Ac
	18:2	27.4 ± 2.0	27.4 ± 0.6	25.7 ± 0.3b	22.4 ± 1.2c	23.0 ± 1.3c	21.9 ± 0.9c
	18:3	38.7 ± 1.3	40.4 ± 2.0	41.9 ± 0.7	48.4 ± 2.0	48.3 ± 1.0c	50.0 ± 0.8c
	IUFA	179.8	182.9	181.5	193.9c	193.2	195.8

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