PHOTOSYNTHETICA 46 (1): 10-16, 2008

Induction of cyclic electron flow around photosystem 1 and state transition are correlated with salt tolerance in soybean

K.X. LU[*,**], Y. YANG[*], Y. HE[*], and D.A. JIANG[*,***]

State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, 310058 Hangzhou, China[*]
Faculty of Life Science and Biotechnology, Ningbo University, 315211 Ningbo, China[**]

Abstract

We investigated the role of cyclic electron flow around photosystem 1 (CEF1) and state transition (ST) in two soybean cultivars that differed in salt tolerance. The CEF1 and maximum photochemical efficiency (F_v/F_m) were determined under control and NaCl (50 mM) stress and the NaCl-induced light-harvesting complex 2 (LHC2) phosphorylation *in vitro* was analysed in light and dark. NaCl induced the increase of CEF1 more greatly in wild soybean *Glycine cyrtoloba* (cv. ACC547) than in cultivated soybean *Glycine max* (cv. Melrose). The F_v/F_m was reduced less in *G. cyrtoloba* than in *G. max* after 10-d NaCl stress. In *G. cyrtoloba*, the increase of CEF1 was associated with enhancement of LHC2 phosphorylation in thylakoid membrane under both dark and light. However, in *G. max* the NaCl treatment decreased the LHC2 phosphorylation. Treatment with photosynthetic electron flow inhibitors (DCMU, DBMIB) inhibited LHC2 phosphorylation more in *G. max* than in *G. cyrtoloba*. Thus the NaCl-induced up-regulation in CEF1 and ST might contribute to salt resistance of *G. cyrtoloba*.

Additional key words: chlorophyll fluorescence induction; DBMIB; DCMU; electron flow inhibitors; light-harvesting complex; maximum photochemical efficiency; NaCl; phosphorylation; species differences; Western blot.

Introduction

Soil salinity is one of the most severe factors impairing plant growth and productivity globally (Hasegawa and Bressan 2000, Pitman and Läuchli 2002), which causes both hyperionic and hyperosmotic effects in plants, *e.g.* membrane disorganization, metabolic disorder, and genesis of reactive oxygen species (Chaparzadeh *et al.* 2004, Amor *et al.* 2006), *etc.* leading to the inhibition of CO_2 fixation (Hernández *et al.* 1999). To alleviate the damage initiated by salt stress, plants have developed a complex defence system including ion homeostasis, osmolyte biosynthesis, compartmentation of toxic ions, and reactive oxygen species scavenging system (Hasegawa and Bressan 2000, Mittova *et al.* 2004). So far, accumulating evidence demonstrated that over-expression of one single salt tolerance gene may only confer a partially improved salinity tolerance (Maggio *et al.* 2002). Therefore, it is necessary to identify novel salt tolerance determinants and functionally analyse cause-effect relationships between physiological responses and their potential benefits in stress adaptation (Munns 2002).

Our recent research (Yang *et al.* 2007) using *Glycine cyrtoloba* and *G. max* has revealed that salt-induced up-regulation of cyclic electron flow around photosystem (PS) 1 (CEF1) in *G. cyrtoloba* was closely related to its high salinity resistance. One of the mechanisms involved in stimulating CEF1 was the phosphorylation of light-harvesting complex 2 (LHC2) (Joliot and Joliot 2002, Sharkey 2005), which is considered as a basis for state transitions (ST) (Bellafiore *et al.* 2005). Then what is the relationship between LHC2 phosphorylation (ST2) and salt tolerance in soybean? Some reports suggest that ST represent a flexible regulation mechanism to balance excitation energy between two photosystems (Allen 1992, Liu and Shen 2004) and the key LHC2 phosphorylation enzyme (LHC2 kinase) can sense the unbalance between the light reactions and the carbon metabolism (Hou *et al.*

Received 25 May 2007, *accepted* 13 August 2007.
[***]Corresponding author; fax: 86-571-88206485, e-mails: deanjiang@zju.edu.cn; lukaitong@nbu.edu.cn
Abbreviations: CEF1 – cyclic electron flow around PS1; Chl – chlorophyll; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB – 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; LHC – light-harvesting complex; PS – photosystem; ST – state transition.
Acknowledgements: This work was supported by the National Natural Science Foundation (30471051), and Natural Science Foundation of Zhejiang Province (Y306213). We also acknowledge Dr J.L. Yang for critical reading of the manuscript.

2002). Recently, the function of ST on stress tolerance has been proved in *Oryza sativa* to chill (Moll and Steinback 1986), in *Zea mays* to cold (Bergantino *et al.* 1995), in *Gossypium hirsutum* to high temperature (Wise *et al.* 2004), or in *Dunaliella salina* to salt stress (Liu and Shen 2004, 2005). However, the function of ST on salt tolerance in higher plants was less reported.

The aim of the present study was to explore the functions of CEF1 and LHC2 phosphorylation in salt tolerance of soybean by comparing a salt-tolerant soybean *G. cyrtoloba* with a salt-sensitive *G. max* under different conditions.

Materials and methods

Plants and growth conditions: Seeds of *Glycine cyrtoloba* (cv. ACC547) and *Glycine max* (cv. Melrose) were germinated and cultivated in Zijingang campus of Zhejiang University as described previously (Yang *et al.* 2006). The seedlings were grown in complete solution including 5 mM $Ca(NO_3)_2$, 1.75 mM K_2SO_4, 0.25 mM KCl, 1.25 mM $MgSO_4$, 25 mM H_3BO_3, 1.5 mM $MnSO_4$, 1.5 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 0.25 mM KH_2PO_4, 25 µM $(NH_4)_6Mo_7O_{24}$, 20 µM $FeSO_4$, and 20 µM EDTA-Na_2H_2O (Neumann *et al.* 1999) till the sixth leaves grew out. Then all seedlings were divided into two groups: one group was grown continuously in the complete solution as the non-salt stressed control while the other one was grown in complete solution plus 50 mM NaCl as the salt-stressed treatment. The nutrient solutions were renewed twice a week and the pH was adjusted to about 5.5 during the culture period. The measurements of CEF1 and chlorophyll (Chl) fluorescence were conducted in the sixth leaves attached to the main stem after 10-d treatment. The plants used for phosphorylation measurements were dark adapted at least for 16 h before collection of fully expanded leaves. The leaves were immediately frozen in liquid nitrogen and stored at –80 ºC till the isolation of thylakoid membranes.

Post-irradiation transient in Chl fluorescence was measured using a portable photosynthesis system with an integrating Chl fluorometer (*LI-6400–40* leaf chamber fluorometer, *LI-COR*, USA) according to the procedure described by Yang *et al.* (2006). After dark-adaptation of the sample for 1 h, the minimum (dark) fluorescence yield (F_0) was obtained with a weak measuring beam (<0.1 µmol m^{-2} s^{-1}). A 600-ms saturating flash (>7 000 µmol µmol m^{-2} s^{-1}) was applied to measure the maximum fluorescence yield (F_m). Immediately, the "actinic light" (1 000 µmol µmol m^{-2} s^{-1}) was turned on. After 3 min, the "actinic light" was turned off for the relaxation of fluorescence and fluorescence yield changes were continuously recorded.

Thylakoid membrane isolation was made according to Rintamäki *et al.* (1996) with some modifications as follows: Frozen leaves were homogenized in a mortar with liquid nitrogen and ice-cold isolation buffer containing 50 mM HEPES-NaOH, pH 7.5, 300 mM sucrose, 5 mM $MgCl_2$, 1 mM Na-EDTA, 10 mM NaF, and 1 % (m/v) bovine serum albumin. The homogenates were filtered through *Miracloth* and centrifuged at 1 500×*g* for 4 min. The pellets were washed with 10 mM HEPES-NaOH (pH 7.5), 5 mM sucrose, 5 mM $MgCl_2$, and 10 mM NaF, and centrifuged at 3 000×*g* for 3 min. Thylakoid pellets were re-suspended in assay buffer consisting of 50 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 5 mM NaCl, 10 mM $MgCl_2$, and 10 mM NaF at a final Chl concentration of 0.4 kg m^{-3}. The isolated thylakoid membranes were immediately used for the next procedure. The preparations were protected from light and kept ice-cold during the isolation procedure.

***In vitro* phosphorylation** of the isolated thylakoid proteins started in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM NaF, and 400 µM ATP with 50 g(Chl) m^{-3} (Liu and Shen 2004, 2005). These mixtures were incubated at 25 °C for 20 min in the presence or absence of 0.4 mM ATP, NaCl, or electron transport inhibitors DCMU (*Sigma*) and DBMIB (*Sigma*) under either dark or light [about 200 µmol(photon) m^{-2} s^{-1}]. The samples were then centrifuged at 3 000×*g* and 4 °C for 4 min and the pellets were re-suspended in isolation buffer.

Detection of thylakoid phosphoproteins by polyclonal phosphothreonine antibody: The isolated thylakoids were solubilized in 0.5 M Tris-HCl (pH 6.8), 7 % SDS, 20 % glycerol, and 2 M urea, and then incubated at 100 °C for 5 min. Non-solubilized materials were removed by centrifugation at 8 000×*g* for 8 min. SDS-PAGE was made according to Laemmli *et al.* (1970) using 15 % (m/v) acrylamide, 0.5 % bisacrylamide, and 6 M urea with 0.75×6.00×8.00 cm slabs on the miniprotein three cell system (*Bio-Rad*). Each sample contained 2.5 µg Chl. For immunoblotting the proteins were transferred to a polyvinylidene difluoride membrane (*Millipore*, Bedford, MA, USA). The membranes were then blocked with fat-free milk, incubated with rabbit polyclonal phosphothreonine antibody (*Cell Signalling Technology*, Beverly, MA, USA), since all the main LHC proteins were phosphorylated at an N-terminus threonine residue (Rintamäki *et al.* 1997). The blots were incubated with alkaline phosphatase-conjugated secondary antibody [AP-goat anti-rabbit IgG (H+L) conjugate] and visualized using *NBT-BCIP* colour developing kit (*Boehringer*, Mannheim, Germany).

Chl was extracted with 80 % acetone solution and determined spectrophotometrically as described by Porra *et al.* (1989).

Results and discussion

Chl fluorescence: *G. cyrtoloba* had higher peak of Chl fluorescence than *G. max* under normal condition (Fig. 1). Furthermore, salt stress induced the post-irradiation transient increase more greatly in *G. cyrtoloba* than in *G. max*, indicating that the dark reduction of PQ pool was promoted by salinity in salt-resistant soybean as reported in other plants (Burrows *et al.* 1998, Cournac *et al.* 1998). Compared to the control plants, the increase in height of fluorescence peak induced by salt stress was greater in *G. cyrtoloba* than in *G. max*. The transient post-irradiation increase in Chl fluorescence is due to the back flow of electrons from NADPH or other stromal reductants to intersystem chain. After dark-adaptation, PS2 is in the open state and the level of fluorescence is low (F_0) because the majority of photon energy absorbed by PS2 is used to reduce PQ pool and to drive trans-membrane electron transfer. Upon irradiation, F_m can be reached quickly after saturating flash as the PQ pool is fully reduced and the PS2 reaction centre is closed. Fluorescence is then subsequently quenched as the oxidation of PQ pool by the downstream electron transport pathway. Turning off "actinic light" breaks the reduction of PQ *via* PS2 photochemistry, and fluorescence is quenched as the PQ pool is oxidized instantaneously. The subsequent transient increase in fluorescence is due to the dark reduction of PQ pool leading to closure of PS2 reaction centre through reverse electron flow. So it has been widely accepted that the transient post-irradiation increase in Chl fluorescence could reflect the CEF1 (Deng *et al.* 2003, Ma and Mi 2005). This result is in accordance with our previous reports that, using dark reduction of the oxidized primary electron donor in PS1 ($P700^+$), salt stress stimulated the increase of CEF1 much more in *G. cyrtoloba* than in *G. max* (Yang *et al.* 2007) and *G. cyrtoloba* could maintain higher non-photochemical quenching due to state transition under salt treatment (Yang *et al.* 2006). Though the function of CEF1 on temperature tolerance has been proved in some other higher plant species including cotton (Schrader *et al.* 2004, Wise *et al.* 2004), barley and maize (Egorova *et al.* 2002); tobacco (Wang *et al.* 2006), wheat (Apostol *et al.* 2006), the function of CEF1 on salt tolerance

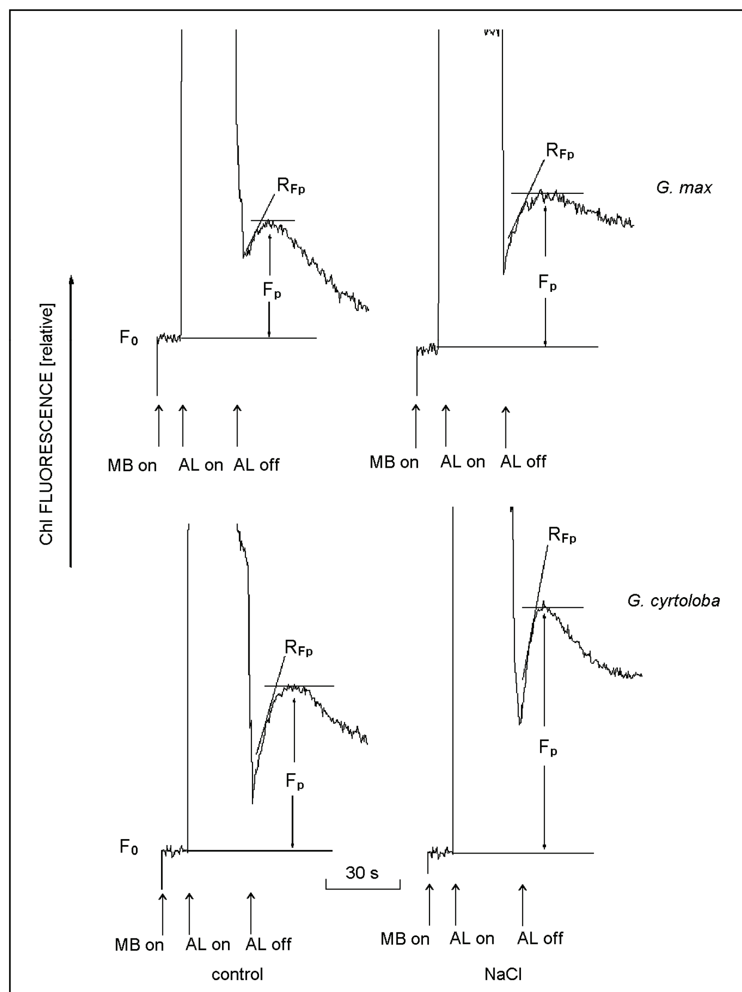

Fig. 1. Effects of salt stress on the height of post-irradiation transient increase in Chl fluorescence in the leaves of *G. cyrtoloba* and *G. max* after 10 d of treatment. MB – measuring beam (<0.1 µmol m^{-2} s^{-1}); AL – "white actinic light" (1 000 µmol m^{-2} s^{-1}); F_0 – minimum Chl fluorescence yield when photosystem 2 reaction centres are open; F_p – the height of post-irradiation fluorescence increase.

has only been reported by Liu and Shen (2006) with halotolerant green alga *Dunaliella salina*. Our results showed that CEF1 might play an important role in tolerance of soybean *G. cyrtoloba* to salt stress.

NaCl-induced greater increase in CEF1 in *G. cyrtoloba* was accompanied by a lesser decrease in maximal photochemical efficiency estimated by the F_v/F_m ratio than in *G. max* (Fig. 2). In comparison with the control plants, F_v/F_m of *G. cyrtoloba* declined much less than that of *G. max* did after 10 d of 50 mM NaCl stress. The former was only decreased by 1.9 %, while the latter was significantly decreased by 5.3 %. We suggest that less photoinhibition in *G. cyrtoloba* might partly explain why *G. cyrtoloba* had higher resistance to salt stress because the maximal photochemical efficiency is a sensitive parameter for photoinhibition (Krause and Weis 1991).

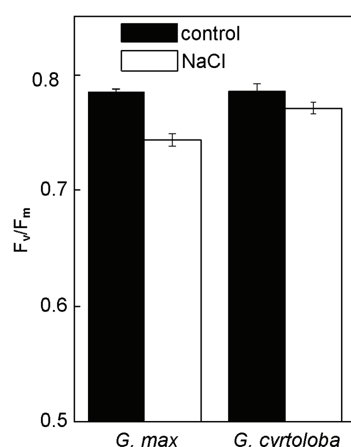

Fig. 2. Effects of salt stress on maximal photochemical efficiency (F_v/F_m) in the sixth fully expanded leaves of ACC547 and Melrose. Attached leaves were kept in dark for adaptation for 1 h before the measurement of chlorophyll fluorescence. Means±standard error of four replications.

LHC2 phosphorylation: The function of CEF1 is to generate a pH gradient across the thylakoid membrane and this ΔpH helps generate ATP (Heber and Walker 1992), which is necessary for many other physiological responses including ST (Schrader *et al.* 2004). We further studied the effect of NaCl stress on transition of ST2, which could be reflected by the level of LHC2 phosphorylation in isolated thylakoid membrane. We found that NaCl could induce LHC2 phosphorylation in *G. cyrtoloba* even in the dark and its level was similar to that induced by irradiation (Fig. 3, lanes *2* and *3*). Furthermore, NaCl had an additive effect on light-induced LHC2 phosphorylation in *G. cyrtoloba* thylakoid membrane (Fig. 3, lane *4*). The experiments for dependence of the NaCl-induced LHC2 phosphorylation levels on NaCl content showed (Fig. 4) that the level in light-stimulated *G. cyrtoloba* thylakoid membrane was obviously enhanced with the increasing NaCl content in the range from 0.1 to 0.3 M in the protein phosphorylation reaction medium (Fig. 4*A*) as reported for *D. salina* (Liu and Shen 2004, 2005). In *G. max*, however, NaCl treatment almost completely inhibited the LHC2 phosphorylation in dark-adapted thylakoid membrane (Fig. 3*B*, lane *7*) and NaCl slightly inhibited the light-induced phosphorylation (Fig. 3*B*, lanes *6* and *8*). In contrary to that in *G. cyrtoloba*, the LHC2 phosphorylation in *G. max* was repressed with increase in NaCl content (Fig. 4*B*). Thus the difference NaCl-induced LHC2 phosphorylation ability between *G. cyrtoloba* and *G. max* may act as an adaptation mechanism to salinity. All these results further proved that NaCl-induced up-regulation of CEF1 coupled with the improvement of ST could contribute to the high salinity tolerance in *G. cyrtoloba* and such improvement of ST was reported in other stress-induced plants (Egorova and Bukhov 2002, Hou *et al.* 2003), especially in *Chlamydomonas reinhardtii* (Finazzi *et al.* 1999, 2002).

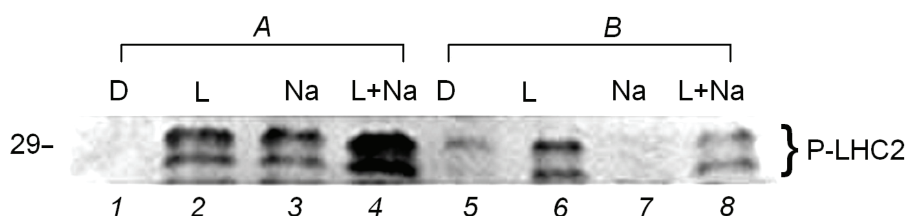

Fig. 3. NaCl-induced different phosphorylation of LHC2 in isolated thylakoid membranes of *G. cyrtoloba* (*A*) and *G. max* (*B*). Thylakoid membrane proteins from dark-adapted *G. cyrtoloba* or *G. max* were phosphorylated at 25 °C for 20 min under the different indicated conditions: D, L, Na, and L+Na – dark, light [about 200 μmol(photon) m^{-2} s^{-1}], 0.3 M NaCl, and light+0.3 M NaCl, respectively. The positions of phosphorylated LHC2 recognized by the Thr(P) antibody at 29 kDa are indicated.

Response of LHC2 phosphorylation to ATP and photosynthetic inhibitors: The state transition in *Chlamydomonas* represents an adaptive response to the intracellular ATP content (Bulté *et al.* 1990). We investigated the effect of exogenous ATP on LHC2 phosphorylation. The level of LHC2 phosphorylation in dark-adapted intact leaves of both soybean genotypes was fairly low (Fig. 5, lanes *1* and *5*). The exogenous ATP slightly improved the phosphorylation level in *G. max* in the dark (Fig. 5, lane *8*), but no effect was observed in *G. cyrtoloba* (Fig. 5, lane *4*), indicating that the regulation of LHC2 phosphorylation in *G. max* might be dependent

much more on ATP than in *G. cyrtoloba*. Irradiation induced LHC2 phosphorylation in both species in the presence of exogenous ATP (Fig. 5, lanes *3* and *7*). In the dark, LHC2 phosphorylation in *G. max* had been masked by the presence of NaCl (Fig. 5, lane *6*), while the phosphorylation in *G. cyrtoloba* was enhanced by the presence of NaCl (Fig. 5, lane *2*).

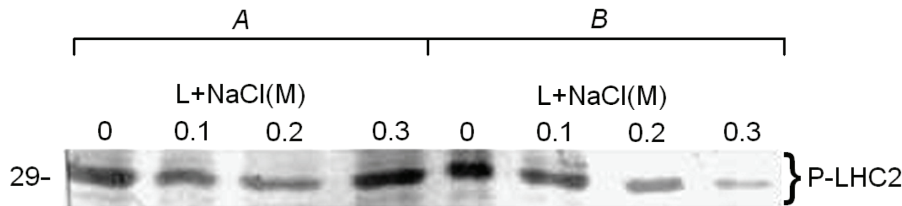

Fig. 4. NaCl-induced different phosphorylation of LHC2 in isolated thylakoid membranes of *G. cyrtoloba* (*A*) and *G. max* (*B*). Thylakoid membrane proteins from dark-adapted *G. cyrtoloba* or *G. max* were phosphorylated at 25 °C for 20 min under light either in the absence (0) or in the presence of the indicated NaCl concentrations. The positions of phosphorylated LHC2 recognized by the Thr(P) antibody at 29 kDa are indicated.

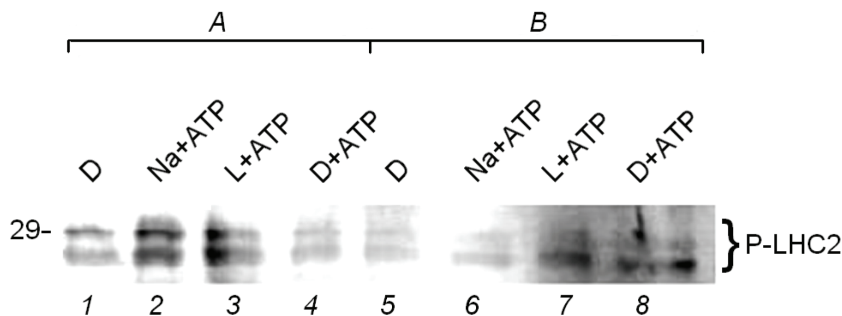

Fig. 5. Different effects of irradiation and 0.3 M NaCl on LHC2 phosphorylation in isolated thylakoid membranes of *G. cyrtoloba* (*A*) and *G. max* (*B*). Thylakoid membrane proteins from dark-adapted *A* and *B* were phosphorylated at 25 °C for 20 min in the absence (lanes *1* and *5*) or in the presence (*left lanes*) of 400 µM ATP under different conditions: D – dark control; L and Na – light- and NaCl-induced LHC2 phosphorylation, respectively. The positions of phosphorylated LHC recognized by the Thr(P) antibody at 29 kDa were indicated.

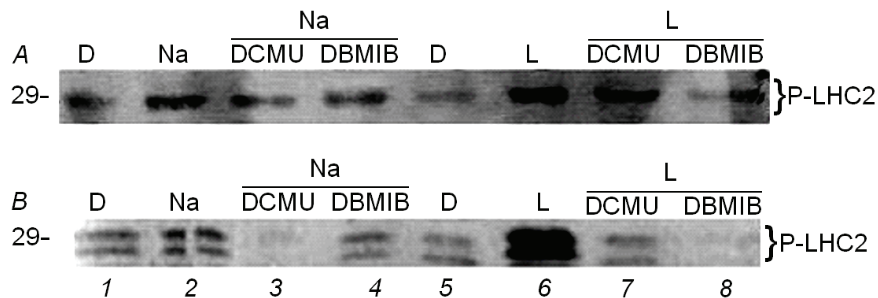

Fig. 6. Different effects of DCMU and DBMIB on light- and NaCl-induced LHC2 phosphorylation in isolated thylakoid membrane of *G. cyrtoloba* (*A*) and *G. max* (*B*). Thylakoid membrane proteins from dark-adapted *G. cyrtoloba* or *G. max* were phosphorylated at 25 °C for 20 min in the light (lanes *6*) and light plus 10 µM DCMU (lanes *7*), 5 µM DBMIB (lanes *8*) or in darkness with the addition of 0.3 M NaCl (lanes *2*), 0.1 M NaCl plus 10 µM DCMU (lanes *3*) or 5 µM DBMIB (lanes *4*). D – dark control (lanes *1* and *5*); L and Na – light- and NaCl-induced LHC2 phosphorylation, respectively. The positions of phosphorylated LHC2 recognized by the Thr(P) antibody at 29 kDa were indicated.

To check whether the LHC2 phosphorylation in *G. cyrtoloba* and *G. max* depends on photosynthetic electron transport or not, we examined the effects of inhibitors: In *G. cyrtoloba* DCMU and DBMIB hardly inhibited NaCl-induced LHC2 phosphorylation (Fig. 6*A*, lanes *3* and *4*) and slightly decreased the irradiation-induced LHC2 phosphorylation (Fig. 6*A*, lanes *7* and *8*). However, both inhibitors decreased the LHC2 phosphorylation induced by irradiation or NaCl in *G. max* (Fig. 6*B*). Hence the LHC2 phosphorylation might be dependent less on redox state of photosynthetic electron carriers in *G. cyrtoloba* than in *G. max*. There may be

different pathways and/or mechanisms to phosphorylate LHC2 in *G. cyrtoloba* than in *G. max*. In the experiments of Liu and Shen (2005) with *D. salina* these inhibitors did not inhibit the NaCl-induced LHC2 phosphorylation, though they could abolish the light-induced LHC2 phosphorylation. So these authors concluded that the NaCl-induced LHC2 phosphorylation was redox-independent, while the light-induced one was redox-dependent. A further research is needed to test this hypothesis. Surely, the loss of DCMU or DBMIB sensitivity in *G. cyrtoloba* may also be explained by the following hypothesis: the electron inhibited by DCMU and DBMIB would be replaced by that coming from CEF1 because of NaCl-induced switch from linear to cyclic electron flow in *G. cyrtoloba* just as found in some algae (Finazzi *et al*. 1999, Rochaix 2002).

In conclusion, we found that CEF1 and ST are up-regulated by NaCl stress in *G. cyrtoloba*, but they are absent and even down-regulated in *G. max*. Therefore CEF1 as well as ST may act as mechanisms of adaptation to salt stress in *G. cyrtoloba*; such mechanism may also be employed by other salt-tolerant species.

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