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Coordinate expression of light-harvesting chlorophyll *a*/*b* gene family of photosystem II and chlorophyll *a* oxygenase gene regulated by salt-induced phosphorylation in *Dunaliella salina*

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Abstract

As a stress factor, salt induces the phosphorylation of light-harvesting chlorophyll (Chl) *a/b* proteins (LHCII) in *Dunaliella salina*. In this study, we found that the salt-induced phosphorylation of LHCII was not affected by phosphatase, and that salt simultaneously regulated both the phosphorylation of LHCII and the expression of genes encoding light-harvesting Chl *a/b* proteins of photosystem II (*lhcb*) and the gene encoding Chl *a* oxygenase (*cao*) in dark-adapted *D. salina*. The mRNA accumulation patterns of *lhcb* and *cao* were similar, which further affected the size of LHCII and the ratio of Chl *a* to Chl *b*. Therefore, we inferred this simultaneous regulation is one of the mechanisms of *D. salina* to adapt to the high-salinity environment.

Additional key words: *Dunaliella salina*; high salinity; light-harvesting chlorophyll *a*/*b* proteins; phosphorylation; simultaneous regulation.

Introduction

As one of the most important life activities on earth, photosynthesis in higher plants or algae is regulated by the surrounding environmental factors, such as temperature, light, nutrients, and water. In plants, many strategies are used to prevent damage, and the reversible protein phosphorylation in LHCII plays a crucial role in this process. Bennett (1977) first reported light-dependent phosphorylation of proteins in thylakoid membranes, and many successive studies confirmed that the reversible phosphorylation of LHCII is the basis of state transition, which functions to balance light distribution in two photosystems (PS) to efficiently utilize light energy or avoid photodamage elicited by excessive light (Lunde *et al*. 2000).

Besides regulating the LHCII proteins *via* phosphorylation or dephosphorylation in PSI and PSII, light may also induce kinase activity, which causes the signal transduction in the nucleus and further modulates the size of LHCII. The experiment indicated that the redox state of plastoquinol (PQ) pool was closely associated with the phosphorylation of LHCII through the cytochrome b_6f complex located in the vicinity of LHCII kinase (Bennett *et al*. 1988). Expression of nuclear genes encoding LHCII was also adjusted by the redox state of PQ pool (Escoubas 1995). The study on the simultaneous regulation of LHCII phosphorylation and transcription of *lhcb* genes at different irradiances and temperatures in white rye (Pursiheimo *et al*. 2001), and especially the discovery of a thylakoid-bound protein TSP9, which was phosphorylated by LHCII kinase and possibly activated downstream kinases (Carlberg *et al*. 2003), suggested that LHCII phosphorylation kinase may be involved in the regulation of LHCII nuclear gene expression of LHCII (Zer and Ohad 2003). However, the signal transduction process remains unclear and more evidences are needed to clarify the mechanism of this phosphorylation process. As to the studies of the effect of other environmental factors on LHCII modification, Liu and Shen (2004, 2006) found that salt was able to induce LHCII phosphorylation and state II transition in *D. salina*, and more interestingly, salt-induced phosphorylation of LHCII was not regulated by the redox state of PQ (Liu and Shen 2005).

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Abbreviations: *cao* – the gene encoding chlorophyll *a* oxygenase; cDNA – DNA complementary to RNA; Chl – chlorophyll; *lhcb* – genes encoding light-harvesting chlorophyll *a/b* proteins of photosystem II; LHCII – chlorophyll *a/b* proteins of photosystem II; PS – photosystem; PQ – plastoquinol; SDS –sodium dodecyl sulfate.

Under different irradiances, plants can adjust the ratio of Chl *a* to Chl *b* in order to respond to the changing environmental conditions (Leong and Anderson 1984). Chl *a* oxygenase is a sort of important enzyme which catalyzes the conversion of Chl *a* to Chl *b*. Similar variation occurs in *cao* and *lhcb* mRNA levels under the irradiance shift in *D. salina*. These transcription regulations may be needed for the optimal assembly of LHCII to adapt to the environmental oscillations (Masuda *et al*. 2002, 2003).

D. salina, a unicellular green alga lacking a rigid cell wall and adapting to a wide range of salt concentration, is an ideal model organism for the research of photosynthesis. Up to now, four *lhcb* genes have been cloned, which allows us to study the relationship between the surrounding environmental conditions and the transcriptions of *lhcb* gene family of *D. salina*.

The purpose of this study was to elicit whether salt-induced phosphorylation regulates the expression of *lhcb* and *cao*, and whether the activity of phosphatases is inhibited by high salinity.

Materials and methods

Strain and growth condition: The unicellular green alga, *D. salina* (dunal) Teod (strain number 435), was obtained from the Institute of Hydrobiology, the Chinese Academy of Sciences, and cultivated in a greenhouse under 16/8h light/dark cycle [photon flux density of 250 μmol(photon) m^{-2} s^{-1}] in hypersaline medium (pH 7.5) (Pick *et al*. 1986) containing 1.5 M NaCl. In addition, $NaHCO_3$ was added as a supplemental inorganic carbon source (initial concentration 25 mM). For the study of the hypersaline response cultures were dark-cultured for 12 h in the logarithmic growth phase [approximately 5×10^5(cell) dm^{-3}], and then NaCl was added at a final concentration of 3 M. Cells were cultured in the dark and collected at different time points.

RNA isolation and real-time PCR: Total RNA was prepared from *D. salina* using the TRIZOL reagent. With *PrimeScript™ RT* reagents kit (*TaKaRa*, Kyoto, Japan), single-stranded cDNA was synthesized from 0.75 μg of total RNA in 15 μl of reaction mixture, according to the manufacturer's manual. Real-Time Quantitative PCR was performed in an *iCycler iQ* (*BIO-RAD*, CA, USA) with *SYBR®Premix Ex Taq™* reagents kit (*TaKaRa*, Kyoto, Japan), and the PCR reaction system and program were optimized referring to the protocol. In order to analyze four *lhcb* genes and *cao*, the primers listed in Table 1 were used to amplify the target segments. The relative Ct ($2^{-\Delta\Delta C_T}$) method was used to determine the relative abundances of the four *lhcb* genes and *cao* genes, and 18S rRNA was used as the internal control. The reaction programs were performed as follows: 1 cycle of 95°C for 10 s; 5 s at 95°C, and 30 s at 60°C for 40 cycles.

Table 1. Primers used in real-time PCR.

Gene	Primer sequence
lhcb1	F *5′TGGTTGAGAGTTTGGACACCGA3′* B 5'GATGGCTTTGTGGGCTTCG3'
lhcb2.1	F 5'TTCTT *GACCGGACCTTAGTTGAGC3′* B 5'TTCTT GACCGGACCTTAGTTGAGC3'
lhcb2.2	F *5′TTGATTCTGTGCCTTAAGACCTGG3′* B 5'CACGCA AGCAGTTACTTCTCACAG3'
lhcb3	F *5′CAGGCTTGAGTGCACCTTGTAG* 3' B 5'ATGGCTGGGTGCTGCTTCG3'
cao	F *CTTGGTGGTTCAAT* GGTTCCTG3' B 5'CAACAGTATGCA TGGTGGGACAG3'
18SrRNA	F *5′TTGGGTAGTCGGGCTGGTC3′* B 5'CGCTCGTTCTTCATCGTT3'

Preparation of total protein and thylakoid membrane: Total protein extract was obtained and the isolation of thylakoid membrane proteins was performed as previously described LaRoche *et al*. (1991), Liu and Shen (2005). The harvested cells were suspended in 0.2 cm^3 fresh medium and stored at liquid nitrogen until all samples were collected. Samples were then transferred to sonication buffer (pH 6.8) containing 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinyl pyrrolidone K30, 3 mM aminocaproic acid, 1 mM aminobenzamidine and 0.2 mM phenylmethanesulfonyl fluoride, and the samples were sonicated 2 min on ice. Unbroken cells and other large fragments were removed by centrifugation at 3,000 × *g* for 3 min at 4°C, and the supernatant was centrifuged at 40,000 × *g* for another 30 min at 4°C. Ultimately, the thylakoid membrane pellets were resuspended in sonication buffer at 1 mg(Chl) cm^{-3}. The total protein extract collected after sonication and thylakoid membranes were used for the analysis of LHCII and its phosphorylation level.

Protein gel electrophoresis and blot analysis: Before protein gel electrophoresis, protein concentration was determined according to the method using Coomassie brilliant blue G-250, then total protein extract was processed with solutions containing 0.5% SDS, 0.05% bromothymol blue, 10% glycerol and 0.5 M Tris-HC1, 7% SDS, 20% glycerol and 2 M urea (pH 6.8). Equal amounts of proteins were loaded and resolved by protein gel electrophoresis (15% acrylamide, 0.5% bisacrylamide and 4 M urea), and then transferred to nitrocellulose membrane for immunoblot analysis (LaRoche *et al*. 1991). Specfic rabbit polyclonal antibody raised against

Lhcb3 of *D. salina* fusion protein expressed in *E. coli* was used to detect LHCII polypeptides (Kim *et al.* 1993). Phosphorylated LHCII was recognized by mouse monoclonal anti-phosphothreonine (P-Thr) (*CST*, CO, USA) (Liu and Shen 2005). The relative amounts of P-LHCII proteins were quantified using a software *Gel-Pro analyzer 3.0*.

Pigments and cell density determinations: For pigments determination, the equal amounts of cells were suspended in 80% acetone, and the debris were removed by centrifugation at 10,000 × *g* for 5 min. The quantification of Chl was performed according to the absorbance of samples at 663 nm and 645 nm, and the amounts of Chl *a* and Chl *b* were calculated using the equation of Arnon (1949). Cell counting was carried out with a haemocytometer.

The determination of phosphatase activity of the isolated thylakoid protein treated with salt was carried out according to the method by Yang *et al*. (1987). The total volume of the reaction system was 1.5 cm^3 and contained 33.3 mM citric acid buffer (pH 6.0), 0.67 mM $MgCl_2$, 0.67 mg cm^{-3} nitrophenylphosphate and 100 mg of thylakoid membrane protein. After incubation for 30 min at 37°C, the reaction mixture was added with 0.2 cm^3 NaOH (2 M). The absorbance was measured at 405 nm and used for calculation of the phosphatase activity and the standard curve made with nitrophenolate was used.

Results

Salt-induced phosphorylation of LHCII in intact cell of *D. salina*: The polyclonal antibodies were produced in rabbit using fusion protein heteroexpressed in *E. coli* using PET-32a vector with inserted *lhcb3* of *D. salina*. Cells were harvested after cultivation in the dark and the total protein extract was separated by gel electrophoresis, electroblotted and tested for the presence of LHCII by specific antibodies. The 29 kDa band was recognized by the antibody (Fig. 1) which was subsequently used for detecting LHCII and its phosphorylation in the dark-cultured *D. salina* cells after being treated with 3 M NaCl for 1 h (Fig. 2). To analyze the effects of the long-term high salinity stress, the LHCII phosphorylation was followed for 12 h after the addition of NaCl to the final concentration of 3 M. As shown in Fig. 3, the phosphorylation level of LHCII obviously increased within the initial 0.5–2 h of the salt treatment but then declined during the subsequent 6 h reaching an increased relative level at 12 h of the hypersaline treatment.

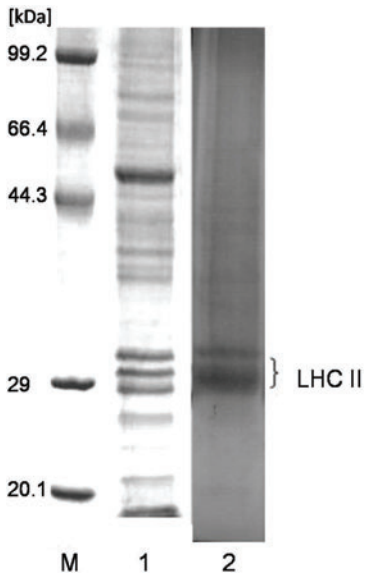

Fig. 1. The detection of Lhcb3 of *D. salina* using specific antibodies. M – marker; 1 – total protein of *D. salina* was stained with Coomassie blue; 2 – immunoblot by specific polyclonal antibody.

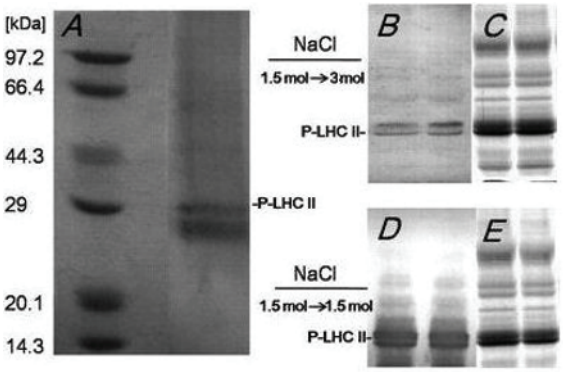

Fig. 2. Salt-induced phosphorylation of LHCII in dark-cultured *D. salina*. The assessment of the LHCII phosphorylation before (*A*) and after incubation of the cells in 3 M and 1.5 M NaCl for 1 h (*B* and *D*, respectively). The equal loading of the gel is documented by general protein staining (*C*,*E*).

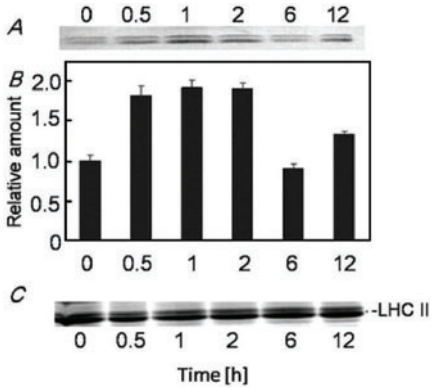

Fig. 3. The phosphorylation level of LHCII in *D. salina* treated with 3 M NaCl. Phosphorylated LHCII proteins in cells harvested in 0.5, 1, 2, 6, and 12 h of the NaCl treatment were detected by anti-phosphothreonine antibody (*A*). The relative amount of P-LHCII proteins were obtained using a software *Gel-Pro analyzer 3.0* (*B*). The equal loading of the gel is documented by general protein staining (*C*). *Vertical bars* indicate the standard deviation of three independent data collections.

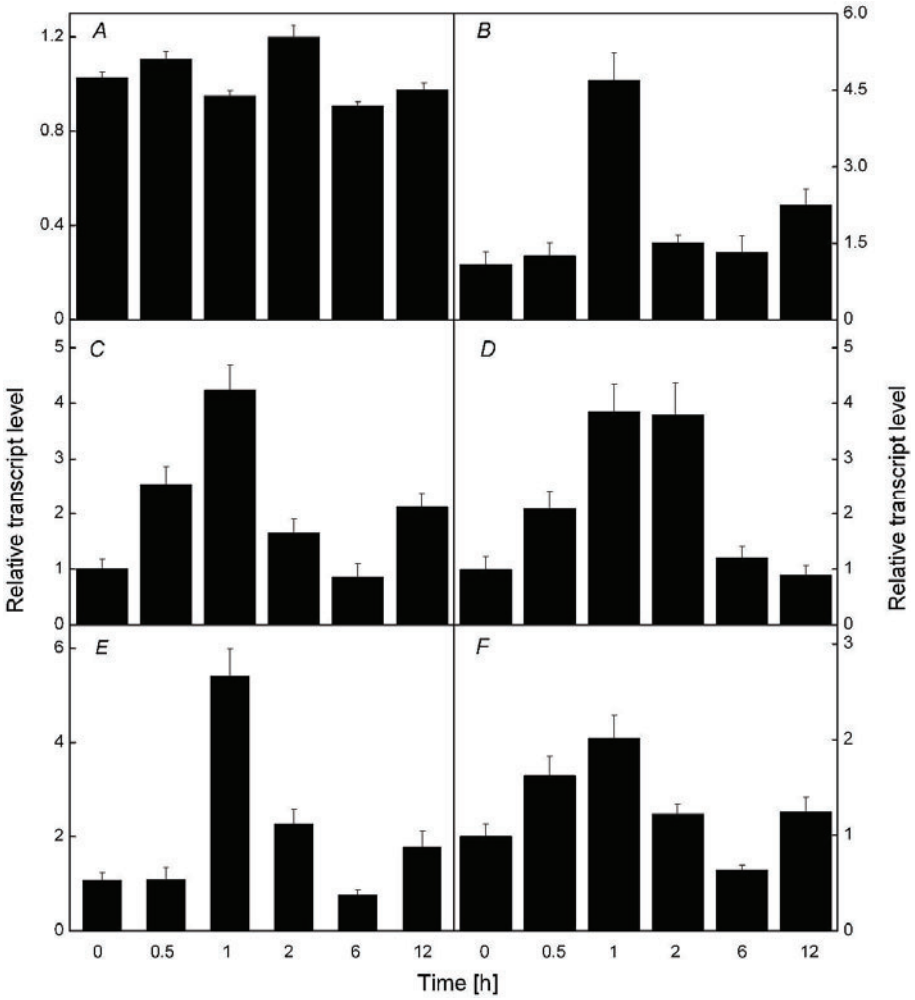

Fig. 4. Effects of salt on the expression of *lhcb* and *cao*. The transcription levels of *lhcb* of *D. salina* in dark condition when the cells were transferred to the medium containing 1.5 M NaCl (*A*). Changes in accumulation of *lhcb1* (*B*), *lchb2.1* (*C*), *lhcb2.2* (*D*) *lhcb3* (*E*) and *cao* (*F*) transcript in cells treated with 3 M NaCl for 0.5, 1, 2, 6, and 12 h. *Vertical bars* indicate the standard deviation of three independent experiments. Note: the different scales for different transcripts (the initial transcript level was recorded for 1).

Changes in the mRNA accumulation of *lhcb* and *cao* in *D. salina* treated with high salinity: To test the effect of high salinity on the accumulation of *lhcb* transcripts, the mRNA levels of four *lhcb* genes, *lhcb1*, *lhcb2.1*, *lhcb2.2* and *lhcb3* in *D. salina* were investigated by the real-time PCR. Under the growth conditions (1.5 M), the *lhcb1* mRNA accumulation was showed in Fig. 4*A*. When dark-cultured cells were treated with salt (Fig. 4*B–E*), the initial transcript level of *lhcb1* (Fig. 4*A*) increased at 0.5 h of the treatment reaching the maximum at 1 h. Then it declined to the relative higher level compared to the control cells (0 h). Similar to that of *lhcb1*, the transcription level of *lhcb2.1* and *lhcb3* exhibited analogous trends except that it declined to the lower level compared with the control cells (0 h) in 6 h. In relation to the level of the *lhcb1* transcript, the amount of *lhcb2.2* transcript was lower at 12 h compared with the control (0 h). Therefore, the expression of all four tested *lhcb* genes was induced by the high salinity from 0.5 h to 2 h and declined at 6 h. Afterwards, the mRNA amount increased at 12 h except *lhcb2.2*. Pattern of the mRNA accumulation of *cao* under high salinity (Fig. 4*F*) was similar to that found for *lhcb3*.

Changes in LHCII, Chl *a* to Chl *b* ratio and phosphatase activity during high salinity stress: After 3M NaCl treatment, cells were harvested and LHCII proteins were quantified in total protein extracts isolated from cells treated for 24 h. Fig. 5 shows that the amount of LHCII was stable in the control cells (Fig. 5*A*) but slightly increased during treatment with 3M NaCl (Fig. 5*B*) except a minor decrease after 6 h. The ratio of Chl *a* to Chl *b* gradually declined during initial 2 h and then remained stable (Fig. 6*A*).

Determination of phosphatase activities revealed their oscillations during high-salinity treatment (Fig. 6*B*). The activity was slightly inhibited at 0.5 h, and then additional enzyme activity was induced between 1 and 12 h of the treatment. In the similar tests, high concentrations of KCl, $NaNO_3$, and KNO_3 increased the phosphatase enzyme activities (data not shown).

Discussion

As to the phosphorylation of thylakoid membrane proteins, there were about 32 phosphorylation sites identified in plants exposed to different environmental conditions (Vener 2007). Eight of them belonged to LHCII. As an important regulation system, reversible phosphorylation regulates the size of LHCII, state transition, and nuclear gene expression related to changes in the environmental conditions (Lunde *et al*. 2000, Masuda *et al*. 2003).

Until now, light, pH, temperature, and metabolites were found to be capable of affecting the phosphorylation level of LHCII (Bennett 1977, Hou *et al*. 2002). Liu and Shen (2004) reported that NaCl and several other salts induce LHCII phosphorylation both *in vitro* or *in vivo* under dark condition in *D. salina* but not in spinach. Further evidence indicates that electron inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, which are capable of controlling the redox state of PQ, have no effect on the salt-induced phosphorylation level suggesting that the phosphorylation mechanism induced by salt is different from that induced by light and low pH (Liu and Shen 2005). In this work, we found that the activity of phosphatases was not inhibited but induced by high-salinity stress. The observed parallel high-salinity-induced increase in phosphorylation of LHCII is in agreement with proposed activation of relevant kinases or alteration in the conformation of LHCII (Liu and Shen 2004).

As shown in the previous studies, the light-induced phosphorylation is controlled by the redox state of PQ which also regulates the nuclear gene expression through unknown signal pathway (Carlberg *et al*. 2003). Transfer of white rye growing in natural environmental conditions to different irradiances and temperatures leads to similar kinetics of LHCII protein phosphorylation and *lhcb* mRNA accumulation (Pursiheimo *et al*. 2001). Such a similarity in time course of phosphorylation levels of LHCII and *lhcb* mRNA accumulation was also found in high-salinity treated cells of *Dunaliella*. Therefore, we believe that salt-induced kinase activity may simultaneously regulate thylakoid membrane phosphorylation and gene expression of *lhcb* genes in dark-cultured cells of *D. salina*, and this regulation is independent of the redox state of PQ.

In the process of Chl synthesis, Chl *a* oxygenase catalyzed the conversion of Chl *a* to Chl *b*. Unlike other enzymes upstream the Chl biosynthesis pathway, *cao* was simultaneously regulated with *lhcb* expression during light shifts (Masuda *et al*. 2003). In our experiments, salt regulated the mRNA accumulation of *cao* and *lhcb* in a similar way. Consequently, we deduced that this might

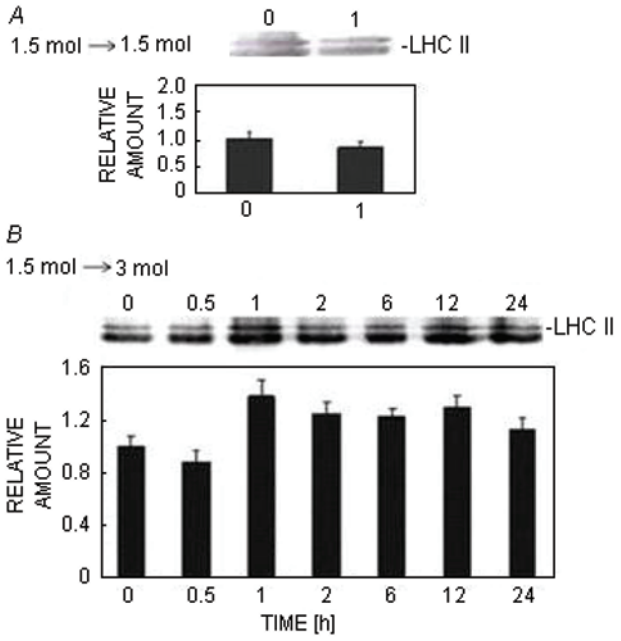

Fig. 5. The abundance of LHCII in cells of *D. salina* treated with 1.5 M or 3 M NaCl. The variation in LHCII abundance when cells was treated with 1.5 M NaCl for 1 h (*A*), Equal total protein extracts obtained from cells harvested after 0.5, 1,2, 6, 12, and 24 h were analyzed by Western blot using LHCb3 specific antibodies (*B*). The relative amounts of LHCII proteins were obtained using a software *Gel-Pro analyzer 3.0*. *Vertical bars* indicate the standard deviation of three independent data collections.

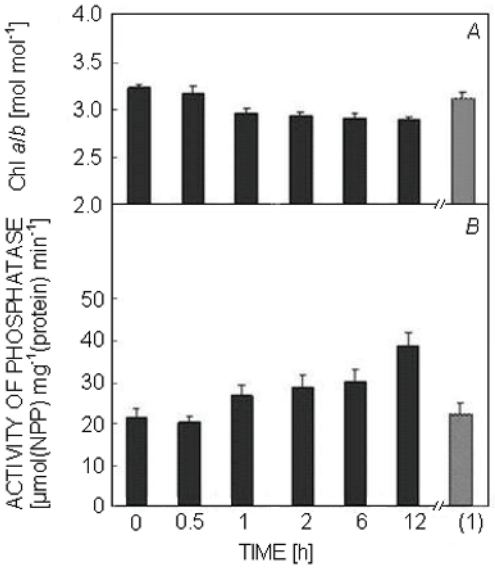

Fig. 6. Changes in the Chl *a/b* ratio after treatment of *Dunaliella* cells with 3 M NaCl for 0.5, 1, 2, 6, and 12 h or 1.5 M NaCl for 1 h. Chlorophyll was extracted using 80% acetone and quantified in equal number of cells (*A*); The phosphatase activity of thylakoid protein extract from *D. salina* cells treated with 3 M NaCl for 0.5, 1, 2, 6, and 12 h or 1.5 M NaCl for 1 h (*B*). *Vertical bars* indicate the standard deviation of three independent experiments.

arise from the need of the optimal assembly of LHCII to adapt to the environmental changes. Actually, the ratio of Chl *a* to Chl *b* declined during the Chl accumulation with the increase in the abundance of the *lhcb* transcripts. This result was in agreement with Tanaka *et al*. (2001) who also reported that *cao* over-expression caused an enlargement of the Chl antenna size of PSII in *Arabidopsis*.

In conclusion, in this work we analyzed high-salinity-induced kinetics of change in LHCII phosphorylation, in the expression of *lhcb* and *cao* genes and in the activity of phosphatases in cells of *D. salina*. Compared with the results obtained with salt-tolerant wheat maintaining photosynthetic activity by suppressing sodium uptake (Muranaka *et al*. 2002), the salt-induced, simultaneous regulation of LHCII phosphorylation and the expression of *lhcb* and *cao* genes in *D. salina* may be the responses of cells to the high-salinity stress to enhance the cyclic electron flow to synthesize more ATP and advance the Na^+ extrusion (Gilmour *et al*. 1985).

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