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Composition and regulation of thylakoid membrane of Antarctic ice microalgae *Chlamydomonas* sp. ICE-L in response to low-temperature environment stress

WANG YI-BIN[1,2], LIU FANG-MING[1,2], ZHANG XIU-FANG[3], ZHANG AI-JUN[1,2], WANG BIN[1,2], ZHENG ZHOU[1,2], SUN CHENG-JUN[1] AND MIAO JIN-LAI[1,2]

[1]First Institute of Oceanography, State Oceanic Administration, Qingdao 266061, China, [2]Key Laboratory of Marine Bioactive Substances, SOA, Qingdao 266061, China, [3]Qingdao Hiser Hospital, Qingdao 266033, China

Ice algae have successfully adapted to the extreme environmental conditions in the Antarctic, however the underlying mechanisms involved in the regulation and response of thylakoid membranes and chloroplast to low-temperature stress are still not well understood. In this study, changes in pigment concentrations, lipids, fatty acids and pigment protein complexes in thylakoid membranes and chloroplast after exposure to low temperature conditions were investigated using the Antarctic ice algae Chlamydomonas *sp. ICE-L. Results showed that the chloroplasts of* Chlamydomonas *sp. ICE-L are distributed throughout the cell except in the nuclear region in the form of thylakoid lamellas which exists in the gap between organelles and the starch granules. Also, the structure of mitochondria has no obvious change after cold stress. Concentrations of Chl* a*, Chl* b*, monogalactosyl diacylglycerol, digalactosyl diacylglycerol and fatty acids were also observed to exhibit changes with temperature, suggesting possible adaptations to cold environments. The light harvesting complex, lutein and β-carotene played an important role for adaptation of ICE-L, and increasing of monogalactosyl diacylglycerol and digalactosyl diacylglycerol improved the overall degree of unsaturation of thylakoid membranes, thereby maintaining liquidity of thylakoid membranes. The pigments, lipids, fatty acids and pigment-protein complexes maintained the stability of the thylakoid membranes and the normal physiological function of* Chlamydomonas *sp. ICE-L.*

Keywords: Antarctic ice microalgae, *Chlamydomonas* sp., chloroplast, thylakoid membrane, cold stress response

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INTRODUCTION

Bunt and Wood first reported the existence of ice algae in the Antarctic seawater and sea ice in 1963 (Bunt & Wood, 1963). Since then, several researchers have carried out studies investigating their diversity, physiological adaptations and ecology (Priscu *et al.*, 1989; Thomas & Dieckmann, 2002; Gudynaite-Savitch *et al.*, 2006; Morgan-Kiss *et al.*, 2006; Dolhi *et al.*, 2013; Lyon & Mock, 2014). Ice algae are the main contributors to primary productivity in sea ice ecosystems associated with their ability to grow rapidly despite low temperature conditions (Arrigo *et al.*, 1997; Thomas & Dieckmann, 2002). Most microalgae isolated from the polar regions are psychrophilic, being able to grow at 0°C or lower but unable to grow at temperatures above 15°C (Teoh *et al.*, 2004; Morgan-Kiss *et al.*, 2006). Ice algae are also capable of reproducing and photosynthesizing in light-limited environments, particularly in snow-covered areas which only receive less than 1% incident photosynthetically active radiation (PAR) transmittance (Morgan-Kiss *et al.*, 2006; McMinn *et al.*, 2010). This photosynthetic adaptation under both low temperature and light conditions requires the rapid regulation of photosynthetic pigments, as well as the composition and structure change of thylakoid membranes.

The adaptation of plants to temperature stress mainly depends on the stability of the biological membrane system, especially the plasma and thylakoid membranes (Staehelin, 2003). The chloroplast thylakoid membrane is a lipid bilayer that has all the functional components required in processing light energy to transform it into chemical energy. Currently, the structure and function of thylakoid membranes are well investigated in the research area of photosynthetic mechanisms (Gounaris *et al.*, 1986; Allen & Forsberg, 2001; Staehelin, 2003). All chlorophyll and most carotenoids in chloroplasts are embedded in the thylakoid membranes and bound to proteins by non-covalent binding (Staehelin & Arntzen, 1983; Staehelin, 2003). The content change of chlorophyll directly affects the light reaction during photosynthesis, thus determining the chlorophyll involved is an important step towards understanding photosynthesis and resistance of plants to stress (e.g. light and nutrient limitations). Stability of the thylakoid membranes' structure has a direct effect on photosynthetic efficiency and other biological membranes which in turn have implications on the amplification of stress signal (Xu & Siegenthaler, 1996). Fatty acids, an

Corresponding author:
M. Jin-Lai and W. Yi-Bin
Email: miaojinlai@fio.org.cn; wangyibin@fio.org.cn

important component of membranes, play a key role in maintaining the stability of the organelle (Gounaris *et al.*, 1986). The extent of unsaturation of the fatty acids in membrane lipids plays a major role in avoiding membrane rigidification (Morgan-Kiss *et al.*, 2006). The monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are the major lipid constituents of chloroplast membranes and make the thylakoid membranes highly specific, anchoring the majority of protein components of the photosynthetic apparatus (Takeshi *et al.*, 1971; Morgan-Kiss *et al.*, 2006; Aronsson *et al.*, 2008). Many studies have indicated that decreased temperatures could result in higher proportions of unsaturated or short-chain fatty acids, resulting in increased tolerance and resistance to colder temperatures (Nagashima *et al.*, 1995; D'Amico *et al.*, 2006; Chen *et al.*, 2012).

The underlying mechanisms against stress of higher plants have been widely studied, but the regulation and response of chloroplast and thylakoid membrane to low-temperature environment stress in the unicellular algae, especially in Antarctic ice algae, are still not well understood. *Chlamydomonas* sp. are unicellular eukaryotic algae that are widespread in the Antarctic and are the same species as the model organism *C. reinhardtii* (Gudynaite-Savitch *et al.*, 2006; Dolhi *et al.*, 2013). This species has been widely used as a model species in a number of studies especially on microalgal physiology (Gudynaite-Savitch *et al.*, 2006; Liu *et al.*, 2006; Eddie *et al.*, 2008; Dolhi *et al.*, 2013), making it an ideal test species to study adaptations in the Antarctic ice environment. In this study, changes of pigment profiles, lipids, fatty acids and pigment protein complexes in chloroplast and thylakoid membranes of Antarctic isolated *Chlamydomonas* sp. ICE-L that responded to low-temperature stress were analysed. Furthermore, the mechanisms responsible for stress response, specifically low temperature, were also studied by looking at the composition of chloroplast and thylakoid membranes. This research contributes significantly to the understanding of the low temperature adaptation in harsh and extreme conditions such as the Antarctic ice.

MATERIALS AND METHODS

Algae species and culture media

The Antarctic ice algae *Chlamydomonas* sp. ICE-L was isolated and purified from the floating ice near the Zhongshan Station of Antarctica (China), which was collected during the Chinese 18th Antarctic Science Exploration during 2001 to 2002 (Liu *et al.*, 2006).

Cultures were grown in Provasoli seawater medium (Provasoli, 1968) in a $(5 \pm 1)^{\circ}C$ temperature-controlled environment, illuminated with 20∼30 μM photons $m^{-2}\,s^{-1}$ using cool white fluorescent bulbs under a 12:12 light/dark cycle. The ICE-L was subcultured every 14 days to a new medium to maintain the logarithmic growth of cells.

Cold stress treatment and algae cells fragmentation

To test for the range of temperature tolerance of the isolate, cultures were exposed subsequently to a temperature gradient. A total 1500 ml of *Chlamydomonas* sp. ICE-L was first grown in 3000 ml Erlenmeyer flasks for 10 days at 5°C. Then, it was transferred in a refrigerator for 2.5 h set at −25°C and subsequently transferred again to an incubator for 5.5 h at 5°C. This cycle was repeated three times in one day. The ice content of the medium was maintained at 25–50%. It was shaken three times a day during the lit period.

After 48 h, some samples were collected for microscopic observation and morphological assessment using transmission electron microscopy (HITACHIH-7000 type, Japan). After the cyclic temperature treatment, cells were collected by centrifugation at 6000 × ***g*** for 5 min with maintained temperature at 4°C. Then, the medium was removed and the algal pellets were preserved at −80°C until use.

Cells were fragmented to facilitate extraction of subcellular components. The pellets were mixed with a ratio of 1:3 (W/V) in separation buffer (0.33 mol l^{-1} sorbitol, 50 mmol l^{-1} Hepes, 2 mmol l^{-1} EDTA, 1 mmol l^{-1} $MnCl_2$, 1 mmol l^{-1} $MgCl_2$, 0.5% BAS, 5 mmol l^{-1} DTT, pH was adjusted to 7.5 with KOH). Next, samples were homogenized by ultrasonic fragmentation method at 400 W while in an ice bath for a total of 4 min. Each reaction was for 5 s with an interval of 3 s.

Preparation of chloroplast and thylakoid membranes

Chloroplasts were extracted and separated by sucrose gradient centrifugation method. The crude chloroplast extracts were added to the sucrose gradient (30%/45%/60%, 30 ml) and centrifuged at 4°C, 40,000 × ***g*** for 5 min. Next, bands between 45 and 60% of sucrose gradient (∼5 ml) were absorbed and then cleaned by adding 3× the volume of pre-frozen washing buffer solution (0.33 mol l^{-1} sorbitol, 25 mmol l^{-1} Hepes, pH was adjusted to 7.5 with KOH). The solution was then centrifuged again at 4°C, 2000 × ***g*** for 2 min, the precipitate was the intact chloroplast.

The chloroplast was mixed with breaking liquid (50 mmol l^{-1} Hepes-KOH, 1 mmol l^{-1} $MgCl_2$, pH 8.0) at a volume ratio of 1:7 at 4°C and shaken for 30 min in subdued light. The thylakoid was finally harvested by centrifugation of the breaking liquid at 20,000 × ***g*** for 2 min at 4°C, added with an equal volume of preservation liquid (10% glycerin, 50 mmol l^{-1} Hepes, 0.5% BAS, 5 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF, pH was adjusted to 7.5 with KOH), and stored at −80°C until use.

Determination of the content of chlorophyll

Chlorophyll concentrations (*a* and *b*) were measured following the method suggested by Jeffrey (Jeffrey & Humphrey, 1975) using high-performance liquid chromatography (HPLC). The absorbance of each chlorophyll *a* and *b* were measured at 647 and 664 nm, respectively and repeated three times. The chlorophyll (Chl *a* and Chl *b*) content of the algae were calculated as follows:

$$Chl\,a = 11.98 \times A_{664\,nm} - 1.93 \times A_{647\,nm}$$
$$Chl\,b = 20.36 \times A_{647\,nm} - 5.50 \times A_{664\,nm}$$
$$Chl = Chl\,a + Chl\,b$$

HPLC analysis system used the Agilent 1100 series, PDA detector, C18 reverse phase column (4 × 250 mm, 5 μm),

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the mobile phase was methylene chloride – acetonitrile – methanol – water (22.5:9.5:67.5:0.5, V/V/V/V); flow rate, 1.0 ml min^{-1}; injection volume, 20 μl; and the detection wavelength of 450 nm. The sample was filtered by PTFE membrane (PTFE 0.45 μm) before HPLC analysis.

Determination of the galactosyl diacylglycerol in thylakoid membrane

Samples of galactosyl diacylglycerol in the thylakoid membrane were determined by taking 0.5 ml of each sample and added with 3 ml solvent (methanol – chloroform – water, which the volume ratio was 1:1:0.7). The extracts were further purified by silica gel column chromatography (silica gel −60 as the adsorben, name of brand). Separation was carried by first eluting the pigment and the neutral lipids in chloroform, and then with acetone to elute the galactolipids. The sample was finally filtered through a PTFE membrane (PTFE 0.45 μm) before doing HPLC analysis (Bligh & Dyer, 1959).

HPLC analysis system used the Agilent 1100 series, PDA detector, C18 reverse phase column (4 × 250 mm, 5 μm), the mobile phase was methanol – water – acetic acid (95:5:0.5, V/V/V); flow rate, 2.0 ml min^{-1}; injection volume, 10 μl, and the detection wavelength of 205 nm. Measurements were done in triplicates.

The relative content of the monogalactosyl diacylglycerol and the digalactosyl diacylglycerol were calculated by normalization method.

Determination of the content of fatty acids in thylakoid membrane

A modified one-step extraction method was used to extract the fatty acids (Bligh & Dyer, 1959; White *et al.*, 1979). The solvent solution was made up of chloroform – methanol – hydrochloric acid with a volume ratio of 1:10:1. A total of 0.2 ml sample of thylakoid membrane was transferred into a screw cap test tube and added with 5 ml solvent solution, and then incubated in a water bath at 75°C for 1 h. Samples were then flash frozen by immersing in liquid nitrogen. After cooling, an additional 5 ml of water was added to terminate the reaction. The fatty acids were then finally extracted with 1 ml of hexane.

Separation and purification was accomplished by column chromatography (HP-5MS, 30 m × 0.25 mmID × 0.25 μm) under the following conditions: column temperature, 280°C; injection port temperature, 250°C; split injection, split ratio 10:1 and injection volume, 0.1 μl. Mass spectrum conditions: EI ion source; multiplier voltage, 1200 V; ion source temperature, 230°C; quadropole temperature, 150°C. Mass scan range was 45–500 AMU. Mass spectrum identified the chemical structure of substances and calculated the relative content of each fatty acid. All tests were done with three replicates.

Mild gel electrophoresis of thylakoid membrane

Chlorophyll concentrations of the thylakoid membrane of samples that were treated for cold stress for 48 h and the control group were all normalized at 0.8 g l^{-1}, and then added with 10% Triton-100 to a final ratio of 1:15. Samples were continuously stirred for 30 min in an ice bath in subdued light.

Electrophoresis with Serva G staining was then employed to determine the size of the fragments (Peter & Thornber, 1991; Li *et al.*, 2003). In brief, samples from chl *a*-triton mix were collected by centrifugation as described in the previous section. This was followed by the addition of 1% Serva G into the supernatant and then mixed thoroughly. The electrophoresis was then carried out at 4°C while avoiding exposure to light. Each well was filled with 15 μl of the stained sample, and bands were separated in the separating gel and stacking gel at 15 and 12 mA, respectively (Li *et al.*, 2003).

Another electrophoresis method using $AgNO_3$ as the stain was also used. Following the collection of pigments as described above, protein markers were added into the supernatant and mixed thoroughly and separated using the same previous conditions. Lastly, $AgNO_3$ was added to stain after electrophoresis.

RESULTS

Morphology and structure of *Chlamydomonas* sp. ICE-L under cold stress

Images captured through TEM revealed that the structure of ICE-L cells (Figure 1A & B). Both in control group and stress group, the cell wall of cells were thicker and uniform, most cells also exhibited plasmolysis, evident in between the cell wall and membrane which were connected by an extension of the plasma membrane system of the protoplast. Diameter of the nuclear regions ranged from 4.4 ∼ 5.1 μm which contained a complete nucleolus (1.5 ∼ 1.9 μm) in the centre and encapsulated by a double membrane. The chloroplasts were mainly composed of irregularly shaped thylakoid lamellas while starch granules occupied most of the volume of the cells. In addition starches, lipid particles which were significantly less in number, were also present. The red eye spots, which were typical of green algae, responsible for photosensitivity were found in between the cell membrane and thylakoid lamella. These were composed of two rows of bodies which contained carotenoids. Most mitochondria were characterized by their ellipsoidal shape and elongated strip-type, while somatotypes were fewer. These organelles were widely distributed throughout thylakoid lamellas and starch granules.

No significant change was observed in the structure of the mitochondria after cold stress (Figure 2). Comparing the two pictures the thylakoid lamellas have less change, such as blurred, distorted and enlarged, but not obvious.

Changes in chlorophyll content and pigment composition

Changes in the chlorophyll concentrations of ICE-L in both the control and stress groups are shown in Figure 3. Results suggest that chlorophyll content in the control group continuously increased during the experiment, peaking at 2 days, and became stable afterwards. Meanwhile, chlorophyll levels in the stress group decreased within the first 24 h of exposure to cold temperature, but slowly increased again and levelled off after 32 h.

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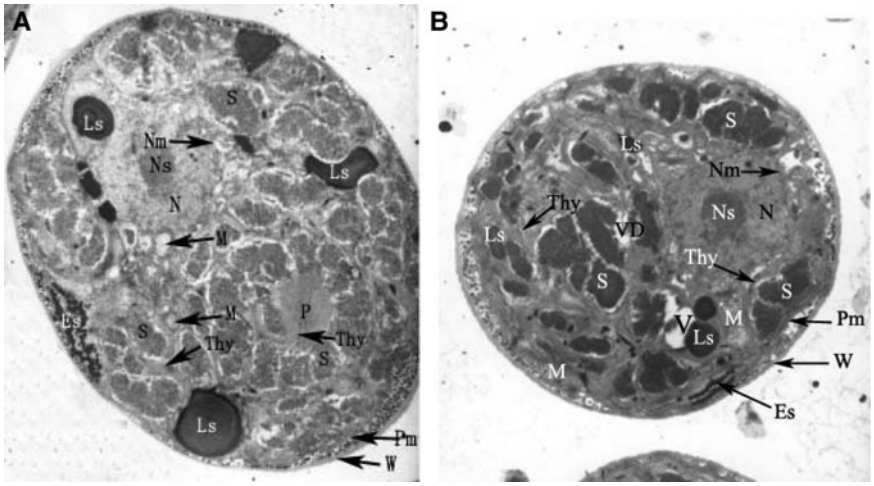

Fig. 1. Transmission electron micrographs of Antarctic ice microalgae *Chlamydomonas* sp. ICE-L. A, Control 48 h (×3500); B, Cold-stress 48 h (×3000). Es, eyespot; Ls, liposome; M, mitochondria; N, cell nucleus; Nm, nuclear membrane; Ns, nucleolus; P, pyrenoid; Pm, plasmic membrane; S, starch granule; Thy, thylakoid; V, vacuole; VD, vacuolar deposit; W, cell wall.

Figure 4 shows the changes in ratio of Chl *a*/*b* in ICE-L during the treatment. The ratio in the control group was stable, only fluctuating between 2.83 ~ 2.86, but a significant change was observed in the treatment group. Consistent with the total chlorophyll concentrations, the Chl *a*/*b* ratio was low but change was marginal in the first 8 h, and then it drastically further decreased towards the end of the first day, but it quickly recovered after 24 h to 32 h and steadily increased with passing days.

Results from HPLC (Figure 5) on the pigment composition of thylakoid membranes in ICE-L revealed the separation effects of lutein, α-carotene, chlorophyll *a* and *b* with very high repeatability. However, β-carotene did not separate clearly.

In all the pigments detected (Figure 6), Chl *a* was the highest occurring followed by Chl *b*. Lower, although not significantly, were the other pigments lutein and α-carotene. Chl *a* and α-carotene in the thylakoid membranes decreased in the first 24 h of cold stress and then began to increase and maintained stability after 32 h, similar to the trends of total chlorophyll. Chl *b*, and similarly lutein, were relatively stable and decreased only a little until the end of the experiments.

Galactosyl diglyceride

The MGDG and DGDG contents of the cells spiked up after treatment in the cold stress, however, the timing of their production varied (Figure 7). For example, MGDG increased slightly from 0 h to 24 h before stabilizing, while DGDG content rapidly increased fast within 32 h before levelling off. Comparatively, DGDG had longer time of production than the MGDG.

Fatty acids

Six major fatty acids in the thylakoid membranes of ICE-L were identified using GC-MS analysis (Table 1). These include hexadecanoic acid (16:0, $CH_3(CH_2)_{14}COOH$), stearic acid (18:0, $CH_3(CH_2)_{16}COOH$), △3-trans-hexadecenoate (16:1ω3t, $CH_3(CH_2)_7CH = CH_3(CH_2)_5COOH$), octadecenoic-9-acid (18:1ω9, $CH_3(CH_2)_7CH = CH_3(CH_2)_7COOH$), octadecadienoic-

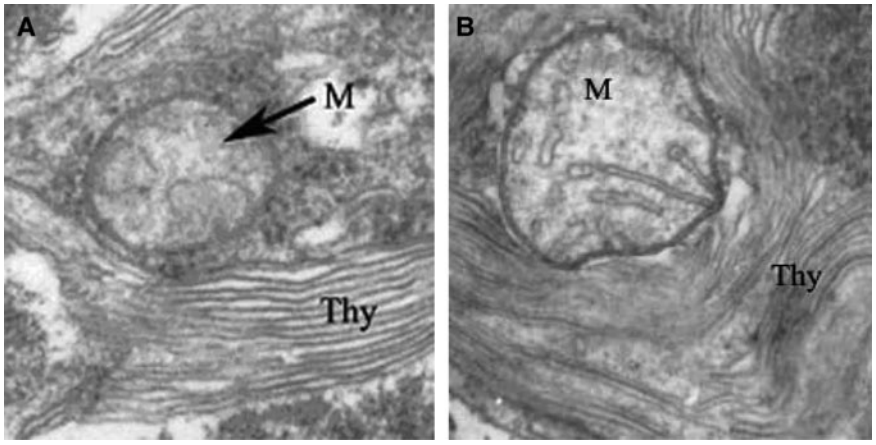

Fig. 2. Ultrastructure of mitochondria and thylakoids in Antarctic ice microalgae *Chlamydomonas* sp. ICE-L (×25,000), A, Control 48 h; B, Cold stress 48 h; M, mitochondria; Thy, thylakoid.

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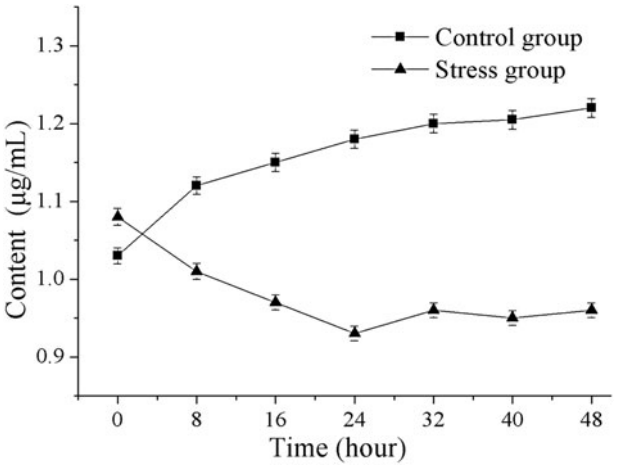

Fig. 3. Content changes of chlorophyll in *Chlamydomonas* sp. ICE-L during cold stress treatment.

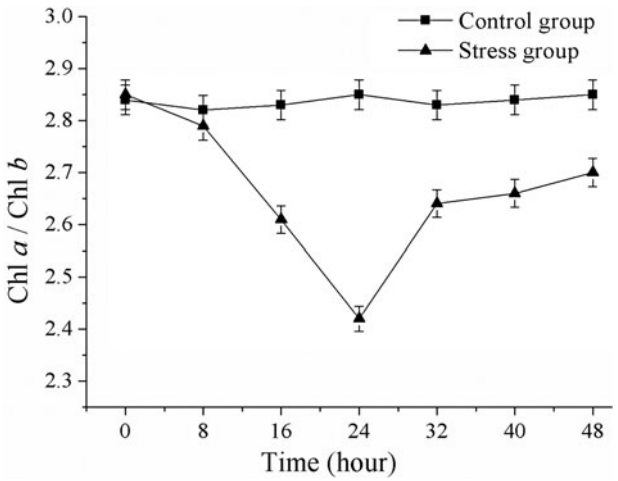

Fig. 4. Changes in Chl *a*/Chl *b* ratio of *Chlamydomonas* sp. ICE-L during cold stress.

9,12-acid (18:2ω9, $CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$) and octadecatrienoic-9,12,15-three acid (18:3ω9, $CH_3CH_2(CH=CHCH_2)_3(CH_2)_6COOH$). Based on Table 1, it can be noted that the saturated fatty acids decreased with cold stress while the index of unsaturated fatty acid increased.

Mild gel electrophoresis of the thylakoid membrane

In Figure 8, there were six bands on the gel which separated corresponding to components of photosystem I, photosystem II centre protein, ATP synthase, cytochrome b_6f, ribulose 1,5-bisphosphate carboxylase (RuBPase) and light harvesting complex II. Few free pigments appeared in the separation process.

Band 1 was the complete complex of photosystem I with a molecular weight of about 540 ku and a dark green colour in the blue-green gel. Band 2 was the ATP synthase and cytochrome b_6f complex (450 ku). The cytochrome b_6f contained Chl *a* so it fluoresced as light green in the gel. The pigment-lacking RuBPase (430 ku) and photosystem II centre protein complex (260 ku) were in bands 3 and 4, respectively. Band 5 was light harvesting complex II (LHC II) mainly composed of Chl *a* and *b*, and combinations of proteins. The molecular weight of LHC II was about 180 ku. Lastly, the band 6 was centrin PsaA/B of photosystem I which was the smallest at 80 ku.

DISCUSSION

The chloroplast in *Chlamydomonas* sp. ICE-L is not simply 'cupped' as normally, it is distributed throughout the cell (except in the nuclear region) in the form of thylakoid lamellas which are localized in the gaps between organelles and the starch granules. Thus, we speculated that this structure can ensure that photosynthesis would be carried out efficiently irrespective of the orientations of the algal cells. It was also found out that only minor changes in mitochondrial structure occurred, and that it maintained relative stability after cold stress allowing stable supply of energy to fuel metabolism and growth of ICE-L in low temperature.

The continued increase in concentrations of chlorophyll in the control group during the experiments indicates that the cells of ICE-L sustained growth and reproduction even when reaching logarithmic growth. Meanwhile, in the stress

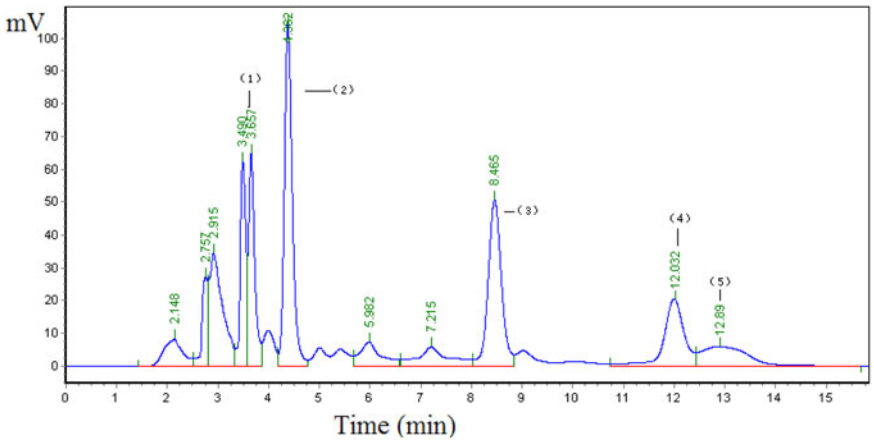

Fig. 5. HPLC chromatogram of pigment extract from *Chlamydomonas* sp. ICE-L thylakoid membranes (1) lutein; (2) chlorophyll *b*; (3) chlorophyll *a*; (4) α-carotene; (5) β-carotene.

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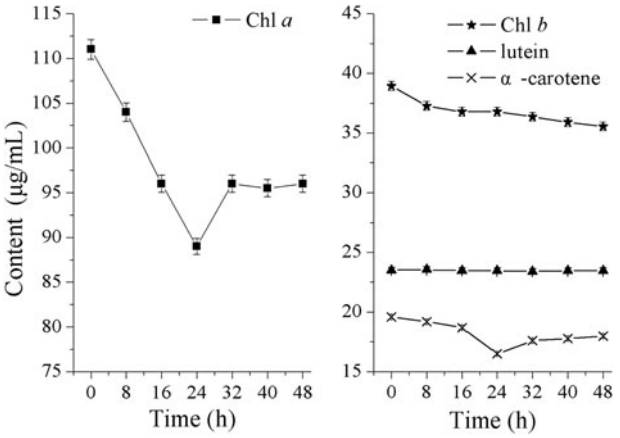

Fig. 6. Changes of pigment content extract from *Chlamydomonas* sp. ICE-L thylakoid membranes during cold stress.

group, the fluctuating trend in the main photosynthetic pigment may suggest that the cells were probably damaged at first by cold stress but immediately adapted to cold conditions.

Chlorophyll *a* and *b* play different functions in photosynthesis and their distribution in the thylakoid membrane also varies. In general, Chl *a* is found in all plants, and Chl *b* is found in higher plants and Chlorophyta algae, but not all marine phytoplankton have Chl *a* and Chl *b*, some have Chl *c*, such as Bacillariophyta and Phaeophyta species (Macpherson & Hiller, 2003). ICE-L belonged to the *Chlamydomonas* sp. of Chlorophyta (Liu *et al.*, 2006), so it has Chl *b* in thylakoid membranes. Their content changes under cold stress provide an important insight into the possible antifreeze mechanisms of the ice algae. Studies have shown that Chl *b* is mainly localized in LHC II, the outer antenna of PS II (Rüdiger, 2002), and Chl *a* in the core complex of PS I and PS II. Thus, change in the ratio of the two pigments could reflect degree of sensitivity and inhibition or potential damage to the antenna components and core complexes of the photosystems under certain stressful conditions like low temperature stress. In this study, Chl *b* was relatively more stable while Chl *a* varied widely, suggesting that the LHCs in the chloroplast of ICE-L played an important role in ensuring its growth in low temperature and light conditions of sea ice.

A major adaptation of metabolic function influencing growth and photosynthesis at low temperatures is the maintenance of membrane fluidity (Morgan-Kiss *et al.*, 2006). Studies have also shown that carotenoid may be regulated as important biological adaptation to low temperature (Hetherington & Smillie, 1984; Jagannadham *et al.*, 2000). Specifically, carotenoid could change the membrane fluidity to surmount the negative effects of low temperatures (D'Amico *et al.*, 2006). Similarly, Jagannadham reported that β-carotene was involved in regulating the membrane fluidity of Antarctic bacteria to adapt to extreme low-temperature environments (Jagannadham *et al.*, 2000). Thus, it could then be speculated that non-polar carotenoids such as lutein and β-carotene may also be contributing to the same function in the Antarctic green algae ICE-L.

Fatty acids MGDG and DGDG are abundant in plant plastid membranes, where they account for 75% of the total lipids in thylakoid membranes, with MGDG being the most abundant (about 40–50%) (Morgan-Kiss *et al.*, 2006; Aronsson *et al.*, 2008). A comparison of chloroplast lipids with *C. reinhardtii, C.* UWO241 has significantly higher levels of polyunsaturated fatty acids for the chloroplast galactolipids MGDG and DGDG (Dolhi *et al.*, 2013). The major MGDG fatty acid species in UWO241 was found to be 18:4 (Dolhi *et al.*, 2013), whereas ICE-L was enriched in 18:3 fatty acids. There contains a high proportion of polyunsaturated fatty acids (PUFA) in these two kinds of galactolipids that is mostly linolenic acid. These two galactolipids contribute directly to various photosynthesis-related processes (Aronsson *et al.*, 2008). The increasing PUFA can reduce some of the temperature-related characteristics of the membrane (D'Amico *et al.*, 2006), such that when the ratio of PUFA is higher, the phase transition temperature is lower and the cold resistance of the plant cell is stronger. High concentrations of PUFA are important for the survival of psychrophilic organisms in extreme environments as such fatty acids maintain the fluidity of the membrane lipids (Morgan-Kiss *et al.*, 2006; Teoh *et al.*, 2013). Chen *et al.* (2012) found that elevated nitrate reductase activity and photosynthetic rates at low temperatures together with the high proportion of unsaturated fatty acids allow the green alga *Stichococcus* species to thrive in Antarctica. The increase in lipids in response to low temperature stress has been reported in other species such as *Scenedesmus* (Li *et al.*, 2011). In the current study, results

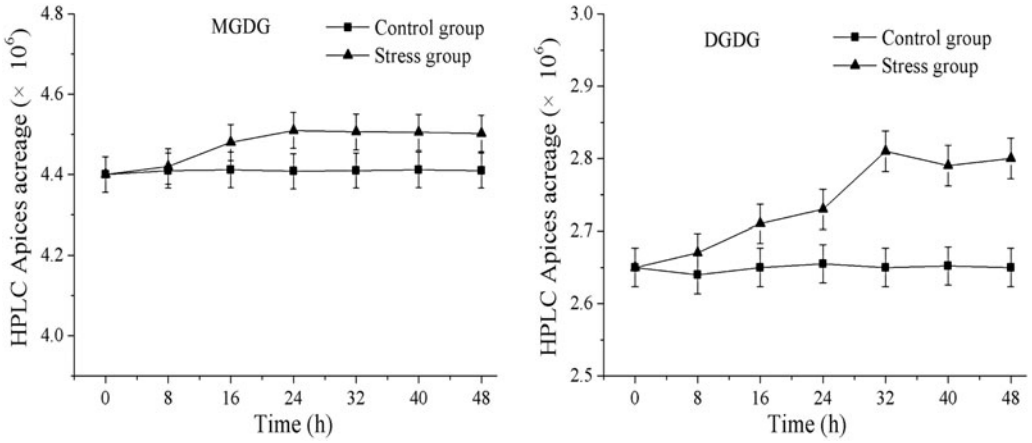

Fig. 7. Changes in Apices acreage of DGDG and MGDG extract from *Chlamydomonas* sp. ICE-L thylakoid membranes during cold stress.

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Table 1. Fatty acid content of *Chlamydomonas* sp. ICE-L thylakoid membranes (%).

Components of fatty acid	Stress 0 h	Stress 24 h	Stress 48 h
16:0	11.63	10.07	10.37
18:0	3.26	2.69	2.64
16:1ω3t	4.36	4.23	3.85
18:1ω9	2.64	2.38	1.32
18:2ω9	7.72	8.56	4.33
18:3ω9	70.39	72.07	77.49
IUFA	233.61	239.94	246.03

IUFA(index of unsaturated fatty acid) = [(16:1)*t* + (18:1) + (18:2) × 2 + (18:3) × 3] × 100.

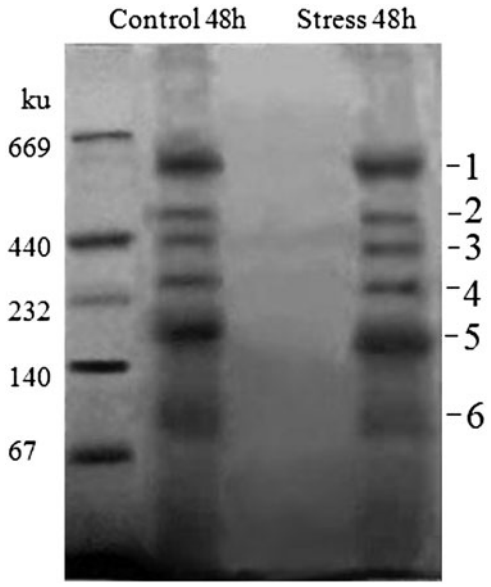

Fig. 8. Blue-native gel electrophoresis analysis of *Chlamydomonas* sp. ICE-L thylakoid (1) PS I, (2) ATPase and cyt b_6/f complex, (3) RuBPase, (4) PS II centre protein complex, (5) LHC II, (6) PsaA/B.

showed that the contents of MGDG and DGDG in ICE-L thylakoid membranes were elevated under cold stress and the unsaturation of fatty acid in thylakoid membranes increased along with stress time, thereby maintaining the liquidity of thylakoid membranes.

Studies by Routaboul (Routaboul *et al.*, 2000) suggested that triethenoid fatty acids are required to maintain chloroplast function of plant cells at low temperature. This type of fatty acid not only maintains liquidity of thylakoid membranes but also promotes the rate of re-synthesis of protein D_1 assembly to the reaction centre of PS II (Vijayan & Browse, 2002). Investigations on the responses toward temperature stress of three *Chlamydomonas* sp. from Antarctic, temperate and tropical regions showed that only the Antarctic algae *C.* UMACC 229 predominantly produced PUFA, especially 16:3, which ranged from 47.0 to 57.2% of the total fatty acids (TFA) (Teoh *et al.*, 2013). In a freshwater *Chlamydomonas* sp. which was isolated from an acidic mining lake, polyunsaturated fatty acids including α -linolenic acid (18:3ω3) and 16:4ω3 along with palmitic acid (16:0) being most abundant at 8°C, was as similar as that described for some psychrophilic bacteria (Poerschmann *et al.*, 2004). In this study, the content of octadecatrienoic-9,12,15-three acid (18:3ω9) increased continuously under cold stress which means that such change was necessary to maintain the normal photosynthetic functions in the thylakoid membranes of ICE-L.

△3-trans-hexadecenoate (16:1ω3t) is a peculiar fatty acid in plants and has been a hot research topic related to antifreeze mechanisms (Vijayan & Browse, 2002). It has been widely recognized that 16:1ω3t has a decisive influence on phase transition temperature of membrane lipid (Thompson, 1996). The results of the cold acclimation research of winter rye by Huner (Huner *et al.*, 1987) also showed that the decrease of 16:1ω3t at low temperature caused the depolymerization of LHC II oligomer resulting in a decline in light-harvesting efficiency. The content of 16:1ω3t in thylakoid membranes of ICE-L decreased with cold stress, consistent with previous results and observations.

From the results obtained so far, it seems that content changes of 16:0 and 18:2ω9 inflection point occurred at 24 h of cold stress which were similar with that of Chl *a*, Chl *a*/*b* and MGDG. These phenomena showed that there was interaction and mutual coordination between the pigments, lipids, fatty acids and pigment-protein complexes, synergistically maintaining the stability of the thylakoid membranes. All of these played a role to maintain the normal physiological function of ICE-L, so that the thylakoid membranes were able to complete photosynthesis and ensure the normal metabolism in low temperature and light conditions.

The conversion of light energy into chemical energy in chloroplast is performed by various protein complexes working together, which include RuBPase in chloroplast stroma and PS I, PS II, ATPase and complex of cytochrome b_6f which combine with thylakoid membranes (Wollman *et al.*, 1999; Allen & Forsberg, 2001). From the results of exposing cells to cold stress, the changes in the complexes of PS I, ATPase, cytochrome b_6f and RuBPase were insignificant. This means that such changes had little or minor (undamaging) effects on these photosynthetic apparatuses which remained stable in the cold environment, thus ensuring normal light and dark reactions. The protein complex of PS II in stress group became clear in the gel signifying that we conjectured cold stress promoted the dissociation of LHC II from PS II. Meanwhile, the distance travelled by LHC II in the gel increased which could probably suggest that the oligomer of LHC II reduced and monomer increased. Complex of cytochrome b_6f was an important electron transit mediator in thylakoid membranes, it plays a key role together with LHC II, PS I and PS II (Staehelin & Arntzen, 1983). Based on these observations, we believed that the compactness of LHC II and PS II protein complex may play an important role in the resistance to cold stress of ICE-L.

Murata *et al.* (1990) argued that the decrease of LHC II oligomer caused decline in the light-harvesting efficiency and it was thought that this might be the adaptive response of plants to low temperature in order to reduce the degree of light inhibition. This is consistent with the results of this paper, and it is further confirmed that the change of the structure of LHC II is an important mechanism to guarantee survival under low temperature adaptations.

CONCLUSIONS

The experiments documented here demonstrate that ice algae *Chlamydomonas* sp. ICE-L has adapted to the Antarctic

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environment, and it can quickly respond to changes particularly under low temperature stress. The structure of mitochondria has no obvious change after cold stress, but the thylakoid lamellas were blurred, distorted and enlarged. Chl *a*, Chl *b*, MGDG and fatty acid contents changed under cold stress. The contents of Chl *a* and α-carotene decreased at first and then increased after 24 h, but did not recover to the level before treatment, Chl *b* and lutein were relatively stable. The increasing trend in MGDG and DGDG improved the overall degree of unsaturation of thylakoid membranes in low temperature, and the proportions of pigments and proteins, protein complexes and the structure of pigment protein complex were affected by low temperature stress. It was found that pigments, lipids, fatty acids and pigment-protein complexes synergistically maintained the stability of the thylakoid membranes and the normal physiological function of *Chlamydomonas* sp. ICE-L.

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Correspondence should be addressed to:
M. Jin-lai and W. Yi-bin
First Institute of Oceanography, State Oceanic Administration, Qingdao 266061, China
email: miaojinlai@fio.org.cn; wangyibin@fio.org.cn

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