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Tolerance to freezing stress in cyanobacteria, *Nostoc commune* and some cyanobacteria with various tolerances to drying stress

Yufang Lin, Manabu Hirai, Yasuhiro Kashino, Hiroyuki Koike, Satoru Tuzi and Kazuhiko Satoh*

[1] *Graduate School of Science, Himeji Institute of Technology, 3–2–1, Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1297 (*satohkaz@sci.himeji-tech.ac.jp)*

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Abstract: Tolerance to and effects of the freezing stress in a desiccation-tolerant, terrestrial cyanobacterium, *Nostoc commune*, in cultivated strains of *N. commune*, and in desiccation-sensitive species, *Synechocystis* sp. PCC6803 and *Fischerella muscicola*, were studied by measuring their photosynthetic activities and fluorescence emission spectra. The results showed that a strain or species with higher desiccation tolerance was more tolerant to freezing stress than one with lower desiccation tolerance, which is consistent with the idea that tolerance to freezing stress is related to resistance to drying stress. Under freezing conditions, light energy absorbed by photosystem (PS) II complexes was dissipated to heat energy in *N. commune*, which may protect the cells from photoinactivation. *N. commune* encountered cellular dehydration due to ice formation outside the cell under freezing conditions. But NMR data showed that relatively high amounts of water still remained in a liquid state inside the cells at −36°C when *N. commune* colonies were fully wetted before freezing. High PSI activities measured by P700 photooxidation also support the result that non-freezing water remains within the cells. Besides, 5% methanol enhanced the resistance to freezing stress in the sensitive species. This effect seems to be related to maintenance of the PSI activity and pigment-protein complexes in their functional forms by methanol.

key words: energy transfer, freezing stress, *Nostoc commune*, photosynthetic activity, photosystems I and II

Introduction

Nostoc commune, a terrestrial cyanobacterium, is spread all over the world, including dry regions and the Antarctic Continent (Whitton and Potts, 2000). *N. commune* shows very high desiccation tolerance (Cameron, 1962), and changes in photosynthetic systems during dehydration are supposed to be important for the desiccation tolerance (Satoh *et al*., 2002). The changes in photosynthetic systems are as follows: (1) light energy absorbed by phycobilisomes (PBS), photosystem (PS) I complexes, and PSII core complexes is dissipated as heat energy, (2) both the PSI and PSII activities are deactivated, (3) quenching of PSII fluorescence occurs later than deactivation of the PSII activity, and (4) energy transfer from PBS to the anchor protein and the anchor to the PSII core complexes is inhibited. When photosynthesis is inhibited under various stresses, the absorbed light energy becomes excessive and damages the cells by producing

strong oxidants, strong reductants, or active oxygen species (Anderson and Barber, 1996). Therefore, knockout of the photochemical reaction center activities and dissipation of the absorbed light energy to heat under stress conditions must be critical for photosynthetic organisms to survive under severe conditions. The degree of dehydration, which causes each change mentioned above, has also been reported in a preceding paper (Satoh *et al*., 2002).

Because *N. commune* inhabits the Antarctic Continent (on soils and at the edge of streams, Vincent, 2000), it must have tolerance to freezing stress as well. Most previous research on *N. commune* has focused on its high desiccation tolerance, which enables *N. commune* to survive extreme dryness (Shirky *et al*., 2000). However, very little is known about the mechanism of survival from freezing stress in this cyanobacterium. Most plants are injured when they experience freezing conditions; only a few species can safely recover after freezing and thawing. Many hypotheses on the cause of freezing injury have been proposed. Among them, intracellular ice formation and cellular dehydration appear to be the main causes of freezing injury (Steponkus, 1984). When cells are frozen rapidly, ice forms inside the cells; this is believed to mechanically damage the cells (Mazur, 1969). However, when the freezing rate is low, water outside the cells freezes first because of its lower solute concentration than those inside the cells. Then, water inside the cells is forced out by water potential differences, which damages the cell membranes (Steponkus, 1984; Hällgren and Öquist, 1990). If the freezing damage is the result of freeze-induced cellular dehydration, then the resistance to water loss may be an important prerequisite for the resistance to freezing stress.

To unveil the relationship between freezing- and desiccation-tolerance, we compared the effects of freezing stress in various cyanobacteria, which have different sensitivities to drying stress, including a desiccation-tolerant species, *N. commune*, and an aquatic drought-sensitive species, *Synechocystis* sp. PCC6803.

In the present work, we measured photosynthetic activities by fluorescence emitted by chlorophyll (Chl) *a* and by light-induced redox changes of P700 (a reaction center Chl dimer of PSI) to detect freezing injury. Furthermore, changes in photosynthetic systems during and after freezing in the presence of methanol, which has been reported to act as a cryoprotectant for cyanobacterial cells (Bodas *et al*., 1995), were also examined in *Synechocystis* PCC6803.

Materials and methods

Colonies of *N. commune* were collected on the campus of Himeji Institute of Technology, Hyogo, Japan (134.5°E, 35°N), mainly from August to October, 2002. The colonies were cut into small pieces, and fully wetted samples were used. Two strains of *N. commune*, UTEX584 (N584) and UTEXB1621 (N1621) from the University of Texas Culture Collection, were cultivated in BG11 medium (Rippka *et al*., 1979) at 25°C. *Synechocystis* PCC6803 and *Fischerella muscicola* Gomont were cultured in BG11 under 3% CO_2 at 30°C for 3 days and 1 week, respectively. The filamentous aquatic species, *F. muscicola*, was collected at Gunai Hot Spring, Hokkaido, Japan. Where indicated, wild type *N. commune* colonies cultured at 5°C for more than 2 months were used. In this case, the colonies were fully rehydrated with 10-fold-diluted BG11 medium

at first, and then were left under dry conditions. Distilled water was added to the colonies once a week.

The samples in aluminum cuvettes were cooled by dry ice to the respective temperatures at slow rates (0.5°C /min around 0°C) and were incubated at those temperatures for 5 min, then were warmed up to room temperature. For *Synechocystis* PCC6803 and *F. muscicola*, 2 m*l* of the culture was used. During these treatments, various fluorescence parameters were detected. In some experiments with *Synechocystis* PCC6803, 5% methanol was added before freezing treatments to observe the effect of alcohol on resistance to freezing stress. The concentrations of *Synechocystis* PCC6803 cells corresponded to about 26.2 μg Chl ml^{-1}.

Chl fluorescence was measured with a pulse-modulated fluorometer, PAM 101/103 (Walz, Germany), as reported by Yamane *et al*. (1997). Samples were placed in a round aluminum cuvette and initially exposed to a modulated measuring beam, followed by continuous actinic light (70 μmol photons $m^{-2} s^{-1}$) through a glass column. The intensity of the measuring light was 0.17 μmol photons $m^{-2} s^{-1}$. Chl fluorescence is a non-destructive and useful tool to measure photosynthetic activities in intact cells (for review, see Lazár, 1999). For example, the Fv/Fm ratio in higher plants is known to reflect the quantum yield of PSII. In this case, Fo and Fm are the minimum and maximum levels of Chl fluorescence in dark-adapted samples, and Fv is a variable part of Chl fluorescence (Fv = Fm − Fo). Under actinic light, the fluorescence intensity changes, and the (Fm' − Ft)/Fm' value is found to reflect electron flow through PSII under light (Genty *et al*., 1989). In this case, Fm' and Ft levels show maximum and stationary fluorescence levels under the actinic light, respectively. Fv or Fv' (Fm' − Ft) is usually very small in cyanobacteria compared to higher plants. This is believed to be due to strong fluorescence from phycobiliproteins, which increase Fo with no effect on Fv. However, changes in Fv/Fm and (Fm' − Ft)/Fm' values still reflect changes in the quantum yield of PSII and electron flow through PSII under actinic light in cyanobacteria (Inoue *et al*., 2000).

Redox changes of P700 were also measured with the PAM101/103 fluorometer, equipped with a dual-wavelength emitter-detector unit, ED-P700DW.

Fluorescence emission spectra at various temperatures were measured with a laboratory-constructed fluorescence spectrophotometer. The actinic light from a Techno Light (100 W halogen lamp, Kenko) was passed through a Corning glass filter, 4–96, which passes light ranging from 360 nm to 600 nm. For measurement of emission spectra at 77K, pretreated samples were quickly dipped into liquid nitrogen and then fluorescence was detected.

The nuclear magnetic resonance (NMR) signals were measured by a JEOL ECA-600 NMR spectrometer (^{1}H: 600.1723046 MHz). Small pieces of *N. commune* colonies were stuffed into a glass tube, cooled down to desired temperatures, incubated at those temperatures for 5 min, and then signals from liquid water in *N. commune* colonies were measured.

Results and discussion

Freezing tolerance in various cyanobacteria

We first compared tolerance to freezing stress in various cyanobacteria (Table 1).

Starting from 25°C, the samples were slowly cooled down to −78°C, kept at this temperature for 5 min, and then warmed to the original temperature. We chose this temperature because it is easy to keep the samples at −78°C by using dry ice. Cooling to −20 or −40°C also had the same effect as shown in Table 1 (data not shown). The Fv/Fm and (Fm'−Ft)/Fm' ratios were measured before and after the freezing treatment. Among the cyanobacteria tested, *N. commune* collected on the Harima Science Garden City Campus showed the highest tolerance to freezing stress; both the maximum quantum yield of PSII (Fv/Fm) and the rate of electron flow through PSII under actinic light ((Fm'−Ft)/Fm') were recovered more than 80%. In higher plants, it is well known that low-temperature treatments of the plants increase their tolerance to freezing stress (Steponkus, 1984). Therefore, we also tested *N. commune*, which had been cultivated at 5°C for 2 months. However, cultivation of *N. commune* at 5°C had little effect on the freezing tolerance (Table 1), suggesting that the protective mechanism in *N. commune* is different from that in higher plants. Although the three strains are thought to belong to the same species, the other two strains, *N. commune* UTEX584 (N584) and *N. commune* UTEXB1621 (N1621), had lower freezing tolerance than the naturally growing strain. Their tolerance was similar to that of *F. muscicola*, an aquatic filamentous cyanobacterium. N584 and N1621 were collected in Scotland and Texas, respectively, but the main characteristic is that they had been cultured in a liquid medium for a long time; that is, they had long been under non-drying conditions. An aquatic single cell cyanobacterium, *Synechocystis* PCC6803, is very sensitive to the freezing stress; although the Fv/Fm value recovered to some extent, photosynthetic electron flow was totally inactivated by the freezing treatment (Table 1). The protective effect of 5% alcohol in this cyanobacterium will be discussed later.

Judging from photosynthetic activities, *F. muscicola* and *Synechocystis* PCC6803 were drought sensitive, and N584 and N1621 were found to be less resistant to drying stress than naturally growing *N. commune* (Hirai *et al*., in preparation). Therefore, the

Table 1. Maximum quantum yield (Fv/Fm) of and electron flow through photosystem II ((Fm'−Ft)/Fm') before and after freezing treatments at −78°C in *N. commune*, *F. muscicola* and *Synechocystis* sp. PCC6803.

	Fv/Fm			(Fm'−Ft)/Fm'		
	Before freezing treatments	After freezing treatments	Recovery (%)	Before freezing treatments	After freezing treatments	Recovery (%)
N. commune	0.219	0.198	90.5	0.204	0.167	81.8
*N. commune**	0.176	0.149	84.7	0.154	0.135	87.7
N1621	0.360	0.228	63.3	0.072	0.036	50.0
N584	0.458	0.328	71.5	0.100	0.076	76.1
F. muscicola	0.571	0.286	50.0	0.104	0.067	64.0
Synechocystis	0.456	0.191	41.8	0.237	0.00	0.00
*Synechocystis**	0.456	0.313	68.6	0.237	0.198	83.5

*N. commune**, cultured at 5°C; *Synechocystis**, 5% methanol was added; N1621, *N. commune* UTEXB 1621; N584, *N. commune* UTEX584.

results mentioned above are consistent with freezing tolerance being closely related to drought tolerance (Steponkus, 1984).

Fluorescence emission spectra at 77K before and during the freezing treatments

A colony of *N. commune*, which had been kept at 25°C, was quickly frozen to 77K, and the fluorescence emission spectrum was measured (Fig. 1A, line 25°C). The fluorescence having a peak at 695 nm (F695) from PSII core complexes was much higher than those of F735 (from PSI complexes) and F645 and F665 (from phycobiliproteins). Fluorescence emission spectra provide useful information because they suggest routes of energy transfer and the fate of light energy absorbed by each pigment-protein complex. Usually, fluorescence from PSI can be observed only at very low temperatures. Furthermore, quick freezing of samples to 77K fixes the pigment-protein complexes in the state just before the samples are dipped into liquid nitrogen. This is why we measured 77K fluorescence emission spectra in this experiment. The spectrum did not change very much after chilling down to 0°C, but F695 decreased considerably when the sample was cooled down to −10°C. After the sample was frozen at −20 or −40°C, F695 became much smaller than F735, and fluorescence from phycobiliproteins also became smaller, while F735 seemed not to be changed by the freezing. The PSII to PSI fluorescence ratio changes from sample to sample, but even when PSII fluorescence was lower than PSI fluorescence, this quenching was clearly observed (data not shown, but see Satoh *et al*., 2002). These results suggest that the light energy absorbed by phycobiliproteins or PSII core complexes is quenched (changed to heat energy) under freezing stress. This characteristic seems very important for cells to avoid freezing injury because, when photosynthesis is inhibited at freezing temperature, light energy absorbed by PSII causes damage to the cells by producing strong oxidants or reductants (Yamamoto, 2001). The quenching of fluorescence recovered when *N. commune* colonies were warmed to room temperature (data not shown) as can be supposed from the data shown in Table 1. The quenching of light energy absorbed by PBS or PSII core complexes also takes place when *N. commune* is subjected to air-drying (Satoh *et al*., 2002).

Figure 1B shows fluorescence emission spectra at 77K in *Synechocystis* PCC6803 before and during freezing treatments. It is clear that no quenching of fluorescence from phycobiliproteins and PSII core complexes was induced by the freezing treatments. This result does not necessarily mean that *Synechocystis* PCC6803 cells are not dehydrated by the freezing treatment, because removal of water from the cells did not cause quenching in this species (Hirai *et al*., in preparation). But this seems to be a typical phenomenon for species sensitive to drought. For example, Heber *et al*. (2000) showed that drying stress caused much smaller fluorescence quenching in higher plants than in desiccation tolerant lichens and mosses.

PSI activities before, during and after freezing treatments

In the presence of an inhibitor of PSII, 3-(3,4-dichorophenyl)-1,1-dimethylurea (DCMU), light-induced oxidation of P700 can be observed (Fig. 2, line 25°C). After 1 s of illumination, P700 was returned quickly to the original reduced level by the electrons through the cyclic electron path around PSI. At low temperatures such as −20, −40,

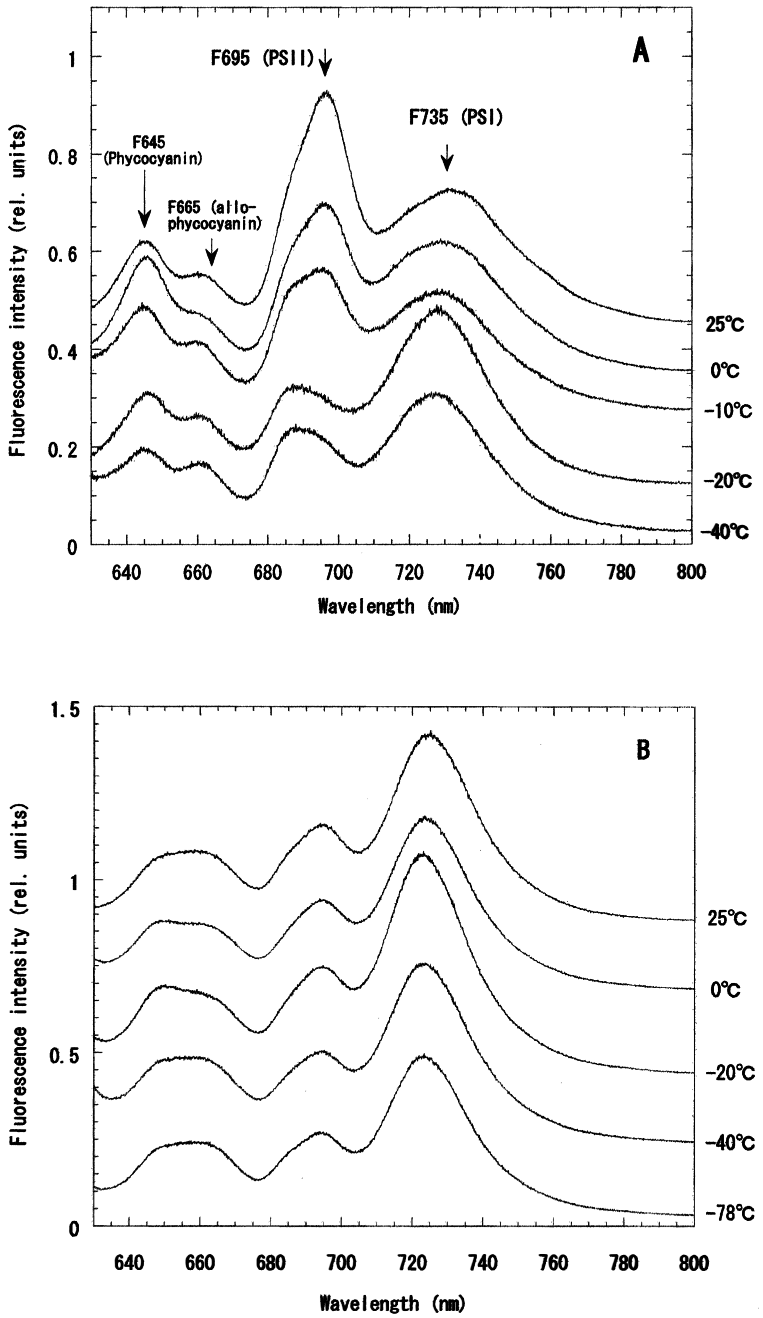

Fig. 1. Fluorescence emission spectra at 77K in fragmented *N. commune* colonies (A) and in *Synechocystis* PCC6803 (B). *N. commune* colonies fixed on glass fiber and *Synechocystis* PCC6803 cells in a brass cuvette were cooled down to the temperatures shown in the figure at slow rates and incubated at those temperatures for 5 min, then quickly frozen at 77K by dipping the samples into liquid nitrogen.

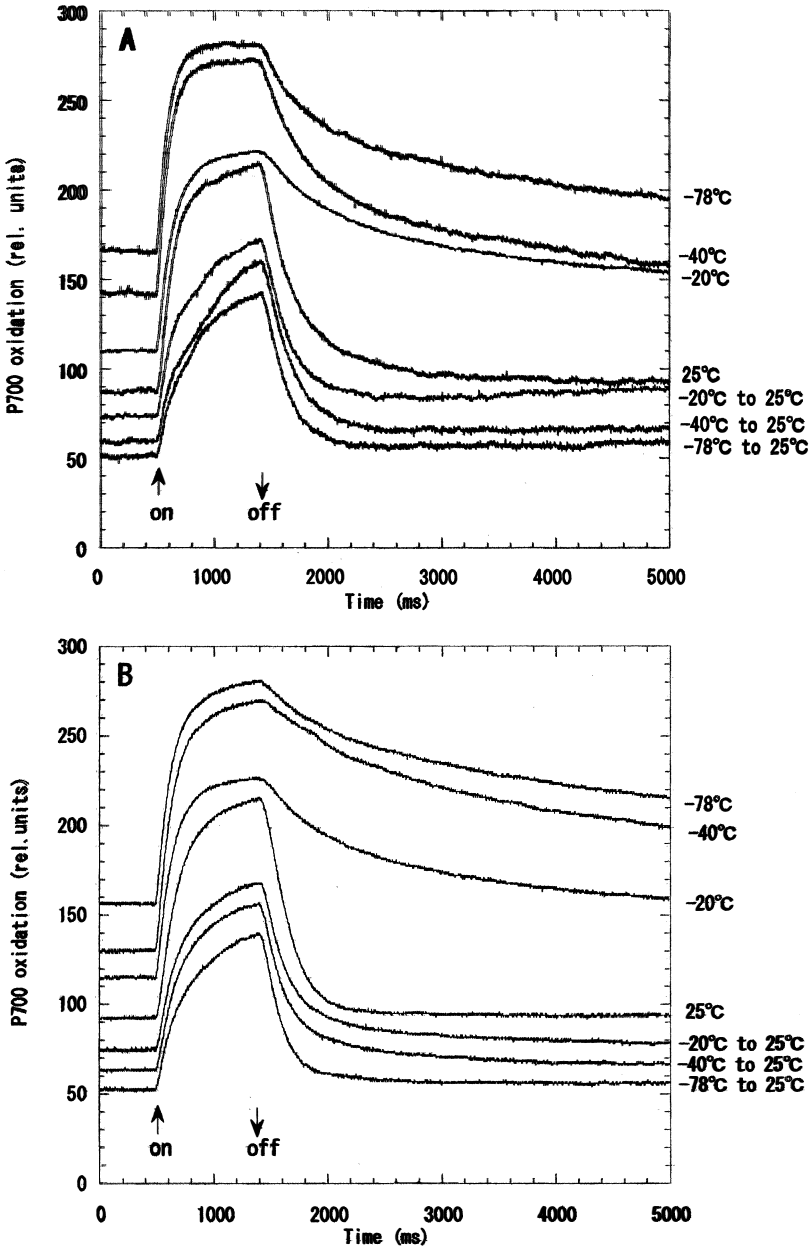

Fig. 2. Light-induced redox changes of P700 in *N. commune* (A) and *Synechocystis* PCC6803 (B) under freezing conditions. Samples to which 10 μM DCMU was added were slowly cooled down to -20, -40, or -78°C, further incubated at that temperature for 5 min, then rewarmed to room temperature. Samples were illuminated for 1 s by white light.

and −78°C, the extent of photooxidation of P700 remained the same or increased in both *N. commune* (Fig. 2A) and *Synechocystis* PCC6803 (Fig. 2B). Dark re-reduction of P700 became slow, but there remains a rapid component in the case of *N. commune* (Fig. 2A), showing that some cyclic electron transport activity through PSI remainins. When the temperature was increased to 25°C after freezing, re-reduction rates of P700 returned to the control level, suggesting little damage to PSI by freezing in either species.

Amounts of liquid water under freezing conditions in *N. commune*

Fragments of *N. commune* colonies were cooled down to various temperatures, incubated at those temperatures for 5 min, and then the NMR signals from liquid water were measured (Table 2). The signal increased with lowering temperature due to the increase in the Boltzmann factor $(kT)^{-1}$, but below −5°C the signal started to decrease, and after incubating the sample for 5 min at −10°C the value became one twentieth of the value at −5°C. This shows that about 95% of water in the colonies can easily become frozen at this temperature. Below −10°C, the NMR signal decreased slowly with decrease in the incubation temperature. The quickly and slowly freezing water (each corresponding to 95% and 5% of the total water content) can be thought to correspond to the water outside and inside the cells, respectively. This is consistent with the ability of *N. commune* colonies to retain water corresponding to about 22 times their dry weights (Satoh *et al*., 2002), with the considerable amount of space outside the cells in the colonies, and with the fact that the amount of water that restores the full photochemical activities nearly equals the dry weight of the colony. These results suggest that the amount of water inside the cells is about 5% (one out of 22) of the total water of fully rehydrated colonies. The amount of remaining liquid water decreased slowly with decrease of the incubation temperature, but was still about 3% (compared to the weight of fully wetted colonies) of the water remaining in a liquid state even at −36.3°C (Table 2). Because ice formation inside cells usually damages the cells (Mazur, 1969), the loss of liquid water may be due to movement of cytoplasmic water to outside the cells due to the difference of water potential across the plasma membranes. The water moved to the outside may become frozen quickly. The time course of light-induced redox changes of P700 at −40°C (Fig. 2) is almost the same as that in *N. commune* colonies when water corresponding to 0.66 times the dry weight of the colonies was added. This amount of water equaled 3.0% of water in fully wetted colonies (Satoh *et al*., 2002).

Table 2. Amounts of liquid water in *N. commune* colonies at various temperatures.

	Temperature						
	25°C	0°C	−5°C	−10°C*	−10°C	−20°C	−36.3°C
NMR signal (rel. units)	538.36	596.84	613.09	287.94	33.38	26.17	23.65
Amount of liquid water (%)	100	100	100	46.18	5.35	4.04	3.44

−10°C*, signals were measured during temperature decrease to −10°C. The increase of the signal by lowering the temperature was took into account in calculation of the amount of liquid water.

Effects of alcohol on freezing tolerance in *Synechocystis* sp. PCC6803

Alcohol is known to inhibit ice formation and is used for cryopreservation of cyanobacteria (Bodas *et al*., 1995). As shown in Table 1, the photosynthetic activity ((Fm'−Ft)/Fm') was not damaged greatly, and recovery of the maximum quantum yield of PSII (Fv/Fm) was increased by the addition of 5% methanol in the freezing-sensitive *Synechocystis* PCC6803. The fluorescence time courses before and after the

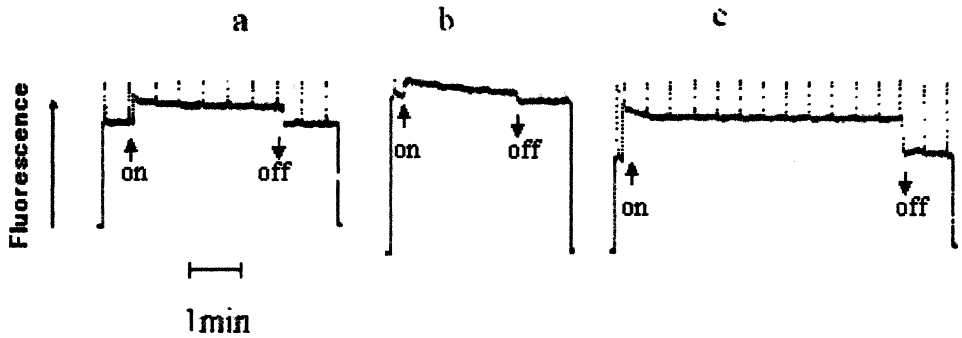

Fig. 3. Fluorescence time courses of *Synechocystis* PCC6803 before and after freezing treatments in the presence and absence of 5% methanol. Samples were slowly cooled down to −78℃ and incubated for 5 min, then rewarmed to 25℃. Fluorescence was measured as mentioned in Materials and Methods. Arrows show where the actinic light was turned on or off. Saturating pulses were fired every 30 s. Trace a, before the low-temperature treatment and without methanol; trace b, pretreated at −78℃ in the absence of 5% methanol; trace c, pretreated at −78℃ in the presence of 5% methanol.

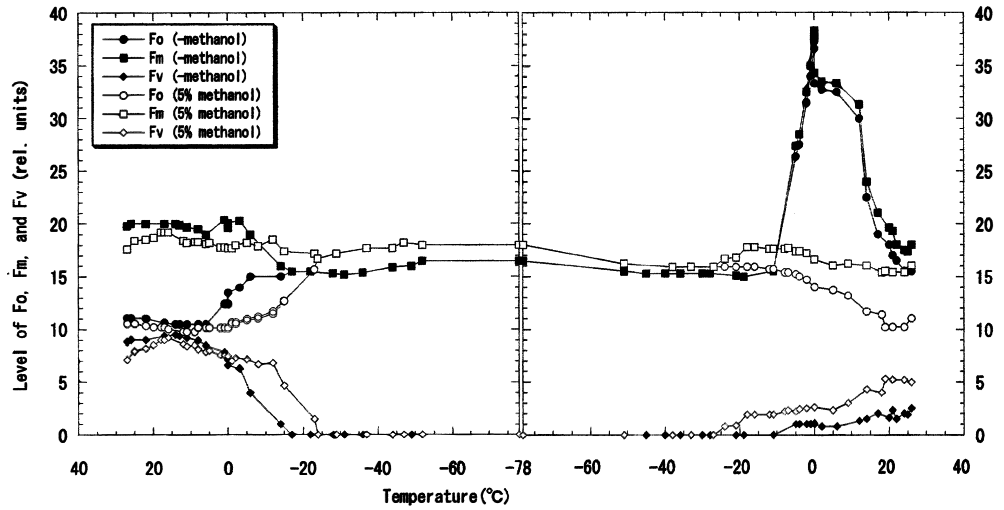

Fig. 4. Changes in Fo and Fm levels during freezing and rewarming of *Synechocystis* PCC6803 cell suspension in the presence and absence of 5% methanol. Cells were cooled slowly from room temperature to −78℃, incubated for 5 min and then warmed up to room temperature. During the temperature treatment, a saturating light pulse was fired every 30 s to observe the Fm level.

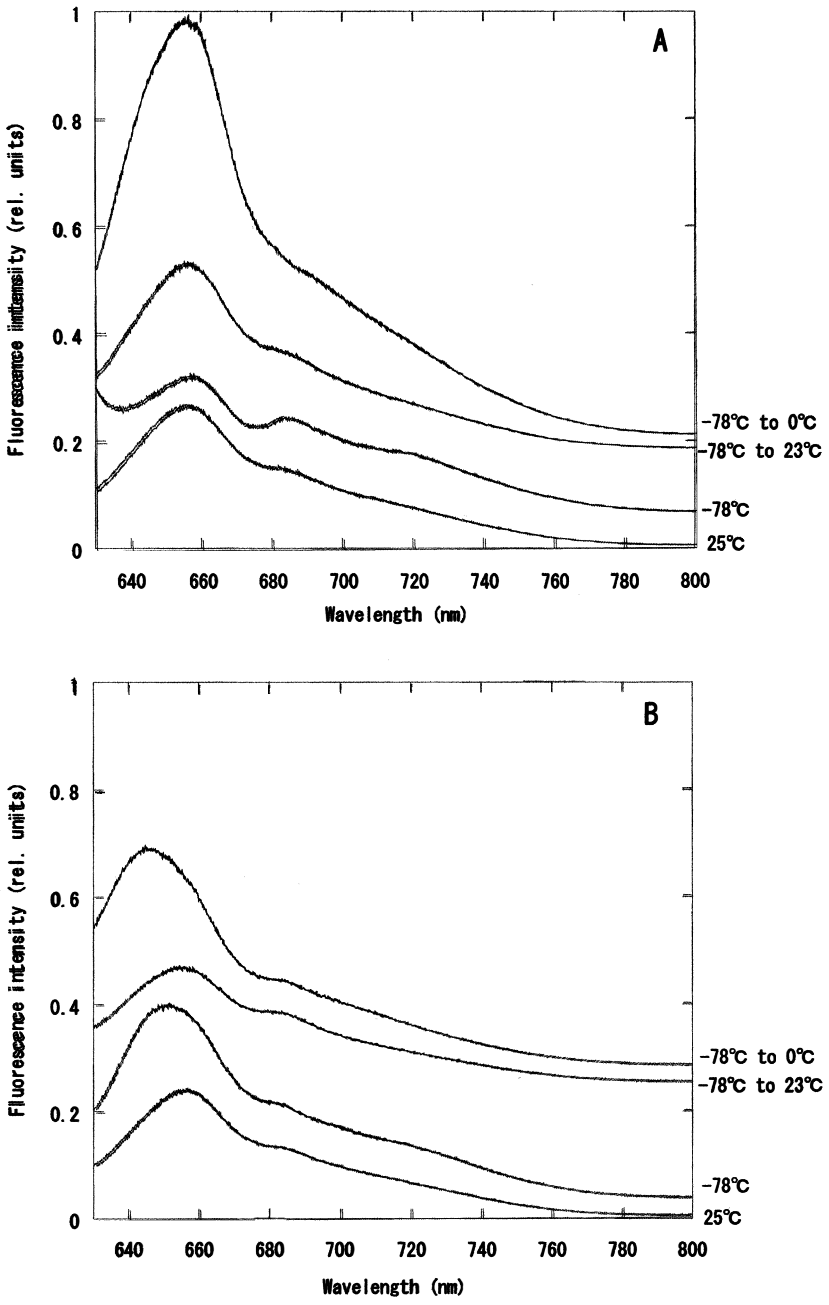

Fig. 5. Fluorescence emission spectra at various temperatures in *Synechocystis* PCC6803 cells. Cells were frozen at −78℃, warmed to 0℃, and then warmed to 20–25℃ in the absence (A) and presence (B) of 5% methanol. Fluorescence emission spectra were measured at the indicated temperatures. "−78℃ to 0℃" means that cells frozen at −78℃ were rewarmed to 0℃ and then the fluorescence emission spectrum was measured at 0℃.

freezing treatment in the presence and absence of methanol are shown in Fig. 3. Addition of 5% methanol had little effect on the time course measured before freezing (data not shown). After the treatment, the fluorescence time course in the sample with alcohol (trace c) was almost the same as that before the treatment (trace a), while no saturating-pulse-induced increase in fluorescence under actinic light was observed in the sample without alcohol (trace b), showing crucial damage to the photosynthetic activity.

In order to find out how alcohol protects *Synechocystis* PCC6803 cells from freezing injury, we measured Fo and Fm levels during freezing and thawing treatments of the cells in the presence or absence of 5% methanol (Fig. 4). The Fo level began to increase at around 0°C due to inhibition of electron flow from Q_A to plastoquinone, which can be deduced from the decrease of decay of fluorescence induced by saturating pulses (data not shown). Five percent methanol protected against the inhibition of electron flow at around 0°C. However, the freezing temperature of *Synechocystis* PCC6803 was decreased only slightly by the addition of methanol (data not shown). A striking difference was observed when the samples were warmed up to around 0°C. When 5% alcohol was absent, there was a big, transient fluorescence increase at around 0°C, which was totally eliminated by addition of methanol (Fig. 4). This transient fluorescence increase was not observed in freezing-tolerant *N. commune* (data not shown).

This transient increase in fluorescence can be attributed to transient and functional disconnection of allophycocyanin in the phycobilisome because F655 increased transiently in cells without addition of methanol (Fig. 5). In the presence of 5% methanol,

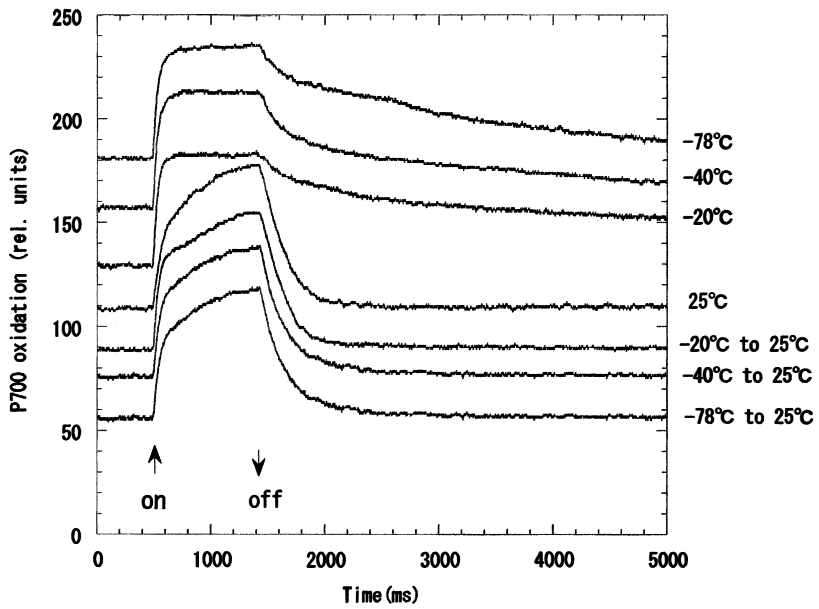

Fig. 6. Light-induced redox changes of P700 in *Synechocystis* PCC6803 in the presence of 5% methanol before, during, and after freezing treatments. A cell suspension containing 10μM DCMU was slowly cooled down to −20, −40, or −78°C, further incubated at the respective temperatures for 5 min, then rewarmed to room temperature. Samples were illuminated for 1 s.

there was a smaller change in fluorescence from phycocyanim (F645). Because a PAM 100/103 fluorometer detects fluorescence only longer than 730 nm, small changes in F 645 could not be observed. This was confirmed by fluorescence spectra at 77K, which further showed that energy transfer from the phycobilisome anchor protein to the PSII core complex was also irreversibly inhibited if 5% methanol was absent (data not shown).

Figure 6 shows time courses of light-induced redox changes of P700 in *Synechocystis* PCC6803 in the presence of 5% methanol. Although dark re-reduction of P700 was slowed down at low temperatures, there remains a rapidly decaying component as seen in freezing-tolerant *N. commune* (Fig. 2A). When the temperature was increased to 25°C, the re-reduction rate increased to the control level both in the presence and absence of 5% methanol (Figs. 2B, 6). However, rapid re-reduction of P700 in the presence of alcohol at low temperature suggests that 5% methanol has the function of keeping not only PBS in functional forms but also PSI complexes active at freezing temperatures. These effects of 5% alcohol seems to be related to its protective effects on freezing injury in freezing-sensitive species.

Concluding remarks

In this paper, it is clearly shown that *N. commune* living in a moderate climate had tolerance to freezing stress, that dehydration of cells occurs during freezing treatments even when we used fully wetted colonies of *N. commune*, and that freezing tolerance is related to drought tolerance in cyanobacteria. The result that cultivation of *N. commune* at 5°C had little effect on the freezing tolerance suggests that there is a new protective mechanism for freezing stress in this cyanobacterium.

The amount of water remaining in a liquid state at freezing temperatures (such as −36°C) insides the cells might decrease if we used not fully wetted samples. However, the light energy absorbed by PSII is mostly quenched at freezing temperatures even when the starting material is fully wetted. It can be easily imagined that, if we use half-dry colonies, the amount of liquid water remaining inside the cells may become much smaller.

The protective effect of 5% methanol against freezing injury in sensitive species seems not only due to lowering of the freezing temperature but due to maintenance of protein complexes in their native forms under freezing conditions. Further work to clarify this mechanism is in progress.

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