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The Action In Vivo of Glycine Betaine in Enhancement of Tolerance of *Synechococcus* sp. Strain PCC 7942 to Low Temperature

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The cyanobacterium *Synechococcus* sp. strain PCC 7942 was transformed with the *codA* gene for choline oxidase from *Arthrobacter globiformis* under the control of a constitutive promoter. This transformation allowed the cyanobacterial cells to accumulate glycine betaine at 60 to 80 mM in the cytoplasm. The transformed cells could grow at 20°C, the temperature at which the growth of control cells was markedly suppressed. Photosynthesis of the transformed cells at 20°C was more tolerant to light than that of the control cells. This was caused by the enhanced ability of the photosynthetic machinery in the transformed cells to recover from low-temperature photoinhibition. In darkness, photosynthesis of the transformed cells was more tolerant to low temperature such as 0 to 10°C than that of the control cells. In parallel with the improvement in the ability of the transformed cells to tolerate low temperature, the lipid phase transition of plasma membranes from the liquid-crystalline state to the gel state shifted toward lower temperatures, although the level of unsaturation of the membrane lipids was unaffected by the transformation. These findings suggest that glycine betaine enhances the tolerance of photosynthesis to low temperature.

Various organisms accumulate compatible solutes in their cells under stressful conditions, such as high salt (12, 17, 26), dehydration (37), and low temperature (15). Among such compatible solutes, glycine betaine (hereafter referred to as betaine) is widely distributed in higher plants (27), animals (7, 16), and bacteria (5). Betaine is a bipolar compound with both positive and negative charges within a single molecule (26). The physiological functions of betaine have not been unequivocally defined, and it has been suggested that betaine protects the cells from salt stress by maintaining osmotic balance with the environment (27) and by stabilizing the quaternary structure of complex proteins (13, 18, 20, 25, 30, 35). For example, betaine prevents the dissociation of ribulose-1,5-bisphosphate carboxylase-oxygenase (13) and the photosystem II complex (24, 25) under high-salt conditions. However, since betaine is not the only compound that is synthesized in cells under salt or dehydration stress, the possibility remains that betaine might not have a direct effect in the protection of cells against such stresses.

To examine whether the effect of betaine in vivo is direct, we established a system for the biosynthesis of betaine in cells of a cyanobacterium, *Synechococcus* sp. strain PCC 7942, by transformation with the *codA* gene for choline oxidase from *Arthrobacter globiformis*, which oxidizes choline to betaine (6). The resultant transformed cells accumulated betaine at an intracellular level of 60 to 80 mM and acquired the ability to tolerate salt stress. Therefore, it appeared that betaine directly protected these cells against salt stress. A similar result was obtained by Nomura et al. (21), such that the transformation of the same cyanobacterial cells with the *bet* gene operon of *Escherichia coli* allowed them to accumulate 45 mM betaine and to tolerate salt stress. We have also transformed *Arabidopsis thaliana* with the *codA* gene. The resultant plants were tolerant of the salt stress (11).

Ko et al. (15) demonstrated that the tolerance of *Listeria monocytogenes* to osmotic and low-temperature stresses was enhanced under osmotic and low-temperature stresses in parallel with the accumulation of betaine in cells. However, so far as we know, there is no analysis for the molecular mechanism in which the accumulated betaine protects the cells from low-temperature stress.

In this study, we examined the effect of synthesis of betaine in vivo on the ability of the transformed cells to tolerate low temperature. The results demonstrated that the biosynthesis of betaine allowed the cyanobacterial cells to tolerate strong light at low temperature by accelerating the recovery of the photosynthetic machinery from the low-temperature photoinhibition.

MATERIALS AND METHODS

Organisms and culture conditions. *Synechococcus* strains PAM (a control strain that had been transformed with the plasmid vector alone) and PAMCOD (a strain that had been transformed with the plasmid vector that included the *codA* gene) were obtained as described previously (6).

Cells were grown, unless otherwise mentioned, at 30°C in BG11 medium (32) supplemented with 1 mM choline chloride (Katayama Chemical, Osaka, Japan) under illumination from incandescent lamps at 70 μE m^{-2} s^{-1}, with aeration by air that contained 1% CO_2. Cells at the exponential phase of growth were used for all experiments. Growth was monitored in terms of turbidity at 730 nm. Cells at a density that corresponded to a chlorophyll concentration of 3 to 4 μg ml^{-1} were used for light treatments. For measurements of the tolerance of photosynthesis to low temperature, the cell density was adjusted to a chlorophyll concentration of 5 to 10 μg ml^{-1}.

Measurement of photosynthetic activity. Photosynthetic oxygen-evolving activity was measured by monitoring the concentration of oxygen at 30°C in BG11 medium that contained 1 mM $NaHCO_3$ with a Clark-type oxygen electrode (Hansatech, Kings Lynn, United Kingdom). The activity of the photosystem II-mediated transport of electrons was measured in a similar way in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. Red actinic light at 2,000 μE m^{-2} s^{-1} was provided from an incandescent lamp after passage through a heat-absorbing optical filter (HA50; Hoya, Tokyo, Japan) and a red optical filter (R-60; Toshiba, Tokyo, Japan).

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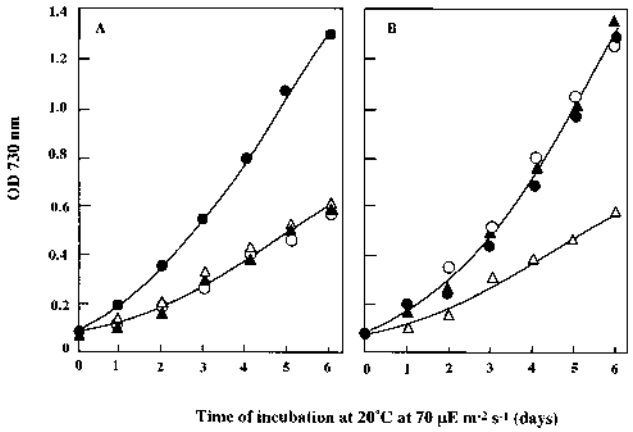

FIG. 1. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on growth at 20°C. (A) Effects of choline chloride. PAM and PAMCOD cells that had been grown at 30°C at 70 μE m^{-2} s^{-1} in the absence of choline chloride were preincubated at 30°C at 70 μE m^{-2} s^{-1} in fresh BG11 medium which did and did not contain 1 mM choline chloride for 24 h. Then, the cells were further incubated at 20°C at 70 μE m^{-2} s^{-1}. △, PAM cells in the absence of choline chloride; ▲, PAM cells in the presence of choline chloride; ○, PAMCOD cells in the absence of choline chloride; ●, PAMCOD cells in the presence of choline chloride. (B) Effect of betaine. PAM and PAMCOD cells were grown at 30°C at 70 μE m^{-2} s^{-1} in the presence of 1 mM choline chloride. Then, the cells were incubated at 20°C at 70 μE m^{-2} s^{-1} with and without supplementation of 3 mM betaine. △, PAM cells in the absence of betaine; ▲, PAM cells in the presence of betaine; ○, PAMCOD cells in the absence of betaine; ●, PAMCOD cells in the presence of betaine. OD, optical density.

Analysis of fatty acid, glycerolipid, and protein compositions. Plasma and thylakoid membranes were isolated from the PAM and PAMCOD cells that were grown at 30°C in the presence of 1 mM choline chloride as described by Omata and Murata (22). Polar lipids were extracted from the plasma and thylakoid membranes by the method of Bligh and Dyer (3). The fatty acid and glycerolipid compositions of membranes were determined as described by Sato and Murata (31). The protein compositions of plasma membranes, thylakoid membranes, and a soluble fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Assessment of the physical phase of membrane lipids. The lipid phase of plasma membranes in intact cells was monitored by noting changes in A_{388} (23, 34). Cells were harvested by centrifugation at 3,000 × *g* for 15 min at 30°C. Pelleted cells were suspended in fresh BG11 medium at a concentration that corresponded to 25 μg of chlorophyll ml^{-1}, and then the suspended cells were incubated at designated temperatures for 60 min. Difference absorption spectra were recorded with a spectrophotometer (model UV-300; Shimadzu Corporation, Kyoto, Japan), and the increase in A_{388} was adopted as a measure of the lipid phase transition.

RESULTS

Growth at low temperature. Figure 1 shows the effects of exogenously supplemented choline chloride and betaine on the growth of PAM and PAMCOD cells during incubation at 20°C. Growth of the PAMCOD cells was remarkably accelerated by the presence of choline chloride (Fig. 1A). By contrast, there was no such effect of the presence of choline on the growth of the PAM cells. The growth profile of the PAMCOD cells in the absence of choline chloride was similar to that of the PAM cells in the presence and absence of choline chloride (Fig. 1A). These results demonstrated that PAMCOD cells could grow better at low temperature than the PAM cells when choline was exogenously supplemented.

Figure 1B shows the effect of exogenously added betaine on the growth of PAM and PAMCOD cells at 20°C. The presence of betaine accelerated the growth of PAM cells, and their growth profile was similar to that of PAMCOD cells in the presence of choline chloride. The presence of betaine had no effect on the growth of PAMCOD cells when choline was present in the culture medium. These observations indicated that the PAMCOD cells incorporated choline into the cells and converted it to betaine by the expressed choline oxidase and that the PAM cells incorporated betaine. They also indicated that the conversion of choline to betaine in the PAMCOD cells enabled these cells to grow well at low temperature.

Inactivation of photosynthesis at low temperatures in the light. To examine the effect of light on the inactivation of photosynthesis at low temperatures, PAM and PAMCOD cells which had been grown at 30°C in the presence of 1 mM choline chloride were incubated at 20°C in the light or in the dark. During the incubation, photosystem II in both cell types remained fully active in darkness. When the cells were illuminated at 500 μE m^{-2} s^{-1} for 120 min, the photosystem II activity of the PAM cells decreased to 35% of the original level (Fig. 2A). Although photosystem II in the PAMCOD cells was also inactivated in the light, the rate of inactivation was much lower than that in the PAM cells (Fig. 2A). These results indicated that photosystem II of the PAMCOD cells was more resistant to photoinhibition than that of the PAM cells.

Photoinhibition of photosystem II results from the competition between the light-induced inactivation of the D1 protein and the restoration of the photosystem II complex by incorporation of newly synthesized D1 protein (1, 2). To determine whether the enhancement of tolerance to light stress at low temperature in PAMCOD cells might be related to suppression of the inactivation or to acceleration of the synthesis of the D1 protein, photoinhibition was induced in the presence of lincomycin, an inhibitor of protein synthesis (Fig. 2B). In darkness, lincomycin did not affect the photosystem II activity of PAM and PAMCOD cells. In the light, the inactivation of the photosystem II complex in both cell types occurred at an identical rate (Fig. 2B). This result suggested that the enhancement of tolerance to light stress at low temperature in the PAMCOD cells was not related to suppression of the inactivation of the D1 protein.

Recovery from photoinhibition. The recovery of the photosystem II complex from photoinhibition was evaluated in the PAM and PAMCOD cells by means of the oxygen-evolving activity. Cells were illuminated with light at 3,500 μE m^{-2} s^{-1} in order to photoinhibit the photosystem II complex to 15% of

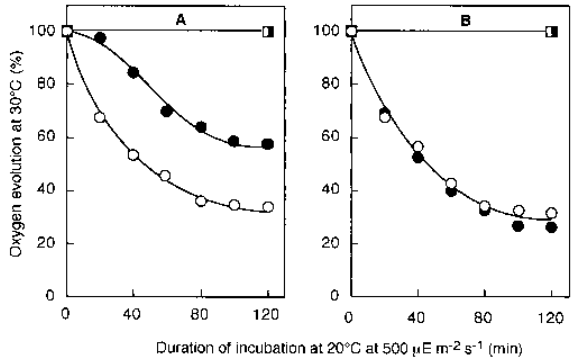

FIG. 2. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on photoinhibition at 20°C of the photosystem II-mediated transport of electrons. Cells were incubated at 500 μE m^{-2} s^{-1} in the absence (A) or in the presence (B) of 400 μg of lincomycin per ml. The evolution of oxygen due to photosystem II-mediated transport of electrons was measured at 30°C at a light intensity of 2,000 μE m^{-2} s^{-1} in the presence of 1 mM 1,4-benzoquinone. Absolute values of maximal activities (100%) in PAM and PAMCOD cells were 708 ± 60 and 698 ± 32 μmol of O_2 per mg of chlorophyll per h, respectively. Values are the averages of results from three independent experiments; deviation was ±8% at most. ○, PAM cells in the light; ●, PAMCOD cells in the light; □, PAM cells in darkness; ■, PAMCOD cells in darkness.

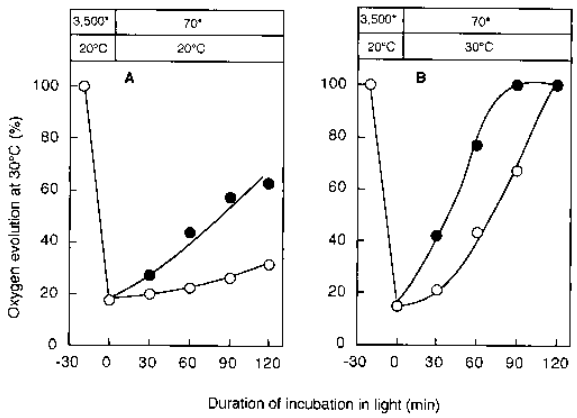

FIG. 3. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on recovery of the photosystem II-mediated transport of electrons from low-temperature photoinhibition. Cells were first incubated in the light at 3,500 μE m^{-2} s^{-1} at 20°C for 20 min, and then they were incubated at 70 μE m^{-2} s^{-1} at 20°C (A) or at 30°C (B). The evolution of oxygen due to photosystem II-mediated transport of electrons was measured as described in the legend to Fig. 2. Absolute values of maximal activities (100%) of photosystem II in PAM and PAMCOD cells were 732 ± 78 and 801 ± 100 μmol of O_2 per mg of chlorophyll per h, respectively. Values are the averages of results from three independent experiments; deviation was ±12% at most. ○, PAM cells; ●, PAMCOD cells. *, 3,500 and 70 μE m^{-2} s^{-1}.

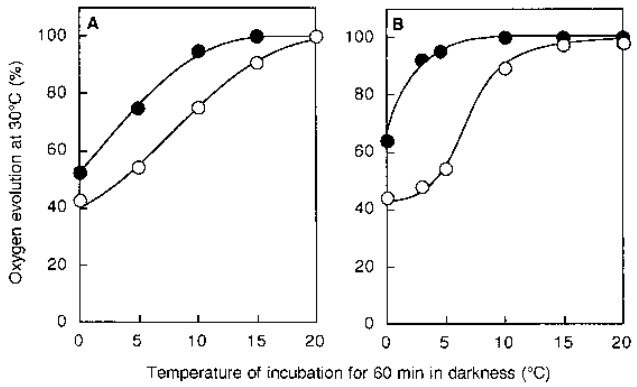

FIG. 4. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on the low temperature-induced inactivation in darkness of the photosynthetic evolution of oxygen. Cells were incubated at designated temperatures for 60 min in darkness and were then incubated at 30°C for 5 min. The photosynthetic oxygen-evolving activity was measured at 30°C at a light intensity of 2.0 mE m^{-2} s^{-1} in the presence of 1 mM $NaHCO_3$ (A) and in the presence of 1 mM 1,4-benzoquinone (B). Absolute values of maximal activities (100%) of the photosynthetic evolution of oxygen in the presence of CO_2 for PAM and PAMCOD cells were 387 ± 23 and 379 ± 19 μmol of O_2 per mg of chlorophyll per h, respectively, and those in the presence of 1,4-benzoquinone were 802 ± 36 and 740 ± 82 μmol of O_2 per mg of chlorophyll per h, respectively. Values are the averages of results from three independent experiments; deviation was ±10% at most. ○, PAM cells; ●, PAMCOD cells.

the original activity. Then, the cells were incubated at 20 or 30°C in light at 70 μE m^{-2} s^{-1} (Fig. 3).

During incubation at 20°C, the recovery of photosystem II complex from photoinhibition occurred to only a minimal extent in PAM cells. By contrast, the photosystem II activity of the PAMCOD cells was restored to 60% of the original level after 120 min (Fig. 3A). During incubation at 30°C, the photosystem II activities of both types of cells were completely restored after a 120-min incubation. However, the rate of recovery in the PAMCOD cells was much higher than that in the PAM cells (Fig. 3B).

Inactivation in darkness of photosynthesis at low temperature. The tolerance of PAM and PAMCOD cells to low-temperature stress was examined by incubating cells in darkness at low temperatures. Figure 4 shows the effects of various low temperatures on the inactivation of net photosynthesis and photosystem II-mediated transport of electrons in PAM and PAMCOD cells. The photosynthetic oxygen-evolving activity of PAMCOD cells was more resistant to low temperature than that of PAM cells (Fig. 4A). Similar results were obtained for the photosystem II-mediated transport of electrons. The activity in PAM cells dropped to 50% of the original level at 5°C, whereas the activity in PAMCOD cells remained at almost the control level at 5°C, starting to decrease only below 5°C (Fig. 4B). These results suggested that photosynthesis in PAMCOD cells was more tolerant to low temperature than that in PAM cells.

Phase transition of plasma membrane lipids. The suppression of growth and photosynthetic activity that occurs when cyanobacterial cells are exposed to low temperatures is caused initially by the transition of the lipid phase of the plasma membrane from a liquid crystalline state to a phase-separated state (19). Therefore, we examined whether the enhancement of tolerance to low temperature of PAMCOD cells was related to changes in the phase behavior of the membrane lipids.

The transition of the lipid phase of plasma membranes can be examined with the aggregation of zeaxanthin by monitoring the changes in A_{388} in cells of *Synechococcus* sp. strains PCC 7942 and PCC 6301 (previously referred to as *Anacystis nidulans*) (4, 8, 19, 23, 34, 36). Figure 5 shows that the phase transition of the membrane lipids of the PAM cells first became apparent at 10°C and ceased at 2°C, with a mid-point temperature at about 6°C. By contrast, the phase transition of membrane lipids of PAMCOD cells began at 5°C. These results demonstrated that the transition of the lipid phase of plasma membranes of the PAMCOD cells occurred at a temperature 5°C lower than that of the PAM cells.

We examined the effect in vitro of 0.1 M and 1 M betaine on the phase transition of plasma membranes that had been isolated from the PAM cells. But no distinct shift of the phase transition temperature was observed. This result might suggest

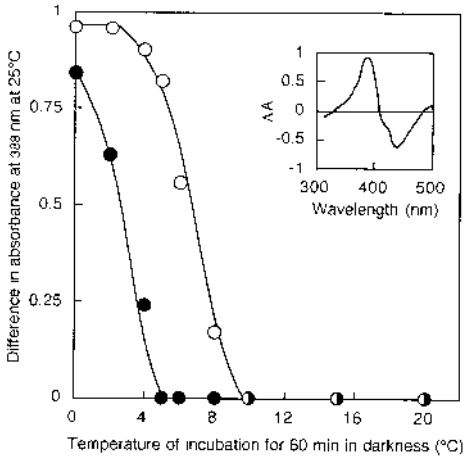

FIG. 5. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on the low temperature-induced transition of the lipid phase of plasma membranes, monitored in terms of changes in A_{388} (23, 34). Cells at a concentration that corresponded to 25 μg of chlorophyll ml^{-1} were incubated at designated temperatures for 60 min in darkness, and then difference absorption spectra of carotenoids at 25°C were recorded. ○, PAM cells; ●, PAMCOD cells. Inset, the difference absorption spectrum at 25°C for PAM cells after incubation at 4°C for 60 min.

TABLE 1. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on the composition of fatty acids[a]

Cell type and membrane	Value for the following fatty acid compositions (mol%):						
	14:0	14:1	16:0	16:1	18:0	18:1(9)	18:1(11)
Plasma membranes							
PAM	1	1	55	36	3	2	2
PAMCOD	2	2	53	37	2	2	2
Thylakoid membranes							
PAM	1	2	52	40	1	2	2
PAMCOD	1	2	50	41	2	2	2

[a] PAM and PAMCOD cells were grown at 30°C in the presence of 1 mM choline chloride. Values are the averages of results from two independent experiments, and the deviations were within ±3% at most. Abbreviations of fatty acids: 14:0, tetradecanoic acid; 14:1, Δ9-tetradecenoic acid; 16:0, hexadecanoic acid; 16:1, Δ9-hexadecenoic acid; 18:0, octadecanoic acid; 18:1(9), Δ9-octadecenoic acid; 18:1(11), Δ11-octadecenoic acid. All of the double bonds were in *cis* configuration.

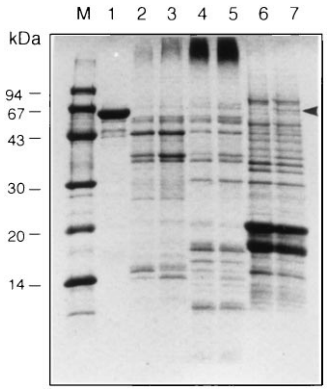

FIG. 6. Effects of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on the composition of proteins in plasma membranes, thylakoid membranes, and a soluble fraction. Lanes: M, molecular mass markers; 1, choline oxidase (purchased from Sigma Chemical Co., St. Louis, Mo.); 2, plasma membranes from PAM cells; 3, plasma membranes from PAMCOD cells; 4, thylakoid membranes from PAM cells; 5, thylakoid membranes from PAMCOD cells; 6, soluble fraction from PAM cells; 7, soluble fraction from PAMCOD cells. Arrow, the expected position of choline oxidase.

that the action of betaine in the system in vivo was not mimicked in the system in vitro.

Changes in membrane lipids and proteins. The phase-transition temperature of membrane lipids in cyanobacterial cells is related to the degree of unsaturation of the fatty acids and the composition of lipid species (19). Therefore, we examined whether the fatty acid and glycerolipid compositions in the membrane lipids of PAMCOD cells were affected by the transformation. Tables 1 and 2 show the compositions of the fatty acids and glycerolipids in the plasma and thylakoid membranes of PAM and PAMCOD cells. There were no significant differences between the two types of cell.

Figure 6 shows the electrophoretic patterns of proteins in membranes and soluble fractions that were prepared from the PAM and PAMCOD cells. There were minor differences in the composition of plasma membrane proteins between the PAM and PAMCOD cells. The level of a 14-kDa protein was increased, whereas the level of a 16-kDa protein was decreased in the plasma membrane of the PAMCOD cells (Fig. 6, lanes 2 and 3). With the exception of a band at 60 kDa that was expected to be choline oxidase in the soluble fraction of PAMCOD cells, the profiles of the soluble proteins from both types of cells were very similar.

DISCUSSION

Effects of choline and betaine on the growth at low temperature. The rate of growth of *Synechococcus* sp. PCC 7942 cells depends on temperature. In the absence of choline in culture medium, the PAM and PAMCOD cells grew slowly at the same rates (Fig. 1A). The presence of choline in culture medium accelerated the growth of the PAMCOD cells but not of the PAM cells (Fig. 1A). These results can be explained by a mechanism in which the exogenous choline was incorporated into the cytoplasm and then converted to betaine by the action of overexpressed choline oxidase (6). By contrast, the presence of betaine in culture medium accelerated the growth of the PAM cells at 20°C (Fig. 1B). It is likely that the exogenous betaine was incorporated into the cytoplasm and functioned to sustain the growth at the low temperature. These observations suggest that the intracellular betaine, which was synthesized from choline in the PAMCOD cells or incorporated from the culture medium in the PAM cells, was effective in sustaining growth of the cyanobacterial cells at 20°C.

TABLE 2. Effect of transformation of *Synechococcus* sp. strain PCC 7942 with the *codA* gene on the glycerolipid compositions of thylakoid and plasma membranes[a]

Lipid class[b]	Value for tylakoid membranes (mol%)		Value for plasma membranes (mol%)	
	PAM	PAMCOD	PAM	PAMCOD
MGDG	54	53	56	55
DGDG	22	23	19	19
SQDG	14	14	15	15
PG	10	10	10	11

[a] Membranes were from PAM and PAMCOD cells that were grown at 30°C in the presence of 1 mM choline chloride. Values are the averages of the results of two independent experiments, and the deviations were within ±2% at most.

[b] MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol.

Biochemical characteristics of the transformed cells. PAMCOD cells accumulated intracellular betaine at 60 to 80 mM, whereas PAM cells did not accumulate betaine (6). The biochemical analyses in the present study demonstrated that transformation with the *codA* gene did not affect the fatty acid unsaturation of membrane glycerolipids and the lipid species composition (Tables 1 and 2). Moreover, the transformation did not significantly modify the protein composition of the plasma and thylakoid membranes (Fig. 6).

Changes in tolerance of low temperature. The effect of transformation with the *codA* gene on the ability of the photosynthetic machinery to tolerate low temperature can be summarized as follows: (i) the transformation enhanced growth of the cells at 20°C; (ii) the transformation enhanced tolerance of low-temperature stress in the light (photoinhibition) at 20°C; and (iii) the transformation enhanced tolerance of low-temperature stress in the dark at temperatures from 0 to 10°C.

Tolerance of low temperature in the light. The extent of photoinhibition in vivo results from the balance between two processes, the initial damage to the D1 protein and the subsequent repair of the photosystem II complex by incorporation of newly synthesized D1 protein (1, 2). Photoinhibition of photosynthesis in vivo is more noticeable at low temperatures than at normal growth temperatures because the recovery from photoinhibition is markedly depressed at low temperatures (9, 10,

14, 33). Therefore, the rate of recovery determines the sensitivity to low-temperature stress.

The results of the present study demonstrated that the photosystem II complex of PAMCOD cells was more tolerant of photoinhibition than that of PAM cells (Fig. 2A). The presence of lincomycin reduced the low-temperature tolerance of photosystem II in PAMCOD cells (Fig. 2B), suggesting that it is the recovery process that is accelerated in the latter cells. Direct measurements of the restoration of photosystem II activity (Fig. 3) supported this suggestion. Our findings indicate that the depression of growth of PAM cells at 20°C was caused initially by low-temperature photoinhibition and that betaine, accumulated in the cytoplasm, sustained the growth of PAMCOD cells at 20°C by accelerating the recovery process.

Tolerance of low temperature in the dark. We showed previously that the inactivation of photosynthesis at low temperatures in the dark in *Synechococcus* sp. strain PCC 6301 (previously termed *A. nidulans*) is caused by the transition of the lipid phase of plasma membranes from the liquid crystalline state to the phase-separated state (19). Since changes in the absorption spectra of carotenoids in intact cells are a good indicator of changes in the lipid phase in *Synechococcus* (4, 8, 19, 23, 34, 36), we were able to determine the profiles of temperature dependence of the lipid phase of the plasma membranes in PAM and PAMCOD cells. The results demonstrated that the phase transition of the plasma membranes of PAMCOD cells was shifted toward lower temperatures, compared with that of PAM cells (Fig. 5). Since transformation with the *codA* gene did not alter the fatty acid unsaturation, the glycerolipid composition, or the major protein components of PAMCOD cells (Tables 1 and 2; Fig. 6), it is likely that the enhancement of the low-temperature tolerance of the photosynthetic machinery in PAMCOD cells was related to the action in vivo of the accumulated betaine. However, betaine at a concentration which corresponded to that in PAMCOD cells (6) had no effect on the phase transition temperature of the isolated plasma membranes. In contrast, it has been demonstrated with artificial membrane systems that 1 M betaine decreases the temperature of the phase transition from the liquid crystalline state to the gel state (28, 29). Thus, the question remains as to whether the presence of betaine in the cytoplasm enhanced the tolerance of cyanobacterial cells to low temperature in darkness by decreasing the temperature for the phase transition.

ACKNOWLEDGMENTS

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