Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 1363–1371
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbabio

Glycinebetaine alleviates the inhibitory effect of moderate heat stress on the repair of photosystem II during photoinhibition

Suleyman I. Allakhverdiev [a,b], Dmitry A. Los [a,c], Prasanna Mohanty [a,d], Yoshitaka Nishiyama [a,e], Norio Murata [a,*]

[a] National Institute for Basic Biology, Okazaki 444-8585, Japan
[b] Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia
[c] Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia
[d] Regional Plant Resource Centre, Bhubaneswar, and Jawaharlal Nehru University, New Delhi, India
[e] Cell-Free Science and Technology Research Center, Ehime University, Bunkyo-cho, Matsuyama 790-8577, Japan

Received 25 June 2007; received in revised form 2 October 2007; accepted 15 October 2007
Available online 22 October 2007

Abstract

Transformation with the bacterial gene *codA* for choline oxidase allows *Synechococcus* sp. PCC 7942 cells to accumulate glycinebetaine when choline is supplemented exogenously. First, we observed two types of protective effect of glycinebetaine against heat-induced inactivation of photosystem II (PSII) in darkness; the *codA* transgene shifted the temperature range of inactivation of the oxygen-evolving complex from 40–52 °C (with half inactivation at 46 °C) to 46–60 °C (with half inactivation at 54 °C) and that of the photochemical reaction center from 44–55 °C (with half inactivation at 51 °C) to 52–63 °C (with half inactivation at 58 °C). However, in light, PSII was more sensitive to heat stress; when moderate heat stress, such as 40 °C, was combined with light stress, PSII was rapidly inactivated, although these stresses, when applied separately, did not inactivate either the oxygen-evolving complex or the photochemical reaction center. Further our studies demonstrated that the moderate heat stress inhibited the repair of PSII during photoinhibition at the site of synthesis *de novo* of the D1 protein but did not accelerate the photodamage directly. The *codA* transgene and, thus, the accumulation of glycinebetaine alleviated such an inhibitory effect of moderate heat stress on the repair of PSII by accelerating the synthesis of the D1 protein. We propose a hypothetical scheme for the cyanobacterial photosynthesis that moderate heat stress inhibits the translation machinery and glycinebetaine protects it against the heat-induced inactivation.

Keywords: *codA* gene; Glycinebetaine; Heat stress; Photoinhibition; Photosystem II; Protein synthesis; Repair

1. Introduction

Heat stress is one of the main environmental factors that limits the growth and productivity of plants [1,2]. It seems likely that photosynthesis is most sensitive to such heat stress among various physiological processes [3]. Inhibition of photosynthesis under heat stress is common to plants in tropical and subtropical regions and the temperature zones where plants are exposed periodically to high temperatures [4]. Among various machineries of photosynthesis, the photosystem II complex (PSII) is particularly sensitive to heat, and even a short period of exposure to high temperatures irreversibly inactivates the oxygen-evolving complex of PSII [5,6]. The photochemical reaction center of PSII is also inactivated by heat, but is less sensitive to heat than the oxygen-evolving complex [5,7].

We have shown that photoinhibition of PSII is stimulated by abiotic stress, such as low-temperature [8], high-salt [9–11], and oxidative stress [12,13]. The CO_2 limitation stress also enhanced the extent of photoinhibition in PSII [14,15]. Since the extent of photoinhibition is a result of balance between the rate of light-induced inactivation (photodamage) and the rate of repair, we investigated the effect of abiotic stress on the rates of photodamage and repair separately. The results demonstrate that abiotic stress, such as cold [8,16,17], salt [11] and oxidative

Abbreviations: BQ, 1,4-benzoquinone; Chl, chlorophyll; PAM, Synechococcus sp. PCC 7942 transformed with the empty vector (control); PAMCOD, *Synechococcus* sp. PCC 7942 transformed with a vector that contains the *codA* gene

* Corresponding author. Fax: +81 557 85 5205.
E-mail address: murata@nibb.ac.jp (N. Murata).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2007.10.005

[12,13], inhibits the repair of PSII, but does not affect the photodamage to PSII [18,19]. The step that is affected by abiotic stress is the translation of *psbA* mRNA [11–13].

Glycinebetaine (*N*,*N*,*N*-trimethylglycine; hereafter betaine) is accumulated in a variety of plants and microorganisms under abiotic stress, such as low temperature, salinity and drought [20–23]. We previously transformed the cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter, *Synechococcus*) with the *codA* gene for choline oxidase from *Arthrobacter globiformis*, which synthesizes betaine from choline [24,25]. Transformed cells, designated as PAMCOD [25] synthesized betaine *in vivo* from exogenously supplied choline and accumulated betaine at levels of 60 to 80 mM in the cytoplasm. We further explored that the accumulation of betaine protected PSII against photoinhibition under low-temperature stress and salt stress [24,25]. A recent study demonstrated that betaine counteracted the inhibitory effect of salt stress on the translation of *psbA* mRNA [26].

A similar system was developed in higher plants by genetic engineering with the *codA* gene for biosynthesis of betaine in *Arabidopsis thaliana* and we observed that the resultant transgenic plants accumulated betaine in leaves at levels of about 1 mM [27]. We have shown that the transformation enhanced the tolerance to moderate heat stress during germination of seeds and growth of plants [28,29]. Transformation with cDNA for betaine aldehyde dehydrogenase also allowed *Nicotiana tabacum* plants to synthesize betaine and enhanced the tolerance to moderate heat stress [30,31].

In the present study, we demonstrated, using PAMCOD cells of *Synechococcus,* that moderate heat stress inhibited the synthesis of the D1 protein of PSII and that the accumulation *in vivo* of betaine counteracted the inhibitory effects of moderate heat stress in the synthesis of the D1 protein. In addition, the betaine accumulation protected the oxygen-evolving complex and the photochemical reaction center when *Synechococcus* cells were incubated at high temperatures in darkness.

2. Materials and methods

2.1. Organisms and culture conditions

In brief, *Synechococcus* sp. PCC 7942 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 for choline oxidase (X84895)) were obtained as described previously [24]. Cells were grown photoautotrophically as described previously in glass tubes (25 mm i.d × 200 mm) at 30 °C under constant illumination from incandescent lamps at 70 μmol photons $m^{-2}s^{-1}$ in BG-11 medium [32] supplemented with 20 mM HEPES–NaOH (pH 7.5) in the presence of 1.0 mM choline chloride. Cultures were aerated with sterile air that contained 1% CO2 [33].

2.2. Exposure of cells to heat and light stress

Cells from 3-day-old cultures were harvested by centrifugation at 8000×*g* for 10 min at 30 °C and resuspended in fresh BG-11 medium at a Chl concentration of 5 μg ml^{-1} [10,34]. The resultant suspensions of cells were incubated at 30 °C for 1 h in 100-ml glass tubes in growth chambers under conditions identical to the original culture conditions. When photoinhibition was induced, cells were exposed to light from incandescent lamps at 500 or 1,000 μmol photons m^{-2} s^{-1}. In some experiments, protein synthesis was blocked by inclusion in the medium of 250 μg ml^{-1} lincomycin (Sigma Chemical Co., St. Louis, MO), which was added to the culture medium 10 min before the start of incubation. Heat treatment was carried out at different temperatures in 100-ml glass tubes in growth chambers under normal growth conditions.

2.3. Measurement of photosynthetic activity

We measured the activity of PSII in intact cells by monitoring the oxygen-evolving activity at 30 °C with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, U.K.) in the presence of 1.0 mM 1,4-benzoquinone (BQ) as the electron acceptor and an inhibitor of respiration [33,35]. The sample, in a 3-ml cuvette, was illuminated by light that had been passed through a red optical filter (R-60; Toshiba, Tokyo, Japan) and an infrared-absorbing filter (HA-50; Hoya Glass, Tokyo, Japan). The intensity of light at the surface of the cuvette was 2000 μmol photons m^{-2} s^{-1}.

2.4. Kinetics of changes in the fluorescence of Chl a

The light-induced quenching of the fluorescence of Chl *a* due to reduction of pheophytin [36–38] in intact cells was monitored with a fluorometer (PAM-101; Heinz Walz, Effeltrich, Germany) in the pulse-amplitude modulation mode. The light-induced quenching of Chl fluorescence was measured at 30 °C in the presence of 1 mg ml^{-1} sodium dithionite after the continuous exposure of the sample to actinic light ($\lambda > 520$ nm) from an incandescent lamp (KL-1500 Electronic; Schott Glasswerke, Wiesbaden, Germany) at 2700 μmol photons m^{-2} s^{-1}. The concentration of Chl was determined as described by Arnon et al. [39]. Other experimental details are given in the legends to figures.

2.5. Isolation of thylakoid membranes and immunoblotting analysis of the D1 protein

Thylakoid membranes were isolated from PAM and PAMCOD cells, as described previously [10,34]. Thylakoid membranes were solubilized by incubation for 5 min at 65 °C in 60 mM Tris buffer (pH adjusted to 6.8 with HCl) that contained 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol, and then proteins were separated by polyacrylamide gel electrophoresis [12.5% (w/v) polyacrylamide] in the presence of 0.08% (w/v) SDS and 6 M urea, as described previously [11]. Molecular markers (Kaleidoscope pre-stained standards; Bio-Rad Laboratories, Hercules, CA) were used for estimations of the molecular masses of immunoreactive proteins. After electrophoresis, the separated proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) in a semi-dry transfer apparatus (Atto, Tokyo, Japan). Then, the D1 protein was detected immunologically with an ECL Western blotting kit according to the protocol supplied with the kit (Amersham International, Buckinghamshire, UK).

The total D1 protein was detected with antibodies raised in rabbits against a synthetic oligopeptide that corresponded to the DE loop of the D1 protein (amino acids 234–242, counted from the amino terminus of the D1 protein from spinach). These antibodies recognize the products (D1) of the *psbAI*, *psbAII* and *psbAIII* genes because the amino acid sequence of the AB loop is exactly the same in the products of these genes. As second antibodies, we used horseradish peroxidase-linked antibodies raised in donkeys against rabbit immunoglobulin G (Amersham International).

The antibodies raised in rabbit against D1 were kindly provided by Prof. Kimiyuki Satoh (Department of Biology, Okayama University, Okayama, Japan). A digital camera system (LAS-1000; Fuji Photo Film Co., Tokyo, Japan) was used to monitor signals from blotted membranes and to quantify the D1 protein.

2.6. Northern blotting analysis

Total RNA was isolated from cells and Northern-blotting analysis was performed as described previously [11,40]. Equal amounts of RNA (4 μg) from each sample were loaded in individual wells of the gel and rRNA was visualized by staining with ethidium bromide. Hybridizations were performed at 55 °C according to standard procedures. Two oligonucleotides of 66 bp, one corresponding to the untranslated sequence of the *psbAI* gene for D1:1 and the other to the similar untranslated sequences of *psbAII* and *psbAIII* genes for D1:2, were used as transcript-specific probes for *psbAI* and *psbAII–psbAIII*

mRNAs, respectively [41]. An internal gene probe (BstEII fragment of *psbAI*) was used to detect all three types of *psbA* mRNAs [41]. After hybridization, blots were soaked in CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNAs were detected with the digital camera system.

3. Results

3.1. Glycinebetaine alleviated the heat-induced depression of growth

To examine the effect of the *codA* transgene on the growth of *Synechococcus*, we compared proliferation of PAM and PAMCOD cells at a physiological temperature, namely, 30 °C, and at moderately high temperatures, such as 38 °C, 40 °C, 42 °C, 44 °C and 46 °C (Fig. 1). At 30 °C, both types of cell grew well at similar rates (Fig. 1A). At 38 °C, the growth rate was slightly lower than that at 30 °C, and PAMCOD cells grew faster than PAM cells (Fig. 1B). At 40 °C, the growth rate markedly decreased as compared with that at 30 °C and this inhibitory effect was less significant in PAMCOD cells than in PAM cells (Fig. 1C). At 42 °C, the growth of cells was slower than that at 40 °C in each type of cell and PAMCOD cells grew better than PAM cells (Fig. 1D). When the temperature was raised to 44 °C and 46 °C, the PAMCOD cells grew slowly after

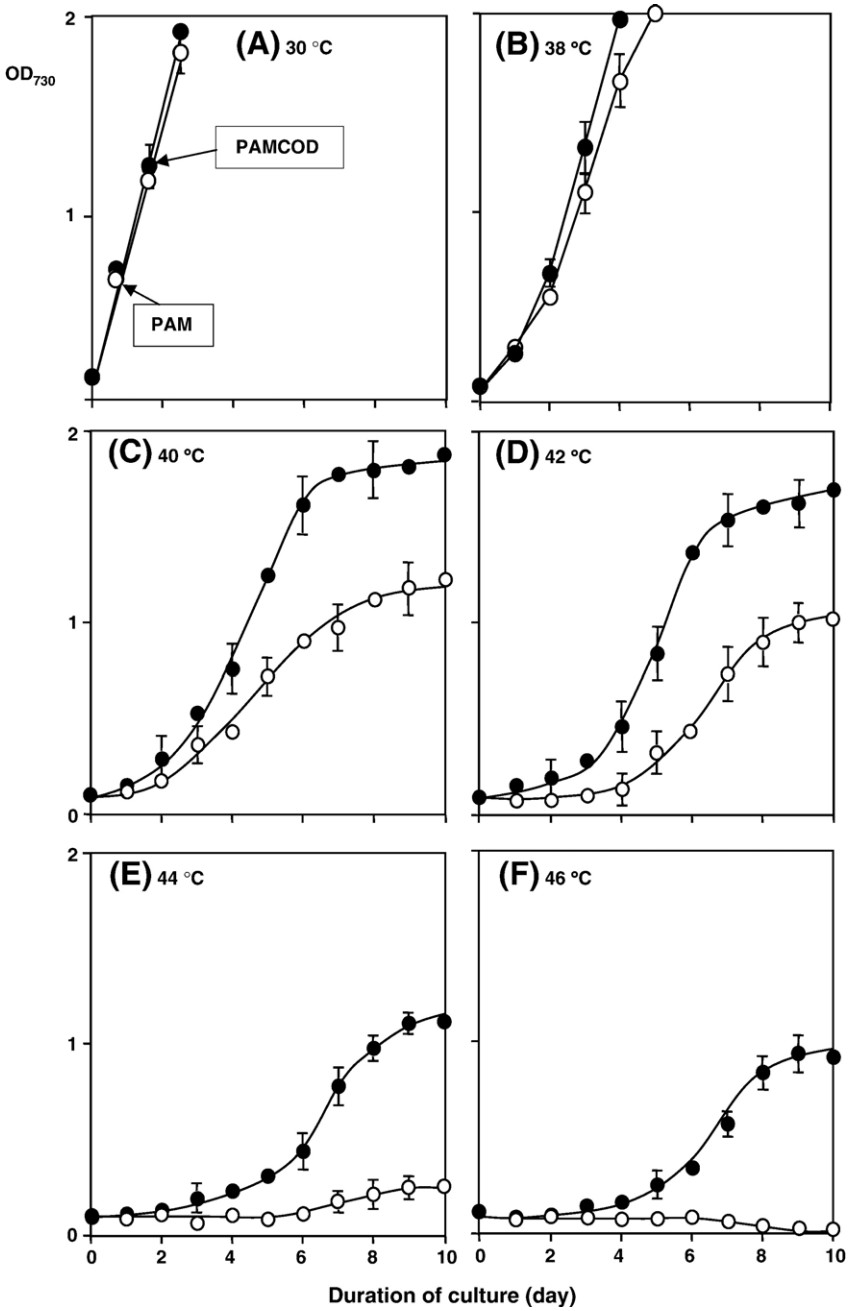

Fig. 1. Effects of various temperatures on growth of PAM and PAMCOD cells of *Synechococcus* sp. PCC 7942. Cells were grown in BG-11 medium supplemented with 1 mM choline chloride at 30 °C (A), 38 °C (B), 40 °C (C), at 42 °C (D), at 44 °C (E) and at 46 °C (F). Optical density at 730 nm was monitored at intervals of 24 h. Each point and bar represents the average ± SE of results from four independent experiments.

a significant lag period of 4 days, whereas PAM cells grew only marginally at 44 °C (Fig. 1E) but failed to grow totally at 46 °C (Fig. 1F).

These results suggested that the *codA* transgene and thus the accumulation of betaine in *Synechococcus* cells alleviated the inhibitory effect of heat stress on growth, being consistent to our previous observation that the *codA* transgene in *A. thaliana* supported the growth at moderately high temperatures [28]. In the following, we attempted to investigate the mechanism for the action of betaine in protecting *Synechococcus* cells at high temperatures.

3.2. Glycinebetaine protected the oxygen-evolving complex of photosystem II against moderate heat stress

We examined the effect of moderate heat stress on the stability of PSII in PAM and PAMCOD cells. Fig. 2 shows the effect of incubation of these cells for 30 min at various high temperatures on the oxygen-evolving activity of PSII. In PAM cells, the activity started to decline at 40 °C and it disappeared at 52 °C with a half inactivation temperature of 46 °C. In PAMCOD cells, the activity began to decline at 46 °C and was completely lost at 60 °C with a half-inactivation temperature at 54 °C. This result demonstrated that the accumulation of betaine in PAMCOD cells protected the oxygen-evolving complex from heat-induced inactivation.

3.3. Glycinebetaine protects the photochemical reaction center of photosystem II against moderate heat stress

The activity of photochemical reaction centers of PSII can be examined by monitoring the light-dependent quenching of chlorophyll fluorescence due to the reduction of pheophytin in the presence of dithionite [36,37]. We applied this method to examine the sensitivity of the photochemical reaction centers of PSII in PAM and PAMCOD cells. Fig. 3 shows that the ability of the light-induced quenching of chlorophyll fluorescence in PAM and PAMCOD cells decreased at moderately high temperatures. The activity started to decline at 44 °C and disappeared at 55 °C with half inactivation at 51 °C in PAM cells, whereas it started to decline at 52 °C with half inactivation at 58 °C in PAMCOD cells (Fig. 3B). These results suggested that the presence of betaine in PAMCOD cells protected the photochemical reaction center of PSII and also that the photochemical reaction center was more resistant to heat stress than the oxygen-evolving complex in PSII. These results are consistent with our previous observations

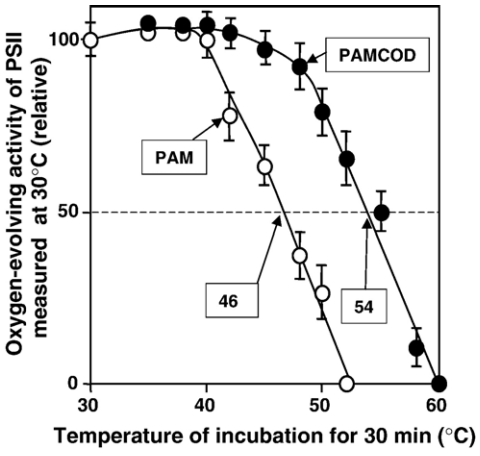

Fig. 2. Effects of incubation of PAM and PAMCOD cells of *Synechococcus* sp. PCC 7942 at various high temperatures on the oxygen-evolving activity of PSII. Cells suspension (5 μg Chl ml^{-1}) were incubated in fresh BG-11 medium at designated temperature for 30 min in light at 70 μmol photons m^{-2} s^{-1} and then the oxygen-evolving activity of cells was measured at 30 °C with 1 mM BQ as the artificial acceptor. The oxygen-evolving activities of PAMCOD and PAM cells that corresponded to 100% were 613±32 and 610±36 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Each point and bar represents the average±SE of results from four independent experiments.

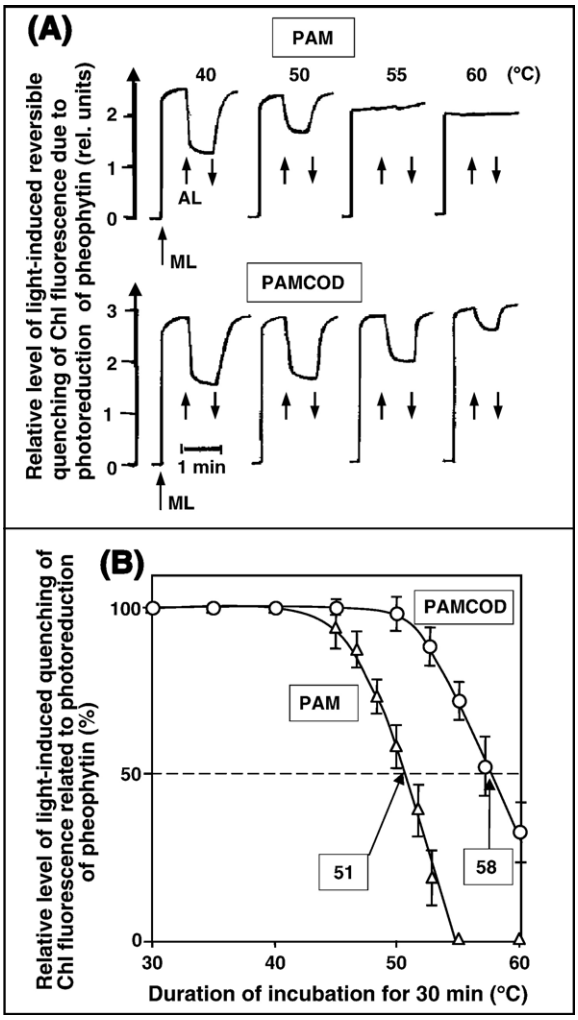

Fig. 3. Effects of incubation of PAM and PAMCOD cells of *Synechococcus* sp. PCC 7942 at various high temperatures on the light-induced quenching of Chl *a* fluorescence. Cells were suspended at a density of 5 μg Chl ml^{-1} and were incubated in fresh BG-11 medium at designated temperature for 30 min in light at 70 μmol photons m^{-2} s^{-1} and then the light-dependent quenching of Chl *a* fluorescence due to reduction of pheophytin of PSII reaction centers was measured after addition of 1 mg ml^{-1} sodium dithionite at 30 °C with strong actinic light (2,700 μmol photons m^{-2} s^{-1}). The relative level of light-induced quenching of Chl fluorescence related to reduction of pheophytin in PAMCOD and PAM cells at 40 °C corresponded to 100%. Each point and bar represents the average±SE of results from five independent experiments. (A) Kinetics of light-induced quenching of Chl fluorescence; (B) dependence on temperature of light-induced quenching of Chl fluorescence.

that betaine enhanced the stability of photochemical performance of the reaction center in thylakoid membranes [42], in PSII core complex [7], and in D1/D2/Cytb559 complexes [43].

3.4. Moderate heat stress enhanced the extent of photoinhibition of photosystem II by inhibiting the repair

We investigated the effect of moderately high temperature, such as 40 °C, on the photoinhibition of PSII in PAM cells (Fig. 4A). As described above, incubation of PAM cells at 40 °C in darkness had no effect on the oxygen-evolving activity. However, when PAM cells were exposed to strong light at 500 μmol photons m^{-2} s^{-1} at 40 °C, the PSII activity declined very rapidly (Fig. 4A). However, at 30 °C, such light stress did not affect the PSII activity (data not shown). These observations suggested that heat stress appeared to accelerate the photoinhibition in PAM cells.

Since the extent of photoinhibition is the balance between the rate of light-induced damage to PSII and the rate of repair of PSII [44–46], the acceleration of photoinhibition at 40 °C could be caused by stimulation of the light-induced inactivation and/or inhibition of repair. Thus, we compared the light-induced inactivation of PSII at 30 °C and 40 °C in the presence of lincomycin, which inhibits the repair of PSII. The result demonstrated that the light-induced inactivation was unaffected by the moderate heat stress (data not shown), suggesting that the repair might have been inhibited by the heat stress.

The repair of PSII can be examined when photoinhibited cells are transferred to normal growth conditions, namely, under weak light. In this experiment, we exposed PAM cells to strong light of 1,000 μmol photons m^{-2} s^{-1} to decrease the PSII activity to approximately 10% of the original and then followed the repair of PSII at 30 °C and 40 °C in light at 70 μmol photons m^{-2} s^{-1}. Fig. 4C shows that the repair at 40 °C was much slower than that

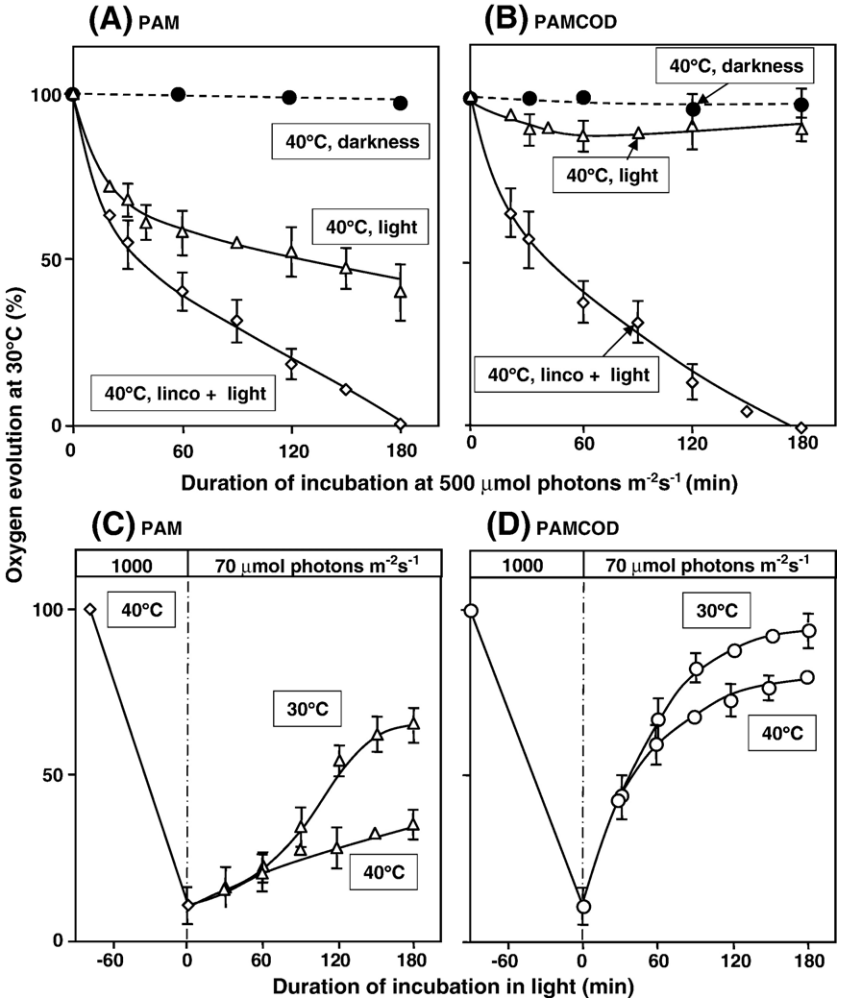

Fig. 4. Photoinhibition at 40 °C and repair at 30 °C of photosystem II in PAM and PAMCOD cells. Cells were grown at 30 °C for 3 days in light at 70 μmol photons m^{-2} s^{-1} in BG-11 medium supplemented with 1 mM choline chloride plus 30 μg ml^{-1} spectinomycin. Then the cells were harvested by centrifugation and resuspended in fresh BG-11 at a density of 5 μg Chl ml^{-1} and incubated in darkness or in light at 500 μmol photons m^{-2} s^{-1} at 40°C in the presence of 250 μg ml^{-1} lincomycin or in its absence, or incubated in light at 1,000 μmol photons m^{-2} s^{-1} for approximately 80–90 min to decrease the PSII activity to 10% of the original level and then at 70 μmol photons m^{-2} s^{-1}. At designated times, aliquots were withdrawn and oxygen-evolving activity was measured after addition of 1 mM BQ to the suspension. The oxygen-evolving activity that corresponded to 100% was 598±32 μmol O_2 mg^{-1} Chl h^{-1}. (A) PAM cells at 500 μmol photons m^{-2} s^{-1}. (B) PAMCOD cells at 500 μmol photons m^{-2} s^{-1}. (C) PAM cells at 1,000 μmol photons m^{-2} s^{-1} and then 70 μmol photons m^{-2} s^{-1}. (D) PAMCOD cells at 1000 μmol photons m^{-2} s^{-1} and then 70 μmol photons m^{-2} s^{-1}. Each point and bar represents the average±SE of results from five independent experiments.

at 30 °C. These observations indicated that the enhancement of photoinhibition at 40 °C was due to the inhibition of repair in PSII.

3.5. Glycinebetaine alleviated the inhibitory effect of moderate heat stress on photoinhibition

Exposure of PAMCOD cells to light at 500 μmol photons m^{-2} s^{-1} at 40 °C did not induce the photoinhibition of PSII (Fig. 4B). However, in the presence of 250 μg ml^{-1} lincomycin, the PSII activity decreased as in PAM cells (Fig. 4A and B). These results suggested that the rate of the light-induced inactivation of PSII was unaffected by the *codA* transgene, namely, the accumulation of betaine.

The repair of PSII in PAMCOD cells was monitored during exposure of PAMCOD cells to light at 70 μmol photons m^{-2} s^{-1} after photoinhibition in light of 500 μmol photons m^{-2} s^{-1}. The rate of recovery was much faster than in PAM cells (Fig. 4C and D) and that, in PAMCOD cells, the rate of repair at 40 °C was similar to that at 30 °C. These observations suggest that the presence of betaine in PAMCOD cells counteracts the effect of heat stress in inhibiting the repair of PSII.

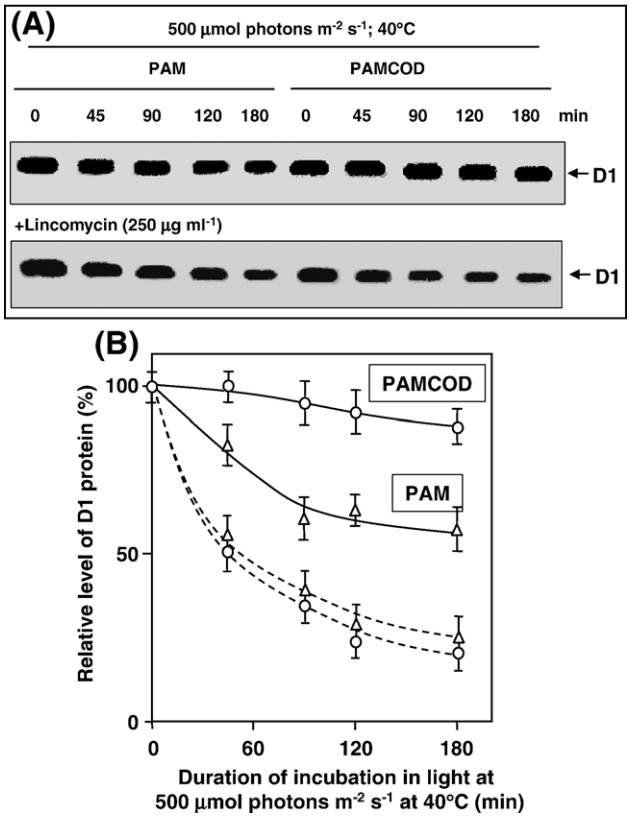

Fig. 5. Effect of photoinhibition of photosystem II in PAM and PAMCOD cells with light at 500 μmol photons m^{-2} s^{-1} at 40 °C on the level of the D1 protein. Cells were grown at 30 °C for 3 days in light at 70 μmol photons m^{-2} s^{-1} in BG-11 medium supplemented with 1 mM choline chloride plus 30 μg ml^{-1} spectinomycin. Then the cells were harvested by centrifugation and resuspended in fresh BG-11 at a density of 5 μg Chl ml^{-1} in light at 500 μmol photons m^{-2} s^{-1} at 40 °C. At designated times, aliquots were withdrawn and thylakoid membranes were isolated from cells and subjected to Western blotting analysis. (A) Results of Western blotting in the absence and in the presence of 250 μg ml^{-1} lincomycin. (B) Quantification of the results shown in (A), in the absence (solid lines) and in the presence of lincomycin (dashed lines). Values are means± SE (bars) of results from three independent experiments.

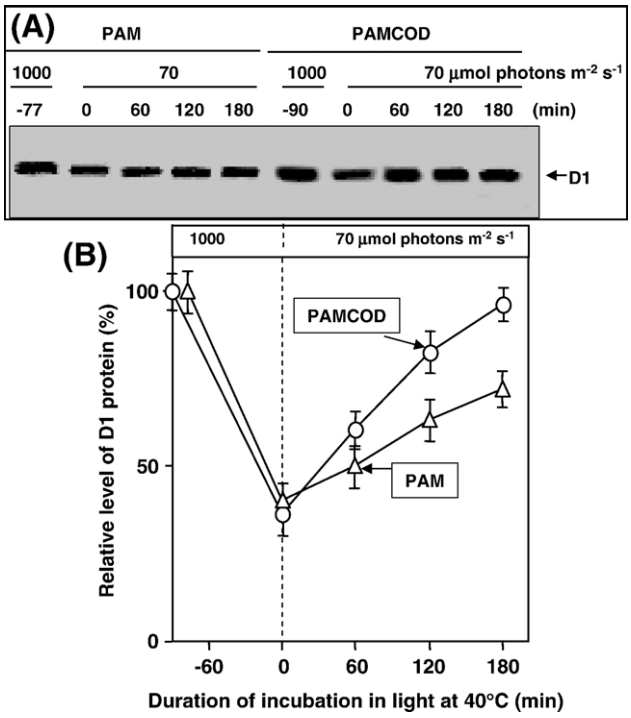

Fig. 6. Effect of photoinhibition of PSII in PAM and PAMCOD cells with light at 1000 μmol photons m^{-2} s^{-1} at 40 °C and recovery at 70 μmol photons m^{-2} s^{-1} at 40 °C in the level of the D1 protein. All other details are as in Figs. 4 and 5. (A) Results of Western blotting. (B) Quantification of the results shown in (A). Values are means±SE of results from four independent experiments.

3.6. Moderate heat stress inhibited the synthesis of the D1 protein

We also monitored the level of the D1 protein during incubation of PAM and PAMCOD cells in light at 500 μmol photons m^{-2} s^{-1} at 40 °C by Western blotting (Fig. 5A). When PAM cells were incubated in light, the level of the total D1 protein decreased gradually to less than 60% of the original level during incubation for 180 min whereas, in PAMCOD cells, the light-dependent decrease in the level of D1 was only 10% (Fig. 5B). These results suggested that degradation of the D1 protein in photodamaged PSII was much faster in PAM cells. However, in the presence of 250 μg ml^{-1} lincomycin, the light-dependent decrease in the level of D1 protein was completely similar in both types of cell (Fig. 5B, dashed lines), suggesting that the rate of degradation of the D1 protein was unaffected in PAMCOD cells and that the synthesis *de novo* of the D1 protein was faster in PAMCOD cells than in PAM cells.

To monitor directly the effect of the *codA* transgene on the recovery of PSII activity at high temperatures, PAM and PAMCOD cells were exposed to 1000 μmol photons m^{-2} s^{-1} at 40 °C to decrease the level of the D1 protein to approximately 40% of the original level and then to 70 μmol photons m^{-2} s^{-1} (Fig. 6). During subsequent repair in the weak light, the level of

D1 returned to the original level, reflecting the repair of PSII. However, the rate of recovery of the D1 protein was markedly faster in PAMCOD cells than in PAM cells (Fig. 6B). These observations clearly demonstrated that the presence of betaine in PAMCOD cells counteracted the effect of moderate heat stress on inhibition of the D1 protein synthesis.

3.7. Accumulation of glycinebetaine did not affect the level of *psbA* transcripts

To determine whether moderate heat stress inhibits the synthesis of the D1 protein at the transcriptional level, we monitored the changes in the level of *psbA* transcripts by Northern blotting. Transfer of PAM and PAMCOD cells from light at 70 μmol photons m^{-2} s^{-1} to 1000 μmol photons m^{-2} s^{-1} caused the rapid accumulation of *psbA* transcripts in both types of cell (Fig. 7). During subsequent exposure of cells to light at 70 μmol photons m^{-2} s^{-1}, the level of *psbA* transcripts decreased similarly in both types of cell (Fig. 7). These results revealed that changes in the level of *psbA* transcripts was similar in the two types of cell, suggesting that the site of regulation for the synthesis of the D1 protein is not the transcription of the *psbA* genes and/or the stability of the *psbA* transcripts.

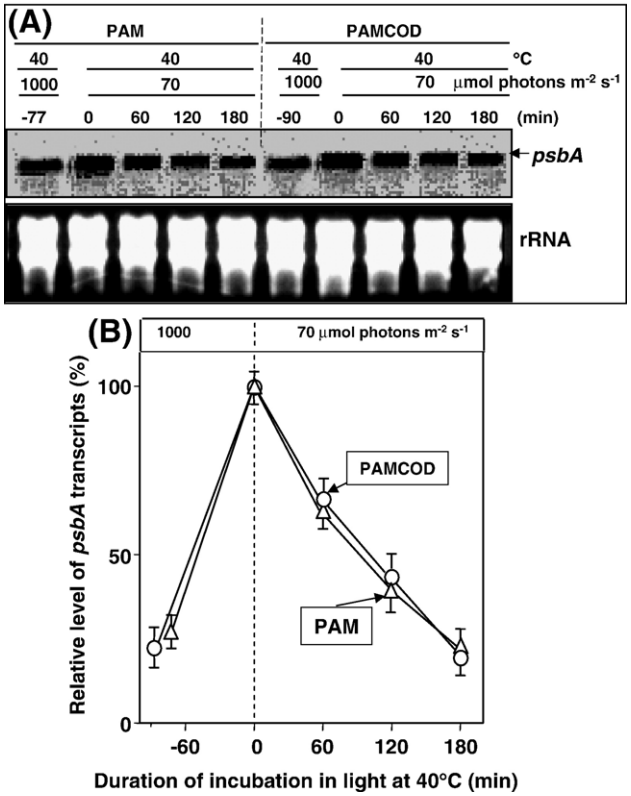

Fig. 7. Effect of photoinhibition of PSII in PAM and PAMCOD cells with light at 1000 μmol photons m^{-2} s^{-1} at 40 °C and recovery at 70 μmol photons m^{-2} s^{-1} at 40 °C on the level of *psbA* transcripts. All other details are as in Figs. 4 and 5. (A) Results of Northern blotting. (B) Quantification of the results shown in (A). Values are means ± SE of results from four independent experiments.

4. Discussion

4.1. Glycinebetaine enhanced the growth of Synechococcus cells under moderate heat stress

In a previous study, we estimated that the level of betaine in the cytoplasm of PAMCOD cells was 60–80 mM [24]. Although this concentration was much lower than that (namely, 1.0 M) used in studies *in vitro* on stabilization of the oxygen-evolving complex of PSII [47] or on the protection of the oxygen-evolving complex against high concentrations of NaCl in thylakoid membranes isolated from spinach [see 48], such a low level of betaine in PAMCOD cells was very effective in supporting the growth in a temperature range of 40–50 °C (Fig. 1). Similar protective effects of betaine at low levels on growth at moderately high temperatures were observed in betaine-synthesizing transgenic plants of *Arabidopsis* [29] and tobacco [30].

4.2. Glycinebetaine protects photosystem II against moderate heat stress in light and darkness

The photochemical reaction center of PSII was relatively resistant to high temperature. The activity of the photochemical reaction center, which was monitored by a light-dependent decrease in Chl fluorescence due to the reduction of pheophytin, decreased in the temperature range of 44–55 °C with a half-inactivation temperature of 51 °C. The accumulation of betaine in PAMCOD cells shifted this temperature range to 52–63 °C with a half inactivation at 58 °C (Fig. 3). This observation clearly suggests that betaine protects the photochemical reaction center of PSII against heat-induced inactivation.

The oxygen-evolving complex was more sensitive to high temperature than the photochemical reaction center. Its activity, monitored by the light-dependent evolution of oxygen, decreased in the temperature range of 40–52 °C with the half-inactivation temperature at 46 °C. The accumulation of betaine in PAMCOD cells shifted this temperature range to 46–60 °C with the half-inactivation at 54 °C (Fig. 2), suggesting that betaine also protects the oxygen-evolving complex *in vivo* against the heat-induced inactivation. This could be attributed to the effect of betaine in stabilizing the binding of 33 kDa manganese-stabilizing protein to the oxygen-evolving complex [47,49].

The present study demonstrated that moderately high temperatures stimulated the photoinhibition of PSII by inhibiting the repair of PSII but not by accelerating the light-induced inactivation (Figs 4 and 5). It also demonstrated that the *codA* transgene, which resulted in the accumulation of betaine, counteracted this inhibitory effect of moderate heat stress. These results suggest that accumulation of betaine in *codA*-transformed cells of *Synechococcus* endows the cells to repair the damaged PSII rapidly and efficiently even at moderately high temperatures.

4.3. A possible mechanism for the protective effect of glycinebetaine on the repair of photosystem II

Recent studies have suggested that, among various processes of photosynthesis, the CO_2 fixation is most sensitive to heat

stress due to the inhibition of activation of Rubisco via Rubisco activase [50–53]. Thus, at the moderately high temperatures, ROS are produced and they inhibit the translation of *psbA* transcripts [see 45,46]. Furthermore, Yang et al. [31], using transgenic tobacco, which had been transformed with a gene for betaine aldehyde dehydrogenase from spinach, suggested that, under moderate heat stress, betaine maintained the activity of Rubisco by preventing the dissociation of Rubisco activase from Rubisco and thus enhances the tolerance of CO_2 fixation to heat stress and protected the translation of *psbA* transcripts via reducing the production of ROS.

It is possible to draw a similar scenario for cyanobacterial photosynthesis such that the CO_2 fixation is a process that is most sensitive to moderate heat stress. The accumulation of betaine may protect Rubisco against the destabilization at moderately high temperatures. However, this scenario is very unlikely in *Synechococcus*, because Rubisco activase does not exist in this cyanobacterium (the genome database for cyanobacteria at http://bacteria.kazusa.or.jp/cyano/cyano.html)and cyanobacterial Rubisco is relatively tolerant to heat stress [54]. Therefore, it seems likely that a different mechanism operates in the protection of the cyanobacterial PSII against moderate heat stress.

An alternate mechanism is also possible: The translational machinery is a large protein complex with many subunits. In most cases, high temperatures dissociate subunits from such large protein complexes and, as a result, inactivate them. The translational machinery in *Synechococcus* may be inactivated at moderately high temperatures, leading to the inhibition of the protein synthesis (Figs. 5 and 6). The accumulation of betaine is likely to prevent the disintegration and the inhibition of the translation machinery under moderate heat stress. A similar type of mechanism for the protection of betaine against the photoinhibition of PSII in *Synechococcus* was proposed for the salt stress-stimulated photoinhibition and the protection of the translation machinery by the *codA* transgene [26].

Northern-blotting analysis reveals that levels of psbA transcripts were similar between PAM and PAMCOD cells (Fig. 7). However, Western-blotting analysis demonstrated that *de novo* synthesis of the D1 protein was markedly faster in PAMCOD cells than in PAM cells (see Figs. 5 and 6). Therefore, the translational machinery appears to be the most sensitive and the primary target of moderate heat stress. Betaine might be able to stabilize the translational machinery under such conditions. Translation of *psbA* transcripts is a strongly regulated process and many regulatory factors are probably involved in initiation and mainly in elongation (see [44–46]).

Acknowledgments

The authors are grateful to Professor Kimiyuki Satoh for his generous gift of antibodies against the D1 protein. This work was supported, in part, by grants to S.I.A. and D.A.L. from the Russian Foundation for Basic Research (nos: 05-04-49672, 05-04-50883 and 06-04-48900) and the Molecular and Cell Biology Programs from Russian Academy of Sciences, by a Grant–in–Aid for Scientific Research to Y.N. (no: 16013237) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the National Institute for Basic Biology Cooperative Research on Stress Tolerance of Plants. P.M. acknowledges the support of INSA and JNU.

References

[1] L. Levitt, Responses of plants to environmental stresses, in: T.T. Kozlowski (Ed.), Physiological Ecology, Academic Press, New York, NY, 1980, pp. 347–348.
[2] J.S. Boyer, Plant productivity and environment, Science 218 (1982) 443–448.
[3] J.A. Berry, O. Björkman, Photosynthetic response and adaptation to temperature in higher plants, Annu. Rev. Plant Physiol. 31 (1980) 491–543.
[4] W. Larcher, Ecophysiology and stress physiology of functional groups,, in: W. Larcher (Ed.), Physiological Plant Ecology, Springer-Verlag, Berlin, Germany, 1995, pp. 340–353.
[5] D. Nash, M. Miyao, N. Murata, Heat inactivation of oxygen evolution in photosystem II particles and its acceleration by chloride depletion and exogenous manganese, Biochim. Biophys. Acta 807 (1985) 127–133.
[6] I. Enami, M. Kitamura, T. Tomo, Y. Isokowa, H. Ohta, S. Katoh, Is the primary cause of thermal inactivation of oxygen evolution in spinach PSII membranes release of the extrinsic 33 kDa protein or Mn? Biochim. Biophys. Acta 1186 (1995) 52–58.
[7] S.I. Allakhverdiev, Y.M. Feyziev, A. Ahmed, H. Hayashi, J.A. Aliev, V.V. Klimov, N. Murata, R. Carpentier, Stabilization of oxygen evolution and primary electron transport reactions in photosystem II against heat stress with glycinebetaine and sucrose, J. Photochem. Photobiol., B Biol. 34 (1996) 149–157.
[8] Z. Gombos, H. Wada, N. Murata, The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8787–8791.
[9] S.I. Allakhverdiev, Y. Nishiyama, I. Suzuki, Y. Tasaka, N. Murata, Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 5862–5867.
[10] S.I. Allakhverdiev, A. Sakamoto, Y. Nishiyama, M. Inaba, N. Murata, Ionic and osmotic effects of NaCl-induced inactivation of photosystem I and II in *Synechococcus* sp. Plant Physiol. 123 (2000) 1047–1056.
[11] S.I. Allakhverdiev, Y. Nishiyama, S. Miyairi, H. Yamamoto, N. Inagaki, Yu. Kanesaki, N. Murata, Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*, Plant Physiol. 130 (2002) 1443–1453.
[12] Y. Nishiyama, H. Yamamoto, S.I. Allakhverdiev, M. Inaba, A. Yokota, N. Murata, Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery, EMBO J. 20 (2001) 5587–5594.
[13] Y. Nishiyama, S.I. Allakhverdiev, H. Yamamoto, H. Hayashi, N. Murata, Singlet oxygen inhibits the repair of photosystem II by suppressing the transport elongation of the D1 protein in *Synechocystis* sp, PCC 6803, Biochemistry 43 (2004) 11321–11330.
[14] S. Takahashi, N. Murata, Interruption of the Calvin cycle inhibits the repair of photosystem II from photodamage, Biochim. Biophys. Acta 1708 (2005) 352–361.
[15] S. Takahashi, N. Murata, Glycerate-3-phosphate, produced by CO_2 fixation in the Calvin cycle, is critical for the synthesis of the D1 protein of photosystem II, Biochim. Biophys. Acta 1757 (2006) 198–205.
[16] S.I. Allakhverdiev, P. Mohanty, N. Murata, Dissection of photodamage at low temperature and repair in darkness suggests the existence of an intermediate form of photodamaged photosystem II, Biochemistry 42 (2003) 14277–14283.
[17] S.I. Allakhverdiev, N. Tsvetkova, P. Mohanty, B. Szalontai, B.Y. Moon, M. Debreczeny, N. Murata, Irreversible photoinhibition of photosystem II is caused by exposure of *Synechocystis* cells to strong light for a prolonged period, Biochim. Biophys. Acta 1708 (2005) 342–351.
[18] S.I. Allakhverdiev, N. Murata, Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage-repair cycle of

photosystem II in *Synechocystis* sp. PCC 6803, Biochim. Biophys. Acta 1657 (2004) 23–32.
[19] S.I. Allakhverdiev, Y. Nishiyama, S. Takahashi, S. Miyairi, I. Suzuki, N. Murata, Systematic analysis of the relation of electron transport and ATP synthesis to the photodamage and repair of photosystem II in *Synechocystis*, Plant Physiol. 137 (2005) 263–273.
[20] J. Gorham, Betaines in higher plants—biosynthesis and role in stress metabolism, in: R.M. Wallsgrove (Ed.), Amino Acids and their Derivatives in Higher Plants, Cambridge University Press, Cambridge, UK, 1995, pp. 171–203.
[21] A. Sakamoto, N. Murata, Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance, J. Exp. Bot. 51 (2000) 81–88.
[22] A. Sakamoto, N. Murata, The role of glycinebetaine in the protection of plants from stress: clues from transgenic plants, Plant Cell Environ. 25 (2002) 163–171.
[23] T.H.H. Chen, N. Murata, Enhancement of tolerance to abiotic stress by metabolic engineering of betaines and other compatible solutes, Curr. Opin. Plant Biol. 5 (2002) 250–257.
[24] P. Deshnium, D.A. Los, H. Hayashi, L. Mustardy, N. Murata, Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress, Plant Mol. Biol. 29 (1995) 897–907.
[25] P. Deshnium, Z. Gombos, Y. Nishiyama, N. Murata, The action in vivo of glycine betaine in enhancement of tolerance of *Synechococcus* sp. strain PCC 7942 to low temperature, J. Bacteriol. 179 (1997) 339–344.
[26] N. Ohnishi, N. Murata, Glycinebetaine counteracts the inhibitory effects of salt stress on the degradation and synthesis of the D1 protein during photoinhibition in *Synechococcus* sp. PCC 7942, Plant Physiol. 141 (2006) 758–765.
[27] H. Hayashi, Alia, L. Mustardy, P. Deshnium, M. Ida, N. Murata, Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress, Plant J. 12 (1997) 133–142.
[28] Alia, H. Hayashi, T.H.H. Chen, N. Murata, Transformation with a gene for choline oxidase enhances the cold tolerance of *Arabidopsis* during germination and early growth, Plant Cell Environ. 21 (1998) 232–239.
[29] Alia, H. Hayashi, A. Sakamoto, N. Murata, Enhancement of the tolerance of *Arabidopsis* to high temperatures by genetic engineering of the synthesis of glycinebetaine, Plant J. 16 (1998) 155–161.
[30] X. Yang, Z. Liang, C. Lu, Genetic engineering of the biosynthesis of glycinebetaine enhances photosynthesis against high temperature stress in transgenic tobacco plants, Plant Physiol. 138 (2005) 2299–2309.
[31] X. Yang, X. Wen, H. Gong, Q. Lu, Z. Yang, Y. Tang, Z. Liang, C. Lu, Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants, Planta 225 (2007) 719–733.
[32] R.Y. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and properties of unicellular blue-green algae (order *Chroococcales*), Bacteriol. Rev. 35 (1971) 171–205.
[33] T. Ono, N. Murata, Chilling susceptibility of the blue-green alga *Anacystis nidulans* I. Effect of growth temperature, Plant Physiol. 67 (1981) 176–181.
[34] S.I. Allakhverdiev, A. Sakamoto, Y. Nishiyama, N. Murata, Inactivation of photosystem I and II in response to osmotic stress in *Synechococcus*: contribution of water channels, Plant Physiol. 122 (2000) 1201–1208.
[35] Y. Tasaka, Z. Gombos, Y. Nishiyama, P. Mohanty, T. Ohba, K. Ohki, N. Murata, Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*: evidence for the important roles of polyunsaturated membrane lipids in growth, respiration and photosynthesis, EMBO J. 15 (1996) 6416–6425.
[36] V.V. Klimov, S.I. Allakhverdiev, V.G. Ladygin, Photoreduction of pheophytin in photosystem II of the whole cells of green algae and cyanobacteria, Photosynth. Res. 10 (1986) 355–361.
[37] S.I. Allakhverdiev, V.V. Klimov, V.G. Ladygin, Photoreduction of pheophytin in photosystem II reaction centers of intact cells of green algae and cyanobacteria under anaerobic conditions, Biofizika (Moscow) 33 (1988) 442–447.
[38] B. Ke, The transient intermediate electron acceptor of photosystem II, pheophytin, in: B. Ke (Ed.), Photosynthesis: Photobiochemistry and Photobiophysics, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2001, pp. 305–322.
[39] D.I. Arnon, B.D. McSwain, H.Y. Tsujimoto, K. Wada, Photochemical activity and components of membrane preparations from blue-green algae: I. Coexistence of two photosystems in relation to chlorophyll *a* and removal of phycocyanin, Biochim. Biophys. Acta 357 (1974) 231–245.
[40] D.A. Los, M.K. Ray, N. Murata, Differences in the control of the temperature-dependent expression of four genes for desaturases in *Synechocystis* sp. PCC 6803, Mol. Microbiol. 25 (1997) 1167–1175.
[41] S.S. Golden, J. Brusslan, R. Haselkorn, Expression of a family of *psbA* genes encoding a photosystem II polypeptide in the cyanobacterium *Anacystis nidulans* R2, EMBO J. 5 (1986) 2789–2798.
[42] M.A. Mamedov, H. Hayashi, H. Wada, P. Mohanty, G.C. Papageorgiou, N. Murata, Glycinebetaine enhances and stabilizes the evolution of oxygen and the synthesis of ATP by cyanobacterial thylakoid membranes, FEBS Lett. 229 (1991) 271–274.
[43] S.I. Allakhverdiev, H. Hayashi, Y. Nishiyama, A.G. Ivanov, J.A. Aliev, V.V. Klimov, N. Murata, R. Carpentier, Glycinebetaine protects the D1/D2/Cytb559 complex of photosystem II against photo-induced and heat-induced inactivation, J. Plant Physiol. 160 (2003) 41–49.
[44] Y. Nishiyama, S.I. Allakhverdiev, N. Murata, Inhibition of the repair of photosystem II by oxidative stress in cyanobacteria, Photosynth. Res. 84 (2005) 1–7.
[45] Y. Nishiyama, S.I. Allakhverdiev, N. Murata, A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II, Biochim. Biophys. Acta 1757 (2006) 742–749.
[46] N. Murata, S. Takahashi, Y. Nishiyama, S.I. Allakhverdiev, Photoinhibition of photosystem II under environmental stress, Biochim. Biophys. Acta 1767 (2007) 414–421.
[47] G.C. Papageorgiou, Y. Fujimura, N. Murata, Protection of the oxygen-evolving photosystem II complex by glycine betaine, Biochim. Biophys. Acta 1057 (1991) 361–366.
[48] G.C. Papageorgiou, N. Murata, The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex, Photosynth. Res. 44 (1995) 243–252.
[49] W.P. Williams, K. Gounaris, Stabilization of PSII mediated transport in oxygen-evolving PSII core preparations by the addition of compatible co-solutes, Biochim. Biophys. Acta 1100 (1992) 92–97.
[50] U. Feller, S.J. Crafts-Brandner, E. Salvucci, Moderately high temperatures inhibit ribulose-1,5-disphosphate carboxylase/oxygenase activase-mediated activation of Rubisco, Plant Physiol. 116 (1998) 539–546.
[51] M.E. Salvucci, S.J. Crafts-Brandner, Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis, Physiol. Plant. 120 (2004) 179–186.
[52] M.E. Salvucci, S.J. Crafts-Brandner, Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments, Plant Physiol. 134 (2004) 1460–1470.
[53] P. Haldimann, U. Feller, Inhibition of photosynthesis by high temperature in oak (*Quercus pubescens L.)* leaves grown under natural conditions closely correlates with a reversible heat-dependent reduction of the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase, Plant Cell Environ. 27 (2004) 1169–1183.
[54] Y. Marcus, H. Altman-Gueta, A. Finkler, M. Gurevitz, Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation, J. Bacteriol. 185 (2003) 1509–1517.