Dissection of the Mechanisms of Growth Inhibition Resulting from Loss of the PII Protein in the Cyanobacterium *Synechococcus elongatus* PCC 7942

Takayuki Sakamoto¹, Nobuyuki Takatani¹, Kintake Sonoike², Haruhiko Jimbo ³,⁴, Yoshitaka Nishiyama ³ and Tatsuo Omata ¹,⁴

¹Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464-8601 Japan
²Faculty of Education and Integrated Arts and Sciences, Waseda University, Tokyo, 162-8480 Japan
³Graduate School of Science and Engineering, Saitama University, Saitama, 338-8570 Japan
⁴Present address: Graduate School of Arts and Sciences, University of Tokyo, Tokyo 153-8902, Japan

*Corresponding author: E-mail: omata@agr.nagoya-u.ac.jp. Fax: +81-52-789-4107.

(Received 28 December 2020; Accepted 18 February 2021)

In cyanobacteria, the PII protein (the *glnB* gene product) regulates a number of proteins involved in nitrogen assimilation including PipX, the coactivator of the global nitrogen regulator protein NtcA. In *Synechococcus elongatus* PCC 7942, construction of a PII-less mutant retaining the wild-type *pipX* gene is difficult because of the toxicity of uncontrolled action of PipX and the other defect(s) resulting from the loss of PII *per se*, but the nature of the PipX toxicity and the PipX-independent defect(s) remains unclear. Characterization of a PipX-less *glnB* mutant (PD4) in this study showed that the loss of PII increases the sensitivity of PSII to ammonium. Ammonium was shown to stimulate the formation of reactive oxygen species in the mutant cells. The ammonium-sensitive growth phenotype of PD4 was rescued by the addition of an antioxidant, confirming that photo-oxidative damage was the major cause of the growth defect. A targeted PII mutant retaining wild-type *pipX* was successfully constructed from the wild-type *Synechococcus* sp. strain (SpC) in the presence of α-tocopherol, confirming that photo-oxidative damage was the major cause of the growth defect. A targeted PII mutant retaining wild-type *pipX* was successfully constructed from the wild-type *Synechococcus* sp. strain (SpC) in the presence of α-tocopherol. The resulting mutant (PD1X) showed an unusual chlorophyll fluorescence profile, indicating extremely slow reduction and re-oxidation of QA, which was not observed in mutants defective in both *glnB* and *pipX*. These results showed that the aberrant action of uncontrolled PipX resulted in an impairment of the electron transport reactions in both the reducing and oxidizing sides of QA.

**Keywords:** Ammonium • Cyanobacteria • Oxidative stress • PII • PipX • Reactive oxygen species.

**Introduction**

PII is a signaling protein present in plants, eubacteria and archaea that detects intracellular nitrogen–carbon balance and controls enzymes and transporters involved in nitrogen assimilation and carbon metabolism (Leigh and Dodsworth 2007, Huergo et al. 2012, Forchhammer and Selim 2020, Selim et al. 2020). Both cyanobacterial and plant PII have a common function of activating N-acetyl-l-glutamate kinase (NAGK), a key enzyme in arginine synthesis (Heinrich et al. 2004, Maheswaran et al. 2004, Sugiyama et al. 2004, Chen et al. 2006), and suppressing acetyl-CoA carboxylase (ACC), which is a key enzyme in fatty acid biosynthesis (Feria-Bourrellier et al. 2010, Gerhardt et al. 2015, Hauf et al. 2016). In addition to NAGK and ACC, genetic and biochemical studies have shown that cyanobacterial PII regulates various enzymes and transporters involved in the uptake and assimilation of inorganic nitrogen sources, such as nitrate reductase and the nitrate/nitrite, ammonium and urea transporters (Lee et al. 1998, Kobayashi et al. 2005, Chang et al. 2013, Watzer et al. 2019). PII acts as a sensor for 2-oxoglutaric acid (2-OG), which serves as the acceptor of newly acquired nitrogen to form glutamate. A rapid increase and a decrease in cellular 2-OG level were observed in response to deprivation and addition, respectively, of ammonium in *Synechocystis* sp. PCC 6803 (Muro-Pastor et al., 2001). Since the action of PII on its target proteins is blocked by 2-OG binding, PII enhances the activity of NAGK and suppresses those of ACC, nitrate reductase and the nitrate/nitrite transporter under the nitrogen-replete conditions with low 2-OG levels. PII is involved in the regulation of cyanobacterial nitrogen assimilation not only by direct interaction with enzymes and transporter proteins but also by controlling the transcriptional levels of genes. The major regulator of nitrogen assimilation-related genes in cyanobacteria is the transcriptional activator NtcA (Vega-Palas et al. 1992, Herrero et al. 2001), which requires 2-OG as a coinducer (Tanigawa et al. 2002). In addition to 2-OG, a protein called PipX acts as a coactivator of NtcA during nitrogen deficiency (Espinosa et al. 2006, Espinosa et al. 2007). PipX is a small protein that interacts with PII in a competitive manner with 2-OG. Therefore, it binds to PII when nitrogen is sufficient but is released from PII to activate NtcA when nitrogen is deficient. Recent studies have pointed out that...
PipX may have various functions in addition to NtcA activation (for a review, see Labella et al. 2020). For example, the PII–PipX complex has been shown to interact with a GntA-family transcriptional activator PlmA and may regulate the genes belonging to the putative PlmA regulon (Labella et al. 2016). In addition, the fact that PipX is conserved in all cyanobacteria suggests its importance. However, PipX-deficient strains show little growth phenotype, and the overall picture of the role of PipX remains unclear.

Interestingly, it was the analysis of PII-deficient strains that revealed the large impact of PipX on the growth of cyanobacteria. To genetically analyze the function of PII, PII-deficient mutants were constructed in Synechococcus elongatus PCC 7942 and Synechocystis sp. PCC 6803 by targeted inactivation of glnB, the structural gene for the homolog of PII (Forchhammer and Tandeau de Marsac 1995, Kobayashi et al. 2005). Surprisingly, it was later shown that all mutants constructed from the PCC 7942 strain also carried a mutation in pipX (Espinosa et al. 2009). Since the attempts to isolate glnB mutants that do not have any mutation in pipX were unsuccessful, it was deduced that the ‘free’ PipX protein is lethal to cells and that PII is essential for suppressing this toxicity via its interaction with PipX (Espinosa et al. 2010, Laichoubi et al. 2012). Using the NA3 mutant (Maeda and Omata 1997), which was constructed from the PCC 7942 strain by deleting the four genes (nrtABCD) encoding the ABC-type nitrate transporter (NRT), as a genetic background, Chang et al. (2013) later succeeded in construction of a PII-deficient mutant retaining wild-type pipX, wherein pipX interacts with NtcA but not with PII. Growth of the mutant was severely inhibited in ammonium-containing medium (i.e. under nitrogen-replete conditions), while PipX is supposed to dissociate from NtcA. The ammonium sensitivity of the mutant was partially rescued by inactivation of pipX, confirming that the ammonium sensitivity was due in part to the toxic effects of ‘free’ PipX. The results also showed that there is PipX-independent toxicity of ammonium due to the loss of PII (Chang et al. 2013).

This study was initiated to better understand the ammonium sensitivity of the PII/PipX double mutant. We show that the growth defect is due to photo-oxidative stress that can be suppressed by exogenously added α-tocopherol. In the presence of the antioxidant, a PII-deficient mutant retaining wild-type PipX was successfully constructed from the wild-type strain under normal growth conditions. Analysis of chlorophyll fluorescence of the mutant colonies suggested that the free PipX protein causes severe impairment of the photosynthetic electron transport even in the absence of ammonium.

Results

PII deficiency increases the sensitivity of PSII to ammonium

To analyze the effects of PII deficiency on PSII in the absence of the toxic effect of PipX, the previously constructed mutants PX2 (ΔNRT, ΔPipX) and PD4 (ΔNRT, ΔPipX, ΔPII) and the parental NA3 strain (ΔNRT) were grown in nitrate (60 mM)-containing medium. After 66 h of cultivation, the PSII activities of the cultures in the late logarithmic phase of growth were essentially the same, being 576 ± 20, 553 ± 17 and 546 ± 38 μmol O2 mg−1 Chl h−1 for NA3, PX2 and PD4, respectively. Addition of ammonium to these cultures decreased the PSII activity by 30 to 40% in 2 h (Fig. 1A). While there essentially was no further decrease in the PSII activity in NA3 and PX2, PD4 showed a further decrease in the activity thereafter to reach a level significantly lower than those of the other strains in 4 h. When the nitrate-grown cells were incubated without addition of ammonium, there also was a decrease in PSII activity, but the decrease was small and no significant difference was detected between PSII activities of the three strains (Fig. 1B). These results showed that the loss of PII increased the sensitivity of PSII activity to ammonium treatment.

Ammonium promotes reactive oxygen species generation in the PD4 strain

The superoxide anion (O2−), hydrogen peroxide (H2O2) and the hydroxyl radical (·OH) are the reactive oxygen species (ROS) generated by the Mehler reaction, disproportionation of the superoxide anion and the Fenton reaction, respectively. In this study, 5- (and 6-) chloromethyl-2, 7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was used to detect the three ROS in the NA3 and PD4 cells (Fig. 2). There was no significant difference in ROS levels between the nitrate-grown cells of NA3 and PD4 (Fig. 2, t = 0). While the intensity of fluorescence in the NA3 strain did not increase in 24 h after the addition of ammonium, a gradual increase in the fluorescence intensity was observed in the PD4 strain after 6–12 h of the incubation with ammonium. These results suggested that the generation of ROS was promoted in the PD4 strain by the addition of ammonium.

Singlet oxygen (1O2) is the ROS produced by the interaction of molecular oxygen with triplet chlorophyll. The rate of 1O2 production was determined as reported by Rehman et al. (2013), by comparing the rates of oxygen evolution measured in the absence and presence of 5 mM histidine (Fig. 3). When the NA3 and PD4 cells in the linear phase of growth were compared, there was no significant difference between their rates of 1O2 formation or those of photosynthetic O2 evolution (Fig. 3A, B, t = 0). After the addition of ammonium, the rate of 1O2 formation increased in PD4 to reach a rate of ∼25 μmol mg−1 Chl h−1 in 4 h, which was significantly higher than the rate in NA3 (Fig. 3A), while the rate of photosynthetic O2 evolution decreased in PD4 to a level significantly lower than that in NA3 (Fig. 3B).

Exogenously added α-tocopherol alleviates the sensitivity of PD4 to ammonium

Oxidic phototrophs including some cyanobacteria synthesizer tocopherols, which scavenge free radicals and ·O2 (Maeda and DellaPenna 2007). Since S. elongatus PCC 7942 lacks some of the genes in the tocopherol biosynthetic pathway and does not synthesize detectable amounts of α-tocopherol (Nowicka and Kruk 2016), we examined the effects of exogenously added α-tocopherol on PD4. Irrespective of the presence or absence of
added α-tocopherol, NA3 grew well, with the growth rate being slightly higher in ammonium-containing medium than in nitrate-containing medium (Fig. 4A, B). In the absence of α-tocopherol, the growth of PD4 was impaired in ammonium-containing medium but not in nitrate-containing medium as reported previously (Chang et al. 2013) (Fig. 4C, D). The growth phenotype of the PD4 mutant was rescued when 1 mM of α-tocopherol was added to the ammonium-containing medium (Fig. 4D). These results indicated that the ammonium-induced growth inhibition of PD4 was due to oxidative stress caused by ROS.

**PD4 is hypersensitive to methyl viologen**

Fig. 5A compares the effects of 2.5 μM of methyl viologen (MV) on the growth of NA3 and PD4 in nitrate- or ammonium-containing medium. MV is reduced by PSI to its radical, which promotes ROS generation. Irrespective of the nitrogen source used, growth of NA3 was hardly affected by MV, whereas that of PD4 was inhibited (Fig. 5A). The inhibitory effect of MV was more pronounced in the ammonium-containing medium than in nitrate-containing medium, presumably as a result of combined effects of MV-induced and ammonium-induced ROS generation. The inhibitory effects of MV on PD4 in the nitrate-containing medium were further confirmed by comparison of the growth curves of the NA3 and PD4 cells in the presence and absence of MV (Fig. 5B, C). Since the ROS levels in nitrate-grown NA3 and PD4 cells were not significantly different (Figs. 2, 3A, t = 0), the higher sensitivity of PD4 to MV than NA3 suggested that PD4 is defective in the defense mechanisms against ROS.

**P1r-deficient mutant carrying wild-type pipX was isolated on an α-tocopherol-containing medium**

Isolation of P1r-deficient mutants without mutation in pipX has been extremely difficult due to both the toxicity of the unregulated action of PipX and the PipX-independent disorders caused by the loss of P1r (e.g. reduced cellular tolerance to ammonium). In a previous study, Chang et al. (2013) used the nitrate transport-deficient mutant (NA3) as a parental strain to construct such a mutant (PD3) under strictly N-limited growth...
Fig. 3 Ammonium induces \( \cdot \)O\(_2\) generation in PD4 cells. Cells of NA3 (circles) and PD4 (diamonds) were inoculated into nitrate (60 mM)-containing medium to give an OD\(_{730}\) of 0.1, and after cultivation for 48 h, ammonium was added to give a final concentration of 15 mM. The subsequent changes in the rate of \( \cdot \)O\(_2\) formation (A) and photosynthetic O\(_2\) evolution (B) are shown. Data shown are the mean ± SE from three independent experiments. Asterisks indicate significant differences between NA3 and PD4 (\( P < 0.05 \), Student’s t-test).

Fig. 4 Exogenously added \( \alpha \)-tocopherol mitigates the ammonium-promoted growth inhibition in PD4. Growth curves of NA3 (A and B) and PD4 (C and D) were obtained in the presence (closed circles) or absence (open circles) of 1 mM \( \alpha \)-tocopherol in the nitrate-containing medium (A and C) or ammonium-containing medium (B and D). Data shown are the means ± SE from three independent experiments. Asterisks indicate significant differences between the presence and the absence of \( \alpha \)-tocopherol (\( P < 0.05 \), Student’s t-test).
conditions, in which the toxicity of PipX is supposed to be minimized by the interaction with NtcA. Since exogenously added \( \alpha \)-tocopherol was found to mitigate the growth defect resulting from the loss of \( P_{II} \) per se (Fig. 4), we attempted to inactivate \( glnB \) in the presence of \( \alpha \)-tocopherol, using the WT strain (SPc) under the normal growth conditions. The pPD3 plasmid (Chang et al. 2013) was used to transform SPc cells into spectinomycin-resistant mutants by replacing the chromosomal copy of \( glnB \) with the plasmid-borne copy of \( glnB \) interrupted with the spectinomycin resistance gene cassette (Fig. 6A). Since cyanobacteria have multiple copies of chromosomal DNA, the transformants were grown with consecutive transfers onto the solid medium containing 15 mM nitrate, 15 \( \mu \)g ml\(^{-1}\) spectinomycin and 1 mM \( \alpha \)-tocopherol to promote segregation of homozygous mutants. After two streak transfers onto a new medium, a number of small colonies were formed (Fig. 6B). After subsequent several streak purifications, five of the small colonies were isolated. PCR amplification of the \( glnB \) region of the transformants gave rise to the 3.0-kb fragment corresponding to the mutated \( glnB \) region but not the 1.0-kb fragment corresponding to the wild-type \( glnB \) region (Fig. 6C). One of the five transformants was named PD1X and used for the subsequent studies. Nucleotide sequence analysis confirmed the absence of mutations in the \( pipX \) region of the PD1X strain.

In a growth test on solid media, the \( P_{II} \)-defective strains PD1X and PD1 were practically unable of growth on ammonium-containing medium (Fig. 6D). On nitrate-containing medium, growth of PD1, which is defective in PipX, was similar to that of the WT strain, but PD1X, which retains PipX, could grow only poorly, indicating the toxicity of PipX. These phenotypes of the PD1X strain were similar to the phenotypes of the PD3 strain constructed from the NA3 strain (Chang et al. 2013).

**PipX affects the chlorophyll fluorescence kinetics of the \( P_{II} \)-deficient mutant**

Although the PD1X mutant formed colonies on solid medium supplemented with \( \alpha \)-tocopherol, it was hard to establish liquid cultures of the mutant to be used for the determination of the rates of photosynthesis or ROS formation. We therefore evaluated the photosynthetic activity of the mutant colonies by measuring the chlorophyll fluorescence yield, using a pulse-amplitude modulation (PAM) fluorometer that allows for imaging under a microscope. In the case of the WT colonies, application of saturating flashes to dark-adapted colonies increased the fluorescence yield to a high level (Fig. 7A). The onset and cessation of the actinic light resulted in rapid increase and decrease, respectively, in the fluorescence yield, representing reduction and re-oxidation of Q\(_{a}\), respectively (Fig. 7A). The results were consistent with the fluorescence profiles reported for liquid cultures of wild-type cyanobacteria (Ogawa et al. 2017). In the case of the PD1X colonies, by contrast, saturating flashes caused little, if any, change in fluorescence yield when applied on dark-adapted colonies (Fig. 7B). The onset of the actinic light did not cause rapid increase in the fluorescence, either, but brought about a slow, gradual increase.

---

**Fig. 5 Sensitivity of PD4 to methyl viologen.** (A) Appearance of the NA3 and PD4 cultures grown in nitrate (60 mM)- or ammonium (7.5 mM)-containing medium in the presence or absence of 2.5 \( \mu \)M MV. The photos taken in the linear phase of growth (\( t = 72 \) h) are shown. Growth curves of NA3 (B) and PD4 (C) obtained in the presence (closed circles) or absence (open circles) of 2.5 \( \mu \)M MV in the nitrate-containing medium. Data shown are the mean ± SE from three independent experiments. Asterisks indicate significant differences between the presence and the absence of methyl viologen (\( P < 0.05 \), Student’s \( t \)-test).
in the fluorescence yield (Fig. 7B), indicating that the reduction in QA is slow in the mutant. The fluorescence decrease after cessation of the actinic light was also slow in the PD1X mutant, indicating slow re-oxidation of QA. Taken together, the present results showed that the electron transport reactions in both the reducing and oxidizing sides of QA are severely impaired in the PD1X mutant.

Fig. 8 compares the fluorescence profiles of the NA3, PX2 and PD4 colonies as measured by the imaging PAM microscopy. All the strains showed rapid reduction and re-oxidation of QA in response to the onset and cessation of the actinic light, respectively. The ‘wild-type’ fluorescence kinetics of PD4 as well as PX2 indicated that the loss of Pii does not result in the impairment of photosynthetic electron transport, provided that PipX is absent.

Discussion

Tocopherols are lipophilic antioxidants, which act as scavengers of free radicals and 1O2. Studies on an engineered Synechocystis sp. PCC 6803 mutant defective in tocopherol biosynthesis revealed that these compounds protect the repair of PSII from oxidative damage and thereby mitigates photoinhibition of PSII (Inoue et al. 2011). In this study, exogenously added α-tocopherol was shown to rescue the inhibitory effect of ammonium on growth of the glnB::pipX double mutant PD4 (Fig. 4). This indicates that the PipX-independent ammonium toxicity, which is ascribed to the loss of Pii, was caused by ROS. Synechococcus elongatus PCC 7942 does not have the tocopherol biosynthetic pathway (Nowicka and Kruk 2016) and hence should rely on other quenchers and antioxidant enzymes for the elimination of ROS. It is deduced that the capacity of the ROS-eliminating mechanisms is exceeded by the ROS production rate in the PD4 cells growing in the ammonium-containing medium. In Synechocystis sp. PCC 6803, a Pii-less mutant was shown to exhibit several times higher rate of ammonium uptake than that of the WT strain (Takatani and Omata 2006) and PII has been shown to interact with the Amt1 ammonium uptake transporter (Watzer et al. 2019). It is therefore likely that the loss of Pii resulted in de-regulation of ammonium uptake in PD4, leading to the accumulation of excess ammonium in the cell. Ammonium can act as an uncoupler of photophosphorylation and may have enhanced ROS production at PSI in PD4. Another possibility is that the loss of Pii has resulted in the
reduced activity of the ROS scavenging mechanisms. No proteins of this category have been detected as a target of PII in spite of the extensive survey for the PII-interacting proteins (Watzer et al. 2019), but the interaction of PII–PipX with the PlmA transcriptional factor (Labella et al. 2016) may suggest possible indirect interaction of PII and the proteins involved in ROS elimination.

It has been impossible to obtain a PII-null mutant retaining PipX from the wild-type strain of *S. elongatus* PCC 7942, but the physiological reason remained unknown. The successful segregation of the mutant in the presence of α-tocopherol (Fig. 6) shows that oxidative stress was a major problem in the isolation of the PII mutant. Even in the presence of α-tocopherol and the absence of ammonium, however, growth of the resultant mutant (PD1X) was very slow, forming tiny colonies (Fig. 6B). This phenotype is due to the uncontrolled action of PipX because it is not observed in the PD1 mutant deficient in both PII and PipX (Fig. 6D). In the same way, the unusual chlorophyll fluorescence profile of the PD1X mutant (Fig. 7B) is ascribed to the action of ‘free’ PipX (Fig. 8). The slow reduction and re-oxidation of QA in the PD1X mutant indicate suppression of the electron transfer reactions in both the reducing and oxidizing sides of QA. PipX is not a very abundant protein, whose number has been estimated to be 4,500 molecules (i.e. 1,500 trimers) per cell of *S. elongatus* PCC 7942 (Guerreiro et al. 2014). The number of PSII has been reported to vary considerably according to the environmental conditions.
Fig. 8 Loss of PII does not affect the chlorophyll fluorescence profile in the absence of PipX. Chlorophyll fluorescence profiles of the pipX- and glnB mutants constructed from the NA3 strain defective in NRT were compared. (A) NA3, the parental strain. (B) PX2, the pipX-deficient strain. (C) PD4, the double mutant defective in both pipX and glnB. Fluorescence from three colonies of each strain was measured by imaging PAM microscopy as in Fig. 7.

conditions during growth of the cyanobacterium, ranging from 68,000 to 110,000 molecules per cell (MacKenzie and Campbell 2005). PipX is thus unlikely to affect the photosynthetic activity by direct interaction with the photosystem(s). Based on the transcriptome analysis of pipX mutants, it has been suggested that PipX forms at least three types of regulatory complexes other than PipX-NtcA (Espinosa et al. 2014). The interaction partners need to be identified but may include regulators of transcription, signaling, or posttranslational regulation (Espinosa et al. 2014). Therefore, the loss of PII can lead to aberrant activation or inactivation of the PipX partner(s), which may result in the inability to maintain a normal photosynthetic system. It should be noted, however, that the above-mentioned estimation of the number of PipX molecules (Guerreiro et al. 2014) was performed in the wild-type cells. While PipX is known to enhance transcription of the structural gene of PII, glnB (Espinosa et al. 2006), it has not been investigated whether PII regulates the expression of pipX. Therefore, it cannot be completely ruled out at the moment that pipX expression may be deregulated in the PII-less mutant, providing sufficient PipX protein to exert detrimental effects on the photosynthetic activities via protein–protein interaction. Further studies are needed to clarify this point.

The toxicity of ‘free’ PipX is strongly enhanced by ammonium, preventing the growth of the pipX+/glnB+ mutants in ammonium-containing medium (Fig. 6, see also Chang et al. 2013). Ammonium is a good nitrogen source for cyanobacteria, and indeed wild-type cells of S. elongatus PCC 7942 and Synechocystis sp. PCC 6803 grow well in the presence of high concentrations (10–20 mM) of ammonium. However, ammonium enhances PSII photo-inhibition even under low-light conditions (Drath et al. 2008, see also Fig. 1), promoting photodamage to the oxygen-evolving complex of PSII without affecting the repair of the photosystem (Dai et al. 2014). Since the extent of photo-inhibition depends on the balance between photodamage and repair (Nishiyama and Murata 2014), the ability of the wild-type cells to tolerate high ammonium concentrations relies on an efficient PSII-repair mechanism; mutations that impair the repair mechanism render the cells ammonium sensitive (Drath et al. 2008, Dai et al. 2014). It is therefore likely that the strong toxicity of the ‘free’ PipX in the presence of ammonium also resulted from disturbance of the PSII-repair mechanism. It is a very interesting problem that a protein ubiquitously present in cyanobacteria adversely affects photosynthesis under certain conditions. Further research is required to elucidate the biochemical basis of the aberrant effects of PipX on photosynthesis.

**Materials and Methods**

**Strains and growth conditions**

The strains used for the experiments are shown in Table 1. A ‘Small-Plasmid-cured’ derivative of S. elongatus strain PCC 7942 (SpC) (Kuhlman et al. 1981) is the genetic background of all the mutants used in this study. If not otherwise specified, all the strains were photautotrophically grown at 30°C under continuous illumination of 50 μmol photons m⁻² s⁻¹ provided by a white fluorescent lamp and aeration with air containing 2% CO₂. The media used in this study were modifications of the BG11 medium (Stanier et al. 1971), containing either nitrate (15 or 60 mM of potassium nitrate) or ammonium (3.75 or 7.5 mM of ammonium sulfate) prepared as described previously (Suzuki et al. 1995). The solid medium was prepared by adding 1.5% Bacto Agar (BD, Franklin Lakes, NJ, USA) to the liquid medium. All the media were buffered with 20 mM HEPES-KOH (pH 8.2). When appropriate, kanamycin (15 μg ml⁻¹) and/or spectinomycin (15 μg ml⁻¹) were added to the medium. 0.1 mM α-tocopherol was added to the solid medium for cultivation of the PDIX strain.

**Detection of reactive oxygen species**

Cell cultures were collected by centrifugation and resuspended by fresh medium to give an OD730 value of 0.5 (time zero). The cell suspensions were supplemented with 60 mM KNO₃ or 3.75 mM NH₄SO₄, and cultivated under illumination at 70 μmol photons m⁻² s⁻¹ at times up to 24 h. Aliquots of 100 μl of the cell suspension were sampled and ROS in the samples was determined fluorometrically according to Zhou et al. (1997) and Sae-Tang et al. (2016) as...
Table 1 Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Reference or source |
|------------------|--------------------------|---------------------|
| Strains          |                          |                     |
| SPc              | S. elongatus strain PCC7942 cured of pUH24 plasmid, wild type | Kuhlmeier et al. (1983) |
| NA3              | S. pl./ritABCD, lacking genes encoding ABC-type nitrate/nitrite transporter | Maeda and Omata (1997) |
| PX2              | NA3 pipX:nptII Km\(^\text{r}\) cassette | Chang et al. (2013) |
| PD4              | NA3 glnB:Ω Sp\(^{-}\)-Sm\(^{-}\) cassette pipX:nptII Km\(^\text{r}\) cassette | Chang et al. (2013) |
| PX1              | S. pl. pipX:nptII Km\(^\text{r}\) cassette | Chang et al. (2013) |
| PD1              | S. pl. glnB:Ω Sp\(^{-}\)-Sm\(^{-}\) cassette pipX260delC | Kobayashi et al. (2005) |
| PD1X             | S. pl. glnB:Ω Sp\(^{-}\)-Sm\(^{-}\) cassette | This study |
| Plasmids         |                          |                     |
| pPD3             | Plasmid to inactivate glnB by inserting Ω spectinomycin/streptomycin resistance gene cassette | Chang et al. (2013) |

Table 2 Primers used in this study

| Name            | Sequence                                      |
|-----------------|-----------------------------------------------|
| Gup-2F          | 5'-GAGCAGACACGTCTAGCTATATTG-3'                |
| Gdn-2R          | 5'-CAATGGCTTCTCGTAGAAGTG-3'                   |
| PipX-Fw         | 5'-TGGTACAGAAGAGCTGACCTGGC-3'                 |
| PipX-Rv         | 5'-TGGACTGGCTCCATCCGAA-3'                     |

follows. 1.5 μM CM-H\(_2\)DCFDA (Molecular Probes, Inc., Eugene, OR, USA) was added to the cell suspensions and incubated in the dark for 30 min on a 96-well microplate. The fluorescence intensity of the reaction product 5- (and 6-) chloromethyl-2,7-dichlorodihydrofluorescin was measured by using a fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific Inc, Waltham, MA, USA), with excitation at 485 nm and emission at 538 nm. Each sample was measured four times and the results were averaged.

Measurement of the rate of singlet oxygen formation

Rates of singlet oxygen formation were measured as described by Rehman et al. (2013). Cell cultures were diluted with fresh medium to give an OD\(_{730}\) value of 0.1 and cultivated for 48 h under illumination at 70 μmol photons m\(^{-2}\) s\(^{-1}\). The cell cultures were supplemented with 7.5 mM (NH\(_4\)\(_2\))\(_2\)SO\(_4\) and cultivated for 4 h. Aliquots of 1 ml of the cell suspensions were taken after the addition of ammonium and the rate of oxygen evolution was measured in the absence or presence of 5 mM histidine. The rate of singlet oxygen formation was quantitated by subtracting the rate of oxygen evolution in the presence of histidine from the rate in its absence.

Insertional inactivation of glnB in the WT cells

Cells of the WT strain (SPc) were collected by centrifugation and resuspended in the fresh medium. The cell suspension was supplemented with the pPD3 plasmid (Table 1, Chang et al. 2013) and incubated overnight in the dark. The cells were subsequently spread onto solid medium containing 15 mM KNO\(_3\), 15 μg ml\(^{-1}\) spectinomycin and 0.1 mM D-α-tocopherol and cultivated for 4 weeks in the light. The transformants that formed small colonies were isolated. KOD-Plus-Neo (Toyobo, Osaka, Japan) and the primer pair Gup-2F/Gdn-2R (Chang et al. 2013) were used for PCR amplification of the glnB (Synpcc7942_0321) region of the mutant strains. The primer pair PipX-Fw/PipX-Rv (Table 2) was used for the nucleotide sequence analysis of the pipX (Synpcc7942_2061) region.

Measurement of the chlorophyll fluorescence yield

The PD1X cells inoculated on agar medium containing 0.1 mM D-α-tocopherol were cultivated under 50 μmol photons m\(^{-2}\) s\(^{-1}\), 30°C and 2% CO\(_2\) conditions. The SPc cells inoculated on agar medium without α-tocopherol were cultivated at the same condition. Colonies of each strain (~0.1 mm in diameter) were used in this experiment. For the detection of chlorophyll fluorescence, a microscopy type imaging PAM from Walz (Eichenberg, Effeltrich, Germany) was used. A 10× objective lens and a 10× eyepiece were used to observe the colonies. An orange LED (IMAG-L625M) was used as the excitation LED. In addition, saturated pulsed light and actinic light at >2,000 or 47 μmol photons m\(^{-2}\) s\(^{-1}\), respectively, were irradiated. ImagingWinGigE (Walz) was used for the analysis of the obtained data. Four independent experiments were conducted and similar data were obtained for each strain.

Other methods

Chlorophyll was determined as described by Mackinney (1941). The rate of oxygen evolution was measured by using an oxygen electrode (Oxygraph Plus, Hansatech Instruments Ltd, King’s Lynn, Norfolk, England). For the determination of the PSI activity, 1 mM D-α-benzoquinone was used as an electron acceptor. The growth test of NA3 and PD4 was performed on the liquid medium containing nitrate or ammonium as follows. Cell cultures grown to the late logarithmic phase of growth were diluted with the fresh medium containing 60 mM KNO\(_3\) or 3.75 mM (NH\(_4\)\(_2\))\(_2\)SO\(_4\) to give an OD\(_{730}\) value of 0.01, and after addition of 1 mM D-α-tocopherol or 2.5 μM methyl viologen, the cells were cultivated under illumination at 70 μmol photons m\(^{-2}\) s\(^{-1}\). The growth test of the newly constructed P\(_7\) mutant (PD1X) was performed on solid media. Cells cultivated on the solid nitrate (15 mM)-containing medium for 7 d were collected and resuspended in fresh medium to give an OD\(_{730}\) value of 1.0, 0.1 and 0.01. Aliquots of 5 μl of the cell suspensions were spotted onto the solid medium containing 15 mM KNO\(_3\) or 3.75 mM (NH\(_4\)\(_2\))\(_2\)SO\(_4\) and incubated for 6 d under illumination at 50 μmol photons m\(^{-2}\) s\(^{-1}\) and aeration with air containing 0.04% CO\(_2\).

Funding

JST-Mirai program [Grant Number JPMJMI17EE to T.O.] from Japan Science and Technology Agency and Grant-in-Aid for Scientific Research in Innovative Areas [Grant Numbers JP16H06552 and JP16H06553 to K.S.] from Japan Society for the Promotion of Science.

Disclosures

No conflicts of interest are declared.

References

Chang, Y., Takatani, N., Aichi, M., Maeda, S. and Omata, T. (2013) Evaluation of the effects of α-tocopherol and the toxicity of PipX on growth
characteristics of the P$_{I}$-less mutant of the cyanobacterium Synechococcus elongatus. Plant Cell Physiol. 54: 1504–1514.

Chen, Y.M., Ferrar, T.S., Lohmeir-Vogel, E., Morrice, N., Mizuno, Y., Berenger, B., et al. (2006) The PII signal transduction protein of Arabidopsis thaliana forms an arginine-regulated complex with plastid N-acetylglutamate kinase. J. Biol. Chem. 281: 5726–5733.

Dai, G.Z., Qiu, B.S. and Forchhammer, K. (2014) Ammonium tolerance in the cyanobacterium Synechocystis sp. strain PCC 6803 and the role of the pibA multigene family. Plant Cell Environ. 37: 840–851.

Drath, M., Kloft, N., Batschauer, A., Marin, K., Novak, J. and Forchhammer, K. (2008) Ammonia triggers photodamage of photosystem II in the cyanobacterium Synechocystis sp. strain PCC 6803. Plant Physiol. 147: 206–215.

Espinosa, J., Castells, M.A., Laichoubi, K.B. and Contreras, A. (2009) Mutations at pipX suppress lethality of P$_{II}$-deficient mutants of Synechococcus elongatus PCC 7942. J. Bacteriol. 191: 4863–4869.

Espinosa, J., Castells, M.A., Laichoubi, K.B., Forchhammer, K. and Contreras, A. (2010) Effects of spontaneous mutations in PipX functions and regulatory complexes on the cyanobacterium Synechococcus elongatus strain PCC 7942. Microbiology 156: 1517–1526.

Espinosa, J., Forchhammer, K., Burillo, S. and Contreras, A. (2006) Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate-dependent manner with PII and NtcA. Mol. Microbiol. 61: 457–469.

Espinosa, J., Forchhammer, K. and Contreras, A. (2007) Role of the Synechococcus PCC 7942 nitrogen regulator protein PipX in NtcA-controlled processes. Microbiology 153: 711–718.

Feria-Bourrellier, A.B., Valot, B., Guillot, A., Ambard-Bretteville, F., Vidal, J. and Hodges, M. (2010) Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc. Natl. Acad. Sci. USA 107: 502–507.

Forchhammer, K. and Selim, K.A. (2020) Carbon/nitrogen homeostasis in cyanobacteria. FEMS Microbiol. Rev. 44: 33–53.

Ferre-Bourrellier, A.B., Valot, B., Guillot, A., Ambard-Bretteville, F., Vidal, J. and Hodges, M. (2010) Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc. Natl. Acad. Sci. USA 107: 502–507.

Forchhammer, K. and Selim, K.A. (2020) Carbon/nitrogen homeostasis control in cyanobacteria. FEMS Microbiol. Rev. 44: 33–53.

Gerhardt, E.C.M., Rodrigues, T.E., Forchhammer, K. and Contreras, A. (2007) Mutations at pipX suppress lethality of P$_{II}$-deficient mutants of Synechococcus elongatus PCC 7942. J. Bacteriol. 191: 4863–4869.

Espinosa, J., Castells, M.A., Laichoubi, K.B., Forchhammer, K. and Contreras, A. (2010) Effects of spontaneous mutations in PipX functions and regulatory complexes on the cyanobacterium Synechococcus elongatus strain PCC 7942. Microbiology 156: 1517–1526.

Lee, H.-M., Flores, E., Herrero, A., Houmard, J. and Tandeau de Marsac, N. (1998) A role for the signal transduction protein P$_{II}$ in the control of nitrate/nitrite uptake in a cyanobacterium. FEBS Lett. 427: 291–295.

Leigh, J.A. and Dodsworth, J.A. (2007) Nitrogen regulation in bacteria and archaea. Annu. Rev. Microbiol. 61: 340–377.

Mackenzie, G. (1941) Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315–322.

Maeda, H. and DellaPenna, D. (2007) Tocopherol functions in photosynthetic organisms. Curr. Opin. Plant Biol. 10: 260–265.

Maeda, S.I. and Omata, T. (1997) Substrate-binding Lipoprotein of the Cyanobacterium Synechococcus sp. Strain PCC 7942 Involved in the Transport of Nitrate and Nitrite. Journal of Biological Chemistry 272: 3036–3041.10.1074/jbc.272.5.3036

MacKenzie, T.D.B. and Campbell, D.A. (2005) Cyanobacterial acclimation to rapidly fluctuating light is constrained by inorganic carbon status. J. Physiol. 41: 801–811.

Møro-Pastor, M.I., Reyes, J.C. and Florencio, F.J. (2001) Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J. Biol. Chem. 276: 38320–38328.

Nishiyama, Y. and Murata, N. (2014) Revised scheme for the mechanism of photoinduction and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. Appl. Microbiol. Biotechnol. 98: 8777–8796.

Nowicka, B. and Kruk, J. (2016) Cyanobacteria use both ammonia and nitrogenous compounds to signal nitrogen status. Front. Microbiol. 7: 215.10.3389/fmicb.2016.00215

Ogawa, T., Misumi, M. and Sonoike, K. (2017) Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynth. Res. 133: 63–73.

Rehm, A.U., Cser, K., Sass, L. and Vass, I. (2013) Characterization of single oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium Synechocystis PCC 6803 by histidine-mediated chemical trapping. Biochim. Biophys. Acta 1827: 689–698.

Sae-Tang, P., Hihara, Y., Yumoto, I., Orikasa, Y., Okuyama, H. and Nishiyama, Y. (2016) Overexpressed superoxide dismutase and catalase act synergistically to protect the repair of PSI degradation in Synechococcus elongatus PCC 7942. Plant Cell Physiol. 57: 1899–1907.

Selim, K.A., Ermilova, E. and Forchhammer, K. (2020) From cyanobacteria to Archeplastida: new evolutionary insights into PII signalling in the plant kingdom. New Phytol. 227: 722–731.
Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35: 171–205.

Sugiyama, K., Hayakawa, T., Kudo, T., Ito, T. and Yamaya, T. (2004) Interaction of N-acetylglutamate kinase with a PII-like protein in rice. Plant Cell Physiol. 45: 1768–1778.

Suzuki, I., Kikuchi, H., Nakanishi, S., Fujita, Y., Sugiyama, T. and Omata, T. (1995) A novel nitrite reductase gene from the cyanobacterium Plectonema boryanum. J. Bacteriol. 177: 6137–6143.

Takatani, N. and Omata, T. (2006) Effects of PII deficiency on expression of the genes involved in ammonium utilization in the cyanobacterium Synechocystis sp. strain PCC 6803. Plant Cell Physiol. 47: 679–688.

Tanigawa, R., Shirokane, M., Maeda, S., Omata, T., Tanaka, K. and Takahashi, H. (2002) Transcriptional activation of NtcA-dependent promoters of Synechococcus sp. PCC 7942 by 2-oxoglutarate in vitro. Proc. Natl. Acad. Sci. USA 99: 4251–4255.

Vega-Palas, M.A., Flores, E. and Herrero, A. (1992) NtcA, a global nitrogen regulator from the cyanobacterium Synechococcus that belongs to the Crp family of bacterial regulators. Mol. Microbiol. 6: 1853–1859.

Watzer, B., Spät, P., Neumann, N., Koch, M., Sobotka, R., Macek, B., et al. (2019) The signal transduction protein PII controls ammonium, nitrate and urea uptake in cyanobacteria. Front. Microbiol. 10: 1428.

Zhou, M., Diwu, Z., Panchuk-Voloshina, N. and Haugland, R.P. (1997) A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal. Biochem. 253: 162–168.