

Impaired Photosynthesis in Phosphatidylglycerol-Deficient Mutant of Cyanobacterium *Anabaena* sp. PCC7120 with a Disrupted Gene Encoding a Putative Phosphatidylglycerophosphatase¹

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Phosphatidylglycerol (PG) is a ubiquitous phospholipid in thylakoid membranes of cyanobacteria and chloroplasts and plays an important role in the structure and function of photosynthetic membranes. The last step of the PG biosynthesis is dephosphorylation of phosphatidylglycerophosphate (PGP) catalyzed by PGP phosphatase. However, the gene-encoding PGP phosphatase has not been identified and cloned from cyanobacteria or higher plants. In this study, we constructed a PG-deficient mutant from cyanobacterium *Anabaena* sp. PCC7120 with a disrupted gene (*alr1715*, a gene for Alr1715 protein, GenBank accession no. BAB78081) encoding a putative PGP phosphatase. The obtained mutant showed an approximately 30% reduction in the cellular content of PG. Following the reduction in the PG content, the photoautotrophical growth of the mutant was restrained, and the cellular content of chlorophyll was decreased. The decreases in net photosynthetic and photosystem II (PSII) activities on a cell basis also occurred in this mutant. Simultaneously, the photochemical efficiency of PSII was considerably declined, and less excitation energy was transferred toward PSII. These findings demonstrate that the *alr1715* gene of *Anabaena* sp. PCC7120 is involved in the biosynthesis of PG and essential for photosynthesis.

Thylakoid membranes of cyanobacteria and chloroplasts of plants and algae are the sites of the oxygenic photosynthesis, where highly organized pigment-protein complexes are embedded in the lipid matrix. Thylakoid membranes are characterized by its unique lipid composition, which is in contrast with animal, bacterial, and nonchloroplastic cell membranes dominated by phospholipids. The major lipids of thylakoid membranes are the uncharged galactolipids, namely, monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG). These galactolipids account for at least 50 mol% of the total thylakoid lipids. In addition, thylakoid membranes are specific in that there is a negatively charged sulfolipid, sulfoquinovosyl diacylglycerol (SQDG), which seems not to be present in nonphotosynthetic organisms. The only significant phospholipid is phosphatidylglycerol (PG), rather than phosphatidylcholine or phosphatidylethanolamine (Murata and Siegenthaler, 1998).

PG is an anionic phospholipid that is also extensively distributed in the functional domains of thylakoid membranes, suggesting that PG is critical for the structure and function of thylakoid membrane. Indeed, increasing biochemical and genetic studies provided evidence that PG plays an important role in photosynthesis of thylakoid membranes. It has shown that PG is indispensable for the dimerization of PSII (Kruse et al., 2000) and trimerization of the light-harvesting complex of PSII (Nußberger et al., 1993; Hobe et al., 1994; Liu et al., 2004). PG is also essential for the oligomerization of PSI reaction center of cyanobacterium (Domonkos et al., 2004), consistent with the fact that PG is an integral component of PSI (Jordan et al., 2001). It was found that PG is required for the biogenesis of PSII core complex in *Chlamydomonas reinhardtii* mutants *mf1* and *mf2* with the decreased rate of translation of the D1 and CP47 owing to PG deficiency (Pineau et al., 2004), compatible with a previous suggestion that PG might bind to the D1 protein, where PG could sustain the optimal conformation of D1 polypeptide (Kruse and Schmid, 1995). The *PGP1* mutants of Arabidopsis (*Arabidopsis thaliana*) defective in biosynthesis of PG obtained through chemical or T-DNA insertional mutagenesis disrupted in the *PGP1* gene for phosphatidylglycerophosphate (PGP) synthase in chloroplasts showed an impairment of photosynthesis and inhibition of growth and chloroplast development, indicating that PG is essential for growth and chloroplast development (Hagio et al., 2002; Xu

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et al., 2002; Babiychuk et al., 2003). The function of PG has also been studied with *Synechocystis* sp. PCC6803 mutants with the disruption of the *pgsA* or the *cdsA* gene, encoding a PGP synthase and a CDP-diacylglycerol synthase, respectively (Hagio et al., 2000; Sato et al., 2000), which have severe defects in growth and photosynthesis. The growth and concomitant photosynthetic activity of these mutants have an absolute requirement for PG supplementation and vary with the level of PG that can be manipulated in the mutant cells by exogenous addition of PG, indicating the spe-

cific dependence of the function of photosynthesis on PG concentration. Deprivation of PG induced the impairment of the electron transfer from the primary electron-accepting plastoquinone of PSII to the secondary electron-accepting plastoquinone of PSII (Q_B) within PSII and the conformational change at and/or in the environment of the Q_B -binding site (Gombos et al., 2002), consistent with the in vitro observations that the PSII activity decreased with depletion of PG by phospholipase A_2 (remove the β -positioned fatty acyl chains of the phospholipid molecules) treatment

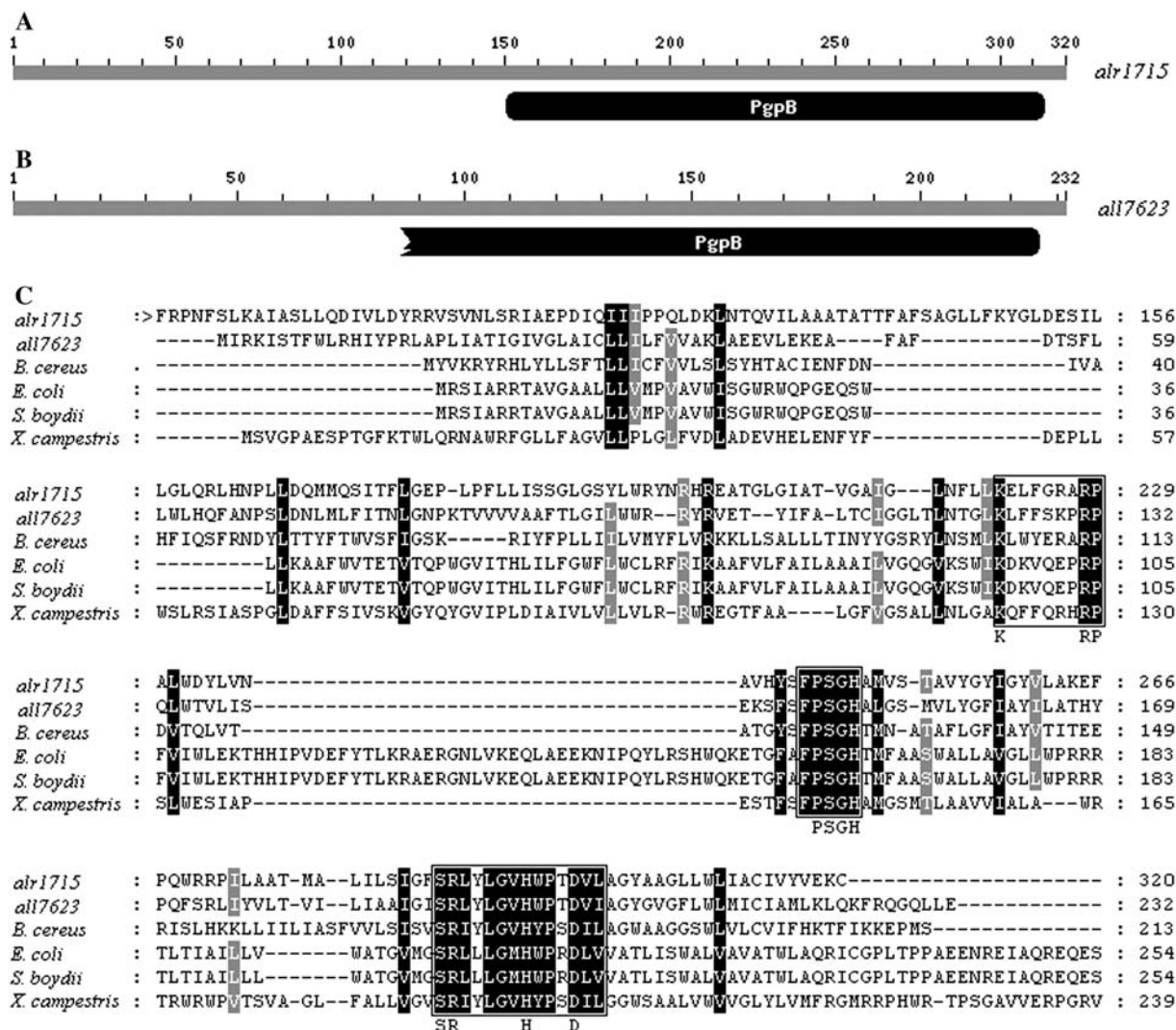


Figure 1. A and B, The National Center for Biotechnology Information conserved domain database-identified domains for a putative phosphatidylglycerophosphatase in *Anabaena* sp. PCC7120. The gray line represents the amino acid sequence of *alr1715* or *all7623* gene of *Anabaena* sp. PCC7120. Domains are represented in black. PgpB represents the domain of phosphatidylglycerophosphatase B, a membrane-associated phospholipid phosphatase involved in phospholipid metabolism. C, Sequence alignments of *alr1715* and *all7623* of *Anabaena* sp. PCC7120 with *pgpB* genes of nonphotosynthetic bacteria. The amino acid residues are numbered on the right. Identical residues are highlighted in black. Residues conserved in 80% are shown in gray. Gaps (indicated by dashes) were introduced to optimize sequence alignment. The symbol > indicates that parts of the protein of *alr1715* have been omitted because they show no similarity to *pgpB*. The amino acid sequence *alr1715* gene exhibits 37% identity with that of *all7623* and 32% to 34% identity with the sequences of other nonphotosynthetic bacterial *pgpB* genes: *E. coli* (32%), *B. cereus* (33%), *S. boydii* (32%), and *X. campestris* (34%). Conserved phosphatase domains (1, 2, and 3) are boxed. Amino acids within those domains indicated below the box are believed to be essential for catalytic activity (Hemrika et al., 1997; Neuwald, 1997; Stukev and Carman, 1997).

(Jordan et al., 1983; Siegenthaler et al., 1987; Kruse et al., 1994). These findings demonstrate that PG is indispensable for the functioning of PSII.

In all organisms, PG is synthesized from phosphatidic acid (PA) in three steps. CDP-diacylglycerol (CDP-DG) is first synthesized by transfer of CMP from CTP to PA with the action of CTP: phosphatidate cytidylyl-transferase (EC 2.7.7.41; Carter and Kennedy, 1966). CDP-DG is then converted to PGP catalyzed by PGP synthase (EC 2.7.8.5; Hirabayashi et al., 1976; Bleasdale and Johnston, 1982), which transfers the phosphatidyl group from CDP-DG to *sn*-glycerol-3-P. The last step is the rapid dephosphorylation of PGP by PGP phosphatase (EC 3.1.3.27; Chang and Kennedy, 1967; Moore, 1982; Andrews and Mudd, 1986) to produce PG. The genes encoding CDP-DG synthase and PGP synthase have been identified for cyanobacteria and higher plants (Kopka et al., 1997; Hagio et al., 2000, 2002; Sato et al., 2000; Xu et al., 2002; Babiychuk et al., 2003), but the gene for PGP phosphatase has not been cloned from photosynthetic organisms.

Although biochemical and genetic results have established that PG is indispensable for photosynthetic organisms and is necessary for photosynthesis process, it has not as yet been unambiguously demonstrated. There still exists the possibility that either the precursor of PG biosynthesis, PGP, or the metabolite of PG biosynthesis, cardiolipin, which remains to be clarified in thylakoid membranes, serves as an essential component for photosynthetic organisms.

It is thus of particular interest to clone the gene involved in the biosynthesis of PGP. Two genes encoding for PGP phosphatase, *pgpA* and *pgpB*, have been characterized in the nonphotosynthetic bacterial strain *Escherichia coli* (Icho, 1988a, 1988b). Through a similarity search of the DNA sequence of the genome of a filamentous, nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC7120 (Kaneko et al., 2001), we found that two open reading frames, *alr1715* and *all7623*, show a significant sequence similarity to *pgpB* of *E. coli* and probably encode the PGP phosphatase in *Anabaena* sp. PCC7120. In this study, we produced a mutant by inactivation of the gene *alr1715* through homologous recombination and have determined the photosynthetic characteristics of this mutant.

RESULTS

Disruption of the Gene *alr1715* in *Anabaena* sp. PCC7120

It was demonstrated that there are at least two genes, *pgpA* and *pgpB*, encoding for membrane-associated phospholipid phosphatases in *E. coli* (Icho, 1988a, 1988b). The *pgpA* gene product is specific for PGP, whereas the *pgpB* gene product has the ability to dephosphorylate PGP, PA, and lysophosphatidic acid (Icho and Raetz, 1983). The product of *E. coli* *pgpB* gene also exhibits diacylglycerol pyrophosphate phosphatase activity (Dillon et al., 1996). Using the gene products of *pgpA* and *pgpB* from *E. coli* in a similarity

search for *Anabaena* sp. PCC7120 genomic sequences, we observed two proteins encoded, respectively, by the genes *alr1715* and *all7623* showing the remarkable similarities to that of *pgpB* in *E. coli*. The sequence alignment indicated that the amino acid sequence deduced from the gene *alr1715* exhibits about 32% identity and 46% similarity over 320 amino acids with that of *E. coli* *pgpB* gene, while the gene *all7623* has 40% identity and 56% similarity over 232 amino acids to the *E. coli* *pgpB* gene product. *alr1715* and *all7623* have conserved domains from the 151st to 314th and from the 65th to 226th amino acid residue, respectively, at their C terminus with phosphatidylglycerophosphatase B (PgpB), as revealed by the result of a conserved domain search in the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih>).

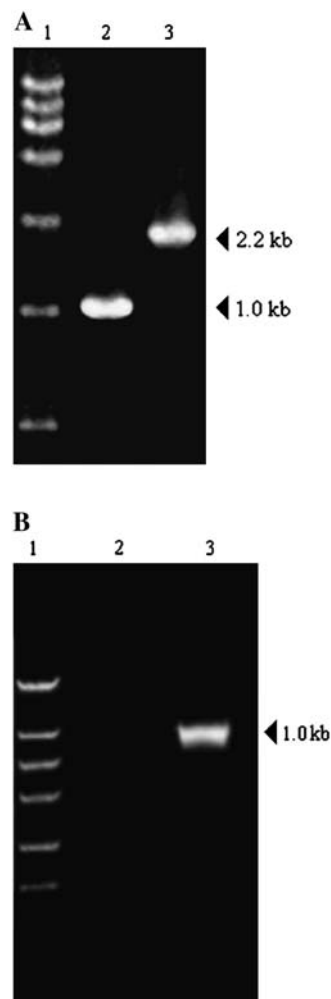


Figure 2. Confirmation of a disruptant of *alr1715* from *Anabaena* sp. PCC7120. A, PCR analysis of *alr1715* in the wild type (lane 2) and the mutant of *Anabaena* sp. PCC7120 (lane 3). Lane 1 is the DNA marker DL15 000 (TaKaRa Bio), with sizes of 15, 10, 7.5, 5, 2.5, 1, and 0.25 kb from top to bottom. The sizes of amplified fragments are indicated on the right. B, Confirmation of the *pgpB* gene mutation by RT-PCR. Lane 1, marker DL 2 000, with sizes of 2, 1, 0.75, 0.5, 0.25, and 0.1 kb from top to bottom; lane 2, the *pgpB* mutant; lane 3, the wild type.

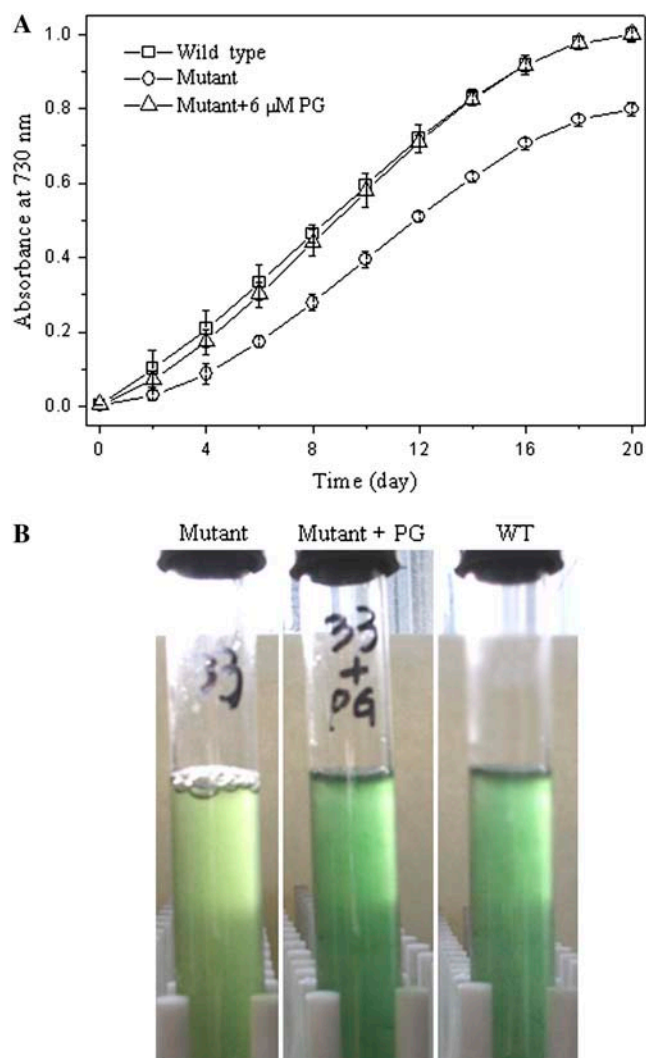


Figure 3. Growth curves of the wild-type strain and the *pgpB* mutant cultured in liquid medium BG11 with and without the addition of PG at 30°C. A, Samples (1 mL) were taken and their optical density at 730 nm was measured. The data are presented as the means for four independent measurements. B, Cells of the wild type (right), mutant (left), and mutant with the addition of exogenous PG (middle) were grown in liquid medium BG11 for 10 d.

gov/structure/cdd/wrpsb.cgi; Fig. 1, A and B). The polypeptide encoded by the gene *alr1715* or *all7623* was also found to be homologous to PGP phosphatases of other nonphotosynthetic bacterial strains such as *Bacillus cereus* (Ivanova et al., 2003), *Shigella boydii* (Yang et al., 2005), and *Xanthomonas campestris* (da Silva et al., 2002; Fig. 1C). Several regions of the deduced amino acids of *alr1715* and *all7623* were observed to be conserved to the nonphotosynthetic bacterial PGP phosphatases, as indicated in Figure 1C. The conserved regions contain a larger sequence motif (boxed in Fig. 1C) that is shared by a superfamily of phosphatase enzymes. This phosphatase signature motif has three conserved domains with the consensus sequences KXXXXXXRP (domain 1, the sequence nearest

the N terminus), PSGH (domain 2, the centrally located sequence), and SRXXXXHXXXXD (domain 3, the sequence nearest the N terminus), where X is any amino acid (Hemrika et al., 1997; Neuwald, 1997; Stukey and Carman, 1997). Separating each of the three domains are protein segments ranging from 12 to 54 amino acids in length. Amino acids within this larger motif are believed to be essential for catalytic activity (Brindley and Waggoner, 1998; Sigal et al., 2005). Indeed, we found that the product of the gene *alr1715* or *all7623* belongs to the superfamily of type 2 PA phosphatase, including the enzyme PAP2, Glc-6-P (EC 3.1.3.9), PgpB (EC 3.1.3.27), and bacterial acid phosphatase (EC 3.1.3.2).

In this work, we have investigated the mutation of the gene *alr1715* of *Anabaena* sp. PCC7120 and characterized the photosynthetic properties of this mutant. Owing to remarkable sequence similarity to the *pgpB* gene of *E. coli*, this gene *alr1715* is therefore also designated *pgpB* in this study. The gene *alr1715* was disrupted by insertion of a kanamycin-resistant cassette into the coding region of *alr1715*, as described in "Materials and Methods." The transformation was conducted with the construct pPGP3 that was transformed into the wild type of *Anabaena* sp. PCC7120 through homologous recombination using the triparental mating method (Elhai and Wolk, 1988).

To check whether or not all copies of this gene were disrupted in the genome of the mutant with the neomycin-resistant phenotype, we carried out PCR analysis of the wild type and the mutant, with primers for the 5'- and 3'-terminal regions of the putative *pgpB* gene. For the wild type, an approximately 1.0-kb DNA fragment corresponding to the native *pgpB* gene was amplified. In the case of the mutant, a 2.2-kb DNA fragment corresponding to the *pgpB::Kan^r* gene owing to insertion of the 1.2-kb cassette was observed, but a 1.0-kb DNA fragment corresponding to the native *pgpB* gene was not amplified (Fig. 2A). To determine if any transcript for the gene is still present in this mutant, reverse transcription (RT)-PCR was performed to examine *pgpB* expression in the wild type and *pgpB* mutant. Figure 2B shows RT-PCR results using total RNA isolated from the wild-type and *pgpB* mutant cells. No *pgpB* transcription product was detected in

Table 1. Cellular lipid composition of the wild type and the *pgpB* mutant of *Anabaena* sp. PCC7120

Each value represents the means of two independent experiments.

Strain	Lipid				
	PG	MGDG	DGDG	SGDG	PG
	mol%				
Wild type	+ ^a	37.0	19.8	17.0	26.2
	- ^b	34.0	22.5	19.5	24.0
Mutant	+	36.6	21.0	20.8	21.6
	-	31.9	23.6	28.5	16.0

^aCells were grown in the presence of 6 μM PG for 10 d. ^bCells were grown without exogenous PG.

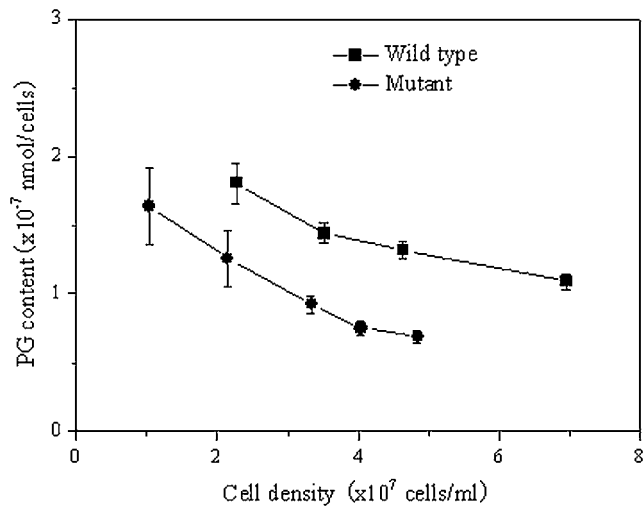


Figure 4. Changes in PG content of the *pgpB* mutant during the growth in liquid medium BG11. The data are presented as the means for three measurements.

the *pgpB* mutant cells (lane 2). This was in contrast to the wild-type cells, where an approximately 1.0-kb transcription product was observed (lane 3). Thus, the *pgpB* gene was successfully knocked out.

Growth Phenotype of the Mutant

To investigate the role of the putative *pgpB* gene in growth, the growth profiles of the wild type and the *pgpB* mutant under photoautotrophic growth condition were investigated. Figure 3 shows the growth of the wild-type and *pgpB* mutant cells in the liquid growth medium BG11. It can be clearly seen that although both the wild type and *pgpB* mutant could grow, a different growth existed between the wild type and the mutant. The mutant showed a slower growth than the wild type (Fig. 3A). Even after lengthy growth, the mutant was pale green in appearance, while the wild type remained normal blue green (Fig. 3B). However, similar growth curves were observed for the wild type and the *pgpB* mutant when the growth medium was supplemented with exogenous PG at concentrations above $2 \mu\text{M}$ (Fig. 3A). In this situation, the mutant appeared blue green (Fig. 3B).

Lipid Phenotype of the Mutant

To explore the effect of the *pgpB* gene on the lipid composition of cyanobacterial cells, lipids from both the wild type and the mutant were extracted and analyzed. Table I shows the composition of lipid classes from intact cells of the wild type and the *pgpB* mutant cultured in the liquid medium BG11. In the wild type, MGDG was the most abundant lipid, making up 34% of the total lipids. DGDG and PG were second, each accounting for about 23% to approximately 24%. The amount of SQDG was only 20%. A major finding from the data is the clear changes in the contents of two acidic lipids, SQDG and PG. The relative amount of

PG was reduced from 24 mol% in wild-type cells to 16% in the *pgpB* mutant. Concomitantly, the content of SQDG was increased in the mutant (29%) compared with the wild type (20%). The relative amount of MGDG and DGDG were slightly changed in the mutant with respect to the wild type, indicating that the *pgpB* mutant is little affected as to galactolipid metabolism in *Anabaena* sp. PCC7120.

The changes in the content of PG in the *pgpB* mutant cells with growth were also determined. Figure 4 shows changes in the content of PG in intact cells of the wild type and the *pgpB* mutant. During incubation with the culture medium BG11 for 10 d, the content of PG in the wild-type cells slightly decreased from 1.8 to 1.1×10^{-16} mol cells $^{-1}$. By contrast, the content of PG in the *pgpB* mutant cells markedly decreased from 1.6 to 0.6×10^{-16} mol cells $^{-1}$. However, the *pgpB* mutant cells with the addition of exogenous PG showed a near

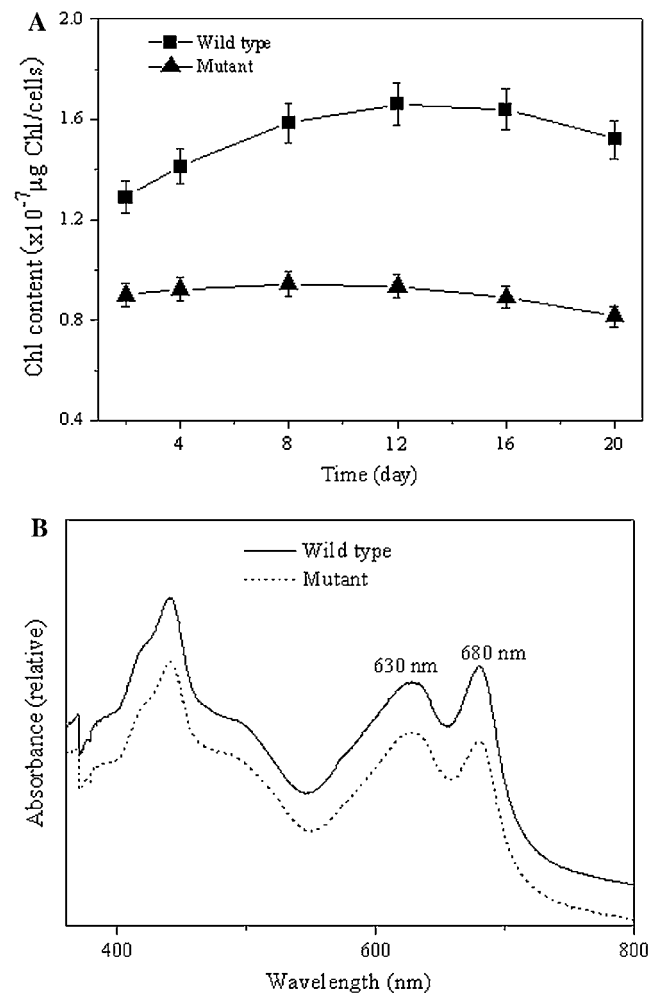


Figure 5. Chl content and absorption spectra of the wild type and the *pgpB* mutant. A, The values of Chl content are on a per-cell basis. The data are presented as the means for four independent measurements. B, The spectra were measured using whole cells of the wild type and mutant. Cells were grown in BG11 for 10 d. The spectra of cells are normalized at A_{730} .

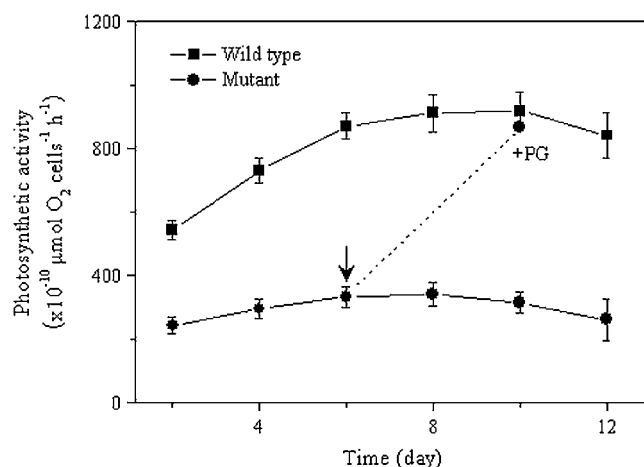


Figure 6. Changes in the photosynthetic activities of the wild-type and *pgpB* mutant cells. The wild-type and *pgpB* mutant cells were washed with BG11 and resuspended in fresh BG11 medium for measurement of photosynthetic activity with 10 mM NaHCO₃. The values are the means of three independent measurements. The arrow indicates the time of addition of 6 μM PG.

recovery of PG content to the level of PG in the wild type (Table I). These results suggest that the synthesis of PG in the *pgpB* mutant is affected and the *pgpB* gene is involved in the biosynthesis of PG.

Characteristics of Photosynthetic Pigments in the Mutant

To check the effect of the *pgpB* mutation on the photosynthetic pigments, the cellular content of chlorophyll (Chl) *a* and phycobilisome pigments were investigated for the wild type and *pgpB* mutant. A reduction in Chl *a* levels was observed in the mutant. During the growth phase, the Chl *a* content on a per-cell basis increased slightly for the wild type, whereas it changed little for the *pgpB* mutant, being only about 54% of the level in the wild type (Fig. 5A). In the meantime, the phycobilisome pigments such as phycocyanin and allophycocyanin in the mutant were little affected throughout growth and maintained at almost the same levels, similar to the wild type (data not shown). These findings indicate that the contents of Chl-protein complexes were reduced and the levels of phycobilisomes were nearly constant for the *pgpB* mutant cells following the decrease in the content of PG.

In accordance with the above results, the *pgpB* mutant cells showed the lower Chl absorbance, with a decrease in the ratio of Chl absorption at 680 nm to phycocyanin absorption at 630 nm, as compared to the wild type (Fig. 5B).

Photosynthetic Characteristics of the Mutant

To characterize the effect of a decrease in the PG content on the oxygenic photosynthesis, the photosynthetic oxygen evolution activity (net photosynthesis, water to carbon dioxide) was determined for the *pgpB* mutant (Fig. 6). During the growth in liquid culture

medium BG11, the photosynthetic activity on a per-cell basis of intact cells of the wild type increased gradually. By contrast, the activity of intact cells of the *pgpB* mutant remained almost constant. However, the CO₂-dependent photosynthesis activity in the wild type was always higher than that in the *pgpB* mutant at the same culture time (Fig. 6A). The reduced photosynthetic activity of the mutant cells could, however, reach the similar level of the activity of the wild-type cells following addition of 6 μM PG to the growth medium for a further 4 d. These results indicate that the decrease in PG content brought about reduction in the cellular content of Chl *a* and, consequently, in the photosynthetic activity of a cell.

The effect of the reduced content of PG on PSII activity with an artificial electron acceptor, 2,6-dichloro-*p*-benzoquinone (DCBQ), was also determined (Table II). As shown in Table II, the PSII activity in intact cells of the wild type and the *pgpB* mutant was essentially the same in the presence of PG. However, the *pgpB* mutant, in comparison with the wild type, showed about 73% of decrease in PSII activity when the cells were grown without the added PG. It is known that DCBQ shows an affinity for accepting electrons from the primary electron-accepting plastoquinone of PSII site of the D2 protein in the PSII reaction center (Schneider et al., 2004). These data suggest that the changes in the conformation of the PSII reaction center are induced by the decrease of the PG content in the *pgpB* mutant and the affinity of DCBQ to Q_B site is thereby changed. Consistent with the above results, the *pgpB* mutant showed decreased values in dark-level fluorescence yield (F_0), the maximum fluorescence yield (F_m), and therefore a lower variable fluorescence yield (F_v). Consequently, the ratio of F_v/F_m , which is a measure of the photochemical efficiency of PSII in the dark-adapted state, was decreased in the mutant (Table III).

To investigate further alterations of the pigment-protein complexes and the excitation energy transfer to the reaction centers, low temperature fluorescence spectra were measured for the wild type and the *pgpB* mutant. The 77 K fluorescence spectra of wild-type and mutant strains obtained after Chl *a* excitation at

Table II. Net photosynthetic and PSII activities in intact cells of the wild type and the *pgpB* mutant of *Anabaena* sp. PCC7120

Net photosynthetic activity was measured for cells in BG11 medium containing 10 mM NaHCO₃, while PSII activity was assayed in BG11 medium containing 0.25 mM DCBQ and 0.25 mM ferricyanide at 30°C. All values represent the means (±SE) of three independent measurements.

Strain	PG	Net Photosynthetic Activity		PSII Activity	
		μmol O ₂ 10 ⁻¹⁰ cells ⁻¹ h ⁻¹		μmol O ₂ 10 ⁻¹⁰ cells ⁻¹ h ⁻¹	
Wild type	+ ^a	895 ± 40	1,432 ± 60		
	- ^b	918 ± 50	1,515 ± 80		
Mutant	+	868 ± 55	1,142 ± 60		
	-	314 ± 15	414 ± 20		

^aCells were grown in the presence of 6 μM PG. ^bCells were grown without exogenous PG.

Table III. Chl fluorescence parameters obtained with intact cells of the wild type and the *pgpB* mutant of *Anabaena* sp. PCC7120

Samples in BG11 medium were adjusted to equal numbers of cells that corresponded to 5 μg Chl/mL for wild-type cells. ($F_v = F_m - F_0$). All values represent the means of four independent measurements.

Parameters	Wild Type	Mutant
F_0	400 \pm 4	312 \pm 5
F_m	632 \pm 10	395 \pm 6
F_v	232 \pm 3	83 \pm 3
F_v/F_m	0.37 \pm 0.05	0.21 \pm 0.04

436 nm are shown in Figure 7. There are three distinct emission maxima at 684, 693, and 728 nm. The large emission peak at 728 nm is attributed to emanate primarily from PSI antenna, whereas the two peaks at 684 and 693 nm can be ascribed to emit mainly from the PSII core antenna complexes CP43 and CP47, respectively (Sato et al., 1995). No position shift in the Chl fluorescence emission maxima was observed for both strains, indicating the complete assembly of PSII and PSI complexes in both wild type and mutant (Hartel et al., 1998). However, the ratio of PSII-associated fluorescence to PSI fluorescence was markedly reduced in the mutant cells as compared to the wild type. It reflects that there is a decrease in the number of PSII units, while the number of PSI units remains almost the same in the mutant (Krause and Weis, 1991). In addition, the magnitude of fluorescence emission at 684 nm slightly decreased relative to the 693 nm fluorescence emission in the *pgpB* mutant. These findings demonstrated that the molecular environment of the PSII core complexes and PSI is almost not affected, but more excitation energy is redistributed toward PSI in the mutant.

DISCUSSION

In this study, we isolated a mutant of *Anabaena* sp. PCC7120 through the disruption of a gene, *alr1715*. This gene shares considerable sequence identity with the *pgpB* genes of several nonphotosynthetic bacterial organisms (e.g. *E. coli* and *B. cereus*). A 320-amino acid sequence of this gene has a conserved domain at its C terminus with PgpB. In addition, there is very little similarity between the amino acid sequence of this gene and that of the PGP synthase (*all4063*) or the phosphatidate cytidylyltransferase (*all3875*) of *Anabaena* sp. PCC7120 (data not shown). Furthermore, inactivation of this gene could bring about a 30% reduction in PG content of intact cells, and supplementation of the *alr1715* mutant with exogenous PG could restore a near wild-type state. Taken together, we believe that the *alr1715* gene is involved in the pathway of PG biosynthesis in *Anabaena* sp. PCC7120 and most likely encodes a PGP phosphatase that catalyzes the formation of PGP.

The *alr1715* mutant has the PG content reduced to only about 70% of that found in the wild type; the majority of PG was still present in this mutant. In

addition, there is no transcript for this gene in the mutant as confirmed by RT-PCR, revealing that it is unlikely that the disrupted gene is still partially functional. These facts indicate that there is at least one other gene that encodes PGP phosphatase and thus accounts for the presence of residual PG in the *pgpB* mutant of *Anabaena* sp. PCC7120. Indeed, we found that one open reading frame, *all7623*, is highly homologous to the putative *pgpB* gene *alr1715* through a similarity search of the genome of *Anabaena* sp. PCC7120 (37% identity and 53% similarity over 320 amino acids). The question of whether there is gene superfamily for PGP phosphatase in *Anabaena* sp. PCC7120 needs to be clarified and is currently under investigation in our group. In *E. coli*, at least two genes, *pgpA* and *pgpB*, and gene products responsible for the in vivo conversion of PGP to PG were identified (Icho, 1988a, 1988b). A third gene locus for PGP phosphatase was proposed to exist (Funk et al., 1992). These results suggested that dephosphorylation of PGP has more than two independent genes associated with a single metabolic activity in *E. coli*.

Although only 30% of PG content in the *pgpB* mutant was reduced in comparison with the wild type, the visible phenotype and photosynthetic activity were considerably affected. The *pgpB* mutant showed a reduced photoautotrophic growth and a pale blue-green appearance. This is similar to the null PG-deficient strains of *Synechocystis* sp. PCC6803 (Hagio et al., 2000; Sato et al., 2000), where PG supplementation was required for the growth. In the presence of a relatively lower concentration of exogenous PG or shift to PG-free medium upon preincubation with PG supplementation, these mutants of *Synechocystis* sp. PCC6803 exhibited a slow growth and even stopped growing.

The alteration of visible phenotype in the *pgpB* mutant could be related to the changes in pigment

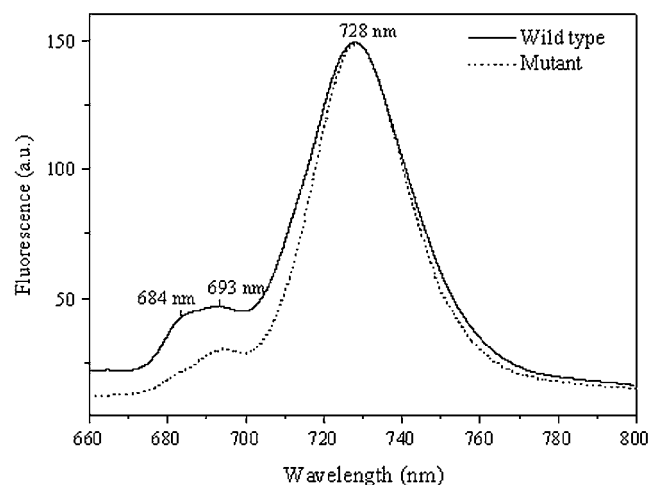


Figure 7. The 77 K fluorescence emission spectra of *Anabaena* sp. PCC7120 wild type and *pgpB* mutant following Chl *a* excitation at 436 nm. Spectra were normalized to the large emission maximum at 728 nm.

composition and photosynthetic light utilization. Indeed, as compared to the wild type, the *pgpB* mutant had a reduction in the content of Chl *a* on a per-cell basis, the sole Chl species of PSI and PSII complexes, with phycobilisome pigments being less affected, suggesting the decreased levels of Chl-protein complexes in mutant cells with decreasing PG content.

Low temperature fluorescence measurements suggest that the PG deficiency in the mutant led to the structural modifications of the reaction center/core antenna complex of PSII. The mutant showed no change in the PSI and PSII fluorescence maxima positions following Chl *a* excitation at 436 nm, suggesting that the molecular environment or binding environment of the Chl *a* emitting from the core antenna proteins CP43 and CP47 of PSII core antenna complexes and the Chl *a* from PSI core antenna complexes are not affected by the PG reduction in the mutant. However, the mutant spectrum is characterized by a decrease in the magnitude of the fluorescence emission of PSII. This effect reflects that PG deficiency causes the structural changes (reduction) of the PSII antenna and/or redistribution of excitation energy more in favor of PSI.

The decrease in the low temperature Chl fluorescence ratio of PSII to PSI together with the decreased Chl content in the *pgpB* mutant indicated a decreased number of PSII (Vermaas et al., 1994; Wilde et al., 1995). The reduction in the CO₂-dependent photosynthesis activity and oxygen evolution activity of PSII under saturating light was also observed for the mutant. Together, these results may explain the decreased variable Chl fluorescence yield of PSII in the mutant (Chl fluorescence at room temperature; Table II), because the decrease in the variable Chl fluorescence yield could arise from either a decreased activity of PSII reaction centers or a decreased number of PSII as well as an increased number of PSI reaction centers (Vermaas et al., 1994; Wilde et al., 1995). Corresponding to the decrease in F_v/F_m that reflects a decrease in the quantum yield of photochemistry of PSII, the function of the PSII reaction center was also impaired by PG reduction in the mutant, as the Q_B-binding site of PSII was affected, which may be caused by depletion of PG from PSII subunits such as of D1 protein in the *pgpB* mutant, further indicating the modifications in the conformation of the PSII complex.

The *pgpB* mutant exhibited a decrease in PG content, which was accompanied concomitantly by an increase in the content of SQDG, the other anionic lipid of cyanobacteria and chloroplasts. It has been thought that photosynthetic organisms require constant anionic lipid content by keeping a certain total level of PG and SQDG; if necessary, the two anionic lipids could substitute for each other to some extent, such that the photosynthetic apparatus can function properly and normally. It was found that several photosynthetic organisms, depending on available sources of nutrition, could reciprocally adjust the PG and SQDG contents and maintain the level of the sum of these anionic

lipids (Benning et al., 1993; Sato et al., 1995; Guler et al., 1996; Essigmann et al., 1998; Yu et al., 2002; Aoki et al., 2004). However, the increased SQDG in this *pgpB* mutant could not recover its decreased CO₂-dependent photosynthetic activity and DCBQ-dependent PSII activity, suggesting the different role for PG and SQDG in maintenance of thylakoid properties in *Anabaena* sp. PCC7120, as suggested by Aoki et al. (2004).

In summary, we generated and characterized a PG-deficient mutant from *Anabaena* sp. PCC7120 with the disrupted *alr1715* gene. The inactivation resulted in a reduction by 30% in the PG content and had a marked effect on growth, Chl levels, photosynthetic oxygen evolution, and photochemistry, particularly associated with PSII activity. The phenotype of the mutant was similar but less severe than those for other *Synechocystis* PG biosynthesis mutants that have reductions in PG by as much as 80% to 90% and require exogenous PG supplements for growth. These findings indicate that the *alr1715* gene of *Anabaena* sp. PCC7120 is involved in the biosynthesis of PG and is essential for photosynthesis. Therefore, this mutant provides a promising opportunity toward understanding the biosynthesis and function of PG in photosynthetic organisms.

MATERIALS AND METHODS

Cell Culture

Wild-type and mutant cells of *Anabaena* (*Nostoc*) sp. strain PCC 7120 (in this study referred to as *Anabaena* sp. PCC 7120) were grown photoautotrophically in BG11 medium (Allen, 1968) at 30°C under continuous illumination by white fluorescent light at mean light intensity of 35 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. For growth on plates, the medium was solidified with separately autoclaved 1.5% (w/v) agar. When appropriate, antibiotics were added to plates at the following final concentrations: erythromycin, 30 $\mu\text{g/mL}$; and neomycin, 25 $\mu\text{g/mL}$. For growth in liquid cultures, wild-type and mutant cells were shaken in flasks, but mutants were grown in the presence of 100 to 150 μg neomycin/mL.

Isolation of DNA

Cells of *Anabaena* sp. PCC7120 grown in the mid-log phase of growth were harvested by centrifugation (5,000g, 10 min, 4°C) and suspended in a buffer with 10 mM Tris and 0.1 mM EDTA, pH 7.5. DNA was isolated from these cells as described in Cai and Wolk (1990) and Goyal (1992).

Construction of Mutant Strains with Disruption of the Putative *pgpB* Gene

Two primers were designed for PCR to clone the putative *pgpB* gene on the genome of *Anabaena* sp. PCC7120, with upper primer CCGCAATGGTTT-TACTCTGTGTTTATCCTCTATC (*Nco*I) and lower primer CGTCTGCAGTTA-GAGGCACAAATTCTAGAGGTA (*Pst*I). Both primers have three extra bases and one *Nco*I or *Pst*I restriction site (underlined) at the 5' end, respectively. PCR was performed according to standard procedures using Taq DNA polymerase with a DNA Thermal Cycler (Perkin-Elmer) under the following thermocycle conditions: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, and 10 min at 72°C at end to allow complete elongation of all product DNA.

A 4,822-bp DNA fragment containing the gene *alr1715* with a 962-bp length was amplified by PCR with the genomic DNA of *Anabaena* sp. PCC7120 as a template and then ligated into pMD18-T vector at its *Eco*RV restriction site for production of the plasmid pPGP1. At one restriction site, *Xba*I of the *alr1715*

gene of the pPGP1 plasmid, we inserted the kanamycin phosphotransferase gene (1.2 kb) from pUC4K by digestion with *Sall* to yield a plasmid, pPGP2. The pPGP2 plasmid containing the disrupted putative *pgpB* gene was then cloned to the *NcoI/SphI*-digested conjugative plasmid pRL271, a cloning vector for *sacB*-mediated positive selection for targeted gene replacement and double recombinants in cyanobacteria (Black et al., 1993). The resultant plasmid was designated as pPGP3 and conjugated into *Anabaena* PCC 7120 as previously described (Cai and Wolk, 1990). The initial conjugants were selected by screening for resistance to 25 $\mu\text{g}/\text{mL}$ of neomycin and were then cultured on BG11 plates containing 5% (w/v) Suc to select for double recombinants lacking the *sacB* gene (Black et al., 1993). Double recombinants were identified by the Suc- and neomycin-resistant and erythromycin-sensitive phenotype. In all cases, the genomic DNA of the resultant mutant strain was checked by PCR and Southern analysis.

RNA Isolation and RT-PCR

RNA from *Anabaena* sp. PCC 7120 was prepared as described previously (Golden et al., 1987). The resulting RNA preparations were digested with RNase-free DNase to eliminate residual DNA. RT-PCR was performed using the RT-PCR kit (Promega).

Lipid Analysis

The total lipids were extracted from the cells of the wild type and the mutant according to the method of Sato and Murata (1988). Individual lipid classes were separated from the total lipid extract by two-dimensional thin-layer chromatography, with a solvent system consisting of chloroform/methanol/water (65:25:4, v/v/v) for the first dimension and chloroform/methanol/ammonia (65:35:5, v/v/v) for the second dimension. Individual lipids were used to prepare fatty acid methyl esters with 5% (v/v) H_2SO_4 in anhydrous methanol. The methyl esters were analyzed by gas chromatography, and the lipid contents were calculated with heptadecanoic acid as an internal standard.

Determination of Pigment Contents

Chl was extracted with 100% methanol from cell pellets obtained from 1 mL of the wild-type and mutant cell cultures, and the Chl content was determined according to Porra et al. (1989). The concentrations of phycocyanin, allophycocyanin, and phycoerythrin were determined using the method of Tandeau de Marsac and Houmard (1988). All absorption spectroscopic determinations were performed on a UV8500 UV-VIS spectrophotometer (Scientific Instrument Network of China).

Measurement of Photosynthetic Activity

Photosynthetic O_2 evolution coupled with CO_2 fixation (net photosynthesis) was measured for intact cells equivalent to 2.5 μg Chl/mL in culture medium containing 10 mM NaHCO_3 as the electron acceptor with a Clark-type oxygen electrode (DW 1 Liquid Clark electrode, Hansatech Institute). PSII activity was measured in BG11 medium containing intact cells equivalent to 2.5 μg Chl/mL, 0.25 mM DCBQ was used as the electron acceptor, and 0.25 mM ferricyanide added to keep the quinones in an oxidized form. For each measurement, an aliquot of cell culture was spun down and resuspended in 1 mL of fresh BG11 medium. The reaction mixture was kept at 30°C and illuminated under saturating white light (approximately 1,000 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$).

Chl Fluorescence Measurements

Low temperature (77 K) Chl emission fluorescence spectra were recorded in a spectrofluorimeter (F-4500, Hitachi) with 5 nm/2.5 nm (excitation/emission) slit widths. For fluorescence measurements, cells were diluted in BG11 medium containing 20% glycerol to a final Chl concentration of 5 $\mu\text{g}/\text{mL}$ and frozen in liquid nitrogen (Schneider et al., 2001).

Room temperature Chl fluorescence was determined with a pulse amplitude modulation fluorometer (PAM101, Walz) according to the procedure of Clarke et al. (1993) and Schneider et al. (2004).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers BAB78081/Q8YWA0.

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