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Bifunctional enzyme FBPase/SBPase is essential for photoautotrophic growth in cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

From a random insertion mutant library of *Synechocystis* sp. PCC 6803, a mutant defective in photoautotrophic growth was obtained. The interrupted gene was identified to be *slr2094* (*fbp1*), which encodes the fructose-1,6-biphosphatase (FBPase)/sedoheptulose-1,7-biphosphatase (SBPase) bifunctional enzyme (F-I). Two other independently constructed *slr2094* mutants showed an identical phenotype. The FBPase activity was found to be virtually lacking in an *slr2094* mutant, which was sensitive to light under mixotrophic growth conditions. These results indicate that *slr2094* is the only active FBPase-encoding gene in this cyanobacterium. Inactivation of photosystem II by interrupting *psbB* in *slr2094* mutant alleviated the sensitiveness to light. This report provides the direct genetic evidence for the essential role of F-I in the photosynthesis of *Synechocystis* sp. PCC 6803.

Keywords: FBPase; Photoautotrophic growth; Light sensitivity; *Synechocystis* sp.

1. Introduction

In oxygenic photosynthetic organisms, the conversion of fructose-1,6-biphosphate to fructose-6-biphosphate is connected with Calvin cycle, oxidative pentosephosphate cycle and gluconeogenesis, representing an important regulatory step in each of these pathways. The hydrolytic dephosphorylation of fructose-1,6-bisphosphate is catalyzed by fructose-1,6-bisphosphatase (FBPase). In plants, two different FBPase forms are found in chloroplasts and cytoplasm [1,2]. In certain cyanobacterial species, such as *Synechococcus leopoliensis* [3,4] and *Synechococcus* sp. PCC 7942 [5], there are also two forms of FBPase. In other cyanobacterial species, such as *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120 and *Plectonema boryanum*, genes encoding the two FBPase forms are also found in genomes, but only one form of FBPase, F-I, is detectable with antiserum, while the second form F-II is probably not expressed under normal conditions [6].

Sedoheptulose-1,7-bisphosphatase (SBPase), which converts sedoheptulose-1,7-bisphosphate into sedoheptulose-7-phosphate, is another key enzyme in all photosynthetic organisms [7]. From sedoheptulose-7-phosphate, the CO_2 acceptor ribulose-1,5-bisphosphate (RuBP) is regenerated. The balance between export, regeneration of RuBP and starch storage is controlled by conversion of fructose and sedoheptulose bisphosphates into their monophosphate forms [8]. In plants, FBPase and SBPase are separate enzymes [8]. In cyanobacteria, F-I form FBPase also hydrolyzes sedoheptulose-1,7-bisphosphate at the carbon 1-ester [5,6].

Synechocystis sp. PCC 6803 (hereafter called *Synechocystis* 6803), a unicellular cyanobacterium, is naturally transformable and incorporates exogenous DNA into its genome via double homologous recombinations. Its entire genomic sequence has been available for more than a decade [9]. In addition to photoautotrophic growth, this cyanobacterium can perform heterotrophic growth. *Synechocystis* 6803

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mutants impaired in photosystems can grow on glucose under photoheterotrophic or light-activated heterotrophic growth conditions. Therefore, *Synechocystis* 6803 has been widely used as a research model for photosynthesis studies. By random insertion of a kanamycin resistance cassette into the genome of *Synechocystis* PCC6803, we constructed a mutant library of this cyanobacterium. Some mutants of this library were impaired in photoautotrophic growth. A mutant disrupted in *slr2094* lost the activity of fructose-1,6-/sedoheptulose-1,7-bisphosphatase bifunctional enzyme and photoautotrophic growth capability. Under mixotrophic conditions, this mutant showed sensitiveness to light.

2. Materials and methods

2.1. Strains, culture conditions and growth rates

Synechocystis sp. PCC 6803 from J. Zhao of Peking University was cultured in BG11 [10] under continuous illumination of 25 μE m^{-2} s^{-1} at 30 °C without shaking. The three growth conditions used in this study were defined according to Astier et al. [11] as photoautotrophic (light, BG-11), mixotrophic (light, BG-11, 5 mM glucose) and photoheterotrophic (light, BG-11, 5 mM glucose, 5 μm DCMU). Mutants were grown in the medium supplemented with kanamycin (10 μg/ml) or erythromycin (10 μg/ml). To prepare solid medium, 1.4% agar, 8 mM TES (pH 8.2) and 0.3% $Na_2S_2O_3$ were added to BG11. In addition to *Synechocystis* 6803, a derivative strain with a Km^r or Em^r marker inserted within a neutral platform [12] was referred to as the wild-type. The derivative strains (wt-1188) were identical to the starting strain (wt) in growth under different conditions.

To study the growth of the cyanobacterial strains, cells were grown under three different conditions at 30 °C starting from OD_{730} 0.05. The turbidity (OD_{730}) was measured every 24 h. Data presented were averages of measurements from three parallel cultures with standard deviation.

2.2. Recombinant DNA manipulations

DNA manipulations were performed according to standard protocols. Molecular tool enzymes were used, following the manufacturer's instructions. *Escherichia coli* DH5α was used as the transformation recipient. DNA fragments were retrieved from agarose gel with a glass milk kit (MBI). The purified PCR products were cloned using the T-vector pMD18-T (Takara). The Km^r marker, C.K2, used in the targeted interruption of a gene was excised from pRL446 [13] with PvuII. C.CE2, a chloramphenicol and erythromycin resistance cassette, was excised from pRL598 [14] with XbaI and purified.

2.3. Generation of cyanobacterial photoautotrophic mutants and identification of mutated genes from random insertion mutants

To select the photoautotrophic mutants, a random insertion DNA library of mutagenesis of *Synechocystis* 6803 was generated basically as previously described [15,16] with some improvements. The transformation of *Synechocystis* 6803 was performed according to Williams [12]. Photoautotrophic mutants were selected under photoheterotrophic and photoautotrophic conditions. Mutants showing a similar growth to the wild-type strain under photoheterotrophic conditions but little or no growth under photoautotrophic conditions were considered to be defective in photoautotrophic growth. The inserted genes were identified by inverse PCR using primers CK2-8 (5′-CC TCTTCCGACCATCAAGCA-3′) and CK2-15 (5′-TTGA GACACAACGTGGCT-3′), or CK2-16 (5′-ACTGGCA GAGCATTACGCTG-3′)/CK2-21 (5′-TCACCGAGGC AGTTCCATAG-3′) as previously described [15,16].

For targeted insertion of a gene, *Synechocystis* 6803 was transformed with plasmids or mutant genomic DNA. Transformants were repeatedly streaked on plates and cultured in liquid medium under the selective pressure of antibiotics until complete segregation was confirmed by PCR.

To inactivate *slr2094*, a 2.0 kb fragment generated by PCR using primers slr2094-1 (5′-TGCCTGGGGATGATA ACCG-3′) and slr2094-2 (5′-CTCGTAGTTTGCTTCGC CA-3′) was cloned into pMD18-T, and the C.K2 or C.CE2 cassette was inserted into the KpnI site of *slr2094*, resulting in plasmids pHB1485 and pHB1486, respectively. Sticky ends were blunted with T4 DNA polymerase before insertion with the C.K2 or C.CE2 cassette. *Synechocystis* 6803 was transformed with the two plasmids separately, resulting in mutants DRHB1485 and DRHB1486.

To inactivate *slr0906* (*psbB*) in the mutant DRHB1486, the genomic DNA of the *slr0906*::C.K2d mutant was used to transform the mutant DRHB1486 and the complete segregation was confirmed by PCR using primers slr0906-1 (5′-GCTAGCTTTGCCGCTTTCGT CGTG-3′) and slr0906-2 (5′-CTCGAGGAAGCCTTCACCCAAATGA-3′).

2.4. Fructose-1,6-bisphosphatase assays

Fructose-1,6-bisphosphatase activity was measured following the methods of Tamoi et al. [6] with modifications. Ten milliliters of cells grown under mixotrophic conditions was harvested by centrifugation (6000*g*, 10 min), and the resulting pellets were washed once with 50 mM potassium phosphate buffer (pH 8.0), then resuspended in 2 ml of solution A (50 mM potassium phosphate buffer (pH 8.0) containing 2.5 mM dithiothreitol, 1 mM glutathione and 10% sucrose). Cells were disrupted by sonication with a Sanyo Soniprep 150 sonicator equipped with a tapered micro-tip at 10 mm amplitude for a total of 1 min with five intervals of 10 s each. Centrifuge tubes containing the cells were immersed in ice bath to avoid heating from the sonication. Unbroken cells and cell debris were removed by centrifugation (4 °C, 12,000*g*, 10 min) and the supernatant containing crude extracts was used for the enzyme assays. One hundred microliters of the crude extract was directly added to a cuvette containing 2.9 ml of reaction buffer B (100 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.5 mM

EDTA, 0.4 mM Na_2NADP, 0.2 mM fructose-1,6-bisphosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase, 1.5 U/ml phosphoglucose isomerase) to initiate the reaction. The same mixture without the fructose-1,6-bisphosphate was used as the control. Enzyme activities were determined by measuring the production of NADPH in the reaction mixtures as a change in absorbance at 340 nm. Protein concentrations were determined with the Bradford method [17]. Data presented were averages of three independent assays with standard deviations.

3. Results and discussion

In order to identify genes required for photoautotrophic growth, we screened a random insertion mutant library of *Synechocystis* 6803 and obtained many mutants with impaired photoautotrophic growth. About 6000 random insertion colonies of the mutant library were maintained in BG11 with glucose using 96-well or Petri-dish plates. The mutants were tested in BG11 without glucose for autotrophic growth capability. Mutants showing no or little growth under photoautotrophic conditions were further screened. Interrupted genes in confirmed mutants, including *slr0906* (*psbB*) and *slr2094* (*fbp1*), were identified using inverse PCR and sequencing. *slr2094* encodes the fructose-1,6-/sedoheptulose-1,7-bisphosphatase bifunctional enzyme (F-I) [6]. In mutant 691-72, C.K2 was inserted at the HaeIII site 763 bp away from the 5′ end of *slr2094* (Fig. 1).

To exclude the possibility that the phenotype of mutant 691-72 might be caused by a second mutation, we independently constructed another *slr2094* mutant by targeted insertion. *Synechocystis* 6803 was transformed with pHB1485, in which the cloned *slr2094* was inserted at the KpnI site by C.K2, resulting in mutant DRHB1485 (Fig. 1). The complete segregation of the mutant was confirmed by PCR using primers slr2094-1 and slr2094-2 (Fig. 1). We assayed the fructose-1,6-bisphosphatase activity in the wild-type strain and the mutant DRHB1485. The mutant showed little fructose-1,6-bisphosphatase activity (1.33 ± 0.29 nm NADPH mg protein^{-1} min^{-1}), relative to the wild-type (62.27 ± 1.47 nm NADPH mg protein^{-1} min^{-1}). The targeted insertion mutant DRHB1485 showed a similar phenotype to the mutant 691-72. Under photoautotrophic conditions, DRHB1485 grew at 0.04 ± 0.01 doublings day^{-1}, while the wild-type at 0.89 ± 0.06 doublings day^{-1}. Under photoheterotrophic conditions, the mutant and the wild-type grew at similar rates (1.03 ± 0.01 and 1.11 ± 0.01 doublings day^{-1}, respectively). We also interrupted *slr2094* with C.CE2 at the KpnI site by transforming *Synechocystis* 6803 with pHB1486. The resulted mutant DRHB1486 showed the same phenotype as 691-72 and DRHB1485 (data not shown). Because three independent mutants showed an identical phenotype, we conclude that the impaired phototrophic growth must be attributed to the null mutation of *slr2094*. The two genes *slr2095* and *slr2096* downstream of *slr2094*, both encoding putative transposases, are unlikely required for the photoautotrophic growth. Polar effects could be excluded.

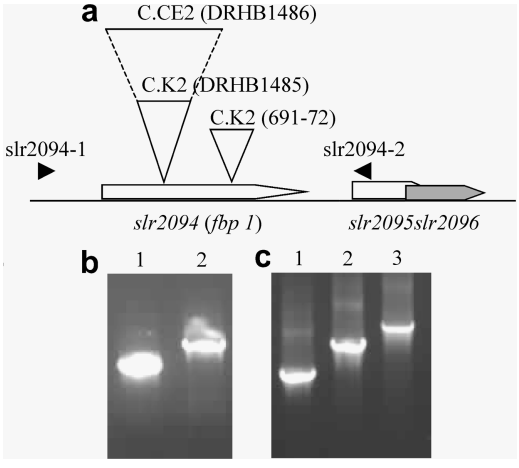

Fig. 1. Inactivation of *slr2094* (*fbp1*) in *Synechocystis* 6803. (a) Locations of C.K2 and C.CE2 in three independent mutants. (b) PCR analyses of the wild-type (1) and mutant 691-72 (2) using primers slr2094-1 and slr2094-2. (c) PCR analyses of the wild-type (1), mutants DRHB1485 (2) and DRHB1486 (3) using the same primers.

The products of reactions catalyzed by fructose-1,6-bisphosphatase or sedoheptulose-1,7-bisphosphatase in the Calvin cycle are precursors of many metabolites. Consequently, the activities of these two enzymes affect the partitioning of carbon sources among end products. Miyagawa et al. overexpressed the FBPase/SBPase gene *fbpI* from *Synechococcus* PCC 7942 in tobacco chloroplasts [18]. Compared with the wild-type, the transgenic plants showed higher photosynthetic CO_2 fixation, accelerated growth rate, increased levels of intermediates of the Calvin cycle and carbohydrates. The results reflected the role of FBPase/SBPase in the regulation of carbon flux in plant cells. Consistent with reports concerning higher plants, our results directly showed the role of FBPase/SBPase in photosynthesis in a cyanobacterium: inactivation of *slr2094* led to loss of photoautotrophic growth. Previously, it was proposed that F-I rather than F-II was expressed in *Synechocystis* 6803 [6]. We found that a very low or virtually no activity of FBPase remained in the F-I mutant DRHB1485. This finding indicated that the gene encoding F-II was silenced no matter with or without active F-I in *Synechocystis* 6803.

In higher plants, the expression of FBPase could be regulated by light [19,20]. In *Synechocystis* 6803, cells grown under light-activated heterotrophic growth conditions showed greatly reduced FBPase activity [6]. We assayed the FBPase activity of the wild-type strain grown mixotrophically under different light intensities and found that the activity remained basically unchanged in the range of 2–150 μE m^{-2} s^{-1} (Fig. 2). Therefore, the FBPase activity is not regulated by light in the cyanobacterium.

We compared the mixotrophic growth of mutant DRHB1486 and the wild-type at different light intensities. The mutant grew at a similar rate as the wild-type at a

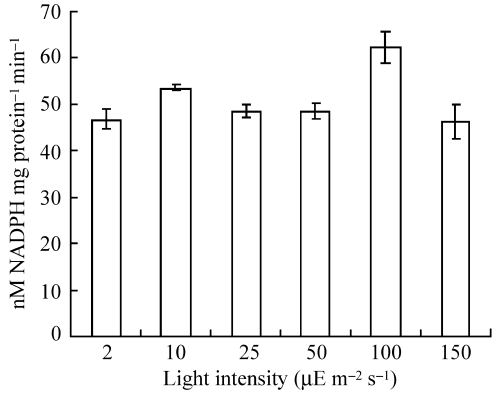

Fig. 2. The fructose-1,6-biphosphatase activity in *Synechocystis* 6803 grown mixotrophically under different light intensities.

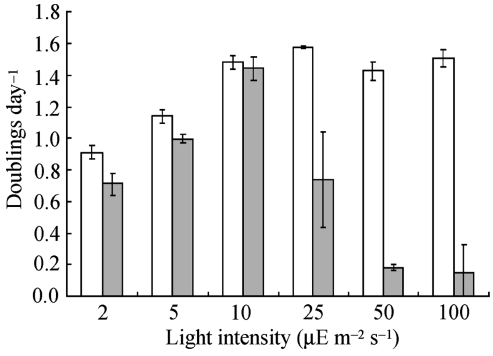

Fig. 3. Mixotrophic growth of *Synechocystis* 6803 wild-type strain and mutant DRHB1486 under different light intensities. Empty bars, the wild-type; grey bars, the mutant.

low light intensity but much more slowly at a high light intensity (Fig. 3). Because the *slr2094* mutant was unable to grow under photoautotrophic conditions and its mixotrophic growth was inhibited at relatively high light intensities, we hypothesized that certain accumulated intermediates (such as fructose-1,6-biphosphate and sedoheptulose-1,7-bisphosphate) were harmful to cyanobacterial cells and inhibited the mixotrophic growth of the mutant. We inactivated *psbB* in the DRHB1486 mutant. The *psbB* gene encodes the chlorophyll-binding protein CP-47 of photosystem II [21,22]. The resulted double mutant DRHB1486/*psbB*::C.K2 was unable to grow under photoautotrophic conditions as the single mutant DRHB1486, but showed significantly increased mixotrophic growth in the light of 25 μE m^{-2} s^{-1} compared with DRHB1486 (Fig. 4). In the presence of DCMU, an inhibitor of photosystem II, the three strains showed similar growth on glucose (Fig. 4). The resumption of mixotrophic growth of the *slr2094* mutant by inactivation of *psbB* supported our hypothesis. Based on this finding, it should be possible to enrich mutant cells defective in photosystem II with an *slr2094* null mutation in the future.

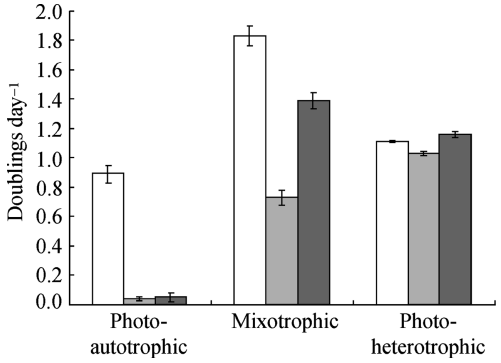

Fig. 4. Growth rate of *Synechocystis* 6803 wild-type strain (empty bars), DRHB1486 mutant (light grey) and DRHB1486/*psbB*::C.K2 double mutant (dark grey) under autotrophic, mixotrophic and photoheterotrophic conditions.

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