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Changes in photosynthesis and pigmentation in an *agp* deletion mutant of the cyanobacterium *Synechocystis* sp.

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Abstract

The *agp* gene encoding ADP-glucose pyrophosphorylase is involved in cyanobacterial glycogen synthesis. By *in vitro* DNA recombination technology, *agp* deletion mutant (*agp*$^-$) of cyanobacterium *Synechocystis* sp. PCC 6803 was constructed. This mutation led to a complete absence of glycogen biosynthesis. As compared with WT (wild type), a 60% decrease in ratio of the c-phycocyanine/chlorophyll *a* and no significant change in the carotenoid/chlorophyll *a* were observed in *agp*$^-$ cells. The *agp*$^-$ mutant had 38% less photosynthetic capacity when grown in light over 600 μmol m^{-2} s^{-1}. Under lower light intensity, the final biomass of the mutant strain was only 1.1 times of that of the WT strain under mixotrophic condition after 6 d culture. Under higher light intensity, however, the final biomass of the WT strain under mixotrophic conditions was 3 times that of the mutant strain after 6 d culture and 1.5 times under photoautotrophic conditions. The results indicate that there is a minimum requirement for glycogen synthesis for normal growth and development in cyanobacteria.

Introduction

Plant productivity is governed by photosynthetic activity and the capacity for utilizing photosynthate. It is therefore important to understand the environmental and genetic factors affecting these processes (Sun *et al.* 1999). In general, photosynthesis is limited by light harvesting and assimilatory power under low light and by CO_2 fixation and photorespiration under low CO_2. Under saturating light and CO_2, however, photosynthesis may be controlled by processes that convert triose phosphate into starch and sucrose. The rate of starch accumulation regulates the rate of carbon import, decrease in starch accumulation is sometimes accompanied by a loss of photosynthetic rate (Stark *et al.* 1992).

Starch synthesis in higher plants and glycogen formation in bacteria proceed through similar pathways (Salamone *et al.* 2000). ADP-glucose pyrophosphorylase (AGPase) catalyzes one of the main regulatory steps in the biosynthesis of starch or glycogen. This enzyme mediates the synthesis of ADP-glucose and inorganic pyrophosphate from ATP and glucose 1-phosphate; the product, ADP-glucose, serves as the glucosyl donor in α-glucan synthesis (Iglesias *et al.* 1991). There are significant differences between the plant and bacterial AGPase. The enzyme from bacteria is homotetrameric, encoded by a single gene (*agp* gene), inhibited by AMP and ADP and activated by other glycolytic intermediates, such as fructose 1,6-bisphosphate, fructose 6-phosphate, or pyruvate (Iglesias *et al.* 1991). In higher plants, AGPase is mainly activated by 3-phosphoglyceric acid (3PGA) and inhibited by orthophosphate; the enzyme is heterotetrameric, composed by two regulatory large subunits and two catalytic small subunits, encoded by two distinct genes (Iglesias *et al.* 1991, Salamone *et al.* 2000). Cyanobacteria are prokaryotic organisms with a distinctive chloroplast type of photosynthesis that bears a strong resemblance to that occurring

in higher plants (Smith 1982). However, the former organisms synthesize glycogen as the major carbohydrate reserve, in a similar manner to what is observed in bacteria. AGPase from cyanobacteria exhibits intermediate properties, being homotetrameric in structure but regulated by 3-PGA and P_i and immunologically more related with the plant enzyme (Iglesias *et al.* 1991, Salamone *et al.* 2000).

Mutations reducing the activity of the enzyme lead to starch deficiency, as demonstrated in *Arabidopsis* and maize (Dickinson & Preiss 1969, Lin *et al.* 1988), and result in a reduction in growth (Sun *et al.* 1999). This suggests that there is a minimum requirement for starch synthesis for normal plant growth and development. Glycogen does not appear to be necessary for bacterial growth. Some mutants of *E. coli, Salmonella typhimurium*, and *Clostridium pasteurianum*, deficient in glycogen synthesis, grow as well as their normal parent strains (Preiss 1984). There is no information available regarding the *agp* deletion and its effects on the physiological features of the mutant in cyanobacteria. Therefore, in the present study we analyzed the changes in photosynthesis, pigmentation and cell growth in *Synechocystis* sp. PCC 6803 after *agp* deletion.

Materials and methods

Cloning and plasmid construction

A 1.5 kb *agp* fragment of *Synechocystis* 6803 genomic DNA was amplified using PCR, and cloned in pUC118, the resulting plasmid was named pUCA (Figure 1A). A 0.3 kb fragment within this *Synechocystis* 6803 gene was deleted and replaced by an erythromycin resistance cassette. The resulting plasmid, designated as pUCAE (Figure 1B), remained *agp* sequence on both sides of the deletion (upstream and downstream). The pUCAE plasmid was used to transform the *Synechocystis* 6803 wild type strain. Transformants were selected by screening for resistance to erythromycin. The mutant was designated as agp^- (Figure 1C).

Culture conditions

The strain of *Synechocystis* sp. PCC 6803 was provided by Dr Wim F. J. Vermaas. Cells were cultured photoautotrophically at 30 °C (±1) in BG11 medium (Iglesias *et al.* 1991). For the mixotrophic growth, 5 mM glucose was added to the medium. Continuous illumination at intensities of 82 μmol m^{-2} s^{-1} was provided by cool white fluorescent tubes. Aeration was provided by bubbling air at regular pressure. Cells were propagated on BG11 agar plates in the presence of 5 mM glucose, 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate at 30 °C (±1) in continuous light at intensities similar to those used for liquid culture. For the propagation of the mutant cells, 30 μg erythromycin ml^{-1} was added to the solid medium. In the growth experiment, two different light intensities were used as follows: lower light intensity (45 μmol m^{-2} s^{-1}) and higher light intensity (82 μmol m^{-2} s^{-1}). Cell growth was determined by the dry weight (mg ml^{-1}) of the biomass.

PCR analysis of agp^- *mutant*

The genomic DNA from WT cells and agp^- mutant cells were extracted respectively according to the method of Williams (1988). Two primers were designed for PCR amplification as follows: 5′-CAGCTCCGGATCCCAACGGCG-3′ (P1) and 5′-GGCCGAGCTCAGGGATTTTACTCGTTT-3′ (P2). The PCR products were verified by agarose gel electrophoresis.

Enzyme extraction and assay

Synechocystis 6803 cells were harvested by centrifugation, washed twice with 20 mM potassium phosphate buffer, pH 7.5, containing 5 mM DTT, and stored at −80 °C. The crude extracts were prepared according to the method of Iglesias *et al.* (1991). AGPase activity of the crude extracts were assayed at 25 °C. The reaction mixtures contained in 5 ml: 0.4 mM Hepes/NaOH buffer (pH 7), 25 μM $MgCl_2$, 1 mg BSA, 5 μM ADPglucose, 3 μM NAD^+, 0.05 μM glucose 1,6-bisphosphate, 50 μM 3-PGA, 10 μM sodium pyrophosphate, and 10 units each of phosphoglucomutase and glucose-6-phosphate dehydrogenase. Assays were initiated by addition of sodium pyrophosphate. The absorption of the formed NADH was recorded at 340 nm (absorption coefficient, 6.22 mM^{-1}) (Sun *et al.* 1999). One unit of activity is defined as the amount of enzyme catalyzing the production of 1 μM glucose-1-P/min under the specified conditions (Iglesias *et al.* 1991).

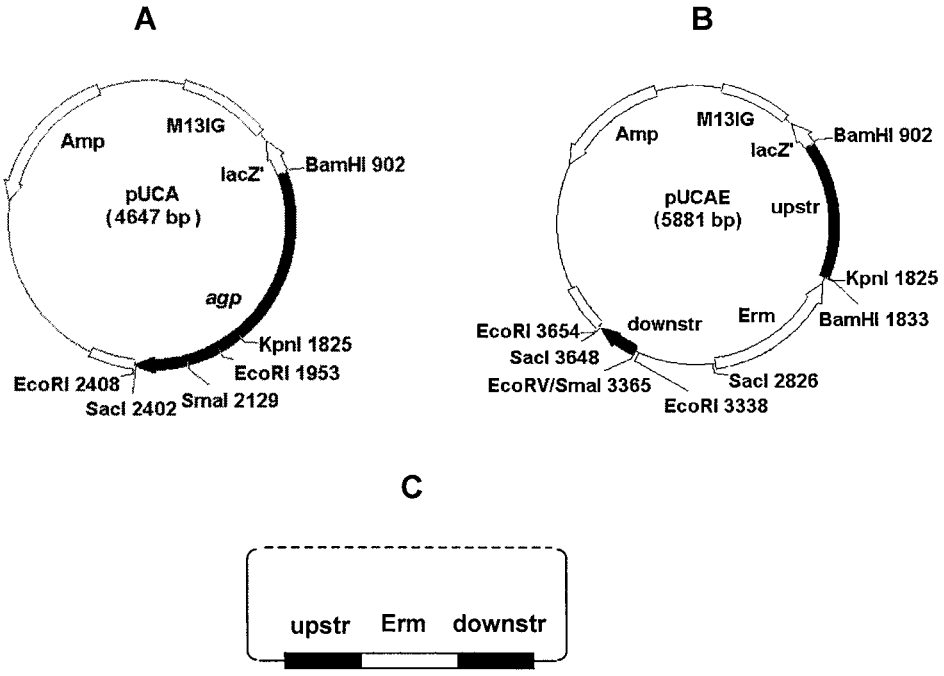

Fig. 1. Mutation of the *agp* gene homolog in *Synechocystis* sp. PCC 6803. The PCR-amplified *Synechocystis* 6803 DNA fragment was digested with *Bam*HI and *Sac*I, and cloned in pUC118. The resulting plasmid was named pUCA (A). A 0.3 kb *Kpn*I-*Sma*I fragment within this *Synechocystis* 6803 *agp* gene was deleted and replaced by an erythromycin resistance cartridge (Erm) from plasmid pRL425. The resulting plasmid was designated as pUCAE (B). Genomic DNA in cells of *agp*$^-$ deleted mutant (C).

Pigment analysis

Chlorophyll *a* (Chl *a*) was determined from the absorbancy of the methanol extracts at 666 nm (Mackinney 1941). The c-phycocyanin (PC) was extracted according to the method of Su *et al.* (1992) and its concentration was calculated by the equation $PC = [A_{615}\text{-}0.474\ (A_{652})]/5.34$ (Harold *et al.* 1978). For the determination of carotenoids, samples were harvested by centrifugation, and the pellet was saponified by suspension in 30% (v/v) methanol containing 5% (w/v) KOH. The remaining pellet was neutralized by addition of 70% (v/v) acetic acid, then carotenoids were extracted by addition of pure dimethylsulphoxide and maintained at 70 °C for 5 min. The absorbance of the supernatant was measured at 490 nm and the concentration of carotenoids was calculated using the specific absorption coefficient $E_{1\%} = 2200$ (Davies 1976).

Measurement of O_2 evolution

Evolution of O_2 was measured using a Clark-type electrode (Hansatech, Kings Lynn, UK). Cells were harvested by centrifugation at 1150 g for 15 min and resuspended in 25 mM Hepes/NaOH buffer, pH 7.

Table 1. AGPase activity and glycogen content in *Synechocystis* sp. PCC 6803 wild type and *agp* deletion mutant cells after 168 h cultivation at illumination of 82 μmol m^{-2} s^{-1}. Glycogen content is based on the dry cellular weight. Mean ± SD, $n = 3$.

Strain	AGPase activity (units dry wt g^{-1})	Glycogen content (%)
WT	480 ± 49	19 ± 1
agp$^-$	0	0

Light-response curves of photosynthesis were obtained for cells by measuring the rate of O_2 evolution at different photosynthetic photon flux densities.

Results and discussion

PCR amplification and AGPase activity

A 0.3 kb fragment within *Synechocystis* 6803 *agp* gene (1.5 kb) was deleted and replaced by a 1.5 kb erythromycin-resistance cassette in the construction of the *agp*$^-$ mutant. A 1.5 kb PCR-amplified DNA

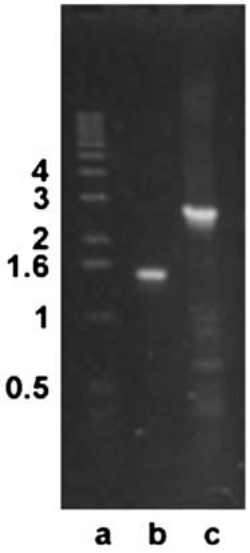

Fig. 2. Electrophoresis of PCR products. A 1.5 kb PCR-amplified DNA fragment from WT genomic DNA and a 2.7 kb fragment from *agp*$^-$ mutant were predicted. The result of agarose gel electrophoresis of PCR-amplified DNA fragments indicated appropriate deletion and insertion had indeed occurred. a. Ladder, b. wild type, c. *agp*$^-$.

fragment from WT genomic DNA and a 2.7 kb fragment from *agp*$^-$ mutant were predicted. The result of agarose gel electrophoresis of PCR-amplified DNA fragments (Figure 2) indicated appropriate deletion and insertion had indeed occurred. Further analysis of AGPase activities failed to detect AGPase activity in the mutant strains (Table 1). In contrast, in the wild-type cells the activity of AGPase was as high as 480 units g^{-1} dry wt . Again, no glycogen was found in the mutant, while in the WT strain glycogen was up to 19% of the dry cell weight after 7 d (Table 1).

Photosynthesis and pigments

The rate of photosynthesis was monitored as O_2 evolution by the intact cells. At an irradiance of 50 μmol m^{-2} s^{-1} (low irradiance), the mutant had almost the same rate of photosynthesis as the WT (Figure 3). With an irradiance above 80 μmol m^{-2} s^{-1} (high irradiance), the WT had higher rate of photosynthesis than the mutant, however. As the irradiance was increased, the photosynthesis of the mutant became light-saturated at 300 μmol m^{-2} s^{-1}, while the WT was saturated at 600 μmol m^{-2} s^{-1} (Figure 3). The maximum rate of photosynthesis of the mutant was 72% of that of the WT (Figure 3). The chlorophyll fluorescence values of PSII yield correlated very well with O_2 evolution (data not shown).

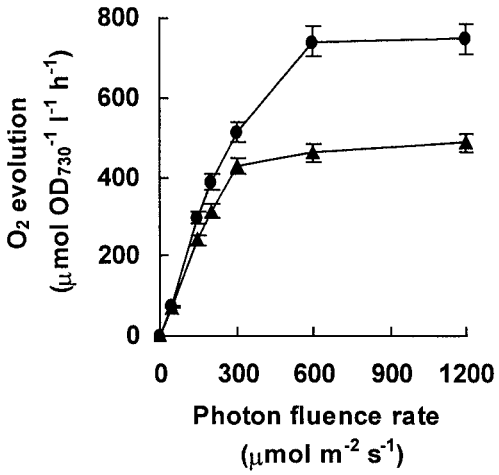

Fig. 3. Light-response curve of photosynthesis. The rate of photosynthesis was monitored as O_2 evolution in intact cells. (●) Wild type, (▲) *agp*$^-$. Mean ± SD, $n = 3$.

These results indicate that there is feedback regulation of electron transport by deficiencies in glycogen synthesis. Similar observations were described for the tobacco plastid phosphoglucomutase mutant and *Arabidopsis* starch mutant (TL25), which are defective in starch synthesis and have a lower rate of electron transport (Eichelmann & Laisk 1994, Sun *et al.* 1999). By analysing the pigment composition, a decrease in carotenoids, and especially phycocyanin content was shown, with little change in Chl *a* (Table 2) after *agp* deletion. The changes in pigment composition were best visible after calculation of the pigment ratios (Table 2). The ratio of phycocyanin/Chl *a* decreased significantly after *agp* deletion, whereas the ratio of carotenoid/Chl *a* showed no significant change in *agp*$^-$ cells. These results suggest that changes in pigment composition after *agp* deletion may have an effect on light absorption. Decrease in the ratio of phycocyanin/Chl *a* would possibly reduce the light absorption flux in PSII, electron transport and, thus, decrease the rate of photosynthesis. Meanwhile, because of a decrease in antenna pigment, phycocyanin, the cells could not capture more energy of a photon with the increased of the light intensity. Thus, the photosynthesis of the mutant became light-saturated earlier at 300 μmol m^{-2} s^{-1} compared with the wild-type which was at 600 μmol m^{-2} s^{-1}.

Inhibition of starch biosynthesis can lead to a reduction in the expression of storage proteins in the potato tuber and modification of one pathway can also have a profound effect on others (Muller-Rober *et al.* 1992). In our study, the mutant had a sharp decrease in

Table 2. Pigment contents per cells and ratios of carotenoid/chl *a* and c-phycocyanin/chl *a* in cells of *Synechocystis* sp. PCC 6803 wild type and *agp* deletion mutant strain after 144 h cultivation under illumination of 82 μmol m^{-2} s^{-1}. Mean ± SD, $n = 3$. Numbers in parentheses are percentages of the control value.

Strain	Chl *a* (μg OD_{730}^{-1})	Carotenoid (μg OD_{730}^{-1})	PC (μg OD_{730}^{-1})	Carotenoid/Chl *a*	PC/Chl *a*
WT	4.87 ± 0.26	0.88 ± 0.05	1.88 ± 0.07	0.18 ± 0.02 (100)	0.4 ± 0 (100)
agp$^-$	4.64 ± 0.35	0.69 ± 0.01	0.8 ± 0.11	0.15 ± 0.01 (83)	0.16 ± 0.01 (40)

phycocyanin content, which suggested that the inhibition of glycogen biosynthesis might have an effect on the expression of the proteins in the thylakoid.

Cell growth

The *agp* deletion did not inhibit O_2 evolution until much higher light intensities (above 80 μmol m^{-2} s^{-1}) (Figure 3). This suggests light intensities might have different effects on the growth of the mutant strain. To confirm this, a lower light intensity (45 μmol m^{-2} s^{-1}) and a higher light intensity (82 μmol m^{-2} s^{-1}) were used for growth. Under the lower light intensity, the mutant strain propagated a little bit faster than the WT strain (Figure 4), the final biomass of the mutant strain was only 1.1 times of that of the WT strain under mixotrophic condition after 144 h (Figure 4A), while under photoautotrophic condition, the final biomass of the mutant strain was almost the same as that of the WT strain (Figure 4B). In contrast, the WT strain grew much faster than the mutant strain under the higher light intensity, the final biomass of the WT strain was 3 times under mixotrophic condition (Figure 4A) and 1.5 times under photoautotrophic condition (Figure 4B) of that of the mutant strain after 144 h.

As shown in Figure 3, under the lower light intensity (45 μmol m^{-2} s^{-1}), the mutant had almost the same rate of photosynthesis as the WT. Therefore, upon the block-up of the glycogen biosynthesis pathway under the irradiance of 45 μmol m^{-2} s^{-1}, the glucose and the chemical energy formed in the photosynthesis would probably be utilized more efficiently for cell growth. As a result, the growth of the mutant strain was enhanced (Figures 4A,B). However, under conditions of rapid photosynthesis (e.g. in higher light intensity of 82 μmol m^{-2} s^{-1}), defective glycogen synthesis would lead to an inhibition of photosynthesis (feedback inhibition) (Figure 3) (Heldt *et al.* 1977) and thus cause a sharp decrease in the growth of

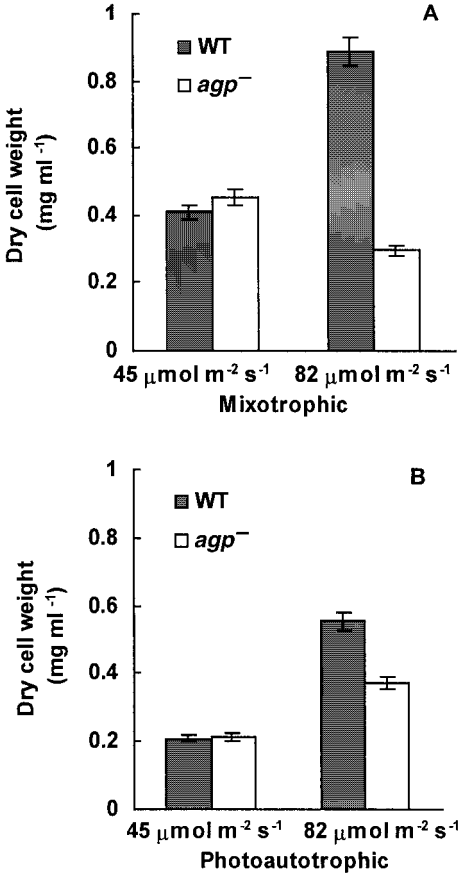

Fig. 4. Comparison of the cell growth of the mutant strain with that of the wild type strain under mixotrophic (A) and photoautotrophic growth (B) at lower light intensity (45 μmol m^{-2} s^{-1}) and higher light intensity (82 μmol m^{-2} s^{-1}) after 144 h. Cells were grown at 30 °C (± 1) on BG11 medium (photoautotrophic growth). For the mixotrophic growth, 5 mM glucose was added to the medium. Cell growth was determined by the dry weight (mg ml^{-1}) of the biomass. Mean ± SD, $n = 3$.

the mutant cells (Figures 4A,B). In contrast, the WT had a higher capacity for glycogen synthesis and thus could maintain higher electron transport and prevent feedback inhibition under the higher light intensity (Figures 3 and 4). Meanwhile, the *agp*$^-$ mutant under mixotrophic condition was the one which grew fastest under lower light intensity (Figure 4A) but the biomass (0.454 mg ml^{-1}) after 144 h was only 51% of that of the WT strain (0.89 mg ml^{-1}) under mixotrophic condition at higher light intensity (Figure 4A).

These results suggest that there is a minimum requirement for glycogen synthesis for normal growth and development of cyanobacterium *Synechocystis* 6803 which is different from bacteria. Glycogen does not appear to be necessary for bacterial growth as many mutants grow as well as their normal parent strains (Preiss 1984). In addition, compared with mixotrophic growth, the mutant strain grew faster during photoautotrophic growth under higher light intensity (Figure 4), which suggested that the glucose in the media inhibits its growth. Under higher light intensity of 82 μmol m^{-2} s^{-1}, glucose accumulation and the addition of glucose to the culture media would probably cause the feedback inhibition, lead to an inhibition of photosynthesis and, thus, decrease the rate of growth.

In conclusion, the results presented in this study demonstrate that glycogen metabolism has a very significant effect on the photosynthetic capacity of the cyanobacteria, the synthesis of which can ameliorate any potential reduction in photosynthesis caused by feedback regulation, which is different from bacteria but similar to the plants.

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References

Davies BH (1976) Carotenoids. In: Goodwin TW, ed. *Chemistry and Biochemistry of Plant Pigments*, 2nd edn. London: Academic Press, pp. 38–165.

Dickinson DB, Preiss J (1969) ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize endosperm. *Plant Physiol.* **44**: 1058–1062.

Eichelmann H, Laisk AH (1994) CO_2 uptake and electron transport rates in wild-type and a starchless mutant of *Nicotiana sylvestris*: the role and regulation of starch synthesis at saturating CO_2 concentrations. *Plant Physiol.* **106**: 679–687.

Harold W, Siegelman, Kycia JH (1978) In: John AH, ed. *Handbook of Phycological Method*. Cambridge: Cambridge University Press, pp. 72–79.

Heldt HW, Chon CJ, Maronde D, Herold A, Stankovic ZS, Walker DA, Kraminer A, Kirk MR, Heber U (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol.* **59**: 1146–1155.

Iglesias AA, Kakefuda G, Preiss J (1991) Regulatory and structural properties of the cyanobacterial ADPglucose pyrophosphorylase. *Plant Physiol.* **97**: 1187–1195.

Lin TP, Casper T, Somerville CR, Preiss J (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose pyrophosphorylase activity. *Plant Physiol.* **86**: 1131–1135.

Mackinney G (1941) Absorption of light by chlorophyll solution. *J. Biol. Chem.* **140**: 315–322.

Muller-Rober B, Sonnewald U, Willmitzer L (1992) Inhibition of ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar storing tubers and influences tuber formation and expression of tuber storage protein genes. *EMBO J.* **11**: 1229–1238.

Preiss J (1984) Bacterial glycogen synthesis and its regulation. *Annu. Rev. Microbiol.* **38**: 419–458.

Salamone PR,Greene TW, Kavakli IH, Okita TW (2000) Isolation and characterization of a higher plant ADP-glucose pyrophosphorylase small subunit homotetramer. *FEBS Lett.* **482**: 113–118.

Smith AJ (1982) Modes of cyanobacterial carbon metabolism. In: Carr NG, Whitton BA, eds. *The Biology of Cyanobacteria*. Oxford: Blackwell Scientific Publications, pp. 47–85.

Stark DM, Timmerman KP, Barry GF, Preiss J, Kishoret GM (1992) Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. *Science* **258**: 287–292.

Su X, Fraenkel PG, Bogorad L (1992) Excitation energy transfer from phycocyanin to chlorophyll in an *apcA*-defective mutant of *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **267**: 22944–22950.

Sun JD, Okita TW, Edwards GE (1999) Modification of carbon partitioning, photosynthetic capacity, and O_2 sensitivity in Arabidopsis plants with low ADP-glucose pyrophosphorylase activity. *Plant Physiol.* **119**: 267–276.

Williams JGK (1988) Construction of specific mutations in the photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Meth Enzymol.* **167**: 766–778.