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Increased cellulose production by heterologous expression of cellulose synthase genes in a filamentous heterocystous cyanobacterium with a modification in photosynthesis performance and growth ability

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ABSTRACT. Cellulose and sugar from microalgae can be utilized for the production of biofuel ethanol. Increasing cellulose and sugar synthesis capacity is key for high yield production of this biofuel. To enhance cellulose production, we transferred *acsAB*, responsible for cellulose synthesis in bacterium *Acetobacter xylinum*, into the cyanobacterium, *Anabaena* sp. strain PCC7120, by conjugation. PCR confirmed the presence of *acsAB* in *Anabaena* sp. strain PCC7120 exconjugates. RT-PCR demonstrated the up-regulation of *acsAB* expression. Production of extracellular cellulose secreted from *Anabaena* sp. strain PCC7120 carrying *acsAB* was revealed using Calcofluor white staining and cellobiohydrolase I (CBHI)-FITC labeling. Further evidence obtained from the digestion of cellulose to glucose demonstrated that the amount of glucose released from cellulose was significantly increased in *Anabaena* sp. PCC7120 cells carrying *acsAB* as compared to the wild type. The photosynthetic efficiency and growth rate were increased in the transgenic strains. Cellulose synthesis is thus enhanced in *Anabaena* sp. strain PCC7120 expressing *acsAB* and does not negatively impact photosynthesis and growth.

Keywords: Cellulose; Cellulose synthase; Cyanobacteria; Photosynthesis.

INTRODUCTION

Renewable energy sources, notably ethanol from biomass, are currently being developed as eventual replacements for present non-renewable energy sources such as petroleum. The limiting factors for producing ethanol from biomass include their high cost and the limited availability of biomass sources, such as corn, sugar cane and cellulose from plants (Sánchez and Cardona, 2008; Balat and Balat, 2009). The cellulose of plant cell walls is intimately associated with lignin and hemicelluloses, forming a complex polymer composite that is exceptionally recalcitrant to mechanical and biological degradation (Sun and Cheng, 2002; Lynd, 1996). Cellulose purification is both energetically expensive and environmentally unfriendly (Pimentel and Patzek, 2008). Additionally, the cultivation of cellulose crops, e.g. cotton for textiles or switchgrass for biofuels, entails the extensive use of arable land (Sun and Cheng, 2002), fertilizers and pesticides, as well as the consumption of fresh water for irrigation (Pimentel and Patzek, 2008). Considering Taiwan's rich and vast microalgal resources, microalgal-derived cellulose stands as a promising alternative to traditional plant cellulose sources.

Cyanobacteria, also known as blue-green algae, are a group of photoautotrophic prokaryotes that perform plant-like oxygenic photosynthesis. These organisms have simple growth requirements and efficiently use light, CO_2, and inorganic elements to produce biomass. Many of them are non-toxic, have high nutrient value, and can be easily cultivated for mass commercial production (Kay and Barton, 1991). They are attractive systems for the bioconversion of solar energy and CO_2 into valuable products. The genetic characteristics of cyanobacteria are similar to *E. coli,* in that their genetic manipulation is relatively easy. This has

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made them model systems in molecular biological studies. The fact that most cyanobacterial strains produce no endotoxin makes them attractive hosts for the production of recombinant products since the purification process is easier and the production less costly.

In recent years, rapid advancements have been made in the genetic engineering of cyanobacteria. The physiology, biochemistry and genetics of *Anabaena* sp. PCC7120, a filamentous heterocystous cyanobacterium, have been well characterized. *Anabaena* sp. PCC7120 has a well-developed genetic transfer system that has been used to express a number of foreign genes in this organism. For example, expression of the mouse metallothionein-I gene in transgenic *Anabaena* sp. PCC7120 conferred cadmium resistance (Ren et al., 1998), while combinational expression of several toxin genes from *Bacillus thuringiensis* conferred mosquito larvicidal activity to transgenic *Anabaena* sp. PCC 7120 (Khasdan et al., 2003; Khasdan et al., 2003; Manasherob et al., 2002; Wu et al., 1997). The human tumor necrosis factor alpha (hTNF-α) gene was also expressed *in Anabaena* sp. PCC 7120 resulting in cytotoxicity of the crude extracts from the transgenic cyanobacteria (Liu et al., 1999; Dai et al., 2001).

Acetobacter xylinum is a gram-negative bacterium which produces a cellulose membrane called bacterial cellulose (BC) (Marx-Figini and Pion, 1974). This highly-pure and mechanically-strong membrane can be used as a food additive and in medical materials (Czaja et al., 2006). The *acsABCD* operon encodes the enzymes responsible for cellulose biosynthesis in *Acetobacter xylinus* (*Gluconacetobacter xylinus*). The cellulose synthases of *A. xylinum* ATCC23769 and ATCC53582 contain three subunits (AcsAB, AcsC, and AcsD). The deduced amino acid sequences of the cellulose synthases of *A. xylinum* ATCC23769 and ATCC53582 are highly homologous (Saxena et al., 1994; Kawano et al., 2002). The N-terminal half of AcsAB catalyzes *β*-glycosyltransfer using either UDP-glucose or similar nucleotide sugars as a substrate (Saxena et al., 1995). The C-terminal half of AcsAB is involved in the regulation of cellulose biosynthesis, as it interacts with cyclic-di-GMP (Ross et al., 1987), an activator of cellulose production. Although the precise functions of AcsC and AcsD have not yet been determined, it has been proposed that AcsC might form pores in the cell wall through which newly-synthesized cellulose can pass, while AcsD might influence cellulose crystallization (Saxena et al., 1994).

Recently, Nobles and Brown (2008) reported the transfer of a partial cellulose synthesis operon containing *acsABDC*, from the *Gluconacetobacter xylinus* strain ATCC 53582 to the unicellular cyanobacterium *Synechococcus leopoliensis* strain UTCC 100, resulting in the secretion of non-crystalline cellulose and sugar. In the current study, a shuttle expression vector was constructed, harboring the cellulose synthethase gene, *acsAB*, from *Acetobacter xylilum* ATCC23769, under the control of the *psbA* promoter, and introduced by conjugation into the filamentous, heterocystous cyanobacterium *Anabaena* sp. PCC 7120, The production of cellulose, as a result of *acsAB* expression, was detected by Calcofluor white staining and FITC-labelled CBHI. Given the possibility that enhanced cellulose synthesis might inhibit the growth of *acsAB* transgenic *Anabaena* the growth and photosynthetic capacity, as measured by cell density and chlorophyll *a* content, respectively, were compared between the wild type and *acsAB* transgenic *Anabaena*.

MATERIALS AND METHODS

Plasmids and bacterial strains

The bacterial strains and plasmids used are listed in Table 1. *A. xylinum* ATCC23769 was grown in mannitol agar containing: 2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 1.5% agar (ATCC MEDIA #1 Agar: Mannitol Agar) at 28°C. *Escherichia coli* cells were grown in LB medium (Miller, 1972) at 37°C on a rotary shaker. Selection with kanamycin (Km) was done at a concentration of 50 μg/mL. All recombinant plasmid constructions used *E. coli* DH5α as a host. Professor C. P. Wolk of Michigan State University (USA) kindly supplied Plasmid pRL489. *Anabaena* sp. PCC 7120 was obtained from the Pasteur Institute (Paris, France).

Growth conditions

Anabaena sp. strain PCC 7120 was cultured in 50 mL modified BG11 medium (BG-11 supplemented with 2 × Na_2CO_3) in a 125 mL flask containing 15 μg/mL neomycin. Algal cells, with initial OD_{750} of 0.1, were incubated in a rotary shaker (TKS OSI-500R, Kaohsiung, Taiwan) at 28°C at a speed of 130 rpm with continuous irradiance with 50 μmol photons·m^{-2}·s^{-1}. After 15 days of incubation, algal cells were collected and the OD_{750} and chlorophyll *a* content were measured. Photosynthesis and respiration rates were also determined. Transgenic strains grown on plates were maintained on solid agar media containing neomycin at 25 μg/mL.

Cloning *acsAB* genes from *A. xylinum*

Total genomic DNA from *A. xylinum* ATCC23769 was isolated and used as a template to amplify the *acsAB* genes by polymerase chain reaction (PCR) using forward (acsAB-F: 5'-aattggatccatgccagaggttcggtcgtcaacgcagtca-3') and reverse (acsAB-R: 5'-aattggatcctcacgacttgcgcctctcatcctcaagctg-3') primers. The initiation and termination codons of *acsAB* were underlined in the primers. PCR was carried out in a volume of 50 μL with the following parameters: 95°C for 3 min; 30 cycles at 98°C for 20 sec and 68°C for 7 min: and a final extension at 72°C for 10 min using *Takara LA Taq* polymerase (Takara Shuzo, Kyoto, Japan). The PCR product with the expected size (4.6kb) was isolated and cloned into yT&A vector (Yeastern Biotech Co., Ltd, Taipei, Taiwan) resulting in plasmid pAcsAB. Complete nucleotide sequence analysis of the *acsAB* insert in pAcsAB was confirmed by DNA sequencing (Tri-I Biotech, Taipei, Taiwan).

Table 1. Bacterial strains and plasmids used.

Strain or plasmid	Relevant characteristic (s)	Source or reference
Strains of *Anabaena* sp.		
PCC 7120	Wild type	PCC[a]
Strain acsAB-2	PCC 7120 carrying pPacsAB	This study
Strain acsAB-8	PCC 7120 carrying pPacsAB	This study
Strain acsAB-17	PCC 7120 carrying pPacsAB	This study
Strain acsAB-26	PCC 7120 carrying pPacsAB	This study
Strain pRL489-Nm^r	PCC 7120 carrying pRL489-Nm^r	This study
Strains of *E. coli*		
DH5α	Recipient in transformations	
Strains of *A. xylinum*		
A. xylinum ATCC23769	Source of *acsAB* gene	ATCC[b]
Plasmids		
pAcsAB	yT&A containing 4.6 kb *acsAB* gene	This study
pRL489-Nm^r	pRL489 removing *luxAB* gene	This study
pPacsAB	pRL489-Nm^r containing *acsAB* gene	This study
pRL489	Km^r Nm^r; pDU1-based shuttle vector; carries PpsbA-luxAB	Elhai, J.
pRL443	Ap^r Tc^r; conjugative plasmid	Elhai, J. & C. P. Wolk
pRL528	Cm^r; helper plasmid; carries *mob*	Elhai, J. & C. P. Wolk
yT&A	Ap^r; cloning vector	Yeastern Biotech Co.

[a]PCC, The Pasteur Culture Collection of Cyanobacteria, Institut Pasteur, France.
[b]ATCC, American Type Culture Collection, Rockville, MD, USA.

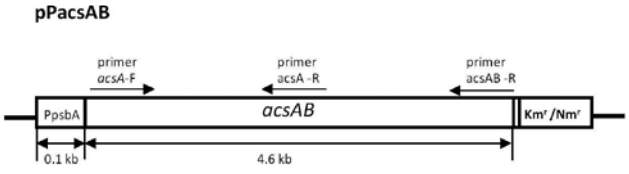

Figure 1. Map of shuttle expression vector pPacsAB.

Shuttle vector construction

A 2 kb *Bam*HI fragment containing the *luxAB* sequence was removed from pRL489 (Elhai, 1993) and a 4.6 kb *Bam*HI fragment from pAcsAB, containing the *acsAB* genes from *A. xylinus* strain ATCC 23769, was ligated into the *Bam*HI site of pRL489 to create a shuttle plasmid pPacsAB, where the *acsAB* sequence was oriented in the same direction as the *psbA* promoter (Figure 1).

The 2 kb *Bam*HI fragment containing the *luxAB* sequence was removed from pRL489 and the remaining fragment was self-ligated to generate plasmid pRL489-Nm^r. Plasmid pRL489-Nm^r was used as a negative control in detecting *acsAB* expression in *acsAB* transgenic cells. The resulting plasmids, pRL489-Nm^r and pPacsAB, were confirmed by restriction digestion, and used for conjugative transfers.

Triparental conjugative transfer

Conjugation between *E. coli* and *Anabaena* was performed as described (Elhai and Wolk, 1988). Briefly, two strains of *E. coli* HB101: one containing conjugation plasmid, pRL443, and the other containing helper plasmid, pRL528, along with either shuttle vector pPacsAB, or pRL489-Nm^r (control), were mixed with *Anabaena* sp. PCC 7120 and spotted onto filter papers laid on BG-11 agar plates without antibiotic. After incubation for 48 h, the filters were transferred to plates supplemented with 25 μg/mL neomycin (Nm) and incubated until Nm^r exconjugant colonies appeared. The cyanobacterial clones, which appeared on the filters after about two weeks, were picked and restreaked on BG-11 plates containing 25 μg/mL neomycin (Nm). Putative exconjugates were transferred to BG-11 liquid medium containing Nm (15 μg/mL) for more rapid cultivation and further analysis.

PCR screens for transgenic cells

Cultures of *Anabaena* sp. PCC7120 harboring either plasmid pRL489-Nm^r or pPacsAB were used in PCR screens to detect *acsAB*. One mL liquid culture of each strain was pelleted by centrifugation, resuspended in 200 μL of TE, pH 8.0 supplemented with 1% Triton X-100, and lysed by heating for 2 min at 95°C (Hagen and Meeks, 1999). The lysate was then extracted twice with an equal

volume of chloroform. Aliquots (5 μL) of the aqueous phase, which contained genomic DNA extracted from *Anabaena* sp. PCC7120 wild type and *Ananbaena* PCC7120 transformed with pRL489-Nm^r or pPacsAB were used in PCR amplifications with acsAB-F and acsA-R primers.

PCR was carried out in a Thermo Hybaid Px2 thermal cycler (Thermo, Franklin, MA, USA) using the following temperature program: 95°C for 5 min; 30 cycles consisting of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min; and 72°C for 10 min. A 2.2 Kb fragment spanning the *acsA* region of the *acsAB* genes was amplified using the primers acsAB-F 5'-aattggatccatgccagaggttcggtcgtcaacgcagtca-3' and acsA-R 5' -aattcccgggtcagacagggcattgccagccgaaggcttg-3' (the underlined regions of the primer sequences indicate introduced restriction endonuclease sites). The resulting PCR products were then detected by agarose gel electrophoresis.

RNA isolation and RT-PCR

Total RNA was isolated from cultures of *Anabaena* PCC7120 wild type and *Anabaena* PCC7120 harboring plasmid pRL489-Nm^r or pPacsAB. Total RNA was extracted from the cells from 15 mL cultures of these strains using the GENEMARK Plant Total RNA Miniprep Purification Kit (Taipei, Taiwan). The RNA quality was analyzed by agarose gel electrophoresis (1%) and the concentration was determined by O.D. at 260 nm (T60 UV-Visible spectrophotometer, PG Instruments, Leicestershire, United Kingdom).

Reverse transcription reactions were carried out with M-MLV Reverse Transcriptase (catalog # M1701, Promega, Madison, Wisconsin, USA) based on the manufacturer's instructions, using random primers and 1μg of DNAse-treated RNA. cDNA produced in reverse transcription reactions was used for PCR reactions with acsAB-F and acsA-R primers. The following PCR program profile was applied: 1 min at 94°C followed by 40 cycles of time and temperature; 10 s denaturation at 94°C, 1 min annealing at 55°C, and 2 min elongation at 72°C; finishing with 7 min at 72°C. Products of the PCR reactions were analyzed on 1% agarose gels. Negative controls in which no reverse transcriptase was added to the RT reaction prior to PCR and reactions in which dH_2O was added to the PCR in place of DNA were included in the experiments.

Calcofluor white staining and microscopy

In order to detect extracellular cellulose, Calcofluor white M2R (Sigma F3543) was used to stain the transgenic strains of *Anabaena* PCC7120. An aqueous solution of Calcofluor white M2R (Sigma F3543) of 0.01% (w/v) was overlaid on cells fixed to microscope slides and incubated for about 5 min at room temperature. After staining, fluorescence and phase contrast micrographs were taken on a LEICA microscope (LEICA DM2500) with an X100 objective using specific emission filter sets (Ex. 377 nm, Em. 447 nm). Images were captured with a cool CCD camera (Leica model DFC420C, Wetzlar, Germany).

Conjugation of Cellobiohydrolase I with fluorescent probes

The conjugation of Cellobiohydrolase I (CBH I) to fluorescein isothiocyanate (FITC) was carried out according to the manufacturer's instructions (Pierce® FITC Antibody Labeling Kit, Thermo Scientific No. 53027, Rockford, USA). One mg CBH I (Cellobiohydrolase I from *Trichoderma* sp., Megazyme Lot 40203a, Sidney, Australia) was mixed with FITC in 0.05 M borate buffer (pH 8.5). After incubation at room temperature for 1 hr in the dark, the FITC labeled Cellobiohydrolase I (FITC-CBHI) was separated from unconjugated dye by chromatography on spin columns.

Specimen fixation and cellulose labeling with FITC-conjugated CBHI

One mL of liquid cultures of *Anabaena* PCC7120 wild type and *Anabaena* PCC7120 harboring plasmid pRL489-Nm^r, pPacsAB were pelleted by centrifugation, and resuspended in 200 μl of ethanol/acetic acid solution (ethanol:acetic acid = 3:1) at room temperature for 1 hr. After centrifugation, the cell pellets were resuspended in 50 μL of 50 mM sodium acetate buffer (pH 5.0). 20 μL of the suspension was spotted onto a poly-L-lysine coated slide. The slides were immersed in CBHI-FITC (1:20 diluted with PBS) and incubated at room temperature for 30 min. They were then rinsed four times for five minutes with PBS. The samples were mounted to a coverslip with ibidi mounting medium (Ibidi, Martinsried, Germany).

After staining, fluorescence and phase contrast micrographs of the CBHI-FITC labeled *Anabaena* samples were taken on a LEICA microscope (LEICA DM2500) with a 25X and a 40X objective using specific emission filter sets (Ex.450-490 nm, Em. 510-550 nm). Images were captured with a CoolSnap ES CCD camera (Roper Scientific, Tucson, AZ, USA) attached to the microscope.

Cellulose hydrolysis and determination of glucose contents

Cells from 15 mL of stationary phase cultures of *Anabaena* PCC7120 wild type, and *Anabaena* PCC7120 cells harboring plasmid pRL489-Nm^r, pPacsAB were collected by centrifugation at 3,000 × g for 10 min. The pellets were resuspended in 0.6 mL of sodium acetate buffer (50 mM sodium acetate, pH 5.0) and broken by shaking for 1 minute using a minibead beater (Biospec Products, Bartlesville, OK, USA) in the presence of 0.2 g sea sand. For total cellulose hydrolysis analysis, 24 μL of sterilized Celluclast 1.5 L and 4 μL of 188 (Novozymes) were added to the broken cells, followed by incubation at 50^{o}C for 48 h. The low-molecular-mass compounds were extracted with 1 mL of 80% (v/v) ethanol for 3 h at 65^{o}C. After centrifugation at 14,000 rpm for 2 min at room temperature, the supernatant was collected and the pellet was washed again with 0.5 mL of 80% ethanol and centrifuged. Both supernatants were combined and incubated for 30 min at 80^{o}C (Higo et al., 2006). The amount of glucose in these

samples was determined with a modified glucose oxidase-peroxidase coupled reaction as previously described (Ou-Lee and Setter, 1985; Cheng, 1994). In brief, PGO reagent (50 mM HEPES containing 3 mg/mL p-hydroxybenzoic acid, 0.1 mg/mL 4-aminoantipyrrine, 0.5 units peroxidase, and 1.5 units glucose oxidase, pH 7.0) was added to the ethanol-extracted supernatant, followed by incubation at room temperature for 15 min. The absorbance at 490 nm was obtained using a Microplate Spectrophotometer (μQuant, Bio-Tek Instruments, Inc. USA). A series of glucose dilutions was included in the assay as a standard.

Determination of algal cell growth rate and chlorophyll *a* contents

Algal cell growth was estimated by OD_{750} in 1 mL of cell suspension using spectrophotometer (HITACHI U-2001, Japan).

To determine the chlorophyll *a* content, 10 mL of algal cell culture was centrifuged at 12,000 ×*g* at 4°C for 5 min, the pellet was ground in liquid nitrogen, extracted with 10 mL of chilled 80% acetone at 4°C and shaken in the dark at 100 rpm for 10 minutes. After centrifugation at 12,000 ×*g* at 4°C for 5 min, the supernatant was collected and the volume was adjusted to 10 mL with 80% acetone. The absorbance of the supernatant was detected at 645 nm and 663 nm for the estimation of chlorophyll *a* contents. The content of chlorophyll *a* was determined using the equation: Chl *a* (μg/mL) = 12.7 (A_{663}) – 2.69 (A_{645}) (Arnon, 1949).

Determination of photosynthesis and respiration rate

Photosynthesis and respiration rate were determined by detecting the amount of O_2 evolution and consumption by algal cells using a Clark-type oxygen electrode fitted with a DW3 chamber (Hansatech, Kings Lynn, Norflok, England) thermostated at 28°C. Ten mL of algal culture was centrifuged at 4,000 ×*g* for 10 min at room temperature. The cells were resuspended in 10 mL of 50 mM HEPES-KOH buffer (pH 7.5) and transferred to a 20 mL square section reaction vessel containing 3 mM $NaHCO_3$. The consumption of O_2 as respiration was determined in the dark for 10 min and then exposed to 100 μmol photons·m^{-2}·s^{-1} for the determination of O_2 evolution as net photosynthesis. The net photosynthesis rate and the respiration rate were expressed as the change in O_2 concentration per hour, and the gross photosynthetic rate was the value of 'net photosynthesis rate-respiration rate'. Three replicates per treatment were measured.

Chemicals and statistical analysis

Chemicals were purchased from Merck (Germany) or Sigma (USA). Statistics were analyzed by SAS (SAS v 9.01, NC, USA). Statistical differences between means (n = 3) were analyzed by Duncan's new multiple range test ($P < 0.05$) following significant analysis of variance (ANOVA, $P < 0.05$).

RESULTS AND DISCUSSION

Construction of expression shuttle vectors of *Anabaena* sp. PCC7120

For cellulose production in *Anabaena* sp. strain PCC 7120, the shuttle expression plasmid pPacsAB was constructed using a pDU1 derivative shuttle vector pRL489 (Elhai and Wolk, 1988). The *acsAB*-containg DNA fragment of *A. xylinum* was amplified by PCR and cloned into the yT&A vector to generate plasmid pAcsAB. DNA sequencing confirmed the cloned *acsAB* sequence which was compared with other known proteins using the NCBI BLAST algorithm (http://blast.ncbi.nlm.nih.gov). As shown in Figure 1, the expression of the *acsAB* genes in plasmid pPacsAB was under the control of the strong *psbA* promoter derived from the chloroplast DNA of *Amaranthus hybris* (Hirschberg and McIntosh, 1983).

Conjugative transfer and the selection of the transgenic cyanobacterial cells

The shuttle expression plasmid pPacsAB was introduced to *Anabaena* cells by triparental conjugaton, and exconjugants were selected on BG-11 agar plates containing neomycin (25 μg/ml). Plasmid pRL489-Nm^r was introduced to *Anabaena* cells by triparental conjugation and a resulting exconjugant was used as a negative control in cellulose production assays. Transgenic cyanobacterial clones forming dense colonies under neomycin selection were transferred to BG-11 liquid medium for further cultivation. DNA samples from axenic cultures of the exconjugate strains were used in PCR screens for *acsAB* using primers which span a segment of *acsA*. As shown in Figure 2, a 2.2 Kb of PCR fragment corresponding to *acsA* was present in all four pPacsAB transgenic strains of *Anabaena* sp. PCC7120 (strain acsAB-2, strain acsAB-8, strain acsAB-17, and strain acsAB-26), but not in *Anabaena* sp. PCC7120 wild type or *Anabaena* sp. PCC7120 cells harboring pRL489-Nm^r vector. The data indicated that the plasmid pPacsAB was successfully introduced into *Anabaena* cells.

RT-PCR transcription analysis

In order to confirm the expression *acsAB* genes in transgenic strains of *Anabaena* sp., RT-PCR-based transcription studies were performed. Four pPacsAB transgenic strains of *Anabaena* sp. strain PCC7120, that had grown well in BG-11 medium containing neomycin (Nm), strain acsAB-2, 8, 17 and 26, were used in RT-PCR analysis. *Anabaena* sp. strain PCC7120 wild type and *Anabaena* sp. strain PCC7120 cells harboring pRL489-Nm^r vector were used as negative controls. Total RNA was isolated from *Anabaena* PCC7120 wild type, and *Anabaena* PCC7120 cells harboring plasmid pRL489-Nm^r, pPacsAB grown under photoautotrophic conditions. cDNA produced in reverse transcription reaction was then used for PCR reactions with acsAB-F and acsA-R primers. As shown in Figure 3, a 2.2-kb band corresponding to *acsA* was detected

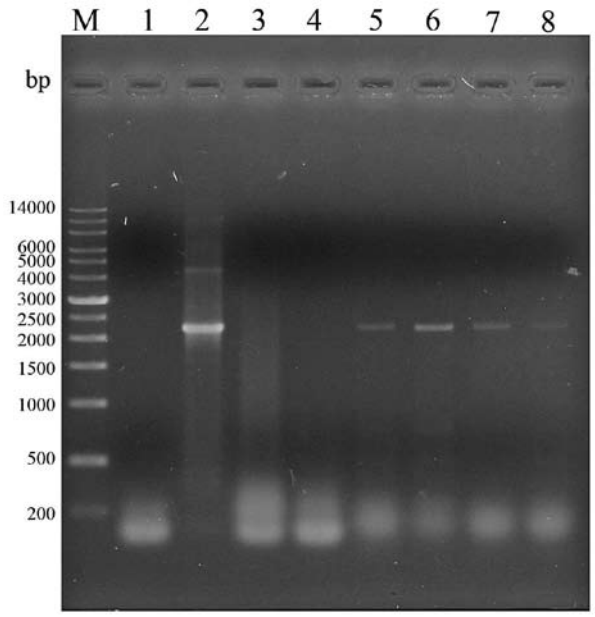

Figure 2. Colony PCR screen of *Anabaena* sp. strain PCC7120 exconjugates. From left to right: Lane M: marker; Lane 1: Negative control. (d·H_2O); Lane 2: pPacsAB plasmid; Lanes 3: *Anabaena* sp. PCC7120 wild type; Lane 4: *Anabaena* PCC7120 carrying plasmid pRL489-Nm^r; Lane 5-8: *Anabaena* PCC7120 carrying plasmid pPacsAB; Lane 5: strain acsAB-2; Lane 6: strain acsAB-8; Lane 7: strain acsAB-17; Lane 8: strain acsAB-26.

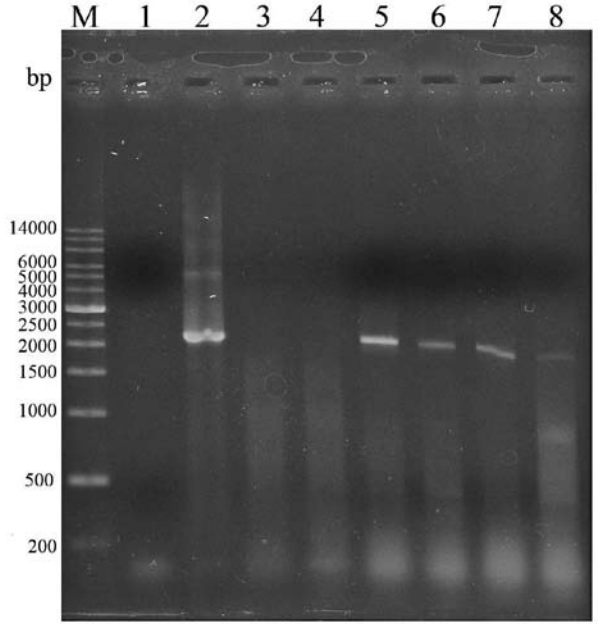

Figure 3. RT-PCR analysis of *acsAB* transcripts in *Anabaena* PCC7120 wild-type, and *Anabaena* PCC7120 cells carrying either pRL489-Nm^r or pPacsAB. From left to right: Lane M: marker; Lane 1: Negative control. (d·H_2O); Lane 2: pPacsAB plasmid; Lane 3: *Anabaena* PCC7120 wild-type; Lane 4: *Anabaena* PCC7120 carrying plasmid pRL489-Nm^r; Lane 5-8: *Anabaena* PCC7120 carrying plasmid pPacsAB; Lane 5: strain acsAB-2; Lane 6: strain acsAB-8; Lane 7: strain acsAB-17; Lane 8: strain acsAB-26.

in all four pPacsAB transgenic strains of *Anabaena* strain PCC7120, but not in *Anabaena* sp. strain PCC7120 wild type, or *Anabaena* sp. strain PCC7120 cells harboring pRL489-Nm^r vector. The results indicate that the *acsAB* genes are transcribed in the *Anabaena* sp. strain PCC7120 transgenic strains.

Determination of the location of cellulose by Calcofluor white staining and FITC-conjugated CBHI

In order to detect the production of cellulose, *Anabaena* PCC7120 wild type and *Anabaena* PCC7120 cells harboring plasmid pRL489-Nm^r, pPacsAB were analyzed by both Calcofluor white staining (Figure 4) and CBHI-FITC labeling (Figure 5). Calcofluor White Stain is a non-specific fluorescent brightening agent that binds to cellulose and chitin and is used extensively for the rapid detection of many yeasts and pathogenic fungi, and in analyzing the cellulosic content of *Crypthecodinium cohnii* cells (Kwok et al., 2007; Monheit et al., 1984). Cellobiohydrolase I (CBHI), from the fungus Trichoderma sp, is capable of binding to cellulose microfibrils or microcrystals (Chanzy et al., 1984) and has therefore been used to visualize the distribution of cellulose in a range of plant species and tissues (Berg et al., 1988; Benhamou, 1989; Berg, 1990; Bonfante-Fasolo et al., 1990; Roy and Vian, 1991; Ferguson et al., 1998).

The results from Figures 4 and 5 show the extracellular matrix stained with Calcofluor white and FITC that was observed in *Anabaena* PCC7120 cells harboring plasmid pPacsAB. Little or no such extracellular matrix staining was observed in either wild type or *Anabaena* PCC7120 harboring plasmid pRL489-Nm^r. The presence of Calcofluor white and CBHI-FITC-stained extracellular material in cells harboring plasmid pPacsAB positively identifies cellulose as a component of the matrix. This is in agreement with previously published results (Nobles and Brown, 2008).

Glucose analysis

Calcofluor white stain is a non-specific fluorescent brightening agent that binds to cellulose and chitin. CBHI is comprised of a cellulose-binding domain linked to a catalytic domain by an extended O-glycosylated interdomain peptide (Van Tilbeurgh et al., 1986; Srisodsuk et al., 1993; Reinikainen et al., 1995). CBHI is capable of binding to cellulose as well as any other plant polysaccharide containing adjacent (1,4)-β-D-glucose residues, including mixed-link (1,3)-(1,4)- β-D-glucans from grasses (Bacic et al., 1988). Thus, in order to unambiguously determine the content of cellulose produced by these transgenic strains further cellulose hydrolysis was carried out.

Cellulose content was measured by determining the glucose levels in extracts of *Anabaena* PCC7120 wild type and *Anabaena* PCC7120 cells harboring either plasmid pRL489-Nm^r or pPacsAB with or without Celluclast hydrolysis. Glucose liberated from cellulose was determined

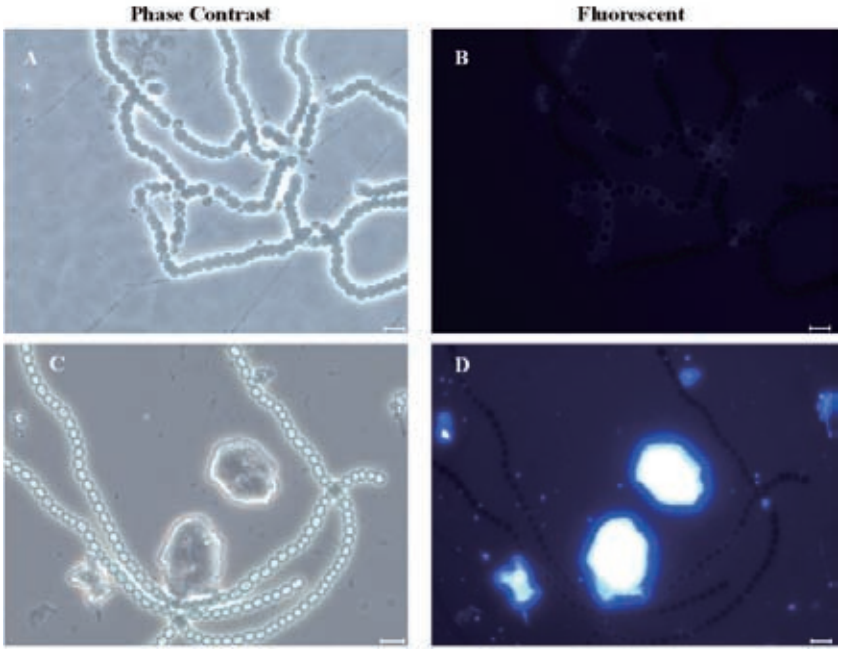

Figure 4. Cellulose staining in different *Anabaena* strains using calcofluor white stain. Bright field: A, C. Fluorescence: B, D. A-B: *Anabaena* sp. PCC7120 wild type C-D: *Anabaena* sp. PCC7120 strains containing plasmid, pPacsAB. Scale bars, 20 μm.

by subtracting the concentration of glucose present in untreated extracts from that of extracts subjected to Celluclast digestion. As shown in Table 2, the glucose content of the *acsAB* transgenic strains acsAB-2 and acsAB-17 was significantly higher than that of either *Anabaena* PCC7120 wild type or a strain harboring pRL489-Nm^r. The data thus demonstrated that the pPacsAB transformants, strain acsAB-2 and acsAB-17, produce a significantly higher amount of cellulosic material than do the controls.

A limited number of studies have reported increased glucose production in transgenic cyanobacteria. One such study, carried out by Nobel and Brown (2008), involved the transfer of cellulose synthesis genes (acsABΔC) from a heterotropic alpha proteobacterium to the cyanobacterium *Synechococcus lepoliensis* strain UTCC 100, resulting in a significant increase in the amount of cellulosic materials. Total glucose (mg ml^{-1} OD_{750}^{-1}) increased from 0.08 to 0.26 (Nobles and Brown, 2008). The results obtained in this study demonstrated that *Anabaena* sp. strain PCC7120 wild type naturally produces much higher total glucose than the *Synechococcus lepoliensis* strain UTCC 100 used in the Nobles study (0.20 vs 0.08 mg ml^{-1} OD_{750}^{-1}). After digestion with celluclast, our *acsAB* transgenic *Anabaena* sp. strain PCC7120 produced much higher levels of total glucose than the transgenic strain made by Nobles and Brown (0.53-0.66 vs 0.26 mg ml^{-1} OD_{750}^{-1}) (Table 2). The reasons for this difference remain to be determined.

Comparison of cell growth, chlorophyll *a* contents, photosynthesis and respiration

In order to examine whether enhanced cellulose synthesis impacts cell growth, the growth, respiration and photosynthesis rates were determined.

A comparison of OD and chlorophyll *a* content shows that the growth of the transformants harboring plasmid pPacsAB was significantly enhanced. After 15 days of incubation, all of the pPacsAB-containing transformants exhibited both higher OD values (Figure 6A) and chlorophyll *a* contents (Figure 6B) than those of the controls.

Table 2. Glucose assays.

Strains of PCC7120	Glucose (mg ml^{-1} D_{750}^{-1})		
	Buffer	Celluclast digestion	Glucose from cellulose*
7120 wild type	0.041 ± 0.042	0.201 ± 0.017	0.160 ± 0.042
pRL489-Nm^r	0.057 ± 0.019	0.315 ± 0.053	0.258 ± 0.052
acsAB-2	0.103 ± 0.025	0.657 ± 0.227	0.553 ± 0.252
acsAB-17	0.093 ± 0.026	0.530 ± 0.097	0.436 ± 0.102

*Glucose liberated from cellulose was determined by subtracting the concentration of glucose present in Sodium Acetate buffer samples from the total glucose obtained from Celluclast digestions. Data are means ± SD (n = 3).

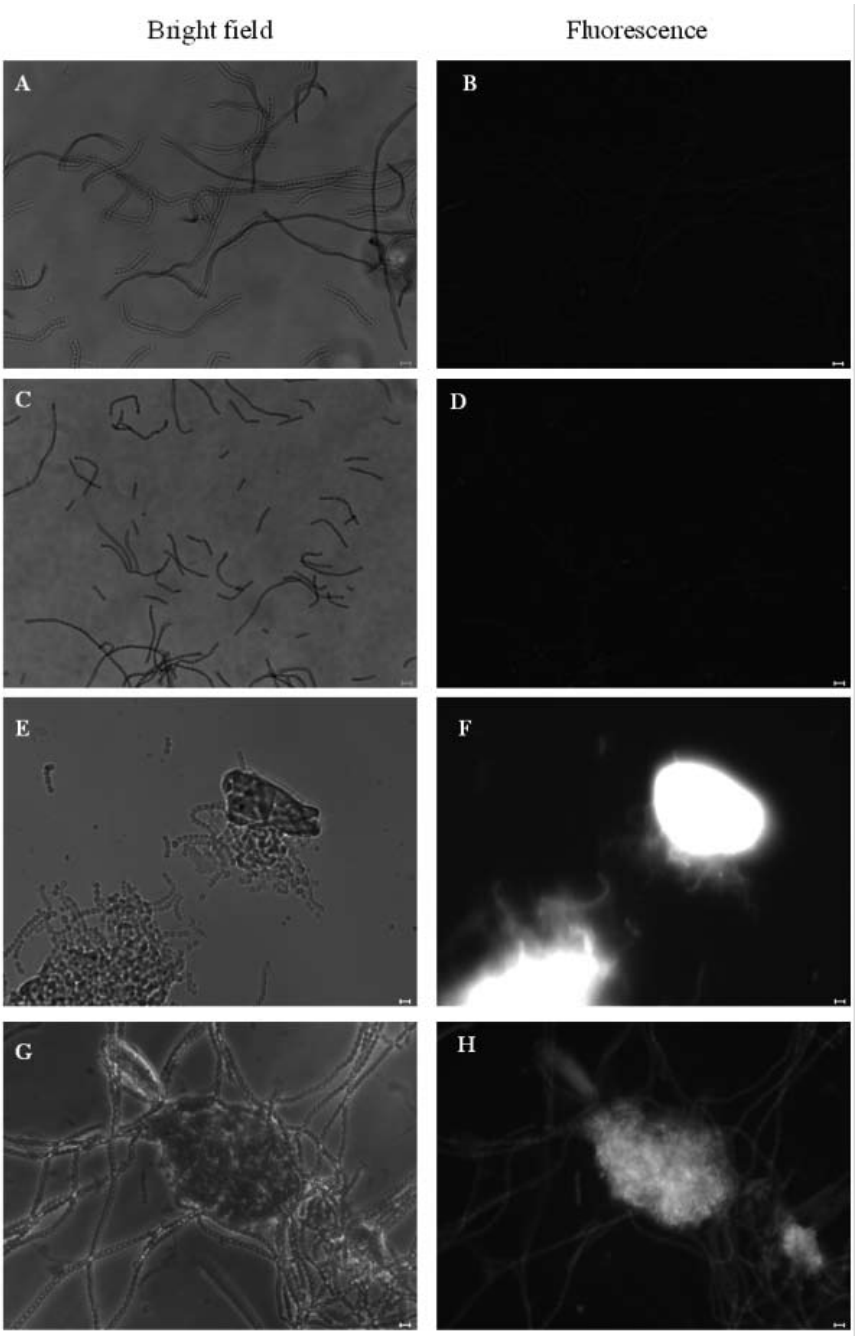

Figure 5. Cellulose staining in different *Anabaena* strains using CBHI-FITC Probes. Bright field: A, C, E, G. Fluorescence: B, D, F, H. A-B: PCC7120 wild type. C-D: *Anabaena* sp. PCC7120 strains containing plasmid pRL489-Nm^r; E-F: strain acsAB-2; G-H: strain acsAB-26. Scale bars, 20 μm.

The net photosynthesis rate of all the transformants was twice as high than that of the wild type control (Figure 7). The respiration rate was significantly depressed in all transformants as compared to that of the controls (Figure 7). The gross photosynthesis rates of transformants *acsAB*-2 and *acsAB*-17 were lower than those of the control, while the gross photosynthesis rates of the other transformants were the same as that of the wild type control (Figure 7).

The growth capacity and the overall photosynthesis capacity, based on O_2 evolution rate, were not negatively affected in the acsAB transgenic *Anabaena* PCC7120 strains. In fact, the net photosynthesis was significantly increased in all the transformants. In contrast, the respiration rate in all tranformants exhibited a ~40% decline relative to the controls. It seems that the higher growth rate of the transformants may result from the down-regulation of respiration. We hypothesize that the overexpression of *acsAB* in *Anabaena* PCC7120 cells not only forces the redistribution of photosynthate for enhanced cellulose production but also reduces the wasteful respiratory utilization of photosynthate. Future studies will test this hypothesis.

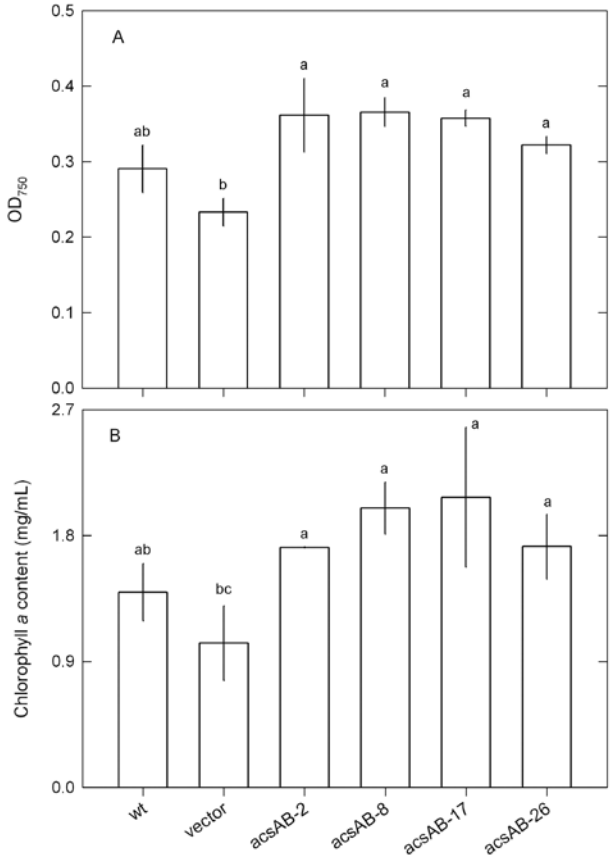

Figure 6. The OD_{750} value (A) and chlorophyll *a* content (B) of *Anabaena* PCC7120 exconjugates and controls. Data are the means ± SD (n = 3). Different symbol indicates significant differences at $P < 0.05$.

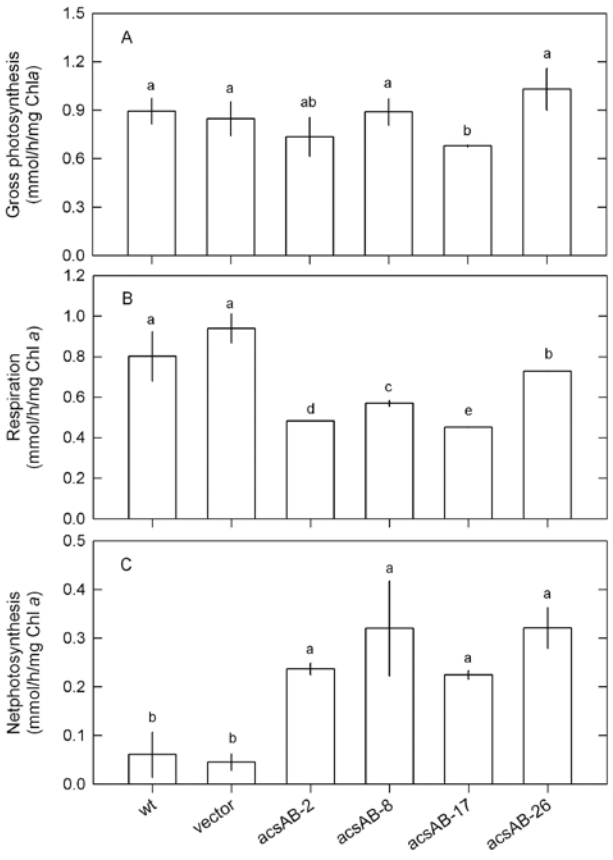

Figure 7. The gross photosynthesis (A), respiration (B) and net photosynthesis (C) rates of *Anabaena* PCC7120 exconjugates and controls. Data are the means ± SD (n = 3) and different symbol indicates significant difference at $P < 0.05$.

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絲狀藍綠菌表現異源纖維素合成酶基因促進纖維素合成、光合作用與生長

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微藻的纖維素與醣可用來生產生質酒精。而提高微藻的纖維素與醣的合成能力是以微藻大量生產生質酒精的關鍵。爲了增加纖維素產量，我們將 *Acetobacter xylinum* 纖維素合成酶基因 *acsAB* 以接合生殖方式轉入藍綠菌 *Anabaena* sp. strain PCC7120 細胞中。PCR 分析結果確認轉殖株具有 *acsAB* 基因，而 RT- PCR 分析亦檢測出轉殖株中 *acsAB* 基因之表現。轉殖株培養液分別以 Calcofluor white 染色，及聯結 FITC 的纖維素水解酶 CBHI-FITC 爲探針檢測纖微素，皆發現 *acsAB* 轉殖株細胞外物質染色後出現螢光，顯示 PCC7120 *acsAB* 基因轉殖株分泌胞外纖維素。而與 wild type 相比，*acsAB* 基因轉殖株經纖維素水解後的葡萄糖含量亦顯著的增加。轉殖株之光合作用效率分析結果顯示轉殖株的光合作用效率及生長速率提高。綜上所述，*Anabaena* sp. strain PCC7120 表達 *acsAB* 不僅提高了纖維素的合成，對其光合作用及生長亦無負面影響。

關鍵詞：纖維素；纖維素合成酶；藍綠菌；光合作用。