Bioresource Technology 143 (2013) 154–162

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Knockdown of PsbO leads to induction of HydA and production of photobiological H_2 in the green alga *Chlorella* sp. DT

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HIGHLIGHTS

- Green alga *Chlorella* sp. DT was delivered with *siRNA-psbO* fragments.
- The *siRNA-psbO* fragments could be driven by *Actin1* promoter.
- PsbO-knockdown mutants had lower PsbO levels in comparison to the wild type.
- The HydA was induced and increased several fold in the PsbO-knockdown mutants.
- The H_2 production increased as much as by 10-fold in a PsbO-knockdown mutant.

ARTICLE INFO

Article history:
Received 25 March 2013
Received in revised form 26 May 2013
Accepted 27 May 2013
Available online 4 June 2013

Keywords:
Green alga
PsbO
Knockdown
HydA
Induction

ABSTRACT

Green algae are able to convert solar energy to H_2 via the photosynthetic electron transport pathway under certain conditions. Algal hydrogenase (HydA, encoded by *HYDA*) is in charge of catalyzing the reaction: $2H^+ + 2e^- \leftrightarrow H_2$ but usually inhibited by O_2, a byproduct of photosynthesis. The aim of this study was to knockdown PsbO (encoded by *psbO*), a subunit concerned with O_2 evolution, so that it would lead to HydA induction. The alga, *Chlorella* sp. DT, was then transformed with short interference RNA antisense-*psbO* (*siRNA-psbO*) fragments. The algal mutants were selected by checking for the existence of *siRNA-psbO* fragments in their genomes and the low amount of PsbO proteins. The *HYDA* transcription and the HydA expression were observed in the PsbO-knockdown mutants. Under semi-aerobic condition, PsbO-knockdown mutants could photobiologically produce H_2 which increased by as much as 10-fold in comparison to the wild type.

1. Introduction

Green algae, which are oxygenic photosynthetic organisms, are able to convert photon energy to produce hydrogen (H_2), a clean and renewable energy (Ghirardi et al., 2000; Melis and Happe, 2001; Kruse and Hankamer, 2010; Skjånes et al., 2013). As early as the 1940s, green algae were observed to occasionally switch from the production of O_2 to the production of biological H_2 during photosynthesis (Gaffron, 1940). In the photosynthetic electron transport chain of the thylakoid membrane, following the absorption of light energy by photosystem II (PSII), H_2O is split into O_2, protons and electrons by the oxygen-evolving complex (OEC), which attaches to PSII (Nelson and Yocum, 2006). Later, the electrons are transferred between PSII and PSI via plastohydroquinone, the cytochrome b_6f complex, and plastocyanin. PSI reduces $NADP^+$ into NADPH in the stroma, mediated by the action of ferridoxin (Fd) and ferridoxin–NADP reductase. Meanwhile the protons are accumulated in the lumen space from which they are used to drive ATP production by the ATP synthase. Under certain conditions, during photosynthetic electron transport, the Fd electron carrier can supply electrons to protons which is converted into H_2 by hydrogenase (Hyd, encoded by nuclear *HYDA* gene): $2H^+ + 2e^- \leftrightarrow H_2$ (Melis and Happe, 2001). Nevertheless, the yield of photobiological H_2 production is very low because the algal HydAis usually inhibited by O_2, a byproduct of photosynthesis (Ghirardi et al., 1997; Happe et al., 1994).

In order to increase algal H_2 production, many innovative H_2-producing *Chlamydomonas reinhardtii* systems have been developed. In particular, genetic manipulation of algal cells to tolerate more O_2 or to reduce O_2 evolution could enhance H_2 yield several fold (Beer et al., 2009; Bonente et al., 2011; Esquível et al., 2011; Kruse and Hankamer, 2010; Melis et al., 2007; Srirangan et al.,

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http://dx.doi.org/10.1016/j.biortech.2013.05.101

2011). Some O_2 tolerant *C. reinhardtii* mutants were therefore selected by either exposure of the cells to high O_2 pressure or rational mutagenesis and found to have higher H_2 production rate than the wild type under the same O_2 partial pressure (Flynn et al., 2002). Some O_2 consuming *C. reinhardtii* mutants were shown to produce quantities of H_2 several fold greater than the wild type (Ruhle et al., 2008; Wu et al., 2011; Xu et al., 2011).

Although H_2 production from the model organism, *C. reinhardtii*, is appealing, a number of *Chlorella* species have been demonstrated as capable of producing H_2 as well. Recently, *Chlorella pyrenoidosa* has been reported to be able to produce H_2 under low illumination and semi-aerobic condition without sulfur-deprivation (Wang et al., 2011). A *Chlorella sorokiniana* strain was demonstrated to produce H_2 despite the high O_2 partial pressure in the photobioreactor (Chader et al., 2011). More recently, a *Chlorella* sp. strain was shown to generate H_2 under aerobic/semi-aerobic/fermentative mixed conditions (Chien et al., 2012).

The attempt of this study was to generate cellular anaerobic conditions by creating an OEC dysfunctional mutant to evolve less O_2 and to produce more H_2. It is known that the PsbO (encoded by nuclear *psbO* gene) protein is the key subunit of the OEC in algae and plants (Enami et al., 2008; Nelson and Yocum, 2006; Rivas and Barber, 2004; Shieh et al., 2011). An *Arabidopsis thaliana psbO*-knockout mutant was shown to evolve less O_2 than the wild type (Bricker and Frankel, 2008). It was also reported that the PsbO-deficient green algal cells of *C. reinhardtii* were unable to oxidize H_2O (Pigolev et al., 2009). These studies suggested that the O_2 evolution of algal cells could be reduced by genetic manipulation of the PsbO subunit.

Therefore, the strategy was to use short interference RNA (siRNA) to knockdown PsbO in *Chlorella* so that it would lead to the depletion of O_2, the induction of HydA, and the sustained production of H_2. The algal DT cells were then transformed with plasmids containing siRNA fragments of antisense *psbO* (*siRNA-psbO*). The *siRNA-psbO* fragments, driven by a rice Actin1 promoter, were delivered into algal cells by agrobacterium (Agro)-mediation. The algal PsbO-knockdown mutants were selected by checking for *siRNA-psbO* fragments in the genomic DNA using PCR. In PsbO-knockdown mutants, the RNA transcription of *HYDA* and the protein expression of HydA were examined. Consequently, the photobiological H_2 production in some PsbO-knockdown mutants was measured and found to have increased several fold in comparison to the wild type.

2. Methods

2.1. Cultures

The green alga, *Chlorella* sp. DT (DT), was a Taiwan native isolate and a generous gift from Prof. Pei-Chung Chen (NCHU) (Chen and Lai, 1996). DT was routinely cultured in 100 mL of Chlorella-medium with the addition of 0.25% glucose in 250 mL flasks with sponge stoppers at 28 °C under continuous illumination at approximately 30 μmol m^{-2} s^{-1} in a rotary shaker at a speed of 120 rpm (Chien et al., 2012). Alternatively DT was maintained on 1.5% agar plates of Chlorella medium.

Escherichia coli strain DH5α (Genemark, Taiwan) was used in all recombinant DNA experiments and grown in Luria–Bertanie (LB) medium (Difco, BD, USA) or on LB agar plates at 37 °C. *Agrobacterium tumefaciens* LBA4404 (Invitrogen, USA) was used for this study. When required, Agro was cultured in LB medium (pH 7.5) or on LB agar plates at 28 °C

2.2. Isolation of plasmid DNA

The procedure of plasmid isolation followed the protocol provided with the Plasmid Miniprep Purification Kit II (Genemark). Isolated plasmids were checked by 1% agarose gel electrophoresis and then stored at −20 °C.

2.3. Isolation of algal genomic DNA and total RNA

Algal cells at the late-logarithmic phase were harvested and washed with ddH_2O by centrifugation at 2000*g* for 3 min (himac CF15RX, Hitachi, Japan). Isolation of algal genomic DNA was performed using a Plant Genomic DNA Purification Kit (Genemark). The isolated genomic DNA was resuspended in sterile ddH_2O and stored at −20 °C. The isolation of algal total RNA was undertaken with the traditional phenol/chloroform method (Sambrook and Russell, 2001). The final isolated total RNA was resuspended with 50 μL of 1 × RNA safeguard reagent (Genemark), treated with 0.1 U μL^{-1} DNase I Amp Grade (Invitrogen) per μg of total RNA to remove DNA, and stored at −80 °C.

2.4. Synthesis of complementary DNA (cDNA) by reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR was followed the standard method with modification (Sambrook and Russell, 2001). The DNase-treated total RNA was mixed with 10 μM random hexamer and 20 μM oligo dT (Sigma, USA). The RNA/dT mixture was incubated at 70 °C for 10 min, and then chilled on ice for 1 min. Then the RNA/dT mixture was resuspended by gentle pipetting with reaction buffer containing 5X First-Strand Buffer (Invitrogen), 2.5 mM $MgCl_2$, and 0.5 mM dNTP. Afterwards, Superscript III Reverse Transcriptase (Invitrogen) was added to the RNA/dT/ reaction buffer mixture for a further incubation at 42 °C for 50 min. The reaction was terminated by incubation at 70 °C for 15 min. The cDNA was stored at −20 °C.

2.5. Primer design, sequencing, and alignment

The primers for detecting *psbO* were designed with Primer3 (http://frodo.wi.mit.edu/) according to the conserved region of *psbO* cDNA from four green algae (Supplementary data Fig. S1). Primers for detecting *HYDA* were designed according to the sequence of *Chlorella fusca HYDA* (AJ298228) and *C. s*. DT *HYDA* (GU354311) (Chien et al., 2012). The primers for detecting the transgenes were designed according to sequences on the siRNA plasmids. The primers as well as the expected sizes of PCR products are shown in Table 1. Sequencing was carried out at the NCHU Biotechnology Center and the sequence alignment was carried out with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.6. Polymerase chain reaction (PCR)

PCR was used to amplify specific DNA fragments, genomic DNA, plasmid DNA, or cDNA. The PCR procedure was as follows: 3 μL of DNA templates, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 U μL^{-1} Prime Taq DNA polymerase (Invitrogen, USA; Genet Bio, Korea) and ddH_2O were added to a final volume of 25 or 50 μL in a PCR tube. The PCR conditions were a pre-denaturation step at 94 °C for 20 min, followed by 30 amplification cycles (denatured at 94 °C for 30 s, annealed at 50 °C for 30 s, and extended at 72 °C for 1 min) and a final extension at 72 °C for 7 min. The amplified fragments were checked by 1% (w/v) agarose gel electrophoresis.

2.7. Construction of siRNA vectors

Two siRNA fragments of the antisense-*psbO-a* (*siRNA-psbO-a*): 3′-TGGCTTTCCTGTTCACCATCA-5′ and antisense-*psbO-b* (*siRNA-psbO-b*): 3′-GTCAGCAGCATCCAGCAAG-5′ were designed in accor-

Table 1
The primers used for PCR.

Primer name	Sequence		Expected size of PCR product
psbO-P	*O*F-373	5′ ATGACCCGCCTGACCTACAC 3′	1.7 kb
	*O*R-816	5′ AGGTCWGTGTCMGAGGGCTG 3′	0.44 kb (cDNA)
psbO 2-P	*O*F-333	5′ GTTCAAGGGTGGCGAGACT3′	0.48 kb (cDNA)
	*O*R-816	5′ AGGTCWGTGTCMGAGGGCTG 3′	
si-psbO-a-P	*A*F-31	5′ TAGGTAGAAGTCGACTCTAGAAGCGCGCGCCA3′	0.5 kb
	*A*R-455	5′ AGGTCWGTGTCMGAGGGCTG 3′	
si-psbO-a-P	*A*F-31	5′ TAGGTAGAAGTCGACTCTAGAAGCGCGCGCCA 3	0.5 kb
	*A*R-455	5′ CATTGTGGCTCCTCCTCTAGAGGCGGTCAGCCGA3′	
si-psbO-b-P	*B*F-101	5′ TAGGTAGAAGTCGACTCTAGACCCAACGACCCCC 3′	0.4 kb
	*B*R-544	5′ CATTGTGGCTCCTCCTCTAGACCGTACACGCCTAC3′	
hydA-P	*H2*F-383	5′ TGACCATTATGGAGGAGGGAA 3′	1.0 kb
	*H2*R-899	5′ACTTCATAGACTGTGCGAAGTGC 3′	0.51 kb (cDNA)
hydA 2-P	*H2*F-811	5′ GCACTTCGCACAGTCTATGAAGT 3′	0.4 kb
	*H2*R-1026	5′ CTTGATGAGCTTCTTGGCATTG3′	0.2 kb (cDNA)
hpt 1-P	*hpt* F-117	5′ GATGTAGGAGGGCGTGGATA 3′	0.8 kb
	hpt R-923	5′ ATTTCTCTACGCCCGACAGT 3′	
hpt 2-P	*hpt* F-117	5′ GATGTAGGAGGGCGTGGATA 3′	0.6 kb
	hpt R-695	5′ GATGTTGGCGACCTCGTATT 3′	
merA-P	*M*F-1179	5′ AACAGGTGCAACCTATGAAC 3′	0.6 kb
	*M*R-1781	5′ GTTAAACCGAATTTCACAGC 3′	
Actin1-siRNA-merA-P	*ASM*F-31	5′ TTGAGAAGAGAGTCGGGATAG 3′	1.7 kb (pHm3A/*psbO-a*)
	*ASM*R-1779	5′ TGCACCCATGTTTTCAAG 3′	1.6 kb (pHm3A/*psbO-b*)

dance with the partial cDNA sequence of *C.s*. DT *psbO* (Supplementary data Fig. S1, this study) using "siRNA Target Finder" software (GenScript, USA). The *siRNA-psbO-a* and *siRNA-psbO-b*, fragments were synthesized and integrated into two plasmids of pR-*siRNA-psbO-a* and pR-*siRNA-psbO-b* by GenScript (Supplementary data Fig. S2). The vectors, pHm3A-*siRNA-psbO-a* and pHm3A-*siRNA-psbO-b*, containing siRNA were constructed from vector pHm3A, containing the RB and LB Agro genes which can be incorporated into plant hosts (Fig. 1A and B). The *siRNA-psbO-a* and *siRNA-psbO-b* inserts containing restriction enzyme cutting sites were generated with the designated primer sets (Table 1). The pHm3A vector was linearized with the *Xba*I (Invitrogen) restriction enzyme and ligated with *siRNA-psbO-a* and *siRNA-psbO-b* inserts following the protocol provided with the Clone EZ kit (GenScript). The ampicillin (Amp) resistance gene, neomycin (Neo) resistance gene, phosphotransferase II (NPTII) gene, hygromycin (Hyg) resistance gene were used as selective markers.

2.8. Algal transformation by Agro-mediation

Agro competent cells were generally prepared according to the method of McCormac et al. (1998). The siRNA vectors were electroporated into Agro competent cells. An 80 μl quantity of Agro competent cells was mixed well with 1 μg of the pHm3A-*siRNA-psbO-a* and pHm3A-*siRNA-psbO-b* plasmids. The mixture was transferred into a 0.2-cm electroporation cuvette and electroporated by using a Gene Pulser II (Bio-Rad, USA) at 2.5 kV, 25 μF capacitance, and 400 Ω resistance. After electroporation, 1 mL ice-cold LB medium was added to the electroporation cuvette, and the tube was incubated at 28 °C in a rotary shaker at a speed of 250 rpm for 60 min. The transformed Agro cells were spread on the LB agar plates containing 50 μg mL^{-1} kanamycin (Kan) and 10 μg mL^{-1} rifampicin (Rif) and incubated at 28 °C for 2 days. Colony PCR was used to examine if Agro-pHm3A-*siRNA-psbO-a* and Agro-pHm3A-*siRNA-psbO-b* contained the transgenes.

Green algal genetic transformation by Agro-mediation was followed the method of Kumar et al. (2004) with modification. The Agro-pHm3A-*siRNA-psbO-a* and Agro-pHm3A-*siRNA-psbO-b* cells were cultivated in 5 mL of LB medium containing 50 μg mL^{-1} of Kan and 10 μg mL^{-1} of Rif at 28 °C in a rotary shaker at a speed of 250 rpm for 16–18 h. The overnight culture was diluted in 5 mL LB (1:10 dilution), and continuously incubated until the OD_{600} reached 0.5. Then 10 mL of 5×10^5 cells mL^{-1} *C.s.* DT culture was mixed with 500 μL of Agro-pHm3A-*siRNA-psbO-a* and Agro-pHm3A-*siRNA-psbO-b* cultures and incubated at 28 °C in a rotary shaker with speed of 250 rpm for 2 days. After washed twice with ddH_2O by centrifugation at 800*g* for 3 min and resuspended with fresh Chlorella-medium, 250 μg mL^{-1} of cefotaxime was added into the co-culture to kill the Agro cells and incubated at 28 °C for 4 h. Then algal cells were collected by centrifugation at 2000*g* and washed twice with ddH_2O by centrifugation at 800*g* for 3 min. The pellet of transformed algal cells was resuspended into 500 μL Chlorella medium. The transformed algal cells were diluted to the final concentration of 5×10^5 cells mL^{-1}. Then 1 mL of transformed algal cells was spread onto selective plates containing 75 μg mL^{-1} Hyg. The Hyg resistant algal colonies were visible after culturing for 6–10 days. The visible green colonies were further subcultured on selective plates or in liquid Chlorella media.

2.9. Extraction of algal total proteins

Algal cells at the late-logarithmic-phase were collected, washed twice with ddH_2O, and washed once with suspension buffer containing 0.5 M glycerol, 10 mM Na^+–MOPS, 2 mM $MgCl_2{\cdot}6H_2O$, and 10 mM KCl by centrifugation at 2000*g* for 3 min (Hitachi himac CF15RX). The pellet was resuspended with 1 mL of suspension buffer with the addition of a Protease Inhibitor for plant cells (Sigma) (1:100 dilution) and then mixed well by pipetting. The mixture was transferred into an eppendorf containing about 0.3 g of 2-mm acid washed glass beads. After vortexing at 4 °C for 30 min (pause-on 10 min; pause-off 5 min), the sample was centrifuged at 3000*g* for 15 min to remove cell debris. The green supernatant containing total proteins was collected and 2 μL of sample was taken for measuring protein concentration using Protein Assay Dye (Strong Biotech, Taiwan) with bovine serum albumin as standard.

2.10. Western blotting analysis of PsbO and HydA

Western blotting was performed as described previously (Chien et al., 2011). Aliquots of 50 μg of total extracted proteins were mixed with sample buffer, loaded onto a 12% SDS–PAGE gel, and electrophoresed on a Hoefer SE 260 gel unit (GE Healthcare, formerly Amersham Pharmacia Biotech) at room temperature. Then the total proteins separated by SDS–PAGE were electrotransferred

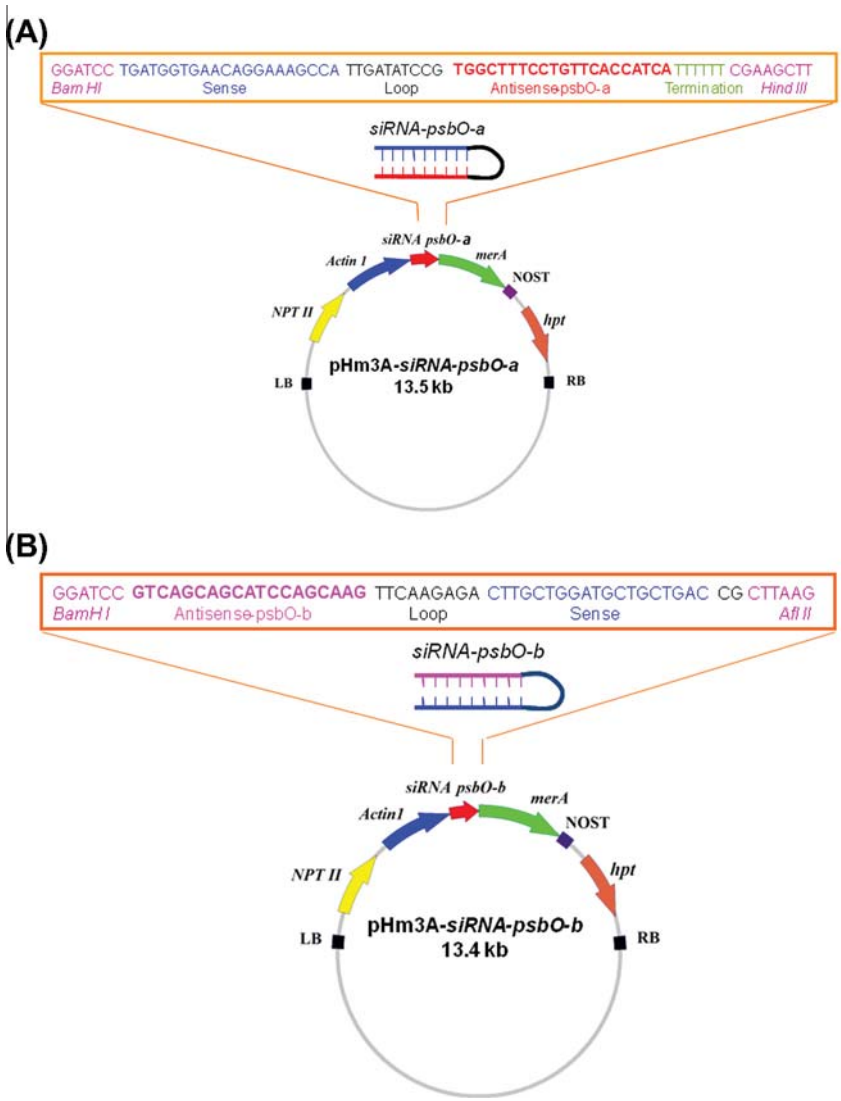

Fig. 1. Maps of the siRNA plasmids containing *siRNA-PsbO-a* and *siRNA-PsbO-b*. (A) The pHm3A-*siRNA-psbO-a* plasmid was cloned with *siRNA-psbO-a* under the control of the rice *Actin1* promoter into the pHm3A cloning vector. The antisense-*psbO-a* was designed in accordance with a partial sequence of *psbO* using "siRNA Target Finder" software (GenScript). The hygromycin phosphotransferase (*hpt*) gene, neomycin (Neo) phosphotransferase (*NPT II*) and mercuric reductase (*merA*) genes were used as selective markers. NOST: 3′UTR of the nopaline synthase gene, a mininator; LB: left border repeat; RB: right border repeat. (B) The pHm3A-*siRNA-psbO-b* plasmid was cloned with *siRNA-psbO-b* under the control of *Actin 1*. The antisense-*psbO-b* was designed in accordance with another partial sequence of *psbO*.

to Hybond P polyvinylidene difluoride (PVDF) membrane (GE Healthcare) at 400 mA using blot-transfer buffer in a Hoefer TE22 transfer tank (GE Healthcare) at 4 °C for 1.5 h. After transferring, the membranes were probed with primary antibodies, anti-PsbO (AS06 142-33, Agrisera, Sweden), anti-synthesized-peptide-HydA (Yoahong, Taiwan) (Chien et al., 2012) or anti-cytochrome oxidase subunit 2 (anti-COX2) (AS04 053A-200, Agrisera). Then the membranes were probed with secondary antibody of anti rabbit horseradish peroxidase (HRP) conjugate. The binding of antibodies was visualized by using diaminobenzidine (DAB) solution. The reaction was stopped by washing the blot with ddH_2O as soon as the specifically stained bands were clearly visible. The blots were then scanned with a TMA 1600 scanner (Microtek, Taiwan). The PsbO and HydA protein levels were quantified with NIH Image J analyzer.

2.11. Measurement of photosystem II (PSII) activity

Photosynthetic activities of algal cells were determined by measuring fluorescence parameters with an FMS1 chlorophyll fluorometer (Hansatech, UK) as described previously (Huang et al., 2006; Chien et al., 2012). A 9 mL volume of algal culture at a cell concentration of 1×10^6 cells mL^{-1} was transferred into a 15 mL-tube and kept in the dark for at least 10–20 min. The fluorescence parameters F_o (minimal fluorescence), F_m (maximal fluorescence), and F_v (variable fluoresce, $F_v = F_m - F_o$) of dark-adapted samples were measured while the PSII activities were determined as F_v/F_m (Baker, 2008).

2.12. Measurement of H_2 production

Measurement of H_2 production was followed as described previously (Chien et al., 2012) (Supplementary data Fig. S3). The DT mutants were cultured in 300 mL Chlorella medium at the initial concentration of 1×10^7 cells mL^{-1} in sealed 350-mL glass bottles and mixed by magnetic stirring. The transformant cultures were kept in the dark for 24 h at 28 °C to reach anaerobic conditions (Wang et al., 2011). Then the cultures were supplied with continuous white illumination at 30 μE m^{-2} s^{-1} for 4 days. The gas mixture in the headspace of the 350-mL glass bottle was sampled

using a syringe with a 4-cm needle. The 200 μL sample of gas was injected into a gas chromatograph (GC-14A, Shimadzu, USA) to measure H_2 concentration. The GC was equipped with a thermal conductivity detector (TCD), nitrogen was used as the carrier gas under 5 bars of pressure, and the temperature of the H_2 column was 60 °C. The temperature of the TCD was set at 100 °C for both the detector and injector. Pure H_2 gas (200 μL) was used as standard gas.

2.13. Statistical analysis

Student's *t*-test was used to analyze the data if there were statistically significant difference between treatment and control measurements. The values of $P < 0.05$ were considered statistically significant difference.

3. Results and discussion

3.1. Design of siRNA-psbO-a and siRNA-psbO-b

In order to design the siRNA, the cDNA fragment of *C. s*. DT *psbO* was partially sequenced. The total RNA was firstly isolated and reversely transcribed into cDNA. The PCR products of the 0.44 kb and 0.48 kb fragments were amplified from cDNA with designated primers (psbO-F333, psbO-F373 and psbO-R816, Table 1) and subjected to sequencing. The partial cDNA of the *psbO* sequence was aligned with sequences of other green algae; *Chlorella ellipsoidea* (*C. e*., AY456395), *C. reinhardtii* (*C. r*. XM_001694647), *Volvox carteri* (*V. c*., AF110780), and *Nephroselmis olivacea* (*N. o*, AB293978) using BioEdit software (Supplementary data Fig. S1). The partial sequence of *C. s.* DT *psbO* was about 74% conserved in comparison to those of other green algae.

The two siRNA fragments, *siRNA-psbO-a* and *siRNA-psbO-b*, were therefore designed from highly conserved regions of *psbO* (Supplementary data Fig. S2). These two siRNA fragments were recombined into the pHm3A plasmid which contained the *hpt* gene, and driven by an Actin1 promoter (Fig. 1A and B). The existence of *siRNA-psbO-a* and *siRNA-psbO-b* fragments in the constructed vectors was examined by sequencing (data not shown).

3.2. Generation of psbO-knockdown DT mutants by transfection with Agro-pHm3A-siRNA-psbO-a and Agro-pHm3A-siRNA-psbO-b

The siRNA vectors of pHm3A-*siRNA-psbO-a* and pHm3A-*siRNA-psbO-b* were firstly transformed into Agro by electroporation. The Agro transformants were selected on Rif and Kan plates, and tested by PCR products with the primer sets as designated in Table 1. In the Agro transformants, a 0.5 kb band containing the *siRNA-psbO-a* fragment (Fig. 2A) and a 0.4 kb band containing the *siRNA-psbO-b* fragment were observed (Fig. 2B).

The Agro-pHm3A-*siRNA-psbO-a* and Agro-pHm3A-*siRNA-psbO-b* transformants were then co-cultured with DT cells in flasks for 2 days. The transfected algal DT cells were selected on 75 μg mL^{-1} Hyg plates. The green Hyg resistant colonies were visualized on the selective plates after cultivation for 6–10 days. Then the algal genomic DNAs of the DT-pHm3A-*siRNA-psbO-a* and DT-pHm3A-*siRNA-psbO-b* mutants were isolated and further tested by PCR. Consequently, the DT mutants, tnOa1, tnOa2, and tnOa3, which contained *Actin1-siRNA-psbO-a-merA* (Fig. 2C), and the DT mutants, tnOb4 and tnOb5, which contained an *Actin1-siRNA-psbO-b-merA* fragment (Fig. 2D), were confirmed.

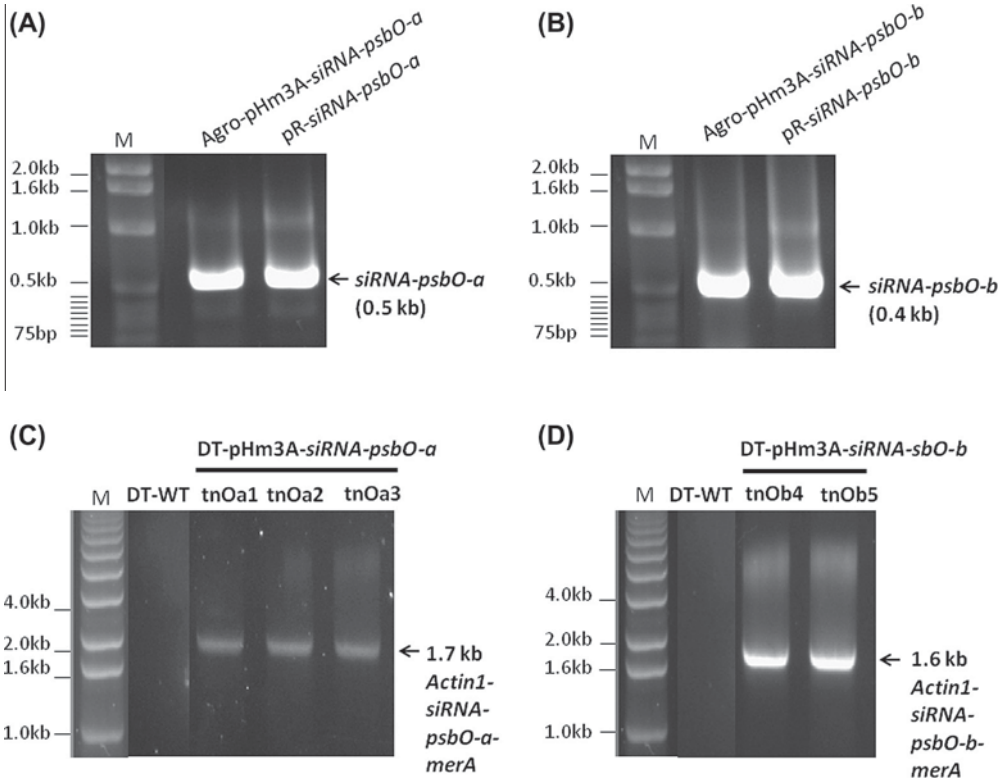

Fig. 2. Generation of DT-pHm3A-*siRNA-psbO-a* and DT-pHm3A-*siRNA-psbO-b* mutants. (A) The 0.5 kb band of the pHm3A-*siRNA-psbO* fragment was observed in Agro transformants by colony PCR. Agro-pHm3A-*siRNA-psbO-a*: Agro transformant containing pHm3A-*siRNA-psbO-a*; pR-*siRNA-psbO-a*: plasmid as the control. (B) The 0.4 kb band of the pHm3A-*siRNA-psbO-b* fragment was observed in Agro transformants by colony PCR. Agro-pHm3A-*siRNA-psbO-b*: Agro transformant containing pHm3A-*siRNA-psbO-b*; pR-*siRNA-psbO-b*: plasmid as the control. (C) A 1.7 kb band was obtained corresponding to the *Actin1-siRNA-psbO-a-merA* fragment amplified from the genomic DNA of DT mutants by PCR. DT-WT: DT wild type; tnOa1, tnOa2, and tnOa3: DT-pHm3A-*siRNA-psbO-a* transgenics. (D) A 1.6 kb band was obtained corresponding to the *Actin1-siRNA-psbO-b-merA* fragment amplified from the genomic DNA of DT mutants by PCR. tnOb4 and tnOb5: DT-pHm3A-*siRNA-psbO-b* transgenics. M: 0.1 μg 1 kb DNA ladder marker.

3.3. Reduced amounts of PsbO protein in DT mutants

The total proteins of the DT mutants were extracted, separated by SDS–PAGE, and subjected to Western blotting. Using anti-PsbO, a detectable PsbO signal was visualized as a protein band of 28 kDa on the blot (Fig. 3A). The PsbO protein expression levels of the tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants were decreased to 42–66% of the levels found in DT-WT (Fig. 3B). The results of western blotting indicated that the PsbO proteins of these mutants were knocked down to different levels.

When cultured in flasks with low illumination under aerobic and S-supplied conditions, the PsbO-knockdown mutants of tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 grew slightly lower than the DT-WT (Fig. 4A and B). In order to understand whether the photosynthetic activity of the PsbO-knockdown mutants was affected, the chlorophyll fluorescence parameters were measured. The F_v/F_m ratio, presenting PSII activity, was determined. The results showed that the F_v/F_m ratios of the tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants slightly decreased to 0.69 ± 0.02 ~ 0.72 ± 0.02 as compared to 0.80 ± 0.01 of the DT-WT (Fig. 4C). It implied that less O_2 would be evolved in the PsbO-knockdown mutants but the electrons could still be transferred from PSII to the electron transport chain. These data contradict a recent report that the antisense reductions in the PsbO protein of Arabidopsis leads to decreased quantum yield but similar photosynthetic rates (Dwyer et al., 2012).

3.4. Induction of HYDA transcripts and HydA proteins in PsbO-knockdown DT mutants

To check the transcription of *HYDA* in the PsbO-knockdown mutants under aerobic condition, the cDNAs from tnOa1, tnOa2, tnOa3, tnOb4 and tnOb5 mutants, which were cultured in the flasks with air and S supplement, were generated and used as templates to amplify *HYDA* fragments. The expected 0.5 kb fragments (amplified by primer set of hydA-F386 with hydA-R899) of *HYDA* transcripts were observed in the tnOa1, tnOa2, tnOa3, tnOb4 and tnOb5 mutants (Fig. 5A). The results showed that the transcription of *HYDA* could be induced in certain mutants under aerobic and S-supplied conditions. Using an anti-synthesized-peptide-HydA, the detectable signals of induced HydA, a protein band of around 46–48 kDa (Posewitz et al., 2004; Chien et al., 2012), was visualized in the tnOa1, tnOa2, tnOa3, tnOb4 and tnOb5 mutants but not in the DT-WT (Fig. 5B). The HydA proteins of the tnOa or tnOb mutants were obviously enhanced, but that of DT-WT was not. After analysis by Image J, in the DT mutants, the induced HydA protein levels were approximately17-fold greater than in the DT-WT (Fig. 5C).

By the way, the algal DT cells have also been electroporated with pR-*siRNA-psbO-a* carrying the *siRNA-psbO-a* (driven by a human nuclear U6 promoter) and pR-*siRNA-psbO-b* carrying *siRNA-psbO-b* (driven by a human cytomegalovirus promoter) (Supplementary data Fig. S2) which both contained cGFP gene. The transformants of u6Oa11, u6Oa12, cmOb3 and cmOb4 could be selected by the green fluorescence light as they carried GFP

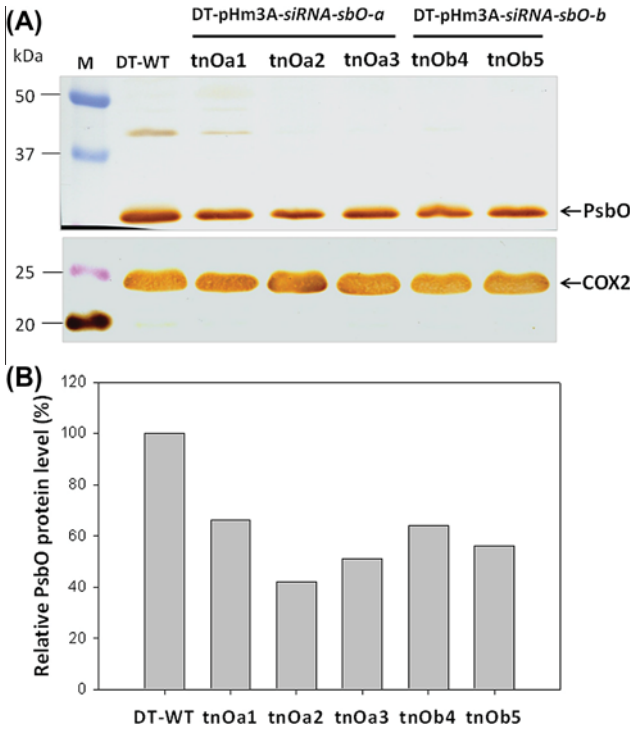

Fig. 3. Knockdown of PsbO protein in DT-pHm3A-*siRNA-psbO-a* and DT-pHm3A-*siRNA-psbO-b* mutants. (A) When probed with anti-PsbO antibodies, PsbO protein (28 kDa on the blot) was detected in all samples. The amount of PsbO in the tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants was lower than in the DT-WT. COX2: 23 kDa protein used as internal loading control. M: pre-stained protein marker (10–250 kDa). (B) The PsbO protein levels were quantified by Image J and referred to the loading control of COX2. The PsbO protein level of DT-WT was considered as 100%.

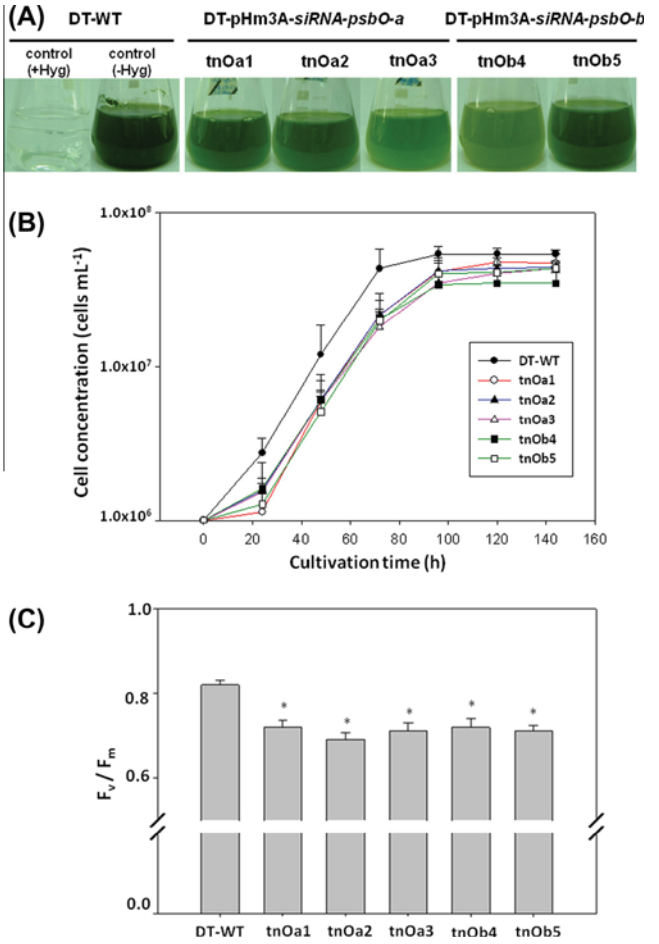

Fig. 4. Cell growth and PSII activity of PsbO-knockdown DT mutants. (A) The tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants with initial concentration at 1×10^6 cells mL^{-1} were cultivated in 100 mL *Chlorella* medium in 250-mL flasks. (B) Cell numbers were counted every day. (C) When the algal cells cultivated for 3 days, the F_v/F_m ratios were measured. Data represented as mean ± SD of three independent experiments. All data were statistically analyzed using the Student's *t*-test. The values of $P < 0.05$ were considered statistically significant difference. The symbol of "*" represented that the F_v/F_m of the mutant is significantly different from that of the DT-WT.

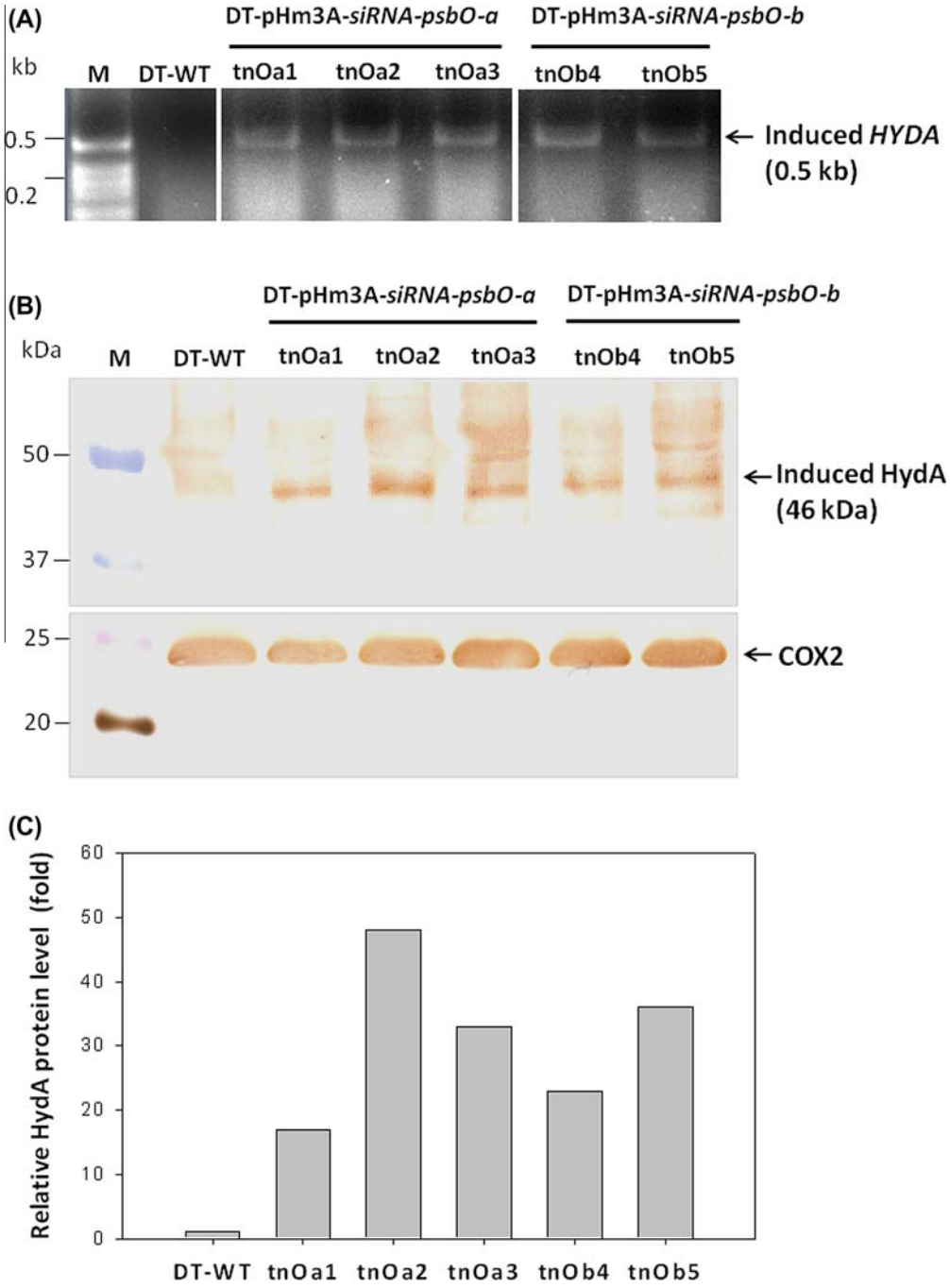

Fig. 5. Induction of *HYDA* transcripts and HydA proteins in PsbO-knockdown DT mutants. The tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants with initial concentration at 1×10^6 cells mL^{-1} were cultivated in 100 mL *Chlorella* medium in 250-mL flasks for 3 days. (A) Induction of *HYDA* in PsbO-knockdown mutants. A 0.51 kb PCR product of the *HYDA* gene fragment amplified from the cDNA of tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants was observed. M: 0.1 μg 1 kb DNA ladder marker. (B) Expression of HydA protein in PsbO-knockdown mutants. A 46–48 kDa band of induced HydA was observed in the tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants. COX2: 23 kDa protein used as a loading control. M: pre-stained protein marker (10–250 kDa). (C) The HydA protein levels were quantified by Image J and referred to the loading control of COX2. The HydA protein level of DT-WT was considered as onefold.

(Supplementary data Fig. S4A). Using the cDNAs of the obtained transformants as templates, the expected *hpt* fragments amplified by PCR were observed in these u6Oa11, u6Oa12, cmOb3 and cmOb4 transformant, indicating that the vector genes were successfully inserted into the algal genome by electroporation (Supplementary data Fig. S4B). The intensities of the PsbO signals from the u6Oa11, u6Oa12 and cmOb4 mutants were also lower than in DT-WT (Supplementary data Fig. S4C). The expected 0.2 kb fragments (amplified by hydA-F811 with hydA-R1026) were observed in the u6Oa11, u6Oa12, cmOb3 and cmOb4 mutants (Supplementary data Fig. S4D). However, the phenotype of the u6Oa11, u6Oa12, cmOb3, and cmOb4 mutants lacked stability after a period of time.

3.5. H_2 production of DT PsbO-knockdown mutants

The H_2 production in the PsbO-knockdown transgenics was subsequently measured using GC (Supplementary data Fig. S3). The H_2 produced by tnOa1, tnOa2, tnOa3, tnOb4 and tnOb5 mutants was detectable and about 3 to 12-fold higher than in the

DT-WT (Fig. 6A). In the DT-WT the H_2 concentration was about 30.3 ± 2.5 mL L^{-1}, whereas in the tnOa1, tnOa2, and tnOa3 mutants the concentrations increased to 98.5 ± 2.9, 352.1 ± 13.3, and 142.3 ± 4.3 mL L^{-1}, respectively. In the tnOb4 and tnOb5 mutants, the concentrations increased to 131.5 ± 4.3 and 113.3 ± 9.8 mL L^{-1}, respectively. These results showed that knockdown of the PsbO protein led to more H_2 gas production. There was some indication that higher levels of HydA might be correlated with increased H_2 production, although the relationship was not strong. There was no significant different effect between *siRNA-psbO-a* and *siRNA-psbO-b* fragment in knocking down psbO. When the transgenics were cultured in bottles with illumination under semi-aerobic and S-supplied conditions during H_2 production, the F_v/F_m ratios of the transgenics were about 0.25 ± 0.03 ~ 0.29 ± 0.02, which is again only slightly lower than the 0.34 ± 0.03 value observed in the DT-WT (Fig. 6B).

The results of this study showed that the PsbO-knockdown mutants retained lower levels of photochemical activity, undertook photosynthesis but evolved less O_2 gas and produced more H_2 simultaneously, whereas the DT-WT could not. In particular, the tnOa2 mutant had the lowest F_v/F_m ratio of 0.25, was supposed to evolve the least amount of O_2, but produced the highest amount of H_2. It can be inferred from this that the knockdown of psbO leads to the induction of HydA and the production of H_2. The results of this study agreed with a previous report that green algal H_2 production is a consequence of partial inactivation of photosynthetic O_2-evolution activity (Ghirardi et al., 2007). Our previous investigation demonstrated that the HydA homologously overexpressed mutants could produce H_2 in the presence of O_2 but DT-WT could not (Chien et al., 2012). The algal H_2 production yield may be further improved if a combined system of the knockdown of psbO and the homologous overexpression of HydA could be established.

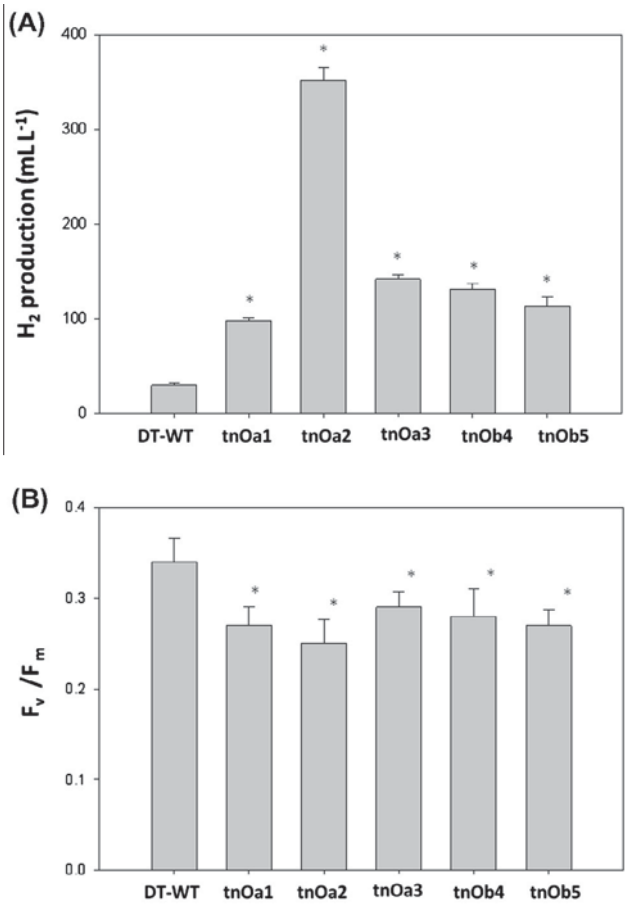

Fig. 6. H_2 production and photosynthetic activity of PsbO-kockdown DT mutants. The tnOa1, tnOa2, tnOa3, tnOb4, and tnOb5 mutants were cultivated at the initial concentration of 1×10^7 cells mL^{-1} in 300 mL Chlorella medium in sealed 350-mL bottles for 4 days. (A) A 200 μL aliquot of gas was extracted from the headspace of the 350-mL glass bottles and analyzed by GC. (B) The changes in the F_v/F_m ratios were detected. Data represented as mean ± SD of three independent experiments. All data were statistically analyzed using the Student's *t*-test. The values of $P < 0.05$ were considered statistically significant difference. The symbol of "*" represented that the concentration of H_2 produced by the mutant is significantly different from that by the DT-WT.

4. Conclusions

Green alga *Chlorella* sp. DT mutants were generated where PsbO was knocked down following transformation with antisence sequences of *siRNA-psbO-a* and *siRNA-psbO-b* driven by the Actin1 promoter. The *siRNA-psbO* fragments are likely to be functional in the mutants as their PsbO expression levels were lower than in the DT-WT. The relative protein levels of the induced HydA in the PsbO-knockdown DT mutants were increased about 3-10-fold in comparison to the DT-WT. Under low illumination and semi-aerobic conditions, photobiological H_2 production in a PsbO-knockdown mutant in particular increased by as much as 10-fold in comparison to the DT-WT.

Acknowledgements

The authors gratefully acknowledge Prof. Pei-Chung Chen (NCHU) for generously providing the *Chlorella* strain. A special thanks goes to Dr. Laurence Cantrill for help in reading the manuscript. This work was supported by the Academia Sinica (to T-Y Feng and L-F Chien), the ATU Plan from the Ministry of Education (to C-C Huang and L-F Chien), and the National Science Council (NSC99-2221-E-005-074 and NSC101-2221-E-005-037 to L-F Chien) of Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.05.101.

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