Available online at www.sciencedirect.com

ScienceDirect

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

Enhanced H_2 photoproduction by down-regulation of ferredoxin-$NADP^+$ reductase (FNR) in the green alga *Chlamydomonas reinhardtii*

Yongle Sun [a,b,1], *Mei Chen* [a,1], *Haomeng Yang* [a], *Jin Zhang* [a,b], *Tingyun Kuang* [a], *Fang Huang* [a,*]

[a] *Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[b] *University of Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 27 June 2013
Received in revised form
27 September 2013
Accepted 2 October 2013
Available online 2 November 2013

Keywords:
Chlamydomonas
H_2 photoproduction
FNR
RNAi
Sulfur depletion

ABSTRACT

Renewable H_2 photoproduction by green algae such as *Chlamydomonas reinhardtii* is a promising system for solar fuels. However, large-scale application of the system has lagged virtually due to lack of high H_2-producing strains. We previously identified ferredoxin-$NADP^+$ reductase (FNR) among the 105 proteins differentially expressed in *Chlamydomonas* during sulfur-deprived H_2 photoproduction. In this work, we used an RNA interference (RNAi) approach to generate *Chlamydomonas* mutant strains with reduced levels of FNR. We found that fnr-RNAi strains exhibited higher rates of H_2 photoproduction (2.5-fold) than wild type under sulfur-deprived condition. To elucidate the basis for this increase, we analyzed the physiological characteristics of the fnr-RNAi strains under such condition. Major changes, due to the down-regulation of FNR, included the lower rates of photosynthetic O_2 evolution (44%), greater reduction of Rubisco amounts (60%) and higher rates of starch degradation (140%). These may result in an earlier onset of anaerobiosis and increased electron supply to the hydrogenases in the mutant strains. The results provide new information of FNR in regulating H_2 metabolism as well as potential strains for further improvement of the organism toward application in solar-powered systems.

1. Introduction

H_2 production by green algae such as *Chlamydomonas reinhardtii* represents one of the promising systems for renewable solar fuels in the future [1]. Under anaerobic condition, the green algal organisms produce a substantial quantity of H_2 using hydrogenases and electrons mainly provided by photosynthetic electron flow generated via water splitting in visible light [2]. The system is advanced over other biological H_2-producing means in terms of the high catalytic activity of the hydrogenases [3] and its direct coupling to the photosynthetic machinery [4]. However, large-scale application of algal H_2-photoproducing system has lagged due to the lack of ideal strains with O_2-tolerant hydrogenases as well as sufficient H_2 yield.

To overcome the limitations, considerable research has been conducted during the past decades to determine

* *Corresponding author*. Tel.: +86 10 62836692; fax: +86 10 62594363.
E-mail address: fhuang@ibcas.ac.cn (F. Huang).
[1] These authors contributed equally to this work.
0360-3199/$ – see front matter
http://dx.doi.org/10.1016/j.ijhydene.2013.10.011

structure and function of hydrogenases and to characterize the H_2 photoproduction metabolism [3,5,6]. A breakthrough progress was made by Melis and co-workers with establishment of an experimental protocol based on sulfur depletion [7]. Using the protocol, the O_2 sensitivity limitation is circumvented by inducing temporal separation of O_2- and H_2-evolution phases. Based on the protocol, in-depth investigations have also been carried out, leading to the discovery of complex regulation of H_2 metabolism [8–10]. Transcriptomic and proteomic analysis in *Chlamydomonas* indicated that a great number of genes/proteins involved in various metabolic pathways were up-or down-regulated during H_2-photoproduction process [11–13]. These findings provide not only detailed information regarding the intricate interplay in H_2 metabolism but also more candidate genes for targeted genetic engineering of *Chlamydomonas* toward improvement of H_2 production.

Ferredoxin-$NADP^+$ reductase (FNR) is the key enzyme catalyzing the reaction of electron transfer from ferredoxin (Fd) to $NADP^+$, leading to production of NADPH mainly used for carbon dioxide (CO_2) fixation in photosynthesis. Under anaerobic condition, FNR has been proposed to be one of the components competing for photosynthetic electrons from Fd with hydrogenases in *Chlamydomonas* [14], though direct evidence is lacking. Recent data from an *in vitro* experiment indicated that, under anaerobic condition supporting H_2 production, there is a significant loss of photosynthetic electrons toward NADPH formation [15]. Using a proteomic approach, we have previously identified FNR as one of the differentially expressed proteins in *Chlamydomonas* that undergo sulfur-deprived H_2 photoproduction process [13]. The question arises as to what extent this gene/protein affects H_2 metabolism in the organism.

RNA interference (RNAi) is an efficient tool to engineer eukaryotic organisms by specifically silencing target genes. In this method, a double-stranded RNA directed against a target gene was introduced into a host cell, leading to the suppression of gene expression [16]. In *Chlamydomonas*, the approach has been used to address fundamental questions in algal cell biology [17,18] as well as to create the mutant strains with biotechnological significance [19,20]. Recently, several RNAi mutant strains such as *Stm6Glc4L01* and *pgrl1-kd* with improved H_2 production were obtained [9,21]. In terms of the *FNR* gene, however, no genetically-engineered mutant strain has been so far reported.

In this work, we have employed RNAi approach to generate *Chlamydomonas* strains with reduced level of FNR. Stable transformants were selected using paromomycin-and 5-FI resistance followed by immunoblot verification. We have analyzed physiological characteristics of fnr-RNAi strains under sulfur-deprived condition. We found that the mutant strains possessed higher H_2-photoproducing capability than wild type. We also found their rates of photosynthetic O_2 evolution and starch degradation decreased and increased, respectively, under sulfur-depletion condition. We conclude that the enhancement of H_2 photoproduction is a cumulative consequence of the earlier onset of anaerobiosis and increased electron supply to the hydrogenases in fnr-RNAi mutant strains.

2. Materials and methods

2.1. Strains, culture conditions and H_2 photoproduction via sulfur depletion

Chlamydomonas reinhardtii wild-type strain, CC400 was obtained from the *Chlamydomonas* Center (www.Chlamy.org). The algal cells were cultured in TAP medium [22] at 25 °C under 100 μmol m^{-2} s^{-1} continuous light. For all experiments, mid-exponentially growing cells (about 3–5 × 10^6 cells ml^{-1}) were harvested as described [13]. Growth was measured as OD_{750} at different time of cultivation [23]. Sulfur-depleted H_2 photoproduction was achieved as described [7,13] with modifications. Briefly, mid-exponentially growing cells were harvested and washed once with TAP-S medium. The culture was transferred to a 100-ml bottle and the cell density was adjusted to 25 μg ml^{-1} initial chlorophyll concentration. To measure the amount of H_2 accumulated, 150 μl of gas was taken from the headspace of the culture and analyzed in a gas chromatograph instrument GC-2014 (Shimadzu; Tokyo, Japan) equipped with a thermal conductivity detector using N_2 as the carrier gas. For protein, mRNA and starch analysis, cells were harvested at different time points by centrifugation and washed once with 0.02 M HEPES/KOH buffer (pH 7.5) before storage at −70 °C.

2.2. Generation and isolation of fnr-RNAi strains

Isolation and purification of total RNA from *Chlamydomonas* cells was performed as described [13]. Reverse transcription reactions using a poly (A^+) specific primer were performed with M-MLV Reverse Transcriptase (TransGen Biotech; Beijing, China). The RNAi construct was generated by introducing *FNR* inverted repeats into the *Maa7/X* IR vector [17]. The *FNR* IR cassette was generated by PCR amplification of two fragments from the *FNR* cDNA (accession no. XM_001697300) with the oligoIR.1 (5′-gaattcGGTGACCACCGATATGTCCAAG-3′) and oligoIR.2 (5′-agatctTTCTTGGCGGGGTCCTCCTTGC-3′), which generate a product of 387 bp (from nucleotide 99–485); and with oligoIR.1 and oligoIR.3 (5′-agatctACGCCCATGAACAGCCAGAACA-3′), which generate a fragment of 612 bp (from nucleotide 99–710). These PCR products were cloned in sense and antisense orientation by digestion with *EcoR* I and *Bgl* II (sites introduced by PCR), and ligation into the *EcoR* I site of *Maa7/X* IR vector. The constructed vector (*Maa7/FNR* IR) was transformed into wild type strain by the glass beads method [24]. Transformants were selected on TAP plates containing 10 μg ml^{-1} paromomycin and 10 μM 5-FI as described [16].

2.3. Production of antiserum against recombinant FNR protein

Gene cloning and heterologous expression were performed as described [25]. Total RNA isolation/purification and reverse transcription reactions were carried out as described above. cDNAs were amplified by PCR with PrimerSTAR HS DNA Polymerase (Takara, Ohtsu, Japan) using specific primers FNR-F-*Nde*I and FNR-R-*Xho*I as listed in Table S1. The amplified fragment was cloned directly into p*Easy*-blunt vector (Beijing

TransGen Biotech, China), which was then transformed into competent *Escherichia coli* DH5α cells. Positive clones containing the recombinant plasmid were selected and sequenced to ensure the authenticity of the ORFs (Beijing Sunbiotech, China). For heterologous expression, the plasmid was digested with *NdeI* and *XhoI*. The fragment was purified and subcloned into similarly treated vector pET-28a (+) (Merck, Germany) to generate pET-*FNR*. The constructed expression plasmid was transformed into expression host *E. coli* Transetta (DE3) (Beijing TransGen Biotech, China). The cells were grown in LB medium containing 50 mg kanamycin l^{-1} at 37 °C. When the culture reached an OD_{600} of 0.6 ~ 0.8, 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and incubated for 6 h at 30 °C. Following the induction, cells were harvested by centrifugation at 4400 g for 5 min at 4 °C and washed once with 10 mM sodium phosphate buffer (pH 7.4). Extraction and purification of *E. coli* proteins were done as described previously [25]. A rabbit serum was produced by MBL (MBL,Nagoya, Japan) using the purified recombinant FNR as immunogen.

2.4. Measurement of rates of oxygen evolution and respiration

Rates of oxygen evolution and respiration were measured with a Chlorolab-2 oxygen electrode (Hansatech, Norfolk, UK) at 25 °C by following the manufacture's instruction. 1 ml of cell suspension supplemented with 20 μl of 0.5 M $NaHCO_3$ (pH 7.4) was used [26]. Measurements were taken with the O_2 electrode, beginning with the registration of dark respiration of cell suspension followed by measurement of the light-saturated rate of oxygen evolution. The rate of each process was recorded for about 5 min.

2.5. SDS-PAGE and immunoblot analysis

Proteins were extracted from *Chlamydomonas* according to Liu et al. [27] with minor modifications. Cells were resuspended in 20 mM HEPES/KOH buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) then disrupted by 4 cycles of freezing-thawing (freezing in liquid N_2 and thawing in ice-water bath). Protein separation by SDS-PAGE and immunoblot analysis were carried out as previously described [28]. Primary antibodies were purchased from Agrisera (Umeå, Sweden) and used by following the instructions. The dilutions for the specific antibodies used in this study were as follows: anti-FNR (1:15000), anti-HydA and anti-RbcL (1:5000). The binding of the cross-reacting antibody was detected using the ECL plus western blotting detection system (GE Healthcare). The blots were scanned using UMAX PowerLook 2100XL scanner (Willich, Germany) at a resolution of 600 dpi. Protein quantification was carried out using ImageJ software (http://rsbweb.nih.gov/ij). Protein content was determined according to Peterson [29] using BSA as a standard.

2.6. Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was carried out as previously described [13] with slight modifications. Reverse transcription reactions were carried out using random primers and SuperScript III Reverse transcriptase from Invitrogen (Carlsbad, California, USA). To detect possible DNA

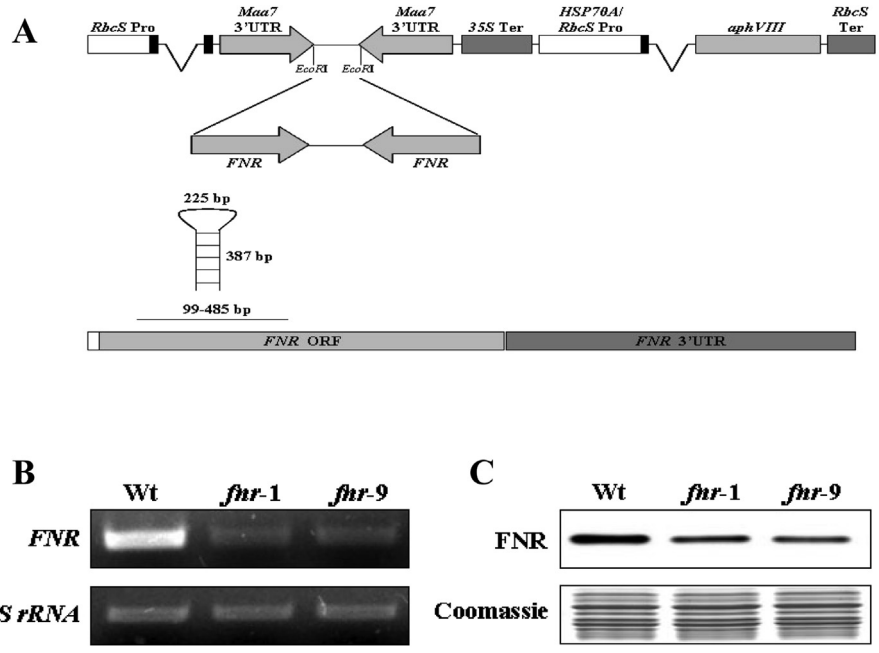

Fig. 1 – RNAi vector construct and selection of FNR down-regulation mutants. (A) Schematic representation of the construct used for the transformation. The *FNR* fragments were cloned in sense and antisense orientation between the *Maa7* inverted repeat. The predicted structure of the resulting hairpin RNA is indicated. (B). RT-PCR analysis of the expression of *FNR* in wild type (Wt) and two independent RNAi strains. Expression of *18S rRNA* was used as an internal reference. (C). FNR protein levels in wild type (Wt) and two independent RNAi strains detected by immunoblotting. Proteins (20 μg, per lane) were separated by 12% SDS-PAGE.

contamination, control reactions were performed without RT. Reverse transcription products were amplified by PCR and analyzed by electrophoresis on 1.5% (w/v) agarose gels. The transcripts selected for this study include: *HydA1* (accession no. XM_001693324); *HydA2* (accession no. XM_001694451); *PsbA* (accession no. NC_005353); *PsbO* (accession no. XM_001694647); *PetC* (accession no. http://www.ncbi.nlm.nih.gov/nuccore/159481437 XM_001698734); *PsaA* (accession no. AB044419); *PsaD* (accession no. http://www.ncbi.nlm.nih.gov/nuccore/159479281XM_001697670); *PetF* (accession no. http://www.ncbi.nlm.nih.gov/nuccore/159469304XM_001692756); *Fdx5* (accession no. http://www.ncbi.nlm.nih.gov/nuccore/159466833XM_001691551); *FNR* (accession no. XM_001697300); *RbcL* (accession no. J01399); *RbcS* (accession no. XM_001702357). The primer sequences were listed in Supplemental Table 1. The *18S rRNA* (accession no. AY665727) was used as a positive control [11]. The log phase of RT-PCR was determined by measuring the amounts of PCR products at different PCR cycles.

2.7. Determination of starch content

Intracellular starch quantification was performed according to [30] with modifications. Cell pellets were resuspended and extracted with 95% ethanol to remove chlorophyll. The air-dried pellets were resuspended in 1 ml of H_2O and incubated in a boiling water bath for 15 min. After centrifugation at 2000 g for 10 min, starch in the colloidal solution was quantified with Lugol solution [31].

3. Results and discussion

3.1. Isolation of fnr-RNAi mutant strains with reduced level of FNR

RNAi approach is an efficient tool to silence target genes in eukaryotic organisms [17,32]. In this work, we used the approach to generate *Chlamydomonas* mutant strains with reduced level of FNR. Inverted repeats corresponding to a sequence of *FNR* gene were introduced into a hairpin-type RNAi vector (Fig. 1A), followed by transformation and mutant selection [17]. Approximate 60 positive clones that exhibited paromomycin-and 5-FI resistance were initially selected (data not shown) followed by RT-PCR analysis (Fig. 1B). To screen for stable mutant strains, antibody against FNR recombinant protein was produced. Heterologous expression and purification of the recombinant FNR protein were performed as described [25]. As shown in Supplemental Fig. 1, the *E. coli* Transetta (DE3) cells produced a substantial amount of expected recombinant protein with an estimated molecular mass of 40 kDa, which corresponds to the molecular mass calculated from the coding sequence of recombinant FNR gene in *Chlamydomonas* (Supplemental Fig. 1A). Total proteins extracted from the *E. coli* cells and wild-type *Chlamydomonas* were analyzed by immunoblot with the antibody. As demonstrated in Supplemental Fig. 1B, no band was detected in the total proteins extracted from the wild-type *E. coli* (Lane 1) while a single band at 40 kDa and 36 kDa was detected in the total protein extracts from *E. coli* expressing the recombinant FNR protein (Lane 2) and *Chlamydomonas* (Lane 3), respectively. The latter corresponds to the molecular mass calculated from the coding sequence of FNR without a predicted transit peptide. This difference of 4 kDa is roughly the sum of molecular mass of the His-tagged peptide (~2 kDa) and the putative transit peptide (~2 kDa) predicted by SignalP version 4.1. Therefore, the antibody showed high specificity to the antigen and recognized mature FNR protein in *Chlamydomonas*. Subsequent quantification of FNR by immunoblot analysis was carried out on the positive clones obtained above. Approximate 40 clones were isolated, in which the level of FNR protein was reduced to different extent in comparison with that in wild type (data not shown). Two of the independent RNAi strains, named *fnr*-1 and *fnr*-9, possessing ca. 25% and ca. 20% of the amount in-wild type strain (Fig. 1C), respectively, were then subjected to further analysis.

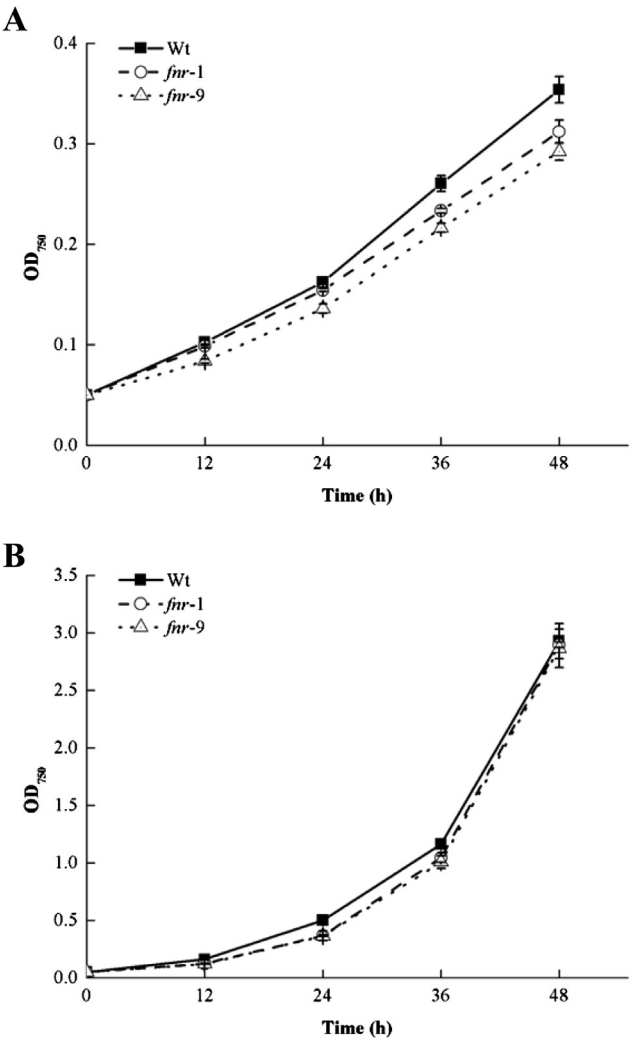

Fig. 2 – Growth of wild type and fnr-RNAi strains. (A) Growth curves of *Chlamydomonas* cultured in HS medium. (B) Growth curves of *Chlamydomonas* cultured in TAP medium. Data are expressed as means (±SD) of three independent experiments.

3.2. *Growth of fnr-RNAi mutant strains under mixtrophic and autotrophic conditions*

To determine whether fnr-RNAi strains are of potential use for large-scale application, growth of wild type and fnr-RNAi strains was at first compared under photoautotrophic (HS medium) and photomixtrophic (TAP medium) conditions, respectively. Consistent with earlier observations in wild-type *Chlamydomonas* [33], we found that the rates of cell growth in HS medium were much lower than that in TAP medium (Fig. 2). These observations may suggest that the deficiency of inorganic carbon source is the major cause limiting the cell growth. Our data also show that the rates of autotrophic growth of fnr-RNAi strains decreased to a greater extent than wild type (Fig. 2A). This may indicate that photosynthetic activity, especially the CO_2 fixation, was affected due to the down-regulation of FNR in fnr-RNAi strains. Considering that FNR is one of the key proteins coupling the light and dark reactions of photosynthesis, larger difference in autotrophic growth between wild type and fnr-RNAi strains could be expected. The small difference (12–18%) in autotrophic growth between wild type and fnr-RNAi strains observed in our experiments (Fig. 2A) may imply that the cells encountered CO_2 deficiency limiting the Calvin–Benson cycle, particularly for the optimal growth in wild-type strain. Our experimental data show that the rates of cell growth of fnr-RNAi strains in TAP medium were almost comparable to wild type throughout the measured period (Fig. 2B). These results could be of support to the assumption above. Because the best system of H_2 photoproduction by sulfur depletion was established using algal cultures grown under mixtrophic condition, rather than that under autotrophic condition [21], the growth performance of the fnr-RNAi cell lines under such condition renders them as potential strains for biotechnological application.

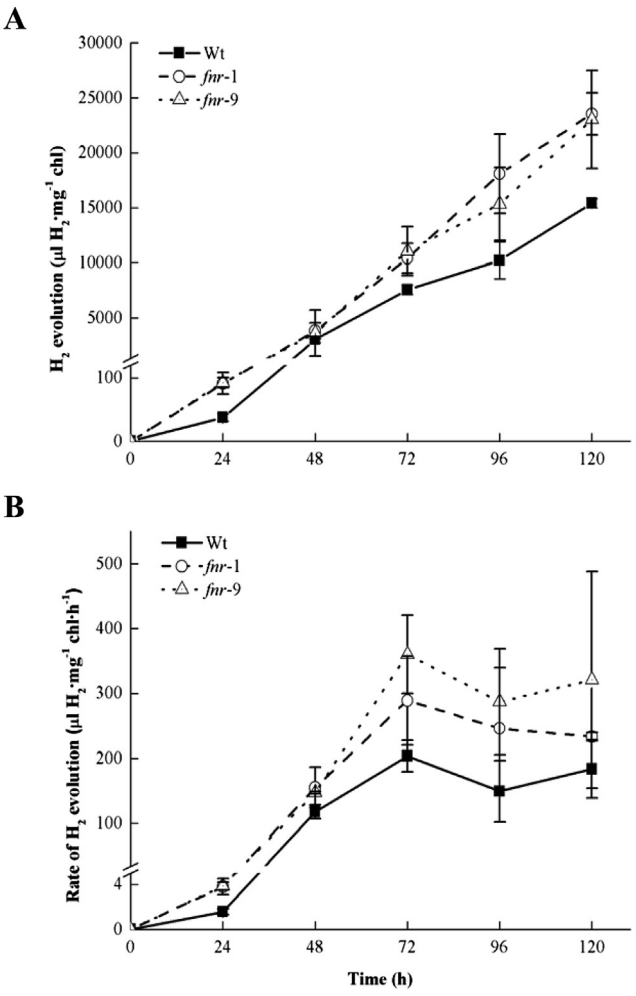

Fig. 3 – Comparison of H_2 photoproduction between fnr-RNAi strains and wild type under sulfur depletion. Amount of H_2 production (A) and H_2 production rate (B) were determined with cultures adjusted to the same chlorophyll content (25 μg ml^{-1}). Data are expressed as means (±SD) of three independent experiments.

3.3. *Increased H_2 photoproduction of fnr-RNAi mutant strains*

Based on our previous proteomic investigation, both protein and mRNA levels of FNR in *Chlamydomonas* decreased during sulfur-deprived H_2 production process [13]. To determine functional significance of FNR involved in the process, we compared the yields of H_2 photoproduction of wild type and fnr-RNAi strains under such condition. Fig. 3A shows that the H_2 yields of fnr-RNAi strains were significantly higher than wild type during the measured period. The total amounts of H_2 evolved from the mutant strains were 23.5 (*fnr*-1) and 23.0 (*fnr*-9) ml mg^{-1} Chl, respectively, whereas that from wild type was 15.4 ml mg^{-1} Chl (Wt). This distinction was most obvious at 24 h of sulfur depletion. The amounts of H_2 evolved from fnr-RNAi strains were 90 (*fnr*-1) and 93 (*fnr*-9) μl mg^{-1} Chl, respectively, which was by average 2.5-fold higher than that from wild type (Fig. 3A). To examine the dynamic profiles of H_2 production under sulfur-depleted condition, the rates of H_2 evolution between the wild type and fnr-RNAi strains were then compared (Fig. 3B). Although the overall pattern of H_2 production rates was similar and reached the peak at 72 h of sulfur depletion, the rates of H_2 production for fnr-RNAi strains were significantly higher than wild type throughout the measured period. The maximal rates of H_2 evolution (72 h) for *fnr*-9 and *fnr*-1 were 360 and 290 μl mg^{-1} Chl h^{-1}, respectively, which was by average 1.6-fold of that for wild type (203 μl mg^{-1} Chl h^{-1}). These results indicated that H_2 photoproduction was significantly enhanced, due to down-regulation of FNR, in fnr-RNAi strains. Negative correlation between FNR abundance and H_2 production revealed from this work was in agreement with our previous observation [13]. Taken together, the experimental data strongly imply that FNR plays an important role in regulating metabolic transition of *Chlamydomonas* cells from photosynthesis to H_2 photoproduction under sulfur-depletion condition.

3.4. *Decreased ratio of photosynthesis/respiration (P/R) in fnr-RNAi mutant strains*

As described above, the greatest enhancement of H_2 photoproduction was observed in fnr-RNAi strains at 24 h of sulfur depletion. To understand the early onset of H_2 photoproduction in fnr-RNAi strains, we then compared the rates of

photosynthetic O_2 evolution and respiratory O_2 consumption between wild type and the mutant strains under sulfur-depletion condition. As shown in Fig. 4, the rates of respiratory O_2 consumption remained constant (~40 μmol O_2 mg^{-1} Chl h^{-1}) and no significant difference was observed between both types of strains during the first 12 h of sulfur depletion. In contrast, the rates of photosynthetic O_2 evolution in the mutant strains were significantly lower than wild type at the beginning (0 h) and at 12 h of sulfur depletion. The initial rates of O_2 evolution in the mutant strains were 92.5 (*fnr*-1) and 96.2 (*fnr*-9) μmol O_2 mg^{-1} Chl h^{-1}, respectively, which were by average 44% lower than of that in wild type. It is well known that in photosynthesis, most of the photosynthetic electrons generated from water oxidation of PSII are used to produce NADPH by FNR [34]. Our experimental data show that the rates of O_2 evolution decreased in fnr-RNAi strains in comparison with that in wild type (Fig. 4). We presume this is largely due to the reduction of the important electron sink related to FNR in *Chlamydomonas*. Consequently, the initial ratios of photosynthesis/respiration (P/R) in fnr-RNAi mutant strains were substantially lower (2.2:1) than that in wild type (3.8:1). At 12 h of sulfur depletion, the rates of O_2 evolution decreased to 43.7 and 47.1 μmol O_2 mg^{-1} Chl h^{-1} in *fnr*-1 and *fnr*-9, respectively, resulting in a *P/R* ratio of 1.2:1 in these mutant strains. In wild type, the P/R ratio was still high (2.4:1) at this time point. This indicates that the attenuation of the P/R ratio to 1:1 could be achieved more efficiently in fnr-RNAi strains than wild type, leading to their earlier onset of H_2 photoproduction. It has been suggested that attenuating the P/R capacity ratio from about 4:1 to 1:1 is the first step to induce expression of hydrogenases toward light-dependent H_2 production in *Chlamydomonas* [5]. Recent *in vitro* experiment reported by Yacoby et al. (2011) indicated the existence of competition between FNR and hydrogenases [15]. In this work, we found that fnr-RNAi strains exhibited higher rates of H_2 production. These results could be of *in vivo* evidence indicating that the competition occurs in *Chlamydomonas* cells. Taken together, we propose that down-regulation of FNR is an efficient module in both inactivation of PSII activity and activation of H_2-producing activity in *Chlamydomonas* under sulfur-deprived condition.

3.5. *Decreased level of Rubisco in fnr-RNAi mutant strains*

It is generally known that, under normal growth condition, FNR catalyzes the reaction of electron transfer from PSI to $NADP^+$, leading to production of NADPH that is mainly used for CO_2 assimilation. However, functional significance of FNR in *Chlamydomonas* cells under sulfur-deprived H_2 production is not clearly determined. To investigate whether the decreased level of FNR influences expression of photosynthetic genes under such condition, a semi-quantitative RT-PCR analysis was carried out using total RNA isolated from both types of strains collected at 0, 12 and 24 h of sulfur depletion (Fig. 5). Nine genes encoding the key photosynthetic proteins, i. e. PSII (*PsbA, PsbO*), PSI (*PsaA, PsaD, PetF, Fdx5*), Cytochromeb_6f (*PetC*) and Rubisco (*RbcL and RbcS*), as well as the two genes encoding hydrogenases (*HydA1* and *HydA2*) were selected for the analysis. As shown in Fig. 5, all the genes encoding the key proteins involved in light reaction of photosynthesis (*PsbA, PsbO, PsaA, PsaD, PetF, Fdx5* and *PetC*) as well as the hydrogenases (*HydA1* and *HydA2*) showed similar kinetic changes in the mutant strains and wild type (Fig. 5). This indicates that the expression of those genes was not significantly affected by down-regulation of FNR in the mutant strains. Noteworthy, the mRNA levels of Rubisco (*RbcL* and *RbcS*), the key enzyme of Calvin–Benson cycle for CO_2 assimilation, were apparently lower in the mutant strains than wild type. This was further confirmed by immunoblot analysis using proteins extracted from the three strains collected at 0 and 48 h of sulfur deprivation (Fig. 6). Equal amounts of cellular proteins were

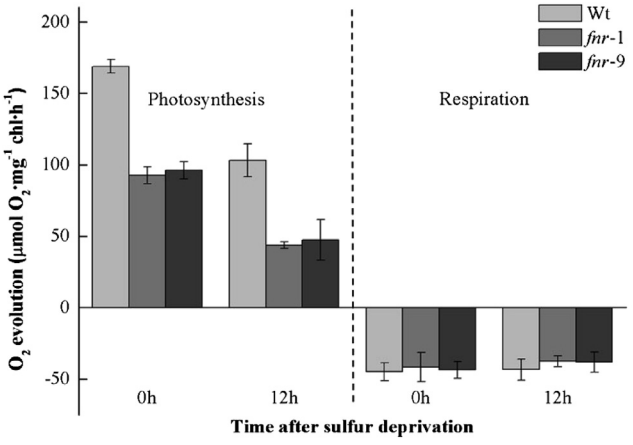

Fig. 4 – Comparison of photosynthetic O_2 evolution and respiration between fnr-RNAi strains and wild type under sulfur depletion. Data are expressed as means (±SD) of three independent experiments.

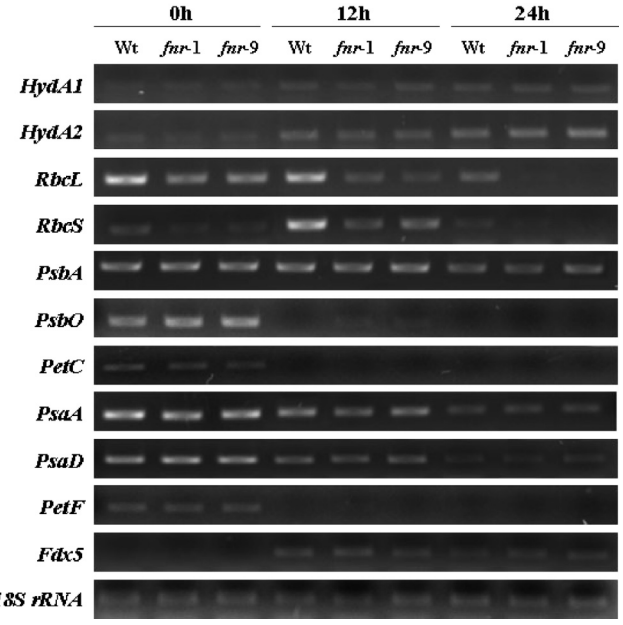

Fig. 5 – Semi-quantitative RT-PCR analysis of expression of selected genes under sulfur depletion. Expression of the *18S rRNA* was used as an internal reference. All experiments were repeated twice with similar results.

analyzed by immunoblotting with antibodies against RbcL, HydA and FNR, respectively. As demonstrated in Fig. 6, the alteration patterns of those proteins are in agreement with that revealed by semi-quantitative RT-PCR analysis. At 48 h of sulfur deprivation, the protein amounts of RbcL in fnr-RNAi strains were approximate 60% of that in wild type. The precise mechanism of how the down-regulation of FNR caused the greater inhibition of Rubisco expression in fnr-RNAi strains is not clear. It has been reported that, in FNR-deficient tobacco leaves, degradation of Rubisco was more pronounced than the wild type [35]. We presume this could be one of the cases occurred in fnr-RNAi strains under sulfur depletion. Because Rubisco represents one of the key enzymes catalyzing irreversible reactions in Calvin–Benson cycle for CO_2 assimilation, the drastic reduction in abundance observed in the present experiments may indicate the efficiency of CO_2 assimilation was decreased to a greater extent in fnr-RNAi strains than wild type. Competition between CO_2 assimilation and H_2 production was earlier demonstrated in the studies using a Rubisco-deficient mutant (CC-2803) as well as Calvin–Benson cycle inhibitor [34,36]. Taken together, we suggest that down-regulation of key enzymes involved in Calvin–Benson cycle for CO_2 assimilation, including Rubisco, in fnr-RNAi strains could be one of the efficient means to further increase their yields of H_2-photoproduction.

3.6. *Enhanced starch degradation of fnr-RNAi mutant strains*

Starch plays an important role in sustaining algal H_2 production under sulfur-depletion condition [37,38]. To investigate whether the enhanced H_2 photoproduction in fnr-RNAi strains was also correlated with their starch levels, starch contents in fnr-RNAi strains and wild type were determined in the duration of 120 h of sulfur-depletion condition (Fig. 7). The experimental data shows that the starch contents in both type of strains increased quickly within 24 h of sulfur depletion, followed by a rapid decline afterward. The pattern was largely consistent with earlier observations [9,39]. Since similar amounts of starch were detected in the two types of strain during the first 24 h of sulfur depletion (Fig. 7), we assume that the ability of starch biosynthesis in the mutant strains was not affected by down-regulation of FNR. After 24 h of sulfur depletion, however, the starch contents in the mutant strains were significantly lower than wild type (Fig. 7). From 24 to 72 h, starch contents in the wild type and fnr-RNAi strains decreased 34.6%, 55.9% (*fnr*-1) and 58.9% (*fnr*-9), respectively. This means that the rates of starch degradation in the fnr-RNAi strains were 1.6-(*fnr*-1) and 1.7-(*fnr*-9)-fold higher than wild type, respectively. However, the reason why the down-regulation of FNR increased starch degradation remains unclear. Based on the observations obtained in *Chlamydomonas* sp. MGA161, starch degradation/mobilization was stimulated by reduced levels of ATP under anaerobic condition [40], we postulate that the enhanced starch degradation observed in fnr-RNAi strains may indicate that ATP levels were lower than wild type under sulfur depletion. It has been initially proposed [37], and later experimentally confirmed that starch degradation takes a predominant part in the indirect pathway of H_2 photoproduction [2,8]. In the indirect pathway, the reducing equivalents generated from starch degradation are injected into the photosynthetic electron transport chain via plastidic NAD(P)H-plastoquinone-oxidoreductase Nda2, an enzyme catalyzes the reaction of electron donation from NAD(P)H to plastoquinone in *Chlamydomonas* [18]. As described in the previous section (3.3), the rates of H_2 photoproduction in the fnr-RNAi mutant strains, in the duration of 24–72 h, were about 1.5-fold higher than wild type. This was comparable to their average rate of starch degradation (1.65-fold) during the same period. Based on these observations, we suggest that the indirect pathway may contribute substantially to the increased H_2 yields, especially at the later stage of H_2-producing process.

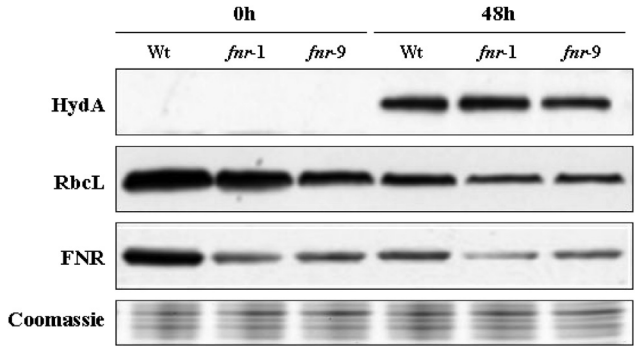

Fig. 6 – Immunoblot detection of selected proteins under sulfur depletion. Proteins (15 μg, per lane) were separated by 12% SDS-PAGE followed by immunoblotting. All experiments were done three times, and similar results were obtained.

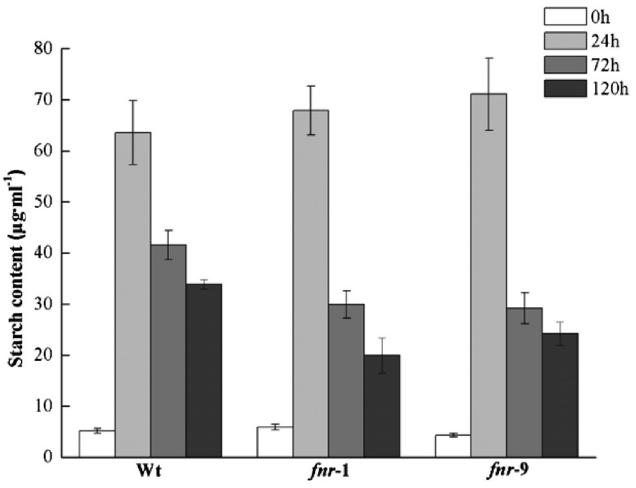

Fig. 7 – Comparison of starch contents between fnr-RNAi strains and wild type under sulfur depletion. Data are presented as means (±SD) of three independent experiments.

4. Conclusions

In summary, we have designed and generated *Chlamydomonas* mutant strains with reduced level of FNR using RNAi

approach. We have also characterized the fnr-RNAi mutant strains in the context of H_2 photoproduction under sulfur-deprived condition. Our experimental data demonstrated, for the first time, the enhanced capability of H_2 photoproduction in the fnr-RNAi mutant strains. The rates of H_2 production in the fnr-RNAi strains were 2.5-fold higher than wild type. Our data also revealed remarkable changes of fnr-RNAi strains as follows: i) decreased rates of photosynthetic O_2 evolution, ii) reduced levels of Rubisco; iii) increased rates of starch degradation, at the beginning or/and during H_2-producing process. These results implicate the increased H_2 production in fnr-RNAi strains is a cumulative consequence of the earlier onset of anaerobiosis and increased electron supply via direct and indirect pathways to the hydrogenases. Based on these physiological implications, we propose that the modulation of FNR is one of efficient perspectives for improving H_2 production both *in vitro* as well as in the algal organism. Together with their optimal growth under mixtrophic condition, we suggest that the fnr-RNAi strains could be used as high-H_2 producing strains directly, or/and served as the potential strains for further genetic engineering that would lead to substantial improvements in light utilization [21,41] and H_2 production for application in industry scale.

Acknowledgments

We thank Dr. Heriberto D. Cerutti (University of Nebraska-Lincoln, USA) for providing the RNAi plasmid, and Dr. Lianwei Peng (Institute of Botany, CAS) for helpful discussions. This work was supported by the Ministry of Science and Technology of China (2009CB220000) and Chinese Academy of Sciences.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijhydene.2013.10.011.

REFERENCES

[1] Melis A, Happe T. Hydrogen production. Green algae as a source of energy. Plant Physiol 2001;127:740–8.
[2] Fouchard S, Hemschemeier A, Caruana A, Pruvost K, Legrand J, Happe T, et al. Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived *Chlamydomonas* cells. Appl Environ Microbiol 2005;71:6199–205.
[3] Ghirardi ML, Posewitz MC, Maness PC, Dubini A, Yu JP, Seibert M. Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. Annu Rev Plant Biol 2007;58:71–91.
[4] Greenbaum E. Energetic efficiency of hydrogen photoevolution by algal water splitting. Biophys J 1988;54:365–8.
[5] Hemschemeier A, Melis A, Happe T. Analytical approaches to photobiological hydrogen production in unicellular green algae. Photosynth Res 2009;102:523–40.
[6] Hemschemeier A, Happe T. Alternative photosynthetic electron transport pathways during anaerobiosis in the green alga *Chlamydomonas reinhardtii*. Biochim Biophys Acta 2011;1807:919–26.
[7] Melis A, Zhang LP, Forestier M, Ghirardi ML, Seibert M. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 2000;122:127–35.
[8] Chochois V, Dauvillee D, Beyly A, Tolleter D, Cuine S, Timpano H, et al. Hydrogen production in *Chlamydomonas*: photosystem II-dependent and -independent pathways differ in their requirement for starch metabolism. Plant Physiol 2009;151:631–40.
[9] Tolleter D, Ghysels B, Alric J, Petroutsos D, Tolstygina I, Krawietz D, et al. Control of hydrogen photoproduction by the proton gradient generated by cyclic electron flow in *Chlamydomonas reinhardtii*. Plant Cell 2011;23:2619–30.
[10] Volgusheva A, Styring S, Mamedov F. Increased photosystem II stability promotes H_2 production in sulfur-deprived *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 2013;110:7223–8.
[11] Nguyen AV, Thomas-Hall SR, Malnoe A, Timmins M, Mussgnug JH, Rupprecht J, et al. Transcriptome for photobiological hydrogen production induced by sulfur deprivation in the green alga *Chlamydomonas reinhardtii*. Eukaryot Cell 2008;7:1965–79.
[12] Toepel J, Illmer-Kephalides M, Jaenicke S, Straube J, May P, Goesmann A, et al. New insights into *Chlamydomonas reinhardtii* hydrogen production processes by combined microarray/RNA-seq transcriptomics. Plant Biotechnol J 2013. http://dx.doi.org/10.1111/pbi.12062.
[13] Chen M, Zhao L, Sun YL, Cui SX, Zhang LF, Yang B, et al. Proteomic analysis of hydrogen photoproduction in sulfur-deprived *Chlamydomonas* cells. J Proteome Res 2010;9:3854–66.
[14] Winkler M, Hemschemeier A, Jacobs J, Stripp S, Happe T. Multiple ferredoxin isoforms in *Chlamydomonas reinhardtii*-their role under stress conditions and biotechnological implications. Eur J Cell Biol 2010;89:998–1004.
[15] Yacoby I, Pochekailov S, Toporik H, Ghirardi ML, King PW, Zhang SG. Photosynthetic electron partitioning between [FeFe]-hydrogenase and ferredoxin: $NADP^+$-oxidoreductase (FNR) enzymes in vitro. Proc Natl Acad Sci USA 2011;108:9396–401.
[16] Kim EJ, Cerutti H. Targeted gene silencing by RNA interference in *Chlamydomonas*. Method Cell Biol 2009;93:99–110.
[17] Rohr J, Sarkar N, Balenger S, Jeong BR, Cerutti H. Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*. Plant J 2004;40:611–21.
[18] Jans F, Mignolet E, Houyoux PA, Cardol P, Ghysels B, Cuine S, et al. A type II NAD(P)H dehydrogenase mediates light-independent plastoquinone reduction in the chloroplast of *Chlamydomonas*. Proc Natl Acad Sci USA 2008;105:20546–51.
[19] Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, et al. Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. Plant Biotechnol J 2007;5:802–14.
[20] Deng XD, Li YJ, Fei XW. The mRNA abundance of pepc2 gene is negatively correlated with oil content in *Chlamydomonas reinhardtii*. Biomass Bioenerg 2011;35:1811–7.
[21] Oey M, Ross IL, Stephens E, Steinbeck J, Wolf J, Radzun KA, et al. RNAi knock-down of LHCBM1, 2 and 3 increases photosynthetic H_2 production efficiency of the green alga *Chlamydomonas reinhardtii*. PLoS One 2013;8:e61375.
[22] Gorman DS, Levine RP. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 1965;54:1665–9.

[23] Mussgnug JH, Wobbe L, Elles I, Claus C, Hamilton M, Fink A, et al. NAB1 is an RNA binding protein involved in the light-regulated differential expression of the light-harvesting antenna of *Chlamydomonas reinhardtii*. Plant Cell 2005;17:3409–21.
[24] Kindle KL. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 1990;87:1228–32.
[25] Zhou JX, Zhou J, Yang HM, Chen M, Huang F. Characterization of two glutaminases from the filamentous cyanobacterium *Anabaena sp. PCC 7120*. FEMS Microbiol Lett 2008;289:241–9.
[26] Melis A, Neidhardt J, Benemann JR. *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. J Appl Phycol 1998;10:515–25.
[27] Liu CM, Willmund F, Whitelegge JP, Hawat S, Knapp B, Lodha M, et al. J-domain protein CDJ2 and HSP70B are a plastidic chaperone pair that interacts with vesicle-inducing protein in plastids 1. Mol Biol Cell 2005;16:1165–77.
[28] Zhao L, Sun YL, Cui SX, Chen M, Yang HM, Liu HM, et al. Cd-induced changes in leaf proteome of the hyperaccumulator plant *Phytolacca americana*. Chemosphere 2011;85:56–66.
[29] Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83:346–56.
[30] Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P, et al. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol 2011;11:7.
[31] Southgate DAT. Determination of food carbohydrates. London: Applied Science Publishers; 1976.
[32] Lermontova I, Koroleva O, Rutten T, Fuchs J, Schubert V, Moraes I, et al. Knockdown of CENH3 in *Arabidopsis* reduces mitotic divisions and causes sterility by disturbed meiotic chromosome segregation. Plant J 2011;68:40–50.
[33] Fischer BB, Wiesendanger M, Eggen RI. Growth condition-dependent sensitivity, photodamage and stress response of *Chlamydomonas reinhardtii* exposed to high light conditions. Plant Cell Physiol 2006;47:1135–45.
[34] Hemschemeier A, Fouchard S, Cournac L, Peltier G, Happe T. Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks. Planta 2008;227:397–407.
[35] Palatnik JF, Tognetti VB, Poli HO, Rodriguez RE, Blanco N, Gattuso M, et al. Transgenic tobacco plants expressing antisense ferredoxin-NADP(H) reductase transcripts display increased susceptibility to photo-oxidative damage. Plant J 2003;35:332–41.
[36] Rühle T, Hemschemeier A, Melis A, Happe T. A novel screening protocol for the isolation of hydrogen producing *Chlamydomonas reinhardtii* strains. BMC Plant Biol 2008;8:107. http://dx.doi.org/10.1186/1471-2229-8-107.
[37] Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, et al. Improved photobiological H_2 production in engineered green algal cells. J Biol Chem 2005;280:34170–7.
[38] White AL, Melis A. Biochemistry of hydrogen metabolism in *Chlamydomonas reinhardtii* wild type and a rubisco-less mutant. Int J Hydrogen Energy 2006;31:455–64.
[39] Zhang L, Happe T, Melis A. Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta 2002;214:552–61.
[40] Maeda I, Hikawa H, Mizoguchi T, Yagi K. Repression of starch degradation under anaerobic conditions by irregularly high levels of ATP in *Chlamydomonas* sp. MGA161. Plant Sci 2001;160:629–34.
[41] Polle JEW, Kanakagiri S, Jin E, Masuda T, Melis A. Truncated chlorophyll antenna size of the photosystems - a practical method to improve microalgal productivity and hydrogen production in mass culture. Int J Hydrogen Energy 2002;27:1257–64.