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Identification of bidirectional hydrogenase genes and their co-transcription in unicellular halotolerant cyanobacterium *Aphanothece halophytica*

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Abstract The halotolerant cyanobacterium *Aphanothece halophytica* has been shown to produce H_2 via dark fermentation of accumulated glycogen under anoxic condition. One set of *hox* genes encoding a bidirectional hydrogenase is present in *A. halophytica*. In this study, the nucleotide sequence and the transcriptional analysis of *hox* genes in *A. halophytica* were investigated. The results revealed that *A. halophytica* contained five structural genes, *hoxE*, *hoxF*, *hoxU*, *hoxY*, and *hoxH*, without an insertion of other open reading frames (ORFs). The conserved cysteine motifs of iron-sulfur clusters involved in an electron transfer were found in all Hox subunits. The nucleotide and deduced amino acid sequences of *hox* genes in *A. halophytica* showed the highest identity and similarity to those of *Halothece* sp. PCC 7418. By reverse transcription polymerase chain reaction (RT-PCR) analysis, *hox* genes in *A. halophytica* were co-transcribed as a single operon. Under nitrogen-deprived condition, the transcripts of *hoxH*, *glgB*, *coxA*, *ndhB*, and *psaA* were upregulated whereas those of *glgP* and *narB* were downregulated which resulted in an increase of H_2 production, H_2ase activity, glycogen content, and dark respiration rate.

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Introduction

Biohydrogen is one of the alternative potential energy carriers which can be produced by various kinds of microorganisms via different processes. Cyanobacterial H_2 production can be performed by either/both a photosynthetic pathway or/and a dark fermentation from the degradation of accumulated carbohydrate in the cells. There are at least three enzymes involved in H_2 metabolism of cyanobacteria. Nitrogenase catalyzes the reduction of atmospheric N_2 to ammonia and simultaneously produces H_2 as a by-product. This reaction requires numerous ATP. Uptake hydrogenase catalyzes the oxidation of H_2, produced by N_2 fixation, to protons. These two enzymes are mainly found in heterocysts of N_2-fixing cyanobacteria. The last enzyme, bidirectional hydrogenase, catalyzes both consumption and production of H_2 and is distributed in both N_2-fixing and non-N_2-fixing cyanobacteria (Tamagnini et al. 2000, 2002).

Bidirectional hydrogenase is a heterotetrameric subunit consisting of hydrogenase structural complex (HoxY and HoxH encoded by *hoxY* and *hoxH*, respectively) and a diaphorase component (HoxF and HoxU encoded by *hoxF* and *hoxU*, respectively). Besides HoxF and HoxU, an additional subunit HoxE encoded by *hoxE* might be found as one of diaphorase components in some cyanobacteria such as *Synechocystis* sp. PCC 6803 (Appel and Schulz 1996), *Synechococcus* sp. PCC 6301 (Boison et al. 1998), and *Anabaena* sp. PCC 7120 (Tamagnini et al. 2002). Nucleotide sequences of these *hox* genes in several cyanobacteria have been reported. The cyanobacterial *hox* gene cluster can be organized as an operon (Appel and

Schulz 1996; Boison et al. 2000) or separated into two gene clusters (*hoxEF* and *hoxUYH*) (Tamagnini et al. 2007). In addition, *hox* gene expression in cyanobacteria differs among various species depending on the environmental condition and their genetic characteristics. The relative transcript levels of *hox* genes in most cyanobacterial species increase under nitrogen starvation/limitation condition (Boison et al. 2000; Sheremetieva et al. 2002; Troshina et al. 2002; Antal et al. 2006; Osanai et al. 2006). Furthermore, an absence or a low level of oxygen can induce both the enzyme activity as well as the relative amount of *hoxH* (Axelsson and Lindblad 2002; Houchins and Burris 1981; Sheremetieva et al. 2002; Schmitz and Bothe 1996).

The unicellular halotolerant cyanobacterium *Aphanothece halophytica* has been shown to be one of the high potential H_2 producers (Taikhao et al. 2013). It is capable of producing high amount of H_2 under nitrogen starvation and dark anaerobic condition. In addition, it could grow in seawater supplemented with as little as 1.76 mM $NaNO_3$ and showed a long-term H_2 accumulation for at least 14 days (Taikhao et al. 2015). To fully understand the mechanisms and functions of bidirectional hydrogenase in *A. halophytica*, genetic characterization and transcriptional analysis of bidirectional hydrogenase genes and other related genes under nitrogen and sulfur deprivation condition were investigated.

Materials and methods

Aphanothece halophytica was grown in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium as previously described with slight modification (Wiangnon et al. 2007). The culture was buffered with 10 mM TES (pH 8.0) and supplemented with Turk Island salt solution (Garlick et al. 1977). Cells were cultivated at 30 °C under a white-light illumination of 30 μmol photons m^{-2} s^{-1} on a rotary shaker with a shaking speed of 120 rpm for 7 days. *Escherichia coli* DH5α used for plasmid DNA manipulation was grown in a 250-mL Erlenmeyer flask containing 100 mL of LB broth (Bertani 1951) at 37 °C with a shaking speed of 250 rpm. For a blue-white screening of correct transformants, *E. coli* cells were selected on LB agar containing 50 μg mL^{-1} kanamycin, 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal).

DNA and total RNA isolation

Genomic DNA of *Aphanothece halophytica* was isolated by using Wizard SV genomic DNA purification system kit (Promega, USA). Plasmid DNA isolation was performed by using High-Speed Plasmid DNA Mini Kit (Geneaid, Taiwan). Total RNA was isolated following the protocol of Gutekunst et al. (2005).

DNA amplification by PCR and DNA walking

First, DNA fragments of partial *hoxUY* and partial *hoxH* of *A. halophytica* were amplified by PCR using conserved primers designed from other cyanobacterial genes (Table 1). The PCR reactions and conditions were performed according to Phunpruch et al. (2006) in a Master gradient thermal cycler (Eppendorf, Germany). The PCR products were subsequently cloned and sequenced. The specific primers of *hoxY* and *hoxH* designed from the obtained sequences were used for PCR amplification of DNA fragment of *hoxYH*, and then its PCR product was cloned and sequenced. Finally, the partial sequence of *hoxU*, the complete sequence of *hoxY*, and the partial sequence of *hoxH* were obtained. DNA fragments of the other genes were amplified by three nested PCR reactions with the target specific primers (Table 1) and the supplied DW-ACP primers using DNA walking speedup premix kit (Seegene, Korea). The final PCR products were purified by using QIAquick PCR Purification kit (Qiagen, Germany) before cloning and sequencing.

Cloning and nucleotide sequencing

The purified PCR products were cloned into pDrive (Qiagen, Germany). The ligation mixture was incubated at 16 °C for 16 h and then transformed to the competent cells of *E. coli* DH5α. The recombinant plasmid was isolated by High-Speed Plasmid DNA Mini Kit (Geneaid, Taiwan) and sequenced with Big-Dye terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) using ABI PRISM 3700 DNA analyzer at First BASE Laboratories (Malaysia). Universal primers T7 and SP6 were used for both strand sequencing. The obtained nucleotide sequences were compared with GenBank nucleotide sequence databases.

Co-transcriptional analysis

Contaminating DNA from 1 μg of total RNA was digested with 6 units of RQ1 RNase-free DNase (Promega, USA) in buffer containing 4 units of RNasin plus RNase inhibitor (Promega, USA) at 37 °C for 1 h before inactivating the enzyme by heating at 65 °C for 10 min. The single-stranded RNA was precipitated by phenol-chloroform extraction and suspended in a buffer containing 4 units of RNasin plus RNase inhibitor, 1 mM dNTPs (Promega, USA), and 1 μM RT-primer RhydApha3 (Table 1) which is located at downstream region of *hoxH*. The mixture was pre-incubated at 70 °C for 10 min and immediately chilled on ice for 10 min before adding 200 units of M-MuLV reverse transcriptase (Thermo Scientific, Lithuania) followed by incubating at

Table 1 Primers used in PCR, genome walking, co-transcriptional analysis, and transcriptional analysis

Primer pair	Gene	Specific organism	Sequence (5′ to 3′)	PCR product size (bp)
PCR				
Rhyd1	*hoxH*	*Synechococcus* sp. PCC	AT(C/T)TG(G/T)GG(C/T)ATTTGT	1189
Rhyd3		6301, *Synechocystis* PCC 6803, *Prochlorothrix hollandica*, and *Anabaena variabilis* ATCC 29413	CC(C/G)GT GAAGAACTTAAACAGGGGTC	
FhoxU1	*hoxU-*	*Synechococcus* sp. PCC	ATTCC(G/C/T)AC(C/T/G)TGCCA	627
RhoxY3	*hoxY*	6301, *Synechocystis* sp. PCC 6803, *Anabaena variabilis* ATCC 29413, and *Nostoc* sp. PCC 7120	TTTAGAAGG AATGACATATGACAGCCAGAACA	
FhoxY4	*hoxY-hoxH*	*Aphanothece halophytica*	GGACAAGATAAAGTTTGCAACG	777
RhoxH1Apha			GGAACTTGGACTGCAAGAATT	
Genome walking				
TSP1hoxUApha	*hoxU*	*Aphanothece halophytica*	GATCCATTCCCACCTCAACGGC	
TSP2hoxUApha			CCCTCCGCAAAGAGCATTTCCACC	
TSP3hoxUApha			TTCCACCAAACAGAGGCGACACGC	
TSP1hoxFApha	*hoxF*	*Aphanothece halophytica*	CCGCCCGACAAGAAATGCACTT	
TSP2hoxFApha			GATCCATGACCACCATGCCACCAG	
TSP3hoxFApha			CCACGGCGGACAGCGATTCGTAAT	
TSP1RhoxFApha	*hoxF*	*Aphanothece halophytica*	GCGAACCGAGGACATTATTGCG	
TSP2RhoxFApha			GGCTAACGGATACTCTGCGCGAAC	
TSP3RhoxFApha			AGCCCGCGATCGCCATTCCTTCTA	
TSP1RhoxEApha	*hoxF*	*Aphanothece halophytica*	GGTACAACAATAGACACGGG	
TSP2RhoxEApha			TGCTTCCTCTGCCCAGTTGT	
Tsp3RhoxEApha			CACTCTCGGTGTCTTGCTGC	
TSP1hoxHApha	*hoxH*	*Aphanothece halophytica*	TCTATCACTATGCGCGGTTAG	
TSP2hoxHApha			GACTTAATGTCGCCTCGGGTGCGT	
TSP3hoxHApha			AGGCATTGGTGTCAGTGAAGCCCC	
Co-transcriptional analysis				
RT-primer:				
RhydApha3	*hoxH*	*Aphanothece halophytica*	CAATGATCAGGT TCACTTTTT	
PCR primer:				
CoFhoxE1Apha	*hoxE-hoxF*	*Aphanothece halophytica*	GTATCGGGGCTTGTGGCATT	1477
TSP1hoxFApha		*Aphanothece halophytica*	CCGCCCGACAAGAAATGCACTT	
CoFhoxF1Apha	*hoxF-hoxU*	*Aphanothece halophytica*	CCAGTATGGTTGGGGTTGCC	434
TSP3hoxUApha		*Aphanothece halophytica*	TTCCACCAAACAGAGGCGACACGC	
CoFhoxUApha	*hoxU-hoxY*	*Aphanothece halophytica*	GCTGTGGAGGCTGGA TCAAC	779
CoRhoxYApha		*Aphanothece halophytica*	GATATCCACGTGCTGGGCG AGGACAA	
FhoxY4	*hoxY-hoxH*	*Aphanothece halophytica*	GATAAAGTTTGCAACG	777
RhoxH1Apha		*Aphanothece halophytica*	GGAACTTGGACTGCAAGAATT	
Transcriptional analysis				
RhydApha1	*hoxH*	*Aphanothece halophytica*	TGTATCGCGTCGGTCCTTTA	340
RhydApha3			CAATGATCAGGTTCACTTTTT	
psaA-FApha	*psaA*	*Halothece* sp. PCC 7418	GGGGTAAGCCTGGACACTTT	460
psaA-RApha			GGACTCCACATTCTGGAACC	
psbA-FApha	*psbA*	*Halothece* sp. PCC 7418	GAGAGCCCGTGATTGGATCA	365
psbA-RApha			TATGGAAGGGGTGCATGAGG	
coxA-FApha	*coxA*	*Halothece* sp. PCC 7418	GGAGTTAGCCACCCCAGAAT	411
coxA-RApha			TACCCCTGTCGCTAACATCG	
ndhB-FApha	*ndhB*	*Halothece* sp. PCC 7418	CTAACCACTGTGGATGACCT	508
ndhB-RApha			GCATCCCAAGTGCCTTTACC	
glgB-FApha	*glgB*	*Halothece* sp. PCC 7418	GGCGCATTTGATCGAAACCG	160

Table 1 (continued)

Primer pair	Gene	Specific organism	Sequence (5′ to 3′)	PCR product size (bp)
glgB-RApha			CTTGGATGCGGAGTTGATAA	
glgP-FApha	*glgP*	*Halothece* sp. PCC 7418	GGCTCCTGCAACGATGGCTA	305
glgP-RApha			CAACTTGCCAACCCTCCTGA	
narB-FApha	*narB*	*Halothece* sp. PCC 7418	GCGGTTAACCGTGATAGTGA	205
narB-RApha			TCATTGCCGTGAGTGACCTG	
Ana16	16S rRNA	*Anabaena variabilis* ATCC 29413, *Anabaena* sp. PCC 9109, *Anabaena cylindrica* UTEX629, and *Anabaena sphaerica* UUTEXB1616	ACCCGTACCCGAAACCGA	288
AnaR			CCGCCGTTCTCCGGGGCT	

42 °C for 1 h. DI water containing 0.1 % (*v*/*v*) DEPC was used instead of M-MuLV reverse transcriptase in a negative control. DNA amplification of *hoxEF*, *hoxFU*, *hoxUY*, and *hoxYH* fragments by PCR using the obtained complementary DNA as template was performed with four primer pairs, CoFhoxE1Apha-TSP1hoxFApha, CoFhoxF1Apha-TSP3hoxUApha, CoFhoxUApha-CoRhoxYApha, and FhoxY4-RhoxH1Apha, respectively (Table 1).

Transcriptional analysis

In this study, transcriptional analysis of *hoxH* and other genes involved in H_2 metabolism was performed by using QIAGEN OneStep reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen, Germany). Total RNA was isolated from cells treated under 24-h nitrogen-deprived and sulfur-deprived conditions, and then the contaminating DNA was digested by DNaseI as above described. The expression of a housekeeping gene 16S rRNA was used as a control for each sample. The sequences and origins of primers are shown in Table 1. The RT-PCR condition was performed as follows: RT reaction at 50 °C for 30 s, initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50–55 °C for 30 s, extension at 72 °C for 90 s, and followed by the final extension at 72 °C for 10 min. The PCR product was analyzed by 1.0 % (*w*/*v*) agarose gel electrophoresis. The intensity of DNA bands was analyzed by GeneTools analysis software (version 4.01.02) from SynGene Genius system.

Determination of bidirectional hydrogenase activity, chlorophyll *a* concentration, glycogen content, and dark respiration rate

Bidirectional hydrogenase activity was determined by measuring H_2 production in the presence of dithionite reduced methyl viologen according to Baebprasert et al. (2010). Chlorophyll *a* was extracted by methanol and determined spectrophotometrically (MacKinney 1941). The glycogen extraction and hydrolysis was performed following the procedure of Ernst and coworkers (1984). Glycogen content was determined as glucose concentration by phenol-sulfuric acid assay (Dubois et al. 1956).The dark respiration rate was measured in a 2-mL cell suspension with a Clark-type oxygen electrode (Oxygraph plus system, Hansatech, UK). All experiments were done in triplicates.

Results

Nucleotide sequences of *hox* genes in *Aphanothece halophytica*

The 4960-bp *hox* gene cluster of *A. halophytica* consisted of five open reading frames (ORFs), namely, *hoxE*, *hoxF*, *hoxU*, *hoxY*, and *hoxH*, without any insertion of ORFs (Fig 1). Their nucleotide sequences were deposited to GenBank under accession number KF885784.1. The upstream gene, *hoxE*, encoding the bidirectional hydrogenase complex protein HoxE consisted of 516 bp and encoded 171 amino acids with a calculated molecular mass of 19.0 kDa (http://web.expasy.org/compute_pi/). The large diaphorase subunit gene, *hoxF*, comprised 1617 bp and encoded 538 amino acids with a predicted polypeptide of 57.8 kDa whereas the small diaphorase subunit gene, *hoxU*, contained 720 bp encoding 239 amino acids with a predicted polypeptide of 26.5 kDa. In addition, the large and small hydrogenase subunit genes, *hoxH* and *hoxY*, constituted 1428 bp encoding 475 amino acids, and 549 bp encoding 182 amino acids, respectively. The HoxH and HoxY of *A. halophytica* showed the estimated molecular masses of 53.1 and 20.1 kDa, respectively. Nucleotide sequences of *hox* genes in *A. halophytica* were compared with those from other cyanobacteria reported in GenBank nucleotide sequence database by ClustalW program (Higgins et al. 1994).The *hox* genes of *A. halophytica* showed approximately 65–81 % nucleotide identity and 77–91 % amino acid similarity to those reported in other cyanobacteria (excluding those for *Halothece* sp. PCC 7418 where 99–100 % nucleotide homology and amino acid similarity were detected).

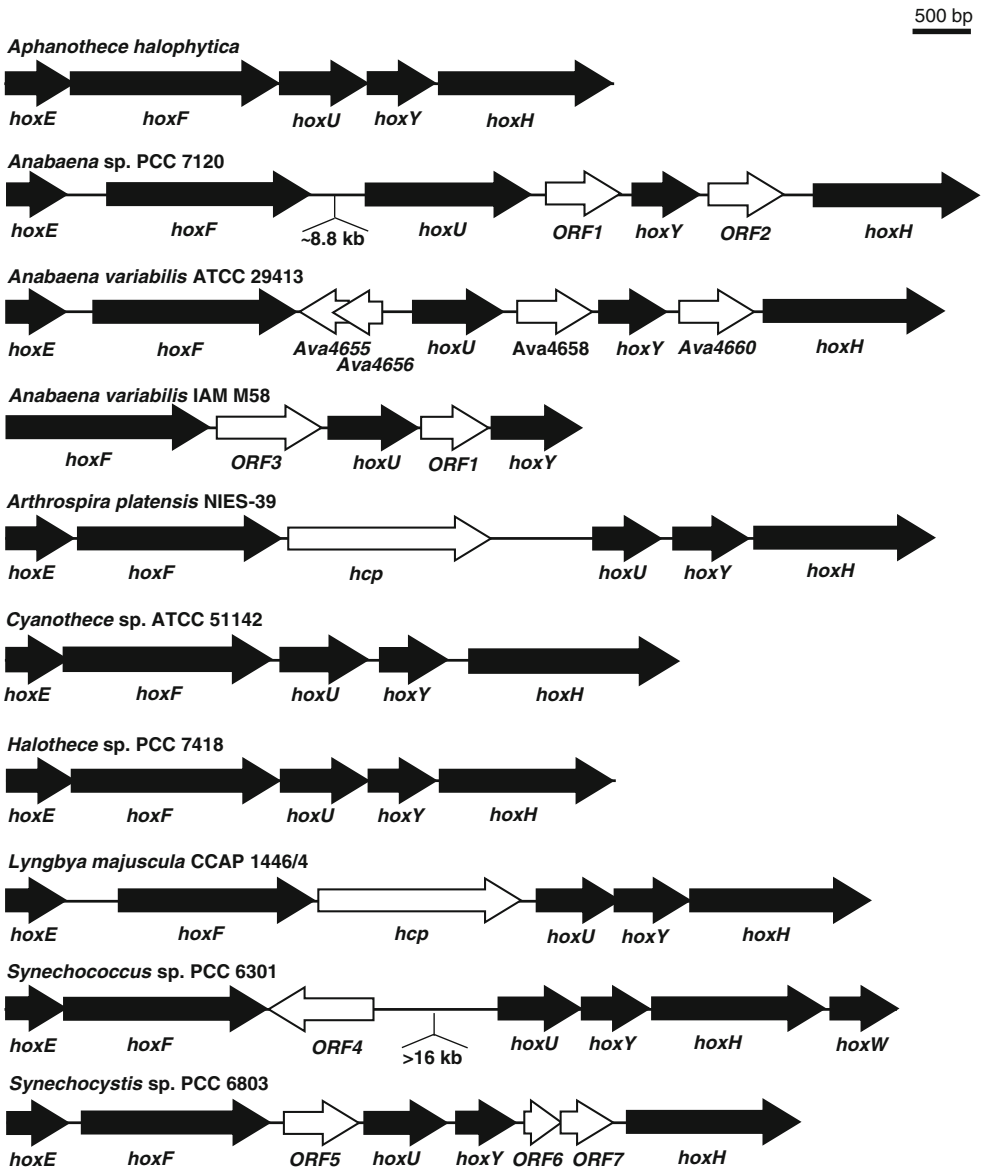

Fig. 1 Physical map of *hox* genes in *A. halophytica* compared with other cyanobacteria

Co-transcriptional analysis by RT-PCR

The co-transcriptional analysis of *hox* gene cluster in *A. halophytica* was performed by RT-PCR. Figure 2a shows the strategy for the analysis in which the single-stranded mRNA was reverse-transcribed into complementary DNA (cDNA) by M-MuLV reverse transcriptase using *hoxH*-specific antisense RhydApha3 as a RT-primer. The obtained cDNA was used as a template for amplification of the DNA fragments of *hoxEF*, *hoxFU*, *hoxUY*, and *hoxYH* using specific primer pairs listed in Table 1. Only one specific RT-PCR product was found in each RT-PCR reaction (Fig. 2b). RT-PCR products of *hoxEF*, *hoxFU*, *hoxUY*, and *hoxYH* showed similar size to those of the expected PCR products of 1477, 434, 779, and 777 bp, respectively. No RT-PCR products were observed in negative RT-PCR reactions (Fig. 2b). The results indicated that all structural genes encoding bidirectional hydrogenase were transcribed as a single operon in *A. halophytica*.

Transcriptional analysis of large subunit bidirectional hydrogenase gene and other related genes in *Aphanothece halophytica*

Aphanothece halophytica grown in BG11 supplemented with Turk Island salt solution was harvested and resuspended in three different media; normal medium (BG11 + Turk), sulfur-free medium (BG11 + Turk (-S)), and nitrate-free medium (BG11 + Turk (-N)). Transcriptional analysis of *hoxH* revealed the upregulation in *A. halophytica* incubated under nitrate-deprived condition for 24 and 48 h (Fig. 3a, b). This was correlated with the increased bidirectional H_2ase activity in cells under nitrate-deprived condition (Fig. 4a). Transcript

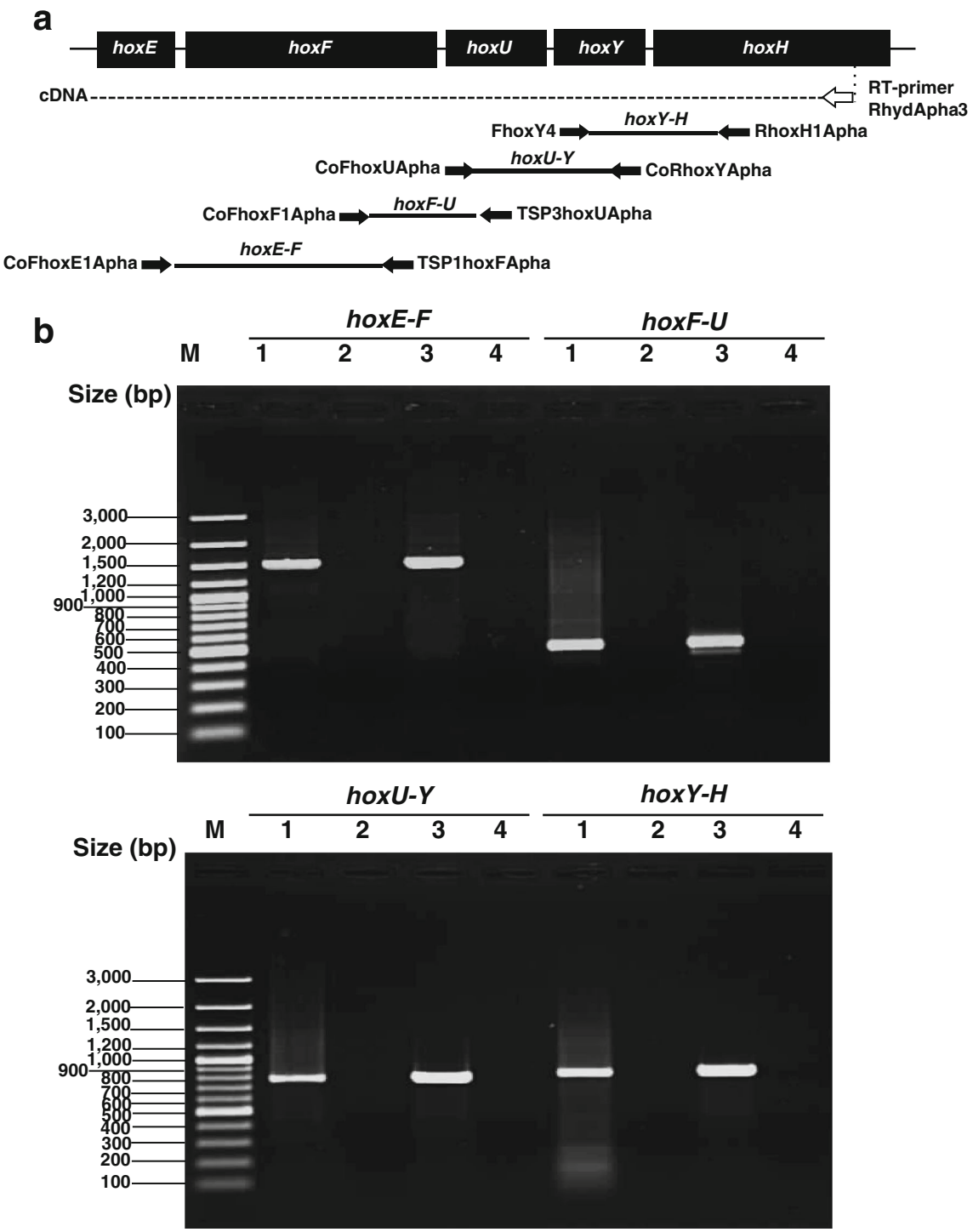

Fig. 2 Strategy of co-transcriptional analysis of *hox* gene cluster in *A. halophytica*. The mRNA was isolated and transcribed into the cDNA using RT-primer RhydApha1. The obtained cDNA was then used as a template for DNA amplification of *hoxEF*, *hoxFU*, *hoxUY*, and *hoxYH* genes by polymerase chain reaction (**a**). Agarose gel analysis of RT-PCR products of *hoxEF*, *hoxFU*, *hoxUY*, and *hoxYH* genes (**b**). *Lane 1*, RT-PCR reaction; *lane 2*, negative RT-PCR reaction without M-MuLV reverse transcriptase; *lane 3*, positive PCR reaction (using genomic DNA as template); *lane 4*, negative PCR reaction (without template DNA); *lane M*, 100-bp DNA ladder

abundance of two genes involved in glycogen metabolism, *glgB* encoding 1,4-α-glucan branching enzyme in glycogen synthetic pathway and *glgP* encoding glycogen phosphorylase in glycogen breakdown pathway, showed the upregulation and downregulation of *glgB* and *glgP*, respectively, in cells under nitrate starvation for 24 and 48 h (Fig. 3a, b). Glycogen content was highest, 55.3±9.7 % of dry cell weight, in cells under nitrate-deprived condition for

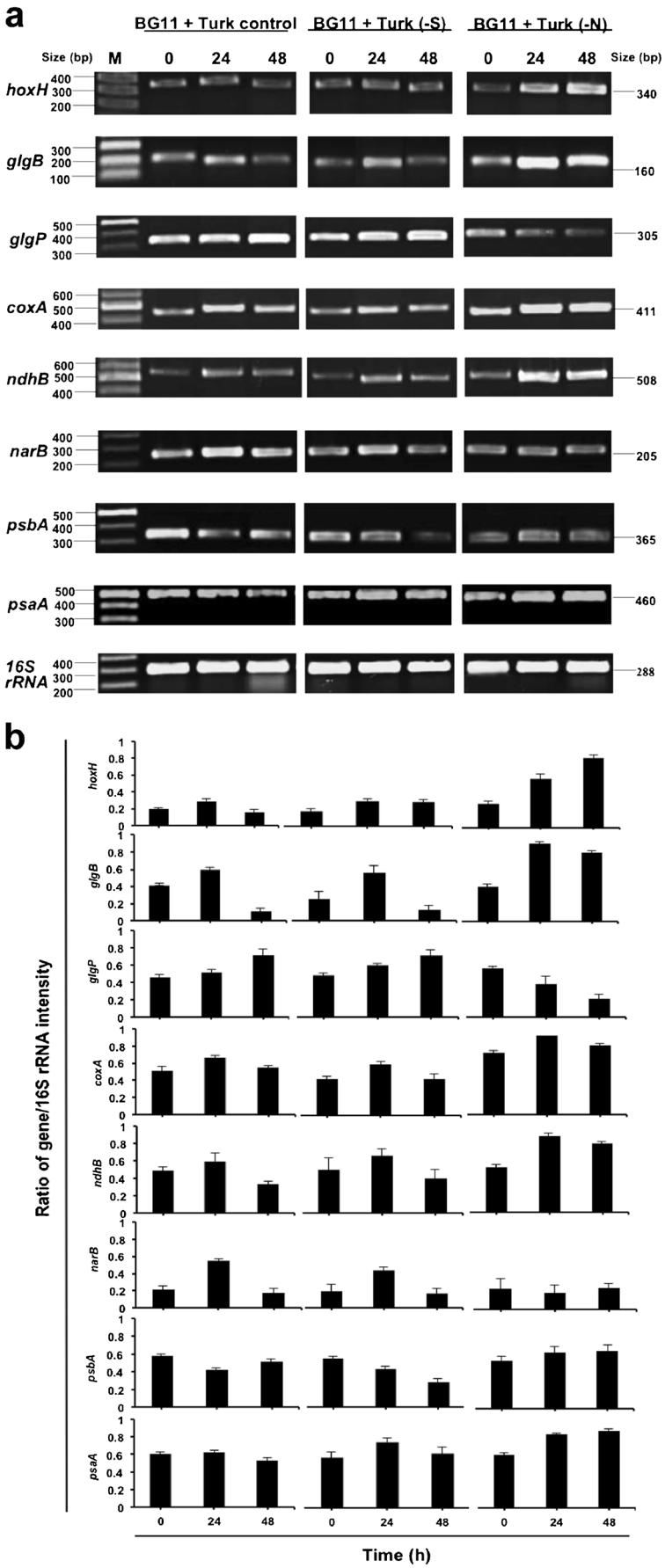

Fig. 3 Relative transcript levels of *hox* genes and other genes involved in H_2 metabolism by OneStep RT-PCR (**a**). Total RNA was isolated from *A. halophytica* incubated in BG11 + Turk (control), BG11 + Turk (-S) (without sulfur), and BG11 + Turk (-N) (without nitrate) for 0, 24, and 48 h. RT-PCR products were analyzed by 1.0 % agarose gel electrophoresis. The expression of a housekeeping gene, 16S rRNA gene, was used as a reference for each sample. Relative quantification of transcriptional level of *hoxH*, *glgB*, *glgP*, *coxA*, *ndhB*, *narB*, *psbA*, and *psaA* of *A. halophytica* was obtained by comparison to transcription level of the housekeeping 16S rRNA gene (**b**)

48 h, a twofold increase compared to cells under normal condition (Fig. 4b). Moreover, the relative transcript levels of *coxA* and *ndhB*, encoding respectively subunit I of cytochrome oxidase and subunit B of NADH dehydrogenase in the respiratory electron transport system, were also upregulated in cells under nitrate deprivation for 24 and 48 h (Fig. 3a, b). This upregulation of *coxA* and *ndhB* caused the increase in dark respiration rate of cells under nitrate deprivation whereas no apparent changes in dark respiration were observed under sulfur deprivation (Fig. 4c). In contrast, a downregulation of *narB*, encoding ferredoxin-nitrate reductase, was observed in cells under nitrate deprivation (Fig. 3a, b). No differences of relative transcript level of *psbA* encoding D1 protein of photosystem II were observed under normal condition. Expression of *psbA* was decreased and increased respectively under sulfur and nitrate deprivation (Fig. 3a, b). Similar results were observed for the expression of *psaA* encoding core protein PsaA of photosystem I with the exception of that under sulfur deprivation showing a slightly increased expression.

Discussion

In this study, we could isolate *hox* gene cluster with complete nucleotide sequences from *A. halophytica*. Previously, bidirectional hydrogenase gene was detected in *A. halophytica* by Southern blot hybridization (Ludwig et al. 2006), and later, Barz and colleagues (2010) reported the partial sequence of a large subunit of bidirectional hydrogenase (*hoxH*) in *A. halophytica*. *Aphanothece halophytica* contains five structural bidirectional hydrogenase genes (*hoxE*, *hoxF*, *hoxU*, *hoxY*, and *hoxH*) similar to those normally found in other cyanobacteria (Fig. 1). After homology search of the entire genes, the nucleotide and amino acid sequences of *A. halophytica hoxEFUYH* showed the highest identity and similarity (99–100 %) to those reported in *Halothece* sp. PCC 7418 whose genome sequences were launched in GenBank in 2013 (accession number CP003945.1). Nucleotide sequences of *hoxF* and *hoxH* in *Halothece* sp. PCC 7418 showed five and one nucleotide differences, respectively, compared to those of *A. halophytica* in the present study, i.e., the amino acid sequences of HoxF and HoxH in both organisms differed in three and one amino acids, respectively. In addition, by phylogenetic tree analysis, all *hox* genes in *A. halophytica* showed a very close relationship with all *hox* genes in *Halothece* sp. PCC 7418 (data not shown). The results suggested that the studied cyanobacterium *A. halophytica* is in the same genus and species as *Halothece* sp. PCC7418 but might be different in strain or at subspecies level.

In *hox* gene cluster of *A. halophytica*, no other ORFs were observed (Fig. 1). Normally, the orientation of *hox* gene cluster in several cyanobacteria is similar, but it might have some differences regarding a direction of *hox* genes or an insertion of ORFs. One or several additional ORFs have been identified between the structural genes in some species of cyanobacteria, for instance *Anabaena* sp. PCC 7120 (Kaneko et al. 2001), *Anabaena variabilis* ATCC 29413 (Schmitz and Bothe 1996), *Anabaena variabilis* IAM M58 (Tamagnini et al. 2002), *Arthrospira platensis* NIES-39 (Zhang et al. 2005a, b), *Lyngbya majuscula* CCAP 1446/4 (Ferreira et al. 2009), *Synechococcus* sp. PCC 6301 (Boison et al. 1998), and *Synechocystis* sp. PCC 6803 (Appel and Schulz 1996) (Fig. 1). In agreement with our results, no ORFs are found between *hox* structural genes in *Cyanothece* sp. ATCC 51142 (Welsh et al. 2008) which showed high potential as H_2 producer (Min and Sherman 2010). Nevertheless, the functions of additional ORFs between structural genes in some cyanobacteria need to be further investigated. The *hoxEFUYH* genes in *A. halophytica* encode five proteins, HoxE, HoxF, HoxU, HoxY, and HoxH, with calculated molecular masses of 19.0, 57.8, 26.5, 20.1, and 53.1 kDa, respectively. Their molecular masses are in good agreement with the data of five dissimilar subunits of about 19, 58, 26, 20, and 53 kDa reported in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 or other cyanobacteria (Appel and Schulz 1996; Ghirardi et al. 2007).

The prediction of conserved motif of *hox* genes in *A. halophytica* was performed according to Tamagnini and coworkers (2002). In all Hox subunits of *A. halophytica*, conserved cysteine motifs, functioning in coordination of iron-sulfur clusters involved in an electron transfer, were found. HoxE in *A. halophytica* contained four conserved cysteine residues, which were likely to harbor a [2Fe-2S] cluster. The pattern of binding of a putative [2Fe-2S] cluster in HoxE of *A. halophytica* was C94-x_4-C99-x_{35}-C135-x_3-C139. A similar result was found in HoxE of *Synechocystis* sp. PCC 6803 harboring a putative [2Fe-2S] cluster that contained cysteine residues at C96, C101, C137, and C141 (Appel and Schulz 1996). Normally, the HoxE harbors a motif for binding an iron center and is thought to couple with the enzyme in the respiratory and photosynthetic electron transport chain on the thylakoid and also possibly at the cytoplasmic membrane (Bothe et al. 2010). In the middle region of large subunit of diaphorase HoxF in *A. halophytica*, typical glycine-rich binding sites for oxidized and reduced nicotinamide adenine dinucleotide (NAD^+ and NADH) and flavin mononucleotide (FMN and $FMNH_2$) were found between amino acid positions from 171 to 180 (G171-x-G173-x_2-G176-x_3-G180) and from 183 to 204 (G183-x-G185-x_4-G190-x_{10}-G201-x_2-G204), respectively, corresponding to the general HoxF glycine-rich binding sites GxGxxGxxxG and GxGxxxxGx$_{10}$GxxG in other cyanobacteria (Schmitz et al. 1995; Bothe et al. 2010). In addition, HoxF also contained a binding [2Fe-2S] cluster (C25-x_4-C30-x_{30}-C61-x_3-C65) at the N-terminus and a binding putative [4Fe-4S] cluster (C455-x_4-C460-x_2-C463-x_{33}-C497) at the C-

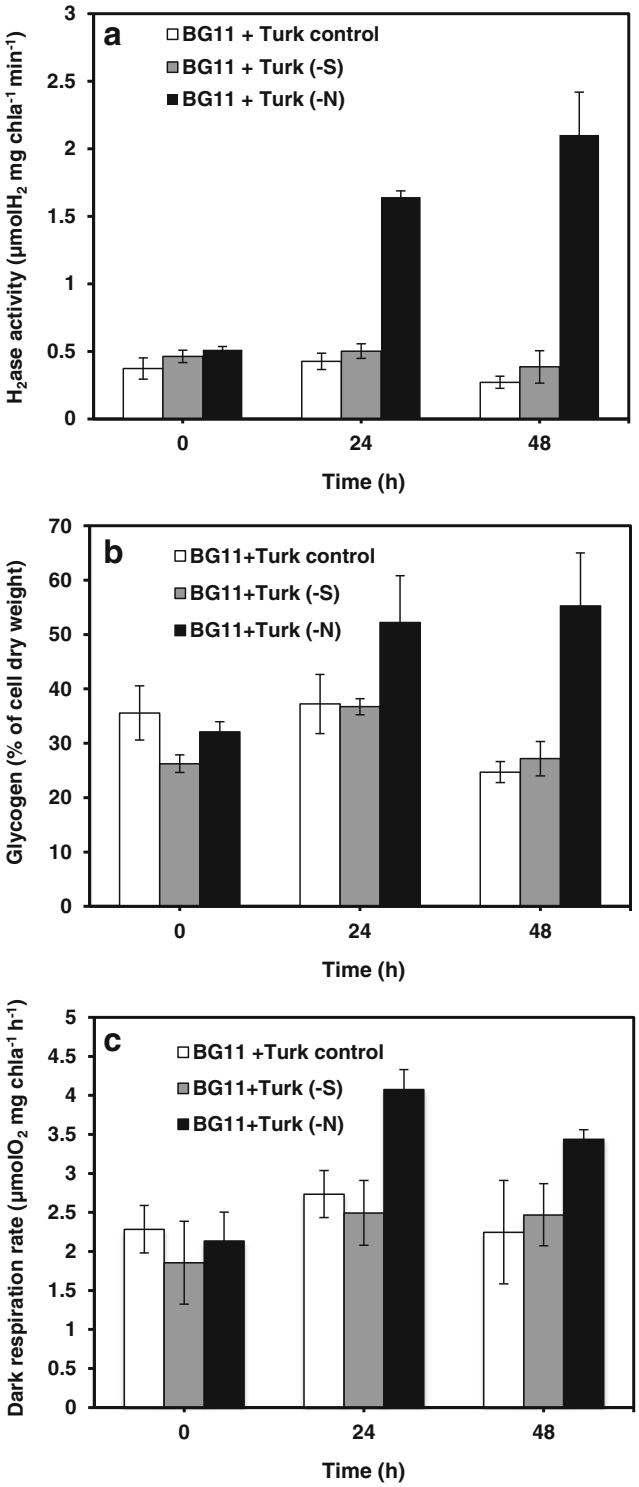

Fig. 4 Bidirectional H_2ase activity (**a**), glycogen content (**b**), and dark respiration rate (**c**) of *A. halophytica* incubated in normal medium (BG11 + Turk control), sulfur-deprived medium (BG11 + Turk (-S)), and nitrate-deprived medium (BG11 + Turk (-N)) for 0, 24, and 48 h. Data are means ± SD (n=3)

terminus. These results were in agreement with those from *Anabaena variabilis* ATCC 29413 (Schmitz et al. 1995) and *Synechocystis* sp. PCC 6803 (Appel and Schulz 1996). The small subunit of diaphorase moiety, HoxU protein, comprised several conserved cysteine residues involved in the binding of a putative [Fe-S] cluster (Appel and Schulz 1996; Schmitz and Bothe 1996). Two motifs were located at the N-terminal part of the protein; the first motif harbored four cysteine residues probably involved in binding a putative [2Fe-2S] cluster (C36-x_{10}-C47-x_2-C50-x_{13}-C64), and the second motif harbored three cysteine residues involved in binding a putative [3Fe-4S] cluster or a putative [4Fe-4S] cluster (C100-x_2-C103-x_5-C109). Moreover, the C-terminal part of HoxU possessed two typical [4Fe-4S] cluster-binding sites with conserved cysteine residues of C149-x_2-C152-x_2-C155-x_3-C159 and C193-x_2-C196-x_2-C199-x_3-C203. A similar result was reported for HoxU of *Anabaena variabilis* ATCC 29413 (Schmitz et al. 1995), *Synechococcus* sp. PCC 6301 (Boison et al. 1996), and *Synechocystis* sp. PCC 6803 (Appel and Schulz 1996). The small subunit of hydrogenase protein (HoxY) in *A. halophytica* contained C14, C17, C87, and C151 as conserved cysteine residues, found to be involved in the binding of a putative [4Fe-4S] (Volbeda et al. 1995). The large subunit hydrogenase protein (HoxH) comprised four conserved cysteine residues (C62, C65, C444, C447) involved in the binding of nickel to the active site of hydrogenase (Volbeda et al. 1995; Schmitz and Bothe 1996).

In *A. halophytica*, five structural genes of bidirectional hydrogenase, *hoxEFUYH*, were clustered and oriented in the same direction. Furthermore, these five structural genes encoding bidirectional hydrogenase were transcribed as a single operon in *A. halophytica* (Fig. 2). In cyanobacteria, structural genes encoding bidirectional hydrogenases are differently organized (Tamagnini et al. 2007). The arrangement of *hox* operon in *A. halophytica* is very similar to that of *hoxEFUYH* genes in *Halothece* sp. PCC 7418 (accession no. CP003945.1) and *Cyanothece* sp. ATCC 51142 (Welsh et al. 2008). In addition, *hox* operon in *A. halophytica* was co-transcribed as a single transcript similar to that reported in *Anabaena variabilis* (Boison et al. 2000), *Lyngbya majuscula* CCAP 1446/4 (Ferreira et al. 2009), and *Synechocystis* sp. PCC 6803 (Gutekunst et al. 2005; Oliveira and Lindblad 2005), whereas *hox* gene cluster was separated into two different clusters, *hoxEF* and *hoxUYH*, in *Synechococcus* sp. PCC 6301 (Boison et al. 1998) and *Anabaena* sp. PCC 7120 (Kaneko et al. 2001).

By relative quantification of RT-PCR products, *hoxH* was upregulated under nitrate starvation (Fig. 3a, b). It has been reported that the relative transcript level of *hox* genes in most cyanobacterial species increased under nitrogen starvation/limitation condition (Boison et al. 2000; Sheremetieva et al. 2002; Troshina et al. 2002; Antal et al. 2006; Osanai et al. 2006; Baebprasert et al. 2011). An increase of H_2ase activity in *A. halophytica* was also observed after 24 h of N-deprived incubation (Fig. 4a), which was in line with the increased transcript of *hoxH* (Fig. 3a, b). This was likely due to the

nitrate-deficiency-induced increase of both NAD(P)H and electrons for promoting H_2 production via bidirectional hydrogenase. Similar observation was reported for *Synechocystis* sp. PCC 6803 disrupted in nitrate assimilation pathway (Baebprasert et al. 2011). In addition, *glgB* encoding 1,4-α-glucan branching enzyme involved in glycogen synthesis was upregulated under N-deprived condition (Fig. 3a, b), which was correlated with an increase in glycogen accumulation in *A. halophytica* (Fig. 4b). In contrast, *glgP* encoding glycogen phosphorylase involved in glycogen degradation was downregulated under N-deprived condition (Fig. 3a, b). Similar results were observed in a freshwater cyanobacterium *Synechocystis* sp. PCC 6803 showing the upregulation of *glgB* after nitrogen starvation for 4 h (Osanai et al. 2006) and for 12 h (Krasikov et al. 2010). The results indicated that cells rather synthesized than degraded glycogen during nitrate starvation. A storage glycogen in the cells might be broken down through glycolysis or other pathways during dark anaerobic fermentation to provide sufficient ATP and NAD(P)H for H_2 production via bidirectional hydrogenase.

The two genes involved in the respiratory electron transport, *coxA* encoding subunit I of cytochrome oxidase and *ndhB* encoding subunit B of NADH dehydrogenase, in *A. halophytica* were upregulated under N-starved condition (Fig. 3a, b), which was supported by the increased respiration rate found in N-starved cells (Fig. 4c). The upregulation of *coxA* and *ndhB* expression in *A. halophytica* was in line with the increased expression of all genes encoding terminal respiratory oxidases under N deprivation in *Synechocystis* sp. PCC 6803 (Krasikov et al. 2010) as well as in *Nostoc* sp. PCC 7120 showing strong expression of *coxA* under N_2-fixing condition (Wünschiers et al. 2007). The upregulation of terminal respiratory oxidase could dissipate reducing equivalents in the thylakoid membranes to protect photosystem II (PSII) from photoinhibition (Krasikov et al. 2010). The storage carbohydrate content and the respiration rate were increased whereas the rates of O_2 evolution and CO_2 fixation were decreased in *Synechocystis* sp. PCC 6308 under nitrogen starvation (Allen et al. 1990). In the N-starved cells of *A. halophytica*, an increased respiration would help eliminate O_2 remaining within the cells, thus providing protection on the enzymes involved in H_2 production against inhibition by O_2.

The relative transcript level of *narB* encoding ferredoxin-nitrate reductase was upregulated under both control and S-deprived conditions after 24 h, whereas no changes of *narB* transcript were detected under N-deprived condition (Fig. 3a, b). This suggested a low nitrate reduction activity in *A. halophytica* under N-deprived condition. On contrary, *narB* in *Synechocystis* sp. PCC 6803 was downregulated under N starvation (Krasikov et al. 2010). Nitrate is transported into the cells by an active transport system and reduced to nitrite by nitrate reductase followed by the action of nitrite reductase (encoded by *nirA*) to yield ammonium (Incharoensakdi 2006). Nitrate reduction to ammonium through the two sequential reactions of nitrate reductase and nitrite reductase requires two electrons and six electrons, respectively (Flores et al. 2005). Therefore, nitrate reduction competes for electrons that would flow to hydrogenase because nitrate

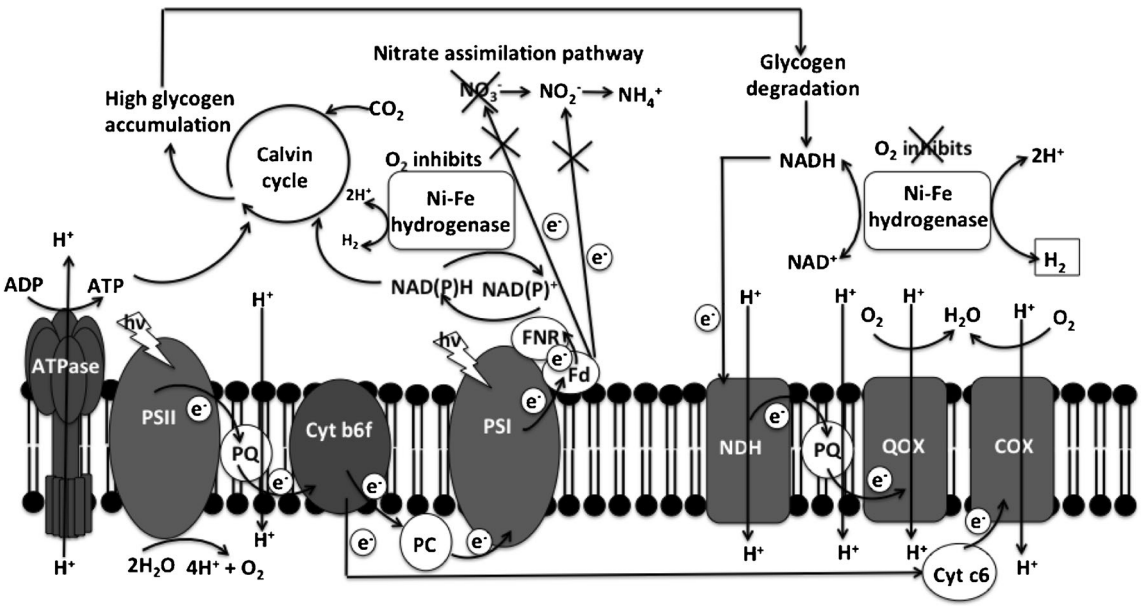

Fig. 5 Schematic mechanism of oxygenic photosynthesis and H_2 metabolism under N deprivation in *A. halophytica*. Abbreviations: *PSII* photosystem II, *PSI* photosystem I, *Pq* plastoquinone, *Cyt b6f* cytochrome *b6f* complex, *PC* plastocyanin, *Cyt c6* cytochrome *c6*, *Fd* ferredoxin, *FNR* ferredoxin/NADP$^+$ reductase, *NiFe hydrogenase* bidirectional hydrogenase, *NDH* NADH dehydrogenase, *QOX* cytochrome-quinol oxidase, *COX* cytochrome oxidase

reduction is a better electron sink than hydrogenase (Ananyev et al. 2008). The availability of $NaNO_3$ in the medium would limit H_2 production.

The relative transcript level of *psbA* encoding D1 protein of photosystem II was downregulated under S starvation (Fig. 3a, b) which was in line with a previous study in *Synechocystis* sp. PCC 6803 (Zhang et al. 2008). The lack of sulfur affected cells by reduction of photosystem II activity, resulting in cessation of O_2 evolution and subsequent reactivation of H_2ase activity (Melis et al. 2000; Zhang et al. 2002). The expression of *psaA* encoding core protein PsaA of photosystem I was slightly increased under N starvation (Fig. 3a, b). The increased activity of photosystem I and II under light condition would produce ATP and NADPH which can then provide electron for the reduction of proton to generate H_2 in *A. halophytica* under N starvation. The schematic mechanism of oxygenic photosynthesis and H_2 metabolism of *A. halophytica* under nitrate deprivation is shown in Fig. 5. Under photosynthetic process, electrons from photosystem II (PSII) to photosystem I (PSI) are transferred through plastoquinone (Pq), cytochrome *b6f* complex (Cyt b6f), plastocyanin (PC), and cytochrome *c6* (Cyt c6). From PSI, electrons can be transferred to ferredoxin (Fd) via ferredoxin/$NADP^+$ reductase (FNR) to generate NAD(P)H. Bidirectional hydrogenase (NiFe hydrogenase) can accept electrons from NAD(P)H, but this enzyme is inhibited by O_2 produced from the water splitting via PSII. Protons transferred across the thylakoid membrane are used to synthesize ATP by the ATPase. Under N deprivation, *A. halophytica* converts the energy of photons into energy in the form of ATP and NAD(P)H to promote an increased accumulation of glycogen, via CO_2 fixation in Calvin cycle, which is the main storage of carbon and energy in cyanobacterial cells. In addition, nitrate assimilation pathway responsible mainly by the expression of *narB* was downregulated under N-deprived condition (Fig. 3a, b), thus further enhancing the flow of electrons toward Fd resulting in an increase of the electrons to generate NAD(P)H. Thereafter, H_2 is generated under dark anoxic conditions as a result of bidirectional hydrogenase reactivation in the absence of O_2 and an over-reduction of NADH during glycogen catabolism. Respiratory electron transfer also includes NADH dehydrogenase (NDH), cytochrome-quinol oxidases (QOX), and cytochrome oxidase (COX), which are involved in the consumption of NADH and O_2. Under N deprivation, the increased expression of *coxA* and *ndhB* in *A. halophytica* was observed. This would benefit *A. halophytica* to maintain the high O_2 consumption rate under anaerobic condition resulting in an increased bidirectional hydrogenase activity due to the protection of enzyme against inhibition by O_2.

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