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Investigating the Link between Fermentative Metabolism and Hydrogen Production in the Unicellular Green Alga *Chlamydomonas Reinhardtii*

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Abstract

In the model green alga *Chlamydomonas reinhardtii*, the electrons required for hydrogen production can come from both the biophotolysis of water and from the fermentation of carbohydrate reserves. Anoxia leads to the activation of several fermentative pathways, which produce a number of end products including formic, malic and acetic acid along with ethanol, carbon dioxide and hydrogen. It has been proposed that by switching off competing fermentative pathways hydrogen production can be increased. Therefore the aim of this study was to devise an experimental strategy to down-regulate the expression of enzymes thought to control *C. reinhardtii's* fermentative metabolism. We demonstrate here that it is possible to use artificial microRNA (amiRNA) technology to generate knock-down mutants with reduced expression of pyruvate formate lyase (PFL1), a key fermentative enzyme in *C. reinhardtii*. This work opens up new possibilities to improve hydrogen yields through metabolic engineering.

1 Introduction

Certain types of green algae and cyanobacteria possess the ability to produce hydrogen, harnessing solar energy through photosynthesis to provide reductant to a [FeFe]- or [NiFe]-hydrogenase [1]. However, current maximum reported yields are low, at 5ml H_2 gas h^{-1} l^{-1} (>90% pure) [2, 3], and therefore must be improved if photobiological hydrogen production is to become an economically viable process [4, 5]. Many of the efforts to improve yields have focused on the unicellular (~10µm), freshwater micro-alga *Chlamydomonas reinhardtii*, due to good basal levels of H_2 production [6, 7], the availability of completely sequenced and transformable mitochondrial, chloroplast and nuclear genomes, detailed information of metabolic pathways and an extensive library of expressed sequence tags [8].

In order to overcome inhibition of the hydrogenase by oxygen evolved during photosynthesis, growth and H_2 production phases can be separated by sulphur depletion. During this process, the rate of oxygen evolution, catalysed by photosystem II (PSII), drops below respiration, driving sealed cultures into anoxia allowing sustained gas evolution over several days [7]. During sulphur depletion, electrons in the photosynthetic electron transport (PET) chain are passed via ferredoxin (Fd) to one of two 49-kDa [FeFe]-hydrogenases, HYDA1 or HYDA2 [9, 10], to produce hydrogen (Figure 1) [1]. Reductant is believed to come from a mixture of residual PSII activity and endogenous catabolism of starch and protein [11], which feeds electrons into the PET at the point of the plastoquinone pool, via a type II NADH dehydrogenase, or directly to ferredoxin by pyruvate ferredoxin oxidoreductase (PFOR) (Figure 1). The process is thought to be limited by supply of reductant [2] making it important to maximize available sources of electrons and down regulate competing e- sinks to improve H_2 evolution.

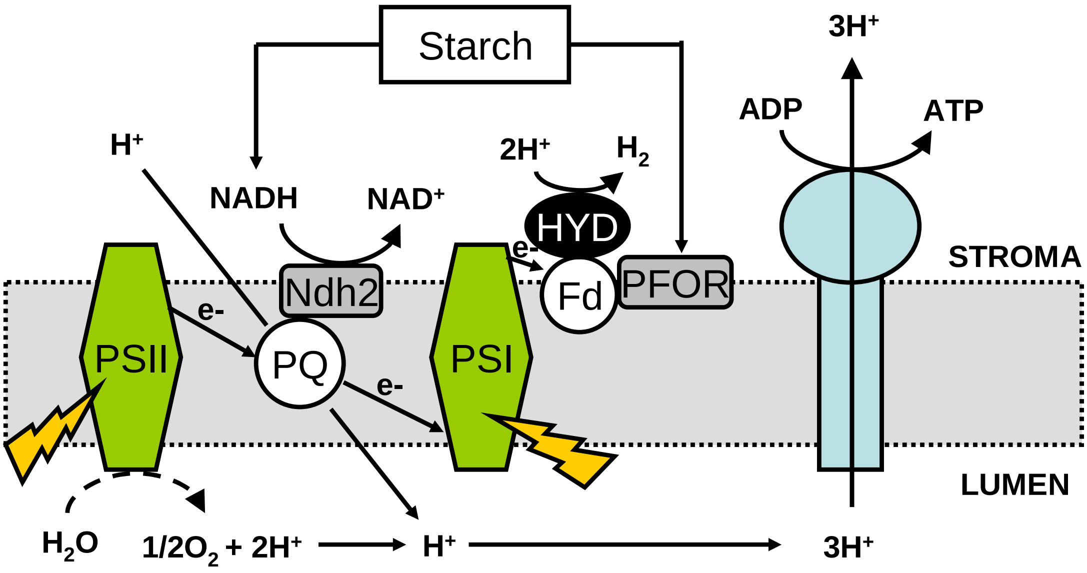

Figure 1: **Diagram showing electron transfer in the photosynthetic electron transport chain (PET) during H_2 production: PSII, Photosystem II; PSI, Photosystem I; PQ, Plastoquinone; Fd, Ferredoxin; HYD, Fe-hydrogenase; Ndh2, type II NAD(P)H dehydrogenase; PFOR, Pyruvate:ferredoxin oxidoreductase.**

Chlamydomonas is known to ferment starch under sulphur deprivation, creating a range of products that accumulate in direct competition with H_2 production [12]. Analysis of the *C. reinhardtii* genome sequence, the products of fermentation and inhibitor studies has suggested the presence of a three branched pathway (Figure 2) [13-15], in which pyruvate can be broken down to produce ethanol, formic acid or hydrogen along with acetic and malic acid further downstream [14, 16].

The predominant fermentative pathway is thought to involve the conversion of pyruvate to acetyl-CoA and formic acid by the enzyme pyruvate formate lyase (PFL1) (XM_001689667.1) [11] which has subsequently been identified as a good candidate for reverse genetic approaches to improve hydrogen production [12].

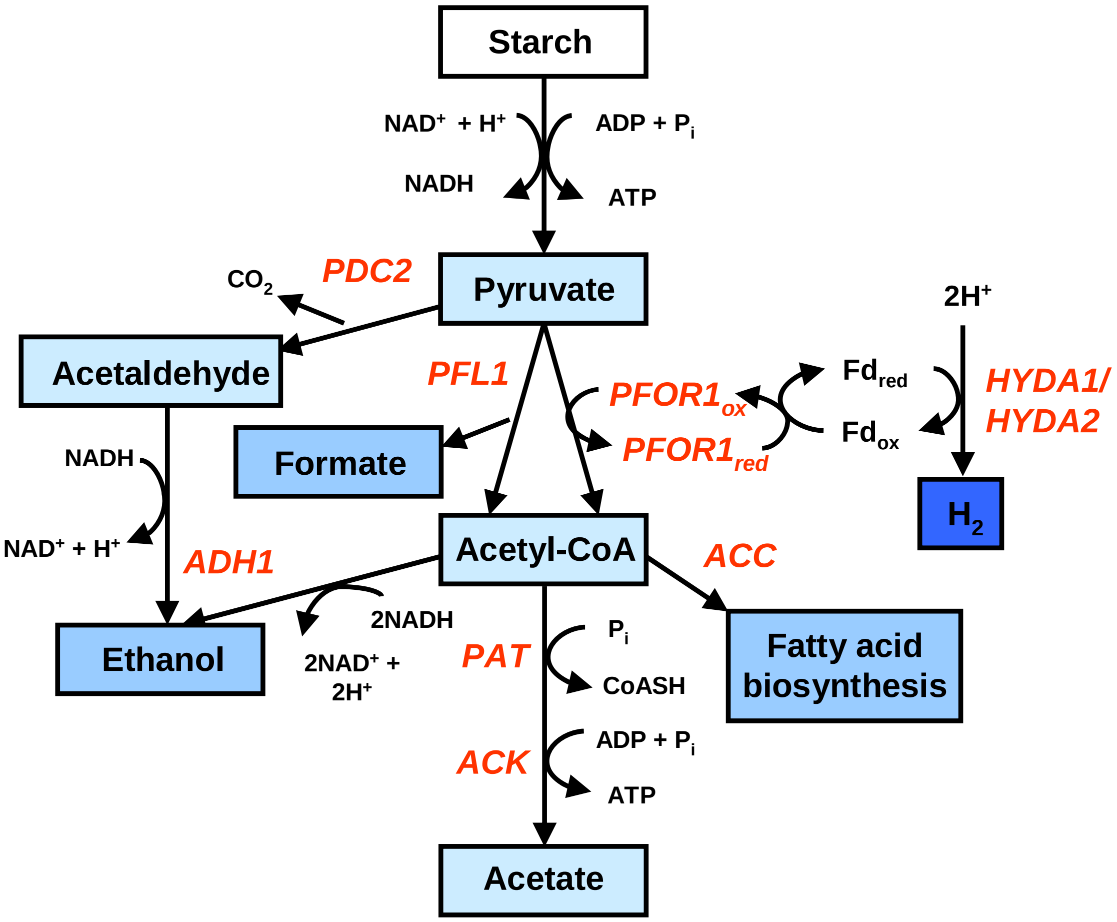

Figure 2: Putative fermentative pathways *C. reinhardtii* (adapted from [14]). ACC, acetyl-CoA carboxylase; ACK, acetate kinase; ADH1, alcohol dehydrogenase; HYDA1/HYDA2, [FeFe]-hydrogenase 1 and 2; PDC2, Pyruvate decarboxylase; PFL1, Pyruvate formate lyase; PFOR, Pyruvate ferredoxin oxidoreductase; PAT, phosphate acetyltransferase.

To date, problems transforming the *C. reinhardtii* nuclear genome by homologous recombination [17], and transcriptional silencing of conventional RNAi constructs, consisting of long dsRNAs [18], have hampered the ability to switch off or down regulate fermentative pathways. However, recent advances in artificial miRNA (amiRNA) technology have opened up the potential for stable metabolic engineering in *Chlamydomonas* [19, 20]. Here we describe the application of this technology to the generation of a strain with reduced levels of PFL1.

2 Experimental Procedures

Strains and culture conditions. Cell wall deficient *C. reinhardtii* strain CC406 (cw15) was grown in Tris acetate phosphate (TAP) media with $MgSO_4$ (or $MgCl_2$ in the case of sulphur-depletion experiments) at 25°C under a constant illumination of $50 \mu Em^{-2}s^{-1}$.

Nuclear transformation of C. reinhardtii. Strains were transformed with ~2µg plasmid DNA by glass bead agitation and positive transformants selected on TAP plates containing paromomycin at 10µg/ml [21].

Construction of artificial microRNA (amiRNA) vectors. Oligonucleotides of 90 bases were designed using the online tool, WMD3, (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) and cloned into amiRNA vector pChlamRNAi3 as described [19].

Real time RT-PCR analysis. RNA was extracted using RNeasy Plant Mini kit (74903; QIAGEN, Germany) according to the manufacturer's protocol. cDNA synthesis was performed using High Capacity DNA Reverse Transcription Kit (4368814; Applied Biosystems, USA) and RT-PCR performed with 2x Taqman Fast Universal PCR Master Mix and analysed on 7500 Fast Real-Time PCR System (Applied Biosystems, USA)

3 Results

Reduction of PFL1 expression was performed by transforming a cell-wall deficient strain of *C. reinhardtii* (strain CC406) with a derivative of plasmid pChlamiRNA3 [19]. Nuclear transformants were selected on the basis of resistance to the antibiotic paromomycin (encoded by *aphVIII*) and the precursor artificial microRNA (amiRNA) was expressed from the *psaD* promoter (Figure 3A). The amiRNA was created by replacing a 90-nucleotide region of a WT pre-miRNA, which incorporates the targeting sequence and hairpin loop structure, by a synthetic construct designed using the online tool WMD3. The plasmid was used to transform the cell-wall deficient strain, CC406, by glass bead agitation. Screening of 30 paromomycin-resistant transformants by immunoblot identified two mutants, 4B23 and 4B24, with 70-80% reduction in PFL1 protein levels (Figure 3B). To check the phenotype was a result of decreased transcript accumulation, RNA was extracted from WT and 4B24 cultures, grown under sulphur depletion conditions, and analysed by real time RT-PCR. The results confirmed a significant knockdown of PFL1 mRNA (Figure 3C).

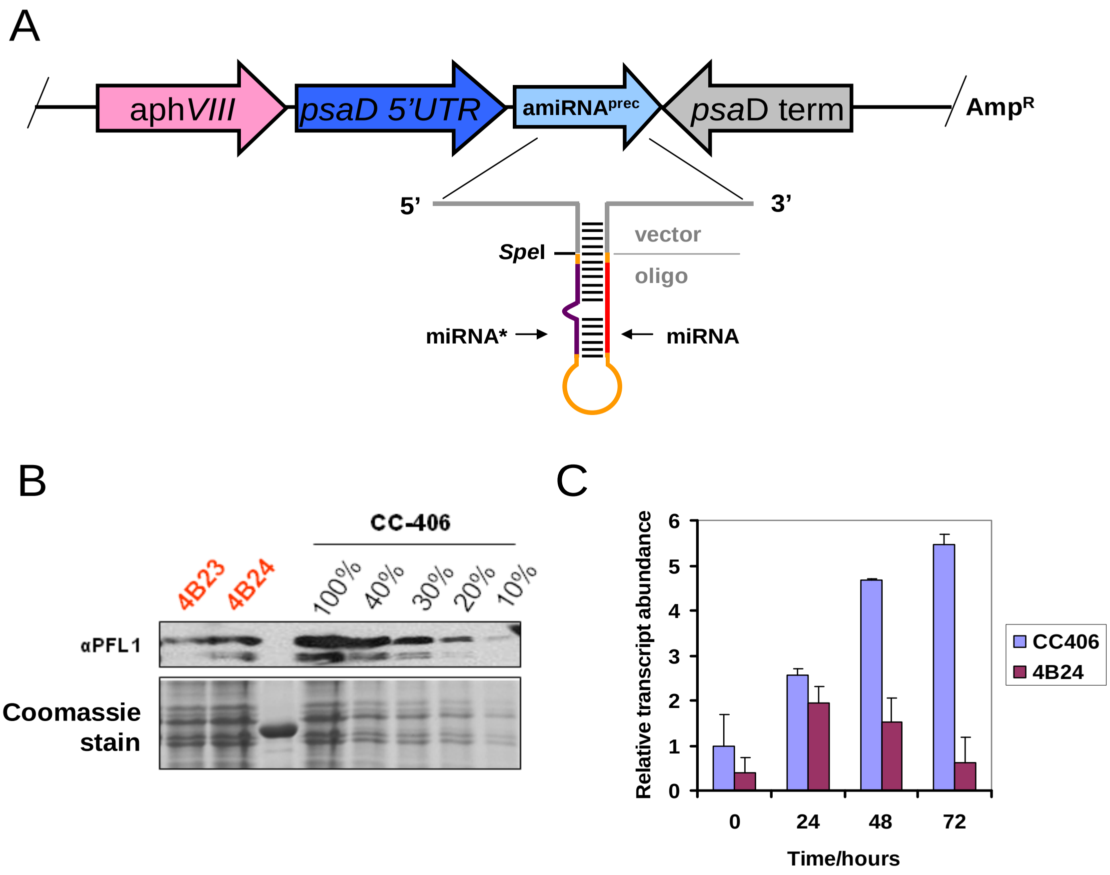

Figure 3: (A) Map of amiRNAi vector pChlamiRNA3 used to transform *C. reinhardtii* strain CC-406. The *aph*VIII resistance cassette allows for selection on paromomycin and a *psaD* promoter was used to drive expression of amiRNAs (Adapted from [19]). (B) Immunoblot screening of transformants for knockdown of PFL1. Strains were grown under standard conditions and harvested at late log phase (OD_{750} 0.9-1). Loading is shown by Coomassie stained gel. (C) Real time reverse transcription-PCR analysis of PFL1 transcript levels, comparing parental strain CC406 to 4B24 at various times after sulphur deprivation. Values are given relative to CC406 t=0 and normalized to transcript levels of RPL10a which encodes the 60S ribosomal protein L10a, all measurements were done in triplicate.

4 Summary

In conclusion, we report here the first successful knockdown of a key fermentative enzyme in *C. reinhardtii*. The application of amiRNA technology opens up exciting new possibilities for increasing hydrogen yields. Analysis of the impact of PFL1 knockdown on metabolite and H_2 yields is ongoing.

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References

[1] Ghirardi, M.L., Posewitz, M.C., Maness, P.C., Dubini, A., Yu, J., and Seibert, M. (2007). Annu Rev Plant Biol *58*, 71-91.

[2] Kruse, O., Rupprecht, J., Bader, K.P., Thomas-Hall, S., Schenk, P.M., Finazzi, G., and Hankamer, B. (2005). J Biol Chem *280*, 34170-34177.

[3] Doebbe, A., Rupprecht, J., Beckmann, J., Mussgnug, J.H., Hallmann, A., Hankamer, B., and Kruse, O. (2007). J Biotechnol *131*, 27-33.

[4] Kruse, O., Rupprecht, J., Mussgnug, J.H., Dismukes, G.C., and Hankamer, B. (2005). Photochem Photobiol Sci *4*, 957-970.

[5] Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., and Hankamer, B. Nat Biotech *28*, 126-128.

[6] Timmins, M., Thomas-Hall, S.R., Darling, A., Zhang, E., Hankamer, B., Marx, U.C., and Schenk, P.M. (2009). J. Exp. Bot. *60*, 1691-1702.

[7] Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L., and Seibert, M. (2000). Plant Physiol *122*, 127-136.

[8] Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L., et al. (2007). Science *318*, 245-250.

[9] Happe, T., and Kaminski, A. (2002). Eur J Biochem *269*, 1022-1032.

[10] Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L., and Seibert, M. (2003). Eur J Biochem *270*, 2750-2758.

[11] Hemschemeier, A., Fouchard, S., Cournac, L., Peltier, G., and Happe, T. (2008). Planta *227*, 397-407.

[12] Matthew, T., Zhou, W., Rupprecht, J., Lim, L., Thomas-Hall, S.R., Doebbe, A., Kruse, O., Hankamer, B., Marx, U.C., Smith, S.M., et al. (2009). J Biol Chem *284*, 23415-23425.

[13] Hemschemeier, A., Jacobs, J., and Happe, T. (2008). Eukaryot Cell *7*, 518-526.

[14] Mus, F., Dubini, A., Seibert, M., Posewitz, M.C., and Grossman, A.R. (2007). J Biol Chem *282*, 25475-25486.

[15] Grossman, A.R., Croft, M., Gladyshev, V.N., Merchant, S.S., Posewitz, M.C., Prochnik, S., and Spalding, M.H. (2007). Curr Opin Plant Biol *10*, 190-198.

[16] Gfeller, R.P., and Gibbs, M. (1984). Plant Physiol *75*, 212-218.

[17] Zorin, B., Lu, Y., Sizova, I., and Hegemann, P. (2008). Gene *432*, 91-96

[18] Rohr, J., Sarkar, N., Balenger, S., Jeong, B.-r., and Cerutti, H. (2004). The Plant Journal *40*, 611-621.

[19] Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. (2009). Plant J. *58*, 165-174

[20] Tao, Z., Wei, W., Xue, B., and Yijun, Q. (2009). The Plant Journal *58*, 157-164.

[21] Sizova, I., Fuhrmann, M., and Hegemann, P. (2001). Gene *277*, 221-229.