

Investigating the link between fermentation
and H₂ production in the green alga
Chlamydomonas reinhardtii

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Declaration

I hereby declare that this thesis, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of Imperial College London, represents my own work and has not been previously submitted to this or any other institute for any degree, diploma or other qualification.

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Abstract

During anoxia, the green alga *Chlamydomonas reinhardtii* activates various ill-defined fermentation pathways leading to the production of hydrogen, formate, acetate and ethanol. A bioinformatic survey was performed to look for *C. reinhardtii* genes potentially involved in fermentation that could compete for carbon and reductant, with a view to increasing H₂ yields by metabolic engineering. Antibodies were raised against the predicted NAD⁺-dependent D-lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC3), pyruvate-formate lyase (PFL1), pyruvate synthase/pyruvate:ferredoxin oxidoreductase (PFOR), pyruvate dehydrogenase E1 α components (PDC1 and PDC2) and a bifunctional acetaldehyde/alcohol dehydrogenase (ADH1). These antibodies were used to improve metabolic models by providing information about localisation, extending previous studies to include analysis of the cytoplasmic fraction and putative LDH. Additionally, it was demonstrated that the majority of enzymes are constitutively present, even during photoautotrophic growth where active lactate, formate and ethanol production pathways were found.

Artificial microRNA technology was used to knockdown *PFL1*, whose gene product catalyses the conversion of pyruvate to acetyl-CoA and formate. *PFL1*-knockdown had no impact upon H₂ evolution during sulphur-deprivation, but caused a decrease in the excretion of formate and ethanol, also revealing a pathway for production of 3-hydroxybutyrate, a metabolite not previously detected in this alga. Additionally amiRNA knockdown was used to provide direct evidence for a PDC3 catalysed pathway during sulphur-deprivation. *PDC3*-knockdown led to the re-direction of flux via PFL1 and a decrease in H₂ production rates. Inhibiting both the PFL1 and PDC3 catalysed pathways did not improve upon wild type yields of H₂ but instead re-directed carbon flux to the production of lactate and alanine.

Overall these data show amiRNA is an effective method for down regulating metabolic pathways controlled by PFL1 and PDC3. However if H₂ yields in *C. reinhardtii* are to be significantly improved by metabolic engineering it will likely require the knockdown of multiple fermentative pathways.

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List of abbreviations

3-HB - 3-hydroxybutyrate

ΔpH - trans-thylakoid proton gradient

A - absorbance

AAT1 - *C. reinhardtii* alanine amino transferase 1

ACAA - acetyl-CoA C-acyltransferase

AcAc-CoA - acetoacetate-CoA,

AcAcO⁻ - acetoacetate

AcALD - acetaldehyde

AcCoA - acetyl-CoA

ACK - acetate kinase

ACK1 - *C. reinhardtii* acetate kinase (plastid)

ACK2 - *C. reinhardtii* acetate kinase (mitochondrial)

AcO⁻ - acetate

ACS - acetyl-CoA synthetase

ADH - alcohol dehydrogenase

ADHE - *E. coli* bifunctional acetaldehyde/alcohol dehydrogenase

ADH1 - *C. reinhardtii* bifunctional acetaldehyde/alcohol dehydrogenase

ADH2 - putative *C. reinhardtii* bifunctional [Fe]-dependent alcohol dehydrogenase

ADH5 - putative *C. reinhardtii* [Fe]-dependent alcohol dehydrogenase

ADP - adenosine-5'-diphosphate

AGO - argonaute nuclease

AIB - anaerobic induction buffer

ALD5 - putative *C. reinhardtii* class II aldehyde dehydrogenase

ALDH - aldehyde dehydrogenase

amiRNA - artificial microRNA

AMP - ampicillin

AOX - alternative oxidase

APS - ammonium persulphate

ASP - aquatic species program

ATP - adenosine-5'-triphosphate

bbl - barrel of oil

BDH - 3-hydroxybutyrate dehydrogenase

BLAST - basic local alignment search tool

BN-AGE - blue native agarose gel electrophoresis

BN-PAGE - blue native polyacrylamide gel electrophoresis

bp - base pair

BSA - bovine serum albumin

CAD - cinnamyl alcohol dehydrogenase

CB - Coomassie blue

cDNA - complementary DNA

CEF - cyclic electron flow

Chl - chlorophyll

CHX - cycloheximide

CoA - coenzyme A

COXIIb - *C. reinhardtii* mitochondrial cytochrome c oxidase subunit II b

CSP - concentrated solar power

$\Delta\Delta C_T$ - cycle threshold method of relative quantification (relating to qRT-PCR)

C_T - cycle threshold (relating to qRT-PCR)

Cyt - cytoplasm

Cytb_{6f} - cytochrome b_{6f}

dNTP - deoxynucleotide triphosphate

D-LDHC - cytochrome-dependent D-lactate dehydrogenase

D1 - photosystem II protein encoded by *psbA*

D2 - photosystem II protein encoded by *psbD*

Da - Dalton

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea

df - degrees of freedom

DLA - dihydrolipoamide acetyltransferase (pyruvate dehydrogenase E2 component)

DLD - dihydrolipoamide dehydrogenase (pyruvate dehydrogenase E3 component)

DNA - deoxyribonucleic acid

ds - double stranded

E - Einstein

EDTA - ethylenediaminetetraacetic acid

EPR - electron paramagnetic resonance spectroscopy

EST - expressed sequence tag

EtOH - ethanol

Eq. - equation

ETR - enoyl thioester reductase

FCCP - carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone

Fdx - ferredoxin

FNR - ferredoxin-NADP⁺ reductase

FOR - formate

FPLC - fast protein liquid chromatography

FUM - fumarase

FMR - fumarate reductase

FNR - ferredoxin-NADP⁺ reductase

GC - gas chromatography

GC/MS - gas chromatography-mass spectrometry

gDNA - genomic DNA

GDP - gross domestic product

GOGAT - ferredoxin-dependent glutamate oxoglutarate aminotransferase

h - hour(s)

H-cluster - [FeFe]-hydrogenase [4Fe-4S] catalytic centre

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMG-CoA - hydroxymethylglutaryl-CoA

HMGL - hydroxymethylglutaryl-CoA lyase

HMGS - hydroxymethylglutaryl-CoA synthase

HP - sodium hypophosphite

HPLC - high pressure liquid chromatography

HR - homologous recombination

HSM - high salt media

HYDA - [FeFe]-hydrogenase

HYDA1 - *C. reinhardtii* [FeFe]-hydrogenase 1

HYDA2 - *C. reinhardtii* [FeFe]-hydrogenase 2

HYDEF - *C. reinhardtii* [FeFe]-hydrogenase assembly factor

HYDG - [FeFe]-hydrogenase assembly factor

J - Joule

KAN - kanamycin

kb - kilobases (DNA)

KD - knockdown
 KO - knockout
 LAC - lactate
 LB - lysogeny broth
 LDH - NAD⁺-dependent D-lactate dehydrogenase
 LDHc – cytochrome dependent D-lactate dehydrogenase
 LHCI - *C. reinhardtii* light harvesting complex I
 LHCII - *C. reinhardtii* light harvesting complex II
 Mit - mitochondrion
 min - minute(s)
 miRNA - microRNA
 MDH - malate dehydrogenase
 MDR - medium chain reductase
 MES - 2-(N-morpholino)ethanesulfonic acid
 MG - methylglyoxal
 MME - malic enzyme
 MOPS - 3-(N-morpholino)propanesulfonic acid
 mtPDH - mitochondrial pyruvate dehydrogenase complex
 NAD⁺ - oxidised nicotinamide adenine dinucleotide
 NADH - reduced nicotinamide adenine dinucleotide
 NADP⁺ - oxidised nicotinamide adenine dinucleotide phosphate
 NADPH - reduced nicotinamide adenine dinucleotide phosphate
 NDA2 - *C. reinhardtii* type II NADH dehydrogenase 2
 NHEJ - non-homologous end joining
 NII2 - ferredoxin-dependent nitrate reductase
 NMR - nuclear magnetic resonance spectroscopy
 NOSEY - nuclear Overhauser effect spectroscopy
 nt - nucleotide
 OD - optical density
 PAGE - polyacrylamide gel electrophoresis
 PAT - phosphate acetyltransferase
 PAT1 - *C. reinhardtii* phosphate acetyltransferase 1 (mitochondrial)
 PAT2 - *C. reinhardtii* phosphate acetyltransferase 1 (plastid)
 PBS - phosphate-buffered-saline

PBS-T - phosphate-buffered-saline-Tween20
 PC - plastocyanin
 PCR - polymerase chain reaction
 PDC1 - putative *C. reinhardtii* pyruvate dehydrogenase E1 alpha component (mitochondrial)
 PDC2 - putative *C. reinhardtii* pyruvate dehydrogenase E1 alpha component (plastid)
 PDC3 - *C. reinhardtii* pyruvate decarboxylase
 PDH - pyruvate dehydrogenase complex
 PDK - pyruvate dehydrogenase kinase
 PEPC - phosphoenol pyruvate carboxylase
 PET - photosynthetic electron transport chain
 PFL - pyruvate formate lyase
 PFL1 - *C. reinhardtii* pyruvate formate lyase
 PFL-AE - pyruvate formate lyase activating enzyme
 PFOR - pyruvate:ferredoxin oxidoreductase
 PGR5 - proton gradient regulator protein 5
 PGRL1 - proton gradient regulator like protein 1
 PHB - polyhydroxybutyrate
 plPDH - plastid pyruvate dehydrogenase complex
 PNS - purple non-sulphur (bacteria)
 PP - pyrophosphate
 PQ - plastoquinone
 PQQ - pyrroloquinoline quinone-dependent (enzyme)
 pre-miRNA - precursor miRNA
 PSI - photosystem I
 PSII - photosystem II
 PTOX - plastid terminal oxidase
 PV - photovoltaic
 PVDF - polyvinylidene fluoride
 PYR - pyruvate
 qRT-PCR - quantitative reverse transcription polymerase chain reaction
 RISC - RNA-induced silencing complex
 RNA - ribonucleic acid
 rpm - revolutions per minute
 RPL10a - RNA polymerase subunit L10a

RT - reverse transcription
 Rubisco - ribulose-1,5-bisphosphate carboxylase oxygenase
 s - seconds
 SAM - S-adenosylmethionine
 SIR - ferredoxin-dependent sulphate reductase
 ss - single stranded
 SDR - short chain dehydrogenase/reductase
 SDS - sodium dodecyl sulphate
 SE - standard error
 SOC - super optimized media catabolite repressed
sta6 - *C. reinhardtii* ADP-glucose pyrophosphorylase deficient mutant
stm6 - *C. reinhardtii* state transition mutant 6
t - student's t- test value
 TADH -tetrameric alcohol dehydrogenase
 TAG - triacylglyceride
 TAE - Tris-acetate-EDTA
 TAP - Tris-acetate-phosphate
 TCA - tricarboxylic acid cycle
 TCD - thermal conductivity detector
 TEMED - N-N-N'-N'-tetramethylethylenediamine
 TPP - thiamine pyrophosphate
 Tris - 2-amino-2-hydroxy-methylpropane-1,3-diol
 TSP - trimethylsilyl-2,2,3,3-tetradeuteropropionic acid
 U - unit(s) (referring to measure of enzyme activity)
 UTR - untranslated region
 UV - ultraviolet
 V - Volts
 v/v - volume per volume
 WCE - whole cell extract
 WT - wild type
 w/v - weight per volume
 XAS - X-ray absorption spectroscopy

Chapter 1: Introduction

1.1 Global context

The pressures of global warming, in addition to energy demand and security, require a shift away from the present carbon-based economy to more sustainable alternatives (Dorian et al., 2006; IPCC, 2007). The Stern Review into ‘The Economics of Climate Change’ estimated that an unchecked increase in CO₂ emissions could result in a global average temperature increase of up to 5°C, leading to rising sea levels, drought, famine and a decrease in GDP of more than 20%. To avoid the worst impacts of climate change it was estimated that a cut of up to 80% in carbon emissions relative to 1990 levels was required (Stern, 2006).

Not only does dependence on fossil fuels need to be cut for environmental reasons, but because the rising demand for energy from newly emerging economies, and a rapidly increasing global population coincide with the predicted peak and decline of oil production within the next decade (Hirsch et al., 2005).

Global energy consumption in 2008 was estimated at ~16 TW-yrs (5.1×10^{20} J) and by 2030 is predicted to rise by 44% to ~23 TW-yrs (7.4×10^{20} J) (IEA, 2010). Therefore if the ambitious target of 80% reduction in CO₂ emissions relative to 1990 levels is to be achieved it is vital that, along with increased energy efficiency, new carbon-neutral sources are developed.

In the UK the decline of North Sea oil reserves means the government is committed to find alternatives (DECC, 2009). Although nuclear power is considered to be the major provider in any move towards a low-carbon economy (BERR, 2008), like fossil fuels it suffers from problems of finite supplies and has the additional hazard of radioactive waste disposal (BERR, 2008). Therefore at best it can be considered as a mid-term solution, forming part of an energy mix along with renewable technologies such as wind, tidal, hydroelectric, and solar (DECC, 2009). It is hoped the development of renewable technologies will both increase the security of supply and aid economic development by promoting business and employment opportunities as the global community moves away from fossil fuels (DECC, 2009).

By far the most abundant renewable energy supply available on earth is in the form of solar radiation, which totals $\sim 5 \times 10^{24}$ J per annum (Miyamoto, 1997). This is

~11,000x more than the total energy demand in 2008, offering the biggest potential to fulfil future global energy demands (Schenk et al., 2008).

Solar energy can be used to generate electricity directly through photovoltaic panels (PV), or by conventional steam turbines in concentrated solar power plants (CSP) (IEA, 2008). However both approaches suffer from limitations: PVs have high capital costs (IEA, 2008) and often contain rare metals of finite supply (Buchert et al., 2009), whereas CSP is effective but is limited to regions of high solar radiation (IEA, 2008) and requires large supplies of water. Despite these drawbacks, the potential benefits have resulted in a rapid growth in the solar economy over the last decade with the help of government subsidies (IEA, 2008), and the EU has shown interest in large-scale projects such as the DESERTEC initiative to bring solar energy from the Sahara desert to Europe.

However, a major problem with many of the current renewable technologies is the emphasis on electricity generation when fuels accounted for ~67% of total energy consumption in 2005 (Schenk et al., 2008). Biofuels provide one solution, utilising solar radiation to fix CO₂ and generate stored energy in the form of biomass. Biofuels are combustible materials created from plants, animals, microorganisms and organic waste that can be used for transportation, heating and electricity generation (Bringezu et al., 2009). By using CO₂, biofuels have the potential to mitigate green house gas emission up to 80% compared to fossil fuels whilst providing substantial energy (Bringezu et al., 2009). This has encouraged governments to foster the development of biofuels which are classified as first, second or third-generation (Bringezu et al., 2009).

First generation biofuels include crops that are used as food or fodder such as wheat, corn, sugar beet, sugar cane and rapeseed, sunflower or palm-oil (IEA, 2008). However there are concerns over the sustainability of using these crops as this can cause competition for arable land, food and water resources, in addition to deforestation and decreased biodiversity as a result of the intensive farming methods required (Bringezu et al., 2009). Second generation biofuels are based on non-food sources (Eisentraut, 2010) such as waste biomass and special energy crops like *Miscanthus* (Dohleman and Long, 2009) thereby avoiding some criticisms levelled against first generation feedstocks, but if widely used will still raise questions of competition for land (Eisentraut, 2010) and a number of technical barriers still remain in the processing of lignocellulosic biomass (Sims et al., 2008).

An alternative to biomass crops is provided in so called third generation or advanced biofuels (Sims et al., 2008) which use algae for the production of bioenergy, overcoming many of the inherent problems faced by traditional biofuels.

1.2 Algal biofuels

Microalgae have a number of advantages over conventional feedstocks used for biofuel production with faster growth rates, better productivity as reviewed by (Waltz, 2009; Kruse and Hankamer, 2010), and the potential for cultivation in saline or waste water, in bioreactors, on marginal land, in lakes or in the sea (Mussgnug et al., 2010) avoiding questions of competition for land or with food production (Rittmann, 2008). The idea of using algae for fuel goes back as far as the 1950s (Meier, 1955) and was investigated extensively in the aftermath of the 1970s fuel crisis as part of the US National Renewable Energy Laboratory's Aquatic Species Program (ASP) (Sheehan et al., 1998).

The ASP ran from 1978–1996 and focused on the production of biodiesel from algae grown in open ponds, utilizing waste CO₂ from coal-fired power stations (Sheehan et al., 1998). A collection of over 300 microalgae species was gathered, consisting mainly of green algae and diatoms, and significant advances were made in lipid yields and carbon capture, with up to 90% sequestration of CO₂ from flue gases achieved through careful control of culturing conditions Sheehan et al. (1998). Despite the progress made, the ASP was closed in the 1990s due to budgetary pressures (Sheehan et al., 1998) and the low cost of crude oil (McGill, 2008).

However, with the current concerns over climate change (IPCC, 2007) and rising fuel prices there has been a renewed interest in algal biofuel production with over \$1 billion dollars invested between 2007–2009 (Mascarelli, 2009), coming from companies such as ExxonMobil and Chevron as well as the US military and the Bill Gates Foundation (Mascarelli, 2009).

Although there is renewed interest in the area, there are still concerns over costs, inflated claims of biomass yields (Mascarelli, 2009; Waltz, 2009), and problems in scaling up from lab-based experiments (Sheehan et al., 1998). However, moderate estimates predict algae oil could be produced with current technology at a cost of \$84 per barrel (bbl) (Huntley and Redalje, 2007), which could be further improved with better cultivation technology and utilisation of non-oil fractions (Stephens et al.,

2010). With the increasing cost of oil production due to limits on carbon emissions and depletion of readily available sources (Verbruggen and Al Marchohi, 2010), along with the greater risk of environmental disasters associated with its extraction and huge potential costs in cleanup and litigation (BP, 2010), it is conceivable oil prices in the future may rise up to and above the record \$147/bbl making algal biofuels a viable alternative (Stephens et al., 2010).

The economics of algal fuels could be further improved by coupling to a range of processes such salt or waste water treatment, carbon capture (Sheehan et al., 1998), human and animal food provision, production of health supplements, biopolymers, cosmetics, high value molecules (Spolaore et al., 2006; Stephens et al., 2010) or therapeutic proteins such as vaccines and antibodies (Rasala et al., 2010). Subsequently any discussion of feasibility should be considered in terms of the bio-refinery concept (Mussgnug et al., 2010), maximising algae's biotechnological potential and utilizing every available fraction of waste biomass.

In addition to biodiesel, algae can be used to produce a range of other fuels including bio-H₂, bio-ethanol or bio-methane (Schenk et al., 2008; Demirbas, 2010; Mussgnug et al., 2010), with H₂ considered a particularly attractive energy carrier as its combustion results only in the release of H₂O (Kruse et al., 2005b). Furthermore synergies exist between the production of various fuels, for example the process of sulphur deprivation used to stimulate hydrogen evolution in certain green algae (Melis et al., 2000) leads to increased lipid accumulation (Timmins et al., 2009b) and low sulphur content which aids biodiesel or bio-gas production (Mussgnug et al., 2010).

1.3 Hydrogen as a fuel source

A carbon-free, 'hydrogen economy' using the gas as a fuel, is proposed as a solution to many of the energy and ecological problems outlined in section 1.1 (Lattin and Utgikar, 2007; Marbán and Valdés-Solís, 2007; McDowall and Eames, 2007; Blanchette Jr, 2008). However unlike oil, gas or coal, hydrogen is a secondary energy carrier, and must therefore be generated from alternate sources. The majority of hydrogen is currently created from fossil fuels which would make any change in energy supply unsustainable (Bartels et al., 2010), but it can also be generated from renewable sources including hydroelectric, wave, wind, solar, geothermal, biomass (Bartels et al., 2010) or biological approaches, which can be classified as dark-

fermentative (Das, 2009; Guo et al., 2010; Hallenbeck and Ghosh, 2010), photo-fermentative (Akkerman et al., 2002; Kars and Gündüz, 2010) or bio-photolytic (Kruse et al., 2005b; Ghirardi et al., 2009; Bothe et al., 2010).

Various artificial, bio-inspired approaches for solar H₂ production have been envisioned. These include the immobilization of hydrogenase and an oxygen evolving photosystem II (PSII) complex onto electrodes, spatially separating oxygen evolution and hydrogen production (Esper et al., 2006), the construction of hybrid systems ranging from coupling of photosystem complexes to Pt nanoparticles for H₂ production (Grimme et al., 2008; Iwuchukwu et al., 2010; Lubner et al., 2010), attachment of bacterial reaction centre complexes to carbon nanotubes for electricity generation (Ham et al., 2010), to the coupling of hydrogenases to TiO₂ nanoparticles (Reisner et al., 2009) or the use of viral particles as scaffolds for the assembly and repair of photocatalytic nanostructures (Nam et al., 2010). Efforts have also been directed at biomimetic design, as reviewed by (Lewis and Nocera, 2006), creating novel water-splitting catalysts based on the [FeFe]-hydrogenase active site (Gloaguen et al., 2001; Ott et al., 2004; Kluwer et al., 2009). There have been significant advances in these fields over the past few years (Kanan and Nocera, 2008) however there are a number of disadvantages inherent in many of these processes including: the short life-span of many hybrid systems containing biological components that are rapidly damaged by light and during the chemistry involved in water oxidation; the requirement for expensive rare metals in the synthesis of many catalysts and the requirement for energy input in some catalytic systems.

The advantages of biological hydrogen production compared to competing technologies is that it is often performed at ambient temperatures and pressures making it less energy intensive than conventional chemical or electrochemical processes, it does not require rare metals and is well suited to small scale, decentralized energy production (Meher Kotay and Das, 2008).

1.4 Biological hydrogen production

H₂ metabolism, involving the reversible oxidation of molecular hydrogen is found in organisms ranging from Archea and Bacteria to Eukaryotes (Adams, 1990; Vignais et al., 2001; Ghirardi et al., 2007) either consuming hydrogen to drive metabolic processes, or evolving molecular H₂ to dissipate excess reducing pressure

(Melis et al., 2000; Böck et al., 2006; Hemschemeier et al., 2008a). The process by which microorganisms create hydrogen is determined by the type of enzyme employed, this can be a nitrogenase (Tamagnini et al., 2002) or a hydrogenase which can be grouped into one of three phylogenetically distinct classes, [NiFe], [FeFe] and [Fe] (Ghirardi et al., 2007; Vignais and Billoud, 2007), the latter of which was initially referred to as metal-free.

The potential to evolve hydrogen has generated significant interest in microbial fuel production which can be broadly classed into dark-fermentative or photo-biological approaches (Benemann, 1996; Nath and Das, 2004).

1.4.1 Dark-fermentative H₂ production

Dark-fermentative hydrogen production can be performed by strict and facultative anaerobic bacteria (Nandi and Sengupta, 1998; Davila-Vazquez et al., 2008) utilising organic substrates ranging from sugars such as cellulose and glucose to low-cost waste materials, including forest and agricultural residues or sewage (Claassen et al., 1999; Davila-Vazquez et al., 2008).

However, problems remain including the inhibition of H₂ evolution at partial pressure of 20% (van Niel et al., 2003), and the redirecting of metabolism to the production of unfavourable products such as lactate, alanine, butyrate, propionate, ethanol or butanol, resulting in low yields (Claassen et al., 1999; Claassen et al., 2010).

Significant progress has been made in increasing substrate to hydrogen conversion efficiencies, through optimisation of culturing conditions (Kraemer and Bagley, 2007; Wang and Wan, 2009; Antonopoulou et al., 2010), inoculum pre-treatment (Wang and Wan, 2008), and genetic engineering of fermentative pathways (Vardar-Schara et al., 2008; Hallenbeck and Ghosh, 2010). Much research has focused on *E. coli* with efforts to redirect carbon flux to a formate hydrogen lyase (FHL) through knockout of competing fermentative pathways (Yoshida et al., 2006; Maeda et al., 2007) and knockout of uptake hydrogenases (Yoshida et al., 2006; Turcot et al., 2008), pushing efficiencies close to the theoretical maximum for this organism of 2 mols H₂ per mol of glucose metabolised (Hallenbeck and Ghosh, 2010).

Although dependent on oil price, previous estimates predicted that a 60–80% substrate to hydrogen conversion efficiencies would be required for the process to

become economically viable (Benemann, 1996). The maximum theoretical yield of hydrogen is 6 mols H_2 per mol of hexose. However maximum achievable yields are lower due to the ‘fermentation barrier’ resulting from the production of waste products, and ultimately yields depend on the process employed (Hallenbeck, 2005);

- (1) Enteric bacteria such as *E. coli* use formate to produce H_2 via the formate hydrogenase lyase complex (Axley et al., 1990), which contains a [NiFe]-hydrogenase, and can produce 2 moles of H_2 per mol of glucose (16.66% efficiency). This is as one molecule of glucose yields two molecules of pyruvate, which are then converted into two molecules of formate and acetyl-CoA by a pyruvate formate lyase (PFL). Two molecules of formate can then be broken down to give two molecules of H_2 . (Hallenbeck, 2005)
- (2) Alternatively in bacteria that use reduced ferredoxin to catalyse the production of H_2 by a [FeFe]-hydrogenase, the 2 molecules of pyruvate produced from glucose can be broken down via the action of a pyruvate:ferredoxin oxidoreductase (PFOR) generating reduced ferredoxin, which is then used to convert four protons to 2 H_2 . Theoretically two additional molecules of H_2 can be produced from the activity of NADH:ferredoxin oxidoreductase, which regenerates NAD^+ reduced by glycolysis, producing reduced ferredoxin to supply the hydrogenase, resulting in a total of 4 moles of H_2 per mole of glucose (33%) (Hallenbeck, 2005).

Various approaches have been proposed to increase the efficiency of substrate utilisation above the fermentation barrier including the use of microbial fuel cells to generate electricity (Logan, 2004) or the coupling of fermentative H_2 and methane production (Chu et al., 2008; Zhu et al., 2008; Wang and Zhao, 2009; Park et al., 2010). Additionally research has begun to focus on two-step H_2 production processes incorporating a second photo-fermentative hydrogen production phase (Nath et al., 2008; Özgür et al., 2010), pushing theoretically achievable molar conversion efficiencies up to 75% (Claassen et al., 2010).

1.4.2 Photo-fermentative H₂ production

Photo-fermentative H₂ production utilises reduced ferredoxin and ATP generated by anoxygenic photosynthesis to produce molecular H₂ via a nitrogenase in the absence of N₂, with electrons coming from the oxidation of organic substrates (Bulen et al., 1965; Liang and Burris, 1988). This process is performed by purple non-sulphur (PNS) bacteria (Akkerman et al., 2002; Kars and Gündüz, 2010). Research has focused on the species of *Rhodobacter*, *Rhodospirillum* and *Rhodopseudomonas* with advancements made through targeting uptake hydrogenases (Ooshima et al., 1998; Kars et al., 2008), inhibiting synthesis of polyhydroxyalkanoic acid storage compounds (Kim et al., 2006) and increasing the efficiency of light utilization through the reductions in pigment content (Vasilyeva et al., 1999; Kondo et al., 2002).

Combining dark and photo-fermentative hydrogen production represents a significant improvement to the overall process, but the requirement for organic substrates and the fact that PNS bacteria can only utilise about 25% of the solar spectrum, has led to proposals that a third bio-photolytic step should be included using green algae or cyanobacteria to increase the efficiency of light capture (Melis and Melnicki, 2006; Ghirardi et al., 2009).

1.4.3 Bio-photolytic hydrogen production

Bio-photolytic H₂ production is the process whereby the light-driven oxidation of water by the Photosystem II (PSII) complex of oxygenic photosynthesis (Eq. 1) is coupled to the enzymatic reduction of protons to H₂ (Eq. 2), to give the net reaction shown in Eq. 3.



Photo-biological hydrogen production seeks to harness solar rather than chemical energy for the creation of bio-H₂ (Melis et al., 2000), and can be performed by a number of different methods; in green algae by a ferredoxin-dependent [FeFe]-hydrogenase (Happe and Kaminski, 2002; Forestier et al., 2003), in unicellular cyanobacteria via a NADPH dependent [NiFe]-hydrogenase (Volbeda et al., 1995)

and in nitrogen-fixing cyanobacteria, in the absence of N_2 via a nitrogenase (Benemann, 1996; Nath and Das, 2004; Bothe et al., 2010).

By utilising photosynthesis, photo-biological approaches have a number of potential advantages over dark-fermentative systems absolving the need for additional carbon substrates and reducing overall CO_2 emissions.

1.5 H_2 production in green algae

Bio-photolytic approaches focus on using algae, as reviewed by (Hemschemeier et al., 2009; Ghysels and Franck, 2010; Kruse and Hankamer, 2010), or cyanobacteria, as reviewed by (Lopes Pinto et al., 2002; Sakurai and Masukawa, 2007; Angermayr et al., 2009; Bothe et al., 2010), for production of hydrogen. The presence of H_2 metabolism in green algae has been known since the 1930s (Gaffron, 1939); incubating cultures under dark anaerobic conditions facilitates hydrogenase expression and low levels of H_2 production, and upon illumination a rapid burst of hydrogen evolution can be observed which lasts for several minutes until coming to a stop (Gaffron and Rubin, 1942). Hydrogen production in algae occurs through the transfer of electrons from the photosynthetic electron transport chain (PET) via ferredoxin to an [FeFe]-hydrogenase (Florin et al., 2001) in a process thought to be limited by supply of reductant (Kruse et al., 2005a). Electrons are believed to come from a combination of photosystem II (PSII)-dependent (Greenbaum et al., 1983), and PSII-independent pathways (Desplats et al., 2008; Jans et al., 2008; Chochois et al., 2009; Chochois et al., 2010) (Figure 1.1).

1.5.1 PSII-dependent pathway

In this process PSII catalyses the photolysis of water, producing O_2 and releasing electrons into the photosynthetic electron transport (PET) chain, ultimately generating reduced ferredoxin that can be used by hydrogenase to reduce protons to hydrogen (Figure 1.1) (Greenbaum et al., 1983). This process offers the maximum theoretical efficiency of converting solar to H_2 energy (Kruse et al., 2005a), but because [FeFe]-hydrogenases are irreversibly inactivated by O_2 (Stripp et al., 2009b), as soon as cells begin to produce oxygen at high rates, hydrogen evolution rapidly stops (Ghirardi et al., 1997) meaning it is not currently a viable process under standard conditions.

1.5.2 PSII-independent pathways

Starch reserves accumulated through photosynthetic activity can undergo autofermentation to produce NADH and pyruvate, both of which can be oxidised to provide electrons for hydrogen production. In the case of pyruvate, a chloroplast-located pyruvate:ferredoxin oxidoreductase (PFOR) is likely to be involved in reducing ferredoxin in the dark, and possibly the light, with the concomitant production of CO₂ and acetyl-CoA (Atteia et al., 2006; Mus et al., 2007) (Figure 1.1). Oxidation of NAD(P)H occurs in the light via NDA2, a thylakoid-associated type II NADH dehydrogenase (Desplats et al., 2008; Jans et al., 2008) which feeds electrons into the PET at the point of the plastoquinone (PQ) pool (Figure 1.1).

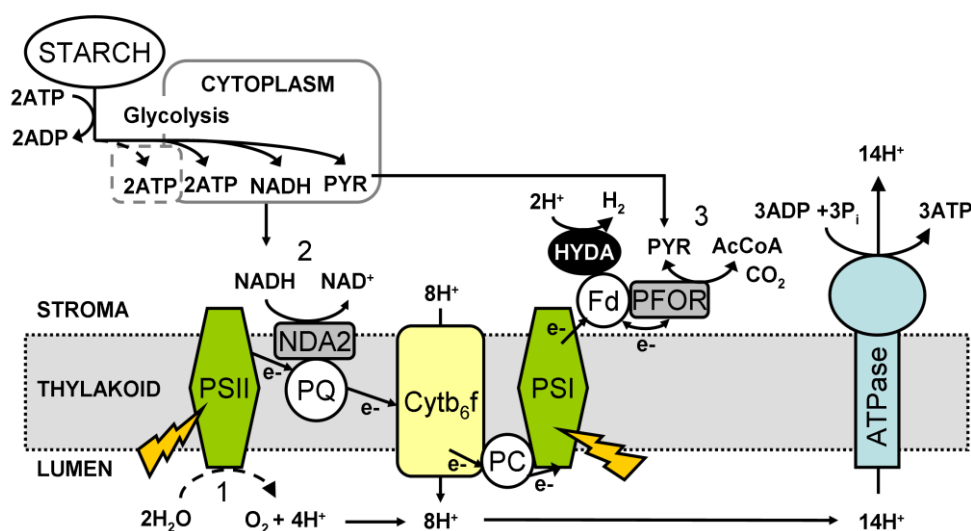


Figure 1.1: Schematic representation of the PET showing potential sources of reductant for hydrogen production in *C. reinhardtii*.

Electrons are provided from (1) the PSII-dependent pathway (2) the PSII-independent pathway (via a type II NADH dehydrogenase) (3) PSII-independent pathway (fermentation). Abbreviations; Cytb₆f; cytochrome *b*₆*f* complex, Fd; Ferredoxin, HYDA; [FeFe]-hydrogenase, NDA2; type II NADH dehydrogenase, PFOR; pyruvate:ferredoxin oxidoreductase, PSI; photosystem I, PSII; photosystem II, PC; plastocyanin, PQ; plastoquinone. For simplicity the Q cycle at the Cyt *b*₆*f* complex is not shown.

Extensive screening of culture collections has revealed that not all green algae possess the ability to evolve hydrogen (Healey, 1970; Stuart and Gaffron, 1972; Ben-Amotz et al., 1975; Greenbaum et al., 1983; Brand et al., 1989; Skjånes et al., 2008; Meuser et al., 2009; Timmins et al., 2009a), and the precise contribution of PSII-dependent and independent pathways is not consistent. For example the addition of

PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) has been found to repress hydrogen production to different degrees in different strains (Healey, 1970; Stuart and Gaffron, 1972), and acetate has been shown to differentially stimulate hydrogen evolution (Healey, 1970).

The model green alga *Chlamydomonas reinhardtii* is currently the main organism for studying algal H₂ production (Timmins et al., 2009a). As well as having one of the highest recorded rates of algal H₂ production (Timmins et al., 2009a), *C. reinhardtii* has a number of benefits for study: firstly strains exist as either mating type positive (mt+), or mating type minus (mt-), which together are capable of sexual reproduction, allowing for classic genetic analysis. Secondly fully sequenced and transformable mitochondrial, chloroplast and nuclear genomes (Boynton et al., 1988; Mayfield and Kindle, 1990; Randolph-Anderson et al., 1993; Vahrenholz et al., 1993; Maul et al., 2002; Merchant et al., 2007), microarrays (Nguyen et al., 2008; Gonzalez-Ballester et al., 2010), a large collection of expressed sequence tags (Asamizu et al., 1999) and mutants are also available to facilitate molecular genetic analysis.

A typical *C. reinhardtii* cell is ~10 µm in diameter and possesses a single large chloroplast in addition to a nucleus, multiple mitochondria and two flagella for motility and mating (Merchant et al., 2007) (Figure 1.2).

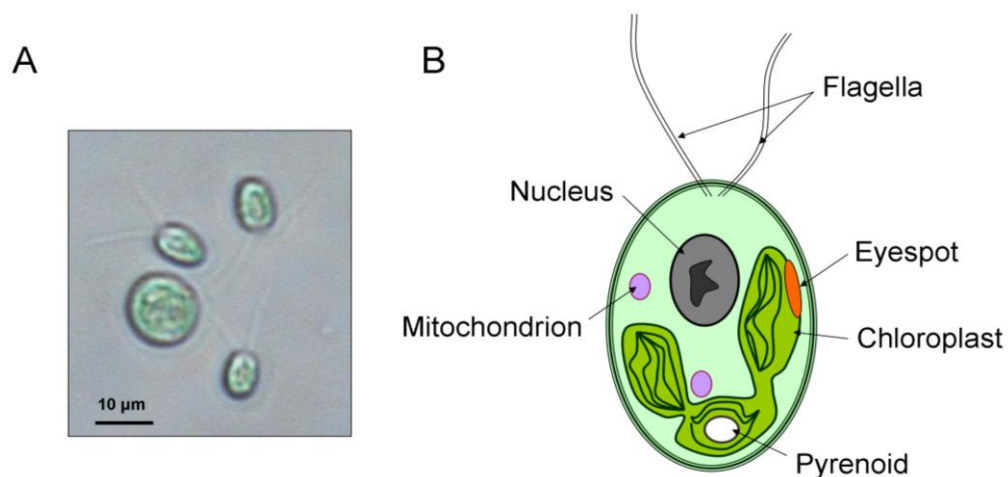


Figure 1.2: The *C. reinhardtii* cell

(A) Light microscope photograph showing four *C. reinhardtii* cells with flagella (B) schematic representation of *C. reinhardtii* cell adapted from Merchant et. al. (2007).

1.5.3 H₂ production during sulphur-deprivation

The problem of hydrogenase sensitivity to O₂ was overcome by Melis and co-workers (Melis et al., 2000) who separated growth and H₂ production stages in the green alga *C. reinhardtii* through sulphur starvation, and managed to achieve sustained hydrogen evolution for several days (Melis et al., 2000). *C. reinhardtii*'s response to sulphur-deprivation can be described as passing through 5 phases: aerobic, oxygen consumption, anaerobic, hydrogen production and hydrogen termination (Kosourov et al., 2002).

The aerobic and O₂ consumption phases last between 20–30 hrs (Kosourov et al., 2002), but are strain (Chochois et al., 2009; Chochois et al., 2010) and condition dependent (Kosourov et al., 2002). Initially cells continue to evolve oxygen (Ghirardi et al., 2000; Melis et al., 2000; Kosourov et al., 2002), and accumulate energy reserves in the form of starch (Zhang et al., 2002; Timmins et al., 2009b) and triacylglycerides (TAGs) (Timmins et al., 2009b), which is a well characterised response to nutrient limitation and can also be seen during nitrogen starvation (Ball et al., 1990) and salt stress (Siaut et al., 2011). These changes are followed by the rapid degradation of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) suggesting a decline in CO₂ fixation rates (Zhang et al., 2002; Zhang and Melis, 2002) and cultures enter a phase of oxygen consumption, as by depriving cells of sulphur, synthesis of the PSII reaction centre protein D1 is impaired, reducing the cell's ability to repair PSII (Wykoff et al., 1998; Nixon et al., 2010). Taken together, the lack of Calvin-Benson cycle activity leading to the over reduction of the PQ pool (Antal et al., 2003), and impaired D1 turnover, cause a decrease in the number of active PSII centres to about 5–10% of normal levels (Zhang et al., 2002), as determined by chlorophyll fluorescence measurements (Zhang et al., 2002; Antal et al., 2003) and the rate of photosynthetic oxygen evolution (Wykoff et al., 1998). Initially respiratory activity in the mitochondrion remains largely unimpaired (Zhang et al., 2002) causing the rate of photosynthesis to drop below respiration and sealed cultures to go anaerobic (Melis et al., 2000). These changes are accompanied by a decrease in photorespiratory and tricarboxylic acid (TCA) cycle intermediates (Timmins et al., 2009b), as O₂ and CO₂ become limiting, and a decline in chlorophyll levels as cells seek to limit photodamage (Melis et al., 2000; Zhang and Melis, 2002).

A brief lag period ensues during which cells consume dissolved oxygen and induce hydrogenase expression, followed by hydrogen evolution which lasts several days (Melis et al., 2000). In the absence of CO₂ fixation, H₂ production acts to allow continued photosynthetic electron transport for the generation of ATP by photophosphorylation albeit at a reduced rate (Melis, 2007). Additionally limited O₂ availability causes reduced rates of oxidative phosphorylation in the mitochondria, resultantly glycolysis and anaerobic fermentation also become major pathways for ATP formation (Timmins et al., 2009b). This suggestion is supported by the decrease in starch concentration during the hydrogen production phase (Zhang et al., 2002) and the excretion of formate (Tsygankov et al., 2002; Hemschemeier et al., 2008b; Timmins et al., 2009b) and ethanol (Kosourov et al., 2003; Hemschemeier et al., 2008b; Timmins et al., 2009b) along with minor amounts of succinate and amino acids with the exception of glutamate (Timmins et al., 2009b), which allows for the re-oxidation of NADH formed by glycolysis. The amino acids excreted are found to originate at least partly from *de novo* synthesis (Timmins et al., 2009b), which coupled to the uptake of ammonium via the glutamine synthetase/glutamate synthase cycle also oxidizes one molecule of NADPH.

In the final period hydrogen production gradually declines to a stop despite the continuing presence of energy reserves in the form of starch, TAG and acetate, which could be due to the toxic nature of the accumulated metabolites or as a result of the long term consequences of sulphur-deprivation (Timmins et al., 2009b).

1.5.4 Contribution of PSII to H₂ production during sulphur-deprivation

In *C. reinhardtii* the precise contribution of the PSII-dependent and independent pathways to hydrogen production is still unclear. A range of values have been proposed (Kosourov et al., 2003; Antal et al., 2009; Chochois et al., 2009) with the most recent suggestion of up to 90% of electrons coming from residual water splitting (Chochois et al., 2009; Chochois et al., 2010), but this value is likely to be strain and condition dependent, which could provide an explanation for the differences reported in the literature (Chochois et al., 2009).

The importance of PSII activity for the process was first discerned from experiments using D1 and D2 deficient mutants which were unable to produce hydrogen (Zhang et al., 2002; Hemschemeier et al., 2008a). Similarly addition of

DCMU at the beginning of sulphur-deprivation led to an inhibition of H₂ evolution (Hemschemeier et al., 2008a). However, if PSII inhibition was delayed until after the aerobic and oxygen consumption phases, hydrogen evolution was observed, although at a vastly reduced level (~10% uninhibited) (Chochois et al., 2009) indicating that, as with light-induced hydrogen production, there is a PSII-independent source of reductant for the hydrogenase. This indirect pathway is dependent on PSII activity during the initial stages of sulphur-deprivation suggesting it is based on degradation of the energy reserves formed in the aerobic phase (Hemschemeier et al., 2008a; Chochois et al., 2009). In addition the indirect pathway was found to be absent in starch-deficient mutants indicating carbohydrate reserves are the sole source of reductant for PSII-independent H₂ production and not protein or TAGs (Hemschemeier et al., 2008a). Further evidence for this theory came from mutants affected in starch catabolism, which showed a general decrease in the amount of PSII-independent H₂ production, and those with a reduced rate of starch degradation which showed delayed H₂ evolution (Chochois et al., 2010).

1.5.5 Role of starch and acetate during H₂ production

NADH produced from starch breakdown appears to play a dual role in hydrogen production: it is oxidized via the mitochondrial respiratory chain, to keep oxygen levels sufficiently low to allow expression of active hydrogenase for the PSII-dependent pathway (Melis, 2007) and it feeds electrons into the PET chain in the indirect pathway (Bamberger et al., 1982; Melis, 2007). However, experiments using the ADP-glucose pyrophosphorylase deficient mutant, *sta6*, which is unable to accumulate starch, found wild-type levels of H₂ evolution during sulphur-deprivation (Chochois et al., 2009). This result suggests that starch is dispensable for PSII-dependent hydrogen production and can be explained by the fact that, unlike *wt* cultures, *sta6* consumes acetate during the hydrogen production phase, which could act as a replacement for carbohydrate in maintaining respiration (Chochois et al., 2009).

Similarly, although acetate is usually included in the growth medium (Melis et al., 2000), it is dispensable for H₂ production when cultures are grown under a defined light regime, with CO₂ bubbling to promote starch accumulation (Tsygankov et al., 2006; Kosourov et al., 2007; Tolstygina et al., 2009). This result suggests the role of

starch or acetate in the maintenance of respiration is interchangeable, although CO₂ bubbling and acetate are required for maximum H₂ yields (Kosourov et al., 2007).

1.6 Improving *C. reinhardtii* H₂ production ability

It has been calculated that, if coupled to the production of high-value products, bio-photolytic energy conversion efficiencies of around 5% will be required for algal bio-H₂ production to become economically viable (Kruse et al., 2005b). Currently achievable light to hydrogen conversion efficiencies, as demonstrated in a laboratory environment, are in the region of 1% post-sulphur-deprivation for *C. reinhardtii* mutant *stm6* (Kruse et al., 2005b). Therefore, as with dark and photo-fermentative processes (Hallenbeck and Ghosh, 2010), further increases in efficiency are still required.

1.6.1 Engineering an O₂-tolerant hydrogenase

Maximum efficiencies are achieved through direct photolysis, therefore the ultimate goal is to engineer an O₂-tolerant hydrogenase. There are a few known examples found in nature of the [NiFe] type (Burgdorf et al., 2005), but they tend to favour H₂ uptake (Maroti et al., 2009). The vast diversity of hydrogenase sequences remains under-represented in databases (Beer et al., 2009), and there may yet be a suitable sequence present in nature. The establishment of metagenomics has allowed the identification of novel hydrogenases for organisms in the environment including analysis of termite gut microbiota (Warnecke et al., 2007) and marine organisms through the Craig Venter Institute's Global Ocean Sequencing program (Rusch et al., 2007; Maroti et al., 2009), but as yet the oxygen tolerance of these enzymes is unknown.

C. reinhardtii contains two highly similar, differentially regulated, oxygen-sensitive [FeFe]-hydrogenases that are ~49 kDa in size - HYDA1 and HYDA2 (Forestier et al., 2003), of which only HYDA1 is thought to play a role in H₂ production (Godman et al., 2010). The assembly and proper functioning of [FeFe]-hydrogenases is an O₂-sensitive process dependent on accessory factors HydE, HydF and HydG (Posewitz et al., 2004a; Nicolet et al., 2010). Resultantly any modified hydrogenase would also require a means of assembly in the presence of oxygen. The active site H-cluster is a [4Fe-4S] cubane linked to a 2Fe sub-cluster via cysteine

thiolate (Adams, 1990; Peters, 1999). The mechanism of assembly is not fully characterised, but HydE and HydG belong to the radical S-adenosylmethionine (SAM) class of enzymes involved in radical based catalysis (Sofia et al., 2001) and are proposed to play a role in H-cluster synthesis (Rubach et al., 2005) while HydF possesses GTPase activity (Brazzolotto et al., 2006) and is thought to act as a scaffold for assembly (McGlynn et al., 2008).

The *C. reinhardtii* [FeFe]-hydrogenase has been expressed heterologously in *E. coli* using the *Clostridium acetobutylicum* hydrogenase assembly factors genes hydE, hydF and hydG (King et al., 2006) opening up the possibility for high-throughput random mutagenesis screening. Examples of research on the algal hydrogenase include random gene shuffling (Nagy et al., 2007) and directed evolution (Stapleton and Swartz, 2010), the latter of which managed to identify a version of *C. reinhardtii* HydA1 with a 4-fold increase in catalytic activity but none yet with improved O₂ tolerance.

In contrast to high-throughput random mutagenesis approaches, it may be possible to use existing structural and mechanistic information to mutate specific amino-acid residues within the hydrogenase structure to improve catalytic properties. For example, it has been suggested that narrowing gas channels leading to the active site (Posewitz et al., 2009) or potentially modifying the protein environment around the H-cluster itself (Stripp et al., 2009b) may be means of preventing inhibition by O₂. There are now a number of X-ray crystal structures of [FeFe]-hydrogenases (Peters et al., 1998; Cohen et al., 2005; Shima et al., 2008) including the partially assembled *C. reinhardtii* enzyme (Mulder et al., 2010). A homology model of the *C. reinhardtii* HYDA2 has been constructed (Chang et al., 2007) and HYDA1 has been isolated and characterised by electron paramagnetic resonance spectroscopy (EPR) (Kamp et al., 2008) as well as by X-ray absorption spectroscopy (XAS) which has revealed the structure of the active site H-cluster (Stripp et al., 2009a), and the means by which O₂ inhibition occurs (Stripp et al., 2009b). However, these data have not yet been successfully applied to making beneficial mutations to reduce the O₂-sensitivity of the algal hydrogenase.

1.6.2 Hydrogenase-ferredoxin interactions

Ferredoxins play a central role in allocating high energy electrons in the chloroplast and are involved in distributing electrons for CO₂ fixation (Kitayama et al., 1994), nitrite (Fernández et al., 1989; Hirasawa et al., 2009) and sulfite reduction (Gonzalez-Ballester et al., 2009), glutamate synthesis (García-Sánchez et al., 2000), cyclic electron flow (CEF) (Finazzi et al., 2002) and reduction of thioredoxins for regulation of biosynthetic pathways (Lemaire et al., 2003), in addition to the role in hydrogen production.

C. reinhardtii encodes six differentially regulated [Fe₂S₂] ferredoxins: PetF (the dominant Fd reduced in the light reactions) and Fdx2-Fdx5 (Mus et al., 2007; Terauchi et al., 2009; Winkler et al., 2010), of which Fdx5 was found to be induced by anoxia as well as copper stress (Mus et al., 2007; Jacobs et al., 2009; Terauchi et al., 2009; Lambertz et al., 2010), but which is unable to donate electrons to HYDA1 (Jacobs et al., 2009). Reduced PetF is thought likely to be the substrate for H₂ production *in vivo* although the Fd specificity of the hydrogenases, HydA1 and HydA2, has yet to be fully evaluated (Happe and Naber, 1993; Lambertz et al., 2010).

In principle, hydrogen production could be enhanced by increasing the specificity of electron transfer from the ferredoxin to the hydrogenase over other competing pathways. Progress has been made in identifying potentially important residues involved in the interaction between Fd and the hydrogenase (Chang et al., 2007; Long et al., 2008; Winkler et al., 2009; Winkler et al., 2010), which opens up the possibility of manipulating the affinity of binding and kinetics of electron transfer.

1.6.3 Dissipating the thylakoid proton gradient

Disrupting the trans-thylakoid proton gradient (Δ pH) through heterologous expression of a proton channel in the thylakoid membrane is another potential way of increasing supply of reductant to the hydrogenase. Δ pH is thought to limit H₂ production by causing a decrease in proton concentration in the stroma and a reduction in electron transport at the point of the cytochrome *b₆f* complex (Kramer and Crofts, 1993; Antal et al., 2009). The proton gradient uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) does indeed stimulate H₂ evolution (Cournac et al., 2002; Lee and Greenbaum, 2003; Antal et al., 2009); however addition prior to anaerobic induction abolished H₂ase activity *in vivo* (Chochois et al.,

2009) suggesting that the proton gradient is also important for hydrogenase expression. Therefore although a potential means of increasing yields, any attempts at decoupling electron transport from ΔpH , such as by integration of a proton channel into the thylakoid membrane (Lee and Greenbaum, 2003), will likely require an inducible element to allow for hydrogenase expression.

1.6.4 Modifying the photosynthetic apparatus

1.6.4.1 Decreasing the size of the light-harvesting antennae

When exposed to high irradiances of light, *C. reinhardtii* dissipates up to 80% of absorbed photons as either heat or fluorescence to protect against photodamage (Polle et al., 2002), in a process known as feedback de-excitation (Holt et al., 2004). This photo-protective mechanism automatically reduces the energy conversion efficiency of PET. Decreasing the light-harvesting capacity of the photosystems reduces excess light absorption by individual cells, thereby increasing photon and energy conversion efficiencies, as well as light penetration in a culture (Melis et al., 1998; Polle et al., 2002; Mussgnug et al., 2007).

Mutants with a truncated light-harvesting antennae (such as the *tla* mutants) have been identified on account of their pale phenotype (Polle et al., 2002; Berberoglu et al., 2008; Melis, 2009; Mitra and Melis, 2010) or through reverse genetic approaches, such as by down regulation of light-harvesting complexes through RNAi (Mussgnug et al., 2007) or over-expression of the RNA-binding protein, NAB1, known to regulate translation of light-harvesting subunits (Beckmann et al., 2009).

Preliminary results have found that the *tla1* mutant could not establish anaerobiosis after sulphur-deprivation in a bioreactor (Kosourov et al., 2011). However, recent results indicate that when immobilized in an alginate film and deprived of both sulphur and phosphorous, H_2 production rates are increased 4-6 times relative to the parental strain at light intensities ranging from 285–350 $\mu E\ m^{-2}\ s^{-1}$, demonstrating the feasibility of this strategy for improving yields under the optimised conditions (Kosourov et al., 2011).

1.6.4.2 Altering photosynthetic rates

The down regulation of PSII activity is seen as a key step to inducing H_2 production, and approaches have been taken to reduce rates of oxygen evolution in an

attempt to decrease internal O₂ levels and shorten the lag phase between growth and H₂ production phases. Mutations in the D-E loop of the Q_B-binding site of D1, which is responsible for optimal electron transport from PSII (Lardans et al., 1998; Johanningmeier et al., 2000; Rose et al., 2008), resulted in substantial increases in hydrogen evolution compared to wild type cultures (Torzillo et al., 2009; Faraloni and Torzillo, 2010). The precise reason for the increase is unclear, but is likely the result of a number of contributing factors including a reduced chlorophyll content, lower levels of dissolved O₂ and an extended H₂ production phase, but mutants also displayed a decrease in photosynthesis to respiration ratios and increased sensitivity to photoinhibition (Faraloni and Torzillo, 2010).

However not all photosynthetic mutations are likely to be beneficial. For example, directly targeting photosynthetic activity appeared to cause a decline in overall hydrogen yields (Makarova et al., 2007), possibly as a reduced water-splitting activity affected starch accumulation in the aerobic phase, and may represent a problem for this approach in certain cases. The difference between these two sets of results likely arises from the type of mutation involved, whereas the D1-R323 mutants were thought to directly affect the functioning of the oxygen evolving complex as a means of down regulating photosynthesis (Makarova et al., 2007), the Q_B-site mutations resulted in multiple effects including an increased sensitivity to photoinhibition but left the maximum quantum yield of PSII (F_v/F_m) largely unaffected (Torzillo et al., 2009; Faraloni and Torzillo, 2010).

1.6.5 Optimising culturing conditions

Under standard conditions, sulphur-deprived cultures of *C. reinhardtii* CC-124 produce H₂ at maximum specific rates of ~4-6 $\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ (Kosourov et al., 2002). Since its establishment in 2000, experimental parameters have been optimised, although mainly in isolation, through synchronization of cultures (Tsygankov et al., 2002), re-addition of low levels of sulphate (Kosourov et al., 2002), optimization of pH (Kosourov et al., 2003), light intensity (Laurinavichene et al., 2004), medium composition (Jo et al., 2006) and growth conditions (Kosourov et al., 2007), with maximum rates of up to 9.4 $\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ reported (Kosourov et al., 2003). The duration of hydrogen evolution has also been extended through cycling between sulphur replete and deprived media (Ghirardi et al., 2000; Laurinavichene et al., 2006;

Kosourov and Seibert, 2009), the use of a two-staged bioreactor setup for continuous production (Fedorov et al., 2005) and immobilisation of cells in an alginate film (Laurinavichene et al., 2006; Kosourov and Seibert, 2009). Immobilising cells also provided a higher tolerance to O₂, greater achievable cell densities and better light utilization, leading to maximum recorded rates of H₂ production at around 12.5 $\mu\text{mol mg Chl}^{-1} \text{ h}^{-1}$ and an increase in light energy conversion efficiency from 0.24% in liquid cultures to ~1% (Kosourov and Seibert, 2009).

1.6.5.2 Light conversion efficiencies

C. reinhardtii also only absorbs part of the solar spectrum which further reduces the light to H₂ conversion efficiency. Therefore a two step process integrating the use of purple sulphur bacteria to capture the near infra-red region of sunlight has been tested (Melis and Melnicki, 2006). This approach resulted in efficiency gains and led to the proposal that a third step incorporating anaerobic fermentative bacteria could be included to further improve the process (Melis and Melnicki, 2006). Similarly a more radical re-engineering of the photosynthetic apparatus has been proposed to further increase the amount of solar spectrum absorbed for increased photosynthetic activity, replacing PSI entirely with a novel reaction centre engineered to contain bacteriochlorophyll *b* and chlorophyll *d* for absorption of low energy red photons as reviewed by (Blankenship et al., 2011).

1.6.6 Improving upon sulphur-deprivation as a means of inducing H₂ production

1.6.6.1 Controlling sulphate uptake

As mentioned in section 1.5.3 sulphur-deprivation is the commonly used process for down regulation of photosynthetic activity, but centrifugation, as used in laboratory protocols as a means of cycling cultures from sulphur replete to sulphur deplete media, is unfeasible for scaleup. One method to circumvent this problem is to restrict sulphate transport into the chloroplast. This was done by targeting the *C. reinhardtii* chloroplast sulphate transporter *SULP* using RNAi, resulting in the establishment of anaerobiosis in stationary phase cultures triggering H₂ production (Chen et al., 2005). Alternatively optimization of the sulphate content in the growth

media would allow cells to reach optimal density for H₂ production at the point when sufficient sulphur is consumed during growth to trigger the starvation response.

1.6.6.2 Regulation of PSII activity through inducible promoters

The disadvantages of sulphur-deprivation are that it ultimately results in cell death, is only suitable as a batch process and requires continuous illumination over several days for maximum H₂ production (Oncel and Sukan, 2011). Therefore, controllable expression of PSII could be used to reduce oxygen evolution to a rate below respiration and drive cultures anaerobic. Surzycki and colleagues used the copper-sensitive cytochrome *c*₆ promoter to repress PSII assembly (Surzycki et al., 2007), thereby inducing anoxia and hydrogen production when cells were transferred from copper free to copper replete media. However it was also found that the promoter used was stimulated by anoxia, even in the presence of copper, and resultantly aerobic conditions were re-established shortly after the initiation of H₂ evolution, bringing the process to a stop (Surzycki et al., 2007). Despite this setback, this approach did demonstrate that controlling photosynthesis through inducible promoters is an effective method of stimulating H₂ evolution.

1.6.6.3 Mutants with altered rates of photosynthesis to respiration

A different approach could be taken to identify mutants with altered rates of photosynthesis to respiration which would automatically go anaerobic when placed in sealed containers. A forward genetics screen was used to identify such strains based on a colorimetric analysis of the dissolved oxygen concentration, finding one, named *apr1* for attenuated photosynthesis to respiration, which showed dramatically reduced photosynthetic rates and a slight increase in respiration (Ruhle et al., 2008). However, despite going anaerobic when placed in sealed containers, H₂ was only produced in the light after the Calvin-Benson cycle was inhibited through the addition of glycoaldehyde (Ruhle et al., 2008). These results suggested down-regulation of the Calvin-Benson cycle, which otherwise acts as a preferential electron sink, is a necessary step for stimulating H₂ production (Ruhle et al., 2008).

1.6.6.4 Rubisco mutants

Mutant CC-2803, which lacks Rubisco, a key enzyme of the Calvin-Benson cycle involved in the fixation of CO₂ has a light-sensitive phenotype and is non-

photosynthetic. Consequently cultures go anaerobic and produce H_2 in sealed containers even in the presence of sulphur (Hemschemeier et al., 2008a).

Control of the Calvin-Benson cycle or Rubisco activity therefore represents a potentially novel method of inducing H_2 production as reviewed by Marin-Navarro et al. (2010), by removing the major sink of electrons for reduced ferredoxin generated by the light reactions. This could be achieved through inducible control of Rubisco or Calvin-Benson cycle enzyme expression or control of CO_2 supply to carbon-concentrating mutants (Spalding, 2008).

Rubisco may also make an interesting target to decrease the specificity of carboxylation to oxygenation reactions as has previously been demonstrated (Chen et al., 1988; Satagopan and Spreitzer, 2004; Genkov et al., 2006), which would result in a higher respiratory rate as well as reduced flux through the Calvin-Benson cycle.

1.6.6.5 Direct reduction in O_2 levels

In addition to altering the specificity of Rubisco to increase the respiratory rate, direct reduction of internal O_2 levels without affecting PSII activity could be achieved by over-expressing O_2 consuming enzymes, such as a plastid/plastoquinol terminal oxidase (PTOX) in the chloroplast or an alternative oxidase (AOX) in the mitochondrion, although it remains to be seen whether the benefits of decreasing O_2 levels would outweigh the loss of electrons which could potentially be fed to the hydrogenase. Another approach, where O_2 scavenging proteins from soya bean root nodules were expressed in *C. reinhardtii*, for the direct reduction of O_2 levels, has been demonstrated to improve yields, causing a 4.5x increase in H_2 production relative to the parental strain during sulphur-deprivation (Wu et al., 2010; Wu et al., 2011).

Other approaches to directly improve hydrogen production have sought to target the competing sinks for electrons derived from photolysis or breakdown of endogenous starch reserves.

1.6.7 Targeting auxillary electron transport pathways

Several auxillary electron transport pathways act to dissipate excess reducing pressure as reviewed by (Cardol et al., 2011; Rochaix, 2011), and H_2 production itself is proposed to act as a 'safety valve' under anaerobic conditions (Melis and Happe,

2001; Hemschemeier et al., 2008a). This therefore suggests that auxillary electron transport pathways act in competition with the hydrogenase, making them targets for future attempts at increasing yields.

Competing pathways include non-photochemical reduction of electron carriers in a process known as chlororespiration as reviewed by (Rumeau et al., 2007), the oxidation of photosystem I (PSI) electron acceptors by O_2 in a process known as the Mehler reaction and cyclic electron flow (CEF) around PSI (Figure 1.3) (Peltier et al., 2010; Rochaix, 2011). The precise effect of the Mehler reaction or chlororespiration on H_2 production is unclear, but several studies have indicated that CEF and H_2 production compete for reductant (Antal et al., 2009).

1.6.7.1 Chlororespiration

Chlororespiration is a proposed respiratory electron transport chain that interacts with the thylakoid PET (Bennoun, 1982). Chlororespiration is now thought to act through the activity of a plastid terminal oxidase (PTOX), distantly related to the alternative oxidase (AOX) of mitochondria (Carol et al., 1999; Wu et al., 1999), and a type II NADH dehydrogenase (Figure 1.2) (Peltier and Cournac, 2002) thought to be NDA2 (Desplats et al., 2008; Jans et al., 2008). Chlororespiration controls the redox state of the PQ pool for optimization of CEF as reviewed by (Rumeau et al., 2007), where electrons would be fed into the photosynthetic electron transport chain by a type II NADH dehydrogenase, and removed by PTOX, taking electrons from PQH_2 to reduce molecular oxygen and form H_2O (Josse et al., 2003). However, PTOX is not considered a major target for increasing yields because the use of *n*-propylgallate, a specific inhibitor of PTOX, was found to have little impact in diverting electrons away from H_2 production (Antal et al., 2009).

1.6.7.2 Mehler reaction

The Mehler reaction has been suggested as the cause for the simultaneous uptake and evolution of O_2 during photosynthesis, occurring at a rate of <15% during steady state conditions in *C. reinhardtii* but reaching up to 80% during photosynthetic induction on minimal media (Franck and Houyoux, 2008). It has been proposed to play a number of roles from ATP generation for CO_2 fixation, activation of signalling pathways via reactive oxygen species, or modulation of state transitions through re-oxidation of the plastoquinone pool (Cardol et al., 2011). The exact enzymes

responsible in *C. reinhardtii* have not been defined, but the alga does contain an ascorbate peroxidase and homologues of cyanobacterial flavodiiron proteins which may be involved (Cardol et al., 2011). However it remains to be seen what effect this pathway has on hydrogen production.

1.6.7.3 Cyclic electron flow (CEF)

For optimal photosynthetic activity, photosynthetic organisms balance the amount of light energy absorbed by the PSII and PSI reaction centres through migration of mobile light-harvesting complexes in a process known as state transitions (Lemeille and Rochaix, 2010). In *C. reinhardtii* this process allows the switch between linear and CEF as reviewed by (Lemeille and Rochaix, 2010). CEF potentially occurs by two pathways in *C. reinhardtii* which can be distinguished by their sensitivity to antimycin A (Ravenel et al., 1994).

The antimycin A-sensitive pathway involves the reduction of PQ using electrons from ferredoxin. The precise mechanism is unclear (Rochaix, 2011) but is mediated by a PSI-LHCI-LHCII-Cytb₆f-FNR-PGRL1 super-complex identified recently in *C. reinhardtii* (Iwai et al., 2010).

The second, antimycin A-insensitive pathway is believed to be controlled by the activity of a type II NAD(P)H dehydrogenase; reducing PQ using NAD(P)H generated via the ferredoxin-dependent NADP⁺ reductase (Rumeau et al., 2007) (Figure 1.3).

The efficacy of disrupting cyclic electron flow as a means of increasing H₂ yields was demonstrated in short-term inhibitor studies using antimycin A, which resulted in a doubling of H₂ production as a result of antimycin A's activity in blocking cyclic electron flow (Antal et al., 2009).

Mutants affected in cyclic electron flow have been identified by chlorophyll fluorescence video imaging, which has allowed the isolation of strains unable to switch between linear and cyclic electron transport (Depège et al., 2003). This screen also led to the discovery of a high hydrogen producing strain deficient in the mitochondrial transcription factor *moc1* known as *stm6* (state transition mutant 6) (Kruse et al., 2005a). *Stm6* is locked permanently in 'state 1' meaning in the process of linear electron transport, thereby inhibiting CEF which was cited as one of the major factors contributing to the high hydrogen phenotype. *Stm6* is also able to accumulate more starch and has a higher respiratory rate due to an up-regulated

alternative oxidase (AOX) which is beneficial for H₂ production because of more favourable rates of oxygen consumption (Kruse et al., 2005a).

For targeted gene knockout (KO), potential candidates in the antimycin-sensitive pathway include protein gradient regulator protein 5 (PGR5) (Munekage et al., 2002; Nandha et al., 2007) and PGRL1 (DalCorso et al., 2008). KO of these genes will also likely have the dual effect of disrupting Δ pH formation to help H₂ evolution.

Identifying the precise enzyme(s) responsible for the antimycin-insensitive CEF pathway in *C. reinhardtii* has been more difficult as the genome encodes six type II NADH dehydrogenases, including a plant type NDB (NDA2), a yeast type NDE (NDA3), one that grouped with plant type NDAs (NDA1) and three cyanobacterial type NDCs (NDA5, NDA6 and NDA7) (Desplats et al., 2008; Jans et al., 2008). *Arabidopsis* also has multiple type II NADH dehydrogenases with differing physiological roles (Michalecka et al., 2003); six out of seven are targeted exclusively to the mitochondria (Michalecka et al., 2003; Elhafez et al., 2006) highlighting the importance of identifying the correct isoform or isoforms for study. In *C. reinhardtii* RNA hybridization only detected transcripts for *NDA2* and *NDA6* under mixotrophic conditions and further analysis revealed *NDA2* was located in the chloroplast. An RNAi study knocking down expression of *NDA2* showed a reduction in CEF and reduced ability for state II transition when oxidative phosphorylation was inhibited (Jans et al., 2008). Despite this, the *NDA2* knockdown resulted in an approximately 50% decrease in H₂ production during sulphur-deprivation (Jans et al., 2008), possibly because *NDA2* may be acting as the point of entry for reductant from the breakdown of starch reserves. Consequently *NDA2* over expression may actually be a means of increasing the supply of reductant for H₂ production (Jans et al., 2008). It is unclear why *NDA2* knockdown resulted in such a dramatic decrease in overall H₂ yields when the indirect pathway is proposed to contribute only 10% of reductant to H₂ production, but this could perhaps be attributed to the knockdown of additional targets by siRNAs generated from the inverted repeated RNAi construct (Jans et al., 2008).

From these studies it is clear more investigation is needed into the physiological role of the individual type II NADH dehydrogenases in *C. reinhardtii* in terms of controlling CEF, state transitions, PSII activity and as a point of entry into the PET for reductant during anoxia, before their role in H₂ production can be fully understood.

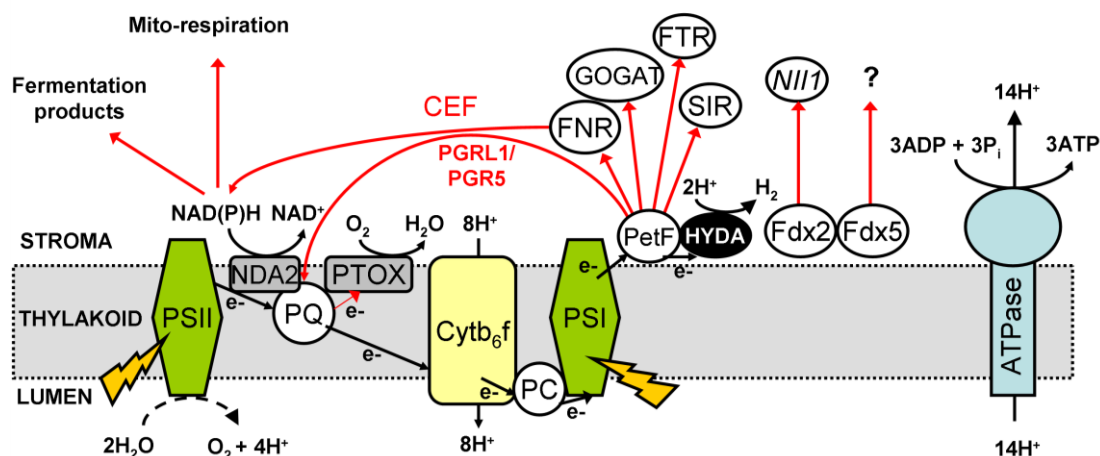


Figure 1.3: Schematic representation of the PET showing processes that potentially compete with the hydrogenase for reductant.

Abbreviations; Cytb₆f; cytochrome *b*₆f complex, Fd; ferredoxin, FNR; ferredoxin-NADP⁺ reductase, FTR; ferredoxin-thioredoxin reductase, GOGAT; ferredoxin-dependent glutamate reductase, HYD; [FeFe]-hydrogenase, NDA2; type II NADH dehydrogenase, NII2; ferredoxin-dependent nitrate reductase, PFOR; pyruvate:ferredoxin oxidoreductase, PGR5; proton gradient regulator protein 5, PGRL1; proton gradient regulator-like protein 1, PSI; photosystem I, PSII; photosystem II, PC; plastocyanin, PQ; plastoquinone, PTOX; plastid or plastoquinol terminal oxidase, SIR; ferredoxin-dependent sulphate reductase. Note: during linear electron transport 8 protons are pumped per 4 electrons coming from the oxidation of one molecule of H₂O. However the operation of both cyclic and linear electron flow will increase the relative number of protons pumped at Cyt *b*₆f complex. Figure adapted from Winkler et al. (2010).

1.6.8 Fermentation and H₂ production in *C. reinhardtii*

During anoxia, in addition to hydrogen production, *C. reinhardtii* activates fermentative pathways to recycle NAD⁺ and allow the continued production of ATP via glycolysis. By consuming reductant that could be fed into the electron transport chain via a type II NADH dehydrogenase, and pyruvate, which is potentially a substrate for the *C. reinhardtii* pyruvate:ferredoxin oxidoreductase (PFOR), fermentation is proposed to act in competition with hydrogen evolution making it a target for genetic manipulation (Mus et al., 2007; Timmins et al., 2009b; Doebe et al., 2010).

The major fermentative products accumulated during dark anoxia include formate, ethanol and acetate at an approximate ratio of 2:1:1 with minor amounts of hydrogen (Gfeller and Gibbs, 1984), glycerol (Klein and Betz, 1978; Gfeller and Gibbs, 1984; Meuser et al., 2009), succinate (Dubini et al., 2009), D-lactate (Gfeller

and Gibbs, 1984; Husic and Tolbert, 1985) and malic acid (Mus et al., 2007) also reported. Likewise during sulphur-deprivation significant quantities of formate and ethanol accumulate (Hemschemeier et al., 2008b; Timmins et al., 2009b).

Key fermentative enzymes are thought to include pyruvate formate lyase (PFL1), a bifunctional acetaldehyde/alcohol dehydrogenase (ADH1) and pyruvate decarboxylase (PDC) (Mus et al., 2007). Figure 1.4 shows a working model of fermentative metabolism constructed from genomic (Grossman et al., 2007), biochemical (Kreuzberg et al., 1987), transcriptomic (Mus et al., 2007; Nguyen et al., 2008), metabolomic (Timmins et al., 2009b; Doebe et al., 2010) and proteomic (Atteia et al., 2009; Chen et al., 2010; Terashima et al., 2010) data.

Pyruvate can be broken down to formate and acetyl-CoA by pyruvate formate lyase (PFL1) (Hemschemeier and Happe, 2005; Atteia et al., 2006; Hemschemeier et al., 2008b), and the acetyl-CoA converted into acetate by a phosphate acetyl-transferase (PAT)-acetate kinase (ACK) catalysed pathway or potentially into ethanol by a bifunctional aldehyde/alcohol dehydrogenase (ADH), consuming two molecules of NADH (Figure 1.4). Additionally pyruvate can be converted into lactate by a D-lactate dehydrogenase (LDH) (Husic and Tolbert, 1985) with the cost of one molecule of NADH (Figure 1.4), or by a pyruvate decarboxylase (PDC3) into acetaldehyde, which could then be converted to ethanol by an alcohol dehydrogenase (ADH) (Mus et al., 2007) catalysed pathway, also consuming NADH (Atteia et al., 2003) (Figure 1.4).

However, the link between fermentation and hydrogen production in *C. reinhardtii* is still not fully understood, and the ability of the alga to redirect metabolism in the absence of certain pathways or under varying environmental conditions has been demonstrated in a mutant deficient in hydrogenase assembly, which resulted in increased production of succinate (Dubini et al., 2009), or by the excretion of lactic acid at extremes of pH and when PFL1 activity is blocked by the inhibitor sodium hypophosphite (Kreuzberg, 1984). Further investigation is required into the relationship between fermentation and hydrogen production, particularly under conditions of sulphur-deprivation before it can be determined if down-regulating fermentation is an appropriate strategy for increasing yields.

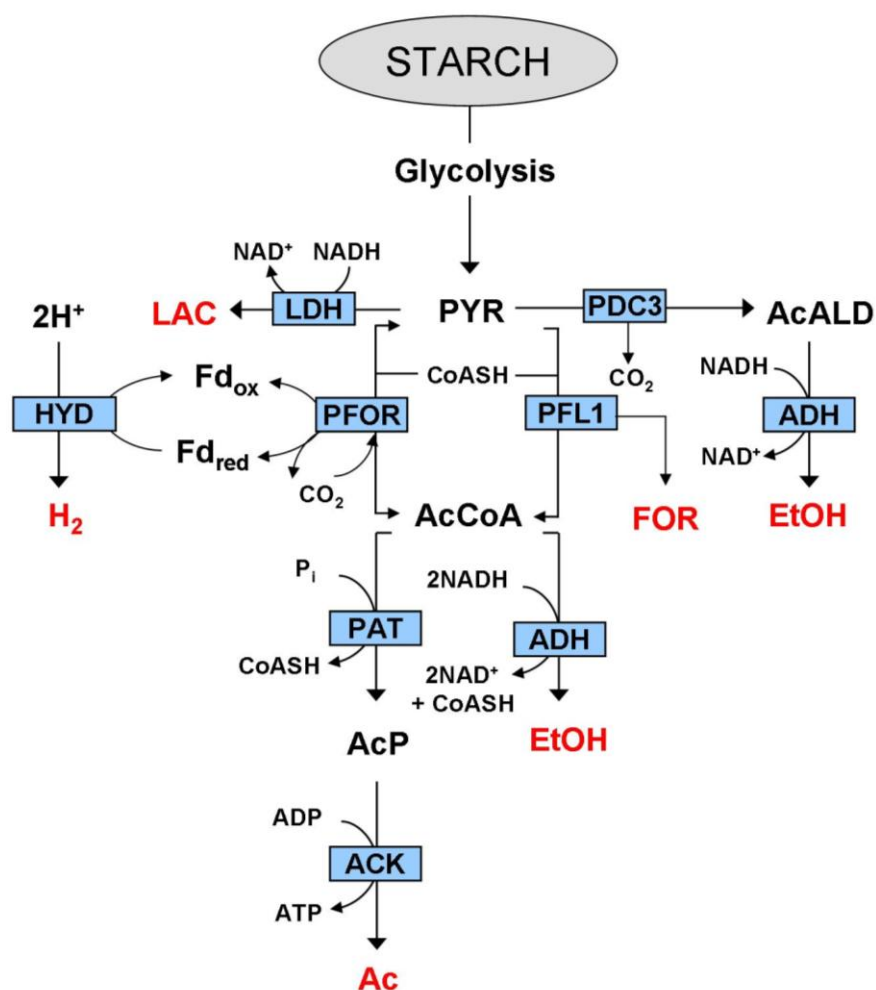


Figure 1.4: Proposed model of fermentative pathways in *C. reinhardtii*.

This model is adapted from Grossman et al. (2007), metabolites are labelled in red, AcALD; acetaldehyde, Ac; acetate, AcCoA; acetyl-CoA, EtOH; ethanol, Fd; ferredoxin, LAC; lactate, PYR; pyruvate, Enzymes present in the *C. reinhardtii* genome are in blue boxes, ACK; acetate kinase, ADH; alcohol dehydrogenase, HYD; [FeFe]-hydrogenase, LDH; lactate dehydrogenase, PAT; phosphate acetyltransferase, PDC; pyruvate decarboxylase, PFL1; pyruvate formate lyase, PFOR; pyruvate ferredoxin oxidoreductase. It must be noted that additional pathways can be activated during anaerobic incubation in mutants, as reviewed by Grossman et al. (2011).

1.7 Reverse genetics in *C. reinhardtii*

The *C. reinhardtii* nuclear genome can be transformed through treatment with glass-beads (Kindle, 1990), electroporation (Shimogawara et al., 1998) or biolistic bombardment (Kindle and Sodeinde, 1994). Biolistic techniques also allow for transformation of the 200 kb chloroplast (Boynton et al., 1988; Maul et al., 2002) and ~15.8 kb mitochondrial genome (Randolph-Anderson et al., 1993; Vahrenholz et al., 1993) through integration of DNA via homologous recombination.

However, problems remain in successfully achieving homologous recombination (HR) in the nucleus at rates sufficient for genetic analysis (Zorin et al., 2009) as DNA predominately integrates by non-homologous recombination (NHEJ) at random loci (Debuchy et al., 1989). Various efforts have been made to improve the ratio of HR to NHEJ including the optimization of transformation conditions, the use of single-stranded DNA and the application of a 'promoter-trap' whereby the resistance cassette is driven by the target promoter (Zorin et al., 2005). This was used effectively to suppress rates of non-homologous integration resulting in ~1:100 transformants arising from a recombination event (Zorin et al., 2009). However, these rates were strain dependent and have not yet been demonstrated for more than one gene of interest, meaning it is not currently a routine laboratory technique.

Although knockout mutants cannot routinely be generated by HR, they can be isolated by PCR screening of random mutagenesis libraries, created by marker gene insertion (Pootakham et al., 2010), but this is a labour intensive process. Alternatively RNA interference techniques based on the expression of inverted repeat constructs have been used successfully to downregulate protein expression in a number of cases (Rohr et al., 2004; Mussnug et al., 2007; Jans et al., 2008; Petroustos et al., 2009). However, this approach suffers from unwanted silencing of off-targets and has problems of knockdown repression. Recent advances in the understanding of micro RNAs in *C. reinhardtii* (Molnár et al., 2007), have opened up the potential to create highly specific knockdown mutants (Molnár et al., 2009; Zhao et al., 2009; Schmollinger et al., 2010), potentially overcoming many of the problems associated with conventional RNAi constructs.

1.7.1 microRNAs

microRNAs (miRNAs) are 21-24 nt small RNAs created from the processing of longer pre-miRNAs (Bartel, 2004) transcribed from non-coding RNA genes (Chen, 2005). They can regulate gene expression through RNA interference (Jones-Rhoades et al., 2006) and are differentiated from small interfering RNAs (siRNAs) by their origin (Chen, 2005). Although miRNAs primarily act as negative regulators, there is also evidence that in certain circumstances they can play a role in stimulating transcription when targeted to promoter regions or in promoting translation through as yet unknown mechanisms (Mendes et al., 2009).

miRNAs have been found in plants, animals and viruses (Mendes et al., 2009), but due to differences in processing, structure, genomic location and transcription are likely to have independently evolved at least twice (Jones-Rhoades et al., 2006). Plant miRNAs predominantly act through cleavage of target mRNAs (Schwab et al., 2005; Jones-Rhoades et al., 2006), binding to sites in the coding region, although some have also been found to target both 5' and 3' UTRs (Mendes et al., 2009). They play roles in plant growth and development and are biased towards targeting transcription factors and regulatory proteins (Chen, 2005; Jones-Rhoades et al., 2006).

Transcribed pre-miRNAs form small hairpin loops with a single base mismatch, which is cleaved by a DICER nuclease releasing a partially double-stranded 21nt miRNA (Figure 1.5). One of the strands (miRNA) is then recruited by an Argonaute nuclease to form an RNA Induced Silencing Complex (RISC) (Figure 1.5). The RISC complex then binds mRNA transcripts either causing a halt to translation or cleaves target mRNAs which are then degraded (Figure 1.5).

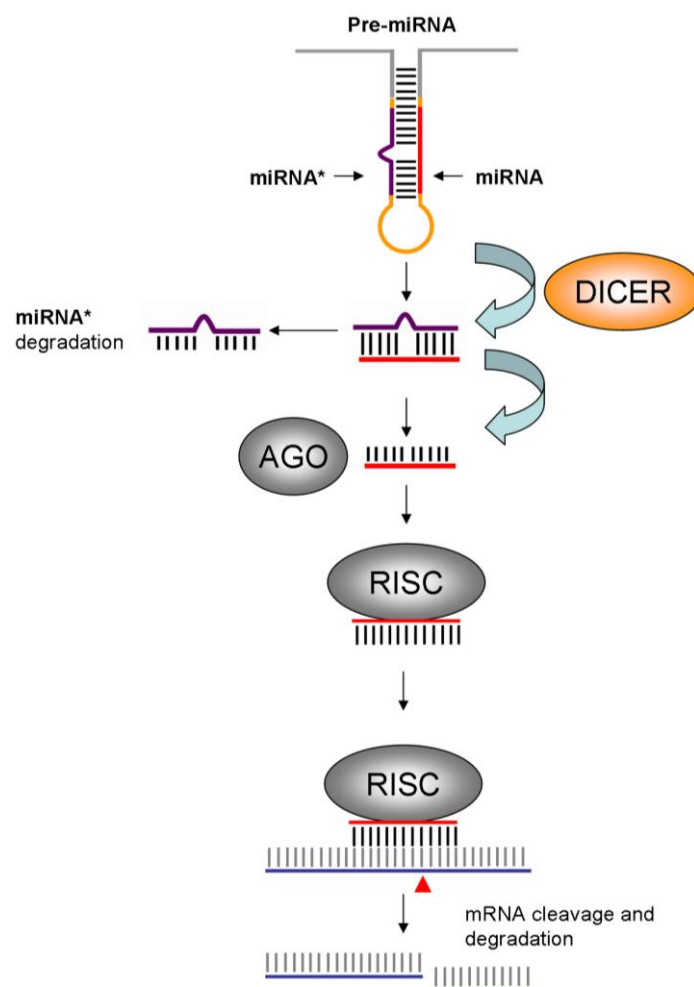


Figure 1.5: Schematic representation of miRNA mode of activity.

Figure adapted from (Jones-Rhoades et al., 2006), abbreviations: AGO; Argonaute nuclease, RISC; RNA induced silencing complex.

It is possible to replace the targeting region of a native pre-miRNA with a novel sequence designed to knockdown a given gene of interest, thereby creating an artificial microRNA construct. This approach has been demonstrated to an effective means of gene regulation in *C. reinhardtii* (Molnár et al., 2009; Zhao et al., 2009) and offers a quick means of performing reverse genetic analysis.

1.8 Aims

Photobiological H₂ production is an attractive approach for generating renewable energy but to become economically viable it is necessary to increase yields along with improvements in culturing, waste biomass utilization and nutrient recycling. The recent application of artificial microRNAs in *C. reinhardtii* has aided the production of specific knockdown mutants and the rapid assessment of target protein function. A number of processes are proposed to compete with hydrogen production during sulphur-deprivation and have been highlighted as potential targets for increasing yields including auxillary electron transport pathways and the fermentative breakdown of pyruvate consuming NADH. Despite extensive genomic analysis, the relationship between fermentation and hydrogen production is still poorly understood, therefore in order to aid future attempts at genetic engineering the aims of the project included:

- Building on the current knowledge of fermentative pathways in *C. reinhardtii* through characterization of fermentative enzyme expression and localisation
- Artificial microRNA knockdown of the fermentative pathways proposed to predominate during sulphur-deprivation through targeting of the enzymes PFL1 and PDC3, and assessing the impact upon metabolite production and hydrogen evolution.

Chapter 2: Materials and Methods

2.1 Bioinformatic analysis

Software, web based programs and databases used in this project for expressed sequence tag (EST) identification, polyclonal antibody production, sequence alignments and phylogenetic analysis are listed below.

2.1.1 Databases

Kazusa DNA Research Institute *C. reinhardtii* EST Database (Asamizu et al., 1999; Asamizu et al., 2004)

(<http://est.kazusa.or.jp/en/plant/chlamy/EST/index.html>)

NCBI (<http://www.ncbi.nlm.nih.gov/>)

Chlamydomonas Centre (<http://www.chlamy.org/>)

Phytozome v5.0 (<http://www.phytozome.net/>)

2.1.2 Protein targeting prediction programs

TargetP (Emanuelsson et al., 2000) (<http://www.cbs.dtu.dk/services/TargetP/>)

ChloroP (Emanuelsson et al., 1999) (<http://www.cbs.dtu.dk/services/ChloroP/>)

Predotar (Small et al., 2004) (<http://urgi.infobiogen.fr/predotar/predotar.html>)

2.1.3 Multiple sequence alignment programs

CLUSTALW (Thompson et al., 1994)

(<http://www.ebi.ac.uk/Tools/clustalw2/index.html>)

2.1.4 Phylogenetic analysis

Phylogeny.fr (Dereeper et al., 2008) (<http://www.phylogeny.fr/>)

2.1.5 Artificial microRNA (amiRNA) design

Web microRNA designer v.3 (Molnár et al., 2009)

(<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>)

2.1.6 qRT-PCR assay design

ABI Custom Taqman® Assay Design Tool

(<https://www5.appliedbiosystems.com/tools/cadt/>)

2.1.7 Graphic tools

pDRAW32 - vector map construction (<http://www.acaclone.com/>)

GeneDoc - multiple sequence alignment editor (<http://www.nrbsc.org/gfx/genedoc/>)

2.2 *Escherichia coli* strains and growth conditions

2.2.1 *E. coli* strains

Name	Source	Genotype	Use
10-β	New England Biolabs Inc.	<i>araD139 Δ(ara-leu)7697 fhuA lacX74 galK (Φ80 Δ(lacZ)M15) mcrA galU recA1 endA1 nupG rpsL Δ(mrr-hsdRMS-mcrBC)</i>	Cloning
KRX	Promega	[F', <i>traD36, ΔompP, proA⁺B⁺, lacI^q, Δ(lacZ)M15</i>] <i>ΔompT, endA1, recA1, gyrA96 (Nal^r), thi-1, hsdR17 (r_K⁻, m_K⁺), e14⁻ (McrA⁻), relA1, supE44, Δ(lac-proAB), Δ(rhaBAD)::T7 RNA Polymerase</i>	Expression

Table 2.1: *E. coli* strains used in this work

2.2.2 *E. coli* growth conditions

For all DNA cloning and protein expression work unless otherwise stated *E. coli* cells were grown in Lysogeny Broth (LB) (Bacto-Tryptone 10g L⁻¹, Yeast Extract 5 g L⁻¹, NaCl 10g L⁻¹) at 37°C, and colonies maintained on 1.5% (w/v) agar LB plates at 4°C with appropriate antibiotics.

2.3 *C. reinhardtii* strains and growth conditions

2.3.1 *C. reinhardtii* strains

#	Strain	Description	Reference
1	CC-124 137c	WT, mt ⁻ , <i>nit1⁻, nit2⁻, agg1</i>	Chlamydomonas Centre
2	D66	mt ⁺ , <i>nit2⁻, cw15</i> ,	(Pollock et al., 2003)
3	<i>pfl1</i> -KD1	CC-124 transformed with plasmid #23, <i>PFL1</i> knockdown, Par ^R	this work
4	<i>pfl1</i> -KD2	CC-124 transformed with plasmid #23, <i>PFL1</i> knockdown, Par ^R	this work
5	<i>pd3</i> -KD	CC-124 transformed with plasmid #25, <i>PDC3</i> knockdown, Par ^R	this work

Table 2.2: Algal strains used and summary of phenotypic markers.

Abbreviations; *agg1*; phototactic aggregation, *cw*; cell wall deficient, *mt*; mating type, *nit1⁻, nit2⁻*; nitrite reductase deficient, Par^R; paromomycin resistant (10 µg ml⁻¹).

2.3.2 *C. reinhardtii* growth conditions

Cells were grown mixotrophically on Tris-acetate-phosphate (TAP) media (Gorman and Levine, 1965) (Tris-HCl [2.42 g L^{-1}], NH_4Cl [0.375 g L^{-1}], $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [0.1 g L^{-1}], $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [0.05 g L^{-1}], K_2HPO_4 [0.10 g L^{-1}], KH_2PO_4 [0.05 g L^{-1}] Hutner's trace elements (prepared according to Harris (1989); NaEDTA [50 g L^{-1}], $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ [22 g L^{-1}], H_3BO_3 [11.4 g L^{-1}], $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ [5.06 g L^{-1}], $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ [1.61 g L^{-1}], $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ [1.57 g L^{-1}], $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ [1.1 g L^{-1}], $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ [4.99 g L^{-1}]) 1 ml L^{-1} , acetic acid [1 ml L^{-1}], pH 7.0) or photoautotrophically in High Salt Minimal (HSM) medium (NH_4Cl [0.5 g L^{-1}], $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [0.246 g L^{-1}], $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [0.01 g L^{-1}], K_2HPO_4 [1.44 g L^{-1}], KH_2PO_4 [0.72 g L^{-1}], Hutner's Trace Elements 1 ml L^{-1} , pH 6.8) bubbled with a 5% CO_2 air mixture (225742-L; BOC, UK) or air. Cells were kept for long term storage on 2% (w/v) agar TAP plates supplemented with appropriate additives at a light intensity of $60 \mu\text{E m}^{-2} \text{ s}^{-1}$.

For sulphur deprivation experiments cultures were washed and re-suspended in modified TAP medium with sulphate salts replaced by their chloride derivatives (Melis et al., 2000).

2.4 Determination of chlorophyll concentration

Chlorophyll concentration was determined according to Porra et al. (1989). Typically 1 ml of algal culture was spun down ($16000g$, 1 min, 25°C ; accuSpin Micro; Fisher Scientific, USA), the supernatant discarded and the pellet re-suspended in 1 ml of 100% methanol to extract chlorophyll. Samples were then spun down ($16000g$, 2 min, 25°C ; accuSpin Micro; Fisher Scientific, USA), the supernatant transferred to a glass cuvette and chlorophyll absorbance measured with a spectrophotometer (UV-1601, Shimadzu, Japan) after zeroing the baseline with 100% methanol at 652 and 665 nm. The concentration of chlorophyll was then estimated using equation (4)

$$\begin{aligned} \text{Concentration of chlorophylls } (a + b) \mu\text{g ml}^{-1} = & \text{Eq. 4} \\ (22.12 A_{652} + 2.71 A_{665}) * \text{dilution factor} & \end{aligned}$$

2.5 *C. reinhardtii* cell number calculation

For routine determination of *C. reinhardtii* cell number a calibration curve was created comparing the optical density (OD) at 750 nm (UV-1601, Shimadzu, Japan) against cell number, counted using a hemocytometer according to Harris (1989). From the equation of the line it was calculated for CC-124 cultures and OD₇₅₀ was equivalent to a cell density of 8×10^6 cells ml⁻¹.

2.6 DNA transformation procedures

2.6.1 *E. coli* transformation

2.5 µl of ligation mix or 50 ng of plasmid DNA was mixed with 50 µl of self prepared 10-β chemically competent cells (New England Biolabs Inc., USA), and incubated on ice for 30 min. The cells were subject to heat shock by incubation at 42°C for 45 s then on ice for 2 min. 450 µl of Super Optimal Broth with Catabolite repression (SOC) medium (2% (w/v) Bacto-Tryptone, 0.5% (w/v) yeast extract, 20 mM glucose, 10 mM MgCl₂, 10 mM MgSO₄, 10 mM NaCl, 2.5 mM KCl) was added and then agitated on an orbital shaker (200 rpm, 1 h; Stuart® SSL1; Bibby Scientific Limited, UK) placed in a 37°C incubator. All the cells were then plated onto 1.5% (w/v) agar LB plates, with appropriate antibiotics (Sigma-Aldrich, UK) and incubated overnight at 37°C.

2.6.2 Biolistic transformation of *C. reinhardtii* nuclear genome

Cultures of *C. reinhardtii* were grown in TAP medium at 25°C, 120 rpm to an OD₇₅₀ of 0.8-1. 50 ml aliquots were pelleted (2500g, 10 min, 25°C; Allegra 6R; Beckman Coulter, USA) and re-suspended in 3 ml of TAP medium, 1 ml of the suspension was pipetted onto 2% (w/v) agar HSM plate and left to dry (2 h; Microflow Advanced BioSafety Cabinet - Class II; BIOQUELL Ltd, UK).

Nuclear transformation vectors were immobilized onto 550 nm gold particles according to the manufacturer's instructions (Seashell Technology; USA), 2 µg of plasmid DNA was used per shot. Particles were then fixed to microcarrier discs (1652335; Bio-Rad Laboratories, Inc., USA) and launched at dried algal lawns on TAP paromomycin 10 µg ml⁻¹ (P10) plates using a gene gun (BIO-RAD-Model PDS-1000/He Biolistic® Particle Delivery System; Bio-Rad Laboratories, Inc., USA). The macrocarrier was loaded in the first slot below the rupture membrane holder which

was fitted with a 1350 psi rupture disc (1652330; Bio-Rad Laboratories, Inc., USA) and fired at plates positioned in the bottom slot of the shooting chamber. Plates were incubated at 25°C with continuous illumination of white fluorescent light (37627; White F58W/35-535; GE Healthcare, USA) 60 $\mu\text{E m}^{-2} \text{s}^{-1}$, putative transformants began to appear after 7–10 days.

2.7 Molecular biology techniques

2.7.1 Preparation of plasmid DNA

For small scale preparations of plasmid DNA typically a single colony of *E. coli* containing plasmid DNA was used to inoculate 3 ml LB medium with appropriate antibiotics and grown overnight at 37°C shaking at 200 rpm. 1.5 ml aliquots of cells were harvested (16000g, 1 min, 25°C; accuSpin Micro; Fisher Scientific, USA) the supernatant discarded and pellet re-suspended in 100 μl of buffer P1 (50 mM Tris-HCl, 10 mM EDTA, 10 $\mu\text{g ml}^{-1}$ RNase A, pH 8.0). 100 μl of P2 solution (200 mM NaOH, 1% SDS (w/v) was added to the sample, mixed by inverting, and incubated at room temperature for 5 min. 100 μl of P3 solution (3 M Potassium acetate pH 5.5) was then added, sample mixed by inverting, incubated on ice for 5 min then spun down to remove cell debris (16000g, 5 min, 25°C; accuSpin Micro; Fisher Scientific, USA). The supernatant was transferred into 1 ml 100% ice cold ethanol, mixed by inverting and left to precipitate for 5 min at room temperature then spun down (16000g, 10 min, 4°C; accuSpin Micro; Fisher Scientific, USA). The supernatant was discarded and the pellet washed in 200 μl of 70% (v/v) ethanol, left to air dry then re-suspended in 30 μl dH₂O.

Plasmid DNA was recovered in mg quantities using a QIA Midi Kit (12143; QIAGEN, Germany) according to the manufacturer's instructions (see Qiagen Plasmid Purification Handbook pg 19–22). The dried DNA pellet was re-suspended in 300 μl or 50 μl of nuclease free water for high and low copy number plasmids, respectively, and stored at -20°C.

2.7.2 DNA sequencing

Purified plasmid DNA was sent for sequencing by Eurofins, MWG, Operon (Germany) and samples prepared in accordance with service instructions. Sequencing was performed with primers listed in Table 2.3.

#	Primer Name	Vector	Sequence
1	M13 Forward (-20)	pENTR/TEV/D TOPO	5'-GTAAAACGACGGCCAG-3'
2	M13 Reverse	pENTR/TEV/D TOPO	5'-CAGGAAACAGCTATGAC-3'
3	T7	pDEST17, pBluescript II SK-	5'-TAATACGACTCACTATAGG-3'
4	RNAi3_Seq_F	pRNAi3, pRNAi6	5'-TATGGGTGTTGGGTTCGGTG-3'
5	RNAi3_Seq_R	pRNAi3, pRNAi6	5'-TAGCGCTGATCACCACCACCC-3'

Table 2.3: List of sequencing primers used in this work.

2.7.3 DNA agarose gel electrophoresis

Gels were made with agarose (final [1-2% (w/v)]; MB1200; Melford Laboratories, UK) in Tris-acetate-EDTA (TAE)-buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.0), supplemented with ethidium bromide (final [1 µg ml⁻¹]) or SYBR® Safe (0.00005% (v/v))(Invitrogen Life Technologies, USA). DNA was loaded into wells after the addition of 6 x loading buffer (40% (w/v) sucrose; 0.25% (w/v) bromophenol blue; final [1x]) and gels run in TAE-buffer at 110 V. Samples were then visualized using a Gel Doc-It TS™ Imaging System with 15 " liquid crystal display (LCD) screen and charge coupled device (CDD) camera (UVP, USA).

2.7.4 DNA purification from agarose gels

All DNA gel extractions were performed using QIAquick gel extraction kit (28704; QIAGEN, Germany) according to the manufacturer's instructions.

2.7.5 Polymerase chain reaction (PCR) enzymes and buffers

Taq DNA polymerase (M0273S; New England Biolabs Inc., USA), BIO-X-Act™ (BIO-21049; Bioline Limited, UK) or Phusion® Hot Start High Fidelity DNA polymerase (F-540S; New England Biolabs Inc., USA), were used for PCR reactions with FailSafe™ Buffer G (FSP995G; Epicentre Biotechnologies: 2x Buffer: 100 mM Tris-HCl pH 8.3, 100 mM KCl, 400 µM dATP, dCTP, dTTP, and dGTP, concentration of MgCl₂ and FailSafe Enhancer not given).

2.7.6 PCR conditions

For cloning of DNA with Bio-X-Act polymerase typically a 50 µl PCR reaction was set up containing 5 ng of template plasmid DNA, 10 µM forward and reverse primers, 10 U BIO-X-Act™ (21049; Bioline, USA; 10 U µl⁻¹) 25 µl 2x FailSafe PCR Buffer G, made up to volume with 10 µl dH₂O. All PCR reactions were

performed on a PeqStar 96 gradient thermocycler (Peqlab Biotechnologie, Germany) and a typical PCR program consisted of one cycle of 92°C (denaturing), then 25 cycles of 92°C for 30 s, 50–70°C for 30 s, and 72°C for 30 s per kb.

#	Primer Name	Sequence
1	EST1_F	5'- <u>CACCATGG</u> TGGTGTCCACCTTCTCCAGCAAG-3', NcoI
2	EST1_R	5'-TTACATGGTGTCTGGAAGGTGCGC-3'
3	EST2_F	5'- <u>CACCATGG</u> TGATGCCGACGCCAAGGGCATG-3', NcoI
4	EST2_R	5'-TTATGCAGCTCGGGCTCGGTGCGGGCC-3'
5	EST3_F	5'- <u>CACCATGG</u> ACAACGAGGCCATCAAGTCCATC-3', NcoI
6	EST3_R	5'-TTAGGTACGGCGAGTCGATCGGGCTGG-3'
7	EST5_F	5'- <u>CACCATGG</u> CGCGCGCCGAGGCCGCGCGCA-3', NcoI
8	EST5_R	5'-CGCGGATCCGCGTTACTTCTCTCGTCCTTCTCCTCC-3', BamHI
9	EST8_F	5'- <u>CACCATGG</u> CCAGCAAGGCCAATCAGAGGAAT-3', NcoI
10	EST8_R	5'-CGCGGATCCGCGTTAGTAGTTGCCCATGCGCACGCGGA-3', BamHI
11	EST11_F	5'- <u>CACCATGG</u> TTGACGGCATGGACGTGCTGGCC-3', NcoI
12	EST11_R	5'-CGCGGATCCGCGTTACGACTTGTAGGTGGGGTCTGAAG-3', BamHI
13	EST12_F	5'-CTAGCGGGATCCATCTACAAGAAGGGCCCCGG-3', BamHI
14	EST12_R	5'-CAGCTAGCTCGAGTTACGACACGACGGCGGTGCC-3', XhoI
15	EST13_F	5'-CAGACGAGGATCCGCCATCTTCAAGTCGCTGCTGTCG-3', BamHI
16	EST13_R	5'-TCGTCTGCTCGAGTTACAGCTTGCCGTCCACAACCACACC-3', XhoI

Table 2.4: List of primers used for cloning.

Suffix denotes forward (F) or reverse (R) primer. Note that primers EST2_R, EST3_R and EST6_R do not code for an in-frame stop codon, restriction sites are underlined with bases in italics, restriction enzyme is indicated at the end.

For cloning reactions with Phusion® Hot Start High Fidelity DNA polymerase PCR reactions were set up as above with the following alterations: 0.5 µM forward and reverse primers, 1 U Phusion® Hot Start High Fidelity DNA polymerase. PCR programs consisted of 98°C for 30 s, then 25 cycles of 98°C for 5 s, 50-70°C for 15 s and 72°C for 15 s per kb with a final extension step of 72°C for 5 min.

2.7.7 Restriction digests

A typical restriction digest for diagnostic purposes contained 200 ng of plasmid DNA, 1.5 µl of appropriate 10x restriction enzyme buffer (New England Biolabs Inc., USA), 1.5 µl of 10x bovine serum albumin (BSA) (New England Biolabs Inc., USA) when applicable, 2.5U of restriction enzyme(s) (New England Biolabs Inc., USA) which never made up more than 10% total volume, made up to 15 µl with dH₂O. Digests were then incubated at specified temperature (New England Biolabs Inc., USA) for 1 h, and half of the reaction analysed on DNA agarose gel.

Restriction digests for the isolation of DNA fragments usually contained 2 µg plasmid DNA, 3 µl 10x restriction enzyme buffer (New England Biolabs Inc., USA), 3 µl of 10x BSA when applicable (New England Biolabs Inc., USA), 20 U of restriction enzyme (New England Biolabs Inc., USA) made up to a total volume of 30 µl with dH₂O. Digests were incubated as described above.

2.7.8 Ligation reactions

A typical reaction setup contained 50 ng of digested, linearised plasmid, a three fold molar excess of insert, 10 µl 2x Quick Ligase Buffer (New England Biolabs Inc., USA; 132 mM Tris-HCl, 20 mM MgCl₂, 2 mM dithiothreitol, 2 mM ATP, 15% polyethylene glycol (PEG 6000) pH 7.6 at 25°C), 1 µl Quick T4 DNA Ligase (M2200S; New England Biolabs Inc., USA) made up to a volume of 20 µl with dH₂O. Ligation was performed according to the manufacturer's instructions (New England Biolabs Inc., USA).

2.7.9 Genomic DNA extraction

C. reinhardtii cultures were grown in TAP medium as described in 2.3.2, 1.5 ml of stationary phase cells were harvested (16000g, 1 min, 25°C; accuSpin Micro; Fisher Scientific, USA) and the supernatant discarded. Pellets were frozen in liquid N₂ then ground with a plastic pestle in a 1.5 ml centrifuge tube. gDNA extraction was then performed using a PeqGOLD Plant DNA Mini kit (12-3486-01; Peqlab Biotechnologie, Germany) according to the manufacturer's instructions.

2.7.10 RNA extraction

C. reinhardtii cultures were grown mixotrophically (as described in 2.3.2.) and a volume of cells containing ~50-100 µg Chl were harvested (16000g, 1 min, 25°C; accuSpin Micro; Fisher Scientific, USA), the supernatant discarded and RNA extracted using RNeasy Plant Mini kit (74903; QIAGEN, Germany) according to the manufacturer's protocol.

2.7.11 DNA and RNA concentration determination

DNA and RNA concentrations were estimated using a spectrophotometer (UV-1601, Shimadzu, Japan), typically 5–10 µl of purified plasmid DNA or RNA was

diluted to 200 μl with dH_2O placed in a Quartz cuvette (105.201-QS; Hellma, UK) and the absorbance measured at 260 nm. Concentrations could then be calculated as an A_{260} value of 1.0 is equivalent to a concentration of 50 $\mu\text{g ml}^{-1}$ dsDNA or 40 $\mu\text{g ml}^{-1}$ RNA (Sambrook, 1989).

2.7.12 Real time RT-PCR analysis

2.7.12.1 cDNA synthesis

cDNA synthesis was performed using High Capacity DNA Reverse Transcription Kit (4368814; Applied Biosystems, USA) according to the manufacturer's instructions. A typical reaction consisted of 2 μl RT Buffer (10x), 0.8 μl dNTP Mix (100 mM), 2 μl RT random primers (10x), 1 μl MultiscribeTM reverse transcriptase, 125 ng RNA and nuclease free dH_2O to a total volume of 20 μl . The reaction was performed using a PeqStar 96 gradient thermocycler (Peqlab Biotechnologie, Germany) with a program of 25°C for 10 min, 37°C for 2 h and 85°C for 10 min. cDNA was then stored at -20°C until further use.

2.7.12.2 Taqman® qRT-PCR analysis

qRT-PCR was performed using the Taqman® Gene Expression Assay System, reactions consisted of 0.5 μl of the relevant Taqman® Gene Expression Assay (Table 2.5), 1 μl of 1:5 diluted cDNA (25ng of RNA equivalent), 5 μl Taqman® Fast Universal PCR Master Mix (4352042, Applied Biosystems, USA) and nuclease free dH_2O to a total volume of 10 μl . Each run was performed in triplicate. Samples were analysed on 7500 Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions, RNA levels were normalised to *RPL10a* mRNA encoding the cytosolic 60S large ribosomal subunit (Hemschemeier et al., 2008b) and relative transcript levels calculated via the comparative C_T method for *PFL1*, or the standard curve method for *PDC3* (see Taqman® Fast Universal PCR Mix x2 protocol, 2005; Applied Biosystems, USA) (Appendix II.1).

Assay	Name of primers and probe	Sequence
<i>RPL10a</i>	CR_RPL10A_F	CCTGCTCCTATCAACAAGAACCT
	CR_RPL10A_R	GAACCTTGATGCTGCACTTGGT
	CR_RPL10A_M	CCAGCACCATCTCCTC
<i>PFL1</i>	Cr_PFL1_F	ACCGCGTGGACGAGATC
	Cr_PFL1_R	GCGGTAGGTGTGCTGCTT
	Cr_PFL1_M	CCTTCTCCAGCAAGCTG
<i>PDC3</i>	Cr_PDC3_F	GGACCAGCTGCTCAAGCA
	Cr_PDC3_R	CGCATTTCAGCTCGTTGCA
	Cr_PDC3_M	ACCAGGGACAGCTCG

Table 2.5: List of Taqman® Custom Gene Expression Assays used in this work.

All assays were designed automatically using online custom Taqman® Assay Design Tool (Section 2.1). Each assay contains 18 μ M forward (F) and reverse primers (R) and 5 μ M Probe (M).

2.8 Protein expression

2.8.1 Cloning expressed sequence tags into the Gateway® vector pENTR/TEV/D TOPO

Name	Source	Purpose	Antibiotic Resistance Cassette
pENTR/TEV/D TOPO	Invitrogen Life Technologies, USA	Cloning	KAN
pDEST17	Invitrogen Life Technologies, USA	Expression	AMP
pRSET A-thr*	Invitrogen Life Technologies, USA,	Expression	AMP

Table 2.6: Commercially available plasmids used in this work

*Adapted by Ernesto Costa (Imperial College) (Appendix II.2)

mRNA sequences of target genes were identified in the Entrez Nucleotide database (National Center for Biotechnology Information, USA), corresponding ESTs were identified and obtained from the *Chlamydomonas reinhardtii* EST index (Kazusa Research Institute, Japan). After plasmid sequencing, fragments were amplified by PCR (see section 2.7.9) and cloned into the Gateway® System entry vector pENTR/TEV/D TOPO (K252520; Invitrogen Life Technologies, USA, UK) according to the manufacturer's instructions (see pENTR™ Directional TOPO® Cloning Kits Manual).

2.8.2 Generating protein expression vectors

Amplified EST fragments were cloned from their entry vectors into pDEST17 (11803012; Invitrogen Life Technologies, USA) by LR recombination according to the manufacturer's instructions (see *E. coli* expression system with Gateway®

Technology manual pg 13–15). Positive transformants were selected on LB ampicillin 50 µg ml⁻¹ plates and the insert orientation was tested by restriction digest.

2.8.3 Small scale protein expression trials

A plate of freshly transformed *E. coli* KRX competent cells (Promega, USA; L3002) was dissolved in 1 ml of LB, inoculated into 100 ml LB media (plus 50 µg ml⁻¹ carbenicillin) and incubated at 37°C, 200 rpm until OD₆₀₀ reached 0.4-0.5. A 5 ml aliquot was taken as an uninduced control and protein expression was induced in the remaining culture by the addition of L-Rhamnose (Sigma-Aldrich, UK; R3875) to a final concentration of 0.1% (w/v). Cells were incubated at 37°C, 200 rpm and after 4 h the OD₆₀₀ was recorded for each sample. 1 ml of un-induced and induced cells was pelleted (16000g, 1 min, 25°C; accuSpin Micro; Fisher Scientific, USA) and re-suspended in 2x SDS sample buffer to allow loading of 10 µl sample equivalent to 1 ml of an OD₆₀₀ of 0.1 on an SDS-PAGE gel. The remaining induced cells were spun down at (2500g, 15 min, 4°C; Allegra 6R centrifuge; Beckman Coulter, USA) and the pellet was used to determine protein solubility (see section 2.8.4).

2.8.4 Determination of protein solubility

Separation of *E. coli* lysate into soluble and insoluble protein fractions for analysis by SDS-PAGE was done with BugBuster® Protein Extraction Reagent (70584; Novagen, USA) according to the manufacturer's instructions. 10 µl samples of soluble and insoluble protein were analysed on an SDS-PAGE gel.

#	Plasmid Name	Description
1	AV389850 ^a	<i>PFL1</i> fragment inserted in plasmid pBluescript SK II-
2	AV637523 ^a	<i>PDC3</i> fragment inserted in plasmid pBluescript SK II-
3	BP093588 ^a	<i>PFOR</i> fragment inserted in plasmid pBluescript SK II-
4	AV389547 ^a	<i>HYDA1</i> fragment inserted in plasmid pBluescript SK II-
5	AV6 26597 ^a	<i>LDH</i> fragment inserted in plasmid pBluescript SK II-
6	BG848457.1 ^b	<i>PDC1</i> fragment inserted in plasmid pBluescript SK II-
7	AV624259 ^a	<i>PDC2</i> fragment inserted in plasmid pBluescript SK II-
8	AV639995 ^a	<i>ADH1</i> fragment inserted in plasmid pBluescript SK II-

Table 2.7: Vectors containing EST sequences used in this work.

^aobtained from *C. reinhardtii* EST index, Kazusa DNA Research Institute, Japan

^bobtained from *C. reinhardtii* EST index, *Chlamydomonas* centre, Duke University USA

#	Plasmid Name	Description
9	pENT-PDC3	<i>PDC3</i> fragment amplified from plasmid #2, with primers 5 and 6 inserted into pENTR/TEV/D TOPO vector (Kan ^R)
10	pENT-PFOR	<i>PFOR</i> fragment amplified from plasmid #3, with primers 3 and 4 inserted into pENTR/TEV/D TOPO vector (Kan ^R)
11	pENT-PFL1	<i>PFL1</i> fragment amplified from plasmid #1, with primers 1 and 2 inserted into pENTR/TEV/D TOPO vector (Kan ^R)
12	pENT-HYDA1	<i>HYDA1</i> fragment amplified from plasmid #4, with primers 7 and 8 inserted into pENTR/TEV/D TOPO vector (Kan ^R)
13	pENT-LDH	<i>LDH</i> fragment amplified from plasmid #5, with primers 9 and 10 inserted into pENTR/TEV/D TOPO vector (Kan ^R)
14	pENT-PDC1	<i>PDC1</i> fragment amplified from plasmid #6, with primers 11 and 12 inserted into pENTR/TEV/D TOPO vector (Kan ^R)

Table 2.8: DNA entry vectors used in this work

#	Plasmid Name	Description
15	pDEST-PDC3	<i>PDC3</i> fragment from plasmid #9 inserted into pDEST17 vector (Amp ^R)
16	pDEST-PFOR	<i>PFOR</i> fragment from plasmid #10 inserted into pDEST17 vector (Amp ^R)
17	pDEST-PFL1	<i>PFL1</i> fragment from plasmid #11 inserted into pDEST17 vector (Amp ^R)
18	pDEST-HYDA1	<i>HYDA1</i> fragment from plasmid #12 inserted into pDEST17 vector (Amp ^R)
19	pDEST-LDH	<i>LDH</i> fragment from plasmid #13 inserted into pDEST17 vector (Amp ^R)
20	pDEST-PDC1	<i>PDC1</i> fragment from plasmid #14 inserted into pDEST17 vector (Amp ^R)
21	pRSET-PDC2	<i>PDC2</i> fragment amplified from plasmid #7 with primers 13 and 14 inserted into pRSET A-thr vector (Amp ^R)
22	pRSET-ADH1	<i>ADH1</i> fragment amplified from plasmid #8 with primers 15 and 16 inserted into pRSET A-thr vector (Amp ^R)

Table 2.9: Plasmids used for protein production

The primers referred to can be found in Table 2.4. Plasmids contained ampicillin (Amp^R) or kanamycin (Kan^R) resistance cassettes.

2.8.5 Large scale protein expression and purification

1 L cultures of *E. coli* KRX cells (L3002; Promega, USA) were set up and induced as in 2.8.3, pelleted (2500g, 15 min, 25°C; Allegra 6R; Beckman Coulter, USA), re-suspended in 25 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM MgCl₂, 5 mM dithiothreitol, 1 tablet Complete EDTA-free protease inhibitor (11873580001; Roche, Germany)), flash frozen in liquid N₂ and stored at -20°C over night.

Thawed cells were broken by three passes through a French pressure cell (FA-078; SLM Aminco, USA) 1200 psi. The resulting lysate was pelleted (2000g, 20 min, 25°C; Allegra 6R; Beckman Coulter, USA), re-suspended in 12.5 ml of binding buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 6 M guanidine HCl, 5 mM imidazole) and left to solubilise for 1 h on ice. 6 ml of Ni²⁺-IDA metal chelate resin (PC-MC100; Prochem, UK) was centrifuged (2500g, 2 min, 4°C; Allegra 6R; Beckman Coulter, USA), the ethanol removed, and washed in binding buffer 3 times. The solubilised

lysate was then centrifuged (2500g, 15 min, 4°C; Allegra 6R; Beckman Coulter, USA) to remove cell debris and the supernatant incubated with Ni²⁺ resin over night at 4°C on a blood tube rotator (SB1; Stuart Scientific, UK) in a 20 ml Ni²⁺ column (Bio-Rad Laboratories Inc., USA).

The protein sample was drawn through the Ni²⁺ using a peristaltic pump (P1; Pharmacia Fine Chemicals, Sweden), and the Ni²⁺ column washed three times with 12.5 ml of washing buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 6 M guanidium hydrochloride, 60 mM imidazole) before eluting with 25 ml of elution buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 6M guanidium hydrochloride, 1 M imidazole). Samples were then dialysed twice for 1.5 h, 4°C in 4.5 L of phosphate-buffered saline (PBS) (1.8 mM Na₂HPO₄, 0.2 mM KH₂PO₄, 150 mM NaCl) to remove any residual guanidium hydrochloride. Dialysed samples including precipitated protein was then centrifuged (2,500g, 20 min, 4°C; Allegra 6R; Beckman Coulter, USA) and the pellet re-suspended in PBS to a concentration of 10–20 mg ml⁻¹ with the addition of 10% (w/v) SDS until pellets dissolved.

2.9 Construction of artificial microRNA (amiRNA) vectors

#	Oligonucleotide name	Oligonucleotide sequence
1	Cr_PFL1_amiRNA_A_For	ctagtACAGGGCATATTGATAAGTCAAtctcgctgatcgccaccatg gggggtgggtgatcagcgctaTGACTTATCAATATGCCCTGTg
2	Cr_PFL1_amiRNA_A_Rev	ctagcACAGGGCATATTGATAAGTCAtagcgctgatcaccaccac cccatggtgccgatcagcgagaTGACTTATCAATATGCCCTGTa
5	Cr_PDC_amiRNA_A_For	ctagtGAACGGGCTTGTTGAGATCGAtctcgctgatcgccaccatg gggggtgggtgatcagcgctaTCGATCTCAACAAGCCCGTTCg
6	Cr_PDC_amiRNA_A_Rev	ctagcGAACGGGCTTGTTGAGATCGAtagcgctgatcaccaccac cccatggtgccgatcagcgagaTCGATCTCAACAAGCCCGTTCa

Table 2.10: Oligonucleotides used for construction of artificial micro RNA vectors used in this work.

In the name the suffix stands for forward (For) or reverse (Rev) strands. In lowercase is the conserved amiRNA sequence including the hairpin loop region, and in capitals is the unique amiRNA targeting sequence.

#	Name	Source	Promoter	Reference
23	pRNAi3	Dr. Attila Molnár (CAM)	PSAD	(Molnár et al., 2009)

Table 2.11: Parental amiRNA vectors used in this work

#	Plasmid name	Description
24	pRNAi3.4A	Oligonucleotide 1 and 2 annealed and inserted into pRNAi3 vector (Par ^R) targeting PFL1.
25	pRNAi3.6A	Oligonucleotide 5 and 6 annealed and inserted into pRNAi3 vector (Par ^R) targeting PDC3.

Table 2.12: Sequence specific amiRNA vectors used in this work.

Oligonucleotides referred to can be found in Table 2.8, all plasmids contain the *aphVIII* gene conferring resistance to paromomycin (Par^R)

2.9.1 Design of amiRNA oligonucleotide sequences

A BLAST search using target mRNAs was performed against the *Chlamydomonas* genome using web based tool WMD3 (See section 2.1). The FASTA definition line was pasted along with the target sequence into the designer tool. The best potential oligonucleotides for knockdown were selected in accordance with the following criteria where possible (personal correspondence with Dr. Attila Molnár); those with a hybridization energy between -45.73 and -40.88 kcal mol⁻¹ and with 0 to 2 mismatches, preferably one at position 3 from the very 3' end of amiRNA). 90nt single-stranded (ss) forward and reverse oligonucleotides were designed for cloning into amiRNA vectors the using the oligonucleotide design tool, sent for synthesis (Sigma-Aldrigh Ltd, UK) and re-suspended to 100 µM in dH₂O.

2.9.2 Generation of double stranded amiRNA oligonucleotides

To anneal complementary single-stranded oligonucleotides, 20 µl of 2x annealing buffer (20 mM Tris-Cl pH 8.0, 2 mM EDTA, 100 mM NaCl) was mixed with 10 µl of forward and reverse ssDNA amiRNA oligonucleotides (100 µM), boiled in a water bath for 5 min and left to cool gradually overnight. The resulting dsDNA oligonucleotides were purified by PCR Cleanup kit (QIAGEN, Germany) according to the manufacturer's instructions, and eluted into a volume of 40 µl dH₂O.

2.9.3 Phosphorylation of amiRNA double stranded oligonucleotides

1 µl ds amiRNA oligonucleotide was phosphorylated in a reaction mix containing 1 µl T4 PNK (M0201S; New England Biolabs Inc., USA; 10 U µl⁻¹), 1 µl 10x ligase buffer (New England Biolabs Inc., USA; 500 mM Tris-HCl, 100 mM MgCl₂, 10 mM ATP, 100 mM dithiothreitol, pH 7.5 at 25 °C) and 7µl dH₂O, incubated at 37°C for 30 min and heat inactivated at 65°C for 20 min.

2.9.4 amiRNA vector digestion and dephosphorylation

1 µg of amiRNA vector was digested in a mix of 20 U of SpeI (R0133S; New England Biolabs Inc.), 5 µl 10x BSA (Final [100 µg ml⁻¹]; New England Biolabs Inc., USA), 5 µl 10x NEB buffer 2 (500 mM NaCl, 100 mM Tris-HCl, 100 mM MgCl₂, 10 mM Dithiothreitol pH 7.9 at 25°C; New England Biolabs Inc., USA) and 18 µl of dH₂O at 37°C for 1 h. To dephosphorylate the linear vector, 20 U of calf intestinal phosphatase (M0290S; New England Biolabs Inc., USA) was added to the restriction digest mix and incubated at 37°C for 30 min. The product was gel purified as described previously (see section 2.7.4) and re-suspended in 25 µl of dH₂O to a concentration of ~20 ng µl⁻¹.

2.9.5 amiRNA vector ligation

1 µl 100x diluted phosphorylated ds oligonucleotide was mixed with 20 ng of digested dephosphorylated vector, 5 µl 2x Quick Ligase buffer (132 mM Tris-HCl, 20 mM MgCl₂, 2 mM dithiothreitol, 2 mM ATP, 15% polyethylene glycol (PEG 6000) pH 7.6 at 25 °C; New England Biolabs Inc., USA), 0.5 µl Quick Ligase (M2200S; New England Biolabs Inc., USA), 2.5 µl dH₂O and incubated at room temperature for 15 min. Transformations were performed as in section 2.6.1 but with the exception that 200 µl of 10-β chemically competent cells (C3019H; New England Biolabs Inc., USA) were transformed per ligation.

2.9.6 Colony PCR screening of amiRNA vector inserts

Individual colonies were picked with sterile tooth picks into PCR tubes filled with 25 µl of dH₂O, and stabbed onto a reference plate (LB plus carbenicillin 50 µg ml⁻¹). 35 µl of PCR mix containing 10 µM forward primer and reverse primers, 1.75 U Taq DNA polymerase (M0273S; New England Biolabs Inc., USA), 40 µl FailSafeTM PCR Buffer G (FSP995G; Epicentre Biotechnologies, USA) and 14 µl dH₂O was added to each tube and run through a PCR program of 95°C for 2 min (cell lysis and DNA denaturation), followed by 35 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 30 s. 10 µl of each PCR reaction was loaded onto a 2% (w/v) agarose gel for analysis, positive clones were selected and sent for sequencing.

2.9.7 Isolation of *C. reinhardtii* knockdown cell lines

amiRNA vectors were transformed into *C. reinhardtii* by biolistic bombardment as described in 2.6.2. Individual paromomycin resistant colonies were picked and grown in TAP media until late log phase. 1 ml of cells were harvested, (16000g, 2 min, 25°C; accuSpin Micro; Fisher Scientific, USA), re-suspended directly in SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v) and 10% β -mercaptoethanol (v/v)) and a sample equivalent to 8×10^5 cells (as calculated by standard curve comparing OD₇₅₀ to cell number) was loaded onto 12.5% polyacrylamide gels for immunoblot analysis.

2.10 Protein analysis

2.10.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared for SDS-PAGE analysis through addition of 2x sample buffer (125 mM Tris-HCl, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v) and 10% β -mercaptoethanol (v/v), pH 6.8) then incubating at 100°C for 5 min followed by centrifugation (16000g, 5 min, 25°C; accuSpin Micro; Fisher Scientific, USA) to pellet insoluble material. For analysis of *C. reinhardtii* cell extracts 1 ml aliquots were spun down (16000g, 2 min, 25°C; accuSpin Micro; Fisher Scientific, USA), the supernatant discarded, and the pellet either resuspended in (25 mM HEPES, 300 mM Sorbitol, 10 mM NaCl, 5 mM CaCl₂, 10 mM NaHCO₃, pH 7.2) and frozen at -20°C or directly in 1x SDS-sample buffer (63 mM Tris-HCl, 10% glycerol (v/v), 2% SDS (w/v), 0.05% bromophenol blue (w/v) and 5% β -mercaptoethanol (v/v), pH 6.8), heated at 100°C for 5 min, spun down (16000g, 5 min, 25°C; accuSpin Micro; Fisher Scientific, USA) then frozen at -20°C. Samples were either stored at -20°C for future use or loaded onto a self cast, 0.75 mm polyacrylamide separation gel (35% (v/v) Acrylogel 2.6 (40% solution; 443745V; BDH), 0.375 M Tris-HCl pH 8.9, 0.01% (w/v) N,N,N',N' tetramethylenediamine (TEMED), 0.2% (w/v) ammonium persulphate (APS)) overlaid with a stacking gel (13.5% (v/v) Acrylogel 2.6 (40% solution; BDH; 443745V), 0.125 M Tris-HCl pH 6.8, 0.01% (w/v) N,N,N',N' tetramethylenediamine (TEMED), 0.8% (w/v) APS). The gels were run at room temperature in the Bio-Rad Mini-PROTEAN III gel system (Bio-Rad Laboratories Inc., USA) (running buffer; 25 mM Tris, 190 mM glycine,

0.1% SDS (w/v), pH 8.3) with a constant voltage of 110 V for 4 h using a Biorad PowerPac BasicTM (Bior-Rad, USA).

2.10.2 One dimensional (1D) blue native-PAGE

BN-PAGE analysis (Schägger and von Jagow, 1991; Schägger et al., 1994), was performed according to Sirpiö et al. (2011). For preparation of samples for BN analysis all steps were done under green light at 4°C, unless otherwise stated. 500 ml of early log-phase *C. reinhardtii* CC-124 cultures, grown in TAP medium, were harvested (2500g, 5 min, 4°C; Allegra 6R, Beckman Coulter, USA), the supernatant discarded and the pellet resuspended in 5 ml of ice-cold breaking buffer, which for membrane fraction analysis was (50 mM HEPES-KOH pH 7.5, 330 mM Sorbitol, 2 mM EDTA, 0.05% BSA, 10 mM NaF) or for soluble fraction analysis was (50mM Tris-HCl, 5ml MgCl₂ pH 7.0), to a chlorophyll content of ~200 µg ml⁻¹. Cells were broken by three passes through a nebulizer (BioNeb; Glas-Col, USA) using N₂ gas at a pressure of ~30 psi, samples were check under a microscope to verify complete breakage. Membranes were spun down (15 min, 100000g, 4°C; Beckman L8-70M ultracentrifuge), the supernatant kept as a soluble fraction. For membrane fraction analysis the pellet was washed with storage buffer (50 mM HEPES-KOH pH 7.5, 100 mM Sorbitol, 10 mM MgCl₂, 10mM NaF) (15 min, 100000g, 4°C; Beckman L8-70M ultracentrifuge). The resulting pellet was re-suspended in buffer 25BTH20G (25 mM BisTris-HCl pH 7.0, 20% glycerol (v/v), 0.25 µg ml⁻¹ Pefabloc, 10 mM MgCl₂, 10 mM NaF) to a chlorophyll concentration of 1 mg ml⁻¹, then diluted to 0.5 mg Chl ml⁻¹ with solubilisation buffer (25 mM BisTris-HCl pH 7.0, 40% (v/v) glycerol, 250 µg ml⁻¹ Pefabloc, 10 mM MgCl₂, 2% (w/v) β-DM)). Samples were incubated on ice for 5 min then spun (16000g, 5 min, 4°C; accuSpin Micro; Fisher Scientific, USA) to pellet insoluble material. The supernatant was transferred to a new centrifuge tube and 10x sample buffer (200 mM BisTris-HCl pH 7.0, 75% (w/v) sucrose, 1 M ε-amino caproic acid and 5% (w/v) Serva blue G) was added before loading. In the case of the soluble fraction, 20% (v/v) glycerol was added to samples with a protein concentration of ~ 2 mg ml⁻¹ prior to addition of 10x sample buffer (200 mM BisTris-HCl pH 7.0, 75% (w/v) sucrose, 1 M ε-amino caproic acid and 5% (w/v) Serva blue G) and ~20 µg protein loaded. 5 to 12.5% (w/v), 0.75 mm continuous polyacrylamide gradient gels were created using a gradient maker (Model 385 gradient former; Bio-Rad

Laboratories Inc., USA). The lighter 5% (w/v) acrylamide solution (5% (v/v) acrylamide (acrylamide/bisacrylamide 32:1 stock solution) 50 mM BisTris-HCl pH 7.0, 500 mM ϵ -amino caproic acid, 0.05% (v/v) TEMED and 0.015% (w/v) APS) was loaded into the first chamber of the gradient maker, and the heavier 12.5% (w/v) solution (12.5% (v/v) acrylamide (acrylamide/bisacrylamide 32:1 stock solution), containing 50 mM BisTris-HCl pH 7.0, 500 mM ϵ -amino caproic acid and 20% (v/v) glycerol, 0.05% (v/v) TEMED and 0.015% (w/v) APS) was loaded into the outer chamber. Gel solutions were continuously mixed by magnetic stirrer and pumped into the bottom of multi-gel caster (165-4110; Mini-PROTEAN 3 Multi-Casting Chamber; Bio-Rad Laboratories Inc., USA) using a peristaltic pump (Model P-1 peristaltic pump, Pharmacia Fine Chemicals) with the low percentage solution entering the gel caster first. In the multi-gel caster acrylamide solutions were overlaid with a thin covering of isopropanol to ensure a flat surface was created. After gels were set the isopropanol was rinsed off, the top of the gel was dried using Whatman paper, and a 4% (w/v) acrylamide gel (50 mM BisTris-HCl pH 7.0, 500 mM ϵ -amino caproic acid, 0.05% (v/v) TEMED and 0.015% (w/v) APS) was applied on top, using a 10 well comb to form sample wells.

Gels were run in a Bio-Rad Mini-PROTEAN III gel system (Bio-Rad Laboratories Inc., USA) with native gels suspended between a coloured cathode buffer (50 mM Tricine, 15 mM BisTris-HCl pH 7.0, 0.02% (w/v) Coomassie Brilliant Blue G-250) and anode buffer (50 mM BisTris-HCl pH 7.0). Gels were run using an electrophoresis power supply (EPS 601; Amersham Biosciences) at 70 V for 30 min, 100 V for 30 min, 125 V for 30 min, 150 V for 30 min. The blue cathode buffer was then replaced with a colourless cathode buffer (50 mM Tricine, 15 mM BisTris-HCl, pH 7.0) and run for a further 30 min at 150 V, followed by 175 V for 30 min then 200 V until the sample reached the end of the gel. Gels were then cut into strips corresponding to a single lane and stored between two sheets cut from A4 clear plastic wallets at -20°C before further analysis.

2.10.3 Two dimensional (2D) SDS-PAGE

Strips cut from 1D BN-PAGE gels (produced as described in 2.10.4) were incubated in solubilisation buffer (0.5 M Tris-HCl pH 6.8, 6 M urea, 23% (v/v) glycerol, 20% (w/v) SDS, 5% (v/v) β -mercaptoethanol) for 60 min at room

temperature. Second dimension gels were 1 mm thick 12% (w/v) polyacrylamide gels prepared in the same manner as for 1D SDS-PAGE analysis (section 2.10.2) with the following exceptions: 6 M urea was dissolved in the separating gel prior to calibrating the pH, the stacking gel had no wells and sufficient space was left above to be overlaid with 1D BN strips. A pre-stained protein marker was applied by soaking a small square of Whatman paper with 5 μ l of ladder and positioning on top of the stacking gel. Both 1D BN strips and ladder were then sealed in place with 0.5% (w/v) agarose dissolved in running buffer (25 mM Tris-HCl, 190 mM glycine, 0.1% (w/v) SDS, pH 8.3). The gel was run at room temperature and a constant voltage of 110 V for 4 h in a Bio-Rad Mini-PROTEAN III gel system (Bio-Rad Laboratories Inc., USA) using a Biorad PowerPac BasicTM (Bio-Rad Laboratories Inc., USA). The gels were then stained with Coomassie-brilliant-blue-R250 (section 2.10.4) prior to performing a silver stain (section 2.10.5), or used for immunoblot (see section 2.10.6).

2.10.4 Coomassie-brilliant-blue-R250 staining solution

Gels were incubated over night with gentle shaking in Coomassie staining solution (40% ethanol (v/v), 10% acetic acid (v/v), 0.2% Coomassie-brilliant-blue-R250 (w/v)), then Coomassie unbound to protein was removed by incubation in destain solution (10% acetic acid). Finally digital photographs were taken using a 8.0 mega pixels Canon PowerShot Pro1 (Canon, UK).

2.10.5 Silver stain

SDS-PAGE gels were incubated in fixing solution (40% (v/v) methanol, 10% (v/v) acetic acid) overnight at room temperature. Gels were washed 3 times in 30% ethanol for 20 min, reduced for 1 min (0.02% (w/v) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) then washed 3 times in H_2O for 20 s. Gels were then incubated in fresh silver solution (0.2% (w/v) AgNO_3 , 0.02% (v/v) formaldehyde) for 20 min, washed 3 times in H_2O for 20 s then incubated in fresh developing solution (3% (w/v) Na_2CO_3 , 0.05% (v/v) formaldehyde, 0.0005% (w/v) $\text{Na}_2\text{S}_2\text{O}_3$) for approximately 3–5 min or until bands become clearly visible. The staining reaction was stopped by discarding developing solution and incubating in 0.5% (w/v) glycine for 5 min. Stained gels were then washed in H_2O for 30 min and a photograph taken as describe in section 2.10.4.

2.10.6 Immunochemical analysis

For immunochemical analysis SDS-PAGE gels were transferred onto polyvinylidene fluoride (PVDF) membranes using an iBlot® transfer stack, regular (IB4010-01; Invitrogen Life Technologies, USA, Life Technologies, USA) on an iBlot® dry blotting system (IB1001UK, Invitrogen Life Technologies, USA, Life Technologies, USA) for 7 min, according to the manufacturer's instructions. Or alternatively, wet transfer of proteins (Towbin et al., 1979; Burnette, 1981) onto nitrocellulose membrane (0.2 µm; 162-0112; Bio-Rad Laboratories Inc., USA) was performed using a mini trans-blot cell (170-3930; Bio-Rad Laboratories Inc., USA), at 100 V, 350 mA for 60 min in transfer buffer (3 mM Na₂CO₃, 10 mM NaHCO₃ and 20% (v/v) methanol).

Membranes were blocked for 1 h with PBS-T (150 mM NaCl, 7.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄ and 0.1% (v/v) Tween 20) with 5% (w/v) milk powder, rinsed with water to remove residual milk then incubated overnight at 4 °C on a shaker with a primary antibody (Table 2.7). On the following day the membrane was washed three times for 20 min in PBS-T then incubated for 1 h at room temperature with a 1:10000 dilution in PBS-T of anti-rabbit IgG secondary antibody conjugated to horse radish peroxidase (NA934V; GE Healthcare, UK). Membranes were then washed four times for 10 min in PBS-T and once for 10 min in PBS to remove residual secondary antibody. Immunodetection was performed by enhanced chemiluminescence procedure (Whitehead et al., 1983). The membrane was incubated for 10 s in a 1:1 mixture of ECL reagent A (100 mM Tris-HCl pH 8.3, 0.4 mM p-coumaric acid, 2.5 mM luminol) and reagent B (100 mM Tris-HCl pH 8.3, 100 mM H₂O₂), put into a clear A4 plastic wallet and exposed for 10 s to 10 min to a X-Ray film (MOL7016; SLS Laboratories, UK). The film was developed on a Curix60 table top processor (AGFA Healthcare, Belgium) according to the manufacturer's instructions.

Antibody Name	Final Bleed #	Antigen	Working Dilution	Source
α PFL1	8219*	<i>C. reinhardtii</i> PFL1	1:3000	This work
α PDC3	8210*	<i>C. reinhardtii</i> PDC3	1:2000	This work
α PFOR	9157*	<i>C. reinhardtii</i> PFOR	1:2000	This work
α HYD1	8217*	<i>C. reinhardtii</i> HYDA1 and HYDA2	1:5000	This work
α LDH	8213*	<i>C. reinhardtii</i> LDH	1:5000	This work
α ADH1	10022*	<i>C. reinhardtii</i> ADH1	1:2000	This work
α PDC2	10024*	<i>C. reinhardtii</i> PDC2	1:10000	This work
α PDC1	9012/ 9013	<i>C. reinhardtii</i> PDC1	1:10000	This work
α ATPase	n/a	<i>C. reinhardtii</i> COXIIb	1:10000	Agrisera
α NAB1	n/a	<i>C. reinhardtii</i> NAB1	1:10000	(Mussgnug et al., 2005)
α D1	n/a	<i>Pisum sativum</i> PsbA	1:10000	P. Nixon

Table 2.13: List of polyclonal antibodies used in this work.

All anti-sera was raised in rabbits and used in conjunction with anti-rabbit secondary antibody for immunodetection. Final bleed number refers to identifier given to the third bleed selected for affinity purification, all were affinity purified with the exception of PDC1. Dilutions were performed in PBS-T.

2.10.7 Protein concentration determination

Samples were treated with 80% (v/v) acetone to precipitate protein, spun down (16000g, 5 min, 4°C; accuSpin Micro; Fisher Scientific, USA) and re-suspended in 0.4% (w/v) SDS. Protein concentration was then determined by Bradford assay (Lowry et al., 1951) (DC Protein Assay; Bio-Rad Laboratories Inc., USA).

2.11 Biochemical Techniques

2.11.1 Isolation of *C. reinhardtii* chloroplasts and mitochondria

The procedure for chloroplast isolation was adapted from Atteia et al. (2006), Mason et al. (2006) and Naumann et al. (2005). *C. reinhardtii* strain *cw15* mt- was grown in TAP medium at 25°C, under 12 h/12 h light/dark cycles for 72 h to a density of $2-6 \times 10^6$ cells ml⁻¹, diluting when necessary. 2 h into the light period of the 3rd cycle cultures were purged for 4 h with Argon for anaerobic induction. Chloroplasts were isolated according to Naumann et al. (2005) and mitochondria were prepared according to van Lis et al. (2005). Briefly cells were re-suspended in buffer A (0.25 M sorbitol, 50 mM Tris-HCl pH 7.2, 50 mM MES-KOH, 3 mM EDTA, 3 mM KH₂PO₄) to a density of 7×10^7 cells ml⁻¹ and lysed with a nebulizer (BioNeb; Glas-Col, USA) using N₂ gas at a pressure of ~20 psi. The resulting lysate was centrifuged to pellet whole cells and chloroplasts (750g, 5 min; Eppendorf 5810R) which were saved for

chloroplast isolation. The supernatant was spun to remove residual chloroplasts and unbroken cells (2500g, 30 min; Eppendorf 5810R), and the supernatant spun down to pellet mitochondria (9000g, 30 min; Sorvall RC6+). The pellet containing mitochondria was re-suspended in 20% Percoll solution (20% Percoll (v/v), 0.25 M Sorbitol, 10 mM MOPS, 0.5% PVP40, 0.1% BSA, 1 mM EDTA, pH 7.2) and spun at 15000g, 4°C, for 40 min. Mitochondria were extracted from the bottom of the gradient, re-suspended in buffer B (0.25 M sorbitol, 10 mM potassium phosphate buffer 1 mM EDTA pH 7.2) and spun down (9000g, 10 min, 4°C; Sorvall RC 6+). The resulting pellet was re-suspended in buffer B and stored at -20°C.

Chloroplasts and unbroken cells were re-suspended in buffer A (0.25 M sorbitol, 50 mM Tris-HCl, 50 mM MES-KOH, 3 mM EDTA, 3 mM KH₂PO₄, pH 7.2), loaded on top of a three step 40:60:80% (v/v) Percoll gradient diluted in buffer A (250 mM sorbitol, 50 mM Tris-HCl, 50 mM MES-KOH, 3 mM EDTA, 3 mM KH₂PO₄, pH 7.2) and centrifuged (8000g, 20 min, 4°C; Model J2-21 centrifuge; Beckman, USA). Intact chloroplasts were harvested from the 40:60% (v/v) interface, diluted in 30 ml of buffer A (250 mM sorbitol, 50 mM Tris-HCl, 50 mM MES-KOH, 3 mM EDTA, 3 mM KH₂PO₄, pH 7.2) and centrifuged (2000g, 10 min, 4°C; Eppendorf 5810R). The supernatant was discarded and chloroplasts were re-suspended in breaking buffer (250 mM sorbitol, 50 mM Tris-HCl, 50 mM MES-KOH, 3 mM EDTA, 3 mM KH₂PO₄, pH 7.2) to the desired concentration.

2.11.2 Isolation of *C. reinhardtii* cytoplasmic fraction

To isolate the cytoplasmic fraction, cells were grown and hypoxia induced as described in 2.11.1. Cells were re-suspended in breaking buffer II (0.25 M Sorbitol, 50 mM Tris, 50 mM MES, 10 mM MgCl₂, 3 mM EDTA, 3 mM KH₂PO₄, pH 7.2) to a density of ~0.2 mg Chl ml⁻¹ and were broken with a single pass through a 27” gauge needle (Mason et al., 2006), centrifuged (650g, 30 min; Eppendorf 5810R) to remove unbroken cells, chloroplasts and thylakoid membranes, the supernatant collected and spun down (9000g for 30 min; Sorvall RC6+) which was repeated to remove any residual contaminating mitochondria. Cytoplasmic fraction was collected from the resulting supernatant by mixing with 20% trichloroacetic acid at a 1:1 ratio and incubated on ice for 20 min and the precipitate was pelleted (16000g, 10 min, 4°C; accuSpin Micro; Fisher Scientific, USA) and washed in acetone (-20°C) before

spinning again (16000g, 5 min, 25°C; accuSpin Micro; Fisher Scientific, USA). Acetone was removed and the pellet air dried before re-suspending in breaking buffer with the addition of 10% SDS solution until the protein solubilised.

2.12 Hydrogen measurements

2.12.1 Transient light-induced H₂ production

H₂ measurements were performed using a modified Clark electrode (S1 electrode disc; Hansatech Instruments Ltd, UK) poised to measure H₂, connected to a computer controlled Oxylab unit by a S1/ADL SMB Mini DIN Cable with reversed polarity (Hansatech Ltd, UK), all data were analysed on Oxylab32 software (Hansatech Instruments Ltd, UK). Samples were stirred at 100 rpm and the electrode chamber maintained at 25°C by a circulating water bath. Calibration measurements were performed by addition of known volumes of H₂-saturated water to 1 ml of argon-purged H₂O, using a gas tight Hamilton syringe. H₂ measurements were performed as in Mus et al. (2007) with a few adjustments. Cells were grown to late log phase (OD₇₅₀ of 1-1.2) and re-suspended in an anaerobic induction buffer (AIB) (50 mM KH₂PO₄ pH 7.0, 3 mM MgCl₂) to a Chl concentration of ~200 µg Chl ml⁻¹. Cells were then placed in 5 ml glass vials sealed with a rubber SubaSeal® (Z124583; Sigma-Aldrich, UK) and wrapped in aluminium foil. Vials were then purged with argon gas for 10 min and the cells incubated anaerobically in the dark, agitated magnetic stirrer at 150 rpm for 4 h. The Clark electrode chamber was filled with 750 µl of 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer pH 6.8, sealed with a silicon septum and de-oxygenated for 10 min by bubbling with argon. Under green light 750 µl of anaerobically adapted cells were transferred to the sealed chamber by a 1 ml gas-tight Hamilton syringe. Samples were stirred at 90 rpm and left in the dark for 2 min, once the signal stabilised cells were illuminated at 1000 µE m⁻² s⁻¹ actinic light to illicit photo-induced H₂ production. H₂ production rate was calculated as described in Appendix II.3.

2.12.2 H₂ measurements following sulphur-deprivation

Cultures were grown to late log phase (OD₇₅₀ ~1.2) spun down (3000g, 10 min, 25°C; Model J2-21 centrifuge; Beckman, USA), washed once then re-suspended in 325 ml TAP-S where the sulphate salts are replaced with chloride

derivatives (Melis et al., 2000) to a chlorophyll content of $\sim 15\text{--}20\ \mu\text{g ml}^{-1}$. Cultures were then placed in custom-made mini-bioreactors (Nguyen et al., 2008) (Cambridge Glassblowing Ltd, UK) (Appendix II.4) and incubated at 25°C under constant stirring and illumination with warm white fluorescent tubes (F18W/29 T8; Koninklijke Philips Electronics N.V.) at an average light intensity of $\sim 140\ \mu\text{E m}^{-2}\ \text{s}^{-1}$. Gas evolution was measured by water displacement, headspace samples were taken with a 1 ml gas tight Hamilton syringe and the concentration of hydrogen determined by a injection onto a GC (GC2014; Shimadzu, Japan) fitted with a gas sampling valve set to an injection volume of $200\ \mu\text{l}$. Using research grade Helium as a carrier gas (285366-L; BOC, UK) at a flow rate of $30\ \text{ml min}^{-1}$, CO_2 , H_2 , O_2 and N_2 were separated by CTR1 concentric packed column (35°C ; 8700; Alltech, UK) and detected by a thermal conductivity (TCD) detector (110°C). Concentration of H_2 was determined by comparison to a calibrant gas standard containing 80% H_2 , 10% N_2 and 10% CO_2 (Appendix II.4).

2.13 Excreted metabolite analysis

2.13.1 Metabolite analysis by nuclear magnetic resonance spectroscopy (NMR)

Samples were prepared for metabolite analysis by passing 5 ml aliquots of *C. reinhardtii* cultures through a disposable filter (46652; $0.2\ \mu\text{m}$ Acrodisc® syringe filters with supor membrane; PALL, USA) to remove whole cells and cellular debris. Analysis of metabolite production was performed by NMR in collaboration with Dr. Jacob Bundy (Imperial College). To each supernatant sample ($450\ \mu\text{l}$) was added 1 M phosphate buffer, pH 7.0 ($30\ \mu\text{l}$) and a D_2O solution ($120\ \mu\text{l}$) containing 5 mM trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP) and 25 mM NaN_3 . Solutions ($550\ \mu\text{l}$) were then transferred to clean 5 mm NMR tubes.

Spectra were acquired on a Bruker Avance DRX600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), with ^1H frequency of 600 MHz. Samples were introduced with an automatic sampler and spectra were acquired following the procedure described by Beckonert et al. 2007. Briefly a one-dimensional NOESY sequence was used for water suppression; data were acquired in 64 K data points over a spectral width of 12 kHz, with 8 dummy scans and 256 scans per sample.

Spectra were processed in iNMR 2.6.3 (Nucleomatica, Molfetta, Italy). Fourier transform of the free-induction decay was applied with a line broadening of 0.5 Hz. Spectra were manually phased and automatic first order baseline correction was applied to all samples. Metabolite peak areas were quantified with the Chenomx NMR suite 7.0 (Chenomx, Inc., Edmonton, Alberta, Canada) relative to TSP and concentrations were normalised to chlorophyll content.

2.13.2 Metabolite analysis by high pressure liquid chromatography (HPLC)

HPLC analysis was performed as previously described (Mus et al., 2007) using a Hewlett Packard 1050 HPLC, with minor modifications. Cells were incubated under dark-anaerobic conditions as described in section 2.12.1. After 4 h the chlorophyll content was determined (see section 2.4), and 1.5 ml of cultures spun down (16000g, 2 min; accuSpin Micro; Fisher Scientific, USA). The supernatant was collected and filtered using a disposable syringe filter to remove residual cell debris (46652; 0.2 µm Acrodisc® syringe filters with supor membrane; PALL, USA), then stored at -20°C. At time of use, samples were thawed and 200 µl aliquots of filtered supernatant samples were injected onto an Aminex ® HPX-87H anion exchange column (300 x 7.8 mm) (Bio-Rad Laboratories, Inc., USA), and eluted with 8 mM H₂SO₄ (at a flow rate of 0.5 ml min⁻¹, 45°C). Organic acids were identified by UV detector and ethanol using a refractive index (RI) detector. Data was processed on Agilent ChemStation software and the concentrations of metabolites were calculated by comparison to known amounts of standards.

Chapter 3: Characterisation of enzymes involved in

C. reinhardtii pyruvate metabolism

3.1 Introduction

When sealed cultures of *C. reinhardtii* are deprived of sulphur in the light, they evolve H₂, with reductant provided by a mixture of photosynthetic water splitting and fermentation of endogenous starch reserves (Melis et al., 2000). One proposed method to increase H₂ yields is to redirect metabolism to H₂ production by switching off competing fermentative pathways (Mus et al., 2007), as has been demonstrated in bacterial H₂ production systems (Maeda et al., 2007).

During anoxia *C. reinhardtii* is known to activate a range of fermentation pathways producing ethanol, formate and acetate in addition to H₂ (Gfeller and Gibbs, 1984; Mus et al., 2007), with lactate accumulation observed when formate production is blocked, or at low pH (Kreuzberg, 1984). Biochemical assays initially suggested the presence of a PFL, at least one ADH, which could utilise both NADH and NADPH, an NAD⁺-dependent D-LDH dehydrogenase, and a PDC (Kreuzberg et al., 1987). Further studies identified a bi-functional alcohol-acetaldehyde dehydrogenase sequence in the *C. reinhardtii* genome (Atteia et al., 2003), then the PFL1 cDNA was isolated and the gene product characterised (Atteia et al., 2006). The release of the *C. reinhardtii* genome sequence (Merchant et al., 2007) allowed for the construction of preliminary models of fermentative metabolism in *C. reinhardtii* (Grossman et al., 2007) (see section 1.6.8), which was further supported by transcript and metabolite analysis during dark-anaerobic incubation (Mus et al., 2007).

However, it is not fully understood what, if any, role is played by fermentative metabolism in *C. reinhardtii* during vegetative growth, how fermentative metabolism is controlled and the link with H₂ production. When attempting to increase H₂ yields by switching off fermentative pathways, it is important to develop a detailed metabolic model and gain a greater understanding of the role of fermentation in *C. reinhardtii*, to make predictions about where metabolic flux may be directed and any potential impact upon cell fitness.

The aims of this chapter therefore included performing an in-depth analysis of the *C. reinhardtii* genome sequence to look for evidence of additional gene products that may be involved in pathways that could compete with the hydrogenase for reductant. This would be followed by selection of key enzymes for immunochemical

analysis, linking gene sequence to protein expression, and probing the role of fermentation in *C. reinhardtii* during different growth conditions and hydrogen production.

3.2 Bioinformatic analysis of pyruvate degrading and fermentative enzymes in *C. reinhardtii*

In order to select fermentative enzymes for further study, the genome was analysed for enzymes involved in pathways that could compete with H₂ production (Table 3.1). Formate metabolism has been well characterised previously and was found to be solely the result of a single pyruvate formate lyase (PFL1), no formate hydrogen lyase sequence appears to be present in the genome (Atteia et al., 2006). Similarly thorough analysis of the genome previously revealed the presence of just two [FeFe]-hydrogenases (*HYDA1* and *HYDA2*) (Forestier et al., 2003). Therefore formate and hydrogen production pathways were not subjected to further bioinformatic analysis.

Enzyme	Gene Name	Phytozyme ID	Chlre v4 Protein ID	Predicted location ChloroP/TargetP	Predicted size (kDa)	IUBMB	EST	Reference
Acetyl CoA synthetase	ACS1	Cre23.g765700	194063	-/-	69.0	EC 6.2.1.1	Y	(Spalding et al., 2009)
Acetyl CoA synthetase	ACS2	Cre23.g768500	-	C/C	50.4	n/a	Y	(Spalding et al., 2009)
Acetyl CoA synthetase	ACS3	Cre07.g353450	196311	-/-	73.2	EC 6.2.1.1	Y	(Spalding et al., 2009)
Acetate kinase	ACK1	Cre09.g396700	129982	C/C	45.5	EC 2.7.2.1	Y	(Dubini et al., 2009; Spalding et al., 2009; Terashima et al., 2010)
Acetate kinase	ACK2	Cre17.g709850	128476	-/S	43.4	EC 2.7.2.1	Y	(Dubini et al., 2009; Terashima et al., 2010)
Alanine aminotransferase	AAT1	Cre10.g451950	206184	-/M	57.8	EC 2.6.1.2	Y	(Chen et al., 1996; Doebbe et al., 2010)
Alanine aminotransferase	AAT2	Cre06.g284700	191703	-/-	56.6	EC 2.6.1.2	N	
Dual function alcohol/ acetaldehyde dehydrogenase	ADH1	Cre20.g758200	133318	C/M	102.2	EC 1.1.1.1	Y	(Kreuzberg et al., 1987; Atteia et al., 2003; Hemschemeier and Happe, 2005; Atteia et al., 2006; Grossman et al., 2007; Mus et al., 2007)
Fe-dependent alcohol dehydrogenase	ADH2	Cre16.g669100	-	C/C	87.0	EC 1.1.1.1	Y	(Posewitz et al., 2009)
Fe-dependent alcohol dehydrogenase	ADH5	Cre16.g669150	-	-/-	35.8	EC 1.1.1.1	Y	(Posewitz et al., 2009)
Aldehyde dehydrogenase	ALD5	Cre12.g500150	135609	-/M	57.9	EC 1.2.1.3	Y	(Dubini et al., 2009; Wood and Duff, 2009)
[FeFe]-hydrogenase	HYDA1	Cre03.g199800	183963	C / C	53.1	EC 1.12.7.2	Y	(Happe and Kaminski, 2002; Winkler et al., 2002; Forestier et al., 2003)
[FeFe]-hydrogenase	HYDA2	Cre09.g396600	24189	C / M	53.7	EC 1.12.7.2	Y	(Forestier et al., 2003)
[FeFe]-hydrogenase assembly factor	HYDEF	Cre06.g296750	145064	C/C	121.9	n/a	Y	(Posewitz et al., 2004a)
[FeFe]-hydrogenase assembly factor	HYDG	Cre06.g296700	378585	-/M	63.7	n/a	Y	(Posewitz et al., 2004a)
NAD(P)H dependent D-lactate dehydrogenase	LDH	Cre07.g324550	182520	C / C	45.6	EC 1.1.1.28	Y	(Gfeller and Gibbs, 1984; Husic and Tolbert, 1985)
FAD dependent D-lactate dehydrogenase	LDHc	Cre10.g434900	-	C/ M	53.3	EC 1.1.2.4	Y	(Beezley et al., 1976)
FAD dependent D-lactate dehydrogenase	LDHc	Cre08.g370550	-	-/M	59.2	EC 1.1.2.4	N	

Table 3.1 (Continued)

Enzyme	Alias	Phytozyme ID	Chlre v4 Protein ID	Predicted location ChloroP/TargetP	Predicted size (kDa)	IUBMB	EST	Reference
FAD dependent D-lactate dehydrogenase	LDHc	Cre10.g434900	11226	C/M	53.3	EC 1.1.2.4	N	
NAD ⁺ dependent malate dehydrogenase	MDH1	Cre03.g194850	137163	-/M	36.6	EC 1.1.1.37	Y	(Dubini et al., 2009)
NAD ⁺ dependent malate dehydrogenase	MDH2	Cre10.g423250	126023	-/-	36.4	EC 1.1.1.37	Y	(Dubini et al., 2009)
NAD ⁺ dependent malate dehydrogenase	MDH3	Cre02.g145800	158129	-/S	38.1	EC 1.1.1.37	Y	(Dubini et al., 2009)
NAD ⁺ dependent malate dehydrogenase	MDH4	Cre12.g483950	60444	C/M	34.4	EC 1.1.1.37	Y	(Dubini et al., 2009)
NADP ⁺ dependent malate dehydrogenase	MDH5	Cre09.g410700	192083	C/M	44.8	EC 1.1.1.82	Y	(Dubini et al., 2009)
Malate dehydrogenase, decarboxylating	MME1	Cre06.g268750	196833	-/M	69.8	EC 1.1.1.39	Y	(Dubini et al., 2009)
NADP ⁺ malic enzyme	MME2	Cre14.g629750	147722	-/-	56.0	EC 1.1.1.40	Y	(Dubini et al., 2009)
NADP ⁺ malic enzyme	MME3	Cre14.g629700	196832	-/-	65.6	EC 1.1.1.40	N	(Dubini et al., 2009)
NADP ⁺ malic enzyme	MME4	Cre14.g628650	196831	-/M	77.4	EC 1.1.1.40	N	(Dubini et al., 2009)
NADP ⁺ malic enzyme	MME5	Cre01.g022500	196351	C/M	81.9	EC 1.1.1.40	Y	(Dubini et al., 2009)
NADP ⁺ malic enzyme	MME6	Cre06.g251400	126820	-/-	65.2	EC 1.1.1.40	Y	(Dubini et al., 2009)
Malate synthase	MAS1	Cre01.g057800	196328	-/S	60.7	EC 2.3.3.9	Y	(Nogales et al., 2004)
Phosphate acetyltransferase	PAT1	Cre17.g699000	11226	C/C	85.6	EC 2.3.1.8	Y	(Dubini et al., 2009; Terashima et al., 2010)
Phosphate acetyltransferase	PAT2	Cre09.g396650	191051	M/C	84.6	EC 2.3.1.8	Y	(Dubini et al., 2009; Terashima et al., 2010)
Phosphoenolpyruvate carboxykinase	PCK1	Cre02.g141400	519867	C/C	67.5	EC 4.1.1.49	Y	(Dubini et al., 2009)
Phosphoenolpyruvate carboxylase	PEPC1	Cre16.g673800	80312	-/-	135.1	EC 4.1.1.31	Y	(Dubini et al., 2009)
Phosphoenolpyruvate carboxylase	PEPC2	Cre03.g171950	182821	-/-	51.1	EC 4.1.1.31	Y	(Dubini et al., 2009)
Pyruvate carboxylase	PYC1	Cre06.g258700	402089	-/-	84.1	EC 6.4.1.1	Y	(Dubini et al., 2009)
Pyruvate decarboxylase	PDC3	Cre03.g165700	127786	-/-	61.9	EC 4.1.1.1	Y	(Hemschemeier and Happe, 2005; Atteia et al., 2006; Grossman et al., 2007; Mus et al., 2007)
Mitochondrial pyruvate dehydrogenase complex	E1 α	PDC1	Cre07.g337650	193810	C/M	43.7	EC 1.2.4.1	Y
	E1 β	PDH1a	Cre01.g063700	139515	-/M	44.7	EC 1.2.4.1	Y

Table 3.1 (Continued)

Enzyme		Alias	Phytozyme ID	Chlre v4 Protein ID	Predicted location ChloroP/TargetP	Predicted size (kDa)	IUBMB	EST	Reference
Chloroplast pyruvate dehydrogenase complex	E2	DLA1	Cre29.g778200	149709	C/M	64.3	EC 2.3.1.12	Y	
	E2	DLA3	Cre06.g252550	187285	-/M	65.2	EC 2.3.1.12	Y	
	E3	DLD1	Cre31.g780600	-	-/M	52.5	EC 1.8.1.4	Y	
	E1 α	PDC2	Cre02.g099850	155587	C/-	43.5	EC 1.2.4.1	Y	
	E1 β	PDH2	Cre03.g194200	190446	C/M	36.3	EC 1.2.4.1	Y	
	E2	DLA2	Cre03.g158900	196500	C/-	43.1	EC 2.3.1.12	Y	
	E3	DLD2	Cre01.g016500	205763	C/C	59.6	EC 1.8.1.4	N	
Pyruvate:ferredoxin oxidoreductase		PFOR	Cre11.g473950	206677	-/-	128.3	EC 1.2.7.1	Y	(Atteia et al., 2006; Grossman et al., 2007; Mus et al., 2007; Dubini et al., 2009)
Pyruvate formate lyase		PFL1	Cre01.g044800	146801	C/C	91.1	EC 2.3.1.54	Y	(Kreuzberg et al., 1987; Mus et al., 2007; Hemschemeier et al., 2008b; Dubini et al., 2009)
Pyruvate formate lyase activating enzyme		PFLA	Cre05.g234650	-	-/-	7.6	EC 1.97.1.4	Y	(Hemschemeier and Happe, 2005)
Pyruvate dehydrogenase kinase		PDK1	Cre06.g252300	196270	C/C	55.1	EC 2.7.11.2	Y	
Pyruvate dehydrogenase kinase		PDK2	Cre06.g278450	196271	-/S	36.6	EC 2.7.11.2	N	
Pyruvate dehydrogenase kinase		PDK3	Cre05.g241750	142512	-/M	44.9	EC 2.7.11.2	Y	
Pyruvate kinase		PYK1	Cre12.g533550	136854	-/C	54.6	EC 2.7.1.40	Y	(Dubini et al., 2009)
Pyruvate kinase		PYK2	Cre06.g280950	196263	-/-	78.2		Y	(Dubini et al., 2009)
Pyruvate kinase		PYK3b, c	Cre05.g234700	107530/ 122254	-/-	237.0		Y	(Dubini et al., 2009)
Pyruvate kinase		PYK4b, c	Cre01.g057900	119280/ 104490	-/-	270.4		Y	(Dubini et al., 2009)
Pyruvate kinase		PYK5	Cre02.g147900	118203	-/-	60.7		Y	(Dubini et al., 2009)

Table 3.1: Bioinformatic analysis of *C. reinhardtii* pyruvate degrading and fermentative enzymes.

Location was predicted using first ChloroP and then TargetP, showing chloroplast (C), mitochondria (M) and cytoplasm (S). EST refers to the availability of corresponding expressed sequence tags in the Genbank database yes (Y) or no (N).

3.2.1 *C. reinhardtii* alcohol dehydrogenase (ADH) sequences

In nature ethanol can be produced or consumed, utilising NAD(P)^+ , by short-chain dehydrogenase/reductases (SDR) (~250 residues), medium chain dehydrogenase/reductases (MDR) (~350 residues) (Persson et al., 2008), [Fe]-dependent alcohol dehydrogenases (~385 residues) (Scopes, 1983; Reid and Fewson, 1994), or by NAD(P)^+ -independent pyrroloquinoline quinone-dependent (PQQ) alcohol dehydrogenases (Reid and Fewson, 1994).

Previous biochemical analysis suggested the possibility of at least two alcohol dehydrogenases in *C. reinhardtii*, with different preferences for NADH or NADPH (Kreuzberg et al., 1987). Three putative [Fe]-dependent alcohol dehydrogenase sequences have been identified in the genome (Atteia et al., 2003; Posewitz et al., 2009) annotated *ADH1*, *ADH2* and *ADH5*, of which *ADH1* appears to be bifunctional acetaldehyde/alcohol dehydrogenase with similarity to the *E. coli AdhE* gene. *AdhE* is thought to have arisen from a gene fusion and contains an acetaldehyde dehydrogenase N-terminal domain and alcohol dehydrogenase C-terminal domain that act in concert to catalyse the sequential conversion of acetyl-CoA to ethanol consuming two molecules of NADH (Membrillo-Hernández et al., 2000) (Eq. 5 and 6).



Only changes in *ADH1* transcription has previously been analysed (Mus et al., 2007; Nguyen et al., 2008; Dubini et al., 2009; Whitney et al., 2010). However, independent evidence of *ADH5* expression was found in the EST database and potentially *ADH2*, suggesting that *ADH5* may also play a role in ethanol production (Appendix III.1). According to the current gene model (Augustus v.10) *ADH5* lacks an N-terminal acetaldehyde dehydrogenase domain so will not catalyse the conversion of acetyl-CoA to ethanol. However the alcohol dehydrogenase domain is intact and could catalyse the second step (Eq. 6) in a fashion similar to other [Fe]-dependent ADHs such as those in *Zymomonas mobilis* (Moon et al., 2011), as demonstrated by a mutated version of the *Entamoeba histolytica* bifunctional ADH (*ADH2*) which

lacked the N-terminal domain but retained the ability to form ethanol when provided with acetaldehyde (Espinosa et al., 2001).

Genome analysis found 12 members of the SDR family in *C. reinhardtii* but based on sequence similarity none are likely to be involved in ethanol metabolism. Several had undefined specificity while others potentially play a role in a variety of processes including glycerophospholipid metabolism, ketone body formation, chlorophyll biosynthesis or fatty acid production (Appendix III.2).

Further analysis revealed the presence of at least 15 sequences belonging to the MDR family in the *C. reinhardtii*, 10 of which are represented in the EST database (Appendix III.3). The MDR superfamily contains a large number of enzymes with a wide variety of activities, with at least 8 large MDR families identified so far (Persson et al., 2008). Two MDR families - the ADH which consists of animal type ADHs, plant type ADHs and glutathione-dependent formaldehyde dehydrogenases (Persson et al., 2008) and TADH family (tetrameric alcohol dehydrogenase) which includes the yeast ADH - contain enzymes that can catalyse the reversible production of ethanol (Persson et al., 2008). However, based on sequence similarity, out of the *C. reinhardtii* MDR sequences only two putative glutathione-dependent alcohol dehydrogenases were identified as belonging to either ADH or TADH superfamilies (Appendix III.3). 8 of 13 *C. reinhardtii* MDRs possess cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) domains, and therefore likely belong to a class of enzymes traditionally associated with lignin biosynthesis in plant cells (Barakat et al., 2009) (Appendix III.3). *C. reinhardtii* is not known to make this compound so their function remains unclear. The role of CADs outside higher plants is less well defined, but they have been shown to possess wide substrate specificity working on alcohols and aldehydes (Mee et al., 2005), and have been proposed to play a role in aldehyde detoxification (Mee et al., 2005) so could potentially perform a similar function in *C. reinhardtii*. The remaining MDR type sequences included enzymes encoding a 2-enoyl thioester reductase (ETR) domain and ETR like domain (Appendix III.3) which catalyse the NADH dependent conversion of trans-2-enol-acyl carrier protein/CoA to acyl-CoA in fatty acid biosynthesis (Airenne et al., 2003). Additionally MDRs of unspecified activity including a quinone-oxidoreductase like sequence were identified (Appendix III.3).

At least 8 genes containing PQQ repeat sequences are present in the *C. reinhardtii* genome. As to the function of these proteins PROSITE analysis yielded no

hits. However, they lacked the conventional tryptophan binding motifs associated with PQQ ethanol dehydrogenases (Keitel et al., 2000), and were of either smaller (~50 kDa) or larger (~110 kDa) size than the convention ADH subunit of PQQ-ADHs (~80 kDa) (Yakushi and Matsushita, 2010) so are unlikely candidates for involvement in ethanol production.

3.2.2 *C. reinhardtii* pyruvate decarboxylase (PDC3)

Three pyruvate decarboxylase sequences, *PDC1*, *PDC2* and *PDC3*, are listed in the *C. reinhardtii* genome (Table 3.1). This annotation is un-helpful as pyruvate decarboxylases (EC 4.1.1.1), which catalyse the production of acetaldehyde releasing CO₂ (Eq. 7), and pyruvate dehydrogenase E1 alpha components (EC 1.2.4.1), which catalyse the production of acetyl-CoA as part of the pyruvate dehydrogenase complex can be described with this designation.

PDC and PDH E1 α components can be differentiated on the basis of their protein sequence. PDC sequences consist of three domains typical of pyruvate decarboxylase and pyruvate decarboxylase-like enzymes: an N-terminal pyrimidine (Pyr)-binding domain, a transhydrogenase dIII (Th3)-like domain consisting of a 2 Rossmann fold and a C-terminal pyrophosphate (PP)-binding domain (Arjunan et al., 1996; Costelloe et al., 2008), which differs from the TPP-binding domain typical of PDH E1 alpha components.

Only *PDC3* was found to encode a predicted pyruvate decarboxylase (PDC) (EC 4.1.1.1) whereas *PDC1* and *PDC2* were found to encode putative pyruvate dehydrogenase E1 α components. Therefore suggesting there is only one PDC catalysed pathway for ethanol production in *C. reinhardtii*.



Eq. 7

Phylogenetic analysis revealed *PDC3* clustered with plant and algal homologues (Figure 3.1). The putative *C. reinhardtii* PDC sequence (Cr_PDC3) was aligned against the four *Arabidopsis thaliana* pyruvate deacboxylase sequences (At_PDC) (Kursteiner et al., 2003), and compared to the *Zymomonas mobilis* enzyme (Zm_PDC) for which a crystal structure is available (Dobritsch et al., 1998) (Figure 3.2) and has been studied extensively (Schenk et al., 1997; Siegert et al., 2005). The

Cr_PDC3 sequence shares 41% sequence identities with the Zm_PDC and 55–58% with the At_PDCs (Figure 3.2). Further analysis of the Cr_PDC3 protein sequence confirmed the presence of characteristic thiamine pyrophosphate (TPP)-binding motif (Hawkins et al., 1989), active-site histidines (Schenk et al., 1997) and conserved substrate recognition residues as found in the *Z. mobilis* enzyme (Siegert et al., 2005) (Figure 3.2).

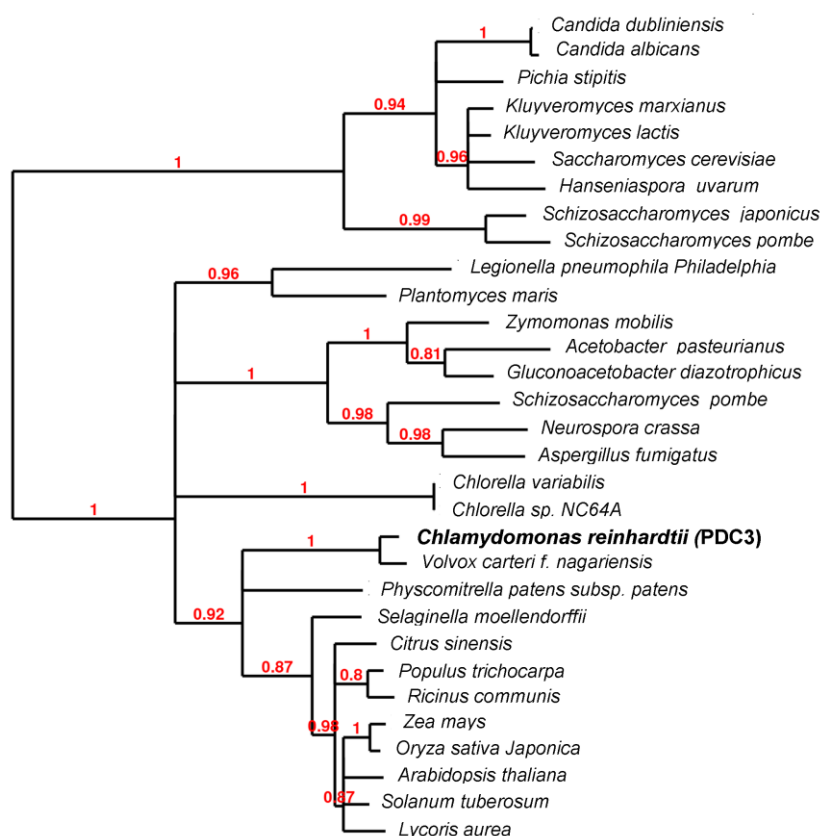


Figure 3.1: Phylogenetic analysis aligning putative *C. reinhardtii* PDC3 with pyruvate decarboxylase sequences.

Branch support values calculated by aLRT test are represented in red (Guindon et al., 2005). *C. reinhardtii* PDC3 protein sequence (EC 4.1.1.1) (XP_001703530.1) is compared to homologous enzymes from *Acetobacter pasteurianus* (AAM21208.1), *Arabidopsis thaliana* (NP_200307), *Aspergillus fumigatus* (XP_753176), *Candida albicans* SC5314 (XP_715589.1), *Candida dubliniensis* CD36 (XP_002420248.1), *Chlorella variabilis* (EFN55335.1), *Citrus sinensis* (AAZ05069.1), *Gluconacetobacter diazotrophicus* (YP_001600462.1), *Hanseniaspora uvarum* (AAA85103.1), *Kluyveromyces lactis* NRRL Y-1140 (XP_454684.1), *Kluyveromyces marxianus* (AAA35267.1), *Legionella pneumophila* (YP_095188.1), *Lycoris aurea* (ABJ99597.1), *Neurospora crassa* (XP_959587), *Oryza sativa Japonica* (NP_001042088.1), *Physcomitrella patens subsp. patens* (XP_001763233.1), *Pichia stipitis* CBS 6054 (EAZ63546.2), *Plantomyces maris* (ZP_01856731.1), *Populus trichocarpa* (XP_002305755.1), *Ricinus communis* (XP_002522545.1), *Saccharomyces cerevisiae* (CAA39398.1), *Schizosaccharomyces japonicus* yFS275

(XP_002174876.1), *Schizosaccharomyces pombe* 972h- (XP_001713041.1), *Selaginella moellendorffii* (XP_002986100.1), *Solanum tuberosum* (BAC23043.1), *Schizosaccharomyces pombe* (NP_595027), *Volvox carteri* f. *Nagariensis* (XP_002951658.1), *Zea mays* (NP_001105645), *Zymomonas mobilis* (AAA27685.1). Protein accessions given according to the NCBI database (<http://www.ncbi.nlm.nih.gov/protein>).

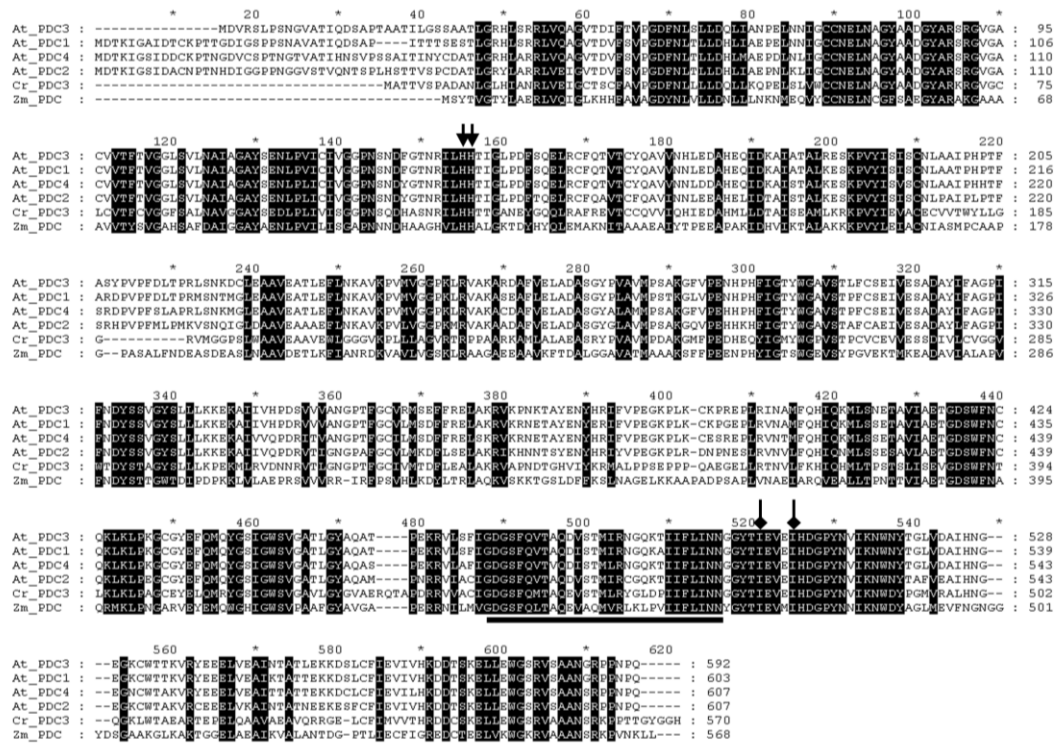


Figure 3.2: Sequence alignment comparing *C. reinhardtii* PDC3 with plant pyruvate decarboxylase sequences.

The *C. reinhardtii* PDC3 sequence (EC 4.1.1.1) (Cr_PDC3; XP_001703530.1), is compared to those of homologous proteins from *A. thaliana* (At_PDC1; AED90327)(At_PDC2; AED96563)(At_PDC3; AAP21263)(At_PDC4; AEE86169) and *Z. mobilis* (Zm_PDC; ADK13058). Underlined is the TPP binding motif GDG-X24-27-NN (Hawkins et al., 1989). TPP substrate recognition residues (black sticks) (Siegert et al., 2005), active site Histidine residues (black arrows) (Schenk et al., 1997). Protein accessions are given according to NCBI database (<http://www.ncbi.nlm.nih.gov/protein>).

3.2.3 *C. reinhardtii* pyruvate dehydrogenase complex (PDH)

The protein sequences of the remaining two pyruvate decarboxylase genes listed in the database (PDC1 and PDC2) were found to cluster with E1 α components of the pyruvate dehydrogenase complex (PDH) (EC 1.2.4.1) (Figure 3.3). The PDH complex is a large multi-enzyme complex (4-11MDa) (Smolle and Lindsay, 2006) that catalyzes the three step oxidative decarboxylation of pyruvate to NADH, CO₂ and

acetyl-CoA (as summarised in Eq. 8) and plays a role in linking glycolysis with the TCA cycle or fatty acid biosynthesis (Mooney et al., 2002).



PDH complexes consist of pyruvate decarboxylase, dihydrolipoyl transacetylase and dihydrolipoamide dehydrogenase subunits, denoted E1, E2 and E3 respectively, with the E1 component in many species constructed of α and β subunits, organised as a heterotetramer (Mooney et al., 2002). The E1 subunit catalyses the TPP-dependent decarboxylation of pyruvate transferring the resulting acetyl group onto the lipoamide moiety of the E2 domain, forming a stable intermediate. E2 then catalyses the transacetylation of CoA forming acetyl-CoA and finally the E3 domain re-oxidizes the E2 component transferring electrons to NAD^+ (Smolle and Lindsay, 2006).

The mammalian PDH is constructed of a pentagonal dodecahedral core comprising of 60 dihydrolipoamide acetyltransferase (DLA) E2 subunits to which is attached 30 E1 α - β heterotetramers and six dihydrolipoamide dehydrogenase (DLD) E3 homodimers when fully assembled making it one of the largest complexes in nature (Smolle and Lindsay, 2006).

BLAST analysis and close inspection of the PDC1 and PDC2 protein sequences suggested both are E1 alpha components of PDH complexes, possessing the requisite active site residues (Markus et al., 2003) and conserved TPP-binding domain (Hawkins et al., 1989) (Figure 3.3 and 3.4). Further analysis of the genome and expressed sequence tag (EST) libraries suggested the existence of genes that could encode for two complete sets of the PDH complex (Table 3.1). Two PDH E1 β subunits are present in addition to three dihydrolipoyl transacetylase (E2) and two dihydrolipoamide dehydrogenase (E3) subunits (Table 3.1), therefore suggesting both *C. reinhardtii* PDH complexes are composed of an E1 heterotetramer attached to an E2 core.

Plants are known to possess two PDH complexes comprising of mitochondrial and chloroplast isoforms (Mooney et al., 2002). In order to test if the situation was similar in *C. reinhardtii*, phylogenetic analysis was performed comparing the sequence of known chloroplast and mitochondrial isoforms of each subunit of the PDH complex to the *C. reinhardtii* gene products.

Analysis of the PDH E1 α components clustered PDC2 with cyanobacterial E1 alpha components along with a subset of plant enzymes, forming a group of proposed chloroplast-located PDH subunits of cyanobacterial origin (Figure 3.5), and PDC1 with a separate set of plant E1 alpha components which were closer aligned to fungal and mammalian isoforms than cyanobacterial, suggesting it belongs to the mitochondrial lineage (Figure 3.5).

A similar separation of *C. reinhardtii* isoforms between putative chloroplast and mitochondrial PDH complexes was seen for each subunit (Figure 3.6–3.8), and suggests overall that *C. reinhardtii* has two distinct PDH complexes, one in the mitochondrion and one in the chloroplast, mirroring the situation found in higher plants (Table 3.1).

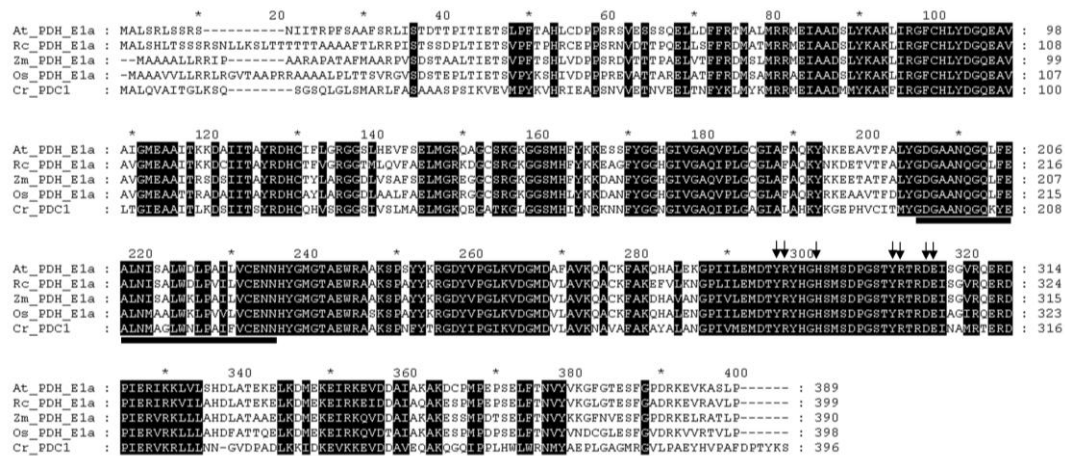


Figure 3.3: Sequence alignment comparing *C. reinhardtii* PDC1 with putative plant mitochondrial pyruvate dehydrogenase E1 alpha components.

The *C. reinhardtii* PDC1 sequence (Cr_PDC1; Cre07.g337650) is compared to homologous proteins from *Arabidopsis thaliana* (At_PDHE1a; At_AAD39331.1), *Oryza sativa* Indica group (Os_PDHE1a; EAZ00344.1), *Ricinus communis* (Rc_PDHE1a; XP_002520198.1), *Zea mays* (Zm_PDHE1a; NP_001150259.1), Active site residues indicated (black arrows) (Markus et al., 2003), underlined is the TPP binding motif GDG-X24-27-NN (Hawkins et al., 1989). *C. reinhardtii* protein accession is given according to the Phytozome database, all the others refer to the NCBI database.

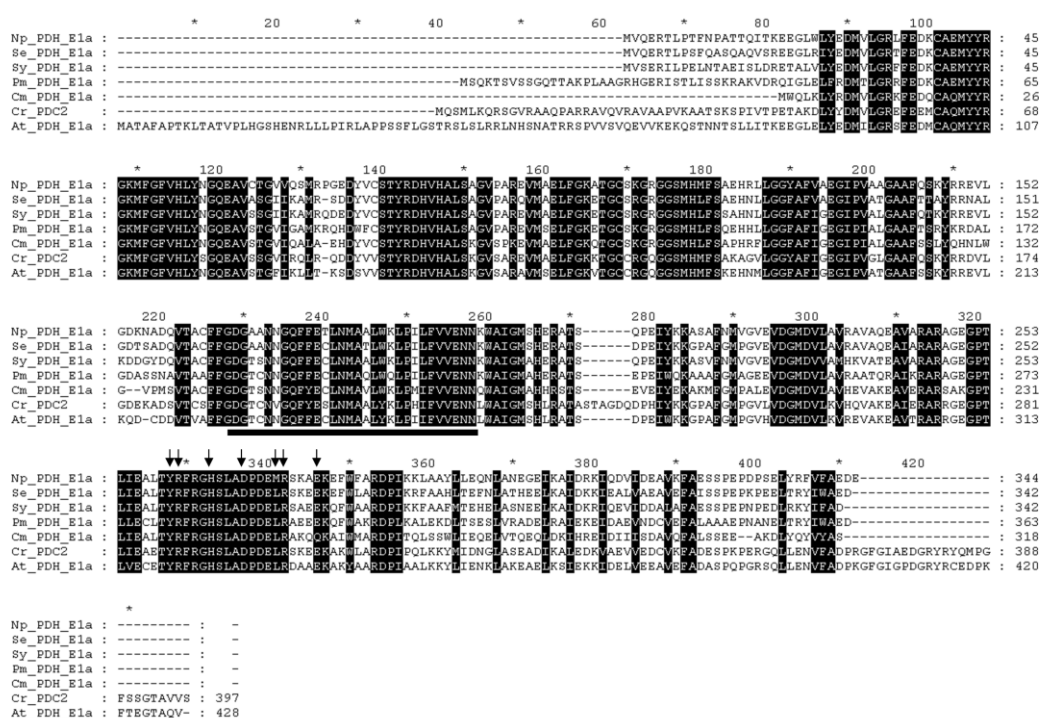


Figure 3.4: Protein sequence alignment comparing *C. reinhardtii* PDC2 with putative plant chloroplast and cyanobacteria pyruvate dehydrogenase E1 alpha components.

The *C. reinhardtii* PDC2 sequence (Cr_PDC2: Cre02.g099850) is compared to those of homologous proteins from *Arabidopsis thaliana* (At_PDH_E1α; NP_171617.1), *Cyanidioschyzon merolae* (Cm_PDH_E1α; NP_849059), *Nostoc punctiforme* (Np_PDH_E1α; YP_001868828), *Prochlorococcus marinus* (Pm_PDH_E1α; YP_001017966.1), *Synechocystis PCC6803* (Sy_PDH_E1α; NP_441914), *Synechococcus elongatus* PCC 6301 (Se_PDH_E1α; YP_172860.1). Active site residues indicated (black arrows) (Markus et al., 2003), underlined is the TPP binding motif GDG-X₂₄₋₂₇-NN (Hawkins et al., 1989). *C. reinhardtii* protein accession is given according to the Phytozome database, all others refer to the NCBI database.

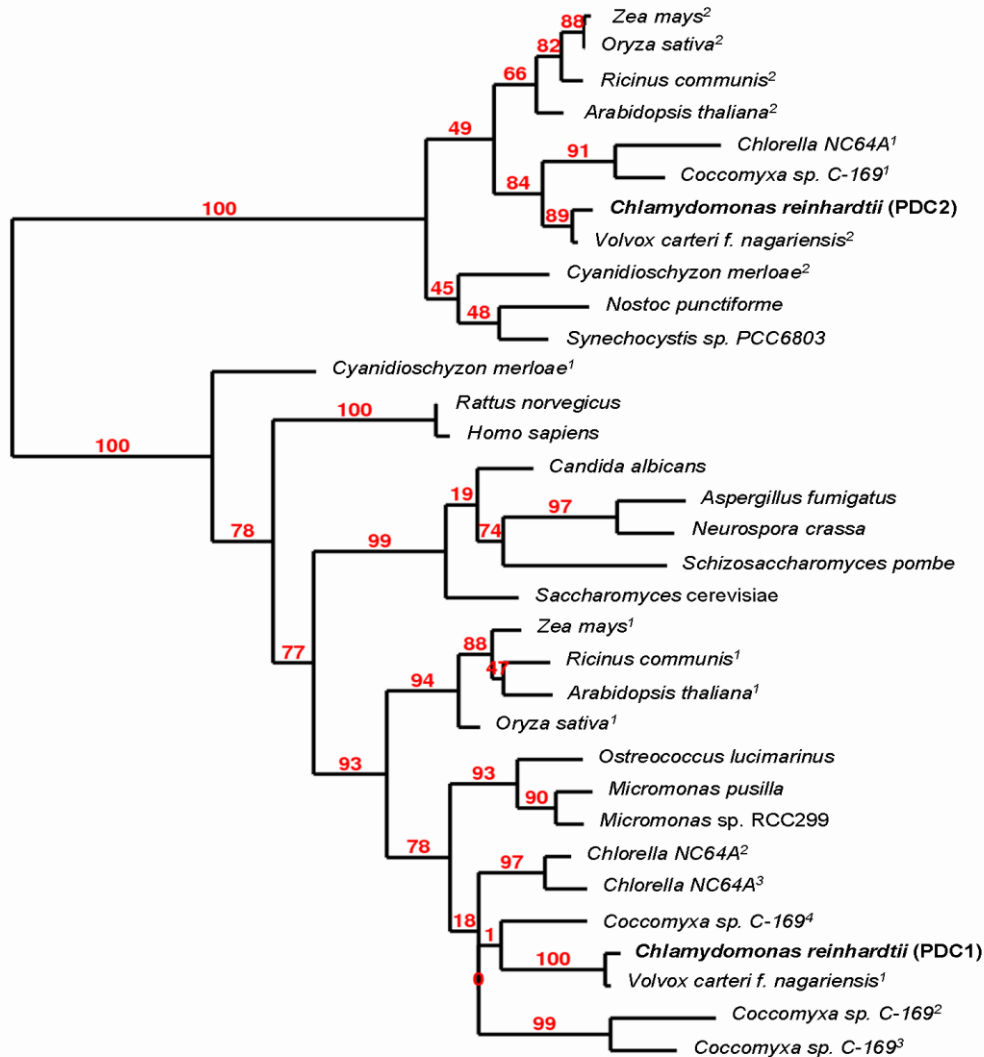


Figure 3.5: Phylogenetic analysis comparing *C. reinhardtii* PDC1 and PDC2 pyruvate dehydrogenase E1 alpha components with plant, fungal and mammal homologues.

Branch support values calculated by aLRT test are represented in red (Guindon et al., 2005). Sequences of the putative *C. reinhardtii* PDH E1 α components (EC 1.2.4.1) PDC1 (Cre07.g337650) and PDC2 (Cre02.g099850) are compared to those of homologous enzymes from *Aspergillus fumigatus* (XP_750445.1), *Arabidopsis thaliana*¹ (NP_171617.1), *Arabidopsis thaliana*² (AAD39331.1), *Candida albicans* (EEQ44823.1), *Chlorella* NC54A¹ (32109^a) *Chlorella* NC54A² (141042^a) *Chlorella* NC54A³ (56254^a) *Coccomyxa* sp. C-1693¹ (33689^a), *Coccomyxa* sp. C-1693² (29303^a), *Coccomyxa* sp. C-1693³ (73729^a), *Coccomyxa* sp. C-1693⁴ (25215^a), *Cyanidioschyzon merolae*¹ (CMT256C^b), *Cyanidioschyzon merolae*² (CMV153C^b), *Homo sapiens* (NP_000275.1), *Micromonas pusilla* (22101^a), *Micromonas* sp. RCC299 (89160^a), *Neurospora crassa* (XP_957122.1), *Nostoc punctiforme* (YP_001868828), *Ostreococcus lucimarinus* (XP_001415646.1), *Oryza sativa Indica group*¹ (CAH65949.1), *Oryza sativa Indica group*² (EAZ00344.1), *Rattus norvegicus* (NP_001004072.2), *Ricinus communis*¹ (XP_002515074.1), *Ricinus communis*² (XP_002520198.1), *Saccharomyces cerevisiae* (EDN63155.1), *Schizosaccharomyces pombe* (NP_594892.1) *Synechocystis* PCC6803 (NP_441914), *Volvox carteri f. nagariensis*¹ (XP_002950542.1), *Volvox carteri f. nagariensis*² (XP_002957475.1),

*Zea mays*¹ (NP_001140759.1), *Zea mays*² (NP_001150259.1). *C. reinhardtii* protein accessions are given according to the Phytozome database, all others refer to the NCBI database (<http://www.ncbi.nlm.nih.gov/protein>) unless ^a given as the JGI protein ID (<http://www.jgi.doe.gov/>), or ^b*Cyanidioschyzon merolae* genome project accession (<http://merolae.biol.s.u-tokyo.ac.jp/>)

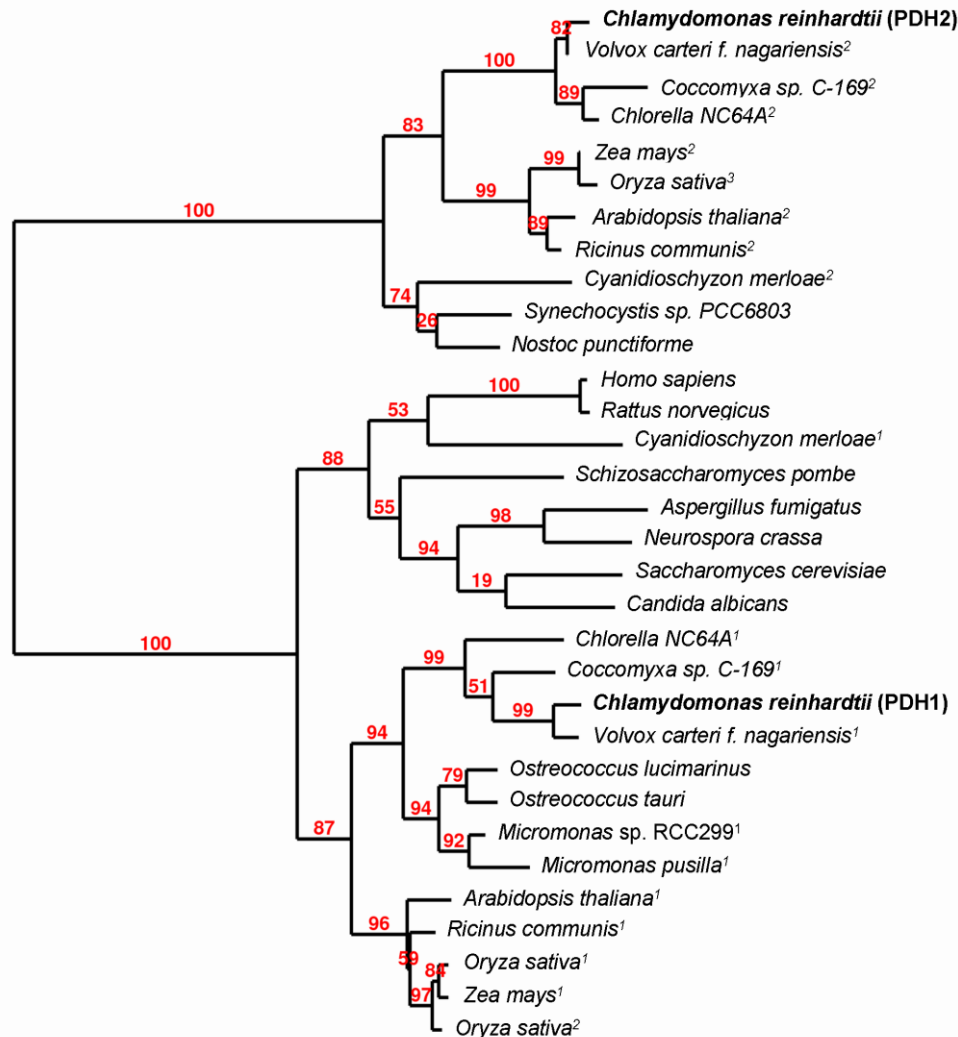


Figure 3.6: Phylogenetic analysis comparing *C. reinhardtii* PDH1a and PDH2 pyruvate dehydrogenase E1 beta components with plant, fungal and mammalian homologues.

Branch support values calculated by aLRT test are represented in red (Guindon et al., 2005). Putative *C. reinhardtii* PDH E1 beta components (EC 1.2.4.1), PDH1a (XP_001699209.1) and PDH2 (XP_001693114.1) are compared to homologous proteins from *Aspergillus fumigatus* (XP_748734.1), *Arabidopsis thaliana* (AAA52225.1), *Arabidopsis thaliana* (AAB86804.1), *Candida albicans* (XP_717098.1), *Chlorella NC54A* (59713)^a, *Chlorella NC54A* (48843)^a, *Coccomyxa sp. C-1693* (21931)^a, *Coccomyxa sp. C-1693* (53517)^a, *Cyanidioschyzon merolae*¹, *Cyanidioschyzon merolae*¹ (CMS327C^b), *Homo sapiens* (NP_000916.2), *Micromonas pusilla* (58821), *Micromonas sp. RCC299* (86033), *Neurospora crassa* (XP_965390.1), *Nostoc punctiforme* PCC 73102 (YP_001867176.1), *Oryza sativa Indica group*¹ (EAZ09701.1), *Oryza sativa Indica group*² (EEC83948.1), *Oryza sativa Indica group*³ (EEC69682.1), *Ostreococcus lucimarinus* (XP_001418477.1), *Ostreococcus tauri* (XP_003079900.1), *Rattus norvegicus* (NP_001007621.1), *Ricinus communis*¹ (XP_002512633.1), *Ricinus communis*² (XP_002519664.1),

Saccharomyces cerevisiae (EDN64829.1), *Schizosaccharomyces pombe* (NP_596272.1), *Synechocystis PCC6803* (NP_440765.1), *Volvox carteri f. nagariensis*(XP_002945988.1), *Volvox carteri f. nagariensis*(XP_002957188.1), *Zea mays*¹ (ACG37759.1), *Zea mays*² (NP_001150473.1). Protein accessions given according to NCBI database (<http://www.ncbi.nlm.nih.gov/protein>) unless ^a given as the JGI protein ID (<http://www.jgi.doe.gov/>), or ^b *Cyanidioschyzon merolae* genome project accession (<http://merolae.biol.s.u-tokyo.ac.jp/>).

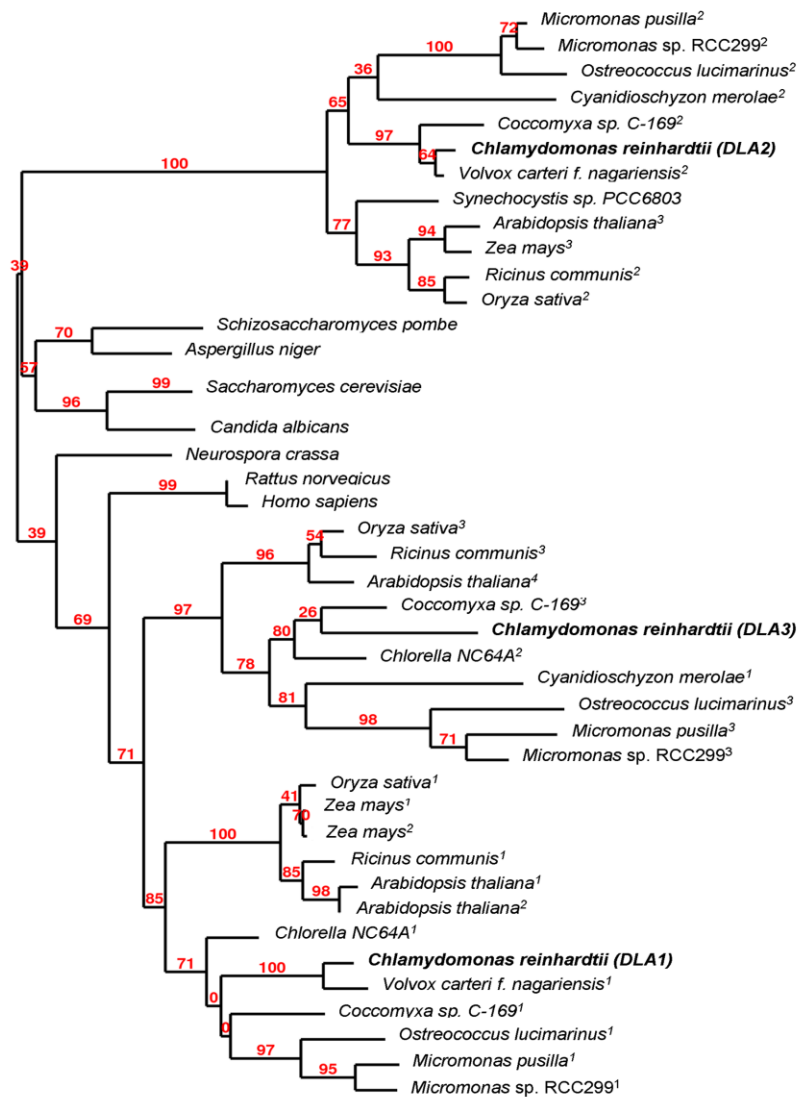


Figure 3.7: Phylogenetic analysis comparing *C. reinhardtii* DLA1, DLA2 and DLA3 pyruvate dehydrogenase E2 components with plant, fungal and mammalian homologues.

Branch support values calculated by aLRT test are represented in red (Guindon et al., 2005). Putative *C. reinhardtii* PDH E2 components (EC 2.3.1.12) DLA1 (XP_001695174.1) DLA2 (XP_001697507.1) and DLA3 (XP_001696403.1) are compared to proteins from *Aspergillus niger* (XP_001391304.2), *Arabidopsis thaliana*¹ (NP_564654.1), *Arabidopsis thaliana*² (NP_566470.1) *Arabidopsis thaliana*³ (NP_189215.1), *Arabidopsis thaliana*⁴ (NP_190788.1), *Candida albicans* (EEQ46961.1), *Chlorella NC54A*¹ (137866) *Chlorella NC54A*² (48412), *Coccomyxa sp. C-1693*¹ (26915), *Coccomyxa sp. C-1693*² (31081), *Coccomyxa sp. C-1693*³ (23293), *Cyanidioschyzon merolae*¹ (CMI273C), *Cyanidioschyzon merolae*² (CMN233C), *Homo sapiens* (AAA62253.1), *Micromonas pusilla*¹ (25687), *Micromonas pusilla*² (42270), *Micromonas pusilla*³ (30461), *Micromonas sp. RCC299*¹ (96637), *Micromonas sp. RCC299*² (68004), *Micromonas sp. RCC299*³ (68004).

(96619^a), *Neurospora crassa* (XP_962786.1), *Oryza sativa Indica group*¹ (EAZ03591.1), *Oryza sativa Japonica group*² (NP_001066319.2), *Oryza sativa Japonica group*³ (EEC80677.1), *Ostreococcus lucimarinus*¹ (XP_001418025.1), *Ostreococcus lucimarinus*² (XP_001416577.1), *Ostreococcus lucimarinus*³ (XP_001420090.1), *Rattus norvegicus*² (NP_112287.1), *Ricinus communis*¹ (XP_002521371.1), *Ricinus communis*² (XP_002526841.1), *Ricinus communis*³ (XP_002526756.1), *Saccharomyces cerevisiae* (EDN62741.1), *Schizosaccharomyces pombe* (NP_587755.1) *Synechocystis PCC6803* (BAA18614.1), *Volvox carteri f. nagariensis*¹ (XP_002947241.1), *Volvox carteri f. nagariensis*² (XP_002950074.1), *Zea mays*¹ (NP_001104936.1), *Zea mays*² (NP_001150860.1), *Zea mays*³ (NP_001145861.1). Protein accessions are given according to NCBI database (<http://www.ncbi.nlm.nih.gov/protein>) unless^a given as the JGI protein ID (<http://www.jgi.doe.gov/>), or *Cyanidioschyzon merolae* genome project accession (<http://merolae.biol.s.u-tokyo.ac.jp/>).

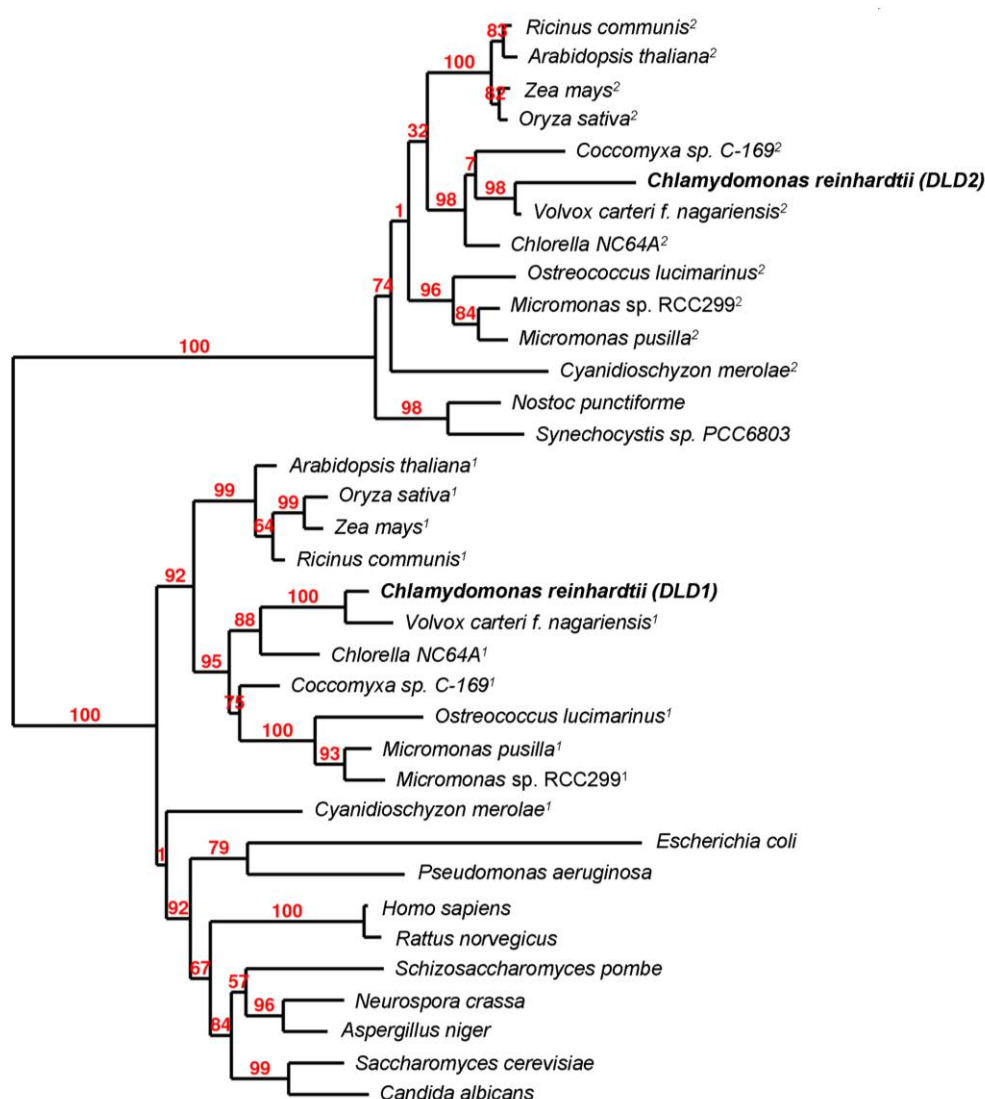


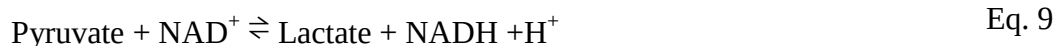
Figure 3.8: Phylogenetic analysis comparing *C. reinhardtii* DLD1 and DLD2 pyruvate dehydrogenase E3 components with bacterial, plant, fungal and mammalian homologues.

Branch support values calculated by aLRT test are represented in red (Guindon et al., 2005). Putative *C. reinhardtii* PDH E3 components (EC 1.8.1.4) DLD1 (XP_001695163.1) and DLD2 (XP_001689920.1) are compared to homologues from *Aspergillus niger* (XP_001391760.1), *Arabidopsis thaliana* (NP_175237.1), *Arabidopsis thaliana* (NP_567487.4), *Candida albicans* (XP_712370.1), *Chlorella NC54A* (48339), *Chlorella NC54A* (58327), *Coccomyxa sp. C-1693* (67663), *Coccomyxa sp. C-1693* (35270), *Cyanidioschyzon merolae* (CMM299C), *Cyanidioschyzon merolae* (CMQ234C), *Homo sapiens* (NP_000099.2), *Micromonas pusilla* (28757), *Micromonas pusilla* (34732), *Micromonas sp. RCC299* (104984), *Micromonas sp. RCC299* (104967), *Neurospora crassa* (XP_959535.2), *Nostoc punctiforme* (YP_001867499.1), *Oryza sativa Japonica group* (EEE62416.1), *Oryza sativa Japonica group* (NP_001042941.1), *Ostreococcus lucimarinus*

(XP_001420369.1), *Ostreococcus lucimarinus*² (XP_001417363.1), *Rattus norvegicus*² (NP_955417.1), *Ricinus communis*¹ (XP_002519286.1), *Ricinus communis* (XP_002519201.1), *Saccharomyces cerevisiae* (EDN59131.1), *Schizosaccharomyces pombe* (NP_593496.1) *Synechocystis* PCC6803 (NP_440075.1), *Volvox carteri* f. *nagariensis*¹ (XP_002954931.1), *Volvox carteri* f. *nagariensis*² (XP_002956693.1), *Zea mays*¹ (NP_001183318.1), *Zea mays*² (NP_001169718.1). Protein accessions are given according to NCBI database (<http://www.ncbi.nlm.nih.gov/protein>) unless ^a given as the JGI protein ID (<http://www.jgi.doe.gov/>), or ^b *Cyanidioschyzon merolae* genome project accession (<http://merolae.biol.s.u-tokyo.ac.jp/>).

3.2.4 Lactate dehydrogenase sequences

Lactate exists in nature as one of two stereoisomers (D or L) and lactate dehydrogenases can be NAD⁺-dependent (Eq. 9), or NAD⁺-independent (cytochrome-dependent) (Eq. 10). Unlike plants *C. reinhardtii* has been shown to produce (Husic and Tolbert, 1985) and oxidize D-lactate (Husic and Tolbert, 1987) but not L-lactate (Beezley et al., 1976), and, accordingly, homologues of the cytochrome-dependent (L-LDHc; EC 1.1.2.3) and NAD⁺-dependent (L-nLDH; EC 1.1.1.27) L-lactate dehydrogenases found in plants, are absent in *C. reinhardtii*.



Lactate formation in *C. reinhardtii* has been proposed to be catalysed by an NAD⁺-dependent D-lactate dehydrogenase (D-nLDH; EC 1.1.1.28), hereafter termed LDH, which belongs to the 2-hydroxyacid dehydrogenase family (Taguchi and Ohta, 1991). Analysis of the genome sequence identified a number of enzymes belonging to the 2-hydroxyacid dehydrogenase family, but only a single likely LDH (Table 3.1). The putative *C. reinhardtii* LDH (Cr_LDH) was aligned to D-lactate dehydrogenase sequences for which crystal structures are available, including *Aquifex aeolicus* VF5 (Aa_LDH) (Antonyuk et al., 2009), *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Lb_LDH) (Razeto et al., 2002) and *Lactobacillus pentosus* (Lp_LDH) (Shinoda et al., 2005) (Figure 3.9). The Cr_LDH possesses 30–33% protein sequence identity with the other LDH sequences, and the sequence contains the conserved catalytic histidine, and substrate-binding arginine residues, in addition to the NAD⁺ coenzyme binding motif GXGX₂GX₁₇D (Wierenga et al., 1986) (Figure 3.9), providing an

indication that this is the gene product responsible for D-lactate formation in *C. reinhardtii*.

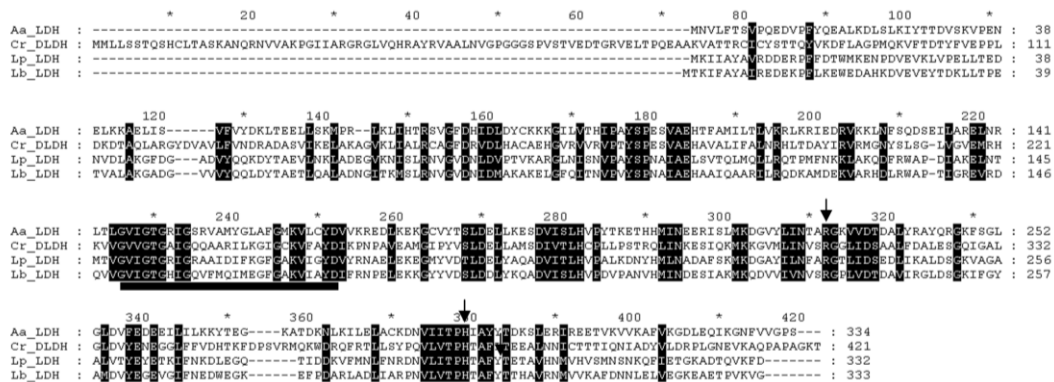


Figure 3.9: Sequence alignment of putative *C. reinhardtii* LDH with NAD⁺-dependent LDHs.

The putative *C. reinhardtii* LDH sequence (Cr_DLHD; Cre07.g324550) is compared to those of homologous proteins from *Aquifex aeolicus* VF5 (Aa_LDH; NP_213499.1), *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (Lb_LDH; P26297.3) and *Lactobacillus pentosus* (Lp_LDH; P26298.1). Annotated is the NAD⁺ binding motif (GXXGXGXGXG₁₇D) (underlined) and active site residues (black arrows) (Lapierre et al., 1999). The *C. reinhardtii* protein accession refers to the Phytozome database (Augustus gene model v.10), the others are given according to the NCBI database.

In addition, three potential FAD-dependent, NAD⁺-independent D-lactate dehydrogenase (D-LDHC; EC 1.1.2.4) sequences are present in the genome (Table 3.1). This class of enzyme acts principally in the direction of pyruvate synthesis, so is unlikely to compete for pyruvate during fermentation (Reed and Hartzell, 1999).

One D-LDHC was represented in the EST database (Table 3.1), the protein sequence of this gene (Cr_LDHC) was compared to that of enzymes which have been investigated experimentally, including *Kluyveromyces lactis* (Kl_LDHC) (Lodi et al., 1994) *S. cerevisiae* (Sc_LDHC) (Lodi and Ferrero, 1993) and *A. thaliana* (At_LDHC) (Engqvist et al., 2009). Cr_LDHC shared 38%, 38% and 44% sequence identities with the Kl_LDHC, Sc_LDHC and At_LDHC respectively. Cr_LDHC was found to possess the FAD-binding domain 4 and C-terminal oxidase domain characteristic of D-LDHCs (<http://pfam.sanger.ac.uk>) (Figure 3.10) (Pallotta et al., 2004; Engqvist et al., 2009) and is predicted to have a mitochondrial location (Table 3.1), so could therefore represent the enzyme responsible for the previously observed uptake of D-lactate by isolated mitochondria (Beezley et al., 1976).

The *C. reinhardtii* sequence (Cr_LDHC, Augustus gene model v.19; Cre10.g434900) is compared to homologous protein sequences from *A. thaliana* (At_LDHC; AED91037), *K. lactis* (Kl_LDHC; CAA50635.1) and *S. cerevisiae* (Sc_LDHC; NP_010107.1). The *C. reinhardtii* protein accession refers to the Phytozome database, all others are given according to the NCBI database (<http://www.ncbi.nlm.nih.gov/protein>).

The putative *C. reinhardtii* PFOR (Cr_PFOR) (Grossman et al., 2007) was aligned with that of *Desulfovibrio africanus*, for which a crystal structure is available (Chabriere et al., 1999) (Figure 3.11). Cr_PFOR shared 51% sequence identity with the *D. africanus* enzyme. Additionally, sequence analysis showed the predicted Cr_PFOR sequence contains the three characteristic [4Fe-4S] binding motifs, two conserved (CX₂CX₂CX₃CP) and one non-conserved (Pieulle et al., 1997), in addition to a TPP-binding motif (G-D-G) (Figure 3.11) (Hawkins et al., 1989), providing good confidence that this gene encodes for a functional PFOR.

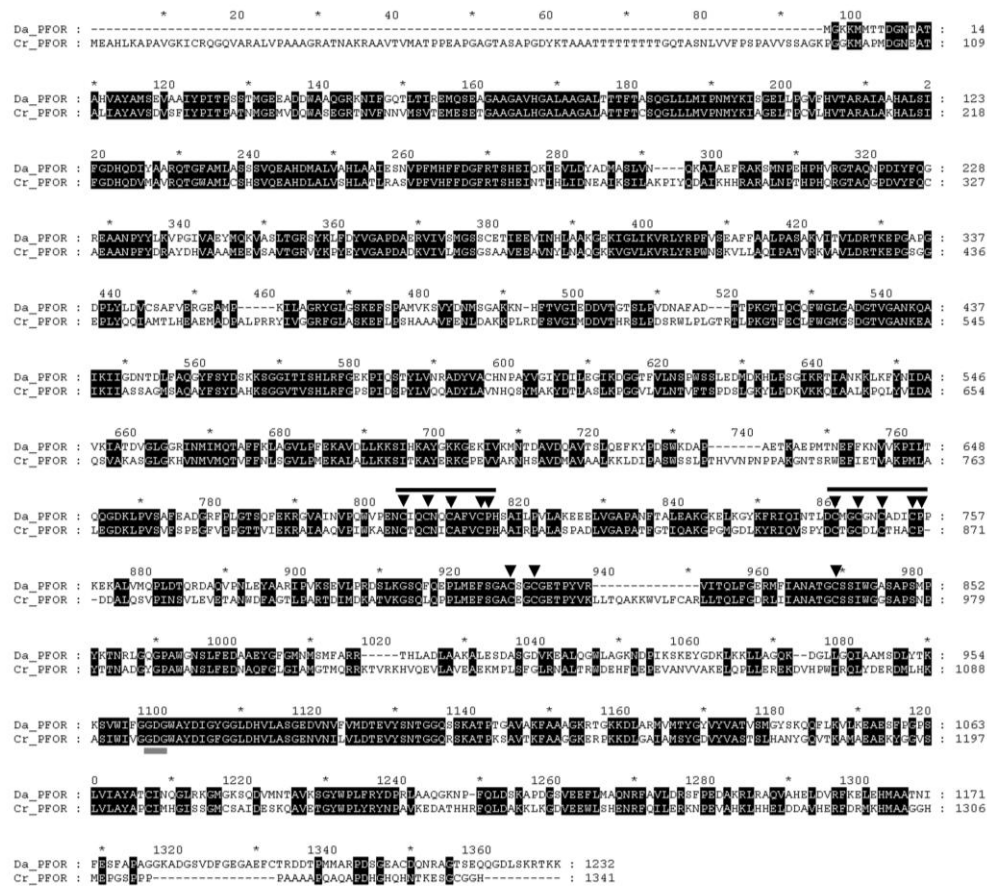


Figure 3.11: Sequence alignment of the putative *C. reinhardtii* pyruvate synthase/pyruvate:ferredoxin oxidoreductase with the *Desulfovibrio africanus* enzyme.

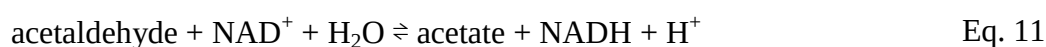
Comparison of the *C. reinhardtii* PFOR sequence (Cr_PFOR; Augustus v.10 gene model; Cre11.g473950) with the *Desulfovibrio africanus* enzyme (Da_PFOR; NCBI; CAA70873.1). Highlighted are the residues that form the three [4Fe-4S] binding motifs (black arrows), two conserved (CX₂CX₂CX₃CP) and one non-conserved (Pieulle et al., 1997), and the TTP binding motif (GDG) underlined in grey (Hawkins et al., 1989).

3.2.5 *C. reinhardtii* aldehyde dehydrogenases

C. reinhardtii contains 8 unique aldehyde dehydrogenase (ALDH) sequences belonging to 7 different ALDH families (Wood and Duff, 2009). The ALDH superfamily consists of NAD(P)⁺-dependent enzymes which catalyse the oxidation of aliphatic and aromatic aldehydes (Kirch et al., 2004) with over 20 families identified in eukaryotes (Sophos and Vasiliou, 2003). Aldehydes are formed as intermediates in a range of metabolism of carbohydrates, vitamins, steroids, amino acid and lipid and are also created in response to environmental stresses such as salination, dessication, cold and heat shock (Kirch et al., 2004). They are highly toxic and have been linked

to cell death in mammalian (Clemens et al., 2002) and plant tissues (Tadege et al., 1998) requiring rapid breakdown to prevent cellular damage.

One *C. reinhardtii* ALDH sequence - ALD5 (EC1.2.1.3) (Table 3.1) is a class II ALDH (Wood and Duff, 2009) whose transcript levels have been shown to be up-regulated in the hydrogenase deficient mutant *hydEF* by a greater extent than in WT cultures after dark-anaerobic incubation (Dubini et al., 2009). This family of enzymes is implicated in acetaldehyde detoxification (Eq. 11) (Wei et al., 2009) and is thought to be active under anaerobic conditions in rice, aiding plant survival (Nakazono et al., 2000) (Table 3.1).



Therefore, in addition to ethanol, acetaldehyde produced by PDC3 activity could be broken down to acetate by a class II ALDH, a route which has been shown to act in concert with an acetyl-CoA synthetase (ACS) as a pyruvate dehydrogenase bypass in yeast (Boubekeur et al., 1999). There is evidence that such a pathway is present in *Arabidopsis*, and is important for growth (Lin and Oliver, 2008) but has not been investigated in *C. reinhardtii*.

ALD5 possesses the characteristic cysteine and glutamate residues found in the active-site of aldehyde dehydrogenases (Figure 3.12) (Jimenez-Lopez et al., 2010). However, an active ALDH pathway during anoxia may be unlikely as this would deplete reserves of NAD^+ , thereby suppressing glycolysis. Furthermore in contrast to experiments with rice, ALDH is thought to be inactive in tobacco leaves in the absence of O_2 (op den Camp and Kuhlemeier, 1997). Therefore, an alternative role for ALD5 could be to oxidise acetaldehyde to acetate as a means of detoxifying ethanol accumulated during anoxic conditions when cells re-encounter an aerobic environment. Such a pathway has been found to be active in plants (Tsuji et al., 2003) where ethanol can be incorporated into lipids (Mellema et al., 2002). To date no extensive work has been done on the *C. reinhardtii* aldehydes dehydrogenases, and therefore before further characterisation is performed it will be difficult to assess the potential importance of these enzymes in cellular metabolism and fermentation pathways.

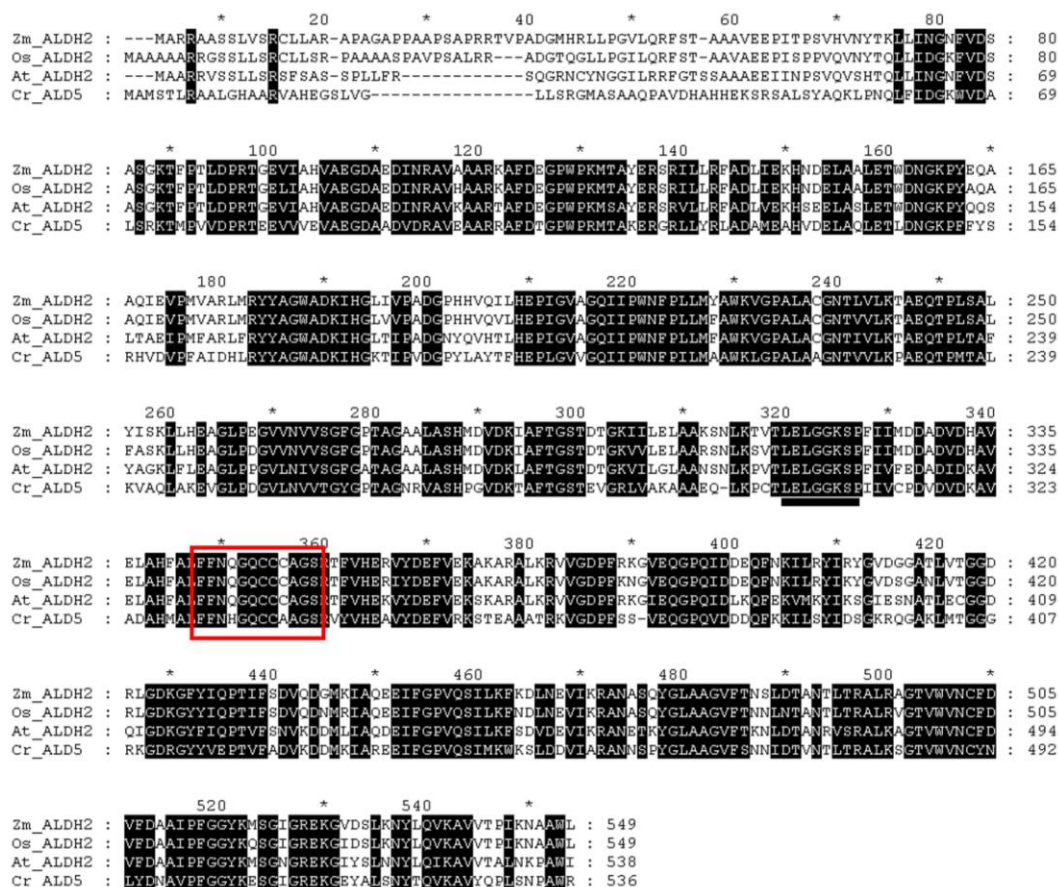


Figure 3.12: Sequence alignment comparing *C. reinhardtii* ALD5 with plant type II aldehyde dehydrogenases.

The *C. reinhardtii* ALD5 sequence (Cr_ALD5; Cre12.g500150) is compared to those of homologous proteins from *Arabidopsis thaliana* (At_ALDH2; NP_190383), *Oryza sativa* (Os_ALDH2; AAF73828.1) and *Zea mays* (Zm_ALDH2; NP_001105891). Conserved motifs are highlighted including; ALDH glutamic acid active site PROSITE (PS00687; black line) ALDH cysteine active site PROSITE (PS00070; red Box). *C. reinhardtii* protein accession refers to the Phytozome database, all others are given according to the NCBI database (<http://www.ncbi.nlm.nih.gov/protein>).

3.2.6 Distribution of fermentative enzymes in algal lineages

With the recent release of a number of algal genomes it was of potential interest to investigate the prevalence of fermentative pathways within the algal lineage (Table 3.2). Homologues of all the *C. reinhardtii* enzymes analysed here were found in the closely related *Volvox catenifer* *f. nagariensis* as well as *Chlorella* sp. NC64A. However some of the enzymes were not conserved across the algal lineage:

Coccomyxa sp. C-169 which belongs to the same class as *Chlorella* lacked *ADH*, *HYD*, *PFL* and *PFOR* homologues (Table 3.2). Additionally the Prasinophyte class of marine algae appeared to lack most of the fermentative enzymes found in *C. reinhardtii* except the putative *LDH* and *PFL1* sequences, the latter of which was the only gene to be found in *Micromonas pusilla* CCMP1545 and *Ostreococcus lucimarinus* CCE9901 genomes, whilst no homologues were identified in the genome of the red alga *Cyanidioschyzon merolae*, which does however contain a [NiFe]-hydrogenase.

Subunits for the putative mitochondrial and chloroplast PDH complexes appeared to be encoded in both green and red algae (Figures 3.5–3.8); the only exception was the apparent absence of chloroplast type *PDH E1* components in Prasinophytes. These marine algae otherwise contained three sets of E2 and two sets of E3 components (Figures 3.7 and 3.8).

In summary, analysis indicated that *C. reinhardtii* was not unique in possessing multiple fermentative pathways, and it is likely the presence of two PDH complexes, one chloroplast, one mitochondrial is wide spread in the algal lineage (Table 3.2).

Enzyme	Organism		Protein Accession
	Class	Genus and species	
ACK	Chlorophyceae	<i>Volvox carteri f. nagariensis</i>	XP_002948613, XP_002952674
	Trebouxiophyceae	<i>Chlorella sp.</i> NC64A	9129 ^a
		<i>Coccomyxa sp.</i> C-169	27864 ^a
ADH	Chlorophyceae	<i>Volvox carteri f. nagariensis</i>	XP_002958353, XP_002958348
	Trebouxiophyceae	<i>Chlorella sp.</i> NC64A	58567 ^a
HYD	Chlorophyceae	<i>Volvox carteri f. nagariensis</i>	XP_002948483, XP_002948487
	Trebouxiophyceae	<i>Chlorella sp.</i> NC64A	34957 ^a , 24040 ^a
LDH	Chlorophyceae	<i>Volvox carteri f. nagariensis</i>	XP_002955115
	Trebouxiophyceae	<i>Chlorella sp.</i> NC64A	55930 ^a
		<i>Coccomyxa sp.</i> C-169	28806 ^a
	Prasinophytes	<i>Micromonas pusilla</i> CCMP1545	90135 ^a
		<i>Micromonas sp.</i> RCC299	31180 ^a
		<i>Ostreococcus lucimarinus</i> CCE9901	XP_001420120
		<i>Volvox carteri f. nagariensis</i>	XP_002948484, XP_002949710
PAT	Chlorophyceae	<i>Chlorella sp.</i> NC64A	141896 ^a
	Trebouxiophyceae	<i>Coccomyxa sp.</i> C-169	54357 ^a
		<i>Volvox carteri f. nagariensis</i>	XP_002951658
PDC	Chlorophyceae	<i>Chlorella sp.</i> NC64A	52583 ^a , 35601 ^a
	Trebouxiophyceae	<i>Coccomyxa sp.</i> C-169	38356 ^a
		<i>Volvox carteri f. nagariensis</i>	NP_001150259
mtPDH E1 α component	Chlorophyceae	<i>Chlorella sp.</i> NC64A	141042 ^a
	Trebouxiophyceae	<i>Coccomyxa sp.</i> C-169	29303 ^a , 73729 ^a , 25215 ^a
		<i>Volvox carteri f. nagariensis</i>	22101 ^a
	Prasinophytes	<i>Micromonas pusilla</i> CCMP1545	89160 ^a
		<i>Micromonas sp.</i> RCC299	XP_001415646
		<i>Ostreococcus lucimarinus</i> CCE9901	XP_002950542
		<i>Volvox carteri f. nagariensis</i>	32109 ^a
plPDH E1 α component	Chlorophyceae	<i>Chlorella sp.</i> NC64A	33689 ^a
	Trebouxiophyceae	<i>Coccomyxa sp.</i> C-169	XP_002958165
		<i>Volvox carteri f. nagariensis</i>	30765 ^a
PFL1	Chlorophyceae	<i>Chlorella sp.</i> NC64A	44800 ^a
	Trebouxiophyceae	<i>Micromonas pusilla</i> CCMP1545	XP_001416311
	Prasinophytes	<i>Ostreococcus lucimarinus</i> CCE9901	

Table 3.2 (Continued)

Enzyme	Organism		Protein Accession
PFOR	Chlorophyceae	<i>Volvox carteri f. nagariensis</i>	XP_002947533, XP_002949465
	Trebouxiophyceae	<i>Chlorella sp.</i> NC64A	134349 ^a

Table 3.2: Distribution of fermentative and pyruvate degrading enzymes in Chlorophyta.

Protein accessions are given according to NCBI database (<http://www.ncbi.nlm.nih.gov/protein>) unless they refer to ^aJGI protein ID, ^bPhytozome accession.

3.3 Protein expression for antibody production

Enzymes were selected for further analysis on the basis of previous studies investigating fermentative product accumulation during dark-anaerobic and sulphur deprived conditions, and transcriptomic analysis (Mus et al., 2007), with a focus on those that potentially compete with hydrogen production through degradation of pyruvate or regeneration of NAD^+ .

Expressed sequence tags (ESTs) for *ADH1*, *LDH*, *PDC1*, *PDC2*, *PDC3*, *PFL1* and *PFOR* were identified by BLAST search against the Kazusa Database (the specific clones used are listed in Table 3.3). The cDNA clones were re-sequenced and confirmed to be correct after checking against the genome annotation. Primers were designed to amplify short segments of the target ESTs and PCR fragments cloned into the Invitrogen Gateway® cloning system DNA entry vector, pENTR/TEV/D TOPO (Invitrogen Life Technologies, USA) (Figure 3.13A) (see section 2.8.1), or directly into pRSET A-thr expression vector (Ernesto Costa, Imperial College) in the case of *ADH1* and *PDC2* (Figure 3.14A).

ESTs cloned into pENTR/TEV/D TOPO were transferred into protein expression vector pDEST17 and integration confirmed by sequencing. All expression vectors added an N-terminal His₆-tag to encoded sequences in order to facilitate protein purification. Vectors were tested by restriction digest to confirm integration of cloned DNA (Figure 3.13B and 3.14B) and validated by sequencing. Sequenced plasmids were then used to transform *E. coli* KRX cells and protein expression was tested in small scale experiments (Figures 3.14C and 3.15).

Once protein expression was confirmed, the solubility was tested by the use of Bugbuster® Protein Extraction Reagent as exemplified in Figure 3.15. All proteins were found in the insoluble fraction at a higher relative concentration than the soluble, presumably in the form of inclusion bodies. Expressed proteins were then purified from 1 L cultures of *E. coli* (Figure 3.15) by immobilised Ni^{2+} affinity chromatography and the quantity and purity of resulting protein estimated by SDS-PAGE in comparison with a BSA standard (Figure 3.16).

The slight differences in molecular weight estimated by SDS-PAGE compared to the expected size for some proteins (Figure 3.17), can potentially be explained by variable SDS-binding (Rath et al., 2009) which is dependent on amino acid sequence

(Ibel et al., 1990). DNA sequencing results were double checked and confirmed to be correct with stop codons in place (data not shown).

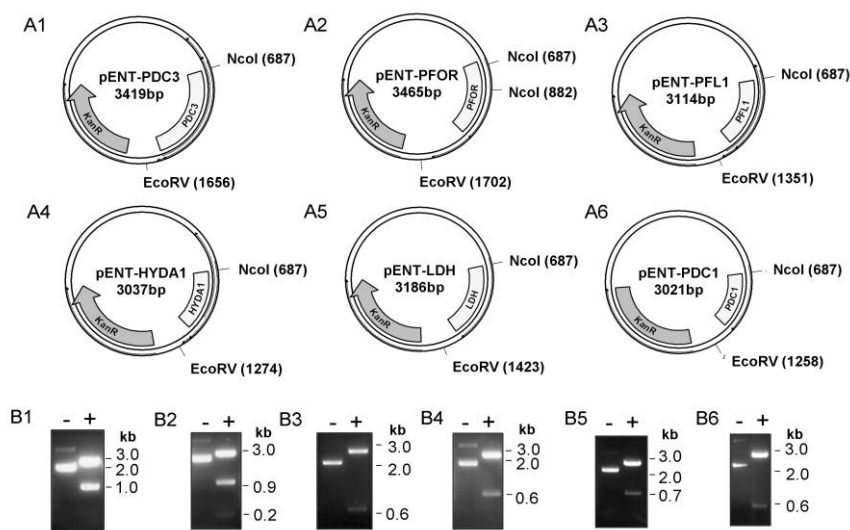


Figure 3.13 Entry vector construction for protein expression.

(A) vector maps of PCR fragments cloned into Invitrogen entry vector pENTR/TEV/D TOPO. (B) Restriction analysis of plasmids, undigested (-) and double digested with NcoI and EcoRV (+), each number corresponds to vector map of the same number.

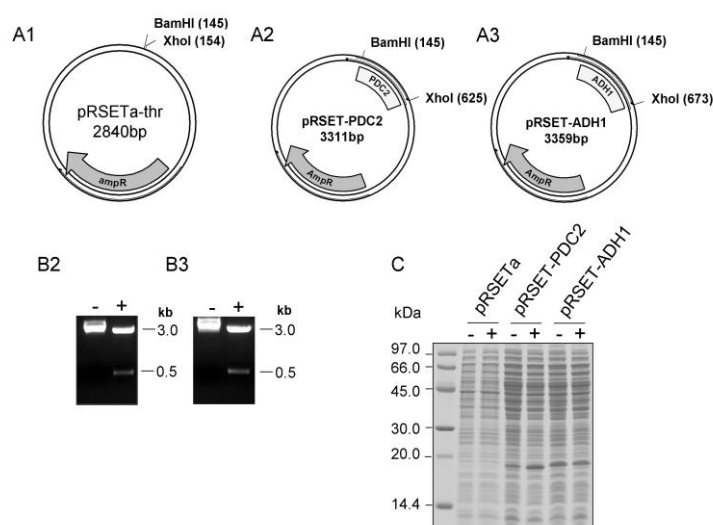


Figure 3.14: Production of pRSET-PDC2 and pRSET-ADH1 EST expression vectors.

(A) Vector maps, PCR fragments cloned directly into BamHI and XhoI sites of protein expression vector pRSET A-thr. Restriction digest analysis of expression vectors showing pRSET-PDC2 (B1) and pRSET-ADH1 (B2), undigested control (-) and BamHI XhoI double digest (+) depicted. (C) Protein expression trials, *E. coli* KRX whole cell extracts containing expression vectors with (+) and without (-) induction of protein expression, pRSET A-thr was used as an empty vector control, samples analysed on Coomassie blue stained 18% SDS-PAGE gel.

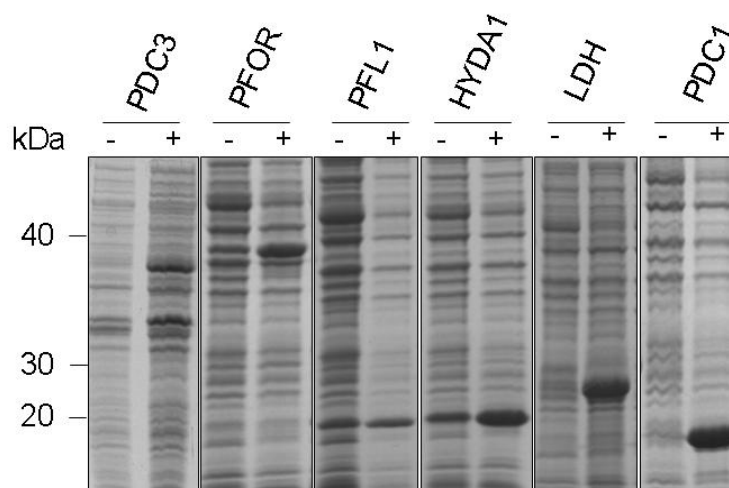


Figure 3.15: EST protein expression trials.

E. coli KRX whole cell extracts containing pDEST17 expression vectors with (+) and without (-) induction of protein expression samples analysed on Coomassie stained 12.5% SDS-PAGE gel.

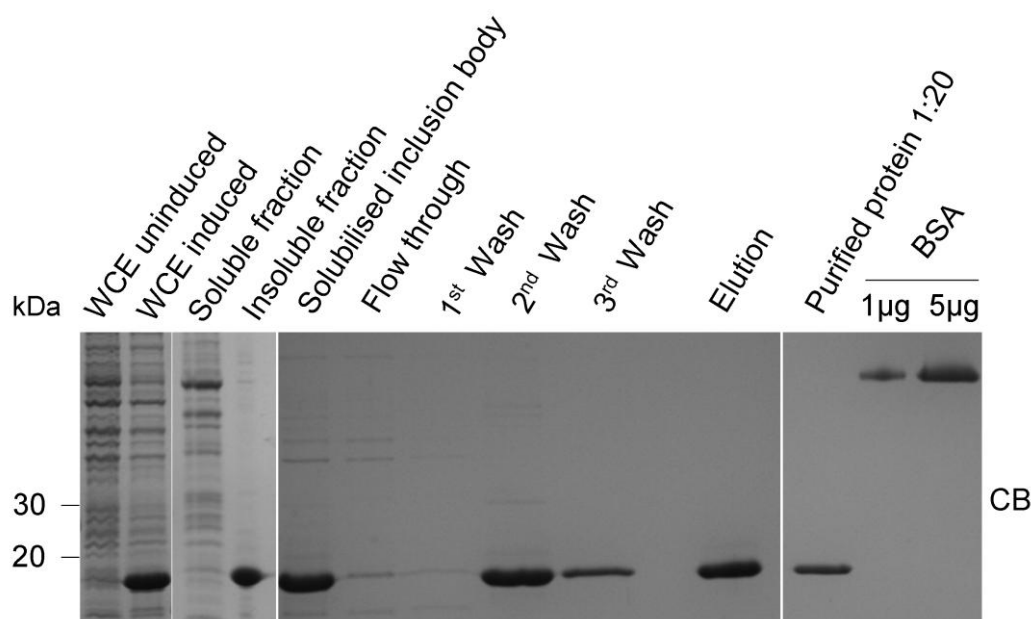


Figure 3.16: His₆-tag purification of *C. reinhardtii* PDC1 protein fragment from *E. coli* whole cell extracts (WCE).

Inclusion bodies were solubilised (see section 2.8.5) then loaded onto a Ni²⁺-IDA metal chelate resin. The column with bound protein was then subjected to wash with binding buffer (1st wash), then 2x binding buffer with 60 mM imidazole (2nd and 3rd wash) before eluting in binding buffer plus 1 M imidazole. Eluted protein was then dialysed in PBS and the precipitated protein re-suspended in PBS followed by the addition of 10% SDS (w/v) until pellet dissolved, to give purified sample. Protein concentration was determined by comparison to fresh BSA standard.

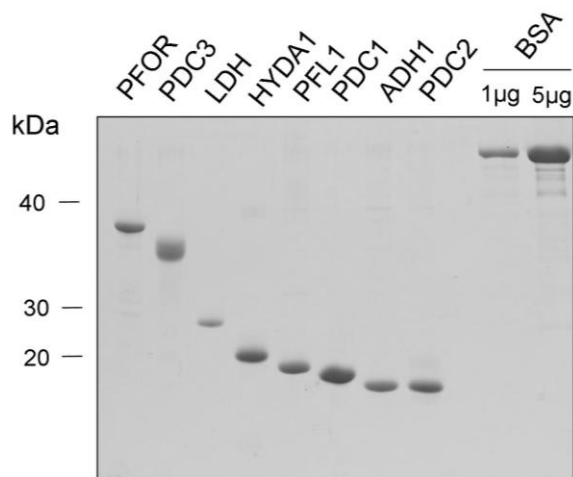


Figure 3.17: Analysis of His₆-tag purified EST protein fragments.

Purified proteins were loaded at a 1:10 dilution in PBS, with the exception of ADH1 and PDC2 which were diluted 1:7.5. Samples were separated on a 15% SDS-PAGE gel and stained with Coomassie blue. BSA was used for protein concentration determination.

Target	Native Protein		Expressed Fragment		
	No. residues	Predicted Size (kDa)	EST Accession	Residues	Expected Size (kDa)
PFOR	1181	128.3	BP093588	180–545	38.3
PDC3	570	61.9	AV637523	241–517	36.2
LDH	421	45.6	AV622264	13–209	24.5
HYDA1	497	53.1	AV389547	350–497	18.9
PFL1	831	91.1	AV389850	655–831	22.5
PDC1	396	43.7	BG848457*	255–396	16.1
ADH1	953	102.2	AV639995	197–368	18.0
PDC2	397	43.5	AV624259	241–397	17.2

Table 3.3: Description of protein fragments expressed in *E. coli* for antibody production.

EST accessions refer to the Kazusa DNA Research Insititute database with the exception of (*) which was obtained from the Duke University collection (see section 2.1).

3.4 Immunochemical analysis

Purified protein was sent to SEQLAB laboratories (Germany) for polyclonal antibody production, all except the PDC1 final bleed underwent affinity purification. Each polyclonal antibody was checked for cross-reaction against the corresponding purified antigen and *C. reinhardtii* cell extracts (Figure 3.18).

Antibodies raised against *C. reinhardtii* PDC3, PFL1 and ADH1 detected proteins of ~60, 90, and ~100 kDa respectively, which was in good agreement with previous experimental data using antibodies raised against pea, and *E. coli* enzymes (Atteia et al., 2006; Hemschemeier et al., 2008b) (Figure 3.18).

The PFL1 antibody was raised against the C-terminal region and included the ~3 kDa sequence that undergoes cleavage upon activation, meaning both inactive and active forms should be recognised. The initial crude antiserum gave two cross reactions at ~90 kDa and ~70 kDa, however after affinity purification the lower band was no longer evident suggesting this was not a genuine cross reaction.

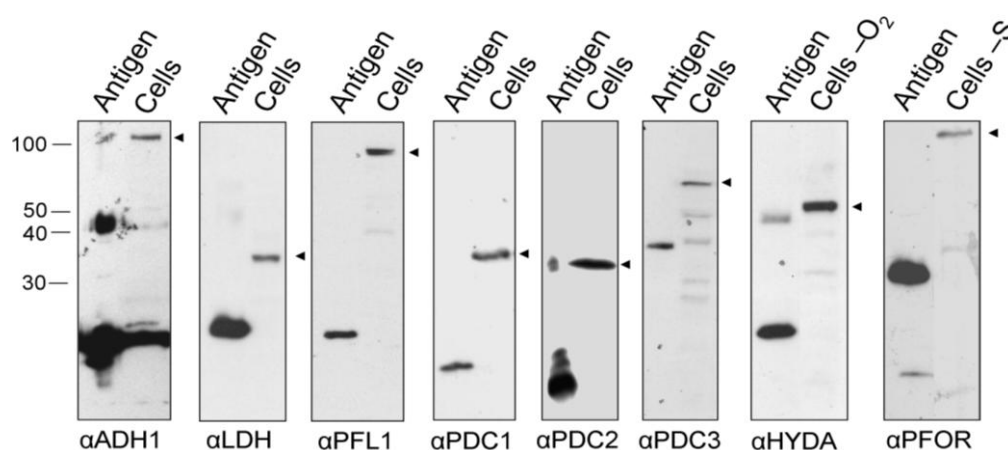


Figure 3.18: Immunoblot analysis testing polyclonal antibodies raised in rabbits. *C. reinhardtii* cells (8×10^5 loaded) and 10 ng of relevant antigen were probed with antibody to the target protein. Bands corresponding to target proteins are highlighted (black arrow). Anaerobic cell extracts were created by 4 h dark-anaerobic incubation under an argon atmosphere ($-O_2$), or in the case of PFOR (-S) 48 h sulphur-deprivation in sealed containers.

PDC1 and PDC2 have a predicted size of ~44 kDa which is similar to other E1 α components. However, immunoblots gave single bands of a slightly smaller size of ~40 kDa which could be a result of transit peptide cleavage (Figure 3.18).

The hydrogenase antibody was raised against the C-terminal portion of HYDA1 and should recognise both HYDA1 and HYDA2 isoforms, giving a cross

reaction at ~50 kDa corresponding to the expected size of both enzymes (Forestier et al., 2003), in some blots a doublet was observed (data not shown), further investigation will be required to ascertain if the bands correspond to HYDA1 and HYDA2, to a degradation product or to apo- and holo-enzyme forms.

PFOR and LDH antibodies detected proteins with a similar mass to their predicted size based on genome annotation, giving bands at a mass of ~120 kDa and 35–40 kDa respectively (Figure 3.18). The LDH antibody was raised against the N-terminal portion of the protein sequence and should include the transit peptide sequence which is predicted to be 44 amino acids long (ChloroP).

Longer exposure times revealed a number of additional cross reactions at lower molecular mass for PFOR, PDC3, PFL1 and ADH1 (data not shown). The identity of these cross reactions were unclear and may be a mixture of breakdown products precurs and unspecific cross reactions.

To check that the harvesting procedure which involved a 2 min centrifugation step (see section 2.10.1) did not induce fermentative enzyme expression, protein samples were taken from cultures of *C. reinhardtii cw10* (CC-406) before and after 4 h dark-anaerobic treatment with or without the addition of protein synthesis inhibitor cycloheximide (CHX) (Figure 3.19). Using the [FeFe]-hydrogenase as a control for anaerobic induction, as expected HYDA was induced after 4 h dark-anaerobic treatment in the absence of CHX but not in the presence of the inhibitor (Figure 3.19). No difference in expression levels could be discerned between samples prior to anaerobic treatment, with or without CHX demonstrating no induction of HYDA occurred during sample preparation (Figure 3.19).

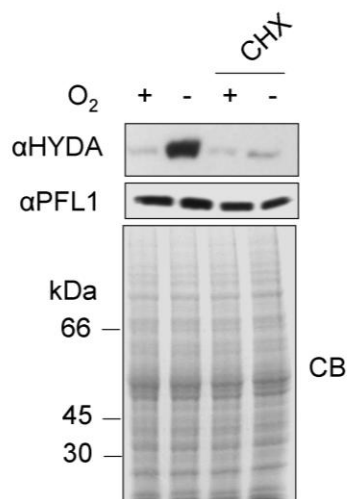


Figure 3.19: Immunoblot analysis of the effect of harvesting procedure on fermentative enzyme induction.

Samples were taken before and after 4 h dark-anaerobic incubation in AIB buffer with or without prior addition of $10 \mu\text{g ml}^{-1}$ cycloheximide (CHX). 8×10^5 cells loaded per lane, Coomassie blue (CB) stained gel provided as loading control.

3.4.1 Enzyme expression under sulphur-deprivation in the light

A widely used protocol for sustained H_2 production involves placing sulphur-deprived cultures of *C. reinhardtii* into sealed bioreactors. To test whether the target enzymes were present under these conditions, extracts were taken at 24 h intervals and analysed by immunoblot (Figure 3.20). Using *C. reinhardtii* strain CC-124, cultures entered hypoxia and began producing H_2 after 24 h of sulphur-deprivation as determined by consumption of O_2 from the headspace of mini-bioreactors and induction of HYDA. Maximum rates of H_2 production were between 24–72 h before declining between 72 to 96 h (Figure 3.20).

PDC1 levels remained consistent throughout the experiment as did LDH. PDC3 levels remained largely unchanged, HYDA was induced after 24 h and remained at a constant level and a slight increase in ADH1 levels was observed in the first 24 h. PFL1 levels decreased after 48 h and PFOR was maximally induced between 24–72 h of sulphur-deprivation. A slight decrease in putative chloroplast PDH E1 alpha component PDC2, was observed hinting at degradation (Figure 3.20)

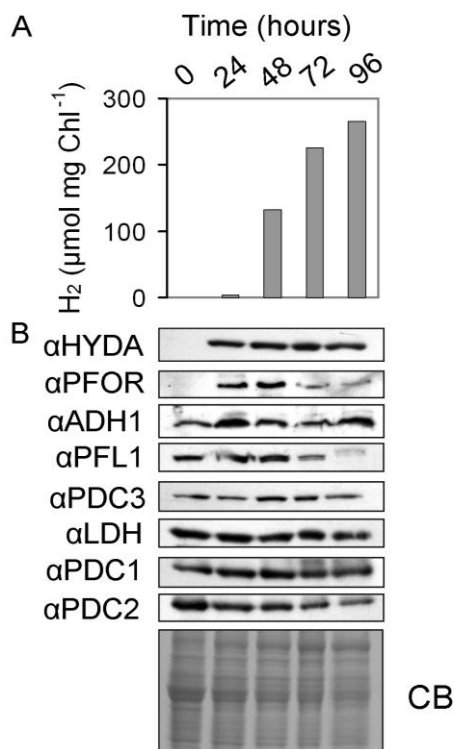


Figure 3.20: Analysis of fermentative enzyme expression during sulphur-deprivation in the light.

(A) H₂ evolution measured by water displacement and analysis by GC (B) Immunoblot analysis. 8×10^5 cells were loaded per lane and protein loading assessed by Coomassie stained gel (CB) (bottom). The zero time point was taken just before sulphur-deprivation to avoid induction of hydrogenase during the washing procedure.

3.4.2 Regulation of fermentative enzymes under varying conditions

3.4.2.1 Fermentative enzyme expression during mixotrophic and heterotrophic growth

Protein extracts were taken from cultures of *C. reinhardtii* during growth in TAP medium under either continuous illumination, or in darkness, and analysed by immunoblot to look for changes in enzyme expression as a function of growth phase (Figure 3.21). The relative intensity of cross-reactions between light and dark-grown cultures was compared from samples loaded on the same protein gel (Figure 3.21), and all bands showed comparable expression with the exception of HYDA and PFOR (Figure 3.21). Relative to dark-grown cultures, HYDA and PFOR proteins were not detected in significant amounts in the light (Figure 3.21A), but were present in high abundance during early growth stages in the dark (Figure 3.21B), before declining at the onset of stationary phase which coincided with the depletion of acetate from the medium, as determined by HPLC analysis (data not shown). All other enzymes were

found to be constitutively present in both the light and the dark and at all stages of growth (Figure 3.21).

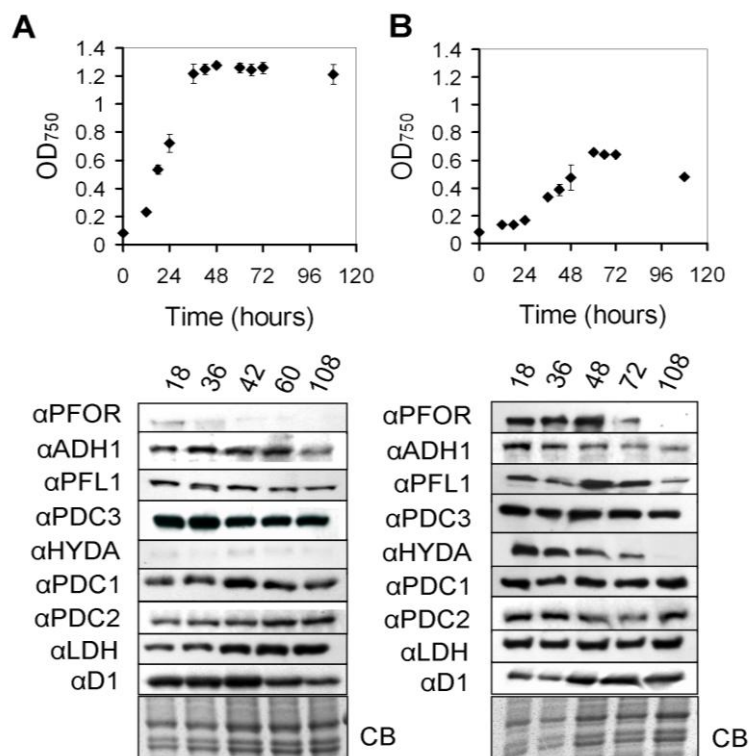


Figure 3.21: Immunoblot analysis of *C. reinhardtii* fermentative enzyme expression during growth on TAP medium.

Cultures were grown in the light (A) or dark (B). Samples were taken from early log to late stationary phase at times indicated error bars are provided $\pm$ SD of three biological replicates. 8×10^5 cells were loaded per lane. Cultures were inoculated from mid-log phase TAP grown cultures under continuous illumination. Protein loading assessed by Coomassie blue (CB) stained gel (bottom).

3.4.2.2 Fermentative enzyme expression during photoautotrophic growth

In order to rule out the possibility that fermentative enzyme expression was the result of anaerobic conditions in the culture vessels used, or as a function of carbon source, CC-124 cultures were grown in minimal medium with continuous aeration (Figure 3.22A). Samples were taken at 24 h intervals and the dissolved oxygen concentration measured by Clark electrode (Figure 3.22B), enzyme expression compared by immunoblot using samples taken from pre-TAP grown samples incubated in anaerobic induction buffer (AIB) in the dark for 4 h with continuous argon purging as a control for anaerobic induction.

A minor dip in O₂ concentrations was observed in samples taken at late log and stationary phase, and is taken to be the result of respiration between sampling and

measurement (Figure 3.22B). As with TAP-grown cultures (Figure 3.21) many of the fermentative enzymes were constitutively present (Figure 3.22C). Significant differences in enzyme expression during growth could only be observed for the putative chloroplast PDH complex protein PDC2 which showed a consistent decrease in levels from early log to stationary phase cultures (Figure 3.22C) (Appendix III.4).

Small differences were seen in fermentative enzyme expression level between aerobic and anaerobically induced samples (Figure 3.22). A cross-reaction for the [FeFe]-hydrogenase was present at low levels in all growth stages (Figure 3.22C), although increased in anaerobic samples (Figure 3.22C) which is more evident in repeat blots (Appendix III.4). Analysis of PFOR was not included, as enzyme levels were very low and a large number of additional cross reactions were present at lower molecular mass (data not shown). Levels of PDC3 and ADH1 actually appeared to decrease (Figure 3.22C) in contrast to previous transcript analysis (Mus et al., 2007). There was a possible appearance of a doublet in PDC1 cross reactions during anaerobic induction (Figure 3.22C) which may hint at post-translational modification but will require further investigation.

As many of the fermentative enzymes were constitutively present it was of interest to see if any fermentative metabolites accumulated in the growth medium. NMR analysis of samples revealed an increase in lactate, formate and ethanol in all the cultures. All metabolites were present in the 0 h cultures, this is proposed to be in part the result of the experimental method - after inoculating HSM with colonies from TAP agar plates, cultures were allowed to grow for 1 day before being diluted ~1:3 to an OD₇₅₀ of ~0.05. The reason for an initial period of growth was to allow cultures to acclimate their metabolism to the altered carbon source (acetate to CO₂) and to ensure all biological replicates began at approximately the same cell density. In future work cells should be spun down and washed once with minimal media before beginning the experiment to remove any metabolites accumulated, but this step was avoided to ensure fermentative pathways were not induced upon centrifugation. Concentrations of formate and ethanol in HSM medium were approximately 4 µM and 2-5 µM respectively, much less than the concentrations seen in the cultures at t = 0 h (Figure 3.22).

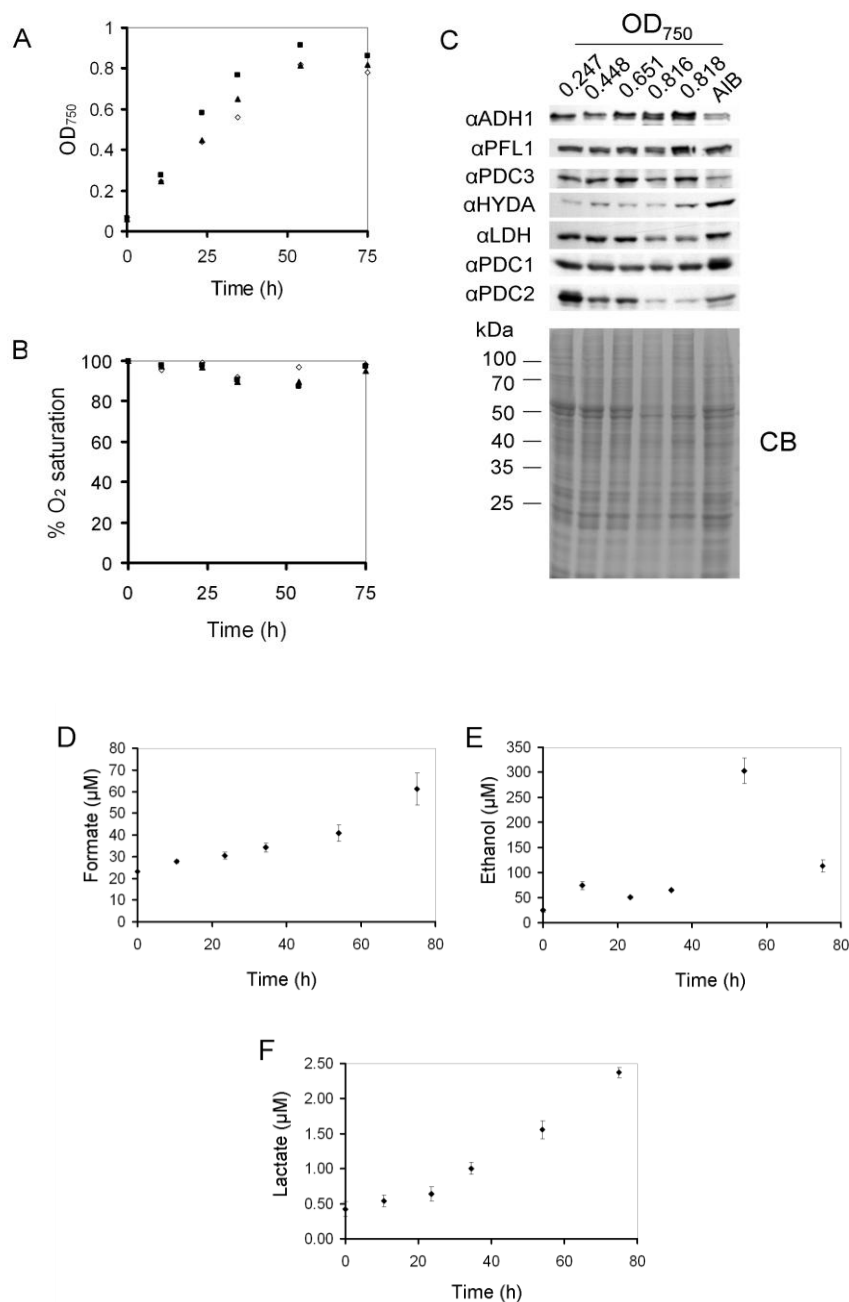


Figure 3.22: Analysis of *C. reinhardtii* fermentative enzyme expression and metabolite production during photoautotrophic growth.

Cultures were grown in HSM under continuous illumination $140 \mu\text{E m}^{-2} \text{s}^{-1}$ with continuous aeration. Mid-log phase HSM inoculated cultures were diluted to OD₇₅₀ ~0.05. (A) Growth curve measuring OD₇₅₀ nm, (B) % O₂ saturation measured by Clark electrode, biological replicates are plotted 1 (squares), 2 (triangles) 3 (diamonds). (C) Immunoblot of fermentative enzyme expression during photoautotrophic growth analysing replicate 3 (diamonds), compared to sample incubated in anaerobic induction buffer (AIB) in the dark for 4 h with continuous argon purging, 8×10^5 cells loaded per lane, loading is provided by Coomassie stained gel (CB). NMR analysis followed formate (D), ethanol (E) and lactate (F) accumulation in the media, results are given $\pm$ SE of three biological replicates.

As the cultures were bubbled with air, there was some evaporation of medium from the flasks, this was not quantified, but would not be expected to account for a decrease of more than a quarter of the total volume, and could therefore also not account for the increase in metabolites observed. Furthermore no bacterial contaminants could be seen in samples of cultures analysed under the microscope. This would therefore suggest that *C. reinhardtii* actively produces formate, lactate and ethanol even during photoautotrophic growth (Figure 3.22D, E, F).

Formate concentrations gradually increased in the medium during the growth then showed the largest increase during the period when cells entered stationary phase (Figure 3.22D). To obtain a rough comparison with the amount of formate accumulated when cultures are deprived of sulphur, the rate of formate production by stationary phase cultures was estimated to be 0.13 nmol per 10^6 cells h^{-1} compared to a maximum rate of 0.24 nmol per 10^6 cells h^{-1} between 24–48 h sulphur-deprivation (Figure 4.10). This was based on the assumption that the cell concentration of stationary phase cultures was measured at $\sim 7.5 \times 10^6$ cells ml^{-1} and $\sim 8 \times 10^6$ cells ml^{-1} in TAP-S experiments. Although additional repeats for more accurate measurements will be required these results suggest a significant rate of formate accumulation in stationary phase, photoautotrophic cultures.

Changes in ethanol levels were more complex, and showed a degree of fluctuation meaning the results should be treated with caution until further repeats are performed (Figure 3.22E). However, these data would suggest ethanol levels show a small increase during growth before peaking at the late stationary phase (Figure 3.22E) which may coincide with wider metabolic changes or nutrient stress caused by depletion of components from the medium (such as nitrogen), but this was not measured. If the maximum concentration of ethanol in photoautotrophic cultures, ~ 40 nmol per 10^6 cells at late stationary phase, is compared to that found after 96 h of sulphur-deprivation ~ 7 nmol per 10^6 cells it would suggest an important role for ethanol metabolism during photoautotrophic growth.

The accumulation of lactate was particularly surprising as this metabolite is not normally produced by cultures under anaerobic conditions (Figure 3.22F). Lactate accumulated to a more significant degree than in TAP-S cultures, produced during stationary phase at a rate $\sim 50\times$ greater (0.0052 nmol per 10^6 cells h^{-1}) than WT cultures during sulphur-deprivation (0.0001 nmol per 10^6 cells h^{-1}), similar to that observed between 72–96 h post-sulphur-deprivation in PFL1 inhibited cultures

(0.0077 nmol per 10^6 cells h^{-1}). These data therefore suggest in WT cultures lactate metabolism may be more important during aerobic than anoxic conditions.

3.4.2.3 Post-translational modification of putative LDH

A slight shift in the apparent molecular weight of LDH was observed between aerobic and anaerobic samples (Figure 3.23). To determine whether this was the result of induction of a novel protein or a post-translational modification, cultures of CC-124 were subject to 4 h dark-anaerobic incubation with or without protein synthesis inhibitor CHX and samples taken at 1, 2 and 4 h (Figure 3.23). HYDA immunoblot was performed as a control for anaerobic induction, indicating CHX treatment inhibited protein synthesis (Figure 3.23). The LDH signal split into a doublet after 1 h dark-anaerobic incubation with the initial LDH band and one of a slightly higher molecular mass in both CHX treated and un-treated samples, suggesting a post-translational modification (Figure 3.23). The LDH appeared to be gradually converted into the higher molecular mass form over 4 h dark-anaerobic incubation with the ratio of lower to upper bands increasing, until little was left of the lower form after 4 h (Figure 3.23).

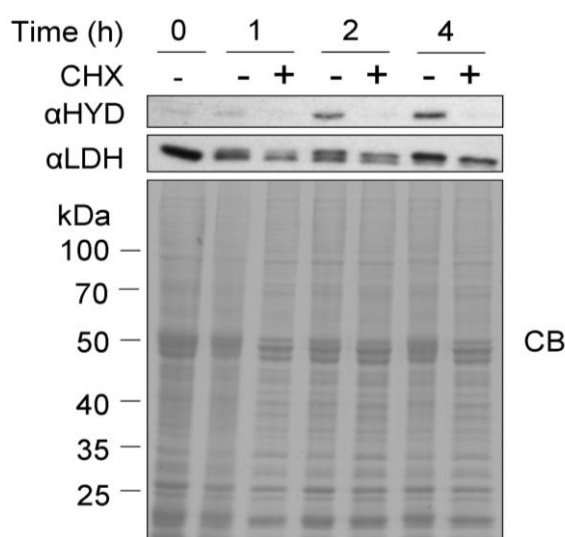


Figure 3.23: Immunoblot analysis of post translational modification of LDH.

Samples were incubated in AIB under dark-anaerobic conditions for 4 h, with (+) or without (-) the presence of $10 \mu\text{g ml}^{-1}$ CHX. 8×10^5 cells loaded per lane, loading control is provided by Coomassie blue (CB) stained gel.

3.4.2.4 Fermentative enzyme expression under dark:light cycles

In the environment, hypoxia is potentially induced in *C. reinhardtii* during dark periods of the diurnal cycle (Whitney et al., 2010). To test whether the oxygen-sensitive [FeFe]-hydrogenase and PFOR protein levels increased during dark periods, so as to provide an indication of their involvement in cellular metabolism at night, samples were taken from TAP-grown cultures undergoing (14/10 h) dark/light cycles. PFOR and HYDA were found to be present in the dark and to decline in subsequent light periods (Figure 3.24A). To rule out the possibility that induction was the result of a light effect, rather than the result of the onset of anaerobic conditions due to cessation of photosynthetic activity, TAP-grown cultures were incubated in sealed containers in the presence of the PSII inhibitor, DCMU, in the light (Figure 3.24B). Under these conditions cells will continue to consume oxygen driving cultures anaerobic. Analysis of enzyme expression revealed a gradual increase in PFOR and HYDA protein levels (Figure 3.24B), indicating expression was independent of light.

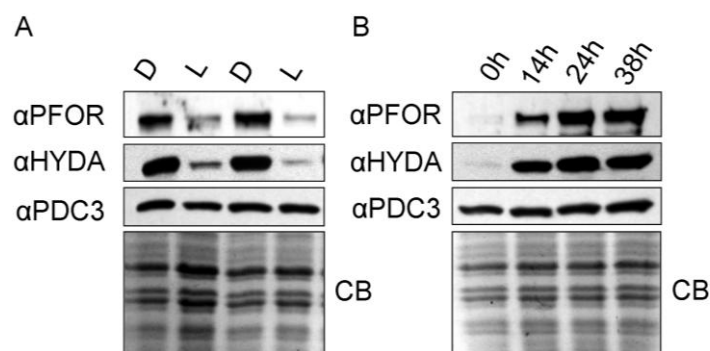


Figure 3.24: Immunoblot analysis of PFOR and HYDA expression.

(A) Cultures grown under 10/14 h dark/light cycles were analysed at the end of the dark; D, and light; L, periods. (B) TAP grown cultures under continuous illumination were treated with 10 μ M DCMU to inhibit O_2 evolution allowing for the establishment of anoxic conditions in the light. 1.2×10^6 cells were loaded per lane, Coomassie blue (CB) stained gel is provided as a loading control.

3.4.3 Size and location fermentative enzymes

3.4.3.1 Subcellar distribution of fermentative enzymes

To determine the sub-cellular localisation of the pyruvate-degrading enzymes, an immunochemical analysis was performed on isolated chloroplast, mitochondrial and cytoplasmic fractions (Figure 3.25). Potential cross-contamination of the mitochondrial and chloroplasts fractions was tested using antibodies specific for the photosystem II D1 reaction center subunit (D1) (chloroplast marker) and the

mitochondrial cytochrome oxidase (COXIIb) subunit (a mitochondrial marker). The results indicated some residual low level mitochondrial contamination of the chloroplast fractions, at similar levels reported in other publications (Terashima et al., 2010).

The PDH E1 alpha components were identified in distinct sub-cellular compartments with PDC1 in the mitochondrion and PDC2 in the chloroplast (Figure 3.25). Previously PFL1 has been suggested to be co-localised to the mitochondrion and chloroplast (Atteia et al., 2006). However, here PFL1 was found to be predominantly present in the mitochondrion. The signal for PFL1 obtained with the chloroplast fraction could potentially be explained by the residual mitochondrial contamination when compared to relative amounts of COXIIb (Figure 3.25).

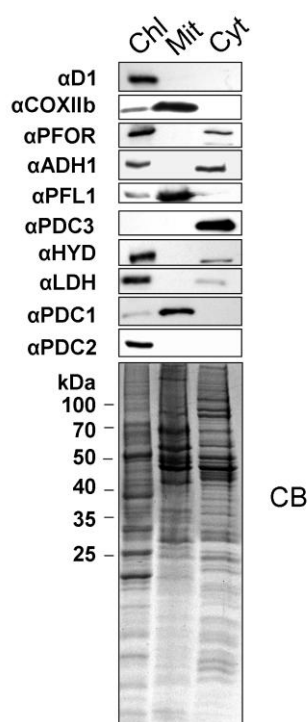


Figure 3.25: Sub-cellular distribution of pyruvate-degrading and fermentative enzymes in *C. reinhardtii*.

Immunoblot analysis of chloroplastic (Chl), mitochondrial (Mit), and cytoplasmic (Cyt) fractions using affinity-purified antibodies. Coomassie Blue (CB) stained SDS-PAGE gel shown at bottom. 8 µg protein loaded per lane.

ADH1, HYDA and PFOR were all located to the chloroplast in agreement with proteomic data (Terashima et al., 2010) along with LDH which had not previously been identified. Interestingly the ADH1 antibody detected bands in both

chloroplast and cytoplasmic fractions, with the cytoplasmic signal corresponding to a protein of a slightly smaller size (Figure 3.25), although this additional band cannot be ruled out as an unspecific cross-reaction without the availability of an ADH1 knockdown or knockout line. PDC3 was localised to the cytoplasm explaining why previous proteomic analysis failed to detect this protein at significant levels in the mitochondrion or chloroplast (Atteia et al., 2009; Terashima et al., 2010). Finally low levels of chloroplast protein contamination in the cytoplasmic fraction were found as indicated by LDH, HYD and PFOR cross-reactions (Figure 3.25).

3.4.3.2 Analysis of fermentative enzyme membrane association

Immunoblotting was used to determine whether the target enzymes were found in the soluble fraction or were membrane associated (Figure 3.26). A D1 immunoblot confirmed separation of thylakoid membrane extracts from the soluble fraction (Figure 3.26).

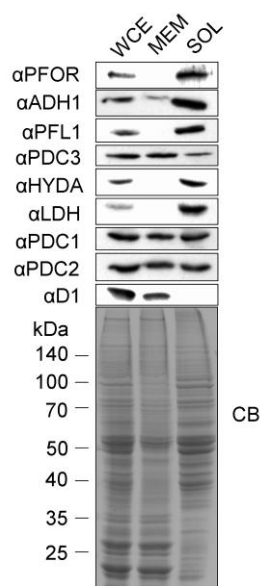


Figure 3.26: Comparison of pyruvate-degrading and fermentative enzyme distribution in *C. reinhardtii*.

Immunoblot analysis of whole cell extract (WCE), membrane (MEM), and soluble (SOL) fractions using affinity-purified antibodies. Coomassie-Blue stained SDS-PAGE gel shown at bottom. 40 µg protein loaded per lane.

PDH E1 alpha components, PDC1 and PDC2, appeared to be equally distributed between membrane and soluble fractions (Figure 3.26). PDC3 was also found in both samples (Figure 3.26). Membrane association of PDC3 is unexpected as has not been demonstrated elsewhere, however this result will have to be repeated as

only one biological replicate has been performed, and analysis of the membrane fraction from a separate preparation by 2D SDS-PAGE failed to provide a cross reaction with PDC3. All other proteins were found exclusively in the soluble fraction. The low-level signal obtained using the ADH antibody in the membrane sample was potentially the result of residual, low level soluble protein contamination, it was also appeared to be of a higher molecular mass and may represent an unspecific cross reaction.

3.4.3.3 2D-SDS PAGE analysis of *C. reinhardtii* fermentative enzymes

C. reinhardtii membrane fractions were solubilised, analysed by blue native gel electrophoresis and probed with anti-PDC1 and anti-PDC2 antibodies to determine whether the putative PDH α E1 components were present in higher complexes to provide further support to their assignment (Figure 3.27).

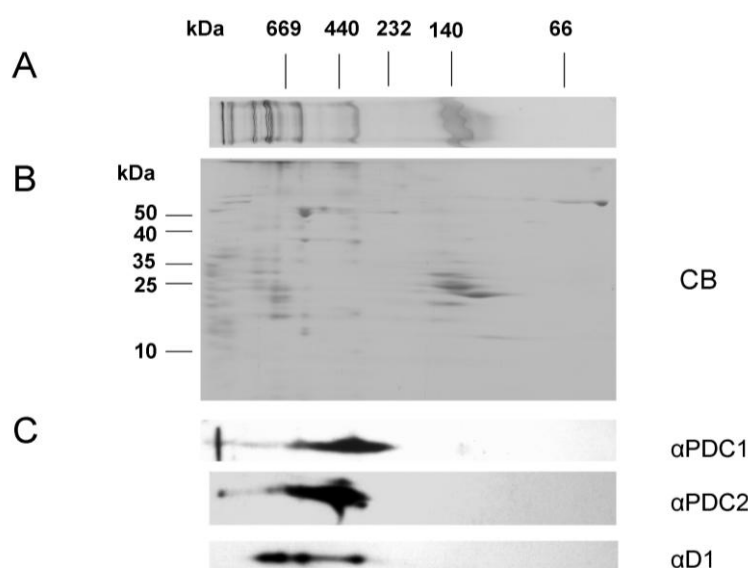


Figure 3.27: 2D-PAGE analysis of the *C. reinhardtii* membrane fraction.

8 μ g Chl loaded per lane (A) Coomassie stained 1D blue native gel (B) 2D coomassie stained SDS-PAGE gel (C) Immunoblot analysis.

A D1 antibody was used as a control for the presence of intact membrane complexes and provided an additional comparison for estimation of complex sizes, cross reactions revealed D1 was present in PSII monomers (230 kDa), dimers (450 kDa) and PSII-LHC supercomplexes (Figure 3.27) indicating the photosynthetic complexes were maintained intact after sample preparation. Immunoblotting

suggested that the portion of both PDC1 and PDC2 proteins that associated with the membrane fraction were part of high molecular weight complexes that ran at the top of the native gel, although the majority of protein was present in lower molecular mass complexes, which may represent dissociated forms (Figure 3.27C). However, the appearance of additional cross reactions for both PDC1 and PDC2 at a lower molecular mass, plus low amount of putative intact PDH complexes relative to dissociated forms means this result should be treated with caution, and further analysis is yet required.

C. reinhardtii soluble fractions were separated by 2D-PAGE to give an indication of complex formation of target proteins in the soluble fraction (Figure 3.28). ADH1 is a homologue of the *E. coli* ADHE which has multiple oligomeric states (Kessler et al., 1992), and was accordingly found distributed along a range which could include dimeric to pentameric forms (Figure 3.28C). PFL1 is known to form dimers (Conradt et al., 1984) and two bands were observed, corresponding approximately to monomeric (~90 kDa) and dimeric forms (~180 kDa).

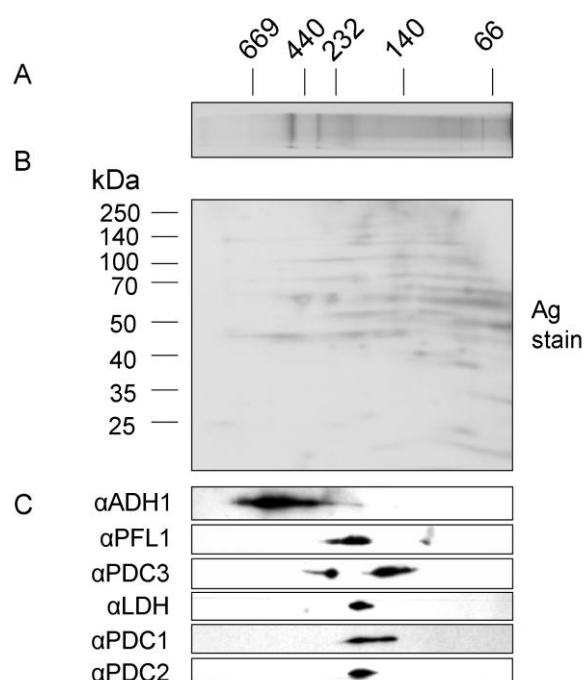


Figure 3.28: BN analysis of *C. reinhardtii* soluble fraction.

Showing (A) 1D BN gel, 40 µg soluble protein loaded per lane, stained with Coomassie Brilliant Blue (R) (B) 12% (w/v) 2D SDS-PAGE silver stained gel (C) immunoblot analysis of second dimension gel.

PDC3 is a pyruvate decarboxylase which conventionally forms homodimers composed of two loosely bound PDC3 dimers (Dyda et al., 1993). Here two bands were observed corresponding approximately to dimeric (~120 kDa) and tetrameric forms (~240 kDa) (Figure 3.28C).

D-lactate dehydrogenases are conventionally homotetrameric (Denicola-Seoane and Anderson, 1990), and putative LDH displayed a single band of approximately the correct size (~160 kDa) (Figure 3.28C). Probing of putative PDH E1 α components PDC1 and PDC2 revealed the components in the soluble fraction were not associated with high molecular weight complexes, potentially representing heterotetrameric PDH E1 complexes consisting of α and β subunits that associate with the E2 core of the PDH complex (Smolle and Lindsay, 2006) (Figure 3.28C).

3.6 Discussion

Fermentative metabolism in *C. reinhardtii* has previously been thought to be activated under anoxic conditions (Mus et al., 2007). Here immunochemical analysis revealed the constitutive presence of all but the highly O₂-sensitive PFOR protein (which could not be properly analysed due to a large number of cross reactions) in *C. reinhardtii* during photoautotrophic growth. Additionally immunochemical analysis of isolated organelle fractions was used to refine current models on genomic, proteomic and transcriptomic data (Grossman et al., 2007; Mus et al., 2007; Dubini et al., 2009; Terashima et al., 2010) and expand upon recent proteomic localisation studies to include analysis of cytoplasmic enzymes (Atteia et al., 2009; Terashima et al., 2010). This allowed for production of a new metabolic scheme (Figure 3.29) that was based largely on immunochemical evidence. The polyclonal antibodies used here were affinity purified (with the exception of PDC2) and assignments were first verified by (1) confirmed cross reaction with EST antigens (Figure 3.18) and (2) the predicted molecular mass of each target (Table 3.1). Further evidence that cross reactions were genuine came from the agreement with predicted localisation performed by mass spectrometry (Figure 3.25) (Atteia et al., 2009; Terashima et al., 2010) and native complex formation (Figure 3.27 and 3.28).

These data provide a reasonable level of confidence in assignments, but results still must be treated with caution until final verification can be provided with the identification of knockdown or KO mutants for each of the enzymes involved. This

was possible for PFL1 (See section 4.3) and PDC3 (See section 4.4), where a reduction in protein level, as identified by immunoblot, could be linked to decreased mRNA accumulation, but will require further experimentation for the other enzymes analysed. Potential future experiments include isolation and cloning of full length cDNA sequences of LDH and ADH5 for expression and characterisation in *E. coli*, to partial purification of intact PDH complexes by FPLC to link cross reactions to enzyme activity.

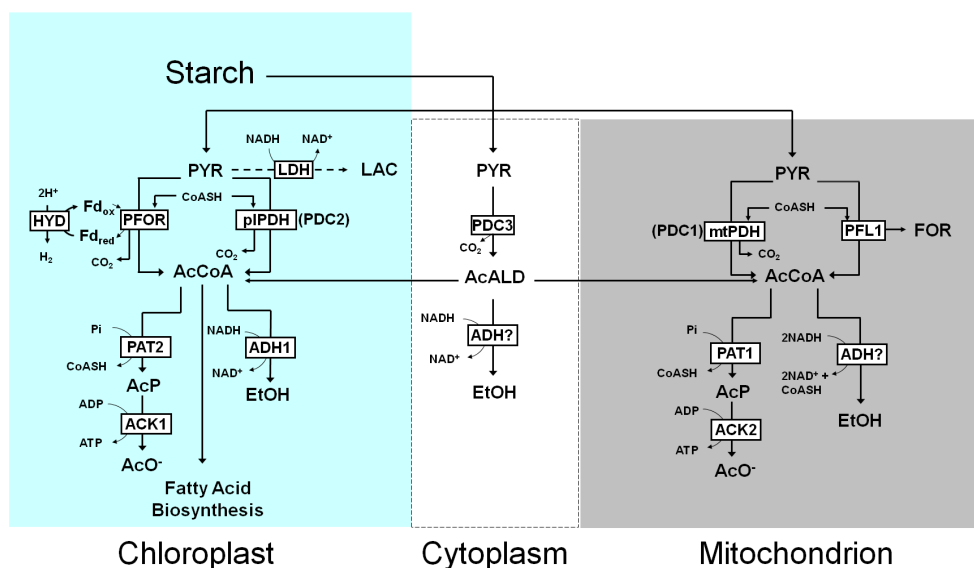


Figure 3.29: Proposed model of the sub-cellular localisation of dark-anaerobic fermentation in *C. reinhardtii*.

The precise routes for metabolite transport are yet to be elucidated in *C. reinhardtii*, aerobic pathways are highlighted (dotted line) although residual activity during anoxia cannot be entirely ruled out. Abbreviations Fd_{ox}; ferredoxin (oxidized), Fd_{red}; ferredoxin (reduced), AcALD; acetaldehyde, AcCoA; acetyl-CoA, AcP; acetyl-phosphate, EtOH; ethanol, FOR; formate, AcO⁻; acetate, LAC; lactate, PYR; pyruvate. The annotated genes are: ACK1; acetate kinase 1, ACK2; acetate kinase 2, ADH1; acetaldehyde/alcohol dehydrogenase, HYDA; [FeFe]-hydrogenase, LDH; D-lactate dehydrogenase, PAT2; phosphate acetyltransferase 2, pIPDH; plastid pyruvate dehydrogenase complex, mtPDH; mitochondrial pyruvate dehydrogenase complex, PDC3; pyruvate decarboxylase 3, PFL1; pyruvate formate lyase, PFOR; pyruvate ferredoxin oxidoreductase. Where specific designations have not been given there are multiple isoforms present in *C. reinhardtii* and the precise enzyme involved has not yet been verified. Localisation enzymes not analysed here is based on proteomic data by Terashima et al. (2010) and Atteia et al. (2009).

3.6.1 Pyruvate dehydrogenase complexes

Evidence was found that *C. reinhardtii* has the potential to express two distinct, complete pyruvate dehydrogenase complexes (Table 3.1). Higher plants are known to possess chloroplast (pIPDH) and mitochondrial (mtPDH) isoforms with

distinct physiological roles; providing acetyl-CoA for the TCA cycle in the mitochondrion and *de novo* fatty acid biosynthesis in the chloroplast (Mooney et al., 2002; Tovar-Méndez et al., 2003). Pyruvate dehydrogenase complexes (PDH) play a central role in aerobic metabolism but are traditionally thought to be inactive during anoxia due to feedback inhibition resulting from the accumulation of acetyl-CoA (Schwartz et al., 1968) and NADH (Hansen, 1966).

Immunochemical analysis of putative *C. reinhardtii* PDH E1 alpha components, PDC1 and PDC2, revealed their location to the mitochondrion and chloroplast, respectively (Figure 3.25). This data coupled to phylogenetic analyses suggests two distinct clades representing mitochondrial, and cyanobacterial-derived chloroplast/plastid isoforms (Figures 3.5–3.8) suggesting the situation in *C. reinhardtii* is similar to that described for higher plants (Figure 3.29) (Mooney et al., 2002; Tovar-Méndez et al., 2003).

Immunoblotting revealed both PDC1 and PDC2 were distributed between soluble and membrane fractions (Figure 3.26); this is in general agreement with previous publications looking at the *Pisum sativum* chloroplast PDC complex (Williams and Randall, 1979); and the mitochondrial PDH complex is known to be associated with the inner mitochondrial membrane (Behal et al., 1993). 2D SDS-PAGE analysis did not conclusively indicate that PDC1 and PDC2 were found in large protein complexes such as the PDH (>4 MDa) (Figure 3.27). However, a literature review subsequently revealed the low level of intact PDH complex could potentially be explained by two factors; (1) previous analysis suggested that intact pyruvate dehydrogenase complexes (4-10 MDa) are too large to enter 4% acrylamide gels (Henderson et al., 2000), and (2) The level of aminocaproic acid used in the native gel (500 mM), was found to cause significant dissociation of PDC E1 components from the E2 core (Henderson et al., 2000). Further experiments could be to repeat native analysis of *C. reinhardtii* membranes using the agarose gel electrophoresis method established elsewhere (Henderson et al., 2000).

PDC1 and PDC2 levels remained constant throughout all growth stages in the presence of acetate (Figure 3.21), yet PDC2 declined at later growth stages in minimal medium (Figure 3.22C), which could potentially reflect the reduced requirement for lipid production in the absence of cell division. Similarly whilst PDC1 levels remained constant during sulphur-deprivation, PDC2 levels declined (Figure 3.22 and Appendix IV.5). TAG synthesis is known to occur during the first 24 h when PDC2

would be required (Timmins et al., 2009b), but may then be degraded as part of a wider decline in overall protein levels (Zhang et al., 2002).

mtPDH activity in plants is thought to be down regulated in response to high ATP levels through the reversible phosphorylation of serine residues on the E1 component by a pyruvate dehydrogenase kinase (PDK) (Patel and Korotchikina, 2006). *C. reinhardtii* possesses three PDK sequences in its genome (Table 3.1) which have been reported as undergoing a small increase in transcription during anoxia (Dubini et al., 2009) and could provide a mechanism for control. Therefore it would be interesting to analyse the phosphorylation state of PDC1 during aerobic and anaerobic conditions.

Plant plPDHs are thought to be controlled by the biochemical environment within the stroma as they are not regulated by phosphorylation (Tovar-Méndez et al., 2003). plPDHs tend to be most active at higher stromal pH and Mg^{2+} levels found during photosynthesis (Camp and Randall, 1985) and are subject to product inhibition resulting from accumulation of NADH or acetyl-CoA (Camp et al., 1988). During anoxia, glycolysis is initiated causing NADH levels to increase and pH levels to drop (Timmins et al., 2009b); it is therefore unlikely that *C. reinhardtii* PDH complexes are active under these conditions. However, there are a few reported examples of PDH activity during anoxia (Snoep et al., 1993; Nakano et al., 1997) and the recent finding that the *E. coli* PDH is active at low levels in the absence of oxygen (Murarka et al., 2010) means residual *C. reinhardtii* PDH activity cannot be ruled out. Perhaps one method of controlling *C. reinhardtii* plPDH activity is through protein degradation, with evidence coming from the reduction in PDC2 levels during late log to stationary phase growth under autotrophic conditions, or after sulphur-deprivation, but will require further investigation.

In plants the control of flux between PDH and PDC is proposed to occur as a function of pyruvate concentration (Rivoal et al., 1990; Tadege et al., 1999). Due to differences in the k_{cat}/K_m values of the two enzymes pyruvate will preferentially be used by PDH complexes under aerobic conditions, therefore it is thought only when PDH complexes are inhibited causing an increase in pyruvate concentration, is the PDC-catalysed pathway activated (Rivoal et al., 1990; Tadege et al., 1999). However, the presence of ethanol in photoautotrophic grown cultures suggests the PDC-catalysed pathway of ethanol production may be constitutively active, making it likely

that flux is controlled primarily by biochemical changes in the cell rather than a transcriptional mechanism.

3.6.2 Pyruvate synthase/Pyruvate:ferredoxin oxidoreductase (PFOR)

C. reinhardtii also codes for an O₂-sensitive PFOR which is usually restricted to anaerobic organisms, potentially providing an extra route for converting pyruvate to acetyl-CoA during anoxic conditions (Atteia et al., 2003; Mus et al., 2007). PFOR accumulation is induced by conditions promoting hypoxia including after sulphur-deprivation in the light. PFOR shows a time-dependent expression, peaking between 24–48 h, coinciding with the maximum rate of hydrogen production (Figure 3.20). This result appears to support a role for PFOR in providing reduced ferredoxin for the hydrogenase, as suggested by a dramatic increases in HYDA and PFOR transcript levels during anaerobic acclimation (Mus et al., 2007) and the down-regulation of PFOR transcription in an *hydEF* mutant lacking a functional hydrogenase (Dubini et al., 2009). However, the PFOR-catalysed reaction is reversible (Furdui and Ragsdale, 2000) working to reduce or oxidize ferredoxin depending on environmental conditions. Hence, in the light during hypoxia, PFOR might conceivably function to oxidize reduced ferredoxin generated by the light reactions to help relieve the excitation pressure in the chloroplast. PFOR could also be responsible for the pyruvate synthase activity previously detected in *C. reinhardtii* (Chen and Gibbs, 1992) and the reverse reaction would explain how *C. reinhardtii* is able to consume hydrogen (Kreuzberg, 1984; Cournac et al., 2002) and photoreduce CO₂ under a hydrogen atmosphere (Maione and Gibbs, 1986).

In anaerobic bacteria, archaea and amitochondriate eukaryotes, which lack PDH complexes, PFOR is involved in the production of ATP by anaerobic respiration or by substrate level phosphorylation (Muller and DasSarma, 2005). In the case of *C. reinhardtii* the acetyl-CoA created by PFOR might be metabolised via a phosphate acetyltransferase (PAT)-acetate kinase (ACK) catalysed pathway to produce ATP in the chloroplast (Figure 3.29), concomitantly reducing ferredoxin and thereby providing electrons for the low level H₂ production observed in the dark (Gfeller and Gibbs, 1984; Kreuzberg, 1984; Cournac et al., 2002). There are two PAT and two ACK homologues found in the genome whose transcription is induced to varying extents during anaerobic acclimation (Mus et al., 2007). ACK2 and PAT1 have been

assigned by mass spectrometry to the mitochondrion (Atteia et al., 2009; Terashima et al., 2010) and ACK1 and PAT2 to the chloroplast (Terashima et al., 2010), although the functionality of both pathways is not confirmed.

The O₂-sensitive PFOR is absent in aerobic conditions and induced under anaerobic conditions which is supported by the previously observed increase in transcript levels (Mus et al., 2007) and is therefore likely to be controlled at the level of transcription. A similar pattern was observed for the HYDA, both enzymes showed periodic expression when subjected to dark:light cycles, accumulating in the dark before being degraded in the light (Figure 3.24). Cyclic induction of PFOR during growth under dark/light cycles (Figure 3.24) is likely to have occurred in response to establishment of anaerobic conditions within the culturing vessel. However, this points to a potential function of PFOR, and the [FeFe]-hydrogenase in maintaining ATP production by glycolysis in the night when O₂ becomes unavailable for oxidative phosphorylation. Under anaerobic conditions pyruvate dehydrogenase complexes are thought to be inactivated, PFOR therefore provides an alternative route for pyruvate degradation in the chloroplast, and the acetyl-CoA produced can be converted into ethanol by ADH1 regenerating NAD⁺ (Figure 3.29).

HYDA and PFOR induction was not however, totally co-regulated, displaying differential expression patterns during sulphur-deprivation in the light indicating slight differences in the pathways which they are involved (Figure 3.20).

3.6.3 Formate metabolism

A key enzyme in *C. reinhardtii* anaerobic metabolism is PFL1 which catalyses the production of formate and acetyl-CoA from pyruvate (Hemschemeier and Happe, 2005; Atteia et al., 2006; Hemschemeier et al., 2008b). In contrast to previous results (Atteia et al., 2006) I only found compelling evidence for PFL1 in the mitochondrion although a minor presence in the chloroplast cannot be totally ruled out. Flux via PFL1 is proposed to predominate during fermentation (Gfeller and Gibbs, 1984) leading to the formation of acetyl-CoA which can then either be converted to acetate with the production of one molecule of ATP via a PAT1/ACK2 pathway in the mitochondrion (Figure 3.29) (Atteia et al., 2009; Terashima et al., 2010), or to ethanol, regenerating one molecule of NAD⁺, via an as yet undefined route.

PFL1 levels showed little change during anoxic conditions, in general agreement with previous reports (Atteia et al., 2006) (Figure 3.22), suggesting transcriptional control (Mus et al., 2007) plays only a minor role in regulating PFL1 activity. PFL1 is also known to be subject to control by post-translational modification through cleavage by PFL1 activating enzyme (PFL-AE) (Knappe et al., 1969) on exposure to anaerobic conditions, observed as a shift in protein size (Atteia et al., 2006). This was not investigated here as gels were of insufficient resolution to see an observable shift.

The data suggesting formate accumulated at a significant rate in photoautotrophic grown cultures was surprising (Figure 3.22D), as PFL1 is rapidly inhibited by oxygen (Wagner et al., 1992). This may therefore suggest formate is produced by alternative metabolic pathways under these conditions (see section 4.5.3.3 for a more detailed discussion), or that during photoautotrophic conditions anoxia is still encountered within at least some cellular compartments such as the mitochondrion.

3.6.4 Ethanol metabolism

A classic pathway for ethanol production is the conversion of pyruvate to acetaldehyde by PDC, followed by reduction to ethanol by an alcohol dehydrogenase. *C. reinhardtii* possesses a single PDC sequence (PDC3) which clustered with plant and algal homologues (Figure 3.1). PDC3 is present under all conditions tested and showed little variation in enzyme levels between aerobic and anaerobic cultures.

PDC3 is also a cytoplasmic enzyme (Figure 3.25) which explains the previous lack of identification in proteomic analysis of chloroplast and mitochondrial compartments (Terashima et al., 2010), and detection of PDC activity in that compartment (Kreuzberg et al., 1987), although it is unclear how observable activity was correctly assigned to PDC as the assay used could also detect LDH or MME activity.

The acetaldehyde produced by PDC3 activity is potentially highly toxic, with the molecule linked to cell death in mammalian (Clemens et al., 2002) and plant tissues (Tadege et al., 1998), and requires rapid breakdown to prevent cellular damage. In plants this is achieved by a cytoplasmic PDC and Zn-dependent alcohol dehydrogenase-catalysed pathway (Davies et al., 1974); however BLAST analysis did

not conclusively identify a plant alcohol dehydrogenase homolog in the genome, instead finding closely related class III type alcohol dehydrogenases belonging to the family of glutathione-dependent formaldehyde dehydrogenases.

C. reinhardtii does, however, possess a potential [Fe]-dependent bifunctional acetaldehyde/alcohol dehydrogenase, designated ADH1 (Atteia et al., 2003), which shows a transient increase in transcript level under anaerobic acclimation (Mus et al., 2007) and has previously been detected in the chloroplast by mass spectrometry (Terashima et al., 2010). Given that PDC3 is located in the cytoplasm, not the chloroplast, it seems likely that a cytoplasmic ADH activity may be present, a theory supported by previous enzymatic assays which suggested the possibility of at least two alcohol dehydrogenase isoforms, with varying preference for NADH or NADPH, in the mitochondrion and cytoplasm, and chloroplast and cytoplasm respectively (Kreuzberg et al., 1987). The *C. reinhardtii* genome contains two homologues of *ADH1* designated *ADH2* and *ADH5*. There may be some evidence for *ADH2* transcription in the EST database (based on the Augustus gene model v.10) although it is currently difficult to discern if some cDNA clones relate to *ADH2* or *ADH1* expression. A cDNA clone matching *ADH5* was found in the EST database (BG855351.1; Table 3.1) making this gene a good candidate for involvement in ethanolic fermentation. Here ADH1 was found in the chloroplast and in the cytoplasm, with an apparently smaller mass, but obtained no evidence to support a mitochondrial location by immoblot (Figure 3.25) in agreement with previous proteomic analysis (Atteia et al., 2009; Terashima et al., 2010). Due to the choice of EST used to raise the anti-ADH1 antibody, neither ADH2 nor ADH5 should be detected based on current gene models (Augustus v.10). The presence of an ADH1 signal in the cytoplasm is unlikely to be explained totally by contamination of the fraction by chloroplast stromal proteins when compared to other enzymes such as HYDA, LDH or PFOR. Therefore this data may suggest that ADH1 is co-localised to the cytoplasm and chloroplast, but the existence of an unrelated cross-reaction in the cytoplasm cannot be entirely ruled out. This leaves a model in which ethanol production can occur in both the cytoplasm and chloroplast under the control of a single protein. However further investigations of ADH2 and ADH5 are required, before firm conclusions can be drawn about the complete nature of ethanolic fermentation in *C. reinhardtii*. The dominance of ethanol fermentation over D-lactate

fermentation in *C. reinhardtii* is intriguing and might reflect a preference to dispose of reducing equivalents with minimum cytosolic acidification.

The observation that ethanol was present in the medium of WT cultures under photoautotrophic growth was unexpected, although further experiments will be required to verify this observation. However, the constitutive presence of both PDC3 and ADH1, which did not appear to undergo post-translational modification, would suggest ethanol production may be possible under these conditions. If this is the case, these data suggest the balance of flux between PDH and PDC is controlled by modification of PDH complex activity (as discussed in section 3.6.1) and biochemical changes within the cell.

3.6.5 Lactate and acetate metabolism

In *C. reinhardtii* the production of lactate had previously only been observed during anaerobic incubation at extremes of pH (<pH 5 or >pH 8) (Kreuzberg, 1984) or when PFL1 is inhibited with sodium hypophosphite (Kreuzberg, 1984; Hemschemeier et al., 2008b) or by mutation (Philipps et al., 2011). A single putative NAD⁺-dependent D-lactate dehydrogenase (Grossman et al., 2007) and several cytochrome dependent D-lactate dehydrogenases are, however, found in the genome (Table 3.1). These enzymes are predominantly of prokaryotic or fungal origin where they can play an important role in anaerobic energy metabolism (Cristescu et al., 2008). LDH was localised to the chloroplast (Figure 3.25) and I showed that the enzyme was present under all conditions providing an extra route for pyruvate degradation. NAD⁺-dependent LDHs are implicated in lactate production, whereas D-LDHcs are considered to work in the direction of pyruvate formation (Goffin et al., 2004). Isolated *C. reinhardtii* mitochondria have been found to take up D-lactate (Beezley et al., 1976). The presence of both D-lactate producing (LDH) and consuming (LDHc) enzymes could provide a mechanism for disposing of reducing equivalents under anoxia then recapturing the lost carbon when oxygen becomes available again, or may even operate as a valve to move excess reducing equivalents between the chloroplast and mitochondrion during photoautotrophic growth, similar to the malate shuttle.

A. thaliana has recently been shown to possess a LDHc which is implicated in catalysing the final step in the methylglyoxal (MG) pathway (Engqvist et al., 2009).

The MG pathway is activated under various stress conditions in plants and it is proposed MG is formed as a by-product of hyperactive glycolysis but is poorly characterised (Kalapos, 1999). MG is a highly toxic molecule and must be rapidly broken down by the activities of glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6) leading to lactate production. In addition to the previously mentioned LDHc, homologues of glyoxylase I (Cre04.g216100) and glyoxylase II (Cre12.g557700) could be found in the *C. reinhardtii* genome and EST database, indicating this pathway is present in *C. reinhardtii* with a potential link to lactate metabolism under stress conditions, but will require further investigation to determine its physiological importance.

Lactate was found to be produced at a more significant rate during aerobic than anaerobic conditions (see section 3.4.4.2). It is unclear how LDH is regulated in *C. reinhardtii*, but appeared to undergo post-translational modification upon exposure to anoxic conditions through an increase in molecular mass, however the type of modification requires further characterisation (Figure 3.23). Although as previously mentioned lactate could potentially be produced by the MG pathway, when taken with observations of lactate accumulation under aerobic conditions, post-translational modification of LDH may suggest an alteration that effectively switches off lactate production under anoxic conditions. However, such a hypothesis requires further investigation.

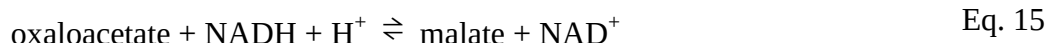
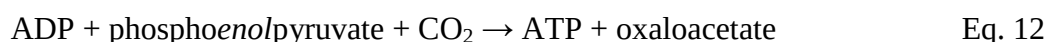
Pathways for both the production and consumption of acetate are also present in *C. reinhardtii*. Acetate can potentially be converted into acetyl-CoA in up to three cellular compartments by one of three acetyl-CoA synthetases (ACS) found in the genome (Spalding et al., 2009), and created from acetyl-CoA by one of two PAT-ACK-catalysed pathways located in the mitochondria and chloroplast (Mus et al., 2007; Atteia et al., 2009; Terashima et al., 2010) or by an ALDH (ALD5) from acetaldehyde, which may be located in the cytoplasm (Kreuzberg et al., 1987).

3.6.6 Additional pathways

In addition to those enzymes investigated here *C. reinhardtii* is known to possess proteins which form a partial reductive carboxylic acid cycle (Dubini et al., 2009). This pathway was activated in the absence of [FeFe]-hydrogenase activity under anaerobic conditions, leading to the production of succinate (Dubini et al.,

2009) and could therefore also act as an alternate route for pyruvate degradation if fermentative pathways are to be switched off. Enzymes include two phosphoenolpyruvate (PEP) carboxylase sequences (Eq. 12), one pyruvate carboxylase (Eq. 13), one malate synthase (Eq. 4), five malate dehydrogenase (Eq. 15), and six NAD(P)⁺ dependent malic enzymes (Eq. 16) (Table 3.1). These enzymes could theoretically work to produce malate which can then be converted in succinate by the concerted activity of fumarase (Eq. 17) and fumarate reductase (Eq. 18).

Producing Malate:



Malate to Succinate

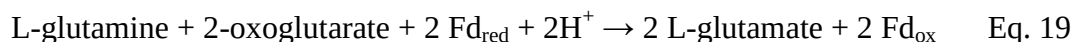


Pyruvate carboxylase is perhaps unlikely to be active during anoxia when energy levels are low as its activity consumes ATP; this enzyme is often involved in gluconeogenesis from alternate carbon sources such as lactate and could provide a route for re-assimilation of carbon after fermentative metabolism (Hers and Hue, 1983).

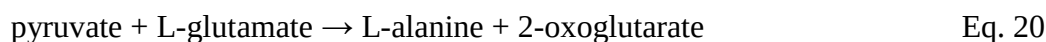
Furthermore *C. reinhardtii* activates a number of metabolic processes in response to nutrient limitation whilst maintaining respiration; consequently levels of metabolic intermediates available for fermentative are affected by a range of processes including respiration, acetate and ammonium assimilation along with amino acid synthesis (Timmins et al., 2009b; Doebbe et al., 2010).

Amongst the range of enzymes that could potentially be involved, good candidates for further investigation include ferredoxin-dependent glutamate synthase

(Eq. 19) which utilises reduced ferredoxin as part of the glutamate synthase pathway of ammonium fixation, potentially competing with the hydrogenase for reductant.



Another route for utilisation of pyruvate is provided by alanine amino transferase (AAT) (Table 3.1) Eq. 20, making it a potential route for re-directing of metabolism as alanine production has been observed during sulphur-deprivation (Doebbe et al., 2010). *C. reinhardtii* possess two AAT sequences, but only one (AAT1) is believed to be expressed (Vallon et al., 2009).



3.6.7 Implications for metabolic engineering

Redirecting fermentative carbon flux in *C. reinhardtii* to PFOR, or reductant in the form of NADH to the thylakoid enzyme NDA2 in the light, are potential ways to improve the yields of H₂ gas. The presence of PFL1, PDC3, LDH and ADH1 during H₂ production makes these enzymes good initial candidates for knockdown.

However, evidence that fermentative enzymes are present during normal growth raises questions as to whether KO or knockdown of key fermentative enzymes will have deleterious effects on cell fitness, and it is as yet unclear what effect knockdown would have on survival during anoxic conditions when these enzymes are most active.

Furthermore, the existence of multiple routes for pyruvate degradation in each compartment, plus the existence of multiple carbon transporters in the genome and EST database means metabolites are able to move between organelles (Peltier and Cournac, 2002). Therefore, if switching off fermentative pathways is to be a successful means of increasing H₂ yields, it is likely that multiple KOs will be required to achieve maximum rates of hydrogen evolution, particularly as it is such a thermodynamically challenging reaction (Hallenbeck and Benemann, 2002). The possibility that metabolism may be redirected to amino acid production would pose problems for efforts at genetic engineering, requiring inducible control of gene expression.

Chapter 4: Isolation and characterisation of *C. reinhardtii* *PFL1* and *PDC3* knockdown mutants

4.1 Introduction

4.1.1 Targeting fermentative pathways

It has been proposed that switching off fermentative pathways in *C. reinhardtii* may be a means of increasing yields of H₂ (Mus et al., 2007). This hypothesis is based on the assumption that increasing the amount of pyruvate available to pyruvate:ferredoxin oxidoreductase (PFOR), or the amount of NADH which could be fed into the PET, would increase the amount of reduced ferredoxin available to the [FeFe]-hydrogenase HYDA1 (Mus et al., 2007; Godman et al., 2010) (Figure 1.1).

To explore this possibility, pyruvate formate lyase 1 (*PFL1*) was chosen for knockdown. *PFL1* is involved in formate production (Atteia et al., 2006), and represents the metabolic pathway thought to predominate during dark-anaerobic conditions (Gfeller and Gibbs, 1984). The availability of a *PFL* inhibitor (sodium hypophosphite) has previously allowed the characterisation of the affect of blocking formate accumulation on H₂, CO₂ and ethanol excretion during sulphur-deprivation (Hemschemeier et al., 2008b). But a complete analysis of excreted metabolite production was not performed, and it remained unclear whether the inhibitor had additional impacts on cellular metabolism. When *PFL1* was inhibited, no increase in H₂ production was observed, which was proposed to be a result of a redirection of metabolism to ethanol production (Hemschemeier et al., 2008b). Therefore the putative *C. reinhardtii* pyruvate decarboxylase (*PDC3*) was also chosen for analysis, in order to determine whether this protein is active in a predicted pathway for ethanol formation (Grossman et al., 2007; Mus et al., 2007). If there is an active, *PDC3* catalysed pathway during anaerobic conditions, it could represent an alternative route for pyruvate degradation and compete with PFOR for pyruvate, impacting on H₂ production.

Generating nuclear mutants in *C. reinhardtii* via targeted gene disruption is currently difficult due to poor rates of homologous recombination (Gumpel et al., 1994; Zorin et al., 2009). Instead KO mutants are being identified by labour-intensive

PCR-based screening methods (Pootakham et al., 2010) or by characterization of insertion mutants with an altered phenotype (Kruse et al., 2005a). Alternatively, genes of interest can be down regulated by sequence-specific RNA interference (RNAi) through expressing long double-stranded RNA (dsRNA) constructs (Fuhrmann et al., 2001; Rohr et al., 2004; Schroda, 2006). However this approach produces many small interfering RNAs that result in unwanted silencing of off targets (Xu et al., 2006), and can suffer from silencing itself (Rohr et al., 2004). An alternative approach is to use artificial microRNA (amiRNA) technology to knockdown the expression of a target gene (Molnár et al., 2009; Zhao et al., 2009). When transcribed, the artificial miRNA sequence is processed by the cellular machinery forming a RNA-induced silencing complex (RISC) that specifically targets the chosen gene of interest (Molnár et al., 2009; Zhao et al., 2009).

The aims of this chapter included testing the efficacy of amiRNA technology as a means of redirecting metabolic flux in *C. reinhardtii*, and analysis of the impact upon fermentation product production concentrating on the *PFL1* and *PDC3* genes. This involved (1) isolation of knockdown cell lines (2) testing the stability of knockdowns under dark-anaerobic and sulphur-deprived conditions (3) testing the long term stability of the knockdowns and (4) analysis of the effect of gene knockdown on fermentative metabolism and H₂ production during sulphur-deprivation experiments by NMR spectroscopy and gas chromatography.

In parallel work, a *C. reinhardtii* *pfl1*-KO mutant was isolated by Happe and colleagues in a genetic screen for insertion mutants showing perturbed hydrogen metabolism (Philipps et al., 2011), albeit in a different parental strain CC-125. The dark metabolite accumulation of the KO mutant was analysed, providing a useful comparison for *pfl1*-KD lines under the same conditions. The data presented here builds on analysis of the *pfl1*-KO strain to assess the effect of reducing PFL1 levels on fermentative metabolism during sulphur-deprivation.

4.1.2 Artificial micro RNAs

The generation of artificial microRNAs involves replacing the targeting region of a pre-miRNA sequence with an artificial oligonucleotide designed to specifically downregulate a chosen gene of interest (Molnár et al., 2009; Zhao et al., 2009).

Plant miRNAs have a high degree of specificity with a maximum of 5 mismatches and include a number of strict structural criteria (Schwab et al., 2005) (Figure 4.1) which can be used to design artificial constructs for use in gene silencing. Ossowski et al. (2008) created an online tool known as Web MicroRNA Designer (WMD3) for this purpose, which takes a target gene and generates all possible specific amiRNAs using the following selection determinants (as displayed in Figure 4.1):

- (1) The region from bases 2-12 is particularly sensitive to mismatches, and generally will not tolerate more than one (Schwab et al., 2005).
- (2) Base 10 is the site of target mRNA cleavage (Schwab et al., 2005), this is either an A or U as these nucleotides are over represented in this position (Mallory et al., 2004), and no-mismatches are tolerated either side of this base (Schwab et al., 2005).
- (3) The 3' region is more tolerant allowing for more than two mismatches but not in a row (Schwab et al., 2005).
- (4) When paired to corresponding mRNA sequences the binding must have a low overall free energy, lower than $-30 \text{ kcal mol}^{-1}$.
- (5) amiRNA must have a relative instability in base pairing at their 5' end compared to their complementary pre-miRNA strand amiRNA* which is later degraded, as this determines which ssRNA is incorporated into the RISC complex (Schwarz et al., 2003), and can be affected by relative low GC content or mismatch.

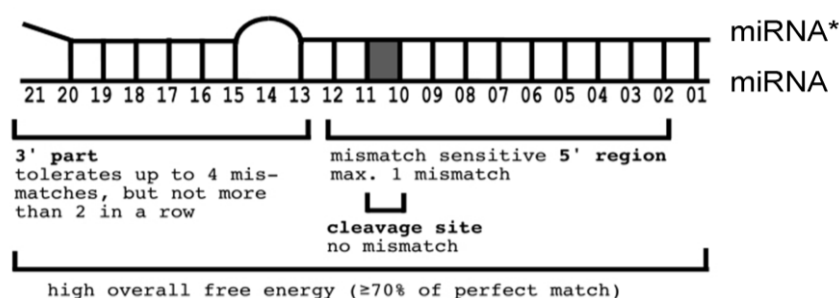


Figure 4.1: Schematic representation of miRNA specificity determinants.

Displayed is a pre-miRNA including miRNA sequence that is incorporated into the RISC complex and miRNA* sequence which is degraded. This figure is taken from Web MicroRNA Designer (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Help;project=stdwmd>)

4.2 amiRNA vector construction

Molnár et al. (2009) constructed several vectors for expression of artificial microRNAs in *C. reinhardtii*. One of these vectors, pChlamiRNAi3, was kindly

provided by Dr. Attila Molnár (University of Cambridge) for use in this work. pChlamiRNAi3 was renamed pRNAi3, and will be referred to as such in this work from this point onwards. pRNAi3 contains the pre-miRNA gene *cre-MIR1157* (Molnár et al., 2009) under the control of the strong, photosystem I, *PSAD* promoter for constitutive expression in the *C. reinhardtii* nucleus (Fischer and Rochaix, 2001) (Figure 4.2A). *cre-MIR1157* has been cut with the restriction enzyme SpeI, to remove the miRNA targeting sequence and the hairpin loop region of the pre-miRNA, to allow for the insertion of amiRNAs (Figure 4.2B). The plasmid also contains the *Streptomyces rimosus aphVIII* gene (Sizova et al., 2001) under the control of the strong hybrid *HSP70A-RBCS2* promoter (Schroda et al., 2000) (Figure 4.2B), to allow for selection of transformants on the antibiotic paromomycin. By utilising antibiotic resistance as a selectable marker rather than the commonly used *ARG7* (Debuchy et al., 1989) or *NIT1* (Kindle et al., 1989) genes, this has the advantage of facilitating transformation of the best H₂ producing WT strains.

4.2.1 Artificial microRNA design

Animal miRNAs predominantly target the 3'UTR of genes, whereas plant miRNAs target the coding region or 3'UTR (He and Hannon, 2004). However, little is currently known about the most effective region on mRNA transcripts to target using artificial constructs in algae, and may be dependent on RNA secondary structure.

Therefore, two separate amiRNAs were designed for each target using web microRNA designer 3, including *PFL1* and *PDC3*, targeting different regions of each transcript, according to the criteria previously outlined (Section 4.1.2). The result was two 90 bp oligonucleotide sequences for each vector, which when annealed form a double stranded (ds) molecule containing a portion of the pre-miRNA sequence flanking the targeting sequence, a 21 bp targeting sequence, the conserved hairpin loop region from *cre-MIR1157* and the reverse complement 21 bp targeting sequence (Figure 4.3A) that forms a hairpin loop sequence when expressed.

For *PFL1*, the oligonucleotide sequences targeted the 5'UTR (used to create vector pRNAi3.4A) and 3'UTR (used to create vector pRNAi3.4B), whereas for *PDC3* both oligonucleotides targeted different regions of the coding sequence (Appendix IV.1). The amiRNAs sequences were sent for synthesis, then upon receipt were annealed to form ds amiRNAs. Oligonucleotide annealing was tested by

restriction digest with *Nco*I, which cuts in the conserved hairpin loop region located in the middle of the 90 bp sequence (Figure 4.3B). As ds but not ss DNA can be cut with restriction enzymes, restriction digest allowed for the differentiation between annealed and un-annealed oligonucleotides via a shift in size on a 2 % agarose gel corresponding to digested ds oligonucleotides (Figure 4.3B).

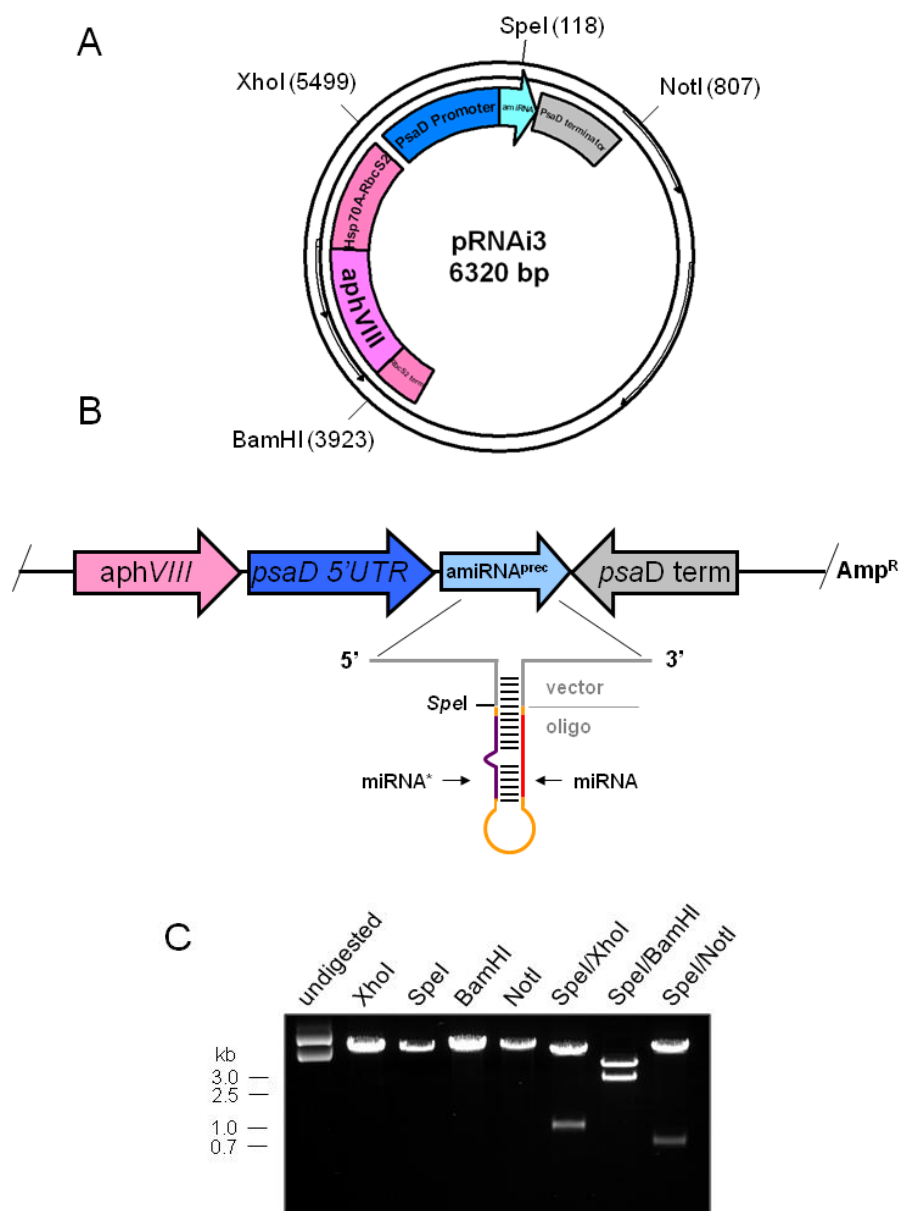


Figure 4.2: Restriction analysis of amiRNA vector pRNAi3.

(A) Vector map showing truncated amiRNA sequence with targeting region removed (cyan), with *PSAD* promoter (blue) and *PSAD* terminator (grey), *aphVIII* resistance cassette for *C. reinhardtii* transformant selection (orange) with *HSP70A-RBCS2* hybrid promoter (orange) and *RBCS2* terminator (purple), ampicillin resistance cassette (white) for *E. coli* selection. (B) Detail of amiRNA precursor region, showing *Spe*I site of insertion with annealed miRNA (C) pRNAi3 restriction digest, samples run on a 1% agarose gel.

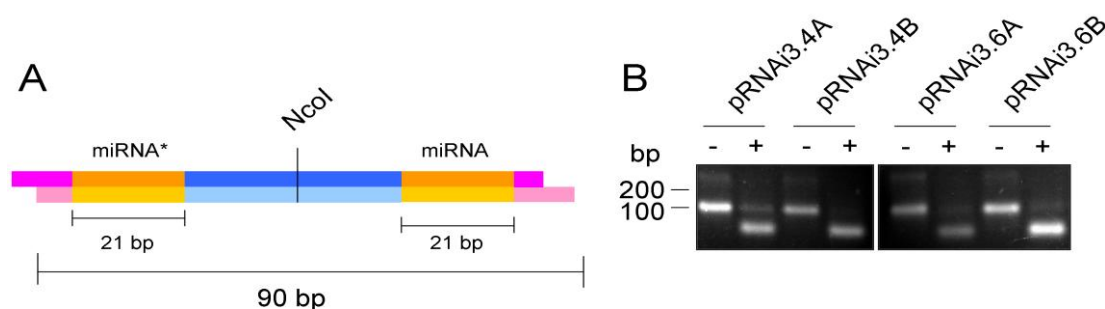


Figure 4.3: Annealing of amiRNA sequences targeting *PFL1* (pRNAi3.4A and 4B) and *PDC3* (pRNAi3.6A and 6B).

(A) Schematic representation of ds amiRNA oligonucleotide (not to scale), flanking regions (pink), targeting sequence (orange, yellow) hairpin loop region (blue) (B) Restriction digest of amiRNA oligonucleotides to test annealing, showing samples undigested (-) and digested with NcoI (+), run on a 2% agarose gel.

Once oligonucleotide annealing was confirmed, sequences were phosphorylated with T4 polynucleotide kinase and cloned into SpeI digested, dephosphorylated pRNAi3. As cloning involved ligating into an SpeI site, *E. coli* transformants could contain self-ligated plasmid, or plasmids with the amiRNA oligonucleotide inserted in one of two orientations. Therefore after ligation, colony PCR was used to identify those *E. coli* transformants containing pRNAi3 with amiRNAs inserted in the correct orientation (Figure 4.4A), using one primer that binds to the vector (pRNAi3_F) and one to the inserted amiRNA sequence (pRNAi3_R) (Figure 4.4B). Colonies providing positive results including; pRNAi3.4A-4, pRNAi3.4B-4, pRNAi6A-3 and pRNAi6B-2 (Figure 4.4A) were then used to prepare purified plasmid DNA, and the final vectors were sent for verification by sequencing.

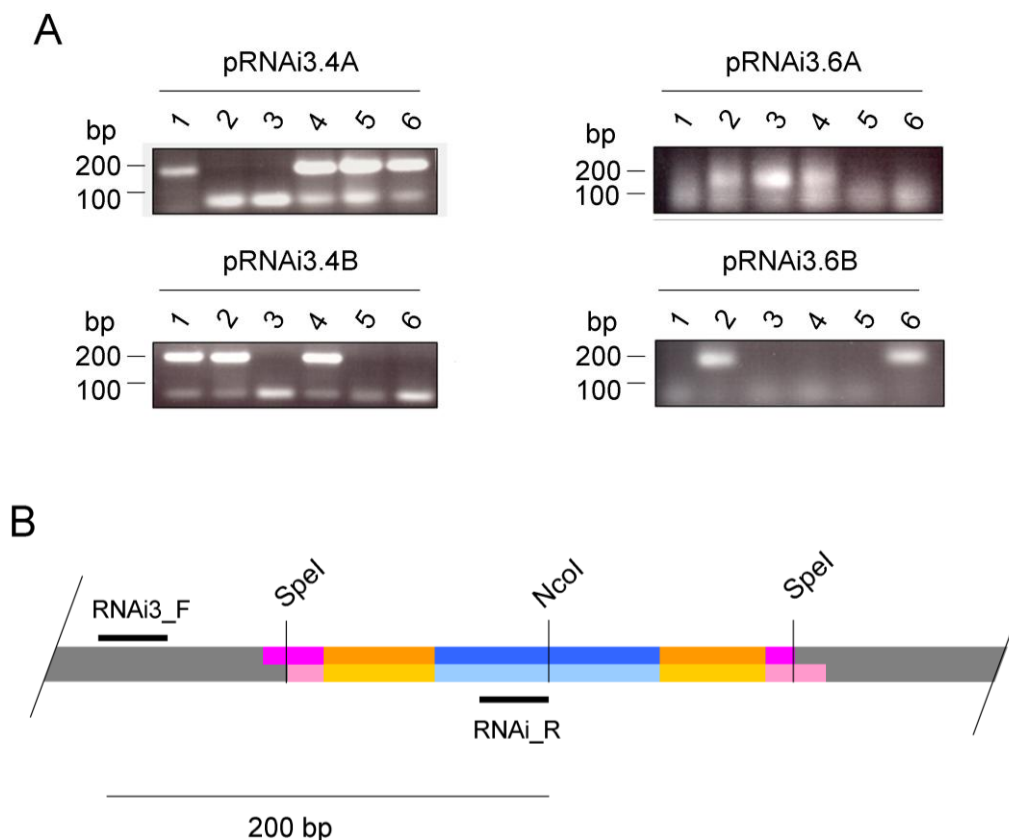


Figure 4.4: Colony PCR screen for correctly ligated amiRNA vectors

(A) *E. coli* colony PCR, screening for insertion of amiRNA oligonucleotides in the correct orientation into vector pRNAi3, targeting *PFL1* (4A and 4B) and *PDC3* (6A and 6B). (B) Schematic representation of amiRNA correctly ligated into vector pRNAi3, depicting *E. coli* colony PCR forward and reverse primer binding sites (Figure not to scale).

4.3 Knockdown of pyruvate formate lyase 1 (*PFL1*)

4.3.1 Isolation of *pfl1*-KD lines and characterisation under dark-anoxic conditions

Plasmids pRNAi3.4A and pRNAi3.4B were shot into *C. reinhardtii* WT strain CC-124 by biolistic bombardment. Transformants were selected by resistance to paromomycin, and for each vector, 70 colonies were screened for a reduction in PFL1 levels by immunoblot. Of the two vectors transformed, one (pRNAi3.4A) provided mutants with a high degree of knockdown. In total, nine mutants were isolated with ~70 to >90% reduction in PFL1 levels relative to WT (Figure 4.5).

Two knockdowns, 4A11 and 4A28 (later labelled *pfl1*-KD1 and *pfl1*-KD2 respectively), were chosen for further analysis.

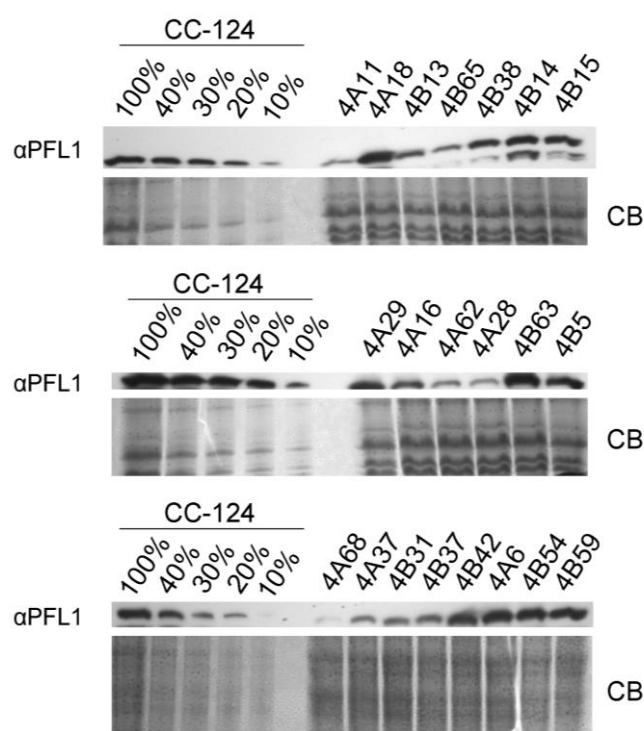


Figure 4.5: Screening transformants for knockdown of *PFL1* by immunoblot.

PFL1 levels in mutant lines are compared against a dilution series of WT (CC-124) cultures. 8×10^5 cells loaded per lane, with the exception of the WT dilution series were percentages of this total are indicated. Loading control is provided by a Coomassie blue stained gel (CB). Mutants are labelled with the prefix 4A for those transformed with (pRNAi3.4A) or 4B (pRNAi3.4B).

Cultures of *pfl1*-KD1 and *pfl1*-KD2 were grown in TAP medium to late log phase, concentrated, re-suspended in AIB, then subject to 4 h dark anaerobic incubation as described in section 2.12.1 to induce dark-fermentative metabolism. By comparison with WT samples, immunoblot analysis revealed ~80–90% decrease in *PFL1* levels in *PFL1*-knockdown lines (Figure 4.6A and Appendix IV.2). Subsequently qRT-PCR analysis revealed a corresponding decrease in *PFL1* mRNA levels in *pfl1*-KD1 (~85%) and *pfl1*-KD2 (~90%) compared to WT (Figure 4.6B). Immunoblotting also suggested hydrogenase induction may be reduced in *PFL1*-knockdown cultures during dark anaerobic incubation (Figure 4.6A) in agreement with analysis of the *pfl1*-KO strain (Philipps et al., 2011) although some variation was seen between biological replicates (Appendix IV.2).

The transient-light induced hydrogen production ability of *pfl1*-KD lines was measured by a reverse polarity Clark electrode, an acceptable level of sensitivity was achieved by using a small coil of platinum wire on top of the platinum electrode (Dr.

Myles Barker personal communication) or replacing the standard PTFE membrane with Nafion, rather than re-plating the platinum electrode as described previously (Ghirardi et al., 1997; Posewitz et al., 2004b; Mus et al., 2007). Recorded rates for WT cultures were slightly lower than previous measurements, with average values of $57.7 \pm 4.1 \text{ H}_2 \mu\text{mol mgChl h}^{-1}$, compared to $135 \pm 10 \text{ H}_2 \mu\text{mol mgChl h}^{-1}$ (Mus et al., 2007) or $\sim 80 \text{ H}_2 \mu\text{mol mgChl h}^{-1}$ (Posewitz et al., 2004b) which could be a result of a combination of variation between experimental setups and laboratory strains. *pfl1*-KD lines showed a slight decrease in hydrogen production ability (Figure 4.6C), in agreement with measurements of the *pfl1*-KO strain (Philipps et al., 2011).

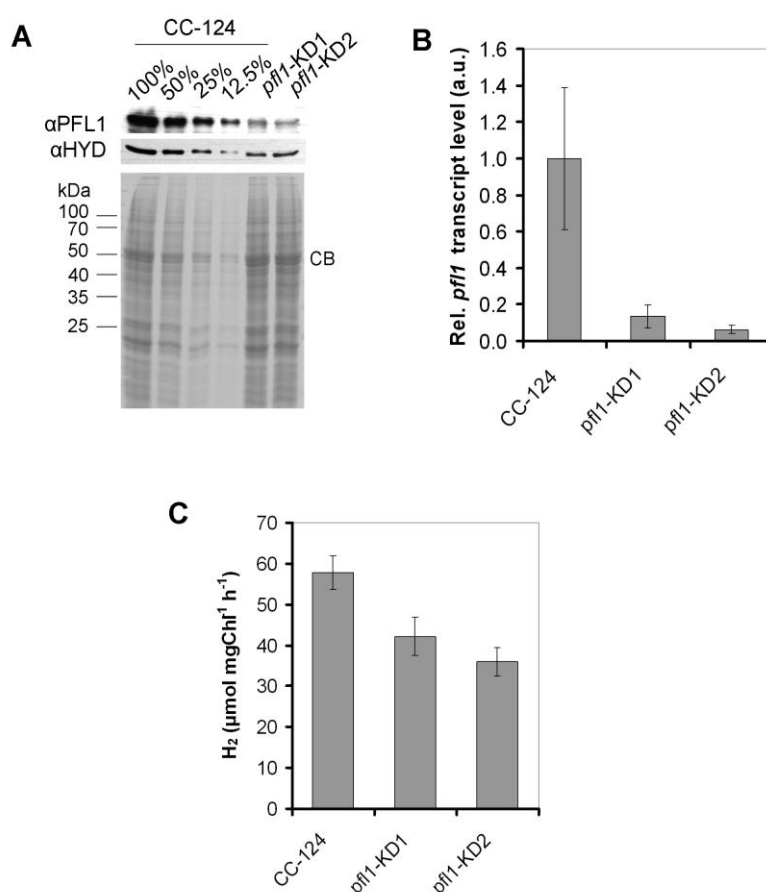


Figure 4.6: Comparison of WT and *PFL1* knockdown cell lines after 4 h dark-anaerobic incubation in AIB.

(A) Immunoblot analysis of target protein levels in WT and knockdown cell lines, 8×10^5 cells loaded per lane (B) Taqman® qRT-PCR analysis comparing *PFL1* mRNA transcript levels after dark anaerobic induction, error bars are given as $\pm$ standard error of the mean (SE) of three biological replicates. (C) Transient light induced H_2 production as measured by Clark electrode, $\pm$ SE (CC-124; $n=18$, *pfl1*-KD1; $n=4$, *pfl1*-KD2; $n=4$).

The degree of *PFL1* knockdown during different growth phases was tested by immunoblot for both *pfl1*-KD1 and *pfl1*-KD2 (Figure 4.7). A reduction in PFL1 levels was visible at all growth phases for both *pfl1*-KD1 and *pfl1*-KD2 (Figure 4.7), with a slight increase in observed knockdown in stationary phase cultures (Figure 4.7).

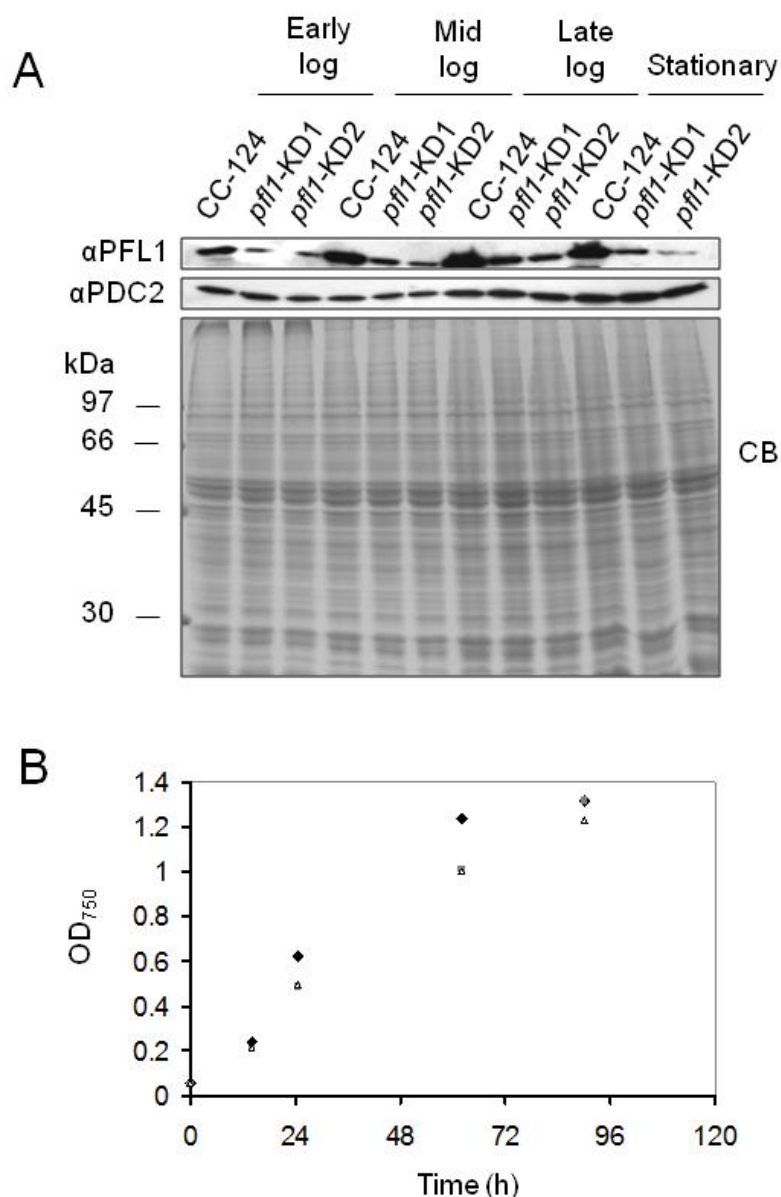


Figure 4.7: Analysis of PFL1 levels in WT and *pfl1*-KD lines during various growth stages.

Cultures were grown in TAP medium under continuous illumination. (A) Immunoblot, 8×10^5 cells loaded per lane with a Coomassie blue (CB) stained gel and PDC2 analysis used as loading controls. (B) Growth curve displaying optical density (OD₇₅₀) against time.

Experiments were performed to assess whether *PFL1*-knockdown had an adverse effect on cell fitness. The growth rate was checked by comparing the

doubling time of cultures grown mixotrophically (in TAP medium, continuous illumination $\sim 40 \mu\text{E m}^{-2} \text{s}^{-1}$) with that of the parental strain. A doubling time of 7.5 h was calculated for CC-124, which is in good agreement with previous measurements (Lee and Fiehn, 2008).

A slight decrease in growth rate was observed for both *pfl1*-KD cultures (Table 4.1), which was determined to be statistically significant by independent two sample t-test (for *pfl1*-KD1; $t=2.47$, $df=22$, $p=0.05$, for *pfl1*-KD2 $t=2.45$, $df=22$, $p=0.05$, degrees of freedom calculated by $2n-2$), suggesting PFL1 activity may be important for optimal growth.

Strain	Doubling Time (hours)
CC-124	7.5 ± 0.2
<i>pfl1</i> -KD1	8.6 ± 0.4
<i>pfl1</i> -KD2	8.3 ± 0.2

Table 4.1: Analysis of effect of *PFL1* knockdown on growth rate during mixotrophic growth.

Cell density was measured using a spectrophotometer (OD_{750}), results are given of twelve biological replicates $\pm$ SE.

To check whether knockdown of *PFL1* affected cell survival in the dark (heterotrophic conditions), where fermentative metabolism is likely to play an important role, plate assays were performed comparing CC-124 and *pfl1*-KD2, finding no significant impact on *pfl1*-KD2 (Appendix IV.3). However *pfl1*-KD2 colonies appeared slightly darker in pigmentation, which cannot be currently explained, but may represent different physiological state such as changes in pH arising from altered fermentation profiles. However, a more thorough analysis will be required to ascertain the effect of *PFL1*-knockdown upon dark growth rates, pH and metabolite production, along with additional repetitions.

4.3.2 HPLC analysis of WT, *pfl1*-KD1 and *pfl1*-KD2 metabolite production during dark-anaerobic incubation

To test if amiRNA knockdown is a suitable technology for redirecting metabolic flux, the excreted metabolites produced by *pfl1*-KD cultures during dark-anaerobic incubation was assessed by HPLC (Figure 4.8), with cultures re-suspended in AIB according to Mus et al. (2007). As a control, CC-124 was incubated with 10

mM sodium hypophosphite (HP) according to Hemschemeier et al. (2008b) which is a known inhibitor of PFL1 (Knappe et al., 1984). HP caused a ~80% decrease formate accumulation (Figure 4.8), the continued low level production of formate even in the presence of HP was in accordance with previous experiments (Hemschemeier et al., 2008b), and would be expected as HP is a competitive inhibitor. A modest decrease in formate accumulation of ~40% was observed for *pfl1*-KD1 and ~65% for *pfl1*-KD2 cultures (Figure 4.8).

Changes in formate production were accompanied by a decrease in acetate formation, the production of lactate and potentially a small increase in ethanol accumulation (Figure 4.8), in agreement with the general trend observed in *pfl1*-KO cultures (Philipps et al., 2011). The presence of low levels of PFL1 in knockdown strains was sufficient to cause significant amounts of formate to accumulate, and would provide an explanation for the lower relative levels of lactate and ethanol produced compared to HP treated cultures (Figure 4.8). The total quantity of metabolites excreted was slightly lower in the absence of PFL1 activity (Figure 4.8), in contrast to analysis of the *pfl1*-KO strain (Philipps et al., 2011), this may be an artefact, or perhaps a hint at reduced rates of glycolysis or the accumulation of intracellular carbon, but will require further investigation.

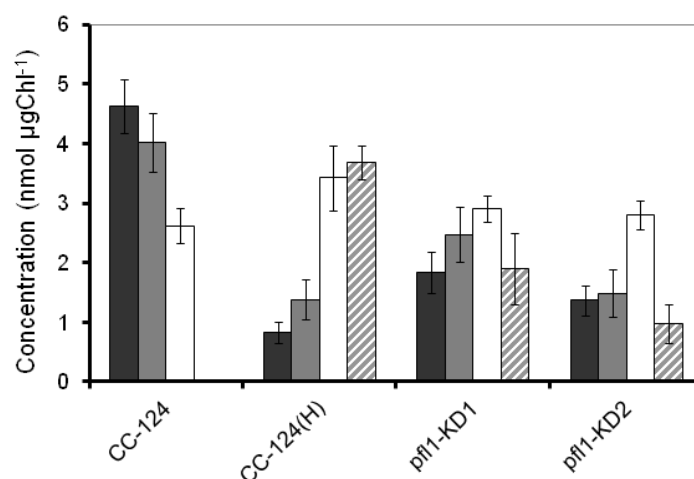


Figure 4.8: HPLC analysis of dark-metabolite accumulation comparing WT, WT + HP and *pfl1*-KD lines.

Showing metabolite formation in WT (CC-124), WT treated with 10 mM HP (CC124(H)) and *pfl1*-KD2 cultures after 4 h dark-anaerobic incubation in AIB buffer. Results are given of five biological replicates $\pm$ SE.

Additionally, cultures were incubated in minimal (HSM) media as opposed to AIB during dark-anaerobic incubation, in order to test if the nutrient content had a significant effect on fermentative metabolite production (Appendix IV.4). Results were analysed by NMR, and found an increased ratio of formate to acetate in WT cultures (Appendix IV.4). Knockdown and inhibition of PFL1 showed reduced lactate production and increased ethanol production compared to AIB incubated cultures (Appendix IV.4).

Unfortunately we were unable to measure dark H₂ production (data not shown) by GC, which may have been the result of the small injection loop volume (200 µl). Similarly, dark H₂ production rates were below detection level on the Clark electrode setup (data not shown). In the future, measurement of dark-H₂ production could potentially be achieved through the use of a membrane inlet mass spectrometry (MIMS) system (Cournac et al., 2002; Hemschemeier et al., 2008a; Tamburic et al., 2011), or increasing the size of injection loop on the GC up from 200 µl.

Overall levels of metabolites produced by WT cultures during dark-anaerobic incubation were slightly less than previously reported, but within the same reported range - approximately half that recorded by Dubini et al. (2009) and Philipps et al. (2011). This finding was consistent between measurements using HPLC or NMR, ruling out potential errors associated with choice of analytical method. The lower levels of metabolite accumulation are most likely a result of laboratory strain variability, which has been shown to vary widely and affect both starch accumulation and rates of catabolism (Chochois et al., 2010), which would impact upon fermentative product formation. Lending credence to this hypothesis, starch levels in the WT strain used here, were found to be slightly lower than strains measured elsewhere (Chochois et al., 2010) with concentrations in TAP grown cultures recorded at ~1 µg starch per 10⁶ cells, compared to ~5 µg starch per 10⁶ cells (Chochois et al., 2010).

4.3.3 Comparison of CC-124 and *pfl1*-KD2 H₂ production during sulphur-deprivation

Based on the results of dark-anaerobic metabolite production (Figure 4.8), the best knockdown *pfl1*-KD2, was chosen for further analysis to compare the effect of *PFL1*-knockdown on H₂ production during sulphur deprivation. As controls, CC-124

cultures incubated either in the absence or presence HP were also analysed. Immunoblotting indicated that knockdown of *PFL1* remained effective under these conditions (Figure 4.9). Induction of the hydrogenase and LDH in *pfl1*-KD2 was not significantly affected (Figure 4.9 and Appendix IV.5), analysis of the other fermentative enzymes suggested there were potentially small increases in PDC3 and PFOR expression (Appendix IV.5), but will require further biological replicates to determine if this is a reproducible trait.

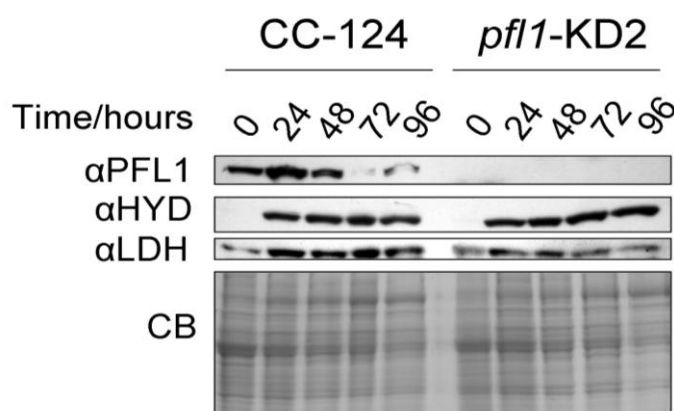


Figure 4.9: Immunoblot analysis of *C. reinhardtii* WT and *pfl1*-KD2 cultures post sulphur-deprivation.

8×10^5 cells loaded per lane with Coomassie blue (CB) stained gel as a loading control.

Sulphur-deprived cultures were incubated in mini bioreactors based on the system used by Dr. Olaf Kruse's group (Beckmann et al., 2009) and H_2 analysed by gas chromatography, which is a standard technique for analysis of headspace gas samples. Recorded yields of H_2 for WT cultures were in good agreement with previous experimental data, finding a total of $280 \text{ nmol } H_2 \mu\text{gChl}^{-1}$ (Figure 4.10A) compared to $\sim 300 \text{ nmol } H_2 \mu\text{gChl}^{-1}$ (Hemschemeier et al., 2008a).

Maximum rates of H_2 evolution were recorded at $\sim 5 \text{ nmol } \mu\text{gChl}^{-1} \text{ h}^{-1}$ for WT cultures (measured between 24–48 h), with gas production coming to a halt $\sim 96 \text{ h}$ post sulphur-deprivation (Figure 4.10A). Knockdown of *PFL1* appeared to have little effect on overall H_2 production rate ($\sim 4.6 \text{ nmol } \mu\text{gChl}^{-1} \text{ h}^{-1}$) or yield, showing only a slight decrease in total H_2 gas volume (Figure 4.10A) (Table 4.2).

CO_2 emission levels for CC-124, *pfl1*-KD2, and HP treated cultures showed no statistically significant differences (Figure 4.10B) (Table 4.2).

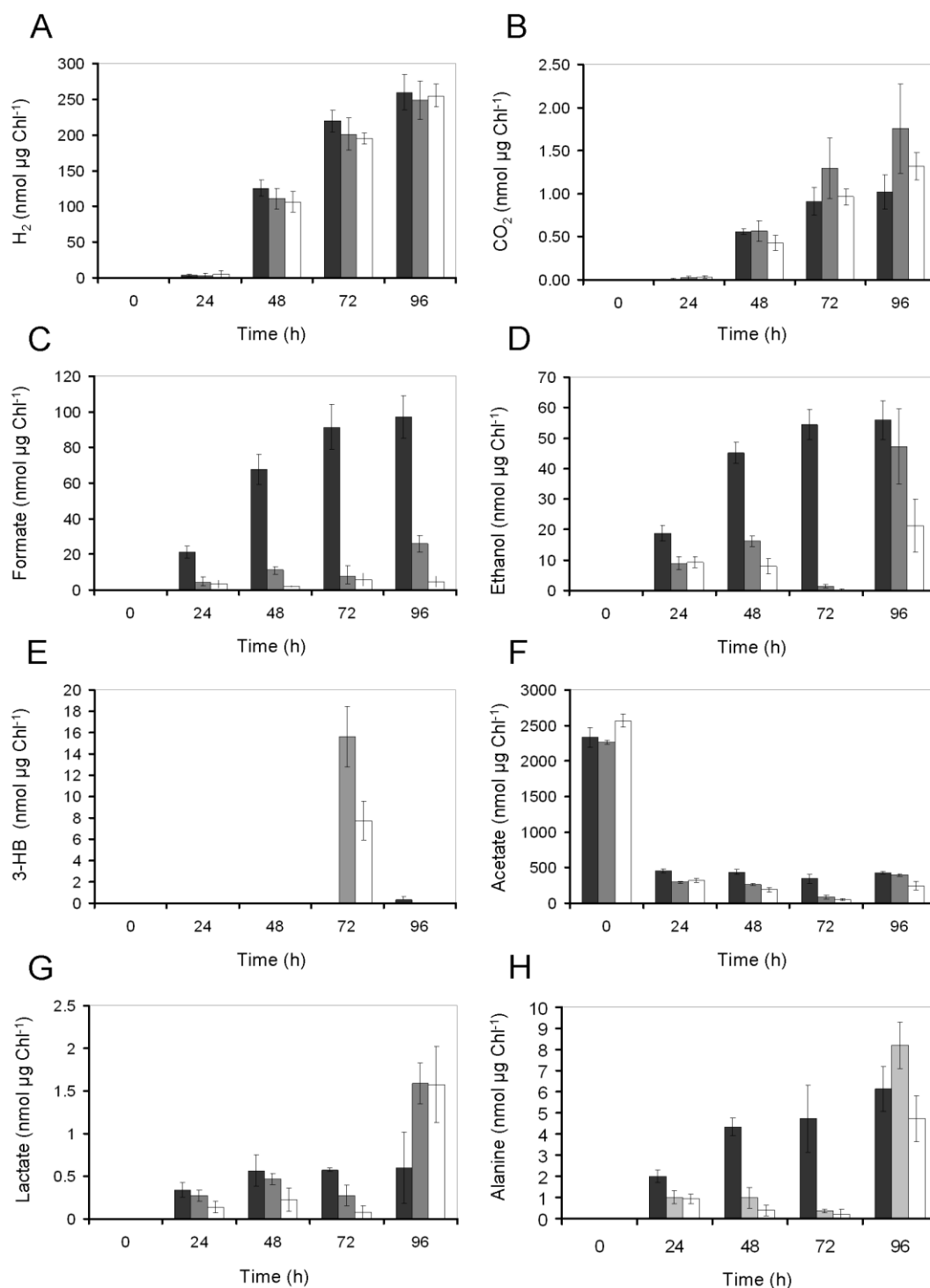


Figure 4.10: NMR analysis of product formation post sulphur-deprivation comparing WT, WT + HP and *pfl1*-KD2 cultures.

Represented is fermentative product accumulation in WT (dark grey bars), *pfl1*-KD2 (light grey bars) WT + 10 mM HP (white bars) cultures, looking at (A) H_2 (B) CO_2 (C) formate (D) ethanol (E) 3-hydroxybutyrate (F) acetate (G) D-lactate, (H) alanine. Error bars are given as $\pm$ SE of three repeat experiments.

Experiment	Total H ₂ yield nmol μgChl^{-1}	Total CO ₂ yield nmol μgChl^{-1}
CC-124	259 $\pm$ 25	1.0 $\pm$ 0.3
CC-124 + 10 mM HP	254 $\pm$ 16	1.3 $\pm$ 0.2
<i>pfl1</i> -KD2	249 $\pm$ 27	1.7 $\pm$ 0.5

Table 4.2: Summary of H₂ and CO₂ evolution by *C. reinhardtii* WT and *pfl1*-KD2 strains during sulphur-deprivation.

Values were calculated from three biological replicates $\pm$ SE.

4.3.4 NMR analysis of CC-124 and *pfl1*-KD2 metabolite production during sulphur-deprivation

NMR spectroscopy was used for metabolite analysis, this is a conventional method used for metabolomic studies and provides quantifiable data for analysis of the complete spectrum of compounds within a sample, requiring no chemical derivatization, and with a sensitivity (1-5 μM) suitable for analysis of major metabolic changes (Wishart, 2008). In this study only the changes in the excreted metabolite profile were analysed, this was due to ease of sample preparation, the fact intracellular and extracellular metabolomes analysed by NMR were previously reported to be very similar, and that 90–95% of metabolites have been found to accumulate in the supernatant during sulphur-deprivation (Timmins et al., 2009b). Previous experiments using NMR to analyse the metabolome of *C. reinhardtii* replaced Tris with a phosphate buffer, this was presumably due to the large signal peak which would make identification of novel compounds in this region of the spectrum difficult. However, by replacing Tris a reduction in overall H₂ yields was observed (Timmins et al., 2009b). We performed preliminary analysis of the excreted metabolite profile from TAP-S samples and found that even in the presence of Tris it was possible to observe changes in formate, ethanol, acetate and lactate in addition to amino acids, and therefore decided to proceed with this approach retaining Tris in the medium.

Cultures of CC-124 showed an approximately linear rate of increase in formate during the first 72 h of sulphur-deprivation, in agreement with previous analysis (Hemschemeier et al., 2008b; Timmins et al., 2009b; Doebe et al., 2010), before declining to 96 h (Figure 4.10C) reaching a total of 97 ± 21 nmol μgChl^{-1} . *pfl1*-KD2 displayed a dramatic reduction in the accumulation of formate up to 72 h post sulphur-deprivation, similar to that observed in the samples treated with HP (Figure 4.10C). However, a late increase in formate levels between 72–96 h was

observed in *pfl1*-KD2 with a final yield equal to ~25% of WT levels (26 ± 8 nmol μgChl^{-1}) (Figure 4.10C). This increase in formate production did not correspond to a significantly reduced efficacy of *PFL1*-knockdown (Figure 4.9).

Ethanol accumulated at a reduced rate in both *pfl1*-KD2 and HP-treated cultures (~3 times slower in *pfl1*-KD2 relative to CC-124) during the first 48 h (Figure 4.10D). In each of three biological replicates, for both *pfl1*-KD2 and HP-treated cultures, ethanol levels appeared to decline between 48–72 h before rapidly accumulating at 96 h, reaching a total of 47 nmol μgChl^{-1} in *pfl1*-KD2 cultures and 21 nmol μgChl^{-1} in the presence of HP, as compared to 56 nmol μgChl^{-1} for CC-124 cultures (Figure 4.10D).

The ratio of fermentation products can be used as a very rough indication for the likely source of metabolites (Gfeller and Gibbs, 1984), as theoretically if ethanol is being produced from acetyl-CoA generated by PFL1 activity, the ratio of ethanol to formate should be 1 or lower (if other pathways such as acetate production compete for acetyl-CoA). The ratio of ethanol to formate production in CC-124 started around 1 after 24 h and declined to around 0.5 after 96 h, whereas in *pfl1*-KD2 cultures this ratio started at 2 after 24 h, indicating an alternative source of carbon for ethanol production, before declining to 0.2 between 24–72 h before an increase to 2 again between 72–96 h. A similar pattern was observed for CC-124 cultures treated with HP, displaying a higher initial and final ratio of ethanol to formate, ~4. From this it can be ascertained that during sulphur-deprivation ethanol was not purely produced from acetyl-CoA generated by PFL1 activity, and additional pathways for ethanol production are active in *C. reinhardtii*, in agreement with previous findings (Hemschemeier et al., 2008b).

The decline of ethanol between 48–72 h was accompanied by the time dependent excretion of 3-hydroxybutyrate (3-HB) in *pfl1*-KD2 and inhibited cultures, a metabolic pathway inferred from proteomic analysis (Atteia et al., 2009), but with no previous experimental evidence in this alga (Figure 4.10E). 3-HB is normally produced as a carbon storage compound under nutrient stress (Anderson and Dawes, 1990), and was unambiguously identified by the appearance of characteristic signals at 1.19 (d), 2.29, 2.40 (2 x dd) and 4.14 (m) ppm in the ^1H NMR spectra (Appendix IV.6).

There was also an alteration in acetate levels when PFL1 activity was reduced (Figure 4.10F). In cultures of CC-124, acetate was rapidly consumed from the

medium in the first 24 h during the initial aerobic and oxygen consumption phases as previously reported (Timmins et al., 2009b), then remained at a largely constant level of $\sim 400 \text{ nmol } \mu\text{gChl}^{-1}$ for the rest of the experiment (Figure 4.10F). In contrast, acetate levels continued to decrease in *pfl1*-KD2 and HP treated cultures from 24–72 h before a late increase (Figure 4.10F).

Limited changes were observed in the remaining excreted metabolite profile, with minor amounts of additional lactate accumulating in *pfl1*-KD2 and CC-124 + HP between 72–96 h post-sulphur-deprivation, but the experimental error was large, meaning further replicates are required before any significance can be attributed to these results (Figure 4.10G). The decrease in formate and ethanol production in *pfl1*-KD2 and inhibited cultures did not result in increases in the excretion of alternate carbon compounds sufficient to account for the altered carbon balance, suggesting either reduced rates of glycolysis or the accumulation of carbon intracellular carbon.

Alanine accumulation, a compound that might have been predicted to increase in the absence of PFL1 activity, was decreased in *pfl1*-KD2 and CC-124 + HP cultures during the first 72 h of sulphur-deprivation (Figure 4.10H), before recovering to WT levels between 72–96 h post-sulphur-deprivation accumulating a total of 8.2 ± 1.0 and $4.8 \pm 1.1 \text{ nmol } \mu\text{gChl}^{-1}$ respectively, compared to $6.1 \pm 1.0 \text{ nmol } \mu\text{gChl}^{-1}$ for WT cultures (Figure 4.10H).

In all measurements succinate and malate levels were low (in the range of $\sim 1 \text{ nmol } \mu\text{g Chl}^{-1}$) and showed a high degree of variability making any quantification difficult (data not shown). The amino acids leucine, isoleucine, valine and phenylalanine gradually accumulated in the medium of WT cultures during sulphur-deprivation (Figure 4.11). In *pfl1*-KD2 or HP treated cultures amino acid excretion appeared to be suppressed during the first 72 h (Figure 4.11), suggesting wider alterations to cellular metabolism, before a late increase between 72–96 h which coincided with increased formate production (Figure 4.10C).

Guanosine levels can be taken as an indicator of release of intracellular debris as a result of cell lysis which occurred predominantly after 96 h sulphur-deprivation (Figure 4.11F), with tyrosine displaying some degree of correlation suggesting the aromatic amino acid is not excreted (Figure 4.11E and F).

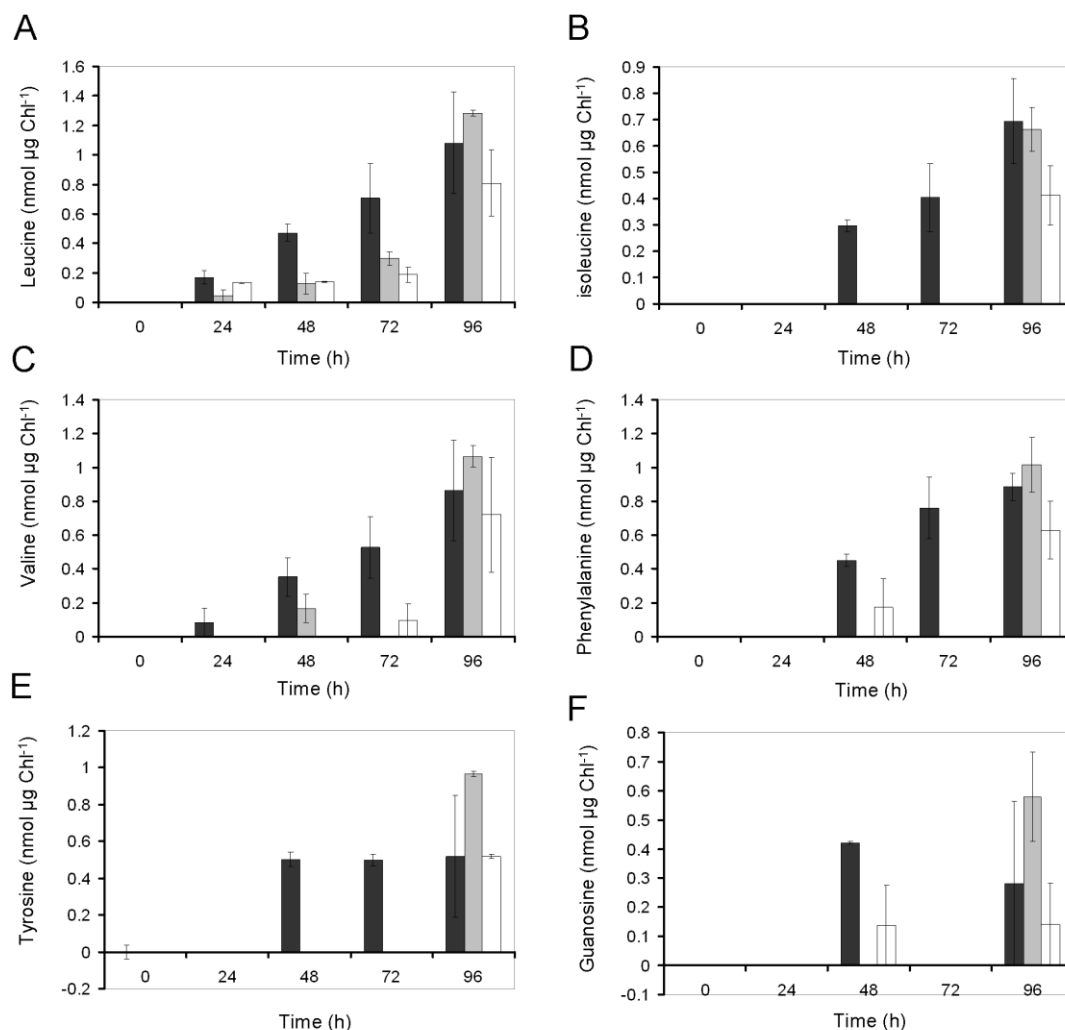


Figure 4.11: NMR analysis of amino acid formation post sulphur-deprivation comparing WT, *pfl1*-KD2 and WT + HP cultures.

Represented is amino acid accumulation in WT (dark grey bars), *pfl1*-KD2 (light grey bars) and WT + 10 mM HP (white bars) cultures, displaying results for (A) leucine (B) isoleucine (C) valine (D) phenylalanine (E) tyrosine (F) guanosine. Error bars are given as $\pm$ SE of three repeat experiments.

4.4 Knockdown of pyruvate decarboxylase 3 (*PDC3*)

4.4.1 Isolation and characterisation of a *pdv3*-KD line during dark-anoxia

Screening 30 transformants for each of the two knockdown constructs created to target *PDC3* (pRNAi3.6A and pRNAi3.6B), isolated only a single mutant colony with over 90% reduction in target protein levels (Figure 4.12A). *PDC3* knockdown proved consistent, with similarly reduced protein levels in all immunoblots (Appendix IV.7), overexposure of membranes in some cases revealed a faint band in the *pdv3*-

KD lane, however it was smeary and unclear whether the cross reaction was genuine or the result of background bands (Appendix IV.7).

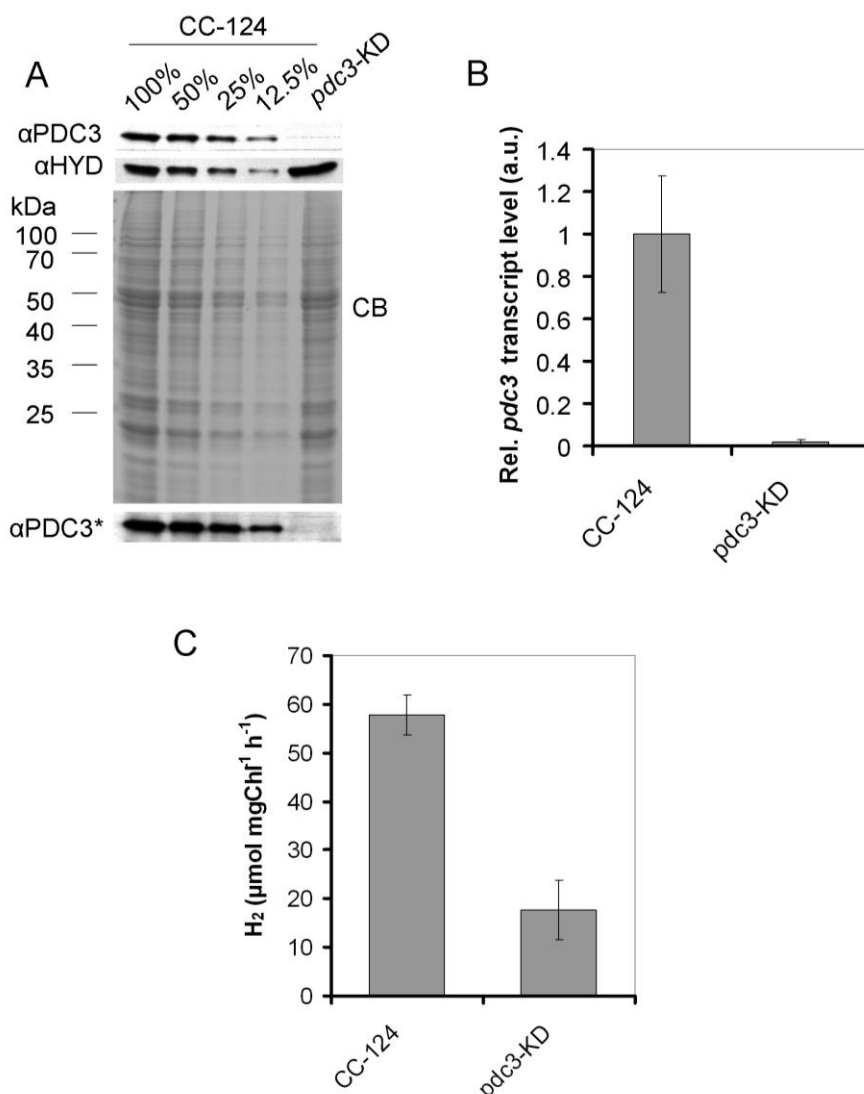


Figure 4.12: Comparison of WT and *pdc3*-KD after dark 4 h dark anaerobic incubation in AIB.

(A) Immunoblot analysis of target protein levels in WT (CC-124) and *pdc3*-KD, *pdc3*-KD* denotes overexposed blot, 8×10^5 cells were loaded per lane, with the expectation of the WT dilution series were the percentages of this total are indicated, loading control is provided by Coomassie stain gel. (B) Taqman qRT-PCR analysis comparing *PDC3* mRNA transcript levels after anaerobic induction relative to CC-124 at $t = 0$ h, error bars are given as $\pm$ SE of three biological replicates. (C) Transient light induced H_2 production as measured by Clark electrode, error bars are given $\pm$ SE (CC-124; $n=18$, *pdc3*-KD; $n=4$).

qRT-PCR revealed a decrease in mRNA levels (Figure 4.12B) in accordance with protein concentrations. The transient light-induced H₂ production ability of *pd3*-KD was tested by Clark electrode, with *pd3*-KD (17.8 ± 6.1 nmol mgChl⁻¹ h⁻¹) consistently displaying a decrease in gas production rate compared to WT. The growth rate of *pd3*-KD was checked by determining the maximum doubling time compared to the parental CC-124 strain in TAP media, a slight increase was observed but was not found to be statistically significant (Table 4.3).

Strain	Doubling Time (hours)
CC-124	7.5 ± 0.2
<i>pd3</i> -KD	7.1 ± 0.3

Table 4.3: Analysis of effect of *PDC3* knockdown on growth rate during mixotrophic growth.

Cell density was measured at OD₇₅₀, results are given of twelve biological replicates $\pm$ SE.

4.4.2 NMR analysis of WT and *pd3*-KD metabolite production during dark-anaerobic incubation

The effect of *PDC3* knockdown on dark-anaerobic metabolite production was analysed by incubating cultures in HSM to avoid changes in metabolism caused by nutrient stress, and purging with argon in the dark for 4 h. NMR analysis revealed *pd3*-KD had little impact on dark-anaerobic product formation, suggesting this pathway may be inactive under these conditions or has only very low activity (Figure 4.13). When *pd3*-KD cultures were incubated with HP to inhibit the PFL1 catalysed pathway, a slight increase in lactate production was observed and ethanol levels appeared to increase relative to WT cultures with and without HP (Figure 4.13).

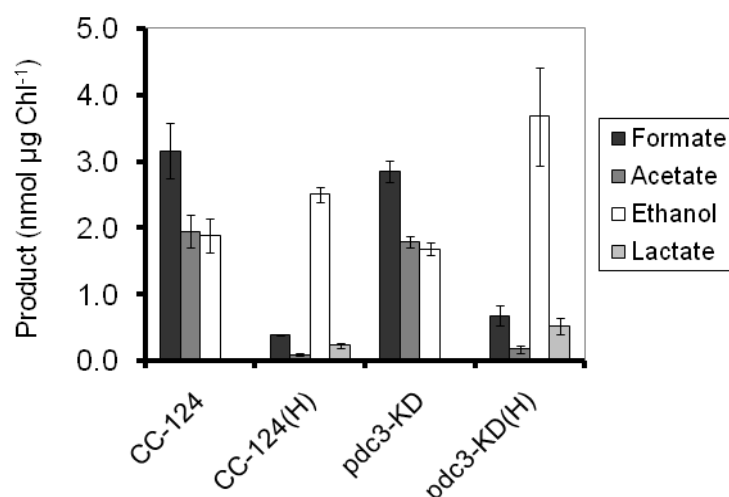


Figure 4.13: NMR analysis of dark-anaerobic metabolite accumulation comparing WT, WT + HP, *pdc3*-KD and *pdc3*-KD + HP cultures.

Represented is WT (CC-124), WT + 10 mM HP (CC124(H)), *pdc3*-KD and *pdc3*-KD + 10 mM HP cultures (*pdc3*-KD(H)). Cells were re-suspended in HSM and incubated under dark-anaerobic conditions for 4 h. Values given as the average of three biological repeats $\pm$ SE.

4.4.3 Analysis of the effect of *pdc3*-KD on H₂ production during sulphur-deprivation

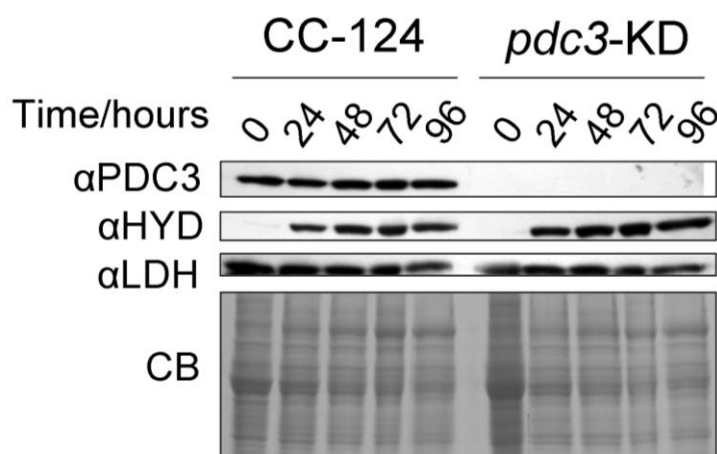


Figure 4.14: Immunoblot analysis of WT and *pdc3*-KD cultures post sulphur-deprivation.

8×10^5 cells loaded per lane. Coomassie blue (CB) stained gel is provided as a loading control.

Knockdown of *PDC3* was found to be stable throughout sulphur-deprivation, knockdown appeared to have little impact upon LDH expression and did not negatively effect hydrogenase levels, which may have been slightly increased (Figure 4.14 and Appendix IV.8). Little change could be seen in other fermentative enzyme levels; a minor increase in PFL1 may have been observed but will require further repeats to determine if this was a reproducible result (Appendix IV.8).

The effect of *PDC3* knockdown on H₂ production during sulphur-deprivation was analysed by GC (Figure 4.15A). *pd3*-KD displayed similar rates of H₂ production to WT over the first 48 h of sulphur-deprivation but evolution declined rapidly between 48-72 h then came to a stop, resulting in a lower overall yield (Table 4.4 and Figure 4.15A). CO₂ accumulation was analysed in headspace samples, *pd3*-KD appeared to produce more CO₂ than WT over the first 48 h, but could not be determined to be statistically significant (Figure 4.15B). Then as the rate of overall gas evolution declined in *pd3*-KD cultures more rapidly than WT, the final amount of CO₂ produced was similar to WT (Table 4.4) (Figure 4.15B).

Experiment	Total H ₂ yield nmol µgChl	Total CO ₂ yield nmol µgChl
CC-124	259 ± 25	1.0 ± 0.2
CC-124 + 10 mM HP	254 ± 16	1.3 ± 0.2
<i>pd3</i> -KD	138 ± 9	1.3 ± 0.1
<i>pd3</i> -KD + 10 mM HP	204 ± 36	0.6 ± 0.1

Table 4.4: Summary of H₂ and CO₂ evolution by WT and *pd3*-KD strains treated with or without 10 mM HP during sulphur-deprivation.

Values were calculated from three biological replicates ± SE.

4.4.4 NMR analysis of CC-124 and *pd3*-KD metabolite production during sulphur-deprivation

4.4.4.1 Formate and ethanol production by *pd3*-KD cultures during sulphur-deprivation

Analysis of excreted metabolites from *pd3*-KD showed both altered dynamics and an increased production of ethanol and formate (Figure 4.15C and 4.15D).

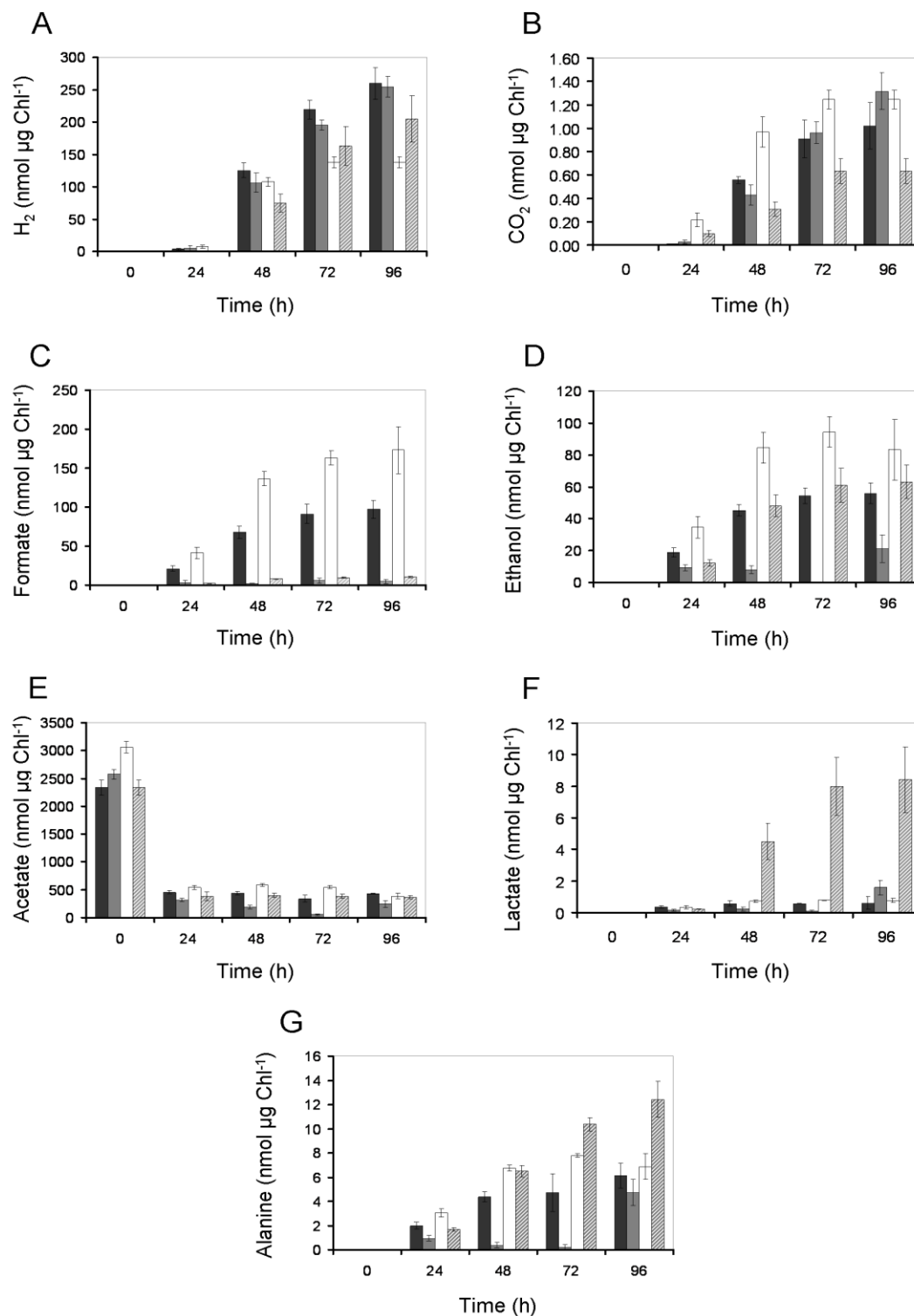


Figure 4.15: NMR analysis of product formation post sulphur-deprivation comparing WT, WT + HP, *pdc3*-KD and *pdc3*-KD + HP cultures

Represented is product formation in WT (dark grey bars), WT + 10 mM HP (grey bars), *pdc3*-KD (white bars) and *pdc3*-KD + 10 mM HP (light grey bars) cultures, displaying (A) H_2 (B) CO_2 (C) formate (D) ethanol (E) acetate (F) D-lactate, (G) alanine accumulation. Results are given for three biological replicates $\pm$ SE (note results for CC-124 cultures are the same as figure 4.9).

Rates of formate and ethanol accumulation more than doubled over the first 48 h in *pd3-KD* cultures before slowing to a stop. The total amount of formate and ethanol accumulated in *pd3-KD* cultures after 48 h was greater than WT cultures after 96 h (136 ± 9 nmol $\mu\text{g Chl}^{-1}$ and 84 ± 10 nmol $\mu\text{g Chl}^{-1}$ respectively) (Figure 4.15C and 4.15D).

4.4.4.2 Miscellaneous metabolite production in *pd3-KD* cultures during sulphur-deprivation

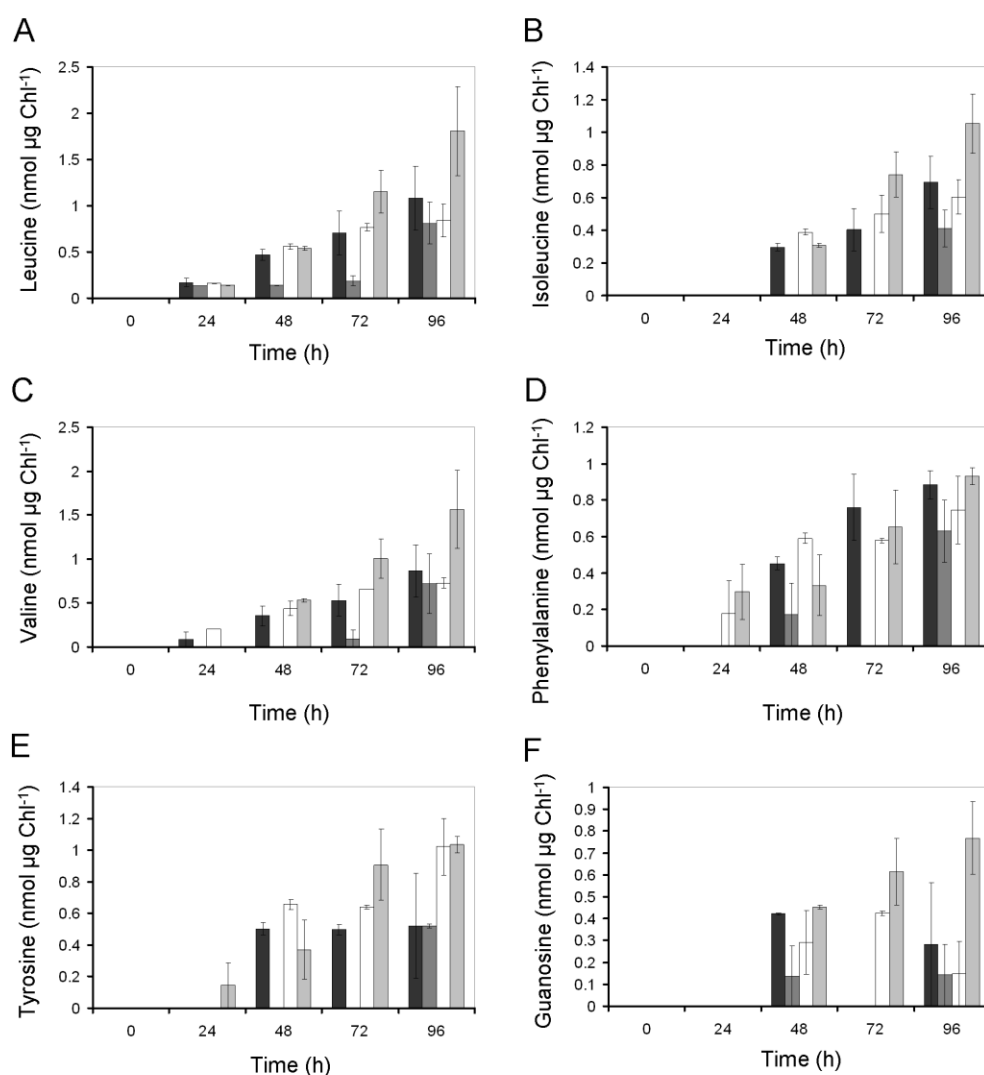


Figure 4.16: NMR analysis of amino acid formation post sulphur-deprivation comparing WT, WT + HP, *pd3-KD* and *pd3-KD* + HP cultures.

Represented is amino acid formation in WT (dark grey bars), WT + 10 mM HP (grey bars), *pd3-KD* (white bars), *pd3-KD* + 10 mM HP (light grey bars) cultures, displaying results for (A) leucine (B) isoleucine (C) valine (D) phenylalanine (E) tyrosine (F) guanosine. Error bars are given as $\pm$ SE of three repeat experiments.

No changes in acetate metabolism were observed in *pd3-KD* relative to CC-124 (Figure 4.14E), amounts of lactate remained low (Figure 4.15F) and 3-HB was not detected. Changes in amino acid excretion were also analysed by NMR (Figure 4.16). There appeared to be no significant difference between amino acid excretion in CC-124 and *pd3-KD* cell lines (Figure 4.16).

4.4.5 Analysis of the effect of inhibition of PFL1 on metabolite production by *pd3-KD* during sulphur-deprivation

Addition of HP to inhibit PFL1 in *pd3-KD* cultures appeared to restore H₂ yields to WT values (Table 4.4 and Figure 4.15A), whereas CO₂ evolution decreased dramatically compared to WT suggesting a reduction in decarboxylation reactions or an increase in CO₂ uptake caused by redirecting of metabolic flux (Figure 4.15B).

Formate production was largely abolished as observed in CC-124 cultures treated with the inhibitor (Figure 4.15C), and no 3-HB was observed (data not shown). Ethanol production decreased relative to untreated *pd3-KD* cultures with rates and yield similar to WT (Figure 4.15C).

Lactate production increased dramatically (8.4 ± 2.1 nmol μgChl^{-1}) relative to WT (0.60 ± 0.42 nmol μgChl^{-1}) and *pd3-KD* cultures (0.75 ± 0.44 nmol μgChl^{-1}) (Figure 4.15F). Additionally alanine production appeared to increase, accumulating a total of 12.3 nmol μgChl^{-1} after 96 h compared to 6.1 nmol μgChl^{-1} in WT cultures (Figure 4.15G).

Additional alterations to amino acid excretion were difficult to assess due to increased levels of guanosine in *pd3-KD* cultures + HP as a result of contamination of samples with intracellular debris caused by cell lysis (Figure 4.16H).

4.4.6 Effect of *pd3-KD* on starch metabolism during sulphur-deprivation

To further investigate the potential causes of decreased H₂ evolution by *pd3-KD* cultures, starch accumulation and catabolism, along with changes in pH were analysed during sulphur-deprivation (Figure 4.17). In this set of experiments H₂ production was lower for both WT and *pd3-KD* than in the previous experiment (Figure 4.17A), the precise reason for this is unclear, but coincided with changes in illumination which may have had an effect. However, the results within this experiment were self-consistent, showing the same general trend as described

previously (Figure 4.17A), with *pd3-KD* cultures showing a reduced rate of H_2 production (Figure 4.17A). CC-124 and *pd3-KD* cultures accumulated similar amounts of starch during the first 24 h ($5.2 \mu\text{g starch per } \mu\text{gChl}^{-1}$), and starch catabolism was unimpaired in mutant cultures, with *pd3-KD* potentially showing a slight increase in the amount of starch broken down (Figure 4.17B). Additionally, both cultures also showed a similar pH profile, rapidly increasing from 7 to 8.1 as acetate is consumed, before slowly decreasing to pH 7.8 through the excretion of formate and acetate (Figure 4.17C), a slight decrease in external pH values was observed in *pd3-KD* cultures, particularly between 48-72 h post sulphur-deprivation which could potentially be explained by increased formate excretion.

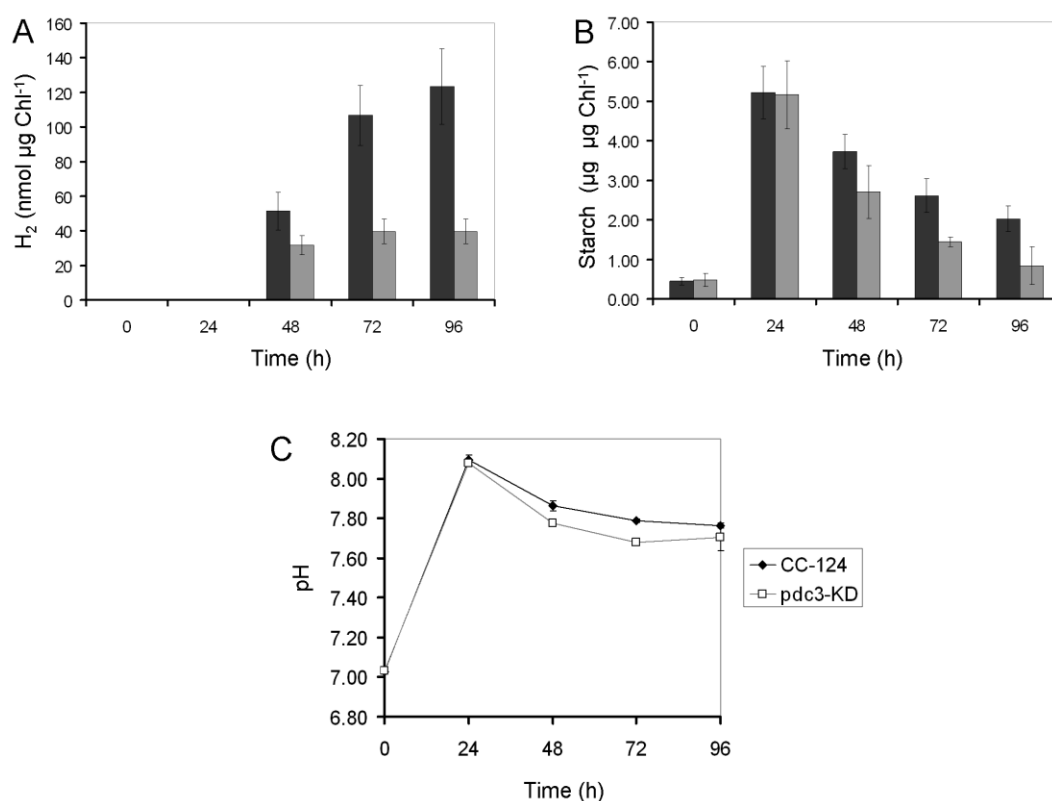


Figure 4.17: Comparison of WT and *pd3-KD* cultures' response to sulphur-deprivation.

Represented are results analysing changes in WT (dark grey) and *pd3-KD* (light grey) cultures during sulphur-deprivation looking at (A) H_2 accumulation, (B) starch levels, and (C) pH of the medium. All values are given of five biological replicates $\pm$ SE.

4.5 Discussion

4.5.1 Investigating the efficacy of amiRNA technology as a tool for redirecting metabolic flux

The results presented here indicate that artificial microRNA technology is a suitable means for redirecting metabolic flux in *C. reinhardtii*. Knockdown analysis provided experimental data for an active PDC3 catalysed pathway in *C. reinhardtii* only previously inferred by transcriptomic analysis and indirect measurements of fermentative product formation. Analysis of metabolite accumulation in *pd3-KD* cultures during sulphur-deprivation showed a re-routing of carbon flux via PFL1 resulting in increased levels of formate production. Additionally, an increase in ethanol production suggests either a more complex alteration to cellular metabolism causing the re-routing of carbon flux to alternative ethanol production pathways, or in contrast to current theory, not all the acetaldehyde produced by PDC3 activity is converted into ethanol.

Analysis of *pfl1-KD* cultures suggested the majority of ethanol produced during sulphur-deprivation in the laboratory strain used here (CC-124) is likely to come from acetyl-CoA produced by PFL1 activity (Figure 4.10D). This is in contrast to previous experiments using a different WT strain where ethanol levels remained unchanged (Hemschemeier et al., 2008b), suggesting differences in how metabolic flux is redirected between the two WTs. Inhibiting formate production also provided evidence for the time dependent production of 3-HB, a metabolic pathway only previously inferred from proteomic analysis (Atteia et al., 2009).

Individual knockdown, and inhibition of PFL1 in *pd3-KD* cultures, all failed to increase H₂ yields during sulphur-deprivation; instead metabolism was redirected in part to the production of lactate and amino acid synthesis, including alanine, making putative LDH and AAT1 interesting candidates for future targeted gene knockdown.

4.5.1.1 Testing amiRNA technology (1): Isolation of knockdown cell lines

Two knockdown constructs were generated to target each gene of interest (see section 4.3.1). For *PFL1*, an initial round of screening identified approximately 1 knockdown with over 80% reduction in protein levels per 15–20 transformants (data not shown).

For *PDC3* after an initial screen of 30 mutants for each amiRNA vector, 60 transformants in total, only a single knockdown was isolated with over 90% reduction in protein level. Screening of a further 60 transformants failed to detect another stable knockdown. Resultantly only one knockdown strain was analysed in this work, and therefore the results presented here must be treated with caution.

The limits of amiRNA knockdown and dependency on amiRNA sequence were demonstrated by the inability to isolate knockdown cell lines showing a substantial reduction in target levels for *LDH*, *PDC1*, *PFOR* and *ADH1* screening two amiRNA constructs for each (data not shown). Further work would involve testing novel amiRNA oligonucleotides for effective knockdown. Out of the number of targets tested, there appeared to be no clear pattern as to a specific region on the mRNA transcript (i.e. 5'UTR, 3'UTR, coding region) that was best to target, and many more knockdowns must be attempted before a firm conclusion can be drawn.

A key area for improving amiRNA technology is therefore to better understand the factors that determine amiRNA binding and activity, so that the current constraints used by the online tool web microRNA designer (WMD3) can be further refined. One potential area of research would be to check the impact of RNA secondary structure on amiRNA binding.

Another factor that should also be taken into consideration when analysing mutant phenotypes is the disruption or deletion of additional nuclear genes caused by the random integration of amiRNA constructs into the genome, sometimes with multiple insertions per transformation. The number and site of insertion of the amiRNA knockdown constructs was not analysed here. The reason for this is that the potential side effects can reasonably be ignored when either; two knockdown cell lines are analysed showing the same phenotype, or if an appropriate specific inhibitor is available. This was the case for *pfl1*-KD, but failure to isolate additional *pdh3*-KD cell lines is a limitation that can only be rectified by further experimentation.

The amiRNA vector itself could potentially be improved in a number of ways; firstly since starting this work a new pChlamiRNAi3 derivative was created, incorporating the *RBCS2* intron 1 into the promoter region (Molnár et al., 2009) which has been shown to enhance expression (Lumbreras et al., 1998). To further increase the strength on knockdown it may be beneficial to replace the targeting region of a different or more abundant native, pre-miRNA sequence as little is currently known about the factors controlling miRNA processing.

Additionally, in circumstances where knockdown has a deleterious effect on cell growth and fitness, it could be beneficial to develop additional inducible amiRNA expression systems. One such vector has already been made, utilising the *NIT1* promoter (Schmollinger et al., 2010), but may be unsuitable for H₂ production due to the requirement for transfer of cultures to nitrate containing medium, potentially activating nitrogen assimilation pathways that could compete with the hydrogenase for reduced ferredoxin.

Finally, the ultimate aim would be to generate multiple knockdowns within the same strain. To facilitate this, a range of vectors with different selection markers would be required. Currently available are vectors with the *ARG* and *aphVIII* cassettes, I constructed derivatives of RNAi3 with the *aphVIII* gene replaced with both the *BLE* (pRNAi4) and *aph7''* (pRNAi5) genes, providing resistance to zeocin or hygromycin respectively, theoretically allowing for the construction of a strain with up to four fermentative enzymes knocked down (Appendix IV.9). Alternatively two knockdown strains with the same resistance marker but in different mating type backgrounds could be crossed and the progeny tested for effective knockdown by immunoblot.

4.5.1.2 Testing amiRNA technology (2): Stability of knockdown

The site of insertion of the amiRNA construct within the genome will have an impact on gene transcription, but can be circumvented by testing multiple transformants for maintenance of knockdown over test conditions. The knockdown lines characterised here remained stable throughout all conditions, and have been maintained for over one year on selective media (see appendix IV.2 and IV.7 with 18 months separating repeat blots A and B of samples isolated after anaerobic induction), indicating amiRNA technology can be used for phenotypic analysis of changes in metabolism. However, other groups have encountered problems regarding the stability of amiRNA knockdowns, such as when targeting CAS1 (Petroutsos, D. personal communication), and this may well explain the inability to isolate strains with a sufficient degree of knockdown of some fermentative enzymes. For example, initial screening of transformants identified mutants potentially displaying reduced levels of LDH, ADH1, and PDC3 (in a repeat screen), but after a second and third test of target knockdown, the effect disappeared. Furthermore when targeting genes that have an important role in cell fitness, and therefore with strong selective pressure to suppress

amiRNA construct expression, amiRNA technology may encounter problems, as suggested by the inability to isolate knockdowns of putative pyruvate dehydrogenase E1 α components PDC1 and PDC2.

Immunochemical analysis of PFL1 levels in *pfl1*-KD1 and *pfl1*-KD2 cultures during various growth stages revealed little change in target protein levels (Figure 4.7) demonstrating the *PSAD* promoter is suitable for expression of the amiRNA construct. Knockdown was effective during anaerobic induction in the dark (Figure 4.6B) and throughout sulphur-deprivation as demonstrated by *pfl1*-KD2 (Figure 4.9), although for the later observation this work could have been improved by overexposing the blot and comparing to a dilution curve of WT cultures, as it is difficult to ascertain the degree of knockdown achieved. *pfl1*-KD1 was not investigated thoroughly under conditions of sulphur-deprivation due to availability of a PFL1 inhibitor, but preliminary results found a significant decrease in formate production similar to *pfl1*-KD2 (Appendix IV.10).

4.5.1.3 Testing amiRNA technology (3): Efficacy for analysing changes in metabolic flux

When WT cultures were incubated anaerobically in the dark in AIB buffer, the fermentation products formate, acetate and ethanol accumulated at an approximate molar ratio of 2:2:1 (Figure 4.8), and a ratio of 2:1:1 in HSM buffer (Appendix IV.4), in general agreement with previous experimental data finding ratios of 2:1:1 when cells were re-suspended in HEPES-Tris buffer, pH 7.5 (Gfeller and Gibbs, 1984), or potassium phosphate buffer (Kreuzberg, 1984), 2.5:2:1 in TAP media (Philipps et al., 2011) and 2:2:1 in AIB buffer (Mus et al., 2007; Dubini et al., 2009) or modified Bristol media (Ohta et al., 1987).

As the ratio of fermentative products produced under dark-anaerobic conditions depended on the choice of buffer, it suggests slight changes in metabolic response depending on the nutrient conditions but will require further investigation. Based on the current metabolic models the production of formate, ethanol and acetate at a ratio of 2:1:1 may suggest the majority of pyruvate is directed via PFL1, producing two molecules of formate and two of acetyl-CoA, the later of which is converted into one molecule of ethanol and one of acetate (Figure 4.18).

The production of equal amounts of formate and acetate at twice the molar ratio of ethanol, suggests that at least two fermentative pathways are active, one via

PFL1 in the chloroplast producing formate and acetyl-CoA which can then be converted to acetate or ethanol (Figure 4.18), plus either PFOR or PDC3 catalysed pathways, although for reasons mentioned previously (See Section 3.6.1) PDH activity cannot be entirely ruled out (Figure 4.18). It is perhaps reasonable to suggest some additional flux is directed to PFOR in an attempt to provide reduced ferredoxin for sulphate or nitrate assimilation by ferredoxin-dependent sulphate and nitrate reductases in response to nutrient stress.

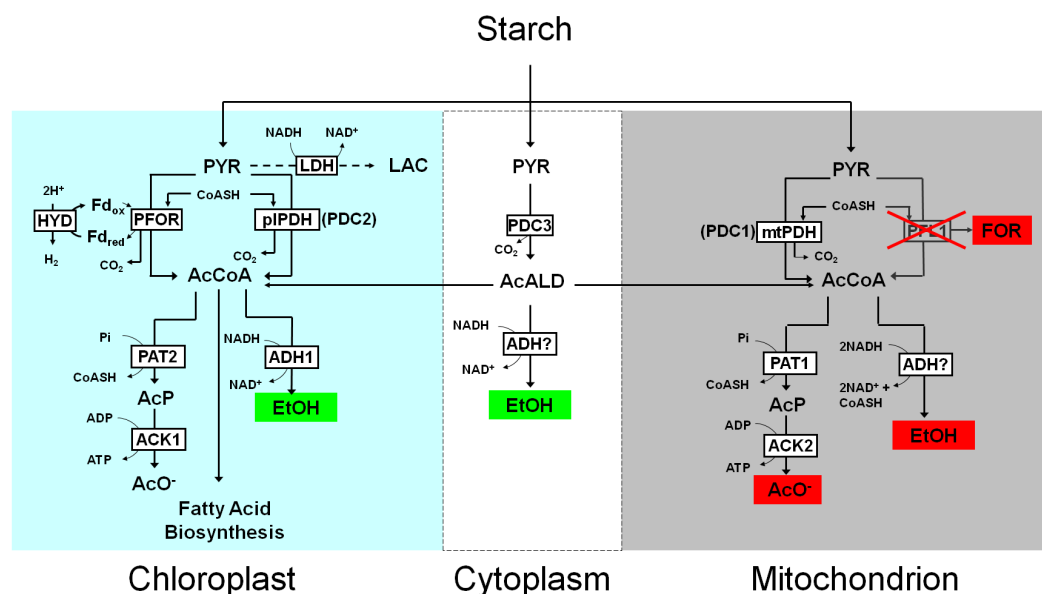


Figure 4.18: Proposed model of dark-anaerobic fermentative metabolism in *pfl1*-KD lines.

Diagram is based on results from cultures incubated in HSM buffer (see Appendix IV.4). Metabolite abbreviations: AcALD; acetaldehyde, AcO⁻; acetate, AcCoA; acetyl-CoA, AcP; acetyl-phosphate, EtOH; ethanol, Fd; ferredoxin, LAC; lactate, OAA; oxaloacetate, PYR; pyruvate. Enzymes are in boxes, ACK; acetate kinase, ADH; alcohol dehydrogenase, HYD; [FeFe]-hydrogenase, LDH; lactate dehydrogenase, PAT; phosphate acetyltransferase, PDC; pyruvate decarboxylase, PFL1; pyruvate formate lyase, PFOR; pyruvate ferredoxin oxidoreductase. Highlighted are metabolites proposed to be reduced (red) or increased (green) in the absence of PFL1 activity. Changes to H₂ production are not displayed as I was unable to measure rates.

amiRNA knockdown of *PFL1* caused a reduction in formate production irrespective of the buffer used, in good agreement with analysis of a *pfl1*-KO strain (Philipps et al., 2011). However, the total amount of formate produced was much higher (~35% of WT) than would be expected if a linear relationship existed between protein levels (10–20% of WT) and formate production. These data indicate that PFL1 may not have a high flux control coefficient, meaning the enzyme level does not fully

control flux through the pathway. This is a potential drawback of RNAi techniques when attempting to redirect metabolism. However, these data do indicate amiRNA may be a useful tool for determining the rate limiting steps of particular pathways for flux balance analysis. The effects of HP on WT cultures were milder than previously reported (Figure 4.8) which could be related to the lower concentration of HP used (10 mM), chosen according to Hemschemeier et al. (2008b), compared to 75 mM in a more recent publication by Philipps et al. (2011).

PFL1 inhibition also caused reduced rates of acetate production (Figure 4.18) as seen in the KO strain (Philipps et al., 2011). However, in this experiment acetate appeared to reduce in a near stoichiometric ratio with formate (Figure 4.8 and Appendix IV.4), suggesting only the mitochondrial PAT1/ACK2 pathway was active, whereas Philipps et al. (2011) found a non-stoichiometric decrease in acetate and formate production, indicating both PAT/ACK catalysed pathways were active in their experiments. This may be attributed to differences in the WT strain used.

Depending on the buffer used, minor increases in lactate (AIB incubated) or ethanol (HSM incubated) were seen, suggesting a redirecting of metabolic flux to LDH and either PFOR or PDC3 (Figure 4.18), although further repetitions are required to provide a level of significance. When compared to previous analysis of the changes in metabolism caused by *pfl1*-KO (Philipps et al., 2011), relative D-lactate and ethanol formation was lower in the *pfl1*-KD strains, which is likely to be the result of incomplete inhibition of PFL1 activity.

Unfortunately we were unable to measure dark H₂ production, GC analysis of headspace gas samples failed to detect traces of H₂, which would help clarify whether flux was redirected to PDC3 or PFOR.

4.5.2 Effect of *pfl1*-KD on transient light-induced H₂ production

A slight decrease in H₂ production rates were observed in *pfl1*-KD2 during transient light induced gas evolution (Figure 4.6C) in agreement with observations of a *pfl1*-KO strain, albeit under slightly different conditions (Philipps et al., 2011). This was consistent with a decrease in hydrogenase expression during dark-anaerobic incubation (Figure 4.6A) as seen in the *pfl1*-KO strain (Philipps et al., 2011), but some variation was observed (Appendix IV.2) which may be the result of a milder phenotype in knockdown lines.

4.5.3 Analysis of fermentative metabolism in *pfl1*-KD2 during sulphur-deprivation

In an attempt to understand the metabolic changes taking place during sulphur-deprivation a refined scheme of fermentative metabolism is presented in Figure 4.19 building on the proposed model in Chapter 3 to incorporate the production of relevant amino acids, as detected by NMR and informed by Timmins et al. (2009) and Vallon et al. (2010), and succinate production pathways as evidenced in *hydEF* strain under dark-anaerobic conditions (Dubini et al., 2009). The situation during sulphur-deprivation is much more complex than that encountered during dark-anoxia, including interactions between fermentation pathways, the TCA cycle and ammonium assimilation. The partitioning of metabolic pathways 1 to 5 between the chloroplast, cytoplasm and mitochondria is not included (Figure 4.19) as this has not been fully verified. However evidence suggests the initial stages of glycolysis, up until the formation of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, occur in the chloroplast, before export to the cytosol (Klein, 1986) and it is thought that valine, leucine, and isoleucine synthesis all occur in the chloroplast, based on transit peptide prediction (Vallon et al., 2009). Aspartate can be synthesised by aspartate transaminase (EC 2.6.1.1; Eq. 21) of which there are five sequences in the *C. reinhardtii* genome distributed between all three cellular compartments (Vallon et al., 2009).



As mentioned previously (see section 3.6.9), there are multiple forms of malate dehydrogenase and malic enzymes present in *C. reinhardtii*, distributed between various cellular compartments, which can act to shuttle carbon, but for ease of representation the various isoforms have not been included here.

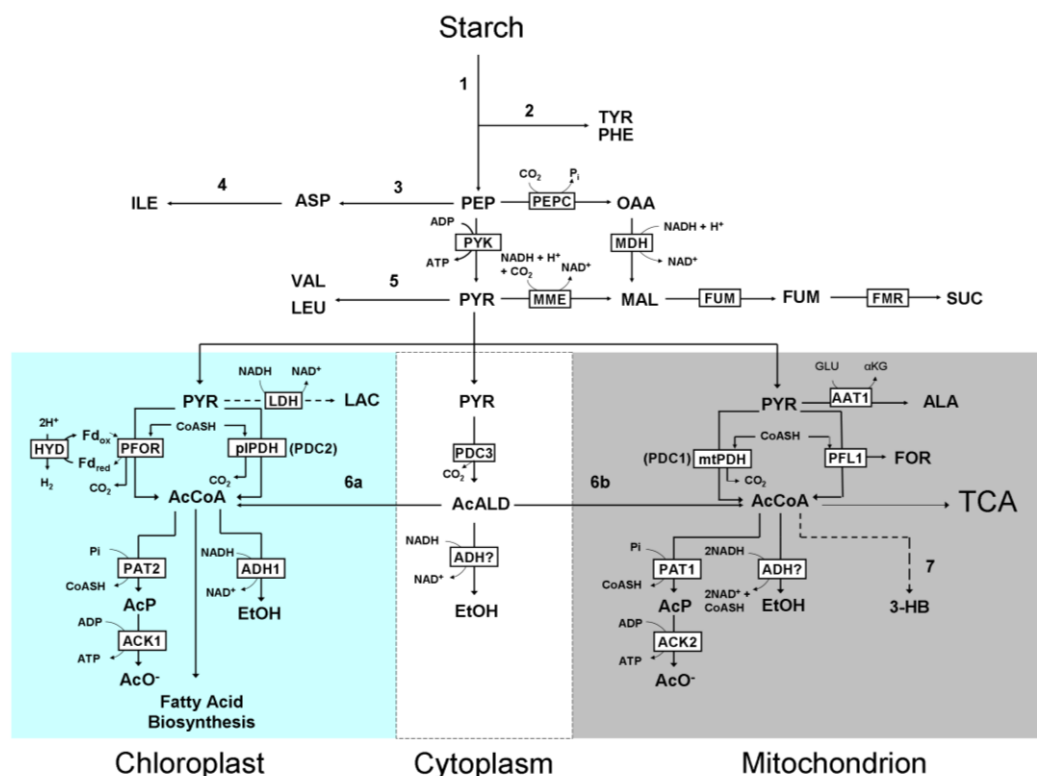


Figure 4.19: Proposed model of *C. reinhardtii* pyruvate degradation, amino acid synthesis and fermentative metabolism during sulphur-deprivation.

Compressed pathways are represented by numbers (1) glycolysis (2) aromatic amino acid synthesis (3) aspartate synthesis (4) isoleucine synthesis (5) branched chain amino acid synthesis (6a and b) proposed routes for acetaldehyde detoxification (7) 3-HB synthesis pathway. Only relevant amino acids are included based on NMR data. Localisation is provided where proven. Metabolite abbreviations: AcALD; acetaldehyde, AcO⁻; acetate, AcCoA; acetyl-CoA, AcP; acetyl-phosphate, ALA; alanine, ASP; aspartate, EtOH; ethanol, Fd; ferredoxin, FUM; fumarate, GLU; glutamate, 3-HB; 3-hydroxybutyrate, ILE; isoleucine, αKG; α-ketoglutarate, LAC; lactate, LEU, leucine, OAA; oxaloacetate, PHE; phenylalanine, PYR; pyruvate, SUC; succinate, TYR; tyrosine, VAL; valine. Enzymes are in boxes, AAT1; alanine amino transferase, ACK; acetate kinase, ADH; alcohol dehydrogenase, FUM; fumarase, FMR; fumarate reductase, HYD; [FeFe]-hydrogenase, LDH; lactate dehydrogenase, MDH; malate dehydrogenase, MME; malic enzyme, PAT; phosphate acetyltransferase, PDC; pyruvate decarboxylase, PEPC; phosphoenol pyruvate decarboxylase, PFL1; pyruvate formate lyase, PFOR; pyruvate:ferredoxin oxidoreductase.

4.5.3.1 Analysis of *pfl1*-KD2 H₂ production ability during sulphur-deprivation

Philipps et al. (2011) revealed an increase in H₂ production by a *pfl1*-KO strain relative to WT cultures under dark-anaerobic conditions, which was proposed to be caused by a redirecting of metabolic flux to PFOR. However, *pfl1*-KO also showed a decrease in H₂ evolution under light anoxic conditions as a result of reduced hydrogenase expression (Philipps et al., 2011).

It was therefore of interest to test the effect of *PFL1* knockdown on H₂ production during sulphur-deprivation. The work here extends previous investigations on HP treated WT cultures (Hemschemeier et al., 2008b) through NMR analysis of the entire excreted metabolome to include acetate, lactate and amino acids, in addition to assessing whether the inhibitor had additional side effects on *C. reinhardtii* cultures.

Analysis revealed HP treated cultures closely mirrored *pfl1*-KD2 in behaviour, suggesting that beyond blocking PFL1 activity, the inhibitor had no significant additional effects on *C. reinhardtii* cells. No significant affect on overall H₂ production rate or yield was observed in *pfl1*-KD2 or WT cultures treated with HP (Figure 4.10A and Table 4.2) in agreement with previously reported inhibitor data (Hemschemeier et al., 2008b). This therefore indicates that the different physiological states arising from dark-anaerobic or microaerobic sulphur-deprived light incubation result in different relationships between the processes of fermentation and H₂ production.

One potential explanation for the failure to improve yields of H₂ is that unlike in the dark, in the light during sulphur stress, carbon flux is not redirected via PFOR. However, analysis of the external metabolome found no increase in alternative metabolites sufficient to account for the reduction in formate production (Figure 4.10). The analysis here was restricted to excreted metabolites so cannot rule out the intracellular accumulation of carbon or alterations to the rate of starch catabolism when PFL1 activity is blocked. Alternatively, if flux is redirected to the chloroplast PDH complex this could result in increased fatty acid synthesis which would not have been observed in our analysis. Therefore research could be extended to include analysis of the internal metabolome, along with analysis of both starch levels in the mutant and lipid accumulation to provide a clear understanding of the metabolic changes taking place.

Two additional possibilities for the failure to increase yields of H₂ include (1) the role of PFOR during sulphur-deprivation may be to act as a pyruvate synthase, as unlike the situation in the dark, reducing pressure in the chloroplast is high and cells activate processes to facilitate photosynthetic electron transport and continued photophosphorylation, or (2) any additional electrons fed into the photosynthetic electron transport chain by PFOR are used by competing processes such as cyclic electron flow. This can only be properly tested through the identification of a PFOR

knockdown or KO cell line and is the key experiment to determine the likelihood of success in terms of increasing H₂ yields during sulphur-deprivation.

4.5.3.2 Analysis of headspace CO₂ levels in pfl1-KD2 during sulphur-deprivation

Levels of CO₂ in the headspace gas can be used as an indirect indicator of changes in carbon flux as a result of alterations to total cellular decarboxylation rates (Dubini et al., 2009). During sulphur-deprivation experiments, degradation of Rubisco (Zhang et al., 2002) and a decline in transcripts of Calvin-Benson cycle enzymes (Nguyen et al., 2008) suggests a lack of active carbon fixation which can be assumed to have no impact on total CO₂ levels. Low rates of mitochondrial respiration will contribute a proportion to overall CO₂ evolution, but will also include the combined activities of PEPC, PDC3, PFOR, PYC and MME (Figure 4.20), either producing or consuming CO₂.

Inhibition of PFL1 activity either in the KD or by treatment with HP did not result in a significant increase in CO₂ levels, in contrast to previous experiments (Hemschemeier et al., 2008b), suggesting no increase in overall decarboxylation levels. This result could potentially be described by differences in WT strain used and indicates a different response to changes in fermentative metabolism during sulphur-deprivation.

Diagram is as in Figure 4.19 with the following exceptions; metabolites which increased are highlighted in green, and metabolites that decreased are highlighted in red.

Formate can also be produced from the decarboxylation of glyoxylate by hydrogen peroxide, and production increased in plants when glyoxylate levels rose through inhibition of glutamate synthesis, glycine metabolism or serine:glyoxylate amino transferase (Igamberdiev et al., 1999). However as photorespiration is dramatically down regulated during hydrogen production phase of sulphur-deprivation (Doebbe et al., 2010) this is an unlikely source of formate under these conditions.

Another possible route of production could come from the de-formylation of various metabolic intermediates. Reactions involving C_1 are important for the synthesis of proteins and nucleotides along with a variety of methylated molecules (Hanson and Roje, 2001). *C. reinhardtii* possesses homologues of the bacterial peptide deformylase (EC 3.5.1.88, Phytozome ID: Cre01.g039750, Cre01.g039650, Cre03.g172100), along with 10-formyl tetrahydrofolate deformylase (EC 3.5.1.10, Phytozome ID: Cre10.g430450), methionyl-tRNA formyltransferase. (EC 2.1.2.9, Phytozome ID: Cre12.g560550) and phosphoribosylglycinamide formyltransferase, which is involved in purine metabolism (EC 2.1.2.2, Phytozome ID: Cre12.g550700) Cre02.g128550). In particular de-formylation of 10-formyl tetrahydrofolate deformylase is proposed as a major route for formate production in the dark, in C_4 plants and in non-photosynthetic tissues (Hanson and Roje, 2001), and could conceivably occur in *C. reinhardtii* under conditions of stress. To test this hypothesis a close analysis of levels of one-carbon pool intermediates would be required.

PFL1 inhibition caused a decrease in ethanol accumulation (Figure 4.10D), in contrast to previous experiments where no change was observed (Hemschemeier et al., 2008), and the situation during dark-anaerobic incubation, where ethanol levels increased (Figure 4.8) as was seen for the *pfl1*-KO strain (Philipps et al., 2011). This therefore suggests the major route for ethanol production in this strain, is from acetyl-CoA generated by PFL1 activity in the mitochondrion (Figure 4.20), but unlike previous experiments with a different WT strain, blocking PFL1 did not result in a significant redirecting of metabolic flux down compensatory ethanol production pathways.

PFL1 was located to the mitochondria (Figure 3.25) (Atteia et al., 2009; Terashima et al., 2010), although some doubt exists about whether PFL1 is also co-localised to the chloroplast (Atteia et al., 2006; Terashima et al., 2010). To date, only ADH1 has been identified as protein that may catalyse ethanol production.

Immunoblot found ADH1 to be in the chloroplast (Figure 3.25) in agreement with proteomic analysis (Terashima et al., 2010), with some potential evidence for an additional cytoplasmic isoform although this cannot be ruled out as a contaminating cross reaction until a KD or KO strain is identified (Figure 3.25), but there was no evidence that ADH1 is found in the mitochondria (Atteia et al., 2009).

Therefore, a simple explanation for the observed results regarding ethanol production in PFL1 inhibited, and *pfl1*-KD2 cultures could be that some PFL1 is localised to the chloroplast, and it is this proportion of the enzyme that is responsible for the acetyl-CoA that leads to ethanol production via ADH1 (Appendix IV.11). On current evidence (Figure 3.25) this theory is not favoured, although changes to PFL1 localisation under different physiological conditions cannot be ruled out as this was not tested here.

An alternative explanation could be that there is an additional alcohol dehydrogenase (potentially ADH5 or ADH2, see section 3.2.1) located in the mitochondria (Figure 4.20) as suggested by enzymatic assay (Kreuzberg et al., 1987). However, no such enzyme has been identified in any proteomic analysis of *C. reinhardtii* mitochondria (Atteia et al., 2009; Terashima et al., 2010), albeit these experiments were not performed on samples from sulphur-deprived cultures, which means the potential induction of a novel ADH under these conditions cannot be ruled out.

A third option is provided by acetyl-CoA transport between the mitochondrion, cytoplasm and chloroplast which could be achieved either through a carnitine-acylcarnitine transport system (Appendix IV.12) (Daniell and Chase, 2004), or by citrate transport (Figure IV.10).

Carnitine can act as a carrier for acetyl-CoA molecules allowing transport between cellular compartments by a specific antiporter (van Roermund et al., 1999), with acetyl-CoA regenerated by the activity of carnitine acetyltransferase (EC 2.3.1.7; Eq. 22).



Isolated pea chloroplasts have been shown to take up acetyl carnitine (Masterson et al., 1990) and carnitine acetyltransferase (CAT) activity has been

detected in plastids (Masterson and Wood, 2000). A number of potential transporters are present in the *C. reinhardtii* genome (Cre20.g757600, Cre06.g295400, Cre04.g223300, Cre04.g222750, Cre01.g069350), some of which were enriched in the EST database during ‘stress II’ conditions, which includes samples extracted from cells under a mixture of conditions, including sulphur-deprivation. However, the precise enzyme displaying carnitine acetyltransferase (CAT) activity, the enzyme required to regenerate acetyl-CoA, in plants has not been identified, and studies in *Arabidopsis* question the existence of a significant CAT catalysed pathway when studying fatty acid respiration (Pracharoenwattana et al., 2005).

Therefore in summary, the pathway between acetyl-CoA generated by PFL1 activity and ethanol remains unclear and requires further investigation, but based on enzymatic assay and PFL1 localisation data at least one additional, uncharacterised ADH is proposed (Figure 4.20).

Ethanol levels were observed to decrease between 48-72 h as if consumed when PFL1 activity was down regulated, in six independent measurements (3 for *pfl1*-KD2 and 3 for CC-124 with HP). The difference with previous experimental data could potentially be explained as a result of strain variation (here CC-124 was used instead of CC-125), as different laboratory strains of the same apparent genotype have been shown to vary dramatically in their response to sulphur-deprivation (Chochois et al., 2010). Uptake of ethanol may be linked with the temporary generation of carbon storage compounds as discussed in section 4.5.3.5.

4.5.3.4 Acetate metabolism in *pfl1*-KD2 during sulphur-deprivation

Changes in the dynamics of acetate consumption and production indicate further changes to metabolic flux when PFL1 activity was blocked. In contrast to WT, acetate levels continued decreasing after the first 24 h of sulphur-deprivation, reaching a low at 72 h (Figure 4.10F). Acetate is believed to be used first for the production of fatty acids during the first 24 h of sulphur-deprivation (Timmins et al., 2009b), then consumed for the maintenance of respiration during sulphur-deprivation (Chochois et al., 2009). In light of this, the stabilisation of acetate levels after 24 h in CC-124 cultures must result from the balancing effect of acetate production by fermentation. The continued decrease in acetate levels when PFL1 is inhibited is therefore likely the result of reduced rates of acetate production in the mitochondrion (Figure 4.20). This suggests that the mitochondrial PAT1/ACK2 pathway is active in

CC-124 cultures (Figure 4.20) but the role of putative chloroplast localised PAT2 and ACK1 remains unclear, and may only become active when ethanol production catalysed by ADH1 is inhibited (Figure 4.20), although the isolation of additional knockdown lines, or treatment with ADH inhibitors such as 4-methylpyrazole will be required to test this theory.

4.5.3.5 Additional metabolite production in *pfl1*-KD2 cultures during sulphur-deprivation

Other compounds that might be predicted to increase in *pfl1*-KD cultures include succinate and malate, as observed in the *hydEF* mutant during dark-anaerobic fermentation (Dubini et al., 2009), or amino acids such as valine, leucine, isoleucine, tyrosine, phenylalanine, lysine, threonine and alanine (Timmins et al., 2009b).

In contrast to these expectations, alanine excretion decreased in *pfl1*-KD2 and HP treated cultures during the first 72 h of sulphur-deprivation (Figure 4.10H), which may provide an indirect indication of reduced carbon flux in the mitochondrion, or reduced breakdown of starch. This was followed by a late increase in alanine levels between 72–96 h, which coincided with increased rates of formate and ethanol production (Figure 4.10C and 4.10D), suggesting carbon flux through mitochondrial pathways increases during this time period.

Additional analysis also provided experimental evidence for a 3-HB production pathway in *pfl1*-KD2 and HP treated cultures (Figure 4.10E and Figure 4.20; for an extended metabolic pathway see Appendix IV.13). 3-HB accumulated exclusively around 72 h post sulphur-deprivation, before disappearing between 72–96 h. 3-HB is a component of ketone bodies usually formed as a carbon store, and is normally produced as the result of acetyl-CoA accumulation caused by inhibition of the TCA cycle, potentially suggesting the transition between microaerobic and anaerobic environment. At this time point two potential explanations why 3-HB accumulated in the absence of PFL1 activity include (1) when PFL1 is inhibited carbon flux increases down the PDC3 catalysed pathway, leading to increased acetyl-CoA or carbon cycle intermediates (Figure 4.20), or (2) as 3-HB production coincided with the uptake of ethanol, the alcohol could be converted into acetyl-CoA by the concerted activity of an ADH, ALDH and ACS, thereby providing a substrate for 3-HB production as a means of balancing NADH ratios between cellular compartments. (Appendix IV.13).

Evidence for a 3-HB production pathway in *C. reinhardtii* comes from both a proteomic study, which identified a 3-hydroxybutyrate dehydrogenase (BDH) in the mitochondrion (Atteia et al., 2009) and genome analysis, which finds genes encoding a putative acetyl-CoA C-acyltransferase, hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA lyase to form the complete pathway (Appendix IV.13). ESTs were identified for all enzymes except hydroxymethylglutaryl-CoA synthase, and therefore further research will be required to verify the details of the enzymes involved.

3-HB disappearance from the medium after 72 h could then be explained as a means of generating energy, degradation could generate ATP through acetate production in the absence of oxidative phosphorylation as evidenced by a late increase in acetate levels, and/or to oxidize NADH for continued glycolysis by producing ethanol.

3-HB is compound of biotechnological interest as it is a precursor for the production of poly-3-hydroxybutyrate (PHB) which can be used for the production of a wide range of products of commercial interest including biodegradable plastics. PHB is a compound belonging to the class of polyhydroxyalkonates which are widely synthesised by gram negative and gram positive bacteria, accumulating up to 90% dry cell weight under nutrient limitation, acting as a sink for carbon and reducing equivalents (Madison and Huisman, 1999). However current bacterial production processes are expensive due to substrate and energy cost and efforts have been directed at using plants for expression of this compound (Poirier et al., 1992; Purnell et al., 2007; Somleva et al., 2008). Various pathways exist for PHB production (Madison and Huisman, 1999), one such route was recently introduced into *C. reinhardtii* utilising intercellular acetoacetate as a substrate through expression of *phbB* and *phbC* genes, however mutants showed a reduction in cell growth rates which could have been the result of PHB accumulation (Chaogang et al., 2010).

4.5.4 Effect of *PDC3* knockdown on transient light-induced H₂ production

pd3-KD appeared to cause a significant reduction in the ability to evolve H₂ during transient light induced H₂ production experiments (Figure 4.12C), which cannot be explained by reduced hydrogenase induction (Figure 4.12A). Although protein levels indicate [FeFe]-hydrogenase expression is unaffected in *pd3*-KD lines

it would be beneficial to test the *in vitro* H₂ase activity to confirm reduced H₂ production rates were not related to the [FeFe]-hydrogenase. In the absence of an additional *pdh3*-KD strain, the possibility that reduced H₂ production is not the result of an additional mutation cannot be ruled out.

4.5.5 Effect of *PDC3* knockdown on dark-anaerobic metabolite production

PDC3-knockdown appeared to have little effect on eventual dark-anaerobic metabolite production (Figure 4.13). This could be because: (1) Flux via *PDC3* is only a minor pathway during dark-anoxia or remains inactive under standard conditions, as any re-directing of metabolism might be expected to increase either formate or acetate, (2) in *pdh3*-KD carbon flux was redirected solely to PFOR, with the acetyl-CoA produced converted to ethanol (Figure 4.20), or (3) the effect of the knockdown was insufficient to cause observable changes. Due to the lack of detectable *PDC3* scenarios (1) or (2) are more likely, but will require measurement of changes to dark H₂ production and analysis of the internal metabolome to discern what changes to metabolism occur.

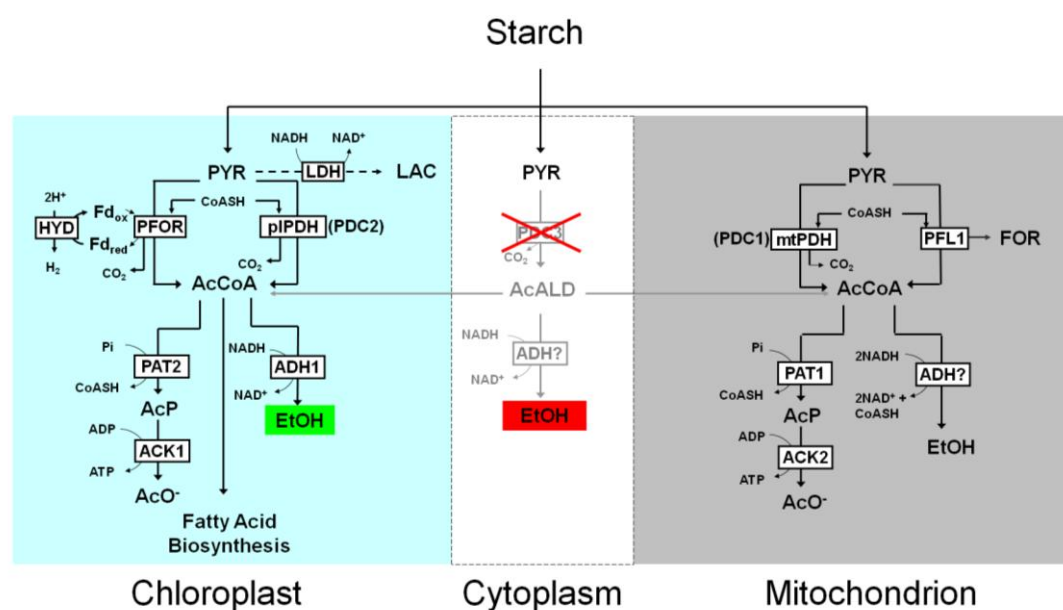


Figure 4.21: Model of potential changes to dark-anaerobic fermentative metabolism in *pdh3*-KD.

Diagram as in Figure 4.18 with the following exceptions; *PDC3* catalysed pathway is downregulated (as indicated by red cross), highlighted are metabolites that are proposed to be reduced (red) or increased (green) in *pdh3*-KD cultures. Changes to H₂ production are not displayed as I was unable to measure rates.

4.5.6 Effect of *PDC3* knockdown on fermentative metabolism during sulphur-deprivation

4.5.6.1. Effect of *PDC3* knockdown on H_2 production during sulphur-deprivation

As with transient light-induced H_2 production, *PDC3* knockdown caused a decrease in overall yields (Figure 4.14A). No dramatic changes in hydrogenase expression (Figure 4.13) or starch metabolism (Figure 4.16B) could be invoked to explain this finding. Unfortunately as no additional knockdown has yet been isolated it is yet unclear whether the reduction in H_2 production ability was the result of *pd3*-KD or additional mutations caused by multiple integrations of the miRNA construct into the host genome. The nature and number of construct insertions in *pd3*-KD could be determined to help clarify the phenotype by using southern blot and genome walker kits, if multiple insertions are found *pd3*-KD could be back crossed with a *mt+* strain to clean up the genotype.

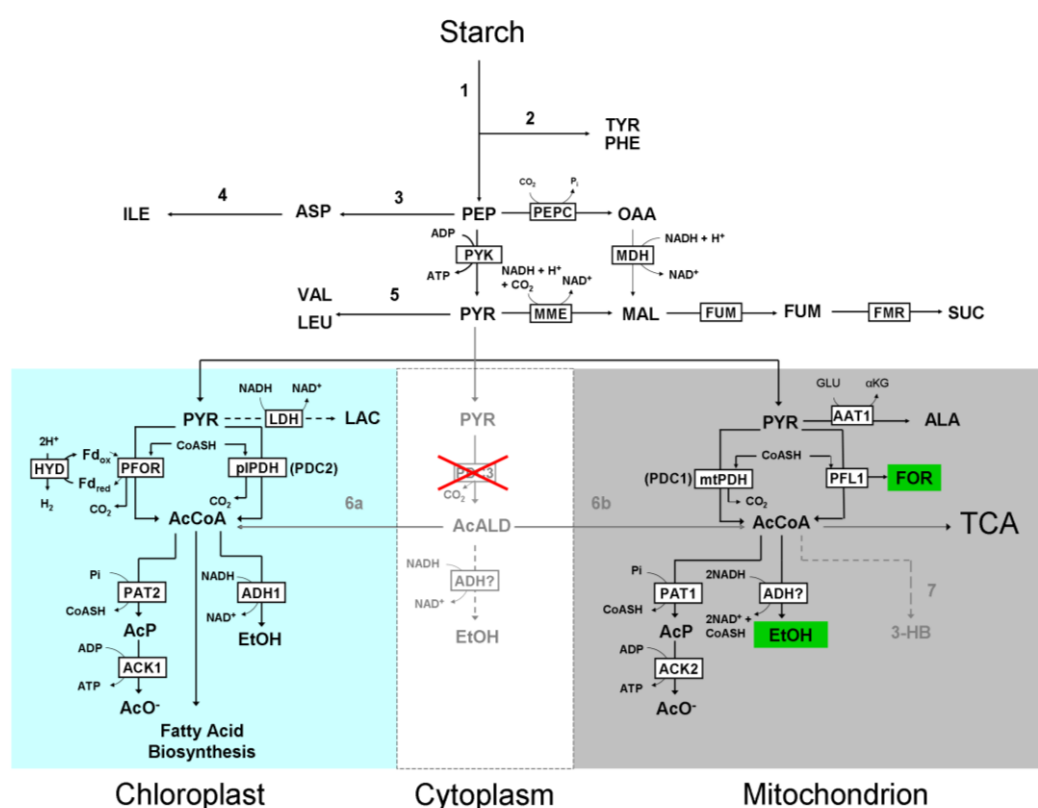


Figure 4.22: Proposed model of pyruvate degradation, amino acid synthesis and fermentative metabolism during sulphur-deprivation in *pd3*-KD.

The diagram is as in Figure 4.19 with the following exceptions; metabolites which increased are highlighted in green, and metabolites that decreased are highlighted in red.

4.5.6.2 Effect of *PDC3* knockdown on formate metabolism during sulphur-deprivation

Although inferred from genomic (Grossman et al., 2007) and transcriptomic analysis (Mus et al., 2007; Nguyen et al., 2008) there was no previous, direct evidence for an active *PDC3* catalysed pathway in *C. reinhardtii*. Knockdown of *PDC3* resulted in increased formate production during sulphur-deprivation, which peaked around 48 h before coming to a stop (Figure 4.10C) (Figure 4.20). This indicates that under these conditions in contrast to dark-anoxia, *PDC3* plays an important role in fermentative metabolism and its inhibition causes the redirecting of metabolic flux to *PFL1* (Figure 4.20).

The almost trebling of initial formate production rates suggests the cytoplasmic *PDC3* catalysed pathway of pyruvate degradation predominates over *PFL1* route in WT cultures by a ratio of ~2:1. The decline in rates of metabolite accumulation and H_2 production between 48–72 h could be the result of accumulation of toxic metabolites including formate and ethanol, and it would be beneficial to perform a cell viability count comparing *pd3-KD* and CC-124 cultures at 24 h intervals post sulphur-deprivation to ascertain if cell survival was effected. However, sustained starch degradation until 96 h sulphur-deprivation would point to continued cell survival (Figure 4.16B).

4.5.6.3 Effect of *PDC3* knockdown on ethanol and acetate metabolism during sulphur-deprivation

Knockdown of *PFL1* suggested that the majority of ethanol in this strain was produced from acetyl-CoA generated by *PFL1* activity, suggesting flux via *PDC3* represented only a minor route of pyruvate degradation if the acetaldehyde produced is converted into ethanol as previously proposed (Grossman et al., 2007; Mus et al., 2007; Dubini et al., 2009; Philipps et al., 2011). Unexpectedly ethanol production almost doubled in *pd3-KD* cultures, showing close correlation with increased formate accumulation (Figure 4.14D), with WT and *pd3-KD* lines maintaining similar formate to ethanol ratios (in WT formate ethanol ratios changed from 1.1 to 1.8 between 24–96 h sulphur-deprivation, and in *pd3-KD* cultures this was 1.2–2.0). This therefore suggests the majority of the additional ethanol is therefore likely to come from increased flux via *PFL1* (Figure 4.21).

Consequently *pd3*-KD either caused dramatic changes in cellular metabolism beyond its own metabolic pathway, re-directing flux from other pathways via PFL1, or not all acetaldehyde produced by PDC3 activity is converted into ethanol.

The fate of carbon directed down PDC3 catalysed pathway therefore remains unclear. One possibility is that in WT cultures acetaldehyde is converted to acetate by an aldehyde dehydrogenase, acetaldehyde is considered to be ‘freely diffusible’ across biological membranes (Stanley and Pamment, 1993) and could therefore be broken down to acetate or ethanol anywhere within the cell. There are three acetyl-CoA synthase genes (ACS) in the *C. reinhardtii* genome, but the localisation of their predicted gene products within the cell is unknown (Spalding et al., 2009). This means acetate could potentially be converted into acetyl-CoA in up to three cellular compartments (Figure 4.21), where it could enter a variety of metabolic pathways.

Another possibility is some acetaldehyde generated by PDC3 activity could be converted into fatty acids via acetyl-CoA in the chloroplast, as has been shown to occur in *Arabidopsis* during ethanol detoxification (Lin and Oliver, 2008). To test for this possibility the lipid content of *pd3*-KD and CC-124 cultures during sulphur-deprivation should be compared.

Alternatively, if acetaldehyde is broken down in the cytoplasm, citrate formation in the peroxisome could provide a potential route for disposal of any acetyl-CoA produced (Appendix IV.12). Acetyl-CoA is imported into the peroxisome by an ABC transporter which in *Arabidopsis* is encoded by the *COMATOSE* gene, of which *C. reinhardtii* possess a homologue (Cre15.g637761), then oxaloacetate and acetyl-CoA can be converted into citrate by an ATP-citrate lyase (EC.2.3.3.8; Eq. 23), thought to be located in the *C. reinhardtii* peroxisome (Terashima et al., 2010)



Citrate can then be transported by a tricarboxylic acid transporter such as those identified in *Arabidopsis* (Picault et al., 2002) into the mitochondria. There is some evidence for citrate uptake into isolate pea chloroplasts (Masterson et al., 1990), but the existence of only single isoforms of citrate synthase and ATP-citrate lyase in *C. reinhardtii* suggests citrate metabolism is divided between the mitochondria and peroxisome (Terashima et al., 2010). Citrate could then be utilised in the TCA cycle,

although if this were to occur additional changes in acetate metabolism would be expected. No significant changes in the dynamics of acetate metabolism could be discerned in *pd3-KD* (Figure 4.14E), suggesting acetaldehyde is not converted to acetate as a final product under conditions of sulphur-deprivation.

4.5.7 Effect of PFL1 inhibition on dark-anaerobic fermentative metabolism in *pd3-KD*

Whereas knockdown of *PDC3* appeared to have no impact upon dark-anaerobic fermentation product formation, potentially suggesting the PDC3 catalysed pathway has very low activity under normal conditions, blocking both PFL1 and PDC3 catalysed pathways appeared to cause an increase in ethanol excretion compared to WT, and WT cultures treated with HP. This could not be verified to be statistically significant due to the low number of repeats but hints that the PDC3 pathway provides an alternative route for carbon degradation in the absence of PFL1 activity (Figure 4.23).

This would suggest that inhibiting both pathways may increase dark H_2 production by re-directing flux to PFOR, with the resulting acetyl-CoA broken down to ethanol by ADH1 in the chloroplast (Figure 4.23), but will require further analysis before this can be determined to be the case. Additionally, as ethanol increased as a result of *PDC3*-knockdown, it may suggest that some of the acetaldehyde produced by PDC3 activity is converted into products other than ethanol.

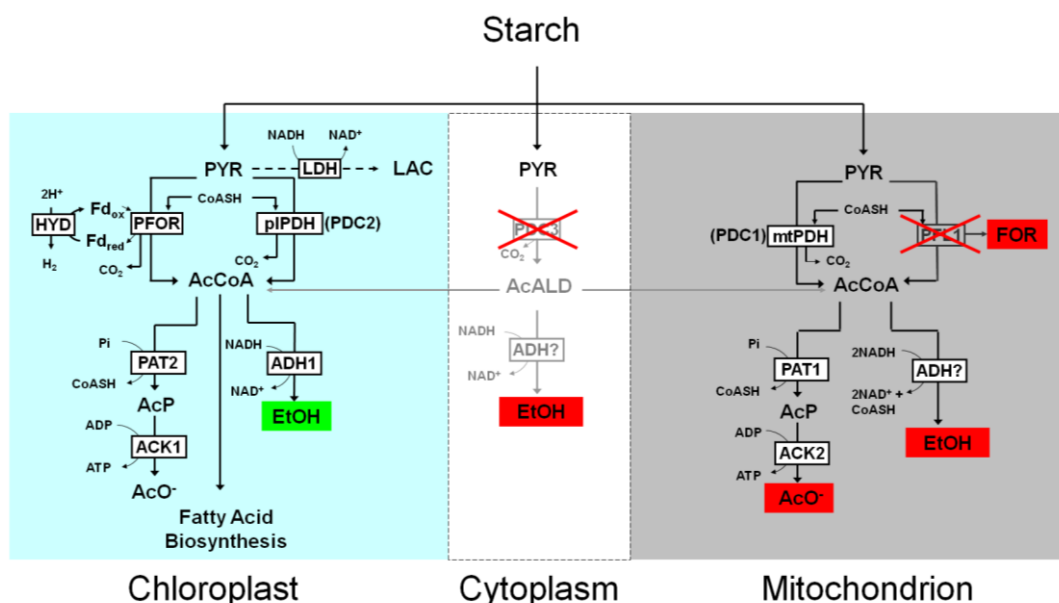


Figure 4.23: Proposed alterations to dark-anaerobic fermentative metabolism when *pdc3*-KD cultures are treated with HP.

Diagram is as in Figure 4.18 with the following exceptions; PDC3 and PFL1 pathways are down regulated (as indicated by red cross), metabolites are highlighted that decreased (red) or increased (green). Alterations to H_2 production could not be determined.

4.5.8 Effect of PFL1 inhibition on *pdc3*-KD fermentative metabolism during sulphur-deprivation

Knockdown of *PDC3* appeared to cause a straight re-directing of metabolic flux to PFL1, observing only significant increases in formate, and ethanol derived from acetyl-CoA generated by PFL1 activity, with no corresponding decrease in any fermentation product, suggesting *PDC3* activity may result in the accumulation of intracellular carbon such as TAGs. Knockdown of *PFL1* caused a decrease in formate production which was unaccompanied by any significant increase in alternate metabolites, which would hint at redirecting flux to intracellular carbon. Inhibition of PFL1 or *PDC3* alone failed to cause an increase in H_2 production, therefore it was possible that inhibiting both pathways could result in increased yields.

Inhibition of PFL1 when *PDC3* was knocked down appeared to cause an increase in overall H_2 yields relative to when *PDC3* was knocked down alone, with totals similar to WT cultures (Figure 4.14A).

4.5.8.1 Effect of inhibition of PFL1 on ethanol metabolism in *pd3-KD* cultures during sulphur- deprivation

When both PDC3 and PFL1 catalysed pathways were blocked, levels of ethanol production recovered to those seen in CC-124 relative to *pfl1-KD2* alone (Figure 4.11C). This therefore suggests flux is being re-directed to form acetyl-CoA in the chloroplast (Figure 4.24), either by PFOR or PDH activity, which can then be converted into ethanol (Figure 4.24). As PDH complexes are thought to be inactive during anoxia (see section 3.6.8) these data favour the hypothesis flux is being re-directed to PFOR (Figure 4.24), although further experimental evidence is required.

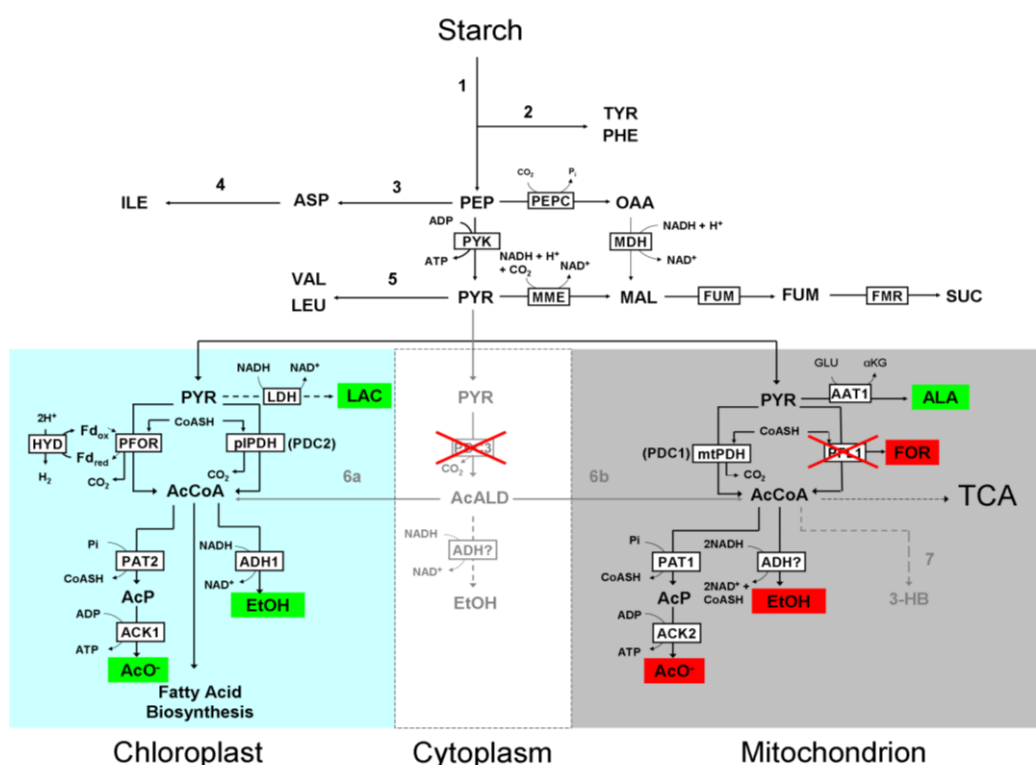


Figure 4.24: Proposed model of pyruvate degradation, amino acid synthesis and fermentative metabolism during sulphur-deprivation in *pd3-KD* plus 10 mM HP.

Diagram is as in Figure 4.19 with the following exceptions; metabolites which increased are highlighted in green, and metabolites that decreased are highlighted in red. The fate of amino acids is unclear due to levels of cellular debris contamination.

4.5.8.2 Effect of inhibition of PFL1 on acetate metabolism in *pd3-KD* cultures during sulphur-deprivation

When both PFL1 and PDC3 activity were down regulated, acetate levels recovered to WT values compared to the phenotype observed when PFL1 was inhibited alone (Figure 4.14F). Only two fermentative pathways for the production of acetate are known, the first is located in the mitochondria and works on acetyl-CoA generated by PFL1 activity. Reducing flux via PFL1 reduced acetate production. Reducing flux via PFL1 and PDC3 had no overall impact on acetate levels, these data could therefore mean increased acetyl-CoA production in the chloroplast from increased PFOR activity, with the resulting acetyl-CoA entering the PAT2/ACK1 pathway (Figure 4.22). An increase in flux to PFOR is further supported by the increase in H₂ evolution seen in *pd3-KD* + HP experiments compared to *pd3-KD* alone.

4.5.8.3 Effect of inhibition of PFL1 on lactate and amino acid metabolism in *pd3-KD* cultures during sulphur-deprivation

Lactate production increased markedly when both PFL1 and PDC3 catalysed pathways are inhibited compared to CC-124 or *pfl1-KD2* cultures (Figure 4.14F). This suggests a re-directing of carbon metabolism to the chloroplast (Figure 4.24) and makes LDH a good target for future efforts at increasing yields. When both PFL1 and PDC3 catalysed pathways were inhibited an overall increase in alanine levels were observed after 96 h (Figure 4.14G), representing a pathway for disposal of excess pyruvate. There were suggestions that additional amino acids increased, however no firm conclusions could be drawn due to levels of contamination due to cell lysis.

4.5.8.4 Effect of joint inhibition of PFL1 and PDC3 knockdown on CO₂ evolution during sulphur-deprivation

When both PDC3 and PFL1 were inhibited a dramatic decrease in overall CO₂ emission levels was observed compared to CC-124 cultures (Figure 4.15B). This could be a combined result of reduced decarboxylation caused by knockdown of PDC3 and redirecting carbon flux to PEPC, PYC or MME thereby increasing the CO₂ consuming pathways (Figure 4.22). These pathways would produce oxaloacetate and malate, which can be converted into aspartate or succinate (Timmins et al., 2009b) (Figure 4.22), the latter of which was described to occur under dark-anaerobic

conditions for the *hydEF* strain deficient in hydrogenase assembly (Dubini et al., 2009). The external metabolome was checked for presence of succinate or malate but no significant increase was observed (data not shown), this does not however rule out intracellular accumulation, due to contamination of samples with intracellular debris it was difficult to assess if aspartate derived amino acids increased. If metabolism had been purely re-directed towards PFOR, CO₂ levels would be expected to increase, as a result of increased decarboxylation of pyruvate that would otherwise be converted to formate (Figure 4.22). Therefore these data indicate that further gains in H₂ yield may be possible, but will require analysis of the intracellular metabolome to determine which additional metabolic pathways compete with H₂ production.

4.6 Summary

Artificial microRNA technology was demonstrated to be an effective means for rapidly analysing the impact of switching off fermentative pathways in *C. reinhardtii*, isolating knockdown mutants affected in *PFL1* and *PDC3* transcription with 80–90% reduction in protein levels.

Knockdown or inhibition of *PFL1*, *PDC3* or both together, failed to increase H₂ yields during sulphur-deprivation. *pd3-KD* cultures displayed a consistent decrease in transient light induced and sulphur induced H₂ production, which appeared independent of [FeFe]-hydrogenase level, starch accumulation/degradation, or oxygen evolution ability, but as only one mutant was analysed it is as yet unclear if this was a result of an additional, unspecific mutation.

Changes in metabolite production indicate in the strain tested here, the majority of ethanol produced is derived from the acetyl-CoA resulting from *PFL1* activity. When combined with immunoblot analysis of enzyme localisation, it is unclear precisely which mechanism results in alcohol formation, either *PFL1* is co-localised to the chloroplast and mitochondria, an additional as yet uncharacterised alcohol dehydrogenase is active in the mitochondria, or acetyl-CoA is exported from the mitochondria by an uncharacterised mechanism.

Metabolite analysis also revealed either wide ranging changes to metabolism during sulphur-deprivation when *PDC3* is inhibited, or that not all acetaldehyde produced by *PDC3* activity results in ethanol formation, as ethanol levels increased.

A significant decrease in CO₂ levels when both PDC3 and PFL1 activity was down regulated suggests an increase in reactions utilising CO₂, such as that catalysed by MME. Therefore in addition to increases in lactate and alanine production, this would point to increased production of malate but will require further investigation.

Chapter 5: Conclusions and Future work

Hydrogen represents an attractive replacement for traditional fossil fuels, with its' combustion resulting only in the release of water vapour. Although technical issues remain, hydrogen's potential to be stored means processes that produce the gas have an advantage over conventional renewable technologies that have tended to focus on electricity generation. Algae have the ability to utilise sunlight, CO₂ and water to produce a range of fuels including H₂. However, for algal biofuel production to become profitable, economic and life cycle analyses have indicated large increases in yields are required (for H₂ a ~5 fold increase upon the current maximum recorded rates produced by *stm6*), in addition to coupling fuel production to a range of processes such as waste water treatment and the production of high value products. To be able to effectively manipulate metabolism towards a chosen molecule of interest it is vital to have an accurate metabolic model in order to make predictions as to the eventual outcome of a particular mutation. Therefore, focusing on H₂ production, the aims of this study were to hone current models of *C. reinhardtii* metabolism through a combination of bioinformatic and immunochemical analysis, and to test amiRNA technology as a means of redirecting metabolic flux.

It had previously been suggested that down regulation of fermentative pathways may be a means of increasing the yields of H₂ obtained from *C. reinhardtii*. It was therefore decided to investigate this possibility through a detailed analysis of fermentative enzymes in the *C. reinhardtii* genome, immunochemical analysis of fermentative enzyme expression and knockdown of key fermentative enzymes, analysing the effect on hydrogen production and excreted metabolite production.

Analysis of the genome found evidence for pathways that could potentially produce and recapture acetate, lactate and ethanol in response to environmental stimuli (Section 3.2). Of particular note was the presence of two distinct pyruvate dehydrogenase complexes which clustered with higher plant chloroplast and mitochondrial PDHs respectively, which to our knowledge had not previously been reported in this alga. It is unclear to what degree PDH activity is maintained under anoxia, and dark-anoxic metabolite formation would suggest that the PDH complexes are inactive under these conditions. However TAG accumulation and continued respiration during sulphur-deprivation make it likely that under these conditions, PDH complexes retain some activity and should be incorporated into metabolic models.

Due to the vast number of enzymes potentially involved in anaerobic metabolism (Table 3.1) it was decided to focus on those pathways that could directly compete for pyruvate and NADH and are thought to predominate during anoxia, including formate, ethanol, acetate and lactate production. Antibodies were raised against fragments of ADH1, HYDA1, LDH, PDC1, PDC2, PDC3, PFL1 and PFOR providing tools for further biochemical and genetic analysis (see section 3.4). Antibodies were used to analyse target enzyme localisation (Figure 3.25), enzyme levels under a range of physiological conditions (Figures 3.20–3.22), and to explore potential methods of regulation including post-translational modification (Figure 3.23). The absence of available knockdown or KO mutants for LDH, ADH, PDC1 and PDC2 to add further credence to the veracity of the observable cross reactions is a limitation of the work here.

Furthermore, enzyme activity has largely been inferred by sequence homology to genes from other species. To verify the catalytic activity of the proteins analysed here a number of experiments could be performed, including partial purification of by ion exchange, size exclusion or affinity chromatography, or expression and purification of full length *C. reinhardtii* fermentative enzyme protein sequences in *E. coli* followed by enzymatic analysis.

The work here identifying enzyme localisation expands upon previous proteomic studies on purified organelles to include analysis of the cytoplasmic fraction, and included the putative LDH (Figure 3.25). PFL1 was located to the mitochondrion, PFOR, LDH, HYDA (potentially including HYDA1 and HYDA2) were located in the chloroplast, PDC3 was found in the cytoplasm whereas ADH1 was potentially found in the chloroplast and cytoplasm. From these data it can be discerned that there is no currently known route for ethanol production in the mitochondrion, however previous biochemical assays suggest the presence of an additional ADH in this compartment, and bioinformatic analysis revealed at least one additional alcohol dehydrogenase (*ADH5*) is present in the EST database, making a good candidate for further investigation.

Localisation studies also provided supporting evidence for the PDC1 (putative mtPDH E1 α component) and PDC2 (putative plPDH E1 α component) assignment, with further indication that these components belong to membrane complexes, like the PDH, coming from association with both the membrane fraction and soluble component (Figure 3.26), the latter of which may represent either complexes

dissociated from the membrane or assembly complexes consisting of E1 heterotetramers (Figure 3.27). BN-PAGE analysis of both soluble (Figure 3.28) and membrane (Figure 3.27) fractions was inconclusive, although small amounts of signal could be detected in very large complexes in the membrane sample, this was suggested to be caused by breakdown of complexes during BN-PAGE analysis, and could potentially be improved by use of BN-AGE instead. PDH activity could be detected in both membrane and soluble components (data not shown), but final verification will likely require partial purification of PDH complexes by size exclusion chromatography, followed by enzymatic assay, to match signals obtained by immunochemical analysis to enzymatic activity.

All fermentative enzymes analysed were found to be constitutively present during photoautotrophic growth (Figure 3.22), with the exception of PFOR which could not be determined to the large number of additional cross reactions. Even the highly O₂-sensitive [FeFe]-hydrogenase was present at low levels, although hydrogenase activity could not be confirmed and may represent an *apo*- form of the enzyme prior to insertion of the H-cluster.

Isolated *C. reinhardtii* mitochondria have been shown to take up D-lactate but not L-lactate, and correspondingly analysis of the genome and EST database suggests lactate metabolism in *C. reinhardtii* will likely involve the action of an NAD⁺-dependent D-lactate dehydrogenase located in the chloroplast, that is thought to work predominately in the direction of lactate production, and a mitochondrial localised FAD-dependent D-lactate dehydrogenase, which is thought to work in the direction of pyruvate synthesis. This system may allow for *C. reinhardtii* to produce lactate as a means of disposing of excess reducing equivalents under anoxic conditions then recapturing the carbon upon aeration.

To investigate potential modes of regulation of pyruvate metabolism, immunoblot analysis of *C. reinhardtii* samples from a range of environmental conditions was performed. In contrast to previous transcriptomic analysis little difference in protein levels could be discerned between aerobic and anaerobic conditions (Figure 3.21, 3.22B), with the exception of HYDA and PFOR (Figure 3.23). HYDA and PFOR levels increased dramatically during anoxic conditions and showed cyclic expression when subject to dark/light cycles, accumulating in the dark period (Figure 3.24) indicating the potential importance of these enzymes during metabolism at night.

Signs of post-translational modification were looked for by immunoblot. LDH was found to undergo a shift in size to a larger molecular mass (Figure 3.22) and PFL1 is known to be activated by post-translational cleavage during anaerobic conditions, but was not analysed here. Precisely how LDH is modified requires further investigation, but could potentially be a means of altering catalytic activity. Levels of PDC2 decreased during sulphur-deprivation (Figure 3.20 and Appendix IV.5) and photoautotrophic growth (Figure 3.22), providing an indication of alterations to protein turnover that could either be the result of changes in transcription/translation or protein degradation, but requires further analysis. mtPDH complexes are known to be controlled by phosphorylation of the E1 α subunit, and the *C. reinhardtii* genome contains putative pyruvate dehydrogenase kinase sequences suggesting this may also be the case in *C. reinhardtii* but will also require further analysis.

Additionally, research is required into the carbon transporters, signalling pathways, transcription factors and response elements involved in regulation of fermentation to gain a more complete understanding of how *C. reinhardtii* alters metabolism in response to environmental changes. It is clear that the metabolic situation in response to nutrient limitation is much more complex than anoxia alone, and can vary depending whether encountered in the light or the dark, presumably as a result of the different redox environment within organelles. This could be particularly important when considering the role of the pyruvate:ferredoxin oxidoreductase - whether it works as a pyruvate reductase or a pyruvate synthase, as the reaction has been shown to be reversible in bacterial systems. This could have clear implications for attempts to improve H₂ production through altering fermentative metabolism and it is vital that the role played by PFOR is determined before further attempts are made, which could best be done through isolation and physiological characterisation of PFOR KO or knockdown cell lines.

In summary these data may suggest the poising of metabolism in *C. reinhardtii* ready for a rapid response to changes in the environment, indicating induction of transcription plays only a minor role in regulating metabolism with the exception of HYDA and PFOR. These results contrast with previous transcript analysis by Mus et al. (2007) which may be explained partly by the fact that in that publication cells were also subject to additional nutrient stresses (-S, -N, -trace metals) and cannot be regarded as a purely anoxic response. Taken together the data

here suggests that control of formate, and potentially lactate production is largely controlled post-translationally, HYDA and PFOR activity is affected by O₂ levels and protein expression, controlled at a transcriptional level, whilst the switch between PDC3, ethanolic fermentation and pyruvate dehydrogenase activity could occur by two means; the first is as a combination of changes in *PDC3* transcription and alterations to the biochemical environment in the cell, with changes in pH and substrate concentrations potential contributing factors. The second possibility is provided by regulation of PDH complex activity by phosphorylation in the case of mtPDH, and control of protein turnover in the case of plPDH.

With only a partial understanding of regulation, or the number of metabolic pathways interacting with H₂ production during sulphur-deprivation, it makes predicting the outcome of switching off fermentative pathways difficult. amiRNA technology has been used to rapidly isolate knockdown mutants in *C. reinhardtii*, and could be a means of refining metabolic models without the requirement for the labour intensive isolation of KO mutants. To see if amiRNA was a suitable technique for redirecting metabolic flux, a thorough test of the system was performed targeting *PFL1*, whose gene product is involved in formate production and is believed to be the predominant fermentative pathway during sulphur-deprivation, and *PDC3* which has been proposed to be part of an ethanol production pathway but with no previous direct experimental evidence.

In parallel work a *pfl1*-KO strain was isolated by Philipps et al. (2011) in a forward genetic screen looking at mutants impaired in light driven H₂ production, and was analysed for changes in dark-anaerobic metabolism (Philipps et al., 2011). This provided a useful comparison for analysis with *pfl1*-KD strains, and the work here extended analysis to look at changes in excreted metabolite production during sulphur-deprivation. The data here also builds on a previous study which assessed the effect of a PFL1 inhibitor on H₂ production during sulphur-deprivation looking at changes in H₂, formate and ethanol, to include analysis of changes to the entire excreted metabolome by NMR (Figure 4.10 and 4.11).

From screening 80 transformants, nine mutants with greater than 80% reduction in PFL1 protein levels were identified (Figure 4.5). Two mutants, *pfl1-KD1* and *pfl1-KD2* were further characterised and found to have a similar reduction in transcript levels (Figure 4.6B). *PFL1*-knockdown strains showed good correlation with the reported phenotypic alterations in the *pfl1*-KO line, displaying reduced

formate and acetate accumulation in the dark (Figure 4.8) and a reduction in transient light induced H_2 production (Figure 4.6C), which was assigned to reduced hydrogenase accumulation. Minor increases in ethanol and lactate were observed (Figure 4.8) - two changes also seen in the KO strain, but the effect was milder due to the presence of residual PFL1 in KD cell lines. This data provided evidence that amiRNA technology could be used for observation of changes in metabolic flux offering comparable results to KO mutants, albeit displaying milder phenotypes.

amiRNA has a number of benefits over conventional reverse genetic techniques in *C. reinhardtii*, compared to PCR screening of random mutagenesis libraries for KO mutants it is a much less labour intensive approach, requiring screening of 40–80 colonies as compared to 50,000 which also makes amiRNA more affordable. Potentially mutants can be obtained and verified in a shorter time span of around 1 month to six weeks from transformation as compared to 3–4 months for KOs. As compared to conventional inverted repeat RNAi constructs, amiRNAs have a much higher degree of specificity and will not result in down regulation of off targets caused by siRNAs generated long dsRNA molecules. amiRNA knockdowns of *PFL1* and *PDC3* remained stable throughout all growth phases (Figure 4.7) and over 18 months (Figure 4.6A and Appendix IV.2).

However, there were a number of disadvantages, KOs are preferred for metabolic engineering as a complete lack of enzyme expression will result in more severe phenotypes than KD cell lines. RNAi can be a useful tool for assessing the likely outcome of gene KO mutations, however if the flux control coefficient of a target enzyme is low, meaning the enzyme has little control over flux through a particular pathway, knockdown techniques are not particularly effective as a means of redirecting metabolism. However, this did not appear to be a significant problem in this study, KD mutants with a greater than 80% reduction in protein levels caused dramatic reductions in accumulation of metabolites, sufficient to provide an indication as to where metabolic flux is redirected. Another drawback is that not all amiRNAs designed provided effective knockdown, and to achieve maximum knockdown multiple oligonucleotide sequences need to be tested. This is still a nascent technology and little is known about the precise factors contributing to amiRNA efficacy, further investigation will be required as to factors such as mRNA secondary structure before this aspect can be improved.

Analysis of changes in the excreted metabolite profile of *pfl1*-KD2, which was closely correlated to results with WT cultures treated with PFL1 inhibitor HP, revealed a reduction in formate and ethanol production, and altered dynamics in acetate metabolism (Figure 4.10). These data suggested that in this WT (CC-124) strain the majority of ethanol is produced from acetyl-CoA generated by PFL1, and in contrast to previous findings using a different laboratory strain (CC-125), PFL1 inhibition did not result in a significant re-directing of metabolic flux down an alternative ethanol production pathway. Although the two WT strains are closely related, large differences in starch metabolism and the H₂ production ability of apparently the same WT strain (CC-124) from different laboratories has been reported, indicating care must be taken in extrapolating results obtained with a particular laboratory strain to all *C. reinhardtii* WT strains.

A decrease in amino acid excretion when PFL1 was blocked or knocked down suggests wider changes to metabolism than simply a reduction in formate production (Figure 4.16). However, no additional metabolites were excreted at a sufficient quantity to compensate for the lower overall amounts of fermentative products accumulated, the reasons for this remain unclear and could point to either reduced starch degradation or accumulation of intracellular carbon, potentially in the form of lipids, which will require further analysis. Inhibition of PFL1 did however reveal the presence of a metabolic pathway only previously inferred from genomic and proteomic analysis in this alga, with the time dependent production of 3-HB (Figure 4.10 and Appendix IV.13).

The dramatic reduction in ethanol production caused by inhibition of PFL1, suggested the possibility that if PDC3 was active, and involved in ethanol production, flux by this route represented only a minor fermentation pathway. To test this theory knockdown constructs were created to target *PDC3*, and a single KD line was isolated with over 90% decrease in protein and mRNA levels (Figure 4.12). Due to the absence of a second KD line the data sets obtained from *pd3*-KD must therefore be regarded with caution, but provide an indication to the effect of *PDC3*-knockdown on fermentative metabolism.

No significant change was observed in dark-anaerobic metabolite production (Figure 4.13) in *pd3*-KD cultures, suggesting this pathway is perhaps inactive under these conditions. However, *PDC3*-knockdown did cause a significant reduction in the transient light-induced H₂ production ability of *C. reinhardtii* cells (Figure 4.12C) that

did not appear to be linked to changes in hydrogenase expression (Figure 4.12A). The reason for this reduction remains unclear, and could potentially be the result of additional mutations resulting from random integration of the amiRNA construct into the host genome, or alterations to cellular redox balances, but it would be useful to perform in vitro hydrogenase assays to rule out an effect on the hydrogenase.

Analysis of excreted metabolite production by *pdc3*-KD cultures provided the first direct evidence for a PDC3 catalysed pathway, and surprisingly knockdown resulted in substantial increases in ethanol and formate production (Figure 4.15). These changes suggest a re-directing of metabolic flux via PFL1, but the increase in overall ethanol excretion can only be explained either as a result of wide ranging changes in cellular metabolism, or if the PDC3-catalysed pathway results in the production of additional products in alternative to ethanol, such as lipids. H₂ production during sulphur-deprivation was also reduced relative to WT, the reason for this was unclear but was not related to changes in starch metabolism, pH or hydrogenase expression. Reduced H₂ production could again be the result of an additional mutation from random insertion of the amiRNA construct, or potentially could have been result of accumulation of toxic metabolites in the culture vessel, such as formate and ethanol but requires further investigation.

When both PFL1 and PDC3 pathways were blocked, H₂ production increased relative to *pdc3*-KD cultures (Figure 4.15A), providing an indication of the redirecting of metabolic flux to PFOR, but remained at yields similar to WT.

Whilst NMR analysis of the supernatant provided a good overview of changes in the levels of key metabolites, it is clear from the result a more detailed analysis of changes in metabolic flux will be required to obtain a clear picture as to the precise alterations resulting from knockdown of fermentative pathways. A number of potential improvements could be made, firstly GC/MS could be used for a more sensitive analysis of metabolic changes, looking at the intracellular metabolome in addition to the excreted profile. This data would have to be coupled to a more complete metabolic model, and could be aided by transcriptomic or RNASeq analysis to search for additional targets to down regulate. Additionally it would be of interest to analyse the changes in lipid content, plus analysis of changes to starch metabolism in all knockdown cell lines would be beneficial to rule out changes in glycolysis.

Fermentation is likely to play an important role in cell metabolism during the night, as well during anoxic stress as evidenced by an increase in HYDA and PFOR

expression during the dark period of day night cycles. Although single knockdowns of *PFL1* and *PDC3* appeared to have no effect on cell fitness when grown in the dark it remains to be seen how cell viability would be affected in *C. reinhardtii* cells lacking a fermentative metabolism, which could potentially pose problems for any future biotechnological processes requiring growth outside during day night cycles.

In conclusion, artificial microRNA can be a useful technique for analysing the impact of redirecting metabolic flux through targeted gene knockdown. However the technique suffers from some disadvantages compared to other available tools for reverse genetic analysis in *C. reinhardtii* and there is potential for further improvement. *C. reinhardtii* possesses a complex metabolism that aids survival by rapid response to changes in environmental O₂ and nutrient levels. It would appear current models are incomplete and questions remain regarding the identity of an additional alcohol dehydrogenase and to the fate of acetaldehyde produced by PDC3 activity. Furthermore regulation of metabolism does not appear to be entirely transcriptional and the constitutive presence of many of the fermentative enzymes here, leaves some questions as to how metabolic flux is controlled. The role of PFOR under varying environmental conditions is still not completely characterised and may have implications for efforts at increasing H₂ yields by re-directing metabolic flux. Single knockdowns of *PFL1* and *PDC3* did not improve H₂ production during sulphur-deprivation and multiple knockdowns will likely be required before the efficacy of this approach can be decided. Promising future targets include chloroplast localised LDH which could compete for pyruvate, and ADH1 which would compete for NADH. However this approach could encounter problems if flux began to be redirected to amino acid production, as suggested by an increase in alanine levels when both PDC3 and PFL1 pathways were blocked, as these pathways could not be knocked down without effecting cell fitness. It is also possible even if extra reduced ferredoxin became available that it would be consumed by competing processes such as cyclic electron flow.

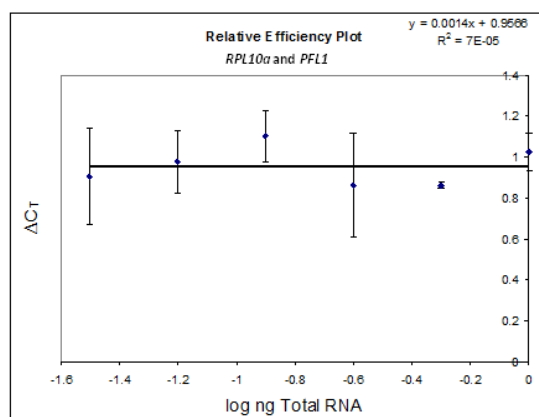
Chapter 6: Appendices

Appendix II.1 - Taqman qRT-PCR assay verification

Custom designed Taqman® qRT-PCR assays were verified for use of the $\Delta\Delta C_T$ method of relative quantification using the RNA polymerase gene *RPL10a* as a reference (Figure II.1) according to the manufacturer's instructions (Applied Biosystems). This is determined by using sample RNA of a known concentration to generate a standard curve, plotting the log value of input RNA amounts against the difference in cycle threshold (C_T) values between test assay (*PFL1* or *PDC3*) and an internal standard (in this case the *rpl10a* gene as used previously (Hemschemeier et al., 2008b)) (Figure II.1). Assays with a slope value of <0.1 when comparing the log input vs C_T were suitable for use by this method, *PFL1* Taqman® assay was found to have a similar binding efficiency to *RPL10a* making the $\Delta\Delta C_T$ method of relative quantification appropriate (Figure II.1A).

However, the *PDC3* assay was not for suitable relative quantification of changes in transcription by the $\Delta\Delta C_T$ method and therefore the relative standard curve method was employed instead, using RNA from CC-124 cultures as a calibrator (Figure III.2).

A



B

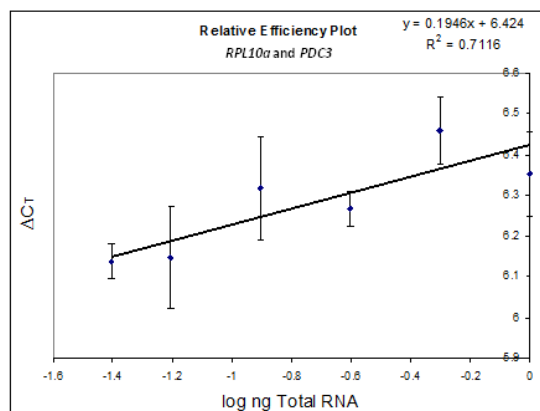


Figure II.2 Determination of the suitability of *PFL1* (A) and *PDC3* (B) Taqman® qRT-PCR assays for quantification of mRNA levels by $\Delta\Delta C_T$ calculation. Error bars represent $\pm$ standard deviation of three technical repeats.

Appendix II.2 - Sequence of adapted pRSET A Cloning Vector

The Xpress epitope tag of the pRSET A commercial expression vector (Invitrogen Life Technologies, USA) has been replaced with a thrombin cleavage site „LVPR“ highlighted (bold, italics)

pRSET A-thr Sequence

```
GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTGTTTAACTTTAAGA
AGGAGATATACATATGCGGGGTTCTCATCATCATCATCATCATGGTCTGGTTCCGCGTGGATCCGAGCTCGAGATCTGCAGCT
GGTACCATTGGAATTCGAAGCTTGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAAC
TAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTGTCTGAAAGGAGGAAGTATATCCGGATCTGGCGTAATAGCG
AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCG
GCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCCCTTCT
CGCCACGTTGCGCGGCTTTCCCGCTCAAGCTCTAAATCGGGGGCTCCCTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACC
CCAAAAACTTGATTAGGTGATGGTTACGTAAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCCCTTGACGTTGGAGTCCACG
TTCTTTAATAGTGGACTCTGTTCACAACTGGAACAACACTCAACCCTATCGCGGTCTATTCTTTTGATTATAAGGGATTTTGCC
GATTTCCGGCTATTGGTTAAAAATGAGCTGATTTAACAAATATTTAACCGGAATTTTAAACAAATATTAACGCTTACAATTTAGG
TGGCACTTTTCGGGGAATGTGCGCGGAACCCCTATTTGTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
AACCTTGATAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCTTTTTTGCG
GCATTTTGCTTCCCTGTTTTGCTCACCCAGAAACGCTGGTGAAGTAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTA
CATCGAAGTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC
TGCTATGTGGCGCGGTATTTATCCCGTATTGACGCGGGCAAGAGCAACTCGGTGCGCCCATACACTATTCTCAGAAATGACTTGGTT
GAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAA
CACTGCGGCCAATTACTTCTGACAACGATCGGAGGACCGAAGGAGTAAACCGTTTTTTGCACAACATGGGGGATCATGTAACCTC
GCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAACGACGAGAGTGACACCACGATGCCTGTAGCAATGCCAACACG
TTGCGCAAACTATTAAGTGGCGAACTACTTACTCTAGCTTCCCGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAAG
ACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTTG
CAGCACTGGGGCCAGATGGTAAGCGCTCCCGTATCGTAGTTATCTACACGACGGGAGTCAGGCAACTATGGATGAACGAAATAGA
CAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAAGTGTGACACCAAGTTTACTCATATATACTTTAGATTGATTTAAA
ACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCC
ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAACAAAA
AAACCACCGCTACCAGCGGTGGTTTGTGTTGCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGTGGCTTCAGCAGAGCGCA
GATACCAAACTAGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAAGTCTGTAGCACCGCCTACATACCTCGCTCTGC
TAATCCTGTACAGTGGCTGCTGCCAGTGGCGATAAGTGTGCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG
CAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGAAGTGAATACCTACAGCGTGA
GCTATGAGAAAGCGCCACGCTTCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGACAGGTCGGAACAGGAGAGCGCACGA
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TCGTCAGGGGGGCGAGCCTATGGAACGCGGACGACGCGGCTTTTACGGTTCCTGGGCTTTTGCTGGCCTTTTGCTGCACAT
GTTCTTTCTGCGTTATCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACGCCGAACGA
CCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAGAGCGCCCAATACGCAACCGCCTCTCCCGCGCGTTGGCCGATTCAATTAA
TGACAG
```

Appendix II.3: Clark electrode H₂ analysis

II.3.1 Adapting a Clark electrode for H₂ measurement

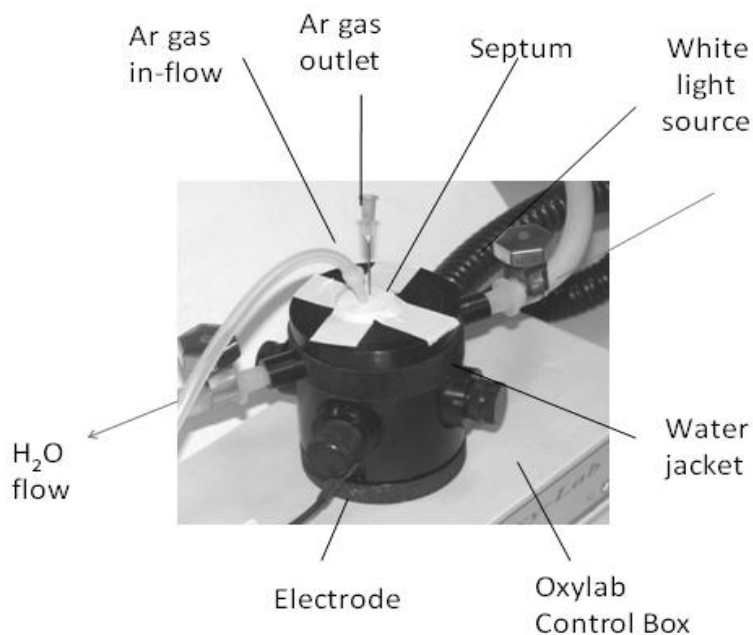


Figure II.3.1: Annotated photograph of the Clark electrode setup used for H₂ measurement.

The first necessary step was to test the efficacy of argon purging of the electrode chamber and culturing vessels, so that cultures are maintained under an anaerobic environment at all times throughout an experiment to facilitate hydrogen evolution. Purging of the electrode chamber was tested using a Clark electrode poised to measure dissolved oxygen concentration, and 1 ml of water, which was the final working volume to be used in hydrogen detection experiments. Dissolved oxygen concentrations were compared after addition of sodium dithionite or purging with argon (Figure II.3.2).

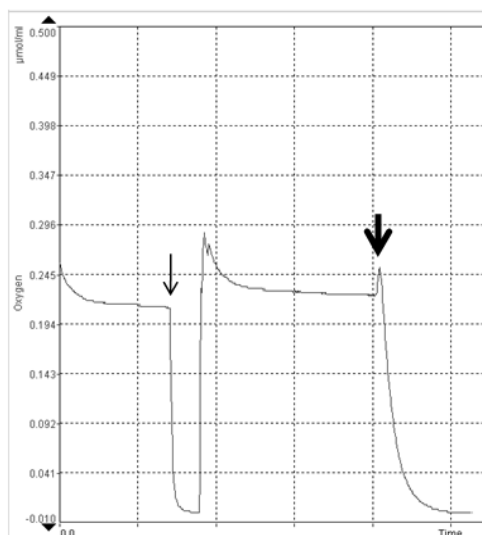


Figure II.3.2: Testing procedure for establishing anoxic conditions in the Clark electrode chamber

Displayed is a screenshot from the Oxylab32 software, showing purging of dissolved oxygen from 1 ml of H_2O by sodium dithionite (thin arrow) and argon bubbling (thick arrow).

It was established that bubbling with argon managed to completely de-oxygenate the water in the electrode chamber after a period of approximately 2 min. Once the water was de-oxygenated the stream of gas was removed and the chamber left for a further 5 min, no increase in oxygen levels was observed indicating the chamber to be sufficiently sealed.

II.3.2 H_2 quantification

To calibrate the Clark electrode, the signal response to known concentrations of H_2 gas was measured. 20 ml glass vials with re-sealable SubSeals were filled with dH_2O and bubbled with H_2 gas for 5 min to saturate the water. To ensure the water was saturated, the change in mV recorded in response to 50 μl of H_2 bubbled water, in a total volume of 1 ml, was recorded after bubbling for 1, 5 and 10 min with H_2 gas. No further significant increase in signal was observed by increasing the duration of bubbling from 5 to 10 min, suggesting the water was saturated with H_2 (Table II.3.I).

A standard curve of change in mV against concentration of hydrogen was created through the addition of various ratios of dH_2O and H_2 saturated water to the electrode chamber, as the mole fraction solubility of H_2 in water is known it was possible to calculate the number of moles of hydrogen added, and hence the concentration.

Duration of bubbling with H ₂ (min)	Change in voltage (mV)
1	0.052 ± 0.001
5	0.083 ± 0.003
10	0.084 ± 0.003

Table II.3.1: Establishing a procedure for generating H₂ saturated water.

The table shows the recorded response of the Clark electrode to 50 µl of water bubbled with H₂ for 1, 5 and 10 min (from a total volume of 50 ml) added to 950 µl of dH₂O, values are the average of 3 results ± SD.

At a partial pressure of gas of 101.325 kPa (one atmosphere) and temperature of 298.15 T/K (25°C) the mole fraction solubility of H₂ gas is 1.411×10^{-5} (X_a) (Lide, 2007), which was calculated from experimental data using equation (1)

$$\ln X_a = A + \frac{B}{T'} + C \ln T' \quad (\text{Lide, 2007}) \quad (1)$$

$$T' = T/100 \text{ K}$$

To calculate the amount of hydrogen gas dissolved in 1 µl of H₂O from the known value of mole fraction solubility we can use equation (2)

$$X_a = \frac{n_a}{n_x} \quad (2)$$

$$\therefore n_a = X_a \times n_x$$

X_a = Mole fraction solubility of H₂

n_a = moles of H₂ gas

n_x = Total moles in mixture of H₂ and H₂O

The total moles in the mixture is approximately equivalent to the moles of H₂O, water has a relative formula mass of 18, and 1 µl of H₂O has a mass of 1 mg

Moles = mass/RFM

Moles of H₂O in 1 µl = 0.001/18

$$= 5.556 \times 10^{-5} \text{ mol (4.s.f.)}$$

Total moles $\approx 5.556 \times 10^{-5} \text{ mol}$

$$n_a = X_a \times n_x$$

Moles of H₂ in 1 µl of saturated H₂O = $1.411 \times 10^{-5} \times 5.556 \times 10^{-5}$

Moles of H₂ in 1 µl of saturated H₂O = 0.78 nmol

The change in signal is proportional to the dissolved hydrogen concentration, as the working volume for all injections and H₂ measurements using *C. reinhardtii* cells will be 1.5 ml the change in signal can be displayed as equivalent to amount of H₂ in nmoles.

To establish the linearity of response to hydrogen concentration by the modified electrode, and reproducibility of measurement calibration curves were performed initially using a PTFE membrane (with 4 mm Pt coil placed on top of cathode to increase surface area), then later with a Nafion NRE-212 membrane. A linear relationship correlating the change in mV with volume of H₂ saturated water added was established for both membranes (Figure A.I.3). The Nafion membrane was found to increase the sensitivity of measurement (Figure A.I.3) due to a greater permeability to hydrogen, but there was a degree of variability between days (data not shown) indicating the necessity for calibration at each occasion of hydrogen measurement.

Once the calibration curve has been established the amount of H₂ evolved by anaerobically adapted cultures of algae can be calculated as proportional to the change in signal (mV) from the equation of the line; where the intercept $c = 0$

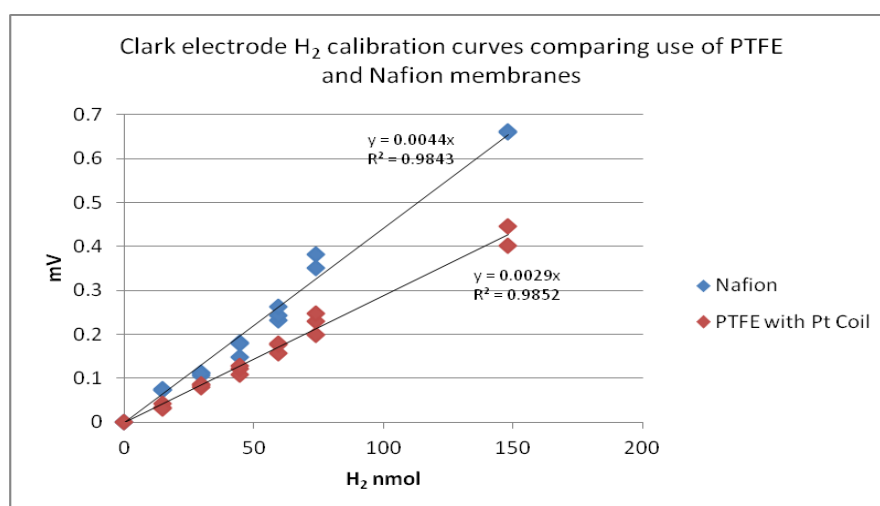


Figure II.3.3: A H₂ calibration curve for the reverse polarity Clark electrode comparing PTFE and Nafion membranes

$$y = mx + c$$

$$x = \frac{y}{m}$$

The concentration dependence of signal response was tested by injecting increasing volumes of H₂ saturated water into the electrode chamber to a constant final concentration, which should result in a constant response. However although values remained similar, signal response did increase slightly with increasing amounts of H₂ saturated water - an effect that became more pronounced with larger volumes. This can perhaps be attributed to incomplete mixing within the electrode chamber.

II.3.3 Testing the protocol for transient induced H₂ measurements using *C. reinhardtii*

Transient light induced H₂ measurements of *C. reinhardtii* have been reported in a number of publications (Ghirardi et al., 1997; Posewitz et al., 2004b; Mus et al., 2007; Dubini et al., 2009). To optimise the procedure for use in our setup (where a higher concentration of cells were required) the light intensity required for maximum H₂ production rates was tested, finding the maximum light intensity provided by the Hansatech light source was required, minor increases in H₂ production rates may have been achieved at a higher light intensity, but any increases would be marginal and can therefore be discounted (Figure II.3.4).

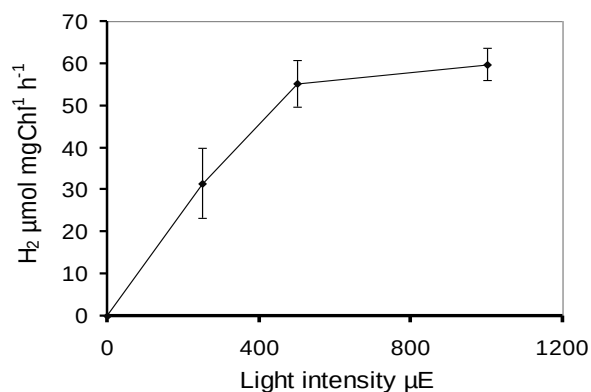


Figure II.3.4: Effect of intensity on the transient light-induced H₂ production rates of *C. reinhardtii* CC-124

Cultures were incubated under dark anaerobic conditions for 4 h as described in section 2.12.1. Actinic light was provided by the Hansatech Clark electrode setup, error bars are provided $\pm$ SE of three technical replicates.

Once the optimal light intensity had been determined, to ensure maximum rates were recorded a time course of the H₂ production ability of anaerobically adapted cells was performed (Figure II.3.4). Peak rates were achieved after 4 h dark

anaerobic incubation, with no further increases at longer durations (Figure II.3.5) which was in good agreement with previous measurements (Posewitz et al., 2004b).

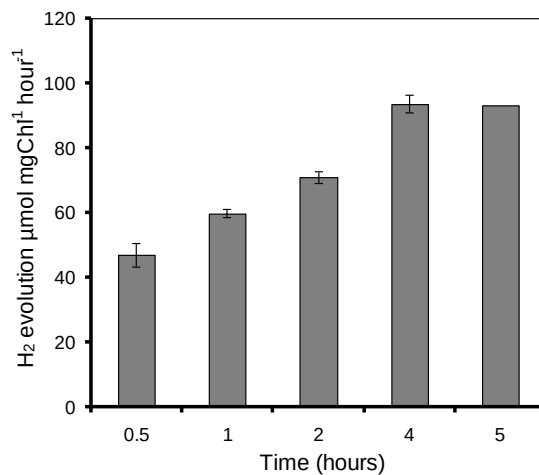


Figure II.3.5: Time course of transient light induced H₂ production ability of dark-anaerobically adapted cultures of CC-124.

Cultures were incubated under dark anaerobic conditions as described in section 2.12.1 and exposed to 1000 $\mu\text{E m}^2 \text{s}^{-1}$ actinic light. Error bars are $\pm$ SE of three biological replicates

Appendix II.4: GC Gas Analysis

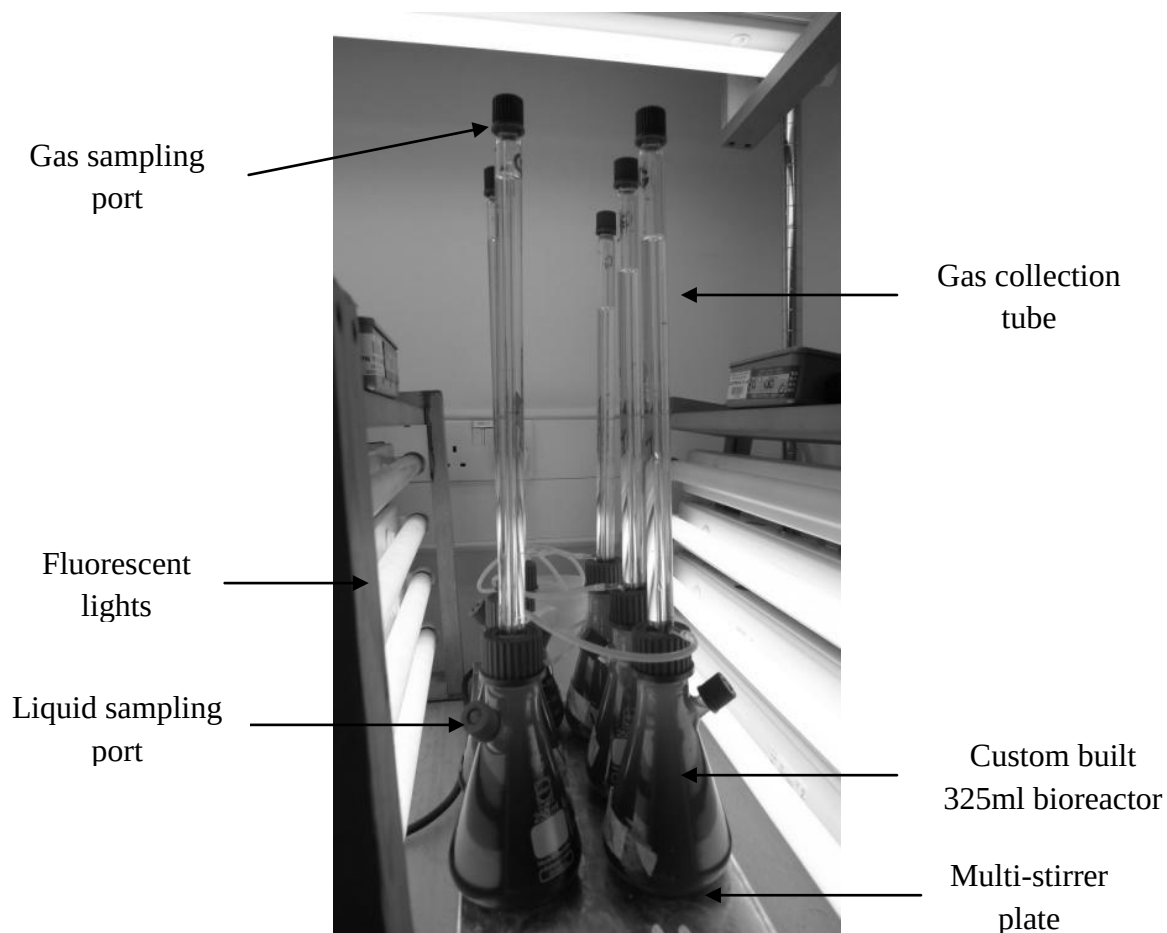


Figure II.4.1: Annotated photograph of experimental setup for TAP-S H₂ measurements.

II.4.1 Calibration of GC

To calibrate the GC, and to determine the amount of gas leakage from the sampling technique, one 323 ml bioreactor fitted with a gas collection tube, filled with H₂O and bubbled via the liquid sampling valve with a calibrant gas mixture containing H₂, CO₂ and N₂.

To ensure a pure calibrant gas mixture was injected for quantification purposes, headspace gas samples were taken using a Hamilton syringe and injected into the GC after 1, 15, 30, 45 and 60 min of purging (Figure II.4.2). Very little change in the signal values detected by TCD for each of the calibrant gases was observed after 30 min purging, suggesting the headspace of the minibioreactor was saturated with calibrant gas and all dissolved gases had been purged from the water chamber (Figure II.4.2). Residual amounts of O₂ were detected in samples even after 1 h of purging, this was assumed to be a result of the experimental technique and subtracted from future calculations (Table II.4.I).

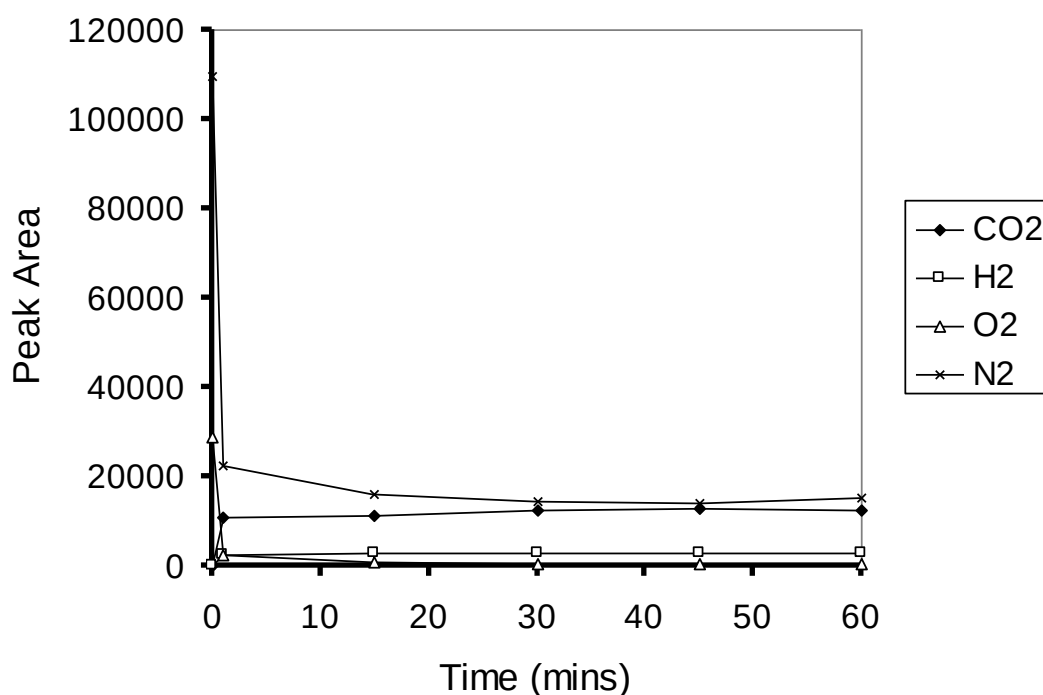


Figure II.4.2: Graph of gas composition versus time of headspace samples from 325ml bioreactor purged with calibrant mixture.

Gas	Area	% Gas	Area per 1%
N ₂	14261	10.0	1427.4
O ₂ *	28029	20.947	1338.1
H ₂	2733	80.3	34.0
CO ₂	12663	9.7	1301.4

Table II.4.1: Average values of calibrant gas signal peak areas measured by TCD.

The injection volume was set at 200 μ l and controlled by gas sampling valve. Values are given as an average of three measurements. *value for O₂ was calculated by injecting air onto the column and measuring the area under the peak.

These data could then be used to calculate the peak area corresponding to 1% of each calibrant gas within a mixture for future elucidation of headspace gas composition.

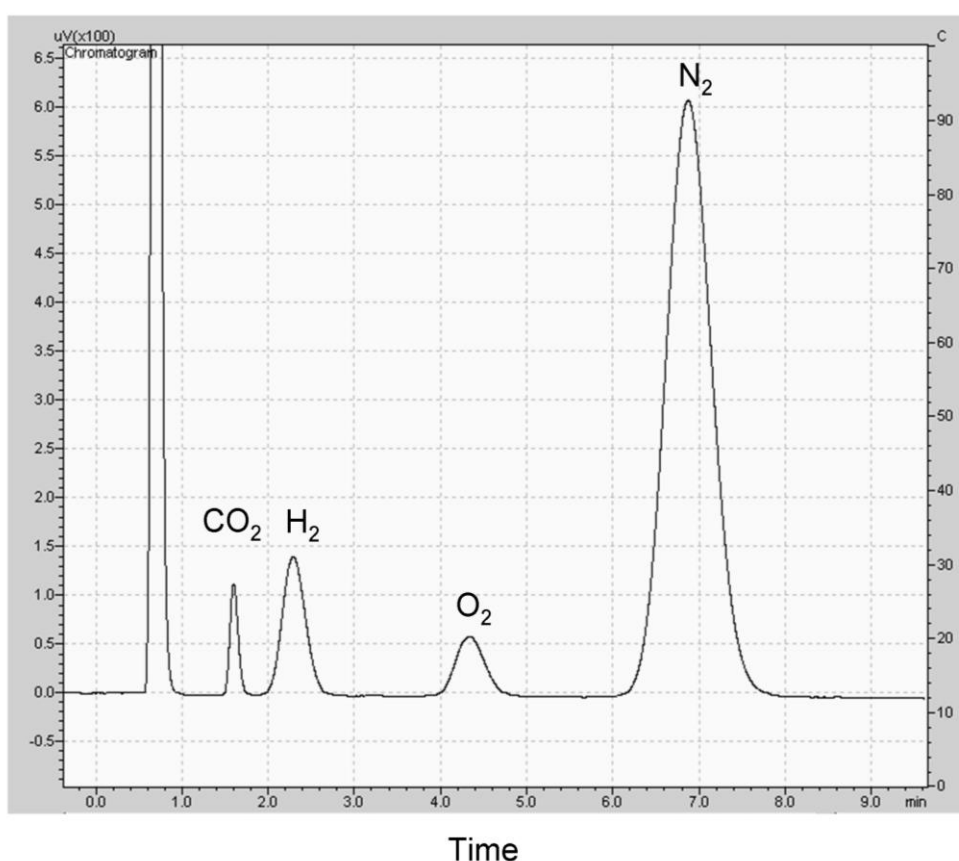


Figure II.4.3: Screenshot of GC analysis by TCD.

Samples were analysed as described in section 2.12.1, peaks and retention times are labelled with their corresponding gases. Sample collected from headspace of a CC-124 culture 72 h post-sulphur-deprivation. Calculated gas percentages: CO₂, 0.63%; H₂, 77.1%; O₂, 1.0%; N₂, 16.5%.

II.4.2 Calculation of moles of H₂ gas

Example:

Total gas volume collected after 24 h sulphur-deprivation was 4.125 ml which was 26.3% H₂ gas. The culture volume was 325 ml and the concentration of cells was 19.6 µg Chl ml⁻¹

(1) Calculate the total gas volume per mg Chl

$$\begin{aligned}\text{Total Chlorophyll (mg)} &= \text{Concentration} \times \text{volume} \\ &= 0.0196 \times 325 \\ &= 6.37 \text{ mg Chl}\end{aligned}$$

$$\begin{aligned}\text{Total Gas per mg Chl} &= 4.125/6.37 \\ &= 0.648\end{aligned}$$

(2) Calculate the volume of H₂ collected per mgChl

$$\begin{aligned}\text{Volume of H}_2 &= \text{Total Gas Volume} \times (\% \text{H}_2) \\ &= 0.648 \times 0.263 \\ &= 0.170 \text{ ml H}_2 \text{ mg Chl}^{-1}\end{aligned}$$

(3) Calculate the number of moles of H₂ using ideal gas equation at 25°C standard pressure

$$pV = nRT$$

$$n = pV/RT$$

$$\begin{aligned}n &= (1 \times 0.00017)/(0.08206 \times 298) \\ &= 6.9 \times 10^{-6} \text{ mol} \\ &= 6.9 \text{ µmol H}_2 \text{ per mg Chl}^{-1}\end{aligned}$$

(using R = 0.08206 L atm K⁻¹ mol⁻¹, P = 1 atmosphere, V = L, T = °K)

Appendix II.5: Additional amiRNA knockdown trials

Vector	Target	amiRNA	Sequence
pRNAi3.1A	<i>PGR5</i>	Cr_PGR5_amiRNA_A_For	ctagtCTCCTTGGCGTTGACAACAAAtctcgctgatcgccaccatgg
		PGR5_amiRNA_A_For	gggtgggtgatcagcgctaTTTGTGTCAACGCCAAGGAGg
			ctagcCTCCTTGGCGTTGACAACAAAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTTTGTGTCAACGCCAAGGAGa
pRNAi3.1B	<i>PGR5</i>	Cr_PGR5_amiRNA_B_For	ctagtCGGGAAGCCATTCAACTACTAtctcgctgatcgccaccatgg
		Cr_PGR5_amiRNA_B_Rev	gggtgggtgatcagcgctaTAGTTGTTGAATGGCTTCCCCg
			ctagcCGGGAAGCCATTCAACACTAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTAGTAGTTGAATGGCTTCCCCa
pRNAi3.2A	<i>NDA5</i>	Cr_NDA5_amiRNA_A_For	ctagtCCCCTGTATACTCCAACATGAtctcgctgatcgccaccatgg
		Cr_NDA5_amiRNA_A_Rev	gggtgggtgatcagcgctaTCATGTTGGAGTATACAGGGGg
			ctagcCCCCTGTATACTCCAACATGAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTCATGTTGGAGTATACAGGGGg
pRNAi3.2B	<i>NDA5</i>	Cr_NDA5_amiRNA_B_For	ctagtGCCGTCGAAGGTTCTCTCTAtctcgctgatcgccaccatgg
		Cr_NDA5_amiRNA_B_Rev	gggtgggtgatcagcgctaTGAGTGAGAACCTTGACCGGCGg
			ctagcGCCGTCGAAGGTTCTCACTCAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTGAGAGAGAACCTTGACCGGCGa
pRNAi3.3A	<i>PSBO</i>	Cr_OEE1_amiRNA_A2_For	ctagtCGTGGCCAGACTGAGTTTGTAtctcgctgatcgccaccatgg
		Cr_OEE1_amiRNA_A2_Rev	gggtgggtgatcagcgctaTACAACTCAGTCTGCCACCGg
			ctagcCGTGGCCAGACTGAGTTTGTAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTACAACTCAGTCTGCCACGg
pRNAi3.3B	<i>PSBO</i>	Cr_OEE1_amiRNA_B_For	ctagtACCCTACTGCTTAGCAAGGTAtctcgctgatcgccaccatgg
		Cr_OEE1_amiRNA_B_Rev	gggtgggtgatcagcgctaTACCATGCTAAGCAGTAGGGTg
			ctagcACCCTACTGCTTAGCATGGTAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTACCTTGCTAAGCAGTAGGGGg
pRNAi3.5A	<i>PFOR</i>	Cr_PFOR_amiRNA_A_For	ctagtGCCCTACTGGGTTTTGTTTTAtctcgctgatcgccaccatggg
		Cr_PFOR_amiRNA_A_Rev	gggtgggtgatcagcgctaTAAAACAAAACCCAGTAGGGCGg
			ctagcGCCCTACTGGGTTTTGTTTTAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTAAAACAAAACCCAGTAGGGGg
pRNAi3.5B	<i>PFOR</i>	Cr_PFOR_amiRNA_B_For	ctagtCACGTCAAGTATCGCAACCAAtctcgctgatcgccaccatgg
		Cr_PFOR_amiRNA_B_Rev	gggtgggtgatcagcgctaTTGGATGCGATACCTTGACGTGg
			ctagcCACGTCAAGTATCGCATCCAAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTTGGTTGCGATACCTTGACGTGg
pRNAi3.7A	<i>LDH</i>	Cr_LDH_amiRNA_A_For	ctagtCGCCTTACGGATGCATACATAtctcgctgatcgccaccatgg
		Cr_LDH_amiRNA_A_Rev	gggtgggtgatcagcgctaTATGTATGCATCCGTAAAGCGGg
			ctagcCGCCTTACGGATGCATACATAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTATGTATGCATCCGTAAAGCGGg
pRNAi3.7B	<i>LDH</i>	Cr_LDH_amiRNA_B_For	ctagtATCGGCATCCCTTACGAAAGAtctcgctgatcgccaccatgg
		Cr_LDH_amiRNA_B_Rev	gggtgggtgatcagcgctaTCTACGTAAGGGATGCCGATg
			ctagcATCGGCATCCCTTACGTAAGAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTCTTTCGTAAGGGATGCCGATa
pRNAi3.8A	<i>HCP</i>	Cr_HCPall_amiRNA_A_For	ctagtCCCCAGGTGATTGAGGCTATAtctcgctgatcgccaccatgg
		Cr_HCPall_amiRNA_A_Rev	gggtgggtgatcagcgctaTATAGCCTGAATCACCTGGGGg
			ctagcCCCCAGGTGATTGAGGCTATAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTATAGCCTGAATCACCTGGGGa
pRNAi3.8B	<i>HCP</i>	Cr_HCPall_amiRNA_B_For	ctagtCCCGTGCTGGTTTGAGGAGAAtctcgctgatcgccaccatgg
		Cr_HCPall_amiRNA_B_Rev	gggtgggtgatcagcgctaTTCTGCTCAAACCAGCACGGGg
			ctagcCCCGTGCTGGTTTGAGCAGAAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTTCTCCTCAAACCAGCACGGGg
pRNAi3.9A	<i>HYDA1</i> and <i>HYDA2</i>	Cr_HYD12_amiRNA_A_For	ctagtCCGCGAATGGTTCTGTGTGAAtctcgctgatcgccaccatgg
		Cr_HYD12_amiRNA_A_Rev	gggtgggtgatcagcgctaTTCACACAGAACCATTGCGGGg
			ctagcCCGCGAATGGTTCTGTGTGAAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTTCACACAGAACCATTGCGGGa
pRNAi3.9B	<i>HYDA1</i> and <i>HYDA2</i>	Cr_HYD12_amiRNA_B_For	ctagtGCTGCCAATGTTTACCTGCTAtctcgctgatcgccaccatgg
		Cr_HYD12_amiRNA_B_Rev	gggtgggtgatcagcgctaTAGCTGGTGAACATTGGCAGCGg
			ctagcGCTGCCAATGTTTACCAGCTAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTAGCAGGTGAACATTGGCAGGg
pRNAi6.15A	<i>PLSB1</i>	Cr_PLSB1_amiRNA_A_Rev	ctagtCCGCCTGGGATTACTTGTATAtctcgctgatcgccaccatgg
		Cr_PLSB1_amiRNA_A_For	gggtgggtgatcagcgctaTATAGAAGTAATCCCAGGCGGg
			ctagcCCGCCTGGGATTACTTCTATAtagcgctgatcaccacc
			ccatgggtccgatcagcgagaTATACAAGTAATCCCAGGCGGg
pRNAi6.15B *	<i>PLSB1</i>	Cr_PLSB1_amiRNA_B_For	ctagtGGGCGCCCGATAACTTAGATAtctcgctgatcgccaccatgg
		Cr_PLSB1_amiRNA_B_Rev	gggtgggtgatcagcgctaTATCAAAGTTATCGGGCGCCCg
			ctagcGGGCGCCCGATAACTTTGATAtagcgctgatcaccacc
			cccatgggtccgatcagcgagaTATCTAAGTTATCGGGCGCCCa

(Table II.5.1 continued)

Vector	Target	amiRNA	Sequence
pRNAi6.16A	<i>DXS1</i>	Cr_DXS1_amiRNA_A_For	ctagtTCGTTGGAACGGCGTCAACTAtctcgctgatcgccaccatgg
		Cr_DXS1_amiRNA_A_Rev	gggtgggtgatcagcgctaTAGTAGACGCCGTTCCAACGAg
pRNAi6.16B	<i>DXS1</i>	Cr_DXS1_amiRNA_B_For	ctagcTCGTTGGAACGGCGTCTACTAtagcgctgatcaccaccacc
		Cr_DXS1_amiRNA_B_Rev	ccatgggtccgatcagcgagaTAGTTGACGCCGTTCCAACGAa
pRNAi6.17A	<i>NADTH</i>	Cr_DXS1_amiRNA_B_For	ctagtTGGCATAATCTGCCCGTAATAAtctcgctgatcgccaccatgg
		Cr_DXS1_amiRNA_B_Rev	gggtgggtgatcagcgctaTATTGTGGGCAGATTATGCCAg
pRNAi6.17A	<i>NADTH</i>	Cr_DXS1_amiRNA_B_Rev	ctagcTGGCATAATCTGCCCAACAATAtagcgctgatcaccaccacc
		Cr_DXS1_amiRNA_B_Rev	cccatgggtccgatcagcgagaTATTACGGGCAGATTATGCCAa
pRNAi6.17A	<i>NADTH</i>	Cr_NADTH_amiRNA_A_Fo	ctagtGAGCCTATTAAGGACTTTATAtctcgctgatcgccaccatgg
		Cr_NADTH_amiRNA_A_Re	gggtgggtgatcagcgctaTATAGGGTCCTTAATAGGCTCg
pRNAi6.17B	<i>NADTH</i>	Cr_NADTH_amiRNA_A_Re	ctagcGAGCCTATTAAGGACCTTATAtagcgctgatcaccaccacc
		Cr_NADTH_amiRNA_A_Re	cccatgggtccgatcagcgagaTATAAAGTCCTTAATAGGCTCa
pRNAi6.17B	<i>NADTH</i>	Cr_NADTH_amiRNA_B_Fo	ctagtCTGCTCCATATCCTTTTGAGAtctcgctgatcgccaccatgg
		Cr_NADTH_amiRNA_B_Fo	gggtgggtgatcagcgctaTCTCTAAAGGATATGGAGCAGg
pRNAi6.17B	<i>NADTH</i>	Cr_NADTH_amiRNA_B_Re	ctagcCTGCTCCATATCCTTTAGAGAtagcgctgatcaccaccacc
		Cr_NADTH_amiRNA_B_Re	ccatgggtccgatcagcgagaTCTCAAAGGATATGGAGCAGa
pRNAi3.18A	<i>MOC1</i>	amiRNAi_MOC1_1F	ctagtCCGCTCGACATTCACACAATAtctcgctgatcgccaccatgg
		amiRNAi_MOC1_1R	gggtgggtgatcagcgctaTATTCTGTGAATGTGCGAGCGGg
pRNAi3.18B	<i>MOC1</i>	amiRNAi_MOC1_1R	ctagcCCGCTCGACATTCACAGAATAtagcgctgatcaccaccacc
		amiRNAi_MOC1_1R	cccatgggtccgatcagcgagaTATTGTGTGAATGTGCGAGCGGa
pRNAi3.18B	<i>MOC1</i>	amiRNAi_MOC1_2F	ctagtAAAGCGAGCCTTTCTGACTAAAtctcgctgatcgccaccatgg
		amiRNAi_MOC1_2R	gggtgggtgatcagcgctaTTAGACAGAAAGGCTCGCTTTg
pRNAi3.18B	<i>MOC1</i>	amiRNAi_MOC1_2R	ctagcAAAGCGAGCCTTTCTGTCTAAAtagcgctgatcaccaccacc
		amiRNAi_MOC1_2R	ccatgggtccgatcagcgagaTTAGTCAGAAAGGCTCGCTTTa
pRNAi6.19A	<i>ADH1</i>	ADH1_A_F	ctagtGGCCAGTCGATTGCGGGCTTAtctcgctgatcgccaccatgg
		ADH1_A_R	gggtgggtgatcagcgctaTAAGTTCGCAATCGACTGGCCg
pRNAi6.19A	<i>ADH1</i>	ADH1_A_R	ctagcGGCCAGTCGATTGCGAACTTAtagcgctgatcaccaccacc
		ADH1_A_R	cccatgggtccgatcagcgagaTAAGCCCGCAATCGACTGGCCa
pRNAi6.19B	<i>ADH1</i>	ADH1_B_F	ctagtCCCCGCGTGTGTAATGTGTAAtctcgctgatcgccaccatgg
		ADH1_B_R	gggtgggtgatcagcgctaTTACTCATTACACACGCGGGGg
pRNAi6.19B	<i>ADH1</i>	ADH1_B_R	ctagcCCCCGCGTGTGTAATGAGTAAtagcgctgatcaccaccacc
		ADH1_B_R	cccatgggtccgatcagcgagaTTACACATTACACACGCGGGGa
pRNAi6.14A	<i>NAC2</i>	Cr_NAC2_amiRNA_A_For	ctagtGAGGTCCAGGATCCTGAGATAtctcgctgatcgccaccatgg
		Cr_NAC2_amiRNA_A_Rev	gggtgggtgatcagcgctaTATCACAGGATCCTGGACCTCg
pRNAi6.14A	<i>NAC2</i>	Cr_NAC2_amiRNA_A_Rev	ctagcGAGGTCCAGGATCCTGTGATAtagcgctgatcaccaccacc
		Cr_NAC2_amiRNA_A_Rev	cccatgggtccgatcagcgagaTATCTCAGGATCCTGGACCTCa
pRNAi6.14B	<i>NAC2</i>	Cr_NAC2_amiRNA_B_For	ctagtATGTATCGAGCTCCCGTAAATAtctcgctgatcgccaccatgg
		Cr_NAC2_amiRNA_B_Rev	gggtgggtgatcagcgctaTTTTTCGGGAGCTCGATACATg
pRNAi6.14B	<i>NAC2</i>	Cr_NAC2_amiRNA_B_Rev	ctagcATGTATCGAGCTCCCGAAAAAAtagcgctgatcaccaccacc
		Cr_NAC2_amiRNA_B_Rev	cccatgggtccgatcagcgagaTTTTACGGGAGCTCGATACATa
pRNAi3.13A	<i>PDC1</i>	Cr_PDC1_amiRNA_A_For	ctagtAGGCCGGCGATTTTGAAGTAAtctcgctgatcgccaccatgg
		Cr_PDC1_amiRNA_A_Rev	gggtgggtgatcagcgctaTTACATCAAAATCGCCGGCCTg
pRNAi3.13A	<i>PDC1</i>	Cr_PDC1_amiRNA_A_Rev	ctagcAGGCCGGCGATTTTGTATGTAAtagcgctgatcaccaccacc
		Cr_PDC1_amiRNA_A_Rev	cccatgggtccgatcagcgagaTTACTTCAAAATCGCCGGCCTa
pRNAi3.13B	<i>PDC1</i>	Cr_PDC1_amiRNA_B_For	ctagtCCCGTTGCAATTACGGCTCTAtctcgctgatcgccaccatgg
		Cr_PDC1_amiRNA_B_Rev	gggtgggtgatcagcgctaTAGACCCGTAATTGCAACGGGg
pRNAi3.13B	<i>PDC1</i>	Cr_PDC1_amiRNA_B_Rev	ctagcCCCGTTGCAATTACGGGCTCTAtagcgctgatcaccaccacc
		Cr_PDC1_amiRNA_B_Rev	cccatgggtccgatcagcgagaTAGAGCCGTAATTGCAACGGGa
pRNAi3.10A	<i>FTSH1</i>	amiRNAi_FTSH1_A_F	ctagtCCGGAGTGATATCCCGTAGAAtctcgctgatcgccaccatgg
		amiRNAi_FTSH1_A_R	gggtgggtgatcagcgctaTTCTGTGGGATATCACTCCGGg
pRNAi3.10A	<i>FTSH1</i>	amiRNAi_FTSH1_A_R	ctagcCCGGAGTGATATCCACAGAAAtagcgctgatcaccaccacc
		amiRNAi_FTSH1_A_R	cccatgggtccgatcagcgagaTTCTACGGGATATCACTCCGGa
pRNAi3.10B	<i>FTSH1</i>	amiRNAi_FTSH1_B_F	ctagtCACCTGGTTTCTCGCACTAAAAtctcgctgatcgccaccatgg
		amiRNAi_FTSH1_B_R	gggtgggtgatcagcgctaTTTACTGCGAGAAACAGGTGg
pRNAi3.10B	<i>FTSH1</i>	amiRNAi_FTSH1_B_R	ctagcCACCTGGTTTCTCGCAGTAAAtagcgctgatcaccaccacc
		amiRNAi_FTSH1_B_R	ccatgggtccgatcagcgagaTTTATGTGCGAGAAACAGGTGa
pRNAi3.11A	<i>FTSH2</i>	amiRNAi_FTSH2_A_F	ctagtCCCCAAAAGGTTATTTTTTAAtctcgctgatcgccaccatgg
		amiRNAi_FTSH2_A_R	gggtgggtgatcagcgctaTTAAGGATAACCTTTTGGGGGg
pRNAi3.11A	<i>FTSH2</i>	amiRNAi_FTSH2_A_R	ctagcCCCCAAAAGGTTATCCTTAAAtagcgctgatcaccaccacc
		amiRNAi_FTSH2_A_R	ccatgggtccgatcagcgagaTTAAAAAATAACCTTTTGGGGGa
pRNAi3.11B	<i>FTSH2</i>	amiRNAi_FTSH2_B_F	ctagtACCCCTTGAGCGTGCATACAAAAtctcgctgatcgccaccatgg
		amiRNAi_FTSH2_B_R	gggtgggtgatcagcgctaTTTGAATGCACGCTCAAGGGTg
pRNAi3.11B	<i>FTSH2</i>	amiRNAi_FTSH2_B_R	ctagcACCCCTTGAGCGTGCATTCAAAtagcgctgatcaccaccacc
		amiRNAi_FTSH2_B_R	cccatgggtccgatcagcgagaTTTGATGCACGCTCAAGGGTa
pRNAi6.20A	<i>PDC2</i>	amiRNAi_PDC2_1F	ctagtGTTGACCGGGCTTTTCAATAAtctcgctgatcgccaccatgg
		amiRNAi_PDC2_1R	gggtgggtgatcagcgctaTTATAGAAAAGCCCGGTCAACg
pRNAi6.20A	<i>PDC2</i>	amiRNAi_PDC2_1R	ctagcGTTGACCGGGCTTTTCTATAAtagcgctgatcaccaccacc
		amiRNAi_PDC2_1R	ccatgggtccgatcagcgagaTTATTGAAAAGCCCGGTCAACa

(Table II.5.1 continued)

Vector	Target	amiRNA	Sequence
pRNAi6.20B	<i>PDC2</i>	amiRNAi_PDC2_2F	ctagtGGTCAGCCGCTCTGAAGGTTTTAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTAAATTTTCAGACGGCTGACCg
		amiRNAi_PDC2_2R	ctagcGGTCAGCCGCTCTGAAAATTTAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTAAACCTTCAGACGGCTGACCg
pRNAi3.22A	<i>HYDA1</i>	amiRNAi_HYDA1_A_F	ctagtGCCCCCTTGAGTCATGACCAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTGGACATGACTCAAAGGGGGCg
		amiRNAi_HYDA1_A_R	ctagcGCCCCCTTGAGTCATGTCCAAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTTGGTCATGACTCAAAGGGGGCa
pRNAi3.22B	<i>HYDA1</i>	amiRNAi_HYDA1_B_F	ctagtCAGGTCCTACCTAGCGCAAAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTTTCCGCTAGGTAGGACCTGg
		amiRNAi_HYDA1_B_R	ctagcCAGGTCCTACCTAGCGGAAAAAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTTTTGCGCTAGGTAGGACCTGa
pRNAi3.23A	<i>HYDA2</i>	amiRNAi_HYDA2_A_F	ctagtCTTGCCATTGCTGAGTGATTAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTAATGACTCAGCAATGGCAAGg
		amiRNAi_HYDA2_A_R	ctagcCTTGCCATTGCTGAGTCATTAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTAATCACTCAGCAATGGCAAGa
pRNAi6.21A	<i>LCA1</i>	amiRNAi_LCA1_A_F	ctagtTGGCCAATGGAGTCGACTTTAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTAAACTCGACTCCATGGCCAGg
		amiRNAi_LCA1_A_R	ctagcTGGCCAATGGAGTCGAGTTTAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTAAAGTCGACTCCATTTGGCCAA
pRNAi6.21B	<i>LCA1</i>	amiRNAi_LCA1_B_F	ctagtCGGGCTGACTATTTCAAACAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTGTATGAAATAGTCAGCCCGg
		amiRNAi_LCA1_B_R	ctagcCGGGCTGACTATTTCATACAAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTTGTTTGAAATAGTCAGCCGCa
pRNAi3.5C	<i>PFOR</i>	amiPFOR_3F	ctagtTCGGATGTGTAACACGAAATAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTATTACGTGTTACACATCCGAg
		amiPFOR_3R	ctagcTCGGATGTGTAACACGTAATAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTATTTCTGTGTACACATCCGAa
pRNAi3.5D	<i>PFOR</i>	amiPFOR_4F	ctagtGAGCGGCATTGATGATATGTAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTACAAATCATCAATGCCGCTCg
		amiPFOR_4R	ctagcGAGCGGCATTGATGATTTGTAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTACATATCATCAATGCCGCTCa
pRNAi6.pgri 1-1	<i>PGRL1</i>	amiRNA_PGRL1_F1	ctagtATGGTTCCCTTACCCGTTGATAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTATCTACGGGTAAGGAACCATg
		amiRNA_PGRL1_R1	ctagcATGGTTCCCTTACCCGTAGATAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTATCAACGGGTAAGGAACCATa
pRNAi6.pgri 1-2	<i>PGRL1</i>	amiRNA_PGRL1_F2	ctagtCGGTCCTGAAGTGACGTTGAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTCATCGTCACTTCAGGACCGg
		amiRNA_PGRL1_R2	ctagcCGGTCCTGAAGTGACGATGAAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTTCAACGTCACCTCAGGACCGa
pRNAi3.7C	<i>LDH</i>	amiRNA_LDH_F3	ctagtAGGCACCTTACGGATGGATAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTATGCATCCGTAAGGTGCCTg
		amiRNA_LDH_R3	ctagcAGGCACCTTACGGATGCATAAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTTATCCATCCGTAAGGTGCCTa
pRNAi3.7D	<i>LDH</i>	amiRNA_LDH_F4	ctagtGTGCTTGCTTATGACAACAAAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTTTGATGTCATAGGCAAGCACg
		amiRNA_LDH_R4	ctagcGTGCTTGCTTATGACATCAAAtagcgctgatcaccaccacc ccatgggtccgatcagcgagaTTTGTGTCATAGGCAAGCACa
pRNAi3.12A	<i>NDA2</i>	amiRNAi_NDA2_A	ctagtCCCGGAGGGGTTATCTCGATAtctcgctgatcgccaccatgg gggtgggtggatcagcgctaTATCCAGATAACCCCTCCGGGg
		amiRNAi_NDA2_rev	ctagcCCCGGAGGGGTTATCTGGATAtagcgctgatcaccaccacc cccatgggtccgatcagcgagaTATCGAGATAACCCCTCCGGGa

Table II.5.1: Table of additional amiRNA oligonucleotides synthesised and corresponding amiRNA vectors that were unused in this work

Appendix III.1 - Identification of an *ADH5* EST

<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- TCCACCTCGGTGTCCACCAACGTGGGCCGAGCACCTGCTGAACATCAAGACCGTCACC	1560
<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- GCGCGCCGCGAGAACATGCTGTGGTTCCGCGTGCCGCCAAGATCTACTTCAAGGGCGGC	1620
<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- TGCTGGAGGTGGCGCTGACCGATCTGCGTGGCAAATCGCGCGCTTTCATTGTCACGGAC	1680
<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- AAGCCGCTTTTGTACATGGGATACGCCACAAGGTCACCCACATCCTGGACAGCATTAAC	1740
<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- GTGCACCACCAGGTGTTCTACCACGTGACCCCCGACCCGACCCTGGCCTGCATTGAGGCG	1800
<i>ADH5</i> BG855351.1 <i>ADH1</i>	----- ----- GGTCTGAAGGAGATCCTGGAGTTCAAGCCCGATGTCATCATCGCGCTGGGTGGTGGCTCG	1860
<i>ADH5</i> BG855351.1 <i>ADH1</i>	-----ATGTGGCTGATGTATGAGTGCCCCGACACCCGCTTCGAC -----GCACGAGGGGCTGATGTATGAGTGCCCCGACACCCGCTTCGAC CCCATGGACGCCGCCAAGATCATGTGGCTGATGTACGAGTGCCCCGACACCCGCTTCGAC	39 43 1920
<i>ADH5</i> BG855351.1 <i>ADH1</i>	GGCCTGGCCATGCGCTTCATGGACATCCGCAAGCGTGTGTACGAGGTGCCGGAGCTGGGC GGCCTGGCCATGCGCTTCATGGACATCCGCAAGCGTGTGTACGAGGTGCCGGAGCTGGGC GGCCTGGCCATGCGCTTCATGGACATCCGCAAGCGCTGTACGAGGTGCCGGAGCTGGGC *****	99 103 1980
<i>ADH5</i> BG855351.1 <i>ADH1</i>	AAGAAGGCCACCATGGTGTGCATCCCCACCACCTCGGGCACC GGCTCCGAGGTGACGCCC AAGAAGGCCACCATGGTGTGCATCCCCACCACCTCGGGCACC GGCTCCGAGGTGACGCCC AAGAAGGCCACCATGGTGTGCATCCCCACCACAGTGGCACC GGCTCCGAGGTGACGCCC *****	159 163 2040
<i>ADH5</i> BG855351.1 <i>ADH1</i>	TTCTCAGTCGTCACGGACGAGCGCCTGAGCGCCAAGTACCCGCTGGCCGACTACGCGCTG TTCTCAGTCGTCACGGACGAGCGCCTGAGCGCCAAGTACCCGCTGGCCGACTACGCGCTG TTCTCGGTGGTCACCGACGAGCGCCTGGCGCCAAGTACCCCTGGCCGATTACGCCCTG *****	219 223 2100
<i>ADH5</i> BG855351.1 <i>ADH1</i>	ACGCCCAGCATGGCCATCGTGGACCCCCAGCTGGTGCTCAACATGCCCAAGAAGCTGACC ACGCCCAGCATGGCCATCGTGGACCCCCAGCTGGTGCTCAACATGCCCAAGAAGCTGACC ACCCCCAGCATGGCCATTGTGGACCCCCAGCTGGTGCTCAACATGCCCAAGAAGCTGACC ** *****	279 283 2160
<i>ADH5</i> BG855351.1 <i>ADH1</i>	GCCTGGGGCGGCATTGACGCCCTGACGCACGCGCTGGAGAGCTACGTGTCCATCTGCGCC GCCTGGGGCGGCATTGACGCCCTGACGCACGCGCTGGAGAGCTACGTGTCCATCTGCGCC GCCTGGGGCGGCATTGACGCGCTACGCACGCGCTGGAGAGCTACGTGTCCATCTGCGCC *****	339 343 2220
<i>ADH5</i> BG855351.1 <i>ADH1</i>	ACCGACTACACCAAGGGTCTATCGCGCAGGCGATCAGCCTGCTGTTCAAGTACCTGCCC ACCGACTACACCAAGGGTCTATCGCGCAGGCGATCAGCCTGCTGTTCAAGTACCTGCCC ACCGACTACACCAAGGGTCTGTCGCGCAGGCCATCAGCCTGCTGTTCAAGTACCTGCCC *****	399 403 2280
<i>ADH5</i> BG855351.1 <i>ADH1</i>	CGCGCCTATGCCGACGGCTCCAACGACTACGTGGCGCGCGAGAAGGTGCACTACGCCGCC CGCGCCTATGCCGACGGCTCCAACGACTACGTGGCGCGCGAGAAGGTGCACTACGCCGCC CGCGCCTACGCCAACGGCTCCAACGACTACCTGGCGCGTGAGAAGGTGCACTACGCCGCC *****	459 463 2340

<i>ADH5</i>	ACGAT---CGGCATGGCCTTCGCCAACGCCTTCCTGGGCATCTGCCACTCCATGGCGCAC	516
BG855351.1	ACGAT---CGGCATGGCCTTCGCCAACGCCTTCCTGGGCATCTGCCACTCCATGGCGCAC	520
<i>ADH1</i>	ACGATTGCCGGCATGGCCTTCGCCAACGCCTTCCTGGGCATCTGCCACTCCATGGCGCAC	2400

<i>ADH5</i>	AAGCTGGGCGCCGCTACACGTGCCGCATGGCCTGGCCAACGCCGCGCTGATCAGCCAC	576
BG855351.1	AAGCTGGGCGCCGCTACACGTGCCGCATGGCCTGGCCAACGCCGCGCTGATCAGCCAC	580
<i>ADH1</i>	AAGCTGGGCGCCGCTACACGTGCCGCATGGCCTGGCCAACGCCGCGCTGATCAGCCAC	2460

<i>ADH5</i>	GTCATCCGCTACAACGCCACCGACATGCCCGCCAAGCAGGCCGCTTCCCCAGTACCAG	636
BG855351.1	GTCATCCGCTACAACGCCACCGACATGCCCGCCAAGCAGGCCGCTTCCCCAGTACCAG	640
<i>ADH1</i>	GTCATCCGCTACAACGCCACCGACATGCCCGCCAAGCAGGCCGCTTCCCGCAGTACGAG	2520

<i>ADH5</i>	TACCCCACTGCCAAGCAGGACTACGCCGACCTGGCCAACATGCTGGGCCTGGGCGGCAAC	696
BG855351.1	TACCCCACTGCCAAG-----	655
<i>ADH1</i>	TACCCCACTGCCAAGCAGGACTACGCCGACCTGGCCAACATGCTGGGCCTGGGCGGCAAC	2580

<i>ADH5</i>	ACCACGGACGAGAAGGTGATCAAGCTGATTGAGGCGGTGGAGGATCTGAAGAAGAAGGTC	756
BG855351.1	-----	
<i>ADH1</i>	ACGGTGGACGAGAAGGTGATCAAGCTGATTGAGGCGGTGGAGGAGCTCAAGGCCAAGGTG	2640
<i>ADH5</i>	GACATCCCGCCCAACCATCAAGGAGATCTTCAATGACCCCAAGACCGACGCCGACTTCCTG	816
BG855351.1	-----	
<i>ADH1</i>	GACATCCCGCCCAACCATCAAGGAGATCTTCAACGACCCCAAGGTGGACGCCGACTTCCTG	2700
<i>ADH5</i>	TCGCACGTGGACGCCCTGGCTGAGGACGCGTTCGACGACCAGTGCACGGGCGCCAACCCG	876
BG855351.1	-----	
<i>ADH1</i>	GCGAACGTGGACGCCCTGGCCGAGGACGCTTCGACGACCAGTGCACGGGCGCCAACCCG	2760
<i>ADH5</i>	CGTACCCGCTGATCGCCGACCTGAAGCAGATCTACCTGGACGCGCACGCCGCGCCATC	936
BG855351.1	-----	
<i>ADH1</i>	CGTACCCGCTCATGGCCGACCTGAAGCAGCTCTACCTGGACGCCACGCCGCGCCATC	2820
<i>ADH5</i>	CTGCCCATCAAGTCGCTGGTGTCTTCTCCAAGATCCACTAG	978
BG855351.1	-----	
<i>ADH1</i>	CTGCCCCTCAAGACCCTGGAGTTCTTCTCCAAGATCAACTAA	2862

Figure III.1.1: Clustalw alignment comparing *ADH1* and *ADH5* mRNA sequences with EST sequence BG855351.1 (Duke University EST stock centre).

Appendix III.2 - Putative *C. reinhardtii* SDR sequences

Enzyme	ID	Phytozyme ID	Predicted location	Predicted size (kDa)	EST
Acylglycerone-phosphate reductase ^a	AYR1	Cre12.g559350	-/-	33.2	Y
Potential Light dependent Protochlorophyllide (Pchl) Oxidoreductase ^b	LPOR	Cre02.g135400	-/M	35.7	Y
Short chain dehydrogenase (cd08936)	SDR	Cre03.g191850	-/C	27.1	Y
3-oxoacyl-[acyl-carrier protein] reductase ^c	FabG	Cre03.g172000	C/M	33.1	Y
Short chain dehydrogenase (cd05355)	SDR	Cre09.g399000	-/-	31.4	N
Short chain dehydrogenase (cd05355)	SDR	Cre09.g399200	-/-	36.9	N
3-hydroxybutyrate dehydrogenase	BDH	Cre07.g352450	-/M	35.1	Y
3-hydroxybutyrate dehydrogenase	BDH	Cre12.g495850	-/M	27.8	Y
Short chain dehydrogenase (cd05325)	SDR	Cre27.g775000	-/-	27.4	Y
Putative serine 3-dehydrogenase (cd05346)	SDR	Cre01.g036400	-/-	26.8	Y
Putative chlorophyll b reductase	SDR	Cre12.g517700	-/-	46.9	Y
Short chain dehydrogenase (cd05332)	SDR	Cre02.g103100	-/S	35.8	Y

Table III.2.1: Short chain dehydrogenase family as identified by BLAST analysis, localisation was given by (ChloroP/TargetP). Where the enzyme was of unknown specificity the NCBI conserved domain identifier is given starting (cd). ^a glycerophospholipid and ether lipid metabolism ^b chlorophyll biosynthesis ^c fatty acid biosynthesis

Appendix III.3 - Putative *C. reinhardtii* MDR sequences

Enzyme	ID	Phytozyme ID	Predicted location	Predicted size (kDa)	EST
Putative zinc binding alcohol dehydrogenase (no complete domain)	ADH3	Cre04.g229650			N
Putative zinc binding alcohol dehydrogenase (ETR like ^a)	ADH4	Cre11.g476550	-/-	36.5	Y
Putative zinc binding alcohol dehydrogenase (QOR1 ^b)	ADH6	Cre16.g674050	-/-	35.6	Y
Putative zinc binding alcohol dehydrogenase (ETR domain ^c)	ADH	Cre01.g035350	-/-	36.3	Y
Putative zinc binding alcohol dehydrogenase (MDR2 like ^d)	ADH	Cre06.g297400	-/-	40.0	N
Putative zinc binding alcohol dehydrogenase class V (CAD1 domain ^e)	ADH	Cre03.g207800	-/-	45.1	Y
Putative zinc binding alcohol dehydrogenase class V (CAD1 domain ^e)	ADH	Cre03.g207550	-M	46.0	Y
Putative zinc binding alcohol dehydrogenase class V (CAD1 domain ^e)	ADH	Cre02.g127750	-M	38.8	N
Putative zinc binding alcohol dehydrogenase class V (CAD1 domain ^e)	ADH	Cre14.g623650	C/C	42.3	Y
Putative alcohol dehydrogenase (CAD1 domain ^e)	ADH	Cre14.g623650	-S	97.8	Y
Putative alcohol dehydrogenase (CAD1 domain ^e)	ADH	Cre03.g207800	-/-	45.1	Y
Putative alcohol dehydrogenase (CAD1 domain ^e)	ADH	Cre03.g207550	-M	46.0	N
Putative alcohol dehydrogenase (CAD1 domain ^e)	ADH	Cre02.g127750	-M	38.8	N
Formaldehyde dehydrogenase	FDH1	Cre12.g543400	-/-	40.4	Y
Formaldehyde dehydrogenase	FDH2	Cre12.g543350	-/-	41.0	Y

Table III.3.1: Bioinformatic analysis of putative medium chain reductase/dehydrogenase sequences in the *C. reinhardtii* genome. Predicted localisation is given (ChloroP/TargetP). ^a2-enoyl thioester reductase-like ^bNADH quinone reductase, zinc oxidoreductase like ^c2-enoyl thioester reductase domain ^dMDR2 Medium chain reductase like protein ^ecinnamyl alcohol dehydrogenase

Appendix III.4: Fermentative enzyme expression during photoautotrophic growth replicate blots

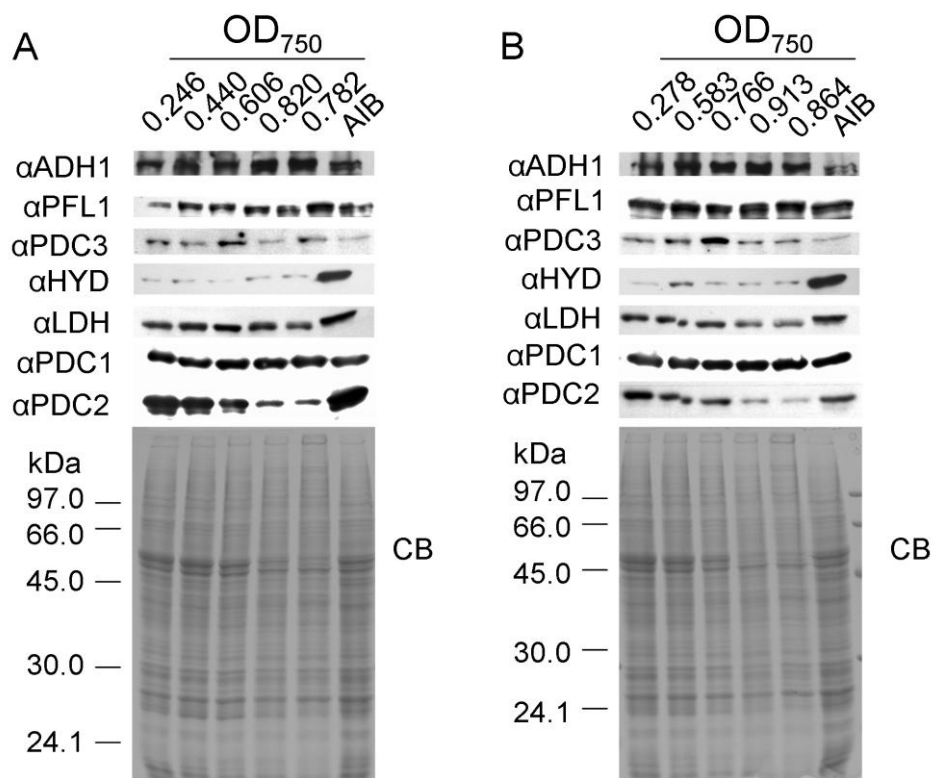


Figure III.4.1: Immunoblot analysis of fermentative enzyme expression during photoautotrophic growth.

Displaying biological replicate blots 2 (A) and 3 (B) of HSM grown cultures, 8×10^5 cells loaded per lane. Coomassie blue (CB) stained gel is provided as loading control.

Appendix IV.1: amiRNA binding sites

Gene: *PFL1*

Phytozome ID: Cre01.g044800

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ACCTTTAAGCTATCCTATCATCAACACCGTCTCGTCAAGTCTGCCAGCGCTTACGCTATGAGCCAGATGCTGCTGGAGAAGACAA
TGGCGCGGGGCTCGCCACGGTCTCGGCCGACGCGAGCTCCGCTGTTGGCCGGCCCATCCCATGGCTGTAGGTGCGCGATGCGC
TCGATGGCCGCTGCCAGCGCGGCCGCTGAGGCCCTCCCGGTGGCACCCAGCCACAGCTGCGCTGCTGACCCCGACAAGCACCCGCA
CCTGCCCGACCCCGCCGAGCGCGCCGTGGACGCGGGCATCAACGTCCAGAAGTATGTGCAGGACAACTACACCGCTTACGCCG
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CTGAGCCGCCCCGCCCAACTCGCGCGAGGCGGTGCAGTGGCTGTACTTCGGCTACCTGGGCGCGCTCAAGGAGCAGGACGGCGC
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CACCTGTACAACCTGGGCCCCGCGCCGAGCCCAACCTGACGGTGTCTGTGAACGACAACCTGCCCCGCGCTTCAAGGAGTTCT
GCGCAAGGTGTCTGCTGGACACCAAGTCCATCCAGTACGAGTCCGACAACCTCATGAGCAAGCTGTTTGGCTCCGACTACTCCATC
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CCCTTTCACAAACACCCACGCGCTAGCCCATAGATTCTCCCATTCGACTCCCTC

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Figure IV.1.1: *C. reinhardtii* *PFL1* mRNA sequence with amiRNA binding sites. Highlighted is the 5'UTR (green), 3'UTR (grey), oligo A binding site (red), oligo B binding site (yellow).

Gene: *PDC3*
Phytozome ID: Cre03.g165700

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 CACTCGCAGCGGTGTCCCAACCGGCGCAGCCAGCGCCACCAAGGCCAAGAGCAGCAGCAGCGCATGGCCACCACCGT
 GTCGCCCCGAGACGCCAACCTCGGCCTGCACATTGC CAACCGGCTGTGTGAGATCGG CTGCACCAGCTGCTTCGCGGTGCCGGCG
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 CTCCTCCACCAGCCTCATCAGCAGGTGGGCGACTCCTGGTTCAACACACTCAAGCTCAAGCTGCCCGCCGGCTGCGAGTACGAG
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 TGTAACCACTGAGAGAGACG

Figure IV.1.2: *C. reinhardtii* *PDC3* mRNA sequence with amiRNA binding sites.
 Highlighted are 5'UTR (green), 3'UTR (grey), oligo A binding site (red), oligo B
 binding site (yellow).

Appendix IV.2: *pfl1*-KD replicate blots

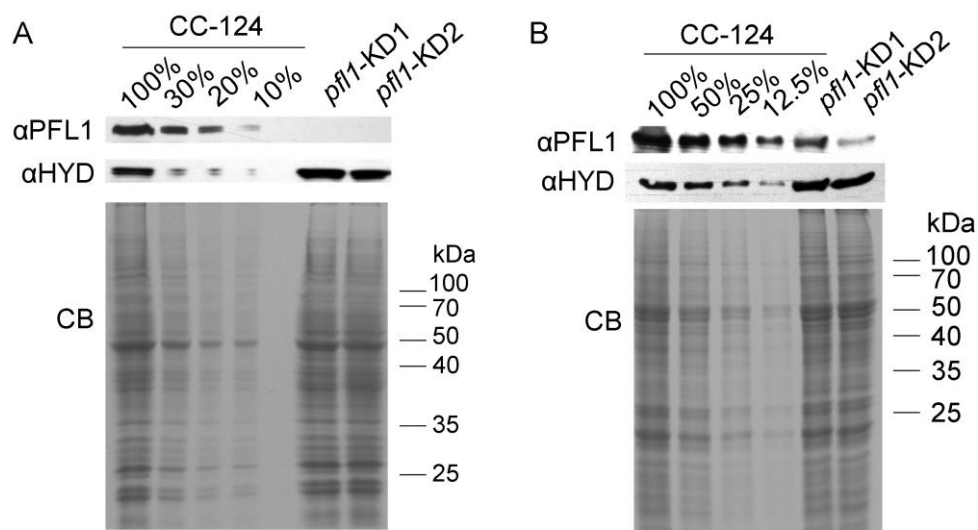


Figure IV.2.1: *pfl1*-KD biological replicate blots after 4 h dark anaerobic incubation in AIB buffer.

Displaying (A) replicate 2 (B) replicate 3 immunoblots. 8×10^5 cells loaded per lane, with the exception of the CC-124 dilution series where the percentage of the total is provided. Coomassie blue (CB) stained gel provides loading control.

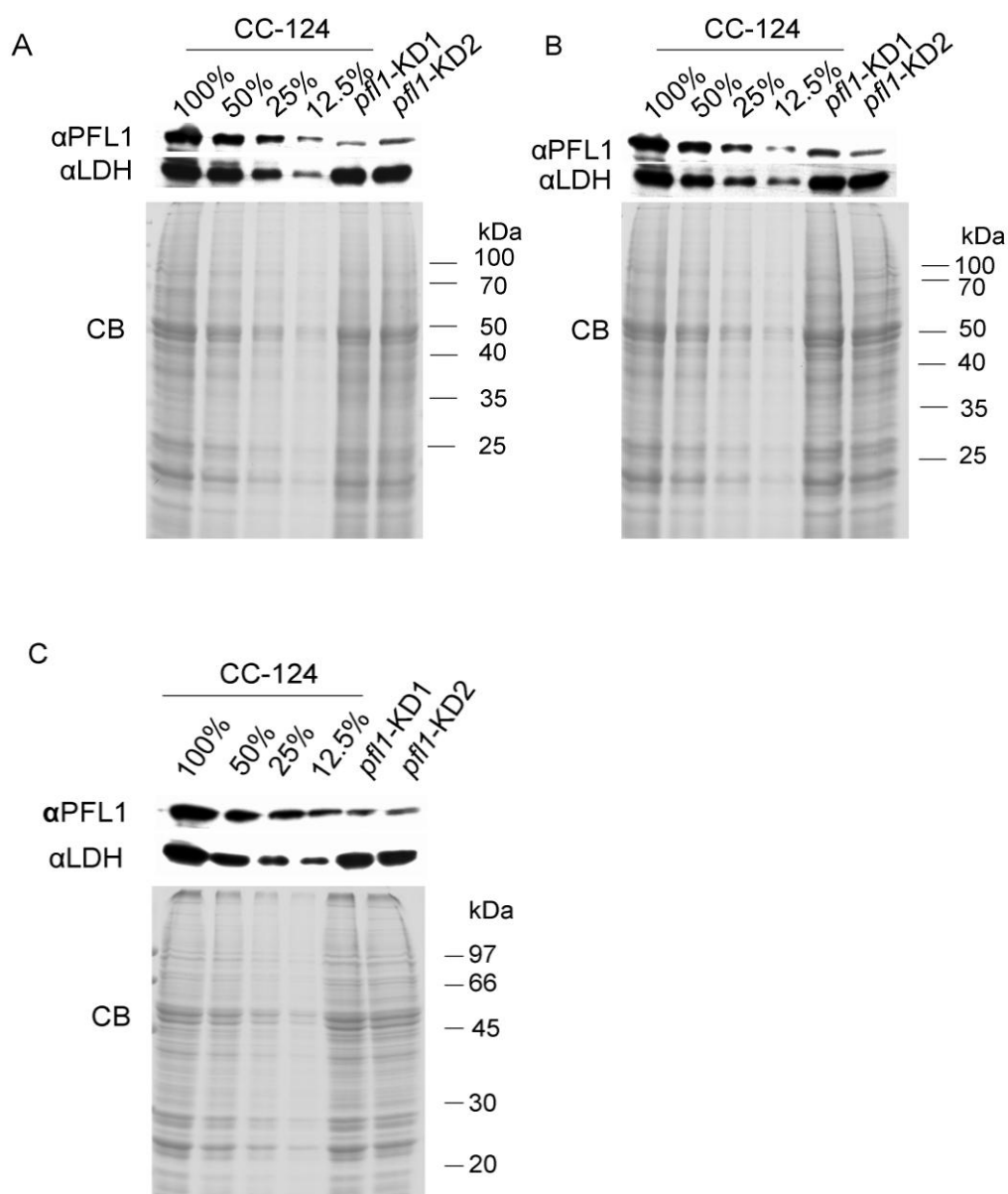
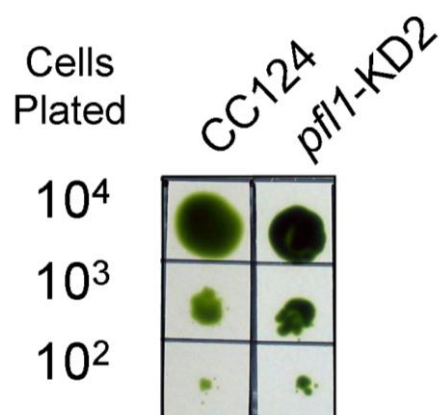


Figure IV.2.2: *pfl1*-KD biological replicate blots after aerobic growth in TAP media.

Displaying (A) replicate 1 (B) replicate 2, (C) replicate 3 immunoblots. 8×10^5 cells loaded per lane, with the exception of the WT dilution series were corresponding percentages are given. Coomassie blue (CB) stained gel provides loading control.

Appendix IV.3: Analysis of *pfl1-KD2* fitness under heterotrophic conditions**Figure IV.3.1: Investigating *pfl1-KD2* fitness in the dark.**

Cells were grown on TAP media in the dark for 22 days, the starting number of cells is indicated (right).

Appendix IV.4: *pfl1*-KD2 dark anaerobic metabolite production in HSM buffer

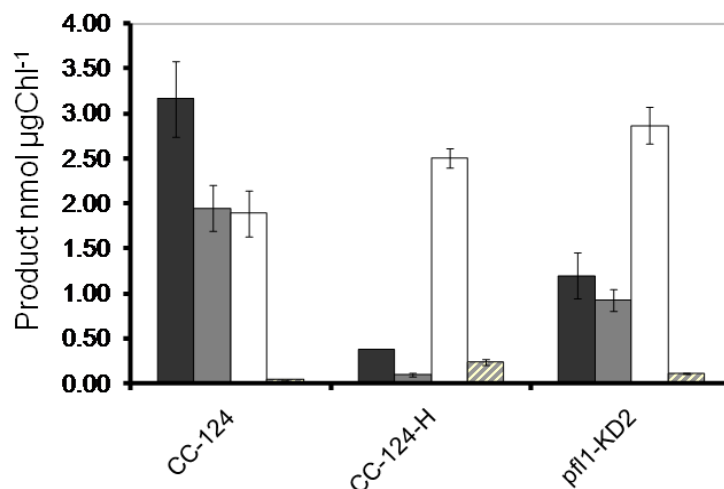


Figure IV.4.1: NMR analysis of dark-metabolite production comparing WT, WT + HP and *pfl1*-D2 cultures incubated in HSM.

Cultures were incubated under dark anaerobic conditions as described in section 2.12.1. Results are given for WT (CC-124), WT + 10 mM sodium hypophosphite (CC124-H) and *pfl1*-KD2 cultures. formate (dark grey), acetate (light grey), ethanol (white), lactate (light grey stripes). All values are given as the average of three biological replicates $\pm$ SE.

Appendix IV.5: Immunochemical analysis of fermentative enzyme expression in *pfl1*-KD2 during sulphur-deprivation

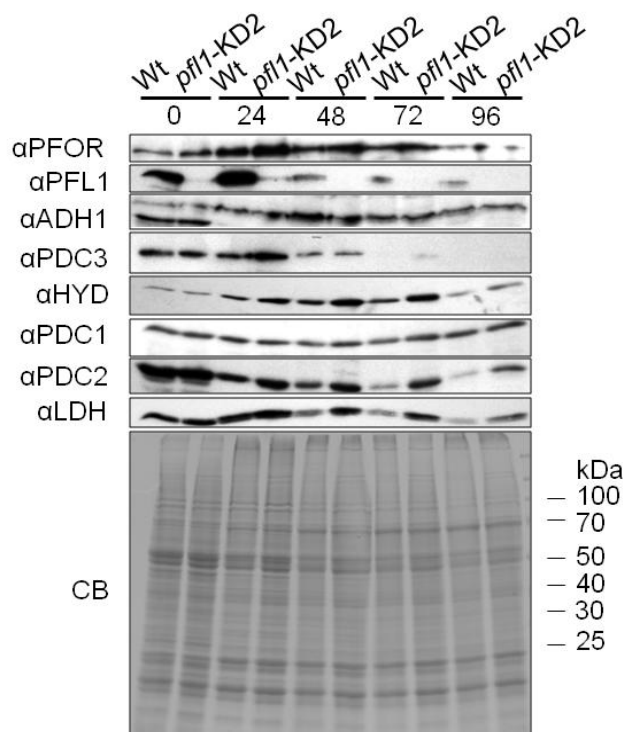
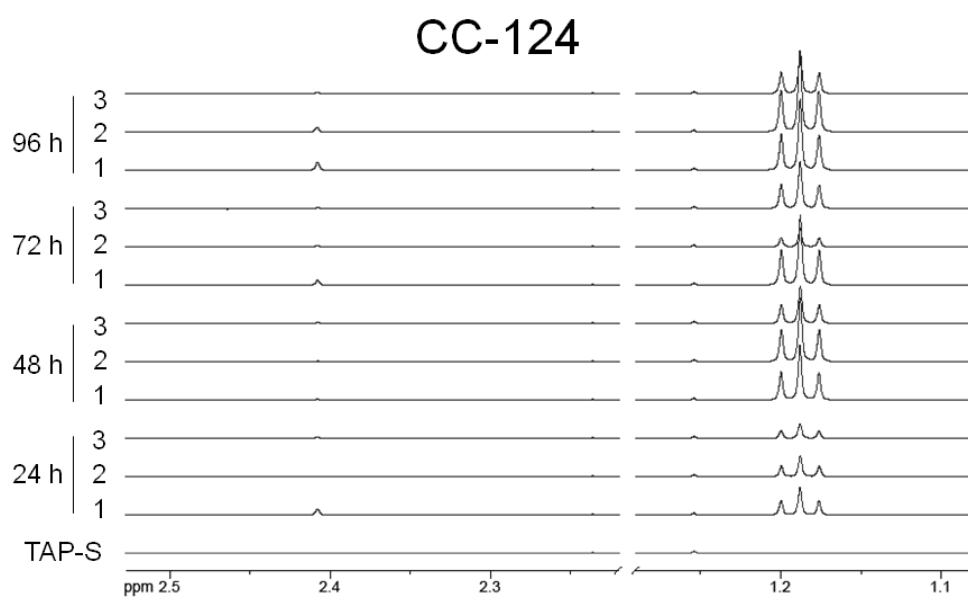


Figure IV.5.1: Immunochemical analysis of fermentative enzyme expression in *pfl1*-KD2 during sulphur-deprivation, volume according to 8×10^5 cells at $t = 0$ h loaded per lane. Loading control is provided by Coomassie blue (CB) stained gel.

Appendix IV.6: NMR spectra of medium samples collected during sulphur-deprivation

3-HB characteristic signals at 1.19 (d), 2.29, 2.40 (2 x dd) and 4.14 (m) ppm in the ^1H NMR spectra are found at 72 h in *pfl1*-KD2 and HP treated cultures which are absent in CC-124 (Figure IV.6.1)



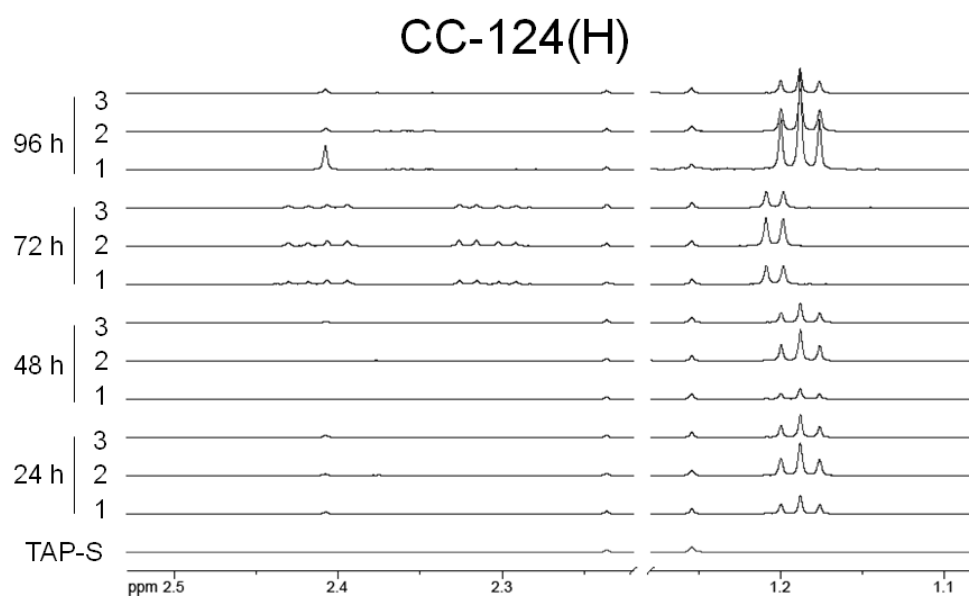


Figure IV.6.1: NMR spectra showing 3-HB accumulation in *pfl1*-KD2 and HP treated cultures at 72 h post sulphur-deprivation which is absent in WT CC-124. Comparison is provided with TAP-S starting medium, time and biological replicate are indicated (right).

Appendix IV.7: *pd3-KD* replicate blots

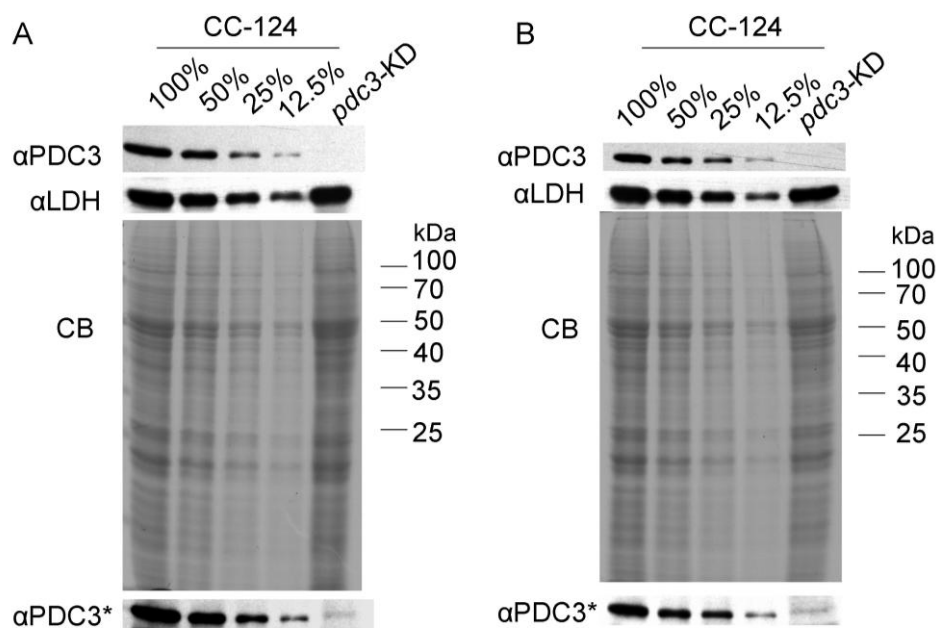


Figure IV.7.1: *pd3-KD* biological replicate blots, of aerobically grown TAP cultures, (A) Replicate 1 (B) Replicate 2, PDC3* denotes overexposed blots. 8×10^5 cells loaded per lane with the exception of WT dilution series were the percentage of total is indicated. Loading is provided by Coomassie blue (CB) stained gel.

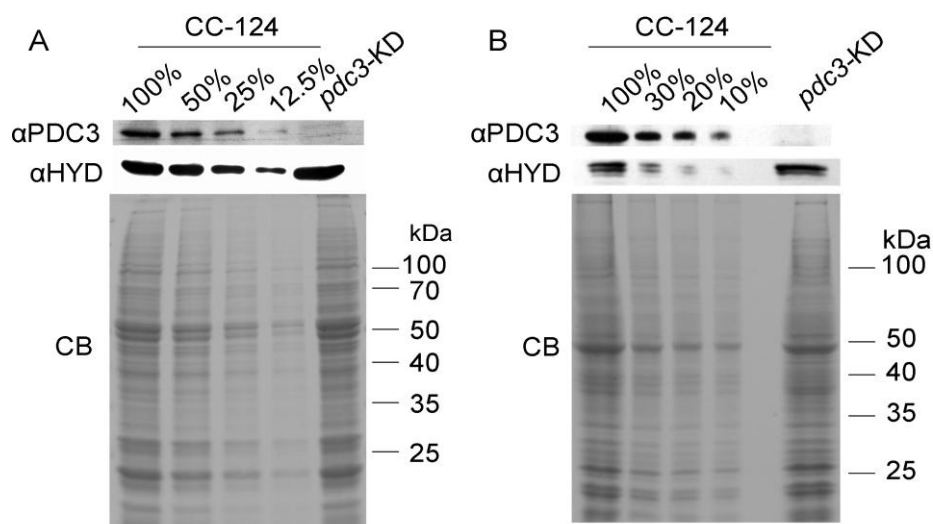


Figure IV.7.2: *pd3-KD* biological replicate blots after 4 h anaerobic incubation in AIB, (A) Replicate 2 (B) Replicate 3. 8×10^5 cells loaded per lane with the exception of WT dilution series were the percentage of total is indicated. Loading control is provided by Coomassie blue (CB) stained gel

Appendix IV.8: Comparison of fermentative enzyme expression in WT and *pdc3*-KD cultures during sulphur-deprivation

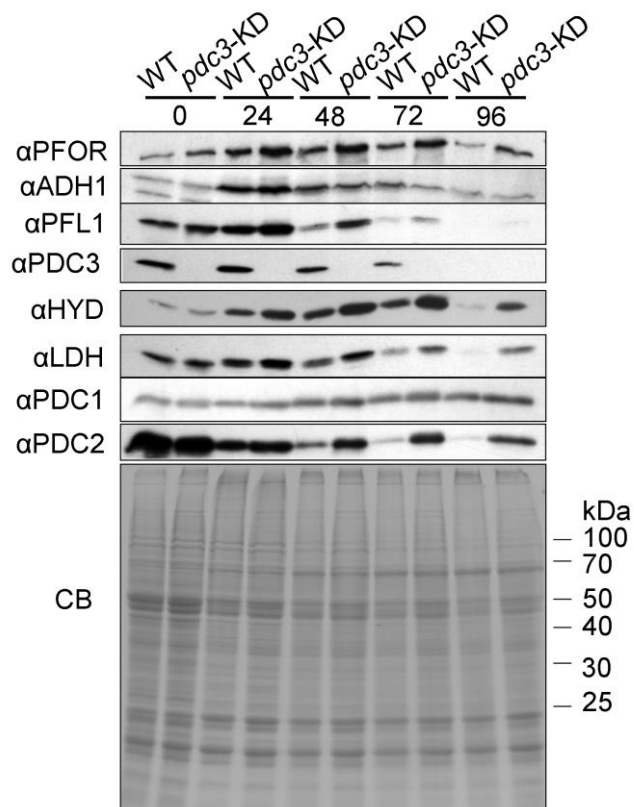


Figure IV.8.1: Immunochemical comparison of fermentative enzyme expression in WT and *pdc3*-KD cultures during sulphur-deprivation, volume according to 8×10^5 cells at $t = 0$ h loaded per lane. Loading control is provided by Coomassie blue (CB) stained gel.

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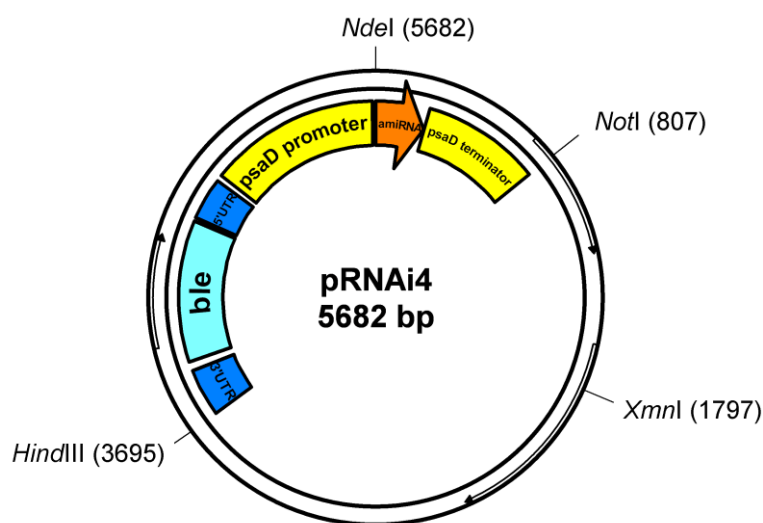


Figure IV.9.1: Vector map pRNAi4, showing *BLE* (zeocin) resistance cassette, and amiRNA cassette (orange) driven by the *PSAD* promoter.

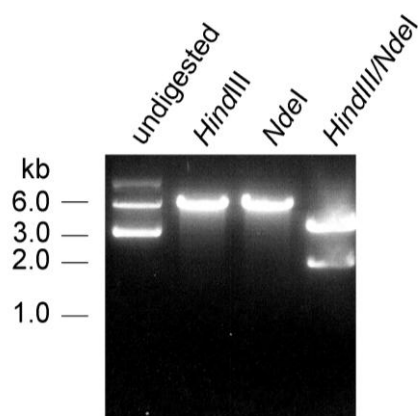


Figure IV.9.2: Restriction digest of vector pRNAi4, expected sizes HindIII (5682bp), NdeI (5682bp), HindIII NdeI double digest (1987bp and 3695bp).

Note: Requires sequencing for final verification


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CCGACACAGTGCTCGCCGAACAGCTTGATCACCGGGCCGGGCTCGCCGACCACTACGGGGTTGGTGCTCTCGCCGGGCACCCGCG
CACCGGCGGCACCGGCAGCCCGAGCTCCTCCAGGGCTCGGCGGGCCAGCGGCTGCAAATGGAACGGCGACGCAGGGTTAGATGCT
GCTTGAGACAGCGACAGAGGAGCCAAAAGCCTTCGTGACACAAATGCGGGCGTTGCAAGTCAAATCTGCAAGCACGCTGCCTGATC
CGCCGGGCTTGCTCGTGCAGTCACTCCAGAATTCCTGGTTCGTTCGCGAGGCTCGCGTAGGAATCATCCGAATCAATACGGTCGA
GAAGTAACAGGGATTCTTGTGTATGTTTGGGGTTGTGACTGAAACGAAGAGGAAGAGGGCCCCCTTAGCGGAGTGCGATCACA
AGCTCGAGTGGCCTGTGTAGAAGTGGTAGTGATCTAGGTGTTTGAATATGGCTTTGGTAGCTCGCTATAATGTCTTTGCAATCGGG
GGCCTGGCTATTTAAACAGCGCTCGCCCTGGAGCGGCATCGGAGCGCCATGCAGCCCCGAAGGAGCTTCGGGGGGTCGAAGCATC
ATCGGTGTTGCATGCAGCGCCGGGAAGCCGTCTCGCAGCCCGCCCTACCTTTTGCTGGAAGTGTATAGCGCAAGAAAGA CACAC
ACCTGCCCCGTCTGCCTGACAGGAAGTGAACGCATGTCGAGGGAGGCGCTCACCAATCGTCACACGAGCCCTCGTCAGAAACAGCTCT
CCGCCACGCTCTCCCTCTCACGGCCGACCCCGCAGCCCTTTTGCCCTTTTCTAGGCCACCGACAGGACCCAGGCGCTCTCAGCATG
CCTCAACAACCCGTA CTCTGCGCAGCGGTGCCCTTGTGCTGGTGATCGCTTGGAAAGCGCATGCGAAGACGAAGGGCGGAGCAGGC
GGCTGGCTGTTGGAAGGGCTCGCCGCCAGTTCGGGTGCCCTTTCCACCGCGCGCTCCACACCTACCGATCGGTGAAGGCGAGGCA
AATGCTCATGTTTGCCTCGAAGTTCGGAGTCTTAAAAAGCCGCTTCTTGTGCTCGTTCGAGACATGTTAGCAGATCGCAGTGCCAC
CTTTCCTGACGCGCTCGGCCCCATATTGGACGCAATTGTCATTGTAGCACAATTGGAGCAAATCTGGCGAGGCAGTAGGCTTTT
AAGTTGCAAGGCGAGAGAGCAAAGTGGGACGCGCGGTGATTATTGGTATTACGCGACGGCCCGCGCGTTAGCGGCCCTTCCCC
AGGCCAGGACGATTATGTATCAATATTGTTGCGTTCGGGCACTCGTGCGAGGGCTCCTGCGGGCTGGGGAGGGGATCTGGGAAT
TGGAGGTACGACCGAGATGCCTTGCTCGGGGGGAGGTTTCTCGCCGAGCAAGCCAGGGTTAGGTGTTGCGCTCTTGACTCGTTGT
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```

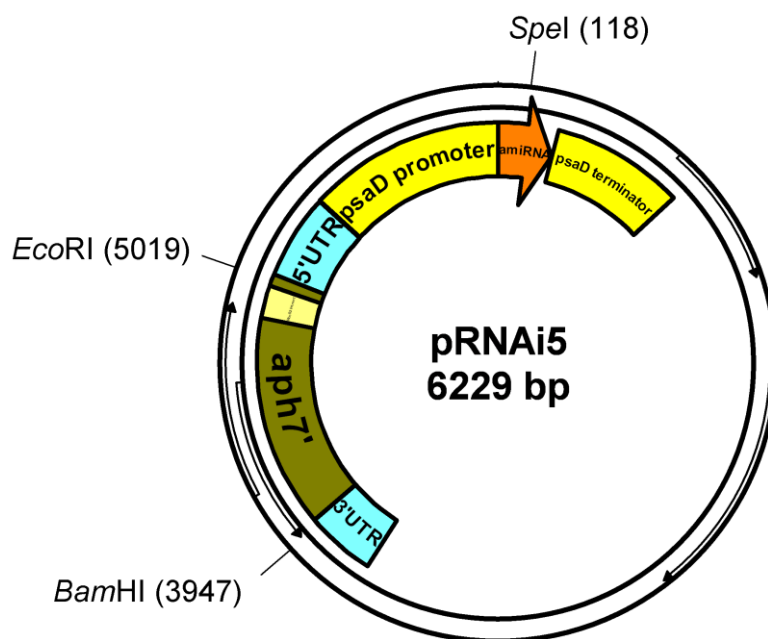


Figure IV.9.3: Vector map of plasmid pRNAi5. Displaying *aph7*^r (hygromycin) resistance cassette (green), amiRNA cassette (orange) driven by PSAD promoter (yellow).

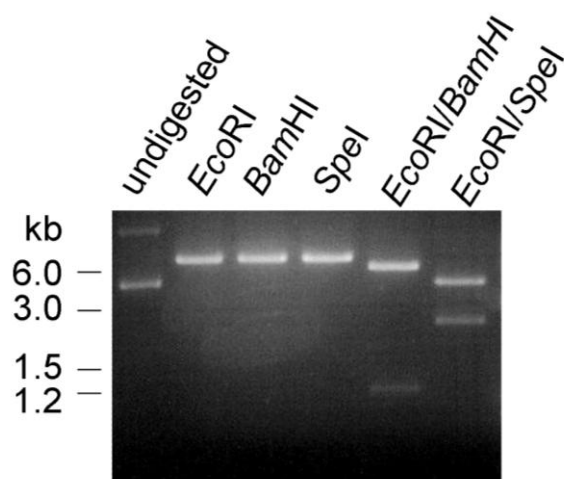


Figure IV.9.4: Restriction digest of artificial microRNA vector pRNAi5. Expected fragment sizes EorRI, BamHI and SpeI single digests (6229bp), EcoRI/SpeI double digest (1072bp and 5157bp) and EcoRI/SpeI double digest (1728bp and 4501bp)

Note: Requires sequencing for final verification

Appendix IV.10: Preliminary analysis of formate accumulation in *pfl1-KD* strain during sulphur-deprivation

Samples were taken from *pfl1*-KD cultures after 100 h sulphur-deprivation and formate content analysed by HPLC as described in materials and methods. Total amounts of formate were in good correlation with later repeat measurements by NMR using samples from different biological replicates, finding 85 nmol μgChl^{-1} formate for WT cultures (compared to 97 nmol μgChl^{-1} as measured by NMR), and 30 nmol μgChl^{-1} for *pfl1*-KD2 (compared to 26 nmol μgChl^{-1} when analysed by NMR).

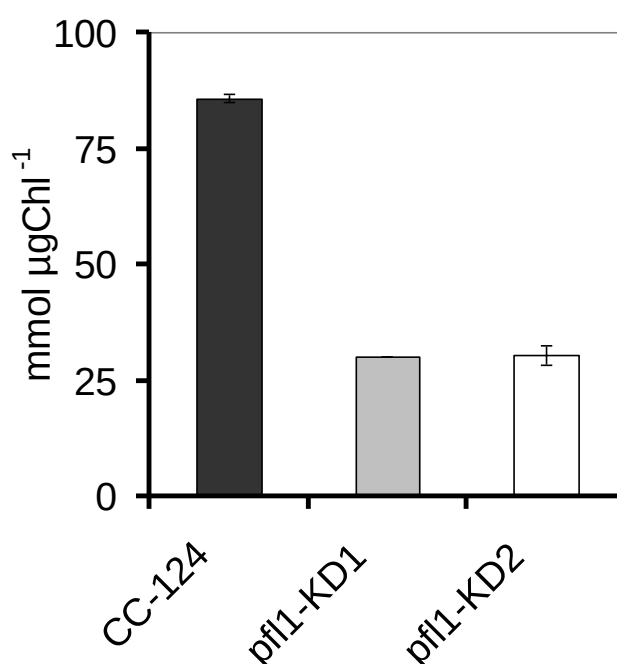


Figure IV.10.1: Graph comparing formate accumulation in *pfl1*-KD cultures after 100 h sulphur-deprivation as measured by HPLC, error bars are given $\pm$ SE of three measurements (with the exception of *pfl1*-KD1 which was only analysed once).

Appendix IV.11: Alternative model of fermentative metabolism during sulphur-deprivation I

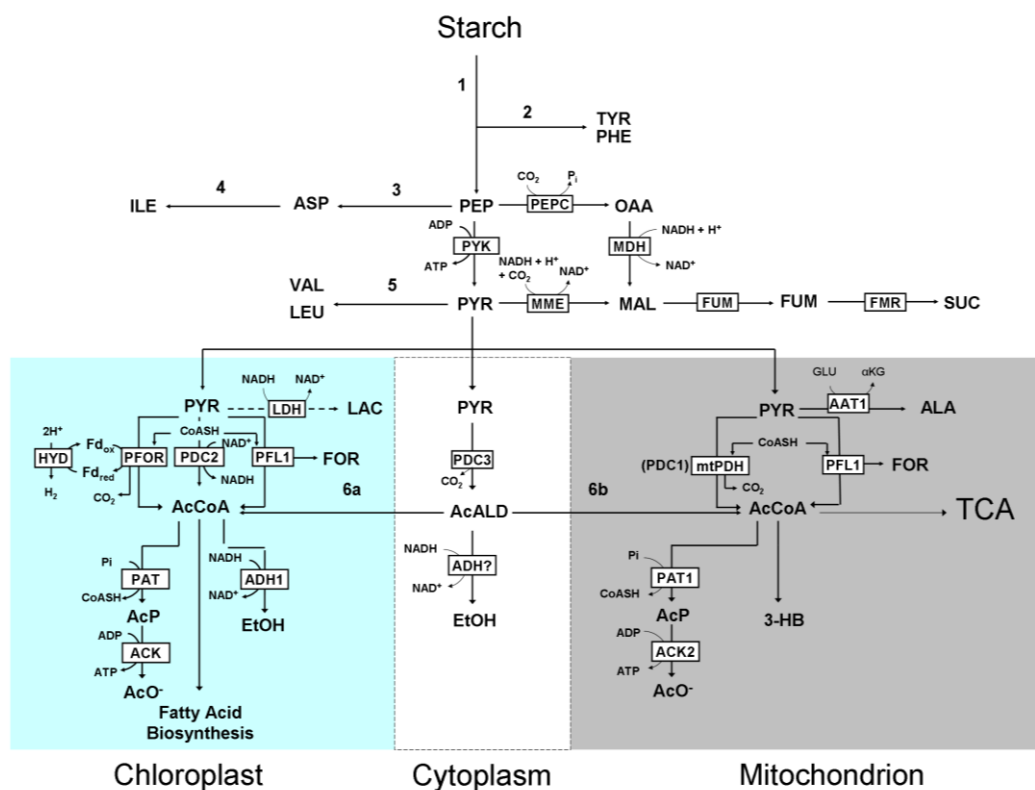


Figure IV.11.1: Alternative model of *C. reinhardtii* pyruvate degradation, amino acid synthesis and fermentative metabolism during sulphur-deprivation displaying potential chloroplast localisation for PFL1 (legend is as for Figure 4.19).

Appendix IV.12: Alternative metabolic pathways for acetaldehyde detoxification and acetyl-CoA transport

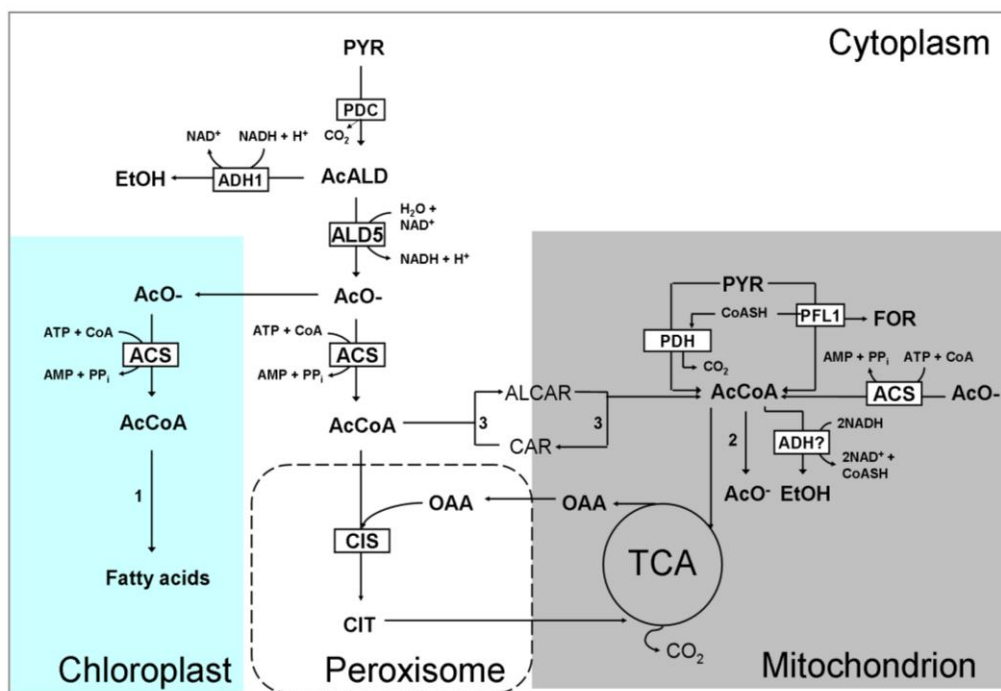


Figure IV.12.1: Alternative metabolic pathways for acetaldehyde detoxification during sulphur-deprivation. Compressed pathways (1) fatty acid biosynthesis (2) mitochondrial PAT/ACK pathway (3) acetyl-carnitine transport. Abbreviations: AcALD; acetaldehyde, AcO⁻; acetate, AcCoA; acetyl-CoA, ALCAR; acetylcarnitine, CAR; carnitine, CIT; citrate, EtOH; ethanol, OAA; oxaloacetate, PYR; pyruvate. Enzymes are in boxes, ACS; acetyl-CoA synthetase, ADH; alcohol dehydrogenase, ALD5; aldehyde dehydrogenase 5, PDC; pyruvate decarboxylase, PDH; pyruvate dehydrogenase complex, PFL1; pyruvate formate lyase.

Appendix IV.13: 3-HB production pathway

Proposed pathway for 3-HB formation in *pfl1*-KD2 and HP treated cultures, including potential route for ethanol uptake (Figure IV.12.1). Ethanol uptake has been located in the cytoplasm based on predicted aldehyde dehydrogenase (ALD5) localisation (Table 3.1).

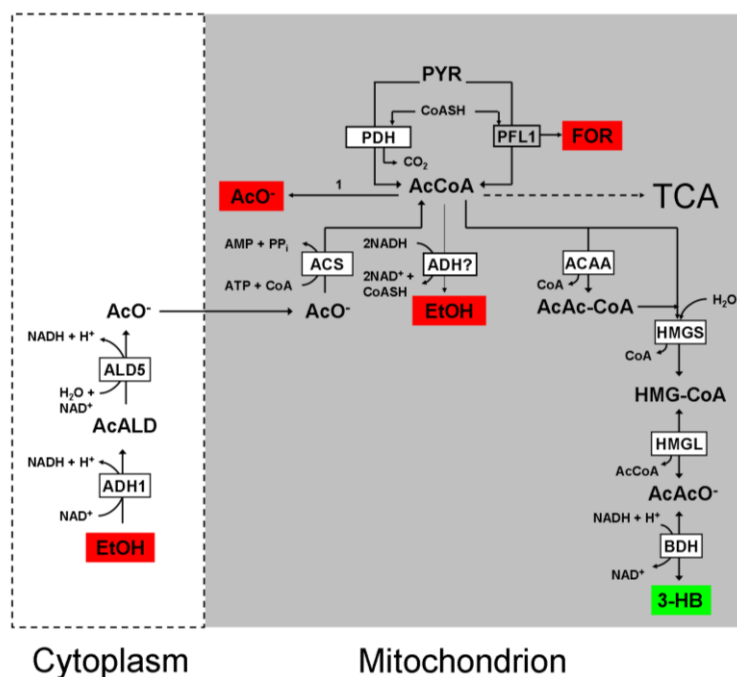


Figure IV.13.1: Alternative model of *C. reinhardtii* pyruvate degradation, amino acid synthesis and fermentative metabolism during sulphur-deprivation I, compressed pathways are represented by numbers (1) glycolysis (2) aromatic amino acid synthesis (3) aspartate synthesis (4) isoleucine synthesis (5) branched chain amino acid synthesis (6a and b) proposed routes for acetaldehyde detoxification (7) 3-hydroxybutyrate synthesis pathway. Only relevant amino acids are included based on NMR data. Localisation is provided where proven. Metabolite abbreviations: 3-HB; 3-hydroxybutyrate, AcAc-CoA; acetoacetate-CoA, AcAcO⁻; acetotacetate, AcALD; acetaldehyde, AcO⁻; acetate, AcCoA; acetyl-CoA, EtOH; ethanol, FOR; formate HMG-CoA; hydroxymethylglutaryl-CoA, PYR; pyruvate. Enzymes are in boxes, ACAA; acetyl-CoA C-acyltransferase, ACS; acetyl-coA synthetase, ADH; alcohol dehydrogenase, ALD5; aldehyde dehydrogenase, BDH; 3-hydroxybutyrate dehydrogenase, HMGS; hydroxymethylglutaryl-CoA synthase, HMGL; hydroxymethylglutaryl-CoA lyase, PDH; pyruvate dehydrogenase complex, PFL1; Pyruvate formate lyase. The mitochondrial PAT/ACK pathway has been compressed and labelled by number (1). In red are metabolites that decrease when PFL1 activity is blocked, in green are metabolites increased.

Chapter 7: References

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