Dissertation

Synthetic rewiring of *Chlamydomonas reinhardtii* to improve biological H_2 production

In accordance with the requirements for the academic degree of
Doctor of Natural Sciences (Dr. rer. nat)

Submitted by
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Completed at the faculty of Biology of Bielefeld University,
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under the supervision of Prof. Dr. Olaf Kruse

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To my mother,

Dakshayani Venkanna

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I would like to dedicate this thesis to my family back in India and thank them for their constant love and support through thick and thin. My German family in Bielefeld for making me feel welcomed and integrating me as a part of their family. Finally, I thank my wife Spoorthy Suneel, for her understanding and support. Any omission in this acknowledgement does not mean lack of gratitude.

Declaration

The following work is a result of my own efforts except where explicitly stated otherwise in the text. The usage of only specified and known resources such as images are accompanied by references. All literature and supplemental sources are cited accordingly.

The work was performed between March 2013 and February 2017 at Chair of Algae Biotechnology and Bioenergy, Universität Bielefeld under the supervision of Prof. Dr. Olaf Kruse.

I declare that this dissertation has been composed solely by me and it has not been submitted in whole or in part, to any other faculty in any previous application for an academic degree. I hereby apply, for the first time, for the degree of Doctor of Natural Sciences at the University of Bielefeld.

Deepak Venkanna

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Contents

Abbreviations

$^1O_2$:	Singlet oxygen
a.u.:	Arbitrary unit
amiRNA:	Artificial microRNA
AMPS:	Ammonium persulfate
APHIII:	Aminoglycoside (3') phosphotransferase III (Kanamycin resistance)
APHVII:	Aminoglycoside (3') phosphotransferases VII (Hygromycin B resistance)
APHVIII:	Aminoglycoside (3') phosphotransferase VIII (Paromomycin resistance)
bp:	Base pair
BSA:	Bovine serum albumin
C terminal:	Carboxyl terminal of a polypeptide
C. reinhardtii:	*Chlamydomonas reinhardtii*
CAH1:	Carbonic anhydrase 1
*c*CA:	Secretion signal of CAH1
cDNA:	Complimentary DNA
chl:	Chlorophyll
CO_2:	Carbon dioxide
Cyt b_6f:	Cytochrome b_6f complex
Da:	Dalton
DBMIB:	2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCMU:	3-(3,4 - dichlorophenyl)-1,1-dimethylurea
DNA:	Deoxyribonucleic acid
dNTP:	Deoxyribonucleoside triphosphate
dsDNA:	Double stranded DNA
DTT:	Dithiothreitol
et al.:	And others
EDTA:	Ethylenediaminetetraacetic acid
ETC:	Electron transport chain
Fd:	Ferredoxin
FNR:	Ferredoxin $NADP^+$ reductase
FNR:	Ferredoxin-NADPH-reductase
GC:	Gas chromatography
*g*Luc:	*Gaussia princeps* luciferase
GTC:	Guanidine thiocyanate

h:	Hour
H_2:	Hydrogen
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HSM:	High salt medium
HSP70A:	Heat shock protein 70A
HydA1/A2:	[FeFe]-hydrogenase A1/A2
i(1/2):	Intron 1 or 2 of RBCS2
IFR1:	Isoflavone reductase like protein in *C. reinhardtii*
kd:	knockdown
l:	Liter
LEF:	Linear electron flow
LHC:	Light harvesting complex
mRNA:	Messenger ribonucleic acid
MV:	Methyl viologen
mVenus:	Monomeric Venus yellow fluorescent protein
N terminal:	Amino terminal of a polypeptide
NADH:	Nicotinamide adenine dinucleotide (reduced form)
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
nm:	Nanometer
NR:	Neutral Red
O_2:	Oxygen
OD:	Optical density
PBR:	Photo-bioreactor
PC:	Plastocyanin
PCR:	Polymerase Chain Reaction
PEG:	Polyethylene glycol
PFL:	Pyruvate formate lyase
PFR:	Pyruvate ferredoxin oxidoreductase
PQ:	Plastoquinone
PsaD:	Gene that Codes for PSI reaction center subunit II
PSI or PSII:	Photosystem I or II
PTOX:	Plastidic terminal oxidase
RTqPCR:	Quantitative real time reverse transcription PCR

RB:	Rose bengal
RBCS (1/2):	Small subunit 1 or 2 of Rubisco
RES:	Reactive electrophile species
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
rpm:	Revolution per minute
RT:	Room temperature
Rubisco:	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S:	Sulphur
SDS:	Sodium dodecyl sulfate
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIR1:	Assimilatory sulfite reductase (ferredoxin)/ Sulfite reductase (ferredoxin)
TAP:	Tris acetate phosphate
TBS:	Tris Buffered Saline
TEMED:	Tetramethylethylenediamine
UTR:	Untranslated region

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Summary

The green algae, *Chlamydomonas reinhardtii* is capable of harvesting sunlight to synthesize energy needs and also evolve hydrogen under stress conditions. Photolysis of water giving rise to protons and electrons as substrates for hydrogen producing enzyme (hydrogenase) backed by cellular respiration ensures establishment of anaerobiosis, which is a pre-requisite for hydrogen production. Due to the properties of hydrogen, it has gained widespread attention as a clean fuel which has also set forth a development in the Chlamydomonas community. Photobiological hydrogen production from green algae is currently not economically viable due to low efficeincy of light to H_2 conversion. It has been shown that using a systematic approach towards genetically engineering strains can improve hydrogen yields. The aim of the following work was to improve hydrogen production via strain egineering. A previous study of transcriptome and metabolome of hydrogen producing culture served as a basis for the following work.

In the following study *C. reinhardtii* wild type CC124, mutant *stm6* and *stm6glc4* were used. CC124 is routinely used as a hydrogen producing wild type strain whereas *stm6* is a high hydrogen producing mutant with a manipulated state transition. *stm6glc4* is a derivative of *stm6* which is capable of taking up glucose and synthesize more starch that can fuel indirect pathway of hydrogen production. Hydrogen production was induced in air tight cultures of Chlamydomonas *via* sulfur deprivation. Potential target genes such as isoflavone reductase like protein (IFR1) and sulfite reductase (SIR1) were identified to be upregulated during H_2 production. A comparison between a high hydrogen producer (*stm6glc4*) and its parental (low hydrogen producing wild type, CC406) showed that the expression of *IFR1* was higher in the wild type. The role of IFR1 has been associated with stress tolerance in maize, rice, etc. but its function in Chlamydomonas is still unknown. SIR1 helps in sulfur assimilation process but by doing so it poses a competition for hydrogenase under sulfur deprived anaerobic hydrogen production conditions. Hence, a reverse genetic approach was adapted to counter these potential target genes.

Artificial microRNA (amiRNA) was used to create *IFR1* and *SIR1* knockdowns. The phenotype of the knockdowns was studied and their positive implication on H_2 production was established. *IFR1* knockdown was first created in CC124 wild type strain. Two knockdown mutants IFR1-1 and IFR1-6 with 35% and 5% of control level proteins were identified and confirmed by western blots. The phenotype of *IFR1*

knockdown mutants was analyzed by performing growth studies such as sulfur and nitrogen starvation, high light stress, ROS and RES stress. An electrophile response element was found in the promoter region of IFR1 which is believed to be under the control of singlet oxygen resistant (sor1) protein. IFR1::YFP fusion protein was done to confirm the cytosolic localization of IFR1. The knockdown mutants were found to be sensitive to RES due to a perturbed RES homeostasis but interestingly showed a prolonged PSII activity (Fv/Fm) under sulfur depletion. The sustained PSII activity resulted in a prolonged phase of hydrogen production (~2fold more hydrogen). The contribution of electrons (~80%) for a direct pathway of hydrogen production from a sustained PSII activity was confirmed by applying a PSII inhibitor (DCMU). Based on these findings, benefits of *IFR1* knockdown was extended to the mutant strain *stm6*. This again resulted in a sustained PSII activity which translated to ~70% more hydrogen production.

The competition for electrons between hydrogenase and SIR1 was overcome by applying amiRNAs in the mutant *stm6glc4*. The amiRNAs were fused to a luciferase reporter to influence the knockdown screening. Two knockdown mutants *sgh2* and *sgh3* with ~20-30% reduced levels of *SIR1* transcript were identified *via* RTqPCR and later confirmed by westernblot. The growth phenotype of the mutants were analyzed under photoautotrophic and photomixotrophic growths. The knockdown mutants were found to be slightly retarded in growth as compared to parental strain due to perturbed sulfur assimilation. Analysis of the hydrogen production phase showed that the knockdown mutants attained anaerobiosis faster than the parental strain and also had an increased rate of H_2 production (~17-35% higher rates compared to parental strain). The mutants retained the ability to take up glucose which contributed to an increase in hydrogen produced via indirect pathway. Though the mutants were more susceptible to sulfur starvation, the higher H_2 production rates boosted the overall H_2 productivity by ~35-55%. This study showed that molecular target such as *IFR1* and *SIR1* could be manipulated genetically to improve biohydrogen production.

1.0 Introduction

The following chapter will provide a brief history on “algae bio-hydrogen”, followed by an introduction to the eukaryotic green algae, *C. reinhardtii*. An overview of hydrogen production in the model organism and the underlining significance of sulfur deprivation will be discussed. Cellular acclimatization to sulfur depletion and changes in transcriptome serve as a foundation for the following study. Putative hydrogen enhancing target gene and a reliable scheme to tackle them with a knockdown strategy is covered. The potential of immobilization to improve robustness of hydrogen production and influence of RES/ROS homeostasis in cellular function will be highlighted.

1.1 General Introduction

The current scenario of global warming is an outcome of a growing global energy demand which is extensively fueled by fossil fuels (Adler et al. 2017; Frame et al. 2017). Usage of fossil fuels is not a sustainable solution due to the rising discrepancy between depleting resource and growing needs. Development of alternative renewable fuels such as solar, wind and hydro energy, biodiesel (Patel et al. 2012), hydrogen (T K Antal et al. 2011), etc is required to meet the energy demands of the future and also improve earth’s climate. Of the several alternative fuels being developed, hydrogen constitutes one of the most promising high energy carrier fuels whose combustion yields water. However, the applicability of H_2 as a fuel gravely depends on the economics of its sustainable large scale production.

Solar energy is an infinite source of renewable energy which could be readily harvested for sustainable production of microalgal biofuels (Norsker et al. 2011). Microalgae are known to effectively harvest and convert light energy into energy of chemical bonds which can be stored as fuels (T K Antal et al. 2011). One of the most direct means of obtaining a clean and promising fuel is production of biohydrogen (Allakhverdiev et al. 2009). Solar driven hydrogen production by microalgae is considered as a green process and a feasible alternative (Allakhverdiev et al. 2009; Ghirardi et al. 2009; Rupprecht et al. 2006) to the conventional method of hydrogen production i.e. steam reforming of natural gas (Kothari et al. 2008).

Hydrogen photoproduction by microalgae is coupled to photosynthesis and involves hydrogen catalyzing enzymes such as [FeFe]-hydrogenase in eukaryotes (Happe et al. 1993) and nitrogenase in prokaryotes (Howarth et al. 1985). Only few

species have evolved the ability to produce hydrogen from sunlight by recombining the protons and electrons generated from photolysis of water (Gaffron et al. 1942; Melis et al. 2000). However, the present means of hydrogen production is not efficient for a sustainable production process, partly because H_2 production is plagued by competition from metabolic processes like photosynthesis, respiration and fermentation (Kruse et al. 2005). The major challenge hindering H_2 process is the inactivation of the hydrogen catalyzing enzymes in presence of oxygen (Ghirardi et al. 2000). A visionary method was applied with eukaryotic green algae (*C. reinhardtii*) to create a temporal separation of water splitting and hydrogen evolution by subjecting cells to sulfur starvation in a sealed environment (Melis et al. 2000). Further improvements in biohydrogen production is required to meet sustainable large scale production which can be realized by tweaking the metabolic pathways *via* molecular biology (refer to chapters 4 and 5).

1.2 A look back on time: Algae hydrogen production

Hydrogen metabolism is predominantly observed in microorganisms belonging to domains of bacteria, archaea and eukaryotes (Ghirardi et al. 2000). Species belonging to the aforementioned domains can either use hydrogen to reduce certain reactions or synthesize hydrogen *via* fine-tuned biochemical pathways that are accompanied with physiological changes (Gfeller et al. 1984; Zhang et al. 2002). Few microalgae can utilize protons and electrons generated by photolysis of water and combine the substrates to give molecular hydrogen. Hydrogen production is catalyzed by enzymes that function under hypoxia such as hydrogenase (Happe et al. 1993) or ATP requiring nitrogenase (Berchtold et al. 1979). Hydrogenase have a higher turnover number compared to nitrogenase and do not require energy derived from ATP to catalyze the reaction. The following chapter will brief about the history and latest reports of molecular biology mediated enhancement of hydrogen production in eukaryotic green algae.

Hydrogen metabolism was first detected in the eukaryotic green algae Scenedesmus, where CO_2 was photo-reduced with the consumption of hydrogen (Gaffron 1939). It was not until 1942, when a landmark in the area of biological hydrogen was attained with the first evidence of photobiological hydrogen production from *Scenedesmus obliqus* (Gaffron et al. 1942). H_2 production was found distributed in several algae such as fresh water *Chlamydomonas reinhardtii* (Melis et al. 2000), *Chlorella vulgaris* (Hwang et al. 2014), marine green algae *Chlorococcum littorale*

(Ueno et al. 1999), *Lobochlamys culleus* (Meuser et al. 2009), etc. The ease of growth and readily available molecular tools favored *C. reinhardtii* to be chosen as a model organism to further pursue hydrogen production. However, the enzyme catalyzing hydrogen production (hydrogenase, HydA1) was reported to be irreversibility inhibited in the presence of molecular oxygen (Happe et al. 1993). Unless photosynthetic oxygen could be spatially entangled from hydrogen production, sustainable H_2 production could not be achieved. Scientists worked on various procedures to establish hydrogen production, eg. dark adaptation followed by illumination yielded 0.02-0.34 $mlH_2\ l^{-1}\ h^{-1}$ (Brand et al. 1989), flushing dark adapted cultures with argon to create anaerobic environment for H_2 production (Happe et al. 1994) and adapting cells to dark/light cycles with an aim to establish a two phase biophotolysis yielded ~0.65-0.85 mlH_2 (Miura et al. 1982). A breakthrough was achieved by Melis and co-workers where for the first time a two stage H_2 production process with rates of 2.05 $mlH_2\ l^{-1}\ h^{-1}$ was established by depriving the cells of sulfur (Melis et al. 2000).

Development of sulfur deprived hydrogen production under continuous illumination served as a platform for future studies. However, the prolonged lack of sulfur limited the operation time to ~120 h because the algal cells could not generate enough energy to sustain (refer to Chapter 1.6). The issue of limited H_2 production phase was addressed by micro feeding sulfur leading to a prolongation of 20-100 days (Kim et al. 2010; Laurinavichene et al. 2006; Oncel et al. 2009). Careful consideration of process parameters such as pH (Kosourov et al. 2003), partial pressure of H_2 (Kosourov et al. 2012), temperature, culture mixing, light regime and intensity (Tamburic et al. 2012) have all shown to play a vital role in improving H_2 production (Wang et al. 2009). Of the several methods available the most direct way to improve hydrogen production is by genetic engineering (Dubini et al. 2014). Significant hydrogen producing mutants developed since 2000 are shown in Table 1.

Table 1: Hydrogen production of genetically engineered eukaryotic green algae

Species / Strain	**Description**	**Total vol. of H_2 [ml/l]**	**Chlorophyll [µg/ml]**	**Reference**
C. reinhardtii pgr5	Knockout of proton gradient regulation like protein 5	850	15	(Steinbeck et al. 2015)
C. reinhardtii pgr5/pgr1	Double knockout of PGR1 and PGR5	610	15	(Steinbeck et al. 2015)
C. reinhardtii Fnr	Knockdown of Ferredoxin-NADP reductase	588	25	(Sun et al. 2013)
Chlorella sp. DT *antiPSBO*	Knockdown of PSBO	350	-NA-	(Lin et al. 2013)
C. reinhardtii stm6glc4L01	Simultaneous knockdown of LHCBM 1, 2 and 3	390	14.5	(Oey et al. 2013)
C. reinhardtii L159I-N230Y	Double point mutation of D1 protein	700	18	(Scoma et al. 2012)
C. reinhardtii pgrl1	Knockout of proton gradient regulation like protein 1	580	15	(Tolleter et al. 2011)
C. reinhardtii stm6glc4	Integration of hexose uptake protein 1 to *stm6*	150% *stm6*	26	(Doebbe et al. 2007)
C. reinhardtii stm6	DNA insertional mutagenesis blocks state transition	540	26	(Kruse et al. 2005)
C. reinhardtii Y67A	Site directed mutagenesis reduces activity of RuBisCo	16	8	(Pinto et al. 2013)
C. reinhardtii tla1	Truncated antenna	~4 folds higher than CC4169	-NA-	(Kosourov et al. 2011)
C. reinhardtii antiSulp	Repression of *Sulp* gene impairs sulfate uptake	~4 folds higher than CW15	-NA-	(Chen et al. 2005)

NA: Not Applicable

1.3 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas reinhardtii (*C. reinhardtii*), is a unicellular, eukaryotic green algae that can grow in light (autotrophic) or in dark (heterotrophic) in presence of acetate (Harris 2009). Chlamydomonas is classified under the kingdom of viridiplantae into class chlorophyceae with several species isolated from many common sources like soil, fresh water, sea, etc. The model organism *C. reinhardtii*, is microscopic ~10 µm in size whose motility is assisted by two apical flagella (fig.1). The cell encompasses a predominant cup-shaped chloroplast which houses the photosynthetic machinery and the chloroplast genome. Several vital metabolic processes such as biosynthesis of starch, lipids, carotenoids and anaerobic process like hydrogen production occur within the chloroplast (Ball 1998; Ghirardi et al. 2000; Libessart et al. 1995; Lohr et al. 2005; Work et al. 2010). Mitochondria, chloroplast and nucleus house their respective genomes. An eyespot helps in phototaxis (Stavis et al. 1973).

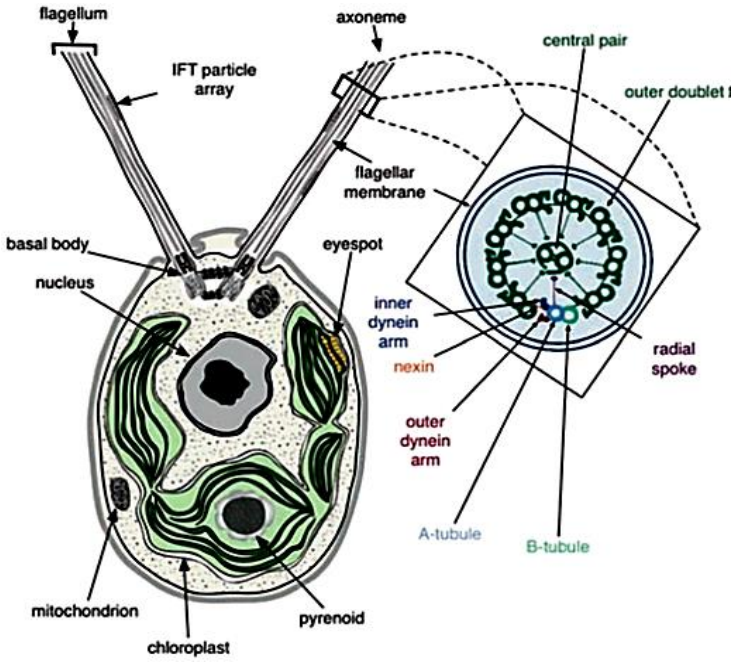

Figure 1: A schematic representation of *C. reinhardtii* cell derived from transmission electron micrograph as shown by (Merchant et al. 2007). A distinct nucleus housing the nuclear genome, mictochondria, eye spot and cup-shaped chloroplast with pyrenoid are shown. Flagella anchored to the basal body along with a cross section of the flagellar axoneme showing nine outer doublets and central pair microtubules is depicted.

Over a billion years ago, green algae belonging to chlorophyceae diverged from land plants with cell walls composed of hydroxyproline-rich glycoproteins (Merchant et al. 2007). The cell wall is associated with sugars such as arabinose, galactose, glucose

and mannose (Ferris et al. 2001; Woessner et al. 1992). Chlamydomonas can reproduce asexually and nitrogen limitation triggers sexual reproduction. In vegetative form the genome of *C. reinhardtii* is haploid, making it a “hot target” for gene manipulation. The genome was successfully sequenced and identified to be ~120 Mb in size, carrying over 15000 genes on 17 chromosomes (Merchant et al. 2007).

C. reinhardtii has served as a model organism for several studies because of the ease with which it can be grown either photoautotrophically, heterotrophically or mixotrophically. The presence of a large chloroplast ensures efficient harvest of solar energy resulting in superior biohydrogen production compared to other chlorophycean algae (Meuser et al. 2009). The availability of well annotated mitochondrial (Vahrenholz et al. 1993), chloroplast (Maul et al. 2002) and nuclear (Merchant et al. 2007) genome, along with transcriptome (Nguyen et al. 2011; Toepel et al. 2013), metabolome (Doebbe et al. 2010; Matthew et al. 2009) and proteome (Chen et al. 2010; Wienkoop et al. 2010) makes chlamydomonas a suitable host for genetic manipulation. These advancements have paved way to genetically improve strains and use them as an industrial biotechnological host (Scaife et al. 2015).

Genetic manipulation of mitochondria (Randolph-Anderson et al. 1993) and chloroplast (Boynton et al. 1988) genomes occur *via* homologous recombination which has led to *C. reinhardtii* being termed as photosynthetic yeast (Rochaix 1995). However, nuclear transformation is a tedious task as it does not comply by the rules of homologous recombination (Zorin et al. 2005), making random insertional mutagenesis (Dent 2005) or UV mutagenesis (Schierenbeck et al. 2015) as preferred means for developing knockout mutants. Genetic transformations can be accomplished by particle bombardment (Boynton et al. 1988), electroporation (Shimogawara et al. 1998; Yamano et al. 2013), glass bead (Kindle 1990) or agrobacterium mediated transformation (Kumar et al. 2004). Distinct selection markers are available to aid selection of transformants with desired phenotype (Debuchy et al. 1989; Goldschmidt-Clermont 1991; Lumbreras et al. 1998; Mayfield et al. 1990). Codon optimized reporter genes such as GFP (Fuhrmann et al. 1999) and luciferase (Fuhrmann et al. 2004) have been shown to assist nuclear gene expression. Numerous native promoters are established and routinely used for expression studies. Frequently used constitutive promoters include *HSP70A* (Schroda et al. 2000), *RBCS2* (Lumbreras et al. 1998) and *PSAD* (Fischer et al. 2001) whereas inducible promoters such as ammonium responsive *NIT1* (Ohresser et al. 1997), CO_2 responsive *CA1* (Villand et al. 1997) and

iron responsive *ATX1* (Fei and Deng 2007) are also available. Knockout mutants have been created for genetic analysis by random insertional mutagenesis (Gonzalez-Ballester et al. 2011; Li et al. 2016; Posewitz et al. 2005; Zhang et al. 2014) or TILLING (Targeting Induced Local Lesions In Genome) (Gilchrist et al. 2005). However, the mutants created need to be screened *via* PCR or phenotypic screening which is labor and time intensive. The only other alternative presently available for a targeted manipulation of genome is by RNA knockdown (Molnar et al. 2009). This method has been successfully proven to downregulate target pathways in *C. reinhardtii* (Burgess et al. 2012; Li et al. 2015; Sun et al. 2013). With such a bulk information and molecular tools, *C. reinhardtii* could be made a work horse for industrial biotechnology.

1.4 Hydrogen production by *C. reinhardtii*

Hydrogen production by microalgae was first reported in the anaerobic adapted green algae *Scenedesmus* (Gaffron 1939; Gaffron et al. 1942). It was shown that green algae possess special enzymes termed hydrogenase which combine protons arising from water photolysis with electrons to yield molecular hydrogen. After several decades of research, *C. reinhardtii* was found to have the highest hydrogen production rates among chlorophyceae (Timmins et al. 2009). Chlamydomonas generates ATP and synthesizes organic compounds during oxygenic photosynthesis. However, subjecting the algal cells to hypoxic conditions leads to the induction of oxygen sensitive FeFe-hydrogenases (*HydA1* and *HydA2*) that catalyze reversible reaction of hydrogen production (Happe et al. 1993). *HydA1* is the most important of the two hydrogenases encoded by Chlamydomonas as shown by a *HydA2* knockdown study (Godman et al. 2010). *HydA1* is highly sensitive to molecular oxygen causing irreversible inactivation within minutes of exposure (Stripp et al. 2009).

Hydrogen production not only helps in generating ATP but also serves as a safety valve in releasing the reductant burden that has built up within the cell during anaerobic conditions (Rupprecht et al. 2006). Hydrogen production in *C. reinhardtii* is known to occur under anaerobic conditions *via* direct (PSII dependent) and indirect (PSII independent) pathways, where both these pathways require the photosynthetic electron transport chain (PETC) for efficient conduction of electrons through PSI to ferredoxin (Fdx) and finally hydrogenase (fig.2). The biggest difference between the two pathways is the source from which the electrons are generated. Direct pathway involves generation of electrons *via* photolysis of water whereas in the indirect pathway

reductant (NADH) arising from starch metabolism fuel electrons into the PETC *via* the enzyme NADPH-plastoquinone reductase (NPQR) (Ghirardi et al. 2009; Melis 2007). Another indirect pathway for hydrogen production involves the direct reduction of ferredoxin by pyruvate ferredoxin oxidoreductase (Grossman et al. 2011; Philipps et al. 2011).

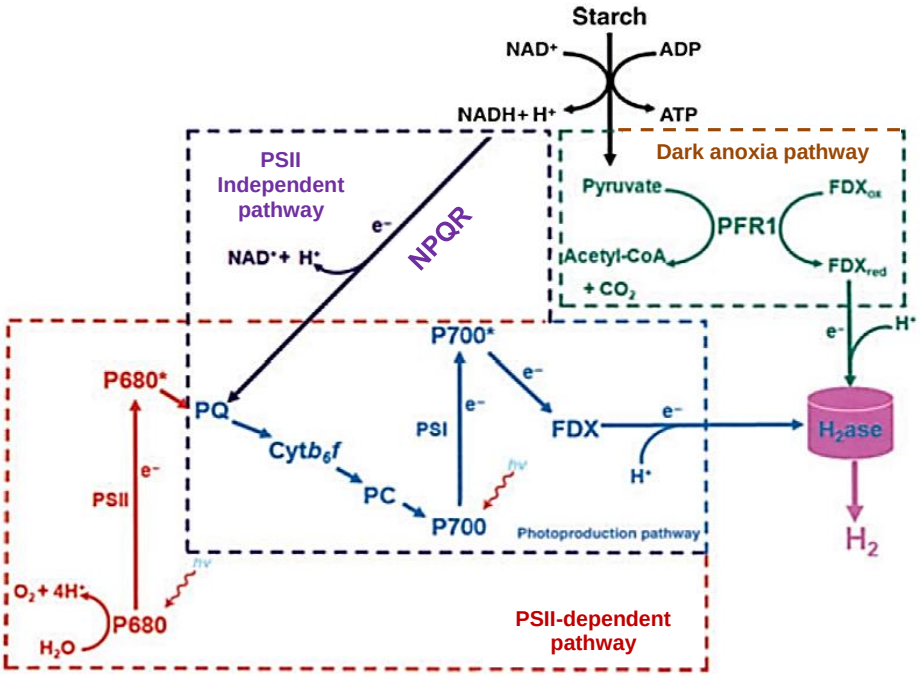

Figure 2: A modified pictorial representation of hydrogen production pathways in *C. reinhardtii* as shown by (Grossman et al. 2011). PSII-dependent direct pathway (red dashed line) and PSII-independent indirect pathway via NADPH-plastoqunione reductase (NPQR) (purple dashed line) for hydrogen production is shown. Both pathways involve reduction of plastoquinone (PQ) pool and transfer of electrons through carriers such as cytochrome b_6f complex ($Cytb_6f$) and plastocyanin (PC) to PSI. At PSI, reduction of ferredoxin (FDX) and subsequent transfer of electrons to hydrogenase (H_2ase) occurs. Another pathway fueling H_2 production under dark anoxic conditions (green dashed line) involves coupling of pyruvate oxidation with FDX reduction by the enzyme pyruvate-FDX-oxidoreductase (PFR1).

1.5 FeFe-hydrogenase drives hydrogen production in *C. reinhardtii*

The first finding of hydrogen production (Gaffron 1939) paved way for several milestones in the field of algae biohydrogen. Studies analyzing several unicellular green algae showed *C. reinhardtii* to be the most efficient of them all (Ben-Amotz et al. 1975; Stuart et al. 1972). The enzyme hydrogenase catalyzes hydrogen production by combining two protons with two electrons to yield molecular hydrogen. Chlamydomonas hydrogenase was characterized (Happe et al. 1993) to comprise of

an Fe metal ion core and the enzyme was reported to be localized in the chloroplast (Happe et al. 1994). Hydrogenase usually contain one or more FeS clusters that assist electron transfer (Armstrong 2004). Chlamydomonas genome encodes two hydrogenases (HydA1, HydA2) of which HydA1 is the predominant enzyme as shown by RNA silencing study (Godman et al. 2010).

HydA1 is highly sensitive to oxygen (Stripp et al. 2009), hence the enzyme is induced and functional only under strict anaerobic conditions (Happe et al. 2002). The enzyme is about 53 kDa, comprising of a [4Fe4S] cluster connected to a [2Fe2S] cluster which is stabilized by a CO and CN ligand (fig.3) (Peters 1999; Posewitz et al. 2009).

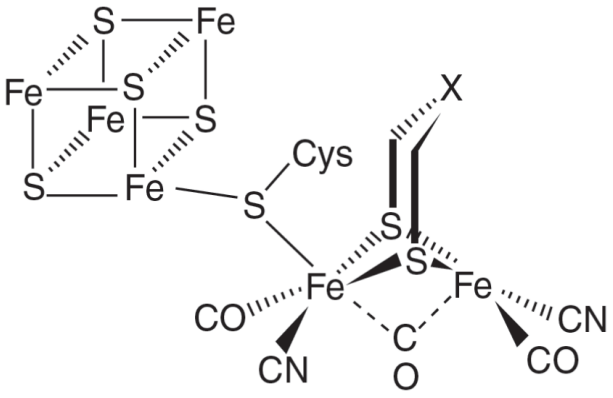

Figure 3: Schematic representation of [FeFe]-hydrogenase core cluster or H-cluster in *C. reinhardtii* as shown by (Posewitz et al. 2009). [4Fe4S] cluster is connected to [2Fe2S] *via* dithiolate bridge.

The [4Fe4S] cluster receives electrons from the reduced ferredoxin and transfers them to [2Fe2S] cluster resulting in reduction of a distal iron atom which later binds to a proton yielding a doubly reduced hydride ion. The hydride ion combines with another proton at the active site generating molecular hydrogen. FeFe-hydrogenase has a high turnover rate of ~9000 enzyme units per second (Ghirardi et al. 2009) but is highly susceptible to oxygen. The enzyme undergoes maturation which is carried out by gene products of *HYDEF* and *HYDG* (Posewitz et al. 2004). *HYDEF* and *HYDG* encode two radical S-adenosylmethionine (Radical SAM) proteins where HYDG and HYDE domain of HYDEF belong to the Radical-SAM superfamily and the HYDF carries a GTPase domain (Ghirardi et al. 2007). Radical-SAM proteins bring about the synthesis of active site precursors of [FeFe]-hydrogenase and catalyze the assembly of metallocluster. The exact maturation mechanism is yet unkown but the transit

peptide in the maturation protein helps in chloroplast targeting of the hydrogenase (Posewitz et al. 2009).

The mechanism of oxygen attack and inactivation of the enzyme's active site is well documented (Lee et al. 2003; Stripp et al. 2009). Oxygen can diffuse into the active site and irreversibly inactivate the H-cluster, which also results in inhibition of hydrogenase expression (Happe et al. 2002). The protein structure of the enzyme provides a certain degree of resistance to the diffusion of oxygen and enables the enzyme to function only under anaerobic conditions (Cohen et al. 2005). Traces of hydrogenase expression was reported as early as 15 min after the establishment of anaerobiosis which was confirmed by reporter gene assays (Stirnberg et al. 2004). A 100-fold increase in the transcript of hydrogenase was observed under anoxic condition (Mus et al. 2007). The starch less mutants *sta6* (Zabawinski et al. 2001) and *sta7* (Posewitz et al. 2004) possess a relatively oxidized PQ pool which resulted in a very low hydrogenase induction compared to the control strain (Posewitz et al. 2009). This finding suggested the necessity of a reduced PQ pool for hydrogenase induction. This shows the stringent regulations governing H_2 production in *C. reinhardtii*. The oxygen phobic hydrogenase makes the hydrogen production process a daunting task. However, this has been overcome by inducing anaerobiosis as a result of sulfur deprivation in cells (Melis et al. 2000) which will be discussed in detail in the next chapter (Chapter 1.6).

1.6 Acclimation to sulfur starvation and H_2 production

The oxygen susceptibility of hydrogenase enzyme makes it impossible to produce hydrogen in Chlamydomonas under aerobic conditions. Over ~80% of hydrogen production mainly arises by light driven PSII dependent direct pathway (Volgusheva et al. 2013) but the presence of light also results in photosynthetic oxygen evolution which causes irreversible inhibition of the enzyme and cessation of hydrogen production (Forestier et al. 2003; Happe et al. 2002; Mus et al. 2007). Several methods have been tested to make an anaerobic environment that can assist hydrogen production, ex. Incubation of cultures in dark, sparging cultures with inert gases (nitrogen, helium or argon) (Greenbaum 1988), using oxygen scavengers or absorbers (Benemann 1997; Hallenbeck et al. 2002), inactivating PSII with chemical inhibitors like DCMU (Bamberger et al. 1982; Healey 1970) and many more. However, the most

reproducible method currently in practice for lab scale H_2 production involves subjecting the cells to sulfur deprivation (Melis et al. 2000).

In the year 2000, Melis and co-workers published their breakthrough results where they successfully separated photosynthetic oxygen evolution and anaerobic hydrogen production into a two-phase biophotolysis process which was induced by sulfur deprivation (Melis et al. 2000). The novel approach involved transferring Chlamydomonas cells into a completely sealed (air free) sulfur deficient environment. Under such conditions, respiration arising mainly due to mitochondria and also chlororespiration help in consumption of photosynthetically evolved oxygen. Over time the rate of respirational oxygen consumption would surpass oxygen evolution resulting in an anaerobic environment leading to induction of hydrogenase and the start of hydrogen production. The key attribute for switch in the algal metabolism from aerobic photosynthesis to an anaerobic hydrogen evolution is triggered by the absence of sulfur.

Sulfur is an essential micronutrient that doubles as a building block of several cellular biomolecules such as amino acids (methionine and cysteine), membrane lipids (sulfoquinovosyl diacylglycerides, SQDG), redox protectant glutathione pool, electron carriers (Fe-S clusters), coenzymes and many more. Chlamydomonas senses sulfur in its environment *via* sulfur acclimation gene (*sac1*) (Gonzalez-Ballester et al. 2008; Ravina et al. 2002; Takahashi et al. 2001) and sulfur depletion induces cascade of reactions involving upregulation of extracellular arylsulfatase that cleave sulfate from aromatic compounds (de Hostos et al. 1989) and overexpression of sulfate transporters (Davies et al. 1994; Yildiz et al. 1994). The sulfur economy in the cell changes by synthesizing sulfur free proteins or proteins with limited sulfur such as Isoflavone reductase like protein (IFR1) and Light Harvesting Complex (LHCBM9) (Grewe et al. 2014; Nguyen et al. 2008). Sulfur is also recycled from the degradation of sulfolipids or by depleting gluthathione pool (Petrucco et al. 1996). The first 24 hours of depletion results in cessation of cell doubling which is followed by an increased starch accumulation (up to 10-fold) (Tsygankov et al. 2006; Liping Zhang et al. 2002). Starch is metabolized during prolonged periods of sulfur starvation to derive energy and also fuel hydrogen production *via* NPQR mediated PSII independent pathway (Antal et al. 2003; Chochois et al. 2009) (fig.4). Photosynthetic machinery also undergoes modification with downregulation of several genes associated with carbon dioxide fixation.

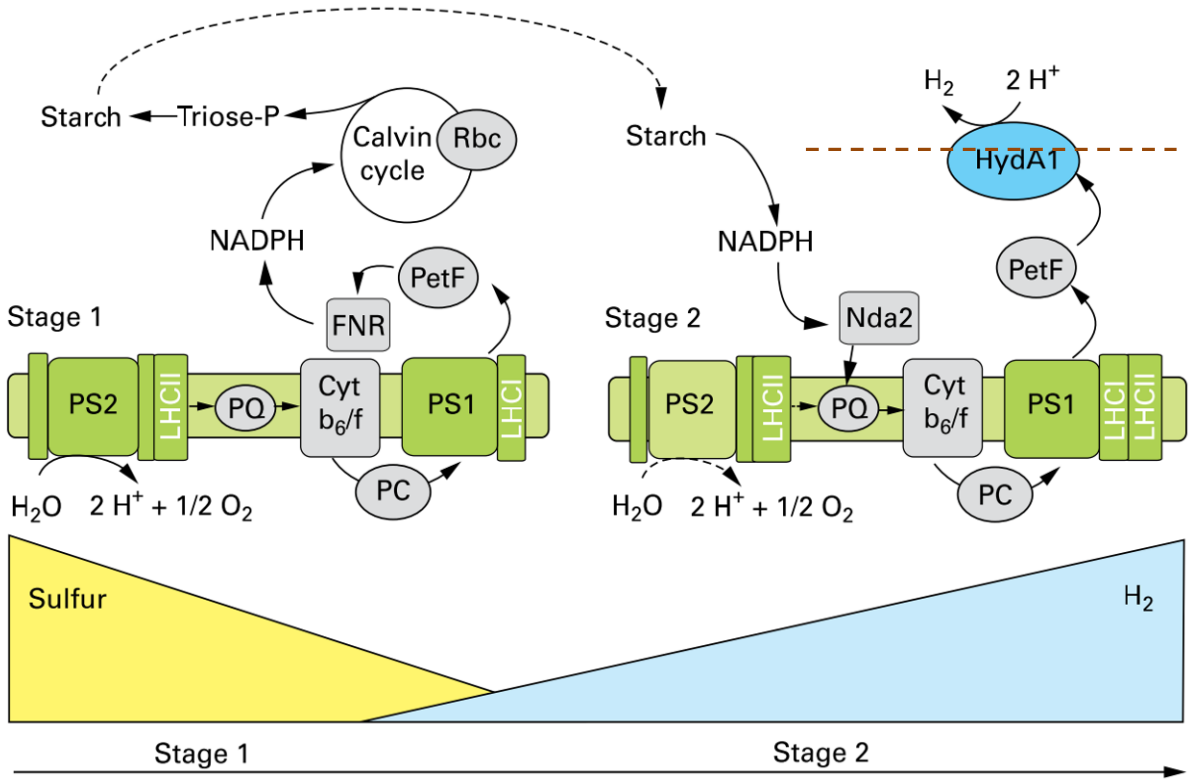

Figure 4: A two-phase biophotolysis hydrogen production process in sulfur deprived *C. reinhardtii* as shown by (Happe et al. 2010). The first stage of decrease in sulfur concentration is represented in yellow (triangle) and the second hydrogen production stage is shown in blue (triangle). During the stage 1, electrons arising from photolysis of water at photosystem two (PS2) is transported through electron carriers plastoquinone (PQ), cytochrome b_6f complex ($Cytb_6f$) and plastocyanin (PC) to photosystem one (PS1). PS1 transfers electrons to ferredoxin (PetF) which further donates it to generate NADPH by ferredoxin NADP+ reductase (FNR). NADPH is used up by ribulose-1,5-bisphosphate carboxylase/oxygenase (RbC) to fix carbon dioxide *via* Calvin cycle. In stage 2, decrease in PS2 photosynthetic activity (light green color and dotted line) and migration of LHCII to PS1 is shown. Cellular respiration leads to anaerobiosis. Electrons arising from residual PS2 activity and degradation of starch are transferred to PetF which is the final electron donor to hydrogenase (HydA1).

The levels of Rubisco decline over time and might be recycled to serve as a sulfur source as observed in *L. minor* (Ferreira et al. 1992). The reduction of Rubisco results in shutdown of CO_2 fixation (Melis et al. 2000; Liping Zhang et al. 2002), thereby ending electron competition faced by hydrogen production (Cinco et al. 1993). Sulfur deprivation also causes a reduction in photosynthetic activity but the cellular respiration (mainly mitorespiration, Antal et al. 2003b) fueled by a carbon source such as acetate

(Endo et al. 1996; Fett et al. 1994) remains unaltered during the early hours (fig.4). The D1 protein of the PSII reaction core undergoes damage under illumination and unless adequately repaired (sulfur replete conditions), results in loss of PSII activity (Komenda et al. 2012; Nixon et al. 2010). The loss in PSII activity was confirmed by assessing the photosynthetic complexes (Volgusheva et al. 2013; Liping Zhang et al. 2002) and an increase of photo protection pigments such as antheraxanthin, and zeaxanthin was also observed (Wykoff et al. 1998). This results in decreased rate of photosynthetic oxygen evolution which is superseded by the rate of respirational oxygen consumption, thereby establishing anaerobiosis in a sealed environment. These conditions create a favorable environment for the induction of [FeFe]-hydrogeanses which catalyzes hydrogen production (Antal et al. 2003). The hydrogen production lasts anywhere between 4 to 5 days depending on the culture density, bioreactor size, light intensity, temperature, pH, etc (Tamburic et al. 2011). Over time, the rate of hydrogen production decreases and finally stops due to loss in cellular functions and accumulation of toxic fermentative products (Doebbe et al. 2010). Micro feeding of sulfur or cycling between sulfur replete and deplete condition is proposed to have a continuous sustainable hydrogen production process (Ghirardi et al. 2000). To summarize, following types of metabolism occur simultaneously during sulfur deprivation: (a) Photosynthetic O_2 evolution, (b) Mitochondrial respiration (to consume oxygen and establish anaerobiosis), (c) H_2 production and (d) Anaerobic starch degradation and generation of fermentative products.

1.7 Improving hydrogen production by genetic engineering

Hydrogen production in *C. reinhardtii* occurs under anaerobic conditions which is driven by a connection between photosynthetic electron transport chain and plastidial hydrogenase (Weber et al. 2014). Hydrogen production functions as a safety valve to protect over reduced plastoquinone (PQ) pool (Tolleter et al. 2011). Research has been focused on optimizing this “hydrogen valve” to obtain a sustainable production process. The following section will provide a brief overview of potential genes or pathways that could be genetically modified to improve hydrogen production in *C. reinhardtii*.

Sulfur deprivation was applied to sealed cultures of Chlamydomonas to attain anaerobic condition which is a prerequisite for H_2 production (Melis et al. 2000). Several key metabolic changes occur within the cells such as increase in starch

accumulation, onset of anaerobiosis, start of hydrogen production, degradation of starch and increase in fermentative metabolites (malate, formate, acetate, etc.) (Antal et al. 2003a; Happe et al. 2002; Zhang et al. 2002). Under anaerobiosis ferredoxin donates electrons not only to [FeFe]-hydrogenase but also to several other metabolic pathways (refer to chapter 5.1). Hydrogenase receives electrons directly from the photosynthetic pathway, indirectly from starch degradation and also form fermentation of pyruvate. The presence of several competing electron sinks hinders hydrogen productivity. One such immediate and extensive competition arising between ferredoxin-NADP reductase (FNR) and hydrogenase was overcome by knocking down FNR, which resulted in a 2.5-fold increase (Table 1) in hydrogen production (Sun et al. 2013). In another approach the competition was countered by point mutation of ferredoxin which lead to a bias towards hydrogenase and yielded five-fold more hydrogen (Rumpel et al. 2014). Potential targets that are considered for improving H_2 production are: (a) Residual PSII activity (Volgusheva et al. 2013), (b) Acclimation to sulfur starvation (Antal et al. 2011; Ghysels et al. 2010), (c) Activity of PSI, (d) Improving cellular starch reserves (Doebbe et al. 2007), (e) Decreasing competitive pathways (Hemschemeier et al. 2011), (f) Decreasing antenna size (Oey et al. 2013), (g) Engineering O_2 tolerant hydrogenase (Bingham et al. 2012) and (h) System biology approach.

Stability of PSII is important for a prolonged H_2 production because it directly contributes to over ~80% of the total H_2 yield (Antal et al. 2003). PSII activity was found to be higher in a state transition mutant *stm6* (Kruse et al. 2005) because high rates of respiration resulted in faster anaerobiosis which helped in preservation of higher residual PSII activity (Volgusheva et al. 2013). The *stm6* mutant was genetically modified further by integrating a membrane hexose uptake protein. The resulting mutant, *stm6glc4* was able to synthesize more starch by metabolizing glucose and yielded 150% more H_2 compared to the parental strain (Doebbe et al. 2007). Reducing antenna size in *smt6glc4* resulted in a mutant *stm6glc4L01*, where a light green phenotype and reduced levels of LHCBM (LHCBM 1, 2 and 3) improved light to H_2 (180%) conversion efficiency (Oey et al. 2013).

Hydrogen production is plagued by competition from several pathways. For instance, competition arising from RuBisCo was overcome by engineering a RubisCo deficient strain Y67A which yielded 10-15fold higher H_2 compared to the parental strain (Pinto et al. 2013). During sulfur deprivation, PQ pool over reduction and non-

dissipation of proton gradient could also lead to electrons being directed towards cyclic electron flow (CEF) (Hankamer et al. 2007). Researchers were able to uncouple the proton gradient from photosynthetic electron transport (PGRL1 mutant, Table1) resulting in an increase in H_2 production (Tolleter et al. 2011). Reductants for hydrogen production also come via the fermentation pathway through oxidation of pyruvate by pyruvate ferredoxin reductase (PFR) and subsequent electron transfer to hydrogenase (Grossman et al. 2011). Fermentation pathway that consume pyruvate such as pyruvate formate lyase (PFL), lactate dehydrogenase (LDH) and pyruvate decarboxylase (PDC) could be downregulated.

As the maximum efficiency of H_2 production is achieved directly through photolysis, engineering an O_2 tolerant enzyme could be a feasible option. Due to the complex nature of the hydrogenase enzyme and its maturation process, designing an oxygen tolerant enzyme and having it function in vivo has still been a daunting task. Some of the methods for modifying the O_2 tolerance are (a) Random mutagenesis (Nagy et al. 2007), (b) Redesigning gas channels near the active site or H-cluster of the enzyme, (c) Searching for novel hydrogenases in other organism, ex. hydrogenases in *Chlorella vulgaris* YSL01 and YSL16 were reported to actively produce H_2 under atmospheric conditions (Hwang et al. 2014). Oxygen sensitivity of hydrogenase could be bypassed with the following options, (a) Overexpressing hydrogenases, (b) Creating a ferredoxin-hydrogenase fusion protein (Eilenberg et al. 2016) or (c) Expressing oxygen sequestering heme proteins within chloroplast (Wu et al. 2011).

Information regarding the transcriptomic, proteomic and metabolomic changes occurring during sulfur deprived hydrogen production is well documented (Chen et al. 2010; Doebbe et al. 2010; Matthew et al. 2009; Nguyen et al. 2011; Toepel et al. 2013). Information from “Omics” can be integrated to create a biological model which would facilitate systematic analysis of bottleneck pathways or target genes. For example, cells undergo stress during hydrogen production which requires acclimatization to reactive oxygen species (ROS) and also maintain cellular energy requirements (Weber et al. 2014). Transcriptomic data (Toepel et al. 2013) reveal stress related genes that could be overexpressed to counter ROS stress ex. Glutathione peroxidase homologous gene (*GPXH/GPX5*) or Glutathione-S-transferase (*GSTS1*). Based on the “Omics” data, the role of sulfite reductase (*SIR1*) and isoflavone reductase like protein (*IFR1*) on hydrogen production have been investigated in the following study (refer to

chapter 4 and 5). To conclude, improving H_2 production by genetic engineering is not limited to the aforementioned targets but a systematic approach is required to achieve a sustainable process.

1.8 Immobilized hydrogen production

Immobilized enzyme or immobilized cells are routinely used in large scale activities which have also been extended to algal systems. Application of immobilized algae (IA) is so far only extended to areas like pollutant removal (de-Bashan et al. 2010; Mallick 2002) and bioremediation of heavy metals (Wilde et al. 1993). However, since the 20th century IA have also gained interest in the area of hydrogen production (Das et al. 2001; Laurinavichene et al. 2006). The following chapter reviews the applicability of immobilized algae system for hydrogen production.

The process of immobilization involves physical confinement of cells within a specific matrix such that the viability is retained to perform the desired catalytic process, ex. hydrogen production. Some of the materials commonly used for immobilizing cells are calcium alginate (Smidsrød et al. 1990), hydrogels (Jen et al. 1996), poly (vinyl alcohol) cryogels (Lozinsky et al. 1998), agarose, polyacrylamide, polyurethane, etc. Among the various materials, Ca-alginate is by far the most frequently used entrapment material. The benefit of entrapment is the use of whole cells instead of purified enzymes and in case of processes dealing with free cells, substitution of free cells with immobilized cells helps in improving process robustness. Use of whole cells is recommended when the enzyme is intracellular (ex. [FeFe]-hydrogenase, see chapter 1.5) as enzymes retain better activity within the cells. Viability quotient of the immobilized cells is very crucial for a successful process because a loss in viability results in a decreased volumetric yield of the product. Hence, the viability of the cells could be better sustained in an entrapped environment by simply replenishing the environment with desired growth medium. The advantages of using immobilized cells against free cells are: (a) Obtain higher cell density (better product yields), (b) Immobilization makes the cells more stable compared to free cells (protection against shear forces), (c) Immobilized cells can be subjected to hostile conditions such as nutrient depletion (sulfur deprived hydrogen production, see chapter 1.6) and then revived easily by exchanging media. Some of the constraints of applying immobilized system include: (a) Hindrance to mass transfer (CO_2 gas or nutrients

supply could be limited), (b) Limitation to light supply (c) pH shifts arising as a result of fermentation could be difficult to control.

The rates of hydrogen production achievable by sulfur deprived process is still significantly lower and could be improved by enhancing process parameters (Tamburic et al. 2011). The current process of hydrogen production involves free cells which are cycled between growth phase and sulfur deplete hydrogen production phase (Melis et al. 2000) making it laborious and cost intensive. This could be overcome by immobilizing the cells which would help in rapid exchange of media without the requirement of energy intensive centrifugation steps. Hydrogen production has been previously reported from immobilized cells of cyanobacteria and purple photosynthetic bacteria (Lambert et al. 1979; Tsygankov et al. 1994). A successful immobilized hydrogen production from *C. reinhardtii* was also reported (Laurinavichene et al. 2006), where cells were immobilized in modified glass matrix (Al-borosilicate). The authors confirmed a prolonged H_2 production phase which lasted for up to 4 weeks and also showed that the algal cells retained specific rate of H_2 which were comparable to those from suspension culture. Hydrogen production was also reported from Chlamydomonas cells immobilized in thin alginate sheets where a light to H_2 conversion efficiency of 1% and a great tolerance to atmospheric oxygen was observed (Kosourov et al. 2008). Another study reported a light to H_2 conversion of 4% at optimized conditions in Chlamydomonas immobilized in TiO_2 shells (Stojkovic et al. 2015). These outcomes highlight the ease of performing a biphasic H_2 production with immobilized cells and also show that an enhanced stability and viability of cells results in a prolonged H_2 production phase. Hence, in the following study a glass like novel silica gel material will be tested for its applicability in immobilized hydrogen production.

1.9 Artificial microRNA

Chlamydomonas is often used as a model organism but lack of targeted nuclear genetic manipulation limits its application. Tools available for reverse genetic analysis such as Tilling, insertional mutagenesis and transposon tagging have not yet been successfully implemented in Chlamydomonas as compared to gene silencing by microRNAs (Molnar et al. 2009). Chlamydomonas encodes endogenous microRNAs (miRNAs) which are 21-22 nucleotide long regulatory elements that are involved in regulating the expression of target genes (Molnár et al. 2007; Zhao et al. 2009). The availability of a highly curated Chlamydomonas genome (Merchant et al. 2007) enables successful application of miRNAs for reverse genetic analysis (Jinkerson et al. 2015).

miRNAs are small RNAs that are processed from long RNA molecules. Two variations of small RNAs routinely used are microRNA and small interfering RNAs (siRNAs). miRNAs are produced as 21-24 nucleotide duplex molecules as a result of the RNAse like enzyme (Dicer) hydrolyzing an imperfectly folded double stranded RNA, whereas siRNAs are produced from a perfectly folded double stranded RNA (Molnar et al. 2009). An argonaute protein complex (AGO) is formed that retains the 5' miRNA strand with low thermodynamic stability (Mi et al. 2008) and the other strand (passenger) is degraded. The resulting RNA-induced silencing complex (RISC) is guided to its target gene and silenced through RNA-RNA interaction. Silencing occurs either at transcriptional level i.e. cleavage of transcribed mRNA or at post-transcriptional level which involves inhibition of translation (Bartel 2004). A partial match between the microRNA and target gene most likely results in translational inhibition, whereas a higher match results in degradation of the target mRNA. In animals, endogenous miRNAs have short complementarity to their target genes (positions 2 to 8) allowing each miRNA to target multiple mRNAs (Brennecke et al. 2005; Farh et al. 2005; Lim et al. 2005; Molnar et al. 2009), whereas in plants miRNAs have very few mismatches resulting in silencing of only few targets (Llave et al. 2002). Artificial miRNAs (amiRNAs) have been successfully applied to enhance hydrogen production by knocking down light harvesting complex (Oey et al. 2013), eliminating competition (Sun et al. 2013), reducing photosynthetic oxygen evolution to aid faster induction of H_2 (Li et al. 2015) and many more.

1.10 Reactive oxygen (ROS) and electrophile species (RES)

ROS and RES are byproducts of oxygenic photosynthesis. ROS, as the name suggests is a hyper-active form of excited state oxygen generated from biotic or abiotic stress and comprise of hydroxyl radical (OH°), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and superoxide anion (O_2^{-}). These reactive species can either serve as messenger molecules (Alboresi et al. 2011) or under lethal concentrations bring about degradation of proteins, nucleic acids, lipids and more (Schmitt et al. 2014). ROS can cause spontaneous uncatalyzed reactions leading to the formation of new species eg. non enzymatic oxygenation of α-linolenic acid (ALA) to form α,β-unsaturated carbonyl groups or other reactive electrophilic groups known as reactive electrophile species (Farmer et al. 2007). RES can be generated either non-enzymatically from ROS or enzymatically such as jasmonic acid (JA), oxophytodienoic acid (OPDA) and 2-E-hexenal (Matsui 2006; Taki et al. 2005). Chemical reactivity of RES with nucleophilic actoms can directly affect cell contents or RES may indirectly damage cells by affecting the cellular reductant pools (Oberschall et al. 2000).

ROS and RES generation within the cells depends on biotic and abiotic factors such as high light, heat, infection from pathogens, etc. (Schmitt et al. 2014). Both the species are also known to function as messenger molecules. It has been proposed that RES liberated from dying cells could activate protection genes in leaves of neighbouring cells (Vollenweider et al. 2000). It was shown that syringolin A (a virulence factor containing α,β-unsaturated carbonyl group) produced by the pathogen *P. syringae*, triggers genes required for the viability of wheat and Arabidopsis (Michel et al. 2006). Depending on the site of origin, ROS species can also function as signalling molecules and either activate responses required for acclimation to stress tolerance or trigger programmed cell death (Galvez-Valdivieso et al. 2010). Acclimatization of *C. reinhardtii* to ROS species such as H_2O_2 and singlet oxygen was shown to activate specific promoters (Shao et al. 2007). ROS activated complex signal pathway in rice and Arabidopsis mediated by mitogen-activated protein kinase (MAPK) (Kovtun et al. 2000). The presence of ROS and RES is mainly controlled by the activity of antioxidant enzymes or antioxidant molecules like glutathione, α/β-tocopherols, carotenoids, flavonoids and ascorbic acid (Apel et al. 2004). Detailed mechanism of ROS and RES detoxification has been previously reviewed (Apel et al. 2004; Farmer et al. 2007) and will not be discussed here.

2.0 Aim and Objectives

C. reinhardtii can use sunlight and water as substrates for the production of biohydrogen. In the following work, genetic manipulation will be employed to further improve H_2 production. The availability of transcriptome, metabolome and proteome data of hydrogen producing Chlamydomonas culture served as a platform for the following work and facilitated the determination of target genes (Chen et al. 2010; Doebbe et al. 2010; Matthew et al. 2009; Nguyen et al. 2011; Toepel et al. 2013). The aim of the following work was to improve the conversion efficiency of light energy to hydrogen by targeting bottleneck or competing pathways or genes. This was achieved by addressing the following objectives.

Objective 1: Knocking down isoflavone reductase like protein (IFR1) to improve hydrogen production

The first objective was to consider a target gene that is upregulated during hydrogen production in a bad hydrogen producer strain as against to a good producer strain. *IFR1* knockdowns will be created by artificial microRNA strategy in CC124 wild type strain. The knockdowns will be analyzed for their phenotype and hydrogen production. On the basis of results obtained from a wild type *IFR1* knockdown, the knockdown strategy will be further extended to a good hydrogen producer mutant strain like *stm6* to boost its hydrogen production.

Objective 2: Redirecting electron flux towards hydrogenase by knocking down sulfite reductase (SIR1)

Transcriptome and proteome data show a competition for electrons between sulfite reductase and hydrogen production enzyme (hydrogenase) under sulfur deplete anaerobic H_2 production conditions. To further enhance H_2 production, *SIR1* knockdowns will be created in high H_2 producing *stm6glc4* mutant strain with the help of artificial microRNA. The H_2 phenotype of the knockdown mutants will be analyzed.

3. Materials and Methods

3.1 Chemicals and equipment

All the chemicals such as general chemicals (acids, bases), chemicals for growth medium, Reactive Oxygen Species (ROS) and Reactive Electrophile Species (RES) inducing agents were purchased from either of the following companies: Carl Roth (Karlsruhe, Germany), Sigma Aldrich (Taufkirchen, Germany), VWR (Langenfeld, Germany), Applichem (Darmstadt, Germany) and Merck (Darmstadt, Germany). Equipment used in the following study along with their manufacturers is outlined in the table below.

Table 2: List of all the instruments used in the following study is reported below

Instrument (manufacturer)	Application
96 well polystyrene microplate (Greiner Bio-One, Germany)	Lowry protein assay
6, 12 and 24 well polystyrene microplate (Greiner Bio-One, Germany)	Algae cultivation and screening
Acculab ALC-210.4 and VIC-3103 (Sartorius, Germany)	Weighing and media preparation
Agilent 3000 Micro FC (Agilent Technologies, Germany)	Hydrogen gas analysis
Autoclave V-150 (Systec, Germany)	Sterilization of media and glassware
BA310 binocular microscope (Motic, Germany)	Microscopic observation of cells
Disposable polystyrene semi-micro cuvettes (Sarstedt, Germany)	Absorption analysis
Disposable vials: 0.5 to 50 ml (Sarstedt, Germany)	PCR reaction, DNA/RNA extraction, solutions
Eppendorf pipette (Eppendorf, Germany)	Pipetting
Filtropur S 0.2 syringe filters (Sarstedt, Germany)	Membrane filtration, sterile inlet of gases
FluorCam (PSI, Czech Republic)	Chlorophyll fluorescence
Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific, Germany)	OD measurement

Glassware (VWR, Germany)	Media preparation
Haemocytometer (Marienfeld-Superior, Germany)	Manual cell count
Infinite M200 multimode microplate reader (Tecan, Germany)	Reading protein absorption (Lowry assay) and bioluminescence (luciferase assay)
LSM780 (Carl Zeiss, Germany)	Confocal laser scanning microscopy
Magnetic stirrer RO 10 (IKA, Germany)	Mixing
Mini PAM (Heinz Walz, Germany)	Photosynthesis yield analysis
Multi-Cultivator MC1000 (Photon System Instruments, Czech Republic)	Parallel air-lift cultivation
Orbital shaker 3017, VKS (GFL, Germany)	Culture growth
Osram Lumilux warm white and cool daylight (Osram, Germany)	Light source
Oxyg1 plus, Clark-type O_2 electrode (Hansatech Instruments, UK)	Photosynthetic oxygen evolution and respiration oxygen consumption rates
pH electrode (VWR, Germany)	pH measurement
Photobioreactor FMT-150 (PSI, Czech Republic)	Algae cultivation
Quartz cuvette 6040-UV (Hellma Analytics, Germany)	Absorption measurement
SIGMA 6-16KS (Sigma, Germany)	Sulfur deprivation
Single channel pipettes (VWR, Germany)	Pipetting
TipOne RPT filter tips (Starlab, Germany)	Sterile work and RNA extraction
Ultratip (Greiner Bio-One, Germany)	Pipetting activities
Z2 cell and particle counter (Beckman Coulter, Germany)	Automatic cell count

3.2 Algae strain and growth conditions

Various wild type (wt) and mutants (mt) of unicellular green algae *C. reinhardtii*, were used in this work. The strains and their description are listed in table 3. All the strains were primarily maintained photoheterotrophically on tris acetate phosphate (TAP) (Gorman et al. 1966; Harris 1989) agar plates under continuous illumination of white light ~40 $\mu Em^{-2}s^{-1}$. Prior to use in any experiments, strains were always transferred from TAP agar plates to TAP liquid medium and grown under similar light intensity at 25°C with 110 rpm of constant shaking. For studies requiring photoautotrophic growth, the strains were first grown in TAP and then transferred to high salt medium (HSM) (Sueoka 1960) and grown with 3% CO_2 to a required cell density. Modified Hutner's trace solution completed TAP and HSM mediums (Kropat et al. 2011).

All the media used in this study were made according to the protocols available at *Chlamydomonas* resource center. A liter of TAP medium was made by dissolving, 2.42 g Tris salt, 25 ml TAP salt solution (15 g NH_4Cl, 4 g $MgSO_4.7H_2O$, 2 g $CaCl_2.2H_2O$ dissolved in 1 L), 0.375 ml P-solution (28.8 g K_2HPO_4, 14.4 g KH_2PO_4 dissolved in 100 ml), 1 ml each of Hutner's trace elements and 1 ml of glacial acetic acid. The final pH was adjusted to 7.2 with 1M HCl. TAP medium is a complete source of micro and macro nutrients along with expensive elements like acetate, phosphate and ammonium. Sulfur free TAP medium (TAP-S) was made by exchanging the $ZnSO_4$ in Hutner's trace solution with an equal concentration of $ZnCl_2$ and by using $MgCl_2$ in place of $MgSO_4$. Nitrogen free TAP medium (TAP-N) was prepared as earlier by replacing NH_4Cl with KCl.

HSM medium is an acetate lacking version of TAP medium, which is primarily phosphate buffered. For photoautotrophic cultivation, 1 L of HSM medium was made by dissolving, 25 ml of TAP salt solution and 1 ml each of Hutner's trace elements in water. The pH was adjusted to 6.8 and 5 ml of sterile P-solution was added to the media after sterilization. Hutner's trace elements used to complete TAP and HSM media were made on the basis of the composition available at the repository of *Chlamydomonas* resource center (Hutner et al. 1950).

Table 3: All the *C. reinhardtii* strains used in this work are listed below

Strain	Description	Purpose	Reference
CC-124	Wild type (wt), mating type minus (mt-), *nit1* and *nit2* mutation	*IFR1* knockdown mutant generation	Chlamydomonas Resource Center (CRC)
CC-406	wt, cell wall deficient (cw15)	Control strain	CRC
CC-1690	Sager's basic wt, mt+	Control strain	CRC
CC-3491	wt, cw15 obtained from CC-125 (mt+) and CC-406	Control strain	CRC
CC-2931	wt, mt-, capable of growing on nitrate	Control strain	CRC
CC-4603 4A	137c background derived from CC-2191 and CC-4051	Control strain	(Dent et al. 2005)
sor1	UV mutant of 4A+	IFR1 expression analysis	(Fischer et al. 2012)
UVM4	A UV mutant generated from CC4350 (cw15, *arg7-8*, mt+)	Expression of IFR1:YFP fusion	(Neupert et al. 2009)
stm6	*MOC1* knockout derived from CC-1618	Generation of *IFR1* knockdown	(Schönfeld et al. 2004)
stm6glc4	*stm6* strain with an integrated glucose transporter HUP1	Generating SIR1 knockdown	(Doebbe et al. 2007)
IFR1-1 IFR1-6	knockdown mutants of *IFR1* derived from CC-124	Assessing *IFR1* knockdown	This work
stm6_IFR1kd	knockdown mutants of *IFR1* derived from *stm6*	Assessing *IFR1* knockdown	This work
sgh2 *sgh3*	knockdown mutants of *SIR1* derived from *stm6glc4*	Assessing *SIR1* knockdown	This work

3.2.1 Growth under nutrient depletion

With an aim to improve hydrogen (H_2) production, all the wt strains and their respective mt derivates were initially analyzed under aerobic TAP-S growth conditions. The strain to be analyzed was always propagated from TAP agar plates (Section 3.2) to a mid-log phase in TAP medium. The cells were harvested (2500g at room temperature for 4 min) and washed (minimum of 3 x 2500g for 4 min) with TAP-S to eliminate sulfur. After the washing, the cells were re-suspended in TAP-S medium to the tune of, optical density (OD_{750}) ~0.8 and placed under continuous illumination of light. Growth was monitored by OD_{750}, OD_{680}, total chlorophyll and cell count. Samples were drawn at defined time points for analytics.

3.3 Bacteria and growth conditions

All the plasmids used in this work were constructed and maintained in *E. coli* strains of DH5α and KRX cells (table 4, single step KRX competent cells from Promega). DH5α strains served as cloning and maintenance host whereas expression of *C. Reinhardtii* Isoflavone reductase like protein (IFR1) was realized in KRX cells. KRX cells were transformed with desired plasmid and cultivated under continuous shaking (180 rpm) at 37ºC in lysogeny broth (LB medium, 10 g/l Tryptone, 5 g/l yeast extract and 10 g/l NaCl). The mutants were selected on LB agar plates supplemented with 50 µg/ml kanamycin. The positive clones were confirmed by colony PCR and the plasmid integrity was assessed by sequencing (Sequencing core facility, CeBiTec, University of Bielefeld, Germany).

Table 4: *E. coli* strains used in the following work

Strain	Description	Reference
DH5α	Cloning host: F', *end A1*, *hsd*R17 *(rk-mk+)*, *sup E44*, *thi-1*, *rec* A1, Δ*lacU169*	(Hanahan 1983)
KRX	Expression host: F', *tra*D36, *pro*A^+B^+, *lac*I^q, Δ(*lacZ*)M15, Δ*omp*T, *end*A1, *rec*A1, *gyr*A96, *sup*E44, Δ(*lac-pro*AB), Δ(*rha*BAD)::T7 RNA polymerase	Promega, Germany

3.4 DNA and RNA techniques

This section provides information on plasmids, primers, methods of DNA and RNA isolation, Polymerase chain reaction (PCR) and Quantitative PCR (qPCR).

3.4.1 Plasmids and primers

All the plasmids created and used in this study were checked by sanger sequencing before use.

Table 5: Following are the plasmids used in this work

plasmid	Description
pGEM-T Easy	Cloning vector (Promega, Germany), amp^R
pChlamiRNA3int	Artificial mRNAi construction vector, amp^R and APHVIII for bacteria and algae selection, respectively (Molnar et al. 2009)
pET-24 a (+)	Bacterial expression vector, kan^R
pOpt_mVenus_Paro	pOptimized vector for *Chlamydomonas* expression, (Lauersen et al. 2015)
pOpt_cCA_*g*Luc_Paro	Chlamydomonas pOptimized vector with *Gaussia princeps* luciferase reporter (Lauersen et al. 2013)

Table 6: Primers used in this work are tabulated below

Primer	Sequence	Purpose
*IFR-Nde*I-*F* *IFR-Bgl*II-*R*	GGCC**CATATG**GCGACTAAGAAGCACACCGTT-GCGGTGATTGGAGGCT GGCC**AGATCT**GGCGTCAGCGAACTGCCAGG-AGGC	*IFR1:YFP* C terminus fusion
IFR-EcoRV-F *IFR-EcoRV-R*	GGCC**GATATC**GCGACTAAGAAGCACACCGTT-GCGGTGATTGGAGGCT GGCC**GATATC**CTAGGCGTCAGCGAACTGCCA-GGAGGCG	*IFR1:YFP* N terminus fusion
IFR-RTQ2-F *IFR-RTQ2-R*	ATGGCGACTAAGAAGCACAC CGAAGCCTGCTCATTGTAGT	*IFR1* RTqPCR

S1-F1 *S1-R1*	CTCCCCCGCAAGTTCAAGAT AAGGTGTCTGCGTCTCTGTG	*SIR1* RTqPCR
S3-F1 *S3-F2*	CACGGACATCGATGCCTTCT GTTGCCGACCAGGAAGCTAT	*SIR3* RTqPCR
RACK1-F *RACK1-R*	TCAACATCACCAGCAAGAAGG CTGGGCATTTACAGGGAGTG	RTqPCR internal control
RPL13-F *RPL13*-R	ATTCTTGCCGGGCAGCAGATTGTG TTGCGCAGGAAGCGGTCATACTTC	RTqPCR internal control
Actin-F *Actin*-R	CGCTGGAGAAGACCTACGAG GGAGTTGAAGGTGGTGTCGT	RTqPCR internal control

3.4.2 Construction of microRNA (miRNA)

Small microRNAs (miRNAs) of 21 to 22 nucleotides in length were constructed to knockdown *C. reinhardtii* genes like *IFR1* and sulfite reductase (*SIR1*). The miRNAs were designed over a web based tool WMD3 (http://wmd3.weigelworld.org). The miRNAs targeting the exons 2 or 4 of the *IFR1* coding region (table 7), were cloned into pChlamiRNA3int (Molnar et al. 2009). For generating SIR1 knockdowns, miRNAs targeting exon 5 located closer to the 5' UTR or the last exon 13 towards the 3' UTR (table 7) were constructed by assembling sequences S1 to S8 as reported earlier (Hu et al. 2014) and cloned into pOpt_cCA_gLuc_Paro (Lauersen et al. 2013). Fermentas Fastdigest restriction enzymes were used according to the manufacturers protocol for all the cloning work. The vector systems were linearized by restriction digestion and dephosphorylated. Forward and reverse single stranded oligonucleotides were annealed to obtain double strand. The phosphorylated double-stranded oligonucleotides were ligated to the dephosphorylated, linearized vectors. The ligation mixture was transformed into KRX competent *E. coli* cells and clones were selected on LB agar plates with kanamycin. The positive clones were checked via colony PCR and their plasmids extracted by mini prep (peqGold plasmid Miniprep Kit I) and sequenced. After verifying the correct sequence, the plasmids were prepared on a large scale and used for nuclear transformation. Two plasmids targeting *IFR1* were constructed and named according to the proximity of the exon to UTRs as *IFR1*(3) and *IFR1*(5). Similarly, two plasmids *SIR1*(3) and *SIR1*(5) targeting *SIR1* were also used.

Table 7: Sequences used to construct amiRNA targeting *IFR1* and *SIR1*

amiRNA	Sequence	Target
5-*amiIFR-F*	ctagtCAGGTCCAGGAGATTGATATA**tctcgctgatcg-gcaccatgggggtggtggtgatcagcgcta**TATAACAATC-TCCTGGACCTGg	
5-*amiIFR-R*	ctagcCAGGTCCAGGAGATTGTTATA**tagcgctgatc-accaccacccccatggtgccgatcagcgaga**TATATCAAT-CTCCTGGACCTGa	*IFR1* exon 2
3-amiIFR-F	ctagtGAGCACGCTATTAAGGTCGTA**tctcgctgatcg-gcaccatgggggtggtggtgatcagcgcta**TACGGTCTTA-ATAGCGTGCTCg	
3-amiIFR-R	ctagcGAGCACGCTATTAAGACCGTA**tagcgctgatc-accaccacccccatggtgccgatcagcgaga**TACGACCT-TAATAGCGTGCTCa	*IFR1* exon 4
S1_*NdeI*	aatt**catatg**AGGAAACCAAGGCGCGCTAG	
S2_*NdeI*	GTACTGCAGCTGGAACACTGCGCCCAGGAAGC-TAGCGCGCCTTGGTTTCCT**catatg**aatt	
S1_*SmaI*	aatt**cccggg**AGGAAACCAAGGCGCGCTAG	Universal
S2_*SmaI*	GTACTGCAGCTGGAACACTGCGCCCAGGAAGC-TAGCGCGCCTTGGTTTCCT**cccggg**aatt	primers for *SIR1* amiRNA
S5	TCTCGCTGATCGGCACCATGGGGGTGGTGGTG-ATCAGCGCTA	construction
S8_*EcoRI*	aatt**gaattc**TCCTGGCAGTGTTCCGGCTGCAGTA	
S3_SIR1_5’	TCCTGGGCGCAGTGTTCCAGCTGCAGTAC<u>TCG-AAATTGGTTCCGAACCCG</u>	
S4_SIR1_5’	CCATGGTGCCGATCAGCGAGA<u>CGGGTTCGGA-ACCAATTTCGA</u>	
	<u>TCGAAATTGGTTCCGAACCCG</u>TAGCGCTGATC-	*SIR1* exon 5
S6_SIR1_5’	ACCACCACCC	
	<u>CGGGTTCGGAACCAATTTCGA</u>TACTGCAGCCG-	
S7_SIR1_5’	GAACACTGCCAGGA**gaattc**aatt	
S3_SIR1_3’	TCCTGGGCGCAGTGTTCCAGCTGCAGTAC<u>TAC-CCAATCTCCAAAGCCCTA</u>	

S4_SIR1_3'	CCATGGTGCCGATCAGCGAGATAGGGCTTTG-GAGATTGGGTA	*SIR1* exon 13
S6_SIR1_3'	TACCCAATCTCCAAAGCCCTATAGCGCTGATC-ACCACCACCC	
S7_SIR1_3'	TAGGGCTTTGGAGATTGGGTATACTGCAGCCG-GAACACTGCCAGGA**gaattc**aatt	

3.4.3 Isolation of genomic DNA

Genomic DNA was isolated from *C. reinhardtii* cells grown to a logarithmic phase of optical density (OD_{750}) 0.6-0.8. 5-10 ml culture was harvested by centrifugation (3000 g for 5 min) and the pellet obtained was lysed with 350 µl of CTAB-buffer (table 8), 100 µl Protienase K and 50 µl 20% SDS. The mixture was incubated for 2 h in a water bath at 55 ºC. After cooling on ice, 50 µl of 5 M potassium acetate was added to the sample and further incubated on ice for 30 min. Supernatant obtained after centrifugation of the sample (13000g for 15 min) was subjected to an equal volume of phenol/chloroform/isoamyl alcohol extraction. The samples were mixed and the resulting supernatant (centrifugation 16000g for 5 min) was transferred to a new tube and incubated with 1 ml of -20 ºC cold ethanol until precipitation of DNA. The DNA was pelleted (16000g for 5 min) and washed with 500 µl of 70% ethanol. The sample was centrifuged twice (10000g for 5 min) to remove any traces of ethanol and finally suspended in 50 µl of TE buffer. DNA concentrations were quantified with a NanoDrop (ND-1000 spectrophotometer) and stored at -20°C until use.

Table 8: Composition of CTAB-buffer

Solution	Composition
CTAB buffer of pH 8	100 mM Tris-HCl
	20 mM EDTA
	1.4 M NaCl
	2% CTAB

3.4.4 Polymerase chain reaction (PCR)

Samples such as plasmid or genomic DNA were used for amplification of required targets via PCR. PCR was performed with Q5 high fidelity DNA polymerase (NEB) and the reaction performed according to the manufacturers protocol. All the

primers required were generated by Primer3plus (Untergasser et al. 2007) and synthesized by Sigma Aldrich. The PCR program comprised of the following cycling conditions: Initial denaturation (98°C for 30 seconds or longer depending on the template size), 35 cycles of denaturation (98°C for 15 seconds) annealing (58-65°C depending on the primers for 10-15 seconds) and elongation (72°C at 30 seconds per kb), followed by a final elongation (72°C for 2 minutes). Based on the PCR product size, the product was analyzed on a 1-2% TAE (tris-acetate-EDTA) agarose gel.

3.4.5 Isolation of total RNA

Total RNA was isolated from Chlamydomonas strains by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski 1987). All the steps were performed on ice unless otherwise mentioned. The cells were pelleted (3000g for 2 min) and lysed in 900 µl denaturation solution (table 9, supplemented with fresh β-mercaptoethanol). The lysate was acidified with 90 µl sodium-acetate buffer and then mixed with 900 µl phenol and 180 µl chloroform. The mixture was incubated (15 min on ice) and later centrifuged (16000g at 4°C for 20 min) to collect 800 µl of aqueous phase. Equal volume of isopropanol was added and the mixture incubated overnight at -20°C. RNA was pelleted (16000g at 4°C for 20 min) and re-suspended in 300 µl each of denaturation solution (with fresh β-mercaptoethanol) and isopropanol. Samples were incubated (-20°C for 30 min) and later centrifuged (16000g at room temperature for 10 min) to obtain the RNA pellet. The pellet was washed with 500 µl of 70 % ethanol and incubated for 15 min at room temperature. The suspension was centrifuged (16000g for 5 min) and the RNA pellet re-suspended in 50 µl DMDC/DEPC treated water. The concentration of RNA was measured by nanodrop and its integrity checked on a denaturizing formaldehyde gel.

Table 9: Composition of denaturation solution

Solution	Composition
RNA denaturation solution of pH 7	4 M Guanidinthiocyanate 25 mM Sodium citrate 0.5% (w/v) N-laurylsarcosin

3.4.6 Quantitative real time PCR (qPCR)

Quantitative real time PCR allowed assessment of the abundance of target mRNA like IFR-1 (Phytozome locus name Cre11.g477200; *C. reinhardtii* v5.5), SIR1 (Phytozome locus name Cre16.g693202; *C. reinhardtii* v5.5) and sulfite oxidase (SO, Phytozome locus name Cre04.g217929; *C. reinhardtii* v5.5). Total RNA was extracted at specific time from cells grown in TAP and TAP-S medium. DNA contamination was overcome by DNase digestion of the samples (as per manufacturer, Promega). The qPCR was done with a SensiFAST SYBR Hi-ROX One step kit (Bioline) and performed in StepOne Real-Time PCR system (Applied Biosystems). qPCR reaction volume was set to 20 µl, which comprised of 10 µl 2x SensiFAST SYBR Hi-ROX One Step Mix (heat activated DNA polymerase, dNTPs, SYBR Green and $MgCl_2$), 0.8 µl each of forward and reverse primers (10 µM), 0.2 µl Reverse transcriptase, 0.4 µl RNase inhibitor and 7.8 µl of 300 ng RNA. The cycling conditions of qPCR were: first cycle of reverse transcription at 45°C for 10 min, which was followed by one cycle of polymerase activation at 95°C for 2 min and finally 40 cycles of 95°C for 15 s: 60°C for 10 s: 72°C for 20 s. The final step of melting curve was performed at 60-90°C for 30 s. cDNA synthesis by reverse transcriptase followed by PCR were completed in one reaction. SYBR Green was the fluorescent dye used for real time monitoring of the reaction. To ensure meaningful results, intron spanning primers and negative controls without reverse transcriptase were also used. The abundance of the target mRNA was calculated by comparing its fluorescence signal to that of an internal control e.g. RACK1 (Receptor of activated protein kinase C 1), RPL13 (60s ribosomal protein L13), Actin or 18s (ribosomal RNA). The relative variances in transcript abundance to control conditions were assessed via $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

3.5 Generation of *IFR1* and *SIR1* knockdown strains

IFR1 knockdowns were generated in CC124 and s*tm6* strains (Kruse et al. 2005) whereas *SIR1* knockdown was generated only in *stm6glc4* (Doebbe et al. 2007). Nuclear transformation of the wild type (wt) strain CC124 was achieved by electrophoresis (Jaeger et al. 2017) and for *Stm6* and *Stm6glc4* it was realized by glass bead transformation (Kindle 1990). 80 µl of a mid-log phase (4×10^8 cells/ml) CC124 strain was transformed with 1 µg DNA (pChlamiRNA3int vector carrying the miRNA targeting *IFR1*) in an electroporation cuvette (Biorad) subjected to a single rectangular wave protocol (1×1500 V cm^{-1} with 8 ms length, GenePulser X cell, Biorad). The cells

were regenerated overnight in ToS (tris-acetate-phosphate medium supplemented with 40 mM sucrose) medium, at low light ~30 µE m^{-2} s^{-1} and selected on TAP agar plates with 10 mg/l paromomycin. *stm6 IFR1* knockdowns were generated by glass bead transformation and also selected on TAP agar plates with 10 mg/l paromomycin. Positive clones (CC124 and *stm6 IFR1* knockdown) were screened by growing them in TAP-S and assessing the IFR1 mRNA and protein levels.

SIR1 knockdowns were created in *stm6glc4* via glass bead transformation. The clones were selected on TAP agar plates supplemented with two antibiotics (TAP-PH) i.e paromomycin (P) and hygromycin (H), (P,10 µg/ml and H, 5 µg/ml). The mutants that survived the double antibiotic selection were screened further on the basis of luciferase activity.

3.6 IFR1 antibody and recombinant protein production

Anti IFR1 polyclonal antibody (αIFR1) was raised in rabbits by an antibody production company (Agrisera, Sweden). 17 aa polypeptide ((NH2)-CRGRTVPLDKAWKSKAH), synthesized de-novo was used as an antigen. Prior to use, the antibody was assessed for cross reactivity against total protein extracted from a *C. reinhardtii* wild type.

Full length *IFR1* cDNA was codon optimized, de novo synthesized (Genscript, USA) and cloned into a pET-24a expression vector. To assist easy purification, the c-terminus was strep-tagged. pET systems were developed primarily for recombinant protein production in *E. coli* (Rocco et al. 2008; Studier et al. 1990). *IFR1* cDNA was cloned under the control of a T7 transcription signal with enzymes NdeI, XhoI and transformed into competent KRX *E. coli* cells. A 20 ml pre-culture was propagated overnight at 37ºC (180 rpm) in LB medium supplemented with 50 µg/ml kanamycin. A 1 l production culture of OD 0.6 was achieved starting from an inoculum size of 5%. The KRX cells were induced with 0.1% (w/v) L-Rhamnose (Sigma) and the protein production was done overnight (37ºC at 180 rpm). The resulting culture broth was centrifuged (10000 g for 3 min) and the pellet re-suspended in 100 ml Strep washing buffer (100 mM Tris, 150 mM NaCl, 1 mM EDTA, Roche complete protease inhibitor tablet). Cells were lysed by 3 consecutive steps of ultra sonification (30 s) on ice followed by 3 freeze thaw cycles. The sample were centrifuged (14000 g at 4ºC for 20 min) and the clear supernatant loaded to a Strep-Tag Superflow high capacity (HC)

chromatography column (Iba Life Science). The washing and elution steps were done as per manufacturer.

3.7 Analysis of knockdown at protein level

The *IFR1* and *SIR1* knockdowns generated in this study were confirmed by western blot analysis. Cells were harvested (10000 g for 2 min) and lysed with the lysis buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol and freshly added 1 mM Pefabloc). The mixture was immediately frozen in liquid nitrogen and thawed on ice. The cell debri was separated by centrifuging at 8ºC (20000 g for 2 min). The total protein was collected into a fresh vial and stored at -80ºC until use. Protein concentration of the samples were measured by a Bio-Rad *DC* protein assay (BioRad), which works on the principle of Lowry assay. Prior to electrophoretic separation, the protein samples were diluted to required concentrations with a loading dye and denatured for 7 min at 65ºC. Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was performed as mentioned before (Laemmli and Favre 1973) with a 12 % resolving gel [40% acrylamide/bisacrylamide, 12.5 % glycerol, 0.95 M Tris-HCl (pH 8), 0.09 % (w/v) SDS, 0.5 % (w/v) AMPS and 0.05 % (v/v) TEMED] and a 4% stacking gel [40% acrylamide/bisacrylamide, 0.7 M Tris-HCl (pH 6.8), 0.07% (w/v) SDS, 1.1 % (w/v) AMPS and 0.05% (v/v) TEMED]. The gels were placed in the electrophoresis chamber (Mini-Protean-II chamber,BioRad) and filled with running buffer [0.2 M Tris-HCl (pH 8), 0.1% SDS]. The gels were visualized by colloidal coomassie staining (CCB) (Candiano et al. 2004). A pre-stained protein ladder (PageRuler, ThermoFisher Scientific) assisted in the analysis of separated proteins.

Samples to be immunodetected were seperated by SDS-PAGE and blotted on to nitrocellulose membranes. After SDS-PAGE, the gel was placed in a “sandwich” in the following order: a layer of sponge, Whatman filter paper, nitrocellulose membrance, gel, Whatman filter paper, sponge layer. All the layers used for sandwiching the gel and membrane were presoaked in transfer buffer. The blot sandwich was placed into the blot chamber (Mini-Protean, BioRad) with the membrane facing the anode and filled with 4°C transfer Buffer. Following the blotting, the membrane was blocked overnight at 4°C with blocking solution [5% Milk powder in TBS with 0.1% tween, where TBS (Tris Buffered Saline i.e 50 mM Tris-HCl, 150 mM NaCl)]. The membrane was incubated at room temperature for 1.5 h with the desired primary antibody, washed and incubated for 1 h with secondary antibody (antibodies used are listed in table 10).

Immunodetection was performed by using an enhanced chemiluminescence kit (Pierce ECL Westernblot substrate: ThermoScientific) as per the manufacturer and fluorescence signal was detected by Fusion-FX7 system (Vilber Lourmat). The bands were analyzed and quantified with MyImageAnalysis software (ThermoScientific).

Table 10: Antibodies used in the following work

Antibody	Detection	Dilution in TBST	Source
Anti-IFR1 (αIFR1)	Isoflavone reductase like protein (IFR1)	1:2500	This work
Anti-SIR1 (αSIR1)	Sulfite reductase (SIR1)	1:2500	(Khan et al. 2010)
Anti-D1 (αD1)	D1 protein of PSII	1:10000	Agrisera (AS05084A)
Anti-Rabbit (2º Ab)	Primary Ab (1º Ab)	1:10000 to 1:15000	Agrisera (AS10845)

3.8 Construction of *IFR1:YFP* fusion protein

Intracellular localization of IFR1 was analyzed by tagging it to a YFP protein. The de novo synthesized *IFR1* cDNA (Genscript, USA) was cloned into pOpt-mVenus_paro vector (Lauersen et al. 2015). C-terminal and N-terminal fusion of the cDNA was done by cloning between *Nde*I::*Bgl*II and *EcoR*V::*EcoR*I of the vector, respectively. Fluorescence imaging was done as before (Lauersen et al. 2016, 2015) with a confocal laser scanning microscope (LSM780, Carl Zeiss GmbH, Germany) fitted with filters for chlorophyll and mVenus.

3.9 Hydrogen production

Hydrogen production was done by the successful sulfur deprivation method (Melis et al. 2000; L Zhang et al. 2002). The desired strains were propagated in TAP medium to a logarithmic phase OD_{750} ~ 0.7, harvested (2500g at room temperature for 4 min) and washed (3 x 2500g at room temperature for 4 min) with sulfur free (TAP-S) medium. The cells were re-suspended in TAP-S to the tune of ~25 µg/ml chlorophyll and filled into unique bioreactors (Doebbe et al. 2007; Nguyen et al. 2011). The bioreactors were continuously illuminated on both sides with cool white light (300 $\mu Em^{-2}s^{-1}$). Samples were harvested at defined time intervals for further analytics.

3.10 Analytical techniques

3.10.1 Gas chromatography (GC) for hydrogen gas analysis

The total amount of H_2 produced was measured volumetrically and the gas quality was analyzed by GC. The unique H_2 bioreactors carried a gas collection column, where the gas produced by the culture were collected by displacement of water. The gas was sampled through a gas tight Hamilton syringe and injected into a gas chromatograph (Micro GC 3000, Agilent). The chromatograph was fitted with a PlotU pre-column (3 cm x 0.32 mm) followed by a MolSieve 5APlot column (10 cm x 0.32 mm). The injected samples were carried by argon gas, which was maintained at 32.5 psi.

3.10.2 Chlorophyll fluorescence measurement

Chlorophyll fluorescence was assessed by monitoring the photosynthetic quantum yield (Fv/Fm of dark adapted cells or ΦPSII in presence of light) of cells by using a MINI-PAM (Waltz). The intensity of measuring, actinic and saturation light was varied according to the experimental conditions. During H_2 production, ΦPSII was measured with the optical sensor by directly applying a saturation pulse of 15000 µmol photons m^{-2} s^{-1} on the surface of the bioreactor. Culture harvested from H_2 bioreactors or TAP-S cultivation were dark adapted for 20 min under continuous bubbling of air prior to Fv/Fm measurement. 1 ml of dark adapted culture was carefully placed in a quartz cuvette (10 x 10 mm base) and mixed at 30 rpm. Ground state fluorescence (F_O) and maximal fluorescence (F_m) were recorded and used for calculation as follows:

$$Fv = \frac{(Fm - Fo)}{Fm}$$

3.10.3 Measuring total chlorophyll

Total chlorophyll content of the samples were quantified by extracting with acetone as reported before (Arnon 1945). 200 µl of sample was mixed with 800 µl of acetone (100%) and vortexed vigorously. The samples were incubated in dark for a minimum of 5 min and centrifuged (20000 g for 2 min) to remove cell debris. The maximum absorbance of the samples was read at 645 and 663 nm. The chlorophyll content was calculated from the following equations:

$$Chlorophyll\ a = \big((0.0127 \times A663) - (0.00269 \times A645)\big) \times dilution\ factor$$

$$Chlorophyll\ b = \big((0.0029 \times A645) - (0.00468 \times A663)\big) \times dilution\ factor$$

$$Total\ Chlorophyll = Chlorophyll\ a + Chlorophyll\ b$$

3.10.4 Optical density and cell count

Optical density (OD) and cell count were measured at defined times for monitoring cell growth. OD_{750} and OD_{680}, were measured with a spectrophotometer (Genesys 10S UV-Vis spectrophotometer, Thermo Fischer Scientific, Germany) and disposable cuvettes (path length of 1 cm, VWR). Cell count was done with a hemocytometer (Neubauer, Hawksley) and automated cell counter (Z2 cell and particle counter, Beckman Coulter).

3.10.5 Analysis of photosynthetic O_2 evolution with a Clark-type electrode

The photosynthetic rate of oxygen evolution and respirational rate of oxygen consumption were measured with a Clark-type oxygen electrode (Hansatech). The electrode was calibrated at 25ºC with air saturated water (100% O_2) and sodium dithionite treated water (0% O_2). 2 ml of cell suspension supplemented with 5 mM $NaHCO_3$ was placed in the measuring cuvette. The suspension was incubated in dark for 2 min, followed by measuring the rate of oxygen evolution under growth light conditions. The rate of oxygen consumption due to respiration was measured under dark. The kinetics were recorded for a minimum of 4 min and finally normalized to chlorophyll content.

3.11 Influence of ROS and RES stress on growth of *IFR1* knockdown mutants

ROS inducing agents like Rose Bengal (RB, 4 µM), Neutral Red (NR, 15 µM) Methyl Viologen (MV, 0.5 µM), Hydrogen peroxide (H_2O_2, 7 mM) and RES inducing agents such as DBMIB (5 µM) and 2*E*-Hexenal (500 µM), were used for a quantitative assessment of *IFR1* knockdown on cell growth. Cells grown to a mid-log phase in TAP medium were harvested and re-suspended in fresh TAP medium to the tune of 2×10^6 cells/ml. The cells were supplemented with a respective ROS or RES agent and their growth monitored (OD_{750}, OD_{680} and cell count). The variations in growth were analyzed in relative to control conditions. At the end of growth analysis, the cells were spotted on TAP agar plates and their recovery monitored.

3.12 Immobilization of *C. reinhardtii* in novel silica gel

C. reinhardtii wild type CC124 and cell wall less MOC1 mutant *stm6* were immobilized in a novel sodium silicate gel. The sodium silicate precursor (2.5 g) was mixed with water (7.5 g) resulting in formation of a 3D network of silica gel with sodium hydroxide as byproduct. The toxicity arising due to sodium hydroxide was overcome by an ion exchanger. The pH of the gel was stabilized to 7.2 with the help of Tris buffer. After attaining the desired pH, known concentration of cells were added to the gelation mixture. The mixture was pipetted on to a non-adhesive surface and allowed to take the form of a lens (gelation time ~ 5 min). The solidified lenses were later transferred to TAP and TAP-S medium and grown under continuous illumination of light 300 $\mu Em^{-2}s^{-1}$. Viability of the immobilized cells were assessed by monitoring their photosynthetic activity (Fv/Fm).

3.13 Qualitative and quantitative bioluminescence analysis

Luciferase activity (*g*Luc reporter) was qualitatively and quantitatively measured by measuring the extracellular bioluminescence. For qualitative analysis, the substrate was sprayed on TAP agar plates with *SIR1* knockdown mutants and bioluminescence recorded (exposure time of 300 s, NightSHADE LB 985, Berthold Technologies). Supernatant harvested at specific time interval of TAP-S growth was used for qunatitative analysis via a micro-titer plate (MTP) assay (50 µl supernatant, 130 µl assay buffer and 20 µl of substrate). The MTP assay comprises of an assay buffer (0.5 M NaCl, 1 mM EDTA and 0.1 M K_2HPO_4, pH 7.6) containing 10 µM coelenterazine (substrate), to which 50 µl of extracellular supernatant is added and the bioluminescence instantly recorded (Shao et al. 2008).

3.14 In silico and statistical analysis

Information about the gene and their respective accession numbers were obtained from Phytozome (*C. reinhardtii* genome v 5.5), Uniprot and NCBI. Homologues of *IFR-1* were searched by BLAST (NCBI) and in-silico protein analysis were done with tools available at Expasy. SWISSMODEL and I-TASSER were used for modelling the protein structure of IFR-1. Significance of all the results obtained in the following study was evaluated with a student's two-tailed *t*-test. The significance threshold (p) was set between 0.05 to 0.1. All the error bars represent standard error (SE). In box plots, whiskers represent variability within the first and third quartile.

4.0 Identification and characterization of *IFR1* knockdown mutants

4.1 Introduction

Carbon neutral fuels are traversing from being a myth of the past to the fuels of the present. Of the several fields explored to meet the ceaseless energy demands of mankind, solar energy seems promising (Lewis 2007). Algae trap solar energy by photosynthesizing to yield biomass, fatty acids, pigments, antioxidant (Vigani et al. 2015) and are also capable of producing hydrogen under specific conditions (Melis et al. 2000). However, the potential of hydrogen as a fuel is poised on the sustainable and economical large scale production. *C. reinhardtii* belongs to an elite group of unicellular eukaryotes capable of solar-driven hydrogen production.

C. reinhardtii possess an efficient [Fe-Fe]-hydrogenase (Happe et al. 1994) that catalyzes the recombination of protons (H^+) and electrons (e^-) to molecular H_2. The substrates for H_2 production come either directly via PSII (~80%,Volgusheva et al. 2013) or indirectly via fermentation of stored reserves (Doebbe et al. 2010). Since H_2 production is not a main metabolic process, direct light to H_2 conversion efficiency is very low and the oxygen susceptibility of hydrogenase makes it a very tricky process. A quintessential progress was achieved by Melis and coworkers where they brought about a separation between O_2 and H_2 evolution by sulfur (S) deprivation (Melis et al. 2000). Illumination of sealed algae cultures in S deprived media lead to anaerobiosis and light driven hydrogen production. Sulphur deprivation slows down D1 repair, resulting in downregulation of O_2 evolution rates below that of respirational consumption followed by anaerobiosis and induction of hydrogen production. Due to the sulfur stress, cells undergo a major metabolic reshuffling (Matthew et al. 2009) and significant changes at transcriptome (Nguyen et al. 2011), proteome (Terashima et al. 2010) and metabolome (Doebbe et al. 2010). The cells show morphological changes and several reactions including photosynthesis are down-regulated causing changes to PSII complex.

Several strategies have been exploited to improve photobiological H_2 production such as, generating mutants locked in state transition (*stm6*) (Kruse et al. 2005), improving starch reserves (*stm6glc4*) (Doebbe et al. 2007), knocking down potential targets (Oey et al. 2013; Sun et al. 2013), improving electron transfer from PETF towards HYDA1 (Rumpel et al. 2014) and many more. Very few reports have highlighted the importance of PSII activity on H_2 production (Antal et al. 2003; Volgusheva et al. 2016, 2013). A repository of genes regulated in *C. reinhardtii* during

H_2 production emphasize the interplay of various pathways and also highlight the potential targets for improving H_2 production (Mus et al. 2007; Nguyen et al. 2008; Terashima et al. 2010; Toepel et al. 2013).

Based on transcriptomic analysis of a H_2 production culture (Nguyen et al. 2011; Toepel et al. 2013), the role of an unreported novel gene, Isoflavone reductase like protein (IFR1) was investigated. *IFR1* belongs to the classical family of short chain dehydrogenases/reductases (SDR's) with a requirement of an NADPH coenzyme (Kallberg et al. 2002) and was shown to be strongly upregulated under hydrogen production conditions (Nguyen et al. 2011). *IFR1* has been reported to be induced under biotic and abiotic stress. It was found to accumulate under UV irradiation in grapefruit (Lers et al. 1998), overexpression of *IFR1* led to ROS tolerance in *O. sativa* (S. G. Kim et al. 2010) and IRL in maize was reported to be expressed under S starvation (Petrucco et al. 1996). *IFR1* shares homology with leguminous Isoflavone reductases (IFRs) which have been involved in isoflavonoid phytoalexin biosynthesis and medicarpin biosynthetic pathway (Guo, Dixon, and Paiva 1994). However, IFR1s from tobacco and maize could not produce isoflavonoids when assayed with IFRs substrates, indicating that they may be more stress related genes (Shoji et al. 2002). Therefore, artificial microRNAs (amiRNA) (Molnar et al. 2009) were applied in the following study to elucidate the phenotype and hydrogen producibility of an *IFR1* knockdown.

4.2 Results

4.2.1 IFR1 belongs to short chain dehydrogenase/reductase (SDR) superfamily

A similarity search (BLAST) with the IFR1 protein sequence (Phytozome, locus Cre11.g477200; *C. reinhardtii* v5.5) followed by a multiple sequence alignment of

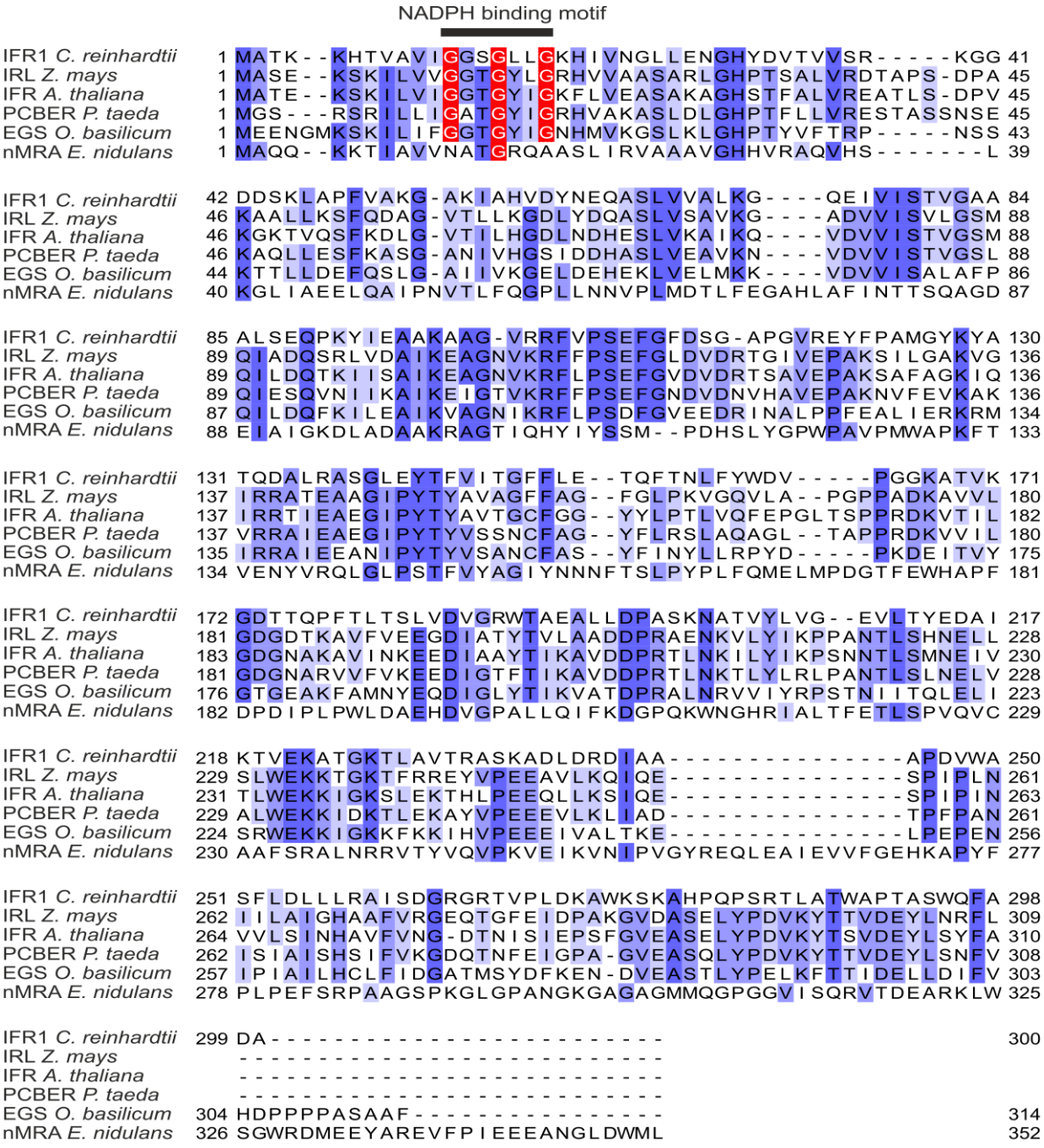

Figure 5: Multiple sequence alignment (MSA) of amino acids of various Isoflavone reductase like protein and atypical SDRs. The conserved glycine rich NADPH binding motif is highlighted in red, whereas other conserved amino acids are highlighted based on the degree of conservation with light or dark blue color. Sequence aligned are as follows: *Chlamydomonas reinhardtii* IFR1, *Zea mays* IRL, *Arabidopsis thaliana* IFR, *Pinus taeda* PCBER (DDCBER1), *Ocimum basilicum* EGS and *Emericella nidulans* nMRA.

homologs were done (fig. 5) to gain functional and structural insights. IFR1 was found to share high similarities with pinoresinol-lariciresinol reductase of western red-cedar (PLR, *T. plicata*, UniProtKB Q9LD14, 30% identity, Min et al. 2003), phenylcoumaran benzylic ether reductase (PCBER, e.g. *P. taeda* DDCBER1, UniProtKB O81651, 30% identity, Min et al. 2003), isoflavone reductase like protein in maize (*Z. mays* IRL, UniProtKB P52580, 28.8% identity, Petrucco et al. 1996), isoflavone reductases in Arabidopsis (*A. thaliana* IFR, UniProtKB P52577, 28.6% identity, Babiychuk et al. 1995), eugenol synthases (e.g. *O. basilicum* EGS, UniProtKB A0A1B2U6R8, 24.5% identity, Louie et al. 2007) and NmrA-like family of proteins that are responsible for nitrogen metabolite repression in fungus (*A. nidulans* NmrA, UniProtKB Q5AU62, Andrianopoulos et al. 1998, 19.8% identity) . All these homologs belong to SDR super family and possess a glycine rich NADPH binding motif (GXGXXG or G[GA]XGXXG) at the N terminal and a small substrate binding domain at the C terminal.

IFR1 gene is translated to a 32 kDa monomer and similar to other IFR1 homologs (Babiychuk et al. 1995; Hua et al. 2013; Min et al. 2003; Petrucco et al. 1996; Wang et al. 2006) it was predicted to be localized in the cytoplasm (LocTree2: Goldberg et al. 2012 and PredAlgo: Tardif et al. 2012). To confirm the cytosolic prediction of IFR1 and understand its cellular localization, IFR1 was C- and N- terminally fused with YFP (mVenus variant, Kremers et al. 2006) and expressed in *C. reinhardtii* cell line UVM4 (Neupert et al. 2009). Two mutants showing stable expression of either full length (~58 kDa) IFR1:YFP (N) or IFR1:YFP (C) were confirmed via immunoblotting (fig. 6).

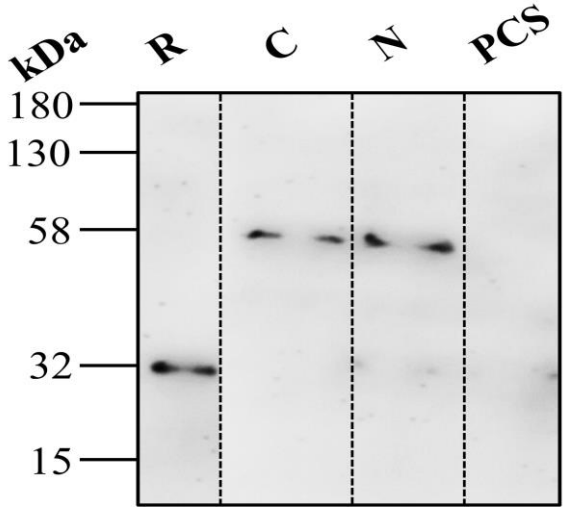

Figure 6: Immunodetection of IFR1:YFP fusion expressing mutants, which were observed by confocal laser scanning microscopy. (40 µg) Total protein extracted from TAP grown parental control strain (PCS, UVM4), mutants with C- or N-terminal IFR1:YFP fusion (C/N) and recombinant IFR1 (R, 32 kDa) were analyzed with anti IFR1 antibody (1:2500).

YFP accumulation in the cytoplasm was observed in both the mutants by confocal laser-scanning microscopy (fig. 7), whereas the parental control strain (PCS) emitted only red signal due to chlorophyll auto fluorescence. Signal from a control strain

(Cyto, Lauersen et al. 2015) expressing YFP in the cytoplasm was similar to the superimposed signal of chlorophyll and YFP obtained from mutants expressing C-terminal and N-terminal fusions. This proved that IFR1 localization was indeed cytosolic.

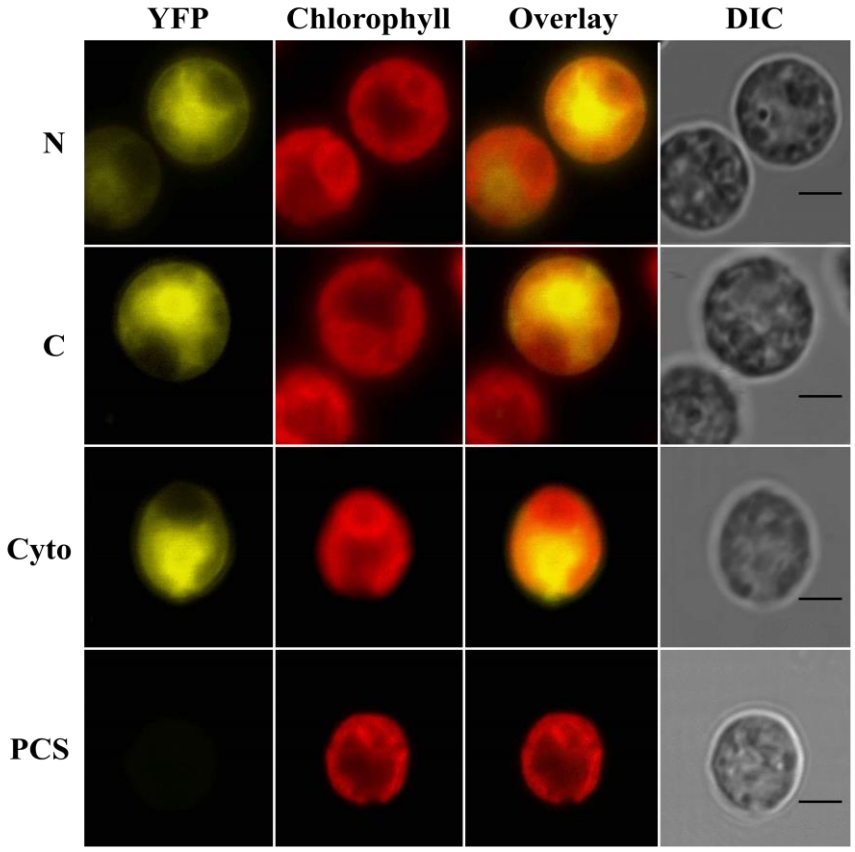

Figure 7: Cytosolic localization of IFR1 as observed by confocal laser-scanning microscopy. Subcellular localization of mVenus reporter fused to C- or N-terminal of IFR1 (C/N) as observed by confocal laser scanning microscopy. Parental control strain (PCS, UVM4), showing no fluorescence signal served as a negative control, while a UVM4 mutant showing cytosolic expression of YFP (Cyto, Lauersen et al. 2015) served as a positive control. The yellow fluorescence signals (YFP) are shown in the first column and autofluorescene of chlorophyll is shown as a red signal. An overlay (YFP and chlorophyll overlay) of the signals are shown indicating cytosolic localization. DIC: Differential Interference Contrast, where the scale bar represent 5 µm.

A 3D structure of the IFR1 protein was modelled with Phyre^2 (Kelley et al. 2015) (fig. 8). The protein structure did not possess any transmembrane motif or localization signal peptides. The 3D structure was compared with other homologs and also utilized for a substrate prediction (ETA: http://mammoth.bcm.tmc.edu/AminErdinetalPNAS/et-

a/) (Amin et al. 2013). However, ETA substrate prediction analysis did not result in prediction of any potential substrates.

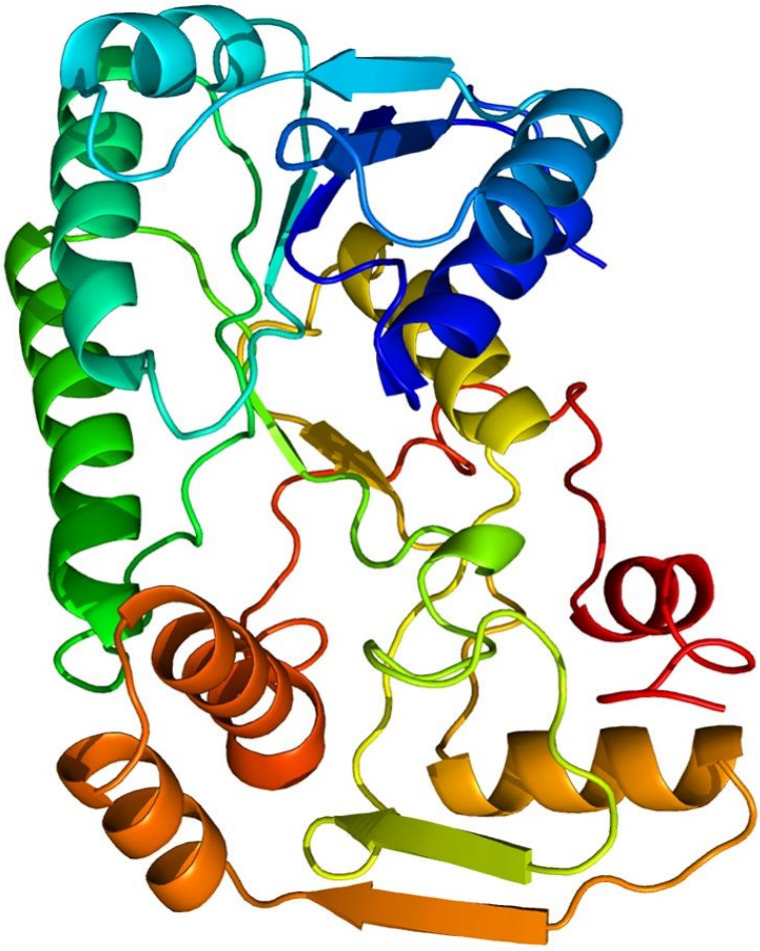

Figure 8: Model of *C. reinhardtii* Isoflavone reductase like protein (IFR1) as generated by the tool Phyre[2].

4.2.2 Abiotic stress induces IFR1 accumulation

Transcriptome from sulfur deprived (Melis et al. 2000) H_2 producing cultures of wild type (wt) CC406 and high H_2 producer mutant *stm6glc4* (Doebbe et al. 2007) were analyzed (Nguyen et al. 2011). The transcriptome data showed the accumulation of *IFR1* transcript (~10-40 fold) in both the strains during peak H_2 production phase. Protein samples were extracted at specific time points from H_2 producing culture of wt CC124 and validated if *IFR1* transcript is indeed translated to protein. IFR1 accumulation was detectable (fig.9) even before the onset of anaerobiosis (within 24 h) which became prominent thereafter. This showed that *IFR1* induction was triggered not by anaerobiosis but because of sulfur depletion which also correlates to the previously reported RNAseq data (~8-fold induction, 6h after sulfur depletion, González-Ballester et al. 2010, gene expression omnibus (GEO) series GSE17970).

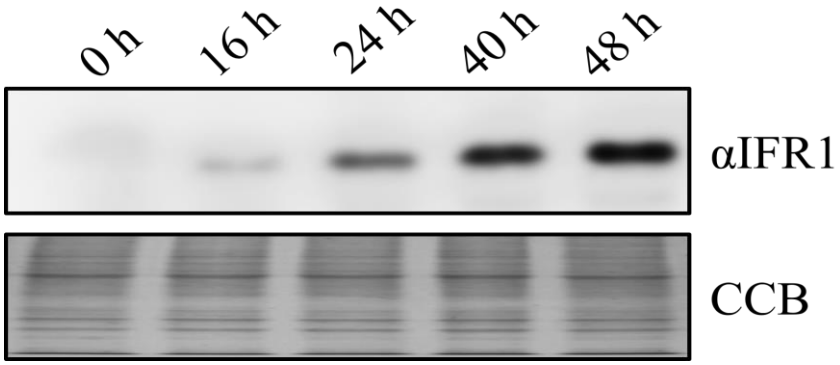

Figure 9: IFR1 expression during sulfur starved hydrogen production. Samples harvested before (0h, Sulfur replete) and during hydrogen production (16 h to 48 h, Sulfur deplete) from CC124 wt are shown. The protein accumulation was detected with an anti IFR1 antibody (αIFR1) and equal protein load was confirmed by colloidal coomassie staining (CCB).

The AlgaePath (Zheng et al. 2014) portal was used to asses *IFR1* RNAseq data derived from various abiotic conditions like carbon dioxide limitation (~2 fold, Fang et al. 2012, GSE33927), nitrogen starvation (~46 fold after 48 h, Miller et al. 2010, GSE24367) and studies reporting hydrogen peroxide stress (~19 fold within 1 h, Blaby et al. 2015, GSE34826). Based on the previous reports, we assessed the impact of macronutrient limitation by growing the wt CC124 under nitrogen depletion. Unlike sulfur starvation (fig. 9), IFR1 protein expression could not be detected (fig. 10) under nitrogen depleted conditions which suggested that IFR1 accumulation could be independent of macronutrient limitation.

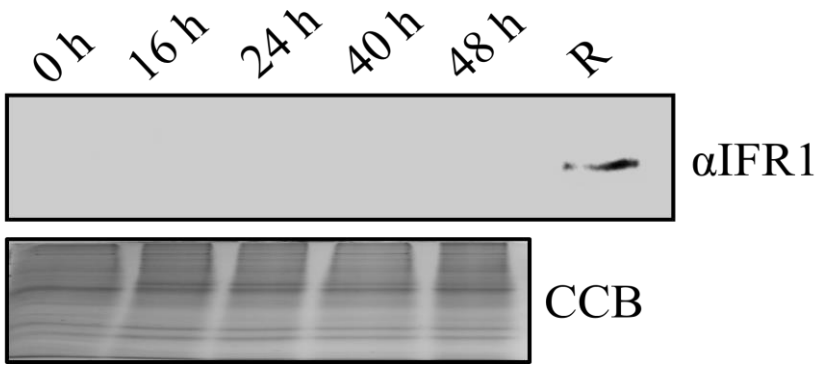

Figure 10: IFR1 expression during nitrogen starvation. Samples taken before (0h, nitrogen starvation) and after (16 h to 48 h) nitrogen starvation. IFR1 protein accumulation cannot be detected. Recombinant protein (R) served as a control and equal protein load is represented by colloidal Coomassie staining (CCB).

IFR1 was also reported as belonging to a certain set of genes that are constantly overexpressed in the singlet oxygen resistant 1 (*sor1*) mutant (~9 fold in *sor1* and undetectable in parental (4A+), Fischer et al. 2012, GSE33548). In the following study, we confirmed the previously observed high expression of *IFR1* in *sor1* via RTqPCR (fig.11A) and later verified if high mRNA level was translated into protein (fig. 11B).

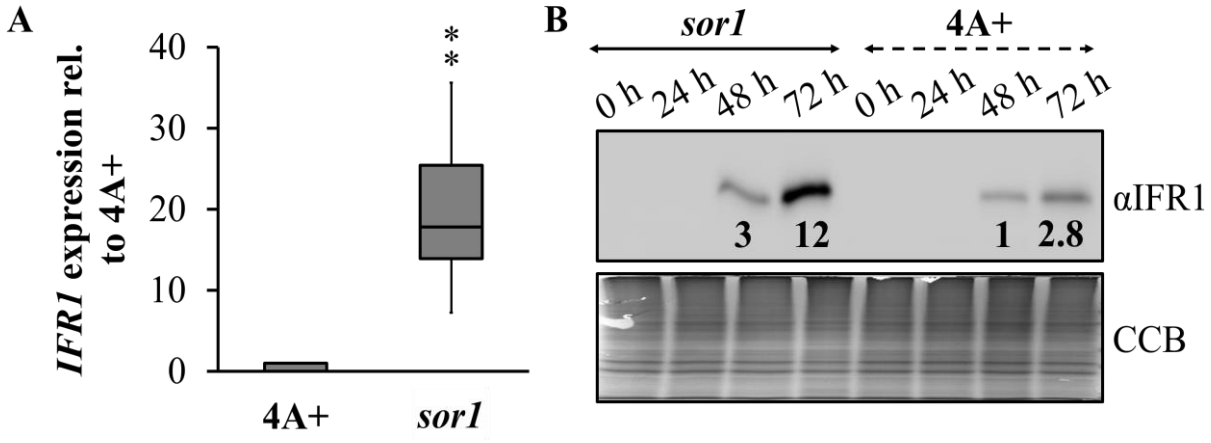

Figure 11: IFR1 expression is driven by SOR1 pathway. (A) RTqPCR analysis showing *IFR1* transcript during early stationary phase (40 h) of TAP grown *sor1* and 4A+. In the plot, *IFR1* transcript level obtained from 4A+ is normalized to 1. Quartile range and median of the box-whisker plot were derived from two biological replicates with nine technical replicates each (n = 18). Asterisk show statistical significance according to a two-tailed student's t-test ($p<0.05$). (B) Immunoblot showing the accumulation of IFR1 in *sor1* and 4A+ grown in TAP. The numbers **3**, **12**, **1** and **2.8** represent denistometric values of IFR1 expression relative to 4A+, where IFR1 expression in 4A+ at 48 h is set to **1**. Lower panel represent colloidal coomassie stained gel as a loading control.

Analysis of IFR1 promoter revealed the presence of an 8 bp palindromic motif (CAACGTTG) (fig. 12A), reported to be an electrophile response element (ERE) that responds to the presence of a reactive electrophile species (RES) via the singlet oxygen resistance (SOR1) pathway (Fischer et al. 2012). Hence, the responsiveness of IFR1 expression in sulfur replete TAP culture of wt CC124 was assessed by adding a RES compound such as DBMIB (2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone). To rule out the impact of photosynthetic inhibitory effects, the experiment was repeated with DCMU [(3-(3,4-Dichlorophenyl)-1,1-dimethylurea, inhibits PSII forward electron transfer, Metz et al. 1986] and also with DBMIB supplemented cultures grown in dark. IFR1 protein accumulated independent of light in cultures supplemented with DBMIB (fig. 12B), whereas no IFR1 expression could be observed in cultures supplemented with 0.1 µM DCMU (Appendix: fig. S1). This suggested that the IFR1 accumulation was indeed due to DBMIB's action as a reactive electrophile species.

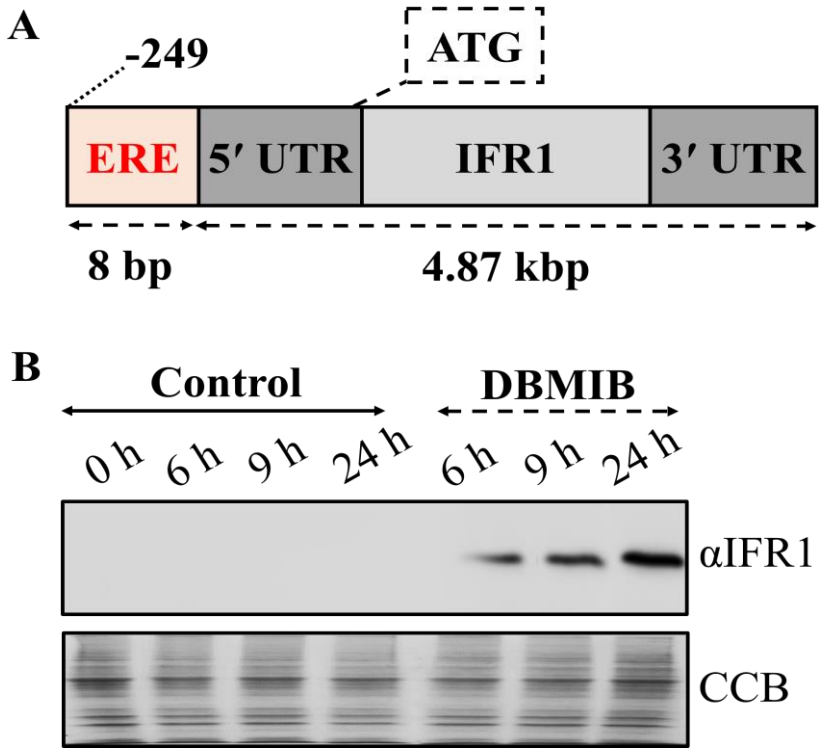

Figure 12: An Electrophile Responsive Element (ERE) drives the expression of IFR1.
(A) Full length (4.87 kbp) IFR1 locus comprising of exons, introns and untranslated regions (UTR) are shown. An 8 bp palindromic sequence regarded as an ERE is shown 249 bp upstream of the start codon. (B) IFR1 accumulation in CC124 wt incubated in dark and grown in TAP supplemented with DBMIB or only solvent (Control).

4.2.3 Isolation of *IFR1* knockdown strains

The previously reported (Molnar et al. 2009) amiRNA approach was applied in this study for functional characterization of *C. reinhardtii IFR1*. The amiRNA targeting *IFR1*'s exon 2 or 4 were analyzed and cloned under the control of a PsaD constitutive promoter (Fischer and Rochaix 2001) into pChlamiRNA3int vector system (Molnar et al. 2009). Selection of paromomycin resistance lead to the isolation of 500 clones. CC124 derived knockdown strains, IFR1-1 and IFR1-6 with ~35% and ~5% of the control level protein (fig. 13), respectively were confirmed by immunoblotting. For unknown reasons, the knockdown strains appeared slightly smaller than the parental control strain (PCS). These strains were used for further physiological analysis.

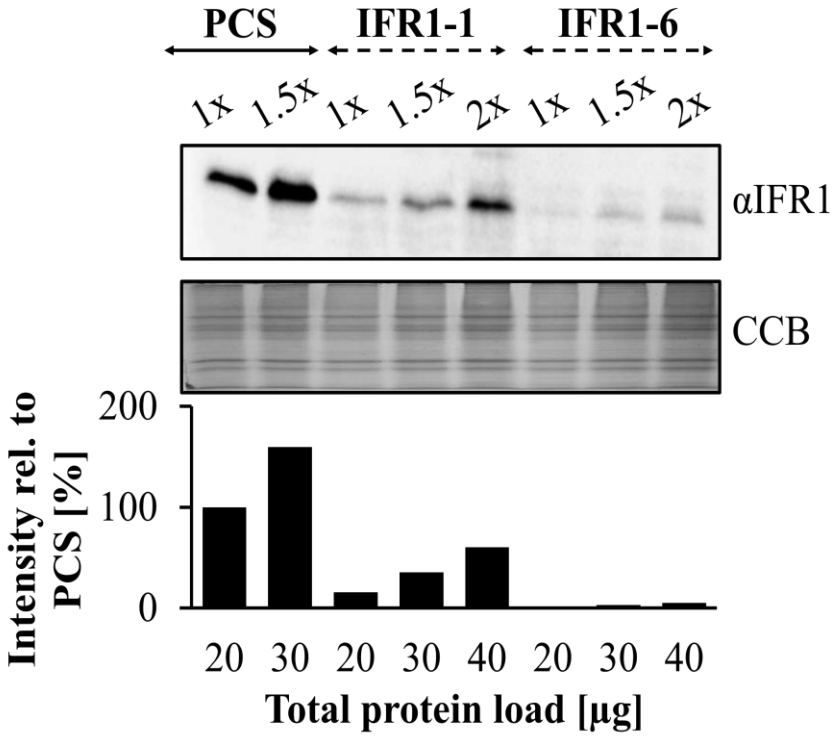

Figure 13: Detection of *IFR1* knockdown mutants. Immunodetection of IFR1 protein in CC124 (PCS) and IFR1 knockdown strains (IFR1-1 & IFR1-6) after 48 h of growth in TAP-S (sulfur depletion). 1x, 1.5x and 2x correspond to 20, 30 and 40 µg of total protein. Colloidal coomassie stained gel served as loading control and densitometric analysis is shown in the graph below (PCS 1x is set to 100%).

4.2.4 Knocking down *IFR1* results in RES sensitivity

IFR1 homologs have been reported to be expressed under various stress conditions (Brandalise et al. 2009; Kim et al. 2003; S. G. Kim et al. 2010; Lers et al. 1998; Luo et al. 2010; Petrucco et al. 1996). Therefore, the growth characteristics of CC124 and its *IFR1* knockdown strains were analyzed under various RES and ROS stress. The accumulation of IFR1 was found to be diminished in both the knockdown strains treated with RES compound DBMIB (artificial RES) and 2-(E)-hexenal (found in higher plants, Mosblech et al., 2009) (fig. 14A and 14B). However, of the two RES agents, 2-(E)-hexenal triggered a stronger IFR1 accumulation in the parental strain as compared to the knockdown strains, which was also evident by the RTqPCR analysis (Fig 14C).

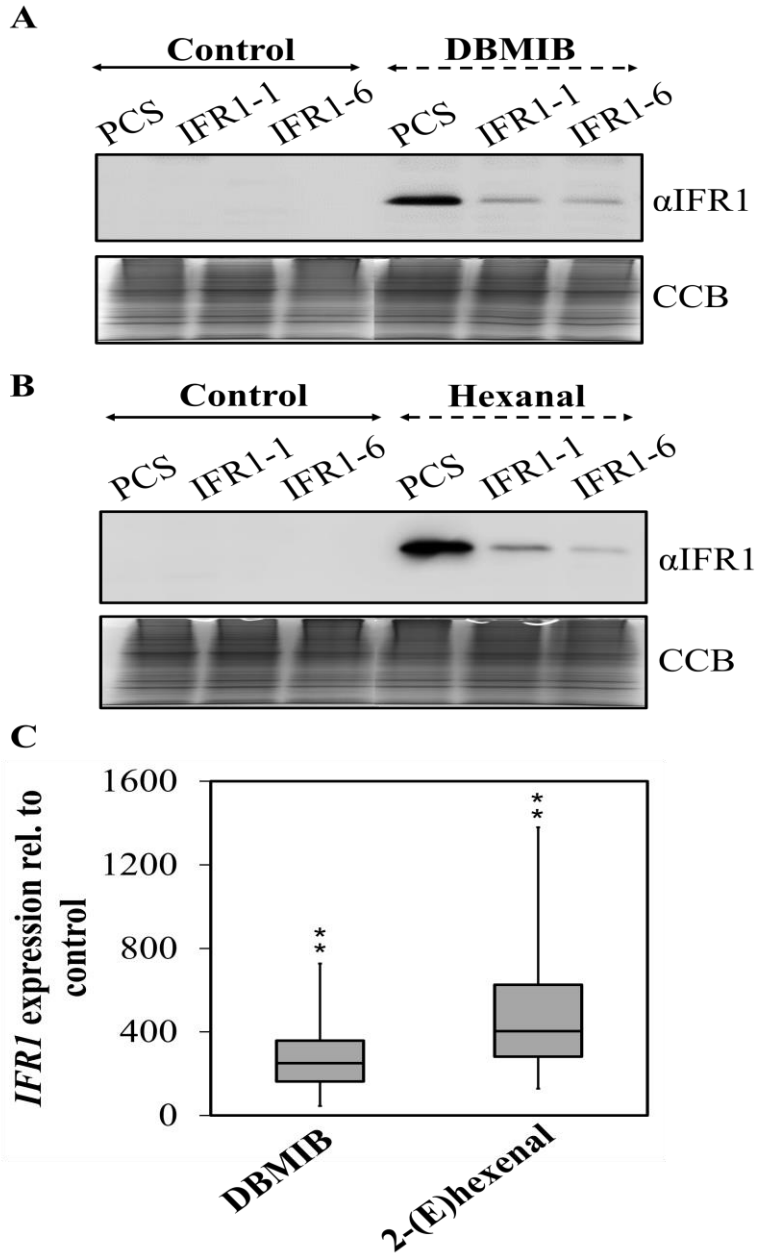

Figure 14: Accumulation of IFR1 in response to RES. Immunoblot analysis of IFR1 expression in PCS and IFR1 knockdown strains grown for 24 h in TAP supplemented with (A) DBMIB (5 µM) or (B) 2*E*-Hexenal (500 µM). Control refers to growth in TAP supplemented with solvent (95% ethanol). (C) RTqPCR analysis showing change in expression of *IFR1* transcript in PCS grown in TAP with the aforementioned RES agents. *IFR1* expression in absence of RES agents was set to 1. Data derived from two biological replicates with three technical replicates each (n = 6). Asterisk show statistical significance according to student's t-test ($p<0.05$).

The growth of *IFR1* knockdown strains in the presence of ROS or RES inducing compounds were analyzed (fig. 15) because of the observed IFR1 accumulation during RES stress (fig. 14) and reports that suggests its role in oxidative stress tolerance in higher plants (S. G. Kim et al. 2010).

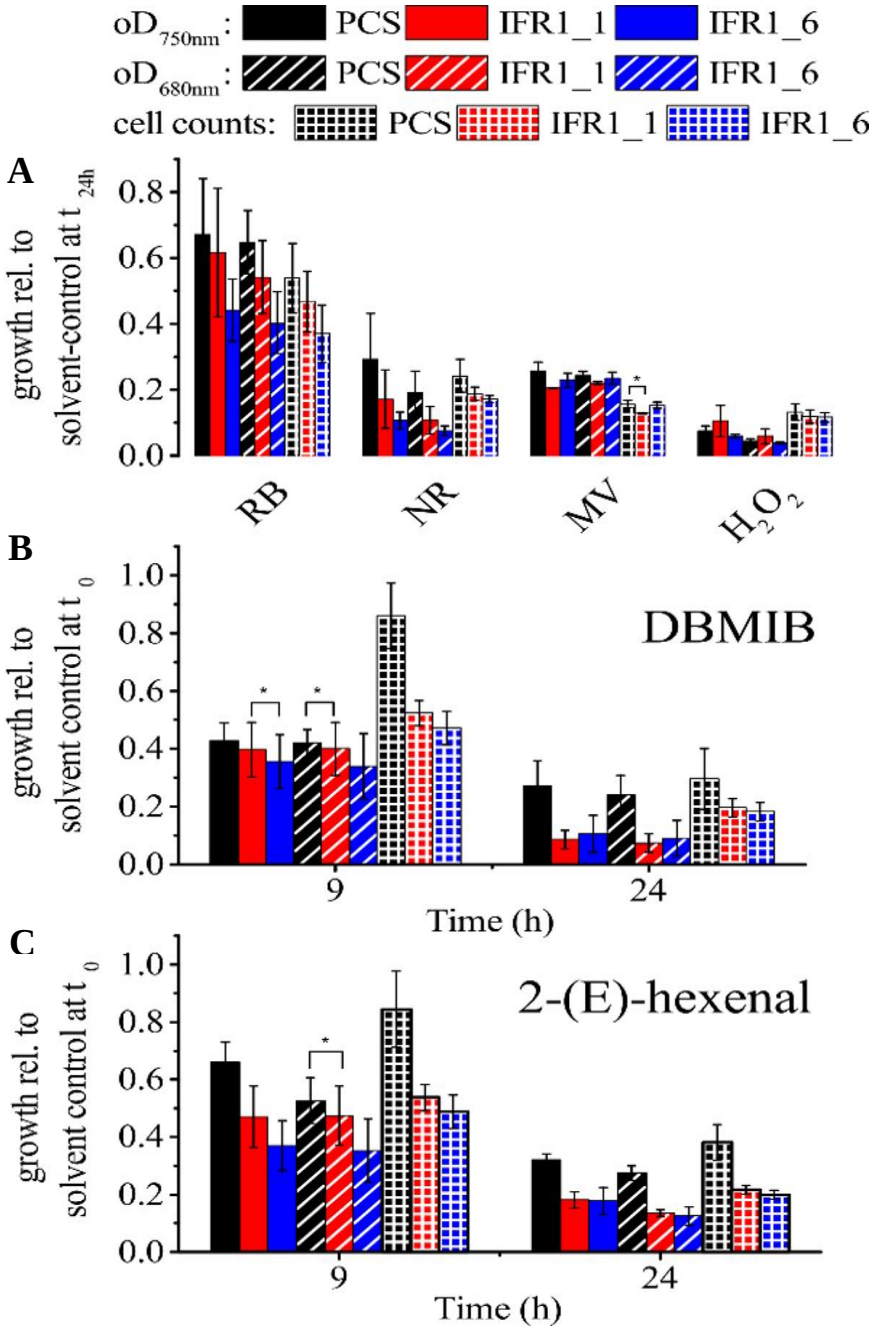

Figure 15: Growth of *IFR1* knockdown strains in presence of ROS and RES compunds. (A) Growth in presence of ROS inducing agents such as 4 µM rose Bengal (RB), 15 µM neutral red (NR), 0.5 µM methyl viologen (MV) or 7 mM hydrogen peroxide (H2O2) are shown. Optical densities (OD_{680} and OD_{750}) and cell counts are shown relative to the control (solvent) which is set to 1. Error bars indicate standard error derived from three biological replicates with technical duplicates (OD_{750}; OD_{680} and cell counts: n=6). Growth in presence of RES agents

such as 5 µM DBMIB (B) and 500 µM 2-(E)-hexenal (C) at 9h or 24h in TAP medium. Error bars indicate standard error obtained from two biological replicates with six technical duplicates (n=12). Asterisks represent statistically significant differences according to a two-tailed student`s t-test ($p<0.05$).

The growth in TAP supplemented with defined concentration of RES (DBMIB and 2-(E)-hexenal) and ROS (Rose Bengal (RB), Neutral Red (NR), Methyl Viologen (MV) and Hydrogen peroxide (H_2O_2)) inducing agents was monitored over 24 h (OD_{680}, OD_{750} and cell count) (fig. 15). Cell viability was assessed at the end of the cultivation by spotting 10 µl of culture on TAP agar plates (fig. 16). Significant growth differences did not arise between PCS and *IFR1* knockdowns grown in presence of superoxide anion (Krieger-Liszkay et al. 2011) triggering agents such as MV or H_2O_2 (fig. 15A). Though growth differences were observable in presence of photosensitizers (Fischer et al. 2004) like RB and NR, the differences were not statistically significant according to two-tailed Student's t-test ($p < 0.05$). However, statistically significant differences were observed in cells treated with RES (fig. 15 B and 15C), which caused a greater growth inhibition in knockdown strains. The high RES susceptibility of knockdown strains was already reflected in the cell count obtained after 9 h of growth, which showed that reduction in *IFR1* hand rendered the knockdown strains sensitive to reactive electrophile species.

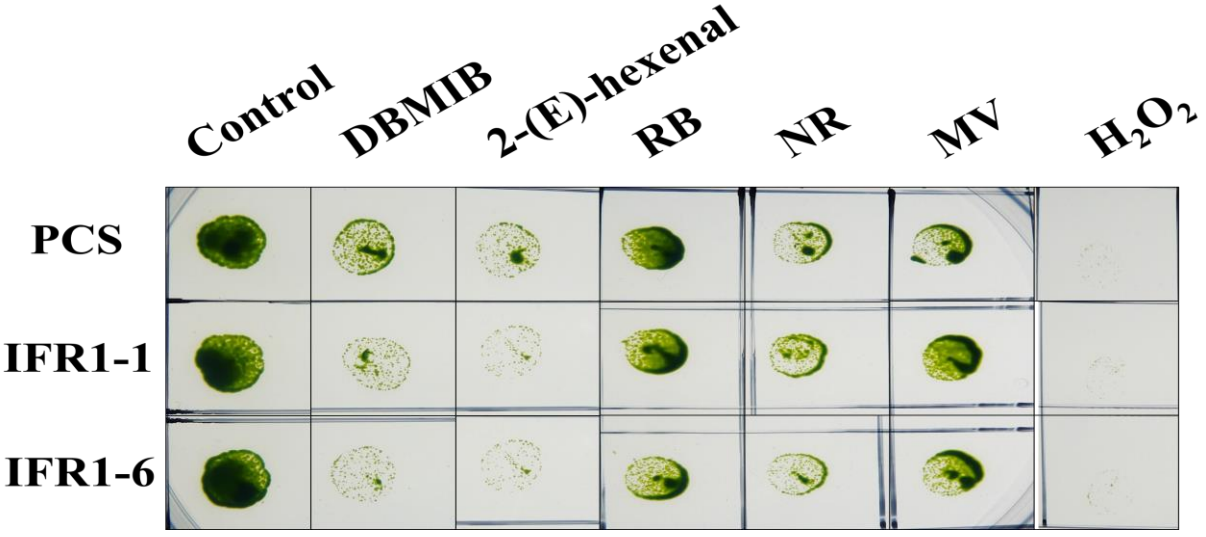

Figure 16: Quantitative growth analyses of CC124 (PCS) and *IFR1* knockdown mutants. Cells grown for 24 h, in liquid TAP complemented with specific concentrations of ROS (RB 4 µM, NR 15 µM, MV 0.5 µM and H_2O_2 7 mM) or RES (DBMIB 5 µM, 2-(E)-hexenal 500 µM) agents were spotted on TAP agar plates for recovery. Cells recovered for 4 days in the presence of low light (100 µmol m^{-2} s^{-1}) is shown.

4.2.5 Prolonged hydrogen production by *IFR1* knockdown mutants

IFR1 was observed to accumulate during sulfur deplete hydrogen production (fig. 9), suggesting its important role during acclimation to sulfur depletion. Hence, the impact of *IFR1* knockdown was determined by looking at the H_2 producibility of the knockdown strains (fig. 17A). Parental control strain (CC124), IFR1-1 and IFR1-6 were grown in sulfur-replete TAP medium to a mid-log phase (OD_{750} ~0.8) and transferred to sulfur-deplete TAP (TAP-S) medium adjusted to same chlorophyll content (~25 µg/ml). Hydrogen production was first detectable in PCS at 24 h but could only be detected after two-days of lag phase in *IFR1* knockdowns. In the beginning (up to 48 h) hydrogen yield by the PCS overtook knockdown strains by ~35 to 40% but beyond 48 h, the rate of H_2 production declined in the PCS (fig. 17B). The H_2 rates increased to a maximum of ~2.25 to 2.5 ml/lh at t_{72h} in the knockdown strains, which declined significantly beyond t_{120h} (fig. 17B). Hydrogen production stopped at 96 h in PCS which translates to a production phase (time between the first detection of H_2 till the end of its production) of 3 days as compared to 5 days in *IFR1* knockdowns. Though the highest rate of H_2 production was reached by PCS, the prolonged phase of hydrogen production observed in the knockdowns led to H_2 yields that were 68 ± 10% (IFR1-1) and 93 ± 12% (IFR1-6) more than the PCS.

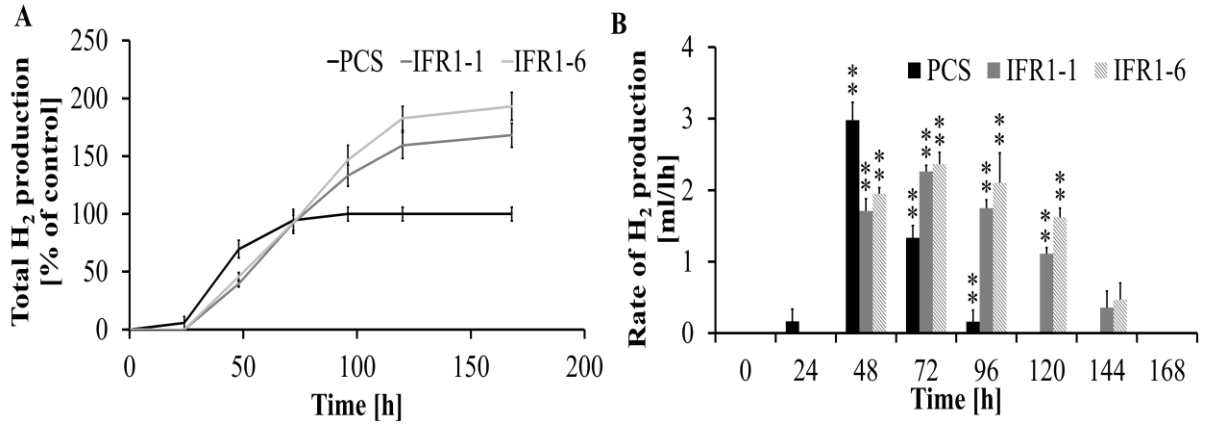

Figure 17: Prolonged phase of hydrogen production by *IFR1* knockdowns as compared to PCS. (A) Comparison of H_2 production between wt CC124 (PCS) set to 100% and *IFR1* knockdowns. Each data curve is derived from an average of three biological with technical triplicates (n = 9), where error bars represent standard error. (B) Rate of H_2 production. Error bars indicate standard error (n = 9) and asterisks represent statistically significant difference among the strains (** $p < 0.05$).

4.2.6 Sustained PSII activity drives prolonged hydrogen production

The mechanism behind prolonged H_2 production was deciphered by monitoring the photosynthetic activity (Fv/Fm) of strains grown under sulfur starvation (TAP-S) and hydrogen production conditions. *IFR1* knockdown strains grown in TAP-S had higher residual PSII activity (fig. 18, $t_{72\text{-}168h}$) as compared to the PCS. This finding correlated to the previous report (Steinbeck et al. 2015; Volgusheva et al. 2013) highlighting the contribution of residual PSII activity on the direct pathway of H_2 production.

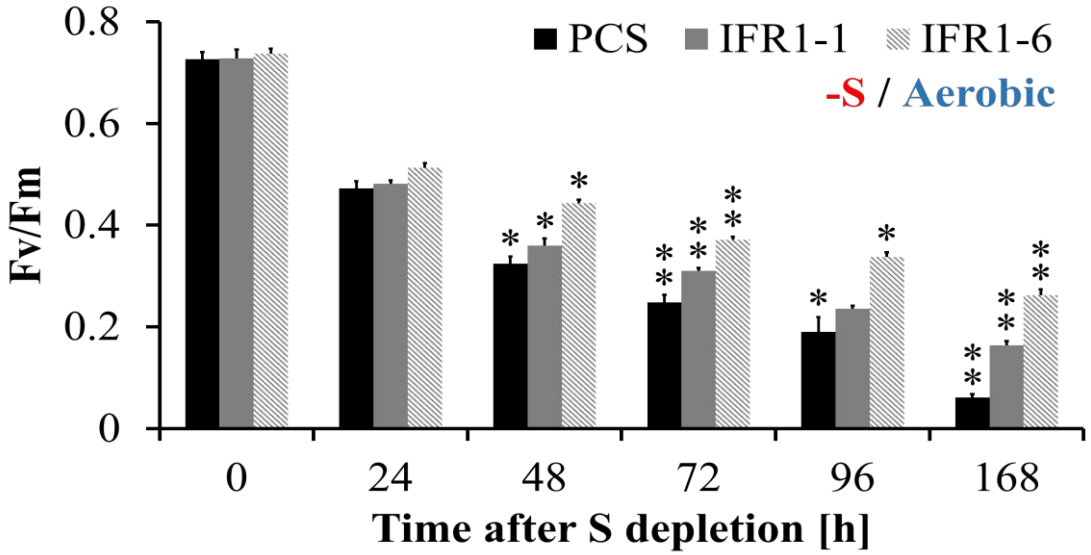

Figure 18: PSII activity of PCS and *IFR1* knockdowns strain during sulfur depletion. Maximum quantum yield (Fv/Fm) of dark-adapted cells before (0 h) and after exposure to aerobic sulfur limitation (t_{24h}-t_{168h}). Error bars indicate standard error from three biological replicates (n = 3) and asterisks represent statistically significant differences (** $p < 0.05$).

The knockdown strain IFR1-6 was chosen to further assess PSII differences observed between the knockdown and PCS under H_2 production conditions (sulfur deprivation and anaerobic conditions). Similar to previous observation, PSII stability was higher in IFR1-6 as compared to PCS (fig. 19A, t_{29h} and beyond) which was also reflected by the decrease in chlorophyll content/cell (fig. 19B, ~30% in IFR1-6 as against 50% in PCS). In accordance to the high PSII activity observed in IFR1-6 knockdown, the decline of D1 (subunit of PSII core) protein was slower in the knockdown as compared to the PCS (fig. 19 C and 19 D).

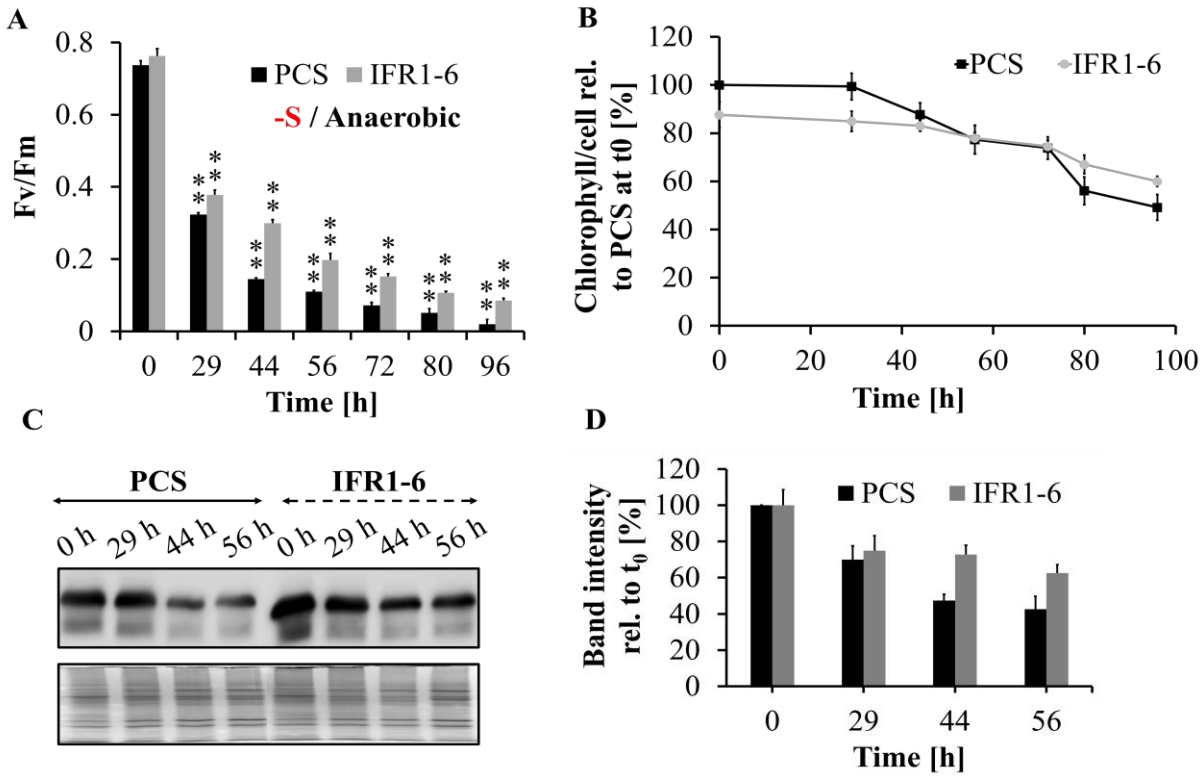

Figure 19: PSII activity of PCS and *IFR1* knockdown strain (IFR1-6) during hydrogen production. (A) Fv/Fm of dark acclimated cells obtained from hydrogen production. Error bars represent standard error derived from three biologicals with technical duplicates (n = 6) and asterisk show statistical significance (** $p < 0.05$). (B) Changes in chlorophyll content/cell of PCS and IFR1-6 during hydrogen production is shown. Chlorophyll content of PCS at t_0 is set to 100%. Standard error is obtained from three biologicals (n = 3). (C) Immunoblot analysis of PSII subunit D1 (upper panel) of PCS and IFR1-6 obtained at specified time of hydrogen production cultures. Colloidal coomassie staining (lower panel) served as a loading control. (D) Densitometry analysis of D1 immunoblot. The intensity of D1 signal obtained at t_0 is set to 100%. Error bars indicate standard error from three biologicals (n = 3).

The forward electron flow of PSII was inhibited with DCMU to assess the contribution of residual PSII activity on prolonged hydrogen production. 20 µM of DCMU was added directly into the H_2 bioreactors 30 h after the onset of sulfur deprivation. DCMU blocks linear electron flow from electron carrier Q_A to Q_B, preventing any direct electron transfer towards hydrogenase which results in inhibition of H_2 production as observed before (Kruse et al. 2005; Scoma et al. 2012; Volgusheva et al. 2013; Zhang et al., 2002). H_2 production dropped in both the strains (fig. 20) and the effect of DCMU inhibition was stronger on *IFR1* knockdown (~73% reduction in PCS as compared to ~163% reduction in IFR1-6). Hydrogen production was 16.6 ± 3.2% in IFR1-6, which was lower compared to 27.1 ± 9.4% from PCS. Thus, it can be

concluded that the prolonged H_2 production caused by *IFR1* knockdown stems from a stabilized PSII fueling the PSII dependent hydrogen production pathway.

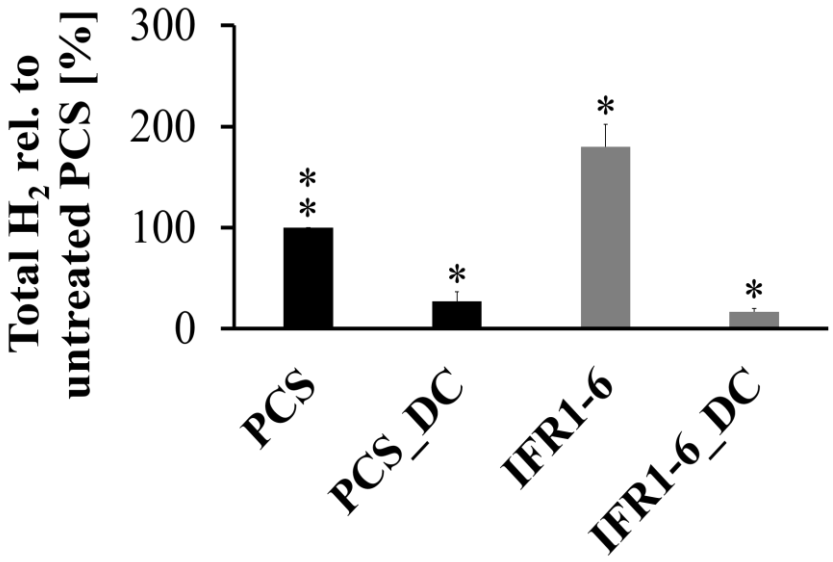

Figure 20: Effect of DCMU on PSII dependent hydrogen production pathway. Total amount of H_2 produced by control strain CC124 (PCS) in the absence of DCMU (solid black bar) is set to 100%. The amount of H_2 produced by DCMU treated samples (PCS_DC and IFR1-6_DC) is shown. Error bars represent standard error derived from two biologicals with technical triplicates (n = 6) and asterisk show statistical significance of the data (** $p < 0.05$, * $p < 0.1$).

4.2.7 Enhancing hydrogen production in *stm6* by knocking down *IFR1*

C. reinhardtii strain *stm6* (Kruse et al. 2005) was selected to test if knocking down *IFR1* could further boost its hydrogen production capacity. The *stm6 IFR1* knockdown strain, *stm6_IFR1kd* showed ~20% IFR1 accumulation as compared to the parental strain (fig. 21A, parental strain 100%). Similar to previous observation (fig. 17A) knocking down *IFR1* led to a prolonged hydrogen production phase in *stm6_IFR1kd* strain (fig. 21B). As compared to the PCS, a ~20 h lag phase preceded the hydrogen production phase in the knockdown mutant. Nevertheless, the H_2 production phase in the knockdown mutant lasted till t_{168h} in comparison the H_2 production phase in PCS plateaued at t_{120h}. A prolonged H_2 production phase with its steep slope in *stm6_IFR1kd*, resulted in ~70% more hydrogen as compared to the PCS. In correlation to the previously observed PSII activity under sulfur limiting conditions (fig. 18), PSII activity of *stm6_IFR1kd* also declined slowly and at t_{96h} reached 0.48 ± 0.01 as compared to 0.35 ± 0.02 in PCS (fig. 21C). These results show that lowering IFR1 results in a sustained PSII activity which significantly fuels

prolonged phase of hydrogen production in *C. reinhardtii*. The relation between cellular amounts of IFR1 and H_2 production was further elucidated by observing low H_2 yields from *sor1* mutant (fig. 11) as compared to its parental strain 4A+ (fig. 21D)

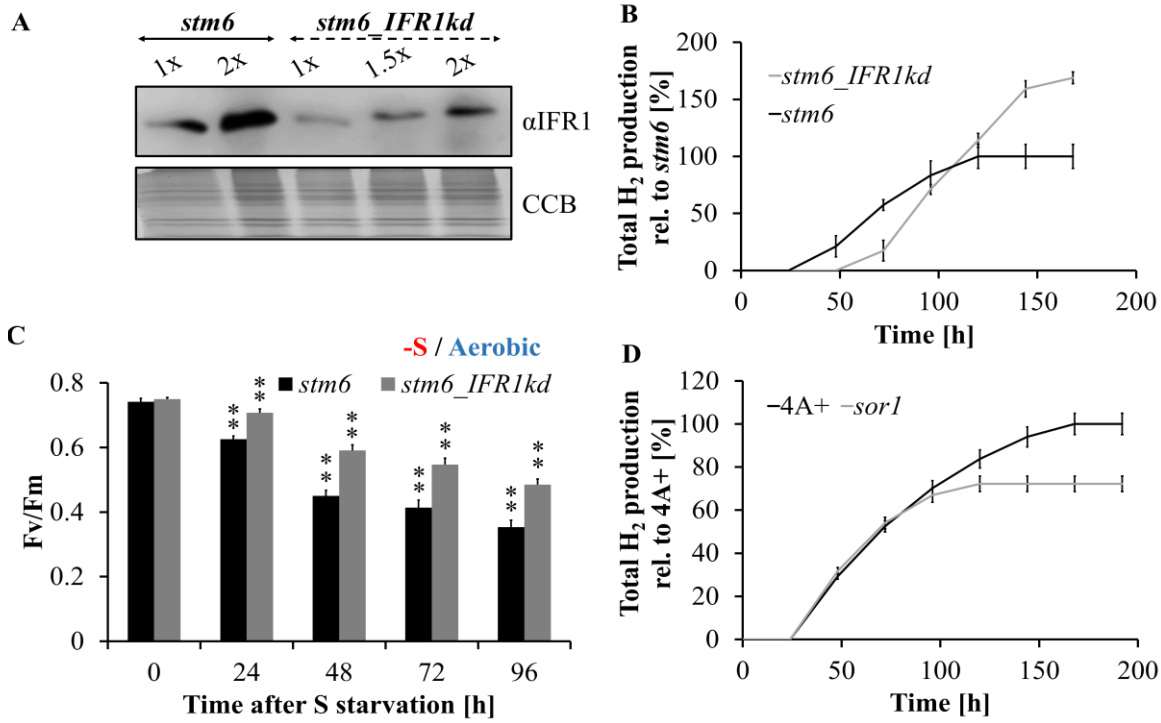

Figure 21: Enhancing hydrogen production by knocking down *IFR1* in *stm6*.

(A) Immunoblot analysis of IFR1 accumulation in *stm6* and *stm6_IFR1kd* grown in sulphur depletion. 1x, 1.5x and 2x represent 20, 30 and 40 µg of total protein. Colloidal coomassie staining served as a loading control. (B) Total H_2 produced by *stm6* (black curve) is set to 100% and H_2 yield obtained from the knockdown strain (grey curve) is shown. Error bars show standard error obtained from three biologicals with technical triplicates each (n = 9). (C) Maximum quantum yield (Fv/Fm) of pSII obtained from cultures grown in sulphur deplete aerobic conditions. Error bars indicate standard error derived from three biological replicates with technical duplicates (n = 6) and asterisk represent statistical significant data ($p < 0.05$). (D) Hydrogen production in *sor1* mutant is ~30% less compared to the parental strain (4A+) which is set to 100%. An average of six replicates is represented by each data curve and the error bars stand for standard error (two biological replicates with technical triplicates, n = 6).

4.3 DISCUSSION

In the following study, the role of Isoflavone reductase like protein (IFR1) in *C. reinhardtii* was investigated. Several studies reported IFR1 homologs to function in response to oxidative stress (Babiychuk et al. 1995), sulfur starvation (Petrucco et al. 1996), reactive oxygen species (Kim et al. 2010) and more. However, there has been no reports regarding function of *IFR1* in Chlamydomonas. In a previous study, deciphering the transcriptome of hydrogen producing cultures (Nguyen et al. 2011; Toepel et al. 2013) showed upregulation of *IFR1* transcripts. In order to gain functional significance, *IFR1* knockdown mutants were created in a wild type hydrogen producing strain (CC124) and further applied to a good hydrogen producing mutant (*stm6*, Kruse et al. 2005). In the following chapter, the physiology of *IFR1* knockdowns will be discussed.

4.3.1 Homologs of *IFR1*

The *C. reinhardtii* isoflavone reductase like protein (IFR1) belongs to atypical short-chain dehydrogenase/reductases (SDR) family of proteins which constitute several enzymes typically sharing 15-30% residue identities (Jörnvall et al. 1995). They are characterized by the presence of an N-terminal Rossmann fold which harbors an NAD(P)H (coenzyme) binding motif and a substrate binding C-terminal (Filling et al. 2002; Kavanagh et al. 2008). IFR1 lack the conserved tyrosine (Tyr152): lysine (Lys156) motif (Tyr-X-X-X-Lys) which is typical for SDR proteins. Hence, IFRs are proposed to use lysine residues at position 56 and 144 for interaction with NADPH and catalytic site, respectively (Min et al. 2003). Nonetheless, IFR1 possess glycine rich coenzyme binding motif G-X-G-X-X-G or G-[G-A]-X-G-X-X-G which is a characteristic of SDR's which also highlights distant proteins (Borras et al., 1989). One of the study classified isoflavone reductase like proteins including the *C. reinhardtii* IFR1 into Nmra-like family of proteins (SDR48A, Persson et al. 2009) whereas another suggested a separate classification belonging to SDR460A family (Moummou et al. 2012).

Local sequence alignment (BLAST) and multiple sequence alignment (MSA) of IFR1 and its homologs were done to figure out structural and functional similarities. An NCBI-BLAST analysis of IFR1 amino acid (Phytozome: Locus Cre11.g477200; *C. reinhardtii* v5.5) identified a conserved phenylcoumaran benzylic ether reductase (PCBER) domain. PCBERs are NADPH dependent aromatic alcohol reductases belonging to SDR superfamily that catalyze lignin biosynthesis (Min et al. 2003).

IFR1 does not possess any signal peptide and the cytosolic prediction by PredAlgo (Tardif et al. 2012) was successfully proven by IFR1:YFP fusion expression (fig. 6 and 7). IFR1 does not possess motifs typically present in sulfur assimilation proteins and carries just three sulfur containing amino acid (cysteine) which is typical for proteins expressed under sulfur limitation (Chen et al. 2010; Gonzalez et al. 2009).

Multiple sequence alignment (fig. 5) showed that IFR1 shares 28.8% identity with isoflavone reductase like protein (IRL, UniProtKB P52580) observed in maize seedlings grown under sulfur deficient conditions (Petrucco et al. 1996). Similar to IFR1, maize IRL is reported to be a 33 kDa monomer localized in the cytoplasm. The lack of transmembrane motifs suggests its exclusion from the role of direct sulfur assimilation. The induction of maize IRL was observed to be inversely proportional to the glutathione pool which suggests that IFR1 may also be involved in restoring the thiol-independent state of the cell by regulating sulfur free antioxidants like carotenoids, α/β-tocopherols and more. IFR1 also shares an identity of 28.6% with isoflavone reductase homolog P3 (IFR, UniProtKB P52577) in *A. thaliana*, whose transcript was shown to be up-regulated under sulfur limiting conditions (Nikiforova et al. 2003). *A. thaliana IFR* was first reported from a study assessing the plants tolerance against oxidative stress and its expression in yeast conferred resistance against thiol-oxidizing drug diamide (Babiychuk et al. 1995). Phenylcoumaran benzylic ether reductase (PCBER) in *P. taeda* (UniProtKB O81651) is an aromatic alcohol reductase that catalyzes lignin biosynthesis and shares 30% identity with IFR1 (Min et al. 2003). There are over 30 residues that are highly conserved between IFR1 and PCBER, suggesting structural importance. Eugenol synthases (EGS) is an enzyme from basil that not only shares 24.5% identity (UniProtKB A0A1B2U6R8) with IFR1 but also possess an NADPH binding site that is homologous to other IFR or IRL proteins. EGS brings about reductive displacement of acetate from the propenyl side chain of substrate to produce phenylpropenes, which act as attractants for pollinator (Louie et al. 2007).

Other important SDRs sharing identity with chlamydomonas IFR1 include Isoflavone reductase like protiens from tobacco (*N. tobacum* IRL, involved in biosynthesis of nicotine, UniProtKB P52579, 29% identity, Hibi et al. 1994), rice (*O. sativa* IRL, overexpression of IRL confers tolerance against ROS, UniProtKB Q8VYH7, 34% identity, Kim et al. 2010), grapefruit (*C. Paradisi* IRL, induced in response to UV irradiation, UniProtKB O49820, 26% identity, Lers et al. 1998) and potato (*S. Tuberosum* IRL, expression enhanced by pollen tube growth which creates

a ROS environment, UniProtKB P52578, 32% identity, van Eldik et al. 1997). Many IFR1 homologs possessing an NAD(P)H binding motif are reported to be expressed in cytosol under ROS stress or during glutathione depletion which is a common occurence under sulfur limiting conditions. In order to otain a correlation with the reported findings, *IFR1* knockdown mutants were assessed under sulfur limitation and RES/ROS stress conditions.

4.3.2 Expression of IFR1 under abiotic stress

A strong induction of IFR1 protein expression was observed in the wild type under sulfur deprived anaerobic H_2 production conditions (fig. 9). The protein expression was not limited to H_2 production conditions but was also detected under sulfur deprived aerobic conditions (data not shown) as reported in maize (Petrucco et al. 1996). Sulfur deprivation resulted in reduction of cellular glutathione pool and an induction of *IRL* expression in maize. In correlation to the observations in maize, *IFR1* induction in Chlamydomonas under sulfur deprived conditions could arise as a result of reduction in the activity of GSH dependent ROS and RES scavanging. IFR1 expression has been reported under various stress conditions (Babiychuk et al. 1995; Brandalise et al. 2009; Kim et al. 2003; Lers et al. 1998; Luo et al. 2010) but no protein expression could be detected either under nitrogen deprivation (fig. 10) or under highlight stress (2000 µE m^{-2} s^{-1}) (Appendix: fig. S2), suggesting IFR1 expression in *Chlamydomonas* to be exclusive to sulfur depletion. As observed in higher plants, sulfur deprivation affected the glutathione pool more drastically than nitrogen limitation (Koprivova et al. 2000). This could explain the absence of IFR1 accumulation in Chlamydomonas under nitrogen starvation.

Sulfur deprivation induces formation of reactive oxygen species (ROS) which lead to lipid peroxidation and indirect formation of reactive electrophile species (RES) (González-Ballester et al. 2010; Zhang et al. 2004). The presence of RES triggers the expression of *IFR1* which is controlled by a *cis* regulatory electrophile response element (ERE, Fischer et al. 2012) located in the promoter (fig. 12A). ERE element is an 8 bp palindromic sequence (CAACGTTG), whose copy number and proximity (distance between ERE element and start codon) determines the expression or overexpression of a gene in the singlet oxygen resistant mutant (*sor1*) (Fischer et al. 2012). Genes involved in detoxification such as glutathiose-S-transferase1 (*GSTS1*), glutathione peroxidase homologous gene (*GPXH/GPX5*) and genes involved in

synthesis of ascorbate were reported to be overexpressed in *sor1*. In the following study *IFR1* was found to be heavily upregulated in *sor1* mutant (fig. 11) and also in CC124 wild type strains treated with DBMIB and 2-E-hexenal (fig. 14). Additionally, *IFR1* knockdown strains were found to be more sensitive to the presence of RES agents as reflected from the growth analysis (fig. 15 B, 15C and 16). These results suggest that *IFR1* may be involved with the detoxification of RES or establish intracellular RES homeostasis similar to a SDR protein (cytosolic aldehyde reductases, CytADRs) involved in detoxification of reactive carbonyls in *A. thaliana* (Yamauchi et al. 2011). CytADRs bring about detoxification by reducing aldehyde groups whereas the mechanism of detoxification by IFR1 is yet to be established.

4.3.3 *IFR1* knockdown strains have a longer phase of hydrogen production

Anerobic conditions is a prerequisite for establishing hydrogen production and can be achieved in air tight Chlamydomonas cultures deprived of sulfur (Melis et al. 2000). Hydrogen production is catalyzed by hydrogenases which receives electrons from the terminal electron donor, ferredoxin. Hydrogen production phase in *IFR1* knockdown mutants (IFR1-1 and IFR1-6 in fig. 17 and *stm6_IFR1kd* in fig. 21B) were found to be prolonged, yielding approximately 2fold more hydrogen compared to the parental strains. During H_2 production several metabolic shifts occur to acclimatize the cell to sulfur starvation (Doebbe et al. 2010; Matthew et al. 2009; Takahashi et al. 2001). RuBisCo is one of the major electron competitor for hydrogenase which is down regulated during sulfur depletion leading to ceasing of CO_2 fixation by Calvin-Benson cycle and generation of ROS. Unless maintained within sublethal levels by scavanging activities (xanthophyll cycle), ROS can lead to the damage of biomoleucles photosynthetic apparatus, proteins, nucelic acids, lipids, etc (Ledford et al. 2007).

Sulfur deprivation decreases the rate of repair cycle of PSII core D1 protein, resulting in a decrease of PSII activity and oxygen evolution. PSII activity of the *IFR1* knockdown mutants also declined but showed significant differences compared to parental strain. PSII activity (Fv/Fm) of *IFR1* knockdowns strains was sustained for a prolonged period of time as reflected by higher Fv/Fm values (fig. 18, 19A and 21C) and slower delcine of D1 protein (fig. 19C and 19D). During sulfur starvation the pool size of PSII reaction centers unable to reduce Qb increase (Wykoff et al. 1998) but a reactivation of such nonreducing PSII centers is also known (Neale et al. 1990). Electrons origniating from the reconstitued or stablised PSII of *IFR1* knockdown

mutants was the main contributor for hydrogen production as depicted by DCMU inhibitor studies (fig. 20). The present findings correlate with previous reports that highlight the importance and impact of residual PSII activity on H_2 production (Volgusheva et al. 2013). These results show that manipulation of RES homoestatis can be used as a tool to improve hydrogen production in *C. Reinhardtii*.

4.4 Conclusion

Isoflavone reductase like protein (*IFR1*) in Chlamydomonas was found to be a soluble, cytosol localized 32 kDa monomeric protein. *IFR1* transcripts were previously reported to be upregulated in a bad hydrogen producer strain as against a good producer strain (Nguyen et al. 2011). This finding promoted the development of an *IFR1* knockdown in CC124 wild type strain. Two knockdown mutants (IFR1-1 and IFR1-6) accumulating very low amounts of IFR1 protein under sulfur starvation were isolated. Just as observed for maize IRL, expression of IFR1 in Chlamydomonas appeared to be exclusive to sulfur stress. Nitrogen or high light stress did not induce any protein accumulation. Presence of an electrophile responsive element (ERE) in the promoter of *IFR1* led to the investigation of RES stress. The knockdowon mutants showed higher sensitivity to RES stress as reflected by their retarded growth. Higher accumulation of IFR1 transcripts and protein in the singlet oxygen resitant 1 mutant (*sor1*) showed that the gene could be controlled by the sor1 protein to detoxify ROS/RES stress. Interestingly, PSII photosynthetic activity (Fv/Fm) of the knockdown mutants was condierably higher under sulfur starvation. Hydrogen production from the knockdown mutants was found to be 2fold higher than the parental strain. Further investigation of the H_2 production cultures showed that the PSII activity of knockdown mutant IFR1-6 was more sustained compared to the control strain as evident from D1 protein decay. H_2 produced by the PSII independent pathway (PSII blocked with DCMU) proved that the prolonged H_2 production by *IFR1* knockdown strains indeed stem from a sustained PSII activity and perturbed RES homeostasis. The beneficial effect of *IFR1* knockdown on enhanced H_2 production was passed on to the good hydrogen producer strain *stm6*. Similar to previous observations, *stm6 IFR1* knockdown strain showed a sustained PSII activity leading to a prolonged phase of H_2 production. However, the question still remains regarding the connection between a cytosolic protein and PSII stability. Following experiments could be performed to decipher the underlying molecular function:

1) *IRL* levels in maize are linked to the concentration of cellular glutathione pools. Hence, a measurement of Chalmydomonas glutathione pools could provide new insights

2) Quantification of PSII reaction centers by paramagnetic resonance spectroscopy and measurement of fluorescence decay could help in better assessment of the prolonged H_2 prodcution phase

3) Substrate screen with the help of recombinant IFR1 protein and NADPH coenzyme

4) A pull down assay *via* immunoprecipitation could assist in the detection of IFR1 interacting partners

Contributions by other people

The production of recombinant IFR1 in *E. Coli* was done by Thomas Baier. The establishment of electoporation procedure and transformation of CC124 wild type strain with *IFR1* amiRNA was done by Daniel Jaeger. Tranformation of *stm6* mutant strain *via* glass bead method was done by Lisa Schierenbeck.

5.0 *SIR1* knockdown improves hydrogen production in *C. reinhardtii*

5.1 Introduction

Hydrogen production in *Chlamydomonas reinhardtii* is catalyzed by the enzyme hydrogenase and can occur directly from the substrates (protons and electrons) generated *via* solar driven water photolysis. The enzyme is an [Fe-Fe]-hydrogenase (Hyda1) which derives electrons from ferredoxin and accomplishes the reversible task of hydrogen production under hypoxic conditions. Sulphur deficiency induces hypoxia (Melis et al. 2000) where H_2 production serves as an alternative electron sink and protects the cell from oxidative damage and over-reduction of PQ pool. Ferredoxins are iron containing terminal proteins that serve as the backbone of reductive metabolism in chloroplast (Terauchi et al. 2009). *C. reinhardtii* genome encodes six plant type ferredoxins of which Fdx1 distributes electrons to various metabolic pathways including H_2 production (fig. 22).

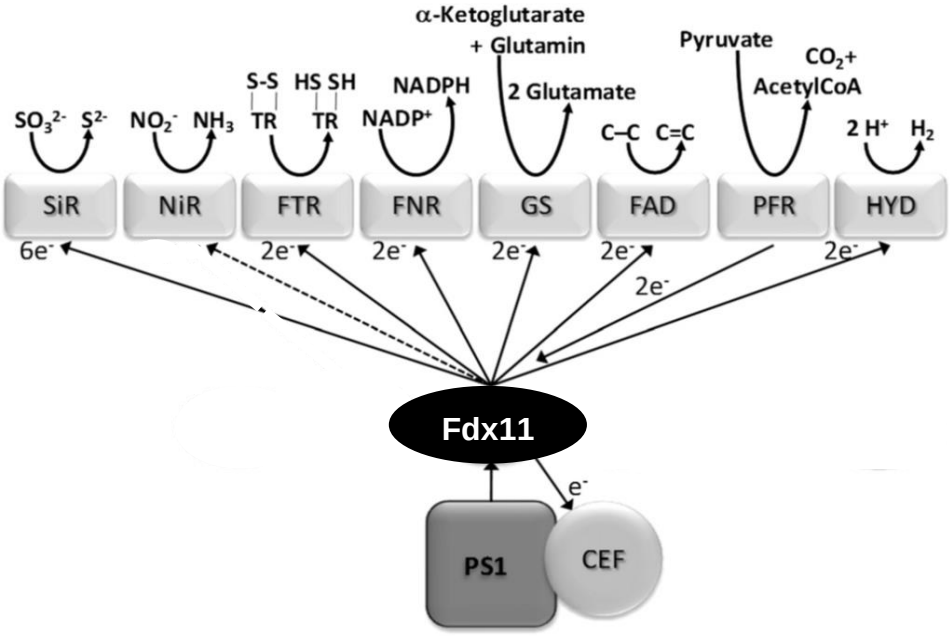

Figure 22: A modified representation of various metabolic pathways dependent on Ferredoxin as described by (Hemschemeier et al. 2011). Fdx1 receives electrons from PS1 and transfers them to various enzymatic pathways such as sulfite reductase (SiR), Fdx-thioredoxin reductase (FTR), Fdx-NADP+ reductase (FNR), glutamate synthase (GS), fatty acid desaturase (FAD), pyruvate ferredoxin reductase (PFR) and hydrogenase (HYD). Studies suggest that nitrite reductase (NiR) receives electrons from Fdx2 (Terauchi et al. 2009) but the role of Fdx1 cannot be undermined (dashed line). The cyclic electron flow (CEF) around PSI is also dependent on Fdx1 for electron flow.

Among the various electron sinks, the electron competition arising in hydrogen production conditions between hydrogenase and FNR (Sun et al. 2013) or hydrogenase and PFR (Noth et al. 2013) have been reported. An increase in the transcript of sulfite reductase (*SIR1*) was observed during sulfur deprived hydrogen production (Nguyen et al. 2011; Toepel et al. 2013), which suggests the competition arising between *SIR1* and hydrogenase cannot be ruled out.

Sulfur forms an essential constituent of various amino acids, cofactors, lipids, etc. and Chlamydomonas encodes several enzymes that work round the clock to accomplish the task of reductive sulfate assimilation. Sulfate (SO_4^{2-}) is first freed from its esters by arylsulfatase (ARS) and then transported into the cells via putative plasma membrane proton/sulfate transporters. Within the cell, SO_4^{2-} is adenylated by ATP sulfurylase (ATS) to adenosine 5-phosphosulfate (APS) which could either face further phosphorylation by APS kinase (APSK) or be reduced by adenosine 5-phosphosulfate reductase (APR) to give the toxic sulfite (SO_3^{2-}). Sulfite is further reduced to sulfide by sulfite reductase, which is then incorporated to cysteine biosynthesis catalyzed by O-acetylserine(thiol) lyase. The reductive assimilation of sulfur from sulfite to sulfide requires 6 electrons (fig. 22) which is contributed by the formation of a transient electron transfer complex between reduced ferredoxin and sulfite reductase (Akashi et al. 1999).

$$Sulfite + 6\,reduced\ Fdx1 + 6H^+ \rightarrow Sulfide\ + 6\,oxidized\ Fdx1 + 3H_2O$$

The sulfide is then combined with O-acetylserine (OAS) catalyzed by O-acetylserine(thiol)lyase (OASTL) to generate cysteine.

Sulfite reductase belongs to the “NIR_SIR superfamily” of protein which possess a characteristic siroheme (4Fe-4S) prosthetic group and catalyze assimilatory reduction of sulfite to sulfide by deriving electrons from reduced ferredoxin (Nakayama et al. 2000). Chlamydomonas genome encodes two ferredoxin type (*SIR1* and *SIR2*) and one bacterial type (*SIR3*) SIR genes (Gonzalez-Ballester et al. 2009). Of the three SIR forms, only *SIR1* and *SIR3* transcripts were detected under sulfur deprivation, whereas only transcripts of *SIR1* were found to be upregulated (Zhang et al. 2004). Chlamydomonas encodes a 68.6 kDa soluble SIR1 protein (Phytozome locus Cre16.g693202, *C. reinhardtii* v5.5; UniProtKB: A8JBI5) which not only shares 39% identity with SIR3 (Phytozome locus Cre03.g180300, *C. reinhardtii* v5.5; UniProtKB: A8IDE3) but also shares a higher degree of identity with ferredoxin dependent sulfite reductases from other species such as *N. tabacum* (SIR1, UniProtKB O82802, 58.3%

identity, Yonekura-Sakakibara et al. 1998), *Z. mays* (SIR, UniProtKB O23813m 57.6% identity, Schmutz et al. 1984), *P. sativum* (SIR, UniProtKB Q75NZ0, 56% identity, Arb et al. 1985) and *A. thaliana* (SiR, UniProtKB Q9LZ66, 54.7% identity, Bruhl et al. 1996). SIR1 also share considerable identity with sulfite reductase found in hydrogen producing cyanobacteria, *S. elongatus* (SIR, UniProtKB P30008, 54.5% identity, Gisselmann et al. 1993). It was observed that feeding O-acetyl serine to nitrogen starved Arabidopsis plants led to an increase in SiR transcripts (Koprivova et al. 2000) and an overexpression of SiR rendered the plants more tolerant to sulfite toxicity (Yarmolinsky et al. 2013). The important role of SiR in oxidative stress was also realized in Arabidopsis grown in presence of methyl viologen (Wang et al. 2016). SIR was reported to be involved in plastid nucleoid compacting (Sekine et al. 2002) in garden pea. Though sulfite reductase is reported to be involved in several vital functions, it has not yet gained attention in Chlamydomonas. In the following study, the impact of *SIR1* knockdown on hydrogen producibility of a high hydrogen producing mutant, *stm6glc4* (Doebbe et al. 2007) will be analyzed.

5.2 Results

An intricately designed enzymatic cascade catalyzes reactions from sulfur acquisition to its assimilation. The role of Sulfite reductase (SIR1) in *C. reinhardtii*, is of great important as it ensures sulfur homeostasis and cysteine metabolism (González-Ballester et al. 2010; Gonzalez-Ballester and Grossman 2009; Zhang et al. 2004). SIR1 catalyzes the final reduction step of sulfite to sulfide by deriving 6 electrons from reduced ferredoxin (Hemschemeier and Happe 2011) which results in a competition for electrons between SIR1 and hydrogenase. Hence, *SIR1* knockdowns were created by using artificial microRNA (amiRNA) to improve the bias of electron flux toward hydrogenase leading to an increase in hydrogen production.

5.2.1 Screening and selection of *SIR1* knockdown mutants

The amiRNA to knockdown *SIR1* were generated (Molnar et al. 2009) and assembled as reported before (Hu et al. 2014). The amiRNA was cloned into pOpt_cCA_gLuc_Paro vector, downstream of the luciferase reporter (*g*Luc) which allowed rapid screening of over 500 transformants. The *stm6glc4 SIR1* knockdown mutants showing resistance to paromomycin (10 mg/l) and hygromycin (5 mg/l) were qualitatively screened for luciferase activity on the plate level (Appendix: fig. S3). Around 14 mutants with relatively high luciferase activities were obtained and based on relative luminescence signal two best mutants (*sgh2* and *sgh3*) were quantitatively analyzed in liquid TAP medium. PCS showed no luminescence but the highest signal was obtained from the knockdown mutant *sgh3* which was two folds more than *sgh2* (fig. 23A). The mutants were further analyzed by RTqPCR and were found to contain ca. 20% and 30% reduced levels of *SIR1* transcripts relative to the PCS (fig. 23B). The *SIR1* knockdown in *sgh3* was highest at ca. 30% in correlation to the observed luciferase activity. As the SIR1 of *C*. *reinhardtii* and *A. thaliana* share ~55% protein identity, a polyclonal antibody produced against *A. thaliana* SIR1 (Khan et al. 2010) (a gift from Prof. Rüdiger Hell, COS Research group, Heidelberg) was used to substantiate the SIR1 protein knockdown. Protein densitometry confirmed the knockdown and showed that the mutants accumulated ca 25-30% lesser amounts of SIR1 protein (fig. 23C).

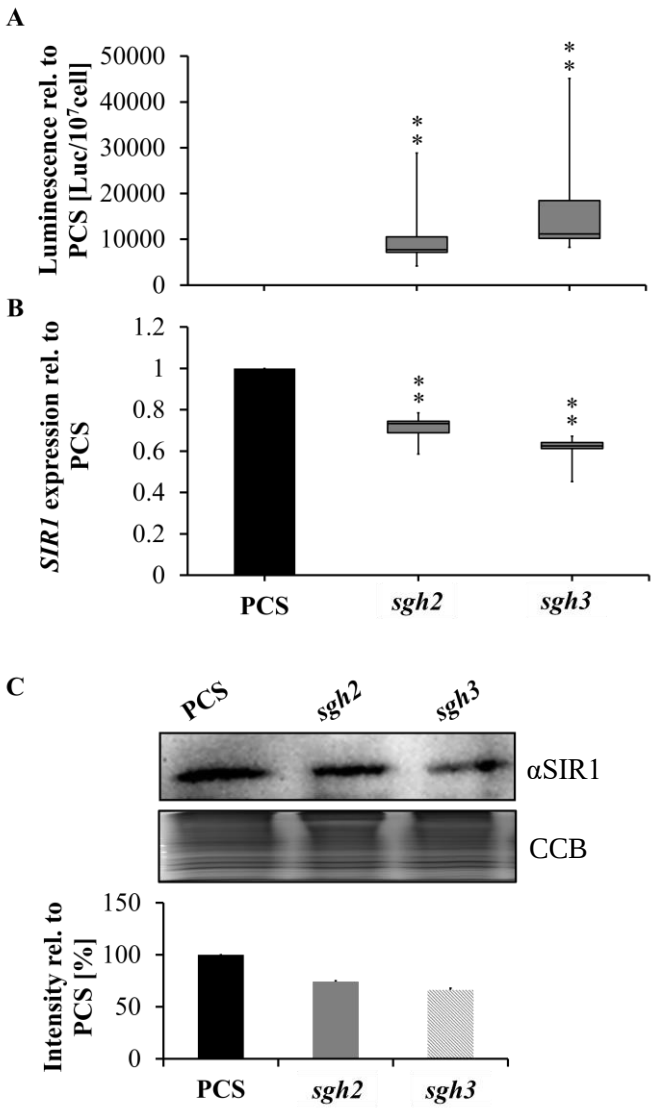

Figure 23: Detection of *SIR1* knockdown mutants. (A) Extracellular luciferase activity of *stm6glc4* (PCS) and *SIR1* knockdowns *sgh2* and *sgh3*. The activity is normalized to cell count and the data is derived from three biologicals with technical duplicates (n = 6), where asterisk indicate statistically significant difference based on a t-test ($p < 0.05$). (B) RTqPCR analysis of *SIR1* transcript in TAP grown cultures where *SIR1* expression from PCS is normalized to 1. Data is derived from three biologicals and two technical replicates (n = 6), where asterisk show statistical significance ($p < 0.05$).
(C) Immunoblot analysis of SIR1 accumulation in PCS and *SIR1* knockdown strains. 60 µg of total protein was blotted and colloidal coomassie staining (lower panel) served as a loading control. Densitometry analysis of SIR1 immunoblot derived from three biological samples (n = 3) is shown.

5.2.2 Knocking down *SIR1* results in growth retardation

The final step of sulfur assimilation is carried out by SIR1 with the help of reduced Ferredoxin (Fdx1), making SIR1 a vital enzyme for cell growth. The level of *SIR1* knockdown (44% and 14% mRNA as compared to control) in *A. thaliana*, was detrimental between plant viability and death (Khan et al. 2010). Therefore, the effect of *SIR1* knockdown was analyzed in mutants grown under photomixotrophic (TAP) and photoautotrophic (HSM) cultivation. High growth rates with high cell densities were observed among all strains grown in TAP medium (fig. 24A) as compared to photoautotrophic conditions. The growth of knockdown mutants was reduced under both tested conditions and in comparison to PCS, reached ca. 19-22% lower cell growth after 48 h in TAP. Under photoautotrophic conditions, PCS grew better than the knockdown mutants (fig. 24B). However, at t_{48} reached only ca.32% cell growth of that

obtained under TAP conditions. This reduction in growth between mixotrophic and autotrophic conditions has been observed as an influence of readily available inorganic carbon. The growth rates of the knockdown mutants were again reduced by ca. 25-28%, similar to the results obtained under TAP conditions. However, no remarkable growth differences could be observed between the knockdown mutants. The retarded growth of *SIR1* knockdown mutants could be associated with perturbed sulfur assimilation.

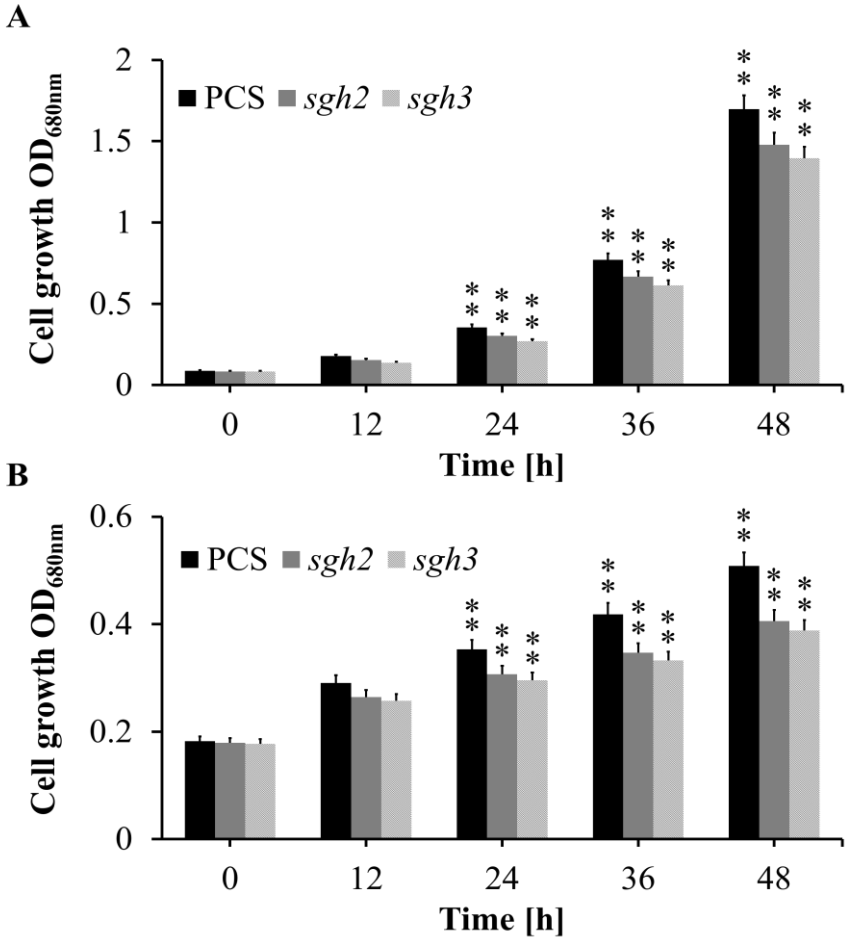

Figure 24: Growth phenotype of PCS and *SIR1* knockdown mutants. PCS and *SIR1* knockdown strains, *sgh2* and *sgh3* grown under (A) photo-mixotrophic (TAP) and (B) photo-autotrophic (HSM) conditions. All strains were grown under the illumination of continuous white light of 100 µE m^{-2} s^{-1}. Error bars represents standard error obtained from three biologicals with technical duplicates (n = 6) and asterisk show statistically significant data ($p < 0.05$).

5.2.3 Sulfite oxidase is upregulated in *SIR1* knockdown mutants

A reduction in SIR1 protein resulting in higher sulfite accumulation and higher sulfite oxidase activity has been previously observed in the leaves of *A. thaliana* (Khan et al. 2010). In correlation to this observation, the transcript abundance of sulfite oxidase (*SO*) was analyzed in two *SIR1* knockdown strains. The accumulation of *SO* transcript was 4 to 6 folds higher than the PCS in *sgh2* and *sgh3*, respectively (fig. 25A). This finding could suggest the possible re-oxidation of excess sulfite to sulfate to overcome sulfite toxicity. An expression of a bacterial type sulfite reductase (*SIR3*) (Gonzalez-Ballester and Grossman 2009) was also observed in *C. reinhardtii* under sulfur depletion. However, there is no evidence that describes if loss in *SIR1* is compensated by *SIR3*. RTqPCR analysis showed that the amount of *SIR3* did not vary significantly between PCS and knockdown mutants (fig. 25B). Hence, on the basis of transcript analysis it could be concluded that *SIR3* does not compensate for *SIR1* knockdown.

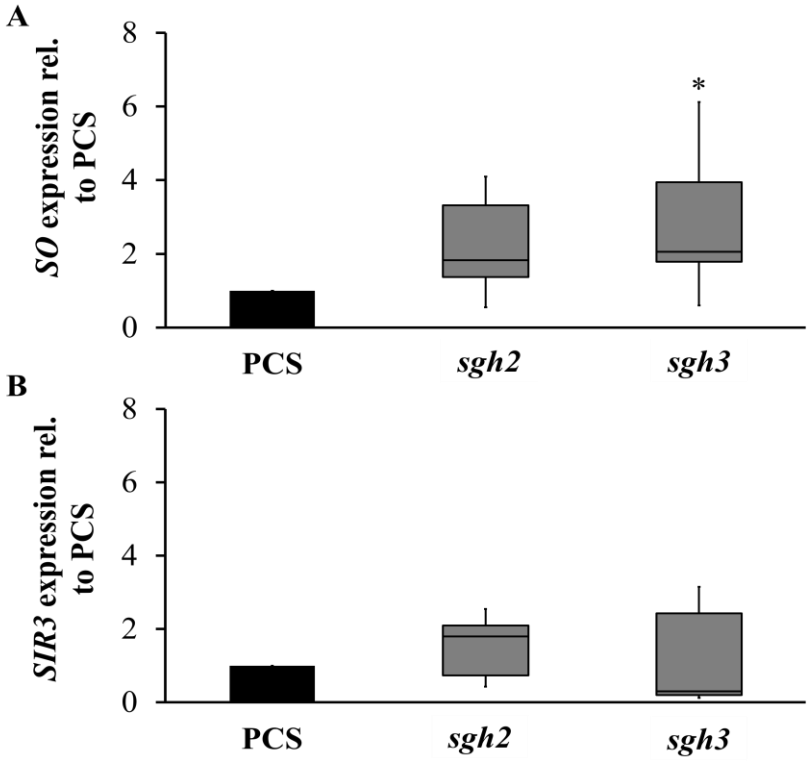

Figure 25: Impact of reduced *SIR1* on the expression of sulfur assimilation genes. Analysis of (A) sulfite oxidase (*SO*) and (B) sulfite reductase (*SIR3*) in PCS and *SIR1* knockdown strains grown in TAP medium. Error bars represent standard error derived from three biologicals with technical duplicates (n = 6) and asterisk show statistical significance ($p < 0.1$).

5.2.4 Rate of hydrogen production is increased in *SIR1* kd mutants

An increase in the level of *SIR1* transcript during sulfur deprived H_2 production has been previously shown (Toepel et al. 2013), which serves as a competing sink for electrons (Hemschemeier et al. 2011; Nguyen et al. 2011). H_2 production between PCS and knockdown strains was done to elucidate the functional significance of *SIR1* knockdown, (fig. 26A). All strains were grown to a mid-log phase in TAP and subsequently transferred to sulfur deplete medium (TAP-S) to the tune of ~25 µg/ml chlorophyll. H_2 production was detectable in both knockdown strains 24 h after sulfur depletion, whereas PCS took twelve additional hours (t_{36}) before hydrogen could be measured (fig. 26B). The H_2 phase (time between earliest detection to termination of H_2 production) was highest in PCS lasting for 108 h whereas it lasted only 96 h in the knockdown mutants. Interestingly, the overall H_2 production was found to be ~35% and ~55% more than PCS in *sgh2* and *sgh3* mutant, respectively.

Rate of H_2 production (fig. 26B) was significantly higher in the knockdown strains until t_{96}. The difference in rates of H_2 production at t_{36} was remarkably ~2 fold higher in knockdown strains compared to PCS. All strains reached their maximum H_2 production rates at t_{48}. In *SIR1* knockdowns, high rates of H_2 production compensated for the reduced production phase leading to a higher volumetric productivity (table 11). It is known that s*tm6glc4* can take up glucose and increase internal starch reserves which contribute to PSII independent hydrogen production (Doebbe et al. 2007). Hence, glucose dependent hydrogen production by *SIR1* knockdown mutants were measured. Addition of glucose led to ~145% more H_2 production in PCS which is in agreement to the previous report (Doebbe et al. 2007), as compared to ~120% increase in the knockdown strains. Within t_{48}, rates of H_2 production did no significantly differ between presence or absence of glucose (fig. 26C) but the differences became significant at t_{72} and beyond, which correlates to previous findings (Doebbe et al. 2010). In the presence of glucose, the H_2 phase did not change in the knockdown mutants but prolonged for an additional day in PCS. These results show that knocking down *SIR1* results in a significant improvement in rate of H_2 production.

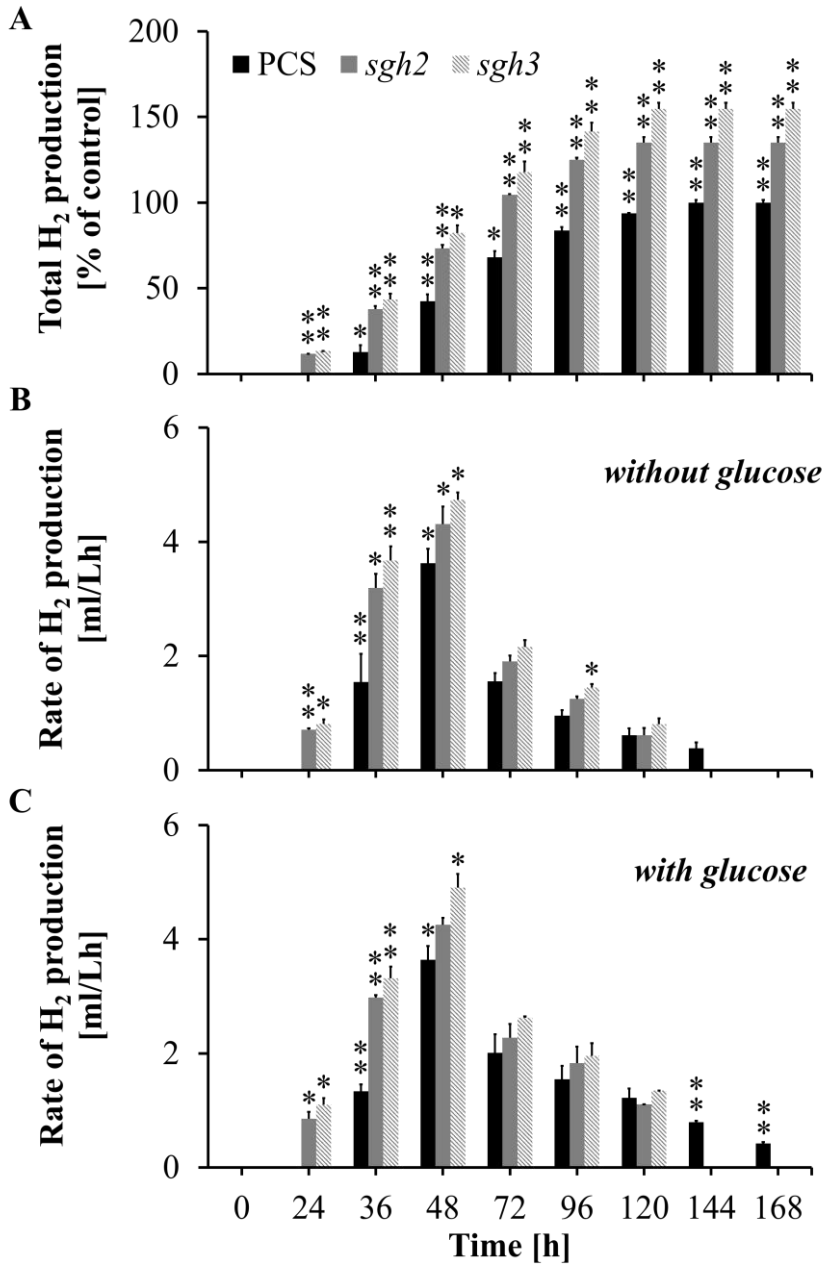

Figure 26: **Analysis of hydrogen production from PCS and *SIR1* knockdown strains.** (A) Total amount of hydrogen produced under sulfur deprivation in the absence of glucose is shown, where PCS is set to 100% and *sgh2, sgh3* produce ~135% and ~155% H_2, respectively. Each data bar represents an average of six replicates (two biologicals with technical triplicates, n = 6), error bars represent standard error and asterisk show statistical significance (**, $p < 0.05$; *, $p < 0.1$). (B) Rate of H_2 production in the absence and (C) presence of glucose is shown. Under both conditions, H_2 production can be noticed by 24 h in the knockdown strains. Error bars denote standard error (n = 6) with statistical significance ($p < 0.05$).

Table 11: Rate and volumetric productivity of H_2 in the presence and absence of glucose

Strain	Glucose	H_2 rate [ml/lh]	H_2 STY* [ml/lh]	H_2 volume [ml/l]
stm6glc4	-	3.62 ± 0.25	1.14 ± 0.04	146.26 ± 3.14
	+	3.88 ± 0.24	1.45 ± 0.1	203.92 ± 12.86
sgh2	-	4.25 ± 0.31	1.65 ± 0.03	197.51 ± 4.67
	+	4.37 ± 0.12	1.94 ± 0.06	232.65 ± 7.71
sgh3	-	4.86 ± 0.12	1.89 ± 0.04	226.21 ± 5.2
	+	5.14 ± 0.24	2.23 ± 0.02	267.39 ± 3.37

*STY (Space Time Yield): Volumetric productivity calculated by considering operation of 120 h.

5.2.5 Rapid decline in ϕ PSII and P/R causes early onset of H_2 production

Chlorophyll fluorescence and ratio of photosynthetic O_2 evolution to respiratory consumption (P/R) were monitored to decipher the phases of hydrogen production in the absence of glucose between PCS and *SIR1* knockdown mutants. ϕ PSII of all the strains were recorded in presence of experimental light conditions with notable differences at t_{12} (fig. 27A). The ϕ PSII of PCS distinctly declined at t_{24} which correlated to previous observation (Doebbe et al. 2010; Nguyen et al. 2011) but the decline was more pronounced in knockdown strains reaching ~8-10% the value of t_{24} PCS. Beyond t_{36} of sulfur starvation, ϕ PSII could no longer be significantly recorded by the PAM instrument. Chlorophyll content of the measured strains were found to decline over the period of H_2 production with significant differences emerging at t_{18} (fig. 27B). By t_{72} chlorophyll/cell in the knockdown mutants had decreased to ~22% of their initial value, whereas the decrease was a meager ~13% in PCS. The ratio of Chl *a/b* at the start of sulfur starvation was ~2.2 in knockdown strains as compared to ~2 in PCS. With time, the Chl *a/b* ratio remained stable in PCS but increased by ~18% to ~2.6 in knockdown strains, which could be due to a higher LHCII degradation as previously reported under similar conditions (Zhang et al. 2002).

Photosynthesis to Respiration (P/R) ratio of samples were analyzed with a Clark-type electrode. At the beginning, rate of photosynthesis was lower in *sgh2* and *sgh3* by ~13% and ~19% of PCS, respectively, but rate of respiration in all strains stood at ~42 µM O_2/mg Chl*h (fig. 27C). The onset of anaerobiosis occurred rapidly by t_{24} in knockdown mutants, reaching a P/R of 0.5:1 and 0.4:1 in *sgh2* and *sgh3*, respectively

as compared to PCS (P/R of 1.1:1). By t_{48}, all strains had attained anaerobiosis and were producing H_2 at maximum rates.

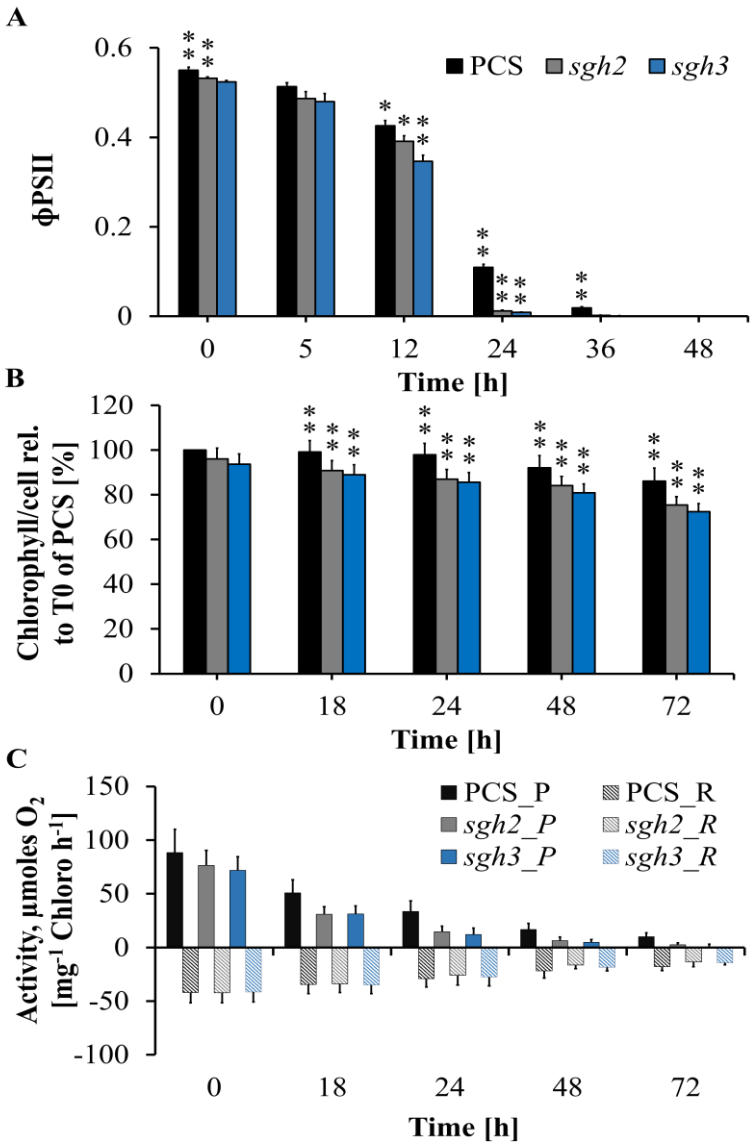

Figure 27: ϕ PSII, chlorophyll and photosynthetic/respiration (P/R) rates of sulfur deprived hydrogen producing cultures. (A) ϕ PSII of strains before (t_0) and after exposure to sulfur limitation (t_5-t_{48}), recorded under experimental light conditions (n = 6, two biologicals with technical triplicates). Error bars show standard error and asterisk show statistical significance (**, $p < 0.05$; *, $p < 0.1$). (B) The chlorophyll content of *stm6glc4* (PCS) at t_0 is set to 100%. Each data point was obtained from two biologicals with technical triplicates (n = 6) where error bars show standard error and asterisk show statistical significance ($p < 0.05$). (C) Comparison of P/R rates from H_2 producing culture, where rate of respiration (striped bar) was measured in the dark followed by photosynthetic rate (solid bar) under experimental light of 350 µE m^{-2} s^{-1}. Error bars represent standard error (n = 6) taken from two biologicals with technical triplicates.

5.3 Discussion

Solar biohydrogen production from green algae is an efficient alternative to other chemical methods. Hydrogenases ([FeFe]-*HydA1*) found in *C. reinhardtii* can catalyze the reversible reaction of hydrogen production by combining the protons released from photolysis of water with electrons derived from reduced ferredoxin (FDx1/PetF), yielding molecular H_2. The activity of Chlamydomonas hydrogenase has been shown to be over 100 times more than that of other hydrogen producers like cyanobacteria and photosynthetic bacteria but the enzyme faces a fierce competition for electrons from other pathways (fig. 22). Sulfite reductase (SIR1) is an essential enzyme participating in the crucial step of sulfur assimilation but by doing so competes with hydrogenase for electrons. Recent advancements in amiRNA technology combined with a luciferase reporter (Hu et al. 2014) was applied in the following study to knockdown the competition arising due to *SIR1*. The growth phenotype and changes observed in hydrogen production of *stm6glc4 SIR1* knockdown strains *sgh2* and *sgh3* will be discussed in this chapter.

5.3.1 Chlamydomonas *SIR1* knockdown mutants are affected in growth and have their sulfite oxidase transcripts upregulated

The *SIR1* knockdown was generated in a high hydrogen producing *C. reinhardtii* strain *stm6glc4* (Doebbe et al. 2007). The knockdown mutants were quantitatively assessed on the basis of luciferase reporter assay and later evaluated by measuring the level of *SIR1* transcripts through quantitative real time reverse transcription PCR (RTqPCR) (fig. 23A and 23B). The mRNA levels in the *SIR1* knockdown mutants *sgh2* and *sgh3* were reduced by ca. 20% and 30%, respectively. The protein abundance was consequently reduced, suggesting the lack of any post-transcriptional or post-translational mechanism governing SIR1 expression (fig. 23C).

Reduction in *SIR1* resulted in a subnormal growth phenotype which correlated to a similar observation reported in an Arabidopsis SiR knockdown plant (Khan et al. 2010). The growth differences between *stm6glc4* parental control strain (PCS) and the knockdown mutants *sgh2* and *sgh3* was evident under the tested growth conditions (photomixotrophic-TAP and photoautotrophic-HSM) (fig. 24). All strains showed high cell growth when grown in TAP due to the readily available carbon (acetate). However, under both growth conditions the growth rates were significantly reduced in knockdown mutants which could be due to perturbed sulfur assimilation. Interestingly, highest

knockdown of *SIR1* resulted in the highest growth retardation as observed in the mutant *sgh3*. This finding suggests the significance of SIR1 for cell viability.

An Arabidopsis sulfite reductase knockdown mutant showing growth retardation due to perturbed sulfate assimilation and rising sulfite toxicity was able to grow by countering the toxicity with increased activity of sulfite oxidase (SO) (Khan et al. 2010). The mechanism of sulfite toxicity and measures taken by the cell is well documented in plants (Brychkova et al. 2007; Yarmolinsky et al. 2013). Based on these reports, an investigation of *SO* transcript revealed an upregulation of sulfite oxidase in both *SIR1* knockdown mutants, suggesting the countermeasure taken by the mutant strains to overcome sulfite toxicity (fig. 25A). A question arises if sulfite reductase (*SIR1*) is indeed the only enzyme catalyzing sulfide production? Therefore, the transcripts of *SIR3* were also analyzed via RTqPCR. *SIR3* transcripts did not change significantly in the knockdown strains and appeared to be similar to that of the parental strain, portraying the importance of *SIR1* in sulfate assimilation of *C. reinhardtii* (fig. 25B).

5.3.3 Rate of hydrogen production is increased in *SIR1* knockdown mutants

Biohydrogen production in Chlamydomonas is catalyzed by hydrogenase which receives electrons from ferredoxin. Of the six ferredoxins encoded by the Chlamydomonas genome, Fdx1 is the predominant electron donor to hydrogenase among other pathways (fig. 22). A recent study with yeast-two hybrid library showed Fdx1 to be supplying electrons to over 18 interacting partners (confident score: moderate) (Peden et al. 2013), which results in a severe electron competition. Redirecting electrons from Fdx1 towards hydrogenase has been done by expressing Fdx1-hydrogenase fusion protein (Eilenberg et al. 2016), knocking down FNR (Fdx1-NADPH Reductase) (Sun et al. 2013) and improving Fdx1 bias towards hydrogenase by site-directed mutagenesis (Rumpel et al. 2014), which have all resulted in an increase in hydrogen production. In the following study, *SIR1* knockdown mutants were generated to decipher their impact on hydrogen production. Hydrogen produced by mutants were detectable as early as t_{24} (fig. 26) due to an early onset of anaerobiosis (fig. 27A and 27C). A closer look into the physiology of the mutants showed a significant increase in the rate of hydrogen production. All the strains reached their maximum H_2 production rates at t_{48} but the knockdown mutants showed a further increase of ~19-26%, due to a decreased level of SIR1 (fig. 26B). It is known that an earlier induction in H_2 production protects residual PSII activity resulting in a prolongation of H_2

production phase (Volgusheva et al. 2013). However, in comparison to the parental strain the phase of H_2 production was shorter by a day in the knockdown mutants lasting for 96 h. Though the reason behind earlier decline of hydrogen production in the *SIR1* knockdown mutants is not clear, it may be because of the inability of the cells to assimilate sulfur and fine tune cellular metabolism under nutrient limited conditions. This in turn could elicit high stress conditions causing severe damage as evident by an increase in Chl a/b ratio from 2.2 (t_0) to ~2.6 (t_{72}). The increase in Chl a/b is known to occur due to an increase in preferential binding of “light harvesting complex stress related protein 3” (LHCSR3) to “chlorophyll a” and reduction of other light harvesting proteins (Bonente et al. 2011). However, an increased rate of H_2 production contributed to an overall higher hydrogen productivity by the knockdown strains (table 11). The *SIR1* knockdown mutants had also retained the ability of glucose uptake which was reflected by an improved hydrogen production rate beyond t_{72} (fig. 26C).

The earlier onset of anaerobiosis in the knockdown mutants as seen by ϕPSII and Clark measurement was the reason behind the early start of hydrogen production (fig. 27A and 27C). Respirational oxygen consumption did not differ among the strains but the rate of photosynthetic oxygen evolution declined rapidly in the knockdown mutants leading to an earlier onset of hydrogen production (fig. 27C). Photosynthetic capacity (ϕPSII) of the mutants decreased more rapidly than the parental strain, which could be due to a decreased rate of PSII repair cycle arising because of a perturbed sulfur acquisition (fig. 27A). The chlorophyll content per cell was also lower in the knockdown mutants which further decreased by ~22% of their initial value at t_{72}. This decay in chlorophyll content was accompanied by an increase in Chl a/b ratio, suggesting higher damage in knockdown mutants under sulfur deprived conditions. These analyses helped in understanding the physiology and adaptation of the *SIR1* knockdown mutants to sulfur deprivation, which resulted in an earlier onset and improved hydrogen production.

5.4 Conclusion

Hydrogen production can be enhanced by elimination of competing pathway to redirect the susbstrate flux towards hydrogenase (Burgess et al. 2012). The prime aim of the following work was to improve hydrogen production in *C. Reinhardtii* by redirecting the electron flux towards hydrogenase by knockdown of sulfite reductase (*SIR1*). With the help of artificial microRNA, competition arising due to *SIR1* was successfully overcome by creating two knockdown mutants in *stm6glc4*. The knockdowns accumulated low levels of SIR1 protein resulting in a pertubed sulfur assimilation as reflected by retarded growth under photoautotrophic and photomixotrophic conditions. The knockdown mutants showed an increase in sulfite oxidase (*SO*) at the transcript level, hinting that *SO* upregulation might help the mutants tolerate sulfite toxicity. Upon sulfur deprivation of air tight cultures, the mutants reached anaerobicity faster than their parental strains and showed an earlier induction of hydrogen production. As postulated at the beginning of this work (Objective 2), *SIR1* knockdown mutants were able to yield more hydrogen due to a higher rate of hydrogen production as compared to their parental strain. However, following things have to be analyzed to elucidate the underlying mechansim of SIR1 competiton so that further improvement of H_2 can be reached:

1) Confirmation of increase in *SO* by enzyme activity assay or protein analysis
2) Increase in H_2 production of knockdown mutants needs to be confirmed by performing PSII independent H_2 production (with DCMU)
3) Reduction in the H_2 production phase of *SIR1* knockdowns has to be analyzed
4) Eliminating *SIR1* led to growth retardation due to perturbed sulfur assimilation. Hence, use of inducible promoters to selectively knockdown SIR1 could be a strategy to have a prolonged H_2 production process

Contributions by other people

The construction and generation of *SIR1* knockdown mutants was equally shared by Thomas Baier. The SIR1 antibody used for the following analysis was a kind gift from Prof. Dr. Rüdiger Hell.

6.0 Viability assessment of *C. reinhardtii* cells immobilized in novel silica gel

One of the aim of the following work was to improve robustness of hydrogen production process by immobilizing good hydrogen producing strains of *C. reinhardtii* in a novel silica gel system. A wild type CC124 and an *stm6* mutant (Kruse et al. 2005) were immobilized in a sodium silicate transparent gel system. Additionally, cells immobilized within calcium-alginate and free cells served as controls as shown in (fig. 28).

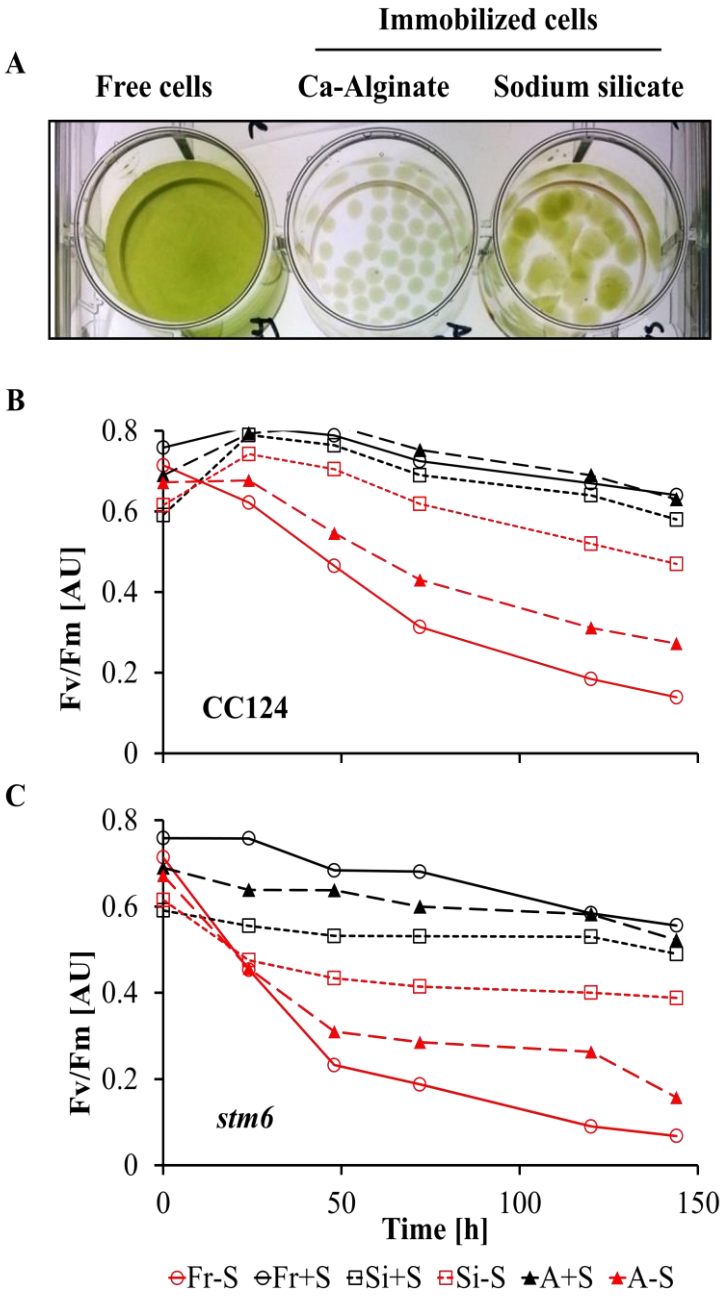

Figure 28: Photosynthetic activity of free and immobilized cells of *C. reinhardtii*.
(A) A picture showing free cells, Ca-alginate and silica gel immobilized cells of CC124 wild type and *stm6* mutant. Strains grown to a late exponential phase were suspended in fresh TAP or TAP-S medium to the tune of ~25 µg/ml chlorophyll. Photosynthetic activity (Fv/Fm) was measured after 3 min of dark incubation. Plots (B) and (C) show changes in photosynthetic activity of CC124 and *stm6* during TAP (sulfur replete, black) and TAP-S (red) growth, respectively. Legends denote the following: Fr: free cells, Si: Novel silica gel immobilized cells, Ag: Ca-alginate immobilized cells, +S: sulfur replete, -S: sulfur deplete. The data points denote average of two biologicals with technical triplicates each (n = 6).

The strains were grown to a mid-log phase and suspended in fresh sulfur replete TAP (+S) and sulfur deplete TAP (-S) medium, represented by black and red lines respectively (fig. 28). Following immobilization in ca-alginate (Fraser et al. 1997) and silica gel, the cells were suspended in +S and –S conditions. Photosynthetic capacity of all the strains were monitored by measuring Fv/Fm after 3 min of dark adaptation. Fv/Fm of free and immobilized CC124 did not differ significantly between the type of immobilization material used and remained high throughout the growth under sulfur replete conditions (fig. 28B). This showed that neither of the immobilization materials posed any toxicity to the cells. In correlation to the previous observation (Zhang et al. 2002), photosynthetic capacity of free cells under –S decreased over time. The decrease in Fv/Fm of alginate immobilized cells was much slower compared to the free cells and could be due to the shading effect caused by the translucent nature of alginate gel (fig. 28B). CC124 immobilized in silica gel retained an Fv/Fm value of 0.47 which was over 3fold and ~2fold higher than that of free and alginate immobilized cells, respectively. The retention of higher photosynthetic activity could help in the contribution of more electron substrate for the hydrogenase (Volgusheva et al. 2013). Similar to the result in wild type strain, the mutant *stm6* immobilized in silica gel also retained higher photosynthetic capacity under –S conditions (fig. 28C). The advantage of immobilization in novel silica gel system is that (a) entrapped cells retain higher viability as seen (fig. 28B and 28C –S conditions) and (b) Immobilization assists in improving robustness of the hydrogen production process by reducing the time and energy requirements of a two-phase process. However, further studies are required to decipher the reason behind improved photosynthetic capacity of silica immobilized cells and test if immobilized cells can produce hydrogen.

7.0 Future perspectives

Several process related parameters have already been tried to improve hydrogen yields but the true potential of light to H_2 conversion efficiency can only be achieved by strain engineering, as shown by this work. The major contribution of PSII on H_2 production has been acknowledged (Volgusheva et al. 2013), which necessitates enhancement of PSII stability or eliminate competing pathways to improve substrate flux for H_2 production. Competition due to essential metabolic pathways like fatty acid desaturase (FAD, fig.22) could be eliminated with the help of a selectively inducible knockdown in order to improve electron flux towards hydrogenase.

The presently used two-phase sulfur deprived hydrogen production process is time and energy intensive. An attractive option could be to have the strains immobilized in a glass like material (as shown by novel silica gel) that allows effective light and mass transfer. Another method could be to look for species that natively express an oxygen tolerant hydrogenase (Hwang et al. 2014).

The quantum efficiency of light conversion could be improved by co-cultivation of Chlamydomonas with other bacteria such as purple sulfur bacteria or purple cyanobacteria. At the present efficiency of hydrogen production (in wild type, light to $H_2 < 0.1\%$, Posten et al. 2012) the cost of recovering hydrogen gas is not profitable (Amos 2004). Therefore, operating an algal bio-refinery that could utilize byproducts of algal growth as animal feed or provide other valuable products such as biodiesel or bioethanol along with H_2 could result in a profitable process.

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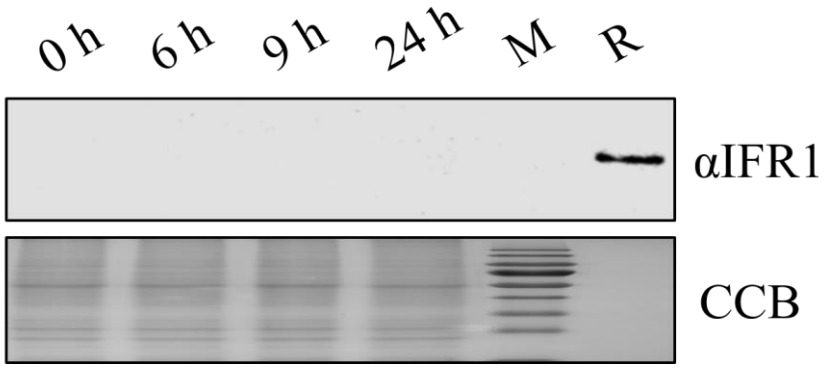

Figure S1: Expression of IFR1 in the presence of DCMU. 0.1 µM of DCMU was added to TAP grown cultures of Chlamydomonas wild type CC124 and protein harvested at regular time intervals as shown. M and R represent protein ladder and recombinant IFR1, respectively. Colloidal coomassie staining (CCB) served as a loading control.

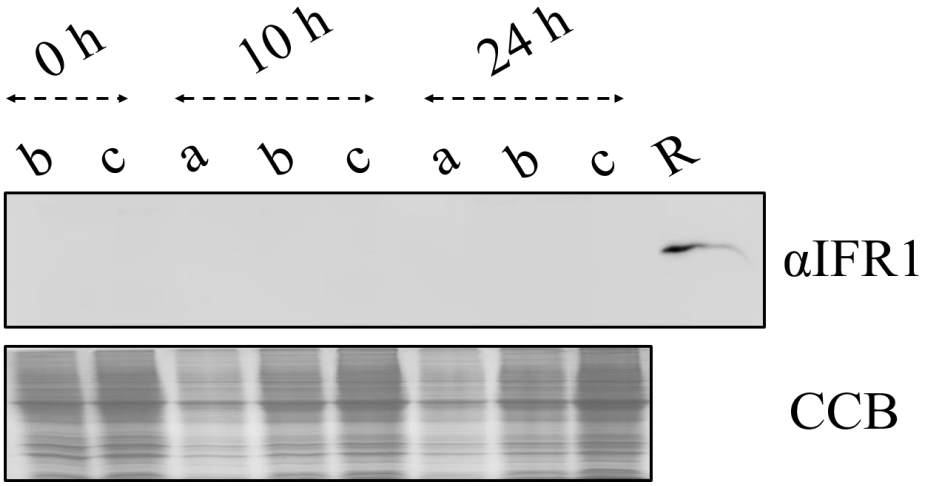

Figure S2: IFR1 expression in Chlamydomonas wild type (CC124) grown photoautotrophically under highlight (2000 µEm^{-2}s^{-1}). a, b and c denote 5 µg, 10 µg and 20 µg of total protein harvested at 0 h, 10 h and 24 h of highlight illumination. Colloidal coomassie staining (CCB) served as a loading control.

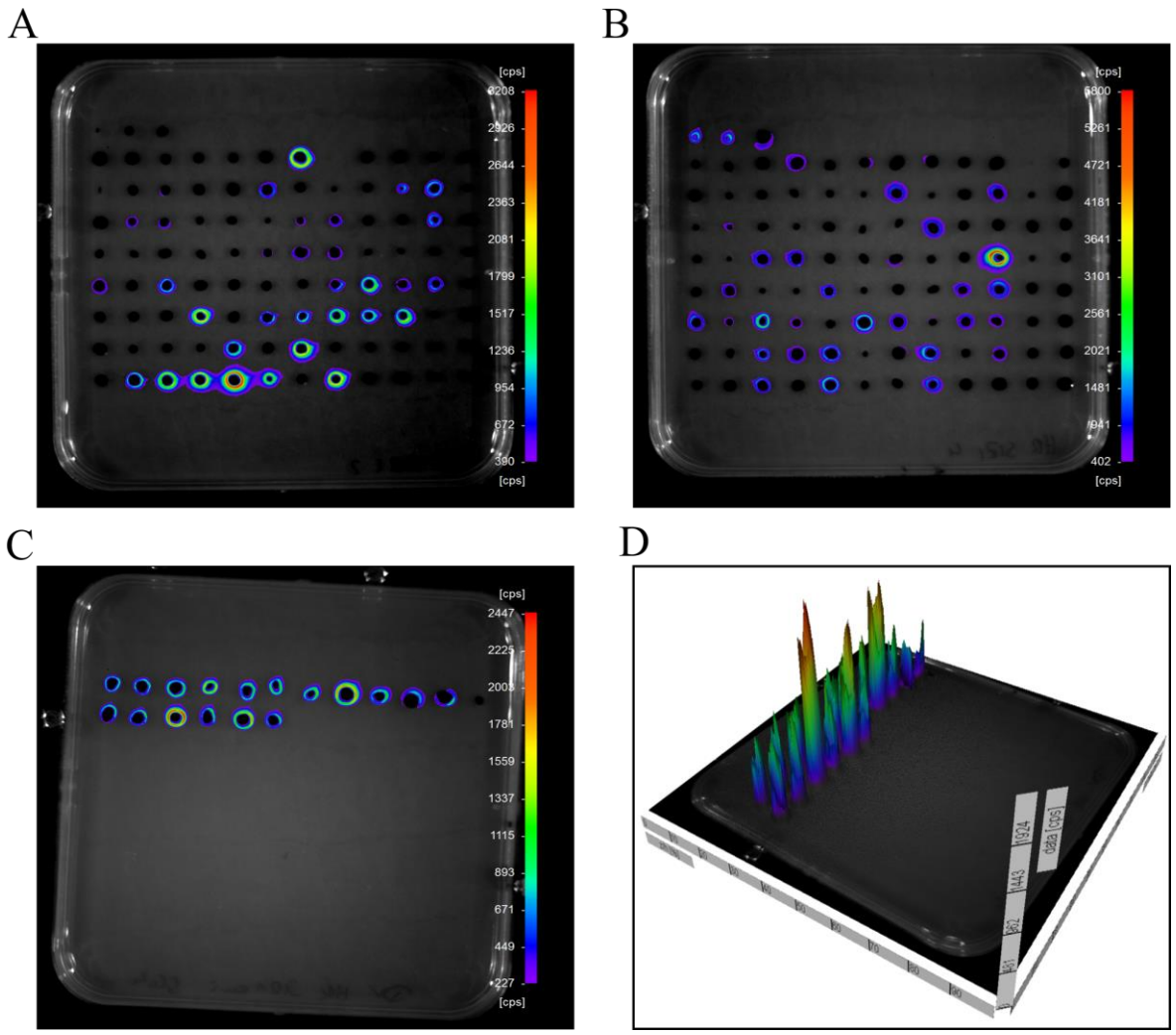

Figure S3: Screening of *SIR1* knockdown mutants by qualitative plate level detection of extracellular luciferase activity. amiRNA constructs targeting *SIR1* were fused to luciferase reporter to facilitate screening of knockdown mutants. (A) and (B) show transformants expressing luciferase on TAP agar plate where intensity of bioluminescence is represented by cps (count per second). (C) 17 positive transformants selected from (A) and (B). (D) Difference in the intensity of bioluminescence between the 17 selected transformants is shown.

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March 2013 – Present **PhD**, Universität Bielefeld, Germany

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- Eukaryotic algae: Cultivation, omics, strain development
- Hydrogen production from eukaryotic green algae
- Use of synthetic polymers to improve process robustness

Dec 2010 – Oct 2011 **Research Assistant, Forschungszentrum Juelich GmbH**

- Bioprocesses and Bioanalytics
- Improved enzyme storage by 10 folds with chemical cocktails
- Reproducible scale up from microbioreactor to pilot scale (30 L)
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- Molecular Biology and Microbiology techniques
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CONFERENCES

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European Networks Conference on Algal and Plant Photosynthesis, **Malta. Speaker**, Title: Doubling Hydrogen production by knocking down an isoflavone reductase like (IRL) protein in *C. reinhardtii*. May 2016
Algal Biotechnology Conference, University of Bielefeld, **Germany**. Poster, Title: Hydrogen photo-production and the potential of immobilization in *C. reinhardtii*. September 2014
ACHEMA, **Germany**. Poster, Title: Automated Microtiter plate based cultivations for optimization of secretory protein expression. June 2012
HTPD-Second International Conference, **France**. Poster, Title: Launching an Automated Microtiter cultivation platform for enhanced Bioprocess Optimization. June 2012
ECAB- First International Conference, **Germany**. Poster, Title: JuBOS: A Bioprocess Optimization system for enhanced development of heterologous protein production. September 2011