NTNU | Faculty of Natural Sciences
Department of Biomedical Laboratory Science

HBIO3001 - Bachelor thesis in Biomedical Laboratory Science

Maturation of plastid cytochrome *c*: A contribution to the study of the function of CCS4 and CCDA in *Chlamydomonas reinhardtii*

By

Ranveig Anita Vinje Natland

Columbus, Ohio, USA, 2022

Department of Molecular Genetics, The Ohio State University

THE OHIO STATE UNIVERSITY

Preface

This thesis was written as the final part of my bachelor's degree in Biomedical Laboratory Sciences from the Faculty of Natural Sciences (NV) at the Norwegian University of Science and Technology (NTNU) in Trondheim. The project was executed in the period between March 11th and June 11th in Dr. Patrice Hamel's laboratory in the Department of Molecular Genetics at The Ohio State University (OSU) in Columbus, Ohio, USA. The Hamel lab's major interest is better understanding biogenesis of energy-transducing membranes, in particular those evolved in mitochondria and chloroplasts.

I want to thank Dr. Patrice Hamel for inviting me into his lab and giving me the opportunity to actively learn about basic research and how it is like to work in a research laboratory. I would also like to give a huge thanks to my supervisor Ankita Das for teaching me new laboratory techniques and having patience with me while I was learning the theory behind every experiment. You have been a huge help through this whole project, and I wish you all the best with the last part of your project. I am also very thankful for the students in the lab Mitchell Ticoras, Kate Kravets, Michelle Mora, Abby Jung and former students Pallavi Chandna and Camina Hebner for being so friendly and welcoming me into the lab.

I would also like to give my thanks to my supervisor at NTNU, Wenche Slettahjell Prestvik for giving me advise and helping me through the whole writing process. I also want to give a special thanks to Kristin Steinsbekk, and Dr. Partice Hamel for making this whole exchange possible through all your hard work and persistence.

I am thankful for all of you and your contribution in giving me this incredibly memorable experience.

June 2022, Columbus, Ohio, USA Ranveig A.V. Natland

Ranveig Anita Vinje Natland

Abstract

Cytochromes *c* are electron carrier proteins with a heme group covalently attached to a CXXCH motif. In the plastid, cytochromes *c* are involved in photosynthesis and their assembly requires the attachment of heme to the cysteines in the motif, a reaction happening in the thylakoid lumen and controlled by assembly proteins called CCS (Cytochrome *C* Synthesis). The proteins CCDA, CCS4 and CCS5 are required to reduce the disulfide bonded CXXCH motif prior to heme ligation. CCDA and CCS5 are thylakoid-bound proteins that transfer electrons from stroma to lumen to directly reduce the disulfide bonded CXXCH via conserved cysteines. CCS4 is a thylakoid-bound protein facing the stroma whose function is unclear because there is no cysteine or motif indicating the protein can transfer electrons. To further study the role of CCS4 in the maturation pathway, we aimed to identify interacting proteins via immunoprecipitation. Transformants producing a tagged CCS4 with 2 or 3 copies of the HA tag in the C-terminal part of the protein were generated and analyzed molecularly for the presence of the tag. Phenotypic analysis showed that such transformants are wild-type like with respect to cytochrome *c* assembly and photosynthetic growth, an indication that the tag does not disrupt the function of CCS4. However, first attempts to detect the CCS4-HA protein using an anti-HA antibody were unsuccessful. We also attempted to create a *CCDA* knock-in mutant using CRISPR/Cas9 technology as there is currently no *ccda* mutant in *Chlamydomonas reinhardtii*. Preliminary results showed that the guide RNAs can cleave the CCDA template in an *in vitro* reaction. An insertional cassette conferring resistance to Hygromycin B was generated via PCR for the *CCDA* knock-in. Optimization is in progress to enhance the yield of the amplicon, a necessary improvement generating the *CCDA* mutation using Cas9 *in vivo*.

Table of Contents

1. Introduction

1.1 *Chlamydomonas reinhardtii* as a model system

Chlamydomonas reinhardtii is a single celled green alga that contains a single chloroplast, which is a closed double membrane (outer and inner membrane) stroma filled cellular compartment [1,2]. In the chloroplast stroma is the thylakoid compartment, a closed membrane network separate from the chloroplast inner membrane and defining a hollow space called the thylakoid lumen [1].

C. reinhardtii uses CO_2 and light energy to perform photosynthesis, a process that ends in the production of biomass, including lipids, glucose, and proteins, that the cell is depending upon for growth. The production of biomass is also a desirable trait on a large-scale industrial platform because of the potential to use microalgae like *C. reinhardtii* to make sustainable biofuels and biomolecules [3]. Microalgae, including *C. reinhardtii*, are attractive organisms not only because high production of biomass can be achieved, but also because it is relatively easy to genetically manipulate them [4,5].

To be able to produce the necessary cell components via photosynthesis, the cell must produce energy in the form of ATP and NADPH via photophosphorylation, a process requiring the thylakoid compartment.

Photophosphorylation is a chemical reaction requiring four thylakoid membrane embedded protein complexes namely Photosystem II (PSII), the cytochrome b_6f complex (b_6f), Photosystem I and ATP synthase shown in Figure 1. Photons carrying light energy are absorbed by PSII and PSI. In PSII the energy is used to split water molecules into O_2 and H^+ (proton) in the thylakoid lumen. Electrons are carried from PSII to b_6f where they fall to a lower energy state and protons from the stroma are pumped into the thylakoid lumen. The low energy electrons are transferred to PSI where the light energy excite the electrons. The electrons are then transferred to the NADP reductase where they are used to reduce $NADP^+$ to NADPH. The movement of electrons enables the establishment of a proton gradient across the thylakoid membrane. ATP synthase is able to use this energy stored in the form of a proton gradient to synthesize ATP from ADP. Synthesis of ATP in the stroma is coupled to the movement of protons flowing from the thylakoid lumen to the stroma [6].

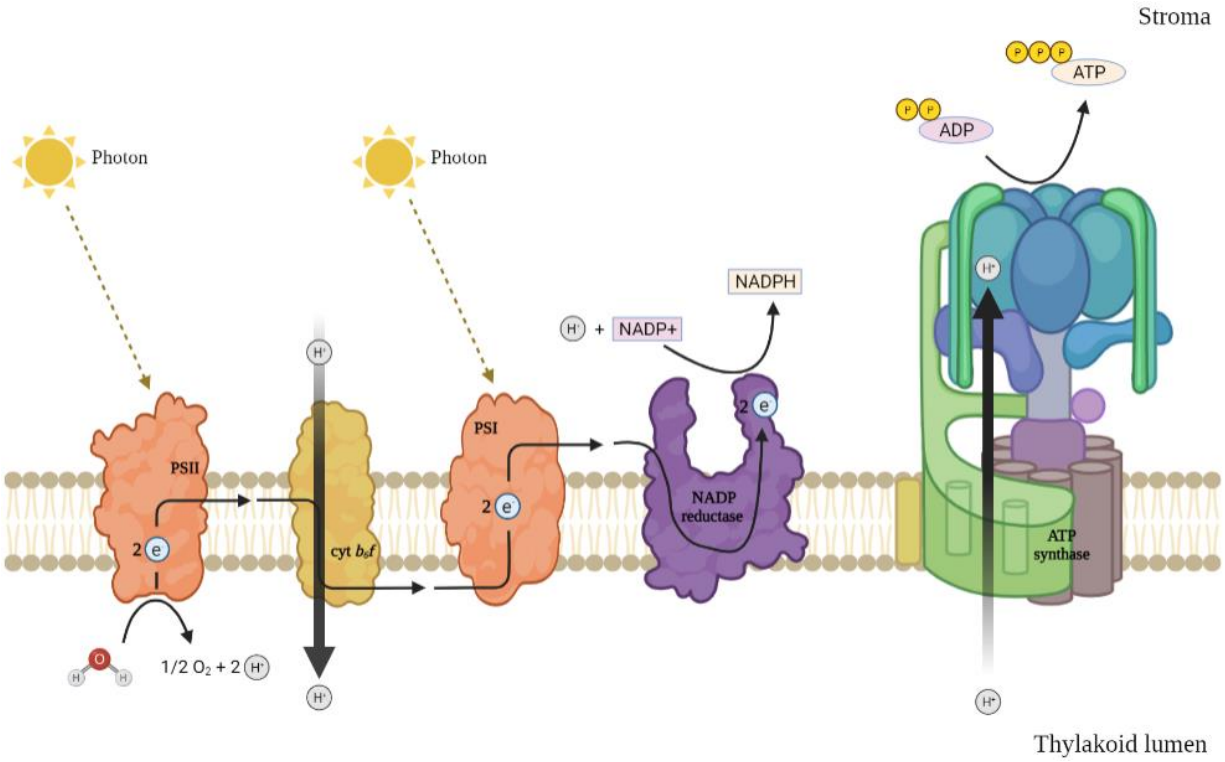

Figure 1: ATP synthesis and electron transport during photosynthesis. *The figure is created in BioRender.com with inspiration from The Cell: A Molecular Approach. 2nd edition Figure 10.22* [7].

Of particular relevance to this study is the cytochrome b_6f complex, a multi-subunit complex named from two of its structural subunits, namely cytochrome b_6 and cytochrome *f* [8]. Cytochrome *f* is a heme containing protein and belongs to the class of cytochrome *c* or *c*-type cytochromes where the "*c*" stands for "covalent" for the covalent bound heme [9,10]. The maturation of plastid cytochrome *c* involves the conversion of apocytochrome *c* to holocytochrome *c*, which is the covalent attachment of heme to the CXXCH motif of the protein. This reaction takes place in the thylakoid lumen and requires 1) the transport of apocytochrome *c* and heme across the thylakoid membrane, 2) the reduction of the heme iron and the disulfide bonded CXXCH, and 3) the attachment of heme via thioether bond (carbon-sulfur) [9,10]. The maturation of plastid cytochrome *c* requires assembly factors called CCS that were discovered genetically in *C. reinhardtii* via the isolation of the *ccs* mutants [9,10]. The *ccs* mutants are deficient for the attachment of heme to apocytochrome *c*.

Because cytochrome *f* is required for the function of the b_6f and photosynthesis, the *ccs* mutants are deficient for photosynthetic growth [9,10].

1.2 Cytochrome *c* maturation: The disulfide reduction pathway

My work was concerned with one aspect of cytochrome *c* maturation which is the preparation of the apocytochrome *c* CXXCH under the reduced form, which requires the disulfide bonded

heme-linking cysteines to be chemically reduced [9–11]. There are two pathways for disulfide reduction in plastid cytochrome *c* maturation in *C. reinhardtii*. The first one uses membrane bound proteins named CCDA (Cytochrome *C* protein A) and CCS5 (Cytochrome *C* Synthesis protein 5) that possess conserved cysteines and transfer electrons from stroma to lumen [12,13] (Figure 2). Electrons transferred across the thylakoid membrane via CCDA to CCS5 are used to directly reduce the disulfide at the heme binding cysteines in apocytochrome *c* in the thylakoid lumen as shown in Figure 2. The second pathway uses CCS4 (Cytochrome *C* Synthesis protein 4), but the biochemical function of this protein still remains unknown.

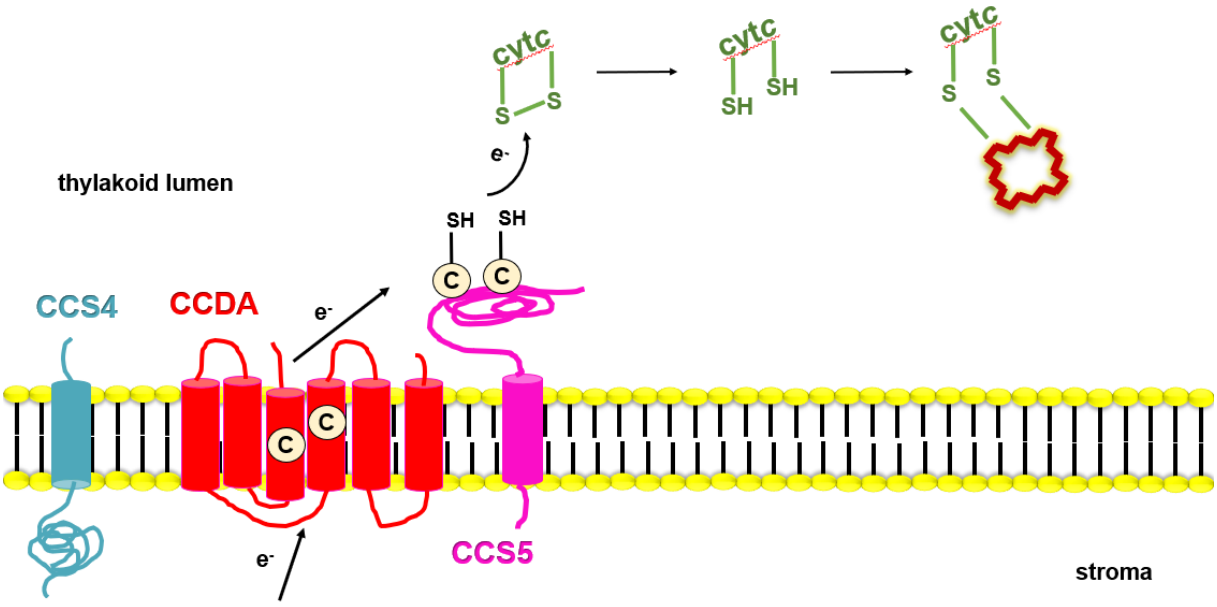

Figure 2: Thylakoid membrane and proteins involved in the maturation of plastid cytochrome c (courtesy of Ankita Das).

The maturation pathway of plastid cytochrome *c* is known to be dependent on the proteins CCDA and CCS5. However, a loss of CCS5 or CCDA function does not completely block plastid cytochrome *c* maturation suggesting there is an additional pathway to reduce the disulfide in apocytochrome *c* [12,13]. Loss of the CCS4 protein affects partially cytochrome *c* maturation but is more severe than loss of CCS5 [14]. Complete loss of function mutations in *CCS5* and *CCS4* abolishes cytochrome *c* maturation suggesting that CCS5 and CCS4 are components of distinct pathways. The *ccs4*-null, *ccs5*-null, or *ccs4*-null *ccs5*-null double mutants can be restored for cytochrome *c* assembly and photosynthetic growth by provisions of reducing agents (reductants). This result indicated the CCS4 and CCS5 proteins both work in the disulfide reducing pathway for cytochrome *c* maturation [14,13]. The function of CCS4 is not obvious based on the fact that 1) the protein does not contain cysteines and has no other motif suggesting it can transport electrons and 2) most of the protein is localized on the stromal side while apocytochrome *c* CXXCH reduction takes place on the luminal side

(Figure 2) [14,15]. While the role of CCS4 in the disulfide reducing pathway in cytochrome *c* assembly is established, the activity of the protein in the assembly process remains unclear.

1.3 CRISPR/Cas9

Here I provide a short description of the CRISPR/Cas9 technology that I used in this work. The clustered regularly interspaced short palindromic repeats (CRISPR) first discovered in archaea, and later in bacteria, are used as a defense mechanism against viral invasions [16]. These repeats are short sequences of genetic code in the cell captured from past invaders [17]. These segments are called CRISPR arrays, and when the cell is attacked by the same virus, it produces RNA segments that recognize and bind to the viral genetic material. The Cas9 (CRISPR-associated protein 9) then detects the CRISPR segment, cuts the viral DNA and destroys the virus.

This natural system has been adapted to make it usable for targeted genome editing through a Cas9-guide RNA (gRNA) complex, RNP (ribonucleoprotein) complex, shown in Figure 3. The gRNA is a short RNA sequence that binds to a target locus to be engineered in the host genome. For the Cas9 to be able to cleave the DNA, a protospacer adjacent motif (PAM) is required. PAM is a short DNA sequence in the target DNA, 2-6 bp long, and is generally located 3-4 bp downstream of the cleavage site generated by Cas9 [18,19]. The CRISPR/Cas9 is a highly precise system with desired reduced off-target cleavage and increased editing efficiencies and was reported to be efficient for gene editing in *C. reinhardtii* [20].

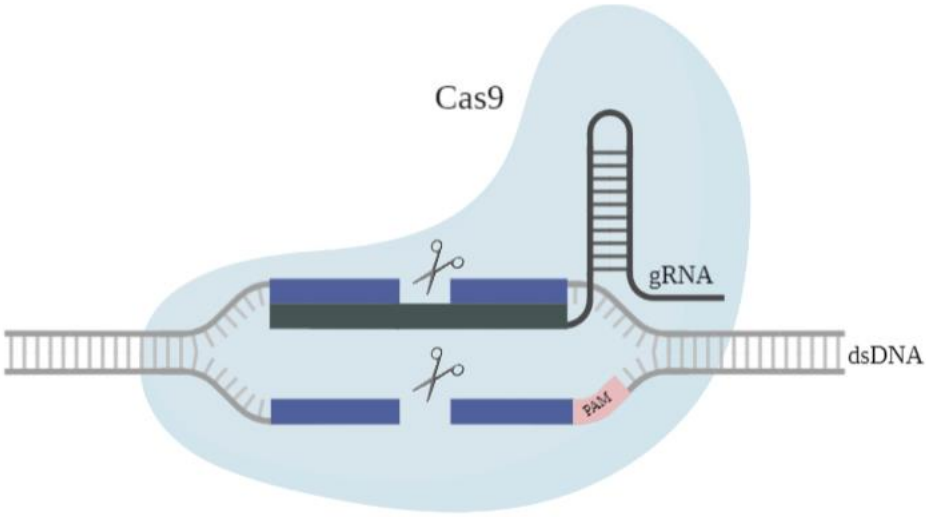

Figure 3: Cas9-gRNA ribonucleoprotein complex, with double stranded DNA and PAM. *The figure is created in BioRender.com.*

In this project, a protocol used by Dhokane et *al*. [20] will be adapted to do *in vitro* CRISPR on amplified CCDA for targeted genome editing of *C. reinhardtii*.

1.4 Aim of study

In this work, I aimed to further explore the disulfide reducing pathway with a focus on the CCS4 and CCDA components. In one project, to get insight into the function of the CCS4 protein, we aim to identify proteins interacting with CCS4. To discover partners of the CCS4 protein, algal strains expressing a tagged version of the *CCS4* gene were generated for subsequent immunoprecipitation experiments. In the second approach, I aim to create a loss of function mutation in *CCDA* in *C. reinhardtii* via CRISPR/Cas9 as the only available mutant is in the plant *Arabidopsis thaliana* [12].

2. Materials and Methods

2.1 Strains and growth conditions

2.1.1 Algal strains

Wild-type (WT) and mutant algal strains were grown mixotrophically (in the presence of light, acetate, and atmospheric CO_2) or phototrophically (in the presence of light and atmospheric CO_2). For mixotrophic conditions, acetate containing media are TAP solid medium (select agar, Invitrogen): Tris-Acetate Phosphate or TARG: TAP + arginine with or without 25 µg/mL Paromomycin (Pm). Paromomycin was used for selection of the transformants containing pSL18-based construct. For phototrophic conditions, minimal medium supplemented with arginine (for arginine auxotrophic strains) was used. All algal strains were maintained on solid medium at 25 °C in continuous low light at 0.3 µE/m^2/s. Strains CC124 (*mt*$^-$) and CC5589 (3A+, *mt*$^+$*arg7-8*) were used as WT, and the CC4589 (*mt*$^-$ *ccs4-.1 arg7-8*) was used as the *ccs4* mutant. The reagents for *C. reinhardtii* media used in this project are listed in Table 1.

Table 1: Reagents for C. reinhardtii media.

Reagents	Concentrations	TAP	TARG	Min + ARG
Beijerinck's Solution (5x)	NH_4Cl (747.8 mM), $CaCl_2 \cdot 2H_2O$ (34.0 mM), $MgSO_4 \cdot 7H_2O$ (40.6 mM)	10 mL	10 mL	10 mL
Phosphate buffer	K_2HPOH_4 (110.1 mM), KH_2POH_4 (78.8 mM)	10 mL	10 mL	50 mL
Tris Acetate Solution	Tris-base (2.0 M), Glacial Acetic Acid (1.7 M)	10 mL	10 mL	-
Trace Elements	$ZnSO_4 \cdot 7H_2O$ (76.5 mM), H_3BO_3 (184.4 mM), $MnCl_2 \cdot 4H_2O$ (25.8 mM), $FeSO_4 \cdot 7H_2O$ (18.0 mM), $CoCl_2 \cdot 6H_2O$ (6.7 mM), $CuSO_4 \cdot 5H_2O$ (6.4 mM), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.9 mM)	1 mL	1 mL	1 mL
Arginine 100 mg/mL	(100 mg/mL)	-	4 mL	0.3 mL
Agar powder		15-16 g	15-16 g	15-16 g
ddH_2O		969 mL	965 mL	938.7 mL

2.1.2 Bacterial strains

Bacterial strain, DH5α, used for plasmid amplification was grown in liquid Miller Luria Broth (LB) medium (VWR, 25 g/L) adjusted to pH 7.2 after autoclaving. For solid LB medium, bacteriological agar (US Biological 15 g/L) was used.

2.3 Plasmid DNA extraction

Plasmid used in this study is pHyg3 (Prof. W. Mages (U. Resenburg, Germany)).

This protocol is adapted from Giannino Del Sal et *al*., [21].

Bacterial cells were grown in 3 mL liquid Miller LB medium containing ampicillin (100 µg/mL) at 37 °C for 14-16 hours with shaking (180 RPM). Cells were spun down in a microfuge tube and resuspended in 400 µL STET buffer (8 % w/v sucrose, 0.1 % v/v TritonX-100, 50 mM EDTA, 50 mM Tris-HCl) pH 8.0. Eight µL lysosome (50 mg/mL in ddH_2O) was added and incubated in room temperature for 5 minutes. The samples were boiled for 45 seconds and centrifuged for 10 minutes at 11,000 x *g*. The pellet was removed using a toothpick and 15 µL of RNaseA (20 mg/mL, Invitrogen) was added and the tube incubated for 15 minutes at 37 °C. A volume of 16 µL CTAB (Cetyltrimethylammonium bromide, 5 % w/v) was added to the tube and incubated for 5 minutes at room temperature followed by centrifugation for 5 minutes at 11,000 x *g*.

The pellet was resuspended in 300 µL NaCl (1.2 M) and the DNA precipitated by adding 750 µL of 100 % ethanol followed by centrifugation for 10 minutes at 11,000 x *g*. The pellet was washed using 1 mL 70 % ethanol. And the tube was centrifuged for 5 minutes at 11,000 x *g*. The air-dried pellet was resuspended in ddH_2O and centrifuged for 5 minutes at 11,000 x *g*. The supernatant was transferred to a new tube and the DNA concentrations was measured using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

2.5 Molecular analysis of algal transformants

Cracking/lysis of *C. reinhardtii* cells was performed to obtain crude extract of genomic DNA. A small amount of cells was collected with a toothpick and transferred to 24 µL of the master cracking mix (Table 2) in VWR™ PCR tubes. The tubes were briefly vortexed and placed in T100 Thermal Cycler for the cracking process showed in Figure 4. This process breaks open the cells to obtain a cell lysate, which was then used as template in the diagnostic PCR.

Table 2: Concentrations and volumes of reagents for Cracking of the pSL18 CCS4-HA transformants.

Reagents	Concentration	Volume (µL)
Colorless Go *Taq* buffer (Promega)	5x	5
Proteinase K (Invitrogen)	20 mg/mL	2.5
ddH_2O		17.5

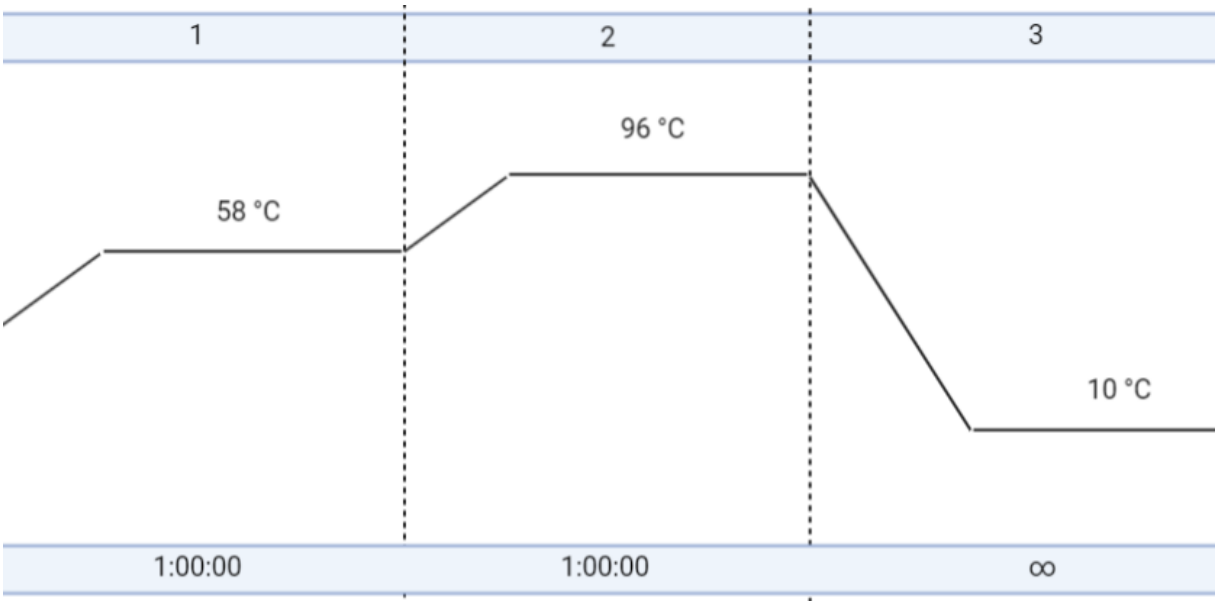

Figure 4: Cracking conditions

For diagnostic PCR, extracts from cracked cells were used as templates in a reaction containing dNTPs, primers, DMSO and polymerase (Table 3) The annealing temperature was adjusted according to the melting temperature (T_m) of the primers (Table 4). The PCR reaction cycle used is showed in Figure 5. Amplicons were analyzed via electrophoresis in ethidium bromide (0.1 µg/mL) containing agarose gel. Following electrophoresis, amplicons were visualized under UV light using Axygen Gel Documentation System.

Table 3: Concentrations and volumes of reagents for Polymerase Chain Reaction

Reagents	Concentration	Volume pr. tube (µL)
Green Go *Taq* buffer (Promega)	5x	4
dNTPs	10 mM	0.4
DMSO		0.54
Forward primer	20 µM	1
Reverse primer	20 µM	1
Template	25 ng/µL	1
Go *Taq* polymerase enzyme	5 units/ µL	0.1
ddH_2O		11.96

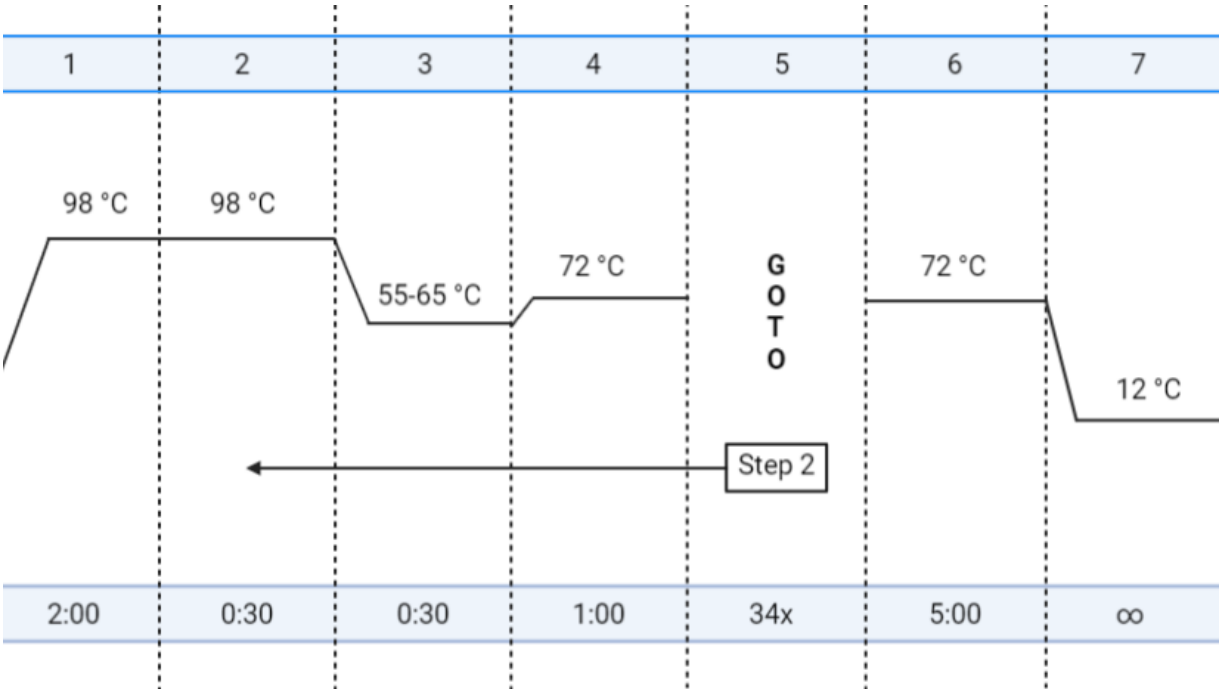

Figure 5: PCR reaction cycle with varying annealing temperatures.

For each PCR reaction different primers were used depending on each template. Every primer used in this study is listed in Table 4.

Table 4: Sequence, T_m and target gene for each primer used in this study.

Primer	Sequence (5'-3')	T_m (°C)	Target gene
CCS4-3HA-NdeI	GCTACTCACAACAAGCCCATATGTCGACTGGCATTGAGGAC	70	*CCS4-3HA CCS4-2HA*
CCS4-3HA-EcoRV	CAGGAATTCGATATCAAGCTTATCGATGGCGTAGTCGGGCACG	70	
pCCS4.10	CGACTGGCATTGAGGACACC	61	*CCS4*
pCCS4.28	ACTTGGTTGCCTGCTCC	57	
CCDA-Fwd1	CACACAGCAGCCAAAATGCAGC	59.7	*CCDA*
CCDA-Rev1	TGCAAGTGAAGGGCCAGTCATTG	60.1	*CCDA*
CCDA-RV1	CCAGGCCGAACGAGAACGACAC	62	*CCDA*
gRNA1-HDR-F1	CTGGATTTGTTTATACAGCTCAGCATTTCAATCTCACTGCAAACATTATGGAATTCGATATCAAGCTTCTTTCTTGC	70	*APHVII*
gRNA1-HDR-R1	GCTTGAACAGTGCCAGCTGACGACTAGGCAGCGCGTACAAGTTGGTGTGGAAGCTTCCATGGGATGACGG	78	*APHVII*
gRNA2-HDR-F2	AGTCGTCAGCTGGCACTGTTCAAGCCATGTCCTGTACGCGTCGCTAGCTCGAATTCGATATCAAGCTTCTTTCTTGC	76	*APHVII*
gRNA2-HDR-R2	CATCAGAATCTCTTTGTGTATTCCGGCCTCGACTGCTGCTTACCTGGCCGAAGCTTCCATGGGATGACGGGCCCG	79	*APHVII*
Nested_F1	TCTCACTGCAAACATTATGGAATTCGATATCAAGCTTCTTTCTTGC	65	*APHVII*
Nested_R1	CGTACAAGTTGGTGTGGAAGCTTCCATGGGATGACGGGC	70.6	*APHVII*
Nested_F2	CTGGATTTGTTTATACAGCTCAGCATTTCAATCTCACTGCAAACATTATGG	65.9	*APHVII*
Nested_R2	GCTTGAACAGTGCCAGCTGACGACTAGGCAGCGCGTACAAGTTGGTGTGGAAG	75	*APHVII*

2.6 Growth assessment on solid medium via serial dilutions

Phenotyping screening of transformants was performed using serial dilution series.
Cells grown on solid medium were collected using wooden sticks and transferred to a microfuge containing 500 µL of ddH_2O. The cell density was measured spectrophotometrically at OD_{750} and normalized to an OD_{750} = 2.0 by dilution. This normalized suspension was used as the starting material [1] for making five serial ten-fold dilutions [10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and, 10^{-5}]. A volume of 5 µL for each dilution was plated on solid medium.

2.7 Protein preparation and analysis

Cells grown on solid acetate containing media for 5-7 days at 25 °C under low light were collected using a wooden stick, washed, and resuspended in 10 mM sodium phosphate (NaPi) buffer (pH 7.0). To extract the chlorophyll, 10 µL of the resuspended cells was mixed with 1 mL 80:20 (acetone:methanol) and then vortexed and centrifuged at 1500 x *g* for 5 minutes. The chlorophyll concentration is measured at 595 nm and the OD_{595} multiplied by 2.9 gives the concentration in mg/mL [22]. The concentration was standardized to 1 mg/mL of chlorophyll and cells were broken via two freeze/thaw cycles (frozen at -80 °C overnight, and then thawed on wet ice). To separate the membrane from the soluble fractions, the cells were centrifuged at 1500 x *g* for 5 minutes. The supernatant was removed, and cells were resuspended in 10 mM NaPi (in the same volume as the volume of the removed supernatant).

For immunoblotting, membrane protein fractions corresponding to 10-20 µg of chlorophyll were electrophoretically separated by SDS-PAGE using a 12.5 % acrylamide gel (Table 5). The gel was transferred to a PVDF membrane at 100 V (at 4 °C) for 90 minutes. The membranes were incubated with α-CF_1 (1:10,000, from Dr. Merchant, UC Berkeley), α-PsbO (1:5000, Agrisera), α-cytochrome f (1:10,000, from Dr. Merchant, UC Berkeley), CCDA (1:1000, a gift from Dr. Motohashi [23]), α-CCS5 (1:3,000) [13]. After the incubation, the membranes were washed in TBS buffer pH 8.0 (160 g NaCl, 5 g KCl and 60 g Tris-base in 1 L ddH_2O).
The immunodetection was performed with Pierce™ ECL Western Blotting Substrate using X-Ray Film for Chemiluminescence Western Blot.

Table 5: Reagents for resolving and stacking gel for 12.5 % acrylamide gels x2.

Reagents	Manufacturer	Resolving gel	Stacking gel
ddH_2O		5.33 mL	2.81 mL
Acrylamide (30 %)	VWR	6.67 mL	0.75 mL
Lower buffer		4 mL	-
Upper buffer		-	1.25 mL
25 % APS	Acros Organics	40 µL	12 µL
Temed	Fisher BioReagents	10 µL	10 µL

The protocol for preparing lower and upper buffer used for the 12.5 % acrylamide gel is showed in Table 6.

Table 6: Reagents with concentrations for lower (pH 8.8) and upper (pH 6.8) buffer.

Reagents	Manufacturer	Lower buffer (pH 8.8)	Upper buffer (pH 6.8)
Tris-base	Fisher BioReagents	18.1 g	6.05 g
10 % SDS	VWR	4 mL	4 mL
ddH_2O		96 mL	96 mL

For the electrophoretic separation, the buffer used was Running buffer 10x. The recipes with the running buffer and that of the Transfer Buffer used for electroblotting are in Table 7.

Table 7: Reagents with concentrations for Running 10x (pH 8.5) and Transfer buffer.

Reagents	Manufacturer	Running buffer 10x (pH 8.5)	Transfer buffer
Tris-base	Fisher BioReagents	54.5 g	6 g
Glycine	Fisher BioReagents	225 g	28.8 g
70 % Ethanol	Fisher BioReagents	-	421 mL
SDS	VWR	15 g	4 mL
ddH_2O		1.5 L	1.575 L

2.9 Construction of an insertional *ccda* mutant using CRISPR-Cas9

The sgRNAs for targeted editing of the *CCDA* gene were designed in the Cloud-based platform for biotech R&D, Benchling [24]. The sgRNAs with the highest on-target and off-target score were selected, and two sets with each gRNA1 and gRNA2 were synthesized (Synthego) For gRNA1 the cleavage is predicted to occur at the beginning of exon 1, while for the gRNA2 the cleavage is predicted to occur at the end of exon 1. As a preliminary assessment of the sgRNA efficiency, *in vitro* CRISPR/Cas9 dependent digestion of a PCR template was performed. For this, sgRNAs were mixed with Cas9 protein (Invitrogen, a gift from the Singh lab at OSU) at 1:1 ratio and incubated at room temperature for 20 minutes to form the RNP complex. An amplicon corresponding to a portion of the *CCDA* gene carrying the site to be cleaved by Cas9 was amplified using primers CCDA-Fwd1, CCDA-Rev1 and CCDA-RV1, *Taq* polymerase and a plasmid carrying *CCDA* as template. The amplicon was gel purified after electrophoresis in 1 % agarose gel. The purified product was added as the template to the RNP mixture and incubated for 15 minutes and overnight at 37 °C for the digestion. Fifty ng of each component (sgRNA, Cas9 and template) was used in the reaction. RNAse (20 mg/mL) was added to get rid of excess sgRNA and the product of the CRISPR/Cas9 reaction was analyzed on an 0.8 % agarose gel. The CRISPR/Cas9 protocol is shown in Table 8.

Table 8: Protocol for CRISPR/Cas9 reaction.

Lane No	sgRNA I/II (µL)	Cas9 (µL)	DNA (µL)	Buffer (µL)	ddH_2O (µL)
1	-	-	1.31	2	16.69
2	-	1.56	1.31	2	15.13
3	1.56	1.56	1.31	2	13.57
4	3.12	1.56	1.31	2	12.01

The Hygromycin B-resistance (HygB) cassette with marker gene *APHVII* for creating the insertional mutation in the *CCDA* gene was generated by PCR using pHyg3 as template and gRNA1-HDR-F1/gRNA1-HDR-R1 and gRNA2-HDR-F2/gRNA2-HDR-R2 as primers.

3. Results

3.1 Identification of CCS4 interacting partners

To identify proteins interacting with CCS4, a functional HA-tagged version of the *CCS4* gene was engineered. The plasmid pSL18 containing the Pm-resistance marker gene (*APHVIII*) [25] for selection was used to generate versions of the *CCS4* gene containing no, 2 or 3 copies of the sequence encoding the HA tag. The sequence encoding the HA tag was engineered at the 3' end of the last coding exon of the *CCS4* gene. The *CCS4-2HA* and *CCS4- 3HA* genes are under the control of the *PSAD* promotor and terminator, which drives high expression of the gene [26] (Figure 6). Transformants were generated by introducing the pSL18-based constructs into a *ccs4* mutant, which is characterized by a complete loss of CCS4 function [14]. These transformants generated by A. Das were selected on acetate-containing medium (containing Pm) and are listed in Table 9.

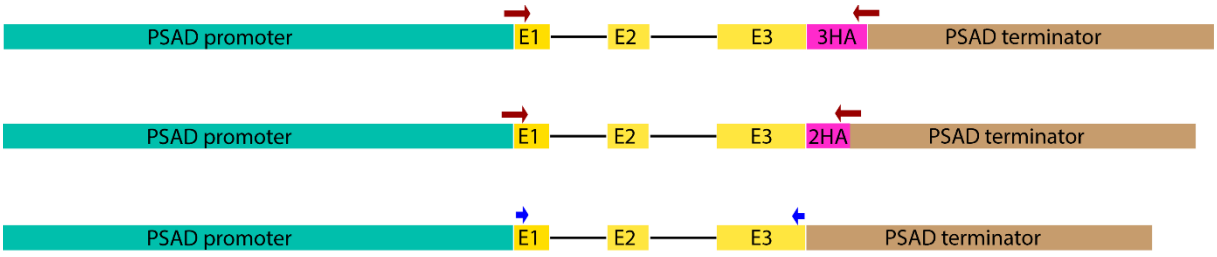

Figure 6: Diagrammatic representation of the CCS4 gene with and without HA tag. *The CCS4 gene is represented with exons (yellow boxes) and introns (line). The positions of the diagnostic primers used for diagnostic PCR is indicated by colored arrows.*

As controls, the pSL18 (empty vector) and pSL18-ORF2L (*CCS4* gene with no HA tag) were used. The pSL18-CCS4 1 kb carries a 1 kb *Sac*II genomic fragment containing the *CCS4* gene under the control of its endogenous promotor and terminator [14].

3.1.1 Generation of *CCS4-HA* expressing strains

The transformants used in this project are listed in Table 9. Transformants were selected in low light conditions and were available for analysis at the beginning of this study.

Table 9: Number of colonies obtained. *Colonies were selected in Pm-containing TARG medium U refers to the undigested plasmid, while L is the plasmid linearized for the transforming DNA.*

Construct	Groups	Transforming DNA	Number of colonies
With tag	1	1U: pSL18-CCS4 x 3HA	3
		2L: pSL18-CCS4 x 3HA	0
	2	3U: pSL18-CCS4 x 2HA	7
		4L: pSL18-CCS4 x 2HA	8
Empty vector	3	5U: pSL18 EV	32
		6L: pSL18 EV	19
With gene, no tag	4	7U: pSL18-ORF2L	9
		8L: pSL18-ORF2L	2
	5	9U: pSL18-CCS4 1 kb	6
		10L: pSL18-CCS4 1 kb	20

3.1.2 Molecular analysis of the *CCS4-HA* and *CCS4* transformants by diagnostic PCR

To test for the presence of the *CCS4-HA* or *CCS4* gene in the transformants, diagnostic PCR was performed on a subset of transformants (Table 9) The general conditions for PCR are described in the Materials and Methods section (Table 3).
All transformants were divided into groups (Table 9) for ease of interpretation of the result.

All the Pm-resistant transformants (groups 1 and 2) generated with the pSL18-CCS4 x 2HA (3 colonies) and pSL18-CCS4 x 3HA (15 colonies) were tested for the presence of the introduced gene via PCR using diagnostic primers. The expected sizes of the diagnostic amplicons are 603 bp for *CCS4-3HA* and 576 bp for *CCS4-2HA*. Because the selected primers are specific for the presence of the tag, one colony from group 3 (empty vector), one colony from group 4 (*CCS4*) (Figure 6) were chosen as negative controls. Figure 7 shows an example of diagnostic PCR

where 2 out of 3 tested colonies were confirmed to contain the *CCS4-3HA* gene while 7 out of 15 tested colonies were found to carry the *CCS4-2HA* gene.

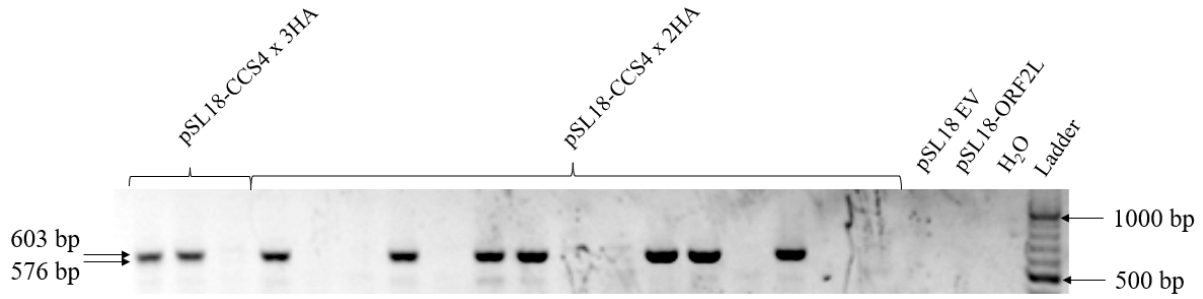

Figure 7: Diagnostic PCR to test for the presence of the CCS4-3HA and CCS4-2HA gene. *Transformants carrying pSL18-CCS4 x 3HA (lanes 1 to 3) and pSL18-CCS4 x 2HA (lanes 4 to 18) were analyzed for the presence of the CCS4-3HA and CCS4-2HA gene via diagnostic PCR. Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

For the screening of the transformants carrying the *CCS4* gene, the diagnostic primers are expected to produce an amplicon of 467 bp. All 11 transformants from group 4 (pSL18-ORF2L) and 13 out of 26 colonies in group 5 (pSL18-CCS4 1 kb) were tested by diagnostic PCR. One colony from group 1 (CCS4 x 3HA), one colony from group 2 (CCS4 x 2HA) were chosen as positive controls. One colony from group 3 (empty vector) serves as negative control.

As shown in Figure 8, 4 out of 6 tested colonies in group 4 (pSL18-ORF2L) contain the *CCS4* gene and 7 out of 14 tested colonies in group 5 (pSL18-CCS4 1 kb) carry the introduced gene. Screening of the remaining transformants identified an additional 5 transformants carrying the *CCS4* gene in group 5 (not shown). Additionally, transformants selected under high light on acetate containing medium and carrying *CCS4-3HA* or *CCS4-2HA* were uncovered by diagnostic PCR (not shown).

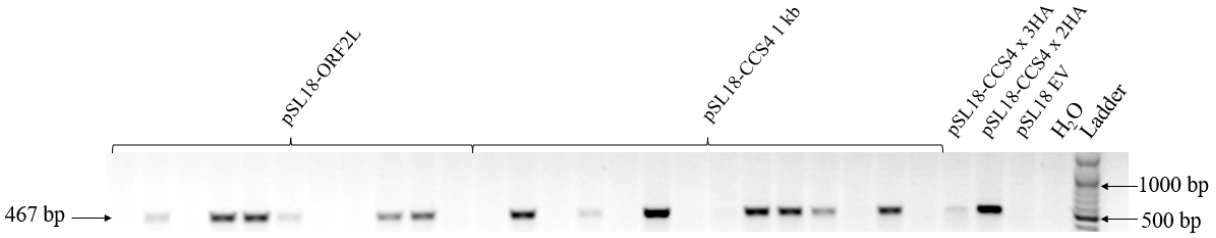

Figure 8: Diagnostic PCR for the presence of the CCS4 gene. *Transformants carrying pSL18-ORF2L (lanes 1 to 11), pSL18-CCS4 (lanes 12 to 25), pSL18-CCS4 x 3HA (lane 26) pSL18-CCS4 x 2HA (lane 27) were analyzed for the presence of the CCS4 gene via diagnostic PCR. Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

3.1.3 Phenotypic screening of the *CCS4* transformants

Because it is possible that the introduced HA tag interferes with the function of the CCS4 protein, we tested the ability of the *CCS4* transformants to grow photosynthetically via serial dilutions on acetate and minimal medium (Figures 9 and 10). All the transformants that showed

restoration of the photosynthetic growth were also positive via diagnostic PCR. The few transformants that did not display restoration of photosynthetic growth were also negative by diagnostic PCR. There were no obvious differences between the photosynthetic growth of transformants expressing the tagged or untagged version of *CCS4*. We concluded the HA tag did not disturb the function of the CCS4 protein.

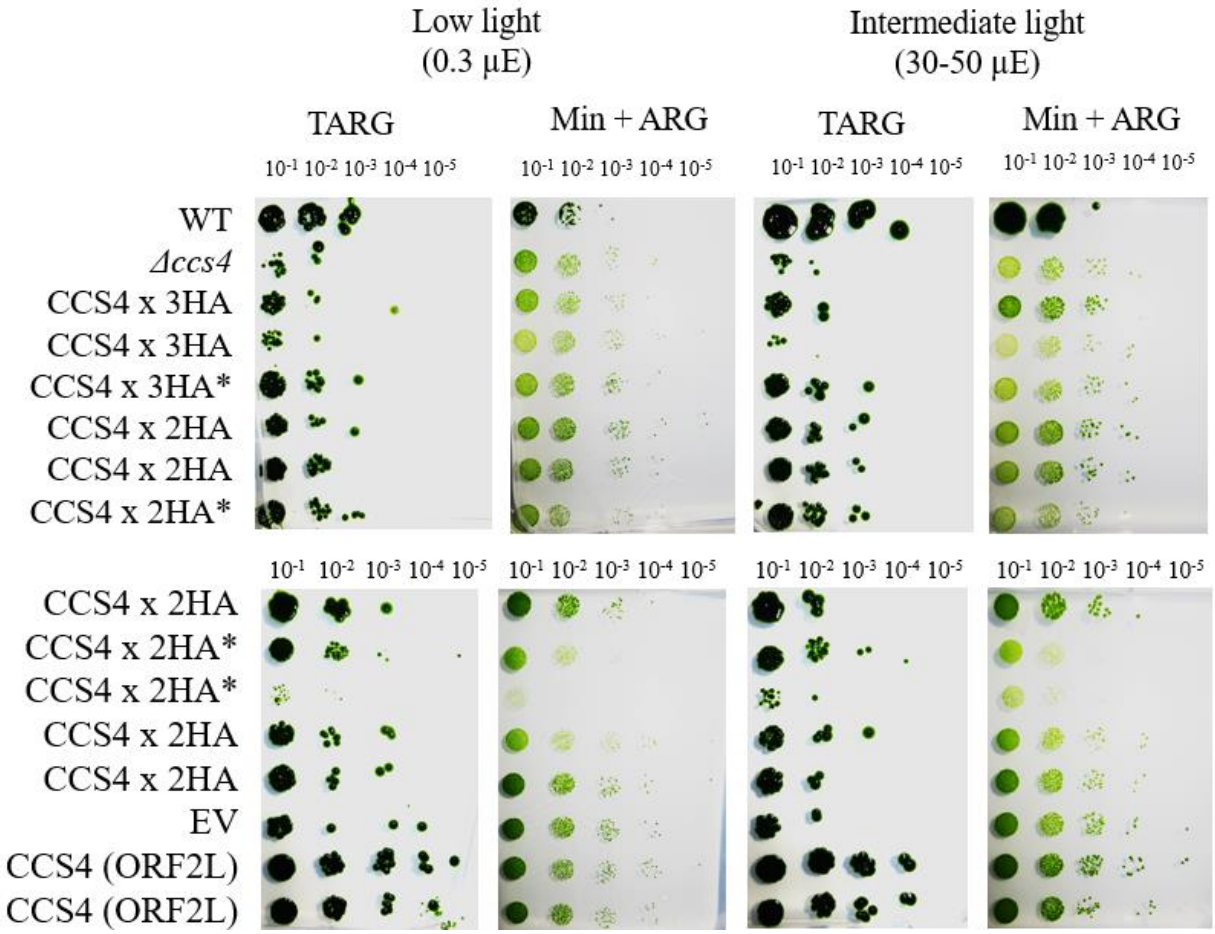

Figure 9: Phenotyping screening of CCS4 transformants. *The image shows growth of C. reinhardtii WT, Δccs4 and CCS4 transformants. Transformants that were screened negative by diagnostic PCR are labeled with "*". The image was taken after 15 days incubation on TARG and Min + ARG media under low light and intermediate light.*

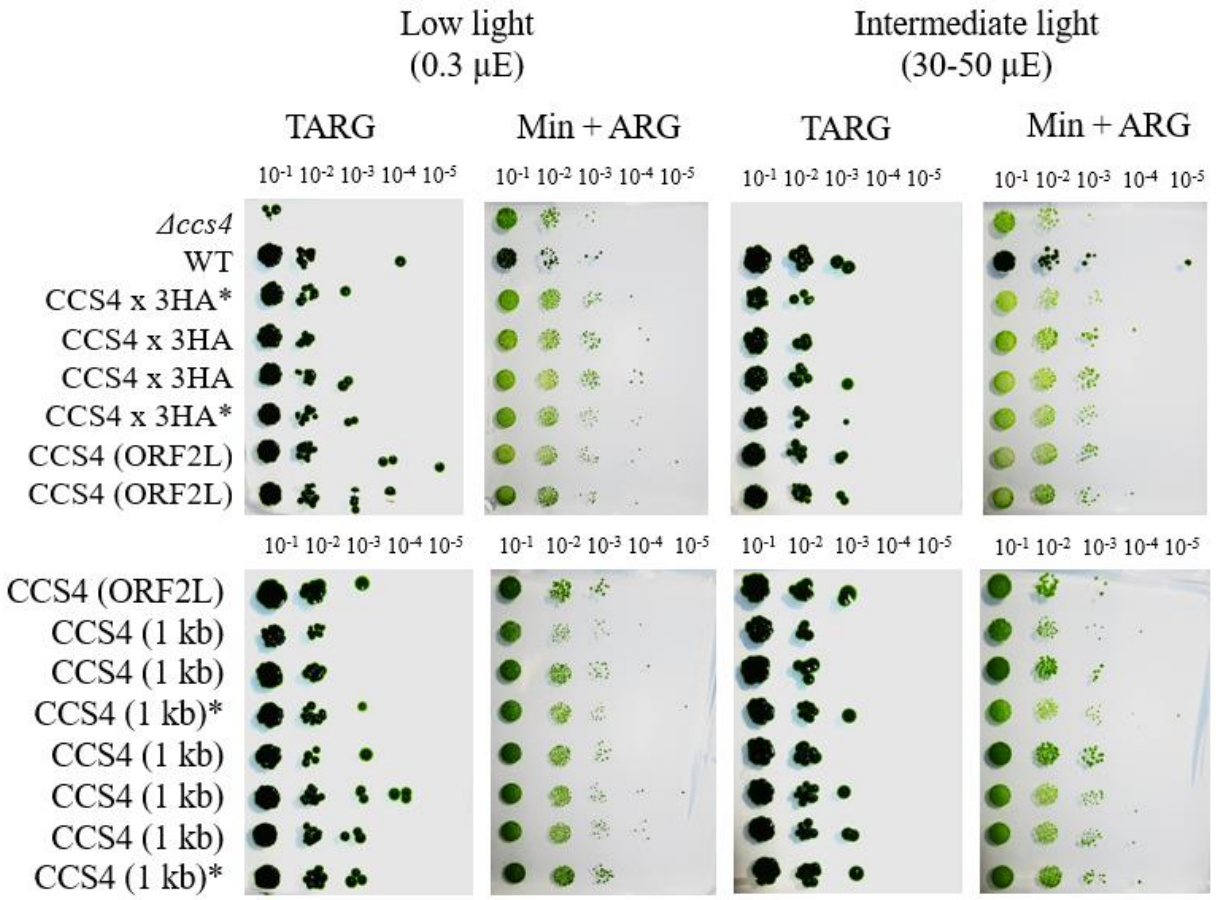

Figure 10: Phenotyping screening of CCS4 transformants. *The image shows growth of Δccs4, C. reinhardtii WT and CCS4 transformants. Transformants that were screened negative by diagnostic PCR are labeled with "*". The image was taken after 15 days incubation on TARG and Min + ARG media under low light and intermediate light.*

3.1.4 Immunoblot detection of CCS4-HA and cytochrome *f*

To test if cytochrome *c* maturation was fully restored in the transformants producing the tagged CCS4 protein, we performed immunodetection of cytochrome *f*, the plastid cytochrome *c* required for photosynthesis (Figure 11). We included transformants that were screened as negative for the presence of the introduced *CCS4* gene (indicated by "-", lanes 2, 4 and 7 Figure 11). All photosynthetic competent transformants carrying the *CCS4*, *CCS4-2HA* or *CCS4-3HA* genes accumulate similar levels if cytochrome *f*, confirming the presence of the tag does not disturb the CCS4 function for cytochrome *c* maturation. As expected, transformants that did not carry the introduced *CCS4* and were not restored for photosynthetic growth were showing residual amount of cytochrome *f*, similar to the *ccs4* mutant [14]. If tagged CCS4 is functional, the CCS4-HA protein must be produced. However, a first attempt to detect the CCS4 protein (expected size 15-17 kDa) using anti-HA antibody failed (Figure 11).

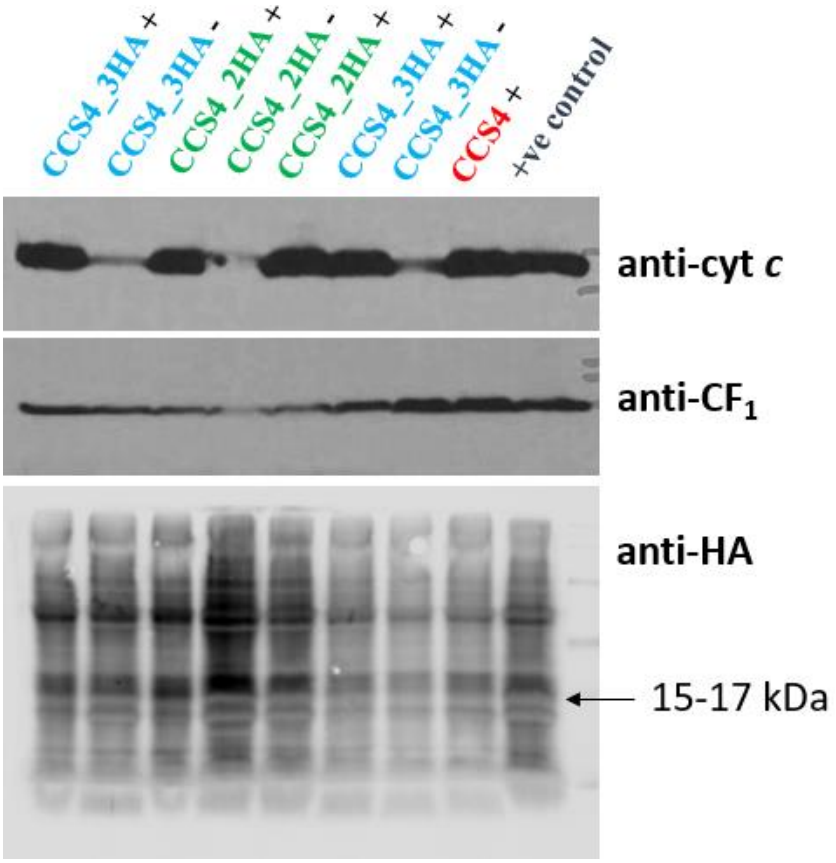

Figure 11: Immunoblot detection of CCS4-HA and cytochrome f. *Transformants that were screened positive by diagnostic PCR are labeled with "+", transformants that were screened negative by diagnostic PCR are labeled with "-". The +ve control shows the same amount of cytochrome f as a WT. CF_1, a protein part of the ATP synthase complex, is used as a loading control. The image was taken after immunodetection with Pierce™ ECL Western Blotting Substrate using X-Ray Film for Chemiluminescence Western Blot.*

3.2 Creating *a ccda*-null mutant through CRISPR/Cas9 via insertion of an antibiotic resistance cassette

In vivo CRISPR/Cas9-mediated knock-in of the *CCDA* gene was attempted and two guide RNAs were designed for this purpose. The efficiency of the guide RNAs was first tested *in vitro* using an amplicon from the *CCDA* gene carrying the cut site.

3.2.1 CRISPR/Cas9 *in vitro* reaction

The general PCR conditions for amplification of the *CCDA* gene are described in Materials and Methods (Table 3). Two pairs of primers (Table 10) were tested to amplify the target DNA. Despite several attempts including use of different templates (genomic DNA or *CCDA* carrying plasmids) and gradient PCR, we failed to amplify the expected size amplicons. Pair 1 produced preferentially an amplicon of 500 bp (instead of 750 bp, Figure 12) even when the template was a plasmid containing the cloned *CCDA* gene (Figure 13).

Table 10: 3 primers and 2 pairs with expected fragment sizes for possible amplification of CCDA.

Pair	Primers	Expected fragment size
1	CCDA-Fwd1 CCDA-Rev1	744 bp
2	CCDA-Fwd1 CCDA-RV1	770 bp

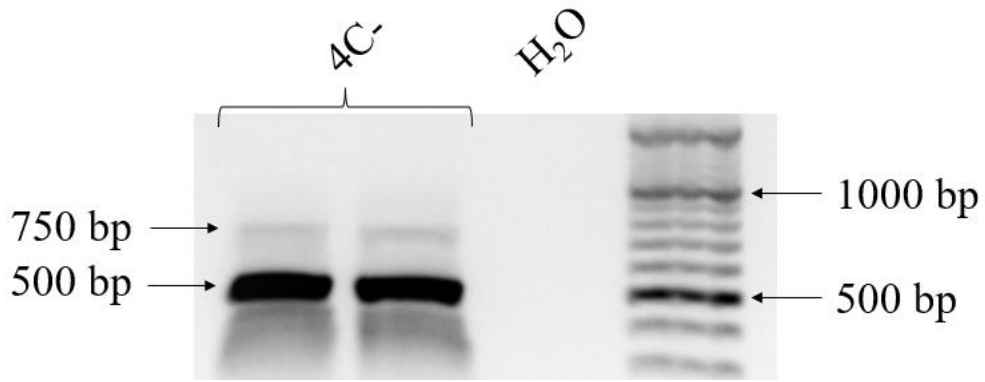

Figure 12: Amplification of CCDA gene from 4C- genomic DNA. *Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

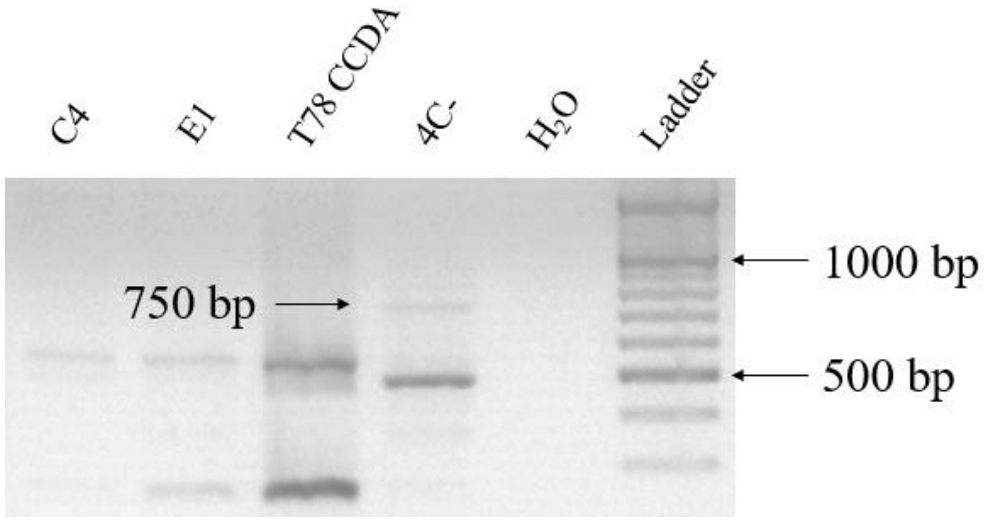

Figure 13: Amplification of CCDA gene from C4, E1, T78 CCDA plasmids and 4C- genomic DNA. *Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

Because the 500 bp amplicon was obtained using both genomic DNA and cloned *CCDA* as templates, we opted to test it in the *in vitro* reaction. The purified amplicon was tested for the *in vitro* Cas9-mediated cleavage using two guide RNAs (Table 8 and Figure 14). While the analysis of the reaction suggests cleavage of the target DNA by Cas9, it is also possible the change in electrophoretic mobility is due to the large amount of gRNA. This experiment was

reproduced by using RNAse to digest the excess gRNA prior to the analysis of the *in vitro* reaction on the gel.

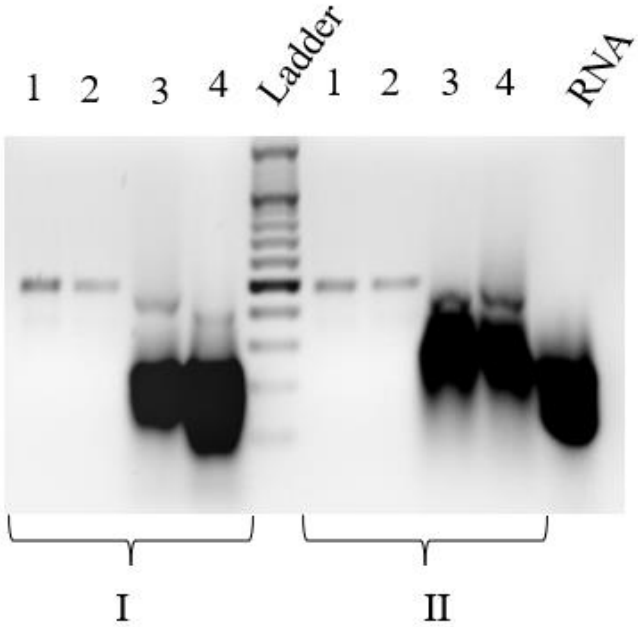

Figure 14: In vitro CRISPR/Cas9 on PCR amplified CCDA from 4C- incubated at 37 °C overnight. *I and II defines the two gRNAs used. Different reaction conditions 1-4 from Table 8. The products of the in vitro CRISPR/Cas9 reaction were analyzed via electrophoresis in 1 % agarose gel for 60 minutes at 130 V. The gel was subsequently imaged. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

Analysis of this second *in vitro* reaction also suggests cleavage of the target DNA by Cas9 (Figure 15). Note that the smear is probably due to residual gRNA bound to Cas9 (and protected from the RNAse treatment). In this experiment no proteinase K was added before analysis of the *in vitro* reaction.

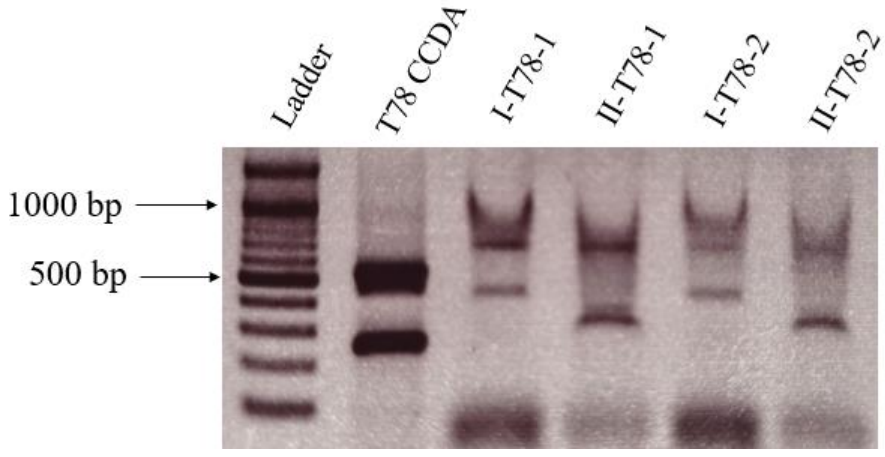

Figure 15: In vitro CRISPR/Cas9 on PCR amplified CCDA from T78 CCDA incubated at 37 °C overnight. *Lane 2 shows uncut CCDA template amplified from the T78 CCDA plasmid. Protocol for the reaction in lane 3-6 shown in Table 8, Lane No 3 with a lower concentration of sgRNA. I and II defines the two gRNAs used. T78-1 and -2 are two repeats of the same reaction. The products of the in vitro CRISPR/Cas9 reaction were analyzed via electrophoresis in 1 % agarose gel (60 minutes at 130 V) which was subsequently imaged. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

3.2.4 Amplification of antibiotic cassette for *CCDA* knock-in.

The HygB cassette was generated using the plasmid pHyg3 as a template with primers (Table 4). The primer pairs used to amplify the cassette (Table 11) contain 20 bp annealing to the cassette and 50 bp complementary to the *CCDA* gene. Different concentrations of the primers were used for both primer pairs but only one primer pair (gRNA1-HDR-F1/gRNA1-HDR-R1) was successful in creating an amplicon (1.8 kb, Figure 16). However, despite several modifications of the PCR protocol, the reaction was not always reproducible, and the yield of the amplicon remained low.

Table 11: 2 primers and pairs with expected fragment sizes for amplification of the antibiotic cassette, HygB.

Pair	Primers	Expected fragment size
1	gRNA1-HDR-F1 gRNA1-HDR-R1	1847 bp
2	gRNA2-HDR-F2 gRNA2-HDR-R2	1847 bp

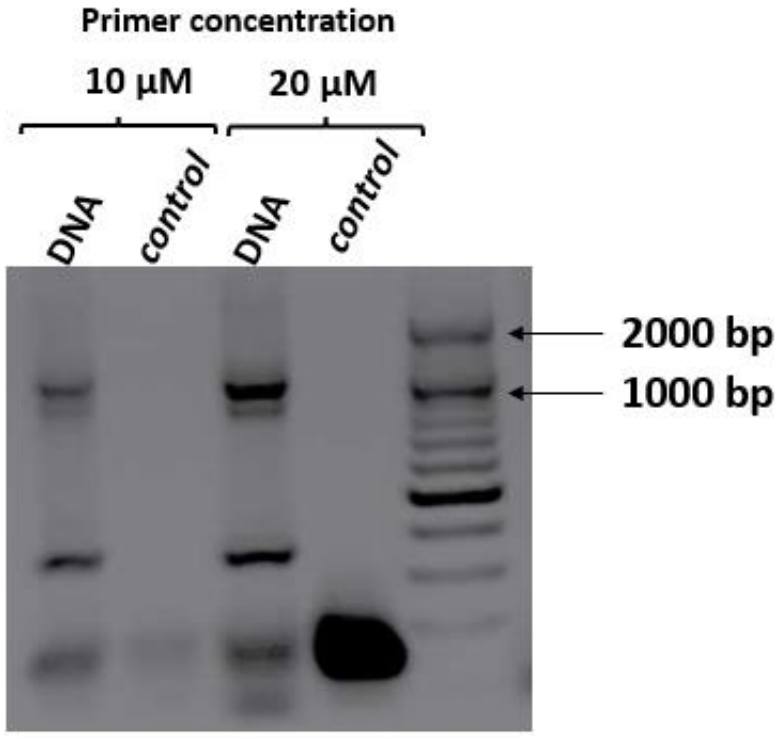

***Figure 16: Amplification of antibiotic cassette, HygB**. The image shows amplification of antibiotic cassette with concentrations 10 µM and 20 µM of primer pair 1 (Table 11). Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. The ladder used is O'RangeRuler 100 bp DNA Ladder from ThermoFisher Scientific.*

To increase the yield of the expected amplicon, a second amplification of the HygB cassette was performed in two steps via nested PCR. Primer pair Nested_F1/Nested_R1 (Table 4) with respectively 19 and 17 bps region homologous to *CCDA* (Figure 17) was used for the first amplification (Figure 18). The amplicons were purified and used as template for the second amplification. Primer pair Nested_F2/Nested_R2 (Table 4) with 50 bps region homologous to *CCDA* (Figure 17) was used for the second amplification of the HygB cassette (Figure 18). The reaction was reproducible, and the yield of the amplicon suitable the *in vitro* reaction. Indeed, published reports recommend the use of 5 µg for the insertional marker to perform the Cas9-mediated knock-in in *C. reinhardtii*.

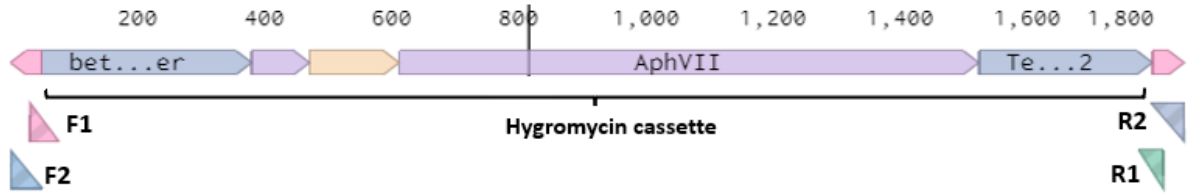

Figure 17: Hygromycin cassette *with primer pair F1/R1 used for the first amplification, and primer pair F2/R2 used for the second amplification.*

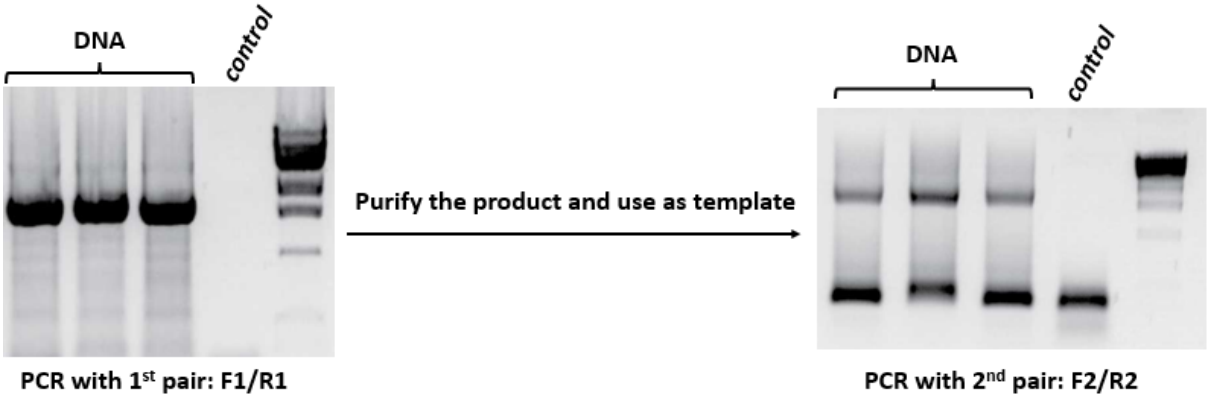

Figure 18: Amplification of the HygB cassette by PCR *performed in two sets with primer pairs F1/R1 and F2/R2. Amplicons were resolved via electrophoresis in 1 % agarose gel for 25 minutes at 130 V which was imaged. H_2O indicates negative control. There are three independent reactions shown for each reaction. The ladder used is λBstEII.*

4. Discussion

In this study, *C. reinhardtii* strains producing a tagged version of the CCS4 protein with 2 or 3 copies of the HA tag were generated and analyzed molecularly and phenotypically. The strains cannot be distinguished from transformants carrying the untagged CCS4 gene with respect to photosynthetic growth and accumulation of plastid cytochrome *c*. We concluded the tag did not disrupt the function of the CCS4 protein and the strains can be used for immunoprecipitation in order to discover interacting partners. The identity of the partners to be discovered might inform about the biochemical function of CCS4.

In this work, we also attempted to generate a CRISPR/Cas9-mediated knock-in mutant in the *CCDA* gene as the only *ccda* mutant available to study plastid cytochrome *c* assembly is in the land plant *A. thaliana*. Preliminary results indicate that *in vitro*, the selected guide RNAs direct the Cas9 dependent cleavage of the expected target in CCDA but the results need to be further corroborated. Generating the insertional cassette for the *CCDA* knock-in is in progress and a two-step PCR (nested PCR) appears a promising approach to produce a high yield of the expected amplicon for the Cas9-dependent *in vivo* editing reaction.

6. Bibliography

1. Engel, B. D. *et al.* Native architecture of the Chlamydomonas chloroplast revealed by in situ cryo-electron tomography. *eLife* **4**, e04889 (2015).
2. Sasso, S., Stibor, H., Mittag, M. & Grossman, A. R. From molecular manipulation of domesticated Chlamydomonas reinhardtii to survival in nature. *eLife* **7**, e39233.
3. Scranton, M. A., Ostrand, J. T., Fields, F. J. & Mayfield, S. P. Chlamydomonas as a model for biofuels and bio-products production. *Plant J* **82**, 523–531 (2015).
4. Radakovits, R., Jinkerson, R. E., Darzins, A. & Posewitz, M. C. Genetic Engineering of Algae for Enhanced Biofuel Production. *Eukaryot Cell* **9**, 486–501 (2010).
5. Grama, S. B., Liu, Z. & Li, J. Emerging Trends in Genetic Engineering of Microalgae for Commercial Applications. *Mar Drugs* **20**, 285 (2022).
6. Cooper, G. M. Photosynthesis. *The Cell: A Molecular Approach. 2nd edition* (2000).
7. Cooper, G. M. Figure 10.22, Electron transport and ATP synthesis during photosynthesis. https://www.ncbi.nlm.nih.gov/books/NBK9861/figure/A1672/ (2000).
8. Malone, L. A., Proctor, M. S., Hitchcock, A., Hunter, C. N. & Johnson, M. P. Cytochrome b6f – Orchestrator of photosynthetic electron transfer. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1862**, 148380 (2021).
9. Gabilly, S. T. & Hamel, P. P. Maturation of Plastid c-type Cytochromes. *Front Plant Sci* **8**, 1313 (2017).
10. Das, A. & Hamel, P. P. Bioenergetics Theory and Components | Cytochrome c Assembly. in *Encyclopedia of Biological Chemistry III (Third Edition)* (ed. Jez, J.) 94–107 (Elsevier, 2021). doi:10.1016/B978-0-12-819460-7.00219-X.
11. Karamoko, M., Gabilly, S. & Hamel, P. Operation of trans-thylakoid thiol-metabolizing pathways in photosynthesis. *Frontiers in Plant Science* **4**, (2013).

12. Page, M. L. D. *et al.* A Homolog of Prokaryotic Thiol Disulfide Transporter CcdA Is Required for the Assembly of the Cytochrome b6f Complex in Arabidopsis Chloroplasts*. *Journal of Biological Chemistry* **279**, 32474–32482 (2004).
13. Gabilly, S. T. *et al.* CCS5, a Thioredoxin-like Protein Involved in the Assembly of Plastid c-Type Cytochromes *. *Journal of Biological Chemistry* **285**, 29738–29749 (2010).
14. Gabilly, S. T. *et al.* A novel component of the disulfide-reducing pathway required for cytochrome c assembly in plastids. *Genetics* **187**, 793–802 (2011).
15. Lennartz, K., Bossmann, S., Westhoff, P., Bechtold, N. & Meierhoff, K. HCF153, a novel nuclear-encoded factor necessary during a post-translational step in biogenesis of the cytochrome b6f complex. *The Plant Journal* **45**, 101–112 (2006).
16. Barrangou, R. *et al.* CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* **315**, 1709–1712 (2007).
17. Bolotin, A., Quinquis, B., Sorokin, A. & Ehrlich, S. D. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**, 2551–2561.
18. What is the PAM Sequence in CRISPR Experiments, and Why is it Important? *Synthego* https://www.synthego.com/guide/how-to-use-crispr/pam-sequence.
19. What are genome editing and CRISPR-Cas9?: MedlinePlus Genetics. https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/.
20. Dhokane, D., Bhadra, B. & Dasgupta, S. CRISPR based targeted genome editing of Chlamydomonas reinhardtii using programmed Cas9-gRNA ribonucleoprotein. *Mol Biol Rep* **47**, 8747–8755 (2020).
21. Del Sal, G., Manfioletti, G. & Schneider, C. A one-tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic Acids Res* **16**, 9878 (1988).

22. Howe, G. & Merchant, S. Heavy Metal-Activated Synthesis of Peptides in Chlamydomonas reinhardtii. *Plant Physiol* **98**, 127–136 (1992).
23. Motohashi, K. & Hisabori, T. CcdA Is a Thylakoid Membrane Protein Required for the Transfer of Reducing Equivalents from Stroma to Thylakoid Lumen in the Higher Plant Chloroplast. *Antioxidants & Redox Signaling* **13**, 1169–1176 (2010).
24. Cloud-based platform for biotech R&D | Benchling. https://www.benchling.com/?utm_campaign=SO-Brand&utm_medium=cpc&utm_source=google&utm_term=benchling&gclid=CjwKCAjwkYGVBhArEiwA4sZLuMnTzII40pRJ7YAohJibhydvEOB4R3DxVs-IOXCs1EpJ_Ye6lDxzmhoCbtcQAvD_BwE.
25. Pollock, S. V., Prout, D. L., Godfrey, A. C., Lemaire, S. D. & Moroney, J. V. The Chlamydomonas reinhardtii proteins Ccp1 and Ccp2 are required for long-term growth, but are not necessary for efficient photosynthesis, in a low-CO2 environment. *Plant Mol Biol* **56**, 125–132 (2004).
26. Fischer, N. & Rochaix, J. D. The flanking regions of PsaD drive efficient gene expression in the nucleus of the green alga Chlamydomonas reinhardtii. *Mol Genet Genomics* **265**, 888–894 (2001).