UNIVERSITY OF CALIFORNIA SAN DIEGO

Optimization of *in vivo* Cre/*lox*-Mediated Recombination in *Chlamydomonas reinhardtii* for Selectable Marker Free Recombinant Protein Production

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2021

The thesis of Chau Hoang Le is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

This thesis is dedicated to my parents.

Thank you for your endless love and support.

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ACKNOWLEDGEMENTS

Firstly, I would like to portray my gratitude to my family who have supported me through all the ups and downs of these wild pandemic years and help me remember I am loved and worthy. L7 for life <3

Thank you to my parents who inspire me to do my best and find my own happiness!

I would also like to acknowledge Dr. Anthony Berndt for his invaluable guidance and encouragement throughout the entirety of my time in the Mayfield lab. He was there to help every step of the way. I would not have accomplished any of this without his scientific brilliance and gracious mentorship!

ABSTRACT OF THE THESIS

Optimization of *in vivo* Cre/*lox*-Mediated Recombination in *Chlamydomonas reinhardtii* for Selectable Marker Free Recombinant Protein Production

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Chau Hoang Le

Master of Science in Biology

University of California San Diego, 2021

Professor Stephen Mayfield, Chair

The Cre/*lox*-mediated recombination system from the bacteriophage P1 is a fundamental genetic tool for the study of cell biology and genetics, as it allows for easy site-directed manipulation of genomes. Application of this system in the green unicellular model algae organism *Chlamydomonas reinhardtii* has not been sufficiently explored or applied *in vivo*. Establishing an efficient method of Cre/*lox*-mediated recombination will transform the way this organism can be

studied and utilized as a bio-factory. A new reporter vector, pLox, was designed to easily screen for both cells with high protein expression—via mClover green fluorescent protein (GFP) detection before Cre recombinase activity—and cells with successful GFP deletion—via Oxyfluorfen herbicide resistance activation after Cre recombinase activity. Experiments were then designed to test if Cre/*lox* recombination is possible *in vivo* in *C. reinhardtii* using two approaches: 1) electroporating in exogenous Cre recombinase and 2) expressing Cre recombinase endogenously. In this thesis, the degrees to which the preliminary experiments and methods showed promise were documented. The design of pLox allows for straightforward verification of deletion, both through observing phenotypic change and PCR genotyping. Commercially supplied Cre recombinase was efficacious in recombining pLox *in vitro*, but our methods showed no positive results *in vivo*. However, one method of stably integrating our Cre recombinase expression cassette shows definite Cre excision, verified by genotyping and sequencing. In conclusion, *in-vivo* Cre/*lox*-mediated recombination in *C. reinhardtii* using our methods is promising, but Cre recombinase delivery must be optimized for efficiency and efficacy.

INTRODUCTION

Cheese, bread, beer, and wine are all goods produced using live organisms, in a process known as biomanufacturing (Rasala & Mayfield, 2015). Historically, food products were the first biomanufacturing products, but as biotechnology has advanced, biomanufacturing has evolved to include the production of recombinant proteins, nucleic acids, and other small molecules, otherwise known as biopharmaceuticals (Kesik-Brodacka, 2018). Those biopharmaceuticals are presently produced by many organisms, or bio-factories, including bacteria, yeast, plants, cell lines, transgenic animals, and algae (Kesik-Brodacka, 2018). However, bottlenecks in scaling up production with certain organisms exist, such as in the purification of toxic byproducts, difficulties in genetic manipulation, slow growth rates, and common pathogens, which makes production in some organisms expensive and inefficient (Rosales-Mendoza et al, 2020).

Green algae have recently been introduced as a bio-factory for pharmaceuticals and metabolic products (Scaife et al, 2015). Algae can recombinantly produce bioactive antibodies, proteins, and enzymes; it is also standard for researchers to manipulate the algal metabolism to generate varying ratios of beneficial macromolecules—lipids, proteins, and carbohydrates—because algae are unicellular and reproduce quickly and densely (Rasala & Mayfield, 2015). Algal recombinant products are more likely to be bioactive in humans because algae are eukaryotic and can carry out post translational modifications of proteins, such as phosphorylation and glycosylation (Dehghani et al, 2020). Though algae are eukaryotic, or similar enough to humans to make effective therapeutics, they are not similar enough to mammals to possess common pathogens (Dehghani et al, 2020). Furthermore, many species of algae do not generate toxic products, so they are deemed edible and can be used as an oral medium for vaccines and therapeutics (Rosales-Mendoza et al, 2020). Additionally, green algae are environmentally

sustainable (Scaife et al, 2015). In contrast to animals, yeast, and bacteria, green algae use photosynthesis to make products, meaning they do not require incubators or constant replenishment of expensive media; they are easily grown to high titers in bioreactors, outdoor ponds, and even in non-potable water (Gimpel et al, 2015). Fostering algal biological development will lead to an even more cost-effective and efficient bio-factory of novel therapeutics.

The green, unicellular microalgae *Chlamydomonas reinhardtii* is one of the best studied model organisms for biopharmaceutical production (Scranton et al, 2015). It is relatively simple to genetically manipulate its multiple compartments, including the chloroplast, mitochondria, and nucleus, which each have their own genome. *C. reinhardtii* is relatively fast growing compared to mammalian and plant cells, as well. It can survive high throughput selection processes using fluorescent markers and cell sorters (Scranton et al, 2015), grow in harsh conditions, and can carry out traditional sexual reproduction, which has many advantages toward biological research (Heitzer & Zschoernig, 2007).

Though *C. reinhardtii* is well studied, more genetic tools must be developed to ease biopharmaceutical production pipelines. Many marker genes can be applied toward developing high expression of products. For example, a fluorescent reporter gene can link with the recombinant gene of interest so that expression levels can be easily detected using techniques such as flow cytometry, fluorescent microscopy, and fluorescent plate reading. High expressors can then be manually sorted out or with fluorescence activated cell sorting (FACS). Antibiotic resistance cassettes are also commonly utilized to ensure the presence of transgenes in the genome, as they sort by survival. After high expressors are identified using the aforementioned methods, they can be isolated and grown for further experiments and potential pharmacological

uses. A common issue in recombinant protein production arises when unwanted marker genes need to be removed, due to biosafety concerns. Before those strains are to be used at commercial scale, the selectable marker genes must be removed in a manner that retains the integrity of the genome. As many uncontrollable genetic and/or environmental factors compound to generate a desired high expressing algal line, a favorable course of action for selectable marker removal while maintaining consistent expression levels is to integrate an *in vivo* site-directed deletion mechanism.

One such internal deletion method that has been widely applied in many organisms, ranging from multicellular to unicellular, is the Cre/*lox* recombination system. The Cre/*lox* system originated from the bacteriophage P1, and it has broad applications toward biotechnology and basic biological research (Zhang *et al*, 2003). This genetic tool can perform a variety of different manipulations, insertions, deletions, and translocations, as shown in Figure 1, and does not require any other co-factors to work, just Cre recombinase enzyme and specific *loxP* sites (Choi et al, 2000). The Cre/*lox* system's modalities for site specific recombination depends on the position, orientation, and sequence of the 34 base-pair (bp) *loxP* sites on the DNA. The *loxP* sites are placed flanking the gene or region of interest in different configurations conditional to the intended manipulation. Cre recombinase, a 38-kDa protein, works by dimerizing at a *loxP* site, tetramerizing and binding a second *loxP* site, performing a double-stranded cut, then ligating the DNA in its new position (Grindley et al, 2006). Other, more advanced uses of the *Cre/loxP* system include inducible recombination (drug, heat-shock, photoactivatable) (Allen et al, 2019), combining with other site-specific recombination systems such as FLP-FRT and CRISPR-Cas9 (Li et al, 2020), and recombinase-mediated cassette exchange (Turan et al, 2013).

If the Cre/*lox* system can be applied in *C. reinhardtii*, it will be an important tool for the removal of fluorescent or other selective markers *in vivo* for biopharmaceutical production.

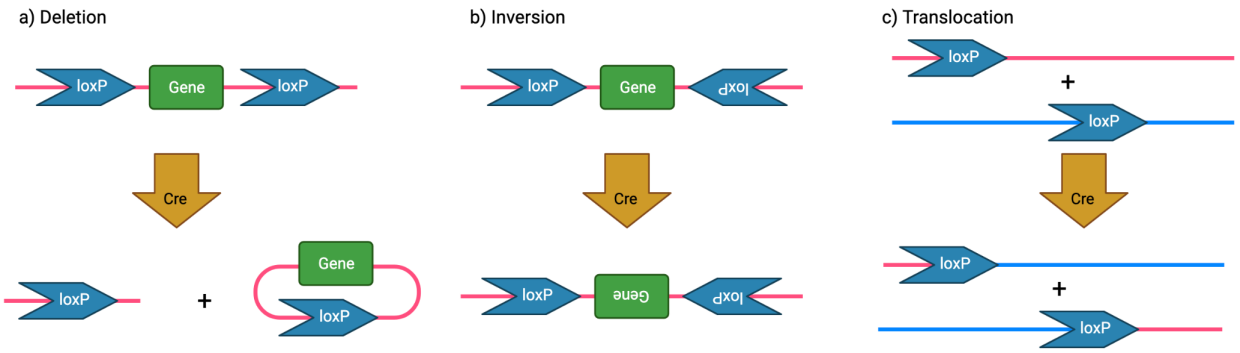

Figure 1. Basic Cre/lox mediated recombination gene manipulations.
A) When the gene of interest is flanked by two identical *loxP* sites facing the same direction, Cre recombinase excises a circular segment of DNA containing one *loxP* site and the gene of interest. B) When the gene of interest is flanked by two *loxP* sites in reverse of each other, Cre recombinase excises the gene and re-ligates it in the opposite direction. C) When there are two *loxP* sites on different DNA molecules, Cre recombinase translocates the segments across the two molecules. (Created with BioRender.com)

There is only one example in the literature of an *in vivo* experiment with Cre/*lox* in *C. reinhardtii.* Kasai and Harayama (2015) showed that Cre/*lox* recombination could potentially occur *in vivo* by testing for paromomycin antibiotic sensitivity. A recombinant algae strain was generated with *loxP* sites flanking a Paromomycin resistance cassette, then a Cre encoding vector was transformed into that strain. The researchers detected Cre activity with Paromomycin^{R} excision, verified by Paramomycin sensitivity, PCR, and sequencing. However, Cre recombination was shown to be inefficient, and the strains that carried out Cre/*lox* recombination were not released for public use. Furthermore, their methods of selection using antibiotic sensitivity and Southern blotting are tedious to replicate. For our lab's purposes, we intend to improve upon the

design (Figure 2) by implementing a selective marker in the reporter vector that allows for further downstream selection, green fluorescent protein (GFP) fluorescence, and simplify the process of confirmation of the transgene's presence. Furthermore, to verify excision more absolutely and to select for potentially very rare events, a frame shift to an Oxyfluorfen herbicide resistance gene that allows for selection by survival was added. Though Kasai and Harayama show there is promise in the application of Cre/*lox* in *C. reinhardtii,* a new experimental design may optimize Cre/*lox* deletion, ease downstream selection of positive clones, and allow for more widespread use of this tool by publicly releasing our algal strains.

To develop a more robust marker removal system using Cre/*lox*, we first designed a new *loxP* reporter vector, pLox. The pLox reporter was designed to be able to answer many questions about Cre/*lox* recombination in *C. reinhardtii*. To facilitate verification of Cre-mediated deletion, pLox contains GFP flanked by *loxP* sites, followed by a stop codon, then followed by an Oxyfluorfen (herbicide) resistance gene. Without Cre recombinase activity, GFP should fluoresce in the flow cytometer or plate reader and be Oxyfluorfen sensitive; after Cre recombinase deletion, there should be no GFP fluorescence, and the cells will be Oxyfluorfen resistant. Using GFP for selection is an efficient and common practice (Lam et al, 2012), and incorporating herbicide survival will select for rare deletion events. The survivors are much more likely to be positive for Cre recombinase-mediated deletion and can easily be picked for analysis. The placement of the Oxyfluorfen resistance cassette also mimics that of a recombinant therapeutic gene, demonstrating the effects of Cre/*lox* recombination on recombinant protein expression.

Besides designing a new reporter vector, we tested two methods of introducing Cre recombinase to the *C. reinhardtii* genome. The first method is to exogenously introduce Cre recombinase enzyme by direct electroporation of the purified protein into the nucleus of the pLox

line. The second method is to construct a Cre recombinase expression vector, pCre, and transiently express it, delivering Cre protein endogenously. In the end, we found that the pLox reporter line is very effective at demonstrating the presence of Cre activity, and *C. reinhardtii* can carry out Cre recombinase-mediated deletion by endogenously expressing pCre. Moving forward, honing the Cre delivery methods to achieve robust Cre-mediated recombination and ensure complete gene excision are the next steps toward establishing this system in *C. reinhardtii*.

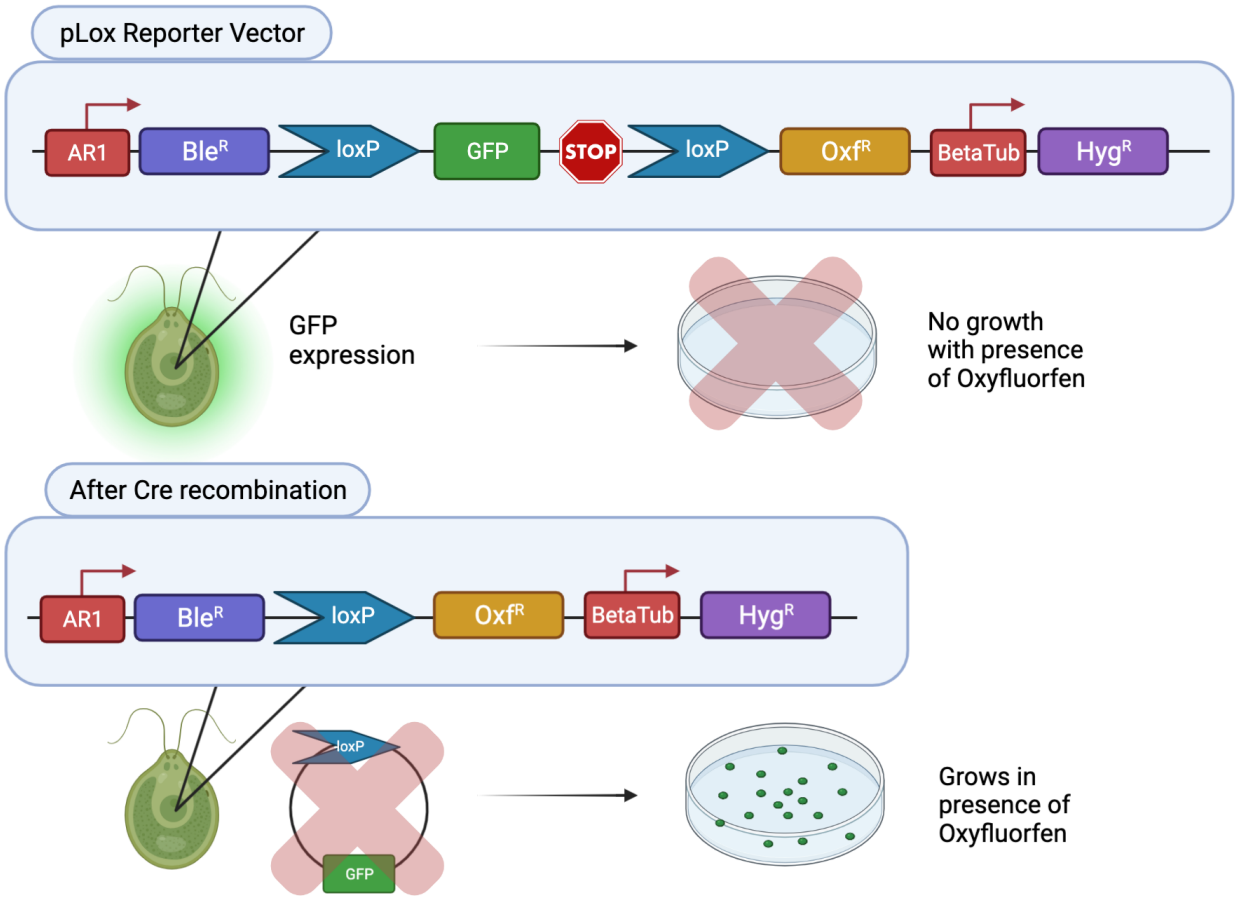

Figure 2. Schematic of expected results of Cre recombinase delivery experiments using pLox in C. reinhardtii.

C. reinhardtii cells transformed with pLox should be GFP positive and Oxyfluorfen sensitive. In the presence of Cre recombinase, either exogenous or endogenous, GFP should be excised and lost as an episomal element, making the cells GFP negative, but Oxyfluorfen resistant. (Created with BioRender.com)

MATERIALS AND METHODS

Strain and Culture Conditions

Throughout all experiments, *Chlamydomonas reinhardtii* strain CC124 was used. Cells grown in culture were in standard TAP media (Gorman 1965). Cells were kept under 24-hour light conditions at a photon flux of 125 μE/m^2/sec at ~22-25 ºC on shaker tables rotating at 110 RPM.

Design and Assembly of Reporter Vector, pLox, and Simulated Deletion Vector, pLOR

The *loxP* sequence (ataacttcgtatagcatacattatacgaagttat) from the bacteriophage P1 was retrieved and incorporated into primers for synthesis (Hoess et al, 1982). All vectors consist of a semi-synthetic AR1 promoter (Rasala et al, 2012) driving a Bleomycin resistance cassette expressing Zeocin antibiotic resistance gene (DQ000653.1). The mClover GFP gene (KM061067.2) in the reporter vector was obtained from previously synthesized plasmids in the lab. The GFP cassette was followed by a STOP codon and flanked by two direct repeats of *loxP* sites (floxxed). To easily select for clones that successfully carried out a Cre-recombinase mediated deletion, the test vector included an Oxyfluorfen herbicide resistant gene downstream of the second *loxP* site. *C. reinhardtii* Oxyfluorfen resistance (OxR) cassette was obtained from Bruggeman et al, 2014 (Supplemental File 1). Two base pairs were added at the 5' end of the Oxyfluorfen gene to keep it in frame after deletion. The hygromycin resistance gene (FR845719.1) driven by a C.re Beta2-tublin promoter (MK357711.1) succeeds the OxR gene to ensure that the whole transgene is present using double antibiotic selection Zeocin and Hygromycin agar plates. This vector was inserted into an *E. Coli* circular backbone and the full sequence can be found in supplemental files 2 and 3.

A simulated deletion cassette, pLOR, was also designed and constructed as a positive control for the assay. If a Cre-recombinase mediated deletion occurred, the gene would be expected to look like pLOR. All components of pLox are present, except for the GFP gene and the 3' end *loxP* site (Figure 3).

Standard PCR for DNA fragments were carried out using NEB Q5® Hot Start High-Fidelity DNA Polymerase and Gibson assembled using the NEBuilder® HiFi DNA Assembly kit (New England Biolabs, Ipswich, Massachusetts). The vector design schematic can be found in Figure 3 and full sequences in the supplemental material.

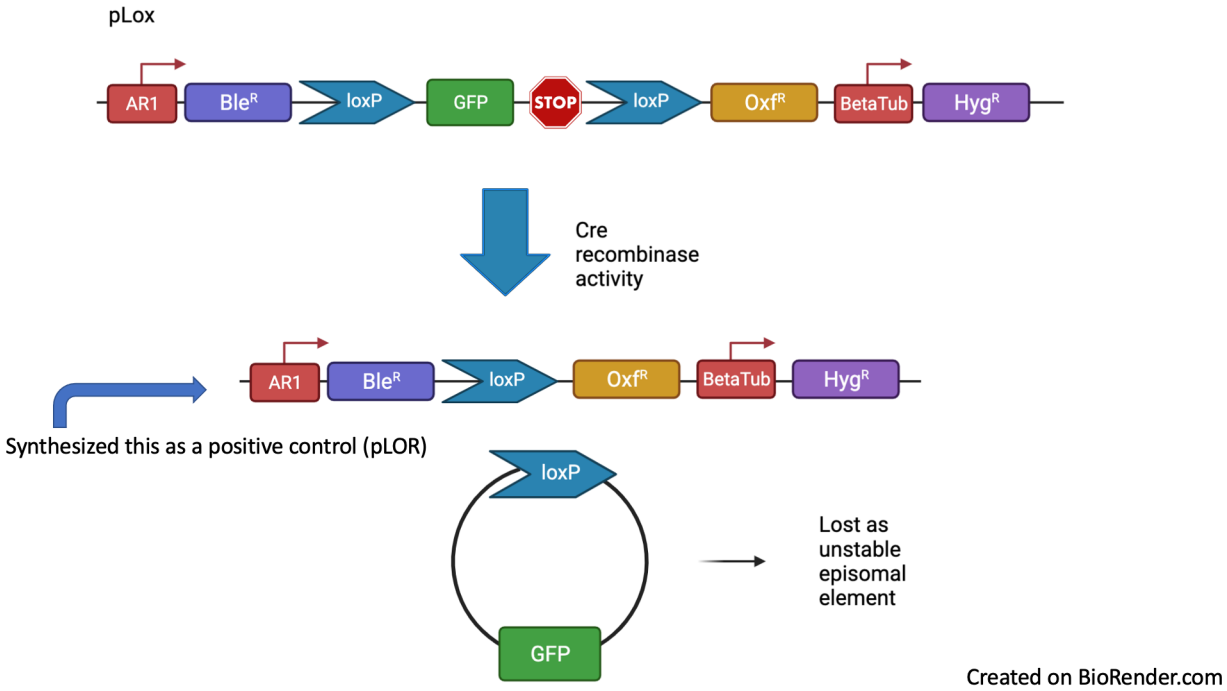

Figure 3. Schematic of expected recombinase activity showcasing pLox reporter vector and pLOR vector designs.

The reporter vector, pLox, was designed as such. Upon Cre recombination, the expected vector would look like pLOR, which was also synthetically generated to use as a positive control.

Design and Assembly of Cre Recombinase Expression Vector, pCre

The Cre recombinase sequence was obtained from the NCBI (NC_005856.1). This sequence was then codon-optimized to *C. reinhardtii* using https://cool.syncti.org/. GC content was set as 63%. A top 400 gene coding regions dataset for *C. reinhardtii* (Ngan et al, 2015) was uploaded as the custom codon usage pattern values. The "constraint" function was used to exclude KpnI, XbaI, XhoI, restriction enzyme sequences and the motif AMMATG, which is the IUPAC degeneracy code for an ideal *C. reinhardtii* Kozak-like sequence. "Enable repeated motif removal" was toggled on, with a length of 9 and minimum number of instances before removal of 2. The best candidate of sequences was submitted to Integrated DNA Technologies, Inc. for gBlock synthesis.

PCR for pCre fragments were carried out using New England Biolabs NEB Q5® Hot Start High-Fidelity DNA Polymerase and assembled using the NEBuilder® HiFi DNA Assembly kit. The vector design can be found in Figure 4 and full sequences in supplemental file 4. The pCre plasmid consists of an AR1 promoter driving a Ble resistance cassette fused to a blue fluorescent protein gene (mTagBFP) from previously published pBR9 (Rasala et al, 2013), followed by a foot and mouth disease 2A ribosomal-skip motif so that the proceeding Cre-recombinase gene is synthesized as a single protein (Rasala et al, 2012), SV40 nuclear localization signal (PKKKRKV) to ensure Cre's import into the nucleus (Guilfoyle & Hagen, 2012), FLAG tag sequence for detection using Western Blotting (Suzuki & Nakayama, 2011), the codon-optimized Cre sequence, and a rbcs2 3' UTR. Proceeding the Cre gene is a APHVIII paromomycin resistance gene (CP045803.1) driven by a C.re Beta2-tubulin promoter (MK357711.1) was placed at the 3' end to ensure an intact transgene when transformants are selected on Zeocin and Paromomycin plates. Paromomycin resistance was chosen because it is

orthogonal to Hygromycin (in pLox) and strains containing pCre can be isolated down the line. This vector was inserted into the same *E. Coli* backbone as pLox.

Figure 4. Diagram of pCre, Cre expression vector.
pCre was designed as such, to be able to resist Zeocin and Hygromycin antibiotics to ensure the presence of the whole transgene when plated on such conditions. TagBFP and a FLAG tag were also included for easy detection of Cre expression using fluorescent detection methods or Western blotting. A nuclear localization signal was also placed on the N terminal to ensure Cre's presence in the nucleus once expressed. (Created using BioRender.com)

Preparing Assembled DNA for Algae Transformation

To scale up the DNA, all vectors, pLox, pLOR, pCre, and pOxR2 were each transformed into NEB® 5-alpha Competent *E. coli* cells using NEB's protocol. The cells were plated onto Luria Broth (LB) and 100 ampicillin µg/ml (Thermo-Scientific) plates and grown at 37°C overnight until single colonies appeared. Individual colonies were grown up in LB and 100 µg/ml carbenicillin (Thermo-Scientific) liquid cultures overnight. The DNA was extracted and purified using the Monarch® Plasmid Miniprep Kit. The junctions of the Gibson Assembly were sequenced by Eton Bioscience, Inc. using the Sanger Method. After sequence confirming, the correct clones were struck out on LB and ampicillin plates and grown overnight at 37°C to isolate into individual colonies. A loop was used to inoculate from warm plates into 60 mL of LB and 100 µg/ml carbenicillin in sterile 250 mL Erlenmeyer flasks and grown in a shaker at 37°C for 16 hours. The QIAGEN Plasmid Midi Kit was used to extract plasmids using manufacturer's

instructions. The plasmids were purified by standard Isopropanol/Ethanol precipitation methods. The full sequences were confirmed by primer-walk. XbaI and KpnI-HF restriction enzymes were used to linearize the plasmids at 37°C in NEB Cutsmart buffer with enough units of enzyme to generate ~5 fold over digest in two hours (New England Biolabs, Ipswich, MA). Standard NaCl/Isopropanol precipitation methods were used to partially purify the DNA, then washed with 70% ethanol three times, and then resuspended in 1 mM Tris-HCl, 0.1 mM EDTA overnight at 4°C.

Electroporation Methods for Cre Recombinase and Vectors

All transformations of Cre enzyme and DNA vectors were carried out using the following methods. *C. reinhardti* strain CC124 was growth in standard TAP media to approximately $3x10^6$ cells/mL (near saturation). This starter culture was diluted back to 3×10^5 cells/ml of TAP media about 24 hours before transformation. The day of transformation, cells, at a density of 0.5-1 $\times 10^6$ cells/ml were harvested by centrifugation at 3000 rcf for 5 minutes at 15°C in sterile disposable conical bottom centrifuge bottles. The supernatant was discarded, and cells were resuspended in GeneArt® MAX Efficiency® Transformation Reagent for Algae (Thermo Scientific, Waltham, MA) and prepared in line with manufacturer's instructions. 2 - 3 ug of linearized vector DNA of each construct were electroporated using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA) in a 4 mm cuvette (Genessee Scientific). For the Cre recombinase enzyme electroporation, 1, 2, 4, and 8 µl of enzyme were electroporated in. Cells recovered for 24 hours in 40 mM sucrose in TAP media. After recovery, the cells were centrifuged at 4000 rcf for 5 minutes at 16°C, the supernatant was discarded, and the cells were resuspended in fresh TAP media. Depending on the experiment, these cells were either plated using 200 µl of cells on

each plate under light conditions at a photon flux of 75-80 μE/m^2/sec for Oxyfluorfen plates, 20-30 μE/m^2/sec for all other agar plates, or transferred to shake flasks with TAP media to continue growing. The plates in each experiment were all TAP+15 g/L agar media unless they had antibiotic or herbicide selection. The concentrations of those selective agents are as followed: 15 μg/ml Zeocin, 30 μg/ml Hygromycin B, 30 μg/mL Paromomycin (Thermo Scientific, Waltham, MA), and 1.7 μM Oxyfluorfen (Sigma-Aldrich).

Genotyping Candidate Colonies

For all experiments, colonies that survived on Oxyfluorfen plates were isolated and genomic DNA was extracted using the Chelex 100 boil method (HwangBo et al, 2010). Multiplex PCR primers (Table 1) were designed to show a clear visual difference between clones that did and did not undergo Cre-recombinase mediated deletion. These primers were used to amplify off the genomic extractions using Q5 DNA polymerase (New England Biolabs). The PCR amplicons were run on a 2% TAE agarose gel stained with SYBR Safe (Thermo Scientific, Waltham, MA). The positive bands that were chosen for sequencing were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI) according to manufacturer's protocol. The purified amplicons were subcloned into pJet1.2 using the CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA) according to manufacturer's protocol. The ligation product was transformed into NEB® 5-alpha Competent *E. coli* cells (New England Biolabs, Ipswich, MA), miniprepped, and sequenced using primer FDX1_seq1 (5'-TAGCGCAGCTTCGCCTACAT-3') by Eton Bioscience, Inc. using the Sanger Method.

Table 1. Sequences of Primers used for Multiplex PCR Genotyping

Primer	Sequence
LG_1	GGTCGTGTCCACGAACTTCC
LG_2	CGACCACGATCACGTCATACAC
LG_5	GGTGGTGCAGATGAACTTCAGG
LG_7	CGATCCTCCGTTGATTTTGG

RESULTS

Testing the Oxyfluorfen resistance cassette and simulated deletion cassette for background activity on Oxyfluorfen selective agar plates

The Oxyfluorfen herbicide is a protoporphyrinogen oxidase inhibitor, which is a class of herbicide that has been used in crops for years (Bruggeman et al, 2014). The Oxyfluorfen resistance plasmid, pOxR2, was obtained from Bruggeman et al. However, the concentrations of oxyfluorfen herbicide used in that paper showed background wild-type growth within 10 days under light. An experiment was set up to test varying concentrations of oxyfluorfen in agar plates and determine the concentrations that showed no background growth of wild-type CC124 cells, no background growth of pLox transformed cells, growth of pOxR2 transformed cells, and growth of transformed pLOR cells.

Arbitrary concentrations were selected to test, as there were no other references in the literature as to what range of amounts are lethal to *C. reinhardtii*. Two concentrations, 0.85 μM and 1.70 μM, showed no CC124 wild-type growth, no pLox growth, but had growth in cells

transformed with pOxR2 and pLOR. pOxR2 and pLOR showed robust colonies after 7 days of growth and pictures were taken 10 days after plating (Figure 5). Since the higher concentration, 1.70 µM, is more likely to guarantee that survival resulted from successful transgene integration and not from spontaneous mutation, it was utilized in future experiments.

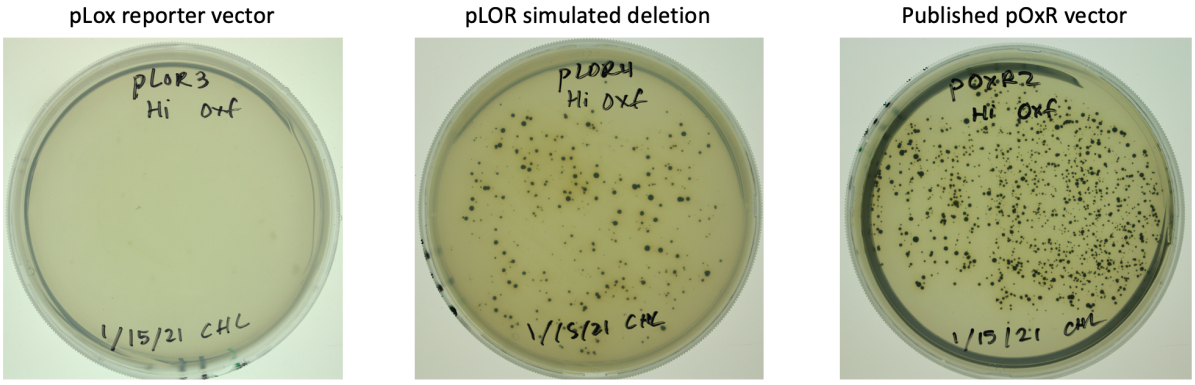

Figure 5. Growth of CC124 transformed with reporter vector, simulated deletion vector, and published Oxyfluorfen resistance vector.
10 days from plating, pLox (renamed from pLOR3) showed no background growth, pLOR (renamed from pLOR4) showed clear growth of large colonies, and pOxR2 had robust growth at about 3 times the amount of pLOR (pLOR4).

In-vitro Cre recombinase test on pLox plasmid DNA

Cre recombinase can be easily and cheaply obtained from a commercial vendor. The Cre enzyme that we purchased for our experiments was from New England Biolabs (NEB). Before carrying out *in vivo* experiments, we needed to verify if it is enzymatically active on the designed pLox plasmid *in vitro*. One unit of Cre recombinase enzyme was each added to 0.25 µg of the positive control provided by the supplier, 0.25 µg of linear pLox DNA, and 0.25 µg of linear pLOR DNA, along with µl of the provided 10X Cre Recombinase Reaction Buffer and water to 50 µl. One unit of enzyme is defined by NEB as the quantity that is required to carry out

maximum site-specific recombination in 0.25 µg of NEB provided positive control DNA *in vitro*. Figure 6 shows that the Cre enzyme successfully carried out recombination at the *loxP* sites in the pLox reporter vector, as demonstrated by the multiple bands on the electrophoresis gel in the presence of enzyme. The Cre enzyme did not induce recombination in the pLOR simulated deletion vector, as the enzyme requires two *loxP* sites to be effective.

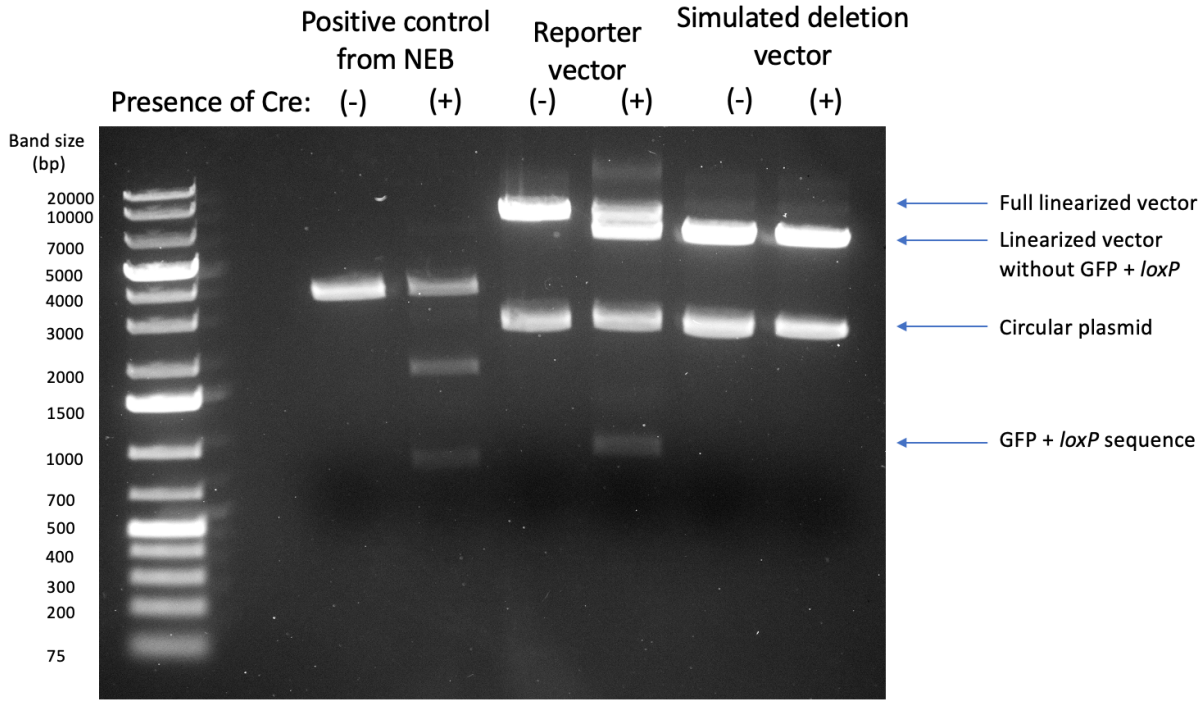

Figure 6. In vitro Cre recombinase assay to verify enzymatic activity.
Photo of electrophoresis gel showing the effects of Cre recombinase on the NEB positive control, pLox reporter vector, and pLOR simulated deletion vector. pLox was cut by Cre recombinase and produced expected sized bands: the full linearized vector at 9987 bp, linearized vector without GFP and a single *loxP* site at 8697 bp, the leftover circular plasmid from previous restriction digest linearization at ~3000 bp, and the GFP and single *loxP* excised fragment at 1290 bp. The simulated deletion vector also performed as expected, as it only has one *loxP* site and cannot undergo excision, showing no change in band size. It is also noted that the pLOR band at 8697 bp is the same size as the band for pLox + Cre.

Generating GFP positive pLox cells

The GFP positive cells were produced by electroporating 3 µg of linearized pLox DNA into CC124. The cells recovered in TAP + 40 mM sucrose media for 24 hours. Recovered cells were plated onto TAP, TAP + Hygromycin, TAP + Hygromycin + Zeocin, TAP + Oxyfluorfen agar plates. Single transformants were selected from the TAP + Hygromycin + Zeocin plates to ensure the presence of the entire transgene. After 10 days of growth, 528 out of 727 colonies were picked into six 96-well plates, passaged into fresh media 3 days later, then analyzed in a fluorescent plate reader 3 days after passaging. Wild type CC124 was used as a negative control. A previously published GFP expressing line was used as a positive control (Fields et al, 2019). mClover and chlorophyll fluorescence were analyzed using previously published methods (Fields et al, 2019). Variances in cell density was accounted for by normalizing mClover to chlorophyll fluorescence. 54 GFP expressing colonies (Supplemental File 5) of varying degrees of fluorescence were picked into one 50 mL baffled flask of TAP media to be grown up for further experimentation.

Electroporation of Cre recombinase enzyme into GFP positive pLox line

After three days of growth, approximately $5x10^8$ of the GFP positive pLox cells were prepped for a second transformation. As there is no documentation of Cre-recombinase enzyme electroporation into *C. reinhardtii* in the literature, a range of amounts were used: 0 (negative control), 1, 2, 4, and 8 units (µl) of enzyme as a preliminary experiment. As stated, one unit (µl) of enzyme is defined as the quantity that is required to carry out maximum site-specific recombination in 0.25 µg of commercially provided positive control DNA *in vitro.* The time constants (TC, time constant = resistance x capacitance) of transformation were as followed:

empty gap—56.5 milliseconds (ms), 0 µl Cre—39.8 ms, 1 µl Cre—39.1 ms, 2 µl Cre—36.9 ms, 4 µl Cre—31.6 ms, and 8 µl Cre—26.8 ms. the After electroporating the respective five amounts of Cre-recombinase enzyme into the GFP positive cells, the cells were struck onto TAP and TAP + Oxyfluorfen plates. The leftover, unplated cells remained in culture media on a shaker table under light for 11 days.

Within the ideal time frame of 10 days after plating, there were no colonies on any TAP + Oxyfluorfen plates with any amount of Cre and no subsequent growth after that time frame. The previously mentioned leftover cells that stayed in culture for 11 days were plated onto Oxyfluorfen in case Cre recombinase takes a longer time to carry out a deletion in *C. reinhardtii in vivo.* There seemed to be no growth after 10 days, but after 23 days, there was growth on the Oxyfluorfen plate with pLox + 8 µl of Cre enzyme (Figure 7). The small size of the colonies and the longer time that it took for them to grow indicated that these may not be true positive cells, but 16 of the largest colonies were picked onto TAP plates for further analysis.

Figure 7. Pinprick colonies of pLox + 8 µl Cre algae cells on Oxyfluorfen agar plate. These cells were grown in shake culture for 11 days after electroporation with Cre recombinase, then plated onto fresh Oxyfluorfen plates. This picture was taken 23 days after growth.

Electroporation of pCre expression cassette into GFP positive pLox line

The next experiment's objective was to evaluate if Cre recombinase could be stably expressed in *C. reinhardtii* and effectively carry out recombination in a pre-transformed transgenic line. The same GFP positive pLox colonies grown for the previous experiment were prepared for pCre DNA electroporation. The cells were electroporated using same method. After 24 hours of recovery in TAP + 40 mM sucrose media, half of the cells were plated onto TAP, TAP + Paromomycin, TAP + Oxyfluorfen, and TAP + Oxyfluorfen + Paromomycin plates. The remaining cells were left to incubate for another 24 hours before plating on the same conditions

of plates. There were no surviving colonies on Oxyfluorfen selective plates from both the first set and second set of transformants after 10 days. The cells were left to grow for another 18 days, until growth was observed in the 48-hour recovery group on TAP + Oxyfluorfen + Paromomycin. The 136 surviving colonies were small pinpricks, so they were plated on fresh Oxyfluorfen plates to make sure they were true positive results. 24 clones grew within 10 days and were isolated for genotype analysis.

Co-electroporation of pLox and pCre into algae

The down-selection to certain GFP expressors in pLox cells may limit the permutations of locations for pLox transgenes that are accessible by Cre recombinase. In this experiment, we skipped the initial GFP-positive selection step by simply co-transforming pLox and pCre into CC124 algae to maximize the combinations of pLox transgene insertion locations. More plating conditions were also used to help screen for these seemingly rare deletion events (Table 2). There was robust growth on the plates with survivors after 10 days. No cells grew on the plates that both contained antibiotic and Oxyfluorfen. The six pLox+pCre colonies that survived on Oxyfluorfen were picked onto TAP plates for further analysis. 32 of the largest pLox+pCre colonies that survived on Hyg + Paro + Zeo were picked onto TAP plates for archival. Leftover biomass of those 32 picked colonies and all remaining colonies were scraped into TAP media and grown in liquid culture for 3 days before spotting onto Oxyfluorfen plates. By 10 days of growth, there were 125 healthy colonies. 32 of those were struck onto TAP plates for further analysis.

Table 2. Plating Conditions vs. Average Number of Colonies Per Plate for pLox and pCre Co-Electroporation Selection Experiment.

The first row outlines the different plating conditions used, Hygromycin, Paromomycin, Zeocin, and Oxyfluorfen are abbreviated as H, P, Z, and O, respectively. The first column outlines the different plasmids and recovery times for each transformation. The average numbers of cells that survived after 10 days of growth for each condition are listed.

	TAP	H + P	H + P + Z	Oxf	H + P + O	H + P + Z + O
No DNA	lawn	0	0	0	0	0
pCre	lawn	0	0	0	0	0
pLox	lawn	0	0	0	0	0
pLOR 24 hr	lawn	0	0	105	0	0
pLOR 48 hr	lawn	0	0	404	0	0
pLox+pCre 24 hr	lawn	74	1	0	0	0
pLox+pCre 48 hr	lawn	559	20	6 (total)	0	0

Genotyping using multi-plex primers and sequencing

All surviving colonies from each Cre delivery method were genotyped using the same methods off genomic DNA, including the controls (Figure 8, lanes 1-4). The surviving transformants from the Cre recombinase protein electroporation experiment did not show positive genotyping results. The surviving transformants from the pCre expression gene electroporation into GFP positive cells experiment did not show positive genotyping results. The co-electroporation experiment of pLox and pCre showed positive genotyping results, designated by the presence of a band at 709 bp, which is the expected result for a deletion (Figure 8, lanes 6-11). There were also bands at 531 bp and 422 bp, which are the expected sizes for a lack of deletion.

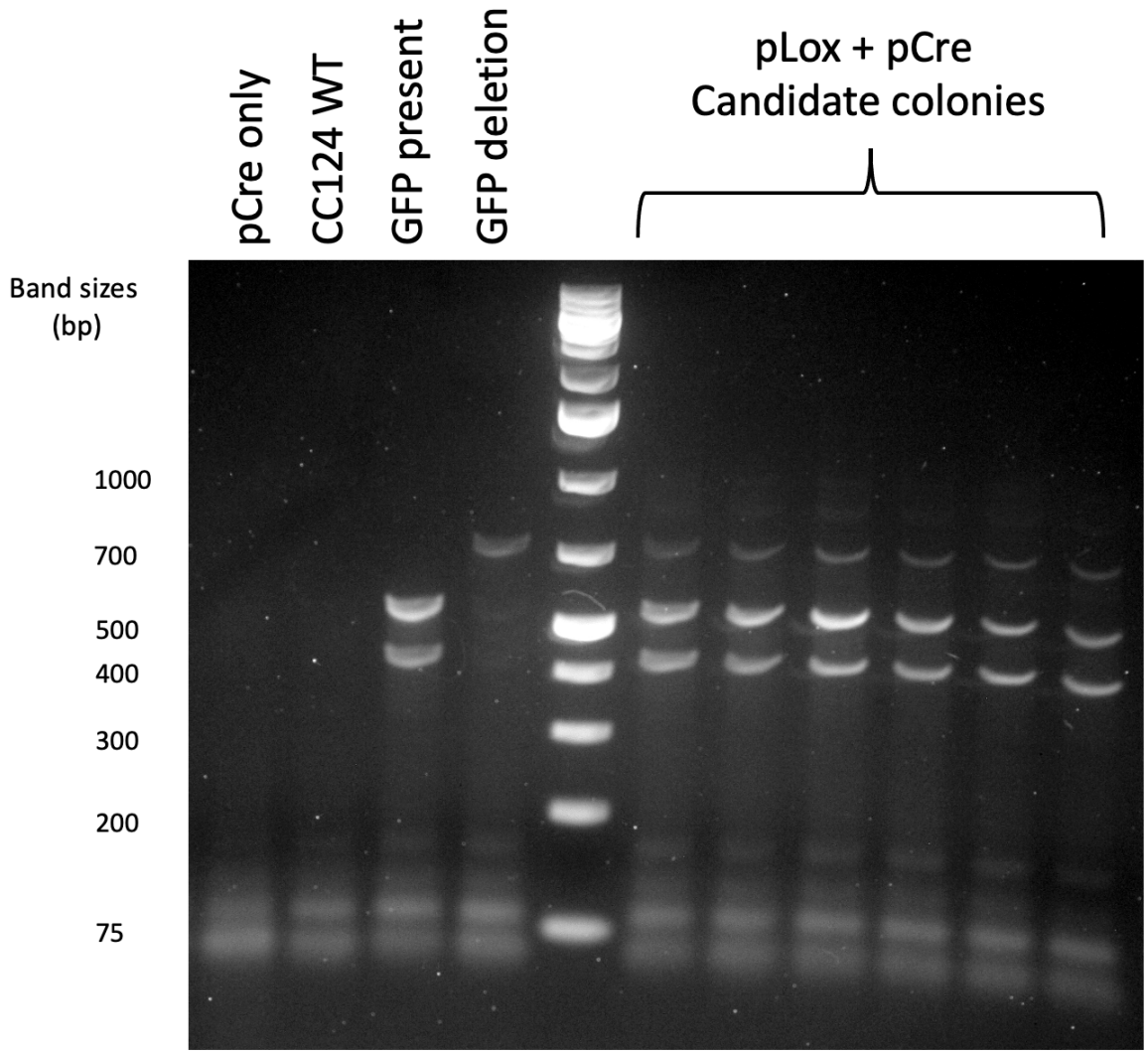

Figure 8. Positive results in pLox + pCre co-electroporation experiment.
Multiplex genotyping primers were used to amplify off of Chelex 100 genomic extractions of algae transformations. The controls in lanes 1-4 on the left are pCre transformed algae, CC124 wild-type algae, pLox transformed algae, and pLOR transformed algae. Lanes 6-11 on the right are 6 of the largest growing pLox + pCre colonies on Hygromycin + Paromomycin + Zeocin to Oxyfluorfen plates. These experimental colonies possess the same sized bands as pLox transformed algae and pLOR simulated deletion algae, indicating the presence of multiple transgenes in the cell.

To ensure that the presence of this band indicates a true deletion, the positive bands at 709 bp from the pLox and pCre co-electroporation were gel extracted, cloned, and sent for sequencing. A ClustalW alignment of the sequencing results to pLOR showed almost perfect alignment (Figure 9), demonstrating that our pCre vector transiently expressed active Cre recombinase and carried out a deletion at the *loxP* sites in pLox *in vivo*.

The genotyping results also indicated that GFP was not fully excised from all cells. To test this, these six positive clones were placed onto a 96-well plate and analyzed by fluorescent plate reader. They all fluoresced at levels comparable to the positive GFP control and above wild-type (Supplemental File 6).

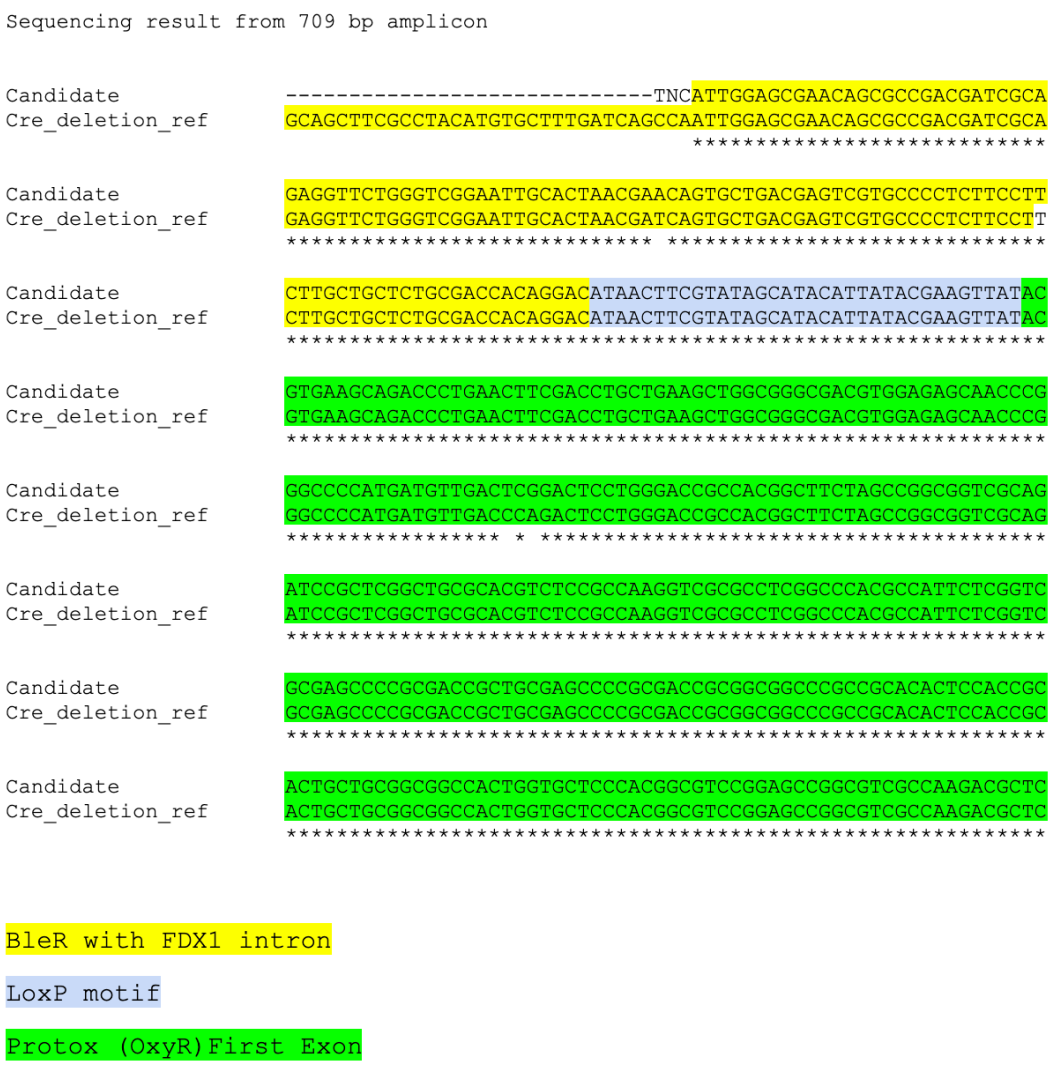

Figure 9. ClustalW sequencing alignment of 709 bp positive band and pLOR Cre deletion reference.

ClustalW was utilized to align the sequencing results with the pLOR simulated deletion sequence. An almost perfect alignment was achieved except for one base-pair discrepancy in the Ble^R FDX1 intron and two base-pairs in the Ox^R coding region.

DISCUSSION

Exogenous delivery of Cre recombinase

The first experiment, in which different amounts of Cre protein were electroporated into a GFP expressing pLox line, may have been unsuccessful due to a variety of factors. The buffer that contains the NEB Cre enzyme consists of 50% glycerol, which affected the transformation into algae. A higher amount than 8 µl of Cre enzyme was not used in the experiment because of the potential of glycerol to affect the conductance in the cuvette, possibly reducing cell viability or preventing pore opening during electroporation. The time constants (TC) in the preliminary experiment showed that the higher the concentration of Cre/glycerol, the lower the TC. The TC is a measure of how long the membrane pores are open, to allow for the influx of molecules (Myers & Tisa, 2003). The more NEB Cre recombinase we introduced to the cells, the less time the membrane pores were open, diminishing the chance the protein can enter the cell. However, there was very slight growth on the plates with 8 µl of Cre enzyme and 11 day incubation time, though no positive band showed up for genotyping. This result is indicative that higher amounts of exogenous NEB Cre delivery may work, and further experiments can be carried out to optimize the process of commercial Cre delivery through electroporation.

Though commercial Cre is easy and convenient to obtain, Cre protein can also be expressed in *E. Coli* and easily purified, then electroporated in through the cell wall to carry out recombination (Furuhata et al, 2019). Since the commercially obtained Cre does not have a NLS, that might have also hindered its ability to get into the nucleus to carry out recombination, so a Cre enzyme with a NLS can be produced instead. Furthermore, electroporation parameters, protein concentration, and electroporation buffers can also be adjusted in these experiments to proficiently deliver Cre protein through the cell wall.

Endogenous delivery of Cre recombinase

The first endogenous delivery experiment, in which GFP positive pLox algal lines were introduced a Cre expression line, there were no survivors within the ideal time frame of 10 days. The growth that was observed 18 days after that did not show any positive genotyping bands, either. The initial down selection to 54 GFP expressing clones for this experiment may have limited the loci in which Cre recombinase could access the *loxP* sites, as it has been shown that exogenous DNA undergo much more epigenetic silencing in *Chlamydomonas*, which could potentially inhibit Cre recombinase activity (Neupert et al, 2020). Thus, a second experiment was designed to combat that.

In the second endogenous delivery experiment, the reporter, pLox, and the Cre expression vector, pCre, were co-electroporated into *C. reinhardtii*, skipping the initial GFP down selection step. This method seemed to work much better to show that Cre recombination can work *in vivo* with our vectors. These cells were recovered in the same manner as previous electroporations but were plated on more selection conditions in which cells were not plated onto Oxyfluorfen straightaway. The only positive colonies that were verified by genotyping and sequencing to have carried out a deletion were first plated on Hyg + Paro + Zeo, given time to grow, and then plated onto Oxyfluorfen, in which those grew within the ideal time frame. For future experiments, cells must not be plated on Oxyfluorfen right after recovery, as it seems to take many days for Cre to be expressed and excise GFP in *C. reinhardtii*. This is substantiated by the fact that there were no positive results on plates with both antibiotic and Oxyfluorfen or Oxyfluorfen by itself, as in all previous Cre delivery experiments.

Though Cre recombination has been demonstrated to work *in vivo* in *C. reinhardtii*, as shown by genotyping and sequencing, the genotyping and plate reader data showed that there

was still GFP DNA and expression in the cells. There can be many explanations for this, such as there being multiple pLox transgenes in each cell or the clones were not pure due to human error, therefore showing up on the gel and by plate reader. Cre recombinase could be inefficient in deleting the multiple inserts because it needs more time to operate in *C. reinhardtii*, or more Cre enzyme must be expressed. This lack of expression may also be because Cre was not perfectly codon-optimized to *C. reinhardtii*; the GC content of the *C. reinhardtii* genome is 67-75% but synthesis of gBlocks with high GC content is prone to error, so we used 63%. An initial down selection step for pLox lines that only have a single insert would be useful to see if a full excision can occur with endogenous Cre, and that can be verified with PCR genotyping. Otherwise, we can first select for a pCre strain that expresses Cre at very high levels, since pCre contains a BFP gene and Flag Tag for quantification of expression. That high expressing strain can be mated with a pLox algae line, or pLox DNA can be electroporated in.

CONCLUSION

Cre/*lox* mediated recombination is one of the most widely used genetic engineering systems, but has not been widely explored in *C. reinhardtii*, the model unicellular green algae organism. There is only one example of its applications in the literature, but the system does not seem to work very efficiently. We were able to design new reporter vectors, new Cre expression vectors, develop an assay to easily verify activity, and demonstrate that Cre/*lox* mediated recombination can occur to generate a phenotypic change. Future experiments will involve increasing the concentration of Cre in the nucleus using methods such as optimizing electroporation parameters, changing buffers, utilizing nuclear localization signals, increasing expression, and limiting transgene insertions. This project has confirmed that the Cre/*lox* system has the potential to be implemented in *C. reinhardtii*, which expands the horizon of research and discovery toward more sustainable bio-manufacturing.

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