

Comparing different delivery systems in site-specific genome editing in *Chlorella vulgaris*

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Microalgae, such as *Chlorella vulgaris*, are among the most important eukaryotic microorganisms due to their ecological, biotechnological, and sustainable energy applications. Although the genome of *C. vulgaris* has been sequenced, its full genetic potential remains underutilized due to challenges in efficient genome editing. In this research, we aim to optimize the CRISPR-Cas9 genome editing system to achieve site-specific knockouts in the *C. vulgaris* genome. Two different delivery methods for CRISPR components plasmid-mediated transformation and direct ribonucleoprotein delivery were compared in terms of efficiency. Three guide RNAs targeting the nitrate reductase gene were designed and tested. The objectives were to assess mutation efficiency, validate phenotypic changes, and develop a genetic toolbox for *C. vulgaris*, enhancing its potential for applications in biotechnology. Phenotypic analysis confirmed successful gene disruption in transformants, while sequencing results highlighted the challenges in genotypic confirmation, emphasizing the need for improved protocols. This study contributes to advancing genetic engineering tools for *C. vulgaris*, providing a foundation for future biotechnological applications.

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List of Abbreviations

AMT	Agrobacterium-mediated transformation
BLAST	Basic Local Alignment Search Tool
Cas9	CRISPR associated protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
DSB	DNA double-strand break
EP	Electroporation
GFP	Green fluorescent protein
gRNA	Guide RNA
HDR	Homology-directed repair
KClO ₃	Potassium chlorate
NEB	New England Biolabs
NH ₄	Ammonium
NHEJ	Nonhomologous DNA end joining
NLS	Nuclear localization signal
NO ₂	Nitrite
NO ₃	Nitrate
NR	Nitrate reductase
OD ₆₈₀	Optical Density at 680 nm
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNP	Ribonucleoprotein
SOC	Super Optimal broth with Catabolite repression
TALEN	Transcription activator-like effector nuclease
TAP	Tris-acetate-phosphate medium
ZFN	Zinc finger nuclease

Chapter 1- Introduction

1.1 I Introduction to Algae and Microalgae

Algae include a diverse group of organisms capable of photosynthesis that inhabit diverse aquatic environments. They include both unicellular microalgae and larger, multicellular forms such as seaweeds. Their ability to produce oxygen through photosynthesis and serve as key producers in aquatic ecosystems underscores their ecological significance, contributing to an estimated 40% of the earth's oxygen supply (S. Kumar et al., 2015). They are primarily classified based on color and nutritional strategies, with three primary categories of algae include green algae (*Chlorophyta*), red algae (*Rhodophyta*), and brown algae (*Phaeophyta*) (Baweja & Sahoo, 2015). This classification also reflects their ecological functions and evolutionary relationships. Green algae, characterized by their green color from chlorophyll a and b, are mainly found in freshwater habitats and contribute to oxygen production and carbon fixation (Arora & Sahoo, 2015). Red algae are notable for their reddish hue, due to phycoerythrin, and predominantly thrive in marine environments (Manzoor et al., 2024). Meanwhile, brown algae, with their distinctive brown coloration from fucoxanthin, are primarily marine and form complex underwater ecosystems, serving as important habitats for various marine species (Hakim & Patel, 2020).

1.2 Importance of algae

Algae are highly important in various fields, playing crucial roles in environmental, energy and health sectors. They contribute significantly to biodiversity and evolutionary studies as they span from cyanobacteria to eukaryotic phytoplankton. Understanding their evolutionary trajectories provides insights into early life on the earth and the evolution of photosynthesis (Khan et al., 2020). In terms of environmental impact, algae are also important in photosynthesis and carbon capture. They are acting as primary producers in the global carbon cycle through their photosynthetic processes (Sharma & Sharma, 2017). In the context of sustainable energy, certain microalgal species rich in lipids are researched as potential sources for biofuel, thus contributing to renewable energy solutions development (Mata et al., 2010). Additionally, *Spirulina spp.* and *Chlorella spp.* are highly valued for their nutritional qualities such as containing high levels of proteins, vitamins, and essential fatty acids making them useful for food and feed industries (Becker, 2007). Besides this, research on algae extends to the production of other health-beneficial compounds like antioxidants and anti-inflammatory

agents potential anti-tumor drugs (Dai et al., 2022). Moreover, some types of algae act as ecological indicators providing information about water quality while others indicate heavy metal contamination or nutrient enrichment (Bhatt et al., 2022).

1.3 Genome editing in algae

Algae, particularly microalgae, are important for their ability to produce a wide array of valuable compounds. This fact underscores the need for advanced gene editing techniques to fully realize and enhance their capabilities. Gene editing is a way of making changes to an organism's DNA; in this vein, scientists can add, remove, or change parts of the DNA. The advancement of gene editing tools, particularly tools like CRISPR-Cas9 system, made this process more precise and efficient, and it is now being used in various organisms, such as algae, enabling targeted improvements in metabolic pathways (Yin et al., 2017).

Algae demonstrate their potential as sources for making pharmaceuticals, nutraceuticals, and bioenergy since they have the natural ability to absorb carbon dioxide and use sunlight (Sharma & Sharma, 2017). Scientist can increase the production of these important compounds in algae by introducing specific changes to their metabolic pathways. For instance, species like *Chlorella spp.*, *Nannochloropsis spp.*, and *Botryococcus braunii* are in focus for their capabilities to accumulate lipids suitable for biofuel production and enhancing their lipid productivity using genetic manipulation (Wijffels & Barbosa, 2010). Additionally, the genomes of many microalgae are comparatively smaller and less complex than that of higher plants, which is beneficial for studies involving genetic manipulation and genome editing. This simplicity facilitates a comprehensive understanding of gene functions, regulations, and the implications of genetic alterations on physiological attributes and metabolic pathways (Matos et al., 2020). Algae, such as *Chlamydomonas reinhardtii*, are used as model organisms to study fundamental biological mechanisms, such as photosynthesis and flagellar movement, due to their simplicity and cost efficiency; in this regard, understanding the genome and ability to make changes on it is a key to grasp a deep understanding in basic life processes (Merchant et al., 2007). Another reason for studying genome editing in algae is to understand their evolution and genome characteristics. As algae become increasingly noticeable in biotechnological, environmental, and nutraceutical fields, there is a growing interest in exploring their biodiversity and utilizing their commercial potential. A comprehensive understanding of the evolutionary, structural, functional, and developmental characteristics of algal genomes is essential for this purpose (Khan et al., 2020). It is of

great importance to note that while there have been advances in genetic manipulation tools for algae, there is limited evidence of genome editing in algae highlighting importance of additional studies to uncover the mechanisms and significance of genome editing in this creature.

1.4 *Chlorella vulgaris*

C. vulgaris, as a single-celled green microalga is spherical, about two to ten micrometers in diameter, and does not have flagella which distinguishes it from many other motile microalgae (Ru et al., 2020). These cells are able to grow in autotrophic, heterotrophic, and mixotrophic environments (Ahmad et al., 2020). *C. vulgaris* belongs to the domain *Eukaryota*, kingdom *Protista*, division *Chlorophyta*, class *Trebouxiophyceae*, order *Chlorellales*, family *Chlorellaceae*, Genus *Chlorella*, and species *vulgaris* (Safi et al., 2014). *C. vulgaris* is capable of photosynthesis, converting sunlight, carbon dioxide, and nutrients into valuable biomass, which consists of proteins, lipids, carbohydrates, and various bioactive compounds (Ahmad et al., 2020; Memon & Memon, 2020).

The genome of *C. vulgaris* has been sequenced and annotated (GenBank assembly accession: GCA_001021125.1, NCBI). It has a nuclear genome that is approximately 39.8 mega bases containing 10724 genes, organized into 16 chromosomes. The genome has 61% GC content and is haploid, which simplifies genetic studies and manipulations (Cecchin et al., 2019; Ru et al., 2020). Its circular chloroplast genome contains 165,504 base pairs, coding 121 genes. The mitochondrial genome of *C. vulgaris* is also sequenced, with 91560 bp coding 48 genes (Cecchin et al., 2019). *C. vulgaris* reproduces asexually through a process called auto-sporulation. In this process, the mother cell divides internally to produce 2 to 32 daughter cells, known as autospores. Once the daughter cells mature, they are released when the mother cell's wall breaks apart providing food for the new cells. This reproduction method helps *Chlorella vulgaris* grow quickly and adapt to various environments (Ru et al., 2020).

1.5 The importance of *C. vulgaris*

Studying *Chlorella vulgaris* as well as developing and refining gene editing tools for this species is very important for biotechnology research. While the genome of many microalgae species has not yet been fully sequenced, *C. vulgaris*' genome has been sequenced and has been the subject of many studies to understand its genomic features. This has made it a valuable species for genome editing studies and has assisted scientists in identifying potential targets for biotechnological manipulation (Cecchin et al., 2019). Moreover, it is worth mentioning that the non-pathogenic nature of *Chlorella* species and its

easy containment, emphasize its safety, mitigating concerns related to environmental or health risks (Aly et al., 2024).

Chlorella vulgaris is widely researched for its potential in biofuel production, nutritional supplements, lipid modification, and biomass generation (Ahmad et al., 2020; Safi et al., 2014). The development of a CRISPR-Cas9-based genome editing tool in *C. vulgaris* has been reported (J. Kim et al., 2021), highlighting its significance as a cell factory for bioproducts. This species is an invaluable part of aquatic ecosystems as a primary producer contributing to the global carbon cycle through photosynthesis (Safi et al., 2014). Developing tools for gene editing in *C. vulgaris* is crucial for harnessing its full biotechnological potential. Studies have used *C. vulgaris* to produce recombinant protein as an oral vaccine for aquatic animals (M.-J. Kim, Kim, Kim, Lee, et al., 2023; M.-J. Kim, Kim, Kim, Yoo, et al., 2023), and identified a salt-inducible promoter from this species (M.-J. Kim, Kim, Abdellaoui, & Choi, 2023), demonstrating the feasibility of genetic manipulation in this alga. However, the importance of its physiological characteristics has been hindered by the absence of an efficient genetic engineering tool; therefore, genome editing techniques, such as CRISPR-Cas9, need to be investigated and developed to overcome this challenge and advance research in this field (J. Kim et al., 2021).

1.6 Current Methods of genome engineering in *C. vulgaris*

1.6.1 Transformation

Many techniques have been developed to introduce DNA into this microalga. The primary methods used include *Agrobacterium*-mediated transformation (AMT), electroporation (EP), polyethylene glycol (PEG)-mediated transformation, and microparticle bombardment (MBP). Each technique has advantages and limitations and can be selected based on the experiment, the type of genetic material being transferred, and the desired efficiency of transformation (Neupert et al., 2012; Niu et al., 2011).

1.6.1.1 *Agrobacterium*-mediated transformation (AMT)

AMT uses the bacterium *Agrobacterium tumefaciens* to transfer DNA fragments into the host genome. This method is beneficial for its ability to transfer large DNA fragments. However, the process can be time-consuming and often results in random integration of the DNA (Cha et al., 2012).

1.6.1.2 Electroporation (EP)

Electroporation is favored for its simplicity and efficiency. This method uses electrical pulses to transiently open pores in the cell membrane, enabling the introduction of DNA into the cell. Electroporation is used for both nuclear and chloroplast transformations in *C. vulgaris* (Bolaños-Martínez et al., 2022). Despite its direct approach and relatively high transformation rates, the DNA typically integrates randomly into the genome (M. Kumar et al., 2018; Shin et al., 2020). Its parameters, such as voltage, resistance, and capacitance are critical variables that have already been adjusted for the transient transfection in *Chlorella spp.* (Yang et al., 2016).

1.6.1.3 Polyethylene glycol (PEG)-mediated transformation

This process begins with protoplasting, which involves creating *C. vulgaris* cells without cell walls. It uses a combination of cell wall-degrading enzymes, such as chitinase, chitosanase, and lysozyme, which work together to break down the components of the cell wall (Gerken et al., 2013). PEG-mediated transformation involves using PEG to help DNA enter protoplasts, and it has been successfully used to transform *C. vulgaris* (Borovsky et al., 2016; Yang et al., 2015).

1.6.1.4 Microparticle bombardment (MBP)

Microparticle bombardment, also known as biolistic transformation, uses high-velocity metal particles coated with DNA to physically penetrate cell walls and deliver genetic material (Lacroix & Citovsky, 2020). This method is applied for both nuclear and chloroplast transformations, and it has successfully been used to transform the chloroplast of *C. vulgaris* (Bolaños-Martínez et al., 2022; K. Wang et al., 2021).

1.6.2 Site-specific genome editing

Site-specific genome editing techniques are used for targeted modification of DNA at specific location. There are several methods to create site specific modifications all of which harness host cell DNA repair mechanisms, allowing researchers can achieve specific editing in the genome (Davis & Maizels, 2014; Gaj et al., 2016). There are two primary types of DNA repair systems in eukaryotes, homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR is typically activated when a double-strand break (DSB) is repaired using a homologous template, while NHEJ is a homology independent method of repairing DNA which often leads to small deletions or insertions at the site of

the break (Lieber, 2010). Many sites directed genome editing techniques rely on homologous recombination/HDR or induce double stranded breaks in a specific location to use NHEJ to induce targeted insertion of DNA.

Currently, only a handful of researchers have reported site-specific genome editing in *Chlorella* spp. One study reportedly used CRISPR-Cas9 to generate random knockouts of nitrate reductase (NR) or adenine phosphoribosyl transferase (APT) in *C. vulgaris* UTEX 395 (J. Kim et al., 2021), while another used CRISPR-Cas9 with a donor fragment for homologous recombination in *C. sorokiniana* to knockout ω -3 fatty acid desaturase (W.-R. Lin & Ng, 2020). Another group has reported several studies using either double or triple homologous recombination to disrupt nitrate reductase and insert genes of interest into *C. vulgaris* (C. Chen & Ward, 2024). A summary of the various site-directed methods of genome editing for eukaryotes are presented below.

1.6.2.1 Zinc finger nucleases (ZFNs)

ZFN are engineered proteins that can create DSBs at specific locations in DNA. Each ZFN has a zinc-finger domain that binds to specific DNA sequences, and a nuclease domain that cuts the DNA. By customizing the zinc-finger domain to recognize certain DNA sequences, ZFNs can make precise cuts at desired locations in the genome. These cuts trigger the cell's natural repair processes, either through NHEJ or HDR to make edits (Mussolino et al., 2011; Zeevi et al., 2008).

1.6.2.2 Transcription activator-like effector nucleases (TALENs)

TALENs, like ZFNs, are tools used to cut specific DNA sequences, but unlike zinc-finger nucleases (ZFNs), TALENs use transcription activator-like effector (TALE) proteins, which are derived from *Xanthomonas* bacteria (K. Chen & Gao, 2013; Joung & Sander, 2013). These proteins naturally bind to specific DNA sequences in host plant cells. TALENs are designed to target almost any DNA sequence by attaching the TALE DNA-binding domain to the FokI nuclease domain, which cuts the DNA. This makes TALENs highly versatile for creating targeted mutations, such as insertions, deletions, or replacements in the genome. Their use has shown high efficiency and specificity across various organisms, demonstrating their broad applicability in genome modification efforts (Joung & Sander, 2013).

1.6.2.3 Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9)

One of the key tools in site-specific genome editing is the CRISPR-Cas9 system, which originated from a bacterial adaptive immune defense mechanism; the system has been engineered to function in different kinds of cells, including human cells, for genome engineering studies (Mali et al., 2013). Clustered regularly interspaced short palindromic repeats (CRISPR) is a family of DNA sequences found in the genome of microorganisms like bacteria and archaea, which can defend them with the aid of a family of CRISPR-associated proteins, Cas proteins (Horvath & Barrangou, 2010). In this process, the Cas9 protein acts as a molecular scissor that can be guided to specific DNA sequences with the aid of a crRNA (gRNA) and tracrRNA molecule (Mali et al., 2013). The crRNA contains a protospacer which complements and binds to the target DNA sequence near a protospacer adjacent motif (PAM) site, directing the Cas9 protein to that specific location (Riesenberg et al., 2022). Once the Cas9 protein binds to the target DNA, it induces a DSB at the targeted site. This process can be harnessed to initiate the cell's DNA repair mechanisms for genome editing (Mali et al., 2013). The natural system was simplified for genome editing by creating a single guide RNA (gRNA) that is can be easily edited to target your gene of interest using a designed protospacer sequence, becoming the leading tool for genome editing in eukaryotes due to its unparalleled specificity, adaptability, and ease of use (Jinek et al., 2012). While older methods like ZFNs and TALENs depend on complex protein-DNA interactions, making them more difficult to design and which sometimes lack the desired precision, CRISPR-Cas9 uses a 20 bp protospacer sequence to find its target. (Gupta & Musunuru, 2014). This makes it straightforward to design and implement and facilitates a more direct and effective targeting strategy. Furthermore, since CRISPR-Cas9 is more cost-effective, it has become the preferred choice for genomic modification (Doudna & Charpentier, 2014; Jinek et al., 2012; Hsu et al., 2014).

1.7 Verification of transformant

One of the common methods for recognizing transformed cells is by assessing the cells for a specific phenotype like an antibiotic resistance marker. Using this method, only those cells that have received the genetic construct, including an antibiotic resistance gene, survive in an antibiotic-containing medium (Clough & Bent, 1998). Although not all antibiotics work with eukaryotic cells like *C. vulgaris*, hygromycin B, geneticin, kanamycin, chloramphenicol, zeocin, and paromomycin have been successfully used in *C. vulgaris* (H.-D. Lin et al., 2013). Moreover, auxotrophy-based selection can be

useful where transformed cells require a specific supplement for growth, which can be controlled. Specially, nitrate auxotrophy has been used successfully in *C. vulgaris*, simplifying the identification of transformed cells and ensuring that only the desired modified strains proliferate (Casado-del Castillo et al., 2021; H.-D. Lin et al., 2013; Yarimizu et al., 2017).

Other methods for verifying the genotype of the transformed cells include PCR amplification of the desired location followed by DNA sequencing. Another method that has been applied to detection of knockouts is the T7 Endonuclease assay. In this assay, the region is PCR amplified from the wild-type cells and suspected transformants. The two PCR products are mixed then denatured and reannealed. Then T7 endonuclease I (T7EI) is added to the resulting product and if a mismatch is present by addition or deletion of one or more base pairs, the DNA is cut at that location resulting in two fragments. This can be easily visualized using DNA electrophoresis, allowing for rapid screening of transformants. Positive transformants can then be sequenced to confirm the deletion/addition.

1.8 Research objectives

The goal of this research is to compare different delivery systems for the CRISPR-Cas9 system in *C. vulgaris*. This research aims to expand our understanding of the efficiency of various genome editing techniques of *C. vulgaris* based on CRISPR-Cas9. The following hypotheses have been proposed to guide this research:

By transforming *C. vulgaris* with Cas9 and gRNA targeted at nitrate reductase, nitrate reductase mutants will be generated.

Several subhypothesis have also been formulated: supplying Cas9 and gRNA through a plasmid and through RNPs will have different genome editing efficiencies. Different gRNAs will have different editing efficiencies, and these knockouts will be stable.

Chapter 2 - Materials and Methods

To assess the efficiency of two different CRISPR-Cas9 delivery strategies in *C. vulgaris* UTEX 395, two core experiment were designed to compare direct delivery of the RNP complexes, and a plasmid vector carrying Cas9 and gRNA encoding region. All cloning reagents were sourced from New England Biolabs (NEB) unless stated otherwise. Additionally, all chemicals used were of ACS grade or higher quality.

2.1 Strains and growth conditions

C. vulgaris strain UTEX 395 was obtained from the University of Texas Culture Collection. This strain was cultivated at 25°C with shaking at 250 rpm and 150 $\mu\text{mol}/\text{m}^2\text{s}$ of continuous photosynthetically active light. They were grown in sterile Tris Acetate Phosphate (TAP) media, or BG-11 media, all of which were sterilized by autoclaving at 120°C for 30 minutes. *E. coli* NEB-5 α was used for cloning purposes. It was grown in LB media. at 37°C and shaken at 150 rpm. The bacteria were kept in a glycerol stock (15% v/v) at -80°C.

2.2 Designing single gRNAs

Initially, for common auxotrophy targets in the *C. vulgaris* genome, Previous studies on *Nannochloropsis* microalgae have demonstrated that NR activity can serve as an auxotrophic marker following CRISPR-mediated gene editing. Similarly, the NR gene has been characterized in *C. vulgaris* (Kilian et al., 2011). Thus, the NR gene was chosen to be targeted in this study because its knockout can be easily assessed using selective media, allowing us to evaluate the effectiveness of CRISPR-Cas9 gene editing in *C. vulgaris*.

To target nitrate reductase gene in *C. vulgaris* UTEX 395, the sgRNAs were designed using CHOPCHOP web tool (Labun et al., 2019). The *C. vulgaris* UTEX 259 nitrate reductase (NR) gene sequence [GenBank; EF201807] was used as a reference for BLAST search against the genome sequence of *C. vulgaris* UTEX 395 to find the genomic sequence of the nitrate reductase gene. The resulting sequence was used on CHOPCHOP to design three different sgRNAs presented in Table 1. The gRNAs were specifically designed to target exons 1, 2, and 3. All designed gRNA were checked for secondary structure using RNAfold webserver (Kerpedjiev et al., 2015), as recent literature suggests that it is the best practice for gRNA design (Corsi et al., 2022).

Table 1. The sgRNAs sequences to target NR gene in *C. vulgaris* UTEX 395

gRNA	Genomic location	Strand	Sequence	PAM	Efficiency score
1	1427	Sense	ACAAGGGCACCAAGGACGAG	TGG	62.73
2	1872	antisense	TGCTCCTCCCCTTAATCTG	CGG	64.21
3	2278	antisense	GTACAGGTCACGTCAACACT	AGG	63.28

2.3 Cloning of Cas9 and sgRNAs

For the plasmids-based system, the pCAMBIA1302 vector was selected as the delivery system for both Cas9 and gRNA genes into *C. vulgaris*. This vector was selected based on our previous results that green fluorescent protein (GFP) can be successfully expressed from this combination of promoter and terminator in *C. vulgaris* (Roushan et al., 2023). Both Cas9 and gRNAs were added to the pCAMBIA-1302 vector, and GFP was removed. This was performed in two steps: initially, the Cas9 gene was integrated into the plasmid in place of GFP and then the individual gRNA fragments, synthesized by TWIST Bioscience and flanked by U6 promoter and terminator sequences, were inserted into the plasmid already containing the Cas9 gene. The constructed plasmid was then delivered into NEB5- α competent cells.

2.3.1 Cloning of Cas9

The pCAMBIA1302 vector is used as a backbone for cloning the Cas9 gene. The *Streptococcus pyogenes* Cas9 (SpCas9) gene, sourced from pET28a plasmid containing Cas9 flanked by nuclear localization signals (NLS) and a polyhistidine-tag (His-tag) [ADD GENE# 98158] (Liang et al., 2017). The target region was amplified using primers designed through NEBuilder, which were synthesized by IDT. PCR amplification was carried out using Q5 polymerase. The pCAMBIA1302 vector was linearized using NcoI and BstEII restriction digests. Then, the pCAMBIA1302 plasmid was created to contain the Cas9 gene using the HiFi assembly master mix. This plasmid was introduced into DH5 α chemical competent cells using heat shock, then the cells were recovered in SOC medium for one hour at 37°C with shaking. To verify successful transformations, colony PCR was performed. A small portion of each colony was suspended in 10 μ L of sterile water and heated at 98°C for 15 minutes to

disrupt the cells. Then, 1 μ L of this lysate was added to the Thermo Scientific Taq DNA polymerase master mix. The amplified PCR products were analyzed via gel electrophoresis to detect positive transformants. Colonies showing the expected band were further cultured, and plasmid DNA was isolated using a mini-prep kit. The extracted plasmids were then sent for sequencing to confirm accurate construct assembly. The whole plasmid sequencing was done by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation. The inserted fragment is shown in Figure 1.

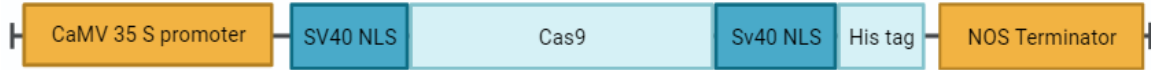


Figure 1. Illustration of the cloned Cas9 cassette into the pCambia1302

2.3.2 Cloning of gRNAs

The gRNA sequences were designed using the CHOPCHOP web tool, flanked by the U6 promoter and terminator sequences, and ordered from Twist Biosciences. The U6 promoter is widely recognized for its strong and consistent transcriptional activity, making it an optimal choice for expressing small non-coding RNAs, including guide RNAs (gRNAs), in eukaryotic systems (Sun et al., 2015). The extensive use of U6 promoters in CRISPR-Cas9 studies demonstrates their effectiveness in driving reliable gRNA expression across various organisms, including microalgae, thereby supporting their selection for this research (Sun et al., 2015; Zheng et al., 2018). After constructing the vector containing the Cas9 expression cassette, each of the three designed gRNAs was inserted into the vector using Gibson assembly. Figure 2 illustrates the position of each fragment within the construct.

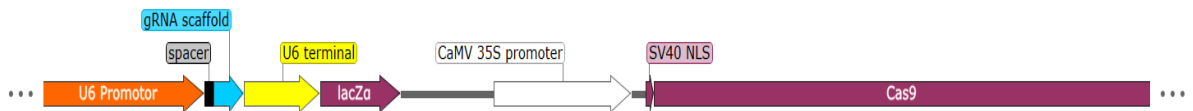


Figure 2. The insertion site of the gRNA fragment into pCambia-1302-Cas9, which is flanked by the U6 promoter and terminator sequences within the vector.

2.3.3 Preparation of competent cells

A 200 mL LB broth culture was prepared by inoculating it with an overnight-grown NEB-5 α culture. The flask was incubated at 30°C with constant shaking at 230 rpm until the OD₆₀₀ reached approximately 0.4. The culture was then placed on ice for 30 minutes. It was divided into two equal 100 mL portions and centrifuged at 4°C for 15 minutes at 2000 $\times$ g. The pellets were resuspended in 100 mL of ice-cold 0.1 M magnesium chloride (MgCl₂) and divided into two 50 mL tubes. After a second centrifugation under the same conditions, the cells were resuspended in 50 mL of chilled 0.1 M calcium chloride (CaCl₂) and incubated on ice for 20 minutes. Following this incubation, the cells were pelleted again by centrifugation at 4°C for 15 minutes at 1000 $\times$ g. Each pellet was then resuspended in 2 mL of a solution containing 85 mM calcium chloride and 15% glycerol. Finally, 50 μ L aliquots of the cell suspension were transferred into sterile microcentrifuge tubes and stored at -80°C for future use.

2.4 RNPs complex

2.4.1 Annealing crRNA and tracrRNA

For the experiments using RNP complexes, the IDT Alt-R CRISPR-Cas9 gRNA system was used. This consists of a crRNA and tracrRNA along with purified Cas9 protein containing a C-terminal SV40 NLS his-tag, and RFP domain. To anneal crRNA and tracrRNA and prepare 10 μ M of the annealed gRNA complex, 1 μ L of 100 μ M crRNA (MW=11748g/mol), 1 μ L of 100 μ M tracrRNA (MW=23759g/mol), and 17 μ L of nuclease-free water or TE buffer containing RNase inhibitor (1 μ L of 100u/ μ L) were mixed. Then the mixture was at 37 °C for 30 minutes and then allowed gradually cool to room temperature.

2.4.2 RNPs preparation

To Prepare the RNP Complex, Cas9 protein and gRNA were combined at a 1:2 molar ratio of Cas9 protein to sgRNA (4 μ g Cas9 and 5 μ l gRNA 10 μ M with molar mass 35507 g/mol were mixed and incubated at 37°C for 5 min or on ice for at least 20 min. These RNPs mixtures were introduced into *C. vulgaris* cells via electroporation.

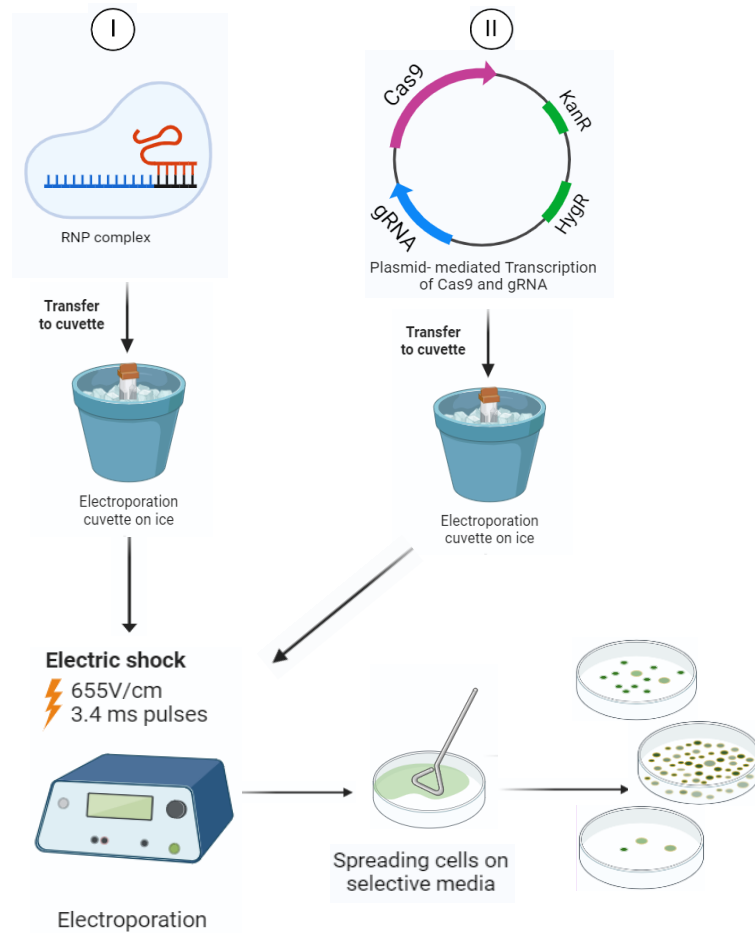


Figure 3. Overview of CRISPR-Cas9 delivery strategies in *C. vulgaris*. The study employs two distinct CRISPR-Cas9 delivery methods: (I) RNP complex, (II) Plasmid-mediated transcription of Cas9 and sgRNA.

2.5 Preparation of *C. vulgaris* cells and Electroporation

A protocol was previously developed that allows for transient expression of pCAMBIA1302 in cells with a cell wall (unreported, Ward Lab). *C. vulgaris* cells in the logarithmic phase (grown in Bg11 media with light for ~36 h to an OD₆₈₀ ~0.5 which corresponds to a cell density of 2×10^7 cells/mL) were treated with 60% polyethylene glycol and 0.1M MgCl₂ to prepare cells for electroporation. Since electroporation settings for *C. vulgaris* vary significantly in the literature, *C. vulgaris* cells were electroporated using 5 µg of miniprep pCAMBIA1302 plasmid containing GFP. Fluorescence was monitored for 24 h using a BioTEK plate reader at Ex. 488nm and Em. 526 nm. The settings resulting in the highest fluorescence were selected for all future experiments: electric pulse at 655 V for 3.4 milliseconds. Cells were rested for 24 h in three different modified BG11 media containing nitrite, ammonia and without nitrogen source, and then were transferred onto the BG11 agar plates containing nitrite and 175-200 mM potassium chlorate for selection. For the RNP experiments 6 µg of RNP complex were used in place of the plasmid as above.

2.6 Selection of transformants

The final step involved plating the electroporated cells onto selective media containing nitrite and potassium chlorate to assess the growth of successfully transfected cells. KClO₃ is reduced to ClO₂⁻ by nitrate reductase, making it toxic to wildtype cells with an intact NR gene. Nitrite was included as the sole nitrogen source to induce nitrate reductase activity in wildtype cells, ensuring the selective pressure works effectively. The absence of ammonium in the media is crucial, as ammonium represses nitrate reductase expression, which would otherwise reduce the selective pressure (Vennesland & Solomonson, 1972). Only cells lacking functional nitrate reductase, due to successful transformation, are expected to survive under these conditions.

2.6.1 Phenotype screening

To confirm the phenotypic changes further, additional plates were used to validate the transformation. These included media containing ammonium (NH₄) and KClO₃, as well as nitrate. Ammonium plates with the presence of KClO₃ allows only the transformants to grow, as it is not toxic to cells lacking functional nitrate reductase. In contrast, plates with nitrate ensure that cells lacking nitrate reductase cannot survive, confirming the successful knockout of the NR gene. By comparing the growth patterns

on these plates, it becomes possible to distinguish between transformed cells and wild-type cells, providing evidence of successful gene editing.

2.6.2 Genotype screening

After this initial screening, colonies exhibiting the transformant phenotype were selected for further validation using the T7 endonuclease cleavage assay. This assay is used to detect insertions or deletions (indels) at the target site.

After genomic DNA extraction from the selected colonies and wild type, using Q5 high-fidelity DNA polymerase the region surrounding the target site was amplified by PCR and the following primers:

Table 2. Primers for NR exon 1-3 amplification

Name	Sequence
T7.gRNA1-F	GCAGCAATGAAAGCCAATGG
T7.gRNA1-R	TCGACGTTGAAGGGATGCC
T7.gRNA2-f	ATTCCTCCTCTGCACCTTCC
T7.gRNA2-R	ATTGTGAATGTGGTGGGCG
T7.gRNA3-F	GCGGTGTATGGCTAGCTCAT
T7.gRNA3-R	CTGACTCAATGCAGCACCG

Following PCR amplification, the products from each colony was combined with the wild type products, then denatured by heating at 98°C for 5 minutes. The mixture was then slowly cooled to 25°C to allow the formation of heteroduplexes, which contain mismatches at the mutation site. These heteroduplexes were subsequently treated with T7 endonuclease I, which cleaves the mismatched regions. The enzyme was incubated with the PCR products at 37°C for 30 minutes. After incubation, the reaction products were assessed using gel electrophoresis on a 1% agarose gel.

Genotype was also assessed by sequencing the PCR fragments by Sanger Sequencing at the TCAG facility at Sick Kids in Toronto. Sequencing primers for each exon are as follows:

Table 3. Primers for NR exon 1-3 sequencing

Name	Sequence
NR-EXON 1-3-FWD	CCCTGCCAACTCCTCCCTCATACTC
NR-EXON 1-3-REV	GGAGCGTTAGTCTGTGACTTGCTGC

Chapter 3- Results

3.1 Identification of exons and designing sgRNA

In this study, we focused on the NR gene in *Chlorella vulgaris*. Nitrate reductase, a cytosolic enzyme in *C. vulgaris*, has a molecular weight of approximately 100 kDa (Solomonson & McCreery, 1986), is encoded by a gene located in the nuclear genome, which is made up of 19 exons (Accession number MN627215.1) (Dawson et al., 1996).

To identify the gene locus, NR gene sequence from UTEX 295 was BLASTed against the *C. vulgaris* UTEX395 genome (Accession number ASM102112v1). For our CRISPR-Cas9 targeting, we selected the first three exons of the NR gene, each targeted by a specific gRNA (figure 4).

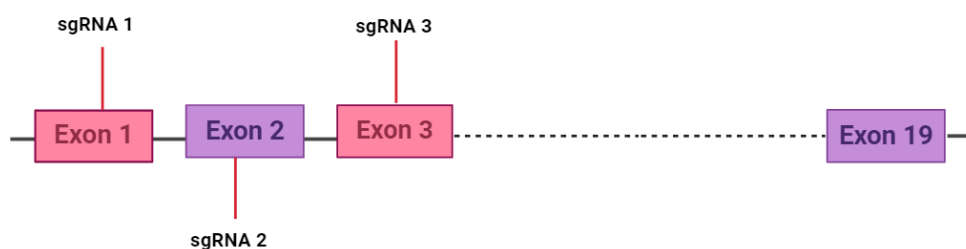


Figure 4. Illustration of nitrate reductase gene and three sgRNAs target sites.

The coding sequence of the NR gene was analyzed using CHOPCHOP web tool to identify PAM sites and crRNA sequences specific to the *Streptococcus pyogenes* Cas9 protein (Labun et al., 2019). The resulting gRNA designs are summarized in Table 1.

3.2 Cloning of Cas9 and gRNAs in pCAMBIA1302

In the first step, Cas9 was inserted into pCAMBIA1302. The *Streptococcus pyogenes* Cas9 (SpCas9) gene, sourced from pET28a plasmid containing Cas9 flanked by nuclear localization signals (NLS) and a polyhistidine-tag (His-tag), was cloned in the pCAMBIA1302 backbone replacing the *mgfp5* gene. To do so, the pCAMBIA1302 plasmid was first linearized using NEB restriction enzymes NcoI and

BstEII. The linearization process was verified by gel electrophoresis, showing distinct bands corresponding to the expected sizes of the non-linearized and linearized plasmids (Figure 5).

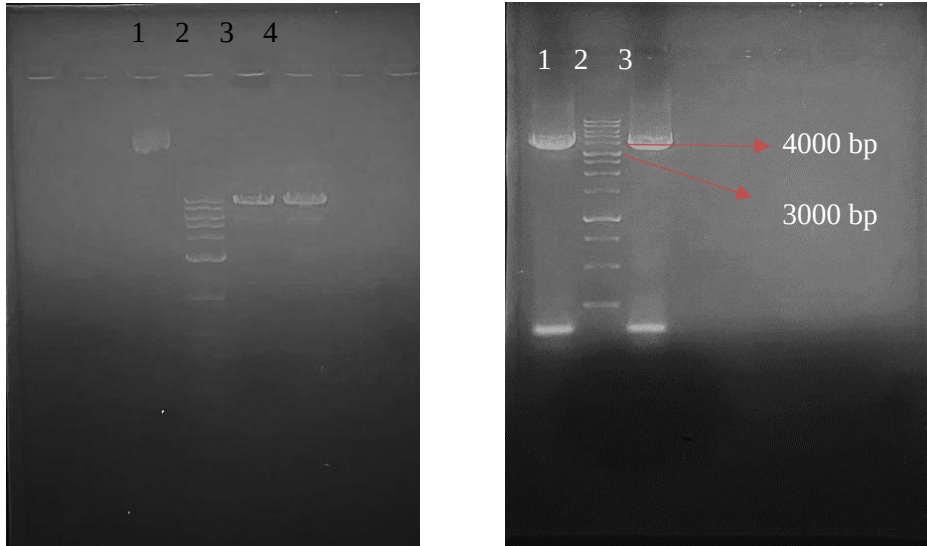


Figure 5. Left) Lane 1: Unlinearized pCAMBIA1302, lane 2: 1 kb ladder (Froggabio), lane 3 and 4: Linearized Backbone using NcoI & BstEII. Right) Lanes 1 and 3 are amplified Cas9 gene 4257 bp long from pET28a plasmid. Lane 2 is 1kb DNA ladder

After assembling the backbone and Cas9 insert using Gibson assembly, the transformants were isolated from plates containing 50 µg/mL kanamycin for further analysis. Figure 6 shows the successful transformation and expression of the plasmid.

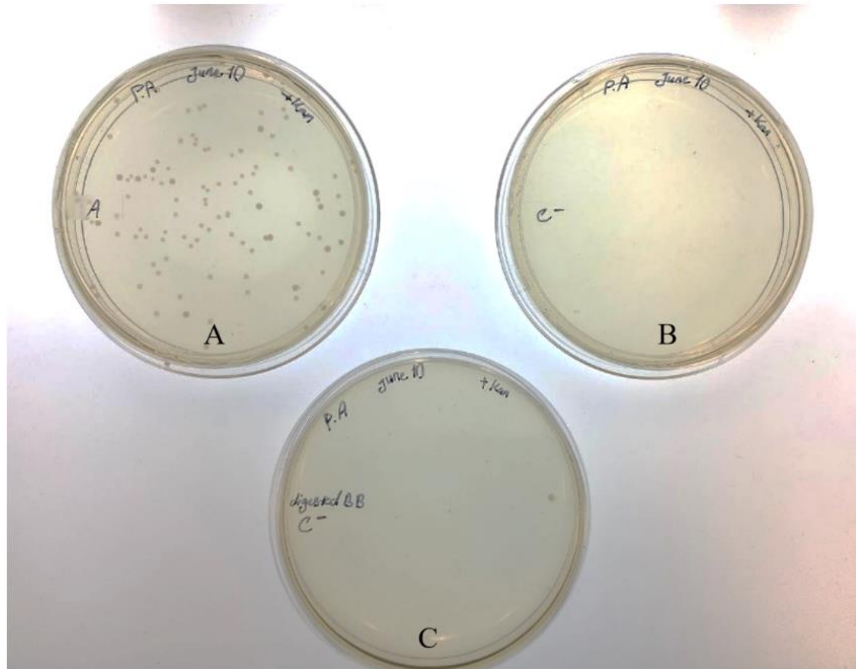


Figure 6. Transformation plates and controls. A: Assembled pCambia1302-Cas9. B: Negative Control (Competent cells without plasmid). C: Negative Control: (Competent cell with linearized backbone).

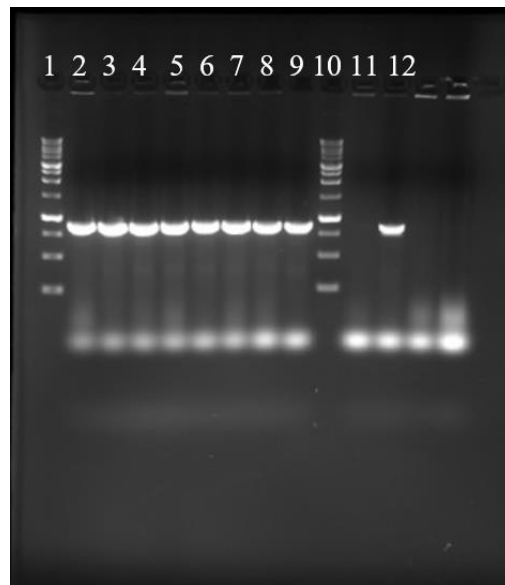


Figure 7. Colony PCR confirmation of expected vector construct. Lane 1 and 10: 1kb ladder (FroggaBio), Lane 2,3,4,5,6,7,8 and 12: 900 bp-long fragments indicating successful construction of Cas9 vector.

To further confirm the successful transformation, 10 colonies were selected for colony PCR. The result confirmed the successful transformation in all of them (Figure 7).

In figure 8, the annotated sequence resulting from whole plasmid sequencing completed by nanopore is shown. The annotated sequence highlights the correct insertion and orientation of the Cas9 gene within the pCambia1302-cas9 plasmid, confirming the successful construction of the vector.

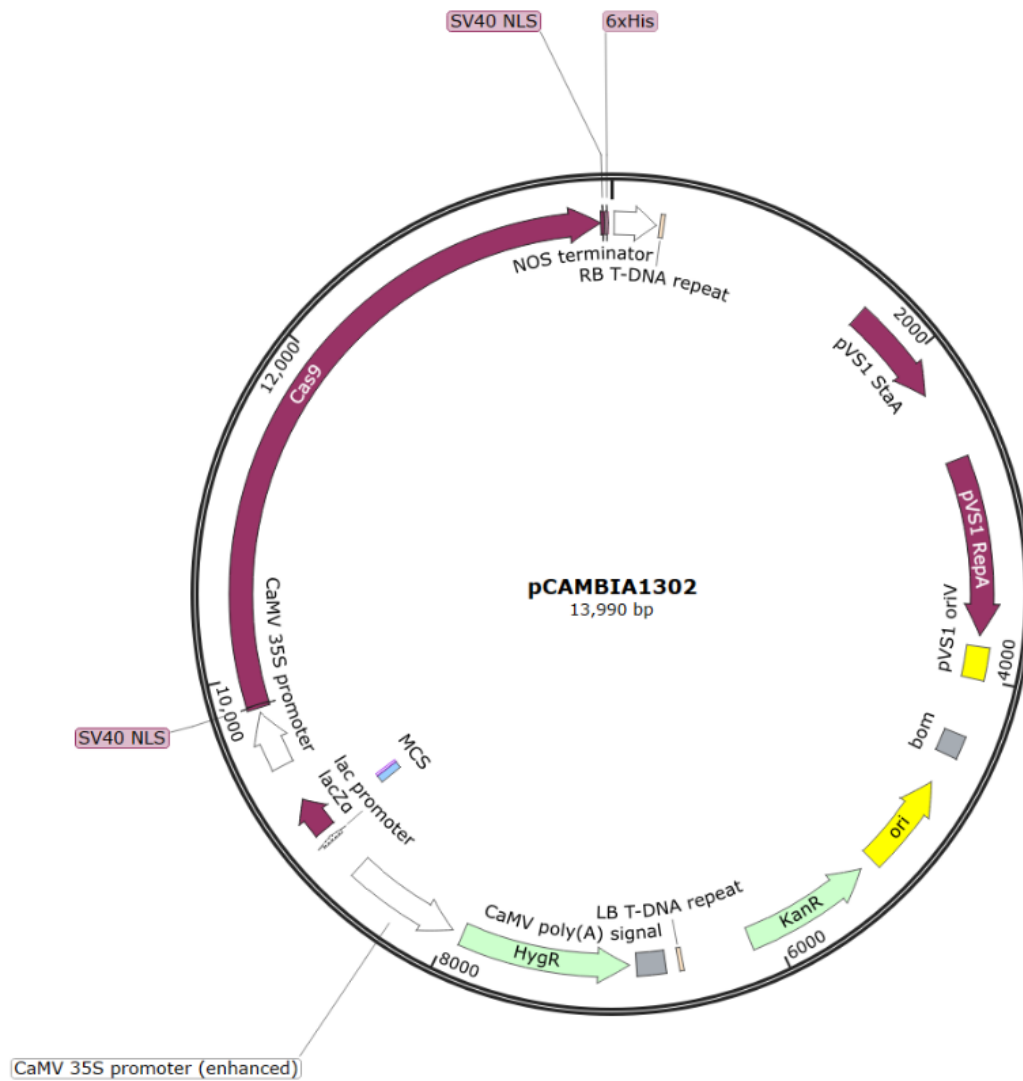


Figure 8. Genetic map of pCambia1302-cas9 plasmid. The Cas9 gene with flanking NLS and a His-tag have been inserted into the backbone.

Following the integration of Cas9 into the vector, the insertion of the sgRNAs was performed. The gRNA fragments, designed using the CHOPCHOP web tool and completed with U6 promoter and terminator sequences, were ordered from Twist Bioscience. After linearizing the plasmid with the SmaI restriction enzyme, each gRNA fragment was PCR amplified with overlaps from the backbone and inserted into the pCambia1302-cas9 plasmid using Gibson assembly. The gRNA sequence was altered to construct three plasmids containing gRNA1, gRNA2, and gRNA3 by altering the primers. Figure 9 shows the linearized backbone.

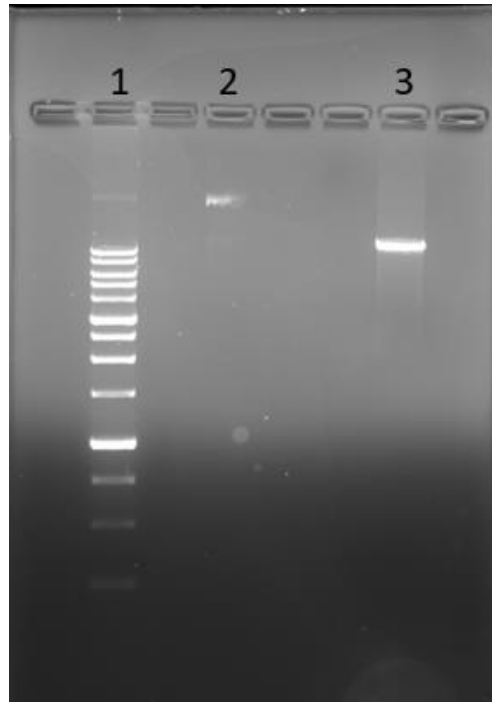


Figure 9. Lane 1: 1 kb ladder (Froggabio), lane 2: Unlinearized pCambia1302, lane 3: Linearized Backbone using SmaI.

Figures 10 shows the successful transformation which were confirmed by nanopore sequencing for all three gRNAs and figure 11 shows the pCambia1302-cas9-gRNA plasmid map confirmed by nanopore sequencing.

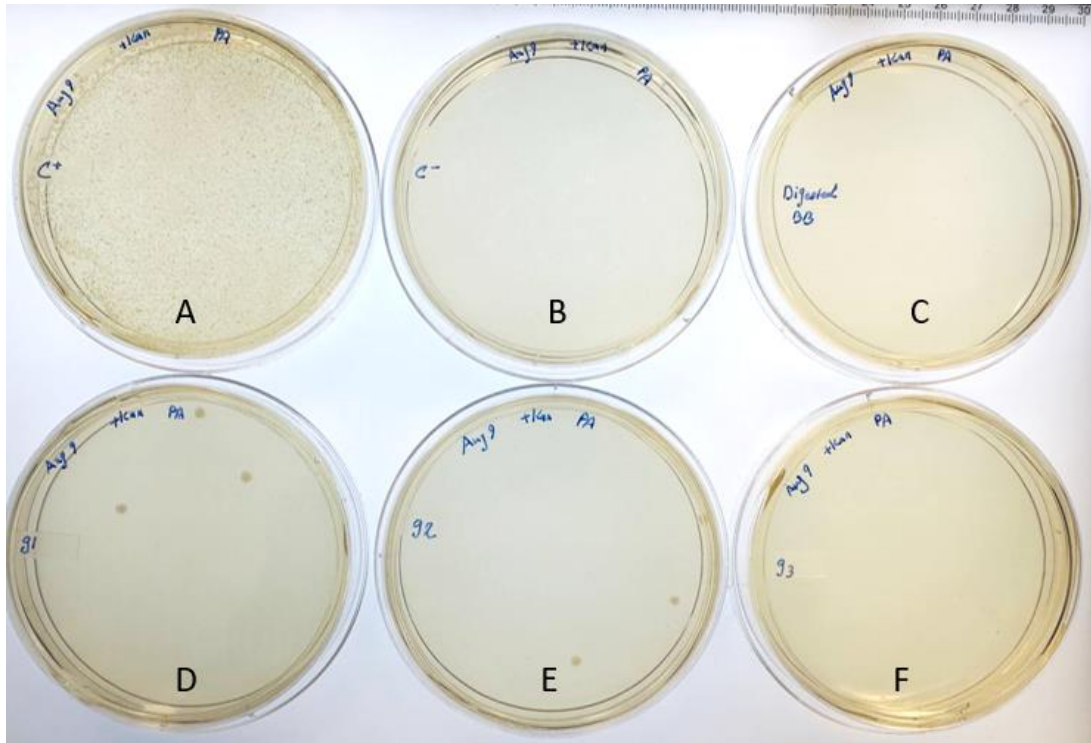


Figure 10. Transformation plates and controls. A: Positive control (Competent cells transformed containing pCAMBIA1302-Cas9. B: Negative Control (Competent cells Without plasmid). C: Negative Control: (Competent cell with linearized backbone). D, E and F: Respectively, transformed cells with plasmid containing gRNA1,2 and 3.

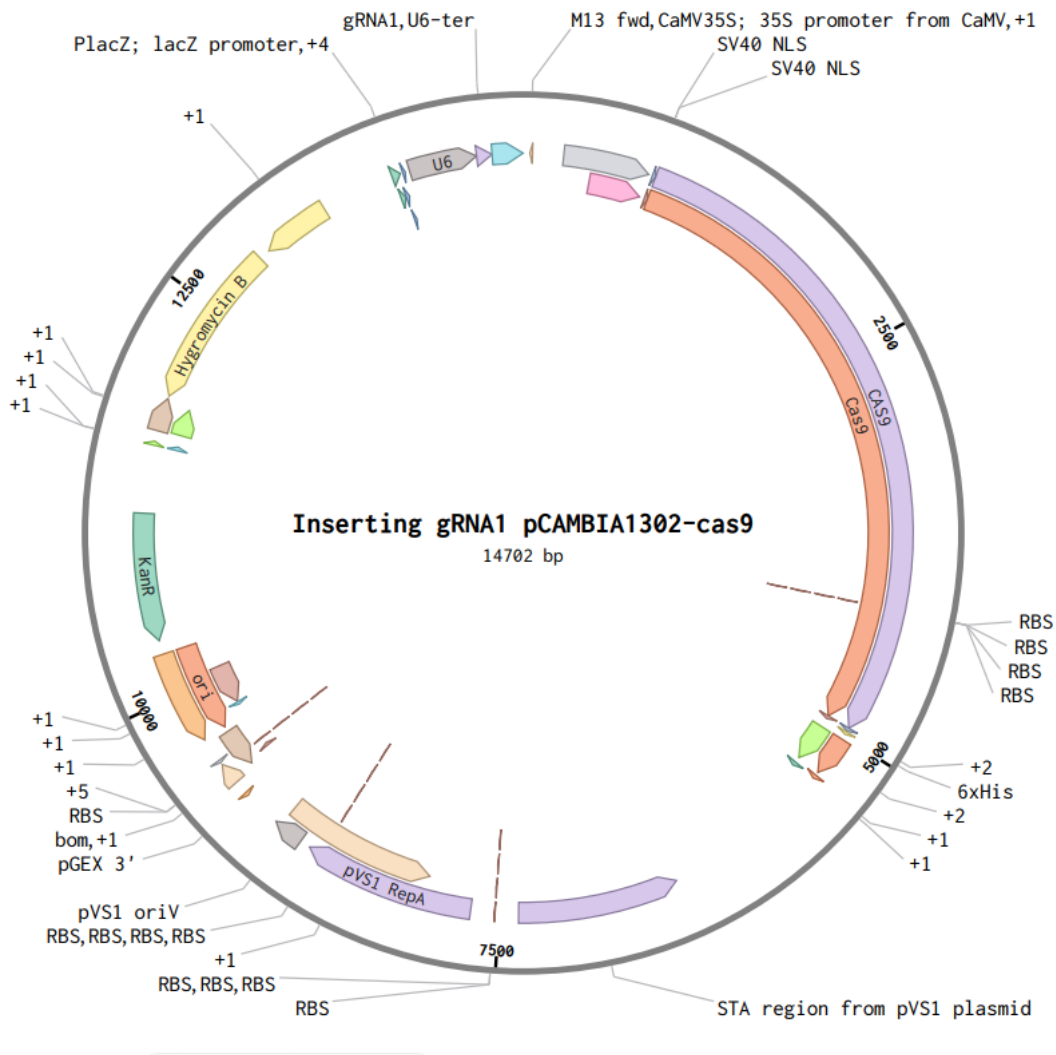


Figure 11. Genetic map of pCambia1302-Cas9-gRNA. Each gRNA flanked by U6 promoter and terminator have been inserted into the backbone.

3.3 Confirming Electroporation Settings

Electroporation settings for *C. vulgaris* vary considerably in literature (Table 2). Therefore, to confirm the correct settings, pCambia1302 containing GFP was electroporated into *C. vulgaris* and fluorescence intensity was recorded to assess GFP expression (Figure 12).

Table 4. Optimized electroporation conditions tested for *Chlorella* transformation. Each condition was evaluated for its impact on transformation efficiency and cell viability in *C. vulgaris*.

	Time constant (ms)	Capacitance (μ F)	Voltage (V)	Resistance (Ω)	References
1	3.4	-	660	-	(M. Kumar et al., 2018)
2	-	25	3300	200	(J. Kim et al., 2021)
3	-	25	360	100	(Ji & Fan, 2020)
4	-	25	1000	200	(Chow & Tung, 1999)

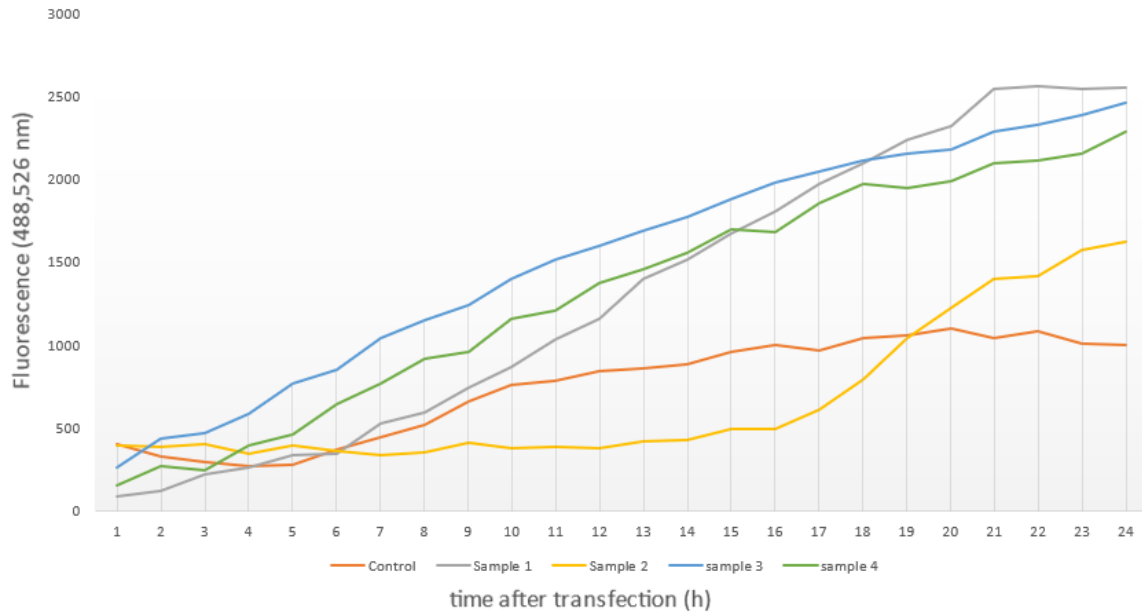


Figure 12. Fluorescence measurements at 488/526 nm for control and electroporated *C. vulgaris* samples under different electroporation conditions. The data represent fluorescence readings over time, highlighting the impact of varied electroporation parameters on GFP expression efficiency. Samples correspond to the electroporation conditions in Table 2 (Control: no plasmid, Samples 2, 3, and 4 correspond to Conditions 1–4, respectively).

Based on the fluorescence results, condition 1 (sample 1) was selected for all future experiments to transform *C. vulgaris*. This condition demonstrated the highest fluorescence intensity over time, indicating greater efficiency in GFP expression. The control represents the growth of *C. vulgaris* wildtype cells which exhibit some absorbance/auto-fluorescence over a large range of wavelengths.

3.4 Selection media confirmation

To confirm the selection method works as expected in *C. vulgaris*, wild-type *C. vulgaris* cells were plated as a lawn on varying concentrations of KClO_3 from 0 to 200 mM and grown at 21°C in the light for 7 days. Figure 13 shows that concentrations of 175 mM and above are sufficient to inhibit growth of wildtype *C. vulgaris*. Therefore 200 mM was selected for all further experiments.

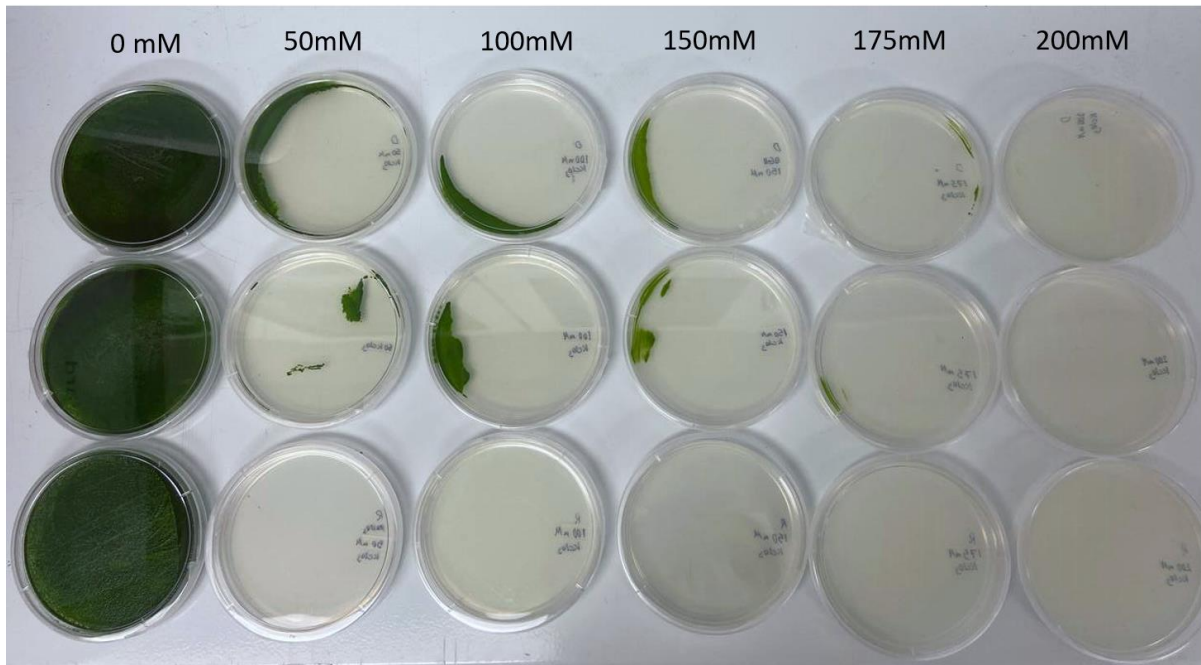


Figure 13. Confirmation of KClO_3 concentration efficacy for selection in *C. vulgaris*. Wild-type *C. vulgaris* cultures were spread across a gradient of KClO_3 concentrations to determine the optimal selection pressure for isolating transformed cells. Concentrations ranged from 0 mM to 200 mM, with increasing KClO_3 levels demonstrating a corresponding decrease in the algal cell growth.

3.5 Transfection

The final stages of this research involved the transfection of constructed plasmids and RNPs into *C. vulgaris* cells via electroporation and plating on selective media for each of the two scenarios described above.

3.5.1 Electroporation of RNPs

The first approach was to deliver RNP complexes into the *C. vulgaris* cells using electroporation. Figure 14 below shows the results of the electroporation process and subsequent plating on selective media for gRNA1-3.

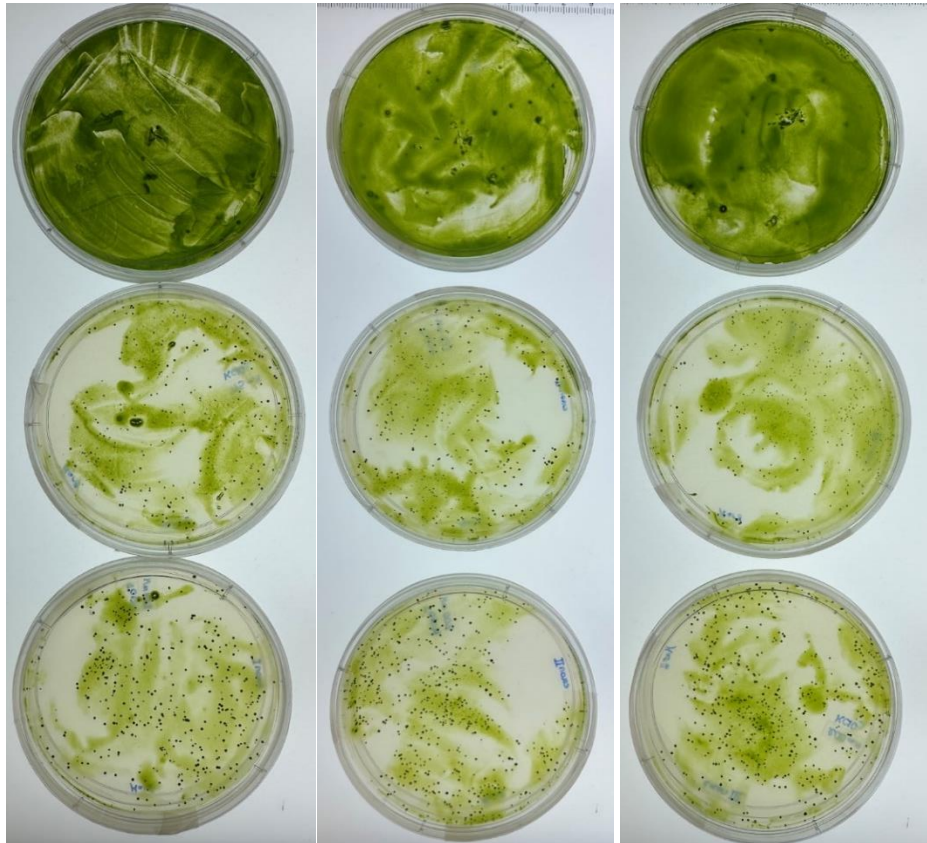


Figure 14. Transformant plates using gRNA1 (left), gRNA 2 (middle), and gRNA3 (right). From top to bottom: BG11 Plates respectively contain nitrite with no KClO_3 , with 175 mM and with 200mM KClO_3 . To compare the efficiency of each gRNA, the number of colonies was counted and summarized in the table below.

Table 5. Colony counts for transformants plates on BG11 containing Nitrite and KClO₃

Sample	175 mM KClO ₃	200 mM KClO ₃
gRNA1	446	498
gRNA2	549	625
gRNA3	456	595

3.5.2 Verification of transformant

To confirm the phenotype, fifty colonies were selected from each 200 mM transformant plate and transferred to two new plates, one contains nitrate (NO₃⁻), and the other one contains ammonium (NH₄⁺). As shown, none of the fifty colonies grew on the nitrite plate for gRNA 1, 2, or 3, whereas the same colonies grew on the ammonium plate (Figure 15, 16, 17), confirming the inability to use nitrate.

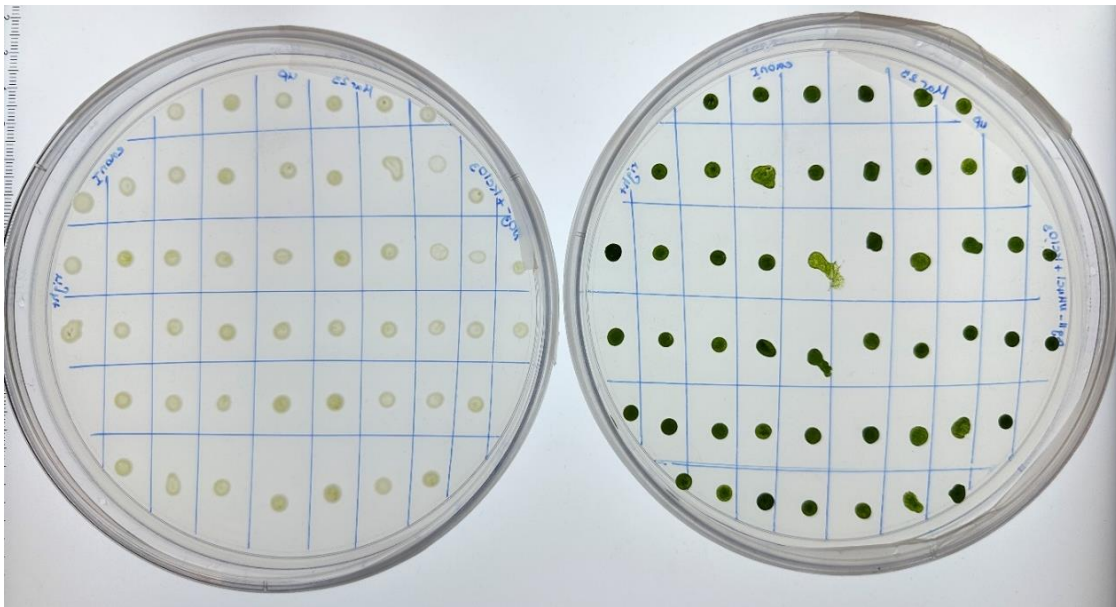


Figure 15. Fifty colonies, in which exon 1 was targeted by gRNA 1, were transferred to plates containing nitrate (NO₃⁻) (top), and ammonium (NH₄⁺) (bottom). None of the fifty colonies grew on the nitrite plate, whereas all the same colonies grew on the ammonium plate.

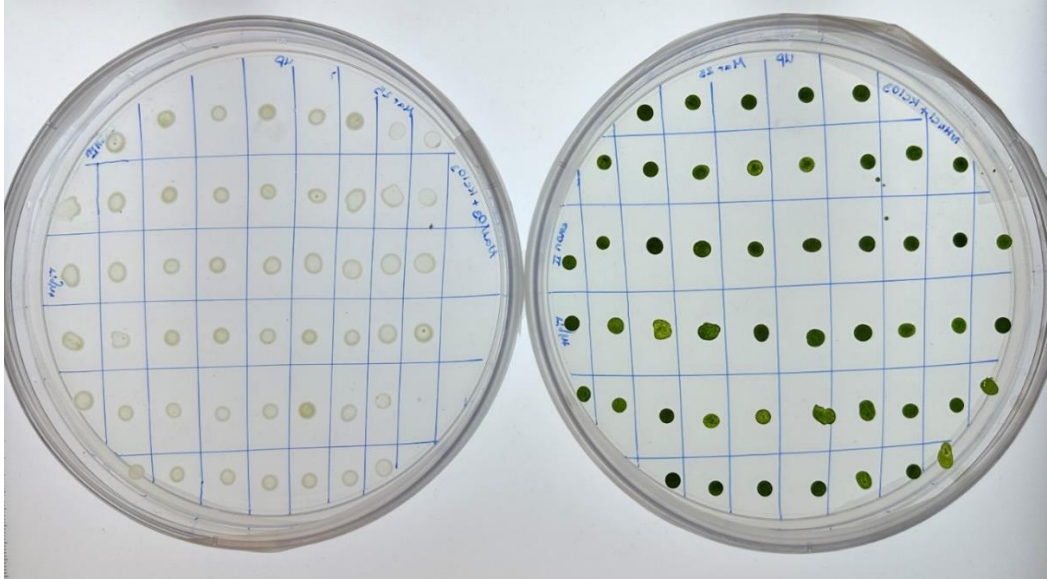


Figure 16. Fifty colonies, in which exon 2 was targeted by gRNA 2, were transferred to plates containing nitrate (NO_3^-) (top), and ammonium (NH_4^+) (bottom). None of the fifty colonies grew on the nitrite plate, whereas all the same colonies grew on the ammonium plate.

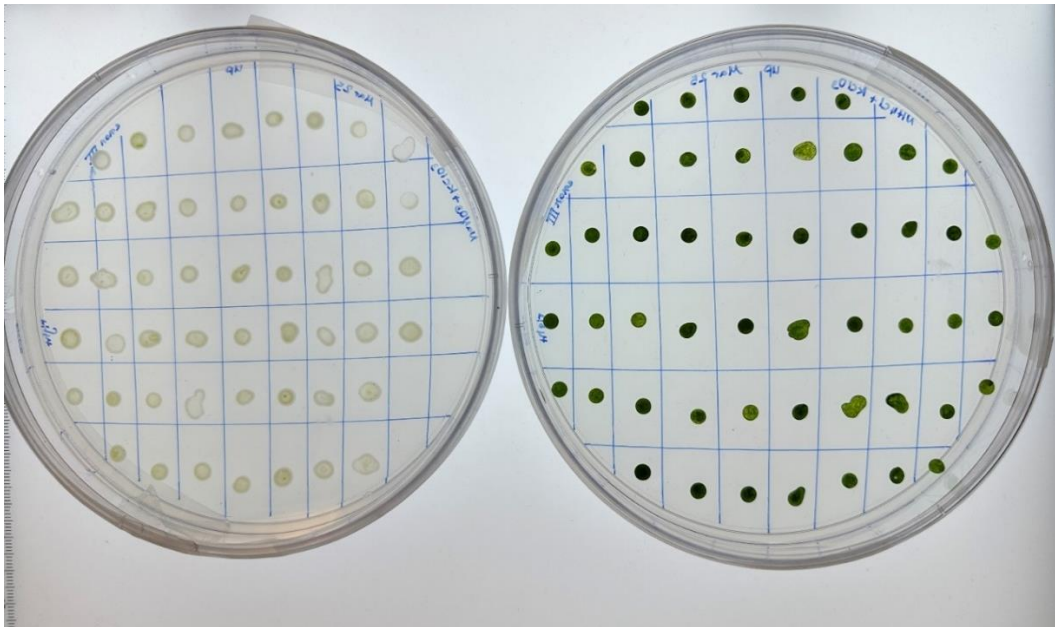


Figure 17. Fifty colonies, in which exon 3 was targeted by gRNA 3, were transferred to plates containing nitrate (NO_3^-) (top), and ammonium (NH_4^+) (bottom). None of the fifty colonies grew on the nitrite plate, whereas all the same colonies grew on the ammonium plate.

3.5.3 T7 cleavage assay

Since the results from the plates confirmed the phenotypes of the transformants, ten colonies were selected for further analysis to confirm their genotypes using the T7EI assay. These three NR exons were successfully amplified from the wildtype *C. vulgaris* using Q5 polymerase (figure 18).

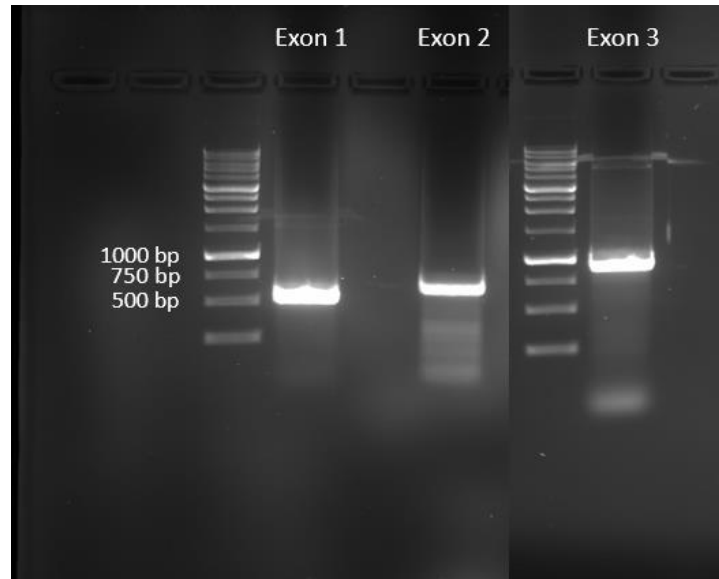


Figure 18. The first three exons amplified with Q5, lane 1 = 1 kb ladder (FrogaBio), Lane 2= exon1 (526 bp), Lane3+ exon 2 (642bp), Lane 4= 1 kb ladder (FrogaBio), Lane 5= exon 3 (886 bp).

Using the transformants, exon 1 of the NR gene of 10 colonies modified using gRNA1 were amplified. Each amplified region was mixed with the wild-type exon 1 fragment and subjected to incubation. Despite multiple attempts, the expected bands were not observed during gel electrophoresis (Figure 19, 20).



Figure 19. Amplification of exon 1 of the NR gene was performed in 10 colonies and used for the T7 cleavage assay. Lane 1: Water, Lane 2: Exon 1 of NR in wild type, Lane 3-7: Transformed colonies, Lane 8: 1kb FroggaBio ladder, Lane 9-13: Transformed colonies.

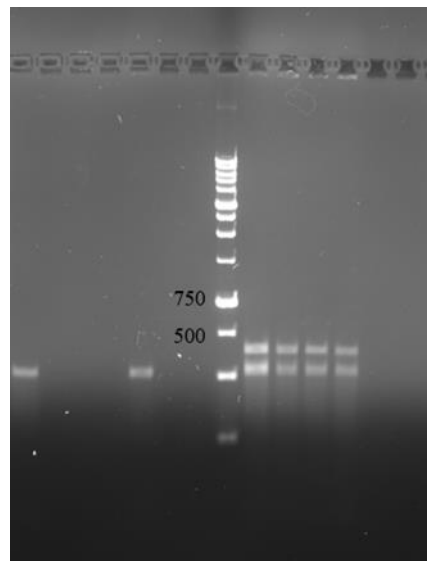


Figure 20. After incubation with T7 endonuclease, three bands of sizes 520, 180, and 350 bp were expected. However, the observed bands on the gel did not match the expectations.

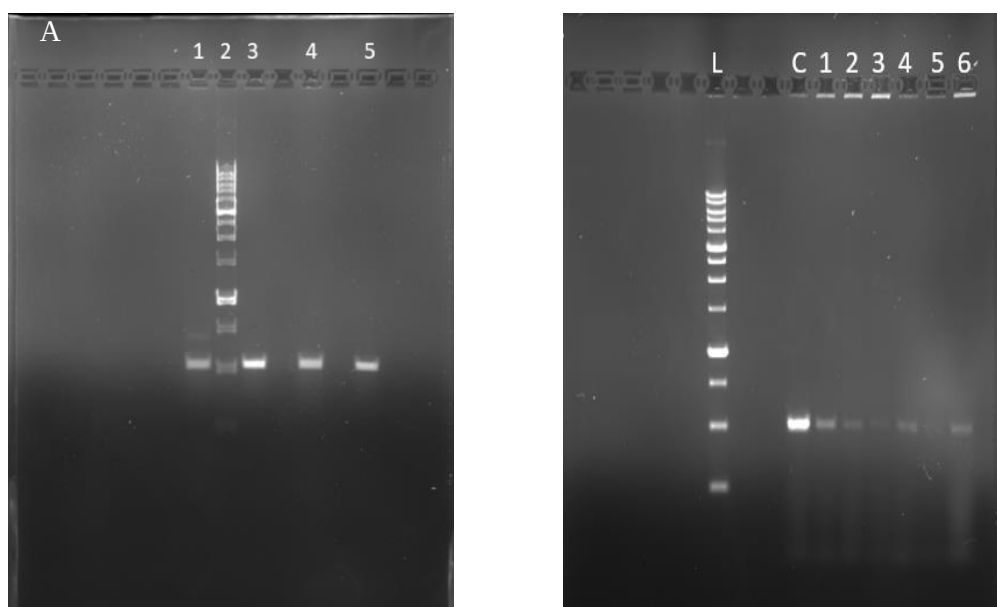


Figure 21. A) Amplification of exon 1 of the NR gene was performed in more colonies and used for the T7 cleavage assay. Lane 1: exon 1 of NR in wild type, Lane 2: 1kb FroggaBio ladder Lane 3,4 and 5: Transformed colonies. B) Lane L: 1kb FroggaBio ladder, Lane C: exon 1 of NR in wild type Lane 1-6: Transformed colonies.

3.5.3.1.1 Sequencing results of targeted exons in NR

After multiple unsuccessful attempts with the T7 endonuclease assay to confirm the mutations within the targeted exon regions, efforts shifted toward amplifying a ~1 kb region surrounding the target site for analysis by DNA sequencing. The amplification process was challenging due to the high GC content of the target region, which hindered efficient extension during PCR. Below, one of many images that illustrate some of the failed attempts related to different conditions to amplify the region, highlighting the experimental difficulties encountered.

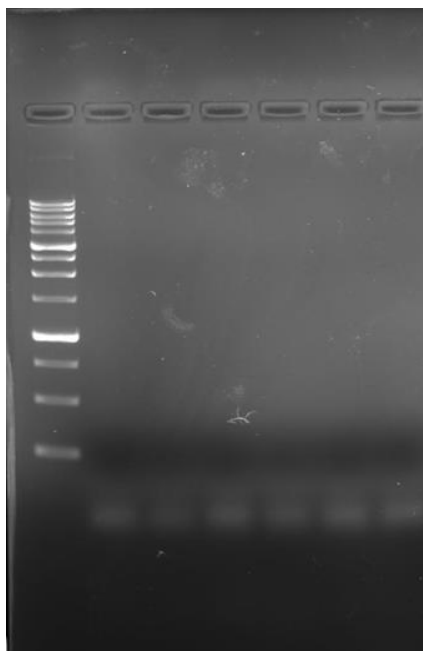


Figure 22. A gel electrophoresis result from many unsuccessful attempts to amplify a ~1 kb region surrounding the target site for sequencing. The high GC content of the region likely contributed to the amplification difficulties observed.

Finally, by designing and utilizing an optimized set of primers as well as Phusion DNA polymerase with GC enhancer, the ~1 kb fragment was successfully amplified in twelve colonies and subsequently submitted for sequencing to confirm the presence of the targeted mutations (Figure 22).

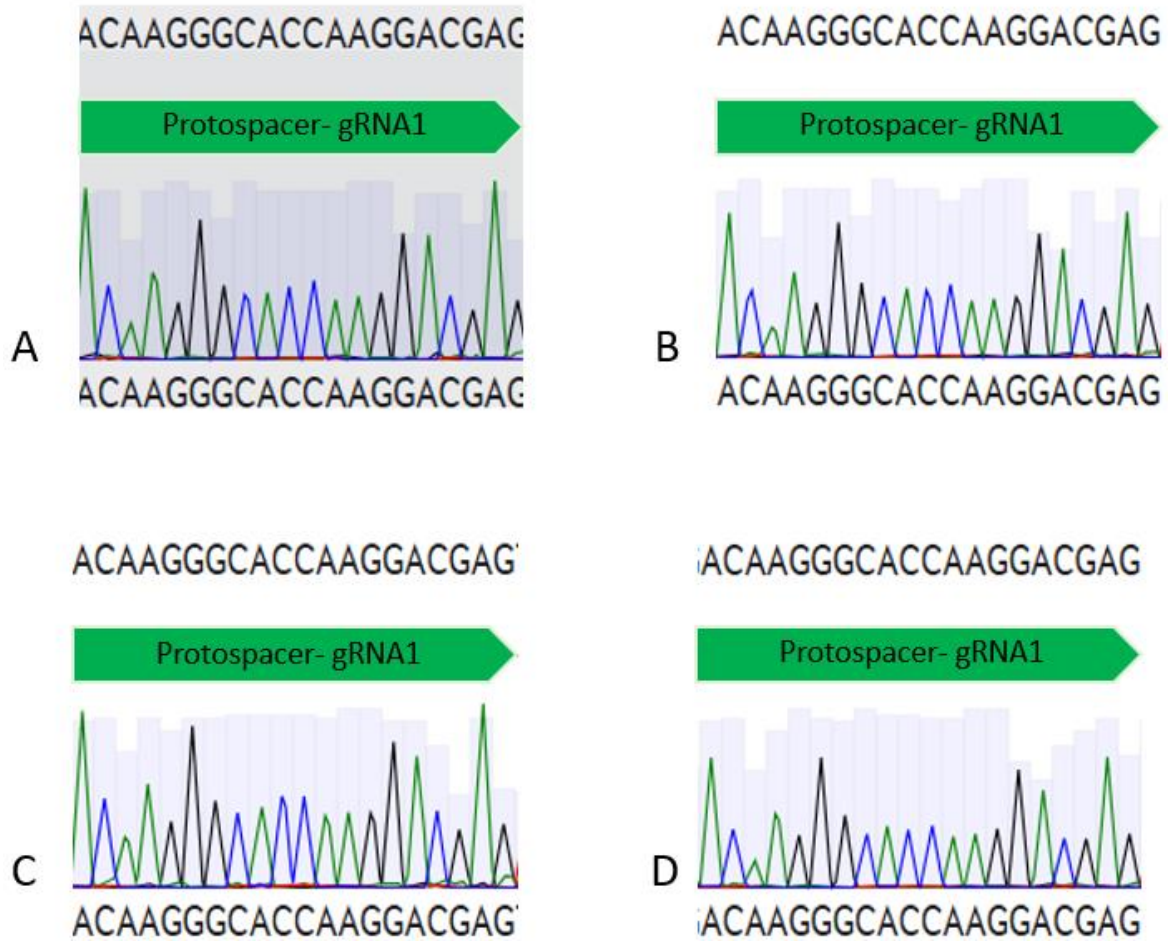


Figure 23. Sequencing data for the wild-type and three transformed colonies: (A) The targeted region in the wild-type, which exactly matches the designed protospacer 1. (B, C, and D) Alignment of protospacer 1 with colonies 1, 2, and 3, respectively.

Despite the observed phenotype, sequencing results revealed no mutations in the targeted sequence. To validate the experiment and the observed phenotype, the procedure was repeated, and further controls were added.

3.6 Repeating the transfection with RNPs

Transfection of *C. vulgaris* cells with RNP complexes was carried out using the previously established protocol, with one modification: the initial cells were inoculated in BG11 medium without a nitrogen source. Since *C. vulgaris* cells can store nitrogen and survive without it for approximately 15 days (C. Chen et al., 2024), the cells were subjected to nitrogen starvation for 10 days. Following this period,

they were inoculated in BG11 medium for 24 hours to reach an OD680 of 0.514, minimizing the impact of accumulated nitrogen on the results. Figure 24 shows the transformant plates.

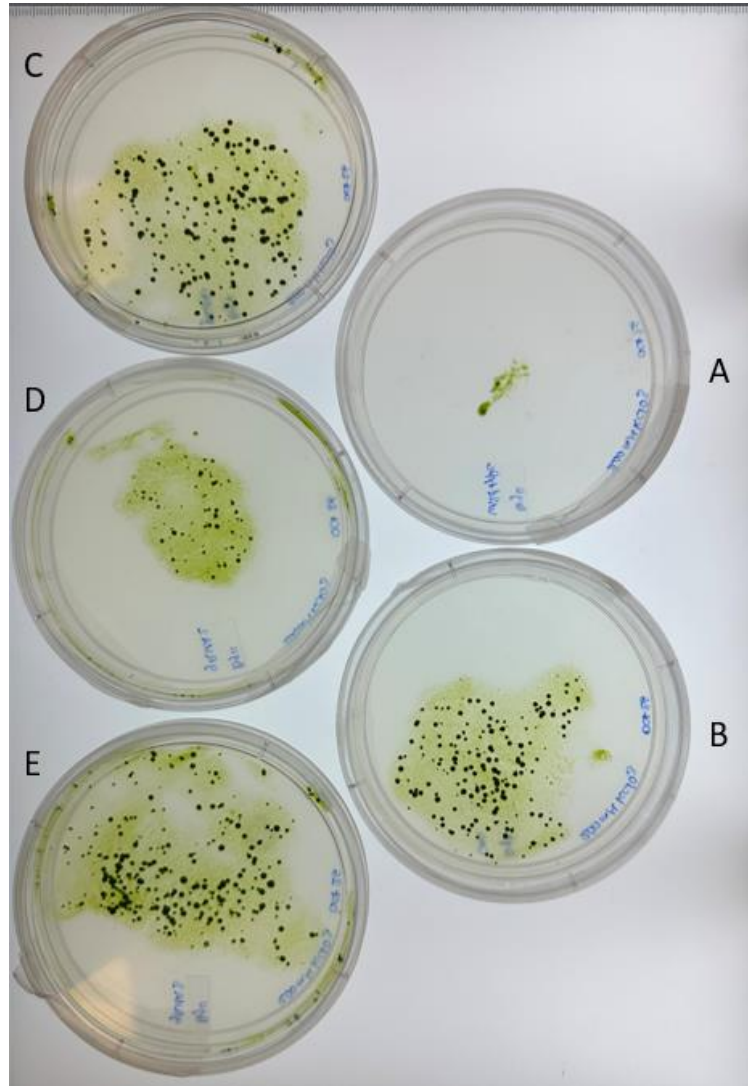


Figure 24. Transformant plates. A) Negative control (wild-type cells) B) Negative control (cells treated with Cas9 only). C, D, and E correspond to RNP1, RNP2, and RNP3 treatments, respectively.

The number of colonies on each plate is presented in Table 4. As shown in Figure 24, the plate containing cells transformed with only Cas9 exhibits colony growth. This could be because of off-target activity of the Cas9 protein, leading to unintended cuts elsewhere in the genome that affect pathways

related to nitrogen assimilation. Such off-target effects might enable the cells to adapt and survive under selective conditions without exhibiting the desired genotype.

Table 6. Colony counts for transformants plates on BG11 containing Nitrite and 200mM KClO₃

Sample	Nitrite with 200 mM KClO ₃
gRNA1	228
gRNA2	94
gRNA3	294
Cas9	185

3.6.1 Verification of transformants

Phenotypic screening was conducted to confirm the phenotype of the transformants. Twenty-five to Fifty colonies were selected to be transferred to plates: one containing nitrate, another with nitrate and 200mM KClO₃, and the last with ammonium and 200 mM KClO₃. Wild-type *C. vulgaris* was added as a positive control and as shown can grow on nitrate only plates while the transformants cannot (Figure 25).

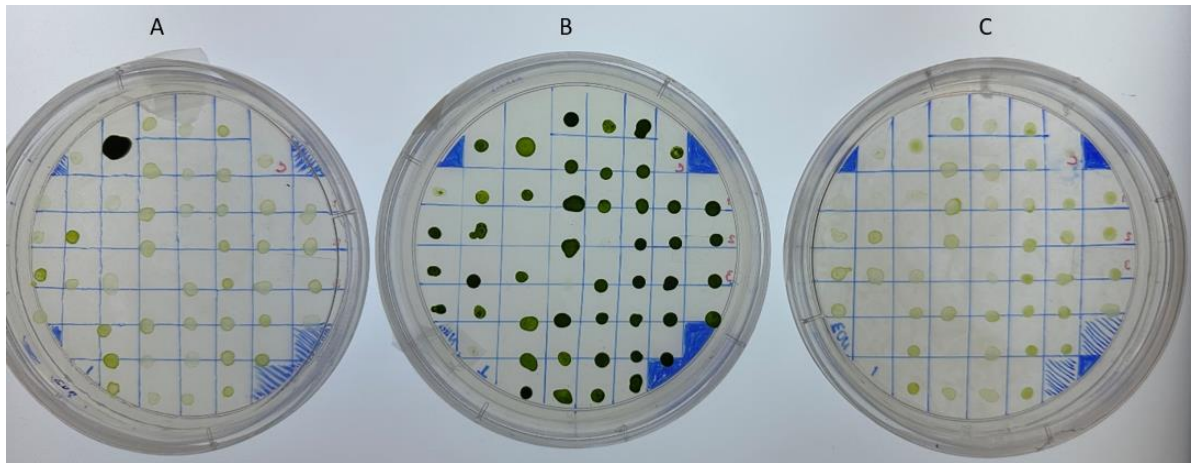


Figure 25. Fifty colonies, in which exon 1 was targeted by gRNA 1, were transferred to plates A) containing nitrate (NO₃⁻), B) ammonium (NH₄⁺) with 200mM KClO₃, and C) Nitrate with 200mM KClO₃. None of the fifty colonies grew on the nitrite plate (except the positive control (wild-type cell)) and nitrate with KClO₃, whereas all the same colonies grew on the ammonium plate.

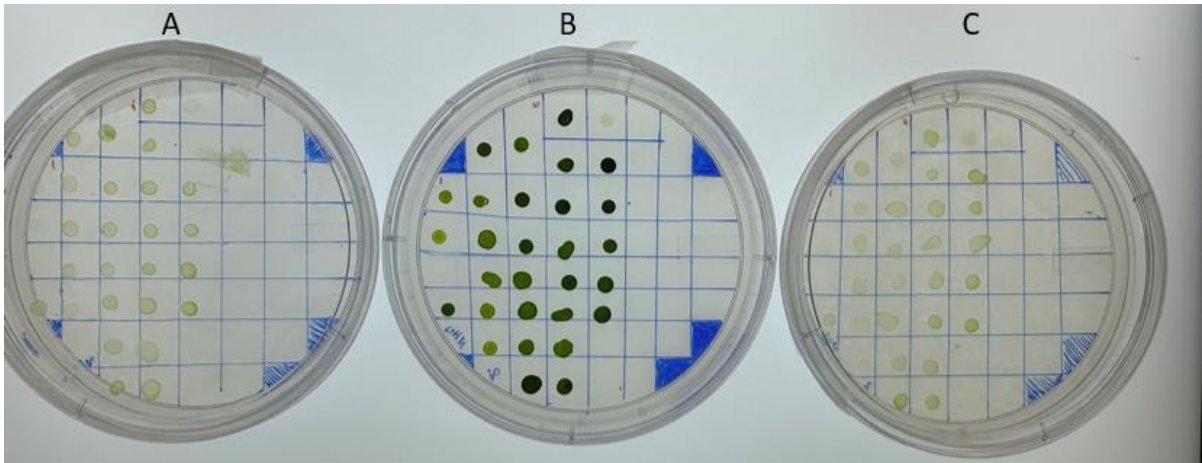


Figure 26. Twenty-five colonies, in which exon 2 was targeted by gRNA 2, were transferred to plates A) containing nitrate (NO_3^-), B) ammonium (NH_4^+) with 200mM KClO_3 , and C) Nitrate with 200mM KClO_3 . None of the fifty colonies grew on the nitrite plate, except for the positive control, which showed limited growth. (wild-type cell)) and nitrate with KClO_3 , whereas all the same colonies grew on the ammonium plate.

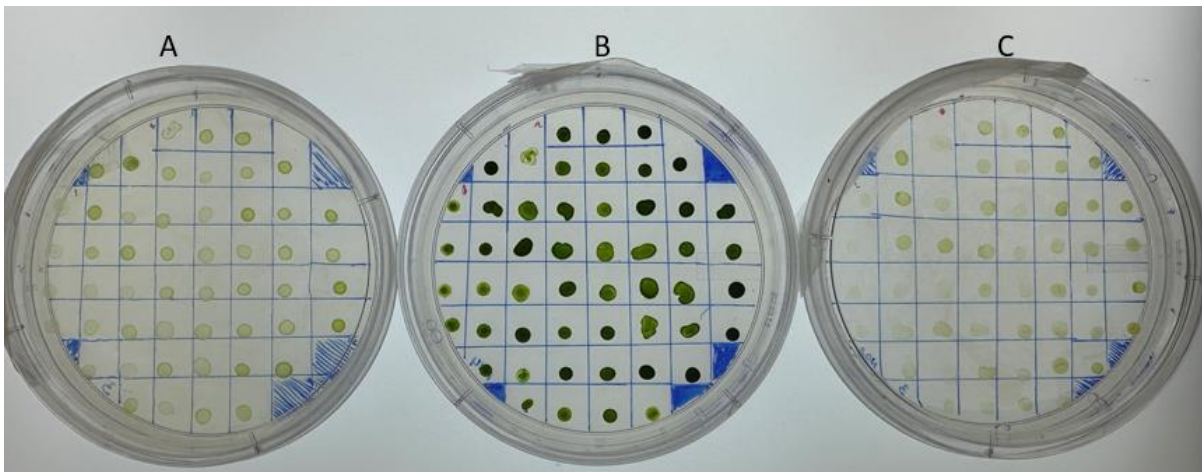


Figure 27. Fifty colonies, in which exon 3 was targeted by gRNA 3, were transferred to plates A) containing nitrate (NO_3^-), B) ammonium (NH_4^+) with 200mM KClO_3 , and C) Nitrate with 200mM KClO_3 . None of the fifty colonies grew on the nitrite plate, except for the positive control, which showed limited growth. (wild-type cell)) and nitrate with KClO_3 , whereas all the same colonies grew on the ammonium plate.

To further evaluate the transformants' phenotypic response, 10 colonies targeted in exon 1 were selected and inoculated into two different BG11 liquid media: one containing ammonium as the nitrogen source and the other containing nitrate. This step was performed to confirm the inability of the transformants to utilize nitrate as a nitrogen source, complementing the results observed on solid media. As shown in Figure 30, after 48 hours, the colonies grew well in flasks containing ammonium but exhibited signs of severe stress and were nearly dying in flasks with nitrate as the nitrogen source.

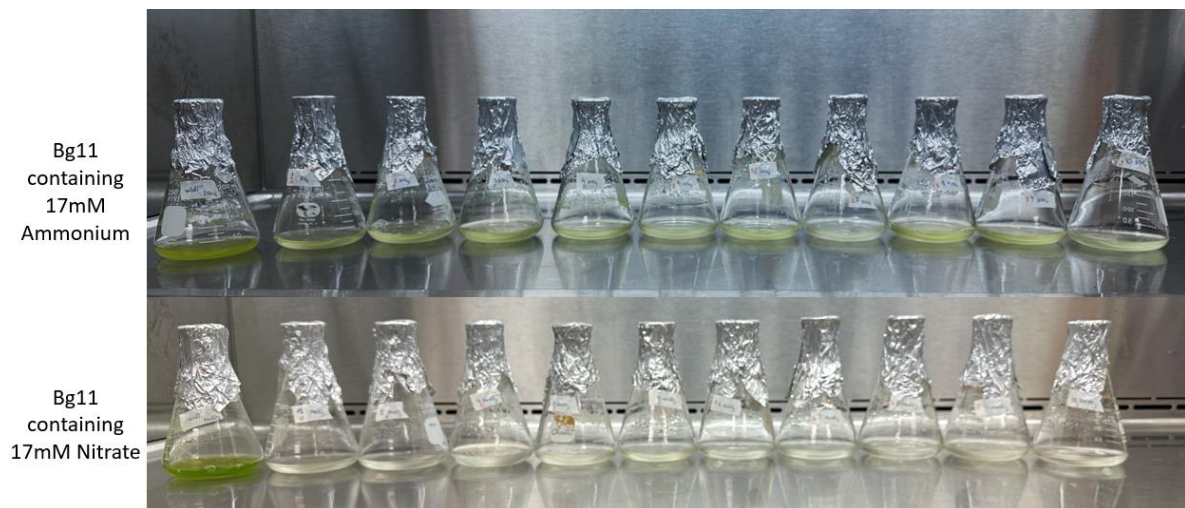


Figure 28. Growth comparison of exon 1-targeted transformants in BG11 liquid media with different nitrogen sources.

In figure 28, the leftmost flasks in both the upper and lower rows represent the wild-type controls. These wild-type cells show healthy growth in both ammonium-containing media (upper figure) and nitrate-containing media (lower figure). From left to right, colony numbers 1 to 10 in the upper figure (ammonium-containing media) demonstrate visible growth after 48 hours but displayed poor growth in flasks with nitrate, indicating their inability to utilize nitrate due to the targeted knockout of the nitrate reductase gene. In contrast, the corresponding colony numbers in the lower figure (nitrate-containing media) show no growth, suggesting that these transformants lack functional nitrate reductase activity. This inability to grow in nitrate media supports the disruption of the nitrate reductase gene, confirming the phenotypic effects of the targeted genome editing.

3.6.2 Transfection using Cas9-gRNA plasmids

Similar to the previous experiments, *C. vulgaris* was electroporated with each of the three plasmids (5 µg). However, no colony growth was observed on the selective plates (Figure 29) indicating that the knockout was not successful. This could be due to a number of reasons, either insufficient expression of Cas9 in the cell, poor nuclear localization, or insufficient expression of gRNA. Further optimization and analysis are needed.

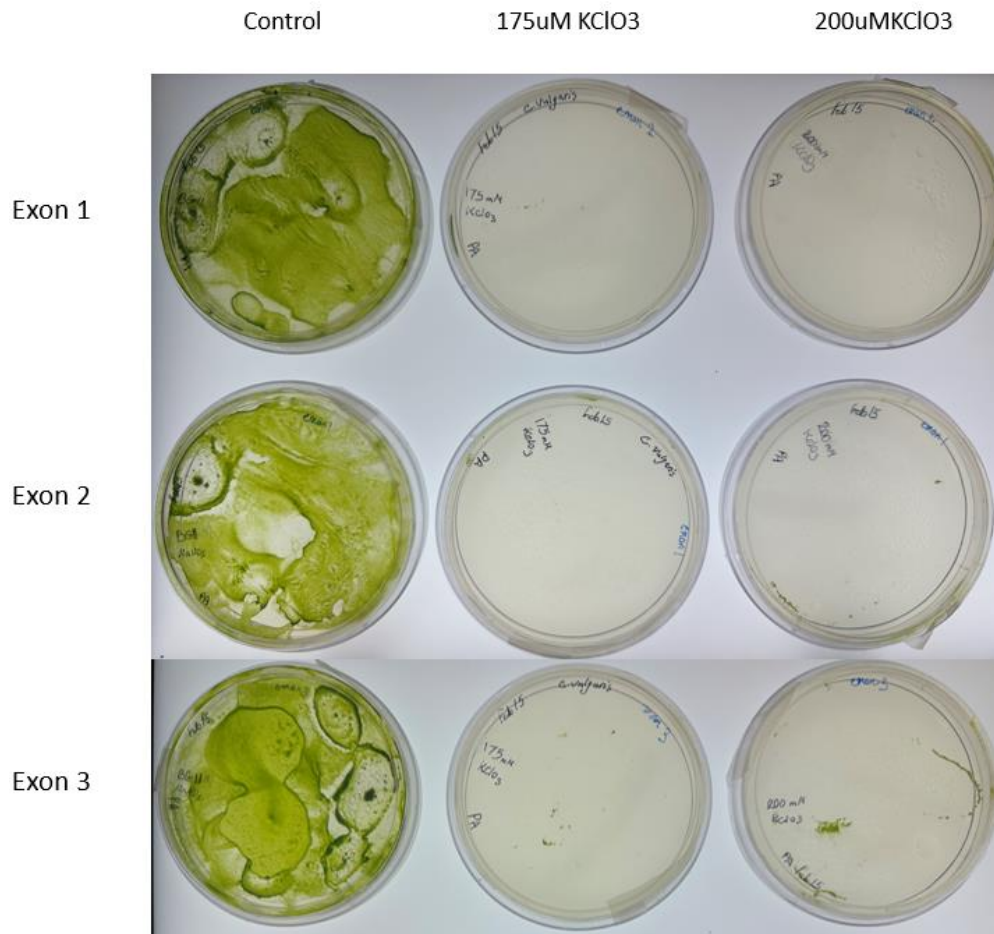


Figure 29. Transformed *C. vulgaris* cells with plasmids containing Cas9 and gRNA constructs. Plates show selective BG11 media containing nitrite and 175 and 200 mM KClO₃. No colony growth was observed.

Chapter 4- Discussion

This research contributes to the field of site-specific genome editing in *C. vulgaris*, a microalga with significant potential for various biotechnological applications (Al-Hammadi & Güngörmüşler, 2024). The study by Kim et al. (2021) represents a foundational effort in developing CRISPR-Cas9 genome editing tools for *C. vulgaris*, successfully targeting the NR and APT genes (J. Kim et al., 2021). Their work demonstrated the utility of both plasmid-mediated and RNP-based approaches for genome editing by generating three confirmed NR mutants from hundreds of colonies resistant to KClO₃ across multiple trials, reflecting a low success rate. While Kim et al. explored the feasibility of genome editing, this study aimed to develop a more efficient and replicable approach to genome editing in *C. vulgaris*. By optimizing transformation parameters and delivery methods, this research sought to ensure greater reliability and adaptability, paving the way for consistent and reproducible results in this microalga. Although RNP-mediated genome editing showed promise, the results from the plasmid-based system remained inconclusive.

The direct delivery of RNP complexes offers several advantages over plasmid-mediated transformation, such as quicker initiation of gene editing and a reduction in off-target effects due to the limited half-life of Cas9 protein and gRNA in the host cell. With RNP delivery, the Cas9 protein and gRNA are directly introduced into the cells, eliminating the need for the cells to transcribe and translate them (Yip, 2020; Zhang et al., 2021). This approach reduces the risk of plasmid integration into the genome, which could lead to unintended genetic modifications (Du et al., 2023). However, plasmid-mediated transformation has its own benefits, its stable and simple that make it a suitable method for experiments that require prolonged gene expression (Vakulskas & Behlke, 2019).

In this study, both delivery methods were tested to evaluate their effectiveness in *C. vulgaris*. The results showed that KClO₃ resistant colonies were obtained by RNP knockout and that these transformants could no longer grow on nitrate on plates or in liquid cultures confirming the expected phenotype. However, DNA analysis showed that these transformants did not have the expected genotype. These results were consistently repeated, suggesting that some other mechanism is responsible for their inability to assimilate nitrate. In the second experiment, the cells were also electroporated with only Cas9 without gRNA which resulted in a similar number of colonies. This suggests that perhaps some off target effects are present and resulting in the observed phenotypes. This issue highlights the technical difficulties involved in genome editing in *C. vulgaris*, emphasizing the

need for improved molecular tools and protocols to achieve consistent and reliable outcomes as nitrate reductase auxotrophy is commonly used as a selectable marker in *C. vulgaris* (J. Kim et al., 2021; M.-J. Kim, Kim, Abdellaoui, & Choi, 2023; M.-J. Kim, Kim, Kim, & Choi, 2023; M.-J. Kim, Kim, Kim, Lee, et al., 2023; M.-J. Kim, Kim, Kim, Yoo, et al., 2023), and *Nannochloropsis oceanica* (Naduthodi et al., 2021; Q. Wang et al., 2016).

In an attempt to address this challenge, cells were nitrogen starved prior to cultivation in nitrate for preparation of electrocompetent cells in order to minimize escaped colonies. As shown by Chen et al. 2024, *C. vulgaris* is capable of growing in media without nitrogen for until 10 days (C. Chen et al., 2024), suggesting that this species can accumulate nitrogen stores which could be used when the KClO_3 selective media is used to circumvent nitrate reductase expression. However, this is just one possible hypothesis for the observations of the desired phenotype without the genotype. CRISPR-Cas9 can result in off target effects, where DSB are induced in other locations throughout the genome. One possibility is that a mutation is occurring downstream of the first 3 exons which were fully sequenced rather than at the targeted location. Another possibility is that the nitrate transporter used by *C. vulgaris* was mutated, resulting in the exclusion of chlorate and nitrate from the cell (Sanz-Luque et al., 2015). This type of mutation would result in the same phenotype without any mutation in the NR gene. DNA sequencing of the nitrate transporter would be required to confirm this hypothesis. The presence of colonies on the Cas9 only plates suggest that some type of off-target effect is responsible for the colonies obtained using this genome editing method.

Chapter 5 – Conclusions and Recommendations

5.1 Conclusion

This work contributes to the growing body of knowledge in CRISPR-Cas9 as a transformative tool for genome editing in *C. vulgaris*. While phenotypic assays confirmed successful generation of nitrate auxotrophs, these transformants lacked the expected genotype emphasizing the need to further refine this experimental technique. These results also demonstrate the challenges in genome editing of microalgae, where very few reports exist and, typically, very few positive colonies are recovered. Without significant improvements to these techniques, microalgae genome engineering will remain the limiting step in realizing the full biotechnological potential of *C. vulgaris*.

5.2 Recommendations

Going forward, it would be valuable to sequence the colonies from the second trial, as they have not yet been sequenced. Sequencing a larger number of colonies, such as 50, would provide a more comprehensive analysis of the mutation efficiency and help confirm whether the observed phenotypic changes are consistently linked to targeted gene disruption or not. Additionally, using protoplasts instead of the unmodified cells could improve transformation efficiency by reducing the challenges posed by the cell wall, which may hinder plasmid or RNP uptake. Redesign of the gRNAs may also increase the specificity of the technique, or use of another gene with less chance of escaped colonies for knock-out might be preferable. Finally, combining CRISPR-Cas9 with the knock-in of an antibiotic selection marker might increase the efficiency of knockouts in *C. vulgaris* by having two selectable markers.

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Appendix A

Table 7. List of primers

Name	Sequence
NR-exon1-fwd	GAGGGTCACCTTCCTTTGGCG
NR-exon1-rev	GAGGCGTTGAGTGGCAAGAGG
NR-EXON1-3FWD	CCCTGCCAACTCCTCCCTCATACTC
NR-EXON1-3-REV	GGAGCGTTAGTCTGTGACTTGCTGC
T7.gRNA1-F	GCAGCAATGAAAGCCAATGG
T7.gRNA1-R	TCGACGTTGAAGGGATGCC
T7.gRNA2-f	ATTCCTCCTCTGCACCTTCC
T7.gRNA2-R	ATTGTGAATGTGGTGGGCG
T7.gRNA3-F	GGCGTGTATGGCTAGCTCAT
T7.gRNA3-R	CTGACTCAATGCAGCACCG
NR-Seq-FWD	TGTCAGGTCATGTGCACAAC
U6.P-FWD	AATTCGAGCTCGGTACCCCGACTTGCCTTCCGCACAATAC
U6.P-REV	AATCACTACTTCGACTCTAGCTGTATATAAAC
gRNA1+Ter-fwd	AGAGTCGAAGTAGTGATTACAAGGGCACCAAGGACGAGGTTTTAG AGCTAGAAATAGCAAG
gRNA2+Ter-fwd	AGAGTCGAAGTAGTGATTTGCTCCTCCCACTTAATCTGGTTTTAGA GCTAGAAATAGCAAG
gRNA3+Ter-fwd	AGAGTCGAAGTAGTGATTGTACAGGTCACGTCAACACTGTTTTAGA GCTAGAAATAGCAAG
gRNA+ter-rev	CGACTCTACAGGATCCCCTATTGGTTTATCTCATCGG
Cas9-NLS-FWd	TGACCATGGTAGATCTGACTCCCAAGAAGAAGAGGAAGGTG
Cas9-NLS-Rev	AATTCGAGCTGGTCACCAATAGCCGGATCTCAGTGGTG

Newcloning(cas)-	CACGACACACTTGTCTAC
Fwd	
Newcloning(cas)-	ATTAAGCGCAAATCCGCTTTATC
Rev	
NR-LH-FWD	GAGCTCTCATACTGCGGCTCAA
NR-LH-REV	ACCATGTTCTCCTCCTTGCGG

Appendix B

Standard curve depicting the relationship between OD680 and cell density of *C. vulgaris* in BG11 medium supplemented with glucose. The curve is based on experimental data showing that an OD680 of ~0.5 corresponds to a cell density of 2×10^7 cells/mL.

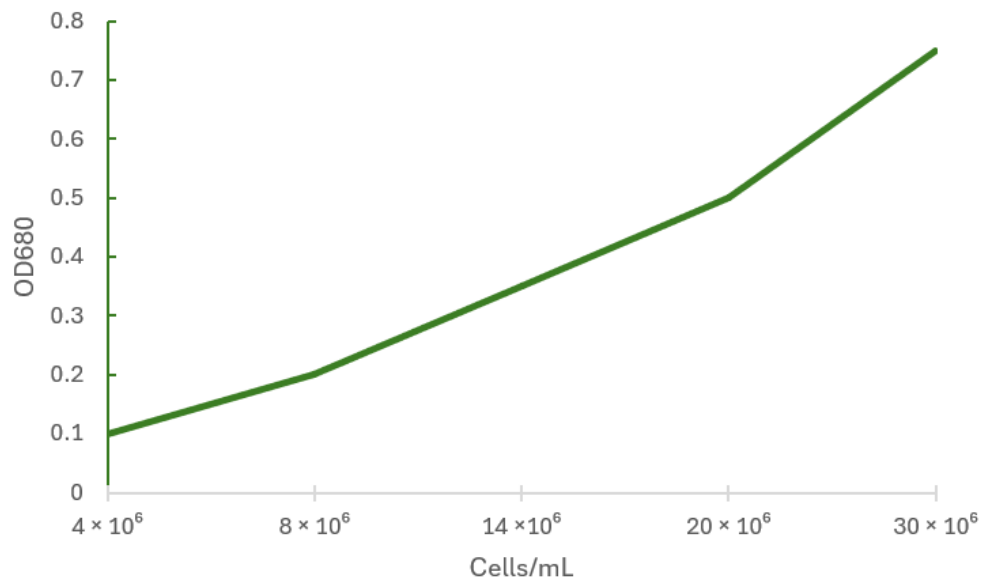


Figure 30. Standard curve showing the correlation between OD680 and *Chlorella vulgaris* cell density