Evaluating phenotype of gene knockout of algeanan biosynthesis related gene PRX and COPOX in *Nannochloropsis oceanica*

Nannochloropsis oceanica Transformation to Disrupt Algeanan Biosynthesis

MONICA WANG
monica.wang@berkeley.edu
Biology of Algae Laboratory PMB 120L, University of California, Berkeley
Berkeley, California

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ABSTRACT

As climate change continues to be a rising concern, discovering alternative biofuel sources as well as methods to sequester carbon has been at the forefront of much research, including this one. For this paper, we will be generating gene knockouts of hypothesized algaenan biosynthesis genes– PRX and COPOX –in the marine microalga *N. oceanica* with CRISPR and observing the generated mutants phenotype using various assays to evaluate how PRX and COPOX genes affect the development of algaenan in *Nannochloropsis oceanica.* While doing such experiments would result in a greater understanding of the algaenan biosynthesis pathway, during the experiments, we ran into unforeseen challenges with our wild-type *N. oceanica* being contaminated by an NPQ mutant which resulted in us being unable to make conclusions about our results. Because of this, further experimentation and a better understanding of the mutation in the supposed wild-type *N. oceanica* must be done before further research can be conducted.

Key Words: Episome, GFP, Hygromycin, algeanan, Nannochloropsis

INTRODUCTION

With the increase in human activities, especially the burning of fossil fuels, the amount of atmospheric CO_2 has risen greatly and has caused measurable global warming. In addition to increasing levels of atmospheric CO_2 being a factor of climate change, increasing CO_2 levels has also led oceans to become more acidic due to increased absorption of CO_2 by seawater which has potential disruptive effects on marine plankton and coral reefs (Sundquist et al., 2008). While there are many ways to address increasing levels of atmospheric CO_2, one proposed method is carbon sequestration. Carbon sequestration is both the natural and deliberate processes by which CO_2 is either removed from the atmosphere or diverted from emission sources and stored in the ocean, terrestrial environments (vegetation, soils, and sediments), and geologic formations (Sundquist et al., 2008). Microalgae is a key player in where carbon sequestration can happen.

For this paper, we will be working with *Nannochloropsis oceanica*, a unicellular marine microalga capable of accumulating large amounts of oil (Kilian et al., 2011). While *N. oceanica* is a member of the Ochrophyta, *N. oceanica* is a non-motile coccoids 2-4 μm in diameter that reproduce by asexual fission during the night period where they also shed their mother cell wall during the process (Gee et al. 2023). Additionally, *N. oceanica* has a cell wall layer also known as algaenan that is extremely resistant to degradation (Gee et al. 2023). While algaenan has no

specific defined chemical structure, it is made up of long hydrocarbon chains, cross linked with ether linkages (Gee et al. 2023). Once the *N. oceanica* algaenan layer is shed from the cell during asexual reproduction, it can persist on the ocean floor for millions of years, offering a potential method of oceanic carbon sequestration (Gee et al. 2023). However, concurrently, the hardy structure of algeanan also poses a barrier to efficient oil extraction from *N. oceanica*. According to prior research on algae potential biodiesel production, *N. oceanica* has gained widespread acceptance as a possible candidate for the production of biodiesel due to its high lipid productivity, rapid growth, excellent resistance to biotic contamination, ability to meet biodiesel quality standards, high photosynthetic efficiency, and the ability to convert carbon dioxide into lipids in the form of triacylglycerol (TAGs) (Senousy et al. 2023). While algaenan provides potential solutions for carbon sequestration and understanding to biofuel extraction, there is still much unknown about algeanan biosynthesis and degradation.

With this in mind, for this paper, we will be generating gene knockouts of two hypothesized algaenan biosynthesis genes– PRX and COPOX –in the marine microalga *N. oceanica* with CRISPR. These two algaenan biosynthesis genes are believed to be involved in two stages of algeanan biosynthesis: precursor synthesis (long-chain diols and long-chain alkenols) and polymerization. Our current model of polymerization of algeanan is based on the polymerization of lignin which requires LCD/LCA hydroxyl groups to be activated into radicals that subsequently react with double bonds to form ether linkages that make algeanan so recalcitrant. It is further hypothesized that this polymerization process is responsible for producing H_2O_2. With these knockout mutants, we will observe the generated mutant phenotype using various assays to evaluate how PRX and COPOX genes affect the development of algaenan in *N. oceanica.* By doing this, we will get greater insight and understanding on the algaenan biosynthesis pathway.

MATERIALS AND METHODS

Episome Construction

The episome construct used was designed prior to the start of our experiments by our GSI, Ethan Boynton. The episome contained hygromycin-resistant gene, cas9 gene, GFP gene, and DNA sequences encoding two gRNAs sequences (Figure 1) for editing of the COPOX1 and PRX1 genes

Algal Harvest

WT *Nannochloropsis oceanica* algal cells were harvested at log-phase growth at low cell density and were repeatedly washed and concentrated. Algal cell harvest was performed before the start of our experiment by our GSI, Ethan Boynton.

Algal Transformation

Concentrated *Nannochloropsis oceanica* algal cells were mixed with 5 ul of provided episomal DNA with genes of interest (PRX1 or COPOX1). 100 ul of DNA-algal cell suspension was transferred and electroporated in an electroporation cuvette. After electroporation, the 100 ul of DNA-algal cell suspension was washed using f/2 liquid media and transferred to a 10 ml f/2 liquid medium tube. After this transfer, the 10 ml f/2 tube was placed in a shaded bag to recover overnight.

After overnight recovery, we centrifuged the 10 ml f/2 liquid medium with transformed algal cells to create cell pellets. We poured off the f/2 medium and resuspended the cell pellet in the remaining f/2 medium. We then aliquoted the cell suspension evenly to two f/2 + hygromycin plates and spread out the cell suspension using glass beads.

Array Subclones and Scoring their Phenotypes

After our initial algal plates of f/2 + hygromycin grew colonies, we randomly selected around 24 isolated transformed colonies using pipette tips and restreaked them in an array on a new f/2 media plate.

After the array plate grew and the sequence were confirmed *Nannochloropsis* transformant sub-clones, we picked up cells from the array patches using a pipette tip and resuspended the cells in 20ul of sterile water. 5ul of the solution were dotted onto 3 different plates: f/2, f/2 with various antibiotics, and f/2 with 3-pentanol. This gave us 3 plates each with 24 dotted samples of 5ul. After a week, we scored the growth of these algae colonies for each plate, expecting that the colonies on the antibiotic and pentanol plate would grow worse.

Algal Mutant Screening – colony PCR and Agarose Gel Electrophoresis

Using PCR, we amplified DNA that allowed us to purify and sequence our genes of interest, identify clones that contain edited sequences. To first generate PCR products, we used pipette tips to pick up cells from the f/2 array plate and resuspended them in 10 ul of sterile water. This solution was our DNA template for PCR reaction. We then prepared a PCR cocktail that contained forward and reverse primers, dNTPs, DNA polymerase, and GC buffer. 22ul of the PCR cocktail was loaded into each PCR tube. The algal colony suspension was added to each

PCR tube separately and lastly. Each PCR tube had a total volume of 25 ul with concentrations of 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 mM dNTPs, 0.1 25 μl Phusion DNA polymerase, 1X Phusion polymerase high GC buffer, and 3μL of your algal suspensions. Once all the PCR solutions were made and added to the PCR tubes, they were placed in the thermocycler that ran the following program: (1) 95°C for 5 minutes, (2) 95°C for 30 seconds, (3) 69°C for 15 seconds, (4) 72°C for 45 seconds, (5) repeat steps 2-4 31 times (for a total of 32 cycles), (6) hold at 10°C.

After generating the PCR product, we ran them through an agarose gel electrophoresis to determine the quality and size of our PCR product. In a medium-sized gel mold with comb, 50ml of premade agarose solution mixed with 5 ul of SyberSafe stain was poured into the gel mold. After pouring the agarose and dye solution into the mold, any air bubbles were removed using the back of a pipette tip and left to cool to a solid gel. After cooling the agarose gel, the comb was removed and the gel was placed in the electrophoresis chamber. 1X electrophoresis running buffer was added into the chamber till it covered the top of the gel by ~3mm. In each gel well, 5 ul of PCR product and 1 ul of 6X loading buffer was added. A DNA size marker was loaded in the first well. Once the gel is set up, the gel was run at 100V for 45 minutes or until the first dye has progressed to almost the end of the gel. Once the first front dye had progressed to the end of the gel,we took the gel out and visualized using a UV illuminator.

PCR Purification and Sequencing

The PCR products were purified by our GSI, Ethan Boynton, using the QIAquick® PCR Purification Kit (QIAGEN Inc., Valencia, California). Our purified PCR products were then sequenced by the UC-Berkeley DNA Sequencing Facility in Barker Hall.

Fluorescent Video Imaging

Measuring NPQ was performed independently by our GSI, Ethan Boynton.

Colony PCR for GFP and Hygromycin Cassette

Colony PCR was performed independently by our GSI, Ethan Boynton.

RESULTS

Generating & Selecting Mutant Nannochloropsis oceanica Transformants

To transform and generate *N. oceanica mutants***,** electroporation was performed on wild-type (WT) *Nannochloropsis oceanica* cells with an episome containing a hygromycin-resistant gene, cas9 gene, GFP gene, and DNA sequences encoding two gRNAs sequences (Figure 1) for

editing of the COPOX1 and PRX1 genes respectively to generate two populations each with one target gene knockout. The time constant from these electroporations for PRX1 and COPOX1 were respectively 22.2 and 24.6. These time constant values provide indication of efficient uptake of the episome into the WT *N. oceanica* cells with the use of CRISPR as algal cell exposure to an electroporation field has the potential for increased membrane permeabilization and higher uptake of the episome.

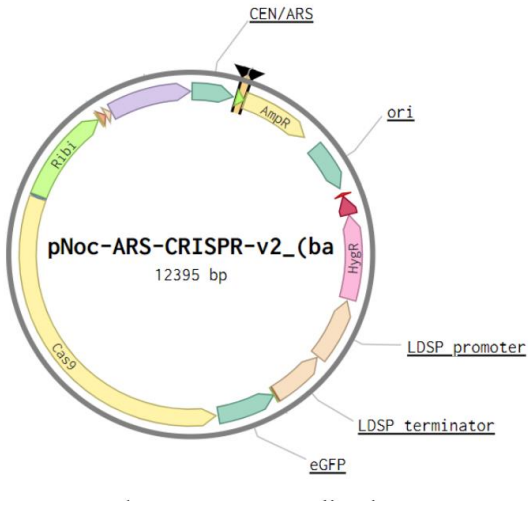


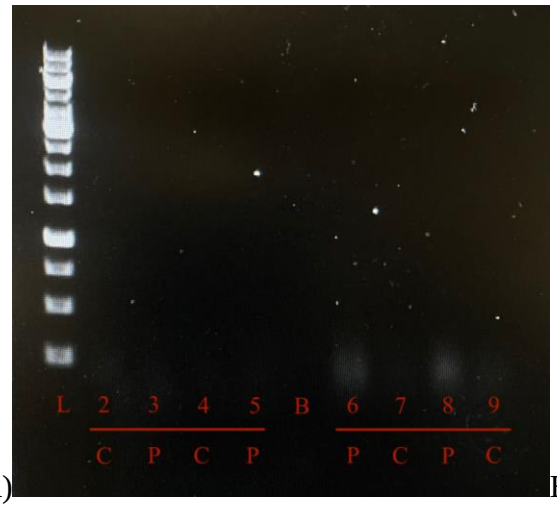
Figure 1. Diagram of the episome used to perform electroporation and transform WT *Nannochloropsis oceanica.* Episome contains CEN/ARS, HygR gene, Ribi promoter, Cas9 gene, and a DNA sequence encoding two gRNAs.

After transformation of WT *N. oceanica* cells, these cells were initially plated on f/2 + hygromycin media plates to select for successfully-transformed colonies resistant to the antibiotic. However, a series of issues arose with the original plates, leading to re-plating the transformation cells. The series of issues faced by the original plates included severe dehydration and potentially old hygromycin stock used in the plate media. After the re-plating, transformed colonies were successfully isolated.

Colony PCR Screening of Transformants

To verify colony transformants in our gene of interest (PRX1 and COPOX1), PCR amplification of CRISPR-edited loci in colonies and agarose gel electrophoresis were performed (Figure 2). According to the JGI database results corresponding to the genes' protein IDs, the expected amplicon length for COPOX1 and PRX1 was approximately 680 base pairs and 3,200 base pairs respectively. However, as seen in Figure 2, there is a lack of bands at the expected amplicon lengths for each gene. While this does not indicate a failure of uptake of episome into

the algal cells, the lack of bands could be due to potential issues in the experimental procedure as with or without the uptake of the episome the algae should have the PRX1 and COPOX1 genes.

A) 

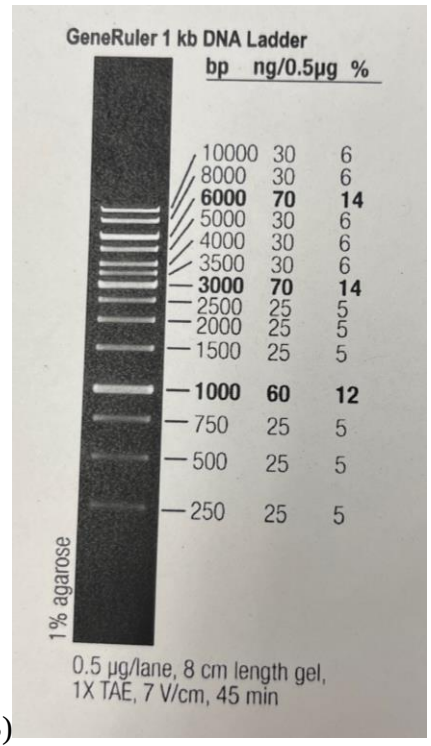
B) 

Figure 2. PCR Confirmation of Transformations. (A) "L" denotes the ladder and "B" denotes a blank lane with no DNA used to separate each set of samples. C and P denote algal cell samples electroporated with episomes containing the genes that target the COPOX1 and PRX1 gene, respectively. The numbers indicate the randomly numbered algal sample used in the PCR amplification. PCR amplification for both COPOX1 and PRX1 targeted genes did not yield any detectable products, as evidenced by the complete absence of bands but visible ladder bands. Non-specific primer amplification can be seen at the bottom of wells 6, 8, and 9. (B) Standard GeneRuler of 1 kb DNA ladder with bands corresponding to bp lengths.

Mutant Genome Sequencing

Due to PCR amplification of CRISPR-edited loci not yielding any detectable products when visualized by gel electrophoresis assay, there were no viable samples available for sequencing analysis. The lack of PCR amplification bands suggests potential issues in the experimental procedure such as issues in primer design or template quality. With no viable samples for sequencing, we cannot verify that our isolated colonies were transformants by analyzing their sequences.

Phenotype Assay of Transformants

Although we were not able to sequence confirmed our *N. oceanica* transformant sub-clones, we continued our experiment by patching WT *N. oceanica* and 24 isolated transformant

algae colonies from the original array selection of each target gene transformant plates onto three various growth media plates: f/2 medium, f/2 containing various antibiotics, and f/2 containing 3-pentanol. After incubation, growth patterns were observed with colony growth on both f/2 plates and f/2 plates containing 3-pentanol while f/2 containing various antibiotics did not show any colony growth for any of the patches (Figure 3). For all COPOX1 gene transformed algal plates, all the transformed patches exhibited similar phenotypic growth of the WT patches. As for PRX1, all the transformed patches exhibited similar phenotypic growth of the WT patches except for patches 5, 9, and 24 in f/2 plate containing various antibiotics where they exhibited a green ring indicating some growth unlike the WT strain in that plate. With these results, further investigation would be required to get a better understanding of the mechanisms responsible for the growth patterns in the PRX1 f/2 plate containing various antibiotics. As a note, slight variation in growth density could be due to variance in initial algal inoculation.

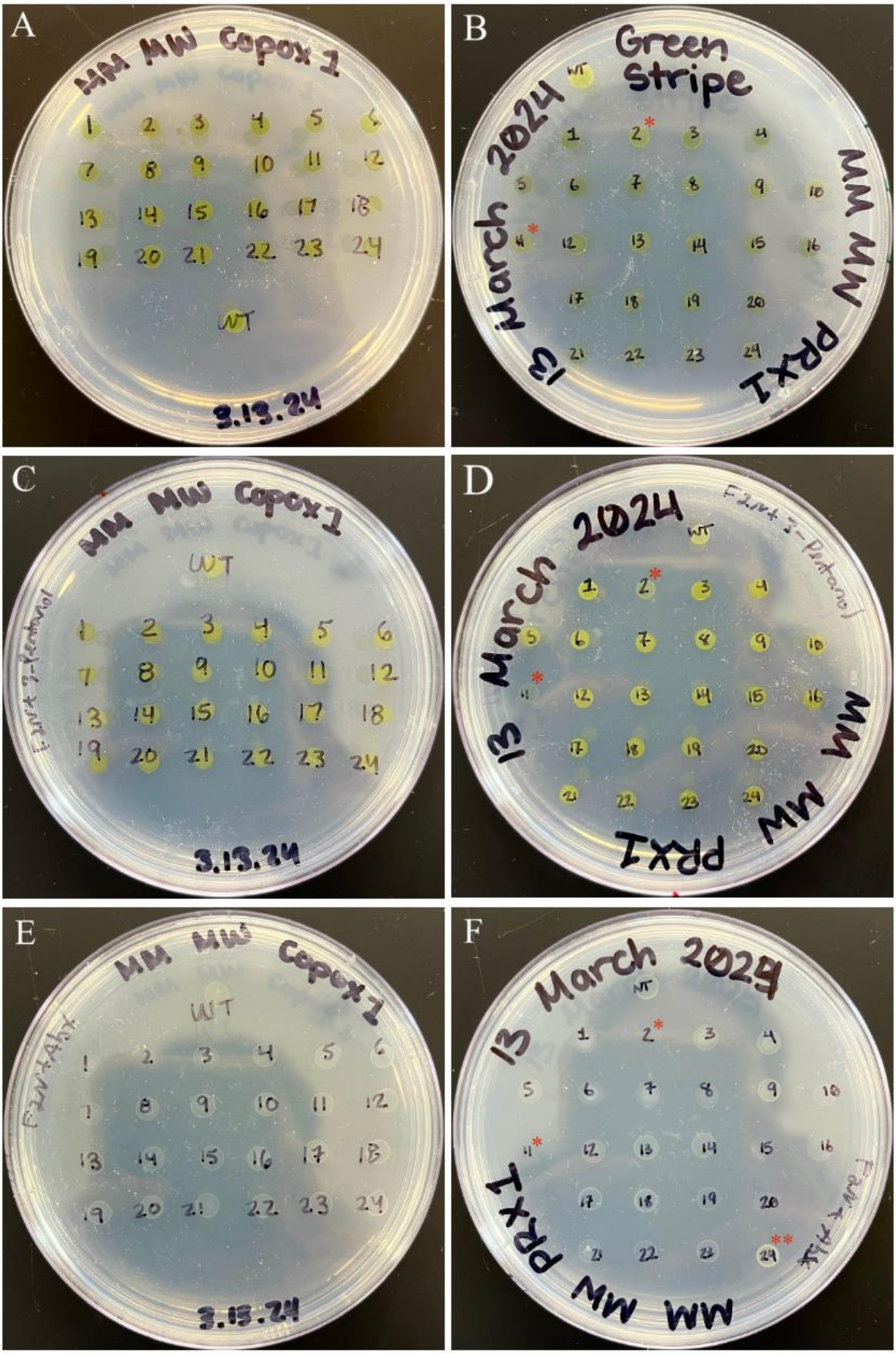

A
MM MW Copox 1
WT
3.13.24
B
Green Stripe
13 March 2024
MW MW PRX1
C
MM MW Copox 1
WT
3.13.24
D
13 March 2024
MW MW PRX1
E
MM MW Copox 1
WT
3.15.24
F
13 March 2024
MW MW PRX1

Figure 3. Transformed *Nannochloropsis oceanica* growth patterns on different growth media. Algae colonies derived from 24 isolated array patch plate for each target gene (PRX1 and COPOX1) were plated on standard f/2 medium (**A, B**), f/2 containing 3-pentanol (**C, D**), and f/2 containing various antibiotics (**E, F**). No distinct growth patterns were observed for COPOX1 mutants, and several colonies exhibited compromised growth on plates containing 3-pentanol compared to wild-type colonies within the same media (denoted by *) for PRX1. Patch 5, 9, 24 from the PRX1 transformation displayed superior growth compared to the WT on the same antibiotic media.

Troubleshooting Our Results

Due to unexpected results such as our initial transformation plate being covered in algae rather than colonies, we performed a PCR reaction to detect two different episomal components (Figure 4) – hygromycin resistance and GFP– as well as fluorescent video imaging of our transformation array plate (Figure 5) to troubleshoot our experiments. In our PCR reaction results, all the samples had hygromycin resistance cassette but no GFP. As for our fluorescent video imaging, every single isolated colony showed the same NPQ defectiveness.

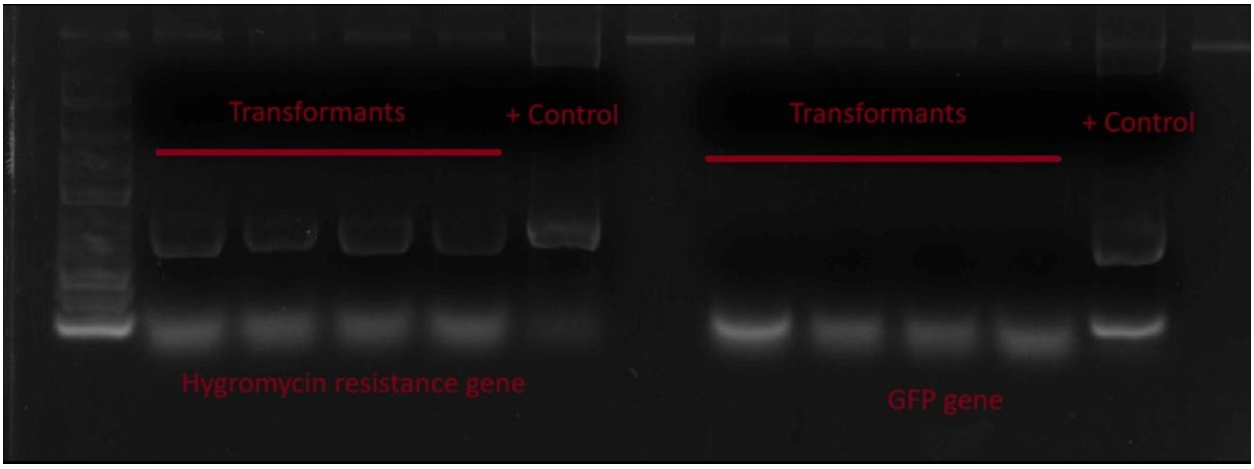


Figure 4. PCR reaction to detect two different episomal components hygromycin resistance and GFP. On the very left lane is the DNA ladder. Transformants all had the hygromycin resistance cassette but no GFP. This indicates that the mutant was not due to the episomal transformation aka the mutation was due to a different origin and not caused by our episomal transformation.

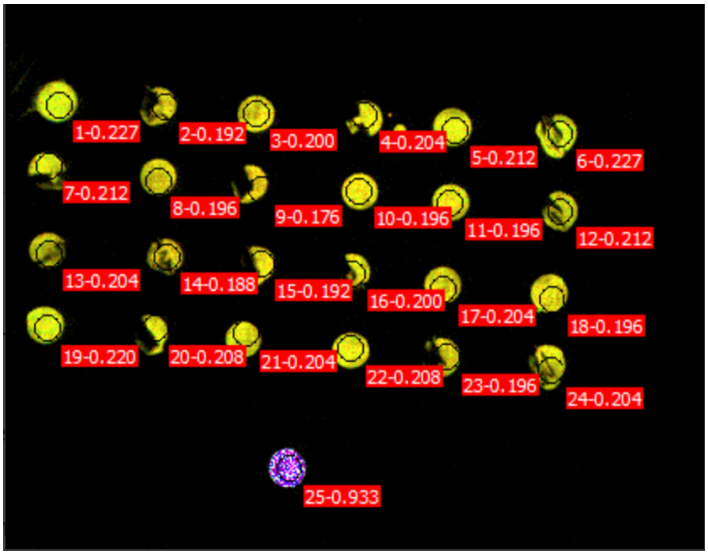


Figure 5. Fluorescent video imaging to measure chlorophyll fluorescence in our COPOX1 transformant plate array. All isolated colonies displayed the same NPQ defective phenotype which is a strong indication that rather than isolating transformants from episomal transformation, we instead isolated NPQ mutants that contaminated our WT *N. oceanica* culture.

DISCUSSION

As briefly mentioned in the results, based on fluorescent video imaging of our COPOX-targeted transformance array plate (Figure 5) and PCR reaction to detect hygromycin resistance and GFP (Figure 4), it was revealed that the WT *N. oceanica* cells were unknowingly transformed prior to this study, resulting in the WT's resistance to the antibiotic hygromycin used in the initial selective growth medium plates following electroporation. Due to all the isolated colonies from the fluorescent video image showing defects in NPQ phenotype, it is most likely that we isolated NPQ mutants that contaminated our WT culture rather than transformants from our episomal transformation. Through the fluorescent video imager conversion of Chl fluorescence signals into false color images, calculation of different photosynthetic parameters and quantitative analyses of the results, we were able to determine that all the isolated colonies displayed similar NPQ defects (Rühle et al. 2018). Because of this, rather than isolating transformants from episomal transformation, we instead isolated NPQ mutants that contaminated our WT *N. oceanica* culture.

In further support that we isolated NPQ mutants that contaminated our WT culture rather than transformants from our episomal transformation, for our transformants to be actual transformation, we should have been able to see two bands in our PCR reaction– one band for hygromycin resistance and another band for GFP– as this would indicate the episomal being

actually uptaken by the algal cells (Science Direct, 2024). However, this result was not seen in our PCR reaction where all the samples had hygromycin resistance genes but no GFP gene, in other words the algal cells did not contain the episome and was not transformed. With this, there is strong evidence that we isolated NPQ mutants rather than episomally transformed transformants. Consequently, the observed colony growth on antibiotic selective plates described in the previous section cannot be indicative of actual genetic transformation caused by electroporation. This unforeseen pre-transformation of the initial WT *N. oceanica* cells compromises any interpretation of potential positive phenotypic or sequencing outcomes.

In conclusion, due to unexpected mutant contamination of our WT, we were unable to achieve conclusive results. In the future, we hope to attempt these experiments again using true WT *N. oceanica* and determine whether or not mutations in PRX and COPOX affect algaenan biosynthesis. If these genes of interest are involved in algeanan biosynthesis, we would gain further insight on how the biosynthetic pathway of algeanan works and could potentially use this knowledge to engineer weakened algeanan N. oceanica that we could grow for biofuel extraction. Despite the experiments not working out as expected, these experiments allowed us to discover the WT contamination issue early on and redirect our experiments towards understanding the WT NPQ mutant.

ACKNOWLEDGEMENTS

The materials, solutions, plates, and light chambers used for the experiments were provided by UC faculty and Niyogi Lab. Fluorescent video imaging and OCR reaction of hygromycin resistance and GFP performed by Ethan. Colony PCR screening and *N. oceanica* transformants plating and araying performed with partner, Marlee Meek.

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Dear Editor,

Thank you for your feedback. I have read through them and have addressed them in my final revised manuscript.

1. The materials and methods are not clear enough to be repeatable. There are things you can add: how and where were the algal cells collected, how the episomes were designed/what was included on them, the washing of the algal cells after electroporation. You did not include purification of our PCR products and sending them to be sequenced. I think you should add a section about the colony PCR and NPQ work that Ethan performed. I think you should also include arraying the subclones and scoring their phenotypes. The point of the paper is to effect algaenan, there needs to be some explanation on the phenotyping so the experiment can be repeatable. You should expand your materials and methods section to include EVERYTHING that was done in the experiment. At the current state, I do not believe that it is adequate for the problems being addressed.
    a. I addressed all the things mentioned in this comment. I added sections to explain about how and where were the algal cells collected, how the episomes were designed/what was included on them, the washing of the algal cells after electroporation, PCR purification and sequencing, colony PCR and NPQ measurements done by Ethan. I also elaborated and fixed wordings of all the sections to make sure that they flowed, made sense, and included all the necessary information for the experiment to be replicated.
2. Your conclusions are clearly supported by what you presented in your results. I suggest that you add a sentence or two talking about next steps, if you could continue working on this project, what would you do? You could add what you would have expected to see if you actually transformed WT cells. The discussion should discuss how to prevent contamination in the future and address anything else that went wrong.
    a. Added a concluding paragraph summarizing our current understanding and what we hope to accomplish from here on out.
3. There needs to be more references. There should be references included in the discussion to tie your work with other work. There is a mistake in the format of your references, the third line of a reference is not indented
    a. I fixed the indentation error in the references and went back to my discussion to include more information that related to outside references. Thus, I used more references for my paper.
4. Figure legends should be single spaced. The results are presented in chronological order, which is logical. I think there are sprinkles of discussion and material/methods in your

result, focusing on “what did we see” in the results. Overall I think your results are relevant to the scientific question being asked, your figures complement that.
    a. Thank you. I changed the figure legends to be single spaced.
5. You need to add a running title. Your title is accurate and informative, I can infer what your experiment was by just reading your title. Your key words represent the transformation aspect of the experiment, but not what we transformed or why. I suggest you add “Nannochloropsis” and “algaenan.”
    a. I added a running sentence and adjusted my key words like you said.
6. The scientific problem was clear, but I feel like its significance was lost due to the blunt transition from paragraph 1 to 2. Maybe a transition sentence to soften it. You described specifically how algaenan could be used for carbon sequestration and biofuel production. Putting the paper in perspective is good for all algal biologists. The way you described algaenan biosynthesis was perfect for a broad audience of algal biologists
    a. I agree. I added a transitory/connecting sentence at the end of the 1st paragraph. This way the big picture issue can better connect with the topic of the paper.
7. Your abstract is short but presents all the information it should.
    a. Thank you.
8. Although the results were not what you expected, I think it is a great learning experience for the scientific community. If the experiment worked, then it would have been of great value to the scientific community.
    a. I agree.
9. The quality of writing was great overall. There were a few unclear sentences. When you were talking about PCR in methods, it was a bit hard to follow. You did a great job explaining what the results meant in your discussion.
    a. Thank you. I reread my paper and adjusted any sentences that felt awkward. I also specifically adjusted wording in the PCR method section.
10. Your subject is relevant for publication in the Journal of Phycology.
    a. Thank you.

Best Regards,

Monica