ENGINEERING THE HYDROCARBON BIOSYNTHETIC PATHWAY FROM THE GREEN MICROALGA *BOTRYOCOCCUS BRAUNII* INTO THE FASTER GROWING HETEROLOGOUS HOST *CHLAMYDOMONAS REINHARDTII*

An Undergraduate Research Scholars Thesis

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VICTORIA YELL

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ABSTRACT

Engineering the Hydrocarbon Biosynthetic Pathway From the Green Microalga *Botryococcus braunii* into the Faster Growing Heterologous Host *Chlamydomonas reinhardtii*

Victoria Yell
Department of Biochemistry
Texas A&M University

Research Advisor: Dr. Timothy Devarenne
Department of Biochemistry
Texas A&M University

Botryococcus braunii is a green microalga that is capable of producing large amounts of liquid hydrocarbons that can be processed into liquid fuels such as gasoline, kerosene and diesel. Lycopaoctaene is a C_{40} hydrocarbon produced by race L of *B. braunii* using the enzyme lycopaoctaene synthase (LOS). LOS catalyzes the head-to-head linkage between two geranylgeranyl diphosphate (GGPP) molecules to yield lycopaoctaene, which is then further reduced to lycopadiene. Lycopadiene is the hydrocarbon that accumulates within the colony extracellular matrix and usually constitutes 8-12% of algal dry weight. The slow growing nature of *B. braunii* is one of the main factors limiting the use of this alga as a viable host for biofuel production. Because *B. braunii* has not been able to be transformed, it would be advantageous to transform a faster growing model alga with hydrocarbon biosynthetic genes. *Chlamydomonas reinhardtii* is a faster growing model green microalga with many genetic tools developed for it, and is being used in our study to express the LOS gene for lycopaoctaene production. In order to increase lycopaoctaene production in *C. reinhardtii*, GGPP synthase (GGPPS) will be expressed

constitutively along with LOS. In order to do this, transformant lines of *C. reinhardtii* will be generated and compared. The four *C. reinhardtii* transformant lines being compared include one overexpressing GGPPS, one overexpressing LOS, the LOS tranformant further transformed with *C. reinhardtii* derived GGPPS, and the LOS line further transformed with *A. thaliana* derived GGPPS11. Using two different sources of GGPPS genes will allow us to compare levels of expression and lycopaoctaene production.

ACKNOWLEDGEMENTS

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NOMENCLATURE

LOS	Lycopaoctaene synthase
GGPP	Geranylgeranyl diphosphate
SSL	Squalene synthase-like
GGPPS	GGPP synthase

CHAPTER I

INTRODUCTION

There has been an influx of interest in the development of biofuels in recent years due to the amount of greenhouse gases, such as carbon dioxide (CO_2), in the atmosphere increasing due to human activities like burning fossil fuels [1]. Increasing CO_2 levels in the atmosphere can lead to a rise in atmospheric temperature due to heat being absorbed, and trapped by CO_2 [1]. This can have negative effects on natural ecosystems and human society. Human society revolves around using fossil fuels to meet day-to-day energy requirements, and fossil fuels will eventually run out [2]. This is why a more sustainable system of obtaining energy is necessary to replace fossil fuels.

Biofuels produced from plants such as corn and soybeans has garnered a large amount of attention, however there are issues associated with using these organisms. Utilizing products derived from plants as a source of fuel leads to the reduction of anthropogenic CO_2 entering into the atmosphere when those fuels replace fossil fuels [3]. One of the major problems with land plant sourced biofuels is the fact that they require much more land than the existing arable land to meet the current energy demand as described for the case of palm, soybean, and corn oils and starches by Fargione et al. [3]. Algae has received increasing attention as a fuel source due to its faster growth and higher efficiency for CO_2 conversion to fuel feedstocks when compared to land plants, and algae do not require arable land for growth [4].

The green microalga *Botryococcus braunii* is able to produce large amounts of hydrocarbon oils, which can potentially be used for biofuel production [5]. These hydrocarbons can be hydrocracked to make gasoline, kerosene, or diesel fuels in a similar way to petroleum, making them drop-in fuels that would not require new fuel production infrastructure [5]. *B. braunii* is classified into three chemical races that are morphologically similar, but can be distinguished based on the types of hydrocarbons they produce [6, 7]. The amounts of hydrocarbons produced by each race vary, but generally constitute between approximately 20-60% of the total dry weight depending on the race [8, 9]. The three main races of *B. braunii* are the A race, B race, and L race which primarily produce alkadienes and alkatrienes, botryococcenes, and lycopadiene, respectively [6, 7]. The percentages of hydrocarbons that make up the algal dry weight produced by races A, B, and L are approximately 25-44%, 30-86%, and 2-8%, respectively [7, 8, 10]. Alkadienes and alkatrienes are derived from fatty acids, while botryococcenes and lycopadiene are both derived from the isoprenoid pathway [6, 7, 11]. Botryococcene is a triterpenoid whereas lycopadiene is a tetraterpenoid [6, 7].

The main limiting factor for using *B. braunii* derived hydrocarbons as a potential feedstock is that it grows very slowly, with a doubling time of up to 8-10 days, making it infeasible to grow commercially [8]. Extensive work has been done to transform *B. braunii*, however all attempts thus far have been unsuccessful. One approach to overcome these limitations could be transformation of other well-studied and faster growing algal species with hydrocarbon producing genes from *B. braunii*. There have been similar studies that have been successful in producing botryococcenes in yeast, the photosynthetic prokaryote *Rhodobacter capsulatus,* and tobacco [12-14]. The enzymes expressed in these studies were squalene synthase-like (SSL)

enzymes 1 and 3, which function similarly to lycopaoctaene synthase (LOS) [12-14]. The SSL-1, SSL-3, and LOS enzymes all use substrates derived from the 2-C-methyl-D-erytheritol 4-phosphate (MEP) pathway, which is the isoprenoid pathway localized to the chloroplast [15]. Because of the similarity in substrate type, many of the aspects of these projects can be applied to the transformation of *C. reinhardtii* with the lycopaoctaene pathway [16].

The biosynthetic pathway for the long chain hydrocarbon C_{40} lycopadiene in race L of *B. braunii* was recently elucidated [16]. The enzyme LOS is responsible for the first committed step in the lycopadiene pathway by condensing two C_{20} geranylgeranyl diphosphate (GGPP) molecules through a head-to-head linkage [16]. Lycopaoctaene is reduced to form lycopadiene, the major hydrocarbon that accumulates in race L. Producing lycopaoctaene in the well-studied green microalga *Chlamydomonas reinhardtii* will be the focus of this project. *C. reinhardtii* is considered a model green microalga and has many genetic tools developed for it, and is also relatively easy to transform [17]. One of the major attractive qualities of *C. reinhardtii* for this project is that it has a much shorter doubling time of approximately 6-8 hours, making it a much more favorable feedstock candidate in terms of potential hydrocarbon production. In order to increase production of lycopaoctaene, a constitutively expressed GGPP synthase (GGPPS) from either *C. reinhardtii* or *Arabidopsis thaliana* (GGPPS11) will be transformed along with LOS to provide an excess of substrate for the LOS enzyme.

CHAPTER II

METHODS

Culturing *Chlamydomonas reinhardtii*

C. reinhardtii strain CC406 cw15 was grown in Tris-acetate-phosphate (TAP) media in flasks with constant orbital shaking and illumination [18, 19]. This strain is a cell wall deficient line of *C. reinhardtii*, and was obtained through the Chlamyconnection.org.

RNA Extraction

For cloning GGPPS from *C. reinhardtii,* RNA was extracted using the Norgen Biotek Plant RNA extraction kit, following the procedures given. Other than this instance the following procedure was used. Between 100 and 200 mg of cells were collected in Eppendorf SafeLock tubes. Approximately 200 µL of steel beads (diameter 0.9-2 mm) and 800 µL of Trizol were added to these tubes and the cell pellet was resuspended with vortexing. These tubes were then placed in a Next-Advance Storm bead beater for 10 minutes on speed 10. The supernatant was transferred into a new tube and another 200 µL of Trizol was added. After mixing, 200 µL of $CHCl_3$ was added and the tubes sat undisturbed for 3 minutes at room temperature before centrifuging for 15 minutes at 12,000 x *g*. The aqueous phase was removed, combined with 500 µL of isopropyl alcohol, and allowed to sit again for 10 minutes at room temperature. After centrifugation and removing the supernatant the RNA pellet was then washed with 75% ethanol. The resulting pellet was washed with 500 µL of 2 M LiCl in order to remove polysaccharide contaminants. This was repeated until the RNA pellet remained the same size. The pellet was dried in a speedvac and then resuspended with 0.5 mL TE buffer (10mM Tris-HCl pH 8, 1 mM EDTA)

and 0.5 mL phenol:$CHCl_3$:isoamyl alcohol (24:4:1). This was centrifuged at 12,000 x *g* for 15 minutes at 4°C and the aqueous phase was placed in a new tube with 0.5 mL $CHCl_3$, then centrifuged again using the same conditions. RNA was again precipitated with 0.1 volumes of 3 M sodium acetate pH 4.7 and 2.5 volumes of 100% ethanol, then incubated at -20°C overnight. The solutions were centrifuged using the same conditions as before and the supernatant was removed. Next 0.5 mL of 75% ethanol was added and the sample was centrifuged again. After removal of the supernatant the pellet was dried in the speedvac and resuspended in 100 µL dH_2O.

Cloning of GGPPS

GGPPS was cloned from *C. reinhardtii* after obtaining the sequence from the previously published genome [20]. Extracted RNA was used for cDNA synthesis in an RT reaction (50°C for 50 minutes, 85°C for 10 minutes, 10°C for 10 minutes) using the reverse transcriptase SuperScript III (Invitrogen), followed by incubating with RNAse H for 20 minutes at 37°C. Using the single stranded cDNA as template, the GGPPS gene was amplified using a gradient PCR 2 minute denaturation at 98°C, (30 sec melting temp 98°C, 1 min extension at 72°C, 30 second annealing at 53.8, 56.2, and 57.5°C) x 33 cycles, followed by 10 minutes of extension at 74°C using GoTaq DNA polymerase mix (Promega). This fragment was ligated into pGEM-T Easy vector (Promega) using T4 DNA ligase (NEB) and incubating overnight at 4° C. The GGPPS forward primer was 5'-ATGCAGATGCAGCAGCAGC-3', and the reverse primer was 5'-TTAGTTTTGCCGGTAGCCGATG-3'.

Gene Construct Design

Two genetic constructs were made containing LOSΔ391 and Δ24GGPPS from *C. reinhardtii*. The LOSΔ391 is a truncated version that lacks the C-terminal trans membrane domain found in wild type LOS [16]. The sequence of LOSΔ391 was cloned from *B. braunii* race L [16] and codon optimized for *C. reinhardtii*. A chloroplast transit peptide (cTP) sequence was derived from *A. thaliana* GGPPS11 and also codon optimized with LOSΔ391 for *C. reinhardtii* [21]. This construct was synthesized by Thermo Fisher Scientific with the cTP at the N-terminus of LOSΔ391, and a His tag at the C-terminus.

The GGPPS cloned from *C. reinhardtii* was truncated at the 24th residue from the N terminus, which was identified as a mitochondrial transit peptide by the TargetP online tool. The sequence for Δ24GGPPS was linked to the *A. thaliana* derived cTP at the N-terminus through overlap extension PCR, and a FLAG tag was added to the C terminus. The cTP-LOSΔ391-His codon optimized construct and Δ24GGPPS OE-PCR were ligated into the vector pHsp70A/RbcS2 separately using T4 DNA ligase (NEB) and incubating at 4°C overnight. Utilizing cre-lox recombination vectors described previously [22], these inserts were combined with either a hygromycin or paromomycin *C. reinhardtii* selective marker containing plasmid [22-24]. The final constructs that resulted from the cre-lox recombination was Δ24GGPPS with a hygromycin selective marker, and LOSΔ391 with a paromomycin selective marker.

Overlap Extension PCR for GGPPS Construct

For the first fragment, the cTP was amplified from the synthesized DNA construct that contained the *C. reinhardtii* optimized LOSΔ391. PCR conditions include 2 minute denaturation at 98°C,

(30 sec melting temp 98°C, 10 second extension at 72°C, 30 second annealing at 63.7°C) x 33 cycles, followed by 10 minutes of extension at 74°C using Phusion DNA polymerase (NEB). The second fragment in the OE-PCR contained the truncated GGPPS, as well as a C terminus FLAG tag, which was added on through the reverse primer.

PCR conditions include 2 minute denaturation at 95°C, (30 sec melting temp 95°C, 1.5 minute extension at 72°C, 30 second annealing at 63.7°C) x 33 cycles, followed by 10 minutes of extension at 74°C using GoTaq DNA polymerase mix (Invitrogen). To combine these two fragments the forward primer for the first fragment and the reverse primer from the second fragment were used in another PCR (2 minute denaturation at 98°C, (30 sec melting temp 98°C, 10 second extension at 72°C, 30 second annealing at 63.7°C) x 33 cycles, followed by 10 minutes of extension at 74°C using Phusion DNA polymerase (NEB)).

The primers used for these PCRs were the following: fragment one forward 5'-CATGCTCGAGATGGCCAGCGTGACCCTGGGC-3', fragment one reverse 5'-CCTTGGCCATGGACACGATGGAGCTGCTG-3', fragment 2 forward 5'-CATCGTGTCCATGGCCAAGGCTGCGCCTGGC-3', fragment 2 reverse 5'-CAGGGATCCTCAAATCTTGTCGTCGTCGTCCTTGTAGTCCATGTTTTGCCGGTAGCCGATGAAC-3'.

Transformation of *C. reinhardtii*

Initially two strains of transformants were made, one with the constitutively expressed *C. reinhardtii* GGPPS and the other with the constitutively expressed LOS. Both of these constructs

were linearized utilizing EcoRV (NEB). *C. reinhardtii* culture with an OD_{750}=0.05 was centrifuged at 1950 x *g* for 5 minutes at room temperature. The pelleted cells were resuspended in fresh TAP media and allowed to incubate at 40°C for 25 minutes while shaking at 80 rpm. Next 300 µL of cells were added to a tube with 300 mg of sterilized glass beads (diameter 0.5 mm). Then 2 µg of linearized plasmid, and 100 µL of 20% PEG-6000 were added to the tubes. These tubes were vortexed at high speed for 15 seconds twice with 1 minute between each vortexing. The liquid from each tube was transferred to a falcon tube with 50 mL of fresh TAP media. These solutions were centrifuged and the pelleted cells were resuspended in TAP media and plated on TAP agar plates containing either 15 µg/mL of hygromycin or 10 µg/mL of paromomycin. One week later colonies were picked from the original plates and restreaked on new plates to test for antibiotic resistance. After an additional week, colonies were grown in liquid cultures using 12-well plates with 2.5 mL of TAP media and the respective antibiotic. This process was repeated using selected strains and the opposite construct, creating a transformant that contained both the LOS and GGPPS construct.

Genomic DNA Extraction

Genomic DNA from both LOS and GGPPS transformants was extracted from 2 mL of *C. reinhardtii* cultures with an OD_{750}=0.2-0.4. The collected cultures were centrifuged at 2000 x *g* for 5 minutes at room temperature, the supernatant was removed, and the pelleted cells were resuspended in 40 µL of dH_2O. Two volumes of both phenol-chloroform and SDS-EB solution (2% SDS, 400 mM NaCl, 40 mM EDTA, 100 mM Tris-HCl pH 8) was added and mixed gently. This mixture was centrifuged and the aqueous top layer was added to a new tube along with two volumes of 100% ethanol to precipitate DNA. This solution was kept at -20°C for 1 hour. The

solution was then centrifuged for 10 minutes at 4°C for 10 minutes and the DNA pellet was washed with 70% ethanol. The pellet was dried with a speedvac and dissolved in 100 μL dH_2O.

Protein Extraction

Between 100 and 200 mg of cells were collected in Eppendorf SafeLock tubes. Approximately 200 μL of steel beads (diameter 0.9-2 mm) and 800 μL of extraction buffer (50 mM MOPS pH 6.8, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 5 mM EGTA, 20% glycerol) were added to these tubes and the cell pellet resuspended with vortexing. These tubes were then placed in the bead beater for 10 minutes on speed 10. The supernatant was moved to a new tube and centrifuged at 9000 x *g* for 10 minutes at 4°C. The supernatant was moved to a new tube and centrifuged again for 20 minutes. This supernatant was then moved to a new tube and stored at -80°C for future use.

Western Blot Analysis

SDS-PAGE and western blotting analysis was conducted using 10% polyacrylamide gel. The SDS-PAGE was run at 120 V for 95 minute, and the western blot transfer was run at 90 V for 2 hours onto an Amersham Hybond PVDF membrane (GE). After the protein was transferred from the SDS-PAGE to the western blot membrane, the membrane was blocked in 5% dry fat free milk in phosphate buffered saline solution containing Tween 20 (PBST, 8mM Na2HPO4, 150mM NaCl, 2mM KH2PO4, 3mM KCl, 0.05% Tween 20, pH 7.4). After blocking with milk, the membrane was washed for 15 minutes in PBST three times and followed by incubating with the primary antibody.

The antibody used for extracts from the GGPPS transformants was the α-FLAG-HRP antibody at a concentration of 1:1000 in PBST. Incubation with this antibody was conducted at room temperature for 2 hours, then the membrane was washed in PBST for 15 minutes three times. The antibodies used for the LOS samples were α-His and then α-mouse-HRP, at concentrations of 1:3000 and 1:5000 respectively. Incubation with the α-His antibody was either conducted at room temperature for 2 hours, or overnight at 4°C. Incubation with the α-mouse-HRP antibody was only done for 2 hours at room temperature. The LOS membrane was washed between incubations, and then again before being visualized.

The western blots were visualized using the Amersham ECL Prime reagent set (GE); 0.5 mL of each reagent was added to membranes for imaging. Western blots were imaged using Amersham Imager 600 (GE).

Hydrocarbon Extraction for Screening Double Transformants for Lycopaoctaene Production

Hydrocarbon extraction of the LOS and GGPPS double transformants was conducted using *n*-hexane. Lyophilized cells from 6 mL of culture grown for one week from a plate culture were transferred to a gas chromatography-mass spectrometry (GC-MS) vial with 1 mL of *n*-hexane, and were vortexed at max speed for 1 minute. Samples were allowed to sit for 1 day, then the *n*-hexane was transferred to a clean GC-MS vial leaving behind all cell debris. The clean supernatant was dried using N_2 gas, resuspended in 100 μL of *n*-hexane, and placed in a glass concentrating insert to allow successful sampling of the small amount of extract.

GC-MS Conditions

Extracted hydrocarbon products were analyzed using GC-MS (Bruker 436-GC-SCION SQ Premium). The column used for this analysis was the 5% phenyl BR-5 ms capillary column, which was 30 m in length, 2.5 mm in diameter, had a film thickness of 2.5 μm, and used He as a carrier gas (flow rate of 2.58 mL per minute). For the GC conditions the temperature was held at 220°C for 1 minute, was increased by 5°C per minute until it reached 280° C, then increased by 2°C per minute until it reached 300°C and was held for 20 minutes. The ionization source for MS analysis was electron ionization at 70 eV, and the temperatures for the injection port, interface, and ion sources were 280°C, 250°C, and 200°C respectively.

CHAPTER III

RESULTS

Identification of Promising Primary Transformants

C. reinhardtii primary transformants containing either the GGPPS construct or LOS construct were screened first through either hygromycin or paromomycin resistance respectively. Approximately 100 colonies from each transformant were picked from the original solid TAP media plates that were made during transformation. Out of these colonies approximately 50% were able to grow on subsequent antibiotic plates and in liquid TAP media with antibiotics. Only 12 lines from each type of transformant were selected for screening of genomic DNA, mRNA, and protein, to detect the presence of the transformed construct.

For the LOS transformant lines the primers used for genomic DNA analysis originated from the sequence of the cTP-LOS insert, resulting in the amplification of the entire insert. Integration of LOS into the genomic DNA was determined to be successful in lines 8C, 10C, 11C, 12C, 14C, 20C, and 22C. These lines were determined to contain the LOS sequence due to bands of expected size (1362 bp) being visualized on the DNA electrophoresis gel shown in Figure 1. The same type of PCR was performed using cDNA from the GGPPS transformants however no fragments were amplified successfully. Different primers used for these PCRs included insert specific, plasmid specific, and wild type *C. reinhardtii* GGPPS specific. The pHsp70A/RbcS2 plasmid originated from *C. reinhardtii* so for the PCRs using primers originating from the plasmid sequence, as well as the native GGPPS, multiple bands were expected to be amplified.

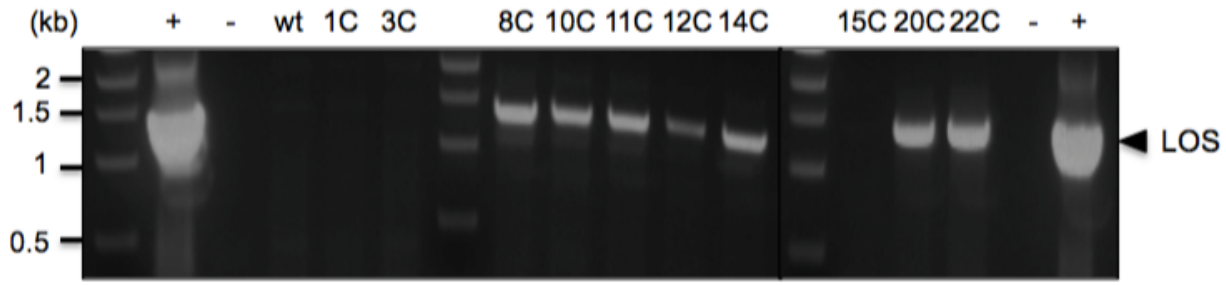

Figure 1 Confirmation of presence of LOS insert in transformant genomic DNA These gels show the amplification of the LOS insert from extracted genomic DNA from the LOS transformants. Labels above the gel lanes indicate arbitrarily named samples. Negative controls in this gel were PCR reactions lacking genomic DNA. The positive controls were linearized purified plasmids containing the LOS insert. The "wt" sample used extracted DNA from untransformed *C. reinhardtii* as template.

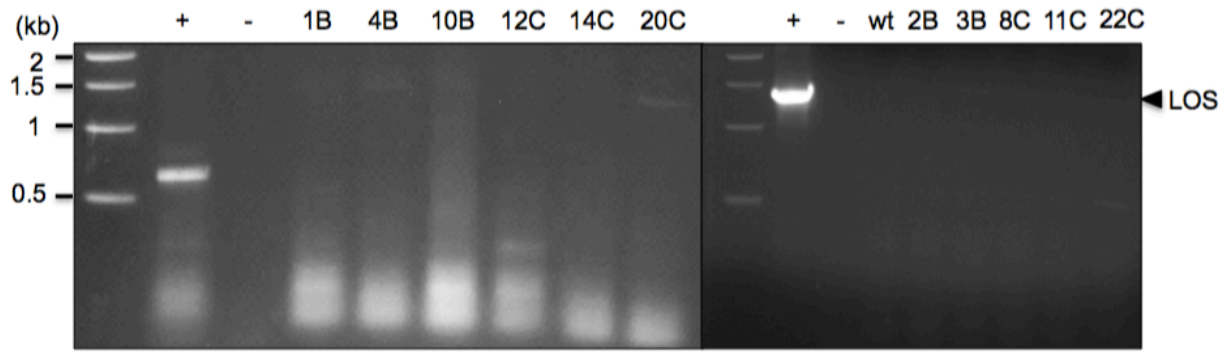

Figure 2 Detecting the presence of LOS in transformant mRNA This gel shows RT-PCR reactions from the LOS transformant lines. Labels above the gel lanes indicate arbitrarily named samples. The leftmost positive control used GGPPS cloning primers to amplify the endogenous GGPPS from a randomly chosen transformant line, while the positive control on the right used a purified plasmid containing LOS as template. The negative control shown is a PCR reaction using no template.

Despite this no bands were seen when these reactions were visualized using DNA gel electrophoresis. This suggests that the PCR failed, rather than all lines being negative transformants for the GGPPS construct. The same results were seen in screening transformant mRNA for the presence of either construct being transcribed (Figure 2). Some LOS transformant mRNA contained the cTP-LOS insert, however no positive results could be seen for the GGPPS lines. The LOS line reactions were visualized on a DNA gel electrophoresis showing bands of expected size (1362 bp) in lines 1B, 4B, 10B, and 20C. Bands seen at the bottom of the gel indicate that a large amount of primer dimers formed.

Screening lines for the presence of expressed proteins did not give clear results due to a large amount of non-specific bands being visualized during western blotting. The cTP-GGPPS construct contained a C-terminal FLAG tag, and the cTP-LOS construct contained a C-terminal His tag. Antibodies specific to these tags were used in western blotting to identify the protein bands of interest. Because the mouse derived His tag antibody used was not conjugated to HRP, a secondary anti-mouse-HRP antibody was used. Bands of the expected size were present in almost all samples but varied in intensity among the different lines (Figure 3). A band of expected size was present in the negative control as well, because of this the intensity of the band was the basis used to identify lines as more likely positive transformants than others. Western blots as well as coomassie blue stained membranes for both types of transformants can be seen in Figure 3A and 3B. In Figure 3A lines 1B, 8C, 11C, 12C, 20C, and 22C appear to have darker bands of expected size (46 kDa) than the negative controls shown. Because of this, these lines were considered to be the most likely positive transformants.

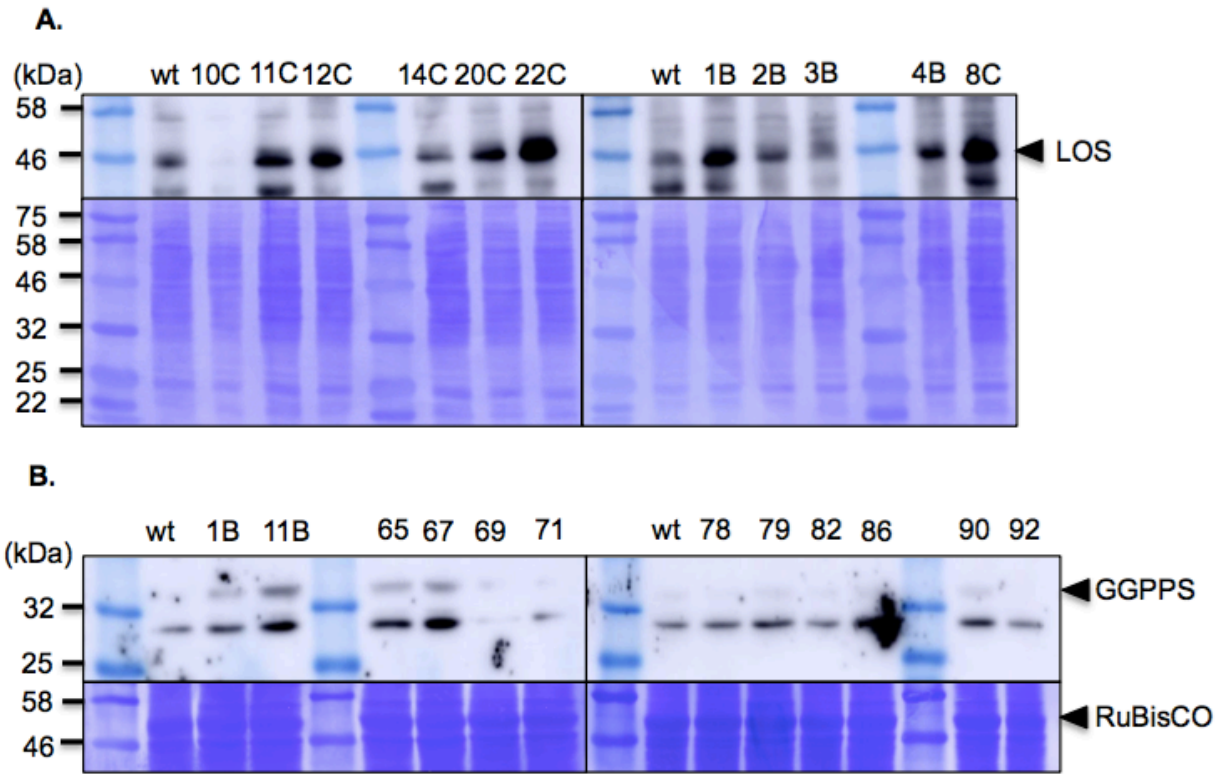

Figure 3 Detecting LOS and GGPPS protein expression in transformants Labels above the gel lanes indicate arbitrarily named samples for both types of transformants. **A.** This western blot was performed using 100 µg of extracted protein from LOS transformant lines. The negative control is denoted on the blot by "wt", which corresponds to non-transformed *C. reinhardtii.* **B.** This western contains 50 µg of protein extracted from GGPPS transformant lines. The negative control is denoted on the blot by "wt", which corresponds to non-transformed *C. reinhardtii.*

An issue with using this method of selection is that the protein amount in each sample was not the same, as indicated by the uneven amount of staining in each lane. This is probably due to using too much protein, and some protein precipitating out of the solution before running through the polyacrylamide gel. This problem was not as prevalent in the GGPPS western blot (Figure 3B), which can be demonstrated by the relatively equal amount of protein stained in each sample lane. This problem was avoided by using 50 μg of protein rather than 100 μg, which was used for the LOS samples. The strongest bands of the expected size (36 kDa) can be seen in lines 1B, 11B, 65, and 67, and some weaker bands of this size can be seen in lines 79 and 90. In each western blot a possibility of two bands could indicate a positive result, a band of expected protein size, or a band of expected protein size in addition to the cTP estimated size of 4 kDa. Theoretically, if the protein was successfully trafficked to the chloroplast, the cTP would have been cleaved off. However, in case the cTP was not cleaved both sized bands would have been considered a positive result. Only bands of the expected protein size were visualized, rather than the cTP-protein band, indicating that the proteins were successfully trafficked to the chloroplasts.

Choosing primary transformant lines to transform with the other construct was done by examining which lines appear to be successfully expressing the original construct they were transformed with. Western blot analysis could give the most insight as to whether the GGPPS or LOS enzymes were being expressed. In addition, genomic DNA and mRNA analysis could give insight into whether either enzyme was incorporated into the genome, and transcribed into mRNA. No lines had positive results for all tests imposed on them.

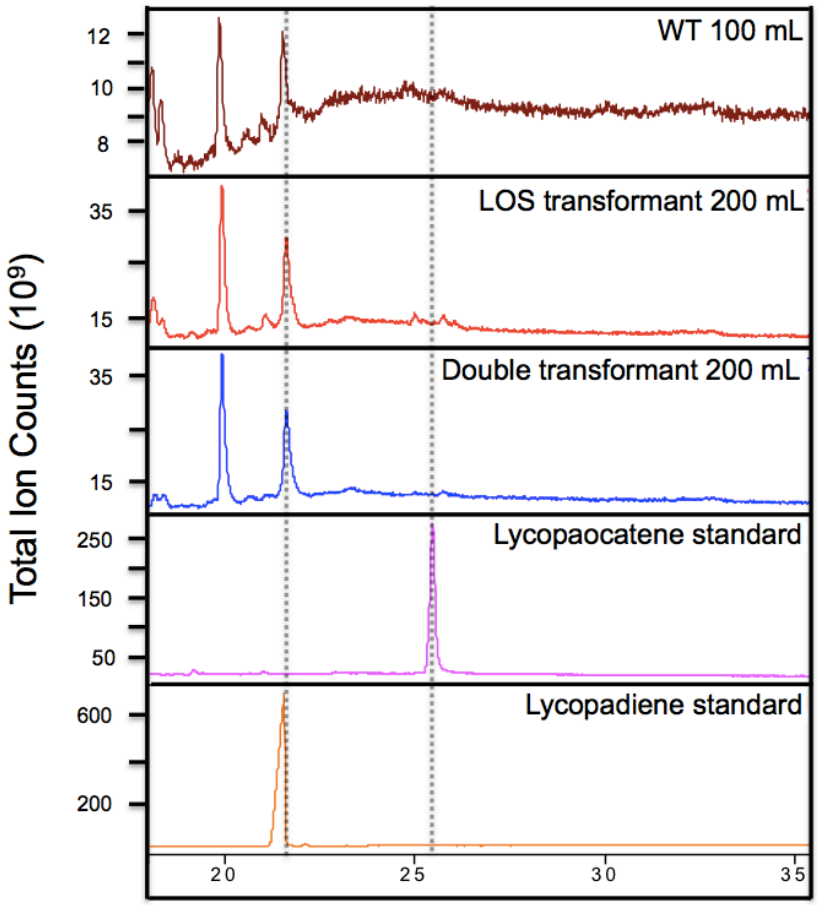

Figure 4 GC-MS spectra of wild type, LOS transformant, and double transformant *C. reinhardtii* Of 74 hydrocarbon profiles of double transformants analyzed only one produced peaks that eluted at the same time as lycopaoctaene. The hydrocarbon profile was generated from a 200 mL culture in order to reduce background peaks, which is shown above by the spectra labeled "Double transformant 200 mL". The LOS transformant, and wild type (WT) both serve as negative controls. Elution times of both lycopaoctaene and lycopadiene are shown in the bottom two spectrums, which were generated using purified hydrocarbons. The peak that appears to elute at the same time as lycopadiene is present in all *C. reinhardtii* samples indicating that this peak comes from a compound other than lycopadiene. Despite the double transformant appearing promising during small scale screening, once the spectra was replicated using a large volume it became apparent that lycopaoctaene was not being produced.

Theoretically, if a positive result was seen for one line in a western blot, it can be assumed that the genomic DNA and mRNA analysis would also give a positive result, which is why more importance was placed on the western blot results. Taking into account all genomic DNA incorporation, mRNA production, and protein expression data, LOS lines 8C and 20C, and GGPPS line 65 were chosen for further transformation with the opposite construct.

Hydrocarbon Extraction Analysis

To analyze successful lycopaoctaene production in transformants containing both the LOS and GGPPS constructs, *n*-hexane extracted hydrocarbons were analyzed using GC-MS. Small cultures of 74 double transformant lines were grown, hydrocarbons extracted, and the extracts analyzed by GC-MS. Out of all lines analyzed one line was potentially interesting, having a peak present at the same elution time as lycopaoctaene. No other double transformants, or negative controls, contained this peak so it was explored further. A larger culture of 200 mL was grown and extracted using similar methods to those used for the 6 mL cultures, but using more *n*-hexane to extract and resuspend for GC-MS analysis (20 mL and 1 mL respectively). This larger culture did not have the same interesting peak present so it was determined that no double transformants containing both the LOS and GGPPS produced lycopaoctaene (Figure 4). A relatively large peak was seen eluting at the same time as lycopadiene, however this peak is also seen in spectra generated from wild type, as well as the LOS only transformant *C. reinhardtii* (Figure 4). When analyzed using mass spectrometry the molecule did have peaks characteristic of an isoprenoid, however a National Institute of Standards and Technology (NIST) library search returned a sterol rather than a linear molecule similar in structure to lycopadiene.

CHAPTER IV

CONCLUSION

During hydrocarbon production analysis of *C. reinhardtii* double transformant lines, no lycopaoctaene production was detected. The hydrocarbon profiles of a yeast line co-expressing LOS and the *A. thaliana* derived GGPPS11 showed a significant increase in lycopaoctaene production (data not shown, from Thapa HR). The same results were seen when GGPPS11 and LOS were expressed in *Escherichia coli* (data not shown, from Thapa HR). These results indicate that GGPPS11 functions proficiently in other organisms to increase available GGPP pools. Due to the inability to successfully amplify GGPPS DNA with PCR in the GGPPS transformants, as well as their slower growth, it is possible that the gene was silenced in *C. reinhardtii* due to the GGPPS cDNA originating from *C. reinhardtii.* Using a GGPPS derived from a different organism such as AtGGPPS11 could reduce the probability of gene silencing. Further work will need to be done to co-express LOS and GGPPS11 in *C. reinhardtii*, increasing the possibility of lycopaoctaene being produced.

In previous studies the *B. braunii* hydrocarbon, botryococcene, was successfully produced in a variety of model organisms [12-14]. Some techniques used in these projects were used in the transformation of LOS in *C. reinhardtii*, such as targeting genes of interest to the chloroplast, the site of isoprenoid synthesis via the MEP pathway [12, 15]. In the previous study by Jiang et al., directing botryococcene-synthesizing enzymes to the chloroplast was shown to have increased the total amount of botryococcene detected [12]. Two enzymes, SSL-1 and SSL-3, are responsible for synthesizing botryococcene [14]. Another technique used for increasing the

amount of botryococcene produced in tobacco was linking the two enzymes with a peptide linker [12]. If transforming *C. reinhardtii* with the GGPPS or GGPPS11 and LOS constructs independently does not yield a significant amount of lycopaoctaene, a possible future direction could be to try and express both enzymes connected by a peptide linker.

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