AUTOTROPHIC PRODUCTION OF LIMONENE IN ENGINEERED MICROALGAE CHLAMYDOMONAS REINHARDTII

A Thesis

by

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ABSTRACT

Biosynthetic alternatives for high-density petroleum derived tactical fuels, like JP-8 and JP-10, remain elusive given their tremendously high energy densities, low temperature viscosity and cloud point. Limonene, a bicyclic terpene abundant in pine resin, may supplant non-renewable aromatic compounds used in current aviation-fuel mixtures. Tractable microbes, such as yeast and *E. coli*, can yield high titers of hydrocarbon, but at the expense of increasing sugar and carbon inputs required by heterotrophs. Here we report a metabolic engineering approach, wherein photosynthetic carbon-flux is rerouted towards limonene in the microalgae *Chlamydomonas reinhardtii.* Conceptually, determining baseline capability of monoterpene production in algae will pave the way for advanced autotrophic hydrocarbon production.

The research thus aims to address the technical barrier of engineering an algae-based autotrophic production system to produce the bicyclic terpene D-limonene to serve as a renewable jet fuel. The broader implication of limonene detected in the headspace of closed photobioreactors is significant on two accounts: First, limonene is emitted from microalgae and volatilized into the airspace above the liquid culture – key for future bioseperations approaches harvesting limonene; and second, redirecting photosynthetic carbon-flux away from crucial sterols/carotenoids/pigments towards volatile limonene will not negatively impact overall biological function and cell viability.

DEDICATION

This work is dedicated to all my family, friends, and committee members for their excellent support and guidance. Thanks go out specifically for Gaston and Estelle Syrenne, Dad and Mary Syrenne, my Mom, Tony, and all my brothers and sisters (Keiko, Kenji, Kobe, Jake, Jed, Hannah, and Emily). I thank my committee members with all my heart for their persistent encouragement, even under difficult circumstance. This work could not have come to fruition without all the generosity and kindness from these folks.

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Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. I would also like to thank my committee chair, Dr. Joshua S. Yuan, and my committee members, Dr. Tim Devarenne, Dr. Susie Dai, and Dr. Elizabeth (Betsy) Pierson, for their guidance and support throughout the course of this research.

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1. INTRODUCTIONS AND LITERATURE REVIEW

Biofuels that are derived from algae could serve to replace many petroleum-based fuels used for transportation. Furthermore, algae do not compete with food crops and could dramatically reduce environmental impact when compared with classic biofuel feedstocks like corn and soybean. Microalgae are unicellular photosynthetic organisms found almost ubiquitously in nature. The energy in fossil fuels originated from photosynthetic algal organisms that exploited energy from the sun, carbon dioxide, and liquid water to grow in biomass and reproduce. In a sense, photosynthesis is inextricably linked to oil, coal, and natural gas, which drive the global hydrocarbon based economy. There are many positive aspects from fuels that are derived from algae, such as; high photosynthetic productivity (Blankenship et al., 2011); reduced competition with food crops (Fargione et al., 2008); use of non-productive/arable land; and diverse use of water sources (wastewater, salt water, fresh water, brackish water) (Dismukes et al., 2008; Downing et al., 2002). Despite the advantages, realization of algae based biofuels has essentially stonewalled due to costs underpinned by major technical challenges that still remain (Wijffels et al., 2013). This research seeks to address several technological challenges associated with algae biofuels. Furthermore, it's important to understand the jet fuel characteristics seeking to be replaced, and why algae offer and a unique advantage when compared to other options and strategies.

1.1 Characteristics of jet fuels

Military and commercial jets require fuels with demanding specifications. Alternatives for high-density petroleum derived tactical fuels remain elusive given their tremendously high energy densities, among other technical challenges, relating to demanding specifications for military jets and missiles. Constraints in thermochemical and thermophysical properties of jet fuel include low cloud point, and high volumetric energy density. For example, missile propulsion fuels (JP-10) have extremely high volumetric energy density requirements that require specialized fuel mixtures and cannot be met by current renewable fuels and processing technology (Meylemans et al., 2012). Cloud point refers is defined as the temperature at which solids precipitate out of a fluid. A high cloud point thereby increases risk of engine malfunction as a result of oxygenated molecules tendency to hold water, and freeze at high altitude. Molecules that lack oxygen do not readily freeze and thus have a lower cloud point temperature. Volumetric energy content is defined as the amount of energy stored in a given unit of mass and is important because the volumetric energy density of a fuel is roughly proportional to the range of an aircraft or missile (Chung et al., 1999). The following section compares traditional and alternative biofuels in terms of their properties and potential for use as jet fuel.

1.2 Comparing traditional and alternative biofuels

First generation biofuels are derived from sugar, starches, vegetable oils and animal fats, and for the most part are typically produced directly from food crops such as corn. Second generation biofuels are produced from fermentation of the sugars in cellulosic plant biomass. Despite tremendous technological progress, 1st and 2nd generation biomass feedstocks are highly complex, containing many oxygenated molecules. Ethanol and biodiesel are two examples of traditional biofuels derived from these feedstocks.

Ethanol has considerably lower energy content and gravimetric density compared to military and commercial jet fuels. The lower energy content of ethanol is a particular disadvantage for use in military jet fuels, as it compromises effective combat radius without use of additional fuel storage capacities. The low energy density of ethanol would require more liquid fuel capacity to equal the energy density of jet fuels currently used by commercial and military aircraft. In addition, optimization of engine components requires extra effort to burn higher ethanol and petroleum fuel ratio mixtures. Ethanol also has a characteristically high cloud point. Combined, the aforementioned reasons pose significant hurdles for bioethanol as a jet fuel, thus making its adaptation unlikely.

Biodiesel is produced by fatty acid methyl transterification of lipid molecules such as vegetable oil and animal fat. Long chain fatty acids derived from triacylglycerol's (TAG's) represent the majority of renewable based algal biofuels –

collectively known as biodiesels. Fatty acid methyl esters (FAMEs), commonly known as biodiesel, offer higher energy content than bioethanol (37.4 MJ/kg) (Bozbas, 2008), but lack other constraints specific for jet fuels. The major disadvantage of FAMEs as renewable jet fuel is their characteristically high cloud point, which refers to the temperature at which solids will precipitate out of the fuel. In the case of both ethanol and biodiesel, this is primarily a result of the presence of oxygenated molecules that could increase the risk of engine malfunction at high altitude.

1.3 Terpenoid based fuels could provide an alternative solution

Aromatic short-chain terpenoids known as monoterpenes are highly sought after in recent years as a drop-in liquid hydrocarbon fuels (Peralta-Yahya et al., 2012). Monoterpenes are naturally produced alkanes and alkenes with properties that overcome the drawbacks of bioethanol and biodiesel renewable fuel mixtures. The unique requirements for jet fuels dictate various molecular criterions as compared to gasoline and diesel. The United States Navy has demonstrated that certain monoterpenes have similar, and in some instances even superior performance characteristics, than petroleum based jet fuel counterparts (B. G. Harvey et al., 2015). For example, the monoterpene limonene exhibits comparable energy density to kerosene derived for military jet fuels like RJ-5, and JP-10 (Brennan et al., 2012). Notably, limonene also serves as the precursor to many bicyclic pinene dimers, whose constrained ring structures offer slightly higher volumetric energy density tactical fuel replacements (Benjamin G.

Harvey et al., 2009). Considering the advantages or terpene-based fuels over tradition biofuels, the overall approach for this work is to engineer limonene production in algae for use as a drop-in aviation fuel.

1.4 Microbial engineering for the production of biofuels

Despite the potential of short-chain terpenoids to serve as fuel intermediates, a sustainable production process is still undeveloped. In fact, heterotrophic terpenoid production from engineered microbial hosts is feasible. For example, a group has successfully manipulated the MVA (mevalonic acid) pathway in *S. cervisiae* to achieve high titer of the immediate precursor to bisabolane, an alternative to D2 diesel fuel (Peralta-Yahya et al.). In contrast to heterotrophic processes, an autotrophic process represents a higher substrate to product conversion efficiency route. In oxygenic photosynthesis, reducing equivalents like carbon dioxide, ATP, and NADPH are consumed to fix carbon into long-chain hydrocarbons which could serve as jet-fuel (Dugar et al., 2011). The following section explains why we chose *C. reinhardtii* as a platform for our proof-of-concept work.

1.5 *C. reinhardtii* as host platform

C. reinhardtii is a eukaryotic green microalgae that has undergone extensive scientific study. Genome sequencing technologies used together with genetically

tractable hosts such as *C. reinhardtii* have developed powerful tools for fundamental biological research. In recent years, a great deal of information regarding the regulation of nuclear transgenes has emerged such as the importance for codon optimization for transgenes. Identification and optimization of native promoter sequences have also demonstrated that nuclear transgene expression can be further improved (Lodha et al., 2008). *C. reinhardtii* also grows well in inexpensive growth medium and requires no serum derived growth factors. The ease of handling and scalability, coupled with inexpensive growth conditions and stable nuclear transformation affirm our use as a host platform for engineering a terpene biosynthesis pathway in microalgae (Lauersen et al., 2012).

1.6 Terpene biosynthesis in microalgae

Apart from higher terrestrial plants, which simultaneously utilize both the MEP (non-mevelonate) and MVA pathways for the production of cellular isoprenoids, some eukaryotic green microalgae possess only the plastidic MEP pathway. Strategically, this offers a unique advantage for algal production of terpenoids. The redox balance favors the MEP pathway for converting photosynthetic derivate (sugars) or glycerol into isoprenoids (Yadav et al., 2012). In comparison, the MVA pathway consumes 1.5 moles of glucose or 3 moles of glycerol for every mole of IPP (isopentenyl diphosphate) produced - in addition to generating excess NADPH which is used for cell growth or biosynthesis of other non-isoprenoid secondary metabolites (Ajikumar et al., 2010;

Dugar et al., 2011). On the other hand, the MEP pathway consumes 1.255 moles glucose or 2.151 moles of glycerol for every mole of IPP produced, generating less reducing equivalents and increasing the overall yield of IPP resulting from the redox balanced catalysis (Dugar et al., 2011). *C. reinhardtii* possess only the MEP pathway for isoprenoid biosynthesis. Linking the more energy efficient MEP pathway with engineered limonene production is potentially significant from an economic standpoint. Generally speaking, as the efficiency to produce a compound increases, the cost of producing that compound decreases.

However heterotrophic production of long chain terpenoids from microbial hosts such as *E. coli* and *S. cerevisiae* features lower product yield per unit of glucose input, an attribute of the MVA energy surplus pathway as compared to the plastid MEP (methyl-erythritol-4-phosphate) energy deficient pathway (Dugar et al., 2011). Overall, the comparatively lower product yield is thought to result from the generation of reducing equivalents, which reduces substrate to product conversion efficiency and ultimately makes MVA derived terpene biofuels cost-inefficient and unsustainable. In contrast to heterotrophic processes, an autotrophic process represents a higher substrate to product conversion efficiency route. In oxygenic photosynthesis, reducing equivalents like carbon dioxide, ATP, and NADPH are consumed to fix carbon into long-chain hydrocarbons which could serve as jet-fuel (Dugar et al., 2011). Microbial production of terpene, the MEP pathway is a more energy efficient route for terpene biosynthesis. The photosynthetic production of terpene biofuel provides a potentially most energy efficient option for hydrocarbon biofuel production.

Notwithstanding significant potential, autotrophic production of monoterpenes has been rarely explored in algal species. Monoterpenes are often pollination or defense signals in higher plants (J. S. Yuan et al., 2008). There is no experimental evidence suggesting that microalgae can produce volatile monoterpenes such as limonene or other pinene dimers. A novel microalgae-based autotrophic hydrocarbon biofuel and bioproduct platform can be enabled by properly addressing these questions. The following sections address these questions and describe the successful engineering of *C. reinhardtii* for production of the volatile monoterpene limonene.

2. RESULTS AND DISCUSSION

Terpenoids comprise the most diverse group of secondary metabolites due to the different combinations, conformations and folding of the five carbon isoprene-based structures (Sacchettini et al., 1997). The diverse chemical structures and characteristics provide the basis for numerous industrial compounds including flavors, fragrances, chemical solvents, rubbers and others (Bohlmann et al., 2008). In particular, short-chain terpenoids such as monoterpene and sesquiterpene are highly sought after in recent years as hydrocarbon biofuels due to their thermophysical and thermochemical properties (Peralta-Yahya et al., 2012). Some C_{10} monoterpenes like limonene exhibit comparable energy density to kerosene derived jet fuels such as RG-5, and JP-10 (Brennan et al., 2012). Notably, limonene also serves as the precursor to many pinene dimers, whose constrained ring structures offer high volumetric energy density required for RJ-5 and JP-10 jet-fuel replacements (Benjamin G. Harvey et al., 2009).

Despite the potential of short-chain terpenoids to serve as fuel intermediates, a sustainable production process is still undeveloped. In fact, heterotrophic terpenoid production from engineered microbial hosts is feasible. For example, a group has successfully manipulated the MVA (mevalonic acid) pathway to achieve high titer of the immediate precursor to bisabolane, an alternative to D2 diesel fuel (Peralta-Yahya et al.). However heterotrophic production of long chain terpenoids from microbial hosts such as *E. coli* and *S. cerevisiae* features lower product yield per unit of glucose input, an attribute of the MVA energy surplus pathway as compared to the plastid MEP (methyl-

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Monoterpenes, such as limonene, are often pollination or defense signals in higher plants. To our knowledge, there is no experimental evidence suggesting that microalgae can produce volatile monoterpenes and other pinene dimers. In relation to the relevant design of a biorefinery, we do not know if monoterpene will primarily evaporate into the PBR headspace or be trapped within the cell. A novel microalgae-based autotrophic hydrocarbon biofuel and bioproduct platform can be enabled by properly addressing these questions. We address the aforementioned questions by describing the successful engineering of the eukaryotic microalgae *C. reinhardtii* for production of the volatile monoterpene limonene.

A rice monoterpene synthase, OsTPS26 (OsO4g27340), was characterized and transformed into *C. reinhardtii.* The enzyme activity and product identification assay followed a modified single-vial analytical method for quantitative GC-MS assays for sesquiterpene synthases (O'Maille et al., 2004). Initial prediction of OsTPS26 function was carried out through phylogenetic analysis and delineated a gene encoding either a monoterpene or diterpene synthase (J. S. Yuan et al., 2008). Further characterization of OsTPS26 via enzyme assay indicated that it is a bona fide limonene synthase, shown in Figure 1. The enzyme catalyzes the conversion of GPP to limonene, where the resulting allylic carbocation attacks a double bond of the substrate to form a cyclic carbocation intermediate and terminates with the loss of a proton to form (-)-limonene (Rajaonarivony et al., 1992). Limonene is the principal product of limonene synthase; however, in vitro enzyme assays also showed production of several other monoterpenes as well. The limonene synthase gene sequence, sequence available from rice genome database (OsO4g 27340), was modified for proper expression in *C. reinhardtii*. Since limonene synthase is a nuclear encoded gene that is targeted to the plastid, the cTP signal from the original sequence was removed and replaced with a *C. reinhardtii* stromal membrane targeting cTP signal and transformed into the nucleus using a modified glass-beads based method (Kindle et al., 1989).

To evaluate heterologous expression of the transgene, we measured *Os*LMS mRNA transcript abundance using qPCR and compared *Os*LMS gene expression against the constitutively expressed *Chlamydomonas* β subunit-like polypeptide (Cblp) reference

gene (Schloss, 1990). Figure 2 shows a comparison among the transgenic *Os*LMS expression from five selected lines.

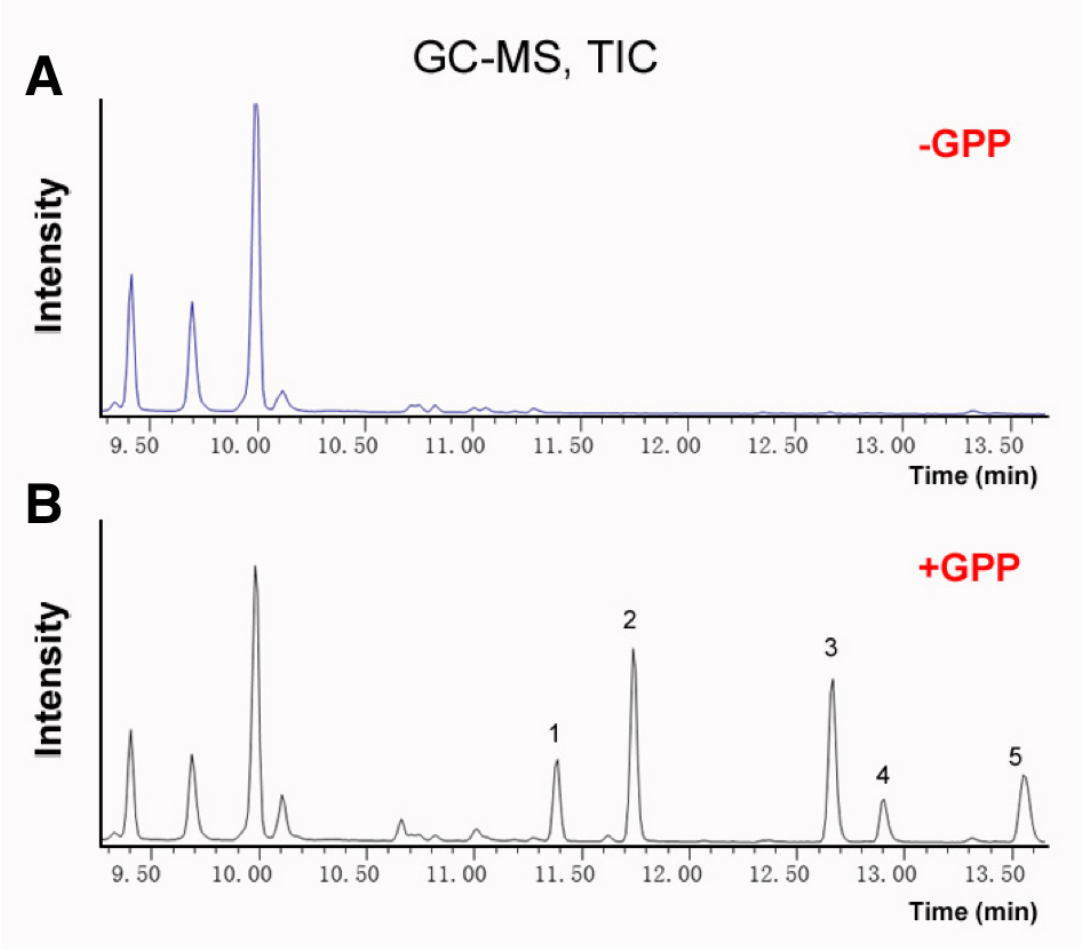

Figure 1 *In vitro* single-vial enzyme product identification assay for *Os*LMS. (A) -GPP; *Os*LMS without substrate. (B) +GPP; *Os*LMS with substrate. Numbered peaks denote monoterpene products produced by rice limonene synthase in the presence of substrate (+GPP), and correspond to (+)-4-carene (1), D-limonene (2), γ –terpinene (3), 4-thujanol (4), and terpinolene (5). GPP, geranyl diphosphate.

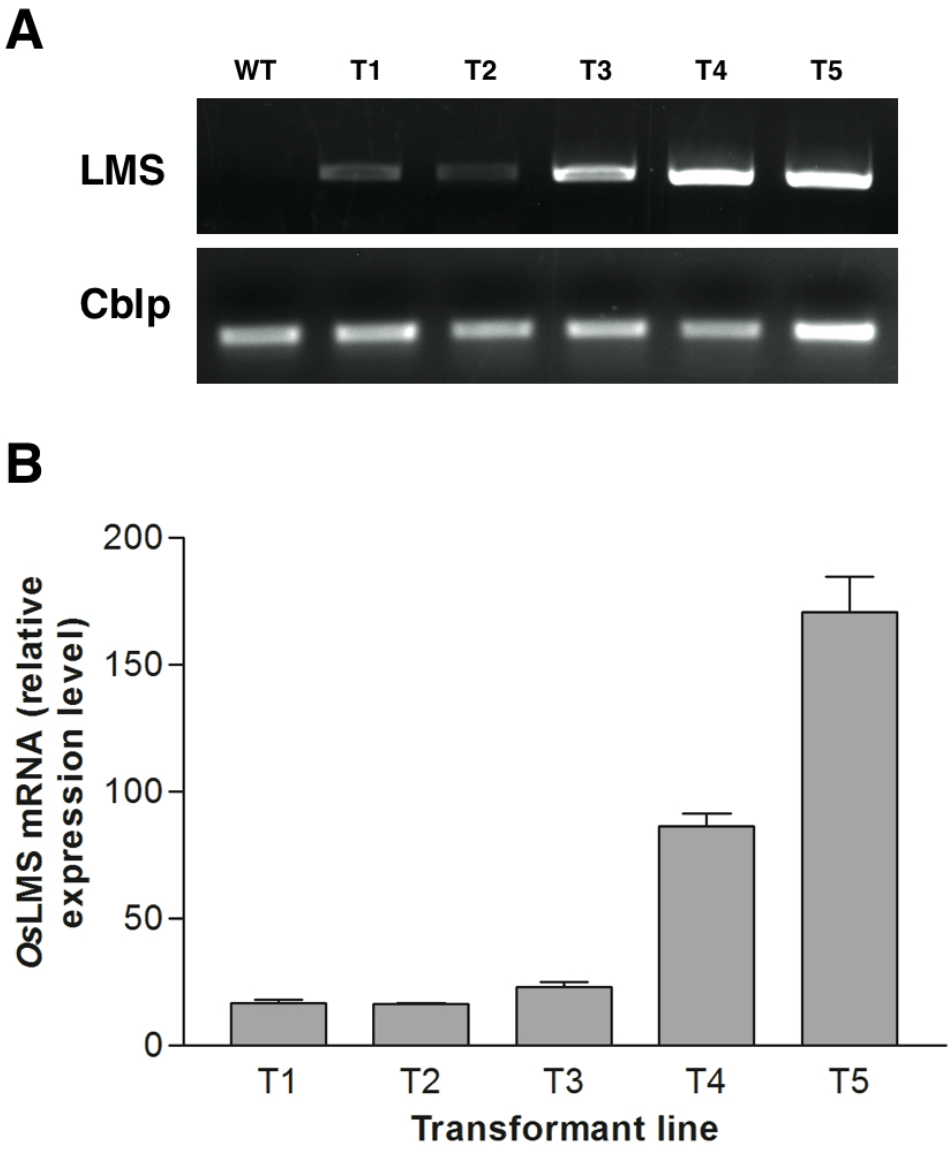

Figure 2 Transgenic expression of limonene synthase. (A) *Os*LMS single strand cDNA synthesis by reverse-transcription; (B) qPCR was used to determine *Os*LMS mRNA transcript abundance for evaluating the relative transgene expression level. Values represent the mean ± S.D. (n = 3).

Gene expression data for *Os*LMS confirmed that the transgene is highly expressed in engineered *C. reinhardtii.* The ΔCt between *Os*LMS and Cblp ranged between 2 to 7. According to standard ΔΔCt method, the results indicated that *Os*LMS was highly expressed, ranging from 4 to 200 times higher than that of Cblp (J. Yuan et al., 2007; J. Yuan et al., 2006). No amplification was observed in any of the wild type controls.

Volatile limonene production in transgenic *C. reinhardtii* was measured in five strains showing varying degrees of *Os*LMS gene expression. In order to evaluate the limonene production level, an in-house flow-through headspace collection system was built by connecting the enclosed photobioreactors with volatile collection traps (VCTs), as depicted in Figure 3. After 48 hours, the VCTs were washed with hexane and analyzed by gas chromatography mass spectrometry. The identity of limonene in corresponding GC peak was established using both reference compound and fragmentation patterns of parent ions. Limonene was absent in wild type cultures, but detected in the headspace of transgenic strains. Besides the headspace collection, limonene was also extracted from the algae biomass and no noticeable amount of limonene was found in the algal cell biomass. The result correlate with previous studies in tobacco that a majority of limonene were emitted to the headspace in transgenic tobacco producing high level of limonene (Wu et al., 2006). Limonene quantification was carried out via a standard curve established with 1-octanol as the internal standard and authentic limonene standard spiked at various concentrations. Detectable limonene accumulation on VCTs ranged from 0.2 - 1.3 μg/g dry weight (DW) over a 48-hour timespan.

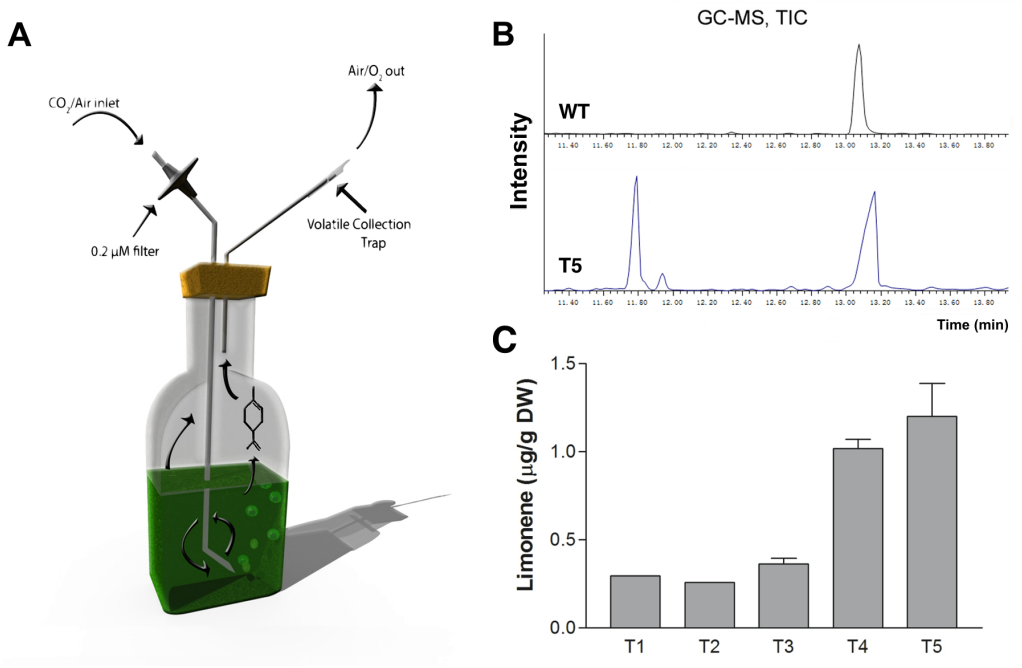

Figure 3 Limonene quantification in the headspace of PBRs containing transgenic algae: (A) Cartoon depicting the typical system for growing algae while simultaneously employing a purge-and-trap method for the collection of limonene on VCTs; (B) GC-MS analysis showing the presence of limonene in transgenic strain T5, and absence in wild type. The inset shows the mass fragmentation pattern for the peak identified as D-limonene. (C) Limonene accumulation in PBR headspace containing transgenic strains. PBR, photobioreactor; VCT, volatile collection trap. Values represent the mean ± S.D. (n = 3).

Limonene production appeared to plateau at around 1.3 µg/g DW, regardless of gene expression level.

It's important to note however that endogenous limonene and limonene trapped within the aqueous media was not evaluated. Heat shock experiments to induce Tlim gene expression (driven by the Hsp70A promoter) were not carried out, as we wanted to

evaluate the basal level of limonene production in transgenic strains. It has been shown that relative gene expression, when under control of the Hsp70A promoter, can increase greater than 4 fold (Lodha et al., 2008). Codon optimization is also known to ameliorate poor transgene expression typical for *C. reinhardtii*, wherein codon optimized reporter genes show 7 fold higher expression when compared to their non-optimized counterpart (Shao et al., 2008). Furthermore, boiling supernatant of transgenic cultures shows significant release of limonene trapped within the aqueous media (data not shown). We speculate, therefore, that our evaluated transgenic strains produce significantly more limonene than what was detected.

These results demonstrate the feasibility and baseline production capability for engineered microalgae to produce limonene; that cultivation in enclosed bioreactors offers unique advantages in sequestering volatile compounds; and using photosynthesis for fixing CO_2 into hydrocarbon biofuels. Maximizing limonene production will require further pathway optimization, codon optimization of heterologous MEP biosynthetic genes, and optimizing culture conditions for facilitating the volatilization and collection of limonene in PBRs. To guide further engineering and optimization of the pathway, we carried out the modeling of production vs. enzyme expression and pathway simulation to elucidate potential metabolic bottlenecks. When limonene yield is plotted against the expression of *Os*LMS, the model followed a logarithm curve, where limonene production saturated at the high gene expression level, Figure 4. The results paralleled the simulation of MEP pathway with limonene synthase as the enzyme for terpene production.

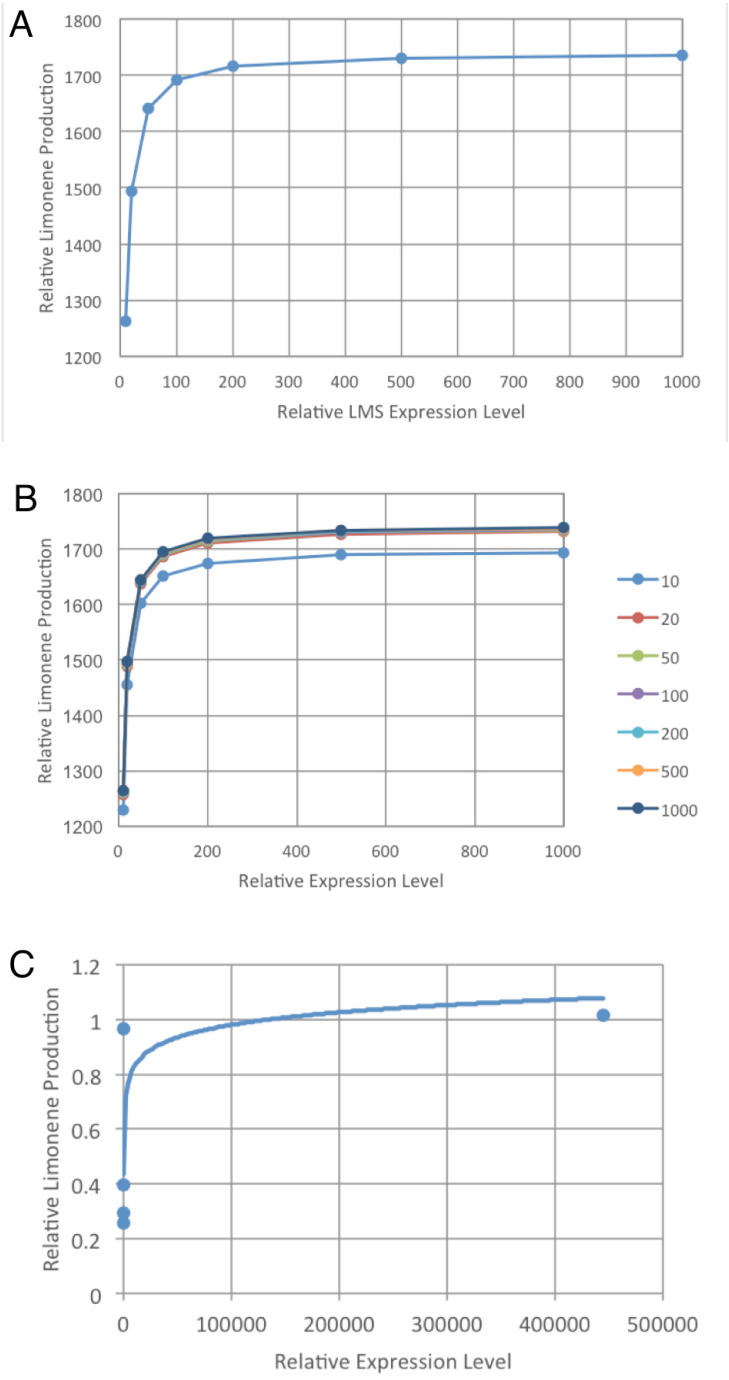

Figure 4 Modeling and simulation of limonene production in response to limonene synthase levels. (A) Plotting limonene production in response to *Os*LMS expression level. (B) Simulation of limonene production in response to the expression level of a generic limonene synthase. (C) Simulation of limonene production in response to the limonene synthase expression level of with varying upstream carbon-flux.

The simulation of terpene yield in response to the expression level of a generic limonene synthase also rendered a similar logarithm curve, indicating that terpene yield is saturated at high limonene synthase expression levels. Both production models and pathway simulation derived the logarithm function for terpene yield in response to gene or enzyme levels. The results indicate that *Os*LMS was one of the metabolic bottlenecks for higher terpene yield. Further simulation indicated that increasing upstream carbon flux would increase limonene yield, but only marginally at high *Os*LMS expression levels. These results are significant by showing that further improvement of limonene yield will require improving both the upstream carbon flux, and substrate turnover for *Os*LMS. An alternative approach is to use a volatile terpene producing enzyme with more rapid turnaround time, or modifying the enzyme itself through rational design.

Overall, the heterologous expression of a single rice limonene synthase gene in *C. reinhardtii* enabled the production limonene. Importantly, we show that limonene was emitted into the headspace of enclosed PBRs, enabling a cheap, continuous harvesting system that does not interrupt feedstock supply. The discovery thus laid down the foundation for a new microalgae-based volatile hydrocarbon biofuel and bioproduct platform with simplified target compound purification. Despite the progresses, more research needs to be carried out to enable a commercial platform. We have identified the both limonene synthase and upstream carbon flux as the metabolic bottleneck to further increase terpene yield. The direct production of volatile hydrocarbon biofuel from photosynthetic algae will simplify industrial processing steps, improve the energy and carbon efficiency for sunlight to fuel, and reduce sugar input costs associated with

heterotrophic organisms. Further optimization of volatile terpene biosynthesis in eukaryotic microalgae could enable a novel alternative for producing advanced hydrocarbon biofuels. We designed a complete pathway, shown in Figure 5 that would theoretically optimize the redirection of photosynthetic carbon flux towards limonene. To our knowledge, the maximum number of genes co-expressed in the nuclear genome of *C. reinhardtii* is two. Extensive efforts that integrated upstream MEP genes to further increase limonene yield showed no significant improvement. Despite insignificant yields, we achieved the first integration of a novel monoterpene pathway in *C. reinhardtii* and demonstrate it's potential as a host platform for metabolic engineering approaches.

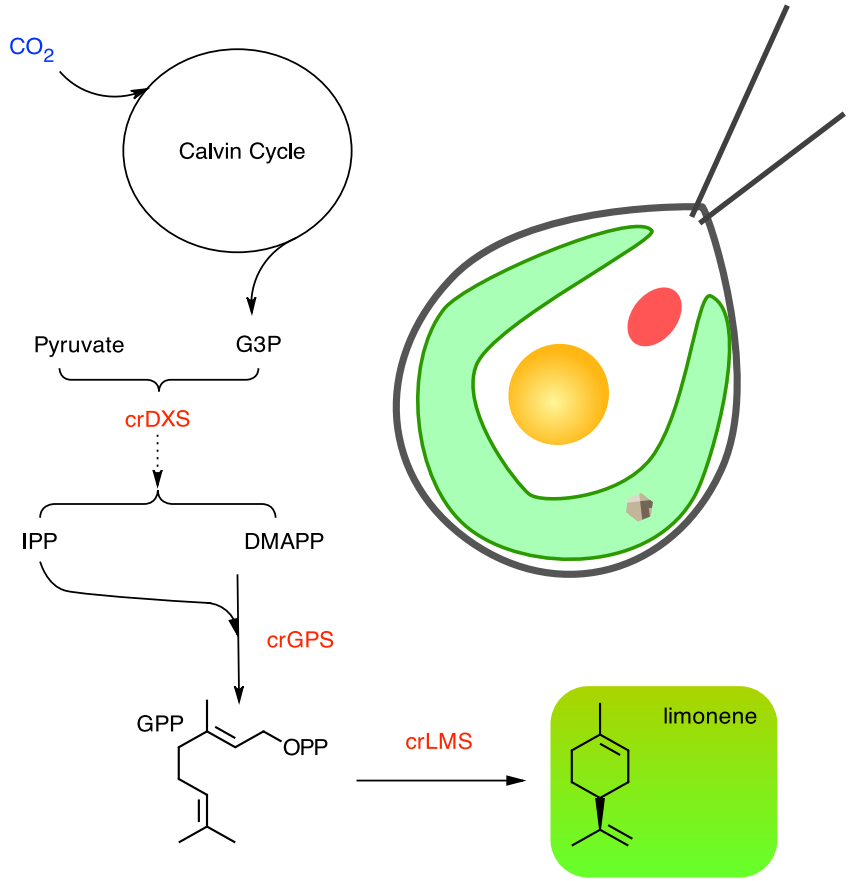

Figure 5 Conversion of CO_2 into terpenoid based hydrocarbon fuel. MEP isoprenoid pathway depicting flow of photosynthetic carbon flux into limonene in engineered *C. reinhardtii*. Isoprenoid biosynthesis begins with the conversion of G3P substrate by *DXPS* and leads to the production of either C_5 IPP or DMAPP, which constitute the building blocks required for all further downstream terpenoid biosynthesis. Red arrow indicates the engineered monoterpene biosynthetic pathway by heterologous expression of *OsLMS*. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; *DXPS*, deoxy-D-xylulose 5-phosphate synthase; G3P, glyceraldehyde-3-phosphate; *GPS*, geranyl diphosphate synthase; GPP, geranyl diphosphate; *GPPS*, geranylgeranyl diphosphate synthase; GGPP, geranylgeranyl diphosphate.

3. METHODS

The pFusion plasmid is a *C. reinhardtii* expression vector resulting from the fusion of a donor and acceptor vector. This is accomplished via cre/lox recombination of the donor vector (pHsp70A/RbcS2-Tlim) and acceptor vector (pKS-aph7”-lox). The acceptor vector provided a Hygromycin antibiotic resistance gene. The cgluc gene present in the original donor vector was removed via XhoI/BamHI restriction and replaced by our Tlim gene. Ligation of Tlim into the donor vector was accomplished by T4 DNA ligase (New England Biolabs) and standard molecular biology techniques. The resulting donor vector is refereed to as pHsp70A/RbcS2-Tlim and is shown in Figure 5.

To generate a modified limonene synthase capable of strong expression in *C. reinhardtii,* we first spliced out the rice cTP signal by designing PCR primers to specifically add the desired cTP signal to the truncated N-terminal region of OsTPS26 and simultaneously add XhoI and BamHI to the 5’ and 3’ ends. The modified rice limonene synthase gene, called Tlim,, was used to replace the cgluc gene in the donor vector. The ligation product between Tlim and the donor vector was then transformed into Pir1 Competant E. coli (Invitrogen) cells and grown on LB supplemented with kanamycin (50 μg/mL) agar plates.

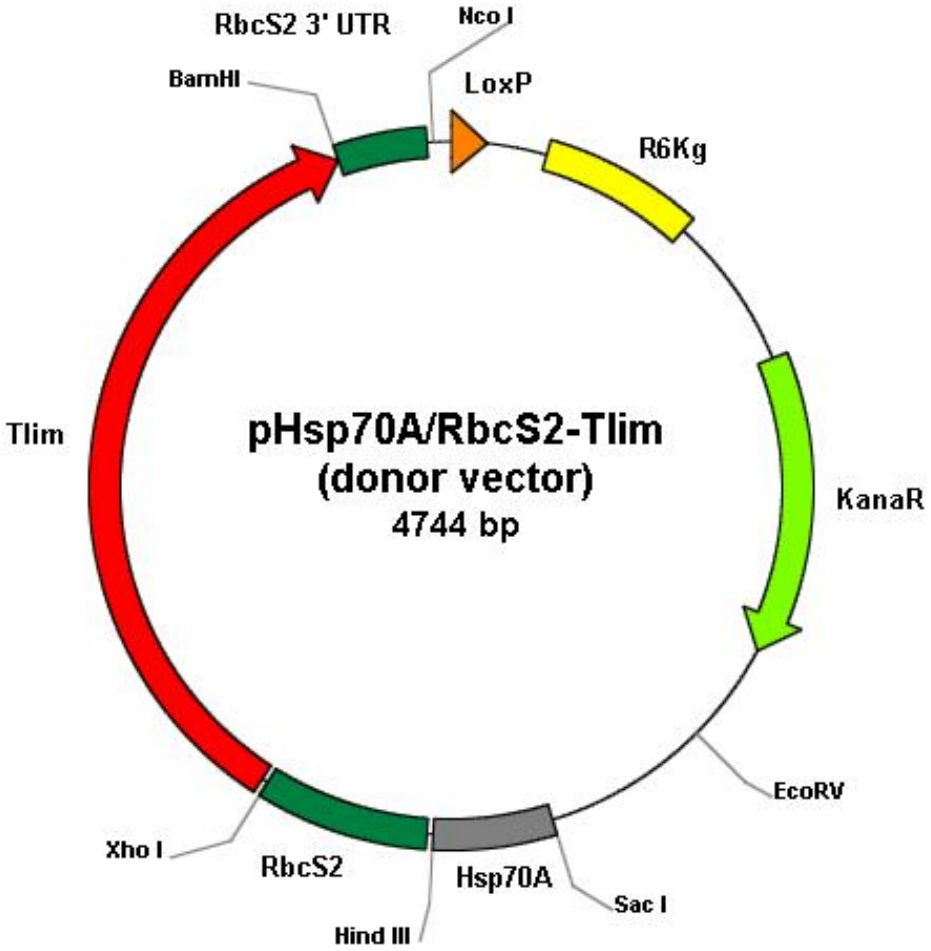

Figure 6 Vector map of pHsp70A/RbcS2-Tlim. The Tlim gene is controlled by a strong constitutive and inducible Hsp70A/RbcS2 heat shock promoter from *C. reinhardtii*. This plasmid serves as the donor vector for cre/lox recombination with pKS-aph7"-loxp acceptor plasmid.

3.1 *C. reinhardtii* expression vector design and construction

Assembly of the pFusion expression vector was carried out using cre/lox recombination of the donor and acceptor vectors. Recombination product DNA was transformed into Mach 1 (Invitrogen) competent *E. coli* and grown using LB supplemented with kanamycin (50 μg/mL) agar plates. Plasmids were screened from single transformant bacterial colonies by diagnostic restriction with EcoRI restriction enzyme, wherein positive clones yielded 2.3 kb and 6.9 kb DNA fragments. Positive clones were purified using a kit (Qiagen Maxi-Prep), and the concentration was determined using a NanoDrop Spectrophotometer. Purified plasmids were stored in -80 °C for future use. All original plasmids were acquired from the Chlamydomonas Collection Center, University of Minnesota.

3.2 Nuclear transformation of *C. reinhardtii*

Transformation experiments were performed using *C. reinhardtii* strain cc503, and cultured in 100 mL Tris-Acetate Phosphate (TAP) liquid media. Inoculated cultures were grown until the cell density reached 1-2 x 10^6 cells/mL, then centrifuged at 4,000 rpm for 5 minutes. The algal pellet was resuspended in 1 mL fresh TAP and shaken horizontally at 40 °C for 25 minutes. After incubation, 1 μg purified pFusion plasmid (shown in Figure 7) was added, in addition to 100 μL 20% polyethylene glycol – 6000 (Sigma Aldrich) and 300 mg sterile concentrated sulfuric acid treated 0.5 mm glass

beads (Sigma Aldrich). The mixture was vortexed for 15 seconds at high speed, and diluted 10 times with TAP media. The cells were then grown for 4-6 hours on an orbital shaker with gentle shaking under constant white light. After recovery, cells were collected by centrifugation at 4,000 rpm for 10 minutes. The pellet was resuspended in 0.5 mL TAP and plated on TAP agar plates containing 25 µg/mL Hygromycin B (Invitrogen) and allowed to grow under constant white light.

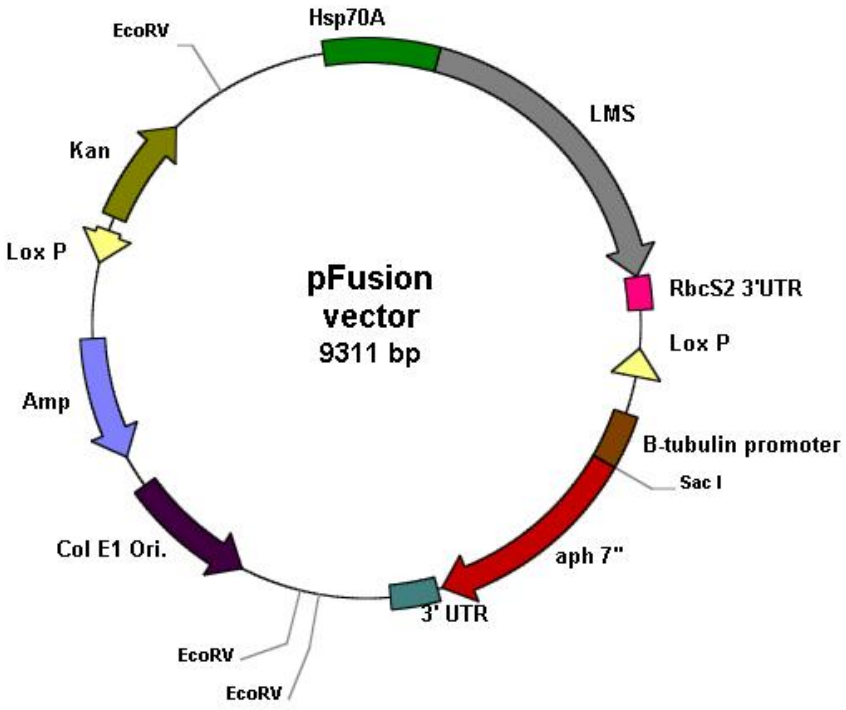

Figure 7 Transformation vector pFusion. Prior to transformation, pFusion was linearized with EcoRV in order to remove superfluous E. coli maintenance genes and increase transformation efficiency.

Single colonies were verified for positive transformants via partial PCR amplification of a 689 bp internal region of *Os*LMS (Figure 8). Single colonies were visible to the naked eye approximately 5-6 days post inoculation. To ensure stable transformation of our expression vector, single colonies were continually sub cultured onto selective plates for at least five generations, then screened for presence of inserted Tlim via PCR amplification using genomic DNA.

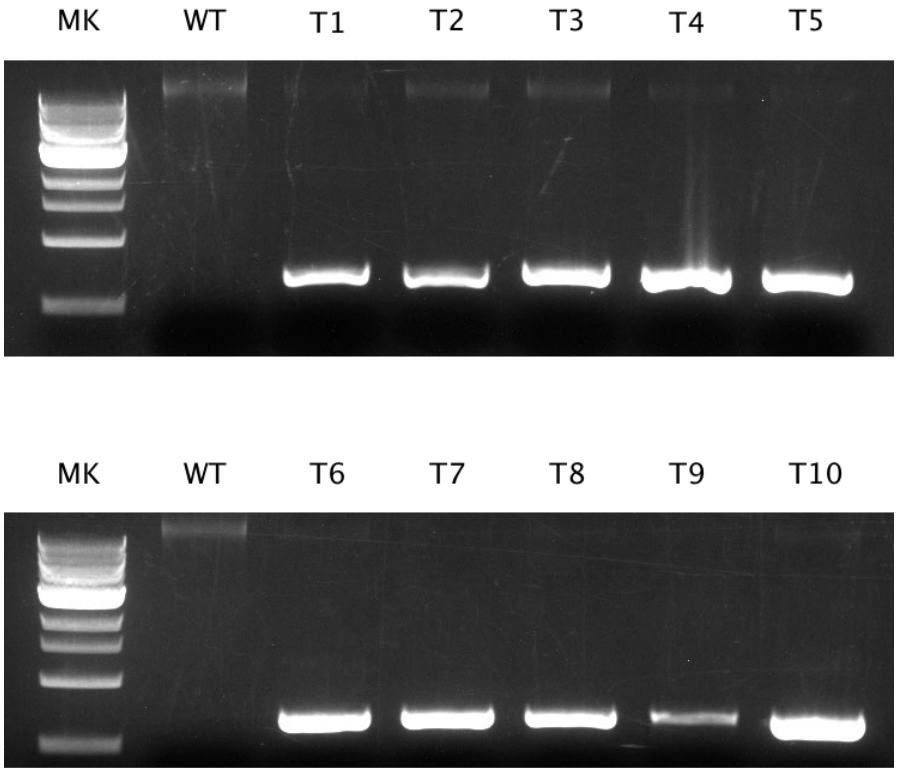

Figure 8 Genomic DNA screen. Amplification of a conserved 689 bp region within Tlim verified positive integration of the transgene into the host. MK, 1 kb ladder; WT, wild-type; T1 to T10, transformant lines.

3.3 RNA extraction and quantitative real-time PCR

To evaluate mRNA transcript abundance for limonene synthase in positive transformants, real-time PCR was utilized for selecting those lines whose LMS gene expression was the highest. High quality total RNA was isolated from 10 transformant lines by modifying the RNeasy Plant Mini RNA Extraction Kit (Qiagen). Fifty milliliters of fresh algae culture was grown until cell density reached ~1 x 10^6 cells/mL, then centrifuged at 4,000 rpm for 5 minutes. The pellet was re-suspended in 500 µL TAP and transferred to a clean 1.5 mL micro centrifuge tube, (Eppendorf). The tube was centrifuged at 14,800 rpm for 10 seconds, and the supernatant was decanted. Two hundred µL RLT buffer from the RNeasy Plant Mini Kit (Qiagen) was prepared immediately prior to use by adding β-ME (Sigma Aldrich), which was then added to the pellet in addition to 30 mg glass beads (Sigma Aldrich). The mixture was vortexed at high speed for 5 minutes before an additional 250 µL RLT+β-ME was added and vortexed again for 2 minutes. The remaining steps were carried out by following the RNeasy Mini protocol (Qiagen), step 4 - page 54. Using this method yielded ~1 µg/µL total RNA which was used in the following qPCR experiments.

Reverse-transcription was carried out using Superscript III Reverse Transcriptase (Invitrogen) and performed using manufacturer's protocol with random primers. The cDNA was diluted to 4 ng/µL before using in qPCR experiments. Individual wells on a 384 well plate each contained 4 µL template first-strand cDNA, 5 µL Power Syber Green PCR Mastermix (Invitrogen), and 1 µL primers. For qPCR, an ABI Prism

7900HT Sequence Detection system was used with the following conditions: stage 1 was 50 °C for 2 minutes; stage 2, 95 °C for 10 minutes; stage 3, 95 °C for 15 seconds, 60 °C for 30 seconds, 68 °C for 30 seconds; stage 4, 95 °C for 15 seconds, 60 °C for 15 seconds, 95 °C for 15 seconds. Stage 3 was done for 40 cycles and a disassociation stage was added. A list of primers used for this body of work is shown in Table 1.

3.4 Algae growth and volatile collection screening system

Each culture was kept in individual 1 L roux flask culture vessels (Pyrex, VWR). Hollow silicone stoppers (Epsi) were used to seal individual vessels. Glass tubes were inserted through the top of the silicone stopper to allow input of mixed gas as well as release displaced air within the culture vessel. A GE Healthcare Vacuguard Filter Device containing a 0.1 μm PTFE membrane was placed upstream of each culture vessel to sterilize the enriched air mixture. Silicone tubing (1/4" Inside Diameter) was used to connect the filter to the glass tube and to connect a pipette tip to the opposite end of the tube inside the vessel. Use of silicone components, as well as the Vacuguard filter device, was functional in design since it allowed the entire assembled culture vessel to be autoclaved prior to adding sterile fresh media and inoculating with algae cultures. To ensure delivery of accurately mixed 5% CO_2 with air, a 2-Gas Adjustable Gas Mixer was used (Superflash). Mixed gas was allowed to flow through a custom-built glass manifold/secondary air mixer with 10 spigots for connecting assembled culture vessels. Headspace analysis of separate culture vessels was accomplished by connecting a 3"

HayeSep-Q volatile collection trap (VCT) from ARS, Inc. VCT's were connected to the vent glass tube using 1/4" ID silicone tubing when the culture density reached 1-3 x 10^6 cells/mL for approximately 12 hours or until the culture reached stationary growth phase. Volatile collections traps were then removed from vessels and placed in individual 1.5 mL centrifuge tubes (VWR) prior to solvent extraction. To wash the volatile compounds off the VCTs for gas chromatography/mass spectrometry identification of limonene, 200 μL hexane containing 1-octanol authentic standard was used (Sigma Aldrich). To ensure a complete wash of the trap, single vial glass inserts were connected to the tip of the VCT via silicone tubing. The VCT plus the insert was then placed near the liquid nitrogen to chill and draw the hexane through the trap and into the insert. The glass insert containing the now washed VCT metabolites in hexane solvent was placed in a 1.5 mL glass chromatography vial for GC/MS analysis.

Table 1 List of Primers.

Application	Name	Sequence (5' -> 3')
Vector construction	Tlim-F	CGACAAAGCAATGCGCATC
Vector construction	Tlim-R	GATGGGGACAGGATTCACAAAC
Vector construction	cTlim-F	ATGCAGGCCCTGTCGTCTCGCGTGAACATCGCGGCCAAGCC
Vector construction	cTlim-R	CCAGCGCGCTCAGCGCCTGGTGGTCCGCGCCGCGAAGGAGGTG
Vector construction	FlimpET_BamH1	AAGGGATCCATGCGACAAAGCAATGCGCATC
Vector construction	RlimpET_Xho I	TCTCTCGAGGATGGGGACAGGATTCACAAAC
Vector construction	FTLimXhoI	ATACTCGAGATGCAGGCCCTGTCGTCTCG
Vector construction	RTlimBamHI	CGCGGATCCGATGGGGACAGGATTCACAAA
Genomic DNA screen	F-Tlim689bp	TGCAAGGCACCACCTCGAATCA
Genomic DNA screen	R-Tlim689bp	GGGTTGACAAAGCAACCCGCT
qPCR	FTlim_140bp	AGCTCACCTCCTTGTCCATGATGA
qPCR	RTlim_140bp	ATGGTAAGTGTAGGGCACGGTTGA

3.5 Limonene synthase enzyme activity and product identification assay

The single vial analytical method utilizes a two-phase separation system: the lower aqueous phase is where catalysis of enzyme and substrate occurs; and the upper organic phase trapped synthesized volatile metabolites. The aqueous phase, or reaction volume, consisted of 18.26 µL GPP substrate (Echelon Bioscience), 16.52 µL extracted crude enzyme, and 465.22 µL enzyme assay buffer (0.1M Bis-Tris pH 7.5, 0.1M $MgCl_2$, water). The total reaction volume was carefully overlaid with 500 µL ethyl acetate (Sigma Aldrich) and the vial cap was affixed. The vial was incubated at room temperature for 1 hour then vortexed for 10 seconds. The upper organic phase was separated into a small glass vial insert and placed into another 1.5 mL glass analytical vial for GC/MS analysis and product identification/quantification.

3.6 Bacterial expression of rice monoterpene synthase

Competent Rosetta cells (Novagen) were transformed with the LimpET32(a) vector for bacterial expression of limonene synthase. For bacterial expression, the cTP signal and stop codon was removed from Tlim before ligating into pET32(a). Single colony transformants were inoculated into 5 mL LB media containing 50 µg/mL ampicillin before sub culturing into 50 mL selective media. After the culture OD_{600} reached 0.6-0.8, it was allowed to cool at 4 °C for at least 1 hour. After cooling, 25 µL IPTG (0.1 M) was added to induce cultures. Water was added to negative controls

instead of IPTG. After induction, cultures were shaken at 150 rpm for 8-10 hours at 18 °C before extraction of crude enzyme.

For crude enzyme extraction, 45 mL of induced cultures was centrifuged at 5,000 rpm for 10 minutes, and the supernatant was decanted. For cell lysis, 3 mL His-Buffer A was added to the pellet and sonicated for a total of 6 minutes (3 minute sonication, 1 minute rest, 3 more minutes of sonication) on ice. A 200 µL fraction of the lysed cells was centrifuged for 10 minutes at 8,000 rpm at 4 °C. Forty microliters of supernatant plus 10 µL SDS loading buffer was boiled for 5 minutes, then visualized on SDS-PAGE. After removing the supernatant, the pellet was resuspended in 200 µL water. 40 µL of the resuspended pellet was mixed with 10 µL SDS loading buffer and boiled for 5 minutes, and then visualized using SDS-PAGE. If limonene synthase was present, then single vial-enzyme product identification was carried out.

3.7 Gas chromatography-mass spectrometry analysis

Analytes were washed from volatile collection traps using hexane spiked with octanol, and were subsequently injected into the gas chromatography-mass spectrometry (GC-MS) using a Combi PAL autosampler (Agilent Technologies, Santa Clara, CA). GC-MS analysis was run on an Agilent 7890 GC (Agilent Technologies) coupled with an Agilent 5975 inert mass spectrometer. The GC analytical column was a HP-5MSI column at 30-m x 0.25-mm I.D., 0.25 um film thickness. The temperature of injection port was set at 250 °C, whereas that of MS transfer line temperature was 220 °C. The

GC oven temperature was programmed as follows: initial temperature 40 °C for 3 min, 40-140 °C at 5 °C/min, and 140-310 °C at 45 °C/min, requiring a total run time of 28 min. Helium was a carrier gas delivered at a constant flow rate of 1.0 mL/min and inlet pressure of 7.0 psi. MS conditions were set to simultaneously acquire scan and a selected ion monitoring (SIM) data for quantification of limonene. The raw chromatography and mass spectrum data were processed with the provided GC/MS software.

4. SUMMARY AND CONCLUSIONS

Despite the potential for algae based biofuels, many challenges technical challenges remain for full realization of an algal autotrophic based liquid hydrocarbon production system. Long chain fatty acids derived from triacylglycerol's (TAG's) represent the majority of renewable based algal biofuels – collectively known as biodiesels (Fatty Acid Methyl Esters FAMEs). However, compared to petroleum-based fuels such as gasoline and diesel, biodiesel lacks certain thermochemical and thermophysical properties intrinsic to its petroleum counterparts – restraining its use in certain applications such as aviation fuel. Limitations related to biodiesel are primarily related to the presence of oxygen molecules in the ester bonds – similarly, ethanol shares this same disadvantage. Limonene differs from biodiesel and ethanol in that it's a pure hydrocarbon – lacking oxygen – therefore offering similar fuel properties to petroleum based fuels.

This study demonstrates that metabolic engineering of the green microalgae species *C. reinhardtii* can be used for the production of limonene ocimene, terpinene, β-caryophyllene, germacrene and other volatile monoterpenes suitable for jet fuel applications. Furthermore, this study demonstrates that closed photobioreactor culture systems can allow harvesting of volatile compounds without harvesting and processing the algal biomass – potentially simplifying downstream production and reducing economic costs associated with traditional algal harvesting for biodiesel production.

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