UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

INGÉNIERIE DES MICROALGUES POUR LA PRODUCTION DE PRÉCURSEURS MÉTABOLIQUES DE HAUTE VALEUR

ENGINEERING OF MICROALGAE FOR THE PRODUCTION OF HIGH-VALUE METABOLIC PRECURSORS

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OSCAR ELIAS MARTINEZ AVILA

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RÉSUMÉ

Les microalgues, tel que *Chlamydomonas reinhardtii*, peuvent produire plusieurs produits d'intérêt commercial et thérapeutique ; en profitant de leur facilité de culture, de leur croissance rapide, de leur entretien relativement abordable et de leur mécanisme métabolique rapide a conduit à une grande connaissance et utilisation de cette algue pour la production de produits de grande valeur. L'utilisation de *C. reinhardtii* en tant que système biosynthétique pour la production hétérologue de cannabinoïdes est relativement nouvelle et il reste encore beaucoup de travail à faire. Par conséquent, la présente mémoire prétend étudier *C. reinhardtii* pour analyser ses effets lorsqu'elle est transformée avec des gènes de la voie de biosynthèse des cannabinoïdes. Depuis l'élucidation de la voie, plusieurs entreprises travaillent avec cette algue et plusieurs procédés et souches ont été brevetés [1]-[7].

L'objectif de cette étude est de caractériser et d'analyser des clones de la microalgue *C. reinhardtii* lorsqu'ils sont génétiquement modifiés avec le gène *Cannabis sativa enzyme activateur d'acyl-1* (*CsAAE1*) et de vérifier s'il y a production de précurseurs de cannabinoïdes. Des centaines de clones transformés ont été criblés après électroporation. Un total de 15 clones transformés sur 886 ont montré des amplifications du gène d'intérêt et plusieurs mois après la transformation, tous ont montré une intégration stable du gène après plusieurs sélections sur des milieux antibiotiques. Le Western blot et le dosage enzymatique n'ont pas révélé de détection de protéines ni d'activité protéique dans nos conditions expérimentales. L'analyse HPLC a révélé une production détectable d'acide olivétolique. Nos résultats suggèrent que quelques clones peuvent être capables de produire ce métabolite, mais à un faible niveau. Par conséquent, une optimisation significative des conditions de croissance, des paramètres d'incubation et de la supplémentation de précurseurs peut être nécessaire pour améliorer la production du métabolite désiré. Nos résultats suggèrent que *C. reinhardtii* est un système biologique fiable, susceptible d'être transformé, et présente une stabilité d'intégration des gènes même plusieurs mois après la transformation génétique, et que ces clones d'algues produisent différents métabolites qui pourraient avoir une relation avec les cannabinoïdes ou leurs précurseurs.

Mots-clés: *C. reinhardtii,* cannabinoïdes, *CsAAE1*, electroporation, gène

SUMMARY

Microalgae, *e.g.*, *Chlamydomonas reinhardtii* can produce innumerable products of commercial and therapeutic interest; taking advantage of their ease of cultivation, rapid growth, relatively cheap maintenance, and rapid metabolic machinery has led to great knowledge and use of this alga for the production of high-value products. The use of *C. reinhardtii* as a biosynthetic system for the heterologous production of cannabinoids is relatively new and there is still a lot of work to be done. Consequently, the present memoire pretends to study *C. reinhardtii* to analyze its effects when transformed with genes from the cannabinoid biosynthetic pathway. Since the elucidation of the pathway, there are several companies that are working with this alga and several processes and strains have been patented [1]–[7].

The objective of this study is to characterize and analyze *C. reinhardtii* clones when being bioengineered with *Cannabis sativa Acyl-activating enzyme 1 (CsAAE1)* gene and verify if there was a production of cannabinoid precursors. Hundreds of transformed clones were screened after electroporation. A total of 15 transformed clones out of 886 showed amplifications of the gene of interest and several months after the transformation all of them showed stable integration of the gene after several rounds of selection on antibiotic media. Western blot and enzymatic assay did not exhibit protein detection nor protein activity under our experimental conditions. HPLC analysis revealed a detectable production of olivetolic acid. Our findings suggest that few clones may be capable of producing this metabolite, but at a low level. Therefore, significant optimization of growth conditions, incubation parameters, and precursor supplementation may be required to enhance the production of the desired metabolite. Our results propose that *C. reinhardtii is* a reliable biologic system amenable to transformation and exhibits gene integration stability even months after genetic transformation and that these alga clones produce different metabolites that could have some relation to cannabinoids or their precursors.

Keywords: *C. reinhardtii,* cannabinoids, *CsAAE1*, electroporation, gene

LIST OF ABBREVIATIONS AND ACRONYMS

APT	aromatic prenyltransferase
BSA	Bovine serum albumin
CBC	Cannabichromene
CBD	cannabidiol
CBDV	Cannabidivarin
CBG	Cannabigerol
CBGA	cannabigerolic acid
CBDA	Cannabidiolic acid
CBN	Cannabinol
CsAAE1	*Cannabis sativa acyl-activating enzyme* 1
DMAPP	Dimethylallyl diphosphate
EDTA	Ethylenediaminetetraacetic acid
FMDV	foot-and-mouth disease virus
GOI	gene of interest
GPP	geranyl pyrophosphate
HA	Human influenza hemagglutinin
HIS	Histidine
HPLC	High performance liquid chromatography
IPP	Isopentenyl diphosphate
HTAL	hexanoyl triacetic acid lactone
OA	Olivetolic acid
OAC	Olivetolic acid cyclase
PDAL	pentyl-diacetic acid lactone
PDVF	Polyvinylidene fluoride
PCR	Polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
ROC	Republic of China

TAG	Triacylglycerol
TCA	Trichloroacetic acid
TAP	Tris-acetate-phosphate
TBST	Tris-buffered saline with Tween 20
THC	Δ^9-tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
TKS	Tetraketyde synthase
USD	United States dollars
UQTR	Université du Québec à Trois-Rivières
WB	Western Blot
THCV	Tetrahydrocannabivarin

LIST OF SYMBOLS

°C	Degree Celsius
G	G force
gr	Gram
H	Hour
Kb	Kilobases
kDa	kilodaltons
Mb	Mega bases
mM	millimolar
ng	Nanogram
rpm	Revolutions per minute
s	Second
w/v	Weight/volume
µL	Microliter

CHAPTER 1

INTRODUCTION

1.1 Algae

Algae are autotrophic organisms that grow in a variety of aquatic habitats such as lakes, rivers, oceans, and streams [8]. They are found in all niches on the planet; therefore, they provide insight into environmental adaptation [9]. All of them have chlorophyll a and carry out a photosynthetic process where carbon dioxide is reduced to sugars using sunlight energy [10]. Taxonomically they are classified into three groups: Phaeophyta, Rhodophyta, and Chlorophyta corresponding to brown algae, red and green respectively (Table 1.0) due to their predominant pigments masking up the other ones [11].

Table 1.0 Main pigments present in the different phyla of algae.

Classification	Common name	Major accessory pigments	Pigments	Reference
Chlorophyta	Green algae	Chlorophylls a and b	Carotenes, xantophylls (lutein, zeaxanthin)	[12]
Rhodophyta	Red algae	Phycoerythrin	Phycobilins, chlorophyll a	[13]
Phaeophyta	Brown algae	Fucoxanthin	Chlorophyll c	[14], [15]

Algae are a source of food for some aquatic organisms and play an important role in the aquatic food web, providing a rich source of polyunsaturated fatty acids for zooplankton, fish, and many other multicellular organisms [16].

For humans, algae extracts and components from algae are used to strengthen the immune system, reduce cholesterol, and sugar levels in the blood, and there's evidence they present analgesic, bronchodilator, and hypotensive activities [17], [18].

1.2 Microalgae and biotechnology

Microalgae are a large and diverse group of unicellular aquatic microorganisms different in sizes going from 0.2 and 200 μm. It's been proposed the existence of 50,000 up to 200,000 species [19], [20]. However, less than thirty thousand species have been studied, along with microalgae being less studied than seaweed (microalgae) showing the great prospects of still unknown compounds microalgae [21], [22] and most of the research has been focused on some species due to their promising commercial exploitation. In the bioeconomy field, the new and pioneering processes are important to manufacture new biomaterials and bioenergy while reducing the consumption of non-renewable raw material [23]. They grow under various environmental conditions and can produce an extensive quantity of products with different applications in the food, nutritional, cosmetic, and pharmaceutical industries. These microorganisms have a simple cellular structure with a large surface-to-volume ratio which provides them the capacity to take up a large amount of nutrients [24].

Microalgae have been used in different biotechnological approaches; nowadays one of the most important concerns is global warming and they have been proposed as an alternative to mitigate the effects of greenhouse gasses [25], [26]; since half of Earth's photosynthesis is run by algae in aquatic environments [27]. They have been proposed to alleviate this problem thanks to their carbon concentration mechanism, where the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible of carbon assimilation has a greater affinity for the CO_2 fixation, thus enhancing its transformation efficiency [28]. This mechanism offers approximately 10-50 times higher CO_2 fixation capacity than vascular plants [29], [30].

Microalgae are a promising sustainable source of lipids and carotenoids. Several microalgae do not produce large amounts of lipids. However, when they come across

environmental stress, such as a lack of nitrogen, they slow down their rate of production and start producing energy-storage products, such as lipids [20].

Genetic engineering in microalgae has been done mostly to produce biofuels and bio-based chemical products. However, despite the advantages present in the production of biofuels and bio-products from microalgae, there are some major challenges such as mass balance to shift composition to obtain the product, higher cost of cultivation and optimization of culture conditions and competitive sale price, with algae-based fuel costing USD $300 whereas fossil fuel is between USD $70 and 80 per barrel. Despite this cost-effective issue, microalgae are a promising alternative to displace or even replace the $2.1 trillion fossil fuel industry [31], the global microalgae market for 2027 is projected to be 4.6 billion USD [32].

1.3 Genus *Chlamydomonas*

Chlamydomonas [Greek: chlamys, mantle; monas one or unity]. This genus was named by German naturalist C.G. Ehrenberg in the 1830s. The features of the genus are two anterior flagella; a cell wall; and a single chloroplast containing one big pyrenoid [33]. This genus is worldwide distributed, and its species have been isolated from freshwater lakes, marine waters, soil, and tundra [33]. In the late 1800s there were proposed 15 species of *Chlamydomonas* and by 1976 there were 459 species [34]. Etll (1976) proposed some classificational traits for the taxonomical division, not considering the habitat nor mode of reproduction for the classification of the *Chlamydomonas* genus, separating the species between one or several traits including presence or absence of apical papilla, body shape, the position of vacuoles, cell wall, etcetera. Although the structure, disposition, form, and shape may vary within *Chlamydomonas* species, the paired apical flagella and one or several pyrenoids inside a chloroplast, is constant [33].

Since the beginning, there have been some difficulties in the classification because many original descriptions and classifications were based on light microscope observations of only some samples without considering the variation of morphological characteristics.

Genus *Chlamydomonas* was considered to be inside the genus *Chloromonas*, whose cell and structure shape are almost identical, but they lack pyrenoids, and their only difference

could remain in the expression level of the gene *CCM1* responsible for the carbon concentrating mechanism [33].

1.4 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a haploid single-cell green alga, with multiple mitochondria, two anterior flagella, and one big chloroplast (Figure 1.0). It was first described by Dangeard in 1888 [35]. With its haploid genome, mutations on *C. reinhardtii* are straight away expressed, and phenotype changes are easily observed [9]. Cells have two mating types: mating type plus (mt+) and mating type minus (mt-)[36]. The vegetative haploid cells reproduce by mitotic divisions, but when there is nitrogen deprivation these cells can differentiate into gametes and the two matin types can fuse to produce a zygote that eventually will go through meiosis producing haploids cells that can continue the vegetative growth stage [37].

C. reinhardtii mitochondrial, chloroplast, and nuclear genomes have been fully sequenced [38], mitochondrial genome has a size of 15.8 kilobases (kb) [39] with a G+C content of 45% approximately, encoding 13 genes without introns. The chloroplast genome is 203.8 kb with G+C content of 35% approximately, encoding 99 genes of which only five have introns [39], [40]. The nuclear genome contains 121 megabases (Mb) with G+C content of 64% approximately [38], [41].

C. reinhardtii is one of the most important alga species that has been used as a model organism to study different cellular processes including plant physiology, photosynthesis, chloroplast biology, cell cycle, flagellar motion, and structure [9]. *C. reinhardtii* can grow mixotrophically; that is, in an autotrophic way (it can produce complex organic compounds using CO_2 as carbon source and energy from light) or heterotrophic (which can use external carbon sources (e.g., glucose) under dark conditions), depending on the availability of the organic source and light intensity. Its genetic transformation (the introduction of exogenous DNA) is relatively easy, at nuclear and chloroplast levels, and the time between the transformation of the microalga and the production of a recombinant protein is variable

between 2-3 days to 7 days, with the potential to reach a scale of up to thousands of liters in a matter of 1-3 weeks [42]–[45].

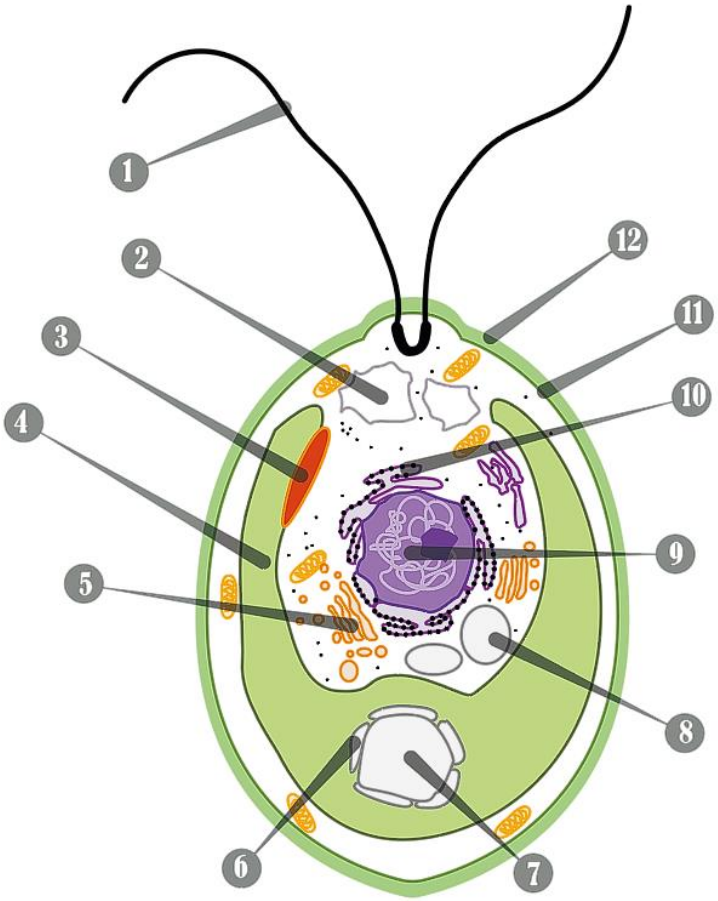

Figure 1.0 Diagram of *C. reinhardtii* in vegetative state. Unicellular oval shape, size about 3 µm width and 10 µm in length. The cell has a pair of cilia of the same length at one pole (1) [9]. (2) Contractile vacuoles located at the anterior end of the cell, this function regulating the water content inside cell, accumulating water and eventually expelling water [46]–[49]. (3) Eyespot, a light-sensitive organelle, composed of red to orange carotenoid pigments [50]. Located in the chloroplast, this organelle uses light shading to get the directionality, and lenses to focus the light [51]–[53]. (4) single cup-shaped chloroplast, the site of photosynthesis and carbon fixation, occupies half of the volume cell. [54], [55]. (5) Golgi apparatus. (6) Starch granules, (7) pyrenoid; when cells divide *C. reinhardtii* accumulates starch around the pyrenoid, a specialized structure composed of the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), responsible for CO_2 fixation [56]. (8) Vacuole. (9) Nucleus, (10) Endoplasmic reticulum, (11) cell membrane, (12) cell

wall, multilayered, an extracellular matrix composed of carbohydrates and 20-25 polypeptides [57]. Based on diagram from Nefronus.

1.4 History and *C. reinhardtii* in biotechnology

Microalgae show broad variation in both cellular structure and biosynthetic capacity, therefore they present a wide range of unique features and characteristics for commercial exploitation. Its unicellular physiology combined with rapid photosynthetic growth in different culture media means they can be more productive per unit land area than plants. Moreover, using microalgae as a substrate for low-cost, high-volume bio-products such as biofuels, helps to avoid several environmental issues that currently affecting the world [58].

Due to their ease of cultivation, manageability, cost-effectiveness, and natural biosynthesis of high-valued products, microalgae, are a promising source of sustainable compounds. The recent advances in molecular biology, genetic engineering, metabolomics, proteomics, and biotechnology, have led to many major breakthroughs for *C. reinhardtii* going from its genome fully sequenced to genome editing as highlighted in Figure 1.1 [59].

Since a long time ago, green algae, especially *C. reinhardtii*, have been the focus of basic and applied research. Therefore, the majority of the techniques and tools developed for transgene expression are specific to this alga species. Current research is targeting microalgae that are of prominent interest on industrial, biotechnological, and environmental applications [20].

Figure 1.1 highlights one of the significant advancements achieved while conducting research on *C. reinhardtii*: since its first transformation at the end of the 80's until the use of the CRISPR/Cas9 technology for gene editing. *C. reinhardtii* is the first photosynthetic organism wherein the nuclear and chloroplast compartments have undergone genetic transformation [60].

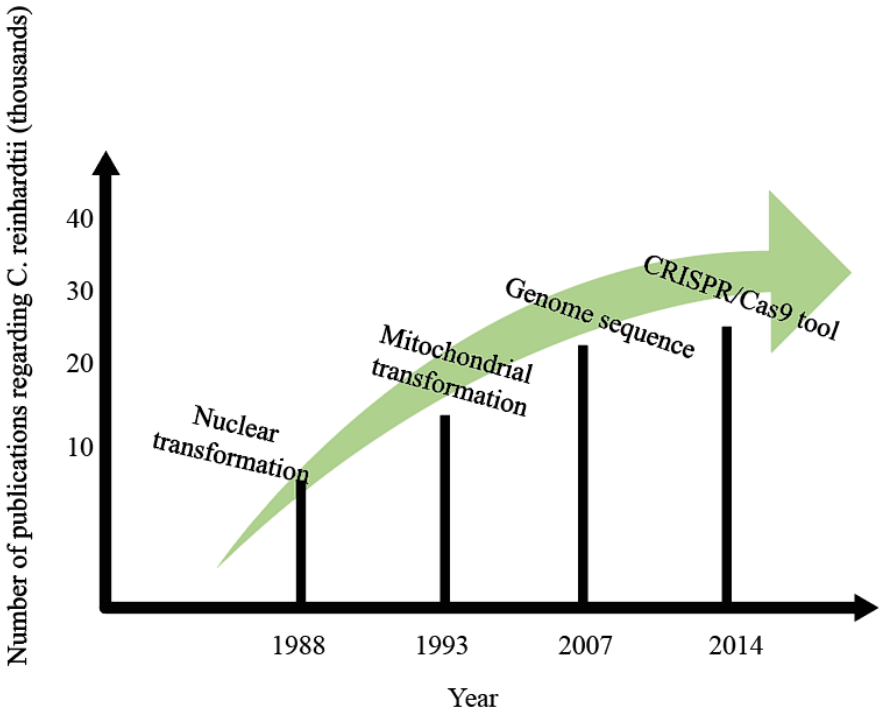

Figure 1.1 Major breakthroughs of *Chlamydomonas reinhardtii* along the years. First nuclear transformation [61]. First mitochondrial transformation [62]. *C. reinhardtii* genome is fully sequenced [38]. CRISPR/Cas9 first successful transformation [63]. Modified from Scaife et al., (2015) [59].

1.5 Heterologous biosynthesis of high-valued compounds in *C. reinhardtii*

The molecular toolkit for *C. reinhardtii* has greatly evolved, including optimized methods and protocols for genetic transformation and recombinant gene expression. The isolation and characterization of genes have led to great advances in biotechnology and the modification of organisms to the production of important metabolites that in normal conditions would be produced but in basal quantities. Molecular tools for this microalga have improved since its first transformations in the mid and late ‘80s [60], [64] and this has allowed efficient transgene expression by the nuclear genomes of microalgae [65]. This includes the generation of mutant strains with great transgene expression, such as UVM4 and UVM11 [66] synthetic genes [67] hybrid and synthetic promoters and use of endogenous introns [68], [69]. Proteins in *C. reinhardtii* can be expressed from either chloroplast [70] or nucleus [65]. It has been shown that the production of recombinant proteins in *C. reinhardtii* is quite advantageous compared to other eukaryotic organisms

(Table 1.1). Proteins encoded inside the nuclear genome can go through post-translational modifications (PTMs) [68], [69]. It has been proven that *C. reinhardtii* has great potential for the production of recombinant human therapeutic proteins in the chloroplast [71].

Table 1.1 Comparison between different recombinant protein systems with microalgae. The table compares various aspects of each system, such as the type of host organism used, yield and quality of the recombinant protein produced, scalability, and any potential challenges associated with the system. Modified from Rivera et al., 2011 [42].

Organism	Production cost	Production time	Scale up capacity	Glycosylation capacity	Final product quality
Bacteria	Low	Short time	High	No	Low
Yeast	Medium	Medium time	High	Yes	Medium
Animal cells	High	Medium time	Low	Yes	Very high
Animals	High	Long time	Low	Yes	Very high
Plant cells	Low	Short time	High	Yes	High
Plants	Very low	Long time	Very high	Nucleus: Yes Chloroplast: No	High
Microalgae	Very low	Short time	Very high	Nucleus: Yes Chloroplast: No	High

1.5.1 High-valued compounds

1.5.2 Lipids

Under normal conditions, algae produce fatty acids mainly for esterification into membrane lipids and these comprise around 20 percent of *C. reinhardtii* dry cell weight [72] and possess a great capacity to produce lipids [73]. The first studies with lipid machinery from *C. reinhardtii* were in 1972 when Levine and Sirevåg found that extracts from the alga catalyzed the incorporation of acetyl-CoA and malonyl-CoA to produce long-chain fatty acids [74]. Enzymes from fatty acid metabolism, glycerolipid biosynthesis, and fatty acid desaturation have been identified and its lipid biosynthesis pathways have been reconstructed [75].

Given that lipids are synthesized in response to various stress factors, including light and nutrient deprivation, lipid metabolism can be altered to increase lipid production [72], [76]. Numerous studies have attempted to enhance microalgal strains' lipid production, with co-culture with *Azotobacter chroococcum* resulting in a 2.3 to 19.4-fold increase compared to control cultures [77]. In a study conducted by Therien et al. in 2014, it was discovered that the growth of a starch mutant strain of *C. reinhardtii* could be optimized by cultivating it in an acetate-free medium, which is crucial for lipid production, in conjunction with a *Synechococcus* sp. mutant [78].
Various genetically modified strains of *C. reinhardtii* have been developed, including several microalgae starch mutants that have been isolated [78], [79]. These strains have shown an increased production of triacylglycerols (TAGs) following genetic modification [80], [81].

1.5.3 Carotenoids

Carotenoids, a subfamily of isoprenoids, are essential compounds to all photosynthetic organisms due to their photoprotective and antioxidant properties, they are produced by plants, fungi, some bacterial species, and algae [82], [83]. Isoprenoids are synthesized by microalgae through the utilization of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [84]. The most plentiful carotenoid is β-carotene, precursor of the vitamin A[85], [86]. Some carotenoids could be required for the survival of microalgae under some stress conditions [87]. For instance, lutein and zeaxanthin showed to be essential for the survival of *C. reinhardtii* under high light stress; as shown in the *npq1 lor1* mutant *C. reinhardtii* lacking these two carotenoids perished [88], [89].

In recent years, the carotenoid biosynthetic pathway in the microalgae *C. reinhardtii* has been thoroughly studied, and the different genes involved in the production of carotenoids have been characterized [90]. With its great carotenoid-producing potential, different ways to obtain large amounts of carotenoids have been studied, especially the most abundant one: β-carotene [90]. It has been found that by subjecting the microalga to constant photoperiod conditions in a bioreactor, greater production of these can be obtained, reaching a concentration of 2.5 g L^{-1} [91]. Napaumpaiporn and Sirikhachornkit (2016)

investigated the role of temperature and light on carotenoid biosynthesis and concluded that higher temperature induces carotenoid accumulation in the presence of light [92].

1.5.4 Cannabinoids

Cannabinoids are prenylated polyketides produced from fatty acids and isoprenoid precursors as part of the specialized metabolism of Cannabis [93], [94]. It has been found in other plants produce cannabinoid-like compounds such as bibenzyl (−)-*cis*-perrottetinene (*cis*-PET) produced by plants from the genus *Radula* sp. [95], [96]. To date they have identified more than 100 different types of cannabinoids [97].

In *Cannabis sativa*, hexanoic acid is transformed to its hexanoyl-CoA form by the enzyme *C. sativa* Acyl-activating enzyme 1 (*Cs*AAE1) [98] (Figure 1.2.). A type III polyketide synthase, tetraketyde synthase catalyzes the condensation of hexanoyl-CoA with three molecules of malonyl-CoA to produce 3,5,7-trioxododecaneoyl-CoA [99], then the enzyme olivetolic acid cyclase performs the cyclization trough aldol condensation of the tetraketide intermediate to yield olivetolic acid (OA) precursor [100]. OA is geranylated to form cannabigerolic acid (CBGA) by adding geranyl pyrophosphate (GPP) catalyzed by the enzyme aromatic prenyltransferase (APT) [101]. The oxidocyclase tetrahydrocannabinolic acid (THCAS) and cannabidiolic acid synthase (CBDAS) catalyze the formation of CBGA to THCA and to CBDA respectively. Finally, the main cannabinoids Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are subsequently derived from their acid forms by non-enzymatic decarboxylation when exposed to heat or light [102]. In relation to microalgae, the heterologous production of cannabinoids is relatively new, but there have been some major advances recently, as several companies have patented their genetically modified microalgae (US11180781B2, WO2021081647A1, WO2019202510A1), as some companies are already using *C. reinhardtii* as a platform for the production of cannabinoids in the chloroplast [103].

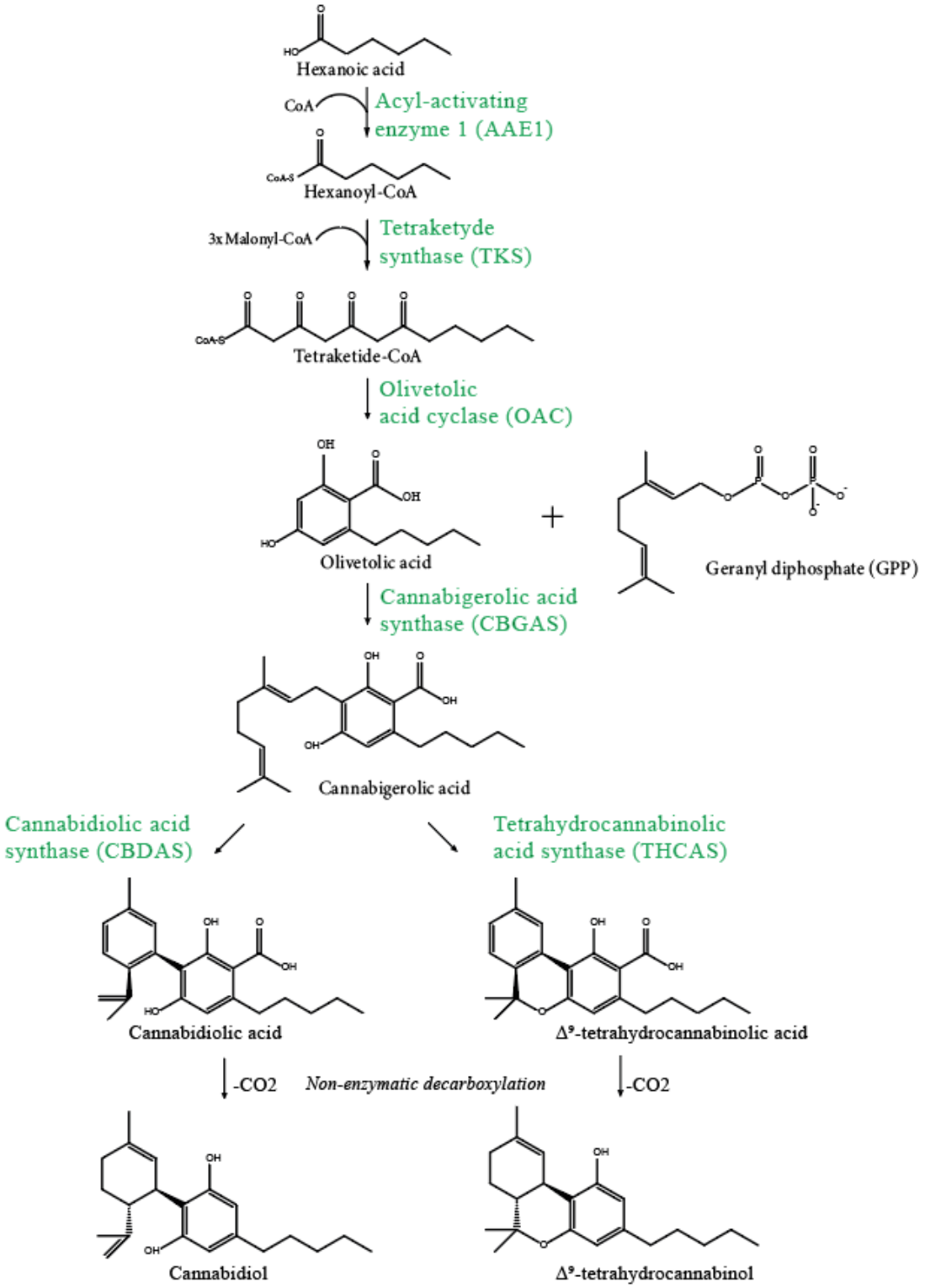

Figure 1.2 Biosynthetic pathway leading to the two main cannabinoids Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD).

1.6 Previous work on *Chlamydomonas reinhardtii* for the production of cannabinoid precursors

In 2016, Prof. Desgagné-Penix's lab started to work with *C. reinhardtii,* and other species of microalgae, focusing on the development of cannabinoid and cannabinoid-precursor-producing clones and their characterization for better understanding of the genetic transformation of *C. reinhardtii* [104]. Numerous patents have been filed for the production of cannabinoids using microalgae, demonstrating the potential usefulness of this system[1]–[7] . Since then, thousands of clones have been obtained using different transformation methods: glass beads, *Agrobacterium*-mediated transformation, and electroporation, to maximize the chance to obtain stable cannabinoid-producing clones, taking into consideration the great challenges of unpredictable transgene expression [105] and gene silencing [106], just to name a few. Added to that, the lack of precursors that can restrain a constant flux production of cannabinoids in *C. reinhardtii*. In an effort to address this issue, our laboratory has conducted several transformations and implemented various constructs. As a result of these efforts, a clone identified as C30 (clone 30) was generated in 2019. This clone demonstrated the production of olivetolic acid in an enzymatic assay conducted *in vitro*. Notably, the production of olivetolic acid has remained consistent since then and these findings are set to be published.

1.8 Research objectives

The goal of this study is to demonstrate if adding *Cannabis sativa* acyl-activating enzyme 1 (Hexanoyl-CoA synthetase) to *C. reinhardtii* boosts the production of cannabinoid precursors, with that, trying to overcome the bottleneck of limited pool of precursors in the production of cannabinoids in *C. reinhardtii*.

1.7.1 Specific objectives

1. To bioengineer *C. reinhardtii* strain CC-125 (control wild-type) and clone C30 (CB-producing strain) with codon-optimized *Cannabis sativa acyl-activating enzyme* 1 (*CsAAE1*) with and without introns to evaluate transformation efficiency.

2. To select positive clones by screens of *C. reinhardtii* transformants using colony PCR to detect amplification of the transgene, RT-qPCR to evaluate transgene expression and protein extraction, electrophoresis followed by blot analyses to observe production of the heterologous *Cs*AAE1 protein of interest.

3. To identify if there is the production of hexanoyl-CoA and/or olivetolic acid in the selected transformants through metabolite detection and enzymatic assay.

In order to address the research objectives, a methodology was employed, which will be elaborated in the methods section. To briefly summarize, electroporation was used to achieve transformation of *C. reinhardtii* CC-125 and clone C30. Following transformation, all clones were cultivated on media supplemented with appropriate antibiotics (zeocin and/or hygromycin, based on the inserted cassette) and subsequently evaluated for gene detection and expression using PCR and RT-qPCR (as shown in Figure 1.3).

To detect the production of a specific protein of interest, Western blot analysis was conducted on a subset of selected clones. Additionally, metabolites were extracted from PCR-positive transformants, which were then grown and subjected to analysis using high-performance liquid chromatography (HPLC) to determine the levels of olivetolic acid and/or hexanoyl-CoA production.

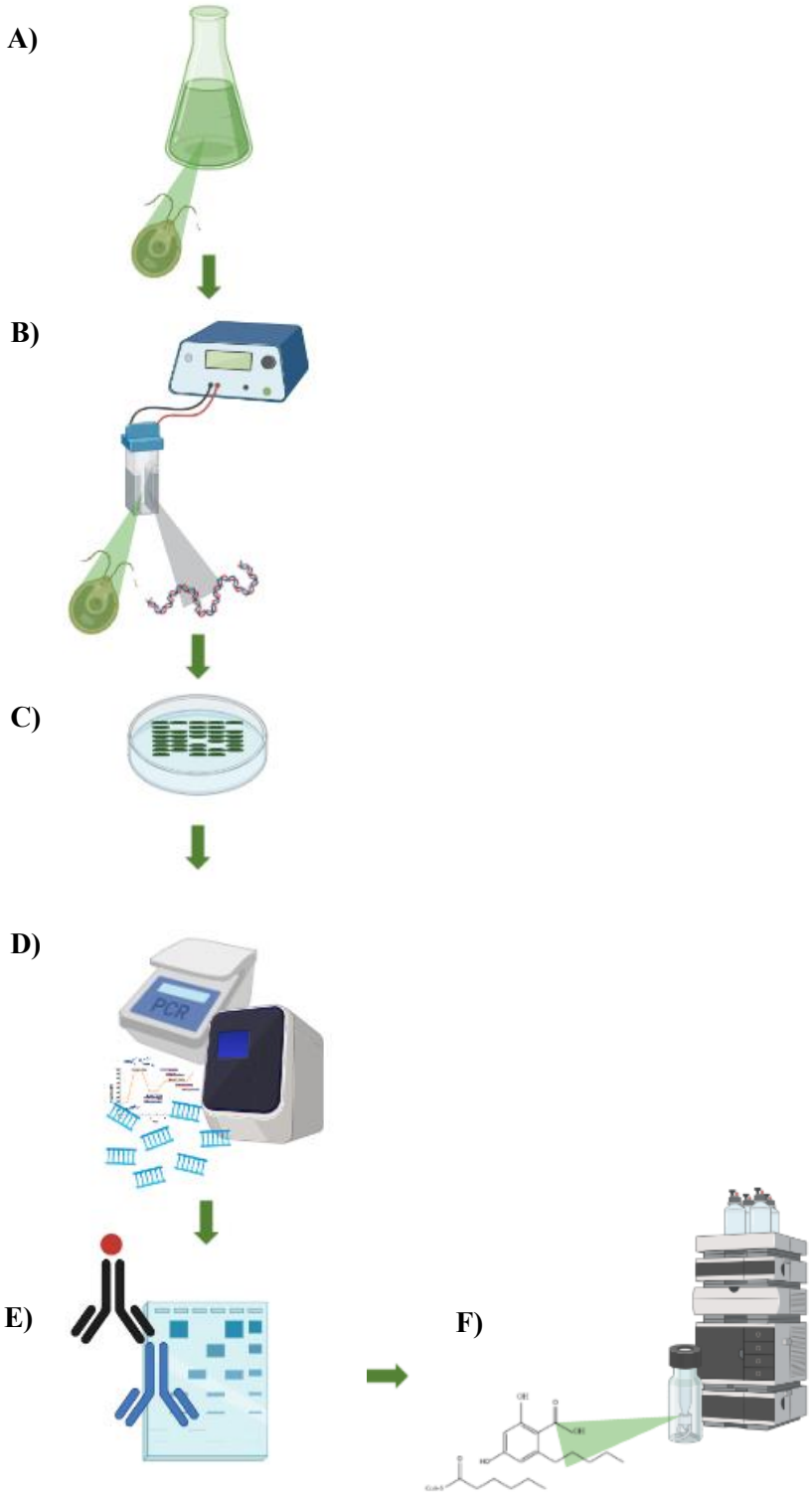

Figure 1.3 Summary of the methodology of this thesis project. A) The cultivation of *Chlamydomonas reinhardtii* in TAP media has resulted in the achievement of exponential growth phase, which is indicated by the appearance of a dark green color in the culture media. B) The electroporation process involves the transformation of microalgal cells

through the integration of genetic material, such as *CsAAE1*, facilitated by reversible pores on the cell membrane. Through non-homologous recombination, the genetic material is able to integrate with the genome of the microalgae. C) Clones are subjected to antibiotic selection media to discern between those that carry the cassette conferring resistance to a particular antibiotic and those that do not. The effectiveness of the selection process may be influenced by several factors, including false positives resulting from overgrowth on the media, variations in tolerance to the antibiotic concentration, and instability of cassette integration into the genome, among other reasons. D) The PCR technique is employed for molecular analysis of the integration of the insert into the genome. Under appropriate conditions, the amplification of the insert should occur, facilitating the eventual detection of the amplicon on an agarose gel. The success of this technique relies on the use of specific primers, specific PCR cycle parameters (including temperature and time), and proper concentration and volumes of reagents. E) After confirmation of PCR-positive clones, protein of interest is detected from the mixture of total cellular proteins using Western blotting technique; proteins are separated by their charge and size, they are transferred to a membrane and protein of interest is found by specific antibodies, eventually the protein is detected by a signal. F) The samples are subjected to high-performance liquid chromatography (HPLC) to enable the selective detection and quantification of the target metabolite within the sample mixture. Conditions and parameters for the experiments are explained ahead. Created with biorender.com

CHAPTER II

ENGINEERING OF MICROALGAE FOR THE PRODUCTION OF HIGH-VALUE METABOLIC PRECURSORS

2.1 Abstract

The green alga *Chlamydomonas reinhardtii* is a reliable model system to study different organelles and study genetic transformation tools. This microalga has been used as a system to produce high-value metabolites such as cannabinoids precursors [107], [108]. The genetic manipulation of this algae has had major drawbacks resulting in gene silencing, random insertion of the gene of interest, production of unknown metabolites, *etc*. [109] Therefore, improvements are essential for the success of the transformation and thus the production of high-valued products. Here, we describe a protocol to transform by electroporation the green cell alga *C. reinhardtii* strains for the insertion of *CsAAE1* gene to increase the pool of precursors of cannabinoids, *i.e*., olivetolic acid. *C. reinhardtii* transformants showing amplification of *CsAAE1* gene after several rounds of selection with antibiotics were analyzed for their protein expression and production. As well as the analysis of metabolite production by HPLC-MS; a low concentration of olivetolic acid was obtained through the experimental process in some clones, and the precise measurement of this concentration was not recorded. Furthermore, the confirmation of olivetolic acid production have yet to be confirmed through subsequent testing and analysis. Also, the gene expression analysis with RT-qPCR could not demonstrate *CsAAE1* expression under our experimental conditions. Further analysis and optimization need to be done in order to obtain and standardize a protocol that results in stable, metabolite-producing clones.

Keywords: *C. reinhardtii;* electroporation; gene; cannabinoids; acyl-activating enzyme antibiotic resistant

2.2 Introduction

Cannabinoids are a group of more than 100 compounds [110] first described in *Cannabis sativa* L. (commonly known as marijuana) [111]. Several cannabinoids have been proposed to have promising clinical applications in managing a range of health conditions and ailments, such as pain, nausea, anxiety, and seizures [103]. As a result, these compounds have been utilized for therapeutic purposes in humans [97], [112]–[115]. These interesting molecules interact with the endocannabinoid system in the body, which plays a crucial role in maintaining homeostasis and regulating various physiological functions [116], [117]. As more research is being done onto the medicinal properties of cannabinoids, more potential therapeutic applications are likely to be unveiled, offering a therapeutical alternative for those suffering from various health conditions.

As the consumption of cannabinoid-containing products increases, there is a corresponding increase in the demand for cannabis products such as marijuana, edibles, and concentrates. This increase in demand leads to a greater need for cannabis cultivation, harvesting, processing, and distribution [118]. The indoor and outdoor cultural production practices of Cannabis uses highly energetic processes causing soil erosion, water pollution, and greenhouse emissions [118], [119]. Also, Cannabis cultivation encounters major challenges such as variable content of cannabinoids, high cost of production, and elevated carbon footprint [119], [120].

Cannabis cultivation faces different problematics that can affect its growth, yield, and environmental impact [121]. Among these challenges are plant diseases, which can greatly compromise the quality and quantity of the harvested product [122]. Cannabis plants are susceptible to various diseases, including powdery mildew, gray mold, and root rot, among others [122]–[124]. These plant diseases can lead to reduced crop yield and increase the need for the use of fungicides and pesticides to control and prevent their spread. Its cultivation leads to variability of cannabinoids content [125], which are the active compounds responsible for the therapeutic and psychoactive effects of the plant. The content of cannabinoids in cannabis plants can vary widely due to factors such as genetics, environmental conditions, tissue type, and cultivation practices [125]–[127]. Moreover, cannabis cultivation can be expensive, requiring significant investment in equipment,

labor, and other resources [118]. Finally, cannabis cultivation can also have a significant environmental impact, particularly in terms of its carbon footprint [118]. Cannabis cultivation requires large amounts of energy for lighting, heating, and cooling, which can contribute to greenhouse gas emissions and climate change [128]. Overall, these challenges highlight the need for sustainable and responsible cannabis cultivation practices that prioritize the health of both the plant and the environment.

Hence, the use of different organisms for heterologous biosynthesis of cannabinoids is a promising alternative to overcome those environmental problems. The identification and discovery of the cannabinoid biosynthetic pathway has been useful because it has led to its reconstruction in heterologous systems [120], [129]. Microalgae has been used as a heterologous host over organisms such as bacteria and yeast due some species are considered GRAS (generally recognized as safe), minimal culture requirements compared to other organisms [130], among many other different advantages that will be further mentioned. Microalgae have huge potential as a high-valued product system and nowadays they represent an excellent system for the heterologous expression of compounds such as recombinant proteins, pigments, oils, lipids, *etc*. [131]–[135]. They present more advantages over conventional production systems like plants since algae don't require a large number of energetic resources in order to grow and depending on the specie, they can double their biomass in a couple of hours [119], [136]–[138]. They also provide low-cost and large-scale production of different important compounds since algae are not high-demanding nutriment organisms[139]. Among the several thousands of algal species, there are some that have been used for their great potential in biotechnology, environmental and industrial applications such as *Schizochytrium* sp., *Thalassiosara pseudonana*, and the freshwater microalga *C. reinhardtii* [140]. This last one has been used as a model organism for various physiological processes, including organelle biogenesis, and photosynthesis genetic studies[141], [142]. *C. reinhardtii* genomes (mitochondria, chloroplast, nuclear) are susceptible for transformation [38], [41], [143], [144] and have been completely sequenced, making genetic screening easier [145].

Metabolic engineering is based on the modification, addition or manipulation of cellular metabolism. One of the key aspects of metabolic engineering is the modification and

optimization of biosynthetic pathways. This involves the manipulation of enzymatic, transport, and regulatory functions within the cell to improve the efficiency of these pathways [146]. Metabolic engineering requires enzymes and precursors to be active, specific, and available to get a desired product. To achieve the desired product, active and specific enzymes and precursors must be available. However, even if these components are present in the organism, metabolic engineering is necessary to increase metabolic flux and exhibit the required yields for industrial production. [147]. The efficiency of biosynthetic pathways could be increased with different strategies for their optimization such as enlarging the pool of precursors, or heterologous enzymes with better characteristics [148], [149]. Cannabinoids biosynthetic pathway in *C. sativa* has been proposed that Hexanoyl-CoA synthetase (*CsAAE1*) supplies the cannabinoids pathway, transforming hexanoate in hexanoyl-CoA[150]. *C. reinhardtii* can produce cannabinoids when genetically modified (patent WO2019202510A1) [1]–[7]. *C. reinhardtii* has a peroxisomal AAE homolog that most likely will be involved in β-oxidation [151]. The aim of this work is to transform *C. reinhardtii* with the Hexanoyl CoA-synthetase gene from *Cannabis sativa* with the purpose of obtaining clones that will be used as a heterologous system for the biosynthesis of cannabinoid precursors.

Despite the advances in algae biotechnology, there are still major challenges to the development of algae as an industrial platform due to low expression of foreign genes in the nuclear genome [152]. One big obstacle is the optimization of culture conditions and parameters to achieve high biomass and greater culture production, which involves the improvement of light, temperature, pH, and nutrients [153]. Another challenge is the cost-effectiveness of large-scale cultivation, harvesting, and downstream processing of algae biomass, which requires the development of efficient and low-cost technologies [154].

Various techniques have been utilized to introduce foreign DNA into algae cells, and each method has its own set of advantages and disadvantages. For instance, electroporation utilizes an electric field to create transient pores in the cell membrane [155], permitting the entry of DNA. Alternatively, foreign DNA can be introduced through mechanical disruption of the cell wall using glass beads [156]. Another method, *Agrobacterium*-mediated transformation [157], employs a plant pathogen to transfer foreign DNA into the

algae cell. Meanwhile, the biolistic particle delivery method utilizes high-pressure helium to shoot DNA-coated microcarriers into the target cells [158]. The selection of the most appropriate method for a particular algae species and intended application should take into account the unique optimization requirements and limitations of each approach [159]–[161].

2.3 Materials and methods

2.3.1. Materials

All reagents used in this thesis were purchased from Thermo Fisher (Waltham, Massachusetts, United States), Sigma Aldrich (Saint Louis, Missouri, United states), Biobasic (Markham, Ontario, Canada), Phytotech Labs (Lenexa, Kansas, United States), Geneaid (Taipei, Taiwan), Bio-Rad (Hercules, California, United States), GenScript (Piscataway, New Jersey, United States). Restriction enzymes were purchased from NEB (Ipswich, Massachusetts, United States). Primers used in this study were synthesized by Integrated DNA technologies (Coralville, Iowa, United States).

2.3.2 Strains and clones

C. reinhardtii strain CC-125 wild type (wt) was obtained from *Chlamydomonas* Resource Center (Minneapolis, MN, USA). *C. reinhardtii* clone C30, an Olivetolic acid positive clone (in vitro enzymatic assay) harboring Tetraketide synthase (*TKS*) and Olivetolic acid cyclase (*OAC*) genes, and an empty vector clone (transformed with pChlamy_3 empty vector) were provided by Bharat Busan Majhi a postdoc fellow.

The expression vector pChlamy_4 was purchased from Thermo Fisher (Waltham, Massachusetts, United States). Cloning of the *CsAAE1* gene from pUC57 into pChlamy_4 was performed. The plasmid pChlamy_4 (conferring resistance to zeocin, *Shble* gene) carrying *CsAAE1* gene, expanded in *E. coli* DH5α, was selected on LB-agar plates with 100µg/mL ampicillin. DNA plasmid was purified using EZ-10 Spin Column Plasmid DNA Miniprep Kit (Biobasic, Canada).

2.3.3 Construction of *C. reinhardtii* expression vectors

Unless otherwise stated *CsAAE1* (GenBank: JN717233) corresponds to a gene with introns and without introns and all experiments were performed with *CsAAE1* in its both versions (Figure 2.0).

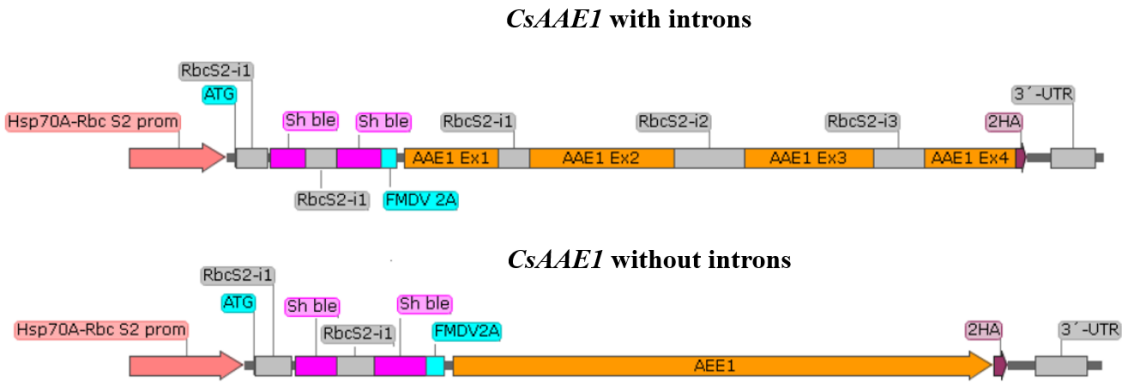

Figure 2.0. *CsAAE1* cassettes. *CsAAE1* with 3 copies of *RbcS2* intron (above) added each 300 nucleotides. Endogenous and hybrid constitutive promoter *Hsp70A-RbcS2*, gene of interest is expressed more abundantly due to the combination of the activator *Hsp70A* and two instances of the RbcS2 intron sequence in *Hsp70A-RbcS2*. Foot-and-mouth disease virus (FMDV) 2A peptide, a self-cleavage sequence between resistance markers and the protein of interest. *CsAAE1* without introns does not bear any copy of *RbcS2* intron.

Cloning vector pUC57 bearing the *CsAAE1* gene optimized for *C. reinhardtii* was synthesized by Biobasic (Markham, Ontario, Canada) with two restrictions sites (EcoRI and BamHI) for directional cloning into the expression vector. The two lyophilized pUC57 containing *CsAAE1* with and without intron, received from Biobasic (Markham, Ontario, Canada), were transformed in *E. coli* DH5α strain in order to amplify them the plasmid DNA. A volume of 50 µL chemically competent cells were used for transformation, 5 µL of DNA plasmid (10 ng/µL) were added followed by a gentle mixing and incubation for 30 minutes on ice and then placed in a 42°C water bath for 45 seconds. After 2 minutes on ice, the cells were transferred to 950 µL of liquid LB broth (Phytotech Labs, Lenexa, Kansas, United States) and left to incubate for 1 hour at 37°C. 250 µL of cells were then evenly plated on 4 different LB-agar plates with ampicillin 100 µg/mL. Left on incubator

at 37°C for 16 hours. After the 16h incubation, growing colonies were labeled and cells were evenly spread on LB-agar plates and left in the incubator at 37°C overnight to allow them grow.

To verify cloning of the plasmid into *E. coli* DH5α cells, plasmid constructs were grown on LB broth with 100 µg/mL, and then a plasmid purification was performed using EZ-10 Spin Column Plasmid DNA Miniprep Kit by Biobasic (Markham, Ontario, Canada) followed plasmid quantification. Plasmid DNA samples were double digested with EcoRI and BamHI (Table 2.0). Digestion profile was confirmed by electrophoresis (Figure 2.1).

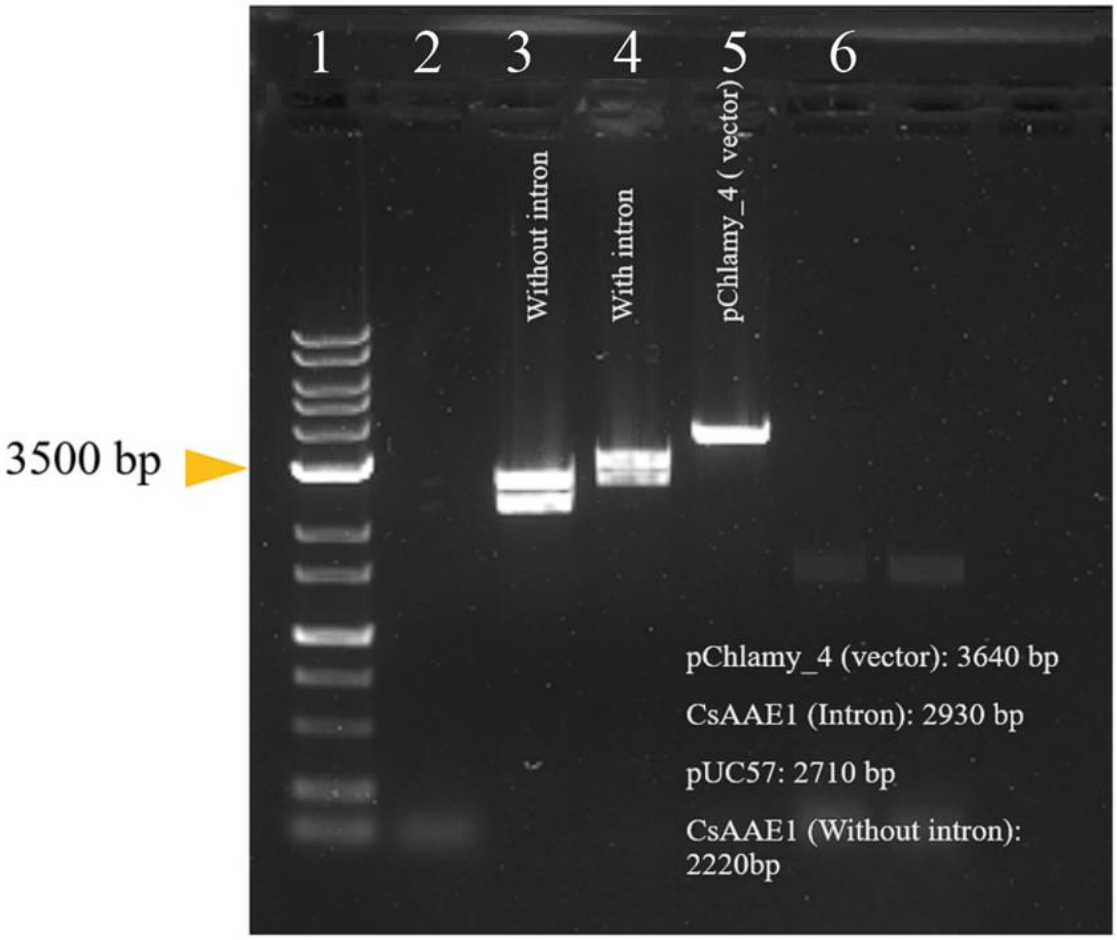

Figure 2.1. Agarose gel showing double digestion BamHI and EcoRI of plasmid DNA samples. Well 3, 4 and 5 represent pUC57-*CsAAE1* without intron, pUC57-*CsAAE1* with intron and Chlamy_4 empty vector respectively. Well 1: 1 kb DNA Ladder (NEB, USA).

Table 2.0. Double-digestion reaction for each plasmid. Numbers are represented in µL. Total volume per reaction is 30 µL. Enzymes EcoRI and BamHI are from NEB (USA).

	***CsAAE1* Intron**	***CsAAE1* without intron**	**pChlamy_4 (empty vector)**
Double digestion reaction	Amount (µL) per reaction		
Water	24	22	23
DNA	1 (500 ng)	3 (500 ng)	2 (1000 ng)
10X cutsmart buffer	3	3	3
EcoR1 (20000 unit/ml)	1	1	1
BamHI (20000 unit/ml)	1	1	1
	30	30	30

For expression vector constructs, the double digested fragments *CsAAE1* DNA with and without intron were extracted from agarose gel and purified using GenepHlow™ Gel/PCR Kit Quick Protocol (Geneaid, Taiwan). Once again, to analyze the correct size of the plasmid and dismiss any star activity, electrophoresis was done.

For ligation, a reaction was performed, as illustrated on Table 2.1, between the purified double digested expression vector pChlamy_4 (Figure 2.2) and the purified insert *CsAAE1* and was incubated for 1 hour at room temperature, followed by 10 minutes at 65 °C, then kept on ice for 5 minutes to stop the ligation reaction.

Table 2.1. Ligation reaction mix for *CsAAE1* and pChlamy_4 empty vector.

	***CsAAE1* Intron**	***CsAAE1* without intron**
Ligation reaction	Amount (µL) per reaction	
Water	7.5	10
Vector DNA	1 (95 ng)	1 (95 ng)
Insert DNA	8.5 (230 ng)	6 (174 ng)
10X ligase buffer	2	2
Ligase (enzyme)	1	1
	20	20

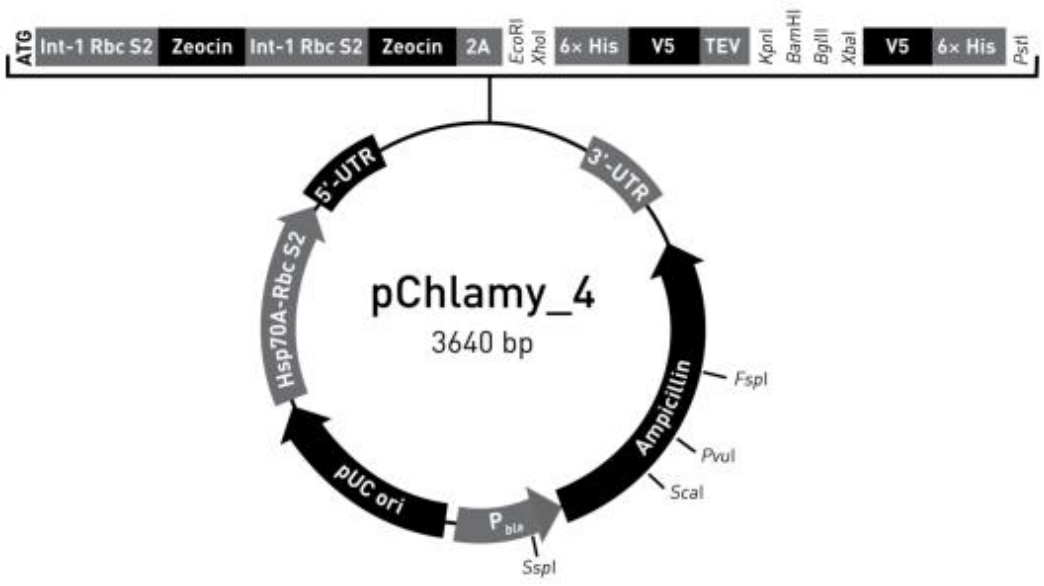

Figure 2.2 Expression vector pChlamy_4 developed to improve transgene expression in *C. reinhardtii* (Thermo Fisher, Waltham, Massachusetts, United States).

The ligated product was transformed into *E. coli* DH5α cells with the same protocol explained on section 2.3.2 with some modifications; after the incubation step for 1h at 37°C the 1 mL culture was centrifuged for 1 minute at 3000 rpm, the supernatant was discarded, then 150 µL of LB broth was added and resuspended by pipetting and all the volume was

plated on LB-agar plate with ampicillin 100µg/mL, left incubated for 16h at 37°C. Ligation was efficient and many clones grew overnight (Figure 2.3).

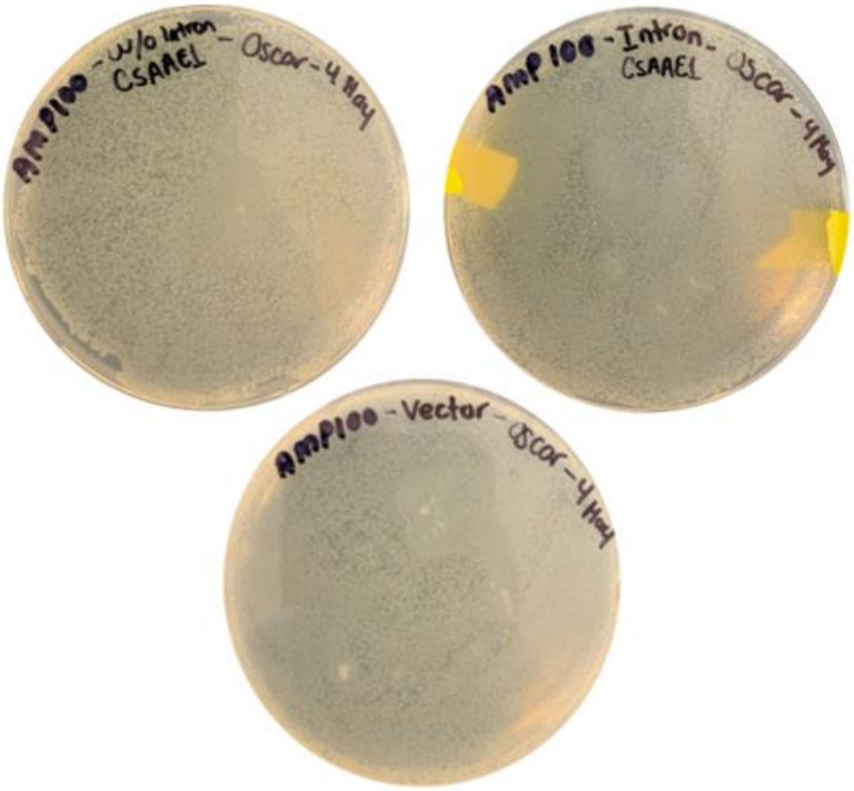

Figure 2.3 Transformation results of pChlamy4 vector bearing *CsAAE1* with or without intron, into *E. coli* DH5α cells.

A number of 25 colonies was selected from each transformation and colonies were streaked on LB-agar plate with ampicillin 100 µg/mL. Colonies from 1 to 5 were inoculated on LB-broth with ampicillin 100 µg/mL and a plasmid extraction protocol (Large Plasmid DNA Extraction Kit, Geneaid, Taiwan) was then performed, and double digestion was conducted as previously explained in this section in order to verify the presence and the size of the insert (Figure 2.4). When sizes were evaluated and confirmed the right sizes we then proceeded for the transformation. We observed that clones 2 to 5 *CsAAE1* without introns

showed a proper size of the insert and vector backbone and all five clones *CsAAE1* with introns showed the right digestion profile.

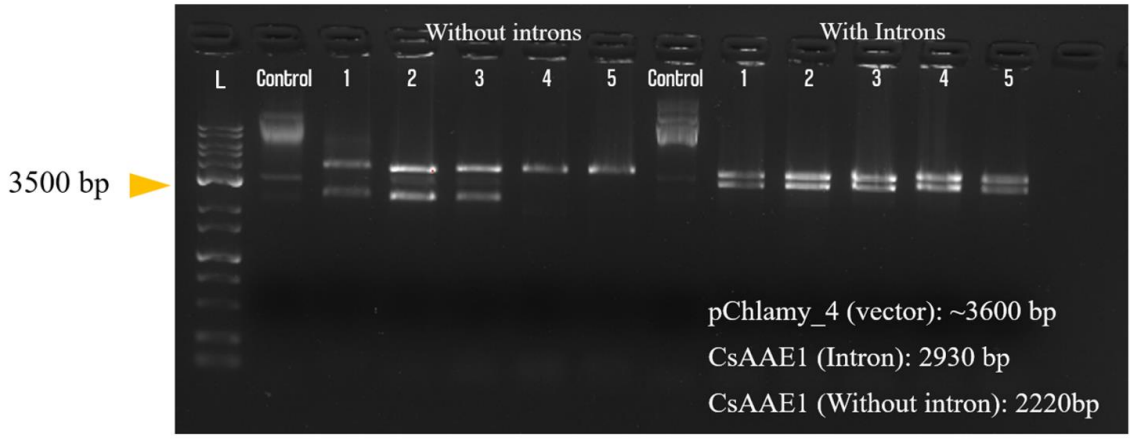

Figure 2.4 Screening of 5 different colonies to verify presence of the insert (*CsAAE1*). Colonies 4 and 5 without introns only present vector backbone. 2 and 3 without introns and 3 and 4 with introns were considered for transformation. L: ladder Quick-Load® Purple 1 kb DNA Ladder (NEB, USA). Control is undigested *CsAAE1* plasmid.

2.3.4 Electroporation of *C. reinhardtii* wild-type strain C-125 and clones already harboring *TKS-OAC* genes.

The electroporation process was performed as described by Nouemssi et al. 2020 [104] with some modifications. *C. reinhardtii* C-125 wild-type strain (WT) and already transformed *C. reinhardtii* clones harboring construct *TKS-OAC* genes (C30) were transformed with the empty vector or the vector harboring the *CsAEE1* construction (Table 2.2.) Transformed cells were cultured in 50 mL liquid Tris-acetate-phosphate (TAP) media and were grown in 125 mL Erlenmeyer flasks with an initial Optical Density (OD750nm) of 0.1 to a final OD750nm of 0.7 (~7 x 10^6 cells/mL) for one week incubation. Cultures were incubated under controlled shaking (100 rpm) conditions at temperature of 22 ± 0.5°C, a 16h ligth:8h dark photoperiod at a light intensity of 50±10 µmol photons m−2 s−1 and relative humidity of 50%, these conditions were used along the electroporation process and after transformation.

Cells were harvested by centrifugation at 3000 rpm for 5 minutes at 4°C, the supernatant was discharged, and the remaining pellet was washed 3 times with 5 mL of MAX

Efficiency™ Transformation Reagent for Algae (Invitrogen, Canada). After the final centrifugation, 250 µL (~1 x 10^5 cells/mL) of each *C. reinhardtii* cell strains were incubated on ice for 5 minutes with 1000 ng of linearized plasmid DNA with ScaI (all linearized plasmids used here were purified). Then, 250 µL of the cell-DNA mix were transferred into an ice-cold 4 mm cuvette for transformation. Electroporation parameters, on the Bio-Rad Genepulser XcellTM electroporation system, were used as follow 0.5 kV, capacitance 5 µF, and resistance 800 Ω. Plasmid DNA pChlamy_4 (without gene) was transformed into *C. reinhardtii* strains and was used as a negative control in future experiments, further discussed. Transformed cells were put in 5 mL TAP liquid media supplemented with sucrose (50 mM) and incubated for 18h at room temperature conditions. After 18 hours, centrifugation at 3500 rpm for 5 minutes was performed. The pellet was washed with 250 µl of MAX Efficiency™ and distributed into a TAP agar media with 5 µg/mL zeocin (Sigma, United States). The incubation period was for two weeks and at the end of the second week, the total number of transformants were counted.

Table 2.2. *C. reinhardtii* strains and the related cassettes used for transformation. 1Selection antibiotic after transformation and targeted metabolite expected to detect.

Algae strain	DNA plasmid pClamy_4-cassette	Selection antibiotic1	Target on metabolite detection
WT C-125	*CsAAE1* Introns	Zeocin	Hexanoyl-CoA
WT C-125	*CsAAE1* without introns	Zeocin	Hexanoyl-CoA
C30	*CsAAE1* Introns	Zeocin and hygromycin	Olivetolic acid
C30	*CsAAE1* without introns	Zeocin and hygromycin	Olivetolic acid
WT C-125	pChlamy_4 empty vector	Zeocin	Negative control
Empty vector (pChlamy_3)	pChlamy_4 empty vector	Zeocin and hygromycin	Negative control

2.3.5 Selection of positive transformants

Zeocin resistant *C. reinhardtii* transformants were selected randomly and colonies were sub-cultured on TAP agar plate media supplemented with 5 µg/mL zeocin and/or hygromycin 10 µg/mL (Sigma, United States), in Petri dishes (150 mm x 15 mm) (Thermo Fisher Scientific, USA), transformants were cultured under conditions as described in section 2.3.4 for one week to verify and ensure their growth stability and avoid that are false positive strains. Wild type C-125 was used as a negative control on each plate to verify the antibiotic activity. New transformants were streaked on TAP media with the appropriate antibiotics as explained in Table 2.2. In the third round of selection, all clones were screened by colony PCR.

2.3.6 DNA extraction and Colony PCR

Colony polymerase chain reaction (cPCR) is utilized to isolate transformants that possess integrated expression cassettes and exhibit antibiotic resistance [104].

Different set of primers were synthesized by Integrated DNA Technologies (Coralville, Iowa, United States) to amplify *TKS-OAC* (2600 bp), *CsAAE1* (2100 bp), and *Shble* genes (523 bp) (Table 2.3).

To select positive transformants, a high-throughput method was implemented as described by Nouemssi et al. 2020; loading dye 10X was added after PCR. After 3 rounds of selection as detailed in section 2.3.5, transformants on TAP agar plates were selected under sterile conditions.

To perform Colony PCR, a master mix with different components (25 µL per reaction) was prepared as shown in table 2.6. Using a 12 multichannel pipette, 23 µL of the master mix solution was dispensed into each of the 96 PCR plate wells, and 2 µL of extracted genomic DNA (~100 ng/µL) was added into each tube. Conditions of the PCR are shown in table 2.4 and 2.5. Following the PCR cycle, 2.5 µL of bromophenol blue and glycerol as explained by Le Gouill and Déry (1991) [162] were added to each of the 25 µL reactions. Finally, the PCR products were loaded onto a 1% agarose gel (w/v) and then analyzed.

Table 2.3 Primers designed for Colony PCR.

Primer I.D.	Gene	Sequence 5'⟶ 3'
Block1_*TKSOAC*_F	*TKS-OAC*	AACGGGTACCAGCGAGTCAGTGAGGGAAG
Block1_*TKSOAC*_R	*TKS-OAC*	ACGCGAATTCGTGTCATAGCGCAAGAAAGACATTC
***CsAAE1*_R**	*CsAAE1*	CTCTAGACCAGATCTTCCGGATCCG
***CsAAE1*_F**	*CsAAE1*	AATTCGAGCTCGGTACCTCG
Zeocin_F	*Shble*	ATGGCCATGCATATGGCCAAGCTGACCA
Zeocin_R	*Shble*	GTCCTGCTCCTCGGCCACGAAGTGCA

Table 2.4 PCR conditions for *CsAAE1* gene amplification. 40 cycles.

Cycle *CsAAE1*	Temperature	Time (minutes)
Initial denaturation	95 °C	5
Denaturation	95 °C	0.5
Annealing	60 °C	2
Extension	68 °C	1
Final extension	68 °C	5

Table 2.5 PCR conditions for *Shble* gene (zeocin) amplification. 40 cycles.

Cycle *Shble*	Temperature	Time (minutes)
Initial denaturation	95 °C	5
Denaturation	95 °C	0.5
Annealing	55 °C	1
Extension	68 °C	1
Final extension	68 °C	5

Table 2.6 Master mix reaction for Colony PCR. *Same concentration and volume for primers *TKS-OAC* and *Shble* (zeocin). Modified from NEB Builder [163].

Reagent	µL per reaction	Final concentration
Thermopol buffer (10X)	2.5	1 X
dNTP's (10 mM)	0.5	200 µM
***CsAAE1* Forward primer (20 µM)**	0.625	0.5 µM
***CsAAE1* Reverse primer (20 µM)**	0.625	0.5 µM
Taq polymerase	0.5	
DNA	2	<1000 ng
Nuclease-free water	Fill up to 25 µL	

2.3.7 Sample collection, RNA isolation, cDNA synthesis, and RT-qPCR

Real-time qPCR was performed to determine and quantify the expression level of our gene of interest. Approximately 0.2 g of wet biomass of microalgae cells was collected and eventually immersed into liquid nitrogen, then stored at -80°C. Monarch® Total RNA Miniprep Kit (New England Biolabs, USA) was used for RNA extraction according to the manufacturer's protocol with some modifications. The cDNA was synthesized from 200 ng of RNA using the M-MLV Reverse Transcriptase kit (Thermo Fisher, Waltham, Massachusetts, United States). Reactions were performed with the iTaq Universal SYBR Green Supermix (Bio-Rad, USA). Primers for RT-qPCR reaction are listed in table 2.7. Phosphoglycerate kinase (PGK) was used as a reference gene. RT-qPCR analysis was performed using three replicates.

Table 2.7 Primers designed for RT-qPCR.

Primer I.D.	Gene	Sequence 5' ⟶ 3'
q*TKS*_F	*TKS*	CAGGACGAGTTCCCCGACTA
q*TKS*_R	*TKS*	GTTGCGCTTGCGGATCATAG
q*OAC*_F	*OAC*	CAAGGACGAGATCACGGAGG
q*OAC*_R	*OAC*	GCGGGGTGTAGTCGAAGATC
qAAE1_F	*CsAAE1*	TCAACATCGCCAACCACA
qAAE1_R	*CsAAE1*	CCTTATAGCAGCCGTAGAACAG

2.3.8 Western Blot

Western blot (WB) method was used for the detection of protein production.

Frozen pellets containing PCR positive *CsAAE1* , wild type, and empty vector constructs were thawed, resuspended, and lysed in 300 µL 1X Laemmli buffer (10% vol/vol β-mercaptoethanol) – 4M urea, then vortexed vigorously, placed 5 minutes at 95 °C, and then centrifuged 1 minute at 14000 rpm, soluble fraction (proteins) was carefully transferred into a new Eppendorf tube. A volume of 30 µL of each sample were loaded and separated on an SDS-PAGE 10% at 120V for 1h, followed by a transfer onto PDVF membrane at 25V for 1 h. After blocking the membrane with 1X TBST + 5% milk. The production of the protein of interest was detected by using two different antibodies. For the detection of OAC-TKS enzymes, a mouse anti-His primary antibody at 1:1000 dilution BSA 3% was used, and to detect Hexanoyl-CoA synthetase enzyme production, an anti-HA in mouse 1:500 in TBST was used. Multiple TAG (GenScript, USA) was used as positive control and *C. reinhardtii* CC125 untransformed was used as a negative control. For rapid protein identification total crude extract was used for western blot, protein purification was not performed in this experiment.

2.3.9 Metabolite detection.

Analytical method such as HPLC was used to detect the targeted metabolites. PCR positive clones harboring *CsAAE1*, and *TKS-OAC*, wild type CC125, empty vector clone (pChlamy_4) and a two-empty vector clone (pChlamy_3 and pChlamy_4) were placed in TAP media for 7 days with the same conditions as explained before electroporation, to eventually perform metabolite analysis aiming to detect olivetolic acid, precursor of two major cannabinoids. One week after, the last subcultures were prepared for HPLC-DAD analysis as outlined herein. The weight of 50 mL conical tubes (Thermo Fisher, Waltham, Massachusetts, United States) were measured before harvesting to know the weight of the final biomass of each clone. 25 mL of cell culture was added and centrifugated at 3500 g for 10 minutes at 4°C, the supernatant was discarded, and conical tubes were inverted to dry. Once dry, the weight of the conical tube was measured again, and the difference between the final and initial weight were obtained to calculate the weight of cells biomass.

A volume of 1 mL of 95% ethanol was added to 100 mg of biomass (ratio 1 mL/100 mg), the sample was vortexed for 10 sec, and incubated overnight at -20°C. The extract (supernatant) was separated from the pellet by centrifugation at 3500 g for 10 minutes at 4°C, eventually samples were filtered using syringe filters (0.22 µm Nylon, Thermo Scientific, cat. no. CH2225-NN) with a volume of Sample: 600µL). HPLC analysis was performed with Shimadzu Prominence-I LC-2030C 3D, column used Poroshell 120 EC-C18 (2.7 µm, 4.6 x 75 mm). Samples were compared with thirteen cannabinoids' standards (Figure 2.5) available in the laboratory. Samples aiming to detect Hexanoyl-CoA were grown for 7 days as stated previously in this section and in the extraction protocol, RNAse-free water was used due the polarity of the Hexanoyl-CoA (ratio 1 mL/100 mg). The sample was vortexed for 10 sec, and incubated overnight at 4°C, then sonication was performed to disrupt the microalgal cell walls with the following parameters 30 s pulses, 30 s pause for 0:03:00 (3 minutes) (6 pulses) with 35% amplitude.

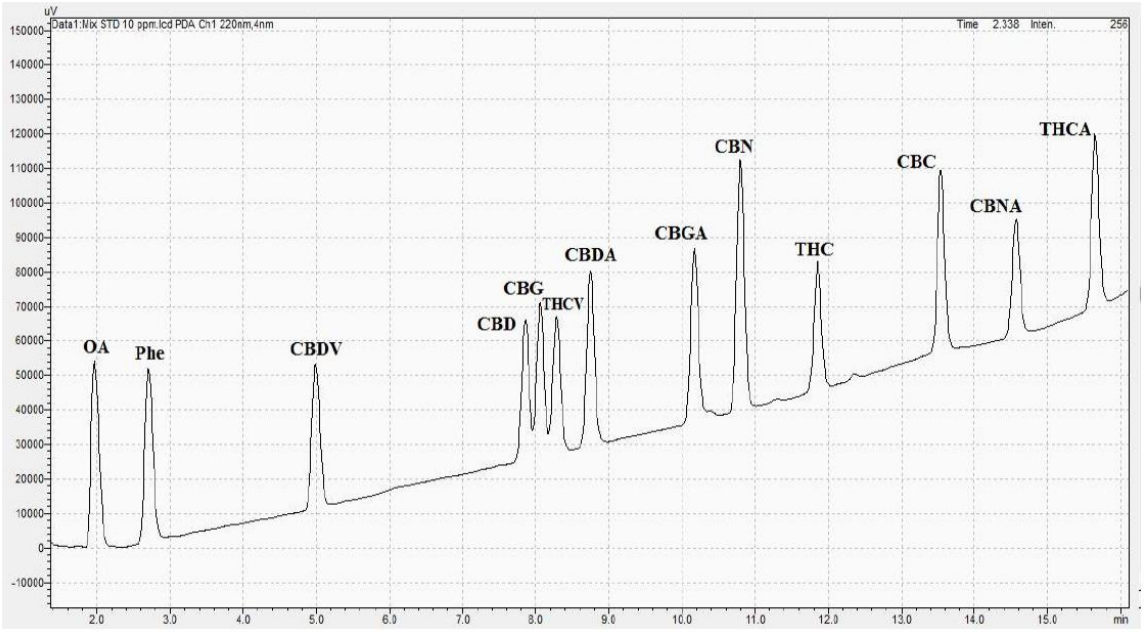

Figure 2.5 HPLC chromatogram of a mix of 13 cannabinoid standards at 10 ppm. Retention time (x-axis) represents the time taken for each standard to travel through the column until its detection. Y-axis represents the absorbance unit at 220 nm, also used to determine the concentration of the standard. This chromatogram was used as a template to compare similarity on retention time between samples and standards.

2.3.10 Enzymatic assay

Algae was cultured in 50 mL liquid Tris-acetate-phosphate (TAP) media and were grown in 125 mL Erlenmeyer flasks with an initial Optical Density (OD750nm) of 0.1 to a final OD750nm of 0.7 (~7 x 106 cells/ml), approximately one week. Temperature condition of 22 ± 0.5°C under a 16 light:8 dark photoperiod at a light intensity of 50±10 µmol photons m−2 s−1 and relative humidity of 50%. Cells were harvested by centrifugation at 3500 g for 5 minutes at 4°C, the supernatant was discharged. Pellet was washed with 500 µL solubilisation buffer (Table 2.8). A volume of 5 µL of protease inhibitor (NEB, cat no# 5871S) was added and 50 µL of PMSF (Sigma, cat no# P7626), prior sonication with same parameters mentioned in section 2.3.9. Sample was then centrifuged at 14000 rpm at 4°C for 30 minutes. After centrifugation, supernatant was transferred to a new Eppendorf tube. Protein quantification was done by using DC Protein Assay (Bio-Rad, USA) according to the manufacturer's protocol.

For the detection of olivetolic acid product, enzymatic assay mix was performed as follows: 200 µM Hexanoyl-CoA, 2 mM Malonyl-CoA, 5 mM DTT, 200 µg total protein extract, 50 mM HEPES buffer (pH 7.5), reaction was left 16h at 25°C, reaction was then stopped by adding 2% TCA. Samples were then analyzed by HPLC.

Table 2.8. Solubilisation buffer preparation.

Reagent	Concentration (mM)
Lithium Dodecyl sulfate	0,75
Glycerol (10%)	2,5
Tris-HCl pH8	51,4 m
EDTA	0,02 mM

3. Results

For this master research project, the aim was to insert *Hexanoyl-CoA synthetase* (*AAE1*) from *Cannabis sativa* aiming to mimic the conversion of hexanoic acid to hexanoyl-CoA in *Chlamydomonas reinhardtii*, thus increasing the pool of precursors of cannabinoids. To find out if *C. reinhardtii* had already any *acyl-activating enzyme* gene we performed a bioinformatic analysis using *Cs*AAE1 as a reference and found two candidates that could be acyl-activating enzymes (Figure 3.0). As shown in the phylogenetic tree analysis the *C. reinhardtii* genes are close in the evolutionary linage (Figure 3.0) but is not still clear if they are AAE1 or if they perform the same reactions as in *C. sativa*, so inserting *AAE1* gene from *C. sativa* into *C. reinhardtii* could boost the conversion of hexanoic acid into hexanoyl-CoA.

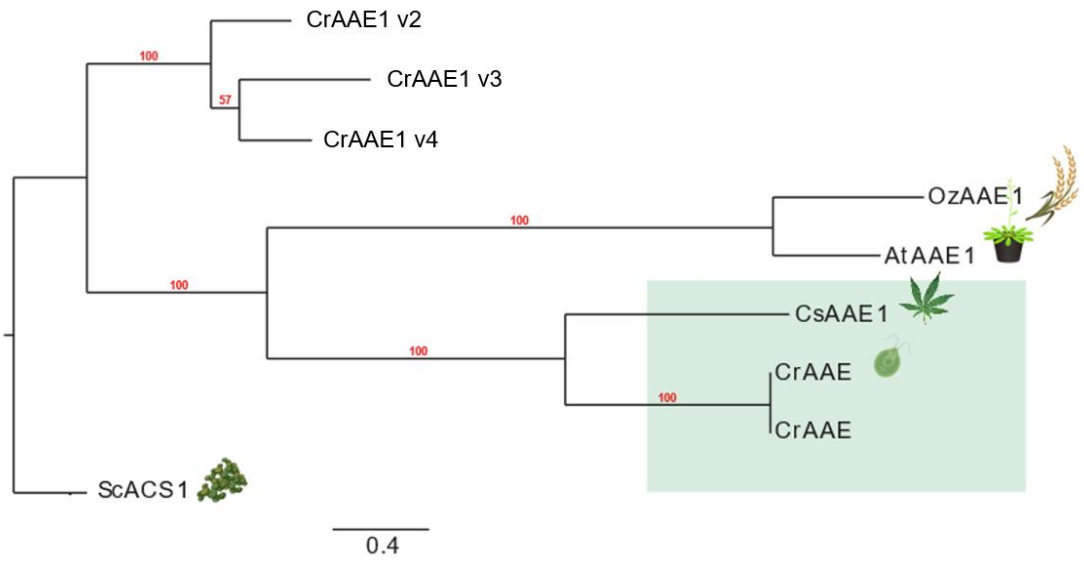

Figure 3.0 Phylogenetic tree analysis of acyl-activating enzyme 1 protein sequence from different species. *Saccharomyces cerevisiae* acetyl-CoA synthase (Q01574) is included as an outgroup. Tree was obtained by maximum likelihood/rapid bootstrapping run on XSEDE (Alexandro Stamatakis, 2008) [164] as implemented in with RAxML-HPC2 version 8.2.12. The numbers on branches designate the percentage support from 100 bootstrap replicates. Phylogenetic tree was made with FigTree v1.4.4. CsAAE1 (*Cannabis sativa* H9A1V3.1), CrAAE1 (*C. reinhardtii* XP_001702039.1 (XP_042914816.1) CrAAE1v2 (*C. reinhardtii* XP_001702039.1), CrAAE1v3 (*C. reinhardtii* XP_001700230.1), CrAAE1v4 (*C. reinhardtii* XP_001700210.2), OzAAE1 (Oryza sativa A2ZST0). AtAAE1 (*Arabidopsis thaliana* Q8H151).

3.1 Transformation efficiency and Colony PCR

After electroporation and one-week incubation on TAP solid media (Figure 3.1 A) with their respective selection antibiotic, more than 705 transformants (*CsAAE1* with and without introns and empty vector clones) were obtained (Table 3.0); transformants were screened by cPCR and 15 clones showed an amplification of *CsAAE1* gene (Figure 3.1 B, C and D). The first round of selection was made with the 705 transformants. For ease of rapid subculturing of colonies, these were cultured in a 96-well plate and eventually streaked on TAP agar plates following the method of Nouemssi et al., 2020. Genomic DNA from transformed clones of 3rd selection and wild type C-125 (negative control) was extracted to use in Colony PCR (cPCR) using the method reported in section 2.3.6. Approximately 1.7% of transformants (15 out of 886) were PCR positive for *CsAAE1* gene (Figure 3.1).

Table 3.0. Summary of clones analyzed after transformation. In total 6 different transformations were made based on strains mentioned in table 2.2. From the total number of clones obtained, 15 clones amplified for the gene of interest, and none of them exhibited production of cannabinoid precursors. Primers used in this study to amplify *CsAAE1*, *TKS*, and *OAC* genes by cPCR are summarized in table 2.3.

Cassettes	Number of clones screened	Number of PCR+ clones	Number of clones tested for metabolite and enzymatic assay	Cannabinoid precursor detection status	Protein detection	mRNA detection	Enzymatic activity
***CsAAE1*i**	235	2	2	ND	ND	ND	ND
***CsAAE1*w/o**	65	2	2	ND	ND	ND	ND
CsAAE1*i + *TKS-OAC	275	11	11	ND	ND	ND	ND
CsAAE1*w/o+ *TKS-OAC	0	0	0	ND	ND	ND	ND
pC4 e.v.	75	2	2	Neg	Neg	Neg	Neg
pC3 e.v. + pC4 e.v.	55	2	2	Neg	Neg	Neg	Neg

ND: not detected. i: introns. w/o: without introns. pC: pChlamy. e.v.: empty vector. Neg: negative control.

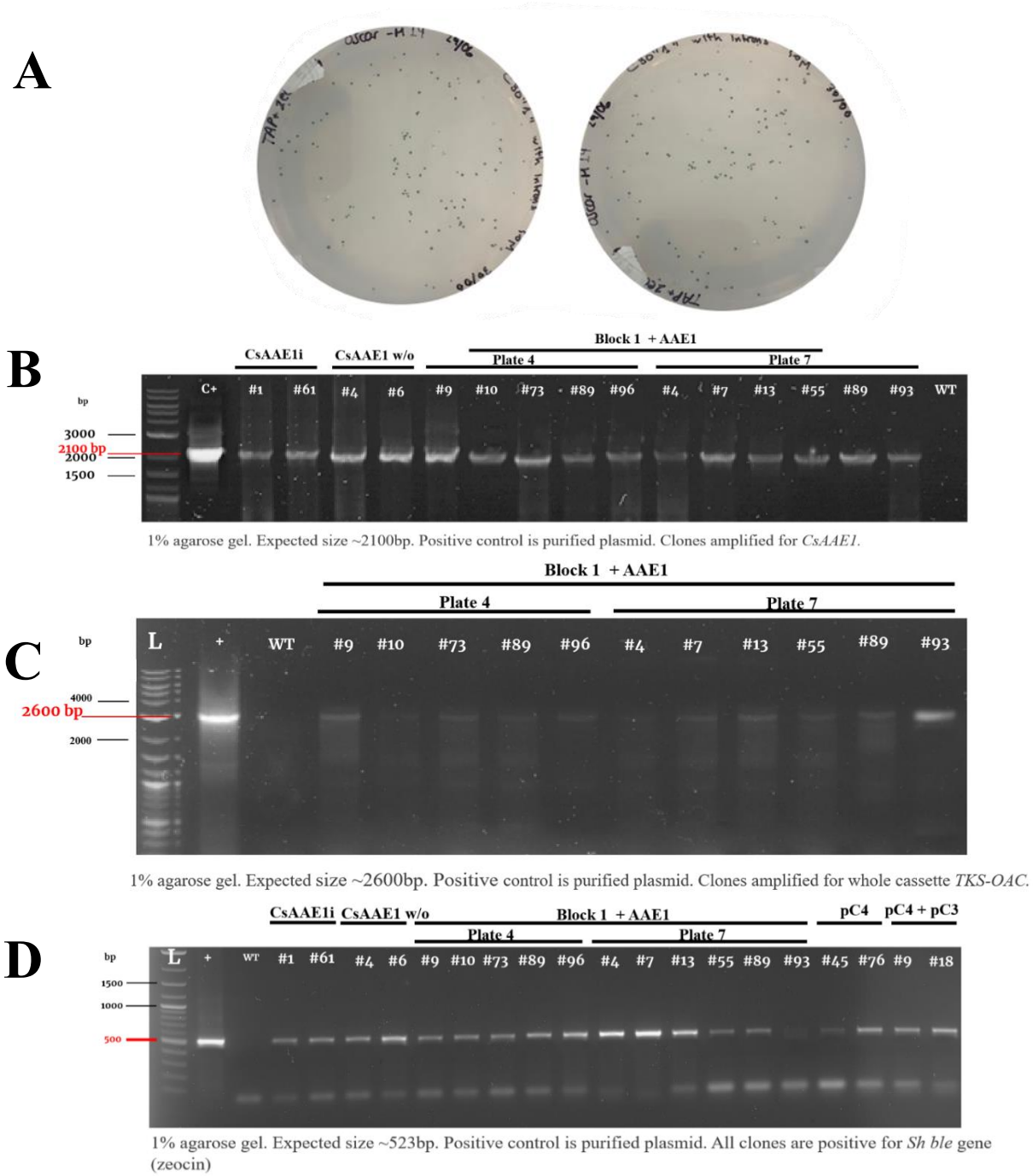

Figure 3.13 Transformation of *C. reinhardtii* and colony PCR of transformants. TAP Agar plates after 14 days of *C. reinhardtii* electroporation (A) *CsAAE1* transformants growing one week after electroporation on TAP agar media 5 µg/mL zeocin. (B) 15 clones showing *CsAAE1* amplification at 2100 bp. C) Gene integration verification of *TKS-OAC*, expected size 2600 bp D) Clones from images B and D amplified *Shble* gene, which confers resistance to zeocin. Empty vectors also showed amplification at 523 bp. Numbers above the gels explain the number of clones and the cassette. M, Quick-Load® Purple 1 kb DNA Ladder (NEB, USA). Positive control (+), purified plasmid pChlamy_4 harboring *CsAAE1* gene. Negative control (-), wild-type strain C-125. Gels A, B, and C are 1% agarose gels.

3.2 Rounds of selection of positive transformants

To obtain stable clones and avoid false positives, several rounds of selection had to be made over five months, as shown in Figure 3.2. After first round of selection 1, hygromycin 10 µg/mL and zeocin 5 µg/mL were used in TAP agar for *CsAAE1-TKS-OAC* clones and double-empty vector clones, instead of only zeocin. 886 colonies out of 1181 were cultured in the first round of selection, 69% *CsAAE1* with introns and 19% *CsAAE1* without introns (Table 3.1) showing that high yield transformation efficiency is better when the cassette of expression has introns in it. Clones *CsAAE1*w/o+ *TKS-OAC* without introns showed the least number of transformants (40) but zero clones grew on the second round of selection. At selection 4, a survival rate of 51% of clones was obtained. On selection 2 of *CsAAE1*w/o+ *TKS-OAC* all clones died. At the end of selection 4 almost half of the clones were growing.

Table 3.1. Summary of total number of colonies obtained after each round of selection in TAP agar media with antibiotics. All rounds of selection had hygromycin 10 µg/mL and zeocin 5 µg/mL, except round one, zeocin only. i: introns. w/o: without introns. pC: pChlamy. e.v.: empty vector.

Cassette	Selection #1	Selection #2	Selection #3	Selection #4
*CsAAE1*i	288	270	235	148
*CsAAE1*w/o	129	103	65	60
*CsAAE1*i + *TKS-OAC*	327	301	275	167
*CsAAE1*w/o+ *TKS-OAC*	40	0	0	0
pC4 e.v.	80	78	75	65
pC3 e.v. + pC4 e.v.	62	58	55	14
Total of transformants	886	810	705	454

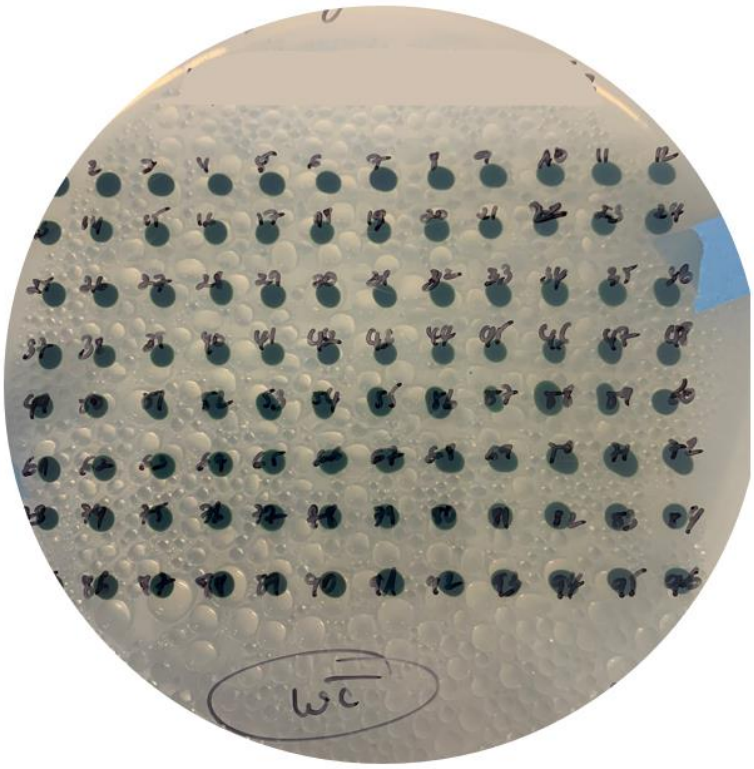

Figure 3.2 Example of streaking on TAP agar plates. 96 different *C. reinhardtii* clones are placed on 150 mm x 15 mm petri dish. Clones are left for growing 7 to 14 days after being transferred to another new fresh media with their respective antibiotic. Temperature condition of 22 ± 0.5°C under a 16L:8D photoperiod at light intensity of 50±10 µmol photons m−2 s−1. *C. reinhardtii* wild type C-125 (untransformed) was used as a negative control.

3.3 Western blot analyses

After four rounds of selection on TAP agar media supplemented with the antibiotics, the PCR positives transformants were tested for the production of our targeted proteins using western blot method. A number of five protein extracts from five transformants (#7, #9, #55, #89 and #4) harboring *TKS-OAC*, with His-Tag at the N-terminus were tested (Figure 3.3). Only five out of the eleven PCR-positive clones were subjected to western blot analysis because they displayed stable growth on liquid media containing antibiotics. The criteria for good growth was stablished when the liquid media turned dark green after being incubated for a week in TAP media. A size of ~ 60 kDa was expected if *TKS-OAC* protein

was not cleaved or ~ 47 kDa if it was cleaved. His-tag protein was not detected in the 5 samples.

Same transformants were tested for the presence of Hexanoyl-CoA synthetase with HA-Tag at the C-terminal of the cassette (Figure 3.4). An expected size of ~ 80 kDa was expected for Hexanoyl-CoA Synthetase. HA-tag protein was not detected in five samples under our experimental conditions.

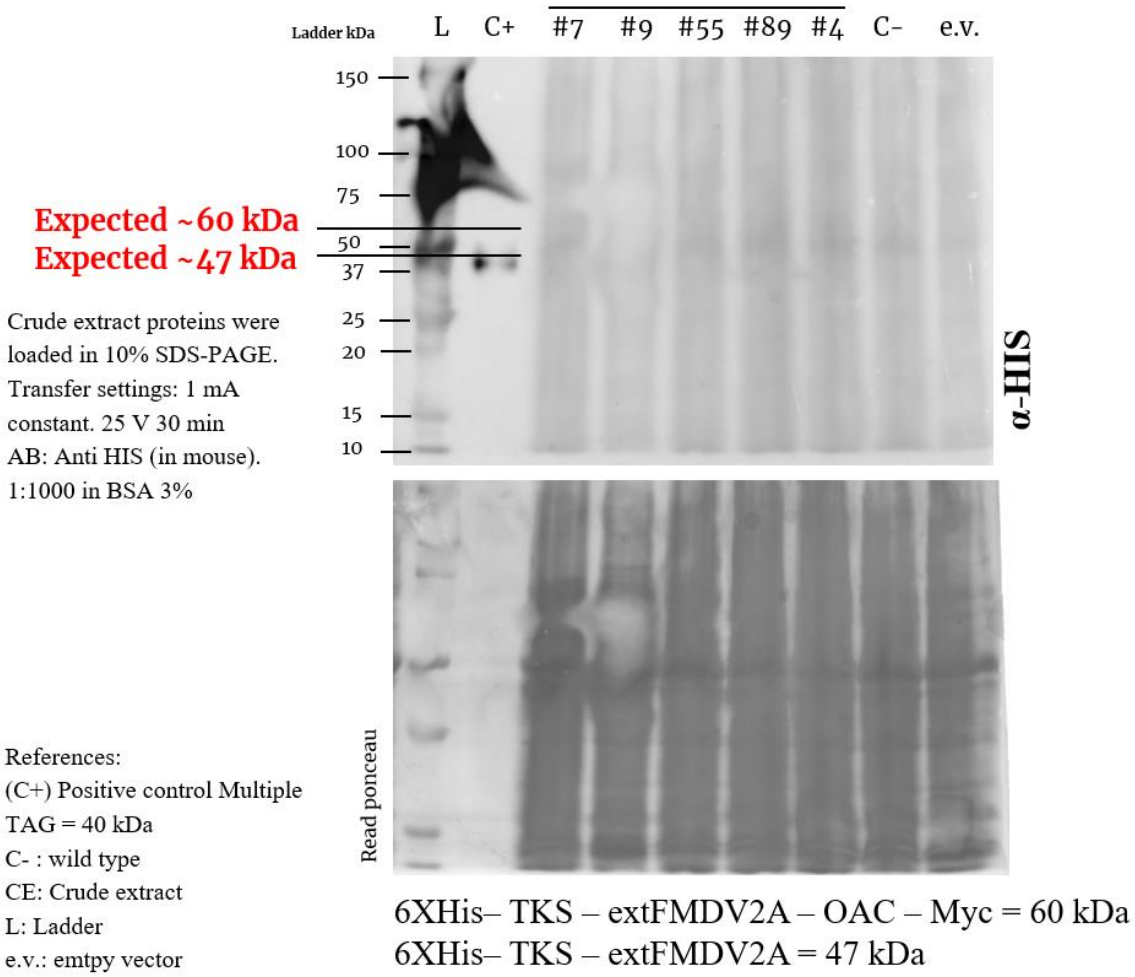

Figure 3.3 Western blot of 5 clones harboring *TKS-OAC*. Total crude extract protein was used in each case. C+, positive control is Multiple Tag (GenScript, USA) has an apparent molecular weight of ~40 kDa. Two negative controls were used: C-, negative control is *C. reinhardtii* wild type C-125 (untransformed). e.v., one clone bearing pChlamy_4 (empty vector). Membrane stained with red ponceau shows total protein from *C. reinhardtii* cells.

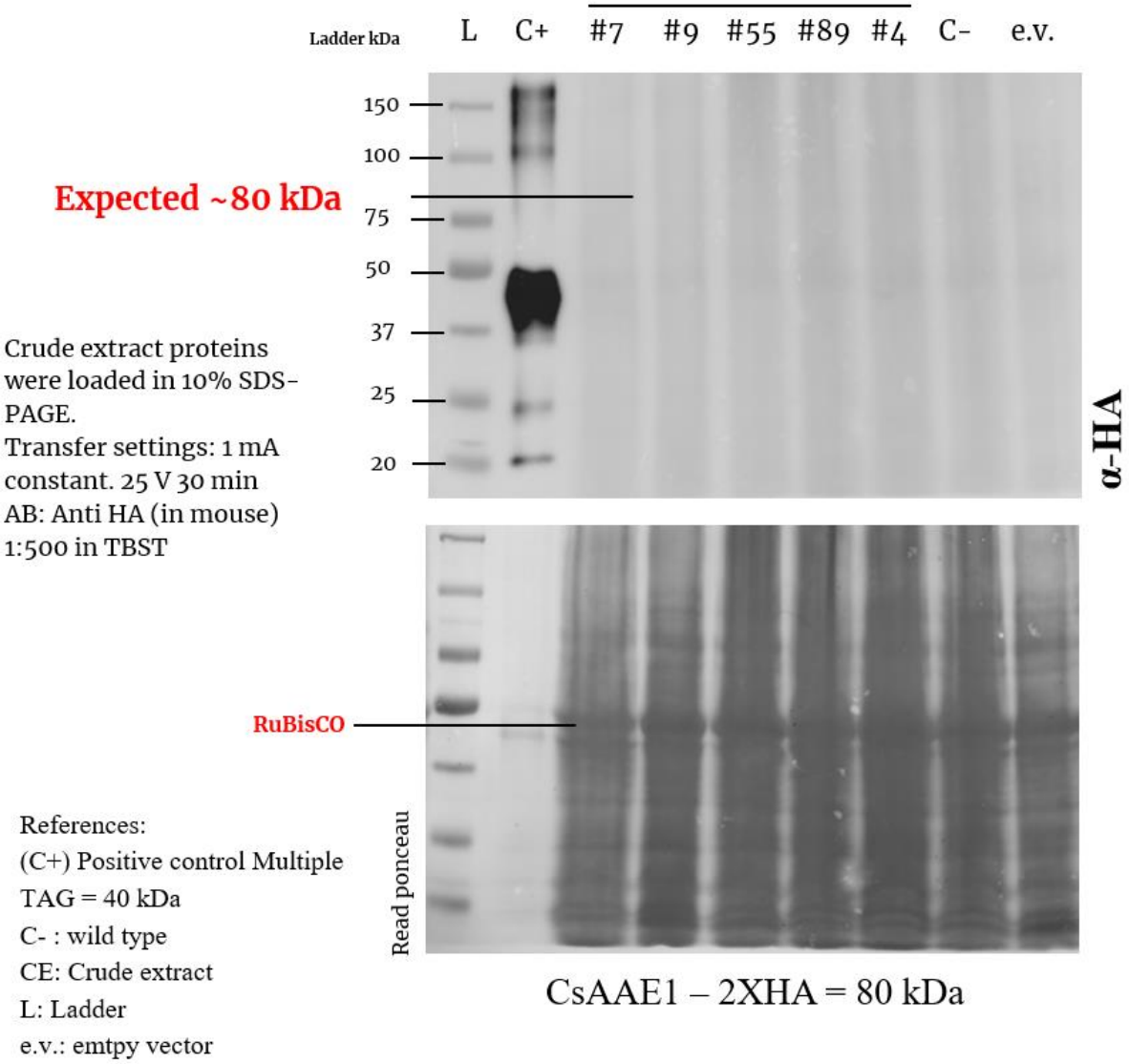

Figure 3.4 Western blot of five clones (same as Figure 3.3) harboring *CsAAE1*. Crude extract protein was used in each case and no protein purification was done. C+, positive control is Multiple Tag (GenScript, USA) has an apparent molecular weight of ~40 kDa. Two negative controls were used: C-, negative control is *C. reinhardtii* wild-type C-125 (untransformed). e.v., one clone bearing pChlamy_4 (empty vector). Membrane stained with red ponceau shows total protein from *C. reinhardtii* cells.

3.4 Detection of olivetolic acid and hexanoyl-CoA by High Performance Liquid Chromatography (HPLC)

A number of eleven PCR-positive clones harboring *CsAAE1-TKS-OAC* were analyzed for the production of olivetolic acid as outlined previously. Unidentified peaks were detected compared to wt and empty vector metabolite extracts (Figure 3.6). HPLC spectra showed that one small peak corresponds to olivetolic acid standard (Figure 3.5 and 2.11). Two clones harboring *CsAAE1+TKS+OAC* supposedly showed production of Hexanoyl-CoA, but wild-type also showed same retention time as standard, under the conditions used in our experiments (Figure 3.7).

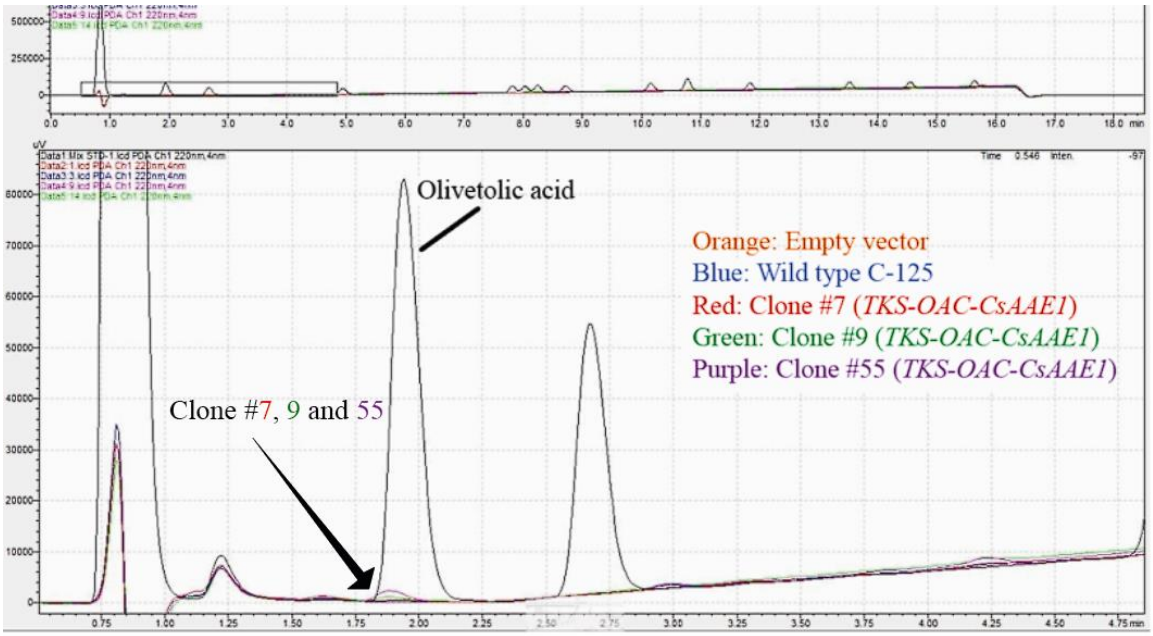

Figure 3.5 Overlay of HPLC chromatograms of three *C. reinhardtii CsAAE1-TKS-OAC* clones (PCR positive clone #7, #9, and #55). Although their concentration was very low, they exhibited a retention time similar to that of olivetolic acid at a concentration of 10 ppm. However, the empty vector and wild type did not demonstrate the same retention time.

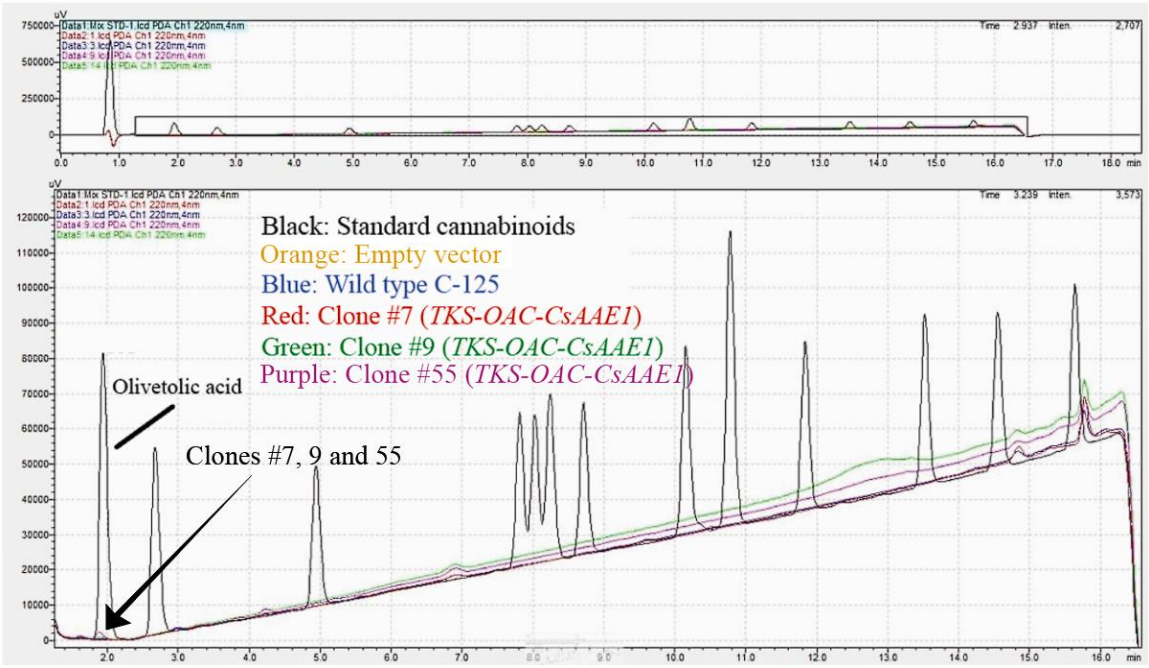

Figure 3.6 HPLC full-view chromatogram of three *CsAAE1* clones (PCR positive). Three clones exhibited a peak at the identical retention time as olivetolic acid, although at a concentration considerably lower than the standard. This is a representative HPLC chromatogram of 11 *CsAAE1* clones (PCR positive). Other chromatograms are not shown.

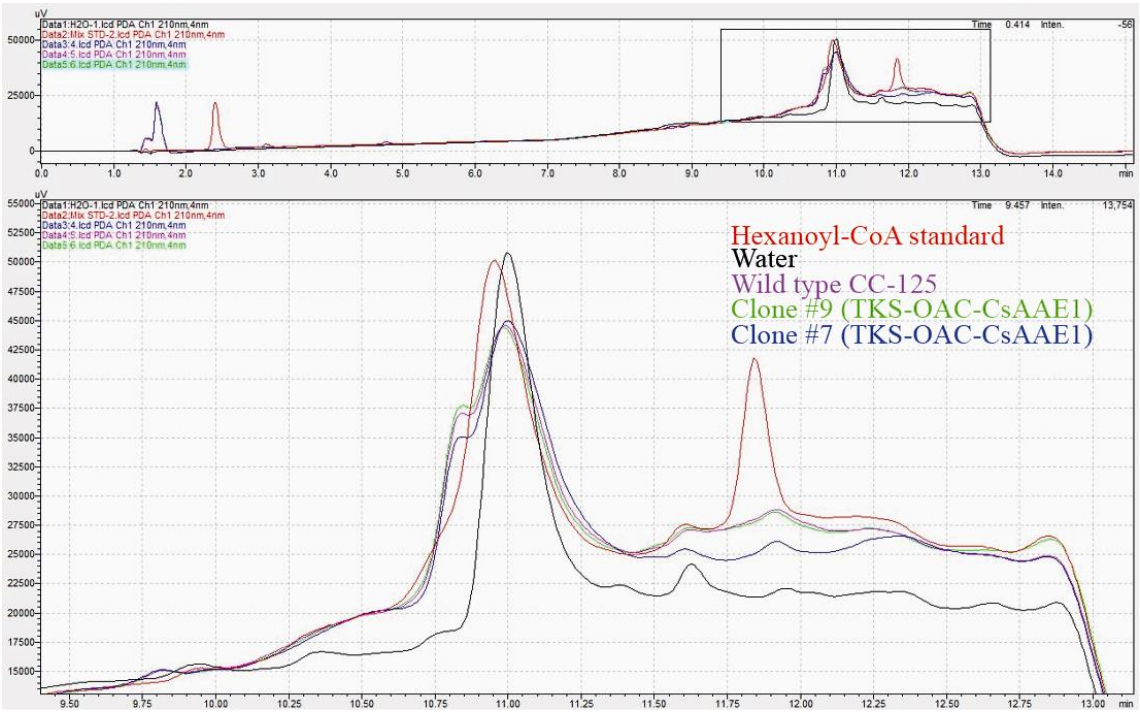

Figure 3.7 HPLC chromatogram of 2 *CsAAE1-TKS-OAC* clones. The two samples obtained from clones, which included two negative controls, wild type and water, exhibited a retention time that closely approximated that of the Hexanoyl-CoA standard. As a result, the outcome was inconclusive.

4. Discussion

We inserted *Hexanoyl-CoA synthetase* (*AAE1*) gene from *Cannabis sativa*, for the purpose of increase the pool of precursors of cannabinoids by mimicking the conversion of hexanoic acid to hexanoyl-CoA [98] since it's been observed that *C. reinhardtii* can synthesize cannabinoids through genetic modification [108]. Augmenting the reservoir of precursors is a crucial aspect since hexanoyl-CoA has been identified as a constraining factor in other organisms such as *Escherichia coli* and *Saccaromyces cerevisiae* [165], [166]. Prior to adding one acyl-activating enzyme in *C. reinhardtii*, a bioinformatic analysis was conducted using *CsAAE1* as a reference to identify if *C. reinhardtii* already had any *acyl-activating enzyme* gene. The analysis revealed two candidate genes that could potentially be acyl-activating enzymes, as shown in the phylogenetic tree analysis (Figure 3.0). It has been reported that *C. reinhardtii* has 14 types of acyl-activating enzymes, but it is not specified the presence of an *acyl-activating enzyme 1* in its genome [167]. These *C. reinhardtii* genes (Figure 3.0) were found to be closely related to each other in the evolutionary lineage, but it is yet unclear for us whether they are *AAE1* or if they perform the same reactions as in *C. sativa*, and to date there have not been thorough studies about this gene in *C. reinhardtii*. In addition, hexanoyl-CoA metabolite was not detected in large amount in *C. reinhardtii* wild-type strains (Figure 3.7). Thus, we had hypothesized that inserting the *AAE1* gene from *C. sativa* into *C. reinhardtii* could potentially increase the conversion of hexanoic acid into hexanoyl-CoA, ultimately leading to an increase in the production of OA. To test the previous hypothesis, multiple clones containing *TKS-OAC-CsAAE1* were examined, and it was observed that there was minimal production of the anticipated compound OA [6] (Figure 3.5 and 2.11). As these clones originated from the same strain that previously produced OA, it was anticipated that a higher production of this compound would be evident. As explained by Carvalho et al., (2017) hexanoyl-CoA synthetase transforms the short-chain fatty acid hexanoic acid in its CoA form, then olivetolic acid is synthesized by a type III polyketide synthase, and polyketide cyclase. Since genes *TKS-OAC* and *CsAAE1* have been inserted into the transformants we expected they were able to produce olivetolic acid, precursor of the main cannabinoids. OA was allegedly produced in small concentration (<1 ppm) by three clones (Figure 3.5 and 2.11).

We had several hypotheses as to why of the production of OA was low; it might have happened because OA could be toxic for *C. reinhardtii*, however, this assertion has not been substantiated in this particular organism, for instance, in *E. coli*, a concentration of 100 mg/L of OA did not exhibit any signs of toxicity. Other hypothesis was because the enzyme TKS produces two lactone compounds hexanoyl triacetic acid lactone (HTAL), and pentyl-diacetic acid lactone (PDAL) [168] (not analyzed in this study), these compounds can be produced by hydrolysis of intermediate polyketide CoAs or spontaneous cyclization [169]. The previous asseveration has been reported in *C. sativa* and currently, there is no information about it in *C. reinhardtii* [168], [170]. Also, OA production might happen but not be detected due to low production, lower than the detection threshold, and might not be to detect trace or lower quantities than the standard cannabinoid (10 ppm).

According to our findings, production of hexanoyl-CoA was allegedly observed in a wild type clone during HPLC analysis of clones that were transformed with *CsAAE1* (Figure 3.7). Considering that this metabolite is known to be present in all organisms [171], it would have been relevant to perform further experiments, such as analyzing the production of hexanoyl-CoA in several replicates using HPLC and confirming the presence of this metabolite through mass spectrometry. This would have allowed us to determine the concentration of hexanoyl-CoA and use it as a precise reference when comparing it to transformed clones. However, due to time constraints, we were unable to perform these experiments, which limits our ability to conclusively determine whether there is a stable production of hexanoyl-CoA in both wild type and transformed clones. One of the primary limitations that can be identified is the absence of verification regarding the synthesis of hexanoyl-CoA in the clones that exclusively contained *CsAAE1*, as well as in the C30 clone. This would have facilitated a better comprehension of the variations and distinctions between the clones that possessed *CsAAE1* and the ones that carried *CsAAE1-TKS-OAC* cassette. Moreover, it would have aided in addressing the search regarding whether *CsAAE1* alone was capable of carry out the reaction for boosting the reservoir of precursors.

All the 15 clones analyzed were shown to harbor *CsAAE1* (Figure 3.1B) which means the gene is integrated into the genome of *Chlamydomonas.* However, in this organism, gene

integration into the nuclear genome is random by non-homologous-end-joining, leading to random insertion and susceptibility to gene silencing, that plays a role hindering heterologous expression in microalgae at transcriptional and post-transcriptional levels [172], [173]. Homologous recombination can happen in *Chlamydomonas reinhardtii* with the integration of the DNA into the chloroplast, using gene gun transformation method with the inconvenience that the chloroplast cannot performs post-translational modifications, important for recombinant proteins [174].

There exist more enzymes that have acyl-CoA synthetase activity rather than *Cs*AAE1, such as *S. cerevisiae* (yeast) fatty acid acyl-CoA (*Sc*FAA2), *Yarrowia lipolytica* fatty acid acyl-CoA (ylFAA1), *Pseudomonas putida* acyl-CoA synthetase (PpLvaE) [175] and according to Jingbo et al., (2022) [175] results, over expression of *PpLvaE* in *Y. lipolytica* had the biggest production of OA, concluding that that enzyme is the best candidate for the transformation of hexanoic acid to hexanoyl-CoA [175] . They have also found that in *Y. lipolytica*, *CsAAE1-OAC-Olivetol synthase*, when supplemented with hexanoic acid, OA production was low. In *S. cerevisiae*, *Cs*AAE1 showed better enzymatic capacity rather than *Y. lipolytica* [165]. This shows the enzymatic versatility on each the organism; we chose *CsAAE1* due transcripts and high expression is high in *C. sativa* [98], the plant where the cannabinoids are predominantly produced [127]. Different AAEs may exhibit superior acyl-CoA synthetase activity in *C. reinhardtii*, depending on the strain, co-expression, and supplementation factors, like demonstrated by Jingbo et al. (2022) [175].

One of the main challenges transforming *C. reindhartii* is gene silencing, which is a molecular mechanism that modifies gene expression (knock-down) and occurs at transcriptional and post-transcriptional levels [176]–[178]. It has been proposed that doing codon optimization and adding endogenous introns in the expression cassette could overcome gene silencing [179], [180]. These last two improvements were used in our experiments. Whilst *CsAAE1* comes from *Cannabis sativa*, codon optimization was done, since it has been proven that adjusting the gene of interest's codons to the natural host codon is beneficial for recombinant protein expression [181]. This is highly advisable to make because even if the redundant nature of nucleotide triplets ensures that one amino acid can be encoded by different triplet codons [182], each organism has their own distinct

usage of the code [183]. *C. reinhardtii* presents a narrow codon bias, the genome has a high percentage of guanine-cytosine content and an average exon length of 240 nucleotides [38]. Baier et al., (2020) showed that by using endogenous introns and codon-optimized cassettes in *C. reinhardtii* to mimic the short exon length, the relative transformation was higher thus gene expression, giving an insight into the importance and relevance of the intron-mediated enhancement and codon optimization [184]. Transformants with introns had a greater number of transformants rather the ones without introns (Table 3.1) this could have occurred because introns regulate gene expression by having transcriptional enhancers or transcription binding sites and enhancing mRNA stability[185].

One of the major challenges while transforming microorganisms is the screening time due to the high number of transformants [104], [186]. Currently, expressions cassettes harbor reporter proteins that assist in the analysis and screening of clones such as mClover, mCherry, mVenus and GFP are suitable for quantitative analysis of recombinant protein expression in biological systems [187]. Our expression cassette does not have a fluorescent reporter protein but in future experiments would be advisable to because reporter proteins expression and accumulation can be observed by microscopy and or flow cytometry, where the signal fluorescence level is proportional to the level of protein [188]. Flow cytometry is a more sensitive technique because of the capacity to measure gene expression in individual cells, rather than a population in a faster and more precise way [189].

Electroporation of *C. reinhardtii* can lead to a high number of transformants and its success depends on several parameters such as concentration, size, and quality of DNA, wavelength pulse in the electroporation, *Chlamydomonas* strains, etcetera [104], [190], [191]. The experiments described here showed electroporation efficiency of 1000 clones per 1µg of DNA, whereas it has been previously shown that the transformation efficiency with standard electroporation varies from 100 to 2000 transformants per µg of DNA [192], but when this method is optimized, it could lead to a yield of 6×10^3 transformants per µg of DNA [192]. Since clones growing on antibiotics could lead to false positives, performing PCR is important to discriminate among the clones harboring the gene and clones that may grow in selection media but do not harbor the gene of interest. This could be due to high cell concentration on the plate that might cause dead cell to overlap the growth of clones

over those cells [193] and a few rounds of selection on antibiotic selection media are advisable to differentiate among false positive clones [194]. Electroporation is until today one of the best methods to insert foreign DNA into the genome of *C. reinhardtii,* we obtained hundreds of transformants harboring *CsAAE1* with and without introns cassettes. Nevertheless, high efficiency does not mean the recombinant protein will be expressed, out of all clones a few showed minor production of OA at a low concentration that was nearly detectable, this shows the main drawback of metabolite engineering; a high number of transformants need to be obtained and these need to be screened to obtain only a few or none clones that could produce the recombinant protein. Also, from all the clones analyzed in western blot (Figure 3.3 and 2.9) no protein was detected in either case. This has been a drawback in different experiments (data not published) where protein is not detected in *C. reinhardtii* clones transformed with genes from the cannabinoid biosynthetic pathway.

Western blotting is a highly sensitive technique capable of detecting protein quantities as low as picograms. However, in cases where protein concentration is not the limiting factor, the detection of the protein of interest may be hindered by its conformational state due to a post-translational modifications [195], [196] such as, phosphorylation where specific binding sites for other proteins are created, thus changing the conformation of the protein and eventual formation of a protein complex [197]. Ghosh et al., (2014) explains that in phosphorylation requires specific antibodies that can selectively recognize and bind to the phosphorylated residues [198]. In the present study, we detected OA at a low concentration level as shown in Figure 3.5, however, no corresponding protein was identified. It is plausible that the protein concentration was below the limit of detection, or the post-translational modification(s) may have resulted in a conformational change that prevented its proper detection.

Mahmood T. and Yang T. (2012) explain that the antibody concentration should be appropriate and, in the case, its too low, the signal may not be visible [199], in our case the dilution was 1/1000; which is a typical dilution and they may vary between 1/250 to 1/4000 but low dilutions may require meticulous quantitative analysis [200]. Another cause for no visible bands is a really low concentration or absence of the antigen, concentrations of at

least 20-30 µg of protein is recommended [199], in our case, since we denatured the protein with urea and laemmli buffer, we could not know the proper concentration of the protein.

5.0 Conclusion

The results of our study provide insights into working with *C. reinhardtii* for the heterologous biosynthesis of cannabinoids. Although our main objective was obtaining cannabinoid-precursor-producing clones, our findings demonstrate that *C. reinhardtii* is a viable candidate for genetic transformation, albeit with crucial considerations that need to be taken into account. Notably, amplification of the transfected gene does not guarantee transgene expression and the production of the desired metabolite. However, the results obtained in this study support the notion that *C. reinhardtii* holds great promise as a non-conventional heterologous host. The stability of gene integration was confirmed after multiple rounds of selection, indicating its potential for use as a platform to produce high-value compounds. Further investigations are warranted to explore gene stability, copy number, random integration, and develop better strategies to enhance the production of metabolite-producing clones. Additionally, complementary culture techniques such as co-culture with other organisms and cell culture methods can be utilized to optimize the use of microalgae. Overall, our study provides a perspective on the use of *C. reinhardtii* in synthetic biology research and as a potential system for producing various high-value compounds, other than cannabinoids.

CHAPITER III

GENERAL CONCLUSION

Our study found that is possible to transform *Chlamydomonas reinhardtii* with *Hexanoyl-CoA synthetase* from *Cannabis sativa* (*CsAAE1*) using 2 different constructs: with introns and without introns. We didn't find a significant difference between clones harboring the two constructs, but we observed that transforming this alga with a codon optimized *CsAAE1* cassette with *rbcS2* introns, the transformation efficiency is higher than using a cassette without introns; higher number of transformants was obtained. Transforming *C. reinhardtii* with electroporation showed to be a reliable method to obtain hundreds of clones, and even the double antibiotic selection was a useful method to discriminate among PCR positive clones, and we used a high throughput PCR screening method to identify them, we still need to perform several molecular techniques in order to better characterize them, for example, southern blot to determine transgene copy number.

Some of the clones after months still harboured the gene of interest and showed stable growth on both antibiotics zeocin (5 µg/mL) and hygromycin (10 µg/mL). Even though clones were able to growth on culture media with antibiotics, we found that double antibiotic selection might be too harsh on *C. reinhardtii* transformants, specially with higher concentrations of zeocin overtime (higher than (5 µg/mL).

It was interesting to see that clones produced different metabolites that had a retention time close to the main cannabinoids and even though this doesn't mean they can produce these compounds; this gives insight into the further analysis of those interesting metabolites. Some transformants harboring The *CsAAE1-TKS-OAC* construct exhibited a limited quantity of putative Olivetolic acid production, as evidenced by its retention time that closely resembled OA standard. This study provides valuable insights into the genetic transformation of *C. reinhardtii* and its potential for use as a platform to produce cannabinoid precursors. The successful integration of the *CsAAE1-TKS-OAC* construct and

its small yet detectable production of putative Olivetolic acid suggest that *C. reinhardtii* is amenable to genetic engineering to produce high-value compounds.

6.0 Limitations and future perspectives

The study of cannabinoid production in *C. reinhardtii* is a nascent technology that is still in the early stages of development. In our laboratory, we have been investigating the feasibility of using genetic engineering techniques to introduce genes from the cannabinoid biosynthetic pathway into *C. reinhardtii*, with the goal of producing cannabinoids in this organism. However, as with any project related to emerging technologies, we have encountered several challenges and limitations. One of the main challenges is the limited knowledge about the regulation of the cannabinoid biosynthetic pathway in *C. reinhardtii,* which makes it difficult to predict the effects of genetic modifications on cannabinoid production. Additionally, the optimization of growth conditions, transformation protocols, and selection methods for *C. reinhardtii* can also be a time-consuming and labor-intensive process. Nonetheless, despite these challenges, the potential benefits of producing cannabinoids in *C. reinhardtii* make this an exciting and promising area of research. With all the objectives that we had, we can mention what we encountered concerning each one. Regarding the first objective, we tried to demonstrate if transforming *CsAAE1* into *C. reinhardtii* could boost the pool of precursors for the main cannabinoids, but several limitations led to the obtention of different compounds that were not cannabinoids or at least that is what we can hypothesize due to the lack of time to analyze all peaks obtained, also the unpredictable results of clones where we could not obtain consistent results while they were analyzed by HPLC (the aim of objective 3); different unknown peaks were obtained when performing experiments in triplicates. Adding to that, the culture time from the starter media until the HPLC analysis was at least two weeks, because *C. reinhardtii* takes at least one week to grow on TAP media, so whenever we repeated an experiment to analyze one ‘interesting peak’ we had to wait at least one month, from the starter culture until the results. And even if in the end we would have obtained a peak that matched our standards we should have had to confirm that peak with mass spectrometry and for further confirmation, nuclear magnetic resonance.

For the second objective, the screening, the time required to screen hundreds and sometimes thousands of clones, even with the high-throughput method [104]. Handling hundreds of samples at a time was quite difficult and several times some experiments had to be repeated due to crossed-contamination and error in the pipetting. Also, we found it quite difficult to find the right set of primers for *CsAAE1*, because we used genomic DNA which makes it difficult to design precise primers due to the specificity of the primers not binding to any region of *Chlamydomonas'* genomic DNA. For the RT-qPCR, I designed primers that were not specific, thus I could not have conclusive results concerning the gene expression.

The clone C30 that was transformed with *TKS-OAC* genes, and our clones obtained along this study need further analysis in order to find the proper culture (and extract) conditions so that the clones could have a stable production of the metabolites of interest.
Further analysis needs to be done for the purpose of verifying if the production of olivetolic acid or another precursor of the main cannabinoids is toxic for *C. reinhardtii;* many times, we hypothesized that maybe its metabolism would not produce those compounds because could be fatal for the alga, or maybe the quantities produced are so low that it can not be detected.

Finally, one of the biggest limitations in the project, was using zeocin as an antibiotic selection marker, because even though in the bibliography they have already used this antibiotic as a reliable marker [201], [202], we found that a concentration (5 µg/mL) was quite high and toxic to the clones, whilst authors propose the use of zeocin concentrations between 20 or 100 µg/mL [201], [202].

REFERENCES

[1] J. Lemay, W. M. A. Carscallen, and I. Desgagné-penix, "engineered microorganism for the production of cannabinoid biosynthetic pathway products," CA3079760C, 2019

[2] W. M. A. Carscallen, I. Desgagné-penix, and J. Lemay, "Engineered microorganism for the production of cannabinoid biosynthetic pathway products," AU2019262112A9, 2021

[3] W. M. A. Carscallen, I. Desgagné-penix, and J. Lemay, "Engineered microorganism for the production of cannabinoid biosynthetic pathway products," US20210189402A1, 2021

[4] W. M. A. Carscallen, I. Desgagné-penix, and J. Lemay, "Engineered microorganism for the production of cannabinoid biosynthetic pathway products," EP3788136A1, 2021

[5] I. Desgagné-penix, F. Awwad, and M. Ghribi, "Engineered microorganism for the production of cannabinoids," WO2021081647A1, 2021

[6] I. Desgagné-Penix, F. Meddeb-mouelhi, R. Beauchemin, and M. Ghribi, "Engineered microorganism for the production of cannabinoid biosynthetic pathway products," WO2021081648A1, 2021

[7] I. Desgagné-Penix, F. Awwad, and M. Ghribi, "Engineered Microorganism for the Production of Cannabinoids," US20230014531A1, 2023

[8] M. I. Khan, J. H. Shin, and J. D. Kim, "The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products," *Microbial Cell Factories*, vol. 17, no. 1. BioMed Central Ltd., Mar. 05, 2018. doi: 10.1186/s12934-018-0879-x.

[9] P. A. Salomé and S. S. Merchant, "A series of fortunate events: Introducing *Chlamydomonas* as a reference organism," *Plant Cell*, vol. 31, no. 8. American Society of Plant Biologists, pp. 1682–1707, Aug. 01, 2019. doi: 10.1105/tpc.18.00952.

[10] F. B. Lopez and G. F. Barclay, "Plant Anatomy and Physiology," in *Pharmacognosy: Fundamentals, Applications and Strategy*, Elsevier Inc., 2017, pp. 45–60. doi: 10.1016/B978-0-12-802104-0.00004-4.

[11] M. C. , S. M. , S. M. , Quitral V., "Nutritional and health properties of seaweeds and its potential as a functional ingredient," *Revista chilena de nutrición*, vol. 39, no. 4, pp. 196–200, 2012.

[12] E. A. Titlyanov, T. V. Titlyanova, X. Li, and H. Huang, “Marine Plants of Coral Reefs,” in *Coral Reef Marine Plants of Hainan Island*, Elsevier, 2017, pp. 5–39. doi: 10.1016/b978-0-12-811963-1.00002-0.

[13] J. Cotas, A. Leandro, D. Pacheco, A. M. M. Gonçalves, and L. Pereira, “A comprehensive review of the nutraceutical and therapeutic applications of red seaweeds (Rhodophyta),” *Life*, vol. 10, no. 3. MDPI AG, 2020. doi: 10.3390/life10030019.

[14] R. S. Alberte, A. L. Friedman, L. Gustafson, M. S. Rudnick, and H. Lyman, “Light-harvesting systems of brown algae and diatoms isolation and characterization of chlorophyll a/c and chlorophyll a/fucoxanthin pigment-protein complexes,” 1981.

[15] S. K. Kim and R. Pangestuti, “Biological activities and potential health benefits of fucoxanthin derived from marine brown algae,” in *Advances in Food and Nutrition Research*, Academic Press Inc., 2011, pp. 111–128. doi: 10.1016/B978-0-12-387669-0.00009-0.

[16] J. Legeżyńska, M. Kędra, and W. Walkusz, “Identifying trophic relationships within the high Arctic benthic community: How much can fatty acids tell?,” *Mar Biol*, vol. 161, no. 4, pp. 821–836, 2014, doi: 10.1007/s00227-013-2380-8.

[17] E. M. Stepanova and E. A. Lugovaya, “Macro- and microelements in some species of marine life from the Sea of Okhotsk,” *Foods and Raw Materials*, vol. 9, no. 2, pp. 302–309, 2021, doi: 10.21603/2308-4057-2021-2-302-309.

[18] O. Babich *et al.*, “Algae: Study of Edible and Biologically Active Fractions, Their Properties and Applications,” *Plants*, vol. 11, no. 6. MDPI, Mar. 01, 2022. doi: 10.3390/plants11060780.

[19] J. Ampofo and Lord Abbey, “Microalgae: Bioactive Composition, Health Benefits, Safety and Prospects as Potential High-Value Ingredients for the Functional Food Industry,” *Foods*, vol. 11, no. 12. MDPI, Jun. 01, 2022. doi: 10.3390/foods11121744.

[20] J. Singh and R. C. Saxena, “An Introduction to Microalgae: Diversity and Significance. Diversity and Significance,” in *Handbook of Marine Microalgae: Biotechnology Advances*, Elsevier Inc., 2015, pp. 11–24. doi: 10.1016/B978-0-12-800776-1.00002-9.

[21] B. Elisabeth, F. Rayen, and T. Behnam, “Microalgae culture quality indicators: a review,” *Critical Reviews in Biotechnology*, vol. 41, no. 4. Taylor and Francis Ltd., pp. 457–473, 2021. doi: 10.1080/07388551.2020.1854672.

[22] V. Dolganyuk *et al.*, “Microalgae: A promising source of valuable bioproducts,” *Biomolecules*, vol. 10, no. 8. MDPI AG, pp. 1–24, Aug. 01, 2020. doi: 10.3390/biom10081153.

[23] C. Caroppo and P. Pagliara, “Microalgae: A Promising Future,” *Microorganisms*, vol. 10, no. 8. MDPI, Aug. 01, 2022. doi: 10.3390/microorganisms10081488.

[24] S. Mandal and N. Mallick, “Microalgae: The Tiny Microbes with a Big Impact,” in *Bioenergy Research: Advances and Applications*, Elsevier Inc., 2014, pp. 171–184. doi: 10.1016/B978-0-444-59561-4.00011-5.

[25] M. Molazadeh, H. Ahmadzadeh, H. R. Pourianfar, S. Lyon, and P. H. Rampelotto, “The use of microalgae for coupling wastewater treatment with CO_2 biofixation,” *Frontiers in Bioengineering and Biotechnology*, vol. 7, no. MAR. Frontiers Media S.A., 2019. doi: 10.3389/fbioe.2019.00042.

[26] M. I. Khan, J. H. Shin, and J. D. Kim, “The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products,” *Microb Cell Fact*, vol. 17, no. 1, p. 36, Dec. 2018, doi: 10.1186/s12934-018-0879-x.

[27] C. Gerotto, A. Norici, and M. Giordano, “Toward Enhanced Fixation of CO_2 in Aquatic Biomass: Focus on Microalgae,” *Frontiers in Energy Research*, vol. 8. Frontiers Media S.A., Sep. 16, 2020. doi: 10.3389/fenrg.2020.00213.

[28] R. Prasad *et al.*, “Role of microalgae in global CO_2 sequestration: Physiological mechanism, recent development, challenges, and future prospective,” *Sustainability (Switzerland)*, vol. 13, no. 23. MDPI, Dec. 01, 2021. doi: 10.3390/su132313061.

[29] C. Caroppo and P. Pagliara, “Microalgae: A Promising Future,” *Microorganisms*, vol. 10, no. 8. MDPI, Aug. 01, 2022. doi: 10.3390/microorganisms10081488.

[30] C. A. Lembi, “control of nuisance algae,” 2003. doi: 10.1016/B978-0-12-741550-5.50025-8.

[31] J. Curtin, C. McInerney, B. Ó Gallachóir, C. Hickey, P. Deane, and P. Deeney, “Quantifying stranding risk for fossil fuel assets and implications for renewable energy investment: A review of the literature,” *Renewable and Sustainable Energy Reviews*, vol. 116. Elsevier Ltd, Dec. 01, 2019. doi: 10.1016/j.rser.2019.109402.

[32] GlobeNewsWire, “ $4.6 Billion Worldwide Microalgae Industry to 2027 - Impact of COVID-19 on the Market.,” 2020.

[33] E. Harris, D. Stern, and G. Witman, *The Chlamydomonas Sourcebook*, Second. 2009.

[34] H. Ettl, “Uber den Teilungsverlauf des Chloroplasten bei *Chlamydomonas* ,” *Protoplasma*, vol. 88, pp. 75–84, 1976.

[35] S. Sasso, H. Stibor, M. Mittag, and A. R. Grossman, “the natural history of model organisms”, doi: 10.7554/eLife.39233.001.

[36] S. K. Dutcher, “Mating and Tetrad Analysis in *Chlamydornonas reinhardtii*,” 1995.

[37] J. D. Rochaix, "*Chlamydomonas reinhardtii*," in *Brenner's Encyclopedia of Genetics: Second Edition*, Elsevier Inc., 2013, pp. 521–524. doi: 10.1016/B978-0-12-374984-0.00230-8.

[38] S. S. Merchant *et al.*, "The *Chlamydomonas* genome reveals the evolution of key animal and plant functions," *Science (1979)*, vol. 318, no. 5848, pp. 245–251, Oct. 2007, doi: 10.1126/science.1143609.

[39] C. Vahrenholz, G. Riemen, E. Pratje, B. Dujon, and G. Michaelis, "Mitochondrial DNA of *Chlamydomonas reinhardtii:* the structure of the ends of the linear 15.8-kb genome suggests mechanisms for DNA replication," 1993.

[40] Higgs DC, *The chloroplast genome. In: Stern DB, Harris EH (eds) The Chlamydomonas Sourcebook.*, vol. 2. 2009.

[41] J. H. Mussgnug, "Genetic tools and techniques for *Chlamydomonas reinhardtii*," *Applied Microbiology and Biotechnology*, vol. 99, no. 13. Springer Verlag, pp. 5407–5418, Jul. 12, 2015. doi: 10.1007/s00253-015-6698-7.

[42] R. Arturo Rivera Solís and S. Peraza Echeverría Virginia Aurora Herrera Valencia, "A La microalga verde nueva alternativa para la producción de proteínas recombinantes de interés médico *Chlamydomonas reinhardtii*," 2011.

[43] L. Stoffels, A. Finlan, G. Mannall, S. Purton, and B. Parker, "Downstream Processing of *Chlamydomonas reinhardtii* TN72 for Recombinant Protein Recovery," *Front Bioeng Biotechnol*, vol. 7, Dec. 2019, doi: 10.3389/fbioe.2019.00383.

[44] E. M. Ramos-Martinez, L. Fimognari, and Y. Sakuragi, "High-yield secretion of recombinant proteins from the microalga *Chlamydomonas reinhardtii*," *Plant Biotechnol J*, vol. 15, no. 9, pp. 1214–1224, Sep. 2017, doi: 10.1111/pbi.12710.

[45] Z. Zhang *et al.*, "Efficient heterotrophic cultivation of *Chlamydomonas reinhardtii*," *J Appl Phycol*, vol. 31, no. 3, pp. 1545–1554, Jun. 2019, doi: 10.1007/s10811-018-1666-0.

[46] K. Komsic-Buchmann, L. Wöstehoff, and B. Becker, "The contractile vacuole as a key regulator of cellular water flow in *Chlamydomonas reinhardtii*," *Eukaryot Cell*, vol. 13, no. 11, pp. 1421–1430, Nov. 2014, doi: 10.1128/EC.00163-14.

[47] P. Luykx, M. Hoppenrath, and D. G. Robinson, "Structure and behavior of contractile vacuoles in *Chlamydomonas reinhardtii*," 1997. [Online]. Available: http://rsb.info.nih.gov/nih-image/.

[48] K. Komsic-Buchmann, L. M. Stephan, and B. Becker, "The SEC6 protein is required for contractile vacuole function in *Chlamydomonas reinhardtii*," *J Cell Sci*, vol. 125, no. 12, pp. 2885–2895, Jun. 2012, doi: 10.1242/jcs.099184.

[49] H. Plattner, “Contractile vacuole complex-its expanding protein inventory,” in *International Review of Cell and Molecular Biology*, Elsevier Inc., 2013, pp. 371–416. doi: 10.1016/B978-0-12-407694-5.00009-2.

[50] N. J. Colley and D. E. Nilsson, “Photoreception in Phytoplankton,” in *Integrative and Comparative Biology*, Oxford University Press, Nov. 2016, pp. 764–775. doi: 10.1093/icb/icw037.

[51] K. W. Oster’ and R. D. Smyth2, “Light Antennas in Phototactic Algae,” 1980. [Online]. Available: https://journals.asm.org/journal/mr

[52] G. Kreimer, “Reflective properties of different eyespot types in dinoflagellates,” *Protist*, vol. 150, no. 3, pp. 311–323, 1999, doi: 10.1016/S1434-4610(99)70032-5.

[53] P. Hegemann, “Algal sensory photoreceptors,” *Annual Review of Plant Biology*, vol. 59. pp. 167–189, 2008. doi: 10.1146/annurev.arplant.59.032607.092847.

[54] B. D. Engel, M. Schaffer, L. K. Cuellar, E. Villa, J. M. Plitzko, and W. Baumeister, “Native architecture of the *Chlamydomonas* chloroplast revealed by in situ cryo-electron tomography,” *Elife*, vol. 2015, no. 4, Jan. 2015, doi: 10.7554/eLife.04889.

[55] R. Sager and G. E. Palade, “Structure and development of the chloroplast in *Chlamydomonas* i. the normal green cell.”

[56] J. Findinier *et al.*, “Deletion of BSG1 in *Chlamydomonas reinhardtii* leads to abnormal starch granule size and morphology,” *Sci Rep*, vol. 9, no. 1, Dec. 2019, doi: 10.1038/s41598-019-39506-6.

[57] S. H. Imam, M. J. Buchanan, H.-C. Shin, and W. J. Snell, “Characterization of the Wall Framework,” 1985.

[58] Y. Chisti, “Biodiesel from microalgae,” *Biotechnology Advances*, vol. 25, no. 3. pp. 294–306, May 2007. doi: 10.1016/j.biotechadv.2007.02.001.

[59] M. A. Scaife, G. T. D. T. Nguyen, J. Rico, D. Lambert, K. E. Helliwell, and A. G. Smith, “Establishing *Chlamydomonas reinhardtii as* an industrial biotechnology host,” *Plant Journal*, vol. 82, no. 3, pp. 532–546, May 2015, doi: 10.1111/tpj.12781.

[60] K. L. Kindle, R. A. Schnell, E. Fermindez, and P. A. Lefebvre, “Stable Nuclear Transformation of *Chlamydomonas* Using the *Chlamydomonas* Gene for Nitrate Reductase.”

[61] K. L. Kindle, “High-frequency nuclear transformation of *Chlamydomonas reinhardtii* (algae/glass beads/transfection),” 1990.

[62] B. L. Randolph-Anderson *et al.*, “Further characterization of the respiratory deficient dum-1 mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation,” 1993.

[63] W. Jiang, A. J. Brueggeman, K. M. Horken, T. M. Plucinak, and D. P. Weeks, "Successful transient expression of Cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*," *Eukaryot Cell*, vol. 13, no. 11, pp. 1465–1469, Nov. 2014, doi: 10.1128/EC.00213-14.

[64] L. Grossman and K. Moldave, *Nucleic acids*. Academic Press, 1967.

[65] R. E. Jinkerson and M. C. Jonikas, "Molecular techniques to interrogate and edit the *Chlamydomona s*nuclear genome," *Plant Journal*, vol. 82, no. 3, pp. 393–412, May 2015, doi: 10.1111/tpj.12801.

[66] J. Neupert, D. Karcher, and R. Bock, "Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes," *Plant Journal*, vol. 57, no. 6, pp. 1140–1150, Mar. 2009, doi: 10.1111/j.1365-313X.2008.03746.x.

[67] M. Fuhrmann, A. Hausherr, L. Ferbitz, T. Scho¨dlscho¨dl, M. Heitzer, and P. Hegemann, "Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene."

[68] A. Eichler-Stahlberg, W. Weisheit, O. Ruecker, and M. Heitzer, "Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii* ," *Planta*, vol. 229, no. 4, pp. 873–883, Mar. 2009, doi: 10.1007/s00425-008-0879-x.

[69] M. A. Scranton *et al.*, "Synthetic promoters capable of driving robust nuclear gene expression in the green alga *Chlamydomonas reinhardtii* ," *Algal Res*, vol. 15, pp. 135–142, Apr. 2016, doi: 10.1016/j.algal.2016.02.011.

[70] S. Purton, J. B. Szaub, T. Wannathong, R. Young, and C. K. Economou, "Genetic engineering of algal chloroplasts: Progress and prospects," *Russian Journal of Plant Physiology*, vol. 60, no. 4. pp. 491–499, 2013. doi: 10.1134/S1021443713040146.

[71] B. A. Rasala and S. P. Mayfield, "The microalga *Chlamydomonas reinhardtii* as a platform for the production of human protein therapeutics," *Bioeng Bugs*, vol. 2, no. 1, pp. 50–54, Jan. 2011, doi: 10.4161/bbug.2.1.13423.

[72] Q. Hu *et al.*, "Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances," *Plant Journal*, vol. 54, no. 4. pp. 621–639, May 2008. doi: 10.1111/j.1365-313X.2008.03492. x.

[73] Y. Li-Beisson, F. Beisson, and W. Riekhof, "Metabolism of acyl-lipids in *Chlamydomonas reinhardtii*," *Plant Journal*, vol. 82, no. 3, pp. 504–522, May 2015, doi: 10.1111/tpj.12787.

[74] R. Sirevhg~ and R. P. Levine, "Fatty Acid Synthetase from *Chlamydomonas reinhardii* SITES OF TRANSCRIPTION AND TRANSLATION*," 1972.

[75] W. R. Riekhof, B. B. Sears, and C. Benning, "Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: Discovery of the betaine

lipid synthase BTA1Cr," *Eukaryot Cell*, vol. 4, no. 2, pp. 242–252, Feb. 2005, doi: 10.1128/EC.4.2.242-252.2005.

[76] I. A. Guschina and J. L. Harwood, "Lipids and lipid metabolism in eukaryotic algae," *Progress in Lipid Research*, vol. 45, no. 2. pp. 160–186, Mar. 2006. doi: 10.1016/j.plipres.2006.01.001.

[77] L. Xu, X. Cheng, and Q. Wang, "Enhanced lipid production in *Chlamydomonas reinhardtii* by co-culturing with azotobacter chroococcum," *Front Plant Sci*, vol. 9, Jun. 2018, doi: 10.3389/fpls.2018.00741.

[78] J. B. Therien, O. A. Zadvornyy, M. C. Posewitz, D. A. Bryant, and J. W. Peters, "Growth of *Chlamydomonas reinhardtii* in acetate-free medium when co-cultured with alginate-encapsulated, acetate-producing strains of Synechococcus sp. PCC 7002," *Biotechnol Biofuels*, vol. 7, no. 1, 2014, doi: 10.1186/s13068-014-0154-2.

[79] V. H. Work *et al.*, "Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains," *Eukaryot Cell*, vol. 9, no. 8, pp. 1251–1261, Aug. 2010, doi: 10.1128/EC.00075-10.

[80] Z. Zhu *et al.*, "The synchronous TAG production with the growth by the expression of chloroplast transit peptide-fused ScPDAT in *Chlamydomonas reinhardtii*," *Biotechnol Biofuels*, vol. 11, no. 1, Jun. 2018, doi: 10.1186/s13068-018-1160-6.

[81] X. Fei, X. Li, P. Li, and X. Deng, "Involvement of *Chlamydomonas DNA* damage tolerence gene UBC2 in lipid accumulation," *Algal Res*, vol. 22, pp. 148–159, Mar. 2017, doi: 10.1016/j.algal.2016.12.019.

[82] R. Yao, W. Fu, M. Du, Z. X. Chen, A. P. Lei, and J. X. Wang, "Carotenoids Biosynthesis, Accumulation, and Applications of a Model Microalga *Euglena gracilis*," *Marine Drugs*, vol. 20, no. 8. MDPI, Aug. 01, 2022. doi: 10.3390/md20080496.

[83] T. Maoka, "Carotenoids as natural functional pigments," *Journal of Natural Medicines*, vol. 74, no. 1. Springer, Jan. 01, 2020. doi: 10.1007/s11418-019-01364-x.

[84] J. Paniagua-Michel, J. Olmos-Soto, and M. A. Ruiz, "Pathways of carotenoid biosynthesis in bacteria and microalgae," *Methods in Molecular Biology*, vol. 892, pp. 1–12, 2012, doi: 10.1007/978-1-61779-879-5_1.

[85] D. Siefermann-Harms, "Carotenoids in photosynthesis. I. Location in photosynthetic membranes and light-harvesting function," 1985.

[86] R. Anand, L. Mohan, and N. Bharadvaja, "Disease Prevention and Treatment Using β-Carotene: the Ultimate Provitamin A," *Revista Brasileira de Farmacognosia*, vol.

32, no. 4. Springer Science and Business Media Deutschland GmbH, pp. 491–501, Aug. 01, 2022. doi: 10.1007/s43450-022-00262-w.

[87] V. Henríquez, C. Escobar, J. Galarza, and J. Gimpel, "Carotenoids in microalgae," *Subcell Biochem*, vol. 79, pp. 219–237, Aug. 2016, doi: 10.1007/978-3-319-39126-7_8.

[88] I. Baroli *et al.*, "Photo-oxidative Stress in a Xanthophyll-deficient Mutant of *Chlamydomonas,*" *Journal of Biological Chemistry*, vol. 279, no. 8, pp. 6337–6344, Feb. 2004, doi: 10.1074/jbc.M312919200.

[89] K. K. Niyogi, O. Bjo¨rkmanbjo¨rkman, and A. R. Grossman, "The roles of specific xanthophylls in photoprotection," 1997. [Online]. Available: www.pnas.org.

[90] T. H. Sun, C. Q. Liu, Y. Y. Hui, W. K. Wu, Z. G. Zhou, and S. Lu, "Coordinated Regulation of Gene Expression for Carotenoid Metabolism in *Chlamydomonas reinhardtii*," *J Integr Plant Biol*, vol. 52, no. 10, pp. 868–878, Oct. 2010, doi: 10.1111/j.1744-7909.2010.00993. x.

[91] S. A. El-Mekkawi, H. S. Hussein, S. A. A. El-Enin, and N. N. El-Ibiari, "Assessment of stress conditions for carotenoids accumulation in *Chlamydomonas reinhardtii* as added-value algal products," *Bull Natl Res Cent*, vol. 43, no. 1, Dec. 2019, doi: 10.1186/s42269-019-0169-8.

[92] P. Napaumpaiporn and A. Sirikhachornkit, "Effects of High Temperature on Carotenoid Accumulation and Gene Expression in the Model Green Alga *Chlamydomonas reinhardtii*," 2016. [Online]. Available: http://epg.science.cmu.ac.th/ejournal/

[93] F. Taura, S. Sirikantaramas, Y. Shoyama, Y. Shoyama, and S. Morimoto, "REVIEW Phytocannabinoids in *Cannabis sativa*: Recent Studies on Biosynthetic Enzymes."

[94] Â. Carvalho, E. H. Hansen, O. Kayser, S. Carlsen, and F. Stehle, "Designing microorganisms for heterologous biosynthesis of cannabinoids," *FEMS yeast research*, vol. 17, no. 4. Jun. 01, 2017. doi: 10.1093/femsyr/fox037.

[95] M. Toyota, T. Kinugawa, and Y. Asakawa, "bibenzyl cannabinoid and bisbibenzyl derivative from the liverwort *Radula perrottetzl*," 1994.

[96] F. Cullmann and H. Becker, "Prenylated Bibenzyls from the Liverwort *Radula laxiramea**," 1999. [Online]. Available: www.znaturforsch.com.

[97] C. Pagano, G. Navarra, L. Coppola, G. Avilia, M. Bifulco, and C. Laezza, "Cannabinoids: Therapeutic Use in Clinical Practice," *International Journal of Molecular Sciences*, vol. 23, no. 6. MDPI, Mar. 01, 2022. doi: 10.3390/ijms23063344.

[98] J. M. Stout, Z. Boubakir, S. J. Ambrose, R. W. Purves, and J. E. Page, "The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-

activating enzyme in *Cannabis sativa* trichomes," *Plant Journal*, vol. 71, no. 3, pp. 353–365, Aug. 2012, doi: 10.1111/j.1365-313X.2012.04949.x.

[99] F. Taura, S. Sirikantaramas, Y. Shoyama, K. Yoshikai, Y. Shoyama, and S. Morimoto, "Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type *Cannabis sativa*," *FEBS Lett*, vol. 581, no. 16, pp. 2929–2934, Jun. 2007, doi: 10.1016/j.febslet.2007.05.043.

[100] S. J. Gagne, J. M. Stout, E. Liu, Z. Boubakir, S. M. Clark, and J. E. Page, "Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides," *Proc Natl Acad Sci U S A*, vol. 109, no. 31, pp. 12811–12816, Jul. 2012, doi: 10.1073/pnas.1200330109.

[101] B. F., F. N. , B. R. Lercker G., "Cannabinoid acids analysis," *Farmaco*, vol. 47, no. 3, pp. 367–378, 1992.

[102] K. B. Walsh, A. E. McKinney, and A. E. Holmes, "Minor Cannabinoids: Biosynthesis, Molecular Pharmacology and Potential Therapeutic Uses," *Frontiers in Pharmacology*, vol. 12. Frontiers Media S.A., Nov. 29, 2021. doi: 10.3389/fphar.2021.777804.

[103] E. Dolgin, "The Bioengineering of Cannabis," *Nature*, vol. 572, pp. S5–S7, 2019.

[104] S. B. Nouemssi, M. Ghribi, R. Beauchemin, F. Meddeb-Mouelhi, H. Germain, and I. Desgagné-Penix, "Rapid and efficient colony-pcr for high throughput screening of genetically transformed *Chlamydomonas reinhardtii*," *Life*, vol. 10, no. 9, pp. 1–13, Sep. 2020, doi: 10.3390/life10090186.

[105] L. Doron, N. Segal, and M. Shapira, "Transgene expression in microalgae—from tools to applications," *Frontiers in Plant Science*, vol. 7, no. APR2016. Frontiers Media S.A., Apr. 22, 2016. doi: 10.3389/fpls.2016.00505.

[106] C. López-Paz, D. Liu, S. Geng, and J. G. Umen, "Identification of *Chlamydomonas reinhardtii* endogenous genic flanking sequences for improved transgene expression," *Plant Journal*, vol. 92, no. 6, pp. 1232–1244, Dec. 2017, doi: 10.1111/tpj.13731.

[107] A. Laban, "Cannabinoid production in algae," WO2019202510A1, 2019

[108] A. Laban, "Cannabinoid production in algae," 2019

[109] J. Neupert *et al.*, "An epigenetic gene silencing pathway selectively acting on transgenic DNA in the green alga *Chlamydomonas,*" *Nat Commun*, vol. 11, no. 1, Dec. 2020, doi: 10.1038/s41467-020-19983-4.

[110] M. Śmiarowska, M. Białecka, and A. Machoy-Mokrzyńska, "Cannabis and cannabinoids: pharmacology and therapeutic potential," *Neurologia i Neurochirurgia Polska*, vol. 56, no. 1. Via Medica, pp. 4–13, 2022. doi: 10.5603/PJNNS.A2022.0015.

[111] M. A. Crocq, “History of cannabis and the endocannabinoid system,” *Dialogues Clin Neurosci*, vol. 22, no. 3, pp. 223–228, Sep. 2020, doi: 10.31887/DCNS.2020.22.3/MCROCQ.

[112] E. A. Levinsohn and K. P. Hill, “Clinical uses of cannabis and cannabinoids in the United States,” *Journal of the Neurological Sciences*, vol. 411. Elsevier B.V., Apr. 15, 2020. doi: 10.1016/j.jns.2020.116717.

[113] K. A. Savage and D. Wielbo, “Pharmacology of legal and illicit drugs.”

[114] A. Klimkiewicz and A. Jasinska, “The health effects of cannabis and cannabinoids,” *Psychiatria*, vol. 15, no. 2, pp. 88–92, Jun. 2018, doi: 10.17226/24625.

[115] “Dialogues ClinNeurosci-19-309”.

[116] H. C. Lu and K. Mackie, “Review of the Endocannabinoid System,” *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, vol. 6, no. 6. Elsevier Inc., pp. 607–615, Jun. 01, 2021. doi: 10.1016/j.bpsc.2020.07.016.

[117] H. C. Lu and K. MacKie, “An introduction to the endogenous cannabinoid system,” *Biological Psychiatry*, vol. 79, no. 7. Elsevier USA, pp. 516–525, 2016. doi: 10.1016/j.biopsych.2015.07.028.

[118] E. Mills, “The carbon footprint of indoor Cannabis production,” *Energy Policy*, vol. 46, pp. 58–67, Jul. 2012, doi: 10.1016/j.enpol.2012.03.023.

[119] Z. Zheng, K. Fiddes, and L. Yang, “A narrative review on environmental impacts of cannabis cultivation,” *J Cannabis Res*, vol. 3, no. 1, Dec. 2021, doi: 10.1186/s42238-021-00090-0.

[120] Â. Carvalho, E. H. Hansen, O. Kayser, S. Carlsen, and F. Stehle, “Designing microorganisms for heterologous biosynthesis of cannabinoids,” *FEMS yeast research*, vol. 17, no. 4. Jun. 01, 2017. doi: 10.1093/femsyr/fox037.

[121] D. Llewellyn, S. Golem, E. Foley, S. Dinka, A. M. P. Jones, and Y. Zheng, “Indoor grown cannabis yield increased proportionally with light intensity, but ultraviolet radiation did not affect yield or cannabinoid content,” *Front Plant Sci*, vol. 13, Sep. 2022, doi: 10.3389/fpls.2022.974018.

[122] Z. K. Punja, “Emerging diseases of *Cannabis sativa* and sustainable management,” *Pest Management Science*, vol. 77, no. 9. John Wiley and Sons Ltd, pp. 3857–3870, Sep. 01, 2021. doi: 10.1002/ps.6307.

[123] C. Balthazar, G. Cantin, A. Novinscak, D. L. Joly, and M. Filion, “Expression of Putative Defense Responses in Cannabis Primed by *Pseudomonas* and/or *Bacillus* Strains and Infected by *Botrytis cinerea*,” *Front Plant Sci*, vol. 11, Nov. 2020, doi: 10.3389/fpls.2020.572112.

[124] Z. K. Punja, D. Collyer, C. Scott, S. Lung, J. Holmes, and D. Sutton, "Pathogens and Molds Affecting Production and Quality of Cannabis sativa L.," *Front Plant Sci*, vol. 10, Oct. 2019, doi: 10.3389/fpls.2019.01120.

[125] O. Aizpurua-Olaizola *et al.*, "Evolution of the Cannabinoid and Terpene Content during the Growth of *Cannabis sativa* Plants from Different Chemotypes," *J Nat Prod*, vol. 79, no. 2, pp. 324–331, Feb. 2016, doi: 10.1021/acs.jnatprod.5b00949.

[126] B. A. Khan, P. Warner, and H. Wang, "Antibacterial Properties of Hemp and Other Natural Fibre Plants: A Review."

[127] C. M. Andre, J. F. Hausman, and G. Guerriero, "Cannabis sativa: The plant of the thousand and one molecules," *Front Plant Sci*, vol. 7, no. FEB2016, Feb. 2016, doi: 10.3389/fpls.2016.00019.

[128] H. M. Summers, E. Sproul, and J. C. Quinn, "The greenhouse gas emissions of indoor cannabis production in the United States," *Nat Sustain*, vol. 4, no. 7, pp. 644–650, Jul. 2021, doi: 10.1038/s41893-021-00691-w.

[129] K. Blatt-Janmaat and Y. Qu, "The biochemistry of phytocannabinoids and metabolic engineering of their production in heterologous systems," *International Journal of Molecular Sciences*, vol. 22, no. 5. MDPI AG, pp. 1–19, Mar. 01, 2021. doi: 10.3390/ijms22052454.

[130] E. A. Cutolo, G. Mandalà, L. Dall'osto, and R. Bassi, "Harnessing the Algal Chloroplast for Heterologous Protein Production," *Microorganisms*, vol. 10, no. 4. MDPI, Apr. 01, 2022. doi: 10.3390/microorganisms10040743.

[131] A. E. Sproles, A. Berndt, F. J. Fields, and S. P. Mayfield, "Improved high-throughput screening technique to rapidly isolate *Chlamydomonas transformants* expressing recombinant proteins," *Appl Microbiol Biotechnol*, vol. 106, no. 4, pp. 1677–1689, Feb. 2022, doi: 10.1007/s00253-022-11790-9.

[132] T. Mogany, V. Bhola, L. Ramanna, and F. Bux, "Photosynthesis and pigment production: elucidation of the interactive effects of nutrients and light on *Chlamydomonas reinhardtii*," *Bioprocess Biosyst Eng*, vol. 45, no. 1, pp. 187–201, Jan. 2022, doi: 10.1007/s00449-021-02651-2.

[133] E. Devadasu and R. Subramanyam, "Enhanced Lipid Production in *Chlamydomonas reinhardtii* Caused by Severe Iron Deficiency," *Front Plant Sci*, vol. 12, Apr. 2021, doi: 10.3389/fpls.2021.615577.

[134] D. K. Y. Lim *et al.*, "Isolation and evaluation of oil-producing microalgae from subtropical coastal and Brackish waters," *PLoS One*, vol. 7, no. 7, Jul. 2012, doi: 10.1371/journal.pone.0040751.

[135] A. J. Berndt *et al.*, “Recombinant production of a functional SARS-CoV-2 spike receptor binding domain in the green algae *Chlamydomonas reinhardtii*,” *PLoS One*, vol. 16, no. 11 November, Nov. 2021, doi: 10.1371/journal.pone.0257089.

[136] I. Krzemińska, B. Pawlik-Skowrońska, M. Trzcińska, and J. Tys, “Influence of photoperiods on the growth rate and biomass productivity of green microalgae,” *Bioprocess Biosyst Eng*, vol. 37, no. 4, pp. 735–741, 2014, doi: 10.1007/s00449-013-1044-x.

[137] M. H. Huesemann, T. S. Hausmann, R. Bartha, M. Aksoy, J. C. Weissman, and J. R. Benemann, “Biomass productivities in wild type and pigment mutant of *Cyclotella* sp. (Diatom),” *Appl Biochem Biotechnol*, vol. 157, no. 3, pp. 507–526, Jun. 2009, doi: 10.1007/s12010-008-8298-9.

[138] J. Sheehan, T. Dunahay, J. Benemann, and P. Roessler, “A Look Back at the U.S. Department of Energy’s Aquatic Species Program: Biodiesel from Algae Close-Out Report,” 1978.

[139] P. A. Salomé and S. S. Merchant, “A series of fortunate events: Introducing *Chlamydomonas as* a reference organism,” *Plant Cell*, vol. 31, no. 8. American Society of Plant Biologists, pp. 1682–1707, Aug. 01, 2019. doi: 10.1105/tpc.18.00952.

[140] S. B. Grama, Z. Liu, and J. Li, “Emerging Trends in Genetic Engineering of Microalgae for Commercial Applications,” *Marine Drugs*, vol. 20, no. 5. MDPI, May 01, 2022. doi: 10.3390/md20050285.

[141] I. K. Blaby *et al.*, “The *Chlamydomonas genome* project: A decade on,” *Trends in Plant Science*, vol. 19, no. 10. Elsevier Ltd, pp. 672–680, Oct. 01, 2014. doi: 10.1016/j.tplants.2014.05.008.

[142] J. E. Maul *et al.*, “The *Chlamydomonas reinhardtii* plastid chromosome: Islands of genes in a sea of repeats,” *Plant Cell*, vol. 14, no. 11, pp. 2659–2679, Nov. 2002, doi: 10.1105/tpc.006155.

[143] J. E. Maul *et al.*, “The *Chlamydomonas reinhardtii* plastid chromosome: Islands of genes in a sea of repeats,” *Plant Cell*, vol. 14, no. 11, pp. 2659–2679, Nov. 2002, doi: 10.1105/tpc.006155.

[144] C. Vahrenholz, G. Riemen, E. Pratje, B. Dujon, and G. Michaelis, “Mitochondrial DNA of *Chlamydomonas reinhardtii*: the structure of the ends of the linear 15.8-kb genome suggests mechanisms for DNA replication,” 1993.

[145] J. E. Maul *et al.*, “The *Chlamydomonas reinhardtii* plastid chromosome: Islands of genes in a sea of repeats,” *Plant Cell*, vol. 14, no. 11, pp. 2659–2679, Nov. 2002, doi: 10.1105/tpc.006155.

[146] J. E. Bailey, “Toward a Science of Metabolic Engineering,” 2014. [Online]. Available: www.sciencemag.org

[147] A. K. Fisher, B. G. Freedman, D. R. Bevan, and R. S. Senger, “A review of metabolic and enzymatic engineering strategies for designing and optimizing performance of microbial cell factories,” *Computational and Structural Biotechnology Journal*, vol. 11, no. 18. Elsevier B.V., pp. 91–99, Aug. 01, 2014. doi: 10.1016/j.csbj.2014.08.010.

[148] L. B. Pickens, Y. Tang, and Y. H. Chooi, “Metabolic engineering for the production of natural products,” *Annu Rev Chem Biomol Eng*, vol. 2, pp. 211–236, Jul. 2011, doi: 10.1146/annurev-chembioeng-061010-114209.

[149] B. M. Bonk, Y. Tarasova, M. A. Hicks, B. Tidor, and K. L. J. Prather, “Rational design of thiolase substrate specificity for metabolic engineering applications,” *Biotechnol Bioeng*, vol. 115, no. 9, pp. 2167–2182, Sep. 2018, doi: 10.1002/bit.26737.

[150] J. M. Stout, Z. Boubakir, S. J. Ambrose, R. W. Purves, and J. E. Page, “The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in *Cannabis sativa* trichomes,” *Plant Journal*, vol. 71, no. 3, pp. 353–365, Aug. 2012, doi: 10.1111/j.1365-313X.2012.04949.x.

[151] J. Chen *et al.*, “Characterization of a Cytosolic Acyl-Activating Enzyme Catalyzing the Formation of 4-Methylvaleryl-CoA for Pogostone Biosynthesis in Pogostemon Cablin,” *Plant Cell Physiol*, vol. 62, no. 10, pp. 1556–1571, Oct. 2021, doi: 10.1093/pcp/pcab111.

[152] B. A. Rasala, P. A. Lee, Z. Shen, S. P. Briggs, M. Mendez, and S. P. Mayfield, “Robust expression and secretion of xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide,” *PLoS One*, vol. 7, no. 8, Aug. 2012, doi: 10.1371/journal.pone.0043349.

[153] J. Sen Tan *et al.*, “A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids,” *Bioengineered*, vol. 11, no. 1, pp. 116–129, Jan. 2020, doi: 10.1080/21655979.2020.1711626.

[154] M. Benedetti, V. Vecchi, S. Barera, and L. Dall’Osto, “Biomass from microalgae: The potential of domestication towards sustainable biofactories,” *Microb Cell Fact*, vol. 17, no. 1, Nov. 2018, doi: 10.1186/s12934-018-1019-3.

[155] H. Potter and R. Heller, “Transfection by electroporation,” *Current Protocols in Molecular Biology*, no. SUPPL. 92. Oct. 2010. doi: 10.1002/0471142727.mb0903s62.

[156] S. Gutiérrez, K. J. Lauersen, M. Fabris, and R. Abbriano, “biology Gene Delivery Technologies with Applications in Microalgal Genetic Engineering,” 2021, doi: 10.3390/biology.

[157] P. Mini *et al.*, "Agrobacterium-mediated and electroporation-mediated transformation of *Chlamydomonas reinhardtii*: A comparative study," *BMC Biotechnol*, vol. 18, no. 1, Feb. 2018, doi: 10.1186/s12896-018-0416-3.

[158] K. A. Jinturkar, M. N. Rathi, and A. Misra, "Gene Delivery Using Physical Methods," in *Challenges in Delivery of Therapeutic Genomics and Proteomics*, Elsevier Inc., 2011, pp. 83–126. doi: 10.1016/B978-0-12-384964-9.00003-7.

[159] P. T. Pratheesh, M. Vineetha, and G. M. Kurup, "An efficient protocol for the *Agrobacterium*-mediated genetic transformation of microalga *Chlamydomonas reinhardtii*," *Mol Biotechnol*, vol. 56, no. 6, pp. 507–515, 2014, doi: 10.1007/s12033-013-9720-2.

[160] P. Mini *et al.*, "*Agrobacterium*-mediated and electroporation-mediated transformation of *Chlamydomonas reinhardtii*: A comparative study," *BMC Biotechnol*, vol. 18, no. 1, Feb. 2018, doi: 10.1186/s12896-018-0416-3.

[161] V. M. Ramesh, S. E. Bingham, and A. N. Webber, "A simple method for chloroplast transformation in *Chlamydomonas reinhardtii*.," *Methods Mol Biol*, vol. 684, pp. 313–320, 2011, doi: 10.1007/978-1-60761-925-3_23.

[162] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, 1982. [Online]. Available: http://nar.oxfordjournals.org/

[163] New England Biolabs, "PCR Protocol for Taq DNA Polymerase with ThermoPol® Buffer (M0267)," Jan. 12, 2023.

[164] "Stamatakis, Alexandros & Hoover, J. & Rougemint, J.. (2008). A rapid Bootstrap algorithm for the RAxML Web Servers. Syst. Biol. 75. 558-771. ".

[165] X. Luo *et al.*, "Complete biosynthesis of cannabinoids and their unnatural analogues in yeast," *Nature*, vol. 567, no. 7746, pp. 123–126, Mar. 2019, doi: 10.1038/s41586-019-0978-9.

[166] Z. Tan, J. M. Clomburg, and R. Gonzalez, "Synthetic Pathway for the Production of Olivetolic Acid in *Escherichia coli*," *ACS Synth Biol*, vol. 7, no. 8, pp. 1886–1896, Aug. 2018, doi: 10.1021/acssynbio.8b00075.

[167] J. Shockey and J. Browse, "Genome-level and biochemical diversity of the acyl-activating enzyme superfamily in plants," *Plant Journal*, vol. 66, no. 1, pp. 143–160, Apr. 2011, doi: 10.1111/j.1365-313X.2011.04512.x.

[168] S. Gagne, "the polyketide origins of cannabinoids in *Cannabis sativa,"* University of Saskatchewan, Saskatoon, 2013.

[169] F. Taura *et al.*, "A novel class of plant type III polyketide synthase involved in orsellinic acid biosynthesis from *Rhododendron dauricum*," *Front Plant Sci*, vol. 7, no. September2016, Sep. 2016, doi: 10.3389/fpls.2016.01452.

[170] H. Morita, I. Abe, and H. Noguchi, *Plant Type III PKS*. Elsevier, 2010.

[171] NCBI, "National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 449118, Hexanoyl-coenzyme a." https://pubchem.ncbi.nlm.nih.gov/compound/Hexanoyl-coenzyme-a. (accessed Apr. 05, 2023).

[172] R. E. Jinkerson and M. C. Jonikas, "Molecular techniques to interrogate and edit the *Chlamydomonas nuclear* genome," *Plant Journal*, vol. 82, no. 3, pp. 393–412, May 2015, doi: 10.1111/tpj.12801.

[173] R. Zhang, W. Patena, U. Armbruster, S. S. Gang, S. R. Blum, and M. C. Jonikas, "High-throughput genotyping of green algal mutants reveals random distribution of mutagenic insertion sites and endonucleolytic cleavage of transforming DNA," *Plant Cell*, vol. 26, no. 4, pp. 1398–1409, 2014, doi: 10.1105/tpc.114.124099.

[174] V. M. Ramesh, S. E. Bingham, and A. N. Webber, "A simple method for chloroplast transformation in *Chlamydomonas reinhardtii*.," *Methods Mol Biol*, vol. 684, pp. 313–320, 2011, doi: 10.1007/978-1-60761-925-3_23.

[175] J. Ma, Y. Gu, and P. Xu, "Biosynthesis of cannabinoid precursor olivetolic acid in genetically engineered *Yarrowia lipolytica*," *Commun Biol*, vol. 5, no. 1, Dec. 2022, doi: 10.1038/s42003-022-04202-1.

[176] T. Zhao, W. Wang, X. Bai, and Y. Qi, "Gene silencing by artificial microRNAs in *Chlamydomonas:* Technical advance," *Plant Journal*, vol. 58, no. 1, pp. 157–164, Apr. 2009, doi: 10.1111/j.1365-313X.2008.03758.x.

[177] N. Shahar *et al.*, "The Integration of Multiple Nuclear-Encoded Transgenes in the Green Alga *Chlamydomonas reinhardtii* Results in Higher Transcription Levels," *Front Plant Sci*, vol. 10, Feb. 2020, doi: 10.3389/fpls.2019.01784.

[178] J. B. Bessler, K. C. Reddy, M. Hayashi, J. Hodgkin, and A. M. Villeneuve, "A role for *Caenorhabditis elegans* chromatin-associated protein HIM-17 in the proliferation vs. meiotic entry decision," *Genetics*, vol. 175, no. 4, pp. 2029–2037, Apr. 2007, doi: 10.1534/genetics.107.070987.

[179] J. Neupert *et al.*, "An epigenetic gene silencing pathway selectively acting on transgenic DNA in the green alga *Chlamydomonas,*" *Nat Commun*, vol. 11, no. 1, Dec. 2020, doi: 10.1038/s41467-020-19983-4.

[180] R. Barahimipour, D. Strenkert, J. Neupert, M. Schroda, S. S. Merchant, and R. Bock, "Dissecting the contributions of GC content and codon usage to gene expression in the model alga *Chlamydomonas reinhardtii*," *Plant Journal*, vol. 84, no. 4, pp. 704–717, Nov. 2015, doi: 10.1111/tpj.13033.

[181] I. Weiner, Y. Feldman, N. Shahar, I. Yacoby, and T. Tuller, "CSO – A sequence optimization software for engineering chloroplast expression in *Chlamydomonas reinhardtii*," *Algal Res*, vol. 46, Mar. 2020, doi: 10.1016/j.algal.2019.101788.

[182] P. M. Sharp and W.-H. Li, "The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications," 1987. [Online]. Available: https://academic.oup.com/nar/article-abstract/15/3/1281/1166844

[183] Z. Han, W. S. Lo, J. W. Lightfoot, H. Witte, S. Sun, and R. J. Sommer, "Improving transgenesis efficiency and CRISPR-associated tools through codon optimization and native intron addition in pristionchus nematodes," *Genetics*, vol. 216, no. 4, pp. 947–956, Dec. 2020, doi: 10.1534/genetics.120.303785.

[184] T. Baier, N. Jacobebbinghaus, A. Einhaus, K. J. Lauersen, and O. Kruse, "Introns mediate post-Transcriptional enhancement of nuclear gene expression in the green microalga *Chlamydomonas reinhardtii*," *PLoS Genet*, vol. 16, no. 7, Jul. 2020, doi: 10.1371/journal.pgen.1008944.

[185] T. Baier, N. Jacobebbinghaus, A. Einhaus, K. J. Lauersen, and O. Kruse, "Introns mediate post-Transcriptional enhancement of nuclear gene expression in the green microalga *Chlamydomonas reinhardtii*," *PLoS Genet*, vol. 16, no. 7, Jul. 2020, doi: 10.1371/journal.pgen.1008944.

[186] A. E. Sproles, A. Berndt, F. J. Fields, and S. P. Mayfield, "Improved high-throughput screening technique to rapidly isolate *Chlamydomonas transformants* expressing recombinant proteins," *Appl Microbiol Biotechnol*, vol. 106, no. 4, pp. 1677–1689, Feb. 2022, doi: 10.1007/s00253-022-11790-9.

[187] S. Y. Kim, K. W. Kim, Y. M. Kwon, and J. Y. H. Kim, "mCherry Protein as an In Vivo Quantitative Reporter of Gene Expression in the Chloroplast of *Chlamydomonas reinhardtii*," *Mol Biotechnol*, vol. 62, no. 5, pp. 297–305, May 2020, doi: 10.1007/s12033-020-00249-9.

[188] F. Delvigne, H. Pêcheux, and C. Tarayre, "Fluorescent reporter libraries as useful tools for optimizing microbial cell factories: A review of the current methods and applications," *Frontiers in Bioengineering and Biotechnology*, vol. 3, no. SEP. Frontiers Media S.A., 2015. doi: 10.3389/fbioe.2015.00147.

[189] M. R. Soboleski, J. Oaks, and W. P. Halford, "Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells."

[190] Y. H. Kim and D. J. Im, "Control of the culture conditions of *Chlamydomonas reinhardtii* for efficient delivery of exogenous materials in electroporation," *Algal Res*, vol. 35, pp. 388–394, Nov. 2018, doi: 10.1016/j.algal.2018.09.010.

[191] M. P. Zhang, M. Wang, and C. Wang, "Nuclear transformation of *Chlamydomonas reinhardtii*: A review," *Biochimie*, vol. 181. Elsevier B.V., pp. 1–11, Feb. 01, 2021. doi: 10.1016/j.biochi.2020.11.016.

[192] L. Wang *et al.*, "Rapid and high efficiency transformation of *Chlamydomonas reinhardtii* by square-wave electroporation," *Biosci Rep*, vol. 39, no. 1, Jan. 2019, doi: 10.1042/BSR20181210.

[193] C. F. Muñoz *et al.*, "Improved DNA/protein delivery in microalgae – A simple and reliable method for the prediction of optimal electroporation settings," *Algal Res*, vol. 33, pp. 448–455, Jul. 2018, doi: 10.1016/j.algal.2018.06.021.

[194] L. Esland, M. Larrea-Alvarez, and S. Purton, "Selectable markers and reporter genes for engineering the chloroplast of *Chlamydomonas reinhardtii*," *Biology*, vol. 7, no. 4. MDPI AG, Dec. 01, 2018. doi: 10.3390/biology7040046.

[195] M. Mishra, S. Tiwari, and A. V. Gomes, "Protein purification and analysis: Next generation western blotting techniques," *Expert Review of Proteomics*, vol. 14, no. 11. Taylor and Francis Ltd, pp. 1037–1053, Oct. 13, 2017. doi: 10.1080/14789450.2017.1388167.

[196] K. J. Lauersen, H. Berger, J. H. Mussgnug, and O. Kruse, "Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*," *J Biotechnol*, vol. 167, no. 2, pp. 101–110, Aug. 2013, doi: 10.1016/j.jbiotec.2012.10.010.

[197] J. W. Mandell, "Phosphorylation State-Specific Antibodies Applications in Investigative and Diagnostic Pathology," 2003. [Online]. Available: http://www.biocompare.com

[198] R. Ghosh, J. E. Gilda, and A. V. Gomes, "The necessity of and strategies for improving confidence in the accuracy of western blots," *Expert Review of Proteomics*, vol. 11, no. 5. Expert Reviews Ltd., pp. 549–560, Oct. 01, 2014. doi: 10.1586/14789450.2014.939635.

[199] T. Mahmood and P. C. Yang, "Western blot: Technique, theory, and trouble shooting," *N Am J Med Sci*, vol. 4, no. 9, pp. 429–434, Sep. 2012, doi: 10.4103/1947-2714.100998.

[200] R. J. Koch *et al.*, "Validating Antibodies for Quantitative Western Blot Measurements with Microwestern Array," *Sci Rep*, vol. 8, no. 1, Dec. 2018, doi: 10.1038/s41598-018-29436-0.

[201] A. E. Sproles, A. Berndt, F. J. Fields, and S. P. Mayfield, "Improved high-throughput screening technique to rapidly isolate *Chlamydomonas transformants* expressing recombinant proteins," *Appl Microbiol Biotechnol*, vol. 106, no. 4, pp. 1677–1689, Feb. 2022, doi: 10.1007/s00253-022-11790-9.

[202] P. Dementyeva *et al.*, "A novel, robust and mating-competent *Chlamydomonas reinhardtii* strain with an enhanced transgene expression capacity for algal biotechnology," *Biotechnology Reports*, vol. 31, Sep. 2021, doi: 10.1016/j.btre.2021.e00644.