UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: GREEN MICROALGAE AS AN ALTERNATIVE FOR PRODUCTION OF PLANT-DERIVED DRUGS

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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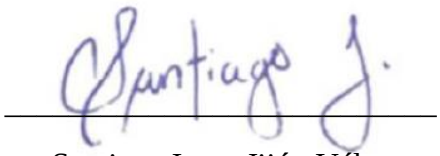

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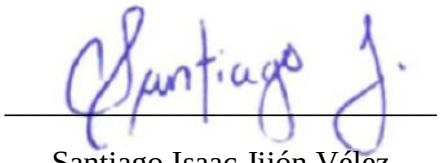

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Resumen

El Paclitaxel es un fármaco obtenido de plantas del género *Taxus.* Es utilizado para tratar varios tipos de cáncer por lo cual hay una alta demanda que actuales medios semisintéticos de producción no pueden cubrir. Por este motivo, la industria apunta su investigación hacia plataformas microbianas para la producción de metabolitos dado que son más eficientes y económicas que las plantas. El uso de microalgas como plataformas alternativas en ingeniería metabólica es un campo sin explorar. Tienen varias cualidades ventajosas como su fotoautotrofía y encapsulación que las vuelven atractivas. El presente trabajo muestra un diseño de ingeniería metabólica en el plastoma de *Chlamydomonas reinhardtii* para producir taxadien-5α-acetato-10β-ol, un precursor avanzado de Paclitaxel. Este estudio aprovecha que parte de la ruta metabólica de la droga se encuentra codificada en el núcleo y localizada en el cloroplasto, lo cual reduce los genes requeridos para producir el compuesto en cinco. Dichas secuencias pueden ser clonadas en el plastoma usando transformación serial ocupando dos plásmidos. El esquema resultante para hacer ingeniería metabólica de *C. reinhardtii* muestra viabilidad para producción del precursor. Futuras investigaciones tendrán que enfocarse hacia mejorar la red metabólica del alga, así como los genes requeridos que optimicen la producción del metabolito. Eventualmente, las plataformas de microalgas podrán competir con bacterias y levaduras y serán consideradas para la producción de otros fármacos derivados de plantas.

Palabras clave:

transplastómica, *Chlamydomonas reinhardtii*, microalgas, paclitaxel, ingeniería metabólica

Abstract

Paclitaxel is a drug obtained from plants of the genus *Taxus*. It is used for numerous anticancer treatments which require a high demand that current semisynthetic means of production are not able to supply. For this reason, industry is directing its research towards microbial platforms to manufacture metabolites which can be more efficient and cost-effective than plant systems. An unexplored field in metabolic engineering is using microalgae as an alternative platform for production of pharmaceuticals. They result advantageous, considering features like photoautotrophy and encapsulation that make it an appealing platform. The present work designs a metabolic engineering trial employing the plastome of *Chlamydomonas reinhardtii* to produce taxadien-5α-acetate-10β-ol, an advanced paclitaxel precursor. This study takes advantage that part of the pathway towards Paclitaxel is already encoded in the nucleus and localized in the chloroplast which reduced the genes required to produce the compound to five. The required sequences could be cloned into the plastome using serial transformation with two plasmids. The resulting scheme for metabolic engineering for *C. reinhardtii* shows feasibility to produce the precursor. Future research will have to focus on improving the metabolic network of the alga as well as the genes required to enhance production of the metabolite. Eventually, microalgal platforms will be able to compete with classical bacteria and yeast and considered for production of other plant-based pharmaceuticals.

Key Words:

transplastomics, *Chlamydomonas reinhardtii*, microalgae, paclitaxel, metabolic engineering

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Abbreviations

GOI	Gene of interest
GRAS	Generally regarded as safe
G3P	Glucose-3-phosphate
IPP	Isopentenyl pyrophosphate
MEP	Non-Mevalonate
MVA	Mevalonate
PTX	Paclitaxel
TAP	Tris-Acetate-Phossphate

CHAPTER 1: INTRODUCTION

Paclitaxel is a renowned drug used to treat several types of cancer (e. g. lung, breast, ovarian cancer) (Sofias, Dunne, Storm, & Allen, 2017). This compound is extracted from *Taxus* trees (Bernabeu, Cagel, Lagomarsino, Moretton, & Chiappetta, 2017). However, the amounts extracted are not sufficient to carry out efficient trials (Sabzehzari, Zeinali, & Naghavi, 2020; Sofias et al., 2017). Currently, it is produced using a semisynthetic technique which produces the drug from a late-stage precursor found in the needles of the plant (Bernabeu et al., 2017). Nevertheless, this procedure results costly and yields toxic residues (Bernabeu et al., 2017). A new method for massive production is required and industry is aiming to use microbial platforms for this purpose (Abdallah et al., 2019; Zhou et al., 2015). However, the furthest that has been reached towards using microorganisms (*S. cerevisiae*) is the precursor taxadien-5α-acetate-10β-ol (Zhou et al., 2015). The intention of these studies is to produce the drug by modifying an advanced precursor or to produce it directly using such platforms.

Microalgae are microbial platforms recognized for producing pigments and polymers (Scaife et al., 2015). They are more efficient than higher plant systems since they require less resources and can adapt to different environments (Scaife et al., 2015). By using controlled conditions of cultivation, a great variety of bioactive compounds can be produced (Scaife et al., 2015). Microalgae are generally regarded as safe (GRAS) (Taunt, Stoffels, & Purton, 2018), and provide advantages like encapsulation, also they could be easily stored in solid or liquid media (Dyo & Purton, 2018). A microalgal cell possesses three different genomes (nuclear, mitochondrial and chloroplast) which can be genetically modified, but the nucleus and the chloroplast are the most attractive for this procedure (Gong, Hu, Gao, Xu, & Gao, 2011).

C. reinhardtii is considered the model organism and has been used for many different research topics including photosynthesis and chloroplast physiology, given its capacity to grow under heterotrophic, autotrophic or mixotrophic conditions (Gong et al., 2011; Scaife et al., 2015; Scranton et al., 2016; Zhang et al., 2019). Moreover, it has also been exploited with engineering purposes for the production of biocommodities (Chen & Johns, 1996; Fields, Ostrand, & Mayfield, 2018; Kliphuis et al., 2012). For these procedures, biolistics is the most common transformation method, although agitation with glass beads may also be utilized; these techniques could be used for transforming either the nuclear or the chloroplast genomes (Scaife et al., 2015).

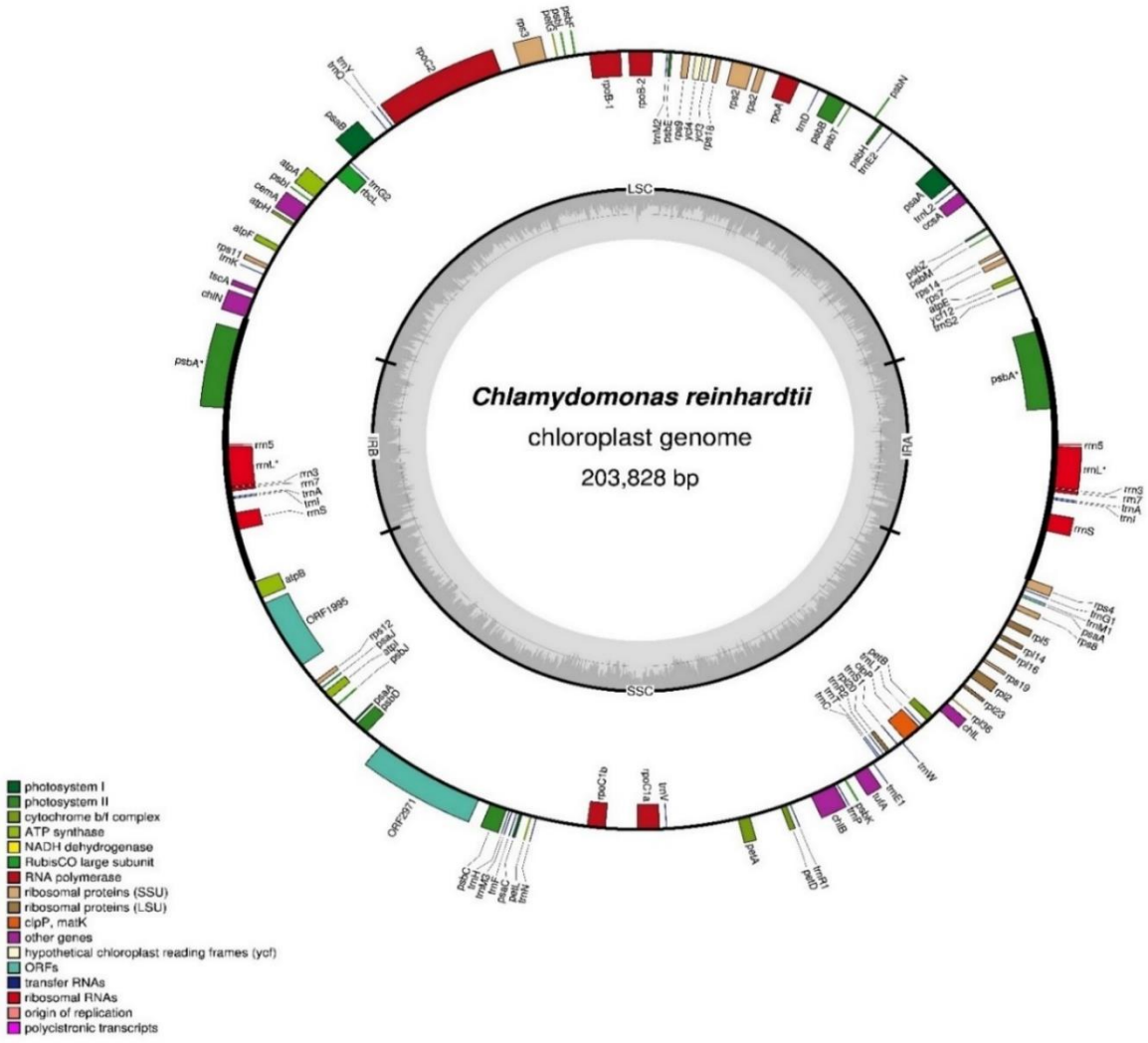

Figure 1: Map of the plastome of *C. reinhardtii*. Genes role are color-coded.

Nuclear engineering is widely used; however, it presents various limitations like random integration of transgenes as well as the existence of silencing mechanisms (Scaife et al., 2015). In contrast, transformation of the chloroplast genome (plastome) shows potential for producing recombinant proteins. The genome sequence is known, and the role of key genes have been identified (Figure 1) (Taunt et al., 2018). Moreover, the chloroplast expression machinery has both prokaryotic and eukaryotic features such as 70S ribosomes and post-transcriptional steps respectively (Dyo & Purton, 2018; Taunt et al., 2018). Furthermore, engineering the chloroplast can be achieved by exploiting the restoration of photoautotrophy, which helps avoid the use of selection methods based on antibiotic selection (Esland, Larrea-Alvarez, & Purton, 2018; Wannathong, Waterhouse, Young, Economou, & Purton, 2016). Expression of a single transgene

is relatively straightforward (Economou, Wannathong, Szaub, & Purton, 2014; Wannathong et al., 2016; Young & Purton, 2014b). However, a standardized method for multigenic engineering the chloroplast genome has not yet been put forward, despite some successful reports (Larrea-Alvarez & Purton, 2020). Moreover, there are only a few examples of successful methods for controlling transgene expression (Specht & Mayfield, 2013). Arguably, metabolic engineering of the plastome could be realized but an appropriate protocol has not been established yet.

Despite the aforementioned limitations, it is always important to suggest potential metabolites that could be produced in the plastid of *C. reinhardtii*. As previously stated, paclitaxel is a drug that required the joint action of various enzymes for its production. Part of the metabolic pathway is already encoded in the nucleus and proteins are targeted to the chloroplast (Lauersen, 2019). A recent report has shown that nuclear engineering of *C. reinhardtii* genome was successful in expressing part of the pathway. Nevertheless, some of the issues reported in this work could be circumvented by using chloroplast engineering. The present work shows a potential experimental scheme aimed at producing taxadien-5α-acetate-10β-ol from the plastome of *C. reinhardtii*.

1.1 Problem statement

Paclitaxel is a useful anticancer drug but current means of production have low yields. Instead, microbial platforms could be used; however, bacteria have limitations and yeast production is not having the efficiency expected. This study proposes an alternative to classic platforms by using microalgae to produce a precursor of the drug and exploiting the advantages of the chloroplast expression system. Expression of multiple genes will be required to integrate this pathway into the metabolic network of *C. reinhardtii.* This will also promote research towards using microalgal platforms for metabolic engineering which can be as resourceful as bacteria and yeast. Even though currently there are some limitations to transform this microorganism, novel tools are being developed that will allow large-scale production to supply demands for drugs like Paclitaxel.

1.2 Objectives

1.2.1 General Objective

Design a potential cloning strategy to express genes required to produce a paclitaxel precursor in the plastome of the green alga *Chlamydomonas reinhardtii.*

1.2.2 Specific Objectives

- Gather information about the drug, its metabolic pathway and previous reports that have attempted to produce it.
- Establish the pathway and genes required for precursor production.
- Design competent devices to clone the required genes into the plastome of *C. reinhardtii.*
- Design a transformation protocol for *C. reinhardtii* using state of the art techniques to ensure precursor production.
- Analyze the possible limitations to the designed scheme.

CHAPTER 2: PACLITAXEL

Paclitaxel (PTX) is a well-known anticancer drug with a complex molecular design (Figure 2). It is widely employed as a chemotherapeutic substance along with others such as cisplatin and doxorubicin (Sofias et al., 2017). It was first formulated as Taxol®, and received FDA endorsement in 1992, which has led to the development of independent formulation by various companies (Sofias et al., 2017). The most successful of these formulations has been Abraxane, which has yielded robust results regarding the functionality and toxicity of the drug (Sofias et al., 2017).

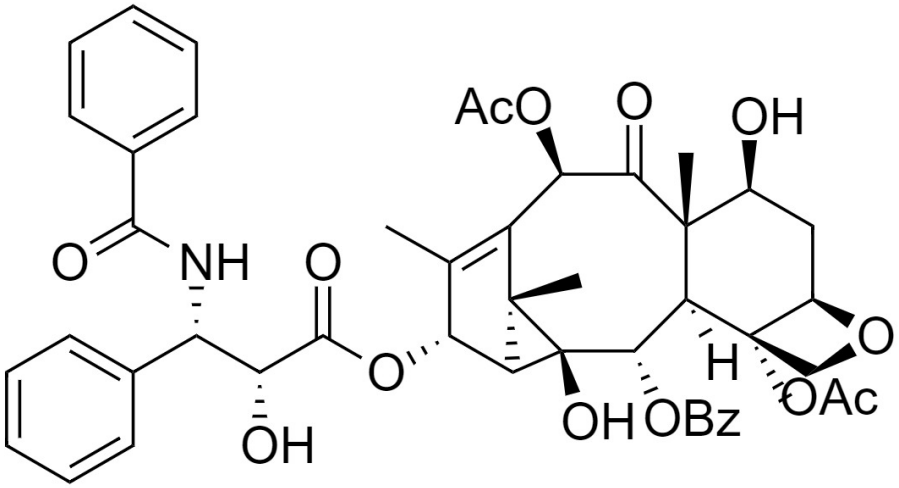

Figure 2: Illustration of the complex chemical structure of PTX. It is organized into a tetracyclic skeleton with seventeen atoms and a tail. The stereochemistry shows that the molecule has eleven stereocenters.

PTX acts in similar fashion to other chemotherapeutic compounds, it triggers apoptosis of the affected cell. This is achieved by disturbing the β subunit of tubulin proteins, which leads to a dysfunctional mitotic spindle (Bernabeu et al., 2017). This will provoke a G2/M phase arrest that kills the cell through caspase dependent or independent pathways (Sabzehzari et al., 2020). Such events are useful to treat tumor progression, even at advanced stages. Therefore, it has been applied in treatments of various cancers. Table 1 lists some types of cancer treated with this drug.

Table 1: Studies treating different forms of cancer using different PTX formulations.

Type of Cancer	Formulation	Phase trial	Reference
Breast Cancer	Nab-paclitaxel	3	(Untch et al., 2016)
Lung Cancer	Abraxane	2	(Green et al., 2006)
Ovarian Cancer	Nab-paclitaxel	2	(Teneriello et al., 2009)
Melanoma	Nab-paclitaxel in combination with carboplatin	2	(Kottschade et al., 2011)
Metastatic pancreatic adenocarcinoma	Nab-paclitaxel in combination with gemcitabine	3	(Von Hoff et al., 2013)

This drug is present in the bark of trees of the genus *Taxus*, but it is generally extracted from *T. brevifolia*. This due because this species shows the least variation in PTX content (Bernabeu et al., 2017). The classic method to extract the drug from the plant consists of fermenting the cells followed by refinement and isolation using chromatography and crystallization respectively (Bernabeu et al., 2017). The yields obtained with this process are generally scarce, which in turn limits the execution of clinical trials (Bernabeu et al., 2017; Sabzehzari et al., 2020). Moreover, a complete treatment cannot be executed from what a single tree produces (Bernabeu et al., 2017). To overcome this problem, PTX is nowadays being produced using a semisynthetic method (Bernabeu et al., 2017). The protocol consists of extracting a precursor in the ultimate steps of PTX production from needles of *T. brevifolia* for posterior laboratory synthesis (Bernabeu et al., 2017; Nazhand et al., 2020). Overall, this methodology can yield 1g of the drug per 3kg of needles (Bernabeu et al., 2017). Furthermore, this procedure is expensive and produces many toxic residues like the presence of osmium tetroxide (Liu, Gong, & Zhu, 2016). Hence, this method is not considered optimal for industrial purposes (Sabzehzari et al., 2020).

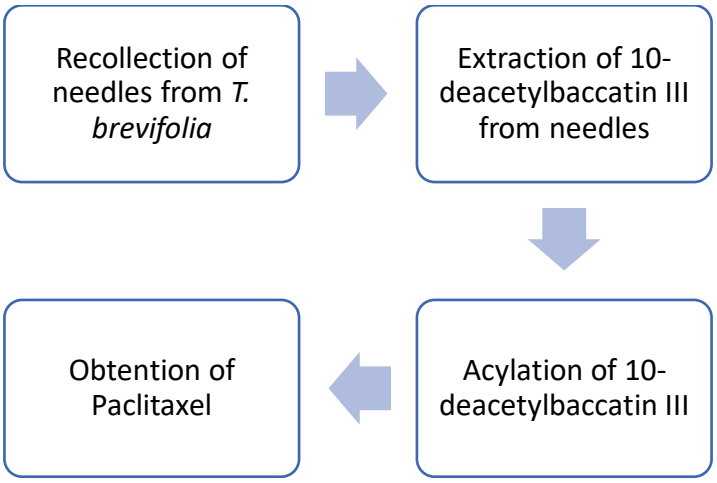

Figure 3: Semisynthetic production of paclitaxel from precursor found in needles of *T. brevifolia.*

Two metabolic pathways have been described which lead to the production of PTX: (i) the mevalonate (MVA) and (ii) the mevalonate-independent (MEP) pathways. These differ on the precursor compound (Kuzuyama & Seto, 2012; Nazhand et al., 2020), MVA begins with Acetyl-CoA while MEP resorts to glyceraldehyde 3-phosphate (G3P) and pyruvate (PYR). These two eventually converge at one point where isopentenyl diphosphate (IPP) is produced (Figure 4). Afterwards, the molecule becomes increasingly complex. Up until now, most of the pathway has been described as well as the molecular composition of the intermediaries. It has been identified that the MVA pathway occurs in the cytoplasm and the MEP pathway is located in the chloroplast of *Taxus* plant cells (Lauersen, 2019).

Due to the aforementioned limitations with synthetic production and natural yields, microorganisms are currently regarded as potential platforms for PTX synthesis, due principally to their high levels of recombinant protein production. Table 2 shows some examples of attempts at producing PTX precursors in such organisms. This approach appears advantageous since microbial production is considered more efficient than plant systems due mainly to its higher productivity per unit land area (Scaife et al., 2015).

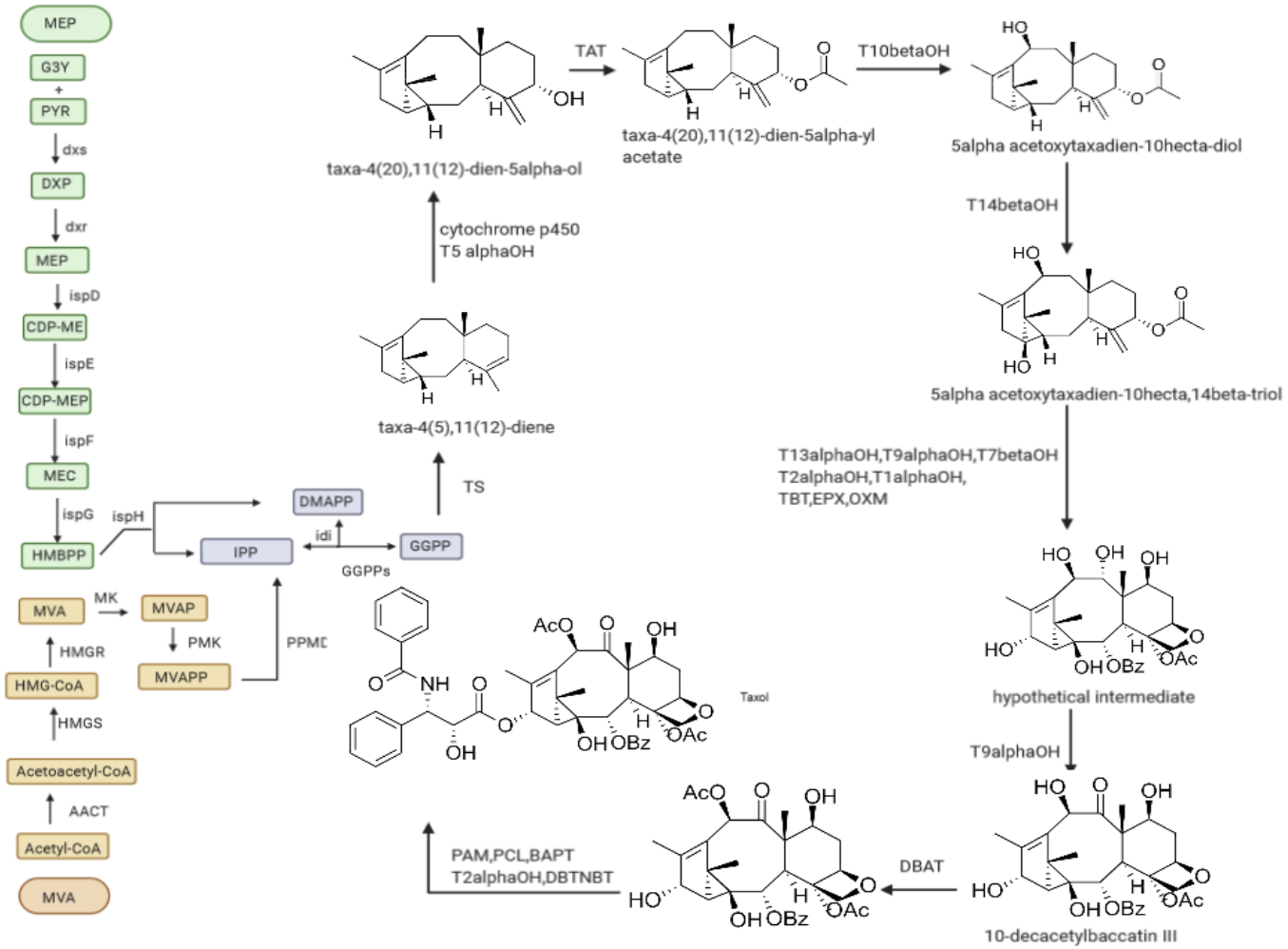

Figure 4: Metabolic Pathway of PTX. Two alternatives of the pathway are presented which may be the MEP or the MVA which differ on the precursors but converge at geranylgeranyl diphosphate (GGPP synthesis). Then, taxadiene is produced which is the most engineered precursor of the pathway. At this point, enzymes that require cP450 genes are required. Both the MEP and the MVA pathway are synthesized in plant cells but the latter is expressed in the cytoplasm while MEP is located in the chloroplast.

Table 2: Examples of metabolic engineering for the production of PTX precursors.

Host for heterologous expression	**Precursor produced**	**Reference**
B. subtilis	Taxadiene	(Abdallah et al., 2019)
N. benthamiana	Taxadiene	(Hasan et al., 2014)
A. thaliana	Taxadiene	(Besumbes et al., 2004)
E. coli	Taxadiene	(Ajikumar et al., 2010)
S. cerevisiae	Oxygenated taxadiene	(Zhou et al., 2015)

Considering the intricacy of the PTX pathways, a first milestone is the production of taxa-4,11-diene, also known as taxadiene. Such production has been achieved in traditional microbial platforms, including *E. coli* and *S. cerevisiae* (DeJong et al., 2006; Wang, Zada, Wei, & Kim, 2017). A potential limitation has been recognized at this stage, since genes of cP450 do not work well in prokaryotes because they lack the machinery to properly express, fold and attach such proteins to the cell membrane (Sabzehzari et al., 2020). To solve this problem, a report showed the production of oxygenated taxadiene using a coculture (Zhou et al., 2015). It consisted on making a coculture using *E. coli* and *S. cerevisiae* for the production of an oxygenated taxadiene. The experiment would need a continuous production of taxadiene by the bacteria while yeast would be transformed to perform the oxygenation of the precursor. Their results were fruitful since they were capable to establish that the gene used was certainly oxygenating taxadiene, therefore, advancing three steps further in the pathway. Nevertheless, their work using a coculture would involve more assays to optimize the variables to maximize the yield of the precursor. For example, inoculum of each participant in the culture, temperature or substrate.

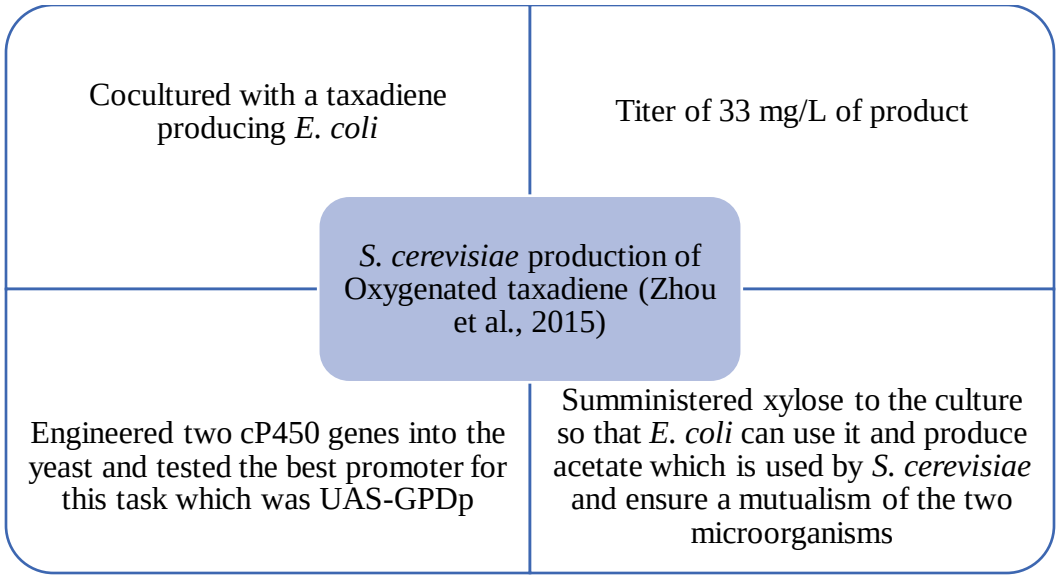

Figure 5: Summary of the results obtained from the experiment by (Zhou et al., 2015) to produce oxygenated taxadiene using *S. cerevisiae.*

In addition, other prokaryotes such as *B. subtillis* have recently been employed for PTX synthesis. In this study, precursor production demonstrated high titers (Abdallah et al., 2019). It included an overexpression of the MEP pathway genes in the bacteria, an enhancement in the production of intermediary GGPPS to further increase yield production and the gene *TXS* cloned into its genome. A detailed schematic of the MEP pathway used in this report is shown in figure 7.

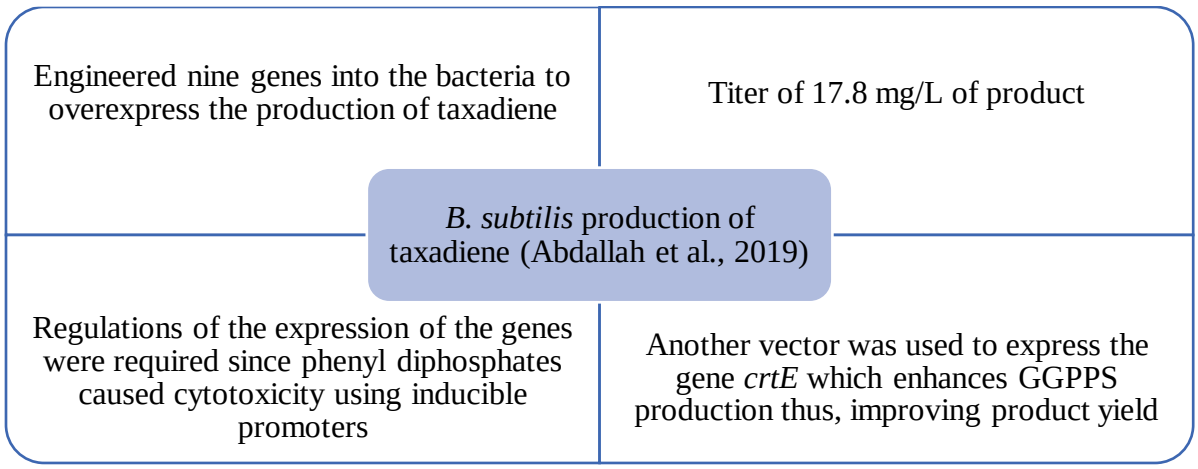

Figure 6: Summary of the results obtained from the experiment by (Abdallah et al., 2019) to produce taxadiene using *B. subtilis.*

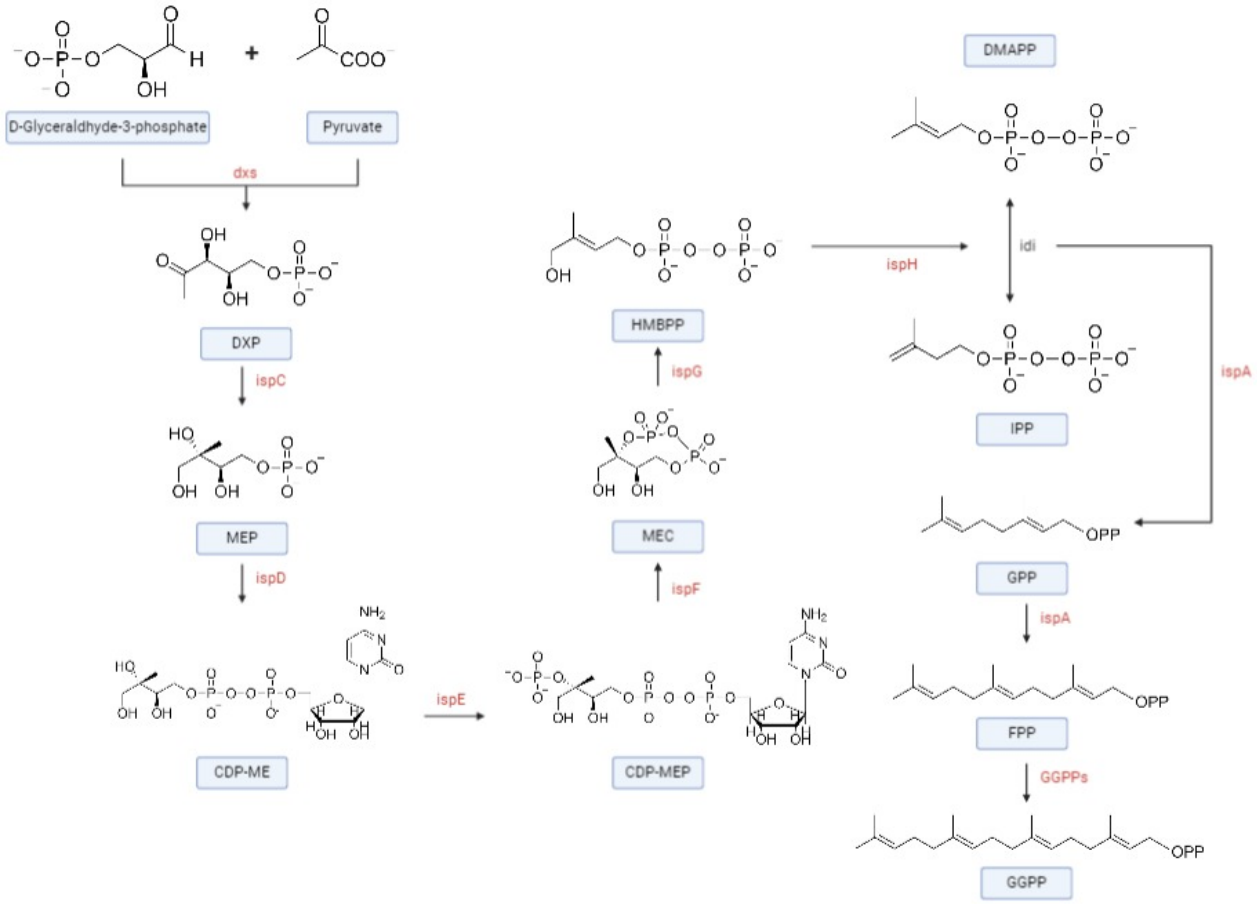

Figure 7: Detailed description of the MEP pathway which was employed for engineering of *B. subtilis* for taxadiene production. A total of nine genes (red colored) of the pathway had their promoters enhanced to induce overexpression which lead to increased GGPP yield. These genes are already a part of the nuclear genome of *B. subtilis*.

The use of model microorganisms for metabolic engineering may present many limitations. For prokaryotes like *B. subtilis* or *E. coli,* the pathway is stopped at the taxadiene oxygenation since these organisms cannot properly express the cP450 genes, which are essential for the remaining steps. Furthermore, promoters are needed to control expression of the pathway since phenyl diphosphates accumulate during the final steps of MEP causing cytotoxicity (Lauersen, 2019). This situation occurs equally for *S. cerevisiae,* although it is able to express cP450 genes thanks to its eukaryotic nature. However, microalgae have a natural advantage over these platforms since the MEP pathway is continuously expressed in their chloroplast thanks to its photosynthetic nature. This leads to an increased GGPP yield which can then be used for production of PTX precursors. This situation was taken advantage in a report by (Lauersen et al., 2018) by cloning

TXS in the nuclear genome of *C. reinhardtii.* This study proved that the precursor could be effectively produced in the alga by expressing the diterpene synthase in the nucleus and then directing it to the chloroplast using target peptides. Additionally, it has been reported that cP450 genes have successfully been expressed in the chloroplast and integrated into its membrane. Specifically, *C. reinhardtii* can follow the model proposed by (Zhou et al., 2015) to produce taxadien-5α-acetate-10β-ol with *S. cerevisiae* and even understand the limitations that occur when using a microalgae metabolic engineering platform. The alga readily possesses the MEP pathway which facilitates the engineering of the organism (Lauersen et al., 2018). Considering the background of heterologous expression in the chloroplast of microalgae and advances in transplastomics, microalgae are a potential resource to biopharmaceuticals production.

CHAPTER 3: MICROALGAE AS AN ENGINEERING PLATFORM

Engineering microbial platforms has gained popularity in recent decades. It offers various alternatives to traditional microorganisms considering their biodiverse cellular architecture, biosynthetic capacity and photoautotrophic growth which lets vast production of bio commodities, such as vaccines or drugs (Scaife et al., 2015). While bacteria and yeast have attracted significant attention, microalgae have emerged as a recently novel platform combining the benefits of bacterial and plant expression systems.

Microalgae is known for delivering greater efficiency considering unit land area and are considered a cheaper alternative for production of pigments or polymers (Scaife et al., 2015). Plants require considerable spaces for development, which implies an extensive use of fertilizers and great amounts of water (Scaife et al., 2015). In this type of production, the soil would require crop rotation, along with the control of run-offs from fertilizers. Additionally, many species of microalgae are known to adapt to diverse environments including soils, glaciers, oceans, lakes and rivers from different altitudes and diverse temperatures (Gupta, Lee, & Choi, 2015; Scaife et al., 2015). On the other hand, microalgae can also be cultured in bioreactors, which offers benefits such as aseptic conditions, along with the ability to manipulate temperature and resources so that biomass production could be enhanced. In particular, the cellular structure of these organisms allows for compartmentalization of chemical processes, which is arguably an advantage over prokaryotic platforms (Scaife et al., 2015).

Interest on microalgae arose mainly due to their use as feedstock for biodiesel production, although the manufacture of other bioactive compounds such as hormones, nutraceuticals and enzymes (e. g. proteases, lipases, laccases) are also attractive (Brasil, de Siqueira, Salum, Zanette, & Spier, 2017; Chisti, 2007; Scaife et al., 2015). Furthermore, studies in microalgae have produced other biocommodities like biohydrogen, vaccines and bioenergetic carriers (Scaife et al., 2015; Work et al., 2013). Although *C. reinhardtii* has been established as the model organism, other species have also been studied for genetic engineering like *C. vulgaris, Dunaliella salina* and *Haematococcus pluvialis* (Anila, Simon, Chandrashekar, Ravishankar, & Sarada, 2016; Norashikin, Loh, Aziz, & Cha, 2018; Scaife et al., 2015). Examples of recombinant expression include compounds derived from proteins such as enzymes, antibodies, antigens, hormones and immunotoxins (Taunt et al., 2018). Moreover, several microalgae species (e.g. *Chlorella,*

Chlamydomonas, Dunaliella or *Haematococcus*) have a Generally Recognized as Safe (GRAS) status which implicates that the recombinant biopharmaceuticals might not necessarily require purification (Taunt et al., 2018). This opens the possibility of direct oral consumption of recombinant vaccines or hormones (Dreesen, Hamri, & Fussenegger, 2010). Encapsulation of recombinant products, within microalgal cells and organelles, protects them from degradation from digestive processes (Dyo & Purton, 2018). Particularly in the case of vaccine manufacturing using, it has been conjectured that its biochemical contents could function as adjuvants (Rosales-Mendoza & Rosales-Mendoza, 2016). Nevertheless, genes encoding for adjuvants could also be engineered to enhance immune response (Yan, Fan, Chen, & Hu, 2016).

The storage of transgenic microalgae is considered relatively simple, given that cells can be maintained on agar or liquid cultures. Bioreactors and open systems can be used for scaling up cultures beyond laboratory levels; in such controlled environments, the most adequate conditions need to be set to optimize biomass yield, which include: temperature, light exposition, pH, substrate and mixing rate (Gupta et al., 2015).

A microalgal cell contains three different genomes, one in the nucleus, one in the chloroplast and one in the mitochondria. All genomes have been demonstrated to be transformable, although nuclear and chloroplast transformation are the most common (Gong et al., 2011). However, there are some key differences between these two. For instance, in nuclear transformation the Gene of Interest (GOI) is arbitrarily integrated, so numerous transformants need to be checked to detect transgenic lines (Scaife et al., 2015). Even modern editing technologies like CRISPR/Cas struggle at producing genetically stable transformants (Scaife et al., 2015; Zhang et al., 2019). Alternatively, chloroplast engineering has also been developed, although to a lesser extent. In this case, gene integration occurs at specific loci, and the recombinant product is maintained within the organelle (Taunt et al., 2018).

3.1 *Chlamydomonas reinhardtii* as a model organism

C. reinhardtii has been used to study diverse areas such as chloroplast physiology, photosynthetic machinery and genetic engineering (Gong et al., 2011; Scaife et al., 2015). Its morphology is characterized by two distinctive flagella and a large chloroplast that occupies a large portion of the cytoplasm (Figure 8) (Scaife et al., 2015). This microorganism is capable of reproducing either sexually or asexually depending on environmental conditions (Atif, Lima, Mirahmadi, & Wong,

2017). Its growth in bioreactors is diverse given its capacity to adapt to resource availability: (i) autotrophic which is achieved by culturing with light cycles and minimum media (Kliphuis et al., 2012), (ii) heterotrophic in contrast requires providing a carbon source such as acetate (Chen & Johns, 1996) and (iii) mixotrophic which combines the two aforementioned types of growth (Fields et al., 2018). Even anaerobic growth is possible for this species, since they are able to generate methane through co-digestion with carbon-rich by-products (Fernández-Rodríguez, de la Lama-Calvente, Jiménez-Rodríguez, Borja, & Rincón-Llorente, 2019). Considering the physiological characteristics mentioned above, this microorganism results appropriate for the production of various biocommodities. For such procedures, either the nuclear or the chloroplast genome can be engineered.

Figure 8: Schematic illustration of *C. reinhardtii*. Abbreviations: Chloroplast (C), flagella (F), Golgi apparatus (GA), mitochondria (M), nucleus (N), pyrenoid (P) and vacuole (V).

For nuclear transformation, the Gene Of Interest (GOI) needs to be cloned accompanied by a promoter/5'UTR, selectable marker, a 3'UTR and target peptides in case the encoded protein requires translocation to organelles. For *C. reinhardtii* nuclear transformation, various methods

are utilized: biolistics, agitation with glass beads, *A. tumefaciens*-mediated cloning or electroporation (Scaife et al., 2015). Biolistics remains the most used technique, despite its costs and difficulty (Scaife et al., 2015). Furthermore, modern technologies for genome transformation like CRISPR-CAS9 have obtained very low efficiencies in experiments to transform the nucleus of this alga and require further studies for optimization (Scaife et al., 2015; Zhang et al., 2019). However, new biosynthetic tools are being developed to enhance nuclear expression like designing synthetic promoters (Scranton et al., 2016). Additionally, even when new promoters, riboswitches and reporter genes are presented, limitations are problematic in this field. Metabolic engineering of the nucleus is further away from being attractive considering the arbitrary integration of the sequences and gene silencing mechanisms it possesses (Scaife et al., 2015). Table 3 shows some examples of nuclear engineering of this microalga to produce different proteins.

Table 3: Examples of recombinant proteins expressed through nuclear engineering of *C. reinhardtii*.

Method for Transformation	**Promoter(s)***	**Marker**	**Gene Expressed and Origin**	**Protein**	**Reference**
Agitation with glass beads	*HSP70A/RBCS2*	*ARG7*	*crEpo* *Homo sapiens*	Erythropoietin	(Eichler-Stahlberg, Weisheit, Ruecker, & Heitzer, 2009)
Agitation with glass beads	*PSAD*	*aphVIII*	*P24* Human immunodeficiency virus	HIV-1 viral particles subunit	(Barahimipour, Neupert, & Bock, 2016)
Electroporation	*HSP70A/RBCS2*	*aph7/sh-ble*	*xyn1* *Trichoderma reesei*	β-1,4-endoxylanase	(Rasala et al., 2012)

*The most used promoter is *RBCS2*, involved in RuBisCo small subunit expression and is fused to *HSP70A* (heat shock protein) which makes expression inducible under light. The *PSAD* promoter induces expression of the docking structure for PSI.

Chloroplast engineering of *C. reinhardtii* has shown promising results for heterologous expression. This species possesses a single chloroplast that occupies most of the cytoplasm (Taunt et al., 2018) (Figure 8). The chloroplast genome (plastome) is smaller and simpler than the nuclear genome, it contains most of the genes encoding for photosynthetic proteins, transcription and translation machineries of the organelle (Taunt et al., 2018). Gene expression shows some prokaryotic characteristics such as expression through operons and translation using 70s ribosomes (Taunt et al., 2018). Yet, some post-transcriptional steps are similar to those found in eukaryotes, which include RNA splicing, stabilization and translation induction (Dyo & Purton, 2018).

Biolistics is the most reliable methods for transplastomic engineering (Purton, Szaub, Wannathong, Young, & Economou, 2013). It consists on shooting DNA-coated gold microparticles with a specialized gun to a lawn of cells (Esland et al., 2018). Alternatively, effective transformations have been achieved using agitation with glass beads, which requires the use of cell wall deficient mutants, although the cell wall can be removed by chemical processes (Purton et al., 2013).

A typical chloroplast transformation vector is schematized in figure 9. These plasmids are assembled using traditional cloning methods (digestion/ligation); nonetheless more sophisticated techniques such as Golden Gate or Gibson assembly are currently being adapted to assemble such plasmids (Scaife et al., 2015). These vectors must contain the following elements: the left arm, promoter/5'UTR, selectable marker, 3'UTR, promoter/5'UTR, GOI, 3'UTR and a right flank. The left and right flank are used for homologous recombination between the construct and the plastome. The promoter, which is typically constitutive, induce expression of the GOI (Scaife et al., 2015). The 5' UTRs allow appropriate ribosome binding to trigger translation, while 3'UTRs provide mRNA stability and avoid mRNA degradation by enzymes (Franklin, Anderson, & Coragliotti, 2014; Scaife et al., 2015; Wannathong et al., 2016). The GOI requires codon optimization since the plastome has a bias regarding A-T content (Esland et al., 2018). Lastly, the selectable marker is required to identify transformed cell lines; the most common markers are antibiotic resistance genes (Figure 10B) (Esland et al., 2018). For example, the gene *aadA* confers resistance to aminoglycosides like spectinomycin and streptomycin that disrupt protein synthesis in microorganisms by inactivating such antibiotics (Kehrenberg, Catry, Haesebrouck, De Kruif, & Schwarz, 2005; Scaife et al., 2015).

Marker expression is not required after successful integration. So, its expression could be considered as a burden when no selection is required. (Esland et al., 2018) have suggested a potential method to remove these markers. It consists on using repetitions to flank the marker, which would be excised through homologous recombination after plating in non-selective medium. However, this technique is still at development and requires further testing. Alternatively, photosynthetic markers are also available. This approach relies on the ability of *C. reinhardtii* to grow heterotrophically, so that photosynthetic genes (e.g. *psbH*) could be disturbed (Figure 10A). The process utilizes mutants that are unable to carry out photosynthesis. The selectable marker confers phototrophy, as it consists of the correct version of the mutated photosynthetic gene (e.g. *psbH*). An obvious advantage is that the cell does not carry any antibiotic resistance genes (Esland et al., 2018; Wannathong et al., 2016). In addition, reporter genes are also available for chloroplast engineering (Scaife et al., 2015). Tables 4 and 5 summarize the different elements available to include in the transformant plasmid.

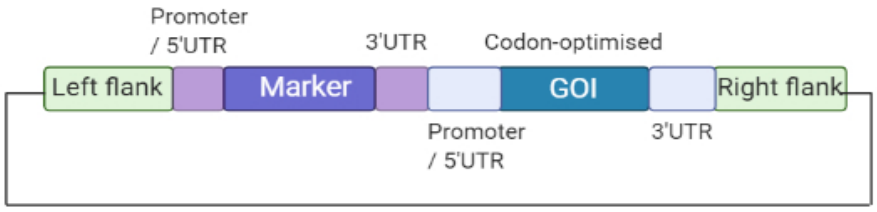

Figure 9: Classical design of a transformant plasmid using a selectable marker. It contains the flanks that will go through homologous recombination with the genome of the recipient cell, a selectable marker which will help detect transformant strains and a codon-optimised GOI.

Table 4: Markers commonly used for chloroplast engineering with their respective genotype and classified by their basis.

BASIS OF THE GENE	GENE	PHENOTYPE	REFERENCE
ANTIBIOTIC RESISTANCE	*aadA*	Resistance to spectinomycin and streptomycin	(Goldschmidt-clermont, 1991)

	aphA6	Resistance to amikacin and kanamycin	(Bateman & Purton, 2000)
ENDOGENOUS FOR ANTIBIOTIC RESISTANCE	Point mutations *rrnL* and *rrnS*	Resistance to erythromycin, spectinomycin and streptomycin	(Newman SM et al., 1990)
ENDOGENOUS	Photosynthetic genes (*atpB, petB, psaA, psbB, psbH, rbcL* and *tscA)*	Confers phototrophy	(Wannathong et al., 2016)
	ARG9	Confers arginine in an *arg9* mutant	(Remade et al., 2009)

Table 5: Elements available for plasmid assembly and transplastomics procedures of *C. reinhardtii.*

TYPE	NAME(S)	DESCRIPTION	REFERENCE
PROMOTER/5' UTR	*atpA, psbA, psbD, psaA-Exon 1, petB*	Constitutive expression. They are derived from ATP synthase α subunit, photosystems I and II and cytochrome gene expression.	(Scaife et al., 2015; Yan et al., 2016)
3'UTR	*rbcL*	Protection for the mRNA against degradation. It is derived from the large chain of RuBisCo	(Yan et al., 2016)
HOMOLOGOUS RECOMBINATION SITES	*p322, atpB-int, pLM7, p72B, p71*	Sequences destined for integration	(Scaife et al., 2015)

REPORTER GENE	*ptxD*	Capability to use phosphite	(Sandoval-Vargas et al., 2019)
	codA	5-fluorocytosine sensitivity	(Young & Purton, 2014a)

For plasmid assembly, two alternatives can be used: (i) restriction enzymes or (ii) "one-step" assembly. The restriction enzyme approach comprises using endonucleases that cut the plasmid at restriction sites which are then bound again using ligases (Raymond, Pownder, & Sexson, 1999). However, it is considered time consuming and requires compatibility for the restriction sites (Raymond et al., 1999). In contrast, "one-step" methods do not rely on compatibility and require less time to be executed. Gibson assembly, for example, involves an exonuclease that produces overhangs which allow insertion of the cassette through homologous recombination (Benoit et al., 2016). Afterwards, a polymerase closes the gap and a ligase seals shut the plasmid. Golden Gate on the other hand, uses type IIS restriction endonucleases which create compatible customizable overhangs between the vector and the insert (Yan et al., 2016). Both of these approaches do not leave scars between genetic elements and only require one reaction. Nevertheless, Gibson is stable only for sequences larger than 200bp (Benoit et al., 2016) or the exonucleases may eliminate part of the transcriptional unit and Golden Gate requires confirmation that IIS sites are not present anywhere else in the genome to avoid other cleavages from the endonuclease (Yan et al., 2016).

Table 6: Examples of recombinant proteins expressed through chloroplast engineering of *C. reinhardtii*.

Method for Transformation	**Promoter**	**Marker**	**Gene(s) expressed**	**Protein**	**Reference**
Biolistics	*psbA*	*aphA6*	*VEGF*	Vascular endothelial growth factor	(Rasala et al., 2010)
Biolistics	*psbA*	*aphA6*	*HMGB1*	High mobility group box 1	(Rasala et al., 2010)

Biolistics	*atpA*	*aadA*	*E2*	Structural protein of swine fever virus	(He et al., 2007)
Agitation with glass beads	*psbD*	*aadA*	*E7GGG*	E7 oncoprotein	(Demurtas et al., 2013)
Biolistics	*psbA*	*aphA6*	*14FN3*	Human fibronectin	(Rasala et al., 2010)
Agitation with glass beads	*psbA* *psaA* *chlL*	*aadA* *psbH*	*hGH*	Human growth hormone	(Wannathong et al., 2016)
Agitation with glass beads	*atpA*	*psbH*	*TPS4*	*Cis*-abeniol synthase	(Zedler, Gangl, Hamberger, Purton, & Robinson, 2015)
Agitation with glass beads	*psbD*	*aadA*	*Co-CcCls*	Copal-8-ol diphosphate synthase	(Papaefthimiou et al., 2019)

(Economou et al., 2014) have described a method that make use of a phototrophic selectable marker. It begins by culturing the alga in Tris-Acetate-Phosphate (TAP) solid medium which is later used to inoculate TAP liquid medium. Then, the culture is grown on light conditions for two or three days to prepare them for transformation. Afterwards, the culture is agitated with glass beads and plated in minimal medium for selection. Finally, PCR is used to check if the colonies have reached a homoplasmic state-i. e., all copies of the plastome in the cell carry the GOI (Wannathong et al., 2016). This method has been used for the expression of various enzymes and the development of genetic tools (Charoonnart, Purton, & Saksmerprome, 2018; Charoonnart et al., 2019; Larrea-Alvarez & Purton, 2020; Sandoval-Vargas et al., 2019).

While genetic engineering involves the manipulation of the genome of an organism for production of a recombinant protein, metabolic engineering gives a purpose to such

transformations. This field studies the possible modifications that can be performed on the metabolic network of an organism to produce a chosen metabolite (e. g. drugs or bioactive compounds) through recombinant DNA technology (Yang, Bennett, & San, 1998). Thus, it has been attractive for the synthesis of different compounds like drugs, polymers and multi sub-unit proteins (Yang et al., 1998). Despite that metabolic engineering is not yet fully explored in the chloroplast (Gimpel, Henríquez, & Mayfield, 2015), some developments are underway. Examples of this are attempts at multigenic engineering of the plastome (Larrea-Alvarez & Purton, 2020) or discovery of innovative regulatory elements for the mRNA (Specht & Mayfield, 2013)

The first bottleneck for achieving transplastomic metabolic engineering is the expression of multiple transgenes at same time, although some examples are available, a standardized methodology has not yet been put forward. The second issue is the regulation of transgene expression, despite that some techniques have shown positive results, they are still far from being useful for such a purpose.

These difficulties do not yet permit to engineer entire pathways in the organelle. However, it seems crucial to rationally design potential pathways that could be targeted to the organelle. First, the plausibility of expressing related enzymes and substrates must be assessed. Then, a minimal number of genes must be determined for expression of a functional metabolite. Finally, a tentative methodology must be designed to incorporate the selected genes.

An example of successful metabolic engineering is the production of anticancer drugs in microbial platforms. In particular, paclitaxel precursors have been produced in *E. coli, S. cerevisae*, and *B. subtilis*. Moreover, recently it has been shown that some key precursors of this drug have been produced in *C. reinhardtii* using nuclear transformation. Despite being successful to some extent there are difficulties with this methodology, including the aforementioned inherent problems with nuclear transformation, target peptides must be added to direct the enzymes into the chloroplast, which adds a layer of complexity. Chloroplast engineering, on the other hand, appears attractive for such a purpose. As previously mentioned, transformation is relatively easy to perform, stable expression is achieved, and a wide variety of synthetic tools are available. Moreover, multigenic engineering (Larrea-Alvarez & Purton, 2020), and some mechanisms to control transgene expression are known to work successfully (Purton et al., 2013; Scaife et al.,

2015). Therefore, transplastomic engineering might ease up the expression of the enzymes required to produce paclitaxel precursors.

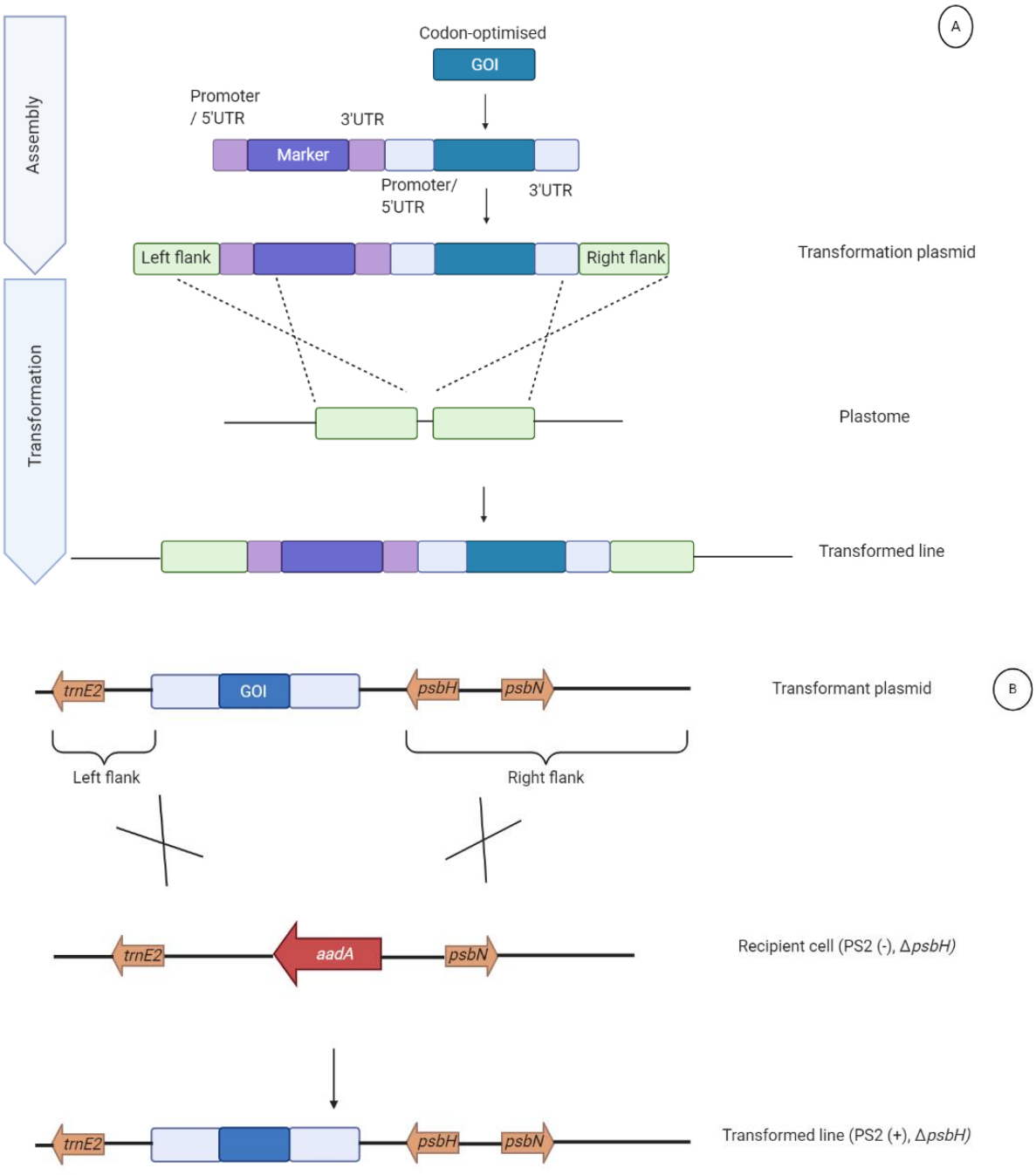

Figure 10: Usual design for chloroplast engineering. A) Utilizing a marker based on antibiotic resistance and B) Cell line with an inactivated photosystem II given the lack of the *psbH* gene, restoring said gene stabilizes the photosystem and allows photoautototrophy.

CHAPTER 4: ENGINEERING THE PLASTOME FOR PACLITAXEL PRECURSOR PRODUCTION

For engineering purposes, a set of elements and procedures must be defined. First, considering that the plastome of *C. reinhardtii* contains the genes required to express the MEP pathway, five additional sequences would be required to produce an oxygenated taxadiene. This compound is the furthest that has been reached regarding PTX production (Zhou et al., 2015). The pathway that would be used for this experiment is illustrated in figure 11. Five coding sequences are needed (Table 7) which include three cP450 genes. Expression of these genes is limited in prokaryotic organisms, but it has been proven that such proteins are able to correctly express, fold and integrate into the thylakoid membrane (Gangl et al., 2015). Thus, this chapter aims at discussing potential strategies that could be useful for integrating the genes required for taxadien-5α-acetate-10β-ol synthesis in the chloroplast of C. reinhardtii. The devices for transgene integration and expression, selection methods and recombinant protein detection will be discussed in detail.

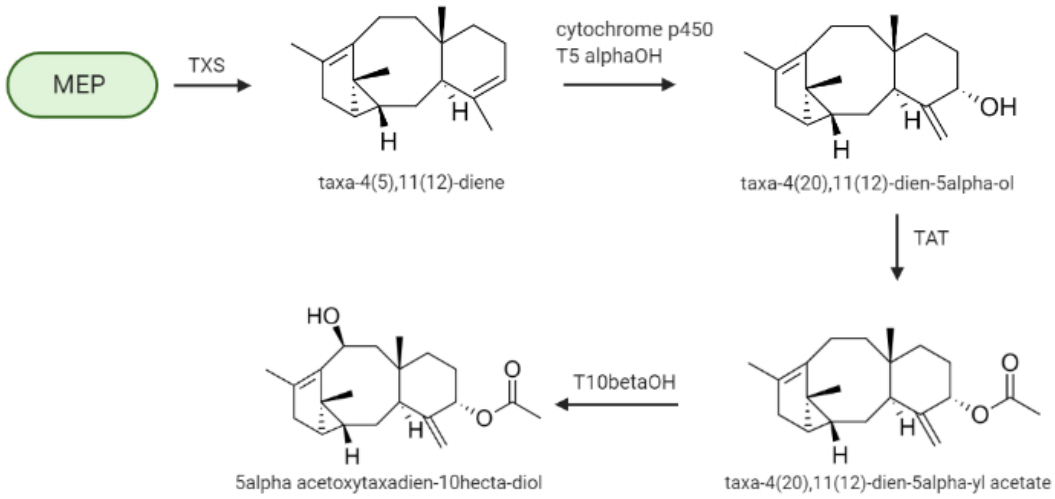

Figure 11: Pathway to be engineered into the plastome of *C. reinahrdtii.* At the end of the MEP pathway in the plastid, GGPP is produced which then forms taxadiene (taxa-4(5),11(12)-diene) using TXS. Lastly, using cP450 enzymes along with TAT, taxadien-5α-acetate-10β-ol (5alpha acetoxytaxadien-10hecta-diol) is made.

4.2 Cloning strategy with Plasmids T5α and T10β

A serial transformation approach would be the most appropriate since at least five genes need to be inserted, such coding sequences are quite large (around 11 kb) which renders transformation with a single plasmid very unlikely (Table 7). For this reason, two sites for insertion should be used: (i) the intergenic region between *trnE2* and *psbN* and (ii) within the *chlL* coding sequence (Figure 12), a gene involved in the production of chlorophyll in a light-independent manner (Wannathong et al., 2016).

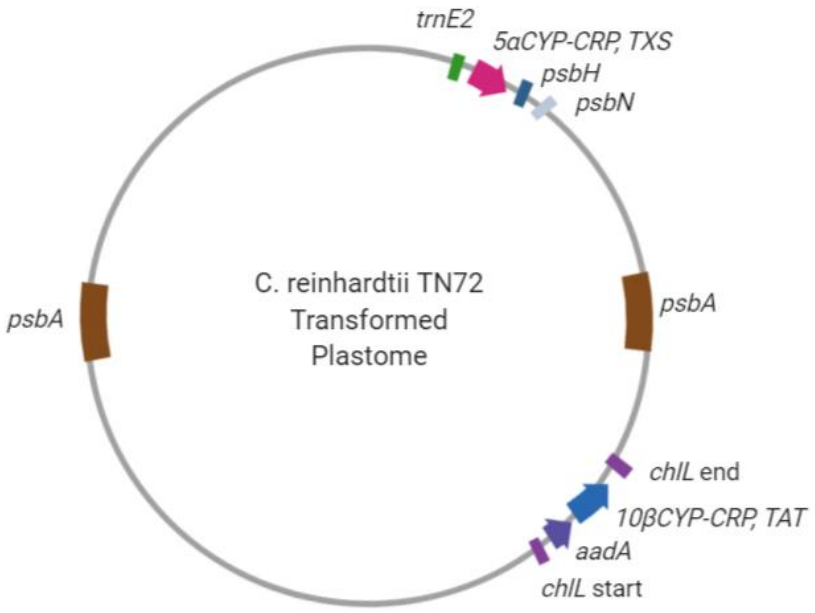

Figure 12: Sites for homologous recombination targeted for a serial transformation strategy for introducing the genes needded for taxadien-5α-acetate-10β-ol production inthe plastome of *C. reinhardtii*. It is noticeable that the inserted transcriptional units will be recombine at sites vastly separated, which reduced the likelihood of recombination between *5αCYP-CRP* and *10βCYP-CRP*.

The genes required to manufacture taxadien-5α-acetate-10β-ol need to be codon-optimized. Many softwares are available for such task, including the widely used Codon Usage Optimizer (http://codonusageoptimizer.org/download/). The codon-optimized sequences of the various genes of interest are shown in Table S1.

Table 7: Genes required for taxadien-5α-acetate-10β-ol production from GGPP at the end of the MEP or the MVA pathway.

Gene name	Description	Encoded Protein Molecular Weight	Protein Accession Number
TXS	Forms a tricyclic compound and separates the two phosphate groups from GGPP	98.32 kDa	Q41594.1
5αCYP	A monooxygenase that catalyzes the first oxygenation step in the pathway	56.57 kDa	Q6WG30.2
CRP	NADPH reductase required after the activity of a CYP enzyme	79.78 kDa	AAT76449.1
TAT	Uses an acetyl-CoA to add an acetyl group to taxadiene	49.09 kDa	Q9M6F0.1
10βCYP	Catalyzes the second oxygenation step in the pathway	56.70 kDa	AFD32419.1

Since the TN72 strain lacks the means to grow in phototrophic conditions, it needs to be cultured in media supplemented with acetate as a carbon source, generally Tris-Acetate-Phosphate (TAP) (Economou et al., 2014). To prepare the cells for transformation, the cells have to be re-streaked repeatedly in solid TAP medium over the course of a week to induce a healthy state. Then, some colonies are used to inoculate liquid media, which must be grown for two to three days under light conditions (20-50 $\mu E/m^2/s$ PAR) (Economou et al., 2014). Then, this process is repeated, but

this time cells are grown until a concentration of 1 or $2x10^6$ cells per ml is reached. Lastly, cells are concentrated in fresh TAP medium to approximately $2x10^8$ cells per milliliter. 3 ml of this culture must be agitated for 15 s with the plasmid TT5α10β and then poured on solid High Salt Minimum (HSM) medium. To avoid phototactic migration of the cells, the plates have to be covered from light for twenty minutes (Economou et al., 2014). Afterwards, the plates have to be incubated at 25 °C under light conditions (50 μE/m^2/s PAR) (Economou et al., 2014). Successful transformants will be able to form green colonies on the agar. This process can be visualized in Figure 13A. Besides, to assure homoplasmicity successive re-streaks should be carried out for approximately 2-3 weeks (Esland et al., 2018). PCR would be used to confirmed such state, and positive colonies would be subjected to Western blot analysis to confirm recombinant protein production. The strain expressing the first group of GOIs (5*αCYP-CRP* and *TXS* genes) ought to be named Cr5α.

The second round of transformation will make the use of Crα5 as a recipient strain. The procedure would be the same as previously described, although selection would be performed in TAP media supplemented with spectinomycin (Figure 13B). Selection must be carried out for two weeks until homoplasmy is achieved. PCR and Western blot would confirm the integration and expression of the GOIs. This resulting strain should be named Cr5α10β, as it should be able to produce the *10βCYP-CRP* and TAT enzymes.

To perform Western blotting, crude extracts from transformed cells would be properly prepared by sonication and addition of a lysis buffer (Zedler et al., 2015); then, this extract is translocated to a 15% acrylamide SDS-PAGE gel, which should be run for 90 minutes with 150 V (Young & Purton, 2014a). Afterwards, the proteins are transferred to a nitrocellulose membrane (Wannathong et al., 2016), using a current of 20V for an hour. Membranes should be then be incubated with respective antibodies against HA and FLAG epitopes which would be attached to the genes that shall be cloned (*TXS* and *5αCYP-CRP* for HA whereas *TAT* and *10βCYP-CRP* are bound to FLAG). Expression of *rbcL* would be a fit loading control since it is expressed ubiquitously in the alga; it is part of RuBisCo which is required for carbon fixation (Johnson, 2011).

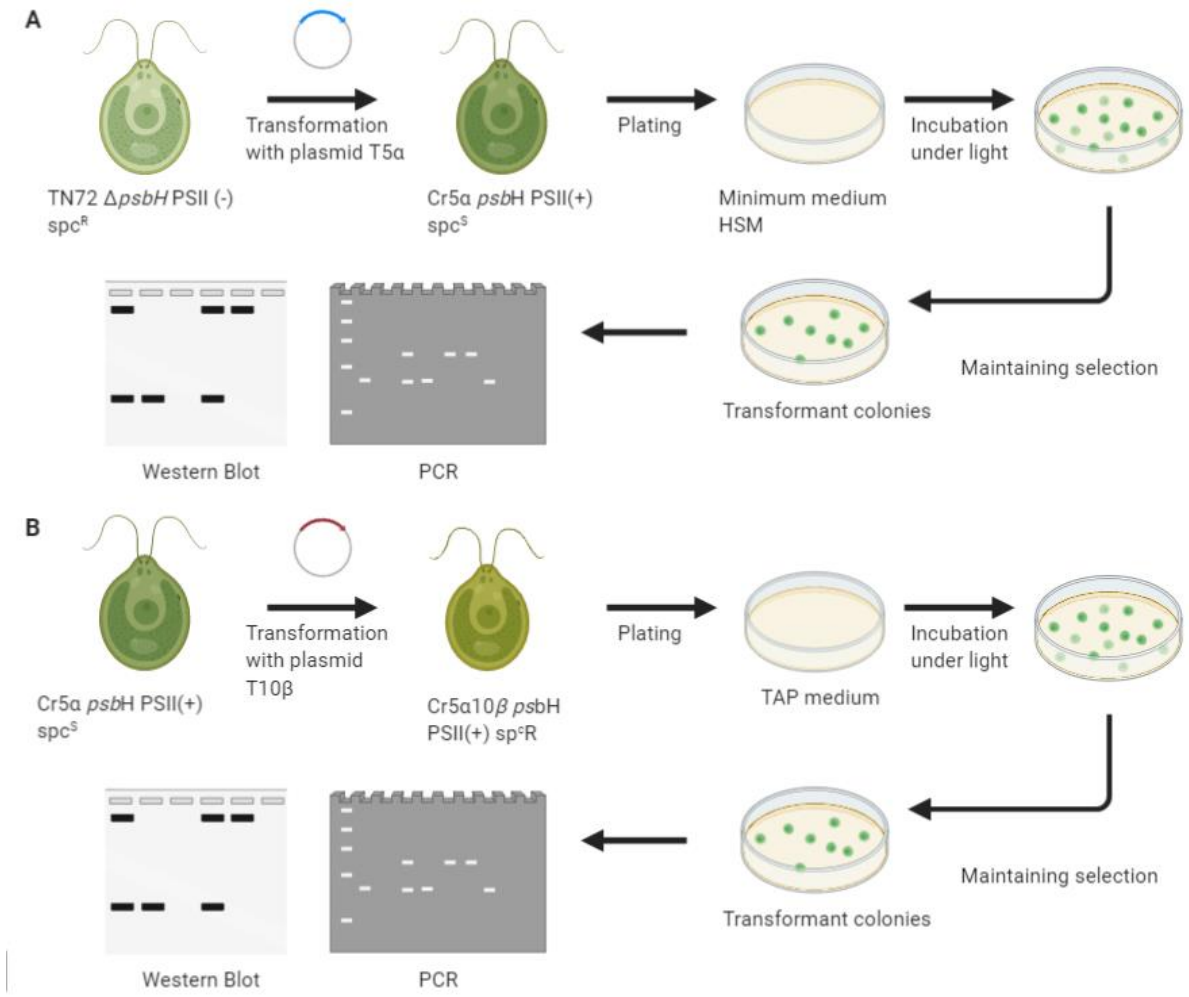

Figure 13: Cloning strategy to engineer the four genes required for producing taxadien-5α-acetate-10β-ol in the chloroplast of *C. reinhardtii*. A) Transformation using plasmid T5α which confers autotrophy by restoring the gene *psbH* but excises the gene *aadA* causing sensitivity to spectinomycin, thus generating strain Cr5α. B) Transformation of the latter strain with plasmid T10β, which confers spectinomycin resistance. The resulting strain would express all enzymes necessary for taxadien-5α-acetate-10β-ol production.

4.3 Devices for Cloning the GOIs

The first plasmid (T5α) would be based on the pSRSapl vector (Economou et al., 2014). It has been shown that expression of cP450 genes (*5αCYP-CRP, 10βCYP-CRP)* does not require targeting techniques to locate the encoded protein into the thylakoid membrane (Gangl et al., 2015). Furthermore, the GOIs can be separated using a Ribosome Binding Site (RBS), known to drive translation and simplify the assemblage by arranging transcriptional units with two genes

and a single promoter (Carrizalez-López et al., 2018). Regarding 3'UTRs, two versions of the *rbcL* (a long of 407bp and a short one of 258bp) should be employed to avoid homologous recombination between them (Larrea-Alvarez & Purton, 2020). Considering that the *5αCYP-CRP* and *10βCYP-CRP* encode proteins of similar weight, two different epitopes for immunoblotting should be used. For this plasmid, the Human Influenza Hemagglutinin (HA) tag would be fused at the 3' end of *5αCYP-CRP* and *TXS*. The *psaA-1* promoter/5' UTR drives the constitutive expression of subunit A from photosystem I (Michelet, Lefebvre-Legendre, Burr, Rochaix, & Goldschmidt-Clermont, 2011). The site for homologous recombination must be the intergenic region between *trnE2* and *psbN*. Figure 14 illustrates the assembled design of T5α.

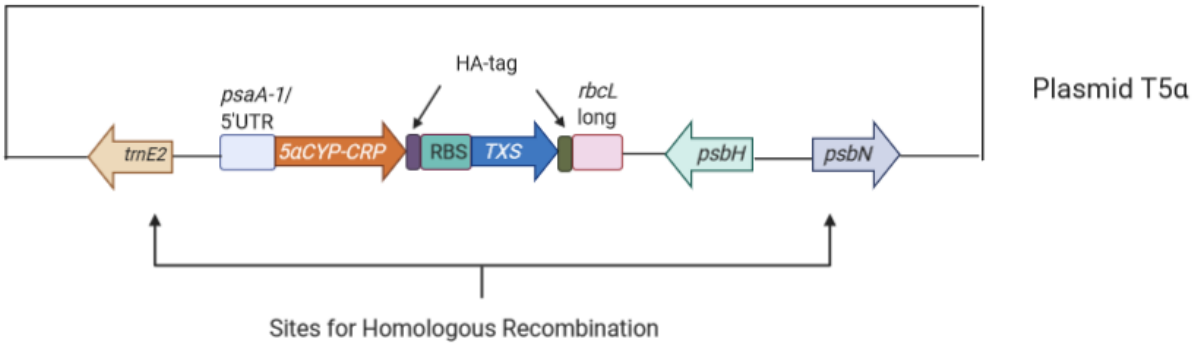

Figure 14: Design of plasmid T5α which carries the genes *5αCYP-CRP* and *TXS*. The psbH gene allows restoration of phototrophy.

The second vector, T10β, should be based upon the pAP plasmid because it targets transgenes to the *chlL* gene (Larrea-Alvarez & Purton, 2020). This site is appropriate for insertion since this gene is not required in cells that are grown under constant light. T10β would require two transcriptional units: one for expression of the marker *aadA* and another for expression of the GOIs. For the *aadA* cassette, expression must be driven by a constitutive promoter such as *atpA*, which drives expression of alpha subunit of the ATP synthase (Scaife et al., 2015). The transcriptional unit of *10βCYP-CRP* and *TAT* would use the *petB/* 5' UTR to drive expression, which is known to produce high levels of protein as it promotes expression of the beta subunit of the cytochrome b6f (Heinnickel et al., 2013). Both GOIs must have a DYKDDDDK epitope, commercially known as FLAG-tag, attached to their 3' ends, which is a synthetic epitope designed specifically for protein localization. Figure 15 illustrates the final assembly of T10β.

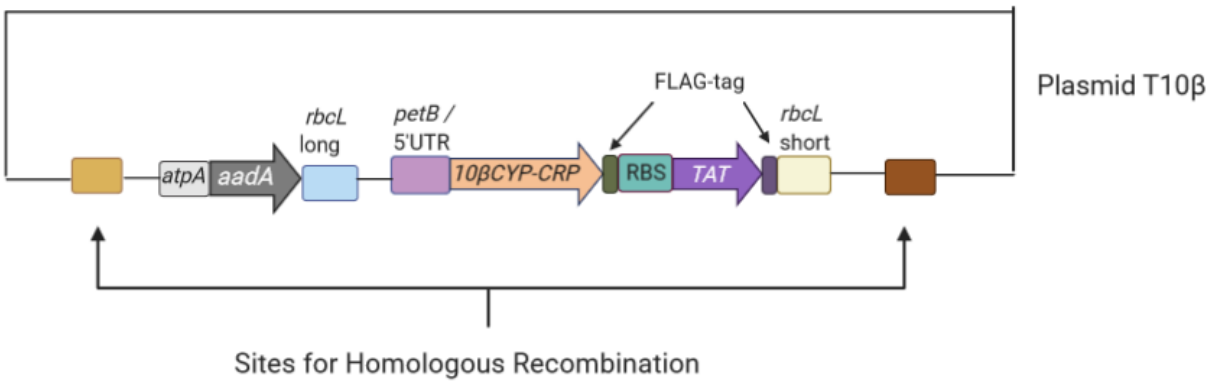

Figure 15: Design of plasmid T10β which carries two transcriptional units: one for the *aadA* marker and another for expression of *10βCYP-CRP* and *TAT*. The left and right flanks of the plasmid allow recombination in the gene *chlL* of Cr5α.

Both plasmids can be assembled by traditional cloning using restriction enzymes. However, it is recommendable to employ methods based on Golden Gate assembly, which allows "one-pot" plasmid construction (Taunt et al., 2018) (Figure 16). Once plasmids are assembled *in vitro*, they should be inserted into *E. coli* DH5α for producing enough copies for chloroplast transformation.

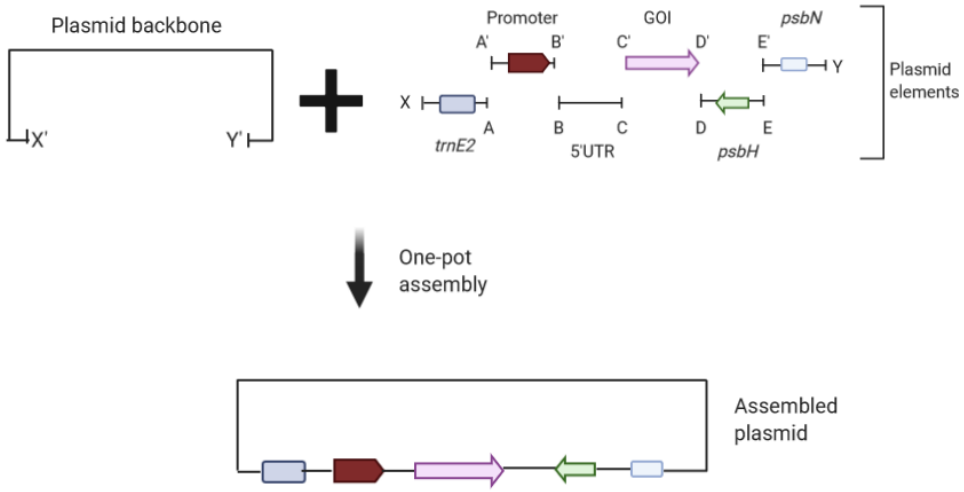

Figure 16: An example of plasmid assembly through Golden Gate which carries the *psbH* gene to rescue autotrophy in the recipient microalgal cell. Elements are designed so that their extremes overlap so that when added in a single tube and a ligase, the plasmid is correctly assembled (i. e.

one- pot assembly). The left and right flanks have an X and Y ends respectively that allow insertion in the plasmid.

4.4 Determination of transformant lines and expression of recombinant proteins

The Cr5α strain would contain the genes *5αCYP-CRP* and *TXS* in the intergenic region between tnrE2 and psbN (Figure 17A). To determine if the synthetic transcriptional unit is correctly integrated, a PCR reaction is required (Figure 17B). A set of three primers would be required (F1, R1 and R2) which will allow to discern homoplasmic and heteroplasmic results. Primers F1 and R1 would produce an amplicon of 0.8kb and be designed upon the original TN72 strain whereas F1 and R2 amplify a greater band at 1.2kb, thus showing introduction of the insert from T5α.

A Western Blot will allow to visualize the expression of the desired proteins as well as how much they are being produced by using antibodies to the HA-tag (Figure 17C). Two bands should be visible for a successful transformant, a lower band for TXS (98 kDa) and an upper one for 5αCYP-CRP (136kDa).

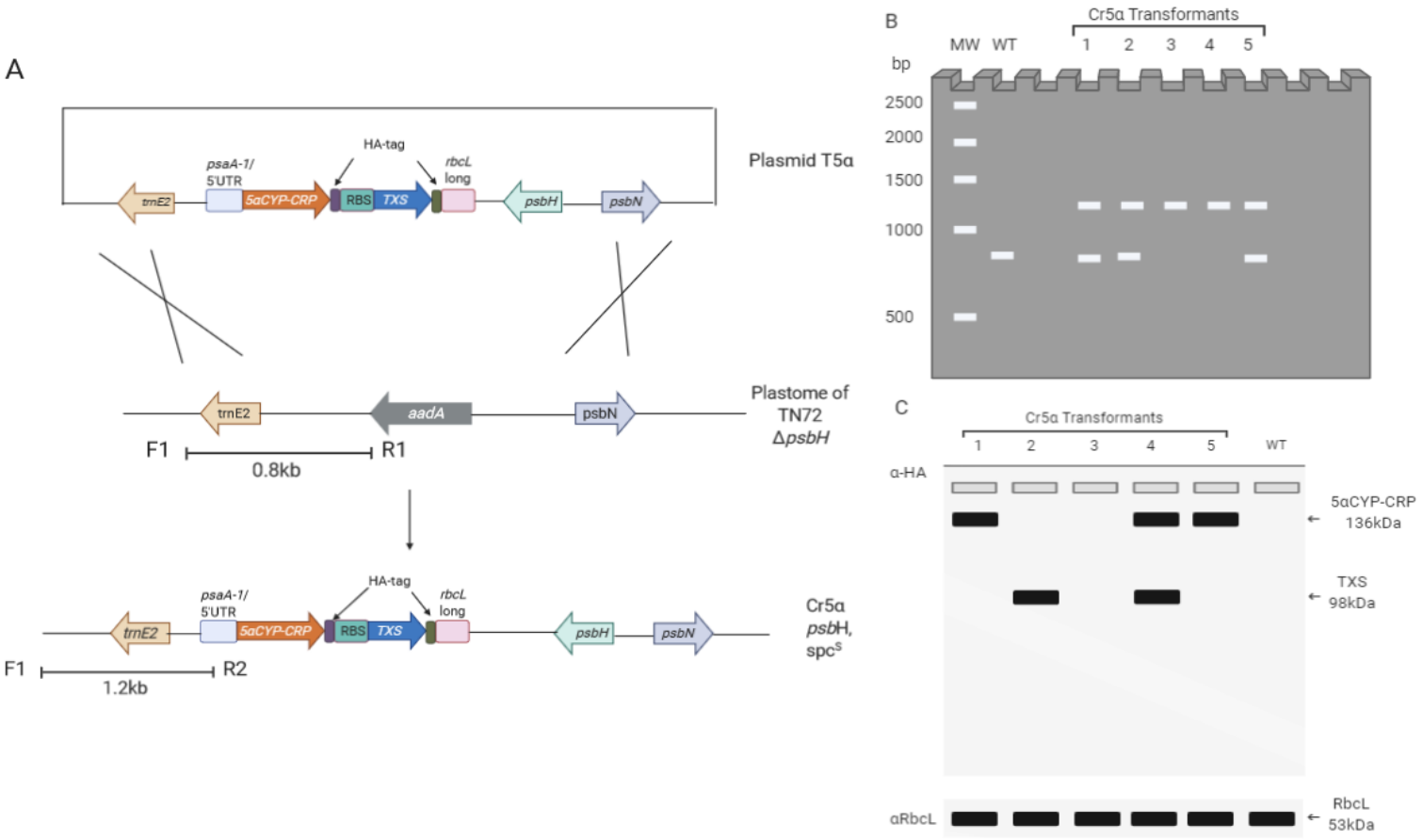

Figure 17: Possible results from transformation of TN72 using plasmid T5α to clone genes *5αCYP-CRP* and *TXS*. A) Transformation scheme whereby autotrophy is restored by rescuing gene *psbH* through the plasmid. HA-tags added at the end of each GOI. B) illustrates the possible results of PCR analysis where only lines 3 and 4 reached homoplasmy, the rest are heteroplasmic. C) Possible Western Blot results of the transformation with T5α where only line 4 is able to express both GOIs; *rbcL* expression would be used as a loading control. The negative control for both PCR and Western Blot is the untransformed TN72 (WT). The new transformed lines would be named Cr5α.

The Cr5α strain must be transformed with plasmid T10β. This step aims at cloning the remaining two genes (*10βCYP-CRP* and *TAT)* into the plastome of Cr5α. The insert would be integrated in the middle of the *chlL* gene and use spectinomycin for selection of colonies (Figure 18A). The approach for transgene determination must be similar to that described for plasmid one (T5α), this step has to make use of a set of three primers (F2, R3 and R4). Primers F2 and R3 would be designed upon the strain Cr5α and amplify a 1.0kb band while F2 and R4 should yield an amplicon of 1.6kb which confirms recombination of T10β with the recipient alga (Figure 18B).

Western Blot would determine expression of the GOIs. In this case, the transgenes would carry the FLAG-tag (Figure 18C). An additional Western Blot analysis should be performed to check if expression of the HA-tagged proteins remained stable.

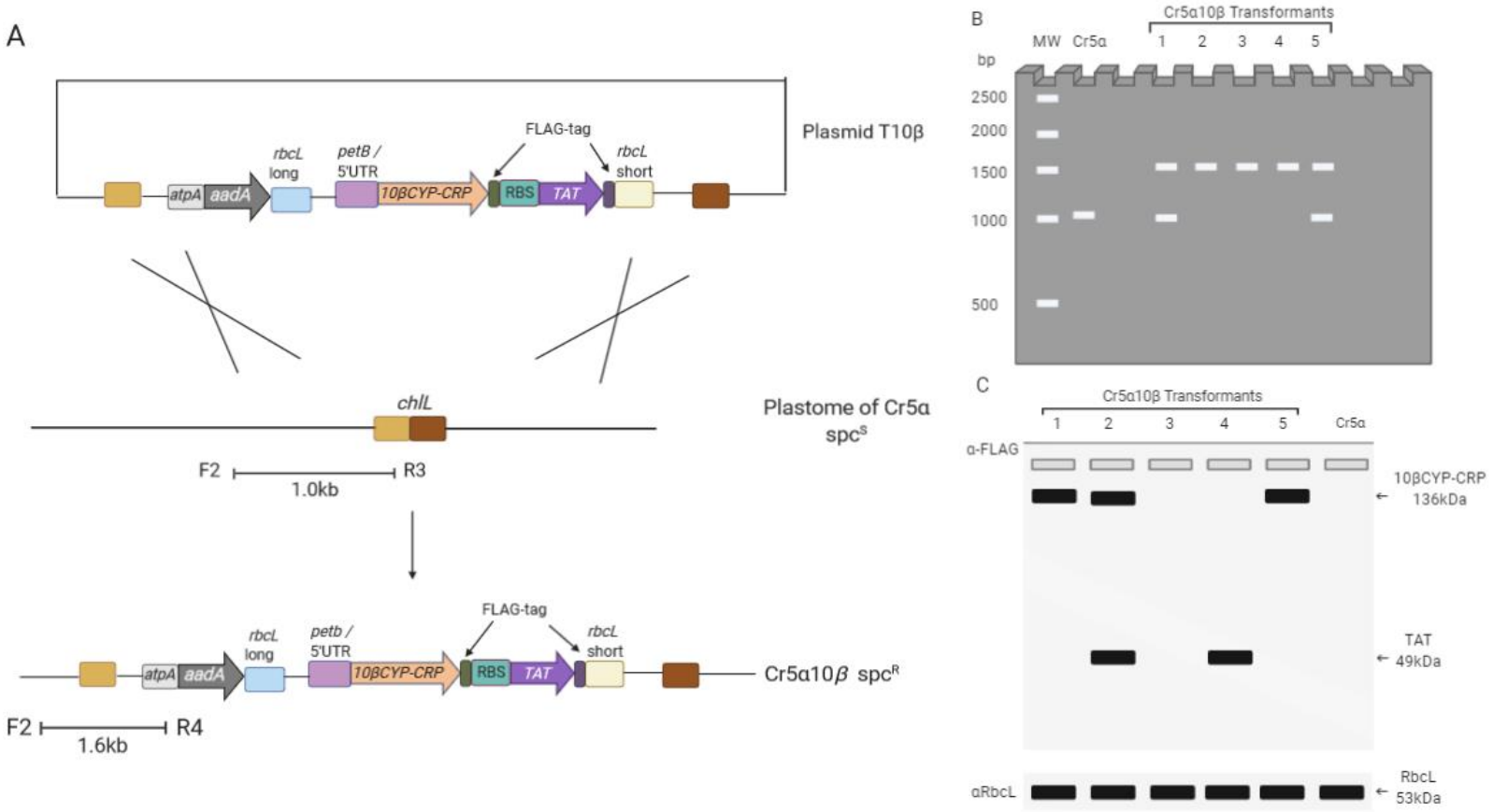

Figure 18: Possible results from transformation of Cr5α using plasmid T10β to clone genes *10βCYP-CRP* and *TAT*. A) shows the transformation scheme where the *aadA* gene is introduced thus granting spectinomycin resistance. FLAG-tags are added at the end of each GOI. B) Putative results of PCR analysis where only lines 2, 3 and 4 reached homoplasmy, the rest are heteroplasmic. C) Possible Western Blot analysis of the transformation with T10β where only line 2 is able to express both GOIs; *rbcL* expression was used as a loading control. The negative control for both PCR and Western Blot is a strain produced using the empty Cr5α vector. The resulting cell lines should be named Cr5α10β.

4.5 Metabolite determination

Metabolite assessment should be performed using cells from the Cr5α10β strain. To determine the presence of taxadien-5α-acetate-10β-ol, cells from transformed Cr5α10β lines should be cultured in TAP medium with a 5% dodecane overlay in constant shaking and under light conditions (100 µE/m^2/s PAR) for six days (Lauersen et al., 2018). Next, the dodecane has to be separated from the culture using centrifugation; two minutes at 18000 G. This layer must be analyzed using gas chromatography mass spectroscopy (GC-MS) (Lauersen et al., 2018) (Figure 19).

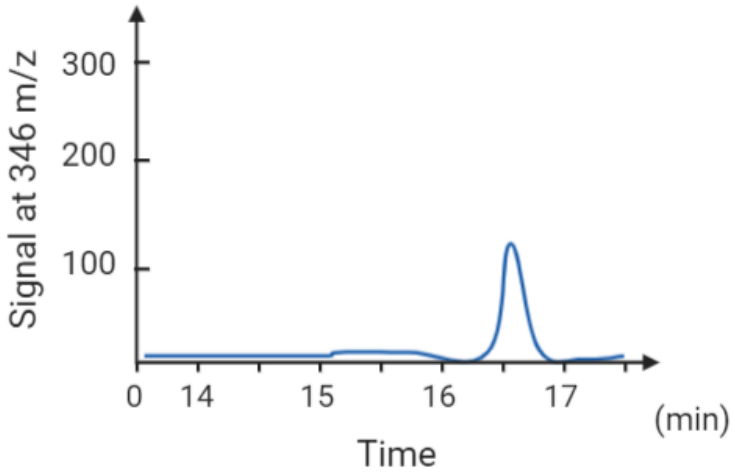

Figure 19: Putative results from analyzing the dodecane overlayer of a Cr5α10β culture using GC-MS. The MS requires to be set to scan on a range of 40-400 m/z. This configuration allows to identify a possible taxadien-5α-acetate-10β-ol at 16 minutes of operation. The Y-axis shows the intensity of the signal form detection of the precursor. Adapted from (Zhou et al., 2015).

CHAPTER 5: DISCUSSION

The metabolic engineering field is dominated by bacterial and yeast platforms. However, microalgae are arising in this field thanks to its photosynthetic features and adaptability. Moreover, tools are constantly being developped as decades pass which will be able to facilitate a standard protocol for metabolic engineering (Scaife et al., 2015).

The present work shows a feasible strategy to produce a complex precursor of PTX using *C. reinhardtii* strain TN72. The design uses serial transformation by employing two plasmids. This option lacks the limitations of using a single plasmid since the repeated sequences are separated when inserted in the genome to reduce the probability of homologous recombination which is illustrated in figure 12. Besides, the GOIs are distributed between the two plasmids which prevents instability from the large insert required. Also, the long and short 3'UTR *rbcL* are distributed as seen in figure 14 and 15 which prevents recombination between these sequences.

To facilitate the assembly of the plasmids, an RBS sequence should be used to encode two genes in each transcriptional unit. This novel method was proposed by (Carrizalez-López et al., 2018) and allows to reduce the number of promoters and 3'UTRs required which simplifies the expression process. It also results beneficial at plasmid assembly since the insert size is minimized. However, it has not been completely researched. It is unknown if it could regulate expression to favour the production of the GOIs or if its limiting. Alternatively, a strategy could be designed where this RBS sequence is not utilized but the genes are separated into different transciptional units composed of their own promoter and 3'UTR. A similar selection approach may be used by using a plasmid that restores phototrophy and inserts three GOIs (*5αCYP-CRP*, *TXS* and *TAT*) while the other plasmid would insert the aadA cassette for spectinomycin resistance and the final GOI (*10βCYP-CRP*). This method could work but requires careful choosing of the 3'UTR sequences as well as the promoters to prevent recombination between similar sequences (Larrea-Alvarez & Purton, 2020).

Assembling the plasmids would be performed using restriction enzymes. This is a time-consuming procedure which can be overcome when techniques like one-pot assembly have been standardized for chloroplast engineering. This will be achieved when a library containing regulated building blocks is established and GOIs are designed to carry type IIS restriction enzyme sites

(Crozet et al., 2018). Currently, this method is only available for nuclear engineering of *C. reinhardtii* (Crozet et al., 2018).

Considering this is a pilot design for the chloroplast, there are no past reports in transplastomics that could help improve this specifical trial. The most similar study was made by (Lauersen et al., 2018) where taxadiene was produced through nuclear engineering of *C. reinhardtii*. However, this diterpene was not the feature production in this report but 13R(+) manoyl oxide. In addition, compared to the study by (Zhou et al., 2015) which reached the furthest precursor of PTX, this trial does not rely on using cocultures which avoids having to manipulate many variables that could enhance the mutualism of the microorganisms used (e. g. substrate, temperature, ratios of inoculation for each species).

The following step after successfully producing taxadien-5α-acetate-10β-ol is enhancing the metabolic network of *C. reinhardtii* to yield a higher titer of precursor. Other platforms like *B. subtilis* (Abdallah et al., 2019) or *S. cerevisiae* already got studies to improve GGPP yield from the MEP pathway (Zhou et al., 2015). As for the alga, the MEP pathway is encoded in the nucleus but is localized in the chloroplast (Lauersen et al., 2018). It has been demonstrated that changing the native GGPP synthase of the alga with a gene from yeast origin (*ERG20)* improves the pathway production (Lauersen et al., 2018). Further improvements to the pathway are still limited since there is a lack of research for cytotoxicity in *C. reinhardtii,* although it is believed that the cell may be able to bear an augmented flux from the MEP pathway (Lauersen et al., 2018). Genes like *TXS* and *DXP* are known to encode proteins that perform a slow catalysis and are scarcely produced (Lauersen et al., 2018) which will require optimization to overcome their limited expression. The use of constitutive promoters for expression of the GOIs should be able to solve this problem, but a continuous production of such proteins have unexpected effects in the alga like toxicity. These observations can be obtained from a deep analysis of the results from trials growing the alga in bioreactors and performing Western blotting to interpret and enhance the genes that are not being adequately expressed. Promising techniques for controlling chloroplast gene expression are currently under development (Carrera-Pacheco, Hankamer, & Oey, 2020) which could potentially solve present limitations at regulation of recombinant protein production.

Production of PTX using microbial platforms is coming closer to a reality. The pathway to produce the drug is almost completely described since there is a theoretical intermediary which

has not yet been confirmed (Nazhand et al., 2020). As for now, it is desired that this experiment could yield a higher or equal taxadien-5α-acetate-10β-ol titer than the report by (Zhou et al., 2015). In any case, the accomplishment of this design will increase interest to settle microalgae as a microbial metabolic engineering platform, not only for future production of paclitaxel but for several other drugs. Moreover, the current semisynthetic means of production of the drug could be eventually surpassed by microbial platforms through higher efficiency and less costs.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

This work has presented an scheme for engineering *C. reinhardtii* chloroplast to produce taxadien-5α-acetate-10β-ol by exploiting its expression machinery, adaptability and photosynthetic nature. An appropiate approach should employ two plasmids for a serial transformation scheme of the at least four genes required for synthesis of the precursor. Modular cloning techniques like Golden Gate which facilitates vector assembly shall be fundamental to carry out these experiments. Emphasis must be made on designing techniques to regulate transgene expression and thus reduce potential risks regarding taxadien-5α-acetate-10β-ol toxicity. Expresion of recombinant proteins must certainly be attained since the scheme is based on methods that have been proven to work. However, the impacts on plastid metabolism have not yet been explored which limits further enhancement of the algal native metabolic pathway towards production of the drug.

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APPENDIX

Table S1: Codon optimized GOIs for expression in *C. reinhardtii* to produce taxadien-5α-acetate-10β-ol.

Gene	Sequence
TXS	ATGGCTCAAT TAAGTTTTAA TGCTGCTTTA AAAATGAATG CTTTAGGTAA TAAAGCTATT CATGATCCTA CTAATTGTCG TGCTAAAAGT GAACGTCAAA TGATGTGGGT TTGTAGTCGT AGTGGTCGTA CTCGTGTTAA AATGAGTCGT GGTAGTGGTG GTCCTGGTCC TGTTGTTATG ATGAGTAGTA GTACTGGTAC TAGTAAAGTT GTTAGTGAAA CTAGTAGTAC TATTGTTGAT GATATTCCTC GTTTAAGTGC TAATTATCAT GGTGATTTAT GGCATCATAA TGTTATTCAA ACTTTAGAAA CTCCTTTTCG TGAAAGTAGT ACTTATCAAG AACGTGCTGA TGAATTAGTT GTTAAAATTA AAGATATGTT TAATGCTTTA GGTGATGGTG

	ATATTAGTCC TAGTGCTTAT GATACTGCTT GGGTTGCTCG TTTAGCTACT ATTAGTAGTG ATGGTAGTGA AAAACCTCGT TTTCCTCAAG CTTTAAATTG GGTTTTTAAT AATCAATTAC AAGATGGTAG TTGGGGTATT GAAAGTCATT TTAGTTTATG TGATCGTTTA TTAAATACTA CTAATAGTGT TATTGCTTTA AGTGTTTGGA AAACTGGTCA TAGTCAAGTT CAACAAGGTG CTGAATTTAT TGCTGAAAAT TTACGTTTAT TAAATGAAGA AGATGAATTA AGTCCTGATT TTCAAATTAT TTTTCCTGCT TTATTACAAA AAGCTAAAGC TTTAGGTATT AATTTACCTT ATGATTTACC TTTTATTAAA TATTTAAGTA CTACTCGTGA AGCTCGTTTA ACTGATGTTA GTGCTGCTGC TGATAATATT CCTGCTAATA TGTTAAATGC TTTAGAAGGT TTAGAAGAAG TTATTGATTG GAATAAAATT ATGCGTTTTC AAAGTAAAGA TGGTAGTTTT TTAAGTAGTC CTGCTAGTAC TGCTTGTGTT TTAATGAATA CTGGTGATGA AAAATGTTTT ACTTTTTTAA ATAATTTATT AGATAAATTT GGTGGTTGTG TTCCTTGTAT GTATAGTATT GATTTATTAG AACGTTTAAG TTTAGTTGAT AATATTGAAC ATTTAGGTAT TGGTCGTCAT TTTAAACAAG AAATTAAAGG TGCTTTAGAT TATGTTTATC GTCATTGGAG TGAACGTGGT ATTGGTTGGG GTCGTGATAG TTTAGTTCCT GATTTAAATA CTACTGCTTT AGGTTTACGT ACTTTACGTA TGCATGGTTA TAATGTTAGT AGTGATGTTT TAAATAATTT TAAAGATGAA AATGGTCGTT TTTTTAGTAG TGCTGGTCAA ACTCATGTTG AATTACGTAG TGTTGTTAAT TTATTTCGTG CTAGTGATTT AGCTTTTCCT GATGAACGTG CTATGGATGA

	TGCTCGTAAA TTTGCTGAAC CTTATTTACG TGAAGCTTTA GCTACTAAAA TTAGTACTAA TACTAAATTA TTTAAAGAAA TTGAATATGT TGTTGAATAT CCTTGGCATA TGAGTATTCC TCGTTTAGAA GCTCGTAGTT ATATTGATAG TTATGATGAT AATTATGTTT GGCAACGTAA AACTTTATAT CGTATGCCTA GTTTAAGTAA TAGTAAATGT TTAGAATTAG CTAAATTAGA TTTTAATATT GTTCAAAGTT TACATCAAGA AGAATTAAAA TTATTAACTC GTTGGTGGAA AGAAAGTGGT ATGGCTGATA TTAATTTTAC TCGTCATCGT GTTGCTGAAG TTTATTTTAG TAGTGCTACT TTTGAACCTG AATATAGTGC TACTCGTATT GCTTTTACTA AAATTGGTTG TTTACAAGTT TTATTTGATG ATATGGCTGA TATTTTTGCT ACTTTAGATG AATTAAAAAG TTTTACTGAA GGTGTTAAAC GTTGGGATAC TAGTTTATTA CATGAAATTC CTGAATGTAT GCAAACTTGT TTTAAAGTTT GGTTTAAATT AATGGAAGAA GTTAATAATG ATGTTGTTAA AGTTCAAGGT CGTGATATGT TAGCTCATAT TCGTAAACCT TGGGAATTAT ATTTTAATTG TTATGTTCAA GAACGTGAAT GGTTAGAAGC TGGTTATATT CCTACTTTTG AAGAATATTT AAAAACTTAT GCTATTAGTG TTGGTTTAGG TCCTTGTACT TTACAACCTA TTTTATTAAT GGGTGAATTA GTTAAAGATG ATGTTGTTGA AAAAGTTCAT TATCCTAGTA ATATGTTTGA ATTAGTTAGT TTAAGTTGGC GTTTAACTAA TGATACTAAA ACTTATCAAG CTGAAAAAGC TCGTGGTCAA CAAGCTAGTG GTATTGCTTG TTATATGAAA GATAATCCTG GTGCTACTGA AGAAGATGCT ATTAAACATA TTTGTCGTGT TGTTGATCGT

	GCTTTAAAAG AAGCTAGTTT TGAATATTTT AAACCTAGTA ATGATATTCC TATGGGTTGT AAAAGTTTTA TTTTTAATTT ACGTTTATGT GTTCAAATTT TTTATAAATT TATTGATGGT TATGGTATTG CTAATGAAGA AATTAAAGAT TATATTCGTA AAGTTTATAT TGATCCTATT CAAGTT
5αCYP-CRP	ATGGATGCTT TATATAAAAG TACTGTTGCT AAATTTAATG AAGTTACTCA ATTAGATTGT AGTACTGAAA GTTTTAGTAT TGCTTTAAGT GCTATTGCTG GTATTTTATT ATTATTATTA TTATTTCGTA GTAAACGTCA TAGTAGTTTA AAATTACCTC CTGGTAAATT AGGTATTCCT TTTATTGGTG AAAGTTTTAT TTTTTTACGT GCTTTACGTA GTAATAGTTT AGAACAATTT TTTGATGAAC GTGTTAAAAA ATTTGGTTTA GTTTTTAAAA CTAGTTTAAT TGGTCATCCT ACTGTTGTTT TATGTGGTCC TGCTGGTAAT CGTTTAATTT TAAGTAATGA AGAAAAATTA GTTCAAATGA GTTGGCCTGC TCAATTTATG AAATTAATGG GTGAAAATAG TGTTGCTACT CGTCGTGGTG AAGATCATAT TGTTATGCGT AGTGCTTTAG CTGGTTTTTT TGGTCCTGGT GCTTTACAAA GTTATATTGG TAAAATGAAT ACTGAAATTC AAAGTCATAT TAATGAAAAA TGGAAAGGTA AAGATGAAGT TAATGTTTTA CCTTTAGTTC GTGAATTAGT TTTTAATATT AGTGCTATTT TATTTTTTAA TATTTATGAT AAACAAGAAC AAGATCGTTT ACATAAATTA TTAGAAACTA TTTTAGTTGG TAGTTTTGCT TTACCTATTG ATTTACCTGG TTTTGGTTTT CATCGTGCTT TACAAGGTCG TGCTAAATTA AATAAAATTA TGTTAAGTTT AATTAAAAAA CGTAAAGAAG ATTTACAAAG

	TGGTAGTGCT ACTGCTACTC AAGATTTATT AAGTGTTTTA TTAACTTTTC GTGATGATAA AGGTACTCCT TTAACTAATG ATGAAATTTT AGATAATTTT AGTAGTTTAT TACATGCTAG TTATGATACT ACTACTAGTC CTATGGCTTT AATTTTTAAA TTATTAAGTA GTAATCCTGA ATGTTATCAA AAAGTTGTTC AAGAACAATT AGAAATTTTA AGTAATAAAG AAGAAGGTGA AGAAATTACT TGGAAAGATT TAAAAGCTAT GAAATATACT TGGCAAGTTG CTCAAGAAAC TTTACGTATG TTTCCTCCTG TTTTTGGTAC TTTTCGTAAA GCTATTACTG ATATTCAATA TGATGGTTAT ACTATTCCTA AAGGTTGGAA ATTATTATGG ACTACTTATA GTACTCATCC TAAAGATTTA TATTTTAATG AACCTGAAAA ATTTATGCCT AGTCGTTTTG ATCAAGAAGG TAAACATGTT GCTCCTTATA CTTTTTTACC TTTTGGTGGT GGTCAACGTA GTTGTGTTGG TTGGGAATTT AGTAAAATGG AAATTTTATT ATTTGTTCAT CATTTTGTTA AAACTTTTAG TAGTTATACT CCTGTTGATC CTGATGAAAA AATTAGTGGT GATCCTTTAC CTCCTTTACC TAGTAAAGGT TTTAGTATTA AATTATTTCC TCGTCCT
TAT	ATGGAAAAAA CTGATTTACA TGTTAATTTA ATTGAAAAAG TTATGGTTGG TCCTAGTCCT CCTTTACCTA AAACTACTTT ACAATTAAGT AGTATTGATA ATTTACCTGG TGTTCGTGGT AGTATTTTTA ATGCTTTATT AATTTATAAT GCTAGTCCTA GTCCTACTAT GATTAGTGCT GATCCTGCTA AACCTATTCG TGAAGCTTTA GCTAAAATTT TAGTTTATTA TCCTCCTTTT GCTGGTCGTT TACGTGAAAC TGAAAATGGT GATTTAGAAG TTGAATGTAC TGGTGAAGGT

	GCTATGTTTT TAGAAGCTAT GGCTGATAAT GAATTAAGTG TTTTAGGTGA TTTTGATGAT AGTAATCCTA GTTTTCAACA ATTATTATTT AGTTTACCTT TAGATACTAA TTTTAAAGAT TTAAGTTTAT TAGTTGTTCA AGTTACTCGT TTTACTTGTG GTGGTTTTGT TGTTGGTGTT AGTTTTCATC ATGGTGTTTG TGATGGTCGT GGTGCTGCTC AATTTTTAAA AGGTTTAGCT GAAATGGCTC GTGGTGAAGT TAAATTAAGT TTAGAACCTA TTTGGAATCG TGAATTAGTT AAATTAGATG ATCCTAAATA TTTACAATTT TTTCATTTTG AATTTTTACG TGCTCCTAGT ATTGTTGAAA AAATTGTTCA AACTTATTTT ATTATTGATT TTGAAACTAT TAATTATATT AAACAAAGTG TTATGGAAGA ATGTAAAGAA TTTTGTAGTA GTTTTGAAGT TGCTAGTGCT ATGACTTGGA TTGCTCGTAC TCGTGCTTTT CAAATTCCTG AAAGTGAATA TGTTAAAATT TTATTTGGTA TGGATATGCG TAATAGTTTT AATCCTCCTT TACCTAGTGG TTATTATGGT AATAGTATTG GTACTGCTTG TGCTGTTGAT AATGTTCAAG ATTTATTAAG TGGTAGTTTA TTACGTGCTA TTATGATTAT TAAAAAAAGT AAAGTTAGTT TAAATGATAA TTTTAAAAGT CGTGCTGTTG TTAAACCTAG TGAATTAGAT GTTAATATGA ATCATGAAAA TGTTGTTGCT TTTGCTGATT GGAGTCGTTT AGGTTTTGAT GAAGTTGATT TTGGTTGGGG TAATGCTGTT AGTGTTAGTC CTGTTCAACA ACAAAGTGCT TTAGCTATGC AAAATTATTT TTTATTTTTA AAACCTAGTA AAAATAAACC TGATGGTATT AAAATTTTAA TGTTTTTACC TTTAAGTAAA ATGAAAAGTT TTAAAATTGA AATGGAAGCT ATGATGAAAA AATATGTTGC TAAAGTT

10βCYP-CRP	ATGGATAGTT TTATTTTTTT ACGTAGTATT GGTACTAAAT TTGGTCAATT AGAAAGTAGT CCTGCTATTT TAAGTTTAAC TTTAGCTCCT ATTTTAGCTA TTATTTTATT ATTATTATTT CGTTATAATC ATCGTAGTAG TGTTAAATTA CCTCCTGGTA AATTAGGTTT TCCTTTAATT GGTGAAACTA TTCAATTATT ACGTACTTTA CGTAGTGAAA CTCCTCAAAA ATTTTTTGAT GATCGTTTAA AAAAATTTGG TCCTGTTTAT ATGACTAGTT TAATTGGTCA TCCTACTGTT GTTTTATGTG GTCCTGCTGG TAATAAATTA GTTTTAAGTA ATGAAGATAA ATTAGTTGAA ATGGAAGGTC CTAAAAGTTT TATGAAATTA ATTGGTGAAG ATAGTATTGT TGCTAAACGT GGTGAAGATC ATCGTATTTT ACGTACTGCT TTAGCTCGTT TTTTAGGTGC TCAAGCTTTA CAAAATTATT TAGGTCGTAT GAGTAGTGAA ATTGGTCATC ATTTTAATGA AAAATGGAAA GGTAAAGATG AAGTTAAAGT TTTACCTTTA GTTCGTGGTT TAATTTTTAG TATTGCTAGT ACTTTATTTT TTGATGTTAA TGATGGTCAT CAACAAAAAC AATTACATCA TTTATTAGAA ACTATTTTAG TTGGTAGTTT AAGTGTTCCT TTAGATTTTC CTGGTACTCG TTATCGTAAA GGTTTACAAG CTCGTTTAAA ATTAGATGAA ATTTTAAGTA GTTTAATTAA ACGTCGTCGT CGTGATTTAC GTAGTGGTAT TGCTAGTGAT GATCAAGATT TATTAAGTGT TTTATTAACT TTTCGTGATG AAAAAGGTAA TAGTTTAACT GATCAAGGTA TTTTAGATAA TTTTAGTGCT ATGTTTCATG CTAGTTATGA TACTACTGTT GCTCCTATGG CTTTAATTTT TAAATTATTA TATAGTAATC CTGAATATCA TGAAAAAGTT TTTCAAGAAC

	AATTAGAAAT TATTGGTAAT AAAAAAAAAG GTGAAGAAAT TAGTTGGAAA GATTTAAAAA GTATGAAATA TACTTGGCAA GCTGTTCAAG AAAGTTTACG TATGTATCCT CCTGTTTTTG GTATTTTTCG TAAAGCTATT ACTGATATTC ATTATGATGG TTATACTATT CCTAAAGGTT GGCGTGTTTT ATGTAGTCCT TATACTACTC ATTTACGTGA AGAATATTTT CCTGAACCTG AAGAATTTCG TCCTAGTCGT TTTGAAGATG AAGGTCGTCA TGTTACTCCT TATACTTATG TTCCTTTTGG TGGTGGTTTA CGTACTTGTC CTGGTTGGGA ATTTAGTAAA ATTGAAATTT TATTATTTGT TCATCATTTT GTTAAAAATT TTAGTAGTTA TATTCCTGTT GATCCTAATG AAAAAGTTTT AAGTGATCCT TTACCTCCTT TACCTGCTAA TGGTTTTAGT ATTAAATTAT TTCCTCGTAG T

Table S2: Resources employed for making the different figures in this work.

Resource	**Description**	**Source**
ChemDraw	Used to draw the different chemical structures	https://chemdrawdirect.perkinelmer.cloud/js/sample/index.html
BioRender	Used to make each pathway, cell, cloning device and cloning strategy	https://biorender.com/
OGDraw	Tool used to draw the plastome map of *C. reinhardtii*	https://chlorobox.mpimp-golm.mpg.de/OGDraw.html
Bioinformatics.org	Used to calculate the molecular weight of the recombinant proteins	https://www.bioinformatics.org/sms/prot_mw.html