New tools and strategies for metabolic engineering of the algal chloroplast

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Declaration

I, Saowalak Changko, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Microalgae are attractive as cell factories for production of bioactive metabolites, therapeutic proteins and high-value metabolites. Of the several microalgae that have been explored as potential biotechnological platforms, the unicellular chlorophyte *Chlamydomonas reinhardtii*, is the most genetically tractable given its long history as a model species for molecular-genetic studies of cell biology. In particular, the chloroplast of *C. reinhardtii* represents a novel sub-cellular compartment for synthesis and accumulation of recombinant products. It possesses a small, genetically tractable genome and lacks any gene-silencing mechanisms. This allows stable and high-level expression of multiple transgenes. However, the exploitation of this microalgal platform requires further advances in the molecular tools available for metabolic engineering, together with new strategies for large-scale cultivation such as simple methods for ensuring 'crop protection' to reduce invasion and growth of contaminating species. Due to the increasing scarcity of phosphate reserve, phosphite is an alternative P source at economical cost and can reduce demand on non-renewable phosphate fertiliser. A key goal of my project was therefore to develop a strain improvement strategy based on the expression in the chloroplast of the bacterial gene *ptxD* encoding an NAD(P)-dependent phosphite oxidoreductase to allow utilization of phosphite as a source of phosphorus (P). The approach is based on the fact that most organisms cannot use phosphite, therefore, growing the transgenic microalga in phosphite provides a selective advantage over competing species. The *ptxD* gene was successfully introduced into chloroplast and shown to produce a functional enzyme that allowed growth on phosphite media. This allows engineered strains to be grown in non-sterile medium without significant spoilage by bacteria or fungi, thereby avoiding costly medium sterilization and culture management. Furthermore, it was demonstrated that *ptxD* can serve as a new non-antibiotic selectable marker for chloroplast transformation, allowing direct selection of transformants for their phosphite-utilising activity. This increases the repertoire of available selectable markers and reduces the use of antibiotics. Having developed

these tools, the metabolic engineering of the chloroplast was attempted by introducing a synthetic gene encoding limonene synthase (LS). Limonene is a high-value terpenoid that has applications as a pharmaceutical, a flavour and a fragrance. Whilst the LS protein was successfully produced in the chloroplast, detailed GC-MS analysis failed to detect limonene synthesis suggesting issues with either functionality of the enzyme or availability of substrate. However, the work adds important new tools to the molecular toolbox for advancing *C. reinhardtii* chloroplast as an expression platform.

Impact statement

Microalgae are promising biotechnological platforms for the production of recombinant proteins and bioactive compounds that can be useful for industry. However, there is an economic barrier to algal production of recombinant proteins, as protein accumulation in the microalgae is still low compared to other established platforms such as bacteria and yeasts. In addition, advanced genetic modification technologies remain under developed. As a result, there is a need to accelerate strain improvement and metabolic engineering to gain higher productivity. Algal cultivation also encounters issues of contamination that lead to the loss of biomass and furthermore requires expensive treatments of media sterilisation and 'crop protection' that is the tool to avoid the growth of other organisms using antibiotics or herbicides to circumvent the problem.

The aim of this project was to develop tools and strategies for genetic manipulation and metabolic engineering of the algal chloroplast, including novel selectable marker for transformant selection and crop protection to reduce the risk of bacterial contamination. The developed tools provide a new selection method for generating the algal transformants that avoids the use of antibiotic-based selection markers. The output of my project may contribute to the reduction of antibiotic selection and increase the number of chloroplast markers available to algal and plant researchers. Additionally, providing the algae with the novel ability to use phosphite instead of phosphate can circumvent an increasing scarcity of phosphate, which is currently supplied by a non-renewable reserve of mine rock. Phi is produced as a by-product of other phosphorus compounds preparation and it is not commonly used as phosphorus fertiliser, so that it may be available as an alternative P source at a lower price than phosphate. Finally, the developed strategy with the algal ability to use phosphite provides an effective crop protection tool to reduce the need for media sterilisation and the risk of contamination that normally increase significantly the cost of productivity.

The findings of this thesis can be applied to other algal species, such as microalga *Nannochloropsis sp* and diatom *Phaeodactylum tricornutum,* for strain improvement by expressing *ptxD* to allow the utilisation of phosphite as a P source and to enable crop protection strategy against the bacterial contamination. This can be used for industrial algal cultivation in phosphite medium under non-sterile environment to produce additive or vaccine for animal and aquaculture as demonstrated in this study.

Abbreviations

aadA	aminoglycoside-3''-adenyl-transferase gene
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CAI	codon adaptation index
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'deoxynucleoside 5'-triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid (disodium salt)
GOI	gene of interest
GMO	genetically modified organisms
GRAS	generally recognized as safe
HSM	high salt minimal medium
IPTG	isopropyl-β-D-thiogalactoside
IgG	immunoglobulin G
LB	luria-bertani medium
mRNA	messenger ribonucleic acid
NHEJ	non-homologous end joining
NCBI	national Centre for Biotechnology Information

NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
OD	optical density
ORF	open reading frame
Pi	Phosphate
Phi	Phosphite
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Psi	pounds per square inch
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TAP	tris acetate phosphate medium
TBS	tris buffered saline
TBS-T	tris buffered saline – tween 20
TE	tris EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
UTR	untranslated region
v/v	volume for volume
w/v	weight for volume
WT	wild type

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Introduction

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1.1 The chloroplast

1.1.1 The origin and evolution of the chloroplast

Photosynthetic eukaryotes first evolved more than 1 billion years ago after a phagotrophic eukaryotic host acquired a cyanobacterial endosymbiont that evolved into a photosynthetic organelle – the chloroplast. This led to the emergence of three primary algal lineages: Glaucophytes, Rhodophytes (red algae) and Chlorophytes (green algae). During secondary and tertiary endosymbiosis, other eukaryotic hosts further engulfed free-living photosynthetic eukaryotes, such as red or green algae, to generate additional photosynthetic eukaryotic lineages in which the captured algal cell was reduced to the level of a complex chloroplast (Figure 1.1) (Kleine, Maier and Leister, 2009; Jensen and Leister, 2014). Primary endosymbiosis resulted in the chloroplasts that have two layers of membrane, whilst complex chloroplasts have one or more additional membranes. It is challenging to track back the membrane history of the organelle. However, the information based on the existence of certain lipids and membrane proteins indicates (Jarvis *et al.*, 2000; Sommer *et al.*, 2011) that the two membranes of primary chloroplast were derived from the two membranes of the Gram-negative cyanobacterial cell (Cavalier-Smith, 1982). The primary endosymbiosis is believed to have occurred via phagocytosis, so the phagosomal membrane that originally enclosed the new endosymbiont was lost at an early stage. The removal of the phagosomal membrane has a great impact on which the host cell recognised the symbiont as ‘organelle’ not ‘food’ (Keeling, 2013). The additional membranes acquired during secondary and tertiary endosymbiosis are believed to arise from the cell membrane of the captured algal symbiont and endomembranes from the host (Keeling, 2013).

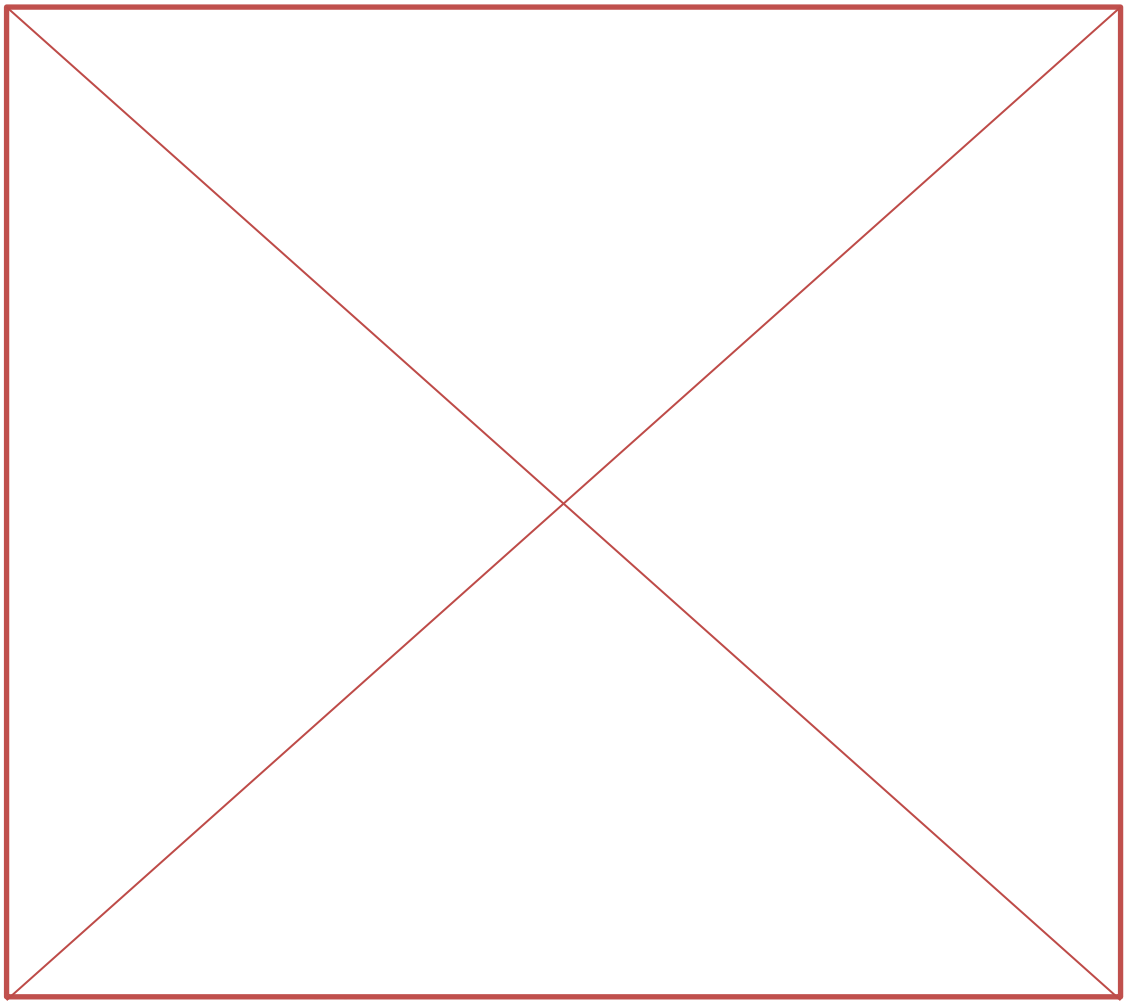


Figure 1.1 Plastid evolution of photosynthetic eukaryotes.

A cyanobacterium was engulfed by an early eukaryotic host and rather than being digested, the cyanobacterium became established as an endosymbiont. This gave rise to three extant lineages of algae: *Rhodophyta*, *Chlorophyta* and *Glaucophyta*. Land plants evolved from within the *Chlorophyta*. Meanwhile, other eukaryotes engulfed early red or green algal cells during secondary endosymbiosis, creating the green secondary lineages *Euglenophyta* and *Chlorarachniophyta*, and the red lineage *Chromalveolata*, that can be divided into four major subgroups: *Heterokontophyta*, *Cryptophyta*, *Haptophyta* and *Alveolata*. The plastid of primary endosymbiosis is surrounded by two membrane layers whereas those of secondary endosymbiosis possess three or four membranes. Figure reproduced from (Marchand *et al.*, 2018).

Throughout the early evolutionary stages of integration of the cyanobacterial endosymbiont into the host cell and its reduction to the level of an organelle, the cyanobacterial genome has undergone a drastic reduction in size, primarily as a consequence of gene loss and the transfer of a large number of genes to the nuclear genome (Martin *et al.*, 2002). Sequenced chloroplast genomes are therefore typically less than 200 kb in size with between 20 and 200 protein-coding genes that mostly encode components of the organelle's transcription-translation apparatus and the photosynthetic apparatus (Simpson and Stern, 2002; Timmis *et al.*, 2004; Jarvis and López-Juez, 2013). This is a marked difference in size and complexity when compared to a typical cyanobacterial genome such as that of *Synechocystis* PCC6803 that is 3.6 Mb and encodes over 3500 protein-coding genes (Kaneko *et al.*, 1995).

As a consequence of the mass chloroplast-to-nuclear gene transfer and the evolution of the chloroplast as an integrated component of the cell, some 2000–3000 different proteins encoded in the nucleus are imported into the chloroplast from the cytosol after synthesis (Sakamoto, Miyagishima and Jarvis, 2008; Li and Chiu, 2010). The proteins are targeted to the chloroplast via the use of an N-terminal extension termed a transit peptide. The recognition and translocation of proteins carrying a transit peptide are mediated through inner and outer membrane proteins of the chloroplast import apparatus, with the transit peptide cleaved off following import (Strittmatter, Soll and Bölter, 2010). For those genes that have remained within the chloroplast, a hybrid genetic system has evolved with the eubacterial-like transcription-translation machinery of the original cyanobacterium overlain with a tight control network mediated by numerous nuclear-encoded protein factors (Kwon et al. 2018). These factors are targeted to the chloroplast and regulate gene expression at post-transcriptional levels such as RNA processing, stability, translation, protein folding and cofactor attachment (Barkan and Eberhard 2002). For example, nuclear factors serve to protect specific chloroplast mRNAs against nucleolytic degradation through interaction with the 5'UTR of the target mRNA, or interact with the 5'UTR and the ribosome to mediate efficient

translation initiation. Such factors have been identified for most of the chloroplast transcripts (Pfalz 2009) and represent the suite of nuclear regulatory mechanisms that coordinate chloroplast gene expression.

1.1.2 Primary production in the algal chloroplast

Chloroplasts are most widely known for carrying out photosynthesis, however, they also catalyse many other vital functions such as nitrogen and sulphur assimilation, the synthesis of carbohydrate (sugars & starch), amino acids, fatty acids and nucleotides together with the biosynthesis of pigments, alkaloids, isoprenoids, hormone precursors and vitamins. Throughout the evolutionary period, the cyanobacterial-derived metabolic pathways of the chloroplast have been refined in order to prevent redundancy in functions with other organelles. The chloroplasts therefore need to interact with the other cell compartments to manage their different functions via exchange and transport of metabolites and ions (Rolland *et al.*, 2012, 2018).

The central process of photosynthesis within chloroplasts takes place on membrane structures known as thylakoids (Figure 1.2). The thylakoid membranes fold and interconnect to form an acidified compartment called the lumen. This whole thylakoid structure is immersed in a fluid stroma that contains enzymes involved in carbon fixation (Meyer and Griffiths, 2015). The overall architecture of the chloroplast and process of photosynthesis is highly conserved between plant and algal cells (Ynalvez, Dinamarca and Moroney, 2018).

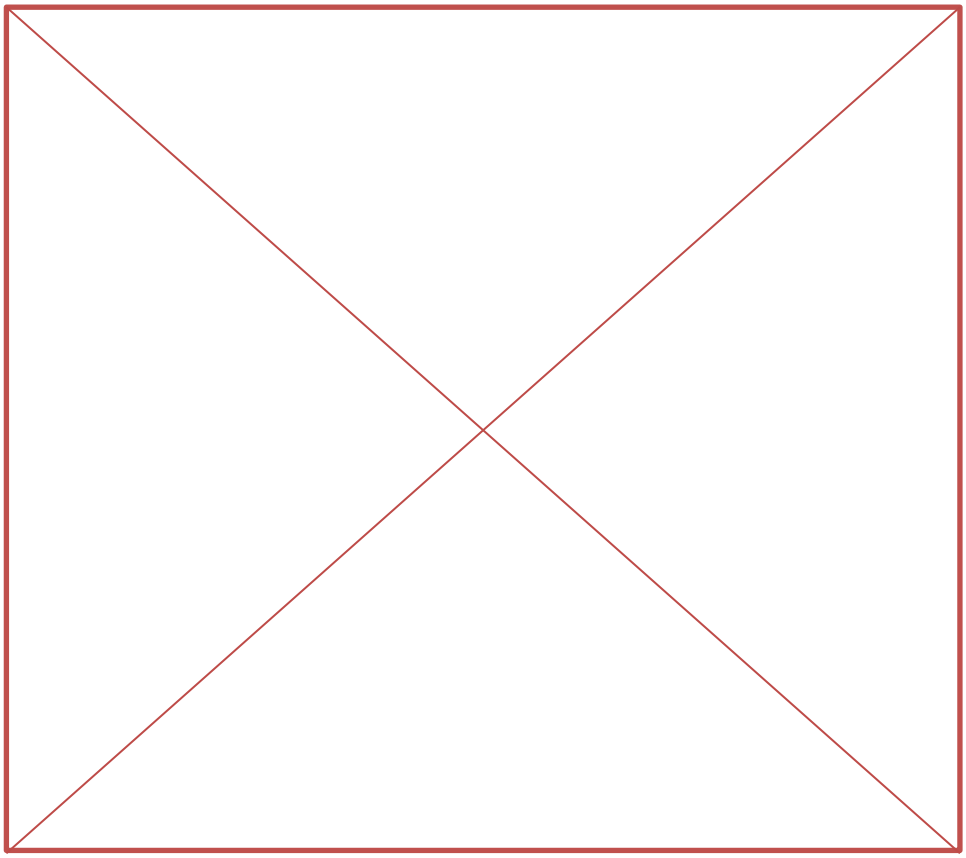


Figure 1.2 The chloroplast in a cell and the thylakoid structure.

The outer membrane (OE) and inner membrane (IE) of the chloroplast are indicated as well as the inter-envelope space (IES). Other labelled structures are nucleus (N), mitochondrion (M), and plasma membrane (PM). Electron microscopy images of thylakoid stacks or the grana are shown on the right and below (Soll and Schleiff, 2004). Figure reproduced from

https://www.nature.com/scitable/topicpage/photosynthetic-cells-14025371/, accessed on 04/01/2021.

Photosynthesis involves two important steps: i) the light-dependent reactions in which light energy is captured and converted into chemical energy in the form of an energised electron transport chain that results in the synthesis of ATP as an energy store and NADPH as a source of reductant; ii) the light-independent carbon fixation reactions in which CO_2 is converted to carbohydrates using the ATP and NADPH (Ynalvez, Dinamarca and Moroney, 2018). The light reactions of oxygenic photosynthesis carried out by cyanobacteria, algae and plants, involve the harvesting of photons via two photosystems that are multi-subunit protein–pigment complexes (Ynalvez, Dinamarca and Moroney, 2018) (Figure 1.3). The photosystem I (PSI) complex resides in the stroma lamella of thylakoids, facilitating electron transfer via a light-driven electron pump from an electron donor (plastocyanin or cytochrome c_6) on the luminal side to an electron acceptor (ferredoxin or flavodoxin) on the stromal side (Harris, Stern and George B., 2009). The photosystem II (PSII) complex, in contrast, is located in the stacked grana system and carries out the oxidation of water and the reduction of plastoquinone (the first molecule in the electron transport chain) (Wise, 2016). Both photosystems consist of a core complex reaction centre (RC) and a peripheral antenna network – light-harvesting complex I (LHCI) for PSI and a light-harvesting complex II (LHCII) that primarily associates with PSII (Harris, Stern and George B., 2009) – working together with chlorophylls and other pigments to harvest the light and transfer the energy to the RC. (Gao *et al.*, 2018). The chemical energy from light-dependent reactions is finally converted to the high-energy molecules nicotinamide–adenine dinucleotide phosphate reduced form (NADPH) and adenosine triphosphate (ATP) (Harris, Stern and George B., 2009).

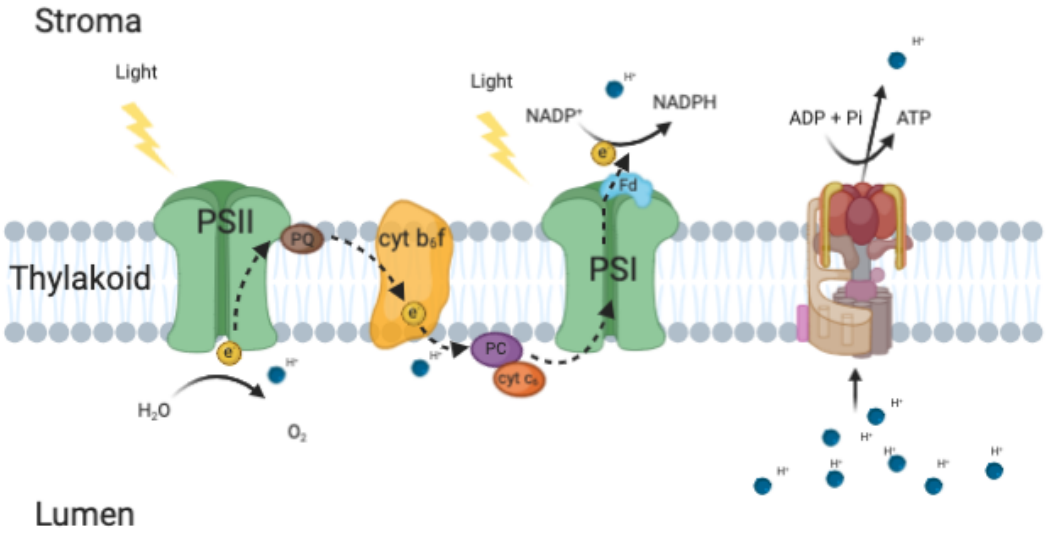


Figure 1.3 Schematic of the photosynthesis reaction in the chloroplast.

Photosystem II complexes located on thylakoid membrane absorb the light energy and this leads to oxidation of water ('water splitting') to produce oxygen (O_2) and H^+. The extracted electrons are then passed to the first electron acceptor, plastoquinone (PQ) to a linear electron transfer pathway (indicated by dotted arrows). In this route, electrons are passed to the cytochrome *b*$_6$*f* complex that is operationally located between PSII and PSI, then to plastocyanin (PC) or cytochrome *c*$_6$ (cyt *c*$_6$), to the Photosystem I complex and then transferred to ferredoxin (Fd) before finally to an electron acceptor NADP+ to generate NADPH used in the Calvin-Benson cycle. The electron transfer also creates a proton gradient across the thylakoid membrane for the synthesis of ATP via ATP synthase (the red complex). ATP is generated from adenosine diphosphate (ADP) and inorganic phosphate (Pi) by dissipation of the proton gradient from the thylakoid lumen to the stroma.

Chloroplasts carry out the Carbon fixation via the Calvin-Benson cycle (also known as the reductive pentose phosphate cycle or C3 cycle) (Figure 1.4), and is the most widely use carbon fixation pathway in autotrophs such as plants and algae (Wang and Lan, 2010). Whilst the Calvin-Benson cycle of eukaryotic algae is almost identical to that found in plants, the process of CO_2 uptake and delivery to the stroma in aquatic organisms is somewhat different (Ynalvez, Dinamarca and Moroney, 2018). In the case of *C. reinhardtii*, it exploits a CO_2 concentrating mechanism or CCM due to a relatively low affinity of ribulose bisphosphate carboxylase oxygenase (Rubisco) for CO_2 during oxygenic photosynthesis (Giordano, Beardall and Raven, 2005; Mackinder, 2018). This CCM mechanism involves importing and concentrating inorganic bicarbonate (HCO_3^-) and CO_2 within the cell. *C. reinhardtii* actively transports HCO_3^- and CO_2 across the cell membrane and passes HCO_3^- to the thylakoids. (Giordano, Beardall and Raven, 2005; Mackinder, 2018). HCO_3^- reacts with H^+ that is formed as a result of the electron transport chain during photosynthesis to generate high CO_2 concentration in the lumen. The CO_2 then diffuses from the lumen to the Rubisco enzyme in the pyrenoid (L. Wang *et al.*, 2016). With such CCM mechanisms, algae are estimated to carry out nearly 50% of global carbon fixation and therefore represent a critical primary producer for the planet (Field *et al.*, 1998).

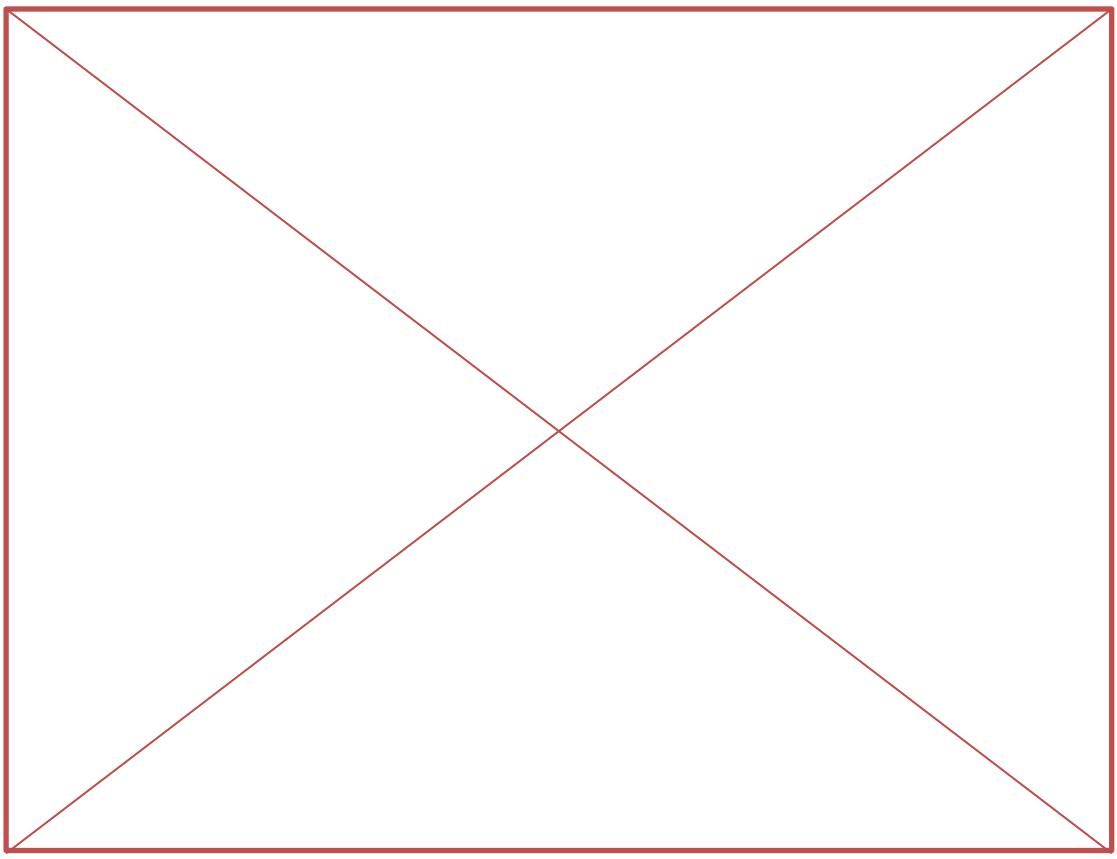


Figure 1.4 The CO_2 concentrating mechanism and Calvin-Benson cycle in *C. reinhardtii* chloroplast.

The Calvin-Benson cycle fixes CO_2 and generates intermediate compounds for producing sugars. This process occurs in the stroma (pale yellow in insert) and in the pyrenoid (purple) that is surrounded by starch sheaths (brown). The CO_2 concentrating mechanism or CCM (at the top) processes the uptake of bicarbonate (HCO_3^-) as a precursor of CO_2 through transporters across the cell membrane and thylakoid membrane (Wang, Stessman and Spalding, 2015). Enzymes such as carbonic anhydrases play a role in conversion of bicarbonate into CO_2 in the thylakoids before diffusing out to Rubisco in the pyrenoid (Aspatwar, Haapanen and Parkkila, 2018). The Calvin-Benson cycle starts with incorporation of CO_2 from Rubisco to 3PGA, then undergoes reduction steps via Triose-P and again regenerates RuBP for the next cycle. Figure is reproduced from (Machingura and Moroney, 2018).

In addition to photosynthesis and carbohydrate biosynthesis, chloroplasts perform other specific biosynthesis and storage functions that are essential for growth and development, including the assimilation of nitrates, sulphates and amino acids. It has been shown that chloroplast can synthesise 17 of the 20 amino acids (Rolland *et al.*, 2012), and ten of these amino acids (including Arg, Lys, Thr, Leu, Ile, Val, Trp, Phe, Tyr, His) are exclusively synthesised in the chloroplasts. On the other hand, the other seven amino acids (Asp, Cys, Gln, Glu, Gly, Ser, Met) can be formed elsewhere in other compartments (Rolland *et al.*, 2018). Biosynthesis of carotenoid pigments also occurs in the chloroplast. These carotenoids are C40 terpenoids that have functions in light harvesting and photoprotection (Sun *et al.*, 2018). They are generated through condensation of the C5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that are described in Chapter 5. Another biosynthesis process located in the chloroplast is that of fatty acid or lipid synthesis as shown in Figure 1.5 (Reyes-Prieto and Moustafa, 2012; Vranová, Coman and Gruissem, 2013; Nielsen *et al.*, 2016; Jensen and Scharff, 2019). It is evident that chloroplasts fulfil many essential functions for cells with the supply of useful compounds and metabolites.

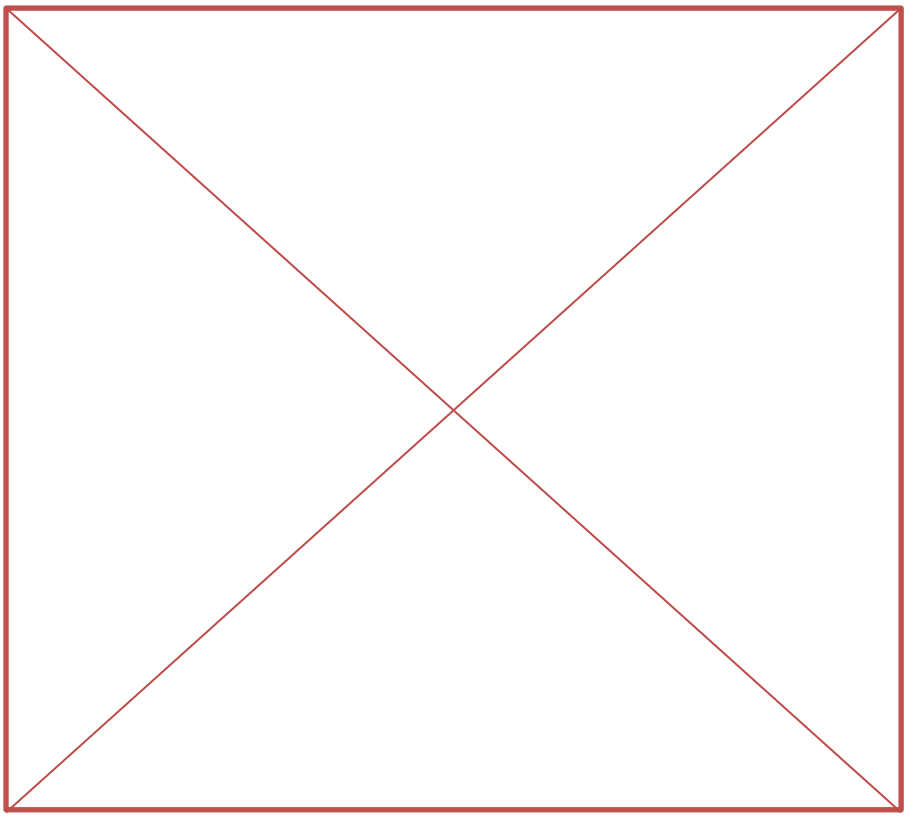


Figure 1.5 Schematic of basic cellular biosynthesis pathway of different compounds produced in the chloroplast and transported to cytosol.

While chloroplasts can function as the main protein factory, the nucleus plays a key role in metabolic regulation (dotted lines). Chloroplasts produce ATP and NADPH from photosynthesis, carbohydrates from the Calvin cycle and other essential compounds such as fatty acids used for membrane and storage lipids including those containing polyunsaturated fatty acids (PUFA). Figure is reproduced from (Rosenberg *et al.*, 2008; Martínez-Francés and Escudero-Oñate, 2018).

1.1.3 The diversity of eukaryotic algae

Algae are a large polyphyletic group of photosynthetic eukaryotes (Blaby-Haas and Merchant, 2019). They are found in almost all environments with different species adapted to a wide range of temperatures, salinities, pH values, light intensities; and water availability – from reservoirs to deserts. They may grow independently or in symbiosis with other organisms (Evangelista *et al.*, 2008). The eukaryotic algae can be divided by size as macroalgae or microalgae. Macroalgae (seaweed) are multicellular organisms that are visible with the naked eye whereas microalgae are single cells or clusters of cells visible only under a microscope (Khan, Shin and Kim, 2018) (Figure 1.6). Some species have been widely studied for fundamental biology and are now being recognised as potential platforms for industrial biotechnology, such as the green algae *Chlorella vulgaris*, *Dunaliella salina* and *Chlamydomonas reinhardtii*., the diatom *Phaeodactylum tricornutum*, and the heterokont *Nannochloropsis sp*. (Feng, Li, *et al.*, 2014; Scaife *et al.*, 2015; Liu and Chen, 2016; Al-Hoqani, Young and Purton, 2017).

Algal species have a wide range of cell architecture, for example, *Nannochloropsis sp*. are unicellular non-flagellate microalgae with a size of 2–4 μm (Al-Hoqani, Young and Purton, 2017). *Nannochloropsis sp*. have a thick cellulosic cell wall and a complex chloroplast structure due to the four membranes derived from the secondary endosymbiosis of a red alga (Janouškovec *et al.*, 2010; Scholz *et al.*, 2014). Whereas the unicellular microalga, *Dunaliella salina* has two flagella, contains a single, large, cup-shaped chloroplast with two membranes and lacks a rigid cell wall structure but rather has a thin elastic plasma membrane that can be easily transformed (Feng, Li, *et al.*, 2014). *Chlorella sp* is spherical with a diameter of 2-15 μm and contains a cup-shaped chloroplast, but does not have flagella and does not undergo sexual reproduction (Liu and Chen, 2016). On the other hand, some algal cells such as *C. reinhardtii* have flagella for motility and to facilitate the mating process (Harris, Stern and George B., 2009). Some algae such as the chlorophyte *Haematococcus pluvialis* can produce high amounts of pigments under stress conditions such as high light. This

species is a unicellular biflagellate living in a freshwater and shown to be an a major producer of the pink carotenoid astaxanthin (Masojídek and Torzillo, 2014). All of these different features in eukaryotic algae underscore the vast algal diversity and provide the opportunity to explore their potential future use.

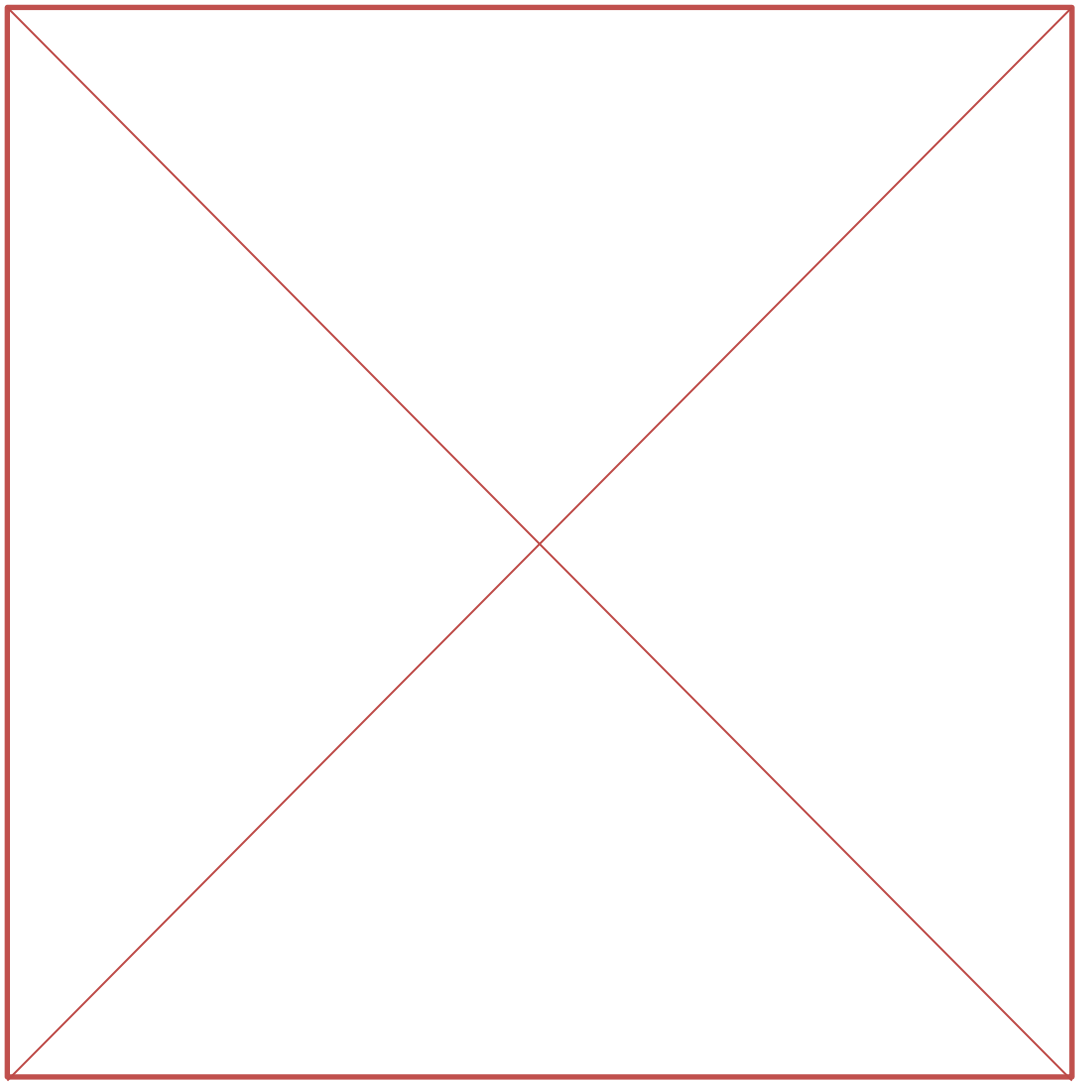


Figure 1.6 The diversity of microalgae and macroalgae.

a) *Haematococcus pluvialis* from (J. Wang *et al.*, 2013) b) *Chlorella vulgaris* from https://tinyurl.com/y49tzby8 c) *Phaeodactylum tricornutum* from (Longworth *et al.*, 2016) d) *Dunaliella salina* (Feng, Feng, *et al.*, 2014) e) *Porphyridium sp.* from https://tinyurl.com/yypapk7o f) *Scenedesmus quadricauda* from https://tinyurl.com/yxkfsele g) *Nannochloropsis oceanica* from https://tinyurl.com/yyqqsxyu h) *Volvox carteri* from (Prochnik *et al.*, 2010) i) *Saccharina latissima* from https://tinyurl.com/yxlvg8y3. Photos a) to h) are representative microalgal species whereas i) is an example of macroalgae commonly known as sugar kelp.

1.1.4 *Chlamydomonas reinhardtii* as a model system

Chlamydomonas reinhardtii has been a useful laboratory model for genetic, physiological and biochemical studies of eukaryotic cell biology for more than 60 years (Salomé and Merchant, 2019). *C. reinhardtii* is a unicellular green microalga, approximately 10 µm in diameter, found in freshwater and soil habitats (Figure 1.7). The cell structure is composed of two anterior flagella and a relatively thick cell wall made of several glycoprotein layers. Several mitochondria are present but only one chloroplast exists in a cup-shaped form. A liquid-like organelle pyrenoid as a microcompartment inside the chloroplast, which is involved in carbon fixation by concentrating Rubisco enzyme and starch accumulation. An eyespot shown as an orange organelle spot near the cell equator contains the carotenoid and facilitates cell movement in response to the light (Harris, Stern and George B., 2009). Several features of *C. reinhardtii* make it a valuable model organism, such as rapid growth rates with a doubling time of approximately eight hours; a haploid nuclear genome and a simple sexual cycle allowing the isolation and genetic analysis of both recessive and dominant mutations, and the ability to grow heterotrophically in the dark in the presence of a fixed carbon source such as acetate (Harris, Stern and George B., 2009). This last feature established *C. reinhardtii* as a model for photosynthetic studies, allowing light-sensitive non-photosynthetic mutants to be isolated and grown in acetate-containing medium (Levine, 1969; Spreitzer and Mets, 1980; Sasso *et al.*, 2018). A range of resources including an extensive mutant collection, cloned genes, genomic data, and protocols can be found at the Chlamydomonas Resource Centre (www.chlamycollection.org), and is supported by over 9,000 publications on *C. reinhardtii* in the PubMed database (pubmed.ncbi.nlm.nih.gov) and 'The Chlamydomonas Sourcebook' written by Harris et al. (2009).

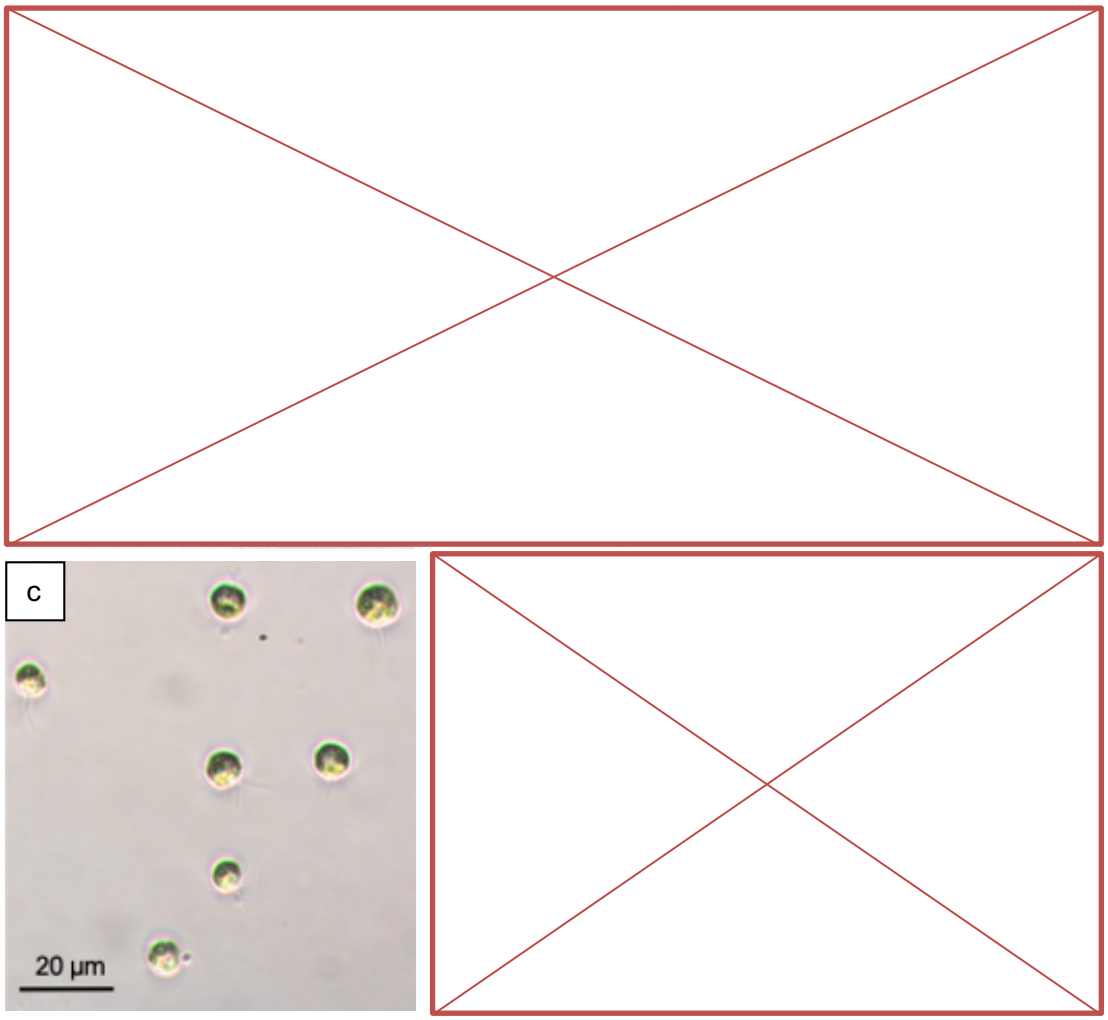


Figure 1.7 Schematics and microscopic images of the green alga *Chlamydomonas reinhardtii.*

a) Transmission electron micrograph (TEM) of a cell (source: CIL:37252, *C reinhardtii*. CIL. Data set: https://doi.org/doi:10.7295/W9CIL37252). b) A simple drawing from the TEM micrograph. The flagella are used for movement of the cell and play a part in the process of mating. Several mitochondria are present but only one chloroplast exists in a cup-shaped form. There is a pyrenoid inside the chloroplast which is involved in carbon fixation. An eyespot allows for tactic cell movements in response to light (Harris, Stern and George B., 2009). c) Light microscope image of live cells at x400 magnification. d) Scanning electron microscope image of the algae swimming with their two long flagella. The scale bar is 2 µm. The photos of a) and b) are reproduced from (Salomé and Merchant, 2019) The photo d) is from online access of Courtesy: Dartmouth College Electron Microscope Facility https://physicsworld.com/a/algae-breaststroke-is-synchronized-from-within/ accessed on 04/08/2020.

The *C. reinhardtii* life cycle is divided into two distinct parts: vegetative and reproductive (Figure 1.8). The cells undergo somatic reproduction in the vegetative cycle if nutrients are abundant with cells dividing by binary fission to create clonal progeny (Salomé and Merchant, 2019). However, they can switch to a reproductive cycle under hostile environment conditions such as nutrient limitation. *C. reinhardtii* cells can possess one of two mating type loci termed mt+ and mt-. Removal of nitrogen or other macronutrients from the growth medium and exposure to light induces gametogenesis in the laboratory with vegetative cells becoming sexually-competent gametes (Sager and Granick, 1954). The haploid gametes are isogamoous with both mating types of the same size and motile, with cells recognising the opposite mating type via glycoproteins on the flagella. This association of mating-type pairs ultimately results in cell fusion that results in a diploid cell that then forms a zygote with a thick cell wall able to withstand the harsh environment. When nutrients subsequently become available the zygote can germinate to yield a ‘tetrad’ of four haploid daughter cells that can re-enter the vegetative life cycle (Salomé and Merchant, 2019).

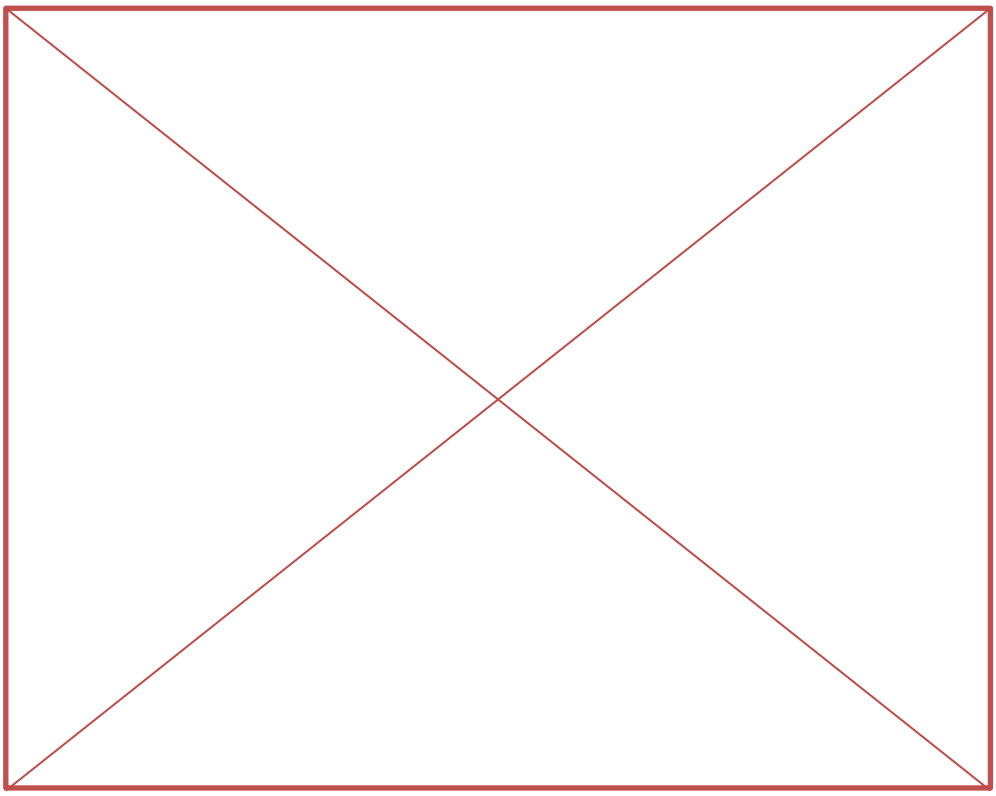


Figure 1.8 Life cycle and sexual reproduction of *C. reinhardtii*.

Gametogenesis is induced by nitrogen deprivation. Opposite mating types mt+ and mt- fuse to form a zygote during the sexual cycle. The flagella are absorbed in the zygote, causing the immobilisation state. In a suitable environment of light and nutrients, the zygote germinates to yield four haploid cells that subsequently develop into vegetative cells during the vegetative cycle (Sasso *et al.*, 2018). Image taken from (Salomé and Merchant, 2019) but originally drawn by Karen VanWinkle-Swift and published in 'The *Chlamydomonas* Sourcebook, Volume 1 (Harris, 2008).

1.2 Algae as phototrophic platforms for industrial biotechnology

Unlike heterotrophic microbial platforms such as yeast and bacteria that are traditionally used for recombinant protein production, phototrophic cells such as microalgae and cyanobacteria offer the advantage of using sunlight as an energy source and carbon dioxide as a source of carbon, rather than acquiring these from sugars or other organic feedstocks (Fabris *et al.*, 2020). In principle, production of recombinants using phototrophic platforms should be less expensive given the abundance of sunlight and CO_2, and more sustainable since they do not require agricultural production and transportation of sugars. Furthermore, many microalgae species that are naturally living under variable light conditions and with insufficient nutrient availability have evolved effective metabolic pathways are well adapted to grow in mass culture (Smetacek, 1999; Litchman, 2007) and with a high photosynthetic efficiency (Bhola et al., 2014). Algae, therefore, have a great potential for the efficient conversion of CO_2 into biomass (Benedetti et al., 2018).

Algae are usually cultivated in closed systems termed photobioreactors (PBRs) that are sited either indoors or outdoors, or in shallow open pond systems (Demirbas, 2009). Unlike crop cultivation that requires fertile land, and is often accompanied by extensive irrigation, the algal cultivation systems can be sited on low-grade land and grown in a simple nutrient medium (Khan, Shin and Kim, 2018). The open ponds are often designed as shallow 'raceways' with mixing achieved using a simple paddlewheel (Figure 1.9). Cultivation is carried out outdoors using natural light for illumination and passive diffusion of CO_2 from the atmosphere into the culture. Therefore, these systems are cheap to install and operate. The open pond system is typically used for the commercial cultivation of species such as *Spirulina* as a health food and *Dunaliella salina* for carotenoid production (Lee, 1997; Carvalho, Meireles and Malcata, 2006). However, open ponds encounter many issues relating to the non-axenic conditions that are contaminated by other foreign species and the high risk of contamination with competing species such as bacteria, fungi and other microalgae, pathogens such as

algal viruses and algal predators (Xu *et al.*, 2009). In contrast, closed photobioreactors (PBRs) (Figure 1.9) can potentially be operated under axenic conditions, illuminated either by sunlight or by artificial lighting panels of fluorescent LED lights. The major drawback of PBRs is the higher cost of construction and operation, and the greater challenge of scale-up when compared to open ponds (Ugwu, Aoyagi and Uchiyama, 2008). Growing microalgae in PBRs also confers a major advantage over open systems of bio-containment, thereby reducing the spread of genetically modified organisms (GMOs) into the ecosystem (Purton *et al.*, 2013). Furthermore, cultivation in PBRs also enables more effective monitoring of contamination and growth conditions (Beacham, Sweet and Allen, 2017).

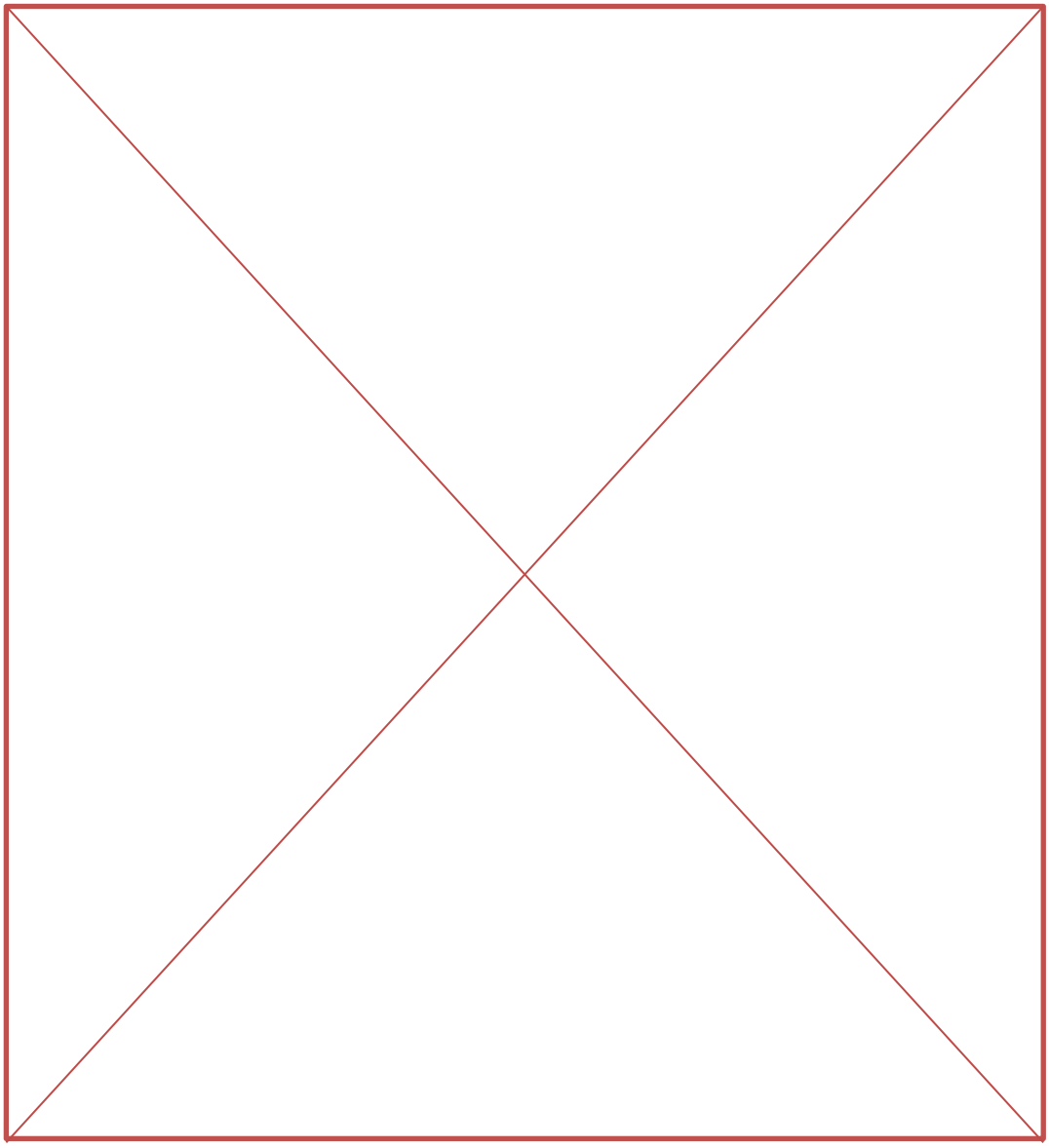


Figure 1.9 Algal cultivation systems from lab analytics to industrial scale.

a) HT24 bioreactor with 24 separate reactors housing 50 ml flasks. Produced by Algenuity, UK. b) Algem photobioreactor with two separate reactors for 1 litre flasks. Algenuity, UK. c) Polythene-based system or 'hanging bag' for indoor cultivation of 40 litres per bags as used commercially by Supreme Biotechnologies Ltd, New Zealand. d) Tubular Photobioreactors at 10,000 litres manufactured by Varicon Aqua, UK e) Raceway open ponds in various sizes >10,000 litres (Handbook of microalgal culture) Photos are taken from https://www.algenuity.com/algem, https://www.variconaqua.com/ and http://www.makebiofuel.co.uk/biofuel-from-algae/

In selecting algal species suitable for industrial biotechnology, there are some considerations to be taken such as growth rates, safety status and the availability of technologies to genetically improve (i.e. 'domesticate') these strains. A further consideration is the ability to control contamination when culturing at industrial scale, and therefore protect the algal 'crop'. Control measures normally add significant to the costs of production, and consequently extremophilic species have attracted increasing interest because they are capable of growing under extreme conditions such as high temperature, high salt or extremes of pH (Varshney *et al.*, 2015; Malavasi, Soru and Cao, 2020), allowing the growth in outdoor open ponds with a low risk of being contaminated by other species, and effectively increasing the economic benefits associated with the use of open ponds (Hirooka *et al.*, 2014; Varshney *et al.*, 2015; D'Alessandro and Antoniosi Filho, 2016). Examples of extremophile species are: *Dunaliella salina* growing in high salt concentrations (Oren, 2014), *Cyanidioschyzon merolae* growing in a thermal (40–56 °C) and acidic (between pH 0.2–4) environment (Toplin *et al.*, 2008), *Acutodesmus sp.* isolated from the wastewater of an oil refinery and has a high tolerance to CO_2 (Varshney *et al.*, 2016). Such extremophile species may have applications in industrial biotechnology, however, most of these species, except *Dunaliella salina,* have not yet been approved for GRAS (generally recognized as safe) status and therefore they are not suitable for the use in the food and animal feed industries. In addition, genetic engineering tools for the representatives of these algal groups, such as *Cyanidioschyzon merolae,* remain at the early stage of development (Fujiwara *et al.*, 2013; Krupnik *et al.*, 2018). In contrast several mesophilic strains are more established algal platforms, that have GRAS status and are already used in the industry, including the chlorophyte species *Chlorella vulgaris*, *H. pluvialis* and *C. reinhardtii* (Spicer and Purton, 2017). For a few algal species, chloroplast transformation has been already developed (Purton *et al.*, 2013) and this provides a new opportunity for high-level transgene expression and strain improvement.

1.2.1 The suitability of the algal chloroplast for genetic engineering

The use of the algal chloroplast as a biotechnological platform for the synthesis of recombinant proteins is highly attractive for multiple reasons. The genetic system of the chloroplast is considerably simpler than that of the nucleus (Purton *et al.*, 2013) with the different features listed in Table 1.1. DNA insertion into the nucleus of *C. reinhardtii* and most other microalgae occurs via non-homologous recombination resulting in transgene insertion at random loci within the genome. This results in varying levels of expression and stability of expression of the transgene as a result of so called 'position effects' (Schroda, 2019). In contrast, transgenes can be targeted into precise loci within the chloroplast genome via homologous recombination (Boynton *et al.*, 1988; Larosa *et al.*, 2012), a process of DNA exchange between strands of similar or identical nucleotide sequence, commonly used for repairing of double-strand DNA breaks (Li and Heyer, 2008) that is highly active in the *C. reinhardtii* chloroplast (Newman *et al.*, 1992). High levels of transgene expression are feasible in the chloroplast due to the polyploid nature of the genome, in the availability of cis elements from highly expressed endogenous genes such as those encoding core subunits of the photosynthetic complexes, and the absence of any silencing mechanisms (Potvin and Zhang, 2010; Purton *et al.*, 2013). Unlike in the nucleus, expression of several heterologous genes as a trans-operon is feasible in the chloroplast (Quesada-Vargas et al. 2005; Macedo-Osorio et al. 2018). Here a polycistronic mRNA is generated using a single promoter before being processed into monocistronic transcripts for translation.

The chloroplast organelle offers a suitable environment for protein folding, along with the availability of some post-translational modifications (Specht, Miyake-Stoner and Mayfield, 2010). For example, disulphide bridge formation appears to occur readily allowing the proper folding and function of proteins such as antibodies (Tran *et al.*, 2009). Furthermore, transgene containment is possible in the chloroplast because the plastid genes are uniparentally inherited, meaning that the chloroplast DNA is obtained only from the mt+ gamete after gametogenesis (Figure 1.8). The uniparental

inheritance is also important and beneficial when using mating to remove undesirable nuclear mutations by crossing back to the wide type (Mussgnug, 2015).

The chloroplast provides a sub-cellular compartment for the contained synthesis and accumulation of recombinant products since there is no mechanism for protein export from the organelle (Jin and Daniell, 2015). In the case of toxic proteins, synthesis in the chloroplast might avoid lethal effects within the cytosol of the accumulated proteins (Doron, Segal and Shapira, 2016). For example, immunotoxins carry a toxin as well as an antibody or growth factor to bind directly to target cell. Immunotoxins are highly inhibitory to cytosolic ribosomes can be accumulated in the *C. reinhardtii* chloroplast without affecting cell growth (Tran *et al.*, 2012, 2013). This natural encapsulation and concentration of toxic and other recombinant proteins, correctly folded and functional, illustrates the potential of the chloroplast as an ideal platform for industrial enzyme production.

Table 1.1 Comparison of transgene expression characteristics in the nucleus and the chloroplast of *C. reinhardtii* *

Criteria	Nuclear expression	Chloroplast expression
Gene insertion	Random (Non-Homologous End Joining as a DNA break repairing pathway)	Targeted (Homologous recombination)
DNA packaging	Extensive chromatin structure	Bacterial-like nucleoids
Silencing mechanisms	Epigenetic-related mechanisms at transcription and post-transcriptional levels	Not present
Positional effect of transgene insertion	Variation in expression level due to random insertion	No observed difference in expression level if gene inserted at a neutral locus
Post-translation modification	Disulphide bond formation and phosphorylation	Disulphide bond formation and phosphorylation
Glycosylation	Available	Not available
Product location	Possible to target to other organelles or secreted into medium	Remains in the chloroplast

*This table is modified from (Taunt, 2014)

1.2.2 A historical perspective on chloroplast engineering research

A key breakthrough that initially progressed the field of chloroplast transformation was the establishment of biolistic technology, which involves the bombardment of living cells and tissues with accelerated DNA-coated microparticles using a 'gene gun' (Boynton *et al.*, 1988). Remarkably, this technology is effective for cells or organelles that are similar in size to the standard microparticles (0.4–1.7 μm) (Bock, 2015).

Several microalgal species have been shown to be transformable via biolistics (Lapidot *et al.*, 2002; Georgianna *et al.*, 2013; Cui, Qin and Jiang, 2014). However, the first successful chloroplast transformation was achieved in *C. reinhardtii* by Boynton et al. (1988). It involved the rescue of a photosynthetic mutant carrying a deletion in the chloroplast gene *atpB.* Biolistic transformation of the mutant with a cloned wild type copy of *atpB* resulted in the restoration of photosynthesis. This technique is not constrained by the presence of cell walls in the recipient cells (Taylor and Fauquet, 2002) and the DNA-coated microparticles are delivered into the organelle through the high-velocity bombardment. The drawback of this technique is the requirement of sophisticated and expensive equipment (Mussgnug, 2015). Furthermore, the size of the microparticles restricts the technique and only works well with species with a relatively large cell size and large chloroplast such as the green algae *C. reinhardtii* (~10 μm diameter), *Platymonas subcordiformis* (~15 μm) (Cui, Qin and Jiang, 2014) and *Dunaliella tertiolecta* (~10 μm) (Georgianna *et al.*, 2013) and the red alga *Porphyridium sp.* (~15 μm) (Lapidot *et al.*, 2002). If the chloroplast is too small, for example, as seen in some industrially relevant species such as *Chlorella* and *Nannochloropsis sp.* (~2 μm cell diameter), transformation becomes intractable and requires a smaller microparticle size (Gan *et al.*, 2018) or other means of DNA delivery.

Alternative transformation methods were later developed, which further promoted chloroplast genome engineering. A simple glass bead transformation pioneered by Kindle et al. (1990) was shown to be a relatively

simple and cost-effective technique for transforming *C. reinhardtii*. This involves brief agitation of a DNA and cell suspension with an appropriate amount of glass beads, resulting in the transient formation of pores on the cell membrane, which allows entry of DNA into the cell or organelle (Economou *et al.*, 2014). Glass bead transformation requires the use of a cell wall-less recipient strain, generated by genetic mutation or autolysin treatment. Whilst feasible for *C. reinhardtii*, removal of the cell wall for some species can be technically difficult as they have a complex cell wall structure (Purton *et al.*, 2013). Other DNA manipulation, for example, electroporation or reversible electrical membrane breakdown, requires exposure of cell membranes to high-intensity electrical field pulses for short periods (Zimmermann, Pilwat and Riemann, 1975; Neumann *et al.*, 1982). Electroporation is often used for nuclear transformation (Spicer and Purton, 2017) delivering the DNA into the cytosol where it can then diffuse through nuclear pores, how delivery of DNA across multiple membranes into the chloroplast compartment is much more challenging. To-date, there have only been two reports of chloroplast transformation by electroporation: one for *C. reinhardtii* (Zhao, Shi and Zhang, 2006). and one for the marine diatom *Phaeodactylum tricornutum* (Xie *et al.*, 2014).

The selection of transgenic algae typically depends on rescue of a key metabolic function or antibiotic-resistance. The early work on chloroplast transformation was achieved by exploiting the use of cloned chloroplast genes as selectable markers. Here a photosynthetic mutant with a deletion in a key chloroplast gene was complemented with the wild type copy: for example, *atpB* (Boynton *et al.*, 1988; Kindle, Richards and Stern, 1991), *tscA* (Goldschmidt-Clermont 1991) and *psbH* (Economou *et al.*, 2014). However, these photosynthetic markers rely on the use of mutant host strains, and as such are less flexible than antibiotic resistance markers that can be used to transform wild-type cells. The most widely used antibiotic resistance gene is *aadA* from *E. coli*, which confers resistance to spectinomycin and streptomycin (Goldschmidt-clermont 1991). This dominant marker has been used extensively for the generation of engineered strains of *C. reinhardtii*, although the presence of an antibiotic resistance gene in the polyploid

plastome raises concern about its potential spread to environmental bacteria, or gut bacteria if the strains are to be used for oral delivery of vaccines, *etc.* (Esland, Larrea-Alvarez and Purton, 2018). Techniques to subsequently remove such markers via recombination between direct repeats flanking the marker are therefore valuable as they solve this problem, and also allow the repeat use of the same selectable marker in subsequent engineering steps (Fischer *et al.*, 1996) Alternatively, metabolic markers can be used for direct selection but such markers can only be used once (Esland, Larrea-Alvarez and Purton, 2018). For further discussion of selectable markers see 4.1.1. Although there have been attempts to develop additional selectable markers for *C. reinhardtii* over the years, the repertoire remains limited and this is one hinderance to the advancement of plastid biotechnology using this alga (Purton *et al.*, 2013; Bock, 2015; Doron, Segal and Shapira, 2016).

The integration of a transgene into plastome requires the proper design of the transformation vector since integration occurs via homologous recombination events mediated by chloroplast DNA sequences flanking the transgene (Purton *et al.*, 2013; Economou *et al.*, 2014; Wannathong *et al.*, 2016; Gan *et al.*, 2018). The optimal length of flanking sequences is between 0.5 and 1.0 kb (Bock, 2015). The transgene itself needs to be an 'expression cassette' that is a unique construction composed of the coding sequence of the target gene and *cis* sequences that mediate expression in the chloroplast. These DNA elements are a promoter, 5'UTR and 3'UTR for efficient transcription and translation (Figure 1.10) (Purton *et al.*, 2013). To avoid disrupting the function of any endogenous gene, the gene cassette is targeted into a neutral site within the plastome by the correct choice of flanking elements. Validated insertion sites for *C. reinhardtii* are listed in Table 1.2.

A second requirement for the establishment of a stable transplastomic line is to ensure that all copies (between 80-100) of the highly polyploid genome in the chloroplast contain the introduced transgene, and that no copies of the untransformed plastome remain (Bock, 2015). It is assumed that the initial integration events following DNA delivery into the chloroplast

results in the transformation of only one or a few copies of the plastome. The chloroplast is therefore initially heteroplasmic with a mixed population of predominantly untransformed plastomes and with a few transformed copies (Day and Goldschmidt-Clermont 2011). Selection for segregation and inheritance of the transformed copies therefore needs to be maintained during consecutive rounds of cell division. Whilst cells are heteroplasmic they are genetically unstable and without selection random segregation may lead to the loss of the transformed copies and a return to the original untransformed homoplasmic state. It is therefore necessary to keep restreaking the cells (approximately 3-4 rounds) on selective media until all untransformed copies have been lost. Once homoplasmy is reached then the selection can be removed (Maliga and Bock, 2011; Bock, 2015).

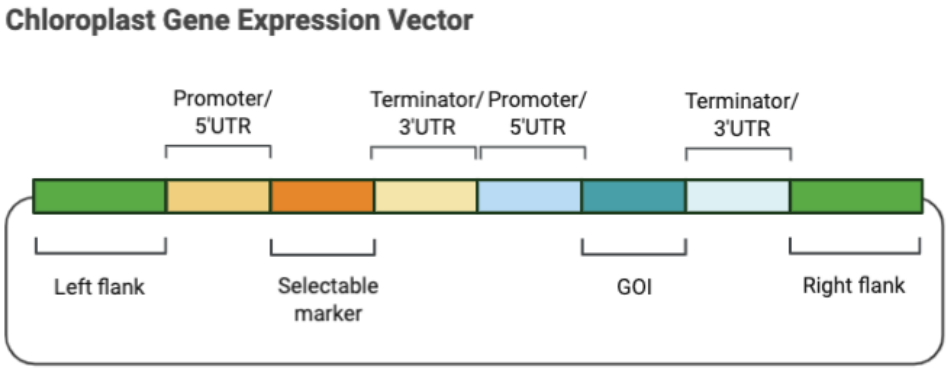


Figure 1.10 Essential elements required for integration of an expression cassette into the plastome.

Left and right flanks are derived from endogenous plastome sequence and mediate the homologous recombination events that define the insertion site within the plastome. The selectable marker and gene of interest (GOI) are placed under the control of a promoter/5'UTRs and terminator/3'UTRs that can be from endogenous or heterologous genes.

Table 1.2 The available integration sites in the *C. reinhardtii* chloroplast for homologous recombination of transgene

Integration site	**Plasmid**	**Reference**
***psbA* – 16S rRNA**	P322	(Barnes et al. 2005; Manuell et al. 2007)
Inverted repeat – *atpB* 3'UTR	patpint-cg11 (atpB-int)	(Nickelsen *et al.*, 1994)
***psbA* – 5S/23S**	pLM7 (IR-int)	(Michelet *et al.*, 2011)
psbH* – *psbN	p72B	(Bateman and Purton, 2000)
***tscA* – inverted repeat**	p71	(Kindle, Richards and Stern, 1991)

1.3 Tools and techniques for algal chloroplast engineering

Algal chloroplast engineering has been most widely demonstrated using *C. reinhardtii* with over 100 different recombinant proteins successfully produced in this species (Dyo and Purton, 2018; Taunt, Stoffels and Purton, 2018). However, whilst molecular tools and techniques for engineering the plastome are now advanced, almost all reports are focussed on monocistronic expression (i.e. only one gene expressed from a single promoter) and attempts to introduce multiple genes or transgenic operons are only just beginning (Macedo-Osorio et al. 2018; Larrea-Alvarez and Purton 2020). Efficient chloroplast expression requires a wide selection of promoters and untranslated regions (UTRs) along with techniques for codon optimisation to match the AT-rich codon preferences within the *C. reinhardtii* chloroplast (Purton *et al.*, 2013; Gangl *et al.*, 2015). Furthermore, mechanisms for inducible and tunable expression of toxic transgenes or those transgenes that place a strong metabolic burden on the cell are a key element in the further development of this platform. To-date, such inducible systems are rather limited but are discussed in section 1.3.2 below. The general procedure for chloroplast engineering is summarised in Figure 1.11.

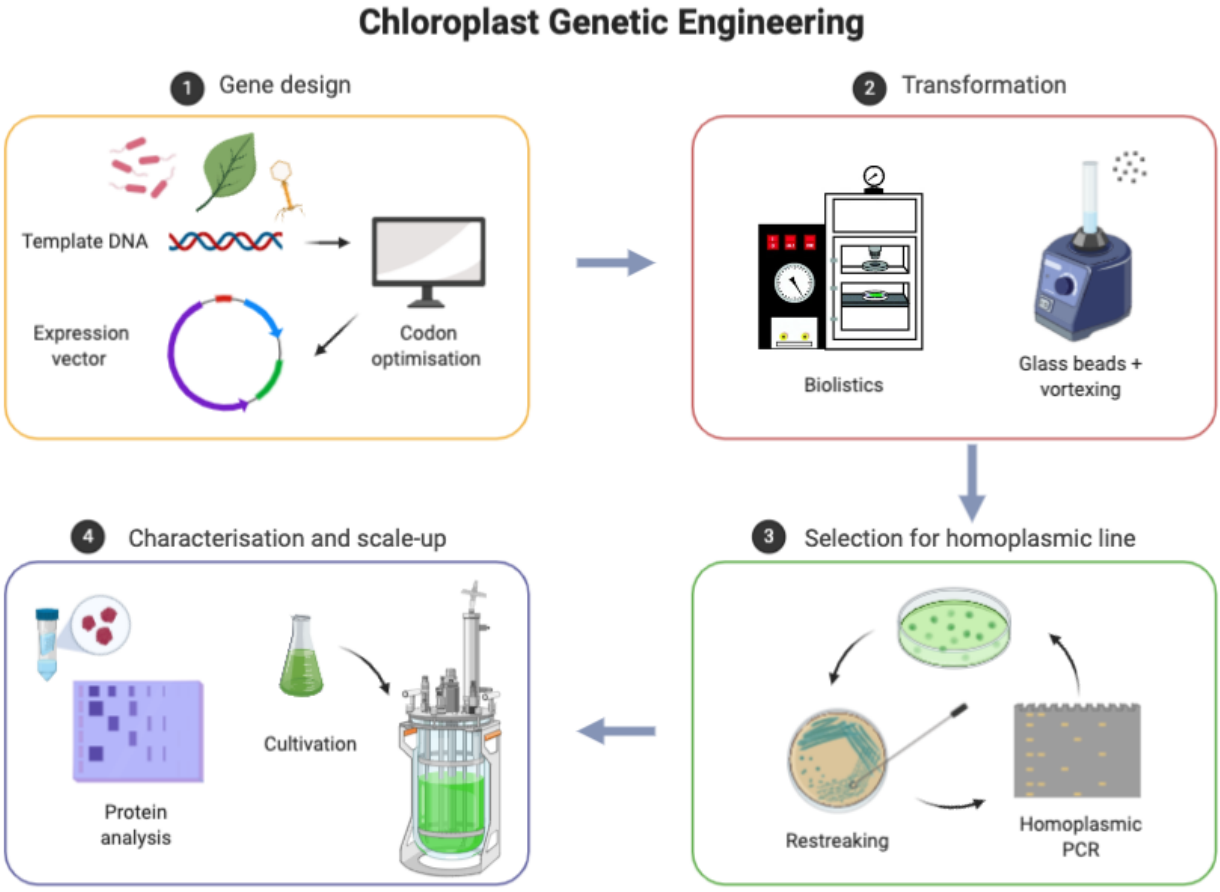


Figure 1.11 Workflow of chloroplast genetic engineering.

The template DNA sequence is based on data from other organisms, codon-optimised for highest expression and then made synthetically. It is then cloned into an established expression vector or assembled with the necessary DNA parts (promoter, UTR's, etc.) using a GoldenGate assembly method. The plasmid can be introduced into the chloroplast of *C. reinhardtii* by two common DNA delivery methods: biolistic or glass bead-mediated transformation. Transformant colonies are selected and restreaked on appropriate medium for several rounds to achieve a homoplasmic state, which is confirmed by PCR. Representative transformants are characterised and verified by protein analysis and can be cultivated at large-scale in a bioreactor.

1.3.1 Promoter selection and the use of UTRs

The most efficient promoters and UTRs are typically derived from highly expressed endogenous genes in the *C. reinhardtii* chloroplast, such as *atpA*, *psaA* and *psbA* (Bateman and Purton, 2000; Sun *et al.*, 2003; Manuell *et al.*, 2007; Mayfield *et al.*, 2007; Rasala *et al.*, 2010, 2011; Michelet *et al.*, 2011). One drawback of using these endogenous elements is the increased possibility of genome instability through homologous recombination with the corresponding sequences on the native gene in the plastome. Another problem is the titrating out of *trans*-acting factors that bind to specific UTR sequences on the transcribed gene and mediate mRNA stability or efficient translation initiation (Dyo and Purton, 2018). Negative feedback regulation of the translation of specific photosynthetic genes might also limit the translation of a transgene using the same 5'UTR. For example, using the *psbA* 5'UTR is not ideal for high transgene expression although *psbA* encodes the abundant D1 protein of photosystem II. A gene of interest (GOI) placed under the control of *psbA* promoter/5'UTR was negatively affected by D1 autoregulation, reducing the translation rate of the mRNA. (Minai *et al.*, 2006; Manuell *et al.*, 2007). It is best to improve the expression with chimeric constructs by using a fusion of promoters and 5'UTRs from different genes. For example, the 16S promoter from the *rrnS* gene fused to the *atpA* 5'UTR was shown to improve heterologous gene expression (Rasala *et al.*, 2011). Available promoters for chloroplast expression are listed in Table 1.3.

As mentioned above, the choice of UTR regions has been shown to affect the level of gene expression through post-transcriptional regulation including mRNA cleavage, mRNA stabilisation and degradation, intron splicing and translation initiation (Del Campo, 2009). The 5' UTRs of many of the most abundant chloroplast transcripts are individually regulated by nuclear-encoded factors that show allow target-specific binding, typically to the 5'UTR (Choquet and Wollman, 2002). For example, NAC2 binds only to the 5'UTR of *psbD* mRNA for the specific control of *psbD* translation (Schwarz *et al.*, 2007). As a general rule, a priority order in terms of choice of

cis elements is 5'UTR > promoter > 3'UTR in order to maximise expression of transgenes (Barnes *et al.*, 2005).

Table 1.3 Endogenous promoters/5'UTR currently available for the *C. reinhardtii* chloroplast gene expression

Promoter	Source	Reference
rbcL	Large subunit of ribulose bisphosphate carboxylase	(Bateman and Purton, 2000; Dreesen, Hamri and Fussenegger, 2010)
psbA	Photosystem II reaction center (D1)	(Rasala *et al.*, 2011)
psbD	Photosystem II reaction center (D2)	(Manuell *et al.*, 2007)
petA	cytochrome b_6f subunit	(Young and Purton, 2014)
petD	cytochrome b_6f subunit	(Sakamoto, Kindlet and Stern, 1993)
atpA	ATPase alpha subunit	(Barnes *et al.*, 2005)
psaA-exon 1	Photosystem I reaction centre	(Michelet *et al.*, 2011)
16S rRNA	*rrnS* gene encoding the 16S rRNA	(Pourmir, Noor-Mohammadi and Johannes, 2013)

1.3.2 Regulation systems for chloroplast gene expression

Whilst transgenic expression in the algal chloroplast has progressed, there is still a lack of development in the regulation of transgenes. This is essential if the product is toxic or induces a metabolic burden on the cells. It can be observed as an inability to select for transgenic lines following transformation or a restriction of growth performance. Inducible expression systems aim to overcome this issue by allowing production of colonies or generation of biomass whilst expression of the transgene is suppressed. Once the biomass is produced, the expression of the transgene can be induced and the product synthesised. Regulatory systems that act at the transcriptional level or translation initiation level have been described by Kato et al. (2007) and Rochaix et al. (2014a) (Figure 1.12). The first method exploits the use of the Lac regulatory system in *E. coli*, in which expression is triggered by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a culture. The IPTG binds to the lac repressor protein synthesised in the chloroplast, and prevents it from binding to operator sequences within the promoter region of a gene cassette. In the absence of IPTG, the binding of the Lac repressor to the promoter region prevents RNA polymerase from initiating transcription. However, this transcriptional repression is relieved after addition of IPTG as an inducer. The second system for expression control involves the use of a nuclear-encoded NAC2 factor where the gene is under the regulation of the *CYC6* promoter. NAC2 is only produced in the absence of copper due to no repression of the *CYC6* promoter. The NAC2 trans-acting factor is targeted into the chloroplast where is essential for the translation of the *psbD* gene. If a transgene is placed under the control of the *psbD* 5'UTR, it is subject to control by NAC2 factor and can be repressed by the addition of copper to a culture (Surzycki *et al.*, 2007; Rochaix, Surzycki and Ramundo, 2014).

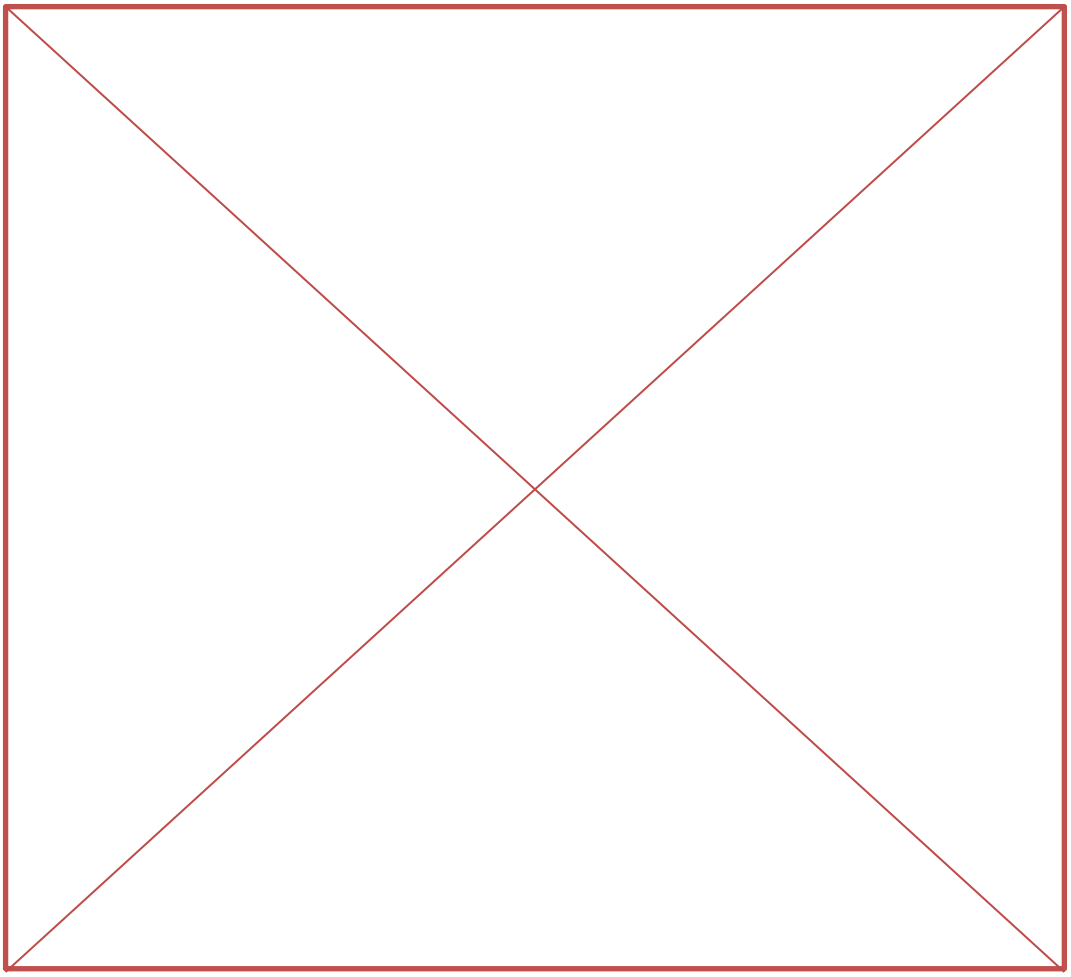


Figure 1.12 Strategies for the regulation of transgene expression in the *C. reinhardtii* chloroplast.

(a) upper panel, represents the NAC2 system in which the gene of interest (GOI) is placed under the control of the *psbD* 5'UTR. Binding of the nuclear-encoded NAC2 is required for the translation of the target mRNA, but NAC2 expression is then regulated by the copper-suppressed *CYC6* promoter. By placing the GOI under *psbD* 5'UTR therefore allows control by the presence or absence of copper in the culture (Surzycki *et al.*, 2007; Rochaix, Surzycki and Ramundo, 2014). (b) lower panel, the synthesis of the GOI is regulated by a modified promoter with an operator sequence as a target site of the LacI repressor. The binding of the LacI repressor to the operator sequence blocks the expression of the GOI. The Lac repressor is encoded by a constitutively-expressed *lacI* gene. However, this repression can be relieved in the presence of IPTG (Kato *et al.*, 2007) that acts as an inducer. Figure is reproduced from (Purton *et al.*, 2013).

An alternative system has been developed by the Purton lab using a codon-reassignment strategy as shown in Figure 1.13 (Young and Purton, 2016), combined with a temperature sensitive tRNA to prevent synthesis of a full-length recombinant protein under the higher temperature used for colony selection or biomass production (Young and Purton, 2018). This system exploits a chloroplast stop codon (UGA) that is not used in the *C. reinhardtii* plastome and repurposes it as a codon for tryptophan by introducing into the plastome a second copy of the tRNA-Trp gene (*trnW*) with an anticodon modified to recognise UGA. This second copy of the tRNA gene ($trnW^{UCA}$) is completely orthogonal as no endogenous chloroplast gene uses UGA and is therefore unaffected by the tRNA. However, replacement of one or two tryptophan codons in the transgene with the stop codon therefore allows the complete translation exclusively in the chloroplast (Young and Purton, 2016). Another modification of $trnW^{UCA}$ was shown to result in a temperature-sensitive version of the tRNA such that it was functional only at the lower temperature (Young and Purton, 2018). The synthesis of a full-length recombinant protein can therefore be regulated by adjusting the culture temperature as demonstrated in Chapter 5.2.2.

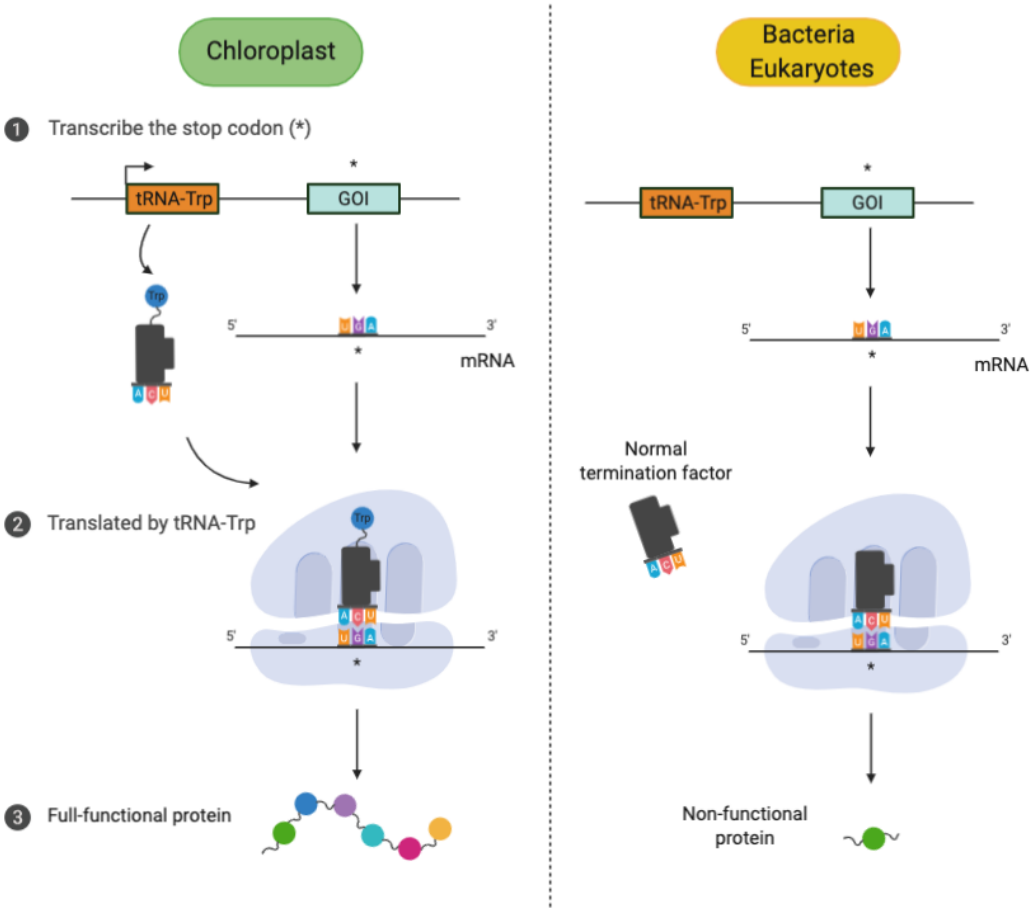


Figure 1.13 Codon-reassignment strategy for the regulation of gene expression.

A tryptophan codon is altered to a stop codon UGA within the gene of interest (GOI). There is a co-expression of a tRNA-Trp that encodes a modified tRNA able to translate the stop codon as a tryptophan, In the chloroplast, the full translation of the mRNA results in the synthesis of a functional protein. In bacteria and other eukaryotes, the tRNA-Trp is not recognised by the translational machinery. The stop codon is read as normal by termination factor and results in incomplete translation and a non-functional protein.

1.3.3 Codon optimisation

Since most amino acids can be decoded by several codons, every organism therefore displays a degree of codon preference in which some codons are used more often than others (Campbell and Gowri, 1990; Mayfield *et al.*, 2007). In the *C. reinhardtii* chloroplast, the codons with the third position as A or T are used much more extensively than those with G or C as the third base (Nakamura, Gojobori and Ikemura, 1998; Mayfield *et al.*, 2007). Efficient translation of transgenes introduced into the chloroplast is therefore assumed to require a similar preference, and therefore a key part of designing a synthetic gene is codon optimisation. The process of codon optimization is normally based on the codon adaptation index (CAI) (Xia, 2007). This is a quantitative analysis of the synonymous codon usage bias for a DNA sequence. The index measures codon usage similarities between the transgene and a reference gene set (Puigbò, Bravo and Garcia-Vallve, 2008). This CAI should be obtained from the dataset of genes that are highly expressed in the chloroplast, rather than the total of codon usage for all chloroplast genes (Surzycki *et al.*, 2009). The index value ranges from 0 to 1, with 1 meaning that a gene contains the most frequently used synonymous codon in the reference set in all codon positions (Puigbò, Bravo and Garcia-Vallve, 2008). It is assumed that a high CAI for a transgene increases the chance of it being translated efficiently by compatible tRNAs and reduces amino acid misincorporation and truncation of the polypeptide chain (Shamriz and Ofoghi, 2016), and several studies comparing non-optimised and optimised transgenes in the *C. reinhardtii* chloroplast support this idea (Franklin *et al.*, 2002; Wu *et al.*, 2011). Design of a transgene coding sequence should therefore utilise optimisation software such as CUO developed in the Purton lab (Purton *et al.*, 2013) or COOL (Chin, Chung and Lee, 2014).

1.3.4 Protein degradation

Even though a transgene cassette is successfully inserted in the plastome and highly expressed, protein accumulation may occasionally not be detected. One of the factors could be the rapid degradation of the

recombinant protein by endogenous proteases. In the chloroplast, there are three types of proteases present and these are responsible for the turnover of native and recombinant proteins (Nishimura, Kato and Sakamoto, 2016). Most of the proteases are similar to ones present in bacteria and shown to be energy-dependent with a need for ATP, such as the Zn-metallo-protease FtsH and the serine-type Clp family (Adam, Rudella and van Wijk, 2006; Liu, Yu and Rodermel, 2010). In contrast, the proteases of the Deg/HtrA family of serine endopeptidases do not require ATP (Huesgen, Schuhmann and Adamska, 2009; Sun *et al.*, 2010). The proteases are nuclear-encoded, except ClpP1 that is encoded in the chloroplast genome in *C. reinhardtii* (Ramundo *et al.*, 2014). Protease activity and its impact on recombinant protein levels in the algal chloroplast has not been studied extensively. However, the role of ATP is revealed by the addition of cyanide m-chlorophenylhydrazone (CCCP) to transgenic cultures. This chemical prevents ATP synthesis and stops the energy supply to proteases, preventing protein degradation. Investigations of ATP-dependent proteases may help to improve the yield and understand the degradation of recombinant proteins (Surzycki *et al.*, 2009). Due to the simultaneous events of protein synthesis and proteolysis, equal attention needs to minimising protein degradation as to maximising gene expression.

1.3.5 Applications and future prospects for algal biotechnology

Algae have been shown to have potential in a wide range of industrial sectors including food, livestock feed, feed for aquaculture, cosmetics, and for production of therapeutic proteins, nutraceuticals and biochemicals (Gimpel, Henríquez and Mayfield, 2015; Doron, Segal and Shapira, 2016; Shamriz and Ofoghi, 2016; Dyo and Purton, 2018; Esland, Larrea-Alvarez and Purton, 2018). The use of the algal chloroplast as a light-driven platform for making high value recombinants is particularly appealing, and some examples of therapeutic proteins made in *C. reinhardtii* are vaccines against malaria (Jones *et al.*, 2013), *Staphylococcus aureus* infection (Dreesen, Hamri and Fussenegger, 2010) and tumours caused by human papilloma virus (Demurtas *et al.*, 2013). In the last two examples, oral administration

appeared to trigger an immune response in mice (Dreesen, Hamri and Fussenegger, 2010; Demurtas *et al.*, 2013). Other therapeutic proteins such as antibodies have been generated in the chloroplast. The first version of chloroplast antibodies was a large single chain structure against the Herpes simplex virus glycoprotein D (Mayfield, Franklin and Lerner, 2003). Human IgG antibodies were further generated in the chloroplast against anthrax and Hepatitis B Virus surface protein (Tran *et al.*, 2009). Similarly, immunotoxins in which an antibody is fused to a cytotoxic protein have also been produced in the *C. reinhardtii* chloroplast. This work was aimed at the treatment of tumours, in which the immunotoxin binds and destroys B cell lymphoma cells (Tran *et al.*, 2012, 2013). All these examples highlight the potential of the algal chloroplast as a low-cost platform for producing therapeutic proteins.

In addition to the medical application for humans, the microalgae are attractive for the low-cost production of orally-delivered vaccines for aquaculture. By incorporating the whole algae carrying the vaccine into the feed, direct injection into the animal is avoided and this allows early vaccination of small, juvenile animals and avoids the stress of handling and injection incurred during conventional vaccination (Charoonnart *et al.*, 2019; Kwon *et al.*, 2019). Furthermore, microalgae contain a high content of natural compounds, such as fatty acid, protein, carbohydrates, terpenoids and vitamins, so whole alga offers nutritional supplements for animal feed, (Figure 1.14) and these could be further improved through metabolic engineering of the chloroplast. These applications have been demonstrated in *C. reinhardtii* and few other species, but many other potential applications of the wide diversity of algae remain unexplored (Doron, Segal and Shapira, 2016; Shamriz and Ofoghi, 2016; Esland, Larrea-Alvarez and Purton, 2018).

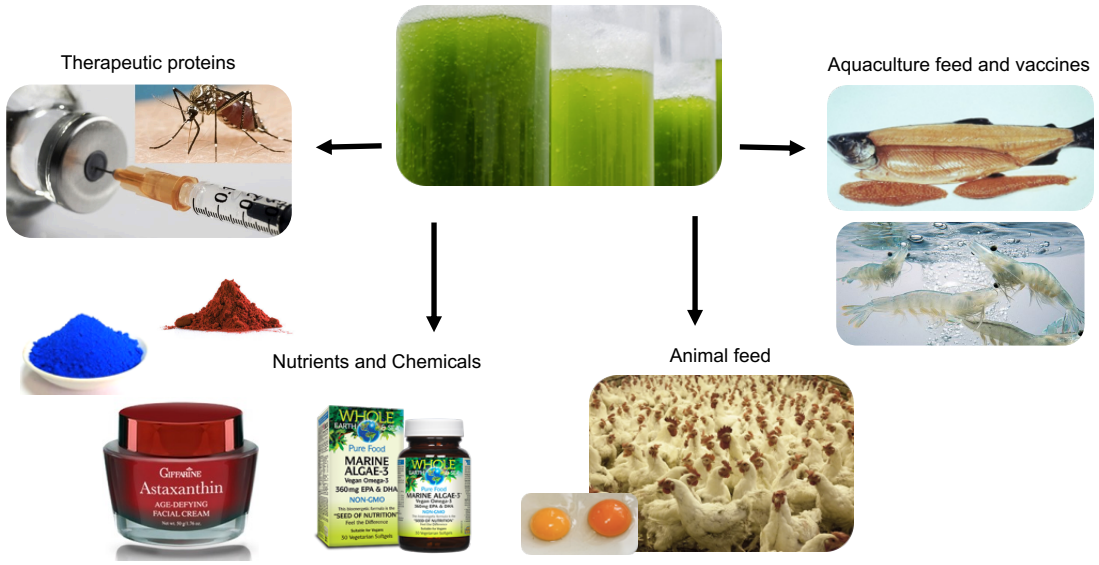


Figure 1.14 Algal applications in a range of products.

The algal chloroplast was used for synthesis of therapeutic proteins such as a subunit vaccine against malaria (Gregory *et al.*, 2012). Freeze-dried microalgae carrying such a vaccine can be mixed with animal feed to protect fish and shrimp from disease (Charoonnart, Purton and Saksmerprome, 2018; Charoonnart *et al.*, 2019). Some high-value chemicals and pigments such as astaxanthin, carotenoids, phycocyanin and antioxidants, produced naturally in algae has been used in cosmetics and as supplementary products (Gangl *et al.*, 2015; Scaife and Smith, 2016), and microalgae with a high nutrient and pigment content are used as a food additive to significantly increase the colour of egg yolk (Zahroojian, Moravej and Shivazad, 2011). Ketocarotenoids from microalgae are used in the aquaculture feed industry as a natural food colouring agent for salmon. Photos are taken from: https://tinyurl.com/y6yhm4n7 and https://face2faceafrica.com/article/malaria-vaccine-.

Over the past decade, there have been efforts to extend the chloroplast technology to a wider range of algal species (Khan, Shin and Kim, 2018). Whilst chloroplast engineering in *C. reinhardtii* has been the most studied, the technology should be transferable in theory from this model organism to other more commercially relevant algal species (Purton *et al.*, 2013). However, barriers to successful transfer include the challenge of DNA delivery that needs to be optimised for a particular species as a result of differences in cell architecture: a thick cell wall or a much smaller size of chloroplast in species such as *Chlorella vulgaris* and *Nannochloropsis* sp. (Purton *et al.*, 2013; Gimpel, Henríquez and Mayfield, 2015). The suitability of selectable markers and the identification of neutral insertion sites in the plastome are also challenges for some species and need to be studied further before chloroplast transformation becomes routine (Al-Hoqani, Young and Purton, 2017).

To advance chloroplast engineering, synthetic biology has been increasingly applied to facilitate the transference of technology to new species or the design and introduction of novel biosynthesis pathways into chloroplasts (Gangl *et al.*, 2015). At a basic level, synthetic biology allows the research community to generate a standard set of design tools, with standardised DNA parts and an agreed assembly procedure that allows rapid construction of transgene cassettes. These parts and assembled cassettes can be deposited in repositories and shared amongst the research community, accelerating research and enhancing the capability for reproducing published results (Peccoud *et al.*, 2011). Furthermore, the tools allow the integration of genetic circuits into the chloroplast to precisely tune and modulate transgene expression (Galdzicki *et al.*, 2014; Gimpel, Henríquez and Mayfield, 2015).

Whilst the application of synthetic biology tools should promote technology transfer among species, another major challenge is applying the technology for the over-production of high value metabolites (Mayfield *et al.*, 2007; Rasala *et al.*, 2011). This can be achieved through knowledgeable metabolic engineering by adjusting the flux of endogenous pathways and

coupling this to new biosynthetic enzymes (Yadav *et al.*, 2018). The technique requires a detailed understanding of cellular pathways for better production of target metabolites. However, it is important to examine the effect of metabolic manipulation on the growth rate and biomass yield as it may have a negative impact on metabolic burden, and sophisticated regulation of the genetic circuitry might be required to produce commercially viable strains (Yadav *et al.*, 2018).

1.4 Aims and Objectives

A review of the literature makes it clear that the green alga *C. reinhardtii* has potential as a low-cost platform for synthesis of recombinant products given the high growth rate and minimal requirements for cultivation. However, there remains a paucity of molecular tools and strategies needed to really advance chloroplast engineering in *C. reinhardtii* and allow its development as a commercial platform for industrial biotechnology.

Most strategies focus on improving the yield of bioproducts but not many approaches have been made to control the contamination via metabolic engineering or genetic engineering. However, one effective method is to metabolically engineer the target organism for the ability to convert a non-metabolisable chemical into the bioavailable form of an essential nutrient so that the contaminating species cannot compete with the target organism. This allows a selective advantage for growing the desirable organism.

Phosphite (Phi) is one of those non-metabolisable chemicals that most organisms cannot use as phosphorus (P) source due to a lack of enzyme for the conversion of phosphite to phosphate. However, expression of the *ptxD* gene that encodes a NAD(P) dependent phosphite oxidoreductase from *Pseudomonas stutzeri* WM88 can allow the genetically modified organism to oxidise phosphite to phosphate. This led to a strain improvement strategy to create an microalga expressing *ptxD* for Phi utilisation.

This study additionally exploits the metabolic engineering to advance the chloroplast of *Chlamydomonas reinhardtii* as commercial platform. One of the potential valuable chemicals is limonene, a monoterpene with a simple structure used in fragrance industry. Limonene is formed by condensation of two common precursors that can be found in the algal chloroplast. Therefore, limonene synthase (LS), an enzyme to produce limonene, was introduced in the *C. reinhardtii* chloroplast to create engineered strains synthesising limonene.

With this in mind, the following aims formed the basis for the research presented in this thesis:

1. To develop a strain improvement strategy enabling the microalga *Chlamydomonas reinhardtii* to grow on non-metabolisable Phi, based on the expression in the chloroplast of the bacterial gene *ptxD* encoding an NAD(P)-dependent phosphite oxidoreductase in the chloroplast.

2. To demonstrate the applications of *ptxD* as a selectable marker for the chloroplast transformation that increases the repertoire of molecular tools, as well as to advance new strategies with *ptxD* for large-scale cultivation such as simple methods for ensuring 'crop protection' under non-sterile condition.

3. To progress metabolic engineering and demonstrate potentials and challenges relating to monoterpene production in the algal chloroplast, the creation of engineered strains for regulated biosynthesis of the valuable compound limonene has been attempted.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Strains and growth conditions

2.1.1 *Escherichia coli*

Escherichia coli DH5α with genotype (F-, (φ80*lacZΔM15*), Δ(*lacZYA-argF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk-, mk+), *supE44*, *thi-1*, *gyrA96*, *relA*, λ) (Hanahan, 1983) was the recipient strain for all bacterial DNA amplification and gene expression work. The strain was supplied by Clonetech (Saint-Germain-en-Laye, France) and was grown on Luria-Bertani (LB) medium containing 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) bacto-yeast extract (Difco) and 0.17 M NaCl (Bertani, 1951). *E. coli* colonies were cultivated on LB plates with 1.5% (w/v) agar, supplemented with appropriate antibiotics when necessary, and incubated overnight at 37 °C. Liquid cultures were inoculated from a single colony with a sterile inoculating loop and grown at 37 °C for 24 h in a shaking incubator at 200 rpm. Long-term storage was achieved by frozen glycerol stocks mixing 700 µl of overnight *E. coli* culture and 300 µl 50% (v/v) glycerol, and were stored at –80 °C.

2.1.2 *Chlamydomonas reinhardtii*

The *C. reinhardtii* strains used in this work are detailed in Table 2.1. Stocks were maintained on 2% agar plates containing Tris-acetate phosphate (TAP) medium (Harris, Stern and George B., 2009) under dim light (5–10 µE/m^2/s) at 20 °C. The strains can be found in the Chlamydomonas Resource Centre (www.chlamycollection.org/) listed under their culture collection codes.

Table 2.1 A summary of *C. reinhardtii* strains in this study

Strain name	Culture Code	Comments	Reference
TN72	CC-5168	Non-photosynthetic recipient used for indirect selection of *ptxD* transformants by glass bead transformation (*cw15*, *psbH::aadA*, mt+)	(Wannathong *et al.*, 2016)
Wild-type	CC-1690	Used to demonstrate direct selection with *ptxD* by biolistic transformation	(Sager, 1955)
cw2	CC-1731	Used to demonstrate direct selection with *ptxD* by glass-bead transformation	www.chlamycollection.org/product/cc-1731-cw2-mt/
H1	N/A	TN72 transformed with empty pSRSapI vector to restore *psbH* function	(Young and Purton, 2014)
***PtxD*1**	N/A	TN72 transformed with *ptxD* gene to confer a capability of phosphite oxidation	(Changko *et al.*, 2020)
TN72::pWUCA2	N/A	TN72 transformed with empty pWUCA2 vector used as a recipient for the direct selection of *ptxD*	(Young and Purton, 2016)

2.1.3 *Chlamydomonas reinhardtii* growth conditions

C. reinhardtii strains were grown mixotrophically in tris-acetate phosphate (TAP) medium or grown phototrophically in Sueoka high-salt (HSM) medium. The media compositions are listed in Appendix 2 and Appendix 3, respectively. TAP medium was prepared by mixing three stock solutions as follows: 4 x Beijerinck salts stock solution (0.3M NH_4Cl, 14mM $CaCl_2$ and 16mM $MgSO_4$), 1M (K)PO_4 stock solution (1M K_2HPO_4 titrated to pH 7.0 with 1M KH_2PO_4) and trace metal mix stock solution (180 Mm H_3BO_3, 77mM $ZnSO_4$, 26mM $MnCl_2$, 18 mM $FeSO_4$, 7mM $CoCl_2$, 6mM $CuSO_4$, 0.1M Na_2EDTA, 0.9mM $(NH_4)_6Mo_7O_2$). The HSM medium was used for phototrophic selection of TN72 transformants, and was prepared in a similar manner to TAP medium but using 2X PO_4 stock solution (80mM K_2HPO_4 and 50mM KH_2PO_4) instead of 1 M (K)PO_4 stock, and adjusted to pH 6.9 with KOH rather than acetic acid.

Liquid cultures of *C. reinhardtii* strains were grown under continuous illumination with a light intensity of 50–100 μE/m^2/s, at 25 °C and shaking at 120 rpm (New Brunswick™ Innova, Germany). The non-photosynthetic mutant strain TN72 is sensitive to the light so was grown under dim light conditions of approximately 5–10 μE/m^2/s. All *C. reinhardtii* strains were restreaked every 6-8 weeks as recommended (Harris, Stern and George B., 2009).

2.2 Quantification of cell density

The measurement of microalgal cell density was carried out using a haemocytometer (Axio Scope. A1, Zeiss) under a light microscope at a magnification of X400. The cell counting procedure required cell immobilization by adding 10 μl tincture of iodine (19.7 mM iodine in 95 % (v/v) ethanol) per 1 ml sample. The cell density was measured in cells per ml calculated by multiplying the average count with the dilution factor and by 10^4. The optical density of liquid cultures of *C. reinhardtii* was measured at

750 nm with a 1 cm path length cuvette using a spectrophotometer (Unicam UV/Vis Spectrometer UV2).

2.3 Growth analysis

2.3.1 'Spot tests' for growth on solid media

The growth of *C. reinhardtii* on compounds of interest, such as Phi or antibiotics, was investigated using a spot test technique. A small volume (~5 µl) of a previously-grown liquid culture was spotted onto an agar plate supplemented with the desirable compounds. The technique allows the comparison of the growth of the transformant lines to that of a control strain. To test whether *PtxD* transgenic lines can grow on Phi medium, liquid cultures were grown in TAP medium for 5 days and then transferred to P-free TA medium for 3 days to deplete internal preserved polyphosphate (Pi) when necessary. The cell samples were prepared to equal optical cell density measured at 750 nm by resuspending in TA medium (Changko *et al.*, 2020). The cells were diluted in ten-fold dilutions (0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and were spotted onto Pi or Phi solid media with 2% agar. The plates were incubated at a light intensity of 50 µE/m^2/s at 25 ˚C.

2.3.2 Small-scale photobioreactor

A commercial lab-scale photobioreactor, Algem® (Algenuity, UK), was used to analyse the growth rate of *C. reinhardtii* transformants in comparison to that of the wild type or control strain. A unit of an Algem® bioreactor contains two equivalent chambers where parameters such as the light intensity, light wavelength, pH and temperature can be precisely controlled. To assess the growth rate in the liquid Pi and Phi media, a stationary-phase inoculum of 25 ml previously grown in the Phi medium was seeded to a flask containing 400 ml of the appropriate media. The conditions were kept consistent with a light intensity of 200 µE/m^2/s, 25 °C, pH 7.0 and shaking at 120 rpm. Cell growth was monitored by the measurement of optical density at 740 nm and the readings were automatically saved every 10 minutes.

Recorded data were subsequently analysed using built-in software or Microsoft Excel for generating graphs.

2.3.3 Laboratory large-scale photobioreactor

The scale-up production was evaluated in a large volume of culture in a 'hanging bag system' originally designed by the Cawthron Institute, New Zealand (Taunt, Stoffels and Purton, 2018). The strain NNV::ptxD was grown in single-use polythene tubular bags (provided by Supreme Health, New Zealand), in which each bag contained 20 litres of Pi or Phi medium. An inoculum of 500 ml was grown to stationary phase in TA-Phi under standard growth conditions. Every bag was later filled with the 500 ml of inoculum, along with 1 ml of a contaminant stock of natural bacteria and fungi that was generated by leaving the TAP medium flask to become unintentionally infected in the open air for several days at 25 °C in the lab (Changko *et al.*, 2020). The hanging bags were sparged at the bottom with pump-sterilised air and illuminated at 100 $\mu E/m^2/s^1$ using Osram Lumilux Cool daylight fluorescence tubes. Cultures were cultivated for 13 days at 25 °C and then the algal/bacterial populations assessed by particle size distribution using a Mastersizer 3000 laser diffraction particle size analyser (Malvern Panalytical Ltd., UK).

2.4 DNA manipulations

2.4.1 Plasmids

A *ptxD* expression plasmid was generated based on the insertion of the *ptxD* coding sequence into the chloroplast expression vector pWUCA2 (Young and Purton, 2016). The synthetic gene was codon-optimised for the chloroplast of *C. reinhardtii* (Appendix 6). The gene was designed with two TGG→TGA codon alterations and generated by GeneArt (ThermoFisher Scientific). Two restriction sites of *SapI* and *SphI* were added immediately upstream and downstream, respectively, of the *ptxD* sequence for cloning purposes (Young and Purton, 2016).

To generate a *ptxD* plasmid for the retro-fitting experiment, the whole gene cassette (*psaA-1* promoter/5′UTR-*ptxD-rbcL* 3′ UTR) was amplified from pWUCA2+*ptxD* plasmid by PCR. This PCR product carried two restriction sites of *Mlu*I and *Mfe*I at the upstream and downstream ends, respectively, allowing the cloning into the *Mlu*I and *Eco*RI sites of the pBa3-AX plasmid (Hallahan et al. 1995). The newly generated plasmid was called pBa3-AX-*ptxD*, and used for integration of the *ptxD* cassette into a neutral region between *psaA-3* and *trnL2*, as illustrated in Figure 4.10. Finally, plasmid pPO3 was created by cloning the *trnW*^{UCA} gene into the *Mlu*I site of pBa3-AX-ptxD for translation of the stop codons (Appendix 8). A summary of the plasmids used in this study is detailed in Appendix 4. Plasmids in this study were propagated in *E. coli* DH5α and extracted at small and large-scale.

2.4.2 Isolation of genomic DNA from algal cells

Genomic DNA was extracted from algal strains using the Chelex 100 method (Werner and Mergenhagen, 1998). A single colony of cells was taken and re-suspended in 10 µl of double-distilled water (ddH_2O). The solution was subsequently mixed with 10 µl of 100% ethanol and incubated for 1 minute at room temperature. A 200 µl aliquot of 5% (w/v) Chelex-100 (Bio-Rad) was then added The cell mixture was heated in a heat block (Thermomixer, Eppendorf) at 99 ˚C for 5 minutes and immediately cooled on ice for 2-3 minutes. The mixture was centrifuged (Heraeus Biofuge Pico) for 2 minutes at 13,000 rpm and the clear supernatant was transferred to a fresh 1 ml microfuge tube. This genomic DNA was stored at 4°C with 1 µl aliquot of the extract used in a 25 µl PCR reaction.

2.4.3 Isolation of plasmids from *Escherichia coli*

Small-scale plasmid isolation was performed by first inoculating 5 ml LB supplemented with the required antibiotic with a single *E. coli* colony and shaking at 200 rpm (Innova 4300 incubator shaker, New Brunswick Scientific) overnight at 37°C. The overnight culture was harvested by centrifugation at 5,000 x g for 15 minutes at 4°C. The supernatant was

collected for plasmid isolation using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, UK). For larger-scale plasmid isolation, a large volume of overnight culture was required (about 30 ml per reaction) and the plasmid was extracted using the QIAfilter Plasmid Midi kit (Qiagen, Venlo, The Netherlands). The concentration of isolated plasmid was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.4.4 Polymerase Chain Reaction (PCR)

The target DNA sequences were amplified by PCR in a 25 µl reaction containing: 1X Q5 Reaction Buffer (NEB), 0.5 unit of Q5 High-Fidelity DNA Polymerase (NEB), 200 µM of deoxyribonucleotide triphosphate (dNTPs), 0.5 µM of each forward and reverse oligonucleotide primers, <1,000 ng DNA template and ddH_2O to reach the final volume of 25 µl (detailed in https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491). The PCR reactions were conducted in a thermal cycler (either Eppendorf mastercycler® personal (Hamburg, Germany) or Techne TC-3000X Thermal Cycler, (California, USA). The conditions of the PCR cycle were based on the amplicon size and primers. The analysis of PCR products was performed on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid and 1mM sodium EDTA) containing 1 µg/ml ethidium bromide. To visualise the results, a UV transilluminator was used and data were captured on black/white thermal paper (UVP Gel Documentation System). The sizes of fragments were estimated by reference to a Gene Ruler DNA ladder Mix (Thermo Scientific, UK). The primers used were synthesised by Eurofins or Integrated DNA Technologies (IDT) and listed in Appendix 5.

2.4.5 PCR purification and gel extraction

The purification of PCR products was required prior to cloning or DNA sequencing. This was performed using the Gene JET PCR purification kit (Thermo Scientific, UK). For gel extraction, the DNA fragment was excised

from the agarose gel using a razor blade and purified using the Gene JET gel extraction kit (Thermo Scientific, UK).

2.4.6 Restriction endonuclease digestion

DNA samples were digested with 10 units of restriction endonuclease per 1 μg of DNA in the recommended buffer following the manufacturer's instructions. To optimise double digestion, appropriate buffers and conditions were identified using the online NEB double digest finder program (https://nebcloner.neb.com/#!/redigest). All restriction endonucleases were supplied from New England Biolabs.

2.4.7 DNA ligation

To perform a ligation reaction, the DNA samples were added in a mix solution with a total volume of 10 µl, including 1 unit of T4 DNA ligase per 1 µg of DNA and 1x DNA ligase buffer (NEB). The DNA ratio of insert to vector was 1:3. The reaction was incubated for 30 minutes at room temperature before being transformed into competent *E. coli* cells by heat shock transformation. The transformed cells were plated on 1.5% (w/v) agar LB medium supplemented with appropriate antibiotic concentration.

2.4.8 DNA sequencing

The correct DNA sequences were confirmed by sequencing of purified plasmids (100 ng/µl) or PCR products (10 ng/µl). Primers were prepared at 3.2 pmol/µl (µM) and 5 µl of each primer were used per reaction. DNA sequencing was carried out by Source BioScience (UK) using the Sanger dideoxynucleotide method, and the sequencing results were analysed using Benchling software (www.benchling.com).

2.5 Genetic transformation

2.5.1 Preparation of *E. coli* competent cells

A glycerol stock of *E. coli* cells (DH5α) was streaked out on a fresh 1.5% (w/v) agar LB plate and incubated overnight at 37°C. A starter culture was then prepared by inoculating a single colony into 5 ml of liquid LB and incubating overnight at 37 °C with constant shaking at 200 rpm. Fresh liquid LB was inoculated with 1% of the overnight starter culture and grown under the same conditions for 2.5 h until an OD_{600} ~ 0.6 (Unicam UV / Vis Spectrometer UV2) was reached. The culture was subsequently cooled on ice for 15 minutes and centrifuged at 4000 x g for 5 minutes. The cell pellet was re-suspended in 40 ml of ice-cold 50 mM $CaCl_2$, and cooled on ice for 30 minutes. The cells were centrifuged again as described previously and re-suspended in 1.5 ml of fresh ice-cold 50 mM $CaCl_2$. The competent cells were aliquoted into microcentrifuge tubes at 200 µl and stored at –80 °C until use.

2.5.2 Transformation of *E. coli*

A 100 µl aliquot of competent *E. coli* cells was mixed with 1 µl of plasmid or 10 µl of ligation mixture. A negative control was set up without the addition of DNA to the aliquot of cells. The cell suspension was cooled on ice for 30 minutes before heating in a heat block at 42 °C for 1 minute. The transformed cells were immediately cooled on ice for 2-3 minutes and mixed with 900 µl of fresh liquid LB. The cells were grown at 37 °C for 1 h and 100 µl of the grown cells were subsequently plated on solid LB agar plates supplemented with appropriate antibiotics for selection. The plates were incubated overnight at 37 ˚C.

2.5.3 Glass bead transformation of the *C. reinhardtii* chloroplast

The glass bead transformation method was used for cell wall-less recipient cells. The method involves brief vortexing of a cell suspension with DNA and small-sized glass beads (~ 425 µm diameter, Sigma), followed by

plating on selective media. This technique was modified from (Kindle, Richards and Stern, 1991). The recipient cells (e.g. TN72, TN72::pWUCA2, *cw2*) were grown in 500 ml of liquid TAP medium to a cell density of 2 x 10^6 cells/ml. The non-photosynthetic mutant TN72 was grown under dim light at 5–10 µE/m^2/s^1 whereas other cell lines were incubated under light intensity of 50 µE/m^2/s^1. The culture was harvest by centrifugation at 4,000 x g (Sorvall Evolution RC, Thermo Scientific) for 5 minutes at 16 °C. The cell pellet was diluted in TAP medium to a final concentration of 2 x 10^8 cells/ml. An additional step of cell washing was performed for direct selection on phosphite. The cell pellet was resuspended in Phi medium to remove trace phosphate, centrifuged to pellet the cells again and resuspended in Phi medium to a final concentration of 2 x 10^8 cells/ml. The cell suspension of 300 µl was pipetted into test tubes containing ~ 0.3 g of pre-sterilised glass beads, followed by the addition of 10 µg of DNA plasmid. The cell mixture was briefly agitated with a Vortex for 15 seconds. Then, 3.5 ml of 0.5% (w/v) molten Phi agar at 42 °C were added into the agitated cell suspension. The cells were poured immediately and evenly onto 2% (w/v) solid Phi agar plates containing sodium phosphite ($Na_2HPO_3 \cdot 5H_2O$, 04283 Sigma-Aldrich) at a final concentration of 1 mM (Appendix 1). The Phi agar plates were prepared using molecular-grade agar (Thermo Fisher Scientific, BP1423-500) to avoid issues with trace phosphate contamination. If the selection is based on restoration of photosynthesis, high salt minimal medium (HSM) agar plates were used instead of Phi, with the medium of the molten agar always the same type as in the agar plate. The transformation plates were incubated under a light intensity of 50 µE/m^2/s^1 at 25 °C for 2–6 weeks until the colonies become visible. For photosynthetic selection, the transformation plates were covered with two layers of tissue overnight before exposure to 50 µE/m^2/s^1 of light, to allow the cells to recover. The putative transformant colonies were selected and re-streaked to single colonies 2-4 times on the same type of selection agar plate to achieve homoplasmy. The correct integration and the homoplasmy were confirmed by PCR and DNA sequencing.

2.5.4 Biolistic transformation of *C. reinhardtii*

The biolistic transformation of *C. reinhardtii* was carried out using the BioRad PDS-1000/He Biolistic® Particle Delivery System (Bio-Rad laboratories Ltd, Hertfordshire, UK). The initial step was the cell preparation, in which the cells were grown in 20 ml of TAP and incubated for 3 days as a starter culture. A larger-sized culture was prepared by inoculating the 1-2% starter culture in 250 ml of TAP and grown for 2 days. For the selection on Phi, the cells were P-starved in TA medium (No P source) for 18 h prior to the transformation. Once the culture reached a cell density of 2-4 x 10^6 cells/ml, the cells were harvested by centrifugation at 4,000 x g (Sorvall Evolution RC, Thermo Scientific) for 5 minutes at 16 °C. The cells were re-suspended in TA medium to a final concentration of 1 x 10^8 cell/ml. To prepare a lawn of algae on an agar plate, 250 µl of cell suspension were mixed with 4 ml of 0.5% (w/v) molten Phi agar. The prepared algae on the agar plates were dried in a laminar flow hood and bombarded with DNA-coated microparticles within 2 h

The second step was to coat the DNA plasmid onto gold particles with a diameter of 0.55 µm using the DNAdel™ gold particles kit (Seashell Technology, San Diego). The gold particles were washed with 100% (v/v) ethanol and re-suspended in absolute ethanol to a final concentration of 5 µg DNA per 1 mg gold particle per shot. This DNA-coated gold suspension was briefly sonicated in a water bath and pipetted onto the centre of sterilised macrocarriers. The final amount of DNA plasmid was 1.5-2.0 µg per macrocarrier. The sterilised macrocarriers were allowed to air dry in a laminar flow for 2-3 minutes before performing biolistic bombardment.

The particle bombardment of the lawn of algal cells was conducted by following the instructions of the PDS-100/He device, using 1350 psi rupture disk (Bio-Rad laboratories Ltd, Hertfordshire, UK). To select for the transformant cells, the plates were incubated under light intensity of 50 µE/m^2/s at 25 °C for 2-3 weeks.

2.6 Protein analysis

2.6.1 Preparation of crude total protein extract from *C. reinhardtii*

The 20 ml cultures of *C. reinhardtii* cells were grown until an OD_{750} of 0.8–1.5 in a shaking incubator. The cells were prepared by harvesting 10 ml of culture at 4000 x *g* for 5 minutes and the cell pellet was diluted in a volume of solution A containing 0.8 M Tris.HCl pH 8.3, 0.2 M sorbitol, 1 % (v/v) β-mercaptoethanol. The solution A was added to each sample at different volume depending on OD_{750} readings to ensure that all samples are equalised to the same concentration. The protein extract samples were aliquoted into microfuge tubes and stored at –80 °C until required.

2.6.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were fractionated based on the molecular weight and movement in response to the electric field using the SDS-PAGE technique. The resolving gel was 15% acrylamide, 1.0 mm thick set up using a Bio-Rad mini-PROTEAN tetra system. To make sufficient for two resolving gels, the chemicals were added in the following order: 5.3 ml of ddH_2O, 5.6 ml of 40% (w/v) acrylamide: bisacrylamide at 37:1, 3.75 ml of 1.5 M Tris-HCl (pH 8.8), 150 µl 10 % (w/v) SDS, 150 µl of 10 % (w/v) ammonium persulphate and 6 µl TEMED. Adding 5 ml of the prepared solution into the pre-set plates and leaving a gap of 1-2 cm at the top for adding of stacking gel. Absolute ethanol was used to overlay the resolving gel to ensure a straight gel level. Gel polymerization was achieved at room temperature over a period of approximately 30 minutes. Once the gel was polymerized, the ethanol overlay was removed and the surface of the gel was cleaned with distilled water.

The next step was the preparation of protein samples from frozen stocks previously-stored at -80 °C. The 50 µl of protein aliquot was mixed with 5 µl of 10% (w/v) SDS followed by heating in a heat block for 1 minute at 99 °C to ensure protein denaturation. The samples were centrifuged for 2

minutes at 13,000 rpm and 15 µl supernatant of each sample was taken for loading on the gel. To identify the molecular weight of each protein, the Color Prestained Protein Standard ladder (NEB, #P7719) was used as a reference, loading at 3 µl. The gel electrophoresis was run in a cold reservoir buffer containing 0.25 M Tris, 1.92 M glycine, 1 % (w/v) SDS pH 8.3 for ~ 90 minutes at 150 V.

2.6.3 Western blot analysis

For blotting the proteins onto a membrane, the semi-dry electrophoresis was carried out to transfer the separated proteins from the polyacrylamide gel to Hybond-ECL nitrocellulose membrane (GE Healthcare). The initial step of gel blotting was to immerse six sheets of 3MM Whatman paper and a sheet of nitrocellulose membrane of equal size in Towbin buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol) at 25 °C for 10 min. Three pieces of the pre-soaked 3MM paper were placed on the semi-dry blotter and the pre-soaked nitrocellulose membrane was laid on the top. Placing the gel on the membrane and followed by the remaining three pieces of 3MM paper. To remove bubbles under the membrane and remove excess buffer the stack was rolled gently using a plastic tube. The protein blotting was achieved by using the Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer system at a constant voltage of 19 V (Fisons FEC 570 powerpac) at 2.5 A run for 1 h.

Membrane blocking was carried out overnight on a shaker at 4 °C in 10 ml of 0.5 % (w/v) skimmed milk powder in TBS-T (20 mM Tris base, pH adjusted to 7.4 with 5M HCl, 137mM NaCl, 0.1 % (v/v) Tween-20). For the washing step, the membrane was soaked in TBS-T for 15 minutes. Primary antibody: α-HA antibody produced in rabbit (Sigma-Aldrich product H6908) was diluted in the 0.5 % (w/v) skimmed milk + TBS-T buffer at ratio of 1:2000 for protein detection. The membrane was incubated with the primary antibody for 1 h at 25 °C with continuous shaking. The membrane was subsequently rinsed with TBS-T buffer for several times prior to detection.

For the Odyssey detection method, the secondary antibody: Goat anti-rabbit IgG, DyLight 800 (Thermo Scientific product 35571, with the excitation/emission at 777/794 nm that can be detected by Odyssey Infrared Imaging method) was prepared in 10 ml of 0.5 % (w/v) skimmed milk + TBS-T buffer at ratio of 1:25,000. The membrane was incubated with the secondary antibody for 1 h at 25 °C on a shaker. As previously, the membrane was washed with TBS-T buffer and briefly rinsed in TBS buffer to remove residual Tween. For detection, the membrane was analysed using the Odyssey Infrared Imaging system (Li-COR Biosciences, Model: Odyssey® CLx Imaging System).

2.7 Large-scale cultivation with 'hanging bag' system

Scale-up cultivation was examined in a photobioreactor 'hanging bag' system originally designed by Cawthron Institute, New Zealand (Taunt, Stoffels and Purton, 2018). The NNV::ptxD strain carrying the fish vaccine was grown in single-use tubular polythene bags (supplied by Supreme Health, New Zealand), each bag filling with 20 litres of Pi or Phi medium. Under standard growth conditions, 500 ml of inoculum were grown to stationary phase in Phi medium. Each bag was subsequently inoculated with the 500 ml of inoculum, along with 1 ml of a contaminant cocktail of natural bacteria and fungi that was produced by allowing a flask of TAP medium to become actively contaminated after leaving it open to the air in the laboratory for 1 week. A source of carbon dioxide was applied to the hanging bags by sparging at the bottom with filter-sterilised air. The culture was illuminated at 100 µE/m^2/s using Osram Lumilux Cool daylight fluorescence tubes. Cultures were grown at 25 °C for 13 days and the algal/bacterial populations were estimated by particle size distribution using a Mastersizer 3000 laser diffraction particle size analyser (Malvern Panalytical Ltd., UK).

2.8 Activity test for limonene production

2.8.1 Toxicity test of limonene in *C. reinhardtii* culture

A liquid culture of *C. reinhardtii* was grown to an OD_{750} of ~ 1.0 and 20 ml was added to each flask. A standard of (S)-(−)-limonene (96%, Sigma Aldrich) was added to individual flasks to a final concentration of 0, 0.02, 0.1, 0.2, 0.4 or 1% (v/v), while 0% was a negative control set up with no limonene added. The cultures were then grown at 25 °C under a light intensity of 50 $\mu E/m^2/s$ for 48 h. Photographs of each flask and the OD_{750} were taken at 4, 8, 24 and 48 h after limonene treatment. $\mu E/m^2/s$

2.8.2 Dodecane overlay test in *C. reinhardtii* culture

A liquid culture of *C. reinhardtii* was grown to an OD_{750} of ~1.0, and 20 ml was pipetted into 12 flasks. Six flasks were overlaid with 2 ml of n-dodecane (Alfa Aesar) whereas the other six flasks did not contain dodecane. Different volumes of standard (S)-(−)-limonene (96%, Sigma Aldrich) were added to the flasks with and without dodecane overlay to reach final concentrations of limonene of 0, 0.02, 0.1, 0.2, 0.4 and 1% (v/v). Photographs of each flask and OD_{750} measurements were taken at 2, 24, 48 and 168 h after limonene treatment.

2.8.3 Two-phase dodecane extraction for the limonene

The *C. reinhardtii* transgenic strains (pWUCA2_LS and pWUCA2_LS) were used to inoculate 20 ml of TAP medium to an OD_{750} of ~ 0.05. The cultures were grown to reach an OD_{750} of ~ 2.0, then overlaid with 2 ml of n-dodecane (dodecane, 99+%, Alfa Aesar) and grown for a further 5 days. The dodecane overlay was harvested and 150 µl was transferred into a GC vial for further analysis on the same day.

2.8.4 Gas Chromatography Mass Spectrometry (GC-MS) optimisation and limonene analysis

A calibration curve for limonene detection was generated by using analytical standard S-Limonene (Sigma Aldrich, #62128). A serial dilution of the S-limonene was prepared in hexane at a concentration range of 0.01, 0.1, 1.0, 10, 100 ng/µl. The 48 µl of each S-limonene concentration were mixed with 2 µl of dodecane, and 1 µl of the mixture was injected to GC-MS.

All dodecane overlay extracted from the culture medium was diluted with hexane at a ratio of 2:48 (v/v) before loading onto GC-MS. The quantitative analysis of limonene present in the dodecane extract was carried out by using ISQ™ Series Single Quadrupole GC-MS Systems and TRACE™ 1300 Gas Chromatograph (Thermo Scientific, UK). The GC injection liners (4.0 mm x 6.3 mm x 78.5 mm, # 453A1925, Thermo Scientific, UK) was used. Methane gas was applied as the carrier gas at a flow rate of 0.30 ml/min. The sample inlet temperature was set at 250 °C with the sample injection volume at 1 µl under the splitless injection mode. The temperature gradient was set as follows: 50 °C for 5 min, raised to 90 °C rate 5 °C /min, held for 2 min, raised to 96 °C rate 2 °C/min, and held for 2 min. After the gradient was completed, the post-run was set to proceed at 260°C for 1 min. The following MS conditions were used: mass range, m/z 45–200; solvent delay, 5 min; SIM mode with m/z 68 and 93. The GC chromatographic peaks were determined using the Thermo Scientific™ Xcalibur™ software linked with NIST/EPA/NIH GC–MS main library.

Chapter 3

Expression of the *ptxD* gene encoding phosphite oxidase in the chloroplast of *Chlamydomonas reinhardtii*

Chapter 3 Expression of the *ptxD* gene encoding phosphite oxidase in the chloroplast of *Chlamydomonas reinhardtii*

3.1 Introduction

3.1.1 Transgenic introduction of the phosphite oxidizing pathway

Phosphorus (P) is an essential nutrient for the biosynthesis of many cellular components: for example, nucleic acids, phospholipids, high energy adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADP) (Manna *et al.*, 2016; Achary *et al.*, 2017). The global demand for P is increasing, primarily as a key fertiliser component for agricultural plants where it is supplied in the form of a soluble salt of phosphate (Pi: PO_4) such as ammonium phosphate. P is primarily obtained from mined rock phosphate, and as such represents a non-renewable resource that is overconsumed globally (Smil, 2000; Neset and Cordell, 2012). When Pi is applied to crop fields, about 80% of the Pi becomes immobilized through precipitation, and hence unavailable for plant uptake (Syers, Johnston and Curtin, 2008). Furthermore, the use of such fertilisers to promote plant growth is non-discriminatory, with both crops and weeds benefiting, and therefore some of the P investment is diverted to supporting growth of weeds. The overuse of Pi also causes environmental issues through eutrophication, where excess Pi is released into rivers and results in algal blooms (Lee *et al.*, 2005; Carpenter, 2008). The issues of P reserve depletion and the massive use of Pi fertiliser need to be addressed by providing an alternative solution for P fertilisation.

Phosphite (Phi: PO_3) is another oxidised form of P (Figure 3.1). This form of P is readily soluble and less likely to react with other components in the soil to form insoluble precipitates. Phi has been commercialised as fungicide and fertilisers and is formed as a by-product of other phosphorus compounds preparation (Gómez-Merino and Trejo-Téllez, 2015). However, unlike Pi, Phi cannot be metabolized by most eukaryotes and prokaryotes. In order to utilise Phi, organisms require a specialised enzyme to convert Phi to

Pi (Metcalf and Wolfe, 1998; Garcia Costas, White and Metcalf, 2001; White and Metcalf, 2007; Achary *et al.*, 2017). Only a few bacterial groups have adapted to grow on Phi and possess the metabolic pathways for selective uptake and oxidation of Phi to Pi. The best characterised pathway is that from *Pseudomonas stutzeri* WM88. The crucial enzyme is phosphite oxidoreductase (PtxD), that oxidises Phi to Pi using NAD+ as a cofactor. This enzyme is encoded by *ptxD* that exists in an operon of five open reading frames: *ptxABCDE* (Garcia Costas, White and Metcalf, 2001; White and Metcalf, 2007). Within this operon, *ptxABC* encodes a binding-protein-dependent transporter responsible for the uptake of Phi, and *ptxE* encodes a possible transcriptional regulator. The PtxD enzyme is sensitive to its substrate Phi with a K_m at ~50 uM. It appears that there is no binding of PtxD to other compounds that are structurally or chemically similar to Phi (Garcia Costas, White and Metcalf, 2001). The activity of PtxD allows the use of Phi as an alternative P source in the organism.

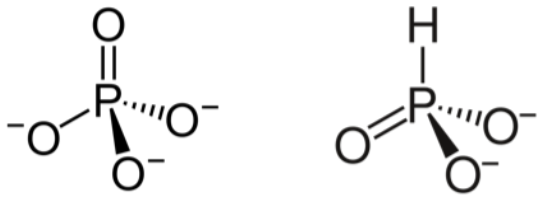


Figure 3.1 A structural comparison of Pi and Phi anions.

One of the oxygens of Pi is replaced by a hydrogen atom in Phi.

The scarcity and non-renewable nature of Pi highlight the need for a new genetic engineering strategy for use of Phi as an alternative source of P. In fact, many plants and other eukaryotes such as green algae appear able to actively import Phi into the cells (presumably via phosphate transporters), however, they cannot use it as a P source and rely on the additional supply of Pi for growth. Lopez-Arredondo and Herrera-Estrella (2012) demonstrated a genetic engineering strategy of expressing *ptxD* in *Arabidopsis* and tobacco such that Phi could be used both as a fertiliser component and a weed control system (López-Arredondo and Herrera-Estrella, 2012). The transgenic lines were able to utilise Phi and needed 30-50% less input of P content for similar growth by a supply of Pi fertiliser. Although high levels of Phi are known to have an inhibitory effect on plant growth and are used for non-selective weed control, the growth of the *ptxD* transgenic lines remained unaffected under Phi treatment. The similar photosynthetic rates of the *ptxD* transformants and the control plants indicated that Phi might not have any detrimental effects on photosynthesis. In terms of weed management, *ptxD*-expressing plants outcompeted weed species in the soil supplemented with Phi. In contrast, the weeds grew much faster than crop plants in soil fertilised with Pi. The same approach was applied to other crop plants such as cotton (Pandeya *et al.*, 2017) and rice (Manna *et al.*, 2016). Also biotechnological microorganisms such as *E. coli* and *S. cerevisiae* have been similarly engineered to grow on Phi as a sole source of P (Shaw *et al.*, 2016; Motomura *et al.*, 2018). This *ptxD*-engineering strategy reveals the possibility of overcoming problems of contamination caused by undesirable species based on the selective advantage provided by the PtxD/Phi system, and the opportunity to exploit Phi as an alternative and low-cost P fertiliser as it is produced as a by-product of other phosphorus compounds preparation (Gómez-Merino and Trejo-Téllez, 2015). The Phi price is two times lower than Pi price, and therefore it could decrease the cost for P supply (Sigma Aldrich, UK).

The expression of *ptxD* in microalgae was demonstrated for the first time using *C. reinhardtii* as a model organism following an earlier study that showed that Phi is not lethal to the microalga, but cannot be used as a P

source (Loera-Quezada *et al.*, 2015). Loera-Quezada et al. (2016) reported the successful genetic engineering of the *C. reinhardtii* with the introduction of *ptxD* into the nuclear genome allowing growth on Phi medium. The authors focused on Phi as a novel nutrient since it is inexpensive and has been widely used and approved in the food industry (Loera-Quezada *et al.*, 2016). By genetic engineering *ptxD* into the nucleus, the transgenic *C. reinhardtii* could outcompete two other microalgae *Scenedesmus obliquus* and *Haematococcus pluvialis* in co-cultures. A similar effect was demonstrated in a culture of the transgenic *C. reinhardtii* that was infected by potential contaminating microbial species such as bacteria and fungi typically found in a non-sterile environment. It was shown that *ptxD* transgenic strains had a strong selective advantage over competing microorganisms in Phi medium and were the dominant species in the mixed culture.

Recently, photosynthetic cyanobacteria were transformed with *ptxD* to allow the utilisation of Phi, and address the need for biosafety and contamination management when using cyanobacteria in biotechnological applications (Motomura *et al.*, 2018). The cyanobacterium *Synechococcus elongatu*s PCC 7942 was transformed with the phosphite oxidoreductase *ptxD* together with the *htxBCDE* gene cluster for the phosphite transporter. The synthesis of the PtxD enzyme and the transporter enabled the uptake and oxidation of Phi by the cyanobacterium. To prevent an escape of the transformant to the environment, the genes for two endogenous Pi transporters, Pit and Pst, were subsequently knocked out. This decreased the ability of the transgenic line to grow on Pi and therefore survive in the external environment. When growing the *ptxD* transgenic strain in a consortium with wild type *S. elongatus*, the PtxD line became the dominated strain in the Phi medium. The study suggests a possible use of this knock-in/knockout strategy for both biocontainment and contamination management when cultivating engineered cyanobacteria in outdoor photobioreactors or open-pond systems.

In this chapter, the expression of *ptxD* in the chloroplast of *C. reinhardtii* is described. This work extends the tools available for engineering

and exploiting this novel sub-cellular compartment as a low-cost biotechnological platform for synthesis and accumulation of recombinant products. Unlike the nucleus, the level of *ptxD* expression was high and stable due to the absence of any silencing mechanisms. The successful expression *ptxD* allows *C. reinhardtii* strains to use Phi as an alternative source of P and provides a strategy for crop protection. This latter feature is discussed further in 4.2.3.

3.1.2 Aims and objectives

- To create transgenic lines of *C. reinhardtii* in which a *ptxD* expression cassette is inserted into a neutral site downstream of *psbH* within the chloroplast genome by using photosynthetic selectable marker.
- To show the expression of *ptxD* in the chloroplast
- To examine the effects of *ptxD* expression on the growth of *C. reinhardtii*

3.2 Results

3.2.1 Design of a synthetic codon optimised version of *ptxD* and creation of the chloroplast expression plasmid pWUCA2+ptxD

An initial experiment was to design a synthetic version of the *ptxD* gene from *Pseudomonas stutzeri WM88* (accession number AF061070). The *ptxD* CDS was codon-optimised for effective expression in the chloroplast of *C. reinhardtii* using the Codon Usage Optimizer software developed in the Purton group. In addition to codon optimization, the CDS was extended at the 3' end to include sequence encoding the haemagglutinin (HA) epitope tag such that this 9-residue peptide sequence would be added to the C-terminus of PtxD (*i.e.* [PtxD]–YPYDVPDYA). This tag would allow protein detection by western blot analysis using commercial anti-HA antibodies. In addition, a stop codon feature was designed in the synthetic *ptxD* CDS by replacing two tryptophan codons (UGG) with stop codons (UGA). This was to allow the full translation of PtxD enzyme only in the chloroplast as described by Young and Purton (2016). Finally, the restriction sites for *Sap*I and *Sph*I were added

to 5’ and 3’ of the gene, respectively, for the cloning to an expression vector (pWUCA2), while avoided in the rest of the CDS. The CDS was synthesized by GeneArt. The synthesized *ptxD* has a length of 1047 bp that includes the HA tag sequence and two stop codons. The molecular mass of the encoded protein was calculated to be 37.51 kDa (and 36.43 kDa without HA tag), excluding the initiating formyl-methionine which is expected to be removed following translation (Giglione and Meinnel, 2001)

The *ptxD* CDS was excised from the synthesised PCR product of GeneArt DNA fragment and inserted into the pWUCA2 plasmid vector as shown in (Figure 3.2) to generate pWUCA2+*ptxD*. This plasmid is designed to drive expression of *ptxD* using native *cis* elements from highly expressed genes in the *C. reinhardtii* chloroplast: namely, the promoter and 5’UTR from *psaA-1* and the 3’UTR from *rbcL*. This pWUCA2 expression vector also carries a chloroplast gene encoding a modified tRNA-Trp that recognises the UGA stop codon as a tryptophan codon (Young and Purton 2016) so that the *ptxD* is translated only in the chloroplast with co-expression of the tRNA gene. The vector has been previously shown to allow high-level expression in the chloroplast of another synthetic bacterial gene: namely CodA from *E. coli* that encodes cytosine deaminase catalysing the hydrolytic deamination of cytosine to uracil (Young and Purton, 2016).

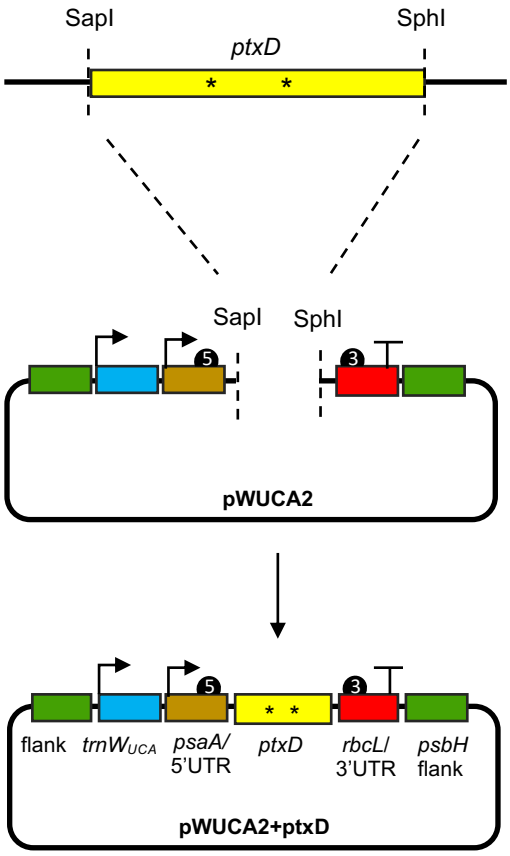


Figure 3.2 Construction of the pWUCA2+ptxD plasmid.

A *ptxD* expression cassette was generated in pWUCA2 by double digestion and ligation of *Sap*I and *Sph*I restriction sites. The CDS was fused to the *psaA* exon 1 promoter/5'UTR and the *rbcL* 3' UTR. The tRNA gene *trnW*$_{UCA}$ is equivalent to the chloroplast *trnW* but its anticodon has been altered to recognize UGA, allowing translational readthrough of the two UGG->UGA codon changes in *ptxD* (shown as*) only in the *C. reinhardtii* chloroplast.

3.2.2 Successful generation of transgenic *C. reinhardtii* lines carrying the *ptxD* cassette

The pWUCA2+ptxD plasmid was transformed into the chloroplast of the recipient strain TN72 via glass bead transformation. TN72 is a non-photosynthetic and cell wall-deficient mutant in which the *psbH* gene encoding an essential subunit of photosystem II was interrupted by the *aadA* cassette (antibiotic selection for creating TN72 strain), resulting in a ∆*psbH*::*aadA* strain (Wannathong *et al.*, 2016). Therefore, the selection of TN72 transformant was based on the restoration of photosynthetic capability using the wild-type copy of the gene carried on the right flanking arm of the plasmid. The flanking elements within pWUCA2 ensure that the *ptxD* cassette is targeted via homologous recombination to a neutral site downstream of *psbH* (Figure 3.3). Since the glass bead transformation method yields only 1-2 colonies per plate (Wannathong *et al.*, 2016) transformation was performed using 10 plates in order to obtain sufficient colonies for further investigation. Five of the resulting colonies were selected for further analysis including confirmation by PCR and DNA sequencing of correct transgene gene integration.

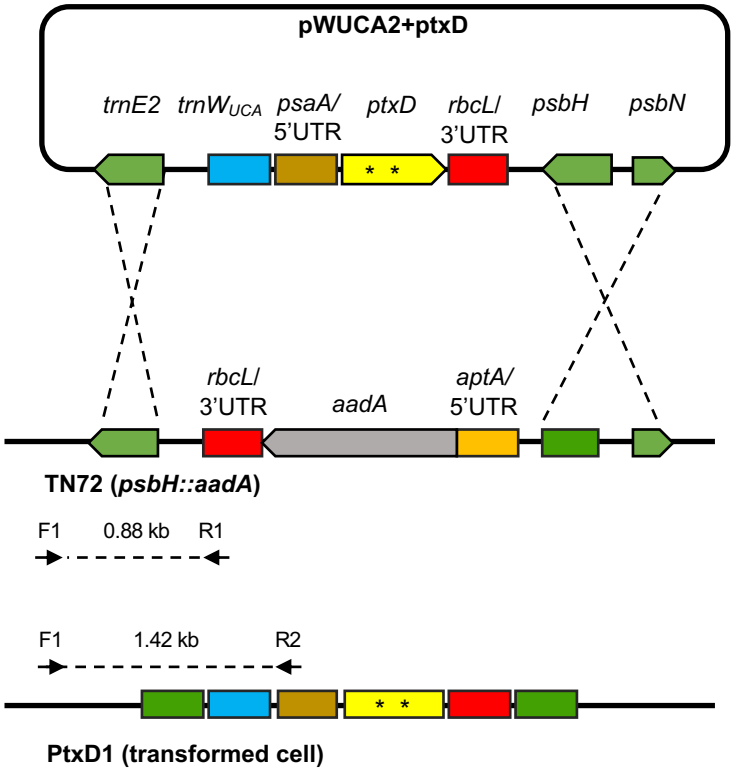


Figure 3.3 Generation of *ptxD* transformants.

Integration of the *ptxD* gene cassette into the chloroplast genome of the non-photosynthetic *psbH* mutant TN72 by homologous recombination (dashed line). A three primer strategy with F1, R1, and R2 was used for PCR confirmation of correct integration into the plastome , and to test whether the polyploid plastome is heteroplasmic (both untransformed and transformed copies) or homoplasmic (all transformed copies). The primer F1 binds to the plastome region outside the insertion site. Primer R1 binds to the *aadA* cassette presented in the TN72 recipient strain, therefore, the combination of F1 and R1 primers gives rise to a band of 0.88 kb to reveal non-transformed copies. Primer R2 binds to the *psaA* promoter area, so the combination of F1 and R2 gives a band of 1.42 kb for the transformed plastome copies.

3.2.3 PCR analysis of transformant lines confirms integration of the *ptxD* cassette and a homoplasmic state.

After chloroplast transformation of the non-photosynthetic TN72 mutant with *ptxD*, the transformant colonies were selected based on the restoration of photosynthesis in the recipient cells. The integration of the *ptxD* gene and the homoplasmicity of the plastome (all ~80 copies transformed) were determined by PCR analysis. Four out of five selected transformant lines (PtxD1, PtxD3, PtxD4 and PtxD5) showed the successful insertion of *ptxD* at the correct locus downstream of *psbH* in the plastome, indicated by a 1.42 kb band. In contrast, TN72 and one selected colony showed a band of 0.88 kb (Figure 3.4). This shorter amplicon of 0.88 kb was not detected in the *ptxD* transformants indicating that there were no remaining copies of the TN72 plastome – i.e. the strains are homoplasmic for the engineered change. Interestingly, this homoplasmic state was achieved after the first round of restreaking on the minimal medium. This contrasts with selection using antibiotic markers such as *aadA* and *aphA6*, where multiple rounds of single-colony isolation and restreaking is required to ensure homoplasmy (Purton, 2007)

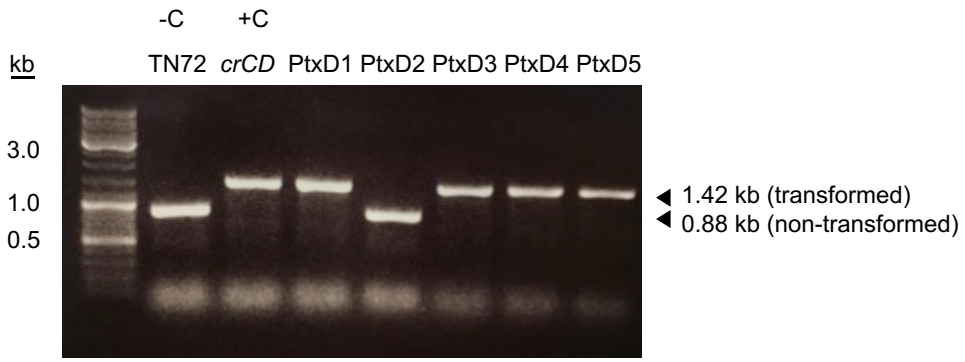


Figure 3.4 PCR screening for the successful integration of the *ptxD* cassette into the plastome of *C. reinhardtii*.

The insertion of the cassette is shown by the presence of a band at 1.42 kb in contrast to the 0.88 kb band from the recipient strain, TN72. A homoplasmic state of the transformant would only show the band of 1.42 kb, whereas a heteroplasmic (both transformant and TN72 plastome copies) transformant would show bands at both 0.88 and 1.42 kb. The *crCD* strain generated by transformation of TN72 with a pWUCA2+codA construct was used as positive control, and TN72 was a negative control. The *ptxD* transformant lines were named PtxD1–5.

3.2.4 Accumulation of PtxD protein in the transformant lines is confirmed by western blot analysis

Whilst the *ptxD* cassette was designed to ensure efficient transcription and translation in the algal chloroplast, there are many aspects of gene expression and protein stability that could impact the successful synthesis and accumulation of the PtxD gene product. For example, i) the choice of codons in the synthetic CDS might inadvertently hinder efficient translation initiation or elongation; ii) the presence of the two internal stop codons might prevent translational read-through despite earlier demonstrations of the successful operation of this codon reassignment system (Young and Purton 2016); iii) the PtxD protein might fail to fold correctly and be rapidly degraded by endogenous proteases in the chloroplast, especially as we have attached the HA epitope to the C-terminus which might affect folding. Consequently, it is important to evaluate the steady-state level of the recombinant protein in the transgenic lines.

To assess the accumulation of PtxD in the lines, a western blot of crude cell lysates was performed using anti-HA antibodies together with IRDye® secondary antibodies and the Odyssey® Infrared (IR) scanner. The western blot showed a band at approximately 37 kDa for each of the four representative transgenic lines indicating the presence of full-length PtxD (Figure 3.5). No band is detected in the negative control, confirming that the band seen in the transformants is the HA-tagged PtxD, and not an endogenous protein that cross-reacts with either the primary or secondary antibodies. The specificity of the antibodies is further demonstrated with a band of 49 kDa seen with the positive control which is TN72 transformed with pWUCA2 carrying a synthetic gene encoding the *E. coli* CodA protein (Young and Purton 2016). The results confirmed the accumulation of PtxD protein in the chloroplast and there appears to be no major issue with respect to the translation readthrough of the two internal stop codons. However, it should be noted that the HA antibody binds to the C-terminus of the PtxD protein, therefore truncated protein terminating at either of the stop codons would not be detected in this western blot.

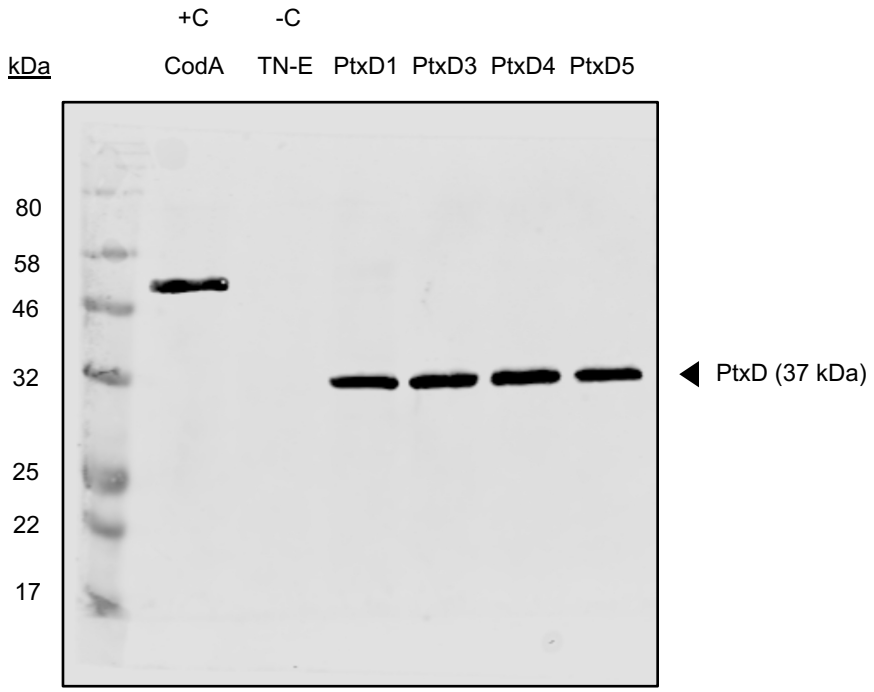


Figure 3.5 Western blot analysis of cell lysates of *ptxD* transformants showing the accumulation of HA-tagged PtxD protein in representative transgenic lines.

The western blot was performed with the cell lysates ([link to methods]). Anti-HA produced in rabbit and anti-rabbit IgG, Dylight 800 IRDye® produced in goat were used as primary and secondary antibody, respectively. The Odyssey® Infrared (IR) imaging system was used for protein detection. HA-tagged PtxD protein is at ~37 kDa shown in equalized loading of cell lysates. The positive control (+C) is HA-tagged CodA protein at 49 kDa, and –C is the negative control, TN-E (TN72 transformed with the empty pWUCA2 vector). Cell lysates were diluted 8 fold prior to loading. Protein sizes were compared to the NEB Color Prestained Protein Standard, Broad Range.

3.2.5 Phosphate levels vary in different sources of water

Pi normally occurs at low concentration in water at about 0.01 mg/L, and it is known that microalgae including *C. reinhardtii* are able to adapt to such low Pi environments, scavenging Pi and accumulating it as intracellular reserves of polyphosphate (Komine *et al.*, 2000). One concern of exploiting the selective advantage offered by the PtxD/Phi system is the presence of small amounts of Pi in the water used for Phi media preparation. The Pi level was therefore measured in two water supplies: the municipal water and the reverse osmosis (RO) water available in the laboratory. TAP medium, which contains 95 mg/litre of Pi (Harris, 1989) was used as a positive control. The water samples were tested using a commercial aquarium kit (JBL Testlab) to determine the level of Pi. Surprisingly, the kit showed a concentration of phosphate around 0.2 mg/l in municipal water when compared to the blue label reference, whereas no Pi was detectable in the RO water, as expected. The TAP medium gave an intense blue colour as expected, since the Pi content exceeds the highest reference by approximately 50-fold (Figure 3.6). This suggests the RO water is suitable for preparing the Pi-free or Phi medium, whereas the municipal water might not be (even though the level is ~500-fold less than that found in TAP).

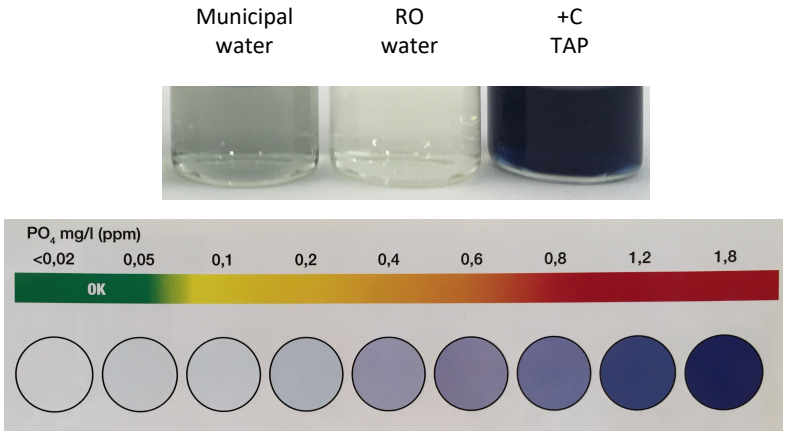


Figure 3.6 Test of Pi level in different types of water using the JBL Testlab kit.

The 5 ml of water was reacted with reagent 1 and 2 provided from the kit. The blue colour of the solution was compared to the colour chart. The sample was moved across the colour chart until it matches as closely as possible. The Pi level is indicated in mg/l (ppm) on the chart.

3.2.6 *ptxD* transformants are capable of active growth on solid medium containing phosphite as the sole source of phosphorus

The next step was to examine whether PtxD was functional in the transgenic lines, and whether the enzyme level obtained results in the capability of the lines to oxidize Phi to Pi at a sufficient rate for normal growth. The PtxD transformants were grown to stationary phase and equalised to the same optical density. Growth tests of the transgenic lines and a control strain (TN72 transformed with empty vector) were performed on solid Tris-acetate medium supplemented with Pi or Phi at 1 mM. 5 µl of each culture were spotted on the medium and incubated for 7 days. If PtxD is active, the transgenic lines should grow on both Pi and Phi whereas the control strain would show a restricted growth on Phi.

The PtxD enzyme was shown to be active as demonstrated by the growth on medium containing Phi as the sole P source. For the initial spot tests, each inoculum was grown in medium supplemented with Pi and then spotted onto the different solid media. All transgenic lines (PtxD1, PtxD3, PtxD4 and PtxD5) were capable of active growth on Phi, although they appeared to grow slightly better on Pi than Phi. There is also limited growth of the negative control on Phi, probably because of intracellular reserves of polyphosphate, but the growth was much lower than that of the PtxD transformants (Figure 3.7). Furthermore, it could be that the cells' phosphate transporters are able to scavenge trace amounts of Pi remaining in the medium, even when in competition with high levels of phosphite.

A second spot test was then performed by first starving cells of Pi by culturing them in Pi-free medium to reduce the effect of internal P storage. The growth of P-starved cells on the solid media was then observed after four days as shown in the lower panels of (Figure 3.7). It was seen that P-starvation hindered the limited amount of growth of the control strain on Phi medium that was observed before, whereas the PtxD transformant lines PtxD1 and PtxD3 grew well. This result therefore confirms that the recombinant PtxD is functional in the algal chloroplast and allows the

transgenic lines to grow on Phi as a source of P. In contrast, the growth of non-transformed strains is limited due to their inability to use Phi.

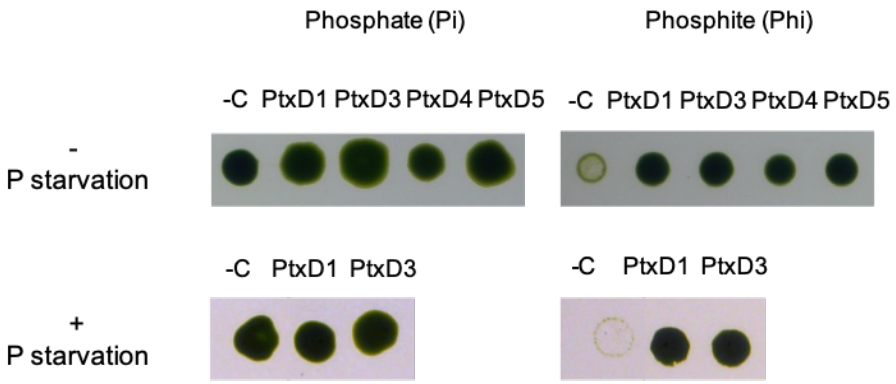


Figure 3.7 Growth assays on solid media to confirm the activity of PtxD in transformants.

The 5 µl of each culture was spotted onto medium containing either phosphate (Pi) or phosphite (Phi) at 1 mM concentration. The cultures were pre-treated through growth either without (– P starvation: upper panels) or with (+ P starvation: lower panels). P starvation was achieved by culturing in TA medium for 3 days. The negative control (-C) was the recipient strain TN72 transformed with the empty pWUCA2 vector. PtxD4 and PtxD5 strains were lost during culture maintenance, therefore, the growth of PtxD4 and PtxD5 was not carried out in (+ P starvation) experiment.

3.2.7 A *ptxD* transformant grows equally well in liquid media containing either phosphate and phosphite

The growth tests on solid media ("spot tests") demonstrate the ability of the *ptxD* transformants to grow using Phi, however, these tests do not give a clear insight into whether the rate of growth rate is comparable on Phi *versus* Pi (and whether the expression of *ptxD* affects growth on Pi when compared to the negative control). The replacement of Pi with Phi in the media may affect the growth rate depending on the efficiency of the PtxD enzyme to covert Phi to Pi. If there was indeed no difference in the growth rate, using Phi instead of Pi would provide an alternative resource of P and a potential strategy for crop protection. In order to investigate this, it was necessary to accurately compare growth rates in liquid cultures under identical conditions of light, temperature and agitation.

PtxD transformant PtxD1 was chosen as a representative line in order to investigate growth in a lab-scale photobioreactor in liquid Tris-acetate medium supplemented with Pi or Phi under standard conditions compared to a control strain. In the first instance, an untransformed cell-wall deficient strain (*cw15*) was used as the control for the first trial. However, subsequently the TN72 strain (that carries the *cw15* mutation) transformed with an empty vector was considered to be a better control than cw15 since it is genetically identical to the PtxD1 except for the absence of the *ptxD* coding sequence. The use of controlled photobioreactors rather than illuminated incubators reduced the effect of variables such as light and temperature because it was possible to keep those factors consistent. For the first trial, the growth assay was performed in three Algem photobioreactors with all six chambers set to the same conditions. The PtxD1 and cw15 inocula were grown for five days as starter cultures and re-inoculated in 400 ml of Tris-acetate medium supplemented with Pi (Tris-acetate-Pi) or Phi (Tris-acetate-Phi) respectively. The starting optical density was normalized to the value at ~0.25 between duplicates of the PtxD1 strain. No replicate was performed for control because there were not enough Algem units available. CO_2 gas was

supplied at a rate of 10 ml/min. The growth was measured every 30 minutes for eight days.

It was found that the growth of PtxD1 was comparative to the control strain in Tris-acetate-Pi medium although the average optical density was slightly lower than that of the control at the exponential phase. PtxD1 reached the same final optical density as seen in the control, *cw15* (Figure 3.8). Although there was some limited growth of the control in the Phi medium, the growth was markedly lower than PtxD1 and plateaued at around OD_{740} 1.00. It could be that *cw15* cells rely on the preserved internal phosphate as P source during P deficient environment. In contrast, the PtxD1 growth curve is very similar to that obtained in the Pi medium, indicating that the transformant line can grow equally well on either source of P.

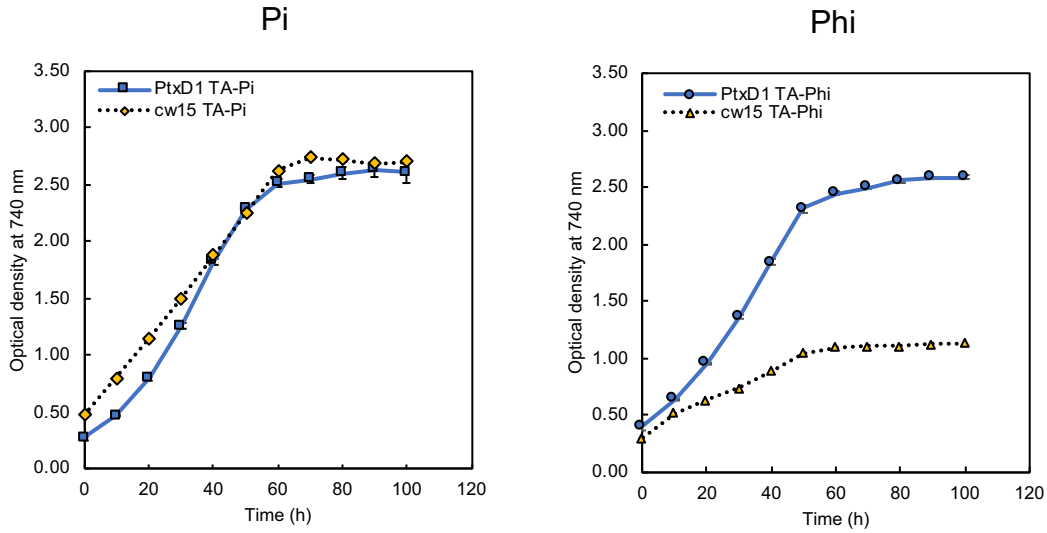


Figure 3.8 Comparison of growth of PtxD1 and the control line cw15 in mixotrophic conditions.

Growth was performed in tris-acetate medium supplemented with phosphate (TA-Pi) or phosphite (TA-Phi). The inoculum was previously grown in the Phi medium and re-inoculated in 400 ml of fresh media, and cultured in Algem photobioreactors (Algenuity, Stewartby (UK)). The standard condition was continuous shaking at 120 rpm, 25°C and with a white light intensity of 200 µmol/m^2/s and CO_2 at 10 ml/min. The OD_{740} reading was recorded automatically every 30 min. Error bars are given for the duplicated PtxD1 flasks as technical repeats, but there were no repeat for the control *cw15* flasks.

The similar results were obtained when the experiment was repeated in an HT24 photobioreactor, and using the TN72 control transformant as the 'wild-type' strain. The HT24 system accommodates 24 culture flasks so allows more units for replicates with all units are in one chamber at the same temperature, but without the supply of CO_2 (Figure 3.9). To address the issue of limited growth of the control strain in the Phi medium due to cellular reserves of polyphosphate, the starter cultures for the HT24 experiments were pelleted, washed and resuspended in a fresh TA medium.

As seen in Figure 3.9, there was almost no growth of the control in Phi medium following this pre-treatment. More importantly, the HT24 data supports further the two key conclusions: i) the presence of PtxD enzyme in the algal chloroplast does not impair the cell's ability to grow using Pi as a source of P (Figure 3.9, upper panel); ii) the PtxD transformant grows equally well on Phi as Pi (Figure 3.9, compare upper and lower panels). This indicates that Phi transport across the cell membrane and chloroplast membrane, and Phi-to-Pi conversion are not limiting factors.

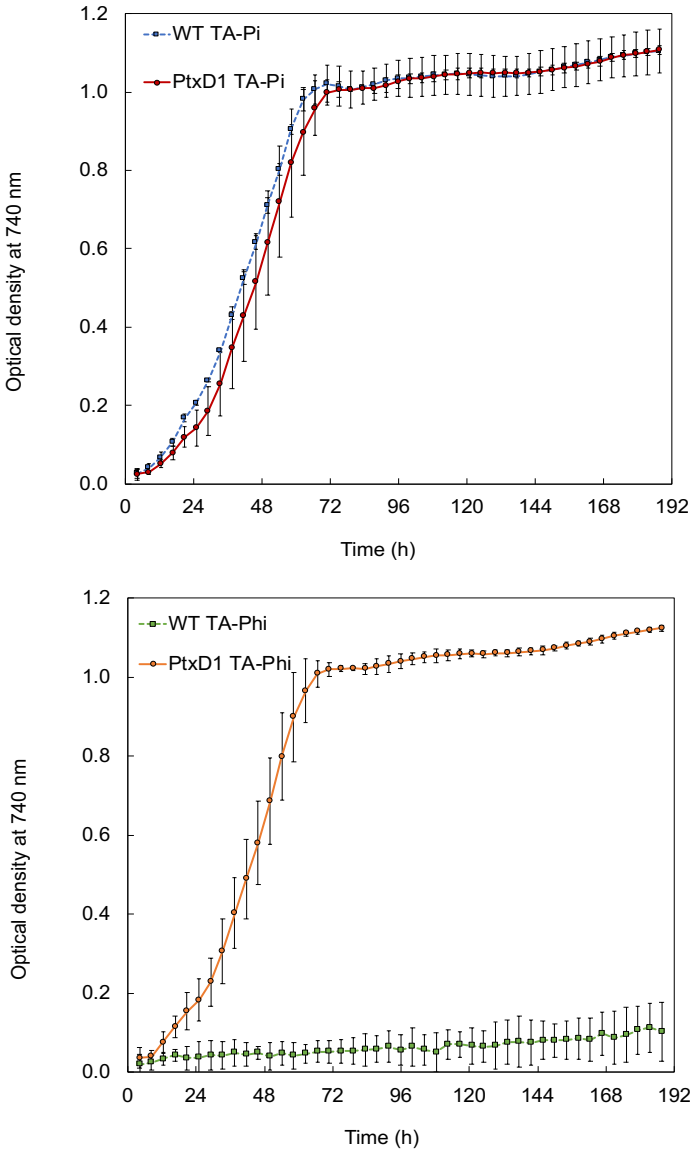


Figure 3.9 Growth of PtxD transgenic line (PtxD1) and the control 'wild type' strain (WT) in Tris-acetate media supplemented with Pi (Tris-acetate-Pi) or Phi (Tris-acetate-Phi).

The inoculum was grown for 5 days and re-inoculated in 20 ml of fresh Tris-acetate medium containing either Phi or Pi to OD_{750} = 0.02 grown in HT24 photobioreactors (a small scale photobioreactor with 24 units of 50 ml flasks from Algenuity, Stewartby (UK)) with controls of light, temperature and mixing. Three technical replicates were performed in each type of medium. Error bars were obtained from standard deviation. The standard condition was continuous shaking at 120 rpm, 25°C and with a light intensity of 200 µmol/m^2/s. The OD_{740} reading was recorded every 10 min. No CO_2 gas was applied to the system, only passive aeration.

3.2.8 Comparison of growth of the *ptxD* transformant in mixotrophic and phototrophic conditions suggests that Pi replacement with Phi has a minimal effect on growth rates.

One potential concern of expressing *ptxD* in the chloroplast is the cofactor requirement for efficient Phi to Pi conversion. The PtxD enzyme requires oxidising equivalents in the form of NAD+ (or NADP+ as a less efficient cofactor). Under mixotrophic growth in which acetate is supplied as a carbon source, the chloroplast may receive sufficient oxidising equivalents from mitochondria as a product of oxidative phosphorylation (Johnson and Alric, 2013). Under phototrophic conditions in which growth relies on the light reaction, the pool of NAD+/NADP+ is more reduced because the latter acts as an electron acceptor in the light reactions. This might be expected to limit the availability of NAD+/NADP+ for the oxidation of Phi, resulting in a reduced rate of Phi to Pi conversion and therefore a limiting level of cellular Pi.

It was hypothesised that this limited Pi availability may affect the growth rate during phototrophic cultivation. To test this, PtxD1 was grown in two different conditions: mixotrophy (acetate supply as a carbon source) and phototrophy (growth relied on photosynthesis) in an Algem photobioreactor. 400 ml of PtxD1 culture was grown under standard conditions with 10 ml/min of 5% CO_2 for three days. The mixotrophic growth of PtxD1 was in Tris-acetate medium either supplemented with Pi (Tris acetate-Pi) or Phi (Tris acetate-Phi). Similarly, the phototrophic growth was in Tris-minimal medium either with Pi (Tris-Pi) or Phi (Tris-Phi). Clearly, growth under both trophic modes would be dependent on the activity of PtxD only in the media containing Phi, and not needed in the Pi media. As seen in Figure 3.10a, when the transformant strain was grown phototrophically in the presence of Phi, a decrease in the growth rate was seen. Under mixotrophic conditions growth was higher than that obtained under phototrophic conditions due to the availability of acetate as a reduced carbon source (Chapman *et al.*, 2015). However, in the Phi medium, a slight growth reduction was observed under mixotrophy towards the end of the exponential phase. It is presumed

that at this point, the acetate became depleted and thus there was a switch from mixotrophy to phototrophy, and hence a growth effect due to the dependency on available oxidising equivalents in Phi medium.

However, despite the indications that the hypothesis might be correct – namely that growth on Phi under phototrophy might be affected by a limited supply of oxidising equivalent, it was subsequently found that there was an uncertainty regarding the temperature control in one of the Algems while performing this set of experiments. Checking the data files revealed that the temperature setting in the Tris-Pi culture was faulty such that the actual temperature was 2°C higher than that in the Tris-Phi culture as shown in Figure 3.10b. Furthermore, under phototrophic conditions the CO_2 bubbling results in a pH crash in the two cultures after three to four days, but that the timing of this crash was different in the two cultures (Figure 3.10c). It cannot be ruled out that the temperature and pH differences were responsible for the observed differences under phototrophic growth.

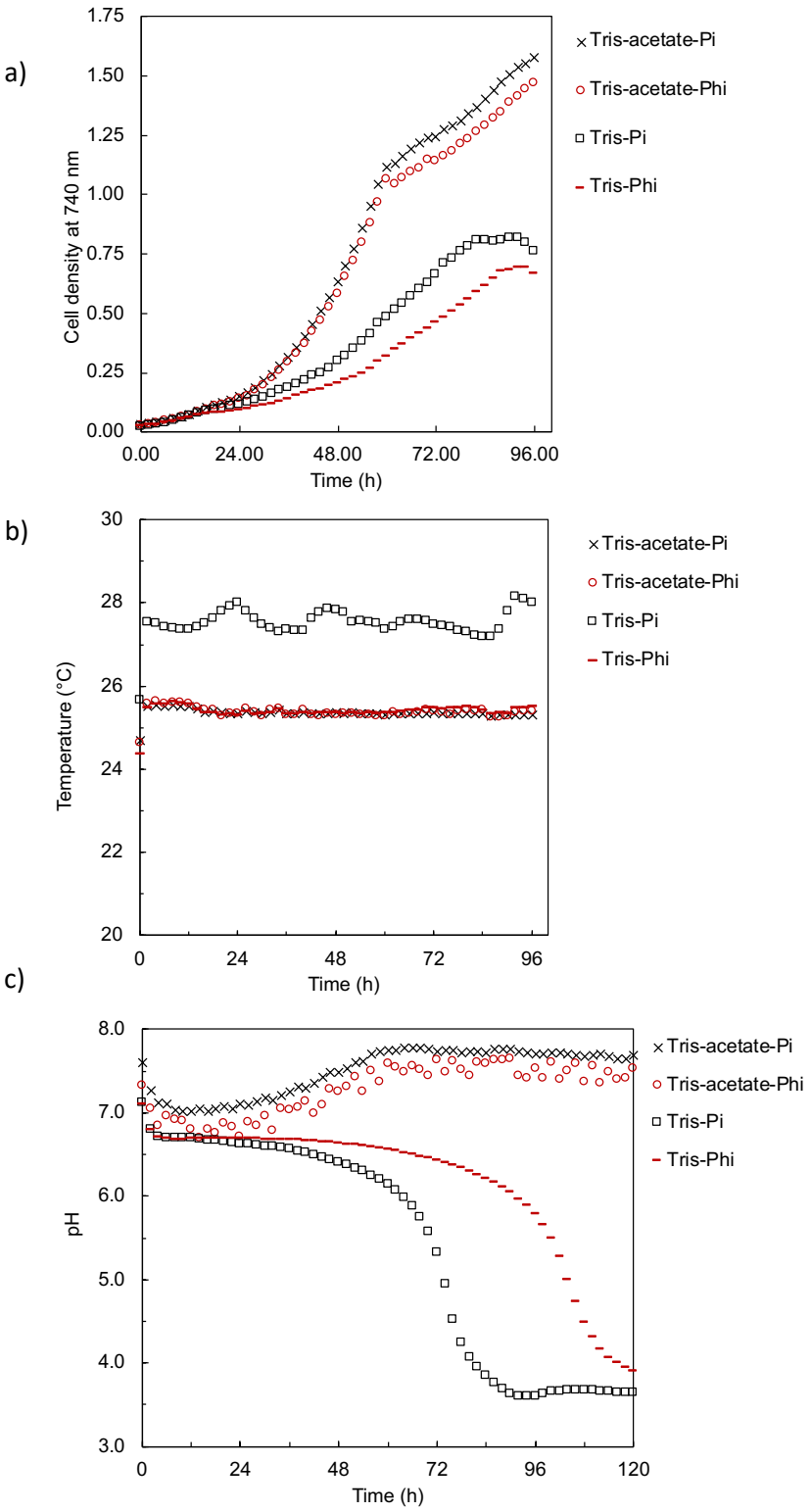


Figure 3.10 Comparison of mixotrophic and phototrophic growth of the PtxD1 transformant in Algem photobioreactors.

The inoculum was grown in Phi medium and used to inoculated 400 ml of fresh Tris-acetate or Tris medium containing either Pi or Phi in Algem photobioreactors with monitoring of growth (a), temperature (b) and pH (c). The standard condition was continuous shaking at 120 rpm, mixing with CO_2 and white light intensity of 200 μmol/m^2/s. The reading was recorded every 30 min. No repeats were performed in the experiments.

To confirm whether PtxD expression has a negative impact on the phototrophic growth as a result of Pi limitation, a repeat experiment was carried out but using the alternative HT24 photobioreactor. Since the HT24 unit is placed in a temperature-controlled incubator, then all 24 culture flasks are maintained at a single temperature. Furthermore, the ability to have 24 parallel cultures allow multiple biological replicates. As shown in Figure 3.11, the results from this experiment reveals no difference in the phototrophic growth of PtxD1 transformant in Tris-minimal medium containing either Pi or Phi. The growth kinetics seem to be comparable between wild type and PtxD.

In this case, the assumption that reducing equivalents might be depleted by photosynthetic activity leading to insufficient Phi-to-Pi conversion for growth appears not to be an issue in the HT24 system. Bear in mind, it is difficult to compare the growth reduction obtained in the Algem with that in the HT24, because of the variation in the two set-ups. Additional CO_2 gas was supplied to Algem system to achieve maximum phototrophic growth, while this was not possible in the small scale HT24 system.

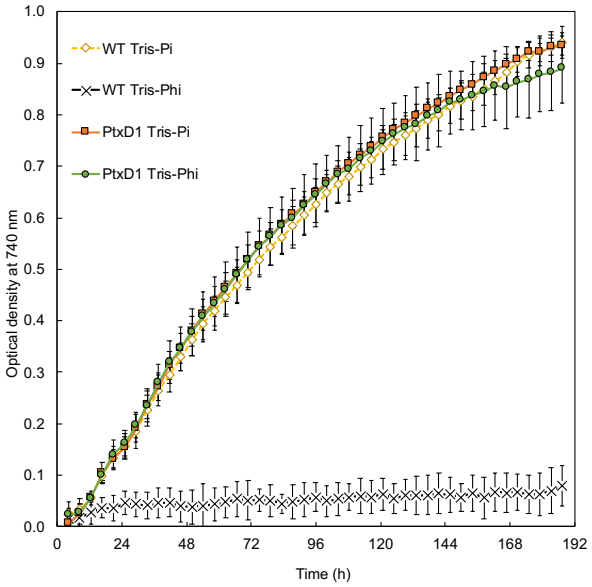


Figure 3.11 Growth of the PtxD transgenic line (PtxD1) and the control 'wild type' strain (WT) in Tris-minimal media supplemented with phosphate (Tris-Pi) or phosphite (Tris-Phi) in the HT24 photobioreactor.

The inoculum was first grown for 5 days, washed with P-free medium and used to inoculate 20 ml of fresh Tris-minimal medium containing either Phi or Pi for growth in the HT24 with controls of light, temperature and mixing. Three technical replicates were performed for each type of medium. Error bars were obtained from standard deviation. The standard condition was continuous shaking at 120 rpm, 25°C and with a light intensity of 200 µmol/m^2/s. The OD_{740} reading was recorded every 10 min.

3.3 Discussion

There is considerable interest in exploiting *C. reinhardtii* as a biotechnological platform, but more advanced molecular tools and cultivation strategies are needed in order to make high-value products and recombinant proteins using this organism (Gimpel *et al.*, 2016; Taunt, Stoffels and Purton, 2018). This chapter is focused on developing a strain improvement strategy to allow the utilisation of Phi in the *C. reinhardtii* chloroplast. The *ptxD* gene encoding a NAD(P)-dependent phosphite oxidoreductase from *Pseudomonas stutzeri* WM88 was introduced into the chloroplast, allowing the microalga to utilise Phi as a P source. After molecular characterization of the *ptxD* transformants, the accumulation of PtxD in the cell was confirmed and its activity showed the ability of the transformant to grow on Phi as the sole source of P. No growth reduction was observed under mixotrophic conditions when compared to equivalent growth on standard Pi-containing medium, nor under phototrophic conditions when cultured without supplemented CO_2.

3.3.1 Stable *ptxD* expression achieved in the *C. reinhardtii* chloroplast

An advantage of expressing transgenes within the chloroplast is that the expression is stable in the absence of selection due to a lack of gene silencing mechanisms, whereas such mechanisms are present in the nucleo-cytoplasmic compartment making nuclear expression more challenging (Sandoval-Vargas *et al.*, 2018a). Furthermore, the ability to target the transgene to a specific chloroplast locus contrasts with the random integration that occurs in the nucleus (Daniell, 2006; Rasala and Mayfield, 2015). However, the algal growth or *ptxD* expression level were not directly compared between the nucleus and chloroplast transformants. The stable integration of *ptxD* into the plastome was confirmed by PCR, as was the establishment of a homoplasmic state for the plastome. Homoplasmicity is essential to ensure that all plastome copies carry the *ptxD* gene and there is no chance of 'loss' of the gene in the strain under study through segregating out of transformed and untransformed plastomes (Purton, 2007). Because of

the strong selective pressure on photosynthetic restoration, homoplasmy was achieved after one to three restreaking (Wannathong *et al.*, 2016). The stable *ptxD* gene integration and expression therefore allow the routine culturing of engineered strains in Pi- or Phi-containing media without the need for antibiotic selection.

Western blot analysis showed the accumulation of PtxD in the transformant cells at an easily-detectable level. The protein is presumed to accumulate inside the chloroplast since there is only very limited evidence for export of any proteins out of the chloroplast (Krause, Oetke and Krupinska, 2012). The lack of silencing mechanisms and positional effects in this organelle (Daniell, Kumar and Dufourmantel, 2005; Daniell, 2006), meant that high and stable expression of *ptxD* was possible. While completing the work, another group reported the successful expression of a separate *ptxD* version in the *C. reinhardtii* chloroplast (Sandoval-Vargas *et al.*, 2018a). However, their synthetic gene differed in its design, lacked the internal stop codons incorporated in our version as a biocontainment feature, and was targeted to a different location within the plastome inverted repeat, between the 23S-5S rRNA genes and exon 5-intron 4 of *psbA*. So far, these are the four reports for the genetic engineering *ptxD* into the *C. reinhardtii* chloroplast to enable Phi utilisation (Sandoval-Vargas *et al.*, 2018a, 2019; Changko *et al.*, 2020; Cutolo *et al.*, 2020).

3.3.2 The expression of *ptxD* allows robust growth on phosphite as a sole phosphorus source

The functionality of PtxD was shown by the growth of PtxD transformants on Tris-acetate medium supplemented with Phi as the sole P source. The normal growth rates demonstrate that the uptake of Phi into the cell and chloroplast occurs readily, presumably via the various phosphate transporters. Several of these transporters are sufficiently promiscuous to transport the toxic anion, arsenate so are also likely to recognise Phi (Murota *et al.*, 2012). Also, the NAD-dependent phosphite oxidoreductase seems to have sufficient oxidising equivalents in the chloroplast despite its preference

for NAD+ to convert a sufficient amount of the accumulated Phi for normal growth. As such, the growth kinetics of PtxD transformants grown in Pi and Phi medium appear to be comparable. When growing PtxD1 in Tris-acetate medium with Phi, the cells showed no reduction in exponential phase growth rate relative to that with Pi. This is in contrast to the report from (Sandoval-Vargas *et al.*, 2018a), in which the doubling time of cells grown in Phi was twice that of cells grown in Pi. They proposed that the cells required more time to transport Phi across the cell membrane and chloroplast, leading to a slower growth.

However, this difference in exponential growth rate could be the result of a lower gene expression level under the control of a weaker *psbD* promoter/5'UTR element in Sandoval-Vargas et al. (2018). In my gene design, *ptxD* expression was driven by the *psaA* promoter/5'UTR element and this has previously been reported to be a stronger element than *psbD* (Michelet *et al.*, 2011). The higher expression of *ptxD* driven by the *psaA* element could increase the level of the enzyme to a point where it is not limiting for exponential growth in Phi media.

Uptake of Phi seems not to be an issue in *C. reinhardtii*. Presumably, endogenous Pi transporters facilitate Phi uptake into the cells and across the chloroplast membrane. Phi is structurally similar to Pi but one oxygen that is bound to P atom in Pi is substituted by an H atom in Phi (Varadarajan, 2002), resulting in a significant difference in its chemical properties (Figure 3.1). Most enzymes processing phosphoryl transfer reactions can distinguish the H atom in Phi from O atom in Pi (McDonald, Grant and Plaxton, 2001; Achary *et al.*, 2017). However, several studies suggest that the uptake of Phi does indeed involve Pi transporters in some plants and yeasts (McDonald, Niere and Plaxton, 2001; Varadarajan, 2002). Phi may compete with Pi for binding sites of Pi transporters due to its structural similarity (Achary *et al.*, 2017). Phi has also been shown to be involved in the modulation of the signal transduction pathway regarding a change of Pi level in the environment (Plaxton and Carswell, 1999). In some species, the introduction of a Phi-specific transporter is required to enable Phi uptake, suggesting that in these

cases the endogenous Pi transporter can discriminate between the two anions. For example, studies of *ptxD* expression in cyanobacteria *Synechocystis sp.* PCC6803 and *Synechococcus elongatus* PCC 7942 demonstrated the need for co-expression of both *ptxD* and genes for the cognate Phi transporter (Polyviou *et al.*, 2015; Motomura *et al.*, 2018). This demonstrates the variation in capability of Phi uptake from one to another organism. Nevertheless, Phi uptake was not an issue in *C. reinhardtii,* so there is no need to requirement for additional Phi transporters in this microalga (Figure 3.12).

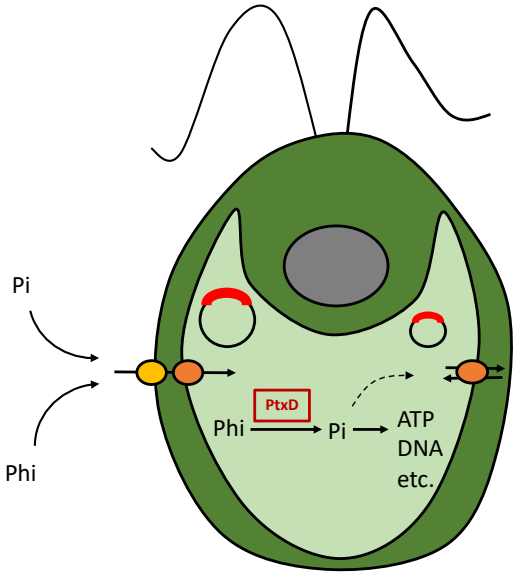


Figure 3.12 Diagram of Phi-utilizing pathway by the chloroplast-localised PtxD in the transformant.

The uptake of Pi and Phi may occur via native Pi transporters in a competitive manner across the cell membrane and chloroplast membrane. Phi is oxidised to Pi by PtxD located within the chloroplast, and then the bioavailable Pi is used in essential cellular functions such as ATP and DNA synthesis and transported out of the chloroplast via putative Pi transporters (Fabiańska, Bucher and Häusler, 2019).

The selective uptake of Pi only by certain organisms could be employed as a biosafety strategy to avoid the escape of PtxD transformants into the environment. The strategy involves the expression of *ptxD* together with the necessary Phi transporter to allow growth on Phi, and then the deletion of the endogenous Pi transporter(s) to prevent the uptake of Pi and only allow the transport of Phi as a sole P source. A study of this Phi-dependent biocontainment approach was described by Hirota et al. (2017). The *ptxABC* (from *Ralstonia sp.* 4506) and *htxABCDE* genes (from P. stutzeri WM88) were inserted into an *E. coli* mutant where all putative Pi transporters had already been knocked out (Hirota *et al.*, 2017). They found that PtxABC can transport both Pi and Phi. In contrast, HtxBCDE (a transporter for hypophosphite (HPhi) that is more reduced form of inorganic P being oxidized to Pi through a phosphite intermediate.) can selectively uptake only Phi but not Pi. They showed that the HtxBCDE hypophosphite transporter is efficient enough to allow normal cellular growth at 1 mM concentration of Phi. They proposed that the conformational change or mode of binding to Phi or Pi by HtxB transporter might be specific and different due to the molecular basis of phosphite and hypophosphite recognition by ABC-transporters (Bisson *et al.*, 2017).

3.3.3 Considerations related to the use of PtxD/Phi system

The work presented in this chapter demonstrated the successful expression of *ptxD* in the chloroplast and showed no significant negative impacts on microalgal growth. However, there are some issues to be considered when applying the PtxD/Phi system to other species.

Loera-Quezada and colleagues showed early on that Phi is not toxic to *C. reinhardtii* at a range of 0.05 to 1 mM Phi concentration, but that wild-type cells cannot utilise Phi as a P source. Consequently, the cells survive until they run out of their internal reserves of P (Loera-Quezada *et al.*, 2015). This may not be true for every species, and indeed Phi has biocidal activities on fungal species, because Phi contributes to an increased level of inorganic polyphosphates that suppress main phosphorylation reactions in fungi. Phi is

also phytotoxic to various crop species at high concentration (Achary *et al.*, 2017). Consequently, the toxic effects of Phi on the growth of other algal species should be examined at a range of concentrations. *C. reinhardtii* is capable of storing Pi as polyphosphate (PolyP) – long polymer chains complexed with calcium or magnesium – found as a main component in the vacuole (Komine *et al.*, 2000). PolyP storage is mainly to support cell fitness by giving free energy from broken phosphoanhydride bonds in PolyP chains through hydrolysis (Werner, Amrhein and Freimoser, 2007). The reserved Pi plays an important role in sustaining growth during P limitation as was shown here by the minor growth of P-replete cells cultured on Phi. In addition to P storage, carryover Pi in the growth medium during transfer to the new medium may contribute to minor growth of the wild type or non-transformed cells (Polyviou *et al.*, 2015). Poly-P storage is also seen in other species such as *Dunaliella tertiolecta* (M. Chen *et al.*, 2011) and *Tetraselmis subcordiformis,* a marine green microalga (Yao *et al.*, 2013), so implementation of the PtxD system in these species needs to take this into account. More significant is a recent report that some green microalgae species such as *Chlorella vulgaris* and *Coccomyxa subellipsoidea* can naturally grow on Phi (Hashizume *et al.*, 2020). This seems to conflict with the current model that enzymatic Phi oxidation is limited to certain prokaryotic groups, and further studies are needed to determine whether the researcher's findings can be attributed to PolyP reserves, or even partial chemical oxidation of the Phi salt in the stocks used for their study ($4H_2PO_3^- + H^+ \rightarrow PH_3 + 3H_2PO_4^-$) (Lovatt and Mikkelsen, 2006; Pasek, Sampson and Atlas, 2014). To ensure the selective advantage of Phi for the PtxD transformants, whether the organisms can assimilate Phi should therefore be examined, and starting any measurement of growth kinetics with a low amount of PolyP through P starvation is essential.

One potential issue of expressing *ptxD* in the chloroplast is that the Phi to Pi conversion needs oxidising equivalents in the form of NAD+ (or NADP+). It was speculated that there is a more reduced NAD+/NADP+ pool under phototrophic growth as a result of the increased activity of ferredoxin:NADP(H) oxidoreductase (Goss and Hanke, 2014), therefore

limiting the conversion of Phi to Pi. This appears not to be a major issue as seen by the equivalent phototrophic growth on Pi and Phi in the HT24 experiments. However, this needs to be explored further with more studies using the Algem bioreactor under a range of light, temperature and CO_2 levels since all of these factors influence photosynthetic rates. Whether the level of NAD+/NADP+ becomes limiting under these conditions is difficult to assay directly since it may be adjusted by supply of oxidant from the mitochondrion (Johnson and Alric, 2013).

3.3.4 Limitations and future works

To understand how the microalgal cells acclimate to P limitation, it is important to consider the inconsistency in the kinetics data of P uptake. The studies require P starvation to obtain a low level of intracellular P content. However, the microalgae are highly efficient at scavenging P through high-affinity Pi transporters, and PolyP stores can support the growth even after a long period of P starvation. There are discrepancies among published data of how P-starvation is performed before P measurement. This ranges from hours to days depending on the species and individual strategy, leading to a difference in P content at the beginning of the measurement. In this project, growth kinetics were performed on P-replete cells in which the amount of P was not at the lowest capacity (Solovchenko *et al.*, 2019). It would be more accurate to examine the growth kinetics in P-starved cells together with the measurement of P remaining in the cells. This shows the need of a standardised method of P deprivation and a reliable assay for cell P content.

Simple and sensitive visualization of P content is a useful tool to monitor how the cells utilize and distribute P. Currently, ^{31}P NMR is used to measure internal P content (Ginzburg, Ratcliffe and Southon, 1988; Živić *et al.*, 2007; Krumova *et al.*, 2008), however, it is a time-consuming method and needs a dense biomass of samples. Some simple and reliable staining methods such as the use of the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) can be used to track the poly-P granules (Kulakova *et al.*, 2011; Gomes *et al.*, 2013; Voronkov and Sinetova, 2019). Recently, a

capillary electrophoresis method was reported for measuring the concentration of Pi and Phi in the media (Hashizume *et al.*, 2020). These monitoring techniques would allow the observation of response to P deprivation and its cellular change.

Finally, it has been reported previously that there is a dynamic change of chloroplast nucleic acid metabolism in response to P deprivation. The chloroplast DNA (cpDNA) content per cell was found to decrease when cells are presented with Phi as a sole P source, presumably as a consequence of limited P. However, the RNA levels remain stable as Phi appears to repress the normal Pi starvation-induced response as a consequence of the cells (mis)sensing Phi as a Pi (Yehudai-Resheff *et al.*, 2007). This suggests the possibility of reducing the chloroplast DNA copy number by P deprivation. This reduction of polyploidy of the plastome might simply be transformed by reducing the time taken to achieve homoplasmic transformants, as discussed further in Chapter 4.2.1.

Chapter 4

Application of *ptxD* as a selectable marker and crop protection tool

Chapter 4 Application of *ptxD* as a selectable marker and crop protection tool

4.1 Introduction

4.1.1 Chloroplast selectable markers in *C. reinhardtii*

Selectable markers for prokaryotic systems have been widely studied over the years (Yan and Fong, 2017), but only a few markers have proved suitable for transformation of the *C. reinhardtii* chloroplast (Esland, Larrea-Alvarez and Purton, 2018). Markers are an essential component of a transformation protocol aimed at introducing transgenes into the plastome due to the low chance of successful DNA delivery into the plastid compartment, with transformation rates typically 10^{-5}-10^{-6} (Spicer and Purton, 2017). A marker allows colonies to form under selective conditions, but limits the growth of non-transformed cells. Each selectable marker has various properties, such as dominance and portability, that offer advantages and disadvantages for chloroplast transformation (Day and Goldschmidt-Clermont, 2011). Markers used in the chloroplast can be divided into three common categories: antibiotic resistance genes, photosynthetic genes and metabolic genes.

For chloroplast transformation of *C. reinhardtii* several portable antibiotic resistance markers have been developed. The bacterial *aadA* gene is currently the most commonly used. The gene encodes an aminoglycoside adenyl transferase (AAD) resulting in resistance to spectinomycin and streptomycin that target the 70S ribosomes of bacteria and chloroplasts (Goldschmidt-Clermont 1991). The *aadA* serves as portable and dominant selectable marker which can be inserted at any desirable locus, and was initially used in the *C. reinhardtii* plastome to study the disruption of the endogenous genes *tscA*, *psaC* and *rpoC2*. The marker has been used to introduce transgenes encoding useful recombinant products such as vaccines (Dreesen, Hamri and Fussenegger, 2010; Jones *et al.*, 2013) and enzymes (Reifschneider-Wegner, Kanygin and Redding, 2014; Dejtisakdi

and Miller, 2016). Some other algal species such as *Euglena gracilis* (Doetsch *et al.*, 2001) and *Haematococcus pluvialis* (Galarza *et al.*, 2018) are naturally sensitive to spectinomycin, and chloroplast transformants can be selected using the *aadA* marker. However, there is a major concern about the presence of antibiotic markers in commercial transgenic strains due to the prospect of undesired horizontal gene transfer to environmental bacteria (EFSA GMO Panel, 2004; Purton *et al.*, 2013; Beacham, Sweet and Allen, 2017). The chloroplast contains multiple copies of its genome, and is of prokaryotic origin as a consequence of endosymbiosis of a cyanobacterium (Keeling, 2013). It is likely that the antibiotic resistance gene cassettes that escape from the chloroplast can be functional if acquired by environmental bacteria. Therefore, the use of such antibiotic-based markers in generating the transformants may inadvertently contribute to the development of antibiotic resistance amongst environmental microbes.

An alternative selection strategy involves using endogenous chloroplast genes that rescue non-photosynthetic mutants carrying mutations in the corresponding chloroplast gene. The selection is based on a restoration of photosynthetic ability in the transformants under light selection on minimal medium. The mutants normally carry point mutations or larger disruption of photosynthetic genes, therefore, they only grow heterotrophically on medium which provides acetate as a fixed carbon source. The transformation with a wild type copy of the photosynthetic gene enables the recovery of functional photosynthesis in the mutants through repair of the genetic lesion. The approach was first described by the rescue of a mutated *atpB* gene encoding the beta-subunit of ATP synthase (Boynton *et al.*, 1988) and the rescue of non-photosynthetic *tscA* mutants that have a mutation in the *tscA* gene involved in the maturation of *psaA* mRNA. (Goldschmidt-Clermont *et al.*, 1991; Kindle, Richards and Stern, 1991).

A current photosynthetic mutant in the Purton lab that is routinely used as an expression platform for generating chloroplast transformants is the TN72 mutant used in the previous chapter. In this mutant a section of *psbH* encoding an essential PSII subunit has been replaced by the *aadA* cassette,

resulting in a Δ*psbH*::*aadA* strain (Wannathong *et al.*, 2016). To rescue the photosynthetic capability, the *psbH* gene was restored by the wild type copy carried in the plasmid. Although the recovery of photosynthetic genes provides a strong selection, often resulting in homoplasmy after a single round of restreaking of single colonies, the use of TN72 as a recipient was problematic as it could take up three to four weeks for colonies to become visible on plates. This is in part due to the requirement for phototrophic growth on minimal medium, which is slower than colony formation via mixotrophic growth on acetate-containing medium.

Whilst the use of selection based on antibiotic resistance and photosynthetic genes in *C. reinhardtii* is most common, metabolic markers can also be used to select for transformants. A dominant metabolic marker would therefore be one in which a non-bioavailable chemical is converted to a bioavailable nutrient essential for growth. As previously described in Chapter 3.2.6, the *ptxD* gene can provide an organism with the novel capability to convert Phi into a bioavailable form of P. The gene could therefore be used for direct selection of transformants, and indeed *ptxD* has been exploited as a marker in several organisms, including cotton (Pandeya *et al.*, 2017), maize (Nahampun *et al.*, 1942) and the yeast, *Saccharomyces cerevisiae* (Kanda *et al.*, 2014). The use of *ptxD* also eliminates the risk of false positive transformation events because, unlike antibiotic resistance, it is highly unlikely that the ability to grow on Phi would arise by spontaneous mutation.

Given that wild-type *C. reinhardtii* can readily uptake Phi but cannot use it as a source of P, growth would be limited on medium containing Phi as the only source of P. However, as shown in Chapter 3.2.7, the expression of *ptxD* in the chloroplast can support normal growth with a sufficient Phi-to-Pi conversion rate and the resulting Pi exported from the chloroplast to feed the whole cell. This highlights the possibility of exploiting *ptxD* as a new metabolic marker for the chloroplast transformation in *C. reinhardtii*. Since *ptxD* encodes an enzyme for Phi metabolism rather than an antibiotic resistance enzyme, this new marker may not limit the use of transgenic lines

as a commercial product. Furthermore, the *ptxD* marker is more suitable as a selection strategy than restoration of a photosynthetic gene as it is portable and can be inserted into any locus within the plastome by flanking the *ptxD* cassette with homologous plastome regions.

4.1.2 PtxD as a crop protection tool

A major economic challenge in the commercial exploitation of microalgae is the need for methods for minimising contamination and spoilage during large-scale cultivation of microalgae. There is often a need for sterility control during media production and culture step-up, and monitoring of the culture to avoid collapse due to uncontrolled growth by opportunistic contaminant species such as bacteria, fungi, protozoa and other microalgal species (Day and Goldschmidt-Clermont, 2011; H. Wang *et al.*, 2013). This sterilisation of medium at massive volume and operating systems for such 'crop protection' contribute significantly to the cost of production. Furthermore, the majority of photobioreactors (PBRs) are relatively simple and low-tech when compared to industrial fermenters (Gupta, Lee and Choi, 2015). Therefore, PBRs are less likely to achieve real-time monitoring and prevent contamination during operation. It is therefore necessary to use antibiotics and other biocides to get rid of undesirable invasive organisms while maintaining the normal growth of the microalgae (H. Wang *et al.*, 2013). An alternative strategy for crop protection is to grow extremophile algae: for example, *Dunaliella salina* that can proliferate at high salinity or *Cyanidioschyzon merolae* that is well adapted to low pH and high temperature. Under these hostile conditions, chance of being contaminated by unfavourable species is reduced. However, the limitations of exploiting these species are the poorly developed molecular tools for genetic engineering, and the additional problems and costs associated with the extreme environments, such as corrosion of metal parts in high salt or low pH systems. Therefore, algal species such as *C. reinhardtii* with well-established genetic tools are often the more preferable platform (Dyo and Purton 2018; Scranton et al. 2015).

Whilst the various strategies described above can be used to keep a microalgal culture free from contamination, exploitation of the PtxD/Phi system offers an alternative crop protection tool. Loera-Quezada et al (2016) reported the successful strategy in genetic engineering of *C. reinhardtii* nucleus with *ptxD*, allowing the alga to grow on Phi. The engineered strain was able to grow and out-compete another algal species when supplied with Phi in the absence of Pi (Loera-Quezada *et al.*, 2016). In this current study, the research into the use of ptxD for crop protection is extended to chloroplast engineering to exploit the benefits of high gene expression levels, no silencing mechanisms in the organelle, and the opportunity for bio-containment of *ptxD* through the stop codons incorporated in the gene to avoid *ptxD* spread in the environment.

4.1.3 Aims and objectives

In this chapter, the aim was to demonstrate the application of *ptxD* as a chloroplast marker in both wild type strains, and in cell wall-less strains since the latter has been used frequently for generating both nuclear and chloroplast transgenic lines. Transformation would be performed using either glass-bead transformation or particle gun bombardment, and selection carried out directly on medium with Phi as the sole source of phosphorus. The potential of *ptxD* as a crop protection tool would then be tested, conferring on the transformants the ability to grow in Phi and outcompete other microorganisms present in the same Phi culture (Figure 4.1). Finally, the 'retro-fitting' of an existing transformant line producing a fish vaccine with PtxD-mediated crop protection would be tested as a proof of concept to demonstrate the potential low-cost production of vaccines for the aquaculture industry.

- To demonstrate the use of *ptxD* as a non-antibiotic selectable marker for chloroplast transformation in *C. reinhardtii*
- To develop a crop protection strategy based on PtxD/Phi system
- To demonstrate a strain improvement strategy by introducing *ptxD* into an existing strain that expresses a fish vaccine

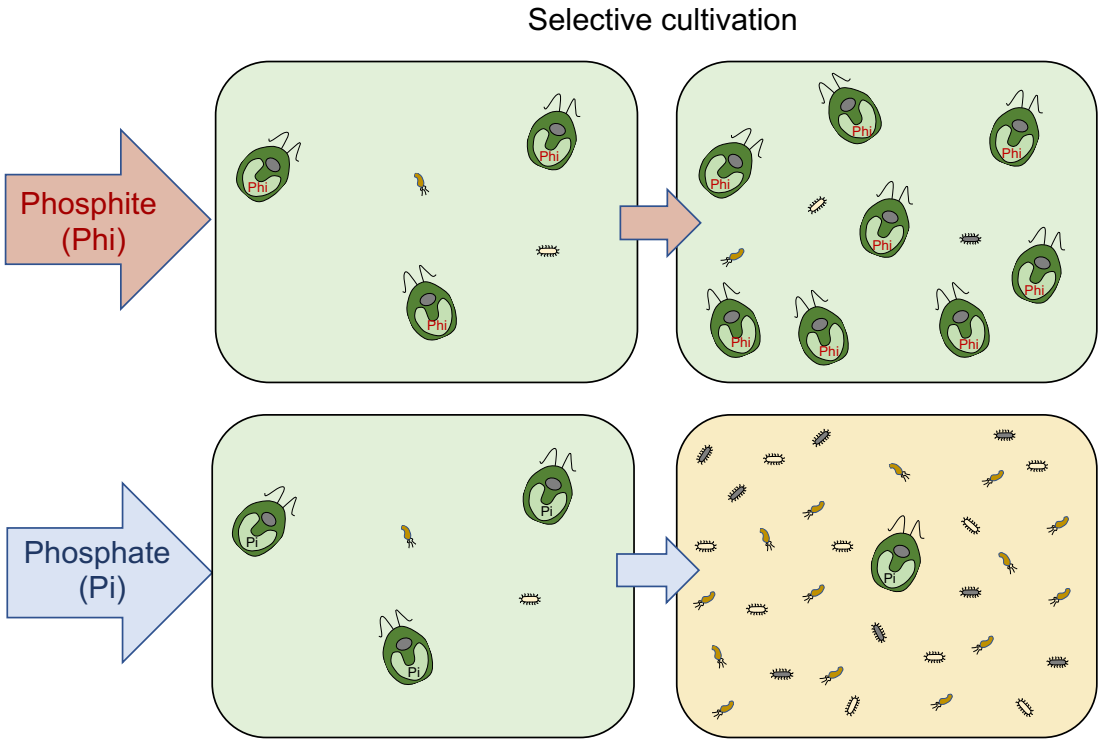


Figure 4.1 Diagram of selective cultivation in non-sterilised medium supplemented with Phi or Pi as a P source.

The *ptxD* engineered microalga has the ability to utilise Phi and out-compete other microbes that have no enzyme to metabolise Phi, demonstrating selective Phi cultivation for *C. reinhardtii*. Providing Pi as a P source results in growth of both microalga and microbes in the non-competitive environment, often resulting in reduced growth of the alga.

4.2 Results

4.2.1 Developing *ptxD* as a non-antibiotic selectable marker for chloroplast transformation

To advance the genetic engineering of the algal chloroplast, multiple selectable markers are needed for such strategies such as serial rounds of transformation. The *ptxD* gene has previously employed as a selectable markers in other platforms (López-Arredondo and Herrera-Estrella, 2013; Manna *et al.*, 2016; Pandeya *et al.*, 2017). This section focuses on demonstrating and optimising *ptxD* as a marker for direct selection of chloroplast transformants.

An initial experiment was to examine the possibility to recover PtxD transformants on Tris-acetate medium supplemented with 1 mM Phi as the only P source. The transformation used the plasmid construct pWUCA2+ptxD described in Chapter 3.2.1, and the negative control transformant line TN72::pWUCA2 as the recipient. This line was previously generated by transforming TN72 with the empty vector pWUCA2, and unlike TN72, this cell line has a recovered photosynthetic ability so is able to grow mixotrophically on acetate-containing media in the light and grow phototrophically on minimal medium due to the restored *psbH* gene. It also has additional flanking sequence provided by the integrated *trnW*$_{UCA}$, promoter, 5' UTR and 3'UTR elements of the expression vector, as illustrated in Figure 4.2.

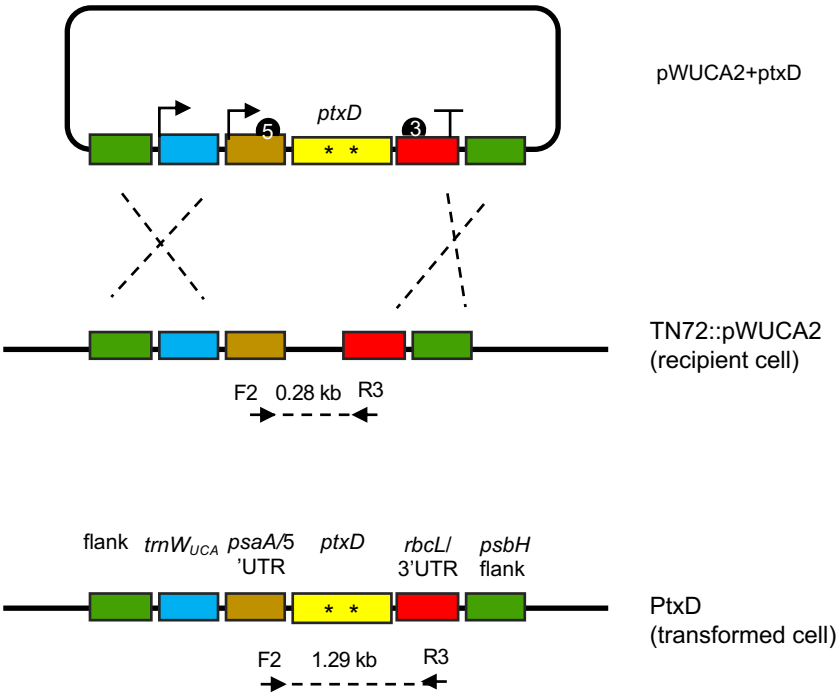


Figure 4.2 Schematic transformation of *ptxD* plasmid into the recipient cell line TN72::pWUCA2.

The plasmid construct carries the *ptxD* gene cassette under the control of the *psaA* promoter and 5'UTR and the *rbcL* 3'UTR. The cassette is flanked by the endogenous chloroplast sequences from the *psbH* locus. The recipient cell TN72::pWUCA2 was created by the transformation of TN72 background with empty plasmid pWUCA2. The coding region of *ptxD* is shown in yellow. PCR analysis was carried out with the use of F2 and R3 primers. The F2 primer binds to the *psaA* 5'UTR region whereas the R3 primer binds to the *rbcL* 3'UTR site. A PCR product of 1.29 kb indicates successful *ptxD* insertion, whereas a 0.28 kb product confirms no integration.

Two colonies of transformed cells appeared after two weeks following glass bead-mediated transformation and plating on TA-Phi plates, and an additional nine colonies became visible after seven weeks (Figure 4.3). The negative control (treated cells with no DNA added) showed a clear background of non-transformed cells to confirm that no colonies was able to grow under Phi selection. This facilitates the identification of genuine transformant colonies on the selective plates. Six representatives colonies were restreaked on a selective Phi medium and verified for correct gene insertion by PCR (Figure 4.4). All six colonies appeared to be homoplasmic as shown by the amplicon of 1.29 kb (as seen in the genetically equivalent positive control strain PtxD1), and there was no detection of the smaller amplicon at 0.28 kb corresponding to the empty locus on the plastome of the TN72::pWUCA2 recipient, indicating a homoplasmic state with no copies remaining of this original plastome. These homoplasmic lines were achieved after just a single round of streaking out on phosphite medium. A strong selection pressure for maximum PtxD activity potentially drives the homoplasmic state to be established quickly.

For the marker to be useful, it should be functional in a variety of different *C. reinhardtii* strains, including those with a wild-type plastome. Another cell wall-deficient mutant, *cw2*, was therefore used as the recipient strain. This strain has a plastome with a wild-type *psbH* locus, unlike TN72::pWUCA2 but should be transformable by the glass bead method using the same pWUCA2+ptxD plasmid. Only a single putative transformant was recovered in this experiment, but the result shows that *cw2* was also able to be transformed with *ptxD* and directly selected on Phi medium. (Figure 4.5). These studies demonstrate the potential of *ptxD* as a novel non-antibiotic marker in cell wall-deficient strains of *C. reinhardtii*. Use of this marker possibly speeds up the transformation process compared to markers such as *aadA* and *aphA6* as only a single round of streaking is needed compared to the several rounds when selecting on antibiotics (Goldschmidt-clermont, 1991; Bateman and Purton, 2000). Selection is also faster than that based on restoration of phototrophy, since the appearance of colonies when using the TN72/*psbH* marker system typically take four weeks. This is because the

colonies must grow phototrophically on minimal medium, rather than mixotrophically as in the case of *ptxD* transformants.

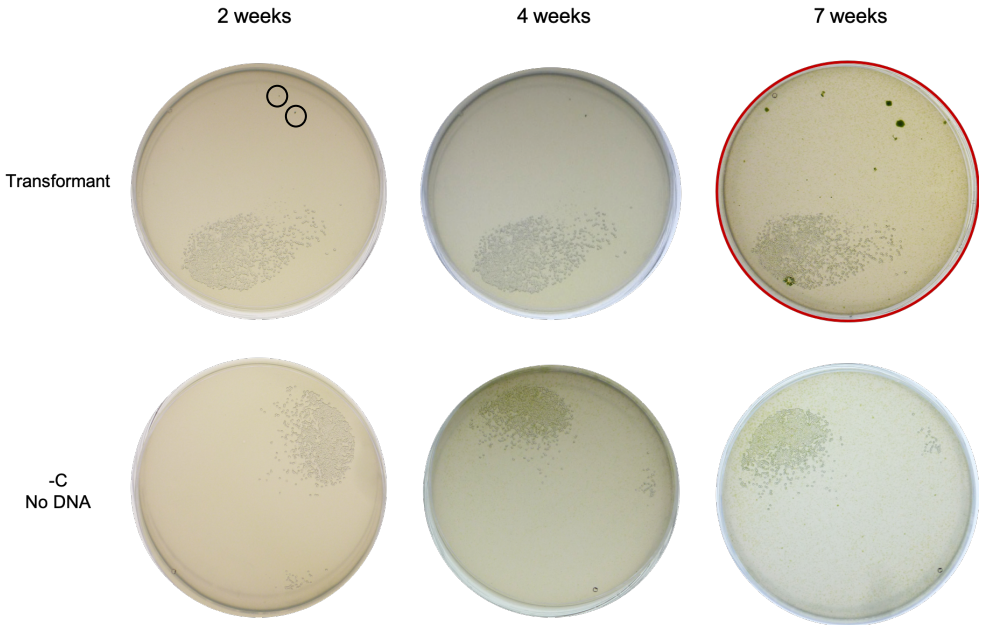


Figure 4.3 Photograph of direct selection when transforming TN72::pWUCA2 with the *ptxD* plasmid.

The cells were resuspended in TA-Phi medium to obtain $2x10^8$ cell/ml and mixed with molten TA-Phi+0.5% agar prior to plating. The selective plates were incubated for 2-7 weeks at 25°C and moderate light conditions (~50 µE white light). -C is the mock transformation without the addition of DNA plasmid as a negative control. The transformant colonies that appeared in week 2 are indicated by circles.

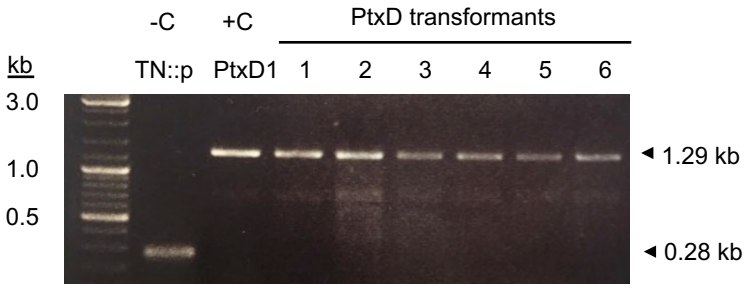


Figure 4.4 PCR analysis confirming successful integration of *ptxD* into TN72::pWUCA2.

The integrated cassette is indicated as a 1.29 kb band. PtxD1 was used as a positive control strain and TN72::pWUCA2 (TN::p) as a negative control strain.

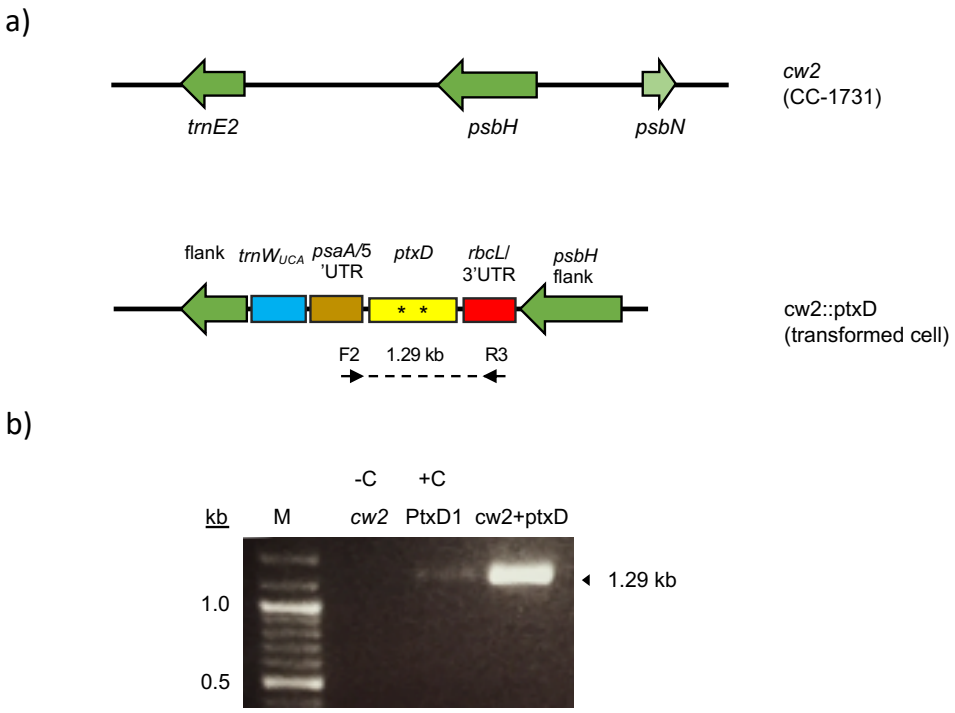


Figure 4.5 Schematic transformation of *ptxD* plasmid into the recipient cell *cw2*.

a) The *ptxD* gene cassette was under the control of *psaA* promoter and 5'UTR and the *rbcL* 3'UTR. The recipient cell was the cell wall-deficient line *cw2* in with no alterations in the *psbH-trnE2* intergenic region. b) PCR analysis confirmed successful integration of *ptxD* into *cw2*. The *ptxD* insertion gives rise to a 1.29 kb band when using primers F2 and R3. PtxD1 was used as positive control strain (+C) and *cw2* recipient line was a negative control (-C).

To enable the broad use of *ptxD* as a portable selectable marker, the next step was to test whether it could be used for biolistic transformation of cells with a wild-type cell wall. Furthermore, it was useful to test another integration site for the marker since the neutral site between *psbH* and *trnE2* is used frequently for insertion of transgenes (Wannathong *et al.*, 2016) and 'retrofitting' these transformants with the *ptxD* gene targeted to the same site would not be possible. A new plasmid was therefore designed in which the *ptxD* cassette was linked to the modified $trnW_{UCA}$ and flanked with homology arms that target the marker to a neutral site between *psaA-3* and *trnL* (Figure 4.6). The gene was targeted to this site because most engineered *C. reinhardtii* strains have not exploited this locus for expressing transgenes, providing a site for *ptxD* insertion. The modified tRNA was included since it is necessary to translate the two UGA stop codons within *ptxD* as tryptophans and allow the accumulation of the full-length PtxD protein within the chloroplast. The plasmid was named pPO3 to reflect its ability to convert any existing strains of *C. reinhardtii* to phosphite metabolism, including non-GMO strains and those with nuclear or chloroplast-engineered genomes.

The use of pPO3 plasmid for the direct selection on Phi was tested using the wild type strain, CC-1690 via biolistic transformation in which DNA was delivered into the chloroplast by high-speed bombardment using DNA-coated gold particles. One starter culture was grown in TA medium that lacks a P source (= P-starved) to minimise the intracellular reserves of polyphosphate which can accumulate in *C. reinhardtii* as a store of Pi (Komine *et al.*, 2000; Solovchenko *et al.*, 2019). Another culture (= P-replete) was grown in TAP medium with a standard amount of phosphate normal to represent the cell line enriched in intracellular polyphosphate. The cells were resuspended in fresh P-free TA medium at a cell density of 1 x 10^8 cell/ml and plated onto TA-Phi 2% agar plates using 0.5% soft agar, to match the conditions used in the glass-bead transformation.

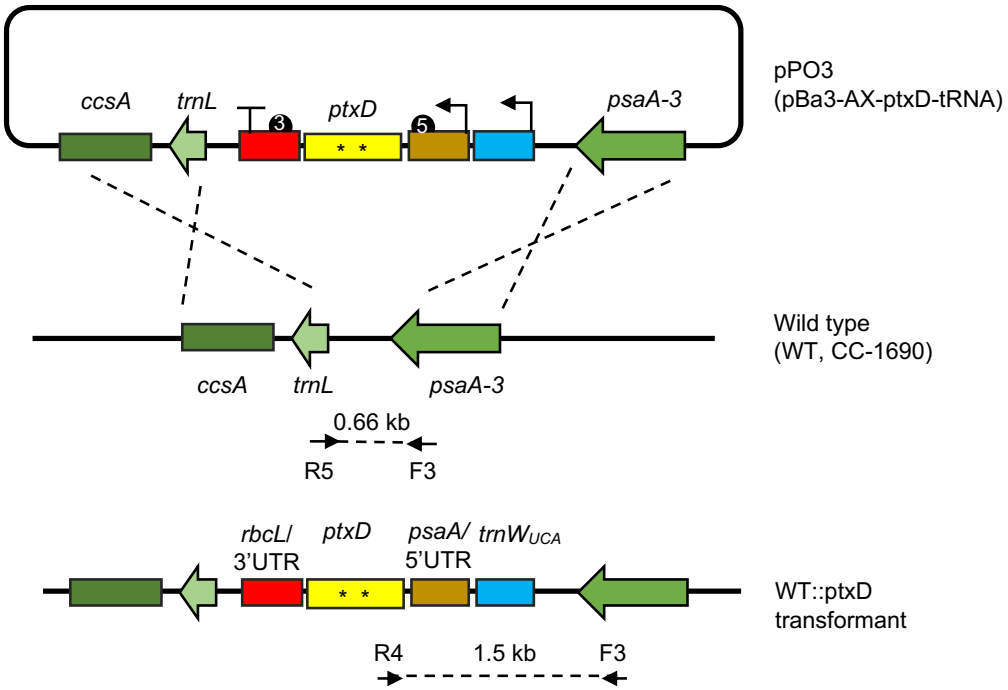


Figure 4.6 Schematic introduction of the *ptxD* gene into the plastome of wild type strain CC-1690.

The cassette is flanked by endogenous chloroplast sequences to mediate insertion into the intergenic region between *psaA* exon 3 and *trnL* genes (green) via homologous recombination (dashed line). Primer F3, R4 and R5 are used to check homoplasmy by PCR analysis. F3 primer binds to *psaA-3* whereas R4 binds to the coding region of *ptxD*, and R5 binds to the *trnL* sequence. A combination of F3 and R4 primers results in a product of 1.5 kb for correct PtxD insertion. In contrast, F3 and R5 primers give rise to a wild type band at 0.66 kb.

Approximately, ten colonies of the transformed wildtype strain were observed within a week for both the P-starved and P-replete grown recipient cells, and more colonies were clearly seen within eight weeks (Figure 4.7). However, it was notable that the P starvation performed for 18 h before the transformation reduced the background of pale green non-transformed cells during the longer incubation time. Whereas no growth of P-replete grown recipient cells appeared outside the biolistic bombardment area after one week, but some minor growth was seen after 8 weeks. It was assumed that the non-transformed cells grew using cell debris as an external source of P rather than having the ability to convert Phi to Pi. In summary, whilst the PtxD/Phi selection can be achieved within P starvation before the transforming process, the background of non-transformants is lower when using P-starved rather than P-replete cells.

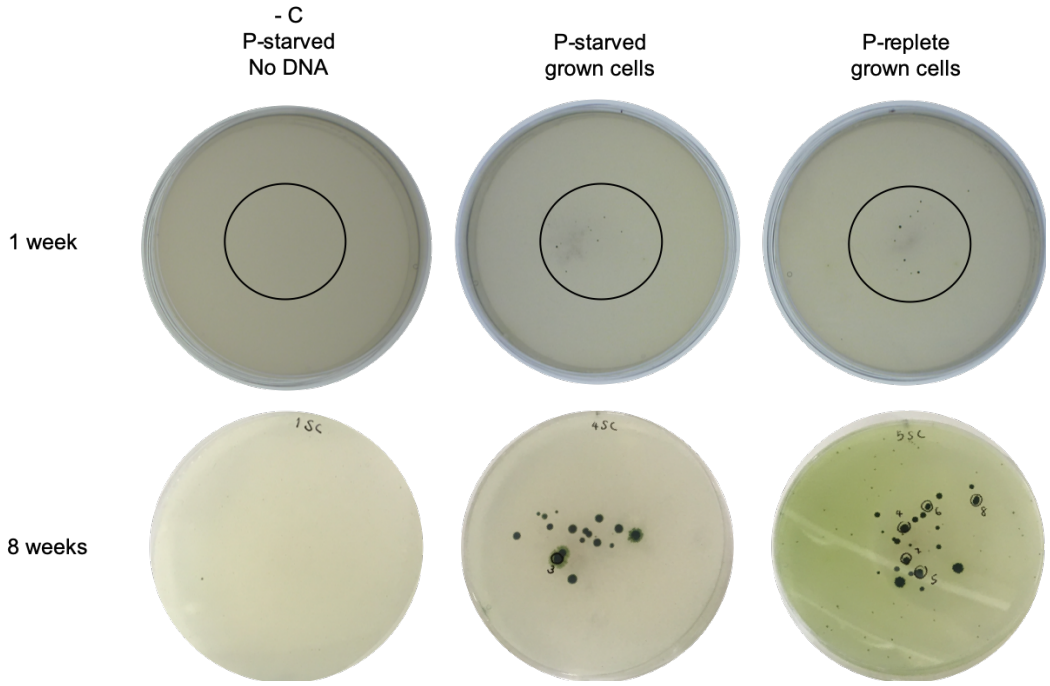


Figure 4.7 Photographs of WT::ptxD transformation plates.

After a week, transformant colonies appeared on phosphite plates when using either P-starved and P-replete grown cells as the recipient line during biolistic transformation. The colonies were seen more clearly after 8 weeks of incubation. – C is the negative control of P-starved cells with no addition of DNA. The circles indicate the working area of biolistic transformation. P starvation was initiated 18 h prior to transformation.

After the first round of restreaking the WT::ptxD colonies, the integration of *ptxD* at the *psaA-3*–*trnL* neutral site and the homoplasmic state of the plastome were examined by PCR using a set of three primers (F3, R4 and R5). In this case, F3 binds to the coding region of *psaA-3,* R4 binds to the coding region of *ptxD* and R5 binds to the *trnL* sequence (Figure 4.6). Therefore, the combination of F3 and R5 primers would give rise to a small amplicon of 0.66 kb from the WT plastome. Conversely, F3 and R4 primers would give rise to a band of 1.5 kb from the transformant plastome, indicating the correct integration of *ptxD*. The result of this PCR (after the first round of restreaking of transformants) confirmed the integration of *ptxD*, but the presence of a faint band at 0.66 kb indicated some remaining wild type copies in the transformant lines – *i.e*. the homoplasmic state was not yet achieved in these transformants (Figure 4.8).

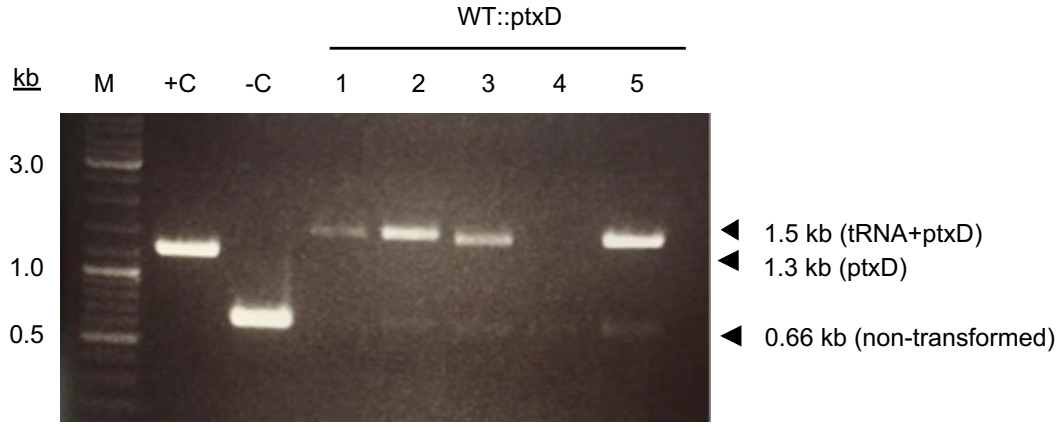


Figure 4.8 Confirmation of *ptxD* integration into the plastome of wild type strain CC-1690 when transformed with the pPO3 plasmid.

Five transformant lines were analysed by PCR after the first round of restreaking. Successful integration of *ptxD* is indicated by a 1.5 kb band arising from a combination of the F3 and R4 primers, and the presence of the wild type plastome shown by a 0.66 kb band amplified by F3 and R5 primers. Positive control (+C) is the NNV:ptxD transformant (described later) given a smaller band at 1.3 kb as a result of the absence of the modified tRNA gene at the insertion locus. The negative control (-C) was the wild type recipient.

The observation above of a faint band in the PCR analysis indicating a heteroplasmic state, prompted the question whether such heteroplasmy might go undetected if only a single copy of the WT plastome remained in the cells of a transformant line. Under mixotrophic conditions, the ploidy of the plastome is ~80 (Gallaher *et al.*, 2018), therefore the colony PCR method was optimised to check for a homoplasmic state at the very low threshold of 1 in 80 plastome copies being a WT copy. This was done by diluting the DNA from the WT strain in a serial dilution of 1/2, 1/10, 1/20, 1/50 and 1/80 and amplifying the 0.66 kb target by PCR over 30 cycles. If the PCR still detected the WT copy at a dilution of 1/80th, it should be sensitive enough to detect the same threshold remaining in heteroplasmic transformants. It was found that the wild type plastome remained detectable from non-diluted up to 80-fold diluted DNA samples. However, the band was very faint when the DNA template was very diluted. (Figure 4.9:a). The number of cycles of PCR was therefore increased to 35 to test whether the band would become more visible, and the transformant lines were re-checked following four rounds of restreaking on TA-Phi medium (Figure 4.9:b). As can be seen, increasing the amplification to 35 cycles led to a clearer band even at 1/80th dilution. The only concern of increasing the PCR cycles was the higher risk of additional bands due to non-specific binding of primers to off-target regions, but it was not seen in this case. That all transformants had achieved a homoplasmic state was confirmed by the amplicon of the 1.5 kb band, and the lack of any amplified 0.66 kb band from a WT plastome. Hence multiple rounds of restreaking leads to the establishment of homoplasmy, and this can be confirmed by colony PCR using the 3-primer strategy.

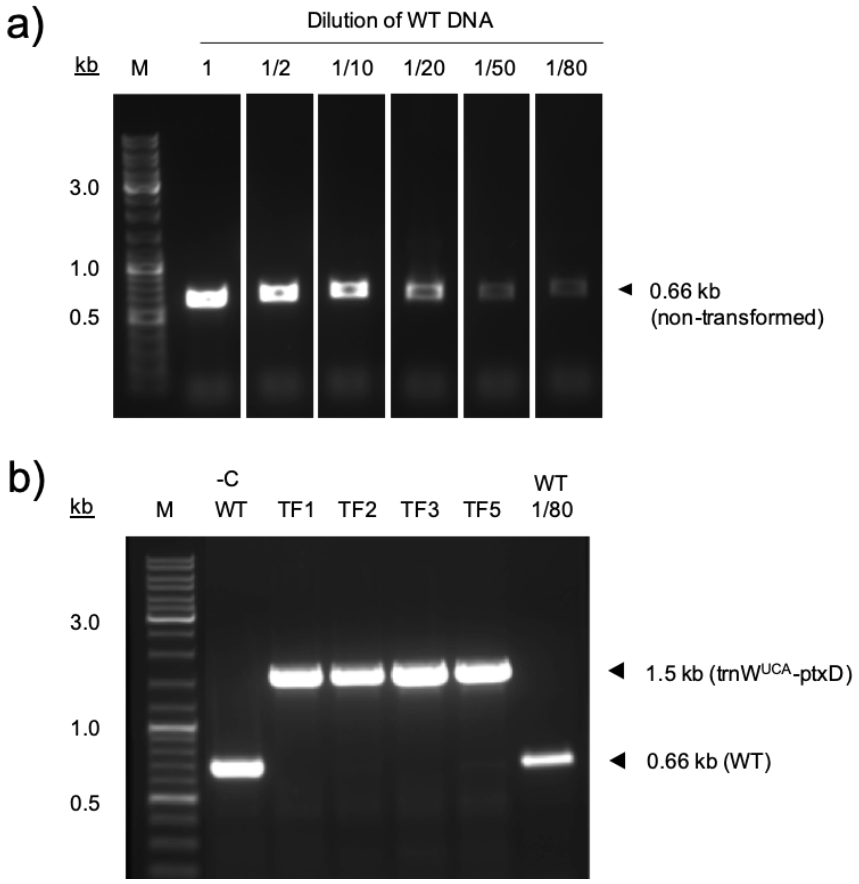


Figure 4.9 Confirmation of homoplasmy in WT::ptxD transformants by PCR after the fourth round of restreaking.

Successful integration of *ptxD* gives a band of 1.5 kb, whereas the untransformed wild type plastome gives a band of 0.66 kb. a) the Chelex-prepared DNA was diluted in serial dilutions of 1/2, 1/10, 1/20, 1/50 and 1/80 to observe the limit of detection of the WT copy by PCR after 30 cycles. M refers to the marker. b) analysis of four transformant lines (TF1, TF2, TF3 and TF5) and the WT recipient by PCR using 35 cycles. The DNA of the transformants was undiluted, whereas the WT DNA template was also diluted 80 fold to mimic the threshold of a single wild type copy present in the chloroplast of a heteroplasmic transformant.

4.2.2 Strain improvement was achieved by introducing *ptxD* into other *C. reinhardtii* strain expressing a fish vaccine

To test whether the *ptxD* cassette could be used as a crop protection tool as well as a selectable marker, a retrofitting strategy was explored in which *ptxD* was introduced into a TN72 strain that had been previously engineered to produce a subunit vaccine against the fish pathogen Nervous Necrosis Virus (NNV) (Rajakumar, 2016) (Figure 4.10). The retrofitting would serve as a proof of concept of how to produce an oral fish vaccine at low-cost for the aquaculture industry. Supplying algal biomass for the aquaculture sector is challenging as contamination issues can arise in the low-cost photobioreactors used for cultivation (Changko *et al.*, 2020). The use of bulk medium sterilization (e.g. via autoclaving or treatment with ultraviolet light or ozone) adds a significant cost to industrial-scale cultivation, and the inclusion of antibiotics or other antimicrobial agents to the cultures to suppress contamination during operation brings further costs as well as product contamination issues.

The experiment was started by generating a NNV::PtxD line expressing both NNV and PtxD from the existing TN72::nnv transgenic line. The *ptxD* expression cassette was inserted into the *psaA*-3–*trnL* neutral site by glass-bead mediated transformation using the plasmid (pBa3-AX-ptxD) that is equivalent to pPO3, except that it lacks the modified *trnW*$_{UCA}$ upstream of the *ptxD* cassette. This was because TN72::nnv already has a copy of *trnW*$_{UCA}$ within its plastome upstream of the *nnv* gene in order to allow translation of the two TGA codons within the *nnv* coding region (Figure 4.11). A representative transformant line was chosen following selection on TA-Phi medium, and was analysed for *ptxD* insertion, homoplasmy and protein accumulation.

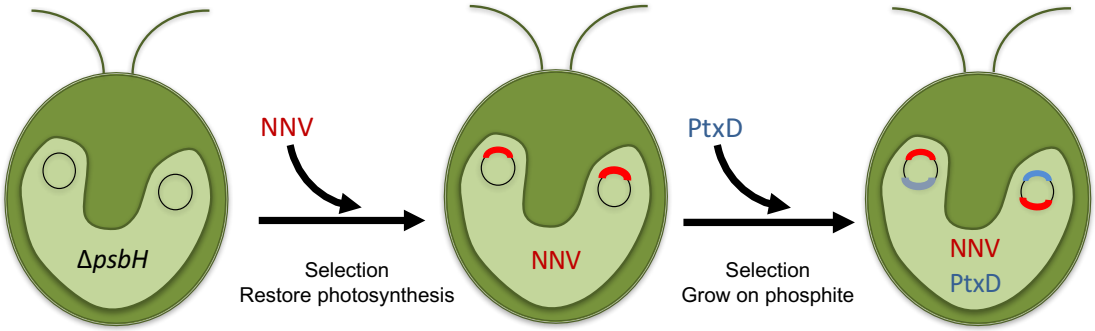


Figure 4.10 Retro-fitting strategy to insert *ptxD* into a *C. reinhardtii* strain previously engineered to synthesise a subunit vaccine against Nervous Necrosis Virus (NNV).

The strain was generated in the TN72 background (Δ*psbH*) with selection based on restoration of photosynthetic capability. This NNV strain was then transformed with *ptxD* and selected based on the ability to grow on phosphite. This created a novel strain which produces the fish vaccine and is able to use phosphite as a P source for crop protection.

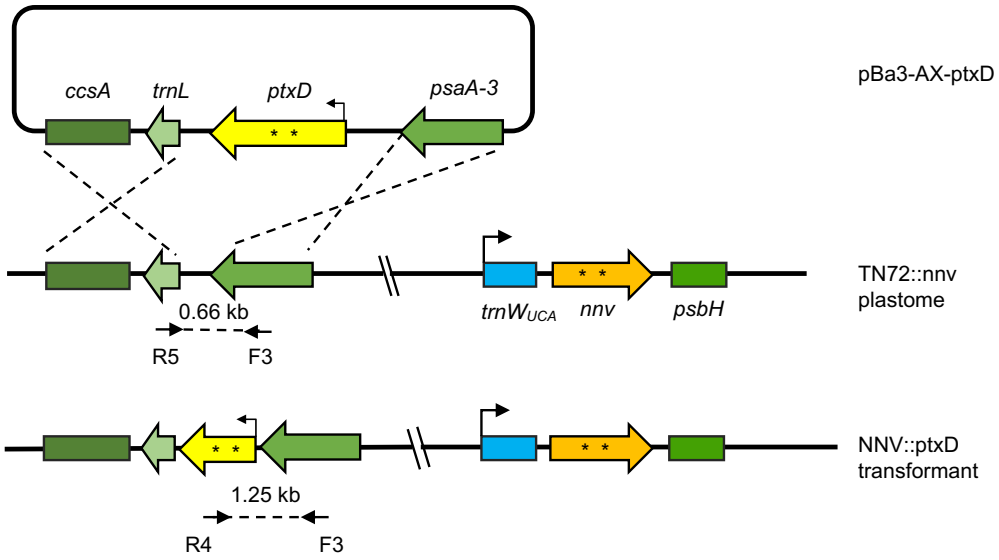


Figure 4.11 Schematic of the introduction of *ptxD* into a neutral site in the plastome of TN72::nnv by transforming with pBa3-AX-*ptxD*.

The modified *trnW*$_{UCA}$ adjacent to the *nnv* cassette is responsible for the translation of stop codons (shown as *) within both *nnv* and *ptxD* located 7.2 kb apart on the plastome. Primers F3, R4 and R5 were used to check homoplasmy by PCR analysis. A combination of F3 and R4 results in a product of 1.25 kb for correct *ptxD* insertion at the *psaA-3–trnL* locus. In contrast, F3 and R5 primers give rise to a band of 0.66 kb in the wild-type locus is present.

The PCR analysis showed the successful integration of *ptxD* at the target locus. (Figure 4.12) The absence of a 0.66 kb band indicates a homoplasmic state in the transformant line.

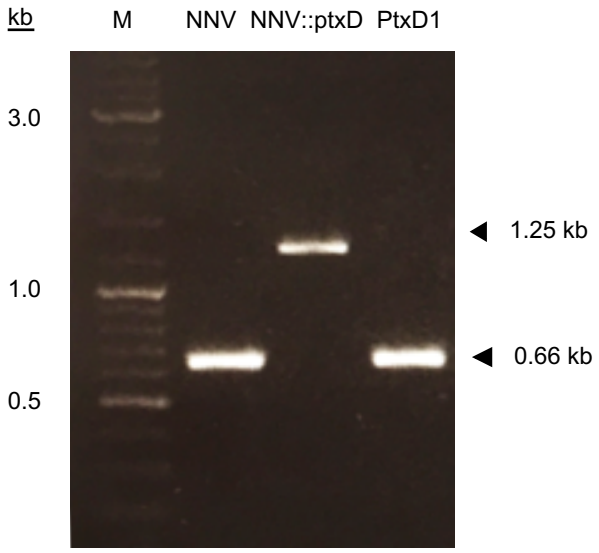


Figure 4.12 PCR analysis confirming the integration of ptxD in the NNV::ptxD transformant.

A combination of the F3 and R4 primers results in a product of 1.25 kb for correct PtxD insertion in the plastome. Whereas, the F3 and R5 primers gives a band at 0.66 kb for the untransformed (wild-type) locus in the TN72::nnv and TN72::ptxD (PtxD1) lines. The lack of this band in the NNV::ptxD strain indicates homoplasmy.

Having established that *ptxD* had successfully integrated into the plastome together with *nnv*, the next stage was to confirm expression of these two genes. One concern was the fact that both genes contain two internal TGA stop codons, and whether this might titrate out the pool of the novel tRNA encoded by the single copy of $trnW_{UCA}$. It was therefore important to test the full readthrough of the *ptxD* and *nnv* transcripts by comparing the accumulation of both proteins to the single transformation lines TN72::nnv (NNV) and TN72::ptxD (PtxD1). The detection of PtxD and NNV proteins was investigated by western blot using crude cell lysates. Since both proteins possess a C-terminal HA tag, an anti-HA antibody was used to detect both the PtxD protein (37 kDa) and the NNV capsid protein (39 kDa) (Figure 4.13:a). Both proteins are detectable in the NNV::ptxD transformant in comparison to the single transformant lines used as positive controls. The levels of both proteins in the NNV::ptxD strain might be marginally lower than that in the positive controls based on the infrared fluorescence signal (Figure 4.13:b), however, the difference might simply reflect loading differences. This NNV::ptxD engineered strain therefore represents a successful retro-fitting of a commercially useful strain: a transgenic line producing a fish vaccine and able to grow in non-sterile phosphite medium as shown below (Figure 4.15).

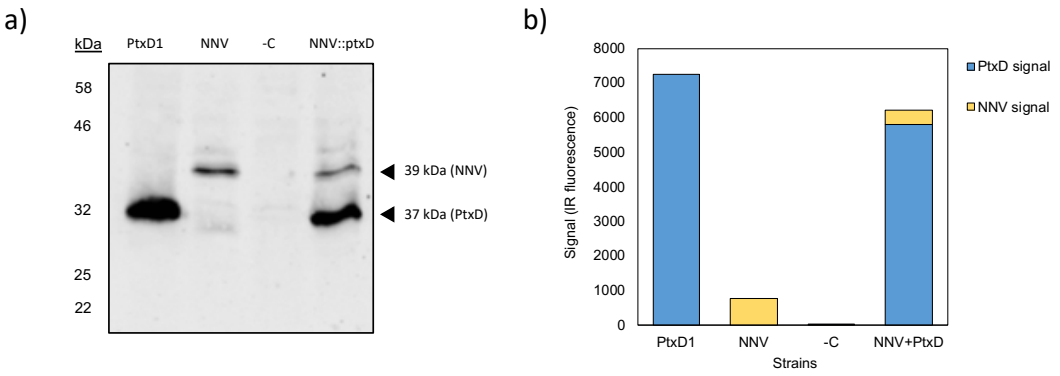


Figure 4.13 Western blot analysis of the NNV::ptxD transformant using anti-HA antibodies to show the accumulation of both HA-tagged proteins.

a) the NNV capsid and PtxD protein are indicated (with predicted sizes of 39 and 37 kDa, respectively). TN72 transformed with the empty pWUCA2 plasmid was used as a negative control (-C). PtxD1 and NNV transformant lines were used as positive controls for the presence of each protein. The lysates were diluted two-fold prior to western blot analysis. b) Quantification shown using the infrared fluorescence signal (IR fluorescence) excited at 785 nm that is suitable for the detection of the secondary antibody: Goat anti-rabbit IgG, DyLight 800 (excitation/emission at 777/794 nm). The detection was performed by Odyssey® Infrared Imaging system (Li-COR Biosciences). All samples are equalised to the same optical density.

4.2.3 Developing *ptxD* as a crop protection tool for large-scale cultivation

Contamination is one of the challenges for microalgal cultivation at large-scale, and the use of *ptxD* as a crop protection tool could potentially minimise undesirable contamination. Genetic engineering *ptxD* into the chloroplast of *C. reinhardtii* would provide a selective advantage – the ability to grow in Phi over other contaminating species. According to Loera-Quezada et al. (2016), strain improvement by expressing *ptxD* in *C. reinhardtii* nucleus supported algal growth in Phi medium under non-sterile conditions, with the growth of the PtxD strain surpassing that of contaminating species including other algae. In this work, *ptxD* has been expressed in the chloroplast. The chloroplast has a relatively simpler genetic system compared to the nucleus and offers higher levels of gene expression and no silencing mechanisms. Furthermore, by flanking *ptxD* with homologous sequences, the gene can be targeted to any specific loci in the chloroplast, as opposed to nuclear transformation that would insert *ptxD* randomly into the genome.

An important feature of the chloroplast *ptxD* gene with regard to crop protection is that it incorporates a biocontainment system to avoid the transgene spreading through horizontal transfer to other microorganisms that would then be able to metabolise Phi. Using the codon reassignment strategy described by Young and Purton (2016), the synthetic *ptxD* carries two internal stop codons that need to be translated as tryptophans by a modified chloroplast tRNA. This tRNA is likely to be non-functional (or toxic if it were functional) if the *trnW*$_{UCA}$ gene were to be transferred together with the *ptxD* gene to a bacterium or fungal genome (Young and Purton, 2016). For the general use of *ptxD* in crop protection, a retro-fitting strategy was illustrated by inserting the gene cassette in a neutral intergenic region between *psaA-3* and *trnL* genes, and this approach was used on the existing chloroplast transformant producing a fish vaccine.

The crop protection strategy was tested initially using the PtxD1 transformant line to see if growth in non-sterilise Phi medium could overcome

the contamination and spoilage issues caused by environmental microbes when microalgae are grown in non-sterile conventional Pi medium. PtxD1 was grown under sterile and non-sterile conditions in 400 ml of different forms of Pi and Phi media (Figure 4.14:a). The mixotrophic growth of PtxD1 was shown in Pi and Phi media containing acetate (+Acetate) as a carbon source. At the beginning of the cultivation, a cocktail of naturally-grown bacteria and fungi (1ml added to each flask of an acetate-containing media that had been left open to the air and then left to form a cloudy growth) was purposely applied to each of the 'non-sterile' flasks to mimic the effect of a culture becoming contaminated. As shown in Figure 4.14:a, no marked difference was observed between the cultures grown in either sterile Pi or sterile Phi media. Importantly, the results also showed that growth in non-sterile Phi media was comparable to that in sterile Phi medium, indicating that algal growth was not grossly affected by the addition of the microbial cocktail when Phi was the source of P. In contrast, there was a clear difference between non-sterile and sterile media when Pi was the source of P. The non-sterile Pi culture experienced heavy contamination as indicated by a pale green culture (Figure 4.14:a). The growth reduction in non-sterile Pi was a consequence of providing Pi as a source of P for the contaminants, allowing them to out-compete the growth of the alga.

The levels of cell density and contamination were also measured by particle distribution analysis (Figure 4.14:b). This technique is able to differentiate particle size by measuring the intensity of scattered light when passed through a culture. A distinct peak at approximately 1 μm diameter was detected in the non-sterile Pi culture, confirming the presence of contaminating bacteria. Moreover, the height of the peak around 10 μm diameter that corresponds to the *Chlamydomonas* is significantly lower in this culture when compare to the other three showing that algal growth is compromised. In contrast, no bacteria is detectable in the non-sterile culture grown on Phi medium. However, the cell size in Phi appears to be slightly smaller than that in Pi under sterile condition. Possibly, Phi may induce the physiological changes due to osmotic or pH shift during cultivation. The cell sizes >10 μm were probably corresponding to clumped cells. These findings

demonstrate that the PtxD/Phi system has potential as an effective crop protection by providing the microalga with a selective advantage over the contaminants.

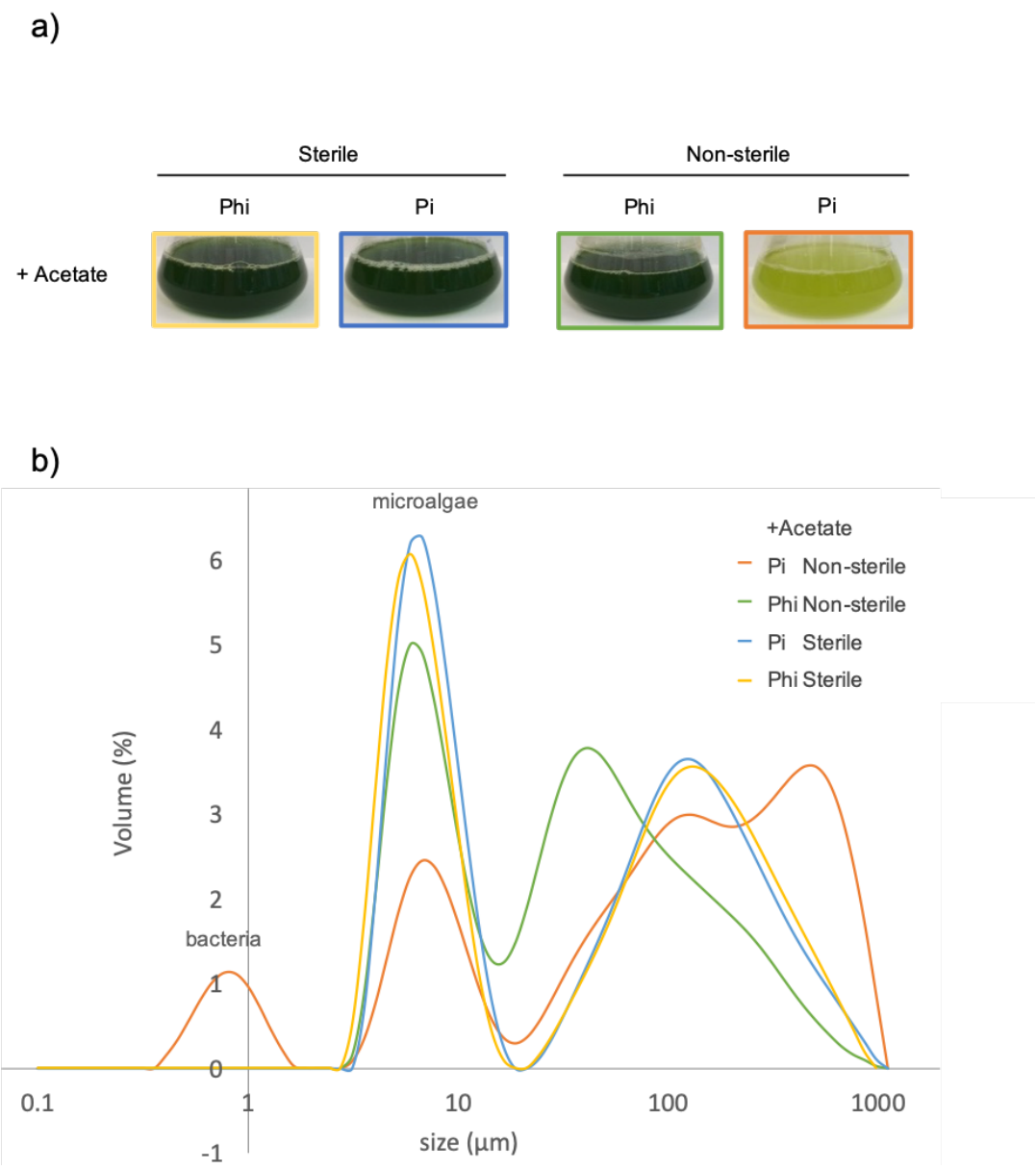


Figure 4.14 Comparison of growth of PtxD transformant (PtxD1) in mixotrophic condition under sterile and non-sterile environments.

a) PtxD1 cultures were grown under sterile and non-sterile conditions. Mixotrophic growth was performed in acetate medium supplemented with Pi or Phi and incubated for 7 days. b) The contamination level in the cultures measured by Mastersizer particle analysis. The bacteria peak is detected at approximately 1-3 µm and *C. reinhardtii* at approximately 10 µm. The peak >10 µm was speculated to be aggregated cells.

The use of microalgae as a platform for fish vaccine production could be challenging when taken to large-scale as the need for massive medium sterilisation would add significantly to the cost of production. As a proof of concept for inexpensive microalgal cultivation, the crop protection system was tested at larger scale using the engineered strain NNV::ptxD that was previously retrofitted with *ptxD*. In this study, the strain was cultivated in a 20 litre hanging-bag system as a pilot-scale model (Figure 4.15). The strain was grown mixotrophically in Tris-acetate medium supplemented with either Pi or Phi as a P source without medium sterilization. A 1 ml of naturally-grown contaminants was inoculated into the cultures to create the potential for contamination. The appearance of both cultures remained similar in the first five days, but a marked difference in colour was seen afterwards. The culture grown in Pi started turning a pale yellow colour while the culture in Phi appeared to be a healthy green. Microscopic photos of Pi and Phi cultures were taken on Day 3 and 13 (Figure 4.16). There was more debris and bacteria observed in the Pi medium compared to the Phi medium. The level of contaminants and algal cells was again estimated by using the Mastersizer technique at Day 13. The Peak at 1-3 µm of bacteria was observed in the Pi but not in Phi culture. In contrast, a higher peak of *C. reinhardtii* at ~10 µm was seen in the Phi culture illustrating healthier algal growth (Figure 4.17). Furthermore, the cells in Pi medium appear enlarged indicating some form of stress on the microalgal cells when exposed to the contamination. However, the protein detection in the NNV strain was not carried out after hanging bag cultivation.

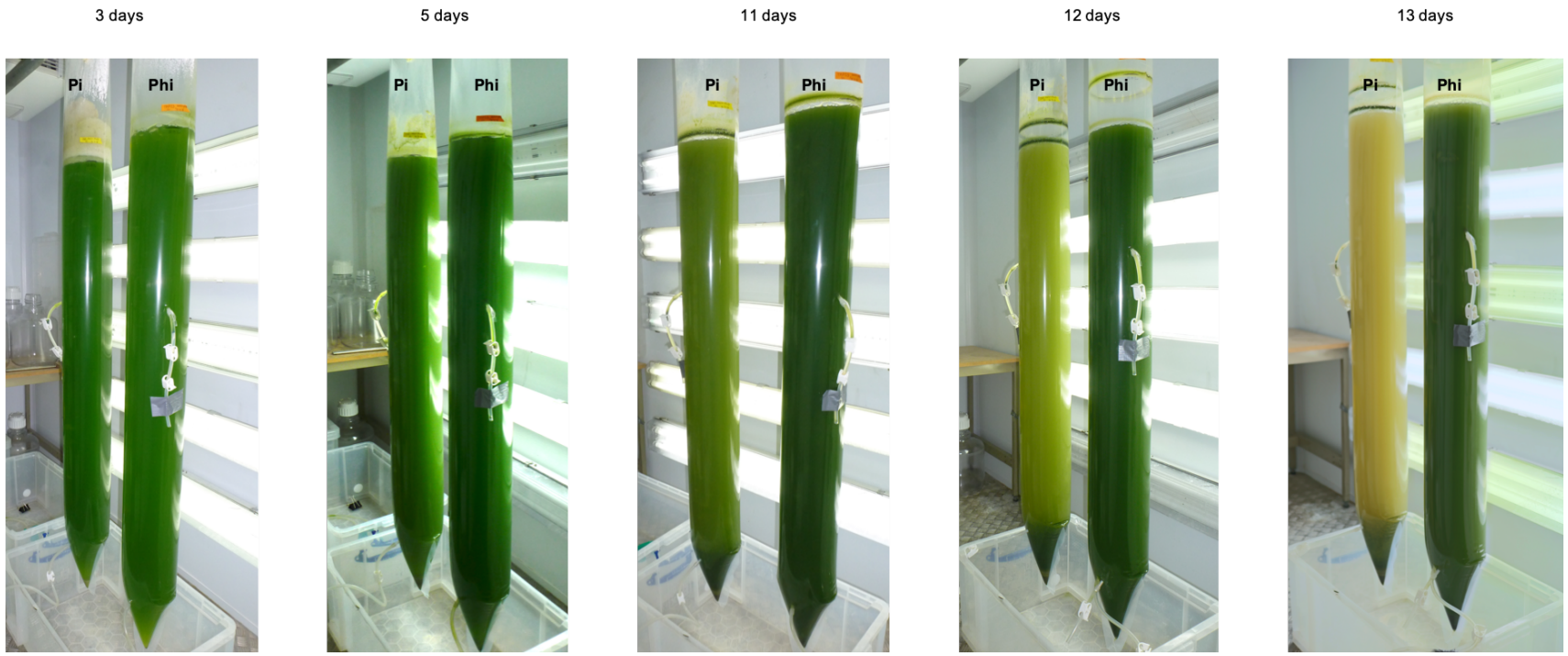


Figure 4.15 Large-scale cultivation of the NNV::ptxD strain in a ‘hanging bag’ system

The cultivation was under non-sterile conditions (no sterilisation of the media or sterile technique during operation set-up). Photograph of the cultures grown in phosphate (Pi) and phosphite (Phi) media over 13 days. Each 20 litre bag was inoculated with a starter culture (2.5 % v/v inoculum) and a cocktail of natural contaminants (0.1% v/v). The cultures were grown mixotrophically with ~100 µE illumination from a fluorescent light panel and bubbling with sterile air at 2.5 L/min.

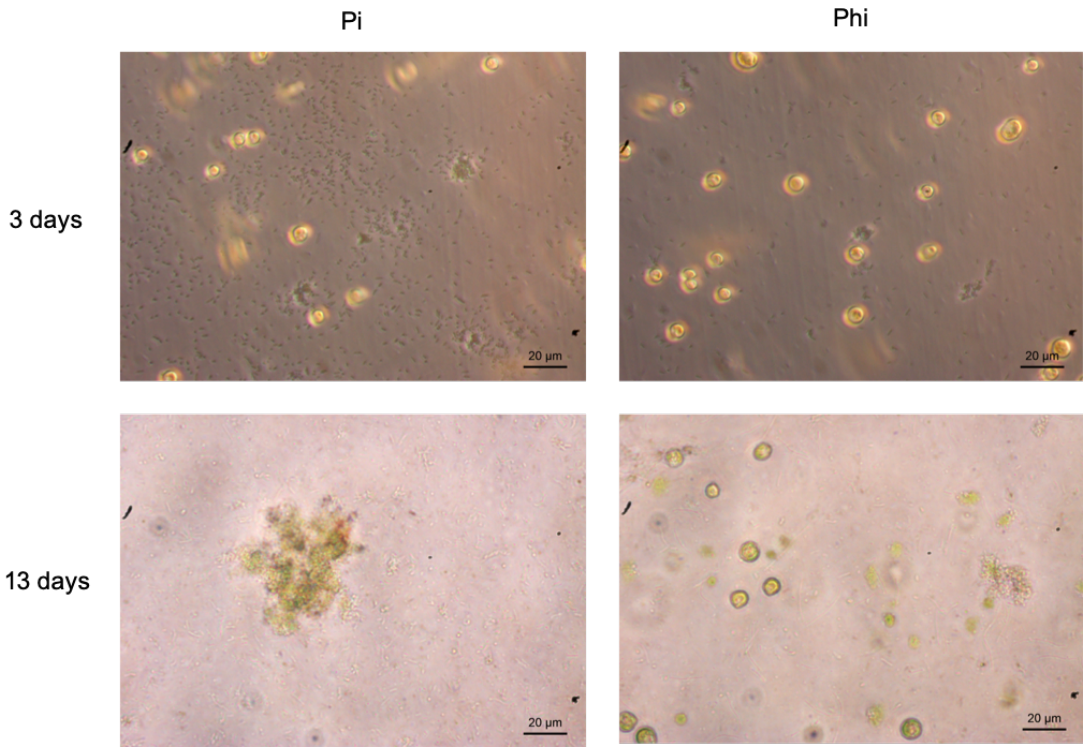


Figure 4.16 Microscopic photos of the NNV::ptxD transformant cultivated in the hanging bag system.

The cells were grown in two different media supplemented with phosphate (Pi) and Phosphite (Phi). The picture taken at Day 3 was observed in Phase contrast 1 and Day 13 in Phase background under x400 magnification.

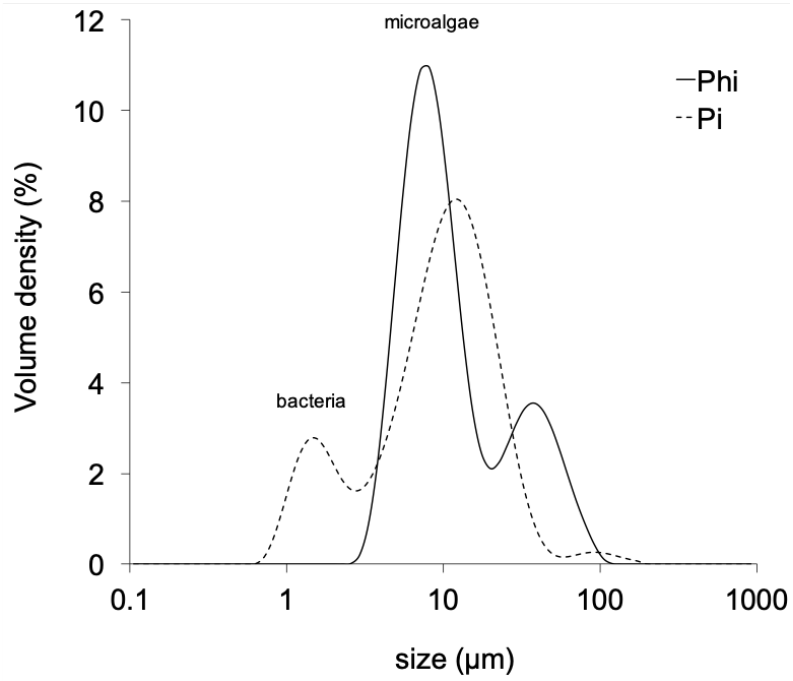


Figure 4.17 Estimation of contamination by Mastersizer analysis in the hanging bag cultures.

The graph is shown as a distribution of bacterial contaminants and *C. reinhardtii* cells at peaks of approximately 1-3 µm and 8-10 µm, respectively. The peak above 10 µm is speculated to be clumped cells as seen in the photograph in Figure 4:16.

4.3 Discussion

4.3.1 Successful use of *ptxD* as a metabolic marker for the chloroplast transformation of *C. reinhardtii*

A large number of recombinant proteins have been produced successfully in *C. reinhardtii* chloroplast using standard methods of transformation and selectable markers (Dyo and Purton, 2018). The introduction of a transgene into the chloroplast requires a suitable DNA delivery technique such as particle bombardment for transforming cells with a wild type cell wall, or glass bead transformation for the cell wall-deficient strains (Esland, Larrea-Alvarez and Purton, 2018). DNA integration in the highly ploidy plastome can be challenging and initially results in a heteroplasmic state with a mix population of non-transformed and transformed plastome copies. However, selective pressure allows the few transformant copies to segregate through several rounds of restreaking until a homoplasmic state is reached. Such selection therefore requires strong selectable markers to achieve a clean background due to die-back of non-transformed cells, but facilitate the development of the transformed colonies. This project has demonstrated *ptxD* as an effective metabolic marker that can serve as an alternative to phototrophic and antibiotic resistance markers.

PtxD transformants of cell wall-deficient lines could be selected using Phi as a sole P source. With sufficient flanking regions (and a modified tRNA gene), the *ptxD* marker was successfully integrated into the plastome via homologous recombination at the first neutral site downstream of *psbH* gene. The *ptxD* marker could also be used with cells possessing a wild type cell wall when DNA delivery is via the biolistic method (Boynton *et al.*, 1988). Furthermore, the marker is portable, and can be targeted to any locus of the plastome such as the neutral region of the *psaA-3-trnL* locus. Hence, *ptxD* is a versatile and portable metabolic marker.

As described in Chapter 3.3.3, *C. reinhardtii* cells can accumulate Pi as long polymer chains of polyphosphate (PolyP) that sustain growth under P-deficient environmental conditions. For efficient transformation it was

important to minimize the level of intracellular PolyP through P starvation of the culture prior to transformation. The P-starvation performed for 18 h before biolistic transformation presumably permitted one to two cell divisions to occur since typical mixotrophic doubling times are 6-8 h (Shimogawara *et al.*, 1998). Each round of DNA replication and generation of biomass would consume the available Pi, so that most cells are assumed to contain significantly less P than those of cells grown in P-replete conditions. This is predicted to provide a strong selective advantage for *ptxD* transformants that obtain Phi metabolism, and reduce the problem of growth of untransformed cells as a background on Phi selection plates when using P-replete grown cells as a recipient. According to Sandoval-Vargas et al. (2019), such conditions in their hands resulted in a pale green background lawn and no transformant colonies appeared even after 60 days of incubation. Since Phi has no lethal effect but cannot support algal growth (Loera-Quezada *et al.*, 2015), a few non-transformed cells may survive on Phi by utilising preserved PolyP and cell debris, unlike most antibiotic selections which actively kill non-transformed cells. It is therefore more effective to deplete PolyP inside the cells before transformation to ensure effective selection for the *ptxD* transformants on Phi.

The generation of stable *C. reinhardtii* chloroplast transformants requires that the plastome achieves a homoplasmic state. The chloroplast has a high polyploidy: it maintains generally about 80-100 DNA copies during mixotrophic growth but around 40 copies in phototrophic growth (Lau, Ren and Wu, 2000). If there is a mix of wild type and transformant copies, random segregation can result in loss of the transgene when no selection is applied. Transgene stability is established only in the homoplasmic state where all plastome copies are transformed. The three-primer PCR strategy was exploited to confirm homoplasmy, and it was shown that the number of rounds of restreaking required varied among strains. For example, transformation of the TN72::pWUCA2 strain needed only a single round of restreaking, but four rounds were necessary for the wild type strain. The reason for this is unclear, but might reflect differences in the DNA delivery method (glass bead v. biolistics) or cell wall (deficient v. wild type). For

example, the presence of a cell wall is speculated to provide an additional P source because PolyP was found to localise within the cell wall and provide the cell protection against toxicity (Werner, Amrhein and Freimoser, 2007). The wild type cell with higher P storage might reduce the selective pressure needed to achieve the homoplasmic state.

When using a metabolic marker such as *ptxD* the level of expression needs to be enough to confer sufficient metabolic conversion, even when the marker exists at few copies as initially obtained in the heteroplasmic state. However, expression should not be so high that there is no further selection to drive the establishment of homoplasmy. In this study, the expression of *ptxD* was regulated by the *psaA* promoter/5'UTR combined with the *rbcL* 3'UTR (as discussed in Chapter 3.2.1). This promoter/5'UTR was previously reported to give high expression level of a transgene (Michelet *et al.*, 2011; Wannathong *et al.*, 2016), stronger than *atpA*, *psbA* and *psbD* promoter/5'UTRs elements (Yoo *et al.*, 2020). In this case, *ptxD* expression under *psaA* promoter/5'UTR was shown to drive sufficient expression for direct selection on Phi and rates of growth comparable with growth on Pi. While finishing this project, there was another report from the Badillo-Corona group expressing a separate version of *ptxD* gene in the chloroplast under the control of the *psbD* promoter/5'UTR (Sandoval-Vargas et al. 2019). They could demonstrate the use of this *ptxD* version as a selectable marker but the transformant colonies only appeared after four weeks of incubation. In contrast, the work in this thesis showed that transformant colonies were visible after one week. It is hypothesised that this reflects the different levels of *ptxD* expression, but direct comparison is needed to confirm this.

The expression of *ptxD* in the *C. reinhardtii* chloroplast instead of the nucleus (as previously achieved by Loera-Quezada et al. 2016) also allows the use of a gene bio-containment strategy developed in the Purton lab. The polyploid nature of the plastome means that the transgene is present as many DNA copies. There is therefore a high chance of transgene DNA being transferred from lysed cells to other environmental microbes via horizontal gene transfer (HGT). Furthermore, the prokaryotic nature of the chloroplast

promoter used to drive *ptxD* expression means that any intact transgene incorporated into a microbial genome has the potential to be functional. To avoid this issue, *ptxD* was designed using a codon reassignment strategy that prevents it from being functional if transferred to other microorganisms. As previously described in Young and Purton (2016), the change of tryptophan codons to UGA stop codons can prevent the full-length translation of the transgene in *E. coli*. This is because the modified chloroplast tRNA that recognises the UGA stop codon as a tryptophan codon is functional only in the chloroplast and not in bacteria. By changing two of the tryptophan codons in *ptxD*, it significantly lessens the chance that microbes that might contaminate an algal culture community could acquire Phi-utilising ability.

The use of *ptxD* as a marker also avoids regulatory issues and public concerns related to the use of antibiotic resistance markers, both in terms of possible HGT and the use of antibiotics to control contamination of algal cultures. The use of Phi as the control measure is preferable since it is unlikely that spontaneous mutations in the genomes of microorganisms would give rises to Phi metabolism under selection, unlike the situation with antibiotic resistance (Esland, Larrea-Alvarez and Purton, 2018). Furthermore, in the case of species that are naturally resistant to antibiotics or herbicides, Phi can be an alternative selective agent. The use of Phi is also safer and less expensive than antibiotics, for example, 25 g of spectinomycin cost £360 whereas 250 g of Phi salt is £15 (prices from Sigma-Aldrich).

4.3.2 Potential use of *ptxD* as a crop protection tool to allow selective growth of the transformant in contaminated cultures

While *C. reinhardtii* has gained attention recently as an industrial platform; a low-cost cell factory for generating protein and metabolites (Gimpel, Henríquez and Mayfield, 2015; Taunt, Stoffels and Purton, 2018; Lauersen, 2019), the major challenge of how to control contamination in large-scale cultures remains poorly studied. Most researchers normally focus on the metabolic engineering for making the products rather than protecting the culture. Current contamination controls normally demand an expensive antibiotic or herbicide treatment to kill the invasive species (Bacellar Mendes

and Vermelho, 2013). The PtxD/Phi system appears to be an alternative and low-cost crop protection tool for *C. reinhardtii* cultivation at the industrial scale (Loera-Quezada *et al.*, 2016; Sandoval-Vargas *et al.*, 2019). Expressing *ptxD* in the chloroplast rather than the nucleus ensures high level and stable gene expression as no silencing mechanisms are found in the organelle, and enables bio-containment to be implemented to avoid *ptxD* spreading. This microalga has been used to express numerous recombinant proteins or products. For example, vaccines for the aquaculture industry (Somchai *et al.*, 2016; Charoonnart *et al.*, 2019), livestock dietary enzymes (Manuell *et al.*, 2007; Erpel, Restovic and Arce-Johnson, 2016), terpenoids as bioactives (Lauersen, 2019), biopolymers (Chaogang *et al.*, 2010) and biofuels (Torzillo *et al.*, 2015; Wichmann *et al.*, 2018), although none of these has yet been commercialised. If these existing strains are engineered to express *ptxD*, they can grow on non-sterilised phosphite medium to lower the cost of production when sterilising of the medium and the cultivation systems is not needed. This would benefit the production of relatively low-valued chemicals such as biopolymers and biofuels that need to be grown at a massive scale. However, it should be noted that whilst the PtxD/Phi allows access to Phi as source of P in a competitive environment, it is not effective against grazers or predators present in the same culture. Such algal consumers would obtain their Pi from that produced by the microalgae. Furthermore, the digestion of the microalgae could lead to the release of Pi into the culture, Similarly, lytic algal viruses could cause the release of significant amounts of Pi into the culture, thereby undermining attempts at crop protection.

Nevertheless, the *ptxD* retrofitting strategy was shown to be successful in a proof of concept experiment by retrofitting the NNV strain that expresses a fish vaccine. In the simple hanging bag photobioreactor using Phi medium, the transformant was able to compete successfully against the bacterial and fungal contaminants and reach a higher cell density compared to Pi medium (Figure 4.17). In terms of construct design, the *ptxD* cassette was regulated by the same *psaA* promoter/5'UTR and *rbcL/* 3'UTR elements as the NNV gene cassette. There was a concern that the use of the same elements would cause a gene replacement with the existing transgene or a looping-out

event through these repeat sequences (Esland, Larrea-Alvarez and Purton, 2018). The sequences in the *ptxD* and *nnv* cassettes corresponding to 285 bp for promoter/5'UTR and 407 bp for the 3'UTR, which are of sufficient length for homologous recombination (Fischer *et al.*, 1996; Iamtham and Day, 2000; Day and Goldschmidt-Clermont, 2011). Nonetheless, the *ptxD* gene was shown to integrate at the predicted site downstream *psaA-3* gene, and the transgenic plastome appeared to be stable, with both NNV and PtxD synthesis in the new NNV::ptxD strain confirmed by western blot result. However, for future retro-fitting in other strains, alternative UTRs might be required to ensure a low risk of a looping-out or gene replacement events.

4.4 Limitation and future experiments

Despite the successful use of *ptxD* as a chloroplast selectable marker in both cell wall-less and wild type *C. reinhardtii*, there is a need to optimise the robust selectivity depending on a specific strain. The first consideration is to examine whether the strain can accumulate intracellular Pi and how long it needs to be starved for minimum P content. In my study, P starvation was performed for 18 h before biolistic bombardment. This might not be optimum. According to Sandoval-Vargas et al. (2019), they achieved more colonies following 14 days of P starvation compared to seven days, but the duration of starvation should be enough to get rid of the bulk of Pi but not to weaken or kill the cells.

The next consideration is whether decreasing the polyploidy in the plastome enhances transformation efficiency. There have been reports of dynamic changes to chloroplast DNA content when cells experience P limitation (Chen *et al.*, 2000; Yehudai-Resheff *et al.*, 2007; Irihimovitch and Yehudai-Resheff, 2008). The DNA was decreased in *C. reinhardtii* when cells were exposed to Phi as a sole P source. In terms of P sensing, the cells recognize Phi as a Pi, resulting in suppression of cell signalling for P limitation. As a consequence, cpDNA synthesis appears to be limited reducing copy number reduction, and this could facilitate the drive to homoplasmy (Kindle, Richards and Stern, 1991; Misumi *et al.*, 1999).

To examine the effect of promoters on *ptxD* expression, a choice of promoters/5'UTRs such as those from *atpA*, *psbA*, *psbD*, and the 16S rRNA gene, *rrnS* could be fused to *ptxD* (Rasala *et al.*, 2011; Yoo *et al.*, 2020). This would enable an investigation of whether different levels of enzyme activity can support sufficient conversion of Phi to Pi for direct selection. Weaker promoters including *atpA* may not contribute enough PtxD activity to support viable growth of transformed colonies on Phi selective plates. In contrast, the strong *16S RNA* promoter probably drives the Phi selection faster with a greater level of enzyme, but may compromise homoplasmic establishment due to a very active enzyme. If the transient expression of *ptxD* gene is required for transformant selection, regulation of protein levels can be achieved by using the temperature-sensitive version of the tRNA that recognised the UGA stop codon, as described in Young and Purton 2018. The modified tRNA allows full-length PtxD synthesis only at the permissive lower temperature (~20 ^{o}C), so the enzyme would be absent at the non-permissive higher temperature (25–30 ^{o}C) during biomass production. Alternatively, inducible expression could be under the control of the nuclear-encoded NAC2 binding factor required for stabilising of any mRNA that has the *psbD* 5'UTR. The NAC2 gene has been engineered to be induced by copper depletion. Hence, for any coding sequence fused to the *psbD* promoter/5'UTR, its expression will be induced by the absence of copper (Surzycki *et al.*, 2007; Rasala and Mayfield, 2015).

Whilst the *ptxD* gene itself had a built in containment feature, it would be good to also have a strategy for containing the genetically engineered alga itself. This could be achieved through disruption of native Pi transporters in the organism and heterologous expression of selective Phi transporters. This would confer a tight control of transgenic lines in which growth in no longer possible on Pi, and depends on Phi as a sole P source. As discussed previously in Chapter 3.3.2, the HtxBCDE transporter selectively transports Phi into an *E. coli* strain that carried knockouts of Pi transporter genes (Hirota *et al.*, 2017). Therefore, the *E. coli* could grow on Phi only. In *C. reinhardtii*, the expression of a Phi transporter was not essential because Phi can be imported via the Pi transport system, and there are as many as ten

transporters responsible for Pi uptake on *C. reinhardtii* with some differentially expressed depending on environmental P levels (Mosley 2006). In theory, the high-affinity Pi uptake that are up-regulated in response to P deprivation could be removed by gene editing to prevent the growth of *C. reinhardtii* on trace amount of Pi and probably limit the survival of transgenic PtxD+HtxBCDE strains into a P-limited environment.

With these strategies, the PtxD/Phi system could be applied to large-scale microalgal cultivation that is currently limited just to some extremophile species. An open pond raceway is one example – normally exploited for growing *Dunaliella* and *Spirulina* at low-cost (Borowitzka, 2013). If media sterilisation is not needed with the PtxD/Phi strategy, this would allow *C. reinhardtii* and other species to be grown at industrial scale in such open ponds. Although Phi is recognised as a safe chemical, some issues related to the environment need to be addressed including how stable is the Phi in the environment and whether it affects the microbial population.

Chapter 5

Limonene biosynthesis in the chloroplast of *Chlamydomonas*

Chapter 5 Limonene biosynthesis in the chloroplast of *Chlamydomonas*

5.1 Introduction

Terpenoids, or isoprenoids, represent a highly diverse class of natural compounds such as carotenoids, sterols and hormones (Pattanaik and Lindberg, 2015). These high-value chemicals have great applications in the area of pharmaceuticals, food additives (colorants and flavourings) and cosmetics. Terpenoid biosynthesis in plants is based on the 5-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 5.1). These building blocks are produced via one of two evolutionarily distinct pathways: the mevalonate (MVA) pathway in the cytosol and 2-c-methyl-D-erythritol 4-phosphate (MEP) in the plastid. The MVA pathway commonly exists in most eukaryotes, whereas the MEP pathway is normally found in bacteria and plastids. However, green algae only contain the plastid MEP pathway having lost the MVA pathway from the cytosol (Lohr, Schwender and Polle, 2012; Vavitsas, Fabris and Vickers, 2018). In terpenoid biosynthesis, one molecule of IPP and DMAPP are fused to form geranyl pyrophosphate (GPP) as a precursor for various monoterpenes by the activity of geranyl pyrophosphate synthase (GPPS) (F. Chen *et al.*, 2011) (Figure 5.2). The consecutive addition of IPP molecules during the sequence of reactions creates the C15 farnesyl pyrophosphate (FPP) as precursor for sesquiterpenes and the C20 genanyl geranyl pyrophosphate (GGPP) for diterpenes (Vavitsas, Fabris and Vickers, 2018). The family of specialized metabolites comprising isoprenes, monoterpenes and diterpenes is further modified by decorating enzymes in a subsequent process to obtain a wide range of terpenoids (Lohr, Schwender and Polle, 2012; Lauersen *et al.*, 2018; Vavitsas, Fabris and Vickers, 2018).

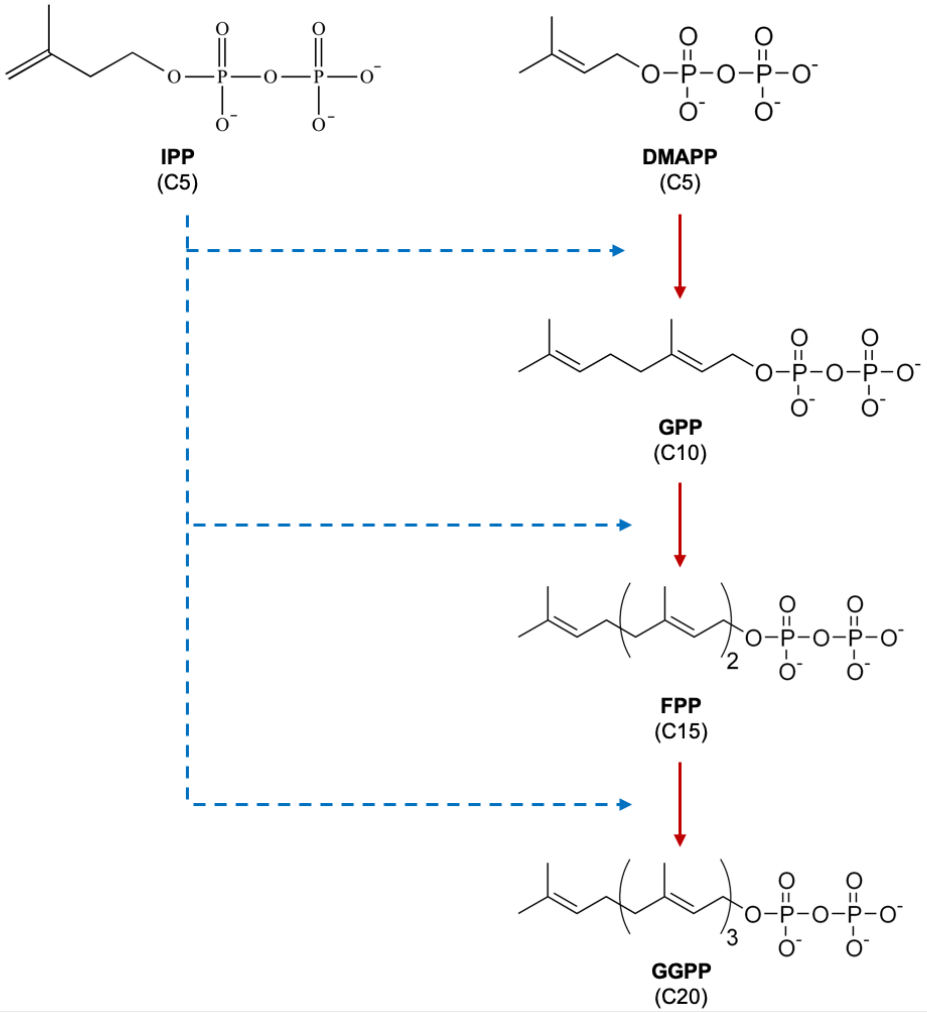


Figure 5.1 Terpenoid biosynthesis in plants

This is based on the 5-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). One molecule of IPP and one of DMAPP are fused to form GPP. The consecutive addition of IPP creates the C15 farnesyl pyrophosphate (FPP) and the C20 genanyl geranyl pyrophosphate (GGPP). The structures used in the figure were obtained from the link: https://tinyurl.com/y2devrst.

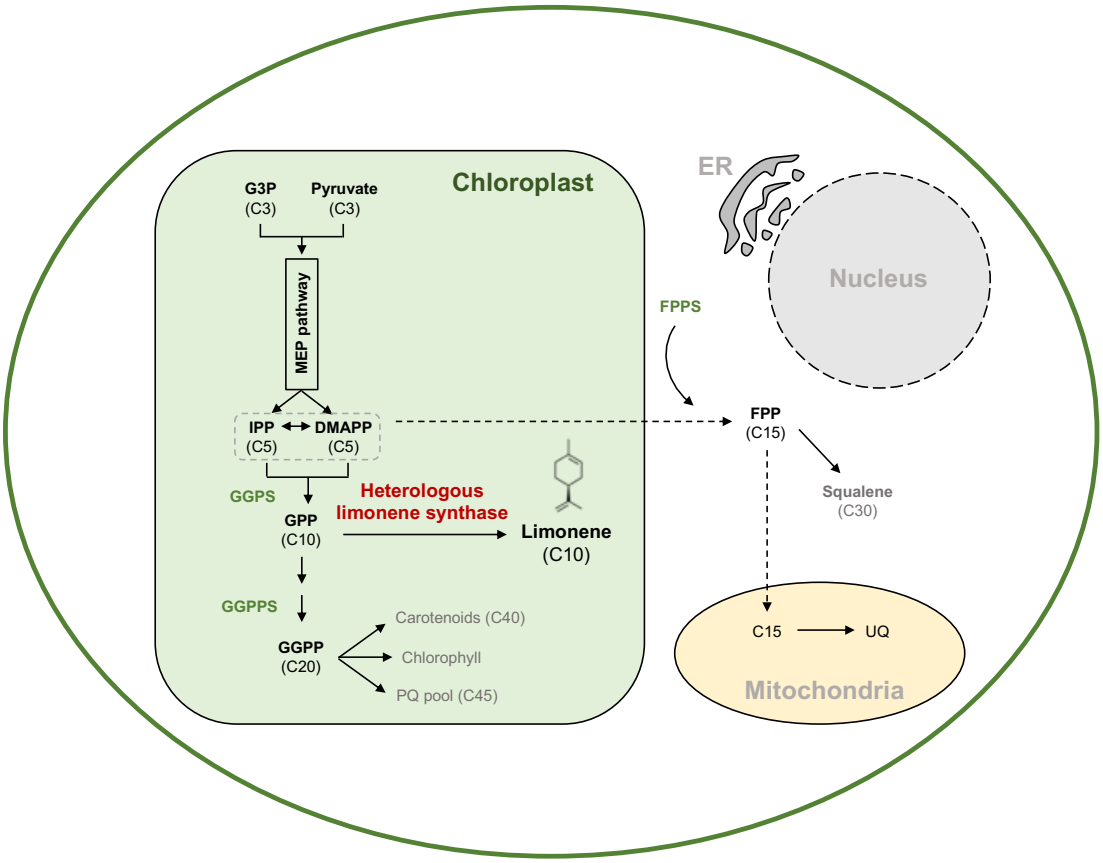


Figure 5.2 Diagram of terpenoid biosynthesis via the MEP pathway in *C. reinhardtii*.

The enzymes in the pathway are nuclear-encoded and targeted to the chloroplast (Lohr, Schwender and Polle, 2012). Glyceraldehyde 3-phosphate (G3P) and pyruvate are processed via the MEP pathway for generation of 5-carbon precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). One molecule of IPP and DMAPP are condensed to form geranyl diphosphate (GPP) as an expected precursor for monoterpenoids in plants. Geranylgeranyl diphosphate synthase forms the precursors (GGPP) for carotenoids, chlorophyll and plastoquinone (PQ). Farnesyl pyrophosphate (FPP) is made by FPP synthase (FPPS) available for the generation of squalene in the cytoplasm and ubiquinone (UQ) in the mitochondria. Heterologous expression of a limonene synthase gene in the chloroplast would allow non-native limonene biosynthesis. Figure modified from (Lauersen *et al.*, 2018).

Of the numerous terpenoids with a high complexity of structures, limonene is a well-known volatile monoterpene with a less complex structure (C10) and has potential applications ranging from an alternative aviation fuel to a perfume fragrance (Figure 5.3) (Jongedijk *et al.*, 2016). In plants, limonene is generated from the GPP precursor by the activity of limonene synthase (LS). Limonene is insoluble in water and could be released from cells due to its volatility. It exists in two forms of naturally occurring enantiomers, R-(-)-limonene that has an orange-like fragrance and S-(-)-limonene with a piney-like odour (Kiyota *et al.*, 2014). The R-limonene is commonly extracted from the peel of citrus fruits while the S-limonene is naturally found as a main volatile compound in oaks and pines (Ciriminna *et al.*, 2014). Limonene synthases are promiscuous enzymes that produce a mixture of monoterpenes, for example, LS from *Mentha spicata* generates S-limonene and trace amounts of α-pinene, β-pinene, and myrcene, but it has the highest specificity of 94% towards S-limonene production (Colby *et al.*, 1993). Limonene production is typically based on plant extraction but the low amount of limonene produced contributes to a non-economically viable production of this compound. Similarly, the chemical synthesis of limonene is also challenging because of the complexity of structure and the slow reaction process since high energy is needed (Pang *et al.*, 2019).

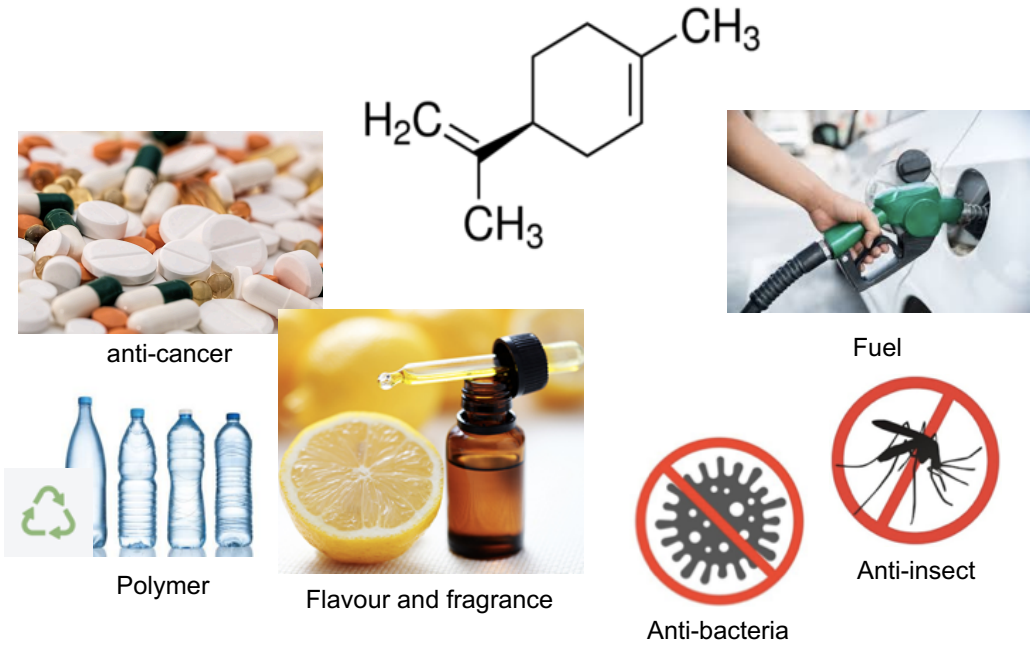


Figure 5.3 Applications of limonene and its derivatives

Limonene has anti-cancer and anti-microbial properties (Inouye, Takizawab and Yamaguchia, 2001; Miller *et al.*, 2010). The lemon-odour of limonene has applications in the fragrance and flavour industry. It is a potential jet fuel and an alternative to conventional solvents for oil and gas production such as benzene and toluene (Jongedijk *et al.*, 2016). Thiols of limonene can aid the production of limonene-based polyesters for sealant and adhesive products (Ciriminna *et al.*, 2014).

As plants naturally produce limonene at a low quantity, it is not cost-effective to use this platform on an industrial scale. With the growing knowledge of terpenoid biosynthesis, there were several attempts to metabolically engineer other hosts including bacteria and yeast as new chassis for the production of limonene (Alonso-Gutierrez *et al.*, 2013; Davies *et al.*, 2014; Kiyota *et al.*, 2014; Jongedijk *et al.*, 2016). The main challenges are to redirect the pool of isoprenoid precursors to heterologous terpenoid pathways at an economically viable level (Vavitsas, Fabris and Vickers, 2018). In *E. coli*, the precursors are supplied by the cytosolic MEP pathway while *S. cerevisiae* employs the MVA pathway to generates the compounds. *E. coli* has been used widely as the target host due to its easy genetic manipulation and high tolerance to the products. Transgenic *E. coli* showed a yield of limonene at 700 mg/L (Willrodt *et al.*, 2014) as a result of the heterologous expression of LS from *Mentha spicata*, GPP synthase from *Abies grandis* and the MVA pathway from *Saccharomyces cerevisiae* with grown mediated using glucose as the carbon source. While limonene was synthesised at the highest yield in *E. coli*, another established host, *S. cerevisiae* was also used to produce limonene with an added advantage of terpene modification via expression of decorating cytochrome P450 enzymes (Jongedijk *et al.*, 2016). However, the disadvantage of these heterotrophic microbes is that they require a fixed carbon source such as glucose or sucrose for growth, so are not a truly sustainable platform for production of limonene or other useful terpenes. This is particularly a problem for large-scale production for use in the polymers or fuels section since the enormous quantities of sugar needed as feedstock would compete with agricultural food and feed production.

There is therefore an interest in developing photosynthetic microorganisms as sustainable platforms for limonene production with the advantage of using sunlight and CO_2 directly as sources of energy and carbon instead of a fixed carbon source. Furthermore, photosynthetic organisms naturally synthesize different kinds of terpenoids and there is the opportunity to redirect some of the precursor flux going to native terpenoids such as the hydrocarbon tail of chlorophyll and carotenoids such as beta-

carotene, towards limonene biosynthesis. This could provide more access to the substrate pool than in yeast and *E. coli* and increase the yield of limonene product (Vavitsas, Fabris and Vickers, 2018). The most widely used example of a photosynthetic host for the production of limonene is cyanobacteria, since several model species such as *Synechocystis* PCC6803 have established molecular toolboxes for metabolic engineering (Vavitsas, Fabris and Vickers, 2018). The LS gene from several plant species including *Mentha spicata*, *Schizonepeta tenuifolia* and *Sitka spruce* has been introduced into cyanobacteria for limonene biosynthesis (Davies *et al.*, 2014; Halfmann *et al.*, 2014; Kiyota *et al.*, 2014; X. Wang *et al.*, 2016; Lin *et al.*, 2017). The highest titre of limonene so far demonstrated is 6.7 mg/L achieved by Lin et al. (2017) when they expressed the LS gene and exploited a strain design algorithm to overexpress the pentose phosphate pathway for higher limonene production (Table 5.1)

Table 5.1 Summary of limonene biosynthesis in cyanobacterial platforms.

Strain	Engineering design	Origin of limonene synthase and accession number	Yield and extraction methods	Reference
***Synechocystis sp*. PCC 6803**	Overexpression of DXS, IDI, and CrtE from *Synechocystis*	*Schizonepeta tenuifolia*, R-limonene synthase, AF282875	56 µg/L culture/day, gas stripping	(Kiyota *et al.*, 2014)
***Synechococcus sp*. PCC 7002**	Wild-type and mutant background of Δ*glgC*	*Mentha spicata* ,S-limonene synthase, Q40322	4mg/L in wild-type background, dodecane organic phase	(Davies *et al.*, 2014)
***Synechococcus elongatus* PCC 7942**	Enhanced carbon flux into the MEP pathway	*Mentha spicata*, limonene synthase, accession number was not clear	885.1 µg/L/OD/day, collection by a hydrocarbon absorbent trap	(X. Wang *et al.*, 2016)
***Synechocystis* 6803**	Pentose phosphate pathway was overexpressed	*Mentha spicata* for S-limonene synthase and *Citrus limon* for R-limonene synthase	6.7 mg/L of limonene in 7 days.	(Lin *et al.*, 2017)

While cyanobacteria are being extensively studied, *C. reinhardtii* as a eukaryotic and photosynthetic microalga has been poorly explored for generating limonene. The advantage of using a eukaryotic green alga may be the fact that it shares distant evolutionary ancestry with land plants and therefore it can be a more suitable host system for heterologous expression of plant terpene synthases, providing more favourable cellular conditions for these enzymes relative to cyanobacteria or microbes. The cultivation of algal cells can be performed at similar temperatures for growing plants, rather than the higher temperatures (32–37^{o}C) needed for growth of yeast or *E. coli*, or the 30^{o}C or higher typically used for cyanobacterial species. The lower temperatures might therefore be closer to optimum for stability and activity of the plant enzymes (Lohr, Schwender and Polle, 2012; Lauersen, 2019). Although there have been a few studies to demonstrate the introduction of novel terpene biosynthesis into the *C. reinhardtii* chloroplast, this has been achieved by expressing synthase genes in the nucleus and targeting the enzymes into the chloroplast (Lauersen *et al.*, 2018). This work demonstrated the use of the native C20 precursor geranylgeranyl pyrophosphate (GGPP) produced via the MEP pathway in the algal chloroplast for the biosynthesis of novel diterpenoids: casbene, taxadiene and manoyl oxide. The microalgal chloroplast could therefore be a site for biosynthesis of monoterpenes such as limonene from the GPP pool provided by the MEP pathway.

This chapter aims to demonstrate the potential for terpene biosynthesis in green algae through chloroplast metabolic engineering, with limonene chosen due to its low complexity of structure and production. Firstly, the inhibitory effect of limonene on *C. reinhardtii* was explored, together with a method for harvesting the volatile terpene by dodecane overlay. Limonene biosynthesis was subsequently introduced into the *C. reinhardtii* chloroplast by introducing into the plastome a codon-optimised gene for the LS enzyme from spearmint *Mentha spicata*. The gene expression was designed to be under the control of the CITRIC cold-inducible system (Young and Purton, 2018) to regulate the production of limonene as a potentially toxic product to the microalga. Finally, the detection

of limonene by gas chromatography-mass spectrometry was optimised to observe limonene production within the engineered LS transformants.

5.1.1 Aims and objectives

- To investigate the toxicity of limonene and explore the use of a dodecane overlay for capturing limonene excreted from algal cells
- To attempt the synthesis of limonene in the *C. reinhardtii* chloroplast under the regulation of a cold-inducible expression system
- To optimise limonene detection using gas chromatography-mass spectrometry technique (GC-MS)

5.2 Results

5.2.1 Limonene is highly toxic to *Chlamydomonas reinhardtii* cultures but toxicity can be overcome by a dodecane overlay

Limonene biosynthesis has been widely introduced into two common microbial platforms, *E. coli* and *S. cerevisiae*, but limonene was shown to be toxic to the cells as it is highly lipophilic and can be accumulated in the cell membrane leading to cell disruption (Griffin, Wyllie and Markham, 1999). Before introducing limonene biosynthesis in *C. reinhardtii*, it was important to determine the minimum concentration of limonene that would inhibit microalgal growth during limonene production. An initial experiment was designed to examine the inhibitory effect of the limonene at a range of concentrations of 0.02, 0.1, 0.2, 0.4 and 1.0 % (v/v) (or approximately 1.2, 6.2, 12.4, 24.8 and 62.0 mM, respectively) by adding limonene externally to cultures at the mid-exponential phase of growth (Figure 5.4). The optical density (OD) was monitored for 4, 8, 24 and 48 h after limonene addition. As shown in Figure 5.4, the cultures started dying as shown by the colourless phenotype after 48 h and the OD decreased at the higher limonene concentrations between 0.1% and 1.0% (v/v). The growth drops immediately within 4 h addition of limonene and the final OD fell by 75% relative to the control after 24 h (Figure 5.5). In contrast, the cells remained unaffected at the very low limonene concentration of 0.02% (v/v) while the growth reached the same level as the control with no limonene added. The observed growth at the lowest limonene concentration might be a result of degradation or loss of limonene from the culture due to its high volatility, but overall the results indicate that at the higher concentrations limonene is toxic and leads to a decrease in growth. It should be noted however that in this experiment the limonene was added exogenously to the culture, and the toxicity of limonene produced within the cells may well be different.

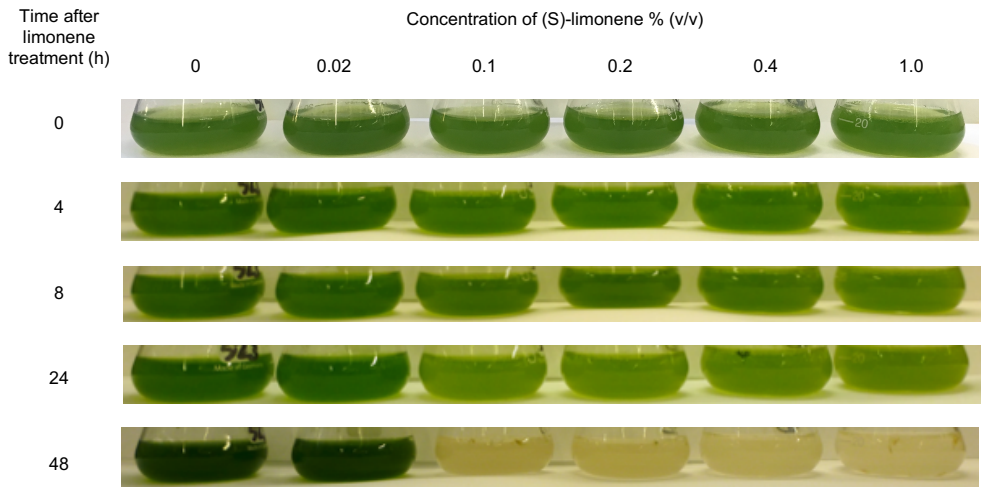


Figure 5.4 The toxic effects of limonene on the growth of *C. reinhardtii*.

Cultures were grown to mid-exponential phase and then treated with (S)-limonene at a range of concentrations % (v/v). The experiment was observed for 48 h after limonene addition.

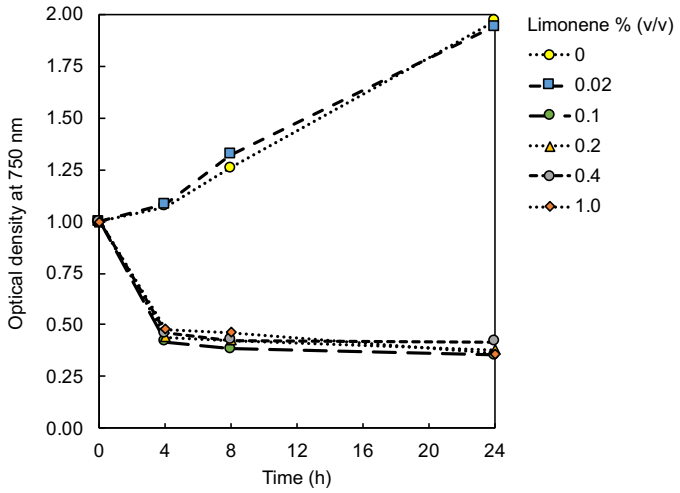


Figure 5.5 Optical density measurements of the cultures shown in Figure 5.4

This is to investigate the growth of *C. reinhardtii* in the presence of limonene. OD_{750} measurements were recorded at 0, 4, 8 and 24 h after limonene treatment. The volume of culture was 20 ml in each flask. No repeats were carried out in this experiment.

Given the evident toxicity of limonene to *C. reinhardtii*, a dodecane overlay strategy was investigated whereby the immiscible dodecane forms a separate layer on top of the culture and the limonene preferentially partitions into the organic layer. This two-phase method should allow continuous harvesting of the synthesised limonene into the dodecane phase, thereby avoid toxic build-up in the aqueous phase containing the algal cells. This strategy has been termed 'algal milking' whereby organic products that are actively or passively secreted from an algal culture are recovered and concentrated using an inert organic solvent without harvesting or destroying the algal cells (Hejazi, Kleinegris and Wijffels, 2004; Doshi, Nguyen and Chang, 2013; Miazek *et al.*, 2017) As dodecane has only a very minor biological effect on cells and a relatively low volatility it represents an ideal biocompatible organic liquid for continuous extraction of limonene during cultivation (Davies *et al.*, 2014; Miazek *et al.*, 2017).

A test of the effect of a dodecane overlay in the extraction of limonene from *C. reinhardtii* cultures was carried out by incubating mid-exponential phase cultures with externally-added limonene as described previously but overlaying each culture with 10% (v/v) dodecane (Figure 5.6). In addition, a control set of cultures with no dodecane overlay was set up to show the lethal effect of the added limonene in the absence of an extraction solvent. As shown in the figure, the rescue of the limonene-treated cultures by the dodecane was observed even at the highest concentration of limonene, with only a slight growth reduction after 148 hours when compared to the control culture with no limonene added (Figure 5.7). Conversely, the control set with no dodecane overlay appeared to be significantly affected by limonene after 24 h post addition as seen previously, except the sustained growth in 1.0% (v/v) until 48 h, which requires more repeats to confirm this limonene effect. Overall, the limonene treatment led to a marked decrease of OD below 0.5 by the end of the experiment.

The use of dodecane overlay therefore appears to be a promising approach both to mitigate the toxic effects of limonene, and to easily and

continuously harvest and accumulate the product during cultivation of an engineered algal strain.

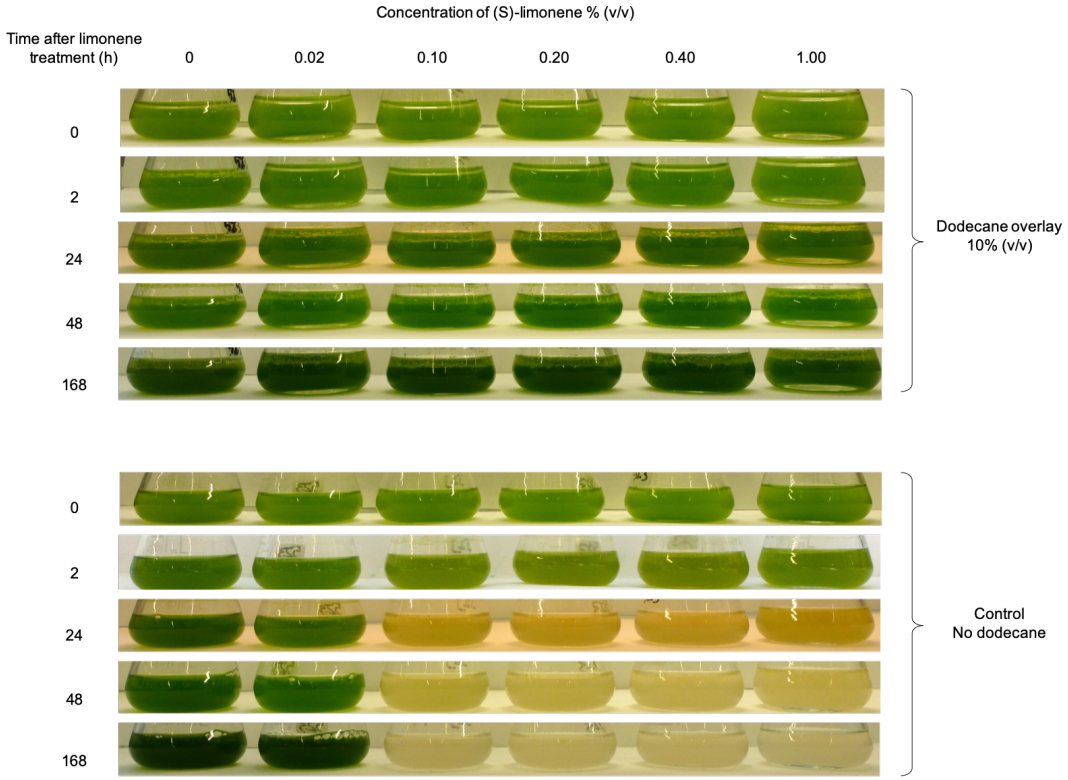


Figure 5.6 Dodecane overlay of *C. reinhardtii* cultures to alleviate the lethal effect of limonene.

The cultures were grown until mid-exponential phase and then treated with (S)-limonene at a range of % (v/v). Dodecane was overlaid at 10% (v/v) and flasks were photographed at the indicated times following the additions. The volume of culture was 20 ml in each flask. No repeats were carried out in this experiment.

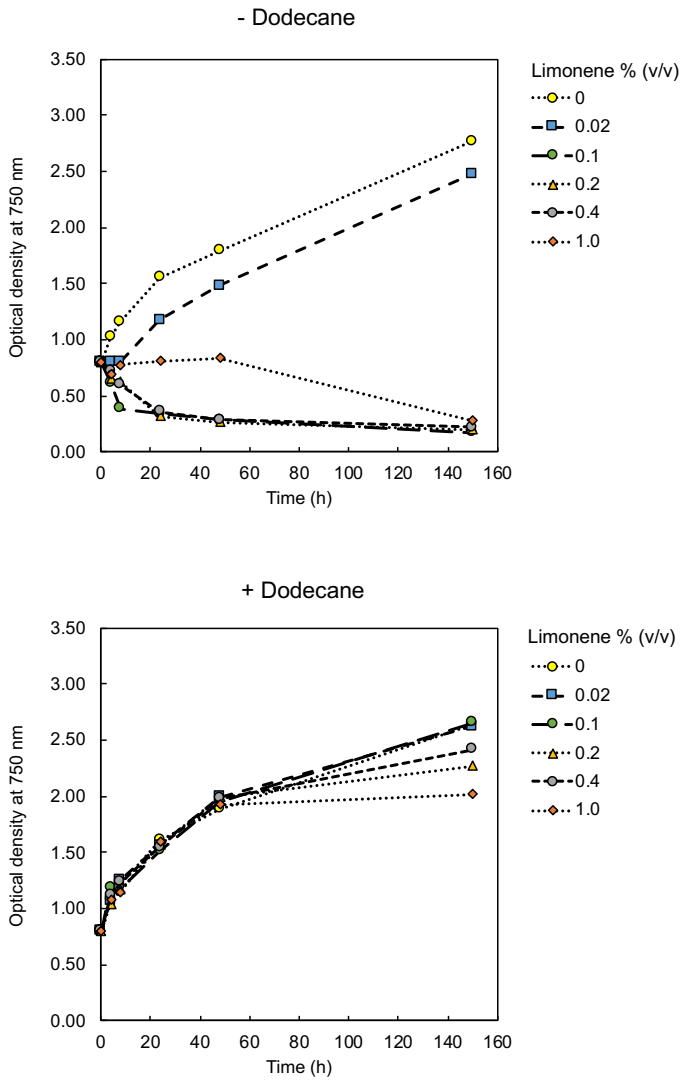


Figure 5.7 Growth measurements to investigate the reduced toxicity of limonene on microalgal growth due to dodecane overlay.

The measurements was recorded at 4, 8 and 24 h after limonene treatment. The volume of culture was 20 ml in each flask. No repeats were carried out in this experiment.

5.2.2 An inducible system for limonene synthesis in the *C. reinhardtii* chloroplast

In previous transgenic studies, the limonene synthase (LS) sequences used to design the transgene have been derived from the various LS found in plants such as spearmint (*Mentha spicata*), or the medicinal herb (*Schizonepeta tenuifolia*) (Davies *et al.*, 2014; Kiyota *et al.*, 2014; X. Wang *et al.*, 2016). Each LS has evolved to produce one or another enantiomer of the monoterpene – *i.e.* R- or S-limonene – although the R-limonene is the more desirable product as it has an orange/lemon odour rather than the turpentine odour of the S form (Morehouse *et al.*, 2017). In this study, the LS from spearmint (*Mentha spicata*, accession number: L13459.1) was chosen since it is highly specific in the synthesis of the S-limonene enantiomer to with a specificity over 90% (Hyatt *et al.*, 2007). This LS has been well characterised (Colby *et al.*, 1993) and previously introduced into *E. coli* to produce a high yield of over 400 mg/L (Alonso-Gutierrez *et al.*, 2013), therefore, it was chosen for introduction into the *C. reinhardtii* chloroplast with the goal of producing the desirable S-limonene as a product.

Since the plant LS is synthesised in the cytosol from a nuclear-encoded gene but then imported into the plastid, the protein initially possesses an N-terminal plastidial transit sequence which is cleaved off upon import to generate the mature protein. This transit sequence is not required for heterologous synthesis in the algal chloroplast, so a gene encoding just the mature sequence of LS was designed whereby the coding sequence for the predicted 99-residue transit sequence (Colby *et al.*, 1993) was replaced with a start ATG codon for translation initiation. In addition, an HA-tag coding sequence was added such that the C terminus would possess this epitope tag for detection by western blot. The LS CDS was codon-optimised for efficient expression in the *C. reinhardtii* chloroplast by using the Codon Usage Optimizer software developed in the Purton group. Finally, the restriction sites for *Sap*I and *Sph*I were added to 5' and 3' of the gene respectively, while avoided in the rest of the CDS. The synthesised LS gene

has a length of 1533 bp including the HA-tag sequence. The molecular mass was calculated to be 59 kDa for the recombinant limonene synthase.

As shown earlier, limonene present at high concentrations has an inhibitory effect on *C. reinhardtii* cell growth, and this would potentially limit the yield of limonene and biomass if LS was constitutively active in the chloroplast. To circumvent this issue of toxic limonene accumulation and achieve sufficient biomass, the LS gene was designed to be under the control of an inducible system termed CITRIC (Cold-Inducible Translational Readthrough In Chloroplasts) (Young and Purton 2018). In this system, the presence of internal UGA stop codons within the transgene CDS is translated as tryptophans only if an orthogonal tryptophan tRNA is present in the chloroplast and able to read UGA as a tryptophan codon. However, this tRNA (encoded by ts-*trnW*$_{UCA}$) has been engineered to be temperature sensitive and will only form the correct tRNA structure at the lower, permissive temperature. Hence, at low temperature (25°C) a full-length LS would be formed, whereas at the high non-permissive temperature read-thorough of the UGA codons is not possible and they serve as stop codons producing non-functional truncated forms of the LS polypeptide (Figure 5.8). The design of limonene synthase under the cold-inducible system would circumvent the toxic issue and provide a tight control for limonene biosynthesis by simply changing the temperature.

In addition to this inducible strategy, constitutive expression of LS was planned to test whether a lack of control of LS synthesis negatively impacts on cell growth or the yield of limonene. The only difference between the two designs is the tRNA gene used – the temperature sensitive version *versus* the 'wild-type' version. Both LS expression systems were driven by the same *psaA* promoter/5'UTR and *rbcL* 3'UTR/terminator, and the LS CDS contained two TGG->TGA replacements at tryptophan positions (Figure 5.9). The two constructs shown in the figure could be achieved by cloning the LS CDS into the expression vectors pWUCA2 and pWUCA4, respectively (Young and Purton 2018).

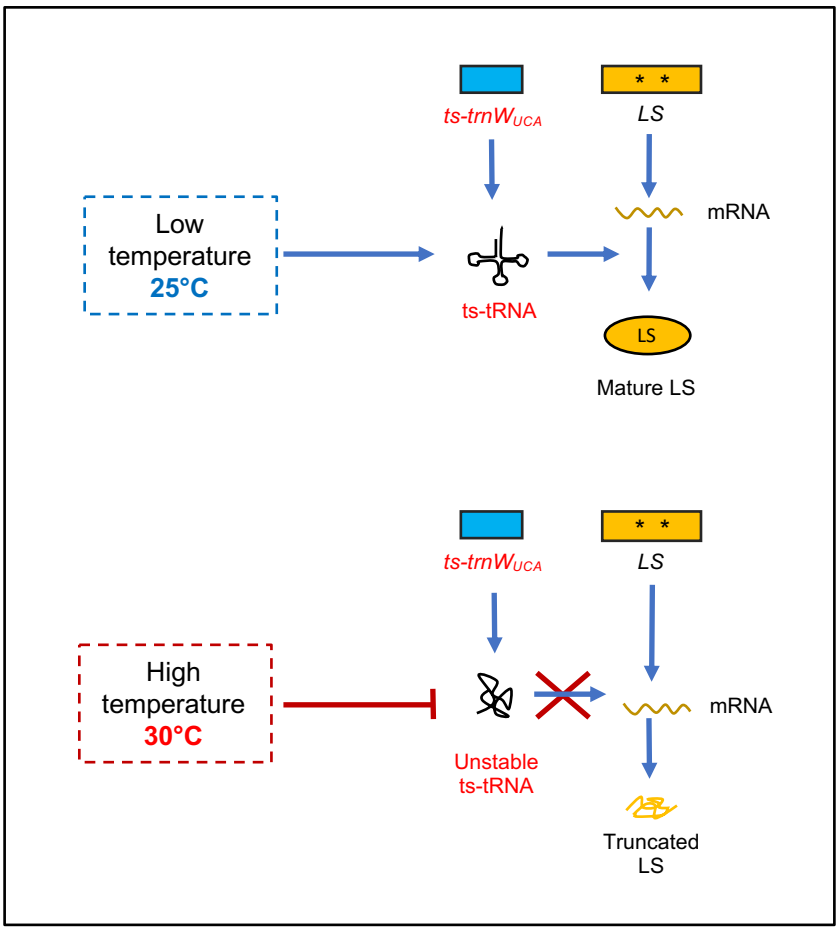
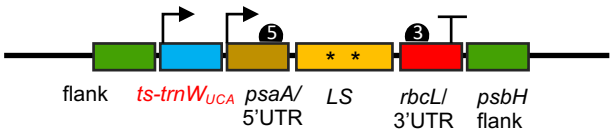


Figure 5.8 Strategy for the cold-inducible regulation of limonene synthase production in the chloroplast.

A lower temperature (<25°C) promotes the correct folding of the modified ts-tRNA for the complete translation of the LS mRNA, while the higher temperature of 30°C destabilizes the ts-tRNA secondary structure and prevents translational readthrough of the internal stop codons.

a) CITRIC cold-inducible system



b) Constitutive system

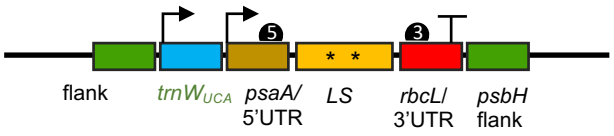


Figure 5.9 Diagram of the limonene synthase gene cassette incorporating the CITRIC cold-inducible and constitutive systems.

Expression of the LS gene is mediated by the *psaA* promoter/5'UTR and *rbcL* 3'UTR/terminator elements. a) CITRIC cold-inducible system: the *ts-trnW*$_{UCA}$ gene encodes a temperature-sensitive tRNA for translating internal stop codons (shown as *) within the LS gene. b) Constitutive system: the *trnW*$_{UCA}$ gene encodes a modified tRNA for translating internal stop codons (shown as *) within the LS gene, regardless of temperature change. In both systems, the flanking regions ensure targeted integration of the foreign DNA via homologous recombination.

5.2.3 Generation of transgenic lines with the limonene synthase gene correctly inserted into the plastome

To generate the transgenic lines producing the limonene synthase, the LS gene was cloned into pWUCA2 and pWUCA4 vectors for constitutive and cold-inducible synthesis respectively (Young and Purton 2018). The resulting plasmids (pWUCA2_LS and pWUCA4_LS) were transformed into the chloroplast of the *C. reinhardtii* strain TN72 via the glass bead transformation method, and the selection was based on the restoration of photosynthetic capability, as described previously in Chapter 3.2.2. Two colonies from each of the pWUCA2_LS and pWUCA4_LS transformation were selected for confirmation of correct transgene integration at the neutral site downstream of *psbH*.

The integration of the LS gene was examined by PCR as previously done for the *ptxD* transformants. As shown in Figure 5.10, all four representative colonies gave rise to a 1.4 kb band confirming correct integration of the LS cassettes. Furthermore, a 0.88 kb band derived from the original TN72 plastome was not detected in the PCR analysis indicating that the lines are homoplasmic for the transgenic plastome.

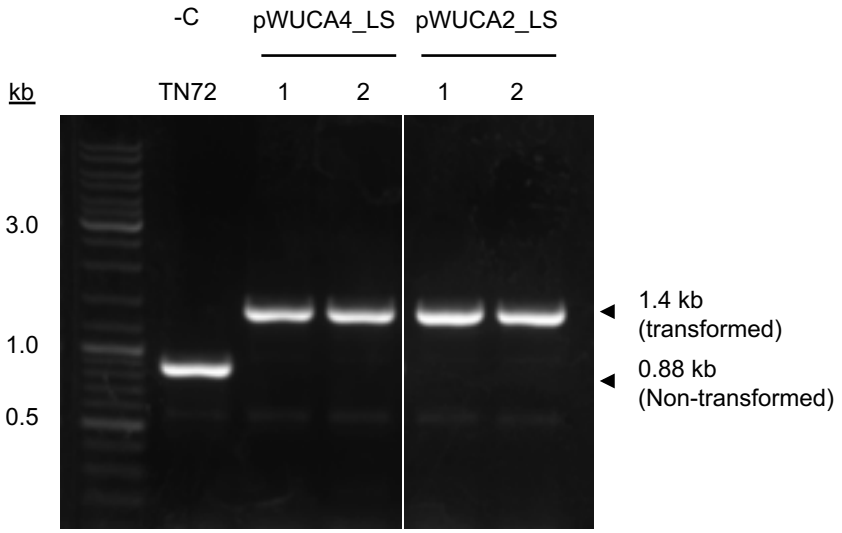


Figure 5.10 PCR screening for successful integration of the limonene synthase (LS) gene cassette into the *C. reinhardtii* plastome.

Transgenic lines were obtained after a single round of streaking on minimal medium. Integration was confirmed by the presence of a 1.4 kb band. The absence of a 0.88 kb band derived from non-transformed copies of the plastome indicates the homoplasmic state of the transgenic lines. The TN72 recipient strain served as a negative control (-C). The pWUCA4_LS lines harbour the inducible system whereas the constitutive system is in the pWUCA2_LS transformants.

5.2.4 Western blot analysis indicates constitutive and inducible LS protein accumulation in the two classes of transgenic lines

Once the successful integration of the LS genes into the plastome was confirmed, the next step was to examine whether full translation of the LS protein was occurring in the chloroplast. Western blot analysis of cell lysates from the transformant lines was therefore performed by using anti-HA antibodies. These antibodies should only detect the full length LS protein (with a predicted size of 60 kDa) and not any truncated forms where translation has stopped at the internal UGA codons, since the epitope is at the C-terminal end of the protein. The two representative clones from the pWUCA2_LS and pWUCA4_LS transformations were grown at both 25°C and 30°C to examine whether the cold-inducible system allowed the tight control of limonene synthase production. As shown in Figure 5.11, several specific bands are seen in the transformant lines that are absent from the negative control transformant. The lower band appears to correspond to the LS since it is seen in all clones and its level is similar for the constitutive transformant clones at both the lower (25°C) and higher (30°C) and for the cold-inducible transformants at the permissive (25°C) temperature. Furthermore, the level is substantially lower for the cold-inducible transformants at the non-permissive temperature (30°C). The photosystem one subunit PsaD was used as a loading control and shows similar loading of each sample, indicating that the difference in levels in the inducible samples was not due to a varied amount of samples loaded into each well.

Nevertheless, there are two issue with the western analysis. Firstly, the size of the LS band appears significantly smaller than the predicted 59 kDa. This is possibly due to unspecific proteolytic cleavage by protease causing the LS to migrate faster in the gel. However, it cannot be ruled out that translation in the chloroplast is occurring at an internal in-frame AUG to produce a smaller HA-tagged product. The second issue is that a second band is seen at a much higher molecular weight. LS is believed to form a homodimer in the plant chloroplast (Hyatt et al. 2007) with the expected size at around 120 kDa, so this might reflect some association of the LS protein

with itself or other proteins in the *C. reinhardtii* extract that, as is not resolved by the SDS.

Despite these anomalies, the western blot analysis demonstrates the successful expression of the LS gene and the induction of enzyme production under the cold-inducible conditions.

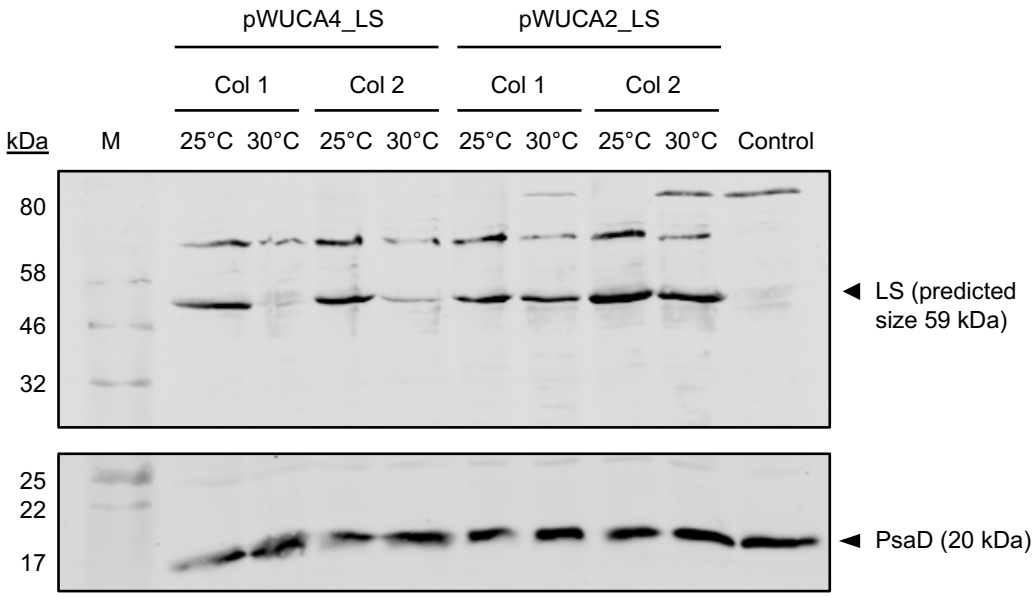


Figure 5.11 Western blot analysis of the LS transformants using anti-HA antibodies to show the accumulation of HA-tagged limonene synthase.

The cultures of colony 1 and 2 (Col1 and Col2) were grown at two different temperatures – 25°C and 30°C. Detection of the chloroplast protein PsaD was used as a loading control at a size of 20 kDa. TN72 transformed with the empty pWUCA2 plasmid was used as a negative control.

5.2.5 Optimisation of a gas-chromatography-mass spectrometry assay to detect limonene

Whilst LS protein was detected in the chloroplast by western analysis, functioning of the enzyme – *i.e*. the GPP pool of substrate is accessible and the LS capable of converting some of this substrate to limonene – needed to be explored next. The study used gas-chromatography-mass spectrometry (GC-MS) analysis for the identification of limonene produced by the LS transformants, as the technique has been well established and widely used for other platforms. In this project, it has also been demonstrated that a dodecane overlay could be employed as the harvesting method by absorbing the product directly from the culture. Using the GC-MS technique would allow a simple and direct analysis of the organic phase since it is possible to distinguish between the dodecane solvent (C12) and the limonene (C10) with no need of further purification.

The transformants were initially grown to mid-exponential phase and treated with dodecane overlay as before to extract the limonene. The cells were examined under a microscope before and after the addition of dodecane (Figure 5.12), and showed no cellular debris after dodecane treatment and no indication of major detrimental effects of the solvent on the algal cells. The harvested dodecane was subjected to the GC-MS analysis for limonene detection with comparison to a standard of pure S-limonene as a positive control.

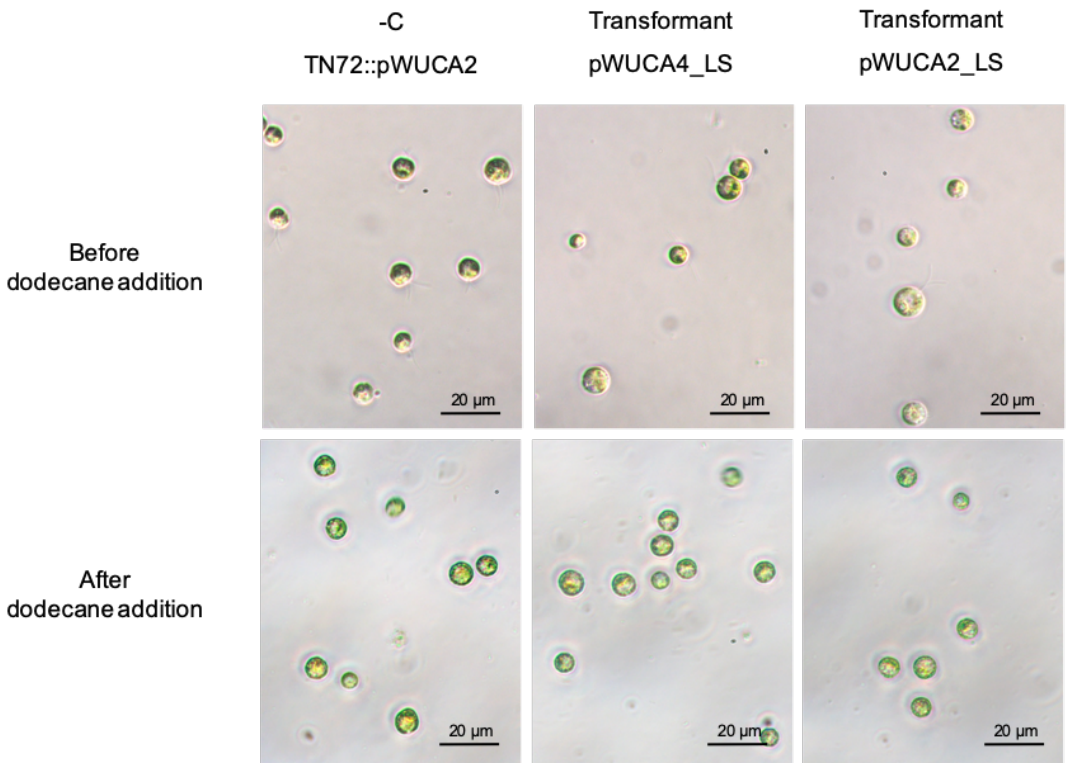


Figure 5.12 Microscopic images of limonene transformants before and after treatment with dodecane overlay.

The cultures were incubated with dodecane overlay at 10% (v/v). The picture taken before treatment with dodecane was from the mid-exponential phase while that after treatment was from the stationary phase. In the pWUCA4_LS transformant LS synthesis was under the control of temperature-sensitive tRNA whereas synthesis is constitutive in pWUCA2_LS. The negative control (TN72::pWUCA2) is TN72 transformed with the empty vector.

The GC-MS technique was initially optimised for limonene detection by using a serial dilution of standard S-limonene as the samples. This was to identify the limit of detection where the lowest quantity of limonene could be distinguished from a dodecane blank by GC-MS, and to determine the minimum limonene concentration could be detected by GC-MS within the extracted dodecane. In this work, GC-MS was performed across a serial dilution of standard S-limonene at a concentration of 1 pg/µl, 10 pg/µl, 1 ng/µl and 100 ng/µl. As previously described in Son et al. (2018), 1 µl of dodecane sample was mixed with hexane at a ratio of 1:49 (v/v) before GC-MS injection to reduce the effect of a broad peak of dodecane when present as such a large proportion of the sample. However, the ratio was adjusted to 2:48 (v/v) as 1 µl was liable to pipetting error. The chromatogram showed the distinct peak of limonene released at retention time 12.73-13.02 min across all concentrations (Figure 5.13a). The lowest limonene level detectable by GC-MS represented as a well-resolved peak was at 1 ng/µl (Figure 5.13b). Lower concentrations of 1 and 10 pg/µl were not distinguishable from the blank, therefore, a second GC-MS analysis was performed with a new GC column to improve the detection limit. The change in the column exhibited an improved chromatogram quality, enabling limonene at 10 pg/µl to be distinguished. A calibration curve for quantitative analysis of limonene concentration was created based on the limonene peak areas in the chromatogram measured at the four different concentrations from 10 pg/µl to 100 ng/ul (Figure 5.14). Therefore, an unknown concentration of limonene in the extracted dodecane could be estimated within these limits by using the calibration curve.

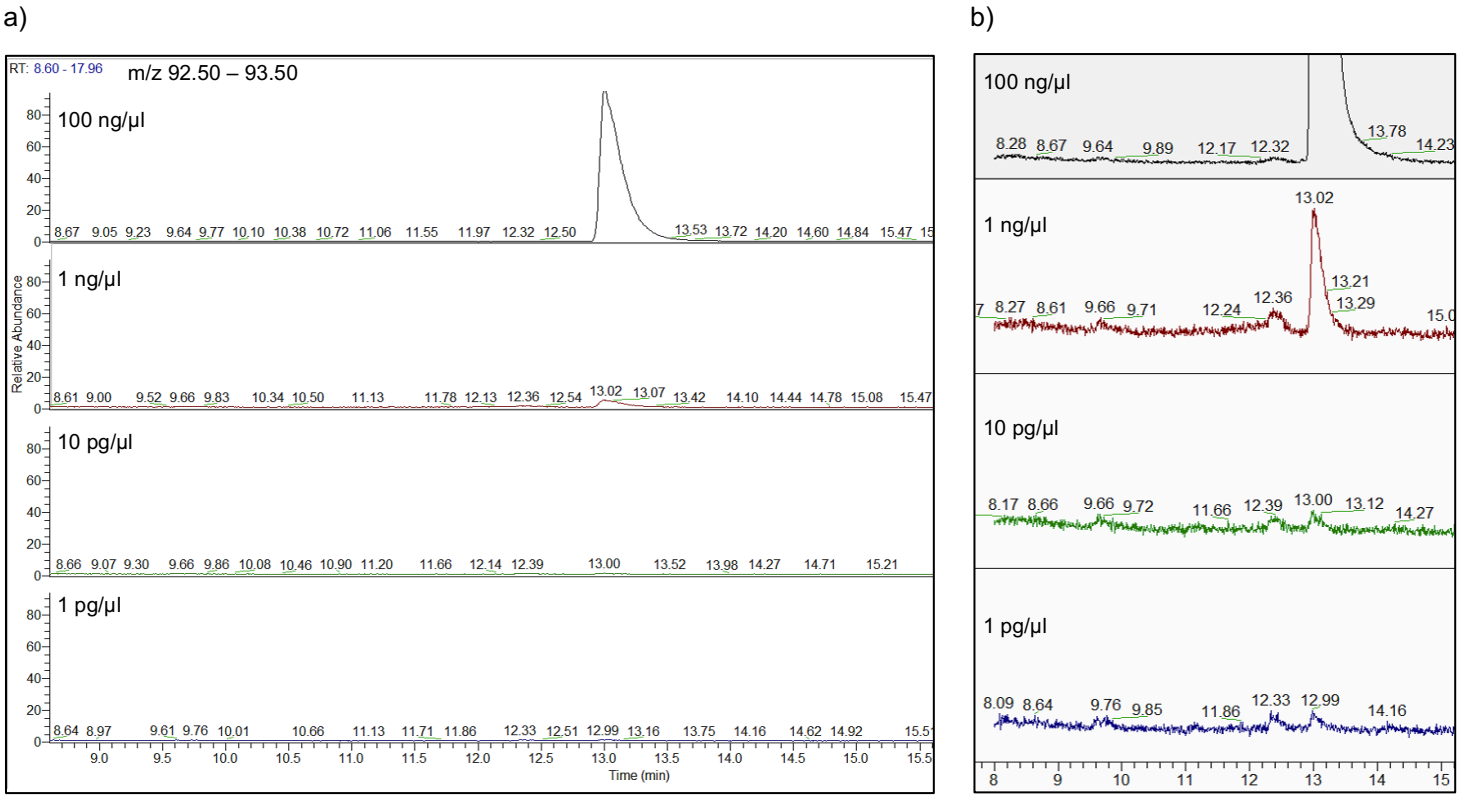


Figure 5.13 GC chromatogram of the serial dilution of S-standard limonene.

a) a clear peak for 100 ng/µl limonene in hexane is seen at retention times of 12.73-13.02 min. b) Zoom-in chromatogram for the limonene peak. This chromatogram was obtained from the first run prior to changing the GC column.

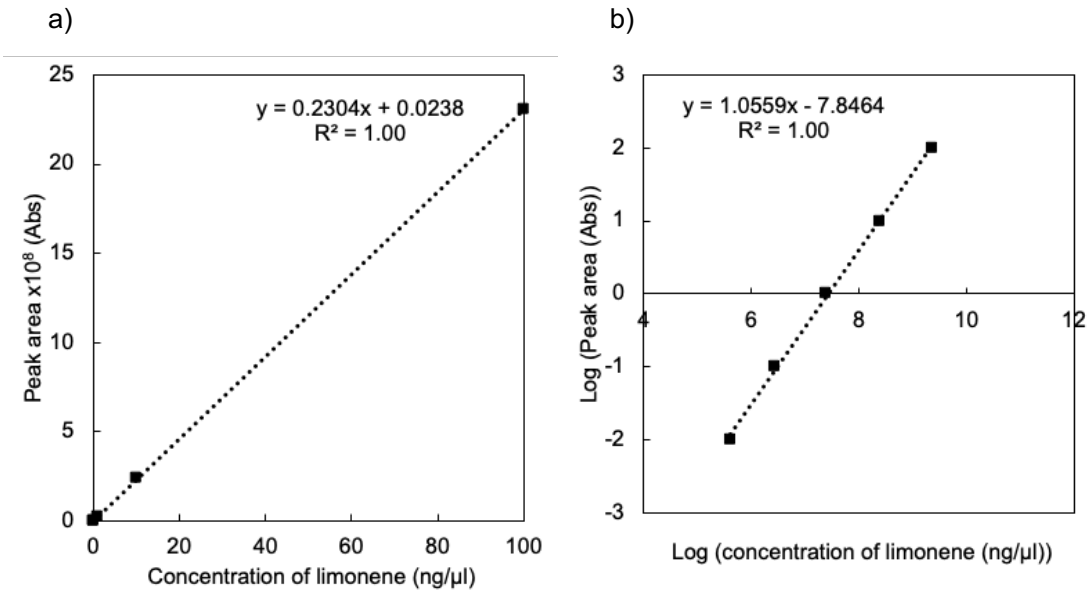


Figure 5.14 Calibration curve for quantitative limonene detection by GC-MS.

The peak area was recorded and used to generate the curve. Data points were obtained from GC chromatograms between a concentration of 0.01 to 100 ng/µl of standard S-limonene. a) the graph of concentration of limonene (ng/µl) against the peak area of GC chromatogram (Abs). b) the graph of the logarithm of concentration of limonene (ng/µl) against the logarithm of peak area of GC chromatogram (Abs).

After the limit of detection was identified, the dodecane collected from the transformant culture was examined for the presence of limonene using the GC-MS technique. The standard S-limonene at 1 ng/µl was chosen as a positive control and was readily detected as a single peak at a retention time of 13.47 min (Figure 5.15). The mass spectra of the chromatographic peak matched the library search of limonene (Figure 5.16). In comparison, dodecane samples from the LS transformants showed no distinct peak of limonene as seen in the negative control. There were peaks observed at around 12.5 and 13.6 min (Figure 5.15), presumably, they might be derivatives of dodecane degradation or oxidation over the harvesting period. However, the key conclusion from this experiment, was that it was not possible to observe any limonene in the dodecane overlays from any of the transformant cultures.

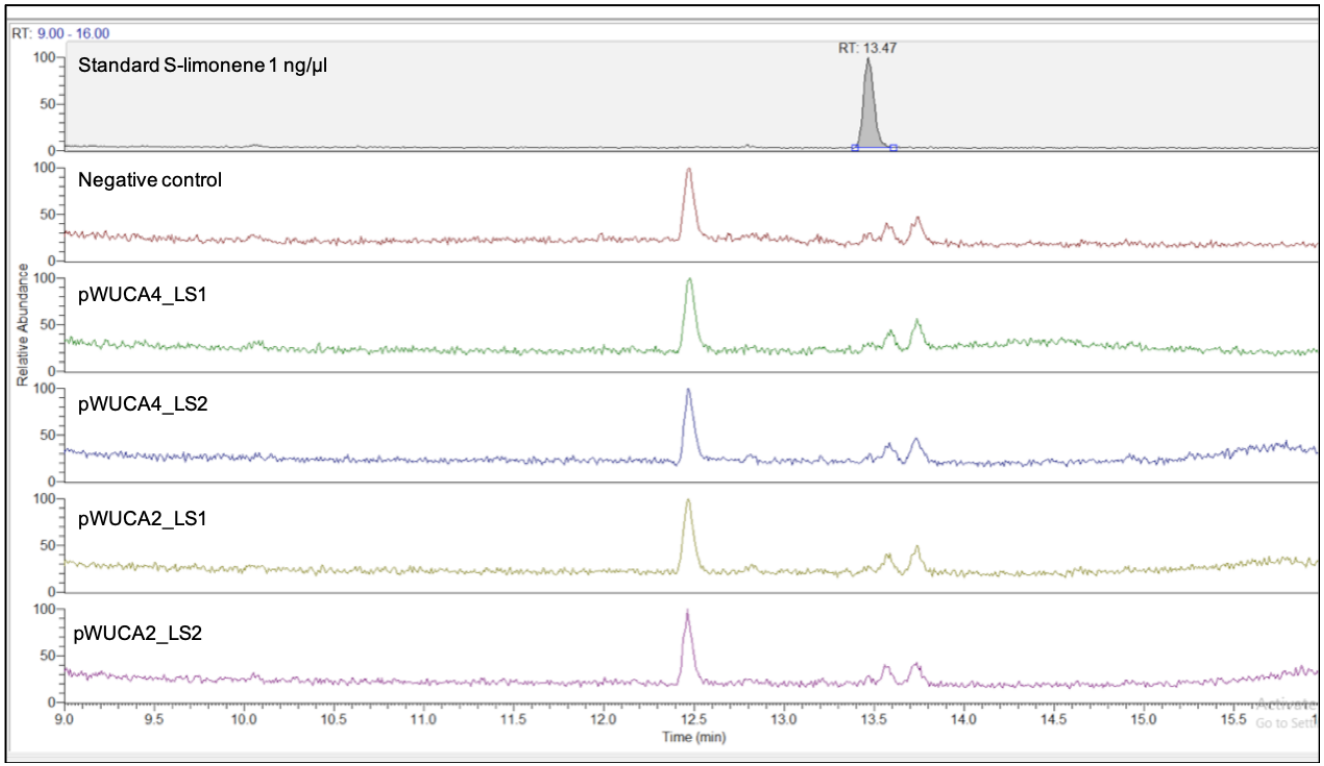


Figure 5.15 GC chromatogram of the dodecane overlay harvested from transgenic *C. reinhardtii* cultures to test for limonene production.

Limonene synthase was constitutively produced in pWUCA2_LS1 and pWUCA2_LS2, or under the control of the cold-inducible system in pWUCA4_LS1 and pWUCA4_LS2 with the cultures grown at 25°C. TN72 transformed with the empty vector pWUCA2 served as a negative control. Limonene peak for 1 ng/µl served as a positive control with a strong signal at retention times of 13.47 min. No limonene was detectable for any of the transformant lines. Peaks observed at around 12.5 and 13.6 min are presumed to be derived from the dodecane.

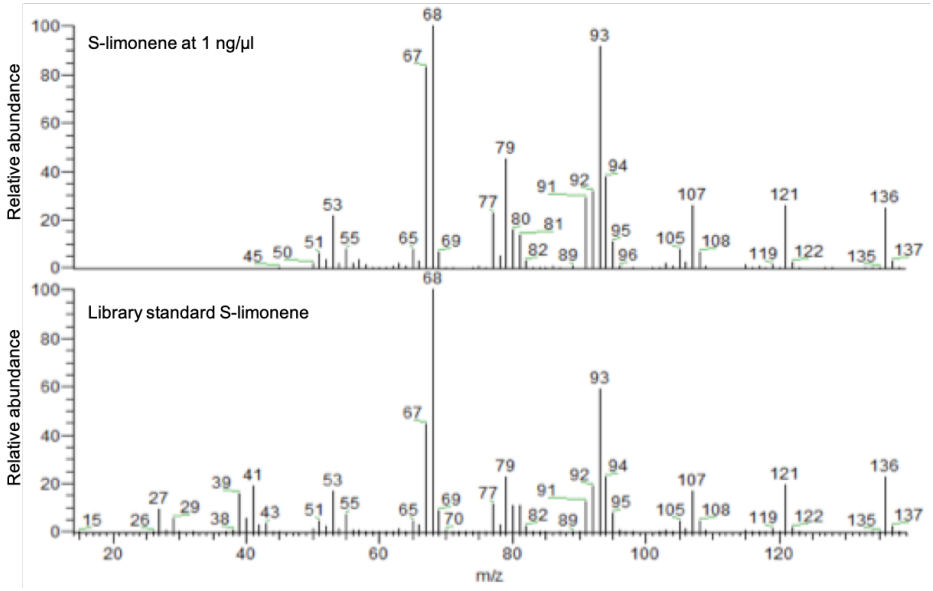


Figure 5.16 Mass spectra of standard limonene at 1 ng/µl in dodecane.

The mass spectra of a substance found in the standard limonene prepared in dodecane at a volume ratio of 2:48 (v/v), detected at 13.47 min retention time. The library of standard mass spectra of S-limonene with the characteristic peaks of 68, 93, 121 and 136 m/z (Byun-McKay *et al.*, 2006).

5.3 Discussion

5.3.1 Toxicity effect of limonene can be alleviated by dodecane overlay

Limonene was shown to be toxic to *C. reinhardtii* at concentrations above 0.02% (v/v), however, a dodecane overlay could be used to minimise toxicity even at a concentration as high as 1% (v/v). Prior studies have noted the importance of correlation of limonene toxicity and its log P_{ow} value. This number is a logarithm of the octanol to water partitioning coefficient of a particular solvent and used to indicate the level of toxicity for microorganisms (Sikkema, de Bont and Poolman, 1994). Organic solvents with a low log P_{ow} between 1.5 and 4.0 are highly harmful to living cells as a result of their potential to partition into the cell membrane, disrupting the structure and damaging vital functions (Ramos *et al.*, 2002). The value of log P_{ow} for limonene was reported as 4.4 (Brennan *et al.*, 2012), so whilst not in the highest toxicity category, it is still predicted to be toxic towards *C. reinhardtii*. The damage probably occurs through cell lysis, however, the killing mechanism is not well understood. In general, compounds with a low P_{ow} are expected to be hydrophobic and can intercalate into the lipid bilayer of the cell membrane to disrupt the membrane function (Sikkema, de Bont and Poolman, 1994). This increases membrane fluidity and causes damage to membrane-bound proteins. Loss of vital chemicals such as ions, metabolites, proteins and lipids to the environment results in impaired cell function and consequently to cell death (Ramos *et al.*, 2002). As seen in the toxicity experiment, the growth of a *C. reinhardtii* culture was inhibited at limonene levels beyond 0.02% (v/v), with no recovery apparent after a longer incubation. At the lowest level of 0.02% it is possible that the lack of an observed effect on the culture over the extended time of the experiment was the result of the trace limonene evaporating owing to its high volatility. Hence, as long as the limonene production in an engineered strain remains below 0.02% (v/v), the culture would probably remain relatively unaffected by the limonene. One consideration to bear in mind is that the limonene was added exogenously in this experiment. Therefore, in *C. reinhardtii* cells synthesising limonene, the intracellular toxicity effect may be different.

Whilst limonene was clearly harmful to the *C. reinhardtii*, the actual cause of toxicity could be oxidation products of limonene such as hydroperoxides. These compounds are easily formed if limonene is exposed to oxygen. A study from Chubukov et al. (2015) showed that exogenously added limonene was harmful to *E. coli* even at a low concentration. They reported hydroperoxides were a major toxic compound that existed in the oxidised limonene. A point mutation in the alkyl hydroperoxidase AhpC of *E. coli* could decrease the inhibitory effect of limonene hydroperoxides by converting them to other less toxic compounds, because the enzyme can catalyse the reduction of organic hydroperoxides to water and alcohols. They also found there was no growth reduction in *E. coli* when applying 2% (v/v) non-oxidised limonene (kept anaerobically) to the culture. However, the impact of limonene hydroperoxides mainly occurs during long-incubating cultures, as noted by the authors. In this project, the detection of limonene hydroperoxides was not performed, but presumably, some harmful hydroperoxides have already formed in the standard S-limonene as it was not stored anaerobically. It is also important to note that chloroplast provides an oxidative environment as a result of photosynthesis, therefore, this could increase the risk of limonene oxidation and hydroperoxide formation.

To relieve the effect of limonene toxicity on microalgal growth, a dodecane overlay was chosen to create a two-phase cultivation. Dodecane has been most commonly used to harvest limonene in other microorganisms such as *E. coli*, *S. cerevisiae* and *Synechococcus sp.* Dodecane is an inert solvent and biocompatible (Brennan *et al.*, 2012), so that it was unlikely to harm *C. reinhardtii* and could be used in the culture at an appropriate concentration. Using the overlay of dodecane, a high load of limonene is possible in the continuous culture without reducing the growth rate. Limonene partitions preferentially in dodecane compared to water because of its low hydrophilicity (Vermaas *et al.*, 2018). Dodecane was used at 10% (volume/culture volume) throughout the project as this seemed sufficient for contacting the cells and medium without compromising growth. Even though the dodecane overlay can serve as simple one-step extraction, this depends on the purity of limonene needed at the end of the process. Another choice

for extraction of volatile organics is headspace collection（a set-up was created to allow trapping of exhaust volatiles during growth at the top of bioreactor) and condensation, and this might be a more appropriate alternative for the pharmaceutical or fragrance industry.

5.3.2 Limonene synthase expression was possible in the *C. reinhardtii* chloroplast although limonene was not detected in the LS transformants through GC-MS analysis

The LS gene was successfully expressed in the chloroplast both constitutively and under the cold-inducible system although limonene synthesis was not observed in this work. With cold-inducible system, the temperature-sensitive tRNA was efficient enough to allow the translation of the LS mRNA at the permissive temperature. This system could be applied to other enzymes that have potentially detrimental effects on the host even at the low product concentration, allowing the build-up of biomass prior to the induction of the toxic product. However, if this system was used for metabolic engineering, it is critical to consider the optimum temperature for the enzyme to work under the inducible conditions, together with the availability of substrate and also product stability at the permissive temperature (Young and Purton, 2018).

One explanation for the failure to observe limonene in the transgenic lines could be a non-functional LS enzyme. Correct protein folding and the presence of co-factors are essential for enzyme activity. As shown for the native LS from *Mentha spicata,* the protein forms a homodimer and needs divalent metal ions of Mg^{2+} or Mn^{2+} for substrate binding and catalysis (Hyatt *et al.*, 2007). The algal chloroplast imports Mg^{2+} as it is the metal ion of chlorophyll and a co-factor of other enzymes in thylakoids (Marchand *et al.*, 2018), so this ion should not be limited for LS activity. The native LS is synthesised with a long plastidial transit peptide of approximately 99 amino acids, this is required for plastid localisation but not for synthesis in the chloroplast (Figure 5.17). The full transit peptide was therefore omitted for the gene design in this project and appeared not to affect the transcription and

translation of the synthetic gene as the tagged LS protein was detectable by western blot analysis. Whether the removal affected the enzyme folding or catalysis is unclear, although previous studies reported that amino acid residues at position 58 and 59 (a tandem pair of arginines - R58/R59) were highly conserved in other monoterpene synthases and might be essential for catalytic function (Williams *et al.*, 1998; Davies *et al.*, 2014). The identity of the transit sequence cleavage site at position 99 might therefore be wrong, and removal of part of the mature protein could impair the enzyme folding or functioning. An enzymatic assay that measures the enzyme activity of purified LS to convert its substrate GPP to limonene would be a key to unlock these catalytic issues of this recombinant LS expressed in the *C. reinhardtii* chloroplast, and whether this organelle provided a suitable environment for LS activity.

Whilst considering the functionality of LS, catalytic efficiency of the enzyme and accessibility to substrate also need to be examined. In cyanobacteria, the GPP substrate for limonene is generated via MEP pathway and available for the heterologous LS for biosynthesis (Davies *et al.*, 2014). However, it might not be the same case in *C. reinhardtii*, where GPP is rapidly converted to GGPP as a precursor for other native terpenoids such as carotenoids (Figure 5.2). Other enzymes might be physically associated with GGP synthase to allow efficient transfer of the product to these enzymes, preventing the recombinant LS from gaining access to the substrate pool. Further analysis of substrate channelling might be required to increase the productivity of limonene within the algal chloroplast.

If limonene was being synthesised in the chloroplast, it could be that the level might be below the limit of detection by GC-MS. Although several studies have exploited GC-MS analysis for limonene detection in other engineered strains such as *E. coli*, *S. cerevisiae*, and *Synechococcus sp.* (Alonso-Gutierrez *et al.*, 2013; Jongedijk *et al.*, 2015; X. Wang *et al.*, 2016; Son *et al.*, 2018), the GC-MS protocol still needed optimisation in this study to detect a low concentration of 1 ng/µl with high sensitivity and develop a calibration curve to estimate the yield of limonene produced in the

engineered algal strain. Despite the improved GC-MS sensitivity, limonene was not detected in any of the transformant cultures. Further optimization is perhaps required for the GC-MS analysis, or for the harvesting method to maximise recovery of limonene in the dodecane phase. This might relate to a variation of limonene accumulation in dodecane across the growth stage. Earlier work on the dynamics of secreted recombinant proteins from *C. reinhardtii* showed that an optimal timeline of product capture was necessary to maximise yield (Lauersen *et al.*, 2015, 2016). The capture of limonene in this study was started after the cells reached late-exponential phase. It would be better to perform the capture at the peak of limonene production or from the start of the cultivation to avoid any limonene loss. The investigation of limonene accumulated in dodecane over the whole incubation period (one week) needs to be addressed to improve the result.

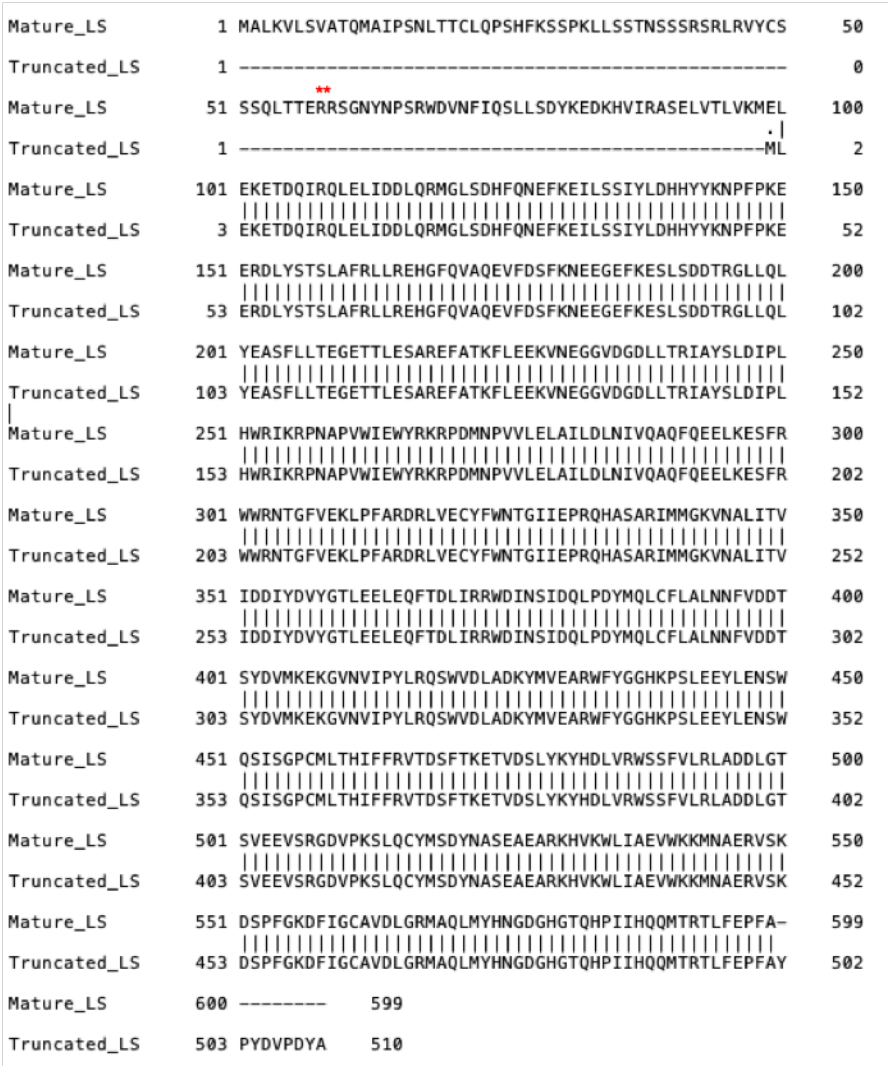


Figure 5.17 Alignment of the native S-limonene synthase from *Mentha spicata* and the protein encoded by the LS gene.

The full-length was shown as Mature_LS including transit peptide from position 1 to 99 whereas the synthesised gene or Truncated_LS did not have this sequence. The arginine tandem for catalytic activity was R58/R59 (shown as **). The Truncated_LS contains HA-tag at C-terminal from position 502 to 510.

5.3.3 Limitation and future experiments

As discussed above, one of several issues may contribute to why no limonene was detected including a low level of limonene below the detection limit of GC-MS and a non-functional LS enzyme being generated. It could be that limonene was synthesised at a very low level as a result of limited access to the substrate or the enzyme being unable to compete with native terpenoid pathways. Also, the dodecane overlay may not be suitable for such a small volume of limonene produced in the transformants, and other harvesting methods such as trapping in the headspace of the culture might be a better approach. As limonene is a highly volatile compound, it may easily evaporate into the headspace during incubation even with a dodecane overlay, and continuous capture directly from a sealed flask needed to accumulate sufficient product. This method also avoids the need for further downstream processing as limonene is not mixed with other compounds with a similar boiling point, however, the complicated set-up of headspace trapping would not be possible for large-scale production (Jongedijk *et al.*, 2015).

Given the failure to detect any limonene, questions remain to be answered whether the LS enzyme was inactive due to the lack of critical arginine residues required for catalysis or correct protein folding. The functioning of the LS could be tested following purification of the enzyme using the C-terminal HA-tag. The addition of GPP substrate to the purified enzyme in the presence of co-factor would allow GGP conversion to generate limonene with product would be extracted into an organic solvent such as hexane. The GC-MS technique could then be employed to monitor the progress of the reaction and the presence of limonene. The use of an internal standard e.g. 1-octanol can further improve the quantitative analysis. By calculating the ratio of GC peaks and using the internal standard concentration, an unknown amount of limonene in the organic solvent can be quantified. The expression in the algal chloroplast of an LS with an N-terminus upstream of the two arginines would be an alternative to investigate the importance of this region on its activity. To conclude, an enzymatic

activity assay, improvements in the limonene harvesting through headspace capture and expression of a longer version of LS may reveal the main factor for a failure to detect limonene biosynthesis in the *C. reinhardtii* chloroplast.

Chapter 6

Final discussion

Chapter 6 Final discussion

6.1 Summary of main findings

An initial aim of this project was to develop a new strain improvement strategy in the algal chloroplast based on the metabolism of phosphite (Phi) to phosphate (Pi). The results presented in Chapter 3 demonstrated the successful introduction and expression into the chloroplast of the bacterial gene *ptxD* encoding an NAD(P)-dependent phosphite oxidoreductase. Expression gave rise to the novel ability to convert Phi to Pi as a P source in *C. reinhardtii*. The chloroplast expression of *ptxD* allows normal rates of algal growth on Phi, and therefore Phi seems to be readily transported into the chloroplast without the requirement of any specific Phi transporters in either the cell or chloroplast membranes. Although growth was not restricted under mixotrophic conditions with rates equivalent to that seen under the same conditions but with Pi as the P source in the medium, there is a need of further investigation to confirm that there is no reduction in phototrophic growth when comparing Phi- and Pi-containing media. Moreover, the conversion of Phi to Pi by PtxD enzyme might be limited by the availability of oxidising equivalent NAD+ (or NADP+) in a reductive environment during photosynthesis.

In Chapter 4, the applications of *ptxD* as both a non-antibiotic selectable marker for chloroplast transformation and as a crop protection tool were demonstrated. Selection based on the PtxD/Phi system was feasible for both strains with and without a cell wall using the glass-bead vortexing and microparticle bombardment delivery systems, respectively. The constructed *ptxD* cassette was shown to work as a versatile and portable metabolic marker that can be inserted at different loci in the plastome. However, there are some considerations regarding the use of metabolic markers such as *ptxD*; they require the synthesis of sufficient enzyme in order to convert enough Phi into Pi so that the transformed colonies grow at a good rate and are visible on selective plates. The efficient expression of *ptxD* involves the selection of a highly active promoter and 5'UTR. Furthermore, the

incorporation of the bio-containment feature of *ptxD* in which internal stop codons prevent its functional translation in other species. This feature greatly reduces the risk of horizontal gene transfer of the phosphite-metabolising phenotype to any environmental microbes.

In addition to the selectable marker application, the results in Chapter 4 demonstrate the value of *ptxD* as a crop protection tool to minimise undesirable contamination with bacteria, fungi, and other protists including other microalgae. The expression of *ptxD* allowed the engineered microalga to effectively outcompete these contaminants when cultured in a medium in which Phi is the only source of phosphorus. This could be an alternative contamination control method to the use of costly antibiotics or herbicides, and decrease production costs due to the lack of a requirement for medium bio-products such as biofuels or bio-plastics. The use of *ptxD* as a crop protection tool is however limited to competitive microbial contaminants, and the strategy cannot deal with contamination by grazer or predators of the algae. To show that the *ptxD* technology could be applied to other strains of *C. reinhardtii* including those already engineered for commercial applications, a strain that produced a fish vaccine against NNV was retro-fitted with *ptxD*. The growth of the newly engineered strain, NNV::ptxD, in non-sterile Phi medium without the serious contamination demonstrated the potential to synthesise the fish vaccine at low-cost.

The research presented in Chapter 5 was an attempt to produce a new engineered strain that synthesised a novel mono-terpenoid in the *C. reinhardtii* chloroplast. Limonene biosynthesis was selected as a test for terpenoid production because it requires expression of only a single gene encoding limonene synthase. The LS gene was successfully expressed under the regulation of the cold-inducible system, such that synthesis of the toxic limonene product would be induced after achieving sufficient biomass. Although the limonene synthase enzyme was present in the chloroplast, the limonene product was not detected. This could be due to an impaired function of the limonene synthase due to the lack of proper folding, or the limonene synthase has no access to the pool of substrate. Small amounts of

limonene might have been generated, however, the concentration present in the dodecane overlay was below the detection limit of GC-MS.

6.2 Future research

6.2.1 Improving the PtxD/Phi system as a better chloroplast selection method

The *ptxD* gene has been successfully demonstrated as a chloroplast marker. However, Phi selection in species capable of storing PolyP presents a challenge. As discussed in Chapter 3 and Chapter 4, *C. reinhardtii* can retain a large pool of cytosolic PolyP (Komine *et al.*, 2000) together with a minor storage of P in the cell wall (Werner, Amrhein and Freimoser, 2007) to support growth under Pi deficiency. This was the main issue in that non-transformed cells still grew on selective plates and this increased the chance of obtaining false-positive colonies. To address the issue, some additional strategies for reducing PolyP storage could be achieved by using of a mutant defective in PolyP accumulation (Aksoy, Pootakham and Grossman, 2014) as a recipient strain for transformation. The lack of PolyP would limit the survival of untransformed cells under Pi-limited conditions because they cannot use Phi or rely on Pi reserve.

If PolyP storage is a critical function for the target algal species, a similar strategy would be to exploit the cells that cannot acclimate to Pi deprivation. In the case of *C. reinhardtii*, a low-phosphate bleaching mutant (*lpb1*) was found to die more quickly than wild-type cells under phosphorus limitation (Chang *et al.*, 2005). If the recipient cell has a knockout of *lpb1* introduced through gene targeting, it would increase the sensitivity to Pi limitation under Phi selection. The function of the *lpb1* gene product remains unclear but it may involve polysaccharide metabolism with an impact on phosphorus metabolism. Another mutant that could be used is that affects in *psr1*, in which the gene encodes a transcription factor required for the regulation of Pi scavenging mechanisms. The *psr1* mutant is additionally sensitive to high light so that the combination of Pi deprivation and high

intensity of light could suppress the growth of non-transformed *psr1* cells under Phi selection. These mutants lacking the ability to acclimatise to P deprivation represent attractive recipient cells for future studies of PtxD/Phi selection.

Alternatively, rather than developing new recipient strains for transformation, the PtxD enzyme itself could be improved. Recent research has reported a mutagenized version of the enzyme for more efficient Phi-to-Pi conversion as a result of a relaxed cofactor binding (Cutolo *et al.*, 2020). This study attempted to address the issue of the limited availability of cofactor NAD+ for PtxD in the chloroplast during photosynthesis. While NADP+ can replace NAD+ *in vitro* for the conversion of Phi to Pi, the overall efficiency was significantly less when comparison to that of NAD+ (Garcia Costas, White and Metcalf, 2001). The researchers therefore introduced the substitution of two adjacent amino acids located in the cofactor-binding pocket of PtxD (glutamic acid-175 and alanine-176 replaced with alanine and arginine residues respectively) to reduce the selectivity of the cofactor binding and so enabling the use of both NAD+ and NADP+ (Woodyer, Van Der Donk and Zhao, 2003). This mutagenized PtxD was demonstrated as an improved version of the chloroplast selectable marker for *C. reinhardtii*.

6.2.2 Studies on Pi transporters for Phi uptake

As discussed previously, the uptake of Phi is assumed to be via Pi transporters, but there are no rigid evidence of how the algal cell and chloroplast transport Phi across the respective membranes. Studies to gain a clearer view on Phi uptake into the algal chloroplast are required and may be achieved through either forward or reverse genetic approaches. If a forward genetic analysis is chosen, the starting cells would be a transgenic line with the capability to use Phi and would be subjected to random mutagenesis by UV irradiation. Then, the mutagenized cells would be grown under rich Pi and selective Phi media. If the mutants were able to grow on Pi but not on Phi media, they may carry mutations affecting the transport or utilisation of Phi. Gene mapping/complementation studies could be done afterwards. However,

this forward genetic analysis is time-consuming and required the laborious screening and identification of affected genes from a large number of mutants.

In contrast, a reverse genetic approach could be taken in which a relevant gene is targeted for mutation in order to search for a function (Griffiths *et al.*, 2000). Since it is assumed that Phi uptake occurs through Pi transporters, candidate genes might be those involved in high-affinity Pi uptake activity such as the PTB genes of the Pi transporter type B family located in the cell membrane (Moseley, Chang and Grossman, 2006). These genes are upregulated up to 20-fold in P-starved cells, especially *PTB2* and *PTB5* (Moseley, Chang and Grossman, 2006) (Figure 6.1). In contrast, Pi uptake across the algal chloroplast membranes might involve the triose-phosphate translocators that exchange carbohydrate from the chloroplast for cytosolic Pi (Weber and Linka, 2011) and the PHT4 family (Karlsson *et al.*, 2015; Marchand *et al.*, 2018). These target transporters genes can be subject to site-directed mutagenesis. If the knockout of a target gene proved lethal when cells were grown on both Pi and Phi due to the loss of a specific Pi transporter, this might suggest that Pi transporter is also required for Phi uptake. This reverse genetic analysis is specific for a set of target genes, but it would be challenging to find other unidentified transporters responsible for Phi uptake.

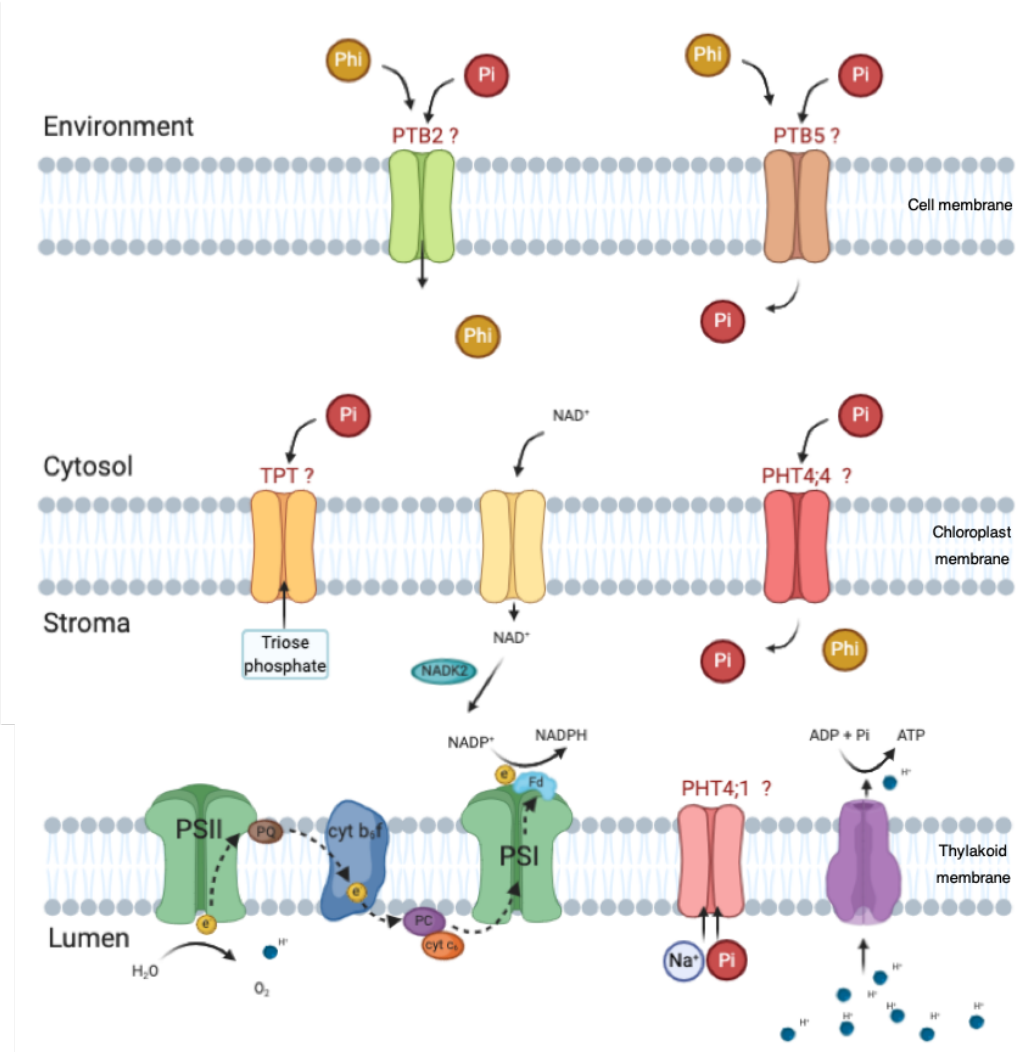


Figure 6.1 Schematic diagram of Phi uptake and NAD+ availability.

PTB2 and PTB5 transporters are localised in the cell membrane and provide the cell with Pi from the environment or medium. The TPT transporter may supply the stroma with cytosolic Pi in an exchange of triose phosphate intermediates. An additional Pi transporter into the stroma is PHT4;4. In the case of the lumen, PHT4;1 is known in plants to supply Pi to the ATP synthase in thylakoids during the photobiochemical reactions and is speculated to facilitate Pi transport via an Na+ gradient, however, no study has yet to support this in algae. The cytosolic NAD+ cofactor is transported into the stroma and converted to NADP+ as the final electron acceptor of the linear electron transfer pathway during photosynthesis.

6.2.3 Further investigations of *ptxD* expression in another cell compartment

To date, a few studies have demonstrated the expression of *ptxD* in *C. reinhardtii*, either in the nucleus or the chloroplast (Loera-Quezada *et al.*, 2016; Sandoval-Vargas *et al.*, 2018b, 2019; Changko *et al.*, 2020). In this study, the results in Chapter 3 suggested that the activity of PtxD in the *C. reinhardtii* chloroplast may compromise algal growth under phototrophic condition, probably due to the reductive environment in the stroma. This issue could limit the use of the transgenic strain in industry in which algal cultivation normally relies on phototrophic growth. Another strategy could be explored through the targeting of PtxD to another compartment within the chloroplast: namely, the thylakoid lumen. Although thylakoid lumen has a relatively small proteome (Kieselbach *et al.*, 1998), it provides a lower pH than stroma and more oxidative environment that is suitable for disulphide formation. This could be useful for some commercial proteins such as antibodies that have multiple disulphide bonds (Zedler, Mullineaux and Robinson, 2016).

Some issues of targeting of the PtxD protein into the lumen that need to be considered are the availability of substrate and cofactor. The first issue of the substrate is the Phi uptake into the lumen. It is unclear how Phi gets into the chloroplast and also no evidence to show luminal uptake of Phi across the thylakoid membrane. In plants, Pi may be transferred from lumen to stroma through the PHT4;1 transporter (Guo *et al.*, 2008) (Figure 6.1). It has been hypothesized that PHT4;1 could transport Pi in or out of thylakoid lumen depending on the direction of the H+ or Na+ gradients (Spetea and Schoefs, 2010). Regarding the previous studies in plants suggested Phi uptake can be achieved via Pi transporters (Varadarajan, 2002; Achary *et al.*, 2017), PHT4;1 may transport Phi to the thylakoid lumen. The second issue is the availability of NAD+ cofactor for PtxD activity in the lumen. The pool of NAD+/NADP+ is mostly present on the stromal side to accept the electron from ferredoxin-NADP reductase during photosynthesis (Gakière *et al.*, 2018), and it is uncertain whether the lumen gains NAD+ from the stromal

pool. Certainly, if a study of targeting of PtxD to the lumen is carried out, it will add more information to the supply of NAD+ cofactor in the lumen and Phi uptake, which will be very useful for the synthesis of other enzymes or proteins in this new compartment.

The genetic engineering approach that would be taken is to use a transit peptide to target PtxD across the thylakoid membrane. A previous study from Zedler et al. (2016) showed the successful translocation of a fluorescent reporter protein (pHRed) and a biopharmaceutical model substrate (scFv) into the thylakoid lumen by using the Tat transit peptide from the TorA protein of *Escherichia coli*. The fusion to the Tat transit peptide normally leads to correct protein targeting and maturation during to transport because the Tat pathway has a proofreading ability to transport the correct folded proteins (Robinson *et al.*, 2011). This strategy could be exploited for PtxD by fusing the TorA transit peptide at the N-terminus of the protein and selection for successful transformants would be based on the ability to grow on Phi.

6.3 Opportunities for developing new tools and strategies for algal chloroplast engineering via synthetic biology

One principle of synthetic biology is the design-build-test approach which involves fast, repeated cycles involving a standardised DNA assembly methodology. Developments in this area have accelerated our ability to modify organisms in a way that would be challenging and time-consuming if based on a bespoke cloning strategy only. Synthetic biology normally requires the use of well-characterised standard DNA components or 'parts' that should be validated under various conditions. These parts (*e.g.* promoters, untranslated regions, coding sequences) can be joined together using an agreed method, allowing rapid assembly of designed transgenes. It also allows the exchange of parts between laboratories thereby facilitating technology transfer and further parts validation. The development in algal biotechnology will be accelerated if it is simpler to acquire and use these

parts for DNA assembly rather than having to design each individual strategy for transgene construction.

A newly developed toolkit for such rapid DNA cloning is a GoldenGate-based method called Start-Stop DNA assembly (Taylor, Mordaka and Heap, 2019). The technique involves using the type IIS restriction endonuclease *Sap*I to generate 3 base overhangs. The overhang designated for a join at the start codon (ATG) is the start codon itself (*i.e*. a DNA join is 'scarless' at this start) and similarly the overhang for a join at the stop codon is the stop codon TAA (Figure 6.2). Several DNA parts can be assembled effectively in the intended order and orientation by the use of these designed cohesive DNA overhangs. The assembly occurs in a single reaction where all enzymes and DNA are incubated under isothermal conditions, leading to a very simple method for DNA assembly. The only requirement for the Start Stop strategy is a large library of parts to cover all possibilities of effective designs.

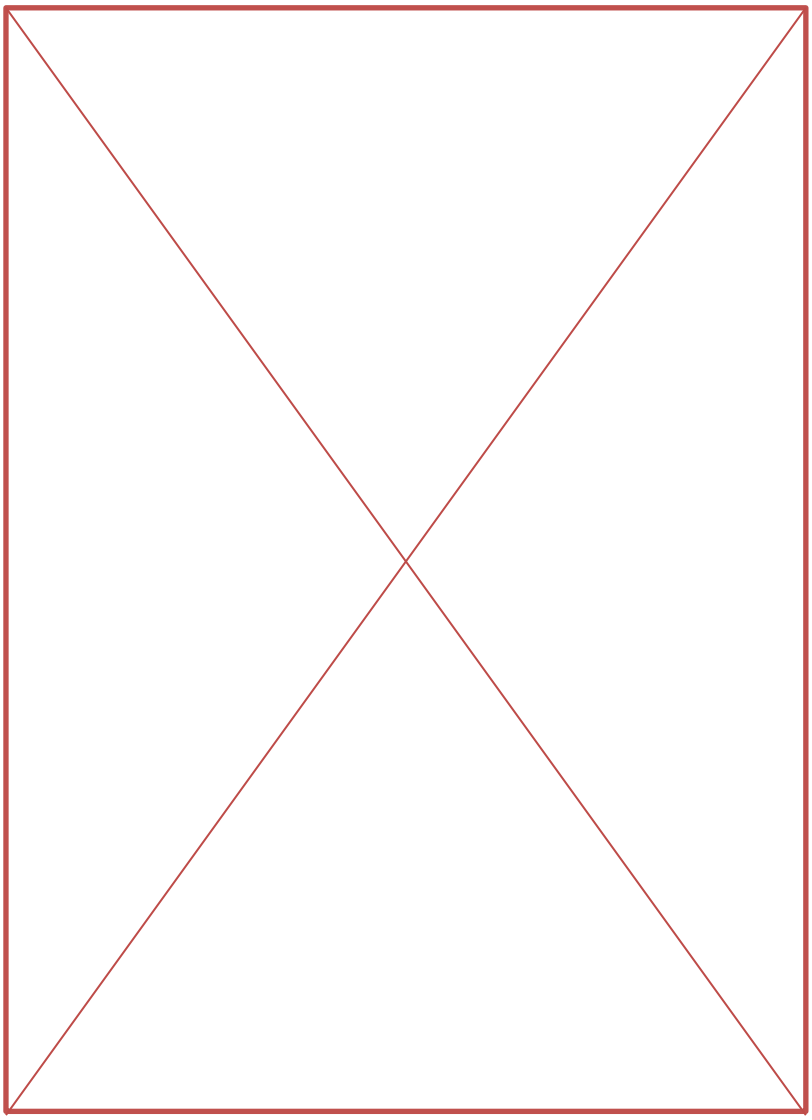


Figure 6.2 Schematic of Start-Stop Assembly for DNA cloning.

A) Expression construct of Level 1 assembly. All parts carried in Level 0 contain inward-facing *Sap*I restriction sites flanked at both sides and the coding sequence (CDS) part in Level 0 was shown in detail. Each required part, such as promoter, RBS, CDS, terminator has unique fusion sites (α-ϵ sites) that allow assembly in the intended order and orientation. The overhang of CDS designated for a join at γ site uses ATG as start codon whereas a join at δ site uses TAA as stop codon. B) Overall workflow of Start-Stop Assembly. All basic parts can be assembled up to 15 expression units in hierarchical order. Figure is reproduced from (Taylor, Mordaka and Heap, 2019)

A library of parts is important to the future of algal biotechnology as there has been an increasing interest in metabolic engineering (Fabris *et al.*, 2020). A large selection of parts would be useful to adjust levels of gene expression via promoters and regulatory elements of different strengths. In addition, the introduction of heterologous pathways that involve many genes could be easier to handle with a suitable cloning strategy such as Start-Stop that allows assembly of multigenic constructs within a few days. In the case of metabolic engineering of *C. reinhardtii* to synthesise limonene, a great demonstration would be to show that the introduction of additional genes to increase the pool of substrates as illustrated in Figure 6.3, and this could be difficult by traditional cloning approach.

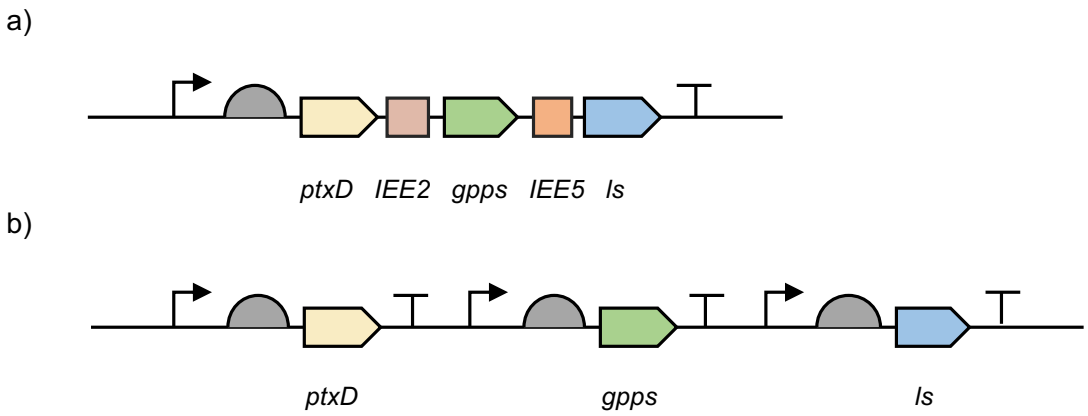


Figure 6.3 Schematic designed constructs for metabolic engineering for improved limonene synthesis.

a) Polycistronic expression of a *ptxD-gpps-ls* gene cassette under the same control of a single promoter, 5'UTR and 3'UTR, facilitated by fusing the coding sequences via to intercistronic expression elements, IEE2 and IEE5 (Macedo-Osorio *et al.*, 2018). The *ptxD* gene enables Phi-to-Pi conversation for the selection of the transformants on phosphite. The *gpps* gene encodes an GPPS enzyme to increase the pool of GPP precursor for limonene production. Then, *ls* encodes limonene synthase to carry out the conversion of GPP to limonene. b) Expression units of *ptxD*, *gpps* and *ls* are under the control of separate promoters, 5'UTRs and 3'UTRs. Both constructs can be assembled by Start-Stop assembly.

6.4 Concluding remarks

This study has demonstrated the successful development of tools and strategies based on the phosphite oxidoreductase gene, *ptxD* and an attempt to introduce a novel terpenoid biosynthesis into the algal chloroplast of *C. reinhardtii*. The *ptxD* expression was achieved in the chloroplast, and this highlighted a novel ability of the organelle to import and convert Phi into Pi. The work showed that the reliance on Phi metabolism did not compromise the mixotrophic growth of the alga, however, a concern is limited cofactor availability in the chloroplast when grown under phototrophic conditions. Successful *ptxD* applications have been demonstrated: namely, a novel non-antibiotic selectable marker and crop protection strategy, which marks a step forwards in chloroplast engineering, strain improvement and cultivation. A proof of concept involving retrofiting *ptxD* into an existing algal strain expressing a fish vaccine has shown the potential for the *C. reinhardtii* chloroplast as a low-cost production platform for aquaculture vaccines. The final attempt of this study for the introduction of novel terpenoid biosynthesis has made some progress on limonene synthase expression in the chloroplast although the limonene product remains undetected, and this requires further metabolic engineering work.

Overall, the work presented here has made some progress on increasing the repertoire of molecular tools and engineering strategies for the algal chloroplast. The next stage in this process is the transfer of these technologies to algal platforms beyond *C. reinhardtii*, and the further adoption of standardised methods for DNA assembly together with detailed metabolic models. Then, the real potential of algal chloroplasts for industrial biotechnology can be explored.

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Appendices

Appendix 1 Composition of phosphite (Phi) medium used in this study (Tris-acetate medium supplemented with phosphite as the sole source of phosphorus).

Component	Concentration
NH_4Cl	0.40 g/L
$MgSO_4 \cdot 7H_2O$	0.10 g/L
$CaCl_2 \cdot 2H_2O$	0.05 g/L
$Na_2HPO_3 \cdot 5H_2O$, 1 M, pH 7.0	1.00 mM
KCl, 1 M, pH 7.0	1.00 mM
Tris	2.42 g/L
Glacial acetic acid, 100%	1.00 ml/l (to pH 7.0)
Trace elements stock solution (see: Kropat et al. 2011)	7.00 ml/l

Appendix 2 Tris-acetate phosphate (TAP) medium modified from (Gorman and Levine, 1965; Harris, 2009)

Component	Concentration
NH_4Cl	0.40 g/L
$MgSO_4 \cdot 7H_2O$	0.10 g/L
$CaCl_2 \cdot 2H_2O$	0.05 g/L
*1 M (K)PO_4, pH 7.0	1.00 mM
Tris	2.42 g/L
Glacial acetic acid, 100%	1.00 ml/L (to pH 7.0)
Trace elements stock solution (see: Kropat et al. 2011)	7.00 ml/L

*1 M (K)PO_4, pH 7.0: 1 M K_2PO_4 titrated to pH 7.0 with 1 M KH_2PO_4

Appendix 3 Composition of Sueoka high-salt (HSM) medium modified from (Harris, Stern and George B., 2009)

Component	Concentration
NH_4Cl	0.40 g/L
$MgSO_4 \cdot 7H_2O$	0.10 g/L
$CaCl_2 \cdot 2H_2O$	0.05 g/L
K_2HPO_4	0.70 g/L
KH_2PO_4	0.40 g/L
Trace elements stock solution (see: Kropat et al. 2011)	7.00 ml/L

Appendix 4 Summary of plasmids used in this study.

Plasmid No.	Plasmid name	Comments	Reference
SCB001	pBa3-AX	Parental plasmid used to construct a plasmid for insertion of the *ptxD* cassette into a neutral region between *psaA-3* and *trnL2*	This study
SCB002	pBa-3-AX-ptxD	Daughter plasmid for an integration of the *ptxD* cassette into a neutral region between *psaA-3* and *trnL2* in NNV strain, in which the readthrough of *ptxD* was achieved with the modified tRNA located in the different expression cassette.	This study
SCB003	pJET1.2_LS	Intermediate cloning vector for expression of limonene synthase gene in *E. coli*	This study
SCB004	pJET1.2_ptxD	Intermediate cloning vector for *ptxD* gene expression in *E. coli*	This study
SCB005	pRY186 or pWUCA4	Chloroplast expression plasmid carrying the cold-inducible tRNA to recognize the TGA stop codon as a tryptophan codon	(Young and Purton, 2018)
SCB006	pRY186_LSmt	Chloroplast expression plasmid carrying the cold-inducible tRNA and mutated version of limonene synthase gene to be transformed in *C. reinhardtii*	This study
SCB007	pSCPTXD	Chloroplast expression plasmid carrying the modified tRNA and *ptxD* gene to be transformed in *C. reinhardtii*	(Changko *et al.*, 2020)
SCB008	pSP124S	Nuclear expression plasmid carrying *ble* gene fused to the 5' and 3' regulatory regions of the *C. reinhardtii* RBCS2 gene	(Stevens, Purton and Rochaix, 1996)
SCB009	pSRSapI	Chloroplast expression plasmid carrying expression cassette with *psaA* exon1 promoter/5'UTR and *rbcL* 3'UTR, based on *psbH* gene as selectable marker	(Young and Purton, 2014)
SCB010	pUC8-atpX-AAD	Expression plasmid carrying *aadA* cassette	(Goldschmidt-Clermont, 1991)
SCB011	pWUCA2	Chloroplast expression plasmid carrying the modified tRNA to recognize the TGA stop codon as a tryptophan codon	(Young and Purton, 2016)

Plasmid No.	Plasmid name	Comments	Reference
SCB012	pWUCA2_LSmt	Chloroplast expression plasmid carrying the modified tRNA and mutated version of limonene synthase gene to be transformed into *C. reinhardtii*	This study
SCB013	pRY186_LS	Chloroplast expression plasmid carrying the cold-inducible tRNA and limonene synthase gene to be transformed into *C. reinhardtii*	This study
SCB014	pWUCA2_LS	Chloroplast expression plasmid carrying the modified tRNA and limonene synthase gene to be transformed into *C. reinhardtii*	This study
SCB015	pBa-3-AX-ptxD-tRNA or pPO3	Chloroplast expression plasmid for integration of *ptxD* into neutral site between *psaA-3* and *trnL,* in which the readthrough of *ptxD* was achieved with the modified tRNA located in the same expression cassette.	(Changko *et al.*, 2020)
SCB016	p72B-SH	Chloroplast expression plasmid for the restoration of *psbH* gene in TN72	(Bateman and Purton, 2000)

Appendix 5 Summary of primers used for PCR or DNA sequencing

Primer	Sequence 5'-3'	Comments
Primers used in plasmid construction		
ptxD.F	AGGTTTCGGATTGAACAATAATGGC	Amplification of *ptxD* gene for cloning into pBa3-AX.
ptxD.R	CCACTACAATTGCGTTAAGATAAACGCGCTACATC	
Primers used to confirm integration and homoplasmy		
F1	GTCATTGCGAAAATACTGGTGC	To confirm the integration of *ptxD* into the plastome. The primer binds to flanking sequence located outside of the transforming pWUCA2 plasmid, therefore, the amplification is successful only if the transgene integrates at the correct plastome locus.
R1	CGGATGTAACTCAATCGGTAG	R1 combines with F1 to generate a PCR product of 880 bp from the untransformed plastome of the TN72 recipient.
R2	ATAGGCTCTTCTCATGGATTTCTCCTTATAATAAC	R2 combines with F1 to generate a PCR product of 1415 bp from the plastome of TN72 transformants.
F2	AACTATTTGTCTAATTTAATAACC	F2+R3 used to confirm introduction of *ptxD* into recipient TN72::pWUCA2 following direct selection. PCR products are 282 bp prior to integration, 1286 bp after integration.
R3	CAAACTTCACATGCAGCAGC	As mentioned above.
F3	TGGTCGTGGTTACTGGCAAGAA	To confirm the integration of *ptxD* at a neutral site between *psaA-3* and *trnL*. F3 binds toward the end of *psaA-3* in both recipient strain and transformant.
R4	CCCAACCTTGTAAACGGTCAGC	R4 is located within *ptxD* and combines with F3 to generate a PCR product of 1251 bp if the cassette integrates at the locus (1532 bp if the cassette includes *trnW*^{UCA}, as for plasmid pPO3).
R5	CGTCTACCATTCCGCCATATC	R5 is located within *trnL* and combines with F3 to generate a PCR product of 657 bp for the WT locus.

Appendix 6 Coding sequence and protein sequence of codon-optimised *ptxD* gene for the *C. reinhardtii* chloroplast. The gene contains two internal TGA stop codons as shown in green colour, which are translated into tryptophan by the modified tRNA in the transgenic line (Young and Purton, 2016).

>*ptxD* (1047 bp)

ATGTTACCAAAATTAGTAATTACTCACCGTGTTCACGACGAAATTTTACAATTATTAGCTCCACACTG
TGAATTAATGACAAACCAAACTGATTCTACTTTAACACGTGAAGAAATTTTACGTCGTTGTCGTGACG
CACAAGCTATGATGGCTTTCATGCCAGATCGTGTTGATGCTGATTTCTTACAAGCTTGTCCAGAATTA
CGTGTAGTTGGTTGTGCTTTAAAAGGTTTCGATAACTTCGATGTTGATGCTTGTACTGCTCGTGGTGT
ATGATTAACATTCGTACCAGATTTATTAACAGTACCAACAGCTGAATTAGCTATTGGTTTAGCTGTAG
GTTTAGGTCGTCACTTACGTGCTGCTGATGCTTTCGTACGTTCAGGTGAATTCCAAGGTTGACAACCA
CAATTCTATGGTACTGGTTTAGATAACGCTACTGTAGGTATTTTAGGTATGGGTGCTATTGGTTTAGC
TATGGCTGACCGTTTACAAGGTTGGGGTGCTACTTTACAATATCACGAAGCTAAAGCTTTAGATACTC
AAACAGAACAACGTTTAGGTTTACGTCAAGTAGCTTGTTCAGAATTATTTGCTTCTTCTGATTTCATT
TTATTAGCTTTACCATTAAACGCTGATACTCAACACTTAGTAAACGCTGAATTATTAGCTTTAGTTCG
TCCAGGTGCTTTATTAGTTAACCCATGTCGTGGTTCAGTAGTAGATGAAGCTGCTGTATTAGCTGCTT
TAGAACGTGGTCAATTAGGTGGTTACGCTGCTGATGTTTTTGAAATGGAAGATTGGGCTCGTGCTGAC
CGTCCACGTTTAATTGACCCTGCTTTATTAGCTCACCCAAACACTTTATTTACACCACACATTGGTTC
AGCTGTTCGTGCTGTTCGTTTAGAAATTGAACGTTGTGCTGCTCAAAACATTATTCAAGTATTAGCTG
GTGCTCGTCCAATTAACGCTGCTAACCGTTTACCAAAAGCTGAACCTGCTGCTTGTGGTTCTTACCCA
TACGATGTTCCAGATTACGCTTAATAA

>NAD:Phosphite oxidoreductase (37.28 kDa)

MLPKLVITHRVHDEILQLLAPHCELMTNQTDSTLTREEILRRCRDAQAMMAFMPDRVDADFLQACPEL
RVVGCALKGFDNFDVDACTARGVWLTFVPDLLTVPTAELAIGLAVGLGRHLRAADAFVRSGEFQGWQP
QFYGTGLDNATVGILGMGAIGLAMADRLQGWGATLQYHEAKALDTQTEQRLGLRQVACSELFASSDFI
LLALPLNADTQHLVNAELLALVRPGALLVNPCRGSVVDEAAVLAALERGQLGGYAADVFEMEDWARAD
RPRLIDPALLAHPNTLFTPHIGSAVRAVRLEIERCAAQNIIQVLAGARPINAANRLPKAEPAACGSYP
YDVPDYA*

Appendix 7 DNA sequence of plasmid pPO3

>pPO3 (9356 bp)

CCCATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGT
CGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTT
AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCC
TTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGAAATTGTAAGCGTTAATATTTTGTTAAAATTCGC
GTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAAT
CAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAAC
GTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC
CTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGAT
TTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGC
GCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCC
GCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCT
AAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAA
AGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCC
TGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGG
GTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA
ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCA
ACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATC
TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCC
AACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCA
TGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCA
CGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCC
CGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC
GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCAC
TGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGAT
GAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGT
TTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCC
TTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTA
GAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAA
ACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTG
GCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG
AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGA
TAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAA
CGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGT
GAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGT
CGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT
TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAAC
GCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGC
GTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCC

GAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTC
CCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA
GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGG
CTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTAC
GCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTC
TAGGTATATACATTCACCCTTTAAGGCTACCCGGCAGTTAGTTACGGCTTACGTTCCATAAAATATTG
GCATATTTTATAAATTATTTTATAGATCATATATTTTGTAAATATATAATATTACGCATAACACATTT
ATTTAAAAACATATAATATTACGCATAACACATTTATTTAAAAACAGCAAAAACTTGCGTCAAATCCC
TATAGGATATTTTTTATTTATGGCACGAGCAGGTTTACACGCTCCGTCAGGACGCCGGCACGTAGTTG
GAAAGTATGTCCCCCTTGCCCGGAAGGGGAAAGGAGGAGACAAATTTATTTATTGTATATAAATAGCA
TGACTTTCCAAGCGAGTTAACATAAACAAACTGCGCGAGTTAACATAAACAAACTTCCTCTCTGGGGA
GGCATGGCAGCAAATGGCACCCTAAATACATGCGCTTTTAACACAGATAATTATAAACAAGCATAGCG
TTAAATCTGCTACCTTGGATTAAATCATTAGAAAGAATTTGAGCCGTGTGCAGTGAAAATTGCATGCA
CGGCTCTTAAGGTTTAAATAAATTTTTAAAGAAGAAAATTTAACTCCTAACTATGTACTTCCATGGTG
CACGTTTTTCAAACTATGAAGCTTGGTTAAGTGACCCTACTCACATTAAACCAAGTGCTCAAGTAGTA
TGGCCTATTGTAGGTCAAGAAATTTTAAACGGTGATGTAGGTGGTGGTTTCCAAGGTATTCAAATTAC
TTCTGGTTTCTTCCAATTATGGCGTGCTAGTGGTATTACTAGTGAATTACAACTTTATACTACAGCAA
TTGGTGGTTTAGTAATGGCTGCTGCAATGTTCTTTGCTGGTTGGTTCCACTACCACAAAGCTGCTCCA
AAACTAGAATGGTTCCAAAACGTTGAATCAATGTTAAACCACCACTTAGGTGGTCTTCTTGGTTTAGG
TAGTTTAGCTTGGGCTGGTCACCAAATTCACGTTTCTTTACCAGTAAACAAATTATTAGATGCTGGTG
TAGATCCAAAAGAAATTCCACTTCCTCATGATTTATTATTAAATCGTGCTATTATGGCTGACTTATAC
CCAAGTTTTGCTAAAGGTATTGCTCCTTTCTTTACTTTAAACTGGAGTGAATACAGTGATTTCTTAAC
ATTTAAAGGTGGTTTAAACCCTGTTACTGGTGGTCTTTGGTTAAGTGATACTGCTCACCACCACGTAG
CTATTGCTGTATTATTCTTAGTAGCTGGTCACATGTATCGTACTAACTGGGGTATTGGTCACAGTATG
AAAGAAATTTTAGAAGCTCACCGTGGTCCATTTACAGGTGAAGGTCACGTTGGTTTATATGAAATTTT
AACAACTTCTTGGCATGCACAATTAGCTATTAACTTAGCTTTATTTGGTTCGTTATCAATTATTGTAG
CTCACCACATGTACGCAATGCCTCCATACCCTTATTTAGCTACTGATTACGGTACACAATTATCATTA
TTTACACACCACACATGGATTGGTGGTTTCTGTATTGTTGGTGCTGGTGCTCACGCAGCTATTTTCAT
GGTTCGTGACTACGATCCTACTAATAACTACAACAACTTATTAGACCGTGTAATTCGTCACCGTGATG
CTATTATTTCTCACTTAAACTGGGTTTGTATTTTCTTAGGTTTCCACAGCTTTGGTTTATACATCCAC
AACGATACAATGAGTGCTTTAGGTCGTCCTCAAGACATGTTCTCAGATACTGCTATCCAACTTCAACC
AGTATTTGCTCAATGGATTCAAAATACACACTTCTTAGCTCCACAATTAACAGCACCAAATGCTTTAG
CTGCTACAAGTTTAACTTGGGGTGGTGATTTAGTTGCTGTTGGCGGTAAAGTAGCTATGATGCCTATT
TCTTTAGGTACTTCTGACTTTATGGTTCACCACATTCACGCTTTCACAATTCACGTAACTGTGTTAAT
TCTTCTGAAAGGTGTTTTATTTGCTCGTAGCTCTCGTCTTATCCCAGATAAAGCTAACTTAGGTTTCC
GTTTCCCTTGTGACGGTCCTGGTCGTGGCGGTACTTGTCAGGTTTCTGCTTGGGACCACGTATTCTTA
GGTCTTTTCTGGATGTACAACAGCTTATCAATTGTAATTTTCCACTTCAGCTGGAAGATGCAATCTGA
TGTTTGGGGTACGGTTACAGCTTCTGGTGTTTCTCACATTACTGGTGGTAACTTTGCACAAAGCGCTA
ACACAATCAACGGTTGGTTACGTGACTTCTTATGGGCACAATCATCACAAGTAATCCAATCATACGGT
TCAGCTCTATCTGCTTATGGTTTAATTTTCTTAGGTGCTCACTTCGTATGGGCATTCTCGTTAATGTT
CTTATTCTCTGGTCGTGGTTACTGGCAAGAACTTATCGAATCAATTGTATGGGCTCACAACAAACTTA
AAGTTGCACCTGCAATTCAACCACGTGCTTTAAGTATTACTCAAGGTCGTGCTGTTGGTGTAGCTCAC

TACCTTTTAGGTGGTATTGCTACTACATGGTCGTTCTTCTTAGCACGTATCATTTCTGTAGGTTAACA
TTTAATACTTTTTAATACATATATGCCTAAGTTTATCTTTAAAGATAAACTTAGCCATATGTGTTAAG
TTATCTAACAAGGTTACCTTTTTATTTCTCTTTAGATATATAAACATTAAAAACTACCGTGATCGTTA
CACTTTAGATAACTGGAAGGGGGAAAAATCATGTATTCGCTGGAAGGCGCACCTCCTACTGCCTACTG
CGCAGCATTAAAATGCTGTAGATATTGGTATCTTACAAAGGACAGTAGTACACAATTAAACGCGTTAA
CCCATGATTAACAACTATATCAATAAAATCAATTTGTAGTGAAATACTCTGATTGACATTAAAATAAT
ACCATGATAAAAATTATAATAACAAATTTT**ACGTCCTTAGTTCAGTCGGTAGAACGCAGGTTTTCAAA**
ACCTGATGTCGTGGGTTCAATTCCTACAGGGCGTGTTTTTCCTAATGTACTTTGTTGTAAAAGTGGCT
GGTTTAACCTTTTTAGGTTTCGGATTGAACAATAATGGCAGTTAAGAGTCACTAAAGCTGCTGTATAG
ACGCGTAAGCTTTCTTAATTCAACATTTTTAAGTAAATACTGTTTAATGTTATACTTTTACGAATACA
CATATGGTAAAAAATAAAACAATATCTTTAAAATAAGTAAAAATAATTTGTAAACCAATAAAAAATAT
ATTTATGGTATAATATAACATATGATGTAAAAAAAACTATTTGTCTAATTTAATAACCATGCATTTTT
TATGAACACATAATAATTAAAAGCGTTGCTAATGGTGTAAATAATGTATTTATTAAATTAAATAATTG
TTATTATAAGGAGAAATCCATGTTACCAAAATTAGTAATTACTCACCGTGTTCACGACGAAATTTTAC
AATTATTAGCTCCACACTGTGAATTAATGACAAACCAAACTGATTCTACTTTAACACGTGAAGAAATT
TTACGTCGTTGTCGTGACGCACAAGCTATGATGGCTTTCATGCCAGATCGTGTTGATGCTGATTTCTT
ACAAGCTTGTCCAGAATTACGTGTAGTTGGTTGTGCTTTAAAAGGTTTCGATAACTTCGATGTTGATG
CTTGTACTGCTCGTGGTGTATGATTAACATTCGTACCAGATTTATTAACAGTACCAACAGCTGAATTA
GCTATTGGTTTAGCTGTAGGTTTAGGTCGTCACTTACGTGCTGCTGATGCTTTCGTACGTTCAGGTGA
ATTCCAAGGTTGACAACCACAATTCTATGGTACTGGTTTAGATAACGCTACTGTAGGTATTTTAGGTA
TGGGTGCTATTGGTTTAGCTATGGCTGACCGTTTACAAGGTTGGGGTGCTACTTTACAATATCACGAA
GCTAAAGCTTTAGATACTCAAACAGAACAACGTTTAGGTTTACGTCAAGTAGCTTGTTCAGAATTATT
TGCTTCTTCTGATTTCATTTTATTAGCTTTACCATTAAACGCTGATACTCAACACTTAGTAAACGCTG
AATTATTAGCTTTAGTTCGTCCAGGTGCTTTATTAGTTAACCCATGTCGTGGTTCAGTAGTAGATGAA
GCTGCTGTATTAGCTGCTTTAGAACGTGGTCAATTAGGTGGTTACGCTGCTGATGTTTTTGAAATGGA
AGATTGGGCTCGTGCTGACCGTCCACGTTTAATTGACCCTGCTTTATTAGCTCACCCAAACACTTTAT
TTACACCACACATTGGTTCAGCTGTTCGTGCTGTTCGTTTAGAAATTGAACGTTGTGCTGCTCAAAAC
ATTATTCAAGTATTAGCTGGTGCTCGTCCAATTAACGCTGCTAACCGTTTACCAAAAGCTGAACCTGC
TGCTTGTGGTTCTTACCCATACGATGTTCCAGATTACGCTTAATAAGCATGCAAGCTTGTACTCAAGC
TCGTAACGAAGGTCGTGACCTTGCTCGTGAAGGTGGCGACGTAATTCGTTCAGCTTGTAAATGGTCTC
CAGAACTTGCTGCTGCATGTGAAGTTTGGAAAGAAATTAAATTCGAATTTGATACTATTGACAAACTT
TAATTTTTATTTTTCATGATGTTTATGTGAATAGCATAAACATCGTTTTTATTTTTATGGTGTTTAGG
TTAAATACCTAAACATCATTTTACATTTTTAAAATTAAGTTCTAAAGTTATCTTTTGTTTAAATTTGC
CTGTCTTTATAAATTACGATGTGCCAGAAAAATAAAATCTTAGCTTTTTATTATAGAATTTATCTTTA
TGTATTATATTTTATAAGTTATAATAAAAGAAATAGTAACATACTAAAGCGGATGTAGATGTAGCGCG
TTTATCTTAACGCAATTCCCATATAGCCAATGGCTTAAGGAGTGTCATAGGAATAACTAGTCATGCAC
ATTTTCCTAAAAATCTAAAATGTTATTAGAAGCTATACAAAAATTAAAAATTATGTTATTATATATTT
ATTAAAATTTAGACTTATCACGGGGATATGGCGGAATGGTAGACGCTACGGACTTAAAATCCGTTCTT
GTGCGAACAAGGTGAGGGTTCAAGTCCCTCTTTCCCCATTTATAAATAAAAATCAAGTCAATATTTTA
ACATTGTAAACTAAAAAATAAGTGTAAAATAACTAGGGTAAATAGTTAAGAATTGGTAGTTTTTGAAC
TAAACAGTGACATAAAACTGGTTAAAAGACATCCCTGTAAGAGAAATGCATATGGTGAATTACACAAT
AAATTTTAAAAATAAAGCTCTGACAAGCCTCTTCCCCTTTGCGATATACATGCTCTGTTAGGATATAA

TTCTGCCTAAGTTCCTAACAAAGGGTTTACTTTTTCTCGGGGAATGGTTTTGCTACTTTGTTTTAAAT
CTTTTTCAAAGAATGTCAATCTCCTATAAAGGGAGGAACATTACTCATGGTCTCACAATTTTAATAAA
CTAGTTTCTTATAATCTATTTTATAAAAATAAATCTAAATATATTTCTTATGAATTTTGTTAATTTAG
AACAAATTGAAAATTCTTTACGTAATGCTACTTTTTGCATGCTTTTTTTAACAACATTTTTATATTGG
TTTTATACTGCATTTTACAGTACAAATCCTCAACAAATTATAAATCCATTGTCATTAACAAACATTAA
AACTAATTTTACTTATCCTGACGGGTTTCAAGCAAACAATATTAATGTTTCTACAACTTATTTACCGA
TTAACCCTGTATTAAATACCGAAAGAGAAGAGCAACCCGAAGCTAACGGAACGAATGGGCTGTTGGGT
GTTTCTTCATTAGTAGTAAATTTAAAATCAATAGCAATCCCACGCATTATGATGGGTGTTTCTAATTT
ATTATTAGTATTATTATTACTTGTTCGTTGGGAAAAATCAGGTCATTTCCCATTAAGTAATTTATATG
AATCTTTAATGTTTTTAGCGTGGTGTTGTACATTTTTATATTTATTATATTGTACAAGTTTTACTTTA
TTGGTTGAAAAAATGTTAGGTTCATTAATTGCACCTTGTAGTTTATTAATGAATGCATTTGCTACTTT
TAGTTTACCAAAAGAAATGCAACAAGCATCACCATTAGTACCAGCTTTACAATCAAATTGGTTAATGA
TGCATGTTACTGTAATGATTATTAGTTATGCCACATTAATTATTGGATCGTTATTGTCGATTTTATTT
TTGATTTTATTTAAACACAAAAAAGGTACACCCAAAAAGTATGATAACTTTATTAACAATTTAGATGC
ATTAAGTTATCGCATTATTGGATTAGGTTTTCCTTTTTTAACTATTGGGATTTTATCTGGGGCTGTGT
GGGCTAATGAAGCATGGGGATCATATTGGAGTTGGGATCC

Key characteristics of the plasmid:

Vector sequence

Coding sequences of chloroplast genes: *psaA-3*; *trnL*; *ccsA*

Element carrying trnW^{UCA} (coding region in **bold**)

ptxD cassette: comprising *psaA-1* promoter/5'UTR:*ptxD* CDS:*rbcL* 3' UTR

Appendix 8 The plasmid pPO3. The bio-contained *ptxD* integration plasmid for the strain improvement strategy to insert *ptxD* into a neutral region of the *C. reinhardtii* plastome between *psaA-3* and *trnL*. The schematic diagram of the plasmid indicates the left and right arms (2.9 kb and 1.5 kb, respectively) flanking the 2.0 kb '*ptxD* cassette' that carries the two elements of the bio-containment system as following: the codon-optimised *ptxD* with two internal TGA stop codons (*) and a chloroplast-expressed tRNA gene modified to recognize the stop codon as a tryptophan codon. Figure is reproduced from (Changko *et al.*, 2020).