

Metabolic engineering of the algal chloroplast for terpenoid production

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Declaration

I, Umaima Hamed Al Hoqani, 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.



Umaima Al Hoqani

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Abstract

Microalgal biotechnology has attracted considerable interest owing to its potential to provide renewable energy and its capacity to produce molecules such as pigments, fatty acids and other high value compounds, which can be used in the biomaterials, cosmetics and pharmaceutical industries. One class of compounds are the terpenoids: a diverse group of molecules derived from C₅ isoprene units that are exploited for their aromatic and bioactive properties. Terpenoid production in microalgae offers an alternative to extraction from plant species or chemical synthesis. However, metabolic engineering technology for microalgae is still in its infancy and far from economic viability. Thus, the aim of this study was to develop engineering tools for the industrial algal species *Nannochloropsis gaditana*, with the goal of manipulating the main terpenoid pathway located in the chloroplast. In parallel, the effects of such manipulation was studied using the laboratory species *Chlamydomonas reinhardtii*, for which chloroplast genetic engineering is already established.

N. gaditana is a robust marine species well suited to industrial scale cultivation. The availability of a draft genomic sequence, nuclear transformation methodology and a high lipid productivity have positioned *N. gaditana* as a promising oleaginous alga for metabolic engineering. However, to develop it as an industrially relevant platform, further molecular tools are needed; in particular a reliable chloroplast transformation method. Thus, the aim of the first project was to develop chloroplast transformation for the alga. This involved optimizing the cultivation conditions for *N. gaditana*, evaluating its sensitivity to herbicides and chloroplast specific compounds in order to identify suitable selectable markers, and to construct chloroplast transformation vectors. In addition, the temporary increase in cell size by inhibition of cytokinesis was investigated in order to facilitate the delivery of DNA into the small chloroplast.

C. reinhardtii is the most developed algal model with well-established tools for genetic manipulation, and can be used to study the effect of chloroplast metabolic engineering in other species such as *Nannochloropsis*. Thus, the second project focused on the manipulation of the terpenoid biosynthetic pathway: specifically, the chloroplast-localized methylerythritol phosphate pathway by over-expressing the rate limiting enzyme; 1-deoxy-D-xylulose-5-phosphate synthase (DXS). An additional *dxs* gene from the cyanobacterium *Synechocystis* 6803 was introduced into the chloroplast genome in the hope of improving the productivity of downstream terpenoid metabolites. A number of transgenic lines were obtained and the successful integration was confirmed by molecular analysis. The effects of up-regulating DXS enzyme activity on overall algal growth and terpenoid profile are studied.

Abbreviations

Amp ^R	ampicillin resistance
ASW	artificial seawater
DABA	3,5-diaminobenzoic acid
DHA	docosahexaenoic acid
DMAPP	dimethylallyl diphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'deoxy nucleoside 5'-triphosphate
DTT	dithiothreitol
DXP	1-deoxy-xylulose 5-phosphate
DXS	1-deoxy-D-xylulose-5-phosphate synthase
ECL	enhanced chemiluminescence
EPA	eicosapentaenoic acid
EDTA	ethylenediaminetetraacetic acid (disodium salt)
FdUrd	5-fluorodeoxyuridine
FPP	farnesyl diphosphate
GPP	geranyl diphosphate
GGPP	geranylgeranyl diphosphate
GOI	gene of interest
HA	haemagglutinin
HPLC	high-performance liquid chromatography
HSM	high salt minimal medium
IPP	isopentenyl diphosphate / isoprene C5 unit
IgG	immunoglobulin G
LB	Luria-Bertani medium
LHRR	left homologous recombination region
MEP	methylerythritol phosphate
mRNA	messenger ribonucleic acid
MVA	mevalonate
PUFA	polyunsaturated fatty acid
NCBI	National Centre for Biotechnology Information
OD	optical density
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
psi	pounds per square inch
Q	quinone
RHRR	right homologous recombination region
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sw	sine wave
TAP	tris acetate phosphate medium
TAG	triacylglycerol
TBS	tris buffered saline
TBS-T	tris buffered saline – tween 20
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	transformant
Tris	tris (hydroxymethyl) aminomethane
UTR	untranslated region
UV	ultraviolet
v/v	volume for volume
w/v	weight for volume
WT	wild type

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CHAPTER 1

Introduction

1. Introduction

1.1. Algae

Algae are a highly diverse and polyphyletic group of organisms that are capable of performing photosynthesis, and range from small unicellular or filamentous organisms (microalgae) that are only a few micrometres in diameter to complex multicellular macroalgae that are tens of meters long such as the giant *Laminaria* seaweeds (Leliaert et al. 2012; Friedl et al. 2012). The cyanobacteria (also known as blue-green algae) are traditionally considered to be algae, although they have a prokaryotic structure and most sources reserve the term “algae” for photosynthetic eukaryotes (Friedl et al. 2012). Algae live in diverse ecological habitats, with most being found in aquatic environments include freshwater, seawater and brackish water, while some algae are adapted to terrestrial environments such as soil, rocks and snow (Friedl et al. 2012; Purton 2001). The majority of algae are free-living cells, however, some algae form symbiotic relationships with multicellular organisms that need to gain access to photosynthetically-fixed carbon. Such symbiotic associations are commonly found in Cnidaria (corals, sea anemones and jellyfish) and the phyla Porifera (sponges) (Bruck & Garbe 2012). The most relevant example is the symbiotic relationship of the dinoflagellates with marine invertebrate such as coral and sea anemones, commonly referred to as zooxanthellae.

In the context of evolution, it is widely accepted that algae originate from primary, secondary and tertiary endosymbiosis that involves the integration of several genomes from the hosts and symbionts to function as a single cell (Keeling 2004). Algae are indeed simple organisms compared to the higher plants, since they lack roots, leaves and other differentiated structures; however, both higher plants and algae possess chloroplasts for photosynthesis by which CO₂ is fixed to synthesise carbohydrates and other cellular compounds using solar energy. All algal chloroplasts contain chlorophyll *a* as a primary photosynthetic pigment, and additional accessory pigments that vary among different algal groups (Purton 2001). The presence or absence of these accessory pigments has traditionally been used as a parameter in the taxonomic classification of algae, for example into green, red and brown algae. Nevertheless, the system of algal classification has changed remarkably with the availability of modern technologies that facilitate the study of evolutionary relationships between algae, through analysis of their ultrastructural characteristics using electron microscopy, together with molecular phylogenetics of nuclear and organelle genomes (Adl et al. 2005).

The biodiversity of algae is remarkable (Figure 1.1), as it is estimated that there are 30,000 – 40,000 distinct algal species that have been described to date, and other algal species as yet undiscovered that may exceed the number of known species (Friedl et al. 2012). Such diversity accounts for the extremely broad variety of natural products that potentially could be exploited for various applications as nutraceuticals, cosmetics, pigments and pharmaceuticals (Table 1.1). Studies also suggest the potential uses of algae in wastewater treatment, bioremediation and in agriculture as biofertiliser (Gangl et al. 2015; Georgianna & Mayfield 2012).

In recent years there has been a considerable interest in the use of algae as a production platform for high value recombinant proteins due to their fast growth rate in comparison to higher eukaryotes and their ability to grow autotrophically thereby making the cultivation process potentially simple and cost effective (Barrera & Mayfield 2013; Georgianna & Mayfield 2012). Furthermore, a number of algal species have been assigned GRAS (Generally Recognized As Safe) status, which means they are safe for human consumption and subsequently there is no need for stringent purification steps for recombinant proteins, or perhaps the algae could be used as edible delivery systems (Barrera & Mayfield 2013; Rasala et al. 2013). In order to exploit these algae as a production platform, progress is being made in the development of genetic transformation methodologies for various algal species from distinctive groups (Kirchmayr & Griesbeck 2012). In addition, we are witnessing the development of modern strategies in reverse genetics to allow strain improvements by targeting endogenous genes through knockouts or knockdown, or knocking-in of foreign genes (Shin et al. 2016; Scaife et al. 2015). However, to establish algae as effective platforms for industrial applications, advanced genetic engineering tools are required for more algal species, particularly those with industrial potential to allow the genetic manipulation of endogenous genes and the introduction of foreign genes (Gimpel et al. 2015; Georgianna & Mayfield 2012), as currently these advanced genetic tools are, to some extent, limited to the model algae *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* (Spicer & Purton 2016; Scaife et al. 2015).

In the light of ongoing interest in algae as a source of renewable energy and natural products, recent efforts have also focused on improving algal cultivation systems at commercial scale (Figure 1.2). At present, the developed cultivation systems mainly fall into two categories: i) open raceway systems and ii) closed photobioreactor systems. In general, the choice of cultivation system depends on the species and the final products, as each of these systems has advantages and disadvantages (Ugwu et al. 2008; Chisti 2008). As such, closed photobioreactor systems offer proper containment for genetically modified algae, with a greater degree of control over growth conditions, and also minimise the contamination that is normally associated with open raceway systems (Wang et al. 2013).

On the other hand, open raceways are cost effective and are commonly used for algal species with extreme growth requirements, as the case for the extreme halophile *Dunaliella salina* (Ugwu et al. 2008).

Figure 1.1 Algae biodiversity

Selected examples of genera and species of algae exhibiting remarkable morphological and ecological diversity. (a) Light micrograph of a colony of the freshwater green alga *Volvox* sp. (b) Light micrograph of *Pinnularia*, a freshwater diatom. (c) Light micrograph of red marine microalga *Porphyridium purpureum*. (d) The brackish green microalgae species with flagella; *Tetraselmis tetraathele*. (e) The freshwater green *Haematococcus pluvialis* at resting stage. (f) *Macrocystis pyrifera* (giant kelp). Images are not on scale. The images (a, b and f) are taken from a website and (c, d and e) from Friedl et al. (2012).

Table 1.1 Examples of microalgae currently used in or potentially suitable for biotechnological applications.

Abbreviations are PUFA: polyunsaturated fatty acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. Table is adapted from Friedl et al. (2012).

Algal species/ genus	Habitats	Compounds and Applications
<i>Chlamydomonas reinhardtii</i>	Freshwater	Recombinant proteins
<i>Dunaliella</i>	Marine	Carotenoids (β -carotene), glycerol
<i>Haematococcus pluvialis</i>	Freshwater	Fatty acids, carotenoids (astaxanthin)
<i>Botryococcus braunii</i>	Freshwater	Oil, carbohydrates
<i>Chlorella vulgaris</i>	Freshwater	Fatty acids, carbohydrates
<i>Nannochloropsis</i>	Marine water and freshwater	Most promising algal EPA producer PUFA (EPA, DHA), lipids; pigments, carotenoids; β -1,3-glucan; poultry feed; aquaculture: rotifer and bivalve feed
<i>Skeletonema costatum</i>	marine	PUFA (EPA); antibacterial activity, β -1,3-glucan; cosmetics: anti-ageing and anti-cellulite effects, aquaculture: mollusc and oyster feed; waste water treatment: detoxification of cadmium and copper, biodegradation of phenolic compounds
<i>Porphyridium cruentum</i>	Marine, brackish and freshwater	PUFA (EPA), pigments (phycoerythrin); antiviral activity, sulfated polysaccharides, antioxidants; poultry feed
<i>Haslea ostrearia</i>	Freshwater	PUFA (EPA), linoleic acid; marennine (colouring agent); isoprenoids, tetra-unsaturated sesterterpenoids (haslenes)
<i>Phaeodactylum tricornutum</i>	Freshwater	PUFA (DHA, EPA), lipids; aquaculture: rotifer feed
<i>Euglena gracilis</i>	Freshwater	PUFA, lipids; β -1,3-glucan (Paramylon), α -tocopherol
<i>Cryptocodinium cohnii</i>	Freshwater	High content of PUFAs (DHA); pigments as colouring agents; extracts used in promoting lactic acid and bifidus bacterial growth; pharmaceuticals; pet foods; aquaculture feed

Figure 1.2 Algal cultivation systems

Examples of different cultivations systems for microalgae; (A) Tubular photobioreactor at AlgaePARC in Wageningen, Netherlands (B) Hanging bags at Supreme Biotechnologies Ltd, located in Nelson, New Zealand, (C) Cascade open raceway cultivation system at Algae for Future (A4F) in Lisbon, Portugal (D) Hanging bag supported with steel-mesh frame at Vitaplankton Ltd, located in North Wales, United Kingdom.

1.2. *Nannochloropsis* species

1.2.1. *Nannochloropsis* biology

Nannochloropsis species are unicellular coccoid microalgae with high photoautotrophic biomass accumulation and high lipid content (Radakovits et al. 2012). As shown in Figure 1.3, they are simple non-flagellated cells with a diameter of 2–4 µm that belong to the class of Eustigmatophyceae within the Heterokontophyta (Sukenik 1999). Each cell has one or more chloroplasts that occupy a large portion of the cell volume and contains chlorophyll *a* but not chlorophyll *b* or *c*. Other accessory pigments in the chloroplast include violaxanthin, β-carotene and vaucheriaxanthin ester (van de Hoek et al. 1995). The chloroplast is derived from the secondary endosymbiosis of a red alga, so it is complex compared to that of chlorophyte algae as it is surrounded by four membranes (Janouskovec et al. 2010). The outermost plastid membrane is connected with the outer nuclear envelope membrane to form a structure called a nucleus–plastid continuum (Murakami & Hashimoto 2009). The cell wall of *Nannochloropsis* species is made of cellulose with sulphated fucans, however, there is variation in the cell wall thickness among *Nannochloropsis* species (Corteggiani Carpinelli et al. 2014; Arnold et al. 2014). Interestingly, the saline growth conditions influence the thickness of the cell wall within a species. This response could be useful for industrial applications, where the growth conditions of these species could be optimised to suit downstream processing (Beacham et al. 2014).

The *Nannochloropsis* genus is traditionally classified as consisting of the species *Nannochloropsis gaditana*, *Nannochloropsis salina*, *Nannochloropsis oculata*, *Nannochloropsis granulata*, *Nannochloropsis oceanica* and *Nannochloropsis limnetica* (Murakami & Hashimoto 2009). However, recent studies have suggested either reclassification of some species within the *Nannochloropsis* genus or addition of a new species such as *Nannochloropsis australis* based on *rbcL* and 18S rDNA sequencing data (Fawley et al. 2015; Starkenburg et al. 2014). Species that are currently classified as *Nannochloropsis* live in marine environments with the exception of *N. limnetica*, which is typically found in fresh and brackish water (Jinkerson et al. 2013). *Nannochloropsis* species have been reported by Kilian et al. (2011) as having a haploid genome, which is potentially useful for the identification of dominant and recessive mutations since both mutations display a phenotype. Nonetheless, there are no reports on the sexual reproduction of *Nannochloropsis* species. The absence of a diploid state limits the possibility of using classical breeding programs to improve desirable traits, map mutations and eliminate undesired mutations.

Several species in the genus *Nannochloropsis* are recognised as being oleaginous due to their ability to produce naturally large quantities of lipids up to 60% of dry weight under various stress conditions, although there is variation in the lipid productivity and fatty acid composition among *Nannochloropsis* species (Beacham et al. 2014; Ma et al. 2014). In general, the lipid content and fatty acid composition of *Nannochloropsis* species can be manipulated by cultivation under various growth conditions such as nitrogen stress (nitrogen-replete and nitrogen-deplete) and sub-saturating light levels. Under nitrogen-deplete conditions, *Nannochloropsis* preferentially accumulates nonpolar storage lipids such as triacylglycerols (TAG), which are enriched with saturated and monounsaturated short fatty acids and it could be ideal as feedstock for biofuel production. On the other hand, both sub-saturating light levels and nitrogen-replete conditions promote the synthesis of photosynthetic and cellular membranes in the form of polyunsaturated fatty acids (PUFA), which are composed of relatively high levels of eicosapentaenoic acid (EPA, C20:5) (Sukenik 1999; Radakovits et al., 2012).

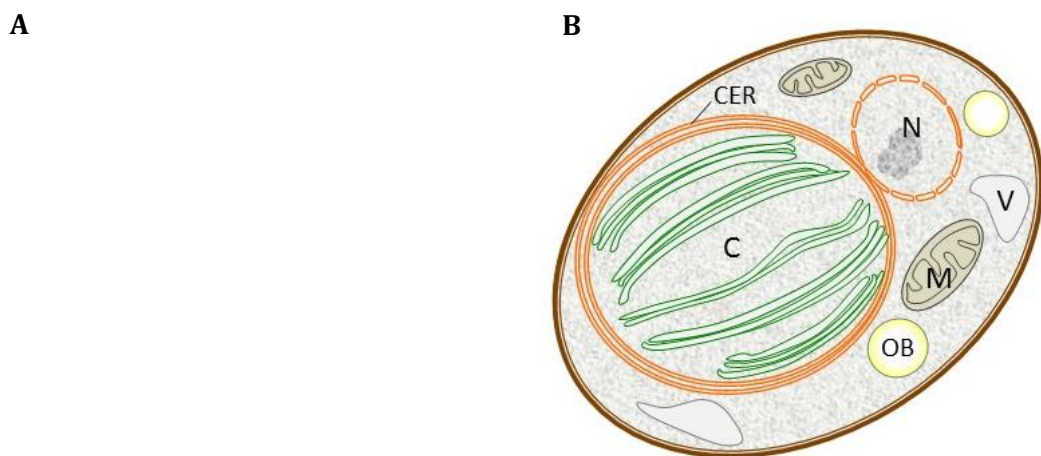


Figure 1.3 Microscopy image and schematic representation of *Nannochloropsis* ultrastructure.

(A) Microscopy image of *N. gaditana*. The image is taken from the Culture Collection of Algae and Protozoa. (B) Schematic diagram of *Nannochloropsis* genus showing the main physiological features under nitrogen replete conditions. Abbreviations are C: chloroplast, CER: chloroplast endoplasmic reticulum; M: mitochondrion; N: nucleus; OB: oil body; V: vesicle. Adapted from Al-Hoqani et al. (2016).

1.2.2. Genetic manipulation tools for *Nannochloropsis*

Nannochloropsis species have attracted considerable interest among algal researchers owing to their natural ability to accumulate large quantities of lipids, high biomass productivity and their successful cultivation at industrial scale. However, the full exploitation of these oleaginous species for biotechnological applications necessitates molecular tools to further our understanding and enable genetic engineering. The first *Nannochloropsis* species to be fully sequenced, including all three genomes, was *N. gaditana*, by Radakovits et al. (2012). Soon thereafter, the nuclear genome sequence for *N. oceanica* became available (Vieler et al. 2012). Furthermore, chloroplast and mitochondrion genome sequences are available from at least one strain of each *Nannochloropsis* species (Wei et al. 2013). The complete genome data of several *Nannochloropsis* species provides a starting point for developing molecular tools, in particular those tools required for genetic engineering of the nuclear genome, as will be discussed in this section. In addition, the available genomic data has enabled researchers to carry out analysis of the transcriptome (Zheng et al. 2013; Tian et al. 2013), proteome (Simionato et al. 2013) and lipidome (Li et al. 2014) to gain basic knowledge about biosynthetic pathways in *Nannochloropsis*.

Chen et al. (2008) first developed nuclear transformation in *Nannochloropsis*, demonstrating the successful expression of functional growth hormones under the control of an inducible promoter using electroporation of protoplasts. Since then, several methods have been developed for the nuclear transformation of *Nannochloropsis* without cell wall removal such as *Agrobacterium*-mediated transformation (Cha et al. 2011), electroporation (Kilian et al. 2011) and biolistics (Kang et al. 2015a). A number of selectable markers have also been developed for the identification of nuclear-transformed cells. The most widely used selectable markers in *Nannochloropsis* are the *Sh ble* and *aph7* genes, which confer resistance to the antibiotics zeocin and hygromycin B, respectively (Radakovits et al. 2012; Vieler et al. 2012). As a potential selectable marker for *Nannochloropsis*, a study reported the successful knockout of two native genes that are essential for the growth on nitrate as a source of nitrogen (Kilian et al. 2011). The nitrate and nitrite knockout cell lines lost the ability to grow on nitrate as the nitrogen source but not on medium containing ammonium. Therefore, complementation of knockout cell lines that are deficient in the expression of either these native genes with a cloned wild-type gene should allow selection on nitrate. A different type of selectable marker that has also been expressed in *Nannochloropsis* is the *chromoprotein* gene from *Stichodactyla haddoni* (*shCP*) that allows visual identification of the transformed cell in the background of non-transformed cells by a distinctive brown phenotype (Shih et al. 2015).

Several reporter genes such as the β -glucuronidase gene (*GUS*) and adapted versions of *green fluorescent protein* (*GFP*) have been developed in *Nannochloropsis* to test promoter efficiency and transformation techniques (Cha et al. 2011; Moog et al. 2015). In addition to this, GFP has been used for *in vivo* localisation studies, demonstrating the possibility of using N-terminal targeting sequences to direct nuclear-encoded proteins of interest into different subcellular compartments of *N. oceanica*; namely, the nucleus, mitochondria, endoplasmic reticulum and chloroplast include the periplastidal compartment and stroma (Moog et al. 2015). A gene encoding a genetically modified mCherry fluorescent protein “sfCherry fluorescent” has been developed recently as a reporter gene in *Nannochloropsis* to overcome the interference signals of endogenous pigments and provide greater brightness with photostability (Kang et al. 2015a).

The development of complementary techniques for engineering the chloroplast genome is not possible at present due to the inability to introduce exogenous DNA into the chloroplast genome, and indeed there is no report of successful chloroplast transformation of *Nannochloropsis*. Therefore, further development of these highly productive oleaginous algae as a potential platform for biofuels and high value compounds has been hampered since the chloroplast houses key metabolic pathways, including biosynthesis of fatty acids, carbohydrates, tetrapyrroles and terpenoids.

1.2.3. Biotechnological applications of *Nannochloropsis*

The ability of *Nannochloropsis* to produce high levels of triacylglycerides and polyunsaturated fatty acids has triggered an interest in exploiting these cellular chemicals as a feedstock for biofuels production, an aquafeed in the aquaculture industry and dietary supplements (Al-Hoqani et al. 2016), as summarised in Figure 1.4. Therefore, a number of studies have investigated the mass cultivation of *Nannochloropsis* for economically sustainable production of the omega-3 fatty acid EPA as a dietary supplement and an aquafeed (Camacho-Rodríguez et al. 2014; Becker 2013; Gressel 2013). Some of these studies have focused on the nutritional value of *Nannochloropsis* under different growth conditions and the transfer of their nutrients through food chains (Fernandez-Reiriz & Labarta 1996; Camacho-Rodríguez et al. 2014), while others have attempted to reduce algal production costs by enriching rotifers with frozen or preserved algae (Camacho-Rodríguez et al. 2015; Lubzens et al. 1995).

The current primary focus of commercial cultivation of *Nannochloropsis* is as a base of the food chain in the aquaculture industry due to the algae’s high content of eicosapentaenoic acid (Ma et al. 2016; Sukenik 1999). For example, concentrates of live cells, or frozen or lyophilised *Nannochloropsis* have been used to enrich aquafeed for cultivation of shrimps, molluscs and marine fish, or alternatively for production of rotifers, which in turn are used

as a food source for fish hatcheries (Camacho-Rodríguez et al. 2014; Lubzens et al. 1995). Furthermore, *Nannochloropsis* has been used as a feed to enrich animal products with PUFA. Such application has been demonstrated by Lemahieu et al. (2013), where the addition of *Nannochloropsis* biomass to the feed of laying hens greatly improved the nutritional value of egg yolk. *Nannochloropsis* has also been used in aquaculture as an oral delivery system for pharmaceutical proteins to improve both the growth rate of fish and survival rate during infection by pathogens. The most relevant example, an engineered *N. oculata* to produce growth hormone, has greatly improved the growth of tilapia larvae upon feeding them with this transgenic line (Chen et al. 2008). Another study reported an improved survival rate of medaka fish infected with a bacterial pathogen as a result of feeding them with an engineered transgenic line that produced the anti-microbial peptide bovine lactoferricin (Li & Tsai 2009).

Nannochloropsis is a potentially important source of dietary omega-3 fatty acids in the human diet as it is a useful component in preventing various human diseases (Pérez-Heras et al. 2016; Doughman et al. 2007). Several studies have therefore investigated the safety aspect of using long chain omega-3 fatty acids from *Nannochloropsis* as a source. These studies reported the safe use of dietary supplements in feeding experiments carried out with animals (Kagan et al. 2014; Kagan & Matulka 2015).

There has been considerable interest in *Nannochloropsis* species for the production of biofuels (Ma et al. 2014; Doan & Obbard 2014; Umdu et al. 2009; Hu et al. 2015; Zhu et al. 2014). However, there are a number of issues that need to be considered before the efficiencies needed for economic production can be achieved (Georgianna & Mayfield 2012). These include i) developing industrial strains with the ability to produce large quantities of triacylglycerols, ii) understanding the effect of lipid productivity on cell growth, iii) improving large-scale cultivation systems and iv) developing cost effective downstream processing techniques. There is also a need to develop more advanced methodologies for *Nannochloropsis* species including genetic engineering approaches for all three genomes.

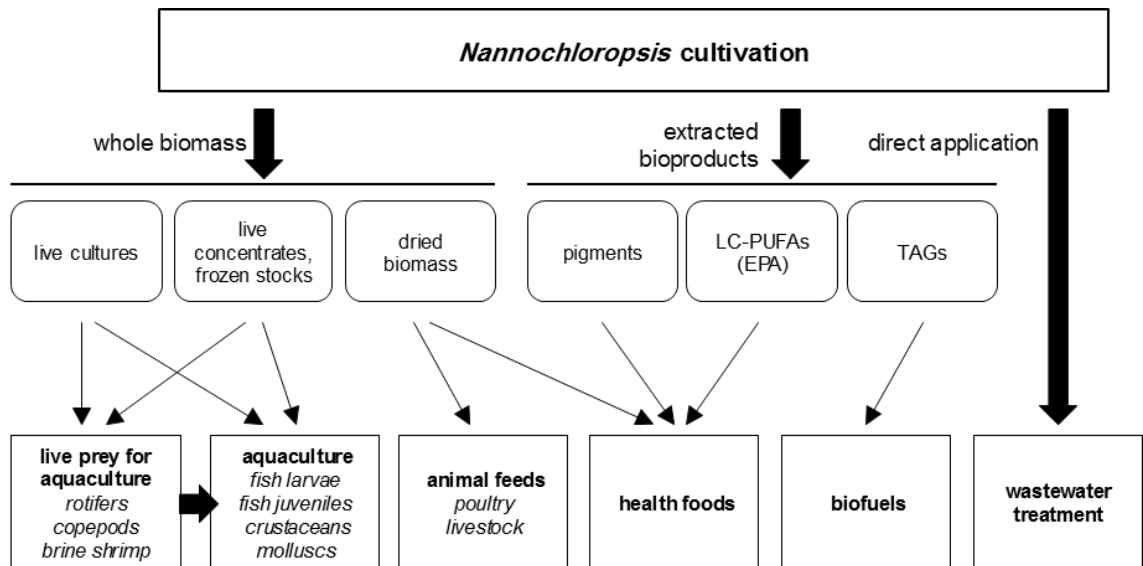


Figure 1.4 Current and potential applications of *Nannochloropsis*, adapted from Al-Hoqani et al. (2016).

1.3. *Chlamydomonas reinhardtii*

1.3.1. *C. reinhardtii* Biology

C. reinhardtii is a unicellular green freshwater microalga with an average diameter of 10 µm and it has a doubling time of 5–8 hours (Harris 1989). It is a member of the green algae (Chlorophyta), the most closely related algal group to the land plants, and it is characterised by the presence of chlorophyll *a* and *b*, and the ability to accumulate starch as a storage compound (Friedl et al. 2012). *C. reinhardtii* has a single cup-shaped chloroplast that occupies approximately two thirds of the cell volume and it contains 50–80 copies of the chloroplast genome (Harris 1989). Other distinguished features of *C. reinhardtii* include a pair of anterior flagella, eye spot and a pyrenoid, as shown in Figure 1.5. The *C. reinhardtii* cell is enclosed within a thick cell wall consisting of seven principal layers made of hydroxyproline-rich glycoproteins. Several cell wall mutants have been isolated with defects in cell wall biogenesis and these mutants have been widely used as recipient strains for transformation with exogenous DNA, as the process is more efficient with cell wall-less cells (Harris 2001).

C. reinhardtii represents an excellent model organism for basic and applied scientific research, as it is genetically well characterised with genomic resources and molecular tools (Scaife et al. 2015). It has a range of exceptional features. *C. reinhardtii* features two animal-like flagella and has a chloroplast that resembles those found in land plants. In addition to its autotrophic nature, *C. reinhardtii* can grow heterotrophically in the dark if supplied with acetate, or even in the case of a disrupted photosynthetic machinery (Harris 2001). These features have made *C. reinhardtii* the system of choice to study photosynthesis (Rochaix 2011), circadian rhythms (Suzuki & Johnson 2001), phototaxis (Foster et al. 1984) and flagella assembly (Ringo 1967).

C. reinhardtii cells are normally haploid, though they can propagate mitotically as a stable diploid (Harris 1989), as illustrated in Figure 1.6. The haploid cells exist as two genetically distinctive mating types; plus (mt+) and minus (mt-) and these cells tend to reproduce asexually by binary fission under favourable conditions. On the other hand, environmental stress conditions, such as nitrogen deprivation, triggers sexual reproduction, where the vegetative cells differentiate into sexually competent gametes as minus or plus mating type. Opposite mating types initiate the fusing by intertwining flagella and then the cell wall is removed upon the secretion of the metalloprotease autolysin (Buchanan et al. 1989; Kinoshita et al. 1992). Following fusion of the cells, the diploid zygote forms a dormant structure termed a zygospore that is capable of withstanding adverse environmental conditions. Under favourable growth conditions, the zygospore germinates and divides by meiosis to form four haploid daughter cells. The daughter cells inherit the mitochondria

from the mating type minus and the chloroplast from the mating type plus. Nuclear genes follow a Mendelian inheritance pattern (Harris 2001).

The existence of haploid and diploid states in *C. reinhardtii* has greatly contributed to genetic studies. The haploid state of *C. reinhardtii* has been useful for identification and isolation of mutant strains, since both dominant and recessive mutations display a specific phenotype, whereas, the diploid state is ultimately important for classical breeding programs, to obtain a combined desirable trait, eliminate any undesirable mutations, and map mutations.

A

B

Figure 1.5 A schematic representation and microscopy image of *C. reinhardtii*.

(A) Illustrative structure of *C. reinhardtii* cell, adapted from Dent et al. (2001). Abbreviations are **N** nucleus, **Nu** nucleolus, **C** chloroplast, **T** Thylakoid membranes, **P** pyrenoid, **ES** eye-spot, **F** flagellum, **V** vacuole, **T** thylakoid membranes, **S** starch grains, and **St** stroma. (B) Light micrograph of *C. reinhardtii* cells, adapted from Protist Information Server.

Figure 1.6 The sexual reproduction cycle of *C. reinhardtii*.

Sexual reproduction starts by gametogenesis when the haploid progeny of either plus or minus mating type becomes haploid gametes and pairs with their opposite mating type. This is followed by fusion of the two cells and formation of a vegetative zygote. The vegetative zygote then forms a zygospore that germinates under favourable growth conditions. The zygospore gives rise to four haploid daughter cells – two mating type plus and two mating type minus. A small percentage of vegetative zygotes fail to initiate zygote maturation and so divide mitotically as stable diploid cells. The diagram is taken from Harris (2009).

1.3.2. Genetic manipulation tools for *Chlamydomonas reinhardtii*

Although *C. reinhardtii* has been used as a model organism to further our understanding in various research fields, there has also been considerable interest in biotechnological exploitation of this microalga. Extensive research has been carried out to develop molecular tools to allow the genetic engineering of *C. reinhardtii* for various purposes, which potentially could be also applied to other microalgae. These efforts have led to the development of numerous molecular resources for *C. reinhardtii*, and include the complete sequence of all three genomes (nuclear, chloroplast and mitochondrial), cDNA libraries, expression sequence tags (ESTs) databases, transcriptomic and proteomics information (Scaife et al. 2015). The wealth of genetic information has greatly contributed to the establishment of robust methods to transform the nucleus, chloroplast and mitochondria, representing a breakthrough in the genetic engineering of *C. reinhardtii*. DNA delivery methods include biolistics (Boynton et al. 1988; Randolph-Anderson et al. 1993), agitation with silicon carbide whiskers (Dunahay 1993), electroporation (Shimogawara et al. 1998) and *Agrobacterium*-mediated transformation (Kumar et al. 2004). One of the most commonly used methods of transformation relies on agitation of a cell wall-deficient strain with glass beads (Kindle et al. 1989; Kindle et al. 1991).

A number of selectable markers have been developed that rely on the expression of a heterologous gene that confers antibiotic resistance or the complementation of mutants that are deficient in the expression of essential genes (Table 1.2). The latter approach has become a preferred selection system for chloroplast transformation in this alga, where transformants can be selected by rescuing non-photosynthetic mutants with a cloned wild-type gene e.g. *psbH* and *atpB* (Young & Purton 2016; Wannathong et al. 2016). On the other hand, antibiotic selection is most commonly used for nuclear transformation and include markers such as the *Sh ble* gene that confers resistance to zeocin (Rasala et al. 2012; Neupert et al. 2009; Stevens et al. 1996). For the purposes of assessing transgene expression levels and identifying the subcellular location of proteins, several reporter genes have been developed such those encoding β -glucuronidase (GUS), green fluorescent protein (GFP), luciferase (*lux*) and red mCherry (Gangl et al. 2015; Scaife et al. 2015; Rasala et al. 2013), as well as codon-optimised versions of both GFP and *lux* for the chloroplast (Mayfield & Schultz 2004; Franklin et al. 2002). With recent advancements in genome editing technologies, several methods have been applied successfully to improve gene targeting of the nuclear genome in *C. reinhardtii*, and therefore allow the creation of a knockout collection (Scaife et al. 2015). The most recent method, called CRISPR-Cas9, has been used successfully in *C. reinhardtii* to create gene knockouts, thereby generating strains with improved photosynthetic productivity (Shin et al. 2016). There are other genome editing methods that have also been used successfully for targeted mutagenesis in *C. reinhardtii*, including transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) (H. Gao et al. 2014; Sizova et al. 2013).

Table 1.2 Selectable markers for *C. reinhardtii* genetic transformation.

Modified from Day & Goldschmidt-Clermont (2011).

Marker	Selection	Compartment	References
Photosynthesis			
<i>atpB</i>	Photoautotrophy	Chloroplast	(Boynton et al. 1988)
<i>tscA, psbA</i>	Photoautotrophy	Chloroplast	(Kindle et al. 1991)
<i>psaA/B</i>	Photoautotrophy	Chloroplast	(Redding et al. 1998)
<i>petB</i>	Photoautotrophy	Chloroplast	(Cheng et al. 2005)
Antibiotic resistance			
<i>Ble</i>	Zeocin	Nucleus	(Stevens et al. 1996)
<i>Aph7</i>	Hygromycin B	Nucleus	(Berthold et al. 2002)
<i>aphVIII</i>	Paromycin Kanamycin Neomycin	Nucleus	(Sizova et al. 1996)
<i>rrnL</i>	Erythromycin	Chloroplast	(Newman et al. 1990)
<i>aphA-6</i>	Kanamycin, amikacin	Chloroplast	(Bateman & Purton 2000)
<i>rrnS</i>	Spectinomycin, streptomycin	Chloroplast	(Kindle et al. 1991; Newman et al. 1990; Roffey et al. 1991)
<i>aadA</i>	Spectinomycin, streptomycin	Nucleus/ chloroplast	(Goldschmidt-Clermont 1991)
Herbicide resistance			
<i>psbA</i>	DCMU, metribuzin	Chloroplast	(Newman et al. 1992; Przibilla et al. 1991)
<i>ALS</i>	Sulphonylurea	Nucleus	(Kovar et al. 2002)
Metabolism			
<i>ARG9</i>	Arg auxotrophy	Chloroplast	(Remacle et al. 2009)
<i>codA</i>	5-fluorocytosine	Chloroplast	(Young & Purton 2016)

1.3.3. Biotechnological applications of *Chlamydomonas reinhardtii*

The exploitation of *C. reinhardtii* as a source of renewable energy has been the main focus of intense biofuel production efforts due to the fast growth rate, ease of cultivation, genetic tractability and ability to utilise sunlight, inorganic nutrients and CO₂ for the production of biological products (Ibáñez-Salazar et al. 2014; Georgianna & Mayfield 2012). The production of algal biofuels from microalgae normally exploits their ability to accumulate storage lipids, such as triacylglycerols. The storage lipid represents the main feedstock for the biofuel production, in which it can be extracted and then can be converted into biodiesel through the transesterification process (Amin 2009). Additionally, triacylglycerols can be converted into either gasoline or jet fuel through distillation or cracking (Luo et al. 2010). Efforts to genetically engineer *C. reinhardtii* to improve biofuel phenotypes have been successful (Work et al. 2010; Li et al. 2010). For example, starchless mutants defective in ADP-glucose pyrophosphorylase rerouted photosynthetic carbon flux towards TAG synthesis and so resulted in a 10-fold increase of TAG accumulation under stress (Li et al. 2010). However, *C. reinhardtii* is not an inherently exceptional producer of biomass or lipids, and therefore extensive genetic modifications are required to achieve the efficiencies that are needed for biofuel applications. Biofuel research has now mostly shifted the focus

to alternative microalgae that are naturally high producers of biomass or lipids, such as *Nannochloropsis* species, and use *C. reinhardtii* as a genetic model for those productive algal species (Rupprecht 2009). Furthermore, the focus is moving towards utilisation of *C. reinhardtii* as a production platform for other industrially relevant compounds such as therapeutic proteins and high value terpenoids. A number of high value recombinant proteins have been produced in the microalga, such as the production of a human single-chain antibody in the chloroplast (Mayfield et al. 2003). A full human monoclonal antibody was also expressed in the *C. reinhardtii* chloroplast, and was demonstrated to have antigen-binding activity similar to the same antibody expressed in mammalian cells (Tran et al. 2009). Selected examples of therapeutic proteins that have been successfully expressed in *C. reinhardtii* are summarised in Table 1.3. Most recently, Lauersen et al. (2016) reported efficient production of a high value sesquiterpenoid patchoulol from *C. reinhardtii* through metabolic engineering of the terpenoid biosynthetic pathway. Several attempts have been made to improve the carotenoid content and/or profile in *C. reinhardtii* (Cordero et al. 2011; León et al. 2007; Tan et al. 2007). A successful example of improving carotenoids in *C. reinhardtii*, the overexpression of a *phytoene synthase* gene from *Dunaliella salina*, resulted in increased levels of endogenous carotenoids such as lutein and β -carotene (Couso et al. 2011). The use of *C. reinhardtii* in wastewater treatment has also been the focus of research over the past decade. A number of studies have suggested the potential use of either immobilised *C. reinhardtii* cells in alginate beads or non-living cells as a bio-sorbent to recover heavy metals for safe disposal (Arica et al. 2005; Bayramoğlu et al. 2006).

Table 1.3 Some examples of therapeutic proteins/dsRNA expressed in the *C. reinhardtii*, modified from Stoffels (2014).

Gene Expressed	Function	Location	Applications	Reference
HSV8-lsc	Human antibody against glycoprotein from herpes simplex virus	Chloroplast	Therapeutic	(Mayfield et al. 2003)
CTB- VP1	Protein VP1 from foot and mouth disease virus fused to CTB	Chloroplast	Vaccine	(Sun et al. 2003)
CSFV-E2	Swine fever virus structural protein E2	Chloroplast	Vaccine	(He et al. 2007)
VP28	White spot syndrome virus protein 28	Chloroplast	Vaccine	(Surzycki et al. 2009)
CTB-D2	D2 fibronectin-binding domain of <i>Staphylococcus aureus</i> fused to CTB	Chloroplast	Oral vaccine	(Dreesen et al. 2010)
dsRNA-YHV*	RNA dependent RNA polymerase of shrimp yellow head virus	Nucleus	Oral vaccine	(Somchai et al. 2016)

*RNA interference approach.

1.4. Terpenoids

Terpenoids are a diverse class of naturally occurring compounds consisting of more than 50,000 structurally different chemicals, which are found in all living organisms (Pateraki et al. 2015). Many terpenoids are of plant origin, and most of them are functionally important in various aspects of cell metabolism, including photosynthesis, membrane permeability and fluidity, respiration, and regulation of growth and development (Bohlmann & Keeling 2008). Another class of plant terpenoids – often referred to as secondary metabolites – is more specialised and limited to certain plant families or genera with functions in defence against plant pathogens, abiotic stress conditions or the attraction of pollinators (Vranová et al. 2012; Marasco & Schmidt-Dannert 2008). An example of such secondary metabolites can be seen in trees of the pine family that accumulate large quantities of viscous oleoresin constituted mainly of monoterpenoids and diterpene acids as defence mechanisms against herbivores and pathogens (Zulak & Bohlmann 2010; Keeling & Bohlmann 2006). Other examples are the volatile floral and fruit scents that are normally emitted by flowering plants to attract pollinators (Gershenzon & Dudareva 2007). Due to their functional diversity, these secondary metabolic terpenoids are of great industrial interest, largely in areas such as pharmaceuticals, agricultural chemicals, flavours and fragrance additives (Bohlmann & Keeling 2008). For example, the plant derived terpenoid, paclitaxel, is used currently as a potent anticancer drug (Li et al. 2015), while the isolated essential oil from wild mint, menthol, is widely used as a flavour and fragrance additive (Caputi & Aprea 2011). Additionally, recent studies indicate that terpenoids also possess properties favourable in fuels, with potential to serve as advanced biofuel precursors (Peralta-Yahya et al. 2011; Harvey et al. 2010). The structural and functional diversity of plant derived terpenoids is illustrated in Figure 1.7.

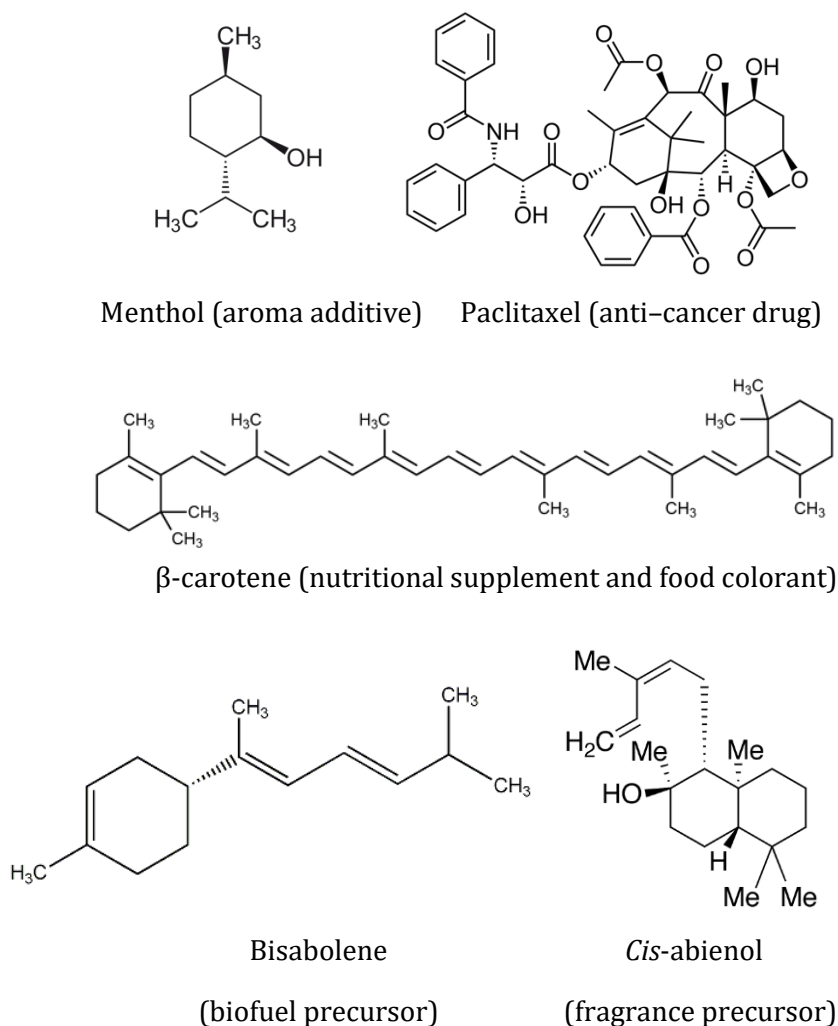


Figure 1.7 Structural diversity of industrially relevant terpenoids in wide range of applications.

1.4.1. Terpenoid biosynthetic pathways in photosynthetic eukaryotes

Despite the enormous structural diversity, all terpenoids are derived from the five carbon precursor isopentenyl diphosphate (IPP), which is assembled repetitively to produce different classes of terpenoid molecules, such as hemiterpene (C_5), monoterpene (C_{10}), sesquiterpene (C_{15}), diterpene (C_{20}), triterpene (C_{30}) and tetraterpene (C_{40}). The biosynthetic pathway of these terpenoid molecules can be divided into four main steps (Figure 1.8), as classified by Chung et al. (2016): i) the synthesis of the isoprene C_5 units (i.e. IPP) ii) the sequential addition of isoprene units to generate linear prenyl diphosphate of varying chain lengths (C_5 , C_{10} , C_{15} , ... etc), which can serve as direct precursors for the synthesis of various types of terpenoid molecules. iii) The formation of various terpene skeletal types through cyclisation reactions by a class of enzyme called terpene synthases and iv) further enzymatic modification of the terpene skeletons by downstream enzymes such as the addition of functional groups by cytochrome P450.

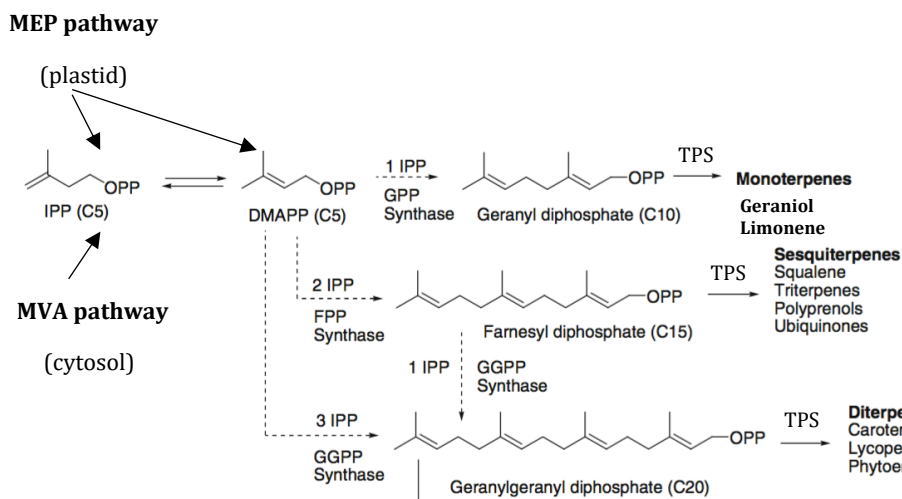


Figure 1.8 Overview of terpenoid biosynthetic pathways in photosynthetic eukaryotes.

The biosynthesis is initiated by the formation of isoprene unit (IPP) by the methylerythritol phosphate (MEP) and mevalonate (MVA) pathways if the organism has both pathways otherwise from either MEP or MVA. Head to tail condensation of DMAPP (dimethylallyl diphosphate) with one or more IPP units leads to the formation of the following direct precursors; geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). The number of added IPP units in the reactions is indicated as 1 IPP, 2 IPP and 3 IPP. Depending on the terpene synthase, these precursors lead to various monoterpene, sesquiterpenes and diterpenes, which are further modified by downstream enzymes into various terpenoid end products. TPS represents terpene synthase. The diagram was modified from Li et al. (2015).

In photosynthetic eukaryotes, the five carbon precursor IPP is synthesised by two alternative pathways; the methylerythritol phosphate (MEP) and mevalonate (MVA) pathways that are localised in different cellular compartments (Cordoba et al. 2009), as depicted in Figure 1.9. The presence of the particular pathway or even both pathways depends on the organism. For example, higher plants and diatoms possess both the MVA and MEP pathways whereas green algae have only the plastidic MEP pathway (Matsushima et al. 2012; Lohr et al. 2012; Cvejić & Rohmer 2000). More examples for the distribution of the MVA and the MEP pathways in various photosynthetic species are summarised in Table 1.4. The classical mevalonate pathway starts with the condensation of three units of acetyl-CoA in the cytosol to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is reduced by HMG-CoA reductase (HMGR) to mevalonate at the ER surface (Vranová et al. 2012). The reaction then proceeds through two successive phosphorylation steps of mevalonate and is followed by a decarboxylation step that leads to the formation of the isopentenyl pyrophosphate (IPP). The formation of its isomer DMAPP is catalysed by isopentenyl diphosphate isomerase (IDI). The isoprene C5 units derived from this pathway provide precursors for the formation of cytosolic and mitochondrial terpenoids such as sesquiterpenes and triterpenes (Bohlmann & Keeling 2008).

On the other hand, plastidic terpenoids are derived from the MEP pathway located in the chloroplast of photosynthetic eukaryotes and it is used for the formation of monoterpenes, diterpenes and tetraterpenes (Vranová et al. 2012; Bohlmann & Keeling 2008). The MEP pathway starts from the condensation of pyruvate and glyceraldehyde 3-phosphate (GA-3P) to form 1-deoxy-D-xylulose-5-phosphate (DXP), catalysed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). The next step involves the intramolecular rearrangement and reduction of DXP to produce MEP, which is catalysed by DXR. The resulting MEP is converted into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) through three enzymatic steps and then followed by a reduction catalysed by 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase (HDS) to produce 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP). In the last step in the MEP pathway, the reaction is catalysed by the isopentenyl diphosphate: dimethylallyl diphosphate synthase (IDS) to produce both IPP and DMAPP as final products (Roberts 2007). In spite the fact that both IPP and DMAPP are made, the IDI enzyme is also found in the MEP pathway presumably to maintain a balanced supply of IPP and DMAPP (Lohr et al. 2012).

Figure 1.9 Biosynthesis of isopentenyl diphosphate in photosynthetic eukaryotes with the cytosolic MVA pathway and the plastidic MEP pathway.

The MVA pathway begins with condensation of acetyl-coenzyme A (CoA) units to generate the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), whereas the MEP pathway begins with the condensation of pyruvate and D-glyceraldehyde 3-phosphate, see the text for further explanations. Transporter (TP) is responsible to transport the isomeric C-5 precursors and prenyl diphosphate across membranes. Dashed arrows indicate a number of steps are involved (not shown). Abbreviations: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HDS, hydroxy-2-methyl-2-(E) butenyl 4-diphosphate synthase; IDS, isopentenyl diphosphate: dimethylallyl diphosphate synthase; IDI, isopentenyl diphosphate isomerase. The diagram is taken from Roberts (2007).

Table 1.4 The distribution of MVA and MEP pathways in some photosynthetic species. This table was modified from Lohr et al. (2012).

Photosynthetic species	Pathway		Reference
	MEP	MVA	
<i>Synechocystis</i> sp. PCC 6714	+	–	(Disch et al. 1998)
<i>Chlamydomonas reinhardtii</i>	+	–	(Lohr 2009)
<i>Chlorella fusca</i>	+	–	(Disch et al. 1998)
<i>Euglena gracilis</i>	+	+	(Disch et al. 1998)
<i>Phaeodactylum tricornutum</i>	+	+	(Cvejić & Rohmer 2000)
<i>Nannochloropsis gaditana</i>	+	–	(Radakovits et al. 2012)
<i>Cyanidium caldarium</i>	+	+	(Disch et al. 1998)
<i>Cyanidioschyzon merolae</i>	+	–	Genome Project ^{&}
<i>Nitzschia ovalis</i>	+	+	(Cvejić & Rohmer 2000)
<i>Tetraselmis striata</i>	+	–	(Schwender et al. 2001)
<i>Arabidopsis thaliana</i> [‡]	+	+	(Lichtenthaler et al. 1997)

[&] Genome project (<http://merolae.biol.s.u-tokyo.ac.jp/>).

[‡] All land plants that have been investigated to date have both pathways

1.4.2. Applications of terpenoids

Many terpenoids accumulate in small quantities in plant tissue, but direct extraction from the plant is not sufficient for commercial production. The biotechnological applications of these terpenoids in various industrial fields has led to an increased interest in understanding the many terpenoid biosynthetic pathways, with a view to enabling sustainable production of terpenoids based on renewable resources (Schrader & Bohlmann 2015; Bohlmann & Keeling 2008). Only by understanding the underlying biosynthetic pathways required to produce terpenoids, can genetic engineering be plausible and potentially facilitate genetic manipulation to produce terpenoids on a commercially viable scale. Pathway manipulation can be undertaken either in their natural host, or in other biological platforms (Li et al. 2015). These attempts are briefly discussed in this section by highlighting the progress being made for selected examples of terpenoids with biotechnological applications. Finally, the current and emerging options for the commercial production of those selected terpenoids will be reviewed.

1.4.2.1 Ambroxide-related diterpenoids

Several naturally occurring diterpenoids of plant origin have been used as starting materials for the synthesis of fragrance molecules with ambergris characters, such as Ambrox®. This semi-synthesis process was originally developed to substitute the use of a biliary secretion of the sperm whales known as ambergris in perfume formulations (Zerbe & Bohlmann 2015). The diterpene alcohol, *cis*-abienol, is an example of a major aromatic oleoresin component from balsam fir (*Abies balsamea*) that serves as a starting material for the industrial synthesis of Ambrox® (Zerbe et al. 2012). Another example is the diterpene diol, mainly obtained from clary sage plants (*Salvia sclarea*) and it is also used as a precursor for Ambrox® or related ambroxide fragrance synthesis (Zerbe & Bohlmann 2015). There is other additional plant derived diterpenoids that could potentially serve as a precursor for the commercial production of Ambrox®, such as labdanolic acid, abietic acid and manool, as illustrated in Figure 1.10. The use of plant-derived terpenoids as starting materials for the fragrance industry, as is the case for many naturally-derived products, is hindered by low yields, high extraction costs and further expensive chemical synthesis requirements (Cordoba et al. 2009).

Given the high value of these diterpenoids, there has been a focus on the identification of the relevant biosynthetic genes and corresponding enzymes which may be genetically engineered to improve production of high value terpenoids (Zerbe et al. 2012; W. Gao et al. 2014; Bruckner et al. 2014). The identification and functional annotation of diterpene synthases that are involved in sclareol biosynthesis resulted in successful production of sclareol in engineered yeast, achieving approximate titres of 400 mg/L (Ignea et al. 2015).

In addition, genetically engineered *E. coli* producing high titres of sclareol up to 1.5 g/L (Schalk et al. 2012) demonstrates a sustainable and cost effective platform. These successful engineered microbial sclareol production systems have been facilitated by the fact that the biosynthesis of sclareol does not require oxidation steps catalysed by P450 enzymes. Transcriptome analysis of balsam fir identified a bifunctional diterpene synthase that produces *cis*-abienol, a terpene of value to the fragrance industry. Given the slow growing and low yielding nature of balsam fir, there exists an opportunity for metabolic engineering in more a feasible expression system for the commercial production of *cis*-abienol (Zerbe et al. 2012).

Figure 1.10 The semi-synthetic route for the production of Ambrox®.

Several plant species produce diterpenoids that serve as a starting material for the synthesis of Ambrox® of high value in fragrance industry, adapted from Zerbe & Bohlmann (2015).

1.4.2.2 Nootkatone

The sesquiterpenoid (+)-nootkatone is a high value aromatic compound that was first isolated from the heartwood of Alaska yellow cedar (*Chamaecyparis nootkatensis*) and later discovered to exist in trace amounts in grapefruit (*Citrus paradisi*), mandarin (*Citrus reticulata*) and pummelo (*Citrus grandis*) (Leonhardt & Berger 2015). It is characterised by the grapefruit odour, a slightly bitter taste and an extremely low odour threshold of approximately 1 µg/L water. These characteristics led to the exploitation of (+)-nootkatone as a flavour and fragrance compound for the food and cosmetic industries (Leonhardt & Berger 2015; Caputi & Aprea 2011). The sesquiterpenoid (+)-nootkatone also possesses insecticidal properties as reported by Miyazawa et al. (2000), where an extract of (+)-nootkatone from *Alpinia oxyphylla* demonstrated insecticidal activity against *Drosophila melanogaster*. Another study reported the use of (+)-nootkatone as a strong repellent and toxicant to termites (*Coptotermes formosanus*), thereby opening the possibility of using this sesquiterpenoid to target other insects such as tick and flea species (Flor-Weiler et al. 2011; Zhu et al. 2001). Interestingly, (+)-nootkatone and its derivatives have also been reported to exhibit therapeutic activities, such as antiplatelet aggregation effects (Seo et al. 2011) and anti-proliferative activity against some cancer cell lines (Gliszczyńska et al. 2011).

However, direct extraction of (+)-nootkatone from its natural sources is not sufficient to meet the industrial demand, mainly due to i) the fact that few species can produce (+)-nootkatone, ii) slow biomass accumulation, iii) poor recovery after extraction and iv) overall low concentrations of (+)-nootkatone (Leonhardt & Berger 2015; Fraatz et al. 2009). The current synthetic route for (+)-nootkatone production relies upon the oxidation of the abundantly available sesquiterpene (+)-valencene, a constituent of orange and mandarin oil (Figure 1.11). This oxidation process normally uses oxidizing agents known to be unsafe such as the carcinogen, chromic acid (Salvador & Clark 2002; Wilson & Shaw 1978). As a result, there is an increasing interest in alternative approaches such as *de novo* (+)-nootkatone synthesis, or the bioconversion of (+)-valencene to (+)-nootkatone (Wriessnegger et al. 2014; Gliszczyńska et al. 2011; Furusawa et al. 2005).

Many studies have reported the possibility of bioconverting (+)-valencene to (+)-nootkatone using whole cell extracts of bacteria, fungi, microalgae and plants (Wriessnegger et al. 2014; Fraatz et al. 2009; Furusawa et al. 2005). The knowledge gained from the bioconversion of (+)-valencene has been utilised for the identification of the enzymes responsible for the production of this compound, and in turn has facilitated its production in different expression systems via metabolic engineering. As such, the use of lyophilisates of the edible mushroom *Pleurotus sapidus* for the bioconversion of (+)-valencene produced more than 250 mg/L of (+)-nootkatone. The oxygenase responsible for such catalytic activity from *P. sapidus* has been identified, and the gene successfully

expressed in *E. coli* (Zelena et al. 2012; Fraatz et al. 2009). Another study screened a large number of cytochrome P450 enzymes from bacteria for their oxidizing activity, and a cytochrome (CYP109B1) from *Bacillus subtilis* was reported to be the most suitable enzyme. This enzyme might be used for the production of (+)-nootkatone, (Girhard et al. 2009). Most recently, Wriessnegger et al. (2014) reported the development of a sustainable route for the production of (+)-nootkatone *in vivo* by metabolically engineered *P. pastoris*, achieving approximate titres of 208 mg/L of cell culture. This was achieved by a combinatorial approach, involving the co-expression of genes for valencene synthase from *Callitropsis nootkatensis*, premnaspirodiene oxygenase from *Hyoscyamus muticus* and cytochrome P450 reductase from *Arabidopsis thaliana*. The successful production of (+)-nootkatone in *P. pastoris* is a promising step to make (+)-nootkatone production practical and environmentally friendly, but there is a need for the development of more cost-effective bioprocess technologies.

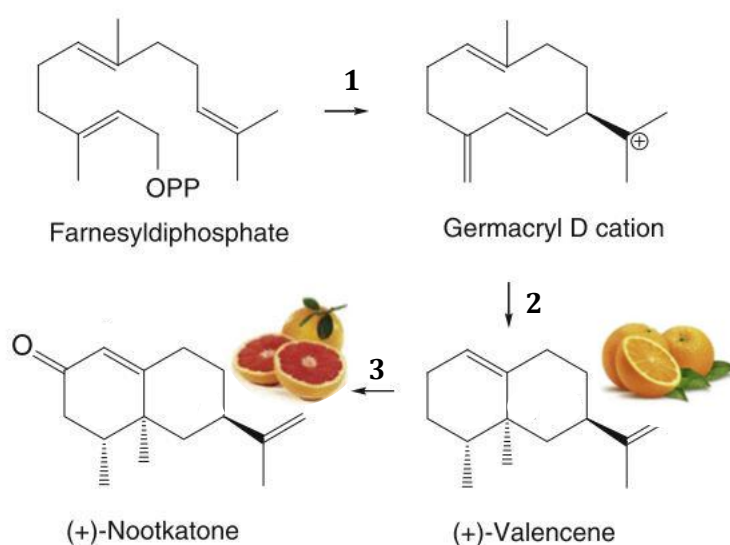


Figure 1.11 The proposed biosynthetic process for (+)-nootkatone from farnesyl diphosphate (C15), modified from Leonhardt & Berger (2015).

1.4.2.3 Carotenoids

Carotenoids constitute a large group of naturally occurring terpenoid pigments with antioxidant properties that are mainly synthesised by plants, algae and photosynthetic bacteria (Sandmann 2015; Fraser & Bramley 2004). Carotenoids are widely responsible for the yellow, orange and red pigments seen in fruits, vegetables, flowers and algae. A number of carotenoids are of high value to humans as food colorants, animal feed and nutraceuticals (Gong & Bassi 2016; Sandmann 2015). These high value carotenoids are shown in Figure 1.12. The best established role of carotenoids in human health is in pro-vitamin activity, where dietary carotenoids, in particular β -carotene, are a precursor for vitamin A which is required for the visual pigment rhodopsin (Sandmann 2015; Fraser & Bramley 2004). Since humans cannot synthesise carotenoids, a deficiency of pro-vitamin A in their diet will ultimately lead to a deficiency in vitamin A, which in turn may cause blindness and even death in severe cases (Black et al. 2016). In addition to β -carotene, other carotenoids are also important for human vision, including zeaxanthin and lutein. A deficiency of these carotenoids may result night blindness, exophthalmos and keratinization of the conjunctiva (SanGiovanni & Neuringer 2012; Bone et al. 2003; Britton 1995). Due to the anti-oxidative properties of carotenoids, several carotenoids are also reported to benefit human health in some chronic diseases (Gong & Bassi 2016), as summarised in Table 1.5.

Figure 1.12 Simplified diagram for the structures and biosynthesis of industrially relevant carotenoids.

The immediate precursor for tetraterpenes including carotenoids is formed by the condensation of two geranylgeranyl pyrophosphate (GGPP), and followed by several enzymatic reactions to form various carotenoids. The colour of the corresponding carotenoid is shown as a coloured circle. The dotted arrow indicates the chemical conversion. This diagram was modified from Sandmann (2015).

Table 1.5 Reported human health benefits of carotenoids, adapted from Gong & Bassi (2016).

Carotenoids	Health benefits	Reference
Astaxanthin	Strong anti-oxidant property Anti-inflammatory effects Anti-cancer Cardiovascular health	(Fasano et al. 2014) (Pashkow et al. 2008) (Chew et al. 1999)
Lutein	Prevents cataract and age-related macular degeneration Anti-oxidant property Anti-cancer Prevents cardiovascular diseases	(Manayi et al. 2016) (Granado et al. 2003) (Bone et al. 2003) (Cha et al. 2008) (Vijayapadma et al. 2014) (Alves-Rodrigues & Shao 2004)
β -carotene	Prevent night blindness Anti-oxidant property Prevents liver fibrosis	(Dufossé et al. 2005) (Virtamo et al. 2014) (Shaish et al. 2006)
Lycopene	Anti-cancer Prevents cardiovascular diseases Radiation protection Anti-oxidant property	(Viuda-Martos et al. 2014) (Srinivasan et al. 2009) (Devasagayam et al. 2004)

As a result of the identified health benefits of carotenoids, there has been a focus upon improving the levels of human consumption of carotenoids. One successful method has been by the genetic manipulation of carotenoid biosynthetic pathways in staple crops. The most common example is “Golden rice”, where the co-expression of genes for phytoene synthase and carotene desaturase in rice endosperm resulted in transgenic rice with a high level of β -carotene (Paine et al. 2005). The staple crop potato, which typically accumulates lutein and violaxanthin, was also targeted for genetic engineering to accumulate zeaxanthin as another important dietary pro-vitamin (Omer et al. 2002).

In addition to fortification of staple crops, carotenoids have been used as food additives in the aquaculture and poultry industries. Crustaceans and pink-fleshed fish such as salmon and trout contain astaxanthin as a result of addition of astaxanthin in the diet (Cardozo et al. 2007). Similarly the addition of carotenoids in poultry feed enhances the nutritional value of eggs as well as the pigmentation of the egg yolk (Sandmann 2015; Tyczkowski & Hamilton 1986). Because of the colour of carotenoids, from yellow to red, these compounds have been exploited as natural colorants in the food industry and in cosmetics. For instance, the use of water soluble norbixin (annatto) provides the yellow pigmentation in dairy products, while lycopene or canthaxanthin provides red pigmentation in food and beverages (Sandmann 2015).

Carotenoids are synthesised chemically to meet increasing demand for these compounds in various applications (Ernst 2002). However, the chemical synthesis process is limited to producing only a few carotenoids; namely β -carotene, astaxanthin, canthaxanthin, lycopene and zeaxanthin. Some natural carotenoids are also commercially available, and are derived from plants and algae such as lutein from the flowers of marigolds, lycopene from tomato fruit, β -carotene from carrot roots, and astaxanthin from *Haematococcus pluvialis*

(Sandmann 2015). Currently, there is an interest in the production of carotenoids by genetic manipulation of microorganisms. These offer a promising alternative for sustainable production of natural carotenoids (Gimpel et al. 2015; Das et al. 2007). In efforts to make this possible, many studies attempted the synthesis of carotenoids in metabolically engineered *E. coli*, reporting concentrations of 6.2 mg/g dry weight β -carotene, 1.4 mg/g dry weight astaxanthin and around 30 mg/g dry weight lycopene (Chen et al. 2013; Lemuth et al. 2011). In addition, engineering non-carotenogenic fungi for the production of carotenoids (for instance, the genetic manipulation of *P. pastoris*) resulted in successful production of lycopene and β -carotene (Araya-Garay et al. 2012). The yeast *Xanthophyllomyces dendrorhous* has also been targeted for genetic engineering in order to improve astaxanthin productivity, as it already has the ability to produce astaxanthin naturally. Combining classical mutagenesis with genetic engineering has resulted in a strain with an improved productivity of astaxanthin, achieving in total approximately 9 mg/g dry weight (Gassel et al. 2014).

1.5. Metabolic engineering of terpenoid biosynthetic pathways in algae

In spite of the major success of plant cell suspension culture in the production of numerous terpenoids in particular high-value pharmaceutical terpenoids such as anti-cancer Taxol® and anti-malarial artemisinin, this approach suffers from low yield and variability of accumulated terpenoids, which in turn complicate the regular supply of these high value compounds at large scale (Ikram et al. 2015; Roberts 2007). Recent genetic engineering efforts therefore have been directed towards finding alternative production platforms for terpenoids other than plants, as genetic manipulations of whole plants often requires in-depth understanding of the metabolic system and regulatory complexity to enable the successful manipulation of the biosynthetic pathway without disturbing other essential processes within the plant (Ikram et al. 2015; Saxena et al. 2014). Terpenoids are indeed important in higher plants for their growth, development and survival (Bohlmann & Keeling 2008). Many studies have demonstrated that genetic manipulations of terpenoid biosynthetic pathways in higher plants is possible, however, these attempts are normally associated with challenges including growth retardation and low yield of the desired terpenoid (Saxena et al. 2014; Farhi et al. 2011). Microalgae may offer an alternative platform for terpenoid production due to their inherently desirable characteristics, in which they combine the high growth rate and ease cultivation of microorganisms with an ability to perform the post-transcriptional and post-translation modifications of plants (Scaife et al. 2015; Gangl et al. 2015). In addition, microalgae have the natural capacity to produce the precursor molecules for bioactive compounds such as terpenoids and fatty acids (Georgianna & Mayfield 2012). As a matter of fact, several algal species have a remarkable capability to accumulate industrially relevant terpenoids under unfavourable condition, as

the case for β -carotene in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis* (Lohr et al. 2012; Eonseon et al. 2006; Ramos et al. 2011). In addition, microalgae have other advantages over higher plants including their rapid growth, the speed of genetic manipulation in term of generating transgenic lines, their potential low cost for large scale production and most importantly their ease of containment in closed photobioreactors (Mayfield et al. 2007).

The genetic manipulation of microalgae as a production platform for terpenoid compounds is fundamentally very similar to that of higher plants, where the metabolic engineering of the pathways can be achieved by inserting transgenes into the nuclear or chloroplast genomes (Gimpel et al. 2015; Kempinski et al. 2015). Nuclear transformation offers the advantage of post-translational modifications and the possibility of targeting the heterologous protein into different subcellular compartments (Purton 2007; Franklin & Mayfield 2004), and indeed most of involved enzymes in terpenoid metabolism in the chloroplast are nuclear encoded (Gimpel et al. 2015). However, the process of transgene integration into the nuclear genome of most algae is essentially random leading to many difficulties associated with positional effects. These include a significant variation in the expression levels of the nuclear transgene and suppression of transgene expression, which is often known as a gene silencing (Franklin & Mayfield 2004). As a consequence, protein accumulation is typically lower compared to transgene expression in the chloroplast where these problems do not arise. Chloroplast transformation proceeds through homologous recombination allowing the precise integration of a transgene into any loci within the chloroplast genome, and therefore, avoids unpredictable integration and the disruption of essential endogenous genes (Purton 2007; Franklin & Mayfield 2004). In addition, the polyploid nature of the chloroplast genome results in a high copy number of the transgene, thereby aiding high level expression. Chloroplast transformation also offers the possibility to express multiple transgenes as an operon (Purton 2007).

Current research has investigated the possibility of exploiting microalgae as cell factories for the production of terpenoids through metabolic engineering by increasing the productivities of the desired terpenoids, improving terpenoids profiles and producing designer terpenoid molecules (Gimpel et al. 2015; Lohr et al. 2012). Since most terpenoid metabolism in microalgae occur in the chloroplast, metabolic engineering has been achieved by nuclear and chloroplast transformation. At present, the primary focus of algal terpenoid metabolism is the metabolic engineering of carotenoid biosynthetic pathways in the model microalga, *C. reinhardtii* (Gimpel et al. 2015). Various approaches have been employed to engineer carotenoid pathways in *C. reinhardtii* by either targeting different endogenous enzymes or introducing new enzymes (Figure 1.13). One such approach relies on the overexpression of genes for rate-limiting endogenous enzymes to increase the

accumulation of the desired product. For example, separate studies successfully demonstrated that the expression of an additional copy of the phytoene synthase from either *D. salina* or *Chlorella zofingiensis* resulted in a significant increase in carotenoid content of the transgenic *C. reinhardtii* (Cordero et al. 2011; Couso et al. 2011), see Figure 1.14. Similarly, the expression of an additional copy of a mutant version of the gene for the endogenous phytoene desaturase in *C. reinhardtii* resulted in a significant increase in carotenoid content (Liu et al. 2013). Another approach was attempted in *C. reinhardtii* using anti-sense RNA to down regulate the endogenous phytoene synthase, however, such manipulation did not result in dramatic changes in the carotenoid profile (Vila et al. 2008). Fukusaki et al. (2003) reported an indirect approach to improving the productivity of carotenoids content in *C. reinhardtii* through increasing the available precursor pool of GGPP by the expression of an additional copy of the GGPP synthase gene from a thermophilic archaeon. However, the additional activity of GGPP synthase in *C. reinhardtii* did not result in measurable changes in the terpenoid profile. As for the introduction of novel enzymes to direct the metabolic flux towards the production of industrially relevant carotenoids, an engineered *C. reinhardtii* expressing a β -carotene ketolase cDNA from *H. pluvialis* resulted in the synthesis of an unexpected novel ketocarotenoid (León et al. 2007).

The genetic manipulation of terpenoids in *C. reinhardtii* is not limited to the carotenoid pathways and there is growing interest in introducing new pathways to harness microalgae in general as cell factories for the production of novel terpenoids. Most recently, Lauersen et al. (2016) reported the successful production of the non-native sesquiterpenoid patchoulol from FPP in engineered *C. reinhardtii* with a patchoulol synthase from the plant *Pogostemon cablin*, achieving productivities up to 922 ± 242 $\mu\text{g/g}$ dry weight in six days. Another study attempted to introduce a bifunctional diterpene synthase from balsam fir into the *C. reinhardtii* chloroplast, where this enzyme catalyses the formation of industrially relevant *cis*-abienol 4 from GGPP (Zedler et al. 2014). This study could not detect *cis*-abienol 4 in spite of the successful heterologous expression of the diterpene synthase gene.

In addition to metabolic engineering attempts using *C. reinhardtii*, there is ongoing efforts to improve the productivity of other biotechnologically relevant microalgae, such as *H. pluvialis* which is the leading commercial producer of natural astaxanthin. A study reported successful stable transformation of *H. pluvialis* using a mutant *phytoene desaturase* gene that confer resistance to norflurazon (Steinbrenner & Sandmann 2006). The additional copy of the mutant gene improved the accumulation levels of astaxanthin in the transformant lines up to 26% compared to the wild type after 48 hours of being stressed with high light. Another study reported a similar approach in *Chlorella zofingiensis* where the transformant lines showed an improved accumulation levels of total carotenoids and astaxanthin up to approximately 32 % and 54%, respectively (Liu et al. 2014).

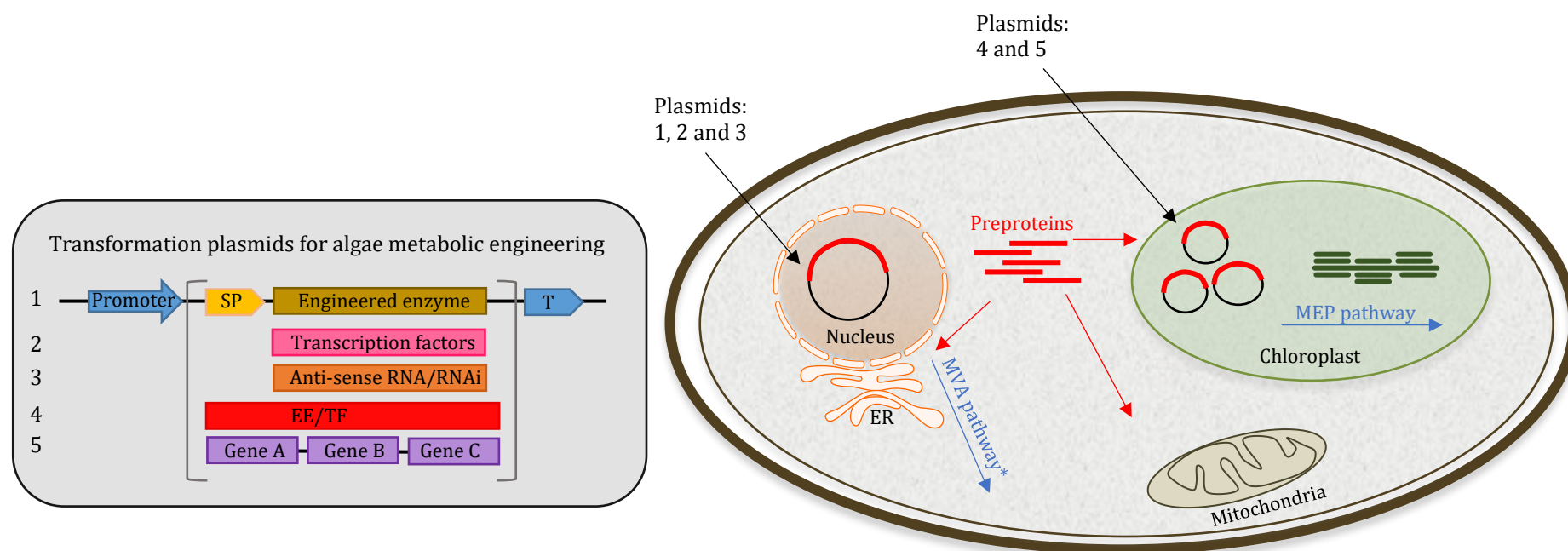


Figure 1.13 Schematic representation of various possible approaches to metabolic engineering of terpenoid biosynthetic pathways in algae.

Genetic engineering of the nuclear genome is carried out by transformation plasmids (1, 2 and 3). The addition of the signal peptide (SP) allows targeting of the proteins into different cellular compartments including the chloroplast, mitochondria and endoplasmic reticulum (ER), as indicated by red arrows. The genetic engineering of the chloroplast genome directly is possible by plasmid 4, which could carry genes either for an engineered enzyme (EE) or transcription factors (TF). Plasmid 5 contains multiple transgenes that could be expressed as an operon in the chloroplast genome. The genetic engineering of terpenoid pathways using transcription factors and multiple transgenes approaches have been reported in the genetic engineering of plants but not in algae. Terminator is abbreviated by T. Asterisk (*) indicates the existence of the MVA pathway depending on the algal species, although this is not found in *C. reinhardtii*.

Figure 1.14 Biosynthetic pathway for astaxanthin in green microalgae.

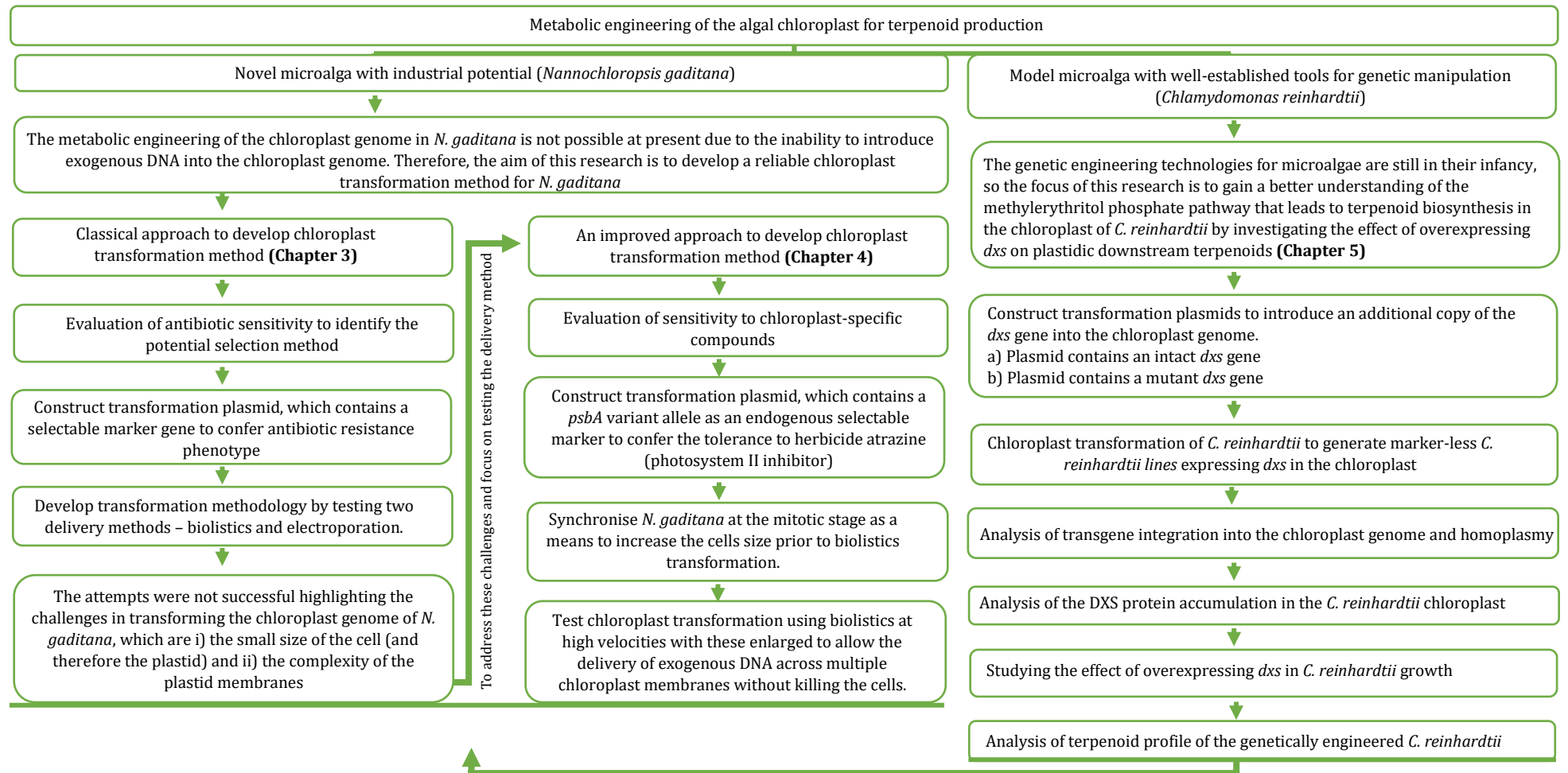
Targeted enzymes for genetic engineering in microalgae are indicated by asterisks. The abbreviations are: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ -carotene desaturase; LYC-B, lycopene β -cyclase; BKT, β -carotene ketolase; CrtR-b, β -carotene hydroxylase. Adapted from Gimpel et al. (2015).

1.6. Aims of the presented work

With recent interest in the use of terpenoids in a wide range of industrial and pharmaceutical applications, many efforts have focused on the commercial production of these high value compounds in novel biological systems using metabolic engineering technologies. As a photosynthetic platform, microalgae may be considered as an attractive alternative to their counterpart plants for terpenoid production due to their minimal growth requirements, their fast growth rate, their containment and potential low cost for large scale production. However, metabolic engineering technologies for microalgae are still in their infancy, and there is a need to develop engineering tools for those novel algal species with industrial potential such as *Nannochloropsis*. At the same time, new strategies for engineering terpenoids need to be tested in established laboratory species such as *Chlamydomonas reinhardtii*. The aims of the research described in this thesis are therefore:

1. The development of a reliable chloroplast transformation method for *N. gaditana* so that the genetic engineering of the plastidic terpenoid pathway would be possible. This work includes:
 - Testing different transformation methods that are commonly employed in the chloroplast transformation of other microalgae (Chapter 3).
 - Exploring new transformation strategies that address some of the uncertainties of chloroplast transformation, focusing on the development of an endogenous selectable marker and the temporary increase in cell size to facilitate the delivery of DNA into the small chloroplast (Chapter 4).
2. The genetic manipulation of the terpenoid biosynthetic pathway in the *C. reinhardtii* to investigate the effect of overexpressing a gene for the rate limiting DXS enzyme on downstream terpenoid levels, and so gain a better understanding of terpenoid biosynthetic pathways in *C. reinhardtii* for further genetic engineering studies (Chapter 5).

1.7. An overview of the presented work and hypothesis



Hypothesis: the genetic manipulation of the plastidic terpenoid biosynthetic pathway in the model microalga *C. reinhardtii* will further our understanding about terpenoid metabolism and rate-limiting steps, which is important for successful terpenoid metabolic engineering. Engineering strategies developed could also be transferred into other microalgae with industrial potential such as *N. gaditana* upon the development of plastome engineering tools including a reliable chloroplast transformation method.

CHAPTER 2

Materials and Methods

2. Materials and Methods

2.1. Strain and growth conditions

2.1.1. *Escherichia coli*

2.1.1.1 *Escherichia coli* strain

Escherichia coli DH5 α (F-, (ϕ 80*lacZ* Δ M15), Δ (*lacZYAargF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk-, mk+), *supE44*, *thi-1*, *gyrA96*, *relA*, λ) was used as a recipient strain for molecular cloning. The *E. coli* strain was supplied by Clontech (Saint-Germain-en-Laye, France).

2.1.1.2 *E. coli* maintenance and growth

E. coli strains were grown on Luria-Bertani (LB) medium, containing 10 g/L bacto-tryptone (Difco), 5 g/L bacto-yeast extract (Difco) and 10 g/L NaCl supplemented with appropriate antibiotics when necessary. Liquid cultures were grown overnight at 37°C in a shaking incubator at 200 rpm. For growth on solid medium, the strain was plated on 1.5% (w/v) LB agar and incubated at 37 °C for 24 hours (Fisher Scientific Incubator). For long-term storage, frozen stocks were prepared by mixing 100 μ l of overnight *E. coli* culture and 100 μ l 50% (v/v) glycerol, and were stored at -80 °C.

2.1.2. *Nannochloropsis gaditana*

2.1.2.1 Strain and growth conditions

Nannochloropsis gaditana CCMP526 was kindly provided by Dr. Matthew Posewitz and was cultivated photoautotrophically in defined artificial seawater medium (ASW). The ASW was prepared as follows: 15 g/L NaCl, 6.6 g/L MgSO₄·7H₂O, 5.6 g/L MgCl₂·6H₂O, 0.5 g/L CaCl₂·2H₂O, 1.45 g/L KNO₃, 0.12 g/L KH₂PO₄, 0.04 g/L NaHCO₃, 0.01 g/L FeCl₃·6H₂O, 0.035 g/L Na₂-EDTA, 0.25 ml/L 3.64 mM MnCl₂·4H₂O, and 0.5 ml/L trace metal mix (20 mg/L CoCl₂·6H₂O, 12 mg/L Na₂MoO₄·2H₂O, 44 mg/L ZnSO₄·7H₂O, 20 mg/L CuSO₄·5H₂O, 7.8 g/L Na₂-EDTA). The pH of the trace metal mix was adjusted to 7.5 and the final pH of the ASW was adjusted to 7.3. The cultures were grown in liquid ASW under continuous light intensity of 30–80 μ E with constant shaking at 120 rpm and at 25 °C (Innova 4340 incubator shaker, Germany). Agar plates were prepared by adding 2% (w/v) Difco™ agar (Becton, Dickinson company, USA). A loopful of culture was streaked on ASW 2% (w/v) agar plates and maintained at light intensity of approximately 20–50 μ E at 25 °C.

2.1.3. *Chlamydomonas reinhardtii*

2.1.3.1 *Chlamydomonas reinhardtii* strains

The *C. reinhardtii* strains used are shown in Table 2.1 and maintained in either an illuminated incubator or a culture room in the Purton laboratory.

Table 2.1 Summary of the *C. reinhardtii* strains used in this study

Strain	Description	Source /Reference	Purpose
TN72	Cell wall deficient and non-photosynthetic <i>psbH</i> mutant	(Wannathong et al. 2016)	Recipient strain for chloroplast transformation of <i>C. reinhardtii</i>
H1	TN72 transformed with empty chloroplast transformation vector (pSRSapI), restoring <i>psbH</i> function.	(Young & Purton 2014)	Control strain for growth analysis, Western blot analysis, enzyme activity assays and HPLC analysis
L1	TN72 transformed with pRY134a. The strain has an additional copy of the <i>dxs</i> gene.	Dr Rosie Young	Transgenic lines that were created to study the effect of overexpressing the <i>dxs</i> gene in downstream terpenoids metabolites in the <i>C. reinhardtii</i>
L3-L5	TN72 transformed with pRY134a. The strains have an additional copy of the <i>dxs</i> gene.	This study	
dl1-dl2	TN72 transformed with pRY134aΔ <i>dxs</i> . The strains have an additional, but mutated, copy of <i>dxs</i>	This study	

2.1.3.2 *Chlamydomonas reinhardtii* growth conditions and maintenance

C. reinhardtii strains were cultivated heterotrophically on tris-acetate phosphate (TAP) medium. The TAP was prepared by mixing three main stock solutions as follows: 4 x Beijerinck salts stock solution (0.3M NH₄Cl, 14mM CaCl₂·2H₂O and 16mM MgSO₄·7H₂O), 1M (K)PO₄ stock solution (1M K₂HPO₄ titrated to pH 7.0 with 1M KH₂PO₄) and trace metal mix stock solution (180 mM H₃BO₃, 77mM ZnSO₄·7H₂O, 26mM MnCl₂·4H₂O, 18 mM FeSO₄·7H₂O, 7mM CoCl₂·6H₂O, 6mM CuSO₄·5H₂O, 0.1M Na₂EDTA, 0.9mM (NH₄)₆Mo₇O₂₄·4H₂O).

All *C. reinhardtii* strains, except photosynthetic mutant strain (TN72), were grown in a shaking incubator, under continuous illumination of intensity approximately 80–100 μE, at 25 °C (New Brunswick™ Innova, Germany). The strains were maintained on 2% (w/v) agar TAP in an illuminated incubator at 80–100 μE at 25 °C. The photosynthetic mutant strain (TN72) was grown shaking under dim light conditions of approximately 5–10 μE at 25 °C and maintained in a culture room at the same conditions. The *C. reinhardtii* strains were sub-cultured every six to eight weeks as suggested by Harris, (1988).

Sueoka's high salt minimal (HSM) medium plates were used to select for *C. reinhardtii* transformants. The preparation of HSM is similar to TAP except that the 1 M (K)PO₄ stock is replaced by 2 x PO₄ stock solution (80mM K₂HPO₄ and 50mM KH₂PO₄, adjusted to pH 6.9 with KOH) and no acetic acid is added.

2.2. Quantification of cell density

The cell density of microalgae was counted by eye under a light microscope (x40 objective) at x400 magnification using a haemocytometer (Axio Scope. A1, Zeiss). In order to count the *C. reinhardtii* cells, they were first treated with 10 µl tincture of iodine (19.7 mM iodine in 95 % (v/v) ethanol) per 1 ml sample to immobilize the cells and facilitate counting. The addition of iodine solution was not necessary to count *N. gaditana* cells, due to the absence of flagella. The number of cells per ml of culture was calculated as follow: average cell count x dilution factor x 10⁴. The optical density of cells was measured at 750 nm with a 1 cm path length using a spectrophotometer (Unicam UV/Vis Spectrometer UV2).

2.3. Growth analysis

A commercial lab scale photobioreactor known as an Algem® (Algenity, UK) was used either to investigate the effect of different parameters on the growth rate of *N. gaditana* or to compare the growth rate among different strains of *C. reinhardtii*. To study the effect of growth conditions on *N. gaditana*, the Algem® was set up to expose the culture (approx. 500 ml) in two chambers simultaneously to different conditions by altering a physical parameter such as the light regime, light intensity, light wavelength or temperature. In contrast, to compare the growth rates between different *C. reinhardtii* strains, the comparison was carried out by growing the strains under identical conditions; a constant temperature of 25 °C, a constant light intensity of 200 µE and constant mixing at 120 rpm. The cell growth for both species of microalgae was monitored by measuring the optical density at 740 nm and the readings were saved automatically every 10 minutes. The recorded data were later retrieved for graph plotting and data analysis using the instrument's built-in software when necessary or using Microsoft Excel.

2.4. Sensitivity test

Sensitivity of *N. gaditana* to various compounds, as tabulated in the appendices (Appendix.1), was evaluated initially by spotting a small volume (5 µl) of culture onto nutrient agar plates (so-called "spot tests"). The spot test is based on measuring algal growth in the presence of the tested compound, in comparison to the growth of a control. The cells were re-suspended in ASW medium and then diluted in ten-fold dilutions (0, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴). The suspension of diluted cells was pipetted onto 2% (w/v) ASW agar supplemented with one of the following concentrations: 0, 50, 100, and 200 µg/ml of the tested compound. For those compounds which inhibited the growth, a high density of cell suspension (> 5x10⁴ cells/plate) was plated on 2% (w/v) agar ASW plates supplemented with the corresponding compound to assure the consistent inhibition of growth at high density as well. The plates were maintained at a light intensity of 20–50 µE at 25 °C.

A further test to confirm the effect of a particular compound was carried as follows: the *N. gaditana* was cultured in liquid ASW medium to log phase (OD₇₅₀ 0.6–0.8) and then the culture was aliquot into a 12 well microplate; 3 ml in each well. The tested compound was added to each well to give a range of concentrations from 10 µg/ml to 300 µg/ml. However, the tested compound was omitted from three wells as a positive control for each row. The culture was maintained in a shaking incubator at a light intensity of 20–50 µE at 25 °C. Four weeks later, the sensitivity of *N. gaditana* to various concentration of compound of interest in liquid state was evaluated by spotting 5 µl of culture onto 1.5% (w/v) ASW agar plates. The plates were maintained at light intensity of 20–50 µE at 25 °C.

2.5. DNA manipulations

2.5.1. Plasmids

A number of plasmids was used in this study and the details of these plasmids is listed in Table 2.2.

Table 2.2 Summary of used plasmids in this study.

Vector	Source	Function / purpose
pJet1.2	Thermo Scientific	A cloning vector for DNA manipulation and genetic engineering.
pSRsapI	Purton's collection (Dr Rosie Young)	A cloning vector to introduce genes into the <i>C. reinhardtii</i> chloroplast under the control of the <i>psaA</i> exon 1 promoter/5'UTR and the <i>rbcl</i> 3'UTR.
pRY134a	Purton's collection (Dr Rosie Young)	A cloning vector to introduce the intact <i>dxs</i> gene into the <i>C. reinhardtii</i> chloroplast.
pRY134aΔ <i>dxs</i>	Modified in this project	A cloning vector to introduce an additional copy of the <i>dxs</i> gene, but mutated, into the <i>C. reinhardtii</i> chloroplast genome.
pARG1.3	Purton's collection	A source of the <i>chloramphenicol acetyl transferase</i> gene (<i>cat</i>)
pUH.NCc2	Made in this project	A cloning vector for introducing the <i>cat</i> gene into the <i>N. gaditana</i> chloroplast under the control of the <i>psaA</i> promoter/ 5'UTR and the <i>psbA</i> 3'UTR. .
pUH.psbA264A	Made in this project	A cloning vector to mutate the wild type <i>psbA</i> gene with an atrazine tolerant <i>psbA</i> allele.

2.5.2. Isolation of genomic DNA from microalgae

Extraction of genomic DNA either from *N. gaditana* or *C. reinhardtii* strains was performed by taking a loopful of cells and re-suspending them in 20 µl of double distilled water (ddH₂O). Then 20 µl of absolute ethanol was added to the mixture and incubated at room temperature for 1 minute. About 200 µl of 5% (w/v) Chelex-100 resin (Bio-Red) suspension was added and the mixture was briefly mixed using a vortex. The mixture was heated in a heating block (Thermomixer, Eppendorf) at 99 °C for 5 minutes. After heating, the mixture was centrifuged (Heraeus Biofuge Pico) at 13,000 rpm for 2 minutes, followed by transfer of the supernatant into a fresh Eppendorf tube. The genomic DNA samples were stored in

the freezer for later use in PCR. A 2 μ l aliquot of extracted genomic DNA was used in a 50 μ l PCR reaction.

2.5.3. Isolation of plasmids from *Escherichia coli*

Small scale plasmid isolation was conducted by initially inoculating 5 ml LB medium, supplemented with the appropriate antibiotic, and incubating overnight at 37 °C with constant shaking at 200 rpm (Innova 4300 incubator shaker, New Brunswick Scientific). The overnight grown cells were harvested by centrifugation at 5,000 $\times g$ for 15 minutes at 4 °C. The plasmids were isolated from the cells using a Gene JET plasmid mini-prep kit following the manufacturer's instructions. A large volume of LB (approximately 30 ml) supplemented with appropriate antibiotic was inoculated for larger scale isolation. The large scale isolation was conducted using the Qiagen plasmid Midi kit following the manufacturer's instructions. The concentration of isolated plasmid was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5.4. Polymerase chain reaction

The amplification of target DNA sequences was performed in a 25 μ l PCR reaction that contained: 1x Phusion® HF or GC buffer (NEB), 1 unit of Phusion® High-Fidelity DNA Polymerase (NEB), 10mM of each deoxyribonucleotide triphosphate (dNTPs), 5 μ M of each forward and reverse oligonucleotide primers (MWG), ~2.5 ng/ μ l DNA template and ddH₂O to make the final volume 25 μ l. The PCR reactions were performed in a thermal cycler (either Eppendorf mastercycler® personal (Hamburg, Germany) or Techne TC-3000X Thermal Cycler, (California, USA). The cycle conditions employed varied based on the size and GC content of DNA fragment to be amplified. The PCR products were analyzed on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid and 1mM sodium EDTA) containing 1 μ g/ml ethidium bromide. The PCR products were visualized on a UV transilluminator and results 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). The primers used were synthesized by Eurofins MWG operon and the primer details for each reaction are listed in the appendices (Appendix.2).

2.5.5. Gibson assembly

A Gibson assembly method, as described by Gibson et al. (2009) was used to assemble multiple overlapping DNA fragments in a single reaction by the action of different enzymes. The reaction was performed in a total volume of 20 μ l and consists of 5 \times isothermal buffer (5% PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 50 mM DTT, 1 mM each of the four dNTPs and 5 mM NAD), 5 μ l of equimolar quantities of each of the DNA fragments and a

three enzyme cocktail (0.64 μl of 10 U/ μl T5 exonuclease, 2 μl of 40 U/ μl *Taq* DNA ligase and 0.5 μl of 2U/ μl Phusion DNA polymerase). The reaction was carried out at 50 °C for 60 minutes. The reaction product was stored in a freezer at -20 °C to be used later.

2.5.6. Restriction endonuclease digestion

DNA samples were digested with 10 units of restriction endonuclease per 1 μg of DNA in the recommended buffer according to the manufacturer's instructions. For a double digestion, the online NEB double digest finder program (www.neb.com) was used to determine the suitable buffer for both enzymes. All restriction endonucleases were supplied from New England Biolabs.

2.5.7. PCR purification and gel extraction

PCR products were purified using Gene Jet PCR purification kit (Thermo Scientific) as per the manufacturer's instructions. For gel extraction, the DNA fragment was visualized on a UV transilluminator and excised from the agarose gel using a razor blade. Then, the DNA fragment was purified using the Gene JET gel extraction kit (Thermo Scientific) as per the manufacturer's instructions.

2.5.8. DNA ligation

The ligation reaction for DNA samples was carried out in a total volume of 10 μl , containing 1 unit of T4 DNA ligase per 1 μg of DNA and 1x DNA ligase buffer (NEB). The DNA ratio of insert:vector was 1:3, respectively. The reaction was incubated for 1-2 hours at room temperature and then the ligated plasmids were transformed into competent *E. coli* cells by heat shock transformation (Sambrook & Russell 2001). Cells were grown on 1.5% (w/v) LB agar medium supplemented with the appropriate antibiotic. Colonies containing the correct plasmid and insert were identified either by colony PCR or restriction digests of the purified plasmid.

2.5.9. DNA sequencing

DNA was sequenced by Source Bioscience Services. DNA samples were supplied either in plasmid form or as a purified PCR product at a concentration of 100 ng/ μl and 1 ng/ μl per 100 bp, respectively. The primers were supplied for sequencing at a concentration of 3.2 μM . The results of the sequencing were analysed using MacVector with assembler (version 12.6.0).

2.6. UV mutagenesis to isolate atrazine tolerant mutants

Cells of *N. gaditana* were grown in 100 ml ASW medium to mid-log phase with a cell density of approximately 1×10^6 cells/ml and then approximately 60 ml of culture was irradiated by UV light at 254 nm wavelength at a distance of 15 cm from a 6W ultraviolet light bulb using an in-house built ultraviolet box. During UV irradiation, the cells were stirred constantly in a large petri dish approximately 14 cm in diameter using a sterilised paper clip as a stirrer. To produce a kill curve for *N. gaditana*, 50 μ l samples were taken over 10 minutes at 1 minute intervals. The cells were left to recover in the dark for 1 hour to limit photo-reactivation of cells. Afterwards, each aliquot was diluted 1 in 100 with fresh ASW medium and plated on 2% (w/v) ASW agar. The plates were scored for colonies after 3–4 weeks and viable cells were calculated. A kill curve was plotted of viable cells against time of exposure in order to estimate the time (t_{10}) of exposure needed to produce a 10% survival of cells.

Once a 10% survival rate was estimated for *N. gaditana*, the procedure was repeated using this time, t_{10} , to screen for chloroplast mutant colonies. However, to induce chloroplast mutations, the cells were treated with a 5-fluorodeoxyuridine (FdUrd) prior to UV mutagenesis in order to reduce the chloroplast genome abundance (Wurtz et al. 1979; Anthony et al. 2014). In order to do this, *N. gaditana* cells were grown in 200 ml in ASW medium to mid-log phase with a cell density of approximately 1×10^6 cells/ml. Sterilised glucose was added to the culture to give a final concentration of 20 mM. Thereafter the culture was split equally into three sterilised flasks, with each flask contains approximately 50 ml culture. The FdUrd was added at two different concentrations; 0.5 mM and 1 mM. The third flask contained no FdUrd and was used as a control. All cultures were maintained in the dark under constant agitation for 20 days until they reach stationary phase. The Fdurd-treated cells were washed twice with ASW medium and re-suspended to a cell density of 2×10^8 cell/ml. An aliquot of cells (500 μ l) was removed from each culture for PCR analysis. The rest of the re-suspended cultures were subjected UV mutagenesis for time t_{10} . The cells were left to recover in the dark for 1 hour and after that, a 500 μ l aliquot of cells was spread onto 20 prepared 2% agar (w/v) ASW plates, supplemented with various concentrations of atrazine (50 μ g/ml, 80 μ g/ml and 100 μ g/ml). The mutant colonies were observed after 6–8 weeks under atrazine pressure and these colonies were chosen for further analysis.

2.7. Genetic transformation

2.7.1. *Escherichia coli*

2.7.1.1 Preparation of *E. coli* competent cells

E. coli (DH5 α) cells were streaked from a glycerol stock on to a fresh 2% LB agar (w/v) plate and incubated overnight at 37 °C. A single colony was used to inoculate the starter culture and the culture was incubated overnight at 37 °C with constant shaking at 200 rpm. 1 ml of starter culture was used to inoculate 100 ml fresh LB medium and grown under the same conditions for 2.5 hours, at which point the OD₆₀₀ was measured to be approximately 0.6 (Unicam UV/Vis Spectrometer UV2). The 100 ml culture was then cooled on ice for 15 minutes followed by pelleting the cells in pre-cooled sterilin tubes at 4000 x *g* for 5 minutes. The pellets were re-suspended in 40 ml of ice-cold 50 mM CaCl₂, then incubated on ice for 30 minutes and centrifuged again at 4000 x *g* for 5 minutes. The pellets were re-suspended in 1.5 ml of fresh ice-cold 50mM CaCl₂ up to a final volume of 8 ml. The re-suspended solution was mixed with 3.5 ml of 50 % (v/v) glycerol. The competent cells were dispensed as 200 μ l aliquots, frozen in liquid nitrogen and stored -80 °C until required.

2.7.1.2 Transformation of *E. coli*

The prepared competent cells were transformed using the heat shock method. The aliquots of competent cells (100 μ l) were thawed on ice, and then either 10 μ l of ligation mixture or 1 μ l of plasmid miniprep, diluted in water by a factor of 10, was added. Another tube of competent cells (100 μ l) was included without the addition of DNA as a negative control. The samples were incubated on ice for 30 minutes and then heat shocked at 42 °C for 1 minute. The samples were returned immediately to ice and 1 ml of LB medium was added to each tube, followed by incubation for 1 hour at 37 °C. The aliquots were spread on 1.5 (w/v) LB agar plates supplemented with the appropriate antibiotic. The plates were incubated overnight at 37 °C.

2.7.2. Transformation of the *Chlamydomonas reinhardtii* chloroplast

The chloroplast transformation method used was adapted from the original glass bead method described by Kindle et al. (1991), with some modifications. The recipient cell wall deficient and photosynthesis deficient strain (TN72) was grown in 500 ml TAP to mid-log phase (with a cell density of 1–2 x 10⁶ cells/ml) under dim light approximately 5–10 μ E. The cells were harvested by centrifugation at 5,000 rpm for 5 minutes at 16 °C (Sorvall Evolution RC, Thermo Scientific) and then re-suspended in fresh TAP to a concentration of 2 x 10⁸ cells/ml. Aliquots of the cell suspension were pipetted into test tubes containing ~ 0.3 g of glass beads (Glass beads, acid washed, 425-600 μ m, Sigma). 10 μ g of DNA was added into each tube containing 300 μ l of cell suspension and then agitated for 15 seconds at maximum speed using a Vortex genie-2. Then 3 ml of molten 0.5% (w/v) HSM agar at 42 °C

was added to each tube and immediately the mixture was poured onto 2% HSM (w/v) agar plates. The plates were incubated under a light intensity of approximately 50 μE at 25 °C for 6–8 weeks, with the selection based on restoration of photosynthesis and the ability to grow autotrophically on 2% (w/v) HSM agar plate. The putative transformant colonies were picked and re-streaked three times on 2% HSM agar (w/v) plates before being screened for homoplasmicity by PCR.

2.7.3. *Nannochloropsis gaditana*

2.7.3.1 Transformation of *N. gaditana* using electroporation

Cells of *N. gaditana* were grown in 500 ml of ASW medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin and 100 $\mu\text{g}/\text{ml}$ ampicillin to late log phase with a cell density of $\sim 1\text{--}2 \times 10^7$ cells/ml. The cells were harvested by centrifugation at 3,500 $\times g$ at 22 °C for 15 minutes and then cells were washed three times with 375 mM sorbitol. After the final wash, the cells were re-suspended in 5 ml to give the final concentration of approximately 1×10^9 cells/ml, following which the cells were dispensed into fresh 1.5 ml tubes as 400 μl aliquots. The 400 μl aliquot of cells was mixed with 5–10 μg of linearized DNA and kept on ice prior to electroporation. The electroporation was performed with a constant time Electroporator (2510 Electroporater, Eppendorf) or (Gene Pluser Xcell™ Electroporation system, Bio-Rad) in 2-mm cuvettes. The suspension of cells and DNA was transferred into a cuvette and subjected to an electrical field strength at set conditions. After electroporation, the cells were allowed to recover in 10 ml of ASW medium for 24 hours at 25 °C with shaking under dim light (approximately 5–10 μE). The cell concentration was adjusted to give $\sim 1 \times 10^8$ cells per plate, and then plated on 2% (w/v) ASW agar plates containing the appropriate selection agent. Successful transformants were scored after 4 weeks and re-streaked onto fresh plates one week later.

2.7.3.2 Biolistic Transformation of *N. gaditana*

The biolistic transformation of *N. gaditana* was performed using the BioRad PDS-1000/He Biolistic® Particle Delivery System. For the cell preparation, the cells were grown in 400 ml of ASW medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin and 100 $\mu\text{g}/\text{ml}$ ampicillin to late log phase with a cell density of $1\text{--}2 \times 10^7$ cells/ml. The cells were then diluted with one-sixth of its original volume and subsequently nocodazole (Sigma-Aldrich) added to give the desired final concentration. The cells were allowed to grow in the presence of nocodazole for 24 hours with light intensity at 50 μE at 25 °C with constant shaking at 120 rpm. After that, the cells were harvested at 3,500 $\times g$ for 15 minutes at 22 °C and the pellet was re-suspended in fresh ASW medium containing the desired concentration of nocodazole to a concentration of 5×10^8 cells/ml. The cells were spread evenly on 2% (w/v) agar ASW in which the cell concentration was adjusted to $1\text{--}5 \times 10^7$ cells per plate. The plated cells were

allowed to dry briefly in a laminar flow hood, and then were bombarded with DNA-coated microparticles within 2 hours.

For DNA preparation, the plasmids were linearised and then coated onto gold particles with a diameter of 0.55 μm using the DNAdel™ gold particles kit (Seashell Technology, San Diego) as per the manufacturer's instructions. The coated microparticles were washed with 70% (v/v) ethanol and re-suspended in absolute ethanol to give a final concentration of 0.5 mg gold particle coated with 5–10 μg DNA per shot. This suspension solution was briefly sonicated in a water bath and then applied to the center of sterilized macro-carriers (Bio-Rad laboratories Ltd, Hertfordshire, UK). Before loading the macro-carriers for bombardment, they were allowed to air dry in a laminar flow for few minutes.

The bombardment of prepared algal plates was performed by following the instructions for the PDS-100/He device, using 1350 psi rupture disk (Bio-Rad laboratories Ltd, Hertfordshire, UK). After bombardment, the cells were allowed to recover by incubating the plates under dim light at 25 °C for 24 hours. Afterwards, the cells were re-suspended in fresh ASW medium using a spreader and were then harvested by centrifugation at 3,500 $\times g$ for 15 minutes at 22 °C. Twice the pellet was washed by centrifugation at 3,500 $\times g$ for 15 minutes followed by pellet re-suspension in 10 ml of sterile ASW medium. Following the washing steps, the cells were left to recover in 10 ml ASW medium for 1 hour at 25 °C with shaking at low light intensity (5–10 μE). The cells were harvested by centrifugation at 3,500 $\times g$ for 15 minutes at 22 °C and re-suspended in 2 ml of sterile ASW medium. About 500 μl of re-suspended cells were spread on 2% (w/v) ASW agar supplemented with the appropriate selection agents. The plates were incubated at 25 °C under constant illumination at approximately 80–100 μE . The putative transformed colonies were observed after 4–6 weeks and re-streaked on fresh selective medium for further analysis.

2.8. Protein analysis

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

Cultures of *C. reinhardtii* cells were grown to log phase (OD_{750} 0.8–1.5) in a shaking incubator and the optical density of cultures at 750 nm was measured using a spectrophotometer. The cells were prepared by centrifuging 20 ml of culture at 5,800 $\times g$ for 5 minutes and then re-suspending the pellet 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 added volume of solution A in each sample depends on OD_{750} readings to ensure that all samples are more or less at the same concentration. Afterwards, 50 μl aliquots of the algal suspension was stored at –80 °C to be used later for western blot analysis.

2.8.2. Preparation of protein extract from *E. coli*

E. coli cells were grown overnight at 37 °C with constant shaking at 180 rpm. The overnight cultures were harvested by centrifugation for 10 minutes at 5,500 rpm at 4 °C. The protein was then extracted from cells using BugBuster® protein extraction reagent (Novagen) following the manufacturer's instructions. The resulting supernatant was transferred into a fresh 1.5 ml tube and stored as 0.5 ml aliquots at -80 °C to be used later in western blot analysis and fluorometric assay.

2.8.3. SDS-polyacrylamide gel electrophoresis

The proteins were resolved on a 15% SDS-PAGE gel. The 1.0 mm thick gel was set up using a Bio-Rad mini-PROTEAN tetra system and the gels (resolving and stacking) were made following Laemmli's gel recipe (Sambrook & Russell 2001). The resolving gel was prepared by adding the chemicals in the following order: 2.8 ml 40 % (w/v) acrylamide: bisacrylamide at 37:1, 1.87 ml 1.5 M Tris-HCl (pH 8.8), 75 µl 10 % (w/v) SDS, 2.65 ml ddH₂O, 75 µl 10 % (w/v) ammonium persulphate and 3 µl TEMED. 4 ml of this solution was added to the pre-set plates, leaving a few centimeters at the top for the stacking gel and then overlain with ethanol. The gel polymerization was allowed to set at room temperature for at least 30 minutes. After polymerization, the ethanol overlay was removed and the surface of the gel was washed several times with distilled water.

The stacking gel was then prepared by adding the chemicals in the following order; 375 µl 40 % (w/v) acrylamide: bisacrylamide at 37:1, 375 µl 1M Tris-HCl (pH 6.8), 30 µl 10 % (w/v) SDS, 2.15 ml distilled H₂O, 30 µl ammonium persulphate and 3 µl TEMED. Approximately 1 ml of this solution was used to fill the plates with stacking gel right to the top. The appropriate comb was inserted carefully into the stacking gel and left for at least for 30 minutes at room temperature for the gel to set.

After setting up the gel, the 50 µl protein extract aliquots previously stored at -80 °C were prepared for loading by adding 5 µl of 10% (w/v) SDS followed by heating on the heatblock for 1 minute at 99 °C. The samples were centrifuged for 2 minutes at 13,000 rpm and a 25 µl volume of supernatant was used in the loading. The molecular weight of proteins was estimated by reference to a PageRuler™ prestained ladder (Fermentas, York, UK). The proteins were run in cold electrophoresis buffer containing 0.25 M Tris, 1.92 M glycine, 1 % (w/v) SDS pH 8.3 for 90 minutes at 150 V until the green chlorophyll was close to the bottom of the gel.

2.8.4. Western blot analysis

The semi-dry electrophoresis method detailed by Sambrook and Russell , (2001) was used to transfer proteins separated by SDS-PAGE to Hybond-ECL nitrocellulose membranes (GE

Healthcare). To set up the gels for transfer, 6 sheets of equivalent sized 3MM Whatman paper and a sheet of nitrocellulose membrane were soaked in Towbin buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol) at room temperature for 10 min. Three sheets of soaked 3MM paper were placed on the semi-dry blotter followed by the nitrocellulose membrane, the gel and then the remaining three sheets of 3MM paper. Excess buffer and air bubbles in the assembled stack were removed by rolling with a plastic pipette.

The transfer of proteins was conducted using the Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer system at a constant voltage of 20 V (Fisons FEC 570 powerpac) for 2 hours. Following transfer, the membrane was blocked overnight in 20 ml of 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) at 4 °C shaking at 50 rpm (Mini-orbital Shaker, Stuart). The membrane was rinsed briefly with TBS-T, followed by two rounds of 15 minute washes in TBS-T while shaking at 50 rpm. Diluted rabbit anti-HA antibody (1:2000) was used as the primary antibody and the membrane was treated with primary antibody for 1 hour at room temperature with shaking at 60 rpm. The membrane was rinsed several times briefly and the membrane was then washed four times in TBS-T for 10 minutes.

For the ECL detection method, a horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare) was used as the secondary antibody. The membrane was incubated in 0.5% (w/v) skimmed milk in TBS-T with the secondary antibody (1:5000 dilution) for 1 hour at room temperature with shaking at 60 rpm and then the membrane was washed as for the primary antibody. The proteins were detected by the ECL method using a luminescence substrate detection kit according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent substrate, supplied by Pierce). The membrane was sealed between polythene sheets using a heat sealer and taped in a developing cassette. In the dark room, a sheet of Hyperfilm ECL (GE Healthcare) was exposed to the membrane and then developed using an automatic film processor (Xograph Compact X4 film developer). The signal strength was adjusted by changing the exposure duration until the protein of interest was visualized at the desirable level.

As for the Odyssey detection method, a goat anti-rabbit IgG Dylight 800 (Thermo Scientific) was used as secondary antibody. The membrane was therefore incubated in 0.5% (w/v) skimmed milk in TBS-T with the secondary antibody (1:2000) for an hour at room temperature with shaking at 60 rpm and then the membrane was washed as for the primary antibody. An additional washing step with TBS was included to remove the excess Tween. Afterwards the protein was detected using the Odyssey® Infrared Imaging system (Li-COR Bioscience, USA).

2.9. Protein activity assay

2.9.1. Preparation of crude extracts for the DXS enzyme activity assay

The *C. reinhardtii* transformant lines were grown to exponential phase (OD_{750} 1.0–2.0) and then the cells were harvested by centrifugation at 5,000 rpm for 10 minutes. The cell pellet for each strain was washed with water and then re-suspended in extraction buffer (40mM Tris-HCl, 5mM 2-mercaptoethanol, 2.5 mM $MgCl_2$, pH 7.5) containing protease inhibitor Roche cOmplete, EDTA-free (Roche, Switzerland). Afterwards, the cells were lysed by a freeze and thaw method. Cells were frozen in liquid nitrogen and thawed in a water bath at 35 °C. The freeze and thaw cycle was repeated three times and then the lysed cells were centrifuged at 5,500 x *g* for 30 minutes. The supernatant was recovered, which is referred as the crude enzyme extract. In some cases, the crude enzyme extract was further centrifuged at 100,000 x *g* for an hour in Sorvall Discovery 90SE ultracentrifuge (Hitachi, USA) to get rid of more cellular debris and non-soluble proteins. The crude enzyme extract was transferred into a new tube and stored as 1 ml aliquots at –80 °C to be used later in a fluorometric assay.

2.9.2. Fluorometric assay for the DXS enzyme

The reaction mixture for the DXS enzyme activity assay consisted of 40 mM Tris-HCl, 2.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1mM thiamine diphosphate, 10mM sodium pyruvate, and 20 mM DL-glyceraldehyde 3-phosphate, and the crude enzyme extract to make the final volume of 100 μ l. The reaction mixture was incubated at 30 °C for 60 minutes. The reaction was stopped by heating the mixture at 80 °C for 5 minutes, and then centrifuged at 13,000 x *g* for 5 minutes. The supernatant was transferred into a new tube and diluted with distilled water to 1 ml. The diluted supernatant was mixed with an equal volume of 10 mM DABA in 5 M phosphoric acid, and then heated in a boiling water bath for 15 minutes. The supernatant containing the enzymatic product 'DXP' was analyzed at an excitation wavelength of 396 nm using a fluorometer (LS 55 Luminescence, PerkinElmer).

2.10. Freeze-drying the algal materials

C. reinhardtii strains were grown in 250 ml of TAP medium to late log phase (OD_{750} 1.0–2.0). The cells were harvested by centrifugations at 5,000 rpm for 15 minutes at 16 °C (Sorvall Evolution RC, Thermo Scientific). The cell pellets were washed with distilled water followed by the transfer of the re-suspended cells in water into 50 ml falcon tubes wrapped with foil. The cells were harvested and frozen in liquid nitrogen. Once the cells were frozen, the freeze dryer (Modulyo Freeze Dryer, Edwards) was set up for the use according to the manufacturer's instructions. The frozen samples were placed into the freeze dryer chamber, where the lid of falcon tubes was kept loose to allow moisture to escape during

the process. The air was evacuated from the chamber, allowing the cells to dry for approximately 48 hours at $-50\text{ }^{\circ}\text{C}$, Pressure $< 1\text{ Pa}$. The cells were removed from the freeze drier chamber according to the manufacturer's instructions, and the lid was tightened properly. The freeze-dried algal materials were kept at room temperature for a few days and then they were sent for HPLC analysis in the laboratory of Professor Paul Fraser, Royal Holloway, London.

2.11. Cell cycle synchronisation using nocodazole

A culture of *N. gaditana* was grown in 200 ml ASW medium supplemented with 100 $\mu\text{g/ml}$ ampicillin and 50 $\mu\text{g/ml}$ kanamycin to log phase (OD_{750} 1.0–1.2) at light intensity of approximately 50–80 μE with constant shaking at 120 rpm. The culture was diluted with ASW medium to one-sixth of its original volume and then DMSO was added to give a final concentration of 1% (v/v). The culture was then divided equally among four culture flasks and nocodazole was added at three different concentrations; 20 ng/ml, 3 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$. One of the cultures did not contain nocodazole and was used as an asynchronous control culture. Afterwards the cultures were grown for 72 hours under continuous illumination (approximately 50 μE) with constant shaking at 120 rpm at $25\text{ }^{\circ}\text{C}$. Throughout 72 hours, the cultures were monitored every 12 hours by measuring the optical density (OD_{750}) as well as microscopically observing the cells (Axio Scope. A1, Zeiss).

2.12. Flow cytometry analysis

The cells grown in the presence of nocodazole at different concentrations and durations, along with the asynchronous control culture were harvested by centrifugation for 15 minutes at $3,500 \times g$. The cell pellets were washed twice with Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4). After the second wash, the pellets were re-suspended in PBS buffer and equalized to an OD_{750} of 0.5. A 500 μl aliquot of suspension from each culture was transferred into a fresh Eppendorf tube and then the cells were fixed by adding 1 ml of 70% (v/v) of ice cold ethanol slowly while sonicating the cells in the water bath. Afterwards, the fixed cells were incubated on ice for 30 minutes, harvested by centrifugation at 13,000 rpm for 5 minutes and washed twice with PBS buffer. After the last wash, the pellet was re-suspended in 0.5 ml PBS buffer and then the cells were treated with 100 $\mu\text{g/ml}$ RNase. The cells treated with ribonuclease were incubated at room temperature for 30 minutes and then all Eppendorf tubes were wrapped with foil as a preparatory step before adding DNA stain. The cells were then stained with propidium iodide (5 mg/ml) and stored at $4\text{ }^{\circ}\text{C}$. Prior to analysis, the cells were passed through a syringe and vortexed briefly. The cells were then analysed using an Accuri™ C6 flow cytometer and Accuri™ C6 software- Cflow Plus (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

CHAPTER 3

Classical approach of developing a chloroplast transformation methodology in *N. gaditana*

3. Classical approach of developing a chloroplast transformation methodology in *N. gaditana*

3.1. Introduction

Microalgae are a highly diverse group of photosynthetic organisms that have attracted attention as a potential platform for biofuels, nutraceuticals, therapeutics and industrial chemicals. In particular the chloroplast within these microalgae, in addition to being the site of photosynthesis, is also the site for synthesis of industrially relevant compounds such as pigments, fatty acids, and starch. Stable transformation of the algal chloroplast was first reported in 1988 using the model *Chlamydomonas reinhardtii* (Boynton et al. 1988). Since then, significant progress has been made in the development of molecular tools and efforts to transfer developed technologies to other industrially relevant species including higher plants. There has been also success in expressing high value recombinant proteins in the *Chlamydomonas* chloroplast such as protein vaccines and therapeutic antibodies (Barrera & Mayfield 2013; Purton et al. 2013).

Nonetheless, there are other algae species yet to be exploited, especially those algal strains with high growth density and suited for large-scale cultivation, such as *Nannochloropsis*. The biotechnological exploitation of these microalgae requires the development of molecular tools and technologies (Al-Hoqani et al. 2016). There is also a need to develop a reliable chloroplast transformation method for these industrially relevant species, so that the genetic engineering of chloroplast metabolism would be possible. Since transformation of the chloroplast for these microalgae is a first step in their biotechnological exploitation, the focus of the research in this thesis is primarily on developing a chloroplast transformation method for *Nannochloropsis gaditana*.

3.1.1. Chloroplast

The chloroplast organelle is the site of photosynthesis within algal and plant cells. Plastids such as chloroplasts also house important biosynthetic pathways such as the synthesis of carbohydrates, fatty acids, carotenoids, tetrapyrroles and terpenoids. The chloroplast has its own genetic material as a result of a cyanobacterial endosymbiosis event that occurred over a billion years ago. The chloroplast has retained many features that reflect its prokaryotic origin including a polyploid circular genome termed the “plastome”, and prokaryotic-like transcriptional and translational machinery such as a prokaryotic-type RNA polymerase and 70S ribosomes. The plastome is relatively small in size; it contains approximately 100–250 genes and these genes are often organized in polycistronic units across the genome. The majority of these genes code for either subunits of the photosynthetic apparatus or components required for the transcription-translational

machinery (Green 2011; Purton 2007). The level of chloroplast gene expression is generally high in actively growing cells, indicating the primary role of these genes in encoding the core components of photosynthetic apparatus: for example, *psbA* which encodes the D1 core subunit of photosystem II. Although the chloroplast has its own genome, chloroplast gene expression is regulated by various nuclear-encoded factors that act on chloroplast mRNA. These factors are involved in post-transcriptional steps such as RNA processing, RNA stability and translation (Stern et al. 1997).

The chloroplast genome represents an attractive target for genetic engineering for a number of reasons: First, the small size (100–200 kb) and low genetic complexity of the genome, with less than 100 protein-coding genes; Second, the high level of gene expression particularly genes for core components of the photosynthetic apparatus; Third, the precise integration of foreign DNA to any loci within chloroplast genome by homologous recombination; Fourth, the absence of gene silencing effects and the possibility to express multiple transgenes as an operon. In addition, the algal chloroplast is a major site of metabolism and product storage (Wang et al. 2009; Wani et al. 2010).

Interestingly, chloroplast transformation in all studied species to date proceeds through double homologous recombination events between chloroplast sequences on the plasmid and corresponding genomic sequences (Figure 3.1). The stable integration of exogenous DNA into the chloroplast genome needs sufficient homologous sequences to the targeted site of insertion, normally around ~ 1 kb of homologous sequence flanking either side of the exogenous DNA to allow recombination events. Therefore, the exogenous DNA can be targeted precisely to any region in the chloroplast genome provided it is a 'silent site' for transgene integration so that it does not interrupt any essential function (Purton 2007).

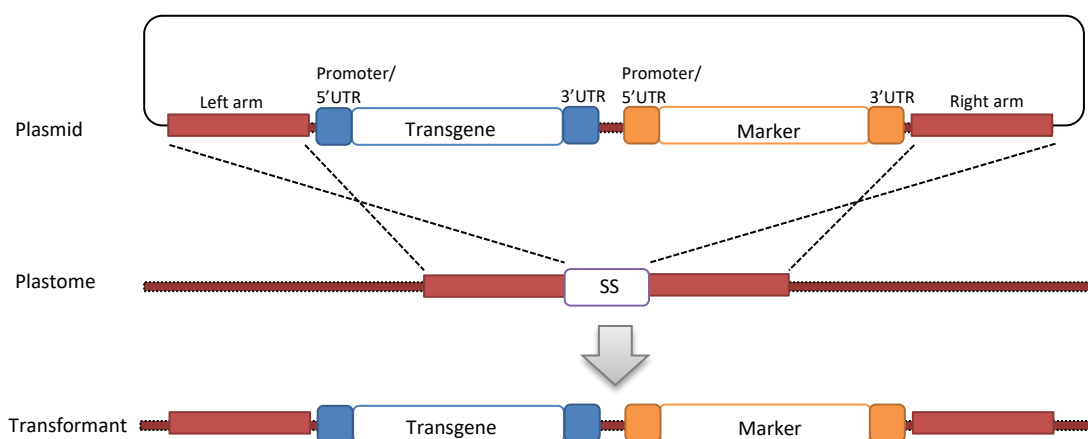


Figure 3.1 Schematic representation of chloroplast transformation.

A typical chloroplast transformation plasmid, consisting of the transgene under the control of an endogenous promoter and regulatory elements, and a selectable marker gene under the control of different endogenous promoter and regulator elements, together with flanking regions (left arm and right arm). The homologous recombination occurs between homologous sequences shared between the plasmid and the plastome, resulting in the integration of the cloned gene/s into the plastome. X-X represents the double homologous recombination events, whereas SS represents the silent site for the transgene integration into the plastome.

3.1.2. The algal chloroplast and genetic transformation

The chloroplast of unicellular microalgae has been the focus of recent genetic engineering efforts as most microalgae contain a single chloroplast per cell. This makes the genetic manipulation of the chloroplast genome much easier compared to the plant leaf for instance, which can have up to a hundred plastids per cell (Day & Goldschmidt-Clermont 2011). The algal chloroplast also has the ability to accumulate significant amounts of starch, lipid or pigments under stress conditions, which can be utilized for the production of industrially relevant products (Radakovits et al. 2010). In addition, some algal species are characterized by high growth rates, low cultivation cost and diverse metabolic capabilities (Purton et al. 2013). Given all these advantages, the genetic engineering of algal chloroplasts can offer an ideal platform for large-scale production of biofuel molecules and high value compounds with nutritional, cosmetic and pharmaceutical applications.

The first stable transformation of the chloroplast genome of a unicellular alga was reported by Boynton et al. (1988) using the biolistic method. This was achieved by rescuing a photosynthetic mutant of *C. reinhardtii* carrying a deletion in the chloroplast gene, *atpB* back to photoautotrophic growth with the cloned wild-type gene. Another DNA delivery method that has been used successfully to transform the chloroplast of *C. reinhardtii* involves agitation of a cell wall deficient strain with glass beads (Kindle et al. 1991). Soon thereafter, the first recombinant protein produced in chloroplasts was reported by Goldschmidt-Clermont (1991), where a bacterial gene was expressed successfully in the *C. reinhardtii* chloroplast. Since then, the molecular tools for genetic manipulation for this alga

have been further improved and refined. These improvements include developing new selectable marker/reporter genes, recipient strains and better understanding for the key elements for the successful chloroplast transformation (Wannathong et al. 2016; Young & Purton 2016; Scaife et al. 2015). As illustrated in Figure 3.2, the availability of molecular tools and resources for genetic manipulation for this green alga in general has increased dramatically over the past 30 years providing significant insights into the chloroplast gene function and expression. With such resources and tools, *C. reinhardtii* has become a model system for chloroplast genetics and molecular biology. Several techniques first developed for chloroplast genome engineering in this species have since been applied in other algal species and higher plants, in particular tobacco (Bock 2014).

The *C. reinhardtii* chloroplast has been exploited as a heterologous protein expression system for the production of high value recombinant proteins such as therapeutic antibodies and protein vaccines (Rasala & Mayfield 2010). In fact, a number of high value recombinant proteins have been produced in microalgae, such as the production of a human single-chain antibody in the chloroplast of *C. reinhardtii* (Mayfield et al. 2003). A full human monoclonal antibody was also expressed in the *Chlamydomonas* chloroplast, and was demonstrated to have antigen binding activity similar to the same antibody expressed in mammalian cells (Tran et al. 2009).

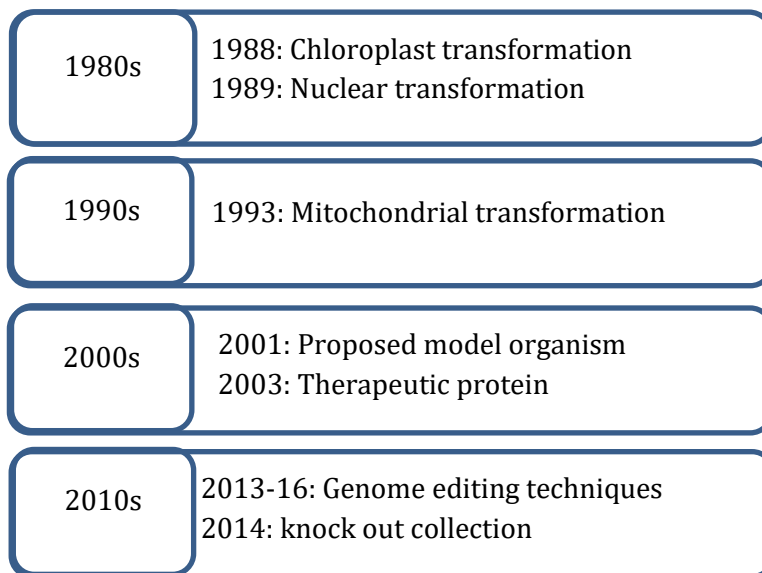


Figure 3.2 Timeline of molecular advances in the development of tools and techniques for engineering *C. reinhardtii*.

Development of chloroplast and nuclear transformation (Boynton et al. 1988; Blowers et al. 1989; Kindle et al. 1989), mitochondrial transformation (Randolph-Anderson et al. 1993), *C. reinhardtii* as a model photosynthetic organism (Harris 2001), proteomics for system analysis (Hippler et al. 2001), first therapeutic recombinant protein to be expressed in the chloroplast (Mayfield et al. 2003), sequencing the nuclear genome (Merchant et al. 2010), development of genome editing techniques (Jiang et al. 2014; Sizova et al. 2013; Gao et al. 2014; Shin et al. 2016), establishment of a knock-out collection (Zhang et al. 2014). Figure modified from Scaife et al. (2015)

Currently, *C. reinhardtii* is the most developed algal species for biotechnology with well-established transformation methods and molecular tools. However, when it comes to its biosynthetic capacity for production at a commercial level, it is still far from reaching economic viability. There are successful attempts to improve biofuel phenotypes of *C. reinhardtii* using genetic engineering (Li et al. 2010; Work et al. 2010), but this alga is not naturally an exceptional producer of either lipid or biomass and so extensive genetic modification will be required before it is suitable for industrial applications. For the biotechnological exploitation of the algal chloroplast, the chosen species should be a naturally exceptional producer of biomass and well-suited for large-scale cultivation. A number of *Nannochloropsis* species have attracted interest due to their high rates of biomass accumulation, their high lipid productivity and their successful cultivation at industrial scale by several companies. Such companies include Aurora Algae, Seambiotic, Solix Biofuels, Hairong Electric Company and Proviro. One species of particular interest is *N. gaditana*, which has a high photoautotrophic biomass rate, and can grow to high densities (> 10 g dry weight/litre) and high lipid content (Radakovits et al. 2012). The availability of the complete genome sequence for *N. gaditana* has facilitated the development of a nuclear transformation methodology and subsequently the development of more tools for nuclear genetic engineering (Al-Hoqani et al. 2016). These tools include electroporation and other methods to deliver DNA into the nuclear genome of *Nannochloropsis* (Kilian et al. 2011; Cha et al. 2011), and the development of various selectable marker and reporter genes to allow the selection of nuclear transformants and assessment of expression levels, respectively. In addition, forward and reverse genetic studies using *Nannochloropsis* species in general have provided some insights into nuclear gene function and expression, especially for those genes involved in lipid biosynthesis (Al-Hoqani et al. 2016).

However, the full exploitation of *N. gaditana* as a biotechnology platform for biofuel production or synthesis of high-value metabolites requires the development of molecular tools and techniques for engineering the chloroplast genome. Chloroplast transformation has yet to be described for *Nannochloropsis*, and indeed the genetic manipulation of the chloroplast is still limited to very few algal species (see Table 3.1), as it has proved challenging due to a number of reasons. Firstly, the exogenous DNA needs to be delivered across a cell wall and multiple membranes – as many as five depending on the species that is being transformed. Secondly, the exogenous DNA needs to integrate into the chloroplast genome and be expressed successfully using the organelles's expression machinery. Thirdly, the chloroplast contains multiple copies of the plastome and so a strong selection strategy is required to recover a stable transgenic cell with all plastome copies carrying the modification (Purton 2001). So, there is a need to develop chloroplast transformation

method that is applicable to more algal strains, especially for those adapted to cultivation for large-scale production.

Table 3.1 Reports of chloroplast transformation in microalgae.

Algal species	Transformation method	Selectable marker	Selection	References
<i>Chlamydomonas reinhardtii</i>	Particle gun	<i>atpB</i>	Photoautotrophy	Boynton et al. 1988
	Glass beads	<i>tscA, psbA</i>	Photoautotrophy	Kindle et al. 1991
<i>Euglena gracilis</i>	Particle gun	<i>aadA</i>	Streptomycin, spectinomycin	Doetsch et al. 2001
<i>Porphyridium sp.</i>	Particle gun	<i>AHAS</i> (W492S)	Sulfometuron methyl	Lapidot et al. 2002
<i>Haematococcus pluvialis</i>	Particle gun	<i>aadA</i>	Spectinomycin	Gutiérrez et al. 2012
<i>Dunaliella tertiolecta</i>	Particle gun	<i>ereB</i>	Erythromycin	Georgianna et al. 2013
<i>Platymonas subcordiformis</i>	Particle gun	<i>bar</i>	Basta	Cui et al. 2014
<i>Phaeodactylum tricorutum</i>	Electroporation	<i>cat</i>	Chloramphenicol	Xie et al. 2014
<i>Cyanidioschyzon merolae</i>	PEG-mediated transfection, particle gun	<i>cat</i>	Chloramphenicol	Zienkiewicz et al. 2016

3.1.3. Aim and objectives

The overall aim of this chapter is to develop a reliable chloroplast transformation method for oleaginous and industrially relevant algae, in particular *N. gaditana*.

Specifically, the aims of the work in this chapter are:

1. Optimisation of cultivation conditions for *N. gaditana* by testing different growth conditions.
2. Evaluation of the antibiotic sensitivity of *N. gaditana* in order to identify suitable selection strategies for chloroplast transformation.
3. Construction of a chloroplast transformation plasmid for the introduction of novel genes into the chloroplast genome.
4. Optimizing a nuclear transformation protocol as a control for testing different DNA delivery methods for *N. gaditana*.
5. Testing different delivery methods including electroporation and biolistics to transform the chloroplast of *N. gaditana*.

3.2. Results

3.2.1. Preliminary studies on *Nannochloropsis gaditana* cultivation

Several groups have cultivated various species of *Nannochloropsis* successfully and it seems each species has different requirements for optimum growth (Rocha et al. 2003; Radakovits et al. 2012; Kilian et al. 2011). Although the growth conditions of *N. gaditana* CCMP526, the strain under be studied in this research, have been already been optimized as reported by Radakovits et al. (2012), this needs to be re-examined to suit our lab facilities and lab scale cultivation. Therefore, a number of important parameters were tested throughout the optimization using a commercial lab scale photobioreactor known as an Algem®. These parameters are temperature, light conditions, additional source of carbon and addition of vitamins. First, different temperatures were assessed for the optimum growth of *N. gaditana*. The result of cell cultivation at different temperatures (Figure 3.3) shows that the fastest log-phase growth for *N. gaditana* in artificial seawater (ASW) is observed at 25 °C.

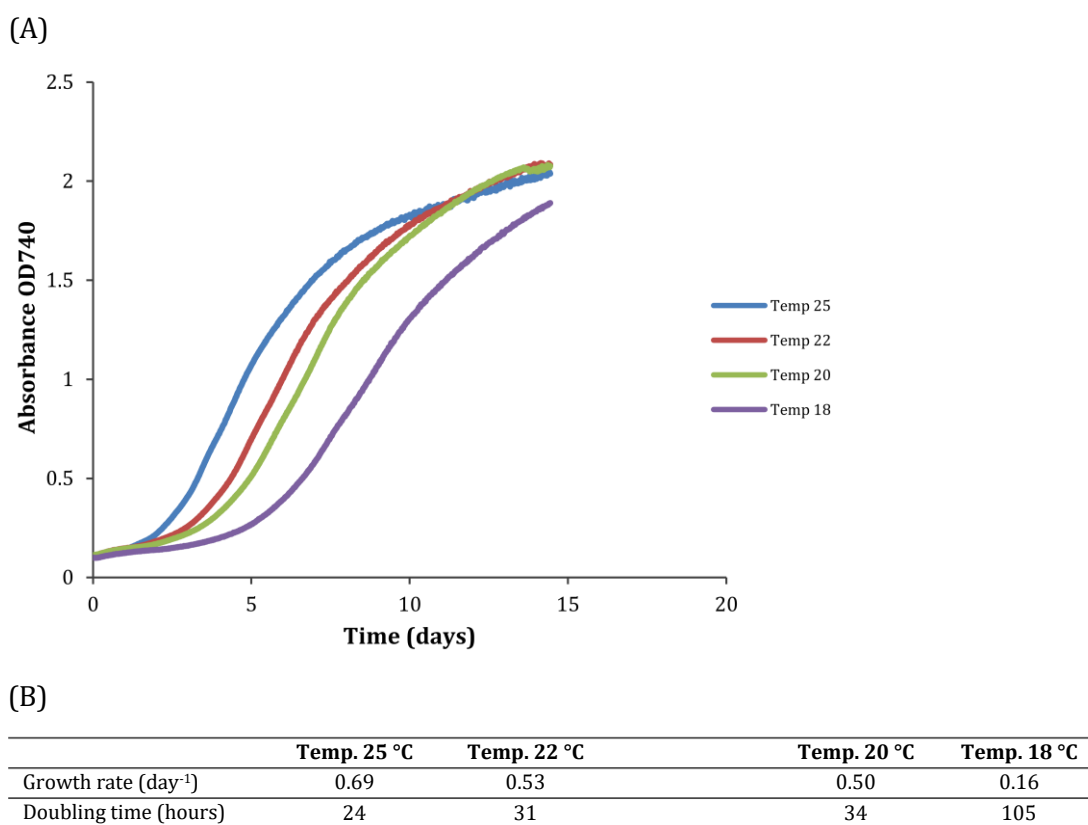


Figure 3.3 Growth of *N. gaditana* at different temperatures using an Algem® photobioreactor.

(A) The growth curves of *N. gaditana* cultures at different temperature for two weeks. These cultures were inoculated from same starter culture. The cells illuminated continuously with 200 μ E. (B) The calculated growth rate and doubling time of *N. gaditana* at different temperatures. The the growth rate of *N. gaditana* was calculated by identifying two points at log phase and using these points into the following equation: specific growth rate (μ)= $(\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_1 and X_2 is the optical density of cells at time (t_1) and time 2 (t_2), respectively. The calculated growth rate was used then into the following equation to calculate the doubling time, where doubling time = $\ln 2 / \mu$.

As for the light conditions, the Algem® was set up to expose the cultures in four chambers simultaneously to different light intensities and regimes: namely: constant light 50 μE ; 100 μE and 200 μE , and a sine wave (sw) cycle from 100–200 μE . The sw cycle in the Algem® is programmed such that it allows a gradual increase of light intensity from 100 to 200 μE , followed by a gradual decrease from 200 to 100 μE . The period of the sw is 12 h. The results (Figure 3.4) show that there is an obvious growth difference among the cultures exposed to constant light intensities. The 200 μE constant light showed an improved growth rate in comparison to constant illumination of 50 μE and 100 μE . However, the sw condition clearly gives a higher growth rate in comparison to constant illumination of 200 μE . Subsequently, the cells were able to accumulate more biomass when harvested on day 14, measured as 1.1 g and 2 g wet weight per liter for the grown culture under constant light illumination of 200 μE and sw illumination of 100-200 μE , respectively.

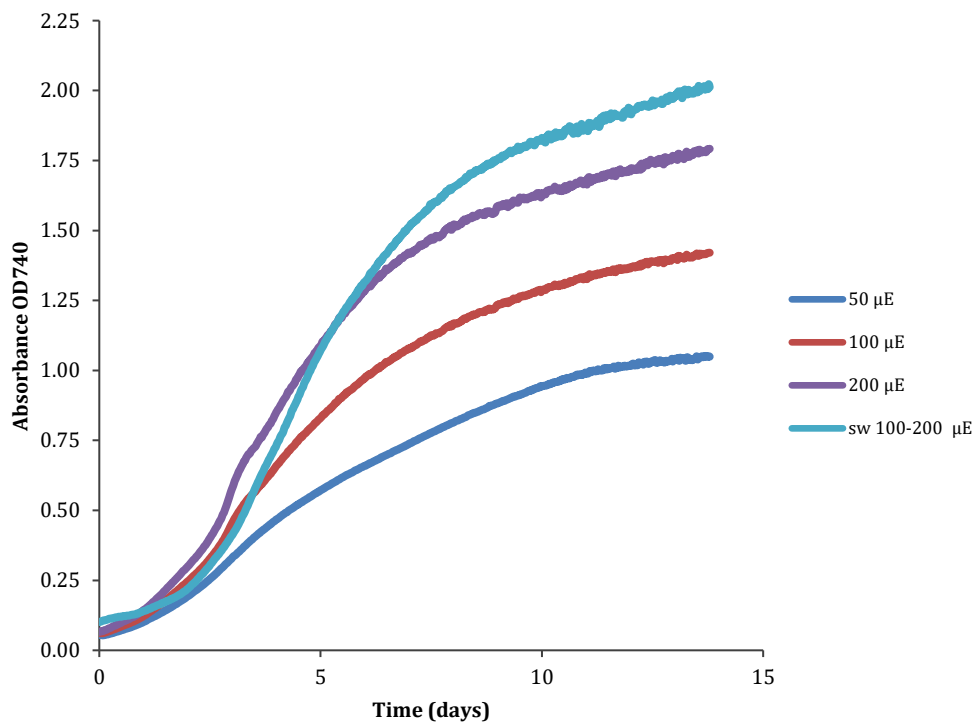


Figure 3.4 Cultivation of *N. gaditana* under different light conditions.

The light conditions are constant illumination of 50, 100 and 200 μE , and sine wave light regime from 100–200 μE . The temperature was set up at 25 °C in the growth chambers. The raw data was retrieved from Algem®.

The effect of adding different carbon sources to *N. gaditana* also was tested by adding either an organic source of carbon or supplying the culture with 5% CO₂. The mixotrophic conditions using various organic carbon sources (i.e. glucose, galactose and sucrose) did not result in any improvement in the rate of growth of *N. gaditana* as shown in Figure 3.5. This result highlights the absence of a hexose symporter in this species that enables glucose uptake from the growth medium (Doebbe et al. 2007), as confirmed by the available genome data (Radakovits et al. 2012), where the hexose symporter gene (HUP1) is absent in the *N. gaditana* genome¹. Although the genome data indicates the presence of a putative galactose-proton symporter gene with accession number Naga_100010g2, the growth evidence suggests that *N. gaditana* does not take up galactose from medium, which is unlikely to be found in the marine environment.

The addition of 5% CO₂ at 50 cm³ per min to the medium showed a deleterious impact on *N. gaditana* growth as shown in Figure 3.6 (right panel). Such a result could be due to either the high flow rate leading to a shear stress effect in the 500 ml cultures, or a marked drop in pH as a result of carbon dioxide uptake, resulting in the inhibition of growth.

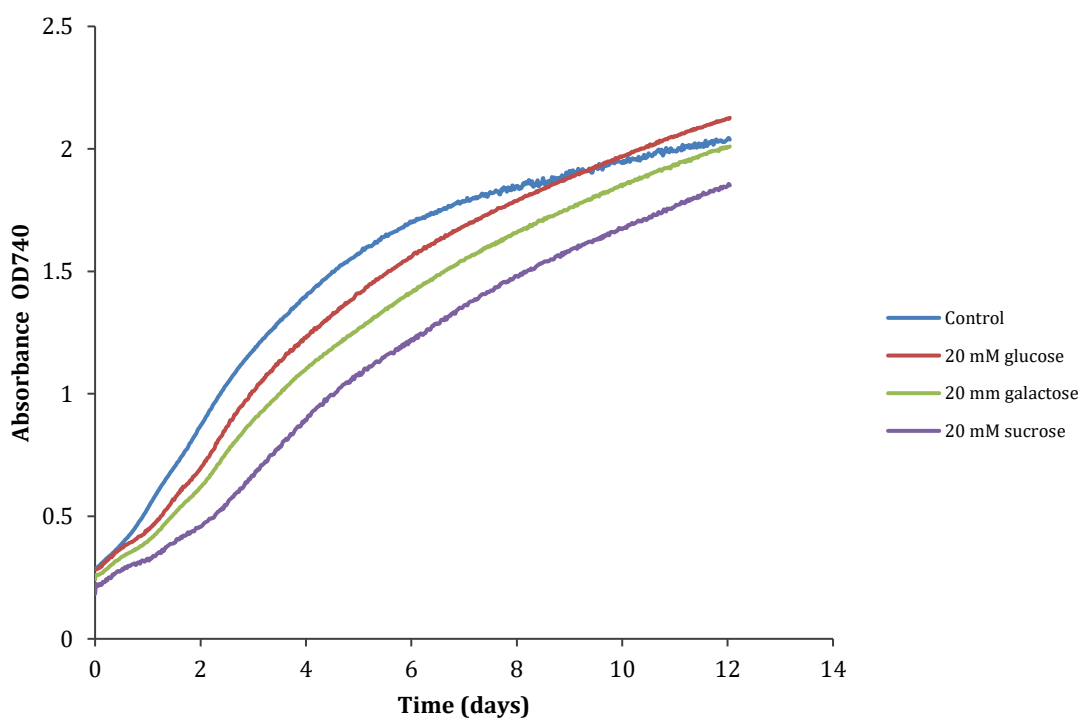


Figure 3.5 Effect of different sources of organic compounds on *N. gaditana* growth

The effect of various organic carbon sources on cell growth. The cells illuminated continuously with 200 μ E at 25 °C. The temperature was set up at 25 °C in the growth chambers.

¹ <http://www.nannochloropsis.org/>

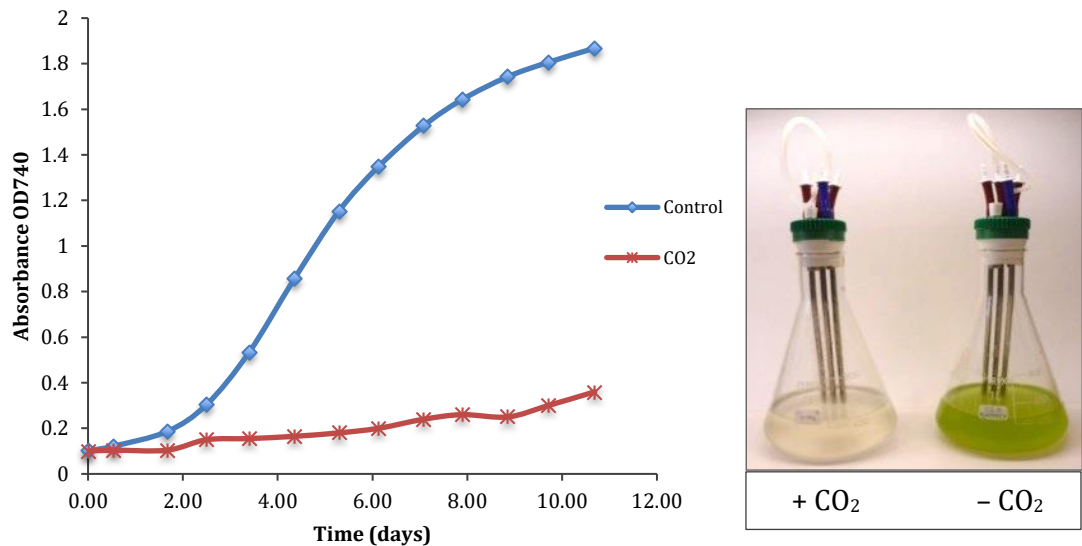


Figure 3.6 The effect of 5% CO₂ on the cultivation of *N. gaditana* in the Algem® photobioreactor.

In left panel; the growth curves of two cultures i) a control culture was grown in ASW without CO₂. ii) a tested culture was bubbled with 5% CO₂ at 50 cm³ per min. In right panel, a visual comparison of the effect of CO₂ after 10 days of cultivation. +5% CO₂ and -5% CO₂ indicate the cultures were grown in presence and absence of CO₂ supply, respectively. The cultures were illuminated continuously with 200 μE at 25 °C in ASW.

As reviewed by Croft et al. (2006) a number of algal species require an exogenous supply of one or more B vitamins for their metabolism: namely, vitamin B₁ (thiamine), vitamin B₁₂ (cobalamin) and vitamin B₇ (biotin). Therefore, the effect of those vitamins has been tested in *N. gaditana* growth. However, the addition of exogenous vitamins did not improve the growth of *N. gaditana* (data not shown). This result is further supported by a BLAST search of the *Nannochloropsis gaditana* genome which identified the *METE* gene (Nga 00266) that encodes the B₁₂-independent methionine synthase. The presence of this gene has been shown to correlate with a lack of dependence for exogenous B₁₂ (Helliwell et al. 2011). In addition, the under representation of genes involved in vitamin acquisition as reported by Radakovits et al. (2012) indicates that *N. gaditana* growth does not require exogenous sources of B vitamins.

After testing different growth conditions, the best achieved specific growth rate of *N. gaditana* in ASW using the Algem® is under sine wave illumination of 100–200 μE at 25 °C and ranged from 0.8 to 0.69 per day (i.e. a doubling time of 21–24 hours, respectively). The preliminary growth studies using Algem® facilitated the identification of the key parameters in *N. gaditana* growth include temperature and light conditions. However, the best light conditions to cultivate *N. gaditana* using the Algem® was not possible to be applied in the subsequent experiments mainly due to i) the absence of the sine wave condition in the standard growth chamber and ii) the growth of cultures in small volumes (≤ 20 ml) can be inhibited by high light intensity (See section 2.3 for the volumes used in the Algem®).

3.2.2 Evaluation of antibiotic sensitivity

In order to identify a suitable selectable marker for chloroplast transformation, the antibiotic sensitivity of *N. gaditana* was evaluated by growth tests on solid medium (i.e. “spot tests”). This technique involved spotting a few microlitres of culture onto nutrient agar plates and then assessing the growth in the presence of various antibiotics compared to a control plate without antibiotic. The spot test results, as summarized in Table 3.2, indicate *N. gaditana* is resistance to different antibiotics even at high concentrations as 200 µg/ml. In particular, *N. gaditana* shows resistance to kanamycin, streptomycin and spectinomycin at tested concentrations from 10 to 200 µg/ml. Sensitivity of *N. gaditana* to erythromycin and paromomycin was observed at 100 µg/ml and higher. Although these five antibiotics cannot therefore be used as a selection method for chloroplast transformant lines, they could be useful for eliminating bacterial or other contaminants in *N. gaditana* cultures. *N. gaditana* is most sensitive by far to chloramphenicol in which cell growth was strongly inhibited from 50 µg/ml as shown in Figure 3.7. Chloramphenicol therefore was initially chosen as a potential selection method for chloroplast transformants. The inhibitory effect of chloramphenicol was further studied by more tests to determine the appropriate concentrations to inhibit the growth of *N. gaditana* at high cell density throughout a period of time. Sensitivity of *N. gaditana* to chloramphenicol was tested by spreading a culture at mid-log phase on ASW plates containing 50, 100 and 200 µg/ml of chloramphenicol and the results, as shown in Figure 3.8, demonstrate a very low frequency of any spontaneous resistance to chloramphenicol with no colonies observed on any of the plates. The minimum concentration of chloramphenicol required to inhibit the *N. gaditana* growth was 50 µg/ml, which is consistent with results reported on-line by PhotoSynLab². However, a number of *Nannochloropsis* strains were found to be resistant to 100 µg/ml of chloramphenicol (Vieler et al. 2012).

Table 3.2 The effect of a number of selected antibiotics on *N. gaditana* based on spot tests.

The concentrations of tested antibiotics are 10, 50, 100 and 200µg/ml. “+” indicates the natural resistance to the respective concentration from low to high. Resistance gene refers to a microbial gene conferring resistance to the corresponding antibiotic.

Antibiotic	Resistance gene	Natural resistance (10, 50, 100 and 200 µg/ml)
Kanamycin	<i>aphA-6</i>	++++
Streptomycin	<i>aadA</i>	++++
Spectinomycin	<i>aadA</i>	++++
Erythromycin	<i>ereB</i>	++
Paromomycin	<i>aphVIII</i>	++
Chloramphenicol	<i>catA1</i>	+

² <http://unitedscientists.org/labs/norway/NTNU/PhotoSynLab/>

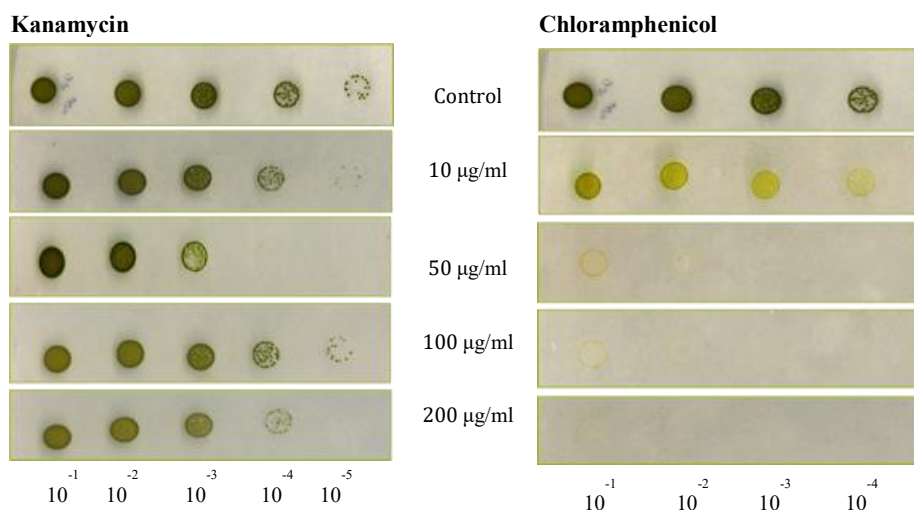


Figure 3.7 The spot test for growth response of *N. gaditana* to different concentrations of kanamycin and chloramphenicol.

Spot tests demonstrated the sensitivity of *N. gaditana* to chloramphenicol in which cell growth was strongly inhibited from 50 µg/ml. Aliquots of *N. gaditana* were spotted on 2% ASW containing increasing concentrations of kanamycin (left panel) or chloramphenicol (right panel), with serial tenfold dilutions left to right. Plates were incubated under 30 µE illumination for three weeks.

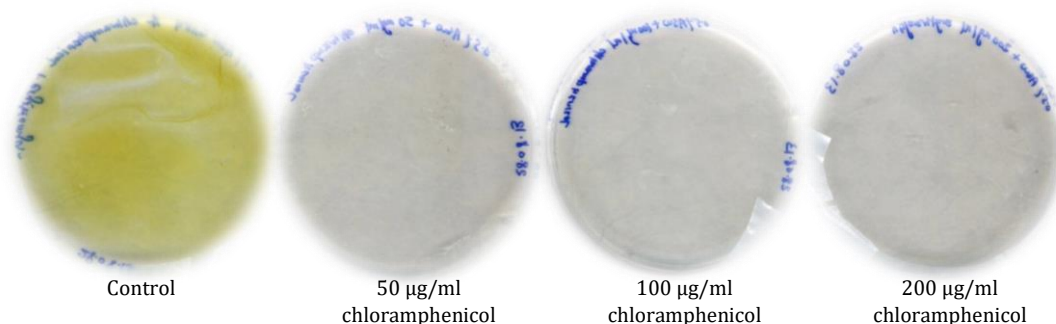


Figure 3.8 Growth response of *N. gaditana* with high cell density to different concentrations of chloramphenicol.

Equal volumes of culture at mid-log phase were spread onto ASW medium (control) and on ASW containing 50, 100 and 200 µg/ml of chloramphenicol. The plates were left to grow for three weeks under 30 µE light at 25 °C. The control plate has shown the growth of *N. gaditana*, whereas the cells on ASW plates containing chloramphenicol were not able to grow.

A further test to confirm the effect of chloramphenicol was carried out in liquid ASW to ensure the evaluation of antibiotic sensitivity was conducted in both agar-solidified ASW and liquid ASW. This test involved growing *N. gaditana* to mid-log phase and then the culture was aliquoted into a 12 well microplate; 3 ml in each well. The chloramphenicol was added to each well to give a range of concentrations from 10 µg/ml to 300 µg/ml. However, the chloramphenicol was omitted from three wells as a positive control for each row. Four weeks later, the sensitivity of *N. gaditana* to increasing concentrations of chloramphenicol

in liquid state was evaluated by spotting 5 μ l of each culture onto a 1.5% ASW plate. The results (see Figure 3.9) show the similar inhibition effect to as indicated earlier by both the spot and spread tests. However, the effect of chloramphenicol in liquid state was more potent compared to the agar-solidified ASW, with the cell growth inhibited at concentrations as low as 10 μ g/ml. In general, the evaluation of sensitivity of *N. gaditana* against a wide range of antibiotics using different test methods confirms that the *N. gaditana* CCMP526 used in this study is most sensitive to chloramphenicol. Therefore, the chloramphenicol acetyl transferase gene (*cat*) represented the most promising selectable marker for chloroplast transformation of *N. gaditana*. This marker has been used successfully for chloroplast transformation of tobacco (Li et al. 2011), although attempts to express the marker in the green alga *C. reinhardtii* were not successful (Wannathong 2012).

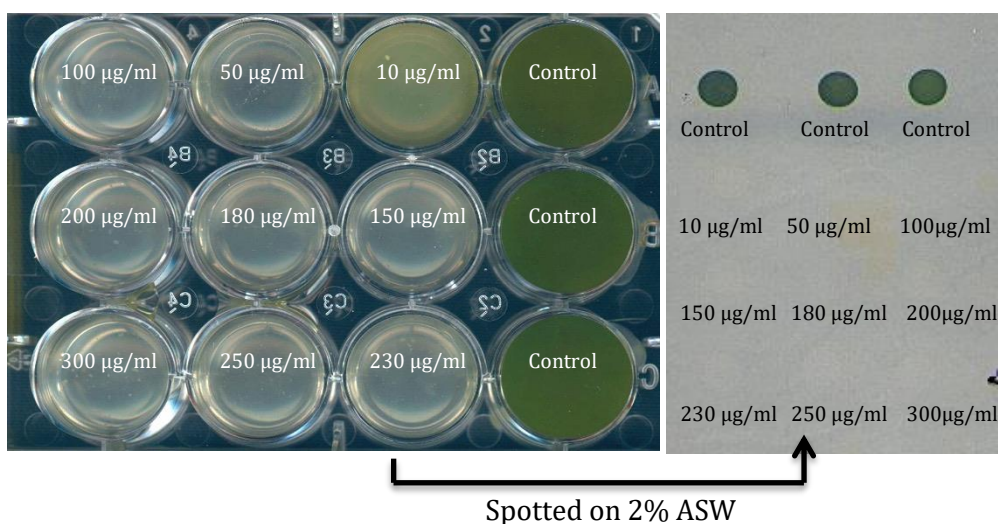


Figure 3.9 Growth response of *N. gaditana* to chloramphenicol in liquid ASW at different concentrations.

An aliquot of 3 ml of a culture at mid-log phase was pipetted into each well of a 12 well microplate (left panel). The cultures in three wells were used as controls, and thus no chloramphenicol was added. Whereas, the rest were treated with different concentration of chloramphenicol from 10 to 300 μ g/ml final concentration. Spotted cells on 2% ASW after being cultivated in ASW or in ASW supplemented with different concentration of chloramphenicol for three weeks (right panel).

3.2.3. Construction of a chloroplast transformation vector

3.2.3.1 Design and construction of a chloroplast transformation vector

The first stage in developing chloroplast transformation technology is to demonstrate that we can introduce foreign DNA, in the form of a selectable marker gene, into the chloroplast genome such that it is expressed and confers an antibiotic resistance phenotype. Chloroplast transformation in plants and algae occurs exclusively via a double homologous recombination event involving endogenous sequences flanking the foreign DNA (the left- and right homologous recombination regions, LHRR and RHRR), so the choice of these elements determines the site of integration of the foreign DNA as shown in Figure 3.10. Since it is desirable that this DNA integrates into a neutral locus in which the expression of endogenous genes is not affected, then the *N. gaditana* chloroplast genome was searched for a suitable insertion site (see Appendix.3 for potential insertion sites in the chloroplast genome of *N. gaditana*). The best site was identified as that shown in Figure 3.10, a region between two tRNA genes that are transcribed in opposite directions. Primers were therefore designed to amplify this region from total genomic DNA to obtain the LHRR and RHRR elements. As shown in figure 3.11A (3), a PCR product of the expected size (2164 bp) was obtained and this was cloned into the standard plasmid vector, pJet. In order to drive expression of the selectable marker gene, it is necessary to fuse it to an endogenous promoter/5' untranslated region (UTR) and a 3' UTR. The next stage of making the chloroplast transformation plasmid is therefore to amplify a suitable promoter/5'UTR. It was decided to use the element from the highly expressed *psaA* gene and this was amplified using suitable primers to give a 276 bp product with appropriate overlaps for Gibson assembly (Figure 3.11A (1)). Finally, the 3'UTR of another gene, *psbA* was amplified to give a 318 bp product, with overlaps (Figure 3.11A (1)). As chloramphenicol was suggested to be a potential selection method, the *cat* gene conferring resistance to chloramphenicol was amplified directly from plasmid pARG1.3 generating a *cat* PCR product of the expected size of 701 bp, with overlaps figure 3.11A (1). After successfully amplifying the key elements that were required for make the expression cassette –P/5'UTR. *cat*. 3'UTR– for constructing the transformation vector, the next step was assembling these overlapping fragments using Gibson assembly (Gibson et al. 2009). The product of the Gibson assembly reaction was used as a template for a second round of PCR to add AflII restriction sites resulting in a 1157 bp fragment, as shown in Figure 3.11A (2). The *cat* cassette was then digested with AflII and ligated into the linearized LHRR and RHRR element cloned into pJet to form the desired construct. The correct construct (pUH.NCc2) was confirmed by digestion and DNA sequencing. The physical map of pUH.NCc2 is shown in Figure 3.11B.

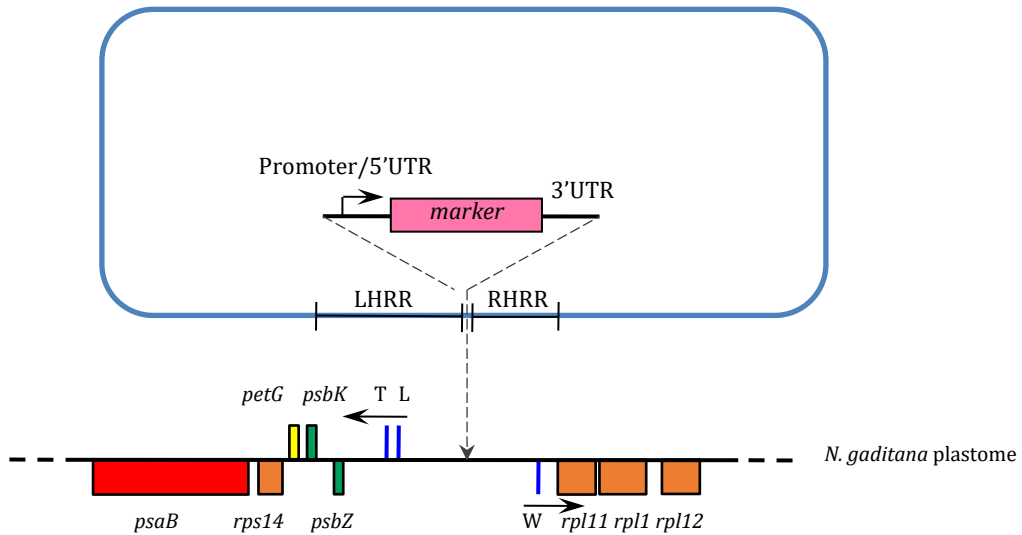


Figure 3.10 Schematic representation of chloroplast transformation by homologous recombination in *N. gaditana*.

A typical chloroplast expression vector containing the essential elements for introducing a foreign gene into the chloroplast genome. The foreign gene in the form of a selectable marker is placed under the control of endogenous regulatory sequences (promoter/5'UTR and 3'UTR). For the precise integration into the chloroplast genome by double homologous recombination event, the cassette is flanked by endogenous sequences called the left and right homologous recombination regions (LHRR and RHRR). The intergenic region between *trnW* and *trnL* may act as a neutral site for cassette integration since *trnW* and *trnL* are transcribed in opposite directions. The transcriptional direction of the genes is depicted by their location on the strand and arrows. The chloroplast genes are colour coded by function; green for photosystem II (*psbK* and *psbZ*), red for photosystem I (*psaB*), yellow for cytochrome *b₆/f* (*petG*), blue for tRNA (*trnW*, *trnL* and *trnT*) and orange for transcription/translational (*rpl11*, *rpl1*, *rpl12* and *rpl14*). Abbreviations are T: *trnT*, L: *trnL* and W: *trnW*

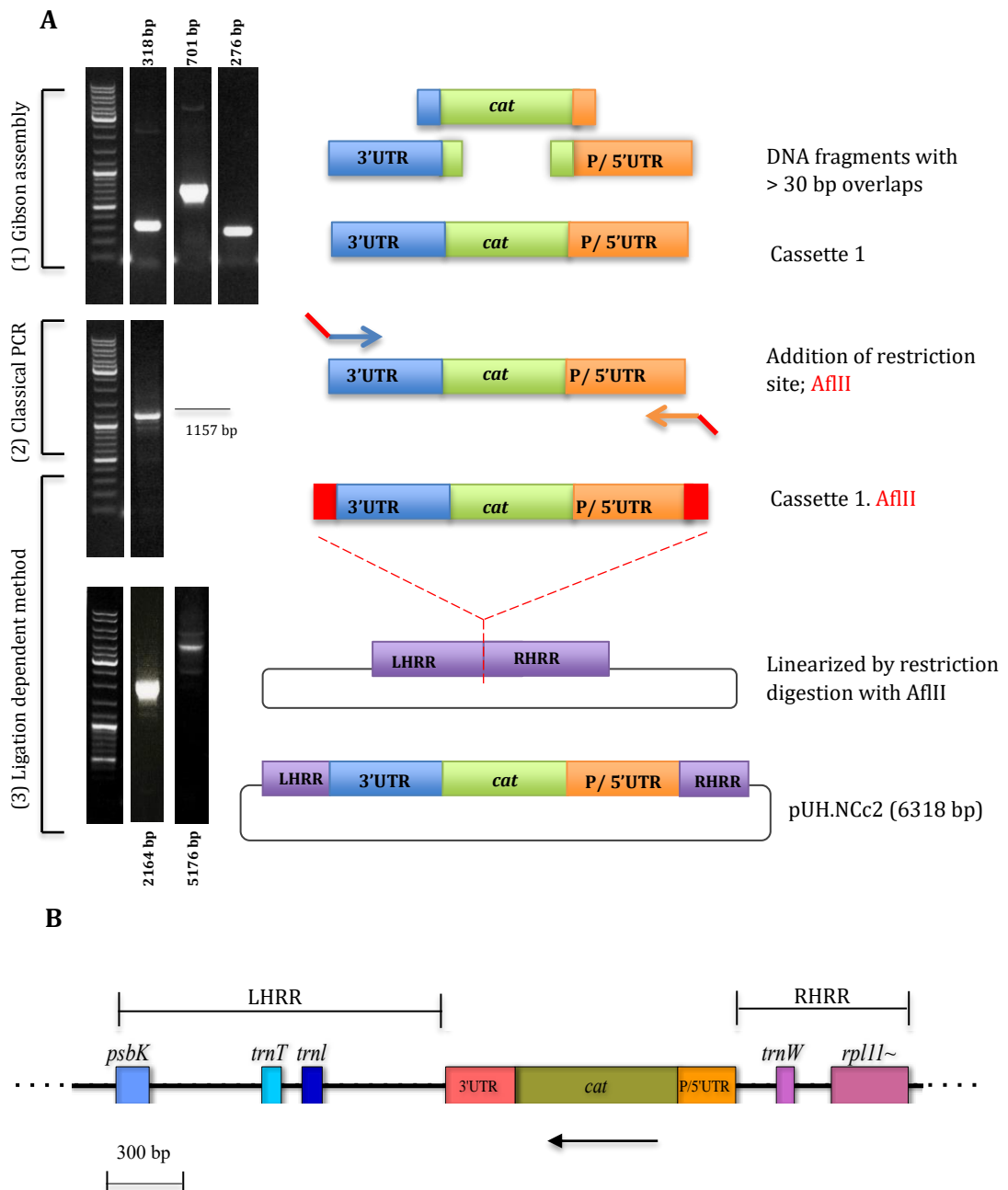


Figure 3.11 A schematic representation of the steps involved in making the chloroplast transformation vector pUH.NCc2.

(A) Schematic diagram of the steps involved in making the chloroplast transformation vector pUH.NCc2. Three parts consisting of i) the promoter and 5'UTR of *psaA*, ii) the *cat* gene, and iii) the 3'UTR of *psbA* were amplified successfully at expected size resulting in three DNA fragments sharing terminal sequence overlaps. These three parts were assembled using a Gibson assembly method. The restriction sites were added to the assembled parts (cassette1) by PCR, resulting in a 1157 bp product. In parallel, the cloned LHRR:RHRR region in the pJet vector was linearized by digestion and the expected size was obtained ~ 5176 bp. The linear vector backbone was ligated to the cassette, forming a construct of 6318 bp; pUH.NCc2. (B) The pUH.NCc2 vector containing the *cat* gene under the control of the *psaA* promoter/5'UTR and the *psbA* 3'UTR. This *cat* expression cassette is bordered by sequence between *psbK* and *trnL* gene as left homologous recombination region (LHRR) and sequence around the *trnW* gene as right homologous recombination region (RHRR). The pJet vector region of the plasmid is depicted as a dashed line.

3.2.3.2 Studying the expression of *cat* gene under the control 5' untranslated of *N. gaditana* chloroplast in *Escherichia coli*.

There are a number of chloroplast genes in microalgae that have maintained bacterial features of regulatory elements such as the -35 and/ or -10 promoter elements and 70S ribosome-binding site. This can be exemplified by the promoter and 5' untranslated region (P/5'UTR) of the highly expressed *psaA* exon 1 gene in the *C. reinhardtii* chloroplast. The P/5'UTR of *psaA* has been used extensively to drive robust expression of foreign genes in the *C. reinhardtii* chloroplast and has also been found to function effectively in *E. coli* (Wannathong et al. 2016). Indeed, a strategy of codon reassignment has been developed to overcome the problem of cloning genes that are toxic to *E. coli* when producing the plasmid vector (Young & Purton 2016).

In order to investigate possible *E. coli* ribosome binding in the 5' UTR of the *Nannochloropsis* *psaA* (referred as *psaAⁿ*) used in the chloroplast transformation vector, the nucleotide sequences around the translational initiation site of *psaA* were analysed. This involved alignment of the 35 nucleotide regions immediately upstream from the start codon of *cat*, *psaA^c* and *psaAⁿ* then comparing these mRNA sequences to a consensus Shine-Dalgarno (SD) sequence from *E. coli* (Figure 3.12A). The *cat* sequence represents the P/5'UTR region found on pARG1.3 that drives the expression of this bacterial gene. The *psaA^c* sequence represents the P/5'UTR of the *psaA* gene from the *C. reinhardtii* chloroplast genome.

As illustrated in Figure 3.12A, the initial analysis of these regions reveals the presence of SD core sequences in the mRNA sequences of *cat* and *psaA^c*, but not *psaAⁿ*. The absence of the core sequences of SD in the mRNA sequences of *psaAⁿ* suggests that translation initiation for the *cat* gene in *E. coli* is not possible using this chloroplast element and therefore, the CAT protein will not be produced in *E. coli*. To support the initial suggestion, a set of *E. coli* cultures carrying different plasmids (i.e. pARG1.3, pSRSapI and pUH.NCc2) were spotted onto solid medium containing increasing amounts of chloramphenicol to test for growth (Figure 3.12B). It is noteworthy that pSRSapI does not contain *cat* gene while pUH.NCc2 lacks of a core SD sequences, hence the growth of these transformants are not expected under chloramphenicol selection. As expected, the growth test shows the growth of a positive control, pARG1.3 transformant, while pSRSapI was not able to grow under such selection. For pUH.NCc2 there is some limited growth observed only at the lowest chloramphenicol and highest plating (10^{-1} dilution), demonstrating that translation of *cat* is occurring very inefficiently. The absence of SD core sequence on the mRNA sequences of *N. gaditana* *psaA*, together with our demonstration that the cells were not able to grow under chloramphenicol selection, shows that ribosome interaction upstream of the *psaA* start in the *N. gaditana* chloroplast is sufficiently different to that in *E. coli*, and therefore efficient translation is not achieved in *E. coli*.

3.2.4. Attempts to transform *Nannochloropsis* chloroplast using pUH-NCc2 plasmid

Although chloroplast transformation is the main goal in this study, nuclear transformation was used as a control along with chloroplast transformation attempts. The initial choice of nuclear selectable marker was based on sensitivity of *N. gaditana* to different antibiotics as demonstrated by Vieler et al. (2012), in which zeocin was shown to be the most effective at low concentration. The *Sh ble* gene confers resistance to zeocin, and therefore this gene was chosen as a selectable marker for the nuclear transformation experiments. This marker has been used successfully for nuclear transformation of *N. gaditana* (Radakovits et al. 2012) and other *Nannochloropsis* species (Kilian et al. 2011). The construct pQYZ1 (4611 bp) was kindly provided by Stephen Slocombe at SAMS. As shown in Figure 3.13A, pQYZ1 is a transformation vector for introducing a *Sh ble* gene into the *Nannochloropsis* nuclear genome under the control of *VCP1* promoter/ 5'UTR and the *FCP* 3'UTR from *Nannochloropsis oceanica* 849/1 and *Phaeodactylum* plasmid pPha-T1, respectively. Several groups have developed an efficient transformation method based on electroporation of intact *Nannochloropsis* cells (Kilian et al. 2011; Radakovits et al. 2012; Vieler et al. 2012) and therefore the described protocol has been adapted initially to test the delivery of pQYZ1 containing *Sh ble* gene into the nuclear genome. For all nuclear transformation experiments, the pQYZ1 plasmid was linearized with *ScaI*, cleaving in the ampicillin resistance gene (*amp^R*).

The electroporation experiment was conducted using the maximum voltage 2,500 V (12,500 V/cm) with either 5 or 10 µg DNA, yielding on average 28 and 70 colonies, respectively. This result is in agreement with other studies, in which increasing the amount of DNA leads to an increase in the number of transformants per transformation but within a specific range (Kilian et al. 2011). The employed electric field to transform *N. gaditana*, 12,500 V/cm, is higher than is typically used for *C. reinhardtii* (approx. 1,800–2,300 V/cm) and *Chlorella pyrenoidosa* (approx. 3500 V/cm) (Run et al. 2016; Shimogawara et al. 1998). The highest transformation efficiency achieved for the pQYZ1 construct in our attempts was $\sim 2.5 \times 10^{-7}$ per µg plasmid using a standard electroporator (Eppendorf). The putative nuclear transformants appeared 3 weeks later after incubation on solid medium supplemented with 3 µg/ml zeocin (Figure 3.13B). These colonies were picked after 4-6 weeks and streaked onto fresh zeocin plates. To confirm the successful transformation of *N. gaditana* and the synthesis of a functional Ble protein, the putative transformants were further tested by spot test and PCR analysis. In the spot test (Figure 3.13B) all selected colonies (T1-T8) grew on medium supplemented with 3 µg/ml zeocin. Meanwhile, genomic PCR demonstrated the successful integration for the *Sh ble* gene into the nuclear genome,

as the transgene was detected in the selected colonies by a band of the expected size of 370 bp, but not in the wild type (Figure 3.13B).

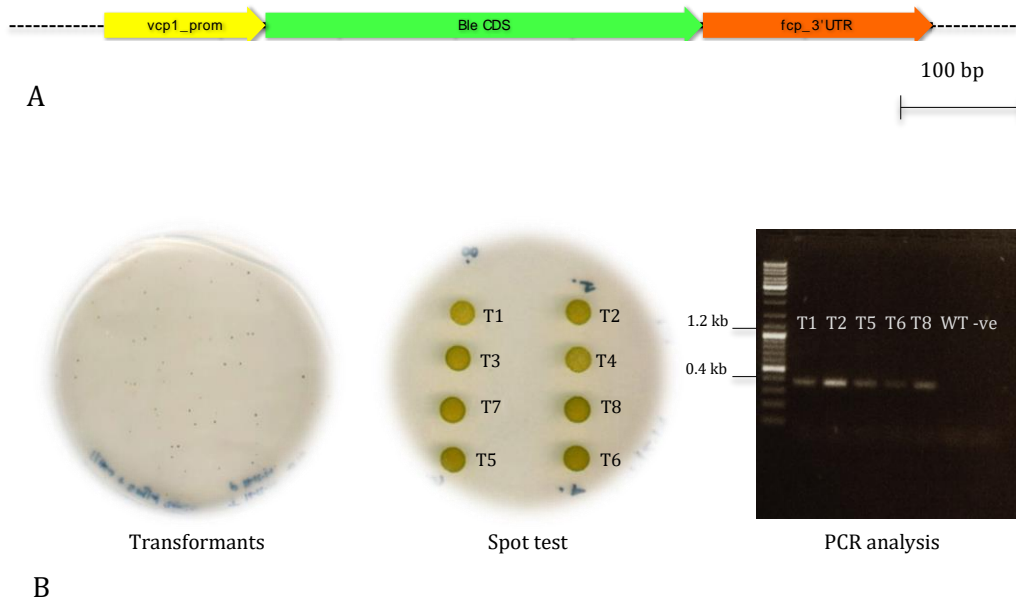


Figure 3.13 Analysis of nuclear transformants obtained with pQYZ1 using electroporation.

(A) The pQYZ1 construct for nuclear transformation, conferring resistance to zeocin, was used as a control along with chloroplast transformation attempts. (B) Left panel: putative nuclear transformants after four weeks incubation on 2% ASW medium supplemented with 3 $\mu\text{g}/\text{ml}$ zeocin. Central panel: a number of putative nuclear transformants was selected and spotted on ASW containing 3 $\mu\text{g}/\text{ml}$ zeocin. All selected putative nuclear transformants (T1 –T8) grew under zeocin selection. Right panel: PCR analysis for the transformant lines (T1-T2, T5-T6 and T8). The *ble* marker gene was detected in all tested transformants by a band at the expected size of 370 bp while no band was present in the wild type sample (WT).

Following the success of optimizing the nuclear transformation for *N. gaditana* using electroporation, the same conditions were initially tested for chloroplast transformation with pUH.NCc2. Several attempts were made using the electroporation system of which none were successful. These attempts are summarized in Table 3.3 and Table 3.4, where different experimental conditions were tested using two different electroporators. In addition to electroporation attempts, a biolistic method was tested to deliver exogenous DNA into the nuclear and chloroplast genome. However, the initial attempts were not successful in generating either nuclear or chloroplast transformants. The unsuccessful attempts could be due to the small size of *Nannochloropsis* cells (approximately 2–4 μm diameter), which is relatively small in comparison to the gold microparticles used for biolistics, around 0.55 μm in diameter.

Table 3.3 Summary of various experimental conditions to test the feasibility to transform the chloroplast of *N. gaditana*.

A standard electroporator (Eppendorf) was used resulting in a nuclear transformation efficiency for the control construct pQYZ1 of $\sim 2.5 \times 10^{-7}$ per μg plasmid. The maximum voltage deliverable by the system, 2500 V, was used with a set resistance of 600 Ω and capacitance at 10 μF .

	Plasmid	DNA (μg)	Plasmid form	MgCl ₂ (mM)	Selection ($\mu\text{g}/\text{ml}$)	Salmon sperm DNA (μg)
A	pUH.NCc2	10	Linear	-	Chloramphenicol (50, 100)	-
B	pUH.NCc2	10	Circular	-	Chloramphenicol (50, 100)	-
C	pUH.NCc2	10	Linear ^Φ	-	Chloramphenicol (50, 100)	-
D	pUH.NCc2	10	Linear	0.1, 0.3, 0.5	Chloramphenicol (50, 100)	-
E	pUH.NCc2	10	Circular	0.1, 0.3, 0.5	Chloramphenicol (50, 100)	-
F	pUH.NCc2	10	Linear ^Φ	0.1, 0.3, 0.5	Chloramphenicol (50, 100)	-
G	pUH.NCc2	10	Linear	-	Chloramphenicol (50, 100)	25 μg
H	pUH.NCc2	10	Linear	-	Chloramphenicol (50, 100)	25 μg ^δ
I	pQYZ1: pUH.NCc2	6: 10	Linear	-	Chloramphenicol (50) zeocin (3)	-
J	pQYZ1: pUH.NCc2	4: 8	Linear	-	Chloramphenicol (50) zeocin (3)	-

^Φ: pUH.NCc2 was double digested with XbaI and XhoI resulting two linear DNA fragments; a cassette with flanking regions ~ 3369 bp and pJet backbone ~ 2949 bp.

^δ: Salmon sperm DNA was denatured by heating at 99 °C for 10 min (Shimogawara et al. 1998)

Table 3.4 Summary of employed conditions in the chloroplast transformation attempts by using a Gene Pulser Xcell™ electroporation system.

The highest transformation efficiency for the control construct pQYZ1 is $4.0 \times 10^{-6} \sim$ per μg plasmid at 2400 V, 500 Ω and 50 μF . The selection was based on using two different concentrations of chloramphenicol; 50 and 100 $\mu\text{g}/\text{ml}$ for each condition.

	Plasmid	DNA (μg)	Voltage (V)	Capacitance (μF)	Resistance (Ω)	MgCl ₂ (mM)
1	pUH.NCc2	5	2400	50	500	0.1, 0.3, 0.5
2	pUH.NCc2	5	2400	50	500	-
3	pUH.NCc2: pQYZ1	8: 4	2400	50	500	0.1, 0.3, 0.5
4	pUH.NCc2: pQYZ1	8: 4	2400	50	500	-
5	pUH.NCc2	5	2400	10	600	0.1, 0.3, 0.5
6	pUH.NCc2	5	2400	10	600	-
7	pUH.NCc2	5	480	50	800	0.1, 0.3, 0.5
8	pUH.NCc2	5	480	50	800	-
9	pUH.NCc2	5	2400	25	200	0.1, 0.3, 0.5
10	pUH.NCc2	5	2400	25	200	-

3.3. Discussion and conclusions

Nannochloropsis gaditana CCMP 526 is a slow growing microalgae with a doubling time range from 21–24 hours, in comparison with the model green alga *C. reinhardtii* that requires 5–8 hours. The slow growth of *Nannochloropsis* species in general could be due to their oleaginous nature, where they tend to accumulate high lipid content and therefore the energy required for the cell division would be diverted for lipid accumulation. Such slow growth is noticeable among microalgae that are able to accumulate a substantial amount of hydrocarbons as is the case for *Botryococcus braunii*, where the doubling time is in excess of 48 hours (Yoshimura et al. 2013). Although, *Nannochloropsis* requires approximately a day to double, its high photoautotrophic biomass accumulation and high lipid content make this species a promising oleaginous microalga for metabolic engineering and therefore, the

direction of this research is to develop a transformation method for the chloroplast as there is no report on successful chloroplast transformation of this species yet.

The key components for a successful chloroplast transformation for any photoautotrophic organism are: the prior knowledge of the plastome sequence; a suitable selection system, and appropriate method to introduce exogenous DNA into the chloroplast. The availability of a plastome sequence as reported by Radakovits et al. (2012) was the basis of designing the chloroplast transformation plasmid for *N. gaditana*, where the essential elements comprised an endogenous promoter, untranslated regions and homologous recombination regions were chosen and amplified from the chloroplast genome (Figure 3.10). The choice of endogenous promoter and 5'UTR was from the highly expressed *psaA* gene to ensure a high level of transgene expression. This promoter and regulatory element of the corresponding *psaA* gene from *C. reinhardtii* have already been demonstrated to allow the successful expression of transgenes in the chloroplast of this model alga (Wannathong et al. 2016). The identification of a neutral site suitable for transgene integration into the chloroplast genome via homologous recombination is also a necessary requirement. A region between two tRNA genes that are transcribed in opposite directions was chosen for transgene insertion, which in theory should not interrupt the function of these chloroplast genes. These endogenous elements; promoter, regulatory elements and flanking regions were assembled along with a selectable marker, forming chloroplast transformation plasmid pUH.NCc2. In a similar approach to Goldschmidt-Clermont (1991), this plasmid would be initially used to demonstrate that we can introduce a foreign gene in the form of a selectable marker into the chloroplast genome, that it is expressed and confers the antibiotic resistant phenotype.

Another important component for a successful chloroplast transformation is the availability of a suitable selection system that facilitate the selection and analysis of transgenic lines. A number of selection systems have been used in algal chloroplast transformation, for example antibiotic resistance, herbicide tolerance, photoautotrophy and the complementation of metabolic mutants (Table 3.5). The initial choice for the selectable marker was based on the sensitivity of *N. gaditana* to different antibiotics as summarized in Table 3.2, where chloramphenicol was shown to be the most effective at low concentration. The *chloramphenicol acetyl transferase* gene (*cat*), conferring resistance to chloramphenicol was chosen as a selectable marker for the initial transformation experiments. This marker has been used successfully for chloroplast transformation of tobacco (Li et al. 2011), although attempts to express the marker in the green model alga *C. reinhardtii* were not successful (Wannathong 2012).

Table 3.5 A number of selectable marker genes are currently used in algal chloroplast technology, modified from Day & Goldschmidt-Clermont (2011).

Marker	Selection	Algal species	References
Photosynthesis			
<i>atpB</i>	Photoautotrophy	<i>Chlamydomonas reinhardtii</i>	(Boynton et al. 1988)
<i>tscA, psbH</i>	Photoautotrophy	<i>Chlamydomonas reinhardtii</i>	(Kindle et al. 1991)
<i>psaA/B</i>	Photoautotrophy	<i>Chlamydomonas reinhardtii</i>	(Redding et al. 1998)
<i>petB</i>	Photoautotrophy	<i>Chlamydomonas reinhardtii</i>	(Cheng et al. 2005)
Antibiotic resistance			
<i>ereB</i>	Erythromycin	<i>Dunaliella tertiolecta</i>	Georgianna et al. 2013
<i>aadA</i>	Streptomycin, spectinomycin	<i>Euglena gracilis</i>	Doetsch et al. 2001
<i>rrnL</i>	Erythromycin	<i>Chlamydomonas reinhardtii</i>	(Newman et al. 1990)
<i>aphA-6</i>	Kanamycin, amikacin	<i>Chlamydomonas reinhardtii</i>	(Bateman & Purton 2000)
<i>rrnS</i>	Spectinomycin, streptomycin	<i>Chlamydomonas reinhardtii</i>	(Kindle et al. 1991b; Newman et al. 1990; Roffey et al. 1991)
<i>aadA</i>	Spectinomycin, streptomycin	<i>Chlamydomonas reinhardtii</i>	(Goldschmidt-Clermont 1991)
Herbicide resistance			
<i>psbA</i>	DCMU, metribuzin	<i>Chlamydomonas reinhardtii</i>	(Newman et al. 1992; Przibilla et al. 1991)
Metabolism			
<i>ARG9</i>	Arg auxotrophy	<i>Chlamydomonas reinhardtii</i>	(Remacle et al. 2009)
<i>codA</i>	5-fluorocytosine	<i>Chlamydomonas reinhardtii</i>	(Young & Purton 2016)

The reported transformation of the *Phaeodactylum tricornutum* plastid with the *cat* gene and *gfp* gene by electroporation (Xie et al. 2014), opens up the possibility of using electroporation as a potential method for introducing exogenous genes into the chloroplast, especially for algal groups that harbour a secondary plastid. Therefore, the focus was testing and optimizing an electroporation transformation method to introduce the *cat* marker into the chloroplast genome of *N. gaditana*. An initial plasmid, pUH.NCc2 carrying a bacterial chloramphenicol resistance marker failed to generate transformants using electroporation. Nonetheless, this method showed the success in delivering pQYZ1 containing *Sh ble* gene into the nuclear genome and therefore generating zeocin resistant transformants. The successful transformation of the nucleus by electroporation necessitates unusual high electric field strength $\sim 12,500$ V/cm, which could be explained by the very robust cell wall of *Nannochloropsis*. Furthermore, this method has been demonstrated by several groups to be effective for stable nuclear transformation in different *Nannochloropsis* species (Radakovits et al. 2012; Kilian et al. 2011) yet there is no report of successful chloroplast transformation. The challenge in delivering the exogenous DNA into the *Nannochloropsis* chloroplast using electroporation methods could be due to the complexity of plastid membranes, where the chloroplast is surrounded by four membranes (Figure 3.14). In this case, for chloroplast transformation, the successful delivery of the exogenous DNA into the chloroplast genome relies on crossing the following biological barriers: cell wall,

cytoplasmic membrane and four plastid membranes. In contrast, the successful delivery of exogenous DNA into the nuclear genome requires overcoming solely two biological barriers: cell wall and cytoplasmic membrane. The question arises here as the possibility of electroporation to create transient pores across all four membranes, beside the plasma membrane and cell wall through which to allow DNA entry to the chloroplast stroma.

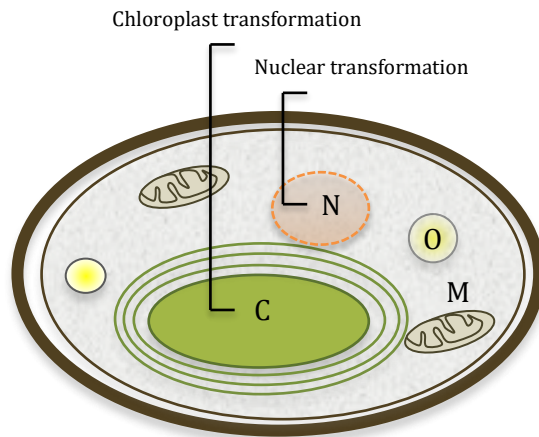


Figure 3.14 Schematic representation of *Nannochloropsis* cell illustrating the biological barriers to the deliver of exogenous DNA.

The biological barriers to exogenous DNA delivery before integration into either the nuclear or the chloroplast genome. The biological barriers for the chloroplast transformation are cell wall, cytoplasmic membranes and four chloroplast membranes. On the other hand, the biological barriers for the nuclear transformation are less, where the transgene has to go through the cell wall and cytoplasmic membranes only. Abbreviations are: C; chloroplast, N; nucleus, M; mitochondrion and O, oil body.

Alternatively, biolistics is an effective method for delivering exogenous DNA across the cell wall and multiple membranes and has been employed successfully for nuclear transformation in many species from green algae, red algae and diatoms (Doron et al. 2016). This method has demonstrated its robustness in delivering exogenous DNA into the chloroplast of algal species other than *C. reinhardtii*, for example *Haematococcus pluvialis* (Gutiérrez et al. 2012), *Dunaliella tertiolecta* (Georgianna et al. 2013), and *Platymonas subcordiformis* (Cui et al. 2014). In our search for an appropriate delivery method for the *Nannochloropsis* chloroplast, this method was tested for delivering the exogenous DNA into the nuclear and chloroplast genome. Surprisingly, our attempts failed to generate either nuclear or chloroplast transformants. An implication of this result is that the invasive nature of this method prevented the recovery of transformants in the case of *Nannochloropsis* due to the small size of cell (approximately 2–4 μm). The recent availability of gold nanoparticles with a diameter of 40 nm might be more convenient for small cells as these tend to cause less tissue damage. O'Brien & Lummis (2011) demonstrated that the use of nanoparticles caused less tissue damage than 1 μm during the biolistic transfection of human and mouse cells. However, the use of such nanoparticles may not be suitable for

chloroplast transformation of *Nannochloropsis* species because such small particles may not penetrate the cellulosic cell wall and plastid membranes efficiently, or may not even carry enough DNA copies to favour recombination.

In general, for *Nannochloropsis*, the main challenges in delivering exogenous DNA into the chloroplast genome using commonly employed methods are the small size of the cell (and therefore the plastid) and the complexity of the plastid membranes. These challenges have been observed clearly in our transformation attempts using these delivery systems. The DNA delivery by electroporation resulted in successful nuclear transformants but not chloroplast, emphasizing the need for a more effective method to deliver DNA across more membranes. Nonetheless, the biolistic method failed to generate even nuclear transformants as the invasive nature of the employed method prevented the recovery of these small cells.

Apart from DNA delivery methods, other aspects of molecular biology might lead to the failure to generate chloroplast transformants. In fact, once a transgene has been stably integrated into the plastome, there are still a number of other issues relating to foreign gene expression such as a failure of transcription, ribosomal stalling and protein misfolding to be overcome before exhibiting the desired phenotype, as is illustrated in (Figure 3.15). This could be the case for the chosen selectable marker, as the bacterial version of the *cat* gene could be affected by codon bias resulting in inefficient translation in the *Nannochloropsis* chloroplast, thereby a codon optimization version of the gene might be required for successful expression. Several studies have shown the importance of codon optimization in improving the heterologous expression of foreign genes in the host cell (Shao & Bock 2008).

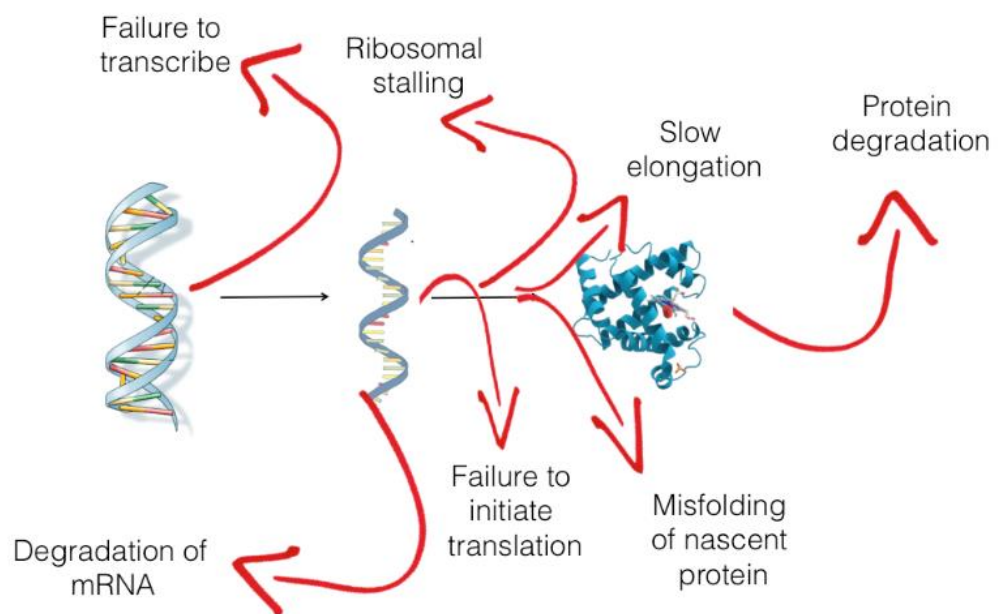


Figure 3.15 The central dogma of gene expression and potential problems.

The figure is taken from Taunt (2013).

As mentioned earlier, attempts to use the *cat* gene as marker in the *C. reinhardtii* chloroplast were not successful although tobacco chloroplast transformation was achieved using this marker (Wannathong 2012; Li et al. 2011). One suggestion for the failure in the case of *C. reinhardtii* is that the mitochondrial translational machinery of this alga is sensitive to chloramphenicol. Expression of *cat* in the chloroplast therefore does not result in a chloramphenicol-resistant phenotype as the antibiotic is still able to kill the cell through its action in the mitochondria. It could be that this is also the case for *N. gaditana*.

Generally speaking, the development of a chloroplast transformation method for a new species often requires significant effort. There are a number of potential pitfalls between introducing a foreign gene into chloroplast genome and successful expression of the gene to exhibit the desired phenotype. In spite of using two different delivery methods (electroporation and biolistics), the attempts were not successful as clearly demonstrated throughout this chapter. The reason behind such failure is normally difficult to determine due to the involvement of several factors. So, the next chapter aims to develop a more controllable approach in order to monitor the steps involved in developing a chloroplast transformation methodology. Such an approach relies on screening for chloroplast-specific compounds as a selection system and then developing an endogenous selectable marker to confer the resistance. This approach should remove some of the uncertainty regarding the expression of the marker and allow the focus to turn to identifying a successful delivery method.

CHAPTER 4

An improved approach for developing chloroplast transformation methodology in *N. gaditana*

4. An improved approach for developing chloroplast transformation methodology in *N. gaditana*

4.1. Introduction

As discussed in Chapter 3, a plasmid carrying a bacterial chloramphenicol resistance marker failed to generate chloroplast transformants despite testing two DNA delivery methods – biolistics and electroporation. The development of a chloroplast transformation method necessitates monitoring of each step, as there are several potential pitfalls between introducing the foreign gene into the chloroplast genome and the successful expression of the gene resulting in the desired phenotype. Therefore, the focus of this chapter is on employing a more controllable approach to investigate these uncertainties, as illustrated in Figure 4.1. In this strategy, the design of the selection system is based on the screening for chloroplast specific compounds that target the photosynthetic machinery instead of the prokaryotic-like translational machinery (which is found in both chloroplast and mitochondria). Furthermore, the strategy involves the development of an endogenous marker based on a chloroplast gene to eliminate issues relating to foreign gene expression. Since the small size of the *Nannochloropsis* cell represented a further issue with respect to biolistic DNA delivery, an additional step – cell synchronisation at the mitotic stage – is included prior to biolistics transformation to increase the cell size of *Nannochloropsis* and allowing the exploitation of this high velocity delivery method with these enlarged cells. This is relevant since high velocities may be necessary to penetrate the multiple chloroplast membranes without killing the cells, as illustrated in Figure 4.2.



Figure 4.1 Simplified flow chart for the proposed approach to develop a chloroplast transformation methodology.

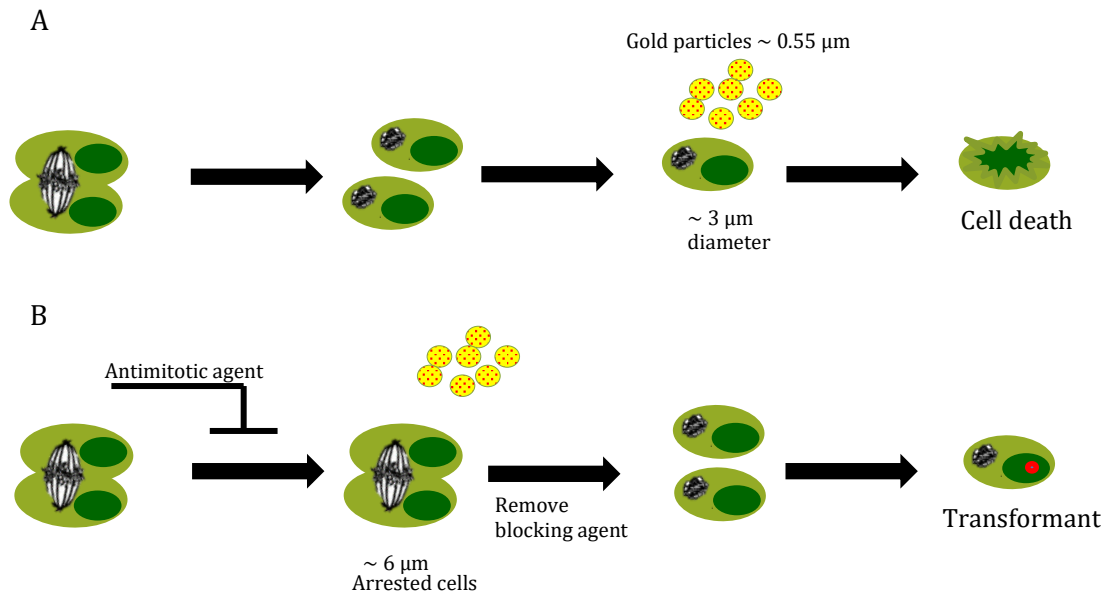


Figure 4.2 Schematic representation of biolistics transformation of *Nannochloropsis* cells using two approaches.

(A) The classical approach to deliver the exogenous DNA into the chloroplast genome using biolistics at high velocities tend to generate intracellular damage and have a higher probability of killing the *Nannochloropsis* cells due to their small size. (B) An alternative approach to deliver the exogenous DNA into the chloroplast genome of reversibly arrested cells at the mitotic stage, where they double their cytoplasmic and DNA content and so increasing the chances of generating chloroplast transformants. The size of gold particles will be used in 0.55 μm – the smallest size commercially available.

4.1.1. D1 as a target site for herbicides

Photosynthesis in cyanobacteria, algae and plants is initiated at photosystem II, a multisubunit protein-chromophore complexes embedded in the thylakoid membrane (see Figure 4.3). The light energy captured by antenna system chlorophylls is transferred rapidly to a unique chlorophyll molecule termed the reaction centre (P680), which initiates electrons transfer through a number of carrier molecules within photosystem II. The first stable electron acceptor is a bound quinone (Q_A), which carries a single electron and is closely associated with the two electron carrier, quinone Q_B , reversibly bound to the D1 protein. The secondary acceptor Q_B transfers the electrons to the mobile plastoquinone pool and then the electrons pass through the cytochrome *b₆f* complex to the copper containing plastocyanin, and then to photosystem I. The end point of electron transfer is reached when NADPH is formed on the stromal side of photosystem I.

Photosynthesis is a distinct biochemical pathway found only in photosynthetic organisms: target sites for chemical inhibition of photosynthetic are therefore unique to these organisms. For example, photosystem II herbicides specifically inhibit the photosynthesis process by disrupting electron transfer within photosystem II. The extensive screening of a large number of synthetic compounds led to the development of several photosystem II herbicide classes such as ureas and triazines (Cobb & Reade 2010). The primary action of

triazine-type herbicides such as atrazine and the chemically unrelated ureas such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU or diuron), is to inhibit photosynthesis by disrupting the electron transfer within photosystem II reaction center by competing with Q_B on its binding site on D1 protein, encoded by the chloroplast *psbA* gene. Interestingly, the D1 protein of photosystem II is also a common target for other chemically unrelated herbicides include bromacil and metribuzin (Mets & Thiel 1989).

Figure 4.3 A schematic representation of the main complexes embedded in the thylakoid membrane of the chloroplast.

These protein complexes are photosystem II (PSII), the cytochrome *b₆f* complex (cyt *b₆f*), photosystem I (PSI) and the ATP synthase complex. Electrons are transferred from photosystem II through cytochrome *b₆f* to photosystem I, as indicated by arrows. The electrons transfer is ended by using the electrons for NADPH synthesis on the stromal side of photosystem I. Protons are generated during the process as a result of water photolysis on the luminal side of photosystem II and plastoquinone driven movement of protons into thylakoid lumen. The generation of such amounts of protons inside thylakoid compartment leads to an electrochemical gradient across the thylakoid membrane. Protons flow down their concentration gradient from the lumen into the stroma through the ATP synthase complex and therefore drives the synthesis of ATP. Each protein complex contains a number of associated subunits, though some of them have been removed for clarity. Each subunit is given a letter based on the name of the gene that encodes; for instance, protein K in PSII is the product of the *psbK* gene. Labeling from left to right: tyrosine residues on D2 (Yz/Yd), unique chlorophyll reaction centre (P680), reaction core proteins (D1 and D2), core antenna proteins CP43 and CP47 (C and B, respectively), quinones bound to D1 and D2 (Q_B and Q_A , respectively), pheophytin (Phe), light harvesting chlorophyll binding proteins (LhcB1-6), mobile plastoquinone pool (PQ), iron-sulphur protein (ISP), plastocyanin (PC), light harvesting chlorophyll binding proteins (LhcA1-4), reaction centre core proteins (A/B), unique chlorophyll reaction centre (P700), electrons acceptor and donor in PSII (A0 and A1), iron-sulphur centres (F_X/F_A and F_B), ferredoxin (Fd) and oligomycin sensitivity conferral protein (OSCP). The diagram is adapted from Pyke (2009).

Natural resistance to photosystem II inhibitors has been observed as a result of single amino acid substitutions in a highly conserved region of the D1 protein (i.e. the binding pocket for Q_B). In higher plants, the atrazine-tolerant biotype has been shown to occur as a result from a single substitution of amino acid at position 264 from serine to glycine (Goloubinoff et al. 1984b). Herbicide tolerance in *C. reinhardtii* and *Synechococcus* sp. PCC 7942 was found to be caused by substituting a serine with alanine in residue 264 (Gingrich et al. 1988). The mutation at residue 264 in *C. reinhardtii* confers strong tolerance to atrazine, but not for *Synechococcus* sp. PCC 7942 where such a change confers strong tolerance to diuron. Changes in residues 219, 251, 255, 256, and 275 have been also reported to result in photosystem II herbicide tolerance in *Chlamydomonas* (Rochaix & Erickson 1988; Rand et al. 1985; Johanningmeier et al. 1987). To date, all these mutations have been shown to cluster in a small region of *psbA* gene around the codon for serine 264. However, the herbicide tolerance phenotype in general varies depending on the location of the mutation within Q_B binding pocket resulting in different level of tolerance towards different photosystem II herbicides. The most potent mutation reported by far is at residue 264 itself. A single amino acid change at residue 264 in *C. reinhardtii* showed a very high level of tolerance to the herbicides atrazine and metribuzin – at a hundred times and five thousand times the wild type level, respectively (Pucheu et al. 1984). However, the magnitude of herbicide tolerance for this mutation (i.e. at residue 264) is variable between different species.

Since many chemically diverse groups of photosystem II herbicides target the same binding site, i.e. the D1 protein, is not surprising that cross resistance is observed to other photosystem II herbicide classes. This is evident from characterization of cross tolerance in herbicide tolerant biotypes for *C. reinhardtii*, *Synechococcus* sp. PCC 794 and *Amaranthus hybridus* (Hirschberg & McIntosh 1983; Rand et al. 1985; Gingrich et al. 1988). Each tolerant biotype showed tolerance to other herbicides though these photosynthetic organisms have never been exposed to other herbicides. For instance, the mutation at residue 264 in *C. reinhardtii* confers tolerance to atrazine at hundred times the wild type level with a cross tolerance to diuron at ten times the wild type level (Rand et al. 1985). More examples of herbicide cross-tolerance are summarized in Table 4.1.

Table 4.1 Summary of reported mutations in the *psbA* gene product that result in herbicide tolerance and cross-tolerance.

The tolerance factors represent relative to the minimal inhibitory concentration for the wild type, where X means times. The table is reproduced from Gingrich et al. (1988). The references are (Hirschberg & McIntosh 1983), (Goloubinoff et al. 1984a), (Golden & Haselkorn 1985), (Johanningmeier et al. 1987) and (Rochaix & Erickson 1988).

Photosynthetic organism	Herbicide tolerance level		Mutation
	Atrazine	Diuron	
<i>Chlamydomonas reinhardtii</i>	100X	10X	264 Ser →Ala
	84X	10X	256 Gly →Asp
	25X	5X	251 Ala →Val
	15X	0.6X	255 Phe →Tyr
	1X	5X	275 Leu →Phe
	2X	17X	219 Val →Ile
<i>Synechococcus</i> sp. PCC 7942	10X	100X	264 Ser →Ala
<i>Synechococcus</i> sp. PCC 7002	7X	2X	211 Phe →Ser
	2X	10X	219 Val →Ile
<i>Amaranthus hybridus</i>	1000X	1X	264 Ser →Gly
<i>Solanum nigrum</i>	1000X	1X	264 Ser →Gly

4.1.2. Development of a selectable marker based on herbicide resistance

Photosystem II inhibitors such as those described above represent an attractive selection system for chloroplast transformation as they target specifically the photosynthetic machinery of the organelle instead of the prokaryotic-like translational machinery. The identification of herbicide-tolerant alleles within the chloroplast gene, *psbA* provide a potential endogenous marker in which introduction of the allele into the chloroplast results in replacement of the wildtype, herbicide-sensitive allele and allows selection for transformant colonies on plates containing the herbicide (Mets & Thiel 1989). The use of *psbA* as a selection system was demonstrated in *C. reinhardtii* by Boynton & Gillham (1993) who used different approaches. The first approach relied on phototrophic rescue of a non-photosynthetic recipient strain having both copies of *psbA* deleted and was transformed with a plasmid carrying a wild type *psbA*. While the second approach relied on using a herbicide tolerant allele as a selectable marker to generate resistant colonies as described above.

In addition to the use of *psbA* alleles for herbicide-based selection of chloroplast transformants of microalgae, several other approaches have been described. These approaches can be divided into two different strategies, as illustrated in Figure 4.4. The first strategy is to isolate a tolerant allele of an endogenous gene and use this to replace the wild-type, sensitive allele; the second is to introduce a foreign gene encoding an enzyme that is involved in detoxification of the herbicide. As another example of the first strategy, Lapidot et al. (2002) reported the isolation of a *Porphyridium* strain that was resistant to

sulfometuron methyl (SMM. SMM is known to target acetohydroxyacid synthase (AHAS)), which is chloroplast-encoded in *Porphyridium*, and the resistance phenotype resulted from a mutation in *AHAS*. This mutant allele was used successfully as a selectable marker for the chloroplast transformation of *Porphyridium*. Alternatively, *bar* gene is an example of a foreign gene that can be used for herbicide detoxification. This gene encodes phosphinothricin acetyl transferase (PAT) and it has been isolated from *Streptomyces hygroscopicus* which is tolerant to its own toxic product, bialaphos. The *bar* gene indeed demonstrated its usefulness as a selectable marker in the chloroplast transformation of *Platymonas subcordiformis* (Cui et al. 2014).

Since the possible markers for chloroplast genetic engineering of *Nannochloropsis* are limited, the use of a herbicide-tolerant *psbA* as a selection system could prove useful. The simplest strategy to achieve this is a forward genetic approach, which depends on random mutagenesis by physical or chemical methods followed by the selection of strains with desirable properties. Such an approach has already been used to isolate nuclear mutants of various *Nannochloropsis* species where the haploid nature of the nuclear genome make the use of this approach more appealing as both dominant and recessive mutations can display a phenotype (Kilian et al. 2011). The *psbA* gene could then be isolated from herbicide resistant strains, checked for the presence of a DNA change and then tested as a selectable marker when introduced into the chloroplast of a wild-type strain. An alternative approach would be a reverse genetics strategy in which one of the *psbA* mutations known to confer herbicide resistance in other organisms (Table 4.1) is engineered into the cloned *Nannochloropsis* gene and then this is tested as a selectable marker.

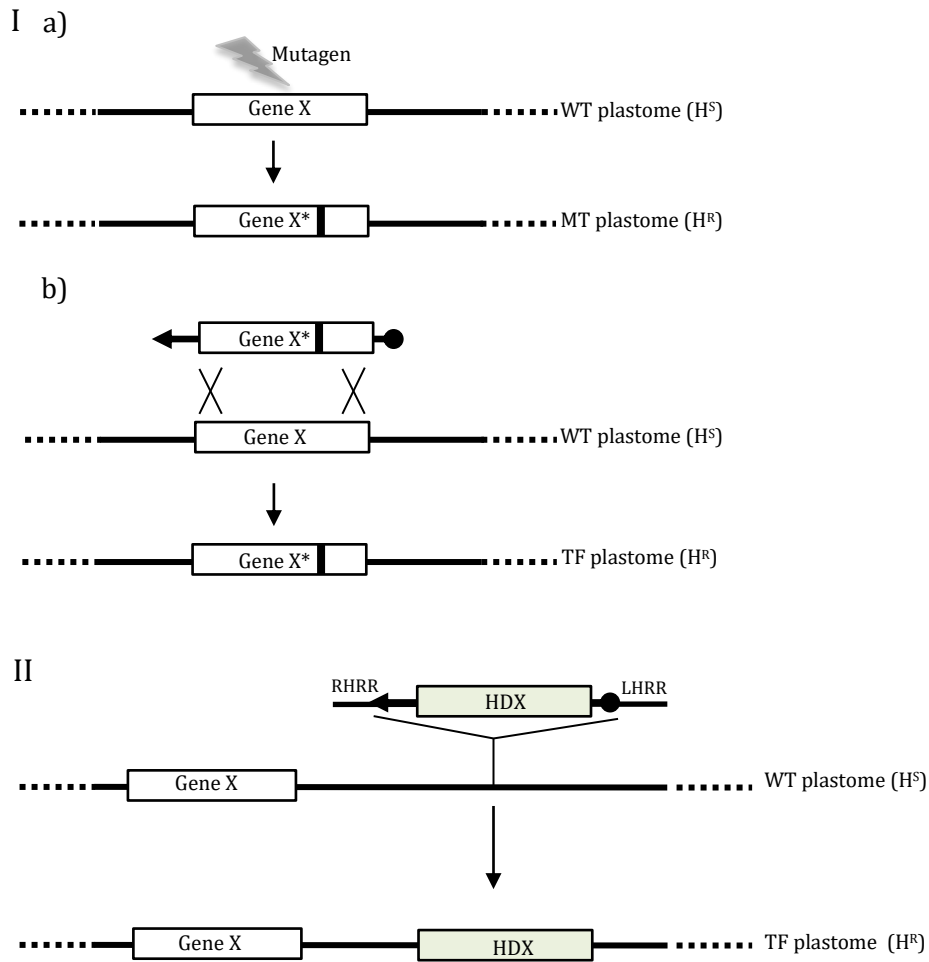


Figure 4.4 Schematic representation of two different cloning strategies to use herbicides as a selection system.

(I-a) A random mutagenesis to isolate herbicide tolerant mutants (MT) and identify the responsible allele/s for such herbicide tolerance. Gene encodes herbicide target protein is referred as X, while the gene encodes altered herbicide site that confers herbicide tolerance is indicated by X*. (I-b) Site directed mutagenesis by homologous recombination (X – X) to replace the gene X in the wild type strain (WT) with X*, allowing the selection for herbicide tolerant transformants (TF). (II) Transformation with a plasmid construct contains a gene encoding (HDX) for an enzyme causing herbicide detoxification. The HDX gene is under the control of endogenous regulatory elements and the cassette is flanked by endogenous sequences called as the left and right homologous recombination region (LHRR and RHRR). The integration of the HDX gene into the WT plastome proceeds through homologous region resulting herbicide tolerant transformants. In both cases, a gene of interest could be added alongside X* or HDX. Abbreviations are H^S : Herbicide sensitive strain, H^R : Herbicide resistance strain, WT: wild type strain, MT: mutant strain and TF: transformant.

4.1.3. Cell synchronisation

Cell synchronisation is a common approach to study the cell cycle regulatory mechanisms in eukaryotic cells and thus a number of methods have been developed to synchronise cells at a specific phase of the cell cycle (Blajeski et al. 2002; Yvon et al. 1999; Matsui et al. 2012). One of the most frequently used methods is a 'block and release' method using chemical agents. This method relies on using either a single chemical or a combination of chemicals to induce the cell synchrony by blocking the cell cycle progression at a specific stage and upon the removal of the chemical/s; the synchronized cells can proceed into the next stage of the cell cycle. In general, the blocking agents arrest the cell cycle by acting on essential proteins involved in cell cycle control such as cyclin-dependent kinase or enzymes involved in DNA replication, or by acting on the mitotic apparatus of the cell cycle (Planchais et al. 2000). Cell synchronisation is also used for other purposes such as improving gene targeting and transfection efficiency (Tsakraklides et al. 2015; Grosjean et al. 2002). In this study, the aim is to use cell synchronisation as a means to increase cell size by arresting cells at the mitotic stage prior to biolistic transformation. Cell cycle progression is size dependent and arrested cells at the mitotic stage tend to be bigger in size because they have doubled their cytoplasmic content and DNA content (see Figure 4.5). Arresting the cells at the mitotic stage is relatively difficult due to the short period of this phase, however, there are a number of agents that have been routinely used to arrest various type of cells, as a summarized in Table 4.2.

Nocodazole is an example of a widely used microtubule inhibitor in the cell cycle synchronization owing to its reversibility, efficiency and specificity compared to other microtubules inhibitors such as colchicine and colcemid, where a poor recovery has been reported (Zieve et al. 1980). The use of nocodazole in cell synchronisation studies is usually coupled with other chemicals that target a different phase in the cell cycle to generate synchrony of high numbers of cells. This is known as a two-step blocking method (Matsui et al. 2012). The most relevant example is the successful synchronisation of HeLaS3 cells in anaphase and telophase by using a combination of the microtubule inhibitor nocodazole and myosin II inhibitor blebbistatin (Matsui et al. 2012). Although a two-step blocking method of cell synchronisation has shown to greatly enhance the efficiency in generating synchronised cells, a one-step method will be used in our initial investigation as there are no reported studies of algal cell synchronisation by blocking agents, with most studies carried out using yeast, mammal and plant cells. At present, the most common method of synchronising the model alga *Chlamydomonas* is by alternating light and dark cycles of a dividing culture growing phototrophically in minimal medium (Bernstein 1960; Kates & Jones 1964). There are other methods also reported to naturally synchronise *Chlamydomonas* such as an overnight dark starvation of the cells, a temperature shift, size

selection by cell sorting and alteration of light of different wavelengths (Rooney et al. 1977; Knutsen et al. 1973; Oldenhof et al. 2006).

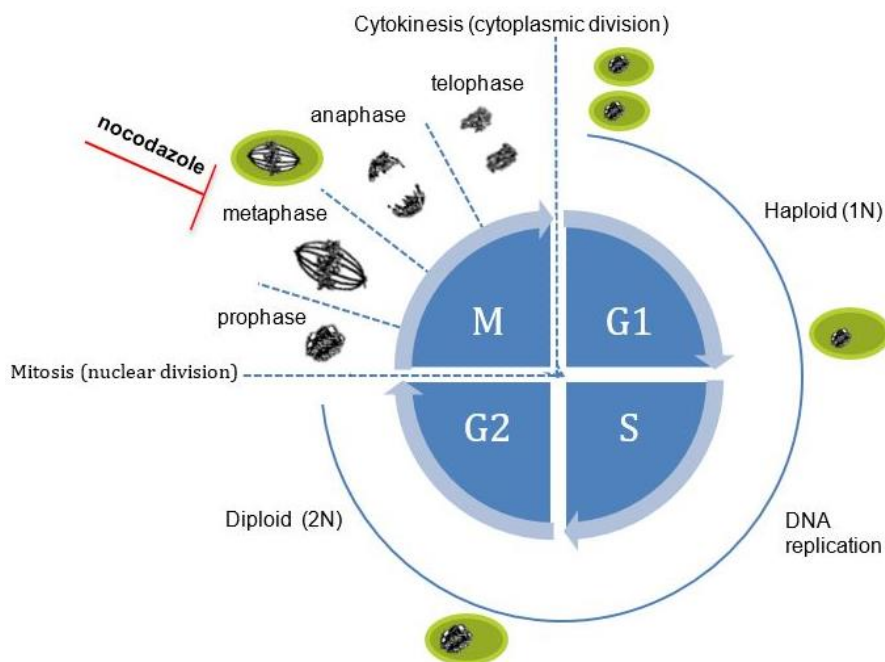


Figure 4.5 Schematic diagram demonstrates the cell cycle of *Nannochloropsis*.

The changes in DNA content and cell size for the cell as progressing through the cycle are shown, where the cell start small in size with 1N and gets bigger with 2 N prior to cytokinesis. The site of action for microtubule inhibitor nocodazole is indicated, as it arrests the cell at metaphase by preventing the microtubule disassembly. The duration of phases (G1, S, G2 and M) does not reflect the actual duration for each phase.

Table 4.2 A number of mitotic agents that are commonly used in cell cycle synchronization.

+: Reversible arrest, -: irreversible arrest, and +/-: reversibility dependent on the duration of the treatment.

Agent	Target	Block	Reversibility	Reference
Colchicine	Microtubule inhibitor	Metaphase	+/-	(Louis C. Morejohn Thomas E. Bureau 1984)
Oryzalin	Microtubule inhibitor	Metaphase	+/-	(Hugdahl & Morejohn 1993)
Propyzamide	Microtubule inhibitor	Metaphase	+/-	(Akashi et al. 1988)
Amiprophos-methyl	Microtubule inhibitor	Metaphase	+/-	(Sree Ramulu et al. 1991)
Taxol	Microtubule inhibitor	Metaphase/anaphase	+	(Yvon et al. 1999)
Nocodazole	Microtubule inhibitor	Metaphase	+	(Matsui et al. 2012)
Blebbistatin	Myosin II inhibitor	Anaphase/telophase	+	(Matsui et al. 2012)
MG132	Proteasome inhibitor	Metaphase/anaphase	-	(Genschik et al. 1998)

4.1.4. Aims and objectives

In order to be able to exploit the unique advantages associated with *Nannochloropsis*, the general aim of this chapter is to continue the investigation into developing a reliable chloroplast transformation method, but this time the focus will be solely on using biolistics as a DNA delivery method. The choice of biolistics as a delivery method to be tested is due to its robustness to deliver exogenous DNA across the multiple membranes.

Specifically the aims of the work in this chapter are:

1. Identify suitable selectable markers by evaluating *N. gaditana* sensitivity toward chloroplast-specific compounds.
2. Develop an endogenous selectable marker based on a variant of the chloroplast *psbA* gene that confers herbicide tolerance using forward and reverse genetics approaches.

Forwards approach

- Develop a protocol for *in vitro* mutagenesis and isolation of chloroplast mutants.
- Study the molecular basis of the mutations that are isolated from herbicide tolerant mutants.

Reverse approach

- Study the sequence homology of D1 sequence with other related species in order to identify the key residues in the algal D1 likely to give a herbicide tolerance phenotype when changed to another residue/s.
3. Use the findings from both forward and reverse genetics approaches to aid the identification of tolerant *psbA* allele/s and therefore incorporate the potential allele into the chloroplast transformation plasmid.
 4. Test the possibility of synchronising *N. gaditana* chemically at the mitotic stage with microtubule inhibitor nocodazole, as a means to increase the cell size prior to biolistic transformation.
 5. Develop a nuclear transformation protocol for nocodazole-treated *N. gaditana* at high velocities as a proof of concept before applying the same conditions for chloroplast transformation.
 6. Test different biolistic conditions to transform the chloroplast of *N. gaditana* by replacing the wild type *psbA* allele with herbicide tolerant one.

4.2. Results

4.2.1. Evaluation of herbicides and D-amino acid sensitivity

Nannochloropsis gaditana demonstrated its robustness in resisting a wide range of antibiotics as demonstrated previously in section 3.2.2. So, in order to identify more selectable markers than those conferring resistance to antibiotics, the susceptibility of *N. gaditana* to herbicides was also evaluated by spot tests. In addition, sensitivity to D-alanine was also examined, since this has been exploited for selection in higher plant chloroplasts (Gisby et al. 2012). The results, as summarized in Table 4.3, indicate that *N. gaditana* is highly resistant to D-alanine at the tested concentrations of 10 to 200 µg/ml. As for the herbicide compounds, *Nannochloropsis* is tolerant to Harvest, glufosinate-ammonium and bialaphos (see Figure 4.6 for bialaphos spot test). In particular, *N. gaditana* shows high tolerance to both glufosinate-ammonium and bialaphos at tested concentrations from 10 to 200 µg /ml, whereas the tolerance to the Harvest herbicide (formerly known as Basta) ranged from 10 to 100 µg/ml. As an example of herbicidal compounds, the tolerance of *N. gaditana* to bialaphos was further confirmed by testing the growth response at higher concentrations in liquid ASW as shown in Figure 4.6. Although the Harvest herbicide has been used successfully in chloroplast transformation of *P. subcordiformis*, such glutamine synthetase inhibitor herbicides cannot be used as a selection method for the chloroplast transformation in *N. gaditana*.

Another category of herbicidal compounds including atrazine and diuron which target specifically the chloroplast as photosystem II inhibitors. *N. gaditana* is sensitive to atrazine and diuron as cell growth was inhibited from 10 µg/ml as shown in Figure 4.7. Although *N. gaditana* is most sensitive to diuron based on the spot tests, atrazine was chosen to be further studied as a potential selection method as it has been used widely to select transgenic plants in atrazine degradation studies (Wang et al. 2010; Wang et al. 2005). In addition to that, two potential marker genes could be used to confer the tolerance against atrazine; a point mutation within an endogenous *psbA* gene and an atrazine chlorohydrolase gene from *Pseudomonas sp.* strain ADP (de Souza et al. 1998; Gingrich et al. 1988; de Souza et al. 1995). Thus, the inhibitory effect of atrazine was further studied by more tests to determine the appropriate concentrations to inhibit growth of *N. gaditana* at high cell density over an extended period of time. Sensitivity of *N. gaditana* to atrazine at high cell densities was tested by spreading a culture at mid-log phase (5×10^8 cells per plate) on ASW containing 50 and 100 µg/ml of atrazine and the results, as shown in Figure 4.8, confirmed the absence of any spontaneous resistance to atrazine.

Table 4.3 The effect of a number of selected chloroplast-specific compounds on *N. gaditana* based on spot test.

The concentration of tested compounds are 10, 50, 100 and 200 µg/ml. “+” indicates the natural resistance to respective concentrations from low to high concentration. Resistance gene refers to a microbial gene conferring the resistance to the corresponding compound except for the mutations in *psbA* gene. The mutation is reported previously in other microalgae.

Compounds	Resistance gene	Resistance
Amino acids		
D- alanine	<i>dao</i>	++++
Herbicides		
Harvest	<i>pat</i>	+++
Glufosinate-ammonium	<i>pat</i>	++++
Bialaphos	<i>pat</i>	++++
Atrazine	mutant <i>psbA</i> or <i>AtzA</i>	+
Diuron (DCMU)	mutant <i>psbA</i>	+

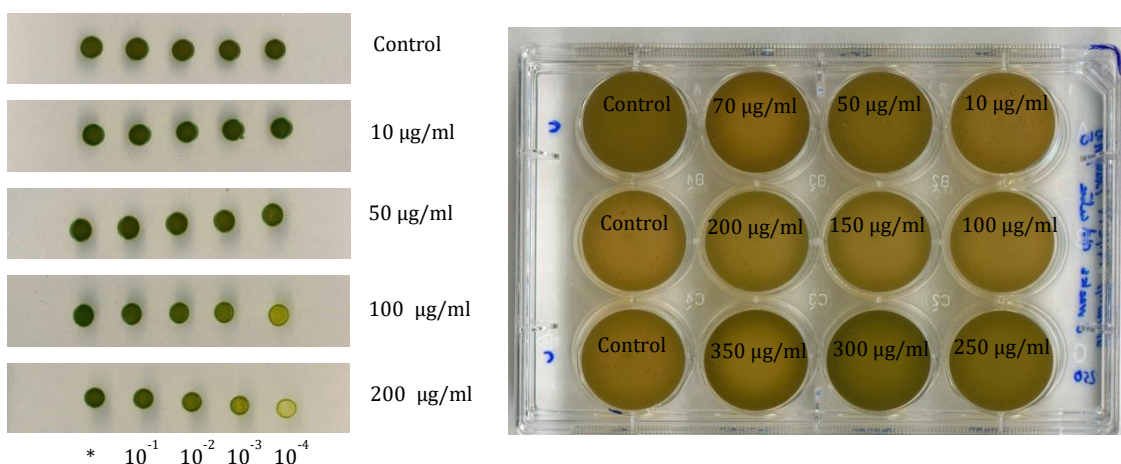


Figure 4.6 Growth response of *N. gaditana* to bialaphos in solid and liquid ASW at different concentrations.

In left panel; aliquots of *N. gaditana* were spotted on 2% ASW medium containing increasing concentrations of bialaphos with serial tenfold dilutions left to right. * indicates non-diluted cells while 10 fold dilutions of cells in ASW are represented as 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . Plates were incubated under 30–50 µE for four weeks. In right panel, an aliquot of 3 ml of a culture at mid-log phase into each well of a 12 well microplate. The cultures in three wells were used as control, and thus no bialaphos was added. Whereas, the rest were treated with different concentrations of bialaphos from 10 to 350 µg/ml. The cultures were incubated for four weeks under 30–50 µE.



Figure 4.7 Growth response of *N. gaditana* at high cell density that were spread onto ASW containing atrazine.

Equal volumes of culture at mid-log phase were spread onto ASW medium (control) and on ASW containing 50 and 100 µg/ml of atrazine. The number of cells per plate was approximately 4.95×10^8 . The control plate has shown the growth of *N. gaditana*, whereas the cells on ASW plates containing atrazine were not able to grow. Plates were incubated under 30–50 µE for four weeks.

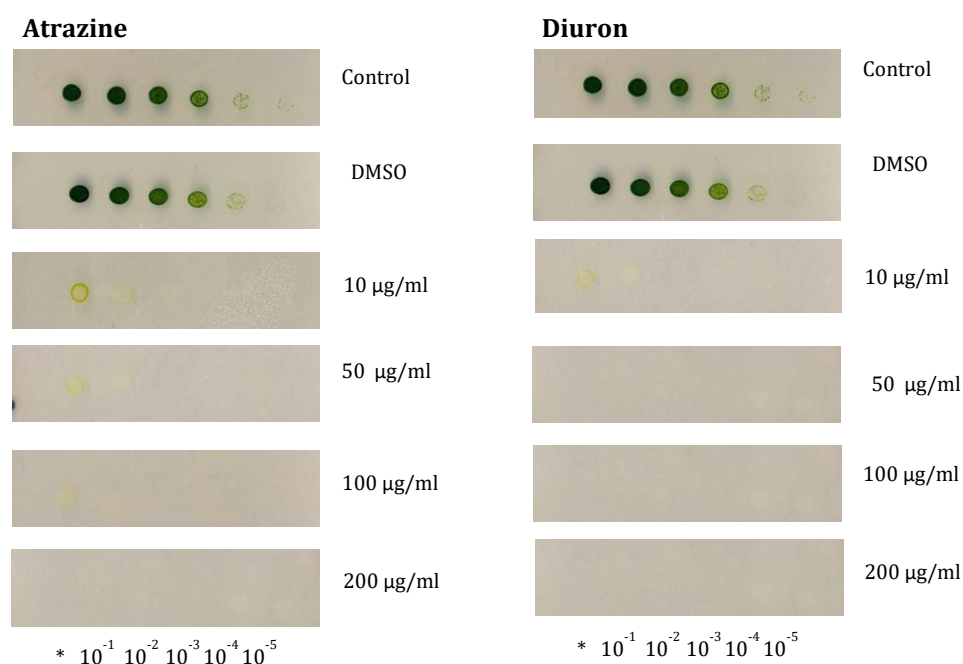


Figure 4.8 Spot test of growth response of *N. gaditana* to different concentration of atrazine and diuron.

Spot test demonstrated the sensitivity of *N. gaditana* to atrazine and diuron in which the inhibition of the cell growth starts from 10 µg/ml. Aliquots of *N. gaditana* were spotted on 2% ASW medium containing increasing concentrations of atrazine (left panel) or diuron (right panel), with serial tenfold dilutions left to right. The asterisk (*) indicates non-diluted cells. An additional spot test was included for the DMSO solvent used with atrazine and diuron. Plates were incubated under ~ 50 µE for three weeks.

An additional test to confirm the effect of atrazine was carried out in liquid ASW to assure the evaluation of atrazine sensitivity has been conducted in both solid and liquid media. This test involved growing *N. gaditana* to mid-log phase and then the culture was split into two flasks. Atrazine was added into one of the flasks to give a final concentration 50 µg/ml, while the other flask was used as a positive control. The growth response of *N. gaditana* to this low concentration of atrazine, as shown in Figure 4.9, has a similar inhibition effect as illustrated earlier by the spot and spread tests results.

The studies above confirmed that *N. gaditana* is sensitive to atrazine and therefore the bacterial gene, *atzA* that encodes the enzyme atrazine chlorohydrolase represents a promising selectable marker for chloroplast transformation. This marker gene was used for the selection of transgenic tobacco plants aimed at developing the plants for phytoremediation of atrazine-contaminated soils (Wang et al. 2005). Alternatively, there is the possibility of inducing several mutations in the *psbA* gene resulting in an atrazine tolerant biotype (Gingrich et al. 1988; Lambreva et al. 2013; Materna et al. 2009). The identification of such *psbA* alleles in *Nannochloropsis* would allow the use of these alleles as endogenous markers.

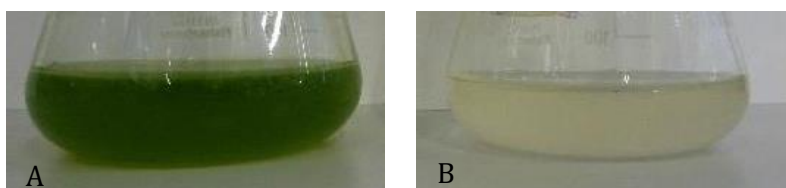


Figure 4.9 Growth response of *N. gaditana* to atrazine in liquid at low concentration.

A) *N. gaditana* grown in ASW as a control, where the culture looks healthy and green B) *N. gaditana* grown in ASW containing 50 µg/ml atrazine as the growth was inhibited due to presence of atrazine. Both cultures were left to grow under ~ 50 µE at 25 °C for 6 weeks.

4.2.2. Development of an endogenous marker using forward and reverse genetics approaches

In order to use atrazine as a potential selection method for transformant clones, reverse- and forward genetics approaches were employed to develop *psbA* as an endogenous marker.

4.2.2.1 Mutagenising *N. gaditana* using a forward genetics approach

To introduce a random mutation into the *N. gaditana* plastome, an appropriate method of mutagenesis was necessary. Several methods have been developed to introduce nuclear and chloroplast mutations, as reviewed (Harris 2009). One of these methods is ultraviolet (UV) mutagenesis, which has been used successfully for the introduction of both nuclear and chloroplast mutations. In one example, UV mutagenesis was used to generate nuclear auxotrophic and pigment deficient mutants in *C. reinhardtii* (Wang et al. 1975). As for the chloroplast mutations, there are only a few cases reported of successfully inducing these in *Chlamydomonas* (Hudock et al. 1979). The challenge of isolating chloroplast mutations is due to the polyploidy of the chloroplast genome and how the newly arising mutation would never reach homoplasmy since the majority of the organelle genomes would carry the wild type allele of the mutation. To reduce the chloroplast genome abundance, a 5-fluorodeoxyuridine (FdUrd) treatment has been shown to work effectively when combined with other mutagens (Gingrich et al. 1988; Anthony et al. 2014). In addition, FdUrd treatment has shown to facilitate the recovery of chloroplast mutants and appears to be mutagenic for chloroplast genes as well (Wurtz et al. 1979). The efficiency of this approach has been demonstrated in creating point mutations, deletions, duplications, and rearrangement of chloroplast DNA of *C. reinhardtii* (Harris 1989). The FdUrd-UV mutagenesis approach therefore was tested in *N. gaditana* to induce chloroplast mutations in the *psbA* gene.

Besides this induced mutagenesis, an initial investigation into whether spontaneous mutagenesis was sufficient to introduce such a mutation into *psbA* was made. In this experiment, an attempt was made to isolate atrazine mutants by plating approximately 10^7 cells per plate on selective ASW medium containing 50 and 100 $\mu\text{g}/\text{ml}$ atrazine. None of these attempts were successful in recovering any resistant colonies (data not shown) suggesting that any spontaneous resistance to atrazine occurs at a frequency well below 10^{-7} , and also confirming the suitability of this herbicide as a potential selection tool for plastid transformation.

In order to use UV mutagenesis to induce random mutagenesis, the optimal dose was first determined by calculating the irradiation time needed to produce a culture with a viability reduced to 10%. This was carried out by irradiating cells with UV light at a distance of 15 cm using an in-house built ultraviolet box, as described in section 2.6. As shown in Figure 4.10, the colony count before mutagenesis was 4240 and upon UV light exposure it significantly reduced. At approximately 4 minutes the culture was reduced to a 10% viable count.

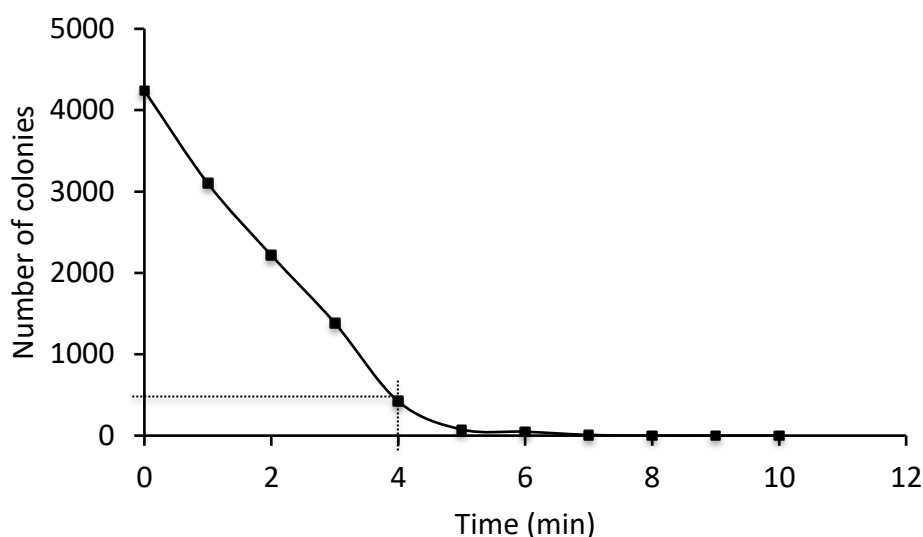


Figure 4.10 Mutagenesis "kill curve" was carried out on *N. gaditana* to determine exposure time (t_{10}) needed to produce a 10% viable culture.

Over the course of 10 minutes, the cells were removed at 1 minute interval starting at 0 minute. The cells were plated on ASW medium and the number of colonies was counted after approximately four weeks. The graph shows that on exposure to ultraviolet light for four minutes, the *N. gaditana* culture was reduced to a 10% viable count (420 colonies).

To determine if FdUrd treatment would reduce the chloroplast content of *N. gaditana* as shown for *C. reinhardtii* (Wurtz et al. 1979), PCR was used to analyse nuclear and chloroplast DNA from exponentially growing cells treated with two different concentrations of FdUrd (0.5 mM and 1 mM). The treatment of cells with 1 mM FdUrd led to an apparent reduction in total amount of chloroplast DNA compared to nuclear as shown Figure 4.11 suggesting that reduction in ploidy of the chloroplast genome can be exploited as a means of increasing the frequency of chloroplast mutations in *N. gaditana*. After determining the optimal dose of UV and confirming the efficacy of FdUrd on *N. gaditana*, a two-step approach was used in which the cells were treated first with 1 mM FdUrd and then subjected to UV mutagenesis. Around 40 putative atrazine tolerant mutants were recovered from FdUrd-UV mutagenesis. Eight mutants were picked and repeatedly re-streaked on media containing atrazine to ensure the mutants recovered are homoplasmic for the chloroplast genome before proceeding to subsequent analysis. These mutants (M1- M8)

were further tested by spot tests and analysed by PCR and sequencing of the entire *psbA*. In spot tests, M1–M8 were spotted onto an atrazine-containing plate; Figure 4.12 shows that all strains were able to grow in the presence of atrazine, unlike the wild type strain. So, the next step was identifying the changes in DNA sequences in these putative mutants by sequencing *psbA*. However, the sequence analysis did not reveal any changes in all of *psbA* gene sequence despite the atrazine tolerant phenotype (data is not shown). These results suggest that the phenotype is the result of other mutations at unknown loci and further investigation is necessary to determine whether these are affecting the uptake, action or stability of the atrazine within the *N. gaditana* cell.

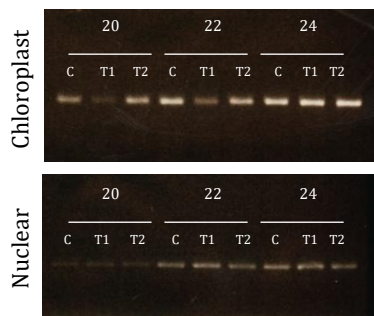


Figure 4.11 PCR to determine the effect of chloroplast DNA synthesis inhibitor on DNA content of *N. gaditana* cells.

Analysis of the effect of chloroplast DNA synthesis inhibitor on DNA content of *N. gaditana* cells that were treated with various concentrations of 5-fluorodeoxyuridine (FdUrd) at late log phase for approximately a month in the dark. The abundance of chloroplast and nuclear DNA content were determined by amplifying *psbA* and a region upstream of the nuclear *TUB* gene during exponential growth, respectively. Treatments are: C, control; T1, cells were treated with 1 mM FdUrd and T2, cells were treated with 0.5 mM FdUrd. The number of cycles used to validate the effect of FdUrd treatment on chloroplast DNA content compared to the nuclear are 20, 22 and 24. The control cells and treated cells were adjusted to equal cell densities before DNA extraction and then equal amount of template DNA from each sample were used in PCR.

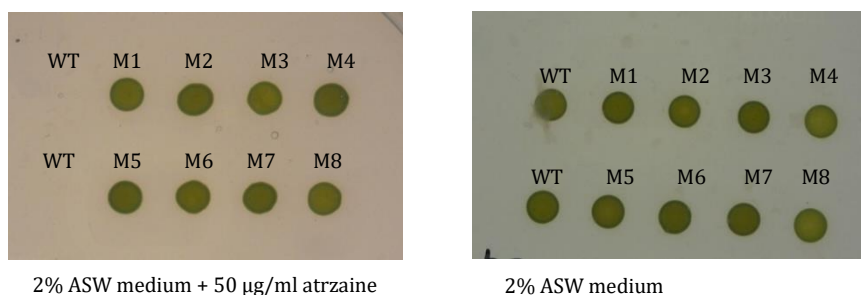


Figure 4.12 Analysis of atrazine tolerant mutants obtained from UV mutagenesis by spot test.

A number of atrazine tolerant mutants were selected, re-streaked and spotted onto ASW agar containing 50 µg/ml atrazine (left panel) and ASW agar (right panel). All selected mutants (M1- M8) were able to grow in presence of atrazine, while the wild type strain (WT) failed to grow. Plates were incubated under 50 µE for 6 weeks.

4.2.2.2 Using a reverse genetics approach to develop an atrazine-resistant *psbA* allele

Resistance to atrazine in higher plant and microalgae has been described previously to occur as a result of single amino acid substitutions in D1, encoded by *psbA* (Block et al. 1985; Hirschberg & McIntosh 1983; Gingrich et al. 1988). A mutation at residue 264 from serine to alanine has been reported by Gingrich et al. (1988) to produce the most profound effect with respect to herbicide tolerance in both *C. reinhardtii* and *Synechococcus* sp. PCC 7942. In order to study the conserved residues involved in the plastoquinone/herbicide binding pocket of D1 protein, the amino acid sequence of D1 from *N. gaditana* was compared to that from *C. reinhardtii* and *Synechococcus* sp. PCC 7002. The comparison involved the sequence retrieval of D1 for those species from the NCBI database and then alignment of amino acid sequences using MacVector. Figure 4.13, shows the alignment, and confirms that the region around serine 264 is highly conserved in all species. In addition, the crystal structure of photosystem II of *Thermosynechococcus elongatus* was used as a model to locate and highlight the interaction of these key residues within the binding pocket, as shown in Figure 4.14. Serine 264 was therefore chosen for site directed mutagenesis with the aim of changing the codon to one encoding alanine and creating a version of *psbA* that can serve as a marker for the selection against atrazine in plastid transformation.

An adapted method from triplet template PCR (Tian et al. 2004) was used to create the *psbA* allele. As shown in Figure 4.15, the gene was amplified into two fragments with primers containing the desired serine to alanine mutation at codon position 264 (i.e. AGT to GCT). The first fragment of *psbA* gene (*psbA'*) starts from approx. 20 bp upstream to the mutation site (i.e. between P 1 and P2), and the second fragment (*psbA''*) contains the rest of the coding sequences and approx. 20 bp of 3'UTR sequences (i.e. between primer P3 and P4). The PCR products of the first round of PCR share terminal sequence overlaps around the mutation site. The purpose of adding terminal sequence overlaps between *psbA'* and *psbA''* is to enable the annealing of these two PCR products together and this can act as a template for the second round of PCR resulting in a full-length *psbA* gene with the desired mutation (*psbA264A*). The *psbA264A* was created successfully and the changes at codon position 264 were confirmed by DNA sequencing.

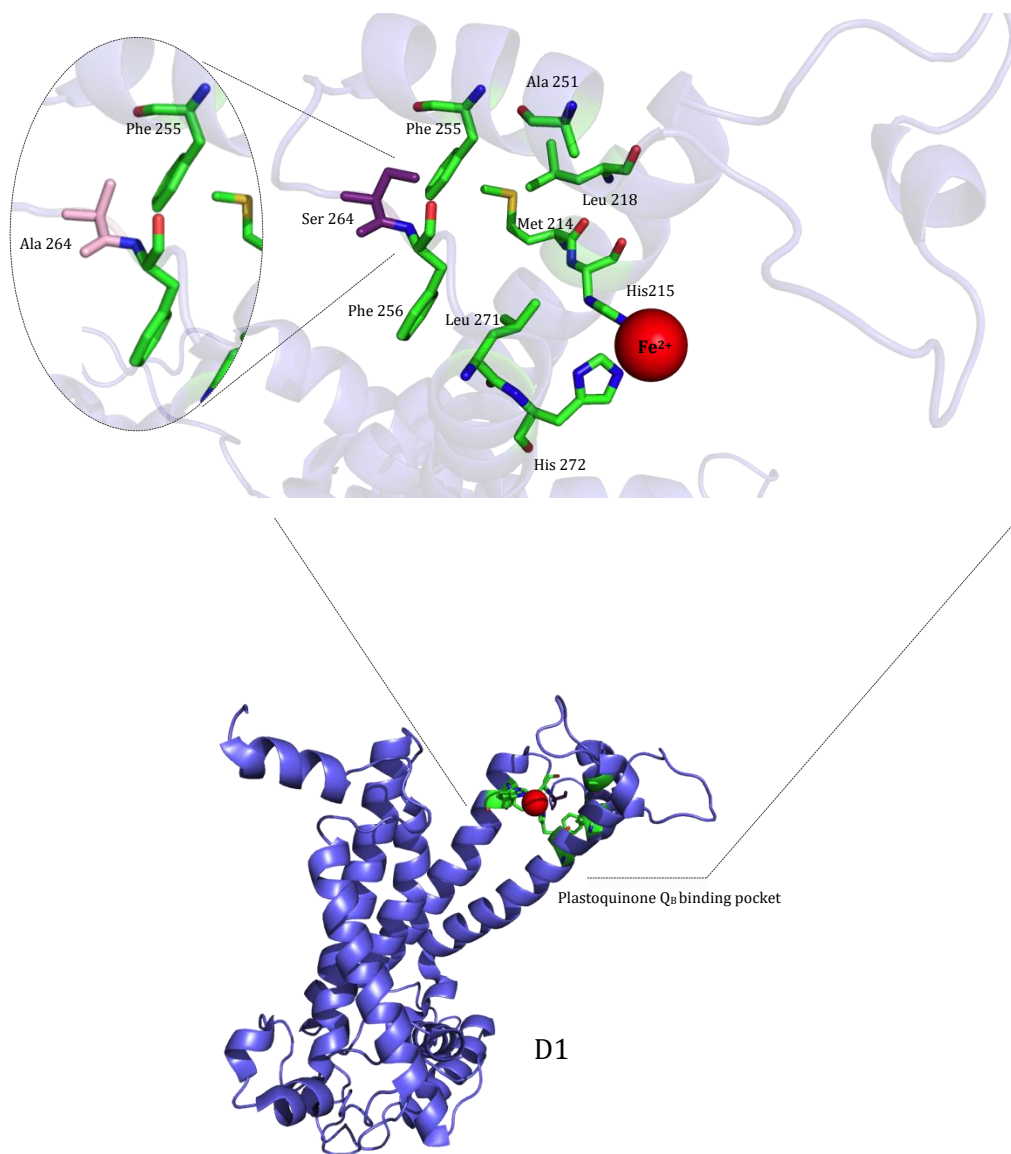


Figure 4.14 Crystal structure of D1 protein shows plastoquinone Q_B binding pocket.

Crystal structure of D1 protein shows plastoquinone Q_B binding pocket. The structure of D1 protein was obtained from the crystal structure of photosystem II based on *T. elongatus* model. The plastoquinone Q_B binding pocket is expanded (zoomed in), and the interacting residues of the binding pocket are labeled. Non-haem Fe^{2+} is depicted as a red sphere. The *Nannochloropsis gaditana* residue 264 (serine) was chosen for site directed mutagenesis and the corresponding plastoquinone Q_B binding pocket of *Thermosynechococcus* is zoomed in, highlighting the changes from serine to alanine. The protein structure was retrieved from the Protein Data Bank (PDB identifier 2axt), visualized and reproduced using PyMOL.

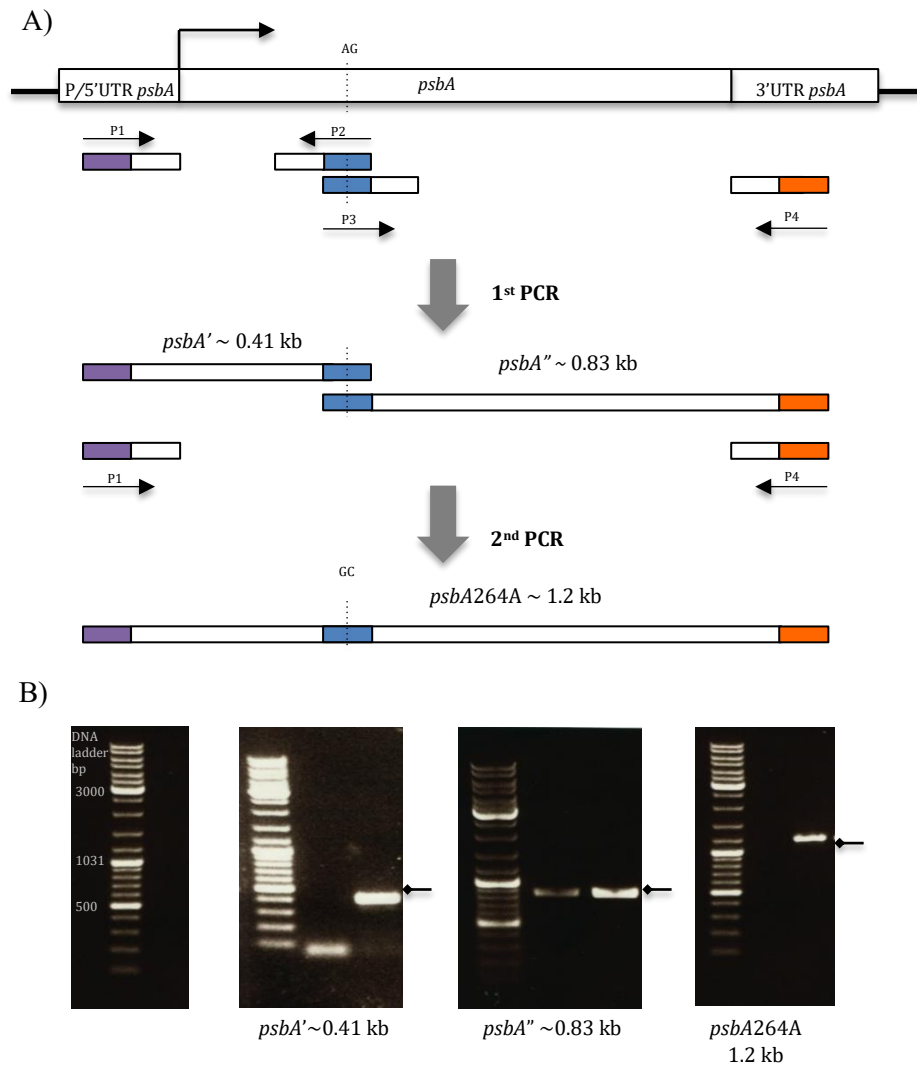


Figure 4.15 Schematic representation of PCR based method to create the herbicide tolerant *psbA* allele.

A) Schematic representation to illustrate how PCR-based method has been used to create a mutant *psbA* by replacing AG with GC at codon position 264. The amplification reactions have been performed in two consecutive steps. In the first step, *psbA* was amplified as two separate pieces using two sets of primers (P1–P2 and P3–P4). The first primer set (P1–P2) amplifies *psbA'* fragment that extends from approx. 20 bp upstream of the transcription start of *psbA* to 20 bp within the mutation site. The second set of primer (P3–P4) amplifies *psbA''* fragment from 20 bp upstream mutation site to approx. 20 bp downstream of *psbA* to include 3'UTR. The resulted PCR products – *psbA'* and *psbA''* – were mixed in the reaction to act as a template for the second PCR reaction producing *psbA* with a mutation at codon position 264 from serine to alanine, (*psbA264A*). To facilitate the subsequent cloning steps of making the construct, primers 1 and 4 incorporate ~20 bp complementary sequences to P/5'UTR *psbA* and 3'UTR *psbA*, respectively. B) The PCR products generated during the amplification reactions to construct *psbA264A*. The arrows point out the expected band.

Having constructed the *psbA264A* allele, the next step was to make a transformation vector that will replace the wild type *psbA* with resistance allele in the *Nannochloropsis* chloroplast via homologous recombination, as illustrated in Figure 4.15. In order to construct the vector, left and right flanking regions (with appropriate overlaps to the *psbA264A* gene) were amplified from the *N. gaditana* chloroplast and then these endogenous elements were assembled together in the pJet plasmid vector using the Gibson assembly method (Gibson et al., 2009). As shown in Figure 4.16, regions of the *rbcS* and *chlN* genes were included into the cassette during PCR amplification of the flanking regions. The final construct (pUH-*psbA264A*) was confirmed by double digestion and DNA sequencing.

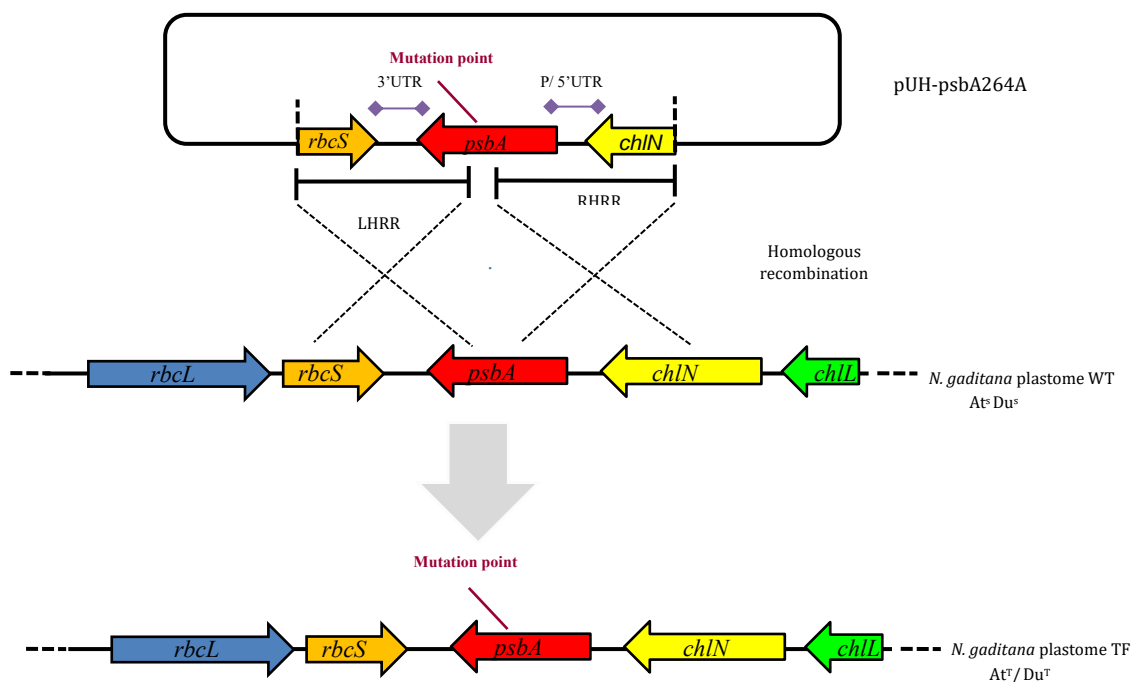


Figure 4.16 Schematic representation of site directed mutagenesis to introduce a mutation into *psbA* gene.

pUH-*psbA264A* comprises the *psbA* variant allele flanked by the 3'UTR and p/5'UTR sequences of *psbA*, together with upstream and downstream sequences for homologous recombination. The *psbA* variant is 1083 bp in length and the mutation has been introduced at codon position 264 by replacing the serine codon with that for alanine, indicated as mutation point. The promoter region and 5'UTR of *psbA* is 213 bp in length and the 3'UTR of *psbA* is 282 bp. Part of both *chlN* and *rbcS* genes were included into the cassette during cloning process. All elements were assembled together using Gibson assembly as one piece and then cloned into pJet. The successful delivery of DNA into chloroplast genome should enable the replacement of wild type *psbA* by double homologous recombination (X-X) generating herbicide tolerant transformants. Abbreviations are WT: wild type, TF: transformant, At^s: atrazine sensitive, Du^s: diuron sensitive, At^T: atrazine tolerance and Du^T: diuron tolerance.

4.2.3. Mitotic cell synchronisation as a means to increase cell size

In order to increase the cell size temporarily prior to biolistic transformation, a microtubule inhibitor was used to test the possibility of arresting *Nannochloropsis* cells at the G2/M phase where cells tend to double their size. To evaluate the initial effect of nocodazole on the *N. gaditana* cell cycle, actively dividing cells were treated with different concentrations (20 ng/ml and 3 µg/ml), either as a single dose or every 24 hours, and the growth of these nocodazole-treated cells was compared to untreated cells. Growth was monitored for a week to investigate the inhibitory and toxic effect of the treatment. As illustrated in Figure 4.17, there is an observed effect on the growth in all treated cultures compared to the control cells starting from ~24 hours, which is typically the time required by *N. gaditana* cells to double. Interestingly, the cells treated with a single dose of either 20 ng/ml and 3 µg/ml nocodazole showed no significant differences in growth. On the other hand, the culture exposed continuously to nocodazole showed more a profound effect in the growth in comparison to the single dose. In general, the tested concentrations and conditions of nocodazole treatments seem to have a limited inhibitory effect on cell growth as the cells continue to grow but slower. However, the single dose is reported to result in recovery of 80% of mammalian cells from a nocodazole block thus allowing the cells to proceed normally through mitosis (Zieve et al. 1980). So, the focus on the subsequent experiments was based on a single dose.

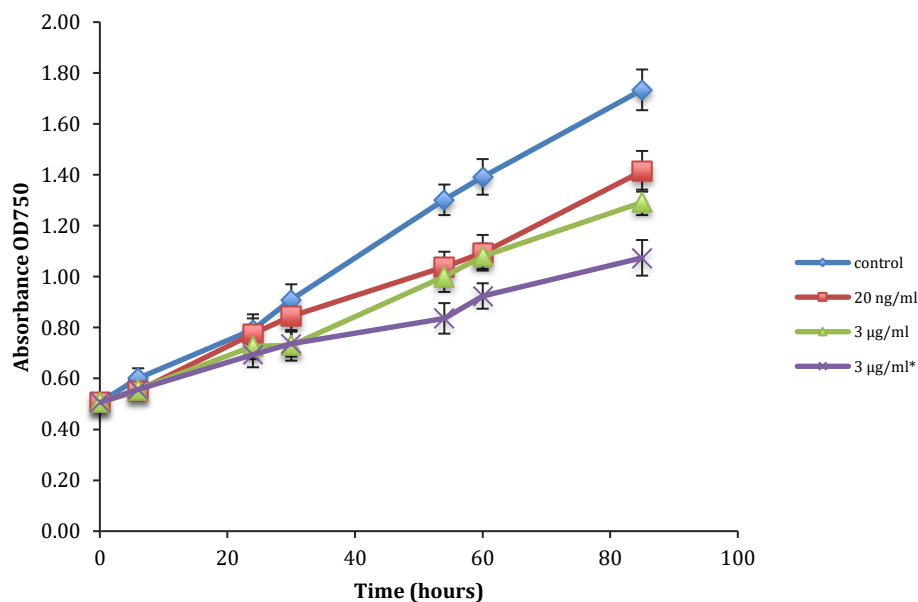


Figure 4.17 The effect of different concentrations of microtubule inhibitor nocodazole on actively dividing *N. gaditana* cells.

All cultures were from the same master culture and nocodazole was added at various concentrations (0, 20 ng/ml and 3 µg/ml). The culture treated with 3 µg/ml nocodazole followed by addition of the same amount every 24 hours, is indicated by asterisk (*). The cultures were incubated at 25 °C with continuous illumination of 80 µE. The error bars represent the standard deviation (n=3).

The next step in evaluating the effect of nocodazole on *N. gaditana* was to observe the morphological features of treated cells at different time intervals by light microscope. This simple method is generally used to determine the cell cycle stage, and widely used in assessing the cycle synchrony of *Saccharomyces cerevisiae* (Walker 2011). During microscopic analysis of *Nannochloropsis* cells, the changes in the chloroplast were monitored as the cell progressed through the cycle in presence of nocodazole, as shown in Figure 4.18. The cell starts small in size and the chloroplast occupies almost the whole cell volume. After 12 hours treatment, the cell gets slightly bigger in size while the chloroplast appears to be less compact within the cell. In the following 12 hours, the chloroplast begins to detach from the plasma membrane as preparatory stage for the formation of daughter plastids. Therefore, after 36 hours of treatment, the two-daughter plastids can be seen clearly within the cells. After 48 hours treatment, the cell is considerably bigger with two daughter plastids. In addition to the observed changes in the chloroplast, the cell size of *N. gaditana* increases as the cell is progressing through the cycle. Therefore, in this study the changes in the cell size and the chloroplast were used as a means to monitor the cell cycle synchrony since the visualization of nuclei and spindle require the use of fluorescence or electron microscopy. Furthermore, the nucleus and the plastid within *Nannochloropsis* species are physically connected forming a nucleus-plastid continuum (NPC) and such physical continuity is presumably maintained throughout the cell cycle as demonstrated by Murakami & Hashimoto (2009).

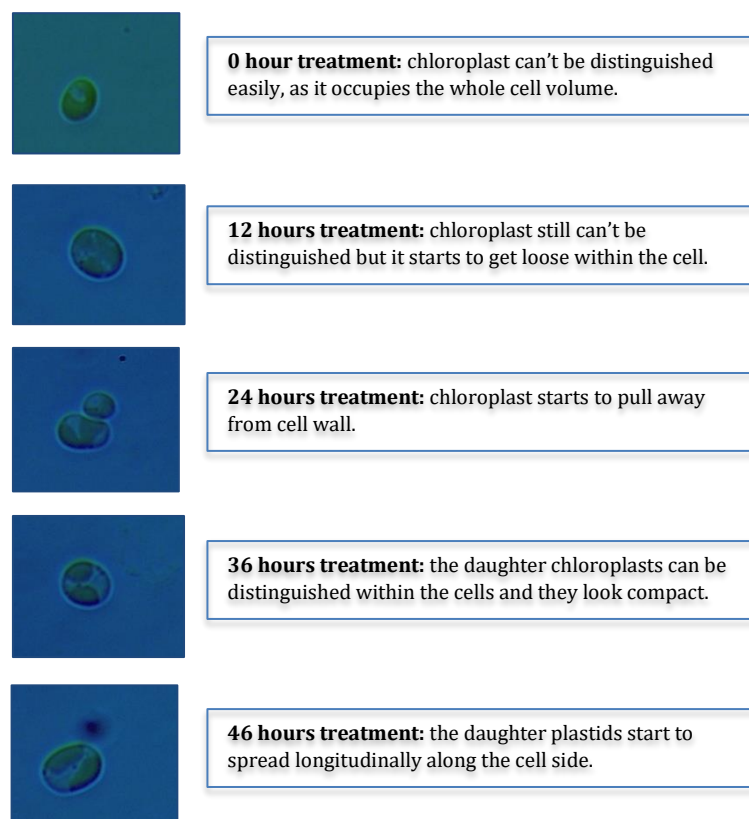


Figure 4.18 Observed changes in the chloroplast as the cell progresses through the cell cycle in the presence of nocodazole.

Although the growth experiment showed that there is little difference between cells treated with 20 ng/ml and 3 µg/ml nocodazole, the cell populations examined under the microscope demonstrated that large number of cells seemed arrested at G2/M phase when treated with 3 µg/ml nocodazole (data not shown due to technical error in zesis camera). So, the effect of treating *N. gaditana* with 3 µg/ml nocodazole upon single exposure was further examined by observing the morphological features of these cells under the microscope, measuring the changes in the cell size and DNA content. Initially, nocodazole was added to a dividing culture of *N. gaditana* to give the final concentration 3 µg/ml and then the morphological changes of nocodazole-treated cells were compared to untreated cells using the microscope. The microscopic analysis of cells treated for 48 hours as shown in Figure 4.19 revealed that most of nocodazole-treated cells become enlarged and display a distinct elongated shape with two daughter plastids. On the other hand, the cells in asynchronous culture (i.e. untreated cells) are a mixture of round and elongated shapes. The observed elongated shape upon nocodazole treatment could indicate a static metaphase spindle configuration of nocodazole-treated cells. In order to determine such changes more accurately, the cell size of 106 cells from each of the untreated and nocodazole-treated images was measured by length. This analysis, as demonstrated in Figure 4.20, illustrates the changes in the cell size and its distribution. The results indicate that nocodazole treatment increased the cell size by approx. 0.5 µm. As for the cell size distribution, there is a general shift in the cell size in particular the cell's size that represents 50% of the population from 2.5 µm in untreated cells to 3.1 µm in nocodazole-treated cells. However, the range of cell size distribution of untreated cells is narrower compared to nocodazole-treated cells, which ranges from 2.2 to 3.1 µm. Such a cell-size distribution of nocodazole-treated cells has been reported previously by Cooper et al. (2006).

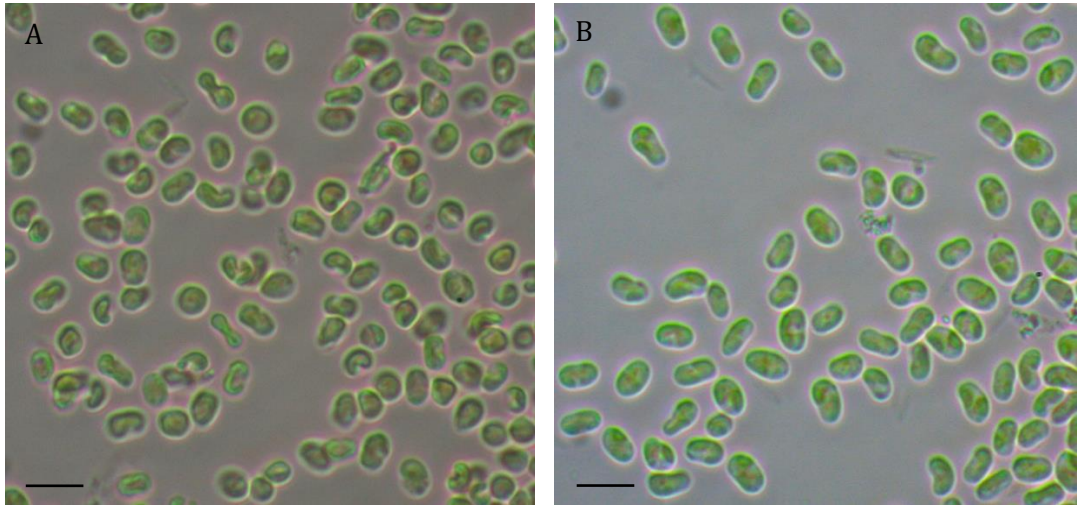


Figure 4.19 Effect of nocodazole on cell division and cell morphology.

A) *N. gaditana* was cultured in ASW medium in absence of nocodazole for 48 hours, as a control. B) *N. gaditana* cells were treated with 3 µg/ml nocodazole for 48 hours appear as enlarged cells with two daughter plastids. The bars represent 5 µm. The images were processed using Carl Zeiss software ZEN (blue edition).

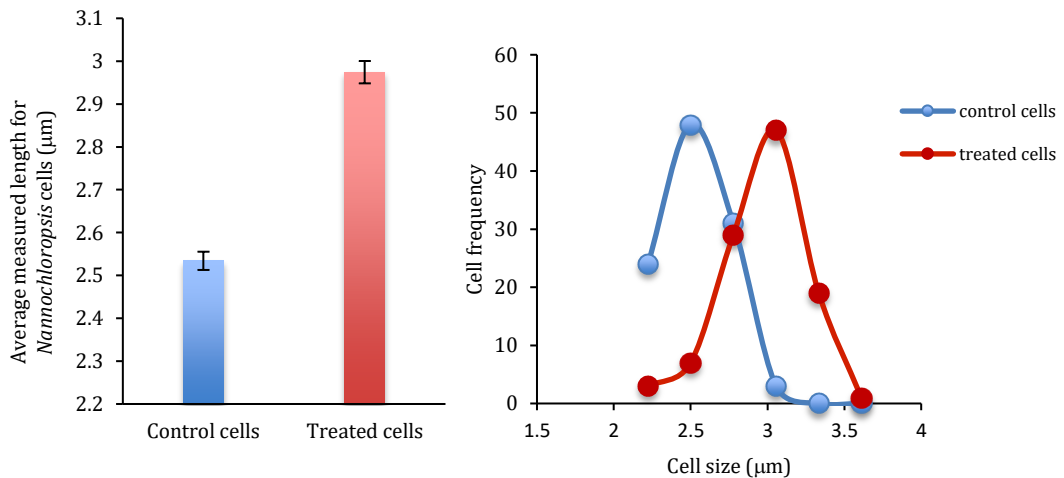


Figure 4.20 The effect of nocodazole on cell size and its distribution.

In the left panel, the effect of nocodazole on *N. gaditana* size. *N. gaditana* cells were treated with 3 µg/ml nocodazole for 48 hours, and the changes in the cell size was compared to untreated cells (i.e. control cells). The total number of measured cells per group is 106 and the error bars represent standard error of the mean. In the right panel, the cell size distribution of measured control cells and nocodazole-treated cells.

Flow cytometric analysis was carried out to investigate if the cells are actually arrested at G2/M phase when treated with nocodazole. This method enables the precise measurement of cellular DNA content, where the DNA content of the individual cells stained with propidium iodide (PI) in the population is measured. In theory, nocodazole arrests the cells at G2/M by preventing the disassembly of microtubules and subsequently the arrested cells tend to be larger with 2N DNA content. The flow cytometric results in Figure 4.21 do not reflect the expected results under the tested conditions, where the relative abundance of 2N DNA content of nocodazole-treated cells is expected to be higher than untreated cells. The results indicate that the percentage of detected major populations of G1, S and G2/M phase were almost the same in untreated cells and 20 ng/ml nocodazole-treated cells. Whereas, the treated cells with 3 µg/ml nocodazole showed a constant distribution of the major populations of G1, S and G2/M phase throughout the different time intervals with G1 the most prominent, representing 50% of the population. There is uncertainty when it comes to flow cytometry analysis though it may suggest there is another effect other than arresting the cells at G2/M phase. Possibly further optimization is required to test the ploidy state of nocodazole-treated cells using flow cytometry. Alternative approaches should be also considered to assess synchrony of the cells, such as studying the mitotic markers of nocodazole-treated cells by western blot.

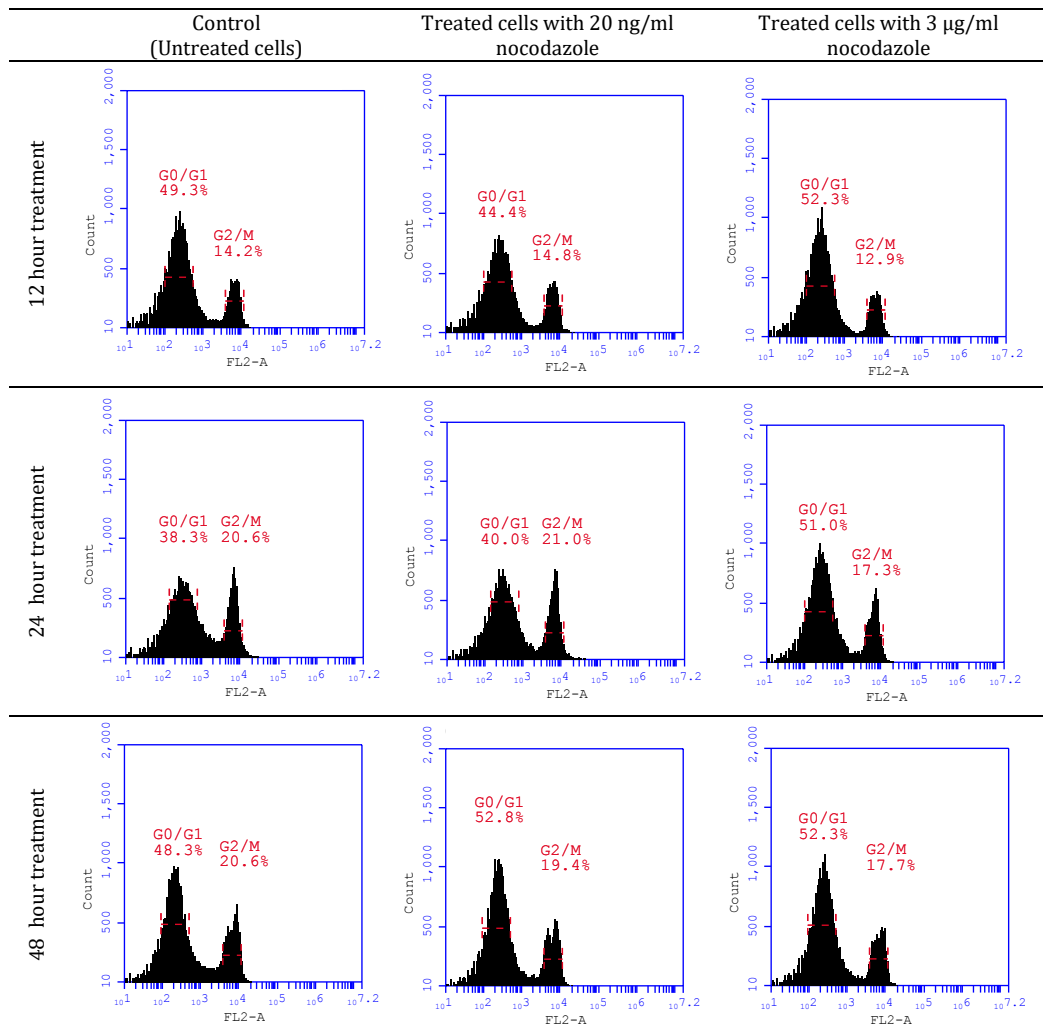


Figure 4.21 The cell cycle profile of untreated cell and nocodazole-treated cells by flow cytometry.

The DNA content of *Nannochloropsis* cells treated with nocodazole was compared to control cells at different treatment intervals; 12, 24 and 48 hours. The analyzed samples were untreated cells as a control (left column), treated cells with 20 ng/ml nocodazole (middle column) and treated cells with 3 µg/ml nocodazole (right column). All cells were fixed in 70% ethanol and stained with propidium iodide (PI) as described in section 2.12. The fluorescence of the PI-stained cells was measured using a BD Accuri C6 flow cytometer. The detected fluorescence by FL2-A (band pass filter) is proportional to the amount of DNA present in the tested samples and thus it represents DNA content, which is mainly sorted into 1N and 2N populations. Each DNA histogram shows an estimated percentage of cells in G1, S, and G2/M phases of the cell cycle. The area between two peaks represents the cells in S phase, as the cells are synthesising DNA.

4.2.4. Development of biolistics transformation methodology for *N. gaditana*

Nuclear transformation was initially used in our attempts as a control to standardize the method being tested on the *Nannochloropsis* cells. Currently, electroporation is the method of choice for nuclear transformation of *Nannochloropsis* species (Radakovits et al. 2012; O. Kilian et al. 2011). This method has been used successfully in this study to generate nuclear transformants, as described earlier in section 3.2.4. However, the electroporation attempt failed to generate any chloroplast transformants. Thus, biolistics is being attempted as the delivery method. It is likely that the principle difficulty of transforming *Nannochloropsis* by biolistics is due to its small cell size (discussed in section 3.3). So, this time a different approach was used in which the cells were treated with the microtubule inhibitor nocodazole for either 24 or 48 hours to increase the cell size temporarily prior to biolistic transformation. The pQYZ1 construct containing the *Sh ble* nuclear marker was used to test the applicability of the biolistics approach since this construct successfully generated nuclear transformants using electroporation (see section 3.2.4).

Biolistic transformation was carried out by bombarding the cells either at 1350 or 1550 psi, 6 cm from launch site with 5 µg DNA-coated gold particles (0.55 µm in diameter). This resulted in a nuclear transformation efficiency of 1.45×10^{-8} and 5.5×10^{-9} per µg plasmid, respectively. Although there is a report on delivering exogenous DNA into the nuclear genome of the *Nannochloropsis* (Kang et al. 2015a) at 500 psi, the focus in our attempts was based on testing higher delivery pressures assuming that the accelerated particles at higher pressure have better penetration power to cross the multiple chloroplast membranes. The success results in delivering exogenous DNA into the nuclear genome using high bombardment pressure are summarized in Table 4.4. The employed biolistic conditions are similar to those used for *P. tricornutum*, where a high pressure was also used to deliver the exogenous DNA into the nuclear genome (Apt et al. 1996).

The highest transformation efficiency achieved for the pQYZ1 construct was 1.45×10^{-8} per µg plasmid by treating cells with 3 µg/ml nocodazole for 24 hours and then bombarding these cells at 1350 psi and 6 cm from launch site with 5 µg DNA-coated gold particles. Putative nuclear transformant colonies appeared three weeks later after incubation on ASW medium containing 3 µg/ml zeocin, as shown in Figure 4.22. After 4–6 weeks, eight of these putative transformants, T1–T8 were picked and re-streaked for further analysis. In order to determine if these colonies were true transformants, they were further tested by spot test and PCR analysis. Figure 4.22 confirms that all the transformants were able to grow in the presence of zeocin. The PCR analysis indicated the successful integration for the *Sh ble* marker gene into the nuclear genome, as the transgene was detected by PCR in all screened strains with a band at the expected size of 370 bp which was absent for the wild type strain.

Table 4.4 A number of tested conditions resulted in nuclear transformants using biolistics.

The variables tested were helium pressure (1350 and 1550 psi) and distance between particles launch site and target cells (3, 6 and 9 cm).

Condition	Rupture disk (psi)	Distance (cm)	Number of transformants	Transformation efficiency* (per μg DNA)
A	1350	3	6	4.0×10^{-9}
B	1350	6	29	1.45×10^{-8}
C	1350	9	13	6.5×10^{-9}
D	1550	6	11	5.5×10^{-9}

*Transformation efficiency is based on colony number per total plated cells per μg DNA. The highest transformation efficiency for corresponding condition is shown in the table.

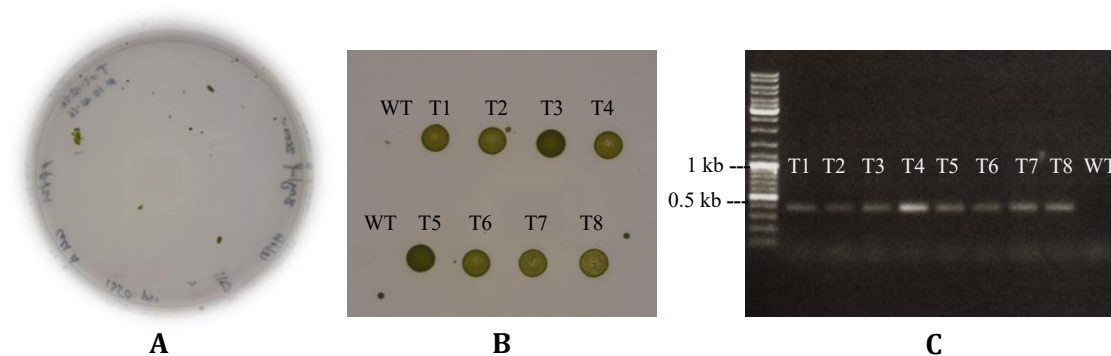


Figure 4.22 Analysis of nuclear transformants obtained with pQYZ1 using biolistics.

(A) Putative nuclear transformants appeared after four weeks' incubation on zeocin containing plates. A number of putative transformants colonies were selected, re-streaked and spotted onto ASW medium containing $3 \mu\text{g}/\text{ml}$ zeocin. As shown in B) All selected putative transformants (T1-T8) were able to grow under zeocin selection when compared to wild type which failed to grow. (C) PCR analysis; the *ble* marker gene was detected in all selected transformants (T1-T8) by a band at the expected size of 370 bp, while no band was detected in the wild type sample (WT).

In order to establish a stable plastid transformation in *N. gaditana*, our attempts aimed to substitute the wild type *psbA* gene with a variant carrying the Ser264→Ala point mutation, as described in section 4.2.2.2. The transformation was initially tested using the same conditions that were developed to transform the nuclear genome by biolistics. In the first attempt, *N. gaditana* cells were treated with $3 \mu\text{g}/\text{ml}$ nocodazole for 48 hours followed by bombardment with DNA-coated gold particles at 1350 psi and 6 cm from the launch site. After seven weeks of incubation on solid medium supplemented with $50 \mu\text{g}/\text{ml}$ atrazine, putative colonies appeared, as shown in Figure 4.23. Nine of these putative colonies were re-streaked and spotted onto an atrazine containing plate along with the wild type strain. Figure 4.23 shows that all putative chloroplast transformants were atrazine tolerant, although there was an observable variation in tolerance levels. To test if the atrazine tolerance phenotype was due to the successful replacement of the wild-type *psbA* allele the target region was sequenced in each line. The sequence analysis as shown in Figure 4.24 reveals changes at codon position 264 in lines T2 and T6, but no changes to *psbA* in lines T1, T3-T5, and T7-T9. However, the observed mutation in both T2 and T6 was a single base

substitution at codon position 264 from G to A resulting in a change in amino acid from serine to asparagine. This mutation is not the point mutation carried on the pUH-psbA264A plasmid, demonstrating that T2 and T6 are not transformants but spontaneous resistance mutants. Nonetheless, this result indicates the involvement of amino acid residue at codon 264 in herbicide tolerance in *N. gaditana*, and suggests that the Ser→Asp variant of *psbA* could be used as a selectable marker. The effect of this mutation on atrazine tolerance levels in strains T2 and T6 was further tested by spotting all strains (T1–T7) on increasing concentrations of atrazine. As shown in Figure 4.25, all the strains were able to grow in the presence of atrazine at the tested concentrations, but strain T2 and T6 were found to grow slowly, suggesting that photosynthetic performance was compromised in these mutants. Mutations to codon 264 have been reported previously to reduce electron transport within photosystem II and so affect the growth rate, which could be the case for *N. gaditana* (Oettmeier 1999; Ohad & Hirschberg 1992). Further attempts were made to obtain true transformants using the pUH-psbA264A plasmid, however none of the obtained resistant lines carried the expected mutation for the successful transformation. In these attempts, a control plate with no DNA was included, where several colonies appeared especially for the nocodazole-treated cells for 48 hours (data not shown).

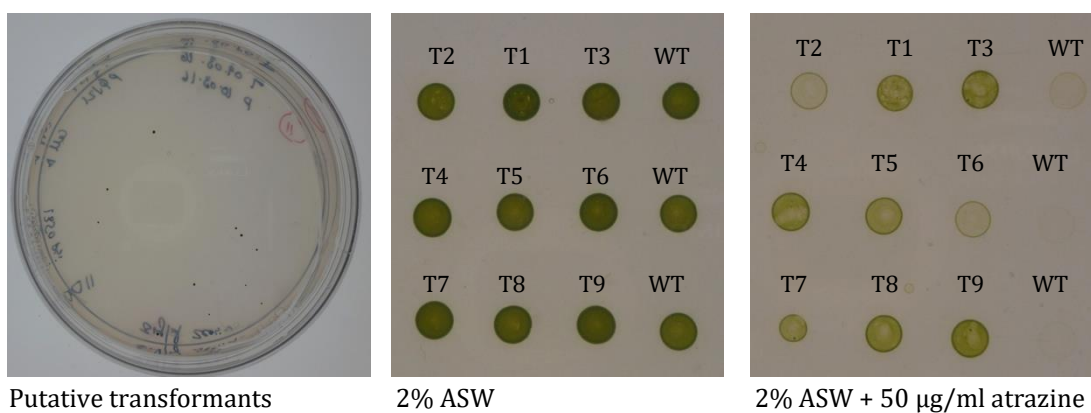
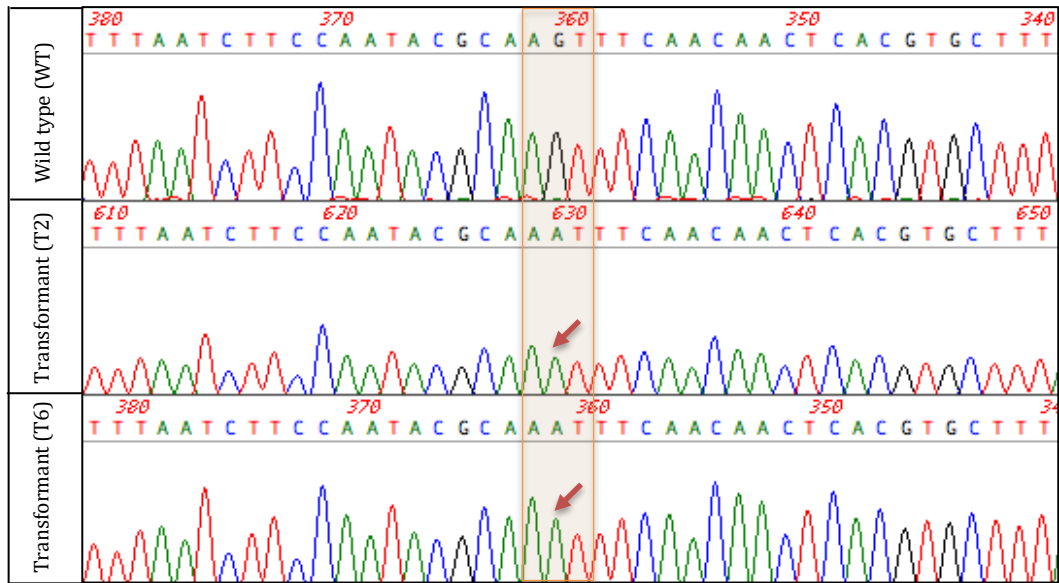


Figure 4. 23 Attempts were made to transform the chloroplast of *Nannochloropsis* using biolistics.

The cells were initially treated with 3 µg/ml for 48 hours and then transformed with pUH-psbA264A. The colonies appeared after 7 weeks incubation on ASW containing 50 µg/ml atrazine. Putative colonies were isolated, re-streaked and spotted onto 2% ASW and 2% ASW containing 50 µg/ml atrazine. All selected transformants, T1–T9, were able to grow under atrazine selection, while the wild type failed to grow in presence of atrazine. Two putative transformants were found to grow slowly, T2 and T6.

A



B

<i>psbA</i> variants	Sequence at codon position 264	Corresponding amino acid
<i>psbA</i> ^{WT}	AGT	Serine
<i>psbA</i> ^P	GCT	Alanine
<i>psbA</i> ^{T2,T6}	AAT	Asparagine
<i>psbA</i> ^{T*}	AGT	Serine

Figure 4.24 Analysis of *psbA* sequence in putative chloroplast transformants in reference to the wild type.

(A) Sanger sequencing chromatograms for *psbA* of wild type and two putative transformants; T2 and T6. The chromatograms of T2 and T6 show a different point mutation from the one expected of chloroplast transformants (GCT). The red arrows show the site of the actual base changes (G→A) and the codon of interest is highlighted by the orange box. (B) Brief summary on the analysis of *psbA* sequence for the selected putative transformants (T1–T9). The changes at codon position 264 have been identified in T2 and T6, but is a different point mutation. T* refers to putative transformants number (1, 3–5, 7–9), and no changes have been detected in *psbA* sequence in spite of an atrazine tolerant phenotype. Abbreviation are WT: wild type, P: predicted by reverse genetics and T2, 6: putative transformant number 2 and 6.

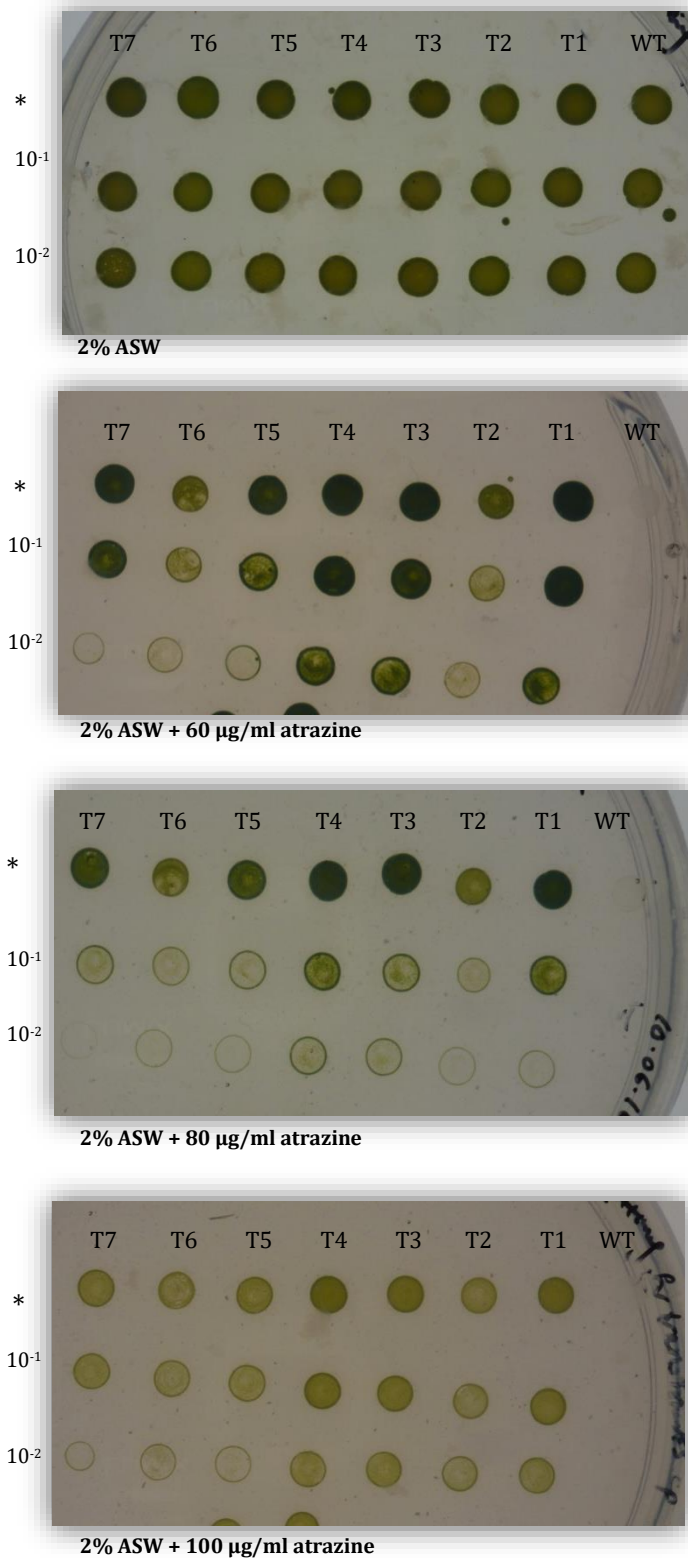


Figure 4.25 Testing the growth response of atrazine tolerant colonies on ASW agar plates containing increasing concentrations of atrazine.

For growth tests, *Nannochloropsis* strains T1–T7 and wild type (WT) were adjusted to equal optical densities and spotted onto 2% ASW agar containing no atrazine (top panel), 60 and 80 µg/ml atrazine (middle panels), and 100 µg/ml (bottom panel), with serial tenfold dilutions. The growth tests demonstrating that strains T1–T7 were able to grow at higher concentrations of atrazine, however, strains T2 and T6 were found to grow slowly. The wild type strain grew on ASW agar but not on atrazine containing plates. Plates were incubated under 50 µE for 6 weeks.

4.3. Discussion

Throughout the initial investigation into developing a chloroplast transformation method - as discussed at length in Chapter 3 (section 3.3)- the main challenges were: i) delivering exogenous DNA into the chloroplast genome of *Nannochloropsis* using commonly employed methods are the small size of the cells and the complexity of the plastid membranes; ii) selection for stable transformant lines. To address these challenges, a second approach was employed this time, which relies firstly on screening for more effective selection systems, secondly developing endogenous marker to overcome issues relating to foreign gene expression and thirdly, investigating the possibility of increasing the cell size so the use of biolistics method would be possible for small size cells such as *Nannochloropsis*. The use of such a powerful transformation method offers the possibility of delivering exogenous DNA across a thick cell wall and several membranes.

A number of selection systems have been developed for algal chloroplast transformation with the most widely used selection systems based on antibiotic resistance markers. However, *Nannochloropsis* has shown high resistance to a wide range of antibiotics (described in section 3.2.2) and therefore there is an obvious need for alternative selection systems that target the chloroplast specifically. So the choice of selectable marker this time was based on the results of sensitivity tests to several herbicides and to D-amino acid, as summarized in Table 4.3. Two herbicides, atrazine and diuron were shown to be effective at low concentrations. These herbicides target this photosynthetic machinery by disrupting the electron transfer within the photosystem II reaction centre. A number of studies have reported algal chloroplast transformation using herbicides as a selection system, including glufosinate (Cui et al. 2014), diuron (Przibilla et al. 1991; Newman et al. 1992) and sulfometuron methyl (Grundman et al. 2012; Lapidot et al. 2002). Atrazine was chosen as a potential selection system in our investigation given the established molecular basis of resistance through changes to the D1 protein of photosystem II encoded by *psbA*. There is a possibility to use a marker gene encoding for an enzyme that inactivates atrazine such as *atrazine chlorohydrolase* gene (*AtzA*), however the focus in this study was principally on developing *psbA* as an endogenous marker to overcome issues related to foreign gene expression in the *Nannochloropsis* chloroplast.

Initially a forward genetics approach was taken. Unfortunately, the isolated atrazine-tolerant mutant strains from FdUrd-UV mutagenesis did not show any changes in *psbA* so it was not possible to identify *psbA* allele/s that could be used as a selectable marker. Such atrazine tolerant phenotypes in the absence of changes in *psbA* suggest that other mutations have occurred at unknown loci. It is likely these strains have developed a way to overcome atrazine either by reducing the permeability of the cells or by direct efflux of atrazine, as is

often the case with bacterial resistance to antibiotics (Llarrull et al. 2010). The unsuccessful attempts in forward genetics approach led us to rely on a reverse genetics approach in which an atrazine tolerant *psbA* allele is designed and tested. In this approach, the study of the sequence homology of the *Nannochloropsis* D1 sequence with other related species aided the identification of a key residue at position 264 in the algal D1 that was likely to give the desired phenotype – atrazine tolerance – when changed from serine to alanine residue. This point mutation i.e. *psbA*:264A was chosen as a selectable marker, presuming to confer the tolerance to atrazine. Therefore, a plasmid carrying this point mutation (pUH-*psbA*264A) was created and used to investigate the possibility of developing a chloroplast transformation method by substituting the wild type *psbA* in the chloroplast genome with the atrazine tolerant allele.

In an attempt to increase the cell size, a microtubule inhibitor nocodazole was used to arrest *N. gaditana* cells at G2/M phase prior to biolistic transformation without killing the cells. Although it was not possible to validate the degree of synchronisation of nocodazole-treated cell using flow cytometry, the microtubule inhibitor nocodazole demonstrated its usefulness in producing larger cells. For this reason, the feasibility of using biolistics on nocodazole-treated *N. gaditana* cells was tested with nuclear transformation vector, pQYZ1. The employed conditions were based on testing high velocities using the smallest gold particle available ~ 0.55 µm in the hope of increasing the chances of particle penetration into the plastid in subsequent chloroplast transformation attempts. Employing these conditions in nocodazole-treated cells successfully generated nuclear transformants. As a proof of concept, this nocodazole-mediated biolistic transformation approach demonstrated its success in delivering exogenous DNA into the nuclear genome. To-date, these conditions have not been reported for *Nannochloropsis*, In fact, all the studies report the use of electroporation as a method of choice for nuclear transformation with the exception of Kang et al. (2015). This study reported the success in generating the nuclear transformants using the biolistics but the DNA-coated particles were accelerated at low velocity using ~ 500 psi rupture pressure.

Nonetheless, there are some key issues to be addressed before considering this approach as a tool for metabolic engineering purposes in microalgae. The chemical treatment prior to transformation might have a mutagenic effect on the cells in spite of the reported minimal effects of nocodazole treatment on cells by Zieve et al. (1980). This is possibly seen in our chloroplast transformation attempts in which false positive colonies appeared, despite the absence of spontaneous mutation to atrazine when plating high densities of cells on the herbicide. The occurrence of these mutants is most likely due to the mutagenicity of the transformation process, either where the cells were treated with nocodazole or bombarded with gold particles. Most of isolated colonies showed no changes in *psbA* sequence.

However, two out of nine *psbA* mutants from transformation attempts showed a change at codon position 264 to that encoding asparagine. This raises the question as to whether a serine to alanine change, as designed in pUH-psbA264A would confer resistance in *Nannochloropsis* and suggests that a serine to asparagine change could be used for successful transformation. In addition, the photosynthetic capacity of the mutants could be further characterized to gain some insight into photosystem II function in *Nannochloropsis*. Similar mutagenesis results were reported by Materna et al. (2009) in which the ultimate aim of the study was to establish stable plastid transformation in *P. tricornutum* by mutating the wild type *psbA* gene with a modified *psbA* allele at codon 264. Their attempts in developing plastid transformation were unsuccessful but instead a number of distinct mutants in *psbA* were isolated and characterized.

4.4. Conclusion and future works

Biolistic transformation for a wide range of algal chloroplasts involves bombarding the cells with DNA-coated particles at different velocities depending on cell size and structure of the organism that is being transformed, (see Table 4.5). The direct use of biolistics is not possible for *N. gaditana* mainly due to the small cell size and therefore an additional step was tested prior to biolistic transformation. The additional step relies on increasing the cell size by treatment with the reversible microtubule inhibitor nocodazole. This transformation approach proved a successful strategy for nuclear transformation of *N. gaditana*. Successful nuclear transformation demonstrated that using high pressure did not kill all the cells, and this is relevant since high pressure may be necessary to penetrate the multiple chloroplast membranes despite the unsuccessful attempts in generating chloroplast transformants using pUH-psbA264A. Further studies are required to isolate and characterize atrazine tolerant mutants, which will help to provide more information about *psbA* alleles and their potential use as selectable markers. Similarly, a codon optimized *atzA* gene that confers tolerance to atrazine could be used.

The process of herbicide selection requires a long time to validate the employed conditions, especially with slow growing microalgae. In addition, the cells are prone to spontaneous mutations. The use of a reporter gene coupled with fluorescence-activated cell sorting (FACS) would facilitate testing various conditions for chloroplast transformation within a reasonably short period of time. There are a number of reporter genes that have developed for the nucleus of *Nannochloropsis* and might be useful for such high throughput screening for chloroplast transformants. These reporter genes include *purple chromoprotein* gene, *β -glucuronidase* gene, *green fluorescent protein* gene and the gene encoding sfCherry fluorescent protein (Shih et al. 2015; F. Li et al. 2014; Kang et al. 2015a; Moog et al. 2015).

Use of such reporters would be particularly useful for *Nannochloropsis* given the limited number of selection systems available due to its high resistance to a wide range of tested compounds that target aspects of chloroplast biology, as demonstrated clearly in both chapter 3 (section 3.2.2) and chapter 4 (section 4.2.1).

Alternative natural approaches could also be considered to increase the cell size of *Nannochloropsis*. One example is studying the effect of the spectral light conditions on *N. gaditana* and examining the possibility of increasing the cell size. For example, cell cycles studies of *Chlamydomonas* demonstrated that blue light has an inhibitory effect on the initiation of cell division resulting in cells growing for longer period associated with an increased in the cell size (Oldenhof et al. 2006; Oldenhof et al. 2004). The chloroplast transformation in microalgae relies on direct transfer of the gene into the chloroplast genome where the transgene integration proceeds through homologous recombination. Nonetheless, given the current lack of chloroplast transformation methodology for *Nannochloropsis*, nuclear transformation with genes encoding chloroplast-targeting proteins might offer an alternative approach for now. Such indirect chloroplast transformation might give some insights into industrially relevant pathways that take place in the chloroplast and the gained insights may also help to develop a chloroplast transformation method. There are a number of studies reporting success in targeting the protein into the chloroplast mediated by endogenous N-terminal transit peptides, thus encouraging further studies in this direction (Gruber et al. 2007; Moog et al. 2015; Sunaga et al. 2014).

Table 4.5 Reports of chloroplast transformation in microalgae using biolistics.

Algal species	Transformation conditions		Selection	References
	Rupture disk (psi)	Reported distance (cm)		
<i>Haematococcus pluvialis</i>	1,350 psi	7	Spectinomycin	Gutiérrez et al. 2012
<i>Chlamydomonas reinhardtii</i>	1,100 psi	6	Photoautotrophy	Randolph-Anderson et al. 1995
<i>Dunaliella tertiolecta</i>	300 psi	4	Erythromycin	Georgianna et al. 2013
<i>Platymonas subcordiformis</i>	900 psi	6	Basta	Cui et al. 2014
<i>Porphyridium sp.</i>	1,350 psi	9	Sulfometuron methyl	Lapidot et al. 2002

CHAPTER 5

**Metabolic engineering of the *C. reinhardtii* chloroplast
as a platform for high value terpenoid production**

5. Metabolic engineering of the *C. reinhardtii* chloroplast as a platform for high value terpenoid production

5.1. Introduction

As discussed in Chapter 1, terpenoids are a diverse class of naturally occurring compounds consisting of more than 50,000 structurally different molecules, and are found in all living organisms (Pateraki et al. 2015). Many terpenoid compounds in particular those isolated from plants are of high value to humans in various aspects such as medicine, agriculture and cosmetics. However, these high value terpenoids are produced in relatively small quantities in their natural hosts and their chemical synthesis for large scale production is indeed costly and complicated (Putignani et al. 2013). Therefore, there is much focus on producing these compounds in biological hosts using metabolic engineering technologies that have been advanced greatly in the past few decades (Misawa 2011). A number of biological systems have been exploited for the commercial production of terpenoids including classical microbial systems and plant cells (Roberts 2007; Putignani et al. 2013). Currently exploited systems have shown some success in producing terpenoids with pharmaceutical applications such as artemisinin, paclitaxel and vincristine (Roberts 2007; Ikram et al. 2015).

In spite of the significant progress that has been made in elucidating, transferring and reconstituting terpenoid biosynthetic pathways in microbial systems and plant cells, there is still a considerable work to be done before terpenoid production becomes economically viable. Moreover, there is a need for alternative biosynthetic production hosts to overcome the encountered challenges with heterologous production of some terpenoids in the existing systems. The exploitation of microalgae as heterologous systems for production of high value terpenoids seems an attractive alternative owing to their inherently desirable characteristics, in which they combine the high growth rate and ease of cultivation of microorganisms with the ability to perform post-transcriptional and post-translational modification of plants. The unicellular green alga *C. reinhardtii* is of particular interest as the most well studied model alga with well-established molecular tools for genetic manipulation (Franklin & Mayfield 2004; Purton 2007). However, the direct manipulation of terpenoid biosynthetic pathways in *C. reinhardtii* necessitates a thorough understanding of the basic terpenoid metabolism. Therefore, the focus of this research is to gain a better understanding of the methylerythritol phosphate pathway that leads to terpenoid biosynthesis in *C. reinhardtii* by investigating the effect of overexpressing *dxs* on plastidic downstream terpenoids, and also determining if this step is one of the rate limiting steps in the MEP pathway of *C. reinhardtii*.

5.1.1. Terpenoid biosynthetic production platforms

In plants, both MEP and MVA pathways are responsible for biosynthesis of secondary metabolic terpenoids of industrial interest as pharmaceuticals, agricultural chemicals, flavour and fragrance additives (Bohlmann & Keeling 2008). Examples of pharmaceutical use of these terpenoids are the anti-malarial artemisinin (sesquiterpenoid) from *Artemisia annua* and the anti-cancer paclitaxel (diterpenoid) from *Taxus brevifolia* (Li et al. 2015; Liu et al. 2006). Although some terpenoids are produced in relatively large quantities from natural sources such as essential oils and waxes, other terpenoids with high impact on human health, also called high value terpenoids, are found in low abundance in nature – normally less than 2-3% of total dry weight (Roberts 2007). The extraction efficiency of these high value terpenoids from plant tissue is variable depending on plant source, location and season of harvest, which is normally associated with high extraction costs and low yield recovery (Maury et al. 2005). This can be exemplified with paclitaxel (or Taxol®): approximately 3 kg of yew tree bark is required to provide 300 mg of paclitaxel, which is approximately a single dose. This is an inefficient production method, considering the destructive nature of the extraction process to the mature yew trees that required 200 years to develop fully (Horwitz 1994). Since the production of terpenoids for human consumption needs large amounts of plant tissue, researchers have developed alternative methods of chemical synthesis (Mukaiyama et al. 1999; Danishefsky et al. 1996). Chemical synthesis has been used successfully to produce some monoterpenes and carotenoids (Ernst 2002). As a matter of fact, the most industrially relevant carotenoids currently available in the market are chemically synthesised though they are also available to a small extent as natural products (Sandmann 2015). Nevertheless, the complexity and chirality of the majority of these compounds have generally prevented the development of cost effective chemical synthesis methods (Putignani et al. 2013; Lange & Ahkami 2013).

Since all living organisms contain the metabolic precursors needed for terpenoid production, the heterologous expression of terpene synthase in microbial hosts may offer sustainable platforms for terpenoid production (Lange et al. 2000). The most popular heterologous hosts used for industrially relevant terpenoids are *E. coli* and *S. cerevisiae*. These organisms in particular are genetically tractable with well-established molecular tools and can be cultured at large scales and high density with the current developed fermentation infrastructure (Kirby & Keasling 2009; Kirby et al. 2015; Gruchattka & Kayser 2015). There has been some success in exploiting these heterologous expression systems for terpenoid supply, where a modified *E. coli* strain has been used to produce an artemisinin precursor called amorphadiene (Tsuruta et al. 2009). The further production of complex terpenoids has been extended to yeast due to the difficulty of expressing plant cytochromes P450 hydroxylases in *E. coli*, which are often required to further modify the

carbon skeleton of complex terpenoid metabolites (Putignani et al. 2013), as illustrated in Figure 5.1. As such, the yeast cells can assist in the hydroxylation reactions by natively providing the required P450 reductase, as demonstrated by the successful biosynthesis of a second paclitaxel pathway intermediate in a yeast system, which needs the activity of a P450 enzyme (Engels et al. 2008). However, in some cases, there is a need to express a plant cytochrome hydroxylase and its redox partner in the yeast system to enable the production of the desired terpenoid or terpenoid precursors, which is feasible only if one plant cytochrome P450 is involved. An example is the successful creation of a *S. cerevisiae* strain that is capable of producing high levels of the anti-malarial precursor artemisinic acid by expressing genes for artemisinic acid biosynthesis along with genes for the corresponding plant cytochrome P450 and its redox partner (Ro et al. 2006). This study reported titres of artemisinic acid that exceeded the expression level in *A. annua*, a natural producer of artemisinin. In spite of the success in heterologous expression of terpene biosynthetic genes in the established microbial systems, some plant terpenoid metabolic pathways are either too complex or still unknown thereby making the use of microbial systems difficult in these cases. Furthermore, the production of complex terpenoids requires a number of cytochrome P450 enzymes to confer bioactivity, and the expression of redox partners for these P450 enzymes is considered a key challenge in microbial systems, limiting the progress of terpenoid production (Kirby & Keasling 2009; Putignani et al. 2013).

Figure 5.1 Schematic representation of engineering the biosynthetic pathway of artemisinin from *A. annua*.

A semi-synthetic route to produce artemisinin by devising strains that are capable of high flux to artemisinin precursors through metabolic engineering. The process starts with metabolic engineering of the relevant terpenoid biosynthetic pathway in microbial hosts to produce artemisinin precursors. The metabolic engineering of *E. coli* was successful to accumulate amorphaadiene but not artemisinic acid due to the difficulty of expressing cytochrome P450, whereas the engineered yeast was successful to accumulate artemisinic acid that can be easily processed to the artemisinin by chemical synthesis in a cost effective process for large scale production. Abbreviations are ADS; amorphaadiene synthase, CPR: cytochrome P540 reductase, CYP71AV1; cytochrome P450 monooxygenase. Multiple arrows indicate the steps carried out by chemical synthesis. The diagram was taken from Bohlmann & Keeling (2008).

In such circumstances, the production of complex terpenoids in plant cells by metabolic engineering of terpenoid biosynthetic pathways is a more viable option compared to the microbial systems, as plants already contain native P450-related biosynthetic pathways involved in decorating the carbon skeleton of complex terpenoid metabolites (Kolewe et al. 2008). Additionally, the lack of complete knowledge of many terpenoid biosynthetic pathways prohibits the transfer of these biosynthetic pathways into the microbial hosts (Markus Lange & Ahkami 2013). Therefore, efforts have focused on producing these compounds in undifferentiated plant cell suspension cultures to drive heterologous terpenoid production. The production of many terpenoid and terpenoid-derived compounds in plant cell culture suspension has been investigated especially for high value pharmaceuticals such as artemisinin from *A. annua* (Liu et al. 2006), taxanes from *Taxus spp.* (Frense 2007; Li et al. 2015) and the terpenoid indole alkaloids from *Catharanthus roseus* (Pasquali et al. 2006). In parallel, a number of cell based culture processes have been developed using plant suspension cultures to make terpenoid production economically viable (Fett-Neto et al. 1994; Xu et al. 1998; Kwon et al. 1998). The most successful example of plant cell culture technology is the commercial production of paclitaxel as a powerful diterpenoid anti-cancer drug using *Taxus spp.* cell suspension culture to supply the precursor that can be readily converted chemically into paclitaxel by a cost effective process (Zhong 2002), and more examples are summarised Table 5.1. The progress in exploiting plant cell culture technologies for more terpenoids with industrial applications is limited due to the fact that undifferentiated cultures normally accumulate less terpenoid metabolites compared to specialised tissues, and in most cases not at all (Kolewe et al. 2008). As for now, the key challenges to the commercial production of terpenoids using plant cell culture technology are low and variable yields of metabolite accumulation (Beum et al. 2004). This has been encountered in the use of *Taxus spp.* cell cultures for the commercial supply of paclitaxel, where a ten-fold difference in the accumulated levels of paclitaxel has been observed within the cell lines over time (Ketchum & Gibson 1996).

Table 5.1 Summary of suspension plant cell culture based pharmaceuticals.

The table is adapted from Eibl & Eibl (2002).

Terpenoid	species	Application	Manufacturer
Scopolamine	<i>Duboisia spp.</i>	Anticholinergic	Sumitomo Chemical Industries (Japan)
Protoberberines	<i>Coptis japonica</i> <i>Thalictrum minus</i>	Antibiotic, anti-inflammatory	Mitsui Petrochemical Industries (Japan)
Rosmarinic acid	<i>Coleus blumei</i>	Anti-inflammatory	Nattermann (Germany)
Shikonin	<i>Lithospermum erythrorhizon</i>	Anti-HIV, antitumor anti-inflammatory	Mitsui Petrochemical Industries (Japan)
Geraniol	<i>Geramineae spp.</i>	Anti-tumour	Mitsui Petrochemical Industries (Japan)

With such interest in the use of terpenoids in a variety of industrial and therapeutic applications, eukaryotic microalgae may offer an attractive alternative to suspension cell culture (Lohr et al. 2012). There are a number of microalgae capable of accumulating industrially relevant carotenoids under adverse environmental conditions, which can be exemplified by β -carotene in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis* (Lohr et al. 2012; Eonseon et al. 2006; Ramos et al. 2011). In addition, microalgae have the ability to achieve high cell densities thereby enabling the production of more compound per unit of land area (Georgianna & Mayfield 2012). A large scale cultivation of several algal species has already proven the possibility of producing high value terpenoids such as carotenoids (Gómez et al. 2013). The natural capacity of terpenoid production in these algal species can be improved by using genetic engineering strategies. However, the metabolic engineering of these microalgae is notoriously difficult due to the limited availability of molecular tools. Furthermore, too little is known about their biosynthetic pathways. From a biotechnological standpoint, *C. reinhardtii* is the most developed algal species with well-established transformation methods and molecular tools (Franklin & Mayfield 2004; Purton 2007). Therefore, a number of metabolic engineering efforts have been attempted in this alga, as summarised in Table 5.2. Most of these attempts focused on proof of concept experiments using simple molecular tools to demonstrate transgene expression and confirm the functionality of heterologous proteins within the cells (Vila et al. 2008; Couso et al. 2011; León et al. 2007). In addition, the fundamental concepts of these attempts for metabolic engineering of terpenoids in the algae are largely based around strategies that were developed for higher plants. Although there is still much to be done before exploitation of microalgae for biotechnological applications, a recent study reported by Lauersen et al. (2016) demonstrated the potential of *C. reinhardtii* as a chassis for sesquiterpenoid production, encouraging more research in this direction.

Table 5.2 Reports on terpenoid metabolic engineering in *C. reinhardtii*.

Metabolic engineering approach	Target genome	Transgene /Target gene (Source)	Notes	Reference
Upregulation	Nuclear	Phytoene desaturase* (<i>Chlamydomonas reinhardtii</i>)	A significant increase in carotenoid content in some transformants	(Liu et al. 2013)
Redirect metabolic flux towards non-native ketocarotenoid	Nuclear	β -carotene ketolase (<i>Haematococcus pluvialis</i>)	Detection of relatively small amount of novel ketolutein	(León et al. 2007)
Upregulation	Nuclear	Phytoene synthase (<i>Dunaliella salina</i>)	A significant increase in carotenoid content ranging from 120% - 260%	(Couso et al. 2011)
Downregulation	Nuclear	Phytoene desaturase **	Minimal change on the carotenoid profile	(Vila et al. 2008)
Upregulation	Nuclear	Phytoene synthase (<i>Chlorella zofingiensis</i>)	A significant increase in carotenoid content ranging from 200% - 220%	(Cordero et al. 2011)
Redirect metabolic flux towards non-native astaxanthin	Chloroplast	β -carotene hydroxylase (<i>Haematococcus pluvialis</i>)	A significant increase in carotenoid synthesis, in particular xanthophylls under high light conditions	(Tan et al. 2007)
Redirect metabolic flux to produce non-native <i>cis</i> -abienol 4	Chloroplast	<i>cis</i> -abienol synthase (<i>Abies balsamea</i>)	Absence of <i>cis</i> -abienol 4 traces	(Zedler et al. 2014)
Redirect metabolic flux to produce non-native patchoulol	Nuclear	Patchoulol synthase (<i>Pogostemon cablin</i>)	Successful production of patchoulol up to 922 μ g/g cell dry weight	(Lauersen et al. 2016)

* Mutant version of endogenous phytoene desaturase in which a leucine residue in codon position 505 is substituted with a phenylalanine to confer tolerance to the herbicide norflurazon.

** The endogenous phytoene desaturase was post-transcriptionally silenced by antisense RNA.

5.1.2. Aims and objectives

The overall aim of this chapter is to genetically modify the MEP pathway in the *C. reinhardtii* chloroplast by overexpressing a heterologous *dxs* gene, and to investigate if the DXS enzyme is able to catalyses this key rate limiting step in the MEP pathway.

Specifically, the aims of the work in this chapter are:

1. Engineer *C. reinhardtii* lines carrying an additional copy of the *dxs* gene from the cyanobacterium *Synechocystis* 6803.
2. Investigate the possible toxicity of *dxs* by mutating the gene and then introducing the mutated *dxs* copy into the chloroplast genome.
3. Study the effect of over-expressing *dxs* on *C. reinhardtii* growth.
4. Test the possibility of measuring DXS enzyme activity *in vitro* using fluorometry.
5. Analyse the terpenoid profile of the genetically engineered *C. reinhardtii* compared to a control strain, and identify if over-expression of *dxs* results in enhanced accumulation levels of plastidic terpenoids.

5.2. Results

5.2.1. Chloroplast transformation of *C. reinhardtii* with plasmid pRY134a

DXS catalyses the first biosynthetic step of the chloroplast localised MEP pathway and has been reported to be a rate limiting step for the production of the terpenoid precursor IPP in bacteria and plants (Khemvong & Suvachittanont 2005; Gong et al. 2006; Estévez et al. 2001; Heider et al. 2014). To investigate the effect of overexpressing this enzyme on downstream terpenoid metabolites in *C. reinhardtii*, a construct (pRY134a) containing the *dxs* gene from *Synechocystis* 6803 under the control of the *C. reinhardtii* *psaA* exon 1 promoter was constructed by Dr Rosie Young in the Purton group (Figure 5.2A). This gene from *Synechocystis* 6803 is more suitable for heterologous expression in the chloroplast rather than the nuclear-encoded native copy from *C. reinhardtii* because of its codon bias and GC content since the nuclear genome of *C. reinhardtii* has a high GC content of 64% compared to the chloroplast genome value of 34% (Grossman et al. 2003).

The initial investigation in the Purton group highlighted the difficulty of generating chloroplast transformants of *dxs*, suggesting that the introduction of an extra copy of *dxs* gene into the chloroplast genome might have a deleterious effect on the *C. reinhardtii* cells, as the high levels of prenyl phosphate precursors (DMAPP/IPP/FPP) was reported to be toxic in *E. coli* cells (Sivy et al. 2011; Martin et al. 2003). To investigate such a possibility, the pRY134a construct was modified so that *dxs* in pRY134a was mutated by deleting 114 bp of the coding sequence. The deletion was performed by digesting the pRY134a with *NcoI* at two sites to drop out the 141 bp region, and then re-ligating the plasmid. The resulting plasmid, called pRY134a Δ *dxs*, was initially checked by test digestion resulting in the expected fragment sizes, 4599 bp and 3488 bp (Figure 5.2B). DNA sequencing confirmed the successful deletion of 114 bp in the gene (Figure 5.2C).

The pRY134a and pRY134a Δ *dxs* were then used to transform *C. reinhardtii* TN72 (a non-photosynthetic *psbH* mutant) by agitating the cells with glass beads in the presence of the DNA, as detailed in section 2.7.2. The expression cassette in both constructs was flanked by homologous sequence that contains an intact copy of *psbH*, so homologous recombination into the chloroplast genome results in restoration of functional *psbH* and integration of the transgene, and subsequently allows the selection of restored photosynthetic transformants on minimal medium in the light (Figure 5.3). In total, six putative chloroplast transformants appeared after four weeks of incubation. Four of these colonies (named L1³, L3, L4 and L5) were obtained from pRY134a and the rest (named dL1 and dL2) were obtained from pRY134a Δ *dxs*. These colonies were picked and then re-streaked for a further three rounds

³ The *C. reinhardtii* line L1 was kindly given by Dr Rosie Young

of phototrophic selection on minimal medium to ensure the homoplasmic state of the chloroplast genome since *C. reinhardtii* has approximately 80 copies per cell. The integration of the transgene and homoplasmy of the genome in all transgenic lines were then examined by PCR using a specific set of three primers, as illustrated in Figure 5.4. The successful transgene integration in all transgenic lines was confirmed by the presence of a 1134 bp band, while the homoplasmic state was indicated by the absence of the 880 bp band that arises from the untransformed copies of the parental plastome. A control strain (H1) was included from previous studies and this strain was created by transforming the TN72 strain with the corresponding vector but without carrying a transgene, referred as an empty pSRSapI vector (Young & Purton 2014). This strain will be used as a control in subsequent experiments throughout the investigation.

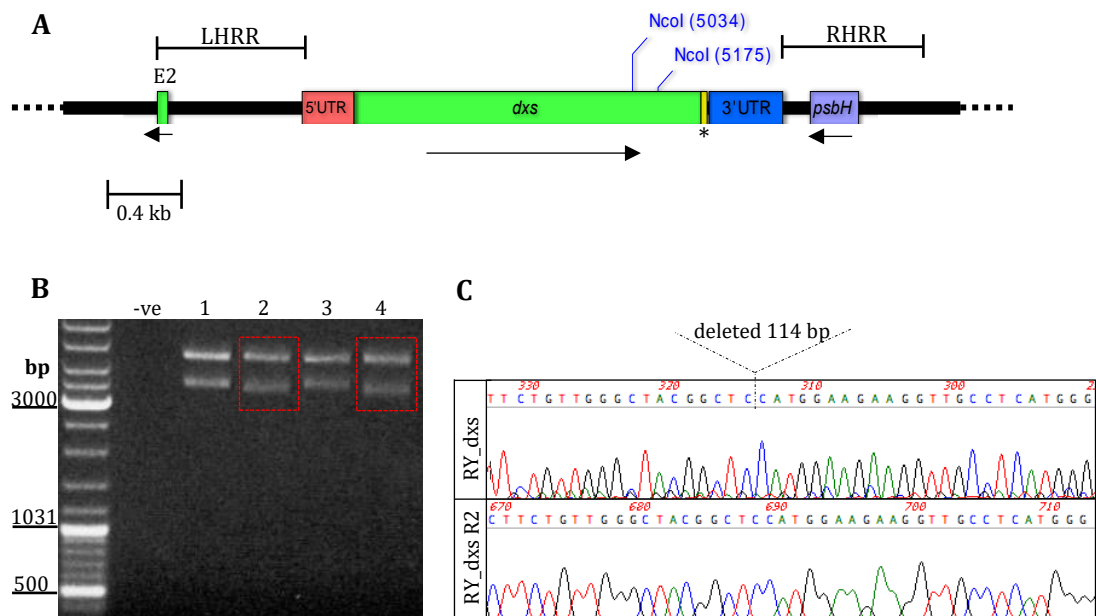


Figure 5.2 Analysis of partial deletion of *dxs* in pRY134aΔ*dxs* plasmid.

A) The schematic representation of pRY134a, a cloning vector for introducing a cyanobacterial *dxs* into the *Chlamydomonas* chloroplast genome under the control of the *psaA* exon 1 promoter and the *rbcL* 3'UTR. The coding sequence of a nine residue haemagglutinin epitope (HA tag) was added to C-terminal of the *dxs* gene before the stop codon so that the Dxs protein could be detected using anti-HA antibody. The HA tag is indicated by an asterisk. The region to be deleted of *dxs* coding sequences is between NcoI restriction sites, as shown in the physical map with their locations. The flanking regions required for homologous recombination within the chloroplast genome is indicated as LHRR and RHRR, and the intact *psbH* acts as a selectable marker. The rest of the pRY134a plasmid is depicted as a dashed black line. *TrnE2* is abbreviated as E2. B) Test digestion showing the successful deletion of 114 bp in *dxs* gene resulting in fragments of the expected sizes, 4599 bp and 3488 bp, as indicated by red box. The lanes 1, 2, 3, and 4 represents different colonies tested for digestion C) Sanger sequencing chromatograph confirms the deletion of 114 bp in *dxs* for the clone number 2 using designated primers. The dashed lines indicate the deletion site within the coding sequence of *dxs*.

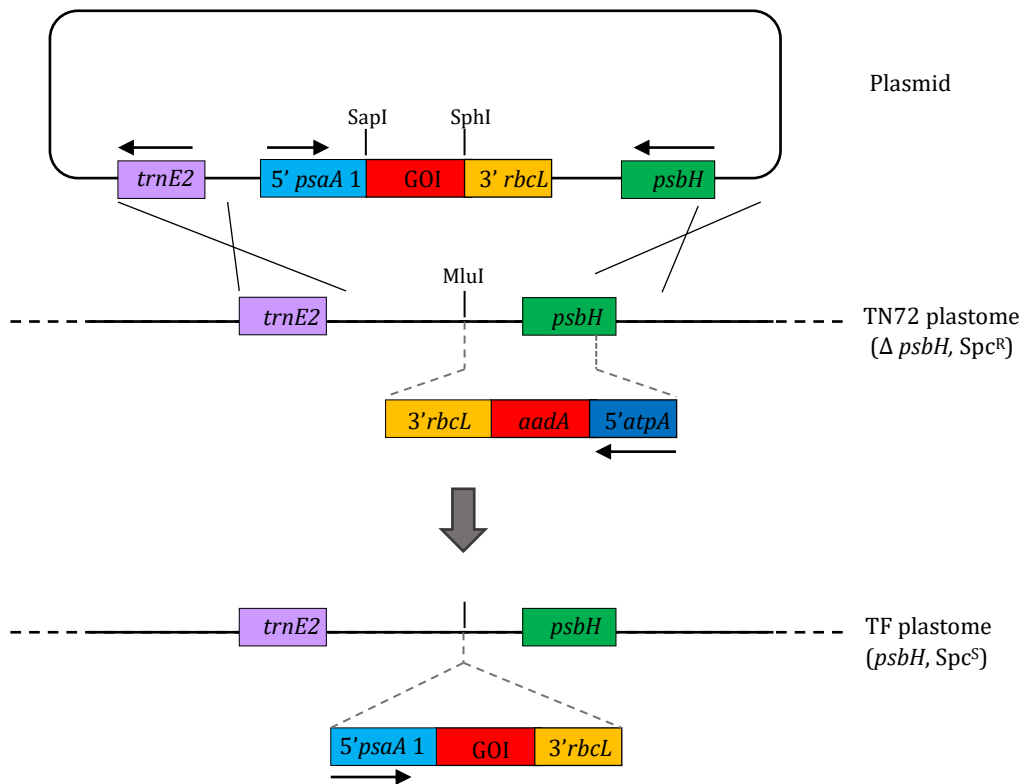


Figure 5.3 Schematic representation for chloroplast transformation strategy to generate marker-less transformants using the TN72 recipient.

Chloroplast transformation plasmid consists of the gene of interest (GOI) under the control of the *psaA* promoter/5'UTR and the *rbcL* 3'UTR. The expression cassette is flanked by homologous sequence that contains wild type *psbH*. The homologous recombination takes place between homologous sequence shared between the plasmid and TN72 plastome, resulting in restoration of *psbH*, loss of *aadA* and the integration of the GOI into the plastome. Thus, the resulting transformants restore their photoautotrophic growth on acetate-free medium but they are sensitive to spectinomycin (Spc^S). Homologous recombination is represented by X-X. The diagram is reproduced from Stoffels (2014).

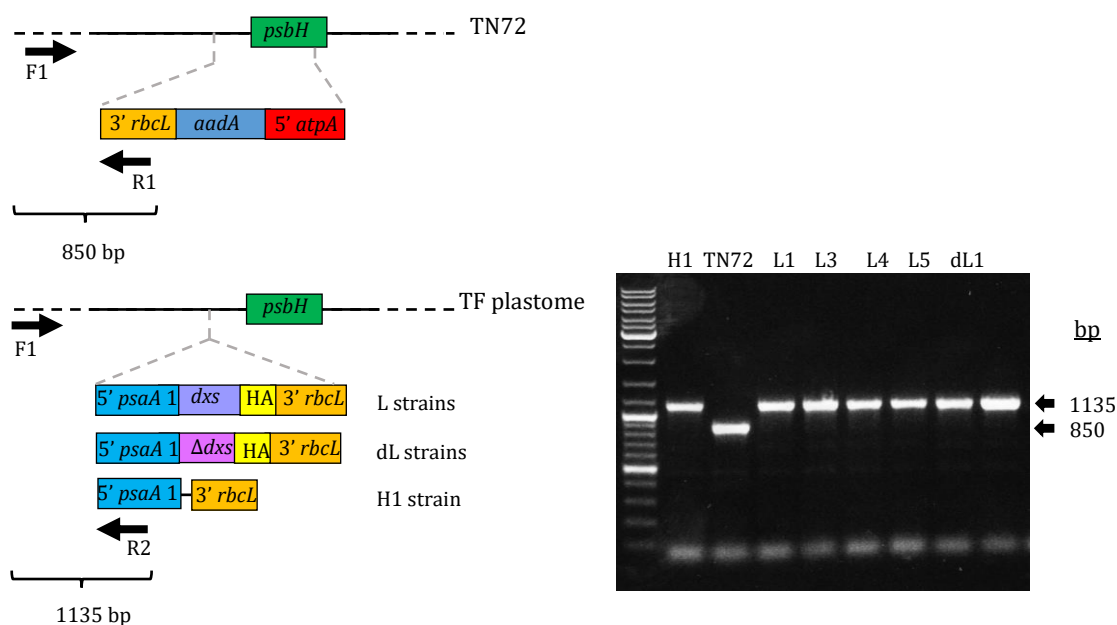


Figure 5.4 Three primer PCR to confirm transgene integration and homoplasmy of transformants.

Left panel: schematic representation of PCR based method to confirm the integration of GOI and homoplasmy of transformants. PCR was performed using three primers in one reaction (F1, R1 and R2). The first primer pair (F1 and R1) amplifies part of the recipient line TN72 plastome, resulting in an 850 bp band, whereas the second primer pair (F1 and R2) amplifies part of the transformant plastome, resulting in a 1135 bp band. The successful integration of the gene of interest is indicated by the presence of a 1135 bp band and the homoplasmy is indicated by the absence of the 850 bp band that normally arises from the untransformed copies of the TN72 plastome. The *C. reinhardtii* lines are TN72 recipient strain, L strains contain the *dxs* gene, dL strains contain the mutant *dxs* gene and H1 strain is an empty-vector as a positive control. TF is an abbreviation of transformant. Right panel: PCR confirming homoplasmic integration of the transgene into the *C. reinhardtii* chloroplast genome.

5.2.2. Analysis of the DXS protein in the *C. reinhardtii* chloroplast

Western blot analysis was performed to check for the accumulation of the DXS protein in all transgenic lines including L1 and L3–L5 for intact DXS protein, dL1–dL2 for mutant DXS protein and H1 as a control. The accumulation of the DXS protein (~ 70.4 kDa) in the L strains was demonstrated clearly by immunoblotting with an anti-HA antibody as shown in Figure 5.5. Surprisingly, there is variation in the expression level of the DXS protein in L lines in which the transgenic lines L4 and L5 showed a high expression level of the protein compared to L1 and L3 lines. These transgenic lines would normally be expected to have similar expression levels, since the transgene was targeted into the chloroplast genome by homologous recombination into a specific locus so they should be genetically identical. This result was further investigated by sequencing the expression cassette of *dxs* amplified from the plastome by PCR, to identify any mutation that might cause the difference in expression level of the protein in the *C. reinhardtii* lines. However, the DNA of the target region from the plastome of the transgenic lines had the expected nucleotide sequence (data not shown).

The variation in the expression levels is presumably due to mutations outside of the *dxs* region. In the dL cell lines, the accumulation of the mutant DXS protein was not detected at 65 kDa though the deletion does not cause any frameshift as shown in Figure 5.6. Nonetheless, such a deletion might result in conformational changes of the protein structure thereby affecting the protein stability and making the protein more vulnerable to degradation.

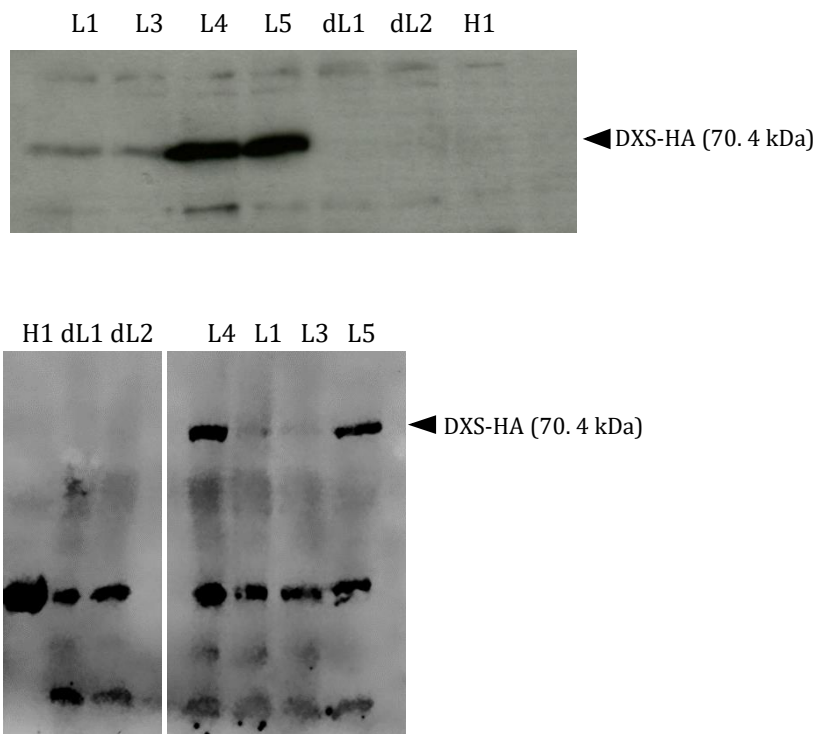


Figure 5.5 Expression of the *dxs* gene in the *C. reinhardtii* lines is demonstrated by Western blot analysis with anti-HA antibody.

Western blot analysis of equalised lysate for L strains containing an intact *dxs* gene, dL strains containing a mutant *dxs* gene and H1 strain as a negative control (i.e. TN72 transformed with an empty vector). Western analysis with anti-HA antibody shows different expression levels of the *dxs* gene in cell lines (L) that is clearly illustrated by a strong band running at an apparent mass of 70.4 kDa in cell lines L4 and L5 but not in L1 and L3. The H1 line does not show any accumulation of the DXS protein, as expected. The immunoblot was visualised by ECL with one-minute exposure (top panel) and Odyssey® Infrared Imaging System (bottom panel). Protein size was determined using the PageRuler™ Prestained Protein ladder (Thermo Scientific).

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DXS 1 MHISELTHPNELKGLSIRELEEVSRQIREKHLQTVATSGGHLGPGLGVVELTVALYSTLD 60
mDXS 1 MHISELTHPNELKGLSIRELEEVSRQIREKHLQTVATSGGHLGPGLGVVELTVALYSTLD 60
*****
DXS 61 LDKDRVIWVDVGHQAYPHKMLTGRYHDFHTLRQKDG VAGYLRKRSERFDHFGAGHASTSIS 120
mDXS 61 LDKDRVIWVDVGHQAYPHKMLTGRYHDFHTLRQKDG VAGYLRKRSERFDHFGAGHASTSIS 120
*****
DXS 121 AGLGMALARDAKGEDFKVVSIIIGDGALTGGMALEAINHAGHLPHTRLMVLINDNEMSISP 180
mDXS 121 AGLGMALARDAKGEDFKVVSIIIGDGALTGGMALEAINHAGHLPHTRLMVLINDNEMSISP 180
*****
DXS 181 NVGAI SRYLNKVRLLSSPMQFLTDNLEEQIKHLPFVGDSLTPEMERVKEGMKRLVVPKVG A 240
mDXS 181 NVGAI SRYLNKVRLLSSPMQFLTDNLEEQIKHLPFVGDSLTPEMERVKEGMKRLVVPKVG A 240
*****
DXS 241 VIEELGFKYFGPIDGHSLQELIDTFKQAEKVP GPVHVSTTKGKYDLAEKDQVGYHAQ 300
mDXS 241 VIEELGFKYFGPIDGHSLQELIDTFKQAEKVP GPVHVSTTKGKYDLAEKDQVGYHAQ 300
*****
DXS 301 SPFNLSGKAYPSSKPKPPSYSKVFAHTLTTLAKENPNIVGITAAAMATGGLDKLQAKLP 360
mDXS 301 SPFNLSGKAYPSSKPKPPSYSKVFAHTLTTLAKENPNIVGITAAAMATGGLDKLQAKLP 360
*****
DXS 361 KQYVDVGI AEGHAVTLAAGMACEGIRPVVAIYSTFLQRGYDQIIHDVCIQKLPVFFCLDR 420
mDXS 361 KQYVDVGI AEGHAVTLAAGMACEGIRPVVAIYSTFLQRGYDQIIHDVCIQKLPVFFCLDR 420
*****
DXS 421 AGIVGADGPTHQGMYDIAYLRCPNLVLMAPKDEAELQQMLVTGVNYTGGAIAMRYPRGN 480
mDXS 421 AGIVGADGPTHQGMYDIAYLRCPNLVLMAPKDEAELQQMLVTGVNYTGGAIAMRYPRGN 480
*****
DXS 481 GIGVPLMEEGWEPLIEGKAEILRSGDVL LLLGYGSMVYPALQTAELLHEHGIEATVNAH 540
mDXS 481 GIGVPLMEEGWEPLIEGKAEILRSGDVL LLLGYGS----- 514
*****
DXS 541 FVKPLDTELILPLAERIGKVVVTMEEGCLMGGF GSAVAEALMNNVLVPLKRLGVPDILVD 600
mDXS 515 -----MEEGCLMGGF GSAVAEALMNNVLVPLKRLGVPDILVD 553
*****
DXS 601 HATPEQSTVDLGLTPAQMAQNI MASLFKTETESV VAPGVS 640
mDXS 554 HATPEQSTVDLGLTPAQMAQNI MASLFKTETESV VAPGVS 593
*****

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Figure 5.6 Sequence alignment of the DXS and mDXS proteins.

Sequence of the DXS protein highlighting the amino acids that were deleted to create the mutant DXS protein, abbreviated as mDXS. The deleted amino acid sequence is highlighted in yellow. The sequence of the protein from *Synechocystis sp.* PCC 6803 was retrieved from the NCBI database (sequence identifier: WP-010871718).

5.2.3. Studying the effect of overexpressing *dxs* on *C. reinhardtii* growth

After confirming the accumulation of DXS protein in the L strains, the next step was to investigate the effect of overexpressing *dxs* on transgenic lines. The growth of the transgenic and control cell lines was therefore compared using the Algem® system. A representative from each group was taken, such as L4 representing transgenic lines that showed high expression level, L3 representing transgenic lines that showed low expression level, and dL1 representing transgenic lines containing non-functional *dxs*. The growth of these representative transgenic lines was compared to a control line H1 that contains was transformed with the empty vector to assure the same genetic background. As illustrated in Figure 5.7, the growth of transgenic lines L4 and L3 was not retarded compared to both the H1 and dL lines, suggesting that the presence of a cyanobacterial DXS protein in addition to the native one does not have a deleterious effect. In fact, all transgenic lines grew normally and the liquid culture of these lines looked healthy. To confirm the obtained result, the experiment was repeated by growing all transgenic lines (L) in comparison with the control H1 strain, as shown Figure 5.8. Clearly the transgenic lines show a normal and healthy

growth pattern. An interesting trend was observed in which the overexpression of *dxs* gene seemed to enhance the growth rate of the transgenic lines in particular in L4 and L5. However, such assumption is too early to state, as there is a need for more analysis. In general, the introduction of *dxs* gene into chloroplast genome did not lead to a reduction of the overall fitness and robustness of the transgenic strain compared to the control strain, so excluding any negative impact of overexpressing the *dxs* gene in the *C. reinhardtii* cells.

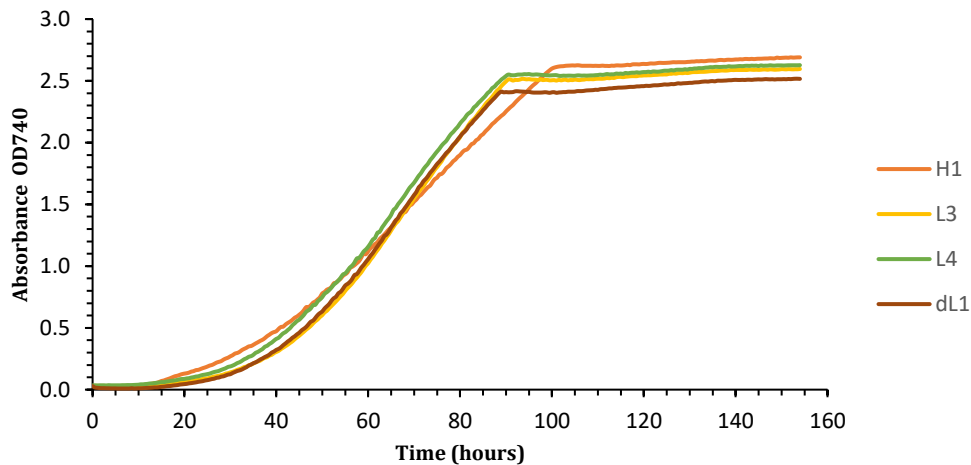


Figure 5.7 The effect of expressing *dxs* gene from *Synechocystis* PCC 6803 on *C. reinhardtii* growth.

The growth curves of *C. reinhardtii* cell lines show similar growth rates compared to the control line H1. All cultures were grown in TAP (acetate containing) medium using the Algem® system and the optical density was measured at 740 nm. The growth conditions were 25 °C, 200 μE light, and continuous shaking at 120 rpm

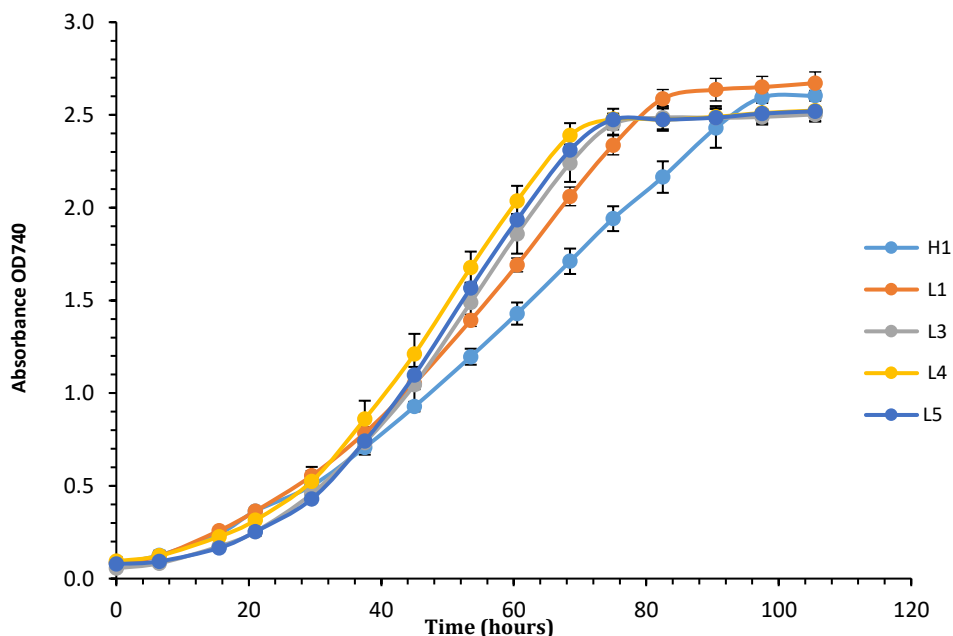


Figure 5.8 Growth curves of *C. reinhardtii* cell lines with and without the *dxs* gene from *Synechocystis* 6803.

All cultures were grown in TAP (acetate containing) medium using the Algem® system and the optical density was measured at 740 nm. The growth conditions were 25 °C, 200 μE light, and continuous shaking at 120 rpm. Error bars represents ± standard deviation of triplicate cultures.

5.2.4. A fluorometric analysis to assess the DXS enzyme activity

A number of methods have been reported for determination of DXS enzyme activity such as fluorimetry and high-performance liquid chromatography (HPLC) (Querol et al. 2001; Han et al. 2003). A fluorometric assay was used in this study to assess the activity of endogenous and 'endogenous+transgenic' DXS enzyme present in the crude extracts from the *C. reinhardtii* strains. This assay relies on the reaction of 1-deoxy-xylulose 5-phosphate (DXP) as reaction product with 3, 5-diaminobenzoic acid in an acidic medium to form a fluorescent quinidine derivative, which produces an emission spectrum with maximum intensity at 510 nm using an excitation wavelength of 396 nm (Querol et al. 2001), as illustrated in Figure 5.9. The activity of DXS enzyme *in vitro* was assessed by using a crude preparation of enzyme, in which the *C. reinhardtii* cells were broken by repeated cycles of freezing and thawing. After centrifugation, the supernatant was obtained as a crude enzyme extract, for more details refer to section 2.9.1. The activity of the DXS protein in the crude enzyme extract was then analysed in three ways: (a) to study the relationship between the amounts of added supernatant and corresponding emission; (b) to compare the activity difference of DXS enzyme between crude extract from the H1 and L strains, (c) to assess the efficiency of a freeze-thaw method for crude extract preparation.

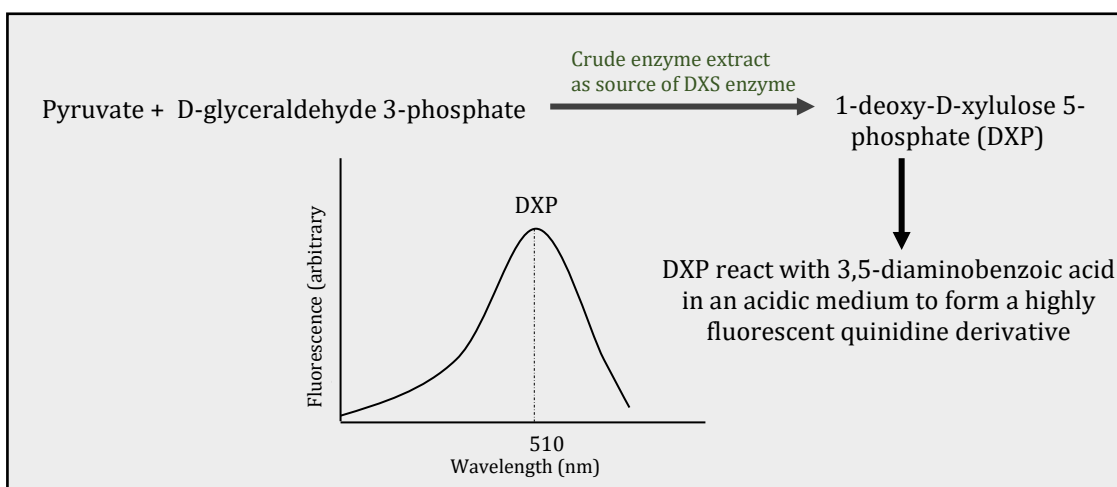


Figure 5.9 Schematic representation of the reaction catalysed by DXS synthase in fluorometric assay.

The DXS activity in the crude extract was firstly assessed by studying the relationship between the amount of added crude enzyme extract of H1 strain and the corresponding emission intensity. Figure 5.10 shows the results of analysing the crude enzyme extract of H1 cells that yielded fluorescent products showing two different maximal fluorescence intensities at different wavelengths: 450 nm and 510 nm. The presence of the emission peak at 510 nm suggests that the DXS enzyme is active, in which DXP was formed and reacted with DABA yielding a fluorescent product that emitted at the expected wavelength. On the other hand, the observed maximal fluorescence intensity at 450 nm in the emission

spectrum may indicate the presence of other fluorescent derivatives in the crude enzyme extract and it is most likely to be the excitation of chlorophyll *b* (Welschmeyer 1994). The relationship between the amount of added crude enzyme extract of H1 strain and the corresponding emission intensity was clearly demonstrated in this experiment, as the intensity of emitted fluorescence increased linearly with the amount of crude extract in the reaction mixture. This result may suggest the activity of the enzyme in the crude extract, whereby increasing the amount of crude extract led to increased catalysis and so formation of more DXP to react with DABA to give more fluorescent derivatives. Negative control results are discussed later in this section.

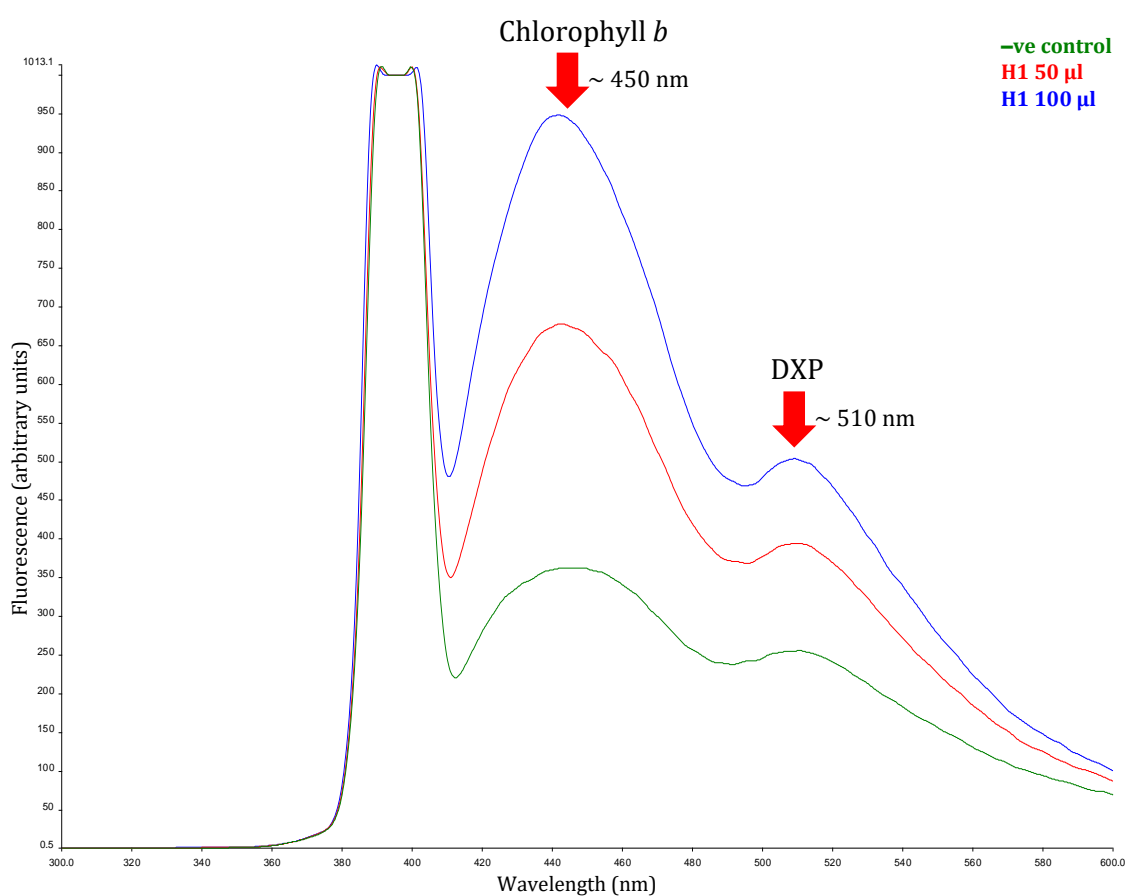


Figure 5.10 Analysis of emission spectra of the reaction product DXP catalysed by the crude enzyme extract of H1.

Emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate in the presence of crude extract of H1 cells. The crude extract of H1 cells was prepared by breaking the cells with repeated cycles of freezing and thawing, and then centrifuging the broken cells. The supernatant fraction, known as crude extract, was used as the source of DXS enzyme and different amounts of crude extract were used in the reaction; 50 and 100µl. The emission spectra show maximal intensities at different wavelength, 450 nm presumed for chlorophyll *b* and 510 nm for DXP. The fluorescence intensities increase linearly with the added amount of crude lysate. The negative control (-ve) does not contain the crude extract. The excitation wavelength was 396 nm.

Although the emission spectrum showed an additional emission peak at 450 nm, the presence of a peak at the expected wavelength (510nm) encouraged more tests to assess the enzyme activity in the *C. reinhardtii* lines. So, the next part to be investigated was whether the L1 strain showed more enzyme activity than the H1 strain, which lacks the additional *dxs* gene. The cell line L1 was used as the only cell line generated with *dxs* at that point. Figure 5.11 shows the level of fluorescence emission of reaction product catalyzed by the H1 strain is more than L1. However, the comparison between the fluorescence emissions was based on the amount of added crude extract rather than actual concentration. Therefore, in order to demonstrate the *dxs* activity difference between the control strain and transgenic strains, further studies are required in which the number of cells is equalised between extracts, and also the cell lines should be used that expressed more DXS. Alternatively, the enhanced activity of *dxs* in the transgenic lines could be demonstrated indirectly by analyzing the profile of downstream terpenoids using HPLC. Such analysis is useful to demonstrate the functionality of DXS *in vivo* and its effect on downstream metabolites at the same time. The use of the HPLC method to assess the effect of overexpressing *dxs* will be discussed in the next section.

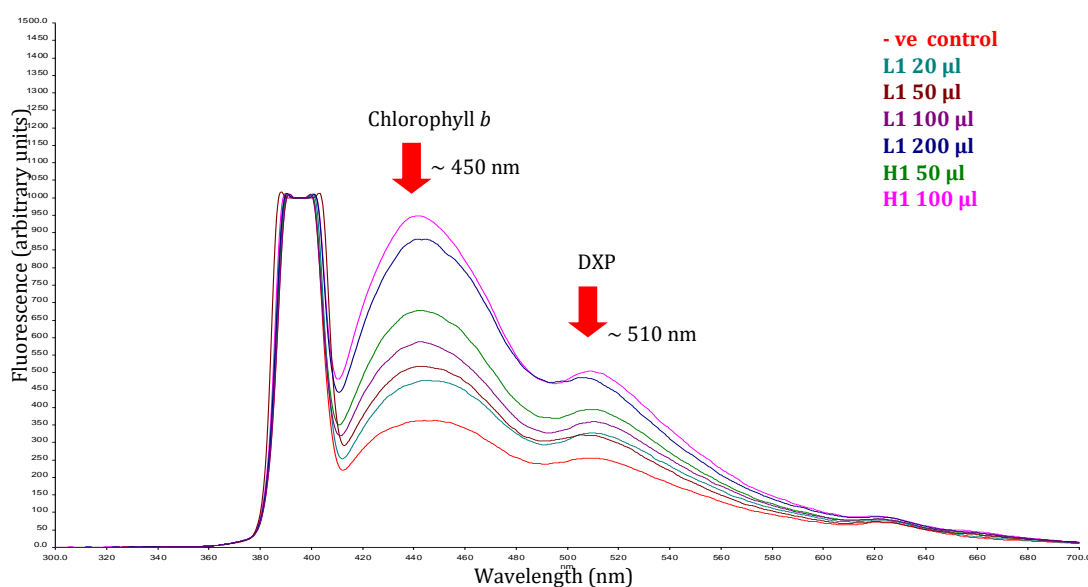


Figure 5.11 Assessing DXS enzyme activity of crude enzyme extracts from H1 and L1 cells.

Emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate in presence of either crude enzyme extract from H1 and L1 strains. The fluorescence intensities increase linearly with the added amount of crude lysate in both H1 and L1 crude enzyme extract. The negative control (-ve) does not contain the crude extract. The excitation wavelength was 396 nm.

The efficiency of freeze and thaw method for crude preparation was also assessed by examining the DXS activity in different cell fractions; lysed cells, pellet and supernatant. As shown in Figure 5.12, the supernatant fraction yielded the highest emission as expected for a soluble enzyme though no clear peak could be detected.

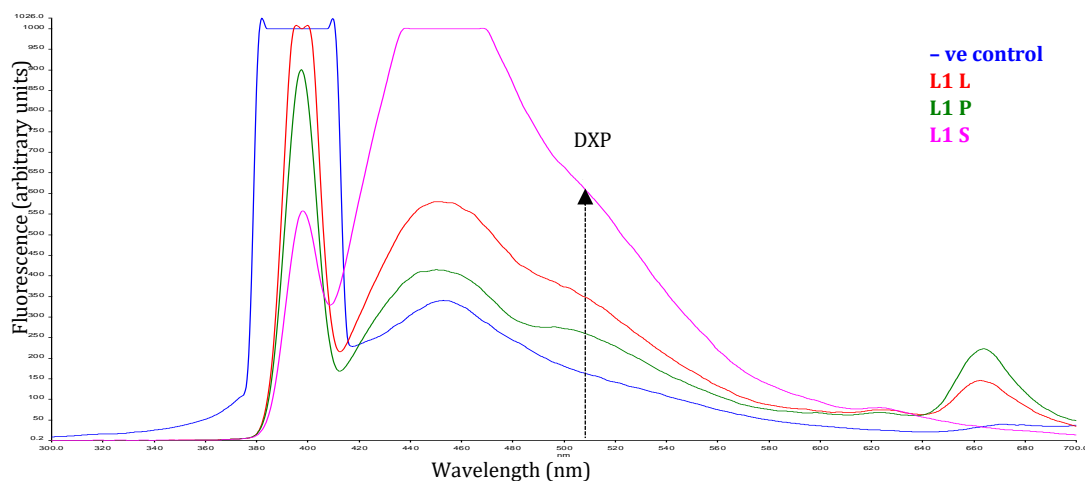


Figure 5.12 Emission spectra for the DXS assay performed using different cell fractions of *C. reinhardtii* cell line L1.

Emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate in the presence of different fractions that resulted during preparing the crude extract of L1 cells. The fractions are lysed cells (L), pellet (P), and supernatant (S). The supernatant is the fraction used in the DXS enzyme assays earlier. The excitation wavelength was 396 nm. The negative control (-ve) does not contain DABA.

To eliminate the possibility that the resulting fluorescence spectrum is due to other components rather than the fluorescent product from DXS activity, two different negative controls were used during the fluorometric analysis. The first negative control included all reaction components except the cell supernatant (Figure 5.10 and Figure 5.11), while the second control contains all reaction components except the DABA in Figure 5.12. Both control samples yielded a fluorescent product showing an emission around the same wavelengths but at lower intensities. In order to identify the component in the reaction mixture that could possibly result in the background emissions in negative control samples, the emission spectrum of reaction mixture without crude enzyme extract were examined by increasing the concentration of DL-glyceraldehyde 3-phosphate from 0 mM to 20 mM. The result in Figure 5.13 suggests indeed that the emission spectrum of negative control samples at low fluorescence intensities is due to other components in the reaction mixture and it is not possible to get a straight line in the negative control spectrum. In addition, the emission spectrum of the negative control sample (without lysate) yielded fluorescence products showing an emission spectrum with similar peak wavelengths to samples containing crude enzyme extract but far less in intensity. Such background emission from the DABA substrate discouraged us from continuing to investigate the DXS activity using this method. Due to the differences of the detected maximal intensities at different wavelength (450 and 510 nm) between the control sample (without lysate) and the sample with crude enzyme extract, a different approach was taken to investigate the activity of the enzyme. The approach relies on introducing the *dxs* gene into the *Escherichia coli* DH5 α to get a chlorophyll free crude enzyme extract for enzyme activity assay. The promoter/5'UTR

of *psaA* used to drive the expression of *dxs* gene has maintained bacterial features of its regulatory elements and therefore the expression of *dxs* gene in *E. coli* is possible, discussed in section 3.2.3.2. So, the following constructs; pRY134a, pRY134a Δdxs and pSRSapI were transformed into *E. coli* DH5 α and western blot analysis with anti-HA antibody was performed to check the accumulation of DXS protein in *E. coli*. The result in Figure 5.14 illustrates that the protein has been expressed successfully as an intense clear band is seen at 70.4 kDa. This result encouraged an enzyme activity assay using crude extract from cells carrying the different plasmids (pRY134a [+*dxs*], pRY134a Δdxs [Δdxs] and pSRSapI [-*dxs*]). It is important to note that the crude extract from pRY134a transformants is expected to give higher fluorescence, whereas less fluorescent product is expected for the extracts from both pRY134a Δdxs and pSRSapI since *E. coli* has an endogenous *dxs*. As illustrated in Figure 5.14, there is no sign of enzyme activity in the emission spectrum for any of the extracts as they all showed similar signal intensities at 510 nm. This suggests that the DXS protein detected in the western blot of the pRY134a extract is either not functional, or that the assay is flawed. The main reason behind using the *E. coli* expression system was to minimize the interfering absorbance related to other components in the algal extracts such as chlorophyll *b*. Nonetheless, the unsuccessful attempts to detect the fluorescent product in emission spectra from the *E. coli* crude extracts leaves open the possibility that the recombinant DXS produced in the *C. reinhardtii* cells is functional.

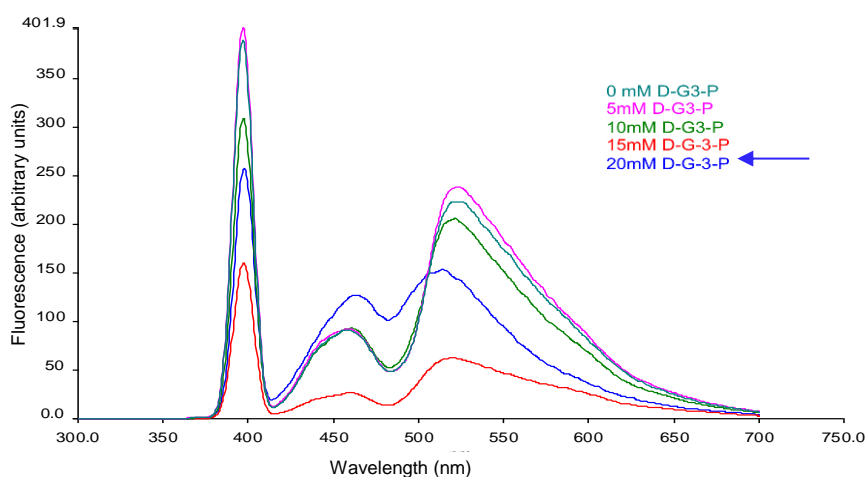


Figure 5.13 Analysis of emission spectra demonstrating the interference absorbance from reaction substrates.

Emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate, without adding any crude extract. Background signal in emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate were examined by using different concentrations of DL-glyceraldehyde 3-phosphate. Arrow indicates DL-glyceraldehyde 3-phosphate concentration that was used in the reaction mixture for DXS assays. The excitation wavelength was 396 nm.

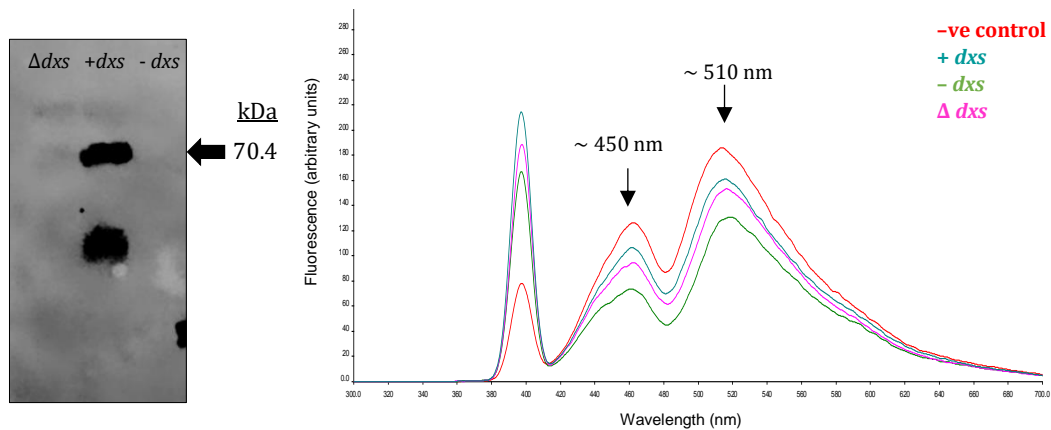


Figure 5.14 Analysis of crude enzyme from *E. coli* transformants.

Left panel: Western analysis with anti-HA antibody show a strong band running at an apparent mass of 70.4 kDa in the crude enzyme extract of pRY134a transformant ($+dxs$), whereas no band was detected in either pRY134a Δdxs transformant (Δdxs) or pSRSapI transformant ($-dxs$). Right panel: emission spectra of the reaction product of DL-glyceraldehyde 3-phosphate and sodium pyruvate demonstrate the absence of the DXS activity in all crude enzyme extracts though a band at 70.4 kDa was detected in Western blot analysis with anti-HA tag antibody in $+dxs$. The negative control does not contain a crude enzyme extract and the excitation wavelength was 396 nm. The wavelengths of peaks are indicated by arrows.

After failing to demonstrate DXS enzyme activity from the *E. coli* extracts, an attempt was made to obtain a clearer form of extract from *Chlamydomonas* by breaking the cells by a freeze-thaw method and then spinning the cells at 100,000 x *g* for 1 hour to pellet membrane material, as details in section 2.9.1. Unfortunately, the attempt was not successful as this treatment resulted in a poor recovery of DXS protein in the soluble extract as illustrated in western blot analysis (Figure 5.15). However, a change in the extract colour was observed as a pale yellow in the control strain H1 and an orange colour in the transgenic strain L4, as shown in Figure 5.16. Such colour change in the crude enzyme extract as a result of overexpressing the *dxs* gene provides a visual indication that the exogenous *dxs* gene is expressed and produces a functional enzyme. Therefore, this result primarily suggests the functionality of DXS enzyme and its effect on increasing downstream metabolites in particular carotenoids. In order to support the observed effect of overexpressing the *dxs* gene in the transgenic lines compared to the control strain H1, the carotenoid level was further analysed using HPLC.

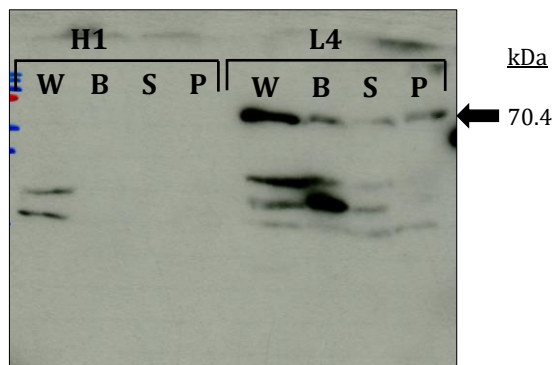


Figure 5.15 Western blot analysis demonstrating a poor recovery of DXS protein in the supernatant after breaking the cells.

The *C. reinhardtii* cells H1 and L4 were broken by repeated cycles of freezing and thawing. The efficiency of lysis process was determined by western blot analysis with anti-HA antibodies. The presence of HA-tagged DXS protein in L4 cell line was detected as a band at 70.4 kDa in the following fractions; whole cell lysate (W), broken cells (B), supernatant (S) and pellet (P). H1 strain was included as a negative control, which as anticipated does not show a band in any fractions.

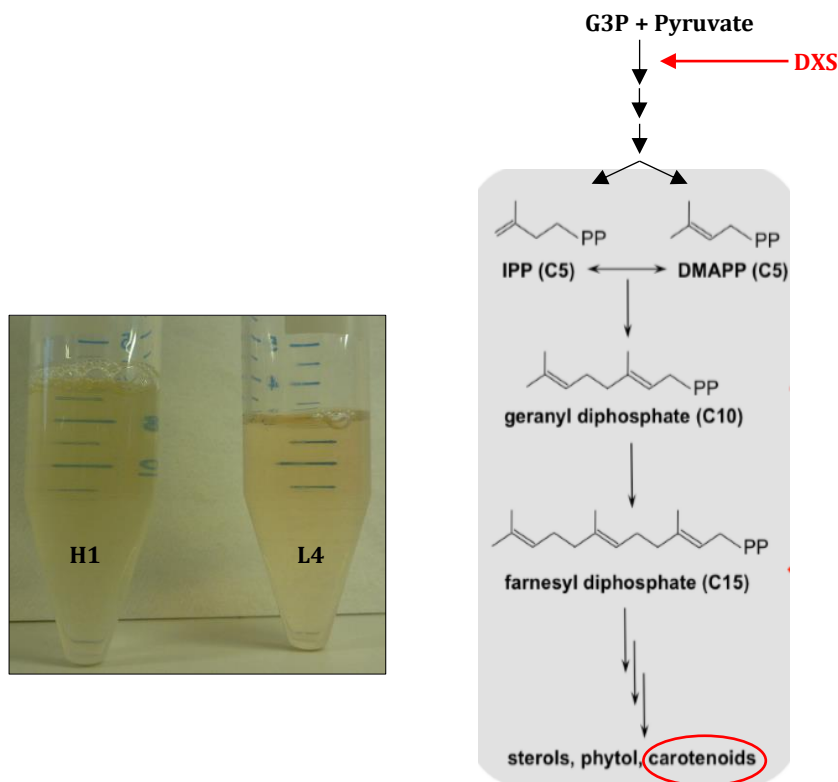


Figure 5.16 The colour changes in the crude enzyme extract of the transgenic line L4 suggesting the activity of the DXS enzyme.

In left panel, the colour differences of the crude enzyme extract from the control strain H1 as a pale yellow and the transgenic strain L4 as an orange. In right panel, schematic representation of terpenoid biosynthetic pathway in the chloroplast. The red arrow indicates the introduced additional copy of *dxs* and its subsequent effect on downstream metabolites in particular carotenoid level, as is indicated from crude enzyme extract colour. Multiple arrows indicate that more steps are involved but not shown.

5.2.5. Analysis of terpenoid profile of the genetically engineered *C. reinhardtii*

In order to determine the impact of expressing an additional *dxs* gene on terpenoid content, the quantity of plastidic terpenoids such as violaxanthin, lutein, α -carotene and β -carotene was measured in the transgenic lines and compared with the levels found in the control line H1. All algal strains were grown in moderate light conditions then harvested and freeze-dried as described in section 2.10. The freeze-dried algal samples were sent to Royal Holloway University of London for HPLC analysis, where carotenoids were extracted by methanol extraction using an equal amount of freeze-dried algal. The results in Figure 5.17 show an overall increase in the carotenoid levels in mg/g DW in all transgenic lines based on peak area. The expression of the additional *dxs* gene results in increased levels of neoxanthin/loroxanthin, lutein, α -carotene, β -carotene and cis- β carotene, though there is variation between the transgenic lines. Interestingly, the increase in lutein and cis- β -carotene levels in transgenic lines is consistent with the accumulated amount of DXS protein detected by western blot analysis in Figure 5.5, where more DXS protein was detected in both L4 and L5 compared to L1 and L3. This result is expected since an additional copy of the *dxs* gene was introduced into the chloroplast genome to increase the supply of IPP/DMAPP and so increase the flux towards downstream metabolites. Such a result was demonstrated for higher plants in which the over-expression of the *dxs* gene is correlated with increased accumulation of β -carotene in the oil palm *Elaeis guineensis* and carotenoid accumulation in *Arabidopsis thaliana* (Khemvong & Suvachittanont 2005; Munoz-Bertomeu et al. 2006; Gong et al. 2006; Estévez et al. 2001). In general, the levels of carotenoid in the transgenic lines indicates the additional activity of DXS in the chloroplast, though further analysis is required to validate the difference.

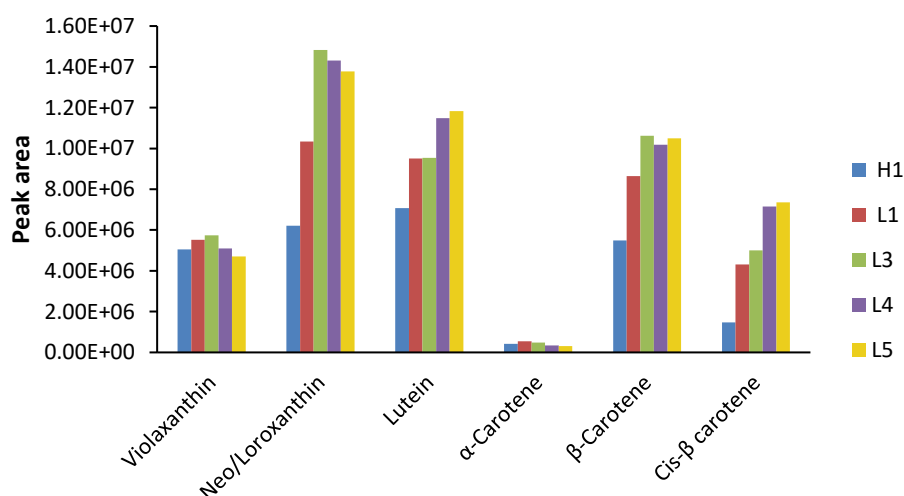


Figure 5.17 Carotenoids analysis of transgenic lines demonstrating the effect of expressing an additional *dxs* gene on downstream metabolites.

Carotenoids level in the *C. reinhardtii* lines were determined by HPLC analysis.

5.3. Discussion and conclusion

Because of the biotechnological application of terpenoids in animal feed, human nutrition, pharmaceuticals and biofuels, this chapter aimed at gaining a better understanding of the MEP pathway that leads to terpenoid production in the green microalga *C. reinhardtii*. A number of studies have proven that the DXS enzyme catalyses one of the rate limiting steps in the MEP pathway in a variety of systems. For example, the overexpression of *dxs* led to increased accumulation of essential oil in lavender, β -carotene accumulation in the oil palm *Elaeis guineensis* and carotenoid accumulation in *Arabidopsis thaliana* (Khemvong & Suvachittanont 2005; Munoz-Bertomeu et al. 2006; Gong et al. 2006; Estévez et al. 2001). Similarly in *Corynebacterium glutamicum*, where the overexpression of *dxs* improved the lycopene accumulation (Heider et al. 2014). All these studies used a common strategy to genetically engineer terpenoid metabolism by enhancing flux to increase the precursor pool of IPP and DMAPP, and thus enhance the accumulation levels of downstream terpenoids. To extend these studies further in the algal chloroplast and investigate the fundamental aspects of flux through the MEP pathway in *C. reinhardtii*, a reverse genetic approach was used to introduce a second *dxs* gene into a neutral site within the chloroplast genome and then study the corresponding changes in the downstream terpenoid profile.

Several transgenic lines were created, in which an additional *dxs* gene from *Synechocystis* PCC 6803 was integrated successfully into the chloroplast genome under the control of the promoter/5'UTR from *psaA* exon 1, and the 3'UTR from *rbcL*. Another set of transgenic lines carrying non-functional *dxs* was also created to examine the possibility that overexpressing the *dxs* gene in *C. reinhardtii* has a toxic effect on the cells, as only low numbers of *dxs* transformants were obtained by the glass bead method. Nonetheless, the growth analysis indicated that there was no toxic effect of overexpressing the *dxs* gene in *C. reinhardtii* as demonstrated by normal growth rate and healthy appearance in all transgenic lines. In principle, the over-accumulation of DXS provides the 'push' to increase the precursor pools for downstream metabolites, as opposed to the 'pull' of introducing a downstream enzyme that competes with other pathways for the same precursor pool. An example of undesirable collateral effects is the introduction of a phytoene synthase cDNA in tomato fruit as a first committed step in a carotenoid biosynthesis pathway. The overexpression of phytoene synthase severely affected the transgenic lines resulting in dwarfism and reduce chlorophyll content due to the alteration in the synthesis of both abscisic acid and phytol from the precursor GGPP shared with carotenoids, as illustrated in Figure 5.18 (Fray et al. 1995). Also, some terpenoids have a toxic effect on the cell; for instance, the diversion of terpenoid intermediates by introducing a geraniol synthase in the grapevine, *Vitis vinifera* to produce geraniol resulted in severely impaired cells and ultimately led to cell death (Fischer et al. 2013).

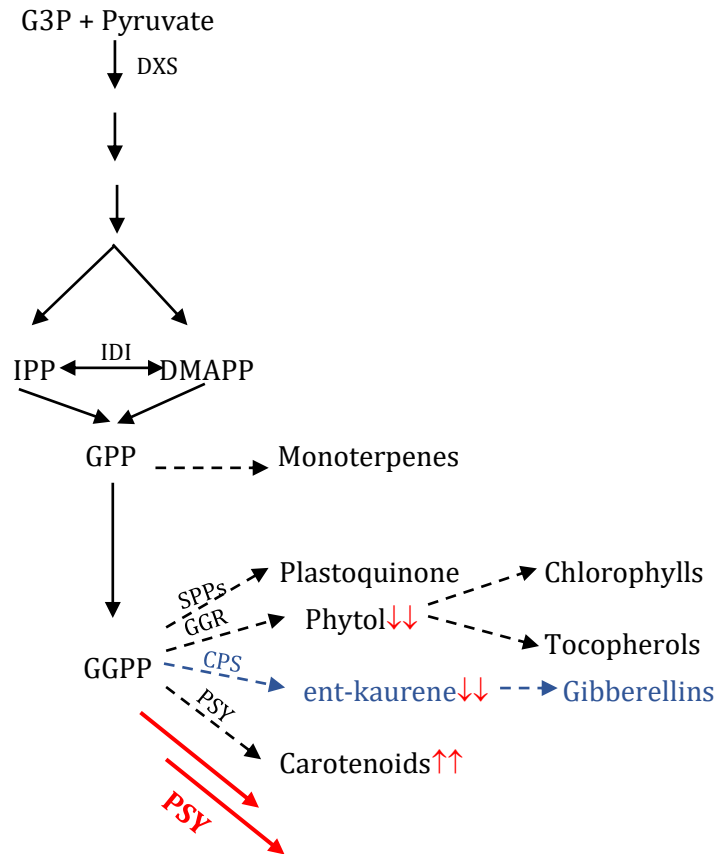


Figure 5.18 Schematic representation of the effect of overexpressing phytoene synthase on other terpenoid biosynthetic pathways in plant.

The overexpression of phytoene synthase (PSY) direct the metabolic flux towards carotenoid production as indicated by red arrows at the expense of ent-kaurene and phytol synthesis that share the same GGPP pool. The increase in carotenoid levels is represented by $\uparrow\uparrow$, whereas the decrease in the level of both phytol and ent-kaurene is represented by $\downarrow\downarrow$. The pathway in blue is found in plant but not in microalgae. The black multiple arrows indicate that more steps are involved but not shown.

The current direction of research on algal terpenoid metabolism is to genetically engineer pathways to increase the productivity or enable the production of novel terpenoids with industrial applications (see Table 5.2). However, most of these attempts relied on targeting non rate-limiting endogenous enzymes to alter the productivity level of the desired terpenoid, resulting in no or little effect. The most relevant example is the genetic manipulation of the carotenogenic pathway in *C. reinhardtii* by heterologous expression of the β -carotene ketolase gene from *H. pluvialis*. This attempt was not successful in obtaining a transgenic microalga capable of synthesising ketocarotenoids (León et al. 2007). Another unsuccessful attempt is reported by Vila et al. (2008) where the post-transcriptional silencing of phytoene desaturase by RNA interference led to under expression of the target gene but did not show any changes on the carotenoid profile. As for producing non-native metabolites in the *C. reinhardtii*, a recent study aimed at producing *cis*-abienol 4 in the *C. reinhardtii* chloroplast genome for its important role in the fragrance industry, in particular

to produce Ambrox®. This study was not successful as transplastomic lines did not show any detectable levels of *cis*-abienol 4 though the recombinant protein was successfully detected (Zedler et al. 2014).

As evident from these attempts, the genetic manipulation of terpenoids has not been free of difficulties as a result of a poor understanding of these biosynthetic pathways. Therefore, in this study a different approach was used to alter terpenoid levels by targeting an enzyme called DXS, which has been reported by a number of studies as a rate-limiting enzyme in the MEP pathway for plastidic terpenoid production in bacteria and plants (Estévez et al. 2001; Heider et al. 2014). In principle, the alteration of *dxs* levels should result in changes in several plastidic terpenoids, unless it is not a rate-limiting step in the MEP pathway of *C. reinhardtii*. The analysis of the transgenic lines that overexpressed *dxs* showed an overall increase in the levels of several plastidic terpenoids such as neoxanthin/loroxanthin, lutein and β -carotene. However, some the plastidic terpenoids include violaxanthin and α -carotene did not show any changes in their levels highlighting the complexity of the pathways and possibility of the presence of co-limiting steps for specific terpenoids.

Generally speaking, an overall increase in a number of downstream terpenoid metabolites as a result of alteration in the *dxs* levels demonstrates that DXS catalyses one of the rate limiting steps in the production of the plastidic IPP (and hence of terpenoids) in *C. reinhardtii*. It is worthy to note that, although each plastidic group of terpenoids has distinct biosynthetic pathways that might be targeted for genetic manipulation, the output of these pathways could be confined by the available amount of IPP that is normally produced in the chloroplast (Estévez et al. 2001). Nevertheless, some studies have shown the success in redirecting the metabolic flux into a single endogenous downstream product, as is the case for the *C. reinhardtii* lines that overexpress phytoene synthase with a resulting 1.8–2.6 fold carotenoid increase (Couso et al. 2011).

The overexpression of *dxs* increases the precursor pools of IPP and DMAPP for the downstream terpenoid metabolites, where three main pathways are most likely to be affected by such increase in both IPP and DMAPP pools. These pathways share the same precursor GGPP and include phytol, carotenoid and plastoquinone. Ent-kaurene is an additional biosynthetic pathway that is found in plants and is involved in the synthesis of a group of plant hormones called gibberellins. However, hormone studies have been neglected in microalgae and there is a possibility of involvement of some these terpenoid hormones in algae but have not been identified yet (Lohr et al. 2012). Although there are three pathways that diverge from the GGPP precursor, and so all three pathways could be assessed upon the upregulation of the DXS enzyme activity in the MEP pathway, the main focus was to analyse the quantitative changes in the carotenoid levels of various classes due

to their industrial applications. Nonetheless, the analysis of other pathways that share the same precursor pools such as phytol – e.g. by analysing phytol-derived chlorophyll compounds – would provide additional information, and therefore increase our chances of genetic engineering these pathways in the algal chloroplast more successfully. The most relevant example that highlighted the significance of understanding these pathways is the initial unsuccessful attempt to generate tomato fruit accumulating more phytoene by overexpressing the phytoene synthase gene (Fray et al. 1995). Though it was unsuccessful, it sheds light on the regulation of particular pathways that share the same precursor pools (GGPP). Such understanding led to a success in a 43-fold average increase of β -carotene in *Arabidopsis* by overexpressing the phytoene synthase gene under the control of a seed specific promoter to avoid the undesirable effects of these metabolites in the plant cells (Lindgren et al. 2003).

In summary, the strategy used here for engineering terpenoid biosynthesis relies on enhancing the flux to increase the precursor pools of IPP and DMAPP by introducing an additional transgenic copy of the gene for DXS, which is involved in catalysing the first step in the plastidic MEP pathway. The overexpression of *dxs* influenced the level of several plastidic terpenoid such as neoxanthin/loroxanthin, lutein and β -carotene suggesting that DXS catalyses one of the rate limiting steps of the MEP biosynthetic pathway in the *C. reinhardtii*. The identification of such a limiting step in the MEP pathway might ultimately enable the use of a combinatorial “push-pull” approach to increase the productivity of endogenous terpenoids without depleting precursor pools and inhibiting other precursor dependent pathways. This would involve engineering the MEP pathway to enhance the precursor pools through overexpressing the rate limiting enzyme, as well as re-directing the pathway toward producing the desired terpenoid molecules by introducing the enzyme involved in the committed step for the specific terpenoid, see Figure 5.19.

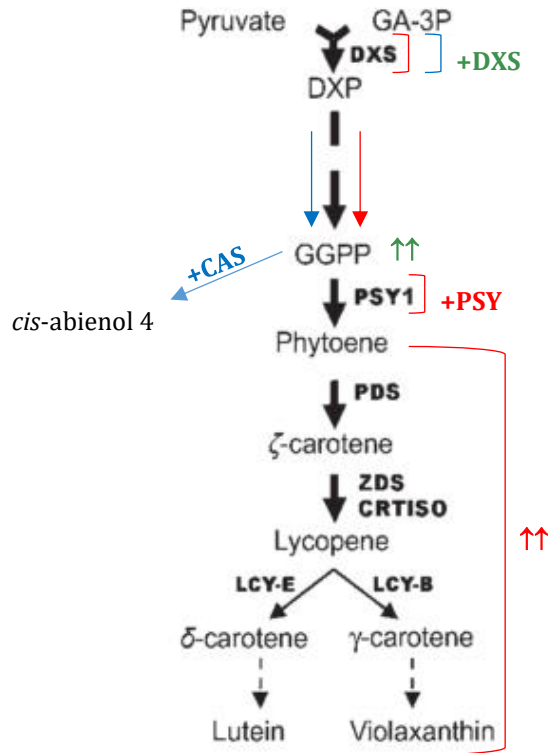


Figure 5.19 Engineering terpenoid synthesis in the *C. reinhardtii* chloroplast.

The terpenoid pathway is indicated with black arrows. Examples of potential pathways that could be coupled with overexpressing the rate-limiting DXS enzyme (green) to direct the metabolic flux to the desired terpenoid products. The endogenous DXS and PSY could be upregulated to direct the metabolic flux to produce more carotenoids (red). Another potential pathway that could be introduced to produce novel terpenoid such as *cis*-abienol (blue), where the upregulation of DXS increase the GGPP pool that required for *cis*-abienol synthesis. The flux increase of the corresponding product/s is represented by ↑↑, while the introduced additional copy of the gene is indicated by +. Abbreviations are DXS: 1-deoxy-D-xylulose-5-phosphate synthase; GGPP: geranylgeranyl diphosphate; PSY: phytoene synthase; CAS: *cis*-abienol synthase. The diagram was modified from Vranová et al. (2012)

CHAPTER 6

Final discussion

6. Final discussion

6.1. Summary of main findings

Nannochloropsis are promising oleaginous species with potential to serve as phototrophic platforms for the commercial production of advanced biofuels and high value compounds. However, the biotechnological exploitation of these microalgae necessitates the development of molecular tools and technologies. Of particular interest is the development of molecular tools for chloroplast engineering, as the organelle is the site of photosynthesis and key biosynthetic pathways for fatty acid and terpenoid production. In an effort to exploit these industrially relevant microalgae, an attempt was made to develop a reliable chloroplast transformation methodology for *Nannochloropsis gaditana*. Results presented in Chapter 3 demonstrated the main steps required for the development of a chloroplast transformation methodology for a new species. These steps were the optimisation of growth conditions, identification of a suitable selectable marker for the chloroplast, the construction of a transformation vector and testing of the different transformation methodologies. This chapter also highlighted the main challenges in delivering exogenous DNA into the chloroplast genome of *N. gaditana* as a result of the small size of the cell (and therefore its plastid) and the complexity of the plastid membranes. Even though the antibiotic chloramphenicol was identified as a potential selectable marker, the chloroplast transformation attempts were not successful in spite of testing two delivery methods: electroporation and biolistics. Nuclear transformation was carried out alongside chloroplast transformation as a control, and transformants were obtained by electroporation, but not biolistics. The preliminary results of these chloroplast and nuclear transformation attempts suggested that the conditions employed for electroporation were not sufficient to allow the passage of the DNA across the plastid membranes, while the invasive nature of biolistics prevented the recovery of viable cells. However, the definite reason behind such unsuccessful attempts was difficult to determine due to the involvement of several factors between introduction of the foreign DNA into the chloroplast genome and successful expression of the gene to exhibit the desired phenotype. As such, the lack of success could be due to poor expression of the chosen selectable marker or the possibility that chloramphenicol adversely affects the prokaryotic-like translational machinery of the mitochondria, thereby preventing the recovery of resistant transformant lines. Or failure could simply reflect the inability to deliver the DNA to the chloroplast, as mentioned above.

Therefore, Chapter 4 focused on developing a more controllable approach to monitor the steps involved in developing the chloroplast transformation methodology and addressing uncertainty regarding gene expression of the marker. This approach relied on i) screening chloroplast-specific compounds to identify alternative selection strategies that would not impact on the mitochondrion, ii) developing an endogenous selectable marker using forward and reverse genetics to eliminate issues associated with foreign gene expression and iii) increasing the cell size temporarily prior to biolistic transformation to maximise the chances of delivering DNA-coated microparticles across multiple barriers without killing the cells. These attempts as presented in Chapter 4 demonstrated the sensitivity of *N. gaditana* to the photosystem II inhibitors atrazine and diuron, and identified a *psbA* mutation that is likely to confer atrazine tolerance (and which may enable the use of atrazine as a selective agent). In addition, the results in Chapter 4 demonstrated the development of a method to increase the average cell size using the microtubule inhibitor nocodazole and, most importantly, demonstrated that the biolistics conditions developed for the best chance of chloroplast transformation allowed nuclear transformants to be isolated, thereby showing that DNA-coated microparticles were able to enter the cell without killing every cell.

The ultimate aim of developing a chloroplast transformation methodology in *N. gaditana* is to allow the genetic manipulation of the key biosynthetic pathways in the chloroplast, such as those for terpenoid and fatty acid production. Since chloroplast transformation of *Nannochloropsis* species is not yet possible, metabolic engineering of the model microalga *C. reinhardtii* is the current option to further our understanding about these biosynthetic pathways until molecular tools become available for those microalgae with industrial potential. Therefore, Chapter 5 describes metabolic engineering of the MEP pathway that leads to terpenoid biosynthesis in the chloroplast of *C. reinhardtii*. The metabolic engineering approach relied on increasing the precursor pools for terpenoid biosynthesis by introducing the cyanobacterial version of the *dxs* gene involved in catalysing the first step in the plastidic MEP pathway. The chloroplast engineering successfully resulted in transgenic lines carrying this additional copy of *dxs* and the expression of DXS was detectable in all transgenic lines in spite of variation in levels of accumulated DXS. Furthermore, these transgenic lines showed normal growth and healthy appearance, exempting any negative effect of such genetic manipulation. The over-expression of the *dxs* gene appeared to show an overall increase in the downstream terpenoid metabolites lutein, β -carotene, cis- β -carotene and neoxanthin/loroxanthin, suggesting that the DXS enzyme catalyses one of the rate limiting steps of the MEP pathway in *C. reinhardtii*.

6.2. Metabolic engineering of the algal chloroplast: challenges and potential

Because of the biotechnological applications of terpenoids in animal feed, human nutrition, pharmaceuticals and biofuels, this study ultimately aimed at exploiting algal chloroplast as a terpenoid production platform through metabolic engineering technologies. However, the biotechnological exploitation of algal chloroplast necessitates the development of genetic engineering tools for more algal species, particularly those with industrial potential to allow the genetic manipulation of terpenoid biosynthesis pathway, at the same time, the development of advanced engineering tools and new strategies to engineer terpenoids pathways in established laboratory algal species such as the model microalga *C. reinhardtii*. With a view to enabling the sustainable production of terpenoids, the genetic manipulation of terpenoid biosynthesis pathway in the algal chloroplast of two different species has been investigated in this study; i) *N. gaditana* as a novel microalga with industrial potential and ii) *C. reinhardtii* as a model microalga with well-established genetic engineering tools.

The biotechnological exploitation of *N. gaditana* chloroplast as a terpenoid production platform is not possible at present due to the absence of a reliable delivery method. The attempts in Chapter 3 and 4 were not successful highlighting the main challenges associated with the development of a reliable chloroplast transformation methodology for this oleaginous microalga as a result of the complexity of the plastid membranes and the small cell size (therefore the chloroplast). This could be an explanation as to why there are no reports yet on successful chloroplast transformation of *Nannochloropsis* species or other microalgae that harbour secondary plastids (with an exception *Phaeodactylum tricornutum*) (Al-Hoqani et al. 2016; Xie et al. 2014). As most of the progress is being made in the plastid transformation technology for those microalgae with a primary plastid such as *C. reinhardtii* and *H. pluvialis* (see Table 3.1 for more examples).

Although there are no reports yet for a successful chloroplast transformation in *Nannochloropsis* species, there is still ongoing interest to improve the intrinsic features of *Nannochloropsis* for biotechnological exploitation through classical mutagenesis and nuclear genetic engineering (Al-Hoqani et al. 2016). In fact, many studies reported their attempts to improve the lipid productivity and/or profile for *Nannochloropsis* species (Table 6.1). One of these studies reported strain improvement in lipid profile by overexpressing *N. oceanica* microsomal-like $\Delta 12$ -desaturase (NoD12) as a committing step for omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) (Huang et al. 2014). While another study focused on improving the light penetration in a dense culture for large scale cultivation by reducing light harvesting apparatus (Perin et al. 2015). This study

successfully isolated a mutant with a reduced light harvesting apparatus demonstrating an improved photosynthetic activity and productivity in the lab-scale cultures.

Table 6.1 Reports on strains improvement and nuclear engineering to improve lipid profile/productivity.

Abbreviations are: EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; PUFAs, polyunsaturated fatty acids; TAG, triacylglycerol. Table was modified from Al-Hoqani et al. (2016)

Species	Approach used for strain improvements	Notes	Reference
<i>Nannochloropsis sp.</i>	Classical mutagenesis (ethyl methanesulfonate)	A 30% increase in palmitoleic acid (16:1) and a 45% decrease of EPA Increase	(Doan & Obbard 2012)
<i>Nannochloropsis salina</i>	Classical mutagenesis (ethyl methanesulfonate)	Increase in total FAME production and reduced levels of PUFAs	(Beacham et al. 2015)
<i>Nannochloropsis oceanica IMET1</i>	Classical mutagenesis (Heavy ion radiation)	High growth rate and 14% increase in TAG	(Ma et al. 2013)
<i>N. salina</i>	The overexpression of <i>basic helix-loop-helix isoform 2</i> gene in the nuclear genome	Increase biomass productivity by 36% under normal condition and FAME productivity by 33% under nitrogen starvation	(Kang et al. 2015b)
<i>N. gaditana</i> †	The heterologous expression of <i>Long-chain acyl-CoA synthetase (LACS)</i> gene in <i>S. cerevisiae</i> as a host	Accumulation of eicosapentaenoic acid and docosahexaenoic acid	(Zheng et al. 2014)
<i>Nannochloropsis strain NIES-2145</i>	The overexpression of <i>Diacylglycerol acyl-CoA acyltransferase type-2</i> gene in the nuclear genome	Increased TAG accumulation by 1.7 fold under phosphorus starvation	(Iwai et al. 2015)

† In this study, the *LACS* gene was isolated from *N. gaditana* to be studied and characterised.

It is also worthy to note that the absence of genetic tools for engineering the chloroplast genome has not prevented the growing sector of exploiting wild type *Nannochloropsis* as a base of the food chain in the aquaculture industry due to the its high lipid content. A number of companies – such as Algaspring, Archimde, Monzon Biotech and Proviron – use the wild type *Nannochloropsis* as a PUFA-rich aquaculture feed for the cultivation of shrimps, molluscs and marine fish, or alternatively for production of rotifers, which in turn are used as a food source for fish hatcheries (Camacho-Rodríguez et al. 2014; Lubzens et al. 1995). The use of *Nannochloropsis* as an aquaculture feed comes up with potential opportunities to use transgenic lines as an oral delivery system for pharmaceutical proteins to improve both the growth rate of fish and survival rate during infection by pathogens. An example of such opportunities has been demonstrated by an improved survival rate of medaka fish infected with a bacterial pathogen as a result of feeding them with an engineered transgenic line that produced the anti-microbial peptide bovine lactoferricin (Li & Tsai 2009). In addition to being used as an aquaculture feed, is also used as a feed for farmed animals to improve the nutritional value such as the addition of *Nannochloropsis* biomass to the feed of laying hens has greatly improved the nutritional value of egg yolk (Lemahieu et al. 2013).

Nevertheless, to establish *Nannochloropsis* as an effective production platform for industrial applications, genetic tools for engineering the chloroplast genome are also required to facilitate the investigation into the chloroplast biosynthetic pathways and allow genetic engineering strategies to further improve this alga. This commercially grown *Nannochloropsis* could be also ‘functionalised’ through chloroplast engineering to produce oral vaccines, hormones, anti-bacterial proteins, etc. More efforts therefore should be focused on the development of genetic engineering tools for the chloroplast, where most efforts currently focused on genetic engineering of the nuclear genome and the development of advanced engineering tools such as CRISPR-Cas9 (Wang et al. 2016; Al-Hoqani et al. 2016). The development of the genetic engineering tools for the nuclear and chloroplast genome should allow the exploitation of *Nannochloropsis* in a wide range of industrial applications. As such, the development of full set of genetic engineering tools for *Nannochloropsis* could further improve the production capacity of secondary metabolites and biomass accumulation (Figure 6.1).

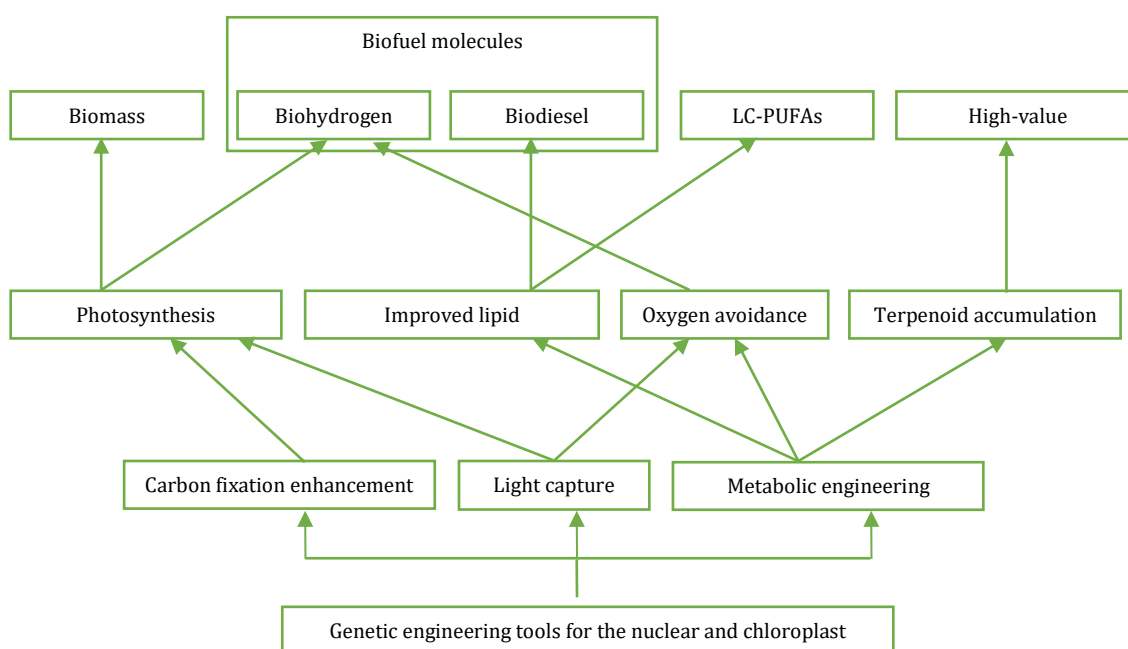


Figure 6.1 The contribution of genetic engineering tools to improve *Nannochloropsis* for industrial applications.

The development of genetic engineering tools for both the nuclear and chloroplast genome could contribute greatly in improving the production capacity of secondary metabolites and biomass accumulation. Diagram was modified from Gimpel et al. (2015).

In contrast to *N. gaditana*, the genetic manipulation of terpenoid biosynthetic pathway in the *C. reinhardtii* chloroplast is possible since the genetic engineering tools for this microalga are highly developed including well-established genetic transformation methods for the chloroplast and standardised expression systems. The advent of genetic engineering tools facilitated the investigation undertaken in Chapter 5 that aimed at gaining a better understanding of the MEP pathway that leads to terpenoid production in the *C. reinhardtii* chloroplast. The results presented in Chapter 5 demonstrated that the over-expression of *dxs* influenced the level of several plastidic terpenoid such as neoxanthin/loroxanthin, lutein and β -carotene suggesting that DXS catalyses one of the rate limiting steps of the MEP biosynthetic pathway in the *C. reinhardtii* (Figure 5.16). The identification of this limiting step in the MEP pathway offer opportunities to use a combinatorial “push-pull” approach to increase the productivity of endogenous terpenoids without depleting precursor pools and inhibiting other precursor dependent pathways (Figure 5.18). It also offers the possibility of transferring the strategy tested for engineering terpenoid biosynthesis pathway to other industrially relevant microalgae such as *N. gaditana* once the molecular toolkit becomes established. It is worth mentioning that *C. reinhardtii* has been used as a model organism for other industrially relevant species including higher plants, where the plastid transformation technologies were initially developed in *C. reinhardtii* and then these technologies were transferred into higher plants (e.g. *Nicotiana tabacum*) (Scaife et al. 2015; Day & Goldschmidt-Clermont 2011). *C. reinhardtii* has also become a model alga for other productive algal species that are naturally high producer of biomass and lipid including *Nannochloropsis* species (Al-Hoqani et al. 2016; Radakovits et al. 2012).

Although *C. reinhardtii* is not an inherently exceptional producer of biomass or lipid, it has potential as a production platform for human therapeutic proteins including human growth hormones, vaccines, antibodies, immunotoxins and antibacterial proteins (Stoffels et al. 2017; Wannathong et al. 2016; Tran et al. 2013; Mayfield et al. 2003). For example, a full human monoclonal antibody was expressed in the chloroplast of *C. reinhardtii*, and was demonstrated to have antigen binding activity similar to the same antibody expressed in mammalian cells (Tran et al. 2009). Most recently, Stoffels et al. (2017) reported a successful synthesis of two antibacterial proteins against a major human pathogen *streptococcus pneumoniae* in the chloroplast of *C. reinhardtii*, and the lytic activity was demonstrated against several serotypes of *S. pneumoniae*. This encouraging progress in the exploitation of the *C. reinhardtii* chloroplast as a production platform for human therapeutic proteins has recently led to the establishment of start-up companies. A company named Triton (<http://www.tritonhn.com/>) exploits an engineered *C. reinhardtii* chloroplast as a viable platform to produce recombinant proteins as nutraceuticals, therapeutics and cosmetics. Because of the GRAS state of microalgae in general including *C. reinhardtii*, Triton

is indeed able to provide its products as an oral dietary supplement: for example, the digestive health comfortin™

The development of *C. reinhardtii* as a production platform is not limited to the production of therapeutic proteins, as several studies investigated also the possibility of engineering biosynthetic pathways in *C. reinhardtii* to allow the production of other industrially relevant molecules such as triacylglycerols and terpenoids (Gimpel et al. 2015; Scaife et al. 2015). The primary focus of these studies is to genetically engineer pathways in *C. reinhardtii* to increase the productivity or enable the production of designer molecules with industrial applications. For example, a recent study was successful in down regulating a key enzyme in the citric acid cycle using a CRISPRi system to direct the carbon flux towards lipid synthesis and therefore, enhance the lipid production in the *C. reinhardtii* (Kao & Ng 2017). Another example is engineering the glycerol acylation step in *C. reinhardtii* by overexpressing endogenous Acyl-CoA:diacylglycerol acyltransferases to improve triacylglycerol production (La Russa et al. 2012). To harness *C. reinhardtii* as a cell factory for the production of novel molecules, a study attempted to introduce a bifunctional diterpene synthase from balsam fir into the *C. reinhardtii* chloroplast, where this enzyme catalyses the formation of industrially relevant *cis*-abienol 4 from GGPP (Zedler et al. 2014). This study could not detect *cis*-abienol 4 in spite of the successful heterologous expression of the diterpene synthase gene. On the other hand, Lauersen et al. (2016) reported the successful production of the non-native sesquiterpenoid patchoulol from FPP in engineered *C. reinhardtii* with a patchoulol synthase from the plant *Pogostemon cablin*, achieving productivities up to 922 ± 242 µg/g dry weight in six days. As evident from the above studies, the conventional metabolic engineering strategy of modifying individual gene/s for the production of desired molecules is not always successful, where sometimes no changes could be detected in the genetically engineered *C. reinhardtii*. This could be mainly due to the involvement of several limiting steps in the pathway especially in the case of secondary metabolites. A transcriptional engineering strategy has therefore recently been developed in algal metabolism as a means to allow the manipulation of multiple targets of a metabolic pathway by engineering regulators such as transcription factors as illustrated in Figure 6.2 (Bajhaiya et al. 2016). Specifically, a patent from Sapphire Energy, Inc demonstrated an overexpression transcriptional factor (SN03) can cause a lipid-trigger effect in *C. reinhardtii* and result in an increase of 11% of total neutral lipids under nutrient-replete conditions (Gimpel et al. 2015). Such transcriptional engineering strategy holds real promise in the metabolic engineering of complex metabolic pathways in *C. reinhardtii* and other algal species with industrial potential.

A

B

Figure 6.2 Schematic representation for different strategies to engineer carotenoid biosynthesis pathway.

Biosynthetic pathways for carotenoids in green microalgae. (A) Conventional metabolic engineering strategy relies on the overexpression of a single gene or multiple genes. (B) Transcriptional engineering strategy can allow the activation of multiple genes in a metabolic pathway by the overexpression of transcription factor (TF), or by the introduction of a mutation in cis element where then the genetic activation would be possible by a specific TF; DNA-binding domain (DBD); Signal-sensing domain (SSD); transactivation domain (TAD). Abbreviation for the key enzymes: phytoene synthase (PSY), phytoene desaturase (PDS), z-carotene desaturase (ZDS), lycopene e-cyclase (LCY-E), lycopene b-cyclase (LCY-B), cytochrome P450 e-hydroxylase (CYP97C3), cytochrome P450 b-hydroxylase (CYP97A5), carotene b-hydroxylase (CHY-B), b- carotene oxygenase (BKT), zeaxanthin epoxidase (ZEP), violaxanthin de-epoxidase (VDE). The diagram is adapted from Bajhaiya et al. (2016).

In general, the current genetic engineering efforts for the manipulation of biosynthetic pathways in *C. reinhardtii* are largely based on proof of concept experiments using simple molecular tools to demonstrate transgene expression and confirm the functionality of heterologous protein within the cells (Couso et al. 2011; Vila et al. 2008; León et al. 2007). These initial experiments are necessary to gain a better understanding of metabolic process and the genetic controls underpinning algal metabolisms. Such understanding, in combination with the development of advanced metabolic engineering strategy (e.g. transcriptional engineering) would allow the successful regulation of complex biosynthetic

pathway in algal metabolism to produce the desired molecules with industrial applications, though it necessitates a lot of research to make this stage possible (Figure 6.3). Most importantly, the advances in the field of metabolic engineering for *C. reinhardtii* could enable the development of genetically engineered strains that suits industrial applications and so make for instance, the production of human therapeutic proteins more practical and economically viable.

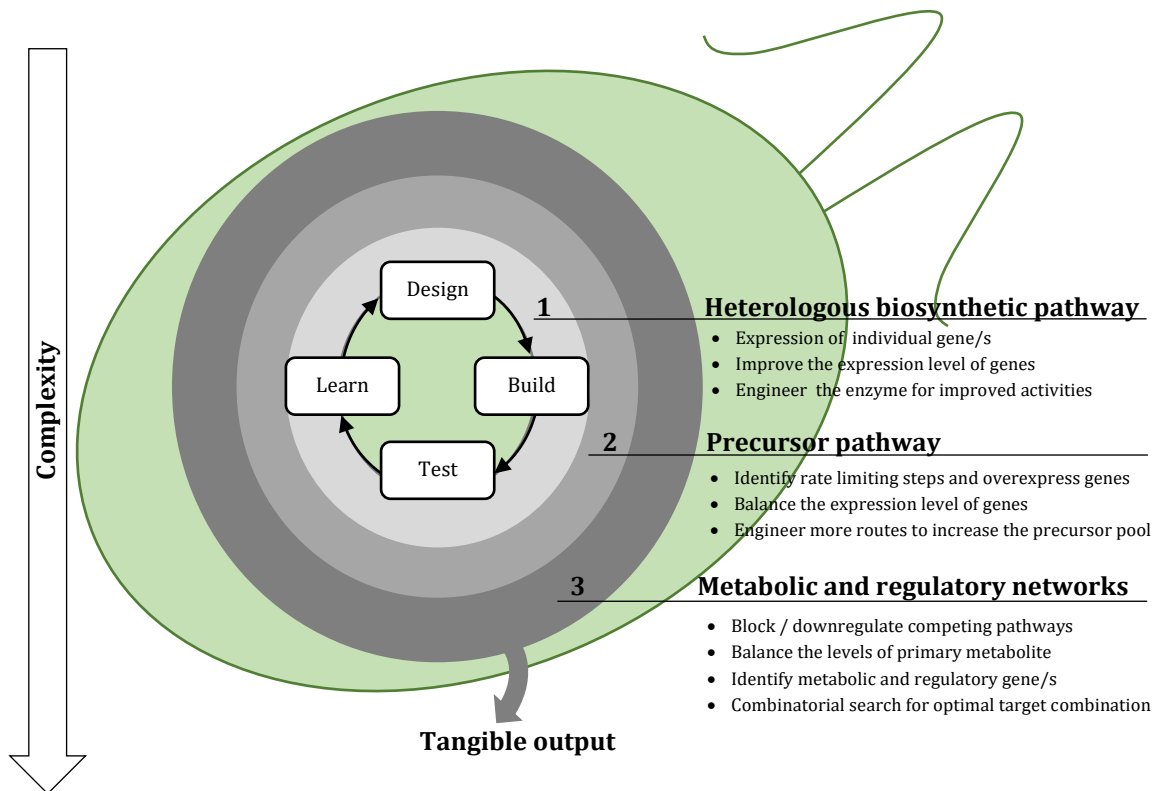


Figure 6.3 Schematic representation of the metabolic engineering stages organised into the levels of increasing complexity.

The essential stages in the manipulation of biosynthetic pathway in *C. reinhardtii* highlighting the efforts and knowledge required to achieve tangible outputs. The core steps for each stage necessitates to follow the cycle of **design** in silico, **build** the target pathway, **test** the productivity of the desired product and the overall effect of pathway manipulation on the host cell and **learn** from the outcomes of engineering the target pathway. Several rounds of such cycle will provide the knowledge required to proceed to the next level of the complexity, where the ultimate goal to achieve the desirable outputs. The diagram was reproduced from Scaife & Smith (2016); Schmidt-Dannert & Pisarchik (2008).

6.3. Next step in engineering the MEP pathway in the algal chloroplast

To further investigate the potential of microalgae as a production platform for terpenoids, the next step is to couple the overexpression of the rate limiting DXS enzyme with terpene synthase enzymes involved in the production of novel terpenoids with industrial applications. Sclareol is an example of an industrially important plant derived-diterpenoid that is used as a precursor for Ambrox® or the related ambroxide fragrance (Zerbe & Bohlmann 2015). Since sclareol is mainly obtained from clary sage plants, the current supply for this terpenoid is not stable. The biosynthetic pathway of sclareol in its natural host relies on the sequential action of two monofunctional diterpenoid synthases, which together form sclareol from GGPP, as illustrated in Figure 6.4 (Schalk et al. 2012).

Figure 6.4 Semi-synthetic route for the production of Ambrox® from sclareol.

The biosynthesis of sclareol in the clary sage plant. The diterpenoid sclareol is used as a starting material to produce Ambrox® by chemical synthesis. The abbreviations are a class II diterpene synthase (SsLPS), labda-13-en-8-ol diphosphate (LPP) and a class I diterpene synthase (SsSCS). Diagram was modified from Ignea et al. (2015). Abbreviation of enzymes according to Zerbe & Bohlmann (2015).

The identification of diterpenoid synthase in sclareol biosynthesis should enable the genetic engineering of the MEP pathway in the chloroplast of *C. reinhardtii* to investigate the possibility of producing sclareol in the algal chloroplast as an alternative platform for commercial production. The reconstruction of this pathway can be undertaken by heterologous expression of a codon optimised version of the diterpene synthases into an engineered strain with an additional copy of the DXS enzyme (Figure 6.5). Alternatively, the heterologous expression of multiple genes (*dxs*, *SsLPS* and *SsSCS*) in the *C. reinhardtii* chloroplast in a single transformation is possible as demonstrated in Saul Purton's group (Marco Larrea-Alvarez, unpublished).

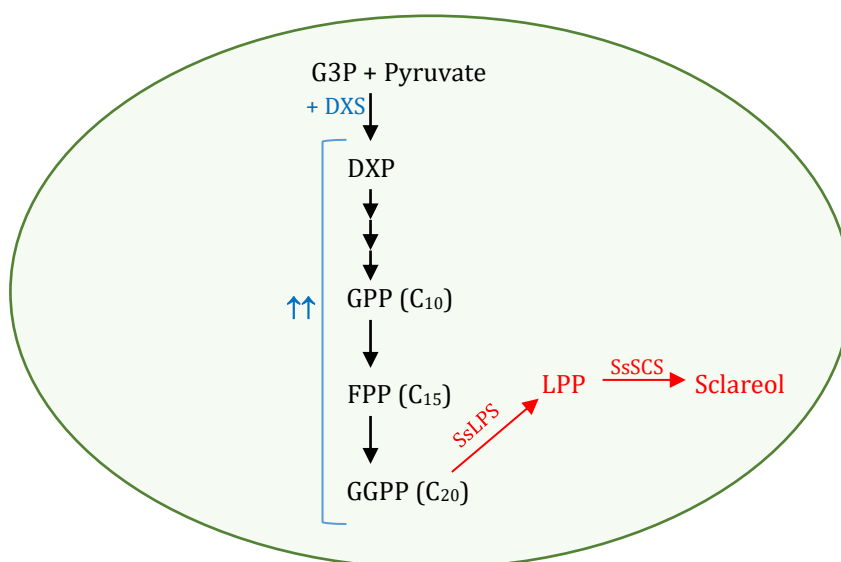


Figure 6.5 Metabolic engineering of the terpenoid biosynthetic pathway for sclareol production in the *C. reinhardtii* chloroplast.

The terpenoid pathway in the *C. reinhardtii* chloroplast is indicated by black arrows. The overexpression of rate-limiting DXS enzyme (blue) to increase GGPP precursor pool that required for sclareol synthesis by introduced pathway (red). The flux increase of the corresponding product/s is represented by $\uparrow\uparrow$, while the introduced additional copy of endogenous gene is indicated by +. Abbreviations are G3P: D-glyceraldehyde 3- phosphate; DXP: -deoxy-D-xylulose-5-phosphate; DXS: 1-deoxy-D-xylulose-5-phosphate synthase; GPP: geranyl diphosphate, FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate; a class II diterpene synthase (SsLPS), labda-13-en-8-ol diphosphate (LPP) and a class I diterpene synthase (SsSCS).

It is worth noting that the reconstruction of the sclareol pathway has been reported in different expression systems demonstrating the feasibility of producing this compound using a heterologous platform (Pan et al. 2015; Ignea et al. 2015; Schalk et al. 2012). However, some of these expression systems are not ideal for diterpenoid production. Genetic engineering of diterpenoid pathways in yeast often necessitates extensive modification to allow the production of a desired terpenoid. This was demonstrated in sclareol production, where an engineered yeast containing farnesyl diphosphate synthase (Erg20p) to produce geranylgeranyl diphosphate was initially used as a chassis for diterpene production and then this was further modified to produce sclareol (Ignea et al. 2015). Metabolic engineering of the terpenoid biosynthesis pathway in the chloroplast of plants is normally associated with side effects, for instance the successful production of sclareol in the moss *Physcomitrella patens* led to growth impairment (Pan et al. 2015). Although *C. reinhardtii* is not an exceptional biomass producer and sclareol production is expected to be far less than 1.5 g/L – the highest titre reported in engineered *E. coli* (Schalk et al. 2012), the reconstruction of the sclareol biosynthetic pathway in *C. reinhardtii* would shed light on the possibility of exploiting the algal chloroplast as a terpenoid production platform.

This gained knowledge could also guide rational design in other algal species with industrial potential like *Nannochloropsis* for sclareol production, which would be possible after the establishment of a reliable chloroplast transformation methodology. The establishment of a chloroplast transformation methodology for such oleaginous algae suited for industrial cultivation is necessary not only to enable the genetic manipulation of biosynthetic pathways including terpenoids, but also to help in building our understanding about lipid biology in response to different growth conditions such as nutrient levels, light, temperature and salinity. Such understanding, together with genetic manipulation tools for both chloroplast and nuclear genomes, can help in genetically modifying various aspects of *Nannochloropsis* biology to improve lipid profiles, productivity levels of desired products or performance for industrial cultivation.

Enzyme engineering is another aspect that should be considered along with pathway engineering in the algal chloroplast to further improve the productivity of the desired terpenoid, as the engineered pathway could be limited by inherent capacity of imported pathway to catalyse conversion of the precursors (Leonard et al. 2010). This could be improved by engineering the recombinant enzymes to achieve the desirable functions such as higher catalytic activity, better substrate affinity and decreased product inhibition (Marcheschi et al. 2013). An example for such a case was reported by Leonard et al. (2010), where the initial attempt to produce the diterpene levopimaradiene in engineered *E. coli* co-expressing GGPP synthase and levopimaradiene synthase resulted in production of a small amount (0.15 mg/L) of levopimaradiene. This was followed by another attempt by the same group to further increase levopimaradiene levels in *E. coli* by overexpressing several rate limiting enzymes in the MEP pathway. This approach was used to improve terpenoid production levels up to 92 mg/L, as further improvement of levopimaradiene levels through metabolic engineering was not possible due to the limited capacity of the downstream pathway. The level of levopimaradiene of engineered *E. coli* was only significantly improved when combined with protein engineering of GGPP synthase and levopimaradiene synthase, achieving a maximum titre of 700 mg/L in a bench scale bioreactor.

It is anticipated that future advances in genetic engineering technologies for microalgae will see similar impressive results in which 'designer algae' are created using a synthetic biology approach of predictive design in silico followed by efficient engineering in the laboratory. This will allow production using a truly sustainable approach in which these photosynthetic organisms produce a valuable metabolite in photobioreactors using just CO₂ and basic nutrients, with the whole process driven by sunlight.

References

- Adl, S.M. et al., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology*, 52(5), pp.399–451.
- Akashi, T. et al., 1988. Effects of propyzamide on tobacco cell microtubules *in vivo* and *in vitro*. *Plant Cell Physiology*, 29(6), pp.1053–1062.
- Al-Hoqani, U., Young, R. & Purton, S., 2016. The biotechnological potential of *Nannochloropsis*. *Perspectives in Phycology*, In Press.
- Alves-Rodrigues, A. & Shao, A., 2004. The science behind lutein. *Toxicology letters*, 150(1), pp.57–83.
- Amin, S., 2009. Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management*, 50(7), pp.1834–1840.
- Anthony, J. et al., 2014. Ultraviolet and 5'fluorodeoxyuridine induced random mutagenesis in *Chlorella vulgaris* and its impact on fatty acid profile: A new insight on lipid-metabolizing genes and structural characterization of related proteins. *Marine Biotechnology (New York)*, 17(1), pp.66–80.
- Apt, K.E. et al., 1996. Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Molecular and General Genetics*, 252(5), pp.572–579.
- Araya-Garay, J.M. et al., 2012. Construction of new *Pichia pastoris* X-33 strains for production of lycopene and beta-carotene. *Applied Microbiology and Biotechnology*, 93(6), pp.2483–2492.
- Arica, M.Y. et al., 2005. Utilisation of native, heat and acid-treated microalgae *Chlamydomonas reinhardtii* preparations for biosorption of Cr(VI) ions. *Process Biochemistry*, 40(7), pp.2351–2358.
- Arnold, A. a. et al., 2014. Identification of lipid and saccharide constituents of whole microalgal cells by ¹³C solid-state NMR. *Biochimica et Biophysica Acta - Biomembranes*, 1848(1), pp.369–377.
- Bajhaiya, A.K., Ziehe Moreira, J. & Pittman, J.K., 2016. Transcriptional Engineering of Microalgae: Prospects for High-Value Chemicals. *Trends in Biotechnology*, 35(2), pp.1–4.
- Barrera, D. & Mayfield, S., 2013. High-value recombinant protein production in microalgae. *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, pp.532–544.
- Bateman, J.M. & Purton, S., 2000. Tools for chloroplast transformation in *Chlamydomonas*: Expression vectors and a new dominant selectable marker. *Molecular and General Genetics*, 263(3), pp.404–410.
- Bayramoğlu, G. et al., 2006. Biosorption of mercury(II), cadmium(II) and lead(II) ions from aqueous system by microalgae *Chlamydomonas reinhardtii* immobilized in alginate beads. *International Journal of Mineral Processing*, 81(1), pp.35–43.
- Beacham, T. A. et al., 2014. Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel production and downstream processing. *Algal Research*, 6, pp.64–69.
- Becker, E.W., 2013. Microalgae for aquaculture: Nutritional aspects. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*. pp. 671–691.
- Bernstein, E., 1960. Synchronous division in *Chlamydomonas moewusii*. *Science*, 131(3412), p.1528–1529.
- Berthold, P. et al., 2002. An engineered *streptomyces hygrosopicus* aph 7" Gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. *Protist*,

153(4), pp.401–412.

- Beum, J.K. et al., 2004. Effect of subculture and elicitation on instability of Taxol production in *Taxus sp.* suspension cultures. *Biotechnology Progress*, 20(6), pp.1666–1673.
- Black, R.E. et al., 2016. Maternal and child undernutrition: Global and regional exposures and health consequences. *The Lancet*, 371(9608), pp.243–260.
- Blajeski, A.L. et al., 2002. G1 and G2 cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *The Journal of Clinical Investigation*, 110(1), pp.91–99.
- Block, M.D. et al., 1985. Chloroplast transformation by *Agrobacterium tumefaciens*. *The EMBO journal*, 4(6), pp.1367–1372.
- Blowers, a D. et al., 1989. Studies on *Chlamydomonas* chloroplast transformation: Foreign DNA can be stably maintained in the chromosome. *The Plant Cell*, 1(1), pp.123–32.
- Bock, R., 2014. Genetic engineering of the chloroplast: Novel tools and new applications. *Current Opinion in Biotechnology*. 26, pp.7–13.
- Bohlmann, J. & Keeling, C.I., 2008. Terpenoid biomaterials. *The Plant Journal*, 54(4), pp.656–669.
- Bone, R.A. et al., 2003. Lutein and zeaxanthin dietary supplements raise macular pigment density and serum concentrations of these carotenoids in humans. *The Journal of nutrition*, 133(4), pp.992–998.
- Boynton, J.E. et al., 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science*, 240(4858), pp.1534–1482.
- Boynton, J.E. & Gillham, N.W., 1993. Chloroplast transformation in *Chlamydomonas*. *Methods in enzymology*, 217(1992), pp.510–536.
- Britton, G., 1995. Structure and properties of carotenoids in relation to function. *FASEB journal*, 9(15), pp.1551–1558.
- Bruck, T. & Garbe, D., 2012. Algae symbiosis with eukaryotic partners. In C. Posten & C. Walter, eds. *Microalgal biotechnology: Potential and Production*. Berlin: De Gruyter, pp. 55–86.
- Bruckner, K. et al., 2014. Characterization of two genes for the biosynthesis of abietane-type diterpenes in rosemary (*Rosmarinus officinalis*) glandular trichomes. *Phytochemistry*, 101, pp.52–64.
- Buchanan, M.J. et al., 1989. Activation of the cell wall degrading protease, lysin, during sexual signalling in *Chlamydomonas*: the enzyme is stored as an inactive, higher relative molecular mass precursor in the periplasm. *The Journal of Cell Biology*, 108(1), pp.199–207.
- Camacho-Rodríguez, J. et al., 2014. A quantitative study of eicosapentaenoic acid (EPA) production by *Nannochloropsis gaditana* for aquaculture as a function of dilution rate, temperature and average irradiance. *Applied Microbiology and Biotechnology*, 98(6), pp.2429–2440.
- Camacho-Rodríguez, J. et al., 2015. Long-term preservation of concentrated *Nannochloropsis gaditana* cultures for use in aquaculture. *Journal of Applied Phycology*. 28(1), pp.299–312.
- Caputi, L. & Aprea, E., 2011. Use of terpenoids as natural flavouring compounds in food industry. *Recent patents on food, nutrition & agriculture*, 3(1), pp.9–16.

- Cardozo, K.H.M. et al., 2007. Metabolites from algae with economical impact. *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology*, 146(1-2), pp.60-78.
- Cassera, M.B. et al., 2004. The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *Journal of Biological Chemistry*, 279(50), pp.51749-51759.
- Cha, K.H. et al., 2008. Antiproliferative effects of carotenoids extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* on human colon cancer cells. *Journal of agricultural and food chemistry*, 56(22), pp.10521-10526.
- Cha, T.S. et al., 2011. Cinnamic acid, coumarin and vanillin: Alternative phenolic compounds for efficient *Agrobacterium*-mediated transformation of the unicellular green alga, *Nannochloropsis sp.* *Journal of Microbiological Methods*, 84(3), pp.430-434.
- Chen, H.L. et al., 2008. Conditional production of a functional fish growth hormone in the transgenic line of *Nannochloropsis oculata* (Eustigmatophyceae). *Journal of Phycology*, 44, pp.768-776.
- Chen, Y.-Y. et al., 2013. Chromosomal evolution of *Escherichia coli* for the efficient production of lycopene. *BMC biotechnology*, 13(1), p.6.
- Cheng, Q. et al., 2005. The *Klebsiella pneumoniae* nitrogenase Fe protein gene (*nifH*) functionally substitutes for the *chlL* gene in *Chlamydomonas reinhardtii*. *Biochemical and Biophysical Research Communications*, 329(3), pp.966-975.
- Chew, B.P. et al., 1999. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer research*, 19(3A), pp.1849-1853.
- Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*, 26(3), pp.126-131.
- Chung, H.-H. et al., 2016. Deciphering the biosynthetic pathways of bioactive compounds in planta using omics approaches. In H.-S. Tsay et al., eds. *Medicinal Plants - Recent Advances in Research and Development*. Singapore: Springer Singapore, pp.129-165.
- Cobb, A.H. & Reade, J.P.H., 2010. Herbicides that inhibit photosynthesis. *Herbicides and Plant Physiology*, pp.87-115.
- Cooper, S. et al., 2006. Nocodazole does not synchronize cells: Implications for cell-cycle control and whole-culture synchronization. *Cell and Tissue Research*, 324(2), pp.237-242.
- Cordero, B.F. et al., 2011. Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. *Applied Microbiology and Biotechnology*, 91(2), pp.341-351.
- Cordoba, E. et al., P., 2009. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. *Journal of Experimental Botany*, 60(10), pp.2933-2943.
- Corteggiani Carpinelli, E. et al., 2014. Chromosome scale genome assembly and transcriptome profiling of *nannochloropsis gaditana* in nitrogen depletion. *Molecular Plant*, 7(2), pp.323-335.
- Couso, I. et al., 2011. Overexpression of an exogenous phytoene synthase gene in the unicellular alga *Chlamydomonas reinhardtii* leads to an increase in the content of carotenoids. *Biotechnology Progress*, 27(1), pp.54-60.

- Croft, M.T. et al., 2006. Algae need their vitamins. *Eukaryotic Cell*, 5(8), pp.1175–1183.
- Cui, Y., et al., 2014. Chloroplast transformation of *Platymonas (Tetraselmis) subcordiformis* with the *bar* gene as selectable marker. *PloS One*, 9(6), e98607.
- Cvejić, J.H. & Rohmer, M., 2000. CO₂ as main carbon source for isoprenoid biosynthesis via the mevalonate-independent methylerythritol 4-phosphate route in the marine diatoms *Phaeodactylum tricoratum* and *Nitzschia ovalis*. *Phytochemistry*, 53(1), pp.21–28.
- Danishefsky, S.J. et al., 1996. Total Synthesis of Baccatin III and Taxol. *Journal of the American Chemical Society*, 118(11), pp.2843–2859.
- Das, A. et al., 2007. An update on microbial carotenoid production: application of recent metabolic engineering tools. *Applied Microbiology and Biotechnology*, 77(3), p.505.
- Day, A. & Goldschmidt-Clermont, M., 2011. The chloroplast transformation toolbox: selectable markers and marker removal. *Plant Biotechnology Journal*, 9(5), pp.540–553.
- Dent, R.M. et al., 2001. Functional genomics of plant photosynthesis in the fast lane using *Chlamydomonas reinhardtii*. *Trends in Plant Science*, 6(8), pp.364–371.
- Devasagayam, T.P.A. et al., 2004. Free radicals and antioxidants in human health: Current status and future prospects. *The Journal of the Association of Physicians of India*, 52, pp.794–804.
- Disch, A. et al., 1998. Distribution of the mevalonate and glyceraldehyde phosphate/pyruvate pathways for isoprenoid biosynthesis in unicellular algae and the cyanobacterium *Synechocystis* PCC 6714. *Biochemical Journal*, 333(2), pp.381–388.
- Doan, Y.T.T. & Obbard, J.P., 2014. Two-stage cultivation of a *Nannochloropsis* mutant for biodiesel feedstock. *Journal of Applied Phycology*, 27(6), pp.10–15.
- Doebbe, A. et al., 2007. Functional integration of the HUP1 hexose symporter gene into the genome of *C. reinhardtii*: Impacts on biological H₂ production. *Journal of Biotechnology*, 131(1), pp.27–33.
- Doetsch, N. a. et al., 2001. Chloroplast transformation in *Euglena gracilis*: Splicing of a group III twintron transcribed from a transgenic psbK operon. *Current Genetics*, 39(1), pp.49–60.
- Doron, L. et al., 2016. Transgene expression in microalgae – from tools to applications. *Frontiers in plant science*, 7, p.505.
- Doughman, S.D. et al., 2007. Omega-3 fatty acids for nutrition and medicine: considering microalgae oil as a vegetarian source of EPA and DHA. *Current diabetes reviews*, 3(3), pp.198–203.
- Dreesen, I.A.J. et al., 2010. Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. *Journal of Biotechnology*, 145(3), pp.273–280.
- Dufossé, L. et al., 2005. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science & Technology*, 16(9), pp.389–406.
- Dunahay, T.G., 1993. Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. *BioTechniques*, 15(3), p.452–455,457–458,460.

- Eibl, R. & Eibl, D., 2002. Bioreactors for plant cell and tissue cultures. In *Plant biotechnology and transgenic plants*. Marcel Dekker, New York, pp.163–199.
- Engels, B. et al., 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metabolic Engineering*, 10(3–4), pp.201–206.
- Eonseon, J. et al., 2006. Secondary carotenoid accumulation in *Haematococcus* (Chlorophyceae): Biosynthesis, regulation, and biotechnology. *Journal of microbiology and biotechnology*, 16(6), pp.821–831.
- Ernst, H., 2002. Recent advances in industrial carotenoid synthesis. *Pure and Applied Chemistry*, 74(11), pp.2213–2226.
- Estévez, J.M. et al., 2001. 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *Journal of Biological Chemistry*, 276(25), pp.22901–22909.
- Farhi, M. et al., 2011. Generation of the potent anti-malarial drug artemisinin in tobacco. *Nature Biotechnology*, 29(12), pp.1072–1074.
- Fasano, E. et al., 2014. Antioxidant and anti-inflammatory effects of selected natural compounds contained in a dietary supplement on two human immortalized keratinocyte lines. *BioMed Research International*, 2014, p.327452.
- Fawley, M.W. et al., 2015. The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis* sp. nov. and *Microchloropsis* gen. nov. *Phycologia*, 54(5), pp.545–552.
- Fernandez-Reiriz, M.J. & Labarta, U., 1996. Lipid classes and fatty acid composition of rotifers (*Brachionus plicatilis*) fed two algal diets. *Hydrobiologia*, 330, pp.73–79.
- Fett-Neto, A.G. et al., 1994. Improved taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *Taxus cuspidata*. *Biotechnology and Bioengineering*, 44(8), pp.967–971.
- Fischer, M.J.C. et al., 2013. Specificity of *Ocimum basilicum* geraniol synthase modified by its expression in different heterologous systems. *Journal of Biotechnology*, 163(1), pp.24–29.
- Flor-Weiler, L.B. et al., 2011. Susceptibility of four tick species, *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* (Acari: Ixodidae), to nootkatone from essential oil of grapefruit. *Journal of Medical Entomology*, 48(2), pp.322–326.
- Foster, K. et al., 1984. A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote *Chlamydomonas*. *Nature*, 311(5988), pp.756–759.
- Fraatz, M.A. et al., 2009. Nootkatone– a biotechnological challenge. *Applied Microbiology and Biotechnology*, 83(1), pp.35–41.
- Franklin, S. et al., 2002. Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal : for Cell and Molecular Biology*, 30(6), pp.733–744.
- Franklin, S.E. & Mayfield, S.P., 2004. Prospects for molecular farming in the green alga *Chlamydomonas reinhardtii*. *Current Opinion in Plant Biology*, 7(2), pp.159–165.
- Fraser, P.D. & Bramley, P.M., 2004. The biosynthesis and nutritional uses of carotenoids. *Progress in Lipid Research*, 43(3), pp.228–265.

- Fray, R.G. et al., 1995. Constitutive expression of a fruit *phytoene synthase* gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *The Plant Journal*, 8(5), pp.693–701.
- Frense, D., 2007. Taxanes: perspectives for biotechnological production. *Applied Microbiology and Biotechnology*, 73(6), pp.1233–1240.
- Friedl, T. et al., 2012. Phylogeny and systematics of microalgae: An over view. In C. Posten & C. Walter, eds. *Microalgal biotechnology: Potential and Production*. Berlin: De Gruyter, pp. 11–37.
- Fukusaki, E.I. et al., 2003. Introduction of the archaeobacterial geranylgeranyl pyrophosphate synthase gene into *Chlamydomonas reinhardtii* chloroplast. *Journal of Bioscience and Bioengineering*, 95(3), pp.283–287.
- Furusawa, M. et al., 2005. Highly efficient production of nootkatone, the grapefruit aroma from valencene, by biotransformation. *Chemical and Pharmaceutical Bulletin*, 53(11), pp.1513–1514.
- Gangl, D. et al., 2015. Biotechnological exploitation of microalgae. *Journal of Experimental Botany*, 66(22), pp.6975–6990.
- Gao, H. et al., 2014. TALE activation of endogenous genes in *Chlamydomonas reinhardtii*. *Algal Research*, 5(1), pp.52–60.
- Gao, W. et al., 2014. Combining metabolomics and transcriptomics to characterize tanshinone biosynthesis in *Salvia miltiorrhiza*. *BMC genomics*, 15, p.73.
- Gassel, S. et al., 2014. Genetic engineering of the complete carotenoid pathway towards enhanced astaxanthin formation in *Xanthophyllomyces dendrorhous* starting from a high-yield mutant. *Applied Microbiology and Biotechnology*, 98(1), pp.345–350.
- Genschik, P. et al., 1998. Cell cycle – dependent proteolysis in plants. Identification of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor mg132. *The Plant cell*, 10(12), pp.2063–2076.
- Georgianna, D.R. et al., 2013. Production of recombinant enzymes in the marine alga *Dunaliella tertiolecta*. *Algal Research*, 2(1), pp.2–9.
- Georgianna, D.R. & Mayfield, S.P., 2012. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature*, 488(7411), pp.329–335.
- Gershenzon, J. & Dudareva, N., 2007. The function of terpene natural products in the natural world. *Nature chemical biology*, 3(7), pp.408–414.
- Gibson, D.G. et al., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6(5), pp.12–16.
- Gimpel, J.A. et al., 2015. In metabolic engineering of eukaryotic microalgae: Potential and challenges come with great diversity. *Frontiers in Microbiology*, 6, p.1376
- Gingrich, J.C. et al., 1988. Genetic analysis of two new mutations resulting in herbicide resistance in the cyanobacterium *Synechococcus* sp. PCC 7002. *Photosynthesis Research*, 16(1), pp.83–99.
- Girhard, M. et al., 2009. Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. *Microbial Cell Factories*, 8, p.36.
- Gisby, M.F., Mudd, E.A. & Day, A., 2012. Growth of transplastomic cells expressing D-amino acid oxidase in chloroplasts is tolerant to D-alanine and inhibited by D-valine. *Plant physiology*, 160(4), pp.2219–2226.

- Gliszczynska, A. et al., 2011. Microbial transformation of (+)-nootkatone and the antiproliferative activity of its metabolites. *Bioorganic & medicinal chemistry*, 19(7), pp.2464–2469.
- Golden, S.S. & Haselkorn, R., 1985. Mutation to herbicide resistance maps within the *psbA* gene of *Anacystis nidulans* R2. *Science*, 229(4718), pp.1104–1107.
- Goldschmidt-Clermont, M., 1991. Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of *Chlamydomonas*. *Nucleic acids research*, 19(15), pp.4083–4089.
- Goloubinoff, P. et al., 1984. Chloroplast-coded atrazine resistance in *Solanum nigrum*: *psbA* loci from susceptible and resistant biotypes are isogenic except for a single codon change. *Nucleic Acids Research*, 12(24), pp.9489–9496.
- Gómez, P.I. et al., 2013. From genetic improvement to commercial-scale mass culture of a Chilean strain of the green microalga *Haematococcus pluvialis* with enhanced productivity of the red ketocarotenoid astaxanthin. *AoB PLANTS*, 5, plt026.
- Gong, M. & Bassi, A., 2016. Carotenoids from microalgae: A review of recent developments. *Biotechnology Advances*, 34(8), pp.1396–1412.
- Gong, Y.F. et al., 2006. Molecular cloning and expression profile analysis of Ginkgo biloba DXS gene encoding 1-deoxy-D-xylulose 5-phosphate synthase, the first committed enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. *Planta Medica*, 72(4), pp.329–335.
- Granado, F., Olmedilla, B. & Blanco, I., 2003. Nutritional and clinical relevance of lutein in human health. *The British Journal of Nutrition*, 90(3), pp.487–502.
- Green, B.R., 2011. Chloroplast genomes of photosynthetic eukaryotes. *Plant Journal*, 66(1), pp.34–44.
- Gressel, J., 2013. Transgenic marine microalgae: A value-enhanced fishmeal and fish oil replacement. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*. pp. 653–670.
- Grosjean, F. et al., 2002. S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi. *Cytotechnology*, 38, pp.57–62.
- Grossman, A.R. et al., 2003. *Chlamydomonas reinhardtii* at the crossroads of genomics. *American Society for Microbiology*, 2(6), pp.1137–1150.
- Gruber, A. et al., 2007. Protein targeting into complex diatom plastids: Functional characterisation of a specific targeting motif. *Plant Molecular Biology*, 64(5), pp.519–530.
- Gruchattka, E. & Kayser, O., 2015. In vivo validation of in silico predicted metabolic engineering strategies in yeast: Disruption of α -ketoglutarate dehydrogenase and expression of ATP-citrate lyase for terpenoid production. *PLoS One*, 10(12), e0144981.
- Grundman, O. et al., 2012. Cloning, mutagenesis, and characterization of the microalga *Parietochloris incisa* acetohydroxyacid synthase, and its possible use as an endogenous selection marker. *Biotechnology and Bioengineering*, 109(9), pp.2340–2348.
- Gutiérrez, C.L. et al., 2012. Chloroplast genetic tool for the green microalgae *Haematococcus pluvialis* (chlorophyceae, volvocales). *Journal of Phycology*, 48(4), pp.976–983.

- Han, Y.-S. et al., 2003. High-performance liquid chromatography assay for 1-deoxy-D-xylulose 5-phosphate synthase activity using fluorescence detection. *Journal of chromatography A*, 986(2), pp.291–296.
- Harries, E.H., 1989. The *Chlamydomonas* Sourcebook. A Comprehensive guide to biology and laboratory use. *Science*, 246(4936), pp.1503–1504.
- Harris, E.H., 2001. *Chlamydomonas* as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52(1), pp.363–406.
- Harris, E.H., 2009. The sexual cycle. In E. H. Harris, D. B. Stern, & G. B. Witman, eds. *The Chlamydomonas Sourcebook (Second Edition)*. London: Academic Press, pp. 119–157.
- Harvey, B.G. et al., 2010. High-density renewable fuels based on the selective dimerization of pinenes. *Energy and Fuels*, 24(1), pp.267–273.
- He, D. et al., 2007. Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in *Chlamydomonas reinhardtii* chloroplasts. *Colloids and Surfaces B-Biointerfaces*, 55(1), pp.26–30.
- Heider, S. et al., 2014. Optimization of the IPP precursor supply for the production of lycopene, decaprenoxanthin and astaxanthin by *Corynebacterium glutamicum*. *Frontiers in bioengineering and biotechnology*, 2, p.28.
- Helliwell, K.E. et al., 2011. Insights into the evolution of vitamin B 12 auxotrophy from sequenced algal genomes. *Molecular Biology and Evolution*, 28(10), pp.2921–2933.
- Hippler, M. et al., 2001. Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*. *The Plant journal*, 28 (5), pp.595–606.
- Hirschberg, J. & McIntosh, L., 1983. Molecular basis of herbicide resistance in *Amaranthus hybridus*. *Science*, 222(4630), pp.1346–9.
- van de Hoek, C. et al., 1995. *Algae : An Introduction to Phycology*. Cambridge: Cambridge University Press.
- Horwitz, S.B., 1994. How to make taxol from scratch. *Nature*, 367(6464), pp.593–594.
- Hu, Q. et al., 2015. The influence of cultivation period on growth and biodiesel properties of microalga *Nannochloropsis gaditana* 1049. *Bioresource Technology*, 192, pp.157–164.
- Huang, Y.-J. et al., 2014. Overexpression of NgAURE01, the gene coding for aureochrome 1 from *Nannochloropsis gaditana*, into *Saccharomyces cerevisiae* leads to a 1.6-fold increase in lipid accumulation. *Biotechnology letters*, 36(3), pp.575–9.
- Hudock, M.O. et al., 1979. A uniparentally inherited mutation affecting photophosphorylation in *Chlamydomonas reinhardtii*. *Biochemical and Biophysical Research Communications*, 87(1), pp.66 –71.
- Hugdahl, J.D. & Morejohn, L.C., 1993. Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. *Plant physiology*, 102(3), pp.725–740.
- Ibáñez-Salazar, A. et al., 2014. Over-expression of Dof-type transcription factor increases lipid production in *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, 184, pp.27–38.
- Ignea, C. et al., 2015. Efficient diterpene production in yeast by engineering Erg20p into a geranylgeranyl diphosphate synthase. *Metabolic Engineering*, 27, pp.65–75.
- Ikram, N.K.B.K. et al., 2015. Stable heterologous expression of biologically active

- terpenoids in green plant cells. *Frontiers in plant science*, 6, pp.129.
- Iwai, M. et al., 2015. Manipulation of oil synthesis in *Nannochloropsis* strain NIES-2145 with a phosphorus starvation-inducible promoter from *Chlamydomonas reinhardtii*. *Frontiers in Microbiology*, 6(September), pp.1–15.
- Janouskovec, J. et al., 2010. A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. *Proceedings of the National Academy of Sciences of the United States of America*, 107(24), pp.10949–10954.
- Jiang, W. et al., 2014. Successful transient expression of Cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 13(11), pp.1465–1469.
- Jinkerson, R. et al., 2013. Genomic insights from the oleaginous model alga *Nannochloropsis gaditana*. *Bioengineered*, 4(1), pp.37–43.
- Johanningmeier, U. et al., 1987. A new mutation in the gene coding for the herbicide-binding protein in *Chlamydomonas*. *FEBS Letters*, 211(2), pp.221–224.
- Kagan, M.L. et al., 2014. Safety assessment of EPA-rich polar lipid oil produced from the microalgae *Nannochloropsis oculata*. *International Journal of Toxicology*, 33(6), pp.459–474.
- Kagan, M.L. & Matulka, R. a., 2015. Safety assessment of the microalgae *Nannochloropsis oculata*. *Toxicology Reports*, 2, pp.617–623.
- Kang, N. kyu et al., 2015a. Heterologous overexpression of sfCherry fl uorescent protein in *Nannochloropsis salina*. *Biotechnology Reports*, 8, pp.10–15.
- Kang, N.K. et al., 2015b. Effects of overexpression of a bHLH transcription factor on biomass and lipid production in *Nannochloropsis salina*. *Biotechnology for Biofuels*, 8(1), p.200.
- Kao, P.-H. & Ng, I.-S., 2017. CRISPRi mediated phosphoenolpyruvate carboxylase regulation to enhance the production of lipid in *Chlamydomonas reinhardtii*. *Bioresource Technology*. In Press.
- Kates, J.R. & Jones, R.F., 1964. The control of gametic differentiation in liquid cultures of *Chlamydomonas*. *Journal of Cellular and Comparative Physiology*, 63, pp.157–164.
- Keeling, C.I. & Bohlmann, J., 2006. Diterpene resin acids in conifers. *Phytochemistry*, 67(22), pp.2415–2423.
- Keeling, P.J., 2004. Diversity and evolutionary history of plastids and their hosts. *American Journal of Botany*, 91(10), pp.1481–1493.
- Kempinski, C. et al., 2015. Metabolic engineering of higher plants and algae for isoprenoid production. *Advances in biochemical engineering/biotechnology*, 148, pp.3–18.
- Ketchum, R.E.B. & Gibson, D.M., 1996. Paclitaxel production in suspension cell cultures of *Taxus*. *Plant Cell, Tissue and Organ Culture*, 46(1), pp.9–16.
- Khemvong, S. & Suvachittanont, W., 2005. Molecular cloning and expression of a cDNA encoding 1-deoxy-d-xylulose-5-phosphate synthase from oil palm *Elaeis guineensis* Jacq. *Plant Science*, 169(3), pp.571–578.
- Kilian, O. et al., 2011. High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proceedings of the National Academy of Sciences*, 108(52), pp.21265–21269.
- Kindle, K.L. et al., 1989. Stable transformation of *Chlamydomonas* using the *chlamydomonas* gene for nitrate reductase. *The Journal of Cell Biology*, 109, pp.2589–2601.

- Kindle, K.L. et al., 1991. Engineering the chloroplast genome: Techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, 88(5), pp.1721–1725.
- Kinoshita, T. et al., 1992. Primary structure and expression of a gamete lytic enzyme in *Chlamydomonas reinhardtii*: similarity of functional domains to matrix metalloproteases. *Proceedings of the National Academy of Sciences of the United States of America*, 89(10), pp.4693–4697.
- Kirby, J. et al., 2015. Enhancing Terpene yield from sugars via novel routes to 1-deoxy-D-xylulose 5-phosphate. *Applied and Environmental Microbiology*, 81(1), pp.130–138.
- Kirby, J. & Keasling, J.D., 2009. Biosynthesis of plant isoprenoids: Perspectives for microbial engineering. *Annual Review of Plant Biology*, 60, pp.335–355.
- Kirchmayr, A. & Griesbeck, C., 2012. Genetic engineering, methods and targets. In C. Posten & C. Walter, eds. *Microalgal biotechnology: Potential and Production*. Berlin: De Gruyter, pp. 87–105.
- Knutsen, G. et al., 1973. Selection synchrony of *Chlamydomonas* using the Rastgeldi threshold centrifuge. *Experimental Cell Research*, 81(1), pp.26–30.
- Kolewe, M.E. et al., 2008. Pharmaceutically active natural product synthesis and supply via plant cell culture technology. *Molecular Pharmaceutics*, 5(2), pp.243–256.
- Kovar, J.L. et al., 2002. Molecular analysis of the *acetolactate synthase* gene of *Chlamydomonas reinhardtii* and development of a genetically engineered gene as a dominant selectable marker for genetic transformation. *Plant Journal*, 29(1), pp.109–117.
- Kumar, S.V. et al., 2004. Genetic transformation of the green alga–*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. *Plant Science*, 166(3), pp.731–738.
- Kwon, I.C. et al., 1998. Enhancement of taxol production by in situ recovery of product. *Process Biochemistry*, 33(7), pp.701–707.
- Lambrea, M.D. et al., 2013. A Powerful molecular engineering tool provided efficient *Chlamydomonas* mutants as bio-sensing elements for herbicides detection. *PLoS One*, 8(4), e61851.
- Lange, B.M. et al., 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proceedings of the National Academy of Sciences of the United States of America*, 97(24), pp.13172–13177.
- Lapidot, M. et al., 2002. Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. *Plant physiology*, 129(1), pp.7–12.
- Lauersen, K.J. et al., 2016. Efficient phototrophic production of a high-value sesquiterpenoid from the eukaryotic microalga *Chlamydomonas reinhardtii*. *Metabolic engineering*, 38, pp.331–343.
- Leliaert, F. et al., 2012. Phylogeny and Molecular Evolution of the Green Algae. *Critical Reviews in Plant Sciences*, 31(1), pp.1–46.
- Lemahieu, C. et al., 2013. Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens. *Food Chemistry*, 141(4), pp.4051–4059.
- Lemuth, K. et al., 2011. Engineering of a plasmid-free *Escherichia coli* strain for improved in vivo biosynthesis of astaxanthin. *Microbial Cell Factories*, 10, p.29.

- León, R. et al., 2007. Metabolic engineering of ketocarotenoids biosynthesis in the unicellular microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, 130(2), pp.143–152.
- Leonard, E. et al., 2010. Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *Proceedings of the National Academy of Sciences of the United States of America*, 107 (31), pp.13654 – 13659.
- Leonhardt, R.H. & Berger, R.G., 2015. Nootkatone. In J. Schrader & J. Bohlmann, eds. *Biotechnology of Isoprenoids*. Cham: Springer International Publishing, pp.391–404.
- Li, F. et al., 2014. High-efficiency nuclear transformation of the oleaginous marine *Nannochloropsis* species using PCR product. *Bioscience, biotechnology, and biochemistry*, 78(5), pp.812–817.
- Li, J. et al., 2014. Choreography of transcriptomes and lipidomes of *Nannochloropsis* reveals the mechanisms of Oil Synthesis in Microalgae. *The Plant Cell*, 26(4), pp.1645–1665.
- Li, S.S. & Tsai, H.J., 2009. Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. *Fish and Shellfish Immunology*, 26(2), pp.316–325.
- Li, W. et al., 2011. Chloramphenicol acetyltransferase as selectable marker for plastid transformation. *Plant molecular biology*, 76(3–5), pp.443–451.
- Li, Y. et al., 2010. *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. *Metabolic Engineering*, 12(4), pp.387–391.
- Li, Y. et al., 2015. Current and emerging options for taxolp. In J. Schrader & J. Bohlmann, eds. *Biotechnology of Isoprenoids*. Cham: Springer International Publishing, pp. 405–425.
- Lichtenthaler, H.K. et al., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Letters*, 400(3), pp.271–274.
- Lindgren, L.O. et al., 2003. Seed-specific overexpression of an endogenous *Arabidopsis phytoene synthase* gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant physiology*, 132(2), pp.779–785.
- Liu, C. et al., 2006. Artemisinin: current state and perspectives for biotechnological production of an antimalarial drug. *Applied Microbiology and Biotechnology*, 72(1), pp.11–20.
- Liu, J. et al., 2013. Engineering of an endogenous *phytoene desaturase* gene as a dominant selectable marker for *Chlamydomonas reinhardtii* transformation and enhanced biosynthesis of carotenoids. *Process Biochemistry*, 48(5–6), pp.788–795.
- Liu, J. et al., 2014. Genetic engineering of the green alga *Chlorella zofingiensis*: A modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker. *Applied Microbiology and Biotechnology*, 98(11), pp.5069–5079.
- Llarrull, L.I. et al., 2010. The future of the β -lactams. *Current Opinion in Microbiology*, 13(5), pp.551–557.
- Lohr, M., 2009. Carotenoids. In *The Chlamydomonas Sourcebook*. Elsevier Science, pp. 799–817.
- Lohr, M. et al., 2012. Isoprenoid biosynthesis in eukaryotic phototrophs: A spotlight on algae. *Plant Science*, 185–186, pp.9–22.

- Lubzens, E. et al., 1995. Potential advantages of frozen algae (*Nannochloropsis sp.*) for rotifer (*Brachionus plicatilis*) culture. *Aquaculture*, 133(3–4), pp.295–309.
- Luo, Y. et al., 2010. The thermal cracking of soybean/canola oils and their methyl esters. *Fuel Processing Technology*, 91(6), pp.613–617.
- Ma, X.N. et al., 2016. Lipid production from *Nannochloropsis*. *Marine Drugs*, 14(4), p.61.
- Ma, Y. et al., 2014. Evaluation of the potential of 9 *Nannochloropsis* strains for biodiesel production. *Bioresource technology*, 167, pp.503–509.
- Ma, Y. et al., 2013. Increased lipid productivity and TAG content in *Nannochloropsis* by heavy-ion irradiation mutagenesis. *Bioresource Technology*, 136, pp.360–367.
- Manayi, A. et al., 2016. Lutein and cataract: from bench to bedside. *Critical reviews in biotechnology*, 36(5), pp.829–839.
- Marasco, E.K. & Schmidt-Dannert, C., 2008. Exploring and accessing plant natural product biosynthesis in engineered microbial hosts. In O. Kayser & W. J. Quax, eds. *Medicinal Plant Biotechnology: From Basic Research to Industrial Applications*. Weinheim: WILEY-VCH Verlag, pp. 287–317.
- Marcheschi, R.J. et al., 2013. Protein engineering for metabolic engineering: Current and next-generation tools. *Biotechnology Journal*, 8(5), pp.545–555.
- Markus Lange, B. & Ahkami, A., 2013. Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes-current status and future opportunities. *Plant Biotechnology Journal*, 11(2), pp.169–196.
- Martin, V.J.J. et al., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology*, 21(7), pp.796–802.
- Materna, A.C. et al., 2009. First induced plastid genome mutations in an alga with secondary plastids: *psbA* mutations in the diatom *Phaeodactylum tricoratum* (bacillariophyceae) reveal consequences on the regulation of photosynthesis. *Journal of Phycology*, 45(4), pp.838–846.
- Matsui, Y. et al., 2012. Enrichment of cell populations in metaphase, anaphase, and telophase by synchronization using nocodazole and blebbistatin: A novel method suitable for examining dynamic changes in proteins during mitotic progression. *European Journal of Cell Biology*, 91(5), pp.413–419.
- Matsushima, D. et al., 2012. The single cellular green microalga *Botryococcus braunii*, race B possesses three distinct 1-deoxy-d-xylulose 5-phosphate synthases. *Plant Science*, 185–186, pp.309–320.
- Mauy, J. et al., 2005. Microbial isoprenoid production: An example of green chemistry through metabolic engineering. *Advances in Biochemical Engineering/Biotechnology*, 100, pp.19–51.
- Mayfield, S.P. et al., 2007. *Chlamydomonas reinhardtii* chloroplasts as protein factories. *Current Opinion in Biotechnology*, 18(2), pp.126–133.
- Mayfield, S.P. et al., 2003. Expression and assembly of a fully active antibody in algae. *Proceedings of the National Academy of Sciences of the United States of America*, 100(2), pp.438–42.
- Mayfield, S.P. & Schultz, J., 2004. Development of a luciferase reporter gene, *luxCt*, for *Chlamydomonas reinhardtii* chloroplast. *The Plant journal*, 37(3), pp.449–458.
- Merchant, S.S. et al., 2010. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*, 318(5848), pp.245–250.

- Mets, L. & Thiel, A., 1989. Biochemistry and genetic control of the photosystem II herbicide target site. In P. Boger & G. Sandmann, eds. *Target sites of Herbicide Action*. Boca Raton: CRC Press, pp. 1–25.
- Misawa, N., 2011. Pathway engineering for functional isoprenoids. *Current Opinion in Biotechnology*, 22(5), pp.627–633.
- Miyazawa, M. et al., 2000. Insecticidal sesquiterpene from *Alpinia oxyphylla* against *Drosophila melanogaster*. *Journal of agricultural and food chemistry*, 48(8), pp.3639–3641.
- Moog, D. et al., 2015. In vivo localization studies in the stramenopile alga *Nannochloropsis oceanica*. *Protist*, 166(1), pp.161–171.
- Morejohn, L. C. et al., 1984. Tubulins from different higher plant species are immunologically nonidentical and bind colchicine differentially. *Proceedings of the National Academy of Sciences of the United States of America*, 81(5), pp.1440–1444.
- Mukaiyama, T. et al., 1999. Asymmetric total synthesis of taxol. *Chemistry – A European Journal*, 5(1), pp.121–161.
- Munoz-Bertomeu, J. et al., 2006. Up-regulation of 1-deoxy- D -xylulose-5-phosphate synthase enhances production of essential oils. *Plant physiology*, 142, pp.890–900.
- Murakami, R. & Hashimoto, H., 2009. Unusual nuclear division in *Nannochloropsis oculata* (Eustigmatophyceae, Heterokonta) which may ensure faithful transmission of secondary plastids. *Protist*, 160, pp.41–49.
- Neupert, J. et al., 2009. Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. *Plant Journal*, 57(6), pp.1140–1150.
- Newman, S.M. et al., 1992. Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: Evidence for a hotspot and an adjacent cold region. *Genetics*, 132(2), pp.413–429.
- Newman, S.M. et al., 1990. Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas*: Molecular and genetic characterization of integration events. *Genetics*, 126(4), pp.875–888.
- O'Brien, J. a & Lummis, S.C.R., 2011. Nano-biostics: a method of biolistic transfection of cells and tissues using a gene gun with novel nanometer-sized projectiles. *BMC biotechnology*, 11(1), p.66.
- Oettmeier, W., 1999. Herbicide resistance and supersensitivity in photosystem II. *Cellular and Molecular Life Sciences*, 55(10), pp.1255–1277.
- Ohad, N. & Hirschberg, J., 1992. Mutations in the D1 subunit of photosystem II distinguish between quinone and herbicide binding sites. *The Plant cell*, 4(3), pp.273–82.
- Oldenhof, H. et al., 2004. Effect of red and blue light on the timing of cyclin-dependent kinase activity and the timing of cell division in *Chlamydomonas reinhardtii*. *Plant Physiology and Biochemistry*, 42(4), pp.341–348.
- Oldenhof, H. et al., 2006. Blue-and red-light regulation of the cell cycle in *Chlamydomonas reinhardtii* (Chlorophyta). *European Journal of Phycology*, 41(3), pp.313–320.
- Omer, S.R. et al., 2002. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metabolic Engineering*, 4, pp.263–272.
- Paine, J.A. et al., 2005. Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nature Biotechnology*, 23(4), pp.482–487.

- Pan, X.W. et al., 2015. Sclareol production in the moss *Physcomitrella patens* and observations on growth and terpenoid biosynthesis. *Plant Biotechnology Reports*, 9(3), pp.149–159.
- Pashkow, F.J. et al., 2008. Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *The American journal of cardiology*, 101(10), p.S58–S68.
- Pasquali, G. et al., 2006. Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: Recent progress related to old dilemma. *Journal of Bioscience and Bioengineering*, 101(4), pp.287–296.
- Pateraki, I. et al., 2015. Cytochromes P450 for terpene functionalisation and metabolic engineering. *Advances in biochemical engineering/biotechnology*, 148, pp.107–139.
- Peralta-Yahya, P.P. et al., 2011. Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications*, 2, p.483.
- Pérez-Heras, A.M. et al., 2016. Long-chain n-3 PUFA supplied by the usual diet decrease plasma stearyl-CoA desaturase index in non-hypertriglyceridemic older adults at high vascular risk. *Clinical Nutrition*, In Press.
- Perin, G. et al., 2015. Generation of random mutants to improve light-use efficiency of *Nannochloropsis gaditana* cultures for biofuel production. *Biotechnology for Biofuels*, 8(1), p.161.
- Planchais, S. et al., 2000. Chemical inhibitors: A tool for plant cell cycle studies. *FEBS Letters*, 476, pp.78–83.
- Przibilla, E. et al., 1991. Site-specific mutagenesis of the D1 subunit of photosystem II in wild-type *Chlamydomonas*. *The Plant Cell*, 3, pp.169–174.
- Pucheu, N. et al., 1984. Metribuzin Resistance and the Herbicide Binding Protein in *Chlamydomonas reinhardtii*. In C. Sybesma, ed. *Advances in Photosynthesis Research*. Dordrecht: Springer Netherlands, pp. 29–32.
- Purton, S., 2001. Algal Chloroplasts. *Encyclopedia of Life Sciences*. pp.1–9.
- Purton, S. et al., 2013. Genetic engineering of algal chloroplasts: Progress and prospects. *Russian Journal of Plant Physiology*, 60(4), pp.491–499.
- Purton, S., 2007. Tools and techniques for chloroplast transformation of *Chlamydomonas*. *Advances in Experimental Medicine and Biology*, 616, pp.34–45.
- Putignani, L. et al., 2013. Engineered *Escherichia coli* as new source of flavonoids and terpenoids. *Food Research International*, 54(1), pp.1084–1095.
- Pyke, K., 2009. Photosynthesis. In *Plastid Biology*. Cambridge: Cambridge University Press, pp. 61–80.
- Querol, J. et al., 2001. A fluorometric assay for the determination of 1-deoxy-D-xylulose 5-phosphate synthase activity. *Analytical Biochemistry*, 296(1), pp.101–105.
- Radakovits, R. et al., 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. *Nature communications*, 3, p.686.
- Radakovits, R. et al., 2010. Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, 9(4), pp.486–501.
- Ramos, A.A. et al., 2011. The unicellular green alga *Dunaliella salina* Teod. as a model for abiotic stress tolerance: genetic advances and future perspectives. *Algae*, 26(1), pp.3–20.

- Rand, S. et al., 1985. Herbicide resistance and cross-resistance: Changes at three distinct sites in the herbicide-binding protein. *Science*, 228(4696), pp.204–207.
- Randolph-Anderson, B. et al., 1995. Sub-micron gold particles are superior to larger particles for efficient biolistic transformation of organelles and some cell types. *Bio-rad Literature On-Line*.
- Randolph-Anderson, B.L. et al., 1993. Further characterization of the respiratory deficient dum-1 mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. *Molecular and General Genetics*, 236(2), pp.235–244.
- Rasala, B.A. et al., 2013. Expanding the spectral palette of fluorescent proteins for the green microalga *Chlamydomonas reinhardtii*. *Plant Journal*, 74(4), pp.545–556.
- Rasala, B.A. et al., 2012. Robust expression and secretion of xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. *PLoS One*, 7(8). e43349.
- Rasala, B. A. & Mayfield, S.P., 2010. The microalga *Chlamydomonas reinhardtii* as a platform for the production of human protein therapeutics. *Bioengineered Bugs*, 2(1), pp.50–54.
- Redding, K. et al., 1998. A systematic survey of conserved histidines in the core subunits of Photosystem I by site-directed mutagenesis reveals the likely axial ligands of P700. *EMBO Journal*, 17(1), pp.50–60.
- Remacle, C. et al., 2009. The *ARG9* gene encodes the plastid-resident N-acetyl ornithine aminotransferase in the green alga *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 8(9), pp.1460–1463.
- Ringo, D., 1967. Flagellar motion and fine structure of flagellar apparatus in *Chlamydomonas*. *Journal of Cell Biology*, 33(3), p.543-571.
- Ro, D. et al., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*, 440, pp.940–943.
- Roberts, S.C., 2007. Production and engineering of terpenoids in plant cell culture. *Nature Chemical Biology*, 3(7), pp.387–395.
- Rocha, J.M.S. et al., 2003. Growth aspects of the marine microalga *Nannochloropsis gaditana*. *Biomolecular Engineering*, 20(4–6), pp.237–242.
- Rochaix, J.-D., 2011. Regulation of photosynthetic electron transport. *Biochimica et Biophysica Acta-Bioenergetics*, 1807(3), pp.375–383.
- Rochaix, J.D. & Erickson, J., 1988. Function and assembly of photosystem II: Genetic and molecular analysis. *Trends Biochemical Sciences*, 13(2), pp.56–59.
- Roffey, R.A. et al., 1991. Photosynthetic electron transport in genetically altered photosystem II reaction centers of chloroplasts. *Proceedings of the National Academy of Sciences of the United States of America*, 88, pp.9122–9126.
- Rooney, D.W. et al., 1977. Synchronization of *Chlamydomonas* division in chemostat cultures by periodic temperature reductions: Effect on cellular ATP. *Journal of Thermal Biology*, 2(3), pp.157–160.
- Run, C. et al., 2016. Stable nuclear transformation of the industrial alga *Chlorella pyrenoidosa*. *Algal Research*, 17, pp.196–201.
- Rupprecht, J., 2009. From systems biology to fuel –*Chlamydomonas reinhardtii* as a model for a systems biology approach to improve biohydrogen production. *Journal of Biotechnology*, 142(1), pp.10–20.

- La Russa, M. et al., 2012. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, 162(1), pp.13–20.
- Salvador, J.A.R. & Clark, J.H., 2002. The allylic oxidation of unsaturated steroids by tert-butyl hydroperoxide using surface functionalised silica supported metal catalysts. *Green Chemistry*, 4(4), pp.352–356.
- Sambrook, J. & Russell, D.W., 2001. *Molecular cloning: A laboratory manual* Third. New York: Cold Spring Harbor Laboratory press.
- Sandmann, G., 2015. Carotenoids of biotechnological importance. In J. Schrader & J. Bohlmann, eds. *Biotechnology of Isoprenoids*. Cham: Springer International Publishing, pp. 449–467.
- SanGiovanni, J.P. & Neuringer, M., 2012. The putative role of lutein and zeaxanthin as protective agents against age-related macular degeneration: Promise of molecular genetics for guiding mechanistic and translational research in the field. *The American journal of clinical nutrition*, 96(5), p.1223S–1233S.
- Saxena, B. et al., 2014. Metabolic engineering of chloroplasts for artemisinic acid biosynthesis and impact on plant growth. *Journal of Biosciences*, 39(1), pp.33–41.
- Scaife, M.A. et al., 2015. Establishing *Chlamydomonas reinhardtii* as an industrial biotechnology host. *Plant Journal*, 82(3), pp.532–546.
- Schalk, M. et al., 2012. Toward a biosynthetic route to sclareol and amber odorants. *Journal of the American Chemical Society*, 134(46), pp.18900–18903.
- Schmidt-Dannert, C. & Pisarchik, A., 2008. Biosynthetic Pathway Engineering Strategies. In *Protein Engineering Handbook*. pp. 849–876.
- Schrader, J. & Bohlmann, J., 2015. *Biotechnology of Isoprenoids (Vol. 148, Advances in Biochemical Engineering/Biotechnology)*, Cham: Springer International Publishing.
- Schwender, J. et al., 2001. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. *Planta*, 212(3), pp.416–423.
- Seo, E.J. et al., 2011. Antiplatelet effects of *Cyperus rotundus* and its component (+)-nootkatone. *Journal of Ethnopharmacology*, 135(1), pp.48–54.
- Shaish, A. et al., 2006. 9-cis beta-carotene-rich powder of the alga *Dunaliella bardawil* increases plasma HDL-cholesterol in fibrate-treated patients. *Atherosclerosis*, 189(1), pp.215–221.
- Shao, N. & Bock, R., 2008. A codon-optimized luciferase from *Gaussia princeps* facilitates the *in vivo* monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*. *Current Genetics*, 53(6), pp.381–388.
- Shih, C. H. et al., 2015. Purple chromoprotein gene serves as a new selection marker for transgenesis of the microalga *Nannochloropsis oculata*. *PloS one*, 10(3), e0120780.
- Shimogawara, K. et al., 1998. High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, 148(4), pp.1821–1828.
- Shin, S. E. et al., 2016. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. *Scientific Reports*, 6, p.27810.
- Simionato, D. et al., 2013. The response of *Nannochloropsis gaditana* to nitrogen starvation includes de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. *Eukaryotic Cell*,

12(5), pp.665–676.

- Sivy, T.L. et al., 2011. Evidence of isoprenoid precursor toxicity in *Bacillus subtilis*. *Bioscience, Biotechnology, and Biochemistry*, 75(12), pp.2376–2383.
- Sizova, I. et al., 2013. Nuclear gene targeting in *Chlamydomonas* using engineered zinc-finger nucleases. *Plant Journal*, 73(5), pp.873–882.
- Sizova, I. et al., 1996. Stable nuclear transformation of *Chlamydomonas reinhardtii* with a *Streptomyces rimosus* gene as the selective marker. *Gene*, 181(1–2), pp.13–18.
- Somchai, P. et al., 2016. Use of microalgae *Chlamydomonas reinhardtii* for production of double-stranded RNA against shrimp virus. *Aquaculture Reports*, 3, pp.178–183.
- de Souza, M.L., Wackett, L.P. & Sadowsky, M.J., 1998. The atzABC genes encoding atrazine catabolism are located on a self-transmissible plasmid in *Pseudomonas* sp. strain ADP. *Applied and Environmental Microbiology*, 64(6), pp.2323–2326.
- de Souza, M.L. et al., 1995. Cloning, Characterization, and Expression of a Gene Region from *Pseudomonas* sp. Strain ADP Involved in the Dechlorination of Atrazine. , 61(9), pp.3373–3378.
- Spicer, A. & Purton, S., 2016. Genetic engineering of microalgae: Current status and future prospects. In *Microalgal Production for Biomass and High-Value Products*. CRC Press, pp. 139–163.
- Sree Ramulu, K. et al., 1991. Mitotic blocking, micronucleation, and chromosome doubling by oryzalin, amiprofos-methyl, and colchicine in potato. *Protoplasma*, 160(2), pp.65–71.
- Srinivasan, M. et al., 2009. Lycopene: An antioxidant and radioprotector against gamma-radiation-induced cellular damages in cultured human lymphocytes. *Toxicology*, 262(1), pp.43–49.
- Starkenburg, S.R. et al., 2014. A pangenomic analysis of the *Nannochloropsis* organellar genomes reveals novel genetic variations in key metabolic genes. *BMC genomics*, 15(1), p.212.
- Steinbrenner, J. & Sandmann, G., 2006. Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. *Applied and Environmental Microbiology*, 72(12), pp.7477–7484.
- Stern, D.B., Higgs, D.C. & Yang, J., 1997. Transcription and translation in chloroplasts. *Trends in Plant Science*, 2(8), pp.308–315.
- Stevens, D.R., Rochaix, J.D. & Purton, S., 1996. The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Molecular and General Genetics*, 251(1), pp.23–30.
- Stoffels, L., 2014. *Microalgae as a novel production platform for antibacterial proteins*. PhD thesis. London: University College London.
- Stoffels, L. et al., 2017. Synthesis of bacteriophage lytic proteins against *Streptococcus pneumoniae* in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal*, pp.1–11.
- Sukenik, A., 1999. Production of eicosapentaenoic acid by the marine eustigmatophyte *Nannochloropsis*. In Z. Cohen, ed. *Chemicals from Microalgae*. Taylor and Francis, pp. 41–53.
- Sun, M. et al., 2003. Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. *Biotechnology Letters*,

25(13), pp.1087–1092.

- Sunaga, Y. et al., 2014. Chloroplast-targeting protein expression in the oleaginous diatom *Fistulifera solaris* JPC DA0580 toward metabolic engineering. *Journal of Bioscience and Bioengineering*, 119(1), pp.28–34.
- Surzycki et al., 2009. Factors effecting expression of vaccines in microalgae. *Biologicals*, 37(3), pp.133–138.
- Suzuki, L. & Johnson, C.H., 2001. Algae know the time of day: Circadian and photoperiodic programs. *Journal of Phycology*, 37(6), pp.933–942.
- Tan, C.P. et al., 2007. Expression of B-carotene hydroxylase gene (crtR-B) from the green alga *Haematococcus pluvialis* in chloroplasts of *Chlamydomonas reinhardtii*. *Journal of Applied Phycology*, 19(4), pp.347–355.
- Taunt, H.N., 2013. *The Synthesis of Novel Antibacterial Proteins in the Chlamydomonas reinhardtii Chloroplast*. PhD thesis. London: University College London.
- Tian, G. T. et al., 2004. High-throughput fluorescent tagging of full-length *Arabidopsis* Gene Products in Planta. *Plant Physiology*, 135(1), pp.25–38.
- Tian, J. et al., 2013. Cloning and stress-responding expression analysis of malonyl CoA-acyl carrier protein transacylase gene of *Nannochloropsis gaditana*. *Gene*, 530(1), pp.33–38.
- Tran, M. et al., 2013. Production of unique immunotoxin cancer therapeutics in algal chloroplasts. *Proceedings of the National Academy of Sciences*, 110(1), pp.E15–E22.
- Tran, M. et al., 2009. Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. *Biotechnology and Bioengineering*, 104(4), pp.663–673.
- Tsakraklides, V. et al., 2015. Improved gene targeting through cell cycle synchronization. *PLoS One*, 10(7), e0133434.
- Tsuruta, H. et al., 2009. High-Level Production of Amorpha-4,11-Diene, a Precursor of the Antimalarial Agent Artemisinin, in *Escherichia coli*. *PLoS One*, 4(2), e4489.
- Tyczkowski, J.K. & Hamilton, P.B., 1986. Absorption, transport, and deposition in chickens of lutein diester, a carotenoid extracted from marigold (*Tagetes erecta*) petals. *Poultry science*, 65(8), pp.1526–1531.
- Ugwu, C.U. et al., 2008. Photobioreactors for mass cultivation of algae. *Bioresource Technology*, 99(10), pp.4021–4028.
- Umdu, E.S., Tuncer, M. & Seker, E., 2009. Transesterification of *Nannochloropsis oculata* microalga's lipid to biodiesel on Al₂O₃ supported CaO and MgO catalysts. *Bioresource Technology*, 100(11), pp.2828–2831.
- Vieler, A. et al., 2012. Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. *PLoS Genetics*, 8(11). e1003064.
- Vijayapadma, V. et al., 2014. Protective effect of lutein against benzo(a)pyrene-induced oxidative stress in human erythrocytes. *Toxicology and industrial health*, 30(3), pp.284–293.
- Vila, M. et al., 2008. Carotenoid content in mutants of the chlorophyte *Chlamydomonas reinhardtii* with low expression levels of phytoene desaturase. *Process Biochemistry*, 43(10), pp.1147–1152.
- Virtamo, J. et al., 2014. Effects of alpha-tocopherol and beta-carotene supplementation on

- cancer incidence and mortality: 18-year postintervention follow-up of the Alpha-tocopherol, Beta-carotene Cancer Prevention Study. *International journal of Cancer*, 135(1), pp.178–185.
- Viuda-Martos, M. et al., 2014. Tomato and tomato byproducts. Human health benefits of lycopene and its application to meat products: a review. *Critical reviews in food science and nutrition*, 54(8), pp.1032–1049.
- Vranová, E. et al., 2012. Structure and dynamics of the isoprenoid pathway network. *Molecular Plant*, 5(2), pp.318–333.
- Walker, J.M., 2011. *Cell cycle synchronization methods and protocol*, Humana Press.
- Wang, H. et al., 2013. The contamination and control of biological pollutants in mass cultivation of microalgae. *Bioresource technology*, 128, pp.745–750.
- Wang, H. et al., 2010. Transgenic tobacco plants expressing *atzA* exhibit resistance and strong ability to degrade atrazine. *Plant Cell Reports*, 29(12), pp.1391–1399.
- Wang, H.H. et al., 2009. Advances in chloroplast engineering. *Journal of Genetics and Genomics*, 36(7), pp.387–398.
- Wang, L. et al., 2005. Biodegradation of atrazine in transgenic plants expressing a modified bacterial atrazine chlorohydrolase (*atzA*) gene. *Plant Biotechnology Journal*, 3(5), pp.475–486.
- Wang, Q. et al., 2016. Genome editing of model oleaginous microalgae *Nannochloropsis* spp. by CRISPR/Cas9. *Plant Journal*, pp.1071–1081.
- Wang, W. et al., 1975. Genetic control of chlorophyll biosynthesis in *Chlamydomonas*: analysis of a mutant affecting synthesis of δ -Aminolevulinic Acid. *Cell*, 6(1), pp.75–84.
- Wani, S.H. et al., 2010. Plant plastid engineering. *Current genomics*, 11(7), pp.500–512.
- Wannathong, T., 2012. *Chloroplast genetic engineering in microalga Chlamydomonas reinhardtii: Molecular tools and applications*. PhD thesis. London: University College London.
- Wannathong, T. et al., 2016. New tools for chloroplast genetic engineering allow the synthesis of human growth hormone in the green alga *Chlamydomonas reinhardtii*. *Applied Microbiology and Biotechnology*, 100(12), pp.5467–5477.
- Wei, L. et al., 2013. *Nannochloropsis* plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. *BMC Genomics*, 14, p.534.
- Welschmeyer, N.A., 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. *Limnology and Oceanography*, 39(8), pp.1985–1992.
- Wilson, C.W. & Shaw, P.E., 1978. Synthesis of nootkatone from valencene. *Journal of Agricultural and Food Chemistry*, 26(6), pp.1430–1432.
- Work, V.H. et al., 2010. Increased lipid accumulation in the *Chlamydomonas reinhardtii* *sta7-10* starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. *Eukaryotic Cell*, 9(8), pp.1251–1261.
- Wriessnegger, T. et al., 2014. Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. *Metabolic Engineering*, 24, pp.18–29.
- Wurtz, E.A. et al., 1979. A specific increase in chloroplast gene mutations following growth of *Chlamydomonas* in 5-fluorodeoxyuridine. *Molecular and General Genetics*, 170(3),

pp.235–242.

- Xie, W.H. et al., 2014. Construction of novel chloroplast expression vector and development of an efficient transformation system for the Diatom *Phaeodactylum tricorutum*. *Marine biotechnology (New York)*, 16(5), pp.538–546.
- Xu, J.F. et al., 1998. Self-immobilized aggregate culture of *Taxus cuspidata* for improved taxol production. *Biotechnology Techniques*, 12(3), pp.241–244.
- Yoshimura, T. et al., 2013. Culture of the hydrocarbon producing microalga *Botryococcus braunii* strain Showa: Optimal CO₂, salinity, temperature, and irradiance conditions. *Bioresource Technology*, 133, pp.232–239.
- Young, R.E.B. & Purton, S., 2016. Codon reassignment to facilitate genetic engineering and biocontainment in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal*, 14(5), pp.1251–1260.
- Young, R.E.B. & Purton, S., 2014. Cytosine deaminase as a negative selectable marker for the microalgal chloroplast: A strategy for the isolation of nuclear mutations that affect chloroplast gene expression. *Plant Journal*, 80(5), pp.915–925.
- Yvon, A.C. et al., 1999. Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Molecular Biology of the Cell*, 10(4), pp.947–959.
- Zedler, J.A.Z. et al., 2014. Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*. *Journal of Applied Phycology*, 27(6), pp.2271–2277.
- Zelena, K. et al., 2012. Functional expression of a valencene dioxygenase from *Pleurotus sapidus* in *E. coli*. *Bioresource Technology*, 108, pp.231–239.
- Zerbe, P. et al., 2012. Bifunctional cis-abienol synthase from *Abies balsamea* discovered by transcriptome sequencing and its implications for diterpenoid fragrance production. *Journal of Biological Chemistry*, 287(15), pp.12121–12131.
- Zerbe, P. & Bohlmann, J., 2015. Enzymes for synthetic biology of ambroxide-related diterpenoid fragrance compounds. *Advances in biochemical engineering/biotechnology*, 148, pp.427–447.
- Zhang, R. et al., 2014. High-throughput genotyping of green algal mutants reveals random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of transforming DNA. *The Plant Cell*, 26(4), pp.1398–1409.
- Zheng, M. et al., 2013. Transcriptome sequencing, annotation and expression analysis of *Nannochloropsis sp.* at different growth phases. *Gene*, 523(2), pp.117–121.
- Zhong, J.-J., 2002. Plant cell culture for production of paclitaxel and other taxanes. *Journal of Bioscience and Bioengineering*, 94(6), pp.591–599.
- Zhu, B. et al., 2014. Large-scale biodiesel production using flue gas from coal-fired power plants with *Nannochloropsis* microalgal biomass in open raceway ponds. *Bioresource Technology*, 174, pp.53–59.
- Zhu, B.C. et al., 2001. Nootkatone is a repellent for Formosan subterranean termite (*Coptotermes formosanus*). *Journal of Chemical Ecology*, 27(3), pp.523–531.
- Zienkiewicz, M. et al., 2016. Transformation of the *Cyanidioschyzon merolae* chloroplast genome: prospects for understanding chloroplast function in extreme environments. *Plant Molecular Biology*, 93(1), pp.171–183.
- Zieve, G.W. et al., 1980. Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor Nocodazole: Nocodazole accumulated mitotic

cells. *Experimental Cell Research*, 126(2), pp.397–405.

Zulak, K.G. & Bohlmann, J., 2010. Terpenoid biosynthesis and specialized vascular cells of conifer defense. *Journal of Integrative Plant Biology*, 52(1), pp.86–97.

Appendices

Appendix. 1 Compounds used in the sensitivity tests for *N. gaditana*

Compound	Mode of action	Resistance gene
Antibiotics		
Kanamycin	Protein synthesis inhibitor	<i>aphA-6</i>
Spectinomycin	Protein synthesis inhibitor	<i>aadA</i>
Streptomycin	Protein synthesis inhibitor	<i>aadA</i>
Paromomycin	Protein synthesis inhibitor	<i>aphVIII</i>
Erythromycin	Protein synthesis inhibitor	<i>ereB</i>
Chloramphenicol	Protein synthesis inhibitor	<i>catA1</i>
Herbicides		
Harvest	Inhibitor of glutamine synthetase activity	<i>pat</i>
Glufosinate-ammonium	Inhibitor of glutamine synthetase activity	<i>pat</i>
Bialaphos	Inhibitor of glutamine synthetase activity	<i>pat</i>
Atrazine	Inhibitor of photosystem II	Point mutation in <i>psbA</i> or <i>AtzA</i>
Diuron	Inhibitor of photosystem II	Point mutation in <i>psbA</i>
Amino Acids		
D-alanine	Interfere with amino acid metabolism	<i>dao</i>

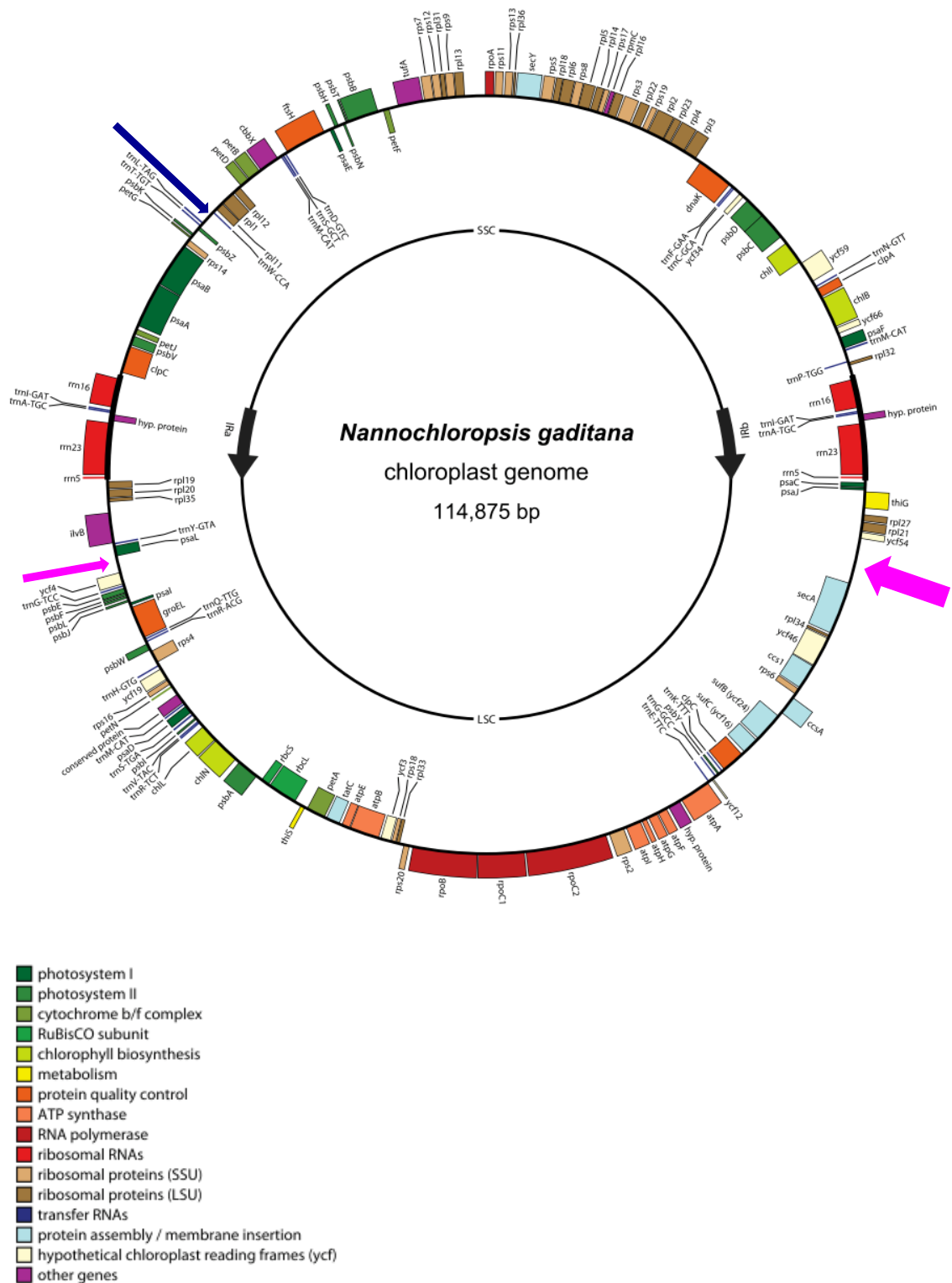
Appendix. 2 Details of primers used for either PCR or DNA sequencing

Microalgae	Primer name	Primer sequence (5'→3')
<i>N. gaditana</i>	UH-NCc 1. F	AGT AAG CAG ACA CAA ATA GGC
<i>N. gaditana</i>	UH-NCc 2. R	GCC TTG ACG ACT AAT TGA ACC
<i>N. gaditana</i>	UH-NCpA.1F	TGG TAG ATT AGA CGA TAG CG
<i>N. gaditana</i>	UH-NCpA.1R	AGAGAATTGACCAGGTTTGG
<i>N. gaditana</i>	UH-NC3pbA.1F	GCTTTTGATAATGCTCGTGGAAC
<i>N. gaditana</i>	UH-NC3pbA.5R	TTAGACCAATGCCGTGCAGC
<i>N. gaditana</i>	UH- GA 1F	ACAAGAAAGGCTTTAAAGGGTGGTAGATTAGACGA
<i>N. gaditana</i>	UH- GA 1R	TATCCAGTGATTTTTTCTCCATGTTTCGTTTGTGAATAAAATC
<i>N. gaditana</i>	UH- GA 2F	GATTTTATTCACAAACGAACATGGAGAAAAAATCACTGGATA
<i>N. gaditana</i>	UH- GA 2R	CTCATATGTAATTTAAATATTCTTACGCCCGCCCTGCC
<i>N. gaditana</i>	UH- GA 3F	GGCAGGGCGGGCGTAAGAATATTTAATTACATATGAG
<i>N. gaditana</i>	UH- GA 3R	GTAATTGAATGAGACATATTCTATAATACATAACAACATA
<i>N. gaditana</i>	UH- GA 4F	TAGTTGTTATGTATTATAGAATATGTCTCATTCAATTAC
<i>N. gaditana</i>	UH- GA 4R	TCGTCTAATCTACCACCCTTTAAAGCCTTTCTTGT
<i>N. gaditana</i>	CafIII. 1F	AGTATCCTTAAGGGTGGTAGATTAGACGATAG
<i>N. gaditana</i>	CafIII. 1R	GAAGAACTTAAGCTATAATACATAACAACACT
<i>N. gaditana</i>	UH-CAS 1F	CTTAAGGGTGGTAGATTAGACG
<i>N. gaditana</i>	UH-CAS 1R	CCAACTGAACTAAAGACGC
<i>N. gaditana</i>	UH-mCAS1F	CGCAAGATGTGGCGTGTAC
<i>N. gaditana</i>	UH-mCAS1R	GTAACACGCCACATCTTGCG
<i>N. gaditana</i>	UH- psbA 1F	AGACCTAACACATTGTACG
<i>N. gaditana</i>	UH-psbA 1R	CCC TAT TTA TTC TAG TACTC
<i>N. gaditana</i>	UH-psbA SEQR	GCAATGCACGGTTCTCTTGT
<i>N. gaditana</i>	UH-psbA SEQF	ACA AGA GAA CCG TGCATTGC
<i>N. gaditana</i>	pJET1.2 F	CGACTCACTATAGGGAGAGCGGC
<i>N. gaditana</i>	pJET1.2 R	AAGAACATCGATTTTCCATGGCAG
<i>N. gaditana</i>	TUBpPst1_F	ATGCCTGCAGACTGCGCATGGATTGACCG
<i>N. gaditana</i>	TUBpMsc1.R	GCATTGGCCATGCTTCACAAAAAGACAGCTTCTTGAT
<i>C. reinhardtii</i>	Flank 1	GTCATTGCGAAAATACTGGTGC
<i>C. reinhardtii</i>	atpA.F	CAAGTGATCTTACCACTC
<i>C. reinhardtii</i>	rbcL.R	CAAACCTTCACATGCAGCAGC
<i>C. reinhardtii</i>	RbcL fn	CGGATGTAACCTCAATCGGTAG
<i>C. reinhardtii</i>	RY-psaR	ATAGGCTCTTCTCATGGATTTCTCCTTATAATAAC
<i>C. reinhardtii</i>	RY-psaSEQ	AACTATTTGTCTAATTTAATAACC

<i>C. reinhardtii</i>	RY-dxsF2	CCATGTTTCCACCACCAAG
<i>C. reinhardtii</i>	RY-rbcR2	GAACGAATTACGTCGCCACC
<i>C. reinhardtii</i>	RY123a	GGAGGGAAGTAGGCAGTAGC
<i>C. reinhardtii</i>	psbH.R	GCAACAGGAAGTCTAAAGC
<i>C. reinhardtii</i>	UH-5'DXS R 1	AAT ACT TGT GGA AGC GTG CC
<i>C. reinhardtii</i>	UH-5'DXS R 2	CTT GGT GGT GGA AAC ATG G
<i>C. reinhardtii</i>	UH-DXS 3'UTR F1	AAT TAC TCC ACG AAC ACG GC
<i>C. reinhardtii</i>	UH-DXS 3'UTR R1	TCC ACT TAA CTC AGA AGC AGG
<i>C. reinhardtii</i>	UH-mDXS F	GGCACGCTTCCACAAGTATT
<i>C. reinhardtii</i>	UH-mDXS 1R	CCATAGCAGCCGTGATACCG

Appendix. 3 Potential neutral sites for transgene integration into the chloroplast genome of *N. gaditana*.

Chloroplast genome of *N. gaditana* CCMP526, where the genes located on the inside are transcribed clockwise and the genes on the outer side are transcribed anti-clockwise. The chloroplast genome map is taken from Radakovits et al. (2012). The insertion site used in this study for the chloroplast transformation attempts is indicated by blue arrow, whereas, other insertion sites which potentially could be used for the transgene integration are indicated by magenta arrows.



Appendix. 4 Primers used to sequence the *psbA* gene from *N. gaditana* plastome.

Key: *rbcS* (green), *psbA* on reverse orientation (red), *chlN* on reverse orientation (blue). Primers used are highlighted in yellow.

GCTTCTGGTGCAACAGCGAATCGTGTGGCTATGGAATGTGTACTTTTAGCTAAATACGAAGGTAAAGATTACATTAACGAAGGACCAAAAAATTTTACGTG
 CTGCTGCAGAAAGCTGTGCTCCATTACGTACTGCTTTAGATCTTTGGAAAGATATTGCTTTCAACTATACATCAACAGATACTGCAGATTACGTTGAAACA
 ACAACTAAACAGTAATCGTAAAAACAATATCAATCAGTTAAACATACTAAAGGAGTATTGAAGAGTGAGATTAACACAAGGTGCATTTTCGTA
 CCAGATTTAACAAACGAACAAAATTTTAGGACAGCTTAAGTATATTTGTAGCAAAGGATGGGCTTGTAGTATTGAATGGACAGAAGATCCCTCATCCGCGTA
 ACACTTACTGGGAGCTTTGGGGTATGCCTTTATTCGATATTAAGACGCTTACAGTAATTATGTATGAGTTAGACCAATGCCGTGCAGCTCACCTACAACG
 TACATCAAAATCAATGCTTTTGATAATGCTCGTGGAAGTAAAGCTGCGCATTATCTTTTCATTGCTCAACGCTCCTACGAAGAACCAGGTTTCTACTTAGA
 ACGTCAAGAAACTGAAAGTCGTAACATTCGTTACACTATTTCATAGTTATGTTGTTAATAAGTATCCTCCAGGAGAAGCTTACGTTTATAATCTATAATA
 CATAACAATAAAACCAACAAACCCACTATTCCATCCTTTACTTGTATGAAATAGTGGGTTTAAATTTTTTAAATTAATATAAATAACAAAGAAAATC
 TGAATATATAAATATACAAATGCTACTTTACTTGGAGACTTTAGATTTTAAATGTTTTCATACAGGATTGTAAGACCTAACACATTGTACGACATAAT
 ATTTTTATCAGTCAATCTAAAAAATATACATACGAGTGATTAATAATCACTCATATGTAATTAATATCTTAAAGCATTAAACAGCTGGAGCGTTTAA
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 AAGTGTACATAAAACGCTTTAATTCATATAGTCATTTAAATGCGCGGATATATCAGCTTCTCCAGTTCGCAATGATATCTCGGTTCCAGCAAAATATCA
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 CTTTTAATCTTTTCAAAATTTAGTTAGTTGTTAAAAAGTCCATTTCTAAATCTTCAACATTTTGCTCAGAATTAGCAGGTTTTAAATAAAA

Details of the primers highlighted in yellow:

Primer	Sequence (5'→3')	Tm (°C)
UH-psbA 1F	AGACCTAACACATTGTACG	53
UH-psbA 1R	CCCTATTTATTTCTAGTACTC	52.3
UH-psbA seq F [§]	ACAAGAGAACCGTGCATTGC	58.4
UH-psbA seq R [§]	GCAATGCACGGTCTCTTGT	58.4

§ Primers anneal to the same sequence

Appendix. 5 Plasmids used in this study

A) Plasmid pUH.NCc2

Key: *psbk* on reverse orientation (red), *trnT* (purple), *trnL* (blue), P/5'UTR *psaA* (highlighted in green), *cat* (highlighted in yellow), 3'UTR *psbA* (highlighted in turquoise), *trnW* (pink) and part of *rpl11* (orange), AflIII restriction sites (underlined and bolded).

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GCCCTGCAGCCGAATTATATTATTTTGGCCAAATAATTTTTAACAAAAGCTCTGAAGTCTTCTTCAATTTAAATCTTAGATGATACCTTCATCTGAAAAAT
TGTCCTCAATTAGTAGCATCAGCTGTGAGTAAGTCTTAAACCATTTTTTATTGTTGTATTATCTCTAATCTTACTACTCGATGAGTTTTTCGGTATTATCT
CTATTTTTAACTTGGAGCAGGTTCCATTCTGTTTTTTTCATCATAGTGAATAAAATCAACTGCTTTAACACTTGTGCTGAACACCATATCCATCCGGC
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GCCTAATACTAAGATTTCGTATTAGACAATCAGAAATTTAAACAAGCAATGAACTAATGAACTCAGGGTATAAATTAGATCAAAATAGGATTAATTTACCTTTATG
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GCCCCCTGACGAGCATCAAAAAATCGACGCTCAAGTCAAGGTTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCTGGAAAGCTCCCTC
GTGGCTCTCTGTTCCGACCTGCGGCTTACCAGTACCTGTCCGCTTTCTCCCTTCCGGAAGCGTGGCGTTTTCTCATAGCTCAGCTGTAGGTATCTC
AGTTCCGTTAGTGTGCTTTCGCTCAAGCTGGGCTGTGTGACGAAACCCCGTTTACGCCGACCGCTGCGCTTATCCGGTAACTATCGTCTTGAAGTCAAC
CCGGTAAAGACGACTTATCGCCACTGGCAGAGCCACTGGTAAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGTACAGAGTTCTTGAAGTGGTGGCC
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GCCTGACTCCCGTCTGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCAGTGTGCAATGATACCGGAGACCCAGCTCACCGGCTCCAGAT
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AACGTTCTTCCGGCGCAAACTCTCAAGGATCTTACCCTGTTGAGATCCAGTTCCGATGTAACCCACTCTGCACCAACTGATCTTTCAGCATTTTTACTT
TCACCAGCGTTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAATTTGTTGAATACTCATACTTCCCTTT
TCAATATAATGAAGCATTTATCAGGTTTATGCTCATGAGCGGATACATATTTGAAATGATTTAGAAAAATAACAAAATAGGGGTTCCCGGCACATTT
CCCCAAAAGTGGCCACTGACGCTTAAAGAAACCATTTATTATCATGACATTTAACTTAAAAAATAGGGCTATCAGGATC
```

B) Mutant version of *psbA* gene used in plasmid pUH-psbA264A

I) DNA sequence of *psbA* gene, AGT→GCT codon alternation highlighted in yellow.

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ATGACAGCAACTTTAGAAAAGAAGAGAAAAGTACTAGCTTATGGGAGCGCTTCTGCTCTTGGATCACTAGCACAGAAAAC
CGTTTATACATCGGTTGGTTCCGGTGTTTAATGATCCCTACATTATTAACAGCTACAAGCTGCTTCATCATCGCTTTCA
TCGCAGCACCTCCAGTAGATATCGATGGTATCCGTGAACCAGTGGCTGGATCACTTTTATACGGAAACAACATTATCA
CAGGTGCGGTTATTCCAAGTTCTAACGCTATTGGTATTCACCTTCTACCCAGTTTGGGAAGCTGCGTCTGTAGACGAAT
GGCTTTACAACGGTGGTCCCTTACCAACTTGTAGTATTACACTTCTTATTAGGTGTAGCTTGCTACATGGGACGTGAAT
GGAACTTAGCTACCGTTTAGGGATGCGTCCTTGGATTTTCGTAGCTTCTCAGCTCCAGTAGCTGCAGCAAGTGCAGT
ATTCTTAATTTACCCAATCGGACAAGGTAGCTTCTCTGATGGTATGCCTTTAGGTATCTCTGGTACTTTCAACTTCATG
TTAGTATTCCAAGCAGAACACAACATTTCTTATGCACCCATTCCATATGGCTGGTGTGCTGGTGTGTTTGGTGGATCT
TTATTCAGTGCAATGCACGGTTCTCTTGTAAACATCTAGTTTAAATCCGTGAAACTGAAGACGGTGTATCTGCTAACTAC
GGTTACAAATTTGGACAAGAAGAAGAACTTACAACATCGTAGCTGCACACGGTTACTTTGGACGTTTAAATCTTCCAA
TACGCA GCTTTCAACAACCTCACGTGCTTTACACTTCTTCTTAGCTGCTTGGCCAGTTGTAGGTATCTGGTTAACTGCTC
TTGGAATTAGCACAATGGCATTCAACTTAAACGGATTCAACTTCAACCAATCAGTTGTTGATAGCCAAGTCCGTGTGA
TCAACACTTGGGCTGACATTATCAACCGTGCTGATTTAGGTATGGAAGTAATGCACGAACGTAACGCCGACAACTTCC
CTCTAGATTTAGCGGTATCTAATGTTCTTCCAGTTGCATTAACGCTCCAGCTGTTAATGCTTAA
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II) Translated sequence of mutated *psbA* at codon position 264 from serine (S) to alanine (A), which is highlighted in yellow.

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MTATLERRESTSLWERFCSWITSTENRLYIGWFGVLMIPTLLTATSCFIIAFIAAPPVDDIDGIREPVAGSLLYGNNIITGAVI
PSSNAIGHFYPPVWEAASVDEWLYNGGPYQLVVLHFLLVACYMGREWELSYRLGMRPWIFVAFSAPVAAAASAVFLIYP
IGQGSFSDGMPLGISGTFNFMVLFQAEHNILMHPFHMAGVAGVFGGSLFSAMHGLVTSLLIRETEDGVSANYGYKFGQE
EETYNIVA AHGYFGRLIFQYA A FNNSRALHFFLAAWPVVGIWLTALGISTMAFNLNGFNFNQSVVDSQGRVINTWADII
NRADLGMEVMHERNAHNFPLDLAVSNVLPVALNAPAVNA-
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C) Plasmid pUH-psbA264A

Key: part of *rbcS* (red), 3'UTR *psbA* (green), *psbA264A* on reverse orientation (blue), P/5'UTR *psbA* (purple) and part of *chlN* (orange).

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GCCCTGCGAGCCGAATTATATTATTTTGGCAAATAAATTTTAAACAAAAGCTCTGAAGTCTTCTCATTAAATTTCTAGATGATACCTCATCTGGAAAAT
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CTATTTTAACTTGGAGCAGGTTCCATTATTGTTTTTTTCATCATAGTGAATAAAATCAACTGCTTAAACACCTGTGCCTGAACACCATATCCATCCGGC
GTAATACGACTCACTATAGGGAGAGCGGCCCGCAGATCTCCGGATGGCTCGAGTTTTTCAGCAAGATCTTTTCATTGCTCAACGCTCTACGAGAACCA
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CCACCTGAGCTTAAGAAACCATTTATCATGACATTAACCTATAAAAAATAGGGTATCAGAGGCC
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D) Plasmid pRY134a

Sequence of pRY134a plasmid, containing *dxs* gene amplified from *Synechocystis* 6803 in pSRSapl backbone. Key: P/5'UTR *psaA* exon 1 (red), *dxs* gene (blue), two stop codons (highlighted in turquoise), HA tag (highlighted in red) and 3'UTR *rbcl* (green).

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AGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCCTGGCGTTACCCAACCTAATCGCCTTGCAGCACATCCCCCTTCGCCAGCTGGCGT
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E) Plasmid pRY134aΔdxs

This plasmid is identical to pRY134a except for the partial deletion of *dxs* gene. The deleted 141 bp from the *dxs* gene is underlined. Key: NcoI restriction site (highlighted in yellow), HA tag (highlighted in red) and two stop codons (highlighted in turquoise).

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Appendix. 6 List of conferences

- **Conference - Poster preparation**
Algae Europe 2015 Conference-Lisbon, Portugal
Date: 01/12/2015 – 03/12/2015
- **Conference - Poster Preparation**
BioProNET 2nd Annual Scientific Symposium, Manchester Midland Hotel, UK
Date: 22/10/2015 – 23/10/2015
- **Conference - Attendance**
PHYCONET Annual Conference 2015 Bordeaux Suite, Novotel London West, UK
Date: 26/08/2015
- **Conference - Poster Presentation**
Algae around the world Symposium, Cambridge University, UK
Date: 19/03/2015
- **Conference - Poster Preparation**
Chloroplast Biotechnology, Ventura, California, United States
Date: 18/01/2015 – 23/01/2015
- **Conference - Attendance**
Algal Biotechnology Symposium 2014, University of Sheffield, Sheffield, UK
Date: 26/08/2014
- **Conference - Poster Preparation**
PHYCONET symposium, Darwin Building, University College London, UK
Date: 14/04/2014
- **Conference - Presentation**
Young Algaeneers Symposium 2014, Montpellier-Narbonne, France
Date: 03/04/2014 – 05/04/2014
- **Conference - Poster Preparation**
Alg'n'Chem 2014: Which future for algae in industry, Montpellier, France
Date: 31/03/2014 – 03/04/2014
- **Conference - Poster Preparation**
Sustainable Chemicals from Microalgae: Encompassing Biocrude through to Fine Chemicals
Burlington House, London, UK
Date: 19/11/2013 – 19/11/2013
- **Conference - Attendance**
High-Value Chemicals from Biomass, RSC, Burlington House, London, UK
Date: 17/09/2013 – 17/09/2013
- **Conference - Presentation**
Plastid Preview 2013, University of Greenwich, London, UK
Date: 09/09/2013 – 10/09/2013
- **Conference - Poster Preparation**
The 2013 Algal Biotechnology Symposium, Cambridge University, UK
Date: 02/09/2013 – 02/09/2013



The biotechnological potential of *Nannochloropsis*

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With 2 figures and 5 tables

Abstract: Oleaginous microalgae have commercial potential as photoautotrophic cell factories capable of producing advanced biofuels and high-value speciality oils. One genus of particular interest is *Nannochloropsis*, which includes a number of robust marine species well suited to industrial-scale cultivation. Advances in bioprocess technology, together with strain enhancement through traditional mutagenesis or genetic engineering approaches, now offer the possibility of improving the economics of oil production from *Nannochloropsis*. In this review we describe the current and potential industrial applications of this genus, consider the present status of genetic enhancement methods, and highlight the need for new advances in this area – including the development of techniques for engineering the chloroplast genome.

Keywords: biofuels; eicosapentaenoic acid; genetic engineering; plastome; transformation; *Nannochloropsis*; *Microchloropsis*

Introduction

Nannochloropsis species are unicellular microalgae that belong to the class of Eustigmatophyceae within the Heterokontophyta and are recognised for their high photoautotrophic biomass productivity, their natural ability to accumulate high lipid content, and their successful cultivation at industrial scale (Radakovits et al. 2012). They are simple, non-flagellate, and spherical to slightly ovoid cells measuring 2–4 µm in size (Fig. 1A), making them difficult to distinguish from chlorophyte species (Sukenik 1999). Each cell has one or more yellow-green chloroplasts that occupy a significant part of the total cell volume and contain chlorophyll *a* as the only chlorophyll. Violaxanthin is the main accessory pigment, with β-carotene, vaucherixanthin esters and several minor xanthophylls as additional accessory pigments (van den Hoek et al. 1995). The chloroplast is complex compared to that of chlorophyte algae because it is surrounded by four membranes derived from the secondary endosymbiosis of a red alga (Janouskovec et al. 2010). The outermost plastid membrane is connected with the outer nuclear envelope membrane to form a nucleus-plastid continuum (Murakami & Hashimoto 2009) as illustrated in Figure 1B. Data from the genome sequencing projects and from NMR studies suggest that *Nannochloropsis* cell walls are cellulosic and contain sulphated fucans (Arnold et al. 2014, Corteggiani Carpinelli et al. 2014).

The *Nannochloropsis* genus is traditionally recognised as comprising the six species *Nannochloropsis gaditana*, *Nannochloropsis salina*, *Nannochloropsis oculata*, *Nannochloropsis granulata*, *Nannochloropsis oceanica* and *Nannochloropsis limnetica* (Fig. 1C) (Murakami & Hashimoto 2009). However, a recent study based mainly on *rbcL* and 18S rDNA sequencing data (Fawley et al. 2015) has proposed that a new species, *Nannochloropsis australis*, be added and that *N. gaditana* and *N. salina* should be reclassified into a new genus named *Microchloropsis*. Another study suggested that *N. gaditana* could be reclassified as a strain of *N. salina*, owing to the 98.4% nucleotide identity and identical gene synteny between the two chloroplast genomes (Starkenburg et al. 2014). Reliable organellar phylogenetic markers for the inter- or intra-species phylotyping of *Nannochloropsis* have recently been determined by Wei et al. (2013) using systematic analysis of full organellar genome sequences. All *Nannochloropsis* species are found in marine environments except *Nannochloropsis limnetica*, which is found in fresh and brackish water (Jinkerson et al. 2013).

The high lipid productivity, abundance of polyunsaturated fatty acids and robust growth of *Nannochloropsis* species, together with the availability of genome sequences and molecular-genetic tools for various strains, make this genus attractive as cell platforms for the production of lipid molecules of industrial interest. Here we review the potential of *Nannochloropsis* in the aqua feed, food and green energy

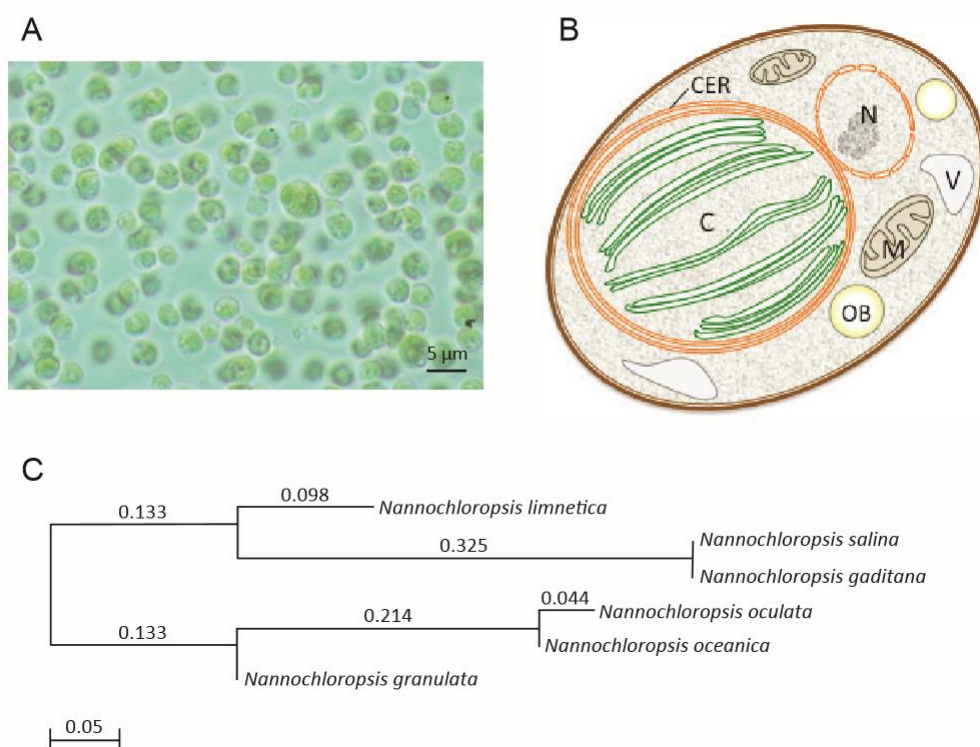


Fig. 1. (A) Light micrograph of *Nannochloropsis oceanica* under 100X magnification. (B) Illustrative cell structure for the *Nannochloropsis* genus under nutrient replete conditions, adapted from a figure by Lubián (1982). Abbreviations are C chloroplast, CER chloroplast endoplasmic reticulum; M mitochondrion; N nucleus; OB oil body; V vesicle. Under nutrient limitation stress, the oil body volume within the cell increases significantly (see, for example, Simionato et al. 2013). (C) Rooted neighbour-joining phylogenetic tree showing the relationship between different *Nannochloropsis* species based on whole alignment of their chloroplast genomes. Other phylogenetic trees for *Nannochloropsis* are based on 18S rDNA (Radakovits et al. 2012, Vieler et al. 2012); on 18S rDNA and *rbcL* (Fawley et al. 2015) and the mitochondrial genome (Wei et al. 2013).

industries. We consider the present status of genetic enhancement methods, and highlight the need for new advances in genetic engineering, including the need for a reliable method for engineering the chloroplast genome.

Biotechnological applications for *Nannochloropsis*

Potential biotechnological applications for *Nannochloropsis* species are summarised in Figure 2A. At present, the predominant commercial use is as the base of the food chain in the aquaculture industry. Companies such as Algaspring,

Archimede, Greensea, Monzon Biotech, Phycopure and Proviron grow and sell wild-type *Nannochloropsis* as aquafeed for the cultivation of marine fish, molluscs and shrimps, or for the production of zooplankton such as rotifers that are in turn used to supply fish hatcheries and nurseries, with the feedstock supplied as concentrates of live algae, or as frozen or lyophilised algae (Lubzens et al. 1995, Camacho-Rodriguez et al. 2016). The main attraction of *Nannochloropsis* for the aquafeed industry is its favourable fatty acid composition, which includes a relatively high content of eicosapentaenoic acid (EPA) (Sukenik 1999, Ma et al. 2016). EPA is a highly unsaturated omega-3 fatty acid (20:5) and is a useful dietary component in preventing several human diseases. Although

EPA is commonly called a 'fish oil', marine fish cannot synthesize this molecule and rely on a dietary source. For *N. gaditana*, EPA productivity can reach 30 mg l⁻¹ day⁻¹ in outdoor photobioreactors (Camacho-Rodriguez et al. 2014) and can represent as much as 27% of total fatty acids under nutrient-sufficient conditions (Ferreira et al. 2009). The nutritional value of *Nannochloropsis* under different growth conditions and the transfer of their nutrients through food chains has been extensively investigated (Fernandez-Reiriz & Labarta 1996, Ferreira et al. 2009, Camacho-Rodriguez et al. 2014), and the use of marine microalgae in the aquaculture industry has been the subject of several detailed reviews (Becker 2013, Gressel 2013).

The use of *Nannochloropsis* as an aquaculture feed also offers potential opportunities for creating transgenic lines for oral delivery of pharmaceutical proteins that improve fish growth rates and reduce loss through pathogens. Chen et al. (2008) reported significant improvements in growth of tilapia larvae when the feedstock of *N. oculata* was replaced with a transgenic line engineered to produce fish growth hormone. In a separate study, the same species was engineered to produce the anti-microbial peptide bovine lactoferricin. Feeding medaka fish with this transgenic line greatly improved their survival rates when subsequently infected with a bacterial pathogen (Li & Tsai 2009). There is also interest in using microalgae such as *Nannochloropsis* for the oral delivery of antigens to the many viral, bacterial and fungal pathogens that plague the aquaculture industry, thereby providing a simple low-cost method of vaccine delivery (Siripornadulsil et al. 2007).

Exploitation of *Nannochloropsis* PUFA-rich feed is not limited to aquaculture and there is growing interest in its use in other animal feeds to improve the nutritional value of farmed foods. For example, a recent study demonstrated such improvement in the nutritional value of egg yolk by adding *Nannochloropsis* biomass to laying hens' feed (Lemahieu et al. 2013). Another growing sector is the production of microalgae for use directly in the human diet: Qualitas Health produces liquid capsules containing EPA-rich oil from wild type *N. oculata* grown in shallow ponds in Texas, while Optimally Organic sells *N. gaditana* dried powder as a nutritional supplement. The safety of both the oil from *N. oculata* and whole cells of the alga has been assessed in toxicology studies and determined safe for use as a dietary supplement (Kagan et al. 2014, Kagan & Matulka 2015). Such products therefore represent vegetarian sources of EPA that avoid the problems of declining fish stocks and potential heavy metal contaminants found in fish oils.

The development of *Nannochloropsis* as a feedstock for biofuel production has also been the focus of much research over the past decade, but there are some key issues to be addressed before algal biofuel production becomes cost effective and competitive with current fuel supplies (Umdu et al. 2009, Doan & Obbard 2015, Zhu et al. 2014, Ma et al. 2014, Hu et al. 2015). These include: i) developing strains

that produce high quantities of triacylglycerols (TAGs) with the desired chain lengths and degree of saturation for conversion to fungible biofuels (Taleb et al. 2015); ii) understanding the link between growth conditions and lipid productivity; iii) developing large-scale cultivation facilities (Fig. 2B), and iv) refining oil extraction techniques. In addition to TAGs, alka(e)nes including heptadecane, heptadecene and pentadecane were recently identified in several *Nannochloropsis* species (Sorigué et al. 2016) and may be suitable for inclusion in jet fuels and diesel fuels.

Fossil fuels and first-generation biofuels derived from land-based energy crops are already available on the market in large quantities, whereas commercial production of algae-derived biofuels are expected to require more advanced technologies (Hannon et al. 2010, Medipally et al. 2015). One aspect of this is the economics of large-scale, outdoor cultivation – as illustrated by Vree et al. (2015) in a comparative study of *Nannochloropsis* growth in four different production systems. The highest areal productivities were achieved in a closed vertical photobioreactor and the lowest in an open pond system, whilst the capital costs for the former are considerably higher than the latter.

Enhancing the lipid profile in *Nannochloropsis*: strain choice, growth conditions and mutant selection

Several species in the genus *Nannochloropsis* are recognized as oleaginous algae owing to their ability to accumulate large quantities of lipid. However, there is inter- and intra-species variation in the lipid productivity and fatty acid composition (Ma et al. 2014, Beacham et al. 2014). In a study of nine *Nannochloropsis* strains, specific growth rates were found to range from 0.07 to 0.21 day⁻¹ while the lipid content varied from 37 to 60% of dry weight (Ma et al. 2014). The predominant fatty acids in most of these strains were 16:0, 16:1 and 18:1. The most suitable strain depends on the desired product; strains for fish oil production should have high levels of EPA either in polar lipids for good bioavailability or as neutral TAGs for ease of extraction, whereas those for biodiesel need shorter saturated and monounsaturated fatty acids in TAGs. Significant variation is also seen in cell wall thickness among *Nannochloropsis* species, which could impact on both the efficiency and cost of lipid extraction, as well as the ease with which a strain can be genetically engineered (Beacham et al. 2014). Furthermore the salinity of the culture medium also influences the thickness of the wall within a species, so growth conditions could be optimized to favour downstream processing (Beacham et al. 2014).

Growth conditions are also key to maximizing lipid yield. Nitrogen deprivation and other stress conditions are typically used to induce increased lipid content in *Nannochloropsis* and other microalgae. However, these conditions result in impeded cell growth and photosynthesis, affecting the

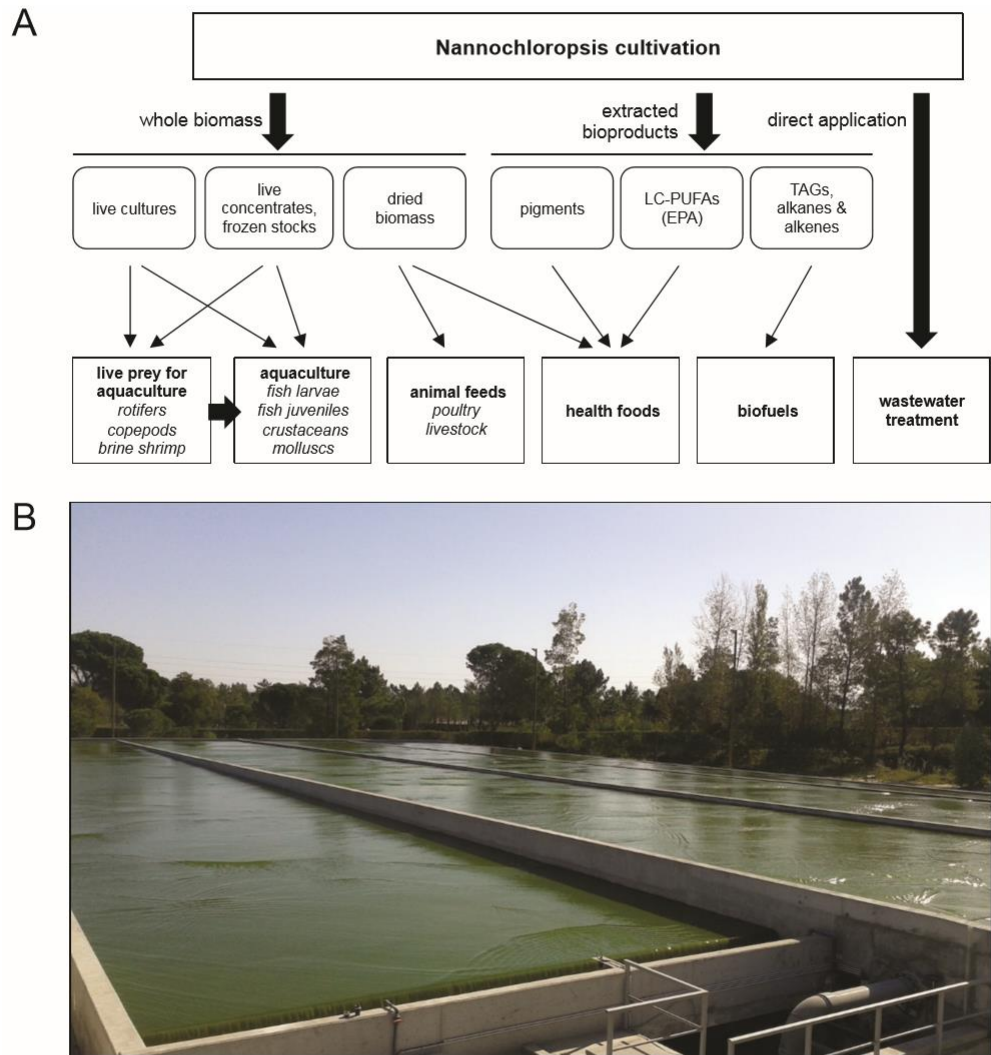


Fig. 2. (A) Current and potential biotechnological applications of *Nannochloropsis*. (B) Cascade raceway used in the E.U. BIOFAT project for *Nannochloropsis* cultivation (reproduced with permission from BIOFAT, www.biofat-project.eu).

biomass productivity and making the system less commercially viable (Radakovits et al. 2012, Corteggiani Carpinelli et al. 2014). The nitrogen starvation response has been studied in detail and includes a reorganization of the photosynthetic apparatus (Simionato et al. 2013). There have been several attempts to reach high lipid content without

losing high biomass productivity, using for instance a two-stage cultivation process (Su et al. 2011) and conventional mutagenesis approaches (Schneider et al. 1995, Beacham et al. 2015). Franz et al. (2013) screened small molecules for their ability to increase intracellular lipid levels as measured by Nile red staining, finding that quinacrine was effective

for *N. oculata* and epigallocatechin gallate for *N. salina*. In addition, exposure to UV-C radiation was found to lead to a two-fold increase in total EPA content in *Nannochloropsis* sp. cultures (Sharma & Schenk 2015).

Following the selection of robust lipid producer strains among *Nannochloropsis* species and adjusting growth conditions to achieve the best yield, genetic approaches can be used to further optimize a strain: i.e. domesticating a wild isolate through phenotypic improvements that make it more suited to industrial application. The simplest strategy is a 'forward genetics' approach involving random mutagenesis by classical physical or chemical methods, followed by the selection of strains with higher lipid productivity or other desirable phenotypes. This genetic approach is aided by the fact that *Nannochloropsis* species appear to be haploid (Kilian et al. 2011), and therefore both dominant and recessive mutations display a phenotype. However, there are as yet no reports of sexual reproduction in *Nannochloropsis*, and therefore classical breeding programmes aimed at combining desirable traits, eliminating undesired mutations from strains, and mapping mutations may not be possible. Genome sequencing failed to identify meiosis-specific genes in the *N. oceanica* genome (Pan et al. 2011) although, actively transcribed meiosis-specific genes were reported for *N. gaditana* B-31 (Corteggiani Carpinelli et al. 2014). The capacity for sexual reproduction, if present at all, may therefore vary between *Nannochloropsis* species and may require more than one mating type. Nonetheless, mutagenesis screens have led to the successful isolation of mutant strains with smaller antenna size, and therefore increased light-use efficiency under bulk cultivation conditions (Perin et al. 2015). In addition, there are a number of reports of strain improvements in lipid profile or productivity through classical mutagenesis as summarized in Table 1.

Progress on genetic engineering of the *Nannochloropsis* nuclear genome

Whilst forward genetic screens can lead to enhanced phenotypes, the improvement of *Nannochloropsis* strains for industrial applications also requires robust and routine technologies for genetic engineering. These then allow 'reverse-genetic' strategies in which endogenous genes are manipulated, or foreign genes introduced into the genome to give desired new phenotypes. Most efforts to date have focused on genetic engineering of the nuclear genome, as discussed in this section. However, the development of complementary techniques for engineering the chloroplast genome is also necessary, as discussed in the subsequent section. The first successful nuclear transformation of *Nannochloropsis* was reported by Chen et al. (2008), who used electroporation of protoplasts to introduce a gene encoding fish growth hormone under the control of an inducible promoter. Subsequently, reports appeared describing the

nuclear transformation of *Nannochloropsis* without cell wall removal using either *Agrobacterium* (Cha et al. 2011), electroporation (Kilian et al. 2011) or microparticle bombardment (Kang et al. 2015a, c). A number of selectable markers and reporter genes have been developed that allow selection of transformant lines and assays of transgene expression levels (Table 2). The most effective markers to date are those that confer resistance against antibiotics such as zeocin (the *Sh.ble* gene) and hygromycin B (*aph7*). On the other hand, two native genes that are required for growth on nitrate as the sole nitrogen source have been successfully knocked out in one *Nannochloropsis* strain (Kilian et al. 2011), opening up the possibility of using the genes as endogenous selectable markers. The nitrate reductase and nitrite reductase knock-out cell lines cannot grow on nitrate as the nitrogen source but can be maintained on medium containing ammonium. Hence, re-introduction of the gene into the corresponding mutant should allow selection on nitrate.

Another marker that has been expressed successfully in *Nannochloropsis* is the purple chromoprotein gene from *Stichodactyla haddoni* (shCP), which is not directly selectable but produces a distinctive brown phenotype that can be easily identified in the background of non-transformed cells (Shih et al. 2015). Reporter genes such as the β -glucuronidase gene (*GUS*) and adapted versions of the gene for green fluorescent protein (GFP) have been used to test promoters and transformation techniques in *Nannochloropsis* (Cha et al. 2011, Moog et al. 2015). In addition, the first *in vivo* localization study of *Nannochloropsis* has been reported by Moog et al. (2015) using GFP, highlighting the possibility of using N-terminal targeting sequences to target nuclear-encoded proteins of interest to different cellular compartments such as the nucleus, mitochondria, endoplasmic reticulum or chloroplast. A recent study has developed a reporter gene to overcome the interference of autofluorescent signals from cells and maintain greater brightness with photostability by using the genetically modified mCherry fluorescent protein "sfCherry fluorescent" (Kang et al. 2015a).

Interestingly, a study shows that homologous recombination in the *Nannochloropsis* sp. W2J3B nucleus can occur when transgenes are flanked with homologous genomic sequence (Kilian et al. 2011). This could allow both the precise and predictable insertion of transgenes into defined nuclear loci, and the systematic manipulation and functional analysis of specific endogenous genes. However, efficient integration of exogenous DNA into the nuclear genome via homologous recombination has yet to be reported in any other *Nannochloropsis* strain, and it appears that for most transformation events in other strains the transgenes insert into the genome at apparently random loci, and sometimes in multiple copies. This can lead to 'position effects' in which the level and stability of transgene expression varies between transformant lines. For overexpression studies or the introduction of foreign genes, the introduction of episomal plasmids via bacterial conjugation may be an option; such a

Table 1. Reports of classical mutagenesis used for strain improvement. Abbreviations: EMS, ethyl methanesulfonate; FAME, fatty acid methyl esters; UV, ultraviolet.

Species	Isolated phenotype	Mutagen	Notes	Reference
<i>Nannochloropsis</i> sp.	Mutant with improved total fatty acid content for biodiesel	EMS	A 30% increase in palmitoleic acid (16:1) and a 45% decrease of EPA	Doan & Obbard 2012
<i>Nannochloropsis oculata</i>	Mutant resistance to quinalofop with enhanced EPA	EMS	Increase in TAG, linoleic acid (18:2), arachidonic acid (20:4 n-6) and EPA (20:5 n-3)	Chaturvedi & Fujita 2006
<i>Nannochloropsis salina</i>	Mutant with enhanced lipid content	EMS	Increase in total FAME production and reduced levels of PUFAs	Beacham et al. 2015
		EMS and UV	Decrease in growth rate compared to the wild type control but with significantly elevated levels of total lipid and a reduction in PUFAs	
<i>Nannochloropsis gaditana</i>	Photosystem II mutant with reduced antenna size	EMS*	Improved biomass productivity in lab-scale cultures	Perin et al. 2015
<i>Nannochloropsis</i> sp.	Mutant with improved growth rate	EMS	Increase in total lipid productivity as result of changes in chlorophyll a content and an increase in growth rate	Anandarajah et al. 2012
<i>Nannochloropsis oculata</i>	Xanthophyll aberrant mutant	EMS	Two- to three-fold increase in violaxanthin and zeaxanthin content, but lowered lutein content	Lee et al. 2006
<i>Nannochloropsis oceanica</i> IME11	Mutant with improved growth rate	Heavy ion irradiation	High growth rate and 14% increase in TAG	Ma et al. 2013
<i>Nannochloropsis</i> sp.	Mutant deficient in EPA	Gamma rays	Increase in TAG associated with four-fold reduction in lipid membranes. Decrease in growth rate	Schneider et al. 1995

* In this study, insertional mutagenesis was used to create mutant strains in parallel with classical mutagenesis.

Table 2. DNA delivery methods and selectable markers used for nuclear transformation of *Nannochloropsis*.

Strain	Transformation Method	Transformation efficiency*	Selectable marker	Promoter	Reference
<i>Nannochloropsis oculata</i>	Electroporation	NR	Red fluorescent protein gene (<i>DsRed</i>)	<i>C. reinhardtii</i> HSP70A/RBCS2 dual promoter	Li & Tsai 2009
<i>Nannochloropsis</i> sp. (strain UMT-M3)	<i>Agrobacterium</i> mediated	NR	β -glucuronidase gene (<i>GUS</i>)	CaMV 35S promoter	Cha et al. 2011
<i>Nannochloropsis</i> sp. (strain W213B)	Electroporation	2.5×10^{-6} **	<i>Sh.ble</i> , <i>bxr</i> (blasticidin resistance) and <i>hygR</i> (hygromycin resistance) genes	Native VCP2 bidirectional promoter	Kilian et al. 2011
<i>Nannochloropsis gaditana</i> CCMF526	Electroporation	12.5×10^{-6}	<i>Sh.ble</i> gene	Native TUB/HSP/UEP promoters	Radakovits et al. 2012
<i>Nannochloropsis oceanica</i> CCMPI779	Electroporation	1.25×10^{-6}	<i>aph7</i> gene	Native LDSP promoter	Vieker et al. 2012
<i>Nannochloropsis salina</i> MBIC10063	Electroporation	61.2×10^{-6}	<i>Sh.ble</i> and <i>GUS</i> genes	Native TUB promoter	Li et al. 2014a
<i>Nannochloropsis oceanica</i> CCMPI779	Electroporation	NR	<i>Sh.ble</i> and <i>gfp</i> genes	Native VCP2 bidirectional promoter	Moog et al. 2015
<i>Nannochloropsis oculata</i> NIES-2146	Electroporation	6×10^{-9}	<i>shCP</i> gene	<i>C. reinhardtii</i> HSP70A/RBCS2 dual promoter	Shih et al. 2015
<i>Nannochloropsis salina</i> CCMPI776	Microparticle bombardment	5.9×10^{-8}	<i>Sh.ble</i> gene and gene encoding sfCherry fluorescent protein	Native TUB and UEP promoters	Kang et al. 2015a, c

* Highest transformation efficiency reported based on transformant number per total plated cells per μ g DNA.**Transformation efficiency is reported as 2500 transformants per μ g. NR: not reported.

Table 3. Reports of strain engineering to improve the lipid profile in *Nannochloropsis*. Abbreviations are: TAG, triacylglycerol; PUFA, polyunsaturated fatty acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid.

Gene	Protein	Gene source	Host	Notes	Reference
<i>AUREO1</i>	Aureochrome-1	<i>N. gaditana</i>	<i>S. cerevisiae</i>	Increased lipid content by 1.6 fold	Huang et al. 2014
<i>Nod12</i>	<i>N. oceanica</i> microsomal-like $\Delta 12$ -desaturase	<i>N. oceanica</i>	<i>N. oceanica</i>	Increased n-6 PUFA, LA and AA content in TAG under nitrogen starvation	Kays et al. 2015
<i>DGTT4</i>	Diacylglycerol acyl-CoA acyltransferase type-2	<i>N. oceanica</i>	<i>S. cerevisiae</i>	Increased LA by converting endogenous yeast OA to LA	
<i>LACS</i>	Long-chain acyl-CoA synthetase	<i>C. reinhardtii</i>	<i>Nannochloropsis</i> strain NIES-2145	Increased TAG accumulation by 1.7 fold under phosphorus starvation	Iwai et al. 2015
<i>bHLH2</i>	Basic helix-loop-helix isoform 2	<i>N. gaditana</i>	<i>S. cerevisiae</i>	Accumulation of eicosapentaenoic acid and docosahexaenoic acid	Zheng et al. 2014
		<i>N. salina</i>	<i>N. salina</i>	Increase biomass productivity by 36% under normal condition and FAME productivity by 33% under nitrogen starvation	Kang et al. 2015b

Table 4. Reports of chloroplast transformation in microalgae.

Algal species	Transformation method	Selectable marker	Selection	References
<i>Chlamydomonas reinhardtii</i>	Particle gun	<i>atpB</i>	Photoautotrophy	Boynnton et al. 1988
	Glass beads	<i>tscA, atpB</i>	Photoautotrophy	Kindle et al. 1991
<i>Haematococcus pluvialis</i>	Particle gun	<i>aadA</i>	Spectinomycin	Gutiérrez et al. 2012
<i>Dunaliella tertiolecta</i>	Particle gun	<i>ereB</i>	Erythromycin	Georgianna et al. 2013
<i>Platymonas subcordiformis</i>	Particle gun	<i>bar</i>	Basta	Cui et al. 2014
<i>Porphyridium sp.</i>	Particle gun	<i>AHAS</i> (W492S)	Sulfometuron methyl	Lapidot et al. 2002
<i>Euglena gracilis</i>	Particle gun	<i>aadA</i>	Streptomycin and spectinomycin	Doetsch et al. 2001
<i>Phaeodactylum tricornutum</i>	Electroporation	<i>cat</i>	Chloramphenicol	Xie et al. 2014

system has recently been developed for diatoms (Karas et al. 2015).

Strain engineering for increased lipid productivity requires knowledge of the relevant biosynthetic pathways so that particular genes can be chosen for knockout, over-expression or introduction. The identification and characterization of lipid metabolic pathway genes, including those involved in fatty acid biosynthesis, TAG assembly, lipid activation and degradation could be used as a guide for rational genetic engineering of *Nannochloropsis*. Several research groups have sequenced and annotated the genomes of different *Nannochloropsis* species and in some cases investigated their metabolic pathways (Radakovits et al. 2012, Vieler et al. 2012, Corteggiani Carpinelli et al. 2014, Wang et al. 2014), providing a rapid and effective way to gain the basic knowledge for further studies. The integration of available genomic data with transcriptome (Tian et al. 2013, Zheng et al. 2013), proteome (Simionato et al. 2013) and lipidome (Li et al. 2014b) data of various *Nannochloropsis* species assists in elucidating the genes involved and how transcriptional changes modulate increased metabolic flux within the biosynthesis pathways under a variety of physiological growth conditions. Further utilization of these data for modelling and overexpression studies will provide a better understanding of lipid biosynthesis in oleaginous algae and opportunities for increasing the production of specific lipids through metabolic engineering. Although the over-expression of genes involved in lipid biosynthesis does not always lead to the predicted outcomes (La Russa et al. 2012), recent transgenic studies have resulted in increased TAG content in *Nannochloropsis*, or the elucidation of gene function through the over-expression of *Nannochloropsis* genes in the model yeast, *Saccharomyces cerevisiae* (Table 3). Other genetic engineering approaches that should be considered for increased lipid production include increasing photosynthetic efficiency under nitrogen deprivation, blocking competing pathways and reduction of TAG catabolism, and decoupling TAG accumulation and stress conditions such as nitrogen deprivation (Klok et al. 2014).

Perspectives for chloroplast genomic engineering

The chloroplast is the site of primary energy production within the algal cell and also houses key metabolic pathways such as those involved in the biosynthesis of carbohydrates, fatty acids, tetrapyrroles and terpenes. The full exploitation of *Nannochloropsis* and other microalgae as a biotechnology platform for biofuel production or synthesis of high-value metabolites therefore requires the development of methods for engineering the chloroplast genome (=plastome). This would allow the manipulation of endogenous chloroplast genes involved in energy transduction and carbon fixation, and the introduction of foreign genes encoding novel metabolic enzymes (Purton et al. 2013). Progress is being made in the development of chloroplast transformation methodology for a number of microalgal species (Table 4). However there are no reports as yet of successful chloroplast transformation of *Nannochloropsis* or other microalgae that harbour secondary plastids, with the exception of *Phaeodactylum tricornutum* (Xie et al. 2014). In this section we consider the three key prerequisites for achieving chloroplast transformation in *Nannochloropsis*.

(i) *Prerequisite 1: plastome sequence for the chosen species*
Integration of DNA into the plastome occurs via homologous recombination, allowing site-directed modification and the precise insertion of foreign DNA into predetermined loci. Consequently, prior knowledge of the plastome sequence is required in order to manipulate the target chloroplast gene or flank foreign DNA with homologous elements. Furthermore, successful expression of foreign genes typically requires the use of endogenous genetic elements such as promoters and untranslated regions (Purton et al. 2013). Wei et al. (2013) sequenced plastomes from at least one strain of each *Nannochloropsis* species; the plastomes were found to range from 115–118 kb in size, contain 123–126 predicted protein-coding genes and had a GC content in the range of 33.0–33.6%. The chloroplast genome sequences of other

strains are also publicly available (Radakovits et al. 2012, Corteggiani Carpinelli et al. 2014, Starkenburg et al. 2014). These data provide the starting point for the design of chloroplast genetic engineering strategies.

(ii) *Prerequisite 2: a suitable selection system*

Development of a successful chloroplast transformation protocol relies on the availability of an effective selectable marker gene that facilitates the growth of transformant cells on selective media. Traits that have been widely used in algal chloroplast transformation as selection methods include restoration of photoautotrophy, resistance to antibiotics, tolerance to herbicides, and the complementation of metabolic mutants (reviewed by Potvin & Zhang 2010, Day & Goldschmidt-Clermont 2011). Heterotrophic growth using glucose or ethanol as an organic carbon source has been reported for one *Nannochloropsis* strain (Fang et al. 2004), suggesting that chloroplast mutants defective in photosynthesis could be isolated and the corresponding wild-type gene used as a marker to restore photoautotrophy. However, no non-photosynthetic mutants have yet been described nor have any auxotrophic mutants been described that are defective in a key metabolic pathway within the organelle. Antibiotics and herbicides that target aspects of chloroplast biology have been screened by ourselves (unpublished data: see Table 5) and others (Vieler et al. 2012, Chernyavskaya 2014) for their effect on various *Nannochloropsis* species. Most compounds tested were found to have little effect on

cell growth even at high concentrations. This could be due to the complexity of plastid membranes, preventing easy access for those molecules into the chloroplast stroma and thus failing to exhibit an inhibitory effect. However, chloramphenicol and the photosystem II inhibitors, DCMU and atrazine show promise as selective agents (Table 5), as does paromomycin for *N. oceanica* but not for other *Nannochloropsis* species (Vieler et al. 2012).

(iii) *Prerequisite 3: A method to introduce exogenous DNA into the chloroplast*

DNA delivery into the algal chloroplast has been achieved using microparticle bombardment (= biolistics), glass bead agitation and electroporation (Table 4). Biolistics is generally a reliable method for delivering DNA across cell walls and multiple membranes, and has been employed successfully for nuclear transformation in many algal species (Gangl et al. 2015). However, progress in achieving chloroplast transformation is still limited to relatively few species, not least because of the small cell size of many algae. The gold or tungsten microparticles used for biolistic DNA delivery are typically 0.5–1.7 µm in diameter, which is rather large in comparison to a *Nannochloropsis* cell (approximately 2–4 µm in diameter), with the chloroplast compartment being even smaller. Recently, gold ‘nanoparticles’ with a diameter of 40 nm have become available and may be more suitable; O’Brien & Lummis (2011) demonstrated that these caused much less tissue damage than 1 µm gold particles during the

Table 5. Results from our group on the effect of selected compounds on *N. gaditana* CCMP526. “>” indicates the highest concentration of the respective compound tested.

Compound	Mode of action	Inhibitory level (µg/ml)	Selectable marker or dominant allele conferring resistance
Antibiotics			
Chloramphenicol	Inhibitor of 70S ribosome	50	<i>catA1</i>
Kanamycin	Inhibitor of 70S ribosome	> 200	<i>aphA6</i>
Spectinomycin	Inhibitor of 70S ribosome	> 200	<i>aadA</i>
Streptomycin	Inhibitor of 70S ribosome	> 200	<i>aadA</i>
Herbicides			
Harvest	Inhibitor of glutamine synthetase activity	> 100	<i>pat</i>
Glufosinate-ammonium	Inhibitor of glutamine synthetase activity	> 500	<i>pat</i>
Bialaphos	Inhibitor of glutamine synthetase activity	> 350	<i>pat</i>
Diuron (DCMU)	Inhibitor of photosystem II	30	Point mutation in <i>psbA</i>
Atrazine	Inhibitor of photosystem II	50	<i>atzA</i> or point mutation in <i>psbA</i>
Amino acids			
D-alanine	Interferes with amino acid metabolism	> 400	<i>dao</i>

biolistic transformation of human and mouse cells and were equally as efficient.

Electroporation has also been used successfully for nuclear transformation for a wide range of microalgae, both with and without cell walls (Radakovits et al. 2012, Zhang & Hu 2013). This method relies on subjecting the cells to controlled electrical pulses of high charges for a short period of time (ms), which results in temporary pores in the cell membranes through which the exogenous DNA enters the cells. The reported transformation of the *P. tricornutum* plastid with the *cat* gene by electroporation (Xie et al. 2014) opens up the possibility of using this technique for other algal species whose chloroplasts are surrounded by multiple membranes, such as *Nannochloropsis*.

Given the current lack of methodology to transfer genes directly into the *Nannochloropsis* chloroplast genome, an alternative strategy is to transform the nucleus with genes encoding chloroplast-targeted proteins. This could give insights into industrially relevant biosynthetic pathways that take place in chloroplast and may also enable the introduction of novel pathways. There are relatively few studies in heterokont algae reporting success in targeting foreign proteins into the chloroplast using endogenous N-terminal signal and transit peptides, and more research is required in this direction (Gruber et al. 2007, Sunaga et al. 2014, Moog et al. 2015).

Conclusions and future directions

Nannochloropsis species have great potential as environmentally sustainable sources of biofuels and nutritionally important oils such as long chain omega-3 fatty acids. However, in order to make these products economically viable and competitive, there is a need for both biological improvements to the strains used and the development of more cost-effective and energy efficient bioprocessing technologies including cultivation, harvesting and product preparation. This is particularly true for biofuels where the desired product is high volume/low value. Many recent studies have examined the possibility of using municipal wastewater or industrial effluent to grow *Nannochloropsis* species for biomass production as part of an integrated system of wastewater treatment. For example, *N. oculata* grows well in 20% untreated municipal wastewater diluted in seawater, resulting in the successful removal of 80% of the nitrogen and phosphorus (Sirin & Sillanpaa 2015). Since lipid productivity is influenced by two conflicting factors – nutrient availability (to maximize biomass production) and nutrient deprivation (to increase TAG accumulation), a balance must be struck between a sufficient rate of wastewater treatment, biomass accumulation and lipid accumulation (as demonstrated for *N. salina* by Cai et al. (2013)).

Strain engineering would also help to address such challenges by creating strains with enhanced TAG accu-

mulation under nutrient replete conditions. To do this, we must build on our current understanding of lipid metabolic/catabolic pathways and how these are influenced by growth conditions such as nutrient concentration, salinity, temperature and light. Progress in this direction is starting to be made using systems biology approaches to develop dynamic models of lipid metabolism in *Nannochloropsis* and related algal groups (e.g. Dong et al. 2013, Mühlroth et al. 2013), and to understand at the genome level the transcriptional factors that regulate gene expression (Hu et al. 2014). In addition to such models, a set of molecular tools is needed to enable the genetic manipulation of lipid biology in both the nucleus and plastid, and to modify other aspects of *Nannochloropsis* physiology to enhance performance and productivity under industrial cultivation conditions. For example, Lu & Xu (2015) have proposed that increased biomass productivity and elevated tolerance to abiotic stresses could be achieved by manipulation of endogenous phytohormone levels in the algae. Such multi-trait strain improvement strategies require advanced techniques for predictable and precise genome engineering. New nuclear genome editing techniques that are being applied successfully in other organisms (Hsu et al. 2014, Chandrasegaran & Carroll 2016) would certainly advance the field. These editing approaches rely on double-stranded breaks made at targeted loci using bespoke nucleases such as engineered meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALENs) or the CRISPR/Cas9 system, and are still very much in the early stages of development for microalgae. However, three recent reports of successful targeted insertions and gene knockouts in the diatom *Phaeodactylum tricornutum* using meganucleases, TALENs and CRISPR-Cas9, respectively (Daboussi et al. 2014; Weyman et al. 2015; Nymark et al. 2016) should encourage efforts to develop these technologies for other heterokont algae such as *Nannochloropsis*. Similarly, further studies of how foreign DNA integrates into the *Nannochloropsis* nuclear genome will reveal whether targeted integration via homologous recombination is limited to one or a few species, or could be applied more generally as a genome engineering tool (Weeks 2011). The successful transformation of the *P. tricornutum* plastid (Xie et al. 2014) suggests also that the hurdles for engineering secondary plastids can be overcome, allowing a full suite of technologies for making designer strains of *Nannochloropsis*.

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References

- Anandarajah, K., Mahendrapuram, G., Sommerfeld, M. & Hu, Q. (2012): Characterization of microalga *Nannochloropsis* sp. mutants for improved production of biofuels. – *Appl. Energy* 96: 371–377.
- Arnold, A.A., Genard, B., Zito, F., Tremblay, R., Warschawski, D.E. & Marcotte, I. (2014): Identification of lipid and saccharide constituents of whole microalgal cells by ¹³C solid-state NMR. – *Biochim. Biophys. Acta – Biomembr.* 1848: 369–377.
- Beacham, T.A., Bradley, C., White, D.A., Bond, P. & Ali S.T. (2014): Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel production and downstream processing. – *Algal Res.* 6: 64–69.
- Beacham, T.A., Macia, V.M., Rooks, P., White, D.A. & Ali, S.T. (2015): Altered lipid accumulation in *Nannochloropsis salina* CCAP849/3 following EMS and UV induced mutagenesis. – *Biotechnol. Reports* 7: 87–94.
- Becker, E.W. (2013): Microalgae for Aquaculture: Nutritional Aspects. – In: Richmond, A. & Hu, Q. (ed.), *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, pp. 671–691. – John Wiley & Sons, Ltd., Chichester.
- Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B. & Sanford, J.C. (1988): Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. – *Science* 240: 1534–1538.
- Cai, T., Park, S.Y., Racharaks, R. & Li, Y. (2013): Cultivation of *Nannochloropsis salina* using anaerobic digestion effluent as a nutrient source for biofuel production. – *Appl. Energy* 108: 486–492.
- Camacho-Rodríguez, J., Cerón-García, M.C., Macías-Sánchez, M.D., Fernández-Sevilla, J.M., López-Rosales, L. & Molina-Grima, E. (2016): Long-term preservation of concentrated *Nannochloropsis gaditana* cultures for use in aquaculture. – *J. Appl. Phycol.* 28: 299–312.
- Camacho-Rodríguez, J., González-Céspedes, A.M., Cerón-García, M.C., Fernández-Sevilla, J.M., Acién-Fernández, F.G. & Molina-Grima, E. (2014): A quantitative study of eicosapentaenoic acid (EPA) production by *Nannochloropsis gaditana* for aquaculture as a function of dilution rate, temperature and average irradiance. – *Appl. Microbiol. Biotechnol.* 98: 2429–2440.
- Cha, T.S., Chen, C.F., Yee, W., Aziz, A. & Loh, S.H. (2011): Cinnamic acid, coumarin and vanillin: Alternative phenolic compounds for efficient Agrobacterium-mediated transformation of the unicellular green alga, *Nannochloropsis* sp. – *J. Microbiol. Methods* 84: 430–434.
- Chandrasegaran, S. & Carroll, D. (2016). Origins of programmable nucleases for genome engineering. – *J. Mol. Biol.* 428: 963–989.
- Chaturvedi, R. & Fujita, Y. (2006): Isolation of enhanced eicosapentaenoic acid producing mutants of *Nannochloropsis oculata* ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level. – *Phycol. Res.* 54: 208–219.
- Chen, H.L., Li, S.S., Huang, R. & Tsai, H.J. (2008): Conditional production of a functional fish growth hormone in the transgenic line of *Nannochloropsis oculata* (Eustigmatophyceae). – *J. Phycol.* 44: 768–776.
- Chernyavskaya, O. (2014): Developing molecular tools for the genetic manipulation of *Nannochloropsis*. – MSc thesis. Norges Teknisk-Naturvitenskapelige Universitet, Norway. [<http://brage.bibsys.no/xmlui/handle/11250/246154>]
- Corteggiani Carpinelli, E., Telatin, A., Vitulo, N., Forcato, C., D'Angelo, M., Schiavon, R., Vezzi, A., Giacometti, G.M., Morosinotto, T. & Valle, G. (2014): Chromosome scale genome assembly and transcriptome profiling of *Nannochloropsis gaditana* in nitrogen depletion. – *Mol. Plant* 7: 323–335.
- Cui, Y., Qin, S. & Jiang, P. (2014): Chloroplast transformation of *Platymonas (Tetraselmis) subcordiformis* with the *bar* gene as selectable marker. – *PLoS One* 9: e98607.
- Daboussi, F., Leduc, S., Maréchal, A., Dubois, G., Guyot, V., Perez-Michaut, C., Amato, A., Falciorato, A., Juillierat, A., Beurdeley, M., Voytas, D.F., Cavarec, L. & Duchateau, P. (2014): Genome engineering empowers the diatom *Phaeodactylum tricorutum* for biotechnology. – *Nat. Commun.* 5: 3831.
- Day, A. & Goldschmidt-Clermont, M. (2011): The chloroplast transformation toolbox: selectable markers and marker removal. – *Plant Biotechnol. J.* 9: 540–553.
- Doan, T.T.Y. & Obbard, J.P. (2012): Enhanced intracellular lipid in *Nannochloropsis* sp. via random mutagenesis and flow cytometric cell sorting. – *Algal Res.* 1: 17–21.
- Doan, Y.T.T. & Obbard J.P. (2015): Two-stage cultivation of a *Nannochloropsis* mutant for biodiesel feedstock. – *J. Appl. Phycol.* 27: 2203–2208.
- Doetsch, N.A., Favreau, M.R., Kuscuoğlu, N., Thompson, M.D. & Hallick, R.B. (2001): Chloroplast transformation in *Euglena gracilis*: Splicing of a group III twintron transcribed from a transgenic *psbK* operon. – *Curr. Genet.* 39: 49–60.
- Dong, H.P., Williams, E., Wang, D.Z., Xie, Z.X., Hsia, R.C., Jenck, A., Halden, R., Li, J., Chen, F. & Place, A.R. (2013): Responses of *Nannochloropsis oceanica* IMET1 to long-term nitrogen starvation and recovery. – *Plant Physiol.* 162: 1110–1126.
- Fang, X., Wei, C., Zhao-Ling, C. & Fan, O. (2004): Effects of organic carbon sources on cell growth and eicosapentaenoic acid content of *Nannochloropsis* sp. – *J. Appl. Phycol.* 16: 499–503.
- Fawley, M.W., Jameson, I. & Fawley, K.P. (2015): The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis* sp. nov. and *Microchloropsis* gen. nov. – *Phycologia* 54: 545–552.
- Fernandez-Reiriz, M.J. & Labarta, U. (1996): Lipid classes and fatty acid composition of rotifers (*Brachionus plicatilis*) fed two algal diets. – *Hydrobiologia* 330: 73–79.
- Ferreira, M., Coutinho, P., Seixas, P., Fabregas, J. & Otero, A. (2009): Enriching Rotifers with “Premium” Microalgae. *Nannochloropsis gaditana*. – *Mar. Biotechnol.* 11: 585–595.
- Franz, A.K., Danielewicz, M.A., Wong, D.M., Anderson, L.A. & Boothe, J.R. (2013): Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity. – *ACS Chem. Biol.* 8: 1053–1062.
- Gangl, D., Zedler, J.A.Z., Rajakumar, P.D., Ramos Martinez, E.M., Riseley, A., Włodarczyk, A., Purton, S., Sakuragi, Y., Howe, C.J., Jensen, P.E. & Robinson, C. (2015): Biotechnological exploitation of microalgae. – *J. Exp. Bot.* 66: 6975–6990.
- Georgianna, D.R., Hannon, M.J., Marcuschi, M., Wu, S., Botsch, K., Lewis, A.J., Hyun, J., Mendez, M. & Mayfield, S.P. (2013): Production of recombinant enzymes in the marine alga *Dunaliella tertiolecta*. – *Algal Res.* 2: 2–9.
- Gressel, J. (2013): Transgenic marine microalgae: a value-enhanced fishmeal and fish oil replacement. – In: Richmond, A. & Hu, Q. (ed.), *Handbook of Microalgal Culture: Applied Phycology and*

- Biotechnology, pp. 653–670. – John Wiley & Sons, Ltd., Chichester.
- Gruber, A., Vugrinec, S., Hempel, F., Gould, S.B., Maier, U.G. & Kroth, P.G. (2007): Protein targeting into complex diatom plastids: Functional characterisation of a specific targeting motif. – *Plant Mol. Biol.* 64: 519–530.
- Gutiérrez, C.L., Gimpel, J., Escobar, C., Marshall, S.H. & Henríquez, V. (2012): Chloroplast genetic tool for the green microalgae *Haematococcus pluvialis* (Chlorophyceae, Volvocales). – *J. Phycol.* 48: 976–983.
- Hannon, M., Gimpel, J., Tran, M., Rasala B. & Mayfield, S. (2010): Biofuels from algae: challenges and potential. – *Biofuels* 1: 763–784.
- Hsu, P.D., Lander, E.S. & Zhang, F. (2014): Development and applications of CRISPR-Cas9 for genome engineering. – *Cell* 157: 1262–1278.
- Hu, J., Wang, D., Li, J., Jing, G., Ning, K. & Xu, J. (2014): Genome-wide identification of transcription factors and transcription-factor binding sites in oleaginous microalgae *Nannochloropsis*. – *Sci. Rep.* 4: 5454.
- Hu, Q., Xiang, W., Dai, S., Li, T., Yang, F., Jia, Q., Wang, G. & Wu, H. (2015): The influence of cultivation period on growth and biodiesel properties of microalga *Nannochloropsis gaditana* 1049. – *Bioresour. Technol.* 192: 157–164.
- Huang, Y.-J., Wang, L., Zheng, M.-G., Zheng, L., Tong, Y.-L. & Li, Y. (2014): Overexpression of *NgAUREOL*, the gene coding for aureochrome 1 from *Nannochloropsis gaditana*, into *Saccharomyces cerevisiae* leads to a 1.6-fold increase in lipid accumulation. – *Biotechnol. Lett.* 36: 575–579.
- Iwai, M., Hori, K., Sasaki-Sekimoto, Y., Shimojima, M. & Ohta, H. (2015): Manipulation of oil synthesis in *Nannochloropsis* strain NIES-2145 with a phosphorus starvation-inducible promoter from *Chlamydomonas reinhardtii*. – *Front. Microbiol.* 6: 1–15.
- Janouskovec, J., Horák, A., Oborník, M., Lukes, J. & Keeling, P.J. (2010): A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. – *Proc. Natl. Acad. Sci. U.S.A.* 107: 10949–10954.
- Jinkerson, R., Radakovits, R. & Posewitz, M. (2013): Genomic insights from the oleaginous model alga *Nannochloropsis gaditana*. – *Bioengineered* 4: 37–43.
- Kagan, M.L. & Matulka, R.A. (2015): Safety assessment of the microalgae *Nannochloropsis oculata*. – *Toxicol. Reports* 2: 617–623.
- Kagan, M.L., Sullivan, D.W., Gad, S.C. & Ballou, C.M. (2014): Safety assessment of EPA-rich polar lipid oil produced from the microalgae *Nannochloropsis oculata*. – *Int. J. Toxicol.* 33: 459–474.
- Kang, N.K., Choi, G., Kim, E.K., Shin, S., Jeon, S., Park, M.S., Jeong, K.J., Jeong, B., Chang, Y.K., Yang, J. & Lee, B. (2015a): Heterologous overexpression of sfCherry fluorescent protein in *Nannochloropsis salina*. – *Biotechnol. Reports* 8: 10–15.
- Kang, N.K., Jeon, S., Kwon, S., Koh, H.G., Shin, S.E., Lee, B., Choi, G.G., Yang, J.W., Jeong, B.R. & Chang, Y.K. (2015b): Effects of overexpression of a bHLH transcription factor on biomass and lipid production in *Nannochloropsis salina*. – *Biotechnol. Biofuels* 8: 200.
- Kang, N.K., Lee, B., Shin, S.E., Jeon, S., Park, M.S. & Yang, J.W. (2015c): Use of conditioned medium for efficient transformation and cost-effective cultivation of *Nannochloropsis salina*. – *Bioresour. Technol.* 181: 231–237.
- Karas, B.J., Diner, R.E., Lefebvre, S.C., McQuaid, J., Phillips, A.P.R., Noddings, C.M., Brunson, J.K., Valas, R.E., Deerinck, T.J., Jablanovic, J., Gillard, J.T.F., Beerli, K., Ellisman, M.H., Glass, J.I., Hutchison, C.A. III, Smith, H.O., Venter, J.C., Allen, A.E., Dupont, C.L. & Weyman, P.D. (2015): Designer diatom episomes delivered by bacterial conjugation. – *Nat. Commun.* 6: 6925.
- Kaye, Y., Grundman, O., Leu, S., Zarka, A., Zorin, B., Didi-Cohen, S., Khozin-Goldberg, I. & Boussiba, S. (2015): Metabolic engineering toward enhanced LC-PUFA biosynthesis in *Nannochloropsis oceanica*: Overexpression of endogenous $\Delta 12$ desaturase driven by stress-inducible promoter leads to enhanced deposition of polyunsaturated fatty acids in TAG. – *Algal Res.* 11: 387–398.
- Kilian, O., Benemann, C.S.E., Niyogi, K.K. & Vick, B. (2011): High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. – *Proc. Natl. Acad. Sci. USA* 108: 21265–21269.
- Kindle, K.L., Richards, K.L. & Stern, D.B. (1991): Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. – *Proc. Natl. Acad. Sci. USA* 88: 1721–1725.
- Klok, A.J., Lamers, P.P., Martens, D.E., Draaisma, R.B. & Wijffels, R.H. (2014): Edible oils from microalgae: insights in TAG accumulation. – *Trends Biotechnol.* 32: 521–528.
- Lapidot, M., Raveh, D., Sivan, A., Arad, S.M. & Shapira, M. (2002): Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. – *Plant Physiol.* 129: 7–12.
- La Russa, M., Bogen, C., Uhmeyer, A., Doebe, A., Filippone, E., Kruse, O. & Mussgnug, J.H. (2012): Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. – *J. Biotechnol.* 162: 13–20.
- Lee, M.Y., Min, B.S., Chang, C.S. & Jin E. (2006): Isolation and characterization of a xanthophyll aberrant mutant of the green alga *Nannochloropsis oculata*. – *Mar. Biotechnol. (NY)* 8: 238–245.
- Lemahieu, C., Bruneel, C., Termote-Verhalle, R., Muylaert, K., Buyse, J. & Foubert, I. (2013): Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens. – *Food Chem.* 141: 4051–4059.
- Li, F., Gao, D. & Hu, H. (2014a): High-efficiency nuclear transformation of the oleaginous marine *Nannochloropsis* species using PCR product. – *Biosci. Biotechnol. Biochem.* 78: 812–817.
- Li, J., Han, D., Wang, D., Ning, K., Jia, J., Wei, L., Jing, X., Huang, S., Chen, J., Li, Y., Hu, Q. & Xu, J. (2014b): Choreography of transcriptomes and lipidomes of *Nannochloropsis* reveals the mechanisms of oil synthesis in microalgae. – *Plant Cell* 26: 1645–1665.
- Li, S.S. & Tsai, H.J. (2009): Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. – *Fish Shellfish Immunol.* 26: 316–325.
- Lu, Y. & Xu, J. (2015): Phytohormones in microalgae: a new opportunity for microalgal biotechnology? – *Trends Plant Sci.* 20: 273–282.
- Lubián, L.M. (1982): *Nannochloropsis gaditana* sp. nov., a new marine Eustigmatophyceae. – *Lazaroa* 4: 287–293.
- Lubzens, E., Gibson, O., Zmora, O. & Sukenik, A. (1995): Potential advantages of frozen algae (*Nannochloropsis* sp.) for rotifer (*Brachionus plicatilis*) culture. – *Aquaculture* 133: 295–309.

- Ma, X.N., Chen, T.P., Yang, B., Liu J. & Chen, F. (2016): Lipid production from *Nannochloropsis*. – *Marine Drugs* 14: 61.
- Ma, Y., Wang, Z., Yu, C., Yin, Y. & Zhou, G. (2014): Evaluation of the potential of 9 *Nannochloropsis* strains for biodiesel production. – *Bioresour. Technol.* 167: 503–509.
- Ma, Y., Wang, Z., Zhu, M., Yu, C., Cao, Y., Zhang, D. & Zhou, G. (2013): Increased lipid productivity and TAG content in *Nannochloropsis* by heavy-ion irradiation mutagenesis. – *Bioresour. Technol.* 136: 360–367.
- Medipally, S.R., Yusoff, F.M., Banerjee, S. & Shariff, M. (2015): Microalgae as sustainable renewable energy feedstock for bio-fuel production. – *Biomed. Res. Int.* 2015: 519513.
- Moog, D., Stork, S., Reislöhner, S., Grosche, C. & Maier, U.-G. (2015): In vivo localization studies in the Stramenopile alga *Nannochloropsis oceanica*. – *Protist* 166: 161–171.
- Mühlroth, A., Li, K., Røkke, G., Winge, P., Olsen, Y., Hohmann-Marriott, M.F., Vadstein, O. & Bones, A.M. (2013): Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of *Chromista*. – *Mar. Drugs* 11: 4662–4697.
- Murakami, R. & Hashimoto, H. (2009): Unusual nuclear division in *Nannochloropsis oculata* (Eustigmatophyceae, Heterokonta) which may ensure faithful transmission of secondary plastids. – *Protist* 160: 41–49.
- Nymark, M., Sharma, A.K., Sparstad, T., Bones, A.M. & Winge, P. (2016): A CRISPR/Cas9 system adapted for gene editing in marine algae. – *Sci. Rep.* 6: 24951.
- O'Brien, J.A. & Lummis, S.C.R. (2011): Nano-biologics: a method of biolistic transfection of cells and tissues using a gene gun with novel nanometer-sized projectiles. – *BMC Biotechnol.* 11: 66.
- Pan, K., Qin, J., Li, S., Dai, W., Zhu, B., Jin, Y., Yu, W., Yang, G. & Li, D. (2011): Nuclear monoploidy and asexual propagation of *Nannochloropsis oceanica* (Eustigmatophyceae) as revealed by its genome sequence. – *J. Phycol.* 47: 1425–1432.
- Perin, G., Bellan, A., Segalla, A., Meneghesso, A., Alboresi, A. & Morosinotto, T. (2015): Generation of random mutants to improve light-use efficiency of *Nannochloropsis gaditana* cultures for biofuel production. – *Biotechnol. Biofuels* 8: 161.
- Potvin, G. & Zhang, Z. (2010): Strategies for high-level recombinant protein expression in transgenic microalgae: A review. – *Biotechnol. Adv.* 28: 910–918.
- Purton, S., Szaub, J.B., Wannathong, T., Young, R. & Economou, C.K. (2013): Genetic engineering of algal chloroplasts: progress and prospects. – *Rus. J. Plant Physiol.* 60: 491–499.
- Radakovits, R., Jinkerson, R.E., Fuerstenberg, S.I., Tae, H., Settlage, R.E., Boore, J.L. & Posewitz, M.C. (2012): Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. – *Nat. Commun.* 3: 686.
- Schneider, J.C., Livne, A., Sukenik, A. & Roessler, P.G. (1995): A mutant of *Nannochloropsis* deficient in eicosapentaenoic acid production. – *Phytochemistry* 40: 807–814.
- Sharma, K. & Schenk, P.M. (2015): Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis* sp. by UV-C radiation. – *Biotechnol. Bioeng.* 112: 1243–1249.
- Shih, C.-H., Chen, H.-Y., Lee, H.-C. & Tsai, H.-J. (2015): Purple chromoprotein gene serves as a new selection marker for transgenesis of the microalga *Nannochloropsis oculata*. – *PLoS One* 10: e0120780.
- Simionato, D., Block, M.A., La Rocca, N., Jouhet, J., Maréchal, E., Finazzi, G. & Morosinotto, T. (2013): The response of *Nannochloropsis gaditana* to nitrogen starvation includes de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. – *Eukaryot. Cell* 12: 665–676.
- Şirin, S. & Sillanpää, M. (2015): Cultivating and harvesting of marine alga *Nannochloropsis oculata* in local municipal wastewater for biodiesel. – *Bioresour. Technol.* 191: 79–87.
- Siripornadulsil, S., Dabrowski, K. & Sayre, R. (2007): Microalgal vaccines. – *Adv. Exp. Med. Biol.* 616: 122–128.
- Sorigué, D., Légeret, B., Cuiñé, S., Morales, P., Mirabella, B., Guédény, G., Li-Beisson, Y., Jetter, R., Peltier, G. & Beisson, F. (2016): Microalgae synthesize hydrocarbons from long-chain fatty acids via a light-dependent pathway. – *Plant Physiol.* 171: 2393–2405.
- Starkenburg, S.R., Kwon, K.J., Jha, R.K., McKay, C., Jacobs, M., Chertkov, O., Twary, S., Roca, G. & Cattolico, R. (2014): A pangenomic analysis of the *Nannochloropsis* organellar genomes reveals novel genetic variations in key metabolic genes. – *BMC Genomics* 15: 212.
- Su, C.H., Chien, L.J., Gomes, J., Lin, Y.S., Yu, Y.K., Liou, J.S. & Syu, R.J. (2011): Factors affecting lipid accumulation by *Nannochloropsis oculata* in a two-stage cultivation process. – *J. Appl. Phycol.* 23: 903–908.
- Sukenik, A. (1999): Production of eicosapentaenoic acid by the marine eustigmatophyte *Nannochloropsis*. – In: Cohen, Z. (ed.), *Chemicals from Microalgae*, pp. 41–53. – Taylor and Francis, London.
- Sunaga, Y., Maeda, Y., Yabuuchi, T., Muto, M., Yoshino, T. & Tanaka, T. (2014): Chloroplast-targeting protein expression in the oleaginous diatom *Fistulifera solaris* JPC DA0580 toward metabolic engineering. – *J. Biosci. Bioeng.* 119: 28–34.
- Taleb, A., Pruvost, J., Legrand, J., Marec, H., Le-Gouic, B., Mirabella, B., Legeret, B., Bouvet, S., Peltier, G., Li-Beisson, Y., Taha, S. & Takache, H. (2015): Development and validation of a screening procedure of microalgae for biodiesel production: application to the genus of marine microalgae *Nannochloropsis*. – *Bioresour. Technol.* 177: 224–232.
- Tian, J., Zheng, M., Yang, G., Zheng, L., Chen, J. & Yang, B. (2013): Cloning and stress-responding expression analysis of malonyl CoA-acyl carrier protein transacylase gene of *Nannochloropsis gaditana*. – *Gene* 530: 33–38.
- Umdu, E.S., Tuncer, M. & Seker, E. (2009): Transesterification of *Nannochloropsis oculata* microalga's lipid to biodiesel on Al₂O₃ supported CaO and MgO catalysts. – *Bioresour. Technol.* 100: 2828–2831.
- van den Hoek, C., Mann, D.G. & Jahns, H.M. (1995): *Algae: An Introduction to Phycology*. Cambridge University Press, Cambridge, UK.
- Vieler, A., Wu, G., Tsai, C.-H., Bullard, B., Cornish, A.J., Harvey, C., Rea, I.-B., Thornburg, C., Achawanantakun, R., Buehl, C.J., Campbell, M.S., Cavalier, D., Childs, K.L., Clark, T.J., Deshpande, R., Erickson, E., Armenia Ferguson, A., Handee, W., Kong, Q., Li, X., Liu, B., Lundback, S., Peng, C., Roston, R.L., Sanjaya, Simpson, J.P., TerBush, A., Warakanont, J., Zäuner, S., Farre, E.M., Hegg, E.L., Jiang, N., Kuo, M.-H., Lu, Y., Niyogi, K.K., Ohlrogge, J., Osteryoung, K.W., Shachar-Hill, Y., Sears, B.B., Sun, Y., Takahashi, H., Yandell, M., Shiu, S.-H. & Benning, C. (2012): Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga

- Nannochloropsis oceanica* CCMP1779. – PLoS Genet 8: e1003064.
- Vree, J.H. de, Bosma, R., Janssen, M., Barbosa, M.J. & Wijffels, R.H. (2015): Comparison of four outdoor pilot-scale photobioreactors. – Biotechnol. Biofuels 8: 215.
- Wang, D., Ning, K., Li, J., Hu, J., Han, D., Wang, H., Zeng, X., Jing, X., Zhou, Q., Su, X., Chang, X., Wang, A., Wang, W., Jia, J., Wei, L., Xin, Y., Qiao, Y., Huang, R., Chen, J., Han, B., Yoon, K., Hill, R.T., Zohar, Y., Chen, F., Hu, Q. & Xu, J. (2014): *Nannochloropsis* genomes reveal evolution of microalgal oleaginous traits. – PLoS Genet. 10: e1004094.
- Weeks, D.P. (2011): Homologous recombination in *Nannochloropsis*: a powerful tool in an industrially relevant alga. – Proc. Natl Acad. Sci. USA 108: 20859–20860.
- Wei, L., Xin, Y., Wang, D., Jing, X., Zhou, Q., Su, X., Jia, J., Ning, K., Chen, F., Hu, Q. & Xu, J. (2013): *Nannochloropsis* plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. – BMC Genomics 14: 534.
- Weyman, P.D., Beeri, K., Lefebvre, S.C., Rivera, J., McCarthy, J.K., Heuberger, A.L., Peers, G., Allen, A.E. & Dupont, C.L. (2015): Inactivation of *Phaeodactylum tricorutum* urease gene using transcription activator-like effector nuclease-based targeted mutagenesis. – Plant Biotechnol. J. 13: 460–470.
- Xie, W.H., Zhu, C.C., Zhang, N.S., Li, D., Yang, W.D., Liu, J.S., Sathishkumar, R. & Li, H.Y. (2014): Construction of novel chloroplast expression vector and development of an efficient transformation system for the diatom *Phaeodactylum tricorutum*. – Mar. Biotechnol. (NY) 16: 538–546.
- Zhang, C. & Hu, H. (2013): High-efficiency nuclear transformation of the diatom *Phaeodactylum tricorutum* by electroporation. – Mar. Genomics 16: 63–66.
- Zheng, M.G., Huang, Y.J., Zheng, L., Sun, Z.T. & Wang, L. (2014): Cloning, expression and stress-responsive transcription of long-chain acyl-coenzyme A synthetase cDNA gene of *Nannochloropsis gaditana* and its involvement in the biosynthesis of eicosapentaenoic and docosahexaenoic acids. – Biotechnol. Lett. 36: 141–145.
- Zheng, M., Tian, J., Yang, G., Zheng, L., Chen, G., Chen, J. & Wang, B. (2013): Transcriptome sequencing, annotation and expression analysis of *Nannochloropsis sp.* at different growth phases. – Gene 523: 117–121.
- Zhu, B., Sun, F., Yang, M., Lu, L., Yang, G. & Pan, K. (2014): Large-scale biodiesel production using flue gas from coal-fired power plants with *Nannochloropsis* microalgal biomass in open raceway ponds. – Bioresour. Technol. 174: 53–59.

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